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Differential immune responses in mice infected with the tissue-dwelling nematode *Trichinella zimbabwensis*

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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).

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Trichinella infections are normally acquired through ingestion of raw or undercooked infected meat (Chai *et al.*, 2005; Murrell *et al.*, 2005) or food contaminated with infective muscle larvae (ML) (Slifko *et al.*, 2000; Dabanch, 2003; Pozio & Rossi, 2008). The ingested ML develop into adults that, in the small intestines, release newborn larvae (NBL) that migrate to the muscles (Gao *et al.*, 2014).

The risk of future human *T. zimbabwensis* infection in sub-Saharan Africa is 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 exacerbated 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). Natural *T. zimbabwensis* infections have been reported in a 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). Although there have been no cases of human infections due to *T. zimbabwensis* in sub-Saharan Africa, the parasite remains a public health risk.

Trichinella infection surveillance, control and treatments have been hampered by wide distributions of domestic, synanthropic and sylvatic reservoirs, the lack of a 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, research continues into the testing of parasite-derived somatic antigens and crude extracts in an effort to develop vaccines, identifying putative molecules to be used in the 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 *Trichinella* species in southern Africa, and its infectivity (Hurnikova *et al.*, 2004; Pozio & La Rosa, 2005; Matenga *et al.*, 2006), host range (Mukaratirwa *et al.*, 2003, 2008; Matenga *et al.*, 2006), biochemistry (La Rosa *et al.*, 2003), diagnosis (Ludovisi et al., 2013) and treatment (Mukaratirwa *et al.*, 2015) have been studied extensively. However, there is a paucity of information on the immune responses induced during the phases of its life cycle.

Materials and methods

Experimental infections in mice

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

A crocodile-derived *T. zimbabwensis* (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 & Gamble (2000). Each mouse was orally infected with 500 ML and, at 0, 7, 14, 21, 28 and 35 dpi, groups of six mice were sacrificed humanely and blood was collected for sera.

Immunological protocols

Preparation of *T. zimbabwensis* antigen was performed as described previously by Escalante *et al.* (2004). The ML obtained as described earlier were sonicated at five pulses of 100 W for 30 s each and centrifuged at 40,000 × *g* at 4°C for 60 min. The supernatant was collected. The protein concentration was determined by Bradford assay (Bradford, 1976) then an anti-protease cocktail (Sigma-Aldrich, St. Louis, Missouri, USA) was added. The antigen was diluted to working concentration and stored at −80°C until use.

Trichinella-specific IgG, IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies were measured in sera collected on 0, 7, 14, 21, 28, 35 dpi by enzyme-linked immunosorbent assay (ELISA) in both experimental and control groups. Microplates were coated with 6.0 μg/ml of crude *T. zimbabwensis* larval antigen in 100 μl bicarbonate buffer (4 mM Na_2CO_3, 8 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. The microtitre plates were blocked with 0.05% Tween 20 in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and incubated at 37°C for 2 h. Serum samples at a dilution of 1:200 were added in triplicate wells and incubated at 37°C for 2 h. Horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Santa Cruz Biotechnology, California, USA) antibodies were added at a dilution of 1:2000, and IgG1, Ig2a, IgG2b and IgG3 at a dilution of 1:1000. After 2 h of incubation at 37°C, 100 μl substrate (TMB substrate; KPL, Gaithersburg, Maryland, USA) was added. Optical density (OD) values were measured at 630 nm using a microplate reader (BioTek, Winooski, Vermont, USA).

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

Data analysis

Antibody responses and cytokine concentrations at each day of sacrifice were expressed as means ± standard error (SE) and analysed using a two-way analysis of variance (ANOVA); the levels of significance were determined by Bonferroni post-hoc test analyses using Graph pad PRISM version 5.04 for windows (Graphpad software, San Diego, California, USA) and a P value of < 0.05 was considered to be significant.

Results

Following infection, the mice were found to be seropositive for parasite-specific IgM (fig. 1A), IgG (fig. 1B) and IgG1 (fig. 1C) antibodies. A gradual increase in IgM levels was observed at day 7 pi, which reached peak levels on days 14 and 28 pi (fig. 1A). Significant levels of IgG were observed compared to the control group (ANOVA, $F_{3,29} = 5.01$, $P = 0.001$; Bonferroni post-hoc $P < 0.01$) (fig. 1B). High levels of anti-*T. zimbabwensis*-specific IgG1 levels were observed at day 14 pi (fig. 1C) and declined at day 21 pi (ANOVA, $F_{5,27} = 5.01$, $P = 0.0007$; Bonferroni post-hoc $P < 0.001$). *Trichinella*-specific IgG3 antibody levels were detected from day 21 pi and remained elevated throughout the course of infection (fig. 1D). 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 ($P < 0.001$).

At day 7 pi, significant levels of TNF-α (ANOVA, $F_{5,43} = 181.87$, $P < 0.0001$; Bonferroni post-hoc $P < 0.001$) (fig. 2) and IL-10 (ANOVA, $F_{5,34} = 98.02$, $P < 0.0001$; Bonferroni post-hoc $P < 0.001$) (fig. 3B) were observed in the infected group compared to the control group. Interleukin-10 concentrations showed a gradual decline, attaining low levels at day 21 pi (ANOVA, $F_{5,34} = 98.02$, $P < 0.0001$; Bonferroni post-hoc $P < 0.01$). At days 28 and 35 pi, a gradual increase and decline were observed, respectively. Significantly higher levels of IL-4 were detected at day 21 pi (ANOVA, $F_{4,24} = 382.91$, $P < 0.0001$; Bonferroni post-hoc $P < 0.001$) (fig. 3A), which declined at day 35 pi to the level of the control group. Overall, there were significant differences in the levels of variation amongst infection groups over the infection period for all the parameters.

Discussion

Our findings show that the intestinal stage of *T. zimbabwensis* infection was characterized by the early production of *T. zimbabwensis*-specific IgM and IgG antibodies. This implies that the host intestinal epithelial mucosa elicits a protective innate immune response against the NBL and adult worms (AW) in the intestinal tract (Picherot *et al.*, 2007). Therefore, it is plausible to deduce that parasite-derived antigens are released by NBL and AW to interact with enterocytes and immune cells influencing, in the long run, T-cell polarization and production of parasite-specific antibodies (Yépez-Mulia *et al.*, 2009). The resultant molecular dialogue between the host and the parasite results in the induction of a mixed T-helper 1 (Th1)- and Th2-type immune response, similar to that induced in mice with chronic *T. spiralis* infection (V. Fabre *et al.*, 2009). Our findings also show that there was markedly enhanced production of the Th1-type cytokine, TNF-α, and parasite-specific IgG1 (Th2-type dependent) antibody as early as 7 dpi, which is not the case with *T. spiralis* infection (Beiting *et al.*, 2007; M.V. Fabre *et al.*, 2009). In chronic *T. spiralis* infection, antibodies were measurable at 15 dpi, with low IgG2a levels. Therefore, the enteric phase of *T. zimbabwensis* infection majorly favoured a mixed Th1- and Th2-type response, as opposed to *T. spiralis* and *T. pseudospiralis* infections which favoured a low-level Th1-type response

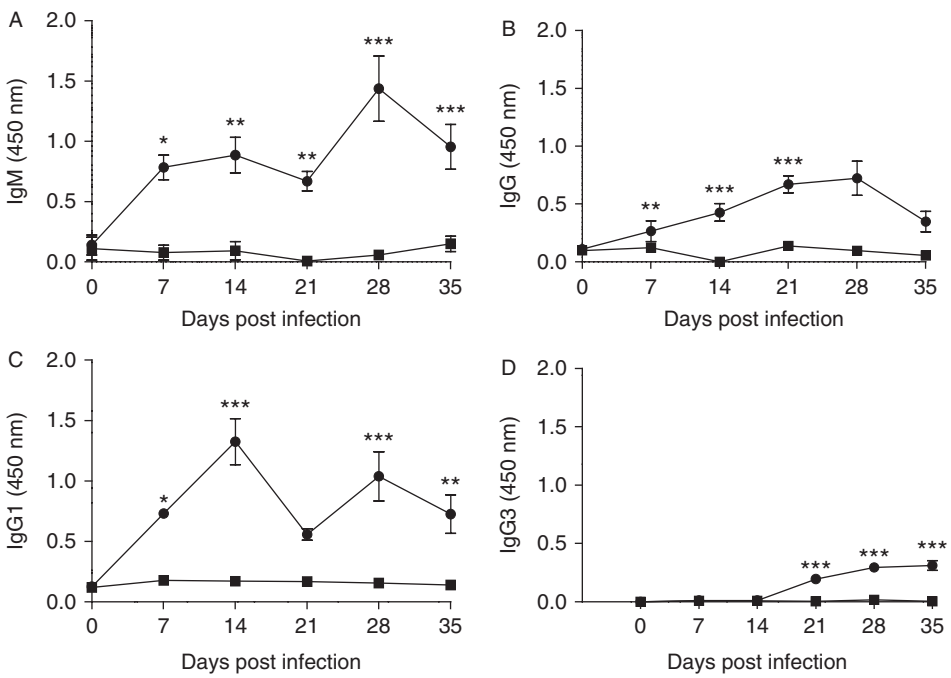

Fig. 1. Optical densities (mean ± SE) of *Trichinella*-specific IgM (A), IgG (B), IgG1(C) and IgG3 (D) antibodies in *Trichinella*-infected mice (●) and control mice (■). Levels of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

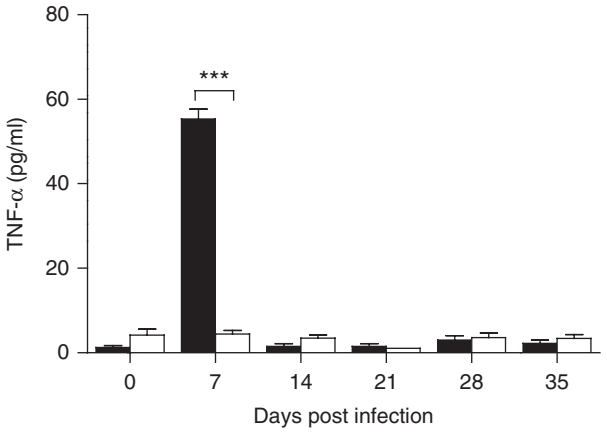

Fig. 2. Tumour necrosis factor alpha (TNF-α) concentrations (mean ± SE) in *Trichinella*-infected (black bars) and control mice (white bars). Levels of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with an earlier Th2-type response (Wakelin *et al.,* 1994). The role of T cells in both parasites has been fully elucidated (V. Fabre *et al.,* 2009) and the differences in immune responses observed between the encapsulating and the non-encapsulating *Trichinella* species may be attributed to the challenge dose, parasite antigenic diversity, animal-host sex and the rate of worm expulsion (Furze & Selkirk, 2005; Fu *et al.*, 2009; Hlaka *et al.*, 2015). The mixed Th1- and Th2-type responses elicited create a pro-inflammatory and anti-inflammatory environment that favours parasite immune evasion and establishment (Hewitson *et al.*, 2009; Gruden-Movsesijan *et al.*, 2011; Ilic *et al.*, 2011; Sofronic-Milosavljevic *et al.*, 2015). The parasite-driven immunosuppression of the host regulatory response offers mutual benefit to the parasite and the host (Ilic *et al.*, 2012; Ashour, 2013). This phenomenon has also been reported during chronic *T. spiralis* infection in mice, where there has been evidence of immune regulatory induction enabling the parasite to survive longer, limiting host pathology and overt inflammatory responses (Beiting *et al.*, 2007). These findings are corroborated by our previous findings, where we established that *T. zimbabwensis* AW persist in the intestinal tract for up to 21 days post infection (Onkoba *et al.,* 2015) as compared to other *Trichinella* parasites that are eliminated in 7–18 dpi (Sadaow *et al.*, 2013). The differences in the persistence of AW suggest that suppressive parasite or host factors may be at work, or that antigenic diversity exists between the non-encapsulating *T. zimbabwensis* and the encapsulating *T. spiralis* parasites (Gao *et al.*, 2012, 2014). In addition, parasite-induced immunomodulatory responses may also be slowing the rate at which the hosts generate immune-mediated worm expulsion and parasite killing (Goyal & Wakelin, 1993; Wakelin *et al.*, 1994; Wakelin & Goyal, 1996). Levels of the TNF-α cytokine at 7 dpi were elevated, suggesting that a protective immune response is initiated against *T. zimbabwensis* AW and NBL, which is geared towards AW expulsion, abrogation of inflammation caused by NBL or parasite killing (Harnett & Harnett, 2010) and eventual tissue healing (Artis *et al.*, 1999; Maizels *et al.*, 2009).

In the course of infection, we observed that the elevated levels of TNF-α at 7 dpi corresponded with a decrease of IL-4, and vice versa at 21 dpi. This implies that the infection orchestrates down-regulation of IL-4, which is a primary driver of the Th2-type response essential for parasite establishment (Wakelin *et al.*, 1994; Wu *et al.*, 2010). In the migrating phase of larval stages, the levels of IL-10 and parasite-specific IgG1 and IgG3 antibodies were significantly elevated. During *T. spiralis* infection, IL-10 levels have also been shown to coincide with IgG1 levels (Beiting *et al.*, 2004). In both wild-type and IL-10 deficient mice, it has been established that the inflammatory response that occurs as a result of migrating larvae is attenuated independently of IL-10 production (V. Fabre *et al.,* 2009). Similarly, during *T. spiralis* infection, IL-10 and transforming growth factor-β (TGF-β) have been indicated to control inflammatory responses (Hübner *et al.*, 2012). However, Beiting *et al.* (2007) have shown that IL-10 alone does not affect parasite survival, but that in combination with TGF-β parasite death occurs. Thus, the decline in the levels of IgM and IgG1 antibodies with significant IgG3 antibody elevation shows that IL-10 in combination with other immune cells down-regulates the production of parasite-specific antibodies.

The mixed cytokine and antibody response shows that the *T. zimbabwensis* parasite does not fully manage to shut off the effects of innate effector cells, evident by the presence of initial parasite-specific antibodies that

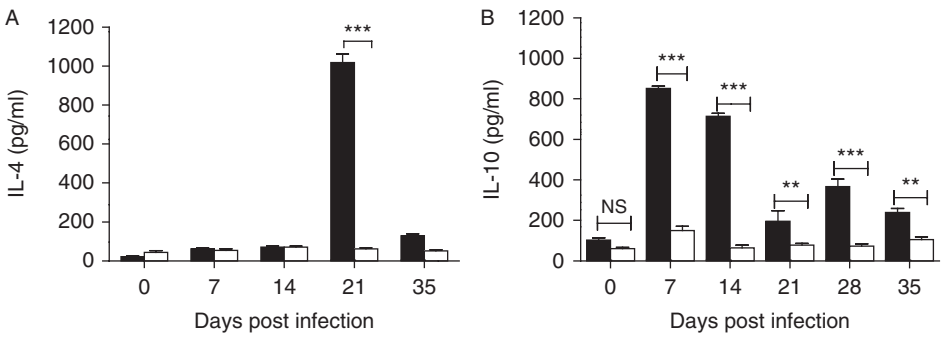

Fig. 3. The concentrations (mean ± SE) of IL-4 (A) and IL-10 (B) of *Trichinella*-infected (black bars) and control mice (white bars). Levels of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

co-exist with either Th1- or Th2-type responses. Therefore, based on our observations, *T. zimbabwensis* does not seem to induce solely either Th1- or Th2-type immune responses (Ilic *et al.*, 2011), similar to a report of soil-transmitted helminth (STH) infections (Anthony *et al.*, 2007). STHs provoke a strong CD4 + Th2-type-mediated resistance that limits the establishment of helminthic infections in the host and the resultant pathologies (Yazdanbakhsh *et al.*, 2001; Maizels *et al.*, 2012).

In the present study, we demonstrate for the first time that *T. zimbabwensis* orchestrates an immunomodulatory response that encompasses mixed Th1- and Th2-type and T-regulatory responses, which are similar to those of other intestinal nematodes during chronic infection (Else & Finkelman, 1998). Our study was not able to elucidate the actual role of *Trichinella*-derived excretory and/or secretory products in T-cell priming, activation and expansion. However, *T. spiralis* secreted products have been shown to have an ability to induce T-cell priming that results in expansion of regulatory T and B cells, and dendritic cell (DC) stimulation in *in vitro* and murine studies (Ilic *et al.*, 2008; Aranzamendi *et al.*, 2012).

In the parasite establishment phase in the striated muscles, our results showed that the presence of ML in the predilection site is associated with high levels of *Trichinella*-specific IgM, IgG, IgG1 and IgG3 antibodies and regulatory cytokine (IL-10). This may be as a result of the muscular inflammation caused by encysting ML and migrating NBL. Studies have shown that the resolution of muscular inflammation is dependent on how successful the enteric-phase immunoregulation was (Helmby & Grencis, 2003). Therefore, we observed that as the number of ML increased in striated muscles, the levels of Th1- and Th2-type cytokines dropped. This is an indication that the successful parasite establishes itself and initiates induction and modulation of host effector mechanisms (Artavanis-Tsakonas *et al.*, 2003; Hewitson *et al.*, 2009). V. Fabre *et al.* (2009) established that nitric oxide is involved in *T. spiralis* parasite killing during the chronic phase of infection, but in the muscle phase the ML are capable of inhibiting inducible nitric oxide synthase (iNOS) to improve their survival and establishment.

We have shown, for the first time, that the non-encapsulated *T. zimbabwensis* parasite induces a mixed Th1- and Th2-type response with a Th2-biased immune response in the parasite establishment phase. Therefore, we can speculate that the NBL may be responsible for the mixed Th1- and Th2-type and regulatory responses in the enteric and migrating phases of the parasite life cycle. The study was not able to elucidate extensively the manner in which the different short-lived immune responses are induced in the body compartments during NBL migration. Further studies are needed to establish the actual immune cells that are involved in anti-*T. zimbabwensis* immunity, immunomodulation and establishment of parasitism.

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Conflict of interest

None.

Ethical statement

All experimental protocols and procedures of the study were reviewed and approved by the Animals Ethics Committee of the University of KwaZulu-Natal (UKZN) (ref no: 114/13/Animal) in accordance with the South African national guidelines on animal care, handling and use for biomedical research. The experiments are reported in accordance with ARRIVE guidelines (Kilkenny *et al.*, 2013).

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