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Research paper

Th2-related cytokines are associated with *Fasciola gigantica* infection and evasion in the natural host, swamp buffalo

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ABSTRACT

The infection of ruminants by *Fasciola* spp. always induces a non-protective Th2-type immune response. However, little is known about changes in the local and systemic immune environment during *F. gigantica* migration in buffalo. In this study, native swamp buffaloes were each infected with 500 viable *F. gigantica* metacercariae. Mesenteric lymph node (MLN), hepatic lymph node (HLN), spleen, and serum samples were collected from control and infected buffaloes at 3, 10, 28, 42, 70, and 98 days post-infection (DPI). The mRNA expression levels of the Th1- and Th2-related cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IFN-γ, TNF-α, and CD4 were measured during different infection stages in the MLNs, spleens, and HLNs using quantitative real-time PCR (qRT-PCR). Levels of the specific anti-ESP isotype antibodies IgG, IgG1, and IgG2 were used to reflect changes in humoral immunity. The results of this study indicated that swamp buffaloes were susceptible to *F. gigantica* infection, and that susceptibility to this infection was closely related to the cytokine environment associated with the Th2-type immune response. The MLNs showed a mixed Th1- and Th2-type immune response during the acute infection stages, after which the production of these cytokines returned to normal. Cytokine expression in the HLNs also expressed a mixed Th1- and Th2-type immune response during the early infection stages. When the infection became chronic, the typical Th2 immune response was induced in the HLNs. At the acute infection stages, the spleen exhibited a Th2 immune response. Nevertheless, cytokines associated with the Th1 and Th2 immune responses were upregulated at 98 DPI. In addition, the total IgG and IgG1 of the parasite-specific antibodies increased. This suggested that the Th2-related cytokines and IgG1 induced by *F. gigantica* infection might mediate successful *F. gigantica* infection in the natural host, swamp buffalo.

1. Introduction

Fasciolosis, an infection caused by the trematode species *Fasciola gigantica* and *F. hepatica*, affects ruminant production and performance worldwide, particularly in developing countries (Mas Coma et al., 2009). Although treatment with triclabendazole (TCBZ) is usually effective against immature and adult *Fasciola* spp., there have been recent reports of drug resistance (Kelley et al., 2016; Venturina et al., 2015). Vaccines, which are safe and highly efficient, might be the best option for the control of fasciolosis (Meemon and Sobhon, 2015). Therefore, further characterization of the relationship between the *Fasciola* parasite and the host would aid the development of intervention strategies to prevent fasciolosis.

During helminth infection, a predominately Th2-type immune response is typically observed, characterized by high levels of IL-4, IL-5, and IgG1. In addition, the expression levels of IFN-γ and IgG2 are typically reduced, consistent with the observed high IgG1/IgG2 and IL-4/IFN-γ ratios (O'Neill et al., 2000; Pleasance et al., 2011). The Th2-type immune response is considered a non-protective immune response; this response may weaken Th1- or Th17-type immune responses, allowing parasites to survive inside host tissues (Anthony et al., 2007; Wynn, 2004). After *F. gigantica* exposure, only Indonesian thin-tail (ITT) sheep (which are resistant to *F. gigantica*) express a strong protective Th1-type immune response (Pleasance et al., 2011). Several studies have

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reported that, during the acute stage of *F. gigantica* infection, peripheral blood mononuclear cells (PBMCs) or serum from cattle, as well as buffalo serum, appeared to elicit a Th2-type immune response (Ingale et al., 2010; Molina, 2005; Zhang et al., 2017a). Other studies have shown that cattle PBMCs and lymph node cells (Mendes et al., 2013), as well as sheep hepatic lymph nodes (HLNs) (Pacheco et al., 2017) and spleens (Pleasance et al., 2011), showed non-protective Th2-type immune responses during the early stage of *F. hepatica* infection. However, when proceeded to a chronic infection, buffalo PBMCs and liver showed a mixed Th1/Th2 immune response (Kumar et al., 2013; Zhang et al., 2006). Unlike *F. gigantica* infection, cattle and sheep infected with *F. hepatica* showed a typical Th2-type immune response (Graham-Brown et al., 2018; Mendes et al., 2013; Pleasance et al., 2011). Unfortunately, few studies have investigated the changes in the local immune microenvironment of host organs in response to *F. gigantica* migration.

This study represents the first attempt to characterize the different life stages of *F. gigantica* in their natural host, swamp buffaloes, and to understand changes in the local immune responses of host organs. The aim of this study was to evaluate the expression of cytokines associated with the Th1- or Th2-type immune response in swamp buffalo infected with *F. gigantica*. Here, qRT-PCR was used to measure the mRNA expression of Th1- and Th2-type immune response-related cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IFN-γ, TNF-α, and CD4) in swamp buffalo MLNs, spleens, and HLNs during different stages of *F. gigantica* infection. The levels of the IgG1 and IgG2 isotype antibodies were used to reflect the strength of the Th1- and Th2-type immune response.

2. Materials and methods

2.1. Experimental design

Thirty-five swamp buffaloes between the ages of 9 and 12 months were used in this study; no *F. gigantica* were identified in any buffalo feces. To confirm that all selected swamp buffaloes were free of *F. gigantica* infection, enzyme-linked immunosorbent assays (ELISAs) (Phiri et al., 2006) were used to examine serum IgG specific for *F. gigantica* excretory and secretory products (ESPs). All calves were purchased from a local buffalo farm in the Guangxi Zhuang Autonomous Region, PR China. Calves were fed *Pennisetum purpureum* Schum, and other coarse green fodder. Buffaloes were kept in an area free of the fascioliasis epidemic. After two weeks of adaptive feeding, all buffaloes were treated with the anti-trematode drug TCBZ (1 mL, 5% per kilogram) to eliminate any potential liver fluke infections. Following a four-week observation period, feces egg tests and ELISA were used to confirm that all calves were free of *Fasciola* spp. Calves were then randomly divided into seven groups (5 calves per group): one control group and six experimental groups. Each experimental buffalo was given 500 viable *F. gigantica* metacercariae orally, while control buffalo was drenched with PBS. At 3, 10, 28, 42, 70, and 98 days post-infection (DPI), animals were killed, and HLNs, spleens, and MLNs were collected. All animals were killed and tissue samples collected in one week (each group was killed in one day), liver fluker infection were staggered using the same batches of *F. gigantica* metacercariae. After collection, tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Serum was isolated from agglutinated blood, and stored at −80 °C for antibody analysis. This study was approved by the Animal Administration and Ethics Committee of Guangxi University, Nanning, Guangxi, 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.

When hosts consume infective metacercariae, the cysts are eliminated by various enzymes in the duodenum. Newly excysted juveniles (NEJs) then migrate to the abdomen, and reach the liver within 4–6 days. Therefore, analysis began three days following ingestion, when the juvenile flukes were estimated to appear in the peritoneal cavity. At 10 DPI, it was hypothesized that the juvenile flukes would have migrated through the liver capsule to the parenchyma. MLN cytokine levels were used to reflect the immune microenvironment of the gut and the peritoneal cavity during the acute stage of *F. gigantica* infection. So we only detecte the MLN cytokine levels of 3DPI, 10DPI and 28DPI in the study. ITT sheep are resistant to *F. gigantica* for the first four weeks after infection (Pleasance et al., 2011). At 42–56 DPI (6–8 weeks), flukes are ˜6–10 mm long and are prepared to enter the bile ducts (Behm and Sangster, 1999). In cattle infected with *F. hepatica*, flukes reach the bile ducts at 70–84 DPI, and then mature. The primary indicator of *F. gigantica* infection in buffalo after 91 days is the presence of eggs in the feces (Yadav et al., 1999). In this study, the earliest discovery of eggs in feces was at 84 DPI. Therefore, 98 DPI was selected to represent a long-term infection.

2.2. RNA extraction and cDNA synthesis

RNA was isolated from approximately 100–200 mg of collected tissue (HLN, spleen, and MN) using the E.Z.N.A Total RNA Kit I (Omega BioTek, Inc., Norcross, GA, USA). RNA quality (260/280) was detected using a NanoDrop 2000c (ThermoFisher Scientific, Waltham, MA, USA), and only RNA samples 260/280 ratios of 2 were used for qRT-PCR. cDNA libraries were generated for each sample using 800 ng of total RNA and the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

2.3. qRT-PCR

PCRs were run on CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA, USA) (primers are given in Table 1). Each 20-μL reaction consisted of 10 μL 2xAceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China), 2 μL of 1:4 diluted cDNA, 0.4 μL of each forward and reverse primer (10 μM), and sufficient water to make 20 μL. The two-step qRT-PCR cycling program was 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. Signal collection was performed at 60 °C. Melt curve analysis was used to ensure that a single product was amplified. If the C_q value was < 35, the data were retained. The qRT-PCR data was exported using Bio-Rad CFX Manager 3.1. The *Bubalus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize gene expression data, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression of each gene of interest (Livak and Schmittgen, 2001). The ratio of IL-4/IFN-γ mRNA expression was calculated using the same method.

2.4. Preparation of ESPs

Vital, adult *F. gigantica* were collected from the cholecysts of freshly killed swamp buffaloes. ESP antigens from adult *F. gigantica* were obtained as described by Estuningsih (2009), with some modifications. Briefly, adult flukes were removed from the cholecysts of naturally infected buffaloes at a local slaughterhouse. Approximately 100 flukes were washed three times with phosphate buffered saline (PBS), then cultured in PBS (1 worm/1 mL of PBS) at 37 °C for 1 h. This incubation period removed host elements (e.g., bile, liver tissue, and blood) from the guts of the flukes. Flukes were then incubated in RPMI 1640 culture medium (GIBICO, Grand Island, US), containing 100 U penicillin and 100 mg/mL streptomycin (Solarbio, Beijing, China), at a density of 1 worm/mL RPMI for 4 h at 37 °C. The supernatant was collected and centrifuged at 3000 rpm for 30 min to remove eggs and large debris, then centrifuged at 10,000 rpm for 1 h at 4 °C. Protein concentrations were quantified using the BCA Protein Assay Kit (CWBIO, Beijing, China). Antigen preparations were stored at −80 °C.

2.5. Antibody detection

The anti-ESP isotype antibodies IgG, IgG1, and IgG2 were detected following the description of Phiri et al (2006), with slight

Table 1
Real-time PCR primers used for the quantification of cytokines and CD4 in the study.

Genes	Primer sense	Product length (bp)	5′–3′sequences	Accession number	Reference
GAPDH	F	106	TTCTGGCAAAGTGGACATCG	XM_006039922.1	Present study
	R		TCCCGTTCTCTGCCTTGACT		
IL-2	F	293	TTTTACGTGCCCAAGGTTAA	AB246354.1	Mingala et al. (2009)
	R		GAGGCACTTAGTGATCAAGTC		
IL-4	F	177	CAGCATGGAGCTGCCT	NM_001009313	Puech et al. (2015)
	R		ACAGAACAGGTCTTGCTTGC		
IL-5	F	164	TGGCAGAGACCTTGACACTGCT	NM_001290894.1	Mingala et al. (2009)
	R		CACAGCATCCCCTTGTGCAGTT		
IL-6	F	191	CTGCAATGAGAAAGGAGATA	AY347710.1	Mingala et al. (2009)
	R		GGTAGTCCAGGTATATCTGA		
IL-10	F	236	CTGTGCCTCTCCCCTAGAGT	NM_001009327.1	Mingala et al. (2009)
	R		GCAGCTAGCTCCACAAGGAA		
IL-12p40	F	214	CAGGGACATCATCAAACCAG	NM_001290887.1	Mingala et al. (2009)
	R		CTTGTGGCATGTGACTTTGG		
IFN-γ	F	253	GTCTCCTTCTACTTCAAACT	KU886026.1	Mingala et al. (2009)
	R		ATTCTGACTTCTCTTCCGCT		
TNF-α	F	218	TAACAAGCCGGTAGCCCACG	KY885010.1	Mingala et al. (2009)
	R		GCAAGGGCTCTTGATGGCAGA		
CD4	F	121	ACACTGAACTGAGCCATC	XM_006065838.1	Present study
	R		GTCTCCACTTCACAGGTAT		

Abbreviations: F forward primer, R reverse primer.

modifications. In brief, a 96-well ELISA plate (JET BIOFIL, Guangzhou, China) was coated with 100 µL *F. gigantica* ESP (2.5 µg/mL) in carbonate buffer (*p*H = 9.6) at 37 °C for 2 h, then incubated overnight in a refrigerator at 4 °C. Plates were then washed three times with PBS containing 0.05% Tween-20 (PBST) for 5 min each time, and then blocked for 2 h at 37 °C with 200 µL/well of 1% gelatin in PBS. After washing for three times, calf serum was diluted (1:200) in PBST and 100 µL was added to each selected well. Plates were incubated for 1 h at 37 °C, washed three times with PBST. Then, 100 µL horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG, IgG1, and IgG2 (AbD Serotec, UK) polyclonal antibodies, diluted 1:40,000 in PBST, were added to each well. Plates were incubated at 37 °C. After three washes, 100 µL/well tetramethylbenzidine TMB (Solarbio, Beijing, China) was added to induce development, and 50 µL/well 2 M H_2SO_4 was added to end the reaction. Plate absorbance at 450 nm was determined using an iMARK Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Statistical analysis

Statistical analyses of the qRT-PCR and ELISA data were performed in GraphPad Prism 6 (La Jolla, CA, USA). One-way analyses of variance (ANOVAs), followed by Dunnett's multiple comparisons tests, were used to evaluate differences among groups. Results are given as means ± the standard error of the mean (SEM). *P* values < 0.05 were considered significant.

3. Results

3.1. Verification of F. gigantica infection in buffaloes

Previous work indicated that buffaloes are highly susceptible to *F. gigantica* infection (Zhang et al., 2006), consistent with other studies (Sanyal and Gupta, 1996; Yadav et al., 1999). Viable liver flukes were identified in buffaloes killed 28 DPI (three buffaloes), 42 DPI (five buffaloes), and 98 DPI (four buffaloes) (Shi et al., 2017). Fluke eggs were identified in the feces of one buffaloes (in the group killed at 98 DPI) at 84 DPI. However, no eggs were identified in the feces of any other buffaloes. Eggs were observed in the bile samples from all infected buffaloes killed at 98 DPI; however, no eggs were observed in the bile samples of the buffaloes killed at 70 DPI. This suggested that, after metacercariae ingestion, *F. gigantica* matured in 70–84 days (Yadav et al., 1999).

3.2. Cytokine expression in the MLNs

The mRNA expression levels of Th1-type cytokines (IFN-γ, IL-12p40, and TNF-α) and Th2-type cytokines (IL-4, IL-10, and IL-6) were significantly elevated at 3 DPI as compared to the control group (Fig. 1). At 10 DPI, the mRNA expression of the type Th1-associated cytokine IFN-γ was markedly upregulated ($p < 0.0001$) (Fig. 1), while the type Th2-associated cytokines IL-10 and IL-6 remained elevated. However, there were no significant differences in IL-4 levels among the groups. This suggested that the Th1 immune response trends to the Th2 immune response. At 28 DPI, the mRNA expression of IFN-γ increased significantly ($p = 0.0289$). CD4 was significantly upregulated in the MLNs ($p < 0.05$) at 3 DPI, but was not upregulated in the MLNs of the control buffaloes at 10 and 28 DPI. No IL-5 or IL-2 mRNA expression was detected in either the infected or the control MLNs.

3.3. Cytokine expression in the HLNs

At 3 DPI, several cytokines were significantly upregulated: IL-12p40 ($p < 0.01$), IL-10 ($p < 0.001$), and CD4 ($p < 0.05$). The expression levels of several others (TNF-α, IL-4, IL-5, and IL-6) were slightly increased. At 10 DPI, the expression levels of IL-12p40, IL-10, and IL-6 were significantly higher in the experimental group than in the control group ($p < 0.05$ for all). Also, IL-4 and CD4 were upregulated in infected animals compared to control animals. At 28 DPI, only IL-12p40 was significantly upregulated ($p < 0.05$). The expression levels of IFN-γ, IL-4, and IL-5 were slightly increased. At 42 DPI, IL-4 and IL-5 were significantly upregulated ($p < 0.05$ for both); IL-6 was also upregulated, but the fold change was not significant statistically when compared to controls (fold change = 2.97, $p = 0.09$). At 70 DPI, cytokine expression in the experimental groups did not differ significantly as compared to the controls, but several cytokines were upregulated: IL-12p40, IL-2, TNF-α, IL-4, IL-5, IL-6, and CD4. At 98 DPI, IL-4 ($p < 0.001$), IL-5 ($p < 0.05$), and IL-10 ($p < 0.01$) were significantly upregulated, while IL-2, TNF-α, IL-6 were slightly upregulated. The IL-4/IFN-γ mRNA ratios in the experimental groups were significantly higher than in the controls.

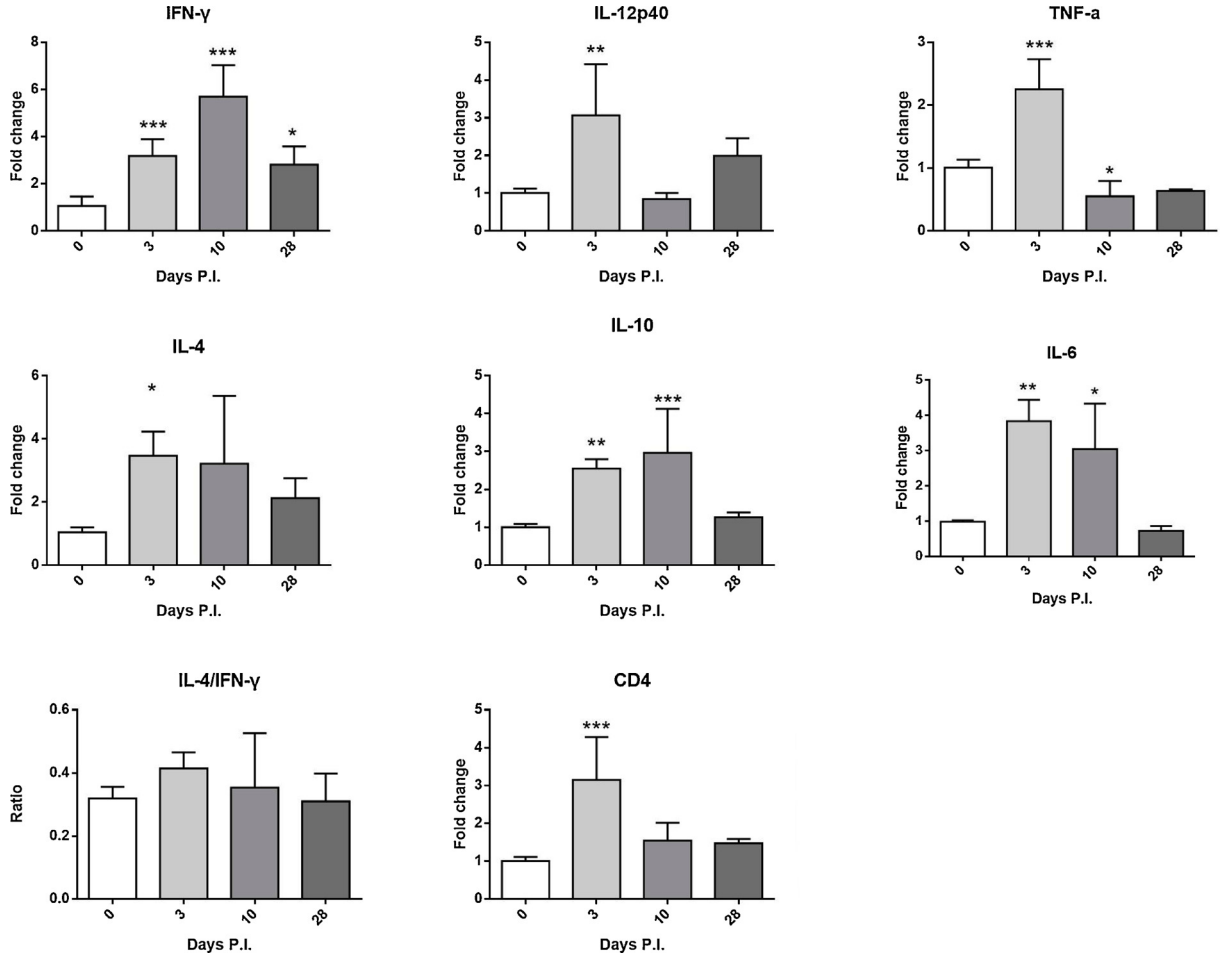

Fig. 1. Expression levels of relative cytokines and CD4 mRNA and IL-4:IFN-γ ratio from MLNs samples isolated from control and infected groups. Values represent the means ± SEM(error bars) ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control groups.

3.4. Cytokine expression in the spleen

At 3 DPI, the mRNA expression levels of the Th1-related cytokines IL-12p40 and TNF-α increased, as did the mRNA expression levels of the Th2-related cytokines IL-5 and IL-10. However, these increases were not significant (Fig. 3). At 98 DPI, the Th1-related cytokines IFN-γ ($p = 0.4$), IL-12p40 ($p = 0.3$), and TNF-α ($p < 0.001$), as well as the Th2-related cytokines IL-4 ($p < 0.001$), IL-5 ($p < 0.05$), and IL-10 ($p = 0.4$), were upregulated in the infected group. The IL-4/IFN-γ ratio was also higher in the infected group ($p < 0.01$). The mRNA expression level of IL-2 in the infected groups was significantly lower than the controls at 3, 10, 28, and 70 DPI, and was not significantly different from the controls at 42 and 98 DPI. This suggested that the cytokines IL-4 and IL-5 were more highly expressed during the acute stages of *F. gigantica* infection, indicated expression of the Th2-type immune response.

3.5. Determination of antibody isotypes in the serum

In the infected buffalo, total Ig levels increased significantly at 10 DPI, and continued to increase until 98 DPI. In contrast, IgG1 levels increased at 28 DPI, and continued to increase until 98 DPI. However, IgG2 responded poorly to *F. gigantica* ESPs, and there no significant changes were observed in any groups. The mean ratios for the IgG1/IgG2 antibody isotypes in the *F. gigantica*-infected buffalo were 1.37 ($p = 1.0$), 1.99 ($p = 1.0$), 3.61 ($p = 0.1$), 5.56 ($p < 0.001$), 8.18 ($p < 0.001$), and 9.17 ($p < 0.001$) at 3, 10, 28, 42, 70, and 98 DPI, respectively.

4. Discussion

The mechanisms by which *F. gigantica* evades the host immune system and establishes an infection are not fully understood. Most helminth infections are characterized by a Th2 host immune response (Emmanuelle and Alain, 2010; Grencis, 2015; Horsnell, 2014). The present study focused primarily on the local and systemic adaptive immune response during the acute and chronic stages of *F. gigantica* infection in swamp buffaloes, a natural host.

Here, the MLN cytokine levels were used to reflect the immune microenvironment of the gut and the peritoneal cavity during the acute stage of *F.* gigantica infection. During the early stage of infection, the MLNs showed a mixed Th1 and Th2 immune response. Clery et al. (1998) found that cattle PBMCs expressed higher level IFN-γ after stimulated with *F. hepatica* immature fluke antigens. Hoyle et al. (2003) measured higher lever production of IL-2, IL-4 and IFN-γin cattle hepatic lymph node cells after culture with *F. hepatica* whole fluke antigen (WFA). Cwiklinski et al. (2018) identified two upregulated genes in transcriptome data that were associated with the lipopolysaccharide (LPS)-response to *F. hepatica* metacercariae. When immature flukes penetrate the intestinal wall, bacteria or LPS adhere to the fluke tegument and are transported to the peritoneal cavity or liver. This may explain the high levels of the Th1-associated cytokines IFN-γ, IL-12p40, and TNF-α produced by the MLN and HLN during early infection (3 DPI and 10 DPI); these high levels of cytokine expression may be associated with parasite antigens (tegument antigens or ESP) and antimicrobial mechanism. Intriguingly, there were no differences in MLN cytokine levels at 28 DPI between the experimental and control groups (except

for IFN-γ). Pleasance et al. (2011) found that ITT sheep infected with *F. gigantica* had MLN levels similar to those of the control group at three weeks post infection. Hoyle and Taylor (2003) applied the *F. hepatica* whole fluke antigen (WFA) culture to cattle HLN cells and mesenteric lymph node cells. It was shown that HLN cells were more sensitive to adult fluke WFAs than MLN cells (Hoyle and Taylor, 2003). This may be because MLN cytokine levels return to normal after the juvenile worm has passed through the duodenal and intraperitoneal regions of the host.

The HLNs were used to reflect the local cytokine microenvironment of the liver during *F. gigantica* infection. CD4 cells are a common marker for Th1 and Th2 cells, and CD4 mRNA expression can be used to determine the bulk of CD4 cells (Galli et al., 2008; Mocellin et al., 2003; Pleasance et al., 2011). In this study, the number of CD4 cells in the HLNs did not differ significantly between the infected groups and the control group (except at 3 DPI). This was consistent with previous studies, which showed that HLNs remain swollen as the infection progresses, but the cell composition shows the inclination to B cells (Meeusen et al., 2010; Pérez et al., 2005). These results were also similar to those of Pleasance et al. (2011), who found no difference in CD4 mRNA expression in the HLNs of *F. gigantica*-infected and control ITT sheep at 3 and 10 weeks post infection (WPI) (Pleasance et al., 2011). Sachdev et al. (2017) suggested that the exhaustion of CD4 cells in chronically *F. hepatica*-infected bovine HLNs likely resulted in a failure to resist the parasite. *Schistosoma japonicum*-infected mice induced T-cells co-expressing IFN-γ(+)/IL-4(+)/CD4(+), but not Th1 or Th2 cells. In addition, the upregulation of IL-10 in the IFN-γ(+)/IL-4(+) cells, which benefits the cells, stimulates the differentiation of these cells into Th2 cells (Chen et al., 2016). The mRNA expression of cytokines IL-4, IFN-γ, and CD4 was upregulated at 3 DPI in HLNs and MLNs (Figs.1 and 2). This indicated that active IFN-γ(+)/IL-4(+)/CD4(+) cells in the HLNs and MLNs might tend to differentiate into Th2 cells during early infection in the presence of high levels of IL-10 and activated T cells. The upregulation of the Th2 cytokines IL-4 and IL-5 and the downregulation of the Th1 cytokines IFN-γ and IL-12p40 during chronic infection supported this possibility.

Crossbred calves infected with *F. gigantica* expressed detectable levels of IL-10 in PBMCs at 10, 30, and 75 DPI (Miller et al., 2009; Ingale et al., 2010). IL-10 is an effective anti-inflammatory cytokine (Kocacik Uygun et al., 2019). During parasite infection, persistent IL-10 immunosuppression can be both favorable to the host (allowing the host to maintain a protective Th2 immune response) as well as to the parasite (by inhibiting inflammatory responses) (Beiting et al., 2007; Larson et al., 2012). IL-12 expression limits the development of *S. japonicum*. Here, worm development in hosts expressing mouse anti-IL-12 was significantly enhanced, and more eggs were produced at 28 DPI as compared to controls. However, these effects were eliminated at 42 DPI (Cheng et al., 2012). Higher mRNA expression of IL-12p40 in the HLNs (Fig. 2) was obvious at the acute stages of infection (3, 10, and 28 DPI). During acute infection, IL-12p40 may limit parasite development to minimize the liver damage caused by parasite migration. During chronic infection, Th1 cytokines may reduce fibrosis and Th2 cytokines may increase fibrosis (Deaton et al., 2014; Figueiredo et al., 2016) when flukes migrate through the liver parenchyma, and settle in the bile duct. The expression of Th1 cytokines (IL-2, IL-12p40, and TNF-α) may regulate the balance between fibrosis and injury repair. At 70 DPI, the expression levels of IL-2, IL-12, TNF-α, and CD4 peaked, indicating that the parasite had evaded host defenses. Both Th1 (IL-12p40 and TNF-α) and Th2 (IL-4, IL-5, and IL-10) cytokines repair damage caused by pathogens or immune defenses.

The levels of Th1- and Th2-type immune response-related cytokines in the buffalo spleens were used to reflect the systemic immune

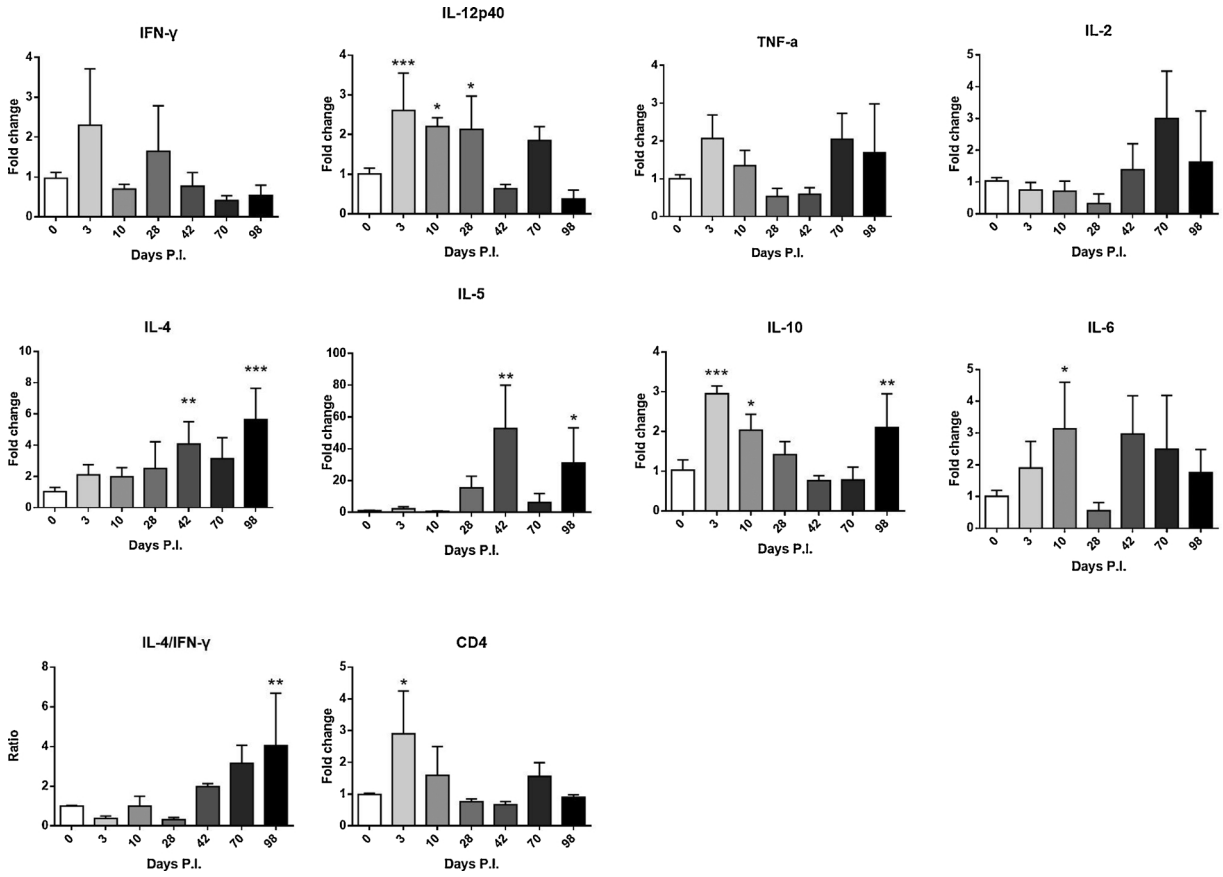

Fig. 2. Expression levels of relative cytokines and CD4 mRNA and IL-4:IFN-γ ratio from LNs samples isolated from control and infected groups. Values represent the means ± SEM(error bars) ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control groups.

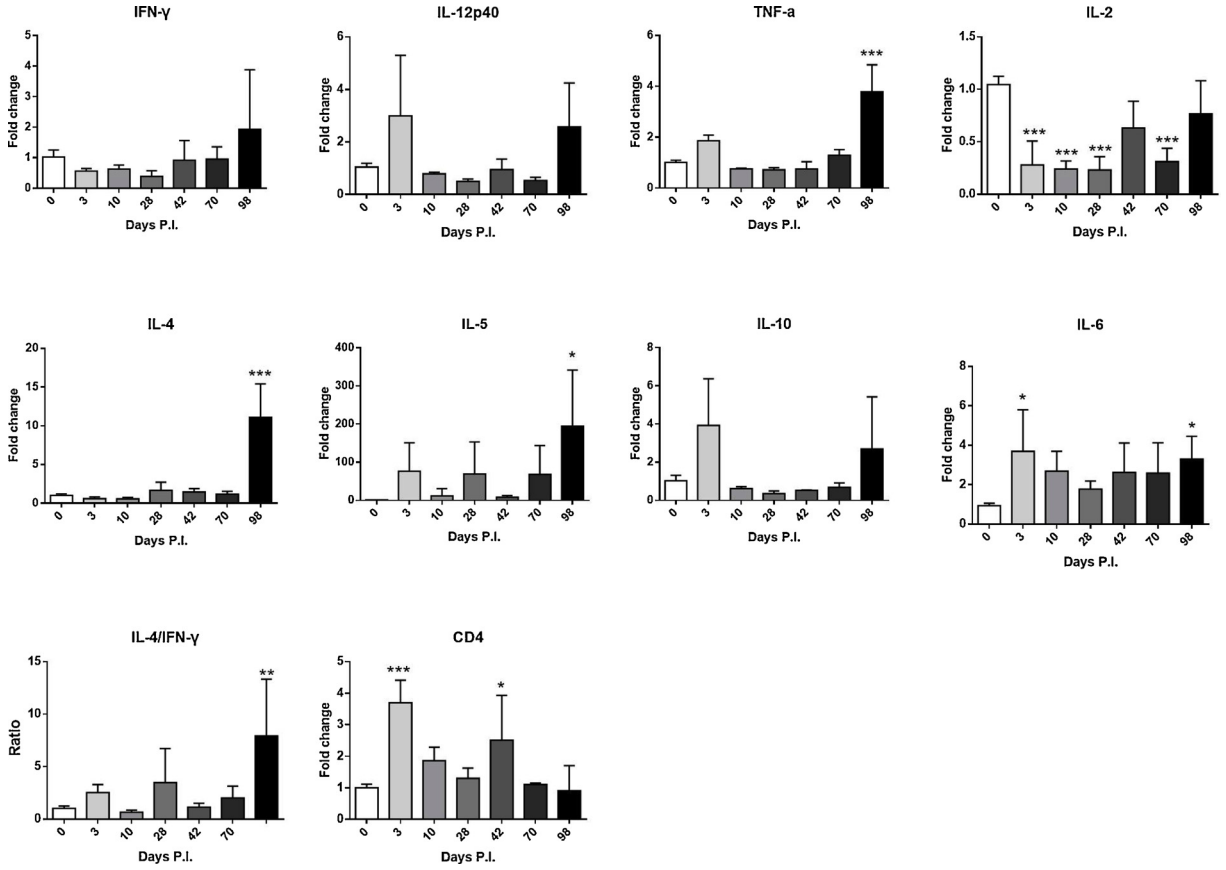

Fig. 3. Expression levels of relative cytokines and CD4 mRNA and IL-4:IFN-γ ratio from Spleens samples isolated from control and infected groups.Values represent the means ± SEM(error bars) ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control groups.

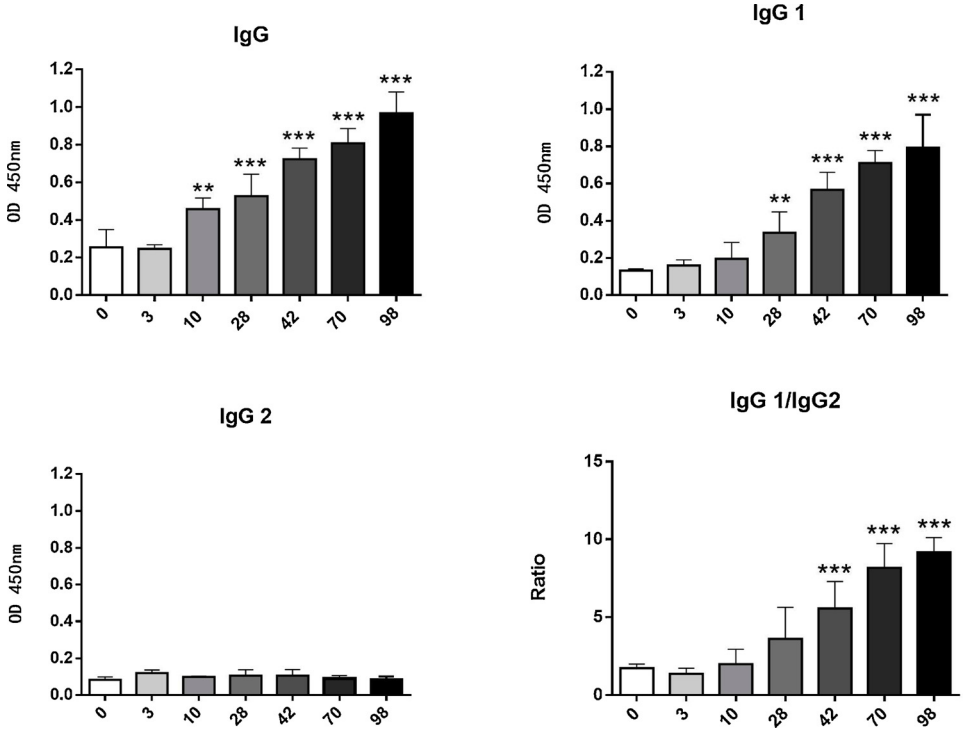

Fig. 4. The adjusted mean ± SEM ELISA OD (450 nm) for total IgG, IgG1, IgG2 and ratio of IgG1:IgG2 of *F. gigantica* infected groups and control groups to *F. gigantica*-ES products. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control groups.

response after infection with *F. gigantica* (Pleasance et al., 2011). Cattle and buffaloes infected with *F. gigantica* invoked a Th2 immune response during early infection (Ingale et al., 2010; Molina, 2005). A Th2-type immune response during the early stage of *F. gigantica* infection in buffalo was recently identified indicated by high serum levels of IL-13 (Zhang et al., 2017b). However, during chronic infection, a mixed Th1/Th2-type immune response was evident. In buffalo challenged with a primary *F. gigantica* infection, PBMCs expressed high levels of the Th1-related cytokines IL-2 and IFN-γ as well as high levels of the Th2-related cytokines IL-4 and IL-6 (Kumar et al., 2013). Here, IL-6 expression was higher in all infected groups than in the control group (Fig. 3). Increased serum levels of IL-4, IL-6, or IL-10 have been observed in cattle, buffalo, sheep, and humans infected with *F. gigantica* or *F. hepatica* (Khalil et al., 1999; Molina, 2005). IL-6 might stimulate a Th2 cell response and inhibit Th1 cell activity (Rincón et al., 1997). IL-6 also plays an important role in B cell maturation and the antibody switch to the IgG1 isotype (Hunter and Jones, 2015). Here, the serum IgG1 titers were also higher in the experimental groups as compared to the control group (Fig. 4). During chronic infection, Th1-related cytokines may also regulate the balance between fibrosis and injury repair, as detailed above.

Through dynamic changes in the cytokine environment in the MLN, HLN, and spleen during *F. gigantica* infection, host expression of Th2-related cytokines IL-4, IL-5, IL-6, and IL-10 accompanied parasite invasion (acute infection) and colonization (chronic infection). Thus, these results suggested that a Th2-type immune response was essential for effective *F. gigantica* infection.

The antibody levels of IgG1 and IgG2 are closely related to the Th1 and Th2 immune responses (Estes and Brown, 2002). In ruminants infected with *Fasciola* spp., high levels of the IgG1 antibody isotype were detected; IgG2 was undetectable in the serum (Hansen et al., 1999; Mulcahy et al., 1998; Phiri et al., 2006; Pleasance et al., 2011). In the buffalo exposed to primary *F. gigantica* infection, specific IgG and IgG1 responses to *F. gigantica* ESPs were observed (Fig. 3). This implied that the production of IgG1 was positively regulated by the Th2-associated cytokines IL-4 and IL-6, while IgG2 was positively regulated by the Th1-associated cytokine IFN-γ (Estes and Brown, 2002; Estes et al., 1994, 1995; Suematsu, et al., 1989; Trigona et al., 1999). Interestingly, the IgG1/IgG2 (Fig. 3) antibody ratio increased with the IL-4/IFN-γ ratio in the HLNs (Fig. 1) at 42, 70 and 98 DPI. These results suggested that buffalo were susceptible to *F. gigantica* infection.

The results of this study suggested that the local Th2-related cytokine environment was associated with the susceptibly of the natural host (swamp buffalo) to *F. gigantica* infection. The Th2-type immune response was conspicuous in the HLNs, spleens, and sera of the buffalo in the chronic infection groups. In addition, Th2-related cytokines accompanied parasite migration in the host, increasing levels of the IgG1 antibody in the serum and repairing the injuries caused by parasite migration. Different immune responses in MLNs, HLNs, and spleens during early infection were also identified. The Th1 cytokines initially produced in the MLNs and HLNs may be connected with antibacterial activity and injury repair. However, changes in the T-cell populations in local and systemic immune organs remain to be demonstrated. It is also unclear whether these cells are functionally important during parasite infection.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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