Veterinary Microbiology 230 (2019) 78–85

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Duck RIG-I restricts duck enteritis virus infection

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ARTICLE INFO

Keywords:
RIG-I
Duck enteritis virus
IFN-β
STAT1
Antiviral response

ABSTRACT

Retinoic acid-inducible gene I (RIG-I) is a nucleic acid sensor that plays a key role in host antiviral defenses. Duck viral enteritis (DEV) is a DNA virus that causes significant economic losses to the poultry industry worldwide. Although RIG-I is known to be involved in a common antiviral signaling pathway triggered by RNA viruses, its role in DEV infection remains unclear. In this study, we demonstrated that DEV infection increased the expression levels of interferon β (IFN-β) and RIG-I in ducks both *in vivo* and *in vitro*. Furthermore, overexpression of duck RIG-I significantly upregulated the expression of interferon-stimulated genes, including myxovirus resistance protein (Mx), Interferon-induced oligodenylate synthetase-like (OASL) and IFN-β. We therefore used overexpression and knockdown methods to determine if RIG-I affected DEV infection in ducks. Viral infection was inhibited by RIG-I, and enhanced by knockdown of RIG-I expression using small interfering RNA. RIG-I overexpression also activated signal transducer and activator of transcription 1 (STAT1), as a member of the JAK-STAT family. The combined results following STAT1 knockdown and RIG-I overexpression suggested that the antiviral activity of RIG-I was STAT1-dependent. Overall, these findings indicate that RIG-I effectively restricts DEV replication and may play a vital role in the host immune response to DEV infection in ducks.

1. Introduction

Duck viral enteritis, also known as called ‘duck plague’, is an acute and highly contagious disease of waterfowl caused by duck enteritis virus (DEV; family *Herpesviridae*, subfamily *Alphaherpesvirinae*), which is an enveloped, double-stranded DNA virus (Gardner et al., 1993; Zhao et al., 2009). Duck viral enteritis is characterized by vascular damage, eruptions on the mucosal surface of the gastrointestinal tract, lesions of the lymphoid organs, and degenerative changes in parenchymatous organs (Dhama et al., 2017). Natural infections occur in ducklings ranging in age from 7 days to mature breeders. Lethal DEV infections can cause 100% mortality in ducklings. The global distribution of the disease means that it causes great economic losses to the waterfowl industry worldwide (Gough and Alexander, 1990; Wang et al., 2013).

The innate immune system plays an essential role in defending the host against viral infection. Specific host pattern recognition receptors recognize various pathogen components and initiate signaling events, resulting in the production of cytokines such as type I interferon (IFN), which in turn induces the expression of a series of ISG transcripts during viral infection and establishes an antiviral state that limits viral replication. Pattern recognition receptors are categorized into distinct families, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and cytosolic viral DNA sensors (Elinav et al., 2011; Kawai and Akira, 2010; Loo and Gale, 2011).

Three members of the RLR family have been identified to date: RIG-I, melanoma differentiation associated factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Yoneyama et al., 2005). RIG-I, which was the first RLR to be identified and remains the best characterized, recognizes short, double-stranded RNA (dsRNA; < 1 kb) and uncapped 5′-triphosphate single-stranded RNA (ssRNA; 5′-ppp-ssRNA). MDA5 senses longer dsRNAs (> 1 kb) and synthetic dsRNAs, such as polyinosinic: polycytidylic acid (poly [I:C]). RIG-I contains a DExD/H-box helicase-like domain containing ATPase and translocase activities, a repressor regulatory domain (RD) at the C-terminus, and two caspase-associated recruitment domains (CARDs) at the N-terminus. A model for RIG-I activation has been proposed based on structural and functional studies. RIG-I remains inactive in the absence of its ligand (non-self RNA generated during viral infection), while binding of dsRNA or 5′-ppp-ssRNA to the basic cleft in the CTD induces a conformational change in RIG-I, which causes the CARDs to be uncovered in the presence of ATP. The CARDs then interact with the adaptor protein MAVS to transduce a signal (Takahasi et al., 2008). The immune response

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https://doi.org/10.1016/j.vetmic.2019.01.014
Received 31 October 2018; Received in revised form 12 January 2019; Accepted 14 January 2019
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triggered by RIG-I is one of the most important lines of defense against viral infection.

In its role as a viral RNA sensor, RIG-I is involved in the recognition of a wide variety of RNA viruses, including Newcastle disease virus (NDV), rabies virus (RV), vesicular stomatitis virus (VSV), Ebola virus, Japanese encephalitis virus (JEV) and Hepatitis C virus (HCV) (Kato et al., 2008, 2006; Spiropoulou et al., 2009). However, few studies have examined the function of RIG-I in DNA virus infections. Chiang et al. demonstrated that the TRIM25-RIG-I-MAVS triad was important for eliciting an antiviral immune response to human papillomavirus type 16 infection (Chiang et al., 2018), but the role of duck RIG-I in DEV infection has not been clarified. In this study, we examined the relationship between duck RIG-I signaling and DEV infection. DEV infection upregulated RIG-I expression both *in vivo* and *in vitro*, and the upregulated RIG-I subsequently restricted viral infection. The antiviral effect of RIG-I was dependent upon STAT1-mediated signaling. The results of this study further our understanding of the role of duck RIG-I in viral infection.

2. Materials and methods

2.1. Cells and viruses

DEF cells were prepared from 11-day-old duck embryos and maintained in complete Dulbecco's modified Eagle medium (DMEM; Gibco, China) supplemented with 5% fetal bovine serum (FBS; Clark, USA) at 37°C in 5% CO_2. HEK 293 T cells were cultured similarly in DMEM supplemented with 10% FBS. The DEV CSC strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China). A mouse monoclonal antibody directed against glycoprotein B (gB) was maintained in our laboratory.

2.2. Plasmid construction and transfection

The duck RIG-I gene was amplified from DEF cDNA using the primers listed in Table 1 and then cloned into the lentiviral vector pLVX-IRES-ZsGreen (Clontech, USA). A recombinant lentivirus was constructed by transfecting HEK 293 T cells with the plasmids pLVX-RIG-I-ZsGreen, psPAX2, and pLP/VSVG. DEF and HEK 293 T cells at 80% confluence were transfected with the recombinant lentivirus at a multiplicity of infection (MOI) of 50. The cells were used for further analysis at 24 h post-infection (hpi).

2.3. Viral $TCID_{50}$ determination

DEV titers were determined by endpoint dilution assay using the Reed-Münch method and expressed as the $TCID_{50}$/ml. DEF cells were

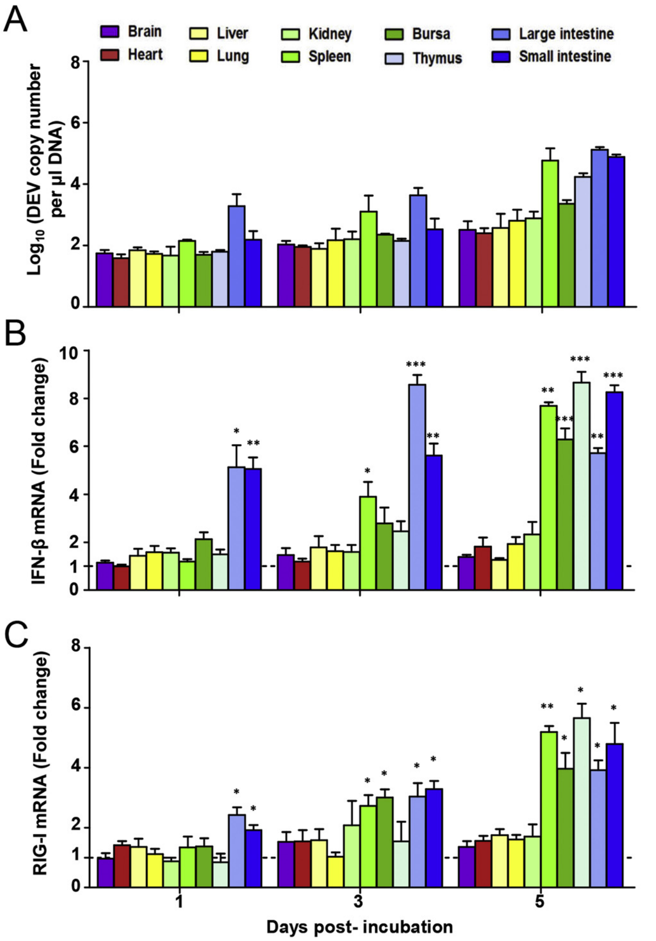

Fig. 1. Effects of DEV on expression of IFN-β and RIG-I *in vivo*. Eighteen 21-day-old specific-pathogen-free ducklings were divided randomly into an infection group (intramuscular injection of 0.2 ml DEV ($TCID_{50} = 10^5$/ml) and a control group (intramuscular injection of 0.2 ml DMEM). Three ducklings from each group were killed and tissue samples were collected at 1, 3, and 5 dpi. (A) Viral DNA loads, (B) IFN-β gene expression levels, and (C) RIG-I gene expression levels were measured in various organs by RT-qPCR. Each sample was run in triplicate. Error bars represent standard deviations. Significant differences in IFN-β and RIG-I expression levels between DEV-infected and mock-infected groups were assessed using the Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Primers used in this study.

Primer	Sequence (5'-3')	Use
Flag-dRIG-I-F	AATACTCGAGATGACGGCGGACGAGAAGCG	Amplification of dRIG-I
Flag-dRIG-I-R	ATAGCGGCCGCCTActtgtcgtcgtcgtccttgtagtcAAATGGTGGGTACAAGT	Amplification of dRIG-I
Q-dIFN-β-F	AGATGGCTCCCAGCTCTACA	QRT-PCR for detection of dIFN-β
Q-dIFN-β-R	AGTGGTTGAGCTGGTTGAGG	QRT-PCR for detection of dIFN-β
Q-dRIG-I-F	GTGTATGGAGGAAAACCCTATTTCTTAACT	QRT-PCR for detection of dRIG-I
Q-dRIG-I-R	GGAGGGTCATACCTGTTGTTTGAT	QRT-PCR for detection of dRIG-I
Q-dDEV-F	TGGGAAGGCTTTCGGTCGC	QRT-PCR for detection of DEV
Q-dDEV-R	CATTCGCGCCTTTGCTAAATTCTCT	QRT-PCR for detection of DEV
Q-dMx-F	TGCTGTCCTTCATGACTTCG	QRT-PCR for detection of dMx
Q-dMx-R	GCTTTGCTGAGCCGATTAAC	QRT-PCR for detection of dMx
Q-dOASL-F	TCTTCCTCAGCTGCTTCTCC	QRT-PCR for detection of dOASL
Q-dOASL-R	ACTTCGATGGACTCGCTGTT	QRT-PCR for detection of dOASL
Q-dSTAT1-F	AGGACCAGAGTCCAGGAACA	QRT-PCR for detection of dSTAT1
Q-dSTAT1-R	TGGGGCTCGTTTTGAGATCC	QRT-PCR for detection of dSTAT1
Q-dβ-actin-F	GATCACAGCCCTGGCACC	QRT-PCR for detection of dβ-actin
Q-dβ-actin-R	CGGATTCATCATACTCCTGCTT	QRT-PCR for detection of dβ-actin

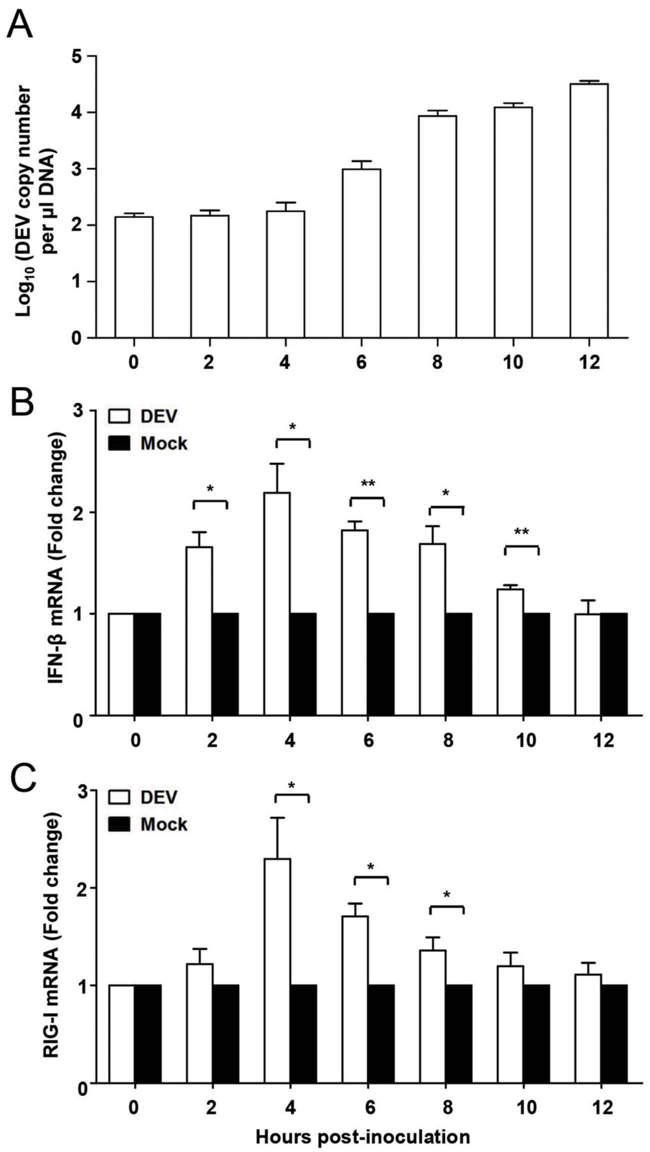

Fig. 2. Effects of DEV on expression of IFN-β and RIG-I *in vitro*. DEF cells were infected with DEV at an MOI of 0.1 for 0, 2, 4, 6, 8, 10 or 12 h and cell samples were collected. (A) Intracellular viral DNA loads. (B) IFN-β gene expression levels and (C) RIG-I gene expression levels in DEV-infected DEF cells were examined by RT-qPCR. Significant differences in IFN-β and RIG-I expression levels between DEV-infected and mock-infected DEF cells were assessed using the Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

seeded in 96-well plates and inoculated with serial 10-fold dilutions of DEV, with eight replicates per dilution. DEV was removed after adsorption for 2 h at 37°C and DMEM with 2% FBS was added to each well. The plates were then incubated for 96 h, and the viral titers were calculated based on the cytopathic effect.

2.4. Real-time quantitative polymerase chain reaction

Viral DNA was extracted from the tissues and DEF cells using a Nucleic Acid Extraction Kit (Axygen, USA) and quantified by TaqMan real-time quantitative polymerase chain reaction (RT-qPCR). The primers and probes are listed in Table 1. RT-qPCR was performed with the following cycling conditions: 95 °C for 30 s for activation, followed by 40 cycles at 95 °C for 5 s and 60 °C for 50 s. Total RNA was isolated from the tissues and DEF cells using an RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed into cDNA with a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). The cDNA generated was analyzed by RT-qPCR with 2 × SYBR Green qPCR Master Mix (S-2014-T) (US Everbright Inc, Suzhou, China). The specific primers used to amplify duck RIG-I, IFN-β, Mx, OASL and β-actin cDNAs have been described previously (Barber et al., 2010; Chen et al., 2018). qPCR was conducted under the following cycling conditions: 95 °C for 30 s for activation, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The relative expression levels of duck RIG-I, IFN-β, Mx and OASL were determined using the comparative Ct ($2^{-\triangle\triangle t}$) method with the β-actin gene as a control.

2.5. Western blotting assay

Whole-cell lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer of the proteins onto nitrocellulose membranes (Millipore, USA) according to the manufacturer's instructions. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated for 2 h at room temperature with the following primary antibodies: rabbit anti-STAT1 (OmnimAbs, USA), rabbit anti-pSTAT1 (OmnimAbs, USA), mouse anti-β-actin (Sigma, Canada), or mouse anti-gB antibody. IRDye-800-CW-COnjugated goat anti-mouse IgG antibody (LI-COR Biosciences, USA) or IRDye-800CW-COnjugated goat anti-rabbit IgG antibody (LI-COR Biosciences, USA) was used as the secondary antibody at a dilution of 1:5000. Signal detection was performed using an Odyssey Infrared Fluorescence Scanning Imaging System (LI-COR Biosciences, USA).

2.6. RNA interference assay

Small interfering RNAs (siRNAs) directed against the duck RIG-I transcript (siRIG-I) were synthesized by GenePharma (Suzhou, China). The siRIG-I sequencewere 5′-GCGAAUUCACAAUCCCUUUTT-3′ (sense) and 5′-AAAGGGAUUGUGAAUUCGCTT-3′ (antisense), the sequences targeting duck STAT1 (siSTAT1) were 5′-GGAGCAAUCACGUUUACUUTT-3′ (sense) and 5′-AAGUAAACGUGAUUGCUCCTT-3′ (antisense), and the non-targeting control siRNA (siNC) sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). DEF cells grown to 80% confluence on a six-well plate were transfected with 200 nM siRNA or siNC using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, USA) according to the manufacturer's instructions. The DEF cells were then infected with DEV at an MOI of 0.1 at 36 h post-transfection. The cell medium was replaced with DMEM containing 2% FBS after 2 h, and the cells were then incubated at 37°C. For 24, 36, and 48 h, respectively, after which the supernatants were collected for analysis. DEF cells were collected for further analysis at 48 h post-transfection.

2.7. Animal infection experiments

Eighteen 21-day-old specific-pathogen-free ducklings were obtained from the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The ducklings were divided randomly into two groups: an infection group that received an intramuscular injection of 0.2 ml of DEV per duck ($TCID_{50} = 10^5$/ml) and a control group that received an intramuscular injection of 0.2 ml of DMEM. Three ducklings from each group were killed at 1, 3, and 5 days post-infection (dpi), respectively, and tissue samples were collected. The viral loads in the different tissues were detected by TaqMan RT-qPCR, as described above, and IFN-β and RIG-I mRNA expression levels were evaluated by qPCR as described above. All animal experiments were performed according to the Guidelines for Animal Experimentation of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

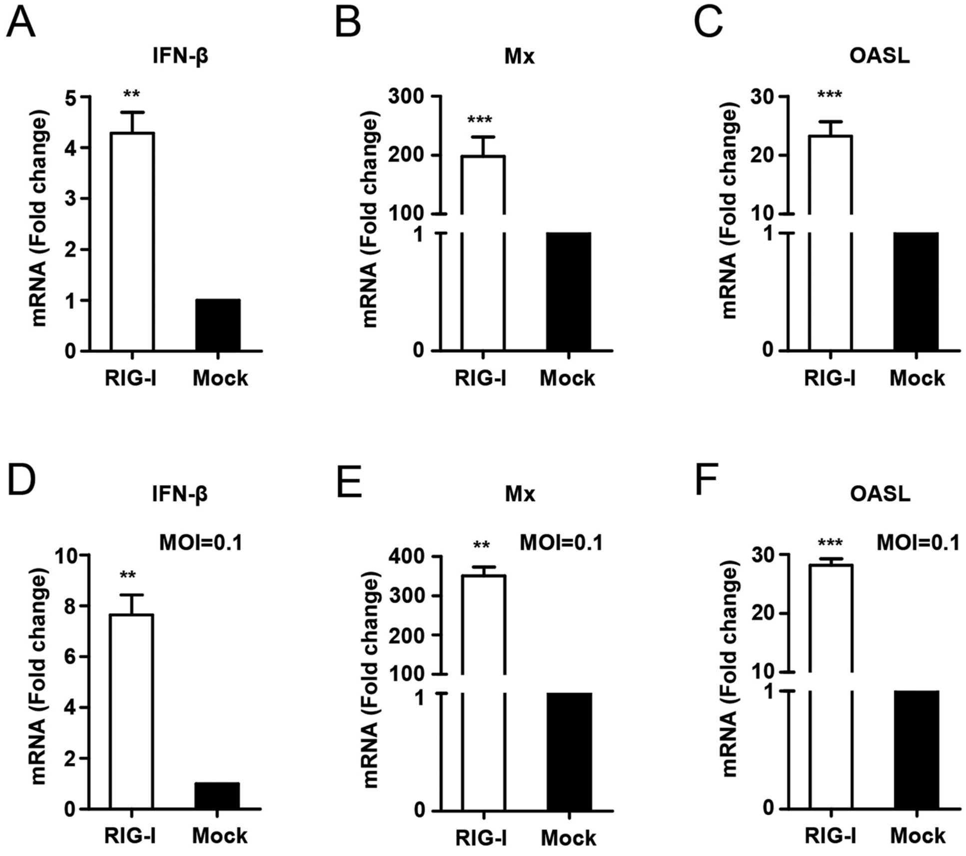

Fig. 3. IFN-β and ISG expression levels in DEF cells. (A–C) DEF cells were transfected with a lentiviral vector expressing duck RIG-I and a lentivirus encoding ZsGreen for 48 h and IFN-β, Mx and OASL gene expression levels were detected by RT-qPCR. (D–F) DEF cells were transfected with a lentiviral vector expressing duck RIG-I and a lentivirus encoding ZsGreen for 24 h, and were then infected with DEV at a MOI of 1 for 24 h. DEF cells were then collected and IFN-β, Mx and OASL gene expression levels were detected by RT-qPCR. Significant differences in IFN-β, Mx and OASL expression levels between RIG-I-transfected and mock-transfected DEF cells were assessed using the Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Effects of DEV on the expression of duck IFN-β and RIG-I in vivo

To determine if DEV infection activated the host innate immune response *in vivo*, we collected brain, heart, liver, spleen, lung, kidney, bursa of Fabricius, thymus, large intestine, and small intestine from infected ducklings at the indicated times, and determined the viral loads in the different tissues using TaqMan RT-qPCR. DEV was detected in all the tested tissues at all three time points in the infected group (Fig. 1A). At 1 dpi, the viral load was highest in the large intestine ($10^{3.29}$ copies), while the load was also high in the small intestine ($10^{2.38}$ copies). The viral load in the spleen reached $10^{2.15}$ copies, but the viral loads in the other organs were all below 10^2 copies. The viral load remained highest in the large intestine at 3 dpi ($10^{3.77}$ copies), while loads in the spleen and small intestine reached $10^{3.64}$ and $10^{2.82}$ copies, respectively, and the load was lowest in the lung ($10^{2.39}$ copies). The viral loads in the various tissues increased at 5 dpi, with the highest load again observed in the large intestine ($10^{5.61}$ copies), followed by the small intestine and spleen. Notably, the viral load in the thymus was $10^{4.27}$ copies, indicating that DEV could replicate quickly in the immune system. No viral DNA was detected in any tissues in the control group. Collectively, these results indicated that DEV replicated quickly in many organs, suggesting that the immune response induced by DEV required further study.

We therefore determined if DEV infection induced the expression of IFN-β, and showed that IFN-β expression was universally upregulated in response to DEV infection in ducks (Fig. 1B). IFN-β was only upregulated in the intestinal tract at 1 dpi, but IFN-β mRNA levels were increased in the large intestine, small intestine, and spleen at 3 dpi, and in immune-related tissues such as the spleen, bursa of Fabricius and thymus at 5 dpi. Notably, IFN-β expression was significantly upregulated in the large intestine and small intestine at all time points. Because RIG-I acts as both a sensor and effector in the IFN antiviral signaling pathway, we hypothesized that RIG-I may also be activated in response to DEV infection. We therefore assessed RIG-I expression in different tissues, and showed that RIG-I mRNA levels followed similar trends to IFN-β mRNA at all three time points (Fig. 1C). Specifically, RIG-I mRNA was significantly upregulated in the intestinal tract and immune organs. The corresponding high viral loads and high IFN-β and RIG-I expression levels in the intestinal tract and immune organs implied the existence of a good correlation between DEV load and the host's innate immune response. DEV infection thus strongly enhanced the transcription of IFN-β and RIG-I in ducks *in vivo*, indicating that duck RIG-I was involved in the recognition of DEV in different organs.

3.2. Effects of DEV on IFN-β and RIG-I expression in vitro

To confirm that DEV infection activated the host innate immune response *in vitro*, we infected DEF cells with DEV and determined the intracellular viral DNA loads and IFN-β and RIG-I mRNA expression levels from 0 to 12 h. Intracellular viral DNA loads increased continuously with increasing duration of DEV infection (Fig. 2A). Meanwhile, IFN-β mRNA levels were increased at 2 hpi, peaked at 4 hpi, and began to decline from 6 hpi (Fig. 2B). RIG-I mRNA levels showed a similar trend to IFN-β mRNA, increasing significantly at 4 hpi and returning to baseline levels at 12 hpi (Fig. 2C). These data demonstrate that IFN-β and RIG-I expression levels increased significantly *in vitro* following DEV infection, indicating that they may play important roles in resisting DEV infection.

3.3. Overexpression of RIG-I induces IFN-β and ISG expression in DEF cells

We further examined the ability of RIG-I to induce IFN-β and ISGs by analyzing the expression levels, of IFN-β, Mx and OASL by qPCR. Overexpression of RIG-I in infected or uninfected DEF cells upregulated

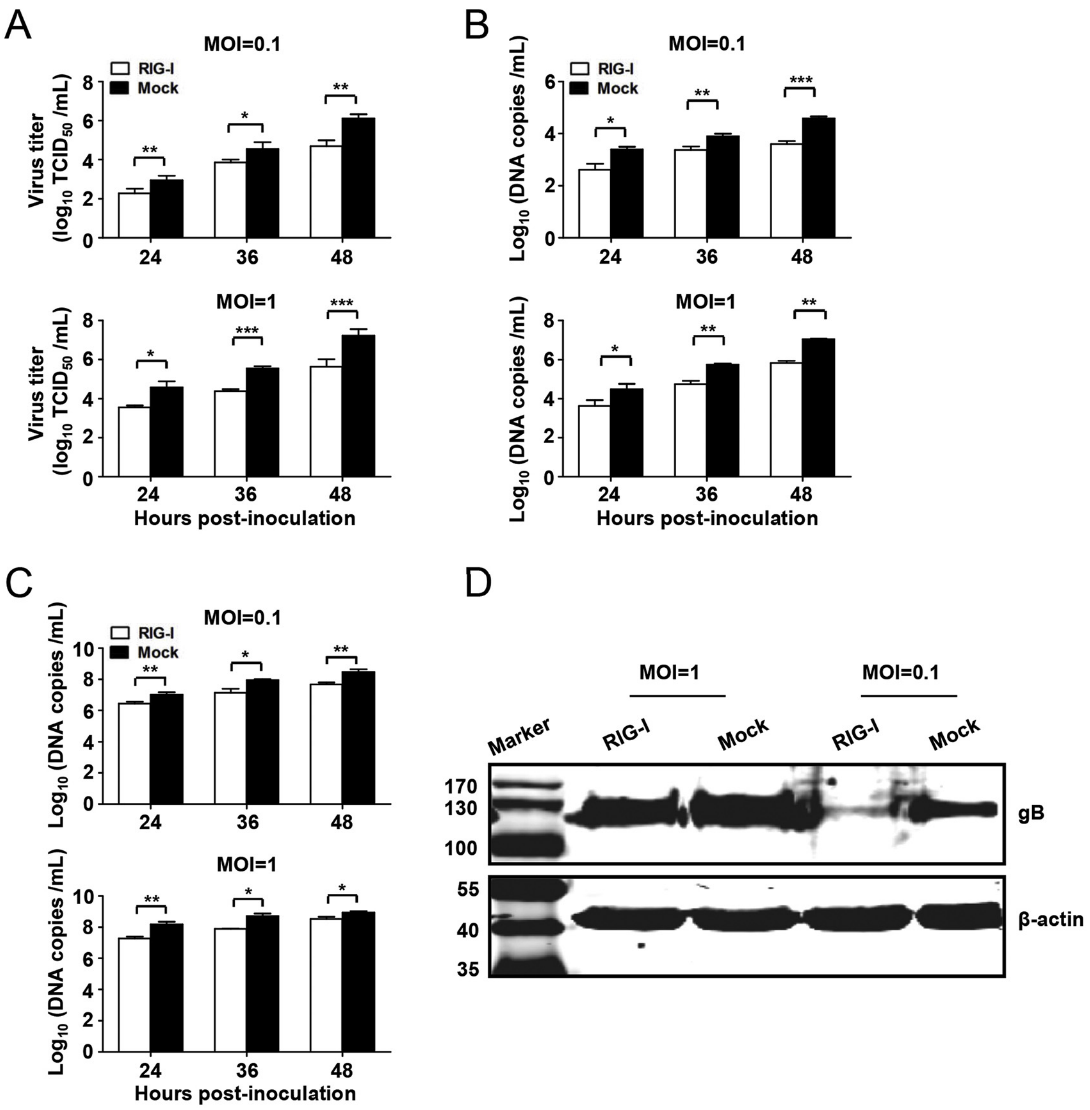

Fig. 4. RIG-I inhibits DEV infection. DEF cells were transfected with a lentiviral vector expressing duck RIG-I and a lentivirus encoding ZsGreen, and were then infected with DEV at an MOI of 0.1 or 1 for 24, 36 and 48 h, respectively. (A) Viral titers in the supernatants were detected by endpoint dilution assay and are presented as $TCID_{50}$/ml. (B) Copies of DEV in the supernatants and (C) Intracellular genomic copy numbers of DEV were quantified by RT-qPCR. (D) Expression of gB in cell lysates was analyzed by western blotting, with β-actin protein as the control. Each sample was run in triplicate. Error bars represent standard deviations. Significant differences in virus titer and DNA copies between RIG-I-transfected and mock-transfected DEF cells were assessed using the Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

IFN-β expression 4.28- and 7.65-fold, respectively ($P < 0.01$; Fig. 3A and D). Notably, Mx expression increased by 197.72- and 350.9-fold, respectively ($P < 0.01$; Fig. 3B and E), while OASL expression increased by 23.26- and 28.15-fold, respectively ($P < 0.001$; Fig. 3C and F).

3.4. Overexpression of RIG-I inhibits DEV infection

To determine if RIG-I plays a role in inhibiting DEV infection, we transfected DEF cells with recombinant lentivirus and then challenged them with DEV at MOIs of 0.1 and 1. The viral titers and the numbers of viral genome copies both decreased in the supernatant of DEFs in RIG-I-transfected compared with vector-treated control DEF cells at 24, 36, and 48 hpi (Fig. 4A and B). We also quantified intracellular viral genome copy numbers in infected cells at various time points after infection, and showed that the viral genome copy number was significantly reduced in RIG-I-transfected compared with vector-treated control DEF cells at 24, 36, and 48 h (Fig. 4C). gB protein expression levels were also reduced in RIG-I-transfected cells (Fig. 4D). These data indicated that RIG-I played an essential role in DEV infection.

3.5. Knockdown of RIG-I promotes DEV infection

We confirmed the effects of RIG-I on DEV infection by analyzing the effects of RIG-I knockdown by siRIG-I on DEV infection in DEF cells (Fig. 5A). The viral titer and viral genome copy number were higher in siRIG-I-transfected compared with siNC-transfected DEF cells (Fig. 5B and C). Western blotting analysis of gB expression also confirmed that DEV infection was enhanced in siRIG-I-transfected DEF cells (Fig. 5D). Collectively, these results suggest that knockdown of RIG-I expression enhanced DEV infection.

3.6. Antiviral activity of RIG-I is abolished by STAT1-knockdown

We investigated the downstream factors *via* which RIG-I activates the antiviral response. Several studies have suggested that RIG-I acts as a tumor suppressor by increasing the activation of STAT1 (Xu et al., 2018). We therefore determined if STAT1 was responsible for the

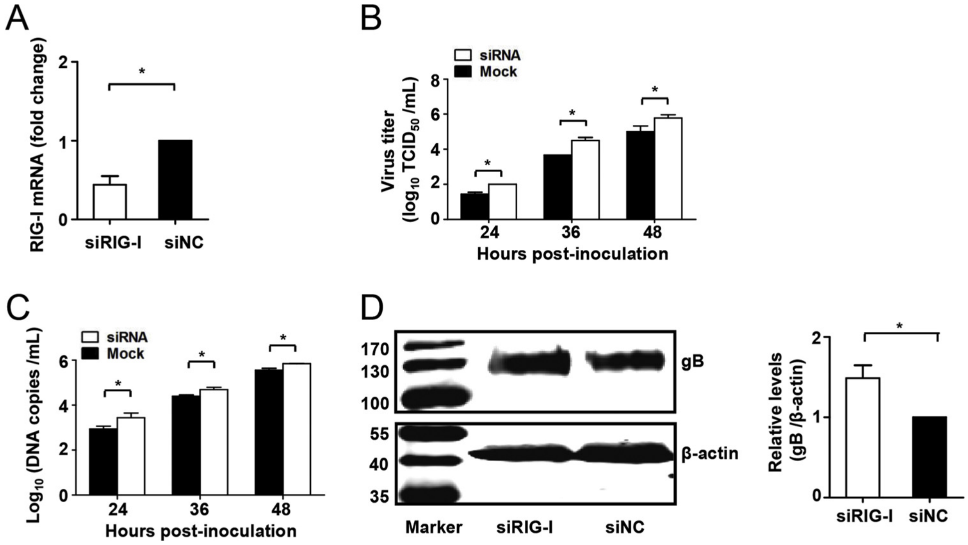

Fig. 5. Knockdown of duck RIG-I promotes DEV infection. (A) DEF cells transfected with siRIG-I or siNC were harvested at 36 h after transfection and the efficiency of RIG-I knockdown was confirmed by RT-qPCR. (B–D) DEF cells were transfected with siRIG-I or siNC mRNA for 36 h, and then infected with DEV at an MOI of 0.1 or 1 for 24, 36 or 48 h, respectively. (B) Viral titers in the supernatants were detected by endpoint dilution assay and are presented as $TCID_{50}$ /ml. (C) Copies of DEV in the supernatants were assessed by RT-qPCR. (D) Expression of gB in the cell lysates was analyzed by western blotting, with β-actin protein as the control. Each sample was run in triplicate. Error bars represent standard deviations. Statistical significance was assessed using the Student's t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

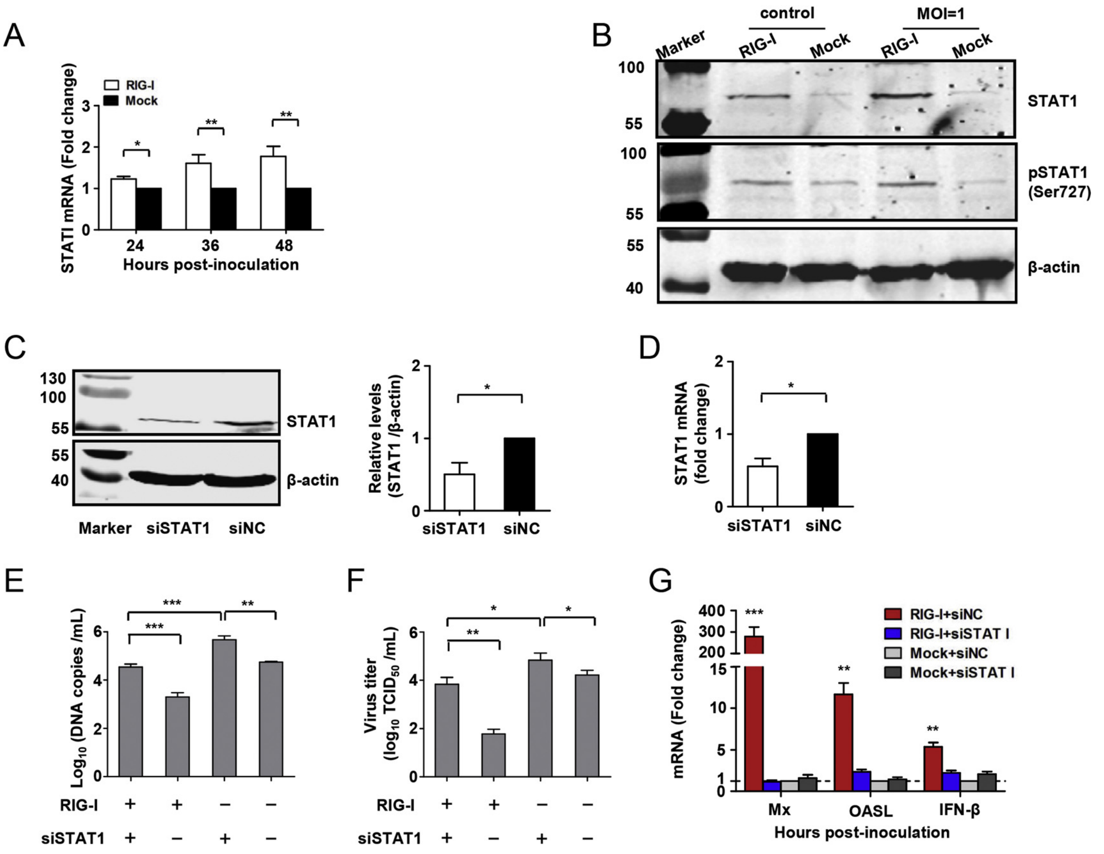

Fig. 6. Antiviral activity of duck RIG-I was abolished in STAT1-knockdown DEF cells. (A) DEF cells were transfected with a lentiviral vector expressing duck RIG-I and a lentivirus encoding ZsGreen for 24, 36 and 48 h, respectively. The cells were then harvested and STAT1 expression levels were detected by RT-qPCR. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.(B) DEF cells were transfected with a lentiviral vector expressing duck RIG-I and a lentivirus encoding ZsGreen for 24 h, and then infected with DEV at an MOI of 1 or mock-infected for 48 h, Expression levels of STAT1 and pSTAT1 in the cell lysates were analyzed by western blotting, with β-actin protein as the control. (C) DEF cells were transfected with siSTAT1 or siNC for 36 h. Cells were harvested and expression levels of duck STAT1 in the cell lysates were analyzed by western blotting, with β-actin protein as the control. (D) DEF cells were transfected with siSTAT1 or siNC for 36 h and the efficiency of STAT1 knockdown was then confirmed by RT-qPCR. (E) DEF cells were transfected with RIG-I or siSTAT1 for 36 h and the copies of DEV in the supernatant were then assessed by RT-qPCR. (F) DEF cells were transfected with a vector expressing RIG-I or siSTAT1 for 36 h and viral titers in the supernatants were then detected by an endpoint dilution assay, and are presented as $TCID_{50}$ /ml. (G) DEF cells were transfected with vector expressing RIG-I or siSTAT1 for 36 h and expression levels of Mx, OASL and IFN-β were then detected by RT-qPCR. Statistical significance were assessed using the Student's t-test, $* P < 0.05$, $**P < 0.01$, $***P < 0.001$.

antiviral effects induced by RIG-I. We showed that overexpression of RIG-I increased the expression of STAT1 mRNA (Fig. 6A). We then examined the effects of RIG-I on the expression levels of STAT1 and phosphorylated STAT1 (pSTAT1). STAT1 and pSTAT1 were both upregulated in DEV-infected or mock-infected DEF cells overexpressing RIG-I (Fig. 6B).

We further clarified if the anti-DEV effects of RIG-I and the induction of ISGs were due to STAT1 activation by silencing STAT1 expression using siRNA. siSTAT1 caused a marked reduction in STAT1 expression in DEF cells (Fig. 6C and D), we then examined the effects of combined siRNA-mediated silencing of STAT1 with overexpression of RIG-I in DEF cells. The reduction in viral infection mediated by RIG-I overexpression was inhibited in siSTAT1-transfected cells, suggesting that the anti-DEV capacity of RIG-I was mediated by STAT1 (Fig. 6E and F). We also examined Mx, OASL and IFN-β mRNA expression levels in RIG-I-overexpressing siSTAT1-transfected DEF cells. Transfection with siSTAT1 significantly reduced the expression of Mx, OASL and IFN-β mRNA induced by RIG-I overexpression (Fig. 6G), indicating that RIG-I-induced ISG activation was reduced by silencing STAT1. These data suggest that STAT1 knockdown blocked the antiviral response induced by RIG-I in DEF cells.

4. Discussion

RIG-I is a key cytosolic sensor of RNA viruses, has been the subject of intensive studies (Kato et al., 2008, 2006). However, the research into its antiviral activity in avian species remains lacking. In this study, we showed that DEV infection activated IFN-β and RIG-I both *in vivo and in vitro*. Further analysis revealed that overexpression of RIG-I significantly upregulated the expression of ISGs, including Mx, OASL and IFN-β. Furthermore, RIG-I inhibited DEV infection, while its knockdown promoted DEV infection. Notably, the antiviral activity of RIG-I and its induction of ISGs depended on the activity of STAT1. These results collectively suggest that RIG-I acts as an anti-DEV ISG, and that its antiviral activity depends on STAT1.

Previous reports have shown that DEV replicates quickly in many tissues, and it is considered as a pantropic virus responsible for pathological lesions in many different organs (Li et al., 2016). The present study found that viral loads continued to increase in various organs in ducklings infected with DEV throughout the period of infection, with the highest viral load in the intestinal tract during the 3-day test period, followed by the spleen. These data are consistent with the results obtained by other researchers (Xuefeng et al., 2008), indicating that DEV primarily replicates in the mucosa of the digestive tract and then spreads to the immune organs.

The epithelial cells and lymphocytes of the lymphoid and intestinal tissues are the main sites of DEV infection, and DEV has been shown to cause lymphoid organ lesions and immunosuppression in infected birds (Qi et al., 2008; Salguero et al., 2002). In the event of viral infection, the host innate immune system is the first defense mechanism invoked to eliminate the virus. Interferons are a group of antiviral cytokines involved in antiviral immunity, and binding of IFNs to their cognate receptors induces the expression of IFNs and ISGs, thus eliciting the antiviral response (Katze et al., 2002). In this study, IFN-β expression was upregulated by DEV infection, both *in vivo* and *in vitro*. Notably, in the intestinal tract and immune organs. These data suggest that IFN-β is involved in recognizing DEV during the early phase of infection. The high levels of IFN-β expression in these tissues are considered to be mainly attributable to high viral replication throughout the collection periods.

Previous studies demonstrated that RIG-I expression was significantly upregulated in ducklings infected with H5N1 avian influenza virus (Barber et al., 2010). However, information on the response of RIG-I to DEV infection has been lacking. In this study, RIG-I expression was upregulated by DEV infection, both *in vivo* and *in vitro*. High levels of RIG-I expression were primarily restricted to the intestinal tract and immune organs, as the target tissues of DEV infection, compared with other tissues. These results indicate that a RIG-I-dependent pathway might be involved in the antiviral immune response in ducks.

Previous studies demonstrated that goose type I, II, and III IFN inhibited the replication of DEV (Chen et al., 2017), while the current study showed that expression levels of IFN-β and RIG-I increased during DEV infection, both *in vivo* and *in vitro*, thus providing evidence of an important role for RIG-I in protecting the duck from DEV. Based on the positive IFN-β and RIG-I responses to DEV infection, we inferred that RIG-I facilitates IFN-mediated defenses against DEV in ducks. IFN-β, Mx and OASL expression levels were upregulated when RIG-I was overexpressed in DEV-infected or mock-infected DEF cells, indicating that RIG-I plays an important role in regulating the expression of IFN-β and ISGs in ducks.

RIG-I has been reported to play an important role in regulating host immunity against various viruses (West et al., 2014; Xing et al., 2012). We therefore speculated that RIG-I may play an important role in DNA viral infection in ducks. We explored this using RIG-I overexpression and knockdown methods, and showed that RIG-I significantly suppressed DEV infection in DEF cells, while RIG-I was knock down by siRNA promoted DEV infection in DEF cells. These data suggest that RIG-I acts as a host antiviral factor during DEV infection in ducks.

Previous reports showed that STAT1 was involved in antiviral signaling induced by IFNs (Katze et al., 2002). We therefore explored the involvement of JAK-STAT signaling in the action of RIG-I against DEV. RIG-I drove the transcription of STAT1, which is a key element of JAK-STAT cascade within the IFN pathway. Moreover, we observed that RIG-I overexpression induced the phosphorylation of STAT1 at the 701 site in DEF cells, which is an indispensable marker of JAK-STAT pathway activation. These observations suggested that knockdown of STAT1 in DEF cells would block the antiviral signal induced by RIG-I. We investigated this hypothesis by knock down of STAT1 using siRNA, and observed that ISG expression and the anti-DEV capacity of RIG-I were dependent on STAT1. These results thus demonstrated that the antiviral mechanism of RIG-I involves the expression and phosphorylation of STAT1, which subsequently activates the JAK-STAT pathway and the transcription of antiviral ISGs.

In conclusion, this study demonstrated that DEV infection induced the expression of IFN-β and RIG-I in DEF cells and in various organs in ducks. Furthermore, RIG-I played an essential role in the induction of IFN-β and ISG expression and in inhibiting DEV infection. Our data also showed that the induction of ISGs and the anti-DEV capacity of RIG-I were dependent on STAT1. The finding that DEV infection induces the host antiviral immune response involving RIG-I may provide important information for the future development of new antiviral drugs. However, further studies are required to clarify the precise mechanism underlying the antiviral activity of RIG-I in ducks.

Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

This work was supported by the Natural Science Foundation of Heilongjiang Province (QC2018033), the Key Program of Natural Science Foundation of Heilongjiang Province (ZD2016006) and Central Public-interest Scientific Institution Basal Fund (NO.1610302017013; NO.1610302018013).

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