Poly (D, L-lactide-co-glycolide) delivery system improve the protective efficacy of recombinant antigen TA4 against *Eimeria tenella* infection

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ABSTRACT *Eimeria tenella* is a protozoan parasite endemic in chickens and is one of the causative agents of avian coccidiosis. The aim of this research was to determine if poly (D, L-lactide-co-glycolide) (**PLGA**) nanoparticles carrying recombinant TA4 protein of *E. tenella* (**rEtTA4**) could improve the level of protective immunity against *E. tenella* challenge. Recombinant TA4 protein was expressed and purified. Poly (D, L-lactide-co-glycolide) loaded with rEtTA4 (**PLGA-rEtTA4**) nanoparticles was prepared and was delivered to 2-week-old layer chickens via intramuscular inoculation. Chickens injected with PBS and PLGA nanoparticles were served as control groups. The rEtTA4 and PLGA-rEtTA4 nanoparticles induced changes of serum cytokines, IgY levels, and T lymphocytes subpopulation, and the protective efficacy against *E. tenella* challenge was evaluated. Results showed that both rEtTA4 and PLGA-rEtTA4 vaccination groups induced significantly higher levels of specific EtTA4 IgY antibody and IL-17 and higher proportion of $CD8^+$ T lymphocytes. However, no significant differences were observed in the proportion of $CD4^+$ T lymphocytes compared with the PBS control. Chickens immunized with rEtTA4 and PLGA-rEtTA4 prominently increased the BW gains and decreased oocyst output compared with chickens immunized with PBS and PLGA after oral challenge with *E. tenella*. Poly (D, L-lactide-co-glycolide) encapsulated rEtTA4 nanoparticles–immunized chickens significantly induced higher levels of interferon gamma, IL-6, and IL-17 and a little bit higher proportion of $CD8^+$ T lymphocytes compared with rEtTA4 subunit vaccine–immunized chickens. Thus, PLGA encapsulated rEtTA4 nanoparticles appeared to have great potential to enhance the immune response and improved the protective efficacy against *E. tenella* infection. Our results provided available protective subunit vaccine rEtTA4 and PLGA loaded with rEtTA4 nanoparticles against coccidiosis and suggested that PLGA nanoparticles could be an effective adjuvant to enhance the protective efficacy of rEtTA4 subunit vaccine.

Key words: *Eimeria tenella*, TA4 antigen, subunit vaccine, PLGA

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INTRODUCTION

Coccidiosis of domestic chicken is caused by single or multiple species of protozoan *Eimeria* and is one of the most important intestinal diseases resulting in huge commercial loss to the poultry industry worldwide (Haug et al., 2007; Shah et al., 2011; Blake and Tomley, 2014; Kumar et al., 2014). Among 7 *Eimeria* species, *Eimeria tenella (**Et**)*, *Eimeria necatrix, Eimeria maxima,* and *Eimeria acervulina* are the main reasons for global economic impact shown by avian coccidiosis (Williams, 1998; Shirley et al., 2004, 2005; Shah et al., 2010a, 2010b; Reid et al., 2014; Song et al., 2015b). *Eimeria* infection is very prevalent in the world owing to the reason that sporulated oocysts can persist for long periods in the environment and chickens can easily catch infection. Infection of *Eimeria* parasite lead to inefficient feed conversion ratios, decreased BW gain (**BWG**), reduced egg production, and even mortality (Song et al., 2015b; Huang et al., 2018).

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Recombinant vaccines consist of subunit vaccines and DNA vaccines are based on protective antigens of *Eimeria*, which have been widely studied as a novel strategy to control coccidiosis (Shirley et al., 2004; Zhu et al., 2012). To date, numerous *Eimeria* antigens have been investigated for their capacity to alleviate enteric lesions, reduce parasite replication, increase BWG, and improve feed conversion in the face of *Eimeria* challenge (Blake et al., 2017; Huang et al., 2018), and many recombinant *Eimeria* antigens showed protective immunity against chicken coccidiosis by inducing both humoral and cell-mediated immune responses (Blake and Tomley, 2014; Jenkins et al., 2018). However, no recombinant vaccine has yet to reach the marketplace (Lillehoj et al., 2000; Shirley et al., 2007; Subramanian et al., 2008). One reason limiting the use of subunit vaccine to the marketplace is the limited capability to elicit long-lasting cellular and humoral immune responses (Sahdev et al., 2014). Researchers have made many attempts to eliminate this limitation, and codelivery strategy is one of them, that is using adjuvants to deliver antigens of *Eimeria*.

Previous studies showed that *Eimeria* antigen codelivery with adjuvants can induce a broad and long-lasting humoral and cellular immunity (Yang et al., 2010; Zhu et al., 2012; Huang et al., 2015). Besides, immunization with antigen in microparticles can prolong the duration of antigen and leads to serum IgG response greater than those induced by antigen in adjuvant (Challacombe, 1983). Poly (D, L-lactide-co-glycolide) (**PLGA**) is a biopolymer approved by the U.S. Food and Drug Administration that can be used as adjuvant and PLGA nanoparticles have been widely used for target specific and controlled delivery of various drugs, proteins, and DNA in various biomedical applications (Mir et al., 2017). Previous study has found that PLGA encapsulated growth factors nanoparticles can protect the protein from degradation in the surrounding microenvironment and provide continuous release of protein (Quadros et al., 2020).

E. tenella is one of the most economically important *Eimeria* species (Shirley et al., 2005). Sporozoite surface antigen 1 of *E. tenella* namely TA4 has been widely investigated as a vaccine antigen (Blake et al., 2017). It has been reported that TA4 is able to bind cultured epithelial cells playing an important role in parasite attachment to the host before invasion, inducing protective immunity against *E. tenella* infection (Jahn et al., 2009; Reid et al., 2014; Song et al., 2015a).

To date, few reports on the use of PLGA nanoparticles delivery system to deliver subunit vaccines against chicken coccidiosis are available. In our laboratory, gene TA4 of *E. tenella* (GenBank accession No. M21088.1) has been cloned to produce the recombinant plasmid pET-28b-TA4 (Song et al., 2015a). In the present study, recombinant EtTA4 (**rEtTA4**) subunit vaccine and PLGA encapsulated rEtTA4 protein (**PLGA-rEtTA4**) nanoparticles were prepared and delivered to chickens intramuscularly. The immune response induced by PLGA-rEtTA4 nanoparticles was investigated. The protective efficacy of PLGA-rEtTA4 nanoparticles in chickens experimentally challenged with *E. tenella* sporulated oocysts was evaluated and compared with nonencapsulated rEtTA4 subunit vaccine.

MATERIALS AND METHODS

Materials

Poly (D, L-lactide-co-glycolide) (molar ratio of D, L-lactide to glycolide, 65:35, MW = 40,000–75,000) and polyinyl alcohol (MW=31,000–50,000, 98–99% hydrolyzed) were purchased from Sigma-Aldrich (St. Louis, MO). The Ni-NTA column was the product of GE Healthcare Life Sciences. The Micro BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL). Horseradish peroxidase-conjugated goat-anti-chicken IgY antibody was purchased from Abcam (Cambridge, UK). Mouse anti-chicken CD3-FITC antibody, mouse anti-chicken CD8α-PE antibody, and mouse anti-chicken CD4-PE were the products of Southern Biotechnology Associates (Birmingham, AL). The Toxin Eraser Endotoxin Removal Kit was the product of GeneScript (Piscataway).

Animals and Parasites

Animals Newly hatched Hy-Line brown layer chickens (commercial breed W-36) were raised in a sterilized room under coccidian-free environment throughout the experiment. Chickens were provided with coccidiostat-free feed and water. The feces of experimental chicken were examined periodically to check and ensure that chickens are free of coccidia. The study was conducted following the guidelines of the Animal Ethics Committee, Nanjing Agricultural University, China. All experimental protocols were approved by the Science and Technology Agency of Jiangsu Province. The approval ID is SYXK (SU) 2017-0007.

Parasites *E. tenella* JS strain was propagated and maintained in the MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University.

Preparation of rEtTA4 Protein

Recombinant *E. tenella* TA4 protein was expressed as a His-fusion protein in *Escherichia coli* BL21 (DE3). The recombinant protein was purified by the Ni-NTA column following the manufacturer's instructions. The purity of rEtTA4 was confirmed by SDS-PAGE followed by Coomassie blue staining. The concentration of rEtTA4 protein was determined using the Micro BCA Protein Assay Kit as per the Bradford procedure (Bradford, 1976). Endotoxins were reduced from recombinant proteins using the Toxin Eraser Endotoxin Removal Kit (GeneScript, Piscataway). The protein was stored at −20°C for later use.

Poly (D, L-Lactide-Co-Glycolide) Nanoparticles Preparation

Poly (D, L-lactide-co-glycolide) nanoparticles were prepared using the double emulsion solvent evaporation method, as described previously, with some modification (Nazarian et al., 2013). Briefly, 50 mg of PLGA was dissolved in 1 mL of dichloromethane. Then, 2 mL of 5% (w/v) polyinyl alcohol solution was added slowly into PLGA solution at a rate of 1 mL/min. The mixture was homogenized in an ice bath for 3 min by ultrasound (200W, work time 5 s, interval time 5s), and the primary emulsion (W1/O) was obtained. After that, 5 mg recombinant EtTA4 protein was added in a dropwise manner to the emulsion and was ultrasonicated for 3 min in an ice bath. Then, 2 mL of 5% polyinyl alcohol solution was added into the emulsion in a dropwise manner and was ultrasonicated continuously for 6 min in an ice bath. After ultrasonication, the secondary emulsion was continually stirred for more than 2 h at room temperature to evaporate dichloromethane. Poly (D, L-lactide-co-glycolide) nanoparticles in the supernatant were collected by ultracentrifugation at 30,000 rpm for 25 min at 4°C. The pellet was collected and freeze-dried for 24 h to get lyophilized powder of PLGA nanoparticles for further use. Blank PLGA nanoparticles were also prepared and used as control.

Poly (D, L-Lactide-Co-Glycolide) Nanoparticles Characterization

Morphology The morphology of lyophilized PLGA-rEtTA4 nanoparticles were examined under a JSM-IT100 series scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at 16.0 kV. Photographs were taken at progressively higher magnifications up to × 10,000.

Determination of EtTA4 Encapsulation Efficiency The encapsulation efficiency of rEtTA4 into PLGA nanoparticles was determined using the Micro BCA Protein Assay Kit. After ultracentrifugation at 30,000 rpm for 25 min at 4°C, unencapsulated rEtTA4 protein was measured in supernatant.

In Vitro Release of EtTA4 from PLGA Nanoparticles Release of rEtTA4 protein from PLGA-rEtTA4 nanoparticles was assayed in vitro performed by monitoring changes of the free rEtTA4 protein in solution. Three milligrams of lyophilized nanoparticles was dispersed in 150 μL sterile PBS (0.1 M, pH 7.4) and placed in a shaker bath (37°C, 120 rpm). At specific time intervals (2, 6, 12, 24, 48, 72, 96, 120, 144, 192, 288 h), the suspension was centrifuged at 12,000 rpm for 15 min, and 60 μL of supernatant was removed and immediately replaced with the same volume of fresh PBS. The concentration of free recombinant EtTA4 protein in the supernatants was determined using the Micro BCA Protein Assay Kit. All analyses were performed in triplicate.

Animal Experimental Designs

Newly hatched 1-day-old chicks were raised under coccidia-free conditions. On day 14, chickens were weighed, and similar-weight chickens were randomly divided into 5 groups of 23 each (Table 1). Experimental groups were inoculated with 100 μg rEtTA4 and 100 μg rEtTA4 in PLGA-rEtTA4 nanoparticles intramuscularly. Chicken immunized with PBS and PLGA nanoparticles served as control groups. Chickens from the challenged or unchallenged control group were given 300 μL of PBS. The PLGA group was given the same volume of PLGA per chicken. A booster immunization was given on day 21 with the same dose of components as first immunization. On day 28, chickens were orally challenged with 50,000 sporulated oocysts of *E. tenella*. The unchallenged control group was given the same volume of PBS orally. On day 28 and day 35, BW of all chickens were weighed to calculate weight gain during the *E. tenella* infection period. Seven day after challenge, all chickens were slaughtered, then average BWG, oocyst decrease ratio, lesion score, and anticoccidial index (**ACI**) of each group were calculated.

Evaluation of Immune Protection

Fifteen chickens were used for the study of immune protection. Immunization efficacy was evaluated on the basis of survival rate, lesion score, BWG, oocyst decrease ratio, and ACI (Morehouse and Baron, 1970; Chapman and Shirley, 1989). Survival rate was estimated by the number of surviving chickens divided by the number of initial chickens. BW gain was determined by the BW of the chickens at the end of the experiments subtracting the BW at the time of *E. tenella* challenge. Lesion scores of the chickens in different treatment groups were observed and recorded as per the methods of Johnson and Reid (Johnson and Reid, 1970). Caecal content of each group was collected separately and oocysts per gram were determined by McMaster's counting technique.

The ACI is a synthetic criterion for assessing the protective effect of a medicine or vaccine and calculated as follows: (survival rate + relative rate of weight gain) - (lesion value + oocyst value) (McManus et al., 1968). An ACI value of ≥180 was considered high performance, ACI value of 160 to 179 was considered as effective, and ACI value of <160 was considered as ineffective, as previously described (Zhang et al., 2014).

Determination of Serum EtTA4-specific IgY Antibody Level by Indirect ELISA

Groups were set as previously mentioned. Serum of preimmunization, 7 d after initial immunization, and 7 d after second immunization was obtained after centrifugation at 3,000 rpm for 5 min at 4°C. Serum from 5 chickens per group was used to determine the EtTA4-specific IgY antibody by indirect ELISA (Huang et al., 2015). Briefly, 96-well microtiter plates

Table 1. Protective efficacy of recombinant EtTA4 protein and PLGA-rEtTA4 nanoparticles against *Eimeria tenella* challenge.

Groups	Survival rate (%)	BW gain (g)	Relative BW gain (%)	Average lesion score in cecum	Oocyst output ($\times 10^6$)	Oocyst reduction ratio (%)	ACI
Unchallenged control	100.00	85.10 ± 8.98[d]	100.00	0.00 ± 0.00[a]	0.00 ± 0.00[a]	-	200.00
Challenged control	86.67	52.82 ± 8.93[a]	62.06	3.2 ± 0.78[c]	10.23 ± 2.44[c]	0.00	86.73
PLGA	93.33	66.28 ± 11.00[b]	77.89	3.07 ± 0.70[c]	9.01 ± 1.49[c]	11.99	120.52
rEtTA4	100.00	71.67 ± 8.98[b,c]	84.21	1.60 ± 0.91[b]	1.83 ± 0.59[b]	82.10	163.21
PLGA-rEtTA4	100.00	79.93 ± 9.89[c,d]	93.93	1.53 ± 0.92[b]	1.72 ± 0.63[b]	83.18	173.63

[a-d]Means within the same column with different superscript letters are significantly different ($P < 0.05$); n = 15. In the same column, no significant difference ($P > 0.05$) between groups is shown with the same letter. Data are presented as mean ± SD.

Chickens of the unchallenged control group did not receive any challenge. Chickens of the challenged control group were orally challenged with *Eimeria tenella* (50,000 oocysts per bird) on day 28. BW was measured in grams per individual bird on day 14, day 28, and day 35. Lesion scores were evaluated and scored on day 35. Caecal content of each group was collected and was used to determine oocysts per gram on day 35, namely 7 d after infection. After that the survival rate, BW gain, relative BW gain, lesion scores, oocyst reduction ratio, and anticoccidial index of each group were calculated.

Abbreviations: PLGA, Poly (D, L-lactide-co-glycolide); PLGA-rEtTA4, Poly (D, L-lactide-co-glycolide) loaded with rEtTA4; rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

(Costar) were coated with rEtTA4 (50 μg/mL) overnight at 4°C. After washing 3 times with 0.01 M PBS containing 0.05% Tween-20, the plates were blocked with 5% skim milk powder in PBS containing 0.05% Tween-20 for 2 h at 37°C, and 100 μL of the serum samples diluted 1:50 in PBS containing 0.05% Tween-20 and 5% skim milk powder was added for 1 h at 37°C. After washing, the plates were incubated with 1:40,000 dilution of horseradish peroxidase-conjugated goat-anti-chicken IgY antibody (Abcam) for 1 h at 37°C. Tetramethylbenzidine (Sigma) substrate was used to develop colors and the optical density at 450 nm was measured on a microplate reader (Thermo Scientific, United States). All serum samples were determined with 3 repeats.

Determination of Serum Cytokine Concentration

All serum samples were used for cytokines detection by ELISA. The concentration of interferon gamma, IL-4, IL-6, IL-10, and IL-17 in serum was detected by double antibody sandwich ELISA with the "chick cytokine ELISA Quantitation Kits" (catalog numbers: JEB-14761, JEB-14771, JEB-14816, JEB-14773, and JEB-15474 for interferon gamma, IL-4, IL-6, IL-10, and IL-17, respectively; Nanjing JinYibai Biological Technology Co. Ltd. China) as per the manufacturer's instructions.

Flow Cytometric Analysis of $CD4^+$ and $CD8^+$

Three chickens from each group were sacrificed 7 d after second immunization, and the spleens were removed. Single cells from spleens were prepared as described (Sasai et al., 2000). One hundred microliter of splenocyte suspension containing 1×10^6 cells was dually stained with mouse anti-chicken CD3-FITC and mouse anti-chicken CD8α-PE or mouse anti-chicken CD4-PE for 30 min at 4°C in the dark. Splenic lymphocytes population was analyzed on a BD FACSVerse flow cytometer (BD Biosciences, New Jersey). Data acquired were processed with FlowJo software, version 10.0 (Tree Star Inc, Ashland, OR).

Statistical Analysis

Statistical significances between the means of different treatment groups were analyzed using 1-way ANOVA with Duncan multiple range tests with SPSS software (IBM SPSS Statistics 25 version; SPSS Inc., Chicago, IL). Differences between means were considered significant at $P < 0.05$.

RESULTS

Expression and Purification of rEtTA4 Protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showed that purified rEtTA4 was expressed as a His-fusion protein with a molecular mass about 29 kDa (Figure 1); along with 4 kDa His-fusion protein in the vector, the molecular weight of EtTA4 protein was predicted approximately to be 25 kDa.

Characterization of Nanoparticles

The PLGA-rEtTA4 nanoparticles were successfully prepared using the double emulsion technique. Scanning electron microscopy was performed to investigate the geometric shape and particle size of PLGA-rEtTA4 nanoparticles. The PLGA-rEtTA4 nanoparticles displayed a spherical geometry with smooth and pore-free surfaces, and the particle size of PLGA-rEtTA4 nanoparticles was 0.225 ± 0.049 μm (Figure 2).

The encapsulation efficiency of PLGA-EtTA4 was 82.40 ± 0.06%, as measured using the Micro BCA Protein Assay Kit. The protein-loaded rate of PLGA-rEtTA4 nanoparticles was 2.00 ± 0.01%.

The release kinetic profile for rEtTA4-loaded nanoparticles revealed a burst during the first 24 h with ∼62.5% of antigen being released (Figure 3). The amount of rEtTA4 released with the first 4 d was around 77.47%. The behavior was followed by a slower release profile over the next 8 d.

Protection Against E. tenella *Challenge Studies*

A significant increase ($P < 0.05$) in BWG during *E. tenella* challenge was observed in rEtTA4 and PLGA-rEtTA4 nanoparticles vaccines–inoculated chickens relative to PBS or PLGA nanoparticles–inoculated controls (Table 1). The BWG in PLGA-rEtTA4 nanoparticles–inoculated chickens was equal ($P > 0.05$) to that observed in non–*E. tenella*–challenged controls (Table 1). Oocyst counts of rEtTA4 or PLGA-rEtTA4 nanoparticles–immunized chickens were significantly lower than those of the challenged control group or PLGA nanoparticles group ($P < 0.05$). Significant alleviations in cecum lesions were observed in rEtTA4 and PLGA-rEtTA4–immunized chickens compared with that of the challenged control group ($P < 0.05$). The ACI of the PLGA-rEtTA4 and rEtTA4–inoculated group was both greater than 160 but the PLGA-rEtTA4–inoculation group showed higher ACI.

Cytokines Concentration and IgY Titers in Sera of Immunized Chickens

The levels of cytokines of each group are illustrated in Figure 4. Serum samples from PLGA-rEtTA4 nanoparticles–immunized chickens showed significant higher concentration of interferon gamma (Figure 4A), IL-4 (Figure 4B), IL-6(Figure 4C), IL-10 (Figure 4D), and IL-17 (Figure 4E) than chickens of the PBS control group ($P < 0.05$). Chickens immunized with rEtTA4 generated higher levels of IL-10 (Figure 4D) and IL-17 (Figure 4E) at 7 d after first immunization when compared with the PBS control group ($P < 0.05$). The significant higher concentrations of IL-6 (Figure 4C) and IL-17 (Figure 4E) were observed in rEtTA4 vaccination group at 7 d after second immunization compared with the PBS control group ($P < 0.05$). The serum of chickens immunized with PLGA-rEtTA4 nanoparticles showed significantly higher levels of interferon gamma (Figure 4A), IL-6 (Figure 4C), and IL-17 (Figure 4E) compared with the rEtTA4-inoculated groups at 7 d after first and second immunization ($P < 0.05$) and generated significantly higher levels of IL-4 and IL-10 than that of the rEtTA4-vaccinated group at 7 d after second immunization ($P < 0.05$). Poly (D, L-lactide-co-glycolide) nanoparticles produced higher level of IL-4 than the PBS control group at 7 d after first immunization ($P < 0.05$).

Serum from chickens immunized with rEtTA4 and PLGA-rEtTA4 showed significantly higher level of EtTA4-specific IgY antibody ($P < 0.05$) than that of the PBS and PLGA nanoparticles control groups (Figure 4F), whereas nonspecific IgY antibody was detected in the PBS and PLGA nanoparticles control groups ($P > 0.05$) (Figure 4F). No significant difference of serum specific-EtTA4 IgY antibody was found between rEtTA4 and PLGA-rEtTA4–vaccinated groups ($P > 0.05$).

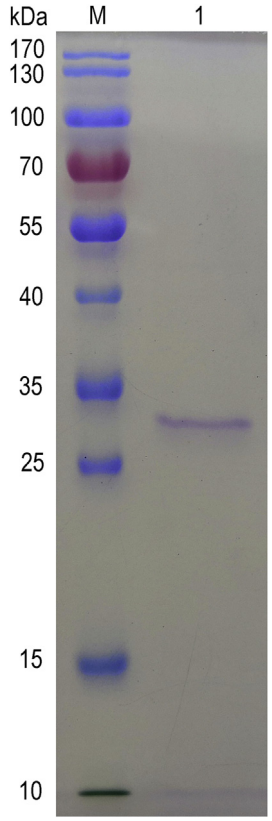

Figure 1. SDS-PAGE analysis of purification of rEtTA4 protein. Ni-NTA column was used to purify rEtTA4 protein and the purified rEtTA4 protein was confirmed by 12% SDS-PAGE followed by Coomassie blue staining. Lanes shown are as follows: M: standard protein molecular weight marker (ordinate values in kDa); Lane 1: purified rEtTA4. Abbreviations: PLGA, Poly (D, L-lactide-co-glycolide); rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

The Percentage of CD4$^+$ and CD8$^+$ of Splenic Lymphocyte

Splenic lymphocytes of immunized chickens were analyzed by flow cytometry at 7 d after second immunization (Figure 5A). No significant differences were observed in the percentage of CD4$^+$ T cells (Figure 5B) among the rEtTA4 group, PLGA-rEtTA4 group, PLGA group, and PBS control group ($P > 0.05$). Subunit vaccine rEtTA4 and PLGA loaded with rEtTA4 nanoparticles–vaccinated chickens showed significantly higher percentage of CD8$^+$ T cells (Figure 5C) than the PBS control group ($P < 0.05$). The percentage of CD8$^+$ T cells of the PLGA-rEtTA4 group was a little bit higher than that of the rEtTA4 group. No significant difference was observed in the percentage of CD8$^+$ T cells of the PLGA injection group compared with the other groups.

DISCUSSION

Coccidiosis is a disease of major global importance to the poultry industry and results in huge economic losses, as estimated in excess of $3 billion per annum (Kis et al., 1989; Shah et al., 2011; Blake and Tomley, 2014). In recent year, recombinant vaccines including subunit

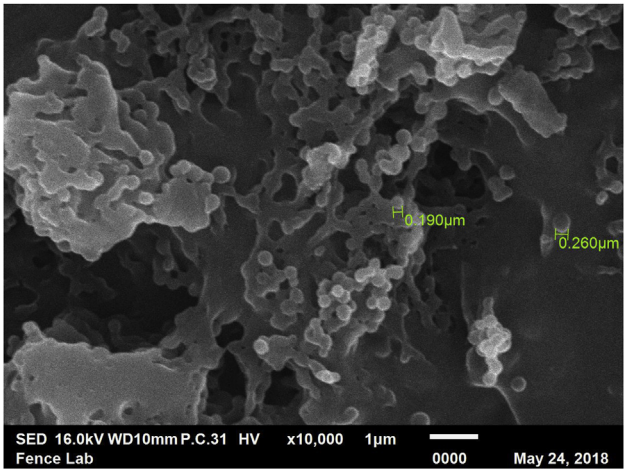

Figure 2. Scanning electron microscope images of PLGA nanoparticles loaded with rEtTA4. The lyophilized powder of PLGA encapsulated rEtTA4 nanoparticles was examined using a scanning electron microscope to observe its morphologic characteristics. Images were taken at 16 kV with a 10,000 magnification times. Scale bars are shown by horizontal lines. Abbreviations: PLGA, Poly (D, L-lactide-co-glycolide); rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

vaccine and DNA vaccine are used as novel strategy to control avian coccidiosis (Blake et al., 2017). This strategy relies significantly on the protective antigens of *Eimeria*, therefore the selection of protective antigens is extremely important. Previous studies in our laboratory suggested that recombinant TA4 can provide partial protection to *E. tenella* infection and TA4 is an effective candidate antigen for vaccine (Xu et al., 2008; Song et al., 2015a, 2015b).

In this study, rEtTA4 subunit vaccine and PLGA encapsulated rEtTA4 nanoparticles were used for vaccination against *E. tenella*. The results suggested that inoculation with EtTA4 could significantly increase the levels of sera EtTA4 specific IgY antibody, generate higher proportion of $CD8^+$ T cells, and promote the production of cytokines such as IL-17. Furthermore, animal challenge experiment showed that vaccination with

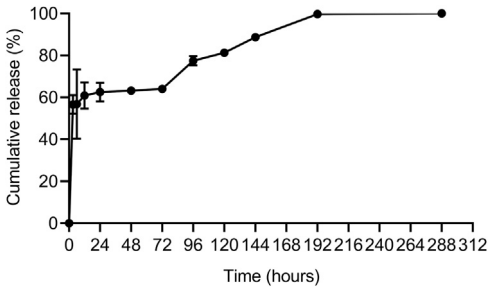

Figure 3. In vitro release profile of rEtTA4 from PLGA nanoparticles in sterile PBS during 12 d. Free rEtTA4 released from PLGA-rEtTA4 nanoparticles in PBS solution at different time point (2, 6, 12, 24, 48, 72, 96, 120, 144, 192, 288 h) was collected, and the concentration of released rEtTA4 protein was determined using the Micro BCA Protein Assay Kit. Cumulative percentage release values of rEtTA4 at each time point are presented as mean ± SEM (n = 3). Abbreviations: PLGA, Poly (D, L-lactide-co-glycolide); PLGA-rEtTA4, Poly (D, L-lactide-co-glycolide) loaded with rEtTA4; rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

EtTA4 could result in ACI of more than 163. These results revealed that inoculation with EtTA4 could provide protective immunity against *E. tenella*. Moreover, PLGA encapsulated rEtTA4 nanoparticles could significantly induce higher levels of cytokines including interferon gamma, IL-4, IL-6, and IL-17 and produced ACI higher than the rEtTA4 group. Besides, PLGA-rEtTA4 elicited a little bit higher proportion of $CD8^+$ T cells. These data demonstrated that PLGA nanoparticles could enhance the immune responses and promote the protective immunity of rEtTA4.

Recombinant TA4 protein was expressed and purified, the molecular weight of TA4 protein was about 25 kDa. This result is consistent with the findings that TA4 protein is a single polypeptide of 25 kDa located in the surface membranes of *E. tenella* sporozoites (Brothers et al., 1988; Xu et al., 2008). Poly (D, L-lactide-co-glycolide) encapsulated rEtTA4 nanoparticles was prepared and the particle size was 0.225 ± 0.049 μm. An initial burst release of rEtTA4 protein from PLGA nanoparticles was observed during the first 24 h with ∼62.5% of rEtTA4 protein being released. This initial burst effect is most likely owing to the rapid release of protein located on or near the surface of the nanoparticles (McGee et al., 1994). Currently, less information is available on the use of PLGA nanoparticles to deliver subunit vaccine of *Eimeria*.

In our study, the immunity induced by subunit vaccine rEtTA4 and rEtTA4-loaded PLGA nanoparticles was evaluated. Our results indicated that inoculation with rEtTA4 and rEtTA4-loaded PLGA nanoparticles both could provide moderate protection against *E. tenella* infection. This finding is consistent with the previous report that recombinant TA4 protein increased the BWG, reduced the oocyst production, alleviated the cecum lesions, and provided a medium protection against *E. tenella* infection (Song et al., 2015a). In addition, rEtTA4-loaded PLGA nanoparticles provided higher ACI than rEtTA4, showing a better protection. This result is in accordance with the findings that 20-nm polystyrene nanoparticles-conjugated *E. maxima* IMP1 antigen (NP-EMaxIMP1) increase the BWG and elicit complete protection against *E. maxima* challenge infection (Jenkins et al., 2018). It is known that the intestine is the specific parasitic site of *Eimeria* and the bursa and spleen are the immune organs. The finding is that polystyrene nanoparticles and the protein it carried can rapidly (within 1–6 h) migrate to the small intestine, bursa, and spleen by oral administration. This study indicates the ability of polystyrene nanoparticles to promote immune recognition at sites of *Eimeria* infection and deliver antigens to the sites of important immune function to improve immune response (Jenkins et al., 2018). However, the migration routes and distribution of PLGA nanoparticles in the chicken intestine, bursa, and spleen has not been investigated yet, and further research is needed.

IgY, equivalent to the mammalian IgG, is produced by lower vertebrates, for example, birds. Previous studies illustrated that nanoparticles larger than 200 nm carry

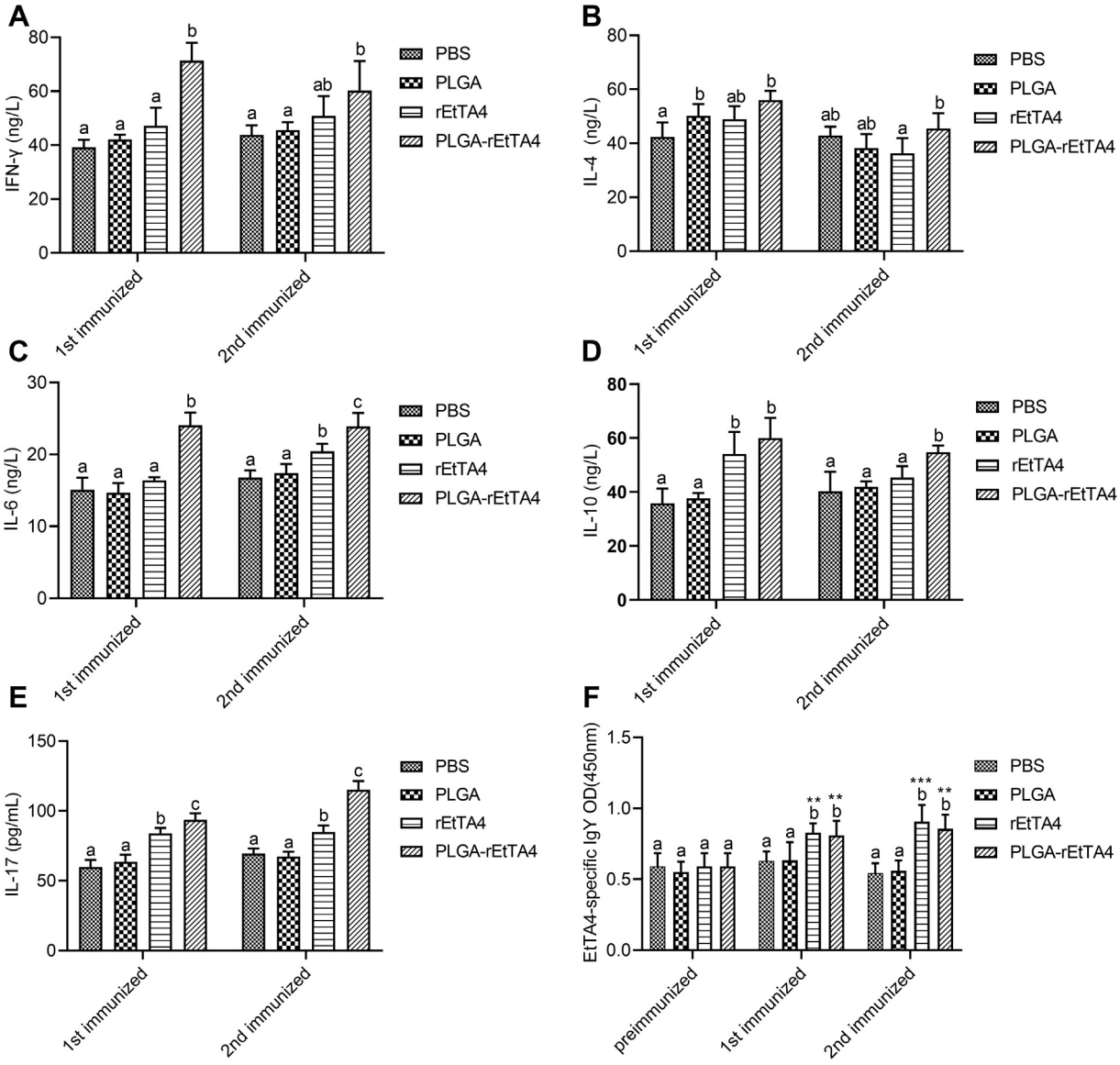

Figure 4. Cytokine concentrations and EtTA4-specific IgY in chicken serum. Chickens were immunized intramuscularly with PBS, PLGA, rEtTA4, and PLGA-rEtTA4. Serum was collected at 3 time point, 14 d (pre-immunization), 21 d (first immunized), and 28 d (second immunized). Serum cytokine concentration and EtTA4-specific IgY antibody levels were detected by ELISA. The significant difference between groups is shown with different letters ($P < 0.05$), and no significant difference ($P > 0.05$) between groups is shown with the same letter. The asterisk designates the difference among the pre-immunization, first immunized, and second immunized group when compared by stimulation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A) IFN-γ; (B) IL-4; (C) IL-6; (D) IL-10; (E) IL-17; (F) Serum EtTA4-specific IgY. Abbreviations: IFN-γ, interferon gamma; PLGA, Poly (D, L-lactide-co-glycolide); PLGA-rEtTA4, Poly (D, L-lactide-co-glycolide) loaded with rEtTA4; rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

larger amount of antigen (Howe and Konjufca, 2015) and immunization with antigen carried by microparticles leads to greater serum IgG response (Challacombe, 1983). In our study, remarkable serum specific EtTA4 IgY antibody response was observed both in rEtTA4 and 0.225 ± 0.049 μm of PLGA-rEtTA4 nanoparticles vaccination. However, no significant difference of serum specific-EtTA4 IgY antibody was found between them. This needs further investigation.

Interferon production is usually used as a measure of T cell responses to antigens of chicken coccidia, and interferon gamma is considered a mark of Th1 immune response (Lillehoj, 1998; Zhu et al., 2012). In addition, interferon gamma is involved in host defense against avian coccidiosis and plays a role to significantly reduce the intracellular sporozoite development without affecting sporozoite invasion of host cells (Lillehoj and Choi, 1998). Similarly, recombinant chicken interferon gamma with incomplete Freund's adjuvant elicits significant protection immunity against *E. acervulina* challenge, increases BWG and decreases the oocyst production (Lillehoj and Choi, 1998). Our results showed that PLGA encapsulated rEtTA4 nanoparticles vaccination generated significantly higher levels of interferon gamma. This maybe one of the reasons that explain the higher ACI of PLGA encapsulated rEtTA4 nanoparticles compared with rEtTA4. Previous studies and our result together confirm that interferon gamma may play an important role in the process against coccidiosis.

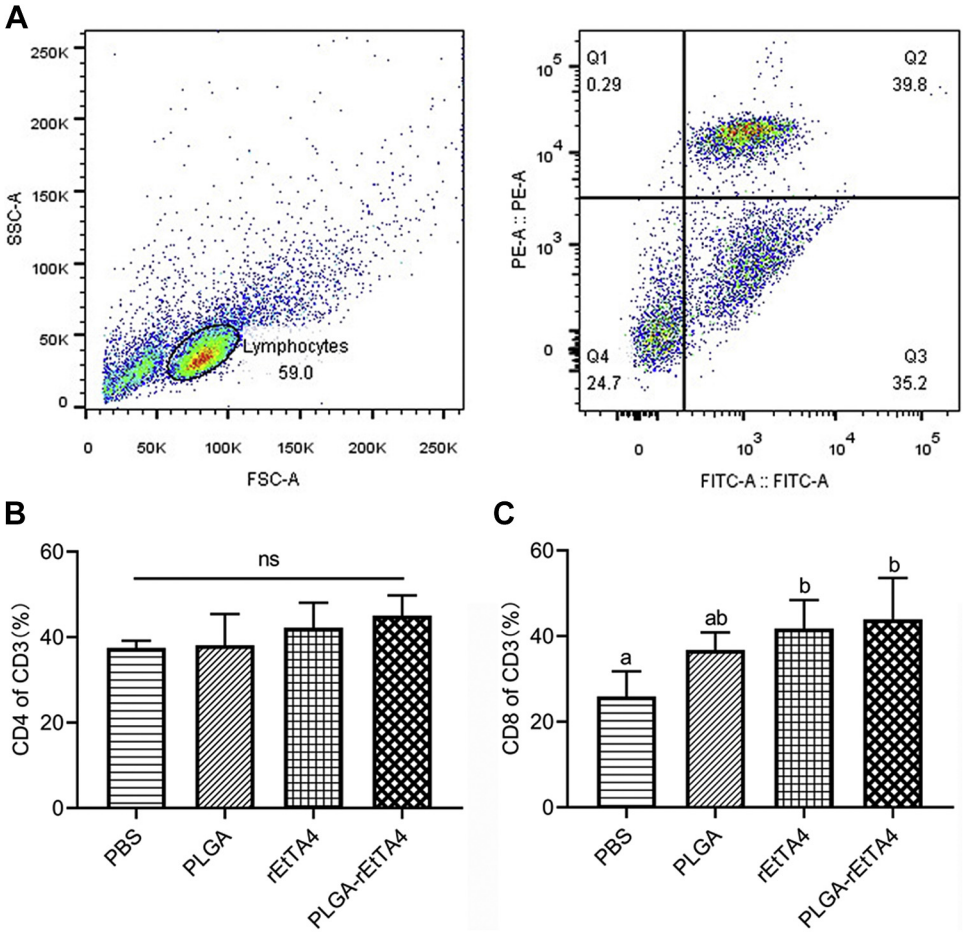

Figure 5. Flow cytometric analysis of spleen $CD4^+$ and $CD8^+$ T lymphocytes. Chickens were immunized intramuscularly with PBS, PLGA, rEtTA4, and PLGA-rEtTA4. Seven day after second immunization, the splenic lymphocytes that were isolated from 3 immunized chickens in each group were stained with mouse anti-chicken CD3-FITC and mouse anti-chicken CD8α-PE or mouse anti-chicken CD4-PE for 30 min. After washing twice, cells were used for flow cytometric analysis. (A) Gating strategy for the T cell subset; (B) $CD3^+$ $CD4^+$ T cells; (C) $CD3^+$ $CD8^+$ T cells. The significant difference between groups is shown with different letters ($P < 0.05$), and no significant difference ($P > 0.05$) between groups is shown with the same letter. Abbreviations: PLGA, Poly (D, L-lactide-co-glycolide); PLGA-rEtTA4, Poly (D, L-lactide-co-glycolide) loaded with rEtTA4; rEtTA4, recombinant TA4 protein of *Eimeria tenella*.

IL-17 is a proinflammatory cytokine produced by activated Th17 cells and plays a functional role in avian immunity as observed in COS-7 cells that transfected with chicken IL-17 cDNA has the ability to induce chicken embryonic fibroblasts to generate IL-6 (Lillehoj et al., 2004; Bischof et al., 2015). IL-6 has the ability to induce the final maturation of B cells into antibody-producing cells (Lillehoj et al., 2004). Moreover, the activity of chicken IL-6–like factor can be detected during the primary infection with *E. tenella*, suggesting IL-6 may play a role in the development of acquired immunity (Lynagh et al., 2000). Similarly, our data displayed significant higher level of IL-6 in rEtTA4 and PLGA encapsulated rEtTA4 nanoparticles immunization groups.

Cell-mediated immune response confers the protective immunity against coccidiosis (Subramanian et al., 2008). $CD8^+$ cells have the ability to simultaneously high levels of interferon gamma and IL-10 in response to parasite, and IL-17 is produced by a type of $CD4^+$ cell line (Zhang et al., 2016). In our research, high levels of interferon gamma and IL-10 were detected in the serum of PLGA-rEtTA4–inoculated group, and higher levels of IL-17 were detected both in the rEtTA4-immunized and PLGA-rEtTA4–immunized groups. In addition, significant differences were observed in the proportion of $CD8^+$ T lymphocytes. However, no significant differences were observed in the proportion of $CD4^+$ T lymphocytes compared with the controls, which need to be further researched.

Taken together, our study suggested that PLGA as a carrying agent promoted the immune response of recombinant TA4 subunit vaccine and provided partial protection to *E. tenella* infection via i.m. injection. However, different immune routes such as oral administration, i.m. injection, intraocular nasal vaccination, and subcutaneous injection may have different affects to the distribution of PLGA encapsulated rEtTA4 nanoparticles. Thus, the delivery routes need to be further studied for the application of PLGA encapsulated rEtTA4 nanoparticles.

CONCLUSION

Our data demonstrates that immunization with rEtTA4 subunit vaccine and PLGA-rEtTA4 nanoparticles can induce strong immune responses and result

in partial protection against *E. tenella* challenge in chickens. Therefore, both rEtTA4 subunit vaccine and PLGA-rEtTA4 nanoparticles are effective vaccine candidates against *E. tenella*. Our result demonstrated that PLGA nanoparticles encapsulation of recombinant rEtTA4 could provide a stronger immune response than rEtA4 alone.

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DISCLOSURES

The authors declare no conflicts of interest.

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