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Transmissible gastroenteritis virus infection induces NF-κB activation through RLR-mediated signaling

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ABSTRACT

Transmissible gastroenteritis virus (TGEV) is a porcine enteric coronavirus which causes lethal severe watery diarrhea in piglets. The pathogenesis of TGEV is strongly associated with inflammation. In this study, we found that TGEV infection activates transcription factors NF-κB, IRF3 and AP-1 in a time- and dose-dependent manner in porcine kidney cells. Treatment with the NF-κB-specific inhibitor BAY11-7082 significantly decreased TGEV-induced proinflammatory cytokine production, but did not affect virus replication. Phosphorylation of NF-κB subunit p65 and proinflammatory cytokine production were greatly decreased after knockdown of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) or its adaptors MAVS and STING, while only slight reduction was observed in cells following silencing of Toll-like receptor adaptors, MyD88 and TRIF. Furthermore, TGEV infection significantly upregulated mRNA expression of RIG-I and MDA5. Taken together, our results indicate that the RLR signaling pathway is involved in TGEV-induced inflammatory responses.

1. Introduction

Transmissible gastroenteritis virus (TGEV) infection causes severe enteritis accompanied by highly contagious in pigs (Garwes, 1988; Penzes et al., 2001). TGEV is an enveloped, positive-sense, single-stranded RNA virus with a genome of 28.5 kb, belonging to the *Coronaviridae* family in the order *Nidovirales* (Eleouet et al., 1995). The genome consists of a 5' untranslated region (UTR), open reading frame 1a/1b (ORF1a/1b), spike (S), envelope (E), membrane (M), nucleocapsid (N) and 3' UTR, arranged in this order, with three accessory genes 3a, 3b and 7 interspersed within the structural genes at the 3′ end of the genome (Putics et al., 2006; Yount et al., 2000). TGEV circulates in pig farms and has been known to mutate, resulting in the S gene deletion mutant now recognized as porcine respiratory coronavirus (PRCV) (Kim et al., 2000; Wang et al., 2010; Wang and Zhang, 2015). It also provides the virus backbone which, in combination with the S gene of porcine epidemic diarrhea virus (PEDV), has generated the recently described chimeric viruses termed swine enteric coronaviruses (SeCoVs) (Belsham et al., 2016). This frequent evolution poses a huge threat to the pig industry.

Virus invasion always triggers an inflammatory response, which is a key mediator of the host response against microbial pathogens (Shrivastava et al., 2016). To monitor and rapidly respond to diverse viruses, germline-encoded pattern recognition receptors (PRRs) are appointed to sense pathogen-associated molecular patterns (PAMPs) and subsequently induce the production of inflammatory mediators with a two-step process: priming and activation (Poeck and Ruland, 2012; Rietdijk et al., 2008). The priming step is mediated primarily by Toll-like receptors (TLRs) or RIG-like receptors (RLRs): TLRs recognize specific viral motifs within the endosomal compartments, then recruit the Toll/IL-1 receptor (TIR) domain-containing adaptors, including myeloid differentiation primary response gene (MyD88) and TIR-domain-containing adaptor-inducing IFN-β (TRIF); RLRs recognize cytosolic viral RNA, then interact with mitochondrial antiviral signaling protein (MAVS, also known as IPS-1/VISA/CARDIF) leading to the recruitment and activation of mitochondria associated complexes (Fukata and Arditi, 2013; Lamkanfi and Dixit, 2009; Takeuchi and Akira, 2010; Tschopp and Schroder, 2010; Yamamoto

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et al., 2003). In addition, the stimulator of interferon gene (STING, also known as MITA) has also been identified as an adaptor involved in the RLR pathway (Ishikawa and Barber, 2008). The activation step is mainly mediated by cytosolic PRRs including nucleotide binding and oligomerization domain-like receptors (NLRs), absent in melanoma 2 (AIM2), retinoic acid inducible gene I (RIG-I), gamma-interferon-inducible protein 16 (IFI16), and pyrin that initiate assembly of the appropriate inflammasome components where receptors recruit apoptosis-associated speck-like protein containing CARD (ASC) proteins and caspase-1 complexes that are required for the secretion and bioactivity of priming step-produced cytokines (Dutta et al., 2015; Latz et al., 2013; Man et al., 2016; Vajjhala et al., 2014; Yu and Levine, 2011).

The transcription of proinflammatory cytokines are regulated by many transcription factors, including nuclear factor kappa B (NF-κB), activating protein 1 (AP-1), interferon regulatory factor 3 (IRF3) and so on (Hagiwara et al., 2009; Lei et al., 2015; Wang et al., 2008). NF-κB is essential in priming inflammasome activation for production of cytokines like TNF-α, IL-6 and also of pro-IL-1β (Lan et al., 2012; Li and Verma, 2002). The NF-κB family is comprised of five mammalian members: RelA (p65), RelB, cRel, p50 (NF-κB1), and p52 (NF-κB2) (Ghosh et al., 1998). A heterodimer composed of p65 and p50 is the most frequently activated form of NF-κB, interacting with inhibitory kappa B (IκB) molecules in resting cells (Malek et al., 1998). The classical NF-κB activation cascade is initiated by stimulus-induced phosphorylation, ubiquitination and degradation of IκBα, releasing NF-κB dimers and promoting their nuclear translocation. As a result, transcription of various NF-κB target genes involved in the inflammatory response are activated, such as IL-6, IL-8, RANTES and TNF-α (Hayden and Ghosh, 2008).

Previous studies demonstrated that virulent TGEV causes significant inflammation in intestinal tissues, and animal death is mainly attributed to the extreme imbalance of Na+ and K+ ions caused by the severity of the clinical symptoms (Cruz et al., 2013; Saif, 1996). Thus, inflammatory injury to intestinal tissue caused by an overactive immune response is a hallmark of TGEV pathogenesis. Additionally, TGEV-infected cells showed enhanced NF-κB activity and high expression of genes associated with inflammation, including RANTES, CCL2 and CXCL16 (Ma et al., 2014), thus suggesting that the inflammatory response plays an important role in the pathogenicity of TGEV. However, the mechanisms utilized by TGEV to induce NF-κB and inflammatory responses remain largely unknown. In this study, we confirm that TGEV infection enhances NF-κB activation and induces expression of molecules associated with inflammation including IL-6, IL-8, TNF-α and RANTES in porcine kidney (PK)−15 cells. We further analyzed the underlying mechanisms and found that TGEV-activated inflammation mainly depends on the RLR pathway and downstream adaptors MAVS and STING.

2. Results

2.1. TGEV infection activates NF-κB in PK-15 cells

To explore whether TGEV infection activates NF-κB in PK-15 cells, we first assessed the ability of TGEV to induce NF-κB-dependent promotor activity (NF-κB-Luc) using a luciferase reporter system. To this end, PK-15 cells were transfected with a NF-κB-Luc reporter plasmid and the internal plasmid pRL-TK, followed by infection with TGEV at different multiplicity of infection (MOI). At 24 h post infection (hpi), cells were collected for dual luciferase activity analysis. As shown in Fig. 1A, TGEV infection significantly increased the activity of the NF-κB-dependent promoter in a dose-dependent manner. We also analyzed NF-κB-dependent promotor activity at different time points after TGEV infection (MOI=0.1). Enhanced NF-κB-Luc activity was detected as early as 12 hpi and continued to increase at 24 hpi and 36 hpi (Fig. 1B). However, this activation was not observed in cells treated with UV-inactivated TGEV (equivalent to 0.1 MOI) (Fig. 1A). These results indicated that TGEV infection could activate NF-κB, and this ability required viral replication. We also tested whether transcription factors IRF3 and AP-1 can be activated after TGEV infection. Similar to NF-κB, TGEV infection activated AP-1 (Fig. 1C) and IRF3 (Fig. 1D) in PK-15 cells.

Activation of NF-κB is usually characterized by degradation of IκBα, phosphorylation of NF-κB subunit p65 and subsequent nuclear translocation (Salminen et al., 2008). Therefore, the expression level of IκBα and phospho-p65 were necessarily detected after TGEV infection in PK-15 cells. At different times postinfection, mock- and TGEV-infected cells were lysed and assayed by immunoblotting using specific antibodies against IκBα, phospho-p65 and total-p65. TNF-α-treated cells were used as a positive control, and PK-15 cells treated with 0.1 MOI UV-inactivated TGEV were used as a negative control. In addition, a specific monoclonal antibody against TGEV N protein was used to monitor viral replication status. As shown in Fig. 2A, the expression of phospho-p65 was increased to 6 fold at 24 hpi in TGEV-infected cells compared with the mock-infected control, and kept 12 fold increase at 36 hpi. Accordingly, TGEV infection induced degradation of IκBα to various degrees throughout the time course, with this being most apparent at 36 hpi. However, UV-inactivated TGEV failed to stimulate IκBα degradation or p65 phosphorylation, indicating that it is not viral particle but live TGEV infection activates NF-κB. Recent study reported the pathogenicity of TGEV is strongly associated with inflammation (Regla-Nava et al., 2015). We compared the ability of virulent and attenuated TGEV to activate NF-κB. PK-15 cells were infected with virulent or attenuated TGEV for 36 h, followed by western blot analysis with antibodies against IκBα, phospho-p65, total-p65. As shown in Fig. 2B, the expression levels of phospho-p65 induced by the attenuated TGEV were significantly low than the virulent TGEV did.

To further determine whether TGEV infection promotes phospho-p65 nuclear translocation, PK-15 cells were transfected with pEGFP-p65, a DNA construct expressing a fusion protein of porcine p65 and green fluorescent protein (GFP). At 24 h post-transfection, the cells were mock-infected or infected with TGEV at 0.1 MOI for 24 h, after which they were fixed and subsequently immunostained with a monoclonal antibody against TGEV N protein and Cy3-conjugated goat anti-mouse IgG. As shown in Fig. 2C, most p65 remained in the cytoplasm in mock-infected cells, while TGEV-infected cells containing the pEGFP-p65 fusion protein showed obvious nuclear accumulation of p65. These data demonstrated that TGEV infection induced phosphorylation and nuclear translocation of p65 and degradation of IκBα.

2.2. Effect of NF-κB on TGEV-induced proinflammatory cytokine expression

To further investigate whether TGEV infection induces proinflammatory cytokine production through the NF-κB signaling pathway, the mRNA expression of various proinflammatory cytokines (IL-6, IL-8, TNF-α and RANTES) were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). To this end, PK-15 cells were pretreated with BAY11-7082, a NF-κB-specific inhibitor, at different doses (1, 2, 5, and 10 μM) or left untreated, but subjected to an equal volume of DMSO, for 1 h, followed by inoculation with TGEV (MOI=0.1). All tested doses of BAY11-7082 used in the present study had no detectable cytotoxicity in PK-15 cells, determined by MTT assay (data not shown). After incubation for 1 h, the supernatant was removed and replaced with cell culture media containing different doses of the inhibitor BAY11-7082.RT-qPCR was performed with total RNA collected from different cell samples at 24 hpi. As shown in Fig. 3A, the levels of IL-6, IL-8, TNF-α, and RANTES mRNAs were significantly upregulated in TGEV-infected cells at 24 hpi, compared with the mock-infected group. Furthermore, the effects of TGEV-induced proinflammatory cytokine expression were reduced in a dose-dependent manner after treatment with the NF-κB inhibitor.