

In vitro effects of 5 recombinant antigens of *Eimeria maxima* on maturation, differentiation, and immunogenic functions of dendritic cells derived from chicken spleen

Muhammad Haseeb, Shakeel Ahmed Lakho, Jianmei Huang, Muhammad Waqqas Hasan, Muhammad Ali-ul-Husnain Naqvi, Zhouyang Zhou, Ruofeng Yan, Lixin Xu, Xiaokai Song, and Xiangrui Li¹

MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, PR China

ABSTRACT *Eimeria maxima* possesses integral families of immunogenic constituents that promote differentiation of immune cells during host-parasite interactions. Dendritic cells (DCs) have an irreplaceable role in the modulation of the host immunity. However, the selection of superlative antigen with immune stimulatory efficacies on host DCs is lacking. In this study, 5 recombinant proteins of *E. maxima* (**Em**), including Em14-3-3, rhomboid family domain containing proteins (**ROM**) EmROM1 and EmROM2, microneme protein 2 (**EmMIC2**), and Em8 were identified to stimulate chicken splenic derived DCs in vitro. The cultured populations were incubated with recombinant proteins, and typical morphologies of stimulated DCs were obtained. DC-associated markers major histocompatibility complex class II, CD86, CD11c, and CD1.1, showed upregulatory expressions by flow cytometry assay. Immunofluorescence assay revealed that recombinant

proteins could bind with the surface of chicken splenic derived DCs. Moreover, quantitative real-time PCR results showed that distinct gene expressions of Toll-like receptors and Wnt signaling pathway were upregulated after the coincubation of recombinant proteins with DCs. The ELISA results indicated that the DCs produced a significant higher level of interleukin (IL)-12 and interferon- γ secretions after incubation with recombinant proteins. While transforming growth factor- β was significantly increased with rEmROM1, rEmROM2, and rEmMIC2 as compared to control groups, and IL-10 did not show significant alteration. Taken together, these results concluded that among 5 potential recombinant antigens, rEm14-3-3 could promote immunogenic functions of chicken splenic derived DCs more efficiently, which might represent an effective molecule for inducing the host Th1-mediated immune response against *Eimeria* infection.

Key words: *Eimeria maxima*, recombinant antigens, chicken spleen, dendritic cell, immunogenic function

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INTRODUCTION

Avian coccidiosis is a protozoan disease caused by the genus *Eimeria*. It virtually reduces production and feed utilization and causes tremendous global economic losses, more than 3 billion US dollars per annum in the commercial poultry setting (Dalloul and Lillehoj, 2006; Kadykalo et al., 2018). Among 7 notorious species of the *Eimeria* genus, *Eimeria maxima* (*E. maxima*) has equal significance; it influences intestinal regions with

the colonization of *Clostridium perfringens* and provokes toward necrotic enteritis in chicken (Collier et al., 2008). Live vaccines and chemoprophylaxis are used to combat against *Eimeria* infection as a most common control strategy, that merely resulted in stern drug resistance in the poultry industry worldwide (Blake and Tomley, 2014; Ahmad et al., 2016). Hence, alternative approaches are needed, which can evade these shortcomings and provide an adequate level of immunological protection against coccidiosis. In this regard, the development of a novel vaccine directed our attention to pinpoint the potential antigen that could be safe and enhance the host immunity against *Eimeria* infection.

Immunity against *Eimeria* infection is mainly T-cells dependent (Dalloul and Lillehoj, 2006). However, the stimulation and activation of T-cells are induced by the immunogenic maturation of dendritic cells (DCs).

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¹Corresponding author: lixiangrui@njau.edu.cn

After antigen uptake, DCs undergo a complex maturation process, marked by the release of cytokines, chemokines, and increased expression of costimulatory molecules (Shrestha et al., 2018). Mature DCs have the ability to prime naïve CD4⁺ T-cells into effector T-cells either T-helper type I (Th1) or Th2 cells, depending on the immunogenic type antigens or invading pathogens (Steinman, 1991; Shrestha et al., 2018). Moreover, various signaling pathways such as Wnt/ β -catenin and Toll-like receptor (TLR) are crucially involved in the regulation, activation, and differentiation of DCs (Kawai and Akira, 2010; Gupta et al., 2015; Suryawanshi et al., 2016; Shrestha et al., 2018). Antigens that induce activation of these signaling pathways in host DCs can enhance the immune response. Thus, Identification of those antigens that potentially stimulate the maturation of DCs would be helpful for an effective vaccine.

Each *Eimeria* species expresses numerous antigens that are involved in the host-parasite interface during infection (Blake and Tomley, 2014). The repertoire of microneme (MIC) proteins is critical for parasite gliding motility and invasion process via parasite actin-myosin system (Bansal et al., 2013; Lai et al., 2009). Furthermore, 2 proteins of EmTFP250 (Em8 and Em6) have been reported as novel members of thrombospondin-related anonymous proteins and are detected during the asexual stage of sporozoites from *E. maxima* (Song et al., 2015). These antigens are associated with parasite gliding motility and highly conserved within apicomplexan MIC proteins (Witcombe et al., 2003). However, in prior work, rhomboid-like gene of *Eimeria tenella*-RHO1 (*EtRHO1*) elicited humoral and cell-mediated immunity and induced partial protection against homologous *Eimeria* infection in the chicken (Li et al., 2012; Yang et al., 2008). The Chaperone-like functional molecule (14-3-3) is spontaneously formed by phosphoserine-threonine-binding modules and involves cell signaling, signal transduction, and immune modulation in host-parasite interfaces (Siles-Lucas and Gottstein, 2003). The 14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells and largely found in the cytoplasmic compartment. However, 14-3-3 proteins can be detected in intracellular organelles and also have important functions in the extracellular environment (Fu et al., 2000; Smith et al., 2011; Kaplan et al., 2017). Within apicomplexan parasites, in *E. tenella*, a single isoform of 14-3-3 seems to be involved in the regulation of the mannitol pathway. Mannitol is believed to be an essential energy source for the sporulation process in this parasite (Liberator et al., 1998). From a drug discovery perspective and control strategies, it is important to note that the mannitol pathway is missing in higher eukaryotes, thus representing an attractive target for the development of selective drug or vaccine (Siles-Lucas and Gottstein, 2003). It has been reported that the 14-3-3 protein of *E. tenella* could interact with the telomerase RNA-binding domain of telomerase reverse transcriptase (Zhao et al., 2014). Previously, it has been proved that 14-3-3 protein can

induce immune protective characteristics and serve as a novel recombinant subunit-vaccine candidate against schistosomiasis (Schechtman et al., 2001). However, elucidation of these recombinant antigens on stimulation of DCs would be supportive to promote cellular immune responses of the host and to identify a novel vaccine candidate against *Eimeria* infection.

The present study was aimed to identify the superlative antigen among 5 potential recombinant antigens: recombinant *E. maxima* (rEm)14-3-3, rEmMIC2, rEm rhomboid-like protein (ROM) 1, rEmROM2, and rEm8 of *E. maxima*, which were involved in immunogenic maturation, stimulation, and differentiation of chicken splenic derived DCs (chSP-DCs) in vitro.

MATERIALS AND METHODS

Ethical Approval

The present study was carried out in strict accordance with the recommendation of the Nanjing Agricultural University Institutional Animal Care and Use Committee guidelines (approval ID is 2012CB120762), and the experiments were approved by the Science and Technology Agency of Jiangsu, Nanjing, PR China.

Experimental Animals

Day-old Hy-Line yellow layer chickens (commercial breed W-36) were conventionally raised in standardized and sterilized bird cages in the coccidia-free environment. The chicken was presented coccidia free during the entire duration of the experiment by monitoring the fecal samples twice per week.

Eight-week-old female Sprague Dawley rats (body weight 150 g) were obtained from the Experimental Animal Center of Nanjing Agricultural University, Nanjing, Jiangsu, PR China (Certified ID: SCXK 2008-0004). Chickens and rats were kept in pathogen-free conditions with a supply of sterilized food and water ad libitum.

Cell Surface Markers and Reagents

The following details concerning the antibodies (Abs) and reagents were used in this study: mouse anti-chicken [MHC-class II⁺ (clone-2G11), CD86⁺ (Clone-GL1), CD1.1⁺ (Clone-CB3), CD11c⁺ (Clone-N418)] with appropriate isotype IgG1-PE, IgG2a-PE/Cy7, IgG1-FITC, IgG-APC, respectively, all were from Southern Biotech (Birmingham, AL). Hiscript II Q RT SuperMix for QRT-PCR (⁺gDNA Wiper) (L/N: 7E312C9) and ChamQ SYBR QRT-PCR Master Mix are from Vazyme. TRIzol (L/N: A304-1) from TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian City, Liaoning, China. The enhanced horseradish peroxidase (HRP)-DAB chromogenic Kit (L/N: S7418) was from TIANGEN Co. Ltd. (Tiangen, China). Freund's adjuvant Complete and Freund's adjuvant Incomplete were from (Sigma Aldrich, Merck KGaA, Darmstadt, Germany).

Lymphocyte separating medium (L/N: LTS1077) was from TBD-science, Tianjin, China. Gibco RPMI Medium-1640 (L/N: 2037577) and Chicken Serum was from Life Technologies, San Francisco, CA. Recombinant chicken-granulocyte macrophage colony-stimulating factor (GM-CSF) was from Abcam, Cambridge, UK, and recombinant chicken interleukin 4 (IL-4) was from Kingfisher, London, UK.

Purification of rEm Antigens

Recombinant plasmids including rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8 with GenBank accession no. XM_013480135.1, CDJ58339, CDJ59687, FR718971.1, and AY239227 (6242–7310 of EmTFP250), respectively, were provided by the Ministry of Education Joint International Research Laboratory, Preventive Veterinary Medicine, Nanjing Agricultural University. The recombinant proteins were purified by the protocol described previously (Huang et al., 2015). Briefly, the recombinant plasmid was transformed into *Escherichia coli* (BL21) and cultured in Luria Bertini medium containing ampicillin (100 µg/mL). The recombinant proteins were expressed by induction with 1-mmol isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich) at an optimal density (OD) of 0.5 at 37°C. The culture was centrifuged at 4,500 rpm for 25 min, and the supernatant was discarded. Pellet was lysed using lysozyme (10 µg/mL; Sigma-Aldrich) followed by sonication. Then, recombinant proteins were purified using a Ni²⁺-nitrilotriacetic acid column (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The fusion protein encoded by Poly His-tag protein of pET-32a was also expressed. The purity of the recombinant proteins was determined by 12% SDS-PAGE with staining by Coomassie Brilliant Blue R-250 dye (Beyotime, China). Protein concentrations were estimated by Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA). Endotoxins were reduced from recombinant proteins by using a Toxin Eraser Endotoxin Removal Kit (GeneScript, Piscataway, NJ). The purified proteins were concentrated and stored at –80°C until further use.

Preparation of Polyclonal Abs Against rEm Antigens

Polyclonal Abs against *E. maxima* recombinant antigens were produced as described previously (Liu et al., 2018a). Concisely, Sprague Dawley rats were injected with 300 µg of each recombinant proteins (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) with 0.5 mL Freund's complete adjuvants. Fourteen days later, 2 doses for each protein were emulsified in 0.5 mL of Freund's incomplete adjuvant and were given subcutaneously with an interval of 1 wk. The antisera were collected after 8 d of the last inoculation and then

stored at –80°C for Western blot and immunofluorescence assay.

Determination of IgG Titration

Indirect ELISA was performed to evaluate IgG titration using anti rEm antigens (anti-rEm-Ags) immunized rat sera as described previously (Poolperm et al., 2017). Briefly, different concentrations (120–0.93 µg/mL) of rEm-Ags (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) were diluted in 0.05M carbonate buffer solution (pH 9.6) and incubated in 96-well binding plates (Cambridge, MA) overnight at 4°. Then, unbound proteins were discarded after washing with phosphate buffered saline (PBS) +0.05% Tween-20, and wells were blocked with 5% bovine serum albumin (BSA) 100 µL/well for 1.5 h at 37°C. Wells were washed with PBS + Tween-20 (PBS+T) 3 times; control and immunized rat sera were diluted (1:50–1:200) in 5% BSA and incubated in known wells for 1.5 h at 37°C. Subsequently, dilute secondary antibody HRP-conjugated goat antichickens IgG (1:4,000) in 5% BSA was added and incubated for 1.5 h at 37°C. Plates were washed with PBS+T after each incubation time. Finally, add tetra-methyl-benzidine (100 µL/well) and incubate the plates for 15 min in the dark at room temperature. After that, 2M H₂SO₄ was added (100 µL/well) to terminate the reaction. Then OD₄₅₀ was measured using a microplate reader (Thermo Fischer Scientific, Waltham, MA). All samples were analyzed in triplicates, and each trial was performed twice.

Immunoblot Assay

Immunoblotting shows a critical role in identifying target protein and evaluates the immunogenicity of recombinant antigens (Naqvi et al., 2019). Western blotting was performed to detect recombinant proteins' specific response in sera of immunized rats. The recombinant proteins of *E. maxima* were separated by reducing on 12% SDS-PAGE and transferred to polyvinylidene difluoride nitrocellulose membrane (MP, USA) using a semi-dry system (Nova-blot, HF) in western blotting transfer buffer (Tris 48 mmol, glycine 39 mmol, SDS 0.0364%, methanol 20%). Obtained membranes were blocked with 5% Skimmed milk diluted in Tris-buffer saline (with 0.05% Tween-20). Five membranes were incubated with primary antibody rat anti-rEm14-3-3 sera with dilution 1:100, rat anti-rEmROM1 sera with dilution 1:150, rat anti-rEmROM2 sera with dilution 1:150, rat anti-rEmMIC2 sera with dilution 1:100, rat anti-Em8 sera with dilution 1:150, and normal rat sera in the negative control group (1:100 dilution) for 1.5 h at 37°C. After washing three times with Tris-buffer saline, membranes were incubated with the secondary antibody HRP-conjugated goat antichickens IgY-Ab6877 (1:4,000) (Abcam Inc., Eugene, OR) for 1.5 h at 37°C. Ultimately, immunoreaction was

visualized using an enhanced Horseradish peroxidase (HRP-DAB) chromogenic kit (TIANGEN) according to the manufacturer's instructions in dark.

Chicken Splenocytes Preparation In Vitro

Spleens of 3 three-week-old birds were collected and isolated from a thin surrounding layer using sterile instruments and placed into a Petri plate with enough sterile PBS. Single-cell suspensions were prepared from spleens by gentle mashing through the nylon mesh filter and mixed with sterile PBS 1:1. Splenocytes were obtained after loading onto an equal volume of cell suspension and lymphocyte separating medium (TBD-science, Tianjin, China) and centrifuging at $600 \times g$ for 20 min. Trypan blue negative cells were counted as viable under a microscope in a hemocytometer chamber, and approximately 2.5×10^6 cells were obtained from each bird.

Generation and Maturation of chSP-DCs

DCs were generated from the splenocytes and matured as previously described with some alteration (Wu et al., 2010; Sun et al., 2017). Briefly, splenocytes were seeded into six-well ultra-low attachment polystyrene plates at the rate of 1.9×10^7 cells/3 mL/well in prewarmed RPMI-1640 complete culture medium with 10% chicken serum (Life Technologies, Carlsbad, CA), penicillin (100 U/mL), and streptomycin (1 μ g/mL), for 7 d at 37°C, 5% CO₂. After 12-hour incubation, nonadherent cells were washed and removed by gentle pipetting with sterile PBS. Adherent cells (splenic derived monocytes) were cultured with recombinant chicken-GM-CSF (20 ng/mL) and recombinant chicken interleukin 4 (IL-4) (10 ng/mL) in the complete culture medium (RPMI) for 6 d. The medium was replaced at an interval of 48 h by aspirating 1.5 mL of media and replacing 1.5 mL of fresh complete culture medium. Subsequently, cells were incubated with stimulation of rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8 antigens (45 μ g/mL) as a treatment group from day 6 to 24 h. The cells of blank control (PBS) and pET-32a tag protein (45 μ g/mL) negative control group were cultured by similar procedures in treatment groups. Procedures in each group were repeated in triplicates. On day 7, the semisuspended and suspended cells were collected by gently pipetting, and adherent cells were harvested by pasture pipettes or cell scraper for further characterization. To induce maturation, in the positive control group, cultured cells were stimulated with *E. coli* lipopolysaccharides (LPS; Sigma-Aldrich) (200 ng/mL) and incubated at 37°C from day 6 to 24 h; finally, chicken splenic derived mature DCs were obtained.

Morphological Observations of chSP-DCs

The DCs' morphology was assessed as described previously (Kalaiyarasu et al., 2016). Briefly, cells were grown in a RPMI-1640 culture medium with 10% chicken serum-supplemented every 48 h with 20 ng/mL GM-

CSF and 10 ng/mL IL-4. The mature and immature state of DCs were differentiated based on their morphological characteristics, that is, great increase in size, asymmetrical shape, multiple dendritic projections, and identification of cell clusters either adherent to the bottom or suspended in the medium. The distinct effects of recombinant antigens and LPS groups in the morphology of DCs were evaluated on day 1, 4, and 7 of culture using an inverted microscope (Olympus, Shibuya, Japan) with a digital camera (Nikon, Tokyo, Japan). Cells from pET32a-tag protein and PBS groups were also observed.

Phenotypic Analysis of chSP-DCs by Flow Cytometry

Phenotypic identification of DCs was showed by flow cytometry, as described previously (Wu et al., 2010). The cells were harvested on day 7, washed with sterile PBS, and the cells' concentration adjusted to 1×10^6 cells/mL. PE (Phycoerythrin)-labelled MHC-II (immunoglobulin G1 [IgG1_k-PE]) and FITC (fluorescein isothiocyanate) labeled CD1.1 (immunoglobulin G1 [IgG1-FITC]) were added in cell suspension to a final concentration of 2 μ g/100 μ L volume, and appropriate concentration of respective isotype were added up to 8 μ g/100 μ L. PE/CY7 (R-phycoerythrin/Cyanine7) labeled CD86 (immunoglobulin G_{2a} [IgG_{2a}-PE/CY7]) and allophycocyanin-labelled CD11c (immunoglobulin G [IgG-allophycocyanin]) were added in cell suspension to a final concentration of 3 μ g/100 μ L, and isotype concentration was 5 μ g/100 μ L and incubated for 30 min at 4°C in dark. However, double-color positive staining cells were labeled with MHC-II in combination with CD1.1, CD86, and CD11c monoclonal Abs. Each sample was measured in triplicates (n = 3). After the final wash with PBS, stained cells were gated forward and side scattered with 10,000 events for each sample and examined by using FACSCalibur (Flow Cytometry, BD Bio-science, CA) for flow cytometry analysis.

The Binding Ability of rEm Antigens to chSP-DCs

Immunofluorescence assay was performed with some modification as described previously (Liu et al., 2018b) to validate the capability of recombinant antigens to bind with DCs. DCs were collected by gentle pipetting, washed with sterile PBS, and centrifuged at $500 \times g$ for 5 min; the prepared concentration was 1×10^4 cells/mL. Cells were permitted to settle onto poly L-lysine-treated glass slides and fixed with 4% paraformaldehyde for 30 min at 37°C. Cells were blocked with 5% BSA for 1.5 h and washed 3 times with PBS+T. Accordingly, cells were incubated with primary antibody rat anti-rEm14-3-3 (1:100), rat anti-rEmROM1 (1:150), rat anti-rEmROM2 (1:150), rat anti-rEmMIC2 (1:100), rat anti-rEm8 (1:150) sera, and normal rat (1:100) sera overnight at 4°C. The next day, cells were washed with PBS+T 3 times

(at 5-min intervals) and incubated with secondary antibody goat antichick (IgY, H&L, Alexa-Fluor 647) labeled with Cy3 (1:600) fluorescein-conjugated for 1 h in the dark. After washing with PBS+T, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) for 5 min. Finally, the slides were washed, air-dried, embedded in a antifade mounting medium (Crystal Mount Aqueous; Sigma-Aldrich), and covered with a glass coverslip. Fluorescence was analyzed with a laser scanning confocal microscope (PerkinElmer, Waltham, MA).

Quantitative Real-Time PCR

Quantitative real-time PCR (QRT-PCR) was performed to analyze mRNA expression of DCs stimulated with rEm-Ags-related Wnt and TLRs pathway genes with primers which are listed in [Supplementary Table 1](#) (supplementary file). Total RNA was extracted from cells (2.1×10^7 cells/mL) through the TRIzol reagent (Invitrogen, Carlsbad, CA) method, as described previously ([Lan et al., 2016](#)). Briefly, the quantity and purity of extracted RNA were measured by Nano-drop spectrophotometry (Eppendorf, Hamburg, Germany). The cDNA from 1- μ g RNA was synthesized with a HiScript-II Q-RT reagent kit with a +gDNA wiper according to the manufacturer's instructions. Total reaction volume of 20 μ L (ChamQ SYBR QRT-PCR master mix) consisting of 10 μ L of 2 \times SYBR QRT-PCR Master Mix, 0.5 μ L of each primer (10 μ M), 0.5 μ L of 50 \times ROX reference dye-I, 1 μ L of cDNA, and 7.5 μ L of DNA/RNA-free deionized H₂O was used in triplicates with the following thermocycler profile, one cycle at 95°C for the 30 s, 40 cycles at 95°C for 10 s, 60°C for 30 s, and one extension cycle at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s according to instructions given. The housekeeping gene β -actin was used for all samples. The mRNA expressions of relative genes were calculated by formula $Ct 2^{-\Delta\Delta Ct}$ from raw cycle thresholds Ct according to the method described before ([Rao et al., 2013](#)). Data were represented by 3 technical repeats, and each bar represents the mean \pm SEM value (n = 3).

Detection of Cytokines by ELISA

After day 7, stimulated and nonstimulated cell supernatants were collected to detect the expression level of IL-12, interferon (IFN)- γ , transforming growth factor (TGF)- β , and IL-10 by ELISA using corresponding enzyme-linked immunosorbent assay kits, according to manufacturers' instructions (R&D system, Nanjing JinYibai Bio-Technology, China).

Statistical Analysis

Statistical data were presented as mean \pm SEM. Flow cytometry results were analyzed by Flowjo software (Tree star Version 10.0.7) of BD FACSVerse. Graphical analysis was performed by using the software package GraphPad Prism 6.01 (GraphPad Prism, San Diego, CA). One-way ANOVA was used followed by Tukey's

multicomparison test. Statistically significant difference values are showed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, and ns stands for nonsignificant ($P > 0.05$). The entire experimental values were repeated at least 3 times and each bar represents mean \pm SEM value (n = 3).

RESULTS

Purification of rEm Antigens

Five recombinant products of *E. maxima* (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) were expressed, and a HisTrap FF column was used to purify histidine-tagged recombinant protein by using immobilized Ni²⁺ ion affinity chromatography system. They were about 53 kDa, 50.5 kDa, 49 kDa, 50.5 kDa, and 71 kDa, correspondingly. The molecular size of the fusion protein poly his-tag in the pET-32a vector was about 20 kDa.

Determination of IgG Titration

The prepared polyclonal Abs against recombinant antigens were collected, and sera titer was measured by indirect ELISA. OD₄₅₀ values derived from checkerboard showed positive/negative anti-rEm14-3-3 (dilution 1:100), anti-rEmROM1 (dilution 1:150), anti-rEmROM2 (dilution 1:150), anti-rEmMIC2 (dilution 1:100), and anti-rEm8 (dilution 1:150) positive sera as shown in [Supplementary Figure 1](#) (supplementary file).

Immunoblot Analysis

Polyclonal Abs were generated in rats through immunization with 300 μ g of rEm-Ags, and sera were collected at the eighth day after the last immunization. Immunoblot assay revealed specific bands of rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8, indicating recombinant proteins were recognized by anti-rat sera ([Supplementary Figure 2](#)). No specific band was detected by incubation with normal rat sera.

Morphological Observations of chSP-DCs

Higher magnification of inverted microscope showed that chSP-DCs were slackly adherent and morphologically distinct and became single rather than colonies after coincubation with rEm-Ags and with LPS. PBS and pET-32a tag protein group cells did not alter their shape clearly ([Figure 1](#)).

Binding Confirmation of rEm Antigens

Immunofluorescence assay was performed to investigate whether rEm antigen (rEm-Ags) can bind with the surface of chSP-DCs. After incubation with rEm-Ags, proteins were labeled with rat-anti-sera and visualized by Cy3 fluorescein-conjugated goat antichick antibody and DAPI. The results of confocal microscopy concluded that blue fluorescence staining was for nuclei of cells

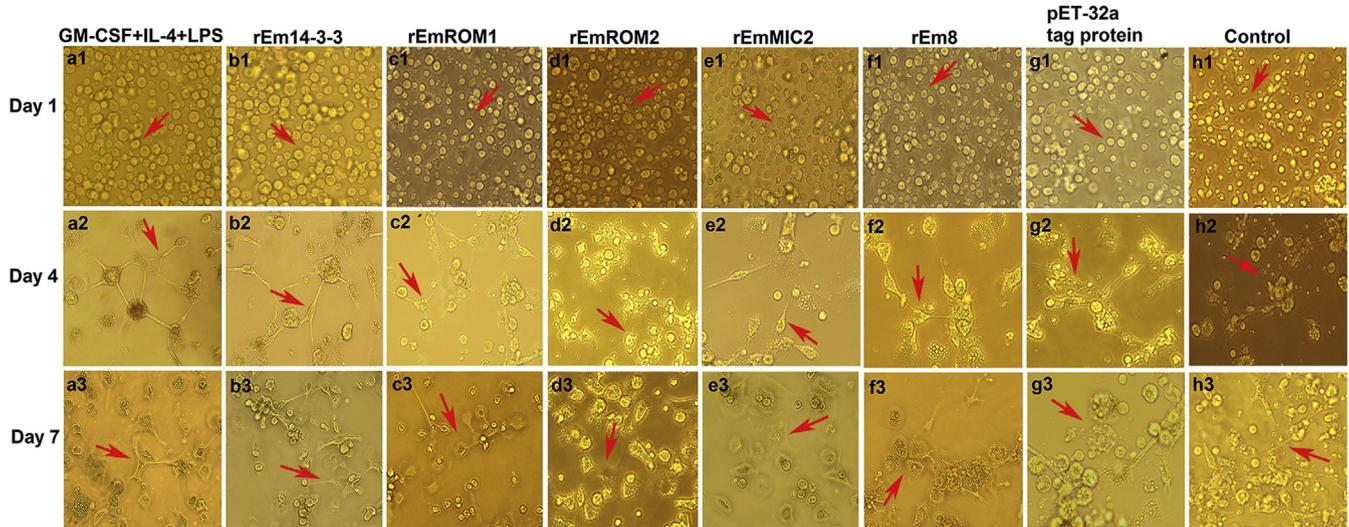


Figure 1. Morphological characteristics of chicken splenic derived dendritic cells (DCs) were observed at Day 1, red arrows indicated that seeded cells were round and smooth in shape. At Day 4, red arrows showed that cell surface projection/veils were seen in complete culture medium (RPMI + chicken serum + recombinant chicken GMCSF + IL-4). After stimulation of rEm antigens or LPS stimulation at day 7, red arrows shown that cultured populations were matured, irregularly shaped cell aggregates with dendrites that became semi-suspended or suspended in the medium and exhibited distinctive morphology of DCs.

(DAPI), and rEm-Ags were bound to DCs that showed red fluorescence (Cy3) staining on cell surface (Figure 2).

Stimulatory Effects of rEm Antigens on DCs' Maturation

Typical cell surface markers expressed on DCs were assessed by flow cytometry. The results of dot plot single-color flow cytometry analysis are shown in

Supplementary Figure 3. The percentage of MHC-II cells was 37.1% in group rEm8 ($P < 0.05$), rEmMIC2 39.6% ($P < 0.01$), rEmROM1 38.2% ($P < 0.01$), and rEm14-3-3 41.6% ($P < 0.0001$), whereas the percentage of the GM-CSF + IL-4+LPS group was significantly higher (53.5%, $P < 0.0001$) as compared with control groups PBS (31.2%) and pET-32a tag protein (32.4%), respectively (Figure 3A; Supplementary Figure 3). However, in comparative analysis, rEm14-3-3 was significantly

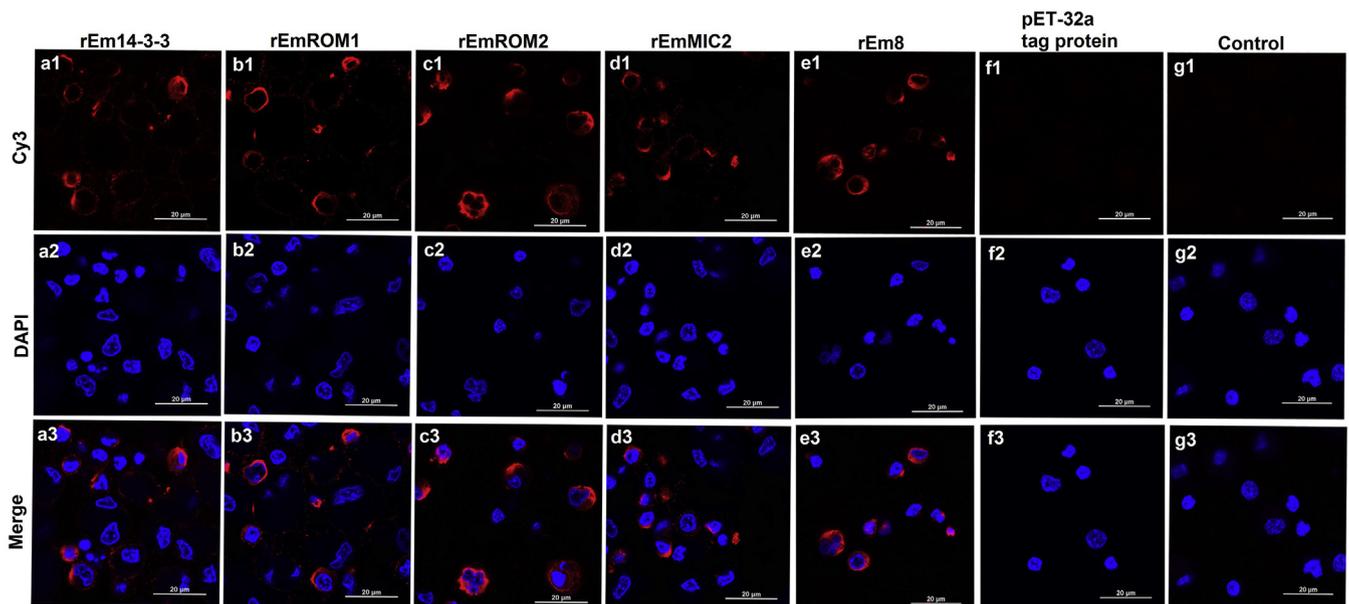


Figure 2. The binding ability of recombinant proteins (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) with chicken splenic derived dendritic cells by immunofluorescence assay. The cells were incubated with rat sera antirecombinant antigens IgG, anti-pET-32a tag protein, and negative rat sera IgG (control). (a1, b1, c1, d1, e1) Staining of target proteins (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) was visualized by cy3-conjugated secondary antibody (red color). (a2, b2, c2, d2, e2) Nuclei of corresponding cells were visualized by 4',6-diamidino-2-phenylindole (DAPI; blue color). (a3, b3, c3, d3, e3) Merged, red, and blue colors overlapped. No fluorescence was observed in control groups. The results were analyzed by a confocal laser scanning microscope with scale bars 20 μ m.

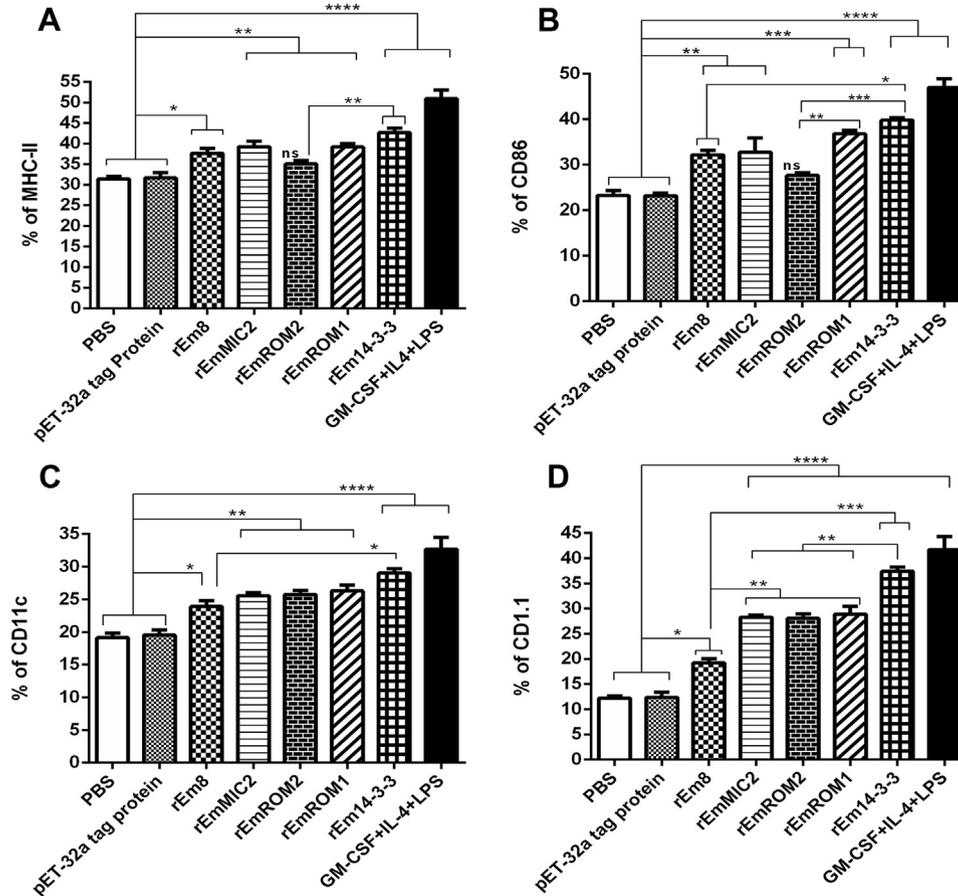


Figure 3. Recombinant antigens of *E. maxima* (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) increased the expression of cell surface markers MHC-II, CD1.1, CD11c, and CD86 in chicken splenic-derived dendritic cells (ChSP-DCs) as determined by flow cytometry. Bar graph representing percentages of A, MHC-II; B, CD86; C, CD11c; D, CD1.1. The data are presented as mean \pm SEM and represented of triplicates experiments ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$) and ns represents nonsignificant ($P > 0.05$).

increased ($P < 0.01$) than rEmROM2. Similarly, proportions of CD86 cells were significantly upregulated in groups rEm8 (33.3%, $P < 0.01$), rEmMIC2 (34.9%, $P < 0.01$), rEmROM1 (37.5%, $P < 0.001$), and rEm14-3-3 (40.6%, $P < 0.0001$), when related with PBS and pET-32a group (21.8%, 23.3%), respectively (Figure 3B; Supplementary Figure 3). Relatively, the CD86 cells of rEm14-3-3 protein were substantially higher ($P < 0.05$), ($P < 0.001$) from rEm8 and rEmROM2 proteins, respectively. Thus, these findings concluded that rEm14-3-3 antigens could accelerate the expression of MHC-II and CD86 percentages and can stimulate chSP-DCs than remaining antigens. In addition to the expressions of CD1.1, those of CD11c were also evaluated. CD11c cells ratio was 24.7% in rEm8 ($P < 0.05$), rEmMIC2 26.3% ($P < 0.01$), rEmROM2 25.5% ($P < 0.01$), rEmROM1 27.9% ($P < 0.01$), and rEm14-3-3 31.1% ($P < 0.0001$) as compared with PBS (19.1%) pET-32a (19.6%) groups, respectively. In comparative analysis, rEm14-3-3 was significantly increased ($P < 0.05$) than rEm8 (Figure 3C; Supplementary Figure 3). Percentage of CD1.1 cells was significantly increased in rEm8 (19.2%, $P < 0.05$), rEmMIC2 (28.2%, $P < 0.0001$), rEmROM2 (26.7%, $P < 0.0001$), rEmROM1 (30.0%, $P < 0.0001$),

and rEm14-3-3 (37.5%, $P < 0.0001$) as compared with PBS (12.1%) and pET-32a (11.8%) groups (Figure 3D; Supplementary Figure 3). While rEm14-3-3 protein markedly upregulated the DCs' proportions in rEm8 ($P < 0.001$), rEmMIC2, rEmROM2, and rEmROM1 groups ($P < 0.01$) relatively.

To characterize the cells' population in more detail, double-color staining was performed by flow cytometry using MHC-II as reference molecule. The dot plot analysis of double-color staining is presented in Supplementary Figure 4. The bar graphs represent the proportions to which CD11c/MHC-II, CD86/MHC-II, and CD1.1/MHC-II cells were upregulated after stimulation with rEm-Ags and LPS as compared with control groups (PBS and pET-32a) Figures 4A–4C, respectively. The expression of CD11c/MHC-II cells was eminently elevated in rEmMIC2 (45.6%, $P < 0.001$), rEmROM1 (49.2%, $P < 0.001$), and rEm14-3-3 (55.0%, $P < 0.0001$) as related to control groups. Comparatively, rEm14-3-3 protein was significantly increased from ($P < 0.001$) rEm8, ($P < 0.001$) rEmROM2, and ($P < 0.01$) rEmMIC2 groups, and rEmROM1 antigen expression was also higher ($P < 0.05$) than rEmROM2 group (Figure 4A; Supplementary Figure 4). CD86/MHC-II cell proportions were also significantly higher

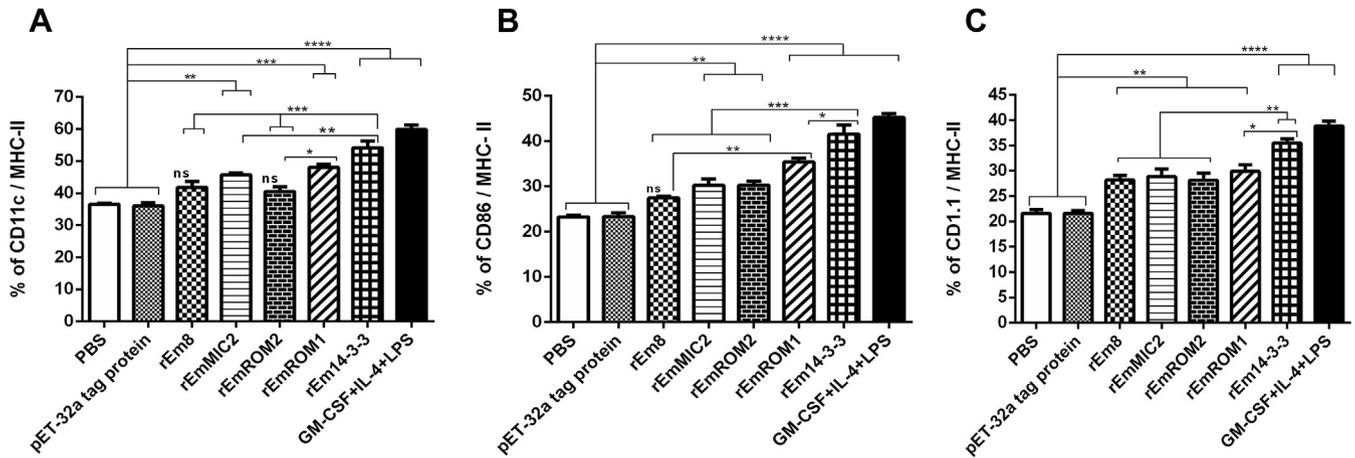


Figure 4. To characterize the cells population in more detail, double-color flow cytometric analysis was performed using MHC-II as a reference molecule. Bar graph representing proportions of cells: A, CD11c/MHC-II; B, CD86/MHC-II; C, CD1.1/MHC-II. The data are presented as mean \pm SEM and represented of triplicates experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$) and ns represents nonsignificant ($P > 0.05$).

in rEmMIC2 (32.6%, $P < 0.01$), rEmROM2 (30.7%, $P < 0.01$), rEmROM1 (36.3%, $P < 0.0001$), and rEm14-3-3 (42.0%, $P < 0.0001$) than those in control groups. Comparatively, rEm14-3-3 protein was substantially increased ($P < 0.001$) than rEm8, rEmMIC2, and rEmROM2 groups (Figure 4B; Supplementary Figure 4). Typical phenotypes of DCs with CD1.1/MHC-II cell proportions were remarkably increased in rEm8 (28.7%, $P < 0.01$), rEmMIC2 (30.3%, $P < 0.01$), rEmROM2 (27.1%, $P < 0.01$), rEmROM1 (31.7%, $P < 0.01$), and rEm14-3-3 (35.8%, $P < 0.0001$) as related to PBS, pET-32a (20.2, 20.6%) groups, respectively (Figure 4C; Supplementary Figure 4). In comparison to rEm antigens, rEm14-3-3 showed significantly higher expression of double-positive cells percentages ($P < 0.001$), when compared with rEm8, rEmMIC2, and rEmROM2 groups (Figure 4). Together, single/double color staining results suggested that recombinant antigens of *E. maxima* can upsurge the proportions of ChSP-DCs.

Genes Expression of Wnt and TLR Pathway

QRT-PCR was performed to investigate mRNA expression of Wnt and TLRs pathway genes after stimulation of rEm-Ags on chSP-DCs. As indicated in Figure 5A, among Wnt pathway genes, the expression level of β -catenin was upregulated with rEm14-3-3; rEmROM1 ($P < 0.01$); and rEmROM2, rEmMIC2, and rEm8 ($P < 0.05$) as compared with the control groups (PBS and pET-32a). Similarly, dishevelled (DVL) expression level was also increased with rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8 groups, which were significant ($P < 0.01$), when compared with control groups correspondingly (Figure 5A). But no significant effects were observed from APC and CK2 gene expression levels.

DCs play an important role in linking the innate and adaptive immune system in TLR stimulatory pathway (Kannaki et al., 2010). QRT-PCR was performed to

estimate the mRNA expression levels of TLR1, TLR3, TLR4, TLR7, TLR15, TLR21, and myeloid differentiation protein 88 (MyD88) with a response to rEm-Ags treated with DCs. Out of the aforementioned genes, TLR (1, 3, 4, 7, 15, and MYD88) were upregulated, and TLR21 expression was not significant (Figure 5B). Our comparative study further revealed that TLR1 expression was substantially elevated ($P < 0.01$) with rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8 as compared with control groups (PBS and pET-32a). TLR3 expression was significantly increased with rEm14-3-3 ($P < 0.001$), rEmROM1, and rEmROM2 ($P < 0.05$), as compared with control groups. In comparative analysis, TLR3 expression was significantly higher with rEm14-3-3 ($P < 0.001$), rEmROM1, and rEmROM2 ($P < 0.05$) from the rEmMIC2 group, respectively. Likewise, TLR4 mRNA expression was substantially upregulated with rEm14-3-3, rEmROM1, rEmMIC2, rEm8 ($P < 0.01$), and rEmROM2 ($P < 0.05$) as compared with control groups. The mRNA expression of TLR7 was significantly increased with rEm14-3-3 ($P < 0.001$) and rEmROM1 ($P < 0.05$) as related to control groups. With comparative analysis, TLR7 expression was substantially upregulated ($P < 0.001$) with rEm14-3-3 from rEmROM2, rEmMIC2, and rEm8 groups. TLR15 was substantially increased with rEm14-3-3 ($P < 0.001$), rEmROM1, rEmROM2, rEm8 ($P < 0.05$), and rEmMIC2 ($P < 0.01$) as related with control groups. MYD88 expression was prompted clearly ($P < 0.001$) with rEm14-3-3 and LPS groups as compared with control groups. Relatively, MYD88 expression in rEm14-3-3 was significantly increased ($P < 0.01$) from rEmROM1, rEmMIC2, rEmROM2, and rEm8 groups (Figure 5B).

Effects of rEm Antigens on Cytokines Production

Upon activation by mature DCs, T-cell-mediated immune response differentiate into Th1 and Th2 cells that

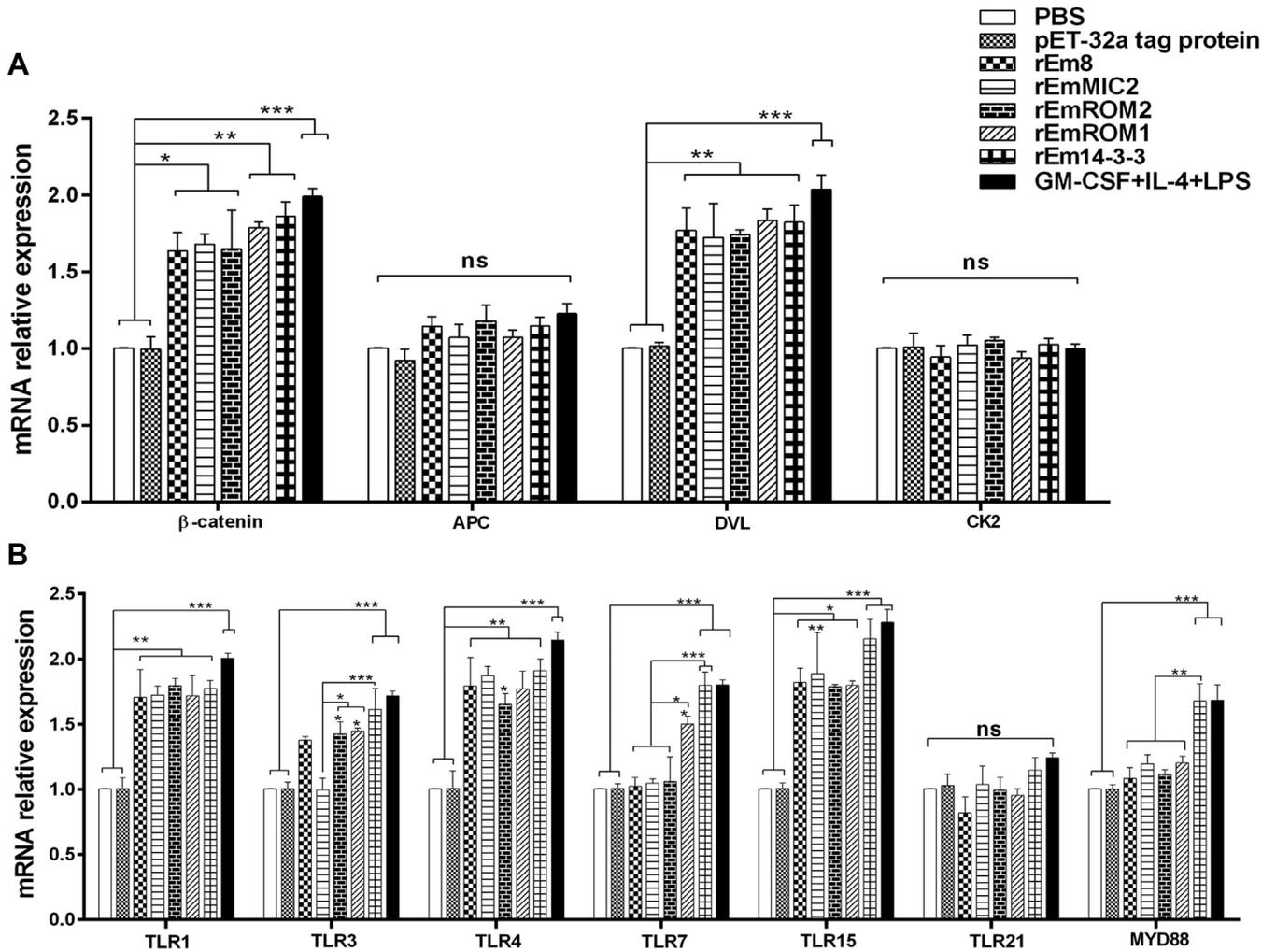


Figure 5. Gene expressions of Wnt and TLRs signaling pathways by QRT-PCR. Total RNA was isolated from chicken splenic derived DCs. A, mRNA expression of key genes β -catenin, APC, DVL, CK2; B, mRNA expression of key genes TLR1, 3, 4, 7, 15, 21, and MYD88. Significant values are obtained as presented ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). Data are presented as mean \pm SEM from 3 independent experiments, with methodical 3 technical triplicates per group, and ns represents nonsignificant ($P > 0.05$).

promote eminent cytokines secretions (Wu et al., 2010; Kalaiyarasu et al., 2016). In this study, ELISA kits were used to evaluate the expression of IL-12, IFN- γ , IL-10, and TGF- β cytokines from the supernatant of DCs stimulated with rEm-Ags, GM-CSF + IL-4+LPS, and control groups (PBS, pET-32a). The obvious production of IL-12 and IFN- γ in cells supernatant receiving rEm-Ags stimulation were significantly increased as shown in (Figures 6A, 6B). IL-12 cytokine was significantly higher in rEm8, rEmMIC2 ($P < 0.05$), rEmROM2, rEmROM1 ($P < 0.01$), and rEm14-3-3 ($P < 0.001$) and GM-CSF + IL-4+LPS ($P < 0.001$) as compared with control groups (PBS and pET-32a). The secretion of IFN- γ cytokine was also upregulated in rEm8, rEmMIC2 ($P < 0.05$), rEmROM2, rEmROM1 ($P < 0.01$), and rEm14-3-3 ($P < 0.001$) as compared with control groups (Figure 6B). Subsequently, the rEm14-3-3 protein was a more significant molecule to produce IL-12 and IFN- γ cytokines than remaining antigens. On the other hand, secretion of TGF- β was also significantly increased with rEmMIC2 ($P < 0.05$),

rEmROM1, and rEmROM2, ($P < 0.01$) as compared with control groups (Figure 6C). No significant effect was observed in the secretion of IL-10 cytokines (Figure 6D).

DISCUSSION

DCs are professional APCs that serve as a passage between innate and adaptive immune response involved in the activation of naïve T-lymphocytes (Del Cacho et al., 2011; Kalaiyarasu et al., 2016; Van Goor et al., 2016). For exploiting DCs in vaccine development, vaccine antigens must induce DCs' maturation because functions of DCs are highly dependent on the level of maturation (Pulendran, 2004). *E. maxima* comprise numerous immunogenic antigens that play a crucial role in host-parasite interaction by modulating the immune response of the host against *Eimeria* infection (Blake et al., 2017; Huang et al., 2018). Hence, identification and screening of potential antigens from the complex biology of the parasite is always a priority for the development of novel

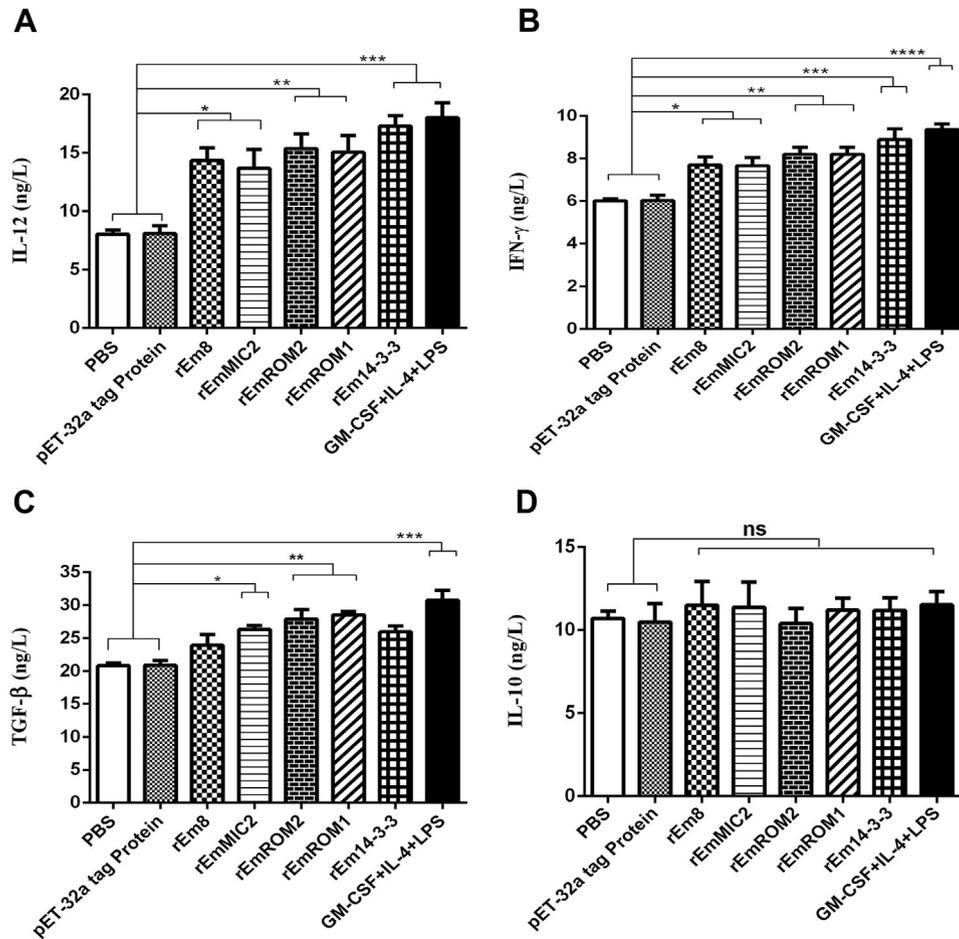


Figure 6. Relative analysis of multiple cytokines produced by chicken splenic derived dendritic cells. After coincubation with recombinant proteins (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8), control groups (PBS, pET-32a), and positive control group (GM-CSF + IL-4+LPS), cytokines secretions in the supernatant of cell culture were measured by ELISA kits. A, Cytokine production level of IL-12; B, cytokine production level of IFN- γ ; C, cytokine production level of TGF- β ; D, cytokine production level of IL-10. The data were representative of 3 independent experiments, and the values presented here were the means \pm SEM (* P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001); ns represents nonsignificant (P > 0.05).

vaccines. Therefore, the present study was designed to probe the immunogenic effects of 5 recombinant antigens (rEm14-3-3, rEmMIC2, rEmROM1, rEmROM2, rEm8) of *E. maxima* on maturation and differentiation of chicken splenic-derived DCs.

The maturation process of DCs reorganizes the MHC molecules from intracellular endocytic compartments to the cell surface and increases in the expression of costimulatory molecules required for the activation of T-cells (Shrestha et al., 2018). The previous investigation proved that MHC-class II, CD86, CD11c, and CD1.1 cell expressions were upregulated in chicken bone marrow-derived mature DCs after stimulation of LPS (Wu et al., 2010; Kamble et al., 2016). Likewise, our results showed that after the coincubation of recombinant proteins rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8 with DCs, cell percentages of MHC-II, CD86, CD11c, and CD1.1 expression were upregulated as compared to control groups. These findings indicated that among 5 immunostimulatory recombinant antigens, rEm14-3-3 promoted maturation and differentiation of DCs proficiently than remaining antigens.

Seminal research stated that the Wnt/ β -catenin pathway might differentially regulate the function of DCs and promote proinflammatory response against protozoan infection (Cohen et al., 2015). Previous study proved that during the regulatory process of Wnt/ β -catenin canonical pathway genes expression, binding of Wnt ligands to its Frizzled receptor, low-density lipoprotein receptor-related proteins 5/6 activate scaffolding protein DVL. The upregulated DVL protein turns into inhibition of complex which includes adenomatous polyposis coli (APC) and casein kinase (CK) proteins, which lead to the phosphorylation process (Rosso and Inestrosa, 2013). In line with these, DCs' maturation is paralleled by downregulation of inhibition complex and upregulation of the β -catenin molecule (Yasmin et al., 2013; Suryawanshi et al., 2016). Given our data, it was demonstrated that mRNA expression of β -catenin and DVL with recombinant Em-Ags was upregulated, and a similar effect was also profound with LPS-stimulated DCs. While APC and CK2 did not show significant changes. Further studies should classify the correlation between Wnt, MAPK, and NF κ B, signaling pathways gene expressions that are paralleled to activate the

complicated mechanisms for driving DCs maturation and T-cells differentiation.

DCs activation plays a significant role to develop linkage between an innate and acquired immune system in TLRs stimulatory pathway. In addition, TLRs-based adjuvant can enhance immunity for vaccine improvement against parasitic infection (Kannaki et al., 2010; Gupta et al., 2014). Ten ch-TLRs and their ligands have been reported: TLR1a, TLR1b, TLR2a, TLR2b, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 (Roach et al., 2005; Jie et al., 2013). Previous studies reported that during TLRs pathway activation process, TLR4, TLR15, MYD88 (Zhou et al., 2013; Kalaiyarasu et al., 2016), TLR7 (Xiao et al., 2016), TLR1/TLR2 (Kokkinopoulos et al., 2005), and TLR3 (Jiao et al., 2012) genes expression were well expressed in immune cells subsets in chickens. Our findings revealed that mRNA expressions of TLR1, 4, and 15 were upregulated in rEm-Ags incubated with DCs as compared to control groups. While the mRNA expression of TLR3, 7, and MYD88 was significantly increased with rEm14-3-3 rather than remaining antigens. Finally, with comparative analysis, we speculate that the rEm14-3-3 molecule may activate the TLRs signaling pathway by stimulation of chSP-DCs in immunogenic but not tolerogenic status. TLR genes are recognized to be allied with resistance to infectious diseases. In addition, TLR ligands could be used as a novel molecular adjuvant with immunogenic antigen. Further studies are necessary to expand the knowledge about TLRs and their role in the chicken immunity; an in vivo approach is needed.

With maturation and activation of DCs, cytokines production has a pleiotropic effect on the immune system, as well as modulating inflammatory cellular immunity in the host (Erf, 2004; Lillehoj et al., 2004). As in previous experiments, the expression level of proinflammatory (IL-1 β , IL-17), Th1 (IFN- γ , IL-12), and TGF- β cytokines was detected in intestinal intraepithelial lymphocytes after *E. maxima*, *E. tenella*, and *E. acervulina* infections (Erf, 2004; Park et al., 2008; Seeger et al., 2015). In this study, binding of recombinant proteins (rEm14-3-3, rEmROM1, rEmMIC2, rEmROM2, rEm8) to the surface of chSP-DCs increased the production level of IFN- γ and IL-12 cytokines. Typically, the IL-10 production from mature DCs mediates suppression of effector T-cells by Treg-response (Boks et al., 2012). According to the research by Arendt et al. (2016), *Eimeria* challenge birds increased intestinal luminal IL-10, and anti-IL-10 was effective at preventing *Eimeria*-induced decrease in body weight. Another previous study indicated that in the context of *E. maxima* and *C. perfringens* acting synergistically, it can cause severe disease phenotype leading to increase in IL-10 cytokine response (Park et al., 2008). Therefore, the anti-IL-10 Abs did not appear to adversely affect adaptive immunity to an *Eimeria* spp. immunization, and the use of an antibody to IL-10 is a novel method to prevent *Eimeria* infection in poultry, which was reported in the study by Sand et al. (2016). Hence, our findings

demonstrated that IL-10 production did not show the obvious modification in experimental and control groups.

Higher secretions of IL-12 and IFN- γ showed that recombinant antigens could promote Th1-cells-mediated immune response, which may induce the host's immunity during host-parasite interaction. With comparative analysis, IL-12 and IFN- γ secretion were more significant with rEm14-3-3-treated DCs instead of remaining recombinant proteins. Although agreeing to the previous study, TGF- β cytokine involves in the regulation of DCs differentiation (Seeger et al., 2015). The earlier study indicated that TGF- β signaling is essential to regulate DC-mediated immune tolerance in health and disease (Esebanmen and Langridge, 2017). Our finding revealed that secretion of TGF- β cytokine was significantly increased with rEmROM1, rEmROM2, and rEmMIC2 but was nonsignificant with rEm8 and rEm14-3-3 after cocubating with DCs. However, the comparative analysis showed that rEm14-3-3 might significantly upregulate the production of IL-12 and IFN- γ than remaining antigens, which could promote Th1 cell-mediated immune response in the host. To unravel the pleiotropic complex mechanism of TGF- β and effector functions of Th17 expressed by TGF- β on immune cells, further investigation should be permitted.

CONCLUSION

The current observations highlighted that immunostimulatory functions of recombinant antigens of *E. maxima* (rEm14-3-3, rEmROM1, rEmROM2, rEmMIC2, and rEm8) significantly promoted maturation and differentiation of DCs in chicken. With comparative analysis, the results suggested that among 5 stimulatory antigens, rEm14-3-3 is an effective immunogenic constituent and plays a crucial role to induce immunity of the host DCs. This research may contribute a novel nominee (Em14-3-3) to act as an effective subunit-vaccine for stimulating the host's immunity against *Eimeria* infection.

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Conflict of Interest Statement: The authors declare that they have no conflict with competing interests.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.07.028>.

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