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Strong inflammatory responses and apoptosis in the oviducts of egg-laying hens caused by genotype VIIId Newcastle disease virus

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Abstract

Background: Newcastle disease virus (NDV) can cause serious damage to the reproductive tracts of egg-laying hens and leads to egg production and quality reduction. However, the mechanism of severe pathological damage in the oviducts of egg-laying hens after NDV infection has not been fully elucidated. In this study, the correlation between the primary pathological lesions and viral load in the oviducts of egg-laying hens infected with the velogenic genotype VIIId NDV strain was evaluated by pathological observation and virus detection. Subsequently, apoptosis, the expression of immune-related genes and lymphocyte infiltration into the infected oviducts were determined to explore the potential causes of the pathological changes.

Results: A higher viral load and severe tissue lesions and apoptotic bodies were observed in the oviduct of NDV-infected hens compared with the control. Immune-related genes, including TLR3/7/21, MDA5, IL-2/6/1 β , IFN- β , CXCL11/2, and CCR5, were significantly upregulated in the magnum and uterus. IL-2 presented the highest mRNA level change (137-fold) at 5 days post infection (dpi) in the magnum. Infection led to CD3⁺CD4⁺ and CD3⁺CD8 α ⁺ lymphocyte infiltration into the magnum of the oviduct. A higher viral load was found to be associated with pathological changes and the elevated expression of proinflammatory cytokines in the NDV-infected hens.

Conclusions: Our results indicate that the severe lesions and apoptosis in the oviducts of egg-laying hens caused by genotype VIIId NDV strains are associated with the excessive release of inflammatory cytokines, chemokines and lymphocyte infiltration, which contribute to the dysfunction of the oviducts and the decrease of egg production in hens.

Keywords: Genotype VIIId NDV, Oviduct, Apoptosis, Inflammatory responses, Lymphocyte infiltration, Egg-laying hens

Background

The Newcastle disease virus (NDV) is highly contagious and widespread among avian species and causes severe economic losses in domestic poultry, especially chickens [1]. It does not only cause the death of chickens but also causes serious damage to the reproductive tracts of egg-laying hens. This leads to an overall decrease in egg production and increases the percentage of deformed, sand-shelled, and soft-shelled eggs [2]. Since

the 1950s, vaccination has been applied for the prevention and control of this disease in many countries, including China. However, it is still enzootic in some areas and is recognized as a major disease that affects poultry [3]. The genotype VII NDV is dominant in Asia and poses a serious threat to its poultry industry [4–6]. Previous studies have revealed that the significant oviduct inflammation and decline of egg production are caused by the variance [2, 7]. However, the inflammatory effects and apoptosis of local oviduct of the virulent NDV genotype VIIId strains were still unclear, which may lead to oviduct dysfunction and drop in egg production.

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Generally, host-virus interactions caused by NDV include a complex interplay of molecular pathways directed by the host to prevent viral replication [8, 9]. These involve intense inflammatory responses in the form of a cytokine storm that are initiated inside infected cells or tissues following viral replication and result in excessive cellular apoptosis and tissue damage. In vivo studies suggested that NDV initiated strong immune responses in the spleen, lymph nodes and peripheral blood of infected chickens, where the mRNA expression levels of proinflammatory and cytokines/chemokines were significantly upregulated [9–12]. Strong innate immune response and cell death were observed in chicken splenocytes infected with genotype VIIId NDV [13]. The cytopathic effect (CPE) and apoptosis were observed in NDV infected cells and viral replication and caspase activation were detected [14–16]. Studies investigating the mechanism underlying severe pathological damages in the oviducts of egg-laying hens after NDV infection are limited. Furthermore, the reproductive tract is a target of both the vaccine strain and field isolates of NDV [7], so the innate immunity roles of oviduct played a critical role in viral pathogenesis, especially, the inflammatory effects and attribution of different immune cells and immune molecules.

In this study, a severe oviduct tissue damage of egg-laying hens were observed in the genotype VIIId NDV strain infected hens. This damage was associated with high levels of virus replication, a strong innate immune response/inflammatory response and lymphocyte infiltration. These responses contributed to oviductal dysfunction and the decline of egg production, which played a critical role in viral pathogenesis.

Results

Clinical signs and histopathological changes

Obvious ND-related clinical signs, including ruffled feathers, depression, and drowsiness, were observed in

NDV-infected birds 2 dpi. At 3 dpi, the infected hens showed typical clinical signs, including dyspnea, depression and greenish feces. The first bird died of NDV infection 3 dpi. At 4 dpi, Four infected hens died from viral challenge-associated causes. The most severe deaths occurred 5 dpi, when seven infected birds died. Furthermore, egg production of the NDV-infected hens started to plunge by approximately 40% on approximately 5 dpi, whereas the controls maintained approximately 90% egg production (Table 1). From 7 dpi, no hens died spontaneously or was moribund, and clinical signs and egg production of the remaining hens began to abate. Upon examination of the gross lesions, the experimental group hens showed proventricular hemorrhage, tracheorrhagia, brain edema and dissolved follicles, while the oviducts showed edema. Hens in the control group did not show any clinical signs or gross lesions throughout the procedure.

Histopathological examinations of infundibulum, magnum, isthmus, uterus, and vagina are shown in Fig. 1. The tissues of the laying hens of the control group appeared normal. On 1 dpi, there were scattered necrotic areas in the mucosal epithelia and lymphocyte infiltration into the infundibulum, magnum, isthmus, and uterus. Occasionally, heterophils were found in the lamina propria of the magnum. On 3 dpi, the uterus presented marked edema. Severe mucosal epithelial necrosis and numerous lymphocytes were seen in the vagina. On 5 dpi, the degree of the lesions in the isthmus was elevated, while mucosal epithelial necrosis, heterophil infiltration, and edema were also observed. Moreover, a large number of lymphocytes were also observed in the vagina. On 7 dpi, the lesions in the infundibulum became less severe. However, heterophil infiltration and mucosal epithelial necrosis persisted. On 15 dpi, some lymphocytes were found in the infundibulum. No significant changes were observed in the control group.

Table 1 Tissue processing and egg production changes in chickens infected with the velogenic genotype VIIId NDV

Day	Control group			Experimental group				
	Total	Sample	Laying rate%	Total	Mortality	Autopsy	Sample	Laying rate %
0	45	0	91.1 (41/45)	55	0	0	0	90.9 (50/55)
1	45	5	87.5 (35/40)	55	0	5	5	88.0 (44/50)
3	40	5	88.6 (31/35)	50	1	5	5	66.7 (30/45)
4	35	0	91.4 (32/35)	45	4	4	0	48.8 (20/41)
5	35	5	90.0 (27/30)	41	7	7	5	38.2 (13/34)
6	30	0	90.0 (27/30)	34	3	3	0	41.9 (13/31)
7	30	5	88.0 (22/25)	31	1	5	5	46.2 (12/26)
9	25	5	90.0 (18/20)	26	0	5	5	52.4 (11/21)
11	20	5	86.7 (13/15)	21	0	5	5	56.3 (9/16)
15	15	5	90.0 (9/10)	16	0	5	5	72.7 (8/11)

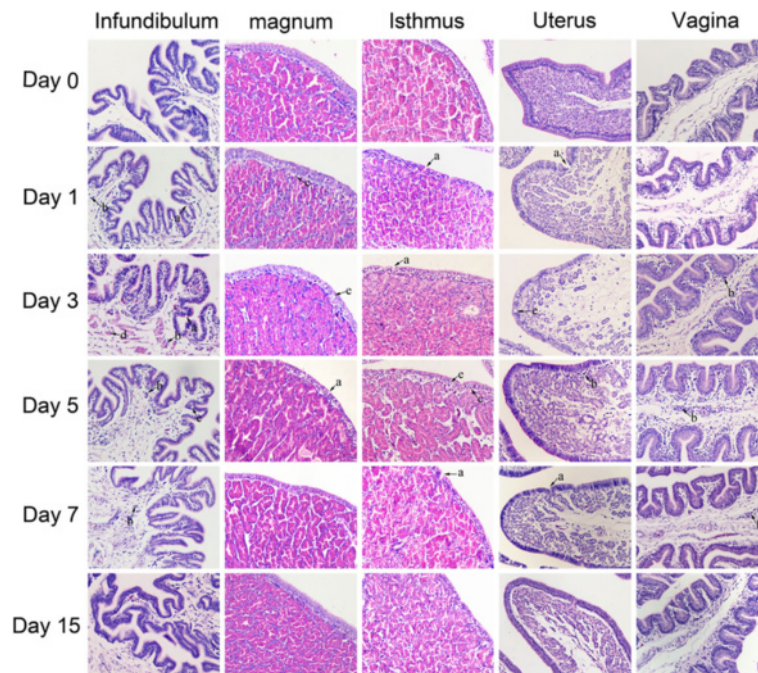


Fig. 1 Histopathology of the oviducts stained with hematoxylin and eosin (400x). (A) Cilia loss. (B) Lymphocyte infiltration. (C) Heterophil infiltration. (D) Pink-stained material in the infundibulum. (E) Mucosal epithelial necrosis

Viral loads in oviduct

To evaluate differences in viral replication in the oviduct of egg-laying hens after NDV infection, we determined the transcriptional levels of the viral matrix (M) gene in the oviducts of hens infected with the velogenic genotype V11d NDV strain by quantitative RT-PCR assay. We detected the viral load from the infundibulum, magnum, isthmus, uterus, and vagina taken at 1, 3, 5, 7 and 9 dpi. The level of

viral RNA is shown in Fig. 2. The viral load was significantly increased in the infundibulum, magnum, isthmus, uterus, vagina and reached the peak at 5 dpi, gradually decreased at 7, 9 and 11 dpi. The magnum presented the highest amount of virus load (8918.6 copies/ml), followed by the uterus (2934.6 copies/ml), vagina (1890 copies/ml), isthmus (1789 copies/ml), and infundibulum (1358 copies/ml) (Fig. 2). No virus was found in healthy oviducts.

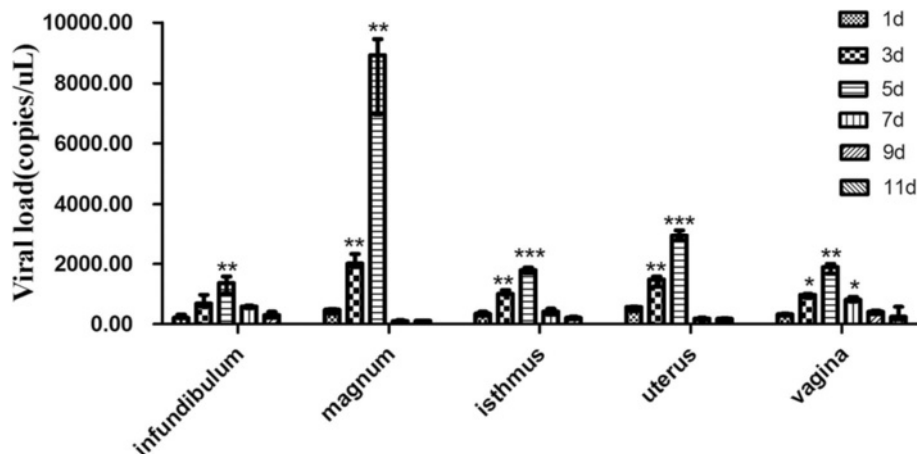


Fig. 2 NDV viral load in oviduct. Primers targeting the M gene of NDV were used to detect virus replication in the oviducts of hens infected with the velogenic genotype V11d NDV strain by quantitative RT-PCR assay with three replications. According to our standard curve, the correlation coefficient (R^2) was 0.9979 with a slope value of -3.3749 ($y = -3.3749x + 37.202$)

Virus distribution along the oviductal segments

Indirect immunofluorescence assays were conducted to confirm the distribution of the NDV HN protein in the oviducts at 5 dpi. Positive staining was not observed in any part of the oviducts of the control hens. However, HN proteins were observed in the glandular epithelial cells of the lamina propria and found scattered in the submucosa of the infundibulum, magnum, and isthmus. Conversely, the HN proteins were present in the submucosa and scattered in the lamina propria in the uterus. In the vagina, they were located in the mucosal epithelium (Fig. 3).

Apoptosis in the oviducts of the infected hens

In this study, caspase-3 activity and TUNEL assays were employed to detect apoptosis in the oviducts of the infected hens on 5 dpi. The caspase-3 activities in the magnum and uterus were found higher compared to the control group (2.41- and 1.98-fold increases, respectively) (Fig. 4). The results of the TUNEL assays revealed that NDV induced apoptosis in the lamina propria of the infundibulum and vagina (Fig. 5). In the magnum, isthmus, and uterus, apoptotic cells were observed in both the lamina propria and the mucosa.

Expression of TLRs, MDA5, cytokines, and chemokines

To estimate the inflammatory response in the oviducts of the infected hens, a real-time quantitative PCR assay was used to quantify the expression profiles of immune-related genes in the magnum and uterus of the oviduct of SPF egg-laying hens infected with genotype VIIId NDV on 1, 3, 5, 7, 9, 11 and 15 dpi. The mRNA levels of TLR3, 7, and 21 were altered in the oviducts of the infected laying hens (Fig. 6) and were upregulated in the magnum and uterus tissues. The mRNA expression levels in the magnum segments peaked at 5 dpi (42.6-, 98.9-, and 27.1-fold, respectively), while in the uterus they peaked on 7 dpi. Moreover, TLR expression levels in the magnum were significantly greater compared to the uterus. This result suggests that the magnum segments were most sensitive to NDV. Another intracellular pattern recognition receptor (PRR), MDA5, was also upregulated, with its highest fold changes of 8.23 and 7.29 detected in the magnum and uterus, respectively (Fig. 6d).

The expression levels of IL-2/6/1 β and IFN- α / β were also examined at the transcript level (Fig. 7). IL-2/6/1 β and IFN- β were significantly upregulated at two instances during the infection process, but the ranges of upregulation were different. IL-2 presented the highest mRNA level

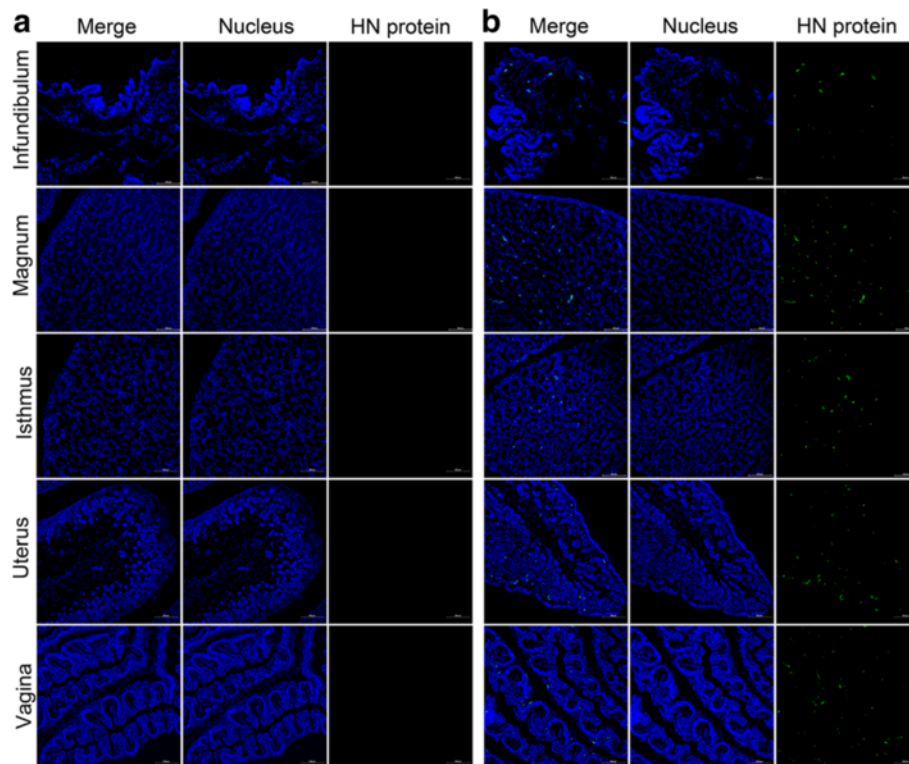
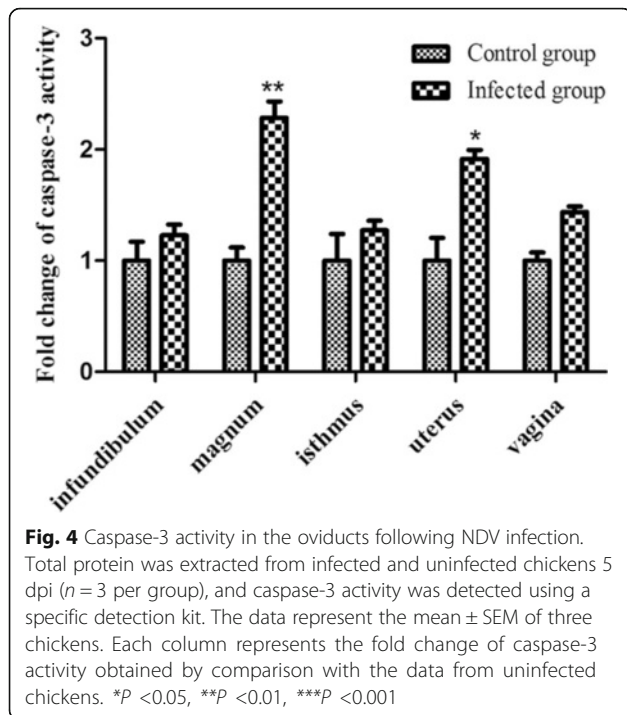


Fig. 3 Detection of the NDV HN protein in the oviducts by IFA at 5 dpi. Distribution of the NDV HN protein was detected by staining with a mouse anti-HN monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (green fluorescence); the nuclei were stained with Hoechst 33342 (blue fluorescence) (400 \times). **a** Control hens. **b** Infected hens

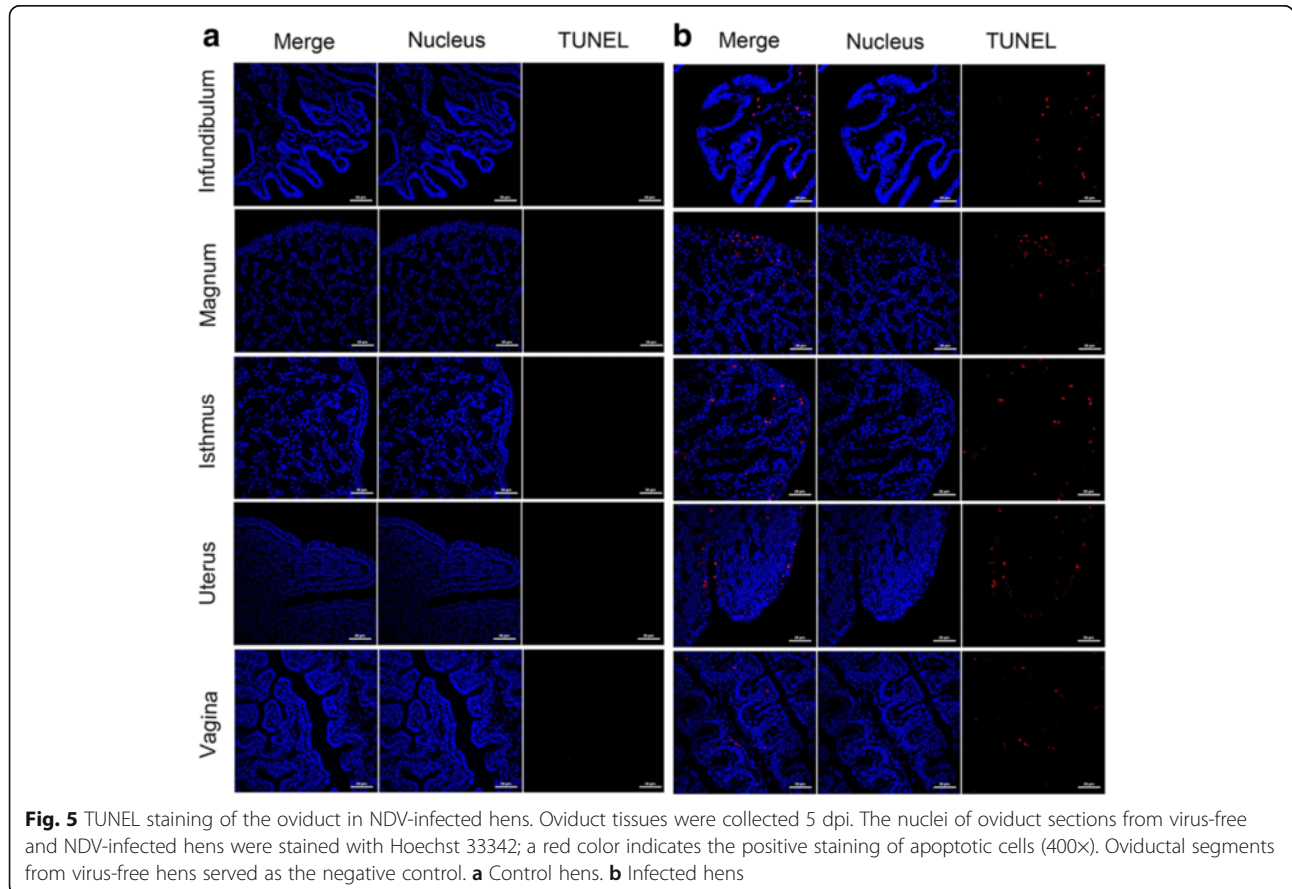


change of 137-fold on 5 dpi (Fig. 7b), followed by IFN- β (95.1-fold) and IL-1 β (15.9-fold). In contrast, IFN- α was downregulated by 0.19-fold 3 dpi and subsequently upregulated by 1.78-fold 5 dpi in the magnum, but not in the uterus (Fig. 7d). Furthermore, IFN- α expression was significantly upregulated and peaked 5 dpi with a 4.56-fold increase in the uterus (Fig. 7d).

We examined three chemokines (CXCLi1/2 and CCR5) (Fig. 8). The mRNA expression level of CXCLi1 was increased in the magnum throughout the infection process, reaching its peak of 20.5-fold on 5 dpi. It was also significantly upregulated (5.48-fold) in the uterus on 5 dpi (Fig. 8a). The chemokine receptor for CXCLi1 (CCR5) was also upregulated during the infection process. This upregulation was higher than CXCLi1 in the magnum (22.1-fold) and uterus (25.5-fold) (Fig. 8b). CXCLi2 was also significantly upregulated and peaked at 5 dpi with a 78.3-fold increase in the magnum (Fig. 8c).

8Dynamic changes in CD3⁺CD4⁺ and CD3⁺CD8 α ⁺ lymphocytes in the oviducts

To study the dynamic changes in the CD3⁺CD4⁺ and CD3⁺CD8 α ⁺ lymphocytes in the oviducts of hens after NDV infection, we selected the magnum, which was the segment with the highest level of viral replication and



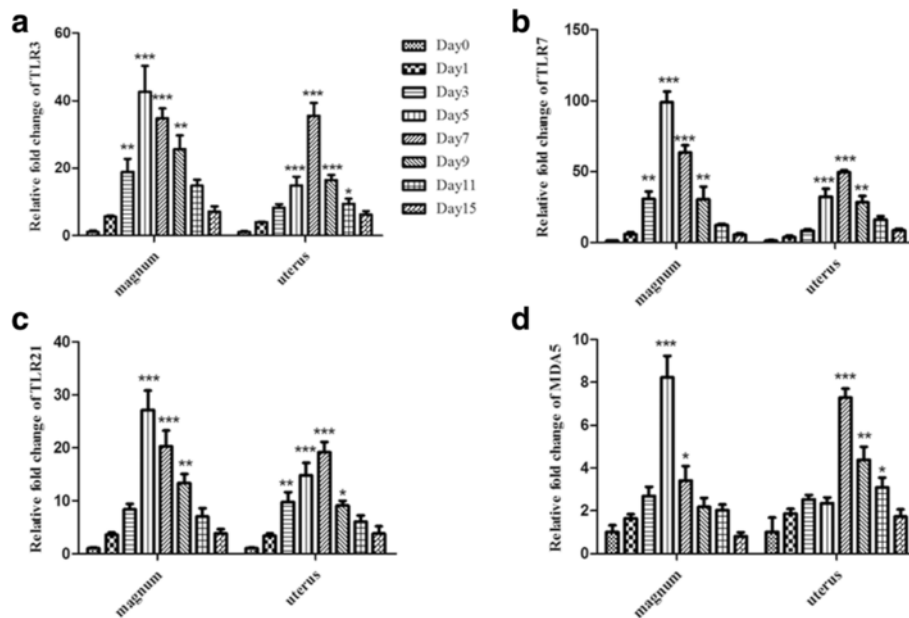


Fig. 6 mRNA expression levels of the TLRs and MDA5 in the magnum and uterus of laying hens infected with NDV. Total RNA was extracted from the infected and control groups ($n = 3$ per group). The TLRs and MDA5 were quantified using appropriate primers specific to the chicken and SYBR Green-based real-time (RT) PCR. The data represent the mean \pm standard error of the mean (SEM) for three chickens. Panels **a**, **b**, **c**, and **d** depict the results for TLR3, TLR7, TLR21, and MDA5, respectively, in the magnum and uterus. Statistical analysis was performed by comparison with the healthy chickens. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

mRNA expression of immune-related factors, for further study. The accumulation of $CD3^+CD4^+$ and $CD3^+CD8\alpha^+$ lymphocytes in the magnum was greater in the experimental than in the control group throughout the course of infection (Figs. 9 and 10). The $CD3^+CD4^+$ and $CD3^+CD8\alpha^+$ lymphocytes began to accumulate 1 dpi and peaked 5 and 7 dpi, respectively (Figs. 9 and 10). $CD3^+CD4^+$ and $CD3^+CD8\alpha^+$ lymphocytes were distributed in the lamina propria of the magnum. Moreover, $CD8\alpha^+$ cells were recruited into the magnum more deeply than the $CD4^+$ cells. The frequency of $CD4^+$ cells in the lamina propria was significantly higher compared to the bottom of the mucosal epithelium. In contrast, the $CD8\alpha^+$ cells localized predominantly at the bottom of the mucosal epithelium. Sporadic distribution of these lymphocytes was detected in the oviducts in the control birds.

Discussion

Newcastle disease is a highly contagious viral disease that causes serious economic damage to the poultry industry [4]. The velogenic genotype VII NDV has been documented as the predominant epidemic genotype and has been responsible for frequent outbreaks on vaccinated farms in China and other Asian countries since 1990 [17, 18]. The strain used in this study was isolated from a vaccinated farm. We observed that the significant clinical signs, pathological changes, and apoptosis induced by velogenic genotype VII NDV infection contributed to

oviduct dysfunction and the decline of egg production in the hens. Our findings also indicated that the severe tissue damage in the reproductive tract was associated with high viral replication, apoptosis, an intense inflammatory response, and lymphocyte infiltration.

In this study, examination of the viral load confirmed that NDV replication occurred in the oviducts of egg-laying hens. At 5 dpi, when the viral load was higher than 1, 3, 7, 11 and 15 dpi, the histopathology were more severe, which may be due to the higher viral load in oviduct at these days. Therefore, the efficiency of viral replication in oviduct of hens appears to be associated with the elevated expression of proinflammatory cytokines in the NDV-infected hens. Furthermore, positive staining for the NDV HN protein in the oviducts revealed that the infected cells were glandular epithelial and mucosa cells. This finding was consistent with the presence of apoptotic cells in the five oviductal segments observed in the current study. We also observed the invasion of inflammatory cells and necrocytosis in the mucosal epithelia in five segments (Fig. 1). We hypothesized that the lesions aroused by NDV infection in the five segments might lead to the poor performance in egg-laying hens, thereby resulting in decreased egg production.

Apoptosis is a critical mechanism of NDV-induced tissue lesions, and caspase-3 plays a critical role in the execution of the apoptotic process [19, 20]. In our study, caspase-3 activity was significantly high in the NDV-

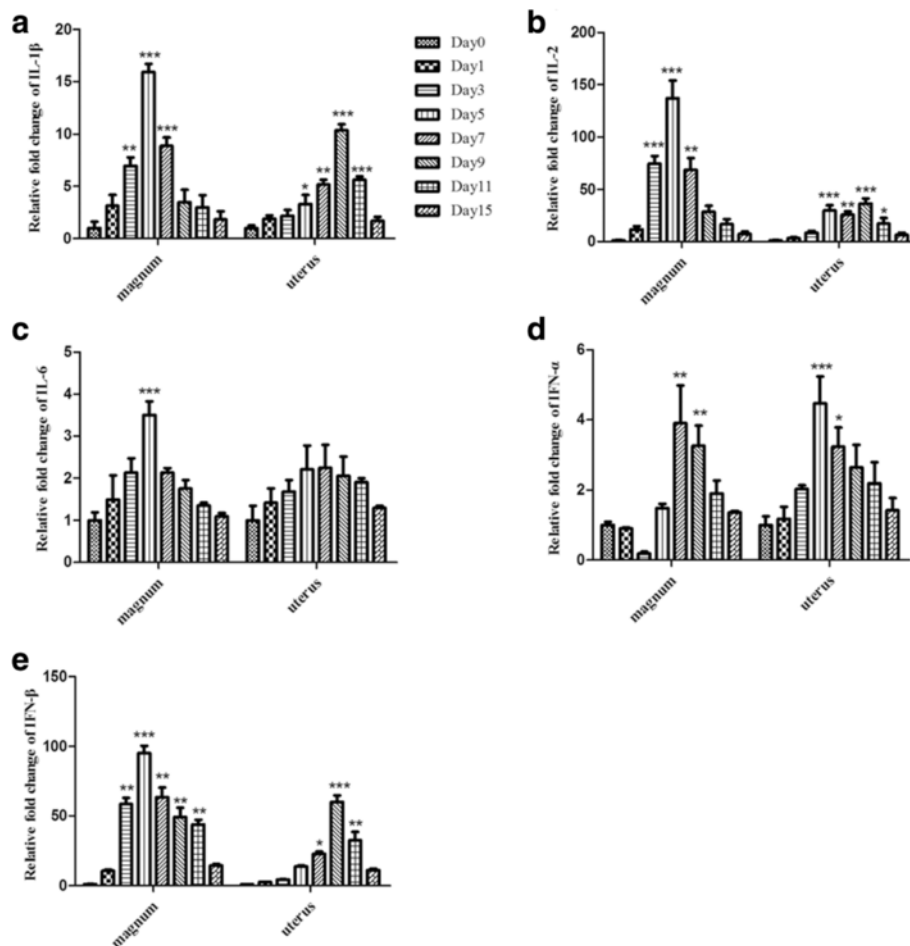


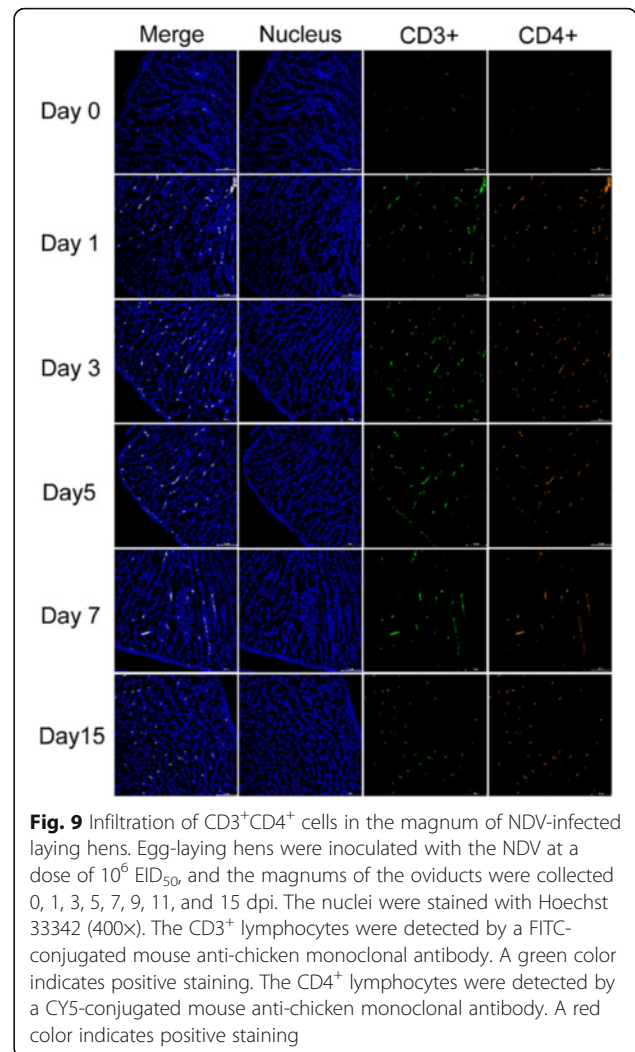
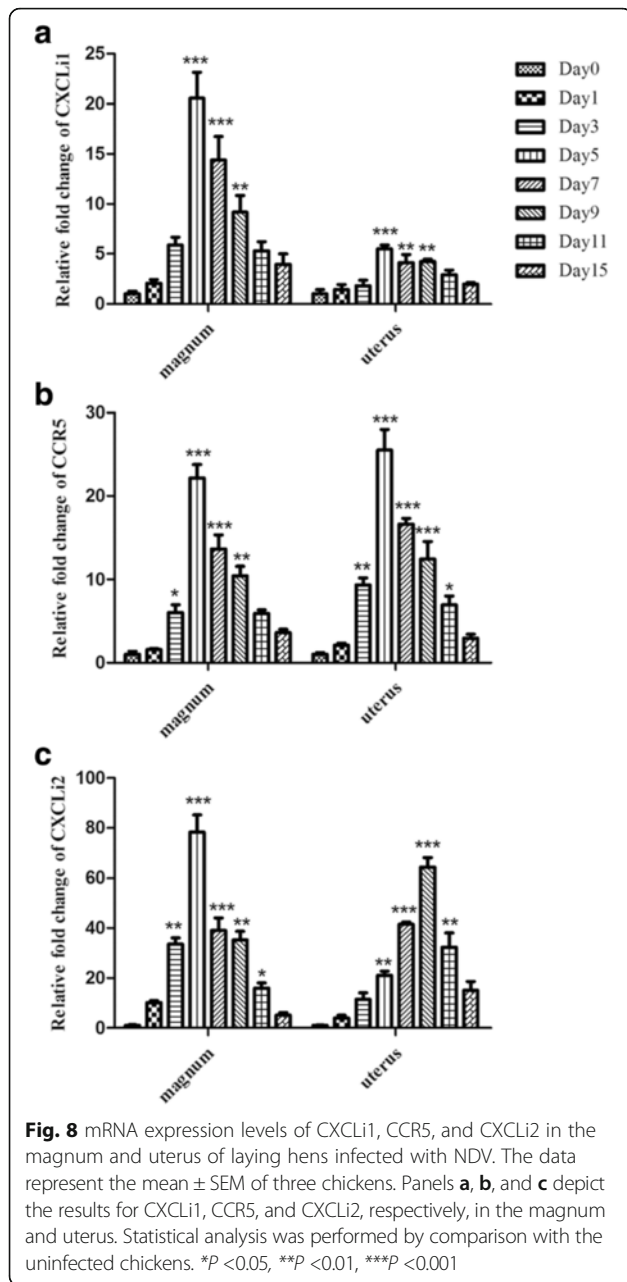
Fig. 7 Expression levels of inflammatory cytokines in the magnum and uterus of laying hens infected with NDV. IL-1 β /2/6 and IFN- α / β were quantified using SYBR Green-based RT-PCR. Panels **a**, **b**, **c**, **d** and **e** depict results for IL-1 β , IL-2, IL-6, IFN- α , and IFN- β , respectively, in the magnum and uterus. Statistical analysis was performed by comparison with uninfected chickens. * P < 0.05, ** P < 0.01, *** P < 0.001

infected layers, which was consistent with the results of a previous study [16]. Furthermore, our TUNEL assays also revealed apoptosis in the five oviduct segments upon NDV infection. Collectively, these results indicate that the oviductal tissues undergo cellular apoptosis both in the early and late stages of NDV infection.

We examined whether pattern recognition receptors (PRRs), inflammatory cytokines and chemokines in the oviduct exhibited altered expression in response to NDV infection. TLRs and MDA5 belong to different PRRs that recognize viral nucleic acids and are central to host antiviral defenses. TLRs are the most important family of PRRs and activate the MyD88-dependent pathway to produce cytokines, MHC molecules, and chemokines. In turn, these molecules trigger an appropriate immune response to eliminate the invading pathogens [21, 22]. In birds, the TLRs 3, 7, and 21 play major roles in the induction of antiviral IFN- β during such infections [23, 24]. In our study, the transcriptional levels of TLR3/7 were

significantly increased post-NDV infection, suggesting the anti-NDV roles of these molecules.

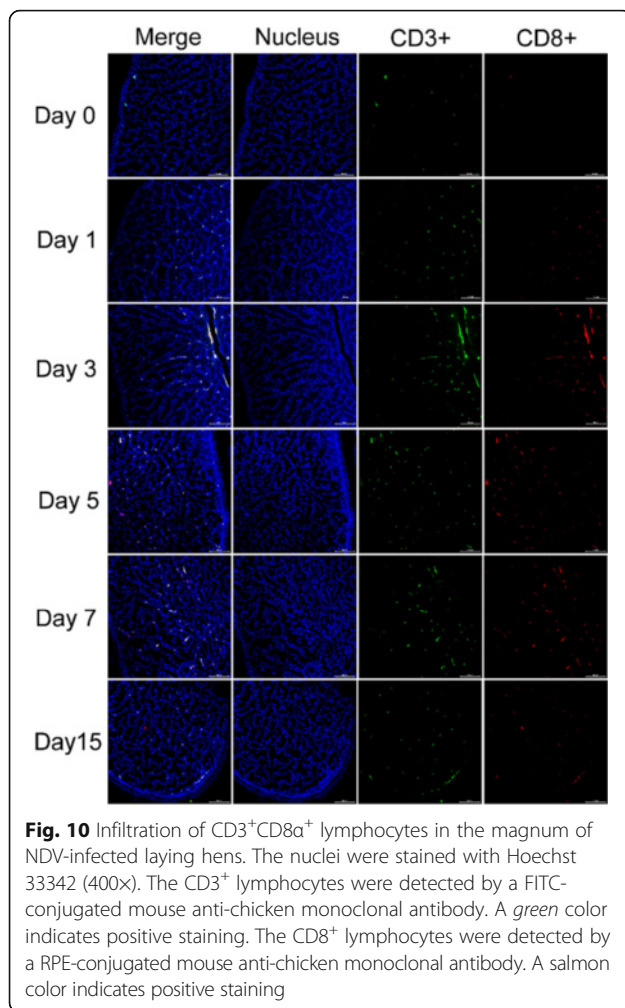
Cytokines and chemokines are major mediators of the host immune response during viral infection [25]. NDV infection induced strong pro-inflammatory responses that played an important role in viral pathogenesis. The IL-2/6/1 β and IFN- β mRNA expression levels were increased in the oviductal tissues of NDV-infected hens; our data are consistent with previous findings [9–12]. The expression of the IL-2 gene in the peripheral blood of F48E9 NDV-infected chickens in a previous study fluctuated and did not exhibit a significant difference compared with the control group during the experimental period [26]. In contrast, the expression of IL-2 in our study increased rapidly and presented the highest mRNA level change (137-fold) at 5 dpi. IFN- α belongs to the type I interferon family and can be produced in most cells after virus infection, whereupon it localizes to the site of viral infection and inhibits viral replication. In



NDV infection in this study, the CXCL12 mRNA expression levels were higher compared to CXCL11. The up-regulation of CXCL11, CXCL12, and CCR5 might potentially attract immune cells, thereby modulating cellular immunity.

Cell-mediated immunity is a specific adaptive immune response mediated by T lymphocytes. It is suggested to be an important factor in the development of protection in chickens vaccinated against NDV and viral clearance [28–30]. T lymphocytes are classified according to their expression of cell surface proteins. The CD3 molecule is the surface marker for mature lymphocytes; most CD4⁺ lymphocytes are helper cells, while CD8⁺ lymphocytes are cytotoxic cells [31]. In this study, immunofluorescence staining in the magnum demonstrated lymphocyte infiltration. CD3⁺CD4⁺ and CD3⁺CD8α⁺ lymphocytes were distributed in the lamina propria and mucosal epithelium of the magnum. Furthermore, the CD3⁺CD8α⁺ cells were located and infiltrated deeper into the bottom of the mucosal epithelium compared with the CD3⁺CD4⁺

this study, IFN-α experienced a decline in the magnum of hen oviducts 3 dpi; the same phenomenon was reported in another study that demonstrated that the expression of IFN-α experienced a sharp decline in the peripheral blood of F48E9 NDV-infected chicken 7 dpi [12], which might contribute to rapid viral replication. The highest levels of expression of the examined genes corresponded with the significant clinical signs and the inflammatory responses in the oviductal tissues. In the chicken, there are two syntenic genes (CXCL11 and CXCL12) that mainly chemoattract heterophils and monocytes, respectively [27]. Interestingly, although both the CXCL chemokines were upregulated following



cells. Thus, infiltration of CD3⁺CD8 α ⁺ lymphocytes may be responsible for the aggravated tissue lesions and mediate viral clearance. A previous study suggested that T cell recruitment in association with the expression of proinflammatory cytokines and the CXCL12 chemokine were upregulated in response to LPS in the lower part of the oviduct [32], our data are consistent with their findings. The high degree of lymphocyte infiltration in the NDV-infected chickens might contribute to the elevated oviductal damage observed during the early stage of infection.

Overall, NDV is a continuing threat to the poultry industry. Our findings revealed that the oviducts of laying hens were potential target tissues for NDV infection. Our results suggest that the strong inflammatory responses and apoptosis induced by NDV infection lead to severe local damage in the oviducts of egg-laying hens. We also measured the eggshell and albumin quality in another study, and our results suggested that NDV caused eggshell thinning and decreases in the expression of ovalbumin (OVA) and calcium-binding protein D28K

(CaBP-D28k) (data not shown), all of which may contribute to the reduced egg production. Given the findings that the magnum is the most sensitive segment to NDV, this sensitivity may contribute to the poor albumen quality of eggs laid by NDV-infected birds. Further studies are needed to determine the mechanisms underlying the apoptosis and immune responses in infected hen oviductal cell lines in vitro.

Conclusions

Infection with the velogenic genotype VIIId NDV triggered apoptosis and production of cytokines and chemokines in oviduct, accompanied by inflammatory responses. It further led to infiltration of CD3 + CD4⁺ and CD3 + CD8 α + lymphocytes in the segments of the oviduct. Our results indicate that the severe lesions in the oviducts of egg-laying hens caused by genotype VIIId NDV strains is associated with excessive release of inflammatory cytokines and chemokines and lymphocytes infiltration, which contribute to the dysfunction of oviducts and the decline of egg production in hens.

Methods

Virus

The velogenic genotype VIIId NDV strain (NDV/Chicken/TC/9/2011) was isolated from Shanxi province, China, in 2011 [33]. The mean death time (MDT) and intracerebral pathogenicity index (ICPI) were previously determined to be 48 h and 1.69, respectively. The virus was cultured in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Green Biological Engineering Co., Yangling, China).

Animals and experimental design

One hundred forty-week-old specific-pathogen-free (SPF) White Leghorn egg-laying hens (Green Biological Engineering Co., Yangling, China) were housed in isolators under negative pressure with food and water provided ad libitum. They were randomly divided into the control group and experimental group. The forty-five hens of control group were used as the NDV-free controls. The fifty-five hens in the experimental group were infected with a total of 0.5 mL (10^6 median embryo infective doses) of the velogenic genotype VIIId NDV strain through combined intraocular and intranasal routes. The infected hens were monitored for clinical signs of disease and egg production. All animals were checked at least twice daily to ensure their health and welfare.

Animals displaying severe clinical distress were euthanized with intravenous sodium pentobarbital at a dose of 100 mg/kg. Five birds from each group was humanely euthanized on days 1, 3, 5, 7, 9, 11 and 15 post-infection (dpi), and various parts of the oviduct were sectioned. Birds that died as a result of infection were included

with the sampled birds on the indicated days. Birds that died on other days were observed by autopsy only. The criteria for euthanasia were somnolence, akinesia and dyspnea. Tissue processing and egg production changes were shown in Table 1. All experiments were performed in laboratories with biosafety level 2 facilities.

Tissue samples of the infundibulum, magnum, isthmus, uterus, and vagina from the control and experimental groups were collected immediately and placed on ice. Some parts of the tissues were preserved in liquid nitrogen for SYBR Green-based real-time (RT)-PCR, and others were fixed in 10% phosphate-buffered formalin for more than 24 hs [34]. The blocks were sectioned at 5- μ m thicknesses for histopathological lesion observation, terminal deoxynucleotidyl transferase-mediated d-UTP biotin nick end labeling (TUNEL) staining, hemagglutinin neuraminidase (HN) protein detection, and CD3⁺CD4⁺ and CD3⁺CD8 α ⁺ lymphocyte detection by immunofluorescence.

Histopathological examination

The infundibulum, magnum, isthmus, uterus, and vagina were collected and fixed in 10% phosphate-buffered formalin, embedded in paraffin wax and cut into sections using routine protocols. Next, the paraffin sections were stained with hematoxylin and eosin, and the tissue changes were observed under a light microscope.

Detection of viral loads in the oviducts

The SYBR Green I (TransGen biotech, China) RT-PCR assay was used to detect the viral loads in the infundibulum, magnum, isthmus, uterus, and vagina. The M gene of NDV was used as the target gene and as a reference for the expression analyses. The primers used are as follows: Forward: 5'-ATCCGAAGAGCCCGTTAG-3' (3939-3956) and Reverse: 5'-ACATCACTGAGCCCGACA-3' (4113-4096). Total RNA was extracted from the reproductive tract tissues using the TRIzol reagent (Takara Biotechnology, Japan). The concentration of RNA in each sample was measured spectrophotometrically at 260 nm versus 280 nm. Next, the RNA was reverse-transcribed using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) according to the manufacturer's instructions. Construction of standard curve was done according to the procedure of [34].

Detection of the viral hemagglutinin-neuraminidase protein in the oviduct by indirect immunofluorescence

The paraffin sections of the oviducts were examined by IFA to detect the viral HN protein. Briefly, the tissue sections were deparaffinized with xylene for 10 min, followed by hydration through a graded series of alcohol to xylol (100%, 95%, 90%, 80%, and 70%) for 3 min at

each step. Then, the sections were blocked with 5% BSA for 1 h at 37 °C. Next, the sections were incubated with 1 mg/ml mouse anti avian monoclonal antibody specific for HN of NDV (Santa Cruz Biotechnology) in PBS overnight at 4 °C. After washing thrice with PBS, the sections were incubated with FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, 1:400) in PBS for 1 h at 37 °C, and then washed thrice with PBS once again and counter stained with Hoechst 33342 (Sigma, USA) for 5 min. Finally, the treated sections were observed under a confocal laser scanning microscope (Nikon, Japan).

Determination of apoptosis in the oviduct

Detection of caspase-3 activity

A total of 0.1 g of oviduct tissue was cut into small pieces, added to 1 mL of radio immunoprecipitation assay lysis buffer, and homogenized in a glass pestle on ice. The tissue homogenates were transferred into 1.5 mL centrifuge tubes and incubated on ice for 5 min. Then, they were centrifuged at 20,000 rpm for 10 min, and the supernatants were collected into pre-cooled centrifuge tubes. The caspase-3 enzyme activity was measured using the detection kit (KeyGen Biotech), and its concentration was determined by the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Nanjing, China).

Quantification of apoptosis by the TUNEL assay

Apoptosis was examined using the one-step measurement of terminal deoxynucleotidyl transferase mediated d-UTP biotin nick end labeling (TUNEL) in the oviducts of the infected hens on 5 dpi. Tissue sections were deparaffinized and rehydrated through an alcohol gradient then rinsed in deionized water. The following steps corresponded to the standard operation staining procedures provided by manufacturer (Vazyme Biotech, Nanjing, China).

Quantification of TLRs, MDA5, and proinflammatory cytokines by RT-PCR

Total RNA extraction was performed as described in the detection of viral loads in the oviducts section. RT-PCR reactions were performed with equal amounts of cDNA samples from all egg-laying hens using a thermal cycler (Bio-Rad, America). Table 2 showed the primers used for PCR, which were designed using Primer Premier 5 software. The specificity of each primer was checked using the NCBI blast program. The expression levels of TLRs, MDA5, and proinflammatory cytokines and chemokines were normalized to chicken β -actin and amplification efficiency were consistent with β -actin. The results of real-time PCR were quantified by the

Table 2 PCR primers used for mRNA expression analysis

Genes	Primer sequences	Accession no.	Location	Product size
IL-2	F: 5'-CTGTATTCGGTAGCAATG-3' R: 5'-ACTCCTGGGTCTCAGTTG-3'	NM_204153.1	87-247	161
IL-1 β	F: 5'-ACCCGCTTCATCTTCTACCG-3' R: 5'-TCAGCGCCCACTTAGCTTG-3'	NM_204524.1	663-836	174
IL-6	F: 5'-GGCATTCTCATTTCTTCT-3' R: 5'-CTGGCTGCTGGACATTTT-3'	NM_204628.1	853-1051	199
IFN- α	F: 5'-GGACATGGCTCCCACTAC-3' R: 5'-GGCTGCTGAGGATTTGAAGA-3'	XM_004937097.1	2896-3044	149
IFN- β	F: 5'-CACCAACCTTCTCCT-3' R: 5'-TGTGCGGTCAATCCAGT-3'	NM_001024836.1	66-321	256
CXCL12	F: 5'-CATCATGAAGCATTCCATCT-3' R: 5'-CTTCCAAGGGATCTTCATTT-3'	NM_205498.1	124-223	100
CXCL11	F: 5'-AACTCCGATGCCAGTG-3' R: 5'-TTGGTGTCTGCCTTGT-3'	NM_205018.1	176-380	205
CCR5	F: 5'-GTGGTCAACTGCAAAAAGCA-3' R: 5'-GCCCGTTCAACTGTGTCG-3'	NM_001045834.1	1126-1315	190
MDA-5	F: 5'-GGACGACCACGATCTCTGTGT-3' R: 5'-CACCTGTCTGGTCTGCATGTTATC-3'	NM_001193638.1	457-535	79
TLR3	F: 5'-GCTATTGAGCAAAGTCGAGA-3' R: 5'-ACAGGGGGCACTTTACTATT-3'	NM_001011691.3	2491-2693	203
TLR7	F: 5'-TCTGGACTTCTAACAACA-3' R: 5'-AATCTCATTCTCATTATCATCA-3'	NM_001011688.2	1824-2010	187
TLR21	F: 5'-AGTTGTGCTCTGTGCTGAGAG-3' R: 5'-AGCAGGTTGTGTTCCACTGTC-3'	NM_001030558.1	1035-1164	130
β -actin	F: 5'-AGACATCAGGGTGTGATGGTTGGT-3' R: 5'-TGGTGACAATACCGTGTCAATGG-3'	NM_205518.1	183-300	118

comparative threshold analysis after deductions of data from uninfected chickens.

Detection of T lymphocytes in the magnum by immunofluorescence

The dewaxing and rehydrating process and BSA treatment was performed as described in the HN protein detection section. After removing the blocking solution, tissue sections were incubated for 1 h at 37 °C with 1 mg/ml mouse monoclonal antibody specific for chicken CD markers, FITC conjugated CD3, CY5 conjugated CD4, or RPE labeled CD8 α (Southern biotech, USA) to detect CD3CD4 and CD3CD8 α glycoprotein of lymphocytes. Following steps were performed as described in the HN protein detection section.

Statistical analyses

The data are presented as the means \pm SEM of three independent experiments with three replicates per

experiment. Analysis of the gene expression levels was performed using one-way analysis of variance with Dunnett's post-test in GraphPad Prism version 5.0 for Windows. *P* values less than 0.05 were considered statistically significant.

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Availability of data and materials

All data supporting the findings is contained within the manuscript.

Authors' contributions

RL and KG performed the majority of the experiments and were involved in manuscript preparation. CL, DT participated in collection of samples and data analysis. Jing W, CT and XH performed immunofluorescence experiment. Jingyu W conceived of the study and participated in its design and coordination. YZ helped with experiments, provided valuable

discussion and modified the final manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Care of laboratory animals and animal experimentation were performed in accordance with the animal ethics guidelines and approved protocols. All animal experiments were approved by the Animal Ethics Committee of Northwest A&F University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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