Research Note: Expression of T cell-related cytokines in chicken cecal and spleen tissues following *Eimeria tenella* infection in vivo

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ABSTRACT The T cell-mediated immune response plays an important role in coccidiosis. To reveal the host T cell immune response following *Eimeria tenella* (*E. tenella*) infection in chickens, we performed quantitative real-time PCR to analyze the dynamic expression of the Th1-related cytokines *IFN-γ*, *IL-2*, and *IL-12*; the Th17-related cytokines *IL-17A*, *IL-17F*, and *IL-22*; and the Treg-related cytokines *IL-10*, *TGF-β*, and *CTLA-4* in the cecum and spleen at 0, 2, 4, 6, 8, and 10 d postinfection **(dpi)**. In the cecal tissue, the expression of the Th1-related cytokine *IFN-γ* was significantly higher at 6 and 8 dpi than at other time points (11.97-fold and 39.78-fold, respectively, compared with 0 dpi, $P < 0.05$). *IL-2* and *IL-12* expression was significantly higher at 6 and 8 dpi than at 0, 2 and 10 dpi ($P < 0.05$). The expression of the Th17-related cytokines *IL-17A* and *IL-17F* at 2 and 4 dpi and *IL-22* expression at 4 dpi were significantly higher than those at 0, 6, 8 and 10 dpi ($P < 0.05$). The expression of the Treg-related cytokines *IL-10*, *TGF-β* and *CTLA-4* was significantly higher at 6 and 8 dpi than at 0, 2 and 4 dpi ($P < 0.05$). In the spleen, *IFN-γ* and *IL-12* expression peaked at 4 dpi, while *IL-2* expression peaked at 10 dpi. *IL-17A*, *IL-17F* and *IL-22* expression was significantly higher at 2 and 4 dpi than at 0, 6, 8 and 10 dpi ($P < 0.05$). Treg-related cytokine *TGF-β* expression was almost unchanged and significantly decreased at only 4 dpi ($P < 0.05$), while *CTLA-4* expression showed an overall decreasing trend from 0 to 8 dpi but increased significantly at 10 dpi ($P < 0.05$). The expression patterns of three T cell subset-related cytokines were different in the cecum and spleen. Furthermore, Th1 and Treg cells participate in the immune response mainly in the latter stage of coccidia infection (6 and 8 dpi), while Th17 cells play a role mainly in the early stages of infection (2 and 4 dpi). Our data will help to deepen the understanding of the complex T cell immune response after coccidia infection.

Key words: *E. tenella*, cecum, spleen, chicken, T cell immune

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INTRODUCTION

Avian coccidiosis is an intestinal parasitic disease caused by *Eimeria* protozoa, and this infection hinders the development of the global poultry industry (Yu et al., 2020). The annual global economic loss in the poultry industry due to coccidiosis exceeds $3 billion (Blake and Tomley, 2014). At present, 7 species of *Eimeria* are known to infect chickens, and the different *Eimeria* species of parasites target different specific regions of the intestine. *E. tenella* parasitizes the cecum and causes cecal epithelium hemorrhage and discharge of bloody stools (Blake and Tomley, 2014). Since the life cycle of coccidia is relatively complex and includes intracellular and extracellular stages, understanding the immune response of chickens will be helpful to prevent and treat coccidiosis.

Studies have demonstrated that T cell immunity plays an important role in the process of coccidiosis. T helper 1 **(Th1)** cells are a subset of $CD4^+$ T helper cells. Early studies have shown that Th1 cell-related cytokines play a major role in the host's resistance to coccidia infection. Th1 cells perform biological functions by releasing cytokines and recruiting monocytes/macrophages and lymphocytes. Th17 cells are a newly emerged subset of CD4+ T cells whose hallmark feature is the release of IL-17 (also called IL-17A). Recent studies have indicated that the IL-17 family of cytokines is involved in various autoimmune diseases and pathogenic infections and participates in host inflammatory

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responses (Iwakura et al., 2011). Min and Lillehoj (2002) isolated IL-17 from intestinal epithelial cells of *Eimeria*-infected chickens and discovered that IL-17 plays a role in coccidia infection as a proinflammatory cytokine. IL-17F is the closest member to IL-17A, with a 50% homology and rather similar biological functions. However, IL-17A has a stronger effect than IL-17F (Min et al., 2013). Furthermore, Kim et al. (2012) revealed that the mRNA expression of *IL17F* was upregulated following chicken coccidia infection. Regulatory T cells (Tregs) are a subset of T cells characterized by a CD4+CD25+ phenotype in avians and play an important role in the regulation of host immune suppression and the protective immune response. When an infection subsides, the Treg-mediated immune system suppresses activated immune cells to prevent excessive immune response from harming the body (Workman et al., 2009). More recent work has indicated that Treg cells can reduce the occurrence of intestinal inflammation by inhibiting Th17 cells, which is essential in host protective immunity against *E. tenella* (Kim et al., 2019). Based on the above research results, this study used qRT-PCR to analyze the dynamic expression of three helper T cell-related cytokines, including Th1 cells, Th17 cells, and Treg cells, in the cecum and spleen during chicken *E. tenella* infection. The objective of this study was to strengthen the understanding of the complex host T cell immune mechanism in the process of coccidiosis and provide references for the prevention and control of coccidiosis in the future.

MATERIALS AND METHODS

Animals

A total of 120 (60 males, 60 females) 1-day-old healthy broiler chicks were randomly selected from Jiangsu Jinghai Poultry Industry Group Co., Ltd. (Haimen, China) and raised in a sterile animal room with free access to antibiotic-free feed and drinking water. Fecal detection determined that the chicks were free from parasitic infection before the experiment. Each chick was orally challenged with 2.5×10^4 *E. tenella* sporulated oocysts at 18 d of age. All protocols for animal sample collection were approved by the Animal Welfare Committee of Yangzhou University (permit number: SYXK (Su) IACUC 2012-0029).

Tissue Collection

At the age of 18 d, samples were taken at the 6 time points of 0, 2, 4, 6, 8, and 10 d postinfection **(dpi)**. Twenty chickens (half males and half females) were euthanized by cervical dislocation at each time point, and the cecal and spleen tissues were collected in cryopreservation tubes, immediately stored in liquid nitrogen, and transferred to a −80°C freezer for storage.

RNA Isolation and Quality Assessment

The TRIzol method was used to extract total RNA from cecum and spleen tissues according to the manufacturer's instructions. Then, the concentration of the RNA was measured by a NanoPhotometer spectrophotometer (Implen, Inc., Westlake Village, CA), and the RNA purity was verified at an optical density ratio of 260 to 280 nm. An RNA Nano 6000 detection kit and an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) were employed to assess RNA integrity. RNA was stored at −80°C until cDNA synthesis.

Primers *and* Reverse Transcription

The primer sequences of *IFN-γ*, *IL-2* and *IL-12* (Th1-related cytokines), *IL-17A*, *IL-17F* and *IL-22* (Th17-related cytokines), *IL-10*, *TGF-β*, and *CTLA-4* (Treg-related cytokines) and reference genes, are presented in Table 1. All primers were synthesized by Dingguo Biotech Co. (Beijing, China).

The extracted total RNA was reverse transcribed to cDNA using the PrimeScript RT Master Mix kit (TaKaRa Bio, Inc., Otsu, Japan). Briefly, the first step was to remove genomic DNA. The reaction system contained 5 × gDNA Eraser Buffer (2.0 μL), gDNA Eraser (1.0 μL), total RNA and RNase-free dH_2O (7 μL). The first reaction condition was 42°C for 2 min. The second step was reverse transcription, which contained 10.0 μL of the step 1 reaction solution, 1.0 μL of PrimeScript RT Enzyme Mix I, 1.0 μL of RT Primer Mix, 4.0 μL of 5 × PrimeScript Buffer 2, and 4 μL of RNase-free ddH_2O. The reaction conditions were 37°C for 15 min and 85°C for 5 s. The cDNA samples were stored at −20°C.

Quantitative Real-Time PCR

The SYBR Premix Ex Taq II kit (TaKaRa, Japan) was used for qPCR analysis as previously described (Yu et al., 2020). Briefly, the reaction system contained 0.8 μL of each upstream and downstream primer, 10 μL of SYBR Premix Ex Taq Ⅱ (Tli RNaseH Plus, 2 ×), 2 μL of cDNA template, 0.4 μL of ROX Reference Dye II (50 ×), and ddH_2O added to a final volume of 20 μL. The PCR program was 94 °C for 30 s, repeated 40 cycles of 95°C for 5 s and 60°C for 34 s. Each sample was analyzed in triplicate, and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The significance of the difference in the target gene expression on different days after *E. tenella* infection was analyzed by one-way analysis of variance (ANOVA) with SPSS 25.0 software, and multiple comparisons were performed using the least significant difference method. Statistical significance was indicated by P-values < 0.05.

Table 1. Quantitative real-time PCR primers used in this study.

T cell subgroup type	Gene	Primer sequence (5'-3')	Accession No.
	β-actin	F: CCTGAACCTCTCATTGCCA R: GAGAAATTGTGCGTGACATCA	NM_205518.1
Th1	*IFN-γ*	F: ATGTAGCTGACGGTGGACCT R: ACGCCATCAGGAAGGTTGTT	NM_205149.1
	IL-2	F: ATCTTTGGCTGTATTTCGGTAG R: CTGGGTCTCAGTTGGTGTGTAG	XM_015276098.2
	IL-12	F: AGATGCTGGCAACTACACCTG R: CATTTGCCCATTGGAGTCTAC	NM_213571803
Th17	*IL-17A*	F: CTCCTCTGTTCAGACCACTGC R: ATCCAGCATCTGCTTTCTTGA	NM-204460.1
	IL-17F	F: GACTGCCTGAACCAA R: GAGACCGATTCCTGATGT	JQ776598.1
	IL-22	F: GGTTGTCTTCTGCTGTTGTTGCTG R: GCCAAGGTGTAGGTGCGATTCC	NM_001199614.1
Treg	*IL-10*	F: CGCTGTCACCGCTTCTTCA R: TCCCGTTCTCATCCATCTTCTC	NM_001004414.2
	TGF-β	F: CATCGAGCTCTTCCAGATCC R: GACATCGAAGGACAGCCACT	NM_205454.1
	CTLA-4	F: GATGGAGCGGATGTACC R: TGGCTGAGATGATGATGCTG	NM _001040091

RESULTS AND DISCUSSION

Expression of Th1-Related Cytokines in the Cecal and Spleen Tissues During E. tenella *Infection*

Th1 cells play a key role in the regulation of cell-mediated immunity by secreting cytokines such as IFN-γ, IL-2 and IL-12. Moreover, IFN-γ is a signature cytokine that mediates the Th1 immune response in avian coccidiosis. Studies have demonstrated that the peripheral blood lymphocytes of birds infected with *Eimeria* will specifically produce IFN-γ to regulate anticoccidial immunity (Breed et al., 1997). As shown in Figure 1, the mRNA expression level of *IFN-γ* in the cecum at 6 and 8 dpi was significantly higher than that at 0, 2, 4, and 10 dpi ($P < 0.05$), and the expression in the spleen at 4 and 6 dpi was significantly higher than that at the other time points ($P < 0.05$). At 6-8 dpi, there is the gametogony stage when a large number of merozoites in the body infect the intestinal mucosa and cause severe damage and bleeding. The significantly increased expression of IFN-γ promotes the host immune response against coccidiosis infection. The mRNA expression level of *IL-2* in the cecum at 4, 6, and 8 dpi was significantly higher than that at 0, 2, and 10 dpi ($P < 0.05$), and the expression in the spleen at 10 dpi was significantly higher than that at other time points ($P < 0.01$), which is also related to the immune response during the infection process. This may be because IL-2 is a necessary immune cytokine for the activation and proliferation of T lymphocytes and promotes the production of NK cells (Boyman et al., 2006). IL-12 induces the production of IFN-γ by stimulating natural killer cells and Th1 cells and plays a regulatory effect on the immune system (Su et al., 2011). The mRNA expression level of *IL-12* in the cecum at 6, 8, and 10 dpi was significantly higher than that at 0, 2, and 4 dpi ($P < 0.05$), while the expression of *IL-12* in the spleen at 0, 2, and 4 dpi was significantly higher than that at 6, 8, and 10 dpi ($P < 0.05$). *IL-12* expression in the spleen seems to be inconsistent with the expression pattern of the cecum, which is worthy of further discussion. In general, the present study found that the Th1 cell-related cytokines *IFN-γ*, *IL-2*, and *IL-12* were expressed at higher levels in the late stage of *E. tenella* infection (6 and 8 dpi). The body is in a more serious stage of infection, and the increase in the expression of three cytokines promotes the immune response of host Th1 cells to resist coccidia infection.

Expression of Th17-Related Cytokines in the Cecal and Spleen Tissues During E. tenella *Infection*

Th17 cells are an emerging T cell subset and are involved in host proinflammatory responses to various diseases and pathogenic infections, including coccidiosis (Kim et al., 2019). Avian Th17 cells mainly produce high amounts of IL-17A, IL-17F, and IL-22. As shown in Figure 2, the mRNA expression level of *IL-17A* in the cecum at 2 dpi was significantly higher than that at other time points after *E. tenella* challenge ($P < 0.01$), while the expression in the spleen at 2 and 4 dpi was significantly higher than that at 0, 6, 8, and 10 dpi ($P < 0.01$). Furthermore, the mRNA expression of IL-17A peaked at 2 dpi in the cecum and at 4 dpi in the spleen. Previous studies have shown that IL-17A plays a key role in mediating inflammation early during the course of an infection and prior to the onset of adaptive T cell responses (Min et al., 2013). Zero to 2 dpi is in the initial stage of coccidia infection, including the ingestion of oocysts and the release of sporocysts. Therefore, this study further shows that IL-17A is involved in the early

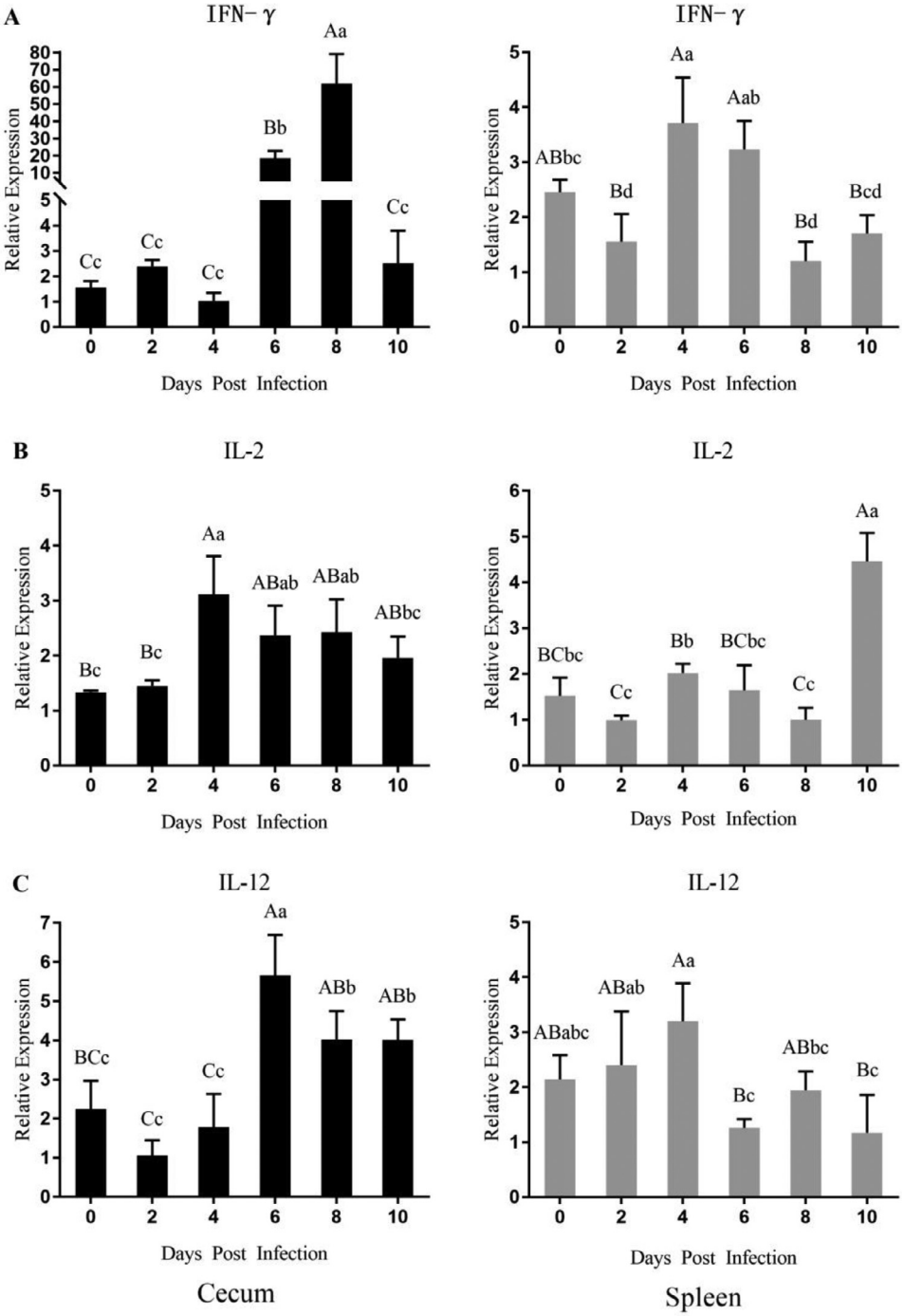

Figure 1. The mRNA expression of Th1-related cytokines at 0, 2, 4, 6, 8, and 10 dpi in the cecum and spleen of birds infected with *E. tenella*. (A), the mRNA expression of *IFN-γ* in the cecum (left), and spleen (right) (B), the mRNA expression of *IL-2* in the cecum (left), and spleen (right) (C), the mRNA expression of *IL-12* in the cecum (left), and spleen (right). Different lowercase letters indicate significant differences ($P < 0.05$), and different capital letters indicate extremely significant differences ($P < 0.01$). This system is used to indicate significance in the other figures.

immune response to coccidia infection. The mRNA expression level of *IL-17F* in the cecum at 2 and 4 dpi was significantly higher than that at 0, 8, and 10 dpi ($P < 0.05$), and the expression in the spleen at 2 and 4 dpi was significantly higher than that at other time points ($P < 0.05$). The mRNA expression of *IL-17F* peaked at 4 dpi in the cecum and at 2 dpi in the spleen. The study also indicates that IL-17F may play a role in the early stage of coccidia infection. The mRNA expression level of *IL-22* in the cecum at 4 dpi was significantly higher than that at 0, 2, 8, and 10 dpi ($P < 0.05$), peaked at 4 dpi, while the expression of *IL-22* in the spleen at 2 dpi was significantly higher than that at other time points ($P < 0.05$) and peaked at 2 dpi. IL-22 is an immune cytokine induced by a variety of pathogen infections and mainly acts on intestinal epithelial cells and enhances mucosal barrier function (Behnsen et al., 2014). After the chicken ingests the sporulated oocysts, the sporocysts are released, and the sporozoites invade the intestinal epithelial cells and multiply to produce merozoites,

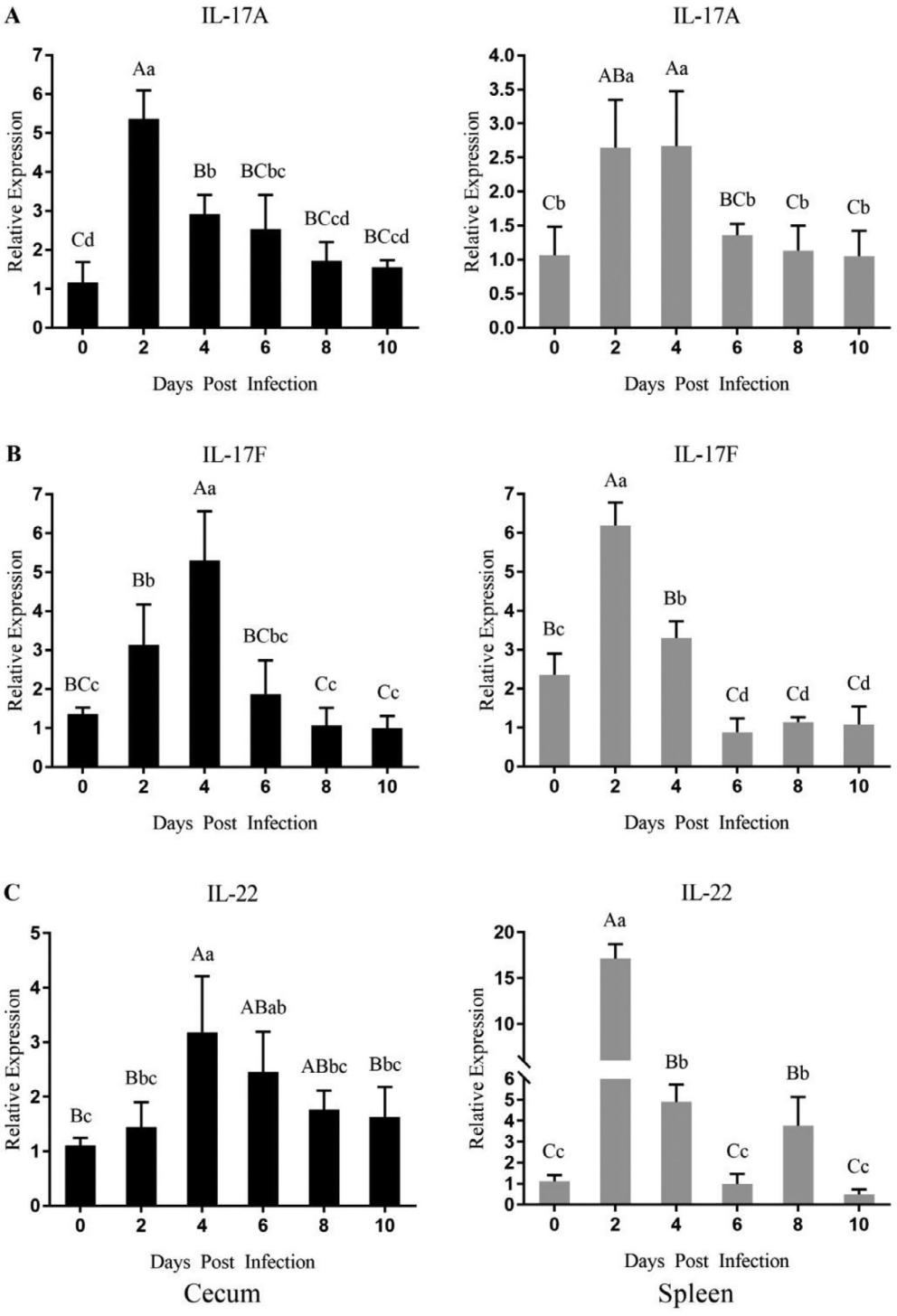

Figure 2. The mRNA expression of Th17-related cytokines at 0, 2, 4, 6, 8, and 10 dpi in the cecum and spleens of birds infected with *E. tenella*. (A), the mRNA expression of *IL-17A* in the cecum (left), and spleen (right) (B), the mRNA expression of *IL-17F* in the cecum (left), and spleen (right) (C), the mRNA expression of *IL-22* in the cecum (left), and spleen (right).

which activate the host's innate immunity and adaptive immune response, resulting in *IL-22* expression reaching a peak in the early stage of infection.

Expression of Treg-Related Cytokines in the Cecal and Spleen Tissues During E. tenella *Infection*

As shown in Figure 3, the mRNA expression level of *IL-10* in the cecum at 6 and 8 dpi was significantly higher than that at other time points after *E. tenella* infection ($P < 0.05$), and the expression in the spleen at 6 dpi was significantly higher than that at 0, 4, 8, and 10 dpi ($P < 0.01$). The mRNA expression level of *TGF-β* in the cecum at 6, 8 and 10 dpi was significantly higher than that at 0, 2, and 4 dpi ($P < 0.05$), and the expression in the spleen at 4 dpi was significantly lower than that at 0, 2, 6, and 10 dpi ($P < 0.05$). The mRNA expression level of *CTLA-4* in the cecum at 6 and 8 dpi was significantly higher than that at 0, 2, 4, and 10 dpi ($P <$

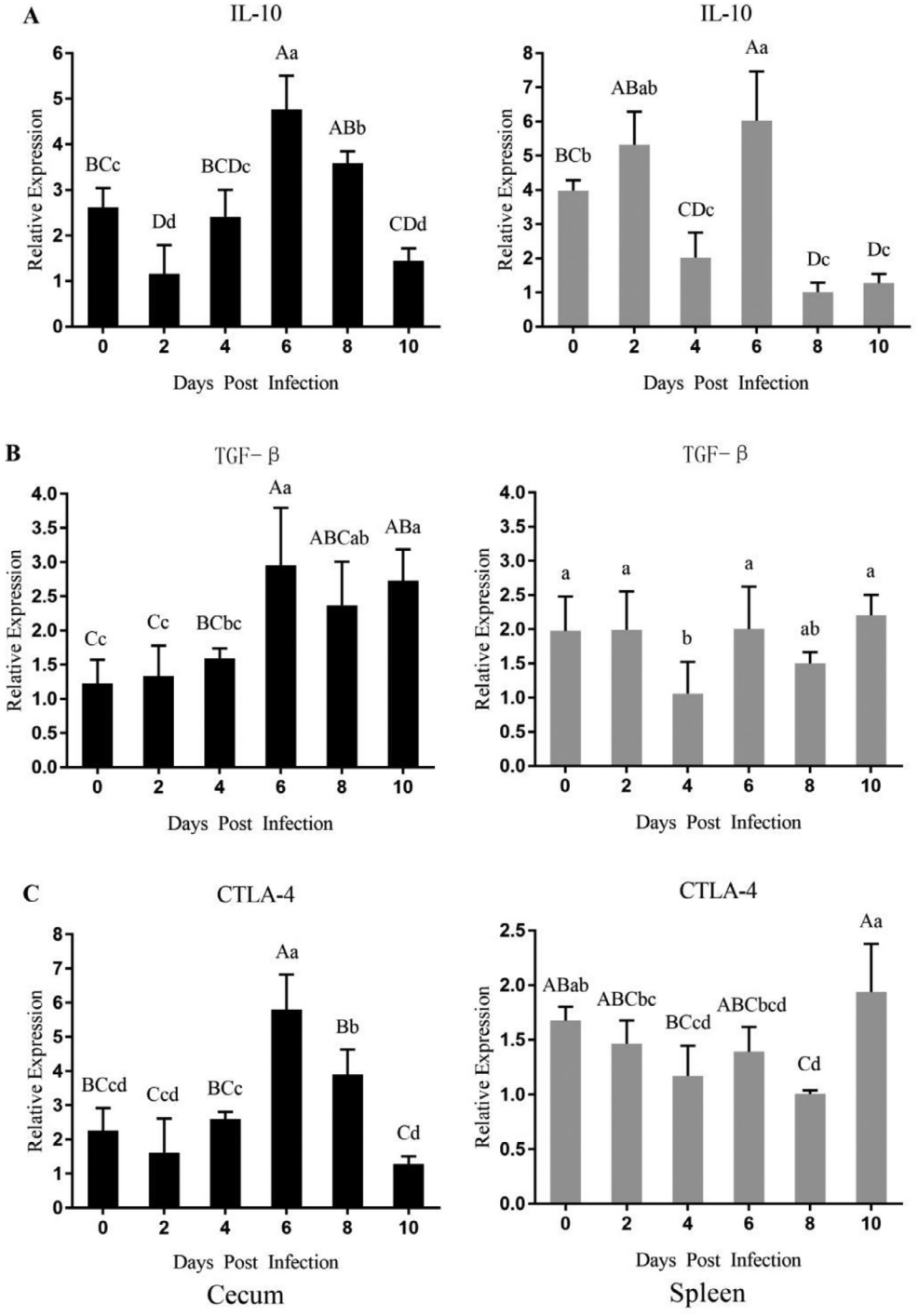

Figure 3. The mRNA expression of Treg-related cytokines at 0, 2, 4, 6, 8, and 10 dpi in the cecum and spleen of birds infected with *E. tenella*. (A), the mRNA expression of *IL-10* in the cecum (left), and spleen (right) (B), the mRNA expression of *TGF-β* in the cecum (left), and spleen (right) (C), the mRNA expression of *CTLA-4* in the cecum (left), and spleen (right).

0.05), while the expression of *CTLA-4* in the spleen gradually decreased from 0 to 8 dpi, but the expression at 10 dpi was significantly higher than that at other time points ($P < 0.05$). Furthermore, the mRNA expression of *IL-10* peaked at 6 dpi in the cecum and spleen, that of *TGF-β* and *CTLA-4* peaked at 6 dpi in the cecum and at 10 dpi in the spleen. Our study found that the expression of *IL-10*, *CTLA-4* and *TGF-β* in the cecum tissue (infected site) showed an upward-regulated trend after chicken *E. tenella* challenge, and the highest expression level was at 6 dpi. At this time, the coccidia was in the gametogony stage, and the host produced a large number of proinflammatory immune responses. To alleviate inflammation that could be deleterious to the host and reduce tissue damage at the site of infection, *IL-10* and other anti-inflammatory factors are highly expressed, thereby inhibiting the production of pro-inflammatory cytokines such as IL-2, IFN-γ and IL-1β, reducing the inflammatory immune response (Arendt et al., 2016), and promoting inflammation and anti-inflammation reach a certain balance as much as possible (Cyktor and Turner, 2011), in which the relevant immune factors produced by Treg cells may play an important role.

In conclusion, the dynamic expression patterns of Th1, Th17, and Treg cell-related cytokines in the cecum and spleen are associated with *E. tenella* infection stages. Th1 and Treg cells participate in the immune response mainly in the latter stage of coccidia infection (6 and 8 dpi), while Th17 cells play a role mainly in the early stages of infection (2 and 4 dpi). The findings of the study can enhance our understanding of T cell immunity against chicken coccidiosis and provide a basis for the prevention and control of coccidiosis.

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DISCLOSURES

The authors declare no conflict of interest.

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