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# MDA5 against enteric viruses through induction of interferon-like response partially via the JAK-STAT cascade

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ABSTRACT

Enteric viruses including hepatitis E virus (HEV), human norovirus (HuNV), and rotavirus are causing global health issues. The host interferon (IFN) response constitutes the first-line defense against viral infections. Melanoma Differentiation-Associated protein 5 (MDA5) is an important cytoplasmic receptor sensing viral infection to trigger IFN production, and on the other hand it is also an IFN-stimulated gene (ISG). In this study, we investigated the effects and mode-of-action of MDA5 on the infection of enteric viruses. We found that MDA5 potently inhibited HEV, HuNV and rotavirus replication in multiple cell models. Overexpression of MDA5 induced transcription of important antiviral ISGs through IFN-like response, without triggering of functional IFN production. Interestingly, MDA5 activates the expression and phosphorylation of STAT1, which is a central component of the JAK-STAT cascade and a hallmark of antiviral IFN response. However, genetic silencing of STAT1 or pharmacological inhibition of the JAK-STAT cascade only partially attenuated the induction of ISG transcription and the antiviral function of MDA5. Thus, we have demonstrated that MDA5 effectively inhibits HEV, HuNV and rotavirus replication through provoking a non-canonical IFN-like response, which is partially dependent on JAK-STAT cascade.

## 1. Introduction

There are over 100 types of viruses excreted in feces, which collectively known as enteric viruses. Among these, hepatitis E virus (HEV) has been recognized as an emerging zoonotic virus, representing a major cause of acute hepatitis worldwide. Although it is often self-limiting, high mortality rate has been reported in pregnant women. Human norovirus (HuNV) and rotavirus are the leading causes of acute gastroenteritis. Interestingly, all three types of viruses can cause chronic infection in immunocompromised organ transplantation patients (Kamar et al., 2008; Zhou et al., 2013; Bok and Green, 2012; Yin et al., 2015), highlighting the essential role of host immunity in determining the outcome of these infections.

Innate immune system is the first line of host defense against viral infection and plays a critical role in clearance of pathogen (Medzhitov and Janeway, 2000). Upon infection, pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptor (PRRs), such as toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) (O'Neill and Bowie, 2010; Jensen and Thomsen, 2012). Subsequently, PPRs initiate downstream signaling pathway to produce a panel of cytokines, in particular, the antiviral IFNs. Mechanistically, type I and III IFN bind to their receptors on the cell surface to initiate signal cascades. This binding triggers the phosphorylation of STAT1 and STAT2, which subsequently bind to IRF9 to form a IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 translocates to the nucleus to drive expression of IFN-stimulated genes (ISGs) to establish antiviral states (Schneider et al., 2014).

Melanoma Differentiation-Associated protein 5 (MDA5) is an

*Abbreviations:* MDA5, Melanoma Differentiation-Associated protein 5; HEV, hepatitis E virus; HuNV, human norovirus; ISG, interferon-stimulated gene; STAT1, signal transducers and activators of transcription 1; Flu, *Photinus pyralis luciferase*; ISRE, interferon-stimulated response element; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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important cytoplasmic receptor, a member of RLRs, which participates in recognition of different RNA viruses. Upon the detection of viral RNA ligand, MDA5 recruits mitochondrial antiviral signaling (MAVS, also known as IPS-1, VISA, or Cardif) (Seth et al., 2005) to activate IRF3/7 and NF-κB, and eventually triggers IFN and inflammatory responses. On the other hand, MDA5 is also an important antiviral ISG. MDA5 has been reported to potently inhibit the replication of Sindbis virus (SINV), West Nile virus (WNV) and Venezuelan equine encephalitis virus (VEEV) (Schoggins et al., 2011). The antiviral mechanisms may directly through pathogen recognition pathway or directly act as an antiviral ISG. However, how MDA5 exactly exerts antiviral action against different types of viruses remains largely under investigated.

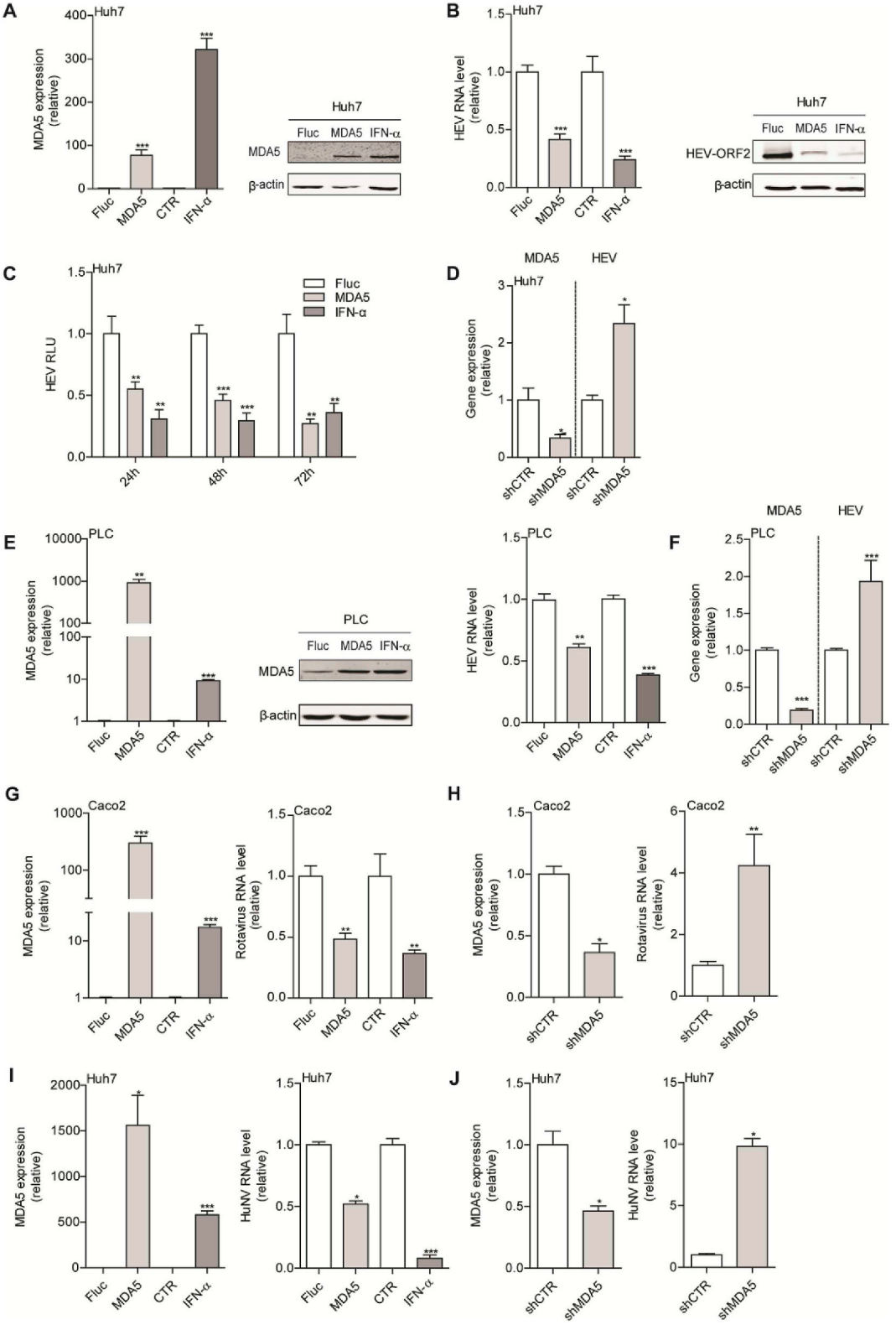


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**Fig. 1. MDA5 inhibits the replication of different enteric viruses.** (A) Quantitative RT-PCR analysis and immunoblot analysis of MDA5 expression in Huh7-p6 cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 13). (B) Quantitative RT-PCR analysis and immunoblot analysis of HEV RNA level and HEV ORF2 protein level in Huh7-p6 cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 6–9). (C) Analysis of HEV-related *Gaussia* luciferase activity in Huh7-p6-luciferase cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 24, 48, or 72 h (n = 6–10). (D) Huh7-p6 cells transduced with lentiviral short hairpin RNA vector targeting MDA5 or scramble control. Quantitative RT-PCR analysis of MDA5 expression level and HEV RNA level in stable MDA5 knockdown or scramble control Huh7-p6 cells (n = 4). (E) Quantitative RT-PCR analysis and immunoblot analysis of MDA5 and HEV expression in PLC-p6 cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 6–9). (F) PLC-p6 cells transduced with lentiviral short hairpin RNA vector targeting MDA5 or scramble control. Quantitative RT-PCR analysis of MDA5 expression level and HEV RNA level in stable MDA5 knockdown or scramble control PLC-p6 cells (n = 10). (G) Quantitative RT-PCR analysis of MDA5 expression and rotavirus RNA level in Caco2 cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 6–14). (H) Caco2 cells transduced with lentiviral short hairpin RNA vector targeting MDA5 or scramble control. Stable MDA5 knockdown or scramble control Caco2 cells were infected with rotavirus for 48 h. Quantitative RT-PCR analysis of MDA5 expression level and rotavirus RNA level (n = 4–6). (I) Quantitative RT-PCR analysis of MDA5 expression and HuNV RNA level in HG23 (Huh7-based HuNV replicon) cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 4–12). (J) HG23 cells (Huh7-based HuNV replicon) transduced with lentiviral short hairpin RNA vector targeting MDA5 or scramble control. Quantitative RT-PCR analysis of MDA5 expression level and HuNV RNA level in stable MDA5 knockdown or scramble control Huh7 cells (n = 4). Data were normalized to the Fluc or untreated control CTR (Fluc/CTR, set as 1) or to the scramble control (shCTR, set as 1). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviation: Flu, Photinus pyralis luciferase; CTR, control; RLU, relative luciferase unit; sh, short hairpin.

Given the importance of MDA5 in innate antiviral defense, we investigated the effects and mechanism-of-actions of MDA5 in enteric virus infections. We found that overexpression of MDA5 effectively inhibited HEV, HuNV, and rotavirus replication. Mechanistically, MDA5 activated STAT1 phosphorylation and the transcription of antiviral ISGs, but dispensable of the production of functional IFNs. Surprisingly, STAT1 deficient only partially impaired the antiviral activity and ISG transcription induced by MDA5. These results have demonstrated the broad antiviral activity of MDA5 against enteric viruses, involving non-canonical mechanisms partially through the JAK-STAT cascade.

## 2. Materials and methods

### 2.1. Reagents

Human IFN-α (Sigma-Aldrich, H6166) was dissolved in PBS. Stocks of JAK inhibitor 1 (SC-204021, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were dissolved in DMSO with a final concentration of 5 mg/ml. Dimethyl sulfoxide (DMSO, Sigma, Zwijndrecht, the Netherlands) was used as vehicle control. Phospho-STAT1 (Tyr701) (58D6, Rabbit mAb, 9167), STAT1 (Rabbit mAb, 9172), RIG-I (D14G6, Rabbit mAb,3743), PKR (D7F7, Rabbit mAb, 12297), MDA5 (IFIH1) (D74E4, Rabbit mAb, 5321) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody (mouse monoclonal, sc-47778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) were also used.

### 2.2. Cell culture

Huh7 and PLC/PRF/5 (PLC) human hepatoma cells, Huh7-STAT1 knockout cells, HEK293T cells and Caco2 cells were cultured in DMEM (Lonza Biowhittaker, Verviers, Belgium) complemented with 10% (v/v) fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin. For the ISRE reporter model (Huh7-ISRE-Luc), Huh7 cells were transduced with a lentiviral transcriptional reporter system expressing the firefly luciferase gene driven by a promoter containing multiple ISRE promoter elements (SBI Systems Biosciences, Mountain View, CA, USA). Luciferase activity represents ISRE promoter activation (Pan et al., 2012).

### 2.3. Viruses and cell culture models

In this study, multiple cell lines were used for HEV replication, including human hepatoma cell lines, Huh7 and PLC. For the full-length HEV model, a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone; GenBank Accession Number JQ679013) was used to generate HEV genomic RNA with the Ambion mMessage mMachine in vitro RNA transcription Kit (Thermo Fisher Scientific Life Sciences). Huh7 and PLC cells were electroporated with full-length HEV genome RNA, to generate consecutive HEV-infected cell models, Huh7-p6 and PLC-p6. For the subgenomic HEV model, a construct containing subgenomic HEV in which the portion of HEV ORF2 was replaced with the in-frame Gaussia princeps luciferase reporter gene to yield p6-Luc (Shukla et al., 2012). Huh7 cells were electroporated with HEV subgenomic RNA to generate an HEV subgenomic model, Huh7-p6-Luc, in which the accumulation of secreted luciferase serves as a reporter for HEV replication. HG23 model is based on Huh7 cells containing a stable subgenomic HuNV replicon (Chang et al., 2006). Gentamicin (G418; Gibco) was added to HG23 culture medium at 1.5 mg/mL for selection before experimentation.

### 2.4. Gene knockdown and overexpression by lentiviral vectors or plasmid

For gene knockdown, pLKO.1-based lentiviral vectors (Sigma-Aldrich) targeting MDA5 and non-targeted control vector (shCTR) were obtained from the Biomics Center in Erasmus Medical Center. Lentiviral pseudoparticles were generated in HEK293T cells. To generate a stable gene-knockdown cell line, PLC cells were transduced with lentiviral particles for 48 h. Subsequently, transduced cells were cultured with medium containing 2.5 mg/ml puromycin (Sigma-Aldrich). Because the vectors also express a puromycin resistance gene, cell lines showing optimal gene knockdown after selection were chosen. pTRIP.CMV.IVSb.ISG.ires.TagRFP-based MDA5 overexpression vector was a kind gift from Prof. Charles M. Rice (Rockefeller University, New York, NY, USA) (Schoggins et al., 2011). Meanwhile, Photinus pyralis luciferase (Fluc) was used as the control (also kind gifts from Prof. Charles M. Rice). Target cell lines were seeded into 12-well plates at a density of $6 \times 10^4$ cells per well and transduced with lentiviral pseudoparticles at 37 °C for 24, 48, or 72 h. The transduction time of each experiment is described in the legend of each figure, along with the control vector used. The MDA5 plasmid was also used for direct transfection to overexpress MDA5.

### 2.5. Quantification of viral replication

HEV, HuNV and rotavirus RNA levels were quantified by SYBR-Green-based qRT-PCR (Applied Biosystems SYBR Green PCR Master Mix; Thermo Fisher Scientific Life Sciences) with the StepOnePlus System (Thermo Fisher Scientific Life Sciences). GAPDH was used as housekeeping genes, and all gene expression levels (relative) were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. For HEV-related Gaussia luciferase analysis (HEV-p6-Luc), the activity of secreted luciferase in the cell culture medium was measured by BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA, USA), according to

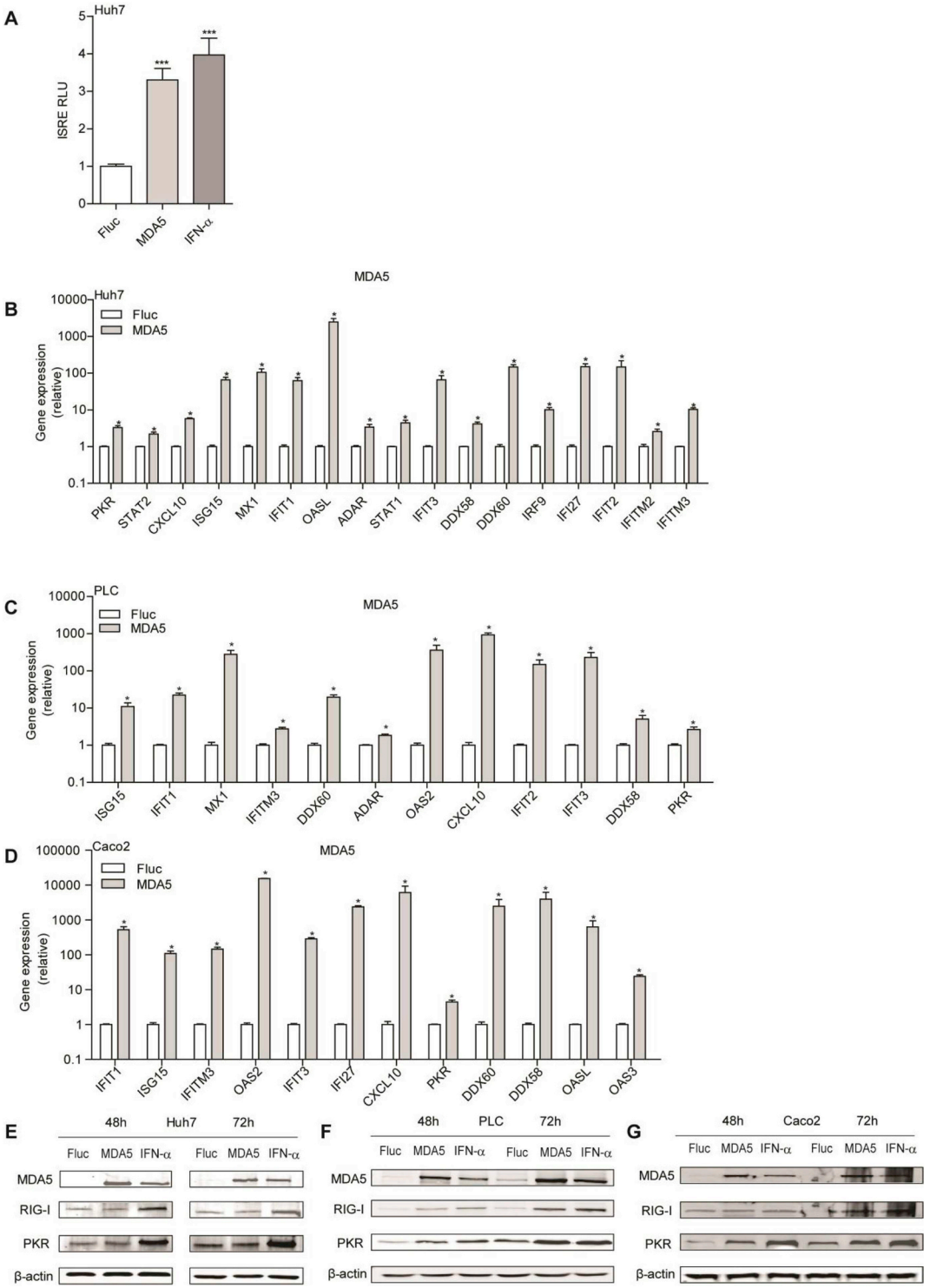


**Fig. 2. MDA5 triggers the transcription of a range of antiviral ISGs.** (A) Analysis of ISRE-related *firefly* luciferase activity in Huh7-ISRE-Luc cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) for 48 h (n = 9). (B) Quantitative RT-PCR analysis of ISG mRNA level in Huh7-p6 cells, (C) PLC-p6 cells, and (D) Caco2 cells transduced with MDA5 or Fluc vector for 48 h (n = 4–6). (E) Immunoblot analysis of ISG protein level in Huh7-p6 cells, (F) PLC-p6 cells, and (G) Caco2 cells transduced with MDA5 or Fluc vector for 48 or 72 h (n = 3). Data were normalized to the Fluc control (Fluc, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviation: Flu, Photinus pyralis luciferase.

the manufacturer's instructions. Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). For the firefly and Photinus pyralis luciferases, luciferin potassium salt (100 mM; Sigma-Aldrich) was added to the cells and incubated for 10 min at 37 ˚C, and luciferase activity was measured.

### 2.6. IFN production bioassay

Cells were seeded into 6-well plates at a density of $1 \times 10^5$ cells per well and transduced with MDA5 or control lentiviral particles at 37 ˚C. After 72 h, lentiviral particles were removed, and cells were washed 3 times with PBS and cultured for another 72 h. The cultured supernatant

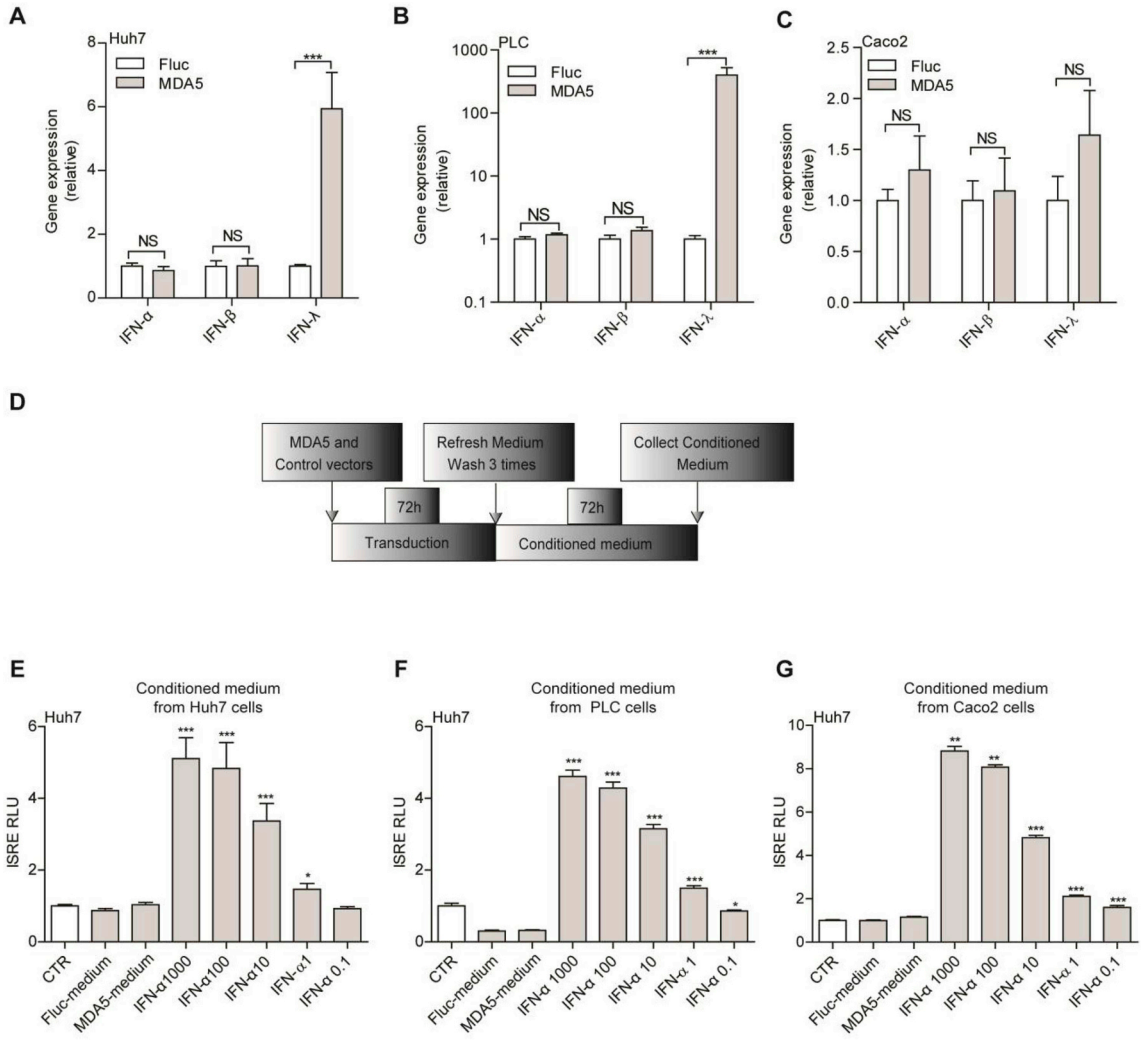


**Fig. 3. MDA5 overexpression does not induce the production of functional IFNs.** (A) Quantitative RT-PCR analysis of IFN gene mRNA level in Huh7-p6 cells (B) PLC-p6 cells and (C) Caco2 cells transduced with MDA5 or Fluc vector for 48 h (n = 6–12). (D) Production of conditioned medium (supernatant). Cells were transduced with MDA5 or Fluc (control) vector for 72 h; then, the cells were washed three times, and medium was refreshed. Cells were cultured for another 72 h, and supernatant was collected as conditioned medium. (E) Analysis of ISRE-related firefly luciferase activity in Huh7-ISRE-Luc cells treated with conditioned medium from Huh7-p6 cells (F) PLC-p6 cells and (G) Caco2 cells or various concentrations of IFN-α for 48 h (n = 9–18). Data were normalized to the Fluc control (Fluc, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; Abbreviation: ns, not significant; CTR, control; RLU, relative luciferase unit.

was subsequently collected and filtered through a 0.45 μm pore size membrane and added to Huh7-ISRE-Luc reporter cells which are sensitive to IFNs.

### 2.7. Immunoblot analysis

Lysate was heated at 95 ˚C for 5 min. Proteins were subjected to a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated at 100 V for 100 min, and electrophoretically transferred onto a PVDF membrane (pore size: 0.45 μm; Thermo Fisher Scientific Life Sciences) for 100 min with an electric current of 230 mA. Subsequently, the membrane was blocked with blocking buffer (Li-Cor Biosciences) in PBS containing 0.05% Tween-20. Membranes were incubated with primary antibodies overnight at 4 ˚C. Rabbit anti-MDA5 (1:1000), p-STAT1 (1:1000), STAT1 (1:1000), RIG-I (1:1000), PKR (1:1000) antibodies or mouse anti-β-actin (1:1000) were diluted in blocking buffer. The membrane was washed 3 times, followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000; Li-Cor Biosciences) at room temperature. β-actin served as the loading standard. The membrane was scanned by Odyssey Infrared Imaging System (Li-Cor Biosciences). Results were visualized with Odyssey 3.0 software. Band intensity data of each immunoblot was also quantified by Odyssey Software.

### 2.8. MTT assay

Cells were seeded in 96-well plates, and 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added. The plate was incubated at 37 ˚C with 5% CO2 for 3 h, then the medium was removed. After adding 100 μL of DMSO to each well, the plate was incubated at 37 ˚C for 1 h. The absorbance was read on the microplate absorbance reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm.

### 2.9. Statistical analysis

GraphPad Prism 5 software was used for data analysis using a Mann-Whitney test. All results were presented as mean ± standard errors of the means (SEM). P values of less than 0.05 (single asterisks in figures) were considered statistically significant; whereas P values less than 0.01 (double asterisks) and 0.001 (triple asterisks) were considered highly significant.

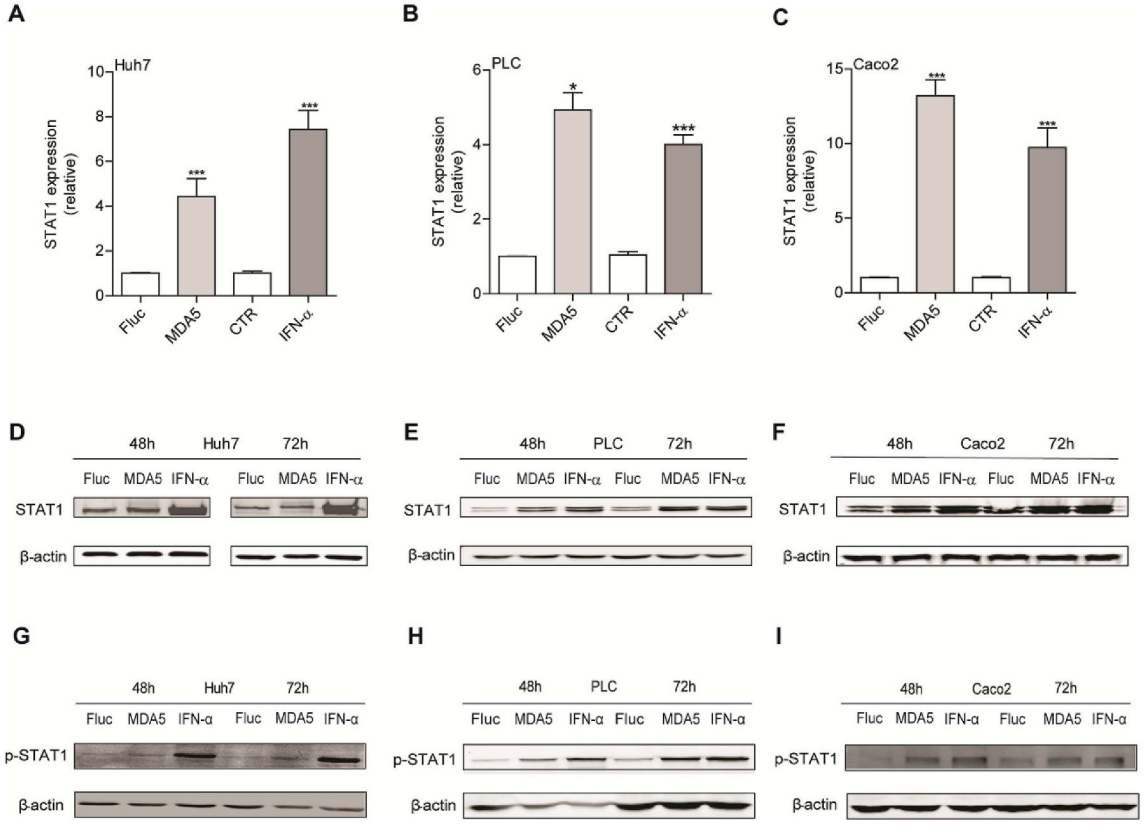


**Fig. 4. MDA5 overexpression activates STAT1 expression and phosphorylation.** (A) Quantitative RT-PCR analysis of STAT1 mRNA level in Huh7-p6 cells (B) PLC-p6 cells and (C) Caco2 cells transduced with MDA5 or Fluc vector for 48 h (n = 4–12). (D) Immunoblot analysis of STAT1 expression in Huh7-p6 cells (E) PLC-p6 cells and (F) Caco2 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/Ml) for 48 or 72 h (n = 3). (G) Immunoblot analysis of p-STAT1 (Tyr701) expression in Huh7-p6 cells (H) PLC-p6 cells and (I) Caco2 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/Ml) for 48 or 72 h (n = 3). Data were normalized to the Fluc/CTR control (Fluc/CTR, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; Abbreviation: Flu, Photinus pyralis luciferase; CTR, control.

## 3. Results

### 3.1. MDA5 potently inhibits HEV, HuNV and rotavirus replication

To evaluate the effects of MDA5 on enteric viruses, we employed multiple cell culture models of HEV, HuNV and rotavirus infections. For HEV, two Huh7-based HEV models (Huh7-p6 and Huh7-p6-Luc) were used. Lentiviral mediated MDA5 overexpression was confirmed in Huh7-p6 cells at mRNA and protein levels (Fig. 1A). In both HEV infectious and replicon models, MDA5 overexpression significantly inhibited HEV replication (Fig. 1B, C). Conversely, gene silencing of MDA5 by RNAi significantly facilitated HEV replication (Fig. 1D) in Huh7 cells. We also confirmed the anti-HEV activity of MDA5 in PLC cells (Fig. 1E, F).

To validate the antiviral ability of MDA5 in other enteric viruses, we overexpressed MDA5 in rotavirus infected Caco2 cells. The results showed that MDA5 potently inhibited rotavirus replication (Fig. 1G), and the antiviral activity is comparable to IFN-α treatment. Conversely, Gene silencing of MDA5 in Caco2 cells significantly increased rotavirus replication (Fig. 1H). Overexpression and knockdown of MDA5 in HG23 cells further demonstrated the anti-HuNV activity of MDA5. These results have demonstrated that MDA5 has a broad antiviral activity against enteric viruses, and encouraged us to further explore the mechanism-of-action.

### 3.2. MDA5 triggers the transcription of a range of antiviral ISGs through IFN-like response

In general, IFNs active ISGF3 complex, which binds to IFN-stimulated response element (ISRE) motifs in nucleus to drive ISG transcription. Usually upon sensing of particular viruses, MDA5 triggers IFN production, which subsequently induce ISG expression. Here, we employed a transcriptional report system that mimics IFN response with a reporter luciferase gene that was driven by multiple ISRE (ISRE-Luc). Surprisingly, similar to IFN-α treatment, MDA5 overexpression in naïve cells already potently increased ISRE-luciferase activity (Fig. 2A). Activation of ISRE leads to the transcription of ISGs that contain this motifs in their promoter regions. Consistently, MDA5 overexpression stimulated the transcription of a large panel of ISGs in Huh7, PLC and Caco2 cells (Fig. 2B, C, and 2D). The transcription level of ISGs induced by MDA5 is similar to that by IFN-α treatment (Supporting Fig. 1A, B, and 1C), whereas the induction patterns are different. The expression of several important ISGs was further confirmed by immunoblotting at protein level (Fig. 2E, F, and 2G). Since all these experiments are based on lentiviral vector mediated MDA5 overexpression, we next attempted to verify these findings by plasmid-based gene delivery. Consistently, transfection of plasmid expressing MDA5 into Huh7-p6 cells resulted in inhibition of HEV replication and induction of many antiviral ISGs (Supporting Fig. 1D). These results suggested that MDA5 induces ISG expression through triggering IFN-like response.

### 3.3. Overexpression of MDA5 does not induce the production of functional IFNs

Since MDA5 induced antiviral ISG transcription through IFN-like response, we examined whether MDA5 overexpression triggers IFN production. At mRNA level, MDA5 overexpression did not induce IFN-α and IFN-β, but moderately induced IFN-λ expression in Huh7, PLC and

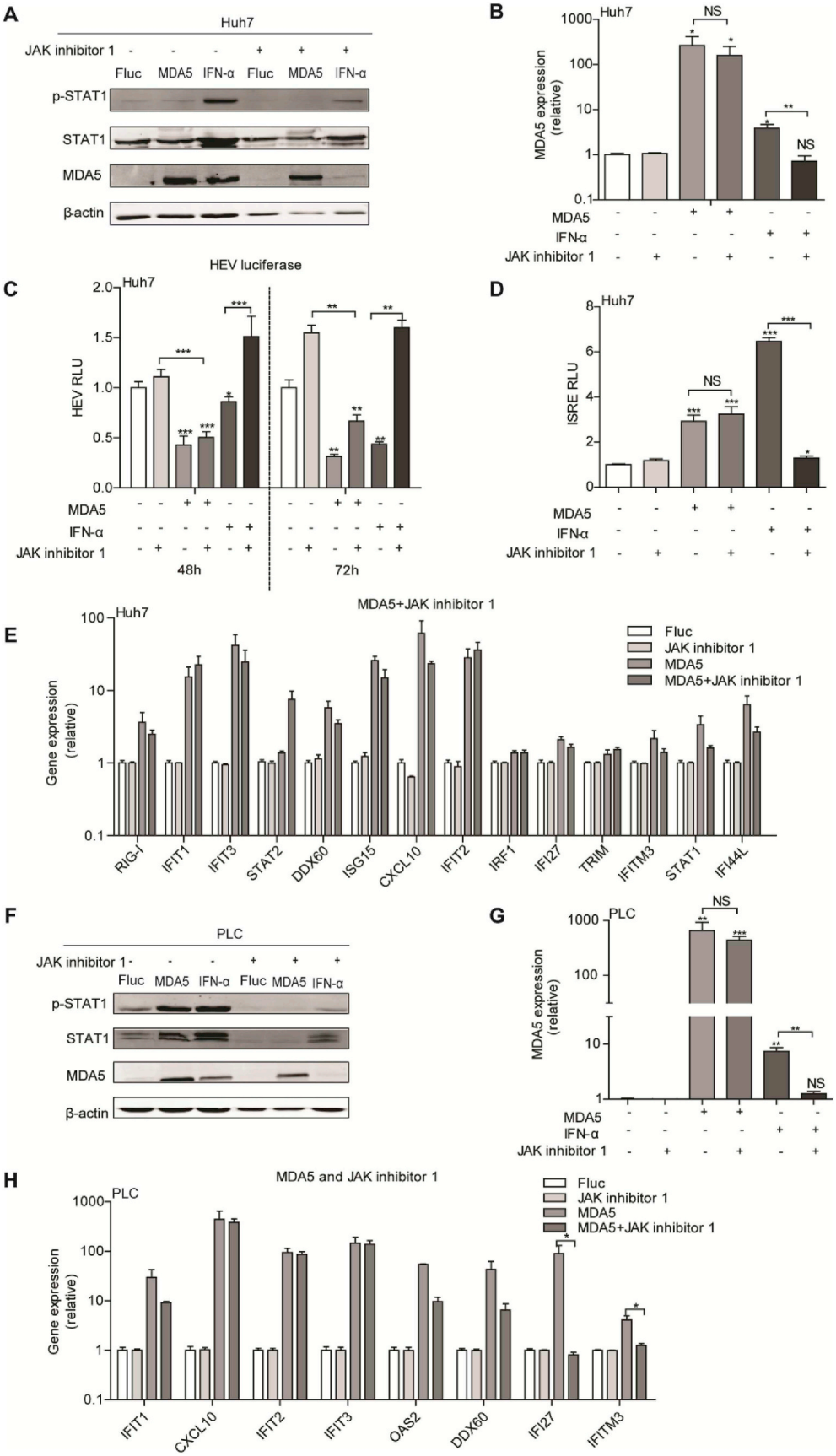


**Fig. 5. JAK inhibitor 1 partially attenuates MDA5-induced ISG transcription and anti-HEV activity.** (A) Immunoblot analysis of p-STAT1 (Tyr701) expression and ISG protein levels in Huh7 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 3). (B) Quantitative RT-PCR analysis of MDA5 mRNA levels in Huh7 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 6–8). (C) Analysis of HEV-related *Gaussia* luciferase activity in Huh7-p6-luciferase cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 6–9). (D) Analysis of ISRE-related *firefly* luciferase activity in Huh7-ISRE-Luc cells transduced with MDA5 or Fluc vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 9). (E) Quantitative RT-PCR analysis of ISG mRNA levels in Huh7 cells transduced with MDA5 vector or Fluc control or JAK inhibitor 1 (10 μM) for 48 h (n = 4–6). (F) Immunoblot analysis of p-STAT1 (Tyr701) expression and ISG protein levels in PLC cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 3). (G) Quantitative RT-PCR analysis of MDA5 mRNA levels in PLC cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 6–9). (H) Quantitative RT-PCR analysis of ISG mRNA levels in PLC cells transduced with MDA5 vector or Fluc control or JAK inhibitor 1 (10 μM) for 48 h (n = 4–6). Data were normalized to the Fluc control (Fluc, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; Abbreviation: ns, not significant; Flu, Photinus pyralis luciferase.

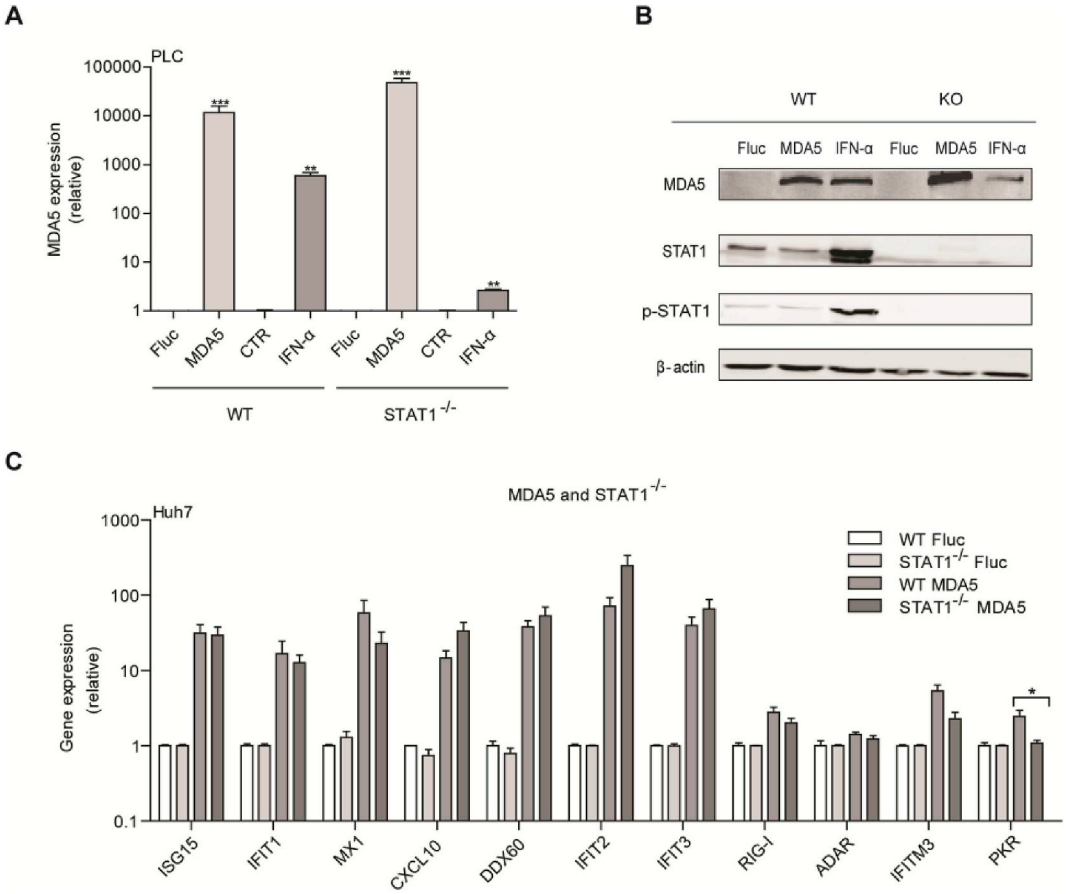


**Fig. 6. MDA5-induced ISG transcription was not affected in STAT1 knockout cells**. (A) Quantitative RT-PCR analysis of MDA5 mRNA levels in WT and STAT1$^{-/-}$ Huh7 cells transduced with MDA5 vector or Fluc control for 48 h (n = 4–9). (B) Immunoblot analysis of ISG protein level in WT and STAT1$^{-/-}$ Huh7 cells transduced with MDA5 vector or Fluc control for 48 h (n = 3). (C) Quantitative RT-PCR analysis of ISG mRNA levels in WT and STAT1$^{-/-}$ Huh7 cells transduced with MDA5 vector or Fluc for 48 h (n = 4). Data were normalized to the Fluc/CTR control (Fluc/CTR, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviation: ns, not significant; Flu, Photinus pyralis luciferase; CTR, control.

Caco2 cells (Fig. 3A 3B, and 3C). More importantly, we investigated whether there is production of functional IFNs by testing conditioned medium (supernatant) from MDA5 overexpressed Huh7, PLC or Caco2 cells (Fig. 3D). In the ISRE-based IFN reporter assay, treatment with these conditioned medium did not trigger ISRE activation (Fig. 3E, F, and 3G). These results demonstrated that MDA5 overexpression does not trigger the production of functional IFNs in these cell models.

### *3.4. Overexpression of MDA5 upregulates STAT1 expression and phosphorylation*

Given the dispensability of IFN production in MDA5-triggered IFN-like response, we next investigated the effect on STAT1, a central component of the JAK-STAT cascade. At mRNA level, similar to IFN-α treatment, MDA5 overexpression significantly upregulated STAT1 expression in Huh7, PLC and Caco2 cells (Fig. 4A, B, and 4C). This was further confirmed at total protein levels of STAT1 in these cell models (Fig. 4D, E, and 4F).

Phosphorylation of STAT1 is hallmark of IFN-triggered activation of JAK-STAT cascade. We found the activation of p-STAT1, at 701 site, by MDA5 overexpression, similar to IFN-α treatment (Fig. 4G, H, and 4I).

### *3.5. The effect of JAK-STAT pathway on MDA5-mediated antiviral ability and triggered ISG transcription*

Because MDA5 activates STAT1 expression and phosphorylation, we further investigated the role of JAK-STAT pathway on MDA5-mediated ISG transcription and antiviral activity. We firstly used JAK inhibitor 1 to pharmacologically block the JAK-STAT pathway. As expected, MDA5 or IFN-α-induced STAT1 phosphorylation was effectively blocked by JAK inhibitor (Fig. 5A), whereas lentiviral-delivered MDA5 overexpression was not affected by the inhibitor in Huh7 cells (Fig. 5A, B). Surprisingly, the anti-HEV activity of MDA5 was only partially attenuated, whereas the anti-HEV activity of IFN-α was totally blocked by JAK inhibitor 1 (Fig. 5C). Consistently, MDA5-induced ISRE activation was hardly affected by this inhibitor, whereas ISRE activation induced by IFN-α was totally blocked (Fig. 5D). Subsequently, we measured the mRNA level of many antiviral ISGs in MDA5-overexpressed Huh7 cells treated with JAK inhibitor 1. Expectedly, the expression of ISGs was minor affected by the inhibitor (Fig. 5E). In contrast, IFN-α-induced ISG transcription was totally blocked by the inhibitor (Supporting Fig. 2A). Similar results were observed in PLC cells (Fig. 5F, G, 5H, and supporting Fig. 2B).

To further confirm, we overexpressed MDA5 in STAT1 knockout (STAT1$^{-/-}$) and wild type Huh7 cells (Fig. 6A, B). Although MDA5 failed to induce p-STAT1 in STAT1$^{-/-}$ cells (Fig. 6B), it induced a similar level of ISG transcription compared to wild type Huh7 cells (Fig. 6C). In contrast, IFN-α failed to induce ISGs in STAT1$^{-/-}$ cells (Supporting Fig. 3A).

Next, we explored the effects of JAK-STAT pathway on MDA5 mediated anti-HuNV and anti-rotavirus activity. Pharmacologically blocking the JAK-STAT pathway by JAK inhibitor 1 in Caco2 and HG23 cells only had minor effect on the anti-HuNV and anti-rotavirus ability of MDA5 (Fig. 7A, B). However, the antiviral ability of IFN-α was

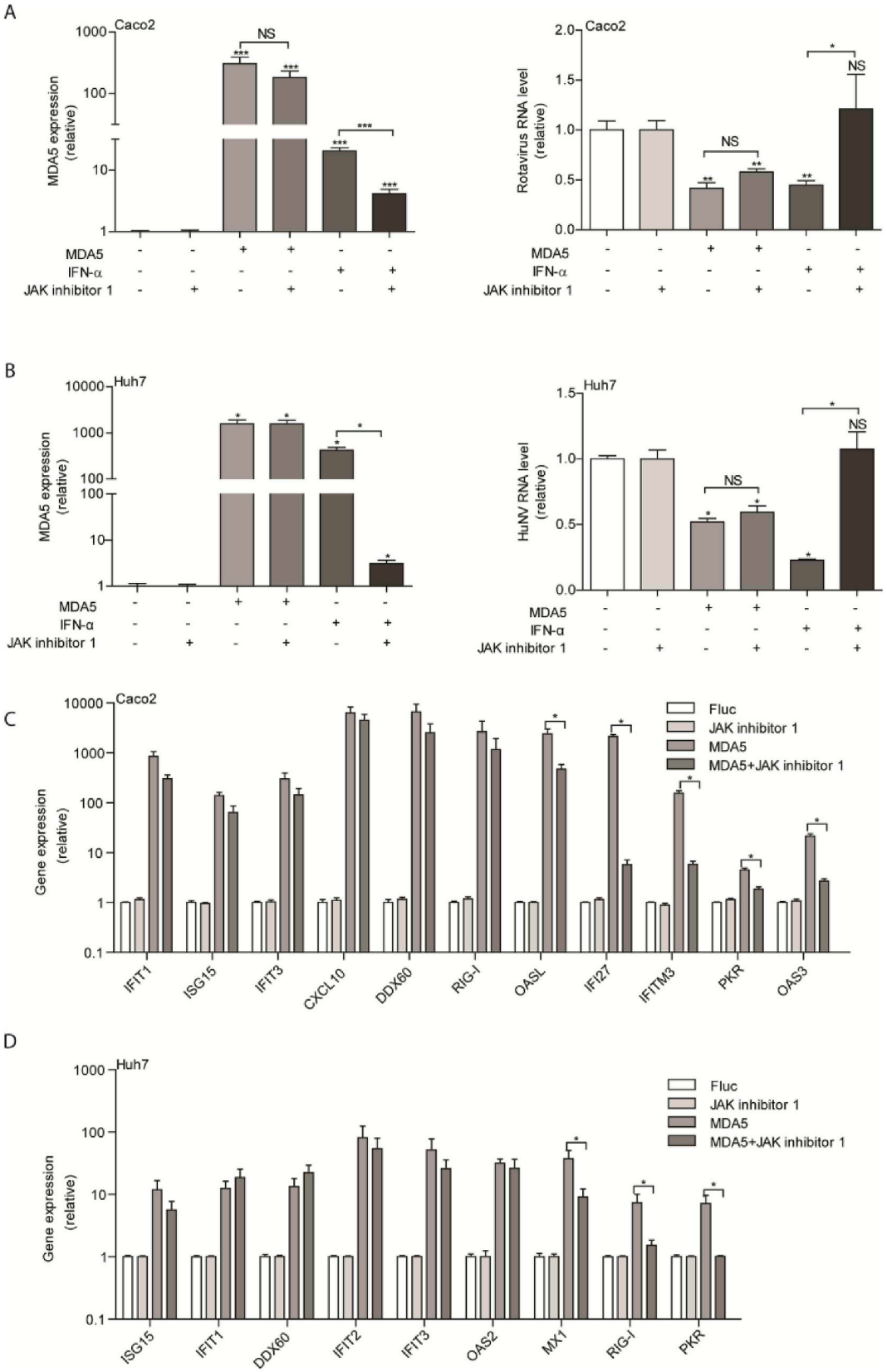


**Fig. 7. MDA5 inhibits rotavirus and HuNV replication and induced ISG transcription partially via JAK-STAT pathway** (A) Quantitative RT-PCR analysis of MDA5 mRNA levels or rotavirus RNA level in Caco2 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 6–9). (B) Quantitative RT-PCR analysis of MDA5 mRNA levels or HuNV RNA level in HG23 cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 4). (C) Quantitative RT-PCR analysis of ISG mRNA levels in Caco2 cells or (D) HG23 (Huh7-based HuNV replicon) cells transduced with MDA5 vector or treated with IFN-α (1000 IU/mL) or JAK inhibitor 1 (10 μM) for 48 h (n = 6). Data were normalized to the Fluc control (Fluc, set as 1). Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviation: ns, not significant; Flu, Photinus pyralis luciferase.

completely blocked by this inhibitor. We observed similar results on the induction of ISGs by MDA5 or IFN-α (Fig. 7C, D, and supporting Fig. 4A, B). Collectively, these results demonstrated that the induction of ISG transcription and the anti-enteric virus activity of MDA5 only partially depend on JAK-STAT cascade.

## 4. Discussion

This study has demonstrated that MDA5 is a abroad antiviral factor against enteric virus infection, including HEV, HuNV and rotavirus. Overexpression of MDA5 induces the phosphorylation of STAT1 and triggers the transcription of a panel of antiviral ISGs, without the requirement of functional IFN production. Knockout or pharmacological inhibition of STAT1 only partially blocked the induction of ISG transcription and the antiviral activity of MDA5.

The innate immune system is the first line of defense against viral infections. One predominant characteristic is the rapid and spectrum of resistance to infection through recognition of viral components by host PRRs, which initiate signal cascade transduction that triggers the production and secretion of IFNs. Subsequently, IFNs stimulate transcription of ISGs through JAK-STAT pathway to establish antiviral state. Among them, only a small subset of ISGs have antiviral effects (Schoggins et al., 2011). Previous studies have shown that MDA5 plays an important role in defending against a wide range of viral infections (Dang et al., 2018; Lu and Liao, 2013; Zhang et al., 2018; Cao et al., 2015). Consist with these reports, we found that MDA5 significantly inhibits enteric viruses replication in multiple cell models.

MDA5 is a main cytoplasmic sensor, which recognizes the viral RNA to trigger downstream antiviral signaling (Zhang et al., 2018; Cao et al., 2015; Kuo et al., 2013; Wei et al., 2014). Classically, viral infection induces MDA5 activation and mediates IFNs production through MAVS-IRF3 signal pathway. Several studies have demonstrated that MDA5 induces IFN-α or INF-β production during viral infection (Zhang et al., 2018; Pham et al., 2016). In turn, IFNs combat viral infection and exert immune regulatory roles through induction of ISGs. In this study, we found that MDA5 induced transcription and protein expression of many ISGs, and most of these have been reported to have antiviral activity (Schoggins et al., 2011, 2014; Xu et al., 2016, 2017). However, the expression pattern of ISGs induced by MDA5 is different from IFN-α treatment. For instance, in Huh7 cells, overexpression of MDA5 highly activates the expression of IFIT2, IFIT3 and CXCL10, whereas the expression of PKR and IFI27 is highly activated by IFN-α treatment. This indicates non-canonical mechanisms of MDA5 mediated ISG transcription independent of IFN. Although IFN has been used as therapeutic agent for treating infectious diseases for many decades (Finter et al., 1991; Rijckborst and Janssen, 2010). However, it is often associated with substantial side effects partially related to the induction of pro-inflammatory ISGs. Furthermore, many patients do not adequately respond to the treatment, which is partially associated with the induction of ISGs functioning as negative regulators. Among these ISGs, the high level induction of ISG15 has been well-recognized to affect the treatment outcome of patients co-infected with HIV and HCV (Katsounas et al., 2013; Nick et al., 2016). The distinct mechanisms of MDA5 in ISG induction may implicate as a new avenue for developing antiviral strategies with better specificity and less side effects.

For rotavirus infection, it has been reported that MDA5 and RIG-I are important sensors to trigger IFN response through MAVS, whereas MDA5 silencing significantly decreased IFN production (Broquet et al., 2011). However, we have previously demonstrated that in culture of intestinal epithelial cell line or organoids, infection of rotavirus increased IFN gene transcription, but not the production of IFN proteins (Hakim et al., 2018). Consistently, in the different models of this study, MDA5 did not induce the production of IFNs, but provoked a non-canonical IFN-like response. Similar actions have been previously observed for RIG-I and IFN regulatory factor 1 (IRF1) in inhibiting HEV replication in epithelial cell lines (Xu et al., 2016, 2017). Mechanistically, this remains an intriguing question that how these broad antiviral factors can trigger ISG transcription without the requirement of IFN production?

It has been previously reported that RIG-I overexpression triggers STAT1 expression and activation (Xu et al., 2017; Jiang et al., 2011). Similarly, we found that overexpression of MDA5 also activates STAT1 expression and phosphorylation at the 701 site. STAT1 phosphorylation is the hallmark of JAK-STAT activation cascade, the downstream cascade of IFN pathway. Interestingly, genetic silencing of STAT1 or pharmacological imbibition of JAK-STAT pathway only partially attenuated ISG induction and antiviral activity of MDA5, whereas completely blocked IFN-α-induced ISG transcription and antiviral activity. It has been reported that these non-classical antiviral mechanisms are also very important in antiviral defense. For instance, there are different forms of ISGF3 complexes, and some function independent of the JAK-STAT pathway. Unphosphorylated ISGF3 (u-ISGF3) complex has been demonstrated to drive antiviral ISG expression and protect from viral infections at homeostatic status (Wang et al., 2017; Sung et al., 2015). The STAT2-IRF9 complex can also regulate antiviral activity and ISG expression without the presence of STAT1 (Blaszczyk et al., 2015). Thus, our findings on MDA5 have added new knowledge to the non-classical IFN responses.

In summary, MDA5 potently inhibits the infection of enteric viruses, including HEV, HuNV and rotavirus. It provokes an antiviral IFN-like response without requirement of IFN production, but is partially dependent on the JAK-STAT cascade. These findings have revealed non-canonical actions of MDA5 on viral infections, and the detailed mechanisms deserve further investigation.

## Declaration of competing interest

The authors declare that no competing interests exist.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2020.104743.

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