**Construction of recombinant baculovirus vaccines for Newcastle Disease Virus
and an assessment of their immunogenicity**

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Abstract

Newcastle disease (ND) is a lethal avian infectious disease caused by Newcastle disease virus (NDV) which poses a substantial threat to China's poultry industry. Conventional live vaccines against NDV are available, but they can revert to virulent strains and do not protect against mutant strains of the virus. Therefore, there is a critical unmet need for a novel vaccine that is safe, efficacious, and cost effective. Here, we designed novel recombinant baculovirus vaccines expressing the NDV *F* or *HN* genes. To optimize antigen expression, we tested the incorporation of multiple regulatory elements including: (1) truncated vesicular stomatitis virus G protein (VSV-GED), (2) woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), (3) inverted terminal repeats (ITRs) of adeno-associated virus (AAV Serotype II), and (4) the cytomegalovirus (CMV) promoter. To test the *in vivo* efficacy of the viruses, we vaccinated chickens with each construct and characterized the cellular and humoral immune response to challenge with virulent NDV (F48E9). All of the vaccine constructs provided some level of protection (62.5-100% protection). The F-series of vaccines provided a greater degree of protection (87.5-100%) than the HN-series (62.5-87.5%). While all of the vaccines elicited a robust cellular and humoral response subtle differences in efficacy were observed. The combination of the WPRE and VSV-GED regulatory elements enhanced the immune response and increased antigen expression. The ITRs effectively increased the length of time IFN- γ , IL-2, and IL-4 were expressed in the plasma. The F-series elicited higher titers of neutralizing antibody and NDV-specific IgG. The baculovirus

system is a promising platform for NDV vaccine development that combines the immunostimulatory benefits of a recombinant virus vector with the non-replicating benefits of a DNA vaccine.

Keywords: Newcastle disease virus; *F* gene; *HN* gene; Baculovirus expression vector system; Immunogenicity

1. Introduction

Infectious diseases, including Newcastle disease (ND), cause major economic hardship in the poultry industry. ND is caused by Newcastle disease virus (NDV; Maas et al., 2003), and is characterized by acute morbidity and high mortality (Lam et al., 2011). A means of controlling the spread of NDV is an urgent unmet need in the global poultry industry.

Current strategies for preventing ND utilize inactivated and attenuated vaccines. However, it is always possible that immune failure can occur and there will be a resurgence in virulent NDV (Kattenbelt et al., 2006). The F protein is one of the major protective antigens in NDV (White et al., 2008; Yin et al., 2006). It acts as a fusion protein and contributes to viral adsorption (Lamb et al., 2007). The HN protein is the other major protective antigen in NDV. The HN protein combines hemagglutinin (HA) and neuraminidase (NA) activities (Takimoto et al., 2002). Previous studies have achieved a 100% rate of protection by immunizing chickens using a recombinant NDV vaccine containing the *F* and *HN* gene using the avian paramyxovirus type III virus (APMV 3) as the vector (Kumar et al., 2011). Similarly, a subunit vaccine developed by Lee et al. using recombinant F and HN protein elicited a good immune response, and the protection rates were 100% and 80%, respectively (Lee et al., 2008). However, it is likely that conventional vaccines will be replaced by genetically engineered vaccines.

Compared with other recombinant expression systems, the baculovirus system has distinct advantages. For example, it can accommodate large fragments of exogenous

genes (Sakaguchi et al., 1998) and post-translationally modify products without causing cytotoxic effects (Li et al., 2009). In addition, baculovirus systems can express multiple genes simultaneously at high levels (Mahonen et al., 2007). The expressed products also retain their biological activity (Hu, 2008). Most notably, the baculovirus expression system is generally considered a very safe way to express exogenous genes.

The baculovirus system has been modified in many different ways to optimize the expression of exogenous genes. For example, mammalian cell promoters such as simian virus 40 (SV40), cytomegalovirus (CMV), and CMV early enhancer/chicken β -actin promoter (CAG) are utilized to optimize the efficiency of exogenous gene expression (Hu, 2006). The CMV promoter is a particularly strong promoter that controls expression from recombinant baculovirus expression platforms in mammalian and poultry cells (Krishnan, 2000). The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) added to the 3' untranslated region of the expressed gene can also improve the expression efficiency of target gene expression (Donello et al., 1998; Mahonen et al., 2007). The transduction efficiency of the baculovirus system can be increased by displaying a truncated vesicular stomatitis virus G protein (VSV-GED) (Kaikkonen, 2006) on the surface of the baculovirus. Finally, inverted terminal repeats (ITRs) from AAV extend the length of time that target genes are expressed *in vivo*. Sustained expression has been observed for up to 90 days *in vivo* from a CMV expression cassette containing adenovirus ITRs (Wang et al., 2006). Thus modified baculovirus systems have the

potential to produce large amounts of recombinant proteins in a sustained manner, suggesting they could be an ideal platform for NDV vaccine development.

The aim of this study was to investigate the effects of the VSV-GED, WPRE, and ITRs regulatory elements on expression of the NDV *F* and *HN* genes controlled by the CMV promoter in a recombinant baculovirus vaccine for NDV. To assess the efficacy of the vaccine we assessed the humoral and cellular immune response *in vitro* to the F and HN proteins in the presence and absence of each regulatory element. We also assessed the level of target protein expression. Finally, the *in vivo* efficacies of the constructs were tested *in vivo*, and the humoral and cellular immune response to vaccination was characterized.

2. Materials and methods

2.1. Ethics Statement

All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of the National Institute of Infectious Diseases (NIID, Japan). Experimental protocols were reviewed and approved by the Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and the Animal Ethics Committee of Heilongjiang Province (SYXK (H) 2006-032).

2.2. Virus, plasmids, and cells

The virulent NDV strain F48E9 was purchased from the China Veterinary Microbiology Culture Collection. The plasmids pLM(-), pLM, pLM-ITRs,

pTYL-HA-F, and pNDV-HN were obtained from the Key Laboratory of Microbiology in the College of Life Science at Heilongjiang University. The F and HN genes were obtained from plasmids pTYL-HA-F and pNDV-HN using Xho I / Sal I and Xho I / Sph I, respectively. Plasmid pLM(-) contains the CMV promoter and simian virus 40 (SV40) poly(A) ; Plasmid pLM contains the elements of WPRE, VSV-GED, CMV promoter, SV40 poly(A) and gp64 signal peptide (gp64sp) sequence; Plasmid pLM-ITRs contains the elements of ITRs, WPRE, VSV-GED, CMV promoter, SV40 poly(A) and gp64sp sequence. The chicken embryo fibroblast cells and Sf9 insect cells were maintained in our laboratory.

2.3. Construction of baculovirus vectors

Six (6) plasmids were constructed: (1) pLM(-)-F, (2) pLM-F, (3) pLM-ITRs-F, (4) pLM(-)-HN, (5) pLM-HN and (6) pLM-ITRs-HN. The *F* or *HN* genes were individually amplified with primers that also inserted a the His tag (The forward primer for the *F* gene was 5'-ATCCTCGAGATGGGCTCCAGACCTTCTACC-3'.

The reverse primer for the *F* gene was:

5'-GGCGTCTGACTCAATGATGATGATGATGAT

GCATTTTGTAGTGGCTCTCATC-3'. The forward primer for *HN* was:

5'-ATCCTCGAGATGGACCGCGCAGTTAGC-3' and the reverse primer for *HN*

was: 5'-CGGGCATGCCTAATGATGATGATGATGATGATGACCAGACCTGGCTTATCT

AACCTAT-3'). F and HN genes were inserted into the plasmids pLM(-), pLM and

pLM-ITRs with *Xho* I / *Sal* I and *Xho* I / *Sph* I endonuclease, respectively.

The *E.coli* DH10 Bac competent cells were prepared using the SEM method (Rong et

al., 2002). The plasmids (pLM(-)-F, pLM-F, pLM-ITRs-F, pLM(-)-HN, pLM-HN and pLM-ITRs-HN, pLM(-), pLM, and pLM-ITRs) were transformed into *E.coli* DH10 Bac competent cells. Positive colonies were identified by blue-white screening and the recombinant Bacmid (rBac) DNA was extracted using the alkaline lysis method. The recombinants were identified as rBac-LM(-)-F, rBac-LM-F, rBac-LM-ITRs-F, rBac-LM(-)-HN, rBac-LM-HN, and rBac-LM-ITRs-HN, rBac-LM(-), rBac-LM, and rBac-LM-ITRs (Fig. 1) by PCR amplification with the M13 primer (CGCCAGGGTT TTCCCAGTCACGAC).

2.4. Cell culture

Sf9 insect cells were cultured in suspension at 27°C in Sf900II SFM medium containing 10% FBS (Gibco, CA, USA) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a 0 mL volumes. Primary avian cells were cultured in a 6-well plate at 37°C in 11-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The primary avian cells were prepared according to a standard protocol (Spector et al., 1988) and were maintained in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, USA) supplemented with 10% FBS (Gibco, CA, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

A third passage (P₃) baculovirus (section 2.5) was used to infect chicken embryo fibroblasts (CEFs) at 80% confluence using a multiplicity of infection (MOI) of 100 in the presence of 10mM sodium butyrate in 2 mL of DMEM for 12h. The media containing virus was replaced by fresh DMEM containing 10% FBS and the cells were incubated for an additional 48 h. The cells were pelleted by centrifugation and

washed three times with PBS then lysed using 200 μ L/well cell lysates buffer (Beyotime, Shanghai, China) in a 6-well culture plate.

2.5. Generation and titering of recombinant baculovirus

Sf9 cells were transfected with the individual baculovirus transfer vectors, such as rBac-LM(-)-F, rBac-LM-F, rBac-LM-ITRs-F, rBac-LM(-)-HN, rBac-LM-HN, and rBac-LM-ITRs-HN, rBac-LM-ITRs, rBac-LM(-), rBac-LM and rBac-LM-ITRs using the liposome-mediated method (Whitt et al., 2001). The co-transfection supernatants were collected after 72 h culture. The viruses were passaged 3 times in Sf9 cells to obtain high titer viral stocks. The viruses were allowed to infect the Sf9 cells (2×10^6 /mL) at room temperature for 30 min, then cultured at 27°C with agitation (70 r/min). The culture supernatants from infected cells were collected once 80% of the cells had been infected. The same process were repeated to obtain second and third passage virus. The viral genome was extracted and amplified with the M13 universal primer, and each reverse primer, to confirm that the target genes were correctly inserted into the recombinant baculoviruses. The recombinant baculoviruses were named BV-LM(-)-F, BV-LM-F, BV-LM-ITRs-F, BV-LM(-)-HN, BV-LM-HN, BV-LM-ITRs-HN, BV-LM-ITRs, BV-LM(-), BV-LM and BV-LM-ITRs, respectively. The titre of the baculovirus stocks were measured by plaque assay (Burlison et al., 1992). Briefly, Sf9 cells were plated in 6-well plates, and 10-fold serial dilutions of the virus stocks were added to the cells. Viruses and cells were allowed to interact for 1 h before the viruses were removed, and the cell monolayers were overlaid with plaquing medium. The cells were incubated for 8 days, stained

with neutral red, and the numbers of plaques present at each dilution were counted.

Viral titers were shown in Table 1.

2.6. Expression of the F and HN proteins from chicken embryo fibroblasts

The recombinant proteins were detected by SDS-PAGE using a 4% stacking gel and 12% separation gel. The expression of recombinant proteins was determined by Western blot. An equivalent volume (30μL) of recombinant protein was loaded in each well of the SDS-PAGE gel. A rabbit anti-His tag antibody (1/100 dilution) was the primary antibody, and a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1/500 dilution) was the secondary antibody (Bioss, Beijing). The Western blot strips were analyzed using a Biology Software Gel-Pro analyzer 4.5 (Media Cybernetics, US). BV-LM(-), BV-LM and BV-LM-ITRs were used as controls. The expression levels of the target protein were compared between baculoviruses.

2.7. Chicken immunization

The SPF chickens were bred and immunized at the Harbin Veterinary Research Institute. Chickens were housed separately in sterile isolators and provided with standard food and water. The health of the chickens was monitored daily. Fourteen (14)-day old chickens were randomly divided into ten groups containing eight chickens in each group (80 total chickens). The chickens were randomly assigned to be immunized with baculoviruses containing the F protein (cohort A: BV-LM(-)-F, B: BV-LM-F, and C: BV-LM-ITRs-F), the HN protein (D: BV-LM(-)-HN, E: BV-LM-HN, and F: BV-LM-ITRs-HN), or a combination (G: BV-LM-ITRs-F+BV-LM-ITRs-HN). The empty vector (H: BV-LM-ITRs) was used

as the vector control. Additional controls were the Lasota commercial vaccine (cohort J; vaccinated control) and PBS (cohort I; unvaccinated control). For the vaccinated groups: the cohorts vaccinated with baculovirus (A-H) received 10^9 pfu recombinant baculovirus and the vaccinated control group (J) received 0.2 mL of the commercial vaccine. The same doses were administered as a boost 14 days after the first immunization. Blood was drawn from each chicken group at days 14, 28, 42, 56 and 70 (Figure 2) to detect their immune response. The timeline was shown in Figure 2. The chickens were challenged with 10^4 TCID₅₀ of virulent NDV F48E9 14 days after the second immunization. The chickens were monitored for clinical symptoms and physiological changes, and the rate of protection was determined.

2.8. Lymphoproliferation assay

Lymphoproliferative responses in peripheral blood mononuclear cells (PBMCs) from vaccinated chickens were measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to assess the cellular immune response to stimulation with NDV F48E9 at 49 days. The peripheral bloods of immunized chickens were collected regularly to prepare chicken peripheral T lymphocytes. ConA and NDV F48E9 were used to stimulate lymphocyte proliferation. 100 μ L of peripheral blood lymphocytes were inoculated into each well of a 96-well plate. RPMI1640 medium (Hyclone, Logan, USA) containing 50 μ L of NDV F48E9 (TCID₅₀= $10^{-4.33}$ /100 μ L) was added to the experimental groups. RPMI1640 medium containing ConA (5 μ g/mL) was added to the positive controls, while only RPMI1640 medium was added to the negative control wells. Each group

was done in triplicate. Cells were cultured at 37°C with 5% of CO₂ for 44h. At 44h, 10 µL of 5 mg/mL CellTiter 96® Aqueous One Solution (Promega) was added to each well and incubated in the dark for 4h. The OD490 values were obtained using a microplate reader (Gene, US) and the stimulation index (SI) of each group was calculated. The calculated stimulation index (SI) was defined as the ratio of the mean counts per minute (cpm) from the wells incubated with NDV F48E9 to the mean cpm of wells incubated with medium alone. Medium alone was used as the negative control and 5 µg/mL concanavalin A (ConA, Sigma) was used as the positive control.

2.9. Serological assays

Blood samples were obtained from six randomly selected chickens in each of the immunized groups 0, 7, 14, 21, 28, 35, 42, 49 and 56 days post the first vaccination. The serum was collected by centrifugation for analysis (Kosaka et al., 1998).

A serum neutralization test was conducted to determine whether the serum was able to neutralize NDV infection *in vitro*. The serum collected from the vaccinated chickens was inactivated at 56°C for 30 min and then serially diluted with PBS in 96-well plates. For the assay, 70 µL of each diluted serum sample was mixed with an equal amount of F48E9 virus suspension (100 TCID₅₀) in a 96-well plate at 37°C in 5% CO₂ for 1 h. The virus/serum mixture was then used to infect 96-well plates of CEFs at 1×10⁶ cells/well at 37°C in 5% CO₂ for 1 h. The controls for the neutralization assay were serum alone and F48E9 virus suspension (100 TCID₅₀) alone. After 1 h, the virus/serum suspension was replaced with DMEM containing 10% FBS and the cells were incubated at 37°C for 96 h in 5% CO₂. The cellular

morphology and cytopathic effects were observed every 12 h. The median protective dose (PD₅₀) was determined using the Reed-Muench method (Tukamoto et al., 2002). The PD₅₀ of each serum sample was calculated, as well as the geometric mean titers (GMT) in each group, and the neutralizing antibody titer. NDV-specific serum IgG levels were determined by ELISA (Abcam, UK). The levels of Interleukin (IL)-2, IL-4, and Interferon- γ (IFN- γ) were determined by ELISA (Abcam, UK), and the concentration was calculated using standard linear regression curves (Meng et al., 2001).

2.10. Statistical analysis

The mean differences between groups were compared and presented as $X \pm SD$ using SPSS Statistics19 software (SPSS, US). Statistical significance was assessed by one-way analysis of variance, $P < 0.05$ was considered significant.

3. Results

3.1. Expression of the NDV F and HN recombinant proteins in CEFs with different regulatory elements

We first assessed whether the F and HN proteins were successfully expressed from the baculovirus expression systems, and compared the effect of different regulatory elements (WBRE, ITRs, VSV-GED) on expression levels of the NDV proteins. The cell lysates from the BV-F-series of recombinant baculovirus transduced primary CEF cells (BV-LM(-)-F, BV-LM-F, and BV-LM-ITRs-F) contained a single band of approximately 55 KDa (Fig. 3A (A)). Similarly, the cell lysates from the BV-HN

(BV-LM(-)-HN, BV-LM-HN, and BV-LM-ITRs-HN) series also contained a single band at approximately 74 KDa (Fig. 3A (B)). The sizes of the bands were consistent with the target proteins F and HN, indicating successful expression from the baculovirus constructs. The protein expression levels from each baculovirus were quantified based on their intensity (Fig. 3B). Compared to the control group BV-LM(-)- F, the expression levels of the F protein from the BV-LM-F and BV-LM-ITRs-F baculoviruses were 3.45 and 3.62 higher, respectively. Similarly, compared to the control group BV-LM(-)-HN the expression levels of the HN protein were 3.99 and 4.60 fold higher from the BV-LM-HN and BV-LM-ITRs-F baculoviruses, respectively. These results indicated that the VSV-GED and WPRE regulatory elements markedly improved the expression of the target protein.

3.2. Testing the in vivo efficacy of the F and HN expressing baculovirus vaccines

To test the *in vivo* protective efficacy of the baculovirus constructs, ten groups (n=8/group) of 14-day old chicks were immunized with the F-series, the HN-series, the combined F-ITRs and HN-ITRs constructs, a commercial vaccine, PBS, or BV-LM-ITRs (as a control); and then challenged with a virulent strain of NDV (F48E9). The vaccine and booster (section 2.7) did not elicit any clinical symptoms in any group.

Following F48E9 challenge, the chickens in groups H (BV-LM-ITRs; empty vector) and I (PBS) appeared depressed, had a suppressed appetite, and passed white stool. In each group, 4 chickens died 3 days post-challenge. In groups A (BV-LM(-)-F), E (BV-LM-HN), and F (BV-LM-ITRs- HN) 1 chicken/group exhibited signs of

depression and eventually died. The same symptoms were also observed in two chickens from group D (BV-LM(-)-HN). There were no obvious clinical symptoms following challenge in groups B (BV-LM-F), C (BV-LM-ITRs-F), G (BV-LM-ITRs-F+BV-LM-ITRs-HN) and J (commercial vaccine) as seen in the survival plot was shown (Figure 4).

Five (5) days after the challenge (47 days after chicken birth), the protection rate in each experimental group was calculated (Table 2). The BV-LM-F, BV-LM-ITRs-F, BV-LM-ITRs-F+BV-LM-ITRs-HN, and commercial vaccine groups all achieved 100% protection, which was markedly higher than the control group of PBS (12.5%) and empty vector group BV-LM-ITRs (12.5%) ($P < 0.05$). Taken together, the results indicated that the recombinant baculovirus vaccines could protect against challenge with a virulent NDV strain. In addition, the protection rate of the F-series immunized groups (mean 95.83%) were higher than the HN-series (mean 75%), suggesting that the F protein elicited stronger protective immunity than the HN protein.

3.3. Assessing lymphoproliferative responses to NDV F48E9 following vaccination

To better understand the mechanism of protection induced by the baculovirus vaccines, we examined the cellular and humoral immune response elicited following challenge. Cellular immunity was measured by assessing the lymphoproliferative responses to F48E9 stimulation in PBMCs collected from vaccinated chickens at 21, 36, 49 and 56 days after the first vaccination. ConA was used as the positive control. The proliferative response to F48E9 and ConA was increased in all of the vaccinated chickens following challenge indicating that the recombinant baculovirus vaccine did

elicit a cellular immune response. Seven (7) days after challenge (49 days after chicken birth) with F48E9, the SI values were highest in the BV-LM-ITRs-F (2.94), BV-LM-ITRs-F+BV-LM-ITRs-HN (2.830), and commercial vaccine (2.73) groups. The SI index in these groups was significantly higher than the BV-LM(-)-F (2.14) and BV-LM(-)-HN (2.190) groups ($P < 0.05$; Fig. 5). Thus, recombinant baculovirus vaccines containing the WPRE and VSV-GED regulatory elements significantly improved the level of cellular immunity.

To determine whether the increased cellular immunity was persistent, we repeated the lymphoproliferative response assay 42 days post the first vaccination (56 days after the chicken birth). The SI values for groups vaccinated with baculovirus constructs containing ITRs (BV-LM-ITRs-F = 2.910; BV-LM-ITRs-HN = 2.212) and the commercial vaccine (2.78) remained significantly higher than the SI values without ITRs (BV-LM-F= 2.06; BV-LM-HN= 1.78; $P < 0.05$). These results indicated that ITRs effectively extended the duration of the cellular immune response to the vaccine. The SI values in the BV-LM-ITRs-F+BV-LM-ITRs-HN group, which provided 100% protection and had one of the highest SI values 42 days post the first vaccination, was slightly lower than the BV-LM-ITRs-F group, but significantly higher than the BV-LM-ITRs-HN group ($P < 0.05$; Fig. 5).

3.4 Neutralizing titer assays

The ability of serum to neutralize virus infection is an important indicator of humoral immunity. We used a serum neutralization assay to determine the residual infectivity of NDV F48E9 after exposure to immune sera to assess the effects of the F or HN

antigen, and the different regulatory elements on humoral immunity. As shown in Figure 6, all of the immunizations elicited peak levels of neutralizing antibodies at 28 days post the first vaccination (42 days after the chicken birth). However, different vector and antigen combinations did not elicit the same neutralizing antibody titer. For example, constructs containing the VSV-GED and WPRE regulatory elements and expressing the same antigen produced higher titers of neutralizing antibodies than the plasmid without the regulatory elements (pLM (-)); for the F-series, the difference in titer of serum neutralizing antibody (GMT) was 1159.64 compared to 413.06, and for the HN-series the difference was 720.17 compared to 383.68 28 days post the first vaccination. Thus, including the VSV-GED and WPRE regulatory elements significantly improved the induction of neutralizing antibodies, consistent with better protection observed in the *in vivo* challenge.

42 days post the first vaccination (56 days after the chicken birth), the levels of neutralizing antibodies from the vectors without ITRs were significantly reduced ($P < 0.05$) compared to the vectors containing the ITRs regardless of F or HN antigen expression. In contrast, vaccination with vectors that contained ITRs elicited neutralizing antibody titers that did not decline significantly 42 days post the first vaccination suggesting that the neutralizing titers elicited by ITRs persisted to 14 days.

Finally, in general the F antigen vaccine series tended to induce a greater humoral immune response in immunized chickens than the HN antigen series, which was consistent with better *in vivo* protection in the F48E9 challenge in chickens

vaccinated with the F-series vaccines.

3.5. Detection of NDV-specific IgG titers in immune serum

The IgG titer is as an important index of humoral immunity in immunized chickens for a response specific to NDV. The BV-LM-F constructs containing the WPRE and VSV-GED regulatory elements had significantly higher NDV-specific IgG titers (1.673) than constructs without the regulatory elements (1.372; $P < 0.05$) 42 days post the first vaccination (56 days after the chicken birth). Similarly, the BV-LM-HN constructs containing the WPRE and VSV-GED elements elicited significantly higher NDV-specific IgG titers than constructs without the regulatory elements (1.265 vs. 1.131; $P < 0.05$; Fig. 7). In addition, ITRs elicited high titers of NDV-specific IgG 56 days post the first vaccination (70 days after the chicken birth) compared to constructs without ITRs (BV-LM-ITRs-F= 1.560; BV-LM-ITRs-HN= 1.173; $P < 0.05$). When the baculovirus constructs containing the same regulatory elements but expressing different antigen (F or HN) were compared to each other, vaccines containing the F antigen elicited more NDV-specific IgG antibodies than the HN gene.

3.6. Cytokine levels in immune serum

The cytokine levels in immune sera can be used to measure the immune state of the host. IFN- γ and IL-2 are secreted by T-helper type 1 (Th1) cells and play important roles in regulating the cellular (T cell) immune response. IL-4 is secreted by Th2 cells and can stimulate B cell proliferation, and antibody production involved in the humoral immune response. Thus, by comparing the levels of cytokine production it is

possible to determine whether the baculovirus constructs are eliciting a predominately Th1 or Th2 response.

The concentration of IFN- γ was significantly ($P < 0.05$) increased at 14 and 28 days post the first vaccination (28 and 42 days after the chicken birth) in the immunized groups compared to the control groups (PBS and BV-LM-ITRs; Fig. 8). The concentration of IFN- γ peaked at 42 days post the first vaccination (56 days after the chicken birth) in all of the immunized groups. The highest concentrations of IFN- γ were observed in commercial vaccine (72.57 ng/mL) and the BV-LM-ITRs-F+BV-LM-ITRs-HN (69.65 ng/mL) groups.

As shown in Figure 9, the mean level of IL-2 increased significantly in all of the groups, except the control groups ($P < 0.05$); providing further evidence that the baculovirus constructs elicited a robust cellular immune response. The levels of IL-2 were significantly higher than the control groups in the commercial vaccine (74.65 ng/mL), BV-LM-ITRs-F (71.26 ng/mL), and BV-LM-ITRs-F+BV-LM-ITRs-HN (63.08 ng/mL) groups 28 days post the first vaccination (42 days after the chicken birth). Different combinations of antigen genes and regulatory elements elicited different levels of IL-2 production. In constructs expressing the same antigen, the levels of IL-2 production were greater when constructs contained the WPRE and VSV-GED regulatory elements than without (F-series: 61.62 ng/mL vs. 33.34 ng/mL; HN-series: 37.69 ng/mL vs. 28.13 ng/mL).

With regard to IL-4, the highest levels of IL-4 were observed in the commercial

vaccine group ($P < 0.05$); followed by the F antigen gene series, BV-LM-ITRs-F+Bv-LM-ITRs-HN, the HN antigen gene series, and the controls (PBS, Bv-LM-ITRs; Fig. 10A). Similar to IFN- γ and IL-2, the levels of IL-4 were highest in the groups vaccinated with baculovirus constructs containing the WPRE and VSV-GED elements.

We also assessed the cytokine levels at 70 days to determine whether the vaccines had any lasting effects on cytokine production. The IFN- γ , IL-2, and IL-4 levels in the Bv-LM-ITRs-F (55.02ng/mL, 44.72 ng/mL, 106.84 ng/mL, respectively) and Bv-LM-ITRs-HN (42.03ng/mL, 21.40ng/mL, 66.74ng/mL, respectively) were significantly ($P < 0.05$) elevated compared to the groups without ITRs (Bv-LM-F: 24.53ng/mL, 17.50ng/mL, 27.21ng/mL; Bv-LM-HN: 20.48ng/mL, 13.85ng/mL, 16.54ng/mL).

Taken together, the cytokine results provided further evidence that the baculovirus constructs elicited a robust cellular and humoral response. In addition, the ITRs consistently improved the magnitude and duration of the cellular and humoral immune response.

4. Discussion

NDV has no effective treatment in part due to the reemergence of virulent strains (Alexander, 2011). Vaccination is the best strategy for preventing and controlling NDV spread (Wu et al., 2006). Here, we have developed a novel baculovirus vaccine series using the F and HN proteins of NDV that efficiently express the

antigen target and elicit a robust immune response. Baculovirus constructs containing the WPRE, VSV-GED, and ITR elements can be directly injected into chickens. The high neutralizing antibody titer, increased IL-4 levels, and increased IFN- γ , and IL-2 levels indicated that the baculovirus vaccine had the dual advantages of recombinant viral vector vaccines and DNA vaccines. The vaccines effectively delivered exogenous antigen to the poultry cells and stimulated the production of humoral and cellular immune responses.

In terms of optimizing the vaccine constructs, the regulatory elements WPRE and VSV-GED increased the amount of antigen protein (F or HN) expressed compared to constructs without these elements. Vaccines containing ITRs also appeared to elicit a longer lasting immunity (up to 70 days) than constructs without ITRs. The SI values (BV-LM-ITRs-F: 2.910 vs BV-LM-F: 2.056; $P < 0.05$) at 56 days, the level of neutralizing antibodies (BV-LM-ITRs-F: 1337.74 vs BV-LM-F: 274.30; $P < 0.05$) at 56 days, and the IgG titer (BV-LM-ITRs-F: 1.560 vs BV-LM-F: 0.635; $P < 0.05$) at 70 days indicated that the ITR elements were helpful to maintain the immune level. In addition, the results of the cytokine levels also demonstrated that the ITRs aided in long-lasting cytokine production. The IFN- γ , IL-2, and IL-4 levels in the BV-LM-ITRs-F (55.02ng/mL, 44.72 ng/mL, and 106.84 ng/mL, respectively) and BV-LM-ITRs-HN (42.03ng/mL, 21.40ng/mL, and 66.74ng/mL, respectively) were significantly ($P < 0.05$) elevated compared to the groups without ITRs (BV-LM-F: 24.53ng/mL, 17.50ng/mL, and 27.21ng/mL; BV-LM-HN: 20.48ng/mL, 13.85ng/mL, and 16.54ng/mL) at 70 days. In other studies, AAV-ITRs have been shown to reduce

expression heterogeneity (Hsiao et al., 2001). Our work supports the benefits of using ITRs. Finally, the F antigen elicited a better immune response and provided a greater degree of protection in the *in vivo* challenge than the HN antigen, which was consistent with previous findings (Kumar et al., 2011). The HN protein series provided the lowest average rate of protection in the *in vivo* challenge (75.0%). Both the F-series (95.8%) and the combination of the F and HN vaccines (100%) provided superior protection. The protection rate of the combination was similar to the live vaccine (Colman et al., 2003).

5. Conclusions

The baculovirus vectors described here have the dual advantages of mimicking natural virus infection by a recombinant viral vector vaccine and the non-replicating characteristic of a DNA vaccine. Therefore, development of genetically engineered vaccines will play an important role in ND prevention and treatment.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

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Fig. 2 Schematic of the Experimental Timeline (since the chicken birth). 14-day old chickens were administered recombinant baculovirus vaccine. The same doses were administered as a boost 14 days after the first immunization. Chickens were challenged with NDV F48E9 14 days after the second immunization.

Fig. 3 Detection of F and HN proteins by Western blot from Cells Transduced with BV-F and BV-HN. Recombinant proteins were detected using a rabbit anti-His tag antibody (1/100 dilution) as the primary antibody and a horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (1/500 dilution) as the secondary antibody. (A) Protein expression using recombinant baculovirus BV-F-series; (B) Protein expression using recombinant baculovirus of BV-HN-series. β -actin was used as control and the molecular weight of F and HN protein were about 55 and 74 KDa. In Fig 3B, the level of protein expression was quantified using the Biology Software Gel-Pro analyzer 4.5 to analyze Western blotting strips. *** indicates a significant difference ($P<0.001$) between the constructs without regulatory elements (BV-LM(-)-F or BV-LM(-)-HN) and the constructs with regulatory elements (BV-LM-F, BV-LM-ITRs-F or BV-LM-HN, BV-LM-ITRs-HN).

Fig. 4 Survival plot of the immunized chickens post challenge with F48E9. The number of survivors post-challenge vaccinated with: A, BV-LM(-)-F; B, BV-LM-F; C, BV-LM-ITRs-F; D, BV-LM(-)-HN; E, BV-LM-HN; F, BV-LM-ITRs- HN; G, BV-LM-ITRs-F+BV-LM-ITRs-HN; H, BV-LM-ITRs; J, vaccinated control; I, unvaccinated control.

Fig. 5 Lymphocyte proliferation at 49 and 56 days. PBMCs were isolated from the blood of chickens one week after infection with NDV F48E9. T cell proliferation in vaccinated chickens was measured in response to F48E9 or concanavlin A (ConA). Bars indicate the mean ($\pm$ SEM) stimulation index calculated for each group of

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Fig. 6 Induction of neutralizing antibodies in the serum following vaccination with recombinant baculovirus vaccines. Serum was isolated from the blood of chickens every two weeks following the initial vaccination with recombinant baculovirus vaccine. (A) The neutralizing antibody titers elicited by the F-series of recombinant baculovirus vaccines; (B) The neutralizing antibody titers elicited by the HN-series of recombinant baculovirus vaccines. Line chart represents the mean ($\pm$ SEM) neutralizing antibody titers calculated for each group of animals (n=8). A) hollow circle: BV-LM(-)-F; solid circle: BV-LM-F; hollow diamond: BV-LM-ITRs-F; solid diamond: Lasota vaccine; hollow triangle: BV-LM-ITRs; solid triangle: PBS. B) hollow circle: BV-LM(-)-HN; solid circle: BV-LM-HN; hollow diamond: BV-LM-ITRs-HN; solid diamond: BV-LM-ITRs-F+BV-LM-ITRs-HN; hollow triangle: PBS; solid triangle: BV-LM-ITRs.

Fig. 7 Serum IgG antibody titers following vaccination with recombinant baculovirus vaccines. IgG antibodies were detected in individual chickens by ELISA. Data are reported as the OD_{450nm} for each sample of immunized serum collected every two weeks. (A) IgG antibody level elicited by the F-series of baculovirus vaccines and the control groups; (B) IgG antibody level elicited by the HN-series baculovirus vaccines and control groups. A) hollow circle: BV-LM(-)-F;

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Fig. 8 Serum IFN- γ concentration following vaccination with recombinant baculovirus vaccines. The IFN- γ concentration present in the blood of chickens was measured by IFN- γ ELISA assay at each time point. (A) IFN- γ concentration in immune serum from chickens immunized with the F-series baculovirus vaccines and the control groups; (B) IFN- γ concentration in immune serum from chickens immunized with the HN-series of baculovirus vaccines and control groups. A) solid circle: BV-LM(-)-F; hollow diamond: BV-LM-F; hollow circle: BV-LM-ITRs-F; solid diamond: Lasota vaccine; solid triangle: BV-LM-ITRs; hollow square: PBS. B) solid circle: BV-LM(-)-HN; hollow circle: BV-LM-HN; hollow diamond: BV-LM-ITRs-HN; solid diamond: BV-LM-ITRs-F+BV-LM-ITRs-HN; solid triangle: BV-LM-ITRs; hollow square: PBS.

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Table 1: Titers of P₃ recombinant baculovirus vaccine strains in Sf9 cells

Virus name-P ₃	Virus titer (pfu/mL)	Dilution	Number of plaques
BV-LM(-)-F	$1.90 \pm 0.26 \times 10^8$	10^{-7}	19
BV-LM-F	$1.11 \pm 0.82 \times 10^9$	10^{-7}	111
BV-LM-ITRs-F	$4.90 \pm 0.41 \times 10^8$	10^{-7}	49
BV-LM(-)-HN	$8.60 \pm 0.97 \times 10^8$	10^{-7}	86
BV-LM-HN	$1.00 \pm 0.34 \times 10^9$	10^{-7}	100
BV-LM-ITRs-HN	$6.10 \pm 0.23 \times 10^8$	10^{-7}	61

Table. 2 Rate of protection following baculovirus immunization and challenge with F48E9

Cohorts	Vaccines	No. deaths after challenge with F48E9					Protection rate
		2d	3d	4d	5d	No. deaths	
						/ total	
						number	
A	BV-LM(-)-F	0	0	0	1	1/8	87.5%
B	BV-LM-F	0	0	0	0	0/8	100%
C	BV-LM-ITRs-F	0	0	0	0	0/8	100%
D	BV-LM(-)-HN	0	0	1	2	3/8	62.5%
E	BV-LM-HN	0	0	1	1	2/8	75.0%
F	BV-LM-ITRs-HN	0	0	0	1	1/8	87.5%
G	BV-LM-ITRs-F+	0	0	0	0	0/8	100%
	BV-LM-ITRs-HN						
J	Lasota attenuated	0	0	0	0	0/8	100%
H	BV-LM-ITRs	1	4	2	0	7/8	12.5%
I	PBS	2	4	1	0	7/8	12.5%