

# NP and NS1 proteins of H5N1 virus significantly upregulated IFITM1, IFITM2, and IFITM3 in A549 cells

Haifeng Wang<sup>1,2</sup>, Lin Chen<sup>2</sup>, Jing Luo<sup>2</sup>, Hongxuan He<sup>2</sup>

1. School of Environmental Engineering, Central Plains Specialty Food Engineering&Technology Research Center, Kaifeng Key Laboratory of Food Composition and Quality Assessment, Yellow River Conservancy Technical Institute, Dong Jing Avenue, Kaifeng 475004, China.
2. National Research Center for Wildlife-Borne Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, 100101, Beijing, China.

## Abstract

**Background:** Avian influenza virus H5N1 causes serious public health concern with significant morbidity and mortality from poultry to humans. Interferon-induced transmembrane (IFITM) proteins usually protect cells from many virus infections by viral entry and replication.

**Objectives:** The purpose of this study was to investigate whether H5N1 viral proteins involved in regulation IFITM1, IFITM2, and IFITM3 following H5N1 infection.

**Methods:** NS1, M1, NP, PB2, HA and NA genes of H5N1 virus were generated by PCR and cloned into pcDNA3.1/myc-His (+) A vector for genes over-expression experiments. Gene expression levels was performed using Real-time PCR.

**Results:** Research displayed that NS1, M1, NP, and PB2 proteins of H5N1 virus increased IFITM1, IFITM2, and IFITM3 expression in A549 cells, only IFITM1 was upregulated by M1 in HEK293T cells. However, our study did not find that HA and NA of H5N1 virus affected IFITM genes family or interferon genes expression.

**Conclusion:** Taken together, our data suggested that IFITM1, IFITM2, and IFITM3 might be directly upregulated via NS1, M1, NP, and PB2 proteins during H5N1 avian influenza virus infection. This study provided new insights into the influence of NS1 and NP proteins on regulation of IFITM1, IFITM2, and IFITM3 expression following H5N1 infection.

**Keywords:** Influenza Virus, H5N1, NS1 protein, NP protein, IFITMs.

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## Introduction

Avian influenza A virus H5N1 is one of the greatest world-

wide pandemic threats to poultry and humans health. H5N1 contains eight negative-sense, single-stranded RNAs that code for eleven proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2)<sup>1,2</sup>. Structural and non-structural proteins of H5N1 are relevant in respect of adaptive and innate immune responses through activating or inhibiting a series of immune-related cytokine. Previous study showed NS1 as the primary influenza virus interferon antagonist with multiple inhibitory effects on host immune pathways<sup>3</sup>. NS1, as a virulence factor of H5N1 due to mutation at the 42<sup>nd</sup> residue within the RNA-binding domain (RBD), dramatically changed the degree of pathogenicity of H5N1 in mice<sup>4</sup>. The IFITM genes belong to a family of interferon-induced transmem-

### Corresponding author:

Hongxuan He,  
National Research Center for Wildlife-Borne Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, 100101, Beijing, China.  
Tel.: +86 10 64807118;  
fax: +86 10 64807118.  
Email: [hehx@ioz.ac.cn](mailto:hehx@ioz.ac.cn)

brane proteins including IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10. Only IFITM1, IFITM2, and IFITM3 were significantly upregulated by I and II type interferons stimulate<sup>5,6</sup>. These proteins as innate antiviral cell-intrinsic restriction factors prevent viruses from traversing the lipid bilayer and accessing the cytoplasm, such as influenza A virus (IAV), Japanese encephalitis virus (JEV), and dengue virus (DENV), et al<sup>7,8</sup>. NP belong to viral nucleoprotein, with viral RNA polymerase together consisting of ribonucleoprotein complexes. Ubiquitination and deubiquitination of NP protein regulated influenza A virus RNA replication<sup>9</sup>.

To assess whether structural and nonstructural proteins of H5N1 virus involved in regulated IFITM1, IFITM2, and IFITM3 expression, we conducted overexpression of NS1, M1, NP, PB2, HA, and NA proteins in order to evaluate the effect of viral proteins on IFITM family. Our study showed that several structural and nonstructural of

H5N1 virus distinctly increased IFITM1, IFITM2, and IFITM3 expression, and especially NS1 and NP protein played a stronger role in A549 cells.

## Materials and methods

### Cell culture

A549 and HEK 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FCS, Gibco), in a humidified 5% CO<sub>2</sub> and 95% air at 37°C.

### Gene cloning and plasmids

The open reading frame (ORF) of influenza virus NS1, M1, NP, PB2, HA and NA genes were generated by PCR and cloned into pcDNA3.1/myc-His(+)-A vector for genes over-expression experiments. All constructs were verified by sequencing, and primers used in this study are listed in Table 1.

**Table 1. Primer sequences of full-length gene annealing temperature used in construct plasmids.**

Gene Symbol	Sequence(5'-3')	Annealing(°C)	plasmid
NS1	TACGAATTCATGGATTCCAACACTGTGTC	54	3.1
	TACCTCGAGCTTTGGAGAGAGTGGAGGTC		
M1	TACAAGCTTATGAGCCTTCTAACCGAGGTCGAA	58	3.1
	TACCTCGAGCTTGAATCGCTGCATTTGCACTC		
NP	CCCGGATCCATGGCGTCTCAGGGCACCAAACGA	56	3.1
	CCCCTCGAGATTGTCATATTCCTCTGCATTGTC		
PB2	CCCAAGCTTATGGAGAGAATAAAAGAATTAAG	56	3.1
	CCCCTCGAGATAGATGGCCATCCGAATCCTTTTG		
HA	TACAAGCTTATGGAGAAAATAGTGCTTCTTCTT	54	3.1
	TACCTCGAGAATGCAAATTCTGCATTGTAACG		
NA	TACAAGCTTGCCACCATGAATCCAAATCAGAAGA	54	3.1
	TAATAAC		
	TCACTCGAGCTTGTCATGGTGAATGGCAAC		

## Virus Culture

Mouse adapted influenza virus A/environment/Qinghai/1/2008 (H5N1) was propagated in 10-day-old embryonated chicken eggs at 37 °C for 2 days. Allantoic fluid was purified by centrifugation and was stored at -80°C until viral infection was performed when cells were 80% confluent. The serum-free DMEM medium containing allantoic fluid was added at 0.5, 1, 2 multiplicities of infection (MOI) for 1h. Then cells were washed thrice by PBS and cultured with DMEM containing 2% FBS. All experiments with this infectious virus were carried out in a Biosafety Level 3 containment laboratory.

## Transfection

Cells were seeded in 12-well plates overnight and transfected with 2 ug plasmids suspended in 3 ul Lipofectamine 3000 (Invitrogen) and 100 ul OPTI-MEM Reduced Serum Medium. After 6 h transfection, the cell medium was replaced by fresh DMEM medium with 10% FBS. A549 and HEK293T cells were transfected for 36 h, then harvested and resolved in TRIzol Reagent for RNA extraction and reverse transcription.

## RNA extraction and cDNA synthesis

Total RNA was extracted from A549 and HEK293T cells using the TRIzol Reagent according to the supplier's specifications. Spectrophotometer (Thermo) was used to determine the concentration of RNA at 260/280 nm. cDNA was synthesized using 5ug of total RNA

from A549 and HEK293T cells, GoScript™ Reverse Transcription System (Promega) was used for first-stand synthesis following manufacturer's instructions and was stored at -80°C until used.

## Real-time PCR

Real-time PCR was performed in a ABI 7500 fast real-time system (Life Technology) with SYBR green real-time PCR master mix (CWBIO). Real-time PCR conditions were as follows: 95°C for 10 min, then 94°C for 30 s, 60°C for 1 min, 40 cycles, followed by 72°C for 7 min. Reactions were performed in a 20ul volume containing 10 ul SYBR green real-time PCR master mix, 8.5 ul double-distilled water, 1 ul forward and reverse primers, and 0.5 ul cDNA template. The primers are listed in Table 2.  $\beta$ -actin as internal reference in order to normalize the mRNA expression levels of target genes, and generation of specific PCR products was confirmed by melting curve analysis.  $2^{-\Delta\Delta CT}$  method was used to quantify the relative mRNA express levels.

## Statistical analysis

Real-time PCR data of IFITM and interferon family genes are expressed as the means  $\pm$  s.e.m. (standard error of the mean). Student's t-test was used to determine the significance of differences between two groups, while one-way analysis of variance (ANOVA) was used when there were more than two groups. P-value <0.05 was considered to be statistically significant.

**Table 2. Primer sequences, amplicon size and annealing temperature used in Real-time PCR assays.**

Gene Symbol	Sequence(5'-3')	Amplicon(bp)	Annealing(°C)
<b><math>\beta</math>-actin</b>	GCGGGAAATCGTGCGTGACATT GATGGAGTTGAAGGTAGTTTCGTG	232	60
<b>IFNA1</b>	AGAAATACAGCCCTTGTGCC TGACCTGGTGTATGAGTCAATAAG	150	60
<b>IFNB1</b>	CAGCTCTTTC CATGAGCTAC CAGCC AGTGC TAGATGAATC	330	60
<b>IFNG</b>	GAGATGACTTCGAAAAGCTGAC ACCTCGAAACAGCATCTGAC	149	60
<b>H5N1</b>	GACCAATCCTGTACCTCTGA GTATATGAGGCCCATRCAACT	251	60
<b>IFITM1</b>	ATCAACATCCACAGCGAGAC CAGAGCCGAATACCAGTAACAG	253	60
<b>IFITM2</b>	TTCATAGCATTTCGCGTACTCC GAATACAGGTCAAGGGCAGAG	296	60
<b>IFITM3</b>	GAGAACCATCCCAGTAACCC CAACCATCTTCTGTCCCTAG	318	60

**Results**

**H5N1 promoted expression of IFITM1, 2, and 3 genes in A549 cells**

H5N1 virus induced death of A549 and HEK293T cells at 0.5, 1, 2 MOI for 1h, IFITM1, IFITM2, and IFITM3 expression increased in A549 cells, only IFITM2 and IF-

ITM3 were upregulated but IFITM1 had no changes in HEK293T cells. In addition, IFITM1 had higher expression levels than that of IFITM2 and IFITM3 in A549 cells. It was a opposite phenomenon with expression level of IFITM1, IFITM2 and IFITM3 a higher expression than that of IFITM1 in HEK293T cells (Figure 1 and Figure 2).

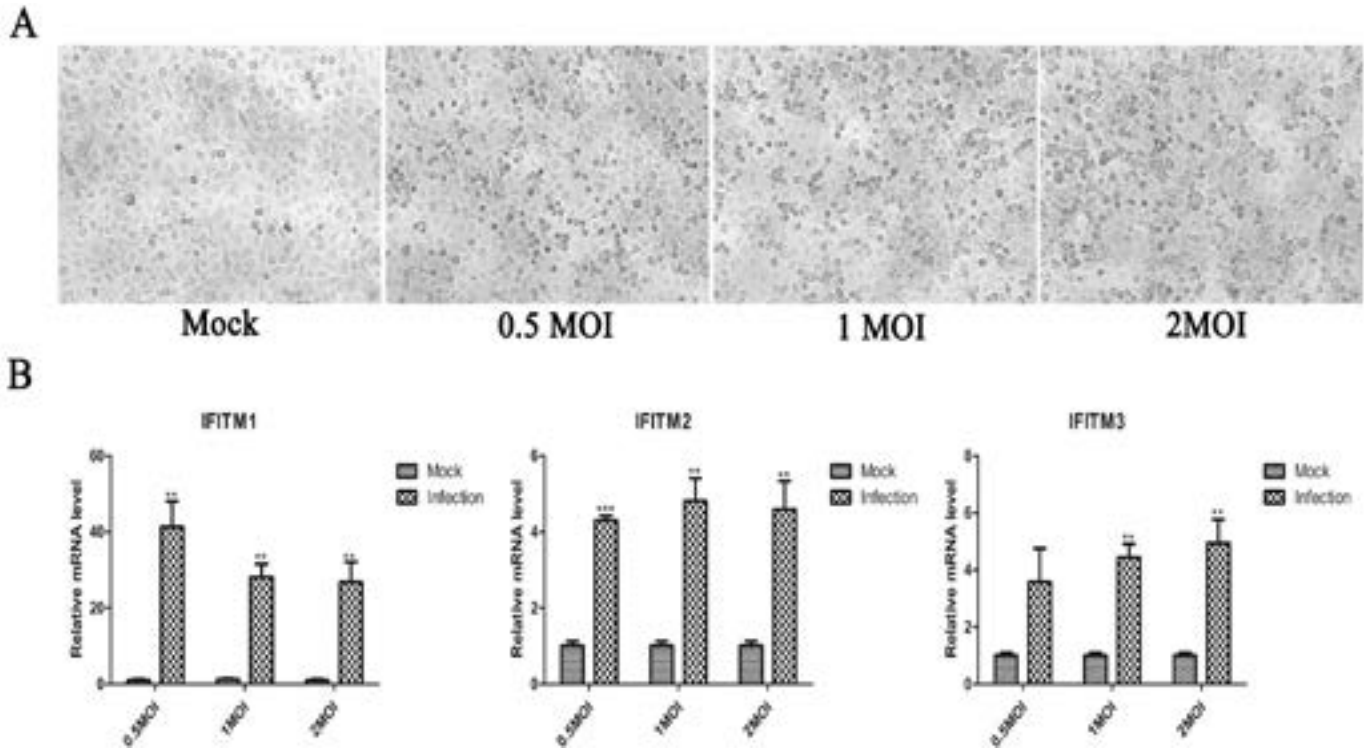


Figure 1. Transcription expression pattern of IFITM1, 2, and 3 in A549 cells by H5N1 influenza virus infection.

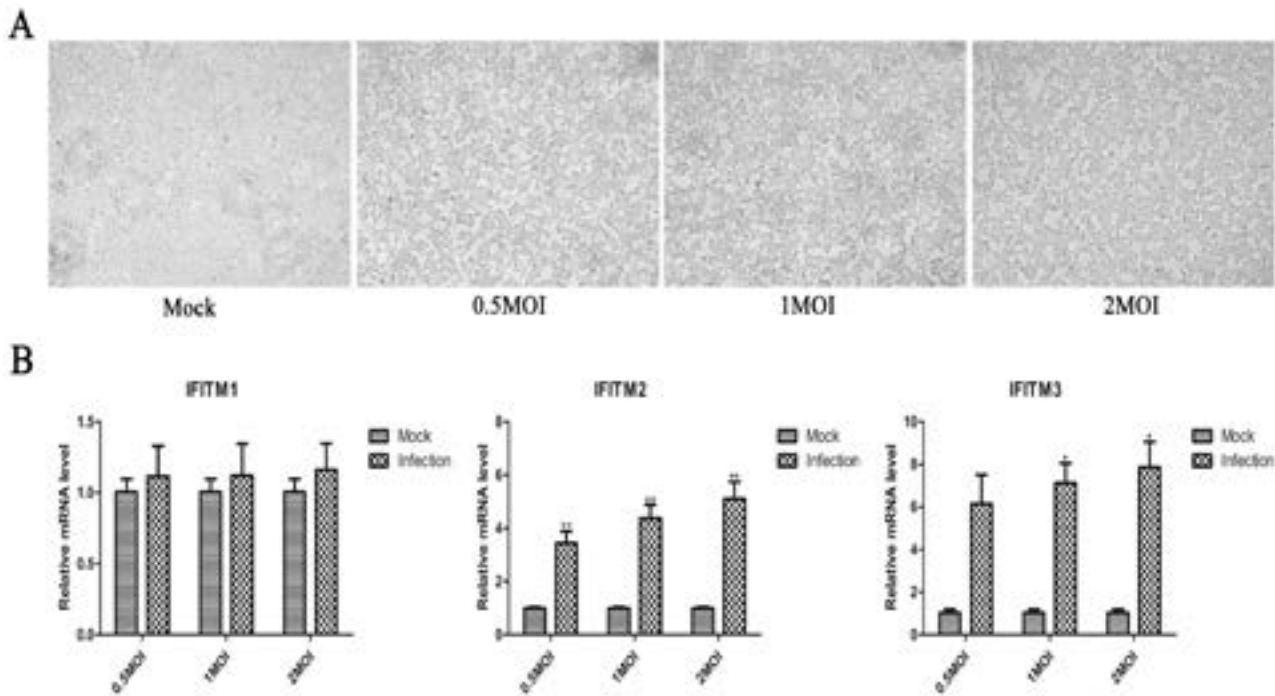


Figure 2. Transcription expression pattern of IFITM1, 2, and 3 in HEK293T cells by H5N1 influenza virus infection.

## IFNA1, IFNB1, and IFNG were upregulated by H5N1 infection

Our study demonstrated that H5N1 avian influenza virus could upregulate IFNA1, IFNB1, and IFNG in both A549 and HEK293T cells (Figure 3). NS1 and NP pro-

teins were found increased IFNA1 expression, NP could increase IFNB1 expression in A549 cells, and simultaneously NS1 upregulated IFNG in HEK293T cells (Figure 4). HA and NA proteins showed no effect on the interferon genes IFNA1, IFNB1, and IFNG expression (Figure 5).

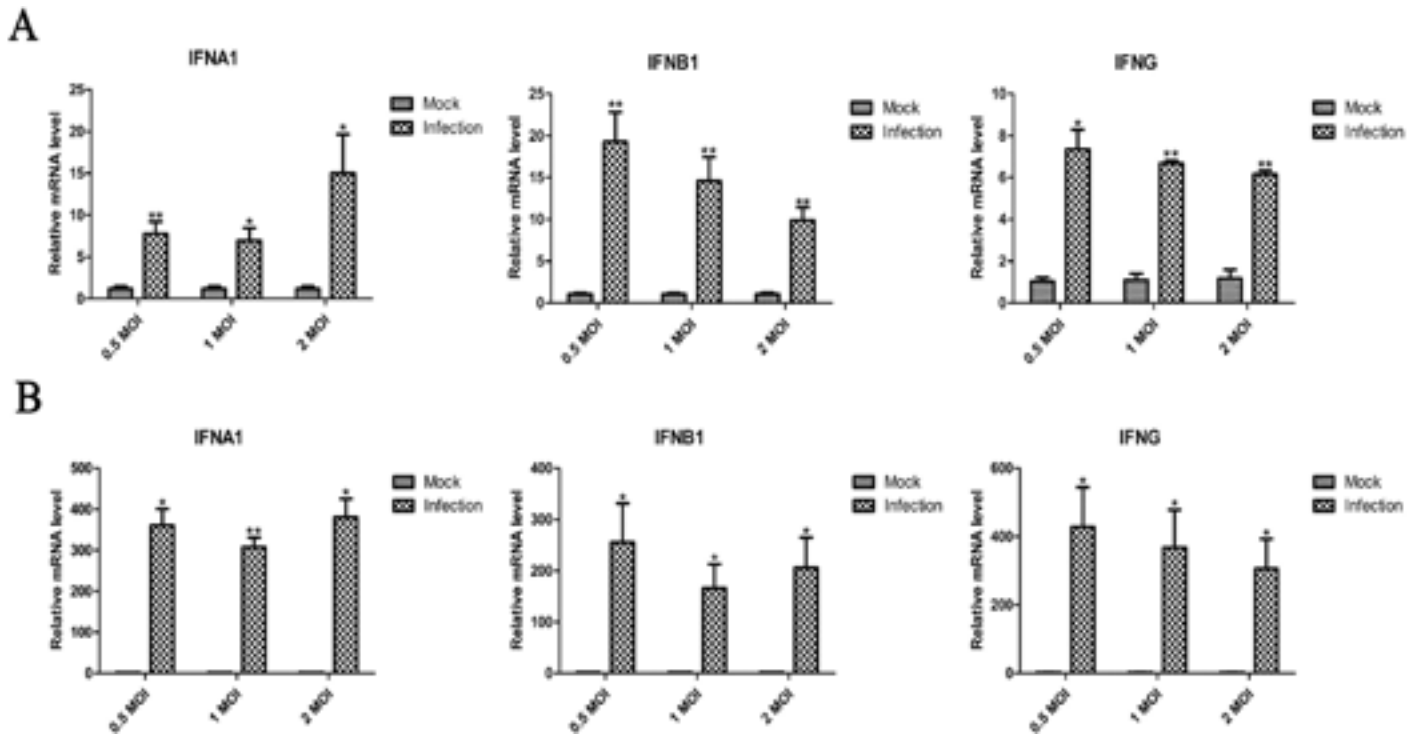


Figure 3. Transcription of IFNA1, IFNB1, and IFNG with H5N1 virus infection.

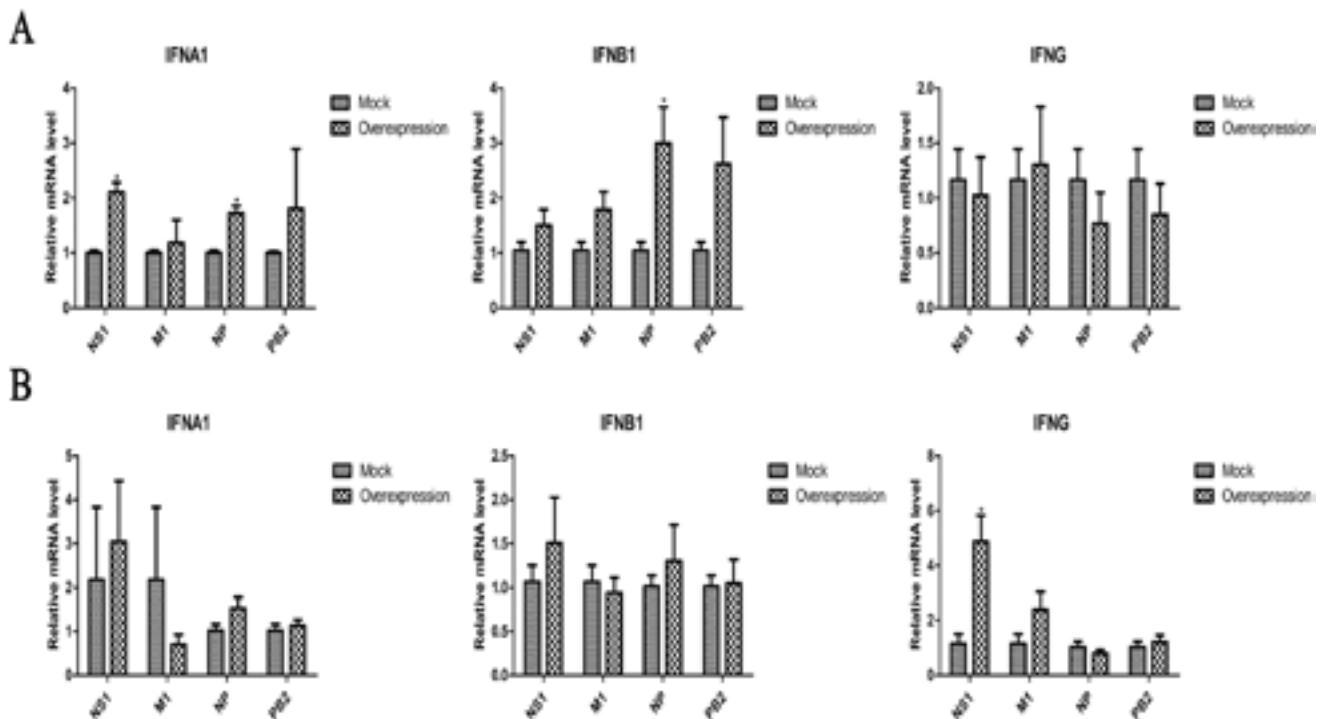


Figure 4. Influences of influenza virus NS1, M1, NP, and PB2 proteins on the expression of interferons.

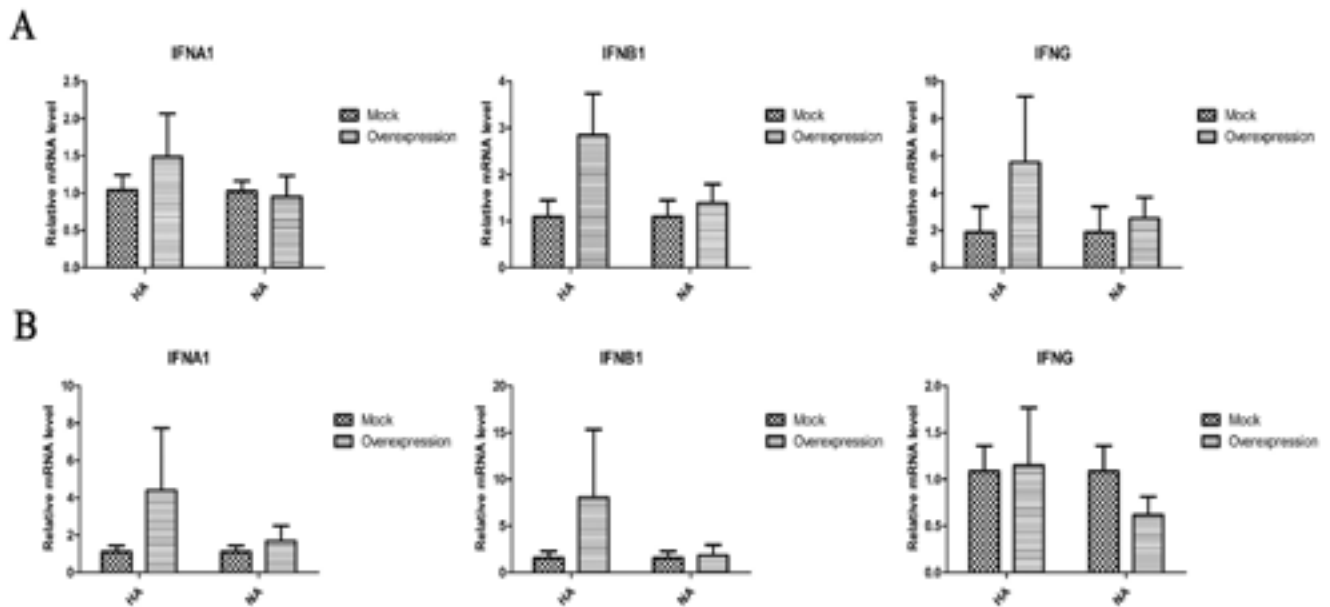


Figure 5. Influences of influenza virus HA and NA proteins on the expression of interferons.

**NS1 and NP proteins increased IFITM1, 2, and 3 expression in A549 cells**

Experiments displayed NS1, M1, NP, and PB2 could promote IFITM1, IFITM2, and IFITM3 expression in A549

cells, only M1 could upregulate IFITM1 expression in HEK293T cells (Figure 6). Either HA or NA showed no effect on the interferon-related genes IFITM1, IFITM2, and IFITM3 (Figure 7).

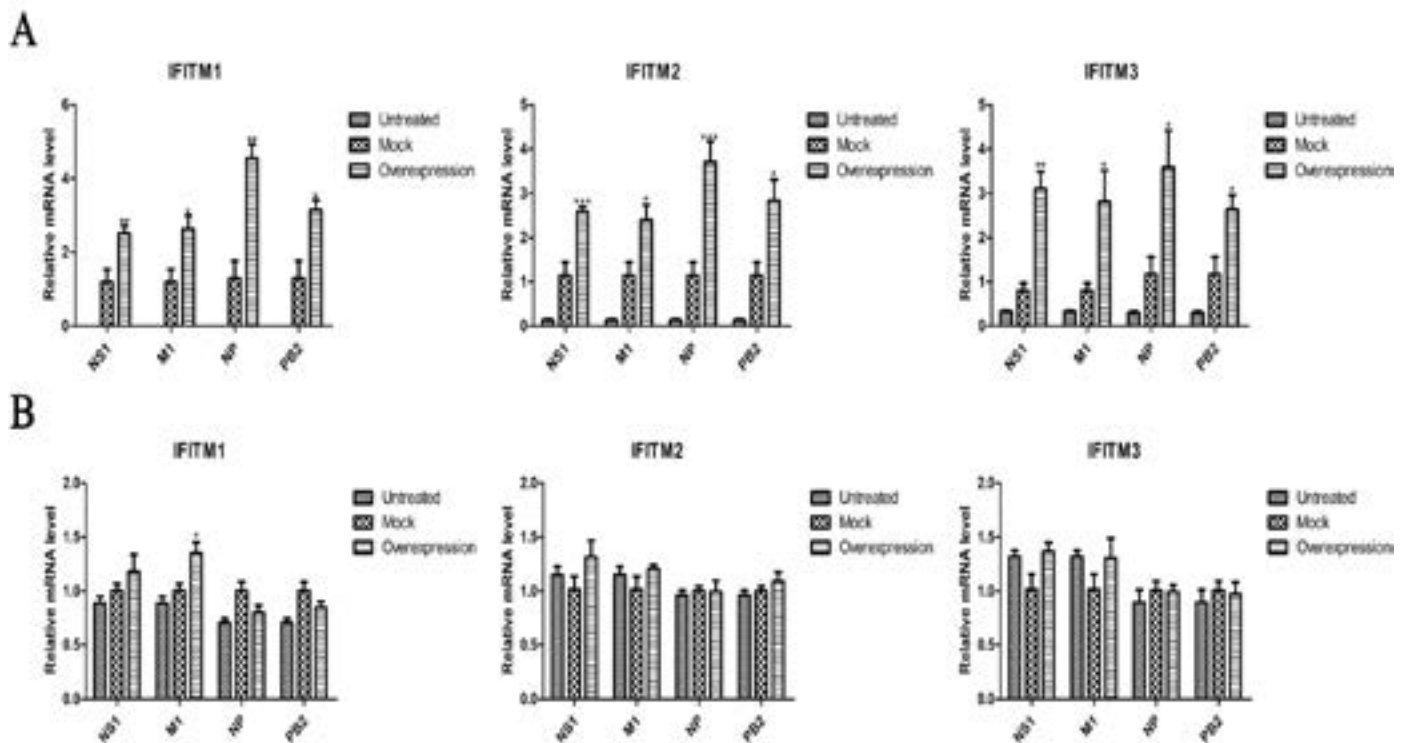


Figure 6. Influences of influenza virus NS1, M1, NP, and PB2 proteins on the gene expression of IFITM family.

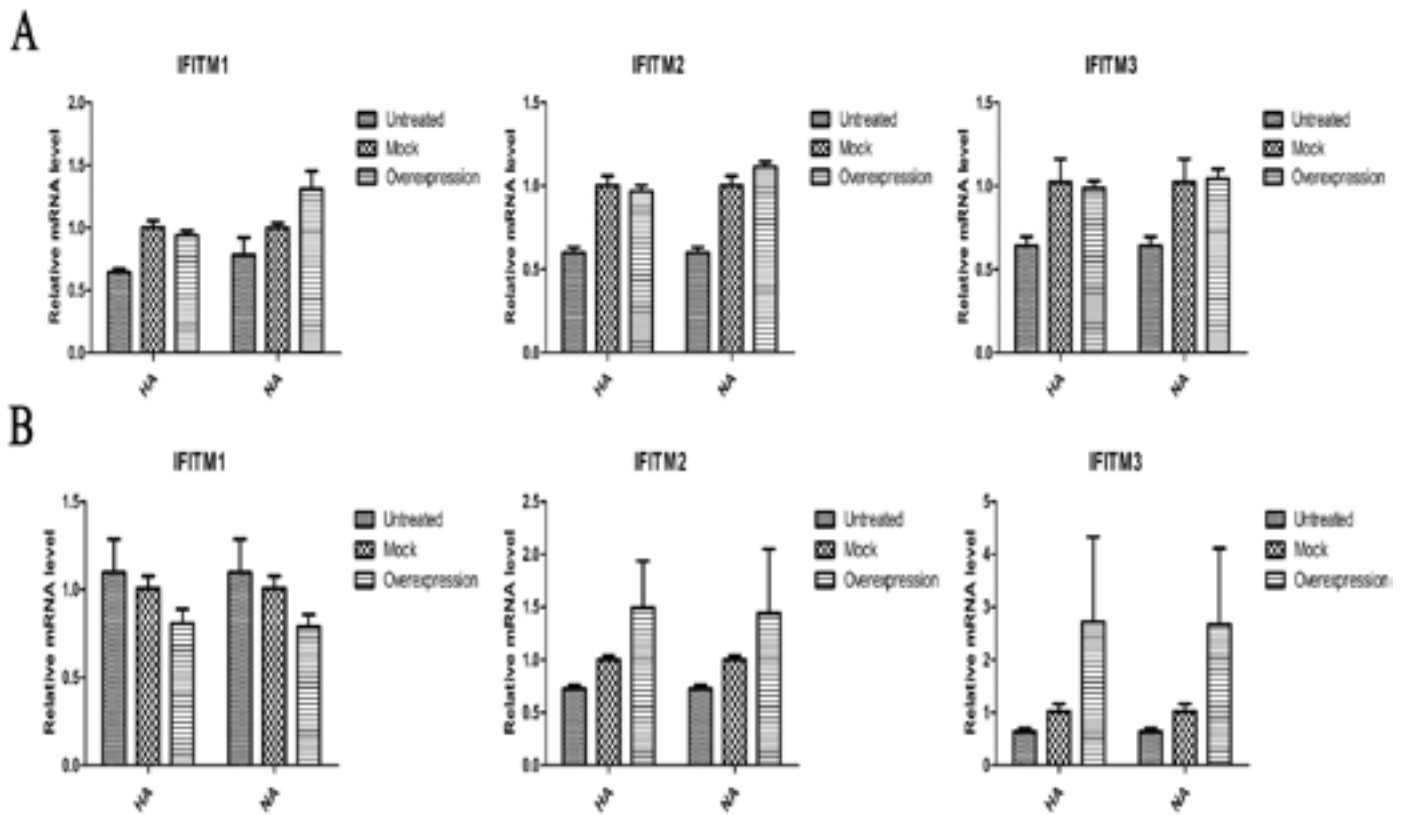


Figure 7. Influences of influenza virus HA and NA proteins on the expression of IFITM family.

## Discussion

Our study revealed that H5N1 virus could upregulate IFITM1, IFITM2, and IFITM3, further showing that H5N1 virus also promoted IFNA1, IFNB1, and IFNG overexpression in A549 cell. Above results illustrated H5N1 itself was probably the optimal immunogenic substances, compared to the individual structural or nonstructural proteins of H5N1 virus. To demonstrate which proteins of H5N1 virus involved in immune-related cytokine production, NS1, M1, NP, PB2, HA and NA proteins were overexpressed in A549 and HEK293T cells, respectively. Our study has shown NS1, M1, NP and PB2 of H5N1 virus could increase IFITM1, IFITM2, and IFITM3 expression, and especially NS1 and NP proteins played a stronger role than M1 and PB2 in A549 cells.

The most diverse IAV genes, HA and NA, have been divided into 17 and 10 subtypes, respectively, differences in sequence motifs have been distinguished between subtypes<sup>10,11</sup>. Previous studies had shown infection with viruses possessing the 1918 virus HA gene caused massive

recruitment of polymorphonuclear cells accompanied by intra-alveolar hemorrhage<sup>12,13,14</sup>. Surprisingly, HA and NA proteins show no effects on the IFNA1, IFNB1, and IFNG expression, and instead NS1 and NP proteins as viral proteins display important regulatory functions in this study. Billharz R et al. revealed 1918 virus NS1 had potent IFN antagonist activities<sup>2</sup>. Many evidence had shown that M1 protein played an important role in influenza virus assembly, as it could interact with the viral envelope proteins HA and NA via their cytoplasmic tails and also could interact with the viral RNP (vRNP), which constituted the viral core<sup>15,16</sup>. NS1 protein of influenza A virus plays important roles in antagonizing the host antiviral response and supporting virus replication, suggesting that the NS1 protein had the ability of suppressing host antiviral defenses at multiple levels<sup>17,18</sup>. Ubiquitin ligase TRIM25, 2',5'-oligoadenylate synthetase (OAS), and protein kinase R (PKR) were identified as target by NS1, further induce antiviral effect, which were key regulators of influenza virus transcription/translation processes<sup>19,20,21</sup>. Our data showed H5N1 virus could promote IFNA1, IFNB1, and



IFNG expression, and upregulate IFITM2 and IFITM3, but had no effects on IFITM1 in HEK293T cells. NS1 increased IFNG expression and M1 increased IFITM1 expression in HEK293T cell. IFITM genes can restrict the entry of a wide range of viruses by I and II type interferons, such IFITM3 as an important restrictor against several influenza A viruses<sup>22</sup>. Moreover, IFN has roles in inhibition of viral replication, stimulation of CTL, increasing MHC I expression, activation of macrophages and neutrophils, and promoting T-cell proliferation<sup>23,24</sup>. A previous study suggested that the presence of IFN- $\gamma$  improves the severity of inflammation and lung damage<sup>25</sup>. Upregulated FAT10 promoted H5N1 viral replication by inhibiting type I IFN<sup>26</sup>. Furthermore, M1, PB2, HA and NA proteins had no significant effect on IFNA1, IFNB1, and IFNG either in A549 cells or HEK293T cells. Above results demonstrated different cells had differences gene expression profiles, and it also revealed IFNA1, IFNB1, and IFNG specifically performed following distinct infection routes of H5N1.

### Conclusion

No articles had reported whether H5N1 virus itself proteins involved in regulation of IFITM1, IFITM2, and IFITM3 with H5N1 influenza virus infection. Our study first found NS1, M1, NP and PB2 of H5N1 virus contributed directly to expression of IFITM1, IFITM2, and IFITM3, revealing a new pathway for understanding activation mechanism of IFITM genes family with influenza virus infection.

### Conflict of interest

The authors declared that there was no conflict of interests.

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