HOST IMMUNE RESPONSES TO *PLASMODIUM BERGHEI* ANKA AND *TRICHINELLA ZIMBABWENSIS* INFECTION IN BALB/c MICE

By

Onkoba Wycliffe Nyamongo, BSc. MLS (Maseno), MSc. (Nairobi)

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Nursing and Public Health, University of KwaZulu-Natal.

Supervisors:

Professor Moses J. Chimbari, College of Health Sciences

Professor Samson Mukaratirwa, School of Life Sciences

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PREFACE

The experimental work described in this thesis was conducted in the School of Nursing and Public Health of the College of Health Sciences and School of Life Sciences of the College of Agriculture, Engineering and Science of University of KwaZulu-Natal, Durban in the Republic of South Africa from July 2013 to April 2015, under the supervision of Prof. Moses J. Chimbari and Prof. Samson Mukaratirwa.

The study described in this thesis is original work done and reported by the author. These studies have not been used in any form, by any person or submitted to any tertiary institution for award of degree or diploma. Some of the work has been published in accredited journals in line with the thesis guidelines of the University of KwaZulu-Natal. Due acknowledgement has been accorded where other people's work has been used in the text.

DECLARATION 1: PLAGIARISM

I, **Onkoba Wycliffe Nyamongo**, do declare that:

i. The research reported in this thesis, except where otherwise indicated is my original research work.

ii. This thesis has not been submitted to any university for any degree award or examination.

iii. This thesis does not contain other people's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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   a) Their words have been re-written but the general information attributed to them has been referenced.

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vii. That there are no competing interests amongst the authors of the publication articles.

3rd September 2015.

Onkoba Wycliffe Nyamongo **Date**

DECLARATION 2: PUBLICATIONS

The publications (in print, in press and submitted) that constitute this thesis and the contribution I made to each of the manuscripts are presented here.

Publication 1:

Onkoba, W.N., Chimbari, M.J., Mukaratirwa, S., 2015. Malaria endemicity and co-infection with tissue-dwelling parasites in sub-Saharan Africa: A review. *Infectious Diseases of Poverty* **4,** 35.

Authors contributions:

My supervisors and I designed the study and I conducted all literature searches and wrote the paper. The supervisors provided valuable input through reviewing all manuscript drafts and providing critical comments

Publication 2:

Onkoba, W.N., Kamau, J.M, Chimbari, M.J., Mukaratirwa, S., 2015. Metabolic and adaptive immune responses induced in mice infected with tissue-dwelling nematode, *Trichinella zimbabwensis*. *Journal of Helminthology In press*

Authors contributions:

I designed, conducted all experiments, processed and analysed samples and data and wrote the manuscript. The supervisors and co-author J.M. Kamau guided the design, provided logistical support during experimentation, reviewed the manuscripts and provided critical comments.

Publication 3:

Onkoba, W.N., Chimbari, M.J., Kamau, J.M., Mukaratirwa, S., 2015. Differential immune responses in mice experimentally infected with tissue-dwelling nematode, *Trichinella zimbabwensis*. *Journal of Helminthology doi:10.1017/S0022149X15000723.*

Authors contributions:

I designed, conducted all experiments, processed and analysed samples and data and wrote the manuscript. The supervisors and co-author J.M. Kamau guided the design and provided logistical support during experimentation, reviewed the manuscript and provided critical comments.

Publication 4:

Onkoba, W.N., Chimbari, M.J, Kamau, J.M., Mukaratirwa, S., 2015. Serum cytokine and anti-*Trichinella* and anti-*Plasmodium* antibody responses induced in mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA. *Veterinary Parasitol*ogy *In press*

Authors contributions:

I designed, conducted all experiments, processed and analysed samples and data and wrote the manuscript. The supervisors and co-author J.M. Kamau guided the design and provided logistical support during experimentation, reviewed the manuscript and provided critical comments.

3rd September 2015.

Signed: _____ _____

Onkoba Wycliffe Nyamongo **Date**

DEDICATION

This thesis is dedicated to fallen wise men whose academic dreams were severed by death.

- The late Mr. James Maina Ichagichu, (Dexta: The Champ) - PhD candidate in Molecular Medicine at the Jomo Kenyatta University of Agriculture and Technology.
- The late Mr. Gilbert Nyambegera Momanyi - BSc. Supplies and purchasing candidate at the Jomo Kenyatta University of Agriculture and Technology.

Friends rest in peace "*Giniwasekao*".

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ABSTRACT

Four objectives were pursued in this study; (i) metabolic and adaptive immune responses induced in BALB/c mice infected with a tissue-dwelling nematode, *Trichinella zimbabwensis* were measured, (ii) differential cytokine and antibody responses induced in mice infected with *T. zimbabwensis* were determined, (iii) cytokines, anti-*Trichinella* and anti-*Plasmodium* antibody responses in mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA were determined and (iv) the effect of anti-helminthic treatment against *T. zimbabwensis* on immunity and malaria disease outcomes was determined. Groups of BALB/c mice were mono- or co-infected with a crocodile-derived *T. zimbabwensis* (Code 1SS1209) and *P. berghei* ANKA parasites. At various time points, metabolic parameters such as levels of water and food intake, glucose and insulin were measured. Cytokine and antibody responses were also measured by ELISA. Parasite burden and survival rates were used to determine malaria disease outcomes. The results showed that primary *T. zimbabwensis* infection was characterised by significantly elevated levels of insulin ($p < 0.001$) that were accompanied with hypophagia, weight loss, altered host compensatory feeding mechanisms. Parasite specific antibodies and Th1/Th2/Th17 and T-regulatory immune responses were elevated. In co-infection, it was observed that *T. zimbabwensis* induced immunomodulation that conferred protection against *Plasmodium* growth and early death. Anti-helminthic treatment enhanced antibody and cytokine production in mono- and co-infection mice ($p < 0.001$) and negatively affected malaria parasite multiplication by improving survivorship of co-infected mice by 42.85% ($p < 0.001$). From the study, it was concluded that *T. zimbabwensis* parasites induce mixed Th1/Th2/Th17 immune responses, alter host glucose metabolism and trigger immunomodulation that ameliorated malaria disease outcome. Anti-helminthic treatment acted as an immunomodulator for cytokine and antibody production, ameliorated malaria infection and improved survivorship of co-infected mice. The study shows that malaria co-infection with *T. zimbabwensis* and anti-helminthic treatment improves survival, enhances immunity and ameliorates malaria. It further shows that deworming may be used as an integrated control measure in areas where malaria and helminths are co-endemic.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Malaria

The World Health Organization (WHO) estimates that 3.3 billion people are at a risk of malaria infections. In 2014, malaria caused 198 million cases and 584,000 global deaths of which 90% occurred in Africa where 78% of them were in children under 5 years of age (WHO, 2014).

Malaria is a deadly infectious disease caused by four human *Plasmodium* parasites species: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* (Ashley *et al.,* 2006) and one simian parasite; *P. knowlesi* transmitted by *Anopheles dirus* in communities living in or near forested areas of South-East Asia (Cox-Singh and Singh, 2008). In sub-Saharan Africa (SSA), *P. falciparum* parasite is transmitted to humans by female anopheline mosquito during a blood meal (Gonçalves *et al.,* 2014). Children under 5 years of age (Were *et al.,* 2006), women in their first pregnancy (Beeson and Duffy, 2005) and travellers from non-malarious areas are mainly affected by the disease (Tang *et al*., 2010).

The WHO recommends an integrated approach for control and prevention of the disease. The interventions include use of insecticide treated bed nets (ITNs) and indoor residual spraying (IRS), chemoprevention and case management of infected persons (White *et al.,* 2014). Globally, these methods have managed to decrease malaria incidences, morbidity and mortality rates (WHO, 2014). However, achievement of the goal towards malaria elimination and eradication is being compromised by the emergence of drug resistant *Plasmodium* parasites and insecticide resistant mosquitoes (White *et al.,* 2014). Armed conflicts, floods, drought, poly-parasitism and co-infections are also hampering the control efforts (Alemu *et al.,* 2012).

Co-endemicity of malaria and helminths have made polyparasitism and co-infections the rule rather than an exception (Bucher *et al.,* 2011). In SSA, soil-transmitted

helminths (STHs) and schistosomes are the major co-infecting parasites alongside other neglected tropical diseases (NTDs) (Brooker *et al.*, 2012).

1.2 Epidemiology of Helminth Infections

Globally, 4.5 billion people are estimated to be at risk of dual co-infections with NTDs where hook worms *(Ancylostoma duodenale*, and *Necator americanus)*, round worms *(Ascaris lumbricoides)*, whip worms *(Trichuris trichura)* and schistosomes *(Schistosoma hematobium and S. mansoni)* are the most ubiquitous co-infecting agents (Ziegelbauer *et al.*, 2012). Communities in developing countries of Africa, Asia and the Americas are vulnerable to these infections and carry the greatest disease burden (Lustigman *et al.*, 2012) due to their socio-economic status, environmental factors, inadequate and poor sanitation systems (Alemu *et al.*, 2012). Long-term co-infections are accompanied with anaemia, blindness, stigmatization, premature death, reduced human productivity (King, 2010) and retardation of physical and cognitive development in children (Ezeamama *et al.*, 2005). Moreover, the co-infections may also affect host-parasite interactions to other NTDs, vaccines (O'Meara *et al.*, 2007) and deworming efficacy (Midzi *et al.*, 2011). High prevalence of *Trichinella zimbabwensis* infections (Mukaratirwa *et al.*, 2008) have been reported among a variety of mammals and reptiles in Southern Africa (Mukaratirwa *et al.*, 2008; 2013). This poses a public health risk of future human epidemic outbreaks of trichinellosis in poor communities who depend on bush meat for protein (De Merode *et al.*, 2004; Mukaratirwa *et al.*, 2013). *Trichinella zimbabwensis* is a tissue-dwelling nematode that has a complex life cycle in one host. The migrating first stage larvae get localized in the host striated muscles for long periods of time (Kapel *et al.*, 2005) contrary to the case of STHs whose larvae settle in the lumen of the gastrointestinal tract. *Trichinella* infection is acquired through ingestion of undercooked or raw meat contaminated with infective muscle larvae (ML). In the small intestines, larvae molt and ecdyse to develop into adult worms. After mating, the female deposits up to 1500 larvae (Despommier *et al.*, 2005) that penetrate the intestinal epithelial mucosa before migrating into vascular and systemic circulation (Gagliardo, 2002). After 21 to 28 days post infection,

the ML get encysted in the striated muscles and persists for a long time until ingested by the next host (Bruschi and Chiumiento, 2011).

1.3 Malaria and Helminths Co-infections

Epidemiological studies and laboratory experiments have been conducted over the last three decades to elucidate the mechanisms and outcomes of parasite-host-parasite and host-parasite interactions in malaria and helminth co-infections but the results obtained do not show a general consensus (Brooker *et al.,* 2007; Degarege *et al*., 2012). Some authors have shown that helminths increase the relative risk of severe malaria attacks (Roussilhon *et al.*, 2010); others have observed no effect (Bejon *et al.*, 2008; Shapiro *et al.*, 2005) while others suggest that helminths confer protective immunity against malaria infections (Lyke *et al.*, 2005). However, there is consensus that co-infecting helminths have the capability to immunomodulate host regulatory network to favour their own multiplication and transmission (McSorley and Maizels, 2012) as well as those of third party antigens (Ilic *et al.*, 2012) without triggering an overt inflammatory reaction (Maizels and Yazdanbakhsh, 2008; Yazdanbakhsh and Wahyuni, 2005). The conflicting findings may be attributed to multiple confounding factors like; discrepancy in study designs, parasite polymorphisms, human population diversity and genetic polymorphisms, exposure history, antibody cross reactivity and nutritional status (Brooker *et al.,* 2012). In addition, the majority of the studies have focused on STHs, schistosomes and filarial worms neglecting the effect of tissue-dwelling helminths and effect of deworming or mass drug administration (MDA) programmes that are in place in malaria endemic areas subtropical and tropical areas.

Clinical trials and epidemiological studies have shown that deworming pre-school and school going children has societal and health benefits in reduction of mortality, child health and growth promotion and school performance improvement (Yapi *et al.*, 2014). However, the manner in which MDA programmes have been implemented raises concerns on the inability of anti-helminthic drugs to confer protective immunity against re-infections

(Anderson *et al.,* 2013), drug safety, efficacy and possible resistance if drug administration is prolonged (Parker and Allen, 2011).

To make improvements in NTDs integrated control there is need to develop effective vaccines, sensitive diagnostic tools and identification of drug candidate molecules through understanding host-parasite interactions elicited during co-infections with tissue-dwelling nematodes like *T. zimbabwensis. Trichinella zimbabwensis* is nematode that can be easily validated and utilized in understanding how tissue-dwelling helminths induce immunomodulation in mono- and co-infections with malaria. The mechanisms of conferring protection against air way respiratory inflammation, type 1 diabetes, auto-immune encephalomyelitis and intestinal colitis by altering immune mediators that favour progression of inflammatory and auto-immune diseases (Aranzamendi *et al.,* 2013; Motomura *et al.*, 2009; Saunders *et al.*, 2007) and their possible effects on NTDs in SSA. It is against this background that the present study was undertaken to establish how a tissue-dwelling nematode, *T. zimbabwensis* induces metabolic and differential immune responses in mice. Further the study explored to establish the effect of *Trichinella* induced immunomodulation and deworming on host immunity and malaria disease outcomes during co-infection with *Plasmodium berghei* ANKA. A crocodile-derived *T. zimbabwensis* parasite (Code 1SS1209) was used in the study because of its prevalence in Southern Africa (Mukaratirwa *et al.*, 2013) and its prone to cause zoonotic infections in SSA. Furthermore, the parasite has a complex life cycle within one host hence lacking several molecular epitope repertoires from intermediate hosts that may mask actual immunological responses. The parasite has been used to successfully infect crocodiles (La Grange *et al.*, 2009), pigs (Matenga *et al.,* 2006), rats (Mukaratirwa *et al.,* 2015) and non-human primates (Mukaratirwa *et al.,* 2008) in our laboratory. Another reason for using *T. zimbabwensis* is the striking paucity of information on its host-parasite interactions during primary infections and with malaria co-infection.

1.4 General Objective

To establish metabolic and differential immune responses induced in BALB/c mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA parasites.

1.5 Research questions

1. What are the metabolic and adaptive immune responses elicited during primary *T. zimbabwensis* infection in BALB/c mice?
2. Which cytokine and antibody responses are induced in BALB/c mice infected *T. zimbabwensis* parasite?
3. Which cytokine and antibody responses are induced in BALB/c mice mono- and co-infected *T. zimbabwensis* and *P. berghei* ANKA parasite?
4. Does chronic *T. zimbabwensis* infection alter malaria disease severity?
5. What effect does anti-helminthic treatment against *T. zimbabwensis* have on differential immunity and *P. berghei* ANKA disease outcomes during co-infection?

1.6 Scope of the study

The present study aimed to establish host-parasite interactions in malaria and tissue-dwelling helminth co-infection using a murine model. The tissue-dwelling nematode, *T. zimbabwensis* and *P. berghei* ANKA parasites were used to induce *Trichinella* and malaria infections in BALB/c mice respectively. BALB/c mice were chosen as appropriate experimental model systems due their availability and ease of manipulation. In addition, we have previously in our laboratories shown that BALB/c mice are susceptible to experimental infection with a crocodile derived *T. zimbabwensis* parasite strain. The study presented here; firstly investigated metabolic and immuno-inflammatory responses induces in mice infected *T. zimbabwensis* and explored potential relationship between infection, insulin signalling pathways and Th17 immune responses **(Study objective 1).** Secondly; determined the differential immune responses induced in mice infected with *Trichinella*

infection **(Study objective 2)** and finally, determined serum cytokine, anti-*Trichinella* and anti-*Plasmodium* antibody responses in mice during mono- and co-infection with *T. zimbabwensis* and *P. berghei* ANKA and explored the effect of anti-helminthic treatment on immunity and malaria disease outcomes **(Study objective 3 and 4).**

1.7 Overview of the thesis

This thesis is divided into six chapters; a general introduction, a systematic review of literature, three data chapters, and a general summary of study findings, conclusion and recommendations. Chapters 2, 3, 4 and 5 are independent publication articles that are in press or published in peer reviewed journals. Chapter 6 thematically links together all study chapters to address the mechanisms and outcomes of parasite-host-parasite and host-parasite interactions in BALB/c mice during mono- and co-infection with *T. zimbabwensis* and *P. berghei* ANKA parasites.

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CHAPTER 2

MALARIA ENDEMICITY AND CO-INFECTION WITH TISSUE-DWELLING PARASITES IN SUB-SAHARAN AFRICA: A REVIEW

Infectious Diseases of Poverty (2015) **4,** 35.

2.1 Abstract

Mechanisms and outcomes of host-parasite interactions during malaria co-infections with gastrointestinal helminths are reasonably understood. In contrast, very little is known about such mechanisms in cases of malaria co-infections with tissue-dwelling parasites. This is lack of knowledge is exacerbated by misdiagnosis, lack of pathognomonic clinical signs and the chronic nature of tissue-dwelling helminthic infections. A good understanding of the implications of tissue-dwelling parasitic co-infections with malaria will contribute towards the improvement of the control and management of such co-infections in endemic areas. This review summarises and discusses current information available and gaps in research on malaria co-infection with gastro-intestinal helminths and tissue-dwelling parasites with emphasis on helminthic infections, in terms of the effects of migrating larval stages and intra and extracellular localisations of protozoan parasites and helminths in organs, tissues, and vascular and lymphatic circulations.

2.2 Introduction

Malaria is a deadly infectious disease and one of the main health problems facing developing countries in Sub-Saharan Africa (SSA) and Asia. Globally, 3.4 billion people are at risk of new malaria infections, and there are around one million deaths annually (Casares and Richie, 2009; Langhorne *et al.,* 2008; World Health Organization, 2012). *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* parasites infect humans under normal conditions (Hayakawa *et al.*, 2008) with *P. falciparum* and *P. vivax* being the major species that cause morbidity and mortality in children under five years of age, pregnant women and travellers from non-malarious areas (Beeson and Duffy, 2005; Warimwe *et al.,* 2013).

In SSA, morbidity and mortality due to malaria is decreasing despite a lack of a malaria vaccine, emergence of parasite resistance to available anti-malarial drugs, the anopheline mosquito being resistant to insecticide residual spraying and a poor socio-

economic situation that hinders malaria control and management (Rasti *et al.,* 2004; ter Kuile *et al.,* 2003; WHO, 2014). Efforts in drug discovery and vaccine development are hindered by limited knowledge of the underlying cellular and molecular mechanisms of host-parasite interactions during co-infection and polyparasitism (Good and Doolan, 2010; Nossal, 2011). This is also aggravated by the emergence of zoonotic *P. knowlesi* malaria infections (Cox-Singh, 2012; Cox-Singh *et al.,* 2008; Fan *et al.,* 2013) as well as other zoonotic infectious diseases (De Meneghi, 2006; Reed *et al.,* 2003). Trichinellosis is an emerging and re-emerging zoonotic disease the geographical distribution of which overlaps with malaria in endemic areas of Tanzania, Uganda, Kenya, Ethiopia, Zimbabwe, South Africa and Mozambique (Gottstein *et al.,* 2009; La Grange *et al.,* 2012, 2010, 2009; Mukaratirwa and Foggin, 1999; Mukaratirwa *et al.,* 2013; Murrell and Pozio, 2011). The development of vaccines against parasitic infections has been complicated due to the fact that co-infecting parasites have life cycles that are either direct or complex. Direct life cycles involve cycling of mature parasites from one definitive host to another while complex life cycles involve cycling of distinct developing life stages through a number of intermediate hosts (Jackson *et al.,* 2009). Parasite cycling within intermediate hosts causes trafficking of molecular epitopes resulting in a generation of parasites variant surface antigens and excretory and/or secretory products that act as host immuno-regulators during co-infections and clinical trials, and hinders the understanding of parasite biology (Abath *et al.,* 1998; Aide *et al.,* 2007; Alving, 2002; Alving *et al.,* 2012; Harnett, 2014; Hartgers and Yazdanbakhsh, 2006; Jones, Trevor and Hoffman, 1994; Schwartz *et al.,* 2012; Su *et al.,* 2006, 2005; van Riet *et al.,* 2007; Wipasa *et al.,* 2002).

Epidemiological studies have shown that the largest burden of malaria infections is felt by communities living in poor regions of developing countries (Boraschi *et al*., 2008; Brooker *et al.,* 2007; Shankarkumar *et al*., 2011). In these areas, high prevalence of soil-transmitted helminthic infections have also been documented (Mwangi *et al.,* 2006). This results in co-infections, multi-parasitism or polyparasitism (Supali *et al.*, 2010). In the past three decades, several studies have been undertaken to establish the nature of interaction that occurs between soil-transmitted helminths (STHs) and malaria during co-infection

scenarios. The studies have mainly focused on immunological aspects and disease outcomes neglecting non-immunological mechanisms that may explain the heterogeneity observed in these studies (Fenton *et al.,* 2014; Knowles, 2011). Varying conclusions have been made from both epidemiological studies and laboratory experiments. Some studies have established that helminths may confer protection against cerebral malaria, others indicate that helminths exacerbate malaria, others report a reduction or increase in prevalence and transmission of malaria, while a few others report no association between the parasites (Bejon *et al.*, 2008; Lyke *et al.,* 2005; Nacher, 2011; Roussilhon *et al.,* 2010). The lack of general consensus in the studies is evidence that malaria immunity is not well understood. However, it is argued that STHs influence clinical malaria disease presentation or confer malaria tolerance through the establishment of chronic infections, induction of adaptive immunity (Taylor *et al.,* 2012) and immunosuppression of immune responses to unrelated antigens and parasites (Segura *et al.,* 2009). These result in an induction of host regulatory immunity, and signalling and effector mechanisms *(Ashour, 2013; Brindley et al.,* 2009; Maizels *et al.,* 2009) that are beneficial to co-infecting parasites. This is mainly due to host's failure to regulate the immune responses induced by the parasites. During co-infections, one parasite does not have direct influence on disease outcome and establishment of another parasite, however, the concept of parasite-host-parasite interactions plays a key role. One parasite influences the host to induce immune responses that will favour its establishment which in the long run, become beneficial to the co-infecting parasite. This immunological phenomenon is parasite-driven to make the host susceptible to infection and not favour the establishment of the co-infecting parasite. The amelioration or exacerbation of the disease outcome of the co-infecting parasites is a spill-over effect.

In the majority of co-infection studies, tissue-dwelling parasites, prevalent in SSA, have not been adequately considered. The hypothetical arguments presented are sketchy, making it difficult to clearly predict disease outcomes during malaria interaction with tissue-dwelling parasites. In this review, we discuss and summarise the available

information and research gaps in studies undertaken on the interactions between malaria and tissue-dwelling parasites.

2.3 Review

2.3.1 Methods

Information sources

The online bibliographic databases, MEDLINE/PubMed, EMBASE, Web of Science, Cochrane Library and Google Scholar were searched for studies on host-parasite interactions of malaria co-infection with tissue-dwelling helminths (up to May 2015). Bibliographic lists and references of the selected papers and previous reviews were used as leads for identification of additional studies.

Literature search

The search was conducted using predefined medical subject heading (MeSH) terms, Boolean operators (OR, AND) and truncation symbols used in combinations of direct key words: malaria, protozoa, co-infection, nematodes, tissue-dwelling parasites, cestodes, trematodes, intracellular parasites, helminths AND all permutations of MeSH terms in all fields.

Study selection

Studies were included in the review if they explicitly reported on immune responses and disease outcomes during malaria co-infection with: (i) tissue and organ-dwelling parasitic protozoa; (ii) migrating parasitic helminths and protozoa; and (iii) vascular and lymphatic circulation dwelling parasites. One hundred and sixty-eight (168) studies were retrieved from the search of published work, of which 13 were excluded because of duplication and 123 were irrelevant because they were dealing with malaria co-infections

and soil-transmitted parasites. Therefore, 32 studies including abstracts, reviews and reports on malaria co-infection with tissue-dwelling parasites were selected and reviewed. No grey literature was included. All articles were managed using Mendeley Desktop reference manager version 1.13.3 (NY, USA). The results of the analysis of the full papers read are described below.

2.3.2 Results

Poor hygiene and sanitary conditions, and dysfunctional health delivery systems in developing countries predispose people living in these areas to STH infections (Fu *et al.*, 2014; Nicoletti, 2013). For example, humans acquire trichinellosis through ingestion of raw or undercooked meat contaminated with infective *Trichinella* larvae (Blaga *et al.*, 2009), or gastrointestinal helminthiases/protozoa infection by ingesting food and water contaminated with embryonated eggs/cysts (Alemu *et al.*, 2012; Girma, 2012; Gottstein *et al.*, 2009). Some STHs and protozoans have complicated life cycles that involve a tissue migration phase where larvae obligately migrate through host vital organs causing tissue damage and myositis (Bruschi and Chiumiento, 2011; Despommier *et al.*, 2005; Mulcahy *et al.*, 2005; Stoltzfus *et al.*, 2000). The tissue migrating larvae (ML) or protozoa trigger induction of immunomodulation (Ilic *et al.*, 2012) through the release of excretory and/or secretory products that act as natural stimuli for stimulation of type 2 skewed immune responses (Maizels and Yazdanbakhsh, 2003). The Th2 immune responses abrogate inflammation, delay worm expulsion and initiate tissue repair (Adisakwattana *et al.*, 2013; Mulcahy *et al.*, 2005). But knowledge of these immunological pathways and signalling are not well described indicating the need for more research to disentangle the underlying immunological scenarios that occur.

Several co-infection studies have been designed and conducted on the assumption that chronic helminths may alter malaria severity and immunity either through Th2/T regulatory lymphocyte immunomodulation, altered antibody dependent cellular inhibition, immunosuppression of pro-inflammatory activity or presence of cross reactive antibodies

(Fairlie-Clarke *et al.,* 2010; Nacher, 2011). On the contrary, Hoeve *et al.,* (2009) established that *P. chabaudi* malaria parasites are capable of altering Th2 immune responses and initiation of pulmonary tissue repair in BALB/c mice co-infected with *Nippostrongylus brasiliensis*. This indicates that the presumption that helminths always alter malaria severity and immunity is not always correct. Several laboratory experiments have also shown that malaria parasites are capable of suppressing helminth-associated immunological activation thereby exacerbating pathological outcomes caused by the ML (Noland *et al.,* 2008). Therefore, this shows that co-infecting helminths influence the host immunity to mediate immune responses that are beneficial to malaria parasites during co-infection. However, the underlying pathophysiological and immunological mechanisms utilised by co-infecting parasites are not completely understood despite the findings being extrapolated to explain disease outcomes in humans. Therefore, it is imperative that considerations are made on non-immunological aspects of infections such as nutrition, immunological status, vector exposure frequency and population genetics to explain the conflicting results. Furthermore, the varied immunological profiles that are elicited by ML stages in various body compartments need to be considered when explaining concepts of immunomodulation. Establishing an animal model for malaria and tissue-dwelling helminth co-infection is of utmost importance, and the use of *Trichinella* sp. as the tissue-dwelling helminth is proposed because of its adaptability to laboratory animals as well as its ease of maintenance. For example, studies could be done on the migratory pathways taken by *Trichinella* sp. and how the *Trichinella* sp. may ameliorate allergic and autoimmune diseases in mono- and co-infections with malaria (Gruden-Movsesijan *et al.,* 2011, 2008; Ilic *et al.,* 2012). To our knowledge there are no studies that have been undertaken to determine the interaction of *Trichinella* sp. with tropical infectious diseases. In SSA, although very few human cases have been reported, trichinellosis is considered as an emerging/re-emerging zoonotic disease that has been reported to infect a variety of mammals (Dupouy-Camet, 2009; Gottstein *et al.,* 2009; Mukaratirwa *et al.,* 2013). Onkoba *et al.* (2015) established that mice co-infected with chronic *T. zimbabwensis* ameliorate and supress *P. berghei* infection. This is attributable to the comparable levels of interferon gamma (IFN-γ) secreted during co-infection and correlated with protective immunity

(McCall and Sauerwein, 2010). However, further research is needed to provide new knowledge and insight into its co-infection with malaria, and the implication on vaccine efficacy and development of diagnostic tools for surveillance and control in case of future outbreaks.

Enteric-dwelling protozoa and malaria

Intestinal protozoans, *Giardia lamblia* and *Entamoeba histolytica* have been reported to be major causes of severe intestinal disorder mostly in children, and HIV/AIDS and immunocompromised patients (Ramirez *et al.*, 2015; Schlagenhauf *et al.*, 2015; Tappeh *et al.*, 2014). The protozoa colonise the intestinal mucosa where they elicit localised innate immune responses against severe forms of the disease (Faubert, 2000). The underlying mechanisms for their unusual migration in the small intestines are still unknown (Carrero *et al.*, 2007).

Coccidian parasites, *Isospora belli*, *Cryptosporidium* sp. and *Cyclospora* sp. also cause severe diarrhoea, morbidity and mortality (Andrews *et al.*, 2014). Despite this, their actual disease burden and prevalence are underestimated in developing countries due to a lack of patient records and sensitive serological assays for disease detection (Faubert, 2000). In developing countries, prevalence of *Cryptosporidium parvum* infections are increasing due to environmental contaminations by pets, poultry, domestic animals and infected humans (Meireles, 2010; Putignani and Menichella, 2010; Wang *et al.*, 2014). In literature, these enteric-dwelling protozoan parasites are only considered as opportunistic infections that are acquired by children, and HIV/AIDS and immunocompromised patients (Adamu *et al.*, 2014; Baroudi *et al.*, 2013; Karp and Auwaerter, 2007; Macpherson, 2005). Their role as potential co-infecting parasites with tropical infectious diseases such as malaria has been neglected despite their prevalence in SSA where malaria is endemic. The risks of potential co-infection of enteric-dwelling parasites with malaria is possible through contaminated drinking and recreational water (Hofstra, 2011; Hofstra *et al.*, 2013), being in overcrowded households, coming into contact with infected calves and maintaining poor

personal hygiene (Adamu *et al.,* 2014). Enteric-dwelling parasitic infections lack specific therapy and vaccines making control of co-infections with malaria a challenge. Persons co-infected with malaria and enteric parasites are expected to exhibit severe diarrhoea, wasting syndrome and reduced quality of life, resulting in a high morbidity and mortality rate in the young and elderly, as well as immunocompromised patients. Co-infections are possible is SSA because susceptible persons live in environments contaminated with sewage, and also share housing with young calves, poultry, cats and dogs that are potential sources of zoonotic transmissions (Meireles, 2010; Robertson *et al.,* 2000).

Blood and tissue-dwelling protozoan parasites

Transmissions of vector-borne parasitic infections are on the rise due to changes in climate and global trends, human behaviour, vector behaviour and prey/host switching (Macpherson, 2005; Torgerson and Macpherson, 2011). The complexity of their life cycles, sophistication in their induction of immune evasion and intricate host-parasite interactions (Sacks and Sher, 2002) have complicated their diagnosis, drug discovery and vaccine development, as described below:

a) Trypanosomes and malaria: In SSA, the tsetse fly transmits extracellular protozoan parasites cause debilitating human African trypanosomiasis (HAT) and nagana in livestock (Fèvre *et al.,* 2008). The diseases have endemic foci in East, Southern and West Africa where they share the same geographical distributions with malaria and STHs, resulting in co-infections and polyparasitism (Blum *et al.,* 2006). Prevalence studies conducted in Kenya, Uganda, Tanzania and Sudan have shown that on average 70% of HAT patients in these countries are co-infected with malaria and STHs (Kagira *et al.,* 2011; Kuepfer *et al.,* 2011; Wangari *et al.,* 2006). This has made diagnosis and management of HAT difficult because both malaria and HAT have common clinical symptoms: intermittent fever, headache, general body pains, sleep disturbances and coma (Nannyonga *et al.,* 2012). Mice concurrently infected with *P. yoelii* or *Trypanosoma brucei* have been shown to block resistance to *Echinostoma revolutum* parasite infection (Christensen *et al.,* 1988). This

suggests that a synergistic interaction exists between protozoan and helminth infections. However, these studies do not provide explicit information on parasite-specific cellular immune and disease outcomes during these interactions, an indication that additional studies are needed. Malaria co-infection with HAT will result in exacerbation of malaria disease outcome with cerebral involvement. Both parasites potentially cross the blood brain barrier and the sequester in micro-vasculature of the brain resulting in cerebral malaria and eventually coma (Baptista *et al.,* 2010; Fèvre *et al.,* 2008; Kuepfer *et al.,* 2011; Renia *et al.*, 2012).

***b) Babesia* sp. and malaria:** Due to increased human-wildlife and livestock-wildlife interactions, a severe recrudescence of malaria-like babesiosis in humans and livestock has been reported (Kjemtrup and Conrad, 2000). In SSA, the actual prevalence and distribution of tick-borne diseases have not been well mapped (Dvoraková and Dvorácková, 2007; Homer *et al.,* 2000; Hunfeld *et al.,* 2008; Vannier and Krause, 2009). In humans, *Babesia* sp. infections might be misdiagnosed as *Plasmodium* sp. because of their overlapping similarities in symptoms (Blevins *et al.,* 2008; Krause, 2003; Krause *et al.*, 2007; Wormser *et al.,* 2006; Zhou *et al.,* 2013). This setback has compromised diagnosis, treatment, management of both diseases and possible development of vaccines (Andrews *et al*., 2014; Kjemtrup and Conrad, 2000; Krause, 2003; Vial and Gorenflot, 2006). During babesiosis infection, the host elicits humoral and cell-mediated immune responses that are responsible for parasite clearance. However, immune-evasion has been suspected during infection (Gray *et al.,* 2010). Clark and Jacobson, (1998) established that both *Babesia* and *Plasmodium* parasites confer cross-protection to mice during co-infection. A human case report from Korea showed that *Babesia* parasites prolonged severity of malaria-induced haemolytic anaemia during co-infection (Na *et al.,* 2014). A child from Ivory Coast co-infected with *Plasmodium* sp. and *Babesia* sp. parasites exhibited markedly enhanced malaria severity (Vermeil *et al.,* 1983). On the other hand, rhesus macaques with chronic *B. microti* infection showed that *B. microti* parasites were able to suppress *P. cynomolgi* infection (van Duivenvoorde *et al.,* 2010). Therefore, studies show that *B. microti* parasites either provoke induction of immune responses that either ameliorates or exacerbates

malaria infection. However, these few available studies have not provided enough insight into immunology and cellular mechanisms that are involved during mono- and co-infection.

***c) Leishmania* sp. and malaria:** In Sudan and Uganda, *Leishmania donovani* complex parasites and malaria have been reported to co-infect humans. The co-infections showed a synergistic immunological interaction characterized by enhanced Th1 immune responses (van den Bogaart *et al.,* 2014, 2012). The *L. donovani* complex parasites naturally colonise macrophages to initiate counter regulation of host immune responses resulting in a release of anergic/dysfunctional T-cells and blocking of intracellular cytokine signalling in macrophages and dendritic cells (van den Bogaart *et al.*, 2012). Currently, the available information on the interaction of visceral leishmaniasis and malaria co-infections among pastoral communities of Kenya, Uganda and Sudan is limited. It does not provide vital information on disease outcomes and immunological interactions. Malaria-infected red blood cells are recognised and internalised macrophages and dendritic cells that are also colonised by *Leishmania* parasites. This shows that during co-infection the control of malaria will be impaired in that the effector cells are used by the *Leishmania* parasites for immunoregulation. This will result in the exacerbation of malaria disease and suppression of *Leishmania* parasites or, conversely, the parasites will impact the host immunity and influence infection and pathophysiological responses of both parasites. The role of *Leishmania* parasite mediators and vector saliva components in mediating immunosuppression of host regulatory immune responses are still unknown (Abdeladhim *et al.*, 2014; Kima, 2014). Therefore, further research should be undertaken to determine disease prevalence and impact on socio-economic and environmental factors in regions where congruency of the two parasites is eminent.

d) *Toxoplasma gondii* and malaria: *Toxoplasma gondii* is a cosmopolitan intracellular apicomplexan parasite that causes ocular, congenital, neurological and systemic infections in approximately one third of the world's population (Guneratne *et al.,* 2011; Jones *et al.*, 2007; Kim and Weiss, 2008). Humans acquire infection through ingestion of sporulated oocysts and trophozoites in undercooked meat, organ transplants from infected donors or

through vertical transmission during pregnancy (Krueger *et al.,* 2014). Stray dogs and cats feeding on offal at abattoirs, poor sewerage systems and sanitation standards, and anthropogenic, climatic and socio-cultural factors have been implicated in human outbreaks of *T. gondii* infections (Flegr *et al.,* 2014; Jones *et al.,* 2007; Marchioro *et al.,* 2014). The severity of infection depends on host immunity and inflammatory foci involved (Lalibert and Carruthers, 2008). A questionnaire study conducted in Kenya established that sources of drinking water and disposal of cat faeces are infection risks amongst subsistence farmers (Ogendi *et al.,* 2013). However, the protozoan parasite is still regarded as an opportunistic agent and not as a causative agent of major infections (Andreani *et al.,* 2012; Chen *et al.,* 2012; Karp and Auwaerter, 2007; Morrissette and Sibley, 2002). This implies that toxoplasmosis and malaria co-infection cannot be ruled out in this malaria-endemic region. In several mono-infection studies, it has been established that *T. gondii* and *Plasmodium* parasites utilise similar cellular mechanisms and biochemical pathways for their nutrition, metabolism, pathology and immunomodulation (Abdi *et al.,* 2014; Adams *et al.,* 2014). This might indicate that during co-infections the parasites will result in competitive establishment that may promote or hamper parasite pathogenicity, and foetal and birth outcomes during pregnancy (Hill and Dubey, 2002), severity of anaemia and mortality (Castro-Filice *et al.,* 2014; Desai *et al.,* 2007; Marchioro *et al.,* 2014; Mustafa *et al.,* 2010), and severity of neurological and cerebral involvement (Da Silva and Langoni, 2009; Renia *et al.,* 2012; Torrey and Yolken, 2003). Malaria and *Toxoplasma* parasites sequester in the placenta resulting in placental disc plate damage thus influencing foetal and pregnancy outcomes (Onditi *et al.,* 2015). Despite these prospects of fatal disease outcomes there is striking paucity of information on immunological and disease outcomes and interactions during co-infections with *T. gondii*.

Lymphatic-dwelling filarial worms and malaria

Lymphatic filariasis (LF) caused by *Wuchereria bancrofti*, *Brugia malayi*, *Onchocerca volvulus* and *Loa loa* are endemic in SSA (Slater *et al.,* 2013; Stensgaard *et al.,* 2011). The filarial nematode worms and *Plasmodium* parasites are transmitted by the same

anopheline mosquito vector making co-endemicity a common phenomenon (Cano *et al.*, 2014; Kelly-Hope *et al.*, 2013) This necessitates implementation of integrated control measures (Stensgaard *et al.*, 2011). Several studies on chronic LF interactions with malaria have been conducted (Slater *et al.*, 2013) and have shown that patent filariasis is able to modify immunological balance to confer protection against malaria severity or exacerbate it (Fernández Ruiz *et al.*, 2009; Graham *et al.*, 2005; Tetsutani *et al.*, 2009). The amelioration malaria severity is achieved by the combined induction of Th1 and Th2 immune responses with increased interleukin (IL)-5 and IFN-γ production (Dolo *et al.*, 2012; Taylor *et al.*, 2012; van der Werf *et al.*, 2013). On the other hand, pre-patent filariasis exacerbates malaria severity through immunosuppression of IFN-γ and initiation of activation of CD4+CD25+FoxP3+ T-regulatory cells (Tetsutani *et al.*, 2009). In epidemiological studies, anti-helminthic treatment against LF has been shown to reduce LF exposure through interruption of its transmission dynamics (Bundy *et al.*, 2013; Njenga *et al.*, 2011). However, in murine studies, it has been shown to exacerbate malaria and sepsis *(Panda et al.*, 2013), thus negating its usefulness in malaria-endemic areas. On the other hand, Aliota *et al.* (2011) established that filarial worms are capable of reducing *Plasmodium* parasite infectivity within the mosquito vector. However, these studies have not determined the immunological changes that occur during deworming and its benefits towards integrated control strategies in malaria-endemic areas.

Taenia solium, Echinococcus granulosus and E. multilocularis, and malaria

Larval stages of some tapeworms cause fatal liver, brain and lung metastasis in humans and livestock (Gillet and Bresson-Hadni, 1991; Wandra *et al.*, 2015). In SSA, exposure risks are attributed to changes in human culinary habits (Macpherson *et al.*, 2000; Macpherson, 2005) and environmental contamination by stray dogs and cats (Ogendi *et al.*, 2013; Pozio *et al.*, 2005; Robertson and Thompson, 2002; Traub *et al.*, 2005). The resultant diseases cause physical damage to vital body organs and tissues, and even lead to neurological and cerebral damage (Utzinger *et al.*, 2010). Active infection favours induction of a Th-2 skewed immune response characterised by markedly elevated levels of

IL-4 and IL-10 (Maizels *et al.,* 2012; Saunders *et al.,* 2007). In several areas of SSA, tapeworm infections are rare due to religious and agricultural practices (Kamuyu *et al.*, 2014). However, isolated cases of human infections have been reported in people working in commercial pig farms (Babalola *et al.,* 2013; Elias *et al.,* 2005), or living in areas where there are no sanitation facilities and the presence of free roaming pigs (Hotez and Kamath, 2009; Magwedere *et al.,* 2012). Information on malaria and cestodes infections is non-existent. Therefore, further studies are needed to determine the actual prevalence, disease burdens and even cases of co-infection with malaria.

Trematodes and malaria

Fasciola hepatica and *F. gigantica* in humans are emerging infections and occur in malaria endemic areas despite the parasites not being considered to have relevance in malaria co-infections (Esteban *et al.*, 2003; Keiser and Utzinger, 2007; Mas-Coma *et al.*, 2005). One of the most important and common snail-borne trematode infections in humans is due to *Schistosoma haematobium* and *S. mansoni*, and these are becoming emerging or re-emerging infections in developing countries of SSA due to climate changes that are influencing spatial distribution of fresh water snails (Mas-Coma *et al.,* 2009; Pedersen *et al.,* 2014). Therefore, the impact of fascioliasis and schistosomiasis on communities demand rapid action and research to define control measures, transmission patterns and epidemiological situations. There is paucity of data on the interaction of fascioliasis with malaria except for shared zonal distributions in Egypt (Keiser *et al.*, 2011). Thus, research on the interactions between both parasites in their shared eco-epidemiological settings is required.

Several laboratory experiments and epidemiological studies have been conducted and meta-analysis reviews have been done to determine the host-parasite interactions of malaria and schistosomes during co-infection (Booth *et al.,* 2008). The studies have shown that clinical outcomes of malaria and immune responses during co-infections with schistosomes are influenced by age, host genetics, immunity and exposure rates in humans

(Diallo *et al.,* 2010; Florey *et al.,* 2012; Legesse *et al.,* 2004; Lemaitre *et al.*, 2014; Mulu *et al.*, 2014, 2013; Pearce *et al.,* 2004; Reilly *et al.*, 2008). In animal models, immunological responses induced also depend on the strain of the parasite and patency of the helminthic infections (Boel *et al.,* 2010; Nacher, 2008, 2002). Research findings show that schistosomes induce Th2 immune responses that are either detrimental or beneficial to the host during co-infection (Imai *et al.,* 2011; Pearce *et al.,* 2004; Sangweme *et al.,* 2010). These findings further show that schistosomes increase malaria susceptibility and transmission (Noland *et al.,* 2007) but have not been able to conclusively explain the underlying mechanism and pathways for immunomodulation.

CONCLUSION

There are few studies that are directed towards elucidating the host-parasite interactions and disease outcomes that are elicited by tissue-dwelling parasites during co-infection with malaria. This has created a glaring paucity of data on understanding the mechanisms and outcomes of tissue-dwelling parasites and mono- and co-infection with malaria. This has also hampered diagnosis, vaccine development, drug discovery, and management and control of these emerging and re-emerging parasites. Therefore, further studies are imperative to address this lack of data and the heterogeneity of results reported during STHs, schistosomes and filarial worm co-infection with malaria. These future studies should be designed and controlled towards elucidating cellular and molecular pathways as well as migratory pathways that are utilised by migrating tissue-dwelling helminths and protozoa. The utilisation of different study designs and approaches, as well as different tissue-dwelling helminths and protozoa will provide vital information that can be extrapolated to humans. These studies and experiments will also provide information on non-immunological aspects, timing and order of parasite infections. The disease outcomes across broad range of hosts and parasites will show evidence of parasite-host-parasite interactions at the phenotypic level. This data will be useful in explaining the actual cellular and molecular mechanisms and signalling pathways that influence conferring of protective immunity, exacerbation and/or amelioration of disease outcomes that have been observed in

concomitant and concurrent infections. In the long term, the studies will provide the thrust for deworming, surveillance, diagnosis, vaccination campaigns and vaccine trials in areas of SSA where tissue-dwelling parasites are co-endemic with malaria.

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CHAPTER 3

METABOLIC AND ADAPTIVE IMMUNE RESPONSES IN BALB/c MICE INFECTED WITH *TRICHINELLA ZIMBABWENSIS*

In press: Acta Tropica.

3.1 Abstract

Tissue-dwelling helminths are known to induce intestinal and systemic inflammation accompanied with host compensatory mechanisms to counter balance nutritional and metabolic deficiencies. The metabolic and immune responses of the host depend on the parasite species and the organs or tissues affected by the parasite. The present study investigated metabolic and immuno-inflammatory responses of mice infected with tissue-dwelling larvae of *Trichinella zimbabwensis* and explored the relationship between infection, insulin signalling pathways and Th17 immune responses. A crocodile-derived *T. zimbabwensis* strain (Code ISS1209) was used in the study. Sixty female BALB/c mice that were 6 to 8 week old were randomly assigned into two equal groups; *T. zimbabwensis*-infected group (n = 30) and the non-infected control group (n = 30). Levels of Th1 (IFN-γ) and Th17 (IL-17) cytokines were measured in the two groups to determine and compare the immune-inflammatory response and the levels of insulin, blood glucose, food and water intake. Body weights were also measured to determine and compare the metabolic response in the two groups. Results showed that during the enteric phase of infection, insulin and IFN-γ levels were significantly higher ($p < 0.001$) in the *Trichinella*-infected group accompanied with hypophagia and weight loss compared with the non-infected control group. During systemic larval migration, food and water intake were significantly altered ($p < 0.001$) and this was attributed to compensatory feeding resulting in weight gain, reduced insulin levels and increased IL-17 levels. Larval migration also induced a Th1/Th17 derived inflammatory response. It was concluded that *T. zimbabwensis* alters insulin signalling pathways instigating host compensatory feeding. Furthermore, we showed for the first time that non-encapsulated *T. zimbabwensis* parasite immunomodulate host Th1/Th17 responses during chronic infection.

3.2 Introduction

Trichinella zimbabwensis is a non-encapsulated zoonotic nematode parasite that is prevalent and widely distributed in Southern Africa where it infects a wide variety of

animals (La Grange *et al.,* 2010; 2009; Mukaratirwa and Foggin, 1999; Mukaratirwa *et al.*, 2013, 2008; Pozio *et al.,* 2002). The parasite is of potential public health risk for future human trichinellosis epidemic outbreaks due to its capability to infect non-human primates, increased globalization, poor sanitation and animal rearing systems, increased animal product movements and human-wildlife-livestock interactions (Gottstein *et al.,* 2009; Mukaratirwa *et al.,* 2013; 2008; Murrell *et al.,* 2005).

Trichinella infection is acquired through ingestion of raw or undercooked meat infected with *T. zimbabwensis* muscle larvae (Nöckler *et al.,* 2000). During the enteric phase of infection, the adult female worm releases up to 1500 new born larvae (NBL) in the intestines which penetrate the intestinal mucosa and migrate to the muscles (Despommier, 1998; Appleton *et al.,* 2001; Gagliardo, 2002). The NBL cause temporary enteric and tissue inflammation during migration to the sites of predilection (Bruschi and Chiumiento, 2011). The migration pattern stimulate both mucosal and systemic immune responses where the parasite utilizes host cell-biological systems to establish its parasitism (Pozio *et al.,* 2004) without an overt inflammatory reaction (Aranzamendi *et al.,* 2013). In mice and human studies, *Trichinella* parasites and worm products have been documented to confer protection against inflammatory and auto-immune diseases through immunoregulation of Th1/Th2/Th17, dendritic cells maturation, T and B regulatory cells as well as monocytes during type 1 diabetes, allergic airway inflammation and encephalomyelitis (Finkelman *et al.,* 2004; Khan and Collins, 2005; Del Prete *et al.,* 2008; Wu *et al.,* 2010; Adisakwattana *et al.,* 2013; Aranzamendi *et al.,* 2013; Rodgers *et al.,* 2014).

In the past two decades, significant studies on the immunological responses and pathological effect of gut-dwelling helminths have identified the importance of Th2 immune responses in their establishment and transmission (Jackson *et al.,* 2009; Maizels and Yazdanbakhsh, 2008; Maizels *et al.,* 2009, 2004). This has not been the case with non-encapsulated tissue-dwelling nematode, *T. zimbabwensis* except with few studies on *T. spiralis* (encapsulated) and *T. pseudospiralis* (non-encapsulated) that are rare in Southern Africa (Pozio and Zarlenga, 2005; Pozio, 2013). Furthermore, the studies have established

that gut-dwelling helminths also suppress host immune effector mechanism hence down regulating Th1 immune responses (Hartgers and Yazdanbakhsh, 2006). This allows for release of IL-6 and tumour growth factor beta (TGF-β) which play a critical role in inflammation (Wu *et al.,* 2010) and IL-17 differentiation (Bettelli *et al.,* 2006; Mihi *et al.,* 2014). T-helper 17 is a CD4+ effector T-cells subset that distinctively produces IL-17 cytokine (Hirota *et al.,* 2007; Hirata *et al.,* 2010). Interleukin-17 cytokine is known to mediate production of regulatory cytokines and chemokines that attract macrophages and neutrophils to the site of tissue injury (Jin & Dong, 2013). However, the role of T-helper 17 (Th17) response during migration of non-encapsulated *T. zimbabwensis* NBL is not fully understood. On the other hand, there is also paucity of studies on metabolic and adaptive immune responses that are induced by *T. zimbabwensis* parasite in infected hosts. This has hampered efforts of its diagnosis, surveillance, management and control. In order to improve the development of vaccines, sensitive diagnostic tools and drug discovery, it is imperative to understand the underlying mechanisms that influence glucose metabolism, the insulin signalling and Th1/Th17 cell type expression during *T. zimbabwensis* infection. Hence, the present study investigated metabolic and immuno-inflammatory responses in mice infected *T. zimbabwensis* and the relationship between infection, the insulin-signalling pathways and Th17-response.

3.3 Materials and methods

Ethical statement

All animal procedures and protocols were approved by University of KwaZulu-Natal (UKZN) Animal Ethics committee (ref. no. 114/13/Animal) in accordance with South African National Legislation Regulations in respect to animal husbandry, experimentation and welfare of laboratory animals for biomedical research. The experiments described herein are in accordance with ARRIVE guidelines (Kilkenny *et al*., 2013).

Study animals

Sixty female BALB/c mice that were 6 to 8 weeks were sourced from University of Cape Town, South Africa. The mice were maintained at the Biomedical Resources Unit (BRU) of the UKZN, Westville campus, under specific pathogen free conditions in individually ventilated metabolic cages (Labotec products, RSA). They were randomly assigned into two groups; control (n = 30) and *T. zimbabwensis* infected (n = 30) and fed daily on heat sterilized pelleted ration (Meadow feeds, RSA) and clean water was *ad libitum*.

Parasite isolation and infection of study animals

Crocodile-*derived T. zimbabwensis* strain (Code ISS1209) larvae were obtained from eviscerated carcasses of stock rats that were digested as described by Kapel and Gamble, (2000). On day 0, each animal in the *Trichinella*-infection group was infected with 500 muscle larvae (ML) through oral lavage as described by Mukaratirwa *et al*. (2001). On day 0, 7, 14, 21 28 and 35 post-infection (pi), six mice from each group were sacrificed, and blood was collected for serum, intestines for adult worms and whole mouse carcasses was digested to obtain ML.

Measurement of food and water intake and body weight

Daily food intake per mouse was derived by daily weighing the food pellets (Meadow Feeds, RSA) before feeding and 24 hours after feeding the animals. After 24 hours from disposing the pellets to the animals the remaining pellets and pellet crumbs were collected and weighed using Boeco balance (Germany) and the difference from the initial weight was calculated as food intake. The volume of water intake per mouse was obtained by measuring the difference between final water volume and initial water volume after 24 hours using a laboratory measuring cylinder on daily basis. Individual mouse body

weight was measured daily using Boeco balance (Germany) and average body weights were calculated for the two groups.

Blood glucose and serum insulin levels

A 25 gauge needle was used to prick the tail vein and approximately 10 µL of blood was collected on glucometer test strip window and used to measure blood glucose using a Contour® TS glucometer (Bayer, Basel, Switzerland).

The sera collected on day 0, 7, 14, 21 28 and 35 pi were used to measure insulin levels using mouse insulin ELISA kit (cat. No. EZRMI-13K, Millipore, Missouri, USA). Briefly, 96 well plates pre-coated with pre-titered mouse monoclonal anti-rat antibodies were washed three times with 300µL of 50mM Tri Buffered Saline containing Tween 20. Each serum sample and a pre-determined standard were mixed with 10µL Assay buffer (0.05M phosphosaline, PH 7.4, containing 0.025 M EDTA, 0.08% sodium azide and 1% BSA), 10µL matrix solution containing charcoal-stripped pooled mouse serum and 80µL of pre-titered biotinylated anti-insulin detection antibody per well. The plates were incubated at room temperature for 2 hours while being agitated. Plates were washed as before and 100µL of pre-titered streptavidin peroxidase conjugate solution was added per well and incubated for 30 minutes at room temperature while being agitated. The plates were washed 6 times as before and 100µL of 3, 3', 5, 5' tetra-methylbenzidine solution added per well and placed at room temperature for 15 minutes. The reaction was stopped by adding 50 µL of 0.3M HCL stop solution. Samples and standard optical densities were obtained by reading the plates at 450nm and 590nm wavelengths. A 4 parameter logistic equation was used to determine serum insulin concentrations using Graph Pad PRISM™ version 5.04 for windows (Graph Pad Software, San Diego, CA).

Measurement of cytokine concentrations

Mouse cytokine specific DuoSet ELISA development system kits (R&D systems, USA) were used to measure IFN-γ (DY485-05) and IL-17 (DY421-05) levels in serum according to manufacturer's instructions. Briefly, NUNC Maxisorp® 96-well ELISA plates were coated with either 4.0 µg/mL of mouse IFN-γ capture antibody or 2.0 µg/mL of mouse IL-17 capture antibody in PBS and incubated overnight at room temperature. Plates were washed three times with 300 µL of PBS containing 0.05% Tween 20 and blocked with 200 µL of 1% BSA in PBS and incubated for 1 hour at room temperature. Further washing was done five times and 100µl of diluted samples and standards were added to the plates. The standards used were serially diluted in incubation buffer and highest concentrations used were 2000pg/ml for IFN-γ and 1000pg/ml for IL-17. The plates were then incubated for 2 hours at room temperature and washed as before. Biotinylated IFN-γ detection antibody was used at a concentration of 600 µg/ml and IL-17 at 400ng/mL per well and incubated for 2 hours at room temperature. Plates were then washed as before and 50µl of horseradish peroxidase -conjugated streptavidin was used at a dilution of 1:2000 and incubated for 20 minutes at room temperature. The reactions were stopped by adding 50 µL of 2N H_2SO_4 and absorbance obtained at 450nm and 540 nm. Cytokine concentrations were extrapolated from standards using a 4 parameter logistic equation in Graph Pad PRISM™ version 5.04 for windows (Graph Pad Software, San Diego, CA).

Statistical analysis

Cytokine, insulin and blood glucose levels, parasite loads and food and water intake measurements were recorded and expressed as mean ± standard error (SE). Student's t-test was used to compare differences between the two groups and a *p*-value of <.05 was considered significant. Graphs showing the burden of *T. zimbabwensis* in mice and metabolic and immunological responses were plotted using GraphPad PRISM™ version 5.04 for windows (Graph Pad Software, San Diego, CA).

3.4 Results

Parasite establishment

Adult *T. zimbabwensis* worms from intestines and larvae from muscles of infected mice are shown in Fig. 3.1. Significantly more adult worms were observed in the intestine at day 7 pi in comparison with day 14 and 21 pi ($p < 0.001$). There were no muscle larvae obtained from day 7 to 14 pi and very few larvae were recovered (average of 0.12 larvae per gram (lpg) of muscle) at day 21. The number of larvae significantly increased to 16 lpg at day 28 ($p < 0.05$) and 20 lpg at day 35 ($p < 0.01$) in comparison to day 21 pi (Fig. 3.1).

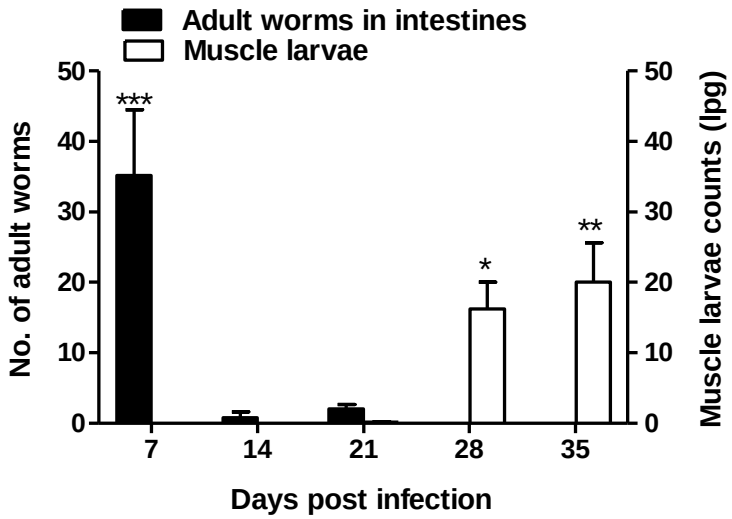

Figure 3.1: Mean number of *Trichinella zimbabwensis* adult worm count in small intestines and muscle larvae count of *Trichinella*-infected BALB/c mice. The *Trichinella* parasite counts are mean ± standard error (SE); Levels of significance * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$.

Food and water intake

At day 3 to 6 pi the infected group developed hypophagia and consumed less food than the control group (Fig 3.2A). At day 7 and 18 pi the food intake of the infected group increased exponentially in comparison with the control group ($p < 0.05$) (Fig. 3.2A). At day 14 pi, the infected group consumed an average of 12 grams of pellets per day compared to the control group which consumed an average of 4 grams. The food intake in the infected group decreased gradually to equal the control which increased on days 19 to 25 pi. At day 26 through 30 post-infection there was no difference on the amount of food intake during this period. The overall food intake by the *Trichinella*-infected group was significantly higher in comparison to the control non-infected group ($p < 0.001$).

There was variation in water intake between the two groups although the infected group overally had a higher water intake. The infected group water intake at day 2 pi was higher than the control group ($p < 0.01$) (Fig. 3.2B). At day 3 to 11 pi the water intake in the infected group was lower than that of the control group.

Daily measurements of body weight are shown in Fig. 3.2C. At day 3 to 11 pi, the infected group significantly lost weight compared to the control group ($p < 0.01$). After day 11 pi the infected group gradually gained weight and at day 15 pi, the weight was similar to that of the control group. The weight gain continued from day 15 pi in the infected group and reached peak at day 27 pi. At the termination of the study (day 30), the body weight change of the infected group was greater ($p < 0.01$) than that of the control group.

Insulin and blood glucose levels

At day 7 pi, *T. zimbabwensis* infected group had significantly high insulin levels (0.931ng/ml) compared to the control group (0.302ng/ml). The insulin levels of the infected group dropped significantly to 0.308pg/ml at 14 day post-infection (dpi), 0.397pg/ml (21

dpi) and 0.297pg/ml (35dpi). Insulin levels in the infected group were significantly higher ($p < 0.001$) compared to the control group (Fig. 3.2D).

After 7 days pi, the infected group had lower blood glucose levels (101.88mg/dL) compared to control group (116.46mg/dL) (Fig. 3.2E) which was not significant ($p > 0.05$). In both groups, a decrease in glucose levels was observed at day 14 pi and later at day 21pi. At day 28 and 35 pi, the glucose levels of the infected group were elevated to an average of 100mg/dL which was higher than that of the control group. Overally, there was a significant difference between the groups at day 28 and 35 pi with the infected group showing high glucose levels ($p < 0.001$).

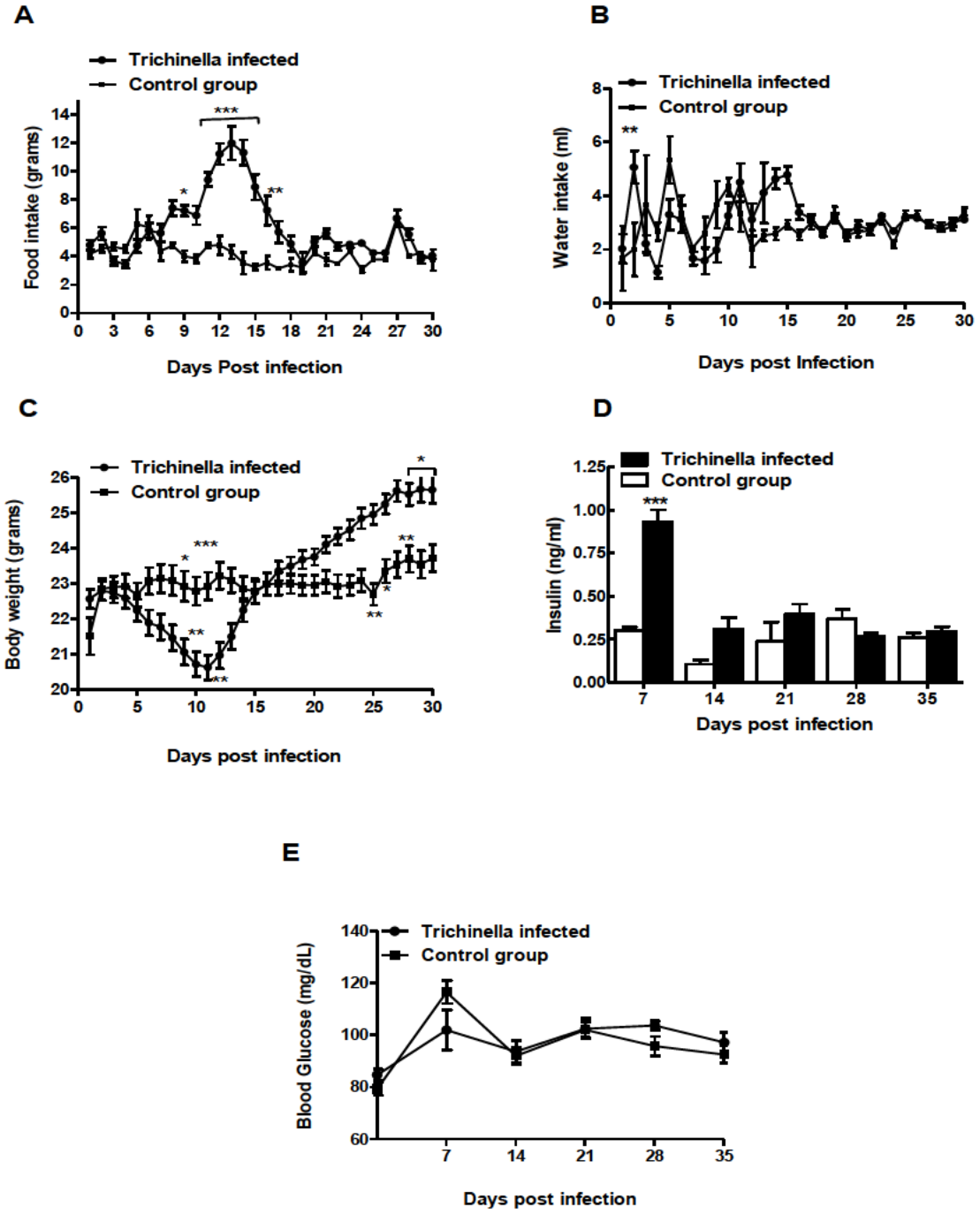

Figure 3:2: Comparison of metabolic parameters of *Trichinella zimbabwensis*-infected and uninfected BALB/c mice. The parameters for each group are presented as means ± standard error (SE) (n = 10); Levels of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Th1/Th17 cytokines

At day 7 pi the infected group had significantly elevated IFN-γ cytokine levels (9.04ng/ml) ($p < 0.01$) which slightly decreased at day 14pi (7.96ng/ml), day 21pi (6.23ng/ml) and day 28 pi (5.85ng/ml) and later increased at day 35 pi to 6.54ng/ml (Fig. 4.3A). The IFN-γ levels of the control group remained at average baseline levels (5.6ng/ml) throughout the experimental period. Overall trend in IFN-γ levels in the infected group was significantly higher than that of the control group ($p < 0.001$).

At day 14 pi, IL-17 levels of the infected group were significantly high (893pg/ml) compared to the control group (17.60pg/ml) ($p < 0.001$) (Fig. 3.3B).

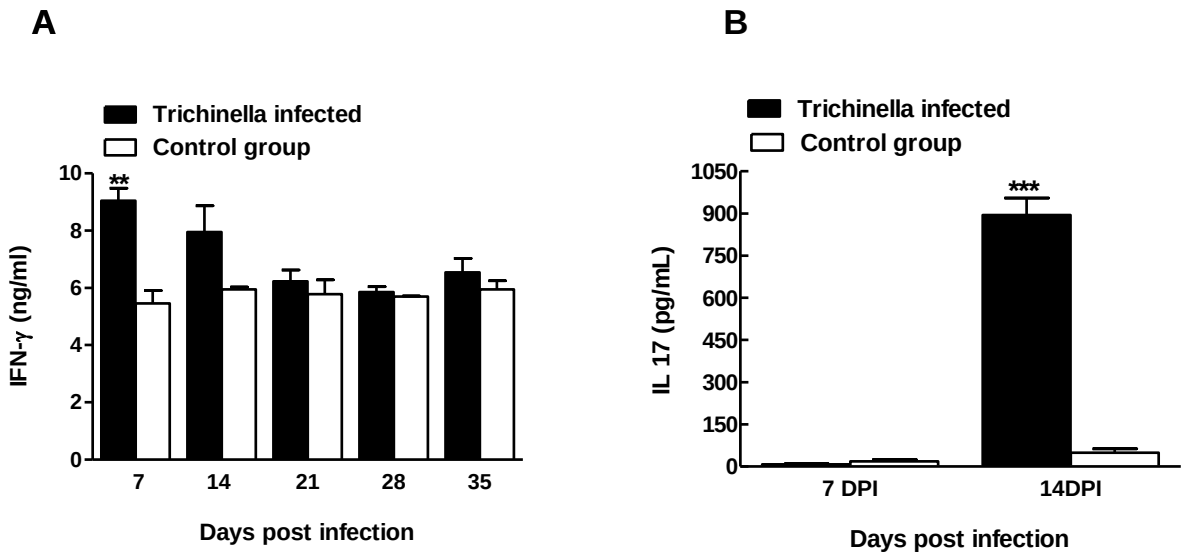

Figure 3.3: Interferon gamma (IFN-γ) (A) and IL-17 (B) cytokine concentrations of *Trichinella zimbabwensis*-infected and non-infected BALB/c mice. The cytokine concentrations for each group are presented as means ± standard error (SE) (n = 10); Levels of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.5 Discussion

The results showed that *T. zimbabwensis* adult worms persisted in the small intestines for up to 21 days post-infection and this is comparable with other studies that used *T. papuae, T. spiralis* and *T. pseudospiralis* in mice (Sadaow *et al.,* 2013). In response to host immunity, the female worms relocate further down the intestinal tract to release new born larvae (NBL) (Despommier *et al.,* 2005). In addition, the delay in worm expulsion may also be attributed to intestinal mucosal epithelia damage resulting in depletion of mucin cells, mucosal mast cells and goblet cells. This is corroborated by Hasnain *et al.* (2011) who reported that worm expulsion was delayed in mucin depleted mice infected with either *T. spiralis* or *Nippostrongylus brasiliensis*.

In our study, we observed reduced food and water intake during the enteric phase of *T. zimbabwensis* development. This shows that inflammation due to NBL penetration causes hypophagic response accompanied with weight loss. The actual mechanism behind this phenomenon is not clear. However, it has been established by Worthington *et al.* (2013) that in *T. spiralis*-infected mice hypophagia occurs and is due to upregulation of cholecystokinin (CCK) hormone. Increase of nutrients in the intestines triggers control of feeding centres in the brain limiting food ingestion, satiety and delay gastric emptying (Rigaud *et al.,* 1994; Schonhoff *et al.,* 2004). We also observed weight loss that may be due to increased nutrients loss in faecal droppings or increased energy expenditure by adult worms and migrating larvae (Montgomery *et al.,* 2003) or the reduced food intake.

We also observed that levels of blood glucose and serum insulin in *Trichinella*-infected mice were elevated compared with non-infected control mice. This may indicate that *T. zimbabwensis* migrating larvae influences expression of insulin signalling factors. Wu *et al.* (2009) establish that mice infected with *T. spiralis*, ML are able to exploit host metabolic pathways by upregulating genes involved in insulin pathway signalling but do not have direct effect in the increasing levels of insulin levels *per se*. However, the alteration in the metabolic responses we observed was short lived until 14 dpi when the

levels of insulin returned to normal suggesting that as enteritis resolved and pancreatobiliary secretomotor functions restored. The migrating larvae favours muscle glucose uptake by inhibiting glucose metabolism, glycogenolysis and controlling feeding behaviour and energy expenditure (Szanto and Kahn, 2000). This results into fat mass accumulation and eventual body weight gain. The accumulated fat mass deposits may influence secretion of TGF-β, TNF-α and IL-6 cytokines that favour parasite establishment (Nehete *et al.,* 2014; Trayhurn and Beattie, 2007) and IL-17 differentiation (Bettelli *et al.*, 2006; Pappu *et al.,* 2011). In the present study, we observed that there were elevated levels of pro-inflammatory cytokines, IFN-γ and IL-17 which indicates that an early tissue-mediated innate immunity is induced against adult *T. zimbabwensis* infection and inflammation due to penetration. After 14 dpi, the levels of IFN-γ were declining and IL-17 were undetectable showing that Th2 immune polarization limit pro-inflammatory cytokine secretion (Wakelin *et al.,* 1994; Wu *et al.,* 2010) This is in agreement with our previous study where we observed decline in the levels of TNF-α and elevated levels of anti-*Trichinella*-specific antibodies (IgG1) and Th2 cytokine (IL-4) and T-regulatory cytokine (IL-10) (Onkoba *et al.,* 2015). This implied that the parasite may be immuno-modulating host immune system to initiates Th2 polarisation that down regulate production of pro-inflammatory cytokines (Harnett, 2014).

The present study could not provide extensive evidence on actual mechanisms involved in Th17 secretion and regulation. Further studies need to be undertaken taking into consideration that *T. zimbabwensis* NBL migrates through various body cavities and tissues with diverse immunological responses that are short-lived. Immunological compartmentalization complicates the understanding of tissue-dwelling host-parasite interactions during infection.

In conclusion, the present study indicated that *T. zimbabwensis* infection alters insulin signalling pathways and host responds by compensatory feeding resulting in muscle glycogen uptake and weight gain. Furthermore, we show for the first time that non-encapsulated *T. zimbabwensis* parasite immunomodulates host Th1/Th17 response during

chronic infection. Further studies are needed to determine immune responses evoked during *T. zimbabwensis* NBL migratory patterns. However, the data generated contributes to new knowledge on the understanding of how tissue-dwelling helminths manipulates and adapts to host metabolic and immune systems to establish parasitism.

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CHAPTER 4

DIFFERENTIAL IMMUNE RESPONSES INDUCED IN MICE INFECTED WITH TISSUE-DWELLING NEMATODE *TRICHINELLA ZIMBABWENSIS*

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4.1 Abstract

To improve diagnostic tools, immunotherapies and vaccine development for trichinellosis surveillance and control there is need to understand the host immune responses induced during infection with *Trichinella zimbabwensis*, a tissue-dwelling nematode. This study was undertaken to determine immune responses induced in mice during *T. zimbabwensis* infection. The parasite strain used (Code ISS1209) was derived from a naturally infected crocodile *(Crocodylus niloticus)*. Sixty female BALB/c mice aged 6 to 8 week were randomly assigned into two equal groups; *T. zimbabwensis*-infected (n = 30) and the non-infected control group (n = 30). Serum tumour necrosis factor-α (TNF-α), Interleukin 10 (IL-10), IL-4 levels and IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 antibody responses were determined using enzyme-linked immunosorbent assay (ELISA). Results showed that during enteric phase of infection, higher levels of IgM and IgG1 ($p < 0.05$), IgG, IL-10 and TNF-α ($p < 0.001$) were observed in the *Trichinella*-infected group compared with the non-infected control group. In the parasite establishment and tissue migration phases, levels of IgG1 and IgG3 were elevated ($p < 0.001$), while those of IgM ($p < 0.01$) declined on day 21 and 35 (pi) compared to the enteric phase. Our findings show that *T. zimbabwensis* infection induces mixed Th1/Th2 immune responses in the enteric, tissue migration and parasite establishment phases. Furthermore, our results show that *T. zimbabwensis* parasite is able to induce immunomodulation that favours its establishment. Further studies are indicated for elucidation of the underlying mechanisms involved.

4.2 Introduction

Trichinellosis is cosmopolitan foodborne zoonosis caused by a parasitic tissue-dwelling nematode of genus *Trichinella* (Murrell & Pozio, 2011). The disease occurs in 66 countries and infects an estimated 11 million people (Dupouy-Camet, 2009; Yang *et al.*, 2010). Scattered outbreaks of human trichinellosis have been reported over time in Asia and Europe (Ranque *et al*., 2000; Khumjui *et al*., 2008; Murrell & Pozio, 2011; European Food Safety Authority, 2011). *Trichinella* infections are normally acquired through

ingestion of raw or under-cooked infected meat (Chai *et al.*, 2005; Murrell *et al*., 2005) or food and water contaminated with infective muscle larvae (ML) (Slifko *et al*., 2000; Dabanch, 2003; Pozio & Rossi, 2008). The ML develop into adults that release new-born larvae (NBL) in the small intestines that migrate to the muscles (Gao *et al.*, 2014).

The risk of future human *T. zimbabwensis* infection are increasing due to poverty, food insecurity, climate change and failure of veterinary controls and surveillance (De Merode *et al.*, 2004; Pozio & Murrell, 2006; Gottstein *et al*., 2009; Magwedere *et al*., 2012; Mukaratirwa *et al*., 2013). Furthermore, globalization has exacerbating the risk through increased movement of people, wildlife and livestock in and out of *T. zimbabwensis* endemic zones of Southern Africa (Mukaratirwa *et al*., 2013). In literature, natural *T. zimbabwensis* infections have been reported in variety of vertebrates (Pozio *et al.*, 2007; La Grange *et al.*, 2009, 2010, 2012). In addition, experimental infections have shown that the parasite can infect non-human primates, pigs and rodents (Mukaratirwa & Foggin, 1999; Pozio *et al.*, 2004; Mukaratirwa *et al.*, 2008).

Trichinella infection surveillance, control and treatments has been hampered by wide distributions of domestic, synanthropic and sylvatic reservoirs, lack of licensed vaccine and efficacious drugs against encysted ML and lack of reliable diagnostic tools for screening early cases of infection (Yépez-Mulia *et al.*, 2007; Gruden-Movsesijan *et al.*, 2008; Feng *et al.*, 2013). However, there is ongoing research of testing parasite derived somatic antigens and crude extracts as efforts to develop vaccines, identifying putative molecules to be used in development of diagnostic tools, vaccines and immunotherapies (Ruangkunaporn *et al.*, 1994; Pozio *et al.*, 2002; Deville *et al.*, 2005; Bień, 2007; Frey *et al*., 2009; Nagano *et al*., 2011; Li *et al*., 2013). The present study was undertaken to determine differential immune responses induced in mice infected with *T. zimbabwensis*. *Trichinella zimbabwensis* is the most prevalent in *Trichinella* species in Southern Africa whose infectivity (Hurnikova *et al.*, 2004; Pozio & La Rosa, 2005; Matenga *et al.*, 2006), host range (Mukaratirwa *et al*., 2003; Matenga *et al*., 2006; Mukaratirwa *et al.*, 2008), biochemistry (La Rosa *et al.*, 2003), diagnosis (Ludovisi *et al.*, 2013) and treatment

(Mukaratirwa *et al.,* 2015) have been extensively studied. However, there is paucity of information on immune responses induced in the phases of its life cycle.

4.3 Materials and methods

Study animals

Sixty female BALB/c mice aged 6 to 8 weeks were sourced from the University of Cape Town, South Africa, and maintained in pathogen free individual ventilated cages at Biomedical Resources Unit of UKZN, Westville Campus. The mice were fed with heat sterilized rodent pellets (Meadow feeds, RSA) and clean water *ad libitum*. The experimental mice were randomly assigned to two groups; *Trichinella*-infected (n = 30) and the non-infected control group (n = 30).

Trichinella zimbabwensis parasite and animal infection

A crocodile-derived *T. zimbabwensis* (Code ISS1209) parasite strain was used. The isolate was maintained in our laboratory by serial passage in Sprague Dawley rats. Muscle larvae were obtained from infected stock rats whole carcasses were digested at 42 days post infection (dpi) according to the digestion method previously described by Kapel and Gamble, (2000). Each mouse was orally infected with 500 larvae ML and at 0, 7, 14, 21, 28 and 35 dpi mice were sacrificed and blood collected for sera.

Trichinella zimbabwensis crude antigen preparation

Preparation of T. zimbabwensis antigen was performed as previously described by Escalante *et al.,* (2004). The ML obtained as described earlier were sonicated at five pulses of 100W for 30 seconds each and centrifuged at 40,000 x g at 4^{o} C for 60 min and supernatant collected. Protein concentration was determined by Bradford assay (Bradford,

1976) then an anti-protease cocktail (Sigma-Aldrich, St. Louis MO) was added. The antigen was diluted to working concentration and stored at -80^{o} C until use.

Parasite specific antibody responses determination

Trichinella-specific IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 antibodies were measured in sera collected on 0, 7, 14, 21, 28, 35 dpi by ELISA in both experimental and control groups. Microplates were coated with 6.0 µg/ml of crude *T. zimbabwensis* larvae antigen in 100 µl bicarbonate buffers (4 mM Na2CO3, 8 mMNaHCO3, pH 9.6) and incubated overnight at 4^{o} C. The microtitre plates were blocked with 0.05% Tween 20 in PBS containing 5% BSA and incubated at 37^{o} C for 2 hours. Serum samples at a dilution of 1:200 were added in triplicate wells and incubated at 37^{o} C for 2 hours. Horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Santa Cruz Biotechnology, CA, USA) antibodies were added at dilution of 1:2000 and IgG1, Ig2a, IgG2b, and IgG3) at dilution of 1:1000. After 2 hours of incubation at 37^{o} C, 100µl substrate (TMB substrate; KPL, Gaithersburg, MD) was added. Optical density (OD) values were measured at 630nm using a microplate reader (BioTek, VT, USA).

Cytokine specific ELISA

Concentrations of IFN-γ, TNF-α, IL-4 and IL-10 were measured in sera by mouse cytokine specific ELISA kits (RnD systems, MN, USA) according to the manufacturer's guidelines. Cytokine concentrations were obtained from standard curves generated by recombinant cytokines.

Statistical analysis

Antibody responses and cytokine concentrations at each day of sacrifice were expressed as means ± standard error (SE). Differences among groups were compared using student's *t*-tests. A *P* value of < 0.05 was considered to be statistically significant. Graphs

were generated using Graph pad PRISM version 5.04 for windows (Graphpad software, San Diego, CA, USA).

4.4 Results

Trichinella zimbabwensis-specific antibody responses during primary infection

Following infection, the infected mice were found to be seropositive for parasite specific IgM (Fig. 4.1A), IgG (Fig. 4.1B), and IgG1 (Fig. 4.1C) antibodies. A gradual increase in IgM levels was observed at day 7 pi which reached peak levels on days 14 and 28 (Fig. 4.1A). Significant levels of IgG were observed ($p < 0.001$) (Fig. 4.1B) compared to the control group. High levels of anti- *T. zimbabwensis*- specific IgG1 levels were observed at day 14 pi (Fig. 4.1C) and declined at day 21 pi ($p < 0.001$). *Trichinella*-specific IgG3 antibody levels were detected from day 21 pi and remained elevated throughout the course of infection. A decrease in IgG, IgM, and IgG1 antibody responses was noted after day 28 pi. Overall, the *Trichinella*-infected group had significantly higher antibody responses as compared to the control group ($p < 0.001$).

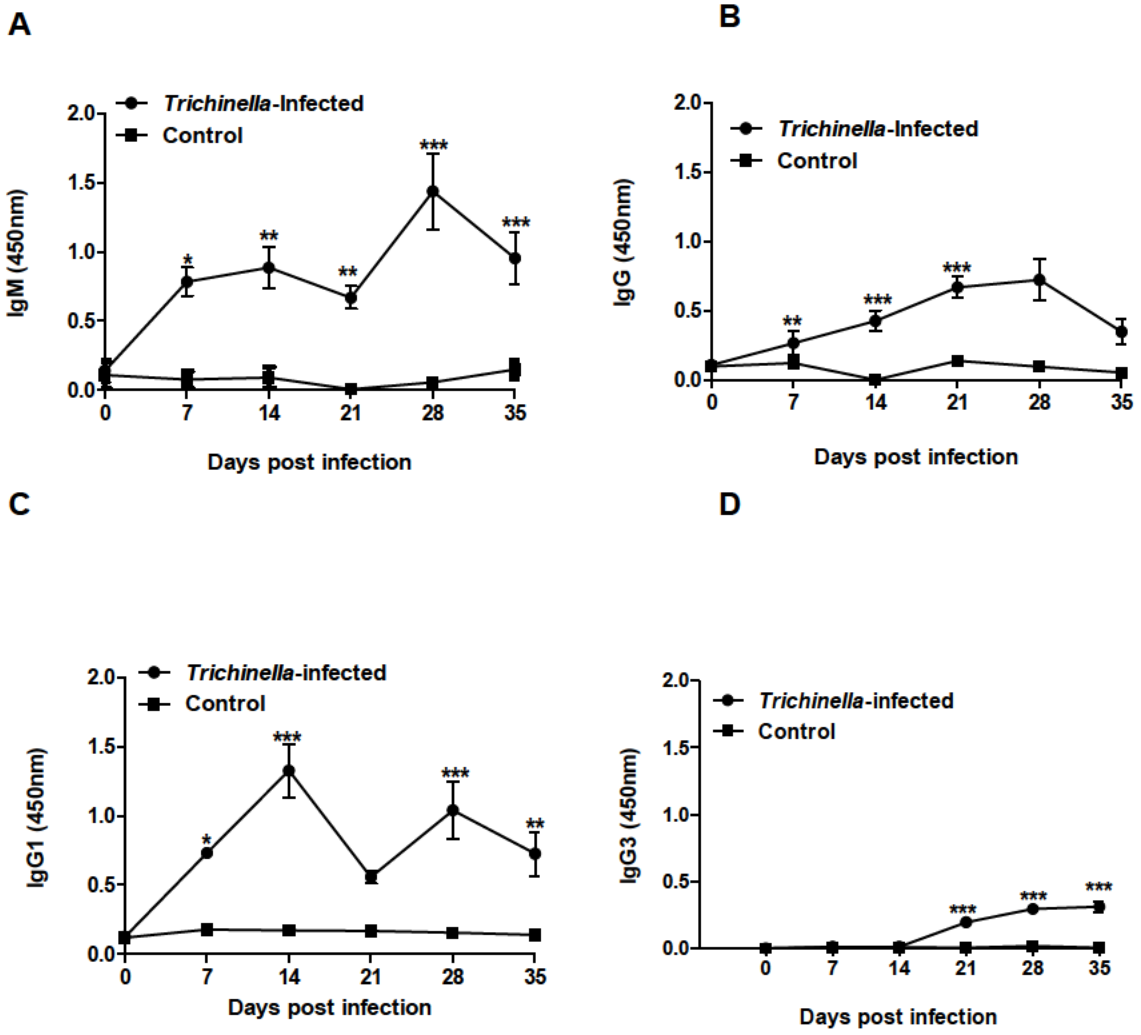

Figure 4.1: *Trichinella*- specific (A) IgM, (B) IgG, (C) IgG1, and (D) IgG3 antibody levels of mice during chronic *Trichinella zimbabwensis* infection. The optical density (OD) shown for each group are mean ± standard error (SE) of antibody levels (n=6); Levels of significance * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$.

Cytokine concentrations

At day 7 pi, significant levels of TNF-α and IL-10 cytokine ($p < 0.001$) were observed in the infected group compared to control group (Fig. 4.2A; 4.2B and 4.2C). Interleukin-10 concentrations showed a gradual decline attaining low levels at day 21 pi ($p < 0.01$). At day 28 and 35 pi, a gradual increase and decline were observed respectively. Significantly higher levels of IL-4 were detected at day 21 pi ($p < 0.001$) (Fig. 4.2 D) and declined at day 35 pi to the level of the control group.

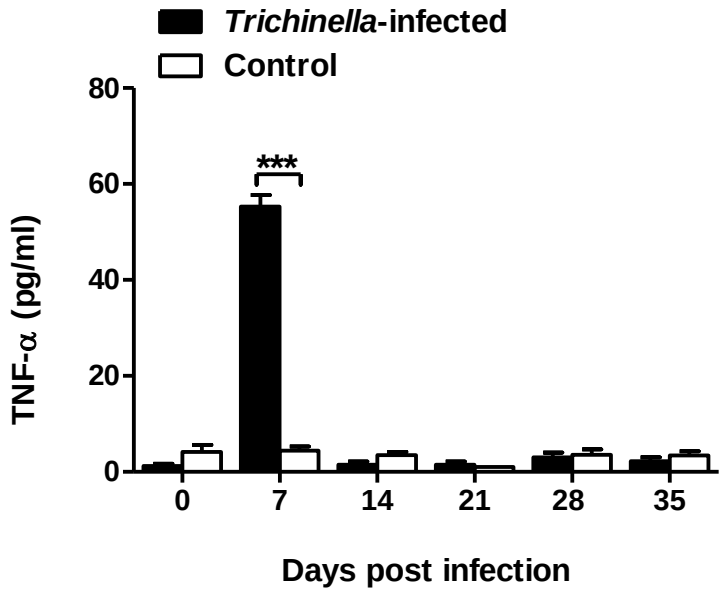

Figure 4.2: Tumour necrosis factor (TNF-α) concentrations during chronic *Trichinella zimbabwensis* infection in mice. The concentrations are means for each group presented as mean ± standard error (SE) (n=6); Level of significance *** $p < 0.001$.

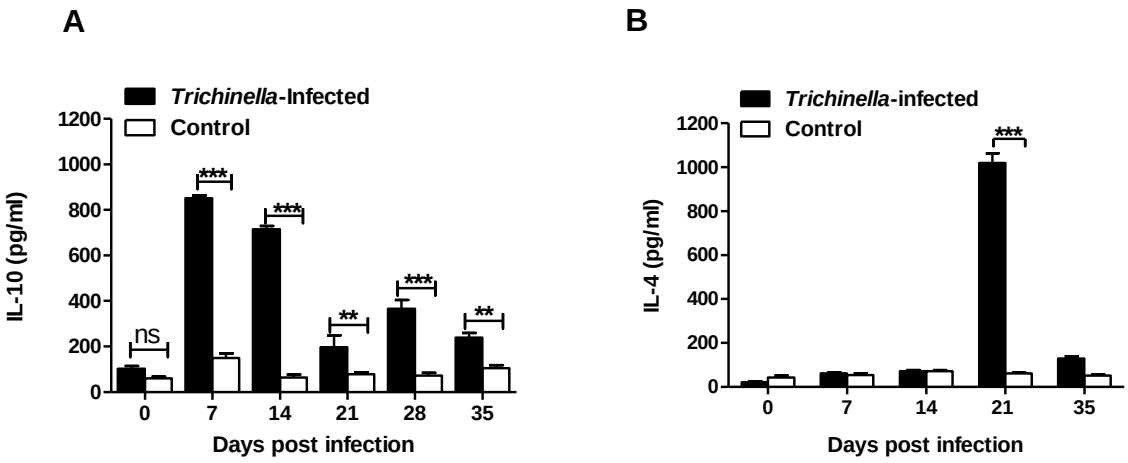

Figure 4.3: Interleukin- 10 (A) and IL-4 (B) concentrations during chronic *Trichinella zimbabwensis* infection in mice. The concentrations are means for each group presented as mean ± standard error (SE) (n=6); Level of significance * $p < 0.01$ and *** $p < 0.001$.

4.5 Discussion

Our findings show that during the enteric phase, *Trichinella*-specific IgM, IgG, and IgG1 antibodies are secreted against adult *T. zimbabwensis*. This may indicate that the mucosal derived antibodies diffuse into systemic circulation or are sequentially secreted in systemic circulation. On the other hand, the systemic response may also be an immunological response to parasite secretory and/or excretory antigens. In *T. spiralis*-infected pigs, intestinal mucosa acts as first line of natural innate defense releasing parasite specific antibodies (Picherot *et al.,* 2007). In our study, we observed that the levels of parasite specific antibodies were elevated with Th2 predominance during the course of infection. We further observed that the levels of TNF-α, a pro-inflammatory cytokine were significantly elevated indicating that the host may be inducing immunoregulatory mechanisms to mediate parasite killing, worm expulsion, abrogate inflammation due to intestinal penetration and initiate tissue healing (Artis *et al.*, 1999; Maizels *et al.*, 2009).

Our previous findings showed elevated levels of IFN-γ and IL-17 cytokine in the enteric phase, an indication of immuno-inflammatory response during infection (Onkoba *et al.,* 2015). In this study, we also observed elevated levels of TNF-α in the enteric phase an indication that the parasite mediates secretion of anti-inflammatory cytokine, IL-4 that down regulate production of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-17) (Wakelin *et al.*, 1994; Wu *et al.,* 2010).

The Levels of IL-10 were significantly elevated as the ML enters lymphatic and systemic circulation. The significant production of IL-10 indicates that the parasite may be immuno-modulating host immune system to down regulate Th-1 immune responses (Harnett, 2014). On the other hand, the *Trichinella* parasite may interfere with dendritic cell maturation via IL-10 secretion (Ashour, 2013). Our findings show that *Trichinella* parasite does not fully manage to shut off the effects of innate effector cells evident by presence of initial humoral response that co-exists with cell mediated immune response. Therefore, based on our observations *T. zimbabwensis* parasite does not seem to induce solely either Th-1 or Th-2 immune responses. Although, it has been reported that soil-transmitted helminths (STHs) infections induce strong Th-2 skewed immune responses for their establishment (Yazdanbakhsh *et al*., 2001; Maizels *et al*., 2012) we have demonstrate that *T. zimbabwensis* induces a mixed Th-1 and Th-2 immune responses.

We observed an elevation in IgG1 and IgG3 cytophilic antibodies with decline in the levels of IgM and IgG1 antibodies as well as significant IgG3 antibody elevation. Therefore, the results showed that migration and presence of ML in their predilection site is associated with high levels of *Trichinella*- specific IgM, IgG, IgG1 and IgG3 antibodies and regulatory cytokine (IL-10). This may be because of the systemic inflammation induced during migration. However, the resolution of muscular inflammation due to ML was reported to be dependent on how successful enteric phase immunoregulation was (Helmby & Grencis, 2003). We further observed as the number of ML increased in striated muscles, the levels of Th-1 and Th-2 cytokines dropped which indicated that the parasite was successful in initiating immunomodulation for its establishment.

We have shown, for the first time that *T. zimbabwensis* infection induces mixed Th-1 and Th-2 immune responses in mice during its migratory phase. However, different short-lived immune responses are elicited in the intestinal mucosa, body compartments, and systemic responses during its migratory pattern. Further studies to establish the actual immune cells involved in anti- *T. zimbabwensis* immunity, immunomodulation and parasitism are indicated. The study findings contribute to new knowledge on the host-immune response against non-encapsulated *T. zimbabwensis* infection insights for development of vaccines and immunotherapies for disease control and management.

4.6 References

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CHAPTER 5

SERUM CYTOKINES, ANTI-*TRICHINELLA* AND ANTI-*PLASMODIUM* ANTIBODY RESPONSES INDUCED IN MICE MONO- AND CO-INFECTED WITH *TRICHINELLA ZIMBABWENSIS* AND *PLASMODIUM BERGHEI* ANKA

In press: Veterinary Parasitology

5.1 Abstract

There is paucity of information on differential immune responses induced by tissue-dwelling helminths during co-infection with malaria. The present study was undertaken to determine cytokines and antibodies responses induced during *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA co-infection and explored the effect of antihelminthic treatment on immunity and malaria disease outcomes. One hundred female BALB/c mice aged 6 to 8 weeks were assigned into five equal groups of 20 mice each; (A) malaria infected, (B) *Trichinella*-mono-infected, (C) *Trichinella*-mono-infected and treated, (D) co-infected and (E) co-infected and treated groups. At day 0 post-infection (dpi), mice in groups B, C, D and E were infected with *T. zimbabwensis*. At 42 dpi, groups C and E and day 0 control groups were treated with fenbendazole three times at 7.5 mg/kg body weight. At 0 dpi group A and at 60 dpi, group D and E were infected with *Plasmodium berghei* ANKA blood stage parasites. Immune responses were determined by measuring circulating levels of IFN-γ, TNF-α, IL- 4 & IL-10 and IgG & IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) responses in serum by ELISA. Malaria disease outcomes were determined by monitoring parasitaemia, survival rates and mortality. Co-infecting *T. zimbabwensis* with *P. berghei* induced mixed Th1/Th2 immune responses that altered survival rate and parasitaemia profiles. We observed that the co-infected mice had prolonged survival and sustained parasitaemia compared with malaria mono-infected group of which all mice died within 5 to 7 dpi. All mice in the co-infected group died by day 13 dpi compared with those of the co-infected-treated group which had a 42.85% survival rate. This showed that chronic *Trichinella* infection and antihelminthic treatment decreased *Plasmodium* parasite load and peak parasitaemia by delaying onset of patent parasitaemia and enhanced survival. The co-infected and treated group had significantly elevated levels of IL-4, IL-10, and TNF-α and parasite-specific IgG, IgG1 IgG2a, IgG2b and IgG3 antibodies compared with the *Trichinella* and malaria mono- and co-infected groups. Co-infection with *T. zimbabwensis* and antihelminthic treatment confers protection against early death and accelerated malaria parasite growth, enhances antibody and cytokine production during mono- and co-infection and ameliorates malaria severity.

5.2 Introduction

Malaria is a parasitic zoonosis characterized by complex immune responses and disease outcomes in the human host (Lyke *et al.,* 2005). *Plasmodium falciparum* and *P. vivax* parasites are the major etiological agents responsible for increased malaria morbidity and mortality in sub-Saharan Africa (SSA) (WHO, 2014). The disease burden is mainly carried by children under 5 years of age and pregnant women in developing countries (Duffy and Fried, 2003; Gonçalves *et al.,* 2014).

Geographical congruency of malaria and soil transmitted helminths (STHs) distributions make co-infections and polyparasitism common phenomenon in SSA (Booth, 2006; Fairlie-Clarke *et al.,* 2010; Malhotra *et al.,* 2011; Midzi *et al.*, 2011; Supali *et al.*, 2010). Several epidemiological and laboratory animal studies have been conducted to elucidate parasite-parasite and/or parasite-host interactions and the findings are inconclusive (Brooker *et al.*, 2012; Degarege *et al.,* 2012). However, there is a general consensus that co-infecting helminths have capability to immuno-modulate host regulatory network to favour their own multiplication and transmission (McSorley and Maizels, 2012) as well as those of third party antigens (Ilic *et al.,* 2012). Studies have shown that STH infections worsen malaria infection (Druilhe *et al.,* 2005; Sokhna *et al.,* 2004) whilst others have actually been shown to ameliorate malaria severity (Nacher *et al.*, 2001) and others suggest that helminths have no influence on malaria disease outcome (Bejon *et al*., 2008; Shapiro *et al.,* 2005). In malaria and STH endemic areas, mass antihelminthic treatment in children is strongly advocated for but its effects in malaria co-infections cases have not been vigorously evaluated (Ndibazza *et al.,* 2010). Mass deworming of pre- and school going children has been shown to have health and societal benefits (Bhoite and Iyer, 2012; Bundy *et al.*, 2013; Fenton, 2013; Hürlimann *et al.,* 2014; Midzi *et al.,* 2011; Mulu *et al.*, 2013; Stoltzfus *et al.,* 1998; Taylor-Robinson *et al*., 2012; Watkins *et al.*, 1996). However, it raises pertinent concerns on its inability to confer protective immunity against re-infections due to continued exposure (Anderson *et al.,* 2013), safety and efficacy, possibility of creating drug resistance if use becomes long term (Parker and Allen, 2011).

Furthermore, mass treatment programmes implemented are aimed at controlling and eliminating STHs. The problem of tissue-dwelling helminths is not addressed by the mass drug administration (MDA). For this reason there is paucity of information on MDA efficacy, safety and immunomodulatory effect on tissue-dwelling helminths like *Trichinella* sp. during mono-and co-infection with major tropical diseases.

Trichinellosis is a food borne zoonosis caused by tissue-dwelling larvae of the genus *Trichinella* (Pozio, 2001). *Trichinella* infection is acquired after ingestion of raw or undercooked meat infected with *Trichinella* larvae (Atterby *et al.,* 2009). The larvae develops into adult worms which mate and deposit new born larvae (NBL) in the small intestines (Thrasher *et al.,* 2013). The NBL penetrates intestinal epithelium to enter vascular and lymphatic circulation where it migrates to its predilection sites (Kapel, 2000; Krivokapich *et al.,* 2012; Pozio, 2013). In the striated muscles, it utilizes its antigenic variants for long term survival and immunomodulation (Gao *et al.,* 2014; Ilic *et al.*, 2012; Mitreva and Jasmer, 2006; Wu *et al.*, 2010). Distinct species exist in different geographical areas (La Rosa and Pozio, 2000; La Rosa *et al.*, 2001; Pozio *et al.*, 2009, 1992). *Trichinella zimbabwensis* is prevalent in Southern Africa and to date, no human infection have been reported despite increasing poverty, animal products trade, hunting, bush meat trade, globalization and surge in number of synanthropic reservoir hosts (Mukaratirwa *et al.*, 2013). Lack of information on the immune responses the parasite stimulates on the host, symptoms, diagnosis and case reporting has complicated its surveillance and control. Therefore there is need for research on parasite-parasite and/or host parasite during mono- and co-infection with malaria, a common tropical disease in SSA.

The present study was undertaken to determine serum cytokine and antibody responses induced in mice mono- and co-infected with *T. zimbabwensis* and *Plasmodium berghei* ANKA and explored the effect of prior anti-helminthic treatment on immunity and malaria disease outcomes.

5.3 Materials and methods

Study animals

Six to eight week old female BALB/c mice kept at the Biomedical Resources Unit (BRU) of the University of KwaZulu-Natal at the Westville campus were used in the experiments. The animals were fed with heat sterilized pelleted food and water was supplied *ad libitum.* All procedures were performed in accordance with South African National Legislation Regulations on laboratory care and use of experimental animals and approved by University of KwaZulu-Natal (UKZN) Animal Ethics Committee (ref. no. 114/13/Animal). The experiments are reported in accordance with ARRIVE guidelines (Kilkenny *et al.*, 2013).

Experimental design

Mice were randomly assigned into five equal groups of 20 mice each; (A) malaria infected, (B) *Trichinella*-infected (C) *Trichinella*-infected and treated (D) Co-infected, and (E) co-infected and treated. At day 0 post infection (dpi), mice in groups B, C, D and E were infected with 500 muscle larvae per mouse through oral lavage as described by Mukaratirwa *et al.*, (2001). At 42 dpi, mice in groups C and E and control group were treated with Fenbendazole three times at 7.5 mg/kg body weight. At day 0 dpi group A and at 60 dpi, group D and E were were inoculated with 1 x 10^5 blood *P. berghei* ANKA parasites according to procedures described by Wang *et al.* (2014). Infected mice were monitored daily for parasitaemia, mortality and survival rates. End points were defined by presentation of the following signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions and coma. Animals with severe malaria were sacrificed using halothane inhalation in line with ethical guidelines (Fig. 5.1).

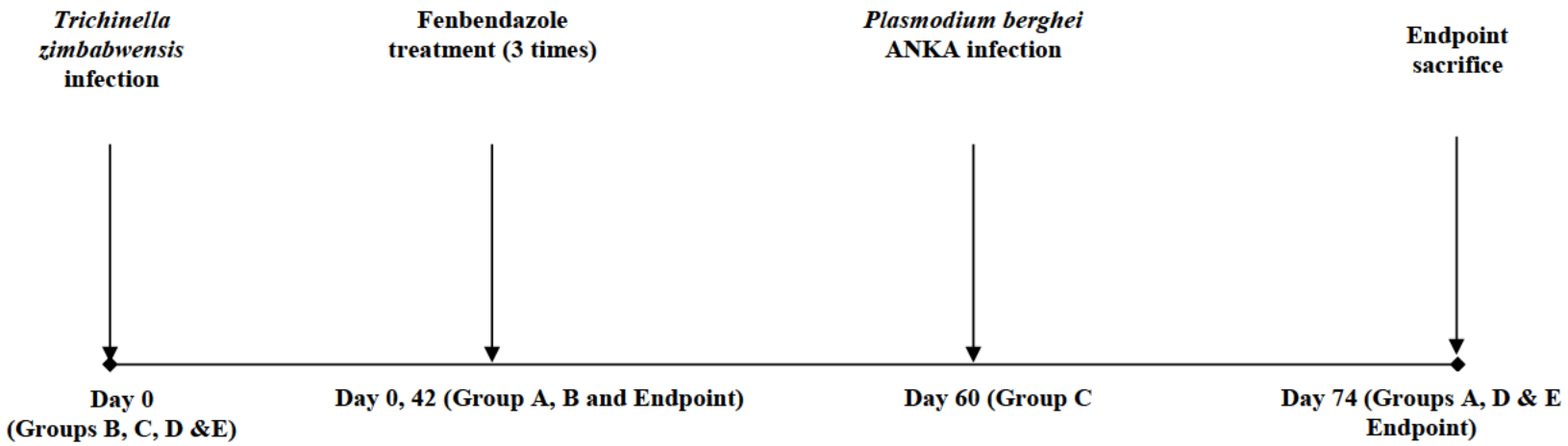

Figure 5.1: Schematic of the experimental design used to determine the cytokine and antibody responses in mice mono- and co-infected with *T. zimbabwensis* and *P. berghei* ANKA. At day 0, mice in groups B, C, D and E were infected with *T. zimbabwensis*. At day 42 post infection (pi), mice in group B were sacrificed; groups C and E were treated three times with fenbendazole at 7.5 mg/kg body weight. At day 60 dpi, mice in group C were sacrificed; groups A, D and E were each inoculated with 1 x 10^5 *P. berghei* ANKA blood stage parasites. After malaria infection, mice were monitored daily for parasitaemia, mortality and survival rates. Horizontal arrows in the schematic show groups A, B, C, D and E while vertical arrows show time points corresponding to days post infection, treatments and sample collection.

Trichinella zimbabwensis infection and treatment

A crocodile-derived *T. zimbabwensis* (Code ISS1209) parasite strain maintained in Sprague-Dawley rats in our laboratory was used in the study. Infective muscle larvae (ML) were obtained from whole carcasses of Sprague-Dawley rats by the standard HCL-pepsin digestion method (Kapel and Gamble, 2000). Each mouse was inoculated with 500 ML *per os* using a lavage needle. Parasite establishment was confirmed by the recovery of adult worms upon necropsy from infected mice intestines at 5 dpi as described by Zocevic *et al.*(2014).

At 42 dpi, mice in group C and E and control group were treated three times with fenbendazole (Panacur® BS 5%, batch number 310005, Intervet, South Africa) at 7.5 mg/kg body weight administered orally.

Plasmodium berghei ANKA infection

At day 0 group A and 60 dpi, group D and E mice were infected with 1 x 10^5 *P. berghei* ANKA blood stage parasites. Prior to infection, cryopreserved *P. berghei* ANKA blood stage parasites were propagated in donor mice to generate infection inoculum. At 10% parasitaemia the donor mice were sacrificed, blood was collected and diluted to give the inoculating dose of 10^5 infected red blood cells (iRBCs) in 100µL per mouse. Malaria infected mice were monitored daily for parasitaemia by Giemsa-stained thin blood smears prepared using tail blood starting at day 3 post-malaria infection. The number of malaria parasites per µL of whole mouse blood was derived from counting the number of iRBCs in at least 10 high power fields.

Ten mice from *P. berghei* ANKA mono- and co-infected groups were used to determine survival rates. Agility, demeanour, appetite, posture, and fur scores were used to determine experimental humane end points according to UKZN animal experimentation standard operating procedures.

Plasmodium berghei parasite antigen preparation

Plasmodium berghei ANKA infected blood was collected from mice and parasitized erythrocytes were obtained by density gradient centrifugation using 60% Percoll solution (Sigma-Aldrich, St. Louis MO). Parasitized erythrocytes collected were washed three times with PBS and presence of white blood cells was determined by Giemsa stained blood smears. Density gradient separation was repeated until white blood cells were removed. The collected parasitized erythrocytes were subjected to four freeze-thaw cycles in the vapour phase of liquid nitrogen. The haemolysed erythrocytes were sonicated by 5 pulses of 30 seconds at 100W and filtered through 0.22 µm. Protein concentration was determined by Bradford assay (Bradford, 1976) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis MO) was added. The antigen was diluted in aliquots of 4.0µg/ml and stored at -80^{o} C until use.

Parasite specific antibody response quantification by ELISA

Circulating parasite specific antibodies were measured in sera by ELISA. Briefly, 96-well Greiner bio-one microplates (Frickenhausen, Germany) were coated with either 6.0µg/ml of *T. zimbabwensis* larvae antigen or 4.0µg/ml of *P. b*erghei ANKA crude antigen in 100 µl bicarbonate buffer (4 mM Na2CO3, 8 mMNaHCO3, pH 9.6) and incubated overnight at 4°C. After blocking with 5% BSA in PBST (containing 0.05% Tween 20), 100-µl volumes of serum dilutions were added to triplicate wells and incubated for 2h at 37° C. Plates were washed with PBST between incubations and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; Santa Cruz Biotechnology, CA, USA) at 1:2,000 dilution. To examine the IgG subclasses of mouse sera, plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Santa Cruz Biotechnology, CA, USA) at a 1:1,000 dilution. Finally, plates were developed with the sureBlue™ TMB peroxidase substrate (Kirkegaard & Perry Laboratories) and absorbance was read at 630 nm using microplate reader (BioTek, VT, USA). Endpoint titres were defined as serum dilutions giving an absorbance higher than the average optical density (OD) at 630 nm of non-infected control serum plus 3 standard deviations (SD).

Quantifications of cytokines

Circulating levels of IFN-γ, IL-4, IL-10, and TNF-α in serum samples were determined using commercially available capture ELISA with antibody capture/detection pairs and standards from (RnD systems, MN, USA) according to the manufacturer's guidelines

Statistical analysis

Statistical analyses were performed in Graph Pad PRISM™ version 5.04 (Graph-pad software, San Diego, USA). Intergroup comparisons were performed with analysis of variance or Kruskal-Wallis test (when samples were not normally distributed). All *p* values less than 0.05 were considered significant.

5.4 Results

Anti- T. zimbabwensis and anti-Plasmodium-specific neutralizing antibody responses

The co-infected group had higher levels of *Trichinella*-specific IgG, IgG1, IgG2a and IgG2b antibodies than the *Trichinella*-infected group (Fig. 5.2) although the differences among the groups were not significant ($p > 0.05$). Overall trends showed that the treated groups had markedly elevated levels of *Trichinella*-specific IgG, IgG1, IgG2a and IgG2b antibodies compared to the *Trichinella*-infected and co-infected groups ($p < 0.001$).

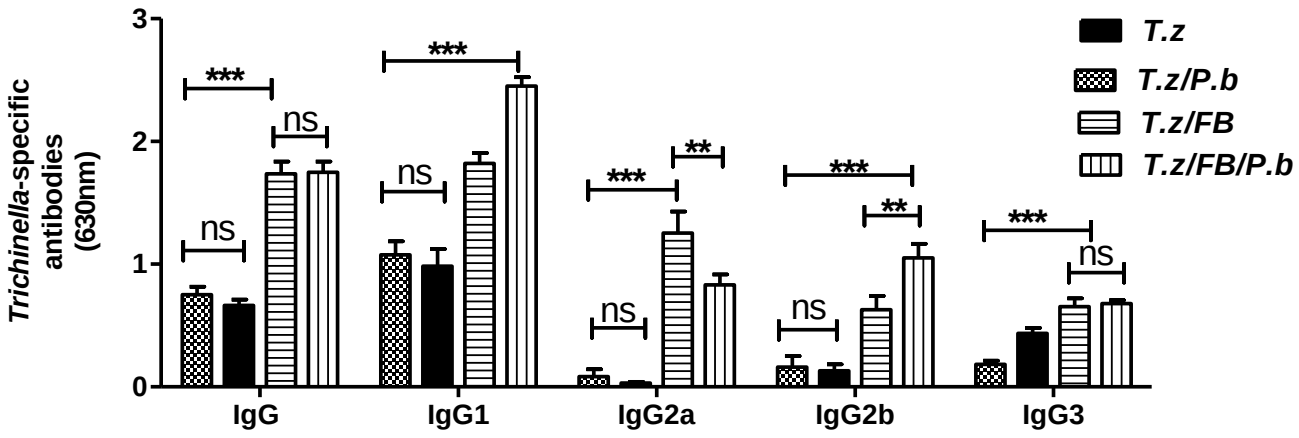

Figure 5.2: End-point anti-*Trichinella*-specific antibody levels of mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei ANKA*. The optical densities for each group are presented as means ± standard error (SE) of antibody levels (n = 10); Levels of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. *T. z = Trichinella zimbabwensis; P. b. = Plasmodium berghei* ANKA; FB = fenbendazole.

As shown in Fig. 5.3, the levels of anti-*Plasmodium*-specific IgG1 ($p < 0.001$) and IgG ($p < 0.01$) antibodies were significantly elevated in the co-infected group compared to the malaria infected group. Generally, the co-infected and treated group had significantly higher levels of *Plasmodium*-specific IgG, IgG1, IgG2a and IgG2b ($p < 0.001$) and IgG3 ($p < 0.05$) antibodies compared to the co-infected group ($p < 0.001$).

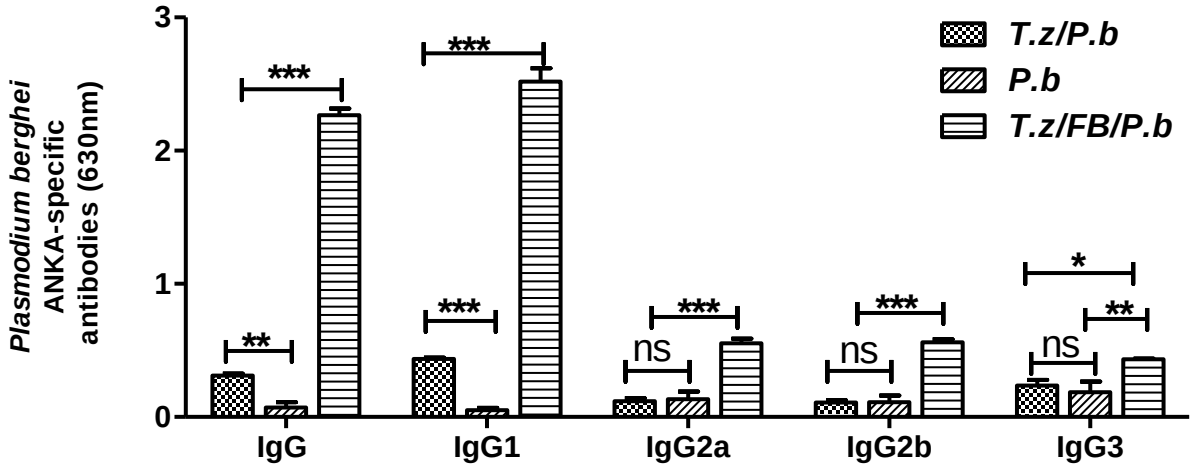

Figure 5.3: End-point anti-*Plasmodium*-specific antibody levels of mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei ANKA*. The optical densities for each group are presented as means ± standard error (SE) of antibody levels (n = 10); Levels of significance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). *T. z = Trichinella zimbabwensis; P. b. = Plasmodium berghei* ANKA; FB = fenbendazole.

Cytokine concentrations

The co-infected group had markedly higher IL-4 ($p < 0.001$), IL-10 ($p < 0.001$) and TNF-α ($p < 0.001$) (Fig. 5.4) levels compared with the malaria infected, *Trichinella*-mono-infected, *Trichinella*-infected and treated and co-infected and treated groups ($P < 0.001$). The levels of IL-4 ($p < 0.01$), IL-10 ($p < 0.001$) and TNF-α ($p < 0.001$) in the malaria infected group were markedly elevated compared with *Trichinella*-infected, and treated *Trichinella*-infected and co-infected groups. The *Trichinella*-infected group had higher levels of IL-4 ($p < 0.05$), IL-10 ($p < 0.001$) and TNF-α ($p < 0.001$) than *Trichinella*-infected and treated and co-infection groups ($p < 0.001$). The levels of IL-10 were elevated in *Trichinella*-infected group compared to the co-infection and treated group ($p < 0.001$). Overall, the co-infected and treated groups had higher cytokine concentrations than the mono-infected groups.

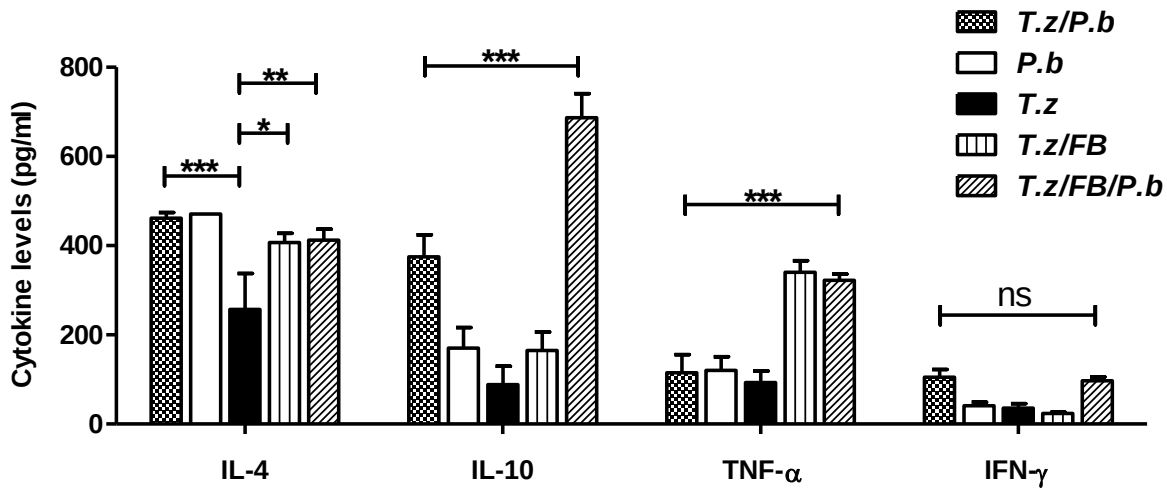

Figure 5.4: End-point levels of IL4, IL-10, TNF-α and IFN-γ cytokines in mice mono- and co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA. The cytokine concentrations for each group are presented as means ± standard error (SE) (n = 10). Levels of significance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). *T. z = Trichinella zimbabwensis; P. b. = Plasmodium berghei* ANKA; FB = fenbendazole.

Parasitaemia and survivorship

Infection of mice with 1 x 10^5 *P. berghei* ANKA parasites resulted in three different infection profiles according to their parasitaemia kinetics and survival rates (Fig. 5.5A & B). A delay in patent parasitaemia was observed in the co-infected and treated group compared with co-infected and malaria infected groups. Peripheral malaria parasites were detected at 5 dpi in the co-infected groups compared to 3 dpi in the malaria infected group. Mice in the malaria infected group showed a rapid-onset of parasitaemia with a peak at day 8 pi and died before day 8pi.The day of death and the slope of parasitaemia increase were significantly different compared to the co-infected and co-infected and treated groups ($p < 0.05$; r = 0.89). Mice in the co-infected group showed a slow increase in parasitaemia, similar to that of the co-infected and treated group, which peaked at 13 dpi with fatal outcome. The trend of parasitaemia increase was significantly different between co-infected and treated and malaria infected groups ($p < 0.0001$; r = 0.97). A 42.85% of mice in the co-infected and treated group survived the infection with low parasitaemia at experimental endpoint (Fig. 5.5A). The surviving mice showed a slow increase in parasitaemia with a peak at 15 dpi ($p < 0.0001$; r = 0.89) (Fig. 5.5A) and infection was fatal between 12 to 15 dpi, when 57.15% of the animals died (Fig. 5.5B).

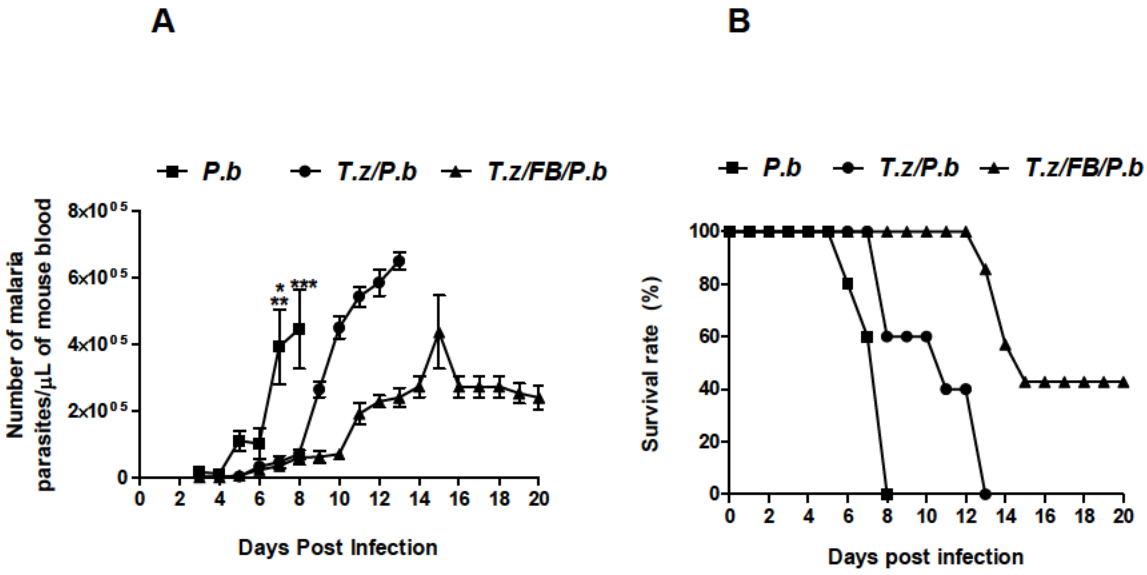

Figure 5.5: Parasitaemia kinetics (A) and survival rate (B) of BALB/c mice mono- and co-infected *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA. The number of parasites per µL of blood and survival rates for each group are presented as means ± standard error (SE) (n = 10) of each group; Levels of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. *T. z = Trichinella zimbabwensis; P. b. = Plasmodium berghei* ANKA; FB = fenbendazole.

5.5. Discussion

This study shows for the first time that immunobiological responses associated with primary *T. zimbabwensis* infection influence the course of malaria infection in mice by induction of protective differential cytokines and neutralizing antibodies. We observed that malaria infection has three different infection outcomes; accelerated parasite growth and early death, sustained parasitaemia and death before day 13 pi and sustained parasitaemia with enhanced survival rate of 42.85%. We observed that early death occurred in the malaria infected group compared with the co-infected group that had sustained parasitaemia

with deaths whilst the co-infected and treated group had sustained parasitaemia and survival. The accelerated parasite growth and early death observed in the malaria infected group may be attributable to lower levels of IFN-γ compared with the co-infected groups. The *Plasmodium* parasite initiates IFN-γ immunosuppression by impairing antigen presentation, dendritic cell maturation and T-regulatory cells induction allowing for parasite multiplication and infection establishment (Normark *et al.,* 2014). Our findings show that the down regulation of IFN-γ observed in the malaria infected group may be associated with infection induced pathogenicity. The findings corroborate with reports from field studies in malaria-endemic areas that have established that IFN-γ is a correlate of protective immunity (McCall and Sauerwein, 2010). However, we observed that reduction in the levels of IFN-γ in the co-infected groups was not pronounced compared with the malaria infected group. This may be because the co-infecting *Trichinella* parasite promotes IFN-γ production which confers protection against accelerated parasite growth and early death. We further observed that the levels of TNF-α were markedly elevated in the co-infected and treated group indicating that prior antihelminthic treatment before malaria co-infection favours production of this pro-inflammatory cytokine. The secreted TNF-α may also be activating macrophages and helping B-cells in producing protective neutralizing antibodies (Wang *et al.*, 2014) or primed immunological action from dead larvae.

The co-infected groups had elevated levels of IL-10 compared with the malaria-mono-infected group. This may be an indication of immunoregulation by the parasite to sustain its establishment and sustained. This suggests that *Trichinella* infection interferes with malaria parasites multiplication by reducing its multiplication which subsequently prolongs the survival of infected mice. Our findings corroborate with those by Moncunill *et al.* (2013) in Mozambican children, adults and European travellers that higher levels of IL-10 are reflective of an immune response towards low parasitaemia. In our previous studies, we showed that *T. zimbabwensis* immunomodulation is characterized by mixed Th1/Th2 responses (Onkoba *et al.*, 2015).

In all the experimental groups, the levels of IL-4 were significantly elevated, indicating that an adaptive Th2 immune response is induced during mono- and co-infection. Interleukin-4 induces B-cells to produce parasite specific neutralizing antibodies and IgG class switching (van Panhuys *et al.*, 2011). This may explain why the treated and co-infected groups had significantly higher levels of parasite-specific antibodies compared with mono-infected groups. This implies that the host controls infections through initiation of rapid innate immunity evident by circulating parasite-specific IgG1, IgG2, IgG2a, IgG2b and IgG3 antibodies in sera. In *Plasmodium* infections, circulating antibodies are known to block merozoite invasion and rosette formation through enhanced opsonization and parasite killing (Wipasa *et al.*, 2011). We have shown that the treated groups had significantly elevated levels of circulating parasite-specific Th1 associated cytophilic antibodies (IgG2a and IgG2b) and Th2 associated non-cytophilic (IgG1 and IgG3b) antibodies. This shows that the co-infecting *T. zimbabwensis* parasite does not fully influence Th2 polarization that is characterized by solely Th2 associated cytokines but mixed Th1/Th2 immune responses, chronic *Trichinella* infection and sustained *Plasmodium* parasitaemia. The elevated levels of cytophilic antibody isotypes (IgG1 and IgG3) during co-infection show that antibody-dependent cell-mediated inhibitions are responsible for control of malaria parasites multiplication and associated symptoms. Our findings agree with those from immuno-epidemiological studies that have established that non-malarial antigens do influence cell-mediated immunity against malaria parasites provided they target cytophilic immunoglobulin G (Jafarshad *et al.*, 2007). However, in human studies, *Plasmodium*-specific antibodies secretion is dependent on chronic parasitaemia (Akpogheneta *et al.*, 2008). Furthermore, cross-reactive antibodies elicited against helminths play a role in immunomodulation of host regulatory systems to ameliorate malaria infection during polyparasitism (Fairlie-Clarke *et al.*, 2010).

In the present study we observed that after anti-helminthic treatment against *T. zimbabwensis* during mono- and co-infection, parasite specific antibody responses and levels of cytokines production were enhanced. This implies that the fenbendazole kills ML and the dead larvae releases excretory and/or secretory products that maintain a continuous

stimulus to the host immune system eliciting immunological responses (Gruden-Movsesijan *et al.*, 2011; Ilic *et al.*, 2012, 2011). Therefore, we deduce that use of fenbendazole as antihelminthic drug has an immunomodulatory effect on parasite-specific antibody production against *T. zimbabwensis* mono- and co-infection with malaria.

We have established that co-infection of *T. zimbabwensis* with *P. berghei* and antihelminthic treatment confers protection against early death from malaria, suppresses *P. berghei* multiplication, enhances antibody and cytokine production and ameliorates malaria severity. The results are an addition to knowledge vital in understanding underlying mechanisms of immunoregulation of malaria, co-infection with tissue-dwelling helminths and protective effect of anti-helminthic treatment on immunity and malaria severity. The results obtained show that mass deworming is useful and should be incorporated as a means of integrated control and disease management strategies in malaria endemic areas.

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General discussion

This chapter focuses on *T. zimbabwensis* infection establishment, metabolic and differential immune responses during primary infection, secondly, the antibody and cytokine responses in mice mono- and co-infected with *T. zimbabwensis* and *P. berghei* ANKA and lastly, the effects of antihelminthic treatment on immunity and malaria disease outcomes. The relevance of the results is discussed in relation to parasite-parasite and/or host-parasite interactions in mono- and co-infection with *T. zimbabwensis* and *P. berghei* ANKA.

6.2 Primary *T. zimbabwensis* infection

6.2.1 Parasite establishment

Five hundred *T. zimbabwensis* ML were used to induce a chronic infection in BALB/c mice that were studied. Confirmation of *T. zimbabwensis* infection was achieved through determining adult worm burden in the small intestines between days 5 to 7 pi. The numbers of gravid female worms obtained corresponded with severity of the infection where animals with large numbers of adult worms succumbed before day 10 pi due to resultant enteric inflammatory reactions. *T. zimbabwensis* adult worms expulsion was delayed for 21dpi compared to *T. papuae*, *T. spiralis* and *T. pseudospiralis* expulsion that occur in 7 to 14 pi in mice (Bell *et al.*, 1979; Sadaow *et al*., 2013). The delay in expulsion may also be attributed to relocation of female worm further down the intestinal tract or depletion of mucin cells (Despommier *et al.*, 2005). This is corroborated by Hasnain *et al*. (2011) who reported that worm expulsion was delayed in mucin depleted mice infected with either *T. spiralis* or *Nippostrongylus brasiliensis*. The ML use their sword-like stylets to create entry holes into vascularized tissues and organs causing intestinal and systemic inflammation (Despommier *et al.*, 2005) reaching their predilection sites between 21 to 28 dpi *(Chapter 3).*

6.2.2 Host feeding

Intestinal inflammation alters host feeding patterns causing hypophagic responses that result in reduced food and water intake accompanied with weight loss. The actual mechanism behind this phenomenon is not clear. However, it has been established by Worthington *et al*. (2013) that in *T. spiralis*-infected mice the resultant hypophagia is due to upregulation of cholecystokinin (CCK) hormone (McDermott *et al.*, 2006; Worthington *et al.*, 2013). Increase of nutrients in the small intestines due to delayed absorption may have triggered control of feeding centres in the brain to limit food ingestion, satiety and delayed gastric emptying (Rigaud *et al*., 1994; Schonhoff *et al*., 2004). Weight loss that may be due to increased nutrients loss in faecal droppings or increased energy expenditure by adult worms and ML (Montgomery *et al*., 2003) or the reduced food intake was observed. In *T. spiralis*-infected mice, weight loss during enteritis may be attributed to reduced levels of Th1 pro-inflammatory adipokine leptin during induction of Th2 immune polarization (Worthington *et al*., 2013).

6.2.3 Metabolic responses

Trichinella zimbabwensis ML and gravid worms require nutrients and energy for their migration, metabolism and reproduction (Montgomery *et al*., 2003; Wu *et al*., 2009); thus they cause alteration of the levels of blood glucose and serum insulin. Therefore, the metabolic alterations observed may indicate that *T. zimbabwensis* ML exploit host metabolic pathways by influencing expression of insulin signalling factors either by upregulating genes involved in insulin pathway signalling but not through directly increasing levels of insulin *per se* (Wu *et al*., 2009). The changes were short lived showing that pancreatobiliary secretomotor functions restored after intestinal inflammation has been abrogation. Increased glucose uptake by infected muscles is achieved through inhibition of glucose metabolism, glycogenolysis and controlled feeding behaviour and energy expenditure (Szanto and Kahn, 2000) resulting in hypoglycaemia. Tissue glucose intake is

regulated by insulin dependent glucose transporter proteins (GLUTs 1 and 4) (Abel *et al*., 2001). However, there is no information on the role of *T. zimbabwensis* parasites on GLUTs regulation and glucose uptake by infected striated muscles. In response to these metabolic changes the host instigates compensatory feeding that result in weight gain.

6.2.4 Differential immune responses

During the enteric phase, systemic anti-*Trichinella*-specific neutralizing antibodies were elevated indicating that immune responses are either mucosal-derived that diffuse into systemic circulation or secretion is sequential with systemic circulation. In addition, the systemic response may be due to immunological response towards somatic, excretory and/or excretory antigens of *T. zimbabwensis* ML or adult worms. However, in *T. spiralis*-infected pigs, it has been established by Picherot *et al.* (2007) that the intestinal mucosa acts as first line of natural innate defense by releasing parasite specific antibodies against ML and adult worms. This corroborates the elevated levels of pro-inflammatory cytokines, TNF-α, IFN-γ and IL-17 which correlated induction of tissue-mediated innate immunity to mediate parasite killing, worm expulsion, abrogate inflammation due to intestinal penetration and initiate tissue healing (Artis *et al*., 1999; Maizels *et al*., 2009). However, the pro-inflammatory cytokine secretion was short-lived indicating that the secretion of Th2 associated cytokine, IL-4 limits their secretion (Brunet *et al*., 1997; Wakelin *et al*., 1994; Wu *et al*., 2010). The sources of IL-17 secretion during *T. zimbabwensis* infection are debatable because it is secreted by a variety of cells like innate lymphoid and mucosal cells and γδT cells *(Chapter 4).*

Migrating *T. zimbabwensis* larvae induce mixed Th1/Th2 immune responses characterized by cytophilic antibodies and IL-4 cytokine (Ashour, 2013; Harnett, 2014). This shows that they are able to induce clear-cut immunomodulation favouring Th2 polarization. On the contrary, gastrointestinal helminths induce a strong Th2 skewed immune responses for their establishment (Maizels *et al*., 2012; Yazdanbakhsh *et al*., 2001). However, these results are inconclusive because ML induces an array of short-lived

immune response in different body cavities and compartments. Therefore, further research to elucidate Th1/Th2/Th17 immune responses differentiation and signalling are indicated.

6.3 *Trichinella zimbabwensis* and *P. berghei* ANKA Co-infection

Concurrent co-infection with malaria during chronic *T. zimbabwensis* infections confers protective immunity against fulminating malaria parasite growth and early death. This was attributed to the comparable levels of IFN-γ during co-infection that were correlated to protective immunity (McCall and Sauerwein, 2010). In *Plasmodium* infections, the parasite initiates IFN-γ immunosuppression allowing for their multiplication and establishment (Normark *et al*., 2014). The elevated levels of IL-10 indicated that co-infecting *Trichinella* parasite-driven immunomodulation influenced malaria disease profile. The study showed that chronic *Trichinella* infection interferes with the rate of malaria parasites multiplication and subsequently prolongs survival of infected mice. This findings corroborate with those by Moncunill *et al.* (2013) who established that high levels of IL-10 in Mozambican children, adults and European travellers were indicative of low parasitaemia *(Chapter 5).*

The increased levels of circulating anti-*Plasmodium*-specific neutralizing antibodies implied that the host controlled the infections via initiation of rapid innate immunity. In humans and animal studies, anti-*Plasmodium* antibodies have been established to block merozoite invasion and rosette formation and they enhance complement fixation, opsonization and parasite killing (Wipasa *et al*., 2011). The mixed Th1/Th2 induced by *Trichinella* parasite influences antibody-dependent cell-mediated inhibition of malaria parasites multiplication (Akpogheneta *et al*., 2008). Our study findings agree with those from immuno-epidemiological studies showing that non-malarial antigens also influence cell-mediated immunity against malaria parasites provided they target cytophilic IgGs (Jafarshad *et al*., 2007) and production of cross-reactive antibodies (Fairlie-Clarke *et al.*, 2010).

6.4 Anti-helminthic treatment

Anti-helminthic treatment against *T. zimbabwensis* during mono-infection and prior to concurrent *P. berghei* co-infection improves anti-*Trichinella*-specific, anti-*Plasmodium*-specific antibodies and cytokines production. This implies that Fenbendazole drug used kills ML and the dead larvae act as an immunomodulator by releasing somatic or excretory and/or secretory products that are processed priming the host immune system mediate production of neutralizing antibodies and cytokines (Gruden-Movsesijan *et al*., 2011; Ilic *et al.*, 2012, 2011). The immune responses induced were able to sustain malaria parasite growth and conferring protective immunity against delayed death to survival rate of 42.85%. This may be attributed to elevation in the levels of TNF-α which is probably involved in macrophages activation, antibody production (Wang *et al.*, 2014) and IFN-γ immunosuppression. In addition, the improvement in IL-4 production may have also induced B cell to enhance production of neutralizing antibodies and IgG class switching (van Panhuys *et al*., 2011). Therefore, anti-helminthic treatment against *T. zimbabwensis* alters differential immune responses and ameliorates malaria during co-infections. In order, to elucidate the actual mechanism employed in immunomodulation and amelioration further studies should be undertaken *(Chapter 5)*.

6.5 Conclusion

The study highlights that primary *T. zimbabwensis* infection is characterized by mixed Th1/Th2/Th17 and T-regulatory immune responses that abrogates immuno-inflammatory reactions and are essential for establishment of parasitism and immune evasion during tissue migratory patterns. The *Trichinella* parasite alters host metabolic responses resulting into hypophagia, weight loss and compensatory feeding patterns that are accompanied with weight gain. The study results indicated that during co-infection with *P. berghei* ANKA the *Trichinella* driven immunomodulation regulates *Plasmodium* parasite multiplication and sustains parasitaemia with death at 13dpi. Antihelminthic treatment

against *T. zimbabwensis* parasite enhances production of pro-inflammatory and anti-inflammatory cytokines and parasite specific neutralizing antibodies. Fenbendazole acts as an immunomodulator that confers protective immunity against early death and severe malaria and suppresses parasite growth resulting in amelioration of malaria disease severity with 42.85% survival rates. These findings provides new knowledge vital in the understanding of underlying mechanisms of *Trichinella* driven immunoregulation, malaria parasites co-infection with tissue-dwelling parasites and immunomodulative and protective effect of antihelminthic treatment against helminths on immunity and malaria disease outcomes. The conclusions drawn here are purely based on the results obtained and may vary depending on animal models, mice strains, *Trichinella* sp. and *Plasmodium* parasite strains as well as experimental design.

6.6 Recommendations

The study did not conclusively establish all the underlying mechanism elicited during the infections, therefore, it recommends for that further research be carried out utilising the baseline information generated to provide information on the actual innate and adaptive immune responses through immune cells immune-typing and profiling. A detailed parasite migration map is needed to understand the actual routes utilised by ML larvae. This is possible through the use of radiolabelled larvae that can be monitored throughout the course of infection. Bioinformatics, metabolomics and molecular tools are paramount is bridging the research gap available by providing new information on mechanisms *Trichinella* sp. parasites use in altering host glucose metabolism, initiating immunomodulation and conferment of protective immunity against co-infecting parasites.

6.7 References

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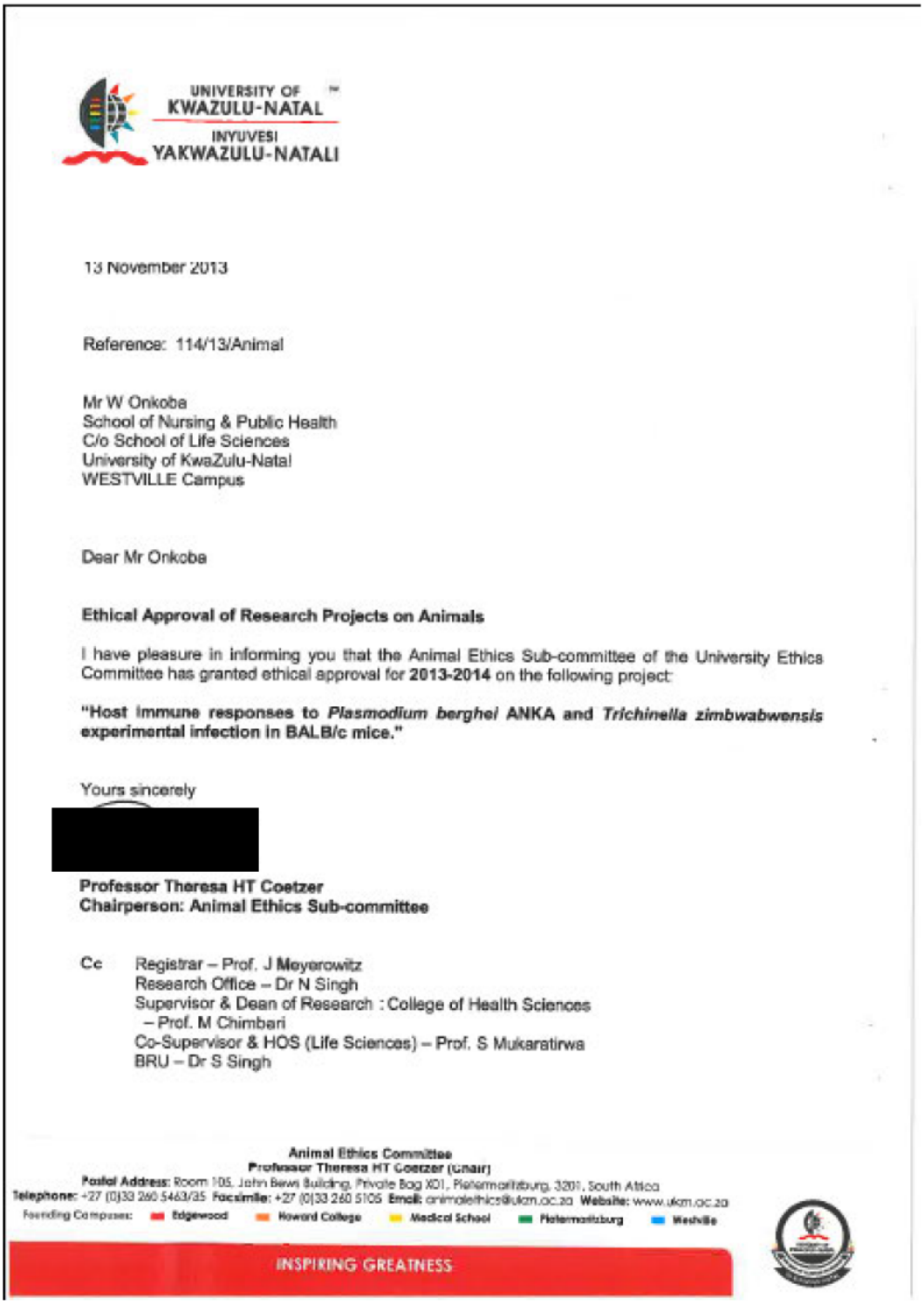
LIST OF APPENDICES

Appendix A1: Ethical approval of project on animals



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 November 2013

Reference: 114/13/Animal

Mr W Onkoba
School of Nursing & Public Health
C/o School of Life Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Onkoba

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for **2013-2014** on the following project:

"Host immune responses to *Plasmodium berghei* ANKA and *Trichinella zimbwabwensis* experimental infection in BALB/c mice."

Yours sincerely

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor & Dean of Research : College of Health Sciences – Prof. M Chimbari
Co-Supervisor & HOS (Life Sciences) – Prof. S Mukaratirwa
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix A2: Postgraduate PhD protocol approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 May 2014

Mr WN Onkoba
Student No 213574350
Discipline of Public Health
School of Nursing & Public Health

Dear Mr Onkoba

PhD PROTOCOL:" *"Host immune responses to Palsmodium berghei ANKA and trichinella Zimbabwensis experimental infection in BALAB/c mice"* ***WN Onkoba*** Student No 213574350

Your protocol has been given final approval of the abovementioned study, on the 12th May 2014. This will be noted at the next Postgraduate and Research & Higher Degrees Committee Meeting.

Please note:

- The Postgraduate Committee must review any changes made to this study.

May I take this opportunity to wish you every success with the study.

Yours sincerely

[redacted]

Mrs Devi Arumugam
School of Nursing & Public Health

CC. Discipline of Public Health Medicine

Postgraduate Administration
School of Nursing and Public Health
University of KwaZulu-Natal
Postal Address: University of KZN, Durban, 4041, South Africa
Telephone: +27 (0) 31 260 2499
Facsimile: +27 (0) 31 260 1543

Founding Campuses:
Edgewood
Howard College
Medical School
Pietermaritzburg
Westville

Appendix A3: Manuscript one submission confirmation

Journal of Helminthology

Metabolic and adaptive immune responses induced in mice infected with tissue-dwelling nematode Trichinella zimbabwensis

--Manuscript Draft--

Manuscript Number:	
Full Title:	Metabolic and adaptive immune responses induced in mice infected with tissue-dwelling nematode Trichinella zimbabwensis
Article Type:	Research article
Corresponding Author:	Nyamongo Wycliffe Onkoba, MSc. Institute of Primate Research Nairobi, Kenya KENYA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Institute of Primate Research
Corresponding Author's Secondary Institution:	
First Author:	Nyamongo Wycliffe Onkoba, MSc.
First Author Secondary Information:	
Order of Authors:	Nyamongo Wycliffe Onkoba, MSc.
	Joseph M Kamau, PhD
	Moses J. Chimbari, PhD
	Samson Mukaratirwa, PhD
Order of Authors Secondary Information:	
Abstract:	Tissue-dwelling helminths are known to induce intestinal and systemic inflammation accompanied with host compensatory mechanisms to counter balance nutritional and metabolic deficiencies. The metabolic and immune responses of the host depend on parasite species and tissues affected by the parasite. The present study investigated metabolic and immuno-inflammatory responses of mice infected with tissue-dwelling larvae of Trichinella zimbabwensis and explored the relationship between infection, insulin signaling pathways and Th17 immune responses. A crocodile-derived T. zimbabwensis strain (Code ISS1209) was used in the study. Sixty female BALB/c mice aged between 6 to 8 weeks old were randomly assigned into two groups; T. zimbabwensis-infection group (n = 30) and the non-infected control group (n = 30). Levels of Th1 (interferon-γ) and Th17 (interleukin-17) cytokines, insulin and blood glucose were determined as well as measurements of body weight, food and water intake. Results showed that during the enteric phase of infection, insulin and IFN-γ levels were significantly higher ($P < 0.001$) Trichinella infection group accompanied with hypophagia and weight loss compared with the non-infected control group. During systemic larval migration, food and water intake were significantly altered ($P < 0.001$) and this was attributed to compensatory feeding resulting in weight gain, reduced insulin levels and increased IL-17 levels. Larval migration also induced a Th1/Th17 derived inflammatory response. It was concluded that T. zimbabwensis alters insulin signaling pathways instigating host compensatory feeding. Furthermore, we showed for the first time that non-encapsulated T. zimbabwensis parasite immunomodulate host Th1/Th17 responses during chronic infection. Keywords: Trichinella zimbabwensis, Enteric phase, Insulin, Larval migration, immune responses, Th1 and Th17

Appendix A4: Publication *(Reproduced with permission from the publisher)*

Onkoba *et al. Infectious Diseases of Poverty* (2015) 4:35
DOI 10.1186/s40249-015-0070-0

INFECTIOUS DISEASES OF POVERTY

SCOPING REVIEW Open Access

Malaria endemicity and co-infection with tissue-dwelling parasites in Sub-Saharan Africa: a review

Nyamongo W. Onkoba[1,2*], Moses J. Chimbari[1] and Samson Mukaratirwa[3]

Abstract

Mechanisms and outcomes of host-parasite interactions during malaria co-infections with gastrointestinal helminths are reasonably understood. In contrast, very little is known about such mechanisms in cases of malaria co-infections with tissue-dwelling parasites. This is lack of knowledge is exacerbated by misdiagnosis, lack of pathognomonic clinical signs and the chronic nature of tissue-dwelling helminthic infections. A good understanding of the implications of tissue-dwelling parasitic co-infections with malaria will contribute towards the improvement of the control and management of such co-infections in endemic areas. This review summarises and discusses current information available and gaps in research on malaria co-infection with gastro-intestinal helminths and tissue-dwelling parasites with emphasis on helminthic infections, in terms of the effects of migrating larval stages and intra and extracellular localisations of protozoan parasites and helminths in organs, tissues, and vascular and lymphatic circulations.

Keywords: Malaria, Tissue-dwelling helminths, Zoonosis, Co-infection, Immunomodulation

Multilingual abstracts

Please see Additional file 1 for translations of the abstract into the six official working languages of the United Nations.

Introduction

Malaria is a deadly infectious disease and one of the main health problems facing developing countries in Sub-Saharan Africa (SSA) and Asia. Globally, 3.4 billion people are at risk of new malaria infections, and there are around one million deaths annually [1–3]. *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* parasites infect humans under normal conditions [4] with *P. falciparum* and *P. vivax* being the major species that cause morbidity and mortality in children under five years of age, pregnant women and travellers from non-malarious areas [5, 6].

* Correspondence: bwonkoba@gmail.com
[1]College of Health Sciences, School of Nursing and Public Health, University of KwaZulu-Natal, Howard Campus, Durban, South Africa
[2]Departmet of Tropical Infectious Diseases, Institute of Primate Research, Karen, Nairobi, Kenya
Full list of author information is available at the end of the article

In SSA, morbidity and mortality due to malaria is decreasing despite a lack of a malaria vaccine, emergence of parasite resistance to available anti-malarial drugs, the anopheline mosquito being resistant to insecticide residual spraying and a poor socio-economic situation that hinders malaria control and management [7–9]. Efforts in drug discovery and vaccine development are hindered by limited knowledge of the underlying cellular and molecular mechanisms of host-parasite interactions during co-infection and polyparasitism [10, 11]. This is also aggravated by the emergence of zoonotic *P. knowlesi* malaria infections [12–14] as well as other zoonotic infectious diseases [15, 16]. Trichinellosis is an emerging and re-emerging zoonotic disease the geographical distribution of which overlaps with malaria in endemic areas of Tanzania, Uganda, Kenya, Ethiopia, Zimbabwe, South Africa and Mozambique [17–23]. The development of vaccines against parasitic infections has been complicated due to the fact that co-infecting parasites have life cycles that are either direct or complex. Direct life cycles involve cycling of mature parasites from one definitive host to another while complex life cycles involve cycling of distinct developing life stages through a number of intermediate hosts [24]. Parasite cycling within

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Appendix A5: Publication *(Reproduced with permission from the publisher)*

Journal of Helminthology, page 1 of 8
doi:10.1017/S0022149X15000723
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Differential immune responses in mice infected with the tissue-dwelling nematode *Trichinella zimbabwensis*

W.N. Onkoba[1,2]*, M.J. Chimbari[1], J.M. Kamau[2,3,4] and S. Mukaratirwa[4]

[1]College of Health Sciences, School of Nursing and Public Health, University of KwaZulu-Natal, Howard Campus, Durban, South Africa: [2]Tropical Infectious Diseases, Institute of Primate Research, Karen, Nairobi, Kenya: [3]School of Medicine, Department of Biochemistry, University of Nairobi, Kenya: [4]School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban, South Africa

(Received 24 April 2015; Accepted 21 July 2015)

Abstract

To improve diagnostic tools, immunotherapies and vaccine development for trichinellosis surveillance and control there is a need to understand the host immune responses induced during infection with *Trichinella zimbabwensis*, a tissue-dwelling nematode. In this study, we sought to determine immune responses induced in mice during *T. zimbabwensis* infection. The parasite strain used (Code ISS1209) was derived from a naturally infected crocodile (*Crocodylus niloticus*) and is the main *Trichinella* species prevalent in southern Africa. Sixty 6- to 8-week-old female BALB/c mice were randomly assigned to two equal groups: *T. zimbabwensis*-infected ($n = 30$) and the non-infected control group ($n = 30$). Levels of serum tumour necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), interleukin-4 (IL-4) as well as parasite-specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibody responses were determined using enzyme-linked immunosorbent assay (ELISA). The cytokines and antibodies provided information on T-helper 1 (Th1)- and Th2-type, T-regulatory and antibody responses. Results showed that during the intestinal stage of infection, higher levels of parasite-specific IgM, IgG, IgG1 ($P < 0.05$) and IL-10 and TNF-α ($P < 0.001$) were observed in the *Trichinella*-infected group compared with the non-infected control group. In the parasite establishment and tissue migration phases, levels of IgG1 and IgG3 were elevated ($P < 0.001$), while those of IgM ($P < 0.01$) declined on days 21 and 35 post infection (pi) compared to the enteric phase. Our findings show that distinct differences in Th1- and Th2-type and T-regulatory responses are induced during the intestinal, tissue migration and larval establishment stages of *T. zimbabwensis* infection.

Introduction

Trichinellosis is a cosmopolitan foodborne zoonosis caused by a parasitic tissue-dwelling nematode of the genus *Trichinella* (Murrell & Pozio, 2011). The disease has been reported to occur in 66 countries and infects an estimated 11 million people (Dupouy-Camet, 2009; Yang *et al.*, 2010). Scattered outbreaks of human trichinellosis have been reported over time in Asia and Europe (Ranque *et al.*, 2000; Khumjui *et al.*, 2008; European Food Safety Authority, 2011; Murrell & Pozio, 2011).

*E-mail: bwonkoba@gmail.com

Appendix A6: Manuscript four submission confirmations

Parasites & Vectors

Serum cytokines, anti-Trichinella and anti-Plasmodium antibody responses in BALB/c mice mono- and co-infected with Trichinella zimbabwensis and Plasmodium berghei ANKA

--Manuscript Draft--

Manuscript Number:	
Full Title:	Serum cytokines, anti-Trichinella and anti-Plasmodium antibody responses in BALB/c mice mono- and co-infected with Trichinella zimbabwensis and Plasmodium berghei ANKA
Article Type:	Research
Funding Information:	Universityof KwaZulu-Natal (CHS) — Mr. Nyamongo Wycliffe Onkoba
Abstract:	Background There is paucity of information on differential immune responses induced by tissue-dwelling helminths during co-infection with malaria. The present study was undertaken to determine cytokines and antibodies responses induced during Trichinella zimbabwensis and Plasmodium berghei ANKA co-infection and explored the effect of antihelminthic treatment on immunity and malaria disease outcomes. Methods One hundred female six to eight weeks old BALB/c mice were assigned into five equal groups of 20 mice each; (A) malaria infected, (B) Trichinella-mono-infected, (C) Trichinella-mono-infected and treated, (D) co-infected and (E) co-infected and treated groups. At day 0 post-infection (dpi), mice in groups B, C, D and E were infected with T. zimbabwensis. At 42 dpi, groups C and E were treated with fenbendazole three times at 7.5 mg/kg body weight. At 0 dpi group A and at 60 dpi, group D and E were infected with Plasmodium berghei ANKA blood stage parasites. Immune responses were determined by measuring circulating levels of IFN-γ, TNF-α, IL- 4 & IL-10 and IgG & IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) responses in serum by ELISA. Malaria disease outcomes were determined by monitoring parasitaemia, survival rates and mortality. Results Co-infecting T. zimbabwensis with P. berghei induced mixed Th1/Th2 immune responses that altered survival rate and parasitaemia profiles. We observed that the co-infected mice had prolonged survival and sustained parasitaemia compared with malaria mono-infected group of which all mice died within 5 to 7 dpi. All mice in the co-infected group died by day 13 dpi compared with those of the co-infected-treated group which had a 42.85% survival rate. This showed that chronic Trichinella infection and antihelminthic treatment decreased Plasmodium parasite load and peak parasitaemia by delaying onset of patent parasitaemia and enhanced survival. The co-infected and treated group had significantly elevated levels of IL-4, IL-10, and TNF-α and parasite-specific IgG, IgG1 IgG2a, IgG2b and IgG3 antibodies compared with the Trichinella and malaria mono- and co-infected groups. Conclusion Co-infection with T. zimbabwensis and antihelminthic treatment confers protection against early death and accelerated malaria parasite growth, enhances antibody and cytokine production during mono- and co-infection and ameliorates malaria severity.
Corresponding Author:	Nyamongo Wycliffe Onkoba, MSc. Institute of Primate Research Nairobi, Kenya KENYA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Institute of Primate Research
Corresponding Author's Secondary Institution:	
First Author:	Nyamongo Wycliffe Onkoba, MSc.