STUDIES ON THE INTERACTION BETWEEN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND ITS NATURAL HOST CELL

BY

WEIYU CHEN

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in VMS-Pathobiology in the Graduate College of the University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

Doctoral Committee:

Professor Federico Zuckermann, Director of Research, Chair
Professor Daniel Rock
Professor Emerita Mariangela Segre
Associate Professor Joanna Shisler
Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus, causes a costly global disease of swine. PRRSV infects their alveolar macrophages (AMΦ) resulting in an interstitial pneumonia. The ability of PRRSV to modulate the production of interferon (IFN)α and tumor necrosis factor (TNF)α by affected AMΦ is implicated in the virus’s pathogenesis. In this regard, infection of porcine AMΦ with PRRSV reduced by >50% the amount of IFN-α otherwise produced following the cells’ exposure to synthetic dsRNA. Interestingly, there was no corresponding impairment of the activation of either interferon regulatory factor 3 (IRF3) or signal transducer and activator of transcription 1 (STAT1), or of the transcription of the IFN-α, IFN-β, or IRF7 genes. Rather, the reduction correlated with the phosphorylation of eukaryotic translation initiation factor 2 (eIF2)α via the protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) and the appearance of stress granules, indicating translational attenuation. Likewise, a TNF-α response to lipopolysaccharide (LPS) was inhibited when this toll-like receptor (TLR)4 agonist was introduced at 6 h post-infection, when peak TNF-α synthesis and eIF2α phosphorylation would coincide. In contrast, a synergistic TNFα response, due to NFkB activation via the inositol-requiring protein-1α (IRE-1α), was observed if LPS exposure occurred 4 h earlier enabling subsequent TNFα production to be unaffected by later eIF2α phosphorylation. Thus, representatives of two branches of the unfolded protein response (UPR) of AMΦ to PRRSV replication, IRE-1α and PERK, can enhance or suppress cytokine production by triggering the activation of NF-κB or eIF2α, respectively. It worth noting that the modulatory activities became critical when the PRRSV-infected cells were super-exposed to secondary stimulation of poly(I:C) or LPS. In which case exacerbated inflammation response in the lung
will develop and produce significant morbidity and death. In the case of PRRSV infection alone, these cytokine responses such IFN-α are barely detected in spite of massive dsRNA produced in PRRSV-infected porcine AMΦ cells. As dsRNA is currently considered to be the most potent type I interferon (IFN) agonist, it has been suggested that sequestration of viral dsRNA may also help viruses evade host innate immune detection by reducing exposure of viral dsRNA to viral nucleic acids cytoplasmic sensors. This predicted type of immune evasion by virus-induced intracellular membrane structures had been confirmed for flaviviruses. By coupling a selective permeabilization technique with immunostaining analysis, a positive correlation between the cytosolic exposure of virus dsRNA and a host type I IFN response was demonstrated. In this case, the extent of flavivirus dsRNA exposure was dependent on both the virus species and host cell type. Despite lacking definitive experimental evidence regarding members of the nidovirales family including arteriviruses and coronaviruses, it has been suggested that these viruses also utilize a similar immune evasion strategy. In this regard, the development of double membrane vesicle (DMV) structures has been associated with the activity of arterivirus nsp2 and its coronavirus equivalent, nsp3. Moreover, in our previous study, the arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV) was shown to be incapable of inducing type I IFN synthesis upon infecting porcine alveolar macrophages. Thus, PRRSV appears to utilize a passive innate immunity evading mechanism. In the present study, in conjunction with the application of selective permeabilization, our immuno-staining results demonstrated for the first time that during PRRSV infection the viral dsRNA is sequestered inside intracellular membranes, which could prevent detection of virus dsRNA by host’s viral nucleic acid sensor. Evidence that it is indeed the case was provided by a laboratory PRRSV strain with a unique and deleterious mutation in the N-terminal region of NSP2, for which the virus losses the ability to
sequester its dsRNA inside intracellular membrane structures and thus away from detection by viral nucleic acid sensors, resulting in a substantial type I IFN response upon infecting alveolar macrophages.
Acknowledgements

The journey of earning the Doctorate degree is long and arduous, and it is impossible to complete my study without the help and supports from the following people.

First and foremost, I would like to thank my wife, Hsiuting, for understanding the absentee husband and father in the past 6 years. Thank you for taking such a good care of me and our family, which allowed me to focus on my research. This dissertation was bound with your love and support. I also thank the rest of my family. To my kids, your smiles are the best stress reliever in the world. To the best, most wonderful parents and sister, your supports makes my life much much easier. The relationship with you is the most precious asset I’ve ever had.

I am very lucky to have Dr. Federico Zuckermann as my PhD advisor. He is very considerate, respectful and probably the least” bossy” advisor I’ve ever worked with. His great personalities reflect on both daily routine communications and academic discussions. Dr. Federico sees me as a person but not some kind of a “data generator”. This is extremely important for a graduate student with family like me. I still remembered that during my first two semesters, I was so stressed out because of school work as well as setting up my family in the US. Thanks to Dr. Federico’s understanding and allowing me some leeway in my benchwork. With secured family and school works, I was able to concentrate on the later research works. From the academic side, every time when we dealt with data frustrations, Dr. Federico always put away the negative emotions and focused on the solutions. He showed exceptional patience going through every detail that might cause problems and redesigned the experiment accordingly. This enabled a positive feedback loop between us, and should be put into the handbook of “How to be a Good Advisor”. I believe it is the attitude dealing with the ugly,
negative data that determines a successful researcher and Dr. Federico is the kind of example I’d like to follow.

I sincerely thank all members in my dissertation committee: Drs. Mariangela Segre, Dr. Daniel Rock, and Drs. Joanna Shisler. Your insight, feedback and advices were influential and essential throughout my PhD training.

For the wonderful people in our lab: Thanks to Drs. Calzada-Nova, who unselfishly shared her expertise in immunology and helped me to troubleshoot all kinds of bizarre results. Thanks to Dr. Mauricio Villamar, for taking care of various lab chores, which allowed me to concentrate on my study. Thanks to Dr. William Schnitzlein for conducting realtime RT-PCR analysis; and together with Dr. Robert Hussmann, proof read my manuscript and dissertation and provided important feedback.

I would also like to express my gratitude to the entire faculty and staff at the department of Pathobiology, especially the graduate program coordinator, Karen and Paula. You were always there when I needed your help.

Last but not least, I would like to thank to all my friends in CU area. You’ve helped me to enrich my life with your love and care.
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CHAPTER 1: LITERATURE REVIEW

1.1 PRRSV, THE VIRUS

1.1.1 Outbreak and epidemiology

In the late 1980’s, an outbreak of a mystery swine disease (MSD) with a syndrome of reproductive failure and respiratory stress was reported in the United States (Keffaber, 1989; Loula, 1991). At the early stage of the outbreak, the clinical outcomes of the disease were frequently described as inappetence, lethargy, fever and breathing difficulty mostly seen in younger animals. At the later stage, a higher mortality rate was observed in younger animals such as suckling and weaning pigs, which usually suffer from nutrition deprivation as well as secondary bacterial infection (Fig. 1.1A). Although the mortality rate was low in grower pigs, the symptom of reproductive failure became more prominent, especially in sows. Other than disruption of the estrous cycle, the pregnant sows tended to farrow the litter prematurely. This increased the number of stillborn or non-viable piglets in each affected litter (Fig. 1.1B) (Gordon, 1992; Hopper, White, & Twiddy, 1992; Keffaber, 1989; Loula, 1991). Around the same time frame, a disease with similar clinical signs devastated the pork industry in several European countries. It first appeared in Germany by the end of the 1990. Within a year, it spread westward from Netherland to Spain ((OIE), 1992). By 1995, the same disease swept across the European continent (Baron et al., 1992; Botner, Nielsen, & Bille-Hansen, 1994; Edwards, Robertson, & Wilesmith, 1992; Pejsak & I, 1996; Plana Duran, Vayreda, & Villarrsa, 1992; Valicek, Psikal, & Smid, 1997) and landed in the countries in Asia (Chang, Chung, & Lin, 1993; Hirose, Kudo, & Yoshizawa, 1995). The epidemic of this disease greatly reduced the pork
production worldwide. As a result, the estimated annual economic loss from this disease in the United States is about $660 million (Holtkamp et al., 2013).

The causative agent that fulfilled the Koch’s postulates for MSD was first isolated by the research group at the Central Veterinary Institute in the Netherland. It had been identified as a previously unknown virus by showing cell-type restriction of propagation and unaffected infectivity after 0.2 micron filtration (Terpstra, Wensvoort, & Pol, 1991; G. Wensvoort et al., 1991). The disease can be reproduced in the SPF-pig with the inoculant prepared from pig alveolar macrophage exposed to the tissue homogenate of affected animals (Terpstra et al., 1991; G. Wensvoort et al., 1991). Shortly thereafter, the etiology causing the MSD outbreak in the United States was also isolated using the continuous cell line, CL2621, to propagate the virus (Collins et al., 1992). The first virus isolated in the Netherland was named Lelystad virus while the US isolate was designed as VR-2332 virus. The two isolates obtained almost identical physical and biological qualities including indistinctive morphology under electron microscopy, same buoyant density, similar clinical outcome, loss infectivity after chloroform treatment and cross-reactivity to either virus by the sera from the affected animal (Benfield et al., 1992; Gert Wensvoort et al., 1992; G. Wensvoort et al., 1991). These evidence suggested that Lelystad and VR-2332 were closely related. The relatedness between two isolates was confirmed later by the molecular evidence showing the same genome organization as well as sequence homology (Benfield et al., 1992; Conzelmann, Visser, Van Woensel, & Thiel, 1993; Meulenberg et al., 1993). The term” Porcine Respiratory and Reproductive Syndrome Virus (PRRSV)” was first introduced by European group to refer to the Lelystad virus and later recognized by other researchers in this field. The PRRS virus was designed as a member of the family of Arteriviridea together with Lactate dehydrogenase elevating virus (LDV), Equine viral arteritis
(EAV) and Simian hemorrhagic fever virus (SHFV) under the order of Nidovirales (Conzelmann et al., 1993; Meulenberg et al., 1993; Pringle, 1996).

The shedding of the PRRSV was primarily done via the body fluid of the infected animals such as saliva, nasal secretion, semen, urine as well as blood (Christianson et al., 1993; Kurt D. Rossow et al., 1994; Swenson et al., 1994; Wills et al., 1997). Although a field study failed to recover the virus from the milk of infected sows, the virus could also be detected from the milk of the sows that was artificially exposed to PRRSV (Wagstrom, Chang, Yoon, & Zimmerman, 2001). Among these body fluids, the contaminated semen caused major concern due to the high demand for artificial insemination in pork industry. The infected boars were usually asymptomatic and could carry the virus in their semen for up to 3 months (Christopher-Hennings et al., 1995), which greatly increased the burden in disease control and the cost in maintaining functions of the reproduction system. Any activities involving body fluid exchange (contact) could be potential transmission routes. The horizontal transmission between individuals was spread via social behaviors of pigs — sharing the contaminated fomite and being exposed to insect vector (Cho & Dee, 2006). For vertical transmission, the PRRSV present in the blood of pregnant sows was able to cross the placenta and enter fetus circulation (Botner et al., 1994; Christianson et al., 1992; Terpstra et al., 1991). The trans-placental transmission was most efficient when viremia occurs at the third trimester of pregnancy which might explain the late-term abortion observed in the affected herd (Lager & Mengeling, 1995; Mengeling, Lager, & Vorwald, 1994).

In spite of the fact that the outbreaks caused similar clinical signs and that they occurred in both continents almost simultaneously, the phylogenetic evidence soon revealed that strains isolated in the same continent were genetically closer to each other and shared only about 60% of
amino acid identity to those isolated in the other continents. Accordingly, the isolates reassembled similar genetic features of Lelystad virus were grouped together and referred to as type I PRRSVs. The other isolates, genetically similar to VR-2332, were grouped and known as type II PRRSVs (M. Shi et al., 2010). The mechanism behind this significant divergence was controversial. However, the early divergence model proposed by Forsberg was most rational. According to the model, the most recent common ancestor of type I and type II PRRSV appeared around late 19th century, which might had been introduced to North America in the early 20th century (Forsberg, 2005). This gave the virus enough time to evolve independently in either continent. The origin of PRRSV was also an enigma. Based on the genetic evidence, Plagemann hypothesized that a particular strain of LDV crossed the species barrier, jumped from mouse host and was adopted in wild hog, sometime during the 19th century in central Europe. Thereafter, this virus circulated in pig population for over a hundred years. The disease caused by PRRSV was not noticeable until late 80’s when intensified farming system was introduced (Plagemann, 2003).

1.1.2 Replication of PRRSV

By using cryo EM analysis, the PRRSV virion can be visualized as a loose core enclosed in a spherical shell with an average diameter of 58 nm. While the lipid bilayer structure of outer-shell can be clearly seen, no clear structure can be defined for the inner core which is composed of a positive stranded genomic RNA encapsidated with viral nucleoprotein (Spilman, Welbon, Nelson, & Dokland, 2009). The replication of the PRRSV, like many arteriviruses, is restricted to the monocyte/ macrophage cell lineage, especially porcine alveolar macrophages during primary infection (Duan, Nauwynck, & Pensaert, 1997a).
The permissiveness of porcine AMΦ cells to PRRSV is rendered by, at least, the presence of heparin sulfate, sialoadhesin as well as cd163 on the surfaces of porcine AMΦ cells. The roles of these surface molecules had been reviewed in detail by Van Breedam et al., (Van Breedam et al., 2010). In brief, the accumulation of the virion on the surface of porcine AMΦ cells is achieved by the initial contact between virion and the ubiquitous surface molecule — heparin sulfate. This gives the virus a better chance to expose its sialic acid, extended from M/GP5 viral protein, to the specific receptor, sialoahesin, which is predominantly displayed by macrophages. Once the virus is captured by sialoadhesin, the PRRSV virion is internalized via the clathrin-mediated endocytosis pathway. The low pH in the early endosome triggers the uncoating process of the virion with the aid of another macrophage-specific surface molecule, CD163, via some unknown mechanism. This leads to the exposure of PRRSV genomic RNA to the cytoplasm, which initiates the viral gene expression as well as genome replication (Van Breedam et al., 2010).

The genome structure of PRRSV resembled the eukaryotic cellular mRNA including a coding region flanked by a capped 5’-UTR and a polyadenylated 3’-UTR. The coding regions of PRRSV genomic RNA are comprised of 10 ORFs (open reading frame). While all the accessory proteins required for viral RNA synthesis are encoded from the two large ORFs (ORF1a and ORF1b) occupying the three quarters of the genome at the 5’ end, the viral structure proteins are exclusively encoded from rest of ORFs (ORF2-7 including 2a and 5a) tightly packed at 3’ end of PRRSV genome (Fig. 1.2A) (Kappes & Faaberg, 2015; Snijder, Kikkert, & Fang, 2013; Snijder & Meulenberg, 1998). The biochemistry behind the genome replication and gene expression is highly conserved among the arteriviruses, therefore, it is acceptable to describe the PRRSV replication using experimental evidence obtained from other arteriviruses, mostly, the prototype
of arterivirus — EAV. Shortly after virion uncoating, the cytosolic genomic RNA is recognized out-of-the-box by host translation complex to synthesize the replicase polyprotein 1a (pp1a) expressed from orf1a as well as polyprotein 1ab (pp1ab) which is itself an extension of pp1a via -1 frameshift at genomic position of 7695 nt [VR-2332], expressed from orf1b (Fig. 1.2A). The odds for the generation of pp1ab via -1 frameshift event are about 1 to 6 (den Boon et al., 1991; Firth & Brierley, 2012; Kappes & Faaberg, 2015). Recently, an additional frameshift site displayed both -1 frameshift and -2 frameshift activity was identified within the orf1a. The resultants derived from -1 frameshift (pp1a-nsp2N) and -2 frameshift (pp1a-NSP2TF) comprise about 7% and 20% of total pp1a population respectively (Fang et al., 2012). Altogether these replicase polyproteins are digested into 14 non-structural proteins (nsp1a/b, nsp2-6, nsp7a/b, nsp8-12) by an array of four intrinsic proteases near the N terminal region, which includes three papain-like cysteine protease for rescue of nsp1a/b, nsp2 and one main serine protease for liberating all other nsps (Z. Chen, Lawson, et al., 2010; den Boon et al., 1995; Y. Li, Tas, Snijder, & Fang, 2012; Snijder, Wassenaar, & Spaan, 1992, 1994; Snijder, Wassenaar, van Dinten, Spaan, & Gorbatenya, 1996; van Aken, Zevenhoven-Dobbe, Gorbatenya, & Snijder, 2006; Leonie C. van Dinten, Rensen, Gorbatenya, & Snijder, 1999; L. C. van Dinten, Wassenaar, Gorbatenya, Spaan, & Snijder, 1996; Ziebuhr, Snijder, & Gorbatenya, 2000). Other than the protease domain, three transmembrane domains reside on nsp2, nsp3 and nsp5 were predicted. The function of these transmembrane nsps were thought to induce the intracellular membrane structure known as double membrane vesicle (DMV) and anchor the whole replication complex on the surface of DMV (Knoops et al., 2012; Snijder, van Tol, Roos, & Pedersen, 2001; van Hemert, de Wilde, Gorbatenya, & Snijder, 2008).
Once the replication complexes are assembled, they interact with the sequences at 3’ UTR of genomic RNA (Beerens & Snijder, 2006, 2007; Verheije, Olsthoorn, Kroese, Rottier, & Meulenberg, 2002) and initiate the synthesis of negative-stranded RNA template for generating either a full-length genomic RNA or a set of subgenomic mRNA (Fig. 1.2B) (Conzelmann et al., 1993; Meng, Paul, Morozov, & Halbur, 1996). In contrast to the straightforward synthesis of full-length template, the synthesis of subgenomic template relies on the “discontinuous RNA synthesis” which is first manifested in coronavirus and well-conserved in all arteriviruses (Pasternak, Spaan, & Snijder, 2006; Sawicki, Sawicki, & Siddell, 2007). A specific sequence known as transcription-regulation sequence (TRS) play an important role in the discontinuous RNA synthesis. For PRRSV, six TRS located upstream of each structure protein coding region are referred to as “body TRSs” which follow the “leader TRS” located at the genomic 5’UTR. The processivity of viral replication complexes is checked each time when it encounters any one of the six body TRS. Either it can pass through the body TRS checkpoint and continue the RNA synthesis or the synthesis reaction is suspended and resumed after reposition nascent RNA to the genomic 5’UTR via the base-paring between complimentary body TRS on the nascent RNA and the leader TRS on the genomic RNA (Fig. 1.2B) (Meng et al., 1996; Pasternak, Gultyaev, Spaan, & Snijder, 2000; Pasternak, van den Born, Spaan, & Snijder, 2001, 2003; van Marle et al., 1999). Later, these negative stranded subgenomic RNAs are transcribed into subgenomic mRNAs for encoding PRRSV structure proteins (Fig.1.2B). Based on this model, not only all subgenomic mRNA shared the identical 5’ and 3’ UTR but also the ORFs towards the 3’ end of genomic RNA get better chances to be expressed (de Vries et al., 1990; Pasternak, Spaan, & Snijder, 2004). Indeed, the three ORFs close to the 3’end of genomic mRNA are used to encode the major structure porteins including nucleocapsid (N), membrane protein (M) and major
glycosylated protein (GP5) while those close to the 5’ end are used to encode minor structure proteins including GP4, GP2 and E protein (Dea, Gagnon, Mardassi, Pirzadeh, & Rogan, 2000; Dokland, 2010; Kappes & Faaberg, 2015; Snijder et al., 2013).

At the last stage, the newly synthesized PRRSV genomic RNAs are encapsidated by nucleoprotein (Tijms, van der Meer, & Snijder, 2002). The encaspidated viral genome acquire the lipid envelop from the smooth ER or golgi complex in which the viral envelop proteins undergo maturation process (Wieringa et al., 2004; Wissink et al., 2005). The enveloped viral particles are released at plasma membrane via the classical exocytosis pathway (Dea, Sawyer, Alain, & Athanassious, 1995).

1.1.3 Pathogenesis of PRRSV

The host pulmonary cells reside in the alveolus and are continuously exposed to a wide range of foreign substances from innocuous antigen to infectious pathogens. Thus an intricate balance must be met to the point where pro-inflammatory response is suppressed to protect the lung from pathological damages in a healthy state yet can still be effectively mounted during the infected condition. This airway immune homeostasis is established via balancing the production of anti-inflammatory and pro-inflammatory cytokines and, to a lesser degree, phagocytic activity by Alveolar macrophage (Hussell & Bell, 2014).

PRRSV primarily targets the alveolar macrophages for replication. Several histological evidence showed that PRRSV infection disrupts the immune homeostasis toward the pro-inflammation state revealed by the increase of infiltrated immune cells (Halbur et al., 1995; K. D. Rossow et al., 1995; S. van Gucht, van Reeth, & Pensaert, 2003) as well as the apoptotic and necrotic pulmonary cells (Costers, Lefebvre, Delputte, & Nauwynck, 2008; Sirinarumitr, Zhang,
Kluge, Halbur, & Paul, 1998; Sur, Doster, & Osorio, 1998). While an adequate inflammatory response is required for faster restoration of the sterile environment, many respiratory virus infection cause unmanageable inflammatory responses which not only exacerbate the lung pathology but also predispose the host to systemic inflammatory response syndrome especially when hosts encounter the secondary infection (Kimura, Yoshizumi, Ishii, Oishi, & Ryo, 2013; K. van Reeth & Nauwynck, 2000).

The interstitial pneumonia is the most recognized pathology observed in PRRSV-infected pigs (Fig. 1.3). Although many pro-inflammatory cytokines possess inflammation activities, TNF-α, IL6 and IL1 have been frequently measured in most cytokine profiling studies. Particularly, studies of respiratory virus infection show that they are crucial for initiation of inflammation and that they are among the first cytokines released by alveolar macrophages. In the case of PRRSV infection, the positive correlation of lung pathology and induction level of TNF-α, IL-1 or IL-6 have been established in some studies suggesting the role of these cytokines in PRRSV pathogenesis. However, the induction phenotype of these cytokines is highly variable and not yet conclusive — that is to say, for any one of these cytokines, the induction level can be anywhere from undetectable in some studies to microgram range in others. This is likely due to the genetics of both viruses (Amarilla et al., 2015; D. Han et al., 2014; K. Han et al., 2013; Martinez-Lobo et al., 2011; Shang et al., 2013; L. Zhang et al., 2013) and their hosts (Ait-Ali et al., 2007; Do et al., 2015).

Regardless of the variation, the induction phenotype of IL-1 and IL-6 had been repeatedly reported in most studies (Gómez-Laguna, Salguero, Barranco, et al., 2010; Labarque, Van Gucht, Nauwynck, Van Reeth, & Pensaert, 2003; C.-H. Liu, Chaung, Chang, Peng, & Chung, 2009; Qiao et al., 2011; S. van Gucht et al., 2003). In contrast, the phenotype of TNF-α is more
unpredictable. For example, while early studies indicate that TNF-α is absent from the bronchoalveolar lavage (BAL) of infected pigs as well as the culture medium of infected porcine AMΦ cells (Roongroje Thanawongnuwech, Young, Thacker, & Thacker, 2001; S. van Gucht et al., 2003; K. Van Reeth, Labarque, Nauwynck, & Pensaert, 1999), some later studies show that TNF-α can be produced by lung macrophages in response to PRRSV infection (Choi & Chae, 2002; Gómez-Laguna, Salguero, Barranco, et al., 2010; Labarque et al., 2003; Qiao et al., 2011). It is possible that this unpredictable TNF-α response is the answer to the also unpredictable clinical outcome by PRRSV. The involvement of these cytokines such as IL-1 and TNF-α in PRRSV pathogenesis have been further confirmed by recent comparative studies with highly pathogenic PRRSV, in which HP-PRRSV displays greater capability in TNF-α and IL-1 induction (Amarilla et al., 2015; D. Han et al., 2014; Xiao et al., 2015; L. Zhang et al., 2013).

Despite above evidence suggest the involvement of TNF-α and/or IL1 in PRRSV pathology, the induction of these cytokines is minimal for most “conventional” PRRSV (Gómez-Laguna, Salguero, Pallarés, et al., 2010; Labarque et al., 2003; C.-H. Liu et al., 2009; Miguel, Chen, Van Alstine, & Johnson, 2010; Qiao et al., 2011; K. Van Reeth et al., 1999). This minimal induction of pro-inflammatory cytokines also explains why most grower pigs infected by conventional PRRSV in field condition are asymptotic and only show mild lung pathology. However, after careful consideration of the priming nature of cytokine response by most immune cells, this minimal cytokine induction by PRRSV should not be underestimated. In other words, even tiny amount of cytokine released by immune cells might exaggerate inflammatory responses, especially when the subject is super-exposed to a secondary stimulant. Indeed, for most conventional PRRSV, the lung inflammation is not detrimental for primary infection unless the infected host is super-exposed to a secondary infection by either bacteria or viruses.
Opriessnig, Gimenez-Lirola, & Halbur, 2011; E. L. Thacker, 2001; Steven Van Gucht, Labarque, & Van Reeth, 2004). In field conditions, the most severe symptoms caused by PRRSV is usually complicated by secondary bacterial infection. Similar observation also have been reproduced in many dual-infection experiments where the results show co-infection of PRRSV and bacteria including Streptococcus suis (W.-h. Feng et al., 2001; Galina et al., 1994; Xu et al., 2010), Bordetella bronchiseptica (Brockmeier, Palmer, & Bolin, 2000), Salmonella choleraesuis (Wills et al., 2000), Mycoplasma hyopneumoniae (Eileen L Thacker, Halbur, Ross, Thanawongnuwech, & Thacker, 1999; R. Thanawongnuwech, Thacker, Halbur, & Thacker, 2004; Roongroje Thanawongnuwech et al., 2001) and Actinobacillus pleuropneumoniae (Pol, Van Leengoed, Stockhofe, Kok, & Wensvoort, 1997) enhanced the severity of the disease in terms of greater clinical score and lung lesion. The exacerbation of the disease associated with greater production of pro-inflammatory cytokines in dual-infected pigs compared to pigs infected either with PRRSV or bacteria alone. The exacerbation of the secondary infection might result from 1) loss of the phagocytic activity via killing the lung macrophages by PRRSV, which benefits the bacterial colonization in lung and increases the risk of systemic infection or 2) excessive inflammation by super-induction of pro-inflammatory cytokines (also known as cytokine storm) in response to bacterial infection. While there is a lack of experimental support for the former mechanism, many experimental evidence indicate the synergistic effect on pro-inflammatory response by PRRSV in response to either bacterial infection (R. Thanawongnuwech et al., 2004; Roongroje Thanawongnuwech et al., 2001) or bacterial lipopolysaccharides (LPS) challenge (Qiao et al., 2011; S. van Gucht et al., 2003). Notably in some studies, despite the fact that PRRSV infection alone failed to induce any detectable amount
of TNF-α, a significant synergistic effect on the same cytokine can be obtained when being super-exposed to LPS (S. van Gucht et al., 2003).

Another important cytokine, type I interferon, together with others choreographs the host innate immune response. Type I interferon is specialized for host anti-viral responses and involved in inflammation to a lesser degree. It is primarily produced by alveolar macrophage, PRRSV natural host cells, during lung infection by respiratory viruses (Yutaro Kumagai et al., 2007) and has been proven to be a potent inhibitor for PRRSV replication (Emmanuel albina, Charles carrat, & Bernard charley, 1998; Buddaert, van Reeth, & Pensaert, 1998; Overend et al., 2007). Like many other viruses, pathogenesis of PRRSV is also dependent of the ability of PRRSV to elicit or modulate the host type I interferon response. Naturally, it is not surprising that PRRSV develops a strategy evading the type I interferon response. The first experimental evidence showing the modulation of the type I interferon by PRRSV was reported by Albina et al., in which PRRSV infection was able to inhibit the type I interferon production induced by TGEV, a potent type I interferon inducer (E. Albina, C. Carrat, & B. Charley, 1998). The compromised antiviral response by PRRSV infection can be further supported by exacerbation of clinical symptoms and lung pathology when PRRSV-infected hosts were superinfected with other swine virus including PRCV(Jung et al., 2009; Renukaradhya, Alekseev, Jung, Fang, & Saif, 2010; Kristien Van Reeth, Nauwynck, & Pensaert, 1996), SIV(Kristien Van Reeth et al., 1996), PRV(Shibata, Yazawa, Ono, & Okuda, 2003) and PCV2(Allan et al., 2000; Harms et al., 2001; Rovira et al., 2002).

At this point, it is safe to conclude that PRRSV pathogenesis is tightly linked to host innate immune responses. Therefore, understanding the molecular mechanism behind the modulation of innate immune response by PRRSV will help us to develop a strategy combating PRRSV.
infection. Based on the principal affecting the host innate immune response, the modulation mechanism can be divided into 1) virus-mediated modulation and 2) stress-mediated modulation. The former mechanism requires the expression of a specific viral protein and has been best described in type I interferon system; while the later mechanism is mediated via a secondary response elicited by virus infection, and the ER stress is one of the best known stress interplayed with host innate immune pathways. This review introduces type I interferon system and ER stress to explain the virus-mediated and the stress-mediated modulations respectively.

1.2 VIRUS-MEDIATED INNATE IMMUNE MODULATION

1.2.1 Type I interferon in brief

In the late 1950s, Isaac and Lindemann observed that an unknown substance produced in the cells treated with inactive virus was able to protect these pretreated cells from a secondary infection by an identical but live virus. This “interferon” was introduced later to refer to this substance for its remarkable interfering activities against viral infection. However, the isolation and purification of this substance was not successful until HPLC technology became practical in the 1970s (Pestka, 2007; Rubinstein et al., 1979).

Interferons are a group of small, secreted glycoproteins which structurally belong to the class II a-helical cytokines and are highly conserved from bonefish to mammals (Schultz, Kaspers, & Staeheli, 2004). They are highly inducible by pathogen infection and are best known for their ability to transduce the danger signals locally to the cells at the site of infection or systematically to the circulating immune cells. As a consequence, the gene expression profile of the interferon-activated cells is reprogrammed into an antiviral state. Depending on the type of
cell activated by interferon, the antiviral phenotype varies from apoptosis of infected cells to activation or proliferation of immune cells (Chawla-Sarkar et al., 2003; Trinchieri, 2010).

Based on the amino acid sequence homology and the physical location on the chromosome, the interferons can be further classified into 3 subfamilies. This includes the largest type I interferon subfamily consisting of more than 20 gene products, type II interferon making up by only one single IFN-γ gene and a recently identified type III interferon. While the antiviral effect of type II interferon and type III interferon is restricted to immune cells and epithelia cells respectively, the type I interferon is able to render the antiviral activity to almost every type of cells due to the universal expression of type I interferon receptor (Mordstein et al., 2010; Pestka, Krause, & Walter, 2004; Schoenborn & Wilson, 2007; Witte, Witte, Sabat, & Wolk, 2010). This powerful and broad antiviral activity by type I interferon attracts great attention in the field of microbiology and immunology. It leads exponential knowledge accumulation to this type I interferon system over the past decades. Regulation under the healthy condition and modulation under the infection condition are the prime examples. The principal of this system, as well as the antagonism by the viruses, will be reviewed as a model for virus-mediated innate immune modulation in the following section.

1.2.2 Induction of Type I interferon

1.2.2.1 Type I interferon agonist

Like most immune responses, the activation of type I interferon is also a double-edged sword. To avoid the adverse effect from unwanted activation, the full activation of this system only occurs under the infection state after recognition of the unique pathogen molecule by host. These molecules are now known as pathogen-associated molecule pattern (PAMPs) which can
be a structural component of pathogen or an intermediate metabolite generated during infection. In the case of virus infection, since there are not many other features that can be distinguis
ded from the host, the relative unique viral nucleic acids were proposed as a type I interferon trigger shortly after discovery of type I interferon (Isaacs, Cox, & Rotem, 1963). Several nucleic acid compounds were screened for their ability to induce interferon. It was revealed that a double-stranded RNA (dsRNA), either virus-derived or chemistry-synthesized, is the most potent activator for type I interferon response (Colby & Morgan, 1971). To date, it is clear that dsRNA not only comprises the genome of dsRNA virus such as rotavirus but is also a common replication intermediate which can be formed via the pairing of sense-antisense viral RNA template during the replication of many, if not all, ssRNA viruses or via pairing of transcripts encoded from overlapped viral ORFs on separate strands for some DNA virus. After discovering dsRNA as a type I interferon activator, several other virus-derived nucleic acids, including single stranded A-U rich RNA, CpG DNA and 5’ triphosphate RNA (5’ pppRNA) have also been identified to obtain the type I interferon induction activity (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004; Hornung et al., 2006; Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003; Pichlmair et al., 2006). One common feature shared by these viral nucleic acids is that they are not the favorable conformation found in host nucleic acids, especially for those residing in the cytoplasm. Thus, they are perfect infection signals picked up by host to activate the innate immune response. Recently the activation of the type I interferon has been observed in cells exposed to cytosolic DNA, suggesting the abnormal cytosolic DNA could be a new member in this category (Stetson & Medzhitov, 2006). However, further investigation reveals that the cytosolic DNA has broader effects on host innate immunity such as formation of Inflammasome. In addition, the activation capability of cytosolic DNA is sequence independent, that is, both the
pathogen and the host endogenous DNA are capable of inducing type I interferon response (Jin et al., 2012; Okabe, Kawane, Akira, Taniguchi, & Nagata, 2005). Altogether, the cytosolic DNA is more like a danger-associated molecule pattern — DAMP, than the professional type I interferon activator such as dsRNA. Therefore, the mechanism by which to activate type I interferon response will not be discussed further in this review.

1.2.2.2 Recognition of agonist-TLRs

The recognition of virus-derived nucleic acids relies on two distinct pattern recognition receptors (PRRs) families, which are membrane-bound Toll-Like Receptors (TLRs) and cytosolic Retinoic acid-Inducible Gene I (RIG-1)-like receptors (RLRs). The TLRs was the first among the identified innate immune sensors. The discovery of the TLRs initiated a new era of innate immunology. The prototype of TLRs is the toll protein identified in drosophila back in late 1980s (Hashimoto, Hudson, & Anderson, 1988). Although the first functional analysis revealed its role in drosophila development (Hashimoto et al., 1988), new insights for drosophila innate immunity was unearthed 10 years later by a research team led by Dr. Hoffmann. Dr. Hoffmann received the 2011 Nobel Prize in Physiology or Medicine for this research. In brief, Dr. Hoffmann’s team found that the drosophila carrying the mutation in toll gene are susceptible to either bacteria or fungi infection. This suggests a critical role of toll protein in innate immunity which is the only immune system existing in the invertebrate organism (Lemaitre, Nicolas, Michaut, Reichhart, & Hoffmann, 1996). Shortly after the disclosure of the immune function in drosophila, the mammalian homologues including human and mouse Toll-like receptors were identified, and their function in innate immune response were also preliminarily confirmed in the same study. For example, the cells transfected with human toll-like protein display greater cytokines responses, and the toll-like gene deficient mice developed better
resistance against LPS-induced sepsis (Medzhitov, Preston-Hurlburt, & Janeway, 1997; Poltorak et al., 1998). Meanwhile the discovery of the mammalian homologues, a family of toll-like protein, was predicted by a computational analysis of humane EST (expression sequence tag) database (Rock, Hardiman, Timans, Kastelein, & Bazan, 1998).

To date, 13 TLRs localized either at plasma membrane or endosome have been identified in human genome. All of them deeply involve in innate immunity. They all share a similar structure with multiple leucine rich repeats (LRRs) domain at N-terminal facing the extracellular environment and Toll/IL-1R homology (TIR) domain at C-terminals facing the cytoplasmic environment. While LRR domains form the pocket to accommodate a variety of PAMP molecules, the N-terminal interacts with adaptors via the hemophilic interaction of TIR domain to transduce the signaling (Kawai & Akira, 2011). Among the 13 TLRs, those localized on the endosome (namely TLR3, TLR7 and TLR9) are best known for their ability to activate the host type I interferon after specific recognition of the viral nucleic acid (Fig. 1.4). Despite endosomal TLRs’ capability to induce type I interferon, the selective utilization of specific TLRs by the host have been observed in many studies. This depends at least on the type of viral ligand and the type of cells acquired the infection signal. In short, TLR3 binds any size of dsRNA present in the endosome lumen and it is expressed in several types of cells including fibroblast, epithelia and many immune cells. In contrast to wider cell tropism of TLR3, the expressions of TLR7 and TLR 9 are restricted to professional antigen-presenting cells (APCs) like macrophages and dendritic cells where the TLR7 binds the AU-rich ssRNA and the TLR9 binds the CpG DNA, respectively in response to RNA and DNA virus infection (Fig. 1.4). The highly evolutionarily conserved TLR indicates the indispensability of this system in response to infection. However, the antiviral potency of TLR system is primarily limited by the routes that
receptors access the PAMPs as well as the narrow cell tropism (Gurtler & Bowie, 2013; Nan, Nan, & Zhang, 2014). That is to say, for those viruses that do not enter the cell by endocytosis such as Vesicular Stomatitis Virus (VSV) and Sendai viruses or for those attack TLR-deficient cells, the best chance for the host to capture these viruses is via phagocytosis or autophagocytosis of infected cells. Otherwise, it’s very like that the viruses can escape from the surveillance of TLR system. Other than the above incompetence of detecting virus infection, the biological significance of this system is debatable. Several studies indicate that the host deficient in TLR3 displays same phenotype of disease progression and interferon production as those observed in wild-type animals under the same infection condition (Edelmann et al., 2004; Johansson et al., 2007; Kato et al., 2005).

1.2.2.3 Recognition of agonist-RLRs

The restrictions of the endosomal TLRs prompted the discovery of RLRs, a cytosolic viral nucleic acid sensor. Unlike the TLRs restricted on the membrane apparatus enriched in immune cells, RLRs are free in the cytoplasm and are universally expressed in almost all types of cells (Fig. 1.4). To date, three RLRs - retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics & physiology-2 (LGP-2) - have been identified in mammalian organism. These RLRs share a consensus DExD/ H helicase box domain forming a branch of DExD/ H helicase family under the helicase superfamily 2. In addition to the center DExD/ H helicase domain’s ability to bind the RNA, C-terminal domain and N-terminal caspase activation and recruitment domains (CARDs) are the other two important domains conserved across RLRs except LGP-2 which does not contain the N-terminal CARDs domains. Since the CARDs domains are required for transducing the signal via the homophilic interaction with the adaptor protein, it is suggested that LGP-2 competitively inhibits the type I
interferon activated by RIG-I and MDA-5 (Baum & García-Sastre, 2010). All RLRs were originally identified via the screening of differential expression genes under specific differentiation conditions of several types of tumor cells (Y. Cui et al., 2001; Huang, Adelman, Jiang, Goldstein, & Fisher, 1999; T.-X. Liu et al., 2000). Although their antiviral activities were not recognized at that time, the links between RLR and host innate immune response had been predicted; as several early studies reported the induction of RIG-I and MDA-5 after treatment of several immuno-stimulators including virus, LPS and interferon (X. F. Cui, Imaizumi, Yoshida, Borden, & Satoh, 2004; Imaizumi et al., 2002; Kang et al., 2002; X. Zhang, Wang, Schook, Hawken, & Rutherford, 2000). This prediction was confirmed in 2004 by Yoneyama et al, who experimentally showed that RIG-1 is capable of binding synthetic dsRNAs and is required for the activation of host type I interferon after a treatment of dsRNA or virus (M. Yoneyama et al., 2004). Shortly after identifying RIG-I as a positive regulator of type I interferon, similar activation phenotype was observed on MDA-5 (but not on LGP-2 which displayed a dominant negative effect on type I interferon activation (M. Yoneyama et al., 2005)). In these earlier experiments, the success of using synthetic dsRNA (poly I:C) to activate type I interferon suggested that dsRNA could be the ligand shared by RIG-1 and MDA-5. It was consistent with previous knowledge of dsRNA as a common virus specific structure recognized by host TLRs. However, later studies soon observed that while RIG-1 is more responsive to negative single-stranded viruses (Kato et al., 2006; Loo et al., 2008; Weber et al., 2013; M. Yoneyama et al., 2005; M. Yoneyama et al., 2004), MDA-5 is preferentially activated by positive single-stranded viruses (Q. Feng et al., 2012; Kato et al., 2006; McCartney et al., 2008), especially those under Picornavirales. It suggests the existence of the ligand selectivity by RLRs. Indeed, after many years of efforts to characterize RLR ligand, it has become clear that a short dsRNA (less than 1
kb) and the panhandle viral genome structure (by complimentary 3’-5’ ends) with 5’ triphosphate overhang are effective substrates for a RIG-1 receptor (Kato et al., 2008; M. Schlee et al., 2009; Strahle, Garcin, & Kolakofsky, 2006) and that a longer dsRNA is suited for MDA-5 recognition (Kato et al., 2008). This finding nicely matched the observation of preferential activation of RLR by specific viral species, where most RIG-1 responsive viruses tend to produce short dsRNA or to form a 5’ppp-panhandle genome structure; whereas most positive single-stranded viruses produce longer linear dsRNA with no specific end structure (Martin Schlee & Hartmann, 2010).

The ligand preference by RIG-I and MDA-5 can also reflect on the different ways that a receptor interacts with ligand. According to crystallographic evidence, despite both receptors used the helicase domains wrapping the dsRNA core, the MDA-5 left both ends open while RIG-1 capped the ends with CTD via the interaction between a positive charged cleft of CTD and a negative charged 5’ triphosphate of dsRNA (Reikine, Nguyen, & Modis, 2014).

1.2.2.4 Induction Signaling of Type I Interferon

The two step induction is a feature of type I interferon production for most cells except for pDC. In short, IFN-β and IFN-α4 gene transcriptions are initially triggered by phosphorylated IRF3 and phosphorylated IRF7 via formation of IRF3:IRF3 homodimer or IRF3:IRF7 heterodimer. This initially produced interferon upregulates the transcription of IRF7 via activating the JAK-STAT pathway. In turn, the induced IRF7 positively feedbacks on a second wave of other IFN-α genes. Nuclear factor-κB (NF-κB) is also required as a cofactor, however, the importance of NF-κB pathway in IFN-α/β production is still much debated (Honda, Takaoka, & Taniguchi, 2006).
Although the signaling pathways initiated by each endosomal TLRs and RLRs converge at the activation of the transcription factors that are important for type I interferon (such as NF-κB, IRF3 and IRF7), causing the same phenotype of type I interferon induction (Fig. 1.4). They can be differentiated by the adaptors that are immediately recruited to the receptors via homophilic interaction. For example, despite all endosomal TLRs use the TIR domain as an adaptor docking site, the adaptor, TIR domain-containing adaptor protein inducing IFN-β (TRIF), is exclusively recognized by TLR3 (Fig. 1.4). In comparison, myeloid differentiation primary response gene 88 (Myd88) is heavily utilized by other TLRs including TLR7 and TLR9 (Fig. 1.4). In contrast to the association with the adaptor freed in the cytosol by TLRs, the RLRs interact with the receptor, mitochondria antiviral signaling (MAVS; also known as IPS-1, VISA, Cardif), that are localized at the outer membrane of mitochondria via CARD-CARD interaction (Fig. 1.4).

Interestingly, although TLR3 and RLRs belong to two different protein families using different adaptor proteins, the signaling pathways descending TLR3/TRIF and RLR/MAVS engagement closely resemble each other as they culminate at the activation of canonical IKK kinase complex (IKKα/β/γ) transmitted by TRAF6 and non-canonical IKK kinase complexe (TBK/IKKe) transmitted by TRAF3 (Fig. 1.4). The activated IKKα/β/γ leads the activation of NF-κB; while the activation of TBK/IKKe directly activates IRF3. The TRAF6- IKKα/β/γ pathway is also shared by TLR7 and TLR9 for NF-κB activation except when using Myd88 as an upstream adaptor (Fig. 1.4). In pDC, the constitutively expressed IRF7 together with IRAK1/4, IKKa, TRAF3/6 forms the signalosome where IRF7 is phosphorylated on site by IRAKs or IKKa and then translocated into nucleus triggering the transcription of IFN-α bypassing a feedback loop mediated by IFN-β. It’s worth noting that many molecules involved in the TLR and RLR pathways undergo a K-63 ubiquitination upon activation. This was proven to be a critical
modification required for a full activation of type I interferon. A K-63 ubiquitination assists the activation of RLR or TLR pathway via, at least, enhancing receptor/adaptor interaction and stabilizing kinase complexes. Genetically mutating the critical ubiquitination sites on these signal molecules or silencing the important Ub ligase expressions greatly reduce the type I interferon response (Davis & Gack, 2015). Recently, the linear activation of RLR signaling pathways (ligand-RLR-MAVS-TBK/IKK-IRF3) was challenged. Evidence now indicate decisive roles of polymerization of receptors and the aggregation of the MAVS molecules for activating the pathway. According to this model, the receptors, RIGI or Mda5, assemble into a filament structure alongside the dsRNA ligand. This filament structure proximately promotes the oligomerization of the CARD domain, which subsequently interacts with MAVS and induces the self-aggregation of MAVS on the mitochondria membrane under a similar chemical principle of prion aggregation. However, this proposed model was based on the in vitro evidence and its importance in virus-infected cells needs to be confirmed (Reikine et al., 2014; Mitsutoshi Yoneyama, Onomoto, Jogi, Akaboshi, & Fujita, 2015).

1.2.3 Response to type I interferon

A classical response to type I interferon is the expression of hundreds of interferon-stimulated genes via the activation of the Janus kinase (JAK)/ Signal Transducers and Activators of Transcription (STAT) pathway in order to establish antiviral state in uninfected cells. When secreted type I interferons bind to their specific dimeric receptor consists of IFNAR1 and IFNAR2, the receptor is subjected to conformational changes which activate the kinases (TYK2 and JAK1) pre-associated on the receptor. This activated kinases phosphorylated specific tyrosine residual on the receptor providing the docking site for transcription factors, STAT1 and STAT2. The docked STAT molecules are phosphorylated by the same activated kinases
followed by the STAT1-STAT2 dimerization in the cytosol. This heterodimer translocates into the nucleus where it associates with another transcription factor, interferon regulatory factor 9 (IRF9), to form the transcription factor complex known as Interferon-stimulated gene factor 3 (ISGF3) (Fig 1.4). An active ISGF3 specifically binds to a consensus DNA sequence of TTTCNNTTTTC known as IFN-stimulated response element (ISRE) and then drives the transcription of hundreds of genes. Although these genes are generalized as interferon-stimulated genes, each gene functions in its own way. For example, while some interferon-stimulated genes (ISGs) directly interfere viral replication such as Mx, 2’-5’ OAS, Viperin, PKR, others regulate on the type I interferon response either positively or negatively including IRF7, SOCSs etc (Fig. 1.4). Still others induce the interleukines or chemokines to maneuver immune cell functions or host inflammation responses. However, the individual function of these ISGs is out of the scope of this review.

1.2.4 Modulation of type I interferon by viruses

Viruses developed numerous antagonist strategies of type I interferon system through million years of co-evolution with the host. Based on the antagonist mechanism, these strategies can be divided into the following categories:

**Prevention of PRR detection**

**Interference of signaling cascade**

**Hijacking the host type I interferon system**
1.2.4.1 Prevention of PRR detection

1.2.4.1.1 Shielding the dsRNA

Because dsRNA is the most potent type I interferon inducer — abundant in virus infected cells — reducing the host PRRs’ exposure to dsRNA seems to be a straightforward strategy to avoid type I interferon activation. Indeed, instead of distributing over the cytosol, the dsRNA generated by members under flaviviridae and nidoviridae family is concentrated inside a virus-induced membrane structure called double-membrane vesicle (DMV) (Ferraris, Blanchard, & Roingeard, 2010; Knoops et al., 2012; Romero-Brey et al., 2012; Snijder et al., 2013). This structure has been suggested to segregate viral ligands such as dsRNA from the host PRRs (den Boon & Ahlquist, 2010; Mackenzie, 2005; Miller & Krijnse-Locker, 2008). This suggestion is further supported by several experimental evidence from recent flavivirus studies. In those studies, the host type I interferon response was correlated with the presence of cytosolic dsRNA leaking out of DMV. It suggests a role of DMV in evading type I interferon system. At the minimum, the intactness of the DMV structure is dependent on the infection stage, species of the cell and the virus strains. The differential capability to develop DMV by cells or to induce such structure by flavivirus might explain the observation of both virus strain- and host- dependent pathogenicity by flavivirus (Överby, Popov, Niedrig, & Weber, 2010; Uchida et al., 2014). Although a similar correlation is not available for nidovirus at this time, the shelter function of DMV was proposed in some coronavirus studies. Results from those studies showed that in the presence of abundant dsRNAs, the coronavirus is unable to induce effective type I interferon or to inhibit the same response induced by synthetic dsRNAs.
1.2.4.1.2 Binding of dsRNA

Instead of hiding dsRNA using rearranged membrane structures, many viruses disrupt the ligand function of dsRNA via an interaction of virus-encoded dsRNA binding proteins such as Ebola virus VP35 (Cárdenas et al., 2006), Bovine Viral Diarrhea Virus (BVDV) Erns glycoprotein (Iqbal, Poole, Goodbourn, & McCauley, 2004), influenza virus NS1 (Min & Krug, 2006) and Vaccina virus E3L (Xiang et al., 2002). These proteins each possess a dsRNA binding motif. The interaction between these proteins and dsRNA greatly suppress the type I interferon production in response to dsRNA stimulation. Mutations in the amino acid residues critical for binding dsRNA restore the host cell responsiveness for type I interferon production. The functions of Ebola VP35 and Influenza virus NS1 are also confirmed in vivo by using the recombinant virus carrying the defective mutation on dsRNA binding domain. Compared to wild type viruses, both NS1 deficient influenza virus and the VP35 deficient Ebola virus are less effective in antagonizing type I interferon response induced by Sendai virus. Notably, animals infected with either recombinant virus display low pathogenicity phenotype (indicated by mild clinical signs), a lower virus titer and less histological lesions (Cárdenas et al., 2006; Min & Krug, 2006).

1.2.4.1.3 Modification of viral genome structure

In addition to the antagonism of type I interferon responses elicited by dsRNA generating during the replication, viruses also need a strategy to make their uncoated genome structure as indistinguishable as possible to avoid activation of host anti-viral response. This can be achieved in several ways. One of the most common approaches used by many RNA viruses is to mimic the host mRNA structure. Assembling a host mRNA structure such as 5’ cap and 3’ polyA tail by
many positive stranded viruses not only benefits from utilization of host translation machinery but also reduces the risk of non-self-recognition. The importance of capturing the host mRNA structure by viruses has been recently demonstrated by Züst et al in a coronavirus study. In this study, the 2’-O-methylation on Cap structure is proved to be a determinant for self/non-self-discrimination. Animals infected with a mutant coronavirus defective in synthesizing this structure produces higher type I interferon in an MDA-5 dependent manner (Züst et al., 2011). Rather than synthesis of Cap structure by many positive stranded viruses, some negative stranded viruses snatch this structure from host mRNA. However, whether this cap-snatch mechanism helps viruses to avoid host PRR detection still needs to be investigated (Dias et al., 2009). Apart from the mimicry of host mRNA structure, the encapsidation of the viral genome is believed to reduce the risk of detection by host PRR. Once again, more evidence are needed to support this notion.

1.2.4.2 Interference of signaling cascade

Instead of passively evading the detection by host PRRs, many viral proteins actively inhibit the type I interferon signaling pathway. Unfortunately, the inhibition activities of many viral proteins are concluded by measuring the activation of the transcription factors (endpoint effector) such as NF-κB, IRF3 or STAT1 without investigating the possible upstream mechanism. For those with clearer mechanisms, based on the inhibition principles, can be categorized, but not limited, to following categories.

1.2.4.2.1 Induce proteasome degradation of signaling molecule

Proteasome degradation is a common mechanism targeted by many viruses. Viruses inhibit the host type I interferon via shortening the half-life of critical signaling molecules. Some well-
known examples are Npro proteins of flavivirus including BVDV and Classical Swine Fever Virus (CSFV), rotavirus nsp1 protein and paramyxovirus V and C protein. To be more specific, several independent studies focused on two different flaviviruses consistently showed cells overexpressed Npro protein displaying the phenotype of loss of IRF3 expression, the same phenotype observed in virus-infected cells. Further, the cells infected with the virus carrying defective Npro gene exhibit a normal expression of IRF3. Altogether, these evidence clearly indicate the roles of Npro in downregulation of IRF3. Their follow-up experiments showed this downregulation is a result of proteasome mediated degradation as using proteasome inhibitor, epoxomicin and MG132, which completely reversed the loss of IRF3 phenotype (Bauhofer et al., 2007; Zihong Chen et al., 2007; Hilton et al., 2006). Using similar approaches including the use of mutant viruses deficient in NSP1 and a treatment of specific proteasome inhibitors, the activity of rotavirus NSP1 in inducing proteasome mediated degradation has been confirmed. Moreover, rotavirus NSP1 has a broader target range for several members in the IRF family including IRF3, IRF5 and IRF7 (Arnold & Patton, 2011; Barro & Patton, 2005; Barro & Patton, 2007). A similar activity has also been identified in C protein of Sendai virus and V protein of Simian virus 5. However, in contrast to block the expression of type I interferon, these two proteins negatively affect IFN-signalling pathway via degradation of the STAT1 (J Andrejeva, Young, Goodbourn, & Randall, 2002; Didcock, Young, Goodbourn, & Randall, 1999; Garcin, Marq, Strahle, Le Mercier, & Kolakofsky, 2002). One common feature shared by IRF3 and STAT1 is that their activities were known to susceptible to post-translational modifications and this may provide a molecular basis for proteasome mediated inhibition.
1.2.4.2.2 Cleave the signaling molecule with viral protease

Many RNA viruses encode a viral protease to liberate the individual functional proteins from a viral polyprotein. This proteolytic process is vital for many RNA virus replications including flavivirus, nidovirus and picornavirus. However, in addition to cleave the viral proteins, several studies show these viral proteases’ ability to inhibit the type I interferon response via cleavage of critical molecules for type I interferon activation. For example, the expression of picornavirus 3C protease or 3C protease precursor can result in a loss of the promoter activity of type I interferon as well as a loss of expression of MAVS and TRIF, which are indispensable adaptors for TLR3- and RLR-mediated activation of type I interferon, respectively (Mukherjee et al., 2011; Neznanov et al., 2005; Qu et al., 2011; Yang et al., 2007). Among these studies, the most convincing evidence came from the studies on coxsackievirus B3 where the researchers not only demonstrated neither promoter activity of IFN-β nor the expression of MAVS is affected when expression of the catalytically inactive C3pro mutant but also showed the same promoter activity is not changed in the cells expressed the C3pro resistant target (Mukherjee et al., 2011). Interestingly, even with different protease activity, the same proteolytic effect on the same molecule (MAVS) is observed in flavivirus NS3/4A serine protease. The inhibition function of NS3/4A protease on type I interferon induction pathway was first reported by Foy et al (Foy et al., 2003). Later studies subsequently confirmed this inhibition activity being rendered by the proteolytic activity of this viral protease attacking cysteine residua at position 508 of MAVS. The cells treated with the serine protease inhibitor, ITMN-C or expressed resistant MAV with substitutive mutation at position 508 prevented the cleavage and preserved the normal IFN-β response (X.-D. Li, Sun, Seth, Pineda, & Chen, 2005; Loo et al., 2006).
1.2.4.2.3 Competitive inhibition

Due to the limited genome size, most innate immune antagonists encoded by RNA viruses are multi-functional. Other than the antagonist function, they are directly involved in virus replication process as an enzyme for macromolecule synthesis or being a part of virion structure. In contrast, DNA viruses with large genome are able to derive a set of genes, whose products have no well-defined enzymatic activities in virus replication but are highly homologue to host antiviral molecules. These viral homologous compete with host molecules in order to disrupt the type I interferon pathway in a dominate-negative fashion. Kaposi’s sarcoma-associated herpesvirus (KSHV) under the Herpesviridae and vaccinia virus under the Poxviridae are the two viruses equipped with this mechanism. IRF3 and IRF7 have been known to be the two decisive transcription factors for type I interferon gene transcription. The promotor binding activity of these two transcription factors require a hetero- or homo dimerization between phosphorylated IRF3 and IRF7, as well as association with other co-factors such as CBP/p300. KSHV disrupt this process via encoded viral homologous of IRF which competes with host IRF3 or IRF7 for 1) co-factor or 2) for promotor binding sites or 3) for the host IRF partner (Joo et al., 2007; R. Lin et al., 2001; Lubyova & Pitha, 2000). Vaccinia virus, another DNA virus with a large genome, develop a similar strategy by encoding viral TIR domain homologue from the early gene A46R to decoy the important adaptors, Myd88 and TRIF, associated with many TLRs via TIR-TIR domain interaction during activation (Bowie et al., 2000; Lysakova-Devine et al., 2010; Stack et al., 2005). Apart from disruptions of type I interferon induction via the A46R viral protein, poxvirus B18R viral protein are functional homologues of host IFNAR, which is secreted and competes with host receptors for type I interferon ligands to abrogate a downstream response
initiated by this molecule (Alcamí, Symons, & Smith, 2000; Colamonici, Domanski, Sweitzer, Larner, & Buller, 1995; Symons, Alcamí, & Smith, 1995).

1.2.4.2.4 Deubiquitination of signaling molecule

Lysine 48-linked ubiquitination usually leads proteasome degradation, which has been introduced in the previous section as a mechanism positively regulated by many viruses to disrupt type I interferon response via degradation of important signaling molecules. In contrast, another type of ubiquitination, lysine 63-link ubiquitination, has been recently recognized as an effector that positively regulates many biological pathways including type I interferon response. Many experimental evidence indicate the full-activation of host type I interferon pathway require lysine 63-linked ubiquitination on several important signaling molecules spanning along the pathway from the upstream receptor, RIG-1 (M. U. Gack et al., 2007) to the intermediate adaptor, TRAF3, TRAF6 (Kayagaki et al., 2007; Lamothe et al., 2007) and to the downstream kinase, TBK1 (C. Wang et al., 2009). It has been shown that many viruses inhibit lysine 63-link ubiquitination to interfere the type I interferon activation. For example, influenza virus NS1 viral protein disrupts the K63 ubiquitination on RIG-I molecule via direct interaction with an upstream ubiquitin ligase, TRIM25. The cells infected with the virus carry the mutation on the critical residue involved in NS1-TRIM25 interaction display normal RIG-I ubiquitination and typical IFN-β phenotype (Michaela Ulrike Gack et al., 2009). Other viruses do not alter the catalytic activity of the specific ubiquitin ligase but encode viral deubiquitinase to reverse ubiquitination of the target molecule. For example, overexpression of papain-like protease (PLP) derived from many coronavirus including mouse hepatitis virus (MHV), human corona virus (HCoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) negatively affect the ubiquitination and the IFN-β promotor activity induced by several type I interferon inducers.
which is consistent with the phenotype observed in cells infected with wild-type viruses. The ubiquitination activity and the IFN-β promoter activity can be reconstituted while transfection of the vector or infection of the virus expressed the mutated PLpro (deficient in DUB activity) in a similar analysis (Clementz et al., 2010; Zheng, Chen, Guo, Cheng, & Tang, 2008). With similar experimental approaches, the involvement of deubiquitination activities in type I interferon inhibition also confirmed in foot-and-mouth disease virus (FMDV) leader proteinase (Wang et al., 2011). Recent HBV study demonstrated that an overexpressed hepatitis B virus (HBV) polymerase caused deubiquitination of the cytosolic DNA sensor — STING. It also inhibits the activation of IFN-β mediated by the same DNA sensor. This suggests that HBV disturbs the ubiquitination process to negatively modulation host type I interferon response. Surprisingly, this deubiquitination activity is associated with reverse transcriptase and RNase H domain of polymerase which lacks any defined proteinase domain or function (Y. Liu et al., 2015).

1.2.4.2.5 Disrupt protein function via interaction

Despite the fact that viruses have evolved to adapt several intricate strategies to block the host type I interferon response, the blockade of this pathway can be sometimes achieved with a simple interaction between viral proteins and host molecules. For example, the V-protein of many Paramyxoviruses including simian virus 5, human parainfluenza virus 2, mumps virus, Sendai virus, and Hendra virus inhibit type I interferon induction via direct interaction between the C-terminal cysteine-rich domain of V protein and MDA5. The infection of the mutant viruses expressing C-terminal truncated V protein fails to inhibit both IRF3 and NF-κB activation (J. Andrejeva et al., 2004). The Z protein of Arenavirus and RSV nonstructural protein NS2 physically associate with the other RLR, RIG-I, to antagonize the IFN-β transcriptional activation.
Some other viral proteins interrupt type I interferon signaling through direct binding to kinase complexes required for activation of the transcription factors, IRF3 or NF-κB. This includes but not limited to Ebola virus VP35 and Dengue virus NS2B/3 binding to IKKε (Anglero-Rodriguez, Pantoja, & Sariol, 2014; Prins, Cardenas, & Basler, 2009), hepatitis C virus (HCV) NS3 and herpes simplex virus (HSV) γ134.5 proteins binding to TBK-1 (Otsuka et al., 2005; Verpoorten, Ma, Hou, Yan, & He, 2009) and Vaccinia virus N1L and B14 binding to canonical IKK complexes (R. A. J. Chen, Ryzhakov, Cooray, Randow, & Smith, 2008; DiPerna et al., 2004). Still, other viruses directly target transcription factors for binding by bypassing all upstream molecules. For example, the Human herpesvirus Epstein-Barr virus (EBV/HHV4) immediate-early protein BZLF-1 and Human papillomavirus 16 (HPV16) E6 oncoprotein respectively binds to IRF-7 and IRF-3 to inhibit its transcriptional activity (Hahn, Huye, Ning, Webster-Cyriaque, & Pagano, 2005; Ronco, Karpova, Vidal, & Howley, 1998).

1.2.4.3 Hijacking the host innate immune system

In contrast to counteract the host innate immune response, some viruses take advantage of the innate immune activation. Most experimental evidence are obtained from studies which investigate the immediate early (IE) gene activities of DNA viruses. For example, several independent studies have shown that the interferon treatment and the activation of TLR by CpG enhanced cytomegalovirus replications and IE gene expressions of the same virus (Iversen et al., 2009; Y. Lee, Sohn, Kim, & Kwon, 2004; Netterwald et al., 2005). This enhancement was likely resulted from hijacking host immune-activated transcription factors such as NF-κB or STATs with the corresponding binding sequences on the ie promoter region, since the loss of that binding sequences greatly reduced the virus replications as well as IE gene expression (Y. Lee et al., 2004; Netterwald et al., 2005). Similar proviral effects by innate immune activation are
observed in a HIV study where the results showed that the HIV LTR promoter activity required
the TLR-mediated activation of NF-κB (Equils et al., 2001; Gringhuis et al., 2010). Moreover,
the IL-6 induced STAT3 activation seemed to be required for optimal promoter activities of
HBV (Waris & Siddiqui, 2002). Indeed, a computational pairwise comparison analysis using
viral promoter sequences derived from seven well-studied mammalian viruses against host innate
immune gene promoter sequences indicated that many immune-activated transcription factors
could be potentially shared between viruses and the host (Kropp, Angulo, & Ghazal, 2014).

1.3 THE STRESS-MEDIATED INNATE IMMUNE MODULATION

1.3.1 The three musketeer of ER stress

As a major protein and lipid processing factory, the function of ER is exploited by viruses
during infection. This includes the protein folding by massive viral proteins, protein
glycosylation by glycosylated viral structural protein and lipid synthesis by a virus-induced
membrane structure or formation of enveloped virion. Evidence increasingly demonstrate
choreography of the ER stress with host innate immunity. Accordingly, to understand the roles of
viruses in this dance will help us develop a better strategy to combat viral infectious diseases.

To date, there are three ER stress sensors identified in Metazoan organism. They are
Inositol Requiring Kinase-1α (IRE-1), Activating Transcription Factor 6 (ATF6), and PKR-like
Endoplasmic Reticulum Kinase (PERK). Like many signaling pathways, an activated ER stress
sensor leads the activation of the corresponding transcription factors which in turn induce the
expression of subset genes. The subset genes mostly function as an ER stress reliever to maintain
the homeostasis of ER condition or as apoptosis inducer if the stress condition persists. In an
unstressed condition, an ER resident chaperon called Bip (aka GRP78) constantly binds to these
sensors and holds them in inactive state. When unfolded proteins accumulated in the ER lumen, the Bip protein is sequestered by these unfolded proteins with higher affinity and dissociated from the stress sensors, initiating a downstream unfolded protein response (UPR) signaling (Fig. 1.5).

The Bip-free IRE1α undergoes oligomerization by which the proximity triggers the trans-autophosphorylation via its own kinase domain located at the cytoplasmic region. This autophosphorylation also enhances activation of ribonuclease domains that are also exposed to the cytoplasm. So far the mRNA encoding X-box binding protein1 (XBP1) is the only substrate for this ribonuclease. The activated ribonuclease removes 26 bp sequence containing premature stop-codon through enzymatic cleavage on the mRNA encoded inactive XBP1 (XBP-1u). The XBP1 translated from full-length, IRE1α-spliced mRNA (XBP-1s) contains a transcriptional transactivation domain which binds to the promoter regions and trigger the transcription of several chaperon genes or genes involved in lipid-biosynthesis or ER-associated degradation, ERAD (Fig. 1.5) (Hetz, Martinon, Rodriguez, & Glimcher, 2011).

Similar oligomerization and auto-transphosphorylation events have also been observed in the activation of PERK sensors. An activated PERK phosphorylates eIF2α, a vital molecule in translation initiation, on Ser-51. This modification converts the eIF2α into a competitive inhibitor of eIF2β, a guanine nucleotide-exchange factor (GEF), required for recycling inactive GDP-bound eIF2α to active GTP bound eIF2α, by which the formation of initiation complexes is reduced by deficiency of active GTP- eIF2α leading the global translation attenuation. However, like most biological responses, this translation attenuation by activating the PERK-eIF2α pathway is negatively regulated via a feedback mechanism (Fig. 1.5) (Wek, Jiang, & Anthony, 2006). The negative feedback loop is initiated by the expression of the activating transcription
faction 4 (ATF4), which is the most effective under the stress condition when the availability of active eIF2α is low. This atypical expression is mediated by an additional inhibitory μORF structure at the 5’ leader region of atf4 flanked by another upstream μORF and atf4 coding region. In an unstressed condition while active eIF2α is abundant, the quick formation of the initiation complex makes a frequent translation re-initiation at this inhibitory μORF. It in turn abrogates the expression of ATF4. In contrast, when the availability of active eIF2α is limited, the time for reconstitution of the initiation complex is increased. This delay makes most re-initiation events take place at initiation site of atf4 as the functional initiation complex cannot be made in time to catch the inhibitor μORF (Vattem & Wek, 2004). As soon as ATF4 is produced, it functions as a transcription factor to activate subset genes transcription. This includes a phosphatase, growth arrest and DNA damage-inducible protein (GADD34), counteracting kinase activity of PERK on eIF2α as well as transcription factor CHOP, regulating wide biological events from cytokine expression to apoptosis.

In contrast to IRE1 and PERK, sensors that transduce signals to downstream effectors, the dual functionalities make ATF6 both an ER stress sensor and an effector. Upon the stress, the dissociation of Bip unmasks the Golgi signal of ATF6 by which ATF6 translocates to the Golgi where it is enzymatically cleaved by proteases, S1P and S2P, resided on the Golgi apparatus. This cleavage releases the cytoplasmic portion of ATF6 containing the bZIP transcription factor domain, which operates as a transcription factor enhancing the transcription of many genes involves in protein foldings (Fig. 1.5) (Adachi et al., 2008).

It’s worth noting that despite these three pathways having their own specific function in an ER stress, they are intertwined with each other. For example, the substrate of IRE1, XBP1, could be induced by activation of ATF6 and PERK pathway (Calfon et al., 2002; Yoshida, Matsui,
Yamamoto, Okada, & Mori, 2001). In addition to PERK, the IRE1 activation also negatively affects the global translation via mRNA degradation (Hollien & Weissman, 2006). Last but not the least, a study on mutant C. elegans deficient either in IRE1-XBP pathway or ATF6 pathway demonstrated a group of overlapped gene expressions is absent from both mutants suggesting redundancies shared between these UPR sensors (Shen, Ellis, Sakaki, & Kaufman, 2005).

1.3.2 The roles of the ER stress in innate immune activation

The hallmark of innate immune response is the activation of NF-κB, which upregulate many innate immune-related genes as a transcription factor. Those genes include those encoding cytokines for inflammatory responses, as well as growth factors for immune cell differentiation. Surprisingly, this activation has also been observed during the ER stress induced by non-infectious agents. To date, accumulated experimental evidence reveal that all three branches of ER stress — IRE1, ATF6 and PERK — possess the activity to activate NF-κB under a stress condition (Fig. 1.5). To be more specific, Kaneko et al., provided the first mechanistic evidence showing that the interaction between ER stress sensor, IRE1, and TNF receptor-associated factor 2 (TRAF2) (important adaptor in TLR signaling) positively regulates NF-κB induced by ER stress agents such as Tunicamycin or Thapsigargin. Expression of the dominant negative mutant of either IRE1 or TRAF2 abolishes the NF-κB activation induced by the same chemical (Kaneko, Niinuma, & Nomura, 2003). A similar phenotype of reducing NF-κB activation was confirmed later by Hu et al. when knocking down the expression of either IRE1 or TRAF2.

Further, this later study suggested recruiting an IKK complex onto an IRE1-TRAF2 complex is important for deactivation/phosphorylation of IκB by which NF-κB is activated, triggering a downstream TNF-α production (P. Hu, Han, Couvillon, Kaufman, & Exton, 2006). The same NF-κB activation can be also rendered by a PERK-eIF2 activation induced by stress treatments.
of thapsigargin or ultraviolet light. However, in contrast to phosphorylation of IκB induced by IRE1 pathway, this NF-κB activation is due to a quick depletion of IκB which is a combined result of the short half-life of IκB and the failure of replenishment of such molecule in a translation shut-off environment created by phosphorylated eIF2 (Fig. 1.5). Relieving the translational stress by expression of Ser-51 phosphorylation-incompetent eIF2 mutant successfully rescues the expression of IκB and diminishes NF-κB activation (Deng et al., 2004; Jiang & Wek, 2005; S. Wu et al., 2004). The ATF6 pathway has also been proposed as a positive regulator for NF-κB activation (Fig. 1.5). Mimicking the ER stress by cleavage of Bip using subtilase leads the NF-κB activation followed the ATF6-Akt/PI3K-NF-κB axis as this NF-κB transcription activity is greatly reduced while including the dominate-negative ATF6 mutant or Akt inhibitor in a luciferase assay (Yamazaki et al., 2009). Besides NF-κB, a sidetrack under the IRE1-TRAF2 pathway is the activation of the c-Jun N-terminal kinases (JNK) mediated by Apoptosis signal-regulating kinase (Ask) (Fig. 1.5) (Nishitoh et al., 2002; Urano et al., 2000). The activated JNK tightly associates with the activation the transcription factors, AP1 or c-jun, which have been long known to co-operate with NF-κB for optimal transcription of many immune-related genes. Accordingly, it is plausible that the IRE1-TRAF2-ASK-JNK activation contributes to ER-induced immune priming although it lacks direct experimental evidence at this time. Another transcription factor critical and specific for type I interferon response, IRF3, can also be activated after treatments of stress agents (Tm and Tg). It’s very likely that this IRF3 activation is a downstream event of ATF6 and that it is independent of IRE1-XBP1 activation. Because the activation is unaffected in cells deficient in XBP1 expression but susceptible to treatments of inhibitors of serine protease required for ATF6 activation (Y.-P. Liu et al., 2012). In addition to aiding the activation of immune-related transcription factors described above, the
chromatin immunoprecipitation (ChIP) results revealed that the ER-stress specific transcription 
factors, XBP1 and C/EBP homologous protein (CHOP), are able to bind the promoter region of 
TNF-α(XBP1), il6(XBP1) and il23 (CHOP) in response to stress agents treatments. This suggests 
a direct involvement of ER stress in setting a pro-inflammatory environment (Goodall et al., 
2010; Martinon, Chen, Lee, & Glimcher, 2010).

Despite the fact that the capability of ER stress in activating immune-related transcription 
factors has been experimentally demonstrated, this activity alone seems insufficient to trigger the 
immune response as the gene expression of the innate cytokines driven by such transcription 
factors have hardly been detected in ER-stressed cells. However, the influence of ER stress on 
the innate immune response turns from imperceptible to drastic when the stressed cells pre- or 
post-conditioned with typical immune activation. The synergistic effect of ER stress on several 
cytokines expressions, including IL-1β, IL-6, IL-23, TNFα and IFN-β, induced by PRR agonists 
LPS or poly I:C has been repeatedly observed in the tunicamycin or thapsigargin-stressed 
immune cells such as macrophages or dendritic cells both at mRNA and protein level. Most of 
the synergistic activities are under the control of IRE1-XBP1 or ATF6 branch of the ER stress, 
as experimental removal of these molecules greatly reduced synergistic phenotypes (F. Hu et al., 
2011; Y.-P. Liu et al., 2012; Martinon et al., 2010; Rao et al., 2014; Smith et al., 2008). Instead 
of amplifying cytokine expressions in response to PRR activation by exogenous stress agents, the 
stress response itself seems to be a part of the TLR activation pathway. It is required for the full 
activation of immune responses mediated by TLR receptor. For example, two independent 
studies performed by Martinon el al and Qiu et al consistently showed that TLR activation of 
macrophage resulted in activation of the IRE1-XBP1 pathway without the treatment of 
exogenous stressor and the loss of either IRE1 or XBP1 gene expression in the same type of cells
greatly reduced the pro-inflammatory cytokine production in response to LPS stimulation (Martinon et al., 2010; Qiu et al., 2013).

One unsolved riddle is that ER stress also leads the global translation shut-off mediated by PERK-eIF2 pathway which is supposed to have negative effect on cytokine protein synthesis. Following this rationale, why are synergistic effects rather than inhibition activities by ER stress that has been observed in most studies? One plausible explanation is that the TLR activation can selectively modulate host ER stress response to keep the pro-immune activities of ER stress yet prevent the adverse effect of translation attenuation mediated by eIF2. This explanation is further credited by Calavarino’s study where they found that unleashing the translation power of il6 and ifnb in mouse embryonic fibroblasts (MEF) cells in response to poly I:C stimulation required a specific induction of GADD34 by the same PRR agonist. As previously mentioned GADD34 is a phosphatase that specifically removes the translation inactive phosphate group from eIF2 and thus restores the translation activity. The genetically mutated cell line lacking GADD34 expression fails to produce the IL6 and IFNB after poly I:C stimulation, even when it’s under the robust transcription of il6 and ifnb (Clavarino et al., 2012). Another possible explanation is that, in contrast to simultaneous activation, the three branches of ER stress are activated in a time-dependent manner. That is to say, at the time while synergistic activities are measured, the translation shut-off mediated by PERK pathway has not been established. Indeed, the step-wise activation of ER stress pathway followed the order of IRE1-ATF6-PERK has been demonstrated by Lin et al (J. H. Lin et al., 2007). Altogether, both hypotheses explained that most synergistic effects by the ER stress are driven by the IRE1 or ATF6 activation as the inhibitory activities of PERK are either selectively disarmed by PRR stimulation or require more time to take the effect.
1.3.3 Modulation of stress response by viruses

1.3.3.1 PERK-eIF2 modulation

Cellular activities in response to ER stress vary from the induction of the chaperon proteins for better ER folding capacity, translation attenuation for reduction of the protein load to apoptosis if the stress is unmanageable. Under a viral infection condition, some of these responses such as induction of chaperon proteins might benefit virus replications while others such as translation shut-off or apoptosis could be detrimental for viruses. Accordingly, as host tends to maximize antiviral effects rendered by ER stress, many viruses develop strategies to modulate the host ER stress in favor of their own replication. Among the three branches of the ER stress, the translation attenuation by PERK-eIF2 is probably the most unwanted response because viruses also rely on eIF2 dependent initiation of translation; even for most viruses use cap-independent translation mechanism. When it comes to counteract the adverse effects of eIF2-mediated translation shut-off, to prevent the eIF2 phosphorylation is perhaps the most common strategy used by viruses.

There are several ways to block this process. For example, while some viruses encode the viral eIF2 phosphatase, i.e. DP17L by African swine fever virus (AFSV), E6 by HPV and g34.5 by HSV, to reduce the eIF2 phosphorylation (Cheng, Feng, & He, 2005; He, Gross, & Roizman, 1997; Kazemi et al., 2004; F. Zhang, Moon, Childs, Goodbourn, & Dixon, 2010), other viruses induce the expression of host factors that negatively regulate the PERK-eIF2 activation. This includes p58IPK which is a PERK/PKR inhibitor. It is induced by Influenza virus (Goodman et al., 2007; Yan et al., 2002) as well as GADD34, the host eIF2 phosphatase induced by infectious bronchitis virus (IBV) and Dengue virus (Peña & Harris, 2011; X. Wang et al., 2009).
In addition to affecting equilibrium of phosphorylation reaction, the Vaccinia virus K3L and the HCV E2 protein act as a pseudo-substrate to compete with host eIF2 for its upstream kinase (Kawagishi-Kobayashi, Silverman, Ung, & Dever, 1997; Pavio, Romano, Graczyk, Feinstone, & Taylor, 2003; Ramelot et al., 2002; Seo et al., 2008). Furthermore, other than reducing the level of phosphorylated eIF2, another strategy used by a small set of viruses is to bypass or reduce the dependence of eIF2-mediated initiation. For example, Cricket Paralysis Virus (CrPV) initiates its viral gene translation without using any known eukaryotic Initiation factors including eIF2 or initiator Met-tRNA by very unique type IV IRES translation (Jan & Sarnow, 2002; Pestova & Hellen, 2003). Further, the alphavirus late S26 mRNA translation bypasses the requirement of eIF2 activity by using another initiation factor, eIF2A. It is worth noting that it used eIF2A (not eIF2 α-subunit) to deliver the Met-tRNA to ribosome stalled at the stem-loop structure of 26S mRNA (Ventoso et al., 2006).

A similar eIF2A-mediated delivery of Met-tRNA to ribosome at the IRES site is also observed in HCV gene translation. Although optimal eIF2 independent initiation of HCV may need the cooperation with another initiation factor, eIF5 (Kim, Park, Park, Keum, & Jang, 2011; Pestova, de Breyne, Pisarev, Abaeva, & Hellen, 2008). The same eIF5 also seems to be involved in the switch from eIF2 susceptible in the early infection to eIF2 resistant in the late infection during the poliovirus replication. The underlined mechanism, however, has not been detailed (White, Reineke, & Lloyd, 2011). The utilization of eIF2-independent translation by these viruses not only make them free from the translation stress mediated by eIF2 but also benefit them since the eIF2-dependent synthesis of the host antiviral molecule is suppressed in such condition.
The role of IRE1-XBP1 pathway in virus-infected cells is more ambiguous. On the one hand, the activation of this pathway can enhance protein synthesis as well as ER expansion benefiting virus replication (Shaffer et al., 2004). On the other hand, the activation of the same pathway also induces the expression of critical genes promoting ER-mediated protein degradation (ERAD) which may have adverse effects on virus replications.

On the pro-viral side, tick-borne encephalitis virus (TBEV) and influenza virus infections have been shown to induce IRE1 activation. And blocking the IRE1 activation with IRE1 specific inhibitor reduced virus replications (I. H. Hassan et al., 2012; Yu, Achazi, & Niedrig, 2013). Although the positive effector of an IRE1 pathway for virus replication has not been determined, several studies on flaviviruses shed light on the underlying mechanism of this pro-viral activity.

The productive replication of HCV, a member of flaviviruses, is known to be positively associated with activation of autophagic pathway, which may provide the machinery for a viral replication factory, DMV, formation (Dreux, Gastaminza, Wieland, & Chisari, 2009). It has been known that autophagy activation can be induced by ER stress via the IRE1 mediated JNK activation (Ogata et al., 2006). An HCV study shows that autophagosome formation and HCV replication are significantly inhibited in the cells treated with IRE1 specific inhibitors (Shinohara et al., 2013). Meanwhile, a separate study on dengue virus demonstrated that the activation of JNK is required for virus replications. The use of JNK inhibitor was shown to negatively affect virus replications. Regrettably the stress response was not discussed in this study (Ceballos-
Olvera, Chávez-Salinas, Medina, Ludert, & del Angel, 2010). Altogether, these results suggest that the pro-viral activities of IRE1 may be a result of the JNK-activated autophagosis.

The other proposed mechanism is the selective degradation of host ER-localized mRNA but not cytosolic viral RNA. This process is dependent on the RNase activity of IRE1 and known as regulated IRE-1 dependent degradation (RIDD). The pro-viral activity of RIDD is suggested in a dengue virus study where the author demonstrated that the viral mRNA but not the host mRNA is resistant to the IRE1 RNase activity upregulated during the infection. The treatment of IRE1 RNase inhibitor reduces the titer of the viruses in the infected cells (Bhattacharyya, Sen, & Vrati, 2014).

From an anti-viral point of view, the activation of the IRE1 pathway displays the inhibitory effect on respiratory syncytial virus (RSV) virus and, interestingly, on HCV virus. Regardless of its unclear mechanism, the inhibitory activity on RSV replication is directly rendered by IRE1 molecules itself other than the downstream effector XBP1. This is because the loss of inhibition activities can only be observed in IRE1 deficient cells but not XBP1 (I. Hassan et al., 2014). In contrast, the inhibitory effect on HCV is mediated by a protein degradation process known as ERAD. ERAD requires the expression of ERAD related genes driven by activated XBP1. In this case, a HCV infection induced the IRE1-XBP1 activation which subsequently, with the transactivation activity of XBP1, triggers the expression of critical ERAD molecules. This molecule targets the viral E2 protein for degradation and thus interferes with the replication. The reversal of the inhibitory phenotype can be obtained when the infection experiment is carried out in the cells knocked down for critical ERAD molecules or treated with ERAD inhibitors (Saeed et al., 2011).
1.3.3.3 ATF6 modulation

The ATF6 branch of ER stress is best known for its capability to induce the expression of ER chaperon genes, to keep the protein quality in check (Adachi et al., 2008). So far, there are no reports of visible antiviral activities associated with this pathway. Accordingly, the preferential activation of ATF6 pathway while leaving other ER pathways such as PERK or IRE1 or both unaffected or suppressed have been observed in many viruses including HCV, West Nile Virus, lymphocytic choriomeningitis virus (LCMV), ASFV and HSV (at early infection) (Ambrose & Mackenzie, 2013; Burnett, Audas, Liang, & Lu, 2012; Galindo et al., 2012; Pasqual, Burri, Pasquato, de la Torre, & Kunz, 2011; Tardif, Mori, & Siddiqui, 2002). More than a simple activation, all preferential activations by these viruses, except HSV, positively regulate virus replications because virus replication is less efficient in the cells treated with the specific inhibitor for ATF6 activation or knocked down/out for ATF6 (Ambrose & Mackenzie, 2013; Galindo et al., 2012; Ke & Chen, 2011; Pasqual et al., 2011). Although the mechanism promoting virus replications has not been detailed, the ATF6-driven expression of chaperon is suspected to play a role in it. For example, knockdown the expression of GRP78, a common chaperone induced by ATF6, significantly impairs the assembly of the Japanese encephalitis virus (JEV) (Y.-P. Wu et al., 2011). In addition to the chaperon induction activity, it has been shown that the WNV-induced ATF6 activation reversely associates with STAT1 activation, suggesting a potential role of ATF6 activation in an antiviral response. However, this association may simply be a result of independent parallel coincidence by multi-functionality of the virus or viral protein; thus needs to be further confirmed (Ambrose & Mackenzie, 2013).
1.4 ANTAGONISM OF INNATE IMMUNE RESPONSE BY PRRSV

1.4.1 Type I interferon modulation by PRRSV

As discussed earlier, PRRSV infection fails to elicit a conventional type I interferon response by plasmacytoid dendritic cells (pDC) (Calzada-Nova, Schnitzlein, Husmann, & Zuckermann, 2011) and by porcine alveolar macrophages (porcine AMΦs) (W. Chen, Calzada-Nova, Schnitzlein, & Zuckermann, 2012). The latter is not only the primary cell targeted by PRRSV for replication (Duan et al., 1997a; Duan, Nauwynck, & Pensaert, 1997b; G. Wensvoort et al., 1991) but also the major interferon producer in lungs (Y. Kumagai et al., 2007). Albina et al. first reported this inadequate type I interferon response to PRRSV. They showed that PRRSV infection failed to induce significant IFN-α production in the lungs of infected pigs and it exhibited only a weak IFN-α response in their serum. In addition, it was shown that PRRSV infection apparently compromised the IFN-α production in alveolar macrophages super-infected with TGEV. It suggested the modulation of host type I interferon response by PRRSV (E. Albina et al., 1998).

Since then, the mechanisms by which PRRSV apparently inhibits the host cell type I interferon response has become a major research topic. Especially at the molecular level, researchers are interested in understanding the PRRSV-host cell interactions.

Several studies have shown that PRRSV non-structural proteins, most prominently described, non-structural protein 1 (nsp1), possess inhibitory activities over the activation of transcription factors required for the initiation of type I interferon genes transcription including CREB/p300, NF-κB, IRF3 and SP1 (Beura et al., 2009; Z. Chen, Lawson, et al., 2010; M. Han, Du, Song, & Yoo, 2013; M. Shi et al., 2010; X. Shi et al., 2011; Song, Krell, & Yoo, 2010;
Subramaniam et al., 2010). These gave a possible explanation for compromised IFN-α responses observed in infected pig host (Table 1).

Nevertheless, most of these studies have been conducted using permissive cells derived from species that are not the natural host to PRRSV and by overexpressing individual viral proteins in cells in combination with reporter assays. Therefore, the results may not necessarily reflect the natural behaviors of PRRSV in the infected animals or in its natural host cell, namely the porcine alveolar macrophage. In fact, using a more naturalistic experiment condition such as PRRSV infection of porcine host or infection of porcine permissive cells, pMo-DC or porcine AMΦ, some studies reported conflicting results showing enhancement of IFN-β transcription activity (Ait-Ali et al., 2011; Z. Chen, Zhou, et al., 2010; Genini et al., 2008; Gudmundsdottir & Risatti, 2009; S. M. Lee, Schommer, & Kleiboeker, 2004; Y. J. Lee & C. Lee, 2012; Loving, Brockmeier, & Sacco, 2007; H. Zhang, Guo, Nelson, Christopher-Hennings, & Wang, 2012). Among these studies, Lee et al. noticed that despite the fact that IFN-α protein production was restricted, the IFN-α mRNA synthesis was virtually increased in the PRRSV infected alveolar macrophages (S. M. Lee et al., 2004). Similar observations have also been reported, later, by Zhang et al. in PRRSV-infected monocyte-derived dendritic cells (H. Zhang et al., 2012). Altogether, both studies suggested the existence of post-transcriptional control over host IFN-α responses by PRRSV. More post-transcriptional regulation evidence can be found in its coronavirus cousin, SARS-CoV and MHV. For example, SARS-CoV 3a protein induces the eIF2 phosphorylation and enhances the degradation of IFNAR1 (Minakshi et al., 2009) and its non-structure protein1 (nsp1) is suggested to promote the degradation of the IFN-β mRNA induced by SeV (Banerjee, An, Zhou, Silverman, & Makino, 2000). Further, Roth-Cross et al. reported that MHV does not inhibit the NDV or SeV -induced IRF3 activation or IFN-β transcription;
instead, significant inhibition of IFN-β production was observed suggesting the post-transcriptional modulation by MHV (Roth-Cross, Martinez-Sobrido, Scott, Garcia-Sastre, & Weiss, 2007).

1.4.2 Stress response by PRRSV

Many steps of the PRRSV replication exploit the host ER function. These include the synthesis of viral proteins, glycosylation of structural proteins and utilization of ER lipids for DMV and virion envelope formation. Following this rationale, Hou et al first provided preliminary yet important results showing that both Marc145 and porcine AMΦ cells infected with PRRSV experienced intensive ER stress (Huo et al., 2013). It is revealed by the activation of PERK-eIF2 as well as IRE1-XBP1 pathway. Furthermore, in the same study, the IRE1 mediated JNK activation seemed to play an important role in sustaining PRRSV replication. The use of IRE1 inhibitor in the infection experiment inhibited the JNK activation as well as the virus replication (Huo et al., 2013). The positive role of JNK activation for PRRSV replication has been suggested in another study where the cells treated with JNK inhibitor greatly reduced the replication of PRRSV (Yoo Jin Lee & Changhee Lee, 2012).

The phenotypes of IRE1 activation and susceptible to JNK inhibitor highly resembled those in HCV-infected cells (Ceballos-Olvera et al., 2010; Shinohara et al., 2013). As mentioned earlier, it has been known that the ER stress-induced IRE1- JNK activation is required for autophagosome formation (Ogata et al., 2006). Such autophagic activities are required for optimal replication activities for both PRRSV and HCV (Q. Chen et al., 2012; Dreux et al., 2009; Q. Liu et al., 2012; Sun et al., 2012). In study of HCV and coronaviruses, it demonstrated that the machinery used for autophagosome formation was exploited by viruses in order to form
DMV, viral replication apparatus (Dreux et al., 2009; Ferraris et al., 2010; Prentice, Jerome, Yoshimori, Mizushima, & Denison, 2004; Reggiori et al., 2010; Sir et al., 2012). Those complexes are again another visible feature shared between HCV and PRRSV (Paul, Hoppe, Saher, Krijnse-Locker, & Bartenschlager, 2013; Romero-Brey et al., 2012; Snijder et al., 2013). All things considered, it is possible that PRRSV benefits from the ER stress by activating IRE1-JNK pathway which in turn up-regulates the autophagic activity promoting the DMV formation.

In addition to the IRE1-XBP1 pathway, the PERK-eIF2 pathway is also activated in the PRRSV-infected cells (Huo et al., 2013). The activation of PERK-eIF2 seems does not perturb the replication of PRRSVs. A similar resistance to eIF2 phosphorylation had also been reported in coronaviruses despite of unknown mechanism (Bechill, Chen, Brewer, & Baker, 2008; Krähling, Stein, Spiegel, Weber, & Mühlberger, 2009). In other word, this leaves open the possibility that PRRSVs may exploit the PERK-eIF2 activation to block the synthesis of host antiviral molecule. The negative effect of eIF2 phosphorylation on antiviral molecule synthesis had already been reported in the study of HCV and Chikungunya virus (Garaigorta & Chisari, 2009; L. K. White et al., 2011). Although the roles of ER stress in PRRSV replication as well as host immune modulations have not been determined at the moment.

In conclusion, the level of a PRRSV-modulated host innate immune response in porcine AMΦ cells was not conclusive. Therefore to clarify the underlying mechanism by which PRRSVs modulate the innate immune response of porcine alveolar macrophages became primary goal in our study. This study is solely performed on PRRSV native host cells, primary culture of alveolar macrophage (porcine AMΦ) and pig alveolar macrophage cell line, ZMAC4 in the context of virus infection. The ultimate goal of this thesis is to depict a clearer picture of PRRSV pathogenesis in terms of the innate immune modulation. We also expected to apply the
knowledge from this research to other virus studies, to help other researchers to develop a better strategy combating not only PRRSVs but also other viral diseases.
1.5 Reference


10.1128/JVI.01326-09


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Fig 1.1 Clinical symptoms by PRRSV (A) The clinical outcomes of the disease were frequently described as inappetance, lethargy, fever and breathing difficulty mostly seen in younger animals. (B) In spite of that the mortality rate was low in grower pigs, the reproductive failure become major threats for pregnant sows. In addition to disrupting the estrous cycle, the pregnant sows tended to farrow the litter prematurely. This includes the stillborns or non-viable piglets in each affected litter.
Fig 1.2 Schematic diagram of the PRRSV genome organization and expression (A) The genome structure of PRRSV resembled the eukaryotic cellular mRNA including a coding region flanked by a capped 5'-UTR and a polyadenylated 3'-UTR. The coding regions of PRRSV genomic RNA are comprised of 10 ORFs (open reading frame). While all the accessory proteins required for viral RNA synthesis are encoded from the two large ORFs (ORF1a and ORF1b) occupying the three quarters of the genome at the 5' end, the viral structure proteins are exclusively encoded from rest of ORFs (ORF2-7 including 2a and 5a) tightly packed at 3' end of PRRSV genome. (B) Model for PRRSV replication and transcription. Continuous minus-strand RNA synthesis yields a genome-length minus strand template for genome replication. Discontinuous minus-strand RNA synthesis results in a nested set of subgenome-length minus strands that serve as templates for sg mRNA synthesis (see text for details). This process required pairing activity between the body TRS at the 3' end of the nascent minus-strand and the genomic leader TRS at the 5' end of genome (see text for details).
Fig 1.3 Lung pathology of PRRS
The interstitial pneumonia is the most recognized pathology observed in PRRSV-infected pigs
Fig 1.4 Schematic of innate immune signaling triggered by viral infection through cytosolic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) or endosomal Toll-like receptors (TLRs). Infection by viruses produces RNA intermediates which in turn are detected by RLR or TLR. RLR. This receptor engagement promotes interaction with downstream adaptor such as MYD88, TRIF or IPS1. The downstream mediated by these adaptors converge at the activation of canonical IKK kinase complex (IKKα/β/γ) or non-canonical IKK kinase complexe (TBK/IKKe) or both. The activated IKKα/β/γ leads the activation of NF-κB, while the activation of TBK/IKKe directly activates IRF3. Once activated, these transcription factors translocate to the nucleus, and together with other transcription factors such as CBP or AP1, induce the transcription of IFN-α and IFN-β. Secreted IFN-β by the infected cells binds to the IFNAR present on the surface of the surrounded cells. Activation of IFNAR induces phosphorylation of Janus kinase (JAK)1 and Tyk2, which can promote the formation of the heterotrimer ISGF3 formed by signal transducer and activator of transcription (STAT)1, STAT2 and IRF-9. Ultimately, translocation into the nucleus of ISGF3 induces hundreds of ISGs.
**Fig 1.5 Endoplasmic reticulum (ER) stress signal transduction** In an unstressed condition, an ER resident chaperon called Bip (aka GRP78) constantly binds to ER sensors and holds them in inactive state. The binding of Bip by ER sensors is outcompeted by increased misfolded protein. The dissociation of the Bip from the ER sensors leads to the activation of Bip. The dissociation of Bip by Bip sensors leads to the activation of Bip sensors including PERK (left), IRE1 (center) and ATF6 (right) located at ER membrane. PERK activation leads to the global translation shut-off via phosphorylation of eIF2. Other than inhibition, the expression of ATF4 translation is facilitated by eIF2 phosphorylation during ER stress, which then induces expression of CHOP and GADD34; GADD34 promotes dephosphorylation of eIF2 as a negative feedback control. PERK activation reduces IκB protein synthesis causing NFκB activation. IRE1 activation results in the splicing of XBP1 mRNA. The transcription factor, XBP1s, encoded from spliced XBP1 mRNA upregulates expression of ER chaperones, as well as components of the ERAD machinery. The recruitment of TRAF2 by activated IRE1 leads to the activation of JNK(via activation of apoptosis signal related kinase; ASK) and NF-κB, which are critical transcription factor for transcription of host inflammatory genes. Activated ATF6 translocates to the Golgi where it is processed by S1P and S2P. The cleaved-off cytoplasmic domain function as a transcription factor to upregulate genes involved in protein folding process. ATF6 can also enhance the activation of NF-κB with the help of the ASK.
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2.1 Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV), an important swine pathogen, infects alveolar macrophages (AMΦ) causing dysregulated interferon (IFN)-α and tumor necrosis factor (TNF)-α production through a mechanism(s) yet to be resolved. Here we show that AMΦ infected with PRRSV secreted a relatively reduced quantity of IFN-α following the cells’ subsequent exposure to synthetic dsRNA. This diminution did not correlate with less IFNA1 gene transcription but rather with two events that occurred late during infection and were indicative of translational attenuation - the activation of eukaryotic translation initiation factor 2 (eIF2)α and the appearance of stress granules. In contrast, the typical, more rapid production of TNF-α of AMΦ in response to encountering lipopolysaccharide (LPS) was suppressed or enhanced by PRRSV infection depending on when stimulation was initiated. If introduction of this agonist was delayed until 6 h post-infection to enable eIF2α phosphorylation by the stress sensor RNA-like endoplasmic reticulum kinase (PERK) to be coincident with abundant TNF-α synthesis, inhibition was observed, presumably due to translational repression. However, a synergistic response, due to earlier NF-κB activation via another stress sensor, inositol-requiring protein (IRE)-1α, was noted if LPS exposure began 4 h earlier, prior to a detectable onset of eIF2α phosphorylation. These results suggest that, depending on when after PRRSV infection an affected AMΦ encounters LPS, the asynchronous actions of two distinct branches of the virus replication-induced unfolded protein response (UPR), IRE-1α and PERK, can increase or decrease TNF-α production via the activation of NF-κB or eIF2α, respectively.
2.2 INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive sense single-stranded enveloped RNA virus, classified within the family Arteriviridae (King, Adams, & Lefkowitz, 2011), and causes the most economically significant infectious malady afflicting pigs in commercial swine farms worldwide (Holtkamp et al., 2013). Following oronasal infection, PRRSV invades the animal’s lower respiratory tract where it exploits alveolar macrophages (AMΦ) for its replication, triggering a massive infiltration with macrophages of the alveolar septa, resulting in interstitial pneumonia (Van Reeth & Nauwynck, 2000). Although there is wide variation in the degree of PRRSV virulence, the associated disease is rarely lethal and begins to resolve within 4 weeks (Thanawongnuwech et al., 2000; Xu et al., 2010). However, in >60% of PRRS cases, complications from bacterial co-infections occur (Zeman, 1996) and the corresponding pneumonias become exacerbated as exemplified by a worsening clinical syndrome and greater mortality (Thanawongnuwech et al., 2000; Xu et al., 2010). During uncomplicated pulmonary PRRSV infections, interleukin (IL)-1 and IL-6 are readily detected in the hosts’ bronchoalveolar lavage (BAL) fluids, while the amounts of IFN-α and TNF-α present are nominal (Albina, Carrat, & Charley, 1998; Van Gucht, Van Reeth, & Pensaert, 2003; Van Reeth & Nauwynck, 2000). As compared to the profile of innate cytokines elicited by other porcine viruses that also cause pneumonia, the relative paucity of these two cytokines could be responsible for the less severe disease produced by PRRSV alone (Van Reeth & Nauwynck, 2000). In contrast, BAL fluids collected from the lungs of animals undergoing a dual infection have been shown to contain relatively large quantities of TNF-α (Guo et al., 2013; Han et al., 2014). Thus, due exposure to PRRSV and bacterial pathogen-associated molecular patterns (PAMP) may elicit enhanced production of this pro-inflammatory cytokine that in turn could
contribute to lung damage. In this regard, pulmonary infection by PRRSV has been shown to sensitize the lung to generate greater amounts of TNF-α upon respiratory challenge with LPS with an accompanying aggravation of respiratory disease (Van Gucht et al., 2003). Moreover, while PRRSV infection of porcine AMΦ stimulates limited TNF-α expression (Van Gucht et al., 2003) the infected cells will release an enhanced amount of TNF-α in response to LPS exposure (Qiao et al., 2011).

Given the critical role that INF-α and TNF-α play in host immunity, the ability of PRRSV to alter the production of these two cytokines has been extensively examined (Gómez-Laguna, Salguero, Pallarés, & Carrasco, 2013; Miller, Lager, & Kehrli, 2009; Wang & Zhang, 2014). Consequently, modulatory properties ascribed to PRRSV have been found to vary from strict inhibition of IFN-α production in response to stimulation with potent type I IFN agonists including a porcine coronavirus (Albina et al., 1998) and synthetic double-stranded RNA (dsRNA) (Miller et al., 2009) to enhancement or inhibition of TNF-α production in response to LPS exposure (Gómez-Laguna et al., 2013). Since the underlying mechanism by which PRRSV infection of AMΦ moderates the cytokine response of its natural host cell is unclear, the method(s) employed by PRRSV to impact the ability of porcine AMΦ to produce IFN-α and TNF-α in response to stimulation with two known agonists, namely a synthetic dsRNA (poly I:C) and LPS, respectively, was systematically examined.

2.3 RESULTS

Permissiveness and growth kinetics of PRRSV in the porcine AMΦ cell line ZMAC.

A difficulty commonly encountered when studying the interaction between PRRSV and its natural host cell is that the permissiveness of different batches of primary porcine AMΦ to
PRRSV infection is highly variable, ranging from 15% to 60% (Duan, Nauwynck, & Pensaert, 1997; Gaudreault, Rowland, & Wyatt, 2009) and thus could contribute to obtaining inconsistent results. To confront this problem, the porcine AMΦ line ZMAC, which is readily infected by PRRSV (Du, Yoo, Paradis, & Scherba, 2011) was used in most of the studies while primary AMΦ served as the host for confirming major observations. The extent of susceptibility of the ZMAC cells to PRRSV was determined by infecting them with PRRSV virus strain P129-GFP, which was engineered to transcribe green fluorescent protein (GFP) as an structural gene in cells productively infected with this virus (Pei et al., 2009). Accordingly, cultures of ZMAC cells were infected with P129-GFP virus at a multiplicity of infection (MOI) of 5 to ensure nearly complete exposure of cells to at least one infectious particle, and the percent of GFP+ cells were scored directly in unprocessed cell cultures. At 4 h post infection (hpi), <1% of the infected ZMAC cells exhibited green fluorescence (Fig. 2.1A). In contrast, at 8 hpi, clear evidence of GFP expression was observed in 73% of the cells and approximately 10% of them exhibited evidence of cytopathic effect (CPE), which was characterized by cell rounding and the random presence of membrane blebs (Fig. 2.1A). By 12 hpi, 80% of the cells were GFP+ and most also exhibited CPE, which was visualized as cell rounding accompanied by the formation of cytoplasmic vacuoles (Fig. 2.1A). At this time, approximately 15% of the cells had membrane blebs, suggesting that these cells were in the late stages of apoptosis, a process triggered by PRRSV infection of AMΦ (Costers, Lefebvre, Delputte, & Nauwynck, 2008). After an additional 8 h (20 hpi), the percent of GFP+ cells had decreased to 50% (Fig. 2.1A). However, at this point, the majority of GFP- cells lacked structural integrity and exhibited extensive disintegration. The nature of the CPE observed at 20 hpi was consistent with an advanced stage of apoptosis termed secondary necrosis, which is known to occur late during PRRSV infection of
AMΦ PRRSV (Couters et al., 2008). Based on these results, ZMAC cells appear to be highly permissive for PRRSV.

Next, the rate of PRRSV replication in ZMAC cells was determined. As shown in the single step growth curve (Fig. 2.1B), after a slight gain in virus titer at 4 hpi, there was an approximately 10⁴-fold increase by 8 hpi with another approximately 20-fold maximum achieved by 8 h later. Thus, the replication cycle of PRRSV in ZMAC cells is completed within 14-16 hpi, at a time similar to the reported 12-h period required for the same process to occur in primary porcine AMΦ (Couters et al., 2008).

**Kinetics of the IFN-α or TNF-α response of porcine AMΦ to the toll-like receptor (TLR) agonists, poly I:C and LPS, respectively.**

To ascertain the effect of known TLR agonists on the cytokine response of AMΦ, the kinetics of IFN-α and TNF-α production by ZMAC cells stimulated with either synthetic dsRNA or LPS, respectively, was examined. Extracellular IFN-α was initially detected at 4 h after cell exposure to poly I:C (Fig. 2.2A) and the quantity of this cytokine had increased 5-fold when measured 4 h later. The detected abundance of IFN-α was preceded by the phosphorylation of IRF3 (p-IRF3), which was evident at 1 h after stimulation of the cells with poly I:C (Fig. 2.2B). In contrast, the TNF-α response to LPS exposure developed much faster and cytokine production had nearly plateaued within 4 h of incubation (Fig. 2.2C). Overall, this response to LPS stimulation was preceded by the phosphorylation of NF-κB, which was detected at 30 min post-infection and appeared to have dissipated by 1.5 h later (Fig. 2.2D). Thus, like primary AMΦ (Miller et al., 2009; Qiao et al., 2011), ZMAC cells readily responded to stimulation by poly I:C and LPS.
Infection of AMΦ with PRRSV inhibits their ability to produce IFN-α in response to stimulation with synthetic dsRNA.

PRRSV infection of porcine primary AMΦ has been shown to result in a decreased cytokine response to type I IFN agonists including synthetic dsRNA (Albina et al., 1998; Miller et al., 2009). To verify a similar situation with ZMAC cells, the influence of PRRSV infection on their ability to produce IFN-α in response to poly I:C exposure was examined. Whereas infectious or inactivated PRRSV failed to elicit the production of IFN-α by ZMAC cells (Fig. 2.3) as previously reported for primary AMΦ (Albina et al., 1998), exposure of the uninfected or UV-treated virus infected cells to poly I:C for 8 h resulted in the release of a similar, abundant quantity of this cytokine. In contrast, a prior 2 h infection of the ZMAC cells with viable PRRSV before stimulation reduced the relative production of IFN-α by approximately 50%. Thus, PRRSV interference with ZMAC responsiveness to this type I IFN agonist appeared to be an active and not a passive event. Similar results were obtained when primary AMΦ which had been known to have a >50% level of permissiveness for PRRSV, were used (data not shown).

PRRSV infection of porcine AMΦ does not inhibit their ability to activate IRF3 and STAT1 or to transcribe the IFNA1, IFNB1, and IRF7 genes in response to stimulation with synthetic dsRNA.

As a mechanism for the inhibition of the IFN-α response by stimulated AMΦ, PRRSV has been reported to have the ability to inhibit the transcription of type I IFN genes by affecting the activation of key transcription factors (TF) via the action of some of its non-structural proteins (Wang & Zhang, 2014). To explore this possibility, the effect of PRRSV infection on the poly I:C-induced activation of TF known to play a key role in the early phase (IRF3) and positive-
feedback regulation (STAT1) of type I IFN gene expression (Honda, Takaoka, & Taniguchi, 2006; Marie, Durbin, & Levy, 1998) was examined. Whereas phosphorylated IRF3 (Fig. 2.4A) or STAT1 (Fig. 2.4B) was not detected in the lysates of uninfected cells, similar quantities were readily found in samples prepared from the poly I:C–treated cells regardless of whether they were left uninfected or infected with one of two different PRRSV strains for 1, 3, or 5 h prior to agonist addition (Fig. 2.4A and 2.4B). Thus, prior PRRSV infection of AMΦ did not appear to influence IRF3 or STAT1 activation in response to stimulation with ds RNA.

Since PRRSV-associated inhibition of IFN-α production by stimulated ZMAC cells occurred at a step past IRF3 and STAT1 activation, a possible effect on the transcription of genes whose products are involved in type I IFN induction was assessed. Initially, an early phase representative of this process, IFNB1, was checked. In this case, virus infection of the ZMAC cells was allowed to proceed 2 h, before the addition of poly I:C for 1, 4, or 7 h. As compared to its undetected activity in the uninfected, untreated cells (data not shown), expression of the IFNB1 gene was observed at 1 h post agonist exposure and was at a similar rate regardless of whether the cells were uninfected or infected with one of two PRRSV strains (Fig. 2.4C). This response appeared to have been mitigated approximately 80% by 3 h later and then may have increased approximately two-fold after an additional 3 h. Since the early phase of type I interferon induction was apparently not being affected by PRRSV, the presence of IRF7 mRNA whose product is a key part of the positive feedback regulation of type I IFN production (Honda et al., 2006) was monitored. In this case, a constitutive expression of the IRF7 gene was detected in the uninfected, untreated cells (data not shown) at a level approximately double of that detected in any of the cells stimulated for 2 h. However, the relative quantities of IRF7 mRNA had approximately tripled by 3 h later and variably increased during the ensuing 3 h (Fig. 2.4D).
At either later time, there was no apparent negative effect on IRF7 gene expression by PRRSV. Since the presence of IRF7 in cells stimulated with dsRNA should result in the induction of IFN-α genes, the expression of one representative, IFNA1, was monitored. Although, like the INFB1 gene, expression of the INFA1 gene in uninfected, untreated cells was not detected (data not shown), relatively similar and small amounts of INFA1 mRNA were measured in all of the cell samples exposed to poly I:C for 1 or 4 h. However, by 7 h post addition, these amounts had similarly increased 4-5-fold (Fig. 2.4E). Since induction of porcine IFNA1 gene expression occurred after that of the IRF7 gene, regulation of this particular IFN-α gene may be like that of the murine delayed IFN-α gene set that has been shown to be dependent on IRF7 for its expression (Marie et al., 1998). In that case, since PRRSV infection did not impede their host cells’ ability to produce IRF7 and IFNA1 mRNA after stimulation with poly I:C, the blockage occurs subsequent to IFNA1 gene expression and would not involve translation arrest until late during the virus’s infectious cycle. Thus, synthesis of the small amount of IFN-β/α necessary to engage the type I IFN receptor and initiate the positive feedback loop of the type I IFN response (Honda et al., 2006) would not be impacted due to the early nature of this action.

**PRRSV infection of porcine AMΦ induces eIF2α activation and stress granule formation.**

One consequence of intracellular virus replication is the elicitation of ER stress (Jheng, Ho, & Horng, 2014) that involves the activation of the stress sensor PERK, which by phosphorylating eIF2α, results in the attenuation of translation (Hetz, 2012). Since the activation of PERK has been observed in PRRSV-infected, primary AMΦ (Huo et al., 2013), temporal screening for the presence of phosphorylated eIF2α (p-eIF2α) in PRRSV-infected ZMAC cells was conducted. Whereas p-eIF2α was either undetected or present in a minor amount in mock-treated, uninfected cells, this activated protein was readily observed in similar, uninfected cells
exposed to DTT, a potent inducer of ER stress, for 1 h. Likewise, phosphorylated eIF2α was found in cells initially infected with either of two PRRSV strains 8 h earlier (Fig. 2.5A). Since eIF2α phosphorylation should cause the accumulation of stalled translation initiation complexes that would associate with RNA binding proteins to create stress granules (SG) (Kedersha, Gupta, Li, Miller, & Anderson, 1999), PRRSV-infected ZMAC cells were screened for their presence by using indirect immunofluorescence with an antibody (Ab) specific for the TIA-1 related protein (TIAR), which is a primary component of these structures (Anderson & Kedersha, 2008).

At 4 hpi, the percentage of TIAR+ cells (3.8%) was comparable to that observed in the uninfected, untreated cells (3.5%). However, by 4 h later (8 hpi), the percentage of TIAR+ cells had increased by approximately 11.8-fold to 72% and this value was similar to that obtained for the DTT-treated, ER-stressed cells (51%) (Fig. 2.5B). Interestingly, the appearance of TIAR+ SG in PRRSV-infected cells temporally coincided with the detection of p-eIF2α, two events indicative of translation arrest (Kedersha et al., 1999).

To determine if the SG-containing ZMAC cells were also productively infected, the cells incubated with anti-TIA-1 Ab were simultaneously probed with an anti-dsRNA Ab in order to detect viral dsRNA produced during PRRSV replication. While not identified in the mock- or DTT-treated cultures, this molecule was observed in 13% and 72% of the virus-infected cells at 4 and 8 hpi respectively (Fig. 2.5B). Although less than half of the ZMAC cells were SG+ in contrast to the majority being virus-infected, all of the former were contained in the latter group suggesting that the perceived translational attenuation was virus-induced.

The PRRSV-mediated inhibition of synthetic dsRNA-induced IFN-α production by porcine AMΦ temporally coincides with eIF2α phosphorylation.
To address further the plausible involvement of translation attenuation on the PRRSV-mediated inhibition of INF-α production, the kinetics of eIF2α phosphorylation and inhibition of IFN-α production were simultaneously determined in the same cultures of PRRSV-infected AMΦ. Accordingly, ZMAC cells or primary porcine AMΦ were infected with PRRSV for 2 h prior to their stimulation with poly I:C and then harvested at various times thereafter. A reduction in the amount of IFN-α released by the infected ZMAC cells as compared to the uninfected cells was apparent at 7 hpi and this difference increased temporally through the ensuing 3 h (Fig. 2.6A). The corresponding amount of p-eIF2α was noticeably greater at each of these times as compared to that in the uninfected cells (Fig. 2.6B and 2.6C) and corroborated the previous demonstration that eIF2α was phosphorylated after 5 but at least by 8 hpi (Fig. 2.5A). Similar results were observed when primary AMΦ served as the host except that a strong inhibition of IFN-α production was first detected at 12 hpi (Fig. 2.6D) when the amount of p-eIF2α was much greater in the virus-infected vs. the uninfected cells (Fig. 2.6E, 2.6F). Thus, for both kinds of porcine AMΦ, the subdued cytokine response to poly I:C exposure post PRRSV infection correlated with an apparent phosphorylation of eIF2α.

**PRRSV infection initially enhances but later suppresses the TNF-α response of porcine AMΦ to LPS.**

Since PRRSV-regulated inhibition of the IFN-α response by stimulated AMΦ appeared to be a delayed and time-dependent effect, the influence of this virus on the expression of a different cytokine that is associated with faster response kinetics was investigated. For this purpose, a system that obtained a half-maximal response within 3 h, namely TNF-α production due to LPS stimulation (Fig. 2.2C), was selected. In this case, PRRSV infection of ZMAC cells was allowed to proceed for 2, 4, or 6 h before the addition of LPS and the presence of TNF-α
was measured 2 and 4 h afterwards, respectively (Fig. 2.7). These time points were selected based on the results in Fig. 2.5A so that the activation of eIF2α should be negligible during the initial stimulation of any of the virus-infected cultures but should proceed within the ensuing 2 h (6-8 hpi). As compared to the amount of TNF-α released by mock-infected AMΦ cultures, an approximately 1.7-fold increase of this cytokine was measured at 4 and 6 hpi in the medium overlaying the ZMAC cells infected 2h prior to LPS exposure. In contrast, when the infection was extended 2 h before LPS addition and measurements made at 6 and 8 hpi, the previously observed enhanced response appeared to be nearly eliminated. A further 2 h delay in the inclusion of LPS in the medium resulting in monitoring at 8 and 10 hpi was associated with an approximately 50% reduction in the measured amount of TNF-α as compared to that made by the treated, uninfected cells (Fig. 2.7). Thus, unlike the delayed negative influence of PRRSV on the INF-α response by stimulated AMΦ, its effect on the cells’ TNF-α production ranged in a spectrum from enhancement to reduction that was dependent on the temporal extent of virus infection prior to stimulation.

The PERK-eIF2α signaling pathway is involved in the inhibitory effect of PRRSV infection on the TNF-α response of porcine AMΦ stimulated with LPS late during the virus’s infectious cycle.

Based on the dependence of agonist exposure occurring near the end of virus replication in porcine AMΦ, the reduction in LPS responsiveness of PRRSV-infected cells might be attributed to eIF2α activation, as accorded to their repressed reaction to poly I:C. In that case, there should be a coincidence between the appearance of p-eIF2α and the decreased cellular responsiveness to LPS. To access this possibility, the influence of the length of PRRSV infection of ZMAC cells on these two events was evaluated. Although not as dramatic as previously observed (Fig. 2.7),
there was a small increase (approximately 10-20%) in the quantity of TNF-α released by 4 hpi from LPS-treated ZMAC cells previously infected for 2 h with either of two PRRSV strains as compared to that secreted by the uninfected cells (Fig. 2.8A). Likewise, reductions (approximately 60-78%) in this parameter were again noted at 8 hpi when the time of infection before LPS exposure was extended 4 h. While there was a negligible, discernable difference between the amounts of intracellular p-eIF2α in the untreated and 4 h virus-infected samples, there was a relative abundance of this protein in the cells at 8 hpi (Fig. 2.8B).

Since the activation of eIF2α during ER stress is mediated by PERK (Hetz, 2012), the effect of the PERK inhibitor (PERKi) GSK2606414 (Axten et al., 2012) on the ability of PRRSV to induce phosphorylation of eIF2α and inhibit TNF-α production was examined. Initially, ZMAC cells were infected with PRRSV for either 4 or 6 h prior to their exposure to PERKi for an additional 5 or 3 h to ensure that harvesting of the cells occurred after the onset of eIF2α activation (Fig. 2.5A). In two independent experiments (Fig. 2.8C), the addition of PERKi at either time post virus infection resulted in the generation of lesser amounts of p-eIF2α as indicated by a ≥50% reduction in their resultant p-eIF2α/eIF2α ratios as compared to that obtained for the untreated, PRRSV-infected AMΦ (Fig. 2.8C). For evaluation of induced TNF-α synthesis, AMΦ were infected for a period of 9 h, with inclusive, delayed 5 and 2 h incubations with PERKi and LPS, respectively. Whereas virus infection alone caused an approximately 38% drop in cellular responsiveness to LPS, this repressive ability was reduced approximately 55% in the presence of the PERKi (Fig. 2.8D). Thus, PERK-mediated phosphorylation of eIF2α, which occurs during the late phase of PRRSV replication in porcine AMΦ, is involved in the virus-mediated inhibition of the LPS-triggered response.
Activation of the IRE-1α-NF-κB signaling pathway is involved in the enhanced effect of PRRSV infection on the TNF-α response of porcine AMΦ stimulated with LPS early during the virus’s infectious cycle.

Based on its dependence on TLR4 agonist exposure occurring close to the beginning of virus replication in porcine AMΦ (Fig. 2.7), an increase in the TNF-α response to LPS responsiveness of PRRSV-infected cells might be attributed to the activation of NF-κB via the IRE-1α signaling pathway. This concept is supported by the demonstrations that TNF-α expression occurring during ER stress is IRE-1α and NF-κB dependent (Hu, Han, Couvillon, Kaufman, & Exton, 2006), by the observation that the activation of IRE-1α occurs in PRRSV-infected AMΦ (Huo et al., 2013) and also by the knowledge that activated IRE-1α can act in concert with TLR to augment pro-inflammatory cytokine production (Martinon, Chen, Lee, & Glimcher, 2010). Accordingly, PRRSV infection of porcine AMΦ should cause an early enhancement of the generation of phosphorylated IRE-1α (p-IRE-1α) and NF-κB (p-NF-κB).

Indeed, this was the case, as the quantity of the former in PRRSV-infected ZMAC cells peaked at 2 hpi (Fig. 2.9A) while the amount of the later somewhat increased at 2-4 hpi with a greater gain noted at 6 hpi as compared to that present in the untreated cells (Fig. 2.9B). Due to the observed temporal overlap in the presence of intracellular p-IRE-1α and p-NF-κB (Fig. 2.9A and 2.9B) and also the known association of the kinase domain of IRE-1α with NF-κB activation (Tam, Mercado, Hoffmann, & Niwa, 2012), it was plausible that PRRSV infection of AMΦ initially promoted the formation of p-NF-κB as a consequence of an induced stress response involving the activation of IRE-1α. In that case, inclusion of the IRE-1α kinase inhibitor, KIRA6 (Ghosh et al., 2014), shown here to disrupt DTT-induced, IRE-1α phosphorylation (Fig. 2.9A), should negatively impact the ability of PRRSV to promote the phosphorylation of NF-κB and
also the synthesis of TNF-α by LPS-stimulated cells. As predicted, the ratio of p-NF-κB/NF-κB was reduced, in this case by >50 %, when KIRA6 treatment was applied to the virus-infected cells (Fig. 2.9C). In contrast, there was no noticeable effect on the relative amounts of the two forms of NF-κB when the LPS-treated cells were incubated with KIRA6 (Fig. 2.9C), presumably because in macrophages LPS signaling through TLR4 causes an early-phase activation of NF-κB via an alternate entity, the intracellular adaptor protein MyD88 (Akira & Takeda, 2004).

Notably, the previously demonstrated augmentation of a combined early stage of PRRSV infection and LPS exposure of AMΦ (Fig. 2.7) was reiterated in that there was still found to be greater production (approximately 30% higher) of TNF-α by the stimulated cells previously infected at 2 or 4 h earlier as compared to the cells only treated with LPS (Fig. 2.9D). Since this enhancement was still apparent when the minor contribution by the non-stimulated, virus-infected cells was considered, it is apparent that a significant synergistic TNF-α response ($P < 0.03$) due to the interaction between PRRSV and LPS was occurring. Interestingly, the observed PRRSV-associated increases in TNF-α production by LPS-stimulated cells were not only abolished by the inclusion of KIRA6 at 2 hpi but were also reduced approximately 14-19% (Fig. 2.9D). Likewise, a similar, approximately 12-14% decrease in the amount of secreted TNF-α was observed when the uninfected, stimulated cells underwent KIRA6 treatment.

Presumably, these diminutions can be attributed to the involvement of IRE-1α in TLR-mediated pro-inflammatory cytokine production by macrophages (Qiu et al., 2013). Thus, the activation of NF-κB during the early stages of PRRSV replication in AMΦ is dependent on IRE-1α phosphorylation resulting in a synergistic TNF-α response to LPS.

**PRRSV protein expression is not affected by the phosphorylation of eIF2α.**
Since the requirement of eIF2α to form the initiation complexes is shared by all the translation mechanism except type IV IRES translation (Jackson, Hellen, & Pestova, 2010), a time-course study using PRRSV-infected ZMAC and primary PAM cells was conducted to determine if viral protein synthesis was being affected by the phosphorylation of eIF2α. Using the GFP-expressing PRRSV, we determined that the production of viral proteins took place concurrently with the highest level of eIF2α phosphorylation observed at 10 hpi (Fig 2.10A) and 12 hpi (Fig 2.10B). This observation suggests that there must be a mechanism by PRRSV protein synthesis overcomes the translational attenuation due to the ER stress induced phosphorylation of eIF2α.

### 2.4 Discussion

As shown previously (Albina et al., 1998; Miller et al., 2009), infection of porcine AMΦ with PRRSV did not promote IFN-α synthesis and instead inhibited their ability to produce this cytokine when stimulated with the appropriate agonist. In this regard, the mechanism by which this repression occurs is considered to be at the transcriptional level via the action of non-structural PRRSV proteins, which seemingly have the ability to block type I IFN signaling pathways (Wang & Zhang, 2014). However, those experiments relied on the overexpression of single viral genes, and thus fail to provide a perspective for a potential, combined action of two or more of the viral proteins. In contrast, the current study was designed to examine the overall effect of PRRSV infection of porcine AMΦ on the signaling pathways involved in the production of type I IFN in response to stimulation with synthetic dsRNA. Here, although the actual production of IFN-α was still markedly inhibited, PRRSV infection was shown to not interfere with the poly I:C-induced activation of IRF3 or STAT1 in AMΦ, or impair the transcription of the IFNB1, IRF7 or IFNA1 genes. These results are in agreement with the
observations by Zhang et al (H. Zhang, Guo, Nelson, Christopher-Hennings, & Wang, 2012), in which the poly I:C induced synthesis of IFN-α by monocyte-derived dendritic cells was curtailed post-transcriptionally. Consequently, one alternate possibility was that the suppression of IFN-α production by PRRSV-infected AMΦ occurred at the translational level, especially since the elicitation of a UPR by PRRSV infection had been demonstrated (Huo et al., 2013). In this regard, RNA virus replication typically places inordinate stress on the protein folding machinery of the host ER. To survive this ER stress, the host mounts a UPR that includes the activation of PERK that enables it to phosphorylate the key translation regulator eIF2α resulting in universal translational attenuation (Jheng et al., 2014). In accordance with this concept, the reduced ability of PRRSV-infected, subsequently stimulated AMΦ to produce IFN-α coincided temporally with the activation of eIF2α and the appearance of SG. Since these two events are hallmarks of stalled translation (Kimball, Horetsky, Ron, Jefferson, & Harding, 2003), it is likely that translation attenuation was responsible for the observed inhibitory effect. This conclusion was further supported by the finding that PRRSV infection could also inhibit TNF-α production by AMΦ in response to LPS contact. However, in this case the timing at which stimulation with the TLR4 agonist was initiated was critical in that it had to occur late during the virus infectious cycle when the bulk of the TNF-α response, which developed approximately twice as fast as the IFN-α response to poly I:C exposure, coincided with an ample supply of activated eIF2α. Otherwise, a relatively, early LPS stimulation of the infected AMΦ promoted an apparent synergistic or unaltered response that could be at least partially attributed to completion of TNF-α synthesis prior to the onset of p-eIF2α regulated, translational blockage. Moreover, a role for p-eIF2α in inhibiting the cellular LPS response was indicated by the reduction of eIF2α phosphorylation and TNF-α production by PERKi-treated, PRRSV-infected AMΦ. Therefore, translational
attenuation via the activation of the ER stress sensor PERK in the late stages of virus infection results in an impaired ability of PRRSV-infected AMΦ to produce cytokines in response to TLR agonists. In addition, our analysis of viral protein synthesis, using GFP protein as a surrogate it seemed not to be affected by phosphorylation of eIF2α. Similar observation had by reported in the studies with coronavirus. However, the underlying mechanism for this event is unknown (Bechill, Chen, Brewer, & Baker, 2008; Raaben, Groot Koerkamp, Rottier, & De Haan, 2007).

In addition to PERK, another stress sensor IRE-1α is activated during PRRSV infection of AMΦ (Huo et al., 2013) as a consequence of ER stress. Following its phosphorylation, its cytosolic effector domain interacts with the C-terminus of the TNF-α receptor associated factor 2 (TRAF2), an adaptor protein in the TNF-α signaling pathway. The IRE-1α-TRAF2 complex recruits IκB kinase (IKK), which in turn leads to the phosphorylation and degradation of IκB, resulting in NF-κB activation and upregulation of its downstream inflammatory pathways (Hu et al., 2006; Kaneko, Niinuma, & Nomura, 2003; Tam et al., 2012). Here, involvement of p-IRE-1α formed in PRRSV-infected AMΦ in the generation of p-NF-κB and the subsequent synergistic TNF-α response to LPS stimulation was demonstrated by the detection of the activated forms of both proteins in untreated cells and the reduction in the quantity of p-NF-κB and the loss of synergy in cells treated with the IRE-1α inhibitor KIRA6. Thus, it appears that successful elicitation of the above-mentioned pathway by PRRSV infection can result in a functional reprogramming of AMΦ activity towards a pro-inflammatory phenotype as evidenced by the enhanced TNF-α response of AMΦ to LPS during the early stages of PRRSV replication.

In their role as sentinels against pulmonary infections (Bowden, 1984), the inflammatory response of AMΦ to cellular debris or to inhaled innocuous particles is relatively limited as compared to a sufficiently strong pro-inflammatory response to respiratory pathogens that is not
so excessive as to compromise the vital gas exchange function of the lung (Hussell & Bell, 2014). However, alterations in the regulatory mechanisms that maintain a delicate balance between a pro- and anti-inflammatory functional phenotype can trigger macrophage-directed immune overreactions resulting in lung immunopathology (Gwyer Findlay & Hussell, 2012). For instance, overly robust pro-inflammatory cytokine responses are thought to be involved in exacerbated lung injury in bacterial co-infections with viruses including human influenza (McCullers, 2014). As the most intensely studied pro-inflammatory cytokine, TNF-α is now considered to be a central factor in acute viral diseases including influenza and is prominently mentioned in cytokine storm literature (Tisoncik et al., 2012). In the respiratory mode, PRRSV targets AMΦ for replication, and produces an interstitial pneumonia that eventually resolves (Halbur et al., 1996). Frequently however, a PRRSV infection becomes complicated with opportunistic bacteria (Opriessnig, Gimenez-Lirola, & Halbur, 2011; Zeman, 1996) that commonly reside in the upper respiratory tract of pigs (Kernaghan, 2014). As a result of pulmonary, dual PRRSV and bacterial infections, a severe clinical syndrome which is characterized by an enhanced pro-inflammatory cytokine response, severe lung tissue damage, high morbidity, hypoxia and often death (Guo et al., 2013; Thanawongnuwech et al., 2000; Xu et al., 2010) ensues. Recently, a relatively large quantity of TNF-α present in the lungs of pigs infected with a highly pathogenic isolate of PRRSV complicated with secondary bacterial infections was credited as the most likely factor responsible for the initiation of a severe pneumonia. This disease was characterized by the presence of co-infecting bacteria, lung consolidation, edema and swelling, infiltration with neutrophils and mononuclear cells as well as vascular injury, epithelial cell death, exudation and hemorrhage, which flooded the alveolar space. The latter changes combined with increased thickness of the alveolar septa resulted in
severe hypoxia and morbidity (Han et al., 2014). An indication that dysregulated cytokine production in the lung could be involved in the severe respiratory syndrome observed during PRRSV-bacterial co-infections is suggested by the observation that this virus sensitizes the lung to respond with enhanced pulmonary pro-inflammatory cytokine production and the resulting respiratory distress worsens upon exposure to LPS (Van Gucht et al., 2003), and also by the greatly increased virulence of PRRSV in the presence of secondary bacterial infections (Xu et al., 2010). Based on the observations presented here and by Van Gucht et al. (Van Gucht et al., 2003), it seems reasonable to propose that the activation of the IRE-1α-NF-κB branch of the UPR during the early stages of PRRSV replication in porcine AMΦ could result in dysregulation of the normally moderate pro-inflammatory cytokine (TNF-α) response to bacterial PAMP derived from commensal but potentially pathogenic bacteria commonly present in the respiratory tract of commercially-raised swine (Kernaghan, 2014). In this scenario, in the absence of PRRSV infection, AMΦ normally produce a regulated pro-inflammatory cytokine response to bacterial PAMPs of sufficient intensity to contain the microbes while maintaining homeostasis between the host and pathogen. In the event of a respiratory infection with PRRSV, those AMΦ in the early stages of PRRSV infection would over react to the presence of bacterial PAMPs, resulting in dysregulated production of TNF-α, promoting the development of severe inflammation and lung dysfunction.

The UPR of ER-stressed cells plays a role in the development of a number of lung diseases (Osorio, Lambrecht, & Janssens, 2013). Here, we provide evidence that during the early stages of PRRSV infection of AMΦ the IRE-1α branch of the UPR is activated resulting in enhanced TNF-α production in response to LPS exposure. These results support the emerging concept that the UPR directly activates pro-inflammatory TF (K. Zhang & Kaufman, 2008), is involved in
microbe sensing by cells of the immune system (Smith, 2014), and that the activation of IRE-1α acted in synergy with TLR activation pro-inflammatory cytokine production in macrophages (Martinon et al., 2010).

2.5 MATERIALS AND METHODS

Cells

The porcine AMΦ cell line, ZMAC-4 (ZMAC), was derived from the lungs of porcine fetuses (Zuckermann, 2008) and consists of phagocytic cells that express several surface markers characteristic of AMΦ (Ezquerra et al., 2009), including CD14, CD45, CD163, and CD172 (Zuckermann, 2008). ZMAC cells have been shown to efficiently support the growth of PRRSV (G. Calzada-Nova, Schnitzlein, Husmann, & Zuckermann, 2011; Du et al., 2011).

PAM were acquired from the lungs of specific-pathogen-free pigs in BAL fluid obtained by infusing their tracheas under aseptic conditions with 50 ml phosphate buffered saline (PBS) per lung. The collected lavages were centrifuged at 500 x g and 4°C for 10 min. The cell pellets were washed twice with Hank’s buffered sterile saline (HBSS) and suspended in RPMI medium supplemented with 10% fetal bovine serum (FBS) (GIBCO®, Invitrogen, Grand Island, NY, USA) and processed for freezing using DMSO. Aliquots of the resulting suspensions were stored in liquid nitrogen until further use. Both PAM and ZMAC cells were cultured in RPMI-1640 medium containing l-glutamine (Mediatec, Herndon, VA, USA) and supplemented with 10% fetal bovine serum (FBS) (GIBCO®), 1 mM sodium pyruvate and 1 × non-essential amino acids (Mediatec), and kept at 37°C in a 5% CO₂ atmosphere. Maintenance of ZMAC cells also required the inclusion of 10 ng/ml recombinant mouse macrophage colony stimulating factor.
(Shenandoah Biotechnology, Inc., Warwick, PA). MARC-145 cells were grown as previously described (Meier et al., 2003).

**Viruses**

PRRSV wild type strains, NADC20 (Harms et al., 2001), and FL12 (Truong et al., 2004), were propagated in MARC-145 cells (Meier et al., 2003). Cell-free preparations of virus were prepared by centrifugation of the medium overlaying infected cell monolayers showing ≥80% CPE at 4°C and 350 × g for 10 min. The medium was then layered on top of a 15% iodixanol (OptiPrep™, Sigma-Aldrich, St Louis, MO, USA) cushion and subjected to ultracentrifugation at 64,100 × g and 4°C for 3 h. The resulting, virus-containing pellets were suspended in TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA). Purified virus stocks were titrated in monolayers of ZMAC cells (50% tissue culture infective dose [TCID₅₀]). When required, purified NADC20 virus was inactivated by exposure to short-wave (254 nm) UV light for 3 min. Loss of viability was verified by the inability of the UV light-exposed viruses to produce CPE in monolayers of MARC-145 cells. GFP-expressing PRRSV (P129-GFP virus) was kindly provided by D. Yoo (University of Illinois) (Pei et al., 2009) and propagated in ZMAC cells. To obtain the single-step virus growth curve, ZMAC cells were infected with PRRSV strain NADC20 at an MOI=5 to ensure a high degree of synchronous viral infection. After 1 h infection at 37°C, the cell cultures were washed twice and thereafter samples of cell-free supernatants were collected at specified time intervals. The amount of infectious virus dose (TCID₅₀) present in them was determined by using ZMAC cells.

**Infection and treatment of porcine AMΦ**
Unless otherwise stated, ZMAC cells were cultured in sterile snap cap tubes (Corning, New York, USA) and were either mock-infected or infected with PRRSV at an MOI=5. Where indicated, uninfected and PRRSV-infected cells were subsequently exposed to either 25 µg/ml poly I:C, 100 ng/ml LPS, 1 µM IRE-1α inhibitor, KIRA6 (Ghosh et al., 2014) (EMD Millipore, Darmstadt, Germany), or 1-2 µM IRE-1a inhibitor, KIRA6 (Ghosh et al., 2014) (EMD Millipore, Darmstadt, Germany), for the indicated lengths of time. As a positive control for eIF2α activation analysis, the AMΦ cultures were treated with 2 mM DTT for 1 h.

Quantitation of IFN-α and TNF-α.

Cell-free culture supernatants were assayed for the presence of IFN-α as previously described (Gabriela Calzada-Nova, Schnitzlein, Husmann, & Zuckermann, 2010). For TNF-α detection, the same procedure was followed except that the wells were coated with 50 µl of 4 µg/ml anti-pig TNF-α monoclonal Ab (mAb) (clone103304, R&D systems, Minneapolis, MN, USA) before addition of the medium and the bound cytokine was detected with 50 µl of 2.5 µg/ml biotin-labeled, anti-pig TNF-α mAb (clone103302 R&D systems). The OD₄₅₀ of triplicate wells were averaged and the amounts of TNF-α were determined based on a standard curve generated from the values obtained with known quantities of this cytokine.

Western blotting of AMΦ lysates

Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with cocktail protease inhibitor (Amresco, Solon, OH, USA) and the protein concentrations of the resulting lysates were determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equivalent protein amounts of each extract (25 to 60 µg per well) were subjected to separation in a 10% SDS-PAGE gel and subsequently transferred onto a 0.2µ
PVDF membrane (Bio-RAD, Hercules, CA, USA) for Western blot analysis. Membranes were incubated in blocking buffer [2% fish gelatin in TBST solution (50 mM Tris, pH 7.5, 500 mM NaCl and 0.5 % Tween 20)] at room temperature (RT) for one h. Afterwards, the membranes were incubated at 4°C overnight with one of the following unconjugated primary Ab (1:1000 dilution of manufacturer’s original concentration in TBST with 5% BSA): anti-IRF3 (clone D83B9, Cell Signaling, Danvers, MA, USA), anti-phospho-IRF3 (Ser396) (clone 4D4G, Cell Signaling), anti-NF-kB p65 (#3034, Cell Signaling), anti-NF-kB p65 (#Sc109, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho NF-kB p65 (Ser536) (clone 93H1, Cell Signaling), anti-STAT1 (sc346,), anti-phospho STAT1 (Tyr701) (SC-7988, Santa Cruz Biotechnology), anti-eIF2 (#9722, Cell Signaling), anti-phospho-eIF2 (#9721, Cell Signaling), or anti-ß-actin (#4967, Cell Signaling). Membranes were then washed four times in TBST solution, and then incubated with horseradish peroxidase (HRP)-conjugated , anti-rabbit immunoglobulin (IgG) secondary Ab (sc2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1: 8000 in blocking buffer) at RT for 1 h. After again being washed, the membranes were incubated with a chemiluminescence reagent (GE Heathcare, Little Chalfont, Buckinghamshire, UK) to enable detection of bound secondary Ab. Screening for the presence of a specific phosphorylated protein was always performed prior to detection of the corresponding, non-phosphorylated form on membranes that had been incubated in stripping buffer (#21059, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 15 min to remove any covalently-bound Ab.

**RNA preparation and real-time RT-PCR**

Samples of $10^5$ uninfected or PRRSV-infected ZMAC cells were cultured in the presence or absence of poly(I:C) for the indicated times. Afterwards, each sample was lysed in buffer RLT, and total RNAs were purified, DNase-treated, converted into cDNA and subjected to real-time
PCR as previously described (G. Calzada-Nova et al., 2011). Primers and probes for the amplification/detection of porcine IFN-α1 and IFNβ1 gene transcripts have been described (G. Calzada-Nova et al., 2011), whereas those associated with the amplification/detection of IRF7 and ribosomal protein (RP) L32 gene transcripts were designed and provided by H. Dawson (USDA, Beltsville, MD) and are described in the DGIL Porcine Translational Research Database (http://www.ars.usda.gov/Services/docs.htm?docid=6065). Changes in the extent of expression of the IFNA1, IFNB1 and IRF7 genes were determined by using the comparative CT method and the formula \(2^{-\Delta\Delta C_T}\) (Livak & Schmittgen, 2001), where RPL32 was used as the reference gene.

**Detection of AMΦ stress granule formation and dsRNA.**

A total of 2 X 10⁵ ZMAC cells were grown in individual wells of a Nunc LabTekII, 8-well chamber slide. At the specified times after just being cultured, exposed to DTT or infected with PRRSV, the monolayers were fixed in PBS containing 4% paraformaldehyde for 20 min at RT, washed with PBS, incubated with blocking buffer (PBS containing 0.3% Triton X-100 and 3% normal goat serum) for 1 h at RT and then incubated in Ab dilution buffer (PBS containing 0.3% Triton X-100 and 1% BSA) containing rabbit anti-TIAR mAb (1:200 dilution of manufacture’s original concentration) (cloneD32D3, Cell Signaling) overnight at 4°C. Afterwards, the cells were washed 3 times in PBS and then incubated with Ab dilution buffer containing 5 ug/ml goat anti-rabbit IgG conjugated to DyLight594 (#35560, Thermo Scientific) for 1 h at RT. After again being washed 3 times with PBS, the monolayers were incubated at RT in blocking buffer for 1 h and then in Ab dilution buffer containing mouse anti-dsRNA monoclonal antibody (1:200 dilution of manufacture’s original concentration) (cloneJ2, Scicons, Szirák, Hungary) for 2 h, washed 3 times with PBS and then incubated with Ab dilution buffer containing 2.5 μg/ml goat anti-mouse IgG conjugated to FITC (#62-6511, Zymed™, Life Technologies) for 1 h at RT.
Afterwards, the chamber was removed and the glass slide was wet mounted in anti-fading medium (Invitrogen™, Life Technologies). Fluorescent signals were observed with an immunofluorescence microscope (Leica DMII 4000B, Wetzler Germany).

**Statistical analysis.**

Unpaired Student’s t test (one-tailed) was used to determine if significant differences existed in regard to the cytokine response exhibited by AMΦ between treatment groups. To determine the presence of statistically significant synergism, the interaction effect between PRRSV, LPS and the IRE-1α inhibitor was tested using a two-way ANOVA (Slinker, 1998). A *P* value of <0.05 was considered statistically significant.
2.6 Reference


2.7 **FIGURES AND LEGENDS**

![Figure 2.1 Kinetics of PRRSV infection of porcine AMΦ](image)

(A) Monolayers of $2 \times 10^5$ ZMAC cells were infected with PRRSV strain P129-GFP (MOI= 5). GFP+ cells in each sample were scored visually at the indicated times (hpi) by using an inverted fluorescence microscope. The outcome of one such infection is sequentially shown in the four panels where the overall mean percentage of GFP+ cells ± SD (n=3) is indicated below each respective panel. (B) Monolayers of $2 \times 10^5$ ZMAC cells were infected with PRRSV strain FL12 (MOI=5). Medium overlaying the infected cells was removed at the indicated times (hpi) and titrated for the amount of infectious virus (TCID$_{50}$/ml) in ZMAC cells. The mean ± SD (n=3) is presented.
Figure 2.2 Kinetics of the IFN-α or TNF-α response of porcine AMΦ to the TLR agonists, poly I:C and LPS, respectively (A) $2.5 \times 10^5$ ZMAC cells were exposed to poly I:C for the indicated times (h). Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The mean ± standard deviation (n = 2) is presented. (B) $10^6$ ZMAC cells were either mock-treated or exposed to poly I:C for 1 h, lysed, and then subjected to Western blotting for the sequential detection of p-IRF3, IRF3, and β-actin. (C) $2.5 \times 10^5$ ZMAC cells were exposed to LPS for the indicated times (h). Afterwards, the amount of TNF-α present in the overlaying media was determined by using a specific ELISA. The mean ± standard deviation (n = 3) is presented. (D) $10^6$ ZMAC cells were either mock-treated for 2 h or exposed to LPS for the indicated times, lysed, and then subjected to Western blotting for the sequential detection of NF-κB and p-NF-κB.
Figure 2.3 PRRSV infection of porcine AMΦ inhibits their ability to produce IFN-α in response to stimulation with synthetic dsRNA. Duplicate samples of 2.5 X 10^5 ZMAC cells were either just cultured (mock) or incubated with UV light-inactivated or viable PRRSV strain NADC20. Two h later, one member of each pair was exposed to poly I:C for an additional 8 h. Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The mean ± standard deviation (n=2) is presented. The statistical comparisons were made between the amount the cytokine present in the overlaying medium of infected vs. un-infected cultures stimulated with poly I:C. Asterisks indicate values that are statistically significant (**, P<0.01); NS indicates differences that were not statistically significant.
Figure 2.4 PRRSV infection of porcine AMΦ does not inhibit their ability to initiate IRF3 and STAT1 activation or IFNB1, IFR7, and IFNA1 gene transcription in response to stimulation with poly I:C (A, B) \(10^6\) ZMAC cells were just cultured for 5 h before an addition 1-h incubation in the presence/absence of poly I:C and then lysed. Other replicate cell sets were infected with either PRRSV strain FL12 or NADC20 for the indicated times and then exposed to poly I:C for 1 h before lysis. Afterwards, all lysates were subjected to Western blotting for the sequential detection of p-IRF3, IRF3, and β-actin (A) or p-STAT1, STAT1, and β-actin (B). (C, D, E) \(10^5\) ZMAC cells were just cultured or infected with either PRRSV strain FL12 or NADC20 for 2 h, and then exposed to poly I:C for the indicated times before being lysed in preparation for RNA extraction. One additional replicate cell set was also just cultured for 3 h before lysis. Total RNA obtained from each sample was subjected to real-time PCR to detect IFNB1, IFR7, and IFNA1 gene transcripts. The fold-changes in the amount of these RNAs present in the virus-infected, poly I:C-stimulated AMΦ as well as the uninfected cells exposed to poly I:C for 4 or 7 h relative to that in the uninfected cells incubated with poly I:C for 1 h were determined by using the formula \(2^{-\Delta \Delta Ct}\), where RPL32 was used as the reference housekeeping gene. RNA fold-increases are not shown for the uninfected, untreated cell sample where IFNB1 and IFNA1 gene transcripts were not detected and IRF7 gene transcripts were approximately 2-fold greater than that observed for the cells exposed to poly I:C for 1 h.
Figure 2.5 PRRSV infection of porcine AMΦ induces the phosphorylation of eIF2α and the formation of SG 
(A) $10^6$ ZMAC cells were cultured for 2 h before an addition 1-h incubation in the presence/absence of DTT or infected with either PRRSV strain FL12 or NADC20 for the indicated hpi and then lysed. Afterwards, all lysates were subjected to Western blotting for the sequential detection of p-eIF2α and eIF2α. (B) Monolayers of $2.5 \times 10^5$ ZMAC cells were cultured for 2 h before an addition 1-h incubation in the presence/absence of DTT, or infected with PRRSV strain FL12 for the indicated hpi. Afterwards, the cells were fixed, made permeable, and stained for the presence of TIAR or dsRNA (red or green fluorescence, respectively). Three-fold crop images are displayed under the original 40X images. The mean percentage ± SD (n=2) is presented and were derived from the examination of 100-cell samples.
Figure 2.6 PRRSV-induced inhibition of IFN-α production by stimulated porcine AMΦ temporally coincides with eIF2α phosphorylation (A-C) $10^6$ ZMAC cells were cultured (mock) or infected with PRRSV strain FL12 for 2 h and exposed to poly I:C for the indicated times. (A) Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The virus-associated reduction in IFN-α synthesis is indicated below the respective time of polyI:C stimulation. The mean ± SD of a representative experiment (n=3) is presented. (B, C) Lysates derived from the corresponding virus-infected (B) and cultured (C) cells were subjected to Western blotting for the sequential detection of p-eIF2α and eIF2α. (D-F) Cultures of $2 \times 10^6$ primary PAM were cultured (mock) or infected with PRRSV strain P129-GFP for 2 h and then exposed to poly I:C for the indicated times. Afterwards, the overlaying media and lysates from the corresponding cells were assayed (D) and probed (E, F), respectively, as described above (Fig.3A-C). For Fig. 3D, the mean ± SD of a representative experiment (n=2) is presented. The statistical comparisons were made between the amount the cytokine present in the medium overlaying the un-infected vs. virus-infected cultures stimulated with poly I:C. Asterisks indicate values that are statistically significant (**, P<0.01, *, P<0.05).
Figure 2.7 PRRSV infection of porcine AMΦ initially enhances but later suppresses the extent of their TNF-α response to LPS. 2.5 x 10⁵ ZMAC cells were cultured for 2 h (mock) or infected with PRRSV strain FL12 for 2, 4, or 6 h and then exposed to 100 ng/ml LPS for the indicated times. Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The statistical comparisons were made between the amount of cytokine present in the medium overlaying infected vs. un-infected cultures stimulated with LPS. The mean ± SD of a representative experiment (n=3) is presented. Asterisk indicate values that are statistically significant (**, P<0.01, *, P<0.05).
Figure 2.8 PRRSV infection of porcine AMΦ requires the PERK-eIF2α signaling pathway to inhibit the cellular TNF-α response to LPS exposure (A, B) 10⁶ ZMAC cells were cultured for 2 h before an addition 2- or 1-h incubation in the presence/absence (mock) of LPS or DTT, respectively. Other replicate cell sets were infected with either PRRSV strain FL12 or NADC20 and then exposed to LPS for 2 h starting 2 (-2 h) or 6 h (-6 h) later. Afterwards, the amount of TNF-α present in the overlaying media was determined by using a specific ELISA. The mean ± standard deviation (n=3, one experiment) is presented. (B) Lysates derived from the corresponding virus-infected and cultured cells were subjected to Western blotting for the sequential detection of p-eIF2α and eIF2α. (C) 10⁶ ZMAC cells were cultured for 2 h before two additional 1-h, sequential incubations in the presence/absence (mock) of 2 μM PERK inhibitor (PERKi) GSK2606414 and DTT and then lysed. Other replicate cell sets were infected with either PRRSV strain FL12 or NADC20 and then exposed to GSK2606414 for 5 or 3 h starting 2 (-2 h) or 6 h (-6 h) later, respectively, before lysis. Afterwards, all lysates were subjected to Western blotting for the sequential detection of p-eIF2α and eIF2α. Densities of the resulting bands, determined by using ImageJ software, were used for calculating the relative amount of eIF2α phosphorylation (p-eIF2α/eIF2α ratios). Results for the cultured and virus-infected cell samples are shown for one or two independent experiments (exp A and B), respectively. In panel A, statistical comparisons were made between the amount of cytokine present in the virus-infected vs. un-infected cultures stimulated with LPS. (D) 10⁶ ZMAC cells were either cultured or infected with PRRSV FL12 for 2 h before an addition 5- and 2- h incubation in the presence/absence of 1 μM GSK2606414 and in the presence of LPS, respectively. Afterwards, the amount of TNF-α present in the overlaying media was determined by using a specific ELISA. Results are shown as the percent reduction in the quantity of TNF-α released by virus-infected versus uninfected, LPS-stimulated AMΦ in the absence/presence of the PERKi. Statistical comparison was made between the inhibitions obtained in the presence vs. the absence of the PERKi. The mean ± SD (n=2) is presented. Asterisk indicate values that are statistically significant (**, P<0.01, *, P<0.05).
Figure 2.9 PRRSV infection of porcine AMΦ requires the IRE-1α-NF-κB signaling pathway to enhance the cellular TNF-α response to LPS exposure

(A) 1.5 x 10^6 ZMAC cells were cultured for 6 h before an addition 1-h incubation in the absence of DTT and the IRE-1α inhibitor, KIRA6 (mock), in the presence of only DTT or in the presence of both DTT and KIRA6 and then lysed. Other replicate cell sets were infected with PRRSV strain FL12 for the indicated times before lysis. Afterwards, all lysates were subjected to Western blotting for the detection of p-IRE-1α. (B) 10^6 ZMAC cells were cultured for 6 h before an addition 1-h incubation in the presence/absence (mock) of LPS or just infected with PRRSV strain FL12 for the indicated times before lysis. Afterwards, all lysates were subjected to Western blotting for the sequential detection of p-NF-κB, NF-κB and β-actin. (C) 1.5 x 10^6 ZMAC cells were cultured for 1 h before an additional 1-h incubation in the absence of LPS and KIRA6 (mock), in the presence of only LPS or in the presence of both KIRA6 and LPS and then lysed. Other replicate cell sets were infected with PRRSV strain FL12 for 3 h in the presence/absence of KIRA6 before lysis. Afterwards, all lysates were subjected to Western blotting for the detection of p-NF-κB and NF-κB. Densities of the resulting bands, determined by using ImageJ software, were used for calculating the relative amount of NF-κB phosphorylation (p-NF-κB/NF-κB ratios). (D) 2.5 x 10^5 ZMAC cells were cultured for 2 h before an additional 4 h incubation in the absence of LPS and KIRA6 (mock) or an addition 2- or 4-h incubation in the presence of both LPS and KIRA6 or just LPS. Other replicate cell sets were infected with PRRSV strain FL12 before an additional 2 or 4 h incubation in the presence of only LPS or both LPS and KIRA6. Afterwards, the amount of TNF-α present in the overlaying media was determined by using a specific ELISA. The mean ± SD of a representative experiment (n=2) is presented. The presence of a statistically significant synergistic TNF-α response caused by the interaction between PRRSV and LPS was determined by using a two-way ANOVA [56]. The test consisted of comparing the amounts of TNF-α present in the overlaying media of cultures that were either mock-infected, PRRSV-infected, LPS-treated, or LPS-treated and infected with PRRSV. The effect of KIRA6 on the synergisms was analyzed in the same way except that the latter two treatment groups consisted cultures performed in the presence or absence of the IRE-1α inhibitor. Asterisk indicate values that are statistically significant (*, P<0.05); NS indicates differences that were not statistically significant.
Figure 2.10 The viral GFP protein synthesis is not affected by phosphorylation of eIF2α ZMAC cells (A) or PAM cells (B) were mock-infected or infected with either PRRSV strain P129-GFP. At the indicated times after infection, WCLs were prepared from the cells and subjected to Western blot to detect the viral GFP. As a positive control for ER stress the cells were treated with 2mM of DTT for 1 hour.
CHAPTER 3: PRRSV VIRUS EVADES THE INNATE IMMUNE DETECTION BY Hiding ITS dsRNA INSIDE INTRACELLULAR MEMBRANOUS COMPARTMENTS.

3.1 ABSTRACT

Most, if not all, positive-stranded RNA viruses induce in its host cells the formation of specialized membranous compartments in order to form what is commonly called “viral replication factories.” For instance, in cells infected with flavivirus or nidovirus, the principal entity is a possible endoplasmic reticulum derivative, the double-membrane vesicle (DMV). When visualized by using electron microscopy, newly synthesized viral RNA as well as a replicative intermediate, namely double-stranded (ds) RNA, has been observed inside these DMV structures. As dsRNA is currently considered to be the most potent type I interferon (IFN) agonist, it has been suggested that sequestration of viral dsRNA may also help viruses evade host innate immune detection by reducing exposure of viral dsRNA to viral nucleic acids cytoplasmic sensors. This predicted type of immune evasion by virus-induced intracellular membrane structures had been confirmed for flaviviruses. By coupling a selective permeabilization technique with immunostaining analysis, a positive correlation between the cytosolic exposure of virus dsRNA and a host type I INF response was demonstrated. In this case, the extent of flavivirus dsRNA exposure was dependent on both the virus species and host cell type. Despite lacking definitive experimental evidence regarding members of the nidovirales family including arteriviruses and coronaviruses, it has been suggested that these viruses also utilize a similar immune evasion strategy. In this regard, the development of DMV structures has been associated with the activity of arterivirus nsp2 and its coronavirus equivalent, nsp3. Moreover, in our previous study, the arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV) was shown to be incapable of inducing type I IFN synthesis upon infecting
porcine alveolar macrophages. Thus, it seemed plausible that to evade detection by the innate immune system, PRRSV simply uses a passive evasion mechanism. In the present study, in conjunction with the application of selective permeabilization techniques combined with immuno-staining for dsRNA we demonstrated for the first time that the PRRSV dsRNA is sequestered inside membranous compartments, which prevent its detection by the host’s viral nucleic acid sensors located in the cytoplasm. This finding was made possible by a PRRSV laboratory strain mutant with a unique and deleterious mutation in the N-terminal region of NSP2, for which the virus loses its ability to keep its dsRNA sequestered inside membranous compartments and thus away from detection by viral nucleic acid sensors, resulting in a substantial type I IFN response upon infecting alveolar macrophages.
3.2 INTRODUCTION

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), the etiological agent of Porcine Reproductive and Respiratory Syndrome (PRRS), majorly impacts the swine industry worldwide, causing about 600 million dollars in losses in the United State per year (Neumann et al., 2005). Together with Equine Arteritis Virus (EAV), Lactase Dehydrogenase Virus (LDV) and Simian Haemorrhagic Fever Virus (SHFV), PRRSV belongs to the family Arteriviridae within the order of Nidovirales, which contains viruses with single stranded, positive-sense RNA genomes with a size of approximately 15 kb including a 5’ cap structure and 3’ poly-A tail (Fang & Snijder, 2010). To date, lack of effective control of this disease is apparently due, for the most part, to antigenic variation among the PRRSV strains (Meng, 2000; Xu et al., 2010) as well as an inadequate immune response elicited by the PRRSV in infected swine hosts (Murtaugh, Xiao, & Zuckermann, 2002).

It has been shown that PRRSV infection fails to elicit a conventional type I interferon response by plasmacytoid dendritic cells (pDC) (G. Calzada-Nova, Schnitzlein, Husmann, & Zuckermann, 2011) and porcine alveolar macrophages (AMΦ) (W. Chen, Calzada-Nova, Schnitzlein, & Zuckermann, 2012). The latter is not only the primary cell targeted by PRRSV for replication (Molitor, Bautista, & Choi, 1997; Snijder & Meulenberg, 1998) but also the major interferon producer in the lung (Kumagai et al., 2007). Albina et al. (Albina, Carrat, & Charley, 1998) first reported this inadequate type I interferon response to PRRSV by showing that PRRSV infection apparently failed to induce significant IFN-α production in the lung of infected pigs and only exhibited a week IFN-α response in their serum. In addition, it was shown that PRRSV infection apparently compromised the IFN-α response in the porcine AMΦ.
superinfected with TGEV, suggesting the modulation of host type I interferon response by PRRSV.

Since then, understanding the mechanisms by which PRRSV apparently inhibits the host cell type I interferon response, especially at the molecular level, has become a major topic of research to understand the interaction between the PRRSV and the host cell. Several studies have shown that PRRSV non-structural protein most prominently described, nsp1, possess inhibitory activity over the activation of transcription factors required for the initiation of type I interferon gene transcription (Beura et al., 2010; Z. Chen et al., 2010; M. Han, Y. Du, C. Song, & D. Yoo, 2013; X. Shi, Wang, et al., 2011; C. Song, P. Krell, & D. Yoo, 2010; Subramaniam et al., 2010; Xu et al., 2010).

For the most part these studies have been conducted using permissive cells derived from species that are not the natural host to PRRSV, and by the overexpression of individual viral proteins in combination with reporter assays in these cells. The observed results from these experiments may not necessarily reflect the natural behavior of PRRSV in the infected animal or in its natural host cell, namely the porcine AMΦ. Accordingly, the numerous mechanisms by which PRRSV mediates type I interferon inhibition in infected alveolar macrophages suggested by these studies could be based on the experimental bias which these techniques introduce.

Our previous studies have shown that in spite of massive dsRNA produced in PRRSV-infected AMΦ, it does not induce a type I interferon response. Moreover, PRRSV infection does not inhibit host type I interferon gene transcription induced by treatment of PRRSV-infected porcine alveolar macrophages with synthetic dsRNA (poly I:C). These findings suggest that dsRNA, produced during a PRRSV infection, is not readily accessible to the dsRNA sensor,
which act as triggers for type I interferon response, located in the cytoplasm of cells of the innate immune system.

Most, if not all, positive-sense RNA viruses induce intensive membrane rearrangement during virus replication. Despite the diversity in morphology and origin of the virus-induced membrane structures, the intimate association of virus replication complex and the virus-induced membrane structures, has become a universally recognized feature shared by positive strand RNA viruses (Paul & Bartenschlager, 2013). This observation is further supported by visualization of concentrated dsRNA and newly synthesized viral RNA inside such structure by using immuno-labeling and metabolic labeling techniques, respectively. Altogether, it is believed that this structure functionally benefits virus replication by providing a surface for assembling virus replication and transcription complexes (RTC) and providing a shelter for replicative intermediates, potent inducers of host type I interferon responses, keeping them from being detected by host dsRNA sensor (den Boon & Ahlquist, 2010; Han et al., 2012; Mackenzie, 2005; Miller & Kijnse-Locker, 2008). The sheltering function of this virus-induced intracellular membrane was preliminarily confirmed in flavivirus studies. Applying selective permeabilization techniques in immunostaining studies demonstrated the correlation between the exposure of cytosolic dsRNA and host type I interferon response. The degree of the dsRNA exposure is dependent on both the virus species, strain, and cell type (L. A. Espada-Murao & K. Morita, 2011; Overby, Popov, Niedrig, & Weber, 2010; Uchida et al., 2014).

Like other RNA viruses with positive polarity genomes, arteriviruses exhibit an exceptional ability to modify intracellular membrane structures in infected cells (Pedersen, van der Meer, Roos, & Snijder, 1999; Pol, Wagenaar, & Reus, 1997; Stueckemann et al., 1982; Wood, Tauraso, & Liebhaber, 1970). Double membrane vesicles are the most evident structure
observed in arterivirus infected cells. These arteriviruses induced structures are the same structures observed in cells infected with a coronavirus (Knoops et al., 2008). Extensive study of DMVs has been conducted in the EAV infection model (Fang & Snijder, 2010; Knoops et al., 2012; Pedersen et al., 1999; Posthuma et al., 2008; Snijder, van Tol, Roos, & Pedersen, 2001). Like other members in this family, EAV replication and transcription complex mainly consists of viral non-structural proteins (nsps) that generated from polyprotein1a or polyprotein1b via a cascade of protease reactions. The association of EAV non-structural proteins with intracellular membranous structures and the co-localization of these proteins with newly synthesized RNA was demonstrated by van der Meer et al., which becomes the first evidence supporting the role of host membrane structure in arterivirus replication (van der Meer, van Tol, Locker, & Snijder, 1998). Recently, the ultrastructure of EAV induced DMV was resolved by applying high-pressure cryofixation techniques in an Electron Microscopy (EM) analysis. The connection of the Endoplasmic Reticulum (ER), and the association of the ribosome with the outer-membrane of DMV, were visualized, indicating the strong relationship between the EAV-induced DMV and the host ER. Moreover, the immunogold labeling analysis in the same study indicates that while most EAV replicative intermediates concentrate within the interior of DMV, the components of viral replication complex have wider distribution on the reticulo-vesicular network (Knoops et al., 2012).

EAV-induced DMV formation mechanisms have not been detailed. EAV nsps contain trans-membrane domains such as nsp2 and nsp3 are likely to be involved in the DMV formation process. By using a transfection approach, the EAV nsp2-nsp3 polyprotein expressed from an alphavirus vector is able to induce DMV-like structures in transfected cells. The association of nsp2 and nsp3 can also be identified by cryo-EM analysis (Snijder et al., 2001). In addition to
EA\(V\), this membrane modification activity has also been observed in Severe Acute Respiratory Syndrome – Corona Virus (SARS-CoV) nsp5s containing trans-membrane domains, including nsp3, nsp4 and nsp6. All of these nsp5s show various degrees of membrane modification capacity when individually or cooperatively transfected in the human kidney epithelial cell line, HEK293T (Angelini, Akhlaghpour, Neuman, & Buchmeier, 2013).

Although the sequestration of viral dsRNA from cytosolic viral nucleic acid sensors has not been proven experimentally for the Nidovirales family, both newly synthesized RNA and dsRNA produced during nidovirus replication have been shown, by immuno-electron microscopy, to be wrapped in double membrane vesicle (DMV) (Gosert, Kanjanahaluethai, Egger, Bienz, & Baker, 2002; Knoops et al., 2012; Knoops et al., 2008). Here we demonstrate for the first time that during its replication in AM\(\Phi\), PRRSV sequesters its dsRNA in membranous compartments that prevent its detection by host Pattern Recognition Receptors (PRRs), and that the functionality of this process is associated with nsp2. This novel observation was made possible with the aid of a unique laboratory strain of PRRSV that carries a unique mutation in the N-terminal domain of PRRSV nsp2 for which the virus lost its ability to hide its dsRNA and as a result it induces a substantial type I interferon response when replicating in AM\(\Phi\).

3.3 RESULTS

The PRRSV strain 1198 exhibits a unique ability to elicit a strong IFN-\(\alpha\) response in porcine AM\(\Phi\).

The 1198 PRRSV strain was isolated in our laboratory by plaque purification from the PRRSV wild-type isolate 46448. The laboratory PRRSV strain 1198 differs from its parental
virus as well as all other PRRSV wild-type strains that we have tested, in that, upon infecting porcine AMΦ, it elicits a strong type I interferon (IFN) response. The IFN-α response induced by the 1198 strain is both dose (Fig. 3.1A) and time (Fig. 3.1B) dependent. A temporal analysis showed that the IFN-α response elicited by the 1198 strain in AMΦ parallels the replication cycle of PRRSV in this type of cell, which is completed within 8 to 12 hours. As compared to the IFN-α response elicited in AMΦ by poly I:C, there was a delay of 0 to 3 hours in the IFN-α response of AMΦ infected with 1198. This delay likely corresponds to the time between virus entry into the cell and the production of the replicate intermediates, namely dsRNA. The earliest time that IFN-α was detected in culture supernatants from virus infected AMΦ was at three hours post infection. This was approximately the same time when the dsRNA from the same culture conditions was be detected by immunostaining analyses (data not shown). These data suggest that the IFN-α production is stimulated by PRRSV dsRNA. The exponential phase of IFN-α production that occurs from 3 to 12 hours after infection parallels the increasing amount of dsRNA present in the cell, which is the result of genome replication and sub-genomic gene transcription. To test the dependency of IFN-α production on virus replication, we tested a UV-inactivated 1198 virus in a similar study. The resulting data clearly showed that the induction of IFN-α production by 1198 PRRSV strain was dependent on virus replication (Fig. 3.1C).

**Infection of AMΦ with PRRSV strain 1198 triggers the activation of eIF2α but also the production of large amounts of IFN-α mRNA.**

Our previous studies have shown that the replication of PRRSV in AMΦ induces ER stress, which triggers the phosphorylation of eukaryotic initiation factor 2α (eIF2α) and the formation of stress granules, indicating the occurrence of translation attenuation. The phosphorylation of eIF2α results in a significant reduction in the ability PRRSV-infected AMΦ
to produce IFN-α in response to their stimulation with poly I:C (Chen et al., submitted for publication). Thus, we set out to examine the status of eIF2α in 1198-infected cells in order to determine if the strong IFN-α production induced by this strain could be the result of a milder activation of this eukaryotic initiation factor, as compared to the strong phosphorylation of eIF2α normally induced by wild-type versions of this virus. As shown in Fig. 3.2A, the 1198 strain efficiently triggered the phosphorylation of eIF2α. The kinetics of eIF2α phosphorylation was comparable to what we have observed when AMΦ are infected with the wild-type PRRSV strains FL12 and NADC20 (Chen et al, submitted for publication).

Next, we reasoned that the intense production of IFN-α by the 1198 strain could be attributed to an over-production of IFN-α mRNA that would compensate for the phosphorylation of eIF2α and the resulting translational attenuation. To test this notion, AMΦ were stimulated with poly I:C or infected with the PRRSV strain 1198, and the levels of IFN-α mRNA quantified by RT-PCR. This analysis demonstrated that at 6 hpi, when the IFN-α secretion stimulated by PRRSV strain 1198 is comparable to that induced by poly I:C (Fig 3.1B), the level of IFN-α mRNA in cells infected with PRRSV strain 1198 is approximately 5-fold higher than the level detected in poly I:C treated cells (Fig 3.2B). This difference was even greater at 10 hpi, at which point the production of IFN-α by 1198-infected cells has greatly increased (Fig. 3.1B) and the amount of IFN-α mRNA present was about 40-fold higher than cells treated with poly I:C (Fig 3.2C). To further investigate a possible compensatory mechanism to explain the intense production of IFN-α by AMΦ infected with the 1198 strain, analyses were conducted using AMΦ infected with different wild-type PRRSV strains, which stimulate a very low production of IFN-α in AMΦ (Fig. 3.1A). In this set of experiments the amount of IFN-α produced and the level of IFN-α mRNA produced after virus infection were determined in the same culture and
used to calculate the translation efficiency of IFN-α. The translation efficiency is based on the IFN-α protein concentrations normalized to relative IFN-α mRNA level. These results showed that although the PRRSV strain 1198 is able to stimulate the production of large amounts of IFN-α in AMΦ, the translational efficiency for IFN-α mRNA, as compared to the one induced by poly I:C, is very low, but it is comparable that observed in cells infected wild-type virus (Fig. 3.2D), which do not elicit significant production of IFN-α or significant amounts of IFN-α mRNA (Fig. 3.1A). From this set of studies we conclude that the ability of the PRRSV strain 1198 to stimulate the production of large amounts of IFN-α despite the abundant presence of phosphorylated eIF2α is most likely due to the large amounts of IFN-α mRNA produced by the infected cell which apparently is able to overcome translational attenuation.

The PRRSV strain 1198 uniquely induces the activation of IRF3, which is associated with its strong ability to stimulate IFN-α production.

IRF3 is perhaps the most critical transcription factor regulating the production of type I interferon. Thus, it was imperative to ascertain whether this transcription factor is activated as a result of the infection of AMΦ with PRRSV strain 1198. Thus, the activation of the IRF3 in AMΦ infected either with strain 1198, the nearly isogenic strains 46448 and 1198B, or wild-type PRRSV, was examined. The results of this experiment showed that the activation of IRF3 was only observed in AMΦ infected with strain 1198 or when stimulated with poly I:C (Fig. 3.3A). During the derivation of the AMΦ cell line ZMAC, a batch of cells became contaminated with Bovine Viral Diarrhea virus (BVDV), which perhaps due to the presence of the BVDV viral protein Npro, which is IRF3-cleaving and inactivates this transcription factor (Zihong Chen et al., 2007; Hilton et al., 2006), resulted in the creation of a version of this cell line, termed ZMAC-9-13, that lost its ability to activate IRF3 and consequently the ability to produce IFN-α
when stimulated with poly I:C (Fig 3.3B). We made use of this BVDV-infected ZMAC cell line to examine the role of IRF3 in the IFN-α response to the 1198 virus in our system. As shown in Fig. 3.3C, the production of IFN-α in ZMAC-9-13 cells infected with the 1198 virus was very low while the same virus strongly stimulated the production of this cytokine in the BVDV-free ZMAC-4 cells. Notably, the growth of the 1198 virus in the ZMAC-4 cells is at least 100-fold lower than in the ZMAC-9-13 cells (data not shown). Together, these results suggest that the copious IFN-α response of the ZMAC-4 cells to the 1198 strain is dependent on the activation of IRF3.

**Comparison of the genomes of 1198 strain and the revertant 1198B virus reveals a single amino acid change in a highly conserved amino acid position of nsp2**

Given the remarkable phenotype of the 1198, its full genome, the genome of the parental strain 46448 as well as the one of the related strain 1198B, which had lost the ability to stimulate IFN-a production, were determined and the resulting amino acid sequences compared. This analysis revealed that the genomic sequences of the 1198 and 1198B strains differ from each other in only a few nucleotides, resulting in single non-conservative amino acid change in nsp2 at position 339 (Tyrosine to Histidine; Fig. 3.4A) as well as the structural viral glycoproteins 3 and 4 (data not shown). The PRRSV strain 1198 was derived from the 89-46448 isolate by plaque purification using the simian kidney cell line MARC-145. In contrast, the mutant 1198B was isolated from the serum of a pig that had been inoculated a few days earlier with the 1198 strain. The fortuitous isolation of the 1198B strain appears to represent a reverting strain of 1198, which displays a mutation, resulting in an amino acid change at position 339 in nsp2, reverting the virus to the original residue displayed by the parental 46448 strain (Fig. 3.4A). As shown in Fig. 3.1A, the reverting 1198B strain was found to have lost the ability to induce IFN-a
production upon infecting AMΦ that is displayed by the 1198 strain (Fig. 3.1A). The uniqueness of this mutation became more apparent in a pairwise alignment analysis that included nsp2 sequences of genetically distant PRRSV strain of type II genotype (Fig. 3.4B). This analysis led us to further investigate the plausible role of the mutation in position 339 of nsp2 in determining the strong IFN-α response elicited by the PRRSV strain 1198.

**The production but not the distribution of dsRNA in AMΦ infected by wild-type PRRSV or the 1198 strain is comparable.**

The PRRSV transcription replication complex (TRC) is comprised of PRRSV nsp2 together with other nsps. It is possible that the unique mutation that we have identified in the nsp2 of strain 1198 increases the replicase activity, of the TRC, which would result in the production of more replicative intermediates, namely dsRNA. An increased amount of dsRNA in the infected cell could result in greater type I interferon response by making it abundantly available for detection by cytoplasmic sensors. To determine whether dsRNA was differentially produced between the PRRSV strains, an immunostaining analysis was performed to visualize the presence of dsRNA in PRRSV infected cells. This analysis demonstrated that all of the PRRSV strains that were examined produced large quantities of dsRNA in the cytoplasm of the infected cells during their replication cycle, and no apparent differences in their amount was noted. This observation suggests that another mechanism was likely involved in determining the ability of strain 1198 to strongly stimulate IFN-α production by infected cells. Notably, while no significant differences in the presence of dsRNA was apparent, the distribution of dsRNA in cells infected by PRRSV strain 1198 was less concentrated at the perinuclear region as compared to the distribution noted in cells infected with either wild-type virus or the reverting strain 1198B (Fig. 3.5). The perinuclear distribution and ER co-localization of PRRSV dsRNA observed in
our immunostaining analysis is consistent with earlier electron microscopic data showing the arterivirus dsRNA concentrates inside the virus-induced double membrane structures called double membrane vesicles (DMVs) which are likely derived from ER and predominantly localized at the perinuclear region (Knoops et al., 2012). The localization of dsRNA in the perinuclear region is consistent with the notion that PRRSV could be passively evading detection by the host’s cytoplasmic viral nucleic acid sensors, possibly via the sequestration of the dsRNA within DMVs. The involvement of Nsp2 in the formation of DMV has been reported. Thus, we reason that the unique mutation displayed by PRRSV strain 1198 could be negatively affecting the ability of the DMV to hide the dsRNA from the cytoplasmic sensors, which could lead to their activation and the resulting activation of IRF3 and production of IFN-α.

**Selective permeabilization of plasma membranes.**

In order to examine the possible leakage of dsRNA in cells infected with PRRSV strain 1198, we needed to use a technique that would enable to discriminate between the localization of dsRNA either free in the cytosol or contained inside a membranous compartment. A selective-permeabilization method coupled with an immunostaining analysis has been applied with success for the selective detection of cytosolic dsRNA in flavivirus (L. A. Espada-Murao & K. Morita, 2011; Overby et al., 2010; Uchida et al., 2014). Among the selective permeabilization methods that we tested, we determined that the freeze-thaw technique reported by Mardones & Gonzalez (2003) was the simplest and most convenient for our purposes (Mardones & Gonzalez, 2003). To validate the selective permeabilization technique, the common ER resident molecule, calreticulin, and the mitochondria outer membrane protein, tom20, were used as intraorganelle and cytosolic markers, respectively. Using these two markers we were able to implement the optimal freeze-thaw conditions that selectively permeabilized the cytoplasmic membrane of the
AMΦ without permeabilizing the membrane of organelles. As shown in Fig. 3.6, while the cytosolic molecule can be detected in both Triton-X100 and freeze-thaw treated cells, the intraorganelle molecule calreticulin could only be detected in the Triton-X100-treated cells. These results indicate the successful use of the freeze-thaw technique to selectively permeabilize the plasma membrane of AMΦ which can be used to differentiate by immunostaining the localization of dsRNA either free in the cytosol vs. inside of a membranous compartment.

The presence of dsRNA free in the cytosol was detected only in cells infected with strain 1198 but not in cells infected with wild-type PRRSV or the revertant virus 1198B.

The localization of dsRNA in AMΦ infected with either wild-type PRRSV, strain 1198, or revertant strain 1198B was examined by immunostaining using selective permeabilization by freeze-thaw, and compared to the staining obtained with identically infected and stained cells that were fully permeabilized with triton-X100. As a control of the performance of the selective permeabilization, the cells were stained, in addition to the dsRNA, with the ER marker calreticulin, which should only be available for staining if the organelle membrane were permeabilized.

Similar to the staining pattern described earlier, the staining of cells permeabilized with triton-X100 treated demonstrated that while most dsRNA in wild-type infected and those infected with the revertant 1198B strain, the dsRNA co-localized with ER marker calreticulin at the perinuclear region (Fig. 3.7; FL12 and 1198B), while the staining pattern of dsRNA in cells infected with 1198 virus was scattered and less prominent at perinuclear region (Fig. 3.7). As compared to the triton-X100 permeabilized cells, cells that were permeabilized by the freeze-thaw the ER marker calreticulin that is localized in the lumen of this organelle was barely
detectable indicating the successful application of the selective permeabilization technique. Comparison of the dsRNA staining in Triton-X100 treated cells to those permeabilized by freeze-thaw, demonstrate that a significant cytosolic dsRNA staining is only observed in cells infected with the 1198 strain. This result indicates that while the sequestration of dsRNA in membranous compartments in cells infected with wild-type and the 1198B revertant is taking place, the sequestration of dsRNA in cells infected with the 1198 strain is not efficient. This observation is consistent with the IFN-α phenotype of these viruses, in which those viruses that effectively hide their dsRNA in membranous compartments do not stimulate IFN-α production, while 1198, which fails to do so, stimulates a potent IFN-α response. To further confirm this notion, we performed a similar experiment using identical experimental conditions, but also performed a temporal analysis to discern the timing at which the dsRNA become available free in the cytosol (Fig. 3.8A). These experiments revealed that the presence of dsRNA outside of membranous compartment in cells infected with strain 1198 was present in 9% of the infected cells at 4 hpi, and increased to 34% at 8 hpi (Fig. 3.8B). In contrast, in cells infected with the reverting strain 1198B only 5% of the cells exhibited the presence of dsRNA outside of the membranous compartment at 8 hpi (Fig. 3.8B). These results explain the minimal yet statistical significant IFN-α production detected in cells infected with nearly-isogenic strains, namely the revertant 1198B, ([IFN-α]=0.461ng/ml), and parental strain 46448 ([IFN-α]=0.568ng/ml) detected at 8 hpi (Fig. 3.1A).

3.4 DISCUSSION

The majority of experimental evidence, suggesting type I interferon inhibition by PRRSV, is obtained from luciferase reporter assays carried out with non-natural host cells transient overexpression of a particular viral protein (Beura et al., 2010; Mingyuan Han, Yijun Du, Cheng
Song, & Dongwan Yoo, 2013; Kim, Sun, Lai, Song, & Yoo, 2010; Patel et al., 2010; Xibao Shi et al., 2012; X. Shi, Zhang, et al., 2011; X. Shi et al., 2013; Cheng Song, Peter Krell, & Dongwan Yoo, 2010; Z. Sun, Chen, Lawson, & Fang, 2010). This approach is confounded by the aberrant enzymatic activity of the viral protein due to overexpression. For example, one common feature shared by PRRSV NSPs - namely nsp1, nsp2, nsp4 nsp11 - displays the significant IFN-β promotor inhibition activities is the inclusion of digestive enzyme activity (Beura et al., 2010; Dong et al., 2015; Huang et al., 2014; H. Li et al., 2010; X. Shi, Wang, et al., 2011; X. Shi, Zhang, et al., 2011; Z. Sun et al., 2010). This implies that the observed inhibition activity may be a result of promiscuous cleavage on type I interferon signaling molecule by viral protease or nuclease under overexpression condition. In our previous study, results suggested that while the PRRSV infection of porcine alveolar macrophage fails to produce the type I interferon, it cannot suppress the same response induced by poly I:C. A similar type I interferon phenotype was also observed in the cells infected with coronaviruses including SARS-CoV and several strains of MHV (Roth-Cross, Martinez-Sobrido, Scott, Garcia-Sastre, & Weiss, 2007; Versteeg, Bredenbeek, van den Worm, & Spaan, 2007; Zhou & Perlman, 2007). In short, the studies on the coronavirus demonstrated that, in the presence of coronavirus dsRNA, the cells fail to elicit the IFN-β transcription activity including mRNA synthesis and IRF3 activation in response to coronavirus infection. Yet the coronaviruses cannot abrogate the same responses induced by poly I:C or SeV infection(Versteeg et al., 2007; Zhou & Perlman, 2007). This suggests the similar mechanism shared by coronavirus, and perhaps PRRSV, can avoid the detection of host viral nucleic acid sensor such as TLRs or RLRs. In the recent studies on flavivirus including TBEV, JEV and Dengue virus, the immune-staining results suggest that the flavivirus’ dsRNA is protected by some intracellular membranous compartments from the detection of viral nucleic
acid sensor as they can only be detected in the cells that are fully permeabilized with triton X100 but not in those with selective plasma membrane permeabilization (Lyre Anni Espada-Murao & Kouichi Morita, 2011; Överby, Popov, Niedrig, & Weber, 2010; Uchida et al., 2014). This concept is further supported by the correlation between the dsRNA leakage and IFN-β response (Lyre Anni Espada-Murao & Kouichi Morita, 2011; Uchida et al., 2014). A structure called double membrane vesicles (DMVs) is likely responsible for this protection activity. It is known that DMVs are induced by flavivirus during the replication and the immuno-labeled dsRNA can be visualized inside this structure using transmission electron microscopic analysis (Romero-Brey et al., 2012; Welsch et al., 2009). The same DMVs structure can also be induced by nidoviruses including coronavirus and arterivirus (Knoops et al., 2012; Knoops et al., 2008). In fact, our study provides the first experimental evidence indicating a role for this structure in evading host immune detection in arteriviruses (Fig 3.7 and Fig 3.8).

While phenotyping the type I interferon response for several different strains of PRRSV, a very unique PRRSV strain 1198 displaying the exceptional capability of type I interferon induction was isolated. The type I IFN induction capability of 1198 requires live virus and it is both moi (multiplicity of infection) and time dependent (Fig 3.1). It is worth noting that, in contrast to a linear, immediate accumulation of IFN-α by poly I:C, the sigmoid IFN-α response induced by PRRSV 1198 occurred with a 3 hour window period (Fig. 3.1B) fitting closely replication of the virus. Also, the IFN-α response induced by 1198 takes place at some point between 3 to 6 hpi and ends by 12 hpi. This matches the detection of minimal PRRSV dsRNA at 4hpi in our immunostaining assay and the replication cycle of 12 hours in AMΦ cells in the one-step growth experiment (data not shown). The IFN-α induction phenotype could result from the loss of the inhibitory activity due to other viral proteins. However, if viral inhibition of a type I
interferon response is redundant, shared by nsp1, nsp2 nsp4 nsp11, (Beura et al., 2010; Dong et al., 2015; Huang et al., 2014; H. Li et al., 2010; X. Shi, Wang, et al., 2011; X. Shi, Zhang, et al., 2011; Z. Sun et al., 2010), the likelihood for 1198 to lose the inhibition activity all at once is highly unlikely, thus suggesting another mechanism is responsible for the 1198 IFN-α induction phenotype.

Besides 1198, two related and nearly isogenic PRRSV strains, 1198B and 46448 fail to induce detectable IFN-α, which is the typical host cell response of porcine AMΦ upon infecting with wild-type PRRS virus. The 1198 PRRSV strain differs from the two other strains by only a few nucleotides, one of which results in a single non-conservative amino acid substitution in position 339 of non-structural protein 2 (nsp2) (Fig. 3.4). PRRSV strain 46448 is the parental virus of 1198. And 1198B strain was recovered from a pig infected with the 1198 strain, which we found had lost its ability to stimulate IFN-α and reverted the mutation in nsp2 to the same amino acid residue as the 46448 strain. Based on these observations, we believe that the mutation in the 339 residue of nsp2 results in the distinct IFN-α eliciting phenotype of the 1198 strain.

The PRRSV nsp2 protein contains a long hypervariable domain (HV) flanked by N-terminal cysteine protease domain and a C-terminal transmembrane domain. While the deletions in the protease domain and TM domain are lethal, PRRSV tolerates deletions in the hypervariable region (Jun Han, Gongping Liu, Yue Wang, & Kay S Faaberg, 2007). This may explain why 1198 carrying that a mutation in the hypervariable region is viable and replication competent. Although the function for the nsp2 hypervariable region is unknown, it is suggested to be involved in PRRSV pathogenicity (Kay S Faaberg, Kehrli, Lager, Guo, & Han, 2010; Y. Li et al., 2007; Tian et al., 2007; Wang et al., 2013). Since the type I interferon induction phenotype
by 1198 is linked to the mutation in this region, it is possible that nsp2-associated pathogenicity is related to the differential type I interferon modulation activity by this molecule.

This study is made possible by successful use of selective permeabilization to distinguish the cytosolic versus intramembranous molecules. (Fig. 3.6). The immune-staining analysis on selective permeabilized cells suggest that the atypical type I interferon response by 1198 is likely a result of dsRNA leakage from intracellular membranous compartments because cytosolic dsRNA only can be detected in the 1198-infected cells but not in the cells infected with revertant or wild-type virus (Fig. 3.7). This result confirms our previous observation that PRRSV dsRNA is sequestered inside the intracellular membranous compartments and it suggests a critical roles of the amino-acid at position 339 of PRRSV nsp2 in modulating membranous compartment formation. The nsp2 from another arterivirus, EAV, and its equivalent, nsp3, from coronavirus have been suggested to play an important role in DMV formation process. The ectopic expression of the EAV nsp2-nsp3 polyproteins and the co-expression of SARS-CoV nsp3, nsp4, nsp5 lead to appearance of DMV-like structures in transfected cells (Angelini et al., 2013; Snijder et al., 2001). This process is highly co-operative as individual expression of any single nsp is not sufficient for DMV formation, although some membrane rearrangement was noted in cells expressing SARS-CoV nsp3 alone (Angelini et al., 2013).

Because of limited knowledge regarding the nsp2 hypervariable (HV) region, it is difficult to hypothesize why this particular mutation negatively impacts the intracellular membranous compartment structures, especially when its location is distant from the C-terminal transmembrane (TM) domain which directly interacts with the membrane. Nevertheless, as mentioned earlier, the DMV formation process requires cooperation between nsps and the region carried this mutation may regulate the interaction with other viral proteins. In fact, the non-
transmembrane region of coronavirus NSP3 (PRRSV NSP2 equivalent) is capable of interacting with several other non-structural proteins including NSP5, another important DMV regulator (Imbert et al., 2008).

This co-operation in DMV formation is not restricted to the interaction between viral proteins alone. The structure of virus-induced DMV resemble the double membrane feature of host autophagosome. Recent evidence suggests involvement of host autophagy signaling in virus-induced DMV formation. First, autophagy marker co-localized with the virus replication complex (Guevin et al., 2010; Prentice, Jerome, Yoshimori, Mizushima, & Denison, 2004; Sir et al., 2012). Second, virus replication as well as DMV formation is reduced in autophagy defective cells (Dreux, Gastaminza, Wieland, & Chisari, 2009; Prentice et al., 2004; Sir et al., 2012). And, the viral proteins capable of DMV induction activity are also capable of inducing autophagosome (Angelini et al., 2013; Cottam et al., 2011; Egger et al., 2002; Su et al., 2011). In vivo study on dengue virus demonstrates that the autophagy enhances replication of virus in neuron. Clinical symptoms or disease caused by this virus can either be aggravated or alleviated by modulating autophagy in cells (Lee et al., 2013). PRRSV replication requires the activation of autophagy (Liu et al., 2012; M. X. Sun et al., 2012). Moreover, LC3 (host autophagy marker) was co-localized with PRRSV nsp2 (M. X. Sun et al., 2012). Although autophagy was not examined in either study, it is plausible that PRRSV nsp2 modulate the intracellular membranous compartments via interaction with host autophagy molecule and the identified mutation at N-terminal of 1198 nsp2 may be critical for this viral-host interaction.

During these studies, we noted that the effect of this particular NSP2 mutation on type I interferon response was cell type dependent. Due to the significant amount of type I interferon produced by 1198-infected PAM cells, 1198 replication was compromised. In fact, we are unable
to rescue the 1198 carrying the same mutation after being reintroduced to the pig host. In contrast, the growth of the 1198 virus in MARC145 cells seems not to be affected. When titering the same virus stock on both PAM and MARC145 cells, the titer of this virus on PAM cells is about 2 log less than it appeared on the MARC145 cells. Actually, the propagation on the MARC145 cells is the only way to keep the type I interferon induction phenotype for this virus. A similar observation was also reported by Faaberg et al. where their study indicated that, in spite of an initial delay, the viruses that contained deletions (nsp2Δ324-523 and nsp2 Δ324-726) which covered the critical mutation identified in our study can eventually replicate to titers of wild-type virus when propagated in MARC145 cells (J. Han, G. Liu, Y. Wang, & K. S. Faaberg, 2007). However, in a subsequent in vivo study, the viruses carrying identical deletions displayed a growth defect in the pig host and could not be isolated (nsp2 Δ324-726) from lung lavage at day 35 after infection (Kay S. Faaberg, Kehrli Jr, Lager, Guo, & Han, 2010). The type I interferon response was not examined in these studies therefore it is unknown whether these deletion mutants retained the type I induction capability as 1198. As the type I interferon response by 1198 is linked to intactness of the virus-induced intracellular membranous compartments, this cell-type dependent type I interferon response suggests the differential capability of cells to develop intracellular membranous compartments. Indeed, with JEV, it has been shown that in contrast to the rapid and robust type I interferon response observed in JEV-infected primate cells, those infection of the porcine-origin cells displayed a weak and delayed type I response. This delayed type I interferon response in porcine cells is not a result of reduction of dsRNA synthesis or defective in RIG-MDA5 pathway but is associated with delayed cytosolic exposure of dsRNA (L. A. Espada-Murao & K. Morita, 2011). Although the
mechanism underlying the “cell type dependent leakage” is unclear, it minimally explains the higher pathogenicity in JEV-infected pig while mild-symptom in most human infections.

In conclusion, our results illustrate how PRRSV virus uses the intracellular membranous compartments to prevent detection of its dsRNA by host PRRs. This process is associated with PRRSV NSP2 activity. A mutant virus carrying a critical mutation on the N-terminal of NSP2 lost the ability to shield its dsRNA in the cell which resulted in a robust type I interferon response.

3.5 MATERIALS AND METHODS

Cells

The porcine AMΦ cell lines ZMAC-1 (ATCC PTA-8764) and ZMAC1107-4 (ZMAC-4), which were derived from the lung of porcine fetuses (Zuckermann, 2008) consist of phagocytic cells that express several surface markers characteristic of porcine alveolar macrophages (Ezquerra et al., 2009), including CD14, CD45, CD163, and CD172 (G. Calzada-Nova, Husmann, Schnitzlein, & Zuckermann, 2012). The ZMAC cell lines can be grown continuously for >70 passages and new fresh cultures are routinely re-established every 4-5 months from low passage cell stocks kept frozen. The ZMAC-4 cell line, which was used for this study has been shown to efficiently support the growth of PRRSV (G. Calzada-Nova et al., 2011) (Du, Yoo, Paradis, & Scherba, 2011). The ZMAC cells were cultured in RPMI-1640 medium containing L-glutamine (Mediatec, Herndon, VA, USA) and supplemented with 10% fetal bovine serum (GIBCO®, Invitrogen, Grand Island, NY, USA), 1 mM sodium pyruvate and 1× non-essential amino acids (Mediatec), and maintained at 37°C in a 5% CO2 atmosphere. The continuous growth of the ZMAC-4 cells was maintained using 10 ng/ml recombinant mouse macrophage
colony stimulating factor (Shenandoah Biotechnology, Inc., Warwick, PA). When required the type I interferon deficient ZMAC cell line, 9-13, was used under same culture condition. MARC-145 cells were grown as previously described (Meier et al., 2003).

**Viruses**

To avoid type I interferon in the inoculant carried to downstream cytokine analysis, PRRSV strains used in this study including wild type strain, NADC20 (Harms et al., 2001) and FL12 (Truong et al., 2004), IFN-α induction strain, 1198 and 1198 isogenic strains, 46448 and 1198B, were propagated in the primate origin MARC-145 cells. Cell-free preparations of virus were prepared by centrifugation of the medium overlaying infected cell monolayers showing ≥80% cytopathic effect (CPE) at 4°C and 350 × g for 10 min. The medium was then layered on top of a 15% iodixanol (OptiPrep™, Sigma-Aldrich, St Louis, MO, USA) cushion and subjected to ultracentrifugation at 64,100 × g and 4°C for 3 h. The resulting, virus containing pellets were suspended in TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA). The purified virus stocks except 1198 were titrated in monolayers of ZMAC cells (50% tissue culture infective dose [TCID₅₀]). The 1198 strain was titrated with type I interferon deficient cell-line, 9-13. When required, the purified 1198 virus was inactivated by exposure to short-wave (254 nm) UV light for 3 min. Loss of viability was verified by the inability of the UV light-exposed viruses to produce a cytopathic effect on monolayers of Marc-145 cells.

**Infection and Treatment of Porcine AMΦ**

Unless otherwise stated, ZMAC cells were cultured in sterile snap cap tubes (Corning, New York, USA) and were either mock-infected or infected with PRRSV at an MOI=5. Where indicated, uninfected ZMAC cells were subsequently exposed to 25 μg/ml poly I:C for the
indicated length of time. As a positive control for eIF2α activation analysis, the AMΦ cultures were treated with 2 mM DTT for 1 h.

**RNA preparation and real-time RT-PCR**

Samples of $10^5$ uninfected or PRRSV-infected ZMAC cells were cultured in the presence or absence of poly I:C for the indicated times. Afterwards, each sample was lysed in buffer RLT, and total RNAs were purified, DNase-treated, converted into cDNA and subjected to real-time PCR as previously described (G. Calzada-Nova et al., 2011). Primers and probes for the amplification/detection of porcine IFN-α1 and IFNβ1 gene transcripts have been described (G. Calzada-Nova et al., 2011).

Changes in the extent of expression of the IFN-α1 genes were determined by using the comparative CT method and the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001), where RPL32 was used as the reference gene.

**Western blotting of AMΦ lysates**

Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with cocktail protease inhibitor (Amresco, Solon, OH, USA) and the protein concentrations of the resulting lysates were determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equivalent protein amounts of each extract (25 to 60 μg per well) were subjected to separation in a 10% SDS-PAGE gel and subsequently transferred onto a 0.2μ PVDF membrane (Bio-RAD, Hercules, CA, USA) for Western blot analysis. Membranes were incubated in blocking buffer [2% fish gelatin in TBST solution (50 mM Tris, pH 7.5, 500 mM NaCl and 0.5 % Tween 20)] at room temperature (RT) for one h. Afterwards, the membranes
were incubated at 4°C overnight with one of the following unconjugated primary Ab (1:1000 dilution of manufacturer’s original concentration in TBST with 5% BSA): anti-IRF3 (clone D83B9, Cell Signaling, Danvers, MA, USA), anti-phospho-IRF3 (Ser396) (clone 4D4G, Cell Signaling), anti-phospho-eIF2 (clone #9722, Cell Signaling), anti-phospho-eIF2 (clone #9721, Cell Signaling), or anti-ß-actin (clone #4967, Cell Signaling). Membranes were then washed four times in TBST solution, and then incubated with horseradish peroxidase (HRP)-conjugated, anti-rabbit immunoglobulin (IgG) secondary Ab (sc2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1: 8000 in blocking buffer) at RT for 1 h. After again being washed, the membranes were incubated with a chemiluminescence reagent (GE Heathcare, Little Chalfont, Buckinghamshire, UK) to enable detection of bound secondary Ab. Screening for the presence of a specific phosphorylated protein was always performed prior to detection of the corresponding, non-phosphorylated form on membranes that had been incubated in stripping buffer (#21059, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 15 min to remove any covalently-bound Ab.

**Quantitation of IFN-α**

Medium used to culture porcine alveolar macrophages that had been left untreated or had been exposed to PRRSV in the presence/absence of poly I:C was examined for the presence of IFN-α by using a specific enzyme-linked immunosorbent assay (ELISA). The IFN-α ELISA procedure had been described before (Gabriela Calzada-Nova, Schnitzlein, Husmann, & Zuckermann, 2010). Briefly, individual wells of a Nunc Immulon II 96-well plate (Thermo Scientific) that had been coated for 16 h at 4 °C with primary anti-pig IFN-α mAb in 0.1 M carbonate buffer (pH 9.6) were washed 3 times with PBS containing 0.05% Tween 20 (PBS–T), and incubated with blocking solution for 1 h at 25 °C. After three washes with PBS–T, 50 μl culture supernatants and IFN-α standards diluted in RPMI complete medium were added to
duplicate wells and placed for 2 h at 25 °C. After washing 5 times with PBS–T, each well was incubated with 50 μl of PBS–T containing 2.5 μg/ml biotin-labeled, anti-pig IFN-α mAb and 0.5% blocking solution at 25 °C for 1.5 h. After 5 washes with PBS–T, each well was incubated with 50 μl PBS–T containing 20 ng/ml SA conjugated to HRP (Biosource™, Life Technologies) for 20 min at 25 °C and then again washed 5 times with PBS–T. Color development was initiated at 25 °C with the addition of 100 μl TMB substrate (KPL, Gaithersburg, MD, USA) per well and terminated with 100 μl 1 M phosphoric acid. Optical densities were determined at 450 nm with a SPECTRAMAX Plus plate reader (Molecular Devices, Sunnyvale, CA). Results were averaged and the amounts of IFN-α were determined by comparison to a standard curve generated from the values obtained with the known quantities of IFN-α.

**PRRSV genomic RNA extraction and sequencing**

RNA was extracted from 140 μl of cell-free virus preparations from MARC-145 and ZMAC cells by using a QIAamp viral RNA minikit (Qiagen Inc., Valencia, CA). Viral RNA was converted into cDNA in the presence of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamers and subsequently amplified with pairs of specific primers and iProof™ High Fidelity DNA Polymerase (Bio-Rad Laboratories, Hercules, CA). The amplicons were electrophoresed in agarose gels, removed and purified in ZYMO gel DNA recovery columns (ZYMO, Orange, CA), and directly sequenced with a BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in the presence of individual specific primers. The reactions were processed by the UIUC Core DNA Sequencing Facility (Urbana, IL) and the resulting chromatograms were visually inspected and edited with the SeqEd program (Applied Biosystems).
Immunoflourescent

A total of 2.5X10^5 ZMAC cells were grown in the each well of 4-well chamber slide (ibidi USA, Madison, WI) and infected with the indicated type PRRSV with a multiplicity of infection (moi) of 5 or mock-infected. After the indicated length of incubation the cell were treated one short freeze-thaw cycle for selective permeabilization at plasma membrane (Mardones & Gonzalez, 2003). Briefly, after rinses twice with PBS, the buffer-free chamber slide was placed on a metal block pre-freezed at -80 °C for 2 to 3 seconds followed by thawing on another metal block prewarmed at 40 °C for 10 seconds. The freeze-thawed cells were fixed in PBS containing 4% paraformaldehyde for 20 min, washed with PBS, and then incubated with blocking buffer (PBS containing 3% normal goat serum) in the presence or absence of Triton X-100, which is used when total permeabilization is required, for 1 hour at 25°C. After blocking, the cells were incubated at 4 °C overnight with one of the following unconjugated primary antibodies (1:200 dilution of manufacture’s original concentration in PBS containing 1% BSA): anti-dsRNA (cloneJ2, Scicons, Szirák, Hungary), anti-Calreticulin (ab2907, abcam) and anti-Tom20 (SC-11415, Santa Cruz). After overnight incubation with primary Ab, the cells were washed 3 times in PBS and then incubated with Ab dilution buffer containing fluorophore-conjugated secondary antibodies for 1 hour at 25°C. The secondary antibodies used were: DyLight594 conjugated goat anti-rabbit IgG (5ug/ml) (#35560, Thermo Scientific) or FITC-conjugated goat anti-mouse IgG (2.5ug/ml) (#62-6511, Zymed™, Life Technologies). After wash 3-times in PBS, the antifade medium were added into the well. When required, after the completion of the detection for first antigen (dsRNA), the sequential co-immunostaining of second antigen (Calreticulin) was performed over again starting with blocking step. Fluorescent signals were observed with a microscope fitted with a UV microscopy apparatus.
**Statistical analysis**

All error bars represent standard deviations generated from at least two biological experiments and calculated using Microsoft Excel module. Unpaired Student’s t test (one-tailed) was used to determine if significant differences existed in regard to the cytokine response exhibited by AMΦ between treatment groups. A $P$ value of $<0.05$ was considered statistically significant.
3.6 REFERENCE


the endoplasmic reticulum via an omegasome intermediate. *Autophagy*, 7(11), 1335-1347. doi: 10.4161/auto.7.11.16642


Fig. 3.1 Replication dependent activation of IFN-α by 1198 strain (A) 2.5 X 10^5 ZMAC cells were infected with indicated PRRSV strain at an moi of either 5 or 1 or 0.2 or 0.04 for 10 h. (B) 2.5 X 10^5 ZMAC cells were exposed to poly I:C or were infected with indicated PRRSV strains for the indicated times (h). The amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The statistical comparisons were made between the amount the cytokine present in the overlaying medium of 1198-infected vs. FL12-infected culture (A, B). (C) Duplicate samples of 2.5 X 10^5 ZMAC cells were either just cultured (mock) or incubated with UV light-inactivated or viable PRRSV strain 1198 or NADC20. The amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The mean ± standard deviation (n = 2) is presented. The statistical comparisons were made between the amount the cytokine present in the overlaying medium of 1198-infected vs. un-infected cultures. Asterisks indicate values that are statistically significant (**, P<0.01); NS indicates differences that were not statistically significant.
Fig. 3.2 Compensation of translation stress by exorbitant transcription activity of IFN-α induced by 1198. (A) 1X10⁶ ZMAC cells were infected with PRRSV strain 1198 at an moi of 5 for the indicated times (h) or treated with 2mM DTT for 1 h. Afterward, the cells were lysed and lysates were subjected to Western blotting for the sequential detection of p-eIF2, eIF2. (B) and (C) 1X10⁵ ZMAC cells were incubated with PRRSV strain 1198, at an moi of 5 or poly I:C at 25 ug/ml for either 6 or 10 hours. Total RNA obtained from each sample was subjected to real-time PCR to detect IFNA1 gene transcripts. The fold-changes in the amount of these RNAs present in the virus-infected AMΦ to that in the uninfected cells incubated with poly I:C for the same time were determined by using the formula 2^{-ΔΔCt}, where RPL32 was used as the reference housekeeping gene. RNA fold-increases are not shown for the uninfected, untreated cell sample where IFNA1 gene transcripts were not detected. The statistical comparisons were made between the amount the cytokine present in the overlaying medium of 1198-infected vs. un-infected cultures stimulated with poly I:C. Asterisks indicate values that are statistically significant (**, P<0.01) (D) 2.5X10⁵ ZMAC cells were infected with PRRSV strains, NADC20, FL12, 1198 or 46448, at an moi of 5 or stimulated with poly I:C at 25 ug/ml. At 6 or 10 hours after virus infection or poly I:C stimulation, the culture supernatants and total RNA were harvested side by side for quantification of IFN-α protein by using ELISA and IFN-α mRNA by using real time RT-PCR, respectively. The secreted IFN-α amount were then normalized to the relative IFN-α mRNA fold change to calculate the translation efficiency.
Fig. 3.3 The association between the IFN-α induction and IRF3 activation in PRRSV 1198-infected cells

(A) 1X10⁶ ZMAC cells were infected with PRRSV strains, FL12, G16X, 46448, 1198B, 1198, at an moi of 5 for 6 h or stimulated with poly I:C at 25 ug/ml for 1h or left untreated for s h. Afterward, the cells were lysed and lysates were subjected to Western blotting for the sequential detection of p-IRF3, IRF3 or β-actin. 

(B) 2.5X10⁵ WT ZMAC cells or type I interferon incompetent 9-13 ZMAC cells caused by persistent BVDV infection were incubated with the medium containing 25ug/ml of poly(I:C) for indicated time. Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. 

(C) 2.5X10⁵ WT ZMAC cells or type I interferon incompetent 9-13 ZMAC cell-line were infected with PRRSV 1198 strain at an moi of 5 for 8 h. Afterwards, the amount of IFN-α present in the overlaying media was determined by using a specific ELISA. The statistical comparisons were made between the amount the cytokine present in the overlaying medium of 1198-infected WT ZMAC vs. infected 9-13 ZMAC. Asterisks indicate values that are statistically significant (**, P<0.01).
Fig. 3.4 Identification of the unique amino acid change at position 339 of nsp2 in 1198 (A) Multiple sequence alignment of the deduced amino acid sequence of 1198 nsp2 with its related strains, 46448 and 1198B was done using the program ClustalX and edited with Genedoc software. A unique mutation at position 339 are indicated with asterisk. (B) The similar alignment analysis was performed by including several WT PRRSV strains. The non-conservative mutation at position 339 were boxed.
Fig. 3.5 The production of dsRNA is comparable among tested PRRSV strain 2.5X10^5 ZMAC cells grown on the each well of the 4-well chamber slide were infected with PRRSV strains, FL12 or 1198 or 1198B at an moi of 5 for 8 hours, or left un-infected. At 8 hpi, the infected cells together with the un-infected cells were fixed, made permeable, and stained for the presence of ER-lumen resident protein, Calreticulin or dsRNA (red or green fluorescence, respectively). Three-fold crop images are displayed under the original 40X images. The nuclei were counterstained with DAPI.
Selective permeabilization at plasma membrane of AMΦ cells by freeze-thaw treatment

2.5X10^5 ZMAC cells grown on each well of the 4-well chamber slide. The whole slides were placed on -80 °C metal block for 3 seconds and then transferred onto the 40 °C metal block for another 10 seconds. After the freeze-thaw cycle, cells were fixed with 4% paraformaldehyde. The fixed cells were further treated either with 0.3% triton X-100 for full-permeabilization or left untreated for selective-permeabilization. The processed cells were immunostained for ER-lumen resident molecule, Calreticulin (red) or for mitochondria outer membrane molecule, Tom20 (green) with specific primary antibodies. Daylight 594-labeled goat anti-rabbit IgG were the secondary antibodies used for both Calreticulin and Tom20. The nuclei were counterstained with DAPI. The calreticulin served as the intracellular membrane marker to indicate the permeability of intracellular membrane and Tom20 served as cytosolic marker to indicate the permeability of plasma membrane.
Fig. 3.7 Cytosolic exposure of 1198 dsRNA  2.5X10^5 ZMAC cells grown on the each well of the 4-well chamber slide were infected with PRRSV strains, FL12 or 1198 or 1198B at an moi of 5 for 8 hours, or mock infected. At 8 hpi, the virus-infected cells together with the mock infected cells were either selectively permeabilized by freeze-thaw treatment or fully permeabilized with additional treatment of 0.3% triton X-100 in conjunction with fixation of 4% paraformaldehyde. The processed cells were immunostained for ER-lumen resident molecule, Calreticulin (red) and for dsRNA (green) with specific primary antibodies. Daylight 594-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG were the secondary antibodies used for detection of Calreticulin and dsRNA, respectively. The nuclei were counterstained with DAPI. The displayed image were cropped from the original 40X magnification image for better visibility. The cytosolic exposure of 1198 dsRNA in selective premeabilized cells were displayed in two view fields.
Fig. 3.8 Time dependent cytosolic exposure of 1198 dsRNA. (A, B) By using similar infection condition as well as analysis approach described in Fig. 3.7, the cytosolic exposure of dsRNA by either 1198 strain or 1198B strain were examined at 4 or 8 hours after infection. The processed cells were immunostained for dsRNA (green) with same set of antibody used in Fig. 3.7. The nuclei were counterstained with DAPI. The displayed image were generated from the original 40X magnification image. Several frames of image were used to calculate the percentage of cytosolic dsRNA positive cells.
As a major protein and lipid processing factory, the function of ER is exploited by viruses during infection for the production of virus progeny, causing the ER stress. This includes the protein synthesis, folding and glycosylation for massive viral proteins and lipid biogenesis for a virus-induced membrane structure and formation of enveloped virion. (Fung & Liu, 2014). ER stress induces the activation of three signaling pathways that constitute the UPR. These are PKR-like ER protein kinase (PERK), inositol-requiring protein-1α (IRE-1α), and activating transcriptional factor-6 (ATF6) pathways. This normal cellular process is aiming at restoring ER homeostasis by reducing the protein synthesis and increasing the chaperon gene expression. (Hetz, 2012).

These three stress sensors are known to activate NF-κB, thus they can affect host innate immune responses (Deng et al., 2004; Hu, Han, Couvillon, Kaufman, & Exton, 2006; Jiang & Wek, 2005; Kaneko, Niinuma, & Nomura, 2003; Nishitoh et al., 2002; Urano et al., 2000; Wu et al., 2004; Yamazaki et al., 2009). Although the activation of NF-κB by the ER stress sensors is not sufficient for the induction of a strong cytokine production, it could affect the responsiveness of cell to immune stimulation of PAMPs. Our results demonstrate that ER stresses activated by PRRSV infection is able to enhance the TNF-α response induced by LPS. This modulatory activity becomes critical for PRRSV when the PRRSV-infected host has a secondary superinfection by either bacteria or viruses. In which case exacerbated inflammation response in the lung will develop and produce significant morbidity and death.

In our studies, the activation of IRE-1α and PERK became evident in PRRSV-infected AMΦ cells, both of which are stress sensors that are known to lead stress-mediated autophagy. (Kouroku et al., 2007; Ogata et al., 2006). Of the two UPR pathways activated by PRRSV, we
believe that early activation of IRE1-TRAF2-JNK pathway is the primary activator of autophagy. This argument is supported by: (I) PERK activation occurs late when the virus replication is completed; (II) several PRRSV studies reported the activation of JNK in infected cells (Huo et al., 2013; Lee & Lee, 2012).

Several independent studies have indicated that the viruses such as PRRSV, coronavirus and flaviviruses require the autophagy pathway for their replication (Dreux, Gastaminza, Wieland, & Chisari, 2009; Liu et al., 2012; Prentice, Jerome, Yoshimori, Mizushima, & Denison, 2004; Sir et al., 2012; Sun et al., 2012). Knockdown analysis of important host genes involved in the autophagy pathway greatly reduce the replication of viruses that use DMV for their replication. (Dreux et al., 2009; Prentice, Jerome, et al., 2004; Sir et al., 2012). Because other than autophagy, there are no other mechanism known to contribute to the formation of the double membrane structure, it is thought that this virus whose replication dependent on DMVs utilize the host autophagy pathway to form their DMV structures. DMV formation is vital for viruses because it provide the surface for assembling the transcription and replication complex (TRC). It is still unclear how the host autophagy pathway co-operates with viral proteins in DMV formation process. However, co-localization of viral replication complex and the autophagosome marker has been reported for HCV, coronavirus and PRRSV (Guevin et al., 2010; Prentice, Jerome, et al., 2004; Prentice, McAuliffe, Lu, Subbarao, & Denison, 2004; Sir et al., 2012; Sun et al., 2012). Infected-cells lacking the expression of autophagy molecule, atg5, displayed lower levels of DMV structure induced by coronavirus (Prentice, Jerome, et al., 2004).

In addition, it has been suggested that sequestration of viral dsRNA inside the DMVs may help viruses evade host innate immune detection by minimizing cytosolic exposure of viral nucleic acid to host sensors. This predicted type of immune evasion by sequestration of viral
dsRNA in intracellular membrane structures had been confirmed for flaviviruses (Espada-Murao & Morita, 2011; Överby, Popov, Niedrig, & Weber, 2010; Uchida et al., 2014). Our results demonstrate for the first time that during PRRSV infection the viral dsRNA is sequestered inside the intracellular membrane structure that prevent the detection of PRRSV dsRNA by the host viral nucleic acid sensors.

To summarize, the interaction between virus infection and each of host response including ER stress, autophagy, DMV formation, innate immune response has been discussed in many studies (Blanchard & Roingeard, 2015; den Boon & Ahlquist, 2010; Hagemeijer, Rottier, & de Haan, 2012; Hoyer-Hansen & Jaattela, 2007; Jackson, 2015; Jheng, Ho, & Horng, 2014; Paul & Bartenschlager, 2013; Versteeg & Garcia-Sastre, 2010). However, the unified concept linking virus infection induced ER stress-, autophagy, DMV formation, and innate immune response to promote virus replication is just emerging. More experiments are needed to clarify the interplays among the each component of this host responses to virus replication. Our results not only provide a clearer picture for virus pathogenesis but also suggest the development of better strategies to control diseases caused by virus replicated in DMVs by targeting the ER stress-mediated autophagy.
4.1 Reference


