Co-administration of chicken IL-7 or NK-lysin peptide 2 enhances the efficacy of *Eimeria* elongation factor-1α vaccination against *Eimeria maxima* infection in broiler chickens

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ABSTRACT This study was conducted to develop a recombinant *Eimeria* elongation factor-1α (**EF-1α**)-vaccination strategy against *Eimeria maxima (**E. maxima**)* infection by co-administering with chicken IL-7 (**chIL-7**) or chicken NK-lysin peptide 2 (**cNK-2**) in commercial broiler chickens. Chickens were divided into the following 5 groups: control (**CON**, no *Eimeria* infection), nonimmunized control (**NC**, PBS plus Montanide ISA 78 VG), Vaccination 1 (**VAC1**, 100 μg of recombinant EF-1α plus Montanide ISA 78 VG), Vaccination 2 (**VAC2**, VAC1 plus 1 μg of chIL-7), and Vaccination 3 (**VAC3**, VAC2 plus 5 μg of cNK-2 peptide). The first immunization except the cNK-2 injection was performed intramuscularly on day 4, and the secondary immunization was given with the same concentration of components as the primary immunization 1 wk later. All chickens except the CON group were orally inoculated with freshly prepared *E. maxima* (1.0×10^4 oocysts per chicken) oocysts on Day 19. The results of the in vivo vaccination trial showed that chickens of all groups immunized with recombinant EF-1α antigen (VAC1, VAC2, and VAC3) showed higher serum antibody levels to EF-1α, and co-injection with chIL-7 further increased the serum IL-7 level in the VAC2 and VAC3 groups. Chickens in the VAC2 group showed significantly ($P <$ 0.01) higher body weight gains at 6 and 9 d post-*E. maxima* challenge infection (dpi) with reduced gut lesions in the jejunum at 6 dpi. The VAC3 group showed reduced fecal oocyst shedding compared to the nonimmunized and infected chickens (NC). At 4 dpi, *E. maxima* infection significantly ($P < 0.05$) up-regulated the expression levels of proinflammatory cytokines (IL-β and IL-17F) and type I cytokines (IFN-γ and IL-10) in the jejunum (NC), but the expression of these cytokines was significantly ($P < 0.05$) down-regulated in the VAC1, VAC2, and VAC3 groups. Furthermore, *E. maxima* challenge infection significantly ($P < 0.05$) down-regulated the expressions of jejunal tight junction (**TJ**) proteins (Jam2 and Occludin) at 4 dpi, but their expression was up-regulated in the VAC2 and VAC3 groups. Collectively, these results show the protective effects of the EF-1α recombinant vaccine, which can be further enhanced by co-injection with chIL-7 or cNK-2 peptide against *E. maxima* infection.

Key words: EF-1α vaccine, *Eimeria maxima*, cytokines, chicken NK-lysin, and IL-7

2022 Poultry Science 101:102013
https://doi.org/10.1016/j.psj.2022.102013

INTRODUCTION

Avian Coccidiosis, a ubiquitous intestinal disease caused by several distinct *Eimeria* species (**spp.**), is one of the costliest diseases in commercial poultry production worldwide (Kim and Lillehoj, 2019). Several distinct species of *Eimeria* infect and propagate within the mucosal epithelial layers in different parts of the gut (Williams, 2005), resulting in enormous economic losses due to intestinal damages including inflammation, bloody lesions, high morbidity, and mortality, and poor nutrition absorption (Williams, 1999; Chapman et al., 2010; Shivaramaiah et al., 2014). In addition, *E. maxima* has been shown to exacerbate the outcome of *Clostridium perfringens* infection to result in necrotic enteritis (Lillehoj et al., 2017; Lee et al., 2018a; Park et al., 2020). In commercial poultry production, coccidiosis is controlled by routine chemo-prevention, such as by ionophores (mostly in broiler chickens), or vaccination with live or attenuated *Eimeria* parasites. (Crouch et al., 2003; Mathis and Broussard, 2006; Chapman et al.,

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Received April 5, 2022.
Accepted June 13, 2022.

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2010). Although live and attenuated parasite vaccines and anticoccidia chemicals have long been used successfully in commercial poultry production, the emergence of drug resistance to anticoccidial drugs and genetic variants of coccidia strains poses a significant threat to chicken welfare and to the sustainability of sound poultry production systems worldwide (Williams, 2006; Lee et al., 2022). Accordingly, there is intensifying public/legislative pressure to reduce the use of antibiotics in poultry production and to find safe alternatives to reduce the economic losses due to coccidiosis (Peek and Landman, 2011; Karavolias et al., 2018; Wickramasuriya et al., 2022). In addition, existing live/attenuated vaccines are relatively expensive due to high production costs, and the process of formulating multiple *Eimeria* spp. limits their scalability (Soutter et al., 2020). Therefore, the development of a cost-effective anticoccidial vaccine is becoming more urgent than ever.

In the efforts to develop novel coccidiosis control measures, recombinant anticoccidial vaccines have been investigated, and many potential candidate *Eimeria* vaccine antigens have been identified (Lin et al., 2017; Tian et al., 2017; Zhao et al., 2020; Lee et al., 2022); in some cases, the combination of more than one immunodominant *Eimeria* antigen mixed in immunostimulatory adjuvants has elicited a substantial level of protective immunity (Lee et al., 2010; Jang et al., 2011). Furthermore, identification and vaccine trials of recombinant coccidial proteins that confer cross-protection against several species of *Eimeria* have also been reported (Lillehoj et al., 2015). For example, Lillehoj et al. (2005) showed that an immunodominant antigen of *Eimeria,* 3-1E (profilin), in combination with recombinant cytokine genes, induced significant cross-protection against different species of *Eimeria* following embryo immunization. In a subsequent in ovo vaccination study, recombinant profilin (3-1E) and *Clostridium perfringens* NetB protein induced significant protection against necrotic enteritis (**NE**) using a co-infection NE challenge model (Lillehoj et al., 2017). Zhao et al. (2020) showed a protective effect of the surface antigen of *E. tenella* (**EtSAG4**) against *E. tenella* challenge infection. Glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**), one of the cross-reactive immunogenic antigens of *Eimeria* shared among *E. tenella, E. acervulina*, and *E. maxima,* induced protective humoral and cellular immune responses against *E. tenella, E. acervulina*, and *E. maxima* (Tian et al. 2017). In a recent in vivo study (Lin et al., 2017), *E. coli*-expressed *E. tenella* elongation factor-1α (**ETEF-1α**) protein elicited protective immunity against *E. maxima* and *E. tenella* challenge infections following intramuscular injection, indicating the importance of cross-protective *Eimeria* antigens in inducing protection against multiple species of *Eimeria* parasites. While the use of cross-protective *Eimeria* antigens as recombinant vaccines to control field coccidiosis offers an advantage over the use of non−cross-protective antigens, there are no commercially available recombinant antigen vaccines currently due to their limited efficacies compared to ionophores or live/attenuated vaccines (Blake et al., 2017). Therefore, novel integrative approaches using recombinant proteins with various immunomodulatory strategies to boost host protective immunity, such as adjuvants, cytokines, antimicrobial peptides, and immune modulators, may improve vaccine efficacies. For these reasons, we conducted in vivo vaccination studies using a cross-protective antigen of *Eimeria* to investigate the adjuvant effects of chicken IL-7 and/or antimicrobial peptides together with a novel immune-enhancing adjuvant to improve the efficacy of EF-1α recombinant vaccine. The efficacies of these vaccines were evaluated using various host immunity and gut integrity biomarkers in commercial broiler chickens following *E. maxima* challenge infection.

MATERIALS AND METHODS

Construction of Recombinant EF-1α, chIL-7, and cNK-2 Peptide

Escherichia coli (BL21)-expressed recombinant *Eimeria tenella* Elongation Factor 1-α protein (EF-1α, GenBank Accession Number KX900609) (Lin et al., 2017) and Chinese hamster ovary (**CHO**)-cell-expressed recombinant chicken IL-7 protein (chIL-7, GenBank Accession number KU02410) were produced commercially (GenScript, Inc., Piscataway, NJ) (Kim et al., 2017; Panebra et al., 2021). The protein concentrations of recombinant EF-1α and chIL-7 were determined using a Bicinchoninic Acid (**BCA**) protein assay kit (Thermo-Scientific-Pierce, Waltham, MA), and their purity was assessed using 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (**SDS-PAGE**). cNK-2 peptide (RRQRSICKQLLKKLRQQLSDALQNNDD) corresponding to the structural domain of chicken NK-lysin with anti-*Eimeria* activity has been previously reported (Kim et al., 2017) and was synthesized by Peptide 2.0 Inc (Chantilly, VA). The cNK-2 peptide concentration was determined by UV spectrometry at a 210 nm wavelength.

Immunoblot Analysis

Recombinant EF-1α and chIL-7 were mixed with an equal volume of SDS-PAGE reduction buffer (0.125 M Tris HCl [pH 6.8], 4.0% sodium dodecyl sulfate [**SDS**], 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue), followed by denaturation at 95°C for 5 min. Both recombinant proteins (each, 3 μg/lane) were resolved with Precision Plus Protein Standards (Bio-Rad, Hercules, CA) on 15% SDS-PAGE, then transferred onto nitrocellulose membranes. The membranes were immediately blocked with 5% SuperBlock Blocking Buffer in PBS (Thermo Scientific, Waltham, MA) for 1 h at room temperature. Subsequently, each membrane was incubated with 1.0 μg of specific rabbit monoclonal antibody (**mAb**) against EF-1α and mouse mAb against chIL-7 overnight at 4°C, followed by washing 5 times with 1X PBS-T. The membranes were

incubated again with a secondary antibody, horseradish peroxidase (**HRP**)-conjugated goat anti-mouse IgG, in 1X PBS-T (1:10,000) for 1 h at room temperature. Each membrane was visualized via a ChemiDoc imaging system (Bio-Rad), using Clarity Western ECL Substrate (Bio-Rad) as a chromogenic substrate.

Il-7-Driven Chicken Thymocyte Proliferation Assay

The thymus was excised from a 3-wk-old healthy broiler chicken and gently pressed through a 100 μm strainer (Becton Dickinson, Franklin Lakes, NJ) with grass syringe plungers to make a single cell suspension. Cells were washed twice with Hanks' Balanced Salt Solution/2% inactivated chicken sera (Sigma-Aldrich, St. Louis, MO) and layered onto Histopaque-1077 (Sigma-Aldrich). Chicken thymocytes were harvested by centrifugation at 250 × g for 10 min and resuspended in RPMI 1640 medium (Gibco NY) containing 10% fetal bovine serum, 5% inactivated chicken sera, 1 mmol sodium pyruvate, and 4 mmol glutamine. Cell viability was determined by trypan blue exclusion. Chicken thymocytes (1×10^7/mL) were seeded in a 96-well plate with chIL-7 in a dose-dependent manner (from 5 ng/mL to 0.005 ng/mL) and kept in a 5 % CO_2 incubator at 41°C for 24 h. Phytohaemagglutinin (**PHA**, Sigma-Aldrich) was used as a positive control (1/10 dilution), and medium was used as a vehicle control. Afterward, the cell concentration was determined using a cell counting kit-8 (**CCK-8**; Dojindo, Japan), and color was measured at 450 nm. Two individual experiments were performed, and data were analyzed in triplicate.

In vitro Eimeria Sporozoite Killing Assay by cNK-2 Peptide

E. acervulina sporozoite killing assay was carried out in vitro as described previously (Kim et al., 2017; Wickramasuriya et al., 2021). Briefly, sporocysts from freshly sporulated *E. acervulina* oocysts were harvested by using a bead beater with 0.5 mm glass and washed twice with cold phosphate-buffered saline (**PBS**). Sporozoites were obtained using excystation solution (0.25% trypsin, 0.014M taurocholic acid) at 41°C for 4 h, then purified using a 10 μm cell strainer (Pluriselect, Germany). One hundred microliters of *E. acervulina* sporozoites (1×10^7/mL) were resuspended in RPMI 1640 medium (Gibco, NY) and incubated with 100 μg/mL of cNK-2 peptide in a 96-well plate for 4 h at 41°C. Medium was used as a vehicle control. After incubation, each sporozoite suspension was stained (v/v) with fluorescence viability dye (AO/PI staining solution, Nexcelom Bioscience LLC, Lawrence, MA), and the viability was determined using a cell counter (Cellometer X2, Nexcelom Bioscience). Each sample was analyzed in triplicate.

Chicken Husbandry and Sample Collection

All procedures were approved by the Beltsville Area Institutional Animal Care and Use Committee (Animal Protocol No. 20-015). One-day-old male broiler chickens (150, 30/group) were purchased from the hatchery (Longnecker Hatchery, Elizabethtown, PA) and housed in Petersime brooder units with feed and water ad libitum. Each group was divided into 6 cages (6 cages per group). Figure 1 depicts the schematic outline of the chicken experimental design used for this study. Individual body weight was recorded at 0 (before *Eimeria* infection), 6, 9, and 12 d post-*Eimeria* infection. The feces for oocyst counting were collected from 6 to 9 dpi and the individual oocyst output was assessed as described (Park et al., 2020). Briefly, feces collected from individual cages were ground and homogenized with 3 L of water. Two subsamples were taken in 50 mL tubes, diluted, and the number of oocysts was counted microscopically using a McMaster chamber. The total number of oocysts were calculated using the following formula: Total oocysts/bird = [oocyst count × dilution factor × (fecal sample volume/counting chamber volume)]/number of birds per cage. For gut lesion scoring, 2 equal 10 cm sections of jejunum (6 chickens per group) were collected at 6 dpi, and the lesions were evaluated on a scale from 0 (none) to 4 (high) by 4 well-trained, independent observers, as described previously (Lee et al., 2018b).

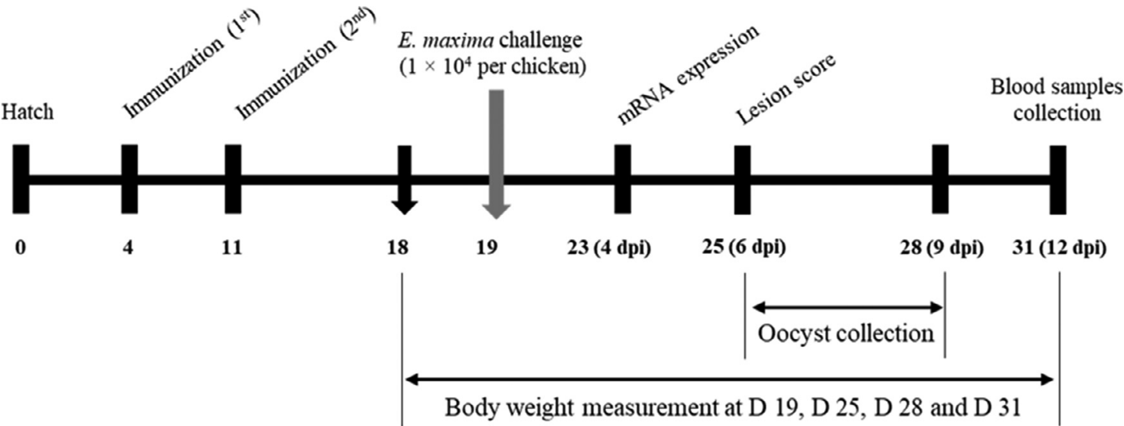

Figure 1. Schematic outline of the experimental design.

Immunization

All immunizations were performed intramuscularly (both sides of the thighs) with Montanide ISA 78 VG adjuvant (Seppic Inc., NJ) in a 70/30 ratio, as recommended by the adjuvant manufacturer. Montanide ISA 78 VG is a mineral oil-based adjuvant which is used for the formulation of Water-in-Oil (W/O) emulsions and contains an immunostimulant. Chickens were administered in the following five groups on Day 4: control (CON, without *Eimeria* infection), nonimmunized control (NC, PBS plus ISA 78 VG), Vaccination 1 (VAC1, 100 μg of recombinant EF-1α plus ISA 78 VG), Vaccination 2 (VAC2, VAC1 component plus 1 μg of recombinant chIL-7), and Vaccination 3 (VAC3, VAC2 component plus 5 μg of cNK-2 peptide) (Table 1). The secondary immunization was administrated with the same concentration of components as the primary immunization 1 wk later. At 7 d postsecondary immunization, all chickens (except CON) were orally infected with 1.0×10^4 sporulated *E. maxima* oocysts.

Anti-EF-1α Serum Antibody Assay

The serum IgG antibody level against EF-1α was measured by indirect ELISA on Day 20 postsecondary immunization, as described previously (Lee et al., 2013b). Briefly, 100 μL of recombinant EF-1α (5 μg/mL) was coated on a high-binding 96-well microtiter plate (Corning, MA) overnight at 4°C. After washing, the plate was blocked with 1% BSA/PBS for 1 h at room temperature. The same volume (100 μL) of each chicken serum (12 chickens per group) diluted 1:10 in 0.1% BSA/PBS was applied on a plate and incubated for 2 h at room temperature on the plate shaker. After washing, the plates were incubated with the same volume (100 μL) of HRP-conjugated rabbit anti-chicken IgG secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:10,000 in PBS/0.1% BSA on a plate shaker at room temperature for 1 h. All processes for plate washing, development, stop reaction, and OD were performed via the same method as for sandwich ELISA (Materials and Methods, Section 2.7).

Serum IL-7 Level by Sandwich ELISA Assay

Blood samples (12 chickens per group) were collected on Day 20 postsecondary immunization, and the IL-7 concentration in serum was measured via an in vitro assay as described previously (Panebra et al., 2021). Briefly, 100 μL of chIL-7-purified mAb (5 μg/mL) was coated on a high-binding 96-well microtiter plate (Corning, MA) overnight at 4°C. After washing, the plate was blocked with 1% BSA/PBS for 1 h. The same volume of each serum diluted 1:5 in 0.1% BSA/PBS was applied on a plate, followed by 2 h of incubation at 37°C on the plate shaker. After washing, the plates were incubated with the same volume of 0.1 mL biotin-labeled detecting mAb (1 μg/mL in 0.1% BSA/PBS) at 37°C for 1 h. The plate was washed again, then incubated with 0.1 mL avidin horseradish peroxidase in PBS/0.1% BSA (Sigma-Aldrich, St. Louis, MO) (1:5,000) on a plate shaker at 37°C for 1 h. The plate was developed with the same volume of TMB solution (Sigma-Aldrich), and the reaction was stopped by the addition of 50 μL of 2 N H_2SO_4 (Sigma-Aldrich). The optical density (**OD**) was determined using an ELx-800 microplate reader (Biotek, Winooski, VT) at 450 nm, and all washing steps were carried out six times with PBS/T. Each sample was analyzed in triplicate.

RNA Isolation and Quantitative RT-PCR

A segment of jejunum tissues (6 chickens per group) was taken on 4 dpi and washed with ice-cold Hank's balanced salt solution (Sigma) to remove the gut contents. The jejunum samples were opened longitudinally, the mucosa layer was scraped away using a surgical scalpel and immediately placed in RNAlater (Invitrogen, Camarillo, CA). For the total RNA extraction, collected mucosa samples were washed with PBS to remove RNAlater and approximately 5 mg tissue sample was homogenized in 1 mL of TRIzol (Invitrogen, Carlsbad, CA) using a homogenizer (TissueRuptor; Qiagen), followed by DNase digestion as described previously (Park et al., 2020). The concentration and purity were assessed using a NanoDrop spectrophotometer (NanoDrop One; Thermo Scientific) at 260/280 nm. cDNA synthesis was carried out with a 1 μg aliquot of total RNA in a 20 μL reaction volume using a QuantiTect Reverse Transcription Kit (Qiagen) as recommended by the manufacturer. cDNA was diluted 1:10 in Rnase-free water (Invitrogen), followed by quantitative RT-PCR analysis with 5 μL of cDNA using SYBR Green qPCR Master Mix (PowerTrack, Applied Biosystems, Vilnius, Lithuania) in triplicate via Applied Biosystems QuantStudio 3 Real-Time PCR Systems (Life Technologies, Carlsbad, CA). cDNA was analyzed under the following PCR conditions: denaturation at 95°C for 10 min, followed by amplification at 58°C for 1 min for 40 cycles. The endogenous control gene (*β-actin*) was used as the reference gene for gene

Table 1. Treatment and group information.

Treatment (Abbreviation)	Description	EF-1α	chIL-7	cNK2	*E. maxima*
CON	–	–	–	–	–
NC	PBS/ISA 78 VG	–	–	–	1×10^4
VAC1	EF-1α/ISA 78 VG	100 μg	–	–	1×10^4
VAC2	EF-1α + IL-7/ISA 78 VG	100 μg	1 μg	–	1×10^4
VAC3	EF-1α + IL-7/ISA 78 VG	100 μg	1 μg	5 μg	1×10^4

Table 2. Oligonucleotide primer sequences used for qRT-PCR.

Type	Target gene	Primer sequence (5′-3′)	PCR product size (Kb)
Reference	β-actin	F-CACAGATCATGTTTGAGACCTT R-CATCACAATACCAGTGGTACG	111
Proinflammatory	IL-1β	F-TGGGCATCAAGGGCTACA R-TCGGGTTGGTTGGTGATG	244
	IL-17F	F-TGAAGACTGCCTGAACCA R-AGAGACCGATTCCTGATGT	117
Th1	IFN-γ	F-AGCTGACGGTGGACCTATTATT R-GGCTTTGCGCTGGATTC	259
	IL-10	F-CGGGAGCTGAGGGTGAA R-GTGAAGAAGCGGTGACAGC	272
TJ proteins	Occludin	F-GAGCCCAGACTACCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68
	JAM2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTCGCTTCA	59
Mucin	MUC2	F-GCCTGCCCAGGAAATCAAG R-CGACAAGTTTGCTGGCACAT	59

expression. The data obtained from six biological replicates were used to evaluate the relative gene expression compared to non-immunized samples by the $2^{-\Delta\Delta Ct}$ method (Schmittgen et al., 2000). All oligonucleotide primer sequences used in this experiment are presented in Table 2.

Statistical Analysis

All data were subjected to one-way analysis of variance (**ANOVA**) using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL) for Windows. The individual chicken was considered the experimental unit for statistical analysis. The mean ± S.D. or mean ± SEM values were compared using Tukey's test, and differences were considered statistically significant when P values were < 0.05.

RESULTS

Western Blot Analysis of EF-1α and chIL-7

As shown in Figure 2, approximately 70.0 kDa of recombinant protein, corresponding to the expected molecular weight (49.1 kDa EF-1α protein, 12.9 kDa thioredoxin protein, 0.9 kDa 6x His-tag, and 4.8 kDa S-tag), was identified. About 19 kDa of recombinant chIL-7 also showed the expected molecular weight (18.4 kDa chIL-7 protein and 0.6 kDa His-tag). The Western blot analysis showed that both recombinant proteins (EF-1α

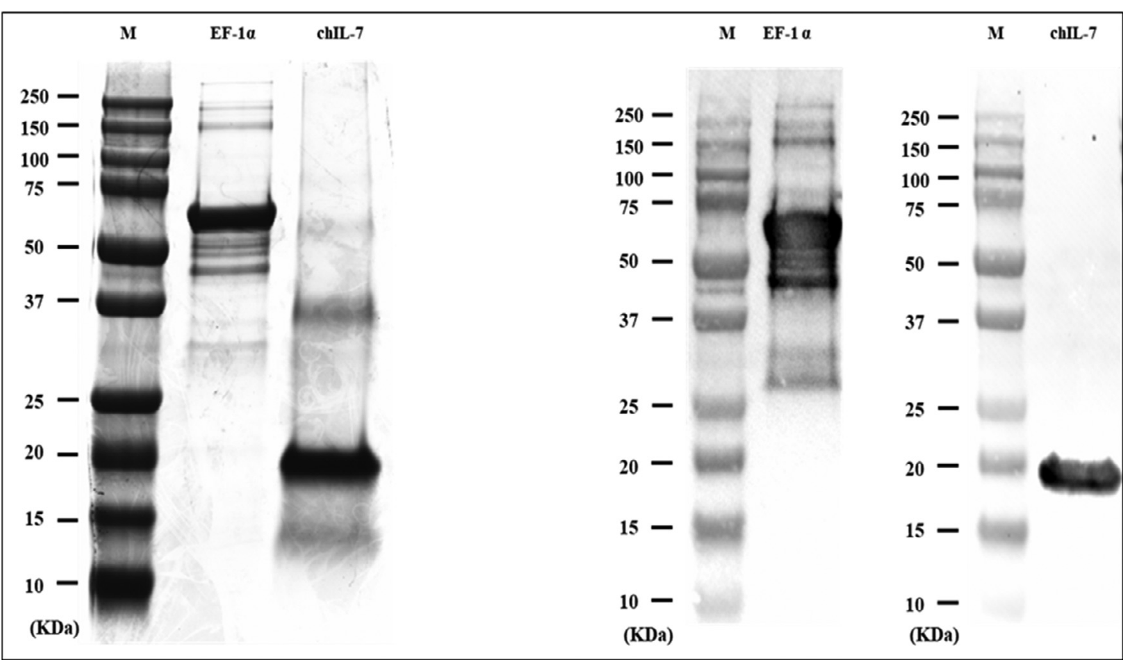

Figure 2. Recombinant EF-1α expressed in *E.coli* and eukaryotic expression of recombinant chIL-7. (A) SDS-PAGE of recombinant EF-1α and chIL-7 respectively. Lane M, standard protein molecular weight marker. (B) Western blot analysis of recombinant EF-1α and chIL-7 proteins. Lane M, standard protein molecular weight marker.

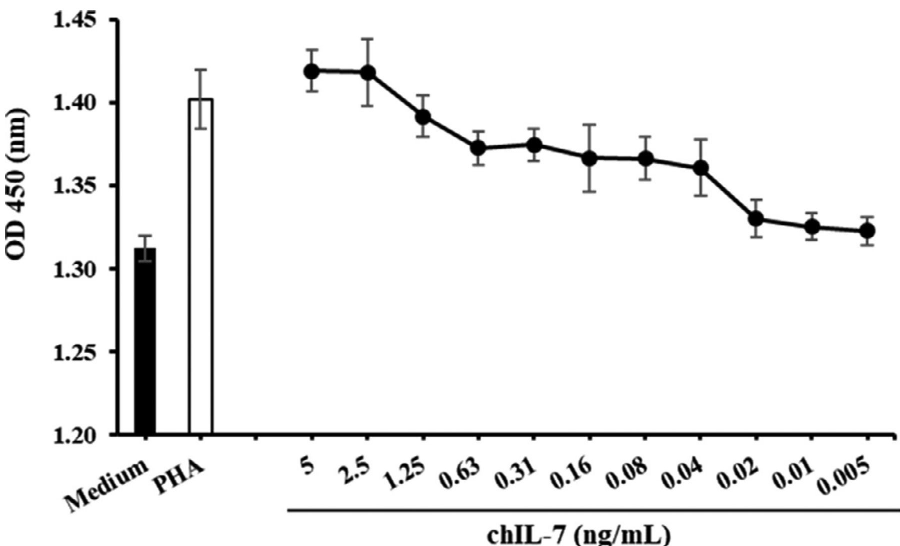

Figure 3. The proliferation of chicken thymocytes by a recombinant chIL-7. The thymocytes were isolated from 3-wks-old chickens by Percoll density gradient centrifugation. The chicken thymocytes (1 × 108/mL) were seeded in a flat bottomed-microplate and stimulated with chIL-7 in a dose-dependent manner (from 5 ng/mL−0.005 ng/mL) for 24 h. Each sample was measured in three replicates and the cells proliferation was determined using a CCK-8 kit. Medium only was used as a negative control and 1/10 dilution of PHA was used as a positive control. Error bars indicate Mean ± SD.

and chIL-7) were specifically recognized by their corresponding mAbs.

Effect of Recombinant chIL-7 on Chicken Thymocyte Proliferation

The proliferation of chicken thymocytes was dose-dependently increased by recombinant chIL-7 stimulation compared to the medium control after 24 h incubation (Figure 3). The chicken thymocyte proliferation showed the highest level when stimulated with 5 ng/mL of recombinant chIL-7, then it slowly decreased in a dose-dependent manner. The stimulation of recombinant chIL-7 at 5 and 2.5 ng/mL induced higher thymocyte proliferation than did PHA, which was used as a positive control.

Anticoccidial Activity of cNK-2 Peptide Against E. acervulina sporozoites

The antimicrobial effect of a cNK-2 peptide against *E. acervulina* sporozoites is presented in Figure 4. Consistent with those in a previous report (Kim et al., 2017), the results show that cNK-2 peptide effectively reduced the viability of freshly prepared *E. acervulina* sporozoites (to the level of about 20%) after 4 h incubation when compared with the medium control. The unbroken sporulated oocysts or sporocysts were not stained by fluorescence viability dye, and only surviving sporozoites were recognized by the cell counter.

Growth Performance of Chickens

Body weight changes according to immunization and *E. maxima* challenge are presented in Table 3. There was no significant change in body weight due to immunization. *E. maxima* challenge significantly ($P < 0.01$) reduced body weights at 6, 9, and 12 dpi. Among the *E. maxima*-challenged group, chickens immunized with recombinant EF-1α and chIL-7 (VAC2) showed significantly improved body weights at 6, 9, and 12 dpi ($P < 0.01$) compared to nonimmunized chickens (NC). Although there was no statistical difference, chickens immunized with VAC1 and VAC3 showed increased weight gains compare to nonimmunized chickens (NC). Chickens immunized with recombinant EF-1α with cNK-2 (VAC3) did not show any enhanced body weight gains compared to those chickens immunized with recombinant EF-1α alone (VAC1).

Level of EF-1α Antibody in Serum of Immunized Chickens

Chickens in the *E. maxima*-challenged group showed increased serum antibody levels against EF-1α compared to nonchallenged chickens (CON) (Figure 5A). Chickens in groups immunized with recombinant EF-1α (VAC1, VAC2, and VAC3) showed enhanced levels of serum EF-1α antibody compared to non-immunized chickens (NC). Co-injection with chIL-7 (VAC2) or cNK-2 (VAC3) did not enhance serum EF-1α antibody production.

Level of chIL-7 in Serum of Immunized Chickens

As shown in Figure 5B, *E. maxima* infection increased overall levels of serum IL-7 compared to those in nonimmunized chickens (CON). Among the chickens in the *E. maxima*-challenged groups, chickens immunized with VAC2 showed a significantly ($P < 0.05$) higher level of

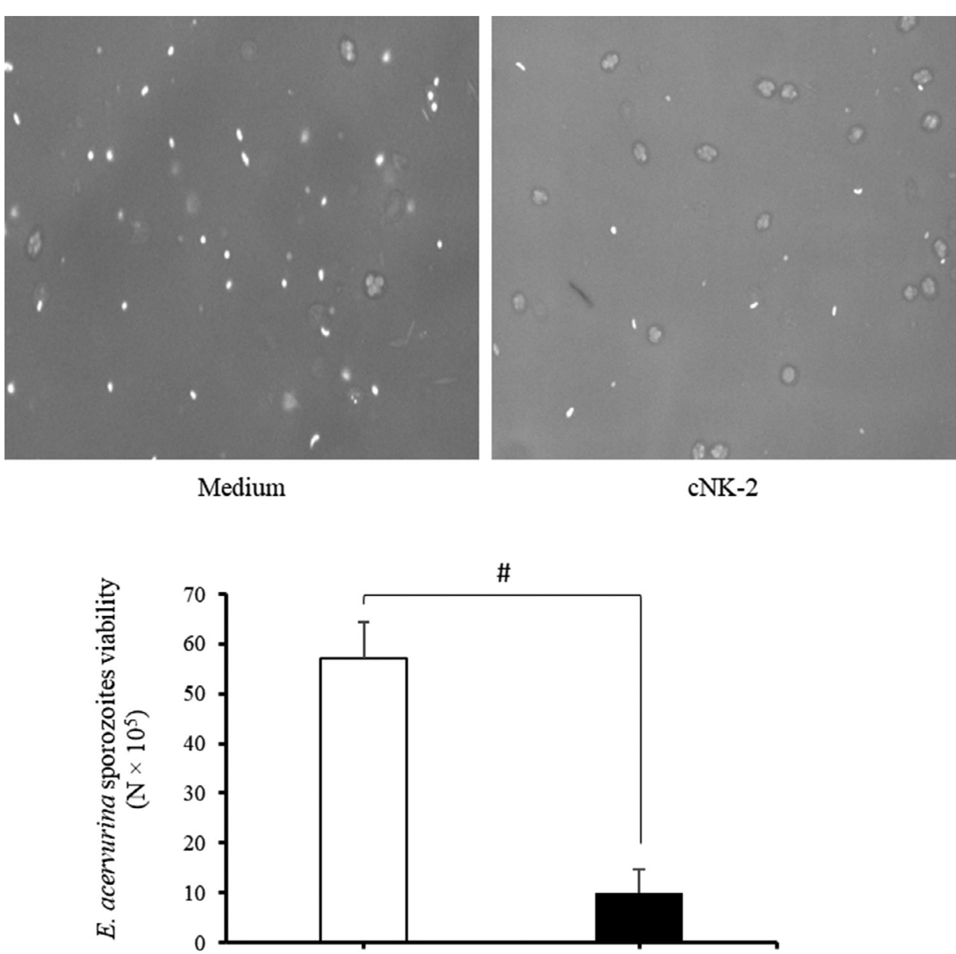

Figure 4. In vitro effect of cNk-2 peptide on *E. acervurina* sporozoites viability. *E. acervulina* sporozoites (5×10^7/mL) were incubated with 100 μg/mL of cNK2 peptide for 4 h at 41°C. Medium only was used as a positive control. The sporozoites were stained with fluorescence viability dye (AO/PI staining solution, Nexcelom Bioscience LLC, Lawrence, MA) and the viability was determined by the cell counter (Cellometer X2, Nexcelom Bioscience, USA). Error bars indicate Mean ± SD values. # $P < 0.01$ when compared with medium control according to Student *t*-test.

serum IL-7 compared to nonimmunized chickens (NC). The serum IL-7 levels of chickens immunized with VAC3 were numerically higher, but there was no statistical difference. Chickens that were immunized with recombinant chIL-7 (VAC2 and VAC3) showed a slightly higher average production of IL-7 compared to those in nonimmunized groups (NC and VAC1). However, chickens co-immunized with cNK-2 peptide (VAC3) showed decreased serum IL-7 levels when compared with VAC2.

Jejunal Lesion Score and Fecal Oocyst Shedding

As shown in Figure 6A, all chickens in the *E. maxima*-challenged groups showed higher gut lesion scores in the jejunum than did chickens in the unchallenged group (CON). Among the chickens in the *E. maxima*-challenged groups, chickens immunized with VAC2 and VAC3 showed less gut lesion formation compared with nonimmunized chickens (NC). Chickens immunized

Table 3. Effects of recombinant EF-1α antigen and in combination with chIL-7 or cNK2 on average body weight (mean ± SD) in *E. maxima*-challenged chickens.

Treatment	CON	NC	VAC1	VAC2	VAC3	SD (±)	*P-value*
BW (g)							
Initial BW	77.2	77.5	77.1	77.3	76.6	0.3	0.413
Before infection	705.7	692.3	680.4	712.9	687.8	13.3	0.319
6 DPI	954.6[a]	803.7[c]	854.5[bc]	891.1[b]	846.3[bc]	56.6	*<0.01*
9 DPI	1,374.9[a]	1,140.5[c]	1,208.3[bc]	1,273.9[b]	1,212.2[bc]	88.0	*<0.01*
12 DPI	1,772.6[a]	1,537.5[c]	1,597.4[bc]	1,667.9[ab]	1,602.6[bc]	89.4	*<0.01*

The results were estimated using one-way ANOVA followed by Tukey's test.
[a-d]means in the same row with different superscripts significantly differ ($P < 0.05$).

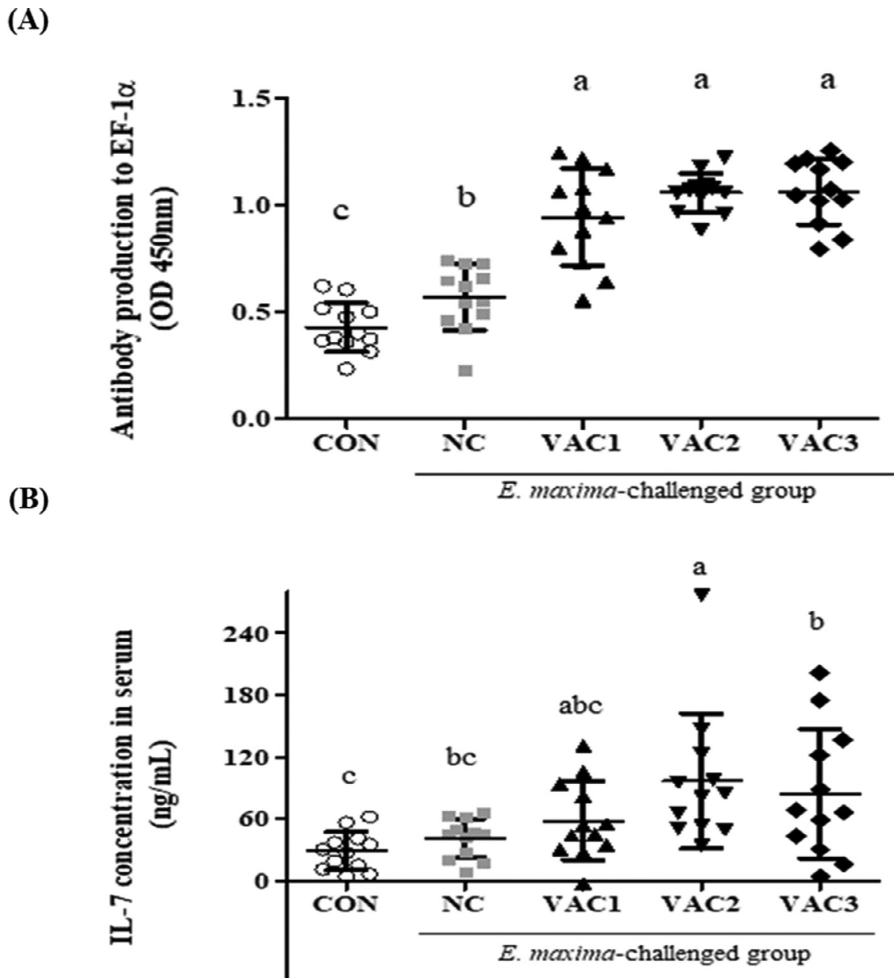

Figure 5. Effect of recombinant EF-1α co-administrated with recombinant chIL-7 or cNK2 on the serum EF-1α antibody and IL-7 production. Each group of chickens was immunized with 100 μg of recombinant EF-1α, 1 μg of recombinant chIL-7, 5 μg of cNK2 peptide or sterile PBS solution, respectively. One week after, the boosting immunization was administrated with the same concentration of components as the primary immunization. Blood samples from each group of birds (n = 12) were collected for determination of antibody to EF-1α (A) and IL-7 (B) levels using indirect or sandwich ELISA methods at 20 d post the secondary immunization. The concentration of the EF-1α (A) and IL-7 (B) were expressed as mean ± S.D. values and different letter between group indicates significant difference according to the Tukey's multiple range test ($P < 0.05$).

with recombinant EF-1α alone (VAC1) also showed slightly reduced gut lesion scores, but there was no significant difference. Fecal oocyst shedding was not observed in the unchallenged group (CON), and only chickens immunized with VAC3 showed significantly ($P < 0.05$) reduced fecal oocyst output when compared with nonimmunized chickens (NC) among the *E. maxima*-challenged groups (Figure 6B).

Expression of Proinflammatory Cytokines

E. maxima challenge significantly ($P < 0.05$) up-regulated the expression levels of proinflammatory cytokines such as IL-1β and IL-17F in the jejunum at 4 dpi, compared to those in unchallenged chickens (CON) (Figure 7). Among the chickens challenged by *E. maxima*, chickens in the recombinant-antigen-immunized groups (VAC1, VAC2, and VAC3) showed a slightly reduced expression level of IL-1β, but it was not statistically significant. The expression of IL-17F showed a similar pattern to that of IL-1β. The overall expression of IL-17F in the jejunum was down-regulated following recombinant EF-1α immunization, and the only group that received recombinant EF-1α vaccine with chIL-7 (VAC2) showed a significant difference ($P < 0.05$) in terms of IL-17F level.

Expression of Th1 Cytokines

Following *E. maxima* challenge infection, all chickens showed enhanced jejunal Th1 cytokines (IFN-γ and IL-10) (Figure 8). However, chickens immunized with VAC2 and VAC3 showed significantly ($P < 0.05$) reduced levels of IFN-γ expression. The expression level of IL-10 was significantly ($P < 0.05$) reduced in a group of chickens immunized with VAC1, VAC2, and VAC3 compared with in nonimmunized chickens (NC).

Expression of TJ and Mucin Genes

E. maxima challenge infection significantly ($P < 0.05$) down-regulated the expression levels of TJ proteins

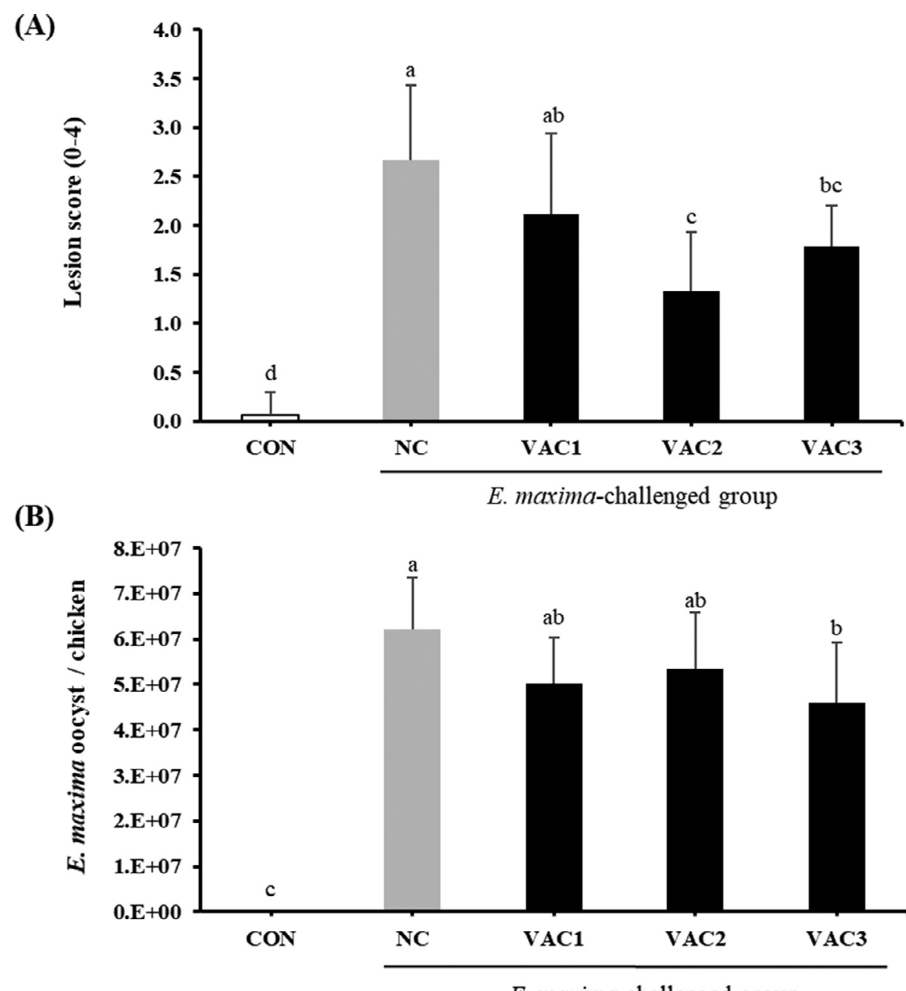

Figure 6. Effect of recombinant EF-1α co-administrated with recombinant chIL-7 or cNK2 on the lesion score and oocyst shedding in *E. maxima*-challenged chickens. Each group of chickens was immunized with 100 μg of recombinant EF-1α, 1 μg of recombinant chIL-7, 5 μg of cNK2 peptide, or sterile PBS solution, respectively. One week after, the boosting immunization was administrated with the same concentration of components as the primary immunization. All chickens except CON were inoculated by oral gavage at day 19 with 1.0×10^4 oocysts/bird of *E. maxima.* (A) lesion score was measured from jejunum at day 25 (n = 6/group), and (B) fecal oocysts numbers were determined between days 25 and 28. Each bar represents the mean ± S.D. values and different letters between group indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

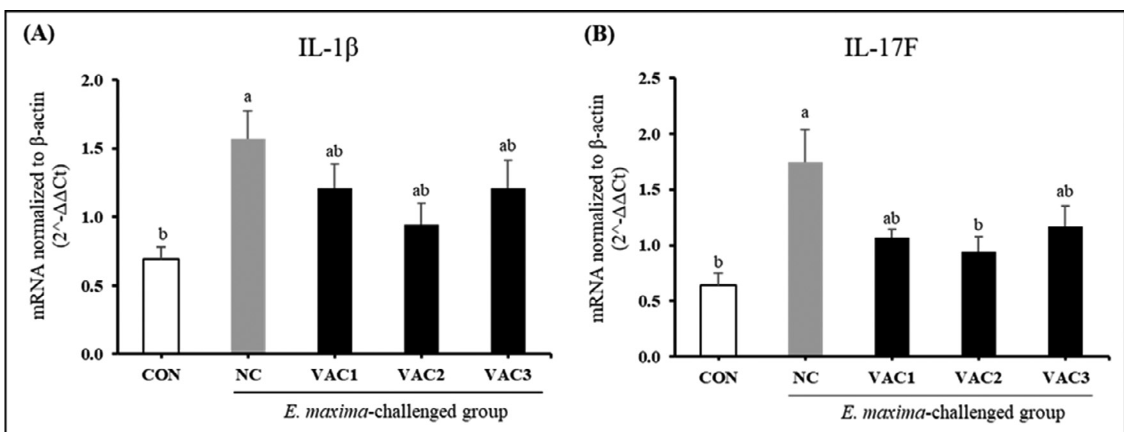

Figure 7. Effect of recombinant EF-1α co-administrated with recombinant chIL-7 or cNK2 on transcripts of proinflammatory cytokines in the jejunum. Each group of chickens was immunized with 100 μg of recombinant EF-1α, 1 μg of recombinant chIL-7, 5 μg of cNK2 peptide, or sterile PBS solution, respectively. One week after, the boosting immunization was administrated with the same concentration of components as the primary immunization. All chickens except CON were inoculated by oral gavage at day 19 with 1.0×10^4 oocysts/bird of *E. maxima.* The data were collected at day 23 (4 d post infection). The level of transcripts for IL-1β and IL-17F were qualified by qRT-PCR and normalized to β-actin transcript levels. Each bar represents the mean ± SEM values and different letters between group indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

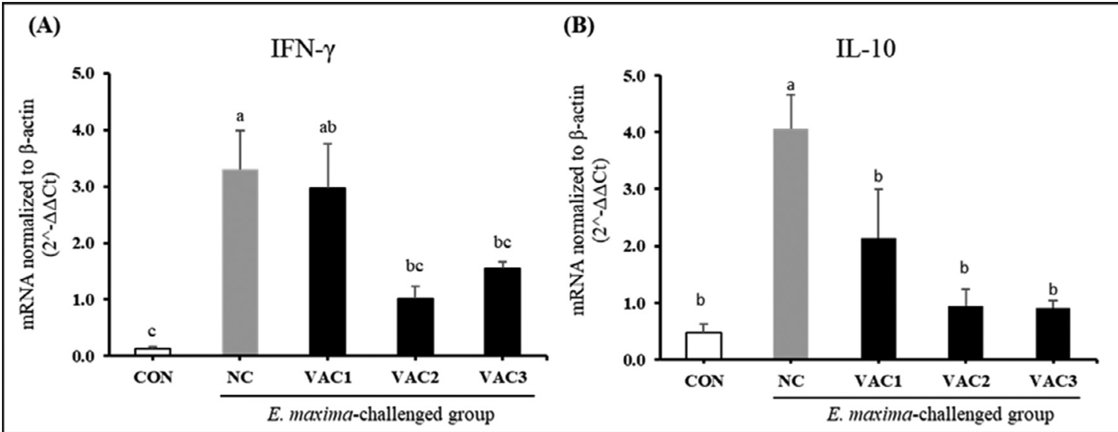

Figure 8. Effect of recombinant EF-1α co-administrated with recombinant chIL-7 or cNK2 on transcripts of Th1 cytokines in the jejunum. Each group of chickens was immunized with 100 μg of recombinant EF-1α, 1 μg of recombinant chIL-7, 5 μg of cNK2 peptide, or sterile PBS solution, respectively. One week after, the boosting immunization was administrated with the same concentration of components as the primary immunization. All chickens except CON were inoculated by oral gavage at day 19 with 1.0 × 10^4 oocysts/bird of *E. maxima*. The data were collected at day 23 (4 d post infection). The level of transcripts for IFN-γ and IL-10 were qualified by qRT-PCR and normalized to β-actin transcript levels. Each bar represents the mean ± SEM values and different letters between group indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

(Jam2 and Occludin) in the jejunum (Figure 9). The expression level of Jam2 was significantly ($P < 0.05$) up-regulated in chickens immunized with VAC2 compared with in nonimmunized chickens (NC). Chickens immunized with VAC3 showed significantly up-regulated levels of Occludin compared to nonimmunized chickens (NC). There were no significant changes in mucin gene (Muc2) expression after *E. maxima* challenge infection.

DISCUSSION

In the present study, we investigated the vaccine efficacy of recombinant EF-1α protein with chIL-7 or cNK-2 peptide against experimental *E. maxima* challenge infection in commercial broiler chickens. In the previous study, Lin et al. (2017) demonstrated that recombinant EF-1α protein can elicit cross-protective immunity against coccidiosis challenge infection with 2 different *Eimeria* spp. as measured by body weight gain, improved antibody production, and reduced fecal oocyst shedding. In this study, we showed that recombinant EF-1α vaccination of young broiler chickens induced a significant level of protective immunity against *E. maxima* challenge, with rapid bodyweight recovery, reduced jejunal lesion formation, and decreased fecal oocyst output. Furthermore, EF-1α-vaccinated chickens showed mitigated proinflammatory cytokine profiles in the gut, where parasites underwent intracellular development following *E. maxima* challenge infection, compared to nonimmunized chickens. More importantly, co-administration of EF-1α vaccine with chIL-7 enhanced protection against coccidiosis challenge and provided beneficial effects in the host protective immune response when compared to the chickens that were immunized with recombinant EF-1α alone.

Interleukin-7 (IL-7) is a cytokine with a central role in the adaptive immune system; it promotes lymphocyte development in the thymus and is involved in the survival of naive and memory T cell homeostasis in the periphery (Gao et al., 2015). These biological functions of IL-7 imply that it can be used as an adjuvant to

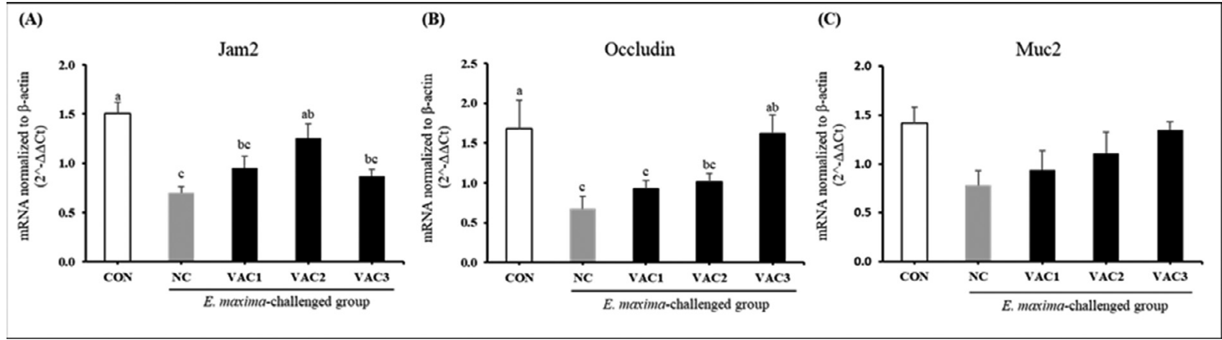

Figure 9. Effect of recombinant EF-1α co-administrated with recombinant chIL-7 or cNK2 on transcripts of TJ proteins (Jam2 and Occludin) and mucin gene (Muc2) in the jejunum. Each group of chickens was immunized with 100 μg of recombinant EF-1α, 1 μg of recombinant chIL-7, 5 μg of cNK2 peptide or sterile PBS solution, respectively. One week after, the boosting immunization was administrated with the same concentration of components as the primary immunization. All chickens except CON were inoculated by oral gavage at day 19 with 1.0 × 10^4 oocysts/bird of *E. maxima*. The data were collected at day 23 (4 d post infection). The level of transcripts for Jam2, Occludin, and Muc2 was qualified by qRT-PCR and normalized to β-actin transcript levels. Each bar represents the mean ± SEM values and different letters between group indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

improve the immune response for various vaccines (Cui et al., 2018; Huo et al., 2019; Logerot et al., 2021). As shown in Figure 3, recombinant chIL-7 effectively enhanced the proliferation of chicken thymocytes compared to the medium control in vitro, indicating its potential adjuvant activity; therefore, we investigated the vaccine efficacy of recombinant EF-1α on coccidiosis when co-injected with chIL-7. As shown in Figure 6A and B, co-administration of recombinant EF-1α and chIL-7 improved the vaccine efficacy of the EF-1α vaccine compared with EF-1α alone. Interestingly, although there was no statistical significance, the co-administration of recombinant EF-1α with chIL-7 (VAC2 and VAC3) enhanced the serum anti-EF-1α antibody levels and increased serum IL-7 production compared with recombinant EF-1α alone (VAC1), as shown in Figure 5. The increase in the serum IL-7 level after recombinant chIL-7 administration (VAC 2 and VAC 3) is expected to be due to the autocrine function of IL-7 (Cattaruzza et al., 2009), but further studies are required to understand its exact mechanism on IL-7 enhancement of host immunity in chickens. Since the main function of IL-7 is to play a pivotal role in the development of B and T cells (Gao et al., 2015; Corfe and Paige, 2012), the increased IL-7 level in serum is expected to improve the protective host vaccinal immune response. cNK-2 is a cationic amphiphilic antimicrobial peptide produced by cytotoxic T cells and natural killer cells (Kim et al., 2017). Previous studies demonstrated that cNK-2 has strong anticoccidial activity against *Eimeria* spp. through its membrane disruptive property, and it exerts an immunomodulatory effect mediated by interaction with host cells (Kim et al., 2017; Lee et al., 2013a; Wickramasuriya et al., 2021). As shown in Figure 4, a strong anti-parasitic effect of NK-2 was confirmed by the direct killing of sporozoites of *E. acervulina*. Firstly, the sporozoites of *E. maxima* were planned to be used for the killing assay as used in animal trial, but we could not secure an enough concentration of *E. maxima*, killing assay was carried out with *E. acervulina* sporozoites instead. Nevertheless, since all *Eimeria* spp. including *E. maxima* and *E. acervulina* are expected to be killed by cNK-2 (Kim et al., 2017), killing effects by NK-2 should be similar with *E. acervulina* sporozoites. Interestingly, co-administration of cNK-2 (VAC3) did not significantly contribute to the efficacy of recombinant EF-1α when compared with the overall results of VAC2. However, administration of cNK-2 (VAC3) significantly reduced fecal oocyst production when compared to other *Eimeria*-infected groups. Consistent with our finding, Lee et al. (2013a) and Wickramasuriya et al. (2021) reported reduced fecal oocyst output in *E. acervulina*-infected broiler chickens that were treated with cNK-2. The findings from the present study showing reduced fecal oocyst shedding provide a strong indication that cNK-2 is a potential anti-infective peptide that can be used for protection against avian coccidiosis.

In this study, the vaccine mixtures (recombinant EF-1α, IL-7, and cNK-2 peptide) were administrated in a mineral-oil-based adjuvant, Montanide ISA 78 VG, in a 70/30 ratio to elicit a long-term immune response and for the visualization of antigen injection. Jang et al. (2011) reported previously that the efficacy of a recombinant profilin subunit vaccine was enhanced when co-administered with mineral-oil-based Montanide ISA 71 VG adjuvant (Seppic Inc., NJ), as measured by increased antibody and improved cell-mediated response in *Eimeria*-challenged chickens. Montanide ISA 78 VG is a new generation water in oil adjuvant dedicated to avian species, based on mineral oil and containing an immunostimulant. Indeed, the Montanide ISA 78 VG adjuvant allowed us to observe that the vaccine mixtures were injected and maintained intramuscularly in the chickens. In addition, it was observed that the residue of first administrated vaccine mixtures remained subcutaneously at the time of second antigen administration (after 7 d). This confirmed that the adjuvant worked well in the current study.

From the results presented in Figure 8, *E. maxima* challenge infection significantly down-regulated the expression levels of TJ proteins (Jam2 and Occludin) in the jejunum when compared to those in uninfected chickens. However, the expression levels of Jam2 and Occludin in the VAC2 and VAC3 groups were up-regulated compared to those in unimmunized chickens (NC). Previous studies showed that the upregulation of these genes correlates with improved gut barrier function in chickens infected with *Eimeria* spp. (Lee et al., 2018a; Chaudhari et al., 2020; Park et al., 2020). Although no significant difference was found, the expression level of Muc2 showed a similar pattern to that of Occludin, which is consistent with the result of Forder et al. (2012).

So far, various recombinant vaccines have been developed as preventive measures against coccidiosis in chickens, with various resulting efficacies (Ding et al., 2005; Mohana Subramanian et al., 2008; Rafiqi et al., 2018), but none has been commercialized (Blake et al., 2017). In the present study, the beneficial effect of immunization of recombinant EF-1α (VAC1) was verified after *E. maxima* challenge infection in commercial broiler chickens, as were, more importantly, the immune-boosting effects of chIL-7 (VAC2) and cNK-2 peptide (VAC3) in recombinant EF-1α vaccination. Future studies using a more efficient delivery system for cNK-2 may improve the quality of protection against coccidiosis, and our recent strategy using a *Bacillus* spore oral delivery system to successively deliver cNK-2 to the gut could be applied to further enhance the EF-1α recombinant vaccine efficacy (Wickramasuriya et al., 2021). Taken together, the results of this study show that the combination of adjuvants including ISA 78 VG, chIL-7, and cNK-2 peptide could boost host vaccinal immunity against coccidiosis in poultry.

ACKNOWLEDGMENTS

This research was supported by USDA/NIFA SAS award 2020-69012-31823; accession number 12023193.

We appreciate to Seppic Inc. for providing a Montanide ISA 78 VG adjuvant for collaboration.

DISCLOSURES

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

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