



OPEN ACCESS

EDITED BY

Chao Yan,
Xuzhou Medical University, China

REVIEWED BY

Yue Xie,
Sichuan Agricultural University, China
Xiao-Xuan Zhang,
Qingdao Agricultural University, China

*CORRESPONDENCE

Run-Min Kang
✉ angelina_0708@hotmail.com
Yong-Gang Ye
✉ yeyg0202@sina.com

RECEIVED 29 May 2023

ACCEPTED 11 July 2023

PUBLISHED 01 August 2023

CITATION

Chen X-D, Xie J, Wei Y, Yu J-F, Cao Y, Xiao L, Wu X-J, Mao C-J, Kang R-M and Ye Y-G (2023) Immune modulation of Th1/Th2/Treg/Th17/Th9/Th21 cells in rabbits infected with *Eimeria stiedai*. *Front. Cell. Infect. Microbiol.* 13:1230689. doi: 10.3389/fcimb.2023.1230689

COPYRIGHT

© 2023 Chen, Xie, Wei, Yu, Cao, Xiao, Wu, Mao, Kang and Ye. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Immune modulation of Th1/Th2/Treg/Th17/Th9/Th21 cells in rabbits infected with *Eimeria stiedai*

Xiao-Di Chen^{1,2}, Jing Xie¹, Yong Wei¹, Ji-Feng Yu¹, Ye Cao¹, Lu Xiao¹, Xue-Jing Wu¹, Cong-Jian Mao¹, Run-Min Kang^{1*} and Yong-Gang Ye^{1*}

¹Key Laboratory of Animal Genetic and Breeding of Sichuan Province, Sichuan Animal Science Academy, Chengdu, China, ²College of Animal and Veterinary Sciences, Southwest Minzu University, Chengdu, China

Introduction: Despite long-term integrated control programs for *Eimeria stiedai* infection in China, hepatic coccidiosis in rabbits persists. Th1, Th2, Th17, Treg, Th9, and Th21 cells are involved in immune responses during pathogen infection. It is unclear whether Th cell subsets are also involved in *E. stiedai* infection. Their roles in the immunopathology of this infection remain unknown. Therefore, monitoring these T-cell subsets' immune responses during primary infection of *E. stiedai* at both transcriptional (mRNA) and protein (cytokines) levels is essential.

Methods: In experimentally infected New Zealand white rabbits, mRNA expression levels of their transcript—TBX2 (Th1), GATA3 (Th2), RORC (Th17), Foxp3 (Treg), SPI1 (Th9), and BCL6 (Th21)—were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR), whereas Th1 (IFN- γ and TNF- α), Th2 (IL4), Th17 (IL17A and IL6), Treg (IL10 and TGF- β 1), Th9 (IL9), and Th21 (IL21) cytokines were measured using enzyme-linked immunosorbent assays (ELISAs).

Results: We found that levels of TBX2, GATA3, RORC, SPI1, and BCL6 in the livers of infected rabbits were elevated on days 5 and 15 post-infection (PI). The concentrations of their distinctive cytokines IFN- γ and TNF- α for Th1, IL4 for Th2, IL17A for Th17, IL9 for Th9, IL21 for Th21, and IL10 for Treg IL10 were also significantly increased on days 5 and 15 PI, respectively ($p < 0.05$). On day 23 PI, GATA3 with its cytokine IL4, RORC with IL17A, Foxp3 with IL10 and TGF- β 1, and SPI1 with IL9 were significantly decreased, but TBX2 with IFN- γ and IL6 remained elevated.

Discussion: Our findings are the first evidence of Th1/Th2/Treg/Th17/Th9/Th21 changes in *E. stiedai*-infected rabbits and provide insights into immune regulation mechanisms and possible vaccine development.

KEYWORDS

E. stiedai, TBX2, GATA3, RORC, Foxp3, SPI1, BCL6

Introduction

Hepatic coccidiosis in rabbits is caused by *Eimeria stiedai*. First discovered more than 100 years ago, it has a high host specificity, infectivity, and lethality. Post-weaning rabbits are particularly susceptible, with mortality as high as 80% (Gomez-Bautista et al., 1987; Pakandl, 2009). Unlike the other 10 species of rabbit intestinal coccidia, *E. stiedai* lives in the rabbit liver to complete its entire development (Duszynski and Couch, 2013). The life cycle of *E. stiedai* occurs both in the external environment and within the host rabbit, and generally experiences three stages, *Sporogony*, *Schizogony* or *Merogony*, and *Gametogony*. Firstly, sporulated oocysts are consumed by rabbits and rupture, then released sporozoites invade the liver and bile duct epithelial cells where merogony occurs. Merozoites multiply within and are released from epithelial cells and continue to increase until they complete a repeat of at least four generations. Lastly, the life cycle enters the gametogony stage, macrogametes and microgametes combine into zygotes and eventually develop into oocysts passed out in the feces. *E. stiedai* causes severe damage to the hepatobiliary epithelial structure, affects the normal hepatobiliary function, leads to slow growth and high mortality in diseased rabbits (Oliveira et al., 2011; Jing et al., 2016), and causes severe harm to the rabbit industry. It is listed as a second-class animal disease by the Ministry of Agriculture of the People's Republic of China.

T helper cells with specific cytokines (IFN- γ , IL4, IL10, IL17A, IL21, IL9, TNF- α , and TGF- β 1) play essential roles in parasitic diseases (Pesce et al., 2006; Seki et al., 2012; Pang et al., 2014; Li et al., 2017; Kalantari et al., 2019; Bai et al., 2021; Al-Masoudi et al., 2021; Parhizgar et al., 2021; Li et al., 2022). Usually, interferon- γ (IFN- γ) is induced by *TBX2*, but in New Zealand white rabbits infected with *Schistosoma japonicum* (*S. japonicum*), it was found that the transcription factor *TBX2* was positively correlated to TNF- α , but not to IFN- γ , which was correlated to another Th1 transcriptional factor, *IRF8* (Farwa et al., 2018); thus, our study aimed to evaluate the correlation between T helper cells, transcription factors (*TBX2*, *GATA3*, *Foxp3*, *RORC*, *SPI1*, and *BCL6*), and cytokines (IFN- γ , IL4, IL10, IL17A, IL21, IL9, TNF- α , and TGF- β 1) in rabbits infected with *E. stiedai*.

The Th1 cytokine IFN- γ sometimes protects against damage, while the Th2 cytokine IL4 enhances fibrogenesis (McManus et al., 2010; Du et al., 2011). Treg cells that produce IL10 cytokines play a crucial role in the immune balance; it acts as an immunosuppressant or a fibrotic inducer (Layland et al., 2010; Adalid-Peralta et al., 2011; Zhou et al., 2015). For example, in *Schistosoma mansoni*-infected mice, higher expression levels of hepatic IL17A were found in CBA mice, suggesting that its lesions may be associated with IL17A produced by Th17 cells (Kalantari et al., 2019). However, lacking the IL21/IL21R axis in *S. mansoni*-infected mice attenuated granulomatous inflammation and liver fibrosis formation in mice (Pesce et al., 2006). There were significantly increased levels of Th9 (IL9) in the liver of C57BL/6 mice infected by *S. japonica* in the early stage, suggesting that IL9 may be involved in liver injury (Li et al., 2017). However, whether these Th subsets and their cytokines also play an immuno-protective or damaging role in *E. stiedai* infection is unknown.

TBX2 induces the production of interferon- γ (IFN- γ) and orchestrates the Th1 cell migration by regulating the expression of chemokines and chemokine receptors. *TBX2* and *GATA3* regulate Th1/Th2 cell fate using a synchronous mechanism, by directly binding to the genetic locus of cytokines: *TBX2* binds IFN- γ whereas *GATA3* binds IL4 and other Th2 cytokines such as IL5 and IL13, leading to the expression of these proteins (Kanhere et al., 2012).

Transcriptional factor *Foxp3* serves as a lineage specification factor of Treg cells, and is induced either during native Treg (nTreg) development in the thymus or in the presence of TGF- β 1 and retinoic acid. High levels of IL17 is expressed by Th17 under the transcriptional regulation of *RORC*. In some studies, it was suggested that the signature transcription factor *RORC* of Th17 cells is also induced by TGF- β 1, thus leading to differentiation of Treg and Th17 lineages. In the absence of a second signal derived from proinflammatory cytokines, *Foxp3* can inhibit *RORC* function and promote Treg differentiation. However, when cells also receive signals from proinflammatory cytokines (e.g., IL6), the function of *Foxp3* is inhibited and Th17 differentiation is induced. Thus, it is the balance between *Foxp3* and *RORC* function that determines the fate of CD4+T cells and the type of immune response that would be generated (Ziegler and Buckner, 2009; Ren and Li, 2017).

PU.1 (*SPI1*) is a transcription factor that belongs to the E-twenty six (ETS) family of proteins and is a key factor for the differentiation of the IL9-producing Th9 subset, in humans and mice (Chang et al., 2010; Ramming et al., 2012). *BCL6* served as a master regulator of Th21 differentiation, and Th21 differentiation does not occur *in vivo* in the absence of *BCL6* (*BCL6* -/-) (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). While *BCL6* is vital in both GC B cells and Th21 cells, it can control Th21 differentiation mainly by regulating genes of GC B cells (Crotty et al., 2010).

In *E. stiedai* infection, it is unclear whether different mechanisms are involved. For these reasons, we measured dynamic changes in Th cells, including Th1, Th2, Th17, Treg, Th9, and Th21, during different stages of *E. stiedai* infection. Our findings provide a theoretical basis to control for protective and therapeutic vaccinations.

Materials and methods

Ethics statement

This study was carried out in full compliance with the framework of noncommercial scientific research, authorized by the Animal Ethics Committee of Sichuan Animal Science Academy (approval no. AECSASA2023-18).

Parasites

The *E. stiedai* strain in this study was provided by the Department of Parasitology at Sichuan Agricultural University. Three coccidia-free New Zealand white rabbits (5 weeks old) were orally infected with *E. stiedai* to obtain more oocysts. Then high numbers of unsporulated oocysts were harvested from liver with

grinding, filtration, and centrifugation. Finally, the oocysts were sporulated in 2.5% potassium dichromate solution at 28°C and stored at 4°C.

Experimental rabbits

We divided 36 female New Zealand white rabbits aged 50 days into a control group ($n = 18$, six rabbits for each time point) and an experiment group ($n = 18$, six rabbits for each time point). They received 1.0×10^5 *E. stiedai* orally and were euthanized on days 5, 15, and 23 post-infection (PI). All rabbits were monitored for 5 days by testing the daily collected fecal samples to confirm the infection. Blood samples were collected for quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assays (ELISAs).

Blood sample collection

We collected 3 ml of blood from the rabbits on days 5, 15, and 23 PI. Serum was also collected and stored at -80°C to evaluate Th1/Th2/Treg/Th17/Th9/Th21-related cellular cytokines.

RNA extraction and cDNA preparation

RNA was extracted from the isolated blood using the TRIzol method (TRIzol reagent provided by Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocols. cDNA preparation was performed in a 20- μl mixture condition using Hifair[®] II 1st Strand cDNA Synthesis SuperMix for qRT-PCR (Yeasen Biotechnology Co., Ltd) as follows: 4 μl of RNA, 2 μl of 5 \times gDNA digester Buffer, 1 μl of gDNA, 10 μl of 2 \times Hifair[®] II SuperMix plus, and 3 μl of sterile RNase-free water. The amplification conditions were 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Primer design and qRT-PCR amplification

CDS sequences of *TBX2*, *GATA3*, *Foxp3*, *RORC*, *BCL6*, and *SPI1* genes and housekeeping *GAPDH* as a reference gene were retrieved from the GenBank database with their accession numbers as illustrated in C 1. For qRT-PCR expression profiles, primer pairs of each target gene were designed by Premier 6.0 software, where their length ranged from 18 to 25 bp with 55%–65% of GC content and different annealing temperatures (Table 1). All primers were synthesized by Tsingke Biotechnology (Chengdu) Co., Ltd. qRT-PCR was performed in a 20- μl mixture condition using Hieff UNICON[®] Universal Blue qPCR SYBR Green Master Mix (Yeasen Biotechnology Co., Ltd) as follows: 1 μl of cDNA, 10 μl of Master Mix, 0.4 μl of forward primer, 0.4 μl of reverse primer, and 8.2 μl of sterile RNase-free water. The amplification conditions started with initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, 57°C for 30 s at annealing temperature, and the melting curve stage at 95°C for 15 s, 55°C for 15 s, followed by 95°C for 15 s.

ELISA

According to the manufacturer's instructions (Shanghai MLBIO Biotechnology Co. Ltd), an ELISA test was performed to measure nine cytokine levels—IFN- γ , TNF- α , IL4, IL10, TGF- β 1, IL17A, IL6, IL9, and IL21—for the control and experiment group. Concentrations of plasma cytokines were calculated by using ELISA Calc v 0.1 software.

Hepatic histopathology

Rabbits were euthanized on days 5, 15, and 23 PI. Partial left lobes of the livers from all rabbits were fixed in 10% neutral buffered formalin and embedded in paraffin for histological examination.

TABLE 1 Characteristics of primers used for qRT-PCR.

Primer name	Accession no.	Sequence (5' → 3')	T_m (°C)	Product size (bp)
TBX2	XM_008271188.1	Forward: CCAACGACATCCTCAAGCTG; reverse: TTGTCGTCTGGTAGGCAGT	58	94
GATA3	XM_002717361.2	Forward: AGAAGGAAGGCATCCAGACC; reverse: AGGACATGTGTCTGGACAGG	58	135
Foxp3	XM_002719910.2	Forward: TTCCGAAACCACCTGCTAC; reverse: AACTCATCCACGGTCCACAC	59	113
RORC	XM_008264444.2	Forward: ACCAGATCGTGCTCCTCAAA; reverse: ATGCCACCGTATTTGCCTTC	59	112
BCL6	XM_008266642.2	Forward: ATCAAAGCCAGTGAAGCGGA; reverse: AAAGGCTCTGCTCTCACACC	59	168
SPI1	XM_017348391.1	Forward: GTTCCCTACGACCGGATCT; reverse: TGTGGCTCTCTCCATCACTG	58	79
GAPDH	NM_001082253.1	Forward: TGACGACATCAAGAAGGTGGTG; reverse: GAAGGTGGAGGAGTGGGTGTC	60	121

Total hepatic oocysts

The total liver weight was determined by electronic weighing, then 10 g of the left liver lobe was ground, dissolved in 50 ml of physiological saline, and filtered with an 80-mesh filter screen. Counts were performed with a hemocytometer.

Statistical analysis

The general linear model procedure in SPSS 22.0 version was used to analyze mRNA expression levels of *TBX2*, *GATA3*, *Foxp3*, *RORC*, *BCL6*, and *SPI1*, as well as plasma cytokine levels of IFN- γ , TNF- α , IL4, IL6, IL10, IL9, IL17, IL21, and TGF- β 1. Differences were considered significantly different when $p < 0.05$. Regression and correlation analyses were performed to detect the relationships between each mRNA transcriptional factor and every cytokine individually. Another set of regression and correlation was performed to determine the relationships between different mRNA transcriptional factors and the relationships between transcriptional factors and oocyst data, all at three time points (on days 5, 15, and 23 PI). $|r|$ values ≥ 0.8 were considered statistically significant.

Results

Hepatic histopathological changes

No rabbit died during the infection period, and the rabbits were euthanized on days 5, 15, and 23 PI. The effect of *E. stiedai* infection on rabbit livers was investigated using stained paraffin-embedded tissues. In control group, as shown in Figures 1A–C and in the experiment group, as shown in Figures 1D–F, in the experiment group, substantial numbers of inflammatory cells had infiltrated around the portal area, collagen depositions were slightly stained, and intrahepatic bile duct epithelia were proliferated in the liver tissue. The intrahepatic bile ducts continued to be exaggerated extensively on days 15 and 23 PI (Figures 1E, F). Several merozoites were observed (black arrow) (Figure 1E). On day 23 PI, the proliferation of bile duct epithelial and numerous eggs of *E. stiedai* cells was observed (Figure 1F).

T helper-1 response: *TBX2* mRNA expressions and IFN- γ and TNF- α serum levels

On days 5 and 15 PI, *TBX2* mRNA expressions and the levels of IFN- γ and TNF- α in serum were significantly increased after the

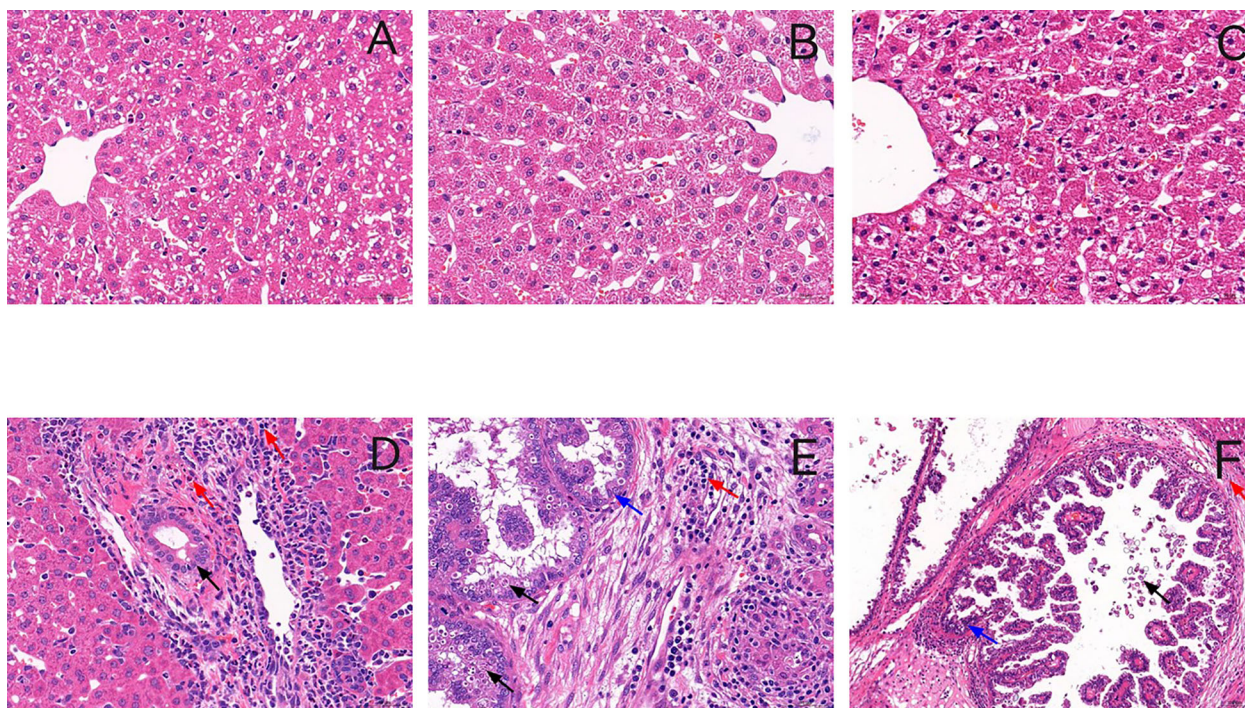


FIGURE 1

Representative histology of liver sections stained with hematoxylin and eosin. (A–C) show liver sections from the control group in three time points, and (D–F) show liver sections from the experiment group in three time points (on days 5, 15, and 23 PI) ($\times 400$). In the experiment group, massive inflammatory cells were infiltrated around the portal areas accompanied by cholangiocyte proliferation on day 5 PI (D). The number of inflammatory cells increased, and bile duct hyperplasia was exaggerated on day 15 PI (E). Many schizonts were observed (black arrow). On day 23 PI (post-infection) bile duct expansion, a proliferation of bile duct epithelial cells and fibrous tissue hyperplasia, and numerous *Eimeria stiedai* eggs appeared (F).

infection compared to the control group. However, on day 23 PI, the infected rabbits showed a significant decrease in $TNF-\alpha$ ($p < 0.05$), whereas $TBX2$ mRNA ($p < 0.05$) expression and $IFN-\gamma$ ($p > 0.05$) were elevated on day 23 PI (Figures 2A, 3A, B).

T helper-2 response: *GATA3* expressions and IL4 serum levels

On day 5 PI, *GATA3* mRNA and IL4 significantly increased after the infection compared to the control. Furthermore, on day 15 PI, IL4 was significantly increased ($p < 0.01$) without any effect on *GATA3* mRNA. On day 23 PI, the infected group showed a decrease in *GATA3* ($p < 0.01$), and IL4 was not significantly different ($p > 0.05$) (Figures 2B, 3C).

T helper-17 response: *RORC* mRNA expressions and IL17A and IL6 serum levels

The levels of *RORC* mRNA ($p < 0.001$) and IL17A ($p < 0.01$) in serum were significantly increased on day 5 PI, but there was no statistical difference in the IL6 serum levels. On day 15 PI, *RORC* mRNA ($p < 0.001$), IL17A ($p < 0.05$), and IL6 ($p < 0.01$) were significantly upregulated. On day 23 PI, *RORC* mRNA ($p < 0.01$) and IL17A ($p < 0.05$) oppositely decreased. Interestingly, IL6 serum levels were upregulated during the three periods in the experiment groups (Figures 2C, 3D, E).

T regulatory response: *Foxp3* mRNA expressions and IL10 and TGF- β 1 serum levels

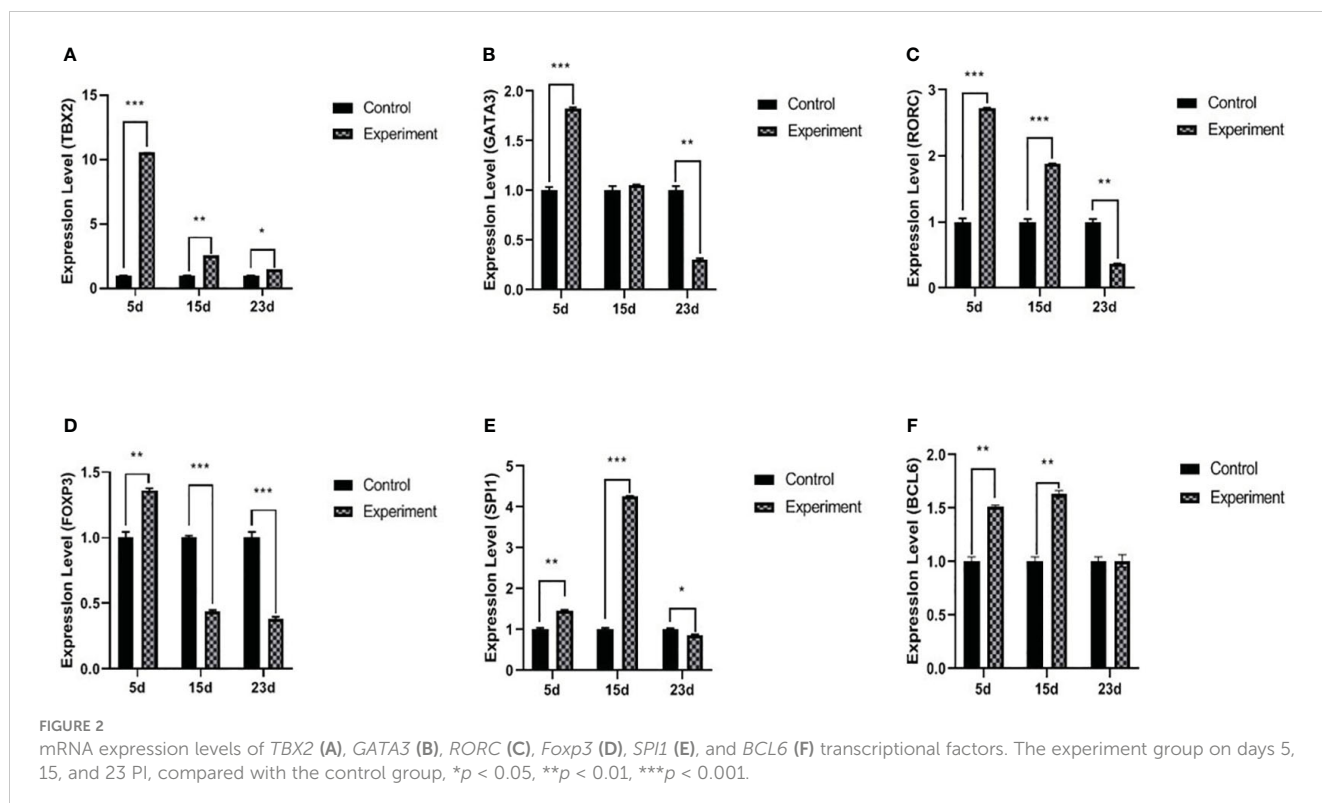
The level of *Foxp3* was significantly increased ($p < 0.001$) on day 5 PI, and IL10 and TGF- β 1 were also significantly increased ($p < 0.01$), compared with normal control. Interestingly, on day 15 PI, the level of *Foxp3* was significantly decreased ($p < 0.001$); however, IL10 and TGF- β 1 were still significantly increased ($p < 0.01$) compared with normal control. On day 23 PI, the levels of *Foxp3* and relative cytokines (IL10 and TGF- β 1) in serum were significantly decreased ($p < 0.05$) (Figures 2D, 3F, G).

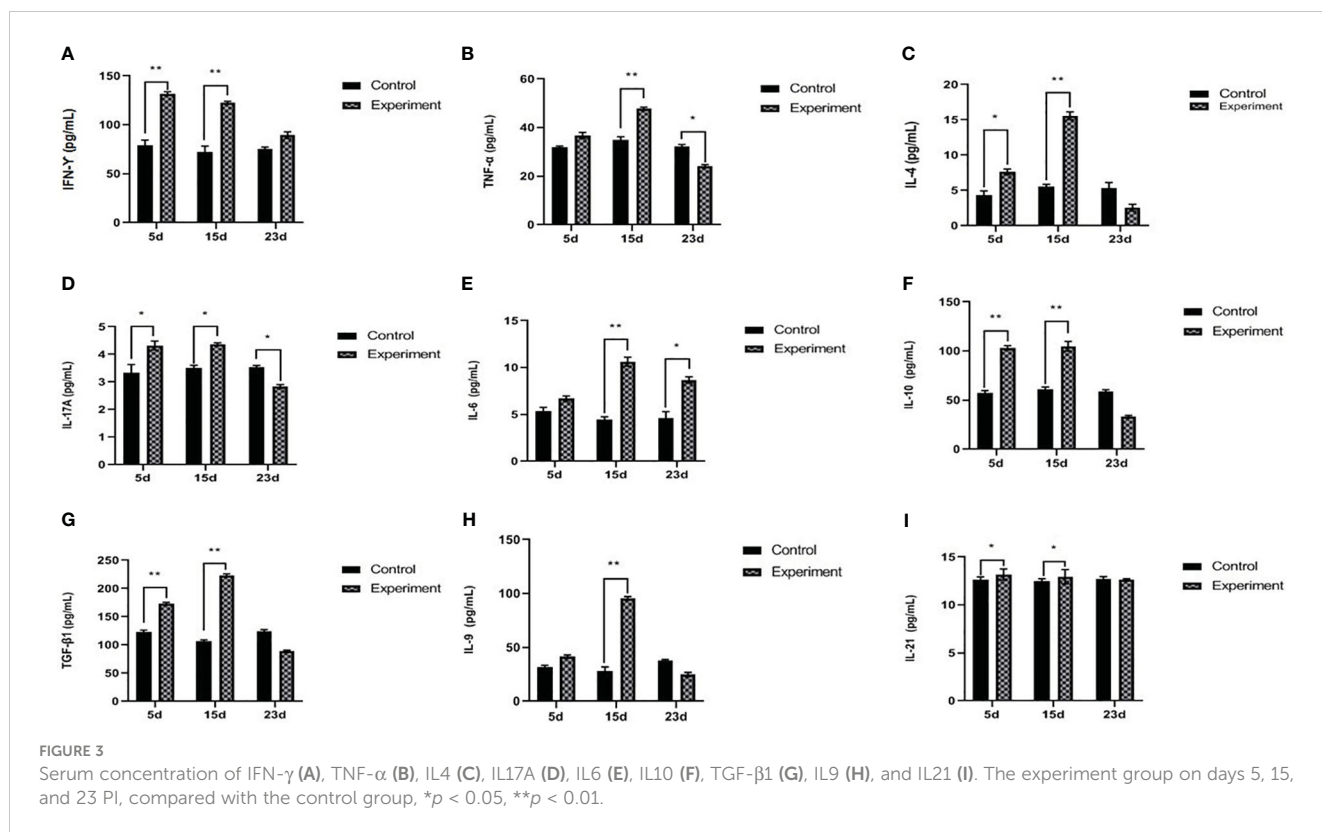
T helper-9 response: *SPI1* mRNA expressions and IL9 serum levels

On day 5 PI, the level of *SPI1* mRNA was significantly increased ($p < 0.01$); however, there was no statistical difference in IL9 ($p > 0.05$). On day 15 PI, *SPI1* mRNA ($p < 0.001$) and IL9 ($p < 0.01$) were significantly elevated. On day 23 PI, the level of *SPI1* mRNA was significantly decreased ($p < 0.05$), but there was no statistical difference in the concentration of IL9 between the normal control and infected groups (Figures 2E, 3H).

T helper-21 response: *BCL6* mRNA expressions and IL21 serum levels

BCL6 mRNA and IL21 were increased with the infection ($p < 0.01$ and $p < 0.05$, respectively) on day 5 PI. This influence was





relatively stable on day 15 PI (*BCL6*, $p < 0.01$ and *IL21*, $p < 0.05$). On day 23 PI, the levels of *BCL6* mRNA and *IL21* showed no statistical difference between the normal control and infected group ($p > 0.05$) (Figures 2F, 3I).

Relationships between different transcriptional factors and their cytokines

There are relationships between Th1/Th2/Th17/Treg/Th9/Th21 transcriptional factors with their cytokine lineages, where positive correlations ($r \geq 0.8$) were found between *RORC* and *IL17A*, *SPI1* and *IL9*, and *BCL6* and *IL21* in the experiment groups throughout the whole period of infection as shown in Table 2. *TBX2* was positively correlated to *TNF-α* on days 5 and 15 PI. Cross-correlations were detected between transcriptional factors and cytokines of other T helper subsets, where *TBX2* was negatively correlated to *IL10* on day 5 PI. *GATA3* was positively correlated to *IL6* during the whole period of infection. *GATA3* was negatively correlated to *IL4* on day 5 PI. *Foxp3* reported a positive correlation with *IL4* and *TGF-β1* ($r \geq 0.8$) on day 5 PI, and *RORC* negatively correlated to *TGF-β1* on days 15 and 23 PI.

The relationships between different transcriptional factors are illustrated in Figure 4.

Hepatic weight and oocyst data are illustrated in Table 3.

Correlation between transcriptional factors and oocyst data are illustrated in Table 4.

Discussion

We studied dynamic changes in Th mRNA expression levels of their transcript—*TBX2* (Th1), *GATA3* (Th2), *RORC* (Th17), *Foxp3* (Treg), *SPI1* (Th9), and *BCL6* (Th21)—and analyzed the potential effects of their imbalance on disease outcomes in the rabbit. Moreover, *E. stiedai* infections provide an excellent model to study the roles of Th cell transcriptional factors. In the early stage of the infection with *E. stiedai* (on day 5 PI), Th1 dominated at both transcriptional and protein levels. We also found a Treg response in the late stage of infection. Furthermore, our study also showed that Th17, Th9, and Th21 were involved in *E. stiedai* infection. However, helminth infection is characterized by Th2-dominated immune responses, which may contribute to chronic infection and the host's inability to clear the parasites (Kim et al., 2012; Uddin et al., 2012). For Th1, the transcription factor *TBX2* was positively correlated to *TNF-α*, but not *IFN-γ*, which Farwa found was correlated to another Th1 transcription factor *IRF8* (Farwa et al., 2018). Mechanistically, *TBX2*-dependent innate *IFN-γ* is essential in *T. gondii* infection T-bet to produce *IFN-γ*, which is essential for host resistance to intracellular infection by maintaining *IRF8* inflammatory dendritic cells at the infection site (Lopez-Yglesias et al., 2021). A report showed that loss of *TBX2* had some effect on *IFN-γ* production in T cells but not parasite-specific T cells (Harms Pritchard et al., 2015).

For Th2, the expression levels of *GATA3* and *IL4* appeared to be elevated on days 5 and 15 PI, and *GATA3* mRNA expression was

TABLE 2 Correlation between transcriptional factors and their cytokines.

		TBX2	GATA3	RORC	Foxp3	SPI1	BCL6
5 days	IFN- γ	—	—	—	—	—	—
	TNF- α	0.998	—	—	—	—	—
	IL4	—	—	—	0.817	—	—
	IL17A	—	—	0.904	—	—	—
	IL6	—	0.807	—	—	—	—
	IL10	-0.900	—	—	—	—	—
	TGF- β 1	—	—	—	0.964	—	—
	IL9	—	—	—	—	0.897	—
	IL21	—	—	—	—	—	0.999
15 days	IFN- γ	—	—	—	—	—	—
	TNF- α	0.800	—	—	—	—	—
	IL4	—	-0.976	—	—	—	—
	IL17A	—	—	0.989	—	—	—
	IL6	—	0.821	—	—	—	—
	IL10	—	—	—	—	—	—
	TGF- β 1	—	—	-0.954	—	—	—
	IL9	—	—	—	—	0.858	—
	IL21	—	—	—	—	—	0.897
23 days	IFN- γ	—	—	—	—	—	—
	TNF- α	0.8113	—	—	—	—	—
	IL4	—	—	—	—	—	—
	IL17A	—	—	0.863	—	—	—
	IL6	—	0.884	—	—	—	—
	IL10	—	—	—	—	—	—
	TGF- β 1	—	—	-0.996	—	—	—
	IL9	—	—	—	—	0.811	—
	IL21	—	—	—	—	—	0.812

uncorrelated to IL4, similar to *GATA3* mRNA expression, which was essential for IL13 production but not IL4 in the experiment group, whereas IL4 appeared to be a STAT6-dependent stimulation (Zhu, 2010). Regarding Treg, the expression levels of *Foxp3* also appeared to be elevated. TGF- β 1 was also induced simultaneously, similar to an observation by Tang et al. (2015) on day 5 PI. *Foxp3* appeared to have elevated expression levels, and the same result appeared in *S. japonicum* infection, which was a potent inducer for *Foxp3* (Finlay et al., 2014). For Th17, *RORC* expression was correlated to IL17A. On days 5 and 15 PI, Th17 mRNA and IL17A expression levels were elevated, similarly in *S. mansoni*-infected mice, in which the liver developed significant hepatic granulomatous inflammation, with higher expression levels of IL17 and *RORC*, suggesting that its lesions may be associated with IL17 produced by Th 17 cells (Kalantari et al., 2019). For Th9, the expression levels of *SPI1* and IL9 appeared to be elevated

on days 5 and 15 PI, and *SPI1* expression was correlated to IL9. The expression of IL9 and its transcription factor *SPI1* in hydatid patients was significantly higher in focal tissue, suggesting the formation of chronic granuloma in hydatid by upregulating the expression of Th9 cells, IL9, and transcription factor *SPI1* during infection (Tuxun et al., 2015). The expression levels of Th21, *BCL6*, and IL21 appeared to be elevated on days 5 and 15 PI, and *BCL6* expression was correlated to IL21. A correlation study of IL21 and transcription factor *BCL6* showed that IL21 was positively correlated to *BCL6*, which was upregulated on Th21 cells, and IL21 is the primary effector molecule of Tfh cells. (Geng et al., 2018).

In addition, many cross-modulations were observed; TBX2 suppressed *GATA3* expression on day 5 PI (Zhu, 2010). Cross-modulations between *GATA3* and *Foxp3* were observed in Treg cells, where *GATA3* expression enhanced the accumulation of Treg in the inflammatory site (Turner et al., 2011). On days 5 and 23 PI,

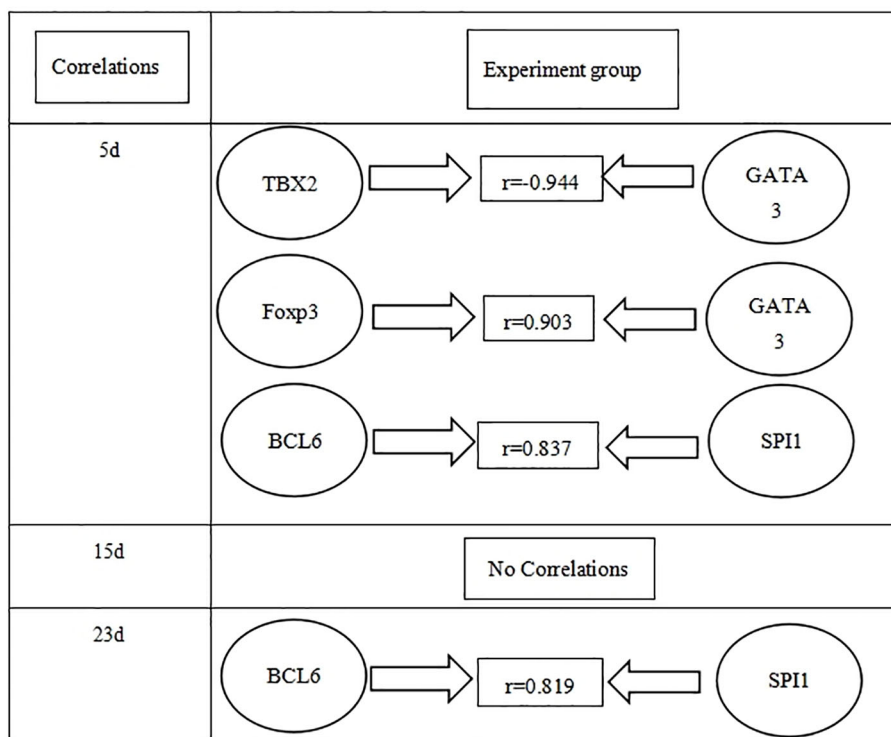


FIGURE 4 Correlations between different transcriptional factors in the experiment group in three time points (days 5, 15, and 23 PI).

TABLE 3 Liver weight and oocysts data in the experiment group at three time points.

Number/Infection time/Liver weight (g)/Oocyst data		1#	2#	3#	4#	5#	6#
5 days	Liver weight(g)	50.21	49.54	48.34	47.51	49.19	47.55
	Oocyst data	0	0	0	0	0	0
15 days	Liver weight(g)	81.85	84.89	84.79	74.72	70.39	86.71
	Oocyst data	1.49×10 ⁶	1.51×10 ⁶	1.50×10 ⁶	1.34×10 ⁶	1.27×10 ⁶	1.53×10 ⁶
23 days	Liver weight(g)	274.24	242.27	235.96	233.75	200.00	257.00
	Oocyst data	1.43×10 ⁹	1.39×10 ⁹	1.27×10 ⁹	1.26×10 ⁹	1.02×10 ⁹	1.40×10 ⁹

we also observed the cross-modulations between *SPI1* and *BCL6*. The expression of B-cell lymphoma protein 6 (*BCL6*) depends on *IRF8* and *SPI1* *in vivo*, which is essential for developing germinal center B cells, and *SPI1* acts as a regulator of *BCL6* expression during GC B-cell development (Wang et al., 2019). *GATA3* promotes Th2 differentiation while inhibiting Th1, and *TBX2* has

been shown to promote the development of Th1 and IFN- γ production (Szabo et al., 2000), but a study contradicted this finding (Cobb et al., 2010). A study showed that the IL21/IL21R signaling axis regulated the effector functions of T-cell subsets (e.g., production of cytokines) by enhancing *TBX2* and *STAT4* expression in human T cells, thereby enhancing IFN- γ production

TABLE 4 Correlation between transcriptional factors and oocysts data.

		TBX2	GATA3	RORC	Foxp3	SPI1	BCL6
Oocysts	5 days	—	—	—	—	—	—
	15 days	—	—	—	—	—	—
	23 days	—	—	-0.968	-0.908	-0.935	—

(Solaymani-Mohammadi et al., 2019). Treg only regulated TGF- β 1 and was independent of type 2 cytokines (Mishra et al., 2014). In general, IL6 combined with TGF- β induced differentiation of Th17 cells from initial CD4+T cells and was also polarized into Treg cells after co-stimulation with IL2 and TGF- β (Kimura and Kishimoto, 2010). Our study found interesting findings: *GATA3*, *Foxp3*, *RORC*, and *SPI1* mRNA and its relative cytokines (such as IL4, IL10, IL17A, and IL9 in the serum) were significantly decreased in the late stage, which suggests that they were suppressed.

In the experiment group, liver weight increased, and the significant proliferation of eggs of *E. stiedai* made liver lesions severe (Table 3). In the liver sections, massive inflammatory cells were infiltrated around the portal areas accompanied by cholangiocyte proliferation on day 5 PI (Figure 1D). *TBX2*, *GATA3*, *Foxp3*, *RORC*, *BCL6*, *SPI1*, and relative cytokine expression levels were elevated, which resisted *E. stiedai* infection. Several merozoites were observed on day 15 PI (Figure 1E) (black arrow). *TBX2*, *RORC*, *BCL6*, *SPI1*, and relative cytokine expression levels were elevated, and *Foxp3* expression levels were decreased, likely due to dynamic Treg/Th17 changes. On day 23 PI, the appearance of numerous eggs of *E. stiedai* was consistent with previous reports (Figure 1F), except *TBX2* expression levels were elevated; *GATA3*, *RORC*, *BCL6*, and *SPI1* expression levels were decreased; and Th1 dominated at both transcriptional and protein levels, but this contradicted a study carried out by Uddin et al. (2012). In addition, *RORC*, *Foxp3*, and *SPI1* were negatively correlated to oocyst numbers on day 23 PI, and expression levels of cytokines including IL17A, IL10, IL9, and TGF- β 1 decreased. No statistically significant difference of relationships between *TBX2*, *GATA3*, *BCL6*, and oocyst output was observed in the experiment groups at three time points (Table 4). In the experiment group, the severe liver damage was observed during the proliferation of *E. stiedai*, likely through suppressing the expression of *RORC*, *Foxp3*, *SPI1*, cytokines (IL17A, IL10, and IL9), and TGF- β 1.

Conclusion

Our findings provide the first evidence of dynamic changes in Th1/Th2/Th17/Treg/Th9/Th21 and confirm an imbalance of these cell populations in *E. stiedai*-infected rabbits. Additional studies are needed to elucidate the detailed roles of these Th cells in the pathogenesis of *E. stiedai*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

References

Adalid-Peralta, L., Fragoso, G., Fleury, A., and Sciotto, E. (2011). Mechanisms underlying the induction of regulatory T cells and its relevance in the adaptive immune

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care Committee of Sichuan Animal Science Academy.

Author contributions

Conceptualization: X-DC, R-MK, and Y-GY. Methodology: X-DC. Software: LX, C-JM, and X-JW. Validation: J-FY, YW, and JX. Formal analysis: X-DC and YC. Resources: Y-GY. Data curation: R-MK and Y-GY. Writing—original draft preparation: X-DC. Writing—review and editing: Y-GY. Visualization: X-DC. Supervision: Y-GY. Project administration: R-MK and Y-GY. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the Sichuan Provincial Financial Operation Special Project (SASA2023CZYX006), the Sichuan Province Regional Innovation Cooperation Project (2023YFQ0037), and the Sichuan Provincial Natural Science Foundation (2022NSFSC0081).

Acknowledgments

We thank the Sichuan Agricultural University for providing us with *E. stiedai*.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

response in parasitic infections. *Int. J. Biol. Sci.* 7 (9), 1412–1426. doi: 10.7150/ijbs.7.1412

- Al-Masoudi, H. K., Al-Hamadani, K. C., and Khairull, I. A. (2021). Interleukin 17 cytokine profiles in patients with cystic Echinococcosis in babilon province, Iraq. *Arch. Razi Inst.* 76 (5), 1493–1500. doi: 10.22092/ari.2021.355855.1730
- Bai, Y., Guan, F., Zhu, F., Jiang, C., Xu, X., Zheng, F., et al. (2021). IL-33/ST2 Axis deficiency exacerbates hepatic pathology by regulating Treg and Th17 cells in murine Schistosomiasis Japonica. *J. Inflammation Res.* 14, 5981–5998. doi: 10.2147/JIR.S336404
- Chang, H. C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G. L., et al. (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat. Immunol.* 11 (6), 527–534. doi: 10.1038/ni.1867
- Cobb, D., Hambright, D., and Smeltz, R. B. (2010). T-bet-independent effects of IL-12 family cytokines on regulation of Th17 responses to experimental *T. cruzi* infection. *J. Leukoc. Biol.* 88 (5), 965–971. doi: 10.1189/jlb.0410238
- Crotty, S., Johnston, R. J., and Schoenberger, S. P. (2010). Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat. Immunol.* 11 (2), 114–120. doi: 10.1038/ni.1837
- Du, X., Wu, J., Zhang, M., Gao, Y., Zhang, D., Hou, M., et al. (2011). Upregulated expression of cytotoxicity-related genes in IFN- γ knockout mice with Schistosoma Japonicum infection. *J. Biomed. Biotechnol.* 2011, 864945. doi: 10.1155/2011/864945
- Duszynski, D. W., and Couch, L. (2013). The biology and identification of the Coccidia (Apicomplexa) of rabbits of the world. *Newnes* 23, 56–23. doi: 10.34/0347-356(67)87-6
- Farwa, A., He, C., Xia, L., and Zhou, H. (2018). Immune modulation of Th1, Th2, and Treg transcriptional factors differing from cytokine levels in Schistosoma Japonicum infection. *Parasitol. Res.* 117 (1), 115–126. doi: 10.1007/s00436-017-5678-5
- Finlay, C. M., Walsh, K. P., and Mills, K. H. (2014). Induction of regulatory cells by helminth parasites: exploitation for the treatment of inflammatory diseases. *Immunol. Rev.* 259 (1), 206–230. doi: 10.1111/immr.12164
- Geng, X. H., Zhang, F. B., Jing, M., Zhao, H., An, M. T., Pang, N. N., et al. (2018). Expression of IL-9, PU.1 and IRF-4 mRNA in liver tissues in patients with alveolar echinococcosis. *Chin. J. Immunol.* 34 (3), 412–416.
- Gomez-Bautista, M., Rojo-Vazquez, F. A., and Alunda, J. M. (1987). The effect of the host's age on the pathology of *Eimeria stiedai* infection in rabbits. *Vet. Parasitol.* 24 (2), 47–57. doi: 10.1016/0304-4017(87)90129-4
- Harms Pritchard, G., Hall, A. O., Christian, D. A., Wagage, S., Fang, Q., Muallem, G., et al. (2015). Diverse roles for T-bet in the effector responses required for resistance to infection. *J. Immunol.* 194 (3), 1131–1140. doi: 10.4049/jimmunol.1401617
- Jing, J., Liu, C., Zhu, S. X., Jiang, Y. M., Wu, L. C., Song, H. Y., et al. (2016). Pathological and ultrastructural observations and liver function analysis of *Eimeria stiedai*-infected rabbits. *Vet. Parasitol.* 223, 165–172. doi: 10.1016/j.vetpar.2016.04.020
- Johnston, R. J., Poholek, A. C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., et al. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Sci. (New York N.Y.)* 325 (5943), 1006–1010. doi: 10.1126/science.1175870
- Kalantari, P., Bunnell, S. C., and Staderker, M. J. (2019). The C-type lectin receptor-driven, Th17 cell-mediated severe pathology in schistosomiasis: Not all immune responses to helminth parasites are Th2 dominated. *Front. Immunol.* 10, doi: 10.3389/fimmu.2019.00026
- Kanhere, A., Hertweck, A., Bhatia, U., Gokmen, M. R., Perucha, E., Jackson, I., et al. (2012). T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nat. Commun.* 3, 1268. doi: 10.1038/ncomms2260
- Kim, E. M., Bae, Y. M., Choi, M. H., and Hong, S. T. (2012). Cyst formation, increased anti-inflammatory cytokines and expression of chemokines support for Clonorchis sinensis infection in FVB mice. *Parasitol. Int.* 61 (1), 124–129. doi: 10.1016/j.parint.2012.07.001
- Kimura, A., and Kishimoto, T. (2010). IL6: regulator of Treg/Th17 balance. *Eur. J. Immunol.* 40 (7), 1830–1835. doi: 10.1002/eji.201040391
- Layland, L. E., Mages, J., Lodenkemper, C., Hoerauf, A., Wagner, H., Lang, R., et al. (2010). Pronounced phenotype in activated regulatory T cells during a chronic helminth infection. *J. Immunol.* 184 (2), 713–724. doi: 10.4049/jimmunol.0901435.2010.4.9
- Li, B., Qi, X., Liu, Y., Yan, Y., Shan, J., Cai, X., et al. (2022). Monocyte-derived macrophages: The supplements of hepatic macrophage in *Echinococcus multilocularis* infected mice. *Immun. Inflammation Dis.* 10 (10), e699. doi: 10.1002/iid3.699
- Li, L., Xie, H., Wang, M., Qu, J., Cha, H., Yang, Q., et al. (2017). Characteristics of IL9 induced by Schistosoma japonicum infection in C57BL/6 mouse liver. *Sci. Rep.* 7, 2343. doi: 10.1038/s41598-017-02422-8
- Lopez-Yglesias, A. H., Burger, E., Camanzo, E., Martin, A. T., Araujo, A. M., Kwok, S. F., et al. (2021). T-bet-dependent ILC1- and NK cell-derived IFN- γ mediates cDC1-dependent host resistance against Toxoplasma gondii. *PLoS Pathog.* 17, e1008299. doi: 10.1371/journal.ppat.1008299
- McManus, D. P., Gray, D. J., Li, Y., Feng, Z., Williams, G. M., Stewart, D., et al. (2010). Schistosomiasis in the people's republic of China: the era of the three gorges dam. *Clin. Microbiol. Rev.* 23 (2), 442–466. doi: 10.1128/CMR.00044-09
- Mishra, P. K., Palma, M., Bleich, D., Loke, P., and Gause, W. C. (2014). Systemic impact of intestinal helminth infections. *Mucosal Immunol.* 7 (4), 753–762. doi: 10.1038/mi.2014.23
- Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., et al. (2009). Bcl6 mediates the development of T follicular helper cells. *Sci. (New York N.Y.)* 325 (5943), 1001–1005. doi: 10.1126/science.1176676
- Oliveira, U. C., Fraga, J. S., Licois, D., Pakandl, M., and Gruber, A. (2011). Development of molecular assays for the identification of the 11 Eimeria species of the domestic rabbit (*Oryctolagus cuniculus*). *Vet. Parasitol.* 176 (2), 275–280. doi: 10.1016/j.vetpar.2011.10.054
- Pakandl, M. (2009). Coccidia of rabbit: a review. *Folia parasitologica.* 56 (3), 153–166. doi: 10.14411/fp.2009.019
- Pang, N., Zhang, F., Ma, X., Zhang, Z., Zhao, H., Xin, Y., et al. (2014). Th9/IL9 profile in human echinococcosis: their involvement in immune response during infection by Echinococcus granulosus. *Mediators Inflamm.* 2014, 781649. doi: 10.1155/2014/781649
- Parhizgar, N., Borji, H., Moazeni, M., Saboor Darbandi, M., and Heidarpour, M. (2021). The effects of Zataria multiflora aromatic water and nano emulsion of Z. multiflora essential oil on the serum level of IFN- γ and IL4 in mice with hydatidosis. *J. Parasit. Dis.* 45 (3), 733–737. doi: 10.1007/s12639-021-01353-6
- Pesce, J., Kaviratne, M., Ramalingam, T. R., Thompson, R. W., Urban J.R., J. F., Cheever, A. W., et al. (2006). The IL21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest.* 116 (7), 2044–2055. doi: 10.1172/JCI27727
- Ramming, A., Druzid, D., Leipe, J., Schulze-Koops, H., and Skapenko, A. (2012). Maturation-related histone modifications in the PU.1 promoter regulate Th9-cell development. *Blood* 119 (20), 4665–4674. doi: 10.1182/blood-2011-11-392589
- Ren, J., and Li, B. (2017). The functional stability of Foxp3 and ROR γ t in Treg and Th17 and their therapeutic applications. *Adv. Protein Chem. Struct. Biol.* 107, 155–189. doi: 10.1016/bs.apcsb.2017.10.002
- Seki, T., Kumagai, T., Kwansa-Bentum, B., Furushima-Shimogawara, R., Anyan, W. K., Miyazawa, Y., et al. (2012). Interleukin-4 (IL4) and IL-13 suppress excessive neutrophil infiltration and hepatocyte damage during acute murine Schistosomiasis Japonica. *Infect. Immun.* 80 (1), 159–168. doi: 10.1128/IAI.05581-11
- Solaymani-Mohammadi, S., Eckmann, L., and Singer, S. M. (2019). Interleukin (IL)-21 in inflammation and immunity during parasitic diseases. *Front. Cell Infect. Microbiol.* 9, doi: 10.3389/fcimb.2019.00401
- Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100 (6), 655–669. doi: 10.1016/s0092-8674(00)80702-3
- Tang, J., Zhu, X., Zhao, J., Fung, M., Li, Y., Gao, Z., et al. (2015). Tissue transglutaminase-regulated transformed growth factor- β 1 in the parasite links Schistosoma japonicum infection with liver fibrosis. *Mediators Inflamm.* 2015, 659378. doi: 10.1155/2015/659378
- Turner, J. D., Jenkins, G. R., Hogg, K. G., Aynsley, S. A., Paveley, R. A., Cook, P. C., et al. (2011). CD4+CD25+ regulatory cells contribute to the regulation of colonic Th2 granulomatous pathology caused by schistosome infection. *PLoS Negl. Trop. Dis.* 5 (8), e1269. doi: 10.1371/journal.pntd.0001269
- Tuxun, T., Apaer, S., Ma, H.-Z., Zhang, H., Aierken, A., Lin, R.-Y., et al. (2015). The potential role of th9 cell related cytokine and transcription factors in patients with hepatic alveolar echinococcosis. *J. Immunol. Res.* 895416.
- Uddin, M. H., Li, S., Bae, Y. M., Choi, M. H., and Hong, S. T. (2012). Strain variation in the susceptibility and immune response to Clonorchis sinensis infection in mice. *Parasitol. Int.* 61 (1), 118–123. doi: 10.1016/j.parint.2012.07.002
- Wang, H., Jain, S., Li, P., Lin, J. X., Oh, J., Qi, C., et al. (2019). Transcription factors IRF8 and PU.1 are required for follicular B cell development and BCL6-driven germinal center responses. *Proc. Natl. Acad. Sci. U.S.A.* 116 (19), 9511–9520. doi: 10.1073/pnas.1901258116
- Yu, D., Rao, S., Tsai, L. M., Lee, S. K., He, Y., Sutcliffe, E. L., et al. (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31 (3), 457–468. doi: 10.1016/j.immuni.2009.07.002
- Zhou, S., Jin, X., Chen, X., Zhu, J., Xu, Z., Wang, X., et al. (2015). Heat shock protein 60 in eggs specifically induces Tregs and reduces liver immunopathology in mice with Schistosomiasis Japonica. *PLoS One* 10 (9), e0139133. doi: 10.1371/journal.pone.0139133
- Zhu, J. (2010). Transcriptional regulation of Th2 cell differentiation. *Immunol. Cell Biol.* 88, 244–249. doi: 10.1038/icb.2009.114
- Ziegler, S. F., and Buckner, J. H. (2009). Foxp3 and the regulation of Treg/Th17 differentiation. *Microbes Infect.* 11, 594–598. doi: 10.1016/j.micinf.2009.04.002