

## NF- $\kappa$ B activation by equine arteritis virus is MyD88 dependent and promotes viral replication

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**Abstract** NF- $\kappa$ B, a family of transcription factors involved in different cell functions and immune responses is targeted by viruses. The mechanism of NF- $\kappa$ B signalling and its role in replication of EAV have not been investigated. We demonstrate that EAV infection in BHK-21 cells activates NF- $\kappa$ B, and this activation was found to be mediated through the MyD88 pathway. Infection of IKK $\beta^{-/-}$  murine embryo fibroblasts (MEFs), which are deficient in NF- $\kappa$ B signalling, resulted in lower virus titre, less cytopathic effect, and reduced expression of viral proteins. These findings implicate the MyD88 pathway in EAV-induced NF- $\kappa$ B activation and suggest that NF- $\kappa$ B activation is essential for efficient replication of EAV.

Equine arteritis virus (EAV) is an enveloped, single-stranded, positive-sense RNA virus that belongs to the family *Arteriviridae*. This family also includes porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase elevating virus (LDV) and simian hemorrhagic fever virus [1]. The families *Arteriviridae*, *Coronaviridae*, and *Roniviridae* comprise the order

*Nidovirales* [2]. EAV causes equine viral arteritis, a debilitating disease of horses manifested as a respiratory syndrome and abortion in adults, and interstitial pneumonia in foals [1, 3, 4].

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) comprises a family of transcription factors that regulate expression of genes involved in cellular functions such as apoptosis, proliferation, immune responses, inflammation and stress responses, by binding to specific DNA sequences [5]. Many viruses modulate NF- $\kappa$ B signalling in terms of activation or suppression, or both, at different phases of the virus life cycle [6]. Porcine respiratory and reproductive syndrome virus, a member of the family *Arteriviridae*, has been shown to activate NF- $\kappa$ B signalling [7]. Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that mediates pathogen-induced NF- $\kappa$ B signalling from toll-like receptors (TLRs) to the nucleus, leading to expression of type I interferon (IFN) and inflammatory cytokine genes [8, 9]. The role of NF- $\kappa$ B signalling in infection with members of the family *Arteriviridae* has not yet been elucidated. Also, the mechanism of NF- $\kappa$ B activation during infection has not been demonstrated with any of the arteriviruses. Therefore, the involvement of MyD88, as a main adaptor molecule in activation of NF- $\kappa$ B signalling, was investigated upon infection with EAV. Also, the effect of NF- $\kappa$ B activation on viral replication was studied in impaired murine cells.

In order to confirm the activation of NF- $\kappa$ B signalling by EAV infection, we used a dual luciferase reporter assay system. Baby hamster kidney 21 (BHK-21, ATCC, CCL-10) cells were co-transfected with an NF- $\kappa$ B reporter plasmid (p-Luc-NF- $\kappa$ B) and pGL 4.74 as transfection control at a concentration of 200 ng and 10 ng, respectively. Six hours post-transfection, the cells were infected

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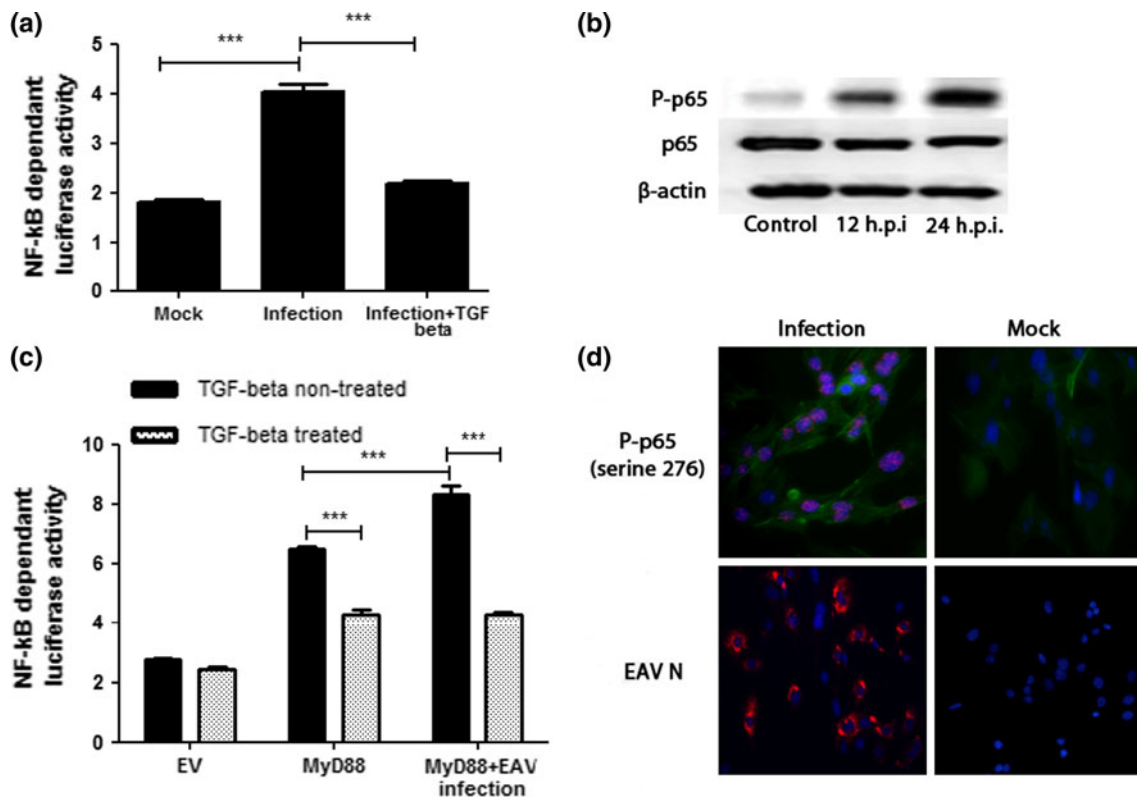
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with EAV strain SP3A at multiplicity of infection (MOI) of 5. Eighteen hours after infection, the cells were lysed and subjected to Dual-Luciferase Reporter Assay (Promega), and NF- $\kappa$ B dependant luciferase activity was measured using a luminometer (GloMax®-Multi Detection System, Promega). Results of three independent experiments showed that NF- $\kappa$ B was significantly activated by EAV infection (Fig. 1a). The activation of NF- $\kappa$ B in EAV-infected cells was also confirmed by immunoblotting at 12 and 24 hours post-infection using a rabbit polyclonal antibody (Santa Cruz Biotechnology) against p65 NF- $\kappa$ B phosphorylated at serine 276 as shown in Fig. 1b.

Moreover, we showed that in presence of MyD88 (200 ng), as an NF- $\kappa$ B stimulus, EAV infection led to a significant increase in NF- $\kappa$ B activity. However, when the cells were pre-treated with TGF- $\beta$ 1 (R&D Systems; 30 ng/ml, 8 hours before cell lysis) EAV infection failed to activate NF- $\kappa$ B (Fig. 1c). TGF- $\beta$  has been shown to inhibit

MyD88-dependent NF- $\kappa$ B activation by causing proteasomal degradation of MyD88 and not TRIF [10]. To show TGF- $\beta$ 1 suppression of the MyD88 pathway in our system, we transfected cells with MyD88 (200 ng) and an NF- $\kappa$ B reporter as before and treated them with TGF- $\beta$ 1 (30 ng/ml). Twenty-four hours after transfection, cells were lysed and subjected to dual luciferase reporter assay. MyD88-induced NF- $\kappa$ B activation was dramatically reduced in TGF- $\beta$ 1-treated cells, indicating that TGF- $\beta$ 1 suppresses MyD88-dependent NF- $\kappa$ B activation in BHK-21 cells (Fig. 1c). Therefore, our results suggest that EAV-induced NF- $\kappa$ B activation is MyD88 dependent, because in the presence of TGF- $\beta$ 1, as a MyD88 inhibitor, EAV could not activate NF- $\kappa$ B signalling. MyD88 is an important mediator in the inflammatory response (e.g., NF- $\kappa$ B-induced cytokine production) to infections, but its role has been controversial when studied in some viral infections using MyD88-knockout mice. It has been demonstrated, for



**Fig. 1** EAV infection activates p65 NF- $\kappa$ B through the MyD88 signaling pathway. (a) NF- $\kappa$ B activation in BHK-21 cells transfected with an NF- $\kappa$ B-dependent luciferase reporter system. Depicted is a representative of three independent experiments, each with three replicates \*\*\*( $p < 0.001$  by unpaired Student's t-test). A significant difference in luciferase activity was seen between infected and mock-infected or infected cells treated with TGF- $\beta$ . (b) Detection of phosphorylated p65 by immunoblotting in EAV-infected cells. Idle p65 was detected in infected and uninfected cells, while the signal for phosphorylated p65 (P-p65) NF- $\kappa$ B was stronger in infected cells.

(c) TGF- $\beta$  suppression of MyD88-induced NF- $\kappa$ B activation. BHK-21 cells were transfected with reporter plasmids as before, plus MyD88 plasmid where indicated, and TGF- $\beta$  was added 8 hours before lysis. A significant reduction in luciferase activity was seen in infected TGF- $\beta$ -treated cells. (d) Nuclear translocation of p-p65 NF- $\kappa$ B in infected but not in mock-infected BHK-21 cells. A proximity ligation assay (PLA) was performed with an antibody recognizing p65 NF- $\kappa$ B phosphorylated at serine 276 (upper panel). EAV infection was confirmed by the signal for expression of the nucleocapsid protein (EAV N) using immunofluorescence (lower panel)

example, that MyD88 is essential for defence against primary influenza A virus infection, since MyD88 knockout mice were significantly more susceptible to primary infection [11]. In contrast, another study demonstrated that functional MyD88 signalling in wild-type mice contributes to coxsackievirus B3 pathogenesis to enhance the virus replication; however, surprisingly, MyD88-deficient mice had higher levels of interferon regulatory factor 3 (IRF3) and interferon  $\beta$ , which are important antiviral elements [12].

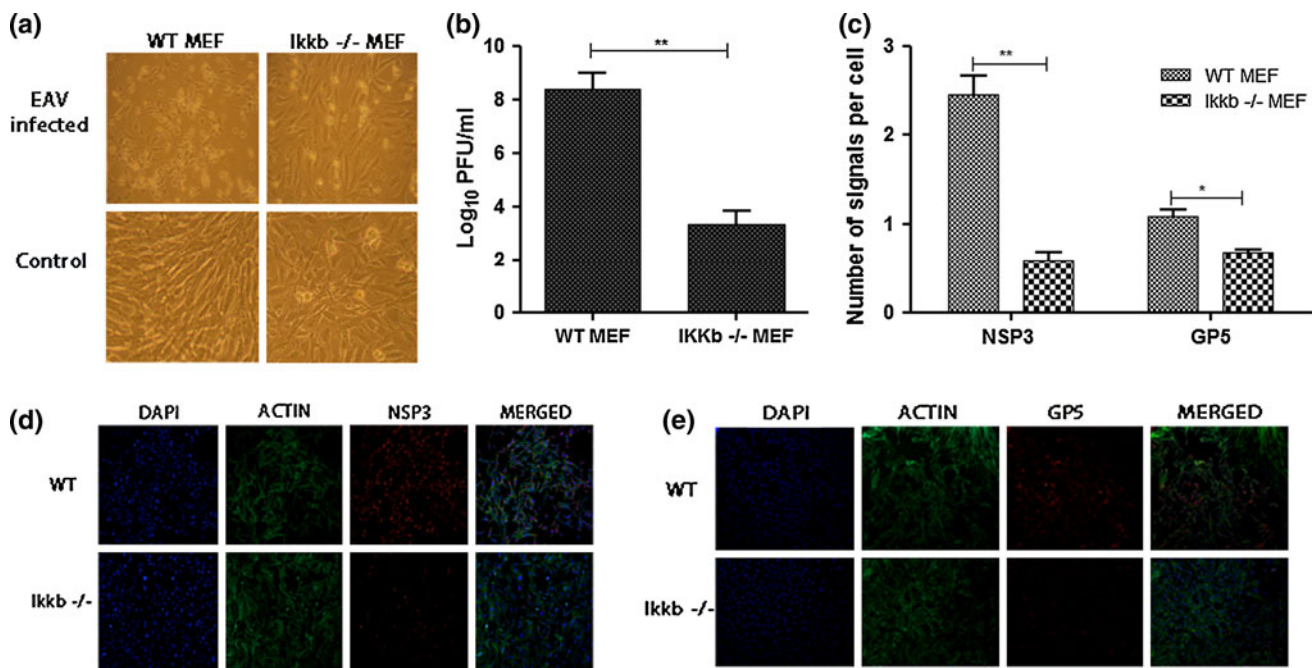
To investigate nuclear translocation of p-NF- $\kappa$ B in EAV infection, BHK-21 cells were infected with EAV SP3A in chamber slides and fixed at 8, 12, 16 and 24 hours post-infection. An *in situ* proximity ligation assay (*in situ* PLA) (Olink Biosciences®) was performed following the manufacturer's protocol and using polyclonal rabbit serum (Santa Cruz Biotechnology) against phosphorylated forms of p65 NF- $\kappa$ B, diluted 1:500 in TBS containing 5 % goat serum (TBS-GS) as blocking buffer. EAV infection was demonstrated by immunofluorescence with a monoclonal antibody targeting the nucleocapsid protein in cells infected in parallel wells of chamber slides and fixed as before. Activation and nuclear localization of p65 NF- $\kappa$ B was seen in cells probed with the anti-p-p65 serine 276 antibody but not in uninfected cells (Fig. 1d). In addition, cells probed with antibodies to forms of p65 NF- $\kappa$ B phosphorylated at serine 536, serine 468, threonine 254 and threonine 435 did not show signals for nuclear translocation (data not shown). The NF- $\kappa$ B family consists of homodimers/heterodimers of five subunits, of which p65 (RelA) is the main subunit of the canonical pathway of NF- $\kappa$ B signalling that is potentially targeted by viruses [6]. In addition, it is known that the phosphorylation status of NF- $\kappa$ B subunits affects their transcriptional activity [13]. The role of p65 (RelA) in the immune response to viral infections and in inducing expression of type I IFN genes has been controversial. Besides, there are only few studies on the phosphorylation status of NF- $\kappa$ B during viral infections. Respiratory syncytial virus has been demonstrated to induce phosphorylation of RelA at serine 276 and serine 536 [14], and that phosphorylation at serine 276 is required for transcription elongation of inflammatory genes [15]. Even though the majority of classical studies agreed on the substantial role of RelA in type I IFN gene expression, recent studies have challenged this perception [16]. One study suggested a crucial role for RelA in induction of an IFN response in the early response to RNA viruses [17]. The outcome of p65 (RelA) activation and its phosphorylation at serine 276 in the context of interferon response during EAV infection needs to be investigated further.

Murine embryo fibroblasts (MEFs) are susceptible to EAV infection [18], and IKK $\beta$ <sup>-/-</sup> MEFs are significantly deficient in NF- $\kappa$ B activity [19]. To study the relevance of

NF- $\kappa$ B signalling in infection with EAV, wild-type (wt) and IKK $\beta$ <sup>-/-</sup> cells were infected with 10-fold dilutions of EAV strain SP3A. Twenty-four hours after infection, cells were observed for cytopathic effect (CPE) using a bright-field microscope. As depicted in Fig. 2a, the CPE was reduced in knockout cells (IKK $\beta$ <sup>-/-</sup> cells), while dramatic in the wild type, suggesting a beneficial role of NF- $\kappa$ B activation in infection.

To further compare EAV replication in NF- $\kappa$ B-competent and deficient cells, the virus titer was determined in wild-type (wt) and IKK $\beta$ <sup>-/-</sup> MEFs using plaque assay. Briefly, cells were incubated with duplicates of 10-fold dilutions of EAV strain SP3A for 30 minutes at 37 °C. Thereafter, cells were washed and incubated in DMEM containing 1 % agar, 2 % fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin. Seventy-two hours later, the cells were stained with crystal violet and plaques were counted. The results showed a significantly higher virus titer in wt MEFs than in IKK $\beta$ <sup>-/-</sup> MEFs (Fig. 2b), indicating a contributing role of NF- $\kappa$ B in EAV replication. Furthermore, the expression of key non-structural (nsp3) and structural (GP5) proteins, indicative of viral replication, was analysed by *in situ* PLA in NF- $\kappa$ B-competent and deficient cells. Following infection of wt and IKK $\beta$ <sup>-/-</sup> MEFs at an MOI of 5, cells were fixed at 8 (for nsp3) and 12 (for GP5) hours after infection. As primary antibodies, rabbit anti-peptide serum recognizing nsp3 (1:1000 in TBS-GS) and GP5 (1:100 in TBS-GS) were used. The signals were quantified using Duolink® Image Tool. As shown in the quantitation plot of Fig. 2c, and in the images of Fig. 2d and 2e, the expression of nsp3 and GP5 proteins of EAV was significantly reduced in NF- $\kappa$ B-deficient cells. These results further confirmed that functional NF- $\kappa$ B signalling is essential for efficient replication of EAV.

Activation of NF- $\kappa$ B in viral infections might promote viral replication [20]. Replication and spread of Theiler's murine encephalomyelitis virus has been shown to be enhanced by virus-mediated NF- $\kappa$ B activation [21]. The presence of IKK $\beta$  was also shown to be essential for lytic replication of murine gamma-hepesvirus 68 [22]. However, Lee and Kleiboeker did not observe any impact of NF- $\kappa$ B activation on replication of porcine respiratory and reproductive syndrome virus, PRRSV (a virus related to EAV) when they suppressed NF- $\kappa$ B activity by overexpression of a negative form of I $\kappa$ B $\alpha$ , which sequesters NF- $\kappa$ B in the cytoplasm [7]. Curiously, this is in contrast with our results using IKK $\beta$ <sup>-/-</sup> cells that are impaired for NF- $\kappa$ B activation. Whether the less-efficient replication of EAV in IKK $\beta$ <sup>-/-</sup> cells is due to impaired NF- $\kappa$ B signalling or due to the direct effect of IKK $\beta$  deficiency warrants further studies. To our knowledge, the present study is the first to reveal the contributing role of the NF- $\kappa$ B activity in



**Fig. 2** EAV infection is impaired in NF-κB-deficient cells. (a) Reduction of cytopathic effect (CPE). Wild-type (wt) and knockout cells were infected with EAV, and after 24 hours, cells were observed for CPE. CPE was reduced in knockout MEFs. (b) Reduction of virus titre. Virus titres were determined by plaque assay in wild-type and knockout (IKKβ<sup>-/-</sup>) MEFs. The virus titre was significantly reduced in NF-κB-deficient cells. (c, d, e) Impaired

replication of EAV in NF-κB-deficient cells. wt and IKKβ<sup>-/-</sup> MEF cells were infected with EAV at an MOI of 5, and expression of replicase (nsp3) and a structural protein (GP5) of EAV was visualized using PLA at 8 and 12 hours post-infection, respectively. Signal quantification showed a significant difference in expression of both proteins between wt and IKKβ<sup>-/-</sup> cells

replication of an arterivirus, a member of a virus order that includes viruses of medical and veterinary importance such as SARS coronavirus and PRRSV. A better understanding of the mechanism and the role of NF-κB activation in infections may lead to identification of potential drug targets for treatment of EVA and related viral diseases.

In summary, we present here the first report on induction of MyD88-dependent NF-κB activation by an arterivirus, EAV. We also demonstrate that EAV infection of cells impaired in NF-κB activity (IKKβ<sup>-/-</sup> MEFs) is less efficient, suggesting that EAV activates NF-κB to enhance its replication. However, the molecular pathways and the mechanisms by which NF-κB activation leads to more-efficient EAV replication deserve further investigation in follow-up studies.

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**Conflict of interest** The authors declare that they have no competing interests.

## References

- Snijder EJ, Spaan WJ (2006) Arteriviruses. In: Knipe DM, Howley PM (eds) Fields virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1337–1355
- Cavanagh D (1997) Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142:629–633
- Glaser AL, de Vries AA, Rottier PJ, Horzinek MC, Colenbrander B (1996) Equine arteritis virus: a review of clinical features and management aspects. Vet Q 18:95–99
- Moore BD, Balasuriya UB, Hedges JF, MacLachlan NJ (2002) Growth characteristics of a highly virulent, a moderately virulent, and an avirulent strain of equine arteritis virus in primary equine endothelial cells are predictive of their virulence to horses. Virology 298:39–44
- Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 8:49–62
- Rahman MM, McFadden G (2011) Modulation of NF-kappaB signalling by microbial pathogens. Nat Rev Microbiol 9:291–306
- Lee SM, Kleiboeker SB (2005) Porcine arterivirus activates the NF-kappaB pathway through IkappaB degradation. Virology 342(1):47–59
- Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4:499–511

9. Medzhitov R (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol* 1:135–145
10. Naiki Y, Michelsent KS, Zhang W, Chen Sh, Doherty TM, Arditi M (2005) Transforming growth factor- $\beta$  differentially inhibits MyD88-dependant, but not TRAM- and TRIF-dependant, lipopolysaccharide-induced TLR4 signalling. *J Biol Chem* 280:5491–5495
11. Seo SU, Kwon HJ, Song JH, Byun YH, Seong BL, Kawai T, Akira S, Kweon MN (2010) MyD88 signalling is indispensable for primary influenza A virus infection but dispensable for secondary infection. *J Virol* 84(24):12713–12722
12. Fuse K, Chan G, Liu Y, Gudgeon P, Husain M, Chen M, Yeh WC, Akira S, Liu PP (2005) Myeloid differentiation factor-88 plays a crucial role in the pathogenesis of Coxsackievirus B3-induced myocarditis and influences type I interferon production. *Circulation* 112(15):2276–2285
13. Zhong H, May MJ, Jimi E, Ghosh S (2002) The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol Cell* 9(3):625–636
14. Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR (2007) Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol* 81(3):1401–1411
15. Brasier AR, Tian B, Jamaluddin M, Kalita MK, Garofalo RP, Lu M (2011) RelA Ser276 phosphorylation-coupled Lys310 acetylation controls transcriptional elongation of inflammatory cytokines in respiratory syncytial virus infection. *J Virol* 85(22):11752–11769
16. Wang X, Hussain S, Wang EJ, Li MO, Garcia-Sastre A, Beg AA (2007) Lack of essential role of NF-kappa B p50, RelA, and cRel subunits in virus-induced type 1 IFN expression. *J Immunol* 178:6770–6776
17. Wang J, Basagoudanavar SH, Wang X, Hopewell E, Albrecht R, Garcia-Sastre A, Balachandran S, Beg AA (2010) NF-kappa B RelA subunit is crucial for early IFN-beta expression and resistance to RNA virus replication. *J Immunol* 185:1720–1729
18. Van Kasteren PB, Beugeling C, Ninaber DK, Frias-Staheli N, Van Boheemen S, García-Sastre A, Snijder EJ, Kikkert M (2012) Arterivirus and nairovirus ovarian tumor domain-containing deubiquitinases target activated RIG-I To control innate immune signalling. *J Virol* 86(2):773
19. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M (1999) The IKK $\beta$  subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 189(11):1839–1845
20. Hiscott J, Kwon JH, Genin P (2001) Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J Clin Invest* 107:143–151
21. Kang MH, So EY, Park H, Kim BS (2008) Replication of Theiler's virus requires NF-kappa B-activation: higher viral replication and spreading in astrocytes from susceptible mice. *Glia* 56(9):942–953
22. Dong X, Feng H, Sun Q, Li H, Wu TT, Sun R, Tibbetts SA, Chen ZJ, Feng P (2010) Murine gamma-herpesvirus 68 hijacks MAVS and IKK $\beta$  to initiate lytic replication. *PLoS Pathog* 6(7):e1001001