Biochemical and Biophysical Research Communications 440 (2013) 437–442

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

An *Eimeria* vaccine candidate based on *Eimeria tenella* immune mapped protein 1 and the TLR-5 agonist *Salmonella typhimurium* FliC flagellin

Guangwen Yin [a,1], Mei Qin [a,1], Xianyong Liu [a,b], Jingxia Suo [a], Xinming Tang [a], Geru Tao [a], Qian Han [c], Xun Suo [a,b], Wenxue Wu [a,b,*]

[a] National Animal Protozoa Laboratory and College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
[b] Key Laboratory of Zoonosis, China Ministry of Agriculture and College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
[c] Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA

ARTICLE INFO

Article history:
Received 12 September 2013
Available online 26 September 2013

Keywords:
Eimeria tenella
Immune mapped protein1
Flagellin
Vaccine

ABSTRACT

Immune mapped protein-1 (IMP1) is a new protective protein in apicomplexan parasites, and exits in *Eimeria tenella*. But its structure and immunogenicity in *E. tenella* are still unknown. In this study, IMPI in *E. tenella* was predicted to be a membrane protein. To evaluate immunogenicity of IMPI in *E. tenella*, a chimeric subunit vaccine consisting of *E. tenella* IMP1 (EtIMP1) and a molecular adjuvant (a truncated flagellin, FliC) was constructed and over-expressed in *Escherichia coli* and its efficacy against *E. tenella* infection was evaluated. Three-week-old AA broiler chickens were vaccinated with the recombinant EtIMP1-truncated FliC without adjuvant or EtIMP1 with Freund's Complete Adjuvant. Immunization of chickens with the recombinant EtIMP1-truncated FliC fusion protein resulted in stronger cellular immune responses than immunization with only recombinant EtIMP1 with adjuvant. The clinical effect of the EtIMP1-truncated FliC without adjuvant was also greater than that of the EtIMP1 with adjuvant, which was evidenced by the differences between the two groups in body weight gain, oocyst output and caecal lesions of *E. tenella*-challenged chickens. The results suggested that the EtIMP1-flagellin fusion protein can be used as an effective immunogen in the development of subunit vaccines against *Eimeria* infection. This is the first demonstration of antigen-specific protective immunity against avian coccidiosis using a recombinant flagellin as an apicomplexan parasite vaccine adjuvant in chickens.

1. Introduction

Eimeria spp. are the most common protozoan pathogens of chickens [1,2]. Infection by *Eimeria* may cause coccidiosis, which results in significant economic losses in the poultry industry. One of the most effective methods in the management of infectious diseases in veterinary practice is through the induction of protective innate and adaptive immunity by vaccination with one or more antigens in combination with an adjuvant.

Immune mapped protein-1 (IMP1) is a newly discovered protein in *Eimeria maxima*, and has been demonstrated to be immunogenic and confer protection against *E. maxima* challenge in chickens [3]. Recently, IMP1 has also been identified as immunoprotective antigens from other apicomplexan parasites, such as *Toxoplasma* and *Neospora* [4,5]. Although the IMP1 gene was also identified in the genome of *Eimeria tenella* [3], there have been no reports evaluating the potential of IMP1 as a vaccine candidate against *E. tenella* infections.

Recent advances in the innate immunity research indicated that pathogen-associated molecular patterns (PAMPs) are promising molecular adjuvants for subunit vaccines. Flagellin, a ligand for toll-like receptor 5 (TLR5), has been shown to be an effective adjuvant for vaccines and immunotherapy [6–8]. As a natural agonist of TLR-5, flagellin is an effective inducer of innate immune effectors such as cytokines and nitric oxide, thereby stimulating the activation of adaptive immune responses [7].

In the present study, we hypothesized that a flagellin may enhance immunogenicity of the IMP1 protein thus providing greater immune protection of chickens from *Eimeria* infection. To prove our hypothesis, we over-expressed EtIMP1 and a fusion protein containing EtIMP1 and a *Salmonella typhimurium* flagellin, FliC, investigated the immunogenicity of *E. tenella* IMP1 (EtIMP1) and adjuvant properties of FliC for the EtIMP1 protein and evaluated protective efficacy of the recombinant EtIMP1-FliC fusion protein in chickens.

* Corresponding author at: National Animal Protozoa Laboratory and College of Veterinary Medicine, China Agricultural University, Beijing 100193, China.
E-mail address: labboard@126.com (W. Wu).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.088

2. Materials and methods

2.1. Parasite propagation and purification

Oocysts of *E. tenella* (BJ strain) were propagated and purified according to established protocols [9]. Sporozoites were prepared from cleaned sporulated oocysts by *in vitro* excystation and were purified by chromatography with columns packed with nylon wool and DE-52 cellulose [10,11]. Freshly prepared sporozoites were resuspended in the cytomix buffer supplemented with 2 mM ATP and 5 mM glutathione [12,13].

2.2. Chickens and cell culture

One-day-old Arbor Acre (AA) broiler chickens were purchased from Beijing Arbor Acres Poultry Breeding Co.Ltd. They were housed in isolators and fed with a pathogen-free diet and water. Primary chicken kidney (PCK) cells were prepared from 2-week-old chickens according to an established method with minor modifications [14], and were used for the propagation of *E. tenella* sporozoites in transient transfection experiments *in vitro*. PCK cells were cultured in DMEM medium supplemented with fetal bovine serum (10%, v/v) and 1000U penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 41 °C.

2.3. Cloning of the EtIMP1 gene

Total RNA was isolated from the *E. tenella* sporozoites using the Trizol reagent (Invitrogen, USA). cDNA was synthesized using random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). According to the EtIMP1 sequence of Houghton strain of *E. tenella* (GeneBank Accession number: FN813229.2), the open reading frame of EtIMP1 was amplified by PCR using EtIMP1-F/EtIMP1-R with introduced EcoRI, XhoI and AvrII sites (underlined)(Table 1). PCR products were electrophoresed on 1% agarose gels and the amplified products were extracted from single bands, purified, and cloned into the pEASY-Blunt Simple Cloning Vector (TransGen Biotech, Beijing, China) for sequencing.

2.4. Design of FliC variant

To utilize the adjuvancy of FliC, a FliC variant was designed to reduce the immunogenicity and antigenicity as previously described [15,16]. FliC (Δ180–400), then named as vFliC, was generated by the deletion of the hypervariable domain using the following primers as vFliC-N-F, vFliC-N-R, vFliC-C-F and vFliC-C-R (Table 1). The deleted region was replaced with a short flexible linker (GAPVDPASPW), whose nucleotide sequence was marked in italics in primer vFliC-C-F.

2.5. Expression and purification of recombinant proteins

EtIMP1 was inserted into the EcoRI and XhoI digested expression vector pET-28a (Novagen, Germany) to create pET-28a-IMPI; and then vFliC was inserted into the AvrII and XhoI digested vector pET-28a-IMPI to create pET-28a-IMPI-vFliC. Then the vectors were transformed into *E scherichia coli* for protein expression. The recombinant His_6-tagged proteins were purified from the soluble fraction of the bacterial lysate using the Hi-Trap metal chelating column (GE Healthcare, USA). Identity and purity of the proteins were evaluated by SDS–PAGE in 12% polyacrylamide gels and western blot analysis.

Table 1
Primers used in this study.

Name	Sequence
EtIMP1-F	5′-GAATTCATGGGGGGGGCTTGCGGGA-3′
EtIMP1-R	5′-CTCGAGACCTAGGAGTTGCTGCCGCCACATTTC-3′
vFliC-N-F	5′-CCTAGGATGCATCATCACCATCACCATGCACAAGTCATTA-3′
vFliC-N-R	5′-CAGGATCCACCGGCGCTCCCTTATATTTTTGTTGCACATT-3′
vFliC-C-F	5′-*GGAGCGCCGGTGGATCCTGCTAGCCCATGGGCTGCTACAACCACC*-3′
vFliC-C-R	5′-CTCGAGACGCAGTAAAGAGAGGACGTTTTG-3′

2.6. Plasmid constructs and transfection

To check the localization of EtIMP1 within the parasite, EtIMP1-RFP fusion protein was constructed and expressed as described below. The double expression-cassette plasmid, pH4-EYFP/ACT-IMP1-RFP, was constructed from the pMIC-EYFP/ACT-RFP plasmid [13] by replacing the *E. tenella* microneme 1 promoter with the *E. tenella* histone 4 promotor and the single RFP gene with the IMP1-RFP fusion fragment. For plasmid transfection, five million sporozoites and 10 μg linearized plasmid DNA together with 5 μl SnaBI were subject to Nucleofector transfection [17]. Electroporated sporozoites were inoculated with PCK cells in 25 cm^2 flasks (Corning, Costar, USA). For the *in vivo* experiment, 2×10^6 electroporated sporozoites were inoculated via the cloacal route [17]. Oocysts in faeces excreted at 6–8 days post-inoculation were collected and examined by fluorescence microscopy [13,18,19].

2.7. Vaccination and parasite challenge infection

To test whether EtIMP1-vFliC vaccination confers effective protection against *E. tenella* infection, three-week-old AA broiler chickens were randomly divided into six groups. The first two groups were immunized intramuscularly in the thigh muscle with 100 μg recombinant EtIMP1 emulsified in Freund's complete adjuvant (FCA) (Group 1), and 100 μg EtIMP1-vFliC without adjuvant (Group 2). The challenged control group (Group 3) and unchallenged control group (Group 4) were injected with PBS. The final two groups were respectively, immunized intramuscularly with 100 μg vFliC (Group 5), and 100 μl FCA (Group 6) as the adjuvants controls. Fourteen days after the primary immunization, birds in each group were boosted with similar dose as the primary immunization. Fourteen days after the final immunization, birds in Groups 1, 2, 3, 5 and 6 were challenged with 2000 virulent *E. tenella* oocysts. Sera were collected prior to each immunization and stored at −20 °C for further analysis.

Body weights were measured at 0 and 8 days post-challenge. Faeces from each group was collected separately at 6–8 days post-challenge. Oocyst shedding per bird was determined using McMaster egg counting chamber [18,20]. At 7 days post-challenge, caecal lesions were scored on a graded scale from 0 (none) to 4 (high) in a blinded fashion by two independent examiners as previously described [21].

2.8. Determination of anti-IMP1 antibodies

Chicken IgG was detected by ELISA as previously described [4,18]. Briefly, 96-well microtiter plates were coated with the recombinant EtIMP1 (4 ug/ml) in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C and blocked for 1 h at 37 °C with 5% milk powder (Difco™skim milk, BD) in PBST (PBS containing 0.05% Tween 20). After washing with PBST, sera were added in a dilution of 1:100 and incubated for 1 h at 37 °C. Antigen specific antibodies were detected using rabbit anti-chicken IgG conjugated to horse-radish peroxidase (1:10^4 dilution). The ELISA was developed using TMB and H_2O_2 as substrates, and optical density was read at 450 nm (A450) with an ELISA reader (Bio-TekEL 680, USA).

2.9. IFN-γ ELISPOT assay

Fourteen days after the final immunization, chicken peripheral blood mononuclear cells (PBMC) were used to determine the levels of IFN-γ secretion. PBMC were prepared as described previously [22]. ELISPOT 96-well plates (Multiscreen Assay System, Millipore, USA) were coated with 5 μg/ml mouse-anti-Chichen IFN-γ capture antibody (Biosource International, USA) in PBS (pH 7.4). The plates were blocked for 2 h with 1% BSA. 1×10^6 PBMCs were added to each well and stimulated overnight at 41 °C in 5% CO_2 in the presence of RPMI 1640 (negative control), phytohemagglutinin (PHA, positive control), or EtIMP1 (5 μg/ml). Subsequently, the cells were washed and incubated for 2 h at 37 °C with 1 μg/ml biotinylated detector antibody (Biosource International, USA). Then streptavidin-HRP conjugate (Biosource International, USA) was added to each well and incubated for 1 h at 37 °C. The plates were washed and treated with 100 μl of AEC substrate solution (Dakewei, China) and incubated at room temperature for 20 min in the dark. The plates were then rinsed with distilled water and dried at room temperature. Spots were counted by an automated ELISPOT reader (Bioreader 4000; Bio-sys, Germany). The results were expressed as the number of spot forming cells (SFC) per 10^6 PBMC cells in the ELISPOT experiment.

2.10. Statistical analysis

All data were statistically analyzed by one-way analysis of variance (ANOVA), complimented by post hoc analysis using the Tukey's HSD test. All statistical analyses were processed by the SPSS13.0 Data Editor software (SPSS Inc., Chicago, IL). The differences between groups were considered to be significant if p values were less than 0.05.

3. Results

3.1. Information of EtIMP1 gene and its encoding protein structure

The target cDNA fragment of 1194 bp was obtained from total *E. tenella* sporozoites RNA by PCR and found to encode a protein of 397 amino acids. By sequence alignment, the cDNA sequence showed 97.24% similarity with the EtIMP1, from Houghton strain of *E. tenella* (GeneBank Accession number, FN813229.2). There were thirty nucleotide insertions in our sequence, resulting in ten amino acid insertions between the residues 115 and 124. The amino acid similarity was 97.23% between the two sequences. The sequence has been deposited in GenBank database (Accession number KC215109).

The obtained IMP1 sequence was analyzed on identification/prediction platforms including SignalP3.0 and online databases, and the results showed the absence of a classical signal peptide. A transmembrane domain was predicted by application of four predictive tools (Fig. S1A in Supplemental Material). The N-terminal part (residues 1–160) of EtIMP1 contains most of the predicted low-complexity regions. Low complexity sequences are a strong indication of disorder, therefore it is not surprising that most of the disordered regions (Fig. S1A & B in Supplemental Material) were also predicted in this fragment. This part of EtIMP1 sequence was also predicted to contain coiled-coil regions (Fig. S1A & C in Supplemental Material). A 3D model of the C-terminal part (residues 161–397) of EtIMP1 was also predicted based on two identified homology structures (Fig. S2 in Supplemental Material). Interestingly, both homology structures are flavin mononucleotide (FMN)-dependent reductases, which may provide insight into possible biochemical functions of EtIMP1.

3.2. Identification of recombinant EtIMP1 and EtIMP1-vFliC

The recombinant EtIMP1 and EtIMP1-vFliC proteins were expressed as a His_6-tagged fusion protein in *E. coli* and purified by Ni^{2+}-affinity chromatography. Protein bands at approximately 60 kDa for EtIMP1 and 110 kDa for EtIMP1-vFliC were visualized by SDS–PAGE (Fig. 1A, left panel). Furthermore, the two proteins could be recognized by both sera from *E. tenella*-infected chickens (1:200 dilution) (Fig. 1A, middle right panel) and a mouse anti-His_6 antibody (Fig. 1A, middle left panel).

3.3. Localization of EtIMP1 in sporozoites and oocysts

To localize EtIMP1 within *E. tenella*, a transient transfection strategy was used to express IMP1 tagged with RFP (EtIMP1-RFP), which was constructed by fusing IMP1 in frame to its C-terminus with the RFP gene and inserted into the pH4-EYFP/ACT-IMP1-RFP plasmid. At 24 h post infection, EYFP and RFP were both expressed in sporozoites transfected with the above described plasmid. As shown in Fig. 1B, red fluorescence appeared on the surface of the transfected sporozoite, while green fluorescence was seen in the cytoplasm (upper panel, PCK cell inoculation). In unsporulated and sporulated oocysts (*in vivo* inoculation), RFP and EYFP also appeared on the surface and in the cytoplasm, respectively (Fig. 1B, lower panel). It is indicated that EtIMP1 may be a membrane protein, consistent with the transmembrane domain prediction result.

3.4. Seroconversion in chickens for recombinant EtIMP1-vFliC

Two weeks after the second immunization, compared to the control animals, significantly ($p < 0.01$) increased levels of EtIMP1-specific IgG were present in EtIMP1 and EtIMP1-vFliC -immunized group (Fig. 2A).

3.5. Cellular immunce responses to recombinant EtIMP1-vFliC

As shown in Fig. 2B, chickens immunized with EtIMP1-FliC had a significantly higher number (45 spots/10^6cells) of IFN-γ-producing T cells in response to EtIMP1 protein stimulation, relative to EtIMP1 emulsified in FCA group (5 spots/10^6cells).

3.6. Protective efficacy of EtIMP1-vFliC protein vaccination against E. tenella in chickens

To determine if responses to flagellin fusion proteins could impart protective immunity, body weight gain, caecal lesion score and the oocyst output were evaluated following virulent *E. tenella* infection (Fig. 3). After challenge, chickens vaccinated with the EtIMP1 or EtIMP1-vFliC gained significantly greater body weight ($p < 0.05$) (Fig. 3A), had significantly decreased cecal lesion ($p < 0.05$) (Fig. 3B), and had reduced numbers of oocysts (reduced by 88%) (Fig. 4) compared with chickens in other groups.

Overall, our results indicated that flagellin as a molecular adjuvant can also produce enhanced EtIMP1-specific immune protective against *E. tenella* in chickens, more efficacious than the protective effect of FCA.

4. Discussion

Our findings confirm our hypothesis that flagellin as an adjuvant for cell-mediated immunity improves the immunoprotective effect of IMPI against *Eimeria* infection. IMP1 was a newly discovered protein in *E. maxima*, and was recognized as a highly conserved antigen in apicomplexan parasites [3,4]. Thus, we

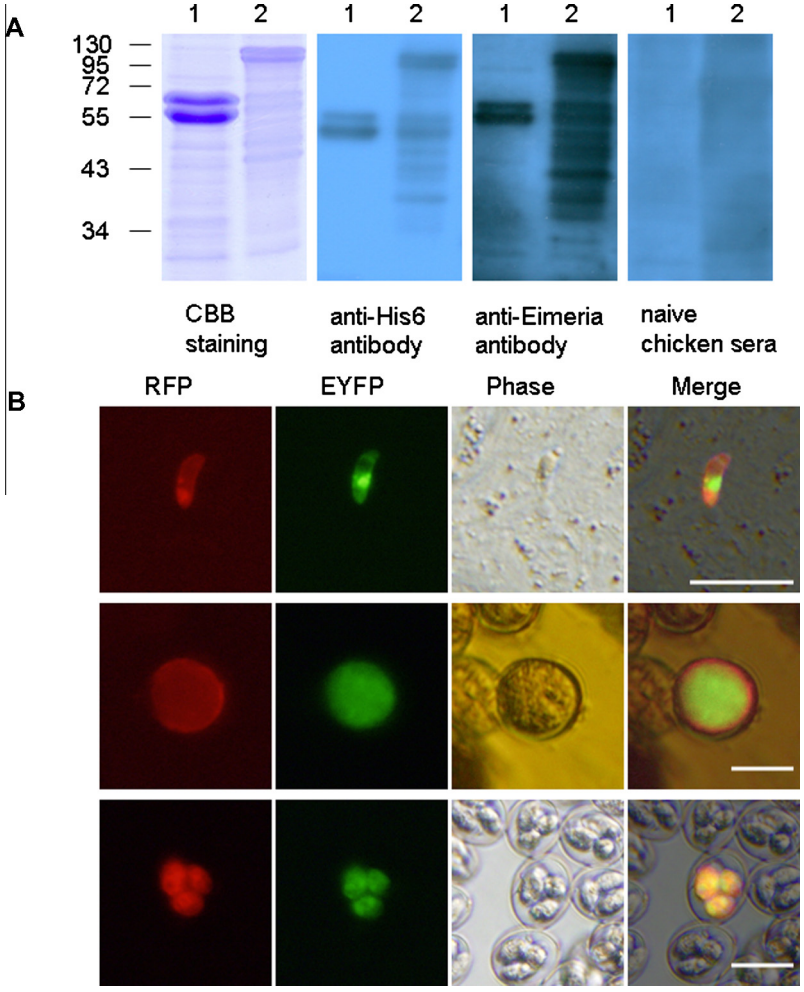

Fig. 1. Expression and localization of EtIMP1. (A) Purified EtIMP1and EtIMP1-vFliC resolved by SDS–PAGE and stained with Coomassie brilliant blue (CBB) (the left first panel). Recombinant EtIMP1and EtIMP1-vFliC were confirmed by western blot. Anti-His_6 monoclonal antibody (the left second panel), sera from *E. tenella*-infected chickens (the right second panel) and were naive chicken sera (the right first panel) used as primary antibody. (B) Localization of the EtIMP1 in *E. tenella*. *E. tenella* sporozoite transfected with the plasmid, pH4-EYFP/ACT-IMP1-RFP for expression of EtIMP1-RFP were inoculated into PCK cells and chickens, respectively. EtIMP1 was showing surface localization in both sporozoite (the upper panel) and unsporulated and sporulated oocysts (lower panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

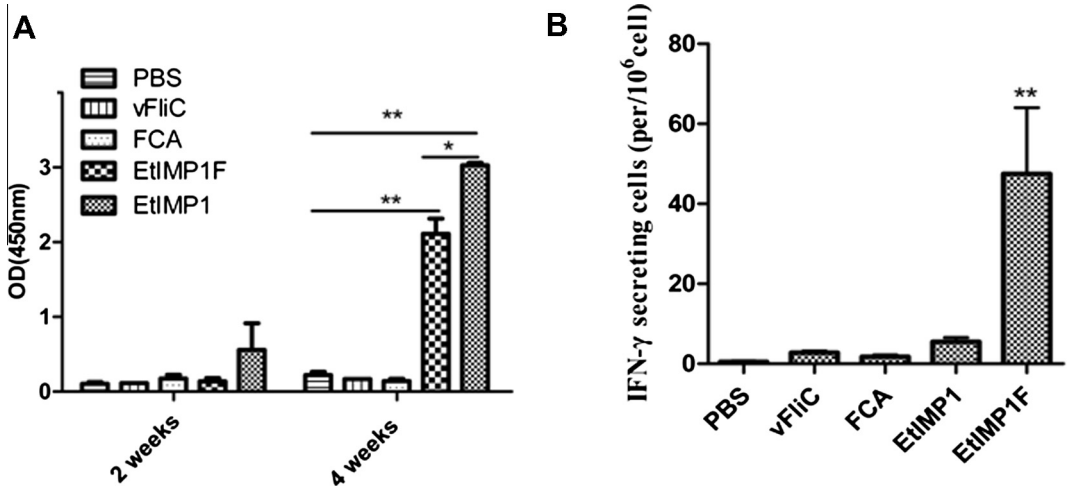

Fig. 2. EtIMP1-vFliC fusion protein induces potent EtIMP1-specific antibody responses and T-cell responses. (A) Antibody responses induced by protein vaccines. Specific anti-IMP1 IgG in the sera of chickens two weeks after immunization twice with EtIMP1, EtIMP1-fliC, vFliC or PBS at a 2-week interval under the same condition. Results are expressed as OD_{450} readings (mean ± S.E, $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$). (B) Antigen-specific cellular responses were examined by the IFN-γ ELISPOT assay. ELISPOT values denote the number of antigen-specific IFN-γ positive spots per 10^6 PBMCs following stimulation with the EtIMP1 protein (mean ± S.E, $n = 6$) ($^{**}p < 0.01$ vs. the control group).

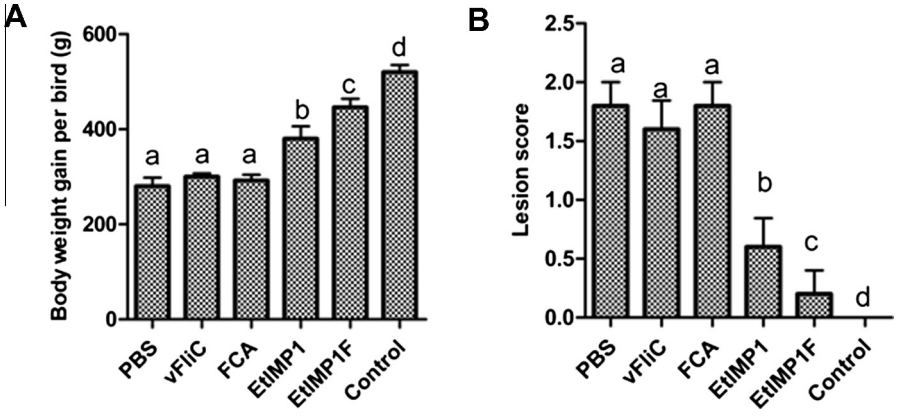

Fig. 3. Efficacy of vaccinations with EtIMP1 and EtIMP1-vFliC. Chickens were immunized with EtIMP1 emulsified in FCA, EtIMP1-vFliC without adjuvant, PBS as the challenged control, and unchallenged controls, or vFliC and FCA as the adjuvants controls, and orally infected with 2000 sporulated oocysts of *E. tenella* 14 days post the second immunization (except for the unchallenged control). Body weight gains between 0 and 8 days post-infection were calculated (A). At 7 days, five chickens from each group were sacrificed for caecal lesion evaluation (B). Bars (mean ± SE) denoted with different letters are significantly different by the Tukey's HSD test ($p < 0.05$).

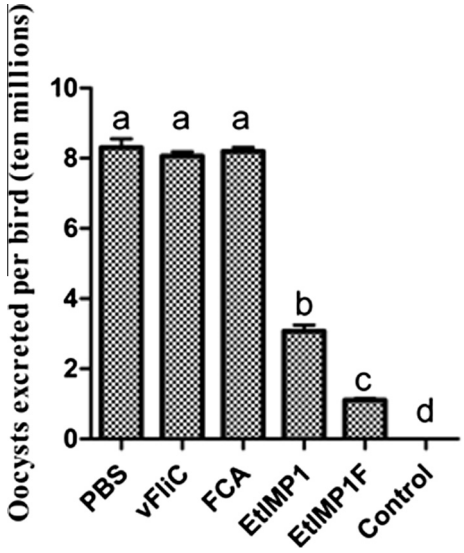

Fig. 4. Oocysts output from PBS, vFliC, FCA , EtIMP1 or EtIMP1-vFliC immunized and *E. tenella* oocysts-challenged birds. Feces from each group were collected between 6 and 8 days post-challenge, and oocyst shedding per gram of feces was determined using a McMaster egg counting chamber. Bars (mean ± SE) denoted with different letters are significantly different by the Tukey's HSD test ($p < 0.05$).

hypothesized that IMP1 from *E. tenella* could also be a candidate protective antigen. In the present study, the EtIMP1 gene was cloned and its protein was over-expressed in *E. coli* cells, with further identification and investigation of protective effect as a vaccine candidate. Immunization of birds with the EtIMP1 protein reduced the oocyst output by 60%, comparable with the protective effects of other antigens, such as EtMIC1, EtMIC11 and profilin [23–25].

The subcellular location of an antigen can influence the immunogenicity and thus protective immune responses of the host. Surface-expressed or secreted antigens generally induce stronger host immune responses than cytoplasmic proteins [26,27]. In studies on the function and localization of the protective asexual-stage antigens in parasite development, it was found that protective antibodies recognized antigens that are mainly localized to the parasite surface or micronemes [28,29]. Our localization study and bioinformatic analysis of EtIMP1 revealed that it may be a membrane protein. The surface location of EtIMP1 suggests that this protein may play an important role in parasite binding or invasion in host cells, and thus antibodies to EtIMP1 could block the function of the protein.

Cell-mediated immunity appears to be the major component of the immune response in eliminating the infection of apicomplexan parasites, including *E. tenella*, but the role of humoral immunity in *E. tenella* infected chickens is ambiguous [30–32]. The vaccination with a recombinant antigen is often not sufficient to elicit a protective immune response against *E. tenella* infection [33–35] . In this study, we demonstrated that the fusion protein of TLR5 ligand, vFliC variant and EtIMP1 reduced oocyst output by 88%, more efficacious than EtIMP1 emulsified in FCA. In addition to having the strong antibody responses, chickens immunized with EtIMP1-vFliC also elicited a specific T-cell response evidenced by a greater IFN-γ production by T-cells in response to EtIMP1 stimulation *ex vivo*. FCA is a standard approved experimental adjuvant, which mainly enhances antibody responses, validated by the higher EtIMPI-specific antibody than EtIMP1-vFliC in this study, while flagellin is a natural agonist of TLR-5, mainly enhancing cell immune responses and produced stronger protective efficacy than FCA adjuvant did. It seems that flagellin, fused to EtIMP1, could increase the uptake and processing of EtIMP1 by antigen processing cells, thus resulting in more efficient stimulation of adaptive immune responses. The effects of flagellin on DCs were demonstrated to be dependent on TLR5 activation [36]. This was consistent with the report that flagellin-EGFP fusion protein can effectively elicit antigen specific T cell responses *in vivo* [37]. Huleatt et al. [38] also reported that immunization of mice with flagellin fusion proteins (STF2.OVA) generated not only high titer antibody responses, but also a potent T-cell response.

In this study we demonstrated that a bacterial flagellin-fused EtIMP1 elicited a stronger protective immune response than EtIMP1 with adjuvant did. This is also consistent with previous studies on the use of recombinant flagellins as vaccine adjuvants with malarial antigens, showing promising results in malaria vaccine development in rodent tests [36,39]. Chickens were used for the first time in this study on the use of a recombinant flagellin as apicomplexan parasite vaccine adjuvants. This demonstrated that flagellins are able to enhance the immunogenicity of antigens and the fusion of flagellins to parasite antigens could be a valuable strategy to improve the immunogenicity of parasite proteins in the stimulation of cell-mediated immunity not only in mammals but also in birds.

In conclusion, our results demonstrate the possibility of feasibly producing an efficacious vaccine against *Eimeria* infection using the novel membrane protein, EtIMP1, fused to flagellin, a molecular

adjuvant, evidenced by significant reduction in the oocyst output, caecal lesion score and body weight decreases in chickens challenged with *E. tenella*. Therefore, EtIMP1-flagellin fusion protein can be used as an effective immunogen in the development of subunit vaccines against *E. tenella* infection, suggesting that flagellin as a subunit vaccine adjuvant in chickens present a more realistic prospect.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (30871862), the National High Technology Research and Development Program of China (2011AA10A209), Yangtze River Scholar and Innovation Research Team Development Program (IRT0945), Research and Innovation Project for College Graduates of China Agricultural University (2013YJ005). We thank Dr. Jin Zhu (University of Canberra, Australian Capital Territory, Australia) for helpful advice and review of the manuscript. All experimentation using chickens was carried out in compliance with the current Chinese legislation and due attention was paid to the welfare of experimental chickens.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.088.

References

[1] X.M. Sun, W. Pang, T. Jia, et al., Prevalence of *Eimeria* species in broilers with subclinical signs from fifty farms, Avian Dis. 53 (2009) 301–305.
[2] M.W. Shirley, A.L. Smith, F.M. Tomley, The biology of avian *Eimeria* with an emphasis on their control by vaccination, Adv. Parasitol. 60 (2005) 285–330.
[3] D.P. Blake, K.J. Billington, S.L. Copestake, et al., Genetic mapping identifies novel highly protective antigens for an apicomplexan parasite, PLoS Pathog. 7 (2011) e1001279.
[4] X. Cui, T. Lei, D. Yang, et al., Toxoplasma gondii immune mapped protein-1 (TgIMP1) is a novel vaccine candidate against toxoplasmosis, Vaccine 30 (2012) 2282–2287.
[5] X. Cui, T. Lei, D. Yang, et al., Identification and characterization of a novel Neospora caninum immune mapped protein 1, Parasitology 139 (2012) 998–1004.
[6] H. Ha, J.H. Lee, H.N. Kim, et al., Stimulation by TLR5 modulates osteoclast differentiation through STAT1/IFN-beta, J. Immunol. 180 (2008) 1382–1389.
[7] S.E. Applequist, E. Rollman, M.D. Wareing, et al., Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin, J. Immunol. 175 (2005) 3882–3891.
[8] S. Agrawal, A. Agrawal, B. Doughty, et al., Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos, J. Immunol. 171 (2003) 4984–4989.
[9] P.L. Long, B.J. Millard, L.P. Joyner, et al., A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis, Folia Veterinaria Latina 6 (1976) 201–217.
[10] L. Jiang, J. Lin, H. Han, et al., Identification and characterization of *Eimeria tenella* apical membrane antigen-1 (AMA1), PloS One 7 (2012) e41115.
[11] D.M. Schmatz, M.S. Crane, P.K. Murray, Purification of *Eimeria sporozoites* by DE-52 anion exchange chromatography, J. Protozool. 31 (1984) 181–183.
[12] M.J. van den Hoff, A.F. Moorman, W.H. Lamers, Electroporation in 'intracellular' buffer increases cell survival, Nucleic Acids Res. 20 (1992) 2902.
[13] G. Yin, X. Liu, J. Zou, et al., Co-expression of reporter genes in the widespread pathogen *Eimeria tenella* using a double-cassette expression vector strategy, Int. J. Parasitol. 41 (2011) 813–816.
[14] T.Y. Shi, X.Y. Liu, L.L. Hao, et al., Transfected *Eimeria tenella* could complete its endogenous development in vitro, J. Parasitol. 94 (2008) 978–980.
[15] F. Liu, J. Yang, Y. Zhang, et al., Recombinant flagellins with partial deletions of the hypervariable domain lose antigenicity but not mucosal adjuvancy, Biochem. Biophys. Res. Commun. 392 (2010) 582–587.
[16] C. Nempont, D. Cayet, M. Rumbo, et al., Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity, J. Immunol. 181 (2008) 2036–2043.
[17] J.D. Clark, K. Billington, J.M. Bumstead, et al., A toolbox facilitating stable transfection of Eimeria species, Mol. Biochem. Parasitol. 162 (2008) 77–86.
[18] X. Huang, J. Zou, H. Xu, Y. Ding, et al., Transgenic *Eimeria tenella* expressing enhanced yellow fluorescent protein targeted to different cellular compartments stimulated dichotomic immune responses in chicken, J. Immunol. 187 (2011) 3595–3602.
[19] X. Liu, J. Zou, G. Yin, et al., Development of transgenic lines of *Eimeria tenella* expressing M2e-enhanced yellow fluorescent protein (M2e-EYFP), Vet. Parasitol. 193 (2013) 1–7.
[20] S.H. Lee, H.S. Lillehoj, R.A. Dalloul, et al., Influence of Pediococcus-based probiotic on coccidiosis in broiler chickens, Poult Sci. 86 (2007) 63–66.
[21] J. Johnson, W.M. Reid, Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens, Exp. Parasitol. 28 (1970) 30–36.
[22] T.S. Dalgaard, L.R.D. Norup, et al., Flow cytometric assessment of antigen-specific proliferation in peripheral chicken T cells by CFSE dilution, Vet. Immunol. Immunopathol. 138 (2010) 85–94.
[23] H.S. Lillehoj, X. Ding, R.A. Dalloul, et al., Embryo vaccination against *Eimeria tenella* and *E. acervulina* infections using recombinant proteins and cytokine adjuvants, J. Parasitol. 91 (2005) 666–673.
[24] K. Sathish, R. Sriraman, B.M. Subramanian, et al., Plant expressed EtMIC2 is an effective immunogen in conferring protection against chicken coccidiosis, Vaccine 29 (2012) 9201–9208.
[25] B.M. Subramanian, R. Sriraman, N.H. Rao, et al., Cloning, expression and evaluation of the efficacy of a recombinant *Eimeria tenella* sporozoite antigen in birds, Vaccine 26 (2008) 3489–3496.
[26] J. Hess, I. Gentschev, D. Miko, et al., Superior efficacy of secreted over somatic antigen display in recombinant Salmonella vaccine induced protection against listeriosis, Proc. Natl. Acad. Sci. USA 93 (1996) 1458–1463.
[27] H. Shen, J.F. Miller, X. Fan, et al., Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity, Cell 92 (1998) 535–545.
[28] A.J. Trees, M.J. Karim, S.B. McKellar, et al., *Eimeria tenella*: local antibodies and interactions with the sporozoite surface, J. Protozool. 36 (1989) 326–333.
[29] D. Jahn, A. Matros, A.Y. Bakulina, et al., Model structure of the immunodominant surface antigen of *Eimeria tenella* identified as a target for sporozoite-neutralizing monoclonal antibody, Parasitol. Res. 105 (2009) 655–668.
[30] B.S. Bhogal, E.B. Jacobson, H.Y. Tse, et al., Parasite exposure elicits a preferential T-cell response involved in protective immunity against Eimeria species in chickens primed by an internal-image anti-idiotypic antibody, Infect Immun. 57 (1989) 2804–2810.
[31] H.S. Lillehoj, J.M. Trout, Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites, Clin. Microbiol. Rev. 9 (1996) 349–360.
[32] M.E. Rose, P. Hesketh, Immunity to coccidiosis: T-lymphocyte- or B-lymphocyte-deficient animals, Infect Immun. 26 (1979) 630–637.
[33] M.S. Crane, B. Goggin, R.M. Pellegrino, et al., Cross-protection against four species of chicken coccidia with a single recombinant antigen, Infect Immun. 59 (1991) 1271–1277.
[34] M.C. Jenkins, Progress on developing a recombinant coccidiosis vaccine, Int. J. Parasitol. 28 (1998) 1111–1119.
[35] G.A. Miller, B.S. Bhogal, R. McCandliss, et al., Characterization and vaccine potential of a novel recombinant coccidial antigen, Infect Immun. 57 (1989) 2014–2020.
[36] A.G. Camacho, L.H. Teixeira, D.Y. Bargieri, et al., TLR5-dependent immunogenicity of a recombinant fusion protein containing an immunodominant epitope of malarial circumsporozoite protein and the FliC flagellin of Salmonella Typhimurium, Mem. Inst. Oswaldo Cruz. 106 (Suppl 1) (2011) 167–171.
[37] C. Cuadros, F.J. Lopez-Hernandez, A.L. Dominguez, et al., Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses, Infect Immun. 72 (2004) 2810–2816.
[38] J.W. Huleatt, A.R. Jacobs, J. Tang, et al., Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity, Vaccine 25 (2007) 763–775.
[39] D.Y. Bargieri, I.S. Soares, F.T. Costa, et al., Malaria vaccine development: are bacterial flagellin fusion proteins the bridge between mouse and humans?, J Parasitol. Res. 2011 (2011) 965369.