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PROTOZOOLOGY - ORIGINAL PAPER

Molecular characterization and immune protection of an AN1-like zinc finger protein of *Eimeria tenella*

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Abstract

Coccidiosis is caused by multiple species of the apicomplexan protozoa *Eimeria*. Among them, *Eimeria tenella* is frequently considered to be the most pathogenic. Zinc finger proteins (ZnFPs) are a type of protein containing zinc finger domains. In the present study, a putative *Eimeria tenella* AN1-like ZnFP (*E. tenella* AN1-like zinc finger domain-containing protein, putative partial mRNA, *Et*AN1-ZnFP) was cloned and characterized, and its immune protective effects were evaluated. The 798-bp ORF sequence of *Et*AN1-ZnFP that encoded a protein of approximately 27.0 kDa was obtained. The recombinant *Et*AN1-ZnFP protein (r*Et*AN1-ZnFP) was expressed in *Escherichia coli*. Western blot analysis showed that the recombinant protein was recognized by the anti-GST monoclonal antibody and anti-sporozoite protein rabbit serum. qPCR analysis revealed that *Et*AN1-ZnFP was highly expressed in unsporulated oocysts and sporozoites. Immunostaining with an anti-r*Et*AN1-ZnFP antibody indicated that *Et*AN1-ZnFP was uniformly distributed in the cytoplasm of sporozoites, except for the refractive body; furthermore, this protein was evenly distributed in the cytoplasm of immature schizonts but seldom distributed in mature schizonts. The results of the in vitro invasion inhibition assay indicated that the antibodies against r*Et*AN1-ZnFP efficiently reduced the ability of *E. tenella* sporozoites to invade host cells. Animal challenge experiments demonstrated that the chickens immunized with r*Et*AN1-ZnFP protein significantly decreased mean lesion scores and fecal oocyst output compared with challenged control group. The results suggest that *Et*AN1-ZnFP can induce partial immune protection against infection with *E. tenella* and could be an effective candidate for the development of new vaccines.

Keywords *Eimeria tenella* · AN1-Like zinc finger protein · Characterization · Vaccine · Chicken coccidiosis

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Background

The protozoan parasite *Eimeria tenella* is a major causative agent of avian coccidiosis, causing considerable economic losses to the poultry industry worldwide (Chapman et al. 2013). At present, the control of coccidiosis relies primarily on prevention and use of anticoccidial drugs and live vaccines. However, *Eimeria* spp. have developed resistance to most commercially available anticoccidial drugs (Chapman 1998). Furthermore, the residues of these drugs have led to food safety concerns, and live anticoccidial vaccines are more costly than anticoccidial drugs (Shirley et al. 2007). Subunit vaccines are much less effective than live ones but are easier to produce, convenient, and safe (Peek et al. 2008). Therefore, novel immunoprotective antigens are urgently needed. However, the coccidial antigens evaluated to date can only induce partial immune protection, probably because of the complex life cycle of coccidia. The immunoprotective antigens identified and characterized to date include apical membrane antigen-1, refractile body proteins, microneme proteins, elongation factor-1α, heat shock proteins, refractile body protein SO7, surface antigens, IMP1, profilin, and so on (Blake et al. 2017; Lin et al. 2017; Liu et al. 2018; Pastor-Fernandez et al. 2018; Plattner et al. 2008; Rafiqi et al. 2018; Yin et al. 2013).

Eukaryotic proteins containing zinc finger domains are known as zinc finger proteins (ZnFPs). ZnFPs play an essential role in the recognition and binding of DNA, RNA, and protein by a unique finger structure (Ghosh and Chatterji 2017; Liu and Heermann 2015). In plants such as rice, soybean, *Arabidopsis*, petunia, wheat, and cotton, ZnFPs are involved in resistance to environmental stress and plant growth and development (Jin et al. 2018). In mycobacteria, *Ms*ZnFP1 and *Ms*ZnFP2 activate transcription by interacting with RNA polymerase (Ghosh and Chatterji 2017). In eukaryotes, ZnFPs are widely expressed and participate in important biological processes, including cell differentiation, proliferation, and apoptosis (Iuchi 2001). In *Toxoplasma gondii*, *Tg*ZNF2 may play a key role in mRNA nuclear export (Gissot et al. 2017). *Eimeria tenella* is one of the most important species causing avian coccidiosis and is frequently used as a model to study species of *Eimeria*. It belongs to the phylum Apicomplexa with a complex life cycle and needs to invade the epithelial cells lining the intestine of chicken to grow and develop. Hence, we speculated that ZnFPs may be involved in these important biological processes in the life cycles of *E. tenella.*

However, to the best of our knowledge, no studies to date investigated ZnFPs in *E. tenella*. In the present study, the putative *E. tenella* AN1-like ZnFP *Et*AN1-ZnFP was cloned, and the recombinant protein GST-*Et*AN1-ZnFP (r*Et*AN1-ZnFP) was produced in an *Escherichia coli* BL21 (DE3) expression system. Anti-r*Et*AN1-ZnFP antibodies were produced in immunized rabbits. These antibodies were used to localize *Et*AN1-ZnFP in parasites by immunofluorescence assay and to assess the inhibitory effect of *Et*AN1-ZnFP using an in vitro assay. The levels of *Et*AN1-ZnFP transcripts at different development stages of the parasite were measured by quantitative real-time PCR (qPCR), and the immunogenicity of *Et*AN1-ZnFP was evaluated in a chicken challenge model. The results of the present study indicate that AN1-ZnFP may participate in parasite growth and development.

Materials and methods

Parasite preparation

The Shanghai strain of *E. tenella* was initially isolated in the 1980s from a sample collected in a farm in Shanghai, China, and was subsequently maintained in our laboratory (Resource Number CAAS21111601) (Huang et al. 1993). The parasites were propagated by passage through coccidian-free 2-week-old chickens, as described previously (Tomley 1997). Each animal was infected by the oral route with 1×10^4 sporulated oocysts (SO). Unsporulated oocysts (UO) were obtained from caeca on day 8 post-infection (p.i.). Feces were collected on days 6 to 8 p.i. and oocysts were sporulated in 2.5% potassium dichromate at 28 °C for up to 96 h (Smith et al. 1993). UO and SO were purified using a standard method, as previously described (Han et al. 2010). Sporozoites (Spz) were excysted in vitro from cleaned sporulated oocysts (Miska et al. 2004). Second-generation merozoites (Mrz) were collected from the cecal contents or mucosa of chickens at 115 h p.i. (Xie et al. 1990).

The chicken fibroblast cell line DF-1 derived from East Lansing Line (ELL-0) chicken embryos was used in animal infection experiments and inhibition and immunofluorescence assays (Jiang et al. 2012).

Amplification and sequence analysis of *Et*AN1-ZnFP

Total RNA was extracted from UO using TRIzol reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. Briefly, a total of 1.0×10^7 UO of *E. tenella* were oscillated and broken in 500 μL of TRIzol with a half volume of 710–1180 μm glass beads (Sigma, St. Louis, MO, USA) for 10 min. Total RNA was precipitated with isopropanol and washed with 75% ethanol and then resuspended in diethyl pyrocarbonate (DEPC)-treated water. Total RNA concentrations were determined by absorbance readings at 260 nm on a UV spectrophotometer (Eppendorf, Hamburg, Germany). The RNA quality was

assessed by electrophoresis on a 1% agarose-formaldehyde ethidium bromide gel. Complementary DNA (cDNA) was synthesized from total RNA using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) with oligo dT primers.

The ORF sequence of the *Et*AN1-ZnFP gene of *E. tenella* (GenBank accession number XM_013374066.1) was amplified using the primers 5′-ATGAGCTCAGAGCAACACG-3′ (forward) and 5′-AAAGCTTCTGGAGTTTGTCTG-3′ (reverse). Conventional PCR was carried out in a 25-μL reaction system using the cDNA of UO as a template. The amplification reactions were performed using the following conditions: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, and extension at 72 °C for 10 min. The amplified PCR products were detected by electrophoresis on a 1.0% agarose gel and purified using the QIAquick® Gel Extraction Kit (QIAGEN, Duesseldorf, Germany). The *Et*AN1-ZnFP fragment was subcloned into the pGEM-T-easy vector (Promega, Madison, WI, USA) using T4 DNA ligase to construct a recombinant plasmid. The size of the recombinant plasmid was confirmed by bidirectional nucleotide sequence analysis with the primers SP6: 5′-ATTTAGGTGACACTATAG-3′; and T7: 5′-TAATACGACTCACTATAGGG-3′ by Sangon (Shanghai, China).

The full-length cDNA sequence was analyzed. The molecular mass and theoretical isoelectric point were predicted using the ProtParam tool at the ExPASy server (http://web.expasy.org/protparam/). Signal peptides, transmembrane motifs, and protein motifs were predicted using the computational tools SignalP (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and Motif Scan (http://hits.isb-sib.ch/cgi-bin/motif_scan), respectively.

Expression and purification of recombinant *Et*AN1-ZnFP protein

The *Et*AN1-ZnFP open reading frame (ORF) was amplified by PCR using the primers 5′-GCGGATCCATGAGCTCAGAGCAACACGAAAACGAAAGGCCTTCTGCTCCGCCCTTGTGTGCGAAGAACTGCGGCTT-3′ (forward) and 5′-GCGTCGACTCAAAGCTTCTGGAGTTTGTCTG-3′ (reverse), incorporating the *BamH*I and *Sal*I restriction sites (underlined), respectively. The amplified fragment and the pGEX-4T-1 vector were digested with *BamH*I/*Sal*I. The *BamH*I/*Sal*I double-digested *Et*AN1-ZnFP fragment and the pGEX-4T-1 vector were gel purified and ligated, and the recombinant pGEX-4T-*Et*AN1-ZnFP plasmid was transformed into *E. coli* BL21(DE3) (Tiangen, Beijing, China). The recombinant protein GST-EtAN1-ZnFP (r*Et*AN1-ZnFP) was expressed in *E. coli* BL21(DE3) as a glutathione S-transferase (GST) fusion protein. The expression of r*Et*AN1-ZnFP was induced in culture at an optical density of 0.6 with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, St Louis, MO, USA) for 8 h at 37 °C. The cell pellets were lysed by sonication on ice, working 2 s to stop for 2 s and lasting 20 min. The r*Et*AN1-ZnFP protein was purified from lysate supernatants using GST-bind resin (Beyotime, Haimen, China). The protein concentration was determined using the BCA protein assay kit (Beyotime, Haimen, China). The purified protein was aliquoted and stored at − 20 °C for further analysis.

Production of anti-r*Et*AN1-ZnFP and anti-sporozoite polyclonal serum

The sporozoite protein was prepared using sonication according to the previous description (Jiang et al. 2012). Two-month-old New Zealand white rabbits were subcutaneously immunized with 200 μg of purified r*Et*AN1-ZnFP or sporozoite protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO, USA). Two weeks later, the rabbits were given a subcutaneous booster injection with the same amount of the protein emulsified in Freund's incomplete adjuvant (Sigma, St Louis, MO, USA). Immunization was carried out once every 7 days, with a total of five immunizations. Seven days after the last immunization, the polyclonal antibody serum was separated from the blood of two rabbits. Negative serum was collected from the rabbits' ear vein before immunization.

SDS-PAGE and western blot for recombinant *Et*AN1-ZnFP

The purified r*Et*AN1-ZnFP proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Merck Millipore, Billerica, MA, USA). After being blocked with 5% (w/v) skimmed milk powder in tris-buffered saline–Tween 20 (TBST), the membranes were incubated with primary antibodies (anti-GST monoclonal antibody and anti-sporozoite protein serum, respectively) for 2 h at 37 °C (dilutions 1:1000 to anti-GST monoclonal antibody, 1:200 to anti-sporozoite protein serum). Secondary antibodies, IRDye® 800CW Goat anti-Rabbit IgG (LI-COR, Lincoln, NE, USA), were added after being washed three times with PBS. Then, the membranes were detected using Odyssey (LI-COR, Lincoln, NE, USA).

Detection of *Et*AN1-ZnFP in different developmental stages of *E. tenella* by quantitative real-time PCR

To detect the expression of *Et*AN1-ZnFP at different developmental stages of *E. tenella* (UO, SO, Spz, and Mrz), total RNAs were isolated by TRIzol reagent (Invitrogen) from four

life stages of *E. tenella* and treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, a total of 1.0×10^7 UO or SO of *E. tenella* were oscillated and broken in 500 μL of TRIzol with a half volume of 710–1180 μm glass beads (Sigma, St. Louis, MO, USA) for 10 min. A total of 1.0×10^7 sporozoites or second-generation merozoites were lysed in 500 μL of TRIzol. Total RNAs were precipitated with isopropanol and washed with 75% ethanol and then resuspended in diethyl pyrocarbonate (DEPC)-treated water. First cDNA strands were synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen, Carlsbad, CA, USA). qPCR was performed on an Eppendorf Mastercycler ep Realplex thermal cycler (Eppendorf, Hamburg, Germany) using the SYBR1 Green I dye and the primers 5′-CGTC GTCAGCAGTTCCCGCAGAGCA-3′ (forward) and 5′-CATCATCAGCAGGTTCTTGCGTAGG-3′ (reverse). A housekeeping gene of *E. tenella* (18S ribosomal RNA) was used as a normalizing control and was amplified using the primers 5′-TGTAGTGGAGTCTTGGTGATTC-3′ (forward) and 5′-CCTGCTGCCTTCCTTAGATG-3′ (reverse). Each reaction was performed in triplicate, and the experiment was repeated three times. The relative expression of *Et*AN1-ZnFP was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Localization of *Et*AN1-ZnFP by indirect immunofluorescence

Indirect immunofluorescence was carried out as described (Jiang et al. 2012) using antiserum to r*Et*AN1-ZnFP. The DF-1 chicken fibroblast cell line was used in indirect immunofluorescence analyses (Jiang et al. 2012). Briefly, 2×10^5 DF-1 cells were cultured in six-well plates (Corning, NY, USA) with precoated glass coverslips in complete medium (CM) (Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 units/mL penicillin/streptomycin, and 2 mM L-glutamine) for 24 h at 37 °C and 5% CO_2. Freshly cleaned *E. tenella* sporozoites (6×10^5 parasites per well) were incubated in CM for 2 h at 41 °C and were used to infect DF-1 cells. The coverslips were collected at 48, 60, and 82 h p.i. All the samples were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 1% Triton X-100 in PBS for 15 min, and then blocked with 2% bovine serum albumin in PBS overnight at 4 °C. The samples were incubated with rabbit anti-r*Et*AN1-ZnFP polyclonal antibody at a dilution of 1:100 for 2 h at 37 °C, followed by the goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody (1:500 dilution) (Sigma, St. Louis, MO, USA) for 1 h at 37 °C in a moist, dark chamber. The cell nuclei were stained with 15 μg/mL 4, 6-diamidino-2-phenylindole (DAPI) (Beyotime, Haimen, China) at room temperature for 30 min in wet and dark conditions. All the samples were washed four times in PBS at the end of each step. After that, the coverslips were mounted on glass slides using 60 μL of Fluoromount Aqueous Mounting Medium (Sigma, St. Louis, MO, USA). The glass slides were examined with a fluorescence microscope (Zeiss LSM800 microscope, Carl Zeiss, Germany). Sporozoites and second-generation merozoites were prepared for immunofluorescence using the same method. Briefly, the second-generation merozoites were evenly spread on glass coverslips. Then, the coverslips were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, and blocked with 2% bovine serum albumin in PBS. The samples were incubated with rabbit anti-r*Et*AN1-ZnFP polyclonal antibody and further incubated with FITC-conjugated antibody. Cell nuclei were labeled with DAPI.

Sporozoite invasion inhibition assay

Flat-bottomed 24-well plates (Corning, NY, USA) were seeded with DF-1 cells (3×10^5 cells per well). These cells were sequentially cultured in DMEM with 10% fetal bovine serum and 300 units/mL penicillin/streptomycin for 16 h at 37 °C and 5% CO_2 and DMEM containing 5% fetal bovine serum, 300 units/mL penicillin/streptomycin for 8 h at 37 °C, and 5% CO_2. Invasion assays were performed as described previously using *E. tenella* sporozoites infecting DF-1 cells (Jiang et al. 2012). Antibodies were purified using protein A+G agarose (Beyotime, Haimen, China), according to the manufacturer's instructions. Freshly purified sporozoites were stained for 15 min using carboxyfluorescein diacetate succinimidyl ester (CFDA SE) (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and incubated with 50, 100, 200, or 400 μg/mL of purified IgG against r*Et*AN1-ZnFP for 2 h at 37 °C. The concentration of IgG was determined by BCA (bicinchoninic acid) Protein Assay Kit (Beyotime, Haimen, China). The same amount of IgG from naive rabbit serum was used as a control. Stained and IgG-untreated sporozoites were used as a blank control. After that, DF-1 cells were infected with sporozoites (6×10^5 parasites per well) in 24-well plates (Corning, NY, USA) and cultured for 12 h at 41 °C and 5% CO_2. The cells were washed, trypsinized, harvested, and analyzed on a flow cytometer (model Cytomics FC500; Beckman Coulter, CA, USA). All assays were performed in triplicate. The deduced percentages of infected cells in the presence or absence of inhibitory antibody were used to calculate inhibition rates, as detailed previously (Jahn et al. 2009).

Immunization experiment design

For the challenge experiment, forty-eight chickens (yellow-feathered broilers, Shanghai Fengxian District, China) were reared in steel cages under coccidia-free conditions from the day of hatching (day 0) until 7 days of age (Gharaibeh and Mahmoud 2013). After that, chickens were randomly divided into four groups of 12 birds. Each animal was immunized at 7 days of age by subcutaneous booster injection with 50 μg or 100 μg of purified r*Et*AN1-ZnFP protein emulsified in Montanide ISA 71 adjuvant (Seppic, Puteaux, France) as a 3:7 mixture (Jang et al. 2013, 2010). Two groups (one challenged and one unchallenged group) were immunized with PBS, at pH 7.2, and served as negative controls. A booster immunization was given 1 week later with the same amount of components as the first immunization. At 7-day post-secondary immunization, chickens of immunized groups and challenged-control group were orally challenged with 1.0×10^4 SO of *E. tenella* whereas birds of the unchallenged control group were given an equal volume of PBS orally.

Evaluation of immune protection of *Et*AN1-ZnFP

Protective efficacy was evaluated by the average body weight gain, mean lesion scores, fecal oocyst output, and oocyst decrease ratio. Body weight was measured on days 0 and 8 post challenge. Fecal samples were collected daily from days 6 to 8 post challenge. The number of fecal oocysts was counted using a McMaster chamber, as previously described. The oocyst decrease ratio was calculated by the formula: (number of oocysts from the challenged − unvaccinated group − number of oocysts from the challenged − vaccinated group)/number of oocysts from the challenged − unvaccinated group × 100% (Rose and Mockett 1983). The caeca of each group were collected separately. Intestinal lesions were scored according to the method of Johnson and Reid (Morehouse and Baron 1970).

Preparation of the serum

The blood samples of day 8 post challenge were collected, incubated at 37 °C for 1 h, and centrifuged at 5000 rpm for 5 min at 4 °C to isolate the serum. The sera were used for the detection of antibody, cytokines, sCD4, and sCD8.

Determination of serum antibody levels

The levels of antibody (serum IgG) against r*Et*AN1-ZnFP were measured by enzyme-linked immunosorbent assay (ELISA) at day 8 post challenge, as previously described (Lee et al. 2013; Lillehoj et al. 2005; Lin et al. 2017; Ding et al. 2004). Briefly, 96-well microtiter plates (Corning, NY, USA) were coated with purified r*Et*AN1-ZnFP (10 μg/well) at 4 °C overnight. All plates were washed with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T) more than three times and blocked with PBS containing 1% BSA for 2 h at 37 °C. Serum samples were added to the plates at a 1:25 dilution (50 μL/well) and incubated for 1 h at 37 °C. After washing five times with PBS-T, 50 μL/well HRP-donkey-anti-chicken IgG antibody (Sigma, St. Louis, MO, USA) with a 1:5000 dilution was added and incubated for 2 h at 37 °C. The plates were washed five times with PBS-T and developed with 3,3′,5,5′-tetramethylbenzidine. Optical densities at 450 nm (OD450) were measured on a microplate spectrophotometer. All assays were performed in triplicate.

Determination of cytokine, sCD4, and sCD8 levels

The immune stimulation effect of r*Et*AN1-ZnFP protein on chickens was evaluated by quantitative ELISA at day 8 post challenge, as previously described (Lee et al. 2013; Lillehoj et al. 2005; Lin et al. 2017; Ding et al. 2004). The cytokines, sCD4, and sCD8 used were soluble cluster of differentiation 4 (sCD4), soluble cluster of differentiation 8 (sCD8), interleukin-17 (IL-17), and transforming growth factor (TGF-β1). Chick Cytokine ELISA Quantitation Kits (catalog numbers CSB-E13114C, CSB-E14317C, CSB-E04607Ch, and CSB-E09875Ch; CUSABIO, Wuhan, China) were used to quantify sCD4, sCD8, IL-17, and TGF-β1, respectively.

Statistical analysis

SPSS version 22 (SPSS, Chicago, IL, USA) was used in body weight gain, mean lesion scores, fecal oocyst output, and oocyst decrease ratio. Microsoft Office Excel version 2016 for Windows (Redmond, WA, USA) was used in record original data of body weight, oocyst count antibody levels, and cytokine levels. GraphPad Prism version 6.0 (GraphPad, La Jolla, CA, USA) was used in these analyses. Data on real-time quantitative PCR (qPCR), invasion inhibition, body weight gain, fecal oocyst output, oocyst decrease ratio, and antibody and cytokine levels were analyzed. Differences between groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test. The lesion scores were compared by the non-parametric Kruskal–Wallis test. p values smaller than 0.05 were considered significant, and p values smaller than 0.01 were considered highly significant. Bars with different letters were significantly different ($p < 0.05$) and the error bars indicate standard deviations.

Results

Characterization of the *Et*AN1-ZnFP sequence

PCR amplification of *Et*AN1-ZnFP resulted in a 798-bp product. Nucleotide sequence analysis showed that the 798-bp

length of ORF sequence encodes a polypeptide of 265 amino acid residues with a predicted molecular mass of 27.0 kDa. The sequence we obtained respectively shared 100%, 92%, and 63% amino acid sequence homology with putative AN1-like ZnFPs from *E. tenella*, *E. necatrix*, and *E. maxima* by BLASTp analysis. The amino acid sequence had 100% homology with a putative *E. tenella* AN1-like ZnFP (Protein ID XP_013229520.1). SignalP program analysis revealed that the protein most likely does not have a signal peptide and a transmembrane domain. Structural module and conservative structure predictions indicated that the protein had an AN1-like zinc finger and an A20-like zinc finger domains. One zinc finger ubiquitin hydrolase domain (204-236 aa), three casein kinase II phosphorylation sites, two N-myristoylation sites, and one protein kinase C phosphorylation site were also predicted (Fig. 1).

Expression and purification of recombinant *Et*AN1-ZnFP

r*Et*AN1-ZnFP was successfully expressed in *E. coli* BL21(DE3) using the expression vector pGEX-4T-1 after induction with 0.8 mM IPTG for 8 h at 37 °C. The purified protein was purified from the supernatant using a GST-Bind Purification kit. The molecular mass of r*Et*AN1-ZnFP fused to the GST-tag was approximately 72 kDa (Fig. 2) but was larger than expected (55 kDa). Western blot analysis showed that the recombinant protein was recognized by the anti-GST monoclonal antibody (Fig. 2) and rabbit sera against sporozoites of *E. tenella* (Fig. 2, lane 2), but was not detected by naïve rabbit serum (Fig. 2C, lane 4).

*Et*AN1-ZnFP transcription at different developmental stages of *E. tenella*

The transcription of *Et*AN1-ZnFP at different developmental stages (UO, SO, Spz, and Mrz) was assessed by qPCR. The relative expression of *Et*AN1-ZnFP was calculated using the $2^{-\Delta\Delta Ct}$ method. Among the four development stages, *Et*AN1-ZnFP transcripts were most abundant in UO, moderately expressed in Spz stages, and much lower in SO and Mrz (Fig. 3). Compared with Spz, the level of *Et*AN1-ZnFP transcripts was significantly higher in UO ($p < 0.05$) and significantly lower in SO and Mrz ($p < 0.05$).

*Et*AN1-ZnFP localization by indirect immunofluorescence during in vitro infection

*Et*AN1-ZnFP was detected in sporozoites and second-generation merozoites, and during first-generation schizogony in vitro using anti-r*Et*AN1-ZnFP polyclonal antibody. In sporozoites incubated with PBS or CM, *Et*AN1-ZnFP was uniformly distributed in the cytoplasm except for the refractive body (Fig. 4A and B). After incubating for 2 h in CM, the

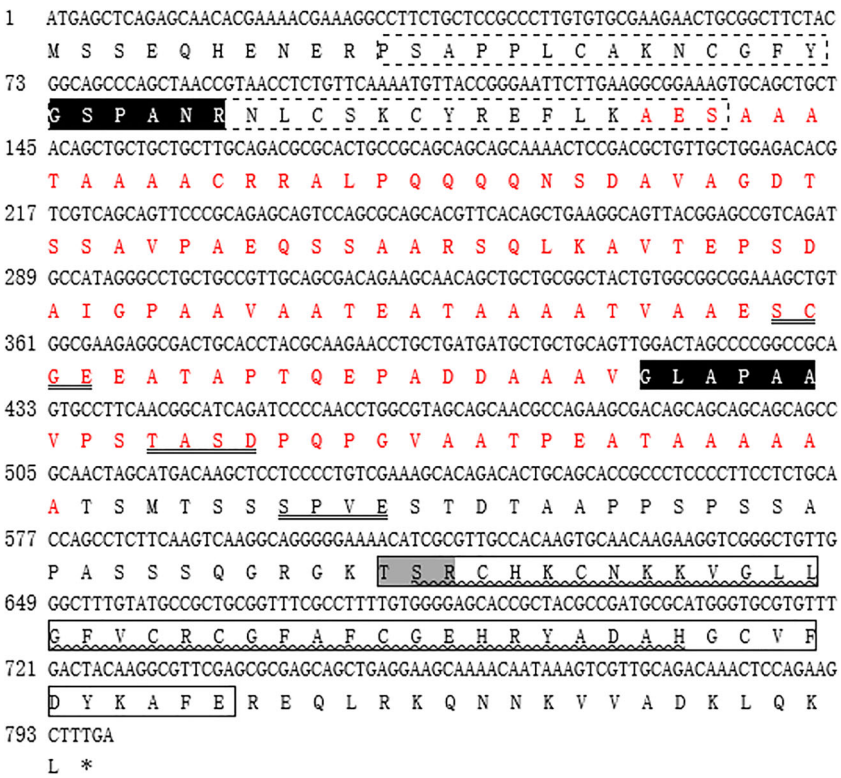

Fig. 1 Nucleotide and predicted amino acid sequences of *Et*AN1-ZnFP. The stop codon is indicated with an asterisk. Casein kinase II phosphorylation sites are double underlined. Zn-finger ubiquitin hydrolases are underlined by a wavy line. N-Myristoylation sites are shaded black with white lettering. Protein kinase C phosphorylation sites are shaded gray. The AN1-like zinc finger is indicated by a black box. The A20-like zinc finger is indicated by a black box with a dotted line. The alanine-rich region is indicated with red lettering

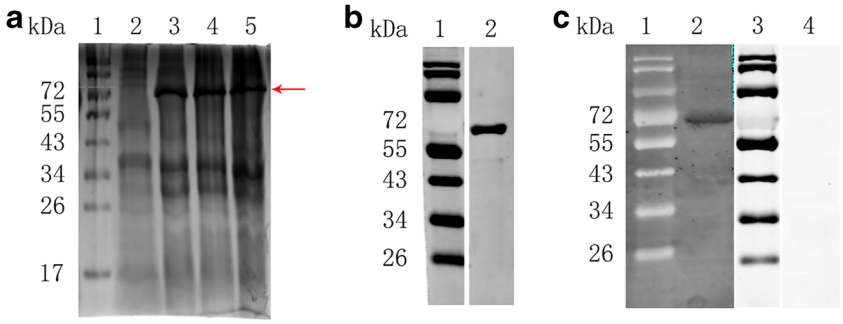

Fig. 2 Expression and purification of r*Et*AN1-ZnFP. (A) SDS-PAGE of the r*Et*AN1-ZnFP negative control (not induced with IPTG) (lane 2), induced with IPTG for 3 to 9 h (lanes 3 to 5). (B) Western blot analysis of purified r*Et*AN1-ZnFP protein recognized by an anti-GST monoclonal antibody (lane 2). (C) Western blot analysis of purified r*Et*AN1-ZnFP protein recognized by rabbit sera against sporozoite of *E. tenella* (lane 2) and incubated with naïve rabbit serum (lane 4). Lane 1 and lane 3 contain protein markers. The sizes of protein markers (kDa) are shown on the left

expression level of *Et*AN1-ZnFP was the same as that expressed in PBS for 2 h. After sporozoite-infected DF-1 cells for 48–60 h, *Et*AN1-ZnFP was evenly distributed in the cytoplasm of immature schizonts (Fig. 4 and). However, after immature schizonts differentiated into first-generation mature schizonts at 82 h p.i., the expression levels of *Et*AN1-ZnFP decreased, and the green fluorescence intensity was lower (Fig. 4). Furthermore, *Et*AN1-ZnFP was primarily located in the membrane and cytoplasm of second-generation merozoites (Fig. 4F).

In vitro invasion inhibition assay

To assess the ability of *Et*AN1-ZnFP to inhibit the infection of *E. tenella*, sporozoites were purified, incubated with different concentrations of purified anti-r*Et*AN1-ZnFP antibody (50, 100, 200, and 300 μg/mL), and used to infect DF-1 cells. Protein function was blocked by pre-incubation of sporozoites with purified antibody against r*Et*AN1-ZnFP before DF-1 cell infection. The infection inhibition rate was up to 30% at 100 μg/mL (Fig. 5). Compared with the same dose of naive rabbit sera IgG (negative control), pretreatment with 50 μg/mL anti-*Et*AN1-ZnFP IgG had no significant effect on the invasion capacity of sporozoites ($p > 0.05$), while pretreatment with 100, 200, and 300 μg/mL significantly decreased invasion ($p < 0.05$). In contrast, naïve rabbit sera IgG did not significantly affect the rate of infection.

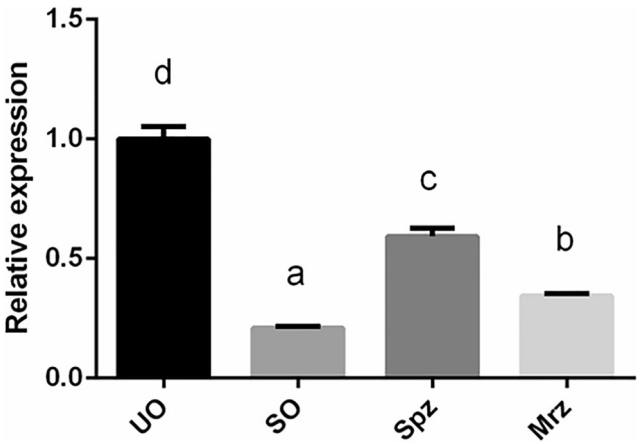

Fig. 3 Quantitative real-time PCR amplification of *Et*AN1-ZnFP mRNA in different life stages of *E. tenella* UO, unsporulated oocysts; SO, sporulated oocysts; Spz, sporozoites; Mrz, merozoites. Bars with different letters indicate significant differences ($p < 0.05$) and the error bars indicate standard deviations

Protective efficacy of vaccination on *E. tenella* challenge

Chickens were immunized twice subcutaneously with 50 or 100 μg of purified r*Et*AN1-ZnFP. The body weight gain in immunized-challenged chickens was higher than that in unimmunized-challenged animals, but the difference was not significant ($p > 0.05$) (Table 1). Body weight gain was lower in the 50-μg– and 100-μg–immunized groups than the unimmunized-unchallenged group, but the difference was only significant ($p < 0.05$) in the latter group. The mean lesion scores and fecal oocyst output in r*Et*AN1-ZnFP-immunized chickens were significantly lower than those in unimmunized-challenged birds ($p < 0.05$). Vaccination with 50 μg of recombinant protein resulted in better protection than vaccination with 100 μg in terms of lesion score and body weight gain. However, oocyst decrease ratio of 100-μg–immunized group is more obvious than 50-μg–immunized group (Table 1).

IgG titers and cytokine, sCD4 and sCD8 concentrations in sera of immunized chickens

The serum IgG titers and cytokine concentrations, sCD4, and sCD8 in sera of chickens following immunization with r*Et*AN1-ZnFP protein are shown in Fig. 6. The IgG titers of both 50 μg r*Et*AN1-ZnFP-immunized group and 100 μg

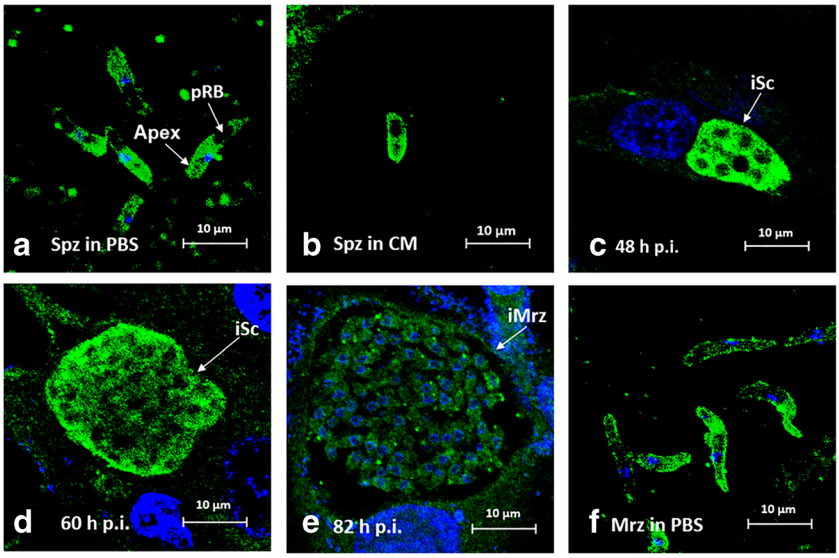

Fig. 4 *Et*AN1-ZnFP localization in infected DF-1 cells by indirect immunofluorescence at different developmental stages of *E. tenella*. Parasites were injected with anti-r*Et*AN1-ZnFP, stained with FITC (green), and counterstained with DAPI (blue). Infected DF-1 cells were harvested at the indicated times. (A) Sporozoites (Spz) in PBS, pRB, posterior refractile body; (B) Spz in complete medium. Infected DF-1 cells were collected at the indicated time points post-infection (pi); (C) immature schizonts (iSC) 48 h pi (hpi); (D) immature schizonts (iSC) 60 hpi; (E) intracellular merozoites (iMrz) 82 hpi; (F) merozoites (Mrz) in PBS

r*Et*AN1-ZnFP-immunized group were significantly higher compared with those of the unimmunized-challenged group ($p < 0.05$) (Fig. 6). As depicted in Fig. 6C, serum from chickens immunized with 50 μg r*Et*AN1-ZnFP and 100 μg r*Et*AN1-ZnFP protein showed significantly high levels of sCD8 ($p < 0.05$) compared with that from unimmunized-challenged group. But no significant differences ($p > 0.05$) of sCD4, IL-17, and TGF-β1 were observed between the r*Et*AN1-ZnFP-immunized and unimmunized-challenged groups (Fig. 6B, D, and E).

Discussion

ZnFPs are formed by a protein structural motif containing conserved amino acid residues tetrahedrally coordinated to one or more zinc ions containing a finger structure, which is a finger-like tetrahedral structure formed by the combination of zinc ions and several conserved amino acid residues. The zinc finger structure of ZnFPs was first discovered by Miller in 1985 in the *Xenopus laevis* transcription factor TFIIIA (Miller et al. 1985). Zinc ions help maintain the function of the protein (Miller et al. 1985). A previous study indicated that plant A20/AN1 ZnFPs serve as an important hub to mediate antiviral immunity (Chang et al. 2018). Zinc finger protein is a kind of transcription factor with a finger-like domain, which plays an important role in life regulation such as gene expression regulation, cell differentiation, and embryo development. Therefore, we speculated that *Et*AN1-ZNFP also may be involved in invasion and survival of the parasite. Whether this protein is related to the virulence or growth of the parasite in the host cells needs to be further researched. Nonetheless, few studies to date investigated ZnFPs in protozoa.

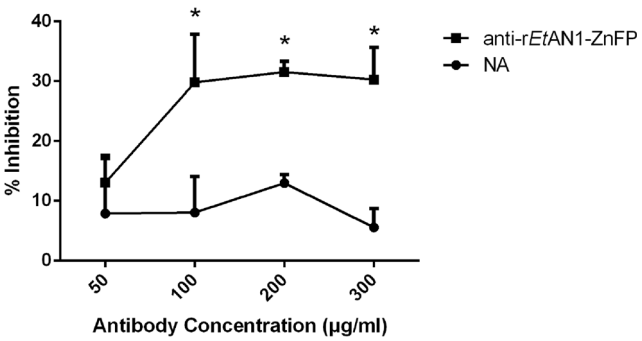

Fig. 5 Inhibition of sporozoite invasion in vitro. Anti-r*Et*AN1-ZnFP, rabbit antiserum against recombinant *Et*AN1-ZnFP protein; NA, naïve rabbit serum. All assays were performed in triplicate. *$p < 0.01$ for differences between treatment with antibody against r*Et*AN1-ZnFP and naïve rabbit serum at the same IgG concentration

The AN1-like zinc finger and A20-like ZnFPs have been described in the literature (Mukhopadhyay et al. 2004; Vij and Tyagi 2008). In this study, we cloned and characterized a ZnFP of *E. tenella*. BLASTp analysis indicated that the cloned sequence shared 100%, 92%, and 63% amino acid sequence homology with putative AN1-like ZnFPs from *E. tenella*, *E. necatrix*, and *E. maxima*, respectively. These results indicate that AN1-like ZnFPs of *E. tenella* and AN1-like ZnFPs of *E. necatrix* have high homology. *E. tenella* and *E. necatrix* are the most pathogenic species among *Eimeria* spp. and they can all reside in cecal mucosa (Sharma et al. 2015).

We successfully expressed r*Et*AN1-ZnFP using a recombinant prokaryotic expression system. The molecular mass of r*Et*AN1-ZnFP was approximately 72 kDa, which is higher

Table 1 Protective effect of r*Et*AN1-ZnFP protein on *E. tenella* infection

Groups	Average body weight gains (g)	Mean lesion scores	Oocyst shedding per bird ($\times 10^7$)	Oocyst decrease ratio (%)
Unchallenged control	258.62 ± 70.26^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	100^{c}
Challenged control	180.87 ± 45.38^{a}	3.20 ± 0.83^{c}	4.43 ± 0.99^{c}	0.00^{a}
r*Et*AN1-ZnFP-50μg	209.00 ± 31.82^{ab}	1.20 ± 0.44^{b}	1.68 ± 0.80^{b}	63.00 ± 13.11^{b}
r*Et*AN1-ZnFP-100μg	184.50 ± 51.02^{a}	1.80 ± 0.83^{b}	1.16 ± 0.81^{b}	75.94 ± 15.12^{b}

[a–d] Values with different letters in the same column are significantly different ($p < 0.05$) according to the ANOVA Duncan test

than that of the deduced amino acid sequence. This difference may be due to several reasons. Muh et al. compared the migration of proteins reduced or not with DTT on SDS-PAGE and found that DTT could affect migration (Muh et al. 2018). Samsó et al. (1995) reported that the large micelles of the complex could cause an abnormal electrophoretic migration of the sodium dodecyl sulfate/histone H5 complex (Samso et al. 1995). Moreover, it is well known that strongly basic or acidic proteins may migrate anomalously on SDS-PAGE gels (Papageorgiou and Soteriadou 2002).

E. tenella undergoes meiosis in the unsporulated oocyst stage when exposed to oxygen and moisture in the environment (Belli et al. 2006). In this study, the results of qPCR showed that the levels of the mRNA transcripts of *Et*AN1-ZnFP were high in unsporulated oocyst and sporozoite. Gissot et al. (2017) have found that the G1 phase is the period of intense production of transcripts in *T. gondii*, and parasite development is blocked in G1 phase when *Tg*ZnFP2 is depleted (Gissot et al. 2017). Additionally, it has been found that ZnFPs can bind to RNA in rice (Jin et al. 2018). *Tg*ZnFP2 and *Et*AN1-ZnFP belong to the zinc finger protein family. We supposed that the high expression of *Et*AN1-ZnFP in unsporulated oocyst may be related to the binding of *Et*AN1-ZnFP to RNA. Immunofluorescence showed that staining with *Et*AN1-ZnFP was stronger in the sporozoite and schizogony stages, and the result of qPCR also indicated that *Et*AN1-ZnFP has a high level of transcription in sporozoite stages; these suggest that this protein may be related to parasite invasion and development. Our hypothesis was confirmed by the results of the invasion inhibition assay. The inhibitory effect of polyclonal anti-r*Et*AN1-ZnFP IgG on sporozoites was gradually increased by up to 30%. Gissot et al. (2017) have demonstrated that *Tg*ZnFP2 is essential for parasite survival in vivo and in vitro (Gissot et al. 2017). Therefore, we postulated that *Et*AN1-ZnFP is involved in *E. tenella* invasion and development. Furthermore, no studies to date investigated ZnFPs in *E. tenella*; then our study provides information on this research. This new antigen might be used to identify novel vaccine targets and improve knowledge of immunogenic proteins in *E. tenella*.

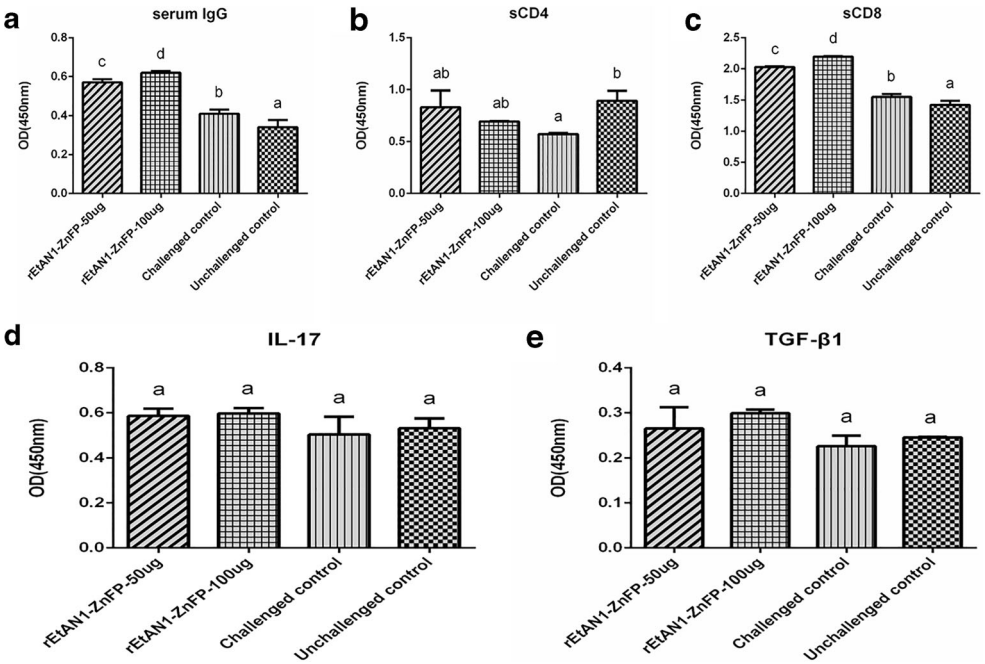

Fig. 6 Levels of IgG (A), sCD4 (B), sCD8 (C), cytokines IL-17 (D), and TGF-β1 (E) in chicken sera were measured using ELISA. Chickens were infected with *E. tenella* except the unchallenged control group. Chickens of group r*Et*AN1-ZnFP-50 μg and group r*Et*AN1-ZnFP-100 μg were immunized with 50 μg or 100 μg of r*Et*AN1-ZnFP protein, respectively. Challenged and unchallenged groups were immunized with PBS and served as controls. The IgG titers and the concentrations of sCD4, sCD8, and cytokines are expressed as mean ± SD. Bars with different letters were considered significantly different ($p < 0.05$)

In previous research, there was a significant decrease in lesion scores and fecal oocyst output after immunization of chickens with recombinant protein or recombinant plasmids (Huang et al. 2015; Zhai et al. 2016). In our study, following challenge infection, chickens vaccinated with r*Et*AN1-ZnFP had significantly lower lesion scores and fecal oocyst output compared with unimmunized birds. Our data showed that immunization with r*Et*AN1-ZnFP could induce partial protection against live *E. tenella* infection. Due to the "crowding effect," an infective dose lower than the crowding threshold should be used for experiments on effects of immunization on oocyst production (Williams 2001). Williams (1973) have pointed that increasing doses of oocysts give rise to progressively higher oocyst yields when the infection dose is less than 2.5×10^4 oocysts per bird (Williams 1973). Our experiment which infected 1×10^4 oocysts per chicken was consistent with this report. This result highlights the importance of AN1-ZnFP in *E. tenella* and demonstrates its vital role in coccidiosis.

In recent years, many researchers have performed immunizations with recombinant proteins and determined the effects of these proteins by measuring cytokine levels in spleen or serum by RT-qPCR or ELISA (Ding et al. 2004; Lin et al. 2017). Kundu et al. (2017) found that chickens immunized with r*Et*IMP-1 produced a significantly strong IgG response (Kundu et al. 2017). Smith et al.(1993) observed that there was a slight increase in IgG levels after primary infection but a strong increase occurred after challenge (Smith et al. 1993). In the present study, the IgG concentrations in the serum of immunized chickens were significantly higher than those in the control groups by using ELISA after 8 days of challenge. A previous report also showed that chickens infected with coccidiosis can stimulate humoral immune response (Wallach 2010).

After chicken infection with coccidia, intestinal intraepithelial lymphocytes (IELs) express high levels of Th1-related cytokines and the Th2 cytokines (Cornelissen et al. 2009). The soluble sCD4 and sCD8 antigens are released by CD4+ and CD8+ lymphocytes, respectively (Zajkowska et al. 2001). The concentrations of sCD4 and sCD8 in serum are consistent with the number of CD4+ and CD8+ lymphocytes (Willsie et al. 1996). And sCD8 is a sensitive and specific parameter of cytotoxic and suppressor T cell activation (Orditura et al. 1998). In a previous study on malaria, patients infected with *Plasmodium* had a gradual increase in sCD8 levels, which was associated with malaria-associated immunosuppression (Harpaz et al. 1992). Liu et al. reported that the levels of sCD4 and sCD8 were higher in the groups immunized with r*Em*SAG compared with the control groups (Liu et al. 2018). The results in our study showed that sCD8 concentrations of the vaccinated groups were significantly higher than the control groups. These results indicate that sCD4 and sCD8 may play a role in immunization against coccidiosis and suggest that *Et*AN1-ZnFP can stimulate cellular immunity.

The Th2-type cytokines IL-17 which produced by Th17 cells are also involved in the immune response to coccidial challenge. Previous studies reported that the induction of Th1 cytokine-producing cells is correlated with a marked reduction in the numbers of Th2 cytokine-synthesizing cells (Bozza et al. 2004; Lu and Zhong 1999). Thus, del Cacho et al. (2012) considered that the immune response is polarized toward a Th1 response following immunization with Ag-loaded exosomes (del Cacho et al. 2012). Further, IL-17 facilitates the immunopathology during *E. tenella* infection and the elevated IL-17 might be harmful to the host (Huang et al. 2015). Two earlier reports showed that serum IL-17 levels were increased in all immunized birds (Huang et al. 2015; Pastor-Fernandez et al. 2018). However, our results showed that serum IL-17 was not significantly different in all immunized chicken compared with that in non-immunized chicken.

TGF-β is another cytokine that related with the immune suppression mechanism (Kehrl et al. 1986). Zhu et al. (2012) and Song et al. (2010) found that TGF-β was significantly higher in chickens immunized with recombinant *Eb*AMA1 (Song et al. 2010; Zhu et al. 2012), which produces Treg cells involved in regulating immune responses (Chen et al. 1995; Fukaura et al. 1996). In the current study, the level of the Treg-type cytokine TGF-β1 in the r*Et*AN1-ZnFP-immunized groups was similar to that in the unimmunized group ($p > 0.05$). We speculate that the discrepancy in the results may be associated with the different parasite strains and proteins used in animal challenge experiments.

In conclusion, Th2-type cytokines regulate the immune response through different pathways, which downregulates the expression level of Th1-type cytokines (Hong et al. 2006). Th1-type cytokines predominate in coccidial infections in chickens (Choi et al. 1999; Laurent et al. 2001). In our study, serum IgG level highly increased following immunization, while high levels of sCD4 and sCD8 were increased. In contrast, IL-17 and TGF-β1 did not increase significantly. Our results corroborated this finding and indicated that Th1-type cytokines were higher after immunization with r*Et*AN1-ZnFP, suggesting that r*Et*AN1-ZnFP elicits strong immune responses to *E. tenella* infection.

Conclusions

This study focused on the amplification of the gene encoding a ZnFP of *E. tenella*, and its nucleic acid and amino acid sequences were analyzed. Moreover, the distribution of r*Et*AN1-ZnFP in the sporozoite and merozoite stages of the parasite was determined, and its polyclonal antibody was used to detect the inhibitory effect of this protein on sporozoite invasion. The antibody against r*Et*AN1-ZnFP could reduce the rate of sporozoite invasion. Animal immune protection assays with recombinant proteins were performed, and the

results were satisfactory. Vaccination with r*Et*AN1-ZnFP could elicit partial protective immunity against *E. tenella*. However, the mechanism of action of r*Et*AN1-ZnFP in coccidial infections needs to be further studied.

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Compliance with ethical standards

The animal experiments, which involved animal immune protection experiments, were performed in accordance with the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (Permit Number:SHVRI-SZ-20180106-3), and were conducted in strict follow the recommendations outlined in the Guide for the Care and Use of Laboratory Animals.

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