



RESEARCH ARTICLE

PDCoV nsp14 interferes with the interferon pathway by degrading MAVS, MyD88, and TRAF3 proteins *via* the autophagy and proteasome pathways

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Highlights

- Porcine deltacoronavirus (PDCoV) nsp14 inhibits interferon (IFN) expression.
- PDCoV nsp14 degrades MAVS, MyD88 and TRAF3 proteins *via* the MARCH8-NDP52-autophagosome and proteasome pathways.
- Host proteins degrade nsp14 protein through selective autophagy pathway.

Abstract

Porcine deltacoronavirus (PDCoV) is a newly identified pathogen that can potentially undergo cross-species transmission to threaten the safety of swine and humans. The mechanism by which PDCoV nonstructural protein 14 (nsp14) inhibits the expression of IFN- β is unknown. In this study, we showed that PDCoV nsp14 degrades the MAVS, MyD88 and TRAF3 proteins in host cells by proteasomal and autophagy pathways. PDCoV nsp14 recruits the E3 ubiquitin ligase MARCH8 for catalyzing MAVS, MyD88 and TRAF3 protein ubiquitination. These proteins were recognized and transported to lysosomes by the cargo receptor NDP52 for degradation to inhibit the expression of IFN- β . Furthermore, MAVS, MyD88 and TRAF3 were also found to degrade PDCoV nsp14 by selective autophagy. These results reveal the dual function of selective autophagy in the PDCoV nsp14 and host proteins, which can promote the ubiquitination of viral particles and host antiviral proteins to degrade them both for regulating the relationship between virus infection and host innate immunity.

Keywords: PDCoV, nsp14, interferon, autophagosome, proteasome

1. Introduction

Porcine deltacoronavirus (PDCoV) is a recently discovered member in genus Deltacoronavirus belonging to family Coronaviridae. It represents the swine enteropathogen causing symptoms of diarrhea, vomiting, and dehydration in pigs, which may contribute to death under severe conditions (Duan 2021; Kong *et al.* 2022). PDCoV was first documented

in Hong Kong in 2012 (Woo *et al.* 2012). Initially, its pathogenic potential was documented in 2014 when it was shown to cause pig diarrheic outbreaks throughout the United States (He *et al.* 2020; Wang *et al.* 2014). PDCoV has since been detected in many countries, including China (Dong *et al.* 2016). It can also infect species other than pigs. Apparently, PDCoV infection can be found among children, strengthening

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its ability for cross-species transmission (Boley *et al.* 2020; Lednicky *et al.* 2021). An epidemic survey performed during 2016–2018 reported that the infection rate of PDCoV in Chinese pig populations reached 13.07%, indicating the possibility that PDCoV is one of the leading pathogens that caused diarrhea outbreaks in swine farms in China (Tang *et al.* 2021). Therefore, effective measures should be developed to prevent and control PDCoV.

PDCoV refers to a positive-sense RNA virus that belongs to the enveloped, single-stranded virus family. The PDCoV genome is around 25 kb long and encodes 4 structural proteins, the spike (S), envelope E, membrane (M), and nucleocapsid (N) proteins, together with 3 accessory proteins, nonstructural protein 6 (NS6), nonstructural protein 7 (NS7), and NS7a, that are interspersed in the structural genes at the 3' end of its genome (Woo *et al.* 2012; Zhu *et al.* 2017). Open reading frame 1a (ORF1a) and ORF1b have been detected in the downstream of the 5'-untranslated region, and they are responsible for encoding 2 polyprotein precursors that are processed into 15 mature nonstructural proteins (nsps) through papain-like protease (PLpro), nsp3, as well as the 3C-like protease nsp5 (Li *et al.* 2014; Chen *et al.* 2015). These PDCoV proteins can be targeted to treat PDCoV infection.

As a conserved intracellular degradation process, autophagy can be induced in cases where host cells are under stressful conditions, including virus invasion (Kong *et al.* 2020). The autophagy pathway involves autophagosome formation, acidic amphisome formation, and degradation with lysosomes. Many coronaviruses can induce autophagy to promote their replication. Meanwhile, some viruses can stop the progression of this pathway before autophagic degradation (Miller *et al.* 2020). As a coronavirus, the porcine epidemic diarrhea virus can play a chess game with host proteins *via* selective autophagy (Kong *et al.* 2020; Jiao *et al.* 2021; Dong *et al.* 2022; Qin *et al.* 2022, 2023; Zhai *et al.* 2023). When host antiviral proteins trigger autophagy, leading to the degradation of viral proteins, viral replication can be hindered. Moreover, viral proteins may also use autophagy for degrading host antiviral proteins, which can suppress the innate immune response and favor viral replication (Duan *et al.* 2021) reported that PDCoV can induce autophagy and facilitate virus replication in piglet models and LLC-PK1 cell models of PDCoV infection (Duan *et al.* 2021). Nevertheless, the crucial PDCoV protein that induces autophagy remains unclear. Elucidating the underlying virus-host interactions plays a vital role in understanding disease pathogenesis, particularly with the worldwide challenges of SARS-CoV-2 and COVID-19. In this case, PDCoV infection triggers IFN family expression *in vitro* or *in vivo* (Duan 2021). The invasion of PDCoV activates antiviral signaling cascades to produce cytokines. However, whether and how PDCoV activates autophagy to antagonize host immunity warrants further investigation.

Considering the significance of the autophagy pathway in immune escape, viruses have evolved targeting mechanisms for hindering interferon (IFN) generation. Previous studies have reported that the viral proteins of coronaviruses target the autophagy pathway through a variety of mechanisms to negatively adjust IFN signaling. The results of the present

study indicated that PDCoV nsp14 can hinder type-I IFN signaling *via* the ubiquitin–proteasome and MARCHF8-NDP52-autophagosome pathways to degrade mitochondrial antiviral signal proteins (MAVS), TNF receptor-associated factor 3 (TRAF3), and myeloid differentiation factor88 (MyD88), indicating a potential role of virulence factor nsp14 in PDCoV pathogenesis. Furthermore, we also found that MAVS, MyD88 and TRAF3 can degrade the PDCoV nsp14 protein through selective autophagy to foster the host antiviral defense response for antagonizing virus infection.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against MAVS (66911-1-Ig), MyD88 (23230-1-AP), TRAF3 (66310-1-Ig), ACTB/ β -actin (66009-1-Ig), GST-tag (10000-0-AP), MARCH8 (14119-1-AP), and NDP52 (12229-1-AP), as well as horseradish peroxidase (HRP)-conjugated anti-mouse (SA00001-1) and anti-rabbit (SA00001-2) IgG antibodies were offered by Proteintech Group, USA. Antibodies against IRF3 (4302S), P-IRF3 (29047S), Bafilomycin A1 (Baf A1; 54645), and HA-tag (3724) were offered by Cell Signaling Technology (CST, USA). Antibodies against ubiquitin (SC-8017), human MARCH8 siRNA (SC-90432), and human NDP52 siRNA (sc-93738) were provided by Santa Cruz Biotechnology (USA). Meanwhile, MG132 (M7449), 3-Methyladenine (3-MA; M9281), the antibody against Flag-tag (F1804), and chloroquine phosphate (CQ; PHR1258) were provided by Sigma-Aldrich (USA). In addition, 4',6-diamidino-2-phenylindole (DAPI; C1002) was obtained from Beyotime Biotechnology (China), while the Dual-Glo Luciferase Assay System (DL101) and ClonExpress II One Step Cloning Kit (C112-02) were obtained from Vazyme Biotech Co., Ltd. (China). Anti-PDCoV nsp14 protein monoclonal antibody was produced in our laboratory.

2.2. Cells

Compared with ST cells, LLC-PK cells usually show a high susceptibility to PDCoV infection (Hu *et al.* 2015; Yang *et al.* 2020). Each PDCoV replication cycle requires 5–6 h (Chen *et al.* 2019). The viral titers and PDCoV genomes peak at 24 h post-infection (hpi), and a plateau is obtained thereafter (Yin *et al.* 2020), so cells were collected at 24 hpi. For this study, Dr. Rui Luo (Huazhong Agricultural University, Wuhan, China) provided LLC-PK1 cells cultivated in minimum essential medium (MEM, Invitrogen, USA, 11095080). The above cells were cultured under incubation conditions of 5% CO₂ and 37°C. Plasmids were transfected into cells that reached 80–90% confluency and were seeded into 6-well plates with the use of Lipofectamine 3000 (Invitrogen, L3000015). In addition, Lipofectamine RNAiMAX (Invitrogen, 13778150) was adopted for transfecting siRNA into cells with 50–60% confluency.

Human embryonic kidney HEK 293T cells (CRL-11268, ATCC, USA) were cultivated in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, D6429) including 10% fetal bovine serum (FBS; Gibco, USA, 10099141), and African green monkey kidney vero cells (CCL-81, ATCC) were cultivated in DMEM (Invitrogen, 12430054) including 10% FBS.

2.3. Virus infection

The PDCoV was isolated and maintained in our laboratory. For PDCoV infection, LLC-PK cells were cultured to over 90% confluency in culture plates. After washing with PBS (Gibco, C20012500BT), PDCoV at a multiplicity of infection (MOI) of 1 or 0.01 was adopted to infect cells for 1 h. After washing with PBS, cells were cultivated in trypsin-containing serum-free DMEM at 37°C for different durations before harvesting. The trypsin concentration used for virus propagation was 0.75 µg mL⁻¹. The Kaerber's method was used for analyzing viral titers, which are indicated as the 50% tissue culture infective dose (TCID₅₀).

2.4. Quantitative real-time PCR (qRT-PCR)

Either a QIAamp Viral RNA Mini Kit (Qiagen, Germany, 52906) or RNeasy Mini Kit (Qiagen, 74104) was used to extract total RNA in accordance with their specific protocols. cDNA was prepared using a PrimeScript™ Reagent Kit (TaKaRa, Japan, RRO47A). SYBR Premix Ex Taq™ (Vazyme Biotech Co., Ltd, China, q711-03) was employed for qRT-PCR. Appendix A shows the primer sequences used in the qPCR. ACTB served as an internal reference.

2.5. Western blotting

After a 5 min incubation using RIPA lysis and extraction buffer (Thermo Fisher Scientific, USA, 89901), including protease (Bimake, USA, B14001)/phosphatase (Bimake, B15001) inhibitor cocktail (Bimake), lysates were obtained and denatured using the 5× SDS-PAGE sample loading buffer for a 10 min period. Thereafter, protein separation was completed through SDS-PAGE prior to transfer onto nitrocellulose membranes (GE Healthcare, USA, 10600001). PBS, including 5% non-fat milk (BD, 232100) and 0.2% Tween 20 (Sigma-Aldrich, USA, P1379), was then added to block the membranes, which were then incubated using primary and HRP-conjugated secondary antibodies. Enhanced chemiluminescence (ECL) was employed for protein measurement (Share-bio, China, SB-WB012).

2.6. Co-IP assay

After plasmid transfection for 1 d, cells were lysed using NP40 cell lysis buffer (Life Technologies, USA, FNN0021), including the protease inhibitor cocktail. Subsequently, the lysates were harvested followed by centrifugation and incubation using anti-Flag-antibody-conjugated Dynabeads Protein G (Life Technologies, 10004D). After washing with 0.02% PBST, cells were resuspended in the glycine elution buffer (50 mmol L⁻¹, pH 2.8). Proteins were evaluated with antibody-based immunoblotting.

2.7. Glutathione S-transferase (GST) affinity-isolation assay

The full-length sequences of PDCoV nsp14, MARCH8, and NDP52 were cloned into the pCold TF (3365) and pCold GST (3372) (Clontech Laboratories, Inc., Japan) plasmids. Thereafter, the expression of those genes was induced within BL21-competent cells (Vazyme Biotech, China, C504-03). In accordance with specific protocols, the GST

Protein Interaction Pull-Down Kit (Thermo, USA, 21516) was employed to investigate any protein interactions.

2.8. Confocal immunofluorescence assay

Transfected cells were immersed in 4% paraformaldehyde (PFA, Sigma-Aldrich, USA, P6148) before permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, T9284) at room temperature. Next, cells were blocked with 5% bovine serum albumin (BSA, Cell Signaling Technology, USA, 9998), followed by another 1 h incubation with the primary antibody. Then, after rising three times with PBS, cells were incubated with fluorescently labeled secondary antibodies for another 1 h based on a previously described protocol (Kong *et al.* 2020). DAPI was then added for 5 min for nuclear staining. Finally, the fluorescence images were observed under a laser scanning confocal immunofluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

2.9. Luciferase reporter assay

HEK 293T cells were cultured in 24-well plates and transfected using target-encoding plasmids by using Lipofectamine 3000. After 24 h, the Dual-Glo Luciferase Assay System (Vazyme Biotech Co. Ltd., China, DL101) was adopted to detect the luciferase activities in the cells, with renilla luciferase as the control.

2.10. Statistical analysis

GraphPad Prism 5 software (GraphPad Software, USA) was used to compare 2 groups by Student's *t*-test (two-tailed). The following *P*-values were regarded to be of statistical significance: * *P*<0.05, ** *P*<0.01, and *** *P*<0.001, with ns suggesting non-significance. The data are shown as averages acquired from three separate assays.

3. Results

3.1. PDCoV nsp14 can inhibit IFN expression

After viral infection, cells can rapidly generate type-I IFNs, with subsequent induction of different IFN-triggered host genes upon entering the antiviral state (Stetson and Medzhitov 2006; Ivashkiv and Donlin 2014). To identify the impact of PDCoV infection on IFN expression, this study determined the IFN-β levels in LLC-PK1 cells coinfecting with SeV and PDCoV at a MOI of 1. The mRNA levels and luciferase activities of IFN-β were significantly down-regulated in PDCoV infected cells compared with those in their PDCoV uninfected counterparts (Fig. 1-A and B), indicating that PDCoV infection can hinder IFN-β expression in host cells. Next, the virus protein that suppresses IFN-β was determined using PDCoV structural proteins (E, M, N) and nonstructural proteins (NS6, NS7, nsps) *via* the IFN-β promoter and IFN-stimulated response element (ISRE)-induced luciferase reporter assay. As shown in Fig. 1-C and D, nsp2, nsp14 and the N protein of PDCoV can significantly reduce IFN-β, and nsp14 was the most effective. The data also indicated that nsp14 suppressed IFN-β activation in a dose-dependent manner (Fig. 1-E and F). These findings indicated that PDCoV nsp14 significantly inhibited IFN expression by PDCoV.

3.2. PDCoV nsp14 downregulates the abundance of MAVS, MyD88, TRAF3, and IRF3

To investigate how PDCoV nsp14 antagonizes IFN-mediated antiviral responses, the interactions of nsp14 with upstream regulators of IFNs were determined. This study co-transfected nsp14 expression plasmids and plasmids encoding key signaling proteins, including the cytoplasmic sensors melanoma differentiation-associated gene 5 protein (MDA5), MAVS, MyD88, TRAF3, TANK binding kinase 1 (TBK1), and interferon regulatory factor 3 (IRF3), which are related to innate antiviral responses. PDCoV nsp14 reduced the ISRE-driven luciferase reporter activity induced by all six promoter regulators mentioned above (Fig. 2-A), and it also reduced the IFN- β -driven luciferase reporter activity stimulated via MAVS, MyD88, TRAF3, and IRF3 (Fig. 2-B). In HEK 293T cells exposed to nsp14 expression plasmids, the mRNA levels of MAVS, MyD88, TRAF3, and IRF3 were significantly reduced compared with the control group (Fig. 2-C). During PDCoV infection, MAVS, MyD88, TRAF3, IRF3, and phosphorylation IRF3 (p-IRF3) were significantly downregulated (Fig. 2-D). To confirm whether the major signaling proteins were downregulated by PDCoV nsp14, PDCoV nsp14 plasmids were transfected into LLC-PK1 and HEK 293T cells. These upstream regulators of IFNs were also significantly reduced in the nsp14 expression cells (Fig. 2-E and F). Based on these findings, the key proteins of the IFN signaling pathway, MAVS, MyD88, TRAF3, and IRF3, can be inhibited by PDCoV nsp14.

3.3. PDCoV nsp14 interacts with MAVS, MyD88 and TRAF3

To elucidate the molecular mechanisms through which PDCoV nsp14 hinders IFN expression, we next determined whether nsp14 interacts with MAVS, MyD88 and TRAF3. A co-immunoprecipitation (co-IP) assay was carried out using the indicated antibodies in HEK 293T cells co-transfected with the MYC-MAVS with Flag-nsp14 plasmids, and MYC-MAVS was precipitated with Flag-nsp14 (Fig. 3-A). Confocal immunofluorescence microscopy demonstrated that MAVS can effectively colocalize with nsp14 in the cytoplasm of HeLa cells (Fig. 3-B). The GST affinity-isolation assay revealed that nsp14 (GST-nsp14) bound directly to MAVS (Fig. 3-C). These findings suggest that the PDCoV nsp14 protein can directly interact with MAVS. Similar co-IP, confocal immunofluorescence microscopy, and GST affinity-isolation assay results were found in MyD88 and TRAF3 binding assays with the PDCoV nsp14 protein (Fig. 3-D–I). Based on these findings, we propose that PDCoV nsp14 interacts with MAVS, MyD88 and TRAF3 to inhibit IFN expression.

3.4. PDCoV nsp14 suppresses MAVS, MyD88 and TRAF3 proteins via ubiquitin-proteasome and autophagy pathways

The SADS-CoV N protein can antagonize IFN- β generation through proteasome-dependent degeneration (Liu *et al.* 2021). Because the PDCoV nsp14 protein downregulates and interacts with MAVS, MyD88 and TRAF3, we

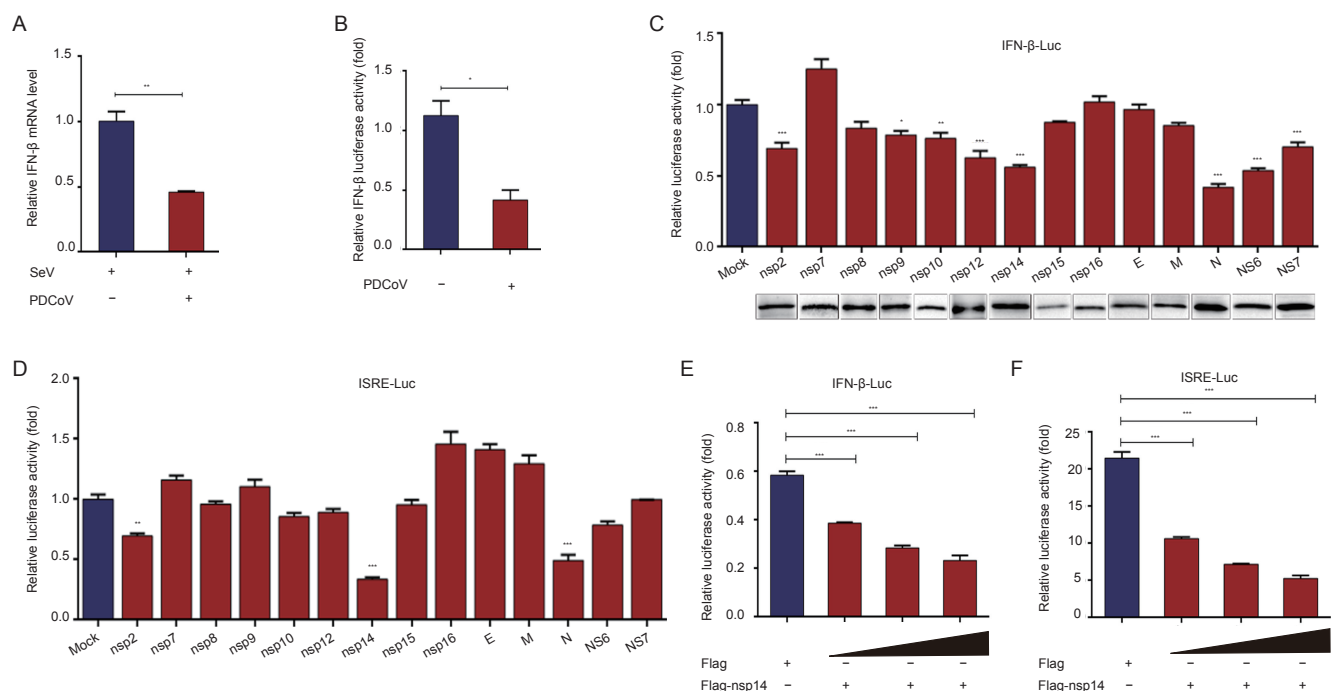


Fig. 1 Porcine deltacoronavirus (PDCoV) infection can hinder interferon (IFN) expression through virus protein nsp14. A, cells were collected at 12 h post-infection (hpi) following infection or negative infection of LLC-PK1 cells with PDCoV and SeV at the multiplicity of infection (MOI) of 1. qRT-PCR was performed to evaluate IFN- β expression. B, LLC-PK1 cells were subjected to transfection with IFN- β luciferase reporter and PDCoV. The cells were analyzed for dual luciferase activity. C and D, plasmids encoding IFN- β or interferon-stimulated response element (ISRE) luciferase reporter along with plasmids encoding PDCoV nsp2, nsp7, nsp8, nsp9, nsp10, nsp12, nsp14, nsp15, nsp16, E, M, N, NS6, and NS7 were co-transfected in HEK 293T cells to analyze dual luciferase activity. E and F, IFN- β or ISRE luciferase reporter was co-transfected with growing doses (wedge) of Flag-nsp14 into HEK 293T cells and were analyzed for dual luciferase activity. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

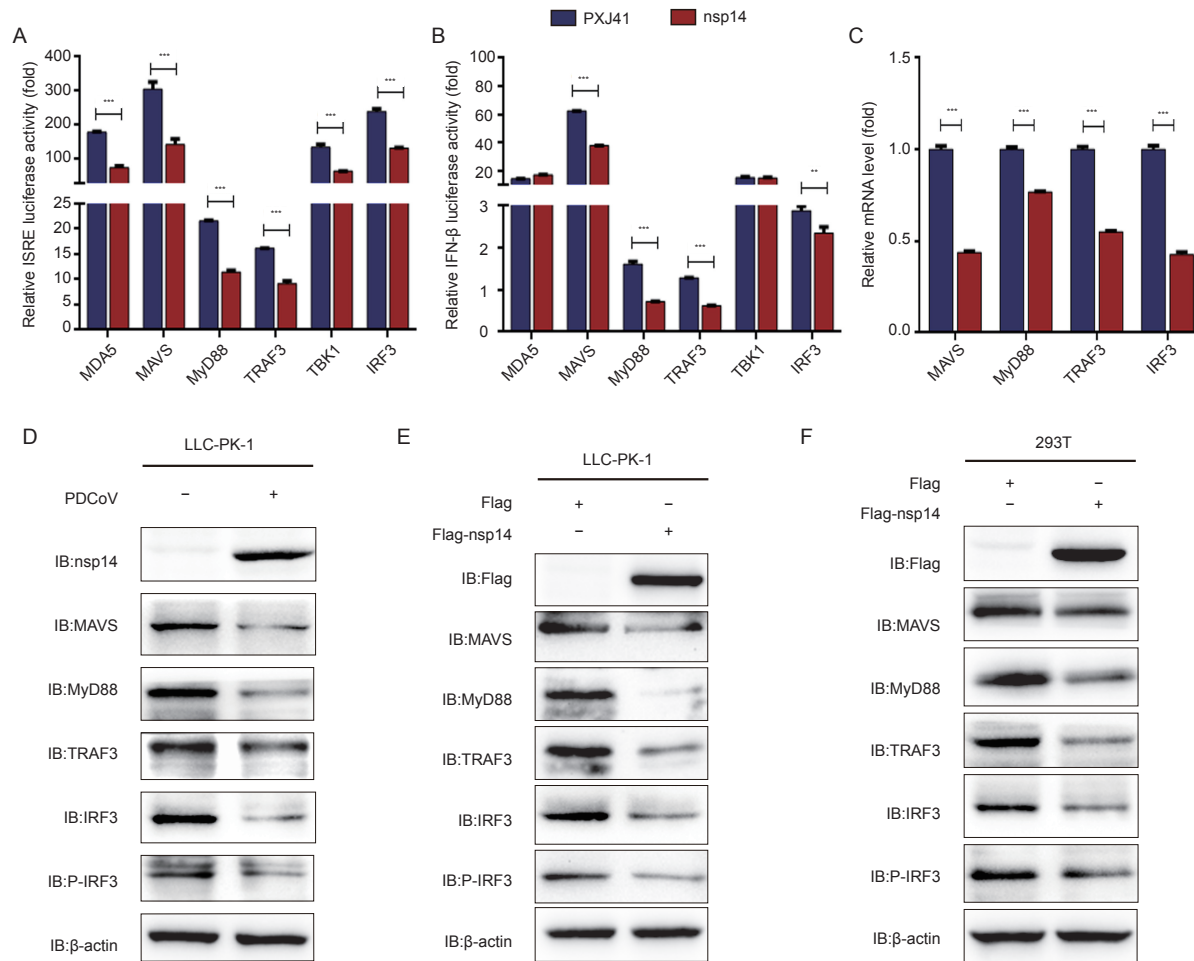


Fig. 2 Porcine deltacoronavirus (PDCoV) nsp14 downregulates the abundance of MAVS, MyD88, TRAF3, and IRF3. A and B, plasmids encoding nsp14 and ISRE or IFN- β luciferase reporter was co-transfected with plasmids encoding MDA5, MAVS, MyD88, TRAF3, TBK1 or IRF3 into HEK 293T cells and were evaluated to perform dual luciferase activity. C, plasmids encoding nsp14 were transfected into HEK 293T cells. The qRT-PCR was conducted to analyze MAVS, MyD88, TRAF3, and IRF3 mRNA expression. D, cells were collected at 12 h post-infection (hpi) after infection or negative infection of LLC-PK1 cells with PDCoV at the multiplicity of infection (MOI) of 1. PDCoV nsp14, MAVS, MyD88, TRAF3, IRF3, or p-IRF3 was evaluated using Western blotting analysis, with β -actin being a loading control for samples. E and F, plasmids encoding nsp14 were transfected into LLC-PK1 or HEK 293T cells. Western blotting was used to determine the levels of RIG-I, MAVS, MyD88, TRAF3, IRF3 and p-IRF3, with β -actin being a loading control for samples. **, $P < 0.01$; ***, $P < 0.001$.

hypothesized that nsp14 may promote host protein degradation pathways to antagonize the IFN signal pathway by degrading MAVS, MyD88 and TRAF3. To identify the predominant degeneration pathway, Flag-nsp14 plasmid was transfected into HEK 293T cells, followed by treatment with either proteasome inhibitor MG132, autophagy inhibitor CQ, or bafilomycin A1 (Baf-A1). The MAVS and MyD88 protein degeneration mediated by nsp14 was elevated in cells subject to treatment with MG132, CQ, or Baf-A1, indicating that PDCoV nsp14 can promote proteasomal and autophagic degradation of the MAVS and MyD88 proteins (Fig. 4-A). Furthermore, TRAF3 protein degeneration was reversed by CQ and Baf-A1, but TRAF3 protein degeneration was not significantly changed in cells treated with MG132, indicating that the PDCoV nsp14 protein only improved autophagic degradation of the TRAF3 protein in host cells (Fig. 4-A).

To further verify whether the PDCoV nsp14 protein

suppresses host proteins through autophagy, MARCH8 (an E3 ubiquitin ligase) and NDP52 (a cargo receptor) were evaluated in Flag-nsp14 co-transfected HeLa cells. Immunofluorescence confocal microscopy analysis revealed the interactions between PDCoV nsp14 and both MARCH8 and NDP52, indicating that nsp14 showed efficient cytoplasmic co-localization with MARCH8 and NDP52 (Fig. 4-B). In addition, MARCH8 coimmunoprecipitated with the nsp14 protein in HEK 293T cells in co-IP assays (Fig. 4-C). Furthermore, a GST affinity-isolation assay indicated that PDCoV nsp14 directly bound to MARCH8 (Fig. 4-D), and similar protein binding results were found between the NDP52 and nsp14 proteins (Fig. 4-E and F). These results showed that PDCoV nsp14 can suppress MAVS and MyD88 based on the ubiquitin–proteasome and autophagy pathways and inhibit TRAF3 *via* the autophagy pathway through interactions with MARCH8 and NDP52.

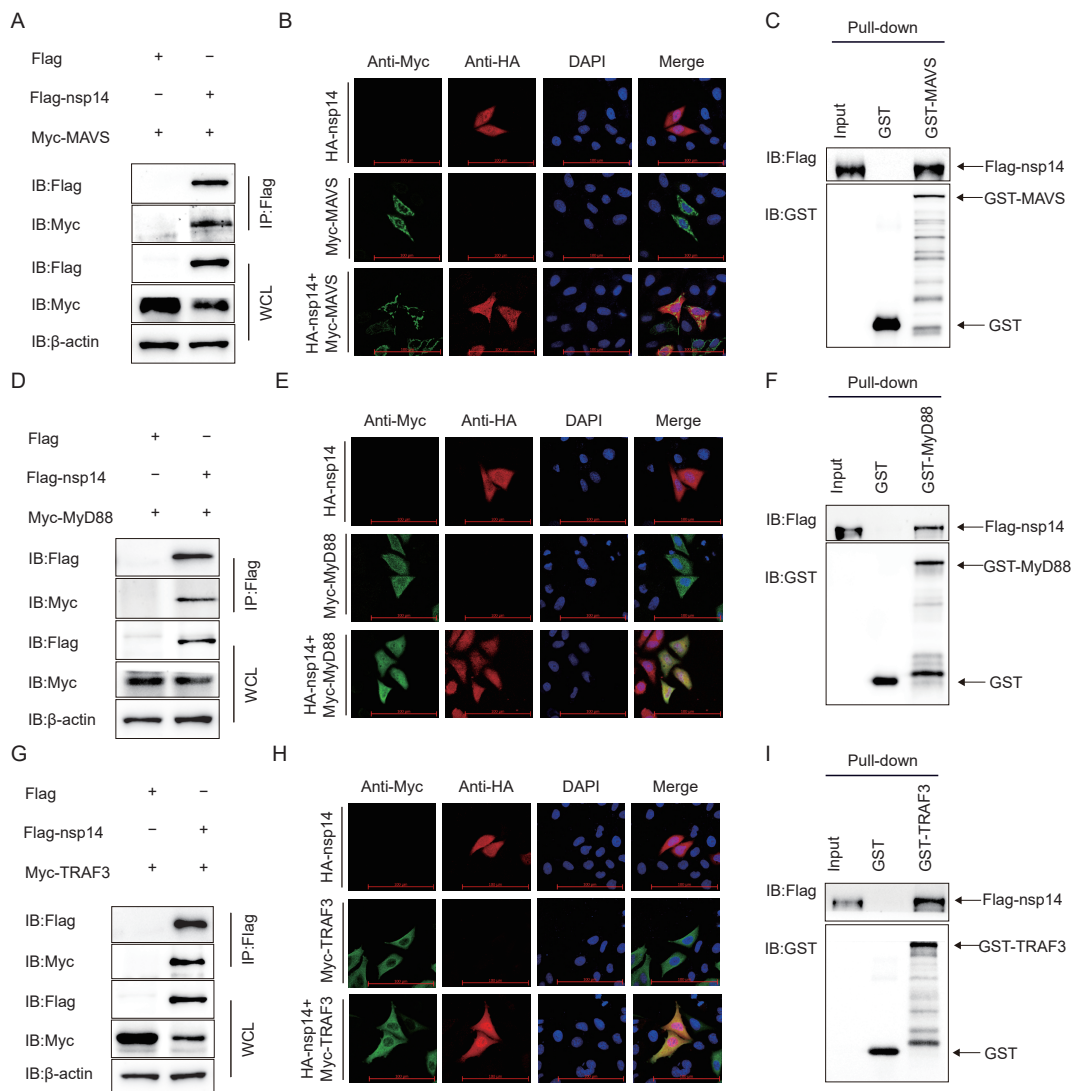


Fig. 3 PDCoV nsp14 shows interaction with MAVS, MyD88 and TRAF3. A, plasmids that encoded MYC-MAVS and Flag-nsp14 were transfected into HEK 293T cells for a 24-h period, then Co-IP assay was conducted using anti-Flag binding beads. Precipitated proteins were evaluated by Western blotting. B, plasmids that encoded MYC-MAVS and HA-nsp14 were co-transfected into HeLa cells for 1 d, followed by incubation using specific primary antibodies and secondary antibodies. Besides, 4,6-diamidino-2-phenylindole (DAPI) was added for nuclear staining. Observation of fluorescent signals was made with the use of confocal immunofluorescence microscopy. Scale bars: 100 μ m. C, nsp14 and MAVS were cloned into pCold GST plasmid and shown in bacterial strain BL21 (DE3) during GST affinity-isolation assay. Eluted proteins were evaluated by Western blotting. D, plasmids that encoded MYC-MyD88 and Flag-nsp14 were transfected to HEK 293T cells, and later Co-IP assay was conducted. E, plasmids that encoded MYC-MyD88 and HA-nsp14 were co-transfected into HeLa cells. Meanwhile, fluorescent signals were observed under the confocal immunofluorescence microscope. Scale bars: 100 μ m. F, the nsp14 and MyD88 combination was analyzed using the GST affinity-isolation assay by performing western blotting analysis. G, plasmids that encoded MYC- TRAF3 and Flag-nsp14 were transfected into HEK 293T cells, and later Co-IP assay was conducted. H, plasmids that encoded MYC-TRAF3 and HA-nsp14 were transfected into HeLa cells. Fluorescent signals were observed under the confocal immunofluorescence microscope. Scale bars: 100 μ m. I, the nsp14 and TRAF3 combination were analyzed using the GST affinity-isolation assay based on Western blotting.

3.5. PDCoV nsp14 degrades MAVS, MyD88 and TRAF3 proteins via MARCH8 and NDP52

To confirm the degradation of the MAVS, MyD88 and TRAF3 proteins via MARCH8 and NDP52, the MYC-MARCH8, Flag-MAVS, and HA-nsp14 plasmids were co-transfected into HEK 293T cells, and the cellular lysates were evaluated using the ubiquitination assay. Furthermore, Baf-A1 was used to inhibit autophagic degradation. Western blotting analysis showed that the overexpressed MARCH8

significantly enabled MAVS protein ubiquitination (Fig. 5-A). The ubiquitination of MyD88 and TRAF3 proteins by MARCH8 was also improved in treated cells (Fig. 5-B and C). Co-IP assays were carried out to further demonstrate the interactions between MARCH8 and NDP52 with MAVS, MyD88 and TRAF3, which showed that MARCH8 and NDP52 coimmunoprecipitated with MAVS, MyD88 and TRAF3 proteins in HEK 293T cells (Fig. 5-D–I). The major role of the MARCH8 and NDP52 proteins in the

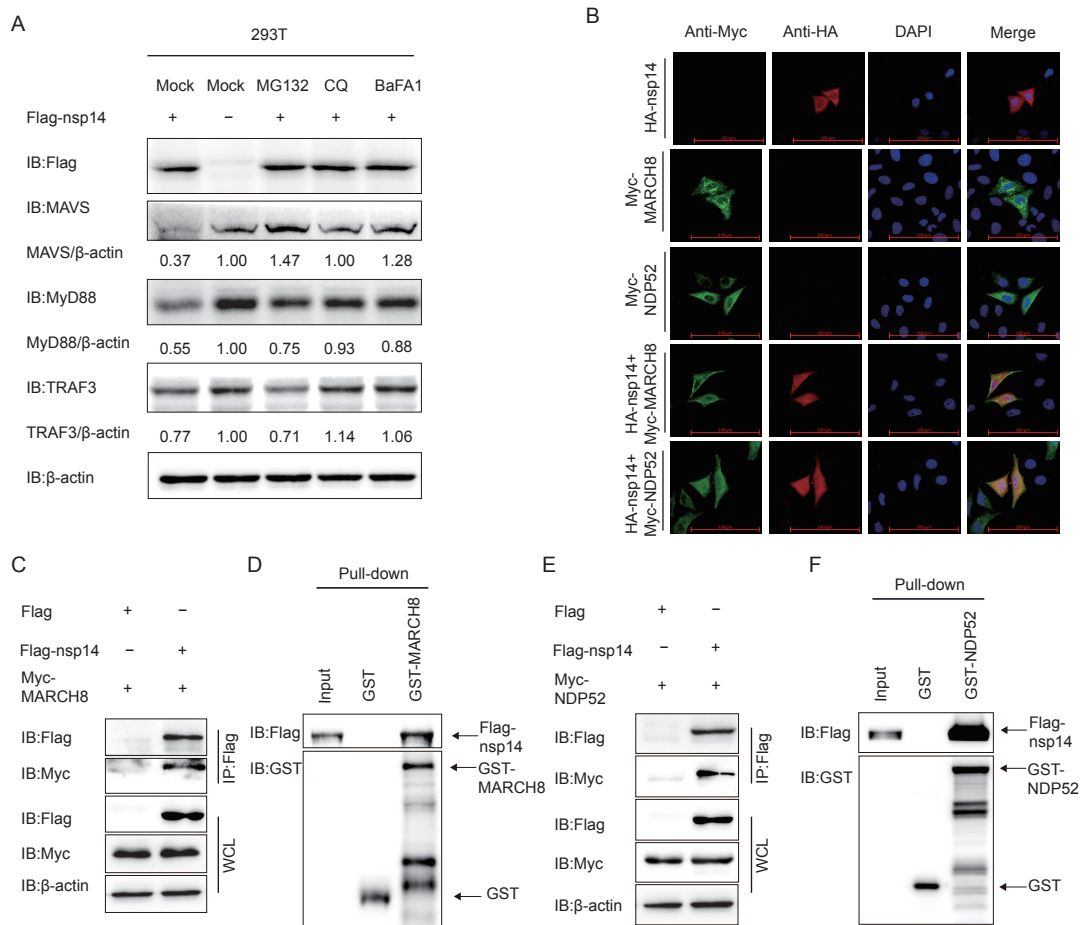


Fig. 4 Porcine deltacoronavirus (PDCoV) nsp14 depresses MAVS, MyD88, TRAF3 via ubiquitin and autophagy pathways.

A, plasmids encoding Flag-nsp14 were transfected into HEK 293T cells, followed by treatment with MG132 (5 $\mu\text{mol L}^{-1}$), chloroquine (CQ) (50 $\mu\text{mol L}^{-1}$) or Baf A1 (50 $\mu\text{mol L}^{-1}$) for 9 h. MAVS, MyD88 and TRAF3 levels were evaluated by performing a Western blotting assay. B, Hela cells were subject to transfection using plasmids that encoded HA-nsp14 and MYC-MARCH8 or MYC-NDP52, followed by incubation using specific primary antibodies and secondary antibodies. Confocal immunofluorescence microscopy was used to observe DAPI-stained cell nuclei and fluorescent signals. In addition, cells were transfected using plasmids encoding HA-nsp14, MYC-MARCH8, or MYC-NDP52 as control. Scale bars: 100 μm . C, plasmids that encoded MYC-MARCH8 and Flag-nsp14 were transfected into HEK 293T cells for a 24-h period prior to co-IP assay using anti-Flag binding beads. Precipitated proteins were later analyzed through Western blotting. D, expression of GST-MARCH8 and Flag-nsp14 was detected within bacterial strain BL21 (DE3), and their purification was completed prior to GST affinity-isolation assay using western blotting analysis. E, plasmids that encoded MYC-NDP52 and Flag-nsp14 were transfected in HEK 293T cells for a 24-h period before co-IP using anti-Flag binding beads. Western blotting analysis was employed to explore precipitated proteins. F, GST-NDP52 and Flag-nsp14 were shown in bacterial strain BL21 (DE3), and their purification was completed before GST affinity-isolation assay by Western blotting analysis.

degradation of MAVS, MyD88 and TRAF3 by nsp14 was verified by employing MARCH8 and NDP52 targeting small interfering RNAs (siRNAs). LLC-PK1 cells were subjected to Flag-nsp14 co-transfection with either MARCH8 siRNA or NDP52 siRNA. Western blotting results showed that interfering with MARCH8 and NDP52 expression could reverse the PDCoV nsp14-induced degradation of the MAVS, MyD88 and TRAF3 proteins compared with the controls (Fig. 5-J). Similar results were also found in HEK 293T cells (Fig. 5-K). These results indicated that PDCoV nsp14 can degrade the MAVS, MyD88 and TRAF3 proteins via MARCH8 and NDP52. Altogether, PDCoV nsp14 protein uses the cellular MARCH8-NDP52 autophagic machinery to inhibit MAVS, MyD88 and TRAF3 to inhibit IFNs.

3.6. Host proteins can utilize the cellular autophagic machinery to degrade PDCoV nsp14

A recent study suggested that PEDV proteins play a chess game with host proteins via selective autophagy (Zhai et al. 2023). The previous sections of this study showed that PDCoV infection considerably suppresses IFN expression via the nsp14 protein, and that PDCoV nsp14 can degrade the MAVS, MyD88 and TRAF3 proteins to inhibit IFNs via the ubiquitin-proteasome and autophagy pathways. Conversely, the PDCoV nsp14 protein can also be degraded via host anti-virus factors. To investigate the dual role of host proteins and selective autophagy and proteasome activity within PDCoV nsp14, HEK 293T cells were co-transfected with the MYC-MAVS and PDCoV nsp14 plasmids (FLAG-nsp14). The Western blotting results revealed that the MAVS protein

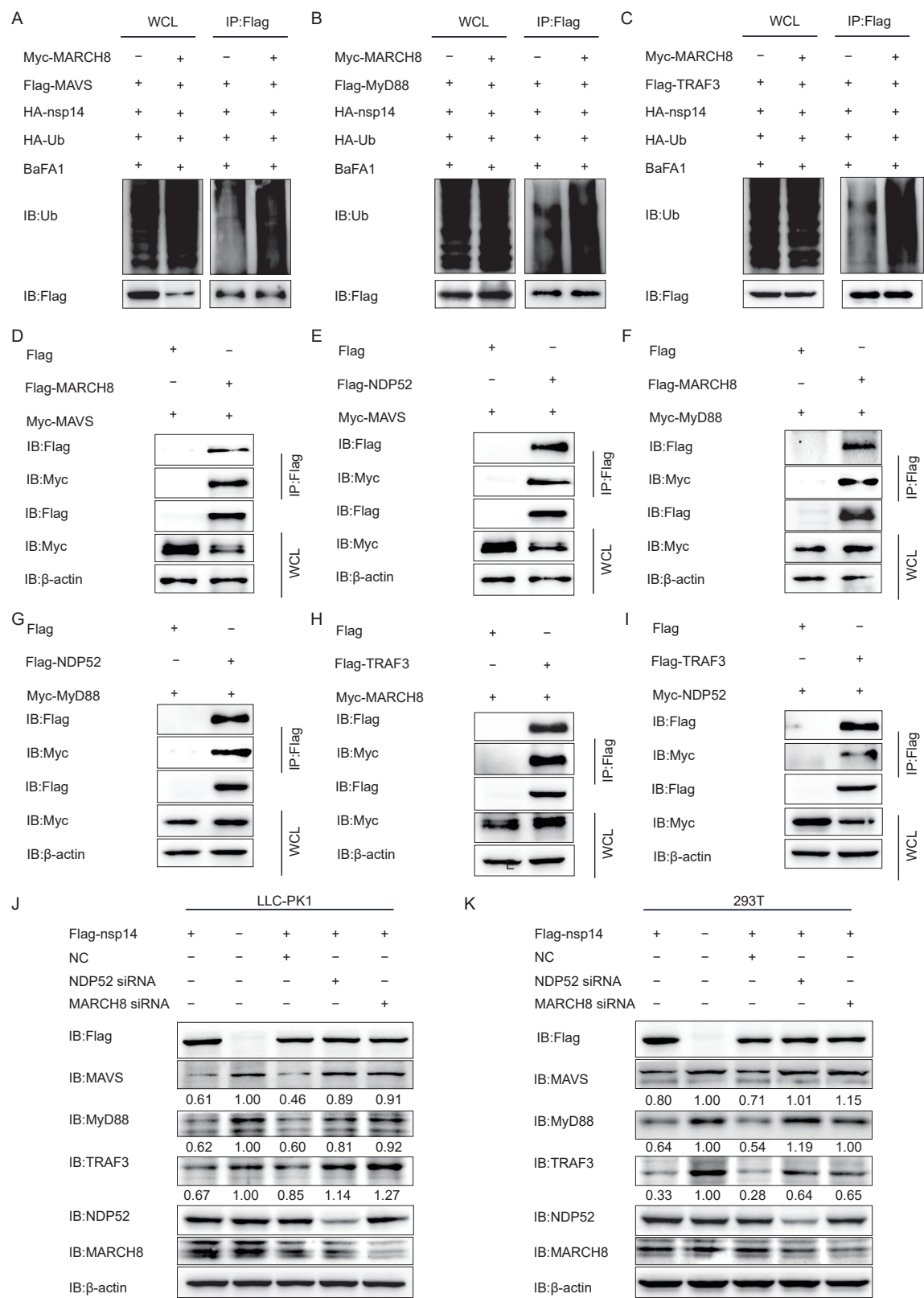


Fig. 5 Porcine deltacoronavirus (PDCoV) nsp14 degrades MAVS, MyD88, TRAF3 proteins via MARCH8 and NDP52. A–C, HEK 293T cells were co-transfected with MYC-MARCH8, Baf A1, HA-nsp14, and Flag-MAVS plasmids (A), Flag-MyD88 plasmids(B), or Flag-TRAF3 plasmids (C), was performed. The cellular lysates were gathered at 24 h following transfection. In addition, an anti-Flag antibody was used for immunoprecipitation of ubiquitinated proteins and evaluated using Western blotting. D–I, plasmids that encoded MARCH8 or NDP52 and MAVS, MyD88 or TRAF3, respectively, were transfected in HEK 293T cells for a 24-h period, and later co-IP was conducted using anti-Flag binding beads. Precipitated proteins were evaluated by Western blotting. J and K, the Flag-nsp14 and siRNA (MARCH8 siRNA or NDP52 siRNA or NC) were transfected in the LLC-PK1 cells and HEK 293T cells. MAVS, MyD88, TRAF3, MARCH8, and NDP52 protein levels were evaluated by Western blotting.

markedly suppressed the overexpression of the nsp14 protein (Fig. 6-A). Then, HEK 293T cells overexpressing nsp14 were treated with proteasome inhibitor MG132, autophagy inhibitor 3-MA, and Baf-A1. The degradation of nsp14 by MAVS protein was significantly improved by 3-MA and Baf-A1 (Fig. 6-B), indicating that the PDCoV nsp14 protein can be degraded by MAVS *via* autophagy pathways. Furthermore, the MyD88 and TRAF3 proteins also significantly suppressed the nsp14 protein (Fig. 6-C and E), and the degradation of virus protein was inhibited in 3-MA and Baf-A1 treated cells co-transfected with the MyD88 and TRAF3 proteins (Fig. 6-D and F). However, nsp14 degradation was not distinctly improved in MG132 treated cells, indicating that MAVS, MyD88 and TRAF3 can use cellular autophagic machinery for degrading the PDCoV nsp14 protein (Fig. 6-B, D and F). Along with our previous studies, we showed that PDCoV infection can degrade host factors MAVS, MyD88 and TRAF3 to suppress the IFN signal pathway to inhibit the innate immune response of the host. Conversely, host proteins MAVS, MyD88 and TRAF3 can prevent virus attack *via* the host autophagy pathway to degrade the viral nsp14 protein.

4. Discussion

During the last two decades, some coronaviruses have infected human beings by crossing the species barrier. These new coronaviruses can cause severe diseases during outbreaks. As a result, improving the global vigilance for resisting coronavirus-associated diseases is necessary. PDCoV is a new swine enteropathogenic coronavirus with global distribution. It mainly threatens the swine industry and

can lead to major financial losses worldwide. Furthermore, in addition to swine, PDCoV can also infect other animals, such as humans, chickens, calves, mice and turkeys, highlighting its role in crossing species barriers. Therefore, the current work focused on investigating how the virulence factor of PDCoV can break through the defense system of the host. Moreover, we provide evidence for a new mechanism through which the PDCoV nsp14 protein can significantly inhibit the MAVS, MyD88 and TRAF3 proteins *via* the ubiquitin-proteasome and MARCH8-NDP52-autophagosome pathways to interfere with IFN response regulation (Fig. 7). Based on this study, MAVS, MyD88 and TRAF3 can degrade the PDCoV nsp14 protein through selective autophagy to foster the host antiviral defense response for antagonizing the viral infection.

PDCoV infection can induce IFN- α , IFN- β , IFN- γ , IFN- λ 1, IL-22, IL-1 β , IL-6, IL-12, TNF- α and IFN- λ 1 expression *in vivo* and *in vitro* (Zhao *et al.* 2020; Duan 2021). *In vitro*, various proteins encoded by viruses can suppress type-I/III IFN responses (Ding *et al.* 2014; Zhang and Yoo 2016). PEDV nsp5 can inhibit IFN- β generation, indicating that an identical IFN antagonistic mechanism is commonly seen among the members of the *Coronaviridae* family (Wang *et al.* 2016). According to this work, PDCoV nsp14 can inhibit IFN expression.

IFN plays a vital role in host innate immunity and can be engaged in the first-line defense when resisting pathogens (Tanji and Ip 2005; Bourgeois *et al.* 2011). IFNs are crucial factors mediating host antiviral immunity by triggering the cellular antiviral state and infected cell apoptosis while adjusting immunocyte subsets that are vital for the antiviral response (Stefan *et al.* 2020). In the process of viral infection,

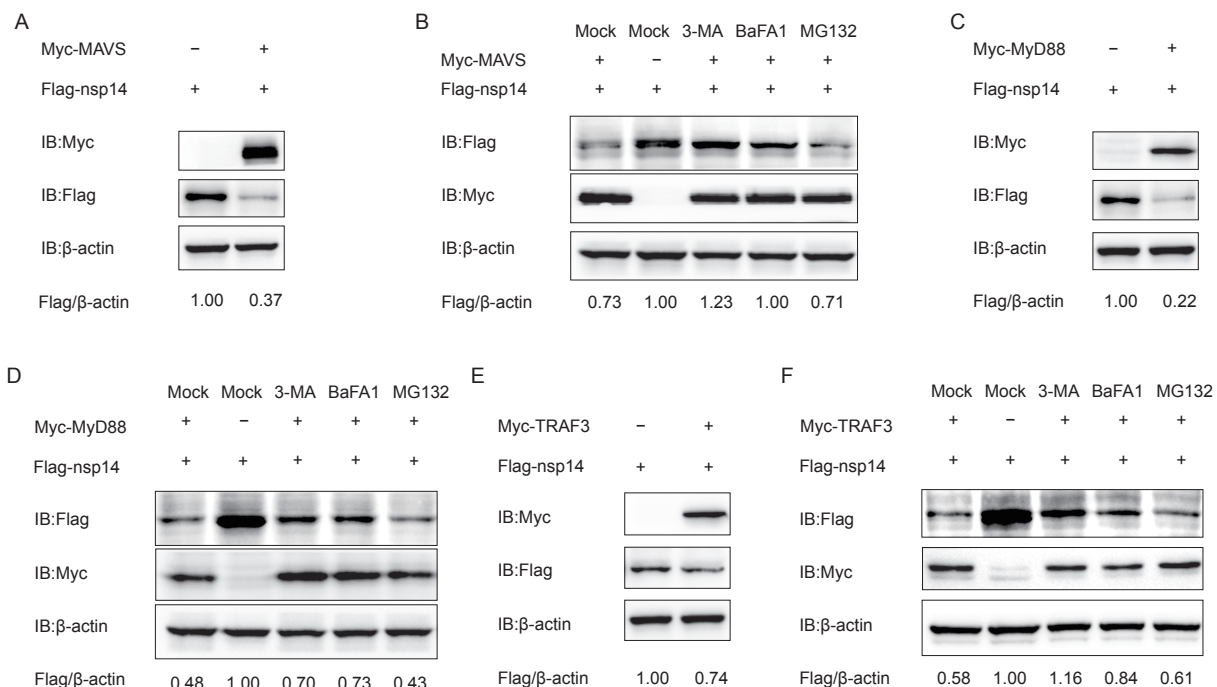


Fig. 6 Host proteins can use the cellular autophagic machinery for degrading porcine deltacoronavirus (PDCoV) nsp14. A, C, and E, plasmids that encoded Flag-nsp14 and MYC-MAVS, MYC-MyD88 or MYC-TRAF3 were transfected into HEK 293T cells. Cellular lysates were detected by Western blotting. B, D, and F, plasmids that encoded Flag-nsp14 and MYC-MAVS, MYC-MyD88 or MYC-TRAF3 were transfected in HEK 293T cells, followed by treatment with 3-MA (1 mmol L⁻¹), Baf A1 (50 μmol L⁻¹) or MG132(5 μmol L⁻¹) for 9 h. Cellular lysates were determined by Western blotting.

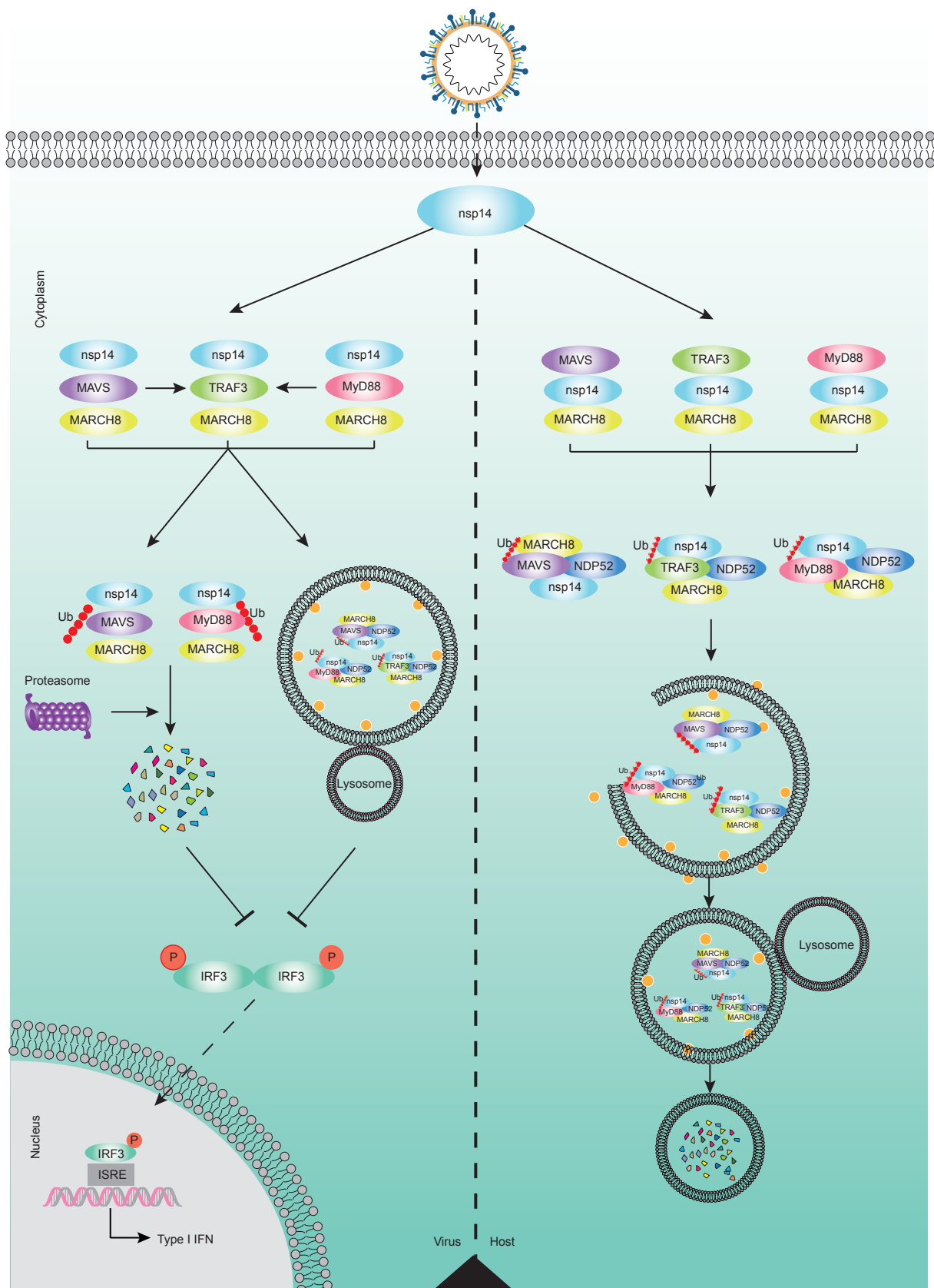


Fig. 7 Porcine deltacoronavirus (PDCoV) nsp14 interferes with interferons (IFNs) by the autophagosome and proteasomal pathways. During PDCoV infection, PDCoV nsp14 facilitates proteasomal and autophagic degradation of MAVS, MyD88 and TRAF3 proteins in host cells. PDCoV nsp14 recruits E3 ubiquitin ligase MARCH8 for catalyzing MAVS, MyD88 and TRAF3 ubiquitination, then the cargo receptor NDP52 can recognize and transfer ubiquitinated proteins into lysosome to degrade them there. Furthermore, the loss of MAVS, MyD88 and TRAF3 prevents IRF3 phosphorylation, dimerization, and nuclear transport to trigger IFN- α/β . Host proteins can use the cellular autophagic machinery to degrade PDCoV nsp14 to inhibit the replication of PDCoV.

pathogen-associated molecular patterns (PAMPs) identify viral RNA through cytoplasmic sensors including MDA5 and RIG-I (Meylan *et al.* 2006). The activated recruitment domains of MDA5 and RIG-I can interact with MAVS, followed by interaction with TRAF3 and the recruitment of I κ B kinase (IKK)-related kinases, including IKK ϵ and TANK-binding kinase 1 (TBK1). IKK ϵ and TBK1 are responsible for phosphorylating IFN regulatory factors 3/7 (IRF3/7) (Fitzgerald *et al.* 2003; Kawai *et al.* 2005; Meylan *et al.* 2005; Seth *et al.* 2005). The phosphorylated and dimerized IRF3/7 is then transferred to the nucleus for the activation of IFN- α/β . During viral infection, the virus adopts several invasive strategies against the IFN response (Kong *et al.* 2020). The interaction between SARS-CoV N protein and RIG-I results in RIG-I ubiquitination, which can, therefore, suppress the host IFN response by triggering proteasome-dependent degradation (Liu *et al.* 2021). In addition, PEDV nsp3 and nsp16 can negatively control the IFN- β level *via* RIG-I and MDA5 (Jaru-Ampornpan *et al.* 2017; Shi *et al.* 2019). PDCoV infection leads to the upregulation of genes related to the RIG-I-like receptor and toll-like receptor (TLR) pathway (Zhou *et al.* 2020). TLRs recruit their downstream adaptor MyD88 for signal transduction, which triggers the generation of chemokines and cytokines (Sun *et al.* 2010; Totura *et al.* 2015; Fitzgerald and Kagan 2020). We suggest that PDCoV nsp14 combines with MAVS, MyD88 and TRAF3, which leads to the ubiquitination of MAVS, MyD88 and TRAF3, thereby inhibiting the generation of IFN and breaking the defensive line of the host.

Note that nsp14 is highly conserved in the family *Coronaviridae* and is related to RNA proofreading. Furthermore, exoribonuclease functional acquisition plays an important role in achieving and preserving the broad genomes of coronaviruses (Eckerle *et al.* 2010; Ma *et al.* 2015). Moreover, nsp14 is related to several additional pathogenicity and virus life cycle processes, which include recombining the viral genome and controlling innate immunity (Gribble *et al.* 2021). In SARS-CoV-2, its 3'-5' exoribonuclease proofreading activity can excise integrated nucleoside analogs, which negates the role of existing medicines to varying extents according to the type of nucleoside analog chemistry (Saramago *et al.* 2021). Therefore, based on these characteristics, nsp14 can be an excellent target for virus inhibitors. As for PDCoV, the nsp5, nsp15, NS6, NS7a, and N proteins have developed diverse escape strategies for interfering with host innate immunity (Duan 2021). PDCoV nsp5 can antagonize type-I IFN signaling through the cleavage of signal transducer and activator of transcription 2 (STAT2) at Q685 and Q758. In addition, the nsp5 protease activity targets STAT2 cleavage (Zhu *et al.* 2017). PDCoV nsp15 hinders IFN- β generation through NF- κ B inactivation according to the endoribonuclease activity-independent mechanism, but it fails to antagonize the activation of interferon regulatory factor 3 (IRF3) (Liu *et al.* 2019). PDCoV NS6 can interact with RIG-I/MDA5, thereby interfering with RIG-I/MDA5 binding to double-stranded RNA (dsRNA), which reduces the generation of RLR-induced IFN- β (Chen *et al.* 2019). PDCoV NS7a completes with TRAF3 and IRF3 for binding with IKK ϵ through simultaneous interactions

with the kinase domain as well as the scaffold dimerization domain in IKK ϵ , which has an antagonistic impact on RLR-induced IFN- β generation (Fang *et al.* 2020). We found that nsp14 inhibited the IFN-related antiviral responses *via* the autolysosome pathway.

During selective autophagy, E3 ubiquitin ligase first ubiquitinates substrate proteins, which are subsequently detected by cargo receptors and transferred into ATG8 family proteins to form autophagosome degradation substrates (Kraft *et al.* 2010). We found that PDCoV nsp14 facilitates the proteasomal and autophagic degradation of the MAVS, MyD88 and TRAF3 proteins. PDCoV nsp14 recruits E3 ubiquitin ligase MARCH8 for catalyzing MAVS, MyD88 and TRAF3 protein ubiquitination; and later, the cargo receptor NDP52 identifies and transfers the ubiquitinated proteins into lysosomes and degrades them there (Kong *et al.* 2020; Jiao *et al.* 2021). The loss of MAVS, MyD88 and TRAF3 proteins reduces both IRF3 phosphorylation and IFN production.

The autolysosome and ubiquitin-proteasome pathways suggest two intracellular protein degeneration-related patterns (Mizushima *et al.* 2010; Mizushima and Komatsu 2011). Autophagy indicates a catabolic degeneration process that delivers cytoplasmic contents into lysosomes. In our previous studies, besides autophagic anti-virus activity, we found that viruses can improve autophagy for destroying host proteins to survive in host-virus interactions (Zhai *et al.* 2023). Host antiviral factors can recruit MARCH8 to catalyze the ubiquitination of virus proteins. Subsequently, the ubiquitinated virus proteins can also be found and transferred into lysosomes to be degraded by NDP52. Meanwhile, viruses can degrade host antiviral proteins *via* the autophagy pathway (Kong *et al.* 2020; Jiao *et al.* 2021). We also found that PDCoV can induce the proteasome and autophagy pathways to improve its replication. At the same time, PDCoV nsp14 can also be inhibited by MAVS, MyD88 and TRAF3 *via* autophagy pathways. Therefore, PDCoV plays a chess game with host proteins in the process of infection. Host cellular reactions have both advantages and disadvantages in PDCoV infection, so the pro-viral and antiviral functions should be perfectly balanced.

5. Conclusion

To summarize, our results indicate that PDCoV nsp14 mediates escape strategies that interfere with the innate immunity of the host by ubiquitinating the MAVS, MyD88, and TRAF3 proteins *via* the MARCH8-NDP52-autophagosome and proteasomal pathways, which further suppresses IFN expression. Hosts can also promote their antiviral defense response to antagonize virus infection by degrading the nsp14 protein through selective autophagy pathways during PDCoV infection. Moreover, the dual role of host proteins and the selective autophagy and proteasome pathways of PDCoV nsp14 can enhance the ubiquitination and degradation of host antiviral proteins and viral particles, thus regulating the association of viral infection with host innate immunity. This study shows a novel mechanism of PDCoV nsp14-regulated viral invasion and provides possible targets for fighting PDCoV infection.

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Declaration of competing interests

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

This article does not contain any studies with human or animal subjects performed by any of the authors.

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