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Enhancement of the immunogenicity of an infectious laryngotracheitis virus DNA vaccine by a bicistronic plasmid encoding glycoprotein B and interleukin-18

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ABSTRACT

A DNA vaccine against infectious laryngotracheitis virus (ILTV) can induce specific humoral and cell-mediated immunity. However, compared to conventional vaccines, DNA vaccines usually induce poor antibody responses. To determine if co-expression of a cytokine can result in a more potent ILTV DNA vaccine, immunogenicity and protective efficacy of a monocistronic vector encoding the glycoprotein B (gB) of ILTV was compared to that of a bicistronic vector separately encoding the gB and chicken interleukin-18.

Humoral and cellular responses induced by the DNA vaccines administered to the quadriceps muscle of chickens were evaluated. There were significant differences in antibody levels elicited by either monocistronic or bicistronic DNA vaccines as determined by ELISA. The percentages of $CD3^+$, $CD3^+CD8^+$ and $CD3^+CD4^+$ subgroups of peripheral blood T-lymphocytes in chickens immunized with the bicistronic DNA vaccine were higher than those in chickens immunized with monocistronic DNA vaccine. When chickens were challenged with a virulent CG strain of ILTV, the protective efficacy was enhanced significantly after immunization with the bicistronic DNA vaccine. These results demonstrated that co-expression of an adjuvant cytokine from a bicistronic DNA vaccine may be an effective approach to increasing ILTV DNA vaccine immunogenicity.

1. Introduction

Infectious laryngotracheitis virus (ILTV) is a member of the Alphaherpesvirinae subfamily of the Herpesviridae family which causes serious respiratory disease worldwide in chickens which affects growth and egg production and may lead to death of the animals. The acute phase of infection lasts between 1 and 2 weeks and is often associated with clinical signs such as respiratory depression, gasping, coughing, expectoration of bloody mucus, conjunctivitis, and high mortality and decreased egg production. Subsequently, an asymptomatic latent infection of the central nervous system can be established (Fuchs et al., 2007). For prevention of disease chickens are immunized with attenuated live-virus vaccines that are suitable for mass application via eye drops, aerosol, or drinking water. As immunogenicity of ILTV is usually correlated with its virulence, almost all modified live ILTV vaccines do not remain sufficiently attenuated and have shown a variety of side effects including spread of vaccine virus to nonvaccinated animals, occurrence of long term "carrier" birds, and increasing virulence during in vivo passages (Dufour-Zavala, 2008). Therefore, a new generation of ILTV vaccines is needed.

Since plasmid immunization was first found to be feasible in 1990 (Wolff et al., 1990), the development of DNA-based vaccines as a potentially safe alternative to live vectors has extensively been explored. DNA immunization is an important vaccination strategy that has many desirable characteristics for an ideal vaccine, including induction of broad immune responses, long-lasting immunity and simple and cheap production. Experimental DNA vaccines against viral, bacterial, and parasitic disease have been described (Tacket et al., 1999; Strugnell et al., 1997; Kalinna, 1997), and DNA vaccines have been licensed for two non-human applications: one for West Nile virus for horses (Powell, 2004) and the other for infectious hematopoietic necrosis virus for salmon (Lorenzen and Lapatra, 2005). DNA vaccines now have become a major focus in the vaccine field. However, DNA vaccines in animals generally induce low antibody levels which can only partially protect from challenge with a lethal dose of pathogen. Therefore, there is an urgent need for enhancing immunogenicity and protection by vaccines. Many studies have shown that immunogenicity of an antigen can be enhanced by cytokines (Gurunathan et al.,

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2000; Thalhamer et al., 2001; Barouch et al., 2004; Stevenson, 2004; Barouch, 2006; Zhang et al., 2007; Tang et al., 2008; Tovey and Lallemand, 2010). Interleukin-18 (IL-18), previously known as interferon-gamma (IFN-γ) inducing factor (IGIF), is an important cytokine with multiple functions in innate and acquired immunity (Dinarello and Fantuzzi, 2003; Marshall et al., 2006). Plasmids expressing IL-18 have been investigated as potential vaccine adjuvants in several studies and have been shown to increase protective immunity by DNA vaccine against pathogens (Chen et al., 2004; Yoon et al., 2006; Mingxiao et al., 2007; Yin et al., 2009).

Glycoprotein B (gB) is the most highly conserved herpesvirus structural glycoprotein and has been demonstrated to be a important target of cell-mediated and humoral immune responses and to confer protective immunity to ILTV. gB has also been investigated as a candidate antigen for recombinant subunit vaccines (York and Fashey, 1991; Tong et al., 2001; Sun et al., 2008). Therefore, we constructed a bicistronic plasmid encoding gB and chicken interleukin-18 and evaluated its immunogenicity and protective effect in chickens. We showed that the delivery of this bicistronic plasmid can accelerate specific antibody induction with an increase T-cell response. The use of a bicistronic vector may enable more efficient delivery of both antigen and cytokine in DNA vaccination and promote synergistic responses.

2. Materials and methods

2.1. Virus, experimental animals

Specific-pathogen-free (SPF) chicken embryos were purchased from the Shangdong Institute of Poultry Science, Shandong, PR China. Chickens were hatched and housed in a specific-pathogen-free environment at the Laboratory Animal and Resources Facility, Henan Agricultural University. In October 2006, a clinical CG isolate of ILTV was recovered from the tracheas of approximately 31-week-old chickens from a chicken farm in China showing respiratory depression, coughing, expectoration of bloody mucus, conjunctivitis, and decreased egg production. The CG isolate of ILTV was inoculated onto chorioallantoic membranes of 10-day-old SPF embryonated chicken eggs, and then chorioallantoic membranes were harvested 168 h post-inoculation. The 50% egg infection dose (EID_{50}) was determined by inoculating serial 10-fold dilutions of virus into 10-day-old SPF embryonated chicken eggs.

2.2. Construction of monocistronic and bicistronic plasmids

The pIRES bicistronic plasmid (BD Biosciences Clontech, USA) has the multiple cloning sites (MCS) located on either side of the internal ribosome entry site (IRES) from the encephalomyocarditis virus (EMCV) and was used to construct monocistronic and bicistronic DNA vaccines. The chicken IL-18 gene was amplified by PCR from the plasmid pGEM-IL-18 reported previously (GenBank accession No. AY775780) as a template and primers (sense: 5′-GACGTCGACATGAGCTGTGAAGAGATC-3′; antisense: 5′-TATGCGGCCGCTTATAG GTTGTGCCTTT-3′. *Sal*I and *Not*I restriction enzyme sites are underlined on the sense and antisense primers, respectively). The DNA fragment yielded in the PCR was digested with *Sal*I and *Not*I, and then inserted into the plasmid pIRES, between the restriction sites of *Sal*I and *Not*I to produce the plasmid pIRES-IL18.

A DNA fragment encoding the full-length gB gene was amplified from the DNA of the ILTV CG strain (GenBank accession No. DQ812546) as a template. A pair of primers (forward primer 5′-GGTACTCGAG ATGGCTAGCTTGAAA-3′ and reverse primer 5′-TGGTACGCGTTTATTCGTCTTCG CT-3′. *Xho*I and *Mlu*I restriction enzyme sites are shown by an underline on the sense and antisense primers, respectively) specific for the gB gene of ILTV were employed. PCR products digested with *Xho*I and *Mlu*I were cloned into the vector pIRES between the restriction sites of *Xho*I and *Mlu*I to give rise to the plasmid pIRES-gB. Meanwhile, the ILTV gB gene digested with *Xho*I and *Mlu*I was ligated into similarly digested pIRES-IL18 to create the bicistronic plasmid pIRES-gB/IL18. Confirmation of the recombinant plasmids was performed by double enzyme digestion and DNA sequencing.

2.3. In vitro expression of plasmid DNA

Six-well tissue culture plates were seeded with chicken embryo fibroblasts (CEF) cells (10^6/well) (Wang et al., 2009), and the cells were grown until they were about 80% confluent. The purified plasmids, pIRES-gB, pIRES-gB/IL18 and pIRES were transfected into the CEF cells with Lipofectamine ™ 2000 Reagent according to the manufacturer's instructions (Invitrogen, CA, USA). The expression products were identified after 36–72 h.

2.3.1. Reverse transcription -polymerase chain reaction (RT-PCR) analysis

The transfected cells were harvested after 36 h and total cellular RNA was prepared from the transfected cells by using TRIZOL reagent (Gibco BRL, USA). The reverse transcription (RT) reaction was performed using 20-μl volumes; the reaction mixture contained 5× Strand buffer, 25 mM of each deoxynucleoside triphosphate (dNTP; Amersham Biosciences Corp., Piscataway, NJ, USA), 2.5 U of RNase inhibitor (Promega Corporation, Madison, WI, USA), 50 pmol/ml random hexamers, Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 5 μl of total cellular RNA and diethyl pyrocarbonate (DEPC)-treated water. RT was performed at 42 °C for 60 min and at 75 °C for 10 min. Polymerase chain reactions (PCR) were then performed with specific primer sets for the gB and IL-18 genes as described above.

2.3.2. Indirect immunofluorescence analysis

The medium was aspirated 48 h after transfection, and the cells were washed twice with phosphate-buffered-saline (PBS), fixed with 100% acetone for 10 min at −20 °C, then washed three times for 5 min each with PBS. Thereafter, transfected cells were incubated at 37 °C for 1 h with ILTV-specific chicken antiserum at a dilution of 1:100. The cells were washed twice for 5 min each with PBS and incubated for a further 1 h at 37 °C with the secondary FITC-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, USA). After washing twice with PBS, the cells were analyzed for the expression of the recombinant plasmid by fluorescence microscopy.

2.3.3. Western blotting analysis of cells transfected with the recombinant plasmids

The biological activities for chicken IL-18 proteins expressed by the recombinant DNA plasmids pIRES-gB/IL18 and pIRES-IL18 were confirmed by Western blotting. Transfected CEF cells were harvested 72 h after transfection. The total cellular lysates were prepared with lysis buffer (10 mM Tris–Cl pH 7.4, 1 mM $MgCl_2$, 0.5% NP40, 20 μg/ml DNase I) and electrophoresed through an SDS–12% polyacrylamide gel. The proteins were then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked in TBS-T (25 mM Tris–HCl, 125 mM NaCl, 0.1% Tween-20, pH 8.0) containing 5% skim milk for 2 h at 37 °C. After washing five times in TBS (25 mM Tris–HCl, 125 mM NaCl, pH 8.0), the membranes were incubated with rabbit anti-chicken IL-18 antibody (The chicken IL-18 antiserum was prepared in our laboratory from New Zealand white rabbits immunized with recombinant chicken IL-18 protein expressed in *Escherichia coli*) at a dilution of

1:100. After washing three times with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (1:3000, Southern Biotechnology Associates Inc., Birmingham, USA) in TBS-T containing 5% skim milk. Tetramethylbenzidine (TMB) substrate (Promega, New Jersey, USA) was added for color development.

2.4. Immunization of chickens with plasmid DNA vaccines

The plasmids pIRES-gB/IL18, pIRES-gB, and pIRES were amplified in *Escherichia coli* DH5α and prepared using an Endofree Plasmid Maxi Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's protocol. The DNA was dissolved in endotoxin-free phosphate-buffered saline (PBS, pH 7.2) and stored at −20 °C until used for immunization. For vaccination, 100 21-day-old chickens were randomly divided into five groups ($n = 20$ each). Chickens in group 1 were injected with 150 μg of plasmid pIRES-gB. Chickens in group 2 were given 150 μg of plasmid pIRES-gB in combination with 150 μg of plasmid pIRES-IL18. Chickens in group 3 were given 150 μg of plasmid pIRES-gB/IL18. Other groups included chickens administered with 150 μg of empty vector pIRES (group 4), and chickens injected with 0.5 ml PBS only (group 5). All groups were inoculated intramuscularly into the quadriceps muscle at 21 days and boosted with an equivalent dose at 2 weeks after the initial inoculation.

2.5. Detection of anti-ILTV-specific antibodies

Five blood samples from each group were collected via wing vein puncture at 0, 1, 2, 3, 4, 5, and 6 weeks after initial immunization. Sera were stored at −20 °C for serologic analysis. Total serum immunoglobulinG (IgG) specific for ILTV was measured by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Wang et al., 2009), with slight modifications: ELISA plates were coated with ILTV lysate at 5 μg/ml in carbonate buffer, pH 9.6, overnight at 4 °C and blocked with 10% non-fat dried milk in PBS at room temperature for 3 h. Serum samples were tested at 1:20 dilution in 10% dried milk in PBS containing 0.25% Tween-20 (PBST). After washing three times with PBST, IgG against ILTV was detected with HRP-labeled goat-anti-chicken conjugate diluted 1:2000 in PBST. The substrate solution used was TMB microwell peroxidase. After 15 min of incubation in the dark, the reaction was stopped by the addition of 100 μl of 2 M H_2SO_4, and the optical density at 450 nm was measured in an ELISA microplate reader. Sera were run in duplicate. Negative and positive control sera were included in each assay. Total serum IgG specific for ILTV are represented by the optical density.

2.6. Analysis of $CD4^+$, $CD8^+$ and $CD3^+$ T-lymphocytes

Five peripheral blood samples from each group were collected via wing vein puncture in 2.5 ml syringes pre-loaded with 0.2 ml of sodium heparin to prevent clotting at 0, 1, 2, 3, 4, 5, and 6 weeks after the vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample by Ficoll–Hypaque density gradient centrifugation. PBMCs were adjusted to 1×10^7 cells/ml. For each sample, 3 tubes containing the following combinations of monoclonal antibodies were set up: 10 μl of mouse anti-chicken CD3-SPRD and 10 μl of mouse anti-chicken CD4-PE; 10 μl of mouse anti-chicken CD3-SPRD and 10 μl of mouse anti-chicken CD8a-PE; and 10 μl of mouse IgG1-FITC and 10 μl of mouse IgG1-PE (BD Biosciences Pharmingen). Each tube received 50 μl of PBMCs suspension, and the contents were gently mixed and then incubated at 4 °C for 20 min in the dark. Then 500 μl of PBS was dispensed into each tube, gently mixed, and left for 10 min at room temperature out of direct light. After 10 min of centrifugation at 800 × *g*, the cells were resuspended with 500 μl of PBS solution, and the percentages of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T-lymphocytes in the PBMCs suspension were determined by flow cytometry (model EPICSXL, American Beckman Coulter, Fullerton, CA).

2.7. Virus challenge experiment

All of the chickens were challenged intratracheally with 100 EID_{50} of the virulent ILTV strain CG in 0.2 ml at 15 days after the boost immunization. For further detection of virus, tracheal swabs were collected every day after challenge and placed in 0.6 ml PBS. The challenged chickens were examined daily for signs of clinical illness such as coughing, sneezing, ataxia, dyspnea or death for 2 weeks. Dead chickens were necropsied to confirm death by ILTV infection. The challenged chickens generally began to show clinical signs from 2 to 10 days after challenge. Chickens in each group were euthanized at 14 days post-infection. Necropsies were performed immediately postmortem and tracheal swabs were also collected for detection of virus.

2.8. Detecting of virus in the tracheas by PCR

The virus in the tracheal swabs of the challenged chickens was detected by PCR. Viral DNA was extracted using a commercial test kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions and subjected to PCR using primers directed to the thymidine kinase (TK) gene (forward primer: 5′-GGGAAACTTGAATGTCGGGAG-3′; reverse primer: 5′-TGG ATTATACGCCGTGCC TGT-3′). Two-μl samples of the supernatant containing extracted DNA were used as the PCR templates. The amplifications were performed in 25-μl reaction mixtures each containing 10.5 μl H_2O and 12.5 μl *Taq* Premix DNA polymerase (TaKaRa, Dalian, China). The reactions were run in a PTC-200 Peltier thermal cycler (MJ Research, USA) under the following conditions: one cycle at 94 °C 3 min, followed by 25 cycles of denaturation at 94 °C for 15 s, primer annealing at 53 °C for 30 s, and extension at 72 °C for 30 s. The PCR was ended with a final extension step at 72 °C for 8 min. The PCR amplification products were analyzed by agarose gel electrophoresis using 1% agarose stained with ethidium bromide and visualized under UV light.

2.9. Statistical analysis

Data were analyzed using the one-sided Student's *t* test. Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Construction of monocistronic and bicistronic DNA vaccines

The monocistronic and bicistronic DNA vaccine plasmids encoding ILTV gB and chicken IL-18 were constructed using the bicistronic plasmid pIRES as shown in Fig. 1. The DNA sequencing results confirmed that the correct sequences of gB and IL-18 were present in the constructed plasmids.

3.2. Transcription of recombinant plasmids in CEF cells

To confirm the transcription of the gB and IL-18 genes from the constructs pIRES-gB/IL18 and pIRES-gB in a eukaryotic system, the plasmids were transfected into CEF cells. Total RNA was extracted from transfected cells at 36 h and analyzed by RT-PCR for the presence of each corresponding mRNA. The predicted RT-PCR products were of 2.6 kb in size for gB and 0.6 kb for chicken IL-18, all of which were confirmed by gel electrophoresis. No specific band of a similar size was seen in any of the mRNA samples in the absence of reverse

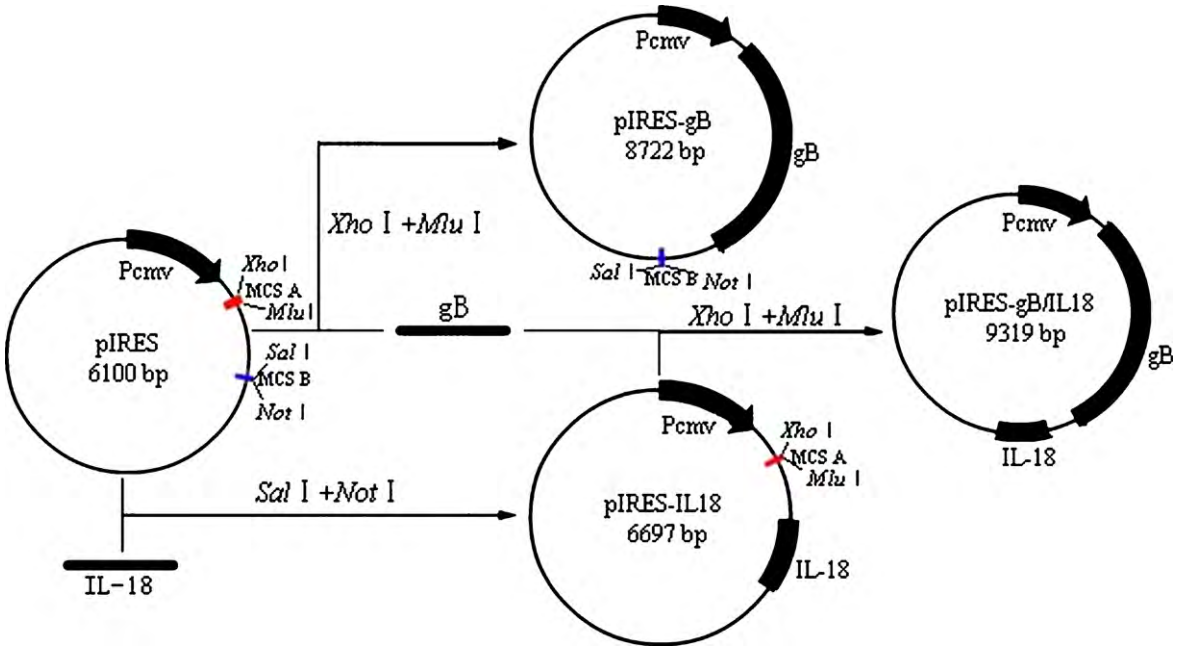

Fig. 1. Strategy for construction of the monocistronic and bicistronic DNA vaccines.

transcription (Fig. 2). The results were further confirmed by DNA sequencing performed on the PCR products. Both PCR and sequencing results showed that the constructs encoding gB and IL-18 genes can be transcribed successfully in the eukaryotic system.

3.3. Expression of recombinant plasmids in CEF cells

The ability of the recombinant DNA plasmids pIRES-gB/IL18 and pIRES-gB to express their gB antigens was evaluated by an indirect immunofluorescence assay in CEF transfected cells. After transfection using Lipofectamine, the cells displayed positive signals for the expressed proteins located in the cytoplasm, where an obvious green fluorescence signal could be observed (Fig. 3A and B) with the pIRES-gB/IL18 and pIRES-gB constructs, whereas no specific fluorescence was detected in control cells transfected with the plasmid pIRES (Fig. 3C). This result showed that the two plasmids could successfully express their target exogenous genes and that the proteins could be recognized by antibodies against ILTV.

In order to demonstrate the appropriate expression of chicken IL-18 protein, transfected CEF cells were harvested 72 h after transfection and the cellular lysates were prepared. The chicken IL-18 proteins were separated by SDS-PAGE and electrophoret-

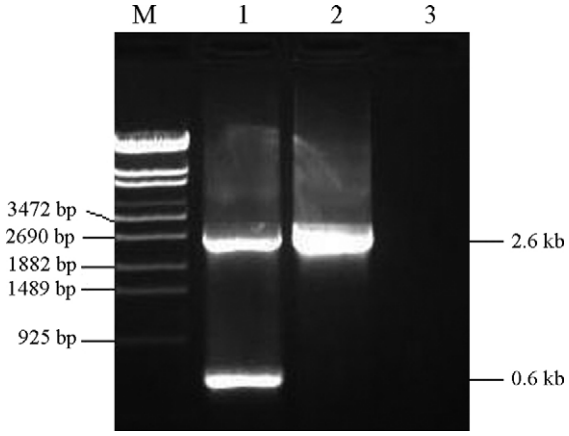

Fig. 2. Detection the transcription of the plasmids by RT-PCR. Total RNA was extracted from CEF cells 36 h after transfection with pIRES-gB/IL18, pIRES-gB. The templates used for RT-PCR were as follows: lane M, DNA marker λ-EcoT14; lane 1, plasmid pIRES-gB/IL18; lane 2, plasmid pIRES-gB; lane 3, negative control.

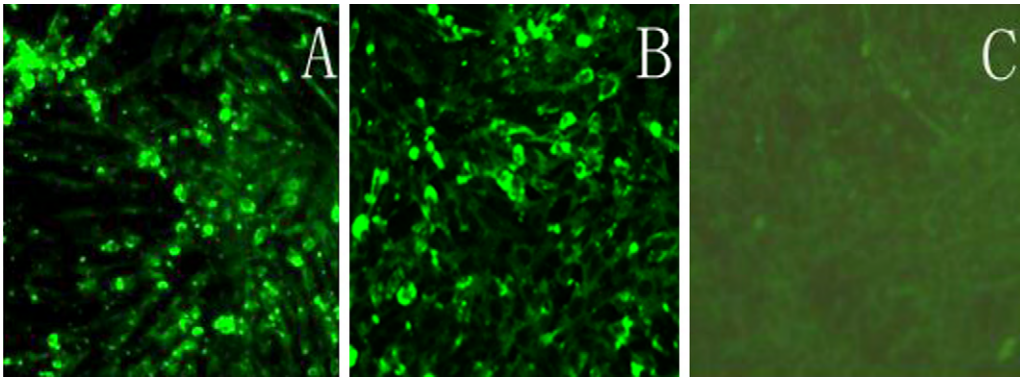

Fig. 3. Indirect-immunofluorescence detection of expressed gB proteins in CEF cell. (A) Cells transfected with the pIRES-gB/IL18 plasmid showed positive results, (B) cells transfected with the pIRES-gB plasmid showed positive results and (C) cells transfected with the pIRES plasmid showed negative results. Scale bar, 30 μm.

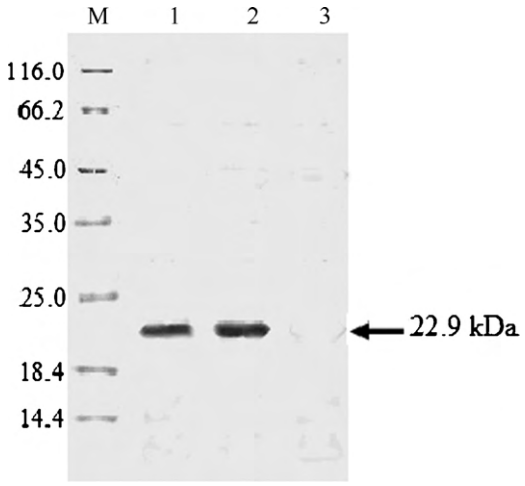

Fig. 4. Western blotting analysis of chicken IL-18 proteins expressed in transfected CEF cells. Lane 1, low molecular weight protein marker; lane 2, CEF cells transfected with pIRES-gB/IL18; lane 3, CEF cells transfected with pIRES-IL18; lane4, CEF cells transfected with pIRES-gB. Arrow at the right indicates the position of chicken IL-18 polypeptide.

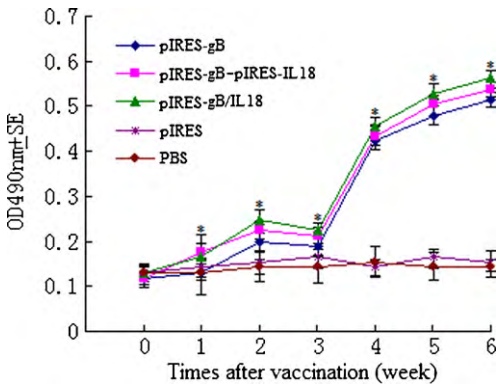

Fig. 5. Detection of antibodies in different vaccine inoculated groups by ELISA ($n = 5$, i.e. number of times the test was repeated). Groups of chickens ($n = 2$) were immunized with 150 μg pIRES-gB alone or in combination with 150 μg pIRES-IL18 at 3 and 5 weeks; chickens in group 3 were given 150 μg of plasmid pIRES-gB/IL18; pIRES-immunized chickens were used as a negative control; PBS-immunized chickens were used as a blank control. Five blood samples from each group were collected via wing vein puncture at 0, 1, 2, 3, 4, 5, and 6 weeks after initial immunization. Antibody was detected by indirect ELISA assay. Values are expressed as mean counts ±standard error. A value > 2.1 was considered as positive by calculating the absolute ratio of post/nai¨ve serum. Statistically significant differences ($P < 0.05$) are indicated by * (compared with negative control or blank control).

ically transferred onto a nitrocellulose membrane. The proteins were probed by a rabbit anti-chicken IL-18 antibody followed by an HRP-conjugated goat-anti-rabbit IgG. The expected band with molecular weight of 22.9 kDa was detected by Western blotting in extracts of cells transfected with pIRES-gB/IL18 and pIRES-IL18. CEF cells transfected with pIRES-gB did not show chicken IL-18 protein expression (Fig. 4).

3.4. Humoral responses to ILTV in chickens vaccinated with recombinant plasmids

To evaluate humoral responses, the antigen-specific antibodies were detected using indirect ELISA. The plasmids pIRES-gB/IL18 and pIRES-gB induced detectable antibodies to ILTV Ag in chickens one week after the initial injection, and compared with the group of chickens receiving PBS and the pIRES plasmid, the levels of IL-18 or gB were significantly increased with subsequent vaccination (Fig. 5; $P < 0.05$). However, the anti-ILTV antibody level of chickens immunized with pIRES-gB/IL18 was slightly higher but not significantly different ($P > 0.05$) than that of chickens immunized with pIRES-gB alone.

3.5. Cellular immune responses induced by recombinant plasmid vaccination

Peripheral blood lymphocytes were analyzed by flow cytometry at 0, 1, 2, 3, 4, 5, and 6 weeks after the vaccination. The percentages of $CD3^+$, $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes of the DNA vaccine-vaccinated groups were significantly greater than those of the groups inoculated with pIRES or PBS ($P < 0.05$). The percentages of $CD3^+$, $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes were significantly higher ($P < 0.05$) in chickens immunized with pIRES-gB/IL18 than in those of the pIRES-gB group (Fig. 6).

3.6. Protection after challenge

Morbidity, mortality, tracheal infection and percent protection after challenge of chickens are summarized in Table 1. Chickens that started to show clinical signs or died from viral infection did so beginning on day 2 after challenge. The chickens immunized with either the plasmid pIRES or PBS were not protected and developed coughing, nasal discharge, expectoration of bloody mucus, conjunctivitis, and dyspnea. Morbidity of the pIRES-gB/IL18

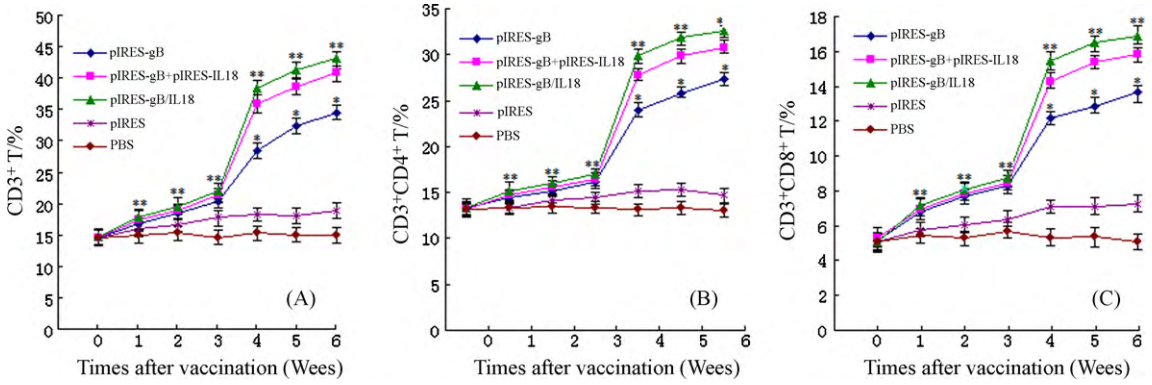

Fig. 6. The percentage of $CD3^+$, $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes of different vaccines inoculated groups ($n = 4$, i.e. number of times the test was repeated). Groups of chickens ($n = 2$) were immunized with 150 μg pIRES-gB alone or in combination with 150 μg pIRES-IL18 at 3 and 5 weeks; chickens in group 3 were given 150 μg of plasmid pIRES-gB/IL18; pIRES-immunized chickens were used as a negative control; PBS-immunized chickens were used as a blank control. Five peripheral blood samples from each group were collected via wing vein puncture at 0, 1, 2, 3, 4, 5, and 6 weeks after initial immunization. Values are expressed as mean counts ±standard error. Statistically significant differences ($P < 0.05$) are indicated by * (compared with negative control or blank control) or ** (compared with pIRES-gB alone).

Table 1
Mortality and protection rate after challenge with the virulent CG strain of ILTV.

Groups	pIRES-gB	pIRES-gB + pIRES-IL18	pIRES-gB/IL18	pIRES	PBS
Morbidity (%)[a]	25 (5/20)	15 (3/20)	5(1/20)*	100 (20/20)	100 (20/20)
Mortality (%)[b]	5 (1/20)	5 (1/20)	0 (0/20)*	65 (13/20)	75 (15/20)
Detectable ILTV in tracheal[c]	6/20	5/20	4/20*	20/20	20/20
Protection rate (%)[d]	70	75	80*	0	0

Statistically significant differences ($P < 0.05$) are indicated by * (compared with pIRES-gB alone).
[a] Morbidity was recorded for each day after challenge and is presented as total number of chickens with any clinical signs in each group.
[b] Mortality was recorded for each day after challenge and is presented as total number of dead chickens in each group.
[c] Detectable ILTV in the tracheal swabs samples was determined by PCR positive bird from dead and euthanized chickens' tracheas.
[d] A bird which was not showing any clinical signs and a negative result for PCR was defined as a protected one. Percent protection was determined by the number of unaffected chickens/total number of chickens.

DNA immunized chickens was only 5%, significantly lower than that of the chickens injected with the pIRES-gB DNA vaccine (25%) ($P < 0.05$). The death rate of the pIRES- and PBS-immunized chickens was 65% and 75% for 14 days after challenge with ILTV, respectively. None of these chickens immunized with the pIRES-gB/IL18 DNA vaccine had died after challenge, whereas 1 of 20 of the chickens injected with the pIRES-gB DNA vaccine had died ($P < 0.05$).

To evaluate the level of protective response after challenge, the tracheal swabs were analyzed by PCR which indicated that 20% and 30% of birds vaccinated with the pIRES-gB/IL18 and pIRES-gB plasmids were positive for the presence of virus in the tracheas, respectively. All chickens immunized with either control vector pIRES or PBS were positive by the PCR test.

A bird which did not show any clinical signs and a negative result for PCR was defined as being protected. The percent of protection in the group that was vaccinated with the pIRES-gB alone was higher than that of the empty vector pIRES or PBS. The group vaccinated with the pIRES-gB/IL18 DNA vaccine (80%) had the highest protection rate among all vaccinated groups. Thus, there was a significant difference in protection rates between the pIRES-gB and pIRES-gB/IL18 groups ($P < 0.05$) suggesting that the plasmid expressing both glycoprotein B and chicken IL-18 offers enhanced resistance against a virulent ILTV challenge.

4. Discussion

DNA vaccination alone is limited in that it often generates only weak immune responses, particularly the cellular response, in the absence of suitable adjuvants. Some cytokines such as IL-1 (Park et al., 2006), IL-2 (Saade et al., 2008), IL-12 (Sin et al., 1999), IFN (Moraes et al., 2003), colony-stimulating factor and TNF, as immunomodulators, have been reported to be effective in animal models or clinical tests. Among the large array of cytokines, IL-18 was initially identified as a potent IFN-γ-inducing factor. IL-18 mRNA is expressed in a wide range of cells, including various types of immune competent cells and non-immune cells (Schijns, 2000; Pollock et al., 2003; Wienhold et al., 2005; Li et al., 2006). Similar to IL-12, the dominant function of IL-18 is to facilitate Th1 immune responses.

In our study we chose to test IL-18 as an adjuvant for the gB antigen expressed from a DNA vector vaccine. We hypothesized that co-expression of the two molecules from the same plasmid might be advantageous. The plasmid pIRES enabled the simultaneous translation of the two genes of interest from the same RNA transcript. Each gene was cloned into one of the multiple cloning sites on either side of the IRES of EMCV. The entire construct is under control of the cytomegalovirus (CMV) immediate-early promoter allowing the expression of two individual proteins from one plasmid. The plasmid pIRES has successfully been used in the construction of DNA vaccine to express protective foreign genes from various pathogens, including infectious bronchitis virus, coxsackievirus and Salmonella typhimurium (Henke et al., 2004; Xu et al., 2007; Tang et al., 2008). Thus, the recombinant bicistronic plasmids pIRES-gB/IL18 and monocistronic pIRES-gB plasmid vaccines were constructed, and our experimental immunization strategy was tested against ILTV. These recombinant plasmids were inoculated in chickens and tested in a protection-challenge experiment, demonstrating that vaccination with the co-expression plasmid pIRES-gB/IL18 can induce stronger immune responses than vaccination with pIRES-gB. Thus, it seems that vaccination with a bicistronic DNA vaccine expressing both ILTV gB protein and IL-18 may elicit a potent immune response.

The $CD4^+$ and $CD8^+$ subsets of T-lymphocytes (both also marked by CD3) are the key cells of cell-mediated immune activity. The $CD4^+$ T-lymphocytes (T-helper cells) can induce and enhance the immune response by secreting cytokines. $CD8^+$ T-lymphocytes (cytotoxic cells) can mediate cytotoxic killing of target cells (Xie et al., 2007). Hence, in order to evaluate the induction of T-cell responses by the recombinant plasmids, peripheral blood lymphocytes were analyzed by flow cytometry. The results indicated that the percentage of $CD3^+$, $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes in pIRES-gB/IL18 vaccinated chickens group were higher than those in the pIRES-gB vaccinated chickens. This demonstrated that chicken IL-18 has the ability to stimulate T-cell growth. The percentages of $CD3^+$, $CD4^+CD3^+$and $CD8^+CD3^+$ T-lymphocytes of the DNA vaccinated groups were significantly greater than those of the groups inoculated with pIRES or PBS ($P < 0.05$). It has been shown that cell-mediated immunity to ILTV is also induced and antigen-specific. It has also been confirmed that cell-mediated immunity to ILTV is believed to be a protective mechanism in ILTV infection.

To investigate the level of protection elicited by pIRES-gB/IL18, vaccinated chickens were challenged with a virulent CG strain of ILTV. Morbidity of the pIRES-gB/IL18 DNA vaccine immunized chickens was only 5%, significantly lower than that of the chickens injected with the pIRES-gB DNA vaccine (25%) ($P < 0.05$). Chickens that received the pIRES-gB/IL18 plasmid DNA were better protected than those administered with the plasmid pIRES-gB. None of the chickens immunized with the pIRES-gB/IL18 DNA vaccine died after challenge, whereas 1 of 20 of the chickens injected with the pIRES-gB DNA vaccine had died. The protection rate of the pIRES-gB/IL18 group was the highest in all the vaccination groups, indicating that the vaccine induced immunity was effective in overcoming the pathogenic effects of the virus. These results suggested that vaccination with the co-expression plasmid of expressing the gB and IL-18 genes conferred increased protection against challenge.

Altogether, our results demonstrated that the group inoculated with pIRES-gB/IL18 displayed stronger cell-mediated immune responses and had better protection against virus challenge than pIRES-gB vaccinated group. This indicated that chicken IL-18 effectively enhances cell-mediated immune responses to some extent. These findings are consistent with the results of other studies, which used IL-18 plasmids as adjuvants in DNA vaccines, and in which IL-18 enhanced the development of Th1-driven antigen-specific T-helper and cytolytic immune responses (Chen et al.,

2004; Salagianni et al., 2007; Yin et al., 2009). Moreover, IL-18 expression plasmid has been shown to have a positive effect upon the magnitude and breadth of the immune response after successive vaccination, particularly with respect to the generation of significant numbers of antigen-specific $CD4^+$ and $CD8^+$ T-cells (Nakanishi et al., 2001; Marshall et al., 2006). Therefore, IL-18 appears to be a broadly effective Th1 adjuvant that could be useful in development of ILTV vaccines. The present study demonstrated that bicistronic DNA vaccine may be an effective approach to increasing ILTV DNA vaccine immunogenicity. Our results showing the induction of both antibody and T-cell responses against the ILTV challenge in chickens demonstrate that the delivery of antigens and cytokines via bicistronic vectors is feasible in the chicken model. However, whether this type of vaccination (DNA) can be utilized in the poultry industry needs to be investigated further.

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