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Lipopolysaccharide restricts murine norovirus infection in macrophages mainly through NF-kB and JAK-STAT signaling pathway

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ABSTRACT

The inflammasome machinery has recently been recognized as an emerging pillar of innate immunity. However, little is known regarding the interaction between the classical interferon (IFN) response and inflammasome activation in response to norovirus infection. We found that murine norovirus (MNV-1) infection induces the transcription of IL-1β, a hallmark of inflammasome activation, which is further increased by inhibition of IFN response, but fails to trigger the release of mature IL-1β. Interestingly, pharmacological inflammasome inhibitors do not affect viral replication, but slightly reverse the inflammasome activator lipopolysaccharide (LPS)-mediated inhibition of MNV replication. LPS efficiently stimulates the transcription of IFN-β through NF-κB, which requires the transcription factors IRF3 and IRF7. This activates downstream antiviral IFN-stimulated genes (ISGs) via the JAK-STAT pathway. Moreover, inhibition of NF-κB and JAK-STAT signaling partially reverse LPS-mediated anti-MNV activity, suggesting additional antiviral mechanisms activated by NF-κB. This study reveals additional insight in host defense against MNV infection.

1. Introduction

Norovirus is a major cause of epidemic nonbacterial gastroenteritis worldwide. Currently, no vaccination or specific antiviral treatment is available except for supportive care and supplementation of fluids, exerting a great global economic burden and significant impact on public health (Karst et al., 2014). The lack of a robust human norovirus (HuNV) cell culture system is a key bottleneck for norovirus research. Recent studies have achieved some success in culturing HuNV in intestinal enteroid or B cells with modest viral replication level (Ettayebi et al., 2016; Jones et al., 2015), and in zebrafish larvae (Van Dycke et al., 2019) and human induced pluripotent stem cell-derived intestinal epithelial cells (Sato et al., 2019), but these systems have not been widely used. A HuNV subgenomic replicon model in Huh7 cell line is widely used, for instance for antiviral drug evaluation (Chang et al., 2006). Murine norovirus (MNV), permissive for replication in both cell culture and small-animal models, shares similar structural, biochemical and genetic features with HuNV, and thus has become a useful model for studying norovirus biology and associated pathology (Wobus et al., 2004, 2006).

Innate immune response provides the first line of defense against viral infection through the recognition of pathogen associated molecular patterns (PAMPs) by its pattern recognition receptors (PRRs) including the recognized Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I like receptor (RLRs) and the cyclic GMP-AMP synthase (cGAS) (Wu and Chen, 2014). During viral replication, the accumulated nucleic acids within cells can be sensed by certain PRRs, and then rapidly activate the following intracellular transcription factors such as nuclear factor-κB (NF-κB) and IFN-regulatory factors (IRF3 and IRF7), leading to secretion of cytokines and chemokines including IFNs (Schneider et al., 2014). The released IFNs bind to their receptors on the cell surface, and in turn activate Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway and further induce transcription of hundreds of IFN-stimulated genes (ISGs). A subset of ISGs are ultimate antiviral effectors limiting viral replication and spread (Schoggins et al., 2011). More recently, the inflammasome signaling has also been reported to exert a central role in the regulation of gastrointestinal health and disease (Man, 2018). Inflammasomes are multiprotein complexes that induce downstream immune responses to specific pathogens and host cell damage (Chen and Ichinohe, 2015). Once activated, inflammasomes can induce the activation of caspases and release of mature interleukin-1β (IL-1β) and IL-18, and execute a form of inflammatory cell death known as pyroptosis (Chen and Ichinohe, 2015; Man, 2018).

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of gram-negative bacteria, acting as a potent

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inflammasome activator. Upon LPS recognition, TLR4 signaling can be activated by undergoing oligomerization and recruiting its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains, which further induces the proinflammatory cytokine expression and IFNs secretion through myeloid differentiation primary response gene 88 (MyD88)-dependent or MyD88-independent pathways (Lu et al., 2008). Moreover, LPS has been reported to associate with non-canonical inflammasome activation by binding with caspase 11 and then cleaving gasdermin D to induce pyroptotic cell death (Broz and Dixit, 2016).

Inflammasome activation is essential in controlling viral infection, such as rotavirus and influenza virus (Ichinohe et al., 2009; Zhu et al., 2017). Previous studies have reported that MNV-1 was rapidly cleared in wild-type mice (Karst et al., 2003), but persisted much longer in NLRP6-deficient mice (Wang et al., 2015), suggesting the potential involvement of inflammasome activation in norovirus infection. A recent study has revealed that MNV infection induced secretion of mature IL-1β by activating NLRP3 inflammasome in primary bone marrow-derived macrophages (BMDMs) that primed with TLR2 agonist, and in STAT1 deficient BMDMs that without TLR2 priming (Dubois et al., 2019). In this study, we investigated whether the IFN and inflammasome machineries interact and collectively respond to norovirus infection, and LPS-mediated anti-norovirus response.

2. Materials and methods

2.1. Reagents

Human IFN-α (Thermo Scientific, the Netherlands) and mouse IFN-γ (ab9922, Abcam) were dissolved in PBS. Lipopolysaccharide (LPS; L6529, Sigma) and adenosine 5-triphosphate disodium salt hydrate (ATP; A3377, Sigma) were dissolved in PBS. Stocks of JAK inhibitor 1 (Santa Cruz Biotech, CA), Bayer 11–7085 (Santa Cruz Biotech, CA), MCC950 (inh-mcc, Invivogen) and Ac-YVAD-cmK (inh-yvad, Invivogen) were dissolved in DMSO. Antibodies against phospho-STAT1 (Ser727) (#9177), STAT1 (#9172), MDA5 (D74E4, #5321) and IRF1 (D5E4, #8478), were purchased from Cell Signaling Technology. GBP2 antibody (11854-1-AP) was purchased from Proteintech. Rabbit polyclonal antisera to MNV NS1-2 (Baker, 2012) was kindly provided by Prof. Vernon K. Ward (School of Biomedical Sciences, University of Otago, New Zealand). β-actin antibody (#sc-47778) was purchased from Santa Cruz Biotechnology. IRDye® 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs (Li-Cor Bioscience, Lincoln, USA) were used as secondary antibodies, as appropriate.

2.2. Cell culture and viruses

RAW264.7 and J774A.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 μg/mL of streptomycin, and 100 IU/mL of penicillin. Human Huh7 hepatocellular carcinoma cells harboring a genotype 1 HuNV replicon (HG23) were kindly provided by Dr. Kyeong-Ok Chang (Kansas State University) (Chang et al., 2006). A neomycin resistant gene was engineered into ORF2, conferring HG23 resistance to neomycin. Gentamicin (G418; Gibco) was added to HG23 culture medium at 1.5 mg/mL for selection before experimentation. Mouse embryonic fibroblast cells (MEFs) (WT, NF-κB$^{-/-}$ and IRF3/7$^{-/-}$) were kindly provided by Dr. Sanna M. Mäkelä (National Institute for Health and Welfare Viral Infections Unit, Helsinki, Finland) with the permission of Dr. Michael J. Gale and Dr. A. Hoffmann (Mäkelä et al., 2015). Wild-type (WT) MEFs and MEFs from STAT1$^{-/-}$ mice were generously provided by Prof. Andrea Kröger (Helmholtz Centre for Infection Research) (Nandakumar et al., 2013).

MNV-1 (murine norovirus strain MNV-1.CW1) was produced by inoculating the virus (kindly provided by Prof. Herbert Virgin, Department of Pathology and Immunology, Washington University School of Medicine) into RAW264.7 cells (Wobus et al., 2004). The MNV-1 cultures were purified, aliquoted, and stored at −80 °C for all subsequent experiments. The MNV-1 stock was quantified three independent times by the 50% tissue culture infective dose (TCID50).

2.3. TCID50

MNV-1 was quantified by TCID50 assay (Hwang et al., 2014). Briefly, 10-fold dilutions of MNV-1 were inoculated into RAW264.7 cells grown in 96-well tissue culture plate at 1000 cells/well. The plate was incubated at 37 °C for another 5 days, followed by observing the cytopathic effect (CPE) of each well under a light scope. The TCID50 was calculated by using the Reed-Muench method.

2.4. MNV-1 infection in macrophages

To determine whether MNV-1 infection could activate the inflammasome, RAW264.7 (1×10^5 cells/well) and J774A.1 (1×10^5 cells/well) cells were plated at 12-well plate and were rested for 24 h at 37 °C. Cells were then either left uninfected or infected with MNV-1 at indicated multiplicity of infection (MOI) for 1 h at 37 °C. The MNV-1 inoculum was removed and the cells were washed three times with phosphate-buffered saline (PBS), and fresh medium was added back onto cells. LPS and ATP treated cells were set as positive control. Total RNA and cell supernatant samples were prepared at indicated time and stored at −80 °C before use.

For MNV-1 antiviral assay, RAW264.7 and J774A.1 cells were either left untreated or treated with LPS, IFN-γ or combination with indicated compounds for 6 h before inoculation with MNV-1 at 1 MOI. After 1 h inoculation, the cells were washed with PBS for three times to remove the residual viruses, and control medium or medium supplemented with LPS or mouse IFN-γ was added back onto cells. After 24 h of infection, total RNA, protein and supernatant samples were collected from the cells for further analysis.

2.5. HuNV antiviral assay

Twenty-four hours before experimentation, HG23 cells were cultured in the medium without gentamicin. HG23 cells were seeded into 48-well tissue culture plates (2.5×10^4 cells per well), treated with different concentrations of LPS or human IFN-α. After 48 h of treatment, total RNA was extracted from cells, and HuNV replication was determined by qRT-PCR analysis.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Supernatant was analyzed for cytokine IL-1β secretion by an ELISA kit (KMC0012; Invitrogen) according to manufacturer's instructions. The absorbance was measured at 450 nm in an automatic microplate reader. Results were calculated based on a standard curve.

2.7. Quantitative real-time polymerase chain reaction

Total RNA was isolated with a Macherey NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and quantified with a Nanodrop ND-1000 (Wilmington, DE, USA). During RNA isolation, DNase was added to remove genomic DNA according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using a cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan). The cDNA of all targeted genes transcript were quantified by SYBR-Green-based (Applied Biosystems) real-time PCR on the StepOnePlus™ System (Thermo Fisher Scientific Life Sciences) according to the manufacturer's instructions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and murine GAPDH genes were used as reference genes to normalize gene expression. The relative expression of targeted gene was calculated as $2^{-\Delta\Delta CT}$,

where $\Delta\Delta C_T = \Delta C_T$sample - ΔC_Tcontrol ($\Delta C_T = C_T$[targeted gene] - C_T[GAPDH]). All primer sequences are listed in Supplementary Table 1 and Table 2.

2.8. Western blotting

Cultured cells were lysed in Laemmli sample buffer containing 0.1 M DTT and heated 5 min at 95 °C, then loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, then proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 μM; Invitrogen) for 2 h with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 mL blocking buffer (Odyssey) and 2.5 mL PBS containing 0.05% Tween 20 for 1 h, followed by overnight incubation with primary antibodies (1:1000) at 4 °C. The membrane was washed 3 times and then incubated with IRDye-conjugated secondary antibody (1:5000) for 1 h. After washing 3 times, protein bands were detected with the Odyssey 3.0 Infrared Imaging System (Li-Cor Biosciences).

2.9. MTT assay

Cells were seeded into 96-well tissue culture plates and cell viability was assessed by adding 10 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, Zwijndrecht, the Netherlands). After 3 h, the medium was replaced with 100 μL of DMSO and was incubated at 37 °C for another 50 min. The absorbance at 490 nm was recorded on the microplate absorbance reader (Bio-Rad, CA, USA).

2.10. Statistical analysis

Data are presented as the mean ± SEM. Comparisons between groups were performed with Mann-Whitney test using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at a P value less than 0.05.

3. Results

3.1. MNV-1 infection did not induce the release of mature IL-1β, but increased its mRNA expression, which was further augmented by inhibition of IFN pathway in macrophages

To investigate whether MNV-1 can activate inflammasome, J774A.1 cells were used because of its permissiveness for MNV-1 infection and competence in inflammasome activation (Hirano et al., 2017; Wobus et al., 2004). We first determined the expression of IL-1β and IL-18 as markers of inflammasome activation in J774A.1 cells treated with LPS (the component in the outer membrane of gram-negative bacteria) and ATP, the potent activators for inflammasome activation. We found that under stimulation by LPS and ATP, the mRNA and protein levels of IL-1β were significantly increased (Fig. 1A–C), and IL-18 mRNA level was moderately elevated (Fig. 1A). Although the IL-1β mRNA level was modestly elevated in MNV-1 infected J774A.1 cells (Fig. 1B), no significant differences in IL-1β protein level were observed in the supernatant of these cells (Fig. 1C). We confirmed active viral infection by detecting the viral NS1/2 protein in J774A.1 cells (Fig. 1D). In another macrophage cell line RAW264.7 cells, the IL-1β and IL-18 mRNA levels were hardly changed upon MNV-1 infection (Supplementary Fig. 1). However, treatment with LPS and ATP also failed to induce the expression of these genes (Supplementary Figs. 1A and 1B), indicating possible defects of the inflammasome machinery in RAW264.7 cells, which is consistent with a previous study (He et al., 2015).

We next investigated whether MNV-1 infection has a role in LPS-induced IL-1β transcription. As expected, the IL-1β mRNA level was further increased in MNV-1 infected J774A.1 cells by LPS and ATP stimulation (Fig. 1E), and this effect was also observed in LPS-pretreated cells (Fig. 1F). A recent study reported that MNV infection stimulated IL-1β RNA transcription and protein release in STAT1 deficient BMDMs (Dubois et al., 2019). Thus, we blocked the JAK-STAT pathway by using JAK-STAT inhibitor (JAK inhibitor 1), and found that inhibition of JAK-STAT pathway attenuated MNV-1 induced ISG transcription (Fig. 1G), but increased MNV-1 RNA level (Fig. 1H) and viral infection-induced IL-1β mRNA transcription (Fig. 1I), which is in accordance with the recent study (Dubois et al., 2019).

3.2. LPS potently inhibits MNV-1 replication largely independent of inflammasome

Norovirus cohabits with bacteria in the intestine. Co-infection of MNV and bacteria in macrophages has been reported to reduce the viral load (Agnihothram et al., 2015; Li et al., 2016). Thus, we further investigated the direct effects of bacteria-derived LPS on MNV-1 replication, and found that treatment with LPS potently inhibits viral replication in J774A.1 cells (Fig. 2A and B). However, this inhibitory effect on MNV-1 is only partially reversed by pharmacological inflammasome inhibitors MCC950 and YVAD-cmk, which did not affect viral replication (Fig. 2A and B) but decreased the release of mature IL-1β triggered by LPS and ATP stimulation in J774A.1 cells (Fig. 2C).

We next evaluated the anti-MNV effect of LPS in RAW264.7 cells, which are incompetent of inflammasome activation (He et al., 2015). IFN-γ was used as a control. Pre-treatment with LPS potently inhibited viral RNA and NS1-2 protein levels in a dose-dependently manner (Fig. 2D). Similar effects were observed when cells were first infected with MNV-1 then subjected to treatment with LPS (Fig. 2E). Moreover, the viral titers were also decreased by LPS and IFN-γ pre-treatment (Fig. 2F).

These results urged us to investigate the antiviral effect of LPS on HuNV replication by using the Huh7-based HuNV replicon cells (HG23). After two days of treatment, LPS failed to but human IFN-α effectively restricted HuNV replication without triggering major cytotoxicities (Fig. 3A). It is widely recognized that IFNs exert antiviral effects via induction of the transcription of ISGs through JAK-STAT signaling pathway. We further revealed that expression of type I IFNs (IFN-α and IFN-β) were not significantly induced by both LPS and IFN-α (Fig. 3B and C). However, IFN-α but not LPS triggered the expression and phosphorylation of STAT1 (Fig. 3D), and induction of ISGs including ISG15, IFIT1 and MX1 in HG23 cells (Fig. 3E). This is consistent with previous findings that hepatic epithelia cells do not respond to LPS stimulation (MacParland et al., 2016). This previous study also showed that LPS suppresses IFN response in hepatocytes by upregulating ubiquitin-like protease 18 (USP18) expression, a negative regulator of type I IFN signaling (MacParland et al., 2016). However, LPS stimulation did not affect USP18 gene transcription in our study (Fig. 3F).

These results have demonstrated that LPS potently inhibits MNV replication in macrophages, but has no significant effect on HuNV replication in HG23 cells. This is probably related to the intrinsic property that epithelial HG23 cells are nonresponsive to LPS stimulation.

3.3. LPS stimulates the transcription of IFN-β and a wide range of ISGs

Given the dispensability of inflammasome in anti-MNV activity of LPS, we explored other possible mechanisms. It is generally recognized that LPS stimulation of macrophages induces production of many cytokines and inflammatory mediators including IRF-1, IL-6 and IFNs (Beutler and Rietschel, 2003; Guha and Mackman, 2001). We found that LPS stimulated the expression of the viral RNA sensor MDA5 in RAW264.7 and J774A.1 cells (Fig. 4A and B), and the increased transcription of IFN-β in both cell lines (Fig. 4C and D), respectively. ISGs are the ultimate antiviral effectors induced by IFNs through the JAK-STAT pathway. We found the activation of JAK-STAT pathway by

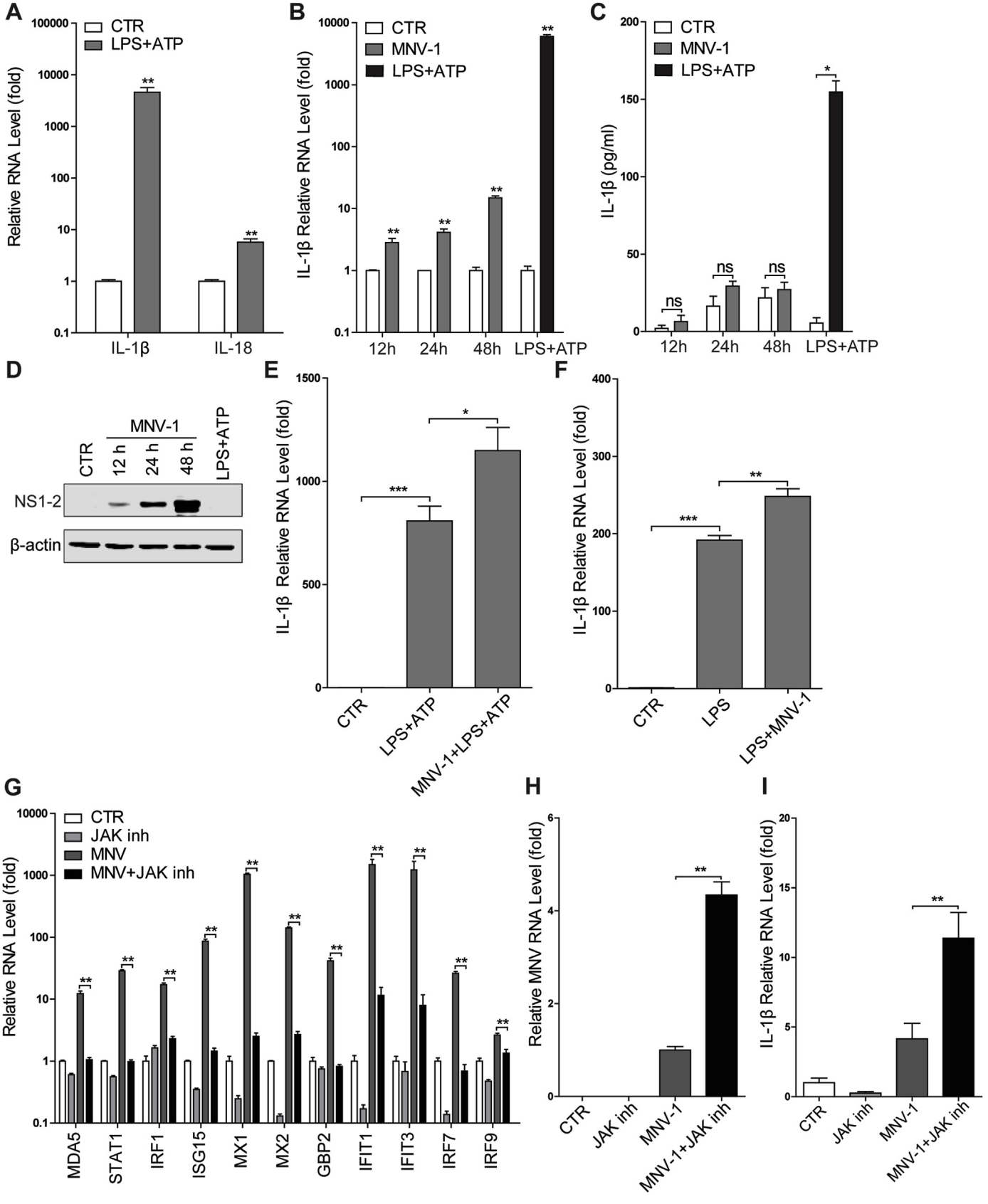

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Fig. 1. MNV-1 infection did not induce the release of mature IL-1β, but increased its mRNA level in macrophages. J774A.1 cells were untreated or treated with LPS (400 ng/μl) for 4 h and then ATP (5 mM) for 40 min, or left uninfected or infected with MNV-1 (MOI 1) for 24 h, and total RNA and cell culture supernatants were collected. mRNA levels of IL-1β (A and B) (n = 6) and IL-18 (A) (n = 6), and protein level of IL-1β (C) (n = 4) were analyzed by qRT-PCR and ELISA, respectively. Western blotting (D) was used to analyze the viral infection in J774A.1 cells at indicated times. (E) J774A.1 cells were uninfected or infected with MNV-1 (MOI 1) for 20 h, then treated with LPS (400 ng/μl) for 4 h and ATP (5 mM) for 40 min mRNA level of IL-1β and cytotoxicity were analyzed by qRT-PCR (n = 8). (F) J774A.1 cells were untreated or treated with LPS (400 ng/μl) for 4 h then uninfected or infected with MNV-1 (MOI 1) for 24 h mRNA level of IL-1β and cytotoxicity were analyzed by qRT-PCR (n = 8). J774A.1 cells were untreated or treated with JAK inhibitor 1 (10 μM) for 6 h then uninfected or infected with MNV-1 (MOI 1) for 24 h mRNA levels of indicated genes (G) (n = 6), MNV-1 RNA (H) (n = 6) and IL-1β mRNA (I) (n = 6) were analyzed by qRT-PCR, respectively. Data were normalized to the untreated control (CTR, set as 1). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. β-actin was used as a loading control.

detecting the increased expression of STAT1 at both the protein (Fig. 4A and B) and transcriptional levels (Fig. 4E and F), respectively. Moreover, the transcription of a wide range of ISGs was also stimulated by LPS (Fig. 4E and F), and IFN-γ treatment also induced the transcription of many ISGs in both cell lines (Supplementary Fig. 2). The induction of several important genes were further confirmed by immunoblotting (Fig. 4A and B).

3.4. NF-κB signaling is important for LPS-induced IFN-β and ISG transcription

It has been reported that recognition of LPS by TLR4 activates downstream transcription factors NF-κB, leading to the transcription of IFNs and ISGs (Moynagh, 2005). Thus, we further investigated whether NF-κB signaling is involved in LPS-induced IFN-β and ISG transcription in macrophages. Treatment with Bay 11–7085, a pharmacological inhibitor of NF-κB activation, largely inhibits LPS-triggered transcription of IFN-β (Fig. 5A and C) and most of the ISGs (Fig. 5B and D) in both macrophage cell lines. Moreover, this inhibitor also attenuated LPS-induced expression and phosphorylation of STAT1, as well as ISG expression such as IRF1 and GBP2 (Fig. 5E and F). Similarly, some ISGs induced by IFN-γ were also affected by Bay 11–7085 treatment in both cell lines (Supplementary Fig. 3).

To further determine the essential role of NF-κB pathway in LPS-induced ISG expression, wild-type (WT) and NF-κB deficient (NF-κB$^{-/-}$) MEFs were used. In NF-κB$^{-/-}$ but not the WT cells, LPS failed to induce the transcription of IFN-β (Fig. 5G). A similar ISG induction pattern was also observed in NF-κB$^{-/-}$ cells. All of the genes detected such as IRF1, ISG15, MX1 and GBP2 were significantly induced by LPS in WT cells but not in NF-κB$^{-/-}$ cells (Fig. 5H). Moreover, transcription of some ISGs induced by IFN-γ were largely decreased in NF-κB$^{-/-}$ cells (Supplementary Fig. 4A). Taken together, these findings revealed that NF-κB pathway is important for LPS-induced IFN-β and ISG transcription.

3.5. IRF3 and IRF7 are important for LPS-induced IFN-β and ISG transcription

LPS as the ligand activates TLR4, which in turn phosphorylates downstream IRF3 and IRF7, leading to their nuclear translocation and induction of type I IFN genes and co-stimulatory molecules (Moynagh, 2005). Moreover, IRF3 and IRF7 have been reported to play an important role in controlling MNV-1 replication (Thackray et al., 2012). Thus, by using WT and IRF3/7 deficient (IRF3/7$^{-/-}$) MEFs, we found that IFN-β was not induced by LPS in IRF3/7$^{-/-}$ cells (Fig. 6A). Moreover, the detected genes induced by LPS in WT cells were reduced in IRF3/7$^{-/-}$ cells (Fig. 6B). Compared to the WT cells, transcription of ISG15, ISG20, MX1 and IFIT1 appeared to be unchanged, while transcription of other ISGs such as IRF1, MX2, GBP2 and GBP5 induced by IFN-γ was decreased in IRF3/7$^{-/-}$ cells (Supplementary Fig. 4B). These results suggested that IRF3 and IRF7 are also important for LPS-induced IFN-β and ISG transcription.

3.6. LPS induces ISG transcription largely through activation of JAK-STAT pathway

The JAK-STAT signaling pathway has been shown to be essential for LPS-mediated gene expression in macrophages (Kovarik et al., 1998; Ohmori and Hamilton, 2001). Consistent with these results, we also observed that LPS induced the expression and phosphorylation of STAT1 in both RAW264.7 and J774A.1 cells, respectively (Fig. 7C and D). To determine whether ISG induction by LPS is through the activation of STAT1, we used JAK-STAT inhibitor to pharmacologically block the JAK-STAT pathway. We found that JAK inhibitor 1 partially blocked LPS induced transcription of IFN-β in both RAW264.7 and J774A.1 cells (Fig. 7A and B), while both the expression and phosphorylation of STAT1 triggered by LPS and IFN-γ were largely attenuated by this inhibitor (Fig. 7C and D). Next, we tested the mRNA level of some ISGs in LPS-stimulated macrophages treated with JAK inhibitor 1, and found that most of the LPS inducible genes were affected by JAK inhibitor 1 (Fig. 7E and F), without triggering major cytotoxicities (Supplementary Fig. 5D). In addition, the mRNA levels of these genes induced by IFN-γ were largely diminished by this inhibitor (Supplementary Figs. 5A and 5B).

To further confirm the ISG induction ability of LPS is partially dependent on the JAK-STAT pathway, we performed experiments with MEFs from WT and STAT1$^{-/-}$ mice to examine the effect of STAT1 on ISG expression (Fig. 7G and H). We found that the abundance of detected ISGs triggered by LPS were also largely decreased in STAT1$^{-/-}$ cells compared to those in WT cells (Fig. 7I). As a control, the transcription of IFN-γ induced ISGs was significantly blocked in STAT1$^{-/-}$ cells (Supplementary Fig. 5C). Together, these results demonstrated that LPS activates ISG transcription largely through the activation of the JAK-STAT pathway.

3.7. The NF-κB cascade contributes to LPS-mediated inhibition of MNV-1 replication partially through the JAK-STAT pathway

In respect to the anti-MNV effects of LPS, we found that Bay 11–7085, the NF-κB signaling inhibitor, partially reversed LPS and IFN-γ-mediated antiviral response against MNV-1 infection in RAW264.7 cells (Fig. 8A and E). Similar results were also observed in J774A.1 cells (Fig. 8B). Interestingly, the inhibitor alone enhanced viral replication (Fig. 8A, B and 8E), suggesting a basal defense mechanism of NF-κB signaling against MNV-1 infection. Moreover, we also determined the role of JAK-STAT pathway in LPS-mediated antiviral activity and found that the anti-MNV activity of LPS was only moderately reversed, whereas the anti-MNV effect of IFN-γ was largely abolished by JAK inhibitor 1 (Fig. 8C–E). Together, these results have demonstrated that NF-κB cascade contributes to LPS-mediated control of MNV-1 replication partially through the JAK-STAT pathway.

4. Discussion

Innate immune response provides the first line of defense against viral infection. Studies have revealed that compared to wild-type mice, mice lacking either type I and type II IFN receptors or STAT1 have higher titers and succumb to lethal MNV infection (Karst et al., 2003; Thackray et al., 2012), indicating the importance of IFNs in controlling

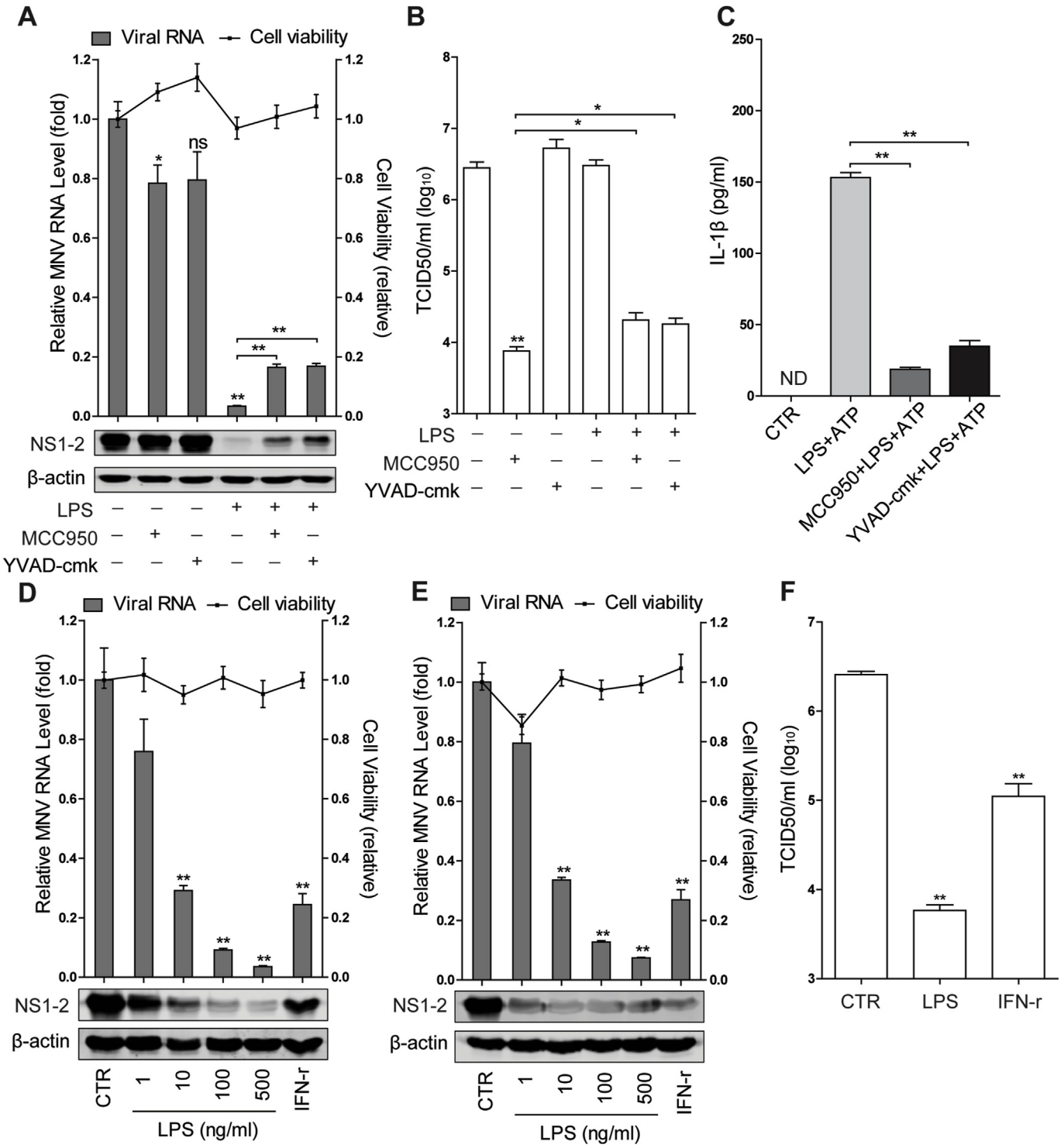

Fig. 2. LPS potently inhibits MNV-1 replication largely independent of inflammasome. J774A.1 cells were treated with LPS (400 ng/μl), MCC950 (5 μM), YVAD-cmk (5 μM), or combination for 6 h, then infected with MNV-1 (MOI 1) for 24 h. (A) The viral RNA, NS1/2 protein and cytotoxicity were analyzed by qRT-PCR (n = 6), western bloting, and MTT (n = 16) assays, respectively. (B) The viral titers were also analyzed by TCID50 assay (n = 4–6). (C) J774A.1 cells were untreated or treated with LPS (400 ng/μl), MCC950 (5 μM), YVAD-cmk (5 μM), or combination for 4 h and then ATP (5 mM) for 40 min. The cell culture supernatants were collected and used for analysis of IL-1β by ELISA assay (n = 6). (D) RAW264.7 cells were untreated or treated with different concentrations of LPS as indicated, or IFN-γ (100 U/ml) for 6 h, then infected with MNV-1 (MOI 1) for 24 h. The viral RNA, NS1/2 protein and cytotoxicity were analyzed by qRT-PCR (n = 6), western blotting and MTT (n = 8) assay, respectively. (E) RAW264.7 cells were infected with MNV-1 (MOI 1) for 1 h, then added fresh medium or medium supplemented with LPS at indicated concentrations or IFN-γ (100 U/ml) for 24 h. The viral RNA, NS1/2 protein and cytotoxicity were analyzed by qRT-PCR (n = 3 independent experiments with 2–3 replicates each), western blotting and MTT (n = 8) assays, respectively. (F) RAW264.7 cells were untreated or treated with different concentrations of LPS as indicated, or IFN-γ (100 U/ml) for 6 h, then infected with MNV-1 (MOI 1) for 24 h. The viral titers were analyzed by TCID50 assay (n = 6). Data were normalized to the untreated control (CTR, set as 1). *P < 0.05; **P < 0.01; ns, not significant. ND, not determined. β-actin was used as a loading control.

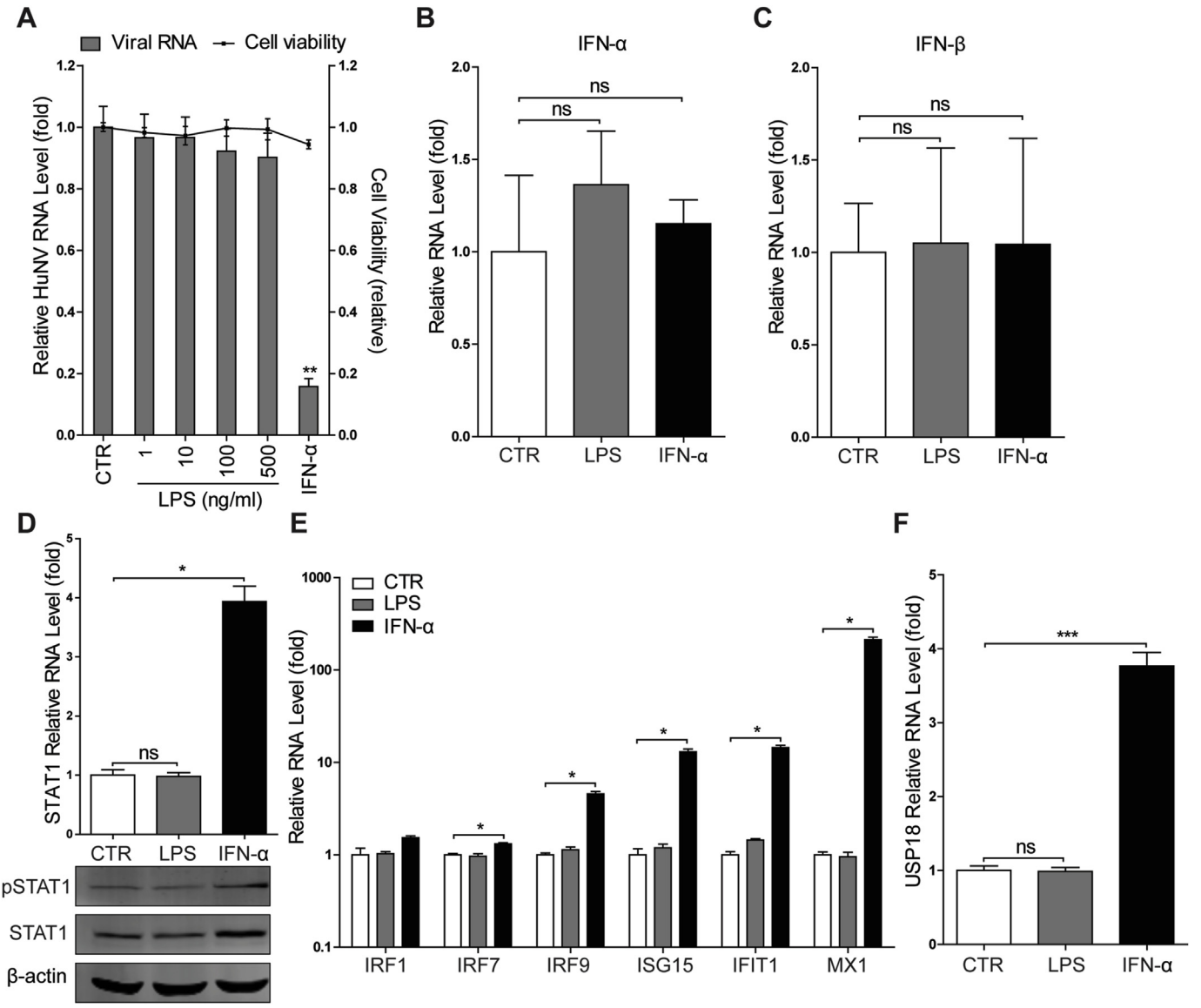

Fig. 3. HuNV replication is not affected by LPS in the replicon model. (A) HG23 cells were untreated or treated with different concentrations of LPS as indicated, or IFN-α (200 IU/ml). After 2 days of treatment the antiviral activities and cytotoxicity were measured by qRT-PCR (n = 6) and MTT assay (n = 16), respectively. qRT-PCR analysis (n = 4) of mRNA levels of IFN-α (B), IFN-β (C), STAT1 (D upper panel), ISGs (E), USP18 (F) and western blotting analysis of STAT1 expression and phosphorylation (D lower panel) in HG23 cells untreated or treated with LPS (100 ng/ml) or IFN-α (200 IU/ml). Data were normalized to the untreated control (CTR, set as 1). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. β-actin was used as a loading control.

norovirus infection. Besides IFNs, inflammasome signaling also has a central role in innate immune response against viral infections (Zhu et al., 2017). Previous evidence indicated the potential role of inflammasome in norovirus replication (Wang et al., 2015). However, in this study, MNV-1 infection failed to adequately activate inflammasome activation in macrophages, although moderate activation of IL-1β transcription was observed in J774A.1 cells. Similar results have been reported that MNV failed to secrete IL-1β in WT BMDMs, but presented caspase-1 processing ability (Dubois et al., 2019). In inflammasome-incompetent RAW264.7 cells, which lack the expression of ASC, one of the components for inflammasome complex formation (He et al., 2015), MNV-1 infection appeared not to affect IL-1β transcription. A recent study reported that MNV infection induced mature IL-1β secretion in STAT1 deficient BMDMs (Dubois et al., 2019). Compared to MNV-1 infection alone, we also observed the increased IL-1β transcription level induced by MNV-1 with JAK-STAT pathway inhibition. Studies have reported the crosstalk between IFN response and inflammasome activation (Kopitar-Jerala, 2017). Here we revealed that inhibition of JAK-STAT pathway reduced MNV-1 infection triggered IFN response, but increased MNV-induced IL-1β transcription. Moreover, decreased IFN response by inhibition of JAK-STAT pathway resulted in increased viral replication, which might further contribute to the upregulation of IL-1β transcription. Thus, whether there is a potential crosstalk between IFN response and inflammasome activation in response to MNV infection needs to be further studied.

Norovirus and bacteria are cohabitants of the intestine. Gram-negative bacteria produce a large amount of LPS, which are naturally potent activators of inflammasome. It has been reported that hepatitis B virus (HBV) infection inhibits LPS-induced inflammasome activation, although HBV alone does not activate inflammasome (Yu et al., 2017). Moreover, studies have revealed that MNV infection can induced mature IL-1β release in primed BMDMs with TLR2 agonist (Pam3CSK4) and TLR4 agonist (LPS) (Dubois et al., 2019), and here we found that MNV-1 augmented LPS-induced IL-1β transcription. Interestingly, we observed that LPS inhibited MNV-1 replication, but this inhibitory effect was only slightly reversed by inflammasome inhibitors, suggesting

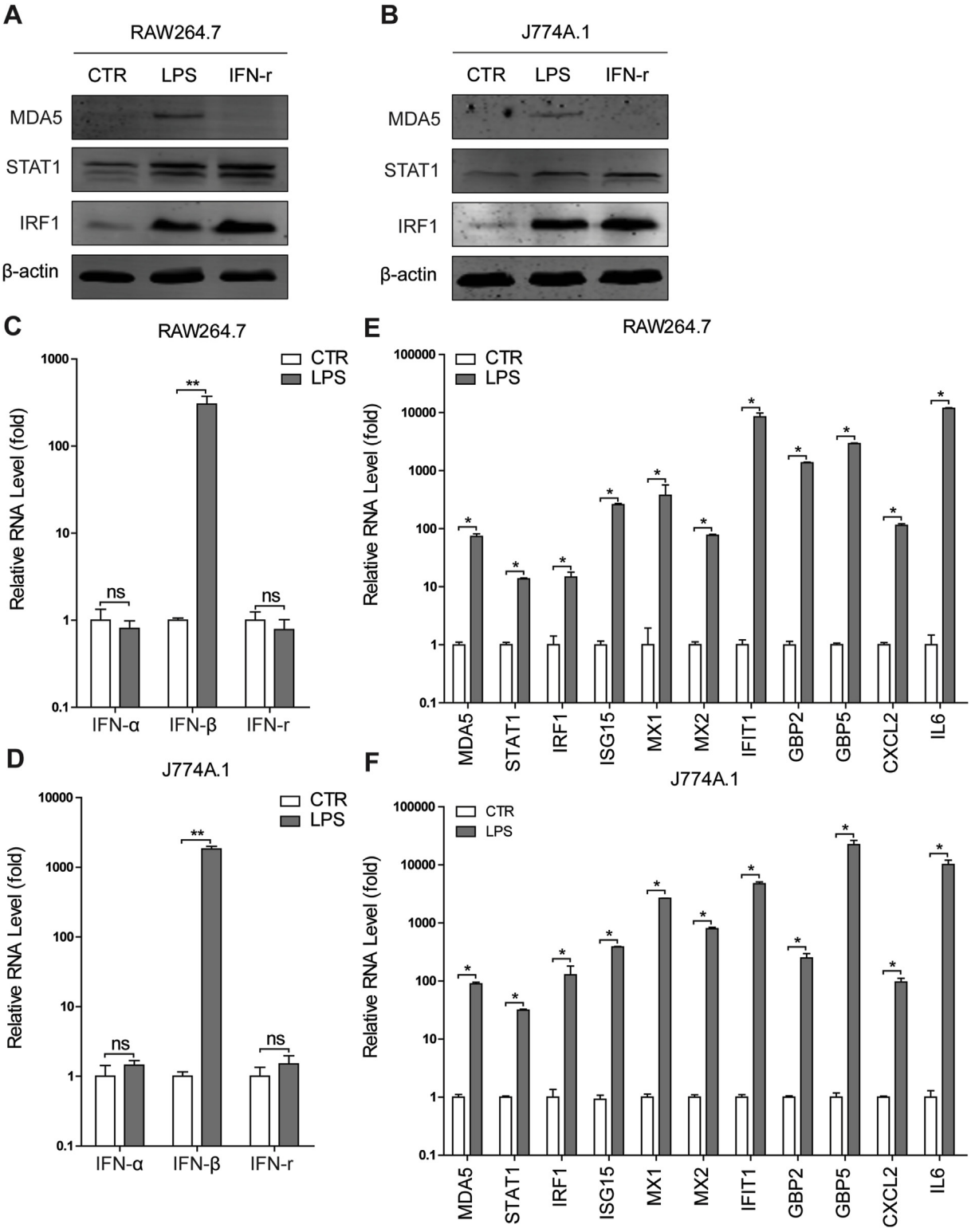

Fig. 4. LPS stimulates the transcription of a wide range of ISGs. Western blotting analysis of ISG protein levels in RAW264.7 cells (A) and J774A.1 cells (B) untreated or treated with LPS (100 ng/ml) or IFN-γ (100 U/ml) for 6 h qRT-PCR analysis of mRNA level of IFNs (C and D) (n = 6) and ISGs (E and F) (n = 4) in RAW264.7 cells and J774A.1 cells that untreated or treated with LPS (100 ng/ml) for 6 h, respectively. Data were normalized to the untreated control (CTR, set as 1). *P < 0.05; **P < 0.01; ns, not significant. β-actin was used as a loading control.

additional mechanisms contributing to the anti-MNV activity of LPS. These inhibitors might have a negative role on LPS-triggered expression of inflammatory factors, which could aid to LPS-mediated antiviral response against MNV infection to some extent. However, we do not exclude that there could be more active interactions of norovirus with inflammasome in other cell types, which deserve to be further investigated. Moreover, blocking NLRP3 inflammasome activation has been reported to reduce MNV-induced immunopathology in STAT1-deficient mice (Dubois et al., 2019). In addition, we found that inflammasome inhibitors alone appear not to affect the viral replication,

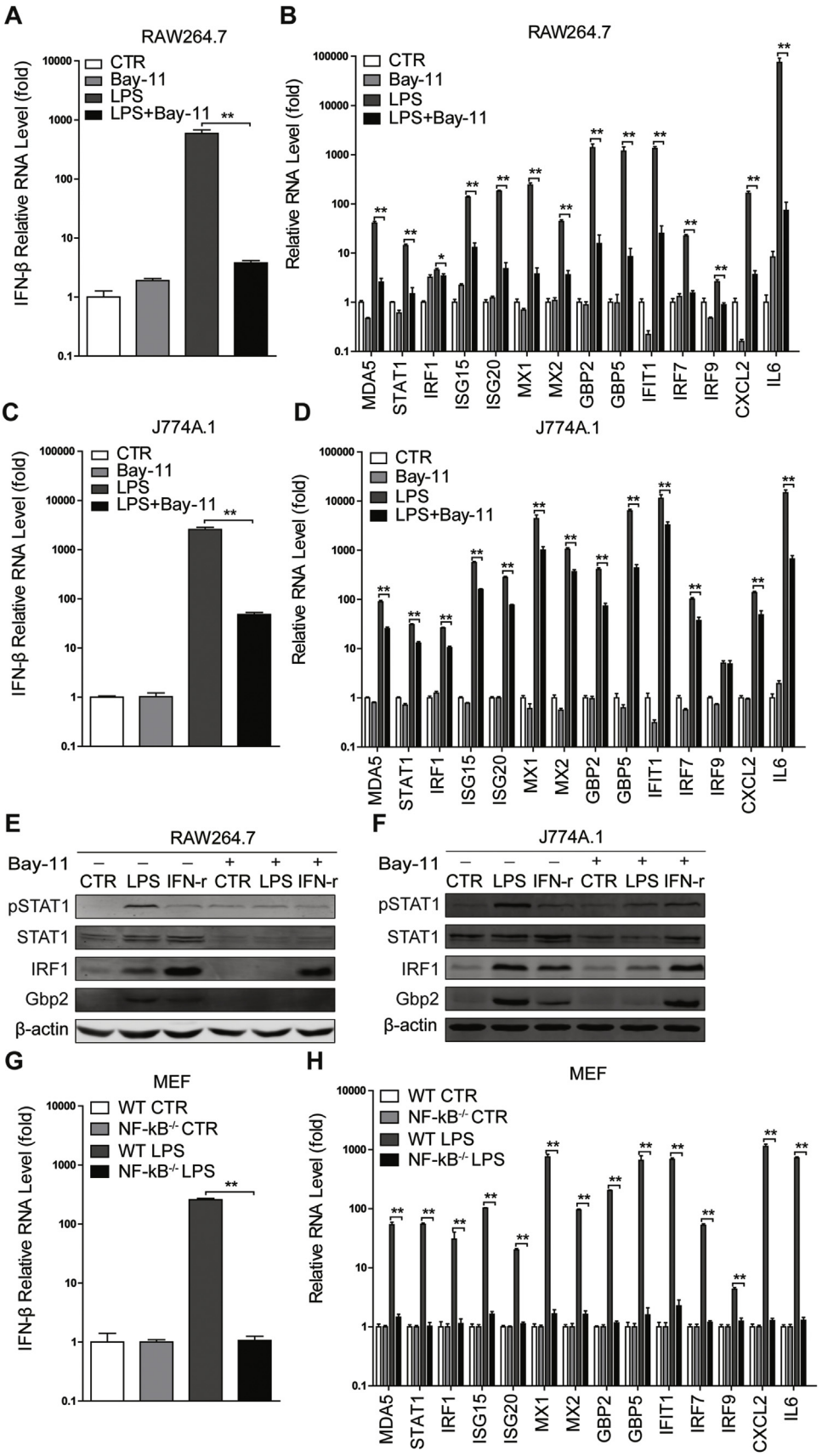

Fig. 5. NF-κB signaling is important for LPS-induced IFN-β and ISG transcription. Cells were untreated or treated with LPS (100 ng/ml), Bay 11–7085 (5 μM) or combination for 6 h qRT-PCR analysis of IFN-β (A) and ISG (B) mRNA levels in RAW264.7 cells (n = 6). qRT-PCR analysis of IFN-β (C) and ISG (D) mRNA levels in J774A.1 cells (n = 6). Western blotting analysis of STAT1 expression and phosphorylation, and expression of IRF1 and GBP2 in RAW264.7 cells (E) and J774A.1 cells (F) untreated or treated with LPS (100 ng/ml), IFN-γ (100 U/ml), Bay 11–7085 (5 μM) or combination for 6 h qRT-PCR analysis of mRNA levels of IFN-β (G) (n = 6) and ISGs (H) (n = 6) in WT and NF-κB−/− MEFs untreated or treated with LPS (100 ng/ml) for 6 h. Data in (A, B, C, D and G) were normalized to the untreated control (CTR, set as 1). Data in (H) were normalized to untreated WT and NF-κB−/− MEFs, respectively (both set as 1). *P < 0.05; **P < 0.01. β-actin was used as a loading control.

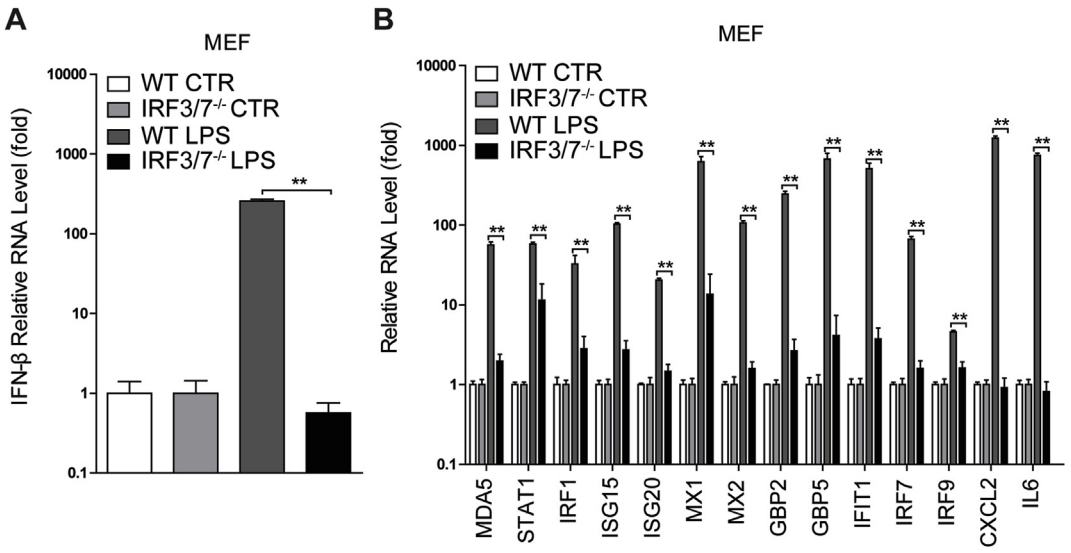

Fig. 6. IRF3 and IRF7 are important for LPS-induced IFN-β and ISG transcription. qRT-PCR analysis of mRNA levels of IFN-β (A) (n = 6) and ISGs (B) (n = 6) in WT and IRF3/7−/− MEFs untreated or treated with LPS (100 ng/ml) for 6 h. Data in (A) were normalized to the untreated control (CTR, set as 1). Data in (B) were normalized to untreated WT and IRF3/7−/− MEFs, respectively (both set as 1). **P < 0.01.

and similar results were also demonstrated that MNV replication rates were not affected in WT or NLRP3$^{-/-}$ BMDMs (Dubois et al., 2019).

As a type of PRRs, TLRs are responsible for the recognition of infectious agents leading to activation of innate immune response (Hertzog et al., 2003). It has been reported that TLR7 activation by its agonists displays potent antiviral effects against norovirus infection by stimulating innate immune response genes (Tuipulotu et al., 2018). As a ligand of TLR4, LPS can activate transcription factors NF-κB and IRFs, leading to the activation of IFN signaling (Moynagh, 2005). IFNs are central to the innate control of MNV infection (Changotra et al., 2009; Hwang et al., 2012; Nice et al., 2015). Here we found that LPS potently induces IFN-β transcription, and pharmacological inhibition of NF-κB signaling significantly blunts LPS-triggered IFN-β transcription, which is further confirmed in NF-κB deficient cells. Moreover, transcription factors IRF3 and IRF7 can regulate IFN-mediated inhibition of MNV-1 replication (Thackray et al., 2012), and we found in this study that IFN-β transcription induced by LPS is largely attenuated in IRF3 and IRF7 deficient cells.

ISGs are regarded as the ultimate antiviral effectors, and several ISGs such as IRF1, ISG15 and GBP2 have been reported to mediate the anti-norovirus effect of IFN (Biering et al., 2017; Maloney et al., 2012; Orchard et al., 2019; Rodriguez et al., 2014). In this study, we found that LPS also potently triggers ISG expression via the transcription factors including NF-κB, IRF3 and IRF7. Moreover, blocking NF-κB signaling attenuates LPS and IFN-γ mediated control of MNV-1 replication. JAK-STAT pathway is essential for IFN-induced ISG expression (Ohmori and Hamilton, 2001; Shuai and Liu, 2003) and host innate immunity against MNV infection (Karst et al., 2003). We revealed that induction of ISGs triggered by LPS or IFN-γ are dramatically decreased by inhibition of the JAK-STAT pathway, which also largely abolishes IFN-γ mediated inhibition for MNV-1 replication. However, blocking this pathway partially reverses LPS-mediated anti-MNV ability. This indicates that LPS-triggered ISGs are partially responsible for its antiviral response against MNV-1 infection in macrophages, and other unknown antiviral mechanisms mediated by LPS may be involved. Furthermore, the non-canonical mechanisms regulating ISG transcription have been reported (Wang et al., 2017). Induction of ISGs via NF-kB cascade might do not require JAK-STAT signaling, and this may account for the partial restoration of MNV replication by the inhibitors.

Accumulating evidences have demonstrated the interaction between gut microbiota and intestinal viruses including norovirus (Baldridge et al., 2015; Jones et al., 2014; Miura et al., 2013). Several mechanisms have been reported on how intestinal viruses interact with bacteria. On one hand, gut microbiota has been revealed to facilitate MNV replication through an antagonistic mechanism to IFN-lambda in the intestine (Baldridge et al., 2015), and prompt HuNV infection in intestinal B cells (Jones et al., 2014). Bacterial component of the enteric microbiome plays an essential role in the establishment of viral persistent infection. For instance, histo-blood group antigen (HBGA)-like substances improve virion stability and protect HuNV from acute heat stress (Li et al., 2015), and infection of B cells (Jones et al., 2014). It has been also reported that LPS binding promotes viral infectivity by enhancing virion stability and cell attachment (Kuss et al., 2011; Robinson et al., 2014), but fails to enhance HuNV infection of B cells *in vitro* (Jones et al., 2014), and inhibits MNV-1 replication in macrophages in this study. On the other hand, previous studies have presented the antagonistic mechanisms that *Enterobacter cloacae*, a member of enteric commensal bacteria specifically binds HuNV, and inhibits virus infectivity in gnotobiotic pigs by enhancing the gut immunity (Lei et al., 2016). In addition, the microbiota-host interaction can also modulate the production of immune system molecules, resulting in anti-MNV effects. Thus, the interactions between intestinal bacteria and viruses collectively modulate the infection outcomes.

In summary, our study demonstrates that MNV-1 moderately triggers IL-1β transcription, and is augmented by inhibition of IFN pathway in macrophages. Importantly, LPS potently inhibits MNV-1 replication, might through inflammasome to some extent but mainly via the NF-κB cascade. Activation of NF-κB by LPS drives the expression of IFN-β through IRF3 and IRF7. This activates the downstream antiviral ISGs via the JAK-STAT pathway. Moreover, inhibition of NF-κB and JAK-STAT signaling partially reversed the anti-MNV activity of LPS, suggesting additional antiviral mechanisms activated by NF-κB, which need to be further studied. Our study has revealed new insight in virus-cell interactions, and may provide rationale for new therapeutic development against norovirus infection.

CRediT authorship contribution statement

Peifa Yu: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Yang Li:** Methodology, Data curation, Writing - review & editing. **Yining Wang:** Methodology, Data curation, Writing - review & editing. **Maikel P. Peppelenbosch:**

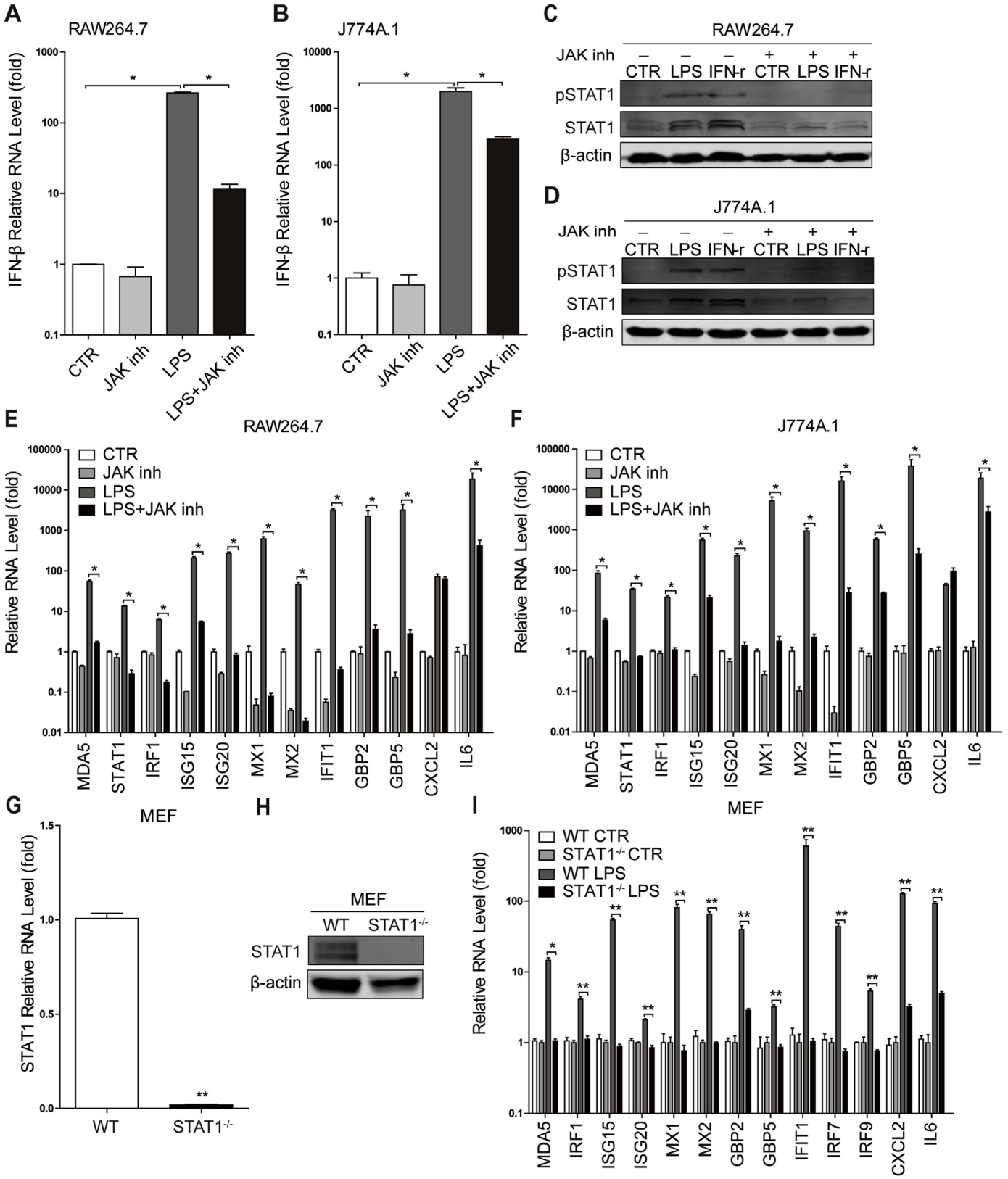

Fig. 7. LPS induces ISG transcription largely through activation of JAK-STAT pathway. qRT-PCR analysis of IFN-β mRNA levels in RAW264.7 cells (A) and J774A.1 cells (B) untreated or treated with LPS (100 ng/ml) or JAK inhibitor 1 (10 μM) for 6 h (n = 4). Western blotting analysis of STAT1 expression and phosphorylation in RAW264.7 cells (C) and J774A.1 cells (D) untreated or treated with LPS (100 ng/ml), IFN-γ (100 U/ml), JAK inhibitor 1 (10 μM) or combination for 6 h qRT-PCR analysis of ISG mRNA levels in RAW264.7 cells (E) and J774A.1 cells (F) untreated or treated with LPS (100 ng/ml), IFN-γ (100 U/ml), JAK inhibitor 1 (10 μM) or combination for 6 h (n = 4). WT and STAT1$^{-/-}$ MEFs were analyzed to determine the relative abundance of STAT1 mRNA level (G) by qRT-PCR (n = 6) and protein level (H) by western blotting. (I) qRT-PCR analysis of ISG mRNA levels in WT and STAT1$^{-/-}$ MEFs untreated or treated with LPS (100 ng/ml) for 6 h (n = 6). Data in (A, B, E, F and G) were normalized to untreated control (CTR, set as 1). Data in (I) were normalized to untreated WT and STAT1$^{-/-}$ MEFs, respectively (both set as 1). *P < 0.05; **P < 0.01. β-actin was used as a loading control.

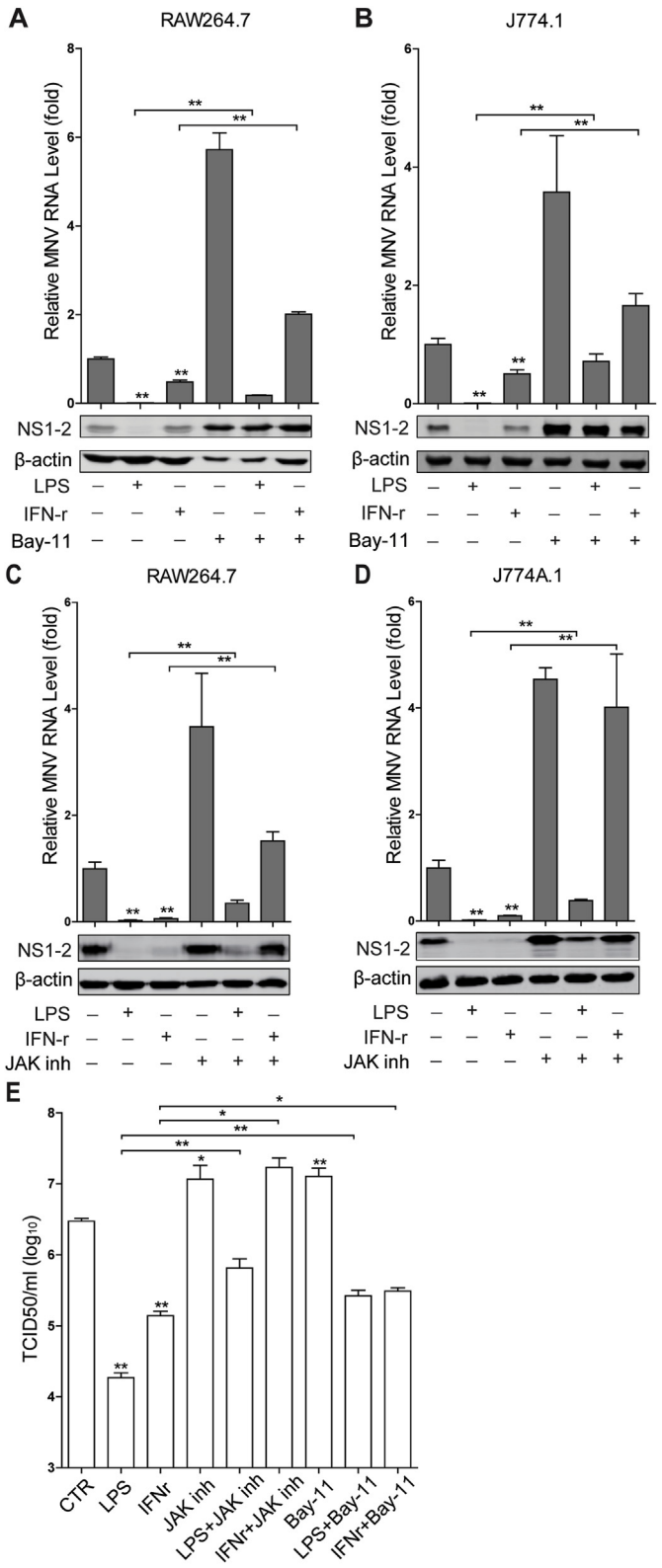

Fig. 8. The NF-κB cascade contributes to LPS-mediated inhibition of MNV-1 replication partially through the JAK-STAT pathway. qRT-PCR (n = 6) and western blotting analysis of MNV-1 viral RNA and protein levels in RAW264.7 cells (A) and J774A.1 cells (B) untreated or treated with LPS (100 ng/ml), IFN-γ (100 U/ml), Bay 11–7085 (5 μM) or combination for 6 h, then infected with MNV-1 (MOI 1) for 24 h qRT-PCR (n = 6) and western blotting analysis of MNV-1 viral RNA and protein levels in RAW264.7 cells (C) and J774A.1 cells (D) untreated or treated with LPS (100 ng/ml), IFN-γ (100 U/ml), JAK inhibitor 1 (10 μM) or combination for 6 h, then infected with MNV-1 (MOI 1) for 24 h. (E) The viral titers were also analyzed by TCID50 assay (n = 4–9). Data were normalized to the untreated control (CTR, set as 1). *P < 0.05; **P < 0.01. β-actin was used as a loading control.

Conceptualization, Supervision, Writing - review & editing. **Qiuwei Pan:** Conceptualization, Methodology, Resources, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.04.010.

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