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Study on endocytosis and hemoglobin uptake in different developmental stages of *Trypanosoma congolense* IL3000 strain

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ABSTRACT

The study aimed to show endocytosis and hemoglobin uptake in *Trypanosoma congolense*. All the different developmental stages of *T. congolense*, IL3000 strain were cultured with dextran Alexa Fluor® 568 10,000 MW, dextran fluorescein 70,000 MW and hemoglobin labeled with Alexa Fluor® 488. The trypanosomes were also incubated in Hoechst® 33342 and observed by confocal laser scanning microscopy. The results showed that non-specific endocytosis was up-regulated in metacyclic form (MCF) and bloodstream form (BSF) whereas receptor-mediated hemoglobin endocytosis was highly activated only in the insect stage epimastigote form (EMF). This might indicate that the rapid uptake of surface-bound molecules in MCF and BSF may be important in the survival of the trypanosomes in the mammalian host. However, there was no observed non-specific endocytosis in the two insect stages procyclic form (PCF) and EMF, which might suggest that these stages totally depend on receptor-mediated endocytosis. Furthermore, the results showed uptake of hemoglobin in EMF but not in other stages. This might explain as to why a haptoglobin-hemoglobin receptor (TcHpHbR) has been specifically expressed in EMF stage of *T. congolense*. Therefore, TcHpHbR mediated hemoglobin requirement of the insect stage is probably higher than other stages because of their habitat and fully activated mitochondrial metabolism.

Key words: endocytosis, haptoglobin-hemoglobin receptor, *Trypanosoma congolense*

INTRODUCTION

The tsetse-transmitted African trypanosomes cause disease of importance to the health of both humans and livestock. Animal African Trypanosomosis, a disease mainly caused by the protozoan parasite *Trypanosoma congolense*, is a major constraint to livestock productivity and has significant impact in the developing countries of Africa (Connor, 1994). The tsetse fly takes up bloodstream form (BSF) trypanosomes from mammalian hosts that initially get established and differentiate into procyclic forms (PCF) in the fly midgut and lose the variant surface glycoprotein (VSG) coat. The notable VSG coat is used to evade the mammalian host's immune system. They migrate to the proboscis where they attach as epimastigote forms (EMF) and finally differentiate into infective metacyclic forms (MCF) that are transmitted to a mammalian host during the next blood meal (Tetley *et al*., 1985; Peacock *et al.*, 2012).

The protective surface coat made from the VSG prevents elimination of the parasites by the host antibody responses (Barry *et al*., 2001). Changes also occur in the energy metabolism pathways and in the trypanosome surface in relation to evasion of the mammalian host's immune response through antigenic variation (Vickerman, 1985; Natesan *et al*., 2007). The coat consists of receptor proteins that allow nutrient uptake, including the haptoglobin-hemoglobin receptor (HpHbR). This receptor is used by the parasite to acquire heme from the haptoglobin-hemoglobin complex which provides cofactor for heme-containing enzymes. In case of *T. brucei*, it was reported that HpHbR was also a component of the innate immune

response of the host to trypanosome infection and functions as the primary receptor by which trypanosome lytic factors (TLF-1, TLF-2), components of human serum that causes trypanosomes to swell and die, are taken up (Jerome *et al.*, 2001; Kieft *et al*., 2010; Whitney *et al.*, 2012).

Extracellular macromolecules enter the trypanosome flagellar pocket through a specific surface receptor or nonspecifically, and internalised by either non-specific endocytosis or receptor-mediated endocytosis (Morgan *et al*, 2002a, b). In non-specific endocytosis, extracellular macromolecules, such as protein complexes and lipoproteins, are brought into the trypanosome by forming an invagination, within small vesicles that fuse with lysosomes to hydrolyse or to break down the macromolecules. On the other hand, receptor-mediated endocytosis is a selective mechanism enabling trypanosomes to ingest large amounts of specific ligands to lysosomes. The trypanosomes use receptors, clustered together in clathrin-coated pits that specifically recognise and bind to the macromolecules.

Receptors within the VSG coat mediate uptake of large ligands from the host, the two best-characterised being *T. brucei* transferrin receptor for iron and HpHbR for heme (Vanhollebeke *et al*., 2008). Although *T. brucei* HpHbR plays an important role in hemoglobin-haptoglobin complex uptake and human serum resistance of the BSF parasite, roles of HpHbR in insect stages (PCF, EMF and MCF) of the parasite are unknown yet. A gene encoding HpHbR has been cloned from *T. congolense* (TcHpHbR) (Matthew *et al.*, 2013). According to the EST and proteome analyses, TcHpHbR was specifically expressed in EMF stage of *T. congolense* (Helm *et al*., 2009; Eyford *et al*., 2011). Hemoglobin requirement of the insect stages is probably higher than BSF because of their habitat and fully activated mitochondrial metabolism. Therefore, the main aim of the study was to show the non-specific endocytosis and receptor-mediated endocytosis in different stages of the *T. congolense* and clarify the importance of hemoglobin uptake in the different developmental stages of the *T. congolense.*

MATERIALS AND METHODS

Trypanosomes

Trypanosoma congolense, IL3000 strain, which is a savannah-type parasite, was used in this study. The PCFs, EMFs and MCFs were maintained *in vitro* in the TVM-1 medium and BSFs maintained in HMI-9 medium as described previously (Hirumi and Hirumi 1991).

Dextran uptake

Dextran Alexa Fluor® 568 10,000 MW anion fixable conjugate, (Life Technologies™) was prepared by mixing 5 mg dextran with 100 µL sterilised phosphate buffered saline (PBS) to a concentration of 50 mg/mL of which 10 µL was diluted 100 times with PBS as a working solution. Dextran fluorescein 70,000 MW anionic lysine fixable conjugate, (Life Technologies™) was prepared by mixing 25 mg dextran with 1 mL sterilised PBS to a concentration of 25 mg/mL of which 20 µL was diluted 50 times with PBS as a working solution. BSF, PCF and EMF were prepared by collecting 1 mL of the culture and centrifuged (860 xg, at 27°C for 5 minutes) and the supernatant discarded, while MCF was purified from EMF culture supernatants by DE52 anion-exchange column chromatography. Labeled dextran conjugate was added to the culture and incubated for 2 hours at 27ºC (PCF, EMF and MCF) and 33ºC (BSF). The culture was centrifuged and washed with 500 µL of PBS containing 1 mM glucose (PSG). The trypanosomes were fixed by adding 100 µL of 0.1% paraformaldehyde and incubated on ice for 5 minutes and then centrifuged at 860 xg, at 27°C for 5 minutes. The supernatant was discarded and 100 µL 1 mg/mL Hoechst® 33342, (Dojindo™) diluted 500 times in PBS was added and incubated for 30 minutes at 37°C. The culture was centrifuged at 860 xg, at 27°C for 5 minutes, the supernatant discarded and 10 µL of PSG added. Wet smears were made and covered with cover glass and observed using confocal laser scanning microscope (Leica Microsystems®, Leica TCS SP5 II) at 630 times magnification. For the negative control experiment, TVM-1 media was added to PCF, EMF and MCF culture and HMI-9 was added to BSF culture. Dextran experiments were divided into three sets of experiments. The first was Dextran Alexa Fluor® 568 10,000 MW anion fixable conjugate incubated

with all the stages of the trypanosomes. The second was dextran fluorescein 70,000 MW anionic lysine fixable conjugate incubated with all the stages of the trypanosomes. The third was a mixture of dextran 10,000 MW and 70,000MW conjugates incubated with all the stages of the trypanosomes.

Hemoglobin labeling, purification and uptake

Bovine red blood cells were separated from venous blood collected from healthy cattle. Bovine red blood cells were separated by centrifugation (1,600 xg, 4^{o}C for 10 minutes) and washed three times in sterile PBS. The cells were lysed and suspended in PBS diluted 100-fold. After hemolysis, the cells were centrifuged at 21,500 xg at 4^{o}C for 10 minutes and the supernatant discarded. Bovine hemoglobin powder was prepared by freeze-drying in a freeze drier (TAITEC™). Hemoglobin solution was prepared by dissolving 2 mg Hemoglobin powder in sterile PBS to 2 mg/ml. The bovine hemoglobin solution was labeled by using Alexa Fluor® 488 (HbA488) protein labeling kit (Invitrogen Molecular probes, Life Technologies™). As a negative control, 2 mg/ml bovine serum albumin (BSA) was labeled with Alexa Fluor® 488 (BSAA488). EMF were attached onto autoclaved cover slides for 3 days in a tight moisture box and washed with TVM-1. PSG was mixed with 20 ng/mL HbA488 up to 200 µL and added 100 µL of the mixture onto cultured cover glass, covered with the parafilm® "M" laboratory film (Pechiney Plastic Packaging) and incubated the covered cover glass for 2.5 hours. The cover glass was washed 3 times with PBS and fixed in methanol for 10 minutes. The cover glass was washed with PBS 3 times and incubated in 1 mg/mL Hoechst® 33342 (diluted 500 times in PBS) for 10 minutes at room temperature. After washing with PBS 3 times, the cover glass was fixed onto the glass slide with mountant, Permafluor®, Thermo Fisher Scientific™, then sealed with nail polish and observed using confocal laser scanning microscope. BSAA488 (2 mg/mL) was added to the culture as a negative control. The other trypanosome stages were prepared by collecting 1 mL of MCF, BSF and PCF and centrifuged at 860 xg at 27^{o}C for 10 minutes and after discarding the supernatant, were re-suspended in 1 mL of PSG. The trypanosomes were incubated (27^{o}C for MCF and PCF, 33^{o}C for BSF) with 200 µL of 20 ng/mL HbA488 for to 2.5 hours. The culture was centrifuged; supernatant discarded and washed 2 times with 1.3 mL PBS. After re-suspending in 30 µL of PBS, the trypanosomes were fixed onto 12-well slide glass with methanol for 10 minutes. The trypanosomes were washed with PBS and 1 mg/mL Hoechst® 33342 added for 10 minutes and washed with PBS. A cover glass was fixed to the 12-well glass slide with mountant, Permafluor®, Thermo Fisher Scientific™ and observed using confocal laser scanning microscope.

RESULTS

The results of BSF and MCF cultured with Alexa Fluor 568 dextran 10,000 MW and dextran fluorescein 70,000 MW conjugates showed increased uptake and accumulation of dextran 10,000 MW and 70,000 MW in the trypanosomes as seen using confocal laser scanning microscope. Live cell imaging of BSFs, MCFs, PCFs, and EMFs are shown in the Figures 1-3. This shows that the internalisation of the fluid phase marker labeled dextran 10,000 MW and 70,000 MW is increased in MCF and BSF as compared to EMF and PCF.

The confocal laser scanning microscopy results of the *T. congolense* cultured with Alexa Fluor® 488 hemoglobin show that there is uptake and accumulation only in EMF compared to other stages (Fig. 4A and 4B). There was no uptake of BSA in all the stages of the trypanosomes (Fig. 4C).

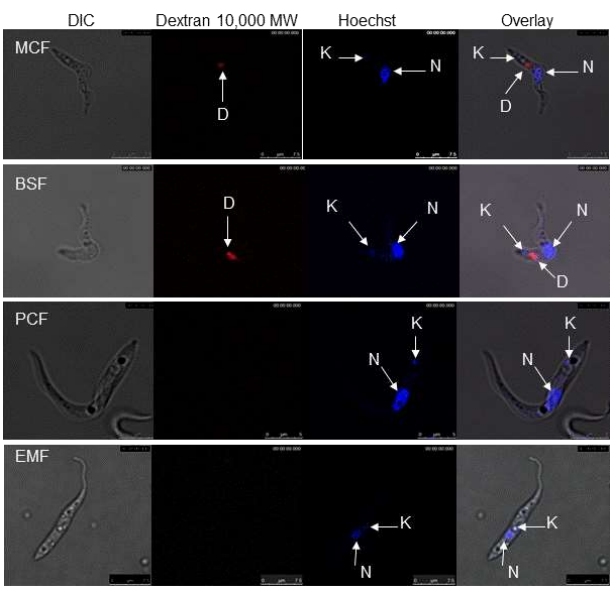

Fig. 1. All life cycle stages of *T. congolense* labeled with Alexa Fluor 568 Dextran 10,000 MW (Red) and Hoechst 33342 (Blue). This shows the uptake of the fluid phase marker dextran 10,000 MW in MCF and BSF.

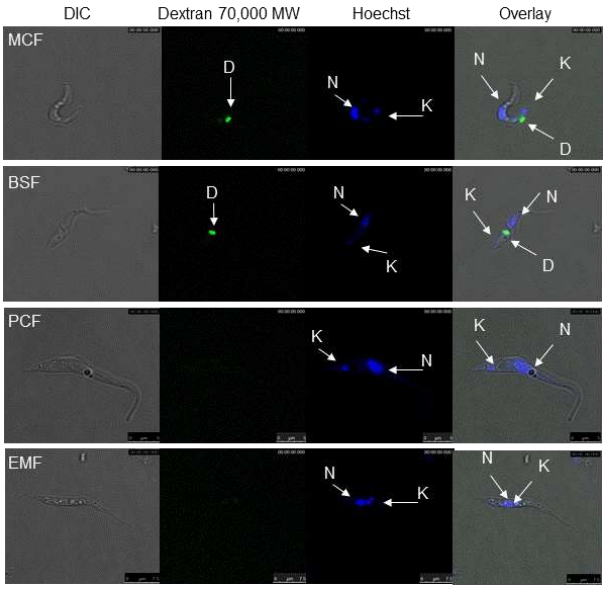

Fig. 2. All life cycle stages of *T. congolense* labeled with Alexa Fluor 568 Dextran 70,000 MW (Green) and Hoechst 33342 (Blue). This shows the uptake of the fluid phase marker dextran 70,000 MW in MCF and BSF.

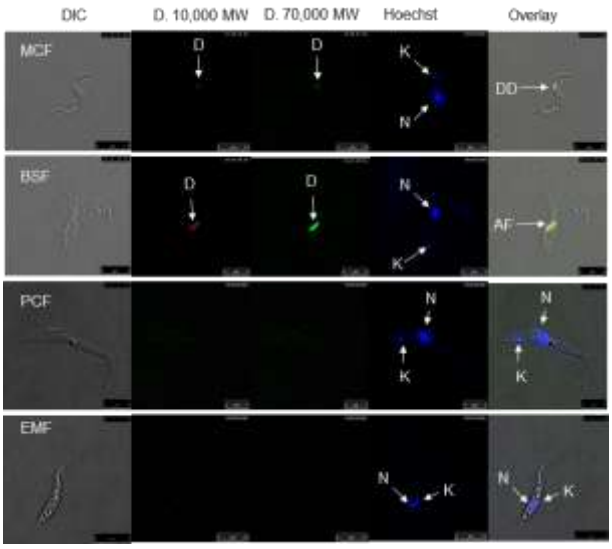

Fig. 3. All life cycle stages of *T. congolens*e labeled with Dextran Alexa Fluor 568 MW 10,000 (Red), Fluorescein MW 70,000 (Green), and Hoechst 33342 (Blue). This shows the uptake of the fluid phase marker dextran MW 10,000 and MW 70,000 simultaneously in MCF and BSF.

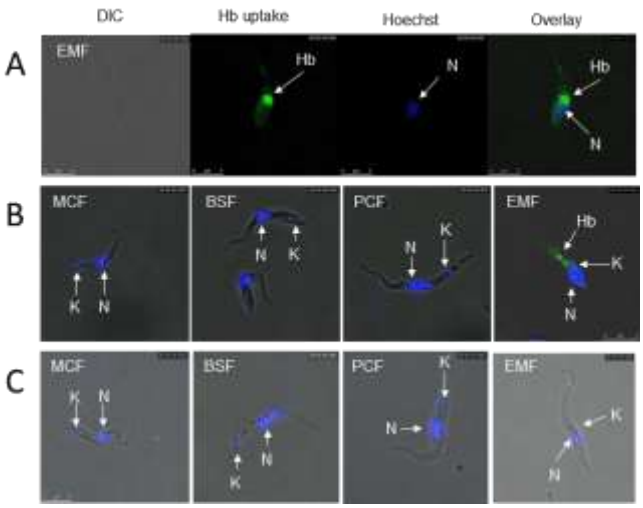

Fig. 4. (A) EMF Trypanosomes Labeled with Alexa Fluor 488-Hemoglobin (Green) and Hoechst 33342 (Blue). This shows the uptake of hemoglobin only in EMF. (B) All life cycle stages of *T. congolense* labeled with Alexa Fluor 488-Hemoglobin (Green) and Hoechst 33342 (Blue). This also shows the uptake of hemoglobin in EMF. (C) All life cycle stages of *T. congolense* labeled with Alexa Fluor 488-BSA and Hoechst 33342 (Blue). This shows lack of uptake of BSA in all trypanosome stages.

DISCUSSION

T. congolense BSF and MCF cultured with Alexa Fluor® 568 dextran 10,000 MW and dextran fluorescein 70,000 MW conjugates showed increased uptake and accumulation as observed using confocal laser scanning microscope, while the two insect stages did not show this feature (Fig. 1-3). This indicates that the internalisation of the fluid phase marker dextran 10,000 MW and 70,000 MW and is increased in MCF and BSF as compared to EMF and PCF. On the other hand, the EMF incubated with Alexa Fluor® 488 labeled hemoglobin showed accumulation of hemoglobin in the flagella pocket of the trypanosomes as seen in the confocal laser scanning microscopy and no other developmental stage showed this accumulation (Fig. 4A and 4B). These results demonstrate that non-specific endocytosis is highly up-regulated in BSF and MCF compared to PCF and EMF, during the gain or loss of the surface glycoprotein coat that accompanies the change in form between these life cycle stages. (Natesan *et al*., 2007). Rapid internalisation of surface-bound immune factors by non-specific endocytosis may contribute to the survival of the trypanosomes in the mammalian host. Such endocytic activity correlates with VSG expression and rapid internalisation of surface-bound antibodies (Nikolay *et al.*, 2012; Vanhollebeke *et al*., 2008; Vanhollebeke *et al*., 2010; Webster P, 1989). Natesan *et al*. (2007) showed that salivary gland MCF dramatically increase expression of endocytosis, indicating that emergence of mammalian infective forms is coupled with re-acquisition of a high-activity endocytic-recycling system. This suggests that non-specific endocytosis in BSF and MCF is an adaptation required for viability in the mammalian host. Non-specific endocytosis by African trypanosomes is also important for nutrient uptake and possibly for processing of intracellular VSG (Webster P, 1989). An understanding of the mechanisms involved may lead to some novel ways to control trypanosomosis.

There is a down-regulation of non-specific endocytic activities that occurs in the trypanosomes upon entering the insect midgut. This expression was explained by the lack of uptake of the dextran 10,000 MW and 70,000 MW labeled conjugates in the PCF and EMF which suggests that non-specific endocytic activity is not required for remodeling the parasite surface or for survival within the tsetse fly. Liu *et al*. (2000) have demonstrated the importance of receptor-mediated endocytosis in PCF trypanosomes. Large coated endocytic vesicles are absent in PCF trypanosomes, and are generally assumed that little or no endocytosis occurs in PCF trypanosomes. It has also been shown that PCF trypanosomes can take up fluid-phase markers but with a rate much slower than that in BSF trypanosomes (Hall *et al.*, 2005). The results also agree with this concept as there was no dextran uptake in the PCF. The different rates of endocytosis between different stages of parasites may be due to differences in morphological and functional properties of the flagellar pocket in different stages of the parasite. Endocytosis from the flagella pocket ceases on transformation to the multiplicative PCF and eventual progressive activation of the mitochondrion culminating in the non-multiplicative PCF (Vickerman *et al.*, 1988).

There is also an indication that receptor-mediated endocytosis was highly activated in the insect stage EMF. There was no non-specific endocytosis observed in the two insect stages of PCF and EMF, which suggests that these stages totally depend on receptor-mediated endocytosis. This was confirmed by uptake and accumulation of hemoglobin in the EMF. Therefore, TcHpHbR mediated hemoglobin requirement of the insect stages is probably higher than mammalian stages because of their habitat and fully activated mitochondrial metabolism. The study results have tallied with Vickerman *et al.* (1988) who have also shown attached and non-attached cytoskeletal proteins association with EMF which may suggest that heme is needed in the EMF development for its attachment process to the endothelium of the proboscis coupled with the activated mitochondrion. Hence the total dependence on receptor-mediated endocytosis through the TcHpHbR mediated hemoglobin.

African trypanosomes are protected by a densely packed surface monolayer of variant surface glycoprotein (VSG). In the primate *T. brucei* BSF, HpHbR within this VSG coat mediates heme acquisition. HpHbR is also exploited by the human host to mediate endocytosis of trypanolytic factor (TLF) 1 from serum, contributing to innate immunity. In their study, Matthew *et al.* (2013) provided an insight into the

role of HpHbR in mediating both nutrient uptake and innate immunity in a balancing act essential for parasite survival. In humans, the *T. brucei* HpHbR also binds hemoglobin–haptoglobin-related protein complexes that are part of the high-density lipoprotein (HDL) particles containing trypanolytic apolipoprotein L1. Thus this HpHbR binds HDLs and contributes to killing of susceptible parasites. Its role in *T. congolense* has not been fully understood and has not been expressed in BSF. This study did not show this trend in the BSF as well. Vanhollebeke *et al.* (2008) and Eyford *et al.* (2011) showed that there is a marked up regulation of a haptoglobin–hemoglobin receptor in EMF. This can perhaps be explained by the requirement for this form of the parasite to acquire heme in the nutrient scarce environment of the tsetse mouthparts. Vanhollebeke *et al.* (2008) also shows that this receptor normally binds the haptoglobin–hemoglobin complex at high affinity, allowing uptake of heme and supporting growth of the parasites. Vanhollebeke *et al.* (2008) data also suggest that African trypanosomes have evolved a receptor specifically designed to acquire heme from Hp-Hb for incorporation into hemoproteins that both increase the trypanosome's growth rate and resistance to the oxidative response of the host. They indicate that *T. brucei* HpHbR recognizes Hp-Hb complexes and in the presence of Haptoglobin related protein on human lytic HDL particles it triggered internalization of the Hb-exposed fraction of these particles, which also contain the trypanolytic factor apoL1. This finding now explains the stimulating effect of Hb on trypanolysis as an indirect mechanism. Thus, in human serum the presence of the Hp-Hb receptor became detrimental to the trypanosomes instead of protective. In turn, synthesis of the apoL1 physical inhibitor serum resistance–associated protein allowed *T. b. rhodesiense* to escape this problem and cause human sleeping sickness. In contrast, this study indicates lack of expression of HpHbR in the *T. congolense* MCF, BSF and PCF except EMF. This hypothetical evolvement could be as a result of the lack of TLF in the *T congolense* host. It only becomes essential in the EMF stage for the acquisition of heme.

This study demonstrates strong correlations between dextran uptake and non-specific endocytosis and also between hemoglobin uptake and receptor-mediated endocytosis. Clear understanding of these molecular changes can influence the development of the transmission blocking vaccine targets.

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REFERENCES

Barry, J.D. and McCulloch, R. 2001. Antigenic variation in trypanosomes: Enhanced phenotypic variation in a eukaryotic parasite. Adv. Parasitol. 49: 1–70.

Connor, R.J. 1994. African animal trypanosomiases. In Coetzer, J.A.W., Thomson, G.R., and Tustin, R.C. (Ed.), Infectious Diseases of Livestock (First., pp. 167–205). Oxford University Press.

Eyford, A.B., Sakurai, T., Smith, D., Loveless, B., Hertz-Fowler C, Donelson, E.J., Inoue, N., Pearson, W.T. 2011. Differential protein expression throughout the life cycle of *Trypanosoma congolense*, a major parasite of cattle in Africa. Mol. Biochem. Parasitol 177: 116–125.

Hall, S.B., Smith, E., Langer, W., Jacobs, A.L., Goulding, D. and Field, C.M. 2005. Developmental variation in Rab11-dependent trafficking in *Trypanosoma brucei*. Eukaryot. Cell 4: 971–980.

Helm, R.J., Hertz-Fowler C., Aslett, M., Berriman, M., Sanders, M., Quail, A.M., Soares, B.M., Bonaldo, F.M., Sakurai, T., Inoue, N. and Donelson, E.J. 2009. Analysis of expressed sequence tags from the four main developmental stages of *Trypanosoma congolense*. Mol. Biochem. Parasitol. 168: 34–42.

Hirumi, H. and Hirumi, K. 1991. *In vitro* cultivation of *Trypanosoma congolense* bloodstream forms in the absence of feeder cell layers. Parasitology 105: 225–236.

Jerome, D., Joseph, R.B., and Stephen, L.H. 2001. Haptoglobin-related protein mediates trypanosome lytic

factor binding to trypanosomes. J. Biol. Chem. 276: 30254–30260.

Kieft, R., Capewell, P., Turner, C.M., Veitch, J.N., MacLeod, A. and Hadjuk, S. 2010. Mechanism of *Trypanosoma brucei gambiense* (group 1) resistance to human trypanosome lytic factor. Proc. Natl. Acad. Sci. USA 107: 16137–16141.

Liu, J., Qiao, X., Du, D., Lee, M.G. 2000. Receptor-mediated endocytosis in the procyclic form of *Trypanosoma brucei*. J. Biol. Chem. 275: 12032–12040.

Matthew, K.H., Olga, T., Alan, B., Jenny, R., Jayne, R. and Carrington, M. 2013. Structure of the trypanosome haptoglobin–hemoglobin receptor and implications for nutrient uptake and innate immunity. Proc. Natl. Acad. Sci. USA 110: 1905–1910.

Morgan, W.G., Hall, S.B., Denny, W.P., Carrington, M. and Field, C.M. 2002a. The Kinetoplastida endocytic apparatus. Part I: A dynamic system for nutrition and evasion of host defenses. Trends Parasitol. 18: 491–496.

Morgan, W.G., Hall, S.B., Denny, W.P., Carrington, M. and Field, C.M. 2002b. The Kinetoplastida endocytic apparatus. Part II: A dynamic system for nutrition and evasion of host defenses. Trends Parasitol. 18: 540–546.

Natesan, S.K.A., Peacock, L., Matthews, K., Gibson, W. and Field, C.M. 2007. Activation of endocytosis as an adaptation to the mammalian host by trypanosomes. Eukaryot. Cell 6: 2029–2037.

Nikolay, G.K., Kiantra, R., George, A.M.C., Elisabetta, U. and Christian, T. 2012. Developmental progression to infectivity in *Trypanosoma brucei* triggered by an RNA-binding protein. Science 338: 1352–1353.

Peacock, L., Cook, S., Ferris, V., Bailey, M. and Gibson, W. 2012. The life cycle of *Trypanosoma* (*Nannomonas*) *congolense* in the tsetse fly. Parasit. Vectors 5: 109.

Tetley, L. and Vickerman, K. 1985. Differentiation in *Trypanosoma brucei*: Host-parasite cell junctions and their persistence during acquisition of the variable antigen coat. J. Cell Sci. 74: 1–19.

Vanhollebeke, B., Muylder, D., Muylder, J.M., Pays, A., Tebabi, P., Dieu, M., Raes, M., Moestrup, K.S. and Pays, E. 2008. A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 320: 677–681.

Vanhollebeke, B., Uzureau, P., Monteyne, D., Pérez-Morga, D. and Etienne, P. 2010. Cellular and molecular remodeling of the endocytic pathway during differentiation of *Trypanosoma brucei* bloodstream forms. Eukaryot Cell 9: 1272–1282.

Vickerman, K., Tetley, L., Hendry, A.K.K. and Turner C.R.K. 1988. Biology of African trypanosomes in the tsetse fly. Biol. Cell 64: 109–119.

Vickerman, K. 1985. Developmental cycles and biology of pathogenic trypanosomes. Br. Med. Bull. 41: 105–114.

Webster, P. 1989. Endocytosis by African trypanosomes. I. Three-dimensional structure of the endocytic organelles in *Trypanosoma brucei* and *T. congolense*. Eur. J. Cell. Biol. 49: 295–302.

Whitney, B., Rudo, K., Paul, C., Nicola, J.V., Annette, M. and Stephen, L.H. 2012. Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum and trypanosome lytic factors. Virulence, 3: 72–76.