Chicken melanoma differentiation-associated gene 5 (MDA5) recognizes infectious bursal disease virus infection and triggers MDA5-related innate immunity

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Abstract The objective of the present study was to determine if chicken melanoma differentiation-associated gene 5 (MDA5) senses infectious bursal disease virus (IBDV) infection to initiate and amplify an innate immune response in the chicken MDA5 (chMDA5) signaling pathway. Chicken embryo fibroblast DF-1 cells were infected with IBDV LP1 at a multiplicity of infection (MOI) of 0.5 or 10. In addition, knockdown and overexpression of chMDA5 were performed by transfecting DF-1 cells with chMDA5-targeting small interfering RNA (siRNA) or chMDA5-expressing DNA. The transfected cells were infected with IBDV LP1 at an MOI of 10. Cell culture supernatants and lysates were collected at 2, 8, 16 and 24 hours postinfection (hpi) for IBDV titer determination and RNA extraction, respectively. IBDV RNA loads and mRNA expression levels of chicken MDA5, interferon-β (IFN-β) promoter stimulator 1 (IPS-1), interferon regulatory factor-3 (IRF-3), IFN-β, double-stranded RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS), myxovirus resistance gene (Mx), and major histocompatibility complex class I (MHC class I) were determined by real-time RT-PCR. The IBDV titer increased up to 1.4×10^7 plaque-forming units (PFU)/mL at 24 hpi, and the IBDV RNA load reached 464 ng/μL at 24 hpi. The mRNA expression levels of chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I in IBDV-infected DF-1 cells exhibited significant ($p < 0.05$) upregulation up to 906-, 199-, 26,310-, 12-, 66,144-, 64,039- and 33-fold, respectively. Expressed chMDA5 from transfection and double-stranded RNA from IBDV infection were localized or colocalized in the cytoplasm of DF-1 cells at 16 hpi. When chMDA5 was knocked down in DF-1 cells, IBDV titers and RNA loads were significantly higher ($p < 0.05$) than those in DF-1 cells without chMDA5 knockdown at 24 hpi. The expression levels of chicken MDA5, IRF-3, IFN-β and MHC class I in chMDA5-knockdown DF-1 cells were significantly lower ($p < 0.05$) at 16 and 24 hpi. DF-1 cells overexpressing chMDA5 by transfection with chMDA5 expressing DNA had significantly lower ($p < 0.05$) IBDV titers and RNA loads at 16 and 24 hpi and showed significantly higher ($p < 0.05$) expression of chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I at 2 hpi. The results indicated that chicken MDA5 recognized IBDV infection and that this interaction resulted in the activation of chMDA5-related innate immune genes and upregulation of chicken MHC class I.

Introduction

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is an acute, contagious and economically important disease in young chickens. The most obvious lesion is bursal atrophy due to IBDV infecting and destroying B cells in the bursal lymphoid follicles. Chickens infected with IBDV at less than 3 weeks of age usually do not have clinical signs but develop severe immunosuppression, which renders these chickens susceptible to secondary infections and vaccination failures [53]. IBDV is a double-stranded, non-enveloped RNA virus belonging to the family *Birnaviridae* [30]. The bi-segmented genome comprises segment A (∼3.3 kilobase pairs [kbp]) and segment B (∼2.9 kbp). Segment A encodes four viral proteins (VP), designated VP2, VP3, VP4 and VP5, in two partially overlapping open reading frames. The longer open

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reading frame in segment A encodes a polyprotein that is further processed into mature VP2 and VP3, which are major structural proteins of the virion, and VP4, which functions as a viral protease [11]. The shorter open reading frame in segment A encodes a non-structural protein, VP5, which has been shown to induce apoptosis in chicken B cells and chicken embryo fibroblasts (CEF) to facilitate virus dissemination [39, 54]. Segment B encodes VP1, which is both an RNA-dependent RNA polymerase and a structural protein that links the ends of two genome segments together [31].

The target cells of IBDV are IgM-bearing pre-B lymphocytes [34]. The cellular machinery used by IBDV to facilitate its internalization is not well understood, but endocytosis is believed to play a role [55]. Once inside the endosome, IBDV releases pep46, a VP2-derived peptide that is present in the virus [5], to perforate the endosomal membrane and gain access to the cytoplasm [7]. It has been proposed that IBDV mRNAs extruded through pores located in the IBDV capsid are translated and replicated in the cytoplasm.

IBDV infection has been demonstrated to cause upregulation of innate and antiviral gene mRNA expression, including the myxovirus resistance gene (Mx), 2′,5′-oligoadenylate synthetase (OAS), type I interferons (IFN) and MHC class I in chicken embryo fibroblasts (CEF) or chicken embryo cells [24, 52]. In addition, chicken bursa infected with IBDV show enhanced mRNA expression of IFN-α and Mx [40]. However, how IBDV is recognized by the innate immune sensors in the host cells is not clearly defined.

Innate immunity is the first line of defense upon infection. Host cells, including immune cells, rely on pattern-recognition receptors (PRR), such as Toll-like receptors (TLR), retinoic acid-induced gene I (RIG-I)-like receptors (RLR) and nucleotide-binding oligomerization domain receptors (NOD)-like receptors (NLR), to detect the presence of pathogen-associated molecular patterns (PAMP), such as lipopolysaccharide (LPS), flagella, single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) on the pathogens [20]. The recognition of these molecules by PRR induces a series of signaling events that culminate in the production of type I interferons (IFN) and proinflammatory cytokines, providing an immediate response to limit the spread of pathogens [16].

RIG-I and melanoma differentiation-associated gene 5 (MDA5), members of the RLR family, serve as dsRNA sensors with different specificity to recognize infection by RNA viruses [37]. When recognizing their ligands, caspase activation and recruitment domain (CARD)-containing MDA5 or RIG-I functions together with IFN-β promoter stimulator (IPS-1) via CARD-CARD interaction, leading to the recruitment and activation of both canonical and non-

canonical inhibitors of NF-κB kinase (IKK) complexes. The canonical IKK complex mediates the phosphorylation of inhibitor of NF-κB (I-κB), which is subsequently degraded by proteasomes. The degradation of I-κB allows functional NF-κB to translocate into the cell nucleus, initiating the transcription of proinflammatory cytokines [56]. On the other hand, the non-canonical IKK complex activates the phosphorylation of interferon regulatory factor 3 (IRF-3) and IRF-7 to form a functional homodimer or heterodimer. The functional IRF dimers enter the cell nucleus to mediate the transcription of type I IFNs, IFN-α and IFN-β [56].

RIG-I and MDA5 play distinct but overlapping roles to recognize virus infection and initiate an innate immune response. RIG-I is known to recognize uncapped 5′-triphosphated RNA and short dsRNA, whereas MDA5 recognizes long dsRNA [14]. In addition, RIG-I has been found to preferentially sense short polyinosinic:polycytidylic acid (poly(I:C)) (300-1000 bp) and short reovirus genomic segments (~1.4 kbp) while MDA5 has been found to recognize long poly(I:C) (>1000 bp) and long genomic segments of reovirus (~3.9 kbp) [15].

Although chickens lack RIG-I [1], chicken MDA5 (chMDA5) has been shown to possess a conserved domain architecture when compared with mammalian MDA5 and to induce IFN-β expression when overexpressed in chicken DF-1 fibroblast cells [12, 23]. Functional characterization of chMDA5 reveals that chMDA5 recognizes dsRNA agonists, regardless of their length, in the cytoplasm and mediates avian influenza virus–induced chIFN-β synthesis [12, 23, 25]. Like mammalian MDA5, chMDA5 expression is upregulated in response to the stimulation by dsRNA and type I IFNs [12].

Type I IFNs induce the expression of MHC class I and numerous antiviral genes, including dsRNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS) and the myxovirus resistance gene (Mx) [12, 45]. Major histocompatibility complex class I (MHC class I) is expressed in most nucleated cells and functions to present endogenous antigens to CD8^{+} T cells, playing an important role in alerting the immune system [50]. dsRNA-dependent protein kinase (PKR) plays a central role in the regulation of protein synthesis in virus-infected cells by catalyzing the phosphorylation of protein synthesis initiation factor-2 (eIF-2), which results in the inhibition of translation [17]. Activation of OAS by dsRNA allows it to synthesize 2′,5′-oligoadenylates from adenosine triphosphate (ATP). 2′,5′-oligoadenylates bind to and activate RNase L, which degrades cellular and viral RNA [38]. Mx shows antiviral activity against various RNA viruses by recognizing nucleocapsid-like structures and restricting their localization within the cell [38]. The expression of the human Mx gene has been shown to provide resistance to two dsRNA

viruses, including IBDV and a mammalian reovirus [32]. The chicken Mx gene was cloned and shown to confer resistance to influenza virus and vesicular stomatitis virus (VSV) infections [19].

Rapid progression of IBD clinical signs and lesions in young chickens underscores the importance of the early innate immune response to IBDV. The PRR-PAMP innate and antiviral signaling is critical to the outcome of the infection, but information regarding the recognition of IBDV infection by PRR to induce innate immunity in chickens is limited. Understanding the interaction and underlying mechanism of innate immunity triggered by IBDV infection may shed light on the development of new approaches for protection against IBDV infection. The objective of the present study was to determine if chMDA5 senses IBDV infection, and if this recognition and inter-action activates the innate immune response in chicken DF-1 cells, which are non-antigen-presenting cells (APC).

Materials and methods

Cell culture and virus

DF-1 (ATCC, Manassas, VA, USA), a chicken embryonic fibroblast cell line [10], was maintained in growth medium containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with $1\times$ sodium pyruvate (Invitrogen), $1\times$ glutamine (Invitrogen), $1\times$ non-essential amino acids (NEAA) (Invitrogen), and 10 % fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO, USA). DF-1 cells were incubated at 37 °C in a 5 % CO_2 incubator (Nuaire, Plymouth, MN, USA). Cell-culture-adapted IBDV LP1 was obtained after four serial passages of IBDV strain variant E (provided by Dr. John K. Rosenberger, University of Delaware, USA) in baby grivet monkey kidney cells (BGM-70) and plaque purified in our laboratory [29].

Experimental design

In study 1 (Figs. 1, 2, 3, 4, 5), 1.5×10^5 DF-1 cells per well were seeded in 24-well plates for 24 h and then inoculated with IBDV LP1 at a multiplicity of infection (MOI) of 10 or 0.5. DF-1 cells added with 100 μL of Opti-MEM® (Invitrogen) served as mock-infected controls. After adsorption for one hour, the virus inoculum was removed and fresh culture medium was added. Cell culture supernatants and cell lysates were collected at 2, 8, 16 or 24 hours postinfection (hpi) for determination of virus titers or determination of mRNA levels of various innate and antiviral genes. To reveal the roles of chicken IPS-1 and IRF-3 in DF-1 cells during IBDV infection, DF-1 cells with chicken IPS-1 or chicken IRF-3 gene knockdown by small interfering RNA (siRNA) were infected with IBDV LP1 at an MOI of 10. Cell lysates obtained at 16 hpi were used for quantification of mRNA levels of chicken IPS-1 or IRF-3 and chicken IFN-β. In addition, DF-1 cells infected with IBDV LP1 at an MOI of 10 were transfected with chMDA5-expressing plasmids [23] for 15 h to determine the localization of IBDV-derived dsRNA and chMDA5 in DF-1 cells.

In study 2 (Figs. 6, 7, 8), DF-1 cells with chMDA5 knockdown by transfecting with siRNA targeting chMDA5 for 24 h were infected with IBDV LP1 at an MOI of 10. Cell culture supernatants and cell lysates were collected at 2, 8, 16 and 24 hpi and subjected to determination of viral titers and quantification of mRNA expression levels of chicken MDA5, IPS-1, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I and IBDV viral RNA loads.

In study 3 (Figs. 9, 10, 11), chMDA5 was overexpressed by transfection of DF-1 cells with chMDA5-expressing
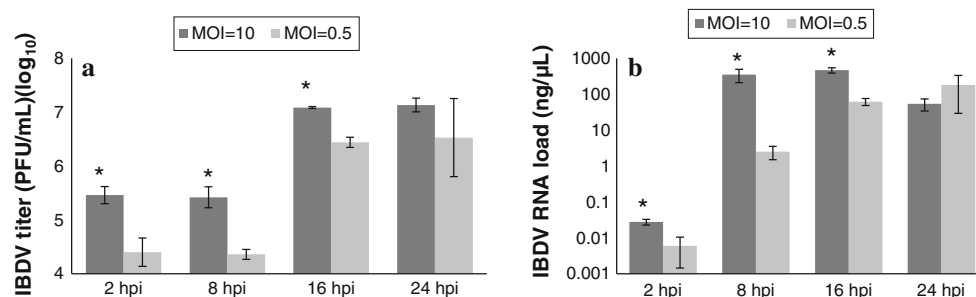

Fig. 1 Infectious bursal disease virus (IBDV) titers in cell culture supernatants (a) and IBDV RNA loads in IBDV-infected DF-1 cells (b). Chicken DF-1 cells were infected with IBDV LP1 at a multiplicity of infection (MOI) of 10 (MOI = 10 group) or an MOI of 0.5 (MOI = 0.5 group). Cell culture supernatants and cell lysates were collected at 2, 8, 16 and 24 h postinfection (hpi). The culture supernatants were used to quantify IBDV titers by plaque assay, and cell lysates were subjected to RNA extraction and subsequent IBDV RNA load determination by TaqMan-based real-time RT-PCR. Bars indicate mean ± standard deviation. Experiments were performed in triplicate. Asterisks (*) indicate $p < 0.05$ between the MOI = 10 and MOI = 0.5 groups at each time point as determined by unpaired Student's t-test
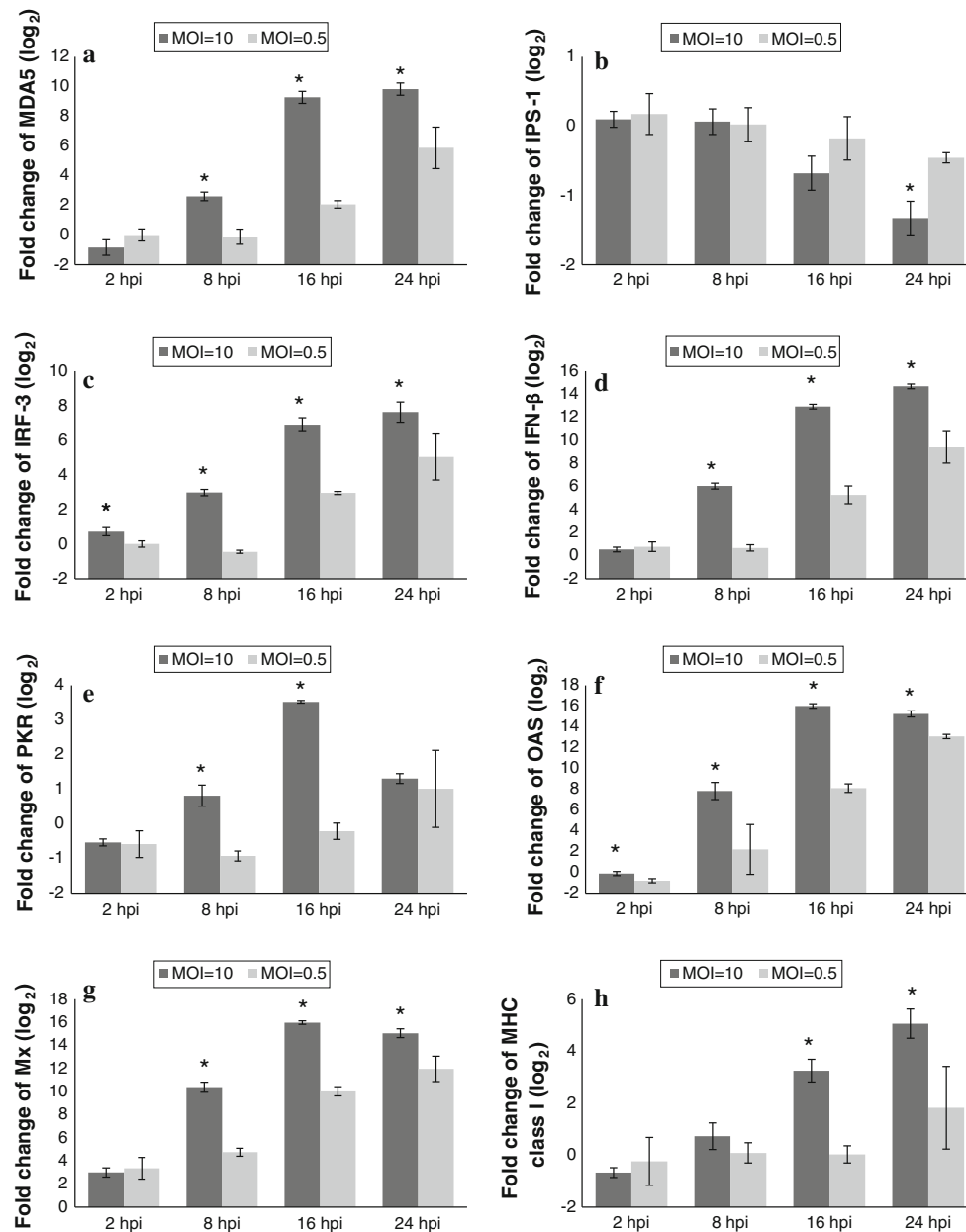
Fig. 2 mRNA expression levels of chicken MDA5 (chMDA5) pathway–related innate and antiviral genes in chicken DF-1 cells infected with infectious bursal disease virus (IBDV), including chicken MDA5 (a), IPS-1 (b), IRF-3 (c), IFN-β (d), PKR (e), OAS (f), Mx (g) and MHC class I (h). DF-1 cells were infected with IBDV LP1 at an MOI of 10 or 0.5. Cell lysates were collected at 2, 8, 16 and 24 hpi and subjected to RNA extraction. The expression levels of chMDA5-related genes were determined by SYBR Green–based real-time RT-PCR. The expression of each target gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content and is presented on a log$_2$ scale as fold change over the level in mock-infected DF-1 cells. Bars indicate means ± standard deviation based on triplicate samples. Asterisks (*) indicate p < 0.05 between the MOI = 10 and MOI = 0.5 groups as determined by unpaired Student's t-test. MDA5, melanoma differentiation-associated gene 5; IPS-1, interferon-β promoter stimulator-1; IRF-3, interferon regulatory factor-3; IFN-β, interferon-β; PKR, double-stranded RNA-dependent protein kinase; OAS, 2′,5′-oligoadenylate synthetase; Mx, myxovirus resistance gene; MHC class I, major histocompatibility class I

plasmids [23] for 24 h. The transfected cells were infected with IBDV LP1 at an MOI of 10. The cell culture supernatants and cell lysates were collected at 2, 8, 16 and 24 hpi and analyzed as described for study 2.

Determination of virus titers by plaque assay

The procedure for the plaque assay was described previously [29]. Briefly, DF-1 cells were seeded in 6-well tissue
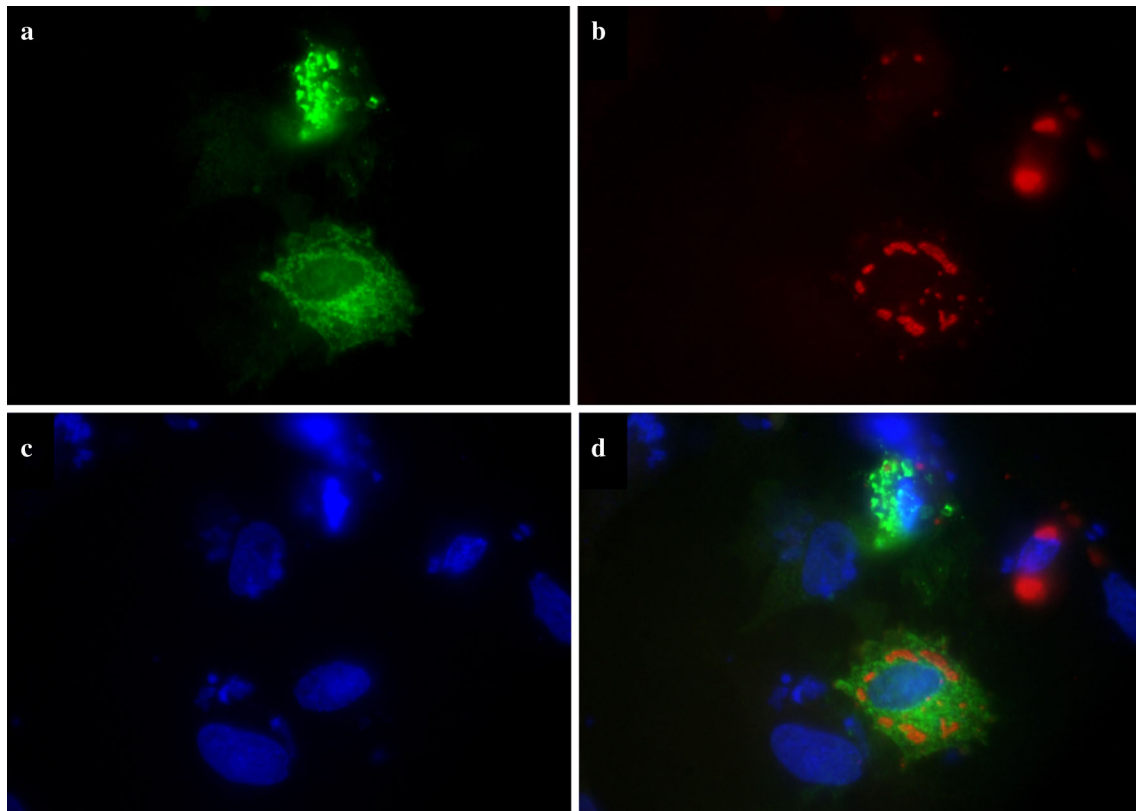
Fig. 3 Representative photomicrographs from fluorescent microscopy showing the location of chicken MDA5 (chMDA5) (a), double-stranded RNA (dsRNA) from infectious bursal disease virus (IBDV) (b), and the cell nucleus (c). A merged image of chicken MDA5 and IBDV dsRNA is shown in panel d. DF-1 cells seeded onto coverslips were infected with IBDV LP1 for 1 h and then transfected with a plasmid for expression of chMDA5. At 15 h post-transfection, the cells were fixed and permeabilized. The cells were incubated with rabbit anti-MDA5 polyclonal antibody and mouse anti-dsRNA antibody (J2), respectively, followed by incubation with fluorescein isothiocyanate (FITC)- and Texas Red–labeled secondary antibodies. 4′, 6′-diamidino-2-phenyl-indole (DAPI) staining was used as a counterstain to reveal cell nuclei. Green, chicken MDA5; red, dsRNA; blue, cell nucleus. Magnification, 1,000× (color figure online)

culture plates at 1×10^6 cells/well for 24 h. The cell culture supernatants collected in studies 1, 2 and 3 were serially diluted tenfold and 0.1 mL of each diluted sample was transferred onto a monolayer of DF-1 cells. The inoculum was removed after adsorption for one hour and the monolayers were overlaid with 3 mL of growth medium supplemented with 0.75 % Agar Noble (Difco Laboratories Inc, Detroit, MI, USA). After incubation for two days, 0.2 mL of 0.1 % neutral red (Sigma, St. Louis, MO, USA) in ddH$_2$O was added to each well to reveal plaques. The plaques were counted, and the virus titer was expressed in plaque-forming units (PFU) per mL.

RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed according to the manufacturer's instructions and have been described previously [23]. The RNA was eluted using 30 μL RNase-free water and stored in a −80 °C freezer. The quality and quantity of RNA was evaluated using a spectrophotometer (GeneQuant 1300, GE Healthcare Life Sciences, Pittsburgh, PA, USA). For cDNA synthesis, 2 μg of total RNA was mixed with 10 nmol of dNTP (Promega, Madison, WI, USA) and 250 ng of random primer (Sigma), heated to 65 °C for 5 min, and immediately placed on ice for 2 min. Following that, the mixture containing 1× first-strand synthesis buffer, 5 mM DTT and 200 units of Superscript III reverse transcriptase (Invitrogen) in 20 μL was heated at 50 °C for 60 min and then at 70 °C for 15 min to deactivate reverse transcriptase. The resulting cDNA was stored in a −20 °C freezer for later use.

Determination of IBDV RNA loads

Real-time RT-PCR using a TaqMan probe was performed on a Rotor-Gene 3000 thermal cycler (Corbett Research, Sydney, Australia) to quantify IBDV RNA loads following a previously published protocol with slight modifications [36]. The oligonucleotide primers and probes used and their corresponding sequences are summarized in Table 1. The vector pCR3.1 (Invitrogen) carrying the open reading frame of the IBDV large segment was serially diluted
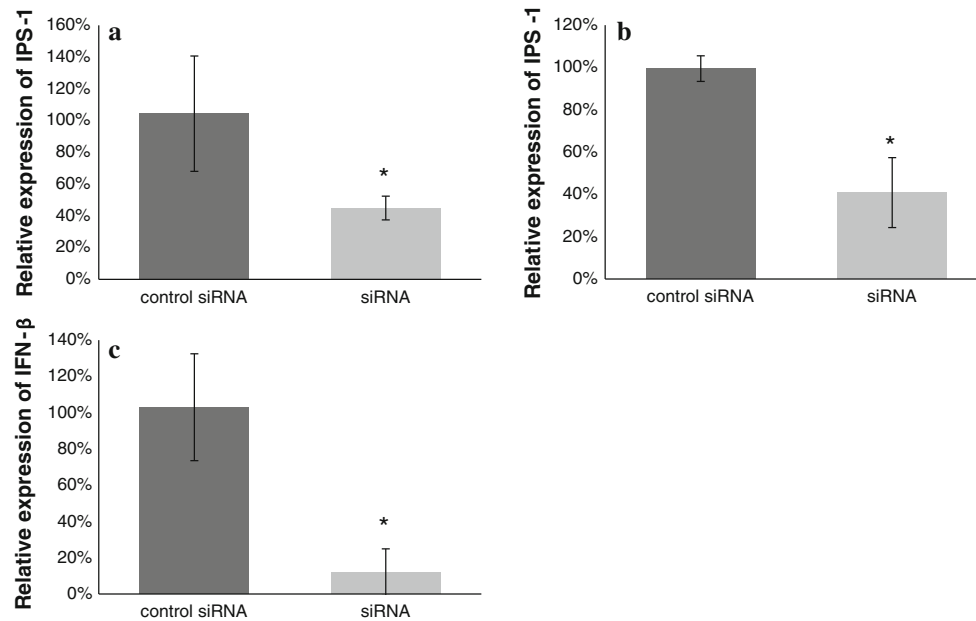
Fig. 4 Expression levels of chicken IFN-β in chicken IPS-1-knock-down DF-1 cells infected with infectious bursal disease virus (IBDV). DF-1 cells were transfected with siRNA targeting chicken IPS-1 (siRNA group) or its control siRNA (control siRNA group) for 24 h, and the expression of chicken IPS-1 was quantified by SYBR Green–based real-time RT-PCR (a). The transfected DF-1 cells were subsequently infected with IBDV LP1 for 16 h, and the expression levels of chicken IPS-1 (b) and IFN-β (c) were determined by SYBR Green–based real-time RT-PCR. The expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content and are presented as fold change relative to the level in control siRNA-transfected mock-infected DF-1 cells. Data are shown as mean ± standard deviation based on triplicate samples. Asterisks (*) indicate a significant difference ($p < 0.05$) determined by unpaired Student's t-test

tenfold and used to generate a standard curve. IBDV RNA loads were calculated from threshold cycle (Ct) values by linear regression using the standard curve.

Quantification of transcript levels of innate and antiviral genes

Real-time RT-PCR using SYBR Green was performed on a Rotor-Gene 3000 thermal cycler (Corbett Research) to determine transcript levels of chicken MDA5, IPS-1, IRF-3, IFN-β, PKR, OAS, Mx, MHC class I and GAPDH using a Rotor-Gene SYBR Green PCR Kit (QIAGEN, Valencia, CA) and the corresponding primer sets (Table 1). These primers were designed using Primer Quest software (Integrated DNA Technologies, Coralville, IA, USA). The reaction mixture, containing 1× Rotor-Gene SYBR Green PCR buffer, 100 nM forward primer, 100 nM reverse primer and 1 μL cDNA, was subjected to the following thermal cycling procedure: 1 cycle of 50 °C for 2 min and 95 °C for 2 min, 40 cycles of 95 °C for 20 s and 60 °C for 1 min, and 1 cycle of 72 °C for 7 min. The melting curve analysis after amplification was performed from 65 °C to 95 °C with a ramp speed of 1 °C/s. The fold changes for each gene were normalized to the GAPDH content in each sample. The relative expression of target gene normalized to GAPDH content is $2^{-\Delta Ct}$,

where $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$. The relative expression in the treatment group versus that in the control group was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{target\ gene}$ in the treatment group $- \Delta Ct_{target\ gene}$ in the control group. The geometric mean of the fold change in expression is reported on a $\log_2$ scale in the figures and was $2^{-(mean\ of\ \Delta\Delta Ct)}$, where the mean of $\Delta\Delta Ct$ was based on three samples (n = 3).

Localization of IBDV dsRNA and chMDA5

DF-1 cells seeded in 24-well plates with sterile coverslips at a concentration of 1.5×10^5 cell/well for 24 h were infected with IBDV LP1 at an MOI of 10. One hour later, the IBDV inoculum was removed, and the DF-1 cells were subsequently transfected with DNA carrying chMDA5 (pCDNA-MDA5#66) [23] for 15 h. DF-1 cells on cover-slips were fixed using 4 % paraformaldehyde for 15 min and permeabilized with 0.5 % Triton X-100 in PBS for 5 min. A double immunofluorescent antibody assay (IFA) was performed on DF-1 cells on cover slips using rabbit-anti-human MDA5 antibodies (1:200) (ProSci, Poway, CA, USA) and mouse-anti-dsRNA (1:200) (English & Scientific Consulting Bt., Balassagyarmat, Hungary) as the primary antibodies followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat-anti-rabbit (1:300)
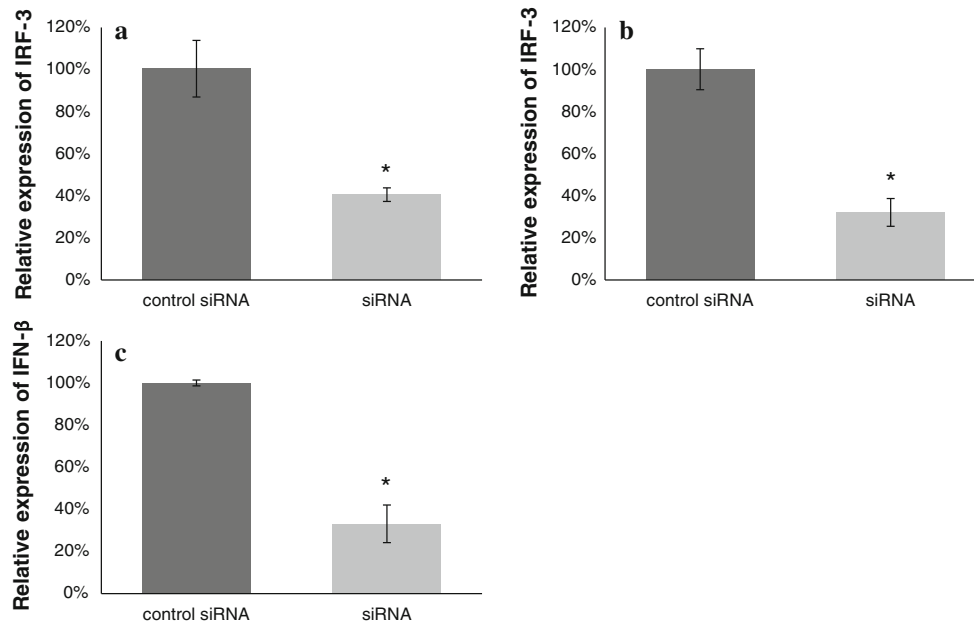
Fig. 5 Expression levels of chicken IFN-β in chicken IRF-3-knockdown DF-1 cells infected with infectious bursal disease virus (IBDV). DF-1 cells were transfected with siRNA targeting chicken IRF-3 (siRNA group) or its control siRNA (control siRNA group) for 24 h, and the expression level of chicken IRF-3 was quantified by SYBR Green–based real-time RT-PCR (a). The transfected DF-1 cells were subsequently infected with IBDV LP1 for 16 h, and the expression levels of chicken IRF-3 (b) and IFN-β (c) were determined by SYBR Green–based real-time RT-PCR. The expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content and are presented as fold change relative to the level in control siRNA-transfected mock-infected DF-1 cells. Data are shown as mean ± standard deviation based on triplicate samples. Asterisks (*) indicate a significant difference (p < 0.05) determined by unpaired Student's t-test
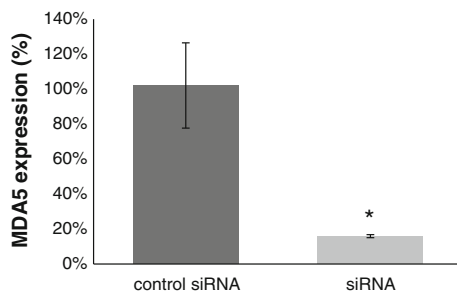

Fig. 6 Knockdown of chicken MDA5 (chMDA5) in DF-1 cells. DF-1 cells were transfected with siRNA targeting chMDA5 (siRNA group) or its control siRNA (control siRNA group) for 24 h, and total RNA was used to determine the chMDA5 expression level by SYBR Green–based real-time RT-PCR. Bars indicate mean ± standard deviation based on triplicate samples. Asterisks (*) indicate p < 0.05 between the siRNA and control siRNA groups as determined by unpaired Student's t-test

(KPL, Gaithersburg, MD, USA) and Texas Red (TXRD)-labeled goat-anti-mouse antibodies (1:200) (Southern Biotech, Birmingham, AL, USA) as the secondary antibodies. In addition, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) was used to counterstain the cell nuclei. The rabbit-anti-human MDA5 polyclonal antibody was shown previously to cross-react with chMDA5 [23]. Green or red fluorescence was examined using a fluorescence microscope (OPTIPHOT-2, Nikon, Melville, NY, USA). Images were taken using a digital microscope camera and SPOT advanced imaging software (SPOT Imaging Solutions, Sterling Heights, MI, USA).

Gene knockdown in DF-1 cells by siRNA

The 21-base pair siRNA duplexes with TT overhangs targeting chicken MDA5, IRF-3 or IPS-1 and their respective control siRNA duplexes were designed and synthesized (Sigma). The siRNA sequence targeting chMDA5 was 5′-GC[mA]AGAAUUGCCACAA[mG]UUdTdT-3′, and its control siRNA, which had a scrambled sequence with the same nucleotide composition of siRNA targeting chMDA5 was 5′-AU[mU]GUCGACUAACAGG[mA]ACdTdT-3′. The sequence of the siRNA targeting chicken IPS-1 was 5′-GG[mG]UUUAUGACUGCUA[mC]CAdTdT-3′, and the sequence of its scrambled control siRNA was 5′-GU[mA]GCUUCUCGAGCGU[mU]AAdTdT-3′. The sequence of the siRNA targeting chicken IRF-3 was 5′-CUGGUUUCACCGUUACAGAdTdT-3′, and the sequence of its scrambled control siRNA was 5′-AGGUCUGUACCUCCUGUAAdTdT-3′. mA, mG, mU and mC indicate methylation at the 2′ position of the ribose (2′-O-methylation) in A, G, U and C, respectively.

DF-1 cells at 1.5×10^5 cells/well in 24-well plates were transfected with 20 picomoles of siRNA complexed with
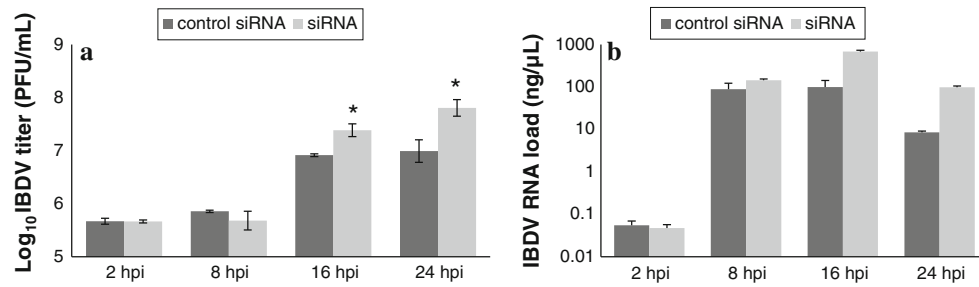
Fig. 7 Infectious bursal disease virus (IBDV) titers (a) and IBDV RNA loads (b) in chicken MDA5 (chMDA5)-knockdown DF-1 cells (siRNA group) and chMDA5-intact DF-1 cells (control siRNA group) during IBDV infection. DF-1 cells seeded in the presence of chMDA5-targeting siRNA or its control siRNA for 24 h were infected with IBDV LP1 at an MOI of 10. Cell culture supernatants and cell lysates were collected at 2, 8, 16 and 24 hpi. The culture supernatants were used to quantify IBDV titers by plaque assay, and cell lysates were subjected to RNA extraction and subsequent IBDV RNA load determination by TaqMan-based real-time RT-PCR. Bars indicate mean ± standard deviation based on triplicate samples. The statistical difference between the siRNA and control siRNA groups at each time point was evaluated by unpaired Student's t-test and is indicated by asterisks (*) ($p < 0.05$)

Fig. 8 mRNA expression levels of chicken MDA5 (chMDA5) pathway–related innate and antiviral genes in chMDA5-knockdown DF-1 cells during IBDV infection. DF-1 cells seeded in the presence of chMDA5-targeting siRNA or its control siRNA for 24 h were infected with IBDV LP1 at an MOI of 10. At 2, 8, 16 and 24 hpi, cell lysates were harvested and expression levels of chicken MDA5 (a), IPS-1 (b), IRF-3 (c), IFN-β (d), PKR (e), OAS (f), Mx (g), and MHC class I (h) were determined by SYBR Green–based real-time RT-PCR. The expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content. Expression levels are presented on a $\log_2$ scale as fold change relative to the level of control-siRNA-transfected mock-infected DF-1 cells. Experiments were performed in triplicate, and each bar indicates mean ± standard deviation. A significant difference between the siRNA and control siRNA groups at each time point was determined by unpaired Student's t-test and is indicated by asterisks (*)
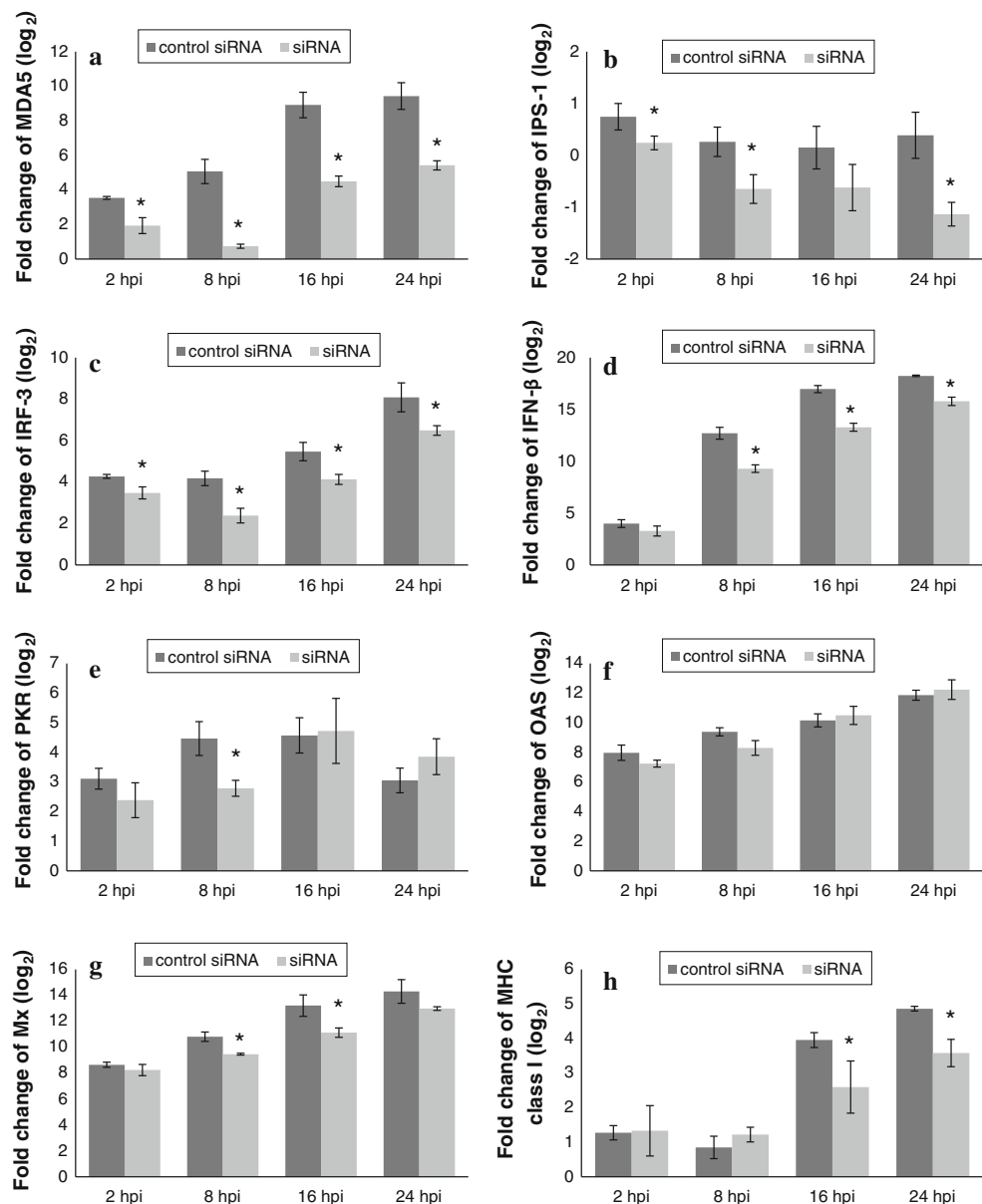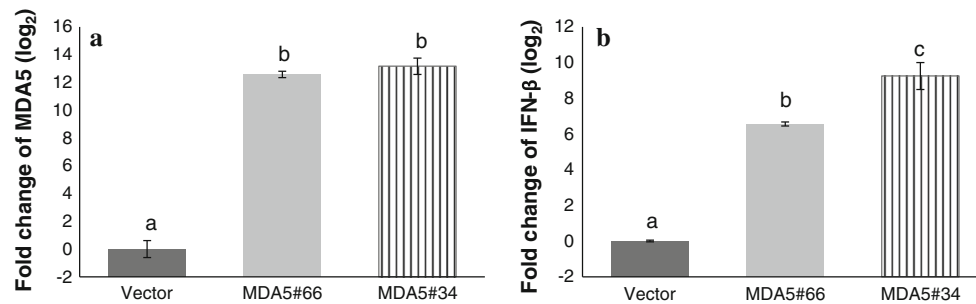
Fig. 9 Overexpression of chicken MDA5 (chMDA5) in DF-1 cells. DF-1 cells were transfected with chicken-MDA5-expressing plasmids, pCDNA-MDA5#66 (MDA5#66 group) or pCDNA-MDA5#34 (MDA5#34 group), or pCDNA vector (vector control group) for 24 h. The chMDA5 protein encoded by pCDNA-MDA5#66 was expressed using an alternative translation initiation site and was therefore 101 amino acids longer than that encoded by pCDNA-MDA5#34 at the N-terminus. Transfected DF-1 cells were subjected to RNA extraction and SYBR Green–based real-time RT-PCR. The expression levels of chicken MDA5 (a) and IFN-β (b) are shown. The expression level of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content and is presented on a $\log_2$ scale as fold change relative to the level of vector-transfected DF-1 cells. Data are shown as mean ± standard deviation based on triplicate samples. Different letters indicate significant difference ($p < 0.05$) as determined by one-way ANOVA
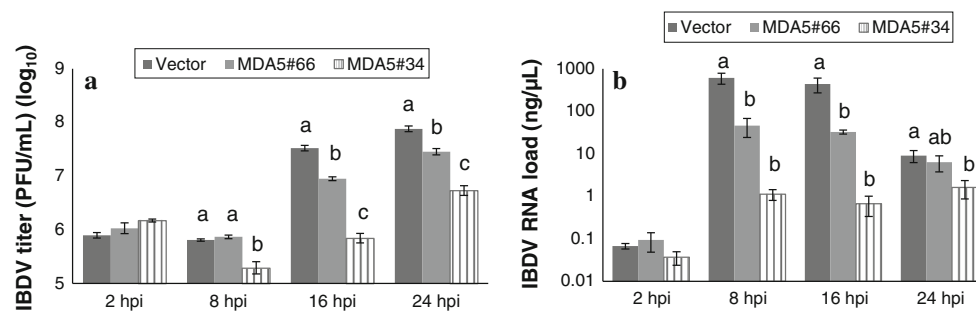

Fig. 10 Infectious bursal disease virus (IBDV) titers (a) and IBDV RNA loads (b) in DF-1 cells transfeted with chicken MDA5 (chMDA5) and infected with IBDV. DF-1 cells transfected with pCDNA-MDA5#66 (MDA5#66 group), pCDNA-MDA5#34 (MDA5#34 group) or pCDNA vector (vector control group) for 24 h were infected with IBDV LP1 at an MOI of 10. Cell culture supernatants and cell lysates were collected at 2, 8, 16 and 24 hpi. The culture supernatants were used to quantify IBDV titers by plaque assay, and cell lysates were subjected to RNA extraction and subsequent IBDV RNA load determination by TaqMan-based real-time RT-PCR. Bars represent mean ± standard deviation calculated based on triplicate samples. Different letters indicate significant differences ($p < 0.05$) among groups at each time point as determined by one-way ANOVA

1 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The knockdown efficiency of each targeted siRNA was evaluated by real-time RT-PCR at 24 h post-transfection. Comparisons of mRNA levels for each gene were made between DF-1 cells transfected with the appropriate siRNA and DF-1 transfected with its corresponding control siRNA. The gene knockdown efficiency by siRNA was determined as the mRNA level of each gene with targeted siRNA relative to that with control siRNA.

ChMDA5 overexpression in DF-1 cells by transfection

DF-1 cells were seeded in 24-well plates at a concentration of 1.5×10^5 cells/well 24 h prior to transfection. Transfection was aided by Lipofectamine 2000 (Invitrogen). One microliter of Lipofectamine 2000 (Invitrogen) was diluted in 50 μL of Opti-MEM® (Invitrogen) and incubated at room temperature for 5 min. Plasmid DNA (0.4 μg) was diluted in 50 μL Opti-MEM®. Two chMDA5-expressing DNA constructs were used: pCDNA-MDA5#66 and pCDNA-MDA5#34 [23]. The insert in pCDNA-MDA5#66 was 101 amino acids longer than that in pCDNA-MDA5#34 at the amino terminus, encoding a longer chMDA5 from an alternative translation initiation site [23]. The diluted Lipofectamine 2000 and plasmid DNA were mixed together and incubated at room temperature for 20 min. The plasmid DNA-Lipofectamine 2000 mixture was added to DF-1 cells with fresh culture medium. At 24 h post-transfection, the DF-1 cells were infected with IBDV LP1 at an MOI of 10. At 2, 8, 16 or 24 hpi, culture supernatants and cell lysates were collected to determine IBDV titers and mRNA levels, respectively.

Statistical analysis

Student's t-test was used in experiments involving comparison between two groups (SPSS, IBM, Armonk, NY). Comparisons among three groups were performed by one-way

Fig. 11 mRNA expression levels of chicken MDA5 (chMDA5) pathway–related innate and antiviral genes in chMDA5-overexpressing DF-1 cells during IBDV infection. DF-1 cells transfected with pCDNA-MDA5#66, pCDNA-MDA5#34 or pCDNA vector for 24 h were infected with IBDV LP1 at an MOI of 10. At 2, 8, 16 and 24 hpi, cell lysates were collected and used to determine the expression levels of chicken MDA5 (a), IPS-1 (b), IRF-3 (c), IFN-β (d), PKR (e), OAS (f), Mx (g) and MHC class I (h) by SYBR Green–based real-time RT-PCR. The expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content. Expression levels are presented on a $\log_2$ scale as fold change relative to the level in vector-transfected mock-infected DF-1 cells. Data are shown as mean ± standard deviation based on triplicate samples. Different letters indicate a significant difference ($p < 0.05$) at each time point as determined by one-way ANOVA
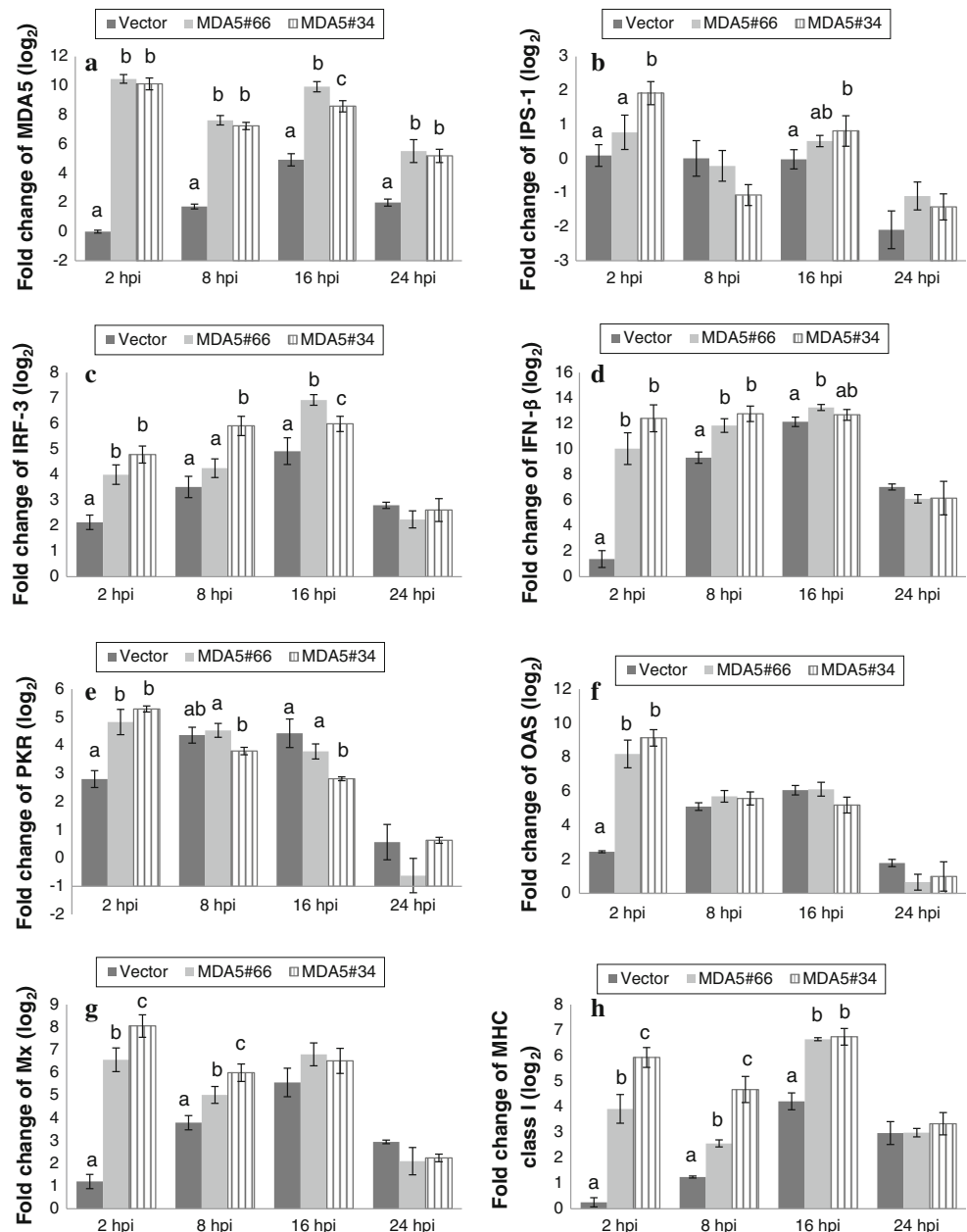
ANOVA, followed by Tukey *post hoc* analysis (SPSS). A p-value less than 0.05 was considered statistically significant and is indicated by an asterisk (*) or different letters in the figures. Data are reported as mean ± standard deviation.

Results

IBDV infection in chicken DF-1 fibroblast cells

IBDV growth kinetics

IBDV titers in the culture supernatants increased throughout the experimental period (Fig. 1a). The greatest increases in IBDV titer occurred between 8 and 16 hpi in both groups. After 16 hpi, IBDV titers in DF-1 cells infected with IBDV at an MOI of 10 (MOI=10 group) remained at around 1×10^7 PFU/mL, while those in DF-1 cells infected with IBDV at an MOI of 0.5 (MOI = 0.5 group) continued to increase from 2.8×10^6 to around 1×10^7 PFU/mL. IBDV titers were significantly higher ($p < 0.05$) in the MOI = 10 group than those in the MOI = 0.5 group at 2, 8 and 16 hpi. IBDV RNA loads in the MOI = 10 and MOI = 0.5 groups increased over time and were dependent on the dose of IBDV inoculum at 2, 8 and 16 hpi (Fig. 1b). The IBDV RNA load in the MOI = 10 group reached the highest level of 460 ng/μL at 16 hpi, while that in the MOI = 0.5 group reached the

Table 1 Sequences of oligonucleotide primers used in the present study

Primer name	Sequence (5′ to 3′)		Target accession number
Real-time PCR:	Forward	Reverse	
GAPDH	ATCAAGAGGGTAGTGAAGGCTGCT	TCAAAGGTGGAGGAATGGCTGTCA	NM_204305
MDA5	TGAAGGCAAAGAGAGATCAGCGTAAGA	CATATCAATTGTGGCAATTCTTGCACAGGA	XM_422031
IPS-1	GCAGTTTGATGCAGAGCAGAAGCA	AGGCTTCAAGGAGGTGTCACAGAA	NM_001012893
IRF-3	ACCACATGCAGACAGACTGACACT	GGAGTGGATGCAAATGCTGCTCTT	NM_205372
IFN-β	ACCAGGATGCCAACTTCTCTTGGA	ATGGCTGCTTGCTTCTTGTCCTTG	NM_001024836
PKR	ACGTGGGACATGATTGAGCCAAAG	TGATGTAGTCAACTGGAGGGAGCA	NM_204487
OAS	GCAGAAGAACTTTGTGAAGTGGCG	TCGGCTTCAACATCTCCTTGTACC	NM_205041
Mx	TTCACGTCAATGTCCCAGCTTTGC	ATTGCTCAGGCGTTTACTTGCTCC	NM_204609
MHC class I	AGTTCCCACCAAGAGGAAATGGGA	ATTCCACATATCTCCGCAGCCACT	NM_001031338
IBDV-2026F	CCCATACCTCCTATTGTGGG		AF133904
IBDV-2161R	GTGGCGAGCTTGGTGCTTCT		AF133904
IBDV probe	FAM-CAATGCTTGTGGCGAGATTGAGAAAGTAAG-BHQ		AF133904

FAM, 6′-carboxyfluorescein; BHQ, Black Hole Quencher-1

highest level of 180 ng/μL at 24 hpi. Unlike IBDV titers, the greatest increases in viral RNA loads occurred between 2 and 8 hpi in both groups. IBDV RNA loads in the MOI = 10 group were higher or significantly higher (p < 0.05) than those in the MOI = 0.5 group at 2, 8 and 16 hpi.

Transcript levels of innate and antiviral genes

The mRNA expression of innate and antiviral genes associated with the chMDA5 signaling pathway increased over time, except IPS-1. The fold changes of chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I were higher or significantly higher (p < 0.05) in the MOI = 10 group at 8, 16 and 24 hpi (Fig. 2a, c-h). The fold increases of chicken MDA5, IRF-3, IFN-β, and MHC class I in the MOI = 10 group reached the highest levels of 906-, 199-, 26,310- and 33-fold at 24 hpi (Fig. 2a, c, d and h), while chicken PKR, OAS and Mx had the highest transcript levels of 12-, 66,144- and 64,039-fold, respectively, at 16 hpi (Fig. 2e-g).

Localization of chMDA5 and dsRNA

Green and red fluorescence representing chMDA5 and dsRNA, respectively, was seen in IBDV-infected, chMDA5-transfected DF-1 cells at 16 hpi (Fig. 3a and b). Some cells showed green and red fluorescence simultaneously, indicating that the cells possessed chMDA5 protein and dsRNA that were derived from IBDV infection. The expression of chMDA5 was mostly scattered in the cytoplasm, while dsRNA formed aggregates in some regions of the cytoplasm without a particular orientation.

IFN-β transcript levels in IBDV-infected IPS-1-knockdown DF-1 cells

The expression of chicken IPS-1 in chicken IPS-1-knockdown DF-1 cells (siRNA group) was reduced to 44.9 % relative to that in chicken IPS-1-intact DF-1 cells (control siRNA group) at 24 hours post-transfection (hpt) (Fig. 4a). Subsequent infection with IBDV in chicken DF-1 cells with IPS-1 knockdown led to a reduction in expression of IFN-β to 12.1 % relative to IFN-β levels in the control siRNA group at 16 hpi (Fig. 4c). The mRNA level of chicken IPS-1 in the siRNA group was reduced to 40.8 % relative to that in the control siRNA group (Fig. 4b).

IFN-β transcript levels in IBDV-infected IRF-3-knockdown DF-1 cells

The expression of chicken IRF-3 in DF-1 cells with chicken IRF-3 knockdown (siRNA group) was reduced to 40.5 % relative to that in DF-1 cells with intact chicken IRF-3 (control siRNA group) at 24 hpt (Fig. 5a). Chicken DF-1 cells with IRF-3 knockdown were subsequently infected with IBDV for 16 h, and the mRNA expression level of IFN-β was reduced to 14.8 % relative to that in the control siRNA group (Fig. 5c). The mRNA expression of chicken IRF-3 in the siRNA group was 12.8 % relative to that in the control siRNA group at 16 hpi (Fig. 5b).

IBDV infection in chMDA5-knockdown DF-1 cells

Knockdown of chMDA5 in DF-1 cells

The expression of chMDA5 transcripts in DF-1 cells with chMDA5 knockdown (siRNA group) at 24 hpt decreased to 15.8 % compared to that in DF-1 cells with intact chMDA5 (control siRNA group) (Fig. 6). The reduction of chMDA5 mRNA was statistically significant ($p < 0.05$).

IBDV growth kinetics

IBDV titers in the culture supernatants increased with time and were significantly higher ($p < 0.05$) in IBDV-infected chMDA5-knockdown DF-1 cells (siRNA group) than in IBDV-infected DF-1 cells (control siRNA group) at 16 and 24 hpi (Fig. 7a). The greatest increase in virus titer was observed between 8 and 16 hpi for both groups. After 16 hpi, the virus titer in the control siRNA group remained at around 1×10^7 PFU/mL, while that in the siRNA group reached 6.7×10^7 PFU/mL. IBDV RNA loads increased between 2 and 16 hpi and started to decline between 16 and 24 hpi in the siRNA and control siRNA groups (Fig. 7b). IBDV RNA loads in the both groups were at similar levels of around 0.05 or 100 ng/μL at 2 or 8 hpi, respectively. However, the siRNA group had significantly higher ($p < 0.05$) IBDV RNA loads of 687 or 99 ng/μL than the control siRNA group did at 16 or 24 hpi. The greatest increase in IBDV RNA loads occurred between 2 and 8 hpi.

Transcript levels of innate and antiviral genes

The mRNA expression levels of innate and antiviral genes associated with the chMDA5 signaling pathway are shown in Fig. 8. The fold changes of chicken MDA5, IRF-3 and IFN-β transcripts were lower or significantly lower ($p < 0.05$) in the IBDV-infected chMDA5-knockdown (siRNA) group than in the IBDV-infected chMDA5-intact (control siRNA) group at 2, 8, 16 and 24 hpi (Fig. 8a, c and d). At 24 hpi, the expression of chicken MDA5, IRF-3 and IFN-β in the siRNA group reached the highest levels at 43-, 90- and 56,397-fold, respectively, significantly lower ($p < 0.05$) than 690-, 271-, and 307,452-fold in the control siRNA group. Although IBDV infection did not cause significant upregulation ($p > 0.05$) of chicken IPS-1 in the control siRNA group, there was significantly ($p < 0.05$) reduced expression of chicken IPS-1 at 8, 16 and 24 hpi in the siRNA group (Fig. 8b). For genes related to the antiviral response, although the fold changes of chicken PKR, OAS and Mx between the siRNA and control siRNA groups were not significantly different ($p > 0.05$), the fold changes were lower in the siRNA group at various time points (Fig. 8e-g). Fold changes of chicken MHC class I transcripts were significantly lower ($p < 0.05$) in the siR-NA group than in the control siRNA group at 16 and 24 hpi (Fig. 8h).

IBDV infection in chMDA5-overexpressing DF-1 cells

Overexpression of chMDA5 in DF-1 cells

Transfection of chMDA5-expressing constructs in DF-1 cells for 24 h resulted in upregulation of chMDA5 transcripts by 6118- and 9567-fold, respectively, in pCDNA-MDA5#66-transfected DF-1 cells (MDA5#66 group) and pCDNA-MDA5#34-transfected DF-1 cells (MDA5#34 group) relative to that in DF-1 cells transfected with pCDNA vector (vector control group) (Fig. 9a). The chMDA5 protein encoded by pCDNA-MDA5#66 was produced using an alternative translation initiation site and was therefore 101 amino acids longer than that encoded by pCDNA-MDA5#34 in the N-terminus [23]. The overexpression of chMDA5 in DF-1 cells induced upregulation of chicken IFN-β by 95- and 649-fold, respectively, in the MDA5#66 and MDA5#34 groups relative to the vector control group (Fig. 9b). The MDA5#34 group induced a significantly higher ($p < 0.05$) level of IFN-β than the MDA5#66 group did.

IBDV growth kinetics

During IBDV infection, IBDV titers in the MDA5#66, MDA5#34 and vector control groups were around 1×10^6 PFU/mL at 2 and 8 hpi, except that the MDA5#34 group had a significantly lower ($p < 0.05$) titer of 2.1×10^5 PFU/mL at 8 hpi (Fig. 10a). IBDV titers increased in the three groups between 8 and 24 hpi. The IBDV titers increased rapidly from around 7×10^6 to 3.4×10^7 and 9×10^6 PFU/mL in the vector and MDA5#66 groups, respectively, between 8 and 16 hpi. The titer of IBDV in the MDA5#34 group also increased from 2.1×10^5 to 7.3×10^5 PFU/mL between 8 and 16 hpi. The IBDV titers in the MDA5#66 and MDA5#34 groups were significantly lower ($p < 0.05$) than that in the vector control group at 16 and 24 hpi. In addition, the MDA5#34 group had significantly lower ($p < 0.05$) virus titers than the MDA5#66 group did at 8, 16 and 24 hpi. The greatest increase in IBDV RNA loads was observed between 2 and 8 hpi (ranging from a 30-fold increase in the MDA5#34 group to a 10,000-fold increase in the vector control group; Fig. 10b). The IBDV RNA loads peaked at 8 hpi and were 610, 46 and 1.1 ng/μL in the vector control, MDA5#66 and MDA5#34 group, respectively. There were significantly lower ($p < 0.05$) IBDV RNA loads in the MDA5#66 and MDA5#34 groups than that in the vector control group at 8,

16 and 24 hpi. In addition, IBDV RNA loads in the MDA5#34 group were significantly lower (p < 0.05) than those in the MDA5#66 group at 8, 16 and 24 hpi.

Transcript levels of innate and antiviral genes

Significantly higher upregulation (p < 0.05) of chicken MDA5, IRF-3 and IFN-β genes was seen in the MDA5#66 and MDA5#34 groups than that in the vector control group at 2, 8 and 16 hpi (Fig. 11a, c and d). The fold changes of chMDA5 expression peaked at 2 hpi and decreased gradually throughout the study period in the MDA5#66 and MDA5#34 groups. The fold changes of chicken IRF-3 and IFN-β were significantly higher (p < 0.05) in groups of chMDA5-overexpressing DF-1 cells than in the vector control group at 2, 8 and 16 hpi, with the highest fold increases of 122- and 9850-fold at 16 hpi (Fig. 11c and d). Chicken IPS-1 showed significantly higher (p < 0.05) fold changes in the MDA5#34 group than in the MDA5#66 and vector control groups at 2 and 16 hpi (Fig. 11b).

Chicken PKR, OAS, Mx and MHC class I gene transcripts had significantly higher (p < 0.05) fold changes, up to 39-, 562-, 265- and 61-fold, respectively, in the MDA5#34 group at 2 hpi compared to the vector control group (Fig. 11e-h). The upregulated mRNA expressions of chicken PKR, OAS and Mx in the MDA5#66 and MDA5#34 groups gradually returned to the baseline level, and their expression levels were not significantly different (p > 0.05) from those in the vector group between 8 and 24 hpi (Fig. 11e-g). The expression level of chicken MHC class I in the chMDA5-overexpressing group were significantly higher (p < 0.05) than in the vector group at 2, 8 and 16 hpi (Fig. 11h).

Discussion

IBDV replicated in DF-1 cells and caused upregulation of chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I in a virus-dose-dependent manner. Chicken MDA5 and IBDV-derived dsRNA were both localized to the cytoplasm of DF-1 cells. During IBDV infection, DF-1 cells with chMDA5 knockdown had significantly lower (p < 0.05) fold changes of chicken MDA5, IRF-3, IFN-β and MHC class I transcripts, and significantly higher (p < 0.05) virus titers and viral RNA loads at 16 and 24 hpi, indicating that chMDA5 was involved in the activation and regulation of these genes in IBDV-infected DF-1 cells. In addition, overexpression of chMDA5 in DF-1 cells led to significantly higher (p < 0.05) expression of chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I transcripts at 2 hpi, and significantly lower (p < 0.05) IBDV titers and RNA loads at various time

points, indicating that the enhanced chMDA5 expression in DF-1 cells suppressed IBDV replication.

Intracellular IBDV loads and extracellular IBDV titers in IBDV-infected DF-1 cells increased in a virus-dose-dependent manner. The increase in viral RNA loads during IBDV infection occurred earlier than that of virus titers, indicating that IBDV replicated within the cells first and then was released to the supernatant.

IFN-β is known to induce the expression of IFN-stimulated genes, including PKR, OAS, Mx and MHC class I, which contribute to antiviral activation and protection against virus infections [2, 13]. Chicken IFN-β has been cloned and expressed in a prokaryotic system and shows antiviral activity against VSV and IBDV [3, 42, 44]. The induction of chicken Mx promoter activity by chicken IFN-β demonstrates the activity of chicken IFN-β to induce IFN-stimulated genes [41]. In the present study, chicken IFN-β showed a virus-dose-dependent upregulation in DF-1 cells upon IBDV infection, suggesting its important role in antiviral defense.

IFN-induced PKR activated by dsRNA facilitates the phosphorylation of protein translation initiation factor eIF2 [21], which leads to termination of protein synthesis in the host cell [51]. This prevents the virus from taking advantage of cellular translation machinery and limits viral protein synthesis. The virus-dose-dependent upregulation of chicken PKR in the present study indicates the activation of chicken PKR upon IBDV infection.

In one study, dsRNA-activated OAS produced 2′, 5′-oligoadenylates, which in turn activated RNaseL to cleave viral RNA. Induction of chicken OAS in IBDV-infected chicken embryo cells (CEC) was demonstrated at 1, 2, 3, 4, 5, 6 and 7 days postinfection (dpi) by microarray analysis, and activation of the OAS-RNaseL system by the IBDV dsRNA genome or its replication intermediates has been suggested [24]. In the present study, the virus-dose-dependent upregulation of chicken OAS in IBDV-infected DF-1 cells was consistent with previous findings [24], indicating the activation of the chicken OAS-RNaseL system by IBDV.

Chicken Mx was previously shown to be upregulated significantly (p < 0.05) in IBDV-infected chicken embryo fibroblasts at 1, 2, 3 and 4 dpi [52] and in IBDV-infected bursa at 1, 2 and 4 dpi [40]. Chicken Mx was demonstrated previously to have antiviral activity [18, 19]. In agreement with previous findings, the present study showed that IBDV infection upregulated chicken Mx expression in DF-1 cells in a dose-dependent manner, indicating the activation of chicken Mx upon IBDV infection.

Increased expression of MHC class I is seen during flaviviral infection [26]. Ducks infected with a highly pathogenic avian influenza virus have upregulation of MHC class I by 1700-fold in the lung, and such

upregulation of MHC class I is most likely due to the enhanced expression of type I IFN upon virus infection [47]. In addition, there is significant upregulation of MHC class I ($p < 0.05$) in IBDV-infected CEC at 1, 2, 3, 4, 5, 6 and 7 dpi [24]. The results of the present study were consistent with the findings from previous studies.

The dsRNA derived from IBDV infection in DF-1 cells was detected using the dsRNA-specific antibody J2, which has been shown to recognize dsRNA produced in cells infected with West Nile virus, encephalomyocarditis virus (EMCV), reovirus and severe acute respiratory syndrome (SARS) coronavirus [6, 49]. In the present study, dsRNA-positive signals were observed as aggregates located in the cytoplasm where chMDA5 was localized or colocalized, suggesting that IBDV dsRNA is recognized by chMDA5 in the cytoplasm, and such recognition and interaction is a starting point for the IBDV-induced chMDA5 signaling pathway.

Chicken IPS-1 has been shown to mediate the induction of chicken IFN-β, downstream of chicken MDA5 and upstream of chicken IRF-3 [25], which is compatible with the signaling order in the mammalian RLR pathway. In previous studies of fish IPS-1, relatively stable expression of IPS-1 was found upon infection with a rhabdovirus or an orthomyxovirus [22, 43]. There was no significant increase of chicken IPS-1 expression in DF-1 cells upon IBDV infection in the present study. However, chicken IPS-1-knockdown DF-1 cells infected with IBDV exhibited significantly reduced IFN-β expression, suggesting that chicken IPS-1 is needed to induce IFN-β in the MDA5 signaling pathway during IBDV infection.

Chicken IRF-3 has been cloned and shown to bind optimal binding sites as a dimer [8, 9]. Its DNA binding domain has been shown to bind an IFN-stimulated response element (ISRE) in the promoter region of the chicken Mx gene. The expression of chicken IRF-3 is induced rapidly by treatment with poly(I:C) or IFNs [8, 27]. In the present study, chicken IRF-3, along with chicken MDA5, was activated by IBDV infection in a dose-dependent manner. In addition, chicken IRF-3 was involved in the induction of IFN-β in DF-1 cells upon IBDV infection. These findings were similar to those reported previously, showing that avian influenza virus infection activated the chicken RLR pathway, including chicken MDA5, IPS-1 and IRF-3, and mediated IFN-β activation [25]. Taken together, IBDV infection in DF-1 cells activated the expression of chicken IFN-β and antiviral genes, involving the MDA5 signaling pathway, including chicken IPS-1 and IRF-3.

Mouse MDA5 has been shown to play a dominant role in the control of EMCV and murine norovirus-1 infection [14, 28]. Significantly enhanced ($p < 0.05$) IBDV replication in chMDA5-knockdown DF-1 cells at 16 and 24 hpi indicated that chMDA5 played a role in the control of IBDV replication in DF-1 cells during IBDV infection.

Chicken MDA5 was shown previously to sense avian influenza virus infection to induce chicken IFN-β mRNA synthesis [25]. Similarly, the knockdown of chMDA5 in DF-1 cells significantly reduced ($p < 0.05$) the activation of chicken IFN-β, indicating that chMDA5 is involved in the recognition of IBDV. However, the expression levels of antiviral genes, including PKR, OAS and Mx, in the siRNA groups were not significantly lower ($p > 0.05$) than those in the control siRNA groups, suggesting that chMDA5 knockdown did not impact the expression of these genes. Possible explanations are as follows: 1. Some DF-1 cells may not be transfected adequately with chMDA5-targeting siRNA due to the inefficient transfection of DF-1 cells. 2. PKR and OAS are not only IFN-stimulated genes, but also dsRNA sensors [33]. Thus, PKR and OAS may be upregulated by directly sensing dsRNA derived from IBDV infection. 3. Other innate immune sensors may have a role in the activation of these antiviral genes upon IBDV infection, as has been shown in the case of avian influenza virus infection [46]. Therefore, chMDA5-mediated signaling was responsible for the recognition of IBDV and the activation of chicken IRF-3, IFN-β and MHC class I in chicken fibroblasts infected with IBDV.

In the present study, transfection of DF-1 cells with chMDA5-expressing constructs (MDA5#66 or MDA5#34) significantly enhanced the expression of chicken MDA5, IPS-1 and IFN-β, which is compatible with a previous report that the expression of chicken MDA5 was increased in DF-1 cells transfected with a dsRNA analog, poly(I:C) [23]. Transfection of DF-1 cells with two chMDA5-expressing constructs induced similar levels of chMDA5 expression, while MDA5#34 induced significantly more chicken IFN-β transcripts than MDA5#66. This suggests that pCDNA-MDA5#66 DNA is a less potent activator of chicken IFN-β than pCDNA-MDA5#34 in DF-1 cells. Possible explanations for the lower expression of chicken IFN-β by pCDNA-MDA5#66 may include the following: 1. The longer N-terminal segment encoded by pCDNA-MDA5#66 may interfere with the signaling by CARD-CARD interaction. 2. Although chMDA5 mRNA transcripts induced by the two DNA constructs were increased to a similar level, less chMDA5 protein was synthesized in the MDA5#66 group, probably due to a lower translation efficiency, as shown in a previous report [48].

Overexpression of Japanese flounder MDA5 has been reported to induce a significantly lower ($p < 0.05$) viral hemorrhagic septicemia virus (VHSV) titer in cells, and lower cytopathic effects in cells infected with infectious pancreatic necrosis virus (IPNV), indicating that overexpression of fish MDA5 suppressed virus infection [35]. Similarly, lower titers of VHSV or alphavirus were shown when rainbow trout MDA5 was overexpressed in fish cells [4]. In addition, enhanced expression of fish Mx gene was

demonstrated when fish MDA5 was overexpressed in fish cells [4, 35]. Consistent with previous findings, overexpression of chMDA5 limited IBDV replication in DF-1 cells in the present study. The lower IBDV titers and RNA loads in DF-1 cells with chMDA5 overexpression could be attributed to the enhanced upregulation of innate and antiviral genes, including chicken MDA5, IRF-3, IFN-β, PKR, OAS, Mx and MHC class I.

Significant upregulation ($p < 0.05$) of chicken MDA5, IRF-3 and IFN-β in the chMDA5-overexpressing cells during IBDV infection suggested the possibility of persistent activation of the chMDA5 signaling pathway. However, similar expression levels of PKR, OAS or Mx in the vector and chMDA5-overexpressing groups at 8, 16 and 24 hpi may be attributable to the following factors: 1. The decreased IBDV replication in the chMDA5-overexpressing group led to decreased activation of antiviral genes, as observed in study 1 in the present report. 2. The upregulation of antiviral genes resulting from the transfection of chMDA5 diminished gradually.

In conclusion, chicken MDA5 recognized IBDV infection in chicken fibroblasts and initiated the MDA5 signaling pathway by activating chicken IPS-1 and IRF-3, leading to the upregulation of chicken IFN-β and MHC class I. Overexpression of chicken MDA5 in chicken fibroblasts infected with IBDV enhanced expression of innate and antiviral genes, contributing to increased inhibition of IBDV replication.

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