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Review article

The viral innate immune antagonism and an alternative vaccine design for PRRS virus

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) remains one of the most economically significant diseases in the swine industry worldwide. The current vaccines are less satisfactory to confer protections from heterologous infections and long-term persistence, and the need for better vaccines are urgent. The immunological hallmarks in PRRSV-infected pigs include the unusually poor production of type I interferons (IFNs-α/β) and the aberrant and delayed adaptive immune responses, indicating that PRRSV has the ability to suppress both innate and adaptive immune responses in the host. Type I IFNs are the potent antiviral cytokines and recent studies reveal their pleiotropic functions in the priming of expansion and maturation of adaptive immunity. Thus, IFN antagonism-negative PRRSV is hypothesized to be attenuated and to build effective and broad- spectrum innate and adaptive immune responses in pigs. Such vaccines are promising alternatives to traditional vaccines for PRRSV.

Contents

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) appeared in pigs in the US three decades ago and became endemic in most pig farming countries globally. Shortly after, two prototype viruses have been identified as etiological agents independently in the Netherlands and the US, and named Lelystad and VR-2332, respectively (Benfield et al., 1992; Wensvoort et al., 1991). PRRSV forms the family Arteriviridae along with equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) (Snijder et al., 2013). Subsequent studies reveal that the genomic sequences of Lelystad and VR-2332 share only approximately 60% identity and thus form the Europe genotype (genotype 1) and the North American genotype (genotype 2), respectively (Allende et al., 1999; Nelsen et al., 1999; Wensvoort et al., 1991). PRRSV has been evolving greatly since its emergence, and a recent variant of the genotype 2 in China shows much higher morbidity and mortality compared to previous isolates (Tian et al., 2007; Xiao et al., 2010). In contrast to the genetic variations of the virus, clinical signs of the disease are similar for both genotypes and exhibit the respiratory disease in young pigs and abortion in pregnant sows and gilts (Benfield et al., 1992; Chand et al., 2012; Magar et al., 1995; Nelsen et al., 1999).

The genome of PRRSV is a single-stranded positive-sense RNA of approximately 15 Kb in length with the 5′-cap and 3′-polyadenylated tail (Meulenberg et al., 1993; Nelsen et al., 1999; Wootton et al., 2000). The PRRSV genome contains 11 open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3 through 7, plus the newly identified ORF5a and ORF2(TF) (Fang et al., 2012; Firth et al., 2011; Johnson et al., 2011). ORF1a and ORF1b take up 75% of the genome in its 5′-proximimity and code for two large polyproteins, pp1a and pp1ab, of which the latter is produced by −1 ribosomal frame shifting in the overlapping region (Den Boon et al., 1991). Pp1a contains four viral proteases: two papain-like cysteine proteases PLP1α and PLP1β, poliovirus 3C-like cysteine proteinase (CP), and serine proteinase (SP) residing in nonstructural protein (nsp) 1α, nsp1β, nsp2, and nsp4, respectively. The processing of pp1a and pp1ab for PRRSV nonstructural proteins is reviewed elsewhere (Fang and Snijder, 2010; Snijder et al., 2013). PLP1α, PLP1β, and PLP2 are responsible for cleaving nsp1α, nsp1β, and nsp2 off from pp1a, while the serine proteinase in nsp4 cleaves the remaining nsps. From the polyprotein processing, a total of 14 nsps are generated including the newly identified nsp2TF and nsp2N encoded by the newly identified ORF2(TF) within the nsp2 coding sequence, which is generated by −1/-2 ribosomal frame shifting (Fang et al., 2012). The remaining quarter of the viral genome consists of structural genes coding for two major membrane proteins (GP5 and M), five minor membrane proteins (GP2a, GP3, GP4, GP5a, and E), and the nucleocapsid (N) protein.

2. Host responses to PRRSV infection

PRRSV infection is comprised of three phases: acute infection, persistent infection, and extinction. During the acute phase, viremia appears as early as 6 to 12 h post-infection and the PRRSV replicates swiftly in alveolar macrophages and dendritic cells in the lungs and the upper respiratory tract. This phase usually lasts for 3 to 4 weeks until the clearance of viremia (Wills et al., 1997). PRRSV can also build a persistent infection (Allende et al., 2000). During this phase, viral replication occurs only in the lymphoid tissues including tonsils and lymph nodes without viremia excluding the spleen (Beyer et al., 2000; Rowland et al., 2003a), and the persistence lasts for an extended period of up to 180 days. Since PRRSV can persist in the lymphoid tissues, the virus spreads easily from infected pigs to naive pigs. Such transmission makes hard to control PRRS in the field. The virus is eventually eliminated from infected hosts (Linhares et al., 2014; Torremorell et al., 2002), indicating that the pigs are able to develop immune responses that finally clear the infection. The response kinetics to eliminate PRRSV is slow and the overall response is delayed. The reason for this delay may be due to multi-phases of infection, poor induction of innate immune response, poor and delayed production of neutralizing antibodies, and/or delayed maturation and proliferation of cell-mediated immune responses (Lunney et al., 2016; Sun et al., 2012a).

2.1. Innate immune response to PRRSV infection

2.1.1. Type I IFN production and signaling

The innate immune response is the first line of defense of a host in response to infection. Type I interferons (IFNs-α/β) are the most potent antiviral cytokines against invading viruses and trigger expressions of IFN-stimulated genes (ISGs) (Schneider et al., 2014). Virtually all nucleated cells have the ability to produce IFNs-α/β, although plasmacytoid dendritic cells (pDC) are the most potent producers of type I IFNs (Liu, 2005). When an RNA virus enters the cell, a series of recognition mechanisms are triggered within the cell. The RNA genome of the virus is sensed by pattern recognition receptors (PRRs) which are consisted of two major categories; retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) distributed in the cytoplasm and toll-like receptors (TLRs) residing in the endosomal membrane. These receptor families work together to maximally sense the “foreigners” despite of their locations (see Wu and Chen, 2014 for innate immune sensing). The signaling cascades are then turned on by PRRs. RIG-I binds to viral RNA and undergoes a conformational change to expose its caspase activation and recruitment domain (CARD) at its N-terminus. The CARD domain then binds to the IFN promoter stimulator-1 (IPS-1) (also known as Cardif, MAVS, or VISA) on the mitochondrial membrane through a CARD-CARD interaction. IPS-1 is an adaptor which recruits the NEMO, TRAF3, and TANK proteins to form a complex, and the complex activates TBK1 and IKKε. They are the kinases responsible for activating IRF3 and IRF7 by phosphorylation. The phosphorylated IRF3 and IRF7 then form a homodimer or heterodimer and are subsequently translocated to the nucleus (Lin et al., 1998). The dimer in the nucleus is recruited by the CREB (cyclic AMP responsive element binding)-binding protein (CBP) to form an IFN enhanceosome (Panne et al., 2007). Inside the enhanceosome, activated IRF3- or IRF7-dimer binds to the positive regulatory domains (PRDs) I–III regions in the IFN promoters which drives IFNs gene transcription (Dragan et al., 2007; Honda et al., 2005; Sun et al., 2012a). Besides, IPS-1 may also trigger the activation of IKKα and IKKβ which phosphorylate IκB to allow degradation through a proteasom-dependent pathway. Degradation of IκB

results in the release of NF-κB which is another transcription factor that binds to PRDs of IFN genes as well as genes coding for pro-inflammatory cytokines (Caamano and Hunter, 2002; Honda et al., 2005; Napetschnig and Wu, 2013). Other than activation of the RIG-I-mediated signaling cascades, TLR3 and TLR7 are also involved in sensing viral RNAs in the endosome (Karikó et al., 2004; Kawai and Akira, 2011). TLR3 binds to dsRNA. During the viral genome replication, dsRNA intermediates are produced, thus TLR3 can recruit the TRIF, NAP1 and TRAF3 adaptors, which leads to the activation of TBK1 and IKKε, like RIG-I does, and activates IRF3 and drives IFN gene expression. Similarly, TLR3 can also activate the NF-κB pathway through the TRAF6, RIP1, TAK1, and IKK (both −α and −β) signaling (Honda et al., 2005; Wu and Chen, 2014).

Once type I IFNs are produced, they are released from the cell and bind to their receptors (IFNAR1 and IFNAR2) on the same cell (autocrine) or neighbor cells (paracrine). The IFN binding to receptors triggers the Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2) activation, which results in phosphorylation and dimerization of signal transducers and activator of transcription-1 (STAT1) and STAT2 to follow the formation of IFN-stimulated gene factor 3 (ISGF3) complex after recruitment of IRF9. ISGF3 then enters the nucleus and binds to IFN-stimulated regulatory response elements (ISRE) in the promoters of IFN-stimulated genes (ISGs). ISGs are the major executors of IFNs to establish an antiviral status of cells and/or to regulate other cellular functions (Ivashkiv and Donlin, 2014; Schneider et al., 2014).

2.1.2. Type I IFN response to PRRSV infection

Porcine alveolar macrophages (PAMs) are the main target cells for PRRSV infection (Duan et al., 1997). Evidence is available to show that DCs are also susceptible for PRRSV replication (Loving et al., 2007). The IFN responses during PRRSV infection have been studied and reviewed previously (Han and Yoo, 2014; Sun et al., 2012a; Yoo et al., 2010). The IFN response is meager in PRRSV-infected pigs (Albina et al., 1998b), and remains low shortly after transient elevation below the detection level in the lungs of pigs where PRRSV actively replicates (Van Reeth et al., 1999). Those studies show that the IFN response against PRRSV is poor, suggesting that the virus may actively suppress the IFN production in vivo. Similarly, the suppression of IFN has also been observed in vitro in PRRSV-infected MARC-145 cells and porcine alveolar macrophages (PAMs) (Albina et al., 1998b; Buddaert et al., 1998; Miller et al., 2004). The poor induction of type I IFN response during infection suggests that PRRSV may have adopted various strategies to suppress the induction and function of IFNs-α/β. Subsequent studies shows that the suppression of IFN production is a major strategy of PRRSV to modulate host antiviral defense. At least six viral proteins (nsp1α, nsp1β, nsp2, nsp4, nsp11, and N) have so far been identified as IFN antagonists, and their mechanisms of action are partially characterized (Han and Yoo, 2014; Lunney et al., 2016; Sun et al., 2012a; Yoo et al., 2010). Type I IFNs in swine are comprised of at least 39 functional subtypes, which is more than double of humans, plus 16 pseudogenes in the type I IFN family (Groenen et al., 2012; Sang et al., 2010). For swine, 17 IFN-α subtypes are found and they contain anti-PRRSV activities (Sang et al., 2010). The second largest subtypes of swine type I IFNs is IFN-δ which contains 11 members, and this population of type I IFNs is growing and rapidly evolving (Dawson et al., 2013; Groenen et al., 2012; Sang et al., 2014a). The rest of swine type I IFNs includes seven subtypes of IFN-ω, and a sole type of IFN-β, IFN-ε, IFN-κ, and IFN-α/ω (Groenen et al., 2012; Sang et al., 2014a, 2010). The antiviral effects of these subtypes are dependent on the cell types and invading viruses. It seems apparent that the type I IFN system in swine is more complicated than that of human, and many subtypes remain unclear in their antiviral actions for PRRSV, which warrants further studies on the interactions between PRRSV and these subtypes. The majority of mechanistic studies for type I IFN antagonism by PRRSV have focused on IFNs-α/β, largely due to their dominant role for antiviral activity.

2.2. Dendritic cells (DCs)

In addition to building an antiviral status by stimulating numerous ISG expressions, type I IFNs can also suppress viral infection indirectly by targeting NK cells, DCs, T cells, and B cell. The production of type I IFNs is in favor of a host to defend infection via three major actions: First, type I IFNs induce intrinsic antiviral activities through the JAK-STAT signaling pathway and the expression of hundreds of antiviral proteins (Schneider et al., 2014). Second, type I IFNs can also enhance antigen presentation in DCs and their migration to lymph nodes. The improved antigen presentation by DCs facilitates the maturation and proliferation of T cells. Third, type I IFNs activate and promote T cells and B cells directly by enhancing their antigen presentation, cytokine productions including IFN-γ, and chemokine production. The importance of type I IFNs in the development of adaptive immunity draws attentions and depicted in Fig. 1.

2.2.1. Regulation of DC functions by type I IFNs

Dendritic cell is the bridge between the innate and adaptive immunity. Type I IFNs regulate the function of DCs through an autocrine manner (Baranek et al., 2012) and activate the immature DCs to functional antigen-presenting cells (APCs) (Gessani et al., 2014). The MHC expression in DCs is elevated, so as in CD80 and CD86 which contribute to the DC activities to stimulate T cells from peripheral blood in humans or bone marrows in mice (Ito et al., 2001; Montoya et al., 2002). During vaccinia virus infection or lymphocytic choriomeningitits virus (LCMV) infection, type I IFNs promote the ability of DCs to cross-present antigens to T cells, as well as modulating the antigen survival and processing by T cells (Le Bon et al., 2003; Spadaro et al., 2012). Bringing viral antigens from the site of infection to lymph nodes and presenting them to T cells is how DCs perform their antigen presentation. Type I IFNs positively regulate the expression of MIP-3β, CCR-7, and Th1-type chemokines, and the DC migration is affected by these chemokines. Thus, it seems clear that type I IFNs promote mature DCs to migrate to lymph nodes, contributing to their antigen-presentation activities as shown in humans and mice for PBMCs (Parlato et al., 2001; Rouzaut et al., 2010). IL-12 is another cytokine that potently regulates NK cells, T cells secreting IFN-γ, and Th1 cells in response to viral infection (Gautier et al., 2005). Bone marrow-derived DCs produce IL-12 in humans or mice, and this activity is tightly regulated by type I IFNs (Gautier et al., 2005). The importance of DCs in regulating normal function of adaptive immune cells is amplified by the fact that, in type I IFN receptor-deficient DCs, the norovirus persistence is increased even in the presence of enhanced adaptive immunity (Nice et al., 2016). In that study, type I IFNs primed DCs, and mature DCs were necessary for adaptive immune response. Not only on the positive regulation of DCs by type I IFNs, an attention needs to be paid on the negative modulation. Thus, depending on the circumstance, type I IFNs can suppress DC maturation or IL-12 secretion (Cousens et al., 1997; Dauer et al., 2003). DCs are the major cell population expressing type I IFNs and connect the innate immunity to adaptive immunity, which makes DCs crucial for activation and maturation of adaptive immunity. Fine tuning on DCs by IFNs can enhance the role in the network important for the both innate and adaptive immune responses (Gommerman et al., 2014).

2.2.2. Porcine DC response to PRRSV infection

Since PRRSV can infect DCs (Loving et al., 2007), the response of DCs to PRRSV infection is of interest. The production of type I IFNs

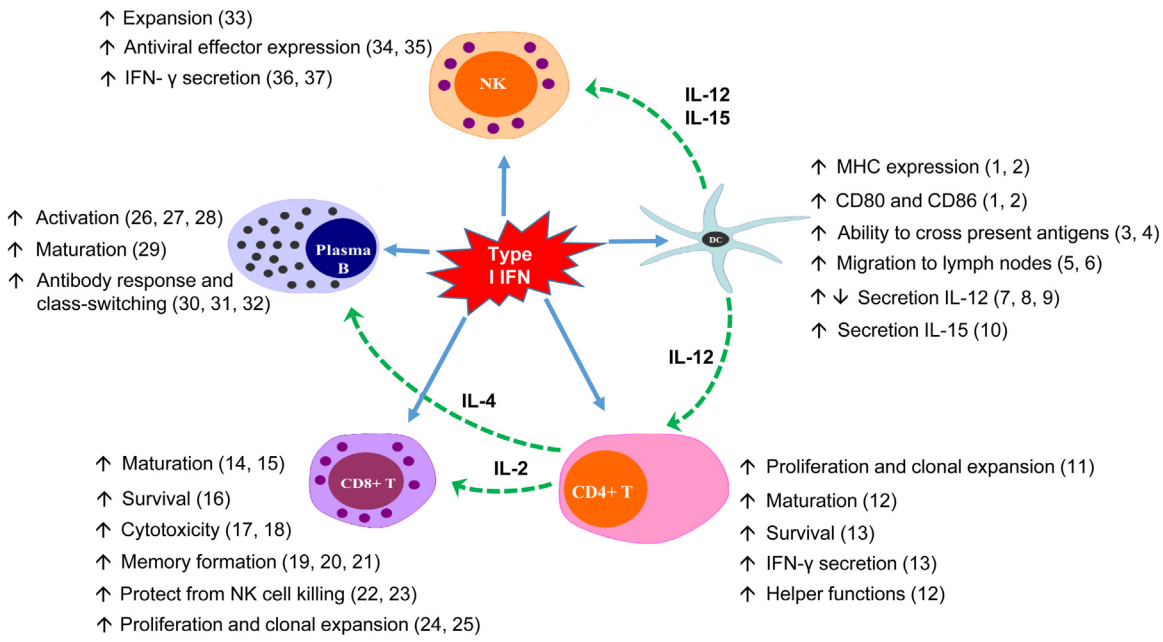

Fig. 1. Pleiotropic roles of type I IFNs for regulation of adaptive immunity. Type I IFNs stimulate dendritic cells (DC), CD4+ T cells, CD8+ T cells, B cells, and NK cells (indicated in solid arrow lines). Type I IFNs also promote the cross-talk of DCs with NK cells and CD4+ T cells. The inter-connection of CD4+ T cells with CD8+ T cells and plasma B cells help the development of adaptive immunity (indicated in dotted arrow lines). Vertical arrows indicate positive regulation (↑) or negative regulation (↓) of cellular functions by type I IFNs. Numbers in parenthesis indicate relevant references. '(h)' associated with references in the figure legend denotes the information derived for humans and '(m)' denotes the information derived from murine models. (1) Ito et al., 2001 (h), (2) Montoya et al., 2002 (m), (3) Le Bon et al., 2003 (h), (4) Spadaro et al., 2012 (h), (5) Parlato et al., 2001 (h), (6) Rouzaut et al., 2010 (m), (7) Gautier et al., 2005 (h, m), (8) Cousens et al., 1997 (m), (9) Dauer et al., 2003 (m), (10) Lucas et al., 2007 (m), (11) Havenar-Daughton et al., 2006 (m), (12) Le Bon et al., 2006b (m), (13) Brinkmann et al., 1993 (m), (14) Le Bon et al., 2006a (m), (15) Pinto et al., 2011 (m), (16) Urban et al., 2016 (m), (17) Marshall et al., 2010 (m), (18) Jennings et al., 2014 (m), (19) Kolumam et al., 2005 (m), (20) Ramos et al., 2009 (m), (21) Thompson et al., 2006 (m), (22) Crouse et al., 2014 (m), (23) Xu et al., 2014 (m), (24) Curtsinger et al., 2005 (m), (25) Keppler et al., 2012 (m), (26) Chang et al., 2007 (m), (27) Coro et al., 2006 (m), (28) Rau et al., 2009 (m), (29) Heer et al., 2007 (m), (30) Bach et al., 2007 (m), (31) Fink et al., 2006 (m), (32) Purtha et al., 2008 (m), (33) Martinez et al., 2008 (m), (34) Lucas et al., 2007 (m), (35) Hu et al., 2014 (h), (36) Hwang et al., 2012 (m), (37) Madera et al., 2016(h).

by pDCs is severely suppressed during PRRSV infection (Baumann et al., 2013; Calzada-Nova et al., 2010). Furthermore, the pDCs activation, which is featured by the increase of CD80/86 expression and cell-type development, is not seen by PRRSV (Calzada-Nova et al., 2011). Since pDCs are not permissive for PRRSV (Calzada-Nova et al., 2011), the failure of pDC activation during PRRSV infection may be due to the lack of type I IFN production, suggesting a role of type I IFN for priming the adaptive response against PRRSV.

2.3. NK cells

2.3.1. Regulation of NK cell functions by type I IFNs

Type I IFNs enhance the survival and function of NK cells directly or indirectly in murine models and human cells (Fig. 1) (Paolini et al., 2015). NK cells kill virus-infected cells directly, or secret IFN-γ and promote other antiviral functions indirectly. In influenza virus-infected or vaccinia virus-infected mice, a deficient production of type I IFNs results in the reduced activation of NK cells, reduced cytolytic effector expressions, and reduced IFN-γ secretion (Hwang et al., 2012; Martinez et al., 2008). Such phenomena are also observed in LCMV-infected mice where type I IFN promotes IFN-γ secretion in NK cells through the STAT4-dependent pathway. Furthermore, anti-LCMV activity of NK cells is also enhanced (Mack et al., 2011). The decision to stimulate cytolytic effectors expression or IFN-γ secretion is dependent on the balance of STAT1 and STAT4 after IFN signaling activation (Hwang et al., 2012). These NK cell activities can also be regulated by IL-15, which is an ISG product after IFN binding to IFNARs on DCs in mouse models (Lucas et al., 2007). In the TLR-stimulated mice, increased accumulation of NK cells is seen in the local lymph nodes. This activity is mediated via IFN-primed DCs to increase the IL-15 production (Lucas et al., 2007). An independent study shows that both pDCs and monocytes (MOs) indirectly stimulate NK cell activation through IFN-α secretion during human herpes simplex virus-1 (HSV-1) infection of PBMC (Vogel et al., 2014). Indeed, type I IFNs can promote the NK cell expansion during murine cytomegalovirus (MCMV) infection (Madera et al., 2016). A worth noting is that the IFN-induced intrinsic antiviral activity of NK cells is less of importance but NK cells respond to IFNs effectively. During MCMV infection, DCs respond to IFNs well by producing IL-15. While NK cells are less responsive to IFNs directly, these cells respond to IL-15 potently (Baranek et al., 2012). A long-term treatment of chronic hepatitis C patients with IFN-α enhances the cytotoxicity of NK cells and results in the decrease of viral RNA in the serum of the patients (Hu et al., 2014). This indicates that IFN-α can fine-tune the NK cell activity through regulating the balance between activation and inhibition to suppress the chronic infection.

2.3.2. Porcine NK cell response to PRRSV infection

Nature killer (NK) cell is an additional arm important in the antiviral innate immune defense. NK cells nonspecifically target virus-infected cells by recognizing "altered-self antigen" characterized by reduced MHC-I expression (Gerner et al., 2009). The NK cell response to PRRSV infection has been reviewed elsewhere (Lunney et al., 2016), and here we will only discuss the negative regulation of NK cell function by PRRSV. The cytotoxic function of NK cells is reduced in PRRSV-infected pigs and the reduced function begins from 2 days after infection and lasts for three to four weeks (Dwivedi et al., 2011a; Renukaradhya et al., 2010). This phenomenon is common for the field isolates and modified-live vaccine (MLV), and also for differently administrated routes of infection (Albina et al., 1998a; Dwivedi et al., 2011b; Renukaradhya

et al., 2010). Notably, experimentally infected pigs show low levels of IFN-α. Even though there is no clear correlation between the low level of type I IFNs and the reduced cytotoxic activity of NK cells, NK cell function is directly regulated by type I IFNs along with other cytokines such as interleukin (IL)-12 and IL-15 (Nguyen et al., 2002).

2.4. T lymphocytes

2.4.1. Regulation of T cell functions by type I IFNs

In addition to modulating T cells through regulating DCs and cytokines release, type I IFNs can also regulate T cells directly through IFNARs on the surface of T cells. Type I IFNs modulate the survival, proliferation, IFN-γ secretion, cytotoxic function, and memory development of T cells (Fig. 1). T cells are divided to CD4+ and CD8+ cells. CD4+ T cells include T-helper 1 (Th1), T-helper 2 (Th2), follicular T-helper (Tfh), and regulatory T (Treg) cells. Each population has a distinct function in the adaptive immune system. All of these commitment of CD4+ T cells make up the crucial role of T cells in the adaptive immunity (Zhu and Paul, 2008). Type I IFNs stimulate CD4+ T cells undergoing clonal expansion during LCMV infection in mice (Havenar-Daughton et al., 2006). CD4+ T cells primed by type I IFNs increases the ability of T cells to help B cells to secrete antibodies (Le Bon et al., 2006b). IFN-α increases the ratio of Th1 cells secreting IFN-γ and inhibits the suppressive effect of IL-4 on IFN-γ production in those cells (Brinkmann et al., 1993). After completing their roles, activated CD4+ T cells undergo either memory or apoptosis. In case of virus infection, IFNs-α/β maintains activated CD4+ T cells live longer and perform their antiviral functions (Marrack et al., 1999). In pigs infected with PRRSV, IL-4 is upregulated at 2 days post-infection (Dwivedi et al., 2012). However, IL-4 does not promote the B cell development in PRRSV-infected pigs and instead suppresses the antibody and IL-6 secretion (Murtaugh et al., 2009). It seems that porcine IL-13 takes over the role of IL-4 in pigs and drives the maturation of monocyte-derived DCs (Bautista et al., 2007). The role of porcine IL-13 in priming B cells is unknown for pigs. This observation leads to a cautious interpretation of immunological data for different animal species.

Studies have focused on the interaction of CD8+ T cells and type I IFNs because these cells lyse virus-infected cells. Type I IFNs regulate CD8+ T cells for their survival, clonal expansion, cytotoxic activity, IFN-γ secretion, and memory cell differentiation (Fig. 1). The ability of type I IFNs to upregulate the survival and clonal expansion of CD8+ T cells has been studied for different viruses. During LCMV and vesicular stomatitis virus (VSV) infections in mice, type I IFNs contribute to the activation and longer survival of CD8+ T cells and a larger number of clonal expansion (Aichele et al., 2006; Curtsinger et al., 2005; Keppler et al., 2012; Kolumam et al., 2005; Le Bon et al., 2006a, 2003; Marrack et al., 1999; Urban et al., 2016). For West Nile virus (WNV) infection, type I IFNs also promote the maturation of CD8+ T cells (Pinto et al., 2011). Additional studies unveil that type I IFNs can enhance the cytotoxicity of CD8+ T cells in mice (Agarwal et al., 2009; Curtsinger et al., 2005; Jennings et al., 2014; Marshall et al., 2010). Interestingly, type I IFNs have both positive and negative effects on IFN-γ secretion by CD8+ T cells for different viruses (Cousens et al., 1999; Nguyen et al., 2000, 2002). Such differences are resulted from the balance between the levels of STAT1 and STAT4. Besides effector CD8+ T cells, memory CD8+ T cells are also regulated by type I IFNs. Type I IFNs promote the differentiation of memory CD8+ T cells via affecting the initial clonal expansion during virus infection (Kolumam et al., 2005; Ramos et al., 2009; Thompson et al., 2006). Reactivation of memory CD8+ T cells to effector CD8+ T cells is dependent on the presence of type I IFNs (Kohlmeier et al., 2010; Martinet et al., 2015; Soudja et al., 2012). Furthermore, type I IFNs can also protect T cells from killing by NK cell by expressing NCR1 (natural cytotoxicity triggering receptor 1; CD335) which is the negative signal for NK cells (Crouse et al., 2014; Xu et al., 2014).

2.4.2. Porcine T cell response to PRRSV infection

The T cell response in pigs to PRRSV is crucial for specific anti-PRRSV activities directly or indirectly. CD4+ T helper 1 (Th1) cells can become memory T cells or improve the killing efficacy of macrophages and the proliferation of CD8+ T cells by secreting IL-2 and IFN-γ. In contrast, CD4+ T helper 2 (Th2) cells can promote the maturation of B cells to plasma B cells and enhance the ability to secrete PRRSV-specific neutralizing antibodies. Alternatively, CD4+ Th2 cells can stimulate B cells to become memory B cells. Cytotoxic T lymphocytes (CTL) cause the death of infected cells specifically and directly. The T cell response of pigs during PRRSV infection is reviewed elsewhere (Loving et al., 2015). During infection, the number of CD4+ T cells decreases quickly in 3–7 days and then returns to normal by 7–14 day of infection (Nielsen and Bøtner, 1997; Shimizu et al., 1996). The number of CD4+ T cells is also limited in the lungs of infected pigs (Tingstedt and Nielsen, 2004). In addition, PRRSV-specific IFN-γ secreting T cells are low at all ages of pigs (Klinge et al., 2009). Such abnormal responses of CD4+ T cell in the host suggest that during PRRSV infection, the normal function of CD4+ T cells is interfered by the virus via indirect mechanisms. A large number of CD4+/CD8+ T cells are seen in pigs and these cells can be recruited to the sites of proliferation (Zuckermann and Husmann, 1996). The role of CD8+ T cells in clearing PRRSV persisting in the tonsils is unknown. In contrast to the crippled CD4+ T cell proliferation, regulatory T (Treg) cells which halt the activity of effector T cells (CD4+ T cells and CD8+ T cells) by producing TGF-β are significantly increased by PRRSV infection (Silva-Campa et al., 2012). The upregulation of IL-10 production by PRRSV also contributes to the Treg cell proliferation (Song et al., 2013; Suradhat et al., 2003).

2.5. B lymphocytes

2.5.1. Regulation of B cell functions by type I IFNs

The B cell-mediated antibody response is an important mechanism of host to eliminate invading viruses. Circulating neutralizing antibodies can potently clear the virus, and the antibody production by B cells is also modulated by type I IFNs (Fig. 1). During influenza virus infection of mice, IFNAR on B cells is necessary for B cell activation, suggesting the role of IFNs for B cell activation in response to influenza virus infection (Chang et al., 2007; Coro et al., 2006; Rau et al., 2009). Type I IFNs also modulate the isotype switching from IgG1 to IgG2a/c in influenza-specific B cells (Heer et al., 2007). Other reports also show the regulation of type I IFNs on B cell activation, antibody secretion, and isotype switching during viral infections, suggesting that type I IFNs regulate the B cells activity generally and deeply in mice (Bach et al., 2007; Fink et al., 2006; Purtha et al., 2008).

2.5.2. Porcine B cell response to PRRSV infection

The poor and delayed B cell-mediated specific antibody response is one of the major obstacles to control PRRSV in pigs. Even though PRRSV-specific antibody appears at early times of 7–9 day post-infection, the efficacy of these antibodies is unclear. Neutralizing antibodies against PRRSV takes a longer time to develop and appears nearly 1 month after initial infection (Labarque et al., 2000; Loving et al., 2015). The role of neutralizing antibodies for protection has been shown by serum transfer experiments, in which the pigs receiving passively transferred neutralizing antibodies are protected from PRRSV infection. However, the passive transfer of PRRSV-specific antibodies does

not confer a full protection against homologous challenge, indicating the PRRSV-specific antibodies do not completely block the infection, and a higher dose of neutralizing antibodies is required for better protection (Lopez et al., 2007; Osorio et al., 2002). The antigenic heterogeneity of PRRSV is an additional challenge for neutralizing antibodies to elicit efficient protection since the neutralizing activity of these antibodies is restricted to the homologous virus (Vu et al., 2011; Zhou et al., 2012).

3. PRRSV proteins and their roles in modulating type I IFN response

Suppression of type I IFN production and signaling is one of the major characteristics of PRRSV, which is a tremendous benefit for the virus to survive in the host. PRRSV has evolved to take several strategies to gain such benefits, and the IFN suppression is polygenic, which is executed by multiple viral proteins (Fig. 2). The viral proteins and their mechanisms of actions to suppress type I IFNs have been identified and reviewed elsewhere (Han and Yoo, 2014; Sun et al., 2012a; Yoo et al., 2010). Here, we briefly discuss the multiple ways that PRRSV modulates such functions. So far, five nonstructural proteins (nsp1α, nsp1β, nsp2, nsp4, and nsp11) and one structural protein (N) have been identified as IFN antagonists (Fig. 2) (Beura et al., 2010; Chen et al., 2010, 2014, 2016; Han et al., 2013; Han and Yoo, 2014; Kim et al., 2010; Patel et al., 2010; Sagong and Lee, 2011; Song et al., 2010; Subramaniam et al., 2010; Sun et al., 2012a,b, 2010; Wang et al., 2013a; Yoo et al., 2010).

3.1. Nsp1 as a negative regulator for type I IFN production

3.1.1. Nsp1α

Among the PRRSV proteins regulating type I IFN response, nsp1 (both nsp1α subunit and nsp1β subunit) is the most potent IFN antagonist. Nsp1 is the first viral protein synthesized and released from the polyprotein during infection. The PLP1α activity resides in the N-terminal portion of nsp1 and its catalytic resides are mapped to C76 and H146. This activity cleaves off the nsp1α subunit and releases it from pp1a (Fig. 2) (Den Boon et al., 1995). The cleavage site by PLP1α is mapped to M180/A181 and confirmed by two independent studies using peptide sequencing and X-ray crystallographic study (Chen et al., 2010; Sun et al., 2009). At the most N-terminal region of nsp1α, a zinc-finger motif (ZF1) of C8-C10-C25-C28 is identified, and this motif together with PLP1α determines the transactivation of viral subgenomic mRNA synthesis (Kroese et al., 2008; Tijms et al., 2007, 2001; Tijms and Snijder, 2003). A second zinc-finger motif (ZF2) of C70-C76-H146-M180 has been identified in the C-terminal portion of nsp1α (Sun et al., 2009). The function of ZF2 remains unknown.

Studies have unveiled the nsp1α-mediated suppression of type I IFN response in cells (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010). Similar to EAV nsp1 (note: EAV nsp1 remains uncleaved), PRRSV nsp1α is distributed in the both nucleus and cytoplasm (Tijms et al., 2002; Yoo et al., 2010). Despite that PRRSV nsp1 is a nuclear cytoplasmic protein, no specific nuclear localization signal (NLS) is found in nsp1α, suggesting that it may enter the nucleus by binding to a cellular nuclear protein. The nuclear localization of nsp1α suggests that the nuclear form of nsp1α may play a regulatory role for host gene expression. Subsequently, nsp1α and nsp1β are found to inhibit type I IFN production and impair the IFN promoter activity when stimulated by a dsRNA analogues (polyinosinic:polycytidylic acid; poly I:C) (Chen et al., 2010). The phosphorylation and nuclear translocation of IRF3 remain unchanged, indicating that nsp1α does not directly inhibit IRF3 function but inhibits the IFN production in the nucleus by blocking other components crucial for IRF3-mediated IFN expression. Subsequently, nsp1α has been found to trigger CBP

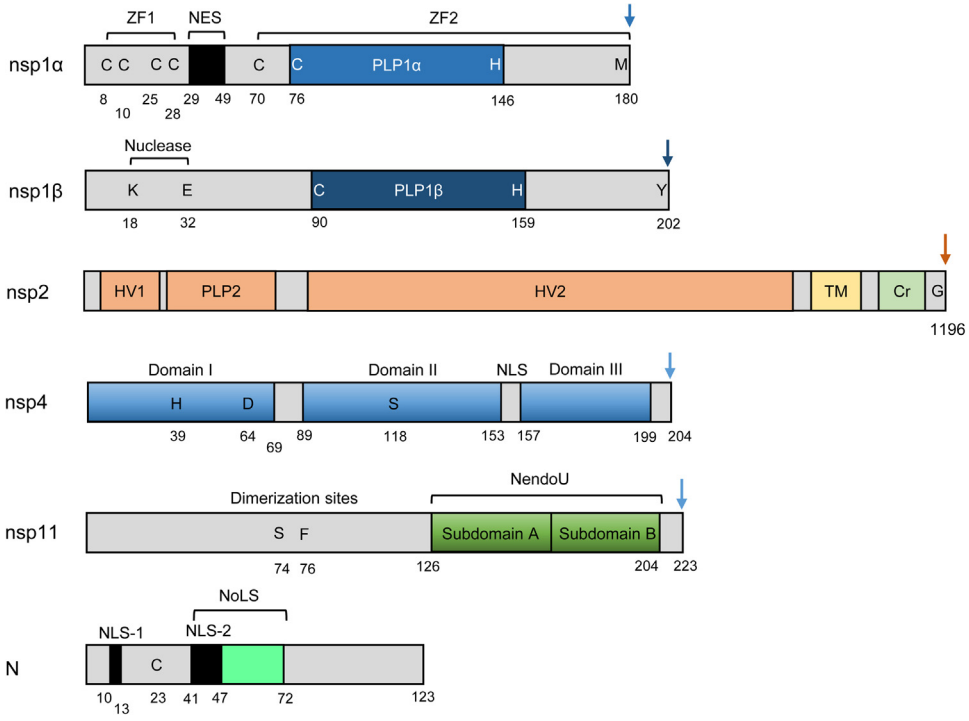

Fig. 2. Functional motifs and domains identified in the nsp1α, nsp1β, nsp2, nsp4, nsp11, and N proteins of PRRSV. Amino acids are presented in single letters. Numbers indicate amino acid positions. ZF1, zinc finger domain of C8-C10-C25-C28; ZF2, zinc-finger domain of C70-C76-H146-M180; PLP1α, papain-like protease domain of C76-H146; PLP1β, papain-like protease domain of C90-H159; HV, hyper-variable region; TM, transmembrane domain; Cr, cysteine-rich domain; NLS, nuclear localization signal; NoLS, nucleolar localization signal; NendoU, nidovirus-specific endoribonuclease; C23 of N, residue for homodimerization; Vertical arrows indicate the cleavage sites specific for PLP1α of nsp1α, PLP1β of nsp1b, PLP2 of nsp2, and serine protease (SP) of nsp4.

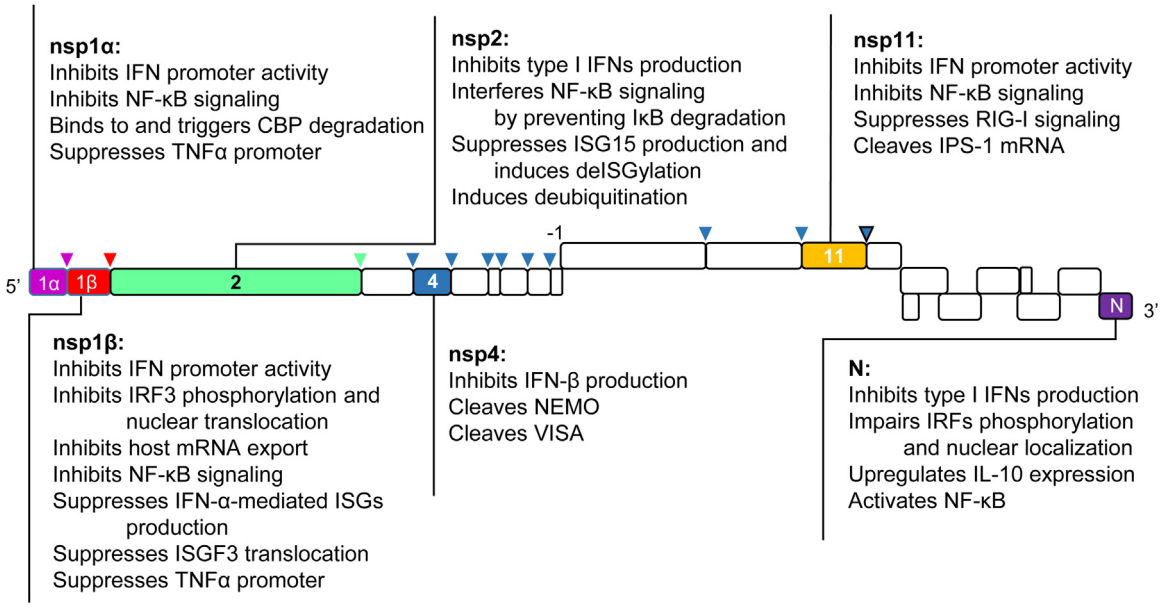

Fig. 3. Coding strategy of the PRRSV genome and type I IFN viral antagonists encoded by PRRSV. Viral IFN antagonists are indicated in magenta for nsp1α, red for nsp1b, light green for nsp2, yellow for nsp11, and purple for N. Vertical arrows indicate the specific cleavage sites for polyprotein processing. Colors match with respective proteinases and cleavage sites. IFN antagonistic functions are illustrated for individual viral proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degradation through the proteasome-dependent pathway (Fig. 3) (Han et al., 2013; Kim et al., 2010). The proteinase activity of PLP1α in nsp1α is no longer active, once is cleaved and released, due to the conformational change (Sun et al., 2009). Thus, the PLP1α activity does not seems to participate in the CBP degradation. The precise mechanism for CBP degradation by nsp1α is currently unknown. Recently, nsp1α of PRRSV has been reported to physically bind to CBP (Chen et al., 2016), which may explain a possible mechanism of nsp1α-mediated CBP degradation. In that study, a nuclear export signal (NES) has been identified in nsp1α and mapped to amino acids positions 29 to 49 (Fig. 2). The NES is crucial for nsp1α-mediated type I IFNs suppression. As discussed above, CBP-mediated enhanceosome formation is critical for both IRF3/7-mediated and NF-κB-mediated IFN productions (Panne et al., 2007). Without CBP, phosphorylated IRF3 in the nucleus does not stably activate IFN production. Interestingly, PRRSV nsp1α triggers the swine leukocyte antigen class I (SLA-I) degradation in a ubiquitin-proteasome dependent manner and this action is mediated by direct binding of nsp1α with both chains of SLA-I (Du et al., 2016). The fact that nsp1α binds to CBP and both chains of SLA-I and thus leading to degradation of CBP and SLA-1 provides new insights into a possibility that nsp1α may possess an ubiquitin E3 ligase-like activity. Such studies are under way.

PRRSV nsp1α also suppresses the NF-κB activation when stimulated by either poly(I:C) or TNF-α (Song et al., 2010; Subramaniam et al., 2010). This suppression is RIG-I dependent. In addition to PRD I/III in the IFN-β promoter for IRF3/7 binding, PRD II and PRD IV are specific for the binding of NF-κB and AP-1, respectively, to activate type I IFN production. The PRD II-specific reporter activity is significantly suppressed by nsp1α upon stimulation (Han et al., 2013). The CBP degradation may also interfere the NF-κB function as a type I IFN inducer in the nucleus. Additionally, nsp1α blocks the phosphorylation and nuclear translocation of NF-κB, suggesting that the cytoplasmic form of nsp1α may regulate the host response (Kim et al., 2010). Thus, nsp1α is a multifunctional protein such that the nuclear form of nsp1α degrades CBP whereas the cytoplasmic form of nsp1α blocks the NF-κB nuclear translocation and activation, making nsp1α as the potent IFN antagonist.

3.1.2. Nsp1β

As with nsp1α, nsp1β also possesses a proteinase activity (PLP1β) and the catalytic residues for PLP1β have been mapped to C90-H159. The PLP1β activity is responsible for the cleavage of nsp1β from nsp2 (Fig. 2) (Den Boon et al., 1995; Snijder et al., 1992; Ziebuhr et al., 2000). Thus, the release of nsp1β is dependent on the activities of PLP1α and PLP1β. PLP1α cleaves off nsp1α at M180/A181 of pp1a, and then PLP1β cleaves off nsp1β at Y382/G383 (Chen et al., 2010). The cleavage sites for PLP1α and PLP1β are highly conserved among different PRRSV isolates. PRRSV whose PLP1β active sites are mutated is non-viable, indicating that PLP1β is essential for virus replication (Kroese et al., 2008). The PLP1β activity in nsp1β also regulates the generation of nsp2TF via −1/2 ribosomal-frameshifting (Fang et al., 2012; Li et al., 2016). A nuclease motif has been identified at K18-E32 in the N-terminal region of nsp1β (Xue et al., 2010), but this motif is irrelevant to IFN suppression (Han et al., 2014).

Although PLP1β loses its activity once cleaving and releasing nsp1β from nsp2 (Sun et al., 2009), it still plays crucial roles in nsp2TF gene expression, type I IFNs suppression, and viral replication. The highly conserved motif in PLP1β plays a critical role in IFN suppression by nsp1β when stimulated with Sendai virus (Li et al., 2013). PRRSV in which mutations are introduced in the conserved motif of PLP1β exhibits attenuated growth, and the suppression of type I IFNs is less potent (Li et al., 2016, 2013). Several studies show that nsp1β suppresses type I IFN production (Chen et al., 2010; Han and Yoo, 2014; Patel et al., 2010). The precise mechanism for nsp1β-mediated IFN suppression remains unclear. A study shows that modified nsp1β, of which 26 amino acids derived from nsp1α are extended at its N-terminus impairs the IRF3 phosphorylation and nuclear translocation (Beura et al., 2010). Whether these 26 amino acids derived from the C-terminus of nsp1α contribute to this activity is unclear. Like nsp1α, nsp1β is also distributed in the nucleus, indicating that nsp1β-mediated IFN suppression is a nuclear event. It is

interesting to note that, although the PLP1β proteinase activity becomes inactive once nsp1β is cleaved, the conserved motif for PLP1β is still a major determinant for nsp1β-mediated IFN suppression. A worth noting is the nuclease activity of nsp1β. Considering the nuclease activity and nuclear distribution of nsp1β, it is presumed that nsp1β may digest the nucleic acids in the nucleus. In addition, nsp1β interrupts the phosphorylation of STAT1 and nuclear translocation of ISGF3, thus leading to the inhibition of JAK-STAT signaling pathway resulting in the inhibition of ISG expressions (Chen et al., 2010; Patel et al., 2010). Nsp1β blocks the ISGF3 nuclear translocation by degrading karyopherin-α1 (KPNA1) (Wang et al., 2013b), which is at least one possible mechanism for nsp1β-mediate ISGs inhibition (Fig. 3). The compensatory activities of nsp1α (for suppression of IFN production) and nsp1β (for suppression of IFN production and JAK-STAT signaling) make the nsp1 protein the most potent type I IFN antagonist of PRRSV. Recent findings from our laboratory show that nsp1β suppresses the host mRNA export from the nucleus to the cytoplasm. The host mRNA nuclear retention is correlated with the IFN suppression of nsp1β. A SAP (SAF-A/B, Acinus, and PIAS) motif is found in nsp1β with the consensus sequence of $_{126}$-LQxxLxxxGL-$_{135}$ residing in the PLP1β domain, and this motif is responsible for the host mRNA nuclear retention and IFN suppression (Han et al., 2017). The SAP-negative mutant PRRSV is attenuated for viral replication in vitro and for virulence in vivo. These findings explicit a specific mechanism of IFN suppression by nsp1β and implies the importance of IFN suppression for viral pathogenesis.

3.2. Nsp2

Nsp2 is the largest protein of PRRSV and five major domains have been identified: 1) hyper-variable region 1 (HV1) at the N-terminal region, 2) papain-like cysteine protease 2 (PLP2) which belongs to the ovarian tumor (OTU) protease family at the N-terminal region, 3) very large hyper-variable region 2 (HV2) in the middle, 4) hydrophobic transmembrane region (TM) near the C-terminus, and 5) a cysteine-rich domain close to the end of nsp2 (Fang et al., 2012; Han et al., 2007) (Fig. 2). The HV1 and HV2 regions in nsp2 account for the major genetic diversity of PRRSV (Music and Gagnon, 2010), and they are the key determinants for differentiation between genotypes 1 and 2 (Fang et al., 2004; Han et al., 2006). Mutations, insertions, and deletions are frequent in HV1 and HV2, whereas other regions in nsp2 are relatively conserved (Fang et al., 2004; Han et al., 2006; Music and Gagnon, 2010). The TM domain is involved in the membrane modification and the formation of a replication complex for PRRSV (Fang and Snijder, 2010). Thus, nsp2 plays an important role in the regulation of PRRSV replication (Wang et al., 2013a, 2012).

The PLP2 activity in nsp2 contributes to inhibiting the IRF3 phosphorylation and nuclear translocation and suppresses the type I IFN production (Li et al., 2010). The PLP2 domain belongs to the ovarian tumor (OTU) protease family which contains the de-ubiquitination (DUB) and de-ISGylation activities (Frias-Staheli et al., 2007). Ubiquitination and ISGylation are important post-translational modifications involved in the RIG-I and TLRs signaling pathways, and the OTU domain interferes them to antagonize type I IFN production. PRRSV nsp2 also inhibits the IκB phosphorylation and degradation when stimulated with TNF-α, resulting in the suppression of NF-κB activation (Sun et al., 2010). In addition to de-ubiquitination, the OTU domain also plays a role to inhibit ISG15 production and ISG15-mediated ISGylation (Fig. 3) (Sun et al., 2012b). Thus, the ISGylation activity of nsp2 interferes with the IFN production and signaling pathways.

3.3. Nsp4

The nsp4 protein of PRRSV is the 3C-like serine proteinase (SP) responsible for cleaving the nonstructural proteins from pp1a and pp1ab, except nsp1α, nsp1β, nsp2 (Snijder et al., 1996). Structural studies unveil three domains in nsp4: domain I (aa 1-aa 69), domain II (aa 89-aa 153), and domain III (aa 157-aa 199) (Tian et al., 2009). The SP activity of nsp4 is located between domain I and domain II and the residues of S118, H39, and D64 constitute the canonical catalytic triad (Tian et al., 2009). Interestingly, a nuclear localization signal (NLS) site is found at positions 154–156 which also serves the hinge between domain II and domain III (Tian et al., 2009). Whether the NLS in nsp4 is functional or not is unclear but it may involve in inhibiting the IFN-β transcription because the residue at 155 (a middle amino acid of the three-residues for NLS) is crucial for nsp4 to suppress type I IFNs response when induced with poly(I:C) (Chen et al., 2014). The nsp4-mediated IFN-β inhibition is NF-κB dependent. The activation of NF-κB is significantly suppressed by nsp4 when stimulated by RIG-I, VISA, TRIF, as well as IKKβ, suggesting that nsp4 interferes with the NF-κB signaling downstream of these molecules. The SP activity of nsp4 is responsible for suppressing IFN-β and NF-κB as shown by mutational studies in domains I and domain II which specifically knock-out the SP activity. Nsp4 appears to cleave NEMO at E349-S350, which is a key regulator for NF-κB signaling (Huang et al., 2014). In addition to the NF-κB suppression and thus NF-κB-mediated IFN suppression, nsp4 also cleaves VISA which is an essential adaptor in the RLR-mediated IFN pathway (Huang et al., 2016).

3.4. Nsp11

Nsp11 of PRRSV has the endoribonuclease activity and is a component of the RNA-dependent RNA polymerase (RdRp) complex for viral RNA synthesis (Fang and Snijder, 2010; Snijder et al., 2013). The endoribonuclease activity of nsp11 is conserved and unique for viruses in the order Nidovirales, designating this domain "NendoU" expanding from L126 to F204 (Fig. 2). The NendoU domain is relative to the XendoU family of Xenopus laevis (Gorbalenya et al., 2006; Ivanov et al., 2004; Laneve et al., 2003). As shown in Fig. 2, the NendoU domain resides near the C-terminus of nsp11 consisting of two subdomains; subdomain A and subdomain B (Posthuma et al., 2006; Shi et al., 2016). Subdomain A contains the nuclease activity of NendoU, and subdomain B serves as a conformational supporter (Shi et al., 2016). Similar to the coronavirus NendoU residing in nsp15 (Bhardwaj et al., 2004; Ivanov et al., 2004), PRRSV NendoU in nsp11 has the uridylate-specific RNA cleaving activity. Dissimilar to coronavirus nsp15, which forms a hexametric structure to achieve optimal enzyme activities (Xu et al., 2006), PRRSV nsp11 exists as a homodimer and elicits the nuclease activity (Shi et al., 2016). While two His residues form an "active site loop" and Val-Thr residues form a "supporting loop" in the NendoU, nsp11 NendoU of PRRSV and nsp15 NendoU of coronavirus are similar enough to be super-imposed (Bhardwaj et al., 2004; Ricagno et al., 2006; Snijder et al., 2003; Xu et al., 2006). The importance of NendoU activity in viral RNA synthesis and virus replication has been shown for EAV and SARS-CoV (Nedialkova et al., 2009; Posthuma et al., 2006).

PRRSV nsp11 is an IFN antagonist and the endoribonuclease activity is critical for IFN suppression when stimulated by poly(I:C) (Shi et al., 2011). PRRSV nsp11 blocks the phosphorylation and nuclear translocation of IRF3, resulting in the inhibition of IRF3-mediated IFN production. PRRSV nsp11 also suppresses the phosphorylation of IκB, and thus activation of NF-κB is suppressed (Sun et al., 2016, 2014). Inhibition of the two pathways leads to IFN suppression. Interestingly, PRRSV nsp11 appears to target MAVS

(IPS-1) and degrades MARV mRNA (Sun et al., 2016). The IRF3 signaling pathway and NF-κB signaling pathway converge at MAVS, and thus MAVS is a crucial adaptor for both signaling pathways. The degradation of MAVS mRNA by PRRSV nsp11 results in the failure of activation of downstream pathways. The nsp11 mutants which specifically lose the NendoU activity do not suppress IFN production, further indicating that the NendoU activity of PRRSV nsp11 is responsible for suppression of type I IFN production (Sun et al., 2012a, 2016).

3.5. Nucleocapsid (N) protein

The nucleocapsid (N) protein of PRRSV is a relatively small protein encoded by ORF7 of the viral genome. N is consisted of 123 amino acids for genotype 2 and of 128 amino acids for genotype 1. In PRRSV-infected cells, N is abundantly expressed to benefit from the discontinuous transcription mechanism (Snijder et al., 2013). The N protein binds to the genomic RNA through its N-terminal region containing positively charged residues (Dokland, 2010). Binding to RNA is one of the major functions of N to form a viral capsid and to protect the viral genome. N is the only protein that forms the viral capsid. The N protein exists as a homodimer through covalent and non-covalent interactions using C23 (Fig. 2) (Wootton and Yoo, 2003). PRRSV of which C23 is mutated loses its infectivity, indicating that the N protein homo-dimerization is critical for virus replication and infectivity (Lee et al., 2005). N is a phosphoprotein and the phosphorylation may be related to RNA binding and/or the dimerization of N even though its exact function remains unknown (Wootton et al., 2002). PRRSV replication occurs in the cytoplasm (Benfield et al., 1992; Mardassi et al., 1994), and thus the cytoplasmic distribution of N fulfills its function to support the particle assembly and replication. In addition it its cytoplasmic distribution, PRRSV N is also distributed in the nucleus and nucleolus in virus-infected PAMs and MARC-145 cells (Rowland et al., 1999). Two NLS sequences are found in the N-terminal half of the N protein and designated NLS-1 at residues 10–13 and NSL-2 at residues 41–47 (Fig. 2) (Rowland et al., 1999; Rowland et al., 2003b). NLS-1 seems non-essential for N protein nuclear translocation, whereas NSL2 is a functional motif. A single amino acid change in the conserved motif of $_{41}$PGKKNKK$_{47}$ for NLS-2 results in the blocking of N to nuclear translocation. (Rowland et al., 1999, 2003b; Rowland and Yoo, 2003). Further studies have unveiled that importin-α and importin-β are the cellular proteins responsible for transporting N to the nucleus (Rowland et al., 2003b; Rowland and Yoo, 2003). A NoLS sequence is mapped to residues 41–72 of N (Fig. 2) (Rowland et al., 2003b; Yoo et al., 2003). The function of N in the nucleolus is not very clear thus far. Several cellular proteins appear to interact with N, including fibrillarin (Yoo et al., 2003), I-mfa domain containing protein (HIC) (Song et al., 2009), protein inhibitor of activated STAT1 (PIAS1), nucleolin, B23, poly-A binding protein (Yoo et al., 2010), importin-α and −β, and exportin (Rowland et al., 2003b). Limited studies show that N protein delays the cell cycle progression and utilizes the cellular machinery for productive infection especially during the early stage of infection.

The list of cellular proteins interacting with N suggests the modulation of host cell functions through their interactions. In studies using NLS-2 knock-out mutant PRRSV, pigs develop a significantly shorter duration of viremia and higher neutralizing antibodies than those of wild-type PRRSV-infected pigs (Lee et al., 2006a,b). This observation suggests that the nuclear localization of N determines the PRRSV pathogenesis and NLS-2 is non-essential for PRRSV replication in vitro (Lee et al., 2006a,b). Overall, NLS-2 is correlated to the virulence of PRRSV and the mutations in NLS-2 confer the attenuation of virus in pigs.

Further studies show that PRRSV N suppresses the type I IFN production by inhibiting the IRF3 phosphorylation and nuclear translocation (Fig. 3) (Sagong and Lee, 2011). IL-10 functions as a negative regulator for host innate and adaptive responses (Couper et al., 2008), and PRRSV increases the IL-10 level in pigs (Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003; Thanawongnuwech and Thacker, 2003). The upregulation of IL-10 results in the suppression of the innate and adaptive immune responses in pigs. Subsequently, N has been identified as the protein upregulating IL-10 in PAMs (Wongyanin et al., 2012). The upregulation of IL-10 by N suggests that N activates NF-κB signaling pathway which tightly regulates the production of IL-10 (Saraiva and O'Garra, 2010). Indeed, the N protein has been shown to activate the NF-κB signaling and increases the expression of proinflammatory cytokines (Fu et al., 2012; Luo et al., 2008). The upregulation of proinflammatory cytokines are associated with PRRSV pathogenesis in pigs (Lunney et al., 2010; Van Reeth et al., 1999, 2002). Together, these findings indicate that PRRSV N, especially the nuclear form of N distributed in the nucleus, regulates the pathogenesis of PRRSV.

4. Alternative approaches to PRRS vaccines

The current vaccines for PRRS elicit protective immunity to some extent but the protection works only against homologous infections and is partial for heterologous infections. Even though PRRSV-specific neutralizing antibodies can protect pigs from homologous challenges to a certain level, higher titers of antibodies are required for complete protection (Lopez et al., 2007). Additional problems associated with existing vaccines include viral persistence and possible reversion to virulence. Incomplete protection against field viruses (heterologous PRRSV) makes current vaccines less valuable in the field (Kimman et al., 2009). Aberrant and delayed cell-mediated immune responses make hard to clear the virus persistently residing in the lymphoid tissues and even harder to control the transmission from persistently infected pigs to naive pigs. Thus, a vaccine that can improve the host anti-viral immune response including the innate immunity, CD4+ T cells, CTLs, and B cells will be of a significant benefit. Immunological hallmarks of PRRS are that the innate immunity (namely type I IFNs) is suppressed and the adaptive immunity is perturbed and delayed. Since type I IFNs prime the proliferation and maturation of adaptive immune responses, it is tempting to speculate that the disturbed and delayed adaptive immune responses against PRRSV is due to the suppression of type I IFNs response in host by PRRSV. A replication-competent recombinant PRRSV co-expressing various type I IFNs increases the IFN levels in pigs and elicits protection against PRRSV, but no significant PRRSV-specific adaptive immune responses are observed in this study (Sang et al., 2014b, 2012). Different observations have been made for an increased and prolonged adaptive immune response when using the porcine IFN-α expressing plasmid as an adjuvant for PRRSV vaccination (Meier et al., 2004). This suggests that the timing and routes to deliver IFNs and how long it will last in animals make a difference in priming the adaptive immune response against PRRSV infection. Studies by Brockmeier et al. (2009, 2012) show the protective role of IFN-α in pigs. In these studies, IFN-α was expressed in vivo by adenovirus-mediated gene delivery. Following virulent challenges with PRRSV, the pigs showed delayed viremia and enhanced PRRSV-specific cell-mediated immune response. These results indicate that a higher level and sustained production of IFNs for an extended period is critical for the control of PRRSV in pigs. During PRRSV infection, a swift response of type I IFNs is observed which may reach a relatively high level of IFN (Albina et al., 1998a,b; unpublished data from our laboratory). However, this response is

only transient and the IFN levels drop quickly as the infection progresses. One of the reasons for this decrease may be due to the potent viral IFN antagonism. Other than exogenous administration of IFNs, promoting endogenous IFNs is also thought to function better to prime the adaptive immune responses. Indeed, the induction of IFN production in vivo has resulted in the increase of adaptive immune responses (Wang et al., 2013c).

Nsp1β inhibits both type I IFN production and signaling potently (Beura et al., 2010; Chen et al., 2010; Patel et al., 2010; Wang et al., 2013b). As discussed above, the conserved sequence motif of $_{123}$-GKYLQRRLQ-$_{131}$ in PLP1β plays a critical role in the both IFN suppression and programming −2/-1 ribosomal frame shifting (Li et al., 2013). Single or double mutations in this motif (R128A, R129A, or RR128/129AA) impair the suppressive activity of nsp1β. Furthermore, PRRSV containing such mutations is attenuated for growth in cells. Pigs infected with the mutant PRRSV have shown lower levels of viremia, higher levels of IFN-α as well as ISGs comparing to pigs infected with wild-type PRRSV (Li et al., 2016). The NK cell function has also been increased, correlating with an increased level of IFN-α in the lungs of pigs infected with mutant virus. Furthermore, the IFN-γ level is also significantly higher in pigs infected with mutant virus. This study nicely demonstrates the role of type I IFNs playing in priming adaptive immune response and ultimate clearance of PRRSV in pigs. However, no direct evidence is available yet that such a mutant PRRSV can elicit protection from heterologous infection or reduced persistence. Such a study is cumbersome because of potential reversion to wild-type or compensatory mutations to restore the function during infection. This phenomenon has also been observed in our laboratory (unpublished data), suggesting the strong selective pressure on the viral IFN antagonism and the favor of IFN suppression for PRRSV replication in pigs. A recent study shows that the deficiency of type I IFN receptor in DCs makes the non-persistent murine norovirus (strain CW3) to systemically persist in mice (Nice et al., 2016). Surprisingly, during the persistent infection of the norovirus, CD8+ T cell function and antibody response are increased, suggesting that the deficiency of IFN responses is the determinant, leading to the virus to persistence in spite of the enhanced adaptive immunity. This finding correlates type I IFNs with viral persistence for the first time, further demonstrating the importance of type I IFNs for inhibition of virus infection either acutely or persistently. Thus, the removal of IFN antagonism from PRRSV seems a reasonable strategy to develop a novel vaccine candidate. Such strategy has been successful for some RNA viruses. Hepatitis C virus (HCV) infects humans and establishes persistence in the liver. During HCV infection, type I IFN response is downregulated, which is mediated by the NS3/4A protease which cleaves MAVS (Li et al., 2005). The suppression of IFN response may be one of the causes for HCV persistence, and the IFN treatment is most effective therapy to control the persistent HCV infection (Manns et al., 2001; Radkowski et al., 2005; Shiffman et al., 1999). In contrast, recent studies show that the LCMV persistence in mice is controlled or lost when type I IFN signaling is blocked, demonstrating the paradoxical role of type I IFNs in viral persistence (Teijaro et al., 2013; Wilson et al., 2013). In these studies, an IFN receptor-neutralizing antibody has been used in mice to block the IFN signaling pathway, and in these animals, the activation of adaptive immunity is diminished and the mice regain the control of persistent infection. These findings demonstrate the dual functions of type I IFNs in virus-host interactions depending on the type of viruses and the species of hosts (for a review, see Snell and Brooks, 2015). Nevertheless, it is logical to hypothesize that a vaccine virus lacking the IFN suppressive function may be more immunogenic and provide improved protection against virulent challenge than the parental virus. Foot-and-mouth disease virus (FMDV) is able to suppress type I IFN production in cattle, and may persist in the tonsils for up to 2 years. N^{pro} is the major IFN antagonist of FMDV, and the mutation of N^{pro} to knock-out the IFN suppression confers viral attenuation in both cells and animals. The IFN antagonism-negative FMDV elicits strong neutralizing antibody response in vaccinated animals, and these animals are completely protected from high doses of wild-type challenge as early as 2 days post-challenge (Díaz-San Segundo et al., 2012). For influenza virus, the NS1 protein is a viral IFN antagonist, and a similar approach has been applied to swine influenza virus (SIV). The IFN suppressive function has been removed from the NS1 gene, and by reverse genetics, mutant SIV has been generated such that the NS1 protein of mutant SIV is no longer able to suppress type I IFN production. This IFN antagonism-negative SIV appears to be attenuated in pigs and is able to stimulate the production of type I IFNs. The mutant SIV grows well in cells and pigs, and its attenuation in pigs is correlated with higher levels of IFNs in vivo (Solórzano et al., 2005). Furthermore, the pigs immunized with the IFN-antagonism-negative SIV are protected from both homologous and heterologous challenges. Induction of heterologous protection by IFN-antagonism-negative virus has also been demonstrated for avian influenza (Marcus et al., 2010). A live attenuated influenza virus vaccine (LAIV) candidate has been isolated from a pool of NS1-truncated mutants, and this virus is able to stimulate a higher level of IFN production than wild-type virus. The avian influenza virus containing a truncated NS1 gene is clinically attenuated and also able to elicit a higher level of IFNs and adaptive immunity (Jang et al., 2016; Marcus et al., 2010; Ngunjiri et al., 2015). Such vaccine candidates induce a good level of protection from heterologous challenges in different species of animals (avian, mice, and pigs), making them alternative vaccine candidates. Equine arteritis virus (EAV) can persist in infected horses, and type I IFN production is suppressed during EAV infection. As discussed above, the nsp2 protein contains the deubiquitinase (DUB) activity in the PLP2 motif, which is responsible for the inhibition of innate immunity during infection. The DUB activity has been separated from PLP2 by mutation, and DUB-negative mutant EAV has been generated. When horses are vaccinated with the DUB-negative EAV, the animals elicit higher levels of IFNs and adaptive immune responses. Upon virulent challenge of these horses, the DUB-negative EAV vaccinated animals are protected at a similar degree as its DUB-positive parental virus (van Kasteren et al., 2015). Therefore, such a strategy is worth trying for PRRSV as an alternative approach to developing a new vaccine. Despite the lack of solid experimental evidence for PRRSV at the present time whether an increased level of type I IFN production will improve the protection from heterologous infection as well as persistence, this is a logical approach to the control of PRRSV, and such studies are in progress by several research groups. Once proven, it will warrant the benefits to elicit effective and protective anti-PRRSV immune responses by vaccination. Such vaccines will be a promising alternative to existing vaccines for PRRSV.

5. Conclusion

The hurdles in controlling PRRSV include viral suppression of innate immunity, delayed adaptive response of host, and antigenic heterogeneity of PRRSV. Poor innate immunity and slow developments of neutralizing antibodies and CTLs result in the failure of clearance of virus, leading to viral persistence in the lymphoid tissues (Zuniga et al., 2015). Type I IFNs are critical components for development and maturation of adaptive immunity, and PRRSV has a potent ability to suppress the type I IFNs production and signaling directly, and to modulate the adaptive immunity indirectly. A key to the successful development of a future vaccine for PRRS is the removal of IFN suppression function from PRRSV such that a replication-competent and IFN antagonism-negative

PRRSV can be generated by reverse genetics. Such virus is anticipated to be attenuated and warrants most effective protection not only from homologous infection but also from heterologous infection.

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