



Research article

Chemokine, cytokine and haematological profiles in Sprague-Dawley rats co-infected with *Plasmodium berghei* ANKA and *Trichinella zimbabwensis*-A laboratory animal model for malaria and tissue-dwelling nematodes co-infectionPretty Murambiwa^a, Ekuyikeno Silas^a, Yanga Mdleleni^a, Samson Mukaratirwa^{a,b,*}^a School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban, 4000, South Africa^b One Health Center for Zoonoses and Tropical Veterinary Medicine, Ross University School of Veterinary Medicine, Basseterre, Saint Kitts and Nevis

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ABSTRACT

Malaria remains a major cause of mortality and morbidity in sub-Saharan Africa (SSA) and tissue-dwelling helminth parasites (TDHPs) are also prevalent in this region presenting a geographical overlap in endemicity. There is paucity of information on the specific host immune responses elicited at different phases of the life cycle by the co-infecting helminth parasites. This study aimed at using a laboratory animal model to determine selected chemokine, cytokine and hematological profiles in Sprague-Dawley rats co-infected with *Plasmodium berghei* ANKA (Pb) and a tissue-dwelling nematode, *Trichinella zimbabwensis* (Tz). One-hundred-and-sixty-eight male Sprague-Dawley rats (90–150g) were randomly divided into four experimental groups; Control (n = 42), Pb-infected (n = 42), Tz-infected (n = 42) and Pb + Tz-infected group (n = 42). *Trichinella zimbabwensis* infection (3 muscle larvae/g body weight *per os*) was done on day 0 while intra-peritoneal Pb infection (10⁵ parasitised RBCs) was done at day 28 of the 42-day experimental study for the co-infection group which corresponded with day 0 of the Pb group on the protocol. Haematological parameters, cytokines (TNF- α , IL-10, IL-4, IL-6), chemokines (CXCL10, CCL5, CCL11) and burden of Tz adult worms and muscle larvae burden were determined as per need for each group. Results showed that Tz infection predisposed the co-infected animals towards rapid development of Pb parasitaemia during co-infection, reaching a higher peak percentage parasitaemia at day 7 post-infection than the Pb mono-infected group at day 6 post-infection. Animals in the co-infected group also exhibited severe anaemia, basophilia, neutrophilia, eosinophilia and lymphopenia at day 7 post Pb infection compared to the control groups. Significant elevation of Pb parasitaemia coincided with elevated pro-inflammatory cytokine TNF- α ($P < 0.001$), regulatory anti-inflammatory IL-10 ($P < 0.001$), and pro-inflammatory chemokines CXCL10 ($P < 0.001$) concentration in comparison to control group, at day 7 post Pb infection. Our results confirm that co-infection of Pb with Tz resulted in increased Pb parasitaemia compared to the control group in the early stages of infection and this might translate to severe malaria.

1. Introduction

Malaria remains a major global health burden, with an estimated 3.4 billion people at risk of new malaria infections, accounting for a total of 0.5–2.5 million deaths annually (WHO, 2008; WHO, 2014). Sub-Saharan Africa (SSA) is the worst affected as 90% of all malaria related deaths occur in this region (Hotez and Kamath, 2009). On the other hand, tissue dwelling and migrating helminth parasites, such as *Ascaris lumbricoides*, *Taenia solium* cysts, *Echinococcus* spp cysts, filarial worms, *Schistosoma*

spp, *Fasciola* spp and *Trichinella* spp are also prevalent in SSA (Onkoba et al., 2015a).

Undoubtedly, there is considerable geographical overlap in the endemicity of malaria and soil-transmitted helminths (STHs) including tissue-dwelling helminth parasites (TDHPs), making co-infection or multiple infection a common phenomenon (Onkoba et al., 2015a). Trichinellosis is an emerging and re-emerging cosmopolitan zoonotic disease caused by a nematode species of the genus *Trichinella* and *T. zimbabwensis* (Tz) is the most prevalent species in SSA (Mukaratirwa

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et al., 2013; Onkoba et al., 2015a). Multiple factors have been reported which may increase the risk of future human Tz infection in SSA, making the parasite an emerging public health risk (Onkoba et al., 2015b). The disease is caused by ingestion of infective muscle larvae in raw or undercooked meat or meat products (Mukaratirwa et al., 2015). Ingested muscle larvae, develop into adult worms in the small intestines, thereafter releasing newborn larvae (NBL) that migrate to striated muscles at approximately ± 28 days post infection (Onkoba et al., 2015b, 2016).

While *Plasmodium* spp and STHs immuno-pathogenesis are fairly understood and well documented as separate infections, there is currently paucity of information and clarity on the immuno-pathogenic disease mechanisms and clinical malaria outcomes during co-infections (Ateba-ngoia et al., 2014). Divergent data has been generated in recent immuno-epidemiological studies of malaria-STHs co-infections in SSA (Hartgers and Yazdanbakhsh, 2006; Mwangi et al., 2006, Ateba-ngoia et al., 2014). Some studies have demonstrated that malaria-helminth co-infections exacerbates clinical malaria outcomes (Nacher et al., 2002; Sokhna et al., 2004; Sangweme et al., 2010; Degarege et al., 2010; Mboera et al., 2011; Lyke et al., 2012; van-den-Bogaart et al., 2014; Kinung'hi et al., 2014; Getie et al., 2015; Adedaja et al., 2015; Anchang-Kimbi et al., 2017), while some researchers have reported that co-infection ameliorates clinical malaria outcomes (Abay et al., 2013; Mulu et al., 2013; Lemaitre et al., 2014). Interestingly, some studies have shown that helminth-malaria co-infections have no effect on clinical malaria outcomes (Shapiro et al., 2005; Degarege et al., 2009; Abanyie et al., 2013; Noone et al., 2013). Such considerable divergent and conflicting data regarding host immune responses induced during co-infection remains a major challenge to date.

It has also been shown that co-infection may alter the cytokine and chemokine secretion patterns in response to co-infecting parasites antigenic molecules. To avert the currently existing paucity of information, a detailed understanding of host-immune responses during co-infection of malaria and tissue-dwelling helminths is indispensable (Onkoba et al., 2015a). Also, the host hematological, cytokine and chemokine profile invoked during co-infection is unclear. It is against this background that the current study is aimed at using a laboratory animal model to determine chemokine, cytokine and hematological profiles in Sprague-Dawley rats co-infected with Tz and *P. berghei* ANKA (Pb) where Tz represents the tissue-dwelling nematode. Our hypothesis was that co-infection with Pb and Tz in Sprague-Dawley rats alter the haematological, cytokine and chemokine profiles of the host. Interpolating results from the study to field situations in areas where malaria and tissue-dwelling helminths are endemic and overlap is envisaged.

2. Methods

2.1. Animals

Male Sprague-Dawley (SD) rats (90–150g) bred and maintained at the Biomedical Research Unit, University of KwaZulu-Natal, South Africa, were used in the study. Experimental animals were allowed free access to standard rat chow (Meadows feeds, Pietermaritzburg, South Africa) and water *ad libitum*, and maintained under standard laboratory conditions of temperature (22 ± 1 °C), humidity ($55 \pm 5\%$), CO₂ (<5000ppm) and illumination (12-h light/12-h dark cycle).

2.2. Ethical statement

The University of KwaZulu-Natal animal ethics committee reviewed and approved all experimental procedures and protocols in this study under the ethical protocol reference number AREC/018/016 PD.

2.3. Induction of Pb infection

A chloroquine susceptible Pb-ANKA strain donated by Professor Peter Smith from the University of Cape Town, Division of Clinical

Pharmacology, South Africa was used in this study. Prior to the study, the parasite had been propagated in Sprague-Dawley rats for 5 generations and frozen Pb stocks stored in liquid nitrogen. The frozen Pb was processed and stock rats were infected to propagate the parasite (Ademola and Odeniran, 2016). After successful induction of Pb in stock rats, each experimental rat was infected with 10^5 parasitised RBCs via intraperitoneal route, while control animals were administered an equal volume of phosphate buffered saline vehicle via the same route as experimental animals.

2.4. Induction and determination of Tz infection

A crocodile-derived Tz parasite strain (Ref: ISS1209) from the International *Trichinella* Reference center (Rome) maintained and passaged in Sprague-Dawley (SD) for 5 generations in our parasitology laboratory was used to infect male SD stock rats. The infected stock rats were humanely sacrificed at day 28 post-infection using isofoor inhalation in a gas chamber and Tz muscle larvae (ML) were harvested from whole rat carcass following a digestion protocol described previously (Kapel and Gamble, 2000). *Trichinella zimbabwensis* infection was induced in the experimental animals by oral gavage at a dose of 3 ML/g of rat body weight. Determination of Tz adult worms (at day 7 and 14 post-infection) and muscle larvae (at day 28, 35 and 42 post-infection) load was done during the course of the experimental protocol. Adult Tz worms from the intestines were recovered using a standard protocol previously described (Mukaratirwa et al., 2003). *Trichinella zimbabwensis* ML load was determined by digesting the whole carcass using a standard artificial digestion protocol as previously described by Kapel and Gamble (2000) and modified by Mukaratirwa et al. (2003).

2.5. Experimental design

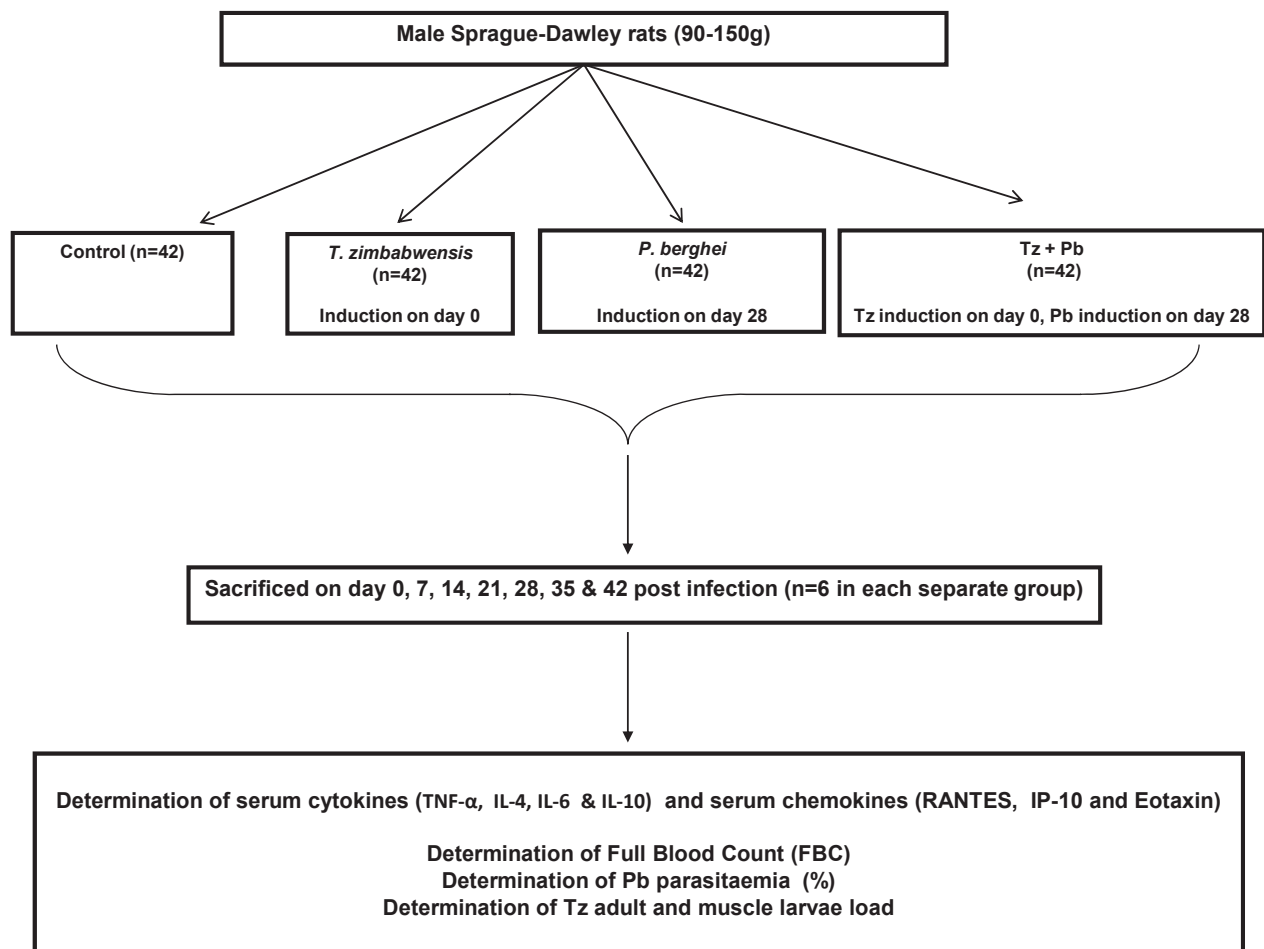
Male Sprague-Dawley rats ($n = 198$) weighing 90–150g were randomly divided into control ($n = 42$), Tz-infected ($n = 42$), Pb-infected ($n = 42$), Pb + Tz-infected ($n = 42$) experimental groups (Figure 1). Percentage parasitaemia was determined by microscopic examination of Giemsa-stained thin blood smears of peripheral tail blood of infected rats. Percentage parasitaemia was measured daily after Pb infection throughout the duration of the experimental protocol according to the formula; % parasitaemia = [(Total number of infected RBC counted/Total number of RBC counted) X 100].

2.6. Terminal studies

Groups of experimental animals were euthanized with CO₂ at day 0, 7, 14, 21, 28, 35 and 42 days post Tz infection and in the co-infected group (Pb + Tz) and Pb mono-infection at day 0 which corresponded with day 28 post Tz infection. Blood for measurement of hematological parameters was collected from animals anesthetized with 2 % isoflurane mixed with 100 % oxygen by cardiac puncture into pre-cooled EDTA tubes, and thereafter animals were euthanized with CO₂. For cytokines and chemokines measurements, blood samples were collected from anesthetized animals by cardiac puncture and sera stored at -70 °C until assayed.

2.7. Determination of hematological profile

Determination of red blood cell (RBC) count, % haematocrit (HCT), white blood cell (WBC) count, neutrophils (NE), basophils (BA), monocytes (MO) and lymphocytes (LY), was done using a calibrated A^C.T. 5diff Beckman coulter counter as per manufacturer specifications. To note is that the coulter counter was calibrated using human standards.



Tz = *Trichinella zimbabwensis*; Pb = *Plasmodium berghei*

Figure 1. Schematic diagram of the experimental design (Control = Not infected, Pb + Tz = *Plasmodium berghei* (Pb) and *Trichinella zimbabwensis* (Tz) co-infection).

2.8. Determination of cytokines and chemokines profile

Measurement of serum cytokines (TNF- α , IL-10 IL-4, IL-6) and serum chemokines (CXCL10, CCL5, CCL11) was done using a commercially available ProcartaPlex Rat Mix and Match, 7 plex immunoassay (invitrogen ThermoFisher scientific, Massachusetts, USA) using magnetic beads as per manufacturer instructions. A calibrated luminex machine (LUMINEX® 100/200™) was used to read the Procarta Plex-Multiplex immunoassay 96 well ELISA plate. The assay upper limit of quantitation (ULOQ) and lower limit of quantitation (LLOQ) for each selected cytokine and chemokine were followed as stipulated in manufacturer's guide.

2.9. Data analysis

Data were expressed as means \pm standard error of means (SEM). GraphPad InStat Software (version 4.00, GraphPad Software, San Diego, CA, USA) was used for statistical comparison of the differences between the means of the experimental groups means with the 95% upper and lower confidence intervals, and/or box blots with the median and the 25% and 75% quartiles. Effects of co-infection (Pb + Tz) on % parasitaemia, adult Tz and ML load between groups was determined using one-way analysis of variance (ANOVA), followed by Turkey-Kramer multiple comparison test. Comparison of co-infection on haematology parameters, cytokine and chemokine levels among experimental groups was done using two-way analysis of variance (ANOVA), followed by

Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of co-infection on Pb parasitaemia

Percentage parasitaemia of the Pb mono-infected group was significantly lower than the % parasitaemia of co-infected group on day 3 ($P < 0.01$) and day 4 ($P < 0.001$) of the experimental period (Figure 2). Peak % parasitaemia of the Pb mono-infected group ($66.8\% \pm 4.6\%$) was reached on day 6 post infection of the experimental period whilst in the co-infected group the peak was at day 7 ($69.2\% \pm 3.80\%$) (35 days post Tz infection of the experimental period) (Figure 2).

3.2. Effect of co-infection on Tz adult worms load and muscle larvae load

Adult worm counts were higher in both experimental groups at day 7 post infection compared to day 14 post infection, while no adult worms were recovered at day 21 post infection (Figure 3). Muscle larvae (ML) counts were relatively higher in the co-infected group at day 42 post infection in comparison to the Tz mono-infected group, although the differences were not statistically significant.

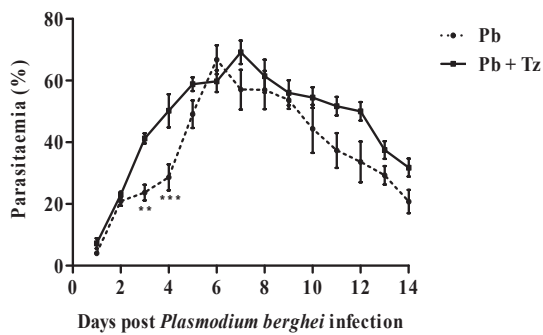


Figure 2. Percentage parasitaemia in male Sprague-Dawley rats infected with *Plasmodium berghei* (only Pb) and co-infected with *P. berghei* and *Trichinella zimbabwensis* (Pb + Tz). Day 0 represents the day of Pb infection when Tz larvae was established in the rat muscle at day 28 post Tz infection. Pb = *Plasmodium berghei*, Tz = *Trichinella zimbabwensis*, Pb + Tz = *P. berghei* and *T. zimbabwensis* co-infection. Values are presented as means and vertical bars indicate standard error of mean (SEM). N = 6 for each group. ** $P < 0.01$, *** = $P < 0.001$.

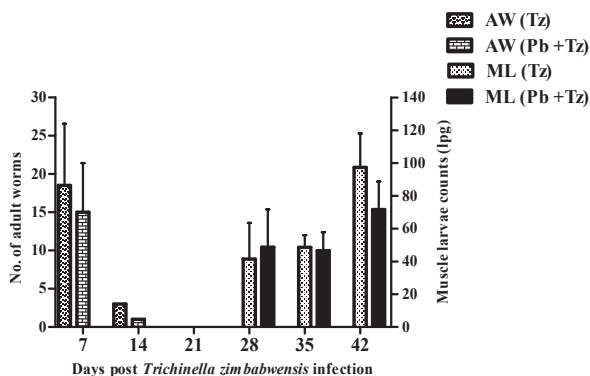


Figure 3. Mean number of intestinal adult worms (AW) and muscle larvae counts (ML) per gram of muscle (lpg) recovered from male Sprague-Dawley rats infected with *Trichinella zimbabwensis* (Tz) only and the group co-infected with *Plasmodium berghei* (Pb + Tz) at day 28 post-infection with Tz when larvae were now established in the rat muscle. Values are presented as means and vertical bars indicate standard error of mean (SEM). AW (Tz) = Adult worms of *T. zimbabwensis* recuperated from a *T. zimbabwensis* infected group, AW (Pb + Tz) = Adult worms of *T. zimbabwensis* recuperated from a *P. berghei* and *T. zimbabwensis* co-infected group, ML (Tz) = Muscle larvae of *T. zimbabwensis* recuperated from a *T. zimbabwensis* group and ML (Pb + Tz) = Muscle larvae of *T. zimbabwensis* recuperated from a *P. berghei* and *T. zimbabwensis* co-infected group. N = 6 for each group.

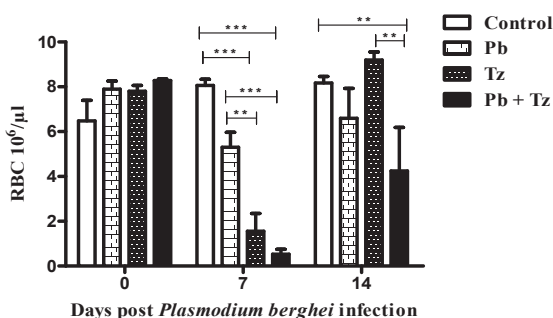


Figure 4. Comparison of the effects of *Plasmodium berghei* (Pb) and *Trichinella zimbabwensis* (Tz) mono-infection and co-infection (Pb + Tz) on RBC concentration in male Sprague-Dawley rats. Day 0 represents the day of Pb infection when Tz larvae were now established in the rat muscle at day 28 post Tz infection. Pb = *Plasmodium berghei* group, Tz = *Trichinella zimbabwensis* group and Pb + Tz = *Plasmodium berghei* and *T. zimbabwensis* co-infected group. Values are presented as means and vertical bars indicate standard error of mean (SEM). N = 6 for each group. ** = $P < 0.01$, *** = $P < 0.001$.

3.3. Effect of Tz and Pb co-infection on haematological parameters

Trichinella zimbabwensis (Tz) mono-infected and co-infected groups had significantly lower RBC levels in comparison to control group at day 7 day post Pb infection ($P < 0.001$) (Figure 4). *Plasmodium berghei* (Pb) mono-infected group had significantly higher RBC count in comparison to Tz mono-infected ($P < 0.01$) and co-infected ($P < 0.001$) groups at day 7 post Pb infection. The co-infected group had significantly lower RBC count in comparison to control ($P < 0.01$) and Tz mono-infected group ($P < 0.001$) at day 14 post Pb infection (Figure 4).

Haematocrit (%) was significantly reduced for Pb mono-infected ($P < 0.05$), Tz mono-infected ($P < 0.001$) and co-infected groups ($P < 0.001$) at day 7 post Pb infection (Figure 5). *Plasmodium berghei* mono-infected group had significantly higher percentage haematocrit in comparison to Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups at day 7 days post Pb infection. However, there were no significant differences for haematocrit (%), 14 days post Pb infection in all experimental groups.

There were no significant differences in the WBC concentration (Table 1) of the Pb mono-infected, Tz mono-infected and co-infected experimental groups with the control group, at day 7 and 14 post Pb infection.

Neutrophils (%) of the co-infected group was significantly elevated ($P < 0.001$) in comparison to control group at day 7 post Pb infection (Table 1). *Plasmodium berghei* mono-infected ($P < 0.001$) and Tz mono-infected ($P < 0.001$) groups had significantly lower percentage neutrophils in comparison to the co-infected group 7 days post Pb infection. However, there were no significant differences in neutrophils (%), at day 14 post Pb infection for all experimental groups.

There were significantly elevated basophils (%) ($P < 0.001$) in Tz mono-infected and co-infected groups compared to control at day 7 post Pb infection (Table 1). Basophils (%) of co-infected group were significantly higher than both Pb mono-infected ($P < 0.001$) and Tz mono-infected ($P < 0.05$) experimental at day 7 post Pb infection. There was a reduction in basophils (%) in all experimental groups at day 14 post Pb infection.

A significant increase in monocytes (%) ($P < 0.001$) was observed in Pb mono-infected group compared to control at day 7 post Pb infection as well as at day 14 post Pb infection (Table 1). Monocytes (%) in Tz mono-infected ($P < 0.05$) and co-infected ($P < 0.01$) groups were significantly lower than the Pb mono-infected at day 7 post Pb infection. Similarly, monocytes (%) in Tz mono-infected ($P < 0.01$) and co-infected ($P < 0.001$) groups were significantly lower than in the Pb mono-infected group at day 14 post Pb infection.

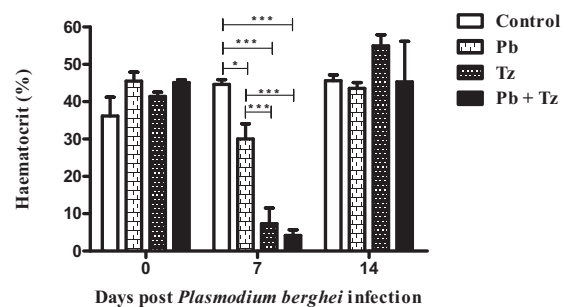


Figure 5. Comparison of the effects of *Plasmodium berghei* (Pb) and *Trichinella zimbabwensis* (Tz) mono-infection and co-infection (Pb + Tz) on haematocrit (%) in male Sprague-Dawley rats. Day 0 represents the day of Pb infection when Tz larvae were now established in the rat muscle at day 28 post Tz infection. Control = Not infected group, Pb = *Plasmodium berghei* infected group, Tz = *Trichinella zimbabwensis* infected group and Pb + Tz = *P. berghei* and *T. zimbabwensis* co-infected group. Values are presented as means and vertical bars indicate standard error of mean (SEM). N = 6 for each group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Table 1. Median values (25%–75% quartiles) of white blood cells concentration and differentials in Sprague-Dawley rats co-infected with *Plasmodium berghei* ANKA (Pb) and *Trichinella zimbabwensis* (Tz) (Pb + Tz), Pb mono-infection and Tz mono-infection. Day 0 represents the day of Pb infection when Tz larvae were now established in the rat muscle at day 28 post Tz infection. N = 6 for each group. Figures with asterisks superscript within a row under experimental groups are significantly different from the control group (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Haematological Parameter	Days post <i>Plasmodium berghei</i> infection	Experimental Groups			
		Control	Pb	Tz	Pb + Tz
WBC ($10^3/\mu\text{l}$)	Day 0	6.10 (4.90–6.97)	6.45 (4.45–7.35)	5.70 (5.00–6.10)	6.55 (5.10–7.10)
	Day 7	5.50 (5.25–6.25)	8.05 (6.15–10.38)	8.15 (5.27–11.73)	7.30 (5.05–10.93)
	Day 14	6.00 (4.77–7.10)	8.75 (5.70–10.20)	7.40 (6.37–9.60)	8.15 (4.50–11.93)
Neutrophils (%)	Day 0	8.30 (00–19.98)	6.95 (4.57–14.45)	5.8 (4.22–7.12)	5.20 (3.95–6.37)
	Day 7	9.45 (4.97–10.38)	7.55 (6.30–12.00)**	0.0 (0.0–18.40)**	24.05 (16.83–28.08)***
	Day 14	11.00 (5.85–11.70)	10.60 (6.67–12.65)	12.65 (11.03–15.73)	7.25 (4.42–11.80)
Basophils (%)	Day 0	0.60 (0.30–1.00)	0.30 (0.15–0.60)	0.60 (0.37–0.73)	0.50 (0.37–0.73)
	Day 7	0.30 (0.17–0.42)	1.55 (0.92–2.42)	2.90 (1.55–4.15)***	4.05 (1.43–6.82)***
	Day 14	0.45 (0.35–0.50)	0.45 (0.23–0.62)	0.25 (0.20–1.03)	0.60 (0.38–1.30)
Monocytes (%)	Day 0	3.15 (2.77–8.10)	3.90 (2.80–7.10)	5.00 (3.20–9.90)	3.75 (3.35–4.85)
	Day 7	5.35 (4.82–6.40)	12.35 (11.18–16.63)***	7.65 (5.02–10.03)	7.90 (4.72–9.30)
	Day 14	5.95 (3.15–8.10)	13.80 (8.85–16.33)***	4.70 (4.20–7.02)	2.95 (0.52–5.85)
Eosinophils (%)	Day 0	3.85 (0.60–13.8)	0.05 (0.0–0.25)*	0.10 (0.10–0.20)*	0.10 (0.0–0.20)*
	Day 7	0.10 (0.10–0.10)	0.25 (0.0–0.53)	13.0 (0.0–16.2)**	12.0 (3.42–26.5)***
	Day 14	0.85 (0.27–2.53)	0.30 (0.08–1.18)	0.60 (0.18–1.80)	0.20 (0.08–1.23)
Lymphocytes (%)	Day 0	70.15 (50.20–83.18)	86.55 (82.35–90.60)	88.60 (84.60–90.35)	90.70 (89.25–91.10)
	Day 7	85.65 (83.75–89.00)	77.45 (69.43–80.63)***	37.10 (30.93–48.45)	52.45 (33.53–66.10)
	Day 14	66.95 (65.53–70.28)	71.55 (51.90–74.40)***	80.35 (76.00–83.70)	85.20 (62.55–85.75)

Percentage lymphocytes were significantly lower in Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups compared to control at day 7 post Pb infection (Table 1). Furthermore, percentage lymphocytes in Tz mono-infected ($P < 0.01$) and co-infected ($P < 0.05$) groups were significantly lower in comparison to Pb mono-infected group at day 7 post Pb infection.

Percentage eosinophils were significantly decreased in Tz and Pb mono-infected ($P < 0.05$) and co-infected ($P < 0.05$) groups compared to control at day 0 and significantly increased in Tz mono-infected ($P < 0.01$) and co-infected ($P < 0.001$) day 7 post Pb infection (Table 1). On day 14 post Pb infection, there were no significant differences in percentage eosinophils in all experimental groups compared to control.

3.4. Effect of Tz and Pb co-infection on serum cytokine concentration

There was a significant elevation of TNF- α concentration in Pb mono-infected group ($P < 0.001$) compared to control at day 7 post Pb infection (Table 2). TNF- α levels in Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups were also significantly lower compared to Pb mono-infected group at day 7 post Pb infection. However, no significant differences were observed for TNF- α levels at day 14 post Pb infection for all groups.

A significant elevation of IL-10 concentration was observed in Pb mono-infected group ($P < 0.001$) compared to control at day 7 days post Pb infection (Table 2). Also, IL-10 concentration in Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups were significantly lower in

Table 2. Median values (25%–75% quartiles) of cytokines TNF- α and IL-10 and chemokines CXCL10 (IP-10), CCL5 (RANTES) and CCL11 (Eotaxin) concentration in Sprague-Dawley rats co-infected with *Plasmodium berghei* ANKA (Pb) and *Trichinella zimbabwensis* (Tz) (Pb + Tz), Pb mono-infection and Tz mono-infection. Day 0 represents the day of Pb infection when Tz larvae were now established in the rat muscle at day 28 post Tz infection. N = 6 in each group. Figures with asterisks superscript within a row under experimental groups are significantly different from the control group (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Cytokine/Chemokine	Days post <i>Plasmodium berghei</i> infection	Experimental Groups			
		Control	Pb	Tz	Pb + Tz
TNF-a (pg/ml)	Day 0	7.51 (4.93–17.68)	7.51 (4.93–17.68)	11.15 (7.91–35.89)	11.15 (7.91–35.89)
	Day 7	7.51 (4.93–17.68)	429.5 (176.0–822.7)***a	9.34 (6.54–58.78)***b	7.85 (6.04–12.44)***b
	Day 14	7.51 (4.93–17.68)	10.32 (7.59–13.15)	7.70 (4.02–11.93)	6.41 (4.08–9.72)
IL-10 (pg/ml)	Day 0	191.3 (82.39–221.7)	191.3 (82.39–221.7)	191.3 (143.0–237.0)	191.3 (143.0–237.0)
	Day 7	191.3 (82.39–221.7)	1490 (375.4–7515)***a	176.3 (111.5–221.7)***b	111.2 (68.18–241.2)***b
	Day 14	191.3 (82.39–221.7)	314.5 (250.4–415.9)	139.2 (105.0–198.0)	169.2 (93.08–291.9)
CXCL10 (IP-10) (pg/ml)	Day 0	415.0 (236.0–488.9)	415.0 (236.0–488.9)	872.6 (726.3–1310)	872.6 (726.3–1310)
	Day 7	415.0 (236.0–488.9)	6518 (5309–34351)***	744.7 (571.0–1281)	644.2 (374.9–665.0)
	Day 14	415.0 (236.0–488.9)	454.5 (280.2–595.1)	923.1 (720.3–1161)	526.7 (399.3–621.6)
CCL5 (RANTES) (pg/ml)	Day 0	7517 (6038–8448)	7517 (6038–8648)	22199 (17148–27999)***	22199 (17148–27999)***
	Day 7	7517 (6038–8448)	23351 (19825–272.48)***	17954 (14354–25337)***	8664 (7359–18941)
	Day 14	7517 (6038–8448)	17664 (13897–24822)**	12048 (10931–13357)	14435 (7365–17342)
CCL11 (Eotaxin) (pg/ml)	Day 0	3803 (1956–8744)	3803 (1956–8744)	12630 (6061–23523)*	12630 (6061–23523)*
	Day 7	3803 (1956–8744)	1527 (1093–2485)	12791 (8239–32466)**	5603 (20140–13490)
	Day 14	3803 (1956–8744)	14308 (7878–19644)	8043 (6074–11793)	6529 (2885–13047)

comparison to Pb mono-infected group at day 7 post Pb infection. However, there was no significant differences for IL-10 concentration at day 14 post Pb infection among all groups. IL-4 and IL-6 concentration were below the detection limit of the Procarta Plex-7 plex bead Multiplex immunoassay.

3.5. Effect of Tz and Pb co-infection on serum chemokine concentration

The median, 25% and 75% quartiles of CXCL10, CCL5 and CCL11 concentration are succinctly summarized in Table 2. A significant increase of CXCL10 concentration ($P < 0.001$) in Pb mono-infected group compared to control group was observed at day 7 post Pb infection (Table 2). Also, in Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups, CXCL10 concentration were significantly lower in comparison to Pb mono-infected group at day 7 post Pb infection. However, there were no significant differences at day 14 post Pb infection among experimental groups.

CCL5 was significantly elevated in Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups compared to control group at day 0 post Pb infection (Table 2). Also, Tz mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups had significantly higher CCL5 levels in comparison to the Pb mono-infected group at day 0 post Pb infection. *Plasmodium berghei* mono-infected ($P < 0.001$) and Tz mono-infected ($P < 0.001$) groups had significantly increased CCL5 concentration compared to control group at day 7 post Pb infection. Co-infected group had significantly lower CCL5 concentration compared to Pb mono-infected ($P < 0.001$) and Tz mono-infected ($P < 0.05$) groups at day 7 post Pb infection. *Plasmodium berghei* mono-infected ($P < 0.01$) remained significantly higher compared to control group until day 14 post Pb infection.

CCL11 concentration was significantly elevated in Tz mono-infected ($P < 0.05$) and co-infected ($P < 0.05$) groups in comparison to control group at day 0 post Pb infection (Table 2). The Tz mono-infected ($P < 0.05$) and co-infected ($P < 0.05$) groups had significantly higher CCL11 concentration compared to Pb mono-infected at day 0 post Pb infection. *Trichinella zimbabwensis* mono-infected ($P < 0.01$) group had significantly increased CCL11 concentration compared to control group at day 7 post Pb infection. *Plasmodium berghei* mono-infected ($P < 0.001$) and co-infected ($P < 0.001$) groups had significantly lower CCL11 concentration compared to Tz mono-infected group 7 days post Pb infection. However, there was no significant differences for CCL11 concentration at day 14 post Pb infection for all groups.

4. Discussion

A high number of Tz adult worms were recovered from the intestines of rats at day 7 post Tz infection compared to the number recovered at day 14 post infection. Adult Tz worms have been reported to persist in the intestinal tract for up to 21 days post infection (Onkoba et al., 2015b). In our study, Tz muscle larvae were observed from the rat muscles as from day 28 post infection until day 42 post infection and previous studies have demonstrated that during the intestinal phases of Tz infection, a protective innate immune response against NBL and adult worms is elicited by the intestinal epithelial mucosa of the intestinal tract (Onkoba et al., 2015b; Picherot et al., 2007). Onkoba et al. (2016) reported increased production of TNF- α at day 7 post infection. A mixed Th1/Th2 host immune response has been reported to occur at day 7 post Tz infection, possibly in response to Tz-derived antigens interacting with host enterocytes and the immune system cells (Onkoba et al., 2016). Elevated anti-inflammatory cytokines and chemokines at approximately day 20–28 post *Trichinella* infection creates a favorable environment for muscle larvae establishment in the muscle cells (Bruschi, 2004; Beiting et al., 2007; Fabre et al., 2008; Bruschi and Chiumiento, 2011).

In this study, co-infection of Pb and Tz elicited a higher percentage of Pb parasitaemia throughout the experimental period when compared with the Pb mono-infected group. Increased percentage parasitaemia of

Pb in co-infection with *Schistosoma mansoni* was also reported by Legesse et al. (2004). Consensus results from a review and meta-analyses of the outcome of helminth-plasmodium co-infection in young African children by Degarege et al. (2016) was associated with an increase in cases of asymptomatic and uncomplicated *P. falciparum* infection and protection from malaria-related anaemia. It has also been shown that even after treatment, the high percentage parasitaemia in co-infected group delayed clearance (Legesse et al., 2004). The observed high percentage parasitaemia in the co-infected group in the current study could probably be due to antagonistic immune responses of the host to the parasite, with an effect of aggravating the malaria disease (Briand et al., 2005; Ateba-Ngoa et al., 2015).

A high percentage parasitaemia at day 7 post Pb infection coincided with significant reduction in RBC concentration and haematocrit (%) in the Pb and Pb + Tz groups. Our results are in agreement with previous studies that demonstrated destruction of infected RBCs by the spleen following Pb infection, with a concomitant decrease in haematocrit (%) (an indicator of anaemia), signifying severe stage of the disease (Kinung'Hi et al., 2014). However, the reduction in RBC concentration and haematocrit (%) in the Tz group at day 35 post-Tz infection, coinciding with day 7 post Pb infection is surprising as Tz infection has not been reported to be associated anemia or RBC destruction. This effect is further observed in the Pb + Tz group where the RBC concentrations and haematocrit (%) were even lower than the Tz group showing the synergistic effect of Tz. However, at day 14 post Pb infection, no significant differences in the RBC concentration and haematocrit (%) were observed across the groups. Mechanisms of anemia development in host following *Plasmodium* spp infection are multi-factorial and remain poorly understood to date and the same should be concluded with Tz infection. Anemia development during Pb infection may be mediated by destruction of parasitized RBCs, shortening the life cycle of non-parasitized RBCs and decreased production of RBCs in the bone marrow (Kinung'Hi et al., 2014). Risk of reduced hemoglobin concentration, RBC concentration and increased anemia has been shown to be higher in school children co-infected with malaria and STHs in comparison to control and mono-infected groups (Kinung'Hi et al., 2014; Mboera et al., 2011).

WBC concentrations were not statistically significant ($P > 0.05$), at day 7 post Pb infection among all the experimental groups. However, differential leukocyte counts showed significant differences in different experimental groups. On the other hand, percentage basophils were significantly elevated in Tz mono-infected and co-infected groups compared to the control and Pb mono-infected groups at day 7 post Pb infection. This observation demonstrates the important role played by basophils as a first line of host cellular (T-cell and macrophage) immune defense mechanisms against continued Tz muscle larvae establishment in the rat muscle.

The co-infected group had significantly higher percentage basophil (%) compared to Tz mono-infected group, possibly demonstrating the effect of co-infection in altering host immune response mechanisms. Our observations are in contrast with previous studies that reported suppression of WBC concentration leading to leucopenia and neutropenia in a co-infection of Pb with *Trypanosoma brucei*, *T. brucei* mono-infection and Pb mono-infection compared to a control group (Ademola and Odeniran, 2016). Differences observed are clearly due to the differences in the pathogenesis of *Trypanosoma brucei* (an extracellular protozoan) and Tz (a tissue-dwelling nematode) as co-infecting parasites.

Percentage monocytes remained significantly elevated in the Pb mono-infected group at day 14 post Pb infection in comparison to control, Tz mono-infected and co-infected experimental groups. Our results are in agreement with previous studies that have reported monocytosis in all experimental groups in comparison to control group (Ademola and Odeniran, 2016). Monocytes are secreted by the bone marrow, whose function in blood is similar to the role of macrophages in tissues (Geissmann et al., 2010; Shi and Pamer, 2011). Macrophages play a pivotal role in phagocytosis, initiating extracellular killing via secretion of toxic chemicals, process and presents antigens to helper T cells (Tacke

and Randolph, 2006; Yona et al., 2012). Monocyte-derived macrophages also secrete pro-inflammatory cytokines that play a major role in inflammatory processes, in activation and differentiation of helper T cells as well as in acute phase response or systemic response to infection or injury cells (Tacke and Randolph, 2006; Geissmann et al., 2010; Shi and Pamer, 2011; Yona et al., 2012; Hu and Korner, 2017). Higher percentage parasitaemia at day 7 post Pb infection coincided with significant elevation in percentage lymphocytes in Pb mono-infected group in comparison to Tz mono-infected and co-infected experimental groups in our study. However, there was a significant suppression of percentage lymphocytes in Tz mono-infected and co-infected experimental groups in comparison to control group, which could imply development of lymphopaenia following Tz chronic infection. This could possibly imply rapid Tz mediated transformation of lymphocytes to plasma cells, leading to anti-Tz antibody production (Ademola and Odeniran, 2016). This observation is consistent with results that demonstrated lymphopaenia following co-infection of *Trypanosoma brucei* and Pb in mice (Ademola and Odeniran, 2016).

In our study eosinophils were significantly higher in the Tz mono-infected ($P < 0.05$) and co-infected ($P < 0.05$) groups (at day 0 and 7) compared to control and this is in agreement with previously reported peripheral blood and tissue eosinophilia that characterizes trichinellosis in humans (Bruschi et al., 2008). Furthermore, *T. spiralis* ML homogenates have been reported to attract eosinophils (Dixon et al., 2006; Bruschi et al., 2008).

Changes in serum cytokine and chemokines concentration during acute and chronic stages of infection were also observed in the current study. Peak parasitaemia in Pb mono-infected group at day 7 post Pb infection coincided with significant elevation of TNF- α , IL-10 and CXCL10 concentrations in comparison to other experimental groups. The antagonistic immune responses are clearly exhibited by peak parasitaemia in the Pb mono-infected group coinciding with significant elevation of Th1 immune response cytokines and chemokines (TNF- α and CXCL10) and Th2 immune response cytokines (IL-10) which occurs towards the terminal stage of acute Pb infection (Onkoba et al., 2016). This could possibly imply to a mixed Th1/Th2 immune response (Onkoba et al., 2015a, 2016). Pro-inflammatory cytokines and chemokines such as TNF- α and CXCL10 play a major role in initial *Plasmodium* spp killing and eventual parasite clearance in the acute or early stages of Pb infection (Hartgers and Yazdanbakhsh, 2006). However, during the chronic or late stages of Pb infection, anti-inflammatory cytokines and chemokines such as IL-10 and TGF- β play a major role in antagonizing the inflammatory effects of pro-inflammatory cytokines. This reduces the potential organ specific pathologies that may lead to development of severe forms of malaria such as cerebral malaria, lactic acidosis, anemia, acute renal failure, hepatomegaly and splenomegaly (Hartgers and Yazdanbakhsh, 2006). In the current study, there was significant elevation of TNF- α , IL-10, and CXCL10 concentration at day 7 post Pb infection in the Pb mono-infected group compared to the control group.

In agreement with observations made in the current study, it has been previously reported that mixed Th1/Th2 host immune response creates a pro-inflammatory and anti-inflammatory environment in the host (Onkoba et al., 2016). This promotes Tz establishment as the parasite evades the host immune-mediated parasite worm killing and expulsion up to day 7 post Tz infection. This is also characterized by the high Tz adult worm load (Wakelin et al., 1994; Onkoba et al., 2015b). Onkoba et al. (2015a) also reported increased regulatory cytokine IL-10 levels during the larval stage establishment in the striated muscle cells, probably in response to inflammation caused by encysting muscle larvae and migrating new born larvae (Onkoba et al., 2015b). However, in the current study, the levels of TNF- α , IL-10, and CXCL10 concentrations observed in the Tz mono-infected group and co-infected group, at day 7 post Pb infection, were not significantly different in comparison to the control group.

It is, however, interesting to note that both CCL5 and CCL11 concentrations were significantly elevated in Tz mono-infected and co-

infected groups compared to control and Pb mono-infected groups at day 0 post Pb infection. This time point in the 42-day experimental protocol coincides with Tz muscle larvae parasite establishment in the striated muscle ± 28 days post Tz infection. Results in the current study are in agreement with previous studies that demonstrated the establishment of Tz larvae in muscle as from day 28 post infection (Onkoba et al., 2016). In comparison to control group, CCL5 concentration remained significantly elevated in the Tz mono-infected at day 7 post Pb infection. Interestingly, CCL5 concentration was also significantly elevated in the Pb mono-infected group at day 7 post Pb infection compared to control group and co-infected group, coinciding with peak Pb percentage parasitaemia. CCL5 concentration was also significantly elevated in Tz mono-infected group compared to control and co-infected groups; at day 7 post Pb infection, coinciding with muscle larvae establishment in the rat muscle. These observations demonstrate the pivotal role of pro-inflammatory chemokines and cytokines in parasite clearance following parasite infection. However, the concentration of CCL5 remained significantly elevated 14 days post Pb infection compared to control group. This observation is in agreement with reports that CCL5 can act both as a pro-inflammatory during acute infection and as an anti-inflammatory chemokine during chronic infection (Appay and Rowland-Jones, 2001; Conti and Digioacchino, 2001).

It has been reported that human eosinophils express receptors for CCL11, which also bind CCL5 and CCL7 chemokines (Baggiolini, 1996). Similar to the pattern observed with CCL5 concentration, CCL11 concentration was significantly elevated in Tz mono-infected and co-infected groups in comparison to control and Pb mono-infected groups at day 0 post Pb infection. Concentration of CCL11 remained significantly elevated at day 14 post Pb infection compared to control, Pb mono-infected and co-infected experimental groups. Observations in the current study are in agreement with studies that have demonstrated the role of eotaxin in chronic inflammatory processes (Bostrom et al., 2015). The elevated percentage eosinophils in Tz mono-infected observed in the current study is in agreement with previous reports where CCL11 has been reported to be a major eosinophil chemo-attractant, promoting migration of eosinophils into tissues mediated via CCR3 receptor (Matthews et al., 2004; Dixon et al., 2006; Bruschi et al., 2008).

5. Conclusions

To the best of our knowledge, this is the first laboratory study that has investigated the effects co-infection of Pb and Tz (a tissue-dwelling helminth), on chemokine profiles in male Sprague-Dawley rats as the laboratory animal model to mimic what may happen in field situations of *P. falciparum* co-infection with tissue-dwelling helminths. The study confirmed that, co-infection of Pb with Tz results in increased Pb parasitaemia in the early stages of infection which might translate to severe malaria and this is in line with findings from other laboratory-based studies involving Pb and the blood fluke, *Schistosoma mansoni* (Legesse et al., 2004). Our results also further strengthen the consensus results from a review and meta-analyses of the outcome of helminth-plasmodium co-infection in young African children by Degarege et al. (2016) which highlighted an increase in cases of asymptomatic and uncomplicated *P. falciparum* infection. However, the protection from malaria-related anaemia mentioned in the review was not confirmed.

Results from our study strongly support that infection with tissue-dwelling helminths such as *Trichinella* sp or others may predispose the host towards rapid development of malaria parasites during co-infection, a point which has already been reported before in previous studies. Furthermore, the infection might also predispose the host to development of severe anaemia, neutrophilia, basophilia, and lymphopenia during the first week of malaria infection. Significant elevation of Pb parasitaemia was also observed to coincide with elevated pro-inflammatory cytokine TNF- α , regulatory anti-inflammatory IL-10 and chemokine CXCL10 at day 7 post Pb infection in the co-infection group. On the other hand, elevated Tz ML load coincided with elevated CCL5 and CCL11 concentration

compared to day 7 and 14 post Pb infection and the results open opportunities for further studies using metabolomics and/or proteomics to identify biomarkers of co-infection using this animal model.

Declarations

Author contribution statement

P. Murambiwa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

E. Silas, Y. Mdleleni: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

S. Mukaratirwa: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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