Serum levels of cytokines in water buffaloes experimentally infected with *Fasciola gigantica*

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ABSTRACT

Fasciola gigantica infection in water buffaloes causes significant economic losses especially in developing countries. Although modulation of the host immune response by cytokine neutralization or vaccination is a promising approach to control infection with this parasite, our understanding of cytokine's dynamic during *F. gigantica* infection is limited. To address this, we quantified the levels of serum cytokines produced in water buffaloes following experimental infection with *F. gigantica*. Five buffaloes were infected via oral gavage with 500 viable *F. gigantica* metacercariae and blood samples were collected from buffaloes one week before infection and for 13 consecutive weeks thereafter. The levels of 10 cytokines in serum samples were simultaneously determined using ELISA. *F. gigantica* failed to elicit the production of various pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-2, IL-6, IL-12, and IFN-γ. On the other hand, evidence of a Th2 type response was detected, but only early in the course of parasite colonization and included modest increase in the levels of IL-10 and IL-13. The results also revealed suppression of the immune responses as a feature of chronic *F. gigantica* infection in buffaloes. Taken together, *F. gigantica* seems to elicit a modest Th2 response at early stage of infection in order to downregulate harmful Th1- and Th17-type inflammatory responses in experimentally infected buffaloes. The full extent of anti-*F. gigantica* immune response and its relation to pathogenesis requires further study.

Keywords:

Buffaloes

Cytokines

Fasciola gigantica

Fasciolosis

Th1/Th2 paradigm

1. Introduction

Fasciola gigantica and *F. hepatica* are digenetic trematodes, which cause economically important global disease of ruminants 'fasciolosis' in the tropical and temperate regions, respectively (Mage et al., 2002). These hepatic flukes are notoriously known for their veterinary medical importance due to the significant economic losses associated with liver fluke infection (Spithill and Dalton, 1998). The tropical liver fluke, *F. gigantica*, imposes a serious threat to buffalo's farming in Asia and Africa because it can adversely affect the vitality and reproductive ability of infected buffaloes (Yadav et al., 1999). In addition to the animal health and economic impact, *Fasciola* spp. impose a zoonotic threat. Several hepatic pathologies attributed to infection with these parasites have been reported in humans (Machicado et al., 2016) and a huge population is at risk especially in Africa, Asia and South America (Hotez et al., 2008).

A mixed Th1 and Th2 immune response has been implicated in the pathogenesis of *F. gigantica* infection in water buffaloes (Kumar et al., 2013; Changklungmoa et al., 2016). The literature showed that *F. gigantica* can modulate host immune response (Molina and Skerratt, 2005), such as induction of Th2 immune response and suppression of Th1-cellular immunity (Molina, 2005; Changklungmoa et al., 2016). In our recent study, we detected downregulation of the MHC-II related genes and suppression of the host pro-inflammatory (Th1) immune response during early *F. gigantica* infection, probably to support the parasite's survival within the host (Zhang et al., 2017). Also, another study reported an association between *F. gigantica* infection and Th2-related cytokines (interleukin [IL]-6 and IL-8), with anti-

74 inflammatory properties (Molina, 2005). In addition, Th0-type response was found to increase
75 during late stage of *F. gigantica* infection and was involved in chronic progression of the
76 disease (Ingale et al., 2008).

77 Because of the important role of Th1/Th2 paradigm in the pathogenesis of *F. gigantica*
78 infection (Molina and Skerratt, 2005; Kumar et al., 2012; Kumar et al., 2013; Changklungmoa
79 et al., 2016) understanding the Th1 and Th2 immune responses can provide the basis for the
80 development of new vaccines or immune-modulatory therapeutic approaches against *F.*
81 *gigantica* infection. However, there have been a few studies correlating immune responses with
82 stages of *F. gigantica* infection in buffaloes (Molina, 2005; Molina and Skerratt, 2005; Kumar
83 et al., 2013; Zhang et al., 2017). Also, although the role of T-helper cells in the pathogenesis of
84 *F. gigantica* has not yet been fully clarified, CD4 T-cells are known to be subdivided into Th1,
85 Th2, Th17, and Treg subsets on the basis of their pattern of cytokine production (Murphy and
86 Reiner, 2002). To this end, the present study was designed to investigate the levels of 10 serum
87 cytokines, representing pro-inflammatory/Th1 (IL-1β, IL-6, IL-2, IL-12, and interferon (IFN)-
88 γ), Th2/anti-inflammatory (IL-4, IL-10, IL-13, and transforming growth factor [TGF]-β), and
89 Th17 (IL-17) immune responses during the course of experimental infection of water buffaloes
90 with *F. gigantica* using enzyme-linked immunosorbent assay (ELISA).

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92 **2. Materials and methods**

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94 *2.1. Ethics statement*

This study was approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, PR China. All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

2.2. Preparation of metacercariae

Eggs of *F. gigantica* were collected from the gall-bladder of buffaloes slaughtered at local abattoirs in Guangxi Zhuang Autonomous Region, PR China, and were incubated at 29 °C for 11 days. The newly-hatched miracidia were used to infect *Galba pervia* snails (Gastropoda: Mollusca). Each snail was infected with 3–5 miracidia and was maintained in a sterile plastic tissue culture plate for 2 hr. The infected snails were incubated in order to allow the miracidium stage to develop into sporocyst, redia and finally to cercariae. After 42 days, fully-developed cercariae emerged from the snails were harvested and developed into metacercariae (ME) on 5 × 5 cm cellophane sheets. The ME on cellophane sheets were washed several times with sterile 1x phosphate buffered saline (PBS) and were used immediately to infect buffaloes as described previously (Phalee et al., 2015). Species identity of the harvested ME was determined by PCR amplification and sequencing of the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) as described previously (Huang et al., 2004), and was confirmed as *F. gigantica* based on 100% homology to the ITS-2 sequence of *F. gigantica* from Guangxi (GenBank accession No. AJ557569).

2.3. Animals and experimental infection

Five buffaloes, 8-10-month-old, were purchased from a local water buffalo's farm in Guangxi Zhuang Autonomous Region, PR China. To rule out any prior infection with *F. gigantica*, faecal examination, and testing for IgG and IgM antibodies against *F. gigantica* were performed using ELISA as described previously (Chauvin et al., 1995). Additionally, all buffaloes were treated with triclabendazole 5% w/v oral suspension in order to eliminate any potential liver fluke infection not detected by screening. Following four weeks of triclabendazole's withdrawal time, each buffalo was orally infected with 500 viable ME as described previously (Molina and Skerratt, 2005).

2.4. Serum collection

Blood samples were collected from all buffaloes one week prior to infection (as a base-line control) and weekly thereafter for 13 weeks. Whole blood was allowed to clot at ambient temperature for 30 min, followed by centrifugation at 1,700 *g* for 10 min at 20 °C. The serum layer was collected, divided into aliquots, and frozen at −20 °C until use. Serum samples were thawed immediately before the experiment.

2.5. Detection of serum cytokines

The levels of 10 cytokines in buffalo's serum were determined by ELISA (Bovine cytokine ELISA kit, Blue Gene Biotech Inc., Shanghai, China) following the manufacturer's instructions. The assay procedure was similar for all cytokines. Briefly, 100 μL of standard or serum sample were added into each well in the antibody pre-coated microtiter plate. Also, 100 μL PBS (pH 7) was added to three wells as a blank control. Then, 50 μL of enzyme conjugate was added into each well, mixed thoroughly and incubated for 1 hr at 37 °C. Following the incubation, the mixture was removed and the wells of microtiter plate were washed 5X with PBS. For color development, 50 μL of each of Substrate A and Substrate B were added to each well including blank wells, followed by 10 min incubation at 37 °C in the dark. Finally, 50 μL of Stop solution was added to each well and mixed with gentle tapping to terminate the reaction. Optical density (OD) of 450 nm (OD_{450}) minus the background of plate absorbance was read on ELISA microplate reader (BIO-RAD, Model 680). All samples were run at least in duplicates.

2.6. Statistical analysis

All statistical analyses were performed in GraphPad Prism (6.0 software, GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test was used to evaluate differences between groups. Two-way ANOVA followed by Bonferroni posttests was used to evaluate differences between the groups during the time course of infection. *P* values of <0.05 were considered statistically significant.

3. Results and discussion

Even though several studies investigated cytokines induced by *F. gigantica* infection (Molina and Skerratt, 2005; Kumar et al., 2013; Changklungmoa et al., 2016), it is still not entirely clear how the immune system of buffaloes respond to *F. gigantica* infection. Here, we simultaneously measured the concentrations of 10 cytokines in the serum of five buffaloes experimentally infected with *F. gigantica* using ELISA. All buffaloes challenged with *F. gigantica* ME were seroconverted at ≥ 4 weeks post infection (wpi).

In our study, the level of IL-4 was not different from the control during the first 3 wpi and thereafter declined, and from 8 wpi to 13 wpi the reduction was statistically significant (Fig. 1), suggesting down-regulation of Th2 immune response at late stage of infection. The level IL-13 cytokine exhibited a similar trend to IL-4. During *Fasciola* infection, Th2 cytokines, IL-4 and IL-13, play important roles in the suppression of Th1-driven inflammatory pathology and in promoting Th2 responses (Donnelly et al., 2008). Also, IL-13 is essential to the induction of M2 macrophages, which promote tissue fibrosis and granuloma formation. Thus, the modest upregulation of Il-13 at early stage of infection might have been triggered by *F. gigantica* to evoke a polarized Th2 response, which antagonizes Th1/Th17, allowing the parasite to establish infection similar to what has been reported in *F. hepatica* and *Schistosoma mansoni* (Donnelly et al., 2008). While IL-4 is needed for the protection against infection, IL-13 can partially compensate for its reduction. However, the decline in the levels of both anti-inflammatory

179 cytokines form 4 wpi onwards (Fig. 1), suggest that active immunosuppression induced by the
180 flukes and/or their secreted products is taking place.

181 In an effort to restrain *F. gigantica* growth, the host suppressed Th2 via activating Th1-
182 biased inflammatory response by increasing the production of the pro-inflammatory cytokines,
183 IL-17 and IL-1β, which occurred, but only slightly and from week four to week six post
184 infection (Fig. 1). A strong correlation exists between Th1/Th17 immune response and the
185 development of inflammation-mediated pathology in helminth-infected mice (Rutitzky et al.,
186 2008). Therefore, regulation of IL-17 is critical to limit the inflammatory damage associated
187 with *F. gigantica* infection. The link between IL-1β and IL-17 cytokines has been reported
188 (Jovanovic et al., 1998), where IL-1β was suggested to enhance IL-17 production (Ilarregui et
189 al., 2016). It is possible that *F. gigantica* has employed an immunomodulatory mechanism,
190 similar to that reported in *F. hepatica*, to downregulate IL-17 production in buffaloes. *F.*
191 *hepatica* protease cathepsin L (rFhCL1) and sigma class glutathione transferase (rFhGST-si)
192 have been shown to attenuate IL-17 production and failed to induce adequate Th2 immune
193 response. These findings suggest that *Fasciola* parasites secrete various molecules, which
194 possess distinct immunomodulatory properties to suppress the inflammatory Th1/Th17
195 response, while permitting a certain level of development of Th2 cells in response to other
196 secretory molecules (Dowling et al., 2010). Further work will be required to explore these
197 possibilities.

198 *Fasciola gigantica* infection seems to attenuate the levels of pro-inflammatory cytokines,
199 INF-γ, IL-2 and IL-12, compared with the control throughout the whole infection period, but

200 the differences were not statistically significant ($P > 0.05$). This reduction in Th1 response
201 allows the parasite to evade host immune defense and promotes its survival (Mendes et al.,
202 2013). The level of IL-6 was also reduced compared with the control and the reduction was
203 statistically significant from four to seven wpi. These data suggest downregulation of pro-
204 inflammatory immune response, in agreement with our recent observation of the
205 downregulation of the MHC-II related genes and suppression of the host pro-inflammatory
206 (Th1) immune response during early *F. gigantica* infection (Zhang et al., 2017). A similar
207 finding was reported in the related liver fluke, *F. hepatica*, where fatty acid binding protein
208 (Fh12) was shown to significantly suppress the expression of TNF-α, IL-12, IL-6, and IL-
209 1β cytokines, inhibit inducible NO synthase-2 in mouse bone marrow-derived macrophages
210 (bmMΦs) and impair the phagocytic capacity of bmMΦs (Martin et al., 2015). IL-1β regulates
211 the pro-inflammatory cytokine IL-8 to attract neutrophils and eosinophils, which are involved
212 in antibody-dependent cell-mediated cytotoxicity (ADCC) pathway and elimination of the
213 parasite (Zhang et al., 2017). Therefore, the modest upregulation of IL-1β at 4-6 wpi reflects
214 the host's endeavor to mount immune response to counter the parasite. Contrariwise, to evade
215 host immune response *F. gigantica* manipulates the host immunity by attenuating the level of
216 IL-1β during early and late infection to ensure their own survival, in agreement with the result
217 obtained in *F. hepatica* infection (Flynn and Mulcahy, 2008).

218 More broadly, as shown in Fig. 2 the levels of the majority of cytokines particularly at late
219 stage of infection (i.e., from 7 wpi to 13 wpi) were not significantly different from the control
220 or even were reduced, indicating an immune-suppressive state of infected buffaloes. This

221 immune-attenuation might be triggered by increased production of IL-10 cytokine between
222 week 4 and week 6. Even though IL-10 has been considered as a Th2-type cytokine, recent
223 findings showed that this cytokine can be produced by Th1 cells and regulatory T cells
224 (Anderson et al., 2007; Jankovic et al., 2007) *in vivo*, and thus can downregulate both Th1-type
225 and Th2-type responses (Hoffmann et al., 2000). Th2-type cytokine, IL-13, which was
226 preferentially expressed during early phase of infection, can also downregulate Th1-type and
227 Th17-type responses and suppress the associated inflammation.

228 IL-12 induces IFN-γ production and simulates Th1 development (Zundler and Neurath,
229 2015). During early infection, the levels of IL-2, IL-6, IL-12, and INF-γ were not significantly
230 different from the control, suggesting a reduced Th1 response. However, at 4-6 wpi, the level
231 of IL-12 was relatively high. It is possible that the modest increase in the level of IL-12 is
232 mediated by the host immune system to potentiate Th1 response to eliminate *F. gigantica*. In
233 line with this assumption, we detected lower level of the anti-inflammatory cytokine,
234 transforming growth factor–β (TGF-β), which might have caused slight increase in IL-4
235 production during the first 3 wpi to counter the migrating juvenile flukes, the most susceptible
236 stage to IL-4-dependent eosinophilia or mastocytosis as suggested in *F. hepatica* (Flynn and
237 Mulcahy, 2008).

238 When the balance is in favor of the parasite, the increased level of IL-10 can induce
239 immune-suppression, probably mediated by the fluke's glycoconjugates similar to *F. hepatica*
240 (Rodriguez et al., 2015), to inhibit Th1 cell proliferation and IL-12 secretion (Cope et al., 2011),
241 as well as the production of pro-inflammatory cytokines, such as IL-6 (Fig. 1). Interestingly,

immune-suppression, mediated by IL-4 and IL-10, was reported in rats experimentally infected with *F. hepatica*, which was proposed as a survival mechanism employed by the parasite to evade the host immune response during the early stage of liver penetration (Cervi et al., 2001). Another study, also in rats, reported a predominance of Th2 response during early chronic *F. hepatica* infection, which was declined as infection progressed to more chronicity leading to a persistent immune suppression in the advanced chronic phase of the infection (Girones et al., 2007).

In defining the buffalo's response to experimental *F. gigantica* infection, our results indicated that this parasite utilizes multiple immunomodulatory mechanisms that affect various facets of buffalo's immune response to ensure their persistence within the host. While early inflammatory (Th1) response is required to prevent the establishment of the juvenile form of *F. gigantica*, strong polarized anti-inflammatory (Th2) response, promoted by the parasite, is needed to suppress the Th1/Th17 immune response and to limit host tissue damage caused by excessive pro-inflammatory cytokines (Zhang et al., 2017). Intriguingly, some molecules secreted by *Fasciola* were found to suppress the differentiation of Th17 cells, independently of Th2 cells, by altering the function of dendritic cells (Dowling et al., 2010). Nevertheless, the outcome of *F. gigantica* infection will always depend on a balance between appropriate and inappropriate induction of Th1 and Th2 mediators.

In this study, we investigated the immune-regulatory mechanisms of *F. gigantica* infection in buffaloes. Our work revealed a polarized Th2 immune response during early infection as indicated by slightly high levels of IL-4, IL-10, and IL-13 cytokines, and reduced levels of IFN-

γ, Il-2, IL-6, IL-12, IL-17, and IL-1β cytokines. Although a complex interplay among Th1, Th2, and Th17 appear to underlie the immune response elicited against *F. gigantica*, the present results suggest that modest Th2-type response early in infection is needed to downregulate harmful Th1- and Th17-type inflammatory responses. Our data also suggest a state of immunosuppression during the late phase of the infection. These findings support continued investigation into immune response mechanisms enabling *F. gigantica* to evade, interfere and suppress host immune defenses. Full understanding of these mechanisms will provide information likely to be critical for the development of effective vaccines.

Competing interests

The authors declare that they have no competing interests.

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Figure legends:

Fig. 1. The effect of *Fasciola gigantica* on the levels of cytokine in the serum of experimentally infected buffaloes. The concentrations of 10 cytokines were quantified one week pre-infection and weekly thereafter for 13 successive weeks. Cytokine measurements were performed by ELISA kit and indicated in each panel. Bars represent the means ± SDs (n = 5). Red and green bars indicate, statistically significant (compared with pre-infection group: $P<0.05$) and non-significant differences, respectively.

Fig. 2. Relative abundance of cytokines in the serum of buffaloes infected with *Fasciola gigantica* over the course of 13 weeks after infection compared to control (1 week prior to infection). Bubble color indicates different cytokines (legend in upper right-hand corner); size indicates the standard deviation of the underlying data (bubbles of higher standard deviation are larger).