EVASION OF AFRICAN TRYPANOSOMES TO HUMAN INNATE IMMUNITY

by

ERIC GLENN DEJESUS

(Under the Direction of Stephen L. Hajduk)

ABSTRACT

Critical to human innate immunity against African trypanosomes are a minor subclass of human high-density lipoproteins, termed Trypanosome Lytic Factors (TLF). Examination of these particles revealed two subsets termed TLF-1 and TLF-2 based on protein and lipid composition. The TLF-1 molecule binds to a haptoglobin-hemoglobin receptor (HpHbR) on the surface of susceptible trypanosomes, initiating a lytic pathway. *T. b. gambiense,* which is split into two groups 1 and 2 based on genetic diversity, causes 97% of all cases of African sleeping sickness, a fatal disease of sub-Saharan Africa. Understanding how *T. b. gambiense* overcomes these factors and infects humans is of major importance in the fight against this disease. Group 1 *Trypanosoma brucei gambiense* causes human African Trypanosomiasis (HAT), escaping TLF-1 killing due to reduced uptake. Previously, it was identified that, when compared to wild type *T. b. brucei*, group 1 *T. b. gambiense* HpHbR (*Tbg*HpHbR) mRNA levels were greatly reduced and the gene contained substitutions within the open reading frame. Here I show that a single, highly conserved amino acid in the *Tbg*HpHbR ablates high affinity TLF-1 binding and subsequent endocytosis, thus evading TLF-1 killing. In addition, we show that over-expression of

*Tbg*HpHbR failed to rescue TLF-1 susceptibility. These findings suggest that the single amino acid substitution present in the *Tbg*HpHbR directly contributes to the reduced uptake and resistance to TLF-1 seen in these important human pathogens. These finding indicate that a failure to take up TLF-1 in *T. b. gambiense* contributes to resistance to TLF-1, although another mechanism is required to overcome TLF-2. Here, in collaboration with Dr. Annette MacLeod, we have examined a *T. b. gambiense* specific gene, TgsGP, which had previously been suggested to be involved in serum resistance. We show that TgsGP is essential for resistance to lysis as deletion of TgsGP in *T. b. gambiense* renders the parasites sensitive to human serum as well as recombinant APOL1. Deletion of TgsGP in *T. b. gambiense* modified to uptake TLF-1 showed sensitivity to TLF-1, APOL1 and human serum. Reintroducing TgsGP into knockout parasite lines restored resistance. The data suggests that TgsGP is essential for human serum resistance in *T. b. gambiense.*

INDEX WORDS: *Trypanosoma brucei brucei, Trypanosoma brucei gambiense,* Trypanosome lytic factor (TLF-1), haptoglobin-hemoglobin, TgsGP, HEK293.

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DEDICATION

To my wife, Megan DeJesus

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First I would like to thank my "bossman", Stephen "Duk" Hajduk. My time in the laboratory has been a period of intense learning and growth. My learning curve was, as I preferred to put it, a brick wall and all the while, Duk was there pushing me to strive to be better. Without acknowledging Rudo Kieft when discussing my development as a scientist would be a huge oversight. He was there to guide me to find the correct answers and also to teach me how to deduce new information on my own: the perfect definition of an independent scientist. As for other lab mates, all of them, in their own unique way, contributed to my development while in this season of life. To them I say thank you.

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Andersen and his wife, Lily, thank you for the time and talks over the years- they are not forgotten and were never taken for granted. I think a great summary sentence would be: To my immediate family, thank you for the tough love cause I know I was tough to love.

The primary reason I am *here* now is due to the concentrated efforts of Dr. Scott Gilchrist and Dr. T.G. Jackson. These two people were mentors in the truest sense of the word. Throughout the process going back to 2002, Scott provided guidance, encouragement and feedback on my goals, interests and desires. When I expressed an interest in the sciences, it was he that suggested challenging myself by taking Dr. Jackson as my Organic Chemistry professor. Notoriously difficult, demanding and sharp tongued; Dr. Jackson was precisely what I needed as a young man thinking about a career in the sciences. My time with TG was invaluable and with his loss one month after starting graduate school (to prostate cancer), I find myself striving to think back on our many long conversations in his office.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Trypanosomatids are single flagellated protozoans that are able to infect a wide range of hosts, stretching from humans, wild game, insects and even plants. African Trypanosomes are responsible for human diseases including: South American trypanosomiasis from infection by *Trypanosoma cruzi*, leishmaniasis resulting from infection by various subspecies of Leishmania, and African trypanosomiasis as a result of *Trypanosoma brucei* infection. Focusing in on the latter, African trypanosomiasis, two subspecies in *T. brucei* are human infective: *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Infectivity of these trypanosomes requires evasion of both the human adaptive and innate immune systems. Each parasite subspecies has evolved unique mechanisms to protect itself from multiple factors in each immune system. While evasion to adaptive immunity has been well characterized through antigenic variation of the VSG coat in all *T. brucei* subspecies (described later), the story for innate resistance in *T. b. gambiense* is evolving.

The goal of this body of work is to describe the mechanism of resistance to specific factors in normal human serum (NHS) by *T. b. gambiense* and to contrast them to *T. b. rhodesiense.* In chapter 1, I outline what mechanisms are currently known in regards to *T. brucei* resistance to NHS. In Chapter 2, I specifically define the impact of a conserved polymorphism present within the haptoglobin-

hemoglobin (HpHb) receptor present within *T. b. gambiense.* It is this HpHb receptor that facilitates the uptake of the human innate immune molecule trypanosome lytic factor-1 (TLF-1) in *T. b. brucei* and *T. b. rhodesiense*. In Appendix A, work is presented that provides insight into another layer of resistance to normal human serum (NHS) in *T. b. gambiense***.** TgsGP, a protein unique to the gambiense genome, was previously identified as a potential player in the resistance that *T. b. gambiense* possesses to human serum [1]. The recent development of successful transfection methods for *T. b. gambiense* allowed us the opportunity to detail the role for TgsGP in resistance to TLF-1. Advancements have been significant in the general understanding of the resistance mechanisms these parasites have evolved to TLF-1. However, there is virtually no data on what prevents human cells from being killed by their own deadly immune particle. In chapter 3, I show initial investigations into the interactions between TLF-1 and mammalian systems. In the final chapter, I discuss the primary findings and implications of the work presented in this dissertation.

HUMAN AFRICAN TRYPANOSOMIASIS

Transmitted by the tsetse fly (Glossina), the protozoan parasite *Trypanosoma brucei* is responsible for human African Trypanosomiasis (HAT) in central and southern Africa. This disease has approximately 50,000 new cases identified each year in the sub-Saharan region of Africa [2]. Two forms of HAT exist and are each caused by a specific subspecies of *T. brucei. T. b. rhodesiense* is responsible for the acute form of eastern HAT while *T. b. gambiense* causes the chronic western HAT.

Eastern sleeping sickness, if left untreated, is lethal within 1-3 years post infection while Western HAT exhibits chronic pathology, taking upwards of 10 years to cause death [2].

Infection begins when a tsetse fly infected with trypomastigotes bites and transmits the parasite into the human host. There are two stages to infection: In Stage I (or the hemolymphatic phase) of HAT, the blood stream form parasites exist in and replicate in the blood and lymph nodes. Without treatment, the parasite eventually crosses the blood brain barrier, a process not well understood, and invades the central nervous system in the neurologic state (Stage II) of the disease. Once beyond this barrier, *T. b. gambiense* and *T. b. rhodesiense* cause neurological symptoms such as dementia and altered sleeping patterns. If untreated, infection culminates in coma and death. The parasite's mechanistic role in late stage symptoms is not understood [2].

TRYPANOSOMA BRUCEI CELLULAR STRUCTURE

Trypanosoma brucei is a eukaryotic parasite with a single flagellum protruding from a specialized region of the cell surface termed the flagellar pocket (FP). These parasites are highly motile, exhibiting a corkscrew type motion that is pivotal for several cellular processes important in cell survival [3]. All endocytosis and exocytosis occurs from the FP due to subpellicular array microtubules throughout the rest of the plasma membrane. This network is absent in the FP and therefore is the only site of content exchange [4-7]. This exchange happens at an exceedingly high rate, recycling all cell surface molecules (approximately 10^7

molecules) in approximately 2 minutes [8, 9]. Trafficking occurs in clathrin-coated vesicles and identified vesicles include early, recycling, sorting and late endosomes, which terminate into a single lysosome. Characteristic RAB proteins have defined each of these vesicles such as RAB5A for early and RAB11 for sorting endosomes [5]. Being a eukaryotic parasite, *T. brucei* contains many standard organelles found among eukaryotes. These include a nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus. Unique to kinetoplastids is a structure called the kinetoplast, which houses the mitochondrial DNA in a region of the mitochondria near the flagellar pocket. Another organelle unique to trypanosomes are glycosomes, which house the glycolytic enzymes pivotal for cell survival (Figure 1.1) [10, 11].

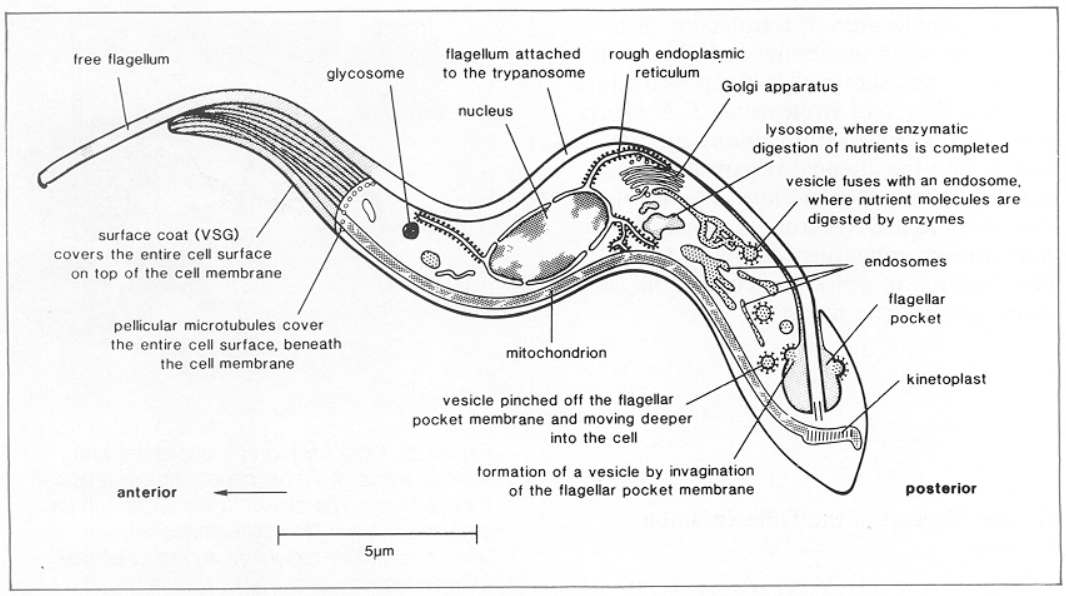

FIGURE 1.1. *Trypanosoma brucei* cellular structure. Representative organelles found in *T. brucei* are identified in the schematic of the bloodstream form of the

parasite. Permission to use image granted from International Livestock Research Institute website (www.ilri.org).

THE *TRYPANOSOMA BRUCEI* LIFE CYCLE

Human African trypanosomes are exquisite in their adaptability in surviving in diverse environments. The two most diverse and well-characterized life stages for African trypanosomes are the bloodstream form within the mammalian host and the procyclic form within the Tsetse fly vector (Figure 1.2). In order to survive the vastly different environments of a Tsetse fly and mammalian blood, the trypanosome undergoes a multitude of physiological changes. Beginning with the bite from an infected Tsetse fly, the trypanosome is transferred to mammalian bloodstream and is rapidly transformed into the long slender bloodstream form. This is a highly proliferative stage of development. Characteristics of this stage include high rates of endocytosis, VSG surface coat expression and high rates of protein synthesis [12]. Energy is supplied through glycolysis in glycosomes, contrary to insect forms, which rely on oxidative phosphorylation in highly developed mitochondria [13, 14]. It is this bloodstream form that is highly virulent and is the hallmark of trypanosome infection. Over a period of time, the long-slender forms can begin to physically transform into visually shortened, slow moving stumpy forms. It is this stage that completes the mammalian part of the cycle as tsetse fly blood meal starts another transformation process [15].

Inside the fly, the trypanosome once again undergoes several developmental changes, with the procyclic (dividing) and metacyclic (non-dividing) forms being the

two predominant stages. Procyclic parasites, residing in the mid-gut of the fly, endure a myriad of phenotypic changes which include an entire change of its surface coat protein from variant surface glycoprotein (VSG) to procyclic acidic repetitive protein (PARP), the slowing of endocytic machinery (low endocytic rates) and the alteration in membrane fluidity, mostly due to the difference in membrane lipid composition [12, 15]. In the metacyclic stage, parasites are found within the proventriculus of the fly and bloodstream form characteristics such as VSG expression and alterations in protein synthesis begin to emerge in preparation for introduction into the mammalian host via blood meal.

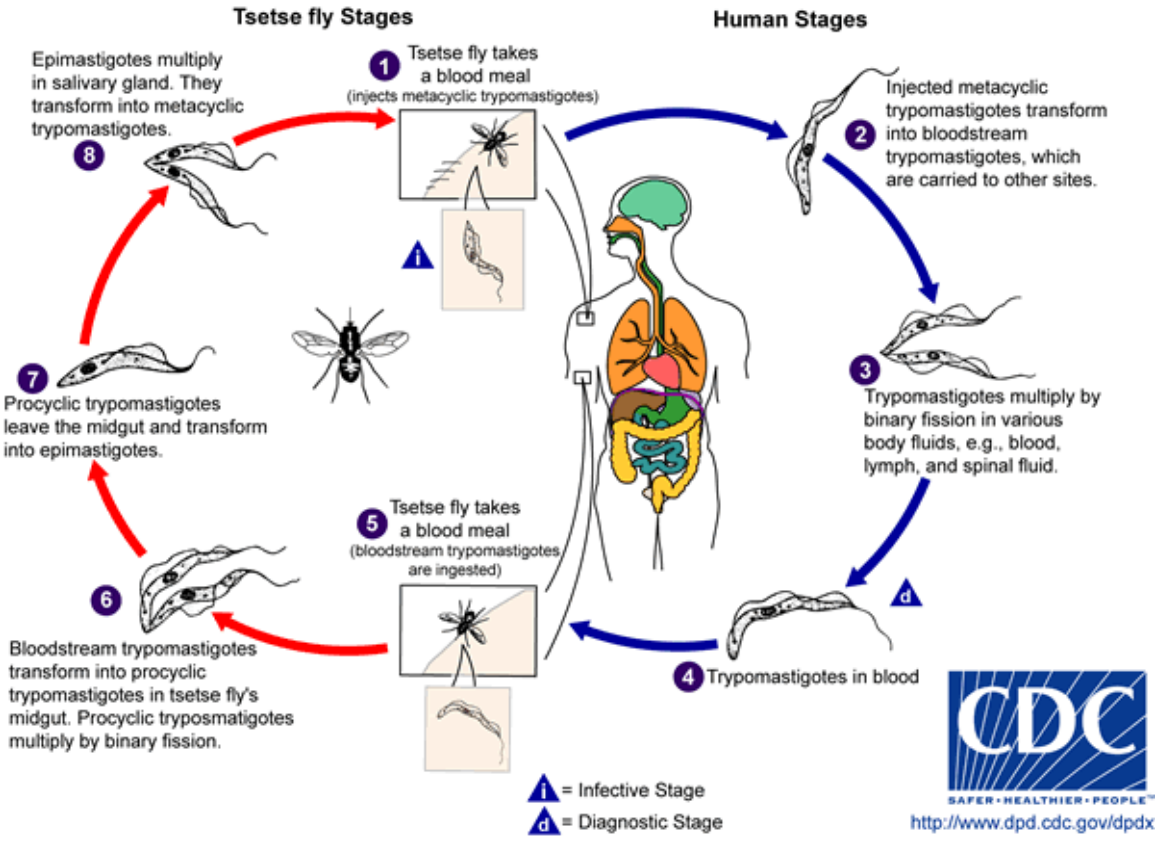

Figure 1.2. Life cycle of *Trypanosoma brucei*. Both primary life stages for *T. brucei* are depicted: Tsetse fly stages; Procyclic and Metacyclic trypomastigotes and Mammalian; Long slender and short stumpy. Image from CDC, open access.

TRYPANOSOME LYTIC FACTOR

In 1902 it was found that humans displayed resistance to specific species of trypanosomes, namely *T. b. brucei* [16]. Early studies aimed to examine human plasma and identified that the serum factor responsible for trypanosome killing was in high density lipoprotein (HDL) fractions after density ultracentrifugation [17]. This factor was later characterized as a minor class of HDLs and was termed the trypanosome lytic factor (later designated TLF-1) [18]. Further analysis revealed the characterization of both TLF-1 and a second particle, TLF- 2 [19]. TLF-1 is a 500 kDa HDL consisting of Apolipoprotein A-I (APOA1), Apolipoprotein A-II (APOAII) and the toxins, haptoglobin-related protein (Hpr) [19] and Apolipoprotein L-1 (APOL1) [20]. TLF-2 is a 1 MDa lipid poor complex with the same proteins contained in TLF-1 but with the addition of IgM [19]. Consistent with all HDLs, synthesis of both TLF particles occurs within the liver. Hpr shares 91% sequence identity to haptoglobin (Hp) and has the ability to bind free hemoglobin (Hb) resulting from hemolysis [21]. Hp is an acute phase serum protein with a similar job as Hpr, binding free-Hb and sequestering the volatile pro-oxidative particle. In mammalian systems, the primary HpHb receptor is CD163 [22]. Expressed specifically in monocytes and macrophages, CD163 binds HpHb but rather interestingly cannot bind Hpr due to a mutation in the CD163 binding loop-region.

Aside from this, Hpr is still capable of binding free Hb with affinity similar to that of Hp [23]. To date, no receptor exists in mammalian cells that efficiently bind TLF-1 with any measureable affinity.

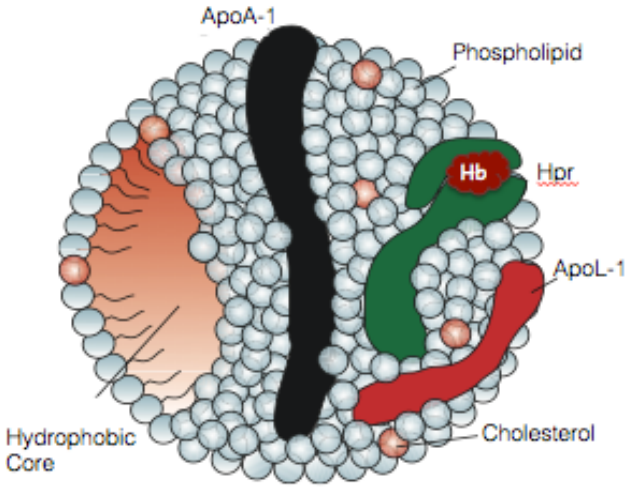

Figure 1.3. Trypanosome Lytic Factor-1. Consistent with all HDL particles is the hydrophobic core, cholesterol and apolipoprotein A1. Exclusive to high primates, TLF-1 contains haptoglobin related protein and apolipoprotein L1.

In order to elucidate the binding and trafficking patterns of TLF-1, gold particles were conjugated to TLF-1 for analysis by electron microscopy (EM). It was found that TLF-1 localized to the FP [24] and specifically bound a receptor with high affinity (described in the next section) [21]. Once endocytosed, clathrin-coated endosomal vesicles trafficked TLF-1 through to the lysosome where the toxins within the particle displayed their respective activities [25, 26]. The low pH of the lysosome has been shown as essential for TLF-1 mediated killing in *T. b. brucei* [27]. Low temperature uptake studies have shown that TLF-1 can bind and traffic to

endosomal vesicles while sparing the trypanosomes [28]. Initial studies on TLF-1 protein components identified Hpr as the primary toxin [29-32]. Data suggested that Hpr, complexed with Hb, in conjunction with low endosomal/lysosomal pH and endogenous peroxides, would initiate lipid peroxidation within the lysosomal membrane [29, 30]. It was hypothesized that this peroxidation of lysosomal lipids was responsible for trypanosome killing. In 2003, APOL1 was identified as another component within TLF-1. It became apparent that APOL1 was able to initiate cell lysis by creating a chloride channel within the lysosomal membrane thereby killing the trypanosome through uncontrolled lysosomal swelling [20, 33]. However, examination of each proteins individual contribution in the lytic mechanism, it was proposed that both Hpr and APOL1 work synergistically to kill *T. b. brucei* with remarkably high efficiency [25].

THE HAPTOGLOBIN HEMOGLOBIN RECEPTOR

The *T. b. brucei* haptoglobin hemoglobin receptor (*Tbb*HpHbR) is responsible for the high affinity binding of TLF-1 in the flagellar pocket of the parasite [34]. To date, this receptor is still only one of two fully identified and characterized receptors in *T. brucei,* with the transferrin receptor (TfR) being the other [35]. Like most cell surface proteins in African trypanosomes, the *Tbb*HpHbR is a GPI-anchored cell surface protein that specifically localizes exclusively to the trypanosome flagellar pocket [34]. At only 200-400 copies per cell, this low abundance receptor has a high affinity for both ligands, HpHb and HprHb (Kd~4 nM, [21, 34, 36, 37]). As the name suggests, it is capable of binding only Hp and Hpr when complexed with Hb. Without

Hb, no binding to the HpHb receptor is observed for either protein [37, 38]. Structurally, the 72 kDa *Tbb*HpHbR is a VSG-like molecule with an N-terminal head-domain that is able to protrude above the trypanosome VSG coat proteins where it can interact with Hp(r)-Hb molecules [34, 36]. This head-domain contains three residues that are important for ligand binding and overall stability of the domain. Mutations of each residue (S29A, K130A and D134A) either reduced affinity or abolished affinity for HpHb in surface plasmon resonance analysis [36].

The fact that the *Tb*HpHbR binds to the Hpr-Hb complex is surprising considering the mammalian HpHb receptor, CD163, cannot bind Hpr-Hb present in TLF-1 [23]. Humans are specifically and effectively protected against *T. b. brucei* infection through TLF-1 uptake via the *Tbb*HpHbR. The *Tbb*HpHbR is postulated to have evolved as a nutrient receptor, functioning for heme acquisition, because trypanosomes are heme auxotrophs [39]. Despite its putative role in nutrition uptake, the *Tb*HpHbR is not essential because RNAi knockdown or genetic knock out of the transcripts was non-lethal [34, 37, 40]. The *Tbb*HpHbR, however, is not the only mechanism for heme uptake in *T. brucei*. Recently, a heme transporter was identified in *T. brucei* that is likely one of the redundant mechanisms in place for heme acquisition [41].

The *Tbb*HpHbR is not the only TLF-1 binding receptor present in T. brucei as an HDL receptor has also been shown to bind TLF-1 through ^{125}I-TLF-1 studies, but efforts to purify and identify it were unsuccessful [21]. It was found to bind the HDLs with low affinity (Kd~1uM) but with a high copy number present in the flagellar pocket (~60,000 receptors). Since African trypanosomes are lipid

auxotrophs, investigations into the manner in which these parasites maintained their lipid composition revealed a scavenging lipid receptor, which was able to bind LDL and HDL including TLF-1 [42]. Since it was only characterized, not fully identified, the number of gene identified receptors present within *T. b. brucei* remains at two.

TRYPANOSOMA BRUCEI MECHANISMS OF RESISTANCE

In order for *T. brucei* to establish an infection in the blood stream of a mammalian host, it must be able to overcome attack from both the adaptive and innate immune response. *T. brucei* has evolved a rather elegant system to handle host antibody response via the expression of its primary surface coat protein termed variant surface glycoprotein (VSG) [43-45]. These VSG's are GPI-anchored homodimers making up approximately 10% of the total *T. brucei* cellular proteins. With approximately 10^7 molecules expressed at a time, per cell, VSG's are densely packed over the entire surface of *T. brucei*. The expression of VSG takes place specifically in telomeric regions termed Bloodstream Expression Sites (BES), of which *T. brucei* contains at least 20. At any given time, only one species of the estimated 1000 VSG genes can be expressed [46, 47]. Other genes can reside in the region of these BES and are termed expression site associated genes (ESAG) [48]. VSG's are highly antigenic and elicit an antibody response from the adaptive immune system. Upon recognition of the VSG by host antigen, the majority of the trypanosome population is killed. However, a small number undergo random VSG switching events are able to evade antigen recognition and killing. This antigenic

variation allows the few survivors, as depicted in Figure 1.4, to then expand out for a period of time before the host antibody response has time to recognize the newly expressed VSG [44]. The mechanisms with which switching occurs include transcriptional switching (inactivating one BES to activate another) and gene conversion (switching VSG gene within an active BES) [49-51].

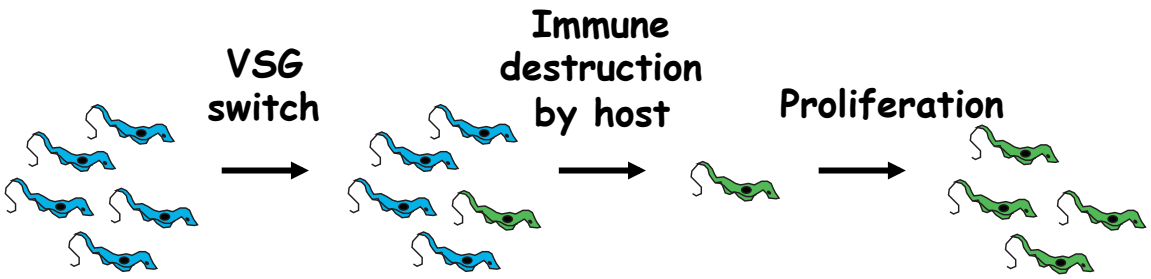

Figure 1.4. Evasion of Adaptive Immune System. Upon recognition of VSG by host antigen, most trypanosomes are killed (blue). Small numbers with switched VSGs are able, then, to proliferate within the bloodstream unaffected by the antigen (green).

Antigenic variation is the primary mechanism in which *T. brucei* overcomes the adaptive immune response. Components of the innate system present another threat, which include the two TLF molecules. In order to successfully establish infection, *T. b. rhodesiense* and *T. b. gambiense*, have evolved resistance mechanisms to TLF-1. In the case of *T. b rhodesiense*, the receptor responsible for binding TLF-1, HpHbR, is identical in sequence to *T. b. brucei* [52]. Therefore, TLF-1 uptake in these cells is identical to that of *T. b. brucei*. Despite this, *T. b. rhodesiense* clones, when incubated with increasing concentrations of NHS, can lose the classic serum

resistance phenotype associated with this pathogen [53, 54]. In cDNA examinations of *T. b. rhodesiense* serum resistant clones, a single transcript was identified and termed serum resistance-associated (SRA), which encoded a VSG-like protein [55]. It was later discovered that *SRA* was an ESAG and was able to confer resistance to otherwise sensitive *T. b. brucei* [54, 56, 57]. Using SRA for NHS affinity chromatography it was found that APOL1 present within TLF-1 interacted with SRA. Mutational analysis suggested that this VSG-like protein contained a α-helical region that was important for NHS resistance [20]. In this finding, the α-helical region of SRA was interacting with a non-essential C-terminal end of APOL1 and mutations performed, in either the α-helix of SRA or the C-terminus of APOL1, reduced binding between the two proteins, elucidating the binding domain interactions [20]. It was therefore determined that this interaction, SRA sequestering APOL1, spared the cells from lysis by TLF-1 neutralization. Additionally, the GPI-anchor of SRA was found to be non-essential in its ability to confer TLF resistance to parasites, and fluorescence microscopy data indicated that SRA trafficked through endosomal vesicles terminating in the lysosome with TLF-1 [20, 28].

Taken together, with the use of antigenic variation for antibody response evasion, *T. b. rhodesiense* uses a direct mechanism to prevent killing by the TLF-1 particle. It is easy to think that SRA would interact with APOL1 in TLF-2 the same way it has been shown to in TLF-1. While not directly tested, SRA is likely functional against TLF-2 considering *T. b. rhodesiense* thrive in NHS and, when SRA is deleted, *T. b. rhodesiense* is rendered NHS Sensitive.

Table 1.1. *Trypanosoma brucei gambiense,* group 1 and group 2

Trypanosoma brucei gambiense			
Group 1 ELAINE	Group 2 STIB386S	Group 2 STIB386R	
-	+	+	*Tb*HpHbR*
+	-	-	*Tbg*HpHbR^{S210L}
+	-	-	*TgsGP* gene
-	+	-	rAPOL1 sensitive
-	+	+	TLF-1 uptake
+	+	+	rAPOL1 uptake

* Group 1 ELAINE mRNA levels suggest that little to no protein would be made. Data generated from [58].

As the causative agent for approximately 97% of reported cases of African sleeping sickness [59], *T. b. gambiense* has proven itself to be highly evolved in evading the human immune system. *T. b. gambiense* has developed several unique mechanisms to deal with exposure to TLF-1 (and 2). Unlike *T. b. rhodesiense*, no strains of *T. b. gambiense* contain the gene for SRA, the protein responsible for TLF-1 resistance in *T. b. rhodesiense* [60]. Within *T. b. gambiense*, two separate and distinct subtypes exist; group 1 and group 2 [58]. Further, group 2 isolates can be divided into sensitive (S) and resistant (R) variants [58]. These groups are defined by a multitude of characteristics unique in both in genotypic and phenotypic traits, some of which are listed in Table 1.

Group 1 *T. b. gambiense* lacks the ability to endocytose TLF-1 and was found to down-regulate the receptor at the mRNA level [40]. Upon sequencing it was revealed that the HpHb receptor contained multiple polymorphisms when compared to *T. b. brucei* [40, 52]. When ectopically expressed in *T. b. brucei* HpHb receptor-null cells, it was revealed that, even with over-expression, the *Tbg*HpHbR was completely unable to bind both TLF-1 and HpHb [37]. In examining the polymorphisms within the *Tbg*HpHbR, it became clear that a serine to leucine substitution at position 210 was responsible for the loss of TLF-1 binding and uptake [37]. This was also confirmed by *in vitro* analysis. Modeling puts this S210L substitution in a position that alters the putative binding pocket for both HpHb and HprHb [36, 61]. When looked at by immunoblotting, the ectopically expressed *Tbg*HpHbR was detected, suggesting either proper trafficking to the flagellar pocket or recycling in endocytic vesicles [37]. To date, studies to decipher the localization of the *Tbg*HpHbR have left localization of the protein unresolved. The finding of no TLF-1 binding strongly suggests that, despite the low mRNA expression levels, the *Tbg*HpHbR itself can be considered a truly dead/non-functional receptor.

In the case of group 2 *T. b. gambiense*, continuous culturing of these cells in the presence of human serum produced two isogenic variants, group 2 *T. b. gambiense* STIB386S (serum Sensitive) and group 2 *T. b. gambiense* STIB386R (serum Resistant). Sequencing revealed that the HpHb receptor (*Tbg*HpHbR) is *T. b. brucei*-like and functionally binds TLF-1 just as *T. b. brucei.* TLF-1 uptake studies did reveal, however, that group 2 *T. b. gambiense* STIB386R endocytosed TLF-1 at a slower rate than group 2 *T. b. gambiense* STIB386S and *T. b. brucei.* To decipher if

this was the only reason for the differences in NHS resistance in group 2 *T. b. gambiense,* analysis with APOL1 was carried out, since, at the time, purified TLF-2 was not available. Recombinant APOL1 (rAPOL1) was found to be endocytosed by all variants of *T. b. gambiense,* a result of general fluid phase endocytosis. Interestingly, despite endocytosis in both subgroups, only group 2 *T. b. gambiense* STIB386S was found to be sensitive to rAPOL1 [58]. It is still under investigation as to what mechanisms are in place in group 2 *T. b. gambiense* STIB386R that confer resistance to APOL1.

Recently, the role of a *T. b. gambiense* specific protein termed *Trypanosoma gambiense*-specific glycoprotein (TgsGP) in TLF-1 resistance was investigated. This protein was found to be instrumental in TLF-1 resistance in-group 1 *T. b. gambiense* [61, 62]. First identified in 2001 while searching for truncated VSG-like molecules similar to SRA, TgsGP was found to lack a VSG C-terminal domain while still containing the putative sequence encoding a glycosylphosphatidylinositol (GPI) anchor [1]. With approximately 2.2 X 10^5 copies per cell, deletion of the gene conferred full sensitivity to serum and TLF-1 in group 1 *T. b. gambiense*. It is worth noting that TgsGP, while *T. b. gambiense* specific, is only present in group 1 variants, not group 2 (both STIB386S and STIB386R) [62]. While the precise mechanism in which *TgsGP* inactivates TLF-1 has not been fully defined, studies have shown that mutations in the amino-terminal region in which two helical patterns exist is sufficient to abolish proper localization and function of the TgsGP [61]. While TgsGP does not bind APOL1, it does colocalize with both APOL1 and TLF-1 in the flagellar pocket, endosomal vesicles and in the lysosome. Nuclear magnetic resonance

(NMR) analysis of recombinant TgsGP on liposomes derived from trypanosome lipids revealed that TgsGP was able to increase membrane rigidity thereby suggesting a model which trypanosome lysosomal membranes are refractory to interaction with TLF-1 [61]. Interestingly, while deletion and adding-back of the gene in group 1 *T. b. gambiense* indicated that this gene is necessary for human serum resistance, addition of this gene to *T. b. brucei* conferred no resistance to human serum or TLF-1 [1, 61, 62]. Therefore the TgsGP gene and the mutated, down-regulated HpHb receptor are not the sole defense mechanism in group 1 *T. b. gambiense*.

The discovery, in 2001, of the chagasin family of inhibitors of cysteine peptidases (ICP) was a result of a search for members of cystatin families, common cysteine peptidase (CP) inhibitors in plants and mammals [63, 64]. It was found that deletion of the *ICP* gene in *T. b. brucei* led to altered surface coat expression, differences in parasitemia and overall cellular differentiation [63]. In looking at the changes to human serum sensitivity in TgsGP knockouts, Uzureau et al. looked at what impact ICP was having within *T. b. gambiense*. Deletion of *ICP* in *T. b. brucei* led to an increase in serum resistance.

Using an *in vitro* liposome system, it was shown that TLF-1 interacts with liposomal membranes in a pH-dependent manner. With a decrease in pH, TLF-1 and liposomal membrane interactions increased as evident by an increase in captured dye release [27]. The pH of endocytic vesicles for *T. b. brucei* and *T. b. gambiense* were found to be 5.34 $\pm$0.09 and 4.85 $\pm$0.05, respectively [61]. It was also shown that the primary *T. b. brucei* CP (tbcatB) was most active at pH 5, with moderate

activity at pH units ±0.5 [65]. Taken together, this data suggests that TLF-1 interacts well with both endocytic and lysosomal membranes in both subspecies of *T. brucei* but that tbcatB can act to inhibit the effects of APOL1 by degradation of the protein. This pH and *ICP* expression data, along with the work presented in this dissertation including the effects of TgsGP expression, work together to build a system of complex, multifaceted defenses used by *T. b. gambiense* against the human innate immune system.

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Chapter 2

A SINGLE AMINO ACID SUBSTITUTION IN THE GROUP 1 *TRYPANOSOMA BRUCEI GAMBIENSE* HAPTOGLOBIN-HEMOGLOBIN RECEPTOR ABOLISHES TLF-1 BINDING

DeJesus E, Kieft R, Albright B, Stephens NA, Hajduk SL. *A single amino acid substitution in the group 1 Trypanosoma brucei gambiense haptoglobin-hemoglobin receptor abolishes TLF-1 binding.* PLoS Pathog, 2013. **9**(4): p. e1003317.

Abstract

Critical to human innate immunity against African trypanosomes is a minor subclass of human high-density lipoproteins, termed Trypanosome 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* HpHb receptor (*Tbg*HpHbR) 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 *Tbg*HpHbR ablates high affinity TLF-1 binding and subsequent endocytosis, thus evading TLF-1 killing. In addition, we show that over-expression of *Tbg*HpHbR failed to rescue TLF-1 susceptibility. These findings suggest that the single substitution present in the *Tbg*HpHbR 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, Trypanosome 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 (*Tbb*HpHbR) [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, *Tbb*HpHbR has been proposed to function as a nutrient receptor providing heme to these parasites [14]. Unlike the mammalian HpHb scavenger receptor (CD163) the *Tbb*HpHbR 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

glycophosphatidylinositol-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 localizes 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 APOL1 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* HpHb receptor (*Tbg*HpHbR) was reduced 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 *Tbg*HpHbR 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 of isolates further revealed a single leucine (L) to serine (S) substitution, at amino acid 210 of *Tbb*HpHbR which was conserved in all group 1 *T. b. gambiense* isolates examined [24]. This led to the suggestion that this substitution might reduce the affinity of *Tbg*HpHbR for TLF-1 [23-25]**.** Recently, the crystal structure of the HpHb receptor was deduced allowing new structure-function analysis. A domain of HpHb receptor, protruding beyond the VSG coat, possesses a hydrophobic core in which ligand binding is predicted to occur. The L210S substitution present in *T. b. gambiense* was predicted to disrupt the core of the head structure of HpHb receptor thus eliminating ligand binding

[26]. Other mechanisms of resistance to TLF-1 and human serum must exist since group 2 *T. b. gambiense* lacks SRA yet expresses the functional HpHbR and takes up TLF-1 [24]. The mechanism of group 2 *T. b. gambiense* resistance to TLF-1 remains unknown.

In the studies reported here, we developed a *T. b. brucei* double knockout line (*Tbb*HpHbR$^{-/-}$) to directly test the function of each of the four non-synonymous amino acid substitutions in the *Tbg*HpHbR. Expression of *Tbg*HpHbR in the *Tbb*HpHbR$^{-/-}$ knockouts did not restore TLF-1 binding or killing. However, the substitution of serine for leucine, at position 210, restored high affinity TLF-1 binding and susceptibility. Changes to the other three substituted amino acids in the *Tbg*HpHbR had no effect on TLF-1 binding, uptake or killing. These results, together with our previous finding, indicate that TLF-1 resistance has exerted strong selective pressure on group 1 *T. b. gambiense,* resulting both in decreased expression levels and loss of function substitutions in the *Tbg*HpHbR.

Methods

Trypanosomes, growth and transfections

Bloodstream form *T. b. brucei* Lister 427 (MiTat 1.2) were grown at 37°C under 5% CO_2 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 S2.1. $3x10^7$/ml trypanosomes were transfected with 5 μg of NotI digested

DNA using the Amaxa nucleofection system according to the manufacturer's instructions. (Human T Cell Nucleofactor 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 *Tbb*HpHbR$^{+/-}$ lines. To examine the effects of amino acid substitution on HpHbR function, stable cell lines expressing ectopic copies of the *Tbb*HpHbR, *Tbg*HpHbR or the individual *Tbg*HpHbR 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 $1x10^4$/ml. Cell counts, determined by hemocytometer, were carried out until stationary phase. Growth curve data is in triplicate.

Epitope tagging of HpHbR

A HA-epitope tag was cloned into the *Tbg*HpHbR 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 ApaI (5 μg total) prior to transfection. Transfections and cloning were carried out as described above.

*Tbg*HpHbR mutant cell lines

The construct used to generate the *Tbg*HpHbR cell line [23] was subjected to site-directed mutagenesis to generate the four *Tbg*HpHbR substitutions of S210L, V293A and GA369-370EG. Mutagenesis was carried out according to manufactures instructions (Agilent Technologies). *Tbb*HpHbR$^{-/-}$ cells were transfected independently with the mutagenized constructs. Transfections and cloning were carried out as described above. Mutant *Tbg*HpHbR 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 *Tbb*HpHbR$^{-/-}$ 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 $1x10^6$/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 transferred to a nitrocellulose membrane (Amersham Hybond-N^+). Pre-hybridization was with a solution containing 40% formamide (Sigma), 3x SSC, 10x Denhardt's, 20 µg/ml salmon sperm DNA, 5% dextran sulfate and 2% SDS at 42°C for three hours. The P^{32} labeled probe, specific for the region upstream of the 5'-flanking regions was added to the hybridization mix and incubated at 42°C overnight. The probe sequence is listed in Table S1. Blots were then washed in a solution containing 3x SSC/0.1% SDS at 55°C for 30 minutes then a final stringency of 0.3x 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 by either fluorescence microscopy or flow cytometry. 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 flow cytometry, cells were grown to mid-log phase, collected, washed and resuspended ($1x10^7$/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 NaP_i, 137 mM NaCl, pH 7.4). The amount of TLF-1 uptake was determined using Cyan 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, pre-chilled Alexa-488 TLF-1/Hb complexes were added to cells in ice-cold HMI-9 supplemented with 1% BSA, 1% glucose and further incubated at 3°C for two hours. Cells were washed two times with ice-cold PBS, kept on ice and subjected to flow cytometry. All binding and uptake experiments analyzed by flow cytometry were done in triplicate. For fluorescence microscopy, approximately 100 cells per data point were analyzed in triplicate. All binding data collected were analyzed using Graphpad Prism software, version 4.0a. To better localize the distribution of TLF-1 binding to the flagellar pocket, PFA fixed and methanol treated trypanosomes were incubated with a mouse anti-paraflagellar rod (PFR) antibody (generously provided by Dr. Diane McMahon-Pratt, New Haven) at a dilution of 1:1,000 followed by a secondary antibody staining

with Alexa Fluor 594 mouse IgG (Invitrogen). Serial image z-stacks were acquired through oil immersion optics with exposure times kept constant for each experiment. Imaging was carried out using a Zeiss Axio Observer inverted microscope equipped with an AxioCam HSm camera and analyzed with the AxioVision v4.6 software (Zeiss). A single stack is shown for each experiment, with individual channels contrasted to the same extent for each image set.

Competition binding studies

Specificity of TLF-1 binding to trypanosomes was analyzed using competition-binding studies with the unlabeled TLF-1 and Hp 1-1. Cells were collected, washed and resuspended ($1x10^7$/ml) in ice-cold HMI-9 supplemented with 1% BSA, 1% glucose then transferred to 3°C for at least 10 minutes. Alexa-488 conjugated TLF-1 (3 nM constant) was complexed with hemoglobin (50 nM) at 4°C for 10 minutes. Increasing concentrations of unlabeled competitor were incubated with Hb (50 nM) for 10 minutes at 4°C. Competing ligands were then mixed with the Alexa-488 conjugated TLF-1/Hb, added to cells at 3°C and allowed to incubate for two hours. Cells were then transferred to ice, washed with ice-cold 1X PBS and analyzed by Cyan 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 HpHb Receptor 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 20ng of total RNA, 6 pmol sense primer, 6 pmol antisense primer, 10 µl SYBR green PCR master mix (Fermentas) in a final volume of 20µl. Real time PCR conditions were: one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. 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 *Tbb*HpHbR$^{-/-}$, Rab5a^{HA} and *Tbg*HpHbR^{HA} cell lines and analyzed, based on cell equivalents, as previously described [22]. Rat monoclonal anti-HA–biotin (Roche Diagnostics, Indianapolis, IN) was used at a dilution of 1:1000, 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 [23, 29]. The resistance phenotype correlated with reduced expression of the *Tbb*HpHbR, susceptibility being restored by ectopic expression of the *Tbb*HpHbR from a different chromosomal locus. We also observed that ectopic expression of the *Tbg*HpHbR failed to restore TLF-1 uptake or susceptibility, suggesting that substitutions to the *Tbg*HpHbR 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 *Tbb*HpHbR [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 *Tbg*HpHbR.

The HpHb receptor knockout cell lines were prepared in *T. b. brucei* 427-221 (Lister 427) cells by sequentially replacing the complete coding sequence for each *Tbb*HpHbR allele with the coding sequences for hygromycin and/or blasticidin (Figure 2.1A). Replacement of the coding sequence for *Tbb*HpHbR, in both single *Tbb*HpHbR$^{+/-}$ (sKO) and double *Tbb*HpHbR$^{-/-}$ (KO) knockouts, was verified by Southern blot hybridization of genomic DNA digested with EcoRI. The expected size restriction fragments were 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 (3.9kb and 4.2kb respectively) (Figure 2.1A). 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 2.1B, Table S2.1). The generation of a stable *Tbb*HpHbR$^{-/-}$ cell line showed that this gene was non-essential in the bloodstream stage of *T. b. brucei* used in these studies. In addition, only a very slight reduction in growth rate was observed (Figure 2.1C).

*Tbb*HpHbR is necessary for TLF-1 binding, uptake and killing

We examined whether the *Tbb*HpHbR$^{-/-}$ cells were deficient in TLF-1 uptake. When WT *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 *Tbb*HpHbR$^{-/-}$ cells (KO) (Figure 2.1D). 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 *Tbb*HpHbR$^{-/-}$ cells were incubated at 3°C with Alexa-488 TLF-1 (Figure 2.1E). 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 *Tbb*HpHbR$^{-/-}$ cells was observed at concentrations up to 66 nM (Figure 2.1E). In addition, *Tbb*HpHbR$^{-/-}$ cells were highly refractory to TLF-1 killing at concentrations of 0.1 nM (Figure 2.1F). These studies showed that the *Tbb*HpHbR was required for high affinity TLF-1 binding and further supports the role of this receptor in trypanosome killing.

Functional analysis of the *Tbg*HpHbR

In order to determine whether the *Tbg*HpHbR was functional in TLF-1 binding and subsequent killing, stable cell lines, ectopically expressing the *Tbb*HpHbR and *Tbg*HpHbR genes, were prepared in the *Tbb*HpHbR$^{-/-}$ background. In addition, to verify HpHbR expression, a HA-epitope tagged variant of the *Tbg*HpHbR (*Tbg*HpHbR^{HA}), in the TbbHpHbR$^{-/-}$ background, was also prepared (Figure 2.2A). Expression of *Tbb*HpHbR, *Tbg*HpHbR and *Tbg*HpHbR^{HA} was determined by nested RT-PCR allowing detection of both endogenous and HA-tagged HpHbR mRNAs (Figure 2.2A). The level of HpHbR mRNA was comparable in all cell lines (Table S2.1). The expression of *Tbg*HpHbR^{HA} was also evaluated by western blot with antibodies specific to the HA-tagged HpHbR (Figure 2.2B). A single band, migrating around 80 kDa, was visible in *Tbg*HpHbR^{HA} cells, but not in the *Tbb*HpHbR$^{-/-}$ cell line (Figure 2.2B). Specificity of the anti-HA antibody was verified with a cell line expressing a HA-tagged Rab5A (Figure 2.2B) [22]. To determine whether the *Tbg*HpHbR^{HA} was functional in TLF-1 binding and uptake, cells were examined for TLF-1 binding and subsequent killing (Figures 2.2C and 2.2D). *Tbb*HpHbR, but not *Tbg*HpHbR or *Tbg*HpHbR^{HA}, restored both TLF-1 binding

and killing in the *Tbb*HpHbR$^{-/-}$ background (Figures 2.2C and 2.2D). We also prepared a HA-epitope tagged variant of the *Tbb*HpHbR (*Tbb*HpHbR^{HA}), in the *Tbb*HpHbR$^{-/-}$ 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 *Tbb*HpHbR^{HA} 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 *Tbb*HpHbR retains the ability to bind TLF-1 while *Tbg*HpHbR and *Tbg*HpHbR^{HA}, while expressed at comparable levels, do not function in TLF-1 binding, uptake or trypanosome killing.

Functional analysis of sequence polymorphisms in the *Tbg*HpHbR

Sequence analysis of a small number of isolates of group 1 and 2 *T. b. gambiense*, *T. b. rhodesiense* and *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 *Tbg*HpHbR [23] (Figure 2.3A). 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 *T. b. gambiense* and was not observed in TLF-1 susceptible *T. b. brucei* or in *T. b. rhodesiense* [24]. To test whether any of the substitutions in the *Tbg*HpHbR could individually restore TLF-1 binding and killing, each of the *T. b. gambiense* specific amino acid substitutions were changed back to the amino acid found in the *Tbb*HpHbR (Figure 2.3A). The steady state levels of *Tbb*HpHbR, *Tbg*HpHbR,

*Tbg*HpHbR^{S210L}, *Tbg*HpHbR^{V293A} and *Tbg*HpHbR$^{GA369\text{-}370EG}$ mRNA s were evaluated by (q)RT-PCR (Figure 2.3B, Table S2.2). 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 *Tbg*HpHbR 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 2.3C and 2.3D). Fluorescence microscopy showed that *Tbb*HpHbR 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 2.3C). Flow cytometry indicated that the amount of TLF-1 taken up by the *Tbb*HpHbR cells was similar to that seen in WT *T. b. brucei* (Figure 2.3D). In contrast, *Tbg*HpHbR cells showed no detectable uptake of TLF-1 either by fluorescence microscopy or flow cytometry analysis (Figure 2.3C and 2.3D). Similarly, *Tbg*HpHbR^{V293A} and *Tbg*HpHbR$^{GA369\text{-}370EG}$ did not take-up TLF-1 and appeared identical to *Tbg*HpHbR cells. However, the single amino acid change at position 210 of the *Tbg*HpHbR, from serine to leucine, restored TLF-1 uptake and localization to levels seen in the *Tbb*HpHbR cells (Figure 2.3C and 2.3D). The specificity of TLF-1 binding in *Tbb*HpHbR and *Tbg*HpHbR^{S210L} was examined by competition binding studies with unlabeled TLF-1 or Hp1-1 in the presence or absence of Hb (Figure 2.3E and 2.3F, respectively). When complexed with Hb both unlabeled Hp 1-1 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 *Tbb*HpHbR, *Tbg*HpHbR, *Tbg*HpHbR^{S210L}, *Tbg*HpHbR^{V293A} and *Tbg*HpHbR$^{GA369\text{-}370EG}$ 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 *Tbb*HpHbR and *Tbg*HpHbR^{S210L} were fully susceptible to TLF-1. *Tbg*HpHbR, *Tbg*HpHbR^{V293A} and *Tbg*HpHbR $^{GA369\text{-}370EG}$ were resistant to TLF-1 killing. Together these studies show that the single amino acid change of serine to leucine at position 210 of *Tbg*HpHbR is sufficient to restore both TLF-1 uptake and killing to levels seen in cells expressing the *Tbb*HpHbR. This finding is consistent with the substitution to the HpHbR in *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 *Tbg*HpHbR 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 1X PBS and the amount and location of TLF-1 binding evaluated by flow cytometry and fluorescence microscopy (Figure 2.4, Figure S2.3). Alexa-488 TLF-1 localized exclusively to the flagellar pocket (Figure 4D) and cell associated fluorescence was concentration dependent in WT *T. b. brucei*, *Tbb*HpHbR and *Tbg*HpHbR^{S210L} cell lines (Figure 2.4A-C). No detectable TLF-1 binding was seen in *Tbb*HpHbR$^{-/-}$, *Tbg*HpHbR,

*Tbg*HpHbR^{V293A}, and *Tbg*HpHbR $^{GA369-370EG}$ cell lines (Figure 2.4C and D). To determine whether the affinity for TLF-1 differed in the TbbHpHbR$^{-/-}$ cell lines expressing *Tbb*HpHbR, *Tbg*HpHbR, *Tbg*HpHbR^{S210L}, *Tbg*HpHbR ^{V293A} and *Tbg*HpHbR $^{GA369-370EG}$ we performed saturation binding studies with Alexa-488 TLF-1 (Figure 2.4A-C). The binding affinity was estimated based on half-maximal binding. Both WT *T. b. brucei* and *Tbb*HpHbR had high affinity for TLF-1 (3.96 $\pm$ 0.31 nM and 4.12 $\pm$ 0.25 nM, respectively). The *Tbg*HpHbR^{S210L} cells also bound TLF-1 with similar affinity (3.96 $\pm$ 0.35 nM). Consistent with the results obtained by microscopic analysis, TLF-1 binding was not observed in the *Tbg*HpHbR^{V293A} and *Tbg*HpHbR $^{GA369-370EG}$ cell lines. Based on these results, the highly conserved amino acid substitution in the *Tbg*HpHbR 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 *Tbg*HpHbR was detected (Figure 2.4B). To determine whether this represented binding to the *Tbg*HpHbR or a low level of background binding we over-expressed the *Tbg*HpHbR and the *Tbb*HpHbR through ectopically expressing the HpHbR, driven by a ribosomal promoter, in the *Tbb*HpHbR$^{-/-}$ cells. This resulted in a 15-fold increase in expression of TbgHpHbR and the *Tbb*HpHbR mRNAs as measured by qRT-PCR (Table 2.1). 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 2.1). 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 *Tbg*HpHbR 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 TbgHpHbR, on TLF-1 binding, uptake and trypanolytic activity we established a *Tbb*HpHbR$^{-/-}$ cell line by replacement of both alleles with drug resistance markers (Figure 2.1A). Using this stable cell line, we tested each amino acid substitution in the *Tbg*HpHbR individually by ectopic expression (Figures 2.3B). 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 2.3, 2.4). 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 HpHb receptor 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* HpHb receptor 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 *Tbg*HpHbR was accessible for antibody detection (Figure 2.2B). In contrast, the HA-epitope, in the stabilized head domain of the *Tbb*HpHbR, 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 HpHb receptor, which showed that the leucine to serine substitution significantly reduced TLF-1 and HpHb binding to the HpHb receptor [26].

A potentially important difference in TLF-1 binding was revealed in the *in vitro* binding studies with recombinant HpHb receptor [26] and the *in vivo* studies reported here (Figure 2.3). The SPR binding results showed a striking difference in the affinity for TLF-1 and HpHb for the *Tbb*HpHbR (5-10mM and 4.5nM

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 *Tbb*HpHbR (Figure 2.3). 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 binding affinity of the heterogeneous TLF-1 particles [30].

Other mechanisms of resistance contribute to human infectivity by group 1 *T. b. gambiense* since TLF-2 kills HpHbR deficient *T. b. brucei* lines, albeit at concentrations approximately 200-fold higher than needed to kill WT *T. b. brucei* [4]. It is possible that group 1 and group 2 *T. b. gambiense* share common, HpHb receptor independent, mechanisms of resistance. Unlike group 1 *T. b. gambiense*, the sequence of the HpHbR gene in subgroup 2 more closely resembles that of *T. b. brucei*. Critically, the group 2 *T. b. gambiense* HpHb receptor has a leucine at position 210 [24, 25]. TLF-1 binding, uptake and trafficking to the lysosome of group 2 *T. b. gambiense* also more closely resemble the *Tbb*HpHbR [24].

The HpHb receptor 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 2.1). 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 HpHb receptor 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 seemly 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 HpHb receptor independent mechanism for inhibition of TLF-1 killing [24]. Since group 2 *T. b. gambiense* express a functional HpHb receptor, 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.

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Author Contributions

EDJ conceived, designed and performed experiments and co-wrote the manuscript. RK conceived, designed and performed experiments and co-wrote the manuscript. BA performed binding and competition studies and edited the manuscript. NAS performed immunoblotting studies. SLH conceived, and designed experiments and co-wrote the manuscript.

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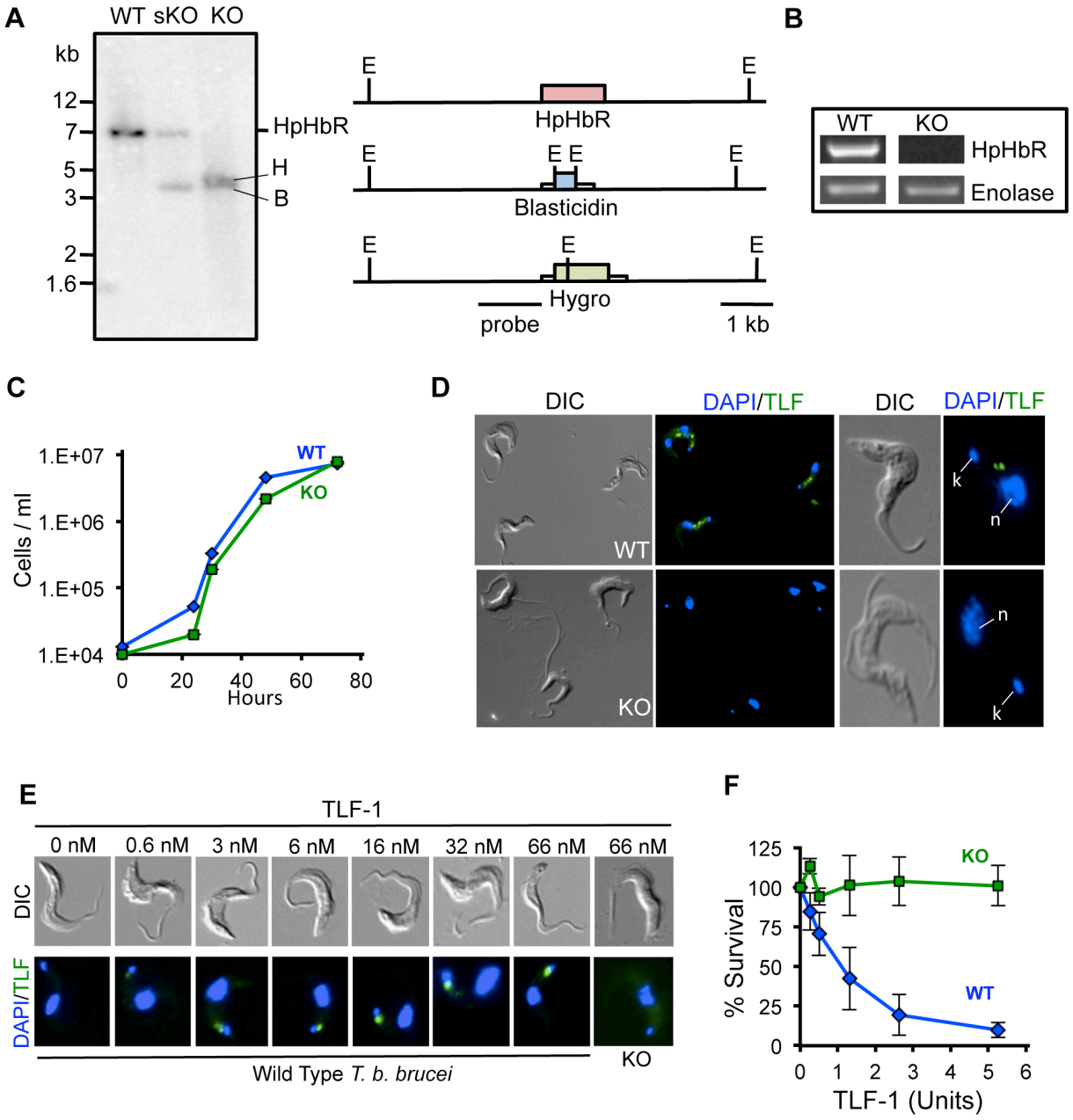
A
WT sKO KO
kb
12
7
5
3
2
1.6
HpHbR
H
B
E
E
HpHbR
E
E E
E
Blasticidin
E
E
E
Hygro
probe
1 kb
B
WT
KO
HpHbR
Enolase
C
1.E+07
1.E+06
1.E+05
1.E+04
Cells / ml
0
20
40
60
80
Hours
WT
KO
D
DIC
DAPI/TLF
DIC
DAPI/TLF
WT
KO
k
n
n
k
E
TLF-1
0 nM
0.6 nM
3 nM
6 nM
16 nM
32 nM
66 nM
66 nM
DIC
DAPI/TLF
KO
Wild Type T. b. brucei
F
125
100
75
50
25
0
% Survival
KO
WT
0
1
2
3
4
5
6
TLF-1 (Units)

Figure 2.1. 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 HpHbR$^{+/-}$ 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) 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.

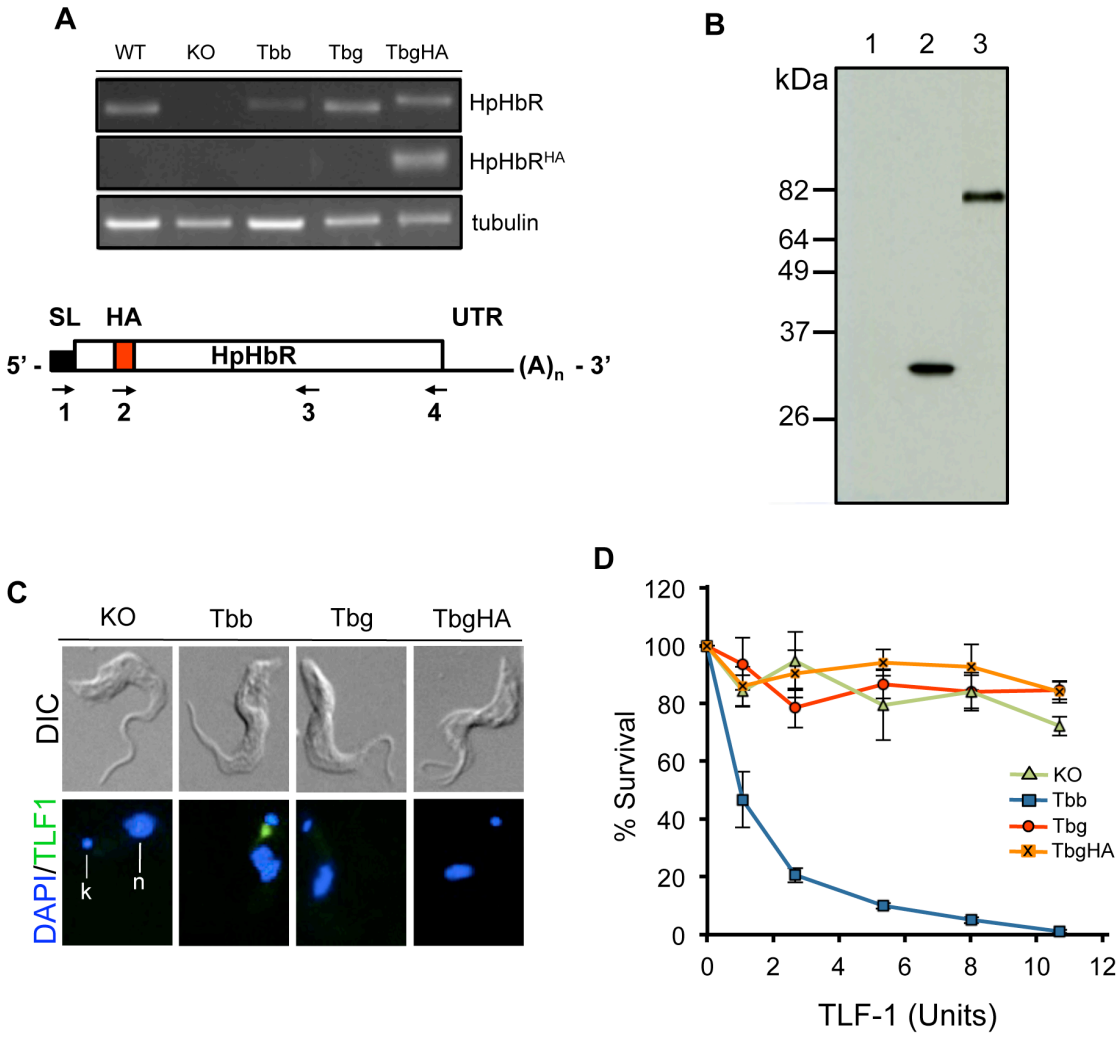
A
WT
KO
Tbb
Tbg
TbgHA
HpHbR
HpHbR^HA
tubulin
SL
HA
UTR
5' -
HpHbR
(A)n - 3'
1
2
3
4
B
1
2
3
kDa
82
64
49
37
26
C
KO
Tbb
Tbg
TbgHA
DIC
DAPI/TLF1
k
n
D
% Survival
TLF-1 (Units)
KO
Tbb
Tbg
TbgHA

Figure 2.2. Generation and functional analysis of the HpHbR in *Tbb*HpHbR$^{-/-}$ cells. **(A)** Expression levels of HpHbR mRNA were determined by RT-PCR from wild type *T. b. brucei* (WT), *Tbb*HpHbR$^{-/-}$ (KO) cells ectopically expressing the *Tbb*HpHbR (Tbb), *Tbg*HpHbR (Tbg) and *Tbg*HpHbR^{HA} (TbgHA) genes. b-tubulin used as the loading control. Schematic of HA-epitope tagged HpHbR mRNA showing the primer binding sites for full length HpHbR mRNA (primers 1 and 4) and HA-tagged HpHbR mRNAs (primers 2 and 3) **(B)** Western analysis of KO, Rab5a^{HA} and TbgHA showing expression of the *Tbg*HpHbR. 1: *Tbb*HpHbR$^{-/-}$, 2: Rab5a^{HA}, 3: *Tbg*HpHbR^{HA} **(C)** Fluorescence microscopy of Alexa-488 TLF-1 binding to *Tbb*HpHbR$^{-/-}$ (KO), *Tbb*HpHbR (Tbb), *Tbg*HpHbR (Tbg) and *Tbg*HpHbR^{HA} (TbgHA). Kinetoplast (k), Nucleus (n). **(D)** Susceptibility of Tbb, Tbg, TbgHA and KO cells to TLF-1 killing determined by a 16 hour incubation at 37°C. In these studies a TLF-1 killing unit = 0.86 nM.

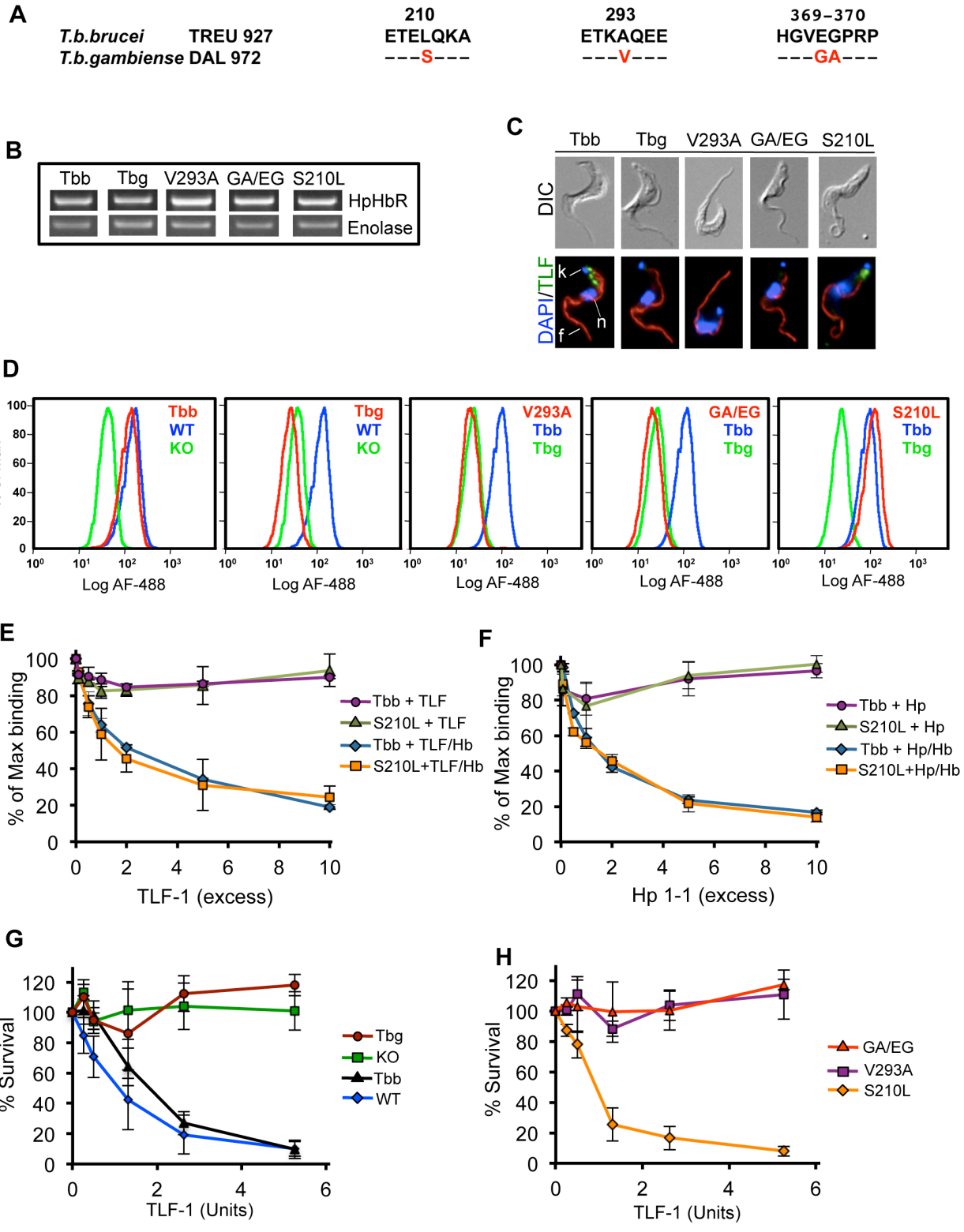
A
T.b.brucei TREU 927
T.b.gambiense DAL 972
210
ETELQKA
---S---
293
ETKAQEE
---V---
369–370
HGVEGPRP
---GA---
B
Tbb
Tbg
V293A
GA/EG
S210L
HpHbR
Enolase
C
Tbb
Tbg
V293A
GA/EG
S210L
DIC
DAPI/TLF
k
n
f
D
% of Max
Log AF-488
Tbb
WT
KO
Tbg
WT
KO
V293A
Tbb
Tbg
GA/EG
Tbb
Tbg
S210L
Tbb
Tbg
E
% of Max binding
TLF-1 (excess)
Tbb + TLF
S210L + TLF
Tbb + TLF/Hb
S210L+TLF/Hb
F
% of Max binding
Hp 1-1 (excess)
Tbb + Hp
S210L + Hp
Tbb + Hp/Hb
S210L+Hp/Hb
G
% Survival
TLF-1 (Units)
Tbg
KO
Tbb
WT
H
% Survival
TLF-1 (Units)
GA/EG
V293A
S210L

Figure 2.3: Effects of amino acids substitutions in *Tbg*HpHbR on TLF-1 uptake and killing. **(A)** Sequence alignment of *T. b. brucei* and *T. b. gambiense* HpHbR. Four amino acids in the *Tbg*HpHbR, positions 210, 293 and 369-370, within the mature coding sequence. **(B)** Expression levels of HpHbR mRNA were determined by RT-PCR from *Tbb*HpHbR$^{-/-}$ cells ectopically expressing the *Tbb*HpHbR (Tbb), *Tbg*HpHbR (Tbg), *Tbg*HpHbR^{V293A} (V293A), *Tbg*HpHbR$^{GA369/370EG}$ (GA/EG) and *Tbg*HpHbR^{S210L} (S210L) genes. **(C)** Fluorescence microscopy of the *Tbb*HpHbR$^{-/-}$ 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)** Flow cytometric analysis of Alexa-488 TLF-1 uptake by wild type *T. b. brucei* (WT), *Tbb*HpHbR$^{-/-}$ (KO), and the *Tbb*HpHbR$^{-/-}$ cell lines ectopically expressing the Tbb, Tbg, V293A, GA/EG and S210L genes. **(E)** Competition for Alexa-488 TLF-1 binding with unlabeled TLF-1 to *Tbb*HpHbR$^{-/-}$ cells ectopically expressing the Tbb and S210L (plus and minus added Hb). **(F)** Competition for Alexa-488 TLF-1 binding with unlabeled Hp 1-1 to *Tbb*HpHbR$^{-/-}$ cells ectopically expressing the Tbb and S210L gene (plus and minus added Hb). **(G)** Susceptibility to TLF-1 killing was determined based on *in vitro* TLF-1 survival assay (16 hour) for WT and *Tbb*HpHbR$^{-/-}$ cells ectopically the Tbb and Tbg genes. **(H)** Susceptibility to TLF-1 was determined for V293A, GA/EG and S210L by an *in vitro* TLF-1 survival assay (16 hour). In these studies a TLF-1 killing unit = 0.019 nM.

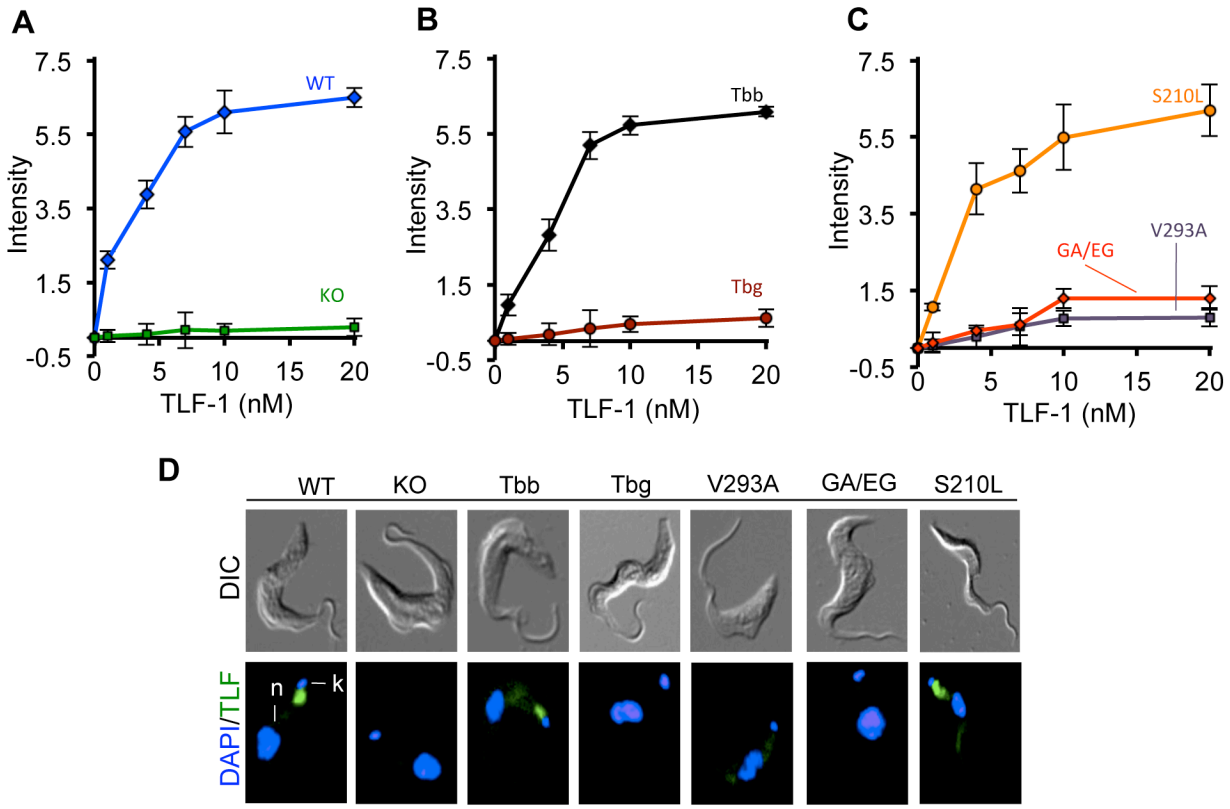

Figure 2.4. Saturation binding studies with TLF-1. **(A)** Binding affinities were estimated based on half-maximal binding at 3°C with varying concentrations of Alexa-488 TLF-1. Binding to *T. b. brucei* (WT) and *Tbb*HpHbR$^{-/-}$ (KO); **(B)** *Tbb*HpHbR$^{-/-}$ cells ectopically, *Tbb*HpHbR (Tbb) and *Tbg*HpHbR (Tbg); and **(C)** *Tbb*HpHbR$^{-/-}$ cells ectopically expressing *Tbg*HpHbR^{V293A} (V293A), *Tbg*HpHbR$^{GA369/370EG}$ (GA/EG) and *Tbg*HpHbR^{S210L} (S210L). Affinities for the Alexa-488 TLF-1 with WT were Kd= 3.96 $\pm$ 0.31 nM, Tbb Kd= 4.12 $\pm$ 0.25 nM, S210L Kd= 3.96 $\pm$ 0.35 nM. No binding was observed for Tbg, KO, V293A or GA/EG cell lines. **(D)** Fluorescence microscopy showed that Alexa-488 TLF-1 binding was seen in the flagellar pocket of WT, Tbb and S210L. Kinetoplast (k), Nucleus (n).

Table 2.1. Over expression of *Tbb*HpHbR and *Tbg*HpHbR

Cell Line	**HpHb mRNA [1]**	**TLF-1 binding [2]**	**TLF-1 killing [3]**
T. b. brucei	1.0	1.0	1.0
KO	N.D.	N.D.	N.D.
*Tbb*HpHbR	1.1 ± 0.2	1.0	1.0
*Tbb*HpHbR (over-expressed)	15.8 ± 2.0 [4]	17.0 ± 0.2 [5]	17.8 ± 2.2 [6]
*Tbg*HpHbR	0.9 ± 0.1	N.D.	N.D.
*Tbg*HpHbR (over-expressed)	11.2 ± 4.0 [4]	N.D. [5]	N.D. [6]

N.D. = Not Detectable
1) determined by qRT PCR (Fig. 1B)
2) determined by Flow cytometry (Fig. 2D)
3) determined by 16 hr. survival assays (Fig. 2G, 2H)
4) determined by qRT PCR (data not shown)
5) determined by Flow cytometry (data not shown)
6) determined by 2 hr. lysis assays (data not shown)

SUPPLEMENTAL FIGURES

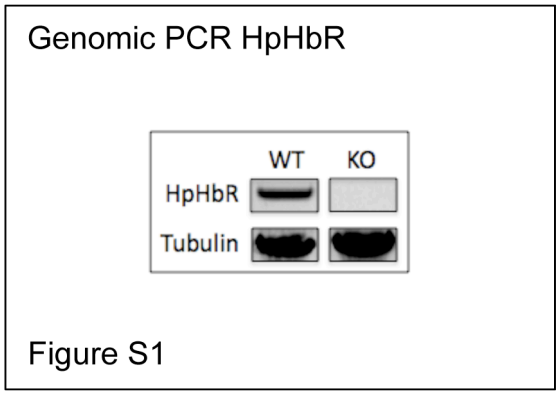

Figure S2.1. Genomic PCR analysis. HpHbR-specific primer PCR analyzed the presence of DNA for the HpHbR in both wild type *T. b. brucei* (WT) and *Tbb*HpHbR$^{-/-}$ (KO). DNA presence is indicated by PCR band appearance with b-tubulin used as the loading control.

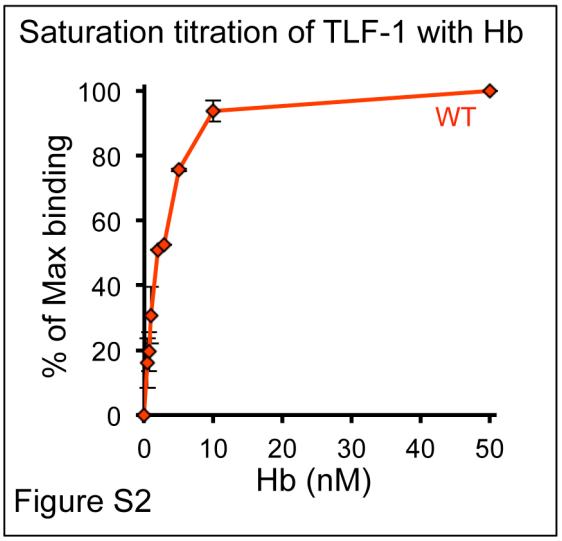

Figure S2.2. Saturation of TLF-1 binding by Hb. To ensure that all TLF-1 was saturated with Hb in the competition binding assays, Hb was added to TLF-1 (3 nM constant) in increasing concentrations. The percentage of maximum binding measured from flow cytometry is plotted versus the concentration of Hb.

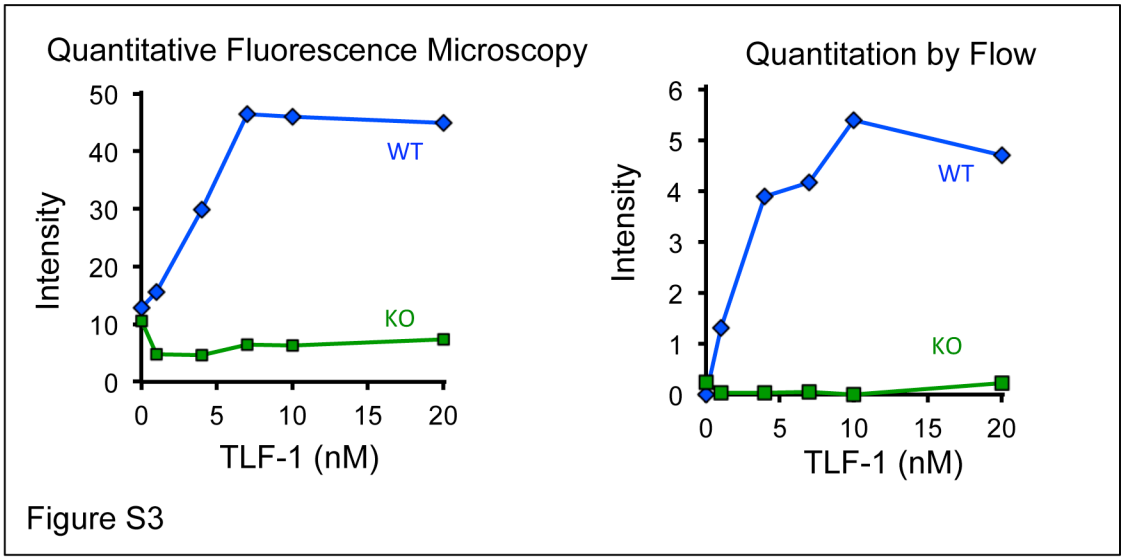

Figure S2.3. Comparative binding curve for TLF-1 measured by fluorescence microscopy and flow cytometry. For both analyses a Kd of 4.06 $\pm$ 0.08 nM (microscopy) and 3.42 $\pm$ 0.52 nM (flow cytometry) was determined.

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

Name	Orientation	Sequence
Oligo dT	antisense	5’-GCTCTAGATTTTTTTTTTTTTTTTTTTT-3’
Splice leader RNA	sense	5’-CCGGAATTCGCTATTATTAGAACAGTTTCT-3’
TbbHpHbR	sense	5’-GATCCCTGCAGGATGGAGAAACCGTCTTGCAG-3’
TbbHpHbR	antisense	5’-GATCGGCGCGCCCTACACCACCACCTGGAGCA-3’
TbbHpHbR (real time)	antisense	5’-CGCCTTCTCAACTTCGTCTTTGGT-3’
Enolase	antisense	5’-CCAACCGGGAAAGCCAAATTTAGC- 3’
Enolase (real time)	antisense	5’-TCTCTGTCGTCACTTCAACCTCCA-3’
5’ UTR HpHbR	sense	5’-ATCGTCTAGAGCGGTTACATGGTGCGACTG-3’
5’ UTR HpHbR	antisense	5’-ATCGTCTAGACAAAGCTGCGACTGCACCCC-3’
3’ UTR HpHbR	sense	5’-ATCGGGGCCCAAAAGACACCGTTTCTTCCA-3’
3’ UTR HpHbR	antisense	5’-ATCGGGGCCCGCGGCCGCCCTCACCGAAAACCGCATGT-3’
5’ UTR HpHbR (probe)	sense	5’-GAATGTTGCTGCTACGGTATG-3’
5’ UTR HpHbR (probe)	antisense	5’-CAAAGCTGCGACTGCACCCC-3’
HpHbR sequence	sense	5’-TTTGGGTTCTGCTTAATGCC-3’
HpHbR sequence	antisense	5’-TTAGACAATTTAAACTTGTTCAGC-3’
TbgHpHbR (S210L)	sense	5’-ACTTCAGAAACTGAGTGCAGAAAGCTATTGACG-3’
TbgHpHbR (S210L)	antisense	5’-GTCAATAGCTTTCTGCCACTCAGTTTCTGAAG-3’
TbgHpHbR (V293A)	sense	5’-GCACATGAAACAAAAGCACAGGAAGAGATCAAGC-3’
TbgHpHbR (V293A)	antisense	5’-CTTCATCTCTTCCTGTGCTTTTGTTTCATGTGC-3’
TbgHpHbR (GA369/370EG)	sense	5’-AAACGTCATGGGGTTGAGGGACCAAGGCCCCGTTGACG-3’
TbgHpHbR (GA369/370EG)	antisense	5’-GTCAACGGGCCTGGTTCCCTCAACCCCATGACG-3’
Tubulin	sense	5’-GGCGAAATCGTCTGCGTTCAGG-3’
Tubulin	antisense	5’-GTATTGCTCCTCTCGTCGAAC-3’
HA primer 1	sense	5’-TACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTGAGGGT-3”
HA primer 2	sense	5’-GCTTTTGTGGTGTTACGGCACCTGTTGCGCTCTACTCCTCCGCCTGATAGTTGAAGCCAG-3”
HA primer 3	sense	5’-ATGGAGAAACCGTCTTGCAGGGGTGCCGGTTGGGCGCAGCTTTTGTGGTGTTACGGC-3”

Table S2. Relative HpHbR mRNA Levels

Cell line	**HpHbR mRNA[1]**
WT	1
*Tbb*HpHbR$^{-/-}$	N.D.
*Tbb*HpHbR	1.05
*Tbg*HpHbR	0.91
*Tbg*HpHbR^{HA}	1.80
*Tbg*HpHbR^{V293A}	2.37
*Tbg*HpHbR$^{GA/EG}$	0.92
*Tbg*HpHbR^{S210L}	0.89

N.D. = Not Detectable
[1]RT-PCR levels normalized to WT *Tbb*HpHbR

Chapter 3

MAMMALIAN TOLERANCE MECHANISMS TO TRYPANOSOME LYTIC FACTOR-1 EXPOSURE

DeJesus E, Dennison L, Albright B and Hajduk SL. *Mammalian Tolerance Mechanisms to Trypanosome Lytic Factor-1 Exposure.*

Upon completion, to be submitted to *PLoS One*

ABSTRACT

Humans and higher primates harbor lethal innate immune molecules circulating within their bloodstream. At nanomolar quantities, both Trypanosome Lytic Factor 1 and 2 (TLF-1, TLF-2) kill specific subspecies of African trypanosomes within hours of exposure. Focusing on TLF-1, this particle contains the apolipoproteins characteristic of all high-density lipoproteins (HDL), apolipoprotein A-1 (APOA1), but also contains two additional proteins unique to higher primates, haptoglobin related protein (Hpr) and apolipoprotein L-I (APOL1). Upon binding of free hemoglobin to Hpr, TLF-1 is able to bind to a haptoglobin-hemoglobin (HpHb) receptor expressed exclusively in *Trypanosoma brucei* (*Tb*HpHbR). Once bound to the receptor, TLF-1 is endocytosed and trafficked to the lysosome where it initiates lysosomal breakdown, resulting in cell lysis. The mechanisms that protect mammalian host cells from the toxic effects of TLF-1 have never been investigated. Here we set out to investigate the effects of TLF-1 on mammalian cells, and what tolerance mechanism(s) the host cells utilize. Using imaging flow-cytometry, we show that TLF-1 is taken up by mammalian cells and localizes to lysosomes. In contrast to the trypanosome uptake pathway, however, uptake of TLF-1 by mammalian cells is not dependent on hemoglobin. Additionally, we identified that it is possible to overwhelm the innate TLF-1 tolerances and inhibit HEK293 growth with super-physiological concentrations of TLF-1. Additionally, we ask whether expressing the *Tb*HpHbR in Human Embryonic Kidney (HEK293) cells will increase TLF-1 sensitivity in a mammalian system.

INTRODUCTION

Representing a minor subclass of high-density lipoproteins (HDL), Trypanosome Lytic Factor-1 (TLF-1) is the one component of the human innate immune system that is responsible for protection against *Trypanosoma brucei brucei* infection [1]. *T. b. brucei* is uniquely susceptible to TLF-1 due, in part, to expression of a high affinity haptoglobin-hemoglobin receptor that binds to TLF-1, facilitating endocytosis of this toxic molecule (*Tbb*HpHbR) [2, 3]. The two human infective forms of the parasite, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* have each evolved separate and distinct mechanisms of resistance to TLF-1 [4-6].

T. b. rhodesiense contains a fully functional HpHb receptor, identical to that of *T. b. brucei*. It does, however, express a single protein termed serum resistance associated (SRA), which is capable of neutralizing the toxic effects of TLF-1 [6]. This protein is necessary and sufficient for protection in *T. b. rhodesiense* as loss of the gene confers susceptibility to TLF-1 activity [7]. Ectopic expression of SRA within T. b. brucei, conversely, introduced TLF-1 resistance within an otherwise sensitive subspecies [8]. Within *T. b. gambiense*, multiple factors work in concert to assist in general evasion of the particle in question. While this pathogen does contain transcripts for a HpHb receptor, levels of mRNA, when compared to T. b. brucei, are down significantly. Also, when sequenced, it was identified that the open reading frame (ORF) contained multiple polymorphisms, one of which was responsible for attributing a complete loss of function to the HpHb receptor. Further investigations

identified a *T. b. gambiense* specific protein, termed TgsGP, which was able to protect against TLF-1 activity.

In reviewing the mechanisms in place for these two subspecies, it becomes evident that protection against this unique toxin is one that comes at a cost [7]. It is surprising, then, that the very cells that not only produce TLF-1 but also associate with the particle constantly are seemingly refractory to its ill effects. Investigations into the mechanisms that allow mammalian cells tolerate TLF-1 have never been carried out. HDL endocytosis has been studied in multiple systems and the precise purpose and efficiency of this process is under debate. The transport of cholesterol is considered to be the primary goal [9]. In terms of TLF-1, the transport of cholesterol by TLF-1 has not been sufficiently analyzed. The question of TLF-1 endocytosis efficiency also has not been thoroughly examined aside from a single observation that TLF-1 was internalized in macrophage infected with Leishmania [10]. In this case, no macrophage cell death was reported, thereby suggesting TLF-1, when at approximate physiological levels in culture, was somehow unable to elicit its toxic effects. By revealing what events take place between TLF-1 and mammalian cells, we can potentially learn how and why this toxin has no impact in its native environment.

Here we show that TLF-1 is endocytosed within human embryonic kidney cells (HEK293) and that localization to low pH vesicles does occur. Unlike human infective trypanosomes, HEK293 cells do not appear to have a specific high-affinity TLF-1 receptor. Additionally, we found no requirement for hemoglobin in regards to TLF-1 binding and uptake. Unsurprisingly, low concentrations of TLF-1 displayed

no negative effect on HEK293 growth but we found that at concentrations reaching 75 μg/ml, TLF-1 can was able to attenuate growth of HEK293 cells.

METHODS

Mammalian Cell Culturing

HEK293, HepG2 and LnLp Prostate Cancer cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich, heat inactivated) and grown at 37°C, 5% CO_2.

TLF-1 particle purification

TLF-1 particles were purified according to protocols previously described. Additionally, all Alexa Fluor 488-TLF-1 labeling was performed on purified TLF-1 with protocols defined my manufacture [11].

TLF-1 Binding and Uptake studies

To measure TLF-1 binding by flow cytometry, cells were grown to mid-log phase, collected, washed and resuspended ($1x10^7$/ml) in DMEM supplemented with 10% fetal bovine serum. Alexa-488 TLF-1, labeled according to manufacture instructions (Invitrogen), was incubated with an excess of Hb for 10 minutes on ice then added to cells in ice-cold complete DMEM and further incubated at 3°C for three hours. Cells were washed two times with ice-cold phosphate buffered saline buffer (PBS) (10 mM NaP_i, 137 mM NaCl, pH 7.4), kept on ice analyzed by flow cytometry. All

binding experiments were done in triplicate with 50,000 cells measured per experiment/data point.

To measure TLF-1 uptake by flow cytometry, cells were grown to mid-log phase, collected, washed and resuspended ($1x10^7$/ml) in DMEM supplemented with 10% fetal bovine serum. Alexa-488 TLF-1 done both with and without hemoglobin, were added to the cells followed by incubation at 37°C for three hours. Uptake was stopped by placing the tubes on ice followed by two washes with ice-cold PBS. The amount of TLF-1 uptake was determined using both Cyan cytometer and Amnis ImageStream cytometer with analysis from FlowJo software. For uptake studies, 20,000 cells were imaged through Amnis ImageStream per experiment, each experiment done in triplicate.

To measure AF488 TLF-1 internalization, the algorithm *Internalization plotting* was utilized. This pre-set within Amnis software is set to decipher localization of fluorescent material recorded via two cameras positioned 90 degrees to the cell stream. The created 3-dimensional image can then be used for calculating the position of endocytosed material.

Competition binding studies

Specificity of TLF-1 binding to HEK293 cells was analyzed using competition-binding studies with the unlabeled non-lytic HDLs and Hp 1-1 (Sigma-Aldrich). Cells were collected, washed and resuspended ($1x10^7$/ml) in ice-cold DMEM supplemented with 10% fetal bovine serum then transferred to 3°C for at least 10 minutes. Alexa-488 conjugated TLF-1 (20 nM constant) was complexed with

hemoglobin (50 nM) at 4°C for 10 minutes. Increasing concentrations of unlabeled competitor were incubated with Hb (50 nM) for 10 minutes at 4°C. Competing ligands were then mixed with the Alexa-488 conjugated TLF-1/Hb, added to cells at 3°C and allowed to incubate for three hours. Cells were then transferred to ice, washed with ice-cold 1X PBS and analyzed by Cyan cytometer and FlowJo software. All competition studies were done in triplicate.

TLF-1 Growth Studies

Briefly, for the survival assays, HEK293 cells were harvested from mid-log phase cultures, washed and re-suspended at a final concentration of $1x10^6$/ml in complete DMEM media. Susceptibility to hemoglobin (Hb) bound TLF was determined over a range of TLF concentrations following incubation at 37°C for 72 hours. The number of surviving cells was determined by hemocytometer count with phase contrast microscopy**.** Additionally, cell viability was quantified by flow cytometry (Cyan) using the LIVE/DEAD Cell viability kit (Invitrogen). All survival assays were done in triplicate.

RESULTS

TLF-1 uptake in HEK293 cells

To establish TLF-1 uptake in mammalian cells, HEK293 cells were incubated with Alexa Fluor-488 conjugated TLF-1 (AF488 TLF-1) and imaged via Amnis ImageStream. As is seen in figure 3.1, AF488 TLF-1 is endocytosed into vesicles inside the HEK293 cells. The quantification of the signal intensity, seen in figure

3.1B, indicates the maximum pixel intensity signifying TLF-1 is taken into the cell at 37°C uptake. No cell surface binding is detectible when the cells are held at 3°C (image data not shown). Using an algorithm termed *Internalization plotting*, we verified that the TLF-1 seen in figure 3.1A is internal and not cell surface associated.

TLF-1 uptake time course in HEK293 cells

Previous studies using HEK293 cells transfected with Scavenging Receptor-BI (SR-BI) have measured SR-BI mediated HDL uptake to reach saturation within three hours [12]. To test if wild type, untransfected HEK293 cellular endocytic machinery could reach equilibrium, a TLF-1 uptake time course was carried out. By three hours, the signal for AF488-TLF-1 plateaued, indicating equilibrium (Figure 3.2). Hemoglobin has been shown to be an essential co-factor for TLF-1 binding and uptake in African trypanosomes [13]. A receptor capable of binding the Hpr present in TLF-1 has not been identified in mammalian systems. In light of this, we looked at whether there was a difference in the rates of uptake when hemoglobin was added. The intensity of the signal of AF488 TLF-1, analyzed using flow cytometry, did not indicate any substantial difference in the rate in uptake (Figure 3.2, single time point and 3.3, time course).

TLF-1 localization studies in HEK293 cells

After confirming that TLF-1 was indeed taken up by HEK293 cells, we next set out to determine TLF-1 cellular localization. Using Amnis ImageStream with live cells, colocalization with AF488 TLF-1 and lysotracker suggests that TLF-1 does, to

some degree, localize to low pH compartments resembling the lysosome (Figure 3.4). This was further confirmed with fluorescence microscopy (data not shown).

Low temperature TLF-1 competition saturation binding studies

To analyze TLF-1 putative binding, a low-temperature binding assay was carried out using a previously developed flow cytometry method [3]. While no high affinity binding was observed (data not shown), high concentrations of AF488 TLF-1 were detectable but not saturable (Figure 3.5A) by flow cytometry. This finding agrees with previously published literature for HDL binding in mammalian cells, including HEK293 cells, that there is no TLF-1 specific receptor in mammalian systems [14]. Additionally, to test whether TLF-1 uptake was due to a specific Hp receptor, a competition-binding assay with unlabeled ligand was done. As indicated in figure 3.5B**,** no competition with increasing amounts (both by molar and mass equivalents) was observed.

TLF-1 Survival Assays

In order to test whether mammalian cells would be resistant to TLF-1 in culture, a 72-hour survival assay was carried out. Concentrations of TLF-1 up to 20 μg/ml showed no inhibition in cell growth (Figure 3.6A). However, incubation with 75 μg/ml TLF-1 for 72 hours caused attenuation of growth (physiological levels of TLF-1 are approximately 10 μg/ml)**.** The difference in cell density between the 24-hour mark and the 72-hour was also recorded by light microscopy (Figure 3.6B).

DISCUSSION

The primary HpHb receptor in mammalian cells, CD163, is present in monocytes and macrophages and plays an important role in scavenging hemoglobin from serum [15]. CD163, however, is unable to bind Hpr and therefore, presumably, has no affinity for TLF-1 [16]. To date, no other mammalian high affinity HpHb receptor has been identified or characterized, which was confirmed by our results showing no hemoglobin dependence for TLF uptake by HEK293 cells. Only at high concentrations were signals measured for TLF-1, indicative of low-affinity binding (Figure 3.5A). No signal for binding was detected for AlexaFluor-488 HpHb indicative of no specific receptor present (data not shown). The manner in which TLF-1 endocytosis occurs in HEK293 cells is likely via bulk phase endocytosis. To further investigate contribution of any putative TLF-1 receptor, a competition-binding assay was performed. The lack of competition from both HpHb and non-lytic HDLs suggests no receptor-mediated endocytosis is occurring for TLF-1 in HEK293 cells (Figure 3.5B).

HDL uptake and resecretion have been thoroughly investigated in regards to reverse cholesterol transport. In the case of SR-BI, HDL endocytic saturation is reached within three hours with full resecretion of over 99% of all HDL within seven hours [12]. In our hands, while TLF-1 saturation was reached by three hours (Figure 3.2), and we did not investigate if TLF-1 was resecreted from cells. We did show that TLF-1 was, to some extent, localized to the lysosome, as is the case in *T. brucei* (Figure 3.4). This was striking since some reports conclude that lysosomal localization of HDL particles, while not impossible, is relatively rare [9].

Numerous studies have set out to define specific HDL features such as binding and endocytosis, trafficking, retroendocytosis, catabolism and turnover [9, 17, 18]. The precise impact of HDL endocytosis on cholesterol homeostasis is under currently under debate [9]. Numerous publications provide data for compensation mechanisms when one pathway is chemically blocked or inhibited by another means indicating multiple, redundant paths for HDL uptake [19]. The work presented here provides initial evidence that the trypanolytic particle, TLF-1, is able to enter into a cell line that possesses no identified HDL or HpHb receptor and traffic through to low pH endosomal vesicles (Figure 3.1, 3.4) [15, 20, 21]. Despite not expressing an HDL-specific receptor, we were able to observe low-affinity binding in HEK293s and define that no contribution from HDL and/or HpHb receptors (Figure 3.5). In searching for how mammalian cells evade toxicity of TLF-1 we first confirmed that HEK293 cells could survive incubation with physiological concentrations of TLF-1. This amount is significantly higher than that required to kill *T. b. brucei*. We were able to confirm that TLF-1 was indeed endocytosed by the cell, in culture, in a manner not dependent on the presence of hemoglobin. This is in contrast to *T. brucei* where TLF-1 uptake is dependent on the presence of hemoglobin to allow binding to the HpHb receptor. Remarkably, we were able to overcome the apparent tolerance mechanisms exhibited by mammalian cells by simply increasing the relative concentration of TLF-1. This data suggests that one reason mammalian systems are not susceptible to the effects of TLF-1 is simply the physiological concentration (approximately 10 μg/ml for TLF-1) of this small subclass of HDL.

Despite this evidence, there are still many questions that stand in regards to TLF-1 tolerance in mammalian systems. Cholesterol content and membrane composition has been brought into question as a possible defense mechanism [22]. When introduced into *in vitro* liposomes, cholesterol increased rigidity of the membranes thereby prohibiting interactions with APOL1 and TLF-1 (Unpublished data). To that end, TLF-1 survival studies involving the cholesterol-inhibiting drug, Lovastatin are underway and will help shed light on the contribution by cholesterol to TLF-1 resistance. Also, pH of endosomal vesicles has been shown to be of some importance for TLF-1 activity [5, 23]. While the pH of endosomal vesicles for *T. brucei* is between 4.7-5.3**,** the pH in mammalian endosomes is approaching 6.5 [24]. Combining this with *in vitro* data on TLF-1 activity and pH, it becomes tempting to suggest that in mammalian endosomes, the pH of endosomal vesicles precludes APOL1 activity. Furthermore, high resecretion rates, membrane cholesterol content, and lack of a high affinity receptor render TLF-1 relatively inactive in mammalian cells at physiological levels.

The lack of an HpHb receptor is one seemingly obvious reason mammalian cells do not specifically bind and target TLF-1 to the lysosome. To that end, addition of the *Tb*HpHbR into HEK293 cells is currently being carried out in order to address whether or not expression of the *Tb*HpHbR would increase TLF-1 sensitivity. Along with the data presented in this chapter, these experiments provide insight into the fate of TLF-1 in mammalian cells.

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Author Contributions

EDJ conceived, designed and performed experiments and co-wrote the manuscript. LD performed binding and competition studies and edited the manuscript. BA performed Amnis uptake and co-localization studies and edited the manuscript. SLH conceived, and designed experiments and co-wrote the manuscript.

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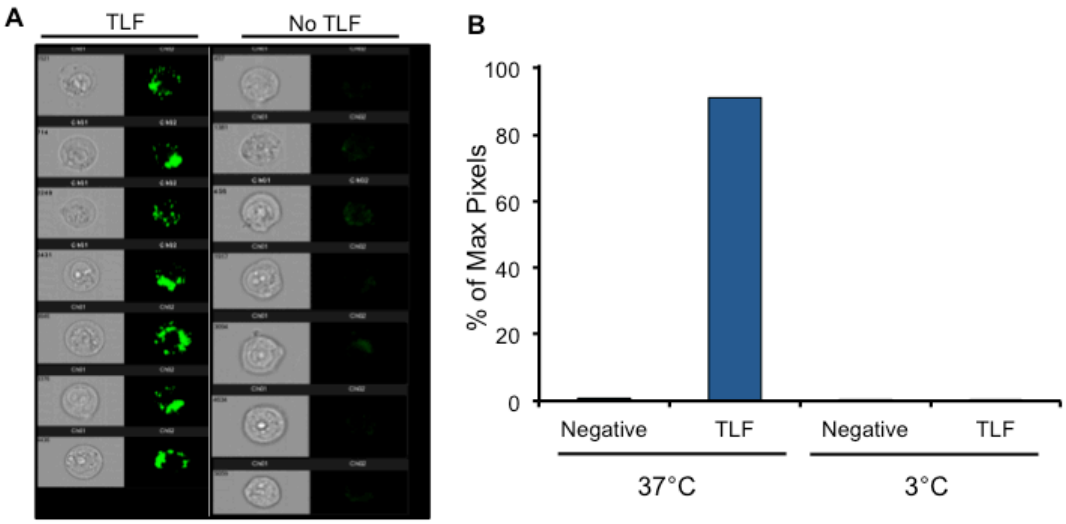

Figure 3.1: TLF-1 Uptake in HEK293 cells. **(A)** TLF-1 uptake (20 µg/ml) by HEK293 cells for two hours. Images captured by Amnis ImageStream at Emory Pediatrics Hospital, Atlanta, Georgia. **(B)** Quantification of images in A. Pixel density was calculated by ImageStream 6.0 Software and plotted as percentage of max pixel density.

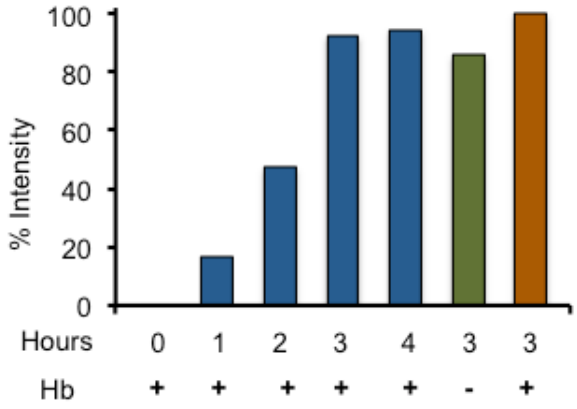

Figure 3.2: Time course of TLF-1 uptake in HEK293 cells. Median intensity of TLF-1 was measured and quantified by FlowJo 9.6.4 software. Data points are indicative of 20,000 cells per point.

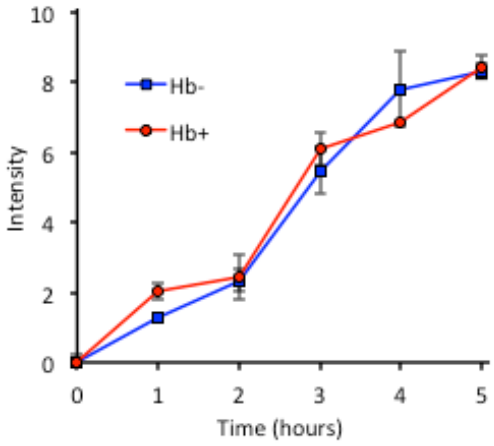

Figure 3.3: Hemoglobin dependence in TLF-1 uptake. Median intensity of TLF-1, with and without hemoglobin, was measured and quantified by FlowJo 9.6.4 software. Data points, indicative of 20,000 cells per point, were taken in triplicate.

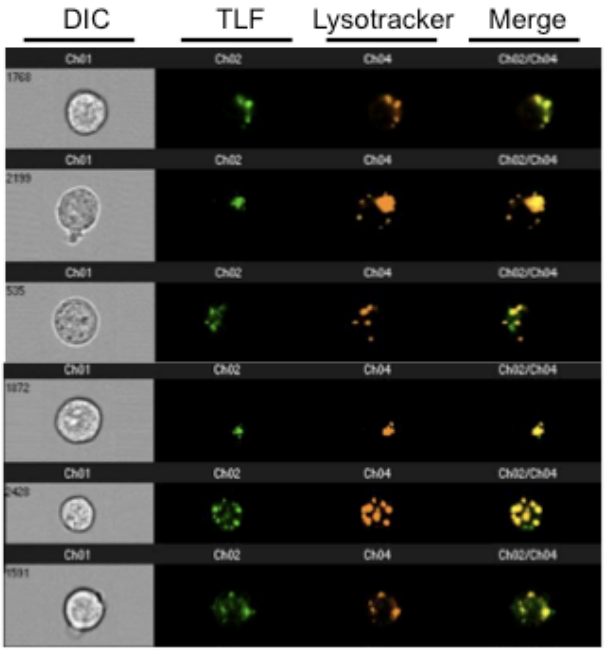

Figure 3.4: Colocalizations of TLF-1 and Lysotracker in HEK293 cells. Images captured by Amnis ImageStream at Emory Pediatrics Hospital, Atlanta, Georgia. Live cells were used with a limit of 10,000 cells through the cytometer per assay.

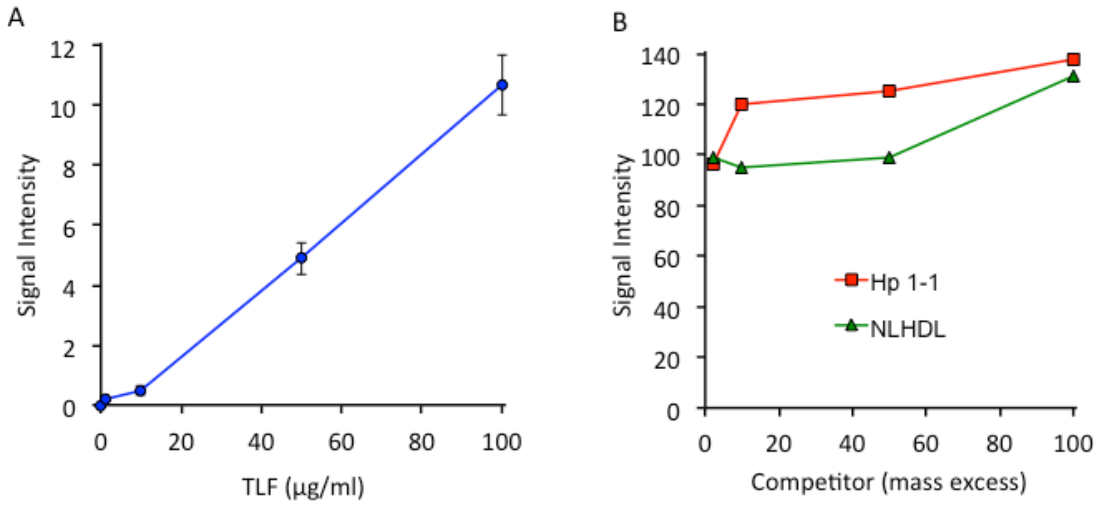

Figure 3.5: TLF-1 binding and competition in HEK293 cells. **(A)** Putative binding estimates were attempted based on half-maximal binding at 3°C with varying concentrations of Alexa-488 TLF-1. No saturation was obtained. 20,000 cells per assay were analyzed in triplicate through flow cytometry. **(B)** For competitions, 2, 10, 50 and 100X unlabeled competitor (non-lytic HDL by mass or Hp 1-1 by molecular weight) were used in low-temperature binding.

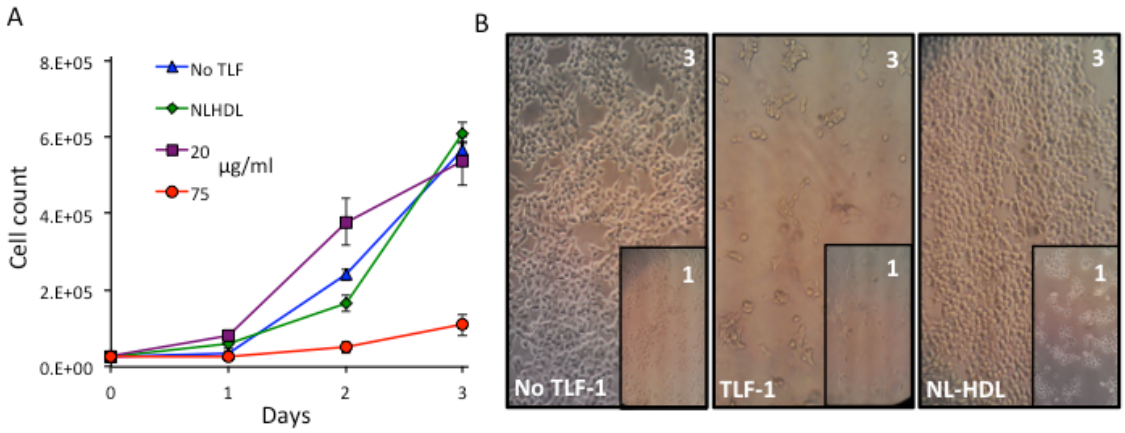

Figure 3.6: TLF-1 Survival Assay in HEK293 cells. **(A)** HEK293 cells were incubated with increasing concentrations of TLF-1 for 72 hours. Cell counts were done every 24 hours with a hemocytometer under light microscopy. **(B)** Cells at 24 hours and 72 hours of assay. 1: Image of cells counted day one (24 hours). 3: Image of cells counted day three (72 hours). NL-HDL: Non-lytic HDLs (1.17 mg/ml).

CONCLUSIONS AND DISCUSSION

The two subspecies of *Trypanosoma brucei* that are responsible for African sleeping sickness induce two very different pathologies: *T. b. rhodesiense* creating acute symptoms, killing within three years and *T. b. gambiense* exhibiting more chronic features, taking upwards of 10 years to kill [1]. While little is known about the cause of the vastly different pathologies, much headway has been made in understanding the reason they can establish infection in the first place. In regards to evasion of the human adaptive immune response, all subspecies of *T. brucei* are able to undergo antigenic variation of its VSG coat thereby cloaking itself from the antigen response. With approximately 20 expression sites for the proposed 1000 VSG genes [2, 3], these trypanosomes are able to evade a wide variation of antigens presented by the adaptive immune system. This adaptation makes the parasitemic waves possible that occur during an infection.

The story for *T. b. rhodesiense* and its evasion of human serum is less complicated when compared to *T. b. gambiense*. Early analysis of *T. b. rhodesiense* clones revealed a transcript that was, when compared to sensitive clones, only present in serum resistant cells [4]. Expression of this protein, termed serum resistance associated protein (SRA), was confirmed to be necessary to confer resistance to both trypanolytic particles, as they thrive in normal human serum (NHS) [4-7]. Knockout of this gene revealed that it is the entire defense system against TLF-1 particles [6] as sequence analysis of the HpHb receptor revealed that it is possess no mutations [8] and is able to bind, endocytose and traffic TLF-1 to the lysosome [9, 10]. Loss of the SRA GPI-anchor was found to be non-essential for NHS

resistance as SRA traveled with TLF-1 through endosomal vesicles terminating in lysosomes thereby inhibiting TLF-1 lysis [9]. Investigations into the mechanism of TLF-1 inactivation rendered by SRA divulged an interaction between a α-helix in SRA and APOL1 in TLF-1. Truncation of this α-helix found it to be essential in binding the C-terminus of APOL1 [11]. This binding interaction is what renders the TLF-1 particle, and likely TLF-2, neutral in *T. b. rhodesiense* [11, 12].

The story for innate immune evasion in *T. b. gambiense* is more complex. No SRA-like gene exists in either form of *T. b. gambiense* (both group 1 nor group 2) and with time it has become apparent that *T. b. gambiense* truly portrays an elegant set of defense mechanisms with a multifaceted approach to establishing human infection [4]. The work presented in this thesis along with work recently published within the field has helped focus the *T. b. gambiense*/NHS resistance story.

In first investigating TLF-1 resistance in group 1 *T. b. gambiense*, data indicated that the mRNA levels for the *Tbg*HpHbR were down-regulated approximately 20-fold when compared to wild type *T. b. brucei* [13]. At the time of this finding, it was known that an SRA-like molecule did not exist in *T. b. gambiense* therefore the lack of *Tbg*HpHbR suggested a first line of defense in TLF-1 killing. To test this hypothesis, we expressed the *Tbg*HpHbR in *T. b. brucei* that had the HpHb receptor genetically knocked out (KO) (Figure 2.2 and 2.3). With transcript levels comparable to *T. b. brucei*, the *Tbg*HpHbR was detected by western (Figure 2.2) indicating that protein was at least being made. Localization studies were, unfortunately, unable to decipher whether or not the *Tbg*HpHbR was being properly trafficked to the flagellar pocket. This is a possible explanation for the fact that the

*Tbg*HpHbR expressing cells were unable to bind and endocytose TLF-1 (Figure 2.3). Overexpression of the *TbgHpHbR*, as measured by qRT-PCR, revealed that it was a truly non-functional, dead receptor as indicated by no TLF-1 binding, uptake or killing (Table 2.1). This finding can be thought of as a primary TLF-1 defense mechanism as HpHb receptor knockout cells in wild-type *T. b. brucei* were able to survive physiological levels of TLF-1 [14].

Upon sequencing the *TbgHpHbR* gene, four non-synonymous polymorphisms were identified in the open reading frame (ORF), and it was asked whether or not these mutations could have some impact on TLF-1 binding function. To test this, we performed site-directed mutagenesis on the *Tbg*HpHbR construct for each mutation and introduced it back into the KO cell line. Initial TLF-1 uptake studies were indicative of a functioning receptor as AF488 TLF-1 was observed in endocytic vesicles (Figure 2.3) in only cells harboring the mutation at position 210 within the ORF of the *Tbg*HpHbR. This change, from a serine in T. b. gambiense back to leucine, which is present in T. b. brucei, was the only mutant capable of allowing AF488 TLF-1 uptake. This finding was further confirmed by live cell flow cytometry (Figure 2.3).

Alexa-Fluor labeling renders the TLF-1 non-lytic due to a high pH step in the preparation process (data not shown). Therefore, incubating this labeled particle with cells is generally harmless. In order to test whether this S210L mutation conferred TLF-1 sensitivity to *Tbg*HpHbR cells, incubation with unlabeled, lytic TLF-1 was carried out. In these assays, the S210L mutation was found to be sufficient in

restoring the uptake and proper lysosomal-targeted trafficking required for TLF-1 associated killing (Figure 2.3).

From the first data on TLF-1 binding [15] to the first identification of the *Tb*HpHbR itself [10], the HpHb receptor has always been defined as a high affinity receptor [14, 16]. With this fact, we set out to address whether the S210L mutation restored the high affinity character found in *T. b. brucei* in the *Tbg*HpHbR. Using a newly developed method for measuring binding affinities, we observed high affinity TLF-1 binding in wild type *T. b. brucei*, *Tbb*HpHbR rescue cells and the *Tbg*HpHbR^{S210L} (Figure 2.4) suggesting that this single point amino acid substitution is necessary and sufficient for restoring the TLF-1 binding nature to the *Tbg*HpHbR. Collectively, this data, in conjunction with the fact that the *TbgHpHbR* is natively down regulated, exhibits a two-way defense scheme aimed at TLF-1 avoidance.

The level of resistance observed in the KO cells is, however, overcome when TLF-1 levels are increased to approximately 10 μg/ml or the HpHb receptor knockout cells are subjected to incubation with either NHS or APOL1 (data not shown). Considering that the *Tbg*HpHbR expressing cells are *T. b. brucei* based, it is correct in stating that these "*T. b. gambiense* cells" would be killed in the presence of NHS as they are essentially no different than the KO cells. Therefore, there must be additional mechanisms in place for NHS resistance in authentic group 1 *T. b. gambiense*. Thus, in collaboration with Annette MacLeod's laboratory at the University of Glasgow, we set out to address the manner in which these cells establish infection. In doing so, we were able to investigate the contribution of a *T. b. gambiense*-specific glycoprotein (TgsGP) to NHS resistance. Prior investigators

identified the protein as *T. b. gambiense* specific but at the time, genetic manipulation in *T. b. gambiense* was not possible *in vitro* [17]. The laboratory of Annette MacLeod, however, recently developed a working transfection system within group 1 *T. b. gambiense* so the question of TgsGP role could be further dissected. As a second mode of defense, the protein TgsGP was able to confer measureable resistance to NHS in *T. b. gambiense.* Knockout of this gene (Figure A.1) and addition of a functional HpHb receptor (Figure A.1 and A.3) revealed *T. b. gambiense* to be sensitive to APOL1, TLF-1 and NHS (Figure A.2). As striking as this was, even more interesting was the lack of resistance when TgsGP was ectopically expressed in wild-type *T. b. brucei* (Figure A.2) [17, 18].

Although we were unable to define a mechanism by which TgsGP confers resistance in *T. b. gambiense*, Uzureau et al. found that an internal hydrophobic region played a role in serum resistance. Invoking *in vitro* data where a hydrophobic peptide was able to increase membrane rigidity in African trypanosomes, thereby killing them [19], this group postulated that TgsGP is perhaps working in a similar manner, that being endosomal/lysosomal membrane ridigification. With the addition of synthetic peptide resembling the hydrophobic region of TgsGP, it was found that at least for a fixed concentration of NHS, both *T. b. brucei* and *T. b. gambiense* TgsGP knockouts were found to posses partial resistance to NHS [18]. Taken together, this clearly indicates that there are redundant mechanisms in place within *T. b. gambiense* to evade the TLF particles.

The fact that ectopic expression of TgsGP has no significant impact on NHS, APOL1 or TLF-1 resistance in *T. b. brucei* [20] opened up discussion on what factors

were still at play in *T. b. gambiense*. Recent published investigations into what mechanisms could be at work revealed some potentially interesting findings. The pH of endosomal vesicles within *T. brucei* was found to differ within *T. b. brucei* (pH: 5.34 $\pm$0.09) while *T. b. gambiense* measured lower (pH: 4. 85 $\pm$0.05). The group hypothesized that while this difference, while not seemingly significant, is enough to create a difference in cathepsin (tbcatB) activity in *T. b. brucei* [18]. This cathepsin has been implicated to have activity at, or below, pH 5 and putatively has the ability to degrade APOL1. Putatively, this action would prohibit APOL1 from intercalating into the endosomal/lysosomal membranes thereby preventing the events necessary for trypanosomes cell lysis [18, 21].

Another facet to the defense story this group invoked came from investigations into the *inhibitor of cysteine protease* (*ICP)* gene in *T. brucei*. Deletion of ICP in wild-type *T. b. brucei* led to changes in VSG-coat expression, cell differentiation, parasitemia, and an increase in resistance to human serum [18, 22]. This was contrary to *T. b. gambiense* where loss of ICP revealed a decrease in serum resistance [18]. These two phenomena (pH and ICP activity), when combined with TgsGP activity and *Tbg*HpHbR data, build the story, for now, of human serum resistance in *T. b. gambiense*. Experiments to test the findings of pH and ICP activity, in regards to human serum resistance, have not been carried out in *T. b. brucei* so whether or not more factors are in play is still a valid question.

Examining the array of tools *T. b. gambiense* uses to evade the immune system and the simplistic approach found in *T. b. rhodesiense*, it becomes equally remarkable that *T. b. brucei* is uniquely sensitive to these trypanolytic particles

present in human serum. Keeping with this thought, it is curious then to think about how mammalian cell systems themselves have evaded the toxic effects of TLF-1 and APOL1. The manner in which higher primates contain the effectiveness of TLF-1 has never before been investigated until now. Insight into the nature of APOL1 in mammalian systems, HEK293 cells in this case, was revealed when APOL1 was ectopically expressed and targeted to the cytosol. There, APOL1 expression was sufficient to induce autophagy indicated by an increase in LC3-II and reduction in LC3-I, both autophagic markers [23]. Canonical HDL transport never has the holo-particle in cytosol; therefore it is easy to imagine that TLF-1 is never delivered to cytosol. Despite this fact, the concept of APOL1 killing mammalian cells was intriguing. On this, we set out to address the question of mammalian systems tolerate TLF-1 exposure.

The first step in this is to address whether we could observe TLF-1 interactions with HEK293 cells. Using AlexaFluor 488-conjugated TLF-1, we were able to confirm that these cells were, in fact, able to endocytose AF488 TLF-1. This was observable by fluorescence microscopy (data not shown), standard flow cytometry (Figure 3.2) and a new imaging flow cytometer, Amnis ImageStream (Figure 3.1). Observing TLF-1 bound and endocytosed by HEK293 cells meant that, in some capacity, APOL1 did have the potential to interact with a lysosomal membrane like it does in *T. b. brucei*. From here, we asked the logical next question: where is this particle localizing? Using a low pH marker, Lysotracker, we identified that TLF-1 was able to partially localize to vesicles of low pH (Figure 3.4). Immunofluorescence microscopy is currently being carried out to further

characterize the precise locations of these particles. Despite this, the initial pieces of data indicate that TLF-1, and therefore APOL1, is potentially in direct contact with lysosomal membranes.

Up to this point, assumptions were that mammalian cells would survive incubation with TLF-1, considering cells are in contact with it *in vivo* daily with no ill effect. Upon more careful analysis in culture, these hypotheses were confirmed when cells survived incubation with TLF-1 at concentrations approaching and matching physiological levels (Figure 3.6A). This was further validated when it was observed that HEK293 cells were able to survive 100 μg/ml TLF-1 in a single 24-hour period, approximately 10 times the amount considered to by physiological (data not shown). It was not until a long-term, high concentration survival assay was carried out that attenuated cell growth was observed (Figure 3.6A and B).

This finding lays the foundation for a myriad of questions. It has been shown that HDLs undergo a high rate of retroendocytosis and resecretion in many mammalian cells as part of reverse cholesterol transport [24-26]. This data, combined with low physiological TLF-1 levels is then perhaps the primary reason that this toxin is non-lethal to the higher primates and humans that make it. Invoking the mechanisms used by *T. b. gambiense*, does endosomal pH and an ICP homolog factor into TLF-1 (and potentially TLF-2) resistance? Studies of endosomal pH report a range of 6.1-6.8 for early endosomes [27]. This high pH has been shown refractory for TLF-1 interactions with lipid membranes and perhaps is another level of defense against TLF-1 attack [28]. Recent unpublished data from the Hajduk laboratory indicates that with increasing cholesterol concentrations in *in vitro*

liposome assays, interaction of TLF-1 and APOL1 with membranes decreases. Thus, as was proposed recently, perhaps cholesterol plays a role in resistance to APOL1 intercalating into lysosomal membranes [29]. To this end, we are currently subjecting HEK293 cells to TLF-1 exposure in the presence of cholesterol inhibiting and sequestering drugs (Lovastatin and metyl-β-cyclodextrin, respectively) to assess any contribution cholesterol lends towards TLF-1 protection.

Finally, would the ectopic expression of the *T. b. brucei* HpHb receptor be able to confer an increased susceptibility to TLF-1 in mammalian cells? Currently, *Tbb*HpHbR expression studies in HEK293 cells are being carried out in order to begin to address these questions. If TLF-1 sensitivity was increased in HEK293 cells with *Tbb*HpHbR expression, this would not only imply sufficient expression and proper protein folding of a trypanosome receptor in a mammalian system but also that receptor/ligand trafficking patterns observed in *T. b. brucei* were occurring in HEK293 cells. This finding would be of significant importance in providing a proof of concept for the hypothesis that cells in a diseased state (cancer cells) could be killed by a naturally occurring toxin present in human serum.

It is becoming increasingly apparent that both *T. b. gambiense* and mammalian cells (in this case, HEK293 cells) have each evolved multiple layers of defense against these cytotoxic components of the human innate system. The findings presented here define both mechanisms of evasion (mutated, down-regulated *Tbg*HpHbR as well as TgsGP expression) and tolerance, in the case of HEK293 cells, to TLF-1 particles. Future studies on HEK293 cells and TLF-1 could prove insightful on to the precise mechanisms in which mammalian cells tolerate

TLF-1 exposure and could lay the foundation for novel therapeutic approaches in treatment of cells in a diseased state.

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Appendix A

THE *TGSGP* GENE IS ESSENTIAL FOR RESISTANCE TO HUMAN SERUM IN

TRYPANOSOMA BRUCEI GAMBIENSE

Capewell P, Clucas C, **DeJesus E**, Kieft R, Veitch N, Steketee PC, Cooper A, Weir W, Hajduk SL and MacLeod A. *The TgsGP Gene Is Essential for Resistance to Human Serum in Trypanosoma brucei gambiense.* PLoS Pathog, 2013. **9**(10): p. e1003686.

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Abstract

Trypanosoma brucei gambiense causes 97% of all cases of African sleeping sickness, a fatal disease of sub-Saharan Africa. Most species of trypanosome, such as *T. b. brucei*, are unable to infect humans due to the trypanolytic serum protein apolipoprotein-L1 (APOL1) delivered via two trypanosome lytic factors (TLF-1 and TLF-2). Understanding how *T. b. gambiense* overcomes these factors and infects humans is of major importance in the fight against this disease. Previous work indicated that a failure to take up TLF-1 in *T. b. gambiense* contributes to resistance to TLF-1, although another mechanism is required to overcome TLF-2. Here, we have examined a *T. b. gambiense* specific gene, TgsGP, which had previously been suggested, but not shown, to be involved in serum resistance. We show that TgsGP is essential for resistance to lysis as deletion of TgsGP in *T. b. gambiense* renders the parasites sensitive to human serum and recombinant APOL1. Deletion of TgsGP in *T. b. gambiense* modified to uptake TLF-1 showed sensitivity to TLF-1, APOL1 and human serum. Reintroducing TgsGP into knockout parasite lines restored resistance. We conclude that TgsGP is essential for human serum resistance in *T. b. gambiense.*

Introduction

Throughout their evolution in sub-Saharan Africa, humans have been under assault by a range of different pathogens. One defining challenge is that posed by African trypanosomes, a species complex of blood-borne protozoan parasites

transmitted by tsetse flies [1]. The principle pathogenic species in Africa are *Trypanosoma brucei, T. congolense* and *T. vivax*, although only *T. brucei* sub-species are able to infect humans. A key feature of these parasites is the ability to undergo antigenic variation by modifying the variant specific glycoprotein (VSG) enveloping the cell that renders the mammalian adaptive immune system largely ineffective [2]. Components of the innate immune system therefore contribute significantly to defense against these organisms [3]. Critical to these defenses is the serum protein apolipoprotein L1 (APOL1) found in some catarrhine primates, including humans [4,5]. The protein is able to kill the majority of trypanosome species in a dose-dependent manner [5]. APOL1 is delivered to parasites in two fractions of the high-density lipoprotein (HDL) component of serum, termed trypanolytic factor 1 and 2 (TLF-1 and TLF-2) [6]. TLF-1 binds to the parasite through an interaction between the haptoglobin-related protein (Hpr) surrounding the TLF-1 particle and the haptoglobin hemoglobin receptor (HpHbR) in the flagellar pocket of the parasite [7–9]. Under the acidic conditions found in the lysosome, APOL1 changes conformation and embeds in the lysosomal membrane, forming pores in the organelle, leading to cell death [5,10]. A proportion of TLF-2 similarly enters trypanosomes via HpHbR, although an alternate route also contributes to uptake [11].

Although TLF-1 and 2 kill the majority of trypanosome species, two sub-species of *T. brucei* have evolved to overcome this innate immunity. *T. b. rhodesiense* and *T. b. gambiense* are both resistant to lysis by APOL1 and establish bloodstream infections in humans [1]. *T. b. rhodesiense* causes an acute form of the disease and is found in East Africa whereas *T. b. gambiense* is found in West and Central Africa. *T.*

b. gambiense causes a more chronic form of the disease and is responsible for 97% of all human cases of trypanosomiasis [12]. The mechanism of human serum resistance for *T. b. rhodesiense* involves the expression of a truncated VSG, termed serum resistance associated (SRA) protein [13,14]. SRA binds to APOL1 in the lysosome, preventing lysis [14]. However, the SRA gene is absent from *T. b. gambiense*, the more prevalent human infective sub-species [15]. The *T. b. gambiense* subspecies consists of two sub-groups (1 and 2) that differ in phenotype, including their associated pathology. Group 1 *T. b. gambiense* parasites are the most prevalent of the human infective trypanosomes and are responsible for the vast majority of cases [16]. Group 1 *T. b. gambiense* can be distinguished by both their reduced efficacy of HpHbR for binding TLF-1, due to a conserved single nucleotide polymorphism [17– 19] and also by the presence of a specific truncated VSG, TgsGP [20]. The TgsGP gene is present in all group 1 isolates examined to date but not in *T. b. brucei*, *T. b. rhodesiense* or group 2 *T. b. gambiense* [20–23]. The specificity of TgsGP to group 1 *T. b. gambiense* and its resemblance to SRA, in that it is a truncated VSG gene, led to a suggestion that this gene may confer human serum resistance to group 1 *T. b. gambiense* [20]. The gene was transfected into *T. b. brucei* where it did not confer increased resistance to human serum. It was hypothesized that if TgsGP was involved in human serum resistance other factors would also be required to confer the phenotype in *T. b. brucei* [20]. Efforts to delete the gene from *T. b. gambiense* were unsuccessful and the function of TgsGP remained unknown [20]. Here we have successfully deleted the TgsGP gene from *T. b. gambiense* and

demonstrated that it is essential for human serum resistance and requires a *T. b. gambiense* genetic background in order to function.

Methods

Trypanosomes strains and maintenance

Bloodstream form *T. b. brucei* Lister 427 (MITat 1.2) was grown at 37uC under 5% CO_2 in HMI9 medium supplemented with 20% fetal bovine serum (Sigma-Aldrich) and 20% Serum-Plus (Sigma-Aldrich). The bloodstream form group 1 *T. b. gambiense* strain ELIANE (MHOM/CI/52/ELIANE) was isolated from a patient infected while in Coˆte d'Ivoire [22]. It was cultured in modified HMI9 [32] supplemented with 20% serum plus (SAFC Biosciences Ltd.). Similar to other group 1 *T. b. gambiense* strains, ELIANE is consistently resistant to lysis by human serum, despite repeated passage.

Transfection of *T. b. brucei* and group 1 *T. b. gambiense*

T. b. gambiense and *T. brucei* strains were transfected using the protocols outlined in [33]. For ectopic expression of TgsGP in T. b. brucei and reinsertion into the TgsGP$^{2/0}$ T. b. gambiense strains, the TgsGP ORF was inserted into the pURAN vector [34] using G418 for selection. Ectopic expression of *Tbb*HbHpR in *T. b. gambiense* was achieved using the tubulin-targeting *Tbb*HbHpR pTub-phelo construct, using phleomycin for selection [17]. For deletion of TgsGP from the genome of *T. b. gambiense* and *Tbb*HbHpR$^{2/+}$T. b. gambiense, 500 base pairs from

both the upstream and downstream regions of TgsGP (sequence AM237444.1, http://www.genedb. org) were inserted into a vector containing a hygromycin resistance cassette. Insertion of TY-tagged TgsGP into the deletion strain *T. b. gambiense* and *T. b. brucei* was performed by inserting a TY tag into a HindIII restriction site at position 1130 of the TgsGP ORF. This sequence was ligated into the pURAN vector [34,35] and transfectants were screened using a G418 selection marker. This insertion site is upstream of the predicted GPI anchor site identified using the big-PI software package [25] and a GPI prediction protocol validated for trypanosomes [26]. Correct integration for constructs was assessed by PCR and/or RT-PCR. All primers used in the studies and their targets, are listed in Table S3.1.

RT-PCR of expressed *Tbb*HpHbR in Group 1 *T. b. gambiense*

Total RNA was isolated from cells using RNeasy kit (Qiagen) according to manufacturers' instruction, with additional DNase steps. 2 mg RNA was subject to a second round of DNase treatment (Invitrogen) prior to cDNA synthesis using Superscript III (Invitrogen), according to manufacturers' instructions. RT- PCR was performed using Taq DNA polymerase and the primers are described in Table S1. For RFLP analysis of HpHpR, the amplified product was cleaned using GeneJet PCR purification column, digested with HpyCh4V and the digested products separated on a 2% agarose gel.

TLF-1 purification

TLF-1 purification, labeling and survival assays were performed as previously described [17,36].

Generation of recombinant APOL1

APOL1 synthesis and purification was performed as previously described [36]. Protein purity was estimated using a Nanodrop spectrometer (Nanodrop) and SDS-PAGE. A Western blot using an antibody raised against an APOL1 peptide (Sigma-Aldrich) was used to verify that the bands present were APOL1.

Lysis survival assays

To assess survival in human serum, trypanosomes were diluted to $5x10^5$ per ml in HMI9 and incubated for 24 hours with 20% human serum or 20% non-lytic fetal bovine serum (FBS). The number of surviving trypanosomes in each well was recorded after 24 hours using a hemocytometer. To assess survival in TLF-1 and APOL1, trypanosomes were diluted to $5x10^5$ per ml in HMI9 with FBS. Cells were incubated with a physiological amount of TLF-1 (10 mg/ml). For the recombinant APOL1 assays, a concentration of 50 mg^{21} ml was used as this had previously been determined to kill 100% of *T. b. brucei* cells in a 24-hour assay [36]. The number of cells in each well was counted with a hemocytometer at 24 hours. There were four replicates for each data point. The numbers of surviving cells for each treatment were compared between each of the *T. b. gambiense* clones and wild-type T. b.

gambiense using the unpaired 2-tailed t-test function of the Minitab 14 Statistics Package (Minitab).

Immunofluorescence assays

TLF-1 immunofluorescence assays were performed as previously described [17,36]. Immunofluorescence localization of TY- TgsGP was performed with approximately 10^6 bloodstream- cultured parasites in mid-log phase. Cells were incubated with 5 mg/ml FITC conjugated Concanavalin A in serum-free HMI9 for 20 minutes at 4uC. The Concanavalin A binds to glycoproteins in the flagellar pocket but is not endocytosed due to the reduced temperature, thus labeling the flagellar pocket [27]. Cells were then fixed by immersion in chilled methanol for 30 minutes. Slides were incubated for 1 hour with 1:500 primary mouse anti-TY antibody (Iain Johnston, University of Glasgow), washed with PBS and then incubated with 1:1000 of AlexaFluor568 anti-mouse secondary (Invitrogen). The slides were mounted using 50% glycerol, 0.1% DAPI and 2.5% DABCO. Parasites were imaged using a Deltavision Core system and SoftWorx package (Applied Precision). Images were composited using the ImageJ software package [37].

Results

Deletion of TgsGP in wild-type group 1 *T. b. gambiense*

To assess whether TgsGP is involved in human serum resistance in *T. b. gambiense*, the gene was deleted from the genome of a group 1 *T. b. gambiense*

strain. All strains of *T. b. gambiense* investigated so far are hemizygous for TgsGP, allowing a complete knockout with just one round of transfection [20–22]. Although it was postulated that TgsGP was an essential gene and could not be deleted [20], several TgsGP$^{2/0}$ clones were generated in this study. One of the clones was selected for analysis and used for subsequent assays. The deletion of TgsGP from the clone was confirmed by PCR (Figure A.1A). The TgsGP$^{2/0}$ *T. b. gambiense* clones was unable to survive in the presence of normal human serum (Figure A.2) or recombinant APOL1 (Figure A.2), with significantly fewer surviving cells compared to wild-type *T. b. gambiense* (human serum t-test p = 0.001, APOL1 t-test p<0.001). The clone grew in the presence of non-lytic serum in a similar manner to wild-type *T. b. gambiense* (t-test p = 0.145). This indicates that TgsGP is involved in protecting against the trypanolytic protein APOL1. The clone was able to grow in the presence of TLF-1 and the number of cells after 24 hours does not differ significantly from that of the wild-type *T. b. gambiense* strain (t-test p = 0.511). Wild- type *T. b. gambiense* is resistant to lysis by TLF-1 due to reduced efficacy of their HpHbR for binding TLF-1. Thus lethal amounts of the lytic particle are not internalized by the parasites [18,19]. It is likely that TgsGP$^{2/0}$ *T. b. gambiense* clones are able to grow in the presence of TLF-1 because it possesses the *T. b. gambiense* HpHbR allele that is less efficient at binding TLF-1.

Deletion and reintroduction of TgsGP in *Tbb*HpHbR$^{2/+}$ *T. b. gambiense*

As previously detailed, group 1 *T. b. gambiense* is characterized by a non-functional HpHbR, which results in a reduced uptake of TLF-1 and to a lesser extent TLF-2 [17–19,24]. To investigate the effect of the loss of TgsGP in combination with TLF-1 uptake, a *T. b. gambiense* strain expressing a functional *T. b. brucei* HpHbR (*Tbb*HpHbR) and lacking TgsGP was created (termed *Tbb*HpHbR$^{2/+}$ TgsGP$^{2/0}$). Expression of both wild type and ectopic TbbHpHbR alleles was confirmed by RT-PCR (Figure A.1B). An allele-specific HpyCh4V restriction site present in the open reading frame of *Tbb*HpHbR, but absent in *Tbg*HpHbR, was used to distinguish between the alleles (Figure A.1B) and demonstrated that both alleles were expressed, although the TbgHpHbR allele exhibits lower expression relative to the *Tbb*HpHbR allele. The strain expresses a fully functional HpHbR and hence takes up TLF-1 to a degree similar to *T. b. brucei*, confirmed by fluorescence microscopy (Figure A.3). *Tbb*HpHbR$^{2/+}$ TgsGP$^{2/0}$ *T. b. gambiense* clones were killed in the presence of normal human serum, recombinant APOL1 or, unlike TgsGP$^{2/0}$ clones, physiological levels of TLF-1 (Figure A.2).

The number of remaining cells at 24 hours was significantly lower than wild-type *T. b. gambiense* (human serum t- test p = 0.001, TLF-1 t-test p=0.001, APOL1 t-test p<0.001). However, the cells were able to grow in the presence of non-lytic serum in a similar manner to wild-type *T. b. gambiense* (t- test = 0.690). A *T. b. gambiense* clone with TgsGP and the functional *Tbb*HpHbR was able to grow in the presence of human serum and APOL1 (Figure A.2) with cell number not significantly

differing from wild-type *T. b. gambiense* (human serum t-test p = 0.936, APOL1 t-test p = 0.465) or in the presence of non-lytic serum (t-test p = 0.972). However, the clone displayed a trypanostatic growth effect in physiological levels of purified TLF-1 with significantly fewer surviving cells compared to wild type (Figure 3.2) (t-test p = 0.001).

To confirm that the loss of resistance to human serum, APOL1 and TLF-1 in *Tbb*HpHbR$^{2/+}$ TgsGP$^{2/0}$ *T. b. gambiense* was due to the loss of TgsGP, the gene was re-introduced into this background. Resistance to human serum, TLF-1 and APOL1 was rescued by the re-introduction of TgsGP, confirming that this gene is essential for resistance to lysis (Figure A.2). When the same TgsGP add-back construct was transfected into a human serum sensitive *T. b. brucei*, it did not confer resistance to any lytic component (Figure A.2), confirming earlier work [20].

Localization of TgsGP

Previous work has shown that TgsGP localizes to the flagellar pocket in *T. b. gambiense* and this is likely to be the site of interaction between TLF and TgsGP [20]. A possible hypothesis for the observation that when TgsGP is transfected into in *Tbb*HpHbR$^{2/+}$ TgsGP$^{2/0}$ *T. b. gambiense* background it restores human serum resistance but does not confer resistance in T. b. brucei [20] (Figure A.2) is that the protein is not trafficked correctly to the flagellar pocket. In order to verify localization, TgsGP was transfected into wild-type *T. b. brucei* with the addition of a TY tag into a HindIII restriction site at position 1130 of the TgsGP ORF, upstream of the predicted GPI anchor sequence [25,26].

Immunofluorescence with anti-TY antibodies shows clear localization of TY-TgsGP adjacent to the kinetoplast and co-localization with fluorescent Concanavalin A, which acts as a marker for the flagellar pocket [27], (Figure A.4). However, these cells were killed in human serum, TLF-1 or APOL1 (Figure SA.1). A similar localization is observed when the TY-tagged TgsGP protein is expressed in *Tbb*HpHbR$^{2/+}$ TgsGP$^{2/0}$ *T. b. gambiense* (Figure A.4), with strong signal close to the kinetoplast and a more diffuse signal closer to the nucleus. In this case, the capacity to grow in human serum, TLF-1 and APOL1 was restored by the reintroduction of the TY-tagged TgsGP (Figure SA.1). As an identical construct was used in both transfections, it is probable that group 1 *T. b. gambiense* possess a protein or mechanism complementing TgsGP that is absent in *T. b. brucei*.

Discussion

This study demonstrates that the TgsGP gene is essential for resistance to human serum in the most clinically important *T. brucei* sub-species, group 1 *T. b. gambiense*. Previous work has shown that TgsGP did not confer resistance to human serum when ectopically expressed in *T. b. brucei* [20], which was confirmed here. As originally hypothesized [20], it appears likely that this is due to other factor(s) or mechanism(s) that works in concert with TgsGP, which are absent in *T. b. brucei*. By removing TgsGP from *T. b. gambiense* itself, we have demonstrated that the gene is necessary for resistance to human serum. Elucidation of a gene essential to human serum resistance in group 1 *T. b gambiense* unlocks new avenues for future

treatment of human African sleeping sickness. These include peptide screens that neutralize the TgsGP protein, targeted antibodies or the possibility of using TgsGP as a vaccine candidate, as expression is required for parasite survival in humans. Additionally, there exists the potential that variants of APOL1 may offer protection against *T. b. gambiense*. Sera from individuals possessing certain APOL1 alleles has been shown to affect the growth of T. b. rhodesiense and it has been suggested that these alleles may be protective against *T. b. rhodesiense* [28,29]. However, this has yet to be confirmed in a case control study. Nevertheless, it is likely that there are variant APOL1 alleles that protect against group 1 *T. b. gambiense* in resistant individuals, such as the reportedly resistant Bambuti people of the Mbomo region in the Democratic Republic of the Congo [30] or recently described asymptomatic and self-cured cases from Coˆte d'Ivoire [31].

One other benefit of our study is the trypanosome research community now possesses a representative group 1 *T. b. gambiense* strain that is easily cultured, is no longer human serum resistant, yet only differs from the wild-type by a single gene. This is a powerful biological resource that could replace *T. b. brucei* as the common laboratory model for the human disease, which maybe useful, particularly as several drugs display different efficacies between sub-species [1]. As such, identifying TgsGP as a gene essential for resistance to human serum in group 1 *T. b. gambiense* will likely be important to future control of the disease.

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Author Contributions

Conceived and designed the experiments: PC CC AM SH AM. Performed the experiments: PC CC ED RK NV PCS AC. Analyzed the data: PC WW AM. Contributed reagents/materials/analysis tools: PC AC RK SH. Wrote and edited the paper: PC CC ED RK WW SH AML.

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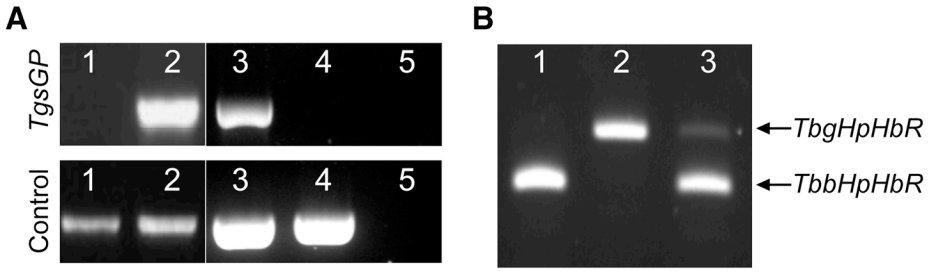

Figure A.1. PCR amplification of *TgsGP* and RT-PCR of *HpHbR* in wild-type and transfected lines. (**A**) Amplification of *TgsGP* and a control gene (cathepsin L) by PCR in [1] wild-type *T. b. brucei* [2], *TgsGP*$^{-/+}$ *T. b. brucei* [3], wild-type *T. b. gambiense*, [4] *TgsGP*$^{-/0}$ *T. b. gambiense* [5] and negative control. (**B**) RT-PCR amplification of HpHbR followed by *Hpy*CH4V restriction digestion of [1] wild-type *T. b. brucei*, [2] wild-type *T. b. gambiense* and [3] *Tbb*HbHpR$^{-/+}$*TgsGP*$^{-/0}$ *T. b. gambiense*.

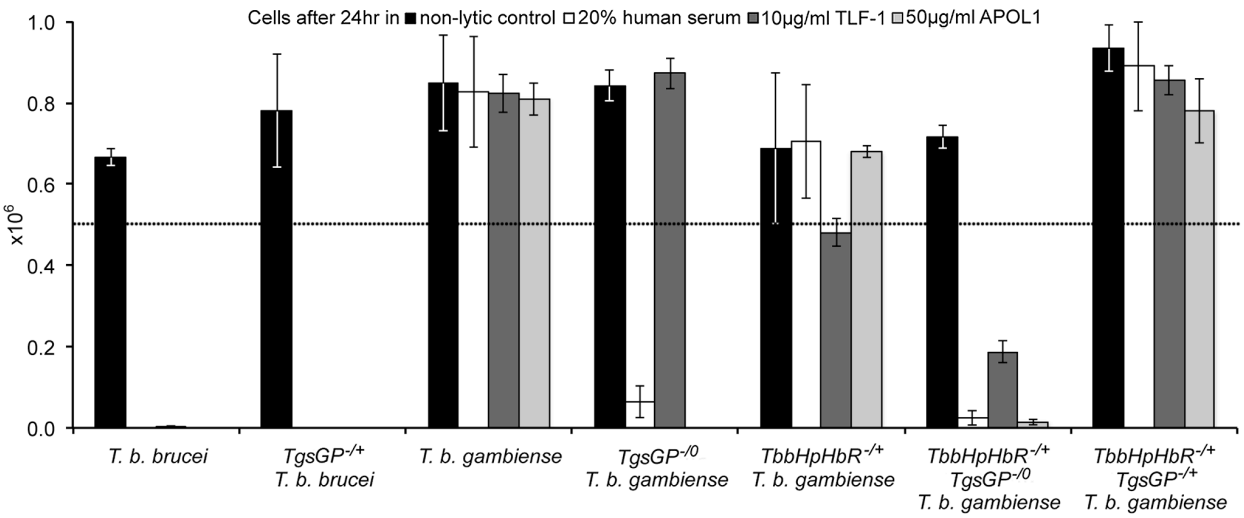

Figure A.2. TgsGP is essential for resistance to human serum in *T. b. gambiense.* The number of surviving cells after 24% human serum (open box), 10 µg/ml TLF-1 (dark grey box), 50 µg/ml recombinant APOL1 (light grey box) or a non-lytic 20% FBS control (black box). The dotted line indicates the starting concentration of 5×10 cells. The cell lines assayed were wild-type *T. b. brucei*; *TgsGP*$^{-/+}$ *T. b. brucei*; wild-type *T. b. gambiense*; *TgsGP*$^{-/0}$ *T. b. gambiense*; *Tbb*HbHpR$^{-/+}$ *T. b. gambiense*; *Tbb*HbHpR$^{-/+}$*TgsGP*$^{-/0}$ *T. b. gambiense* and *Tbb*HbHpR$^{-/+}$ *TgsGP*$^{-/+}$ *T. b. gambiense*. Standard error is shown, n = 4 for each data point.

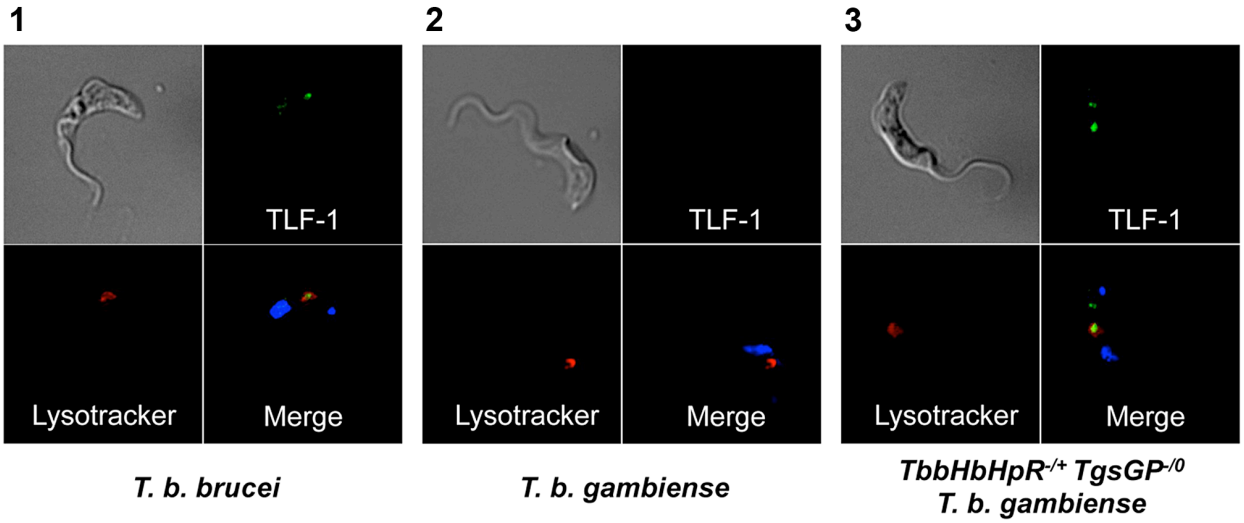

Figure A.3. Uptake of TLF-1 across strains**.** Uptake of TLF-1 after one hour in [1] wild-type *T. b. brucei* [2] wild-type *T. b. gambiense* and [3] *Tbb*HbHpR$^{-/+}$ *TgsGP*$^{-/0}$ *T. b. gambiense* by co-localization of fluorescently tagged TLF-1 (green) with the lysosomal marker Lysotracker (red). The kinetoplast and nucleus were also stained using DAPI (blue).

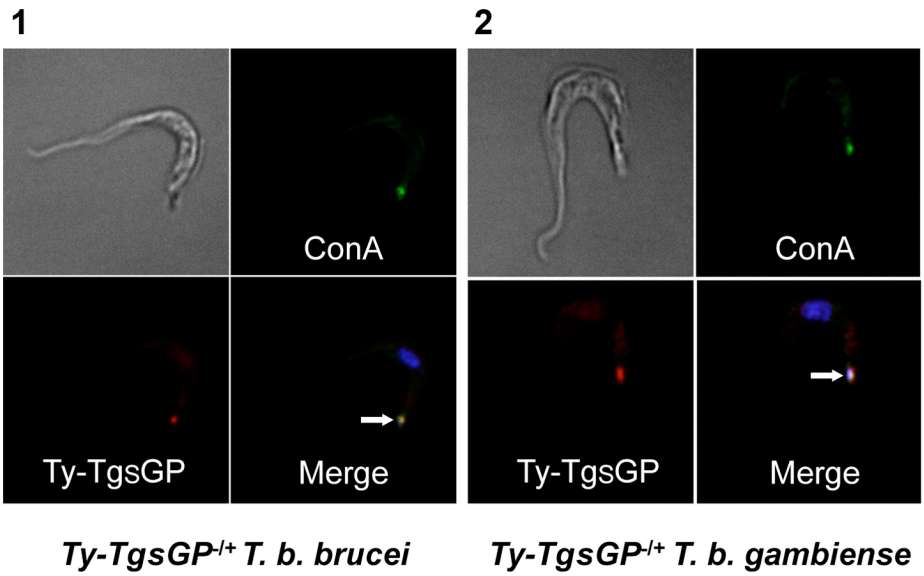

Figure A.4. Localization of TY-TgsGP. Localization of TY-tagged TgsGP (red) relative to un-endocytosed FITC-labeled Concanavalin A bound to glycoproteins in the flagellar pocket (green) and DAPI stained nucleus and kinetoplast (blue) in [1] TY-*TgsGP*$^{-/+}$ *T. b. brucei* and [2] *Tbb*HbHpR$^{-/+}$ TY-*TgsGP*$^{-/+}$ *T. b. gambiense*. The flagellar pocket (revealed by Concanavalin A and kinetoplast position) is indicated with a white arrow.

Supplemental Information

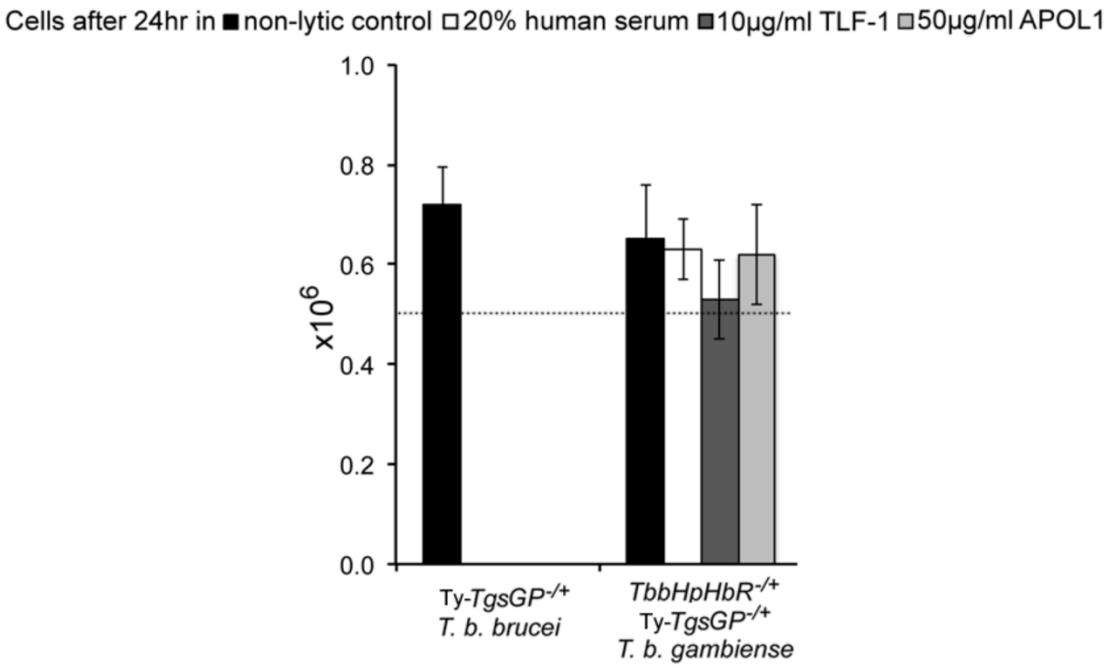

Figure SA.1. TY-TgsGP behaves similarly to TgsGP. The number of surviving cells after 24 hours incubation with 20% human serum (open box), 10 µg/ml TLF-1 (dark grey box), 50 µg/ml recombinant APOL1 (light grey box) or a non-lytic 20% FBS control (black box). The dotted line indicates the starting concentration of $5x10^5$ cells. The cell lines assayed were TY-*TgsGP*$^{-/+}$ *T. b. brucei* and *Tbb*HpHbR$^{-/+}$ TY-*TgsGP*$^{-/0}$ *T. b. gambiense.* Standard error is shown, n = 2 for each data point.

Table SA.1. Primers used and their function.

Oligo	Gene	Sequence 5'-3'	Notes/Use
35-GAPDH	*GAPDH*	TCACAGTGAAATCTGCTGCC	RT-PCR control
36-GAPDH	*GAPDH*	TCGCAATGAAGGTAAGGTCC	RT-PCR control
121-HygF	hygromycin B phosphotransferase	ATGAAAAAGCCTGAACTCAC	Hyg Integration
125HygR2	hygromycin B phosphotransferase	GCTCCATACAAGCCAACCAC	Hyg Integration
126HygR3	hygromycin B phosphotransferase	CTATTCCTTTGCCCTCGGACG	Hyg Integration
224-CatLF	Cathepsin L-like Cysteine peptidase	CAGTGACCCCAGTGAAGGAT	PCR control
225-CatLR	Cathepsin L-like Cysteine peptidase	GAGACATTGGTTTGTGCCCT	PCR control
230-TgsGF	*TgsGP*	CGTCAGCAGCAAAGGTGTTA	PCR
231-TgsGPR	*TgsGP*	CACATCTGCCAGTGTCTGGT	PCR

268_TgGP+EcoRVF	*TgsGP*	CATGGATATCATGTGGCAATTACTAGCAAT	Cloning *TgsGP* into pRM482
264_TgGP_5FlnkF1	*TgsGP* 5' flank	GATGGGCATCATACACAAGG	*TgsGP* KO 5' integration
269_TgGP+EcoRVR	*TgsGP*	CATGGATATCGTGTTAAAATATATCAAATAGCAAAG TTC	Cloning *TgsGP* into pRM482
327_5'tub	*Tubulin* 5'UTR	GAGCTAGTGAGATCAACAGTAC	Tubulin 5' integration
328_3'tub	*Tubulin* 3'UTR	AGCCTGAACGCAGACGATTTC	Tubulin 3' integration
6.440.seq3R	*HpHbR*	CGCTTCATCTCTTCCTGTGC	RT-PCR *HpHbR;* Integration of *HpHbR* into tubulin, RFLP analysis
RealT.6.440B_F	*HpHbR*	ACCTCCGCCAGAGAAAATCTC	RT-PCR *HpHbR;* Integration of *HpHbR* into tubulin, RFLP analysis
TgsGP outerF1	*TgsGP*	TCACGGCCATCAGACGGAGA	PCR and RT-PCR

TgsGP Anti	*TgsGP*	GCCATCGTGCTTGCCGCTC	PCR and RT-PCR
Oligo dT	NA	GCTCTAGATTTTTTTTTTTTTTTTTTT	Target 3' PolyA tail to sequence transcript
Splice leader RNA	NA	CCGGAATTCGCTATTATTAGAACAGTTTCT	Target 5' Splice leader to sequence transcript
TgsGP pURAN_F	*TgsGP*	GATCGGGCCCGAATTCATGTGGCAATTACTAGCAAT	Cloning *TgsGP* into pURAN
TgsGP pURAN_R	*TgsGP*	GATCGCGGCCGCGAATTCTTAAAAAAGCAAAAATGCAAGC	Cloning *TgsGP* into pURAN
QC Ava TgsGP pURAN_F	*TgsGP*	CAATTGAGGCAGGAGCACGAGCGGCAAGCACGATGGC	Verification of *TgsGP* in pURAN
QC Ava TgsGP pURAN_R	*TgsGP*	GCCATCGTGCTTGCCGCTCGTGCTCCTGCCTCAATTG	Verification of *TgsGP* in pURAN
RT TgsGP	*TgsGP*	CGTAGCTTACTGCGGTTACG	Quantifying expression of *TgsGP* using splice leader
RT HpHbR	*HpHbR*	GATCGAATTCCTACACCACCACCTGGAGCA	Quantifying expression of

			HpHbR using splice leader
RT HpHbR nested	*HpHbR*	TTAGACAATTTAAACTTGTTCAGC	Quantifying expression of *HpHbR* using splice leader
RT enolase	Enolase	CCAACCGGGAAAGCCAAATTTAGC	Quantifying expression of the Enolase control using splice leader