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Middle East respiratory syndrome coronavirus-encoded ORF8b strongly antagonizes IFN-β promoter activation: its implication for vaccine design

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Middle East respiratory syndrome coronavirus (MERS-CoV) is a causative agent of severe-to-fatal pneumonia especially in patients with pre-existing conditions, such as smoking and chronic obstructive pulmonary disease (COPD). MERS-CoV transmission continues to be reported in the Saudi Arabian Peninsula since its discovery in 2012. However, it has rarely been epidemic outside the area except one large outbreak in South Korea in May 2015. The genome of the epidemic MERS-CoV isolated from a Korean patient revealed its homology to previously reported strains. MERS-CoV encodes 5 accessory proteins and generally, they do not participate in the genome transcription and replication but rather are involved in viral evasion of the host innate immune responses. Here we report that ORF8b, an accessory protein of MERS-CoV, strongly inhibits both MDA5- and RIG-I-mediated activation of interferon beta promoter activity while downstream signaling molecules were left largely unaffected. Of note, MDA5 protein levels were significantly down-regulated by ORF8b and co-expression of ORF4a and ORF4b. These novel findings will facilitate elucidation of mechanisms of virus-encoded evasion strategies, thus helping design rationale antiviral countermeasures against deadly MERS-CoV infection.

Keywords: accessory protein, interferon beta, MERS-CoV, ORF8b

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV), along with severe acute respiratory syndrome coronavirus (SARS-CoV), belongs to the genus *Betacoronavirus* in the family *Coronaviridae* of the order *Nidovirales* (Chan *et al.*, 2015). Among four genera in the subfamily orthocoronavirinae, only alphacoronaviruses and betacoronaviruses infect mammals, including bats, camels, and humans (Zhou *et al.*, 2018). The first coronavirus isolate was reported in the mid-1960s from the respiratory tracks of patients with common cold (Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966), which was subsequently termed human coronavirus 229E (HCoV 229E) and human coronavirus OC43 (HCoV OC43). Four more human coronaviruses have been isolated and described so far: HCoV NL63 (van der Hoek *et al.*, 2004; Abdul-Rasool and Fielding, 2010), HCoV HKU1 (Woo *et al.*, 2005a; Lau *et al.*, 2006; Vabret *et al.*, 2006), SARS-CoV (Ksiazek *et al.*, 2003; Rota *et al.*, 2003), and MERS-CoV (Corman *et al.*, 2012; Zaki *et al.*, 2012). HCoV 229E and OC43 mostly infect the upper, but rarely the lower, respiratory tracks, thus causing mild, but seldom severe, respiratory diseases, such as common cold (Bradburne *et al.*, 1967; Woo *et al.*, 2005a). On the other hand, HCoV NL63 and HKU1 have been shown to infect not only the upper but also the lower respiratory tracks, thus causing symptoms ranging from mild croup to severe bronchiolitis (Woo *et al.*, 2005b; Abdul-Rasool and Fielding, 2010), of which infections are mostly self-limiting with just few mortality cases reported. The landscape has been widely changing with the emergence of two deadly coronaviruses in humans: SARS-CoV and MERS-CoV. The two newly identified coronaviruses causes severe-to-fatal infection in humans, especially in the presence of preexisting conditions with MERS-CoV cases (Alraddadi *et al.*, 2016; Meyerholz *et al.*, 2016; Nam *et al.*, 2017; Seys *et al.*, 2018). During the SARS-CoV outbreak in 2003, over 8,000 SARS cases were reported in 37 countries resulting in 775 deaths with mortality rate reaching 10% (WHO). Since 2004, no SARS cases have been reported in the human population (Yip *et al.*, 2009; Abdul-Rasool and Fielding, 2010). On the contrary, MERS-CoV is a lingering threat causing sporadic outbreaks since its identification and characterization in 2012. Due to its zoonotic nature of infections from dromedary camels and low-level medical management, MERS-CoV cases are mostly reported in the Arabian Peninsula (van den Brand *et al.*, 2015; Widagdo *et al.*, 2019) with one exceptional outbreak in South Korea in 2015 (Ki, 2015; Lim, 2015; Kim *et al.*, 2017).

The outbreak of MERS-CoV in South Korea is generally considered as a failure of crisis management (Chowell *et al.*, 2015; Fung *et al.*, 2015; Ki, 2015). 28 secondary MERS-CoV infections in a single hospital arose by transmission from a 68-year-old business man (Park *et al.*, 2015) who had returned from the Arabian Peninsula, complaining symptoms similar to those of MERS-CoV infection. MERS-CoV transmission spread to sixteen clinics and hospitals and the chain of transmission via intra- and inter-hospital route

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Table 1. Primers for cloning of MERS-CoV accessary proteins

Name	Sequence (5′→3′)
MERS-ORF3-F	GGCGAATTCAATGAGAGTTCAAAGACCACC
MERS-ORF3-R	GGCGGATCCTTAATTAACTGAGTAACCAACG
MERS-ORF4a-F	GGCGAATTCAATGGATTACGTGTCTCTGCT
MERS-ORF4a-R	GGCGGATCCTTAGTTGGAGAATGACTCCT
MERS-ORF4b-F	GGCGAATTCAATGGAGGAATCCCTGAT
MERS-ORF4b-R	GGCGGATCCTTAAAATCCTGGATGATGTA
MERS-ORF5-F	GGCTCTAGAATGGCTTTCTCGGCGT
MERS-ORF5-R	GGCGGATCCTCACACAATCAGGCTGCTAGG
MERS-ORF8b-F	GGCGAATTCAATGCCAATTCCACCCCTG
MERS-ORF8b-R	GCCTCTAGATTACGCTAGAGGCTCTTGAAG

lasted for two months (Oh, 2016), resulting in 186 confirmed cases of MERS-CoV infections with 38 deaths as well as a huge economic loss (estimated, 8.5 billion US). The MERS-CoV genome from the second patient was extracted and fully sequenced (KT029139.1) (Kim *et al.*, 2015). Characterization and analysis of the genome revealed that it has 99.5% to 99.8% similarity to 53 known MERS-CoVs. Like other MERS-CoVs, the genome of Korean isolate encodes 16 non-structural proteins and 4 canonical structural proteins (S, E, N, and M) (Kindler *et al.*, 2016). As characterized in other coronaviruses, MRES-CoV expresses a unique set of subgenomic RNA's that are translated into accessory proteins, which vary in number and function among conronaviruses (Fehr and Perlman, 2015). MERS-CoV encodes 5 accessory proteins (3, 4a, 4b, 5, and 8b) while SARS-CoV has 8 of them (3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b), suggesting that accessory proteins vary in number as well as function. Indeed, some accessory proteins have been shown to target a number of host cellular processes, especially those involved in innate immunity: 1) ORF4a is shown to antagonize type I interferon (IFN) responses by employing a few different strategies, blocking melanoma differentiation-associated protein 5 (MDA5) (Niemeyer *et al.*, 2013), interacting with protein activator of the interferon-induced protein kinase (PACT) (Siu *et al.*, 2014), or inhibiting IFN-β as well as interferon-stimulated response element (ISRE), promoter activity (Yang *et al.*, 2013). 2) ORF4b interacts in the cytoplasm as well as in the nucleus with TANK binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), disrupting optimal activation type I IFN signaling (Matthews *et al.*, 2014; Yang *et al.*, 2015). 3) ORF5 may also interferes with IFN signaling by blocking nuclear localization of interferon regulatory factor 3 (IRF3), the major transcription factor for the activation of IFN-β promoter.

As viruses have been shown to evolve diverse tactics to evade host IFN responses, it is likely that in addition to antagonistic viral proteins described above, MERS-CoV-encoded proteins may encode other mechanisms to counteract IFN-β promoter induction upon infection in the host cell. With an aim to identify novel virus-encoded antagonist(s), we systematically screen MERS-CoV-encoded accessory proteins against each individual signaling molecule involved in the type I IFN induction pathway: MDA5, retinoic acid-inducible gene I (RIG-I), mitochondrial antiviral-signaling protein (MAVS), TBK-1, IKKε, and IRF3.

Materials and Methods

Cells

HEK293T cells (ATCC) were purchased from the American Type Culture Collection (ATCC) and maintained in a high-glucose (4,500 mg/L D-glucose) Dulbecco's Modified Eagle's Medium (DMEM) (Welgene) including 10% fetal bovine serum (FBS) (Welgene) and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37°C, 5% CO_2 incubator in a humidifying condition.

Antibodies

A mouse monoclonal anti-FLAG antibody (1:5,000) was purchased from Millipore Sigma. HA-Tag mouse antibody (1:1,000), GAPDH Rabbit monoclonal antibody (HRP Conjugate) (1:2,000), Anti-mouse IgG, HRP-linked Antibody (1:4,000) was obtained from Cell Signaling.

Reagents

Polyethylenimine (PEI) were purchased from Millipore Sigma. Luciferase Assay System and Beta-Glo Assay System were procured from Promega. 4X Laemmli Sample Buffer and 2-Mercatoethanol were purchased from Bio-Rad. Amersham ECL Prime Western Blotting Detection Reagent, Amersham ECL Western Blotting Detection Reagent, and Amersham Protran 0.45 NC blotting membrane were purchased from GE Healthcare Life Sciences. The restriction enzymes such as *Eco*RI-

Table 2. Primers for cloning of immune genes

Name	Sequence (5′→3′)
MDA5-F	AGGTGGGTCGGGTGGCGGCGGATCCTCGAATGGGTATTCCACAG
MDA5-R	GGGTTTAAACTCTAGACTCGAGCTAATCCTCATCACTAAATAAACAG
RIG-I-F	GGGTGGCGGCGGATCCTGCAGGACCACCGAGCAGCGACGC
RIG-I-R	GGGTTTAAACTCTAGACTCGAGTCATTTGGACATTTCTGCTGGATCAAATGGTATC
MAVS-F	GGGTGGCGGCGGATCCTGCAGGCCGTTTGCTGAAGACAAGACCTATAAG
MAVS-R	GGGTTTAAACTCTAGACTCGAGCTAGTGCAGACGCCGCCG
TBK1-F	AGGTGGGTCGGGTGGCGGCGGATCCCAGAGCACTTCTAATCATC
TBK1-R	AGCGGGTTTAAACTCTAGACTCGAGCTAAAGACAGTCAACGTTG
IKKε-F	CGCGGATCCCAGAGCACAGCCAATTACC
IKKε-R	CTAGTCTAGATTAGACATCAGGAGGTGCTGGGACTCTAT
IRF3-F	TTGGCGCGCCGGAACCCCAAAGCCACGGAT
IRF3-R	CCGCTCGAGTCAGCTCTCCCCAGGGCCCTGGAAAT

HF, *Bam*HI-HF, *Not*I-HF, *Nhe*I-HF, *Pme*I, *Pml*I, *Xho*I, *Xba*I, and T4 DNA ligase were purchased from New England Biolabs (NEB). Opti-MEM and MAX Efficiency DH5α Competent Cells were obtained from Thermo Fisher Scientific. Pfu Plus DNA Polymerase was purchased from Elpisbio. Complete Mini Protease Inhibitor Cocktail was procured from Millipore Sigma.

Plasmid construction

Multiple cloning site (MCS) of an expression vector, pcDNA-3.1-Hygro(+) (Lee *et al.*, 2018), was modified with a linker DNA to generate an HA-tagged Neo-JY4 vector: 5′-GCTA GCGCCACCATGTACCCATACGACGTCCCAGACTAC GCTAAGCTTTCTGGTGGCGGTGGCTCGGGCGGAG GTGGGTCGGGTGGCGGCGGATCCTGCAGGCGCGC CAGCGCTATCGATATCGATGGCGCCTGGCCAGACC ATCAGTCGAGTGGCGCCACTGGACTAATGGTCCGT ACGCTCGACTGTACAGGCCGGCCTCAGGTTAACAC CGGTACCTCAGCCCGGGCGGCCGCATGCGGGCCC CTCGAGTCTAGAGTTTAAAC-3′. The modified vector, named pcDNA3.1-Hygro-JY4-HAN-GS3 harboring a HA tag and a spacer (3xGGGGS) at the N-terminus of MCS, was used to clone immune genes (see text) by employing sequence and ligation independent cloning (SLIC) (Jeong *et al.*, 2012; Islam *et al.*, 2017). MDA5, RIG-I, MAVS, IKKε, TBK1, and IRF3 were amplified by PCR using Q5 Hot Start High-Fidelity DNA Polymerase (NEB): 98°C for 10 sec, 58°C for 30 sec, 72°C for 30 sec/kb for 30 cycles. Purified PCR products were cloned into the pcDNA3.1-Hygro-JY4-HAN-GS3 vector. All primers for the immune genes are listed in Table 2. Pfu Plus DNA Polymerase (Elpisbio) was utilized to PCR-amplify each MERS accessory gene: 95°C for 20 sec, 58°C for 20 sec, 72°C for 10 sec/kb for 30 cycles. MERS accessary genes were cloned in p3xFLAG-CMV10 vector (Millipore Sigma) by conventional ligation method using restriction enzymes. The sequence of MERS-CoV isolated from a Korean patient was used to construct expression plasmids, which was authorized by Korea Centers for Disease Control (Approval No. 16-RDM-019).

Transfection and luciferase reporter assay

HEK293T cells (4×10^5 cells/well) were seeded in a 6-well plate a day before transfection as previously described (Kang *et al.*, 2018; Kim and Myoung, 2018; Park *et al.*, 2019). Briefly, mixtures, containing 500 ng of Interferon (IFN)-β-luc, 100 ng of β-gal expressing plasmid as the internal control, 500 ng of immune gene stimulating plasmid and 1,000 ng of MERS accessary gene encoding or empty plasmid, were transfected using PEI transfection reagent as ratio of 1:2 (DNA: PEI) in 200 μl of opti-MEM (Thermo Fisher Scientific). At 24 h post-transfection, the transfected cells were lysed using 1 × Reporter Assay Lysis Buffer of the Luciferase Assay System (Promega) with 1 × Protease Inhibitor (Millipore Sigma). Lysates were incubated on ice for 10 min and centrifuged at 150,000 rpm, 4°C for 15 min. The supernatant was transferred to a new tube. 25 μl of sample was mixed with 25 μl of Assay Substrate in the Luciferase Assay System and Beta-Glo Assay System (Promega), and the luciferase or β-gal intensities were measured on GloMax 96 Microplate Luminometer (Promega). Firefly luciferase activity was normalized by β-gal activity, and fold induction of luciferase gene, in the presence or absence of MERS-CoV accessary protein, was calculated.

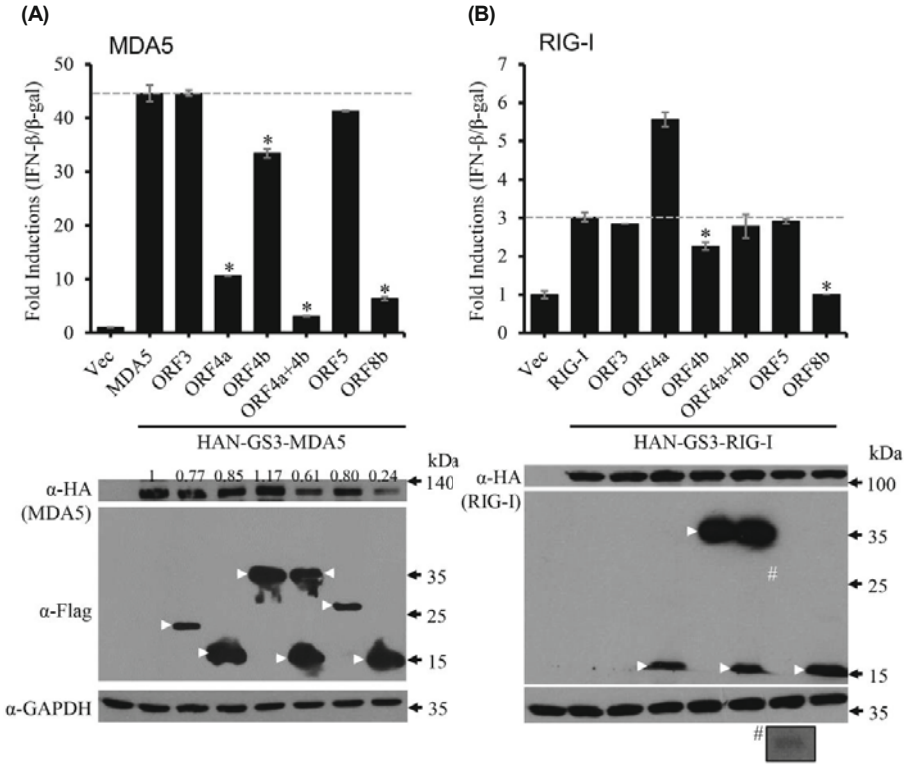

Fig. 1. ORF8b strongly inhibits both MDA5- and RIG-I-mediated induction of IFN-β promoter activity. MERS-CoV accessory genes were co-transfected into HEK293T cells with MDA5 (A) or RIG-I (B) together with IFN-β-luc and β-gal expression construct. Firefly luciferase activities and Western blots are shown at the top and bottom panels, respectively. Band intensities were determined by the ImageJ software and adjusted by that of GAPDH, which were normalized to the band intensity of MDA5 alone control. The arrow heads indicate each MERS-CoV accessary protein with 3XFLAG at the N terminus. # long exposure for ORF5. Data represent the Mean ± SD. *$P < 0.05$.

Western blotting

Protein amount was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) as described before (Cho and Myoung, 2015; Ha *et al.*, 2016; Kang *et al.*, 2016). In brief, 15 μg of protein was loaded and separated on a SDS-PAGE gel and subsequently transferred to a NC blotting membrane (GE Healthcare Life Sciences). Primary antibodies were incubated at 4°C overnight while secondary antibodies for 1 h at RT. For statistical analysis, paired two-tailed Student's *t*-test was performed. Difference between means was considered significant when *P*-value was < 0.05.

Results

ORF8b strongly antagonizes IFN-β promoter activation induced by both MDA5 and RIG-I

The MERS-CoV genome encodes 5 accessory proteins. Although some studies have been conducted (Niemeyer *et al.*, 2013; Siu *et al.*, 2014), detailed mechanisms of how MERS-CoV-encoded accessory proteins interrupt type I IFN induction remain still elusive. A full panel of MERS-CoV accessory genes were cloned into an expression vector (for details, see 'Materials and Methods'). ORF8b was included in this study although it has been neglected in the previous screening (Niemeyer *et al.*, 2013; Siu *et al.*, 2014). Accessory proteins were expressed more or less comparably with albeit lower expression in ORF3 and ORF5. Codon optimization of ORF5 and lysate preparation with sonication did not improve soluble levels of those two proteins. MERS-CoV ORF's were expressed either individually or in combination (ORF4 and ORF4b) to investigate if those two proteins function in the same pathways or they interact synergistically. To examine whether MERS-CoV accessory proteins inhibit RIG-I-like receptors (RLR)-mediated activation of IFN signaling, HEK293T cells were transfected with either MDA5 (Fig. 1, left panel) or RIG-I (Fig. 1, right panel) together with a panel of accessory genes. As expected, ORF4a inhibited MDA5-mediated (Niemeyer *et al.*, 2013), but not RIG-I-mediated (Niemeyer *et al.*, 2013; Siu *et al.*, 2014), induction of IFN-β promoter activity. Of note, ORF4b induced marginal, but statistically significant, downregulation of both MDA5- and RIG-I-mediated activation of IFN-β signaling. In addition, ORF4b cooperated with ORF4a in the inhibition of MDA5-mediated IFN signaling induction in an additive manner (Fig. 1A). A surprise comes with ORF8b. ORF8b suppressed over 80% the activation of IFN signaling induced by both MDA5 and RIG-I (Fig. 1A and B). Furthermore, expression of ORF8b or co-expression of ORF4a and ORF4b led to significant reduction in protein levels of MDA5, but not RIG-I, suggesting for a differential mechanism(s) involved in MERS-CoV protein-mediated inhibition of those two cellular helicases. Taken together, 3 MERS-encoded proteins (ORF4a, ORF4b, and ORF8b) efficiently block IFN induction via blockade of the RLR's.

MAVS- and TBK1-induced activation of IFN-β promoter is not perturbed by MERS-CoV accessory proteins

Next, it was tested if MERS-CoV accessory proteins are also involved in the inhibition of downstream innate immune molecules, namely MAVS and TBK-1 (Fig. 2). As shown, none of MERS-CoV accessory proteins perturb, if any, MAVS- (Fig. 2A) and TBK-1-mediated (Fig. 2B) induction of IFN-β promoter activity. Thus, it appears that the two cellular RNA helicases (MDA5 and RIG-I) are a major target of the accessory protein.

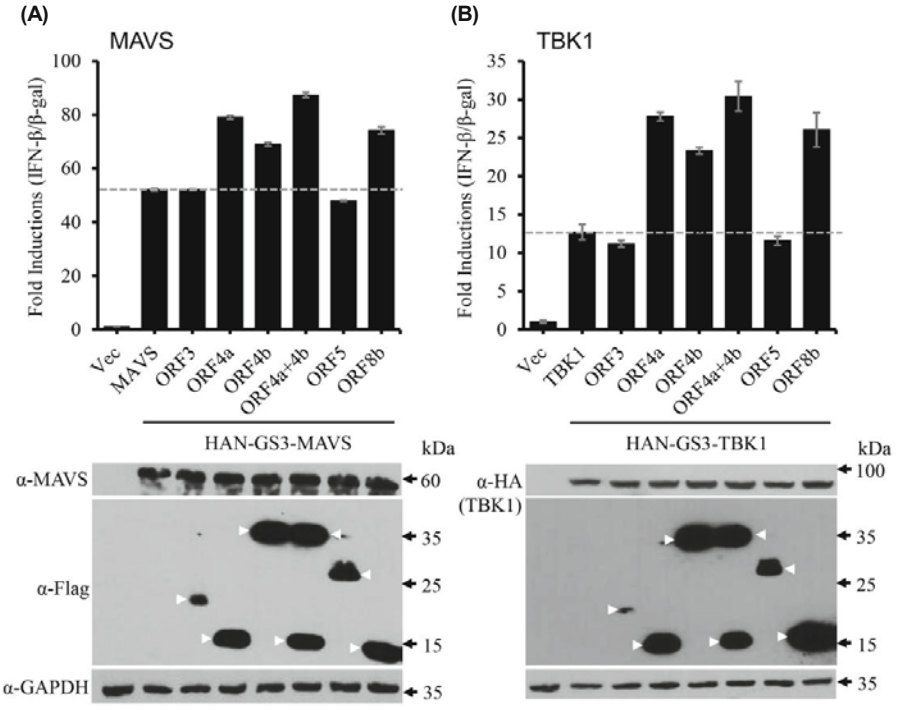

Fig. 2. MAVS- and TBK-1-induced activation of IFN-β signaling were not perturbed by accessory proteins of MERS-CoV. MERS-CoV accessory genes were co-transfected into HEK-293T cells with MAVS (A) or TBK1 (B) together with IFN-β-luc and β-gal expression construct. Firefly luciferase activities and Western blots are shown at the top and bottom panels, respectively. The arrow heads indicate each MERS-CoV accessary protein with 3XFLAG at the N terminus. Data represent the Mean ± SD. **P* < 0.05.

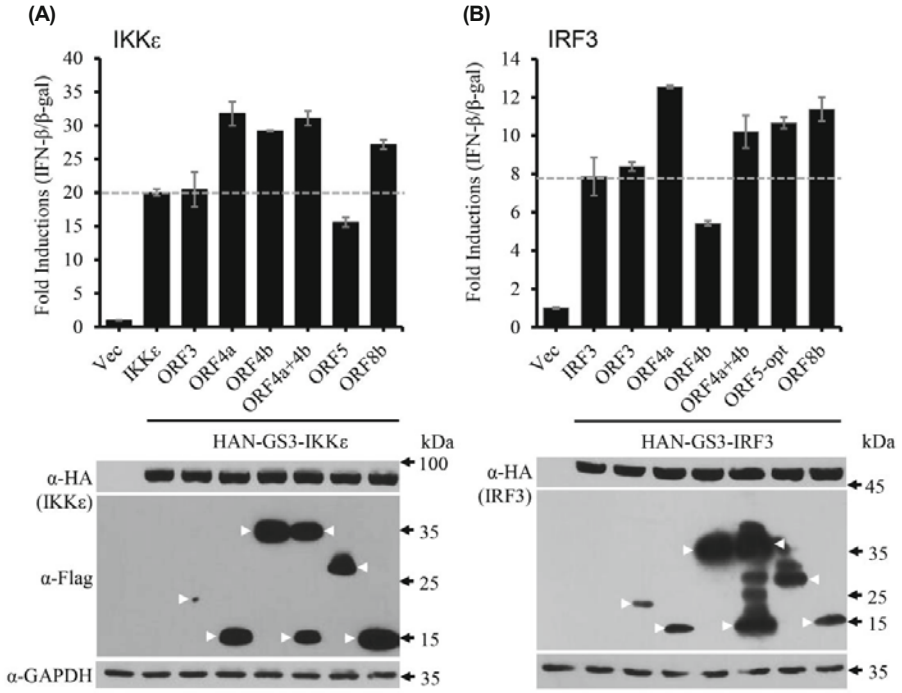

Fig. 3. IKKε- and IRF3-induced activation of IFN-b signaling were not perturbed by accessory proteins of MERS-CoV. MERS-CoV accessory genes were co-transfected into HEK-293T cells with IKKε (A) or IRF3 (B) together with IFN-β-luc and β-gal expression construct. Firefly luciferase activities and Western blots are shown at the top and bottom panels, respectively. The arrow heads indicate each MERS-CoV accessary protein with 3XFLAG at the N terminus. Data represent the Mean ± SD. $^{*}P < 0.05$.

IKKε- and IRF3-mediated activation of IFN-β signaling was left little changed by MERS-CoV-encoded accessory proteins

IRF3, a down-stream signaling molecule of IKKε, is a key transcription factor in the induction of IFN-β (Grandvaux *et al.*, 2002; Honda *et al.*, 2006; Honda and Taniguchi, 2006; Liu *et al.*, 2015). To examine whether MERS-CoV accessory proteins regulate IKKε or IRF3-mediated upregulation of IFN-β, HEK293T cells were co-transfected with IKKε or full-length IRF3 (Fig. 3A) together with individual accessory gene. A marginal, but significant reduction in IFN-β promoter activation, induced by IKKε, was detected by ORF5 (Fig. 3A, upper panel) while the protein level of IKKε was not changed (Fig. 3A, lower panel). On the other hand, IFN-β promoter activation, induced by IRF3, was largely unchanged by accessory proteins of MERS-CoV.

Discussion

Type I IFN responses are a first line of defense against invading viruses (Kang and Myoung, 2017a, 2017b; Kang *et al.*, 2018; Kim and Myoung, 2018; Banerjee *et al.*, 2019). There are 16 different type I IFN's that are known to be expressed in humans (Kindler *et al.*, 2016): IFN-α (13 subtypes), IFN-β, IFN-κ, and IFN-ω (1 type each). Upon viral infection, ligation of viral pathogen-associated molecular patterns (PAMP) with cellular pattern recognition receptors (PRR) initiate a series of activational cascades in cells (Fig. 4). For example, double-stranded RNA (dsRNA) molecules, a byproduct of viral replication and transcription in a cell (Weber *et al.*, 2006; Zielecki *et al.*, 2013), are generally recognized by the RNA helicases (MDA5 and RIG-I), and/or protein kinase R (PKR) in the cytoplasm as well as by toll-like receptor 3 (TLR3) in the endosome. It is now well-known that structurally and

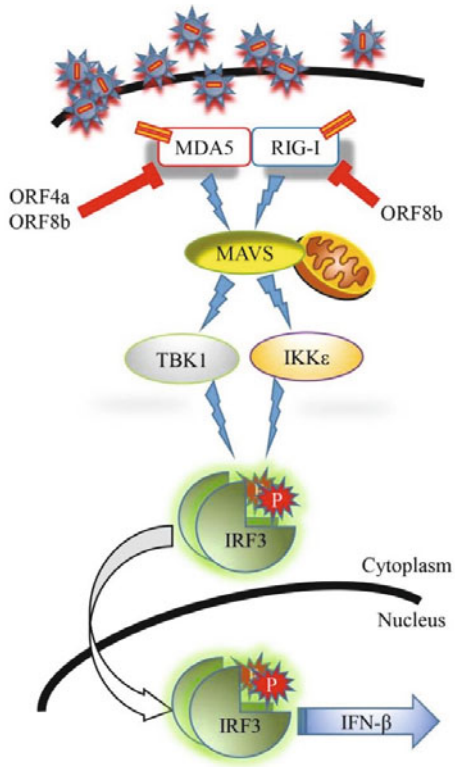

Fig. 4. IFN-β signaling pathway and its modulation by accessory proteins encoded by MERS-CoV. Activation of the cellular helicases (MDA5 and RIG-I), by the ligation with cognate ligands, leads to induction of IFN-β promoter activity via a cascade of signal amplification: aggregation of MAVS on the outer membrane, activation of kinases (TBK1 and IKKε), and phosphorylation and translocation of dimerized IRF3. In this study, we demonstrated that ORF8b strongly inhibits both MDA5- and RIG-I-induced activation of IFN-β signaling. ORF4a-mediated inhibition seems to be limited to that of MDA-5.

chemically distinctive RNA's are recognized by the two cellular helicases (Akira *et al.*, 2006; Medzhitov, 2007): RIG-I senses long or short dsRNA molecules with di- or tri-phosphates at the 5′ end (Goubau *et al.*, 2014) while single-stranded RNAs (ssRNAs) with particular features can also be recognized if the ssRNAs are 3′ phosphorylated or polyU/UC-rich (Malathi *et al.*, 2007). On the other hand, types of RNA that MDA5 binds to include long RNA's with higher-order structures (Runge *et al.*, 2014) as well as some ssRNA's (negative-sense RNA and hypomethylated 5′ capped mRNA molecules) (Luthra *et al.*, 2011; Zust *et al.*, 2011). Upon recognition of these RNA's, RIG-I and MDA5 undergo conformational changes, including oligomerization, and the mitochondrial membrane chaperone 14-3-3ε recruits the oligomers to mitochondria, where caspase activation and recruitment domains (CARD) of the RNA helicases interact with MAVS (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005), leading to induction of its aggregation and activation. Activated MAVS, in turn, triggers activation of TBK-1 and IKKε (Fitzgerald *et al.*, 2003; Hacker and Karin, 2006; Gatot *et al.*, 2007; Chau *et al.*, 2008; Clement *et al.*, 2008), and subsequently phosphorylation and dimerization of IRF3 (Grandvaux *et al.*, 2002; Honda and Taniguchi, 2006; Liu *et al.*, 2015). Dimerized IRF3 translocates into the nucleus, stimulating transcription of IFN-β (Fig. 4).

MERS-CoV, like SARS-CoV, is generally considered to have evolved from bat coronaviruses (Li *et al.*, 2005; Hayman, 2016; Goldstein and Weiss, 2017; Maxmen, 2017). Comprehensive genomic and functional analyses of those two viruses have revealed a few interesting distinctions between them (Kindler *et al.*, 2016): 1) SARS-CoV encodes more number of accessory proteins at the 3′ end of the genome compared to MERS-CoV, 2) More number of SARS-CoV-encoded genes are known to inhibit IFN signaling than those of MERS-CoV, 3) MERS-CoV is more sensitive to IFN-mediated inhibition than SARS-CoV (Zielecki *et al.*, 2013). These results suggest that MERS-CoV might have evolved less means to evade the innate immune responses mediated by IFN. However, exact magnitude or multitude of MERS-CoV-encoded antagonistic mechanisms remain to be elucidated.

To shed light on viral and cellular determinants of IFN evasion by MERS-CoV, a full panel of accessory genes of MERS-CoV (van Boheemen *et al.*, 2012) were cloned into an expression vector: ORF3, ORF4a, ORF4b, ORF5, and ORF8b. Either in combination or individually, these genes were transfected into HEK293T cells together with each molecule that is involved in an IFN signaling: MDA5 and RIG-I (RNA helicases), MAVS, TBK-1, and IKKε (cytoplasmic signaling molecules), or IRF3 (a key transcription factor). The striking finding of current study is that ORF8b was identified as a strong antagonist of two dsRNA sensors (Fig. 1): MDA5 and RIG-I with or without perturbation of protein levels in the cells, respectively (Fig. 1A vs B). Currently, ORF8b-interacting host proteins are being sought. Identity of host interacting partner(s) will hint on the molecular mechanism(s) of ORF8b-mediated suppression. In addition, ORF4a seems to robustly down-regulate MDA5-mediated activation of IFN signaling. This is in line with a previously published study led by Niemeyer *et al.* (2013). ORF4a has a dsRNA-binding motif (Niemeyer *et al.*, 2013; Siu *et al.*, 2014; Comar *et al.*, 2019), thus antagonizing IFN signaling like several virus-encoded proteins, such as influenza A virus NS1 (Chan *et al.*, 2018), herpes simplex virus 1 Us11 (Kew *et al.*, 2013), paramyxovirus V (Motz *et al.*, 2013), and Ebola virus VP35 proteins (Cardenas *et al.*, 2006; Prins *et al.*, 2009, 2010). However, IFN-inhibiting function of ORF4a may be controversial. Siu *et al.* (2014) reported that ORF4a could not inhibit MDA5-mediated initiation of IFN signaling activation, but did suppress an upstream signaling molecule, PACT. PACT binds to dsRNA and recruit it to MDA5 or RIG-I. The authors claimed that ORF4a interrupt dsRNA-PACT interaction with the RLR's (Kok *et al.*, 2011; Ho *et al.*, 2016; Lui *et al.*, 2017). Furthermore, when a recombinant MERS-CoV with ORF4a deletion infects cells, IFN induction was only marginally reduced (Comar *et al.*, 2019). One may envision that after all ORF4a may play a minor role in viral antagonism of IFN signaling and other viral antagonist(s) may exist. In this regard, identification of ORF8b as a virus-encoded antagonist will help elucidate magnitude and multitude of virus-encoded mechanisms of IFN evasion. Or simply, viral dsRNA might not be exposed to antiviral sensors (PACT, MDA5 or RIG-I) (Versteeg *et al.*, 2007; Zhou and Perlman, 2007) as MERS-CoV induces massive membrane re-organization for the formation of double-membrane vesicles for viral transcription and replication (Gosert *et al.*, 2002; Lundin *et al.*, 2014). Elucidation of exact mechanisms, involved in ORF4a antagonism of IFN signaling, awaits further scrutiny.

ORF4b seems to marginally inhibit both MDA5- and RIG-I-induced IFN signaling (Fig. 1) and also to cooperate with ORF4a to further down-regulate MDA5-mediated signaling (Fig. 1A). To our knowledge, this is the first evidence that ORF4a and ORF4b inhibits IFN signaling in an additive manner. As ORF4b harbors nuclear localizing signal (NLS) (Niemeyer *et al.*, 2013; Comar *et al.*, 2019), it is tempting to postulate that it may interact with one or more of transcription factors that are involved in IFN induction.

Taken all together, here we report that ORF8b of MERS-CoV is a potent antagonist of both MDA5- and RIG-mediated activation of IFN signaling (schematic summary in Fig. 4), building up ever-growing list of MERS-CoV evasion strategies against the host innate immune responses. Delineation of molecular mechanisms ORF8b will likely pave way to develop effective protective and/or therapeutic antiviral measures.

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