Effects of *Trypanosoma brucei brucei* on Spatial learning and memory

by

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MSc (Med) in Clinical Science and Immunology

Thesis submitted to the University of Cape Town in fulfilment of the Master of Science Degree

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Declaration

I, Blessing Oluwapelumi Moses, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of Publications

During the course of my Masters' degree, I made a remarkable contribution to the following publications:

1. Brombacher, T. M., Ajonijebu, D. C., Scibiorek, M., Berkiks, I., **Moses, B. O.,** Mpotje, T., & Brombacher, F. (2020). IL-4Rα deletion disrupts psychomotor performance and reference memory in mice while sparing behavioural phenotype associated with spatial learning. *Brain, Behavior, and Immunity*, *92*(August 2020), 157–164. https://doi.org/10.1016/j.bbi.2020.12.003.

2. Brombacher, T. M., Berkiks, I., Pillay, S., Scibiorek, M., **Moses, B. O.,** & Brombacher, F. (2020). IL-4R alpha deficiency influences hippocampal-BDNF signaling pathway to impair reference memory. *Scientific Reports*, *10*(1), 1–8. https://doi.org/10.1038/s41598-020-73574-3

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List of abbreviations

AAT	Animal African Trypanosomiasis
AP	avidin-alkaline phosphatase
BBB	Blood-brain barrier
BSF	Blood stream form
CATT	card agglutination test
cDNA	complementary DNA
CNS	Central Nervous System
Conc	Concentration
CSF	cerebrospinal fluid
DEAE cellulose	Diethylaminoethyl cellulose
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
HAT	Human African Trypanosomiasis
HBBS	Hank's solution
HPRT	Hypoxanthine-guanine phosphoribosyl-transferase
HRP	avidin-horseradish peroxidase
IFN-γ	Interferon gamma
IL-10	interleukin-10
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-6	Interleukin-6
IMDM	Iscove's Modified Dulbecco's Medium
INT	Infected non-trained
IT	Infected trained
LS	long slender
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MWM	Morris water maze
NECT	nifurtimox-eflornithine complex
NINT	Non-infected non-trained

NIT	Non-infected trained
NO	nitric oxide
NTDs	Neglected Tropical Diseases
PBS	Phosphate buffered saline
PBSG	Phosphate buffered saline glucose
Pen-strep	Penicillin-Streptomycin
PMA	Phorbol myristate acetate
RAG	recombination activating gene
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SS	stumpy form
T. b.	Trypanosoma brucei
TNF	tumour necrosis factor
VSG	Variable Surface Glycoprotein
WBC	white blood cell count
WHO	World Health Organization
WT	Wild type

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Abstract

Human African trypanosomiasis (HAT), or sleeping sickness, is a sub-Saharan African disease caused by trypanosome parasites and transmitted through tsetse flies. The HAT is clinically defined by two diagnostic stages, commencing with an early-stage also known as the haemolymphatic stage where parasites appear to be localised in the blood and lymphatic systems, then a late-stage/ meningoencephalytic stage where the parasites are localised in the central nervous system.

In the late stage, the parasite crosses the blood-brain barrier to disrupt the physiological function of the brain which results in marked sleep disorder. Studies on the *Trypanosoma evansi* infection mice model has shown that in addition to the sleep disorder caused by trypanosomiasis disease, learning and memory formation were impaired. Besides, only a little research has been conducted to validate this claim. Thus, this study aims to; i) understand the impact of behavioural Morris water maze (MWM) training test on immune cytokine levels in the brain with or without infection, ii) investigate the impact of *Trypanosoma brucei brucei* infection on spatial learning and memory, also, finally iii) evaluate infection severity with or without the behavioural training test.

To address these objectives, three simultaneous studies were conducted whereby C57BL6 mice were grouped into four and two groups were infected with *Trypanosoma brucei brucei*. At first, we showed that the MWM training task did not affect the basal level of brain immune composition in naïve mice. Secondly, our result revealed that *Trypanosoma brucei brucei* infection resulted in spatial learning and memory impairment. Additionally, the consequential effect of infection resulted in an upregulated pro-inflammatory and anti-inflammatory cytokine (IL-6, IFNγ, TNF, IL-10) immune response in the three brain areas of focus: the prefrontal cortex, hippocampus, and hypothalamus. Lastly, it was interesting to find that the MWM training task reduced the characteristic pathology associated with the disease. In conclusion, the study indicates that the increased immune cytokine composition in the brain above the basal level following *Trypanosoma brucei brucei* infection could contribute to the impairment in spatial learning and memory whereas, training during infection could potentially help to dampen the inflammatory and pathological severity of the disease.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Trypanosomes—the causative agent for African Trypanosomiasis—are amongst the few parasites that gave rise to a major tropical disease in the 20th and 21st century (Ponte-Sucre, 2016). This parasite causes disease in both livestock and humans. Human African Trypanosomiasis (HAT) otherwise called sleeping sickness due to its clinical presentation of insomnia at night and somnolence during the day (Rijo-Ferreira *et al*., 2018) is a vector-borne extracellular, flagellated, haemoparasitic protist disease ranked amongst the top thirteen neglected tropical diseases (NTDs). HAT is endemic to about 36 countries in the sub-Saharan region of Africa with about 60 million people at risk (Simarro et al., 2010; Rodgers *et al*., 2017), however, between the year 2016 and 2017, new cases were only reported from 17 out of the endemic countries (World Health Organization, 2020). There are many sub-species of this parasite, but only two are well known to infect and cause observable pathology in humans; *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) (Moreno *et al*., 2019). It is rather unfortunate that livestock also suffers from the devastating Animal African Trypanosomiasis (AAT) also called Nagana in cattle (Bekele & Begejo, 2015; Muhanguzi et al., 2017). They can as well act as reservoirs for the human trypanosome parasite particularly *T. b. rhodesiense*, adding to the complexity of the disease control (Fyfe et al., 2017).

The utmost consequence of parasitic infections is the involvement of the central nervous system (Figarella et al., 2020). The brain regarded as an immune-privileged site has been challenged in recent times (Louveau et al., 2015) and amongst the few protozoan parasites shown to cross the blood-brain barrier is *Trypanosoma* (Elsheikha & Khan, 2010; Figarella et al., 2020).

Although vaccines are generally an effective and economical means for disease control, there is currently no vaccine against African trypanosomiasis disease due to trypanosomes' ability to switch variable surface glycoprotein (VSG) through the process of antigenic variation to evade the host's immune responses, rendering vaccination strategies futile (T. Jacobs *et al*., 2011; Sima *et al*., 2019). Similarly, antibody-mediated killing of this parasite would have been an alternative route of approach in the vaccine development, however, this parasite has perfected the art of compromising every stage of B-cell development, thus, truncating the adequate production of antibodies by B-cell which in turn favours the parasite survival (Id et al., 2020). Chemotherapy is a treatment method of choice that comes with limitations that include toxicity, parasite resistance, decrease in efficacy, prolonged time of usage, laborious administration and need for trained personnel to administer drugs (Burri, 2010; Babokhov *et al*., 2013; Prayag *et al*., 2020). It is also of note that the HAT epidemic occurs in an undulating manner whereby high incidence rate phases are often followed by periods of seemingly successful eradication. For example, in 1998, a resurgence in infection rate occurred, preceded by a phase of almost reaching complete control in the 1960s (Figure 1). World Health Organization (WHO) has now proposed a new goal to attain sustainable elimination of *T. b. gambiense* through transmission interruption by 2030 (World Health Organization, 2020) upon getting a grasp of the 2020 goal for eliminating HAT as a public threat (Figure 2). However, this requires consistent and persistent effort to prevent rewriting the history of resurgence.

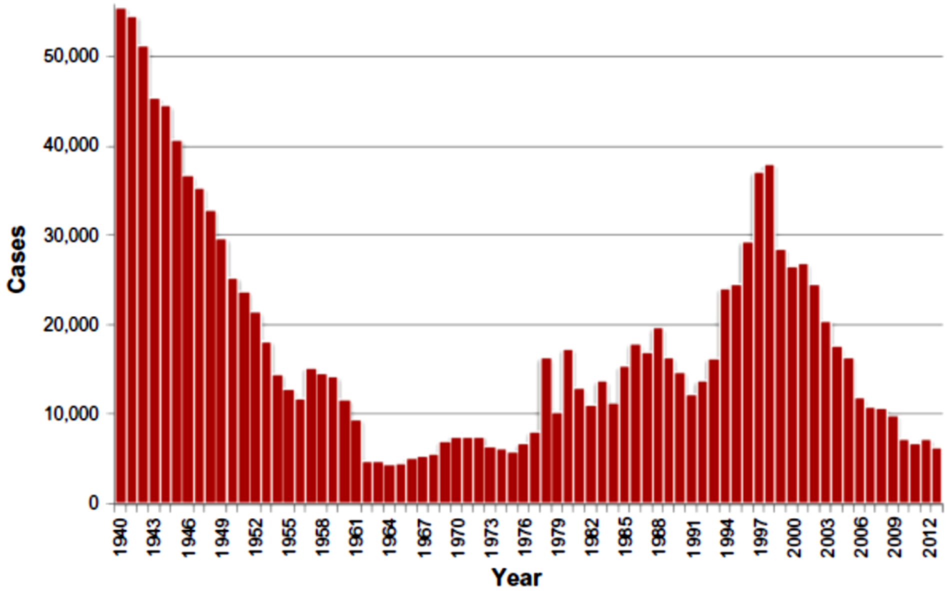


Figure 1: Total number of reported cases of HAT to WHO, 1940-2013. Adapted from (World Health Organization, 2013; Jose R Franco et al., 2014).

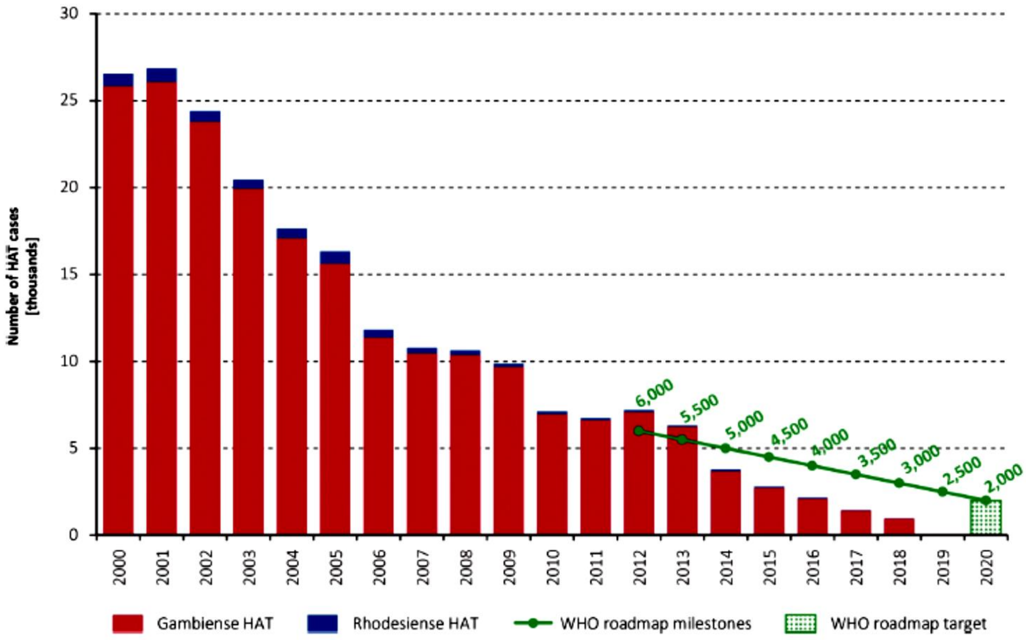


Figure 2: Total number of reported HAT cases, 2000-2018. Green line and bar show the paradigm and set target in WHO Roadmap for HAT elimination. Adapted from (World Health Organization, 2019; José R. Franco et al., 2020).

1.2 The *Trypanosoma* Parasite

1.2.1 Taxonomy

The *Trypanosoma* parasite was first described in 1841 when a flagellate was found in the blood of a brown trout, *Salmo fario*. Reports of a morphologically similar protozoan parasite detected in frogs resulted in the coined genus *Trypanosoma* by Gruby in 1843 based on their distinct motion (Cox, 2004).

1.2.2 Classification of trypanosomes

Trypanosomes are classified under the large kingdom Protista and are members of phylum Sarcomastigophora belonging to the class of Zoomastigophorea (LEVINE et al., 1980). The order Kinetoplastids consists of flagellated motile protozoans, distinguished by the unique DNA-containing region in their mitochondrion known as “Kinetoplast” which is situated close to the basal body of the flagellum (Stuart et al., 2008; Cavalcanti & De Souza, 2018). They are then grouped into the family of Trypanomatidae and genus Trypanosoma. The genus is further subdivided into two sections; Salivaria and Stercoraria based on their form of development in the vector (cecil A. Hoare, 1964). Salivarian develops (mature into metacyclic trypomastigote) in the salivary medium of the foregut of tsetse fly vector (*Glossina* spp) and is transmitted to a host by inoculation. It consists of four main subgenera: *Trypanozoon, Duttonella, Nannomonas, and Pycnomonas. Trypanozoon* consists of 3 species, namely *T. (T) equiperdum, T. (T) brucei and T. (T) evansi*, while *T.(T) brucei* has 3 sub-species viz: *T. b. brucei, T. b. rhodesiense* and *T. b. gambiense* (LEVINE *et al*., 1980; WHO, 1986). Classification of the major groups that cause human and animal African trypanosomiasis is summarised below (Figure 3).

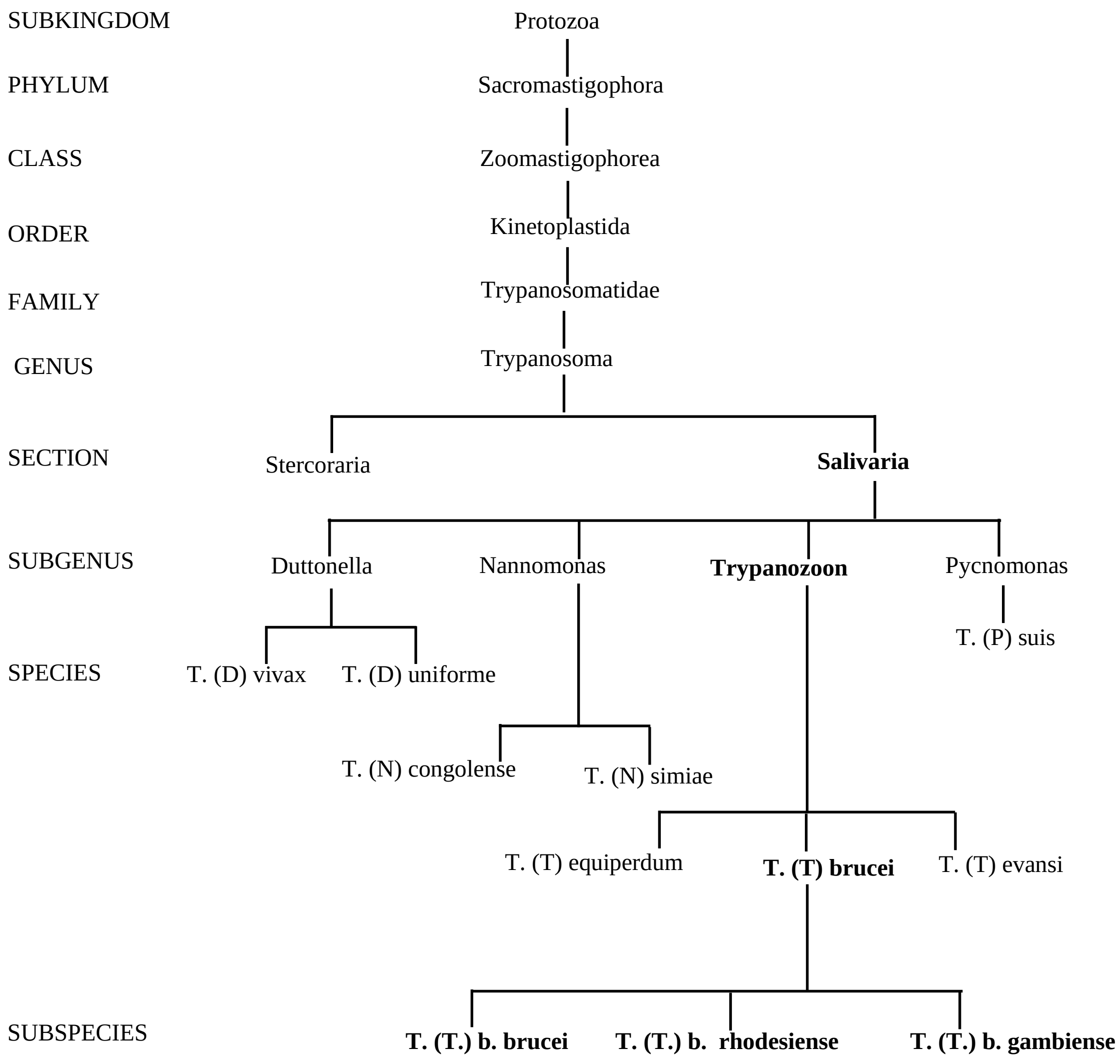


Figure 3: Classification of African trypanosomes. The figure is adapted from (LEVINE *et al*., 1980; World Health Organization, 1986).

1.2.3 Epidemiology

Despite morphological similarities between the two variants of HAT (*T. b. gambiense* and *T. b. rhodesiense*), the geographical distribution, course and severity of the infection are dissimilar (Jose R Franco et al., 2014; Altamura et al., 2020). Approximately 98% of HAT reported cases are caused by *T. b. gambiense* endemic to West and Central Africa, which causes a chronic form of the disease that could lead to death if not treated (Jose R Franco et al., 2014). *T. b. rhodesiense* affects the East and Southern regions of Africa. It progresses much quicker, leading to an acute form of the disease with approximately 2% of reported cases (Jose R Franco et al., 2014; WHO, 2019; José R. Franco et al., 2020).

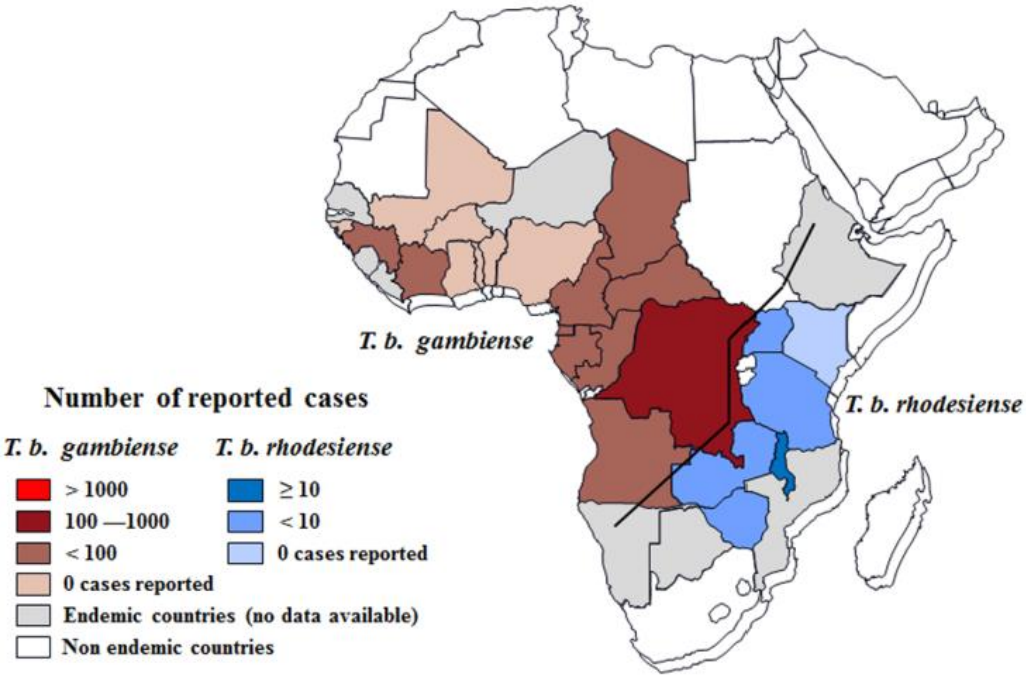


Figure 4: Map of Africa showing geographical distribution of *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, 2018. The figure is adapted from (World Health Organization, 2019; Gao et al., 2020).

1.2.4 Developmental stages of *Trypanosoma brucei* in mammals

African trypanosomes exhibit a complex life cycle (Figure 5) to aid survival due to their constant exposure to host immune response (Magez et al., 2002; Matthews, 2009). Morphological changes involving alteration in the mitochondrion and surface membrane of flagellate occurs at a different developmental stage of trypanosome allowing either parasites evasion of host mounted immune response or aid adaptation to source for energy in the host (Vickerman, 1985; Baral, 2010). Trypanosomes are transmitted when an infected tsetse fly (vector) takes a blood meal and inoculate its mammalian host with metacyclic trypomastigotes characterised by a short flagellum. Upon getting into the bloodstream, it undergoes a morphological transformation into proliferative trypomastigote with a longer flagellum and acquires its variable surface glycoprotein (VSG). The long slender (LS) bloodstream form (BSF) rapidly multiplies by binary fission in the blood, the lymphatic system, and in the cerebrospinal fluid (CSF). As the infection progresses, the proliferative LS form is replaced by a non-proliferative short and stumpy form (SS) irreversibly once the threshold is attained hence, arresting the division process by the incitement of quorum sensing via antigen variation (Zimmermann et al., 2017). Due to the lethality associated with persistently high parasitaemia, this capacity to recognise critical density 'quorum sensing' is a crucial way of regulating its population, thus elongating the survival of its mammalian host (Matthews, 2009; Mony & Matthews, 2015; Szöőr et al., 2020). The SS trypomastigotes are pre-adapted for transmission, infectivity and growth in tsetse fly which is crucial for the life cycle (Rico et al., 2013). During bloodstream stage, the surface of trypanosome is covered in VSG which enables host immune evasion through antigenic variation (Sima et al., 2019) howbeit, non-proliferating stumpy forms do not experience antigenic variation (Vickerman, 1985; Rico et al., 2013). The peak and rescinding cycle of LS and SS BSF of the parasite keeps the life cycle going and this continues until chemotherapy intervention or death.

Tsetse fly gets infected by taking a blood meal and ingesting the SS form trypomastigote. It morphologically transforms into a procyclic trypomastigote in the fly's midgut, characterised by loss of its VSG coat and inability to infect mammalian hosts (Hajduk et al., 1992). At this stage, proliferation occurs by binary fission. It then migrates from the midgut to attach to the lumen epithelium of the salivary gland and transform into an epimastigote. Epimastigotes multiply by binary fission and transform into an infective metacyclic trypomastigote by detaching from the wall of the lumen in the salivary gland, ready to infect its mammalian host (Hajduk et al., 1992).

Despite the great havoc this digenetic disease, trypanosomiasis has caused humans especially in the sub-Saharan region of Africa, tsetse fly is of a fact the definite host while humans and other mammals are just a means of continuity to the lifecycle (Geiger et al., 2014).

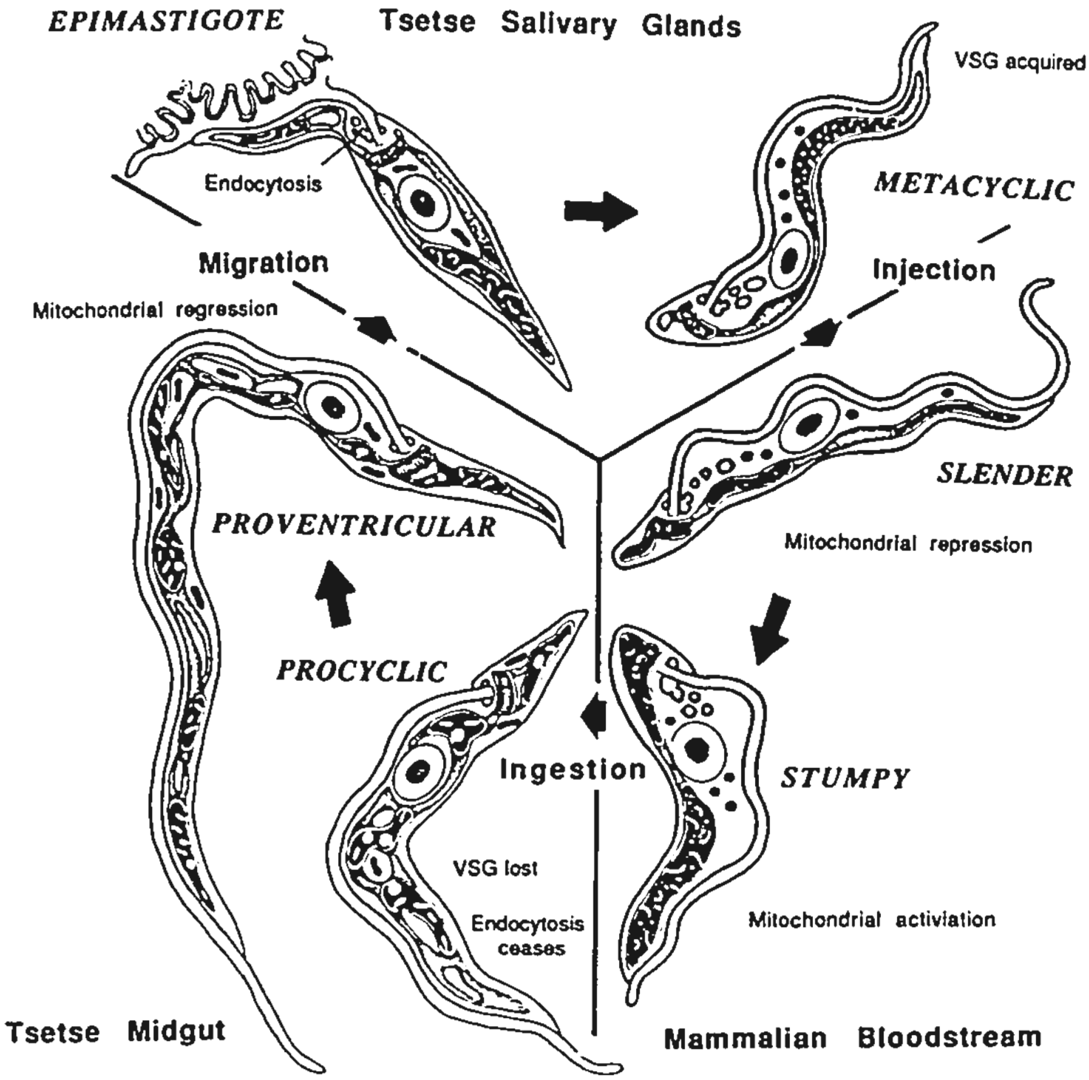


Figure 5: Developmental cycle of *trypanosoma brucei* (Hajduk et al., 1992).

1.3 Clinical signs, symptoms, and stages of the disease in its host

A bite from tsetse fly results in a local inflammatory reaction identified by swelling, tenderness, and redness collectively called a chancre (Vincendeau & Bouteille, 2006). The pathology of the disease worsens as it progresses in 2 sequential stages: the haemolymphatic and meningoencephalytic.

Haemolymphatic stage also known as the first or early stage of the disease is characterised by parasites localised within the bloodstream and lymphatic system. Symptoms associated with this stage are not exclusive for the disease, however, it includes irregular fever caused by the parasitemia wave, headache, joint and muscle pain, tiredness, weight loss, anaemia and pruritus (Brun et al., 2010). With the advancement in disease progression, symptoms get worse and could include posterior cervical lymph node enlargement (Winterbottom sign) mostly seen in *T. b gambiense* infected patients, splenomegaly and/or hepatomegaly, cardiac problems, and reduced fertility (Kennedy, 2013; Carvalho et al., 2018).

Some of the symptoms suffered in the early stage of the disease do not disappear entirely but instead progresses and overlap with the meningoencephalytic stage (Vincendeau & Bouteille, 2006). This meningoencephalytic stage is also referred to as the late or second stage of the disease, distinguished by the parasite circumventing and traversing the blood-brain barrier to get established in the brain. The predominant sleep disturbance at this stage gave rise to the name sleeping sickness. This stage of the disease is also characterised by neuropsychiatric tumult including aggression, confusion and hallucination. Patients suffer from symptoms that include motor skill disability, slurred speech, followed by seizures and eventually death if not treated (Blum et al., 2006; Brun et al., 2010).

1.4 Screening, diagnosis and staging of the disease.

The unspecific and non-exclusive clinical signs and symptoms of the disease impedes a precise disease staging and diagnosis, making it depends mainly on laboratory examination of blood and body fluids which is usually done by a three-step approach that includes screening, diagnostic confirmation and staging of disease (Hainard et al., 2009; Brun et al., 2010). Staging of the disease is very crucial to aid a precise treatment approach. Screening of *T. b. gambiense* requires the use of serological card agglutination test (CATT) while *T. b. rhodesiense* is screened and confirmed by detecting parasites in the blood, lymph nodes and cerebrospinal fluid under a light microscope due to its higher parasite density (Masocha & Kristensson, 2018). Detection of trypanosome in the periphery indicates early stage while its presence in the CSF of either form of disease confirms the second stage of infection.

Another disease staging approach involves white blood cell count (WBC) whereby Count ≤ 5 WBC/µl with no presence of trypanosome in CSF is considered haemolymphatic stage while WBC > 5 WBC/µl with or without detecting trypanosome in CSF is regarded meningoencephalytic stage (World Health Organization, 2019).

1.5 Treatment

Treatment of the early stage of *T. b. gambiense* and *T. b. rhodesiense* rely on the use of pentamidine and suramin, respectively. These drugs are nevertheless ineffective for the late stage of the disease due to their inability to cross the blood-brain barrier (Masocha & Kristensson, 2018). The late-stage used to be managed with melarsoprol which is unfortunately toxic but, was replaced with eflornithine or nifurtimox-eflornithine complex (NECT) to treat the late stage of *T. b. gambiense* (Priotto et al., 2009; Yun et al., 2010). Because the dosage was cumbersome, requiring hospitalization and trained personnel to administer it, fexinidazole was developed and became the first oral drug to treat both stages of *T. b. gambiense* (Neau et al., 2020). This streamlined treatment regimen is easier to use, access, administer, and is cost-effective (Büscher et al., 2017).

1.6 Cytokine immune response to African trypanosomiasis

African trypanosomiasis polarizes either classical (M1) or alternative (M2) activation of macrophages depending on the stage of disease (Stijlemans et al., 2010; Onyilagha & Uzonna, 2019). During the early phase of infection, disease control and parasite clearance are driven by M1-associated type 1 immune response to induce phagocytosis (Cheung et al., 2016) and proinflammatory cytokines production (Drennan et al., 2005). A study conducted by Magez et al. showed that the ability to control parasite growth is driven by a rapid type 1 immune response in an interferon-gamma (IFN-γ) dependent manner to stimulate the production of trypanocidal molecules including tumour necrosis factor (TNF-α) and nitric oxide (NO) by activated M1 macrophage (Stefan Magez et al., 2006). Despite its importance, a persistent type 1 immune response for disease control results in immune hyperactivation leading to severe tissue collateral damage which in turn mediates host susceptibility to disease (Shi et al., 2003; Guilliams et al., 2009).

The increased host survival rate has been linked to the immune system's ability to switch from an initial M1-type 1 immune response to an interleukin-10 (IL-10) anti-inflammatory driven M2-type 2 immune response that dampens pathogenicity caused by pro-inflammatory immune responses during the late stage of infection (Figure 6) (Bosschaerts et al., 2008; Kato et al., 2016; Stijlemans et al., 2020). It has been shown that therapeutic treatment with IL-10 adeno-associated viral vector to induce a sustained IL-10 production in chronically *T. brucei brucei* infected mice dampens TNF and NO production to protect the host from collateral tissue damage (Guilliams et al., 2009).

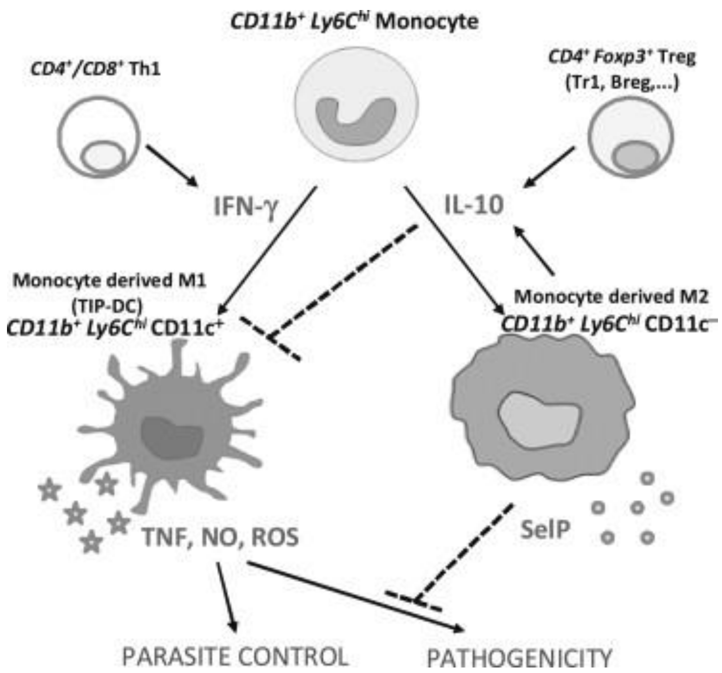


Figure 6: Schematic representation of IFN-γ and IL-10 interaction during African trypanosome infection. The figure is adapted from (Bosschaerts et al., 2009).

1.7 How trypanosome parasite enters the brain in murine models

The route through which *Trypanosoma brucei* parasite enters the brain holds several controversies. Dogma has it that the parasite crosses via the blood-brain barrier (BBB), however, this has been challenged that it is rather through the blood-cerebrospinal fluid barrier (BCB) (Wolburg et al., 2012). It is evident that *T. b. brucei* invasion into the brain depends on the presence of T cells as described by a knockout model of rag1-/- mice lacking both the mature T and B cells presenting with the absence of parasites in the brain. However, upon adoptive transfer of T cells into these rag1-/- mice, the parasite penetration was re-established (Masocha et al., 2004). The Central nervous system barrier consists of an endothelial and parenchyma basement membrane (Sixt et al., 2001; Masocha & Kristensson, 2012) whose integrity during the late stage of *T. b. brucei* is compromised by the induction of T-cell producing IFN-γ wherefore, regardless of the route of the entrance (Mogk et al., 2014), IFN-γ is implicated as a facilitator enabling the parasite to circumvent the central nervous system

(Masocha et al., 2004). A laminin isotype composition of these two basement membranes contributes to white blood cell infiltration to the brain parenchyma (Sixt et al., 2001). In the case of a similar neurological condition causing protozoan Balamuthia mandrillaris known to cause encephalitis, its surface binds to the endothelial cell galactose-containing glycoprotein and laminin leading to parasite attachment to the blood-brain barrier and the degradation of tight junction proteins and basement membrane to enable the parasite crossing into the blood-brain barrier (Matin et al., 2006); Matin et al., 2007). There is also a parasite-derived factor whereby T. brucei-derived cysteine proteases interact with host endothelial cell G protein-coupled to increase in Blood Brain Barrier permeability which promotes parasite crossing (Nikolskaia et al., 2006).

Parasites invasion is also likened to leukocytes infiltration during an inflammatory response, in which case the cells had to pass through the endothelial and the parenchyma membrane of the brain (Sixt et al., 2001). In a disease model of *T. b brucei*, the deficiency in IFN-γ or its receptor (IFN-γ–/– and IFN-γR–/– mice) revealed trapped parasite between the endothelial and parenchyma membrane resulting in a significantly reduced number of the parasites in the CNS when compared to the wild-type counterparts suggesting that successful penetration of the parasite is aided by IFN-γ (Masocha et al., 2004).

Microglia cells on another hand are known to play significant roles during brain infections and inflammation, whereas there has been less attention given to microglia's reaction to this parasite. An in vitro experiment demonstrated that microglia cells respond to the presence of *T. b brucei* by engulfing and eliminating the parasite (Figarella et al., 2018). A recent study revealed microglia-plasma cell crosstalk in the brain during *Trypanosoma brucei* infection mediated by IL-10 and B cell activating factor (BAFF) signalling (Quintana et al., 2022). This demonstrates the importance of this myeloid cell during *Trypanosoma brucei* infection.

1.8 The role of cytokines in cognition

Infections are possible causes of excessive immune response in the brain, but the balance in pro and anti-inflammatory cytokines is said to aid in pathogen elimination (Zheng et al., 2016). A study conducted by Wolkmer et al. and Baldissera et al. using a *T. evansi* strain reported memory impairment caused by infection, with no mention of the role of cytokines concerning the observed cognitive phenotype (Wolkmer et al., 2013; Baldissera et al., 2015). Pro-inflammatory and anti-inflammatory immune responses have been independently shown to play significant roles in learning and memory potentiation in Alzheimer's disease, with thoughts that the same principle could apply to other encephalopathies (Clark et al., 2010). The growing curiosity in the immune system-inflammatory response complex has resulted in identifying an increasing number of pro-inflammatory cytokines implicated in central nervous system-related disorders (Cattaneo et al., 2017). IFN-γ, in particular, has been reported to have both beneficial and detrimental effects on cognitive function. For instance, in an experimental model of Alzheimer's disease, IFN-γ production is shown to support neurogenesis offering neuronal protection and repair (Baron et al., 2008) to aid cognition. On the other hand, research has shown that the absence of IFN-γ enhances cognitive performance (Monteiro et al., 2015) besides overproduction of this cytokine leading to impairment of neural regeneration (Walter et al., 2011), supporting its detrimental effects in cognitive function.

TNF is another inflammatory cytokine implicated in CNS disorders with growing evidence of its detrimental impact on cognition (Liu et al., 2014; Hennessy et al., 2017; Bobińska et al., 2017). Here, the treatment with lipopolysaccharide resulted in an increased TNF level which mediates a memory impairment (Noorbakhshnia & Karimi-Zandi, 2017). Another showed that anti-TNF therapy could help to restore spatial learning cognitive impairment (Balzano et al., 2020). These and more shreds of evidence revealed that the impact of TNF on cognition cannot be overemphasised.

Interleukin-6 is one of the most studied cytokines in the brain due to being versatile and possessing both pro-and anti-inflammatory properties, depending on the cause of physiological manipulation (Hunter & Jones, 2015; Kato et al., 2016). Some report that IL-6 deficiency results in spatial memory impairment because it is essential for recognition memory (Hryniewicz *et al*., 2007; Bialuk *et al*., 2018). Nonetheless, research has also shown that deletion of IL-6 can facilitate learning and memory (Braida et al. 2004). There are also reports that upregulation of IL-6 has detrimental effects linked to memory impairment in neurodegenerative diseases (Bermejo et al., 2008; Wei et al., 2012; Nelson et al., 2012). Notwithstanding, the absence or overexpression of IL-6 does not negate its neuroprotective ability to promote brain repair (Willis et al., 2020) as its implication in cognition is multifaceted, compounded and daunting (Bialuk *et al*.,2019).

Anti-inflammatory cytokine IL-10 is reported to have ameliorative effects on memory potentiation, able to abrogate memory impairment, capable of restoring spatial learning, and can enhance neurogenesis (Lynch *et al*., 2004; Kiyota *et al*., 2012; Donzis & Tronson, 2014), while its absence in an inflammatory condition leads to neurobehavioural impairment (Richwine *et al*., 2009). Therefore, considered the classic immunosuppressive cytokine produced within the CNS (Burmeister & Marriott, 2018). Yet, contrasting evidence has shown that IL-10 promotes amyloidogenesis which causes memory impairment (Chakrabarty *et al*., 2015) and leads to the pathogenesis of dementia (Sathyan et al., 2018), besides its deficiency shown to improve the pathology of Alzheimer's disease (Guillot-Sestier et al., 2015).

Although the role of pro-inflammatory cytokines on cognitive function is well researched, anti-inflammatory cytokines have received less attention. Increasing evidence shows the importance of anti-inflammatory cytokines in learning and memory. IL-4 and/or IL-13 support cognitive behaviour (Kawahara et al., 2012; Boccardi *et al*., 2019) by their neuroprotective abilities, while their absence leads to impaired cognitive function (Shimizu et al., 2008; Derecki *et al*., 2010; Brombacher et al., 2017).

1.9 The Morris water Maze

Spatial learning and memory enable individuals to remember different locations and recognise how various objects relate to that environment which is essential for the survival of humans and other mobile animals (Enrique et al., 2022). This spatial cognition is controlled by the hippocampus such that this area of the brain can be regarded as a central cognitive map of an individual's spatial environment (Epstein et al., 2017; Epstein et al., 2019).

In murine models, the Morris water maze (MWM) is an acknowledged method for its evaluation, ranging from the classic utilising parameters such as latency, distance, and the number of platform crossings, to more complex analysis (Enrique et al., 2022). Besides, there are other behavioural assays such as the Radial arm maze, Barnes maze, Contextual fear conditioning and Open location memory used to evaluate the hippocampal-dependent spatial learning and memory nonetheless, MWM was more appropriate for this study (Patil et al., 2009; Othman et al., 2022).

1.9 Rationale of the study

Although trypanosomiasis invades the brain, there is still limited knowledge of its effects and its resulting immune response on cognition. This study is, therefore designed to investigate the late-stage effects of parasite neuro-infection and cytokine immune responses on cognitive spatial learning and memory following *Trypanosoma brucei brucei* (*T. b. brucei*) infection.

1.10 Aim of the study

This study aims to evaluate the impact of *T. b. brucei* and its resulting inflammatory immune response on cognitive function using mice by:

I. Evaluating impact of the behavioural test (MWM training test) on immune cytokine levels in the brain with or without *T. b. brucei* infection.
II. Investigating the impact of *T. b. brucei* infection on spatial learning and memory.
III. Assessing the histopathological changes in some peripheral visceral tissue.
IV. Investigating the severity of *T. b. brucei* infection in mice with or without the behavioural training test.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

Inbred male wildtype mice of C57BL/6 background aged between 6-12 weeks old, n=4-7 animals per group were used throughout the course of this study. C57BL/6 mice were the strain of choice as they are more resistant to the disease and survive longer than their BALB/c counterpart (Duleu et al., 2004; Antoine-Moussiaux et al., 2008; (Stefan Magez et al., 2020) Mice were divided into 4 study groups, namely non-infected non-trained, non-infected trained, infected non-trained and infected trained and allowed a minimum of 3 days of acclimatisation before the commencement of experiments. Mice were housed in individually ventilated cages under specific pathogen-free conditions at the University of Cape Town, Animal Facility unit, Bio-Safety Level 2 (BSL 2) laboratory. Animal room temperature (RT) was maintained at 22-25 ˚C, provided with 12 hours light/dark cycle whilst food and water were available *ad libitum*.

2.2 Ethics statement

This study was carried out in strict compliance with the South African National Standard (SANS 10386:2008) and University of Cape Town practice for laboratory animal procedures. Experiments were conducted in accordance with approved protocols by the Animal Research Ethics Committee of the Faculty of Health Science, University of Cape Town (FHS-AEC protocol number 018/007).

The humane endpoint to this study was determined by mice body weight measurement and the assessment of their physical performance. Mice that exhibited ≥ 10% loss of their initial body weight, abdominal distension, lethargy, and severe weakness were immediately euthanised by halothane exposure within the confinement of the euthanasia chamber for 5 minutes. Death was confirmed by exsanguination through cardiac puncture then cervical dislocation. These procedures were carried out by trained researchers and South African Veterinary Council (SAVC) authorized personnel.

2.3 Trypanosome maintenance

The pleomorphic *T. b. brucei* AnTat 1.1E of EATRO 1125 stock was originally provided by Dr. N. Van Meirvenne and E. Magnus (Lab. Serology, Institute of Tropical Medicine (ITG), Antwerp, Belgium). Trypanosomes stabilates were maintained at -80 ^{O}C and properly thawed prior to infection.

2.4 Optimization of parasite passage and purification

Trypanosomiasis disease is dependent on infection with trypanosomes. In experimental research, there is a need for accurate quantification of the number of trypanosomes inoculated to aid experiment reproducibility, therefore purifying the parasite from the blood is of importance. Half a century ago, Sheila M. Lanham developed a technique that uses ionic strength in form of column anion exchanger to isolate Salivarian trypanosomes from the blood of infected rodents (Lanham, 1968). This technique works based on an adsorption-elution process whereby the more negatively charged is adsorbed and while the less negatively charged elutes. Under standard buffer condition (pH 8.0), the cell surface of *T. b. brucei* is less negatively charged than the blood. Consequently, Diethylaminoethyl cellulose anion exchange column adsorbs the more negatively charged erythrocytes, platelets and leukocytes while the less negatively charged haemoflagellate is eluted (Lanham & Godfrey, 1970; Lejon et al., 2019). This technique remains the best and the gold standard for isolating parasites as it produces a clear trypanosome suspension that is free of debris and blood cells while maintaining their morphology and viability in their mammalian host (Lejon et al., 2019; Courtois et al., 2019).

2.4.1 Parasite passage

The availability and viability of *T. b. brucei* (AnTat 1.1E) is a principal factor in the commencement of this study. Upon observing the stabilate of *T. b brucei* previously stored for over a decade ago, it presented with mostly dead floating parasites hence, requiring a new passage. The cryopreserved pleomorphic stabilate of T. *brucei brucei* (AnTat 1.1E) was then passaged into immuno-compromised mice of C57/BL6 background to generate new parasites. C57/BL6 mice of background recombination activating gene (rag mice) were used for the parasite passage in other to get an optimum number of parasites whilst bypassing any form of immune system retaliation. The mutation in this mice strain prevents it from producing neither a mature T cell nor B cell thus, creating a suitable environment for parasite culture and enhanced parasite growth.

Stabilates were rapidly thawed at 37 ^{O}C and were assessed for viability, confirmed by motility then diluted in 1X PBS to inoculate mice intraperitoneally with 0.5 ml of the suspension using a 26G needle to prevent shredding of the parasite. A drop of blood was collected with two days interval from mice by a tail prick to monitor parasitemia load. Once the parasite reaches its threshold (assessed by microscopic examination when in its long and slender form) usually between day 5 and 7, mice were exsanguinated, and blood collected in a heparinized cryotube. To each amount of blood, an equal volume phosphate-buffered saline glucose (PBSG) buffer (6.76 g $Na_2HPO_4.2H_2O$, 0.24 g NaH_2PO_4, 1.70 g NaCl, 10 g Glucose, dissolved in 1 L distilled H_2O) containing 20% Dimethyl sulfoxide (DMSO) and Fetal Bovine Serum (FBS) mix was added.

Due to few viable parasites in the previously stored stabilate, the first passage yielded a small amount of parasite (Supplementary Movie 1A) however, the third passage (Supplementary Movie 1B) yielded sufficient parasite to commence research experiments.

2.4.2 Diethylaminoethyl (DEAE) cellulose preparation

The lab protocol previously used in preparing this cellulose requires 2 days before reaching completion, however, this was successfully optimised to a 5 hours protocol. Pre-swollen DEAE Cellulose IonSep Ion-exchange Resins (Biophoretics) was prepared with a method adapted from Courtois (Courtois et al., 2019). 100 grams of DEAE Cellulose was repeatedly washed in a 5 litres conical flask. Washes were done by stirring the pre-swollen cellulose in 4 litres of distilled water after which it was left to settle, and the supernatant decanted until it runs clear and free of fine particles. Then, 3 litres of 2X phosphate-buffered Saline (PBS) was stirred in and the pH of the solution was adjusted to 8.0 with 1 M potassium dihydrogen phosphate (1M KH_2PO_4). The solution was left to settle, and the supernatant was decanted. Washes were done again twice in 4 litres distilled water followed by another two times wash in 3 litres PBSG. The solution was allowed to settle after each wash step before discarding the supernatant. After the last wash step, the slurry was measured and an equal volume of PBSG was added. The prepared solution was aliquoted and stored at 4 ^{O}C for a short time storage and -20 ^{O}C for a longer storage span.

2.4.3 Trypanosome Purification

Harvested trypanosomes were separated from blood using an anion exchange column based on the concept of surface charge; the more negatively charged blood cell is absorbed in the column whereas the less negatively charged parasite is eluted from the column (Souto-padrón, 2002). Purification was performed using this gold standard procedure developed by Lanham recently described by Courtois (Lanham, 1968; Courtois et al., 2019). Using DEAE Cellulose IonSep Ion-exchange Resins (Biophoretics), the column was prepared and about 500 µl of infected blood was carefully layered on it without disturbing the bed then the elution buffer (PBSG supplemented with pen-strep) adjusted to pH 8.0 was used to elute the parasite. Droplets from the column were collected at an interval to monitor the transit of the parasite. The effluent was then centrifuged at 2000 rpm for 10 minutes at 4 ^{O}C to concentrate the parasite at the bottom of the tube. The parasite pellet was resuspended in an equal volume of PBSG containing 20% DMSO and FBS mix, aliquoted and stored at -80 ^{O}C.

The parasites were purified using an anion exchange purification method. This DEAE cellulose isolated and purified parasites using the described column exchange were clear of blood cells, debris and still maintained their motile, active, and viable form (Supplementary movie 2). Viability is not directly correlated to infectivity nonetheless, parasite motility is important for infection (Kisalu et al., 2015)

2.5 Experimental murine infection with *T. b. brucei*

A cryopreserved vial of parasite stabilate was thawed rapidly at 37 ^{O}C and diluted in 1 x PBSG to a concentration of 2 x 10^3 parasites per 100ul of solution. Mice were injected intraperitoneally with either 100 µl 1 x PBSG containing 2000 parasites (Infected non-trained & Infected trained), or 0.9% physiologic saline (Non-infected non-trained & Non-infected trained). Blood was collected from tails (tail prick method) every second day to measure parasitemia by counting under a light microscope.

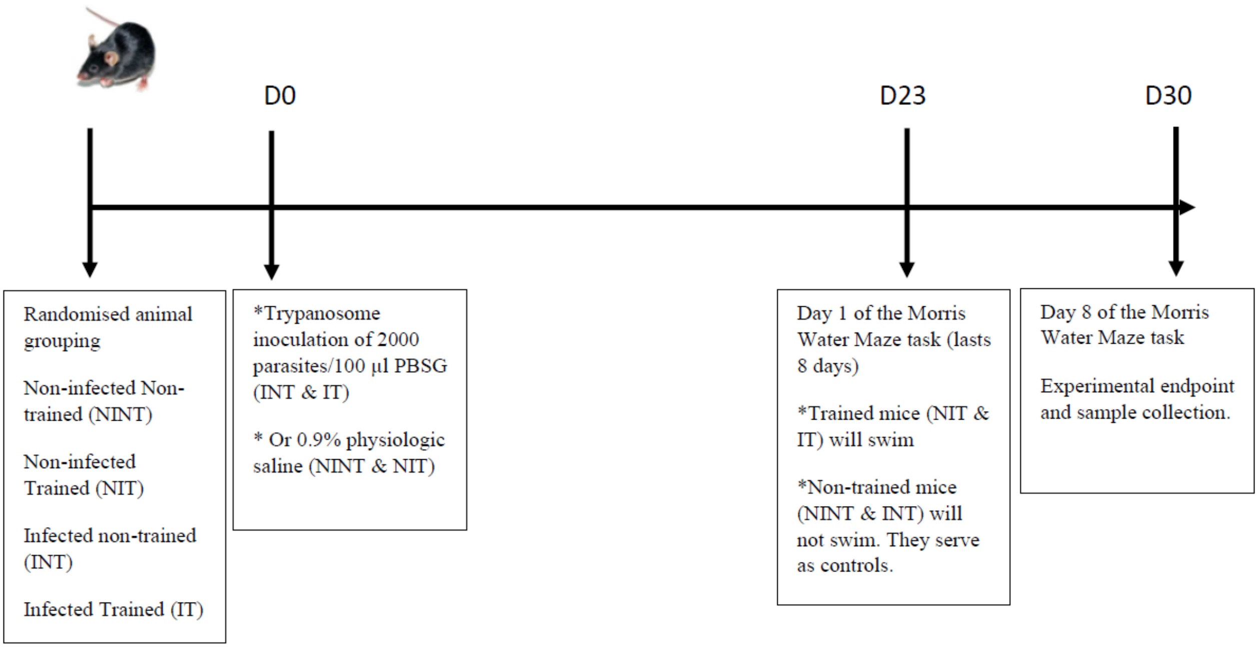


Figure 7: Experimental approach.

2.6 Spatial learning task

The MWM test was used to evaluate the spatial cognitive function of the mice. Here, a circular pool (123 cm in diameter) divided into four imaginary quadrants by a computational tracking system was filled with water and coloured in white with non-toxic paint to allow for colour difference between the black mice and the black pool which enables an accurate tracking. Then, a plexiglass circular platform (10cm in diameter) was submerged 0.5 cm below the water level. Distal extra-maze cues and visual cues were attached to the wall of the room and on the edge of the circular pool along the quadrants to enable the mice to decipher how to locate the hidden platform. Water temperature was maintained at 20-24 OC using an automated water heater. The spatial task which commenced on day 23 post-infection spans for eight days (Vorhees & Williams, 2006). Day 23 post-infection was preferred due to an established 2nd phase/ chronic phase of the disease, and there was little to mostly no appearance of the parasite in the blood during this period. This is to eliminate any possible contributions and alterations of mice behaviour due to the presence of the parasite. The task consists of a learning phase, a probe trial phase, a reversal phase, and a visible platform phase.

During the learning phase, day 23-26 post-infection (day 1-4 of the MWM test), mice were given 4 swim trials per day for the first 4 days of the test, a cumulative of 16 learning trials per mice. Mice were gently released into the water, allotted a maximum of 60 seconds to find and climb onto the platform then allowed ~10 seconds to remain there before returning to a warm home cage fitted with infra-red heat lamps. The entry point of the mice into the maze was changed for each trial using the imaginary quadrant lines as a reference guide. Mice that fail to locate the platform within the stipulated amount of time (60 seconds) were gently guided to the platform and allowed to remain on it for 10 seconds before returning to the home cage (Barnhart et al., 2015). Learning is determined by latency to platform.

A probe trial was performed on day 27 post-infection (day 5 of the MWM test) with the platform removed from the water maze to test for reference memory. Here, each mouse was given a total of 60 seconds to swim freely and recall where the platform was previously located (Brombacher et al., 2018). Since reference memory is determined by latency to the platform, time spent in platform quadrant and the number of platform crossings (Vorhees & Williams, 2006), the path each mouse swam was recorded for these subsequent analysis.

The procedure for the reversal phase is similar to the learning phase. Mice were likewise given 4 swim trials per day; however, the platform was placed in an opposite quadrant and the training only lasted for 2 days to make a total of 8 reversed swim trials. A reversal test was conducted on days 28 and 29 post-infection (days 6 and 7 of the MWM test) for a complex learning test, whereby the platform was placed in a quadrant opposite the original training quadrant.

Finally, on the 30th day post-infection (day 8 of the MWM test), following a similar procedure, the platform was made visible ~0.5cm above water level. It was placed in a third quadrant, different from the other two quadrants in the learning and reversal phase. Groups were run in alternating order on successive days to eliminate time, environmental and endogenous factors. Data were recorded using the EthoVision® XT 8 automated tracking system (Noldus Information Technology, VA).

2.7 Reverse transcriptional evaluation of gene expression in brain tissue

2.7.1 RNA extraction

Brain areas were collected and homogenized in QIAzol Lysis Reagent (QIAGEN Sciences, Maryland, USA). Ribonucleic acid (RNA) extraction was carried out using RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. One-fifth volume of chloroform was added to the homogenate and vortex vigorously for 15 seconds then incubated at RT for 3 minutes. Subsequently, the samples were centrifuged at 1200 g for 15 minutes at 4 ^{O}C leading to the formation of 3 layers. The RNA upper aqueous phase was transferred into a new collection tube, 1.5 volume of absolute ethanol was added and thoroughly mixed by pipetting. Afterwards, up to 700 µl of the mix was transferred into RNeasy Mini spin column stuck in a 2 ml collection tube then spun down at 8000 g for 15 seconds at RT. Flow-through was discarded, 350 µl RW1 buffer was added into the RNeasy Mini spin column then centrifuged at 8000 g for 1 minute at RT thereafter flow-through was discarded. DNase 1 incubation mix was added directly on to the RNeasy membrane and placed at RT to incubate for 15 minutes. Again, 350 µl RW1 buffer was added into the spin column, centrifuged at 8000g for 15 seconds and flow-through was discarded. Thereafter, 500 µl RPE was added to the spin column, centrifuged at 8000 g for 15 seconds at RT then flow-through was discarded. Again, 500 µl RPE was added to the spin column, centrifuged at 8000 g, this time for 2 minutes at RT. The spin column was placed into a new 2 ml collection tube and spun at full speed for 1 minute. RNA was eluted into a new 1.5 ml collection tube from the RNeasy Mini spin column using RNA-free water.

RNA concentration was determined by using Nanodrop One (Thermo Fisher Scientific, USA). Pedestals were wiped clean with RNA free water, 1.5 µl the water was loaded and measured as blank. Afterwards, 1.5 µl of the sample was loaded and measured for their RNA concentration. Samples with RNA $A_{260}/_{A230}$ ratio between 1.9 and 2.1 was considered pure.

2.7.2 Complementary DNA synthesis

The conversion of RNA to complementary DNA (cDNA) was done using Transcriptor First Strand cDNA Synthesis Kit (Roche cat. No. 04 379 012 001). Samples were normalised to the RNA sample with the lowest RNA concentration using RNA free water. Following manufacturer's instruction, 1ul Anchored-oligo (dT) 18 primer 50 pmol/µl (2.5uM conc) and 2 µl random hexamer primer 600 pmol/µl (60uM conc) was added to 10ul of reconstituted RNA samples to make up 13 µl volume. The template-primer mixture was denatured at 65 ^{O}C for 10 minutes in T100^{TM} Thermal Cycler (Bio-Rad, Singapore), then transferred on ice immediately. A first strand synthesis cocktail (1X 8 mM $MgCl_2$ Transcriptor Reverse Transcriptase Reaction buffer, 20 U Protector RNase Inhibitor, 1 mM Deoxynucleotide mix, and 10 U Transcriptor Reverse Transcriptase) was added to the template-primer mix and gently mixed by pipetting up and down. The mix was then incubated in T100^{TM} Thermal Cycler with heated lid (to minimise evaporation) for 10 minutes at 25 ^{O}C, followed by 60 minutes at 50 ^{O}C, then 5 minutes at 85 ^{O}C and the reaction was finally stopped by placing the tubes on ice for 5 minutes. Aliquot of 1/10 dilution was prepared as working solution for later PCR amplification and stored at -20 ^{O}C while the remaining cDNA samples were store at -80 ^{O}C until when needed.

2.7.3 Quantitative real-time PCR amplification

Quantitative real-time PCR (RT-qPCR) was carried out using LightCycler® 480 SYBR Green I Master (Roche, Germany) and gene-specific primers in Table 1. All primers are gotten from Integrated DNA Technologies, USA. Fold change expression was normalised to HPRT (house-keeping gene) and to the Non-infected non-trained or the Non-infected trained control groups.

Table 1. Sequence of primers used for mRNA cytokine expression quantified by qPCR.

Target gene	**Primer sequence**
IL-6-Forward	5'- GTT CTC TGG GAA ATC GTG GA -3'
IL-6-Reverse	5'- TGT ACT CCA GGT AGC TAT GG -3'
IFNγ-Forward	5'- GCT CTG AGA CAA TGA ACG CT -3'
IFNγ-Reverse	5'- AAA GAG ATA ATC TGG CTC TGC -3'
TNF-Forward	5'- TCT CAT CAG TTC TAT GGC CC -3'
TNF- Reverse	5'- GGG AGT AGA CAA GGT ACA AC -3
IL-10-Forward	5'- AGC CGG GAA GAC AAT AAC TG -3'
IL-10-Reverse	5'- CAT TTC CGA TAA GGC TTG G -3'
HPRT-Forward	5'- GTT GGA TAT GCC CTT GAC -3'
HPRT-Reverse	5'- AGG ACT AGA ACA CCT GCT -3'

2.8 Cytokine ELISA assay

2.8.1 Serum preparation

Upon sacrifice, blood samples were collected by exsanguination in a serum separator tube (BD Biosciences) and spun down at 1200 rpm for 20 minutes to separate the serum then stored at -80 ^{O}C until when needed. Serum was measured for the following cytokines: IL-6, IFN-γ (BD

Biosciences), TNF (BioLegend), and IL-10 (BioLegend) using sandwich enzyme-linked immunosorbent assay.

2.8.2 Sandwich enzyme-linked immunosorbent assay

In a bid to quantify cytokine released using sandwich ELISA, 96-Well Microplates (Thermo Fischer Scientific) were coated overnight at 4 ^{O}C with 50 µl of capture antibody diluted appropriately using the recommended concentration in 1X PBS. Plates were washed 4 times with 1x wash buffer (13.4 mM KCl, 5.8mM KH_2PO_4, 684.4 mM NaCl, and 0.25% Tween) (Merck), blocked with 200ul of blocking buffer (2% BSA (Roche)) then incubated in a humidified chamber at 37 ^{O}C for 3 hours. Plates were washed 4 times and standards were appropriated diluted in dilution buffer (1% BSA) and added in a 1:2 serial dilution over 18 wells 50 µl per well. Likewise, 50 µl of samples were added to each well then incubated overnight at 4 ^{O}C. The plates were washed 4 times, 50 µl of biotinylated secondary antibody diluted to the recommended concentration in dilution buffer (1% BSA) was added to each well then, incubated in a humidified chamber at 37 ^{O}C for 3 hours. The plates were washed 4 times with 1X wash buffer, appropriately diluted avidin-alkaline phosphatase (AP) or avidin-horseradish peroxidase (HRP) conjugate was added 50 µl per well and incubated in a humidified chamber for 1 hour at 37 ^{O}C. Plates were washed 4 times then developed at room temperature by adding 50 µl of either 1mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate in substrate buffer or TMB Microwell Peroxidase Substrate (KPL) to each well. The reaction was stopped by adding 50 µl of either 1M NaOH or 1M H_2SO_4. The absorbance and reference filter was read at 405 and 492 or 450 and 540 nm respectively using VersaMax™ Absorbance Microplate Reader (Molecular Devices, California, USA).

2.9 Flow cytometry

2.9.1 Single cell isolation

The hippocampus was collected in 1 ml sterilized Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Waltham, Massachusetts) supplemented with 100 U/ml penicillin-streptomycin (Gibco) and 10% inactivated fetal bovine serum (iFBS, Gibco). The single cell suspension was done on a petri-dish by crushing the sample through a 70 µM nylon cell strainer (Falcon, Corning, MA) using the plunger of a 2 ml sterile syringe. The solution was then transferred into a 15 ml Falcon tube and washed with 3 ml Hank's solution (HBBS) then spun down at 1500 rpm for 5 minutes at 4 ^{O}C. The supernatant was decanted, and the pellet resuspended in 1 ml 1X PBS supplemented with 3% iFBS to allow for viable cell count using trypan blue (Lonza, Walkersville, Maryland) at 1: 10 dilution. After cell number estimation, the samples were then resuspended at 2 x 10^6 cells/ml.

2.9.2 Antibodies

Single-cell suspensions from the hippocampus of the four experimental groups were stained for the following surface and intracellular markers: CD11b (V450, clone: M1/70 BD biosciences); CD45 (biotin, clone: 30-F11 BD biosciences); streptavidin (PE-CF594, BD biosciences); Live/dead (Qdot 605); F4/80 (APC, clone: BM8 eBioscience); IL-10 (FITC, clone: JES5-16E3 BD biosciences); IL-13 (PE-cy7, clone: eBio 13A eBioscience); IL-4 (PE, clone: 11B11 BD biosciences); IFNy (Alexa fluor, clone: XMG 1.2 BD biosciences).

2.9.3 Extracellular staining

Cells were plated 2 x10^6 per 100 µl in a V-bottom 96 well plates then spun down at 1200 rpm for 10 minutes at 10 ^{O}C. The supernatant was discarded then sediment was stained with 50 µl antibody mix of CD11b, CD45 and F4/80 containing 2% inactivated rat serum and 1% FC block to prevent non-specific binding. Samples were mixed with a multichannel pipette and incubated in the dark for 30 minutes at 4 ^{O}C. After incubation, it was centrifuged at 1200 rpm for 10 minutes and unbound antibodies were discarded. The second antibody mix of streptavidin and live-dead marker was added and incubated for 30 minutes at 4 ^{O}C then centrifuged at 1200 rpm for 10 minutes to discard unbound antibodies.

2.9.4 Intracellular cytokine staining

To detect intracellular cytokines, samples were stimulated with 10 µl of the following mix of 2.5 µl phorbol myristate acetate (PMA), 2.5 µl Ionomycin and 1 µl monensin prepared in 500 µl I10 media (IMDM, 10% fetal bovine serum and 1% penicillin streptomycin) and incubated for 8-12 hours at 37 ^{O}C in a humidified chamber. Samples were washed with 200 µl FACS buffer by pipetting up and down then spun down at 1200 rpm for 10 minutes at 10 ^{O}C and supernatant was discarded. Cells were fixed in 100 µl ice cold PBS and 100 µl 4% paraformaldehyde mix and left on ice for 1 hour in the dark. Samples were then washed in 200 µl ice cold PBS and permeabilized with permeabilization buffer (1X PBS containing 1% saponin and 0.1% BSA) for 1 hour at 4 ^{O}C in the dark. The samples were centrifuged at 1200 rpm for 10 minutes at 10 ^{O}C and supernatants discarded then stained for 1 hour with cytokine antibody mix of IFNγ, IL-10, IL-4, and IL-13 containing 2% inactivated rat serum and 1% FC block to prevent non-specific binding. Cells were washed in permeabilization buffer then resuspended in 100 µl FACS buffer and transferred to FACS tubes for BD LSRFortessa™ (BD Biosciences) cell acquisition. Analysis was done using FlowJo™ Software version 10.6.

2.10 Histopathological analysis

Samples of spleen and liver were collected from the four groups, preserved in 4% formalin then paraffin-embedded. Histological sections (5 µm) were stained with Hematoxylin and Eosin (H&E). Slides were examined for microarchitectural changes, inflammatory cell infiltration, splenic and hepatocyte alterations. Images were captured using Olympus-VS-ASW-L100 software on an Olympus VS120-L100 scanner.

2.11 Statistical analysis

The values are presented as mean ± SEM and statistical significance was determined using the unpaired Student's *t*-test, One or Two-Way ANOVA with Bonferroni's post-test (GraphPad Prism). Significance was set at ($p>0.05$: ns, $p\leq0.05$: *, $p\leq0.01$: **, $p\leq0.001$: ***, $p\leq0.0001$: ****) 95% confidence interval.

CHAPTER 3: RESULTS

3.1 The impact of Morris water maze training task on immune responses in the brain.

Training, physical activity or exercise-induced immune benefits has been extensively researched in health but mainly in various disease conditions (Micky et al., 2017). The immune system is believed amenable to training based on the intensity, and span of exertion (Nieman & Wentz, 2019). Studies have shown and compared the effects of the different span of exercise on the peripheral immune system (Campbell & Turner, 2018) like-wise, exercise-induced immune benefits have been highlighted in several brain health-related conditions for instance in schizophrenia (Gómez-Rubio & Trapero, 2019) however it has not been a focus to evaluate training effects on the basal level of immune responses in the brain at a steady-state without any underlying disease condition. Swimming is one of the most used exercises in rodent owning that it is their natural ability (W. Liu et al., 2012). In our case, the MWM training task was considered a form of swimming exercise coupled with its primary function as a spatial learning and memory test. Thus, we investigated immune responses following the MWM training task in naïve groups of mice to serve as a control basis for our subsequent aims in this study.

3.1.1 Cytokine production in the brain following the Morris water maze training task.

To determine the impact of training on cytokine levels in the prefrontal cortex, hippocampus, and hypothalamus as the brain areas of focus, two groups of mice were recruited. One group (Non-infected trained) was subjected to the MWM training task while the other group (Non-infected non-trained) did not perform the task. The mRNA expression of IL-6, IFN-γ, TNF and IL-10 were quantified using quantitative polymerase chain reaction. It is of note that there is usually a basal level of IL-6 in the brain under physiological conditions which functions to providing neurotropic properties which are beneficial to the central nervous system (Trapero & Cauli, 2014; Hunter & Jones, 2015). In this study, we found that both the non-infected non-trained and the non-infected trained group had a comparable level of IL-6 in the three brain areas (Figure 8). In addition, the levels of IFN-γ, TNF and IL-10 were not affected by training as these cytokines were not detected in both groups neither trained nor the non-trained group. This result suggests that the basal level of cytokines in the brain is not affected by training at naïve state.

Figure 8: Cytokine expression in the brain following Morris water maze training task. The non-infected trained group performed the MWM training task while the non-infected non-trained did not. RNA extraction from the prefrontal cortex, hippocampus and hypothalamus converted to cDNA and normalised to HPRT mRNA control was measured for IL-6 production in the (A) prefrontal cortex, (B) hippocampus, and (C) hypothalamus. Representative data of two independent experiments n= 5-7 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's t-test and indicated as p>0.05: ns.

3.1.2 Myeloid cell production following Morris water maze training task.

The production and distribution of myeloid cells (microglia and macrophages) in the brain vary with the state and condition of the brain as they play essential roles in a healthy and diseased CNS state (Herz et al., 2018). To evaluate the composition of training influenced myeloid cells in the brain, a single-cell suspension was obtained from the hippocampus and quantitatively assessed their overall CD11b$^+$ myeloid cell population, CD11b$^+$ CD45^{hi} macrophage cell population as well as CD11b$^+$ CD45^{lo} microglial cell population. This distinction of macrophage and microglial cell population was performed as previously described (Hsieh et al., 2014) when analysed by flow jo. Figure 9 describes the gating strategy used to identify these various immune cells. The MWM spatial training task is mostly hippocampal-dependent (Vorhees & Williams, 2006) hence, the hippocampus being the brain area of focus.

Results showed the percentage and cell number of the CD11b$^+$ myeloid cell population (Figure 10A), CD11b$^+$ CD45^{hi} macrophage cell population (Figure 10B) also the CD11b$^+$ CD45^{lo} microglial cell population (Figure 10C) did not differ with the MWM training task. We as well evaluated the cytokines produced by these myeloid cells and the result showed that the percentage and cell number of the proinflammatory IFN-γ, and anti-inflammatory IL-10, IL-4 and IL-13 cytokines produced by the macrophage (Figure 10D-G) and microglia cell (Figure 10H-K) does not differ with MWM training.

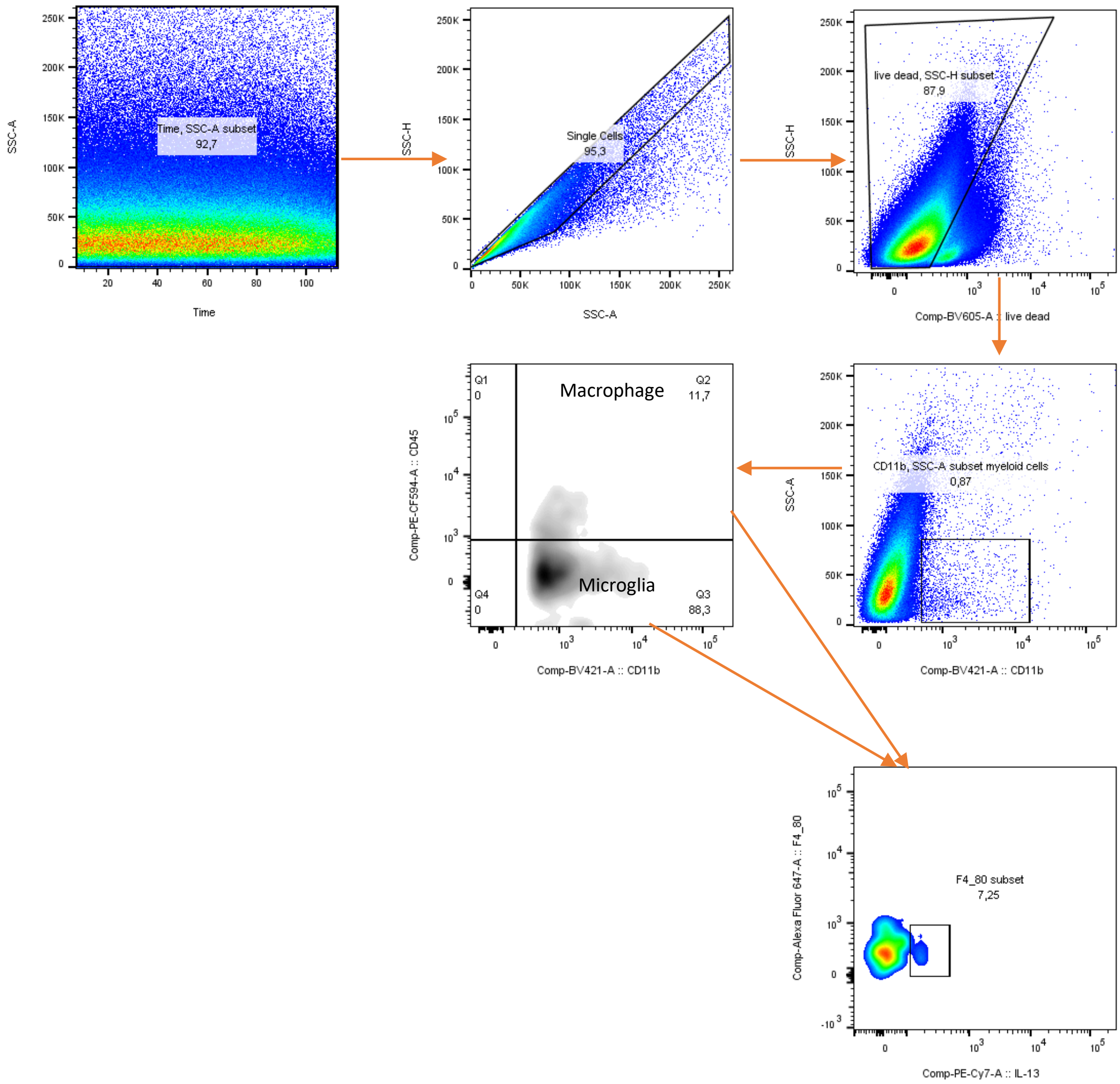
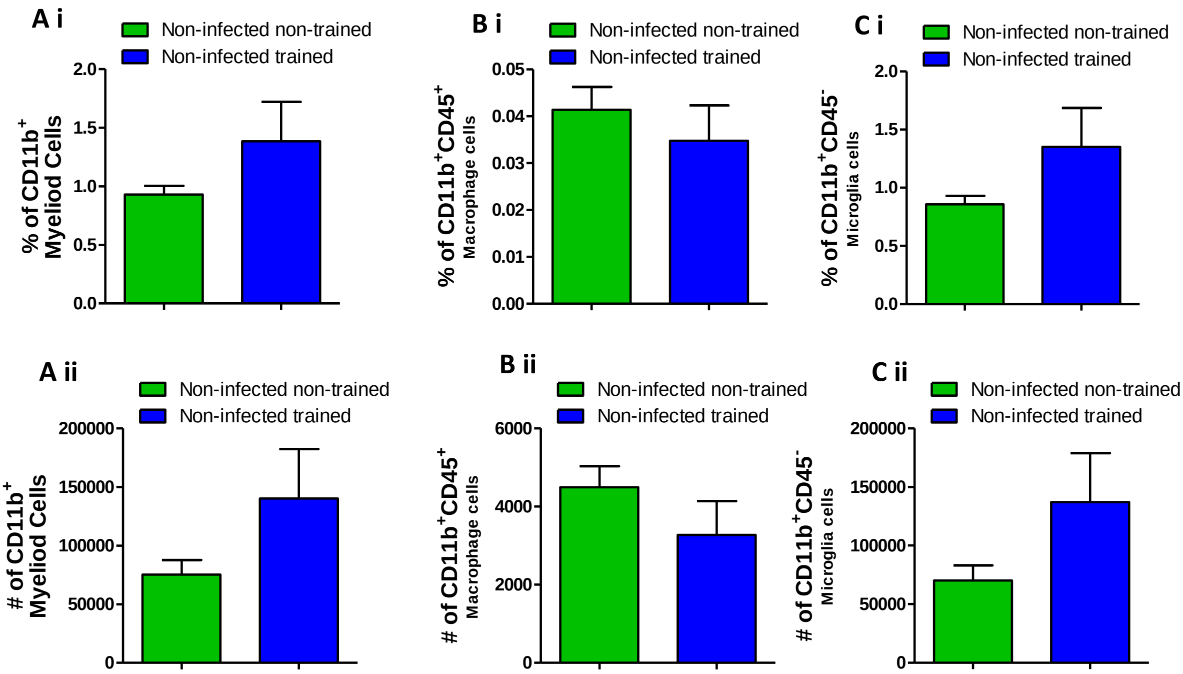


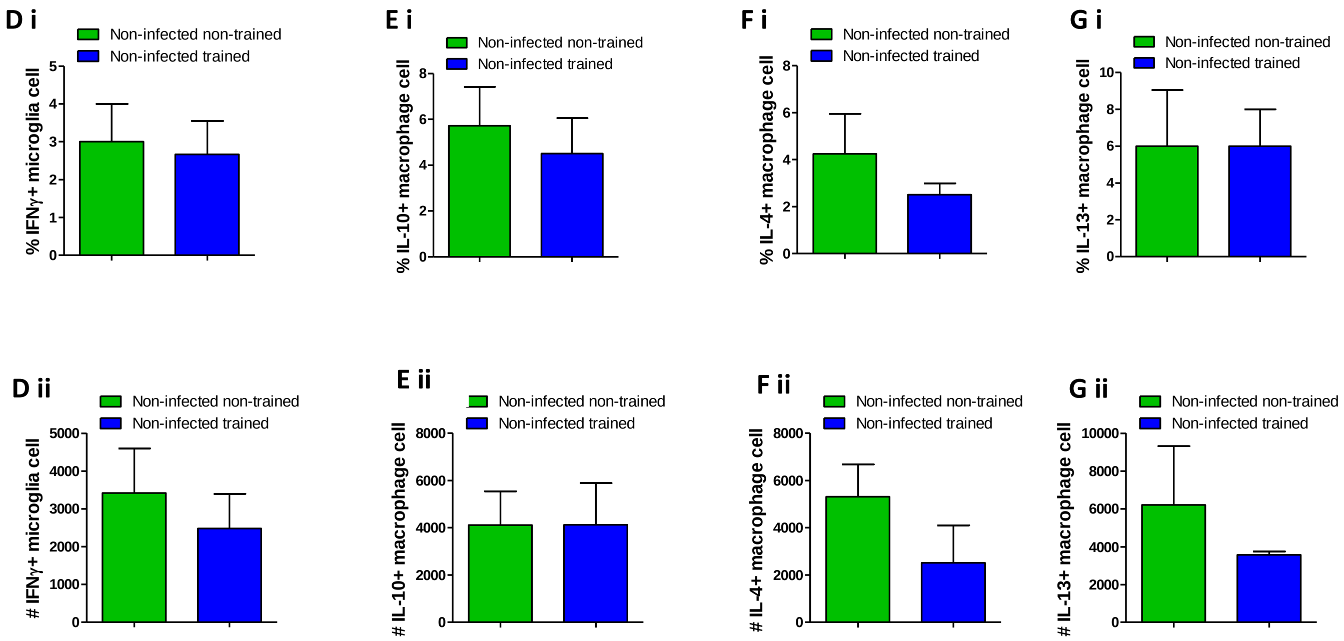
Figure 9: Hippocampus myeloid cell populations and cytokines produced gating strategy. From the time gate, single cells were gated from the SSC-H and SSC-A, then gated for live cells from SSC-H and live/dead. The live cells were next gated for CD11b positive myeloid cells. From the myeloid cell positive population, we gated for macrophages "CD11b positive and CD45 high cells" also microglia "CD11b positive and CD45 low cells". Then, from either the macrophage or microglia cells, we gated for F4/80 positive and cytokines of interest

positive cells (IFNγ, IL-10, IL-4 and IL-13). The analysis was done using FlowJo version 10.6.2.

Myeloid cells



Cytokines produced by macrophage



Cytokines produced by microglia

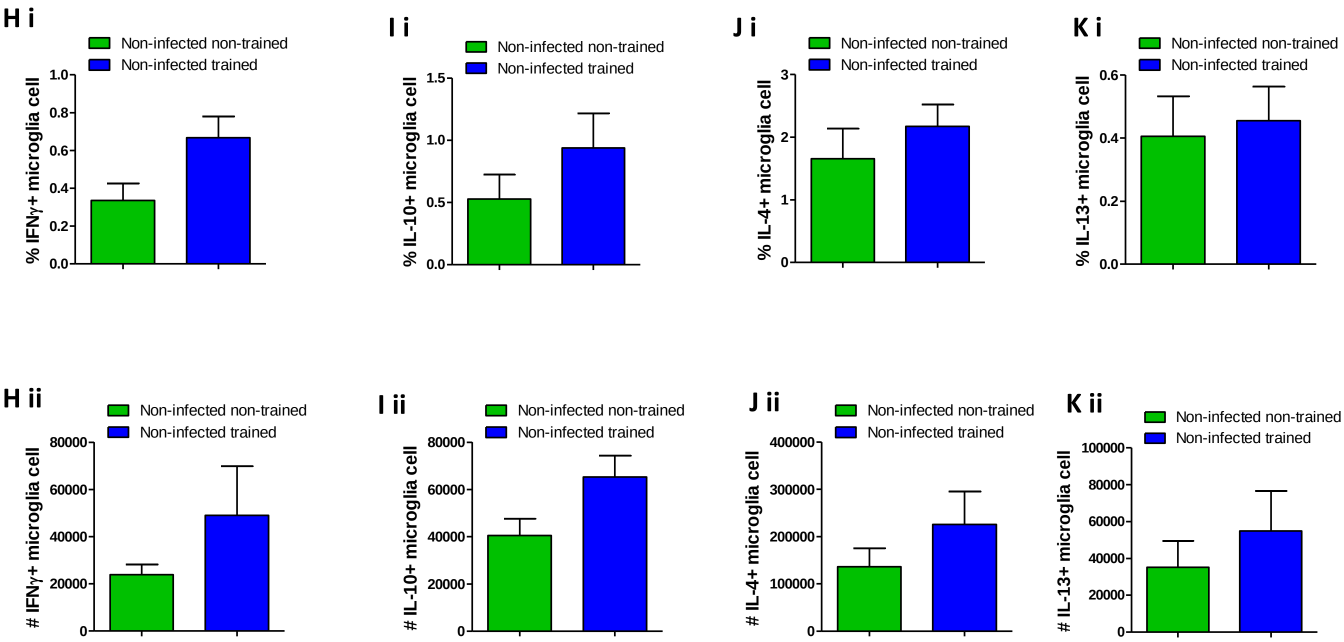


Figure 10: Immune cell and resulting cytokines produced following the Morris water maze training task. The non-infected trained group performed the MWM training task while the non-infected non-trained did not. Hippocampal single-cell suspension from both groups was assessed using flow cytometry to determine the percentage and cell number of the (A) overall myeloid cell, (B) macrophage cell, and (C) microglial cells production. The single-cell suspension was also stimulated for cytokine production and Flow cytometry was used to determine the percentage and number of (D) IFN-γ, (E) IL-10, (F) IL-4, and (G) IL-13 cytokine produced by macrophage cell and likewise (H) IFN-γ, (I) IL-10, (J) IL-4, and (K) IL-13 produced by the microglial cell. *n*= 5-7 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns.

3.1.3 Discussion

To start with, we evaluated training-influenced immune composition in the brain of mice at a naïve state. Here, the expression levels of various cytokines and myeloid cells were observed in the prefrontal cortex, hippocampus, and hypothalamus of the non-infected non-trained and non-infected trained groups. As aforementioned, the immune system responds based on the intensity and duration of the exercise. Mice model of age-induced inflammation has shown that the level of IL-6 and TNF can be reduced with prolonged exercises (Uchida et al., 2019). In its support, long term exercise has also been shown to reduce pro-inflammatory cytokine production in sleep-deprived mice (Chennaoui et al., 2015). Our study found that both experimental groups of the trained and non-trained expression level of IL-6 do not differ, therefore, stipulating that the basal level of this cytokine is not affected by the MWM training task. IFN-γ, TNF and IL-10 were not expressed in both experimental groups, again indicating that the MWM training does not affect the baseline level of these cytokines in the brain.

The myeloid cells including macrophage and microglia are crucial in healthy brain maintenance (Yin et al., 2017). The proportion of myeloid cells can however be impacted by exercise especially with ageing or compromised health condition. For instance, prolonged training reduces the level and activation of macrophage and microglial cell in the brain of aged mice (Kohman et al., 2013; Uchida et al., 2019). High-intensity regular training likewise modulates macrophage and microglia production in the brain hence protecting against neuroinflammation (Zaychik et al., 2021). Also, a prior long term regular swimming exercise before the onset of encephalomyelitis disease ameliorates the disease outcome by modulating the proinflammatory cytokine production (Bernardes et al., 2013). Nonetheless, our work demonstrated that at the naïve state of the experimental mice, levels of myeloid cells and their resulting cytokines (IFN-γ, IL-10, IL-4, and IL-13) produced does not differ with training. This contrary observation might be due to the transient 8 days of MWM training (not enough duration of exercise) as opposed to what was shown in other studies where prolonged/regular exercise was used to achieve an observable result. Also, these previous studies were conducted under an existing inflammatory condition wherefore exercise was used as a therapy option as opposed to the aim of this segment that rather focuses on the impact of exercise on the naïve state of the mice. In a nutshell, it is assumed that an inflammatory condition might be needed before the impact of prolonged/regular exercise can be seen in the brain immune composition.

3.2 The impact of *T. b. brucei* infection on peripheral organs, Spatial learning and memory and the immune responses in the brain.

The *Trypanosoma brucei* infection results in a severe meningoencephalitis disease that emerges after an early haemolymphatic system infection (Ponte-Sucre, 2016). This disease elicits an initial inflammatory immune response then, a counter inflammatory reaction. In mice, several peripheral visceral organs such as the spleen, liver, kidney, heart (Sivajothi et al., 2015), and reproductive system (Carvalho et al., 2018) are affected by the disease but the effect is more pronounced in spleen and liver as evidenced with enlargement. A mice model of *Trypanosoma evansi* has been used to demonstrate a resulting cognitive impairment emerging from the infection (Wolkmer et al., 2013; Baldissera et al., 2015). Observations from several studies indicate that increased levels of inflammatory responses in the brain are associated with cognitive alterations nonetheless, the anti-inflammatory immune reaction has also been shown not to be absolute in rescuing the brain from the inflammatory damage (Chakrabarty *et al*., 2015). Here, we investigated the pathology associated with, and the immune responses elicited following *T. b. brucei* infection in mice.

3.2.1 Parasitemia load during *T. b. brucei* infection

Trypanosomiasis disease is known for its characteristic oscillating blood parasitemia wave (Trindade et al., 2016). To investigate this typical trait, monitor a successful infection, determine the parasitemia burden and as well track disease progression, we infected two of our four experimental groups with *T. b. brucei* and microscopically examined blood smears from these groups (infected non-trained and infected trained) for their parasite load during the experiment at two days interval. Successful infection was validated by the presence of an observable parasite in their blood. This parasite load in the blood got to its peak within 5-7 days from two independent experiments. Afterwards, the mice experienced about 3-4 days of an undetectable parasite subsequently, parasites were detected again with a declined parasitemia burden which connotes the typical parasite wave associated with the disease (Figure 11). This observed wave in parasite load likewise hints transition into the second stage of infection (Ponte-Sucre, 2016). There were no differences in peak and clearance of parasite between the infected non-trained and the infected trained groups.

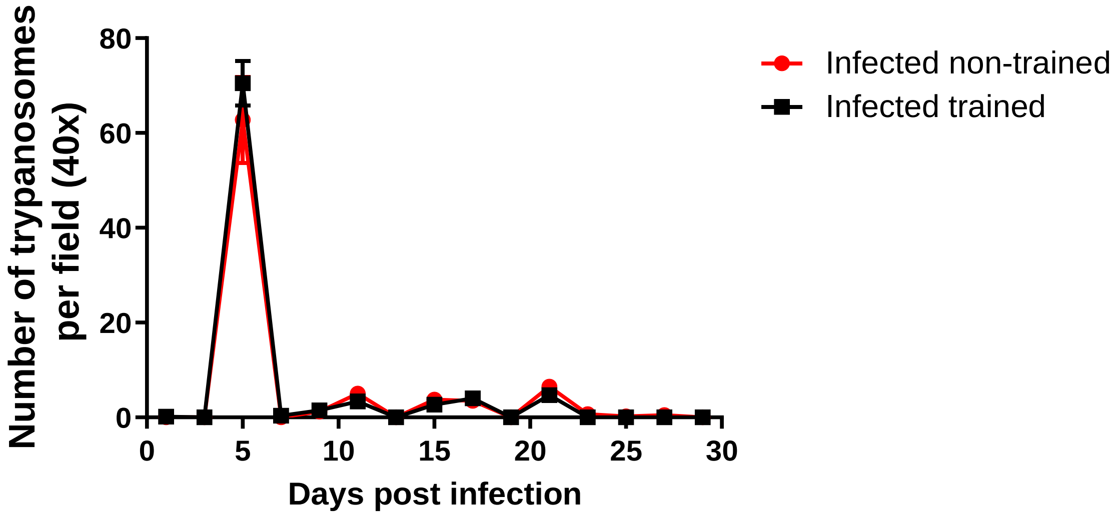


Figure 11: The course and progression parasitemia burden in *T. b. brucei* infected mice. Quantification of the average parasite load in the blood of the infected groups of mice (infected non-trained and infected trained). Parasitemia data are expressed as the means ± SEM of *n* =4-6 mice per group. Statistical significance was determined by two-way ANOVA and indicated as *p*>0.05: ns.

3.2.2 *T. b. brucei* infection results in a loss of body weight.

Having established a successful infection, we aimed to evaluate weight changes resulting from *T. b. brucei* infection keeping in mind the humane endpoint of 10% weight loss. It was observed that after the first parasitaemia peak, infected mice (Infected non-trained and Infected trained) elicited a temporary withdrawal from chow and water intake followed by a decrease in body weight. Eventually, they recovered and returned to their normal food intake although still with reduced body weight compared to the non-infected control group (Figure 12).

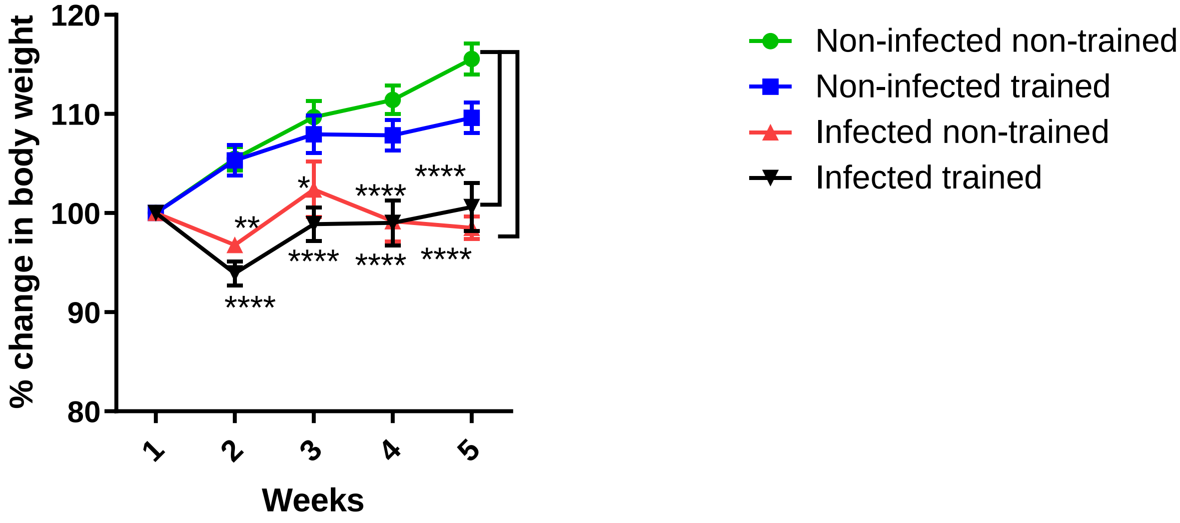


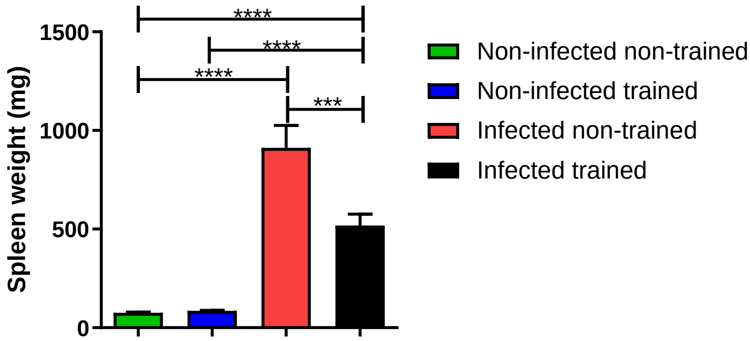
Figure 12: Parasitemia course and progression in *T. b. brucei* infected mice. The four experimental groups of mice (non-infected non-trained, non-infected trained, infected non-trained and infected trained) were evaluated for their percentage change in body weight. Data of body weight changes are expressed as the means ± SEM of n =4- 7 mice per group representative of two independent experiments. Statistical significance was determined by two-way ANOVA and indicated as $p>0.05$: ns, $p\leq0.05$: *, $p\leq0.01$: ** and $p\leq0.0001$: **** Bonferroni's Test: compared to non-infected non-trained control group.

3.2.3 *T. b. brucei* infection causes splenomegaly and spleen histopathology.

The lesion of the spleen is one of the pronounced pathology associated with trypanosomiasis disease (Omotainse & Anosa, 2009). The general pathological observation showed a significant increase in the spleen size (Figure 13 Ai) and weight (Figure 13 Aii) of the infected groups compared to their non-infected counterparts at the experimental endpoint. It was however interesting as well to observe a significant reduction in the weight of the infected trained group compared to the infected non-trained group. We performed a histological examination of the spleen tissue sections to evaluate the extent of pathology caused by the infection. The gross histological appearance of the spleen of the non-infected groups (non-infected non-trained and non-infected trained) presented with clear and distinct marginal zones between the white pulp (white star) and red pulp (red star) (Figure 13 Bi and 13 Ci) however, the histological spleen architecture of the infected groups (infected non-trained and infected trained) (Figure 13 Di and 13 Ei) was altered shown by the lack of a distinct marginal zone between the white and red pulp. Also, the non-infected non-trained (Figure 13 Bii) and non-infected trained (Figure 13 Cii) showed very few and isolated multinucleated giant cells (yellow arrow) overall, with no histopathological alterations. On the other hand, the infected groups have numerous multinucleated giant cells (Figure 13 Dii and 13 Eii, yellow arrows) with a seemingly larger cell in the infected non-trained when compared to its infected trained counterpart. This result suggests a histopathological lesion of the spleen with *T. b. brucei* infection.

Ai

Aii



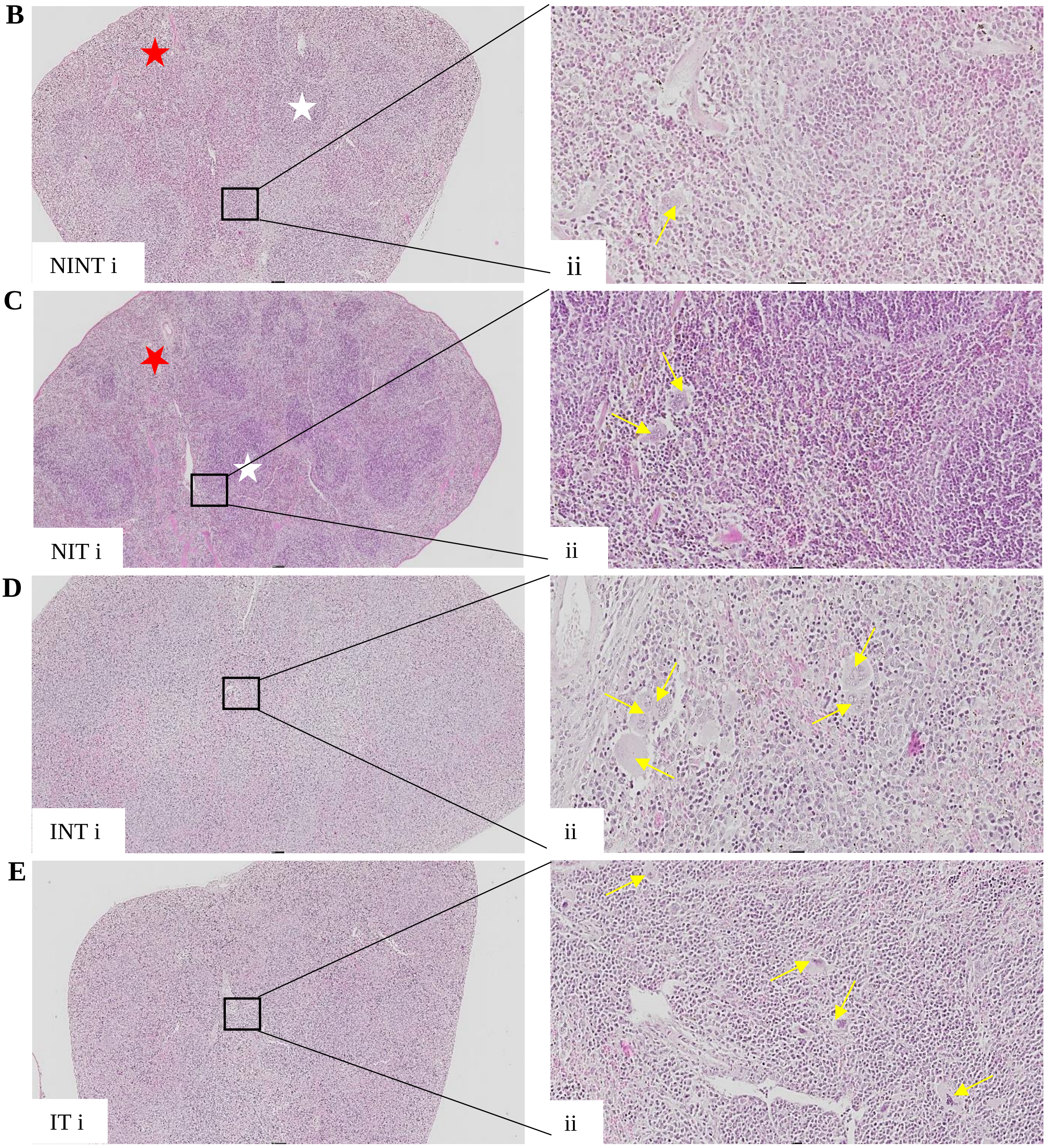


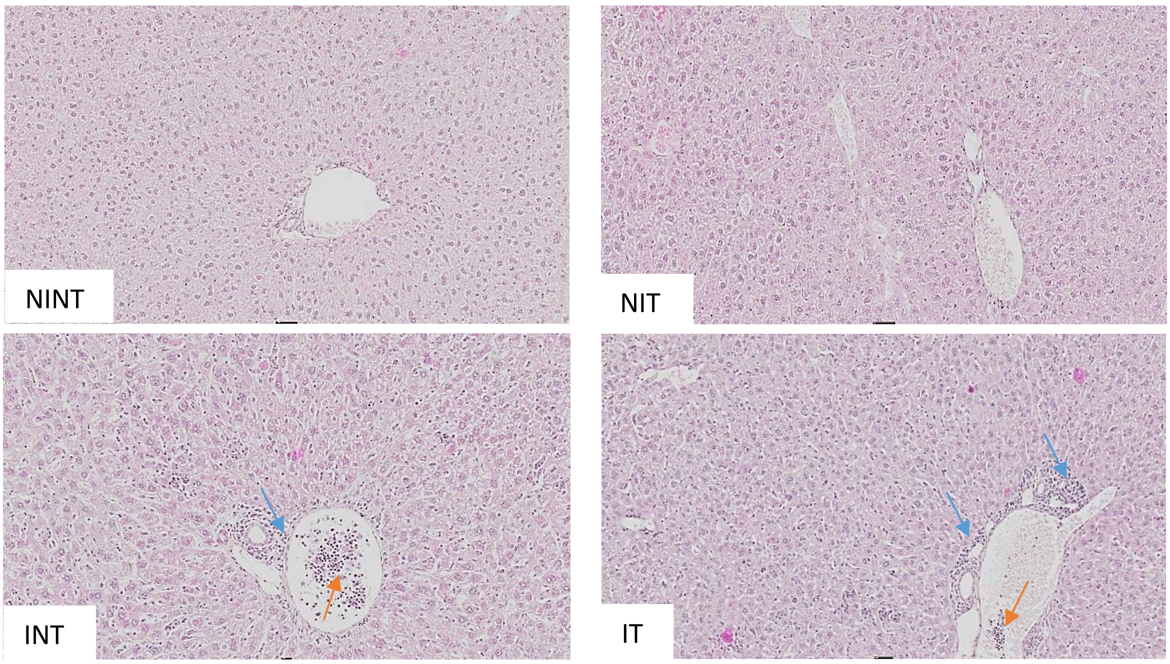
Figure 13: Histopathology and spleen size and weight post *T. b. brucei* infection. Evaluation of the (Ai) Spleen size and (Aii) weight. H&E staining of spleen tissue of the four experimental groups (B) Non-infected non-trained, (C) Non-infected trained (D) Infected non-trained, and (E) Infected trained. The red star, white star, and yellow arrow denotes red pulp, white pulp, and multinucleated giant cell, respectively. Images were captured using Olympus-VS-ASW-L100 software. Scale bars are 20 µm. Representative data of *n*= 4-7 mice per group.

Data are expressed as mean ± S.E.M. Statistical significance was determined by One-Way ANOVA and indicated as $p>0.05$: ns, $p\leq0.001$: *** and $p\leq0.0001$: **** Bonferroni's Test.

3.2.4 *T. b. brucei* infection causes to hepatomegaly and liver histopathology.

We also assessed the liver since hepatomegaly is a known consequence of the disease. The weight of the liver in infected groups was increased significantly compared to the non-infected groups (Figure 14B). Histological examination of the liver tissue sections was performed as well to estimate the expanse of pathology caused during *T. b. brucei* infection as shown in figure 14A. The non-infected non-trained (NINT) and non-infected trained (NIT) groups presented with a normal portal tract free of inflammation without any form of histopathological alterations in their liver tissue. However, the infected groups displayed a portal tract inflammation (red arrow) as well as a perivascular inflammation (blue arrow). The inflammation in the infected trained group (IT) on the other hand presented with milder pathological alterations compared to the infected non-trained group (INT). This result suggests a liver histopathological lesion following *T. b. brucei* infection.

A



B

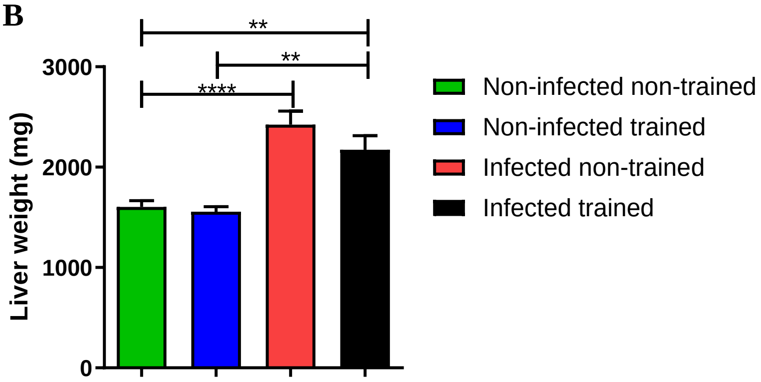


Figure 14: Alterations in liver weight and histology during *T. b. brucei* infection. Evaluation of the (A) Liver weight. H&E staining of liver tissue of the four experimental groups (B) Non-infected non-trained (NINT), Non-infected trained (NIT), Infected non-trained (INT), and Infected trained (IT). Images were captured using Olympus-VS-ASW-L100 software. Scale bars are 20 µm. Representative data of n= 4-7 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by One-Way ANOVA and indicated as p>0.05: ns, p≤0.01: ** and p≤0.0001: **** Bonferroni's Test.

3.2.5 Cytokine blood sera ELISA

We also investigated the peripheral blood following infection since trypanosomiasis disease begins with the blood infection. At the 30th day post-infection (experimental endpoint) blood was taken from the four experimental groups and cytokine ELISA was conducted on its serum. Since IFN-γ, TNF is required for parasite control and IL-6 and IL-10 are necessary for a counter inflammatory response, these cytokines were investigated in the blood sera. During the biological assay, IL-6 and TNF cytokines were undetected in the serum across all groups from two independent experiments performed (data not shown). Besides, IFN-γ (Figure 15A) and IL-10 (Figure 15B) were markedly expressed in the infected non-trained and infected trained group whilst these cytokines remained undetectable in the non-infected groups (non-infected non-trained and the non-infected trained). This result indicates that *T. b. brucei* infection elevates the production of IFN-γ and IL-10 in the periphery.

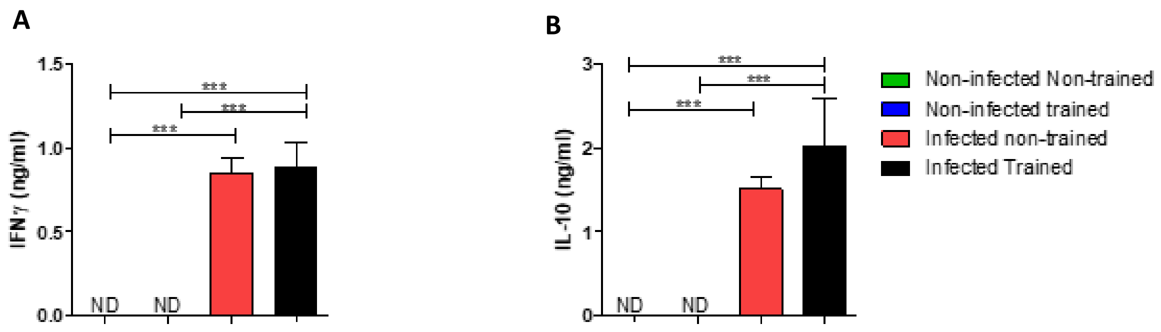


Figure 15: Infection with *T. b. brucei* elevates serum IFNγ and IL-10 level. Quantification of (A) IFNγ and (B) IL-10 serum cytokine concentration by ELISA. Results are shown from two independent experiments n= 4-6 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by One-Way ANOVA and indicated as $p>0.05$: ns, $p\leq0.01$: ** and $p\leq0.001$: *** Bonferroni's Test.

3.2.6 Cytokine production in the brain following *T. b. brucei* infection.

Earlier, we established that the MWM training task does not affect the basal level of cytokines in the brain, now, we sought to evaluate the impact of *T. b. brucei* infection on cytokine profiles in the brain. To address this, two groups of trained mice with differences in infection status (non-infected trained and infected trained) were investigated for IL-6, IFN-γ, TNF and IL-10 levels in the three brain areas of focus (prefrontal cortex, hippocampus, and hypothalamus) knowing that these cytokines are implicated in this disease model. The basal level of Il-6 in the brain which is said to be beneficial to the physiological function of the central nervous system can unfortunately be altered by infection or any form of disturbance to the brain (Bradburn et al., 2018). We found that the level of IL-6 in response to infection was significantly upregulated in all three brain areas of the infected groups compared to their non-infected counterparts (Figure 16 Ai, ii, iii). The pro-inflammatory IFN-γ (Figure 16 Bi, ii, iii) and TNF (Figure 16 Ci, ii, iii) and the anti-inflammatory IL-10 (Figure 16 Di, ii, iii) cytokines evaluated were not detected in the non-infected groups but significantly expressed in all three brain areas of the infected groups. This result indicates that infection with *T. b. brucei* upregulates the level of inflammatory and counter-inflammatory cytokine immune responses in the brain.

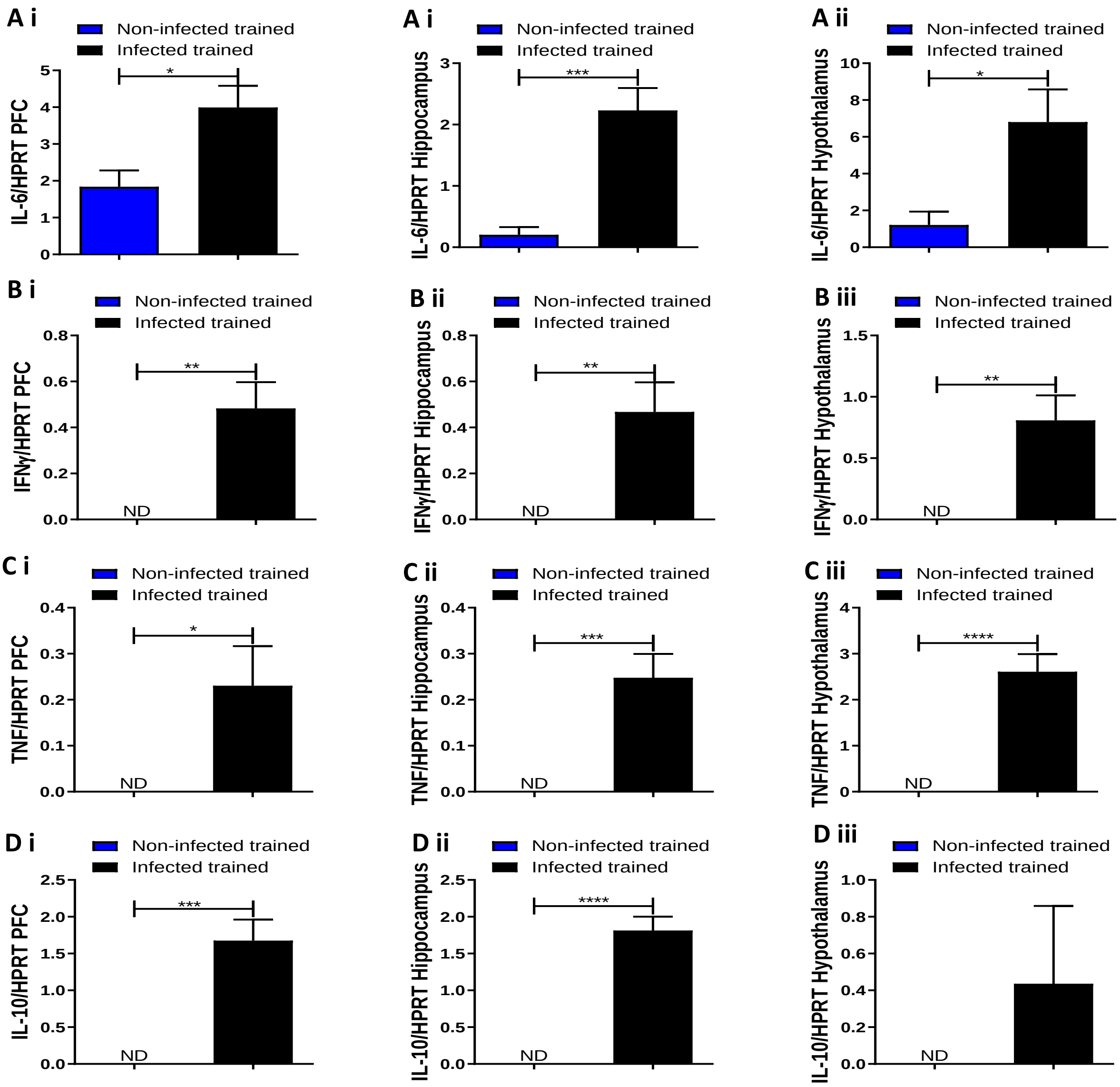


Figure 16: Cytokine immune response in the brain following *T. b. brucei* infection. The infected trained group was inoculated with 2000 *T. b. brucei* parasites while the non-infected trained group was not infected. RNA extraction from the prefrontal cortex, hippocampus and hypothalamus converted to cDNA and normalised to HPRT mRNA control was measured for (A) IL-6, (B) IFN-γ, (C)TNF and (D) IL-10 production. Results are shown from two independent experiments *n*= 4-6 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns, $p>0.05$: *, $p\leq0.01$: **, $p\leq0.001$: ***, and $p\leq0.0001$: ****.

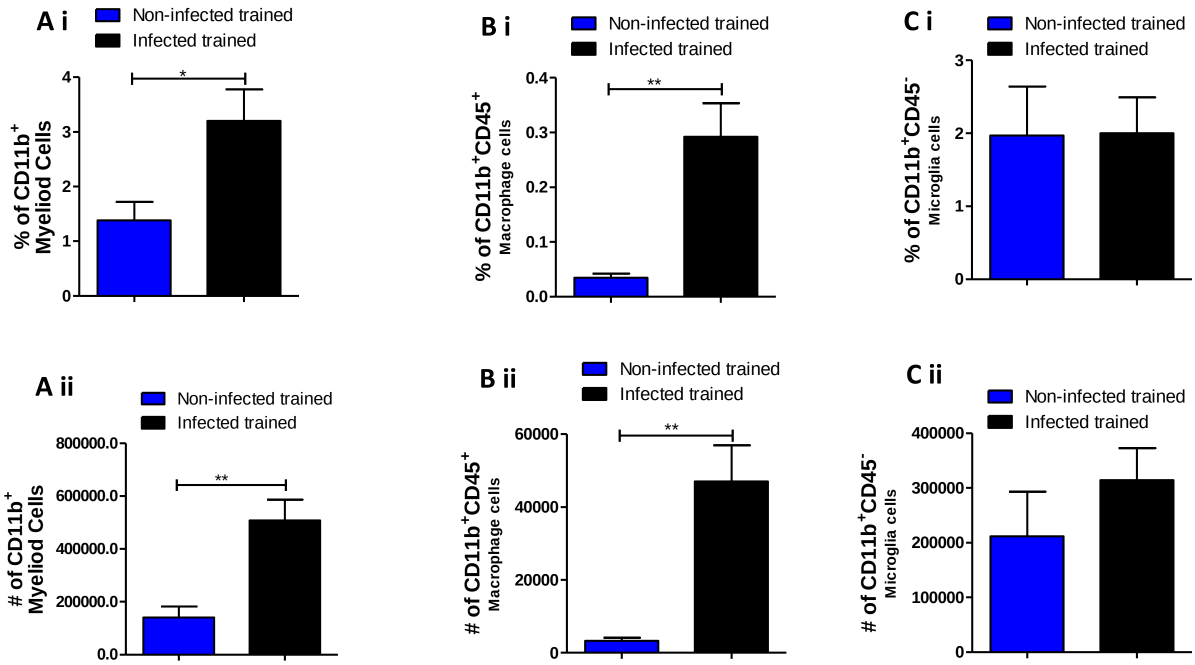
3.2.7 Myeloid cell production in the brain following *T. b. brucei* infection.

The myeloid cells (microglia and macrophages) are equipped to combat the central nervous system invading pathogens (Herz et al., 2018). Having checked that the composition of training-induced myeloid cells (non-infected non-trained compared to the non-infected trained group), we proceeded to evaluate the composition of *T. b. brucei* infection-influenced myeloid cells in the brain. To address that, a single-cell suspension was obtained from the hippocampus and quantitatively assessed their overall $CD11b^{+}$ myeloid cell population, $CD11b^{+}$ $CD45^{hi}$ macrophage cell population as well as $CD11b^{+}$ $CD45^{lo}$ microglial cell population.

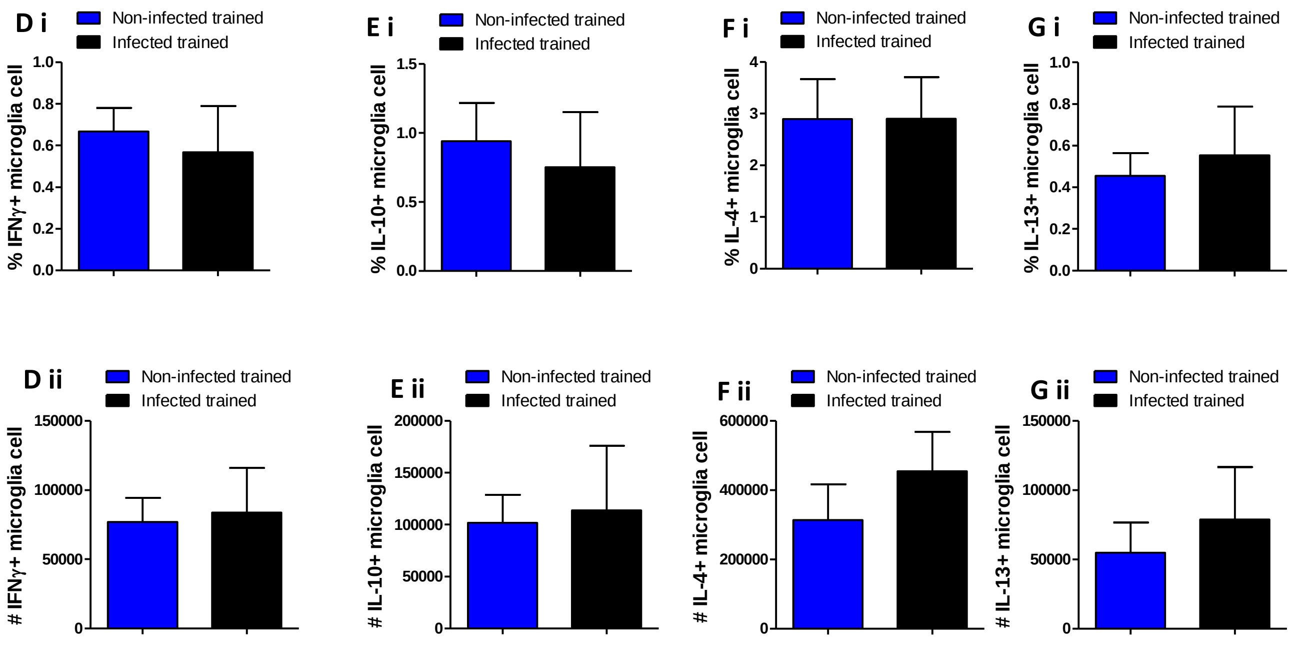
Refer to figure 9 for the gating strategy used to identify these various immune cells.

Results showed the percentage and cell number of the $CD11b^{+}$ myeloid cell population (Figure 17 Ai, ii), and $CD11b^{+}$ $CD45^{hi}$ macrophage cell population (Figure 17 Bi, ii) in the infected group is significantly elevated compared to the non-infected group. On the other hand, this increase in myeloid cell population does not reflect on the $CD11b^{+}$ $CD45^{lo}$ microglial cell population (Figure 17 Ci, ii) as there were no differences between the non-infected and infected groups. We then went further to investigate the cytokines produced by these myeloid cells. Our result showed that the percentage and cell number of the proinflammatory IFN-γ (Figure 17 Di, ii), and anti-inflammatory IL-10 (Figure 17 Ei, ii) cytokines produced by the macrophage cell was significantly higher in the infected group, but IL-4 (Figure 17 Fi, ii) and IL-13 (Figure 17 Gi, ii) production by this macrophage cell was comparable between groups. Consistent with the no differences found in the overall population of microglia cell, IFN-γ (Figure 17 Hi, ii), IL-10 (Figure 17 Ii, ii), IL-4 (Figure 17 Ji, ii) and IL-13 (Figure 17 Ki, ii) cytokines produced by this microglia cell as well does not differ with T. b. brucei infection.

Myeloid Cells



Cytokines produced by macrophage cell



Cytokines produced by microglial cell

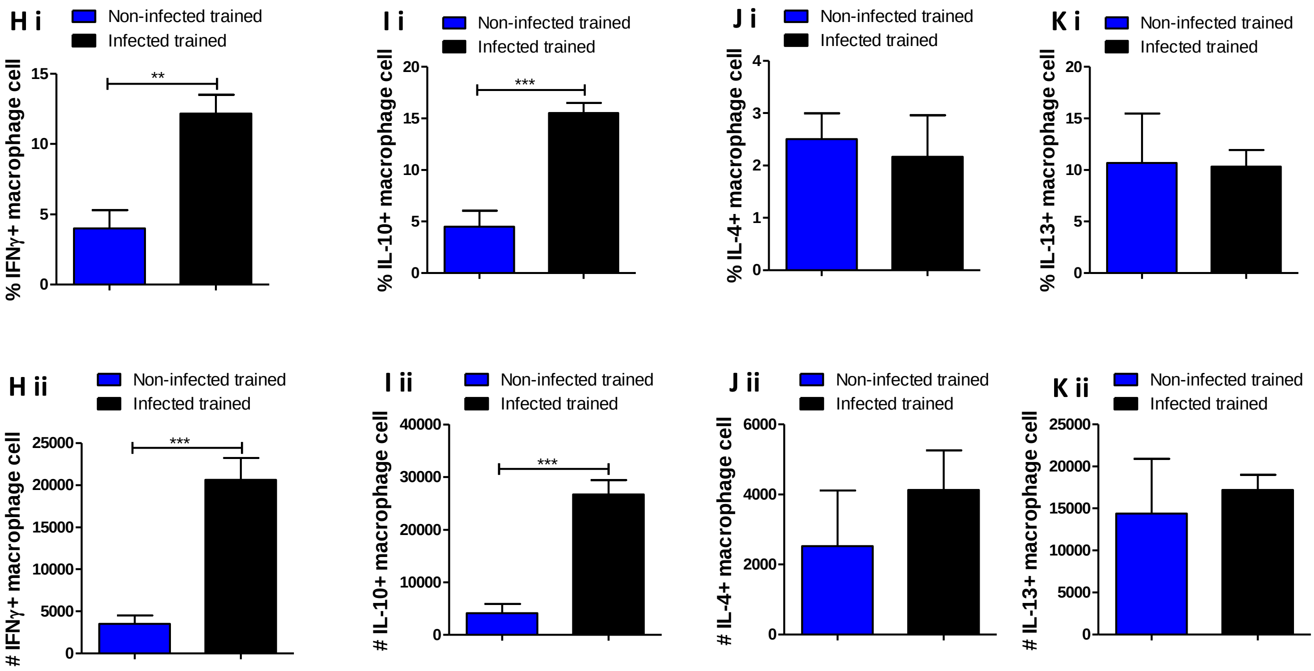


Figure 17: Immune cell and resulting cytokines produced following *T. b. brucei* infection. The infected trained group was inoculated with 2000 *T. b. brucei* parasites while the non-infected trained group was not infected. Hippocampal single-cell suspension from both groups was assessed using flow cytometry to determine the percentage and cell number of the (A) overall myeloid cell, (B) macrophage cell, and (C) microglial cells production. The single-cell suspension was also stimulated for cytokine production and Flow cytometry was used to determine the percentage and number of (D) IFN-γ, (E) IL-10, (F) IL-4, and (G) IL-13 cytokine produced by macrophage cell and likewise (H) IFN-γ, (I) IL-10, (J) IL-4, and (K) IL-13 produced by the microglial cell. *n*= 5-7 mice per group. Data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns.

3.2.8 The Impact of *T. b. brucei* infection on spatial learning and reference memory.

3.2.8.1 *T. b. brucei* infection impairs spatial learning.

To investigate the effects of infection on spatial learning, 12 mice were tested on the MWM task day 23 post-infection over eight days. During the first four days which is the learning phase of the task, both the non-infected and infected groups demonstrated similar velocity (Figure 18A) and distances swam (Figure 18B) in two independent experiments. These analyses served as a control for locomotor activity, ruling out possible contributing factors other than cognition as the reason for any spatial tasks' differences observed between groups.

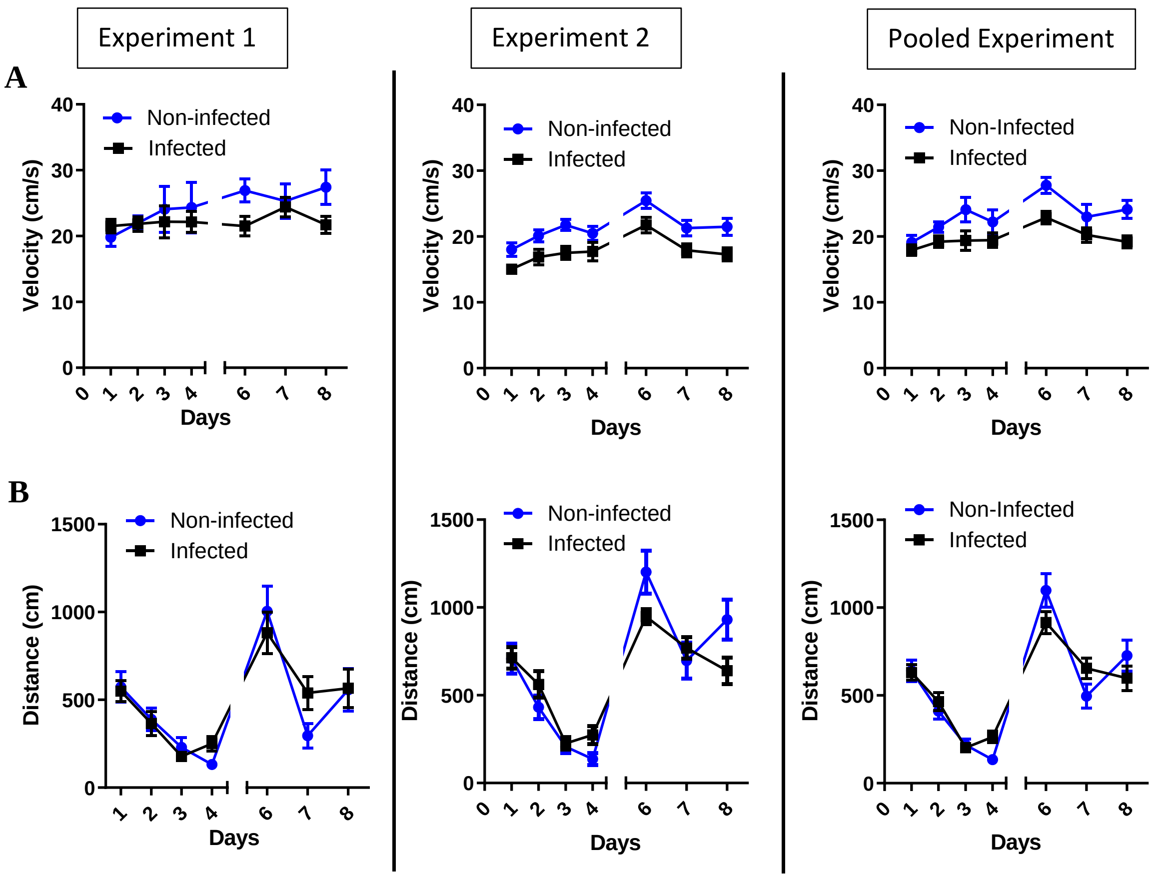


Figure 18: Control parameters used to determine cognitive impact of infection during learning phase of the MWM spatial task. (A) Non-infected and Infected groups performed at a similar velocity. (B) Non-infected and Infected groups swam similar distances. Results are shown from two independent experiments as well as pooled data from both experiments. n=6 mice per group per experiment and data are expressed as mean ± S.E.M. Statistical significance was determined by Two-Way ANOVA and indicated as $p>0.05$: ns.

Acquisition on days 1-4 showed that infected group has an increased latency to platform in experiment 1 (Figure 19A) and experiment 2 (Figure 19B) which is buttressed in the pooled experiment (Figure 19C) presented by the longer time it took them to locate the submerged platform, although this learning gap was non-significant. Besides, the spatial task from reversed phase from days 6 and 7 where complex learning was evaluated presented with a significantly evident learning impairment post-infection with *T. b. brucei*. The visible phase showed no differences with the visual acuity of both groups. Results taken from the two independent experiments and pooled experiment (Figure 19A, B and C) showed that mice in the infected group have a longer latency to locate the submerged platform compared to their non-infected counterparts which indicate a learning impairment with *T. b. brucei* infection.

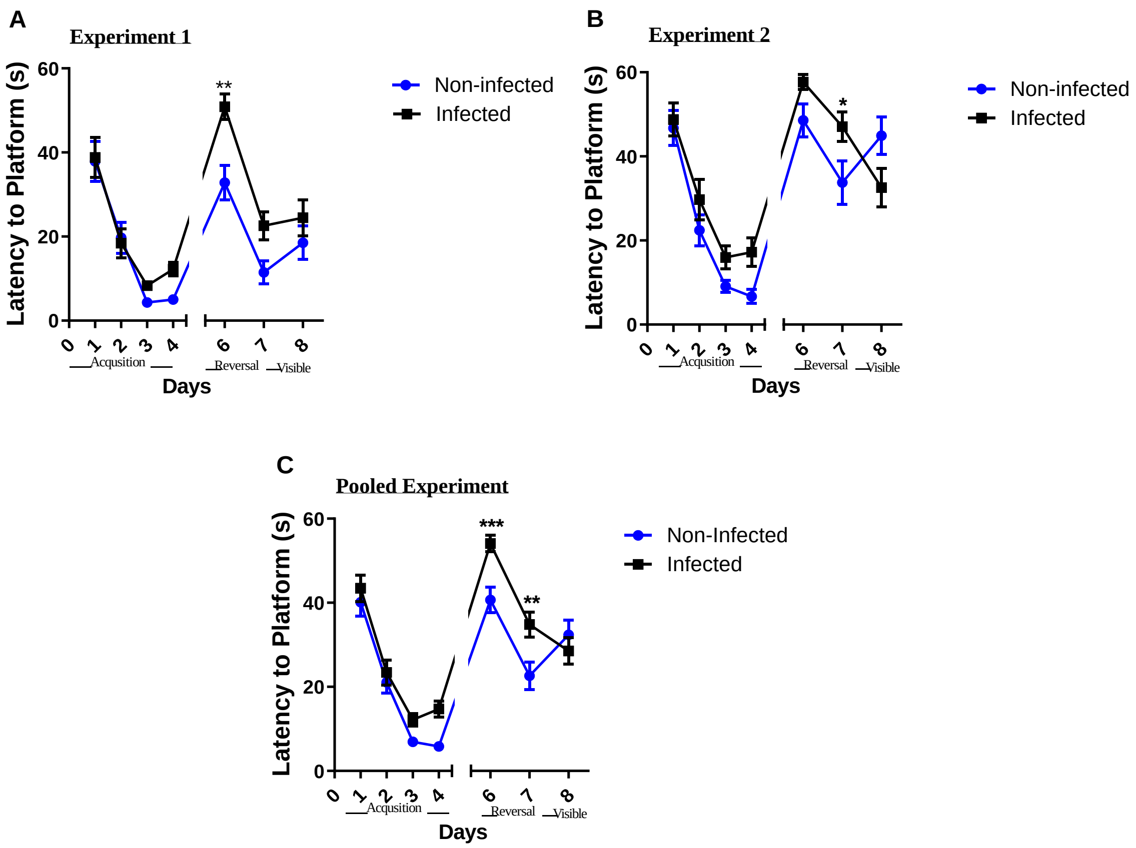


Figure 19: *T. b. brucei* infection causes learning impairment in mice. Non-infected and Infected groups performed the MWM spatial task. Results are shown from (A) experiment 1, (B) experiment 2, as well as (C) the pooled data from both experiments. n=6 mice per group per experiment and data are expressed as mean ± S.E.M. Statistical significance was determined by Two-Way ANOVA and indicated as $p>0.05$: ns, $p\leq0.05$: *, $p\leq0.01$: **, $p\leq0.001$: ***.

3.2.8.2 *T. b. brucei* infection impairs spatial memory formation.

To evaluate the effect of the infection on reference memory, acquisition data from day 5 was assessed for the velocity and distance swam by the non-infected and the infected groups as a control factor for the spatial memory formation. Our result showed that non-infected and infected groups both had similar velocity (Figure 20A) as well as swam similar distances (Figure 20B) validating an intact locomotor activity and as well ruling out other possible contributing factors other than cognition as the reason for any spatial tasks differences observed between groups.

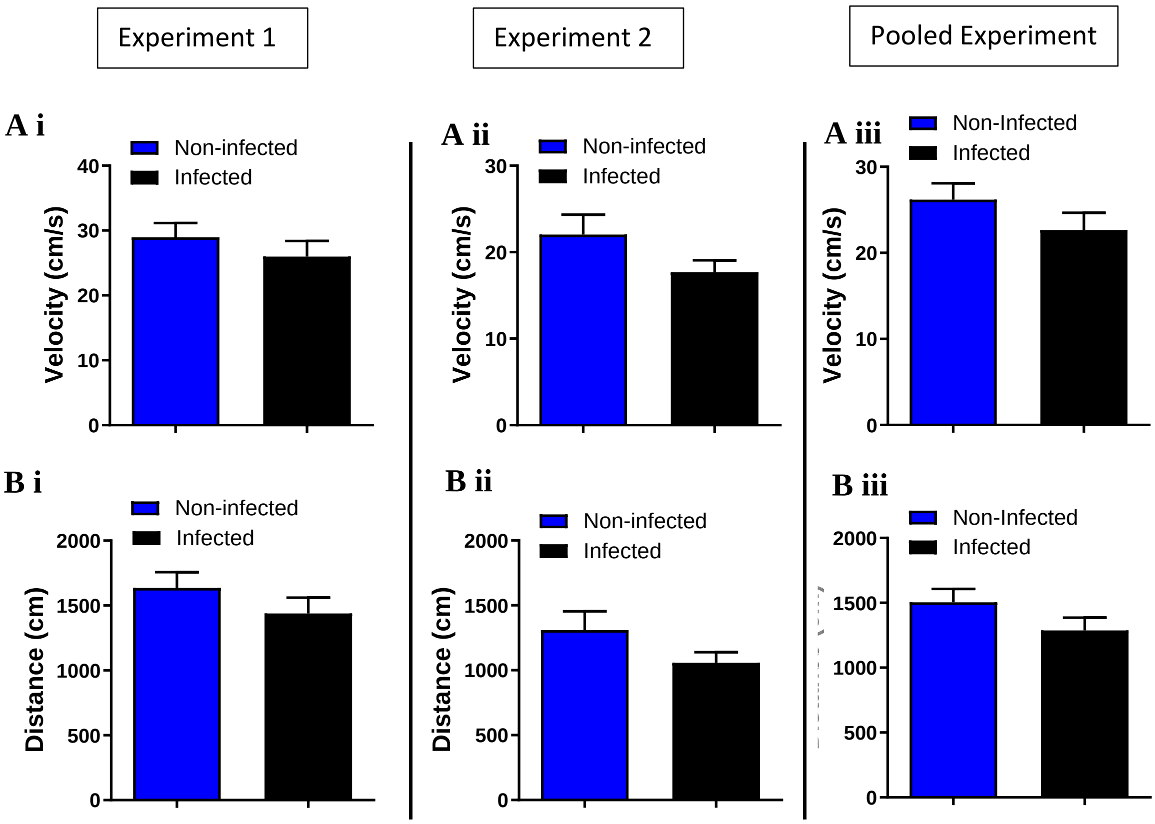


Figure 20: Control parameters to determine the cognitive impact of infection during the probed phase of the MWM spatial task. (A) Non-infected and Infected groups performed at a similar velocity. (B) Non-infected and Infected groups swam similar distances. Results are shown from two independent experiments as well as pooled data from both experiments. n=6 mice per group per experiment and data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns.

The probed spatial task activity performed on 5th-day which measures reference memory was analysed using three parameters: (i) Latency to first platform crossing, (ii) percentage time spent in the quadrant of the previously placed platform and then (iii) the number of platform crossings. Results showed that both infected and non-infected groups used a similar percentage of time in the quadrant (Figure 21A) and likewise have similar latency to the first platform crossing (Figure 21B). Besides, the infected group crossed significantly fewer times over the area of the previously located platform (Figure 21C) meaning that they less remembered where the platform was previously located. As expected, the result suggests a memory impairment following *T. b. brucei* infection. This is consistent with the two independent experiments and as well with the pooled data.

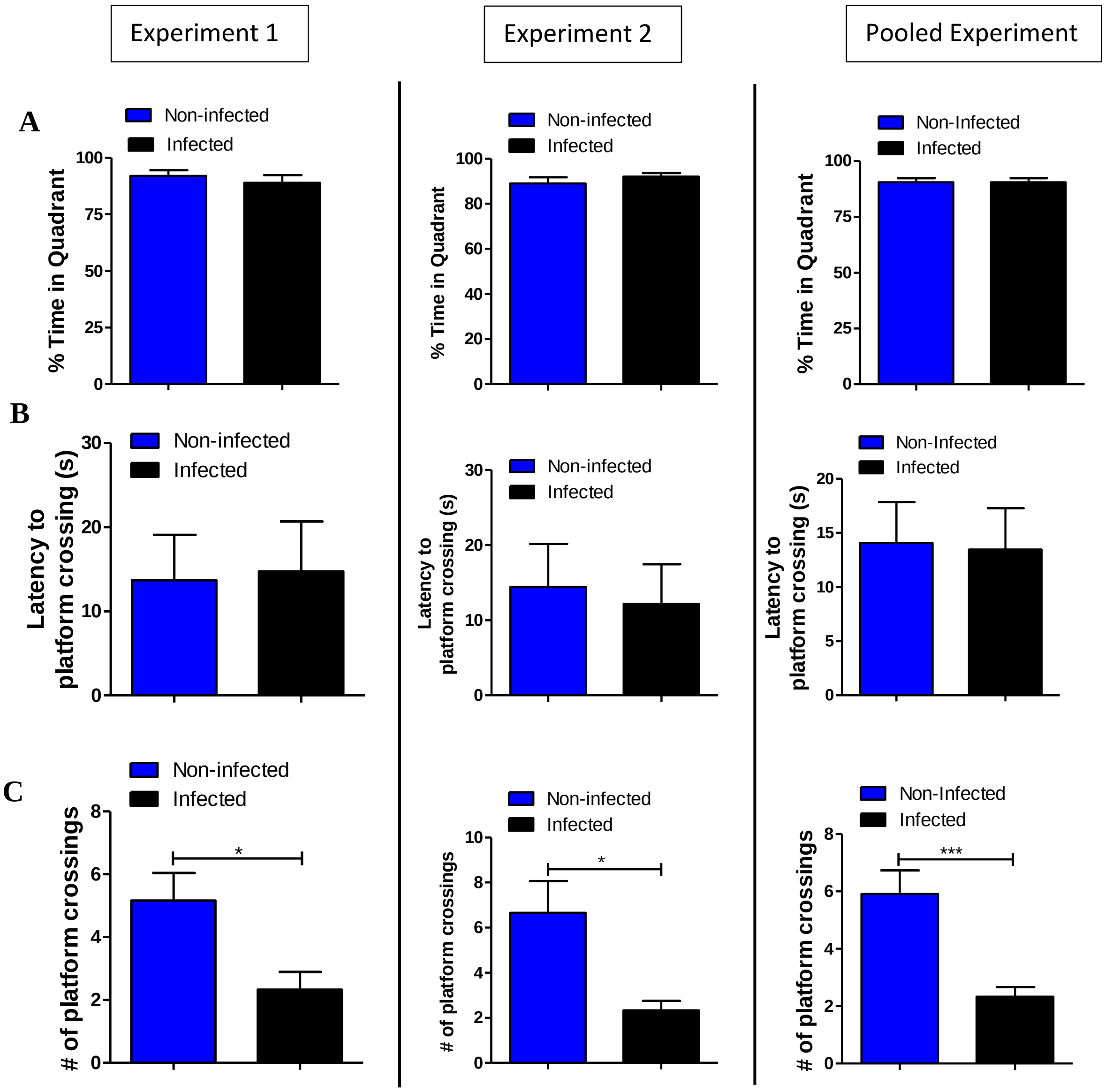


Figure 21: *T. b. brucei* infection causes memory impairment in mice. (A) Non-infected and Infected groups spent similar percentage of time in quadrant of previously located platform. (B) Both groups had comparable latency to platform crossing. (C) Reduced number of platform crossings in the infected group. Results are shown from two independent experiments as well as pooled data from both experiments. n=6 mice per group per experiment and data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns, $p\leq0.05$: *, $p\leq0.001$: ***.

Also, to further substantiate memory impairment observed, anxiety during the experiment (thigmotaxis) which was decided by the duration mice swam within 10 cm of the MWM edges and immobility frequency determined by the rate per second at which there was no limb movement were hypothesised as possible implication in lack of memory consolidation therefore probed (Brombacher et al., 2018). Results showed that neither anxiety (Figure 22A) nor immobility frequency (Figure 22B) had interference with memory impairment. This is consistent with the two independent experiments and as well with the pooled data. Overall, results indicated that memory is impaired with *T. b. brucei* infection.

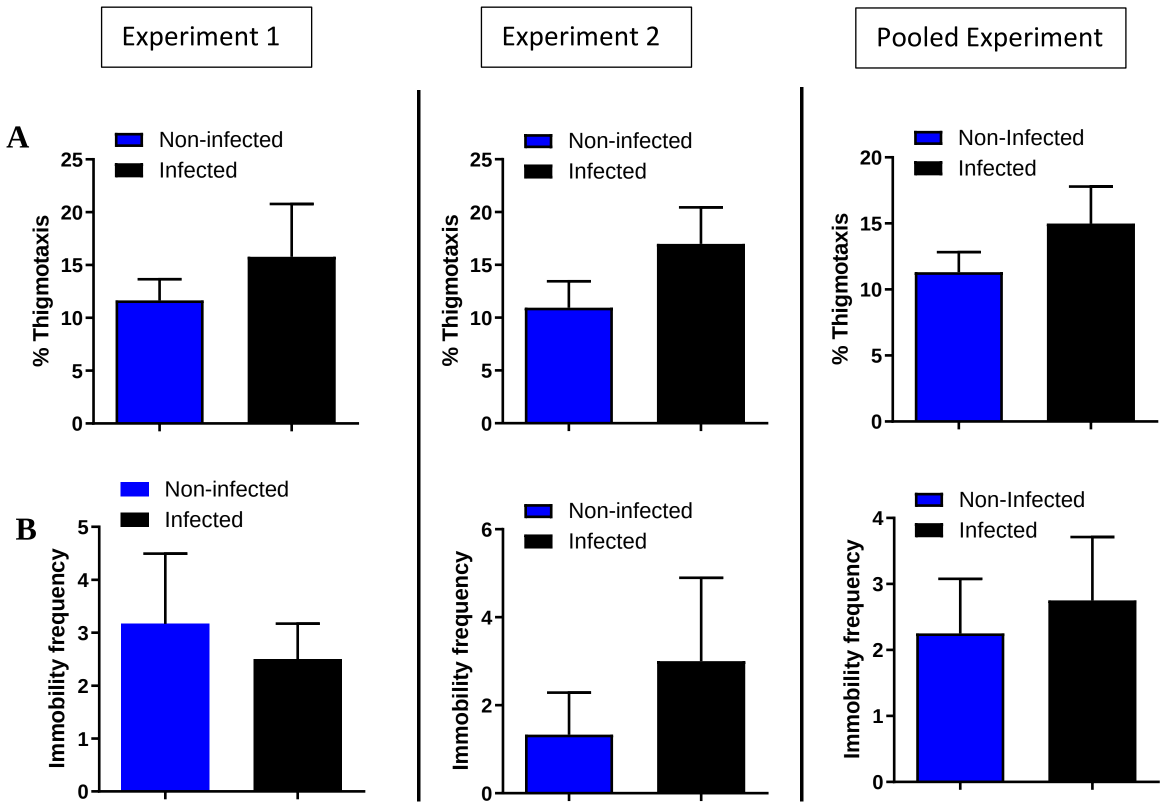


Figure 22: Anxiety and immobility frequency did not influence memory impairment. (A) Non-infected and Infected groups showed a similar percentage of thigmotaxis during the spatial MWM task. (B) Non-infected and Infected groups showed no differences in their rate of immobility. Results are shown from two independent experiments as well as pooled data from both experiments. n=6 mice per group per experiment and data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's *t*-test and indicated as p>0.05: ns.

3.2.9 Discussion

The sequence of event of African trypanosomiasis diseases commences with a blood infection that creates a fluctuating parasitemia wave typical of *Trypanosoma brucei* diseases (MacGregor et al., 2011; Bonnet et al., 2015; Cayla et al., 2019). In this study, an oscillating parasite appearance was observed in the blood of the infected mice consistent with what other studies have shown. Our study further adds that with or without the MWM training, parasitemia peak and clearance in the infected groups of mice progresses similarly, suggesting that this undulating parasitemia wave is not affected by the MWM training task. As a consequence of infection, a drop in food intake was observed around the second week which led to a drastic reduction in the weight of infected mice after the first peak in parasite load. This weight loss might however be due to loss of adipose mass, (Henrique et al., 2022) as fat makes up about 14% of the body weight of a healthy mouse (Jackson Mouse Phenome Database) or the loss of muscle mass. This observation however agrees with what has been previously shown in other studies (Darsaud et al., 2003; Trindade et al., 2016).

In mice, splenomegaly and hepatomegaly are an obvious pathogenic manifestation of African trypanosomiasis (Brun et al., 2010). The increase in size and weight of these organs is caused by an uncontrolled type 1 immune response (Baral, 2010). For instance, this is seen with the infiltration of mononuclear immune cells in the liver during the late stage of the disease (Bosschaerts et al., 2009). On the other hand, the spleen is the largest organ in the immune system as well considered the largest secondary lymphoid organ (Lewis et al., 2019). The vast range of immunological functions carried out by this organ includes but is not limited to filtering pathogens off the blood, in this case, the *T. b. brucei* parasite. This parasite is known to drive an overwhelming inflammatory immune response affecting the spleen at a more significant degree than the liver evidenced by the dramatic enlargement.

A gross examination of these two peripheral organs in the infected groups presented with expected pathological lesions. Intriguingly, the MWM training was observed to be beneficial to the infected mice exposed to the task (infected trained), exhibited by a reduction in pathogenicity, and evidenced in less spleen and liver damage when compared to the infected non-trained group. However, the rapid impact of the parasite and the effect of the MWM task especially on the spleen might be due to its immunological composition and overall function. This observation is supported by findings of exercise/physical activity alleviation of liver and spleen histopathology in several disease conditions (Charrin et al., 2018; Arfianti et al., 2020; Jacob et al., 2021).

The pro-inflammatory cytokine IFN-γ plays an essential role in the progression of the African trypanosomiasis disease. Excessive production of which causes disease susceptibility nonetheless, mice deficient in IFN-γ died earlier than their wildtype counterparts, indicating that an optimum amount of this cytokine is needed to prevent the detrimental inflammatory outcome. Our evaluation of the serum revealed that during the late stage of *Trypanosoma brucei* infection, alongside IFN-γ production, IL-10 is as well produced. This synergistic production of IFN-γ and IL-10 indicates that to achieve a robust benefit from the inflammatory immune response, a downregulatory immune reaction from IL-10 is needed to counter the overt production of IFN-γ (Namangala et al., 2001).

Subsequently, when parasites invade the CNS, an immunological alteration of the brain microenvironment occurs. The brain myeloid cell constitutes a heterogeneous population of microglia and macrophage cells that performs a quite similar function (Murray & Wynn, 2011). Given that microglia which is the brain resident macrophage plays an essential role in the advent of any alteration caused to the brain, we evaluated the changes that might occur in this immune cell when infected with T. b. brucei. Our evaluation at the cellular level substantiated what other studies have shown about the interaction between the pro-inflammatory and anti-inflammatory cytokines by supporting an elevated level of IL-6, IFN-γ, TNF and IL-10 in the prefrontal cortex, hippocampus and hypothalamus following T. b. brucei infection. Further cellular probing of the brain immune responses revealed that T. b. brucei infection elevates the level of macrophage and its resulting IFN-γ and IL-10 production in the hippocampus of the infected mice group.

Morris water maze is a test used in behavioural neuroscience to study spatial learning and memory in rodents (Nunez, 2008). To investigate the effect of *T. b. brucei* infection on spatial cognition, the maze test was performed, and our result revealed an impairment in spatial learning and memory in mice infected with *T. b. brucei*. This data is consistent with what other studies have shown using a *T. evansi* disease model (Wolkmer et al., 2013; Baldissera et al., 2015). We found that neither locomotor irregularities nor anxiety were identified in infected mice during the task which served as basic control for the task. The infected mice took a longer time to locate the platform during the learning phase and likewise displayed a reduced number of platform crossings which describes a learning and memory impairment.

In sum, the influx of immune cells from the periphery into the CNS is limited by the blood-brain barrier in a healthy CNS, nonetheless, this protection can be bridged in the advent of insults, stress, injury or disease that affects the brain (Zhang et al., 2020; Wu et al., 2020; Kadry et al., 2020). It is considerably clear that *T. b. brucei* infection drives an initial inflammatory response and a counter-inflammatory reaction, besides the impairment of spatial reference memory. Reports indicate that increase in IFN-γ production is associated with cognitive defects (J. Zhang et al., 2020). Likewise, an elevated level of TNF has been implicated in several CNS disease conditions including cognitive function (McCaulley & Grush, 2015). There is a previous consensus on proinflammatory cytokines being an instigator of cognitive deficits while anti-inflammatory immune therapy mitigates the harm but, this has been overruled by other research findings (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015; Sathyan et al., 2018). Yes, proinflammatory cytokines are largely implicated in cognitive deficits (Moreira et al., 2015) nonetheless, anti-inflammatory cytokine production might not be sufficient in ameliorating the damage already caused. These findings strengthen our data; hence, it was not surprising that the immune profile we observed during the late stage of *T. b. brucei* infection could not rescue the mice from spatial learning and memory impairment.

3.3 The effects of training during *T. b. brucei* infection on the brain immune composition.

Physical exercise or training is described as a non-pharmacological health intervention that helps to improve general well-being including mental health (Archer et al., 2011; Mahalakshmi et al., 2020). Although its aid is yet to be explored in African trypanosomiasis disease, the benefits of training, physical activity or exercise in various disease conditions have been a focus in many studies (Karacabey, 2005). An experimental animal model of Alzheimer's and Parkinson's disease demonstrates that exercise can mitigate associated pathology by downregulating pro-inflammatory cytokine expression in the prefrontal cortex and hippocampus (Jang et al., 2017; Y. Liu et al., 2020) and by modulating microglial activation (Real et al., 2019). Microglia are CNS resident macrophages trained to identify and initiate immune responses to perturbations caused to the brain. This CNS macrophage population includes the microglia, macrophage of the meningeal, perivascular, circumventricular organs and of the choroid plexus (Perry & Teeling, 2013). During African trypanosomiasis infection, microglial cells are activated to phagocytose trypanosome parasite (Figarella et al., 2018).

Furthermore, amongst the pronounced effects of *T. b. brucei* infection in mice is splenomegaly and hepatomegaly which develops due to the activity of inflammatory immune response. In the case of splenomegaly, a treatment-exercise complex intervention was shown to ameliorate the pathology of an enlarged spleen by the reduction in inflammatory immune response (Buchan et al., 2018). Similarly, exercise was shown to reduce hepatomegaly as well as repress the level of its inflammatory cytokine expression (Gehrke et al., 2019). In a broader overview, exercise was adapted to improve the pathology associated to cancer (Sitlinger et al., 2020), by regulating the immune responses. Since exercise has demonstrated importance in various disease conditions, we investigated the effects of MWM training on immune composition in the brain following *T. b brucei* infection.

3.3.1 Cytokine production in the brain following Morris water maze training during *T. b. brucei* infection.

We have previously been able to show that *T. b. brucei* infection significantly upregulates the production of IFN-γ, TNF, IL-6 and IL-10 cytokines in the prefrontal cortex, hippocampus, and hypothalamus. However, the benefits of training/exercise have been documented in various disease conditions (Karacabey, 2005; Anderson & Durstine, 2019) even in trypanosoma cruzi (Lucchetti et al., 2017) but in trypanosoma brucei disease. Now, we sought to evaluate the influence of the Morris water maze training task conducted during *T. b. brucei* infection on cytokine profiles in the brain. Here, two groups of *T. b. brucei* infected mice with differences in training status (infected non-trained and infected trained) were investigated for IL-6, IFN-γ, TNF and IL-10 levels in the prefrontal cortex, hippocampus, and hypothalamus. In the hippocampus, we found a consistent trend in the reduction of the level of IL-6 (Figure 23 Aii), IFN-γ (Figure 23 Bii), and TNF (Figure 23 Cii) cytokines in the infected trained group compared to their infected non-trained counterpart. On the other hand, there was a comparable level of these cytokines in the prefrontal cortex (Figure 23 Ai, Bi) except with TNF (Figure 23 Ci). Also, the cytokines were at similar level in the hypothalamus of both groups (Figure 23 Aiii, Biii, Ciii). Besides, the level of anti-inflammatory IL-10 cytokine in the prefrontal cortex (Figure 23 Di), hippocampus (Figure 23 Dii) and hypothalamus (Figure 23 Diii) does not differ between the two groups. This reduction in the inflammatory immune response during *T. b. brucei infection* could speculate a beneficial effect of the MWM training task in the brain particularly the hippocampus.

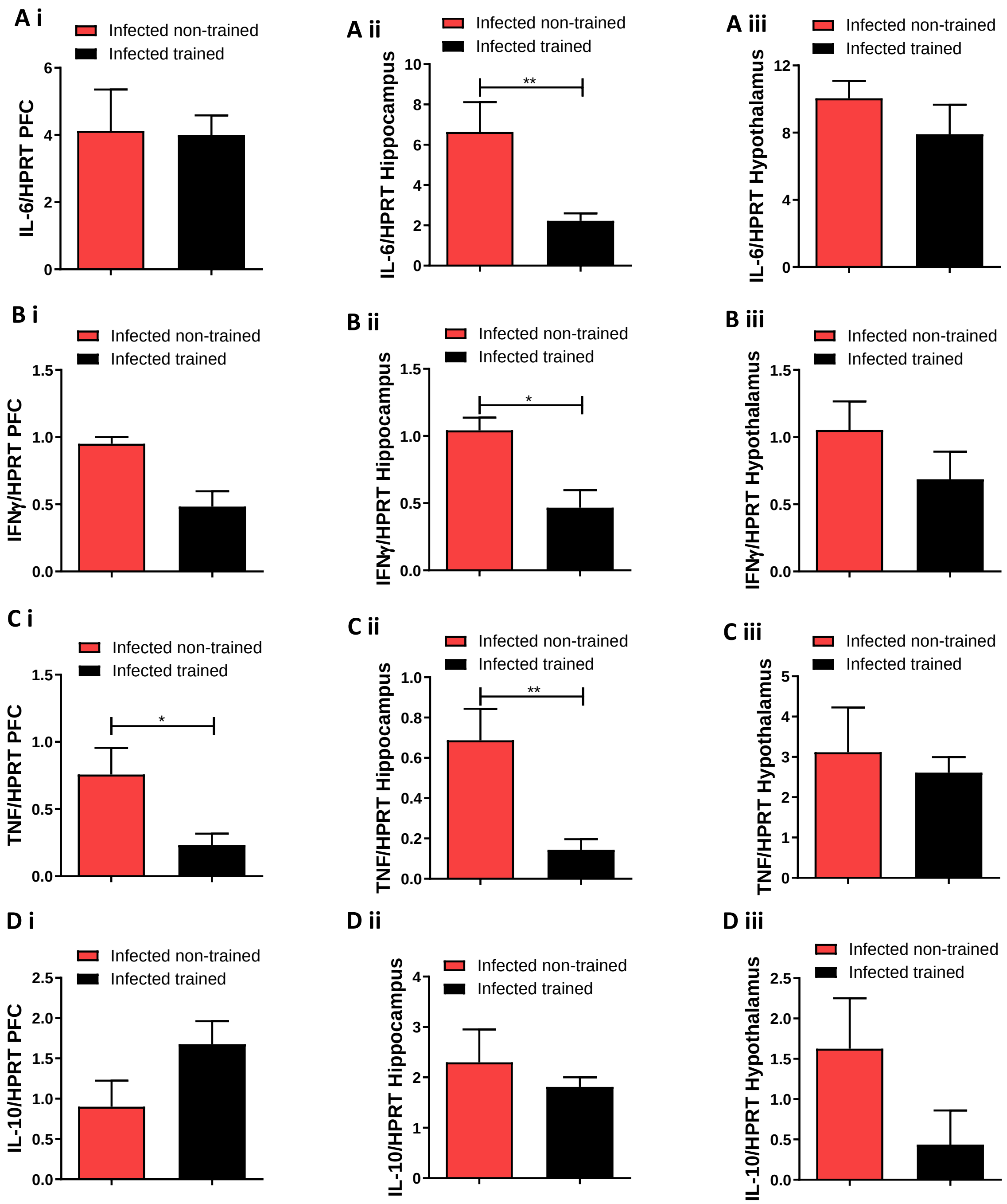


Figure 23: Cytokine immune response in the brain following Morris water maze training during *T. b. brucei* infection. The infected non-trained and infected trained groups were both inoculated with 2000 *T. b. brucei* parasites besides the infected trained group performed the MWM task. RNA extraction from the prefrontal cortex, hippocampus and hypothalamus converted to cDNA and normalised to HPRT mRNA control was measured for (A) IL-6, (B) IFN-γ, (C)TNF and (D) IL-10 production. Results are shown from two independent experiments *n*= 4-6 mice per group. Data are expressed as mean ± S.E.M. Statistical

significance was determined by unpaired Student's *t*-test and indicated as $p>0.05$: ns, $p>0.05$: *, $p\leq0.01$: **, $p\leq0.001$: ***, and $p\leq0.0001$: ****.

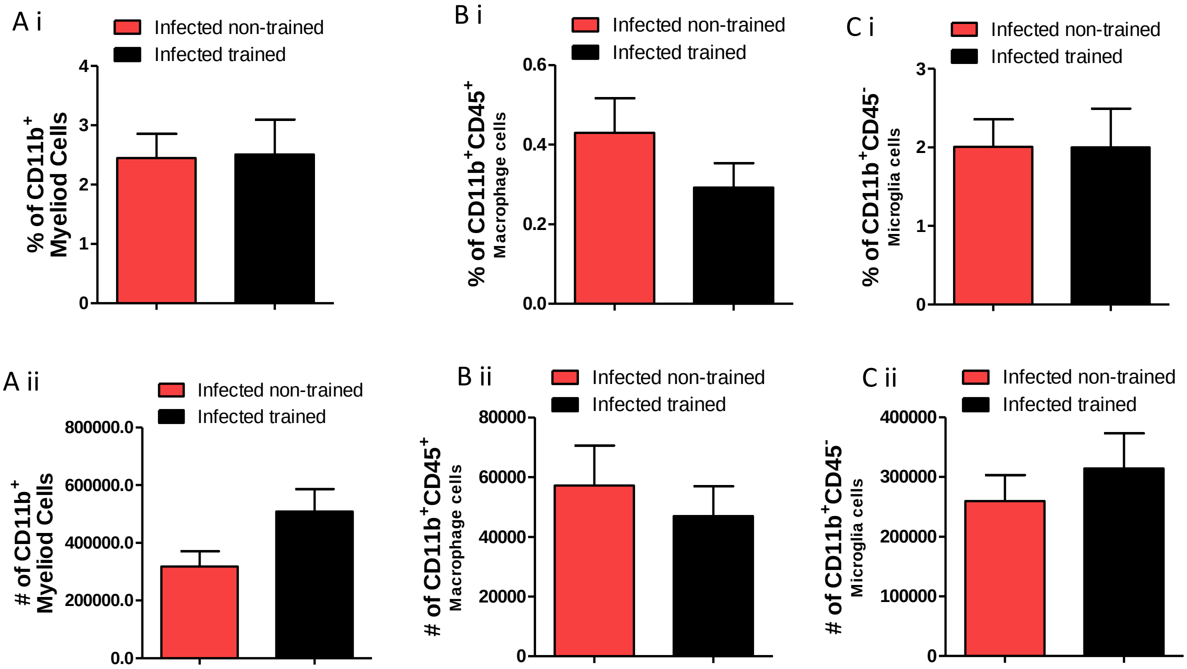
3.3.2 Myeloid cell production in the brain following Morris water maze training during *T. b. brucei* infection.

Having checked that training without infection does not affect the base-line level of myeloid cells and that *T. b. brucei* infection alters the basal level of myeloid cells, we proceeded to evaluate the impact of the MWM training during *T. b. brucei* infection on the composition of myeloid cells in the brain. To address that, a single-cell suspension was obtained from the hippocampus and quantitatively assessed their overall $CD11b^+$ myeloid cell population, $CD11b^+$ $CD45^{hi}$ macrophage cell population as well as $CD11b^+$ $CD45^{lo}$ microglial cell population.

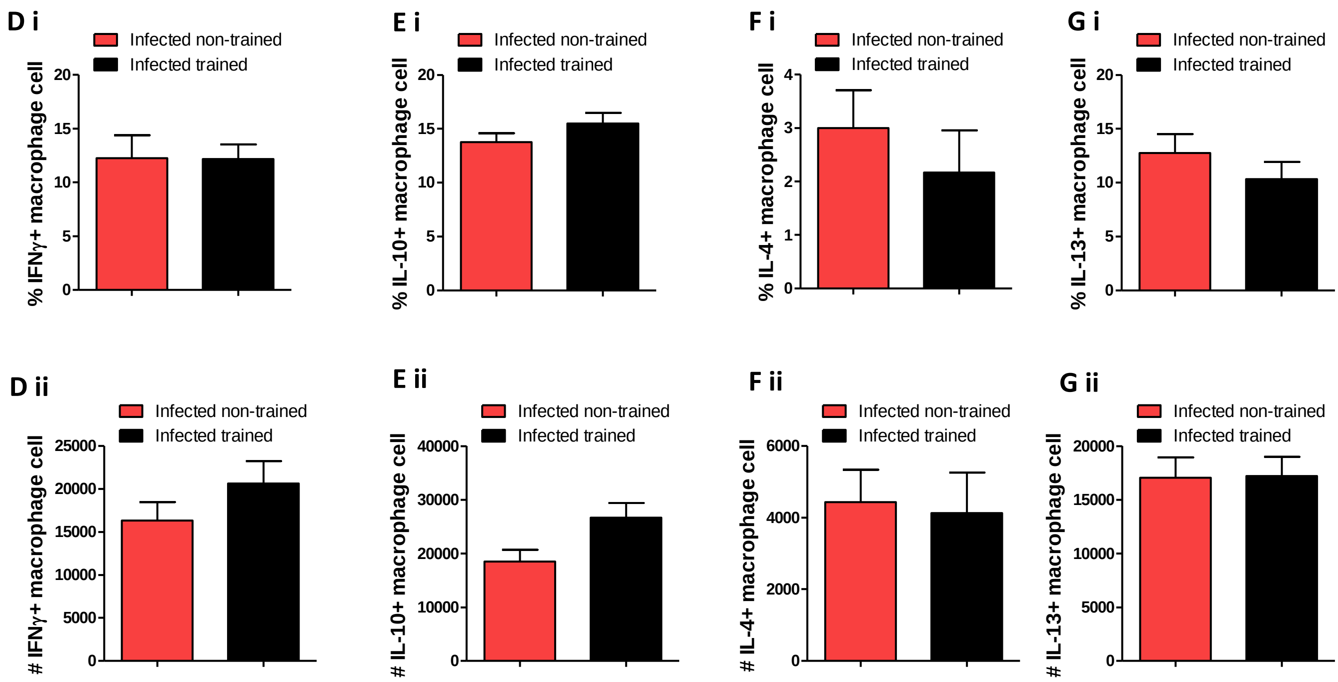
Refer to figure 9 for the gating strategy used to identify these various immune cells.

Results showed the percentage and cell number of the $CD11b^+$ myeloid cell population (Figure 24A), CD11b+ $CD45^{hi}$ macrophage cell population (Figure 24B) also the CD11b+ $CD45^{lo}$ microglial cell population (Figure 24C) did not differ with training of the infected group. We as well evaluated the cytokines produced by these myeloid cells and the result showed that the percentage and cell number of the proinflammatory IFN-γ, and anti-inflammatory IL-10, IL-4 and IL-13 cytokines produced by the macrophage (Figure 24D-G) and microglia cell (Figure 24H-K) does not differ as well between the infected non-trained and the infected trained. This result suggests that training during *T. b. brucei* infection might not have effect on the myeloid cell production.

Myeloid cells



Cytokines produced by macrophage



Cytokines produced by microglia

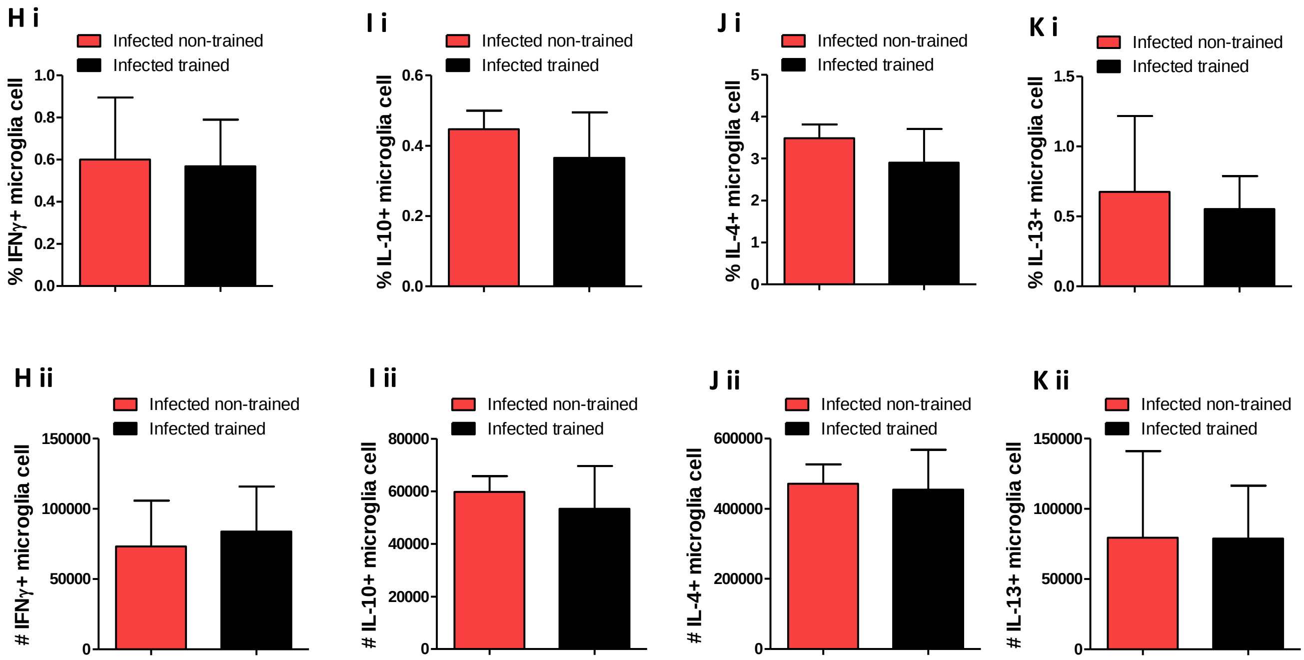


Figure 24: Immune cell and resulting cytokines produced following *T. b. brucei* infection. The infected non-trained and infected trained groups were both inoculated with 2000 *T. b. brucei* parasites besides the infected trained group performed the MWM task. Hippocampal single-cell suspension from both groups was assessed using flow cytometry to determine the percentage and cell number of the (A) overall myeloid cell, (B) macrophage cell, and (C) microglial cells production. The single-cell suspension was also stimulated for cytokine production and Flow cytometry was used to determine the percentage and number of (D) IFN-γ, (E) IL-10, (F) IL-4, and (G) IL-13 cytokine produced by macrophage cell and likewise (H) IFN-γ, (I) IL-10, (J) IL-4, and (K) IL-13 produced by the microglial cell. n= 4-6 mice per group representative of one experiment. Data are expressed as mean ± S.E.M. Statistical significance was determined by unpaired Student's t-test and indicated as p>0.05: ns.

3.3.3 Discussion

To cap it all, we evaluated the effect of MWM training during *T. b. brucei* infection on the brain immune composition in mice. Our study indicates a significantly lowered expression level of the profiled proinflammatory cytokines in the brain of infected mice exposed to the 8 days MWM training task when compared to the infected non-trained group whereas, the level of anti-inflammatory IL-10 cytokine was comparable between both the infected trained and the non-trained groups. Although this study is the first to take into consideration the effect of training during African trypanosomiasis infection and to stipulate that training helps to mitigate inflammatory immune response during *T. b. brucei* infection while potentially reducing the pathology associated with the disease, this mechanism corroborates with what other studies have reported on the benefits of training/exercising during various health and disease conditions. Exercise has been previously demonstrated to dampen inflammatory cytokines wherefore improving the pathological conditions in a traumatic brain injury (Piao et al., 2013), schizophrenia (Gómez-Rubio & Trapero, 2019), lung injury (Rigonato-Oliveira et al., 2018). In fact, exercise training was shown to induce disease resistance in mice infected with *trypanosoma cruzi* by modulating its inflammatory immune response (Lucchetti et al., 2017). These shreds of evidence support our observation that training could potentially help to mitigate the pathology associated with *T. b. brucei* by attenuating the activities of the inflammatory cytokines.

African trypanosomiasis infection activates microglia and macrophages in the brain (Figarella et al., 2018; Campbell et al., 2019). Besides that microglia which is the brain resident macrophage plays an essential role in the advent of any alteration caused to the brain, there are pieces of evidence to show that training/exercise can reduce microglial activation (He et al., 2017). Hence, we investigated the possible differences that might occur between the infected trained and non-trained groups during the course of infection. This study demonstrated that there were no significant differences in the hippocampal microglia and macrophage cell composition, including the cytokines they produce in both the infected non-trained and infected trained groups of mice. A reduced microglia and macrophage activation would have been expected in the infected trained group, however, since most of the studies accentuate 'prolonged exercise', the 8 days MWM training task might have not been sufficient to cause an observable effect.

CHAPTER 4

GENERAL DISCUSSION

Our first set of data showed that MWM training does not affect the basal level of the systemic and CNS immune composition of mice at a naïve state, hence providing us with a negative control for the subsequent results where this training could be a potential contributing factor to the observed changes. Following this, we evaluated the impact of *T. b. brucei* disease and showed that the infection alters immune composition in both the brain and the peripheral system by upregulating the myeloid, pro, and anti-inflammatory cytokine production in the late stage of the disease. Of the cytokines implicated during this disease, IFN-γ and IL-10 are reported from several studies to be highly involved in the disease progression and outcome. Aside from the IFN-γ dependent macrophage production of TNF and NO that induces parasite killing, a study conducted using IFN-γ deficient mice revealed another importance of IFN-γ during the infection as an aid to circumventing parasite through the blood-brain barrier (Masocha et al., 2004). This role is further explained in a human study where the severity of the meningoencephalitic stage of the disease was associated with higher titre values of the IFN-γ cytokine production (MacLean et al., 2007). This suggests that the high IFN-γ production we found in the brain could mean it acts as an anchor for the parasites to enter the brain wherefore the increased TNF production aids in parasite killing. The upregulated IL-10 on the other hand is associated with the mitigation of the pro-inflammatory immune activities (Guilliams et al., 2009). We found an increased IL-10 production in the infected group during the late stage of the disease in contrast to the non-infected group. The described role of these cytokines infers why there was a concurrent increase in both the pro and anti-inflammatory cytokine production in the late stage of the disease. From these studies, it is apparent that immune response during *T. b. brucei* infection plays a crucial role in the disease outcome.

Our result also demonstrated that mice infected with *T. b. brucei* developed spatial learning and memory impairment, a data supported by Wolkermer and Baldissera (Wolkmer et al., 2013; Baldissera et al., 2015). As hitherto explained, the immune response aggravated by this disease is likewise implicated in cognitive decline (Monteiro et al., 2015; Balzano et al., 2020; Chakrabarty et al., 2015). It appears that the disease and a synergistic response from its resulting immune alteration influence spatial learning memory impairment.

Lastly, we showed that the engagement of *T. b. brucei* infected mice in the MWM training task dampens the production of pro-inflammatory cytokines as well as alleviates the spleen and liver histopathology in contrast to their infected non-trained counterparts. This observation of an anti-inflammatory benefit of training on African trypanosomiasis has not been previously reported, nonetheless, researches have shown that training/exercise alleviates the pathologic conditions associated with *Trypanosoma cruzi* (Lucchetti et al., 2017), spleen and liver diseases and even neuroinflammatory diseases (Seo et al., 2019) by downregulating the inflammatory responses. With these pieces of evidence, we can expect that training could as well be beneficial to African trypanosomiasis disease by modulating the proinflammatory cytokines induced. Although, more research needs to be conducted to validate this claim.

CONCLUSION AND FUTURE STUDIES

In summary, the basic finding in this study was that infection of mice with *T. b. brucei* parasite undoubtedly results in a spatial learning and memory impairment. This spatial alignment alteration was demonstrated by the increased time taken for the infected mice to locate the escape platform and its reduced number of platform crossings during the MWM training task. We also reported that the basal immune composition level in naïve mice was not affected by the MWM training task. This is displayed by the comparable cytokines and myeloid cell production. On the other hand, we showed that mice infection with *T. b. brucei* pathogen alters the levels of cytokines and myeloid cell in the brain and periphery.

There is yet no vaccine against African trypanosomiasis plus the current treatment approach is arduous, could be toxic moreover expensive. Considering vaccine development has been rendered futile due to antigenic variation properties of the parasite, it is imperative to focus on some other forms of treatment approach. Given that studies have shown the benefits of exercise/training in various diseases, and by serendipity, we also found that the MWM training task ameliorates the inflammatory immune response associated with *T. b. brucei* infection, we can tentatively hypothesize that training/exercise during African trypanosomiasis infection might be beneficial to the disease outcome. The mechanism with which this happens is not known plus other studies that documented the benefits of training/exercise in other disease scenarios are yet to establish a concrete mechanistic backup (Mee-inta et al., 2019). In-depth research needs to be conducted to validate and establish the benefits of training/exercise on African trypanosomiasis, in same breathe, investigate the mechanism with which exercise mitigates the disease pathology.

Potential direction: We will recruit the use of other test tools like a treadmill to investigate the effect of prolonged exercise during *T. b. brucei* infection on the disease outcome. A loss of function approach using knockout mice would also be used to understand the mechanism by which exercise attenuates the pathological outcome of the disease.

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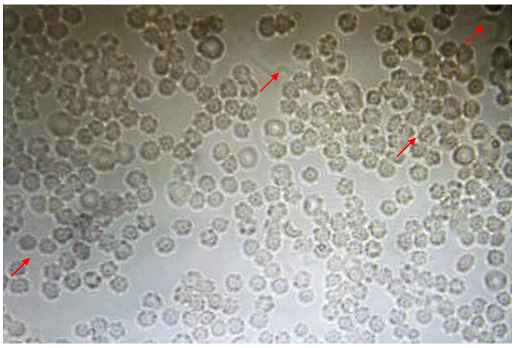
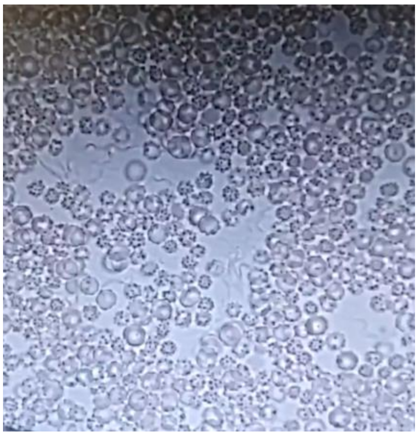
SUPPLEMENTARY DATA.

B

A





Supplementary Movie 1: (A) First *Trypanosoma brucei brucei* parasite passage (B) Third *Trypanosoma brucei brucei* parasite passage. Movies were captured on a Nikon Eclipse 90i microscope. (Please note this is a video but the format allowed for submission does not support video).

Supplementary Movie 2: DEAE cellulose separated *Trypanosoma brucei brucei* parasite. Movie was captured on a Nikon Eclipse 90i microscope. (Please note this is a video but the format allowed for submission does not support video).