Acta virologica **56**: 3 – 9, 2012 doi:10.4149/av_2012_01_3

MINIREVIEW

Mechanisms of suppression of interferon production by porcine reproductive and respiratory syndrome virus

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Received August 22, 2011; accepted January 26, 2012

Summary. – The recently emerged porcine reproductive and respiratory syndrome virus (PRRSV) leads to one of the most economically significant infectious diseases of swine worldwide. The virus modulates the host innate and adaptive immunity to escape its immune response to facilitate the infection. Interferons (INFs) are principal antiviral cytokines, which represent components of the innate immunity and are regarded as a bridge between the innate and adaptive immunity. Currently, accumulating evidence indicates that the virus has developed various strategies to counteract the IFN production. Here, various mechanisms utilized by the virus to antagonize the IFN induction are reviewed.

Keywords: porcine reproductive and respiratory syndrome virus; interferon; nuclear factor-κB; interferon regulatory factor 3

Contents:

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Abbreviations: AP1 = activating protein 1; CBP = CREB-binding protein; IκB = inhibitor of NF-κB; IKKε = inhibitor of κB kinase ε; IFN(s) = interferon(s); IPS-1 = IFN promoter-stimulating factor 1; IRF3 = IFN regulatory factor 3; ISRE = IFN-stimulated response element; MDA5 = melanoma differentiation-associated gene 5; NF-κB = nuclear factor kappa B; NSPs = non-structural polyproteins; PAM = pulmonary alveolar macrophages; PRDs = positive regulatory domains; PRR = pattern recognition receptor; PRRSV = porcine reproductive and respiratory syndrome virus; RIG-I = retinoic acid-inducible protein I; TBK1 = TANK-binding kinase 1; TNF-α = tumor necrosis factor alpha

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), characterized by late-term reproductive failure in sows, and severe pneumonia in piglets and growing pigs, was initially recognized in the United States in 1987 and in Europe in 1990 (Keffaber, 1989; Wensvoort *et al*., 1991; Beura *et al*., 1992; Rossow, 1998). A typical but not often observed sign of the disease is "blue ears". Abortion, and high morbidity and mortality always result in great economic losses to the swine industry (Rossow, 1998). Previous data showed that PRRS causes in America an economic loss of approximately $560.32 million annually (Neumann *et al*., 2005). Now the disease is spread worldwide and tends to be endemic in most populations, though national and regional variations occur.

PRRSV (the family *Arteriviridae*, the genus *Arterivirus* (Meulenberg, 2000)) is an enveloped single-stranded RNA virus with a positive-sense genome of approximately 15.0 kb in length. It was first identified in the Netherlands in 1991 and subsequently in 1992 in the United States (Wensvoort *et al*., 1991; Benfield *et al*., 1992; Collins *et al*., 1992). According

to the genomic nucleotide sequence, the viruses were divided into two genotypes, European genotype (Type I) and North American genotype (Type II) (Allende *et al.*, 1999; Nelsen *et al.*, 1999). Since its emergence, the virus evolved continuously, a new type of PRRSV had been identified in the U.S., and in 2006 a pandemic of highly pathogenic PRRSV in China imposed tremendous economic loss on the farmers (Ropp *et al.*, 2004; Fang *et al.*, 2007; Tian *et al.*, 2007; Tong *et al.*, 2007). Despite an extensive research, a vaccine that protects against all field strains of PRRSV is currently not available.

The host innate immune response is the first line of defense to prevent against viral infection. The defensive cytokines, such as type I IFNs and tumor necrosis factor alpha (TNF-α) produced by macrophages and dendritic cells during virus infection, are critical components of innate immunity (Samuel, 2001; Kawai and Akira, 2006; Haller and Weber, 2007; Randall and Goodbourn, 2008). There are three types of IFNs. Type I IFNs comprise the largest sub-family and include the IFN-α (13 subtypes in humans) and IFN-β, of which IFN-β provides more robust protection against invading pathogens (Taniguchi *et al.*, 2001; Pestka *et al.*, 2004). Type II IFN has just one member, IFN-γ, which is mainly considered a powerful immunomodulatory cytokine. The type III IFNs or IFN-λs have been established to be the ancestral antiviral system of vertebrates (Levraud *et al.*, 2007). Rapid production of IFNs contributes to the termination of early viral replication and the development of an adaptive immune response, thus it is considered to be an essential defense line against invading viruses.

Notably, productive infection predominantly occurs in pulmonary alveolar macrophages (PAM) and monocyte-derived dendritic cells, which are the major source of defensive cytokine production (Wang *et al.*, 2007). During the course of infection, multiple strategies are directly employed by the virus to impair IFNs production and subsequently block the prime of adaptive immune response (Albina *et al.*, 1998; van Reeth *et al.*, 1999). Consequently, the elimination of infection by the immune system is depressed and usually is followed by severe infection or even death. Here, the current knowledge about the mechanism utilized by PRRSV to antagonize IFNs induction, especially for the type I IFNs was dissected and summarized.

2. Some viral non-structural polyproteins (NSPs) block IFN-β promoter

The genome of PRRSV contains 9 ORFs: ORF1a and ORF1b code for two large NSPs PP1a and PP1ab, and ORF2a, ORF2b, and ORF3-7 code for structural proteins GP2a, GP2b (E), GP3, GP4, GP5, M, and N, respectively. PP1a and PP1ab are co- or post-translationally cleaved by 4 putative (auto-) proteases into NSP1α, NSP1β, and NSP2-12 (Meulenberg *et al.*, 1993; Wassenaar *et al.*, 1997; Snijder and Meulenberg, 1998; Wootton *et al.*, 2000).

It has been evidenced that PRRSV infection directly interferes with type I IFNs transcriptional activation (Miller *et al.*, 2004). Further research indicated that the virus codes for proteins antagonizing the type I IFNs response (Bowie and Unterholzner, 2008). It is critical to dissect the detailed interplay between the viral protein and type I IFN induction. Recently, the role of PRRSV NSPs, except NSP6, in mediating IFN antagonism has been identified. In that research, each NSP was cloned into an eukaryotic expression plasmid. The NSP constructs along with interferon regulatory factor 3 (IRF3) and human IFN-β-chloramphenicol acetyl transferase (IFN-β-CAT) were co-transfected into HeLa cells. With subsequent CAT assays, it was found that the modulation of IFN-β promoter by IRF3 was suppressed to various degrees by NSP1, NSP2, NSP4, and NSP11, respectively, among which NSP1 showed the strongest inhibitory effect (Beura *et al.*, 2010). Obviously, the activation of IFN-β promoter by IRF3 was interrupted by some NSPs. The role of NSPs in the inhibition of both IFN-α and the other types of IFNs is not clear. Whether other viral proteins, such as GP2a, GP2b (E), GP3, GP4, GP5, M, or N, which have not been tested, interfere with type I IFN production is unknown. Of note, most of the viral proteins are expressed simultaneously or subsequently in virus infected cells. Hence, whether or not some proteins act synergistically to antagonize type I IFNs induction deserves further research.

3. NSP1 and NSP11 suppress production of type I IFN by interfering with IRF3

Induction of IFNs production can be triggered by many viruses and bacteria, including components of these microorganisms, such as glycoproteins, double-stranded RNA (dsRNA), and DNA containing unmethylated CpG dinucleotides. Toll-like receptor 3 (TLR3), a pattern recognition receptor (PRR), recognizes viral RNA and responds by activating a signaling transduction cascade that culminates in the induction of IFN type I and establishes an antiviral state (Hertzog et al., 2003). The central players of this signaling network are IRF3 and IRF7, which are essential for modulation of the innate immune response (Taniguchi *et al.*, 2001, Miller *et al.*, 2009). The activation of TLR3 by dsRNA leads to the activation of multiple transcription factors, such as nuclear factor κB (NF-κB) and IRF3 (Miller *et al.*, 2009). During the course of PRRSV infection, dsRNA is introduced as a by-product or an intermediate. There is, however, no evidence indicating that the intermediate dsRNA is involved in IFNs induction. The modulation of

type I IFN production by single viral protein is currently being intensely investigated.

The IFN-β promoter contains four positive regulatory domains (PRDs), which are the binding sites for three different transcription factors: IRF3 (PRDs I and III), NF-κB (PRDII), and activating protein 1 (AP1) (PRD IV). Maximal activation of the IFN-β promoter requires binding of the transcription factors to the corresponding sites to form an enhanceosome in the PRDs, which is stabilized by the association with the coactivator, CREB-binding protein (CBP)/p300 (Randall *et al*., 2008). It has been demonstrated that PRRSV suppresses IFN-β transcription by interfering with the activity of IRF3 through the inactivation of IPS-1 (IFN promoter-stimulating factor 1), which serves as an upstream adaptor for the IRF3, but not for NF-κB and AP1 (Luo *et al*., 2008). Subsequently, to investigate the molecular mechanism of the inactivation of IPS-1 by PRRSV, further research with luciferase reporter assay was performed, which indicated that both NSP1α and NSP1β mediate the inhibition of IPS-1 and this results in the suppression of IRF3 (Chen *et al*., 2010).

TBK1 (TANK-binding kinase 1) and IKKκ (inhibitor of κB kinase ε) are essential downstream components of retinoic acid-inducible protein I (RIG-I)/IPS-1 caspase. IRF3 is phosphorylated by the kinases TBK1 and IKKε, dimerizes, and translocates to the nucleus, where it eventually upregulates the transcription of IFN-α/β mRNA (Fitzgerald et al., 2003; Sharma et al., 2003; Sankar et al., 2006). It was observed that during PRRSV infection, the downstream pathway of TBK1/IKKε is still intact, thus, PRRSV interferes with the targets located upstream of the kinases TBK1 and IKKε (Luo *et al*., 2008). Further research confirmed that both NSP1α and NSP1β suppress TBK1- and IKKε-mediated reporter gene expression, which is consistent with previous conclusion (Chen *et al*. 2010).

IRF3, a key transcription factor of IFNs, normally resides in the cytoplasm in an inactivated form and translocates to the nucleus following phosphorylation on Serine/Threonine in response to the stimulation (Weaver *et al*., 1998). The binding and recruitment of IRF3 to the interferon-stimulated response element of promoters is stabilized by CBP/p300, which prevents the IRF3 export from the nucleus. Therefore, CBP is targeted by many viruses to regulate the IFN response, a strategy employed e.g. by Thogoto virus in order to interfere with IFNs production (Jennings *et al*., 2005). It was shown that PRRSV NSP1-induced CBP degradation via the proteasome-dependent pathway results in the suppression of IFR3 (Kim *et al*., 2010). It has been established that IRF3 activity was suppressed by PRRSV NSP1α subunit in a PRD I-III region-specific manner, and that the dsRNA-induced IRF3 phosphorylation and nuclear translocation could be blocked by PRRSV NSP1β (Beura *et al*., 2010; Song *et al*., 2010). Thus, it was suggested that IRF3 plays a role as a direct target for the viral intervention to block IFN production.

Collectively, previous research indicated that PRRSV evolved complicated tactics to directly or indirectly inactivate IRF3 in order to inhibit the IFN-β induction. It was shown that NSP1α and NSP1β are involved in the inhibition of IRF3 phosphorylation and nuclear translocation, which blocks IFN production. An upstream regulator of TBK1 and IKKε (downstream of RIG-I/IPS-1 caspase) serves as a target for this inhibition. For example, IPS-1, one of the activators of TBK1 and IKKε, is inactivated by NSP1. This can also account for the inactivation of the IFN-β promoter.

4. NSP1 affects NF-κB

Virus-host interactions lead to the modulation of complex intracellular signal transduction pathways in a sophisticated manner, resulting in antiviral responses or enhanced viral replication and virulence. In particular, the NF-κB pathway appears to be an attractive common target regulated by multiple viral pathogens (Santoro *et al*., 2003). NF-κB is an inducible transcription factor, which modulates more than 100 genes, such as pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2), and inducible nitric oxide synthase (iNOS), which play a key role in inflammation, innate immune responses and initiating adaptive immunity, the regulation of cell proliferation and cell survival (Keffaber, 1989; Caamano *et al*., 2002; Li and Verma, 2002; Santoro *et al*., 2003). In addition, temporally activated NF-κB confers essential innate antiviral response against cytoplasmic RNA viruses in an IFN-independent manner, showing the importance of NF-κB in initiating the innate antiviral response (Bose *et al*., 2003; Seth *et al*., 2006). Thus, much attention has been focused on the relation between the virus infection and the evasion of host antiviral response and NF-κB signaling.

Previous research showed that NF-κB pathway can be activated by some viruses. For example, hepatitis C virus, dengue virus, and herpes simplex virus have evolved strategies to activate NF-κB to facilitate their replication, to maximize viral progeny production, or to subvert the host immune response (Jan *et al*., 2000; Goodkin *et al*., 2003; Waris *et al*., 2003). It has been demonstrated that PRRSV infection resulted in increased nuclear translocation of NF-κB and increased its DNA binding activity both in MARC-145 cells and PAM cultures. PRRSV-mediated NF-κB activation is initiated by the degradation of the inhibitor of NF-κB (IκB) protein and ROS induction. It was also found that different molecules were involved in NF-κB activation in a PAM culture infected with PRRSV compared to MARC-145 cells. In details, NF-κB activation by PRRSV is mediated, at least

in part, by IκBα degradation in MARC-145 cells, while it was mediated by the degradation of IκBα, IκBβ, and IκBε in PAM cultures. Interestingly, the virions binding to its cognate cellular receptor could not trigger NF-κB activation, thus it was inferred that this activation of viral replication is viral protein expression-dependent (Lee and Kleiboeker, 2005). Coinciding with this observation, another group later reported that PRRSV infection activated two transcription factors, NF-κB and AP1, which are necessary for the IFN-β induction (Luo *et al.*, 2008).

The modulation of NF-κB by NSP1 was examined using tumor necrosis factor alpha (TNF-α) and poly (I:C) as an extracellular and intracellular stimulator, respectively, with the NF-κB-dependent luciferase reporter system (Song *et al.*, 2010). It was observed that NSP1 and NSP1α antagonize both TNF-α- and poly (I:C)-induced NF-κB activation. Furthermore, it was found that the C-terminal 14 amino acids of NSP1α were crucial for this suppression. The transcription factors NF-κB exist as homodimers or heterodimers of five distinct proteins: p50, p52, RelA/p65, RelB, and cRel (Li and Verma, 2002). Its classical form is a heterodimer composed of p50 and RelA/p65, and these proteins are normally sequestered in the cytoplasm through binding to their inhibitor IκBα. Upon stimulation, IκBα is phosphorylated by IκB kinases (IKKs) and targeted for degradation via ubiquitination, causing NF-κB to be released from IκBα and translocated to the nucleus. For NF-κB activation, the p65-p50 heterodimer is required to migrate to the nucleus, where it binds to the DNA sequence to induce the transcription (Senftleben *et al.*, 2001; Liang *et al.*, 2004). Therefore, nuclear translocation of p65 is generally considered to be a marker for NF-κB activation. Previous research indicated that NSP1α inhibits the phosphorylation of IκBα, nuclear localization of p65 is blocked, and thus it aborts the function of NF-κB (Song *et al.*, 2010). In addition, it has been reported that the suppression of NF-κB by PRRSV NSP1α also inhibited TNF-α induction (Subramaniam *et al.*, 2010). This observation contradicts a previous report that NF-κB is activated during PRRSV infection (Lee and Kleiboeker, 2005). Analysis at different time points in their experiments accounts for this discrepancy. The activity of NF-κB was assayed in the late phase of infection (24, 36, 48, and 60 hrs post infection) by Lee and Kleiboeker (2005). As to this issue, we inferred that NSP1, a non-structural viral protein synthesized early during infection, was employed by the virus to suppress NF-κB response. As the infection progresses, a series of viral proteins are translated, and possibly a viral protein strongly activated NF-κB. Which protein acts as the stimulator is unknown. In addition, when MARC-145 cells were transfected with the NF-κB-luciferase reporter and infected with PRRSV, the reporter activity increased at days 1 and 2 after infection, which coincides with the findings of Lee and Kleiboeker (2005) (Song *et al.*, 2010).

Furthermore, Beura *et al.* (2010) observed that both NSP1β and NSP11 could inhibit NF-κB-responsive promoter. The suppressive role of NSP1β in NF-κB-mediated signaling was further confirmed by the evidence that low level of IL-8 is induced in dsRNA-treated HEK293-TLR3 cells stably expressing NSP1β. Besides NSP1β, NSP1α also has been reported to suppress NF-κB activation. (Beura *et al.*, 2010; Song *et al.*, 2010; Subramaniam *et al.*, 2010). This was different from previous results reported by Lee and Kleiboeker (2005). We concluded that the activation of NF-κB is blocked by PRRSV NSP1 at an early stage during the virus infection. In contrast, it is activated in a viral replication-dependent manner in a late phase of the infection.

5. Virus infection interfered with RIG-I and TLR3 signaling pathways

Pathogen-associated molecular pattern (PAMP) in dsRNA is recognized by various pattern recognition receptors (PRRs) including TLR3 and cytoplasmic RNA helicase, such as RIG-I or melanoma differentiation-associated gene 5 (MDA5). RIG-I and MDA-5 are two sensors of intracellular dsRNA containing a caspase recruitment domain (CARD), and they bind to CARD of their adaptor IPS-1 (Yoneyama *et al.*, 2004; Saito *et al.*, 2007). The activated IPS-1 recruits the two downstream kinases, TBK1 and IKKε, which are the common signal molecules in RIG-I and TLR3 signaling pathways and are responsible for the phosphorylation and activation of IRF3 and NF-κB. The coordinately activated transcription factors induce a set of cytokine genes including type I IFNs (Thanos and Maniatis, 1995).

It has been reported that PRRSV interfere with IPS-1 molecule, a component between RIG-I and TBK1/IKKε in the signaling pathway, in response to the dsRNA stimulation. PRRSV also inhibits TRIF, the adaptor molecule of TLR3. But compared to RIG-I signaling pathway, the inhibition effect of PRRSV on TLR3 signaling is minor. Hence, PRRSV mainly interferes with RIG-I signaling pathway, which in turn suppresses IFN-β production and subsequent innate immune response (Luo *et al.*, 2010). In conclusion, PRRSV primarily interferes with RIG-I signaling pathway via inactivation of IPS-1 adapter molecule, thereby suppressing IFN-β production.

6. Conclusions

PRRSV has evolved complicated strategies to fight both innate and adaptive immune response. Series of questions remain to be resolved in PRRSV immunology, such as the delayed appearance of neutralizing antibodies, a short cell-mediated immune response, and a negligible level of

IFNs production (Mateu and Diaz, 2008). IFNs are critically important for the immune response. To initiate or to enhance the powerful IFNs induction is the primary element to be considered in the development of novel vaccine or therapeutics.

Both IRF3 and IRF7 play an essential role in modulating the activity of innate immune response by the regulation of type 1 IFN expression. The absence of IRF7 or combined absence of IRF3 and IRF7 prevents induction of IFN-α and IFN-β following viral infection or TLR-stimulation of dendritic cells (Sato *et al.*, 2000; Honda *et al.*, 2005). Currently, the research focuses mainly on the identification of specific viral proteins responsible for the antagonism of IRF3, and NSP1 was found to be the major player. A definite study addressing the response of IRF7 during PRRSV infection is rare, and it needs additional study.

Acknowledgements. The study was supported by the grant No. U0931003 from NSFC-Guangdong Joint Foundation, grant No. 2009ZX08010-022B from the Ministry of Agriculture of China and grant No. 09XD1405400 from the Excellent Scientist Program of Shanghai.

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