Research paper

Truncated chicken MDA5 enhances the immune response to inactivated NDV vaccine

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ABSTRACT

Melanoma Differentiation-Associated protein 5 (MDA5) is a cytoplasmic sensor for viral invasion and plays an important role in regulation of the immune response against Newcastle disease virus (NDV) in chickens. MDA5 was used as an adjuvant to enhance the humoral immune response against influenza virus. In the current study, truncated chicken MDA5 [1–483 aa, chMDA5(483aa)] expressed by recombinant adenovirus was administered to specific-pathogen-free (SPF) chickens to improve the immune response induced by inactivated NDV vaccine. A total of 156 SPF chickens were divided into six groups, and after two rounds of immunization, the humoral immune response, cell-mediated immune (CMI) response and the protective efficacy of the vaccines against NDV challenge were evaluated. The results showed that co-administration of chMDA5(483aa) expressed by adenovirus increased the NDV-specific antibody response by 1.7 times and chickens received chMDA5(483aa) also gained a higher level of CMI response. Consistently, the protective efficacy of the inactivated NDV vaccine against virulent NDV (vNDV) challenge was improved by co-administrate with chMDA5(483aa), as indicated by the reduced morbidity and pathological lesions, lower levels of viral load in organs and reduced virus shedding. Our study demonstrated that chMDA5(433aa) expressed by adenovirus could enhance the immune efficacy of inactivated NDV vaccine in chickens and could be a potential adjuvant candidate in developing chicken NDV vaccines.

1. Introduction

Newcastle disease (ND) is an acute highly contagious disease which causes severe economic losses in poultry industries around the world. Mortality and morbidity are commonly associated with virulent Newcastle disease virus (vNDV). Newcastle disease virus (NDV), the causative agent of ND, is an enveloped, non-segmented, single-stranded, negative-sense RNA virus with a genome of approximately 15 kb, which encodes six structural proteins, namely, nucleocapsid (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), he-magglutinin-neuraminidase (HN), and RNA dependent RNA polymerase (L). NDV has been reported to infect over 200 bird species, including poultry, waterfowl and wild birds (Gaikwad et al., 2016). Although all NDVs belong to one serotype, at least 18 genotypes have been identified (Snoeck et al., 2013), and they exhibit different virulence among different isolates. Vaccination has been widely applied in the poultry industry to prevent the spread of the disease; however, vaccination failures frequently arise due to the presence of maternally derived antibodies, mismatching of genotypes between vaccine strains and prevalent strains, and continued evolution of NDV (Ganar et al., 2014). Moreover, vNDV strains have been isolated from vaccinated chickens (Diel et al., 2012; Rehmani et al., 2015), indicating that

Table 1
Primers used in the study.

Favorite gene	Primer sequences (5'-3')	Product size (bp)	Reference
ChMDA5	MF:TGTGACCGGCGCCTACGCCACCATGTCGGAGGAGTGCCGAGA	1489	Designed in this study
	MR:GGATCCCGCCCGGGGGAAAGGTGAGGCTGTAAGTCCCA		
IFN-β	F:AGCTCTCACCACCACCTTCTCCT	182	Designed in this study
	R:TGGCTGCTTGCTTCTTGTCCTT		
IL-4	F:GTGCCCACGCTGTGCTTAC	82	Xu et al., 2015
	R:AGGAAACCTCTCCCTGGATGTC		
IFN-γ	F:TGAGCCAGATTGTTTCGATG	152	Sachan et al., 2015
	R:CTTGGCCAGGTCCATGATA		
MHC-II	F:CCACGGACGTGATGCAGAAC	288	Sachan et al., 2015
	R:ACCGCGCAGGAACACGAAGA		
Ch28S	F:GGTATGGGCCCGACGCT	160	Wang et al., 2017
	R:CCGATGCCGACGCTCAT		
NDV P gene	F:CAATAAATCGTCCAATGCTAA	322	Wang et al., 2017
	R:CTCCATCATAGACATCATCGC		

haemagglutination-inhibition (HI) titers of 5 log2 or low are not suitably protective (Kapczynski and King, 2005). In our previous reports, we identified that HI titers of ≥12 log2 are correlated with complete protection against virus challenge and can abrogate all clinical symptoms (Han et al., 2017). However, it is not easy to achieve such high antibody levels through the use of traditional vaccines. MDA5 is a pattern recognition receptor (PRR, recognizing dsRNA) of the RIG-I-like receptor (RLR) family and plays an important role in triggering an innate immune response during infection (Karpala et al., 2011). MDA5 contains two caspase activation and recruitment domains (CARDs) at the N-terminus, an RNA helicase domain and a C-terminus domain (CTD) (Childs et al., 2009). The RNA helicase domain and CTD are responsible for recognition the invasive RNA (Yoandris del et al., 2015), and CARD conveys the recognition signal to the mitochondrial antiviral signaling (MAVS) protein to activate NF-κb pathway and stimulate the production of type I IFN (Kang et al., 2002; Yoneyama et al., 2015; Gebhardt et al., 2017). Chicken MDA5 (chMDA5) has been reported to play an anti-viral role in several chicken-virus infection models, including infection with infectious bronchitis virus (IBV), influenza A virus subtype H9N2 and NDV (Kint et al., 2015; He et al., 2016; Wang et al., 2015; Li et al., 2016). The V protein of NDV, a non-structural protein, acts as an antagonist of the interferon signaling pathway by binding to MDA5 (Steward et al., 1993; Andrejeva et al., 2004; Childs et al., 2007; Takaki et al., 2011; Motz et al., 2013). However, the role of chMDA5 in regulation of immunity during NDV vaccination is currently unknown.

Exogenous expression of the N-terminus of chMDA5 can induce chicken IFN-β (chIFN-β) production in absence of pathogen associated molecular patterns (PAMPs) (Liniger et al., 2012a). Liniger et al. investigated the immune regulation function of chMDA5 (1–483 aa, containing CARD) (Liniger et al., 2012b), and found that 1–483 aa of chicken MDA5 [chMDA5(483aa)] could enhance the humoral immune response to an H5 HA DNA vaccine and could improve the immune protection against H5N1 challenge. In this study, we used chMDA5 as an adjuvant and explored its capacity in elevating cell-mediated immunity and improving the humoral response to NDV vaccination. A recombinant adenovirus expressing chMDA5(483aa) (rAd-chMDA5) was constructed and its effect on improving the efficacy of inactivated NDV vaccine was investigated.

2. Materials and methods

2.1. Embryonated chicken eggs, chickens and ethical statement

Specific-pathogen-free (SPF) embryonated chicken eggs and SPF chickens were purchased from Jinan Sais Poultry Co. Ltd., Jinan, China. The vNDV strain F48E9 was passaged in 9-day-old embryonated chicken eggs and harvested at 24 h post-inoculation by collecting the allantoic fluid. SPF chickens were maintained in separated isolation units (biological security level III) with feed and water ad libitum.

This work was conducted in compliance with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences and The International Council for Laboratory Animal Science. All the animal experiments were approved by the independent Animal Care and Use Committee in Shaanxi Province, China.

2.2. Viruses and cells

The vNDV strain, F48E9, and recombinant adenovirus expressing green fluorescent protein (rAd-GFP) were maintained in our laboratory. HEK293 A cells (ATCC CRL1573) were used for packing, amplification and determining the titer of recombinant replication-defective human adenoviruses serotype5 (Ad5), including rAd-GFP. The chicken fibroblast DF-1 cells were used to assess IFN-β production.

2.3. Construction of shuttle vector and generation of recombinant adenovirus

ChMDA5(483aa) DNA was amplified from the plasmid pCMV-chMDA5-myc (maintained in our laboratory) with primers MF and MR (listed in Table 1) harboring 15 bp recombinant arms. The obtained PCR fragment was inserted into the shuttle vector pHBAd-EF1-MCS-GFP (Hanbio Biotechnology, fused with 3×FLAGs tag) through homologous recombination using a One Step Cloning Kit (Vazyme Biotech Co., Ltd.).

The recombinant shuttle vectors and the adenovirus backbone plasmids pHBAd-BHG were co-transfected into HEK293 A cells with Lipofiter™ (Hanbio Biotechnology). Successfully packed recombinant adenovirus was generated in vitro within 10 days as show cytopathic effect (CPE) in the transfected cells. The recombinant adenovirus was purified three times by picking up single plaque from HEK293 A cells and named as rAd-chMDA5. The virus titer was determined by measuring the 50% tissue culture infective dose (TCID$_{50}$).

2.4. Identification of recombinant adenovirus

2.4.1. Indirect immunofluorescence assay (IFA)

IFA was used to identify the expression of chMDA5(483aa). Briefly, HEK293 A cells were infected with rAd-GFP or rAd-chMDA5 at a multiplicity of infection (MOI) of 5. After 24 h incubation, the cells were washed, fixed with 4% paraformaldehyde (30 min at 25 °C) and incubated with the mouse-anti-FLAG polyclonal antibodies (diluted 1:800) for 1 h at 37°C. Cells were stained with goat anti-mouse IgG H&L (Alexa Fluor®594) (Abcam, ab150116, diluted 1:800) and DAPI (Hoechst33342, Sigma) reagent for 1 h at 37 °C. The cells were then

washed with PBS, and the expression of chMDA5(483aa) was visualized using a fluorescence microscope (OLYMPUS IX73).

2.4.2. Western blot (WB)

HEK293 A cells were infected with rAd-GFP or rAd-chMDA5 at a MOI of 5 and harvested when 50% of the cells showed CPE. Proteins were separated by 12% (w/v) SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with 10% skimmed milk at 4 °C overnight and then incubated with mouse anti-FLAG serum (diluted 1:1000). After washed with PBST (0.05% Tween-20 in PBS), the membrane was incubated with HRP-conjugated rabbit anti-mouse IgG secondary antibody (Abclonal AS025, diluted 1:2000) for 1 h at room temperature. The signals were visualized using enhanced chemiluminescence reagents (PIERCE).

2.4.3. RAd-MDA5 stimulated IFN-β expression in DF-1 cells

The expression of IFN-β was investigated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). DF-1 cells were infected with rAd-GFP or rAd-MDA5 at a MOI of 10. Cells were collected at 36 h post-infection, and the overexpression of chMDA5(483aa) was detected by WB using an anti-FLAG antibody. The total RNA was extracted using TRIzol Reagent, as per the manufacturer's instructions. The first-strand cDNA was synthesized using a HiScript 1 st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.) in a 10 μl reaction system containing 1 μg of total RNA with random oligos and oligo d(T) as primers. qPCR assays were performed using a SYBR Green qPCR kit (Vazyme Biotech Co., Ltd.) in a 20 μl reaction system on a real-time thermocycler system (4-channel; Tianlong, Xi'an). Each sample was analyzed in triplicate. The primers used for detection are listed in Table 1. All data were normalized to the chicken 28S rRNA gene (ΔCt), which is stably expressed in NDV-infected tissues, and compared with a non-infected control (ΔΔCt). The expression level of IFN-β is presented as an n-fold decrease or increase relative to the control.

2.5. Preparation of the inactivated vaccine

The vNDV F48E9 strain was propagated in 9-day-old SPF embryonated chicken eggs. The virus titers were determined by measuring the $TCID_{50}$ on DF-1 cells. The virus in allantoic fluid with a titer of 0.7×10^8 $TCID_{50}$/mL was inactivated in 0.5% formaldehyde for 72 h. The inactivation of the virus was assessed by re-inoculation of the allantoic fluid into 9-day-old SPF embryonated chicken eggs.

2.6. Animal experiment

2.6.1. Chickens immunization

A total of 156 SPF chickens were randomly divided into six groups with 26 in each group; grouping and treatment information is provided in Table 2 and Fig. 1. Immunizations were performed through the intramuscular (IM) route at 7 days old and 21 days old, respectively.

Chickens in group NC were not immunized or challenged. Chickens in group CC, rAd-GFP and rAd-chMDA5 were inoculated with 200 μl PBS, rAd-GFP (containing 5×10^7 $TCID_{50}$ virus) and rAd-chMDA5 (containing 5×10^7 $TCID_{50}$ virus), respectively. Chickens in group InV were given a dose of 200 μl inactivated vNDV F48E9 and those in group rAd-chMDA5 + InV were given a dose of 200 μl inactivated vNDV F48E9 pulsing 200 μl rAd-chMDA5 containing 5×10^7 $TCID_{50}$ virus. RAd-chMDA5 was mixed with the inactivated vaccine by vortex prior to vaccination.

2.6.2. Humoral immune response

Eight blood samples from each group were randomly collected every 7 days post-vaccination until challenge (Fig. 1). The NDV-specific antibody was detected by HI assays according to the OIE standard protocol using 4 haemagglutination (HA) units of homologous antigen (F48E9 NDV virus) (OIE, 2008).

Table 2
Grouping information and chicken treatments.

Groups	Number of birds	Primary Immunization (Day 7)	Boost Immunization (Day 21)	Challenge with 10^4 $TCID_{50}$ vNDV (Day 35)
NC	26	-	-	-
CC	26	PBS	PBS	+
rAd-GFP	26	rAd-GFP	rAd-GFP	+
rAd-chMDA5	26	rAd-chMDA5	rAd-chMDA5	+
InV	26	inactivated NDV vaccine	inactivated NDV vaccine	+
rAd-chMDA5 + InV	26	rAd-chMDA5 and inactivated NDV vaccine	rAd-chMDA5 and inactivated NDV vaccine	+

Note: NC, negative control group; CC, challenge control group; rAd-GFP, immunized with rAd-GFP group; rAd-chMDA5, immunized with rAd-chMDA5 group; InV, immunized with inactivated NDV vaccine group; rAd-chMDA5 + InV, immunized with rAd-chMDA5 and inactivated NDV vaccine group.
–: Chickens were not immunized or challenged, +: Chickens were challenged with vNDV.

2.6.3. Cell-mediated immune (CMI) response

To evaluate the CMI response induced by rAd-chMDA5, three chickens were randomly selected from each group, except group NC, at 3 days-post challenge (dpc) and euthanized to collect spleens for detecting gene expression. The mRNA levels of IL-4, IFN-γ and MHC-II mRNA in spleens at 18 h post-boost immunization were detected by RT-qPCR with primers reported previously (Sachan et al., 2015; Xu et al., 2015; Wang et al., 2017a,b) (Table 1). Total RNA was isolated from 200 μl of tissue homogenates, containing 100 μg tissues and reverse transcription and qPCR assays were performed following previously report (Wang et al., 2017a,b). The qPCR efficiency for the gene-specific primers was confirmed before formal RT-qPCR assays performance as described by Bustin et al. (Bustin et al., 2009). The expression levels of target genes are presented as n-fold decrease or increase to group CC.

2.6.4. Lymphocyte proliferation assay

The lymphocyte proliferation assay was performed to test the stimulated proliferation of lymphocytes. Three anticoagulants were collected from each group at 6 h before challenge, except group NC. The peripheral blood lymphocytes (PBL) were separated with lymphocyte separation medium (P8610; Beijing Solarbio Science & Technology Co., Ltd), as per the instructions. Briefly, the anticoagulants were mixed 1:1 with lymphocyte separation medium and then centrifuged for 20 min at $1,000 \times g$. Lymphocytes in the buffy coat layer were extracted and transferred into 10 ml PBS and then centrifuged at $250 \times g$ for 10 min. After centrifugation, the supernatant was discarded and the lymphocytes in the bottom of the centrifuge tubes were re-suspended in 10 ml PBS. Then, the cells were centrifuged and re-suspended in 2 ml RPMI-1640 cell culture medium. After cell counting, the collected lymphocytes were diluted to 10^6 cells/mL and transferred to 96-well plates with 100 μl per well. Con A (Invitrogen) with a concentration of 125 μg/ml was used to stimulate cell proliferation, and cell proliferation was measured with an EnoGene Cell™ Counting Kit-8 (CCK-8) (Nanjing EnoGene Biotechnology Co., Ltd.). The stimulation index (SI) was calculated with the following formula: (mean OD450 of stimulated cells)/(mean OD450 of non-stimulated cells).

2.6.5. Challenge with vNDV

All chickens, apart from those in group NC, were challenged with F48E9 vNDV at 14 days post-boost immunization (Fig. 1). Chickens were challenged with 5000 $TCID_{50}$ virus in 100 μl PBS via eye drops, and another 5000 $TCID_{50}$ virus in 100 μl PBS via nasal drops (Table 2).
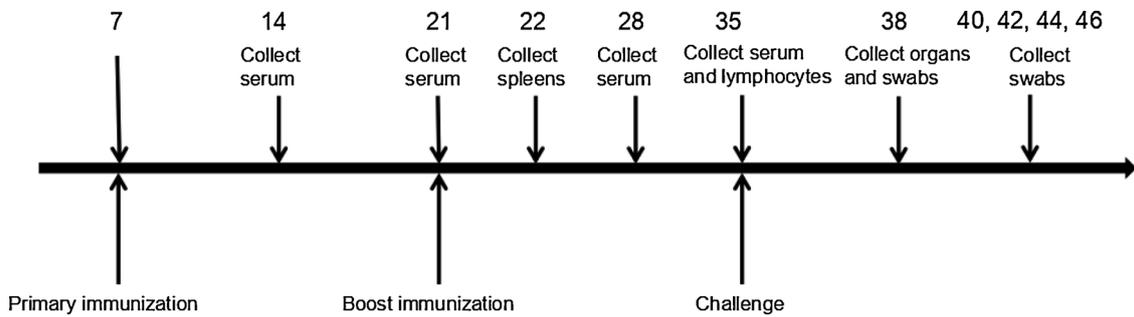
Fig. 1. Timelines for immunization, sample collection and challenge. Numbers represent the age of the chickens (days), and primary immunization was conducted when the chickens were 7 days old.
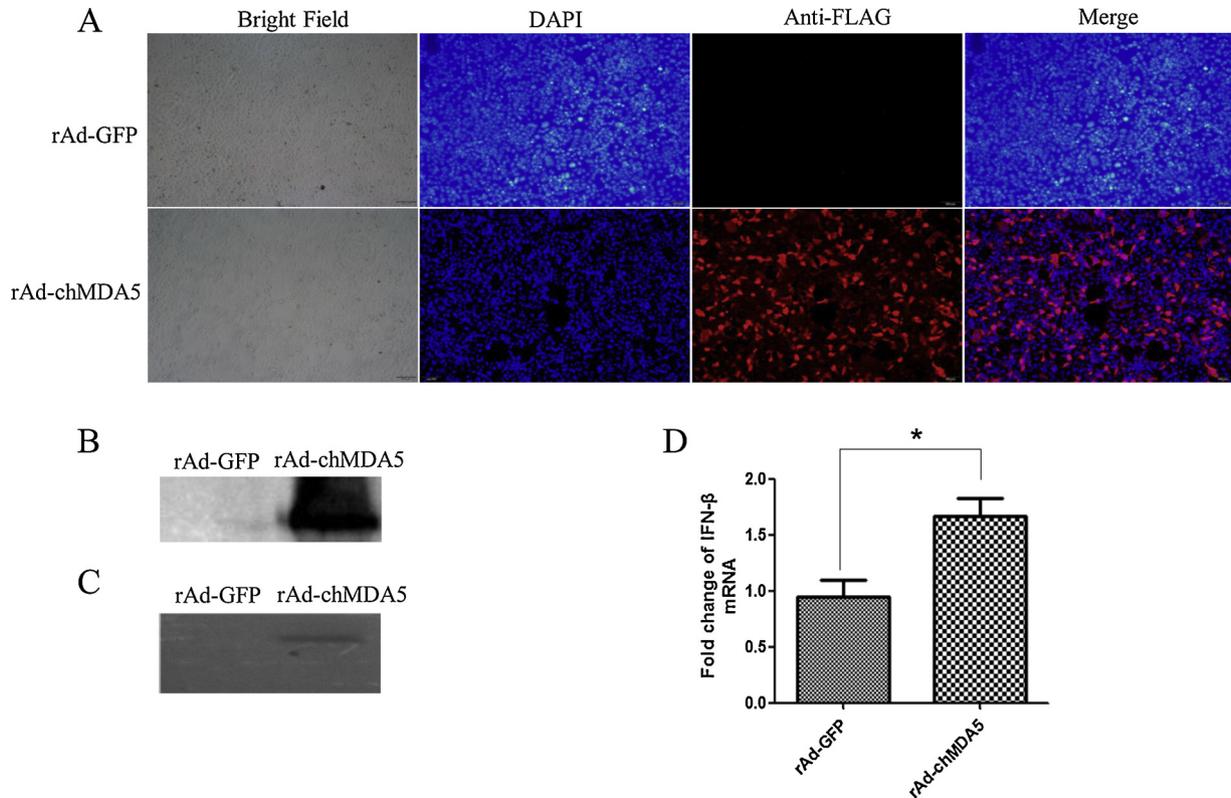

Fig. 2. Identification of the chMDA5(483aa) expressed by recombinant adenovirus. (A) Detection of the chMDA5(483aa) expression in HEK293 A cells by IFA. (B) Detection of the chMDA5(483aa) expression in HEK293 A cells by WB. (C) Detection of recombinant adenovirus mediated chMDA5(483aa) expression in DF-1 cells by WB. (D) Detection of rAd-chMDA5-induced IFN-β expression by RT-qPCR. All assays were performed in triplicate. Data were analyzed by Student's *t*-test and are presented as mean ± SEM. * indicates $p < 0.05$.

Clinical signs, survival rates and morbidities were recorded every day as described previously for a period of 14 days: healthy (0), slightly ill (1), severely ill (2) and dead (3) (Wang et al., 2017a,b). The daily clinical index is represented as the mean value of all chickens in each group.

2.6.6. Pathological examination

Three chickens were randomly selected from each group at 3 dpc and euthanized to collect duodenums and lungs for pathological examination. Duodenums were collected to assess gross-lesions, and lungs were collected and fixed in 4% phosphate buffered neutral paraformaldehyde to generate slices for observing micro-lesions. The histological lesions in the lungs were examined using a previously reported protocol (Wang et al., 2017a,b). A Lung Injury Score (LIS) system was used to evaluate micro-lesions in lungs and took into account five criteria: lymphocyte infiltration, pulmonary interstitial edema, airway epithelial cell damage, hyaline membrane formation and hemorrhage (Liu et al., 2015; Zhan et al., 2018). Five slices were generated from each lung sample and each slice had five scores to assess each criterion: normal (0), minimal change (1), mild change (2), moderate change (3), severe change (4) (Liu et al., 2015; Zhan et al., 2018).

2.6.7. Viral load and shedding

Lungs, spleens, duodenums and bursa of Fabricius were collected at 3 dpc to detect viral loads by RT-qPCR with primers targeting the P gene of NDV (Table 1) (Wang et al., 2017a,b). Cloacal and oral swabs from five birds in each group were collected every two days from 3 to 11 dpc and stored in 50% glycerine solution at −80 °C (Fig. 1). Swab samples were treated and inoculated into the allantoic cavity of 9-day-old embryonated chicken eggs. Allantoic fluid was collected after 24~72 h. In addition, viruses in the allantoic fluid were detected by HA assay (OIE, 2008).

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test, for multiple comparisons, and Student's *t*-test was performed to analyze two-sample comparisons. Daily clinical indexes and LISs were analyzed by Kruskal-Wallis *H* test followed by Nemenyi tests. Survival curves were analyzed by log-rank test. Morbidity and virus shedding ratios were analyzed by Chi-square test. Statistical analyses were performed with SPSS 19.0 software. $P < 0.05$ was considered to be a significant difference.

3. Results

3.1. Construction and packaging of the recombinant adenovirus expressing truncated chMDA5

The sequence of chMDA5(483aa) fused with a 3FLAG tag in a shuttle vector, pHBAd-chMDA5, was confirmed by sequencing and the results showed that it matched the reported sequence (GU570144.1). After co-transfection of the shuttle vector and adenovirus vector, rAd-MDA5 was successfully generated, as indicated by CPE in transfected cells (data not shown).

The expression of chMDA5(483aa) was examined by IFA. Red fluorescence indicating the expression of chMDA5(483aa) was observed in rAd-MDA5 infected cells but not in rAd-GFP infected cells (Fig. 2A). Similarly, western blot (WB) results indicated that chMDA5(483aa) was expressed by rAd-MDA5 (Fig. 2B).

The viral titers of rAd-MDA5 and rAd-GFP were 0.9×10^9 and 1.1×10^9 TCID$_{50}$/mL, respectively. The biological functions of chMDA5(483aa) were tested in DF-1 cells. The expression of chMDA5(483aa), delivered by adenovirus in DF-1 cells, was also confirmed by WB (Fig. 2C). RAd-chMDA5 infection increased IFN-β expression from 0.93-fold (rAd-GFP infected) to 1.66-fold (Fig. 2D) compared with that expressed by non-infected cells. These results suggested that chMDA5(483aa) expressed by the recombinant adenovirus was functional.

3.2. RAd-MDA5 enhanced the immune response induced by inactivated NDV vaccine

3.2.1. Humoral immune response

The NDV HI antibody titers of all the chickens were lower than 4 log2 prior to immunization. Treatments did not induce NDV-specific seroconversion in group CC, rAd-GFP, rAd-chMDA5 and InV at 7 days post-primary immunization (Fig. 3). Seroconversion was observed in one bird in group rAd-chMDA5 + InV. There was no significant difference in the HI titres between groups rAd-chMDA5 + InV and InV prior to boosting. A significantly higher HI titer was detected at day 28 (7 days post-boost immunization) in group rAd-chMDA5 + InV compared
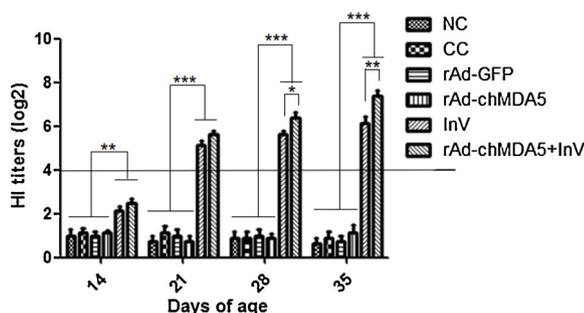
Fig. 3. NDV specific humoral immune response induced by vaccination. Sera (n = 8) antibody titers were detected by HI assay. Data were analyzed by one-way ANOVA and Tukey's post hoc test and are presented as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$.
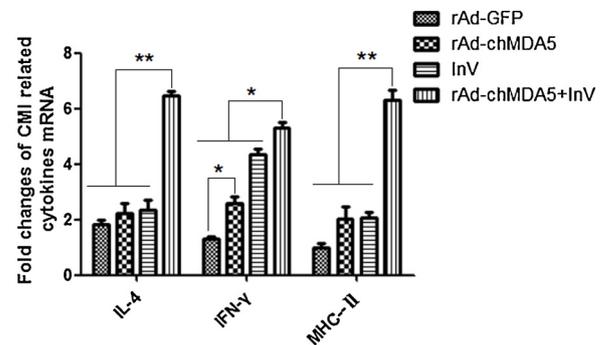
Fig. 4. Detection of CMI related cytokines expression by RT-qPCR. Fold changes were used to demonstrate the mRNA expression changes of the cytokines after immunization. All assays were performed in triplicate. Data were analyzed by one-way ANOVA and Tukey's post hoc test and are presented as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$.

with that from group InV ($p < 0.05$) (Fig. 3). The average HI titers in group rAd-chMDA5 + InV increased to 6.5 log2, while that in group InV was 5.67 log2 at day 28. This difference was even more evident at day 35 (14 days post-boost immunization) ($p < 0.01$) (Fig. 3). Co-administration of inactivated NDV with rAd-chMDA5 resulted in an HI titer of 7.7 log2, while only 6.0 log2 HI titer was detected in chickens immunized with inactivated NDV alone.

3.2.2. Cell-mediated immune response

To investigate the effect of chMDA5(483aa) on the CMI response, spleens were collected at 18 h post-boost immunization (day 22) and the expression levels of IL-4, IFN-γ and MHC-II were analyzed by RT-qPCR. As shown in Fig. 4, chickens treated with rAd-chMDA5 (group rAd-chMDA5) had higher levels (~2.6-fold increase) of IFN-γ mRNA ($p < 0.05$) but similar levels of IL-4 and MHC-II mRNA compared with those treated with rAd-GFP (group rAd-GFP). The expression of IFN-γ was significantly increased in chickens immunized with rAd-chMDA5 and inactivated NDV vaccine (group rAd-chMDA5 + InV) compared with that in chickens in group InV received inactivated NDV vaccine alone ($p < 0.05$). The Th2 immune response was also significantly enhanced, as indicated by the increased expressions of IL-4 and MHC-II in group rAd-chMDA5 + InV ($p < 0.01$), demonstrating that chMDA5(483aa) facilitated the activation of a Th2 immune response when co-administered with NDV antigen.

Lymphocyte proliferation assays were performed to determine the CMI at 6 h before challenge (day 35). The SI values of the chickens immunized with rAd-GFP or rAd-chMDA5 were significantly higher than those of the chickens immunized with inactivated NDV vaccine or PBS (Fig. 5) ($p < 0.01$). The difference in the SI values between group rAd-chMDA5 + InV and the other groups was significant ($p < 0.001$). The SI value was 1.85 in group rAd-chMDA5 + InV compared with the values ranging from 1.02 to 1.23 in the other groups (Fig. 5). Our results suggest that chMDA5(483) could enhance CMI.

3.3. RAd-MDA5 improved vaccine induced protection against vNDV challenge

3.3.1. Clinical symptoms, survivals and morbidities

All chickens apart from those in the control group NC were challenged with the F48E9 strain (10^4 TCID$_{50}$/bird) at day 35 (14 days post-boost immunization). Clinical signs, survival rates and morbidities were monitored for 14 days (Figs. 6A, 6B and 6C). Depression, decreased appetite, green feces and comb cyanosis were observed in chickens from sham-vaccinated groups (CC, rAd-GFP, rAd-chMDA5) from 2 dpc. Chicken mortality commenced from 3 dpc, and all of the chickens in the sham-vaccinated groups died within 5 days. Four chickens in group InV started to show clinical signs at 3 dpc, two died at 5 and 6 dpc, and two
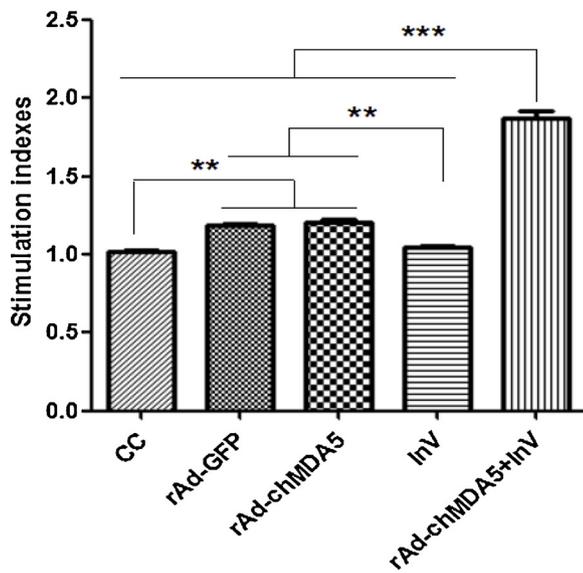
Fig. 5. Lymphocyte proliferative responses. PBLs were collected 6 h before challenge. All assays were performed in triplicate. Data were analyzed by one-way ANOVA and Tukey's post hoc test and are presented as mean ± SEM. ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

survived until 14 dpc (but with overt neural symptoms) (Fig. 6A and Fig. 6B). Chickens inoculated with rAd-chMDA5 and inactivated NDV survived throughout the course of the study and did not show obvious clinical signs. The decreased morbidity of chickens in group rAd-chMDA5 + InV compared with group InV at 6 dpc indicated that chMDA5(483aa) improved the protective efficacy induced by inactivated NDV vaccine (Fig. 6C) ($p < 0.05$).

3.3.2. Histopathology examination

The gross-lesions in the duodenums of each group are shown in Fig. 7A. Hemorrhages were observed in sham-vaccinated groups, and hyperemia was observed in group InV, while no obvious lesions were found in the duodenums from group rAd-chMDA5 + InV and NC. Consistently, no micro-lesions were observed in the lung slices from group rAd-chMDA5 + InV and NC (Fig. 7B). Severe lymphocyte infiltration, pulmonary interstitial edema, airway epithelial cell damage, hemorrhage and moderate hyaline membrane formation were found in the slices from sham-vaccinated chickens (Fig. 7B). The micro-lesions in chickens from group InV were of moderate severity (Fig. 7B). According to LISs analysis, the most serious micro-lesions were found in lungs from the sham-vaccinated groups followed by those from group InV, and then from group rAd-chMDA5 + InV and NC (Fig. 7C).

3.3.3. Viral load in organs and virus shedding

NDV viral RNA was detected in the organs from chickens in the sham-vaccinated groups, and the viral loads varied from $10^{1.46}$ to $10^{4.76}$ copies/0.2 g. Vaccination sharply reduced the viral loads in the organs from inactivated NDV vaccine-immunized groups compared with those in the organs from the sham-vaccinated groups ($p < 0.001$) (Fig. 8). Viral loads in the lungs and spleens from group rAd-GFP were lower than those from group CC ($p < 0.001$). Compared with group rAd-GFP, there were significantly lower viral loads in the spleens, duodenums and bursa of Fabricius from group rAd-chMDA5 ($p < 0.001$, $p < 0.05$ and $p < 0.001$ respectively). Viral RNA was detected in one of three chicken lungs from group InV, and the viral load was 1 copy/0.2 g. The viral RNA in the lungs of chickens from group rAd-chMDA5 + InV was below the detectable threshold. Moreover, the combination of rAd-chMDA5 and inactivated NDV vaccine significantly reduced the viral loads in spleens ($p < 0.05$), duodenums ($p < 0.01$) and bursa of Fabricius ($p < 0.05$) when compared with those from group InV. No virus
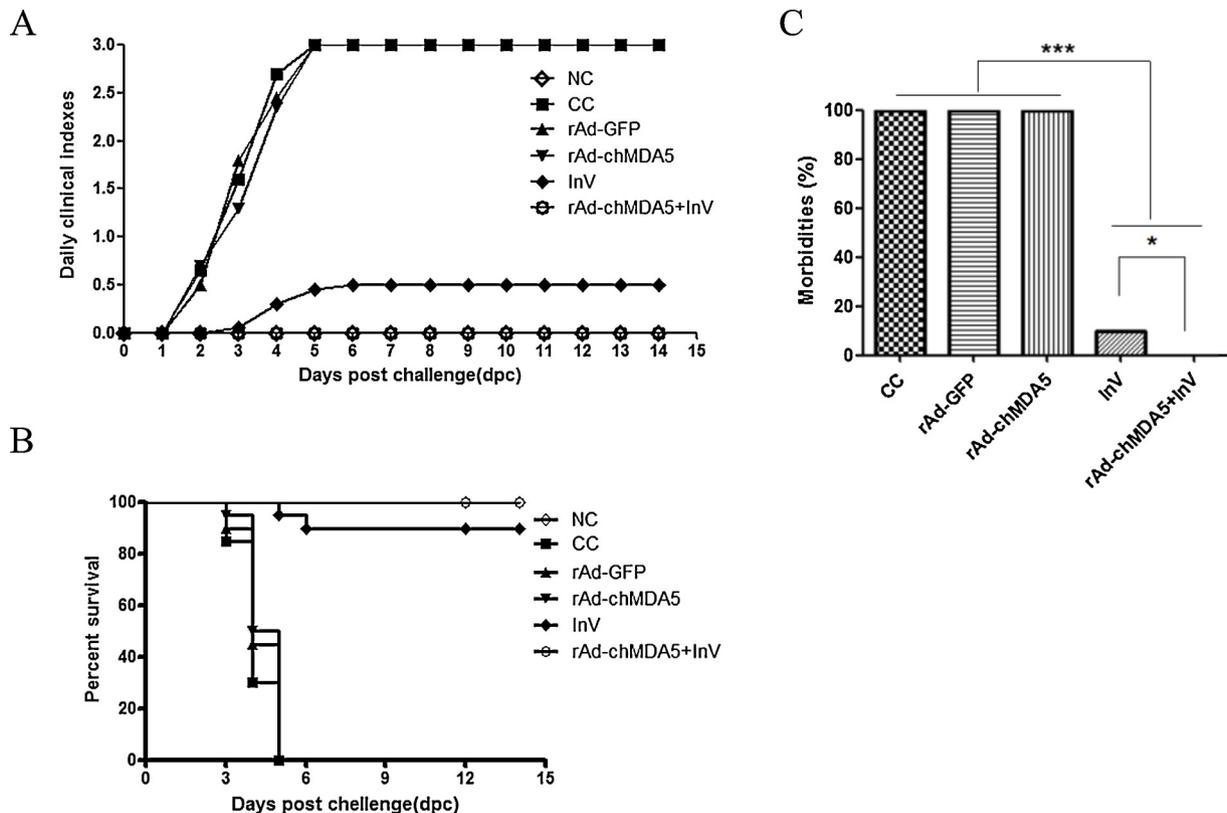

Fig. 6. Protective efficacy induced by vaccination. n = 20. (A) The daily clinical index. Daily clinical indexes were analyzed by Kruskal-Wallis *H* test followed by Nemenyi tests. (B) The survival percentages of chickens in different groups. Survival curves were analyzed by log-rank test. (C) Morbidities at 6 dpc. Morbidities were analyzed by Chi-square test. * indicates $p < 0.05$, *** indicates $p < 0.001$.
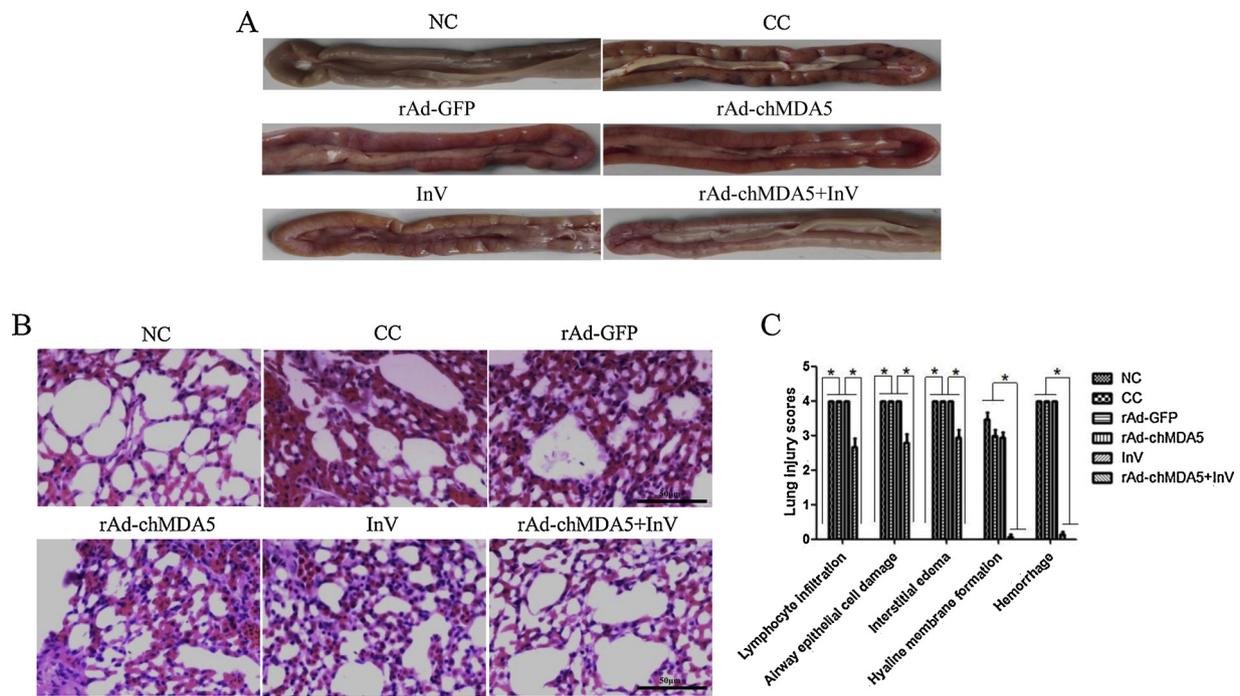
Fig. 7. Pathological lesion observation. Three chickens from each group were euthanized for pathological examination. (A) Gross-lesions in the duodenums of chickens. (B) Micro-lesions in the lungs of chickens. Hematoxylin and eosin staining (HE). (C) Lung lesion scoring in chickens. Lung injury scores were analyzed by Kruskal-Wallis *H* test followed by Nemenyi tests and are presented as mean ± SEM. n = 15, * indicates *p* < 0.05.
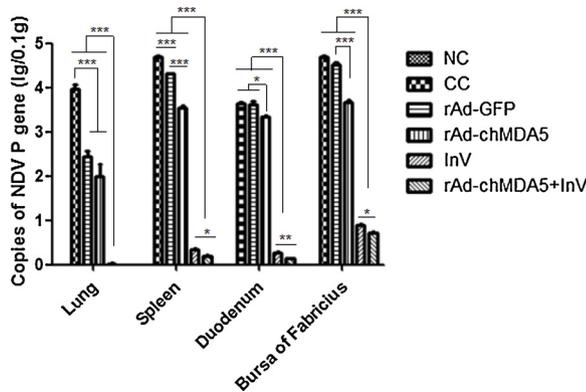

Fig. 8. Viral loads in the lungs, spleens, duodenums, bursa of Fabricius of chickens at 3dpc. All assays were performed in triplicate. Data were analyzed by one-way ANOVA and Tukey's post hoc test and are presented as mean ± SEM. * indicates *p* < 0.05, ** indicates *p* < 0.01 and *** indicates *p* < 0.001.

was detected in the organs from group NC (Fig. 8).

No virus shedding was detected from any birds in group NC (Table 3), while virus shedding was detected in all of the challenged groups. Virus shedding from oral swabs was detected from 3 dpc to 9 dpc in group InV and group rAd-chMDA5 + InV. Virus shedding from cloacal swabs was detected between 3 and 9 dpc in group InV, while shedding could only be detected between 5 and 7 dpc in group rAd-chMDA5 + InV. The virus shedding results suggested that rAd-MDA5 helped to delay and shorten the virus shedding period in the vaccine plus adjuvant group. The virus shedding (from oral and cloacal swabs) ratio of group rAd-chMDA5 + InV was significantly reduced at 3 dpc (1/10) compared with that of group InV (5/10) (*p* < 0.05), which further indicated that chMDA5(483aa) helped to reduce virus shedding.

4. Discussion

Genetic adjuvants, such as PRRs and cytokines, have been widely used to enhance the immune efficacy of vaccines (Matsui et al., 2004; Zeshan et al., 2011). MDA5, one of the PRRs located in the cytoplasm, can trigger an anti-viral immune response and has been used as an adjuvant to improve the humoral immune response induced by a DNA

Table 3
Detection of virus shedding in swabs.

Groups	3 dpc		5 dpc		7 dpc		9 dpc		11 dpc	
	o	c	o	c	o	c	o	c	o	c
NC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
CC	4/5	4/5	5/5	5/5	-	-	-	-	-	-
rAd-GFP	4/5	4/5	5/5	5/5	-	-	-	-	-	-
rAd-chMDA5	4/5	4/5	5/5	5/5	-	-	-	-	-	-
InV	3/5	2/5	4/5	4/5	4/5	2/5	2/5	1/5	0/5	0/5
rAd-chMDA5 + InV	1/5	0/5	3/5	3/5	2/5	1/5	1/5	0/5	0/5	0/5

X/5: the number of shedding swabs/total number of collected swabs.
o: oral swabs, c: cloacal swabs.
–: not detected due to no survivors.
Virus shedding ratio was analyzed by Chi-square test.

vaccine against influenza virus (Liniger et al., 2012b). Liniger and colleagues demonstrated that co-administration of a HA plasmid with plasmid DNA expressing chMDA5(1–483 aa) could result in a ˜10-fold higher HA-specific antibody response in chickens when compared with the treatment with a HA plasmid mixed with empty vector DNA. Accordingly, the protection was enhanced, as reduced clinical symptoms and cloacal virus shedding were found in chickens given chMDA5(1–483 aa)-adjuvanted HA DNA vaccine compared with those received HA DNA vaccine (Liniger et al., 2012b). The authors of the study attributed the enhanced immune response to the activation of the innate immune response by RIG-I like receptors (Liniger et al., 2012b). In our study, chMDA5(483aa)-activated IFN-β expression was also detected.

Chickens lack RIG-I and sense dsRNA through chMDA5, suggesting viral infections in chicken cells are sensed by chMDA5 (Karpala et al., 2011). One previous study reported that plasmid-driven expression of the N-terminal portion of chMDA5 resulted in the induction of the chIFN-β and chMx promoters in the absence of PAMP (Liniger et al., 2012a). ChMDA5 over-expression in HD11 cells has also been shown to significantly reduced Infectious Bursal Disease Virus (IBDV) titers and higher expression levels of chicken IFN-β, IL-6, IL-8 and IL-10 (Lee et al;, 2015). In this study, it was concluded that chMDA5 senses IBDV infection and initiates an innate immune response that in turn activates the adaptive immune response and limits IBDV proliferation (Lee et al;, 2015). In our study we show that the N-terminal 483 amino acids of chMDA5 facilitate an adaptive immune response induced by an inactivated NDV vaccine.

Adenovirus, with its amenable characteristics of stability, safety, high expression level and ease of construction, has been widely used to deliver vaccine antigens (Baden and Dan, 2013; Peters et al., 2013; Ogwang et al., 2013). Here, chMDA5(483aa) delivered from rAd-chMDA5 was used to improve the immune efficiency of an inactivated NDV vaccine. The enhanced immune responses induced by rAd-chMDA5 were detected after a booster immunization, with significantly higher levels of NDV-specific antibodies in group rAd-chMDA5 + InV detected at 7 and 14 days post-boost immunization (Fig. 3). These results suggest that co-administration of chMDA5(483aa) with inactivated NDV vaccine could improve the humoral immune response induced by inactivated NDV vaccine, which is consistent with the previous reports from Liniger et al. (Liniger et al., 2012b). However, CMI was not tested in their study.

CMI has been reported to be a key factor involved in the development of NDV vaccines (Alexander et al., 1999). IFN-γ and IL-4, representing Th1 and Th2 immune responses respectively (Lambrecht et al., 2004), were detected in our study. After boost immunization, expressions of IFN-γ and IL-4 were significantly increased in group rAd-MDA5 + InV, suggesting that chMDA5(483aa) helped to improve both the Th1 and Th2 immune responses induced by inactivated NDV vaccine. In addition, expression of MHC-IIs, representing antigen presentation, was also increased after co-administration of the vaccine antigen with rAd-chMDA5. Accordingly, at 3 dpc, the viral loads were decreased in the spleens, duodenums and bursa of Fabricius of chickens immunized with inactivated NDV vaccine and chMDA5(483aa). Therefore, chMDA5(483aa) would be an excellent adjuvant candidate to strengthen both the humoral immune response and the CMI response when used in combination with inactivated NDV vaccines.

Viral shedding plays an important role in the spread and maintenance of NDV within poultry farming industries. Following an outbreak of ND, surviving birds may shed virus for an extended duration while showing no clinical signs. Minimizing virus shedding is therefore critical to NDV vaccination strategies (Miller et al., 2009). While chMDA5-adjuvanted inactivated NDV vaccine did not completely inhibit virus shedding, a delayed and shortened virus shedding period was observed in group rAd-chMDA5 + InV compared with that in group InV.

In summary, our results showed that chMDA5(483aa) promoted the NDV-specific humoral immune response and CMI and helped to protect chickens against vNDV challenge by reducing the morbidity, tissue lesions, viral loads and virus shedding. These results indicated that chMDA5(483aa) could be an effective molecular adjuvant for NDV inactivated vaccine. In addition, co-immunization with rAd-chMDA5 and inactivated NDV vaccine is a feasible approach to protect birds against NDV infection.

Declaration of interests

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