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Duck STING Mediates Innate Immune Response Induced by H5N8 Subtype Avian Influenza Viruses

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ABSTRACT

***Background*:** The H5N8 highly pathogenic avian influenza virus (HPAIV) poses a significant threat to global poultry and public health due to its high mortality rate and potential for cross-species transmission. As natural H5N8 hosts, Anatidae (e.g., ducks) drive transmission and evolution. STING, a key innate immunity regulator, activates IFN-β via signaling to combat infection. While STING's anti-AIV role is known, its mechanisms in ducks during H5N8 infection remain unclear. This study analyzes STING pathway dynamics in SPF ducks post-infection, revealing its core antiviral role in waterfowl. Findings support HPAIV control strategies.

***Materials, Methods & Results*:** This study utilized sixty 4-week-old specific pathogen-free (SPF) ducks, randomly divided into H5N8-infected (n = 30) and PBS control (n = 30) groups. The experimental group was inoculated via intranasal and oropharyngeal routes with 106 EID50/0.2 mL of A/whooper swan/Henan/SMQ9/2020 (H5N8) viral suspension, while the control group received an equivalent volume of PBS. Euthanasia was performed at 0.25, 1, 2, 3, 5, 7, 10, and 14 days post-infection (dpi) [n = 3-4 per time point], with collection of lung, spleen, and rectal tissues. Clinical observations revealed mild respiratory symptoms in infected ducks, including coughing and nasal secretions. Two SPF ducks died at 3 dpi and 5 dpi. Necropsy demonstrated mucus accumulation in the trachea and pulmonary hemorrhages in the infected group. The control group exhibited no clinical symptoms or histopathological lesions throughout the experimental period. Absolute quantitative fluorescent PCR detected peak viral loads in tissues at 2-3 dpi, with pulmonary viral loads significantly exceeding those in other organs, followed by a gradual decline after 5 dpi. qRT-PCR revealed time-dependent differential changes in the expression of STING pathway immune factors (STING, TBK1, IRF7) as well as TLR7 and IL6. During the early stages of infection, the expression of these immune factors was significantly upregulated, peaking at 2-3 dpi, followed by a gradual decline in expression from 5 dpi onward. Notably, the expression trends of TBK1, IRF7, and IFN-β exhibited a striking similarity to that of STING. SPSS correlation analysis demonstrated a positive correlation between STING and IFN-β expression in the lungs, spleen, and rectum. These results indicate that STING regulates the expression of multiple immune factors and promotes IFN-β production, playing a critical antiviral role in SPF ducks during the early phase of H5N8 avian influenza virus infection.

***Discussion*:** This study elucidates the pivotal role of the STING signaling pathway in coordinating the innate immune response of SPF ducks against H5N8 HPAIV. Our findings demonstrate that the expression of STING and associated immune factors peaked at 2-3 dpi before gradually declining from 5 dpi onward. The STING-mediated TBK1-IRF7 signaling axis was found to promote IFN-β production, as supported by significant positive correlations (SPSS analysis) between STING and IFN-β expression levels in the lung and spleen. These results suggest that enhancing STING activity or stabilizing its expression could augment host antiviral defenses, thereby curbing viral replication during early-stage infections. Collectively, our work establishes a molecular foundation for developing STING-targeted immunomodulatory strategies, such as novel vaccine adjuvants, to improve avian influenza control. This approach not only holds potential to mitigate economic losses in poultry industries but may also reduce zoonotic transmission risks.

Keywords: H5N8, AIV, ducks, STING, IFN-β.

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INTRODUCTION

The Stimulator of Interferon Genes (STING) is the center of the immune response and is a multifaceted adaptive protein. cGAS recognizes double-stranded DNA (dsDNA) and catalyzes ATP and GTP to produce cGAMP, which subsequently activates STING and initiates signal transduction to induce the production of IFN-I. In addition, cGAS can recognize a few RNA viruses and inhibit them. Research has found that after certain strains of influenza A virus enter the body, the fusion peptide (FP) of hemagglutinin (HA) can directly interact with STING to activate the signaling pathway and stimulate IFN-β production [2,6]. The highly pathogenic avian influenza virus H5N8 (HPAIV H5N8) poses severe global threats due to its high mortality in poultry, capacity for cross-species transmission, causing catastrophic economic losses in the poultry industry, endangering wild birds, and mammalian infections, raising public health concerns. Ducks are asymptomatic carriers of AIV and usually do not show obvious clinical symptoms, thus acting as invisible carriers in the virus transmission route. In addition, their interactions with other poultry and wild birds increase the mutation of the virus, thereby posing a significant threat to wild bird populations, the poultry industry, and public health [4].

The aim of the present study was to investigate H5N8 HPAIV dynamics in SPF duck lungs, spleen, and rectum, while analyzing STING signaling components (including TBK1/IRF7) and their regulation of IFN-β production during antiviral responses, providing insights into waterfowl immunity mechanisms.

MATERIALS AND METHODS

Virus

The H5N8 virus strain, designated A/whooper swan/Henan/SMQ9/2020 (Hn/SMQ9), was isolated from a deceased whooper swan in Henan Province in 2020. The virus was obtained by inoculating 10-day-old specific pathogen-free (SPF) chicken embryos at the National Avian Influenza Reference Laboratory of the Harbin Veterinary Research Institute. HA and NA subtypes of avian influenza virus were detected by HA-HI and NA-NI tests. Sequence analysis using the SeqMan program revealed that Hn/SMQ9 possesses multiple basic amino acids (PLREKRRKR↓GLFGAI) at the HA cleavage site, which is characteristic of highly pathogenic avian influenza viruses (HPAIV).

Animal experiment

SPF Shaoxing ducks were used as the experimental animals. All ducks were hatched and raised to 4 weeks of age, with a weight of 750-800 g in the negative pressure isolator at the Experimental Animal Center of Harbin Veterinary Research Institute. The 50% egg infective dose (EID50) of the harvested allantoic fluids of Hn/SMQ9 was determined using the method of Reed and Muench. Sixty SPF ducks were randomly divided into 2 equal groups. These groups were intranasally and orally inoculated with 0.2 mL 10^6 EID50 of Hn/SMQ9 (H5N8 group) and Phosphate-buffered saline (PBS; Control group), respectively. To assess the changes in these factors, ducks were euthanized at 1/4, 1, 2, 3, 5, 7, 10, and 14 dpi. During the experiment, clinical symptoms of ducks were observed and recorded. For each period, 3 SPF ducks were sacrificed in both groups, tissue samples were collected and stored at -80°C.

Quantitative real-time PCR

Tissues (lung, spleen, and rectum) were collected using sterile methods, and RNA was extracted using an RNA extraction kit[1]. The absolute quantification method was used to detect viral loads, with RNA reverse-transcribed into cDNA using Uni12 primers[1]. The target fragment was amplified by qH5N8-F/R primer and cloned into a pMD 18-T vector[1]. The positive plasmid was selected as a standard to construct a standard curve for quantification purposes. The qPCR reactions were conducted using the SLAN®-96S, reaction system: 2×TB Green Premix Ex Taq II 5 μL, ddH_2O 3 μL, cDNA 1 μL, qH5N8-F/R 0.5 μL of each primer. Quantitative real-time PCR was used to detect the expression of STING, IFN-β, TBK1, TLR7, IRF7, and IL6. RNA was reverse-transcribed into cDNA by oligo-dT primers[1]. The qPCR method was consistent with the above. Detailed primer information is in Table 1.

Statistical analysis

An absolute quantitative method was used to detect viral load, and the standard copy number was calculated according to the concentration of the standard substance and the number of bases,equation:

$$Copy\ number = \frac{6.02 \times 10^{14} \times \text{Standard concentration (ng/µl)}}{\text{Standard Base Pari Count} \times 660}$$

Quantitative real-time PCR was used to detect STING, IFN-β, TBK1, TLR7, IRF7, and IL6 Ct values, and they were normalized to the housekeeping gene β-actin, using the $2^{-\Delta\Delta Ct}$ method [1]. Gene expression levels were expressed as fold changes relative to the control group, with the control set to 1. Positive values indicate upregulation and negative values indicate downregulation. Gene expression changes were statistically analyzed with *t*-tests. Statistical analysis and data graphing were performed using GraphPad Prism v. 8.0.1 (GraphPad Software).

SPSS was used to conduct correlation analysis of the relative expression levels of STING and IFN-β in different tissues of SPF ducks following H5N8 infection.

Table 1. Specific quantitative primers for SPF duck cytokines.

Primers	Primer sequence (5'-3')	Product length
qH5N8	F: GACCRATCCTG TCACCTCTGAC R: AGGGCATTYTGGACAAAKCGTCTA	132bp
qSTING	F: CACATCTTGATCCCTCTGAGCT R: CCCTGATTGCATAGAAGCTGT	152bp
qIFNβ	F: ATCAACGCGCACTTTTTCCC R: TGCTTGGACTGCTGAGGATG	120bp
qIL6	F: AGCTGAAATCGGATAAGACCT R: TTCAAATAGCGAACAGCCCTC	106bp
qTBK1	F: GCATCCAAGAGACAGAAATC R: ACTTGCTGACACATTCTTCT	200bp
qTLR7	F:AATTGTGACGCAGTGTGGTTTGTTTGG R:TAATGCATACATGATCAAATAGGAGGTGTC	174bp
qIRF7	F: GCACAGGCCAGCGGGAGGTA R: TGAGGGTCGTCGCTGTTCTTG	134bp
qβ-actin	F: GCAAGTACTCTGGTCTGGATTGGAG R: TTTGCGGTGGACAATGGA	116bp

RESULTS

Clinical manifestations

In the H5N8 group, a few ducks were depressed and had mild respiratory symptoms. Two ducks died at 3 and 5 dpi, respectively. Necropsy findings disclosed that the trachea had mucous and pulmonary hemorrhage. The control group showed no symptoms or changes.

Viral load in tissue

After Hn/SMQ9 virus infection in SPF ducks, the virus exhibits significant temporal and tissue expression changes in the lungs, spleen, and rectum (Figure 1). In the lung, the viral load significantly increases in the early stages of infection and then gradually declines. The viral load in the lung is relatively high at 1/4 and 1 dpi, peaks at 2 dpi, and shows a decreasing trend in 5-14 dpi, remaining at a relatively high level throughout the experimental period. In the spleen, the viral load peaks at 2 dpi, declines at 3 and 5 dpi, but rises again at 7 dpi. From 10 to 14 dpi, the viral load in the spleen shows a decreasing trend. The viral load in the rectum was relatively low at 1/4 and 1 dpi, but significantly increased at 2 dpi. In conclusion, we found that the viral load in the lungs is significantly higher than in the spleen and rectum, and the viral load in the rectum is generally lower than in the lungs and spleen. The viral load peaks at 2 dpi in the early stages of infection.

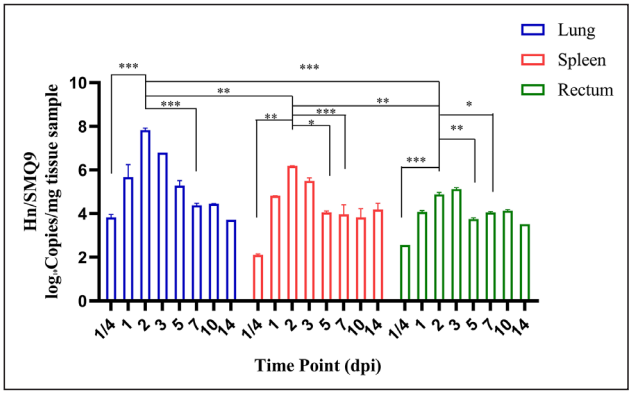

Figure 1. Viral load in tissue. The meter character indicates significant changes(t-text). *means $P < 0.5$; **means $P < 0.01$, and ***means $P < 0.001$.

mRNA expression of STING and related factors

By detecting the expression levels of STING and key immune factors in SPF ducks (Figure 2), we found that in SPF ducks infected with Hn/SMQ9, STING exhibited higher expression levels in the early stages of infection, peaking at 2,3 dpi. The expression trends of IFN-β, TBK1, IRF7, TLR7, and IL6 were similar to that of STING. These immune factors gradually converged with the control group at 5-14 dpi. In the spleen, Hn/SMQ9 stimulated STING and other immune factors to peak at 2 dpi, after which the immune factors showed a declining trend post 3 dpi. The expression changes of immune factors in the rectum were similar to those in the spleen: in the H5N8 group of SPF ducks, STING reached its peak at

2 dpi and gradually returned to normal levels after 5 dpi. In the early stages of Hn/SMQ9 infection in SPF ducks, the viral load significantly increased, and the expression levels of immune factors such as STING and IFN-β also rose in the early stages of infection, peaking at 2 and 3 dpi. As the number of infection days increased, the viral load showed a downward trend, and the expression of immune factors gradually decreased in late-stage infection. In comparison, the rectal viral load and immune factor expression were smaller.

STING and IFN-β comparison in different time and tissues

STING is a key regulator of IFN-β expression. To investigate the link between STING and IFN-β in ducks infected with Hn/SMQ9, we analyzed data at specific time points (Figure 3). Early in the infection, both STING and IFN-β showed significant changes in expression in the lungs, spleen, and rectum of SPF ducks (Figure 3). In the lungs, STING was significantly upregulated at 1/4-1 dpi compared to IFN-β. Both STING and IFN-β were upregulated at 2-3 dpi and then decreased at 5 dpi. In spleens, STING and IFN-β were upregulated at 1/4, 1, 2, and 5 dpi, with the mRNA expression of both STING and IFN-β peaking at 2 dpi. In rectums, STING and IFN-β levels were lower at 1/4-1 dpi, and their mRNA expression peaked at 2 dpi.

Correlation between STING and IFN-β relative expression levels

Correlation analysis using SPSS revealed a significantly positive correlation between STING and IFN-β relative expression levels in lung, spleen, and rectum tissues of SPF ducks infected with H5N8 HPAIV (Figure 4), with statistical significance observed across all 3 target organs ($P < 0.05$).

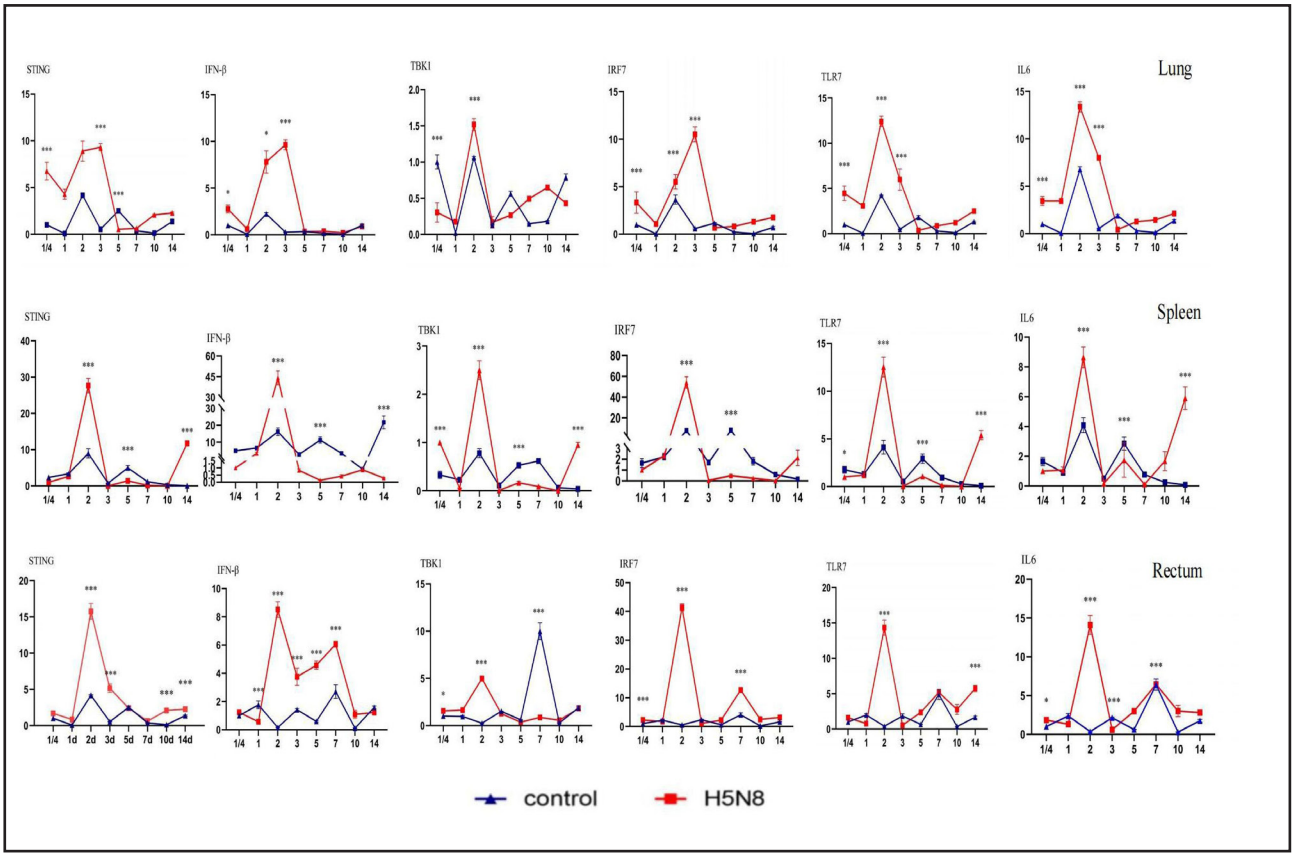

Figure 2. Expression of STING, IFN-β, TBK1, IRF7, TLR7, and IL6 in the lung, spleen, and rectum of SPF ducks in the H5N8 group and the control group. Control stands for the negative control group, and H5N8 stands for the experimental group infected with H5N8. The meter character indicates significant changes(t-text). *means $P < 0.5$; **means $P < 0.01$, and ***means $P < 0.001$.

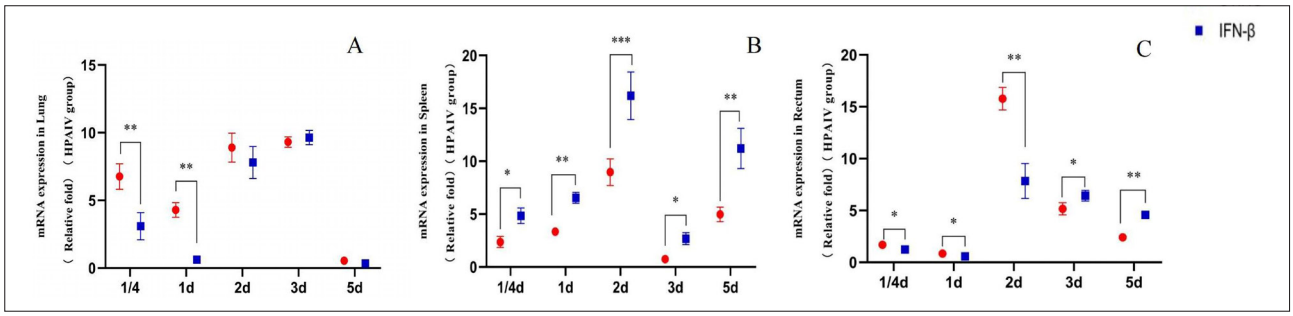

Figure 3. Expression of STING and IFN-β, A in the lung, B in the spleen, and C in the rectum tissues. The meter character indicates significant changes (*t*-text). *means $P < 0.5$; **means $P < 0.01$, and ***means $P < 0.001$.

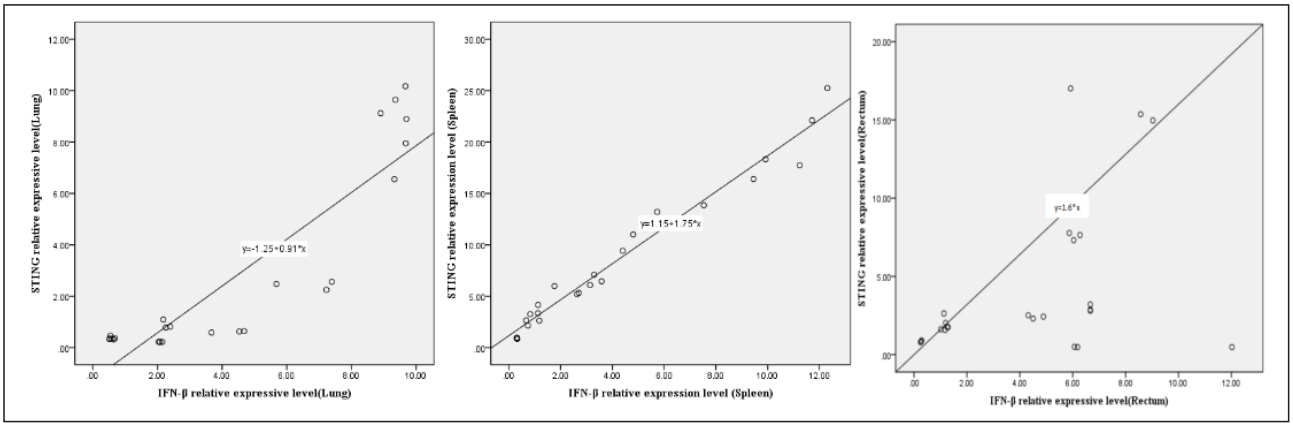

Figure 4. Correlation between the relative expression of STING and IFN-βin SPF ducks infected with H5N8.

DISCUSSION

STING is a crucial factor in modulating the innate immune response by detecting viruses through cGAS and triggering the release of downstream immune factors [6]. The STING signaling pathway to identify the virus and activate TBK-1, phosphorylation and activation of IRF3 induce IFN-I(IFN-β) and other factors (IL6, and TLR7), triggering an immune response [14]. The researchers found that ducks lack IRF3, which is an important type I interferon-induced transcriptional regulator. They speculate that IRF7 as a member of the same family as IRF3 and with a similar structure, may perform a similar function [8,11]. Our study shows that STING and IRF7 expression trends are similar, suggesting that STING may regulate IRF7. IL6 is an important inflammatory cytokine. Studies have found that STING can promote the production of IL-6, and IL-6 can further enhance STING-mediated immune response [1,13]. In SPF ducks infected with HPAIV H5N8, STING, TBK1, and IRF7 were upregulated early, with IFN-β, TLR7, and IL6 significantly increasing at 2 dpi (Figure 2). There was a positive correlation between the expression of STING and IFN-β (Figure 4). STING and IFN-β showed similar expression trends in the early stage of infection (Figure 3), the expression of STING and IFN-β in the spleen and rectum increased significantly at 2 dpi in particular. This synchronously upregulated expression pattern suggests that STING may play a regulatory role in regulating the changes of IFN-β and other immune factors. In the STING signaling pathway, STING promotes the expression of IFN-β by activating TBK1 and IRF3. Although IRF3 is missing in ducks, IRF7 may replace its function to activate IFN-β expression. The significant expression peaks of STING and IFN-β at 2,3 dpi indicate that STING is activated in the early stage of infection and directly or indirectly promotes the production of IFN-β, thus promoting the antiviral immune response.

AIV is a respiratory infectious disease that replicates at different rates in different tissues [4]. The

avian influenza virus initially infects the upper respiratory tract of the host, shows high replication efficiency, and spreads through respiratory secretions [9]. HPAIV can replicate heavily in the respiratory tract, causing acute injury of the trachea, bronchus, and other parts, manifested by pathological changes such as congestion, edema, and bleeding [10]. The intestine is an important organ for the transmission of the virus, through feces to be transmitted. Avian influenza infection in waterfowl is usually asymptomatic, but the virus can be shed and contaminate water sources [3,12]. The spleen is an important immune organ of the body. It contains a large number of lymphocytes and macrophages, which can produce immune responses to invading pathogens. Therefore, the lung, spleen, and rectum were chosen as the experimental targets. By comparing viral load and immune factor expression in tissues, we found that the virus exhibited rapid replication in the lungs, while expression was relatively low in the rectum (Figure 1). This finding is consistent with previous findings suggesting tissue specificity in viral distribution and replication in the host. In addition, the viral load in the lung, spleen, and rectum was highly expressed at 2,3 dpi, while STING, IFN-β, and other immune factors were also expressed highly at the same time (Figures 1 & 2). This pattern suggests that in the early stages of AIV infection, viral replication triggers the release of innate immune factors, particularly STING-mediated immune responses. We found a fluctuating downward trend of viral load after 3 dpi (Figure 1), indicating that host innate immunity (especially STING-mediated interferon response) was activated at the early stage of viral infection and played an effective role in inhibiting viral replication. The immune process suggests that STING-mediated innate immune response plays a key antiviral role in the early stage of host infection, resulting in a significant decrease in viral load.

The study demonstrates the significant involvement of the STING-mediated innate immune response in the antiviral mechanism in SPF ducks following infection with HPAIV H5N8. The positive correlation between STING and key immune factors (IFN-β, TLR7, and IL-6) indicates that STING has a role in activating and maintaining innate immune defense. In the future, we want to use STING-specific inhibitors, such as C-176 or H-151, to assess whether IFN-β and other immune factors are downregulated, thereby better determining the specific effects of STING [5,7]. Additionally, there are numerous pathways involved in the host's antiviral response, such as the RIG-I-like receptor pathway and the Toll-like receptor pathway. In the future, we aim to investigate how these pathways coordinate with each other to better understand the role of STING in the antiviral process.

In summary, viral load and STING-mediated immune factor expression were significantly upregulated at the early stage of infection 2,3 dpi, indicating that viral replication stimulated the STING pathway. After that, the virus showed a downward trend, indicating that STING-mediated innate immunity plays a key role in antiviral. Similar expression trends of STING and IFN-β were found, indicating that STING may regulate IFN-β expression. The finding can provide a deeper understanding of ducks' immunological response to HPAIV H5N8 infections, offering a theoretical basis for improving disease prevention and control measures and enhancing ducks' disease resistance.

MANUFACTURER

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***Ethical approval*.** Our research was approved by the Animal Protection and Ethics Committee of Northeast Forestry University (2023004; Harbin, China).

***Declaration of interest*.** The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this article. Also, they are alone responsible for the content and writing of the paper.

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