

Plasma cytokines quantification among *Trypanosoma brucei rhodesiense* sleeping sickness cases and controls in Rumphu, Malawi

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Abstract

Introduction

Trypanosoma brucei (*T.b.*) *rhodesiense* is the cause of the acute form of human African trypanosomiasis (HAT) in eastern and southern African countries, including Malawi. For a long time, untreated HAT infections were believed to be 100% fatal. However, recent studies show that infection by *T.b. rhodesiense* can result in a wide range of clinical outcomes in its human host. Apart from other factors such as parasite diversity, cytokines have been strongly implicated to play a major role in the outcome of *T.b. rhodesiense* infections.

In this study, we quantify the levels of three cytokines Interleukin-8 (IL-8), Tumor Necrotic Factor alpha (TNF- α) and Interleukin -10 (IL-10) in plasma amongst HAT cases (treated and untreated) and controls recruited during medical survey.

Methods

Two-hundred and thirty-three plasma samples (HAT cases and controls) from Rumphu, one of the endemic areas in Malawi were used. Blood collected was centrifuged, plasma extracted and stored in cryovials at -80°C until processing. Plasma cytokine concentration was measured using ELISA.

Results

Plasma samples for 233 individuals, 76 HAT cases and 157 controls were quantified. Among the cases, nine had their plasma collected before treatment (untreated) and the rest were treated before blood for plasma analysis was collected. Controls had significantly higher mean plasmatic levels of TNF- α (94.5 \pm 474.12 pg/ml) and IL-8 (2258.6 \pm 5227.4 pg/ml) than cases TNF- α (29.35 \pm 181.58 pg/ml) and IL-8 (1191.3 \pm 4236.09 pg/ml). Controls and cases had similar mean levels of IL-10 in plasma. Only IL-8 had statistically significant higher median levels in the untreated than treated HAT cases P=0.006.

Conclusion

Our data suggest that cytokines could be considered as biomarkers of HAT infection and treatment. Further studies with a larger cohort of cases and additional cytokines which are known to be associated with HAT infection outcomes will be required to evaluate these cytokines further.

Keywords; Sleeping sickness, Human African Trypanosomiasis, *Trypanosoma brucei rhodesiense*, Plasma cytokines, IL-8, TNF- α , IL-10

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is one of the neglected tropical diseases that is endemic in sub-Saharan Africa. In the beginning of 20th century millions of people died from the disease and available estimates showed that 36 African countries and over 60 million people were at risk of being infected^{1,2}. HAT is caused by extra-cellular protozoan parasites *Trypanosoma brucei*, *T.b.*, which is transmitted by tsetse flies of the genus *Glossina*. *T. b. rhodesiense* is the cause of sleeping sickness in the eastern and southern Africa, including Malawi. Its reservoirs are in livestock and wildlife. *T.b. gambiense* causes sleeping sickness in central and western Africa, and causes the majority of human cases with the main reservoir being humans^{2,3}.

Recently there has been a reduction in the number of reported new *T. b. rhodesiense* HAT cases as well as deaths⁴. Under reporting has been associated with this reduction since many cases as high as 40% go undetected. *T. b. rhodesiense* HAT being a zoonotic disease still poses as a risk to many people earning a living in or around national parks, forest land and large water bodies^{5,6}. There are two clinical stages for sleeping sickness, the haemolymphatic stage followed by the meningoencephalitic stage. The two

subspecies have different rates of disease progression; *T.b. rhodesiense* infection is typically described as an acute disease with rapid progression to late stage whereas *T.b. gambiense* progresses more slowly³.

For a long time, untreated *T.b. rhodesiense* infections were believed to be 100% fatal, with death occurring within weeks or months of symptoms first appearing^{7,8}. However, there is increasing evidence that infection by *T.b. rhodesiense* can result in a wide range of clinical outcomes with different severity and disease progression in different human hosts⁹⁻¹¹

Some studies in human have demonstrated that there are difference in disease progression in individuals coming from same foci or geographically related foci¹². These differences could be attributed to either parasite diversity or host immune response to infections or both. Since cytokines regulate the host response to infections, trauma, inflammations and immune responses¹³, it has been suggested that cytokines could be the key players in HAT disease processes^{11,12,14}.

Once a human is infected by the African trypanosomes, innate and adaptive immune responses are activated¹⁵. Macrophages are one of the important cells involved in the innate immunity during African trypanosomiasis. Macrophages clear the parasites through phagocytosis¹⁶. The dead trypanosomes release DNA and other molecules

that activate macrophages to secrete pro-inflammatory cytokines/molecules¹⁷.

In HAT infection, some of the known Pro-inflammatory (upregulated during inflammation) cytokines are IL-1, IL-6, IL-12, TNF- α and IFN- γ ¹⁸⁻²² and the anti-inflammatory cytokines include type II cytokines such as IL-4, IL-10 and IL-13. These cytokines down regulate the macrophages to become more anti-inflammatory type, thus controlling both the parasites and pathology²³. Studies have suggested that cytokine responses may influence the outcome of African trypanosomiasis infections^{24,25}. It has been demonstrated that IL-10 and IL-6 are associated with the protection from neuroinflammatory pathology of HAT [26, 27] whereas TNF- α is involved in both parasitemia control and infection-associated pathology²⁸. IL-10 is found to be high in CSF of late-stage patients compared to early-stage patients, because it acts as anti-inflammatory protein and B-cell stimulator. Therefore, it is suggested that CSF IL-10 could be used for staging and treatment monitoring²⁹.

Other cytokines such as IL-8 play an important role in resistance to infection by attracting neutrophils and T cells to infection site³⁰. In a study of *T. b. gambiense* patients, IL-8 was high in both serum in the early stage and CSF in the late stage of infection and dropped after treatment²⁹. Published data³¹ has shown that gambiense sero positive individuals are able to control *T. b. gambiense* infection and that high levels of IL-8 are associated with infection control whereas individuals with high levels of IL10 and TNF α may end up developing the disease.

Methodology

Population and study design

The survey was done from January 2014 to June 2015 at Rumphu district hospital, northern Malawi. Rumphu District has the highest prevalence of HAT cases and is regarded as the epicenter of HAT infections in Malawi³². Sleeping sickness is endemic in areas where other tropical diseases, including malaria are also common¹⁰.

Cases were identified through active and passive surveys. Hospital case files were checked for previously diagnosed HAT patients and were followed up in their communities. All these cases had been treated with Suramin I.V 20 mg/kg body weight for 23 days and Melarsoprol I.V 3.6 mg/kg body weight for 23 days for the early and late stage of HAT respectively according to Malawi HAT Treatment Guidelines (S1 Table). They were then followed up at 3, 6, and 12 months after discharge for review. The time between treatment and sample collection varied from a month to 48 months, but 25% of the samples were collected within the same year the individuals got their treatment.

The active screening was conducted during the follow-up of HAT cases, and nine cases were identified. After taking a history and an examination related to HAT infection, venous blood was collected in heparinized tubes and taken to the laboratory at a temperature of 4°C. Eight capillary tubes were prepared from each sample and the buffy coat was examined by microscopy looking for trypanosomes. Two milliliters of whole blood was kept for DNA extraction. The remaining blood was used for plasma separation. Blood was centrifuged at 5000 revolution per minute (rpm) for 10 minutes in thermo scientific Legend Micro 17R centrifuge to separate plasma from the rest of the blood.

Using pasture pipettes (BRANDR India) the plasma was

separated from red blood cells and stored in -80°C freezer for cytokines analysis. The RBC was also stored in -80°C freezer.

This study is part of a PhD project, which looks at the immunological and genetic factors that contribute to the pathogenesis of *T. b. rhodesiense* in Malawi. Out of 350 people screened, 243 individuals were enrolled into the study^{33,34}. HAT cases were defined as individuals in whom trypanosomes were detected by microscopy in blood, lymphatic fluid or cerebral spinal fluids (CSF). HAT negative controls were defined as individuals with no signs and symptoms suggestive of HAT and no trypanosomes detected from the blood, lymphatic fluid or CSF.

Sample size for this study was calculated based on World Health Organization (WHO) indicated HAT data for Malawi of 41 \pm 13 SD HAT cases annually². To obtain 95% confidence interval at 5% type 1 error ($p < 0.05$), a minimum of 26 HAT cases and 26 controls are required to be included in the study^{35,36}. For this study, 233 samples, 76 HAT cases and 157 controls were available for cytokine measurement and all 233 samples were included in the study.

HAT cases and controls came from the same area and lived in the same area from birth and had been exposed to infection since birth.

Ethics statement

The protocol was approved by the Malawi National Health Sciences Research Committee (NHSRC), protocol numbers NHSRC 15/4/1399 and Malawi 1213. There was also local involvement of all stakeholders, and local leaders gave approval for the study to be conducted in the area. All individuals enrolled in the study were 18 years of age or older. All individuals signed informed consent forms in their native language.

Cytokines Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine concentrations {TNF- α , IL-8, IL-10, (Kit Cat. No. 555212, 555244 and 555157 respectively)} were measured using a solid phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) (BD OptiEIA, BD Biosciences Pharmingen, San Diego, USA) according to the manufacturer's instructions (BD, Biosciences) and as described previously^{12,22}. All assays were done in triplicate. The concentrations were read using a BioTek™ ELx800™ Absorbance Microplate Reader and Gen5 v1.11 reader software (BioTek Instruments, Inc., Winooski, VT, USA) as per manufacturer's instruction and as described previously^{12,22}.

Statistical analysis

Demographic characteristics of the participants were summarized by frequency (percentage), mean and standard deviation while median (interquartile range [IQR]) was used for cytokines. Data distribution normality was analyzed with the Kolmogorov–Smirnov test. Chi-square was used to test for gender differences while age was tested using Student's T-test. Cytokine group differences were explored by Mann–Whitney U test. Correlation analysis was carried out using the Spearman correlation test for non-parametrically distributed variables and Pearson correlation for normally distributed variables. Data entry and sorting were carried out using Microsoft office Excel 2016. Statistical analysis was performed using IBM SPSS software for Windows, version 23 (IBM Corp., Armonk, N.Y., USA.), and we used R version

Table 1: Demographic characteristics of all study participants-HAT cases and controls.

Variable	All (N=233)	Cases (N=76)	Controls (N=157)	P value
Age (years) M±SD	37.45 ± 13.48	39.9 ±13.9	36.3±13.1	0.281
Male gender N (%)	167 (71.7)	54 (71.1)	113 (71.9)	0.884

P values refer to Chi-square test, *p*-values <0.05 are in bold. M-mean, SD-Standard deviation, N (%)-Number (percentage).

Table 2: Cytokine levels of all study participants-HAT patients and controls.

Variable	All (N=233)	Cases (N=76)	Controls (N=157)	P value
TNF-α (pg/ml) M±SD	73.25 ±403	29.35±181.58	94.5 ± 474.12	0.033
IL-10 (pg/ml) M±SD	42.16 ± 88.85	44 ±81.7	41.27 ±92.34	0.734
IL-8 (pg/ml) M±SD	1910.48 ± 4942.35	1191.3±4236.09	2258.6 ±5227.4	0.002

P values refer to *t* test, *p*-values <0.05 are in bold. M-mean, SD-Standard deviation, N (%)-Number (percentage), TNF-α - tumor necrosis factor alpha, IL-10-interleukin 10, IL-8 -interleukin 8.

Table 3: Correlation of Demographic variables, and cytokine levels in all study participants-HAT patients and controls.

	Age	TNF-α pg/ml	IL-10 pg/ml	IL-8 pg/ml
TNF-α pg/ml	0.066 (0.323)	-		
IL-10 pg/ml	0.025 (0.708)	0.215 (0.001)	-	
IL-8 pg/ml	0.086 (0.198)	0.288 (<0.001)	0.110 (0.094)	-

Correlation coefficient refers to Spearman's rho, *p*-values <0.05 are in bold. TNF-α - tumor necrosis factor alpha, IL-10-interleukin 10, IL-8 - interleukin 8.

4.1.0 for data visualization. All reported *p*-values are two-sided, and significance was accepted at *p*<0.05.

Results

Demographic characteristics and levels of plasma cytokines of all study participants-HAT cases and controls.

Two-hundred and thirty-three plasma samples were tested, and these included 76 HAT cases and 157 controls. Among the cases, nine had their plasma collected before treatment (were untreated) and the rest were treated before blood for plasma analysis was collected.

The mean age of HAT cases was higher than controls (Table 1). Seventy-one percent (71%) of the participants in this study were male. Controls had significantly higher mean plasma levels of TNF-α (94.5 ±474.12pg/ml) and IL-8 (2258.6±5227.4pg/ml), than HAT cases (*P*= 0.033 and *P*=0.002, respectively). Mean plasma levels of IL-10 were similar between controls and patients. Cases represented (33%) of all participants (Table 2).

Correlation levels of plasma cytokines and demographic variables of all study participants

There was a statistically significant positive correlation between plasma TNF-α with IL-10 (*p*=0.001) and IL-8 (*p*<0.001) (Table 3). IL-10 and IL-8 were positively correlated but the relationship was not statistically significant (*p*=0.094).

Effect of treatment on cytokine levels.

We further examined the differences plasma cytokine levels between HAT cases who reported being on treatment before

blood collection and those who were not treated before blood collection.

IL-10 median plasma levels were higher among untreated cases than treated cases; however, the difference was not statistically significant (*P*=0.073) as shown in Figure 1.

IL-8 median plasma levels were higher amongst HAT untreated cases than HAT treated cases and were statistically significant (*P*=0.006). For TNF-α median levels, there is no statistically significant difference between the cases who were treated and those who were untreated (*P*=0.520).

Discussion

In the present study, plasma levels of IL-8, TNF-α, and IL-10 were quantified in HAT cases (treated and untreated) and controls. Our study showed statistically significant higher levels of IL-8 in plasma of untreated HAT cases than in treated HAT cases, as well as statistically significant higher levels of IL-8 in plasma of controls than in the HAT cases. IL-8 is a major cytokine that attract neutrophils during innate immune responses. In humans, over 50% of leukocytes are neutrophils, which produce antimicrobial peptides such as cathelicidins, known to be typanotoxic³⁷. The high levels of IL-8 in the untreated HAT cases is in line with the role of IL-8 of attracting neutrophils and mounting an innate immune response to infection after exposure³¹. This supports the findings by Lejon et al in *T. b. gambiense* infection conducted in DRC where it was found that the levels of IL-8 dropped after treatment²⁹. Study of *T. b. gambiense* infection in West Africa showed that “trypanotolerant” (SERO TL+) subjects had elevated levels of IL-8, IL-6, and TNF-α than patients showing a stronger inflammatory response³¹.

TNF-α is a pro-inflammatory cytokine that is upregulated

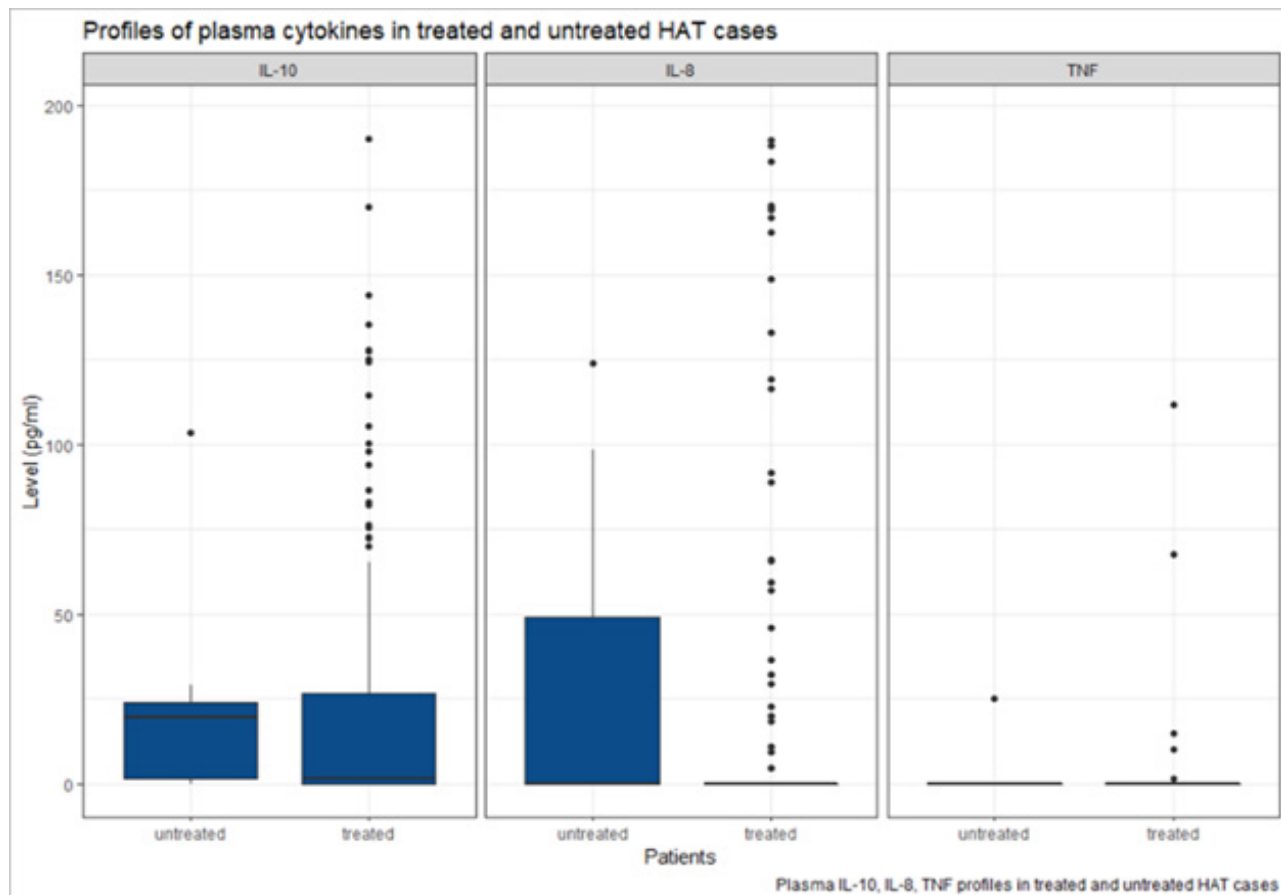


Figure 1. Profile of plasma cytokines in treated HAT cases and untreated HAT cases.

Boxes represent the medians and interquartile ranges, and whiskers represent 10th and 90th percentiles

during infection as described by Maina et al¹⁸. TNF- α is involved in the innate immunity and is produced by macrophages. Its production is induced by soluble VSGs shed by live trypanosomes³⁸. It is known that TNF- α is produced during the first peak of parasitemia in the early stages of the infection³⁹. TNF- α is involved in both parasitemia control and infection associated pathology during HAT infections²⁸. Our data showed statistically significant higher levels of TNF- α in plasma of controls than the HAT cases, but there was no statistically significant difference in the levels of TNF- α in plasma between untreated and treated HAT cases. Contrary to our data, a study by Maclean et al, showed elevated levels of TNF- α in plasma in early stages of *T. b. rhodesiense* infections in Ugandan patients but were normal in the late stages of the infection¹¹. The higher levels of TNF- α in Ugandan HAT cases was associated with rapid disease progression with increased disease severity compared to HAT cases in Malawi, who had low levels. In a similar study by Kato et al, TNF- α was only detected in plasma of two cases, which were early stage, but was not detectable in CNS (late stages)^{11,12,29}. In another related HAT study²², levels of TNF- α in plasma was low in cases and not significantly different from the controls.

IL-10 is a cytokine produced mainly by T-helper (Th) 2 cells⁴⁰. IL-10 is known to play a major role in controlling both innate and adaptive immunity. Its main function is to limit and ultimately terminate inflammatory responses. IL-10 has been shown to protect the host in a number of parasitic diseases, including malaria, toxoplasmosis and in autoimmune encephalitis^{41,42}. IL-10 down regulates the macrophages to become more anti-inflammatory type,

thus controlling both the parasites and pathology⁴³. Data from other studies have shown that IL-10 downregulates the production other cytokines such IFN- γ and TNF- α by classically activated (M1) macrophages⁴⁴⁻⁴⁶. Our data shows the levels of IL-10 in plasma were similar in controls and HAT cases, whereas there were higher levels of IL-10 in plasma of untreated HAT cases than the treated HAT cases. However, the difference was not statistically significant. Previous studies have shown that in both *T. b. rhodesiense* and *T. b. gambiense* infections, levels of IL-10 in plasma are higher in cases than controls and that IL-10 levels in both plasma and CNS were upregulated particularly in the late stage in both *T. b. rhodesiense* and *T. b. gambiense* infections^{11, 12, 29, 47-49}.

Our study has several limitations. Because of the low disease incidence, our study depended on a limited number of samples especially for the untreated HAT cases, and this could prevent the study from finding evidence of small differences in cytokine levels. Furthermore, the inclusion of retrospective HAT cases might not give a true representation of cytokine profiles as some of the cases had already recovered from HAT (duration from time of treatment to sample collection was between a month to 48 months). The study did not screen for other protozoan infections such as malaria (as it was beyond the scope of this study), which may affect levels of the cytokines in plasma in individuals with coinfection^{41,50,51}.

Conclusion

In conclusion, this study gives a preliminary insight of cytokine profiles in HAT individuals in Malawi, which may be used as a basis for further investigation in the future.

Our data suggests that IL-8 could further be evaluated as biomarkers for HAT infection. Further studies with a larger cohort of cases (followed up during treatment), and additional cytokines which are associated with HAT infection outcomes would be useful in this area.

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