Novel approaches to enhance the protective immune responses of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV)

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ABSTRACT

Since late 1980s, porcine reproductive and respiratory syndrome virus (PRRSV) has emerged as the most economically important swine pathogen affecting pig industries worldwide. Vaccination is the principal means that have been used for prevention of PRRSV infection. However, the currently available vaccines for PRRSV are generally considered as not very effective. One of the major obstacles for developing an effective modified live-attenuated vaccine (MLV) with broad protection is the delayed and insufficient immune responses mounted by PRRSV, and the problem is further exacerbated by the antigenic variations of the constantly-evolving field strains of PRRSV.

In order to boost the immune response induced by the MLV vaccine virus, we evaluated the immunogenicity and vaccine efficacy of recombinant PRRSV MLVs expressing porcine IL-15 or IL-18 as adjuvants. The cytokine genes were fused with a GPI modification signal so that they are anchored onto the cell surface upon infection with the recombinant MLV. Both cytokines are successfully expressed on the cell membrane of porcine alveolar macrophage (PAMs) after recombinant MLVs infection in vitro. Subsequently, pigs vaccinated with cytokine-expressing
recombinant PRRSV MLVs had an improved antiviral response of cytotoxic lymphocytes including natural killer (NK) cells and T cells, characterized by increased IFN-γ secretion and/or enhanced CD107a expression. The results offer a novel strategy to incorporate cytokine genes into PRRSV genome as potent bio-active adjuvants expressed by the vaccine virus itself.

Since we showed that PRRSV VR2385 down-regulated swine leukocyte antigen class I surface expression, naturally the next logical question is which viral protein is responsible for this down-regulation. To answer the question, we cloned and expressed all known PRRSV structural and non-structural proteins and examined which protein(s) is involved in SLA-I downregulation. Our results identified the newly-discovered nonstructural protein Nsp2TF of PRRSV as the main mediator in down-regulating SLA-I expression. We also demonstrated that the Nsp2TF-knockout mutant virus lost its function of negatively modulating SLA-I presentation compared to the wild-type virus. The results suggest that disruption of the Nsp2TF’s ability to down-regulate SLA-I expression may improve the existing PRRSV vaccines towards a better CMI response against the virus.
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GENERAL AUDIENCE ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an important swine pathogen, causing enormous economical losses in the pork industry worldwide. However, the vaccine program is not satisfactory, with the insufficient protection against genetically divergent strains and newly emerged strains. One of the most important reasons is that PRRSV is able to suppress immune responses in the host, but the underlying mechanisms are not well known. Therefore, the first dissertation study is to investigate novel strategies of developing live-attenuated vaccines with improved efficacy against PRRSV. In this study, we successfully generated recombinant PRRSV live vaccines that are able to express immuno-activating cytokines as adjuvants. Subsequently, pigs vaccinated with cytokine-expressing PRRSVs had significantly improved anti-PRRSV immune responses when compared to pigs vaccinated with unmodified PRRSV. Those recombinant PRRSVs also provided cross-protection against a heterologous PRRSV challenge.

The second part of dissertation research is to understand the mechanism of immune modulation by PRRSV. Our results showed that one of PRRSV proteins- Nsp2TF contributes to
the PRRSV-induced down-regulation of swine leukocyte antigen (SLA) class I expression. Since SLA class I molecules are essential in the activation of the immune response and required for the clearance of viruses, Our study suggested that knocking-out Nsp2TF could be of great value to generate PRRSV vaccines with a better immune response.
DEDICATION

With my deepest love and appreciation, this dissertation is dedicated to my parents, Zhong Cao and Biqiong Zhang; my parents in law, Guanglu Mu and Cuixia Zhang; and my husband, Qinghui Mu, for their unwavering love and support.
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GENERAL INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), is arguably the most important infectious disease in swine industry worldwide, causing enormous economic losses worldwide every year. The disease was first reported in the United States in 1987 and later in Germany in 1990 and other European countries (1). The clinical manifestations mainly include reproductive failure in sows during the late stage of gestation, and severe pneumonia in nursery and growing pigs with increased mortality and reduced growth performance (2-4). Initially the disease was referred to as “mystery swine disease” or “blue-ear pig disease” due to the blue or purple discoloration of ears as a result of dyspnea. After the causative agent, porcine reproductive and respiratory syndrome virus (PRRSV) was eventually isolated and characterized in 1991 (5-7), the name PRRS was officially adopted to describe the disease.

PRRSV, a positive-sense, single-stranded RNA virus, belongs to the family Arteriviridae in the order of Nidovirales (7). The viral genome is approximately 15 kb in size and composed of at least 10 open reading frames (ORF), designated as ORF1a, 1b, 2a, 2b, and 3-5, 5a, and 6-7 (7). Sequence analyses revealed that PRRSV strains can be divided into two genotypes, European (type 1) and North American (type2), with distinct genetic and antigenic variations (8-10). There is also a high genetic diversity within each genotype (11, 12). For example, at least 9 distinct genotypes are recognized within the genotype 2 PRRSV (13).

Current commercial vaccines are not satisfactory in the prevention of PRRSV infection. Modified live-attenuated vaccines (MLVs) are effective against homologous strains but provide insufficient protection against heterologous strains especially newly emerged field strains. The inactivated vaccines are generally ineffective and even failed to provide solid homologous
protection (14). The ineffectiveness of the commercial vaccines is largely due to the significant antigenic variations among the circulating viruses, and also due to a compromised immune response induced by PRRSV upon exposure or vaccination (15-17). There are still many unknowns in our understanding of the immunological process during PRRSV infection, the role of different immune branches in a long-term protection against PRRSV, the pivotal viral targets of protective immunity, and the mechanisms that PRRSV utilize to dodge from host immune surveillance. Therefore, novel strategies and approaches to develop effective PRRSV vaccines with enhanced immunogenicity and with broad cross-protection against genetically divergent strains are urgently needed.
REFERENCE


epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV.


CHAPTER I

Literature review

Introduction

Since its appearance in 1987, porcine reproductive and respiratory syndrome (PRRS) has arguably been the most economically-important global swine disease, causing immense economic losses worldwide estimated at $664 million annually in the United States alone (1). It usually leads to severe late-phase reproductive failure in pregnant sows and respiratory diseases in pigs of all ages, especially young piglets (2-5). Clinically it is often seen with complications due to secondary bacterial infections and/or co-infections with other viruses such as porcine circovirus type 2, porcine parvovirus, and swine influenza virus (6). The causative agent, PRRS virus (PRRSV), is an enveloped RNA virus that belongs to the family *Arteriviridae* in the order *Nidovirales* (1, 7). Two genotypes of PRRSV, the European type (type 1) and North American type (type 2) (7, 8), were identified with striking genetic variations as well as differences in pathogenicity and antigenicity (9-11). Many potential mechanisms have been proposed to explain how PRRSV employs various strategies to hinder the induction of immune responses and/or evade the effector functions thereafter (12, 13). Understanding how PRRSV modulates immune responses would be of fundamental importance in the rational design and development of effective vaccines.
Genome organization, structure and molecular characteristics of PRRSV

PRRSV is an enveloped virus, with an icosahedral nucleocapsid containing positive-sense single-stranded RNA molecules wrapped by viral N protein. The genomic RNA is capped at 5’ end and polyadenylated at its 3’ end, which is approximately 15 kb in size containing at least 10 open reading frames (ORF): ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3-5, ORF5a, and ORFs 6-7 (14-16). Most the ORFs are overlapped with each other, with exceptions in the non-overlapping region between ORF1b and ORF2, as well as that between ORF4 and ORF5.

Non-structural proteins (Nsps)

ORF1a and ORF1b, composing approximately 75% of the viral genome at the 5’ proximal region, can translate into polyproteins 1a and 1ab, which are further co-translationally processed into at least 14 nonstructural proteins (Nsps) by auto-proteolytic cleavages (17, 18). All the Nsps mainly function in viral replication. For example, Nsp9 is considered the RNA-dependent RNA polymerase, together with Nsp10 which is the helicase, are indispensable in the virus replication. Among all the Nsps, Nsp2 is the largest proteinase released by auto-proteolytic cleavage of polyprotein 1a, and recently reported as a novel structural component of PRRSV particle (19). According to the study on equine arteritis virus, Nsp2 and Nsp3 are also suggested to be involved in the formation of double-membrane vesicles where virus replication and transcription take place (20, 21). Nsp2TF is a recently-discovered non-structural protein encoded in Nsp2 gene coding sequence via a unique -2 programmed ribosomal shifting (-2 PRF) mechanism (22). It has been demonstrated that the “slippery” sequence GGUUUUU at the site of frameshifting, the downstream conserved motif CCCANCUCC, as well as Nsp1β as a trans-activator protein (23) are equally required for generating this -2 PRF product.
Structural proteins

The 3’ portion of the viral genome contains at least 8 ORFs, which encode structural proteins (SPs) including N-glycosylated proteins GP2a, GP3, GP4, GP5a and GP5, non-glycosylated minor E protein and Matrix (M) protein, and nucleocapsid (N) protein. They’re expressed from a nested set of 3’ co-terminal subgenomic mRNAs (24, 25) using the discontinuous mRNA transcription mechanism. This is achieved by the base-pairing between a common 5’ transcription-regulating sequence (TRS) within 5’ UTR of the genome, the so-called “leader,” and the short conserved TRSs embedded in front of the 5’ end of each of the ORFs2-7, the “body TRS”, during transcription of the nascent negative-strand of the virus (26, 27). Additionally, the flanking nucleotide sequences, the secondary hair-pin structure and the relative position of TRSs in the genome may also play an important role in the initiation and the efficiency of this discontinuous transcription mechanism (28-30).

All the membrane proteins are essential for the production of infectious viruses. The heterodimers of major envelop proteins GP5 and M are fundamental building blocks of the viral particle, and are most abundantly presented on the virion, while the minor envelop proteins GP2a, GP3, and GP4 were incorporated into the viral envelope in less abundant amount, more likely as a multimeric complex interacting with each other (31). The minor proteins, other than GP5/M, presumably act as the predominant determinants in viral entry into cells (31, 32). Besides, the N-glycosylation (Salic acid-containing) on viral glycoproteins is also required for PRRSV infectivity, mainly involved in the process of attachment and internalization (33).

E protein is a very small, hydrophobic, integral membrane protein of approximately of 73 amino acids. It not only plays a role in the interacting with and processing of GP2a/3/4 heterotrimers, but may also be involved in the uncoating process as an ion-channel protein of the
virus during entry (34). Nucleocapsid (N) protein is a small basic protein. The homodimers of N comprise 40% of the proteins in the virion, and form icosahedral nucleocapsid core of 20–30 nm diameter, which is surrounded by a lipid envelope containing the envelope proteins (35). N is highly immunogenic, thus has served as the main diagnostic protein to detect PRRSV-specific antibodies in sera (16). In comparison, GP5 is considered to contain neutralization epitopes (36).

As suggested by distantly related coronaviruses (37, 38), the ER–Golgi intermediate compartment (ERGIC) could likely be the budding compartment for PRRSV. It is supported by the observation that the minor glycoproteins were each retained in the ER of the cell until they were all expressed appropriately, and then were collectively carried through the Golgi complex to form viral particles with GP5/M and finally to the plasma membrane (31). After budding of the virions, they are transported along the secretory route and ultimately released by exocytosis, yielding relatively smooth and spherical viral particles of about 60 nm in diameter (16).

Between the two genotypes of PRRSV, N protein of the North American isolate VR2385 share approximately 58% amino acid identity with that in European type Lelystad virus. GP5 is one of the most variable protein between these two isolates, with 54% amino acid homology, while M displays the highest homology as 78% (24, 39, 40). There is even lower amino acid identity in Nsp2 sequence with less than 40% similarity. In addition, nsp2 is also the key region for length difference between two genotypes. More notably, the genetic sequences among strains within each genotype also vary considerably. These huge genetic variations between two genotypes and within each genotype, to a large extent, contribute to the heterogeneity in their virulence, pathogenesis, and antigenicity (41, 42).
**Cell tropism**

The virus has a narrow host range, only infecting pulmonary alveolar macrophages (PAMs), alveolar septal macrophages, and several subsets of DCs including migratory DCs and monocyte derived DCs (MoDCs) that express appropriate receptors. The key receptors for PRRSV infection are considered as porcine CD163, sialoadhesin (pSn), and heparan sulfate, each of which may have a different role in the interaction with PRRSV during infection. Recently, CD163 has been suggested to be the most, or even the only important receptor for PRRSV, as in vivo study revealed that gene-edited pigs without functional CD163 are fully resistant to PRRSV isolate NVSL 97-7895 infection (43), while CD169 acts as an attachment factor but is not fundamentally important for PRRSV entry (44). Additionally, the modified CD163 in which SRCR5 domain that was replaced with a human analog has been shown to support type 2 but not type 1 PRRSV infection both in vitro and in vivo (45). This finding further implied the distinct differences between two genotypes of PRRSV.

*In vitro*, the African green monkey cell line MARC-145 and CL2621 cells (subclones of MA104 monkey kidney cell line) from ATCC are the two non-porcine cell lines that support North American type PRRSV replication (46, 47).

**Modulation of protective immune responses by PRRSV**

Viral infections are commonly controlled in the host within weeks. However, PRRSV typically induces an impaired immune response in the host including a weak innate immunity, delayed cellular responses, and a deferred neutralizing antibody response, and often leads to a prolonged viral shedding (48) even lasting up to 92 days post-infection in experimentally-infected animals. The transmission of PRRSV can be established from persistently-infected pigs to newly-
introduced SPF pigs even after 4 months since initial infection (49). Therefore, many investigations have been made to explore the possible strategies PRRSV utilized to achieve immune suppression. However, one must bear in mind that the innate and adaptive immune responses to highly pathogenic strains of PRRSV may be very different from that to low virulent strains ((50)). Discussed below are among some of the current proposed mechanisms.

Modulation of innate immune response

*Compromised type I interferon responses*

Numerous studies point out that, in the lung and broncholavage fluids, type I IFNs are insufficiently produced, especially during infection with virulent strains (51, 52), and similar results were also obtained from *in vitro* studies in PAMs, PBMCs, and migratory DCs, even though PRRSV is susceptible to type I IFNs (53). Since PRRSV infection is primarily limited in the lung, tonsils, regional LNs and testis, it is possible that, as primary host cells of PRRSV, pulmonary alveolar macrophages (PAMs) that could have potently produced IFN-α are severely damaged by the virus, and pDCs in PRRSV infection could not be activated efficiently (54, 55). This phenomenon is related to the interference in the mediators along the TLRs and RLRs signaling pathways (IRF7, CBP, NF-kB, STAT1 etc.) most likely by PRRSV non-structural proteins such as nsp1α, 1β, 2 and 11 (13). Other studies also suggest a post-transcriptional mechanism of suppression of IFN-α by PRRSV since the relative transcription copies of IFN-α did not correlate with IFN-α protein levels (56). Recently, mutations in Nsp1β protein that abolished -2 PRF and hence Nsp2TF production has been shown to attenuate the viral suppression on IFN-α and ISGs expression, which correlates with increased IFN-γ production in vivo (57).
**Up-regulation of IL-10 and down-regulation of TNF-α**

In addition to IFNα/β, PRRSV modulates the immune response (58, 59) by the upregulation of IL-10, both in PBMCs and bronchoalveolar lavage cells (BALCs). It was shown that the majority of IL-10-producing cells in the BALCs appeared to be attributed to the increased numbers of lymphocytes. Other research groups (35) also suggested the IL-10 production may be a result of the infiltrated monocytes and macrophages with 5-fold increase in numbers. This was supported by many in-vitro studies showing that PAMs produced elevated levels of IL-10 with low amounts of IFN-α and TNF-α. IL-10 is known for its immuno-suppressive activity on phagocytes and proliferation/differentiation of CD4+ T cells (60). It is possible that the migrating activated T cells cannot carry out their effector function in an unfavorable environment especially at site of infection. Moreover, IL-10 appears to play a key role in the differentiation of regulatory T cells (61, 62). Altogether, PRRSV may modulate these innate cytokines at early stage of infection in order to facilitate infection and further dampen the activation of naïve or primed lymphocytes.

By contrast, the recently emerged atypical PRRSV with an extremely high pathogenicity induce massive amounts (63) of serum IL-1, IL-6 and TNF-α, which adversely mediate the bronchial hyper-responsiveness and lung tissue damage with symptoms of high fever, dyspnea and severe gross lung lesions.

**Apoptosis and Autophagy**

Apoptosis is often triggered by host cells in response to the viral replication, thereby limiting viral propagation. An *in vitro* study (64) showed that PRRSV replication in the early stage (<12 hpi) had an anti-apoptotic effect on the PAMs, however subsequently the cells died by apoptosis with necrosis observed as well (24 hpi, 50% PAMs died by apoptosis). It is not clear
whether PRRSV actively induces cell apoptosis, since even though the induction of apoptosis coincides with the virus release from the cell, PRRSV does not necessarily need cell apoptosis for its release. But it is possible that apoptosis of PRRSV-infected cells often results in ineffective T cell activation due to the rapid death of macrophages and APCs. On the other hand, the apoptotic bodies derived from PRRSV-infected PAMs contain infectious virions which are engulfed by neighboring phagocytes. In this way, the virus facilitates its transmission of progeny viruses in the presence of neutralizing antibodies.

Autophagy is one of the evolutionarily conserved intracellular process that delivers misfolded proteins and damaged organelles to lysosomes for degradation and recycling (65, 66). A recent study (67) revealed that PRRSV enhanced its replication by preventing the fusion between autophagosome and lysosome thus increasing the accumulation of autophagosomes. It was also suggested that autophagosomes probably served as a membrane scaffold for effective PRRSV replication.

Modulation of adaptive immunity

Effects on MHC and Co-stimulatory molecules

It was shown in an in vivo study, a European PRRSV strain reduced the number of macrophages as well as their expression levels of MHC-II in the tonsil, retropharyngeal and mediastinal LNs (68). Synergistically with the low production of IL-12 and TNF-α, this probably leads to an inadequate activation of CD4+ T cells, especially a Th1 response, which has been indicated in many field studies.
Several highly pathogenic PRRSV strains of North American type can also downregulate MHC-I on PAMs and MoDCs in vitro (69), and Nsp1α was shown to contribute to this negative modulation. Besides, the recently discovered Nsp2TF was shown to be responsible for this phenomenon, with Nps2TF-knock out mutant viruses losing the function of downregulating MHC-I (70). However, highly pathogenic strains are more likely to induce the down-regulation of MHC class I and MHC class II when compared to low virulent strains in many in vitro studies (68, 69, 71). This downregulation may facilitate PRRSV to escape the induced cytotoxic lysis, since effector CTLs recognize virally-infected cells by the surface MHC I complexed with viral peptides.

Inadequate T cell responses

There is very limited knowledge on the role of T cells in anti-PRRSV immunity even though cytotoxic T cells are considered as the standard anti-viral players in other well-studied viral infections, let alone the roles of different subsets of T cells in cell-mediated response against PRRSV. This is primarily due to the inadequate understanding of porcine immunology, and the lack of proper tools and methods to study PRRSV-specific T cells (50).

As is the case with most respiratory viruses, PAMs remain in the lung without migrating to the regional LNs and do not upregulate their co-stimulatory molecules to a large degree in response to the engulfed viruses and even to IFN-γ (72). Also, the immune-suppressive nature of macrophages in alveolar sacs has been shown to limit antigen-presenting ability of lung DCs by their secretion of nitric oxides and TNF-α. These observations may partially explain the insufficient activation of APCs and T cells by PRRSV. Costers (73) showed that the proliferating CD3+CD8+ cells in vitro failed to exert cytolytic activity towards PRRSV-infected PAMs. This implied that even though cytotoxic T-lymphocytes are activated by the homing of DCs, and
migrate to the site of infection, they may have problems in eliminating PRRSV-infected PAMs. In addition, the suppression of cytotoxicity by natural killer (NK) cells in response to PRRSV-infected PAMs was recently indicated (74). The study suggest that the suppressed NK activity was not related to MHC class I expression or soluble factors released during PRRSV infection, but was associated with the viral protein expression in the PAMs. Since NK cells and CTLs are both cytotoxic lymphocytes that can use the same elimination strategy to destroy intracellular viruses, it is possible that PRRSV infection inhibits both NK and CTL activities.

Regulatory T cells (Treg) were described as immuno-suppressive T helper cells that promote immuno-homeostasis and prevent excess activation of immune responses. In a gnotobiotic pig model, CD4+ helper Tregs that express transcriptional factor Foxp3 were reported to be further separated into CD4+CD25+Foxp3+ and CD4+ CD25-Foxp3+ subpopulations. CD25-Tregs efficiently responded to primary rotavirus infection with decreased IL-10 and TGF-β production, hence facilitating immune activation (75). Silva-Campa and colleagues (76-78) have shown that Tregs were induced by PRRSV-infected DCs and were dependent on the production of TGF-β. Subsequently, they have identified that PRRSV infection induces the proliferation of Tregs with the unique phenotype CD4+CD8+CD25+Foxp3+ (77, 78). This team has also demonstrated that North American PRRSV strains were able to induce iTregs and up-regulate TGF-β expression, but neither TGF-β nor iTregs were induced by the European genotype of PRRSV. Furthermore, up-regulation of IL-10 gene expression and induction of Tregs have been observed when using N protein-pulsed porcine MoDCs, indicating that the PRRSV N protein might play a significant role in the induction of IL-10 and Tregs (79).
Overall, future studies in delineating T cell immunity and addressing its importance against PRRSV are greatly needed, which would help shed light on potential strategies in developing an effective PRRSV vaccine.

Decoy epitopes and ADE

During the course of disease, anti-PRRSV antibody can be detected by 1 week post infection (wpi) including anti-Nsp2 and anti-N; however, these Abs are not neutralizing and what is worse, they promote Ab-dependent enhancement of infection (ADE) of the virus (80, 81). The neutralizing antibodies (nAbs) against GP2, 3, 4 and GP5, do not appear until 4 wpi or later (82-84). There was a report of a decoy epitope close to the neutralization epitope in GP5 but the role of the decoy epitope has been recently debated after evidencing that in vivo, only a minor proportion of circulating PRRSV virions retain such decoy epitopes. Several studies also point out the existence of a secreting form of GP3 (85, 86) in North American type virus, which may play a role in consumption/deprivation of nAb and chemokine-binding.

Glycosylation of viral envelope proteins

N-glycan modifications on the PRRSV envelope proteins enable them to be less immunogenic and shielded from Ab neutralization (87). Several studies showed that the removal of glycosylation sites in the GP5 results in enhanced immunogenicity. However, the deglycosylated virus quickly regains the glycosylation sites through replication in vivo (88), suggesting that a strong selective pressure is exerted at these sites.
Current vaccines against PRRSV

Current commercially available vaccines include both modified live-attenuated vaccines (MLVs) and inactivated vaccines with limited efficacy (89). Only a few inactivated vaccines were licensed in some European countries and other parts of the world, but not in Unites States. It is largely due to the fact that they are unable to provide a solid protection even against homologous PRRSV infections. Some inactivated vaccines have been specifically used in sows and gilts in order to reduce reproductive losses caused by PRRSV (90). On some farms, the inactivated PRRSV were sometimes produced in-house and used as emergency vaccination against newly-emerged virulent field strains.

MLVs are generally effective against homologous or closely-related strains but largely ineffective against heterologous strains. The aforementioned genetic variation and antigenic diversity of circulating PRRSV field strains are thought to be the major obstacles (41, 89). The problem is further exacerbated by PRRSV-induced immune suppression. Another concern in developing MLVs is the risks of reversion of vaccine virus to virulent strains (91, 92). The recombination between/within field and vaccine strains may also lead to the generation of new strains, as reported in the latest outbreaks of PRRSV NADC30-like strain with high pathogenicity in China (93-95).

Since MLVs based on a single strain usually provide no or only partial cross-protection, many efforts have been carried out to develop broadly-protective MLVs. Using the reverse genetic system, chimeric or mosaic PRRSVs containing shuffled envelope genes from genetically divergent strains were successfully developed with both attenuated phenotype in vivo and enhanced cross-protection against heterologous challenge (96-100). In addition, other new
strategies to generate vaccines with enhanced immune response are also being investigated, such as IFN-α-producing recombinant PRRSV (57), DC-targeting subunit vaccines (101), etc.
REFERENCE


52. **Subramaniam S, Kwon B, Beura LK, Kuszynski CA, Pattnaik AK, Osorio FA.** 2010. Porcine reproductive and respiratory syndrome virus non-structural protein 1 suppresses


75. **Wen K, Li G, Yang X, Bui T, Bai M, Liu F, Kocher J, Yuan L.** 2012. CD4+ CD25-FoxP3+ regulatory cells are the predominant responding regulatory T cells after human rotavirus infection or vaccination in gnotobiotic pigs. Immunology **137**:160-171.


80. **de Lima M, Pattnaik AK, Flores EF, Osorio FA.** 2006. Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North


92. Key KF, Guenette DK, Yoon KJ, Halbur PG, Toth TE, Meng XJ. 2003. Development of a heteroduplex mobility assay to identify field isolates of porcine reproductive and


CHAPTER II

Recombinant porcine reproductive and respiratory syndrome virus expressing membrane-bound IL-15 as immunomodulatory adjuvant enhances NK and γδ T cell responses and confers heterologous protection

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ABSTRACT

Cytokines are often used as adjuvants to improve vaccine immunogenicity, since they are important in initiating and shaping the immune response. The available commercial modified live-attenuated vaccines (MLVs) against porcine reproductive and respiratory syndrome virus (PRRSV) are unable to mount sufficient heterologous protection, as they typically induce weak innate and inadequate T cell responses. Here in this study, we investigated the immunogenicity and vaccine efficacy of recombinant PRRSV MLVs incorporated with porcine cytokine IL-15 or IL-18 gene fusing to a Glycophosphatidylinositol (GPI)-anchored signal that can anchor the cytokines to cell membrane. We demonstrated that both cytokines were successfully expressed on the cell membrane of porcine alveolar macrophage (PAMs) after infection with recombinant MLVs. Pigs vaccinated with recombinant MLVs SUV-IL-15 and SUV-IL-18 had significantly reduced lung lesions and viral RNA loads in the lung after heterologous PRRSV challenge with PRRSV NADC20 strain. These two recombinant MLVs also stimulated the proliferation of NK cells when compared to the parental Suvaxyn MLV. Importantly, the recombinant MLV SUV-IL-15 significantly increased the IFN-γ-producing cells in circulation at 49 days post-vaccination (dpv), especially for the IFN-γ-producing NK cells, CD8+ T cells and γδ T cells when compared to the parental Suvaxyn MLV and recombinant MLV SUV-IL-18. Additionally, the MLV SUV-IL-15-vaccinated pigs also had an elevated level of γδ T cell response observed at 7 dpv, 49 dpv and 7 days post-challenge (dpc). These data demonstrate that recombinant MLV expressing membrane-bound IL-15 enhances T cell immune responses after vaccination, and confers an improved protection against heterologous challenge.

Keywords: Modified live-attenuated vaccine (MLV); membrane-bound IL-15; adjuvant; immunogenicity; NK cells; γδ T cells
IMPORTANCE

Porcine reproductive and respiratory syndrome (PRRS) has arguably been the most economically-important global swine disease, causing immense economic losses worldwide. The available commercial modified live-attenuated vaccines (MLVs) against PRRS virus (PRRSV) are generally effective only against homologous or closely-related virus strains but ineffective against heterologous strains, partially due to the insufficient immune response induced by the vaccine virus. To improve the immunogenicity of MLVs, we in this study present a novel approach of using porcine IL-15 or IL-18 as adjuvants by directly incorporating their coding genes into a PRRSV MLV and express them as adjuvants. Importantly, we directed the expression of the incorporated cytokines to the cell membrane surface by fusing the genes with a membrane-targeting signal from CD59. The recombinant MLV vaccine virus expressing membrane-bound IL-15 cytokine greatly enhanced NK cell and γδ T cell responses, and also confers an improved protection against heterologous challenge with PRRSV NADC20 strain.
Porcine reproductive and respiratory syndrome (PRRS) is arguably the most economically important global swine disease, causing immense economic losses worldwide with an estimated annual loss of $664 million in the United States alone (1, 2). The causative agent, PRRS virus (PRRSV), is an enveloped RNA virus that belongs to the family *Arteriviridae* in the order *Nidovirales* (3). The genome of PRRSV is a single-stranded positive-sense RNA molecule of approximately 15 kb in size, consisting of at least 10 open reading frames (ORF): ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3-5, ORF5a, and ORFs 6-7 (4-7). ORF1a and ORF1b at the 5’ proximal region of the genome are translated, and co-translationally processed into at least 14 non-structural proteins by auto-proteolytic cleavage (3, 8, 9). The remaining portion of the viral genome contains at least 8 ORFs which encode viral structural proteins (10). As is the case for all arteriviruses, the structural proteins of PRRSV are expressed from a set of 3’ co-terminal subgenomic mRNA (sg mRNA) (4, 5, 11) using the discontinuous mRNA transcription mechanism (12). This is achieved by base-pairing between a common 5’ transcription-regulating-sequence (TRS) within 5’ UTR of the genome, or the “leader”, and the short conserved TRSs embedded at the 5’ end of each of the ORFs 2-7, or the “body TRS”, during transcription of the nascent negative-strand viral RNA (12, 13). Such a unique transcription mechanism has been strategically utilized by investigators to modify the PRRSV genome with insertion of various foreign genes (14, 15).

Sequence analyses revealed that PRRSV can be divided into two genotypes, European (type 1) and North American (type 2), with distinct genetic variations (5, 16, 17). There is also a high genetic diversity within each genotype, which is often caused by recombination among strains (18). The PRRSV strain ISU-55 was isolated from the lungs of a diseased pig from a PRRS outbreak in Iowa in the early 1990’s (17). The PRRSV ISU-55, with moderate pathogenicity in
pigs (19), belongs to the genetic lineage 5 of type 2 PRRSV that was phylogenetically closely-related to PRRSV VR2385 (11). The Suvaxyn PRRSV modified live-attenuated vaccine (MLV) is a vaccine derived from PRRSV ISU-55 by attenuation of the wild-type virus via serial passages in vitro.

Current commercially available PRRSV vaccines include both MLVs and inactivated vaccines with limited efficacy (20-22). MLVs are generally effective against homologous or closely-related strains but largely ineffective against heterologous strains (23). The ineffectiveness of the commercial vaccines is mainly due to the significant antigenic variations among the circulating viruses, and also due to a compromised immune response induced by PRRSV upon exposure or vaccination. Since innate cytokines or co-stimulatory molecules are critically important in activating antigen-presenting cells (APCs) and shaping adaptive immunity, the use of these molecules as vaccine adjuvants has been explored in numerous studies (24-26). Several cytokines have been utilized as potential adjuvants to improve PRRSV vaccine immune responses, including IL-2 (27), IL-12 (28), and GM-CSF (29), but none was tested for their adjuvant effects in PRRSV MLVs. IL-15, which has been shown to promote the development and function of cytotoxic T cells and NK cells (30), is a good candidate to augment the immune response of PRRSV MLVs. Additionally, IL-18, an IFN-γ-inducing factor similar to IL-12, has also been reported to effectively enhance Th1 immunity and NK cell function (31-33). Therefore, IL-18 may also be an appropriate immuno-modulatory adjuvant to stimulate cellular immune response. The coding regions of bioactive IL-15 and IL-18 are both less than 500 bp in size, thus making them suitable for insertion into PRRSV genome for a more stable expression without affecting the viability of recombinant viruses.
In order to decrease the adverse systemic effects of soluble cytokines in circulation, and to increase the propensity that cytokines act on the same host immune cell as the viral antigens do, it is critically important to express cytokines on the cell surface with the guidance of a plasma membrane-targeting signal. Glycophosphatidylinositol (GPI)-anchored proteins usually concentrate in the lipid raft which is thought to function as a platform for many cell-to-cell contact events (34). Therefore, the GPI modification sequence from porcine CD59, which is constitutively expressed on leukocytes including macrophages, is a logical choice. Furthermore, the transmembrane region of hemagglutinin (HA) from Influenza virus containing the transmembrane domain, the cytoplasmic tail, and a portion of the short stalk region, have already been shown to direct HA protein or fusion proteins to the lipid raft of cell surface (35-37). Therefore, in this study, we incorporated gene sequences of porcine IL15 (pIL-15) or IL18 (pIL-18) fused with either GPI-anchoring signal from porcine CD59, or HA transmembrane region into the PRRSV Suvaxyn MLV infectious clone. These recombinant PRRSV MLVs were rescued and used to infect PAMs, and the expression of pIL-15 and pIL-18 on the cell surface was characterized. Nursery pigs were vaccinated with recombinant PRRSV MLVs expressing cytokines followed by challenge with a heterologous PRRSV strain NADC20. The adjuvant effects of the membrane-bound cytokines for anti-PRRSV protective immunity including NK cell and T cell responses were investigated.

**MATERIAL AND METHODS**

**Cells and viruses.** BHK-21 and MARC-145 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. PBMCs were isolated from heparinized blood of PRRSV-negative pigs and cryopreserved for later use. Upon thawing, PBMCs were cultured with RPMI 1640 medium supplemented with 10% FBS,
25 mM HEPES, and antibiotics. PRRSV Suvaxyn MLV used in this study (kindly provided by Zoetis Inc, Kalamazoo, MI) is a vaccine virus that was attenuated by serial passages in vitro of the wild-type PRRSV strain ISU-55. The PRRSV ISU-55 was isolated from lungs of a diseased pig during a PRRS outbreak in Iowa in the early 90’s (38). PRRSV strain NADC20, a genetic lineage 9 (39), was kindly provided by Dr. Kelly Lager of USDA National Animal Disease Center, Ames, Iowa.

Construction of expression vectors for membrane-bound IL-15 and IL-18. Porcine IL-15 (486 bp) and mature IL-18 (471 bp) were commercially synthesized by Integrated DNA Technologies Inc (Coralville, IA) as gBlock fragments designated as pIL-15 and HAsp-pIL-18, respectively, both containing two flanking restriction enzyme sites (NheI and XhoI) at each of the two ends, as well as a C-terminal flag tag (Table S1). Additionally, HAsp-pIL-18 has an engineered N-terminal signal peptide of 51 bp from the HA of Influenza virus A/WSN/33 (GenBank J02176.1), whereas the pIL-15 retains its own signal peptide. Furthermore, the GPI modification signal from pCD59 (114 bp) and HA transmembrane region (210 bp) were also commercially synthesized as pCD59-GPI or HA TM (Table S1).

To construct plasmids for expressing cytokines with GPI signal, the cytokine gBlock fragment (IL-15 or IL-18) was digested with NheI-HF and XhoI, and the GPI modification signal from pCD59 or HA TM was amplified respectively from gBlock with specific primers (pCD59_XhoI-F and pCD59_NotI-R; HA_XhoI-F and HA_NotI-R) containing XhoI and NotI restriction sites, and subsequently digested with the corresponding enzymes (Table S2). These two flanking fragments were then included in a three-fragment ligation with the NheI-HF and NotI digested vector pIHA (40). The resulting constructs pIL-15-CD59GPI, pIL-18-CD59GPI, pIL-15-
HATM, and pIL-18-HATM contained either a GPI modification signal or a HA transmembrane region at the C-terminus. To construct plasmids for expressing cytokines without GPI signal as controls, two primer sets with an engineered stop codon (IL-15TAA-F and IL-15TAA-R, HAsp-IL-18-TAA-F and HAsp-IL-18-TAA-R) were used to amplify the cytokine gBlock fragment (Table S2), and subsequently cloned into the NheI-HF and Not1 digested vector pIHA. The resulting plasmids were designated as pIL-15TAA and pIL-18TAA, with a flag tag at the C-terminus but without any membrane-targeting signal.

**Determination of the full-length genomic sequence of PRRSV Suvaxyn MLV and sequence analyses.** The sequences of the ORFs 2 to 7 genes, but not ORF1, of the wild-type PRRSV ISU-55 isolate have been published (11, 17). To determine the complete genomic sequence of the PRRSV Suvaxyn MLV, total RNAs were isolated from the Suvaxyn MLV vaccine virus using TRI Reagent (MRC). Reverse transcription and cDNA synthesis were performed at 42°C for 60 min in a 20-µl reaction mixture containing 100 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), 10mM deoxyribonucleoside triphosphate, 100 mM of DTT, 1U of RNasin (Promega, Madison, WI), and 0.5 µg of oligo dT primers (Invitrogen, Carlsbad, CA). A total of 10 overlapping PCR fragments covering the entire genome of the Suvaxyn MLV were amplified, and subsequently cloned into a pCR-2.1 vector (Invitrogen). Three individual clones of each fragment were sequenced. The consensus sequences were assembled and used for sequence analysis utilizing the Lasergene software (Version 10, DNA STAR Inc., Madison, WI). The extreme 5’ end sequence of the viral genome was determined using the GeneRACER kit (Invitrogen, Carlsbad, CA) with two reverse primers SV5RACE1 and SV5RACE2. The extreme
3’ end sequence of viral genome was determined by a nested PCR using the same forward primer SV10F and two reverse primers SV3RACE adaptor_T and SV3RACE primer (Table S3).

**Construction of a DNA-launched infectious clone of the PRRSV Suvaxyn MLV.** After determining the complete genomic sequence at the extreme 5’-end and 3’-end of the viral genome, a total of 5 overlapping fragments (AX, XP, PN, NE, EX) with unique restriction enzyme sites were amplified from the viral cDNA of PRRSV Suvaxyn MLV. Twenty poly (A) nucleotides were introduced immediately downstream of the 3’end of the viral genome. A nested PCR was used to introduce the ribozyme elements to the 5’ and 3’ ends of the viral genome for construction of the DNA-launched infectious clone as previously described (39). Two restriction sites Asc1 and Xba1 were engineered upstream and downstream of the viral genome for genome assembly. A total of 6 individual clones for each fragment were selected for sequencing, and the clone containing the consensus sequence was used for the assembly of the full-length clone. The fragment ACYC-XA from the plasmid pACYC-177 was amplified using primers pACYC-Xbalf (5’AAACCCGATATCAAAACCCTCTAGAGCCCTTCGCCCTTCCGGCTGGCT3’) and pACYC-AscIr(5’GGTTTCATATGGGGTTTAAACGGTGTTGGCGCGCCTCCGGCGT3’) incorporating the unique restriction sites AscI, XhoI, Pme1, Nde1, EcoRV and Xbal, which were then used to ligate the fragment upstream of the viral genome. Among the five fragments, each of PN, NE, and EX fragments was used to sequentially replace the fragments with the same restriction enzyme sites on the modified expression vector pIRES-EGFP2. A three-fragment ligation was used to assemble fragments AX, XP into the vector using restriction sites AscI and Pme1, resulting in the assembly of a full-length genome of PRRSV Suvaxyn MLV, which was then used to replace the PRRSV VR2385 viral genome in pIR-VR2385-CA DNA-launched infectious clone (39) with
restriction sites AscI and XbaI. The final full-length DNA-launched infectious clone of PRRSV Suvaxyn MLV was designated as pIR-SUV.

**Construction of cytokine gene-incorporated recombinant PRRSV MLV infectious clones.** In order to generate the PRRSV MLV expression cassette, an upstream fragment of 3906 bp was amplified from the original pIR-SUV infectious clone backbone with the forward primer Suv-NS_F1 containing NotI restriction site and the reverse primer Suv-NS_R1 containing SbfI and PacI restriction enzyme sites. A nested PCR was used to amplify the downstream fragment of 576 bp with two forward primers (Suv-NS F2-1 and Suv-NS F2-2) containing PacI restriction enzyme site and a synthetic TRS of 40 nt (IDT, Coralville, IA) and the reverse primer (Suv-NS_R2) containing ScaI site. These two flanking fragments were then included in a three-fragment ligation with the NotI and ScaI-digested pIR-SUV infectious clone to generate a new PRRSV MLV infectious clone designated pIR-SUV-2RE, which contains two unique enzyme sites SbfI and PacI and a synthetic TRS that allow for foreign gene insertion and expression.

To insert pIL-15 and pIL-18 together with the GPI signal in pIR-SUV-2RE, the primer set (IL-18_SbfI-F1, IL18-R1, IL18-F2, CD59_PacI-R) and primer set (IL-15_SbfI-F1, IL15-R1, IL15- F2, CD59_PacI-R) were used in the overlapping PCR reaction to amplify the cytokine fused with GPI from the expression plasmids pIL-15-GPI and pIL-18-GPI. The amplified regions and pIR-SUV-2RE were separately digested with SbfI and PacI and subsequently ligated together to generate recombinant PRRSV MLVs SUV-IL-15 and SUV-IL-18 infectious clones. The control recombinant MLVs SUV-GFP, SUV-IL-15TAA, SUV-IL-18TAA were generated similarly with primer sets GFP-F and GFP-R; IL-15_SbfI-F1 and IL15_PacI-R; and IL18mtr_SbfI-F and IL18_PacI-R. All the primer sequences are listed in **Table S4**.
Rescue of recombinant PRRSV MLVs and indirect immunofluorescence assay (IFA).

BHK-21 cells were transfected with respective DNA-launched PRRSV recombinant MLV infectious clone plasmid DNA. The supernatant of transfected cells at 26 hour-post-transfection (hpt) was used to infect MARC-145 cells as described previously (40). Transfected or infected cells were examined by IFA using PRRSV anti-N mAb SDOW17 as described previously (41). To further verify the stability and authenticity of the introduced mutations, viral RNA from the 4th and 5th passages of the recombinant PRRSV MLVs in MARC-145 cells were extracted from supernatants using ZR Viral RNA Kit (Zymo Research), and subjected to RT-PCR amplification (superscript III one-step RT-PCR system) and DNA sequencing analysis for the inserted genes inbetween ORF1b and ORF2a.

Antibodies. The following antibodies against porcine antigens were used in multiparameter FACS analysis: anti-CD8α IgG2a (clone 76-2-11), anti-CD8α IgG1 (clone PT36A), anti-CD4 IgG2b (clone 74-12-4), anti-CD3 IgG2b (clone 8E6-2b3c), anti-γδ T cells IgG1 (clone PGBL31A) were purchased from Washington State University (Pullman, WA). PE anti-human/porcine CD107a and mouse anti-porcine CD3ε-biotin (clone PPT3) were purchased from Southern Biotech (Birmingham, AL). Other primary antibodies included mouse anti-pig CD16 IgG1 (clone G7, Biorad, Hercules, CA), and PerCP-Cy™5.5 Mouse Anti-Pig IFN-γ (clone P2G10, BD eBioscience, San Jose, CA). The secondary antibodies FITC anti-mouse IgG2b, PE-cy7 anti-mouse IgG1, APC Streptavidin, and APC anti-mouse IgG2a used in FACS were purchased from Biolegend (San Diego, CA). Rabbit polyclonal antibodies against porcine IL-15 or pIL-18 were purchased from MyBioSource (San Diego, CA). The SDOW17 antibody against PRRSV N protein used in IFA were purchased from Research Technology Innovation Inc (Brookings, SD).
Flow cytometry. Cells were dispensed into 96-well U-bottom plates (BD Falcon, Bedford, MA) and stimulated with PRRSV strain NADC20 at multiplicity of infection (m.o.i) of 1 in a final volume of 150 µL. As described previously (40, 42), GolgiPlug and GolgiStop (Thermo Scientific, Waltham, MA) were added at a final concentration of 1:1000 at 12 hpi. PE-CD107a was also added at 5 uL per well and incubated for 4 additional hours according to the manufacturer’s instructions. Cells were washed and suspended in FACS buffer (PBS containing 2% FBS, 4°C). They were divided into different panels, incubated with anti-porcine CD3, CD4, CD8 antibodies of different isotypes as panel-1, or separately with anti-porcine CD3 and anti-γδ antibodies as panel-2. This was followed by incubation with isotype-matched PE-Cy7, FITC, and APC-conjugated secondary antibodies. As panel-3, NK cells were incubated with anti-porcine CD3, CD8 and CD16 antibodies of different isotypes followed by isotype-matched PE-cy7, FITC, and APC-conjugated secondary antibodies. After staining these surface markers, cells were fixed with 3.2% paraformaldehyde for 10 min at room temperature, followed by permeabilization in 0.2% saponin for 10 min at room temperature. Cells were incubated with PerCP-Cy™5.5 mouse-anti-porcine IFN-γ for 15 min and subsequently washed with FACS buffer with 0.2% saponin. The fluorescence of each panel was assessed with a BD FACSaria II.

Vaccination and challenge study in pigs. The animal study was approved by Virginia Tech Animal Care and Use Committee (IACUC approval no. 16229). A total of 40 pigs were divided into 5 groups of 8 pigs in each, and vaccinated with recombinant PRRSV MLVs or cell culture medium DMEM (Table 1). At 49 days post vaccination (dpv), all the pigs were either challenged with a heterologous PRRSV strain NADC20 virus or with cell culture medium as control. At 14 days post-challenge (dpc), all pigs were necropsied. At necropsy, the gross lung
pathological lesions were recorded in a blind fashion by a board-certified veterinary pathologist (TL). Samples of lung tissues were also collected during necropsy for histological examination in a blind fashion by a board-certified veterinary pathologist (TL) and quantification of viral RNA loads. Weekly serum samples and PBMCs were also collected from each pig for a total of 9 weeks.

Quantitation of viral RNA loads in sera and lung tissues. Viral RNAs were extracted from serum samples at 7 and 14 dpc using ZR Viral RNA kit (ZYMO RESEARCH, Irvine, CA), and the total RNAs from the lung tissues were extracted using TRI Reagent (MRC, Cambridge, UK), both following the manufacturers’ protocols. The quantification of PRRSV RNA copy number in sera and in lung tissues was performed by qRT-PCR using SYBR Green One-Step qRT-PCR kit (BIOLINE), as described previously (41, 43). The RNA standard used for the qRT-PCR was derived from in vitro transcription of a PRRSV full-length cDNA clone pACYC-VR2385 by mMESSAGE mMACHINE T7 kit (Ambion). Each qRT-PCR reaction was performed in triplicate.

Gross pathological and histological evaluations of lung lesions. All pigs were humanely euthanized by intravenously overdose of pentobarbital (Fatal-Plus, Vortech Pharmaceutical Ltd., Dearborn, MI). At necropsy, the lungs were blindly scored for gross pathology as described previously (19). Five sections of lung tissues were collected from each pig, fixed in 10% neutral-buffered formalin, and processed for routine histopathological evaluation. The criteria for evaluating the gross pathology and histopathology have been well established and described previously (19).

Statistical analyses. The One-way ANOVA was used to evaluate the data for statistical
differences (*P < 0.05, **P<0.01, ***P<0.001). The data were analyzed by GraphPad Prism (version 6.0).

RESULTS

The GPI-anchoring signal from pCD59 and the transmembrane region from Influenza virus HA successfully targeted cytokine expression onto cell plasma membrane surface. We successfully generated expression plasmids in which the coding regions of mature pIL-15 or pIL-18 were individually fused with a short N-terminal signal sequence from HA of influenza virus (Influenza A/WSN/33, GenBank J02176.1) and a C-terminal GPI modification signal from pCD59, or a C-terminal HA transmembrane region (HATM), designated as pIL-15-CD59GPI, pIL-15-HATM, pIL-18-CD59GPI and pIL-18-HATM, respectively. Their plasma membrane-targeting abilities were characterized in the cells transfected with each of these plasmids. As expected, substantial cell surface expressions of IL-15 (Fig. 1A-B) or IL-18 (Fig. 1C-D) were detected in the cells transfected with plasmids containing cytokines fused to either CD59 GPI signal or HATM, while only a minimal surface expression of cytokines was found in cells transfected with the control plasmids pIL-15-TAA- or pIL-18-TAA with only a N-terminal signal sequence proceeding the cytokine genes. The results suggest that both the GPI-anchoring signal from pCD59 and the transmembrane region from HA can serve as an appropriate plasma membrane-targeting sequences for expression of selected cytokines.

Successful rescue of vaccine virus from a DNA-launched infectious clone of PRRSV Suvaxyn MLV. The complete genomic sequence of PRRSV Suvaxyn MLV vaccine virus was determined using primers based on the published partial sequence of the parental strain ISU-55 of
the Suvaxyn MLV and the conserved sequences of type 2 PRRSV strains. The 5’ RACE and 3’ RACE were used to determine the extreme 5’ and 3’ end sequences of PRRSV Suvaxyn MLV genome. A DNA-launched infectious clone of PRRSV Suvaxyn MLV was successfully generated according to similar strategies reported previously (44). The PRRSV Suvaxyn MLV genome fused with ribozyme fragments were successfully assembled in a modified vector pIRES-EGFP2 downstream of a CMV promoter, which is used for in vitro transcription (Fig. S1A). The resulting full-length clone of Suvaxyn PRRS MLV was designated as pIR-SUV. The viable infectious MLV vaccine virus was successfully rescued from this DNA-launched infectious clone and confirmed by IFA with an anti-PRRSV N monoclonal antibody in transfected BHK-21 cells and in infected MARC-145 cells as previously described (44) (Fig. S1B). The sequence of the rescued MLV vaccine virus was confirmed by RT-PCR amplification and sequencing of selected regions of the MLV virus from the supernatant of MARC-145 cells infected with the rescued Suvaxyn MLV virus.

Successful construction and rescue of viable recombinant PRRS MLVs expressing pIL-15 or pIL-18. To determine whether porcine cytokines could be incorporated into the PRRSV genome thus being expressed by the virus in the membrane-bound form, we first generated a new DNA-launched PRRSV MLV infectious clone that allows for foreign gene insertion and expression. Two unique restriction enzyme sites, SbfI and PacI, were introduced into the non-overlapping region between ORF1b and ORF2a of the infectious clone pIR-SUV (Fig. 2A). Therefore, any foreign gene sequence can be inserted into this modified PRRSV MLV infectious clone between SbfI and PacI sites, and subsequently transcribed into sg mRNAs guided by body TRS2 that is embedded in the ORF1b and originally responsible for ORF2a transcription. In order to compensate for ORF2a transcription, a synthetic body TRS with a flanking sequence of a total
40 bp (Fig. 2B) was inserted immediately after the PacI site and upstream the start codon of ORF2a. The resulting PRRSV MLV infectious clone was designated as pIR-SUV-2RE (Fig. 2A). Viable infectious viruses were successfully rescued from the pIR-SUV-2RE (Fig. 3A). Subsequently we demonstrated that pIR-SUV-2RE can also be used as a PRRSV expression cassette for foreign gene insertion, as a GFP sequence was cloned into the vector (Fig. 2C) and a GFP-expressing PRRSV was successfully rescued (Fig. 3A).

The nucleotide sequences of mature pIL-15 or pIL-18 with a short N-terminal signal sequence from HA, as well as the aforementioned C-terminal pCD59-GPI signal or C-terminal HATM were incorporated into pIR-SUV-2RE, respectively. Recombinant MLV viruses, designated as SUV-IL-15-CD59 and SUV-IL-18-CD59 (Fig. 2C), SUV-IL-15-HATM and SUV-IL-18-HATM (Fig. S2A-C) were successfully rescued (Fig. 3B). Two additional control recombinant MLV viruses, SUV-IL-15TAA and SUV-IL-18TAA without a membrane-targeting signal, were also rescued similarly (Fig. 3B). The rescued viruses were sequenced and confirmed to be genetically stable for at least 8 serial passages in cell culture (data not shown).

**Recombinant PRRSV MLVs successfully express membrane-bound pIL-15 or pIL-18 on the plasma membrane of infected porcine alveolar macrophages (PAMs).** In order to determine if the inserted cytokine can be expressed by the recombinant MLVs on the plasma membrane of the infected cells, PAM cells were infected with each of the recombinant PRRSV MLVs at 0.1 multiplicity of infection (m.o.i). At 20 hours post-infection (hpi), the surface cytokine expression as well as the intracellular viral N protein were detected by flow cytometry (Fig. S3). As expected, when compared to the non-infected cells, there is a significant increase of plasma membrane surface expression of IL-15 (Fig. 3C) or IL-18 (Fig. 3D) on PAM cells infected by
recombinant SUV-IL-15-CD59 or SUV-IL-18-CD59. In contrast, only few cells expressing membrane-bound cytokines were detected in cells infected with Suvaxyn SUV-IL-15TAA and SUV-IL-18TAA viruses, the latter two containing cytokine genes but without the membrane-targeting signal. Interestingly, the HATM appeared to be less efficient in terms of tethering cytokines onto the plasma membrane as shown by the results of cells infected with recombinant SUV-IL-15HATM and SUV-IL-18HATM viruses. Therefore, the GPI-anchoring signal from pCD59 successfully tethered cytokines expressed by recombinant PRRSV MLVs onto the cell membrane surface.

Recombinant PRRSV MLVs SUV-IL-15 and SUV-IL-18, but not the parental PRRSV Suvaxyn MLV, activate NK cells after vaccination. To evaluate the immunomodulatory effects of the membrane-bound cytokines expressed by the recombinant PRRSV MLVs as adjuvants, we conducted a vaccination/challenge study in 3-week old piglets. Groups of pigs (Table 1) were vaccinated via a combination of intranasal and intramuscular route with parental PRRSV Suvaxyn MLV, recombinant MLV SUV-IL-15-CD59, recombinant MLV SUV-IL-18-CD59, and DMEM medium, respectively. At 49 days post-vaccination (dpv), the animals were challenged with a high dose of a heterologous PRRSV (strain NADC20) to determine the protective efficacy of the recombinant PRRSV MLVs.

We found that the porcine NK cells (CD3-CD16+) can be further categorized into two subgroups: CD8- NK, and CD8+ NK, according to CD8 expression (Fig. S4), although the functional difference of these two subgroups is not well known. At 7 dpv, NK cells were decreased in the pigs vaccinated with PRRSV Suvaxyn MLV when compared to the non-vaccinated pigs, and the decrease was mainly observed in the subpopulation of CD8+ NK cells (Fig. 4A). However, we
did not find such a reduction of NK cells in recombinant PRRSV MLV SUV-IL-15- or SUV-IL-18-vaccinated pigs.

Additionally, at 7 dpv, there was an increase of IFN-γ-producing CD8-NK cells in recombinant SUV-IL-15-vaccinated group than that in the parental Suvaxyn MLV group (Fig. 4B). Later at 49 dpv, Suvaxyn MLV-vaccinated, but not the recombinant MLV SUV-IL-15- or the SUV IL-18-vaccinated pigs, had significantly lower numbers of total IFN-γ-producing NK cells and IFN-γ+ CD8+ NK cells, when compared to the non-vaccinated pigs (Fig. 4C). These data suggest that the Suvaxyn MLV inhibited the proliferation and IFN-γ production of NK cells, but recombinant MLVs SUV-IL-15 and SUV-IL-18 greatly ameliorated the inhibition.

**Recombinant MLV SUV-IL-15 facilitated long-term γδ T cell response and greatly increased IFN-γ-producing CD8+ T cells during late stages of vaccination.** To evaluate T cell responses, we first analyzed the surface expression of CD107a as an indicator for the lymphocyte degranulation. At 7 dpv, the total CD107a-expressing T cells were comparable among all groups (Fig. S5A-B). However, the Suvaxyn MLV-vaccinated and recombinant MLV SUV-IL-15-vaccinated pigs had substantially increased CD107a+ γδ T cells when compared to non-vaccinated pigs (Fig. 5A). We also found increased total numbers of γδ T cells in these 2 vaccinated groups (Fig. 5B), which may partially explain the enhanced CD107a expression from γδ T cells at 7 dpv. Similarly at 49 dpv, total CD107a+ T cells were comparable among different groups (Fig. 5C), but only recombinant MLV SUV-IL-15 group had an increased level of CD107a+ γδ T cells than that in Suvaxyn MLV group (Fig. 5D).

We also evaluated the IFN-γ production in T cells after vaccination. At 7 dpv, the IFN-γ-producing T cells were comparable among different groups (Fig. S5B). In contrast, at 49 dpv, the
groups vaccinated with recombinant MLV SUV-IL-15, but not with Suvaxyn MLV or recombinant MLV SUV-IL-18, had a significant increase of IFN-γ-producing T cells when compared to other groups (Fig. 5E). Moreover, the recombinant MLV SUV-IL-15-vaccinated pigs had substantially increased IFN-γ+ CD8+ T cells and IFN-γ+ γδ T cells than the Suvaxyn MLV-vaccinated pigs (Fig. 5F). The results demonstrate that recombinant MLV SUV-IL-15, but not recombinant MLV SUV-IL-18, greatly enhanced both the proliferation and anti-PRRSV functions of γδ T cells as indicated by the increased degranulation marker CD107a expression and intracellular IFN-γ production. More importantly, the recombinant MLV expressing IL-15 was able to activate IFN-γ-producing CD8+ T cells at late stages of vaccination.

Recombinant MLV SUV-IL-15 enhances CD107a surface expression in γδ T cells after heterologous PRRSV challenge. To investigate the protective immune response against heterologous challenge with PRRSV strain NADC20, we analyzed both PRRSV-specific T cells and NK cells at 7 dpc. Different from 49 dpv, the CD107a+ T cells at 7 dpc in recombinant MLV SUV-IL-15-vaccinated pigs increased significantly when compared to Suvaxyn MLV-vaccinated pigs, or to the un-challenged control pigs. The IFN-γ+ T cells in the circulation are comparable among the groups (Fig. 6A-B). More specifically, the activated γδ T cells (CD107a+ or IFN-γ+) in the recombinant MLV SUV-IL-15 vaccination group were elevated when compared to that in Suvaxyn MLV vaccination group (Fig. 6C). In fact, the total number of γδ T cells in the circulation was also significantly increased in recombinant MLV SUV-IL-15 group (Fig. 6D). In contrast, there was no significant difference in either CD107a expression or IFN-γ production from T cells in recombinant MLV SUV-IL-18-vaccinated group when compared to the Suvaxyn MLV-vaccinated group.
As to the NK cell response, the non-vaccinated challenged-only pigs had the fewest NK cells in the circulation among the groups (Fig. 6E). The CD107a-expressing NK cells in this challenged-only group were also fewer than the other groups (except for the recombinant SUV-IL-18 group), even though the IFN-γ-producing NK cells were comparable among different groups (Fig. 6F). These results suggested that PRRSV NADC20, similar to PRRSV Suvaxyn MLV vaccine virus, likely inhibited NK cell proliferation and impaired their degranulating activity.

Furthermore, the total circulating cells regardless of cell types were also evaluated for surface expression of CD107a and IFN-γ before and after PRRSV NADC20 challenge (Fig. 7A-B). At 49 dpv, recombinant MLV SUV-IL-15 significantly increased the IFN-γ-producing cells in the peripheral blood when compared to the parental Suvaxyn MLV as well as the non-vaccinated group, suggesting an overall enhanced immunogenicity by recombinant MLV expressing IL-15 after vaccination. However, after heterologus challenge with PRRSV NADC20, there was no significant difference in IFN-γ-producing cells among the groups, while CD107a-expressing cells were increased both in recombinant MLV SUV-IL-15-vaccinated pigs and the un-vaccinated challenged-only pigs.

**Pigs vaccinated with recombinant MLV SUV-IL-15 had significantly reduced lung lesions and reduced viral RNA loads in lungs after heterologous challenge with PRRSV.** In order to measure the replication level of the challenge virus NADC20, viral RNA loads in the sera and lung tissues were compared among different groups. The recombinant MLVs SUV-IL-15 and SUV-IL-18-vaccinated pigs had similar serum viral RNA titers when compared to the parental Suvaxyn MLV-vaccinated against heterologous virus challenge both at 7 dpc and 14 dpc (Fig. 8A-B). In the lung tissues at 14 dpc, there were numerically lower viral RNA loads in recombinant
MLV SUV-IL-15-vaccinated group when compared to parental Suvaxyn MLV and MLV SUV-IL-18 groups (Fig. 9A). All three vaccinated groups had significantly lower viral RNA copy numbers compared to unvaccinated challenged-only group, indicating that the parental Suvaxyn MLV effectively reduced lung viral RNA loads against heterologous PRRSV challenge. The microscopic lung lesion scores in the recombinant MLV SUV-IL-15 group were numerically lower than those in the Suvaxyn MLV, recombinant MLV SUV-IL-18, or unvaccinated challenged-only group (Fig. 9B). There was no significant difference in the gross lung lesions among three vaccinated groups (data not shown). The results suggested that the recombinant MLV SUV-IL-15 exhibited enhanced protection, although not statistically significant, against heterologous challenge, as evidenced by numerically lower lung viral RNA loads and numerically lower lung lesion scores when compared to the parental Suvaxyn MLV. It is important to point out that the parental Suvaxyn MLV is a commercial vaccine, thus any incremental improvement of the efficacy of an existing vaccine is an achievement, as significant improvement of the efficacy of a commercial vaccine is usually very challenging.

**Recombinant MLVs SUV-IL-15 and SUV-IL-18 expressed IL-15 or IL-18 in vivo.** As expected, IL-15 expression in the lung tissues of recombinant MLV SUV-IL-15-vaccinated pigs was significantly higher than that in any other groups at 14 dpc (Fig. 9C). Similarly, IL-18 expression in lung tissues is also significantly higher in recombinant MLV SUV-IL-18-vaccinated group when compared to other groups (Fig. 9D). The data indicates that the recombinant MLVs carrying the cytokine genes successfully expressed IL-15 or IL-18 in pigs.
DISCUSSION

PRRSV infection usually induces insufficient activation of immune response including a weak cell-mediated immune response and a strikingly delayed neutralizing antibody response (45). This is partially due to viral inhibition of type I interferons (46-48), TNFα (49), or other innate cytokines and co-stimulatory molecules (40, 50, 51) that play an important role in priming antigen-presenting cell (APCs) and in bridging adaptive immunity (45, 49, 52, 53). This immune suppression by PRRSV has remained a major challenge for developing effective MLVs against PRRSV (21, 54). Therefore, novel strategies that can improve PRRSV MLV efficacies are needed for effective control of PRRSV.

Cytokines especially those produced in the early stage of viral infection exert significant effects on either priming or directing adaptive immunity (31). Many studies have been carried out to evaluate the use of cytokines as adjuvants to improve the immunogenicity and efficacy of the vaccines, mostly by co-expressing selected cytokines with the viral antigen in subunit vaccines (55-57), recombinant adenoviruses (58), or with the tumor antigens in tumor vaccine development (59, 60). They can be co-delivered along with vaccines in their bioactive form, or alternatively, be fused with DNA sequences of certain vaccines. In this study, for the first time, we investigated the immunogenicity and the protective efficacy of recombinant PRRSV MLVs expressing membrane-bound porcine cytokines as adjuvants. We presented a novel approach of using porcine IL-15 and IL-18 as adjuvants by incorporating their genes into recombinant PRRSV MLVs, in an attempt to induce cytotoxic NK cells in the innate immune response, as well as to enhance adaptive T cell response. At the same time, we also attempted to direct the cytokine expression to the cell plasma membrane surface in order to avoid excessive systemic inflammation. Therefore, the cytokines expressed by the recombinant PRRSV MLV on the membrane of infected cells are more likely to
stimulate immune cells and facilitate cytotoxic lysis due to their proximity to the viral antigen presentation on the same cell.

By using a DNA-launched infectious clone backbone of the commercial PRRSV Suvaxyn MLV, we successfully generated a panel of recombinant Suvaxyn MLVs expressing cytokines. We further demonstrated that the recombinant MLVs can readily infect MARC-145 cells and PAMs in vitro, and express the cytokines of interest during virus infection. Importantly, we fused the GPI modification sequence from porcine CD59 to the cytokine genes in order to target the cytokine expression onto the cell plasma membrane surface. Additionally, we also tested the HA C-terminal coding sequence (HATM) as an alternative to GPI anchor for targeting the cytokine expression to cell surface. The results showed that both GPI signal and HATM can direct cytokine protein expression onto cell plasma membrane surface by expression plasmids. However, the GPI signal from CD59, not HATM, can tether the cytokines on the plasma membrane much more efficiently when they were expressed by the recombinant PRRSV MLVs in PAMs. This is probably due to the fact that the sequence from CD59 is of porcine origin, and thus already adapted for expression in PAMs. When the recombinant MLVs were inoculated into pigs, the MLVs incorporated with cytokine gene fusing with the GPI signal expressed IL-15 and IL-18 in vivo, as demonstrated by an elevated level of IL-18 or IL-15 expression in the lung tissues as measured by qRT-PCR for gene transcription.

After vaccination with the parental Suvaxyn MLV, we observed a significant decrease of total NK cells and IFN-γ+ NK cells in pigs. Similarly, after challenge with a heterologous PRRSV, total NK cells and CD107a+ NK cells were significantly decreased in the un-vaccinated PRRSV NADC20-challenged-only pigs. As demonstrated previously by an in vitro study, the NK cell-mediated cytotoxicity was suppressed in PRRSV-infected PAMs (61), thus our results from this
study reinforce the fact that PRRSV suppresses the stimulation of NK cells. However, PRRSV MLVs SUV-IL-15 and SUV-IL-18 greatly restored the NK cell proliferation and their functions, suggesting that IL-15 and IL-18 expressed by recombinant MLVs as adjuvants can enhance the anti-PRRSV innate immune response.

We also analyzed T cell response after vaccination. We noticed that the γδ T cells are the most abundant among all the porcine T cell subpopulations by making up more than 40% of T cells (Fig.S6A-C). Unfortunately, the γδ T cells have rarely been studied previously in anti-PRRSV immunology. In our study, we demonstrated that the γδ T cells were much more responsive than CD8+, DP, and CD4+ T cells after PRRSV vaccination. This is indicated by the substantially expanded γδ T cells and the significantly increased CD107a+ γδ T cells at early stage of vaccination with Suvaxyn MLV or recombinant MLV SUV-IL-15. However, at late stage of vaccination, the enhanced γδ T cell response was only observed in MLV SUV-IL-15-vaccinated pigs, suggesting that IL-15 as adjuvants improved γδ T cell response in the MLV vaccination. Clearly, further in-depth study is warranted to investigate the role of γδ T cells in anti-PRRSV immunity.

The immune response of other T cell subpopulations after vaccination were generally comparable among the three vaccinated groups. We only found a significantly enhanced IFN-γ production from CD8+ T cells in the recombinant MLV SUV-IL-15 group at 49 dpv. These effector CD8+ T cells, together with the significantly increased IFN-γ+ γδ T cells, contribute to the overall increase of total IFN-γ-producing T cells at 49 dpv observed in the recombinant MLV SUV-IL-15 group when compared to the other 4 groups. Taken together, the recombinant MLV expressing membrane-bound IL-15, not the parental Suvaxyn MLV or the recombinant MLV expressing IL-18, is able to enhance IFN-γ production from NK cells, CD8+ T cells and γδ T cell after vaccination.
At necropsy at 14 dpc, we found that the commercially-used MLV Suvaxyn significantly reduced viral RNA loads in the serum and lung tissues against heterologous PRRSV NADC20. The recombinant MLV SUV-IL-15 group had numerically lower lung viral RNA loads and microscopic lung lesion scores than Suvaxyn MLV group and the recombinant MLV SUV-IL-18 group. We believed that this incremental enhancement of vaccine protection is attributed to the aforementioned improved immune response in the recombinant MLV SUV-IL-15 vaccinated pigs. These data demonstrate that the recombinant MLV expressing membrane-bound IL-15 can enhance the immunogenicity after vaccination, and confer an improved protection against heterologous PRRSV NADC20 strain. Further study with other heterologous PRRSV strains is warranted in order to fully investigate the ability of cross-protection by the recombinant MLV that expresses IL-15. Additionally, it appears that PRRSV is generally less efficient in stimulating IFN-γ production from T cells, since the IFN-γ+ cells were only increased in the recombinant MLV SUV-IL-15-vaccinated pigs as late as 49 dpv. Therefore, it will also be intriguing to evaluate the use of other cytokines or chemokines to be expressed by recombinant MLVs as potential adjuvants through this novel approach.

It’s worth mentioning that CD8+ T cells and DP T cells are two primary T cell populations that are capable of expressing surface CD107a, followed by the γδ T cell population (Fig. 5A&D, Fig. 6C, Fig. S5C). In contrast to γδ T cells, they together compose less than 30% of total T cells (Fig. S6A-C). CD4+ T cells, as expected, were least capable of expressing CD107a. These observations suggested that CD8+ T, DP T and γδ T cells are major players in the lymphocyte degranulation against PRRSV. Meanwhile, NK cells were also capable of expressing surface CD107a at a considerable level when compared to T cells, suggesting their important role in the degranulation and cytotoxicity as well.
In summary, in this study we present a novel strategy of utilizing the discontinuous transcription mechanism of PRRSV to express membrane-bound cytokines as vaccine adjuvants upon infection with recombinant PRRSV MLVs. This is achieved by directly incorporating the cytokine genes and fusing them to a selected plasma membrane-targeting signal into the PRRSV MLV genome. Our results demonstrated that the cytokines of interest are successfully expressed onto the cell plasma membrane surface by the recombinant PRRSV MLVs in vitro and in vivo. Therefore this would potentially eliminate the need for administrating soluble adjuvants along with the vaccines. More importantly, our study demonstrates that cytokine-incorporated MLVs can serve as potential vaccines with an improved immune responses against PRRSV.
ACKNOWLEDGEMENT

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REFERENCE


28. **Carter QL, Curiel RE.** 2005. Interleukin-12 (IL-12) ameliorates the effects of porcine respiratory and reproductive syndrome virus (PRRSV) infection. Vet Immunol Immunopathol **107**:105-118.


hepatitis E virus infection in immunocompromised patients to assess immune correlates during chronicity. Proc Natl Acad Sci U S A 114:6914-6923.


Fig. 1. Characterization of cell plasma membrane-targeting ability of GPI modification signal from pCD59 and HA transmembrane region (HATM). Porcine kidney cell line PK-15 cells were transfected with individual expression plasmids, fused or not fused with respective membrane-targeting sequence. Cell surface cytokines were stained with anti-IL-15 or anti-IL-18 specific antibody and analyzed by flow cytometry. Representative histograms showing the frequency of cells expressing membrane-bound cytokines in individual transfections (A and C). The aforementioned frequency on average of each group was shown in (B and D). Asterisks indicated a statistically significant difference between designated groups.
Fig. 2

A

pIR-SUV

pIR-SUV-2RE

B

C

Suv-GFP

Suv-IL-15-CD59

Suv-IL-18-CD59

Suv-IL-15TAA

Suv-IL-18TAA
Fig. 2. Schematic diagrams of genomic sequences of recombinant PRRSV MLVs incorporated with foreign genes in the vicinity of ORF1b/2 junction region. (A). PRRS MLV pIR-SUV contains a non-overlapping region of one single nucleotide of between ORF1b and ORF2a. pIR-SUV-2RE serves as a PRRSV MLV expression cassette for foreign gene insertion. The introduced 2 unique restriction enzyme sites SbfI and PacI are indicated in bold and underlined in italic, followed by the introduced synthetic body TRS with flanking sequences. The conserved junction motif for the synthetic body TRS is boxed. The asterisks beneath the underlined sequence indicate stop codons of corresponding ORFs. (B). The hair-pin structure of synthesized body TRS and its flanking region are shown. (C). Nucleotide sequences of GFP-inserted recombinant PRRSV and cytokine-incorporated recombinant PRRSV MLVs in the vicinity of ORF1b/2 junction region. The recombinant PRRSV MLV SUV-IL-15-CD59 contains a C-terminal GPI modification signal. The recombinant PRRSV MLV SUV-IL-18-CD59 contains an additional N-terminal signal peptide sequence from HA. The SUV-IL-15-TAA and SUV-IL-18-TAA contain only the cytokine coding regions.
Fig. 3

A  pIR-SUV-2RE
pIR-SUV-GFP

B  Suv-IL15-TAA
Suv-IL15-HATM
Suv-IL18-TAA
Suv-IL18-HATM

C  Infected PAM expressing membrane-bound IL-15
Fold change

D  Infected PAM expressing membrane-bound IL-18
Fold change

NS  Suv-IL15-CDS
Suv-IL15-CDS
Suv-IL15-CDS
Suv-IL15-CDS
Fig. 3. Rescue and characterization of recombinant PRRSV MLVs expressing membrane-bound cytokines. All the recombinant PRRSVs were rescued as live infectious viruses, as confirmed by indirect fluorescence assay (IFA) with PRRSV-specific monoclonal antibody. (A). MARC-145 cells were infected with passage 1 virus rescued from pIR-SUV-2RE, or passage 1 virus rescued from pIR-SUV-GFP, and stained with anti-PRRSV N monoclonal antibody (SDOW17), shown in the top panel of (A). The bottom panel of (A) shows GFP fluorescence in the fresh cells after infection with SUV-2RE or SUV-GFP virus, respectively. (B). Similarly, recombinant MLVs incorporated with IL-15 or IL-18 gene were rescued and confirmed by IFA. (C-D). PAMs were infected with indicated viruses at m.o.i of 0.1, followed by surface staining with anti-IL-15 or anti-IL-18 antibodies and intracellular staining of anti-PRRSV N protein at 20 hours post-infection (hpi). Only PRRSV-infected cells (N protein-positive population as described in Fig. S3) were further analyzed. Frequency of cells expressing surface IL-15 (C) and IL-18 (D) in individual infection were compared with that in non-infected cells. Asterisks indicate statistical significance between designated groups determined by one-way ANOVA.
Fig. 4

A. NK cells

B. IFN$\gamma$+ NK cells

C. IFN$\gamma$+ NK cells

Legend:
- DMEM/DMEM
- DMEM
- Suvaxyn
- SUV-IL-15
- SUV-IL-18
Fig. 4. NK cell response after vaccination at 7 days post-vaccination (dpv) and 49 dpv. PBMCs collected at 7 dpv and 49 dpv were re-stimulated with PRRSV NADC20 virus. Cells were surface-stained with anti-pig CD107a, CD3, CD8 and CD16 antibodies and intracellularly-stained with IFN-γ antibody. (A) NK cells were pre-gated from PBMCs as CD3-CD16+ cells based on lymphocyte morphology. They were further divided into CD8+ and CD8- subpopulations as described in Fig. S4. (B) Total IFN-γ-producing NK cells, IFN-γ-producing CD8+ NK or CD8- NK cells among CD3- PBMCs at 7 dpv. (C) IFN-γ-producing NK cells at 49 dpv. Asterisks indicate statistical significance between designated groups determined by one-way ANOVA.
Fig. 5

A

CD107a+ T cells

% of CD3+ T cells

CD8+ T cells  DP T cells  CD4+ T cells  γδ T cells

7dpv

ns  *  *  ns

B

γδ T cells

% of PBMCs

7dpv

***  **

C

Total CD107a+ T cells

% of PBMC

49dpv

ns

D

CD107a+ T cells

% of CD3+ T cells

CD8+ T cells  DP T cells  CD4+ T cells  γδ T cells

49dpv

ns  ns  ns  *

E

Total IFNγ+ T cells

% of PBMC

49dpv

**  **  **

F

IFNγ+ T cells

% of CD3+ T cells

CD8+ T cells  DP T cells  CD4+ T cells  γδ T cells

49dpv

**  **  ns  ***

DMEM/DMEM  DMEM  Suvaxyn  SUV-IL-15  SUV-IL-18
Fig. 5. T cell response after vaccination both 7 days post-vaccination (dpv) and 49 dpv. PBMCs were collected from each pig at 7 dpv and 49 dpv. T cells were pre-gated as CD3+ lymphocytes, and evaluated for CD107a-expressing, or IFN-γ-producing CD8+ T cells, CD4+CD8+ (DP) T cells, CD4+ T cells and γδ T cells by flow cytometry. (A) Different subpopulations of T cells expressing surface CD107a at 7 dpv. (B) The frequency of γδ T cells among PBMCs at 7 dpv. (C) Total CD3+ T cells expressing CD107a at 49 dpv. (D) Different T cell subpopulations expressing CD107a at 49 dpv. (E) Total T cells producing IFN-γ at 49 dpv. (F) Different T cell subpopulations producing IFN-γ at 49 dpv. Asterisks indicate statistical significance between designated groups determined by one-way ANOVA.
Fig. 6

A. Total CD107a+ T cells
B. Total IFNγ+ T cells
C. CD107a+ γδ T cells
D. γδ T cells
E. NK cells
F. Total CD107a+ NK cells
G. Total IFNγ+ NK cells

---

DMEM/DMEM  DMEM  Suvaxyn  SUV-IL-15  SUV-IL-18
Fig. 6. Anti-PRRSV immune response after heterologous PRRSV NADC20 challenge. PBMCs collected at 7 days post-challenge (dpc) were evaluated for CD107a-expressing and IFN-γ-producing lymphocytes. Total CD3+ T cells expressing CD107a (A) or IFN-γ (B) among PBMCs. (C) γδ T cells expressing CD107a or IFN-γ among PBMCs. (D) The frequency of γδ T cells among PBMCs. (E) NK cells among PBMCs. (F) Total NK cells expressing CD107a or IFN-γ. Asterisks indicate statistical significance as determined by one-way ANOVA.
Fig. 7. CD107a-expressing or IFN-γ-producing cells regardless of cell types in the peripheral blood at 7 dpv, 49 dpv and 7 dpc. PBMCs were collected at indicated time points, and all the cells were evaluated for CD107a expression (A) and IFN-γ production (B). Asterisks indicate statistical significance as determined by one-way ANOVA.
Fig. 8. Serum viral RNA loads after challenge with PRRSV NADC20 strain at 7 dpc (A) and 14 dpc (B). Viral RNAs were extracted from serum samples at 7 and 14 dpc for the quantification of PRRSV RNA copy number in sera by qRT-PCR. The detection limit is calculated as $2 \log_{10}$ copies for each real-time PCR reaction. Samples below the detection limit were considered as negative and labeled as $2 \log_{10}$ copies. Asterisks indicate statistical significance between designated groups as determined by one-way ANOVA.
Fig. 9. Viral RNA loads, microscopic lesions, and cytokine expression in lung tissues after heterologous challenge with PRRSV NADC20 at 14dpc. (A) Viral RNA loads in lung homogenates was determined by qRT-PCR. The detection limit is calculated as $2 \log_{10}$ copies for each real-time PCR reaction. Samples below the detection limit were considered as negative and labeled as $2 \log_{10}$ copies. (B) Microscopic lung lesion scores at 14 dpc after challenge. IL-15 (C) or IL-18 (D) expression in lung tissue homogenates at 14 dpc was quantified by qRT-PCR and normalized to housekeeping gene Ywhaz, shown as a fold change over the Suvaxyn MLV group. Asterisks indicate statistical significance between designated groups determined by one-way ANOVA.
Table 1:

Experiment design for vaccination and challenge study in pigs to evaluate the efficacy of the recombinant PRRSV MLVs expressing membrane-bound cytokines.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pigs</th>
<th>Vaccination(^b) at 0 dpv (5.0×10(^4) TCID(_{50})/pig) with</th>
<th>Challenge(^b) at 49 dpv (5.0×10(^5) TCID(_{50})/pig) with</th>
<th>No. of pigs at necropsy (14 dpc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>Suvaxyn</td>
<td>PRRSV NADC20</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Suv-IL18-CD59(^a)</td>
<td>PRRSV NADC20</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Suv-IL15-CD59(^a)</td>
<td>PRRSV NADC20</td>
<td>7(^c)</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>DMEM control</td>
<td>PRRSV NADC20</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>DMEM control</td>
<td>DMEM</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\)Suvaxyn is the attenuated PRRSV MLV Suvaxyn vaccine virus, rescued from a DNA-launched PRRSV infectious clone pIR-Suvaxyn. Suv-IL15-CD59 and Suv-IL18-CD59 are recombinant PRRSV MLVs expressing plasma membrane (PM)-bound IL-15 or IL-18.

\(^b\)Pigs were vaccinated with virus stocks of respective recombinant PRRSV MLVs by a combination of intranasal (half) and intramuscular (half) inoculation route. They were subsequently challenged with a heterologous PRRSV strain NADC20 by intranasal inoculation. dpv, days post-vaccination. dpc, days post-challenge.

\(^c\)One pig died with intestinal hemorrhage unrelatedly to this study.
Fig. S1. Construction of viable PRRS MLV Suvaxyn virus using reverse genetic system.

(A). Construction of a DNA-launched full-length clone of PRRS MLV Suvaxyn. An Asc I restriction site, ribozyme sequence and 2 guanosines were introduced at 5’ end of fragment AX. An XbaI restriction site, ribozyme sequence, and 20 poly(A) were introduced to the 3’ end of fragment EX. All 5 fragments overlapping with each other contain unique enzyme restriction sites for full-length viral genome assembly. Firstly, these fragment were cloned in order to a modified vector pACYC177-AX with stuffer fragment to generate full-length clone. This full-length clone was then transferred to a modified pIRES-EGFP2 vector downstream of the CMV promoter. The resulting full-length DNA-launched infectious clone for PRRS MLV Suvaxyn is designated as pIR-SUV. (B) IFA staining with anti-PRRSV N antibody of BHK cells transfected with infectious clone of pIR-SUV or MARC 145 cells infected with cell supernatants from BHK-21 transfected with pIR-SUV.
**Fig. S2**: Construction of recombinant PRRSV MLVs SUV-IL-15-HATM and SUV-IL-18-HATM. Nucleotide sequences of SUV-IL-15-HATM (A) and SUV-IL-18-HATM (B) recombinant PRRSV MLVs in the vicinity of ORF1b/2 junction region. The recombinant PRRSV MLV SUV-IL15-HATM contains a C-terminal HATM and the recombinant PRRSV MLV SUV-IL18-HATM contains an additional N-terminal signal peptide sequence from HA. (C) Recombinant PRRSV MLVs SUV-IL-15-HATM and SUV-IL-18-HATM were rescued as live infectious viruses, as confirmed by indirect fluorescence assay (IFA) with PRRSV-specific monoclonal antibody. MARC 145 cells infected with passage 1 viruses SUV-IL-15-HATM or SUV-IL-18-HATM were used in the IFA.
Fig. S3

A

B

Suvaxyn
infected

SUV-IL-15-CD59
infected

SUV-IL-18-CD59-
infected

Non-infected

PRRSV N
APC

C

PRRSV N
positive cells

IL-15-PE
or IL-18-PE

D

PRRSV N
negative cells

IL-15-PE
or IL-18-PE
Fig. S3. Gating strategy for membrane-bound cytokine expressed by recombinant PRRSVs upon infection of PAMs. PAMs were infected with Suvaxyn virus, or each of recombinant Suvaxyn viruses, or mock infected (Non-infected) at 0.1 m.o.i. At 20 hpi, they were stained for both intracellular PRRSV N protein (N) and surface IL-18 or IL-15, and analyzed by multi-color flow-cytometry. (A) According to Aqua live/dead stain, only live cells were further analyzed. (B). PAMs after infection can be divided into PRRSV N positive or negative cell population. (C). The frequency of cells expressing membrane-bound cytokines will be compared only in the PRRSV N positive population. In the Non-infected control group, the frequency of cells expressing membrane-bound cytokines will be calculated in the PRRSV N negative population. Representative dot plots are shown.
Fig. S4. NK cells in PBMCs after vaccination with PRRSV MLVs. Gating strategy of NK cells.

Porcine NK cells were gated as CD3-CD16+ lymphocytes among PBMCs, and further divided into CD8+ and CD8- populations. CD107a and IFN-γ expression was then evaluated in CD8+ and CD8- NK cells at 7dpv.
Fig. S5. Anti-PRRSV T cell analysis after vaccination. (A) Gating strategy of T cells. CD3+ T cells were further divided into CD4+, CD8+, CD4+CD8+ and γδ T cell populations. CD107a and IFN-γ expression were evaluated in these T populations. (B) Early at 7 dpv, CD107a-expressing or IFN-γ-producing T cells (CD3+) were comparable among all groups. (C) CD107a-expressing CD8+, DP, and CD4+ T cells among PBMCs at 7 dpc were comparable between different groups. Statistical significance of difference are determined by one-way ANOVA.
Fig. S6 Composition of T cell subpopulations among CD3+ T cells at 7 dpv (A), 49 dpv (B) and 7 dpc (C). PBMCs were collected at indicated time points, and divided into different T subpopulations by staining with CD3, CD4, CD8, and γδ TCR antibodies. Statistical significance of difference is determined by one-way ANOVA.
Table S1.

**gBlock sequences for cytokines and plasma membrane-targeting signals**

<table>
<thead>
<tr>
<th>gBlock ID</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIL15-flag</td>
<td>AACTCGCTAGCaccatgagaatttttgaacagagtacgtcgaatctggcttcacattgttacttcctgagttattgctt</td>
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<tr>
<td></td>
<td>ctaaatagatcagatctggaagtttcagctacactctcgtggactcgttcctctccatctctcctctctctctctctc</td>
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<tr>
<td></td>
<td>ttgtaaaacttaaacaggtgcgtggtgtgatgttactttatattataatatatatatatatatatatatatatatat</td>
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<tr>
<td>HAsp-pIL18-flag</td>
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<td>ctaaatagatcagatctggaagtttcagctacactctcgtggactcgttcctctccatctctcctctctctctctc</td>
</tr>
<tr>
<td></td>
<td>ttgtaaaacttaaacaggtgcgtggtgtgatgttactttatattataatatatatatatatatatatatatatatat</td>
</tr>
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</table>

a pIL15-flag and pIL18-flag were both flanked with a 5’ end NheI and a 3’ end XhoI enzyme site.

b In the Spliced GPI signals, pCD59-GPI is shown in boldface and HA-TM is shown in italic.
### Table S2.

**Oligonucleotide primers used in the construction of cytokine expression plasmids**

<table>
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<tr>
<th>Primer ID</th>
<th>Sequence (5’&gt;3’)</th>
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</thead>
<tbody>
<tr>
<td>pCD59_XhoI-F</td>
<td>TAACACTCGAGTACAACCTGCTGCGGAA</td>
</tr>
<tr>
<td>pCD59_NotI-R</td>
<td>TAAGGTGCAGCCTTTAGAGACAAAGT</td>
</tr>
<tr>
<td>HA_XhoI-F</td>
<td>CAATACCTCGAGAATGGGACTTATGATTATATCCA</td>
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<tr>
<td>HA_NotI-R</td>
<td>TTAGTGCAGCCTCACTCAGATTCATCTGTGACTGCAAA</td>
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<td>IL-15TAA-F</td>
<td>ACTCGCTAGCACCATGAATTTTGGAAA</td>
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<tr>
<td>IL-15TAA-R</td>
<td>TTAGCTCGAGTTACCTGGCATCGTCGTC</td>
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<td>HAsp-IL-18-TAA-F</td>
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<tr>
<td>HAsp-IL-18-TAA-R</td>
<td>GGATCTCGAGTTACCTTGACTCGTCGTC</td>
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Table S3.

Oligonucleotide primers used in the construction of a DNA-launched infectious clone of PRRS Suvaxyn MLV in this study

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence (5’&gt;3’)</th>
<th>nt position in Suvaxyn virus genome</th>
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<tr>
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<td>SV1R</td>
<td>TTGTGACTGCAAACCGG</td>
<td>1312-1329</td>
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<tr>
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<td>GACCTCCTCAGAAATAAGGTTGA</td>
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<td>SV3F</td>
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<tr>
<td>SV3R</td>
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<td>3595-3616</td>
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<td>SV5F</td>
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<td>5057-5071</td>
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<td>SV7F</td>
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<td>SV10R</td>
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<td>SV5RACE2</td>
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<td>SV3RACE</td>
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For 5' RACE and 3' RACE

<table>
<thead>
<tr>
<th>Primer ID</th>
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<th>nt position in Suvaxyn virus genome</th>
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<td>SV_XPF</td>
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<td>SV_XPR</td>
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<tr>
<td>SV_PNF</td>
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<td>7593-7612</td>
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<td>SV_PNR</td>
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<tr>
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<td>SV_NER</td>
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<td>SV_EXF</td>
<td>GCCACCAGCATGAAGTTT</td>
<td>12007-12026</td>
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<tr>
<td>SV_EXR</td>
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<td>PRV-ASCIF_rz</td>
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*Ribozyme sequences are underlined.*
Table S4.

Oligonucleotide primers used in the construction of a DNA-launched infection clones for recombinant MLVs

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<tr>
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<tbody>
<tr>
<td>Suv-NS_F1</td>
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<tr>
<td>Suv-NS_R1</td>
<td>CGTGCTTAATTAACCTGCAGGTTTCAATTCAACGGCCTTAAAGTTGTTCAA</td>
</tr>
<tr>
<td>Suv-NS_F2-1</td>
<td>CAGGCTTAATTAAGTTCCCGCGAACCCTATAACCAAATTTCAAGCGGAGCAATG</td>
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<tr>
<td>Suv-NS_F2-2</td>
<td>ATAAACAAAAATTTCAAGCGGAGCAATGAAATGGGTTCTATGCAAGAGCCT</td>
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<tr>
<td>Suv-NS_R2</td>
<td>AGCAAGCACCCTGAATTCAAAGTACTATTATACACTAT</td>
</tr>
<tr>
<td>IL-18_SbfI-F1</td>
<td>ACATCCTGCAAGGTAAGGCAACAAACTCTAGGTCTGGTTATATAT</td>
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<td>IL18-R1</td>
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<tr>
<td>IL18-F2</td>
<td>CAAAACAAAGAAACTCAAACGTGCCGGGAGACAGCC</td>
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<td>CD59_PacI-R</td>
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<td>IL-15_SbfI-F1</td>
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<td>IL15-R1</td>
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<tr>
<td>IL15-CD59-F2</td>
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<td>GFP-F</td>
<td>acctcCCTGCAGGATAGTGAAGGAGCAAGGGGAGGAG</td>
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<tr>
<td>GFP-R</td>
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<tr>
<td>IL15_PacI-R</td>
<td>cgtgcTTAATTAAGAGGTTGATGAACATTGGCAGC</td>
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<td>IL18mtr_SbfI-F</td>
<td>acatcCCTGCAGGATGTACCTGGCAAGCTGTTAGAACCCTAAACTCT</td>
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<td>IL18_PacI-R</td>
<td>cgtgcTTAATTAATTACAGGTCTGGTTAGAACATTAGAAGGTTAGAAGCTG</td>
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<tr>
<td>Sequencing-F</td>
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<td>Sequencing-R</td>
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<td>CATTAACCAGGAGACATCAAGCC</td>
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<tr>
<td>RT-pIL-18-R</td>
<td>GCACAGAGATGGTTACTGCGAGA</td>
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CHAPTER III

The non-structural protein Nsp2TF of porcine reproductive and respiratory syndrome virus down-regulates the expression of Swine Leukocyte Antigen class I

Qian Cao, Sakthivel Subramaniam, Yan-Yan Ni, Dianjun Cao, and Xiang-Jin Meng*

Virology, April 2016; 491:115-124

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is arguably the most economically-important global swine pathogen which typically induces sub-optimal immune responses including a delayed induction and impaired effector function of cell-mediated immunity (CMI). Herein we first demonstrated that PRRSV VR2385 down-regulates SLA-I surface expression in three different relevant cell types: porcine alveolar macrophages, PK15-CD163 cells, and monocyte-derived dendritic cells, suggesting the virus-induced inhibition of SLA-I may be partly responsible for the impaired CMI responses against PRRSV. To identify the viral protein(s)
involved in SLA-I down-regulation, we cloned and expressed all 21 PRRSV structural and non-structural proteins, and subsequently identified that the nonstructural viral proteins Nsp1α and Nsp2TF, and structural protein GP3 significantly down-regulated SLA-I expression. Among these three proteins, Nsp2TF showed the greatest effect in this reduction. To further investigate the role of Nsp2TF in SLA-I down-regulation during virus infection, we generated a panel of mutant viruses in which the Nsp2TF protein synthesis was abolished, and found that the two mutants with disrupted -2 ribosomal frameshifting elements and additional stop codons in the TF domain were unable to down-regulate SLA class I surface expression compared to the wild-type virus infection. Collectively, the results from this study indicate a novel function of Nsp2TF in the negative modulation of SLA-I expression. The finding has important implication for future vaccine development, as disruption of the Nsp2TF’s ability to down-regulate SLA-I expression may improve the existing PRRSV vaccines towards a better CMI response against the virus.
IMPORTANCE

Porcine reproductive and respiratory syndrome virus (PRRSV) causes an economically-important global swine diseases. The current modified live-attenuated vaccines (MLVs) and field strains of the virus induce sub-optimal immune responses such as a delayed induction and impaired effector function of cell-mediated immunity, which hinders the vaccine efficacy. In this study, we identified for the first time a novel function of the newly-discovered nonstructural protein Nsp2TF of PRRSV in down-regulating SLA class I expression. Since viral antigen presentation through SLA class I is essential for activation of and recognition by T cells, the Nsp2TF may directly play a role in PRRSV-mediated immune modulation. Therefore, disruption of the Nsp2TF’s ability to modulate SLA-I expression will potentially offer a novel strategy to improve the existing PRRSV vaccines towards better CMI responses against the virus.
INTRODUCTION

Since its appearance in 1987, porcine reproductive and respiratory syndrome (PRRS) has arguably been the most economically-important global swine disease causing severe late-phase reproductive failure in sows and respiratory diseases in pigs of all ages, especially young pigs (1-4). Clinically it is often seen with complications due to secondary bacterial infections and/or co-infections with other viruses such as porcine circovirus type 2, porcine parvovirus, and swine influenza virus (5). The causative agent, PRRS virus (PRRSV), is an enveloped, positive-sense, single-stranded RNA virus that belongs to the family Arteriviridae in the order Nidovirales (6). Two genotypes of PRRSV, the European type (type 1) and North American type (type 2), were identified with striking genetic variations as well as differences in pathogenicity and antigenicity (7-10).

The genomic RNA of PRRSV, which is approximately 15 kb in size, is capped at 5’ end and polyadenylated at 3’ end, containing at least 10 open reading frames (ORF): ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3-5, ORF5a, and ORFs 6-7 (11-13). Approximately 75% of the viral genome at 5’ proximal region can translate into polyproteins 1a and 1ab, which are further cotranslationally processed into at least 14 non-structural proteins (Nsps) by auto-proteolytic cleavages (14, 15). These Nsps mainly function in viral replication and transcription, and some Nsps are also involved in host immune modulation. The Nsp2TF is a newly-discovered non-structural protein encoded within ORF1a and produced via a unique -2 programmed ribosomal shifting (-2 PRF) mechanism (16). The biological function(s) of the Nsp2TF are largely unknown. The 3’ portion of the viral genome contains at least 8 ORFs which encode structural proteins (SPs) including GP2a, E, GP3, GP4, GP5, GP5a, M and N, respectively, translated from a nested set of subgenomic mRNAs (10, 17).
PRRSV infection in pigs typically induces an impaired immune response in the host including a weak innate immunity (18), a deferred neutralizing antibody response (19, 20), and the gradual slow development of cell-mediated immune responses (21, 22). The same scenario also applies to the PRRSV modified live-attenuated vaccine (MLV)-induced immunity even though extensive research efforts have been devoted to the vaccine development against PRRSV (21). Current commercially available vaccines include both MLVs and inactivated vaccines with limited efficacies (23). Inactivated vaccines provide a weak protection, while MLVs are generally effective against homologous or closely-related strains but ineffective against heterologous strains. Antigenic variations and immune evasion of PRRSV are thought to be the major obstacles for developing a more efficacious vaccine against PRRSV (23, 24).

Although cell-mediated immunity (CMI) plays a critical role in the antiviral immunity against PRRSV, little is known about T cell and memory responses against PRRSV. It is frequently observed that PRRSV-specific IFNγ-secreting cells (comprising of classical CD4+CD8αβ+ effector T cells as well as CD4+CD8α+ double positive T cells) were not detected in pigs until 2-3 weeks either post-infection (wpi) or post-vaccination, and their frequency in peripheral blood increased slowly afterwards and remained at low levels (21, 22). It is reported that PRRSV infection reduced the surface expression of swine leukocyte antigen (SLA) class I in macrophages and dendritic cells (25, 26). SLA class I (swine MHC class I) molecules are expressed on the surface of all nucleated cells and present peptide fragments derived from the ‘housekeeping-protein’ of cells or from viral antigens in the context of virus infection. Moreover, SLA class I molecules are essential components of antigen presentation for the subsequent proliferation/differentiation of CD4+ and CD8+ T cells, as well as for the detection of virally infected cells by cytotoxic T cells (27). As a result, it is not surprising that many viruses, especially those establishing persistent or chronic
infections, utilized different strategies of interfering MHC class I pathway in order to evade the
host immune surveillance. In most cases, the viral proteins responsible for such immune evasion
have been identified and characterized, such as the adenovirus E3/19K, human cytomegalivirus
(HCMV, herpesvirus) US2 and US11, and human immunodeficiency virus Nef protein (28). Since
PRRSV also causes persistent infection (29), the inhibition of SLA molecules by PRRSV may be
partly responsible for the impaired CMI responses against the virus in pigs.

In this study, we first demonstrated that PRRSV induced down-regulation of SLA-I expression in three different cell types including a susceptible porcine kidney epithelial cell line stably expressing CD163 (PK15-CD163). Subsequently, we aimed to investigate the mechanism(s) and the specific viral protein(s) involved in the virus-induced down-regulation of SLA-I expression. We found that PRRSV Nsp1α, Nsp2TF and GP3 proteins significantly down-regulated the surface expression of SLA-I. Additionally, we indicated a novel function of PRRSV Nsp2TF in modulating SLA class I expression. The results from this study have important implications for future vaccine design towards a better CMI response.

**MATERIALS AND METHODS**

**Cells and viruses.** BHK-21 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Monkey kidney epithelial cell line, MARC-145 (30, 31), was cultured in low glucose-supplemented DMEM with 10% FBS, and maintained in low glucose DMEM (Invitrogen) with 2% FBS for virus propagation. The porcine kidney cell line (PK-15) that was stably transfected with PRRSV receptor molecule CD163 (PK15-CD163) was previously established in our lab, and the PK15-CD163 is susceptible to PPRSV infection. This cell line was cultured in Minimum Essential Medium (MEM)
supplemented with 0.2 % hygromycin, 10% FBS, non-essential amino acid (NEAA, Invitrogen, GibcoBRL) and sodium pyruvate (Invitrogen, GibcoBRL), and maintained in MEM with 5% FBS after virus infection. Primary porcine alveolar macrophages (PAMs) were prepared from lung lavage fluids of PRRSV-negative piglets as previously described (32)). Primary PAMs were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and incubated at 37°C with 5% CO2, or were cryopreserved in liquid nitrogen for later use.

For culturing monocyte-derived DCs (MoDCs), PBMCs were isolated from heparinized blood of PRRSV-negative piglets and seeded into a 10 mm dish for adherence of monocytes overnight. Non-adherent cells were washed out, and the monocytes were cultured with RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, recombinant porcine granulocyte–macrophage-colony-stimulating factor (GM-CSF) (5 ng/ml) and recombinant porcine interleukin (IL)-4 (2 ng/ml) (R&D Systems, Minneapolis, MN). Half of the culture media was replaced on third day, and cells were collected on the fifth day with cell dissociation buffer (ThermoFisher).

The PRRSV strain VR2385 used in this study was produced by transfection of BHK cells with the PRRSV VR2385 infectious cDNA clone plasmid (pIR-VR2385-CA) (33). The type 2 PRRSV VR2385 was originally isolated from a pig with PRRS in Iowa (10).

**Antibodies.** Antibodies recognizing PRRSV proteins used in this study included a monoclonal antibody SDOW17-A against PRRSV N protein (Mouse IgG1, Research Technology Innovation), a rabbit anti-VR2385 Nsp2TF polyclonal antibody specific to the carboxyl-terminal epitope CFLKVGVKASDLV (GenScript), a rabbit anti-VR2385 Nsp2 polyclonal antibody
specific to the carboxyl-terminal epitope CNGLKIRQISKPSGG (GenScript). For staining cellular surface SLA-I and Sodium/Potassium ATPase in the flowcytometry, an anti-porcine class I monoclonal antibody PT85A (mouse IgG2a) (Washington State University), PE anti-mouse IgG2a antibody (Biolegend), Sodium/Potassium ATPase alpha-1 antibody (mouse IgG1) (Fisher) and FITC anti-mouse IgG1 antibody (Biolegend) were used. APC-conjugated anti-DYKDDDDK Tag antibody (rat IgG2a) (Biolegend) were also used to detect flag-tagged proteins in the flowcytometry. Isotype control antibodies included FITC Mouse IgG1 κ Isotype Ctrl antibody, PE Rat IgG2a κ Isotype Ctrl antibody, and APC Rat IgG2a κ Isotype control (Biolegend). A rabbit polyclonal antibody against a flag tag DYKDDDDK was custom-made by GenScript and used in immunoprecipitation and western blot experiments.

Virus infection. PK15-CD163 cells seeded in 6-well plates reaching approximately 60-80% confluence were infected with PRRSV VR2385 at 2 multiplicity of infection (MOI) for 1 hour, then replaced with fresh medium followed by incubation at 37°C with 5% CO₂ for 60 hours. Mock-infected cells were incubated with medium only. For mutant virus infection, PK15-CD163 cells seeded in 12-well plates were similarly infected with PRRSV VR2385 or each mutant virus at 1 MOI for 1 hour, then replaced with fresh medium followed by incubation at 37°C with 5% CO₂ for 60 hours. PAMs and MoDCs were counted and seeded in 12-well plate and cultured overnight. On the next day, PAMs or MoDCs were infected with PRRSV VR2385 at 0.2 MOI for 1 hour and then replaced with fresh RPMI 1640 and incubated for 24 hour at 37°C with 5% CO₂.

Construction of expression vectors for all non-structural (nsp) and structural proteins (sp) of PRRSV VR2385. A panel of mammalian expression constructs encoding each of the non-
structural and structural PRRSV proteins were constructed by amplifying and cloning the corresponding cDNA sequence of each gene from the PRRSV infectious clone pIR-VR2385-CA into an CMV-driven expression vector pIHA-flag (Courtesy from Dr. Orsorio’s lab) (34). A total of 21 expression constructs including 14 non-structural proteins (Nsp1α, Nsp1β, Nsp2, Nsp2TF, Nsp3-5, Nsp7α, Nsp7β, Nsp8-12) and 7 structural proteins (GP2a, E, GP3, GP4, GP5, M, Npr) were constructed, each with a flag tag expression preceding the amino-terminus. All the 21 expression constructs were confirmed by sequencing and sequence analysis.

For cloning the Nsp2TF, primers containing two mutations in the frameshifting site (GGUUUUU to GGUGUUC) as well as two UU insertion (GGUUUUU to GGUGUUCUU) were used. Primers containing two stop codons at amino acid positions 102 and 105 in the TF domain were used for cloning the gene encoding a truncated Nsp2TF protein (Nsp2TF-truncated). Immediate stop codons at 1st and 3rd amino acid of the TF domain were introduced by the primers in order to generate Nsp2N. All the primers used in this study are listed in Table S1.

**Transfection of PK15-CD163 cells with the PRRSV gene expression constructs.** All the DNA constructs were prepared using the QIaprep Spin Miniprep or Maxiprep kit, and the DNAs were quantified using Nanodrop. The PK15-CD163 cells at approximately 60–80% confluence seeded in a 24-well plate were transfected with 0.35 µg of plasmid DNA in each well, together with Lipofectamine LTX and Plus Reagent (Invitrogen) according to manufacturer’s instruction, followed by incubation at 37°C with 5% CO2 for 18-20 hours.

**Flow cytometry.** The PK15-CD163 cells infected with PRRSV or mock infected with medium at 60 hpi were washed once with PBS, and then incubated with 400 µL TrypLE per well
at 37°C for at least 5 min, and subsequently the cells were collected by adding 1 mL 10% MEM. Cells were pelleted and suspended in FACS buffer (PBS containing 3% FBS, 4°C). After washes with FACS buffer twice, cells were dispensed into 96-well U-bottom plates (BD Falcon) in 100 μL volumes and then incubated with PT85A (1:100) for 30 min on ice, followed by three washes in FACS buffer and stained with PE anti-mouse IgG2a for 15 min. After three subsequent washes in FACS buffer, the cells were fixed by 3.2% paraformaldehyde for 10 min at room temperature, followed by washes and then permeablized in 0.2% saponin for 10 min at room temperature. The cells were then incubated with PRRSV anti-N monoclonal antibody SDOW17 (1:100) for 15 min, washed with FACS buffer with 0.2% saponin, and stained with FITC conjugated anti-Mouse IgG1. The fluorescence intensity was assessed with a BD FACSAria II. For protein over-expression, the PK15-CD163 cells were collected at 20 hours post-transfection and similarly treated as stated above. After permeablization, cells were incubated with APC-conjugated anti-DYKDDDDK flag antibody, and washed three times prior to subsequent analysis. For Sodium/Potassium ATPases surface staining, cells were incubated with the primary antibody Sodium / Potassium ATPase alpha-1 (1:60) and then secondary antibody FITC anti-mouse IgG1, and subsequently treated similarly with SLA class I stained cells.

**Construction of DNA-launched infectious clones of Nsp2TF-deficient mutant viruses.**

All the mutant viruses contained eight point mutations in the frameshifting site and downstream motif in order to disrupt the -2 programmed ribosome frameshifting (PRF) that is required for Nsp2TF translation. For VR2385ΔNsp2TF, two pairs of primers (TF-UP-F-BamH and TF-UP-R; TF-Dn-F and TF-Dn-R-FseI) containing the introduced point mutations were used to amplify two flanking fragments containing BamHI or FseI, respectively, from infectious clone pIR-VR2385-
After overlapping fusion PCR of these two amplified flanking fragments, the fusion product was then used to replace the corresponding region of the infectious clone pIR-VR2385-CA by BamHI and FseI restriction enzyme sites. For generation of ∆Nsp2TF-1UAG mutant which contained an additional stop codon at 105th aa in the TF domain, a similar overlapping fusion PCR with specific primer pairs (TF-UP-F-BamH and 105UAG-TF-UP-R; 105UAG-TF-Dn-F and TF-Dn-R-FseI) and subsequent replacement of the fusion product in VR2385∆Nsp2TF were used. Additionally, in order to generate ∆Nsp2TF-3UAGs which contained three stop codons at 3rd, 4th and 47th aa in the TF domain, three pairs of specific primers (TF-UP-F-BamH and 3UAGs-TF-UP-R1, 3UAGs-TF-Dn-F2 and 47UAG-R2, 47UAG-F3 and TF-Dn-R-FseI) were used to amplify three flanking fragments, followed by three-fragment fusion PCR and subsequent replacement in VR2385∆Nsp2TF. All the mutations were synonymous in ORF1a (0 frame) in order to keep the Nsp2 expression intact. The primer sequences are listed in Table S2.

**Rescue of mutant viruses.** Plasmid DNAs of DNA-launched infectious clones encoding the mutant viruses were prepared using the QIApr ep Spin Miniprep kit, and quantified using Nanodrop. Fresh BHK-21 cells seeded in a 6-well plate at approximately 60-80% confluence were transfected with 3 µg of each plasmid DNA per well using Lipotectamine LTX and Plus Reagent kit (Invitrogen) according to manufacturer’s instruction, followed by incubation at 37°C with 5% CO2. At 48 h post-transfection, cell culture supernatants were harvested and serially passaged onto MARC-145 cells. Transfected or infected cells were examined by indirect immunofluorescence assay (IFA) using PRRSV monoclonal antibody SDOW17 specific to the PRRSV N protein. To further verify the integrity of the introduced mutations, the RNAs of second and third-passage
viruses were extracted from supernatants using ZR Viral RNA Kit (Zymo Research), and then subjected to RT-PCR (superscript III one-step RT-PCR system) and DNA sequencing analysis.

**Virus growth kinetics assay.** To investigate the growth properties of the mutant viruses in MARC-145, a multiple-step growth curve analysis was conducted. Briefly, MARC-145 cells in 12-well plates were infected with each of the mutant viruses as well as parental viruses (passage P2) at a low MOI of 0.1. Starting from 24h to 156 h post-infection (hpi), approximately 150 µl of each cell culture supernatants were collected every 12 hours and subsequently replenished with the same volume of fresh culture medium. Infectious virus titrations were performed in 96-well plates with fresh MARC-145 cells that were inoculated with 10-fold serial virus dilutions (4 replicates per dilution, 100 µl/well) for 1 h, after which the cells were washed with PBS and then incubated with low glucose DMEM supplemented with 2% FBS in a humidified CO₂ incubator. Presence or absence of a cytopathic effect (CPE) was determined at 7 days post-inoculation. Viral infectious titers were calculated using the Reed-Muench method and expressed as the tissue culture infectious dose per milliliter (TCID₅₀/ml). Three independent experiments were carried out for each mutant virus.

**Immunoprecipitation and SDS-PAGE analysis.** Whole cell lysates from virus-infected cells were prepared with RIPA buffer (Pierce) and 100 x protease inhibitor (Life technologies), followed by high-speed centrifugation to remove unresolved pellets. Samples in 500µL volumes were incubated with 5µg specific capture antibodies (anti-Nsp2TF polyclonal antibody) overnight at 4°C with continuously mixing, followed by addition of 35 µL Protein A/G Mix Magnetic Beads (EMD Millipore) and further incubated at least for 2 hours at 4°C. Proteins of interest were
immunoprecipitated according to manufacturer’s instructions, and were then eluted by adding 100 µL 1 x Laemmli buffer (Biorad) with 2.5% β-mecaptoethanol and heating at 95°C for 10 min. Samples were centrifuged to remove unresolved portion and then 35µL of the samples were loaded and separated on a 10% SDS-PAGE gel (Fisher). For cells transfected with Nsp2TF DNA constructs, all the procedures were same as described above, except that the capture antibody was a rabbit polyclonal antibody against the flag tag.

**Western blot analysis.** After separation by SDS-PAGE gel, proteins were transfer onto a nitrocellulose membrane (LICOR biosciences) by a semi-dry blot system (Bio-Rad). The membrane was blocked with 5% non-fat milk in PBST for 30 min at room temperature, and then probed by Nsp2TF or Nsp2-specific antibodies. Rabbit polyclonal antibody against β-actin was also used as an internal control. IRDye 880CW-conjugated donkey anti-rabbit Ab (LICOR biosciences) was used as secondary antibody. The blot imaging were performed using the Odyssey infrared imaging system (LICOR biosciences).

**Indirect immunofluorescence assay (IFA).** The expression of Nsp2 and Nsp2TF in transfected cells was determined by an immunofluorescence assay (IFA). Briefly, cells transfected with Nsp2 or Nsp2TF constructs were fixed at 24 hour post-transfection with a 4% paraformaldehyde. Mock-transfected cells were included as negative controls. After an incubation period of 15 minutes at room temperature, cells were washed three times with PBS, followed by incubation at room temperature with PBS containing 1% Triton X-100 for 10 minutes. After removing the Triton X-100 solution, the cells were washed three times with a PBS-Tween 20 solution (PBS containing 0.02% Tween 20). Subsequently, cells were blocked in 1% BSA at 37°C
for 30 min, washed with PBS and then incubated with a polyclonal antibody against Nsp2TF or Nsp2 (1:1000) previously mentioned at 37°C for 1 h. After extensive washing with PBS, cells were incubated with Alexa fluor594-conjugated goat anti-rabbit IgG (1:1000) for 1 h at 37°C. After washing with PBS, fluorescent signals were visualized using an Olympus inverted fluorescence microscope fitted with a digital camera. To examine the recovery of mutant viruses, the transfected BHK (Passage 0) or infected MARC145 (Passage 1) cells were fixed at 48 h post-transfection or 72 h post-infection with 80% cold acetone for 10 minutes. After washing with PBS, the fixed cells were blocked in 1% BSA at 37°C for 30 min, washed with PBS and then incubated with anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies, Inc.) at 37°C for 1 h. After extensive washing with PBS, the cells were incubated with FITC or alexa fluor594-conjugated goat anti-mouse IgG for 45 minutes at 37°C. After washing with PBS, fluorescent signals were visualized as mentioned above.

**Statistical analysis.** The One-way ANOVA was used to evaluate the data. The data was analyzed by GraphPad Prism (version 5.0).

**RESULTS**

**PRRSV down-regulates SLA class I expression on cell surface.** It has been reported that PRRSV can down-regulate SLA class I molecules *in vitro* (25, 26). In this study, three different cell types were used to confirm this previous finding. The PK15-CD163 cells that stably express porcine CD163 were infected with a pathogenic strain of PRRSV VR2385 at 2 MOI and subsequently subjected to flow cytometry at 60 hours post infection (hpi). Both the intracellular viral N protein (Npr) and cell surface SLA class I molecules were stained (Fig. 1A, 1B) to ensure
that the comparison was between the infected and mock-infected cells. As summarized in Fig. 1C, PRRSV infection reduced the surface SLA class I expression on cells by at least 50%. In addition, porcine alveolar macrophages (PAMs) and monocyte-derived DCs (MoDCs) were also infected with PRRSV VR2385 at 0.2 MOI, and the SLA class I surface expression was similarly evaluated at 24 hpi. Consistently, SLA class I expression was found to be down-regulated by PRRSV infection both in PAMs (Fig. 1D) and MoDCs (Fig. 1E). These findings collectively demonstrated that PRRSV VR2385 down-regulate SLA class I expression in susceptible cells.

PRRSV nonstructural proteins Nsp1α and Nsp2TF and structural protein GP3 are responsible for down-regulating SLA class I expression. In order to determine the mechanisms of the virus-induced down-modulation of SLA class I, we first sought to identify the individual PRRSV protein(s) responsible for the observed down-regulation. First, we constructed a total of 21 mammalian expression constructs including all 14 PRRSV non-structural proteins (Nsps) and all 7 PRRSV structural proteins (SPs). The expressions of each of the 21 nonstructural and structural proteins of PRRSV VR2385 in the PK15-CD163 cells following transient transfection of each individual expression construct were demonstrated by either western blot or indirect immunofluorescence assay (IFA) with anti-flag monoclonal antibody (Fig. S1).

At 20 hr post-transfection with each individual expression construct, cells were subjected to flow cytometry with a dual-labeling analysis for simultaneous detection of both surface SLA class I and intracellular co-expressing flag-tagged individual viral protein. In each individual transfection, cells are divided into viral protein-expressing population, and non-expressing population (Fig. 2C), and the SLA class I expression intensities were compared between the viral protein-expressing population and a separate group transfected with the empty plasmid pIHA-flag
PRRSV nonstructural proteins Nsp1α and Nsp2TF as well as PRRSV structural protein GP3 were found to significantly reduce the surface expression of SLA class I molecules when compared to other viral proteins as well as the empty vector control (Fig. 2B). Additionally, we also evaluated the SLA class I expression in each individual transfection group by comparing between viral protein-expressing population and non-expressing population (Fig. 2C). Similarly, we found that the same three viral proteins were able to significantly reduce SLA class I surface expression, with Nsp2TF showing the greatest reduction to approximately 50% compared to cell only control (Fig. 2D).

To further determine whether these viral proteins worked synergistically in down-regulating SLA class I expression, PK15-CD163 cells were co-transfected with the expression constructs in different combinations (Fig. S2), and the SLA class I surface expression of each group was subsequently measured by flow cytometry as described above. Even though co-transfections of viral protein candidates generally reduce SLA class I surface expression, there was no significant difference among the co-transfection groups. The Nsp5 was also included in this experiment since it had a down-regulating effect on SLA class I expression to some extent but was not statistically significant. Single transfection with each individual Nsp1α, Nsp2TF or GP3 was used as positive controls, and transfection with GP5 construct was used as a negative control.

Furthermore, to exclude the possibility that the decreased SLA class I expression was due to a general suppression of cellular gene expression by PRRSV infection, we evaluated another surface protein, sodium-potassium (Na⁺-K⁺) ATPase, in a similar dual-color flow cytometry analysis. The results showed that none of the viral proteins, especially the three proteins that down-regulate SLA class I expression, had an inhibitory effect on the Na⁺-K⁺ ATPases (Fig. 2E). These results indicated that PRRSV proteins Nsp1α, Nsp2TF and GP3 suppressed SLA class I
presentation on the cell surface.

Among the three identified PRRSV proteins (Nsp1α, Nsp2TF and GP3), the Nsp2TF shows the greatest down-regulation of SLA class I expression on cells. Therefore, we decided to further explore the potential mechanism of Nsp2TF down-regulation of SLA class I expression.

**Loss of Nsp2TF expression by mutant PRRSVs.** The non-structural protein Nsp2TF was produced by PRRSV through a non-canonical -2 programmed ribosome frameshifting (PRF) mechanism in Nsp2 gene coding sequence of PRRSV (16). The sequence GGUUUUUU at the exact frame-shifting site and the downstream conserved motif CCCANCUCC were shown to be required for generating this -2 PRF product. A follow-up study (35) further demonstrated that PRRSV Nsp1β is also required for the efficient ribosome frameshifting as a trans-activator protein in Nsp2TF expression. In addition to -2 PRF, -1 PRF can also occur at the same site with a much lower efficiency, generating -1 PRF product Nsp2N. The frame-shifting site is located within ORF1a just upstream the sequence encoding the Nsp2 putative transmembrane domain. As a result, the Nsp2TF shares the same amino-terminal papain-like protease 2 (PLP2) domain and hypervariable region (HVR) with the Nsp2 while it has a distinct carboxyl-terminal domain referred to as TF domain due to -2 PRF (**Fig. 3A**).

To further determine that the Nsp2TF is indeed responsible for PRRSV-induced down-regulation of SLA class I presentation, we produced three mutant viruses in which the required nucleotide elements for -2 PRF were completely disrupted to abolish the Nsp2TF expression. The VR2385ΔNsp2TF mutant only contained eight point mutations in the frameshifting site and the downstream motif, whereas the other two mutants ΔNsp2TF-1UAG and ΔNsp2TF-3UAGs contained additional stop codons that were introduced in the TF domain by mutagenesis (105th and
3rd, 4th, 47th in the TF domain, respectively) in order to reinforce the Nsp2TF knock out (mutations are shaded in grey and stop codons are indicated as asterisks, Fig. 3B, 3C). All the mutations were synonymous with regards to ORF1a as 0 frame. The mutant viruses were successfully rescued as confirmed by IFA with an anti-PRRSV N monoclonal antibody in transfected BHK cells (Passage 0, P0) or the infected MARC 145 cells (Passage 1, P1) (Fig. S3), and their growth kinetics indicated that the replication of the three Nsp2TF-deficient mutants were impaired in MARC-145 cells when compared to wild-type (WT) virus (Fig. 4A).

The mutant viruses were subjected to immunoprecipitation to verify the lack of Nsp2TF expression. As expected, the -2 PRF product was not detected in the immuno-precipitated portions in all of the three mutant viruses-infected cell lysates using a specific polyclonal antibody against the carboxyl-terminal epitope of the Nsp2TF (Fig. 4B, left panel). Transfection of the flag-tagged Nsp2TF expression vector was included as a positive control. The unbound portions of immuno-precipitated cell lysates were analyzed by western blot with a specific polyclonal antibody against carboxyl-terminal epitope of the Nsp2, showing that all the mutant viruses produced Nsp2 (Fig. 4B, right panel).

**Disruption of Nsp2TF expression in PRRSV restores the surface SLA class I expression after virus infection.** To assess the effect of the mutant viruses lacking the Nsp2TF expression on SLA class I molecule expression, PK15-CD163 cells were infected each with the PRRSV mutants or WT PRRSV, and the surface SLA class I expression as well as intracellular viral N protein were assessed by flow cytometry analysis. The SLA class I expression was compared between the positively infected population in each infection group with the mock-infected cells (Fig. 4C), and the results were indicated as fold changes (Fig. 4D). Both mutants
∆Nsp2TF-1UAG- and ∆Nsp2TF-3UAGs-infected cells expressed similar levels of surface SLA class I molecules when compared to the mock infection, while both WT VR2385 and mutant VR2385∆Nsp2TF infection still significantly reduced SLA class I surface expression, indicating that silencing Nsp2TF in PRRSV restores the SLA class I expression on cells. These mutant viruses still maintained the introduced mutations after the 3rd passage in cells as determined RT-PCR, sequencing and sequence analysis (data not shown). These results indicated that Nsp2TF directly contributed to PRRSV-induced down-regulation of SLA class I expression.

**The TF domain in Nsp2TF is required for the downregulation of SLA class I molecules.**

To fine-map the region in Nsp2TF that is involved in the down-regulation of SLA class I molecule, we constructed a panel of Nsp2TF-related expression constructs for transient expression (Fig. 5A). The full-length Nsp2TF was generated as in-frame control which contains two mutations in the frameshifting site (GGUUUUU to GGUGUUC) as well as two UU insertion for the forced translation in 0 frame (GGUUUUU to GGUGUUCUU). The Nsp2TF-truncated construct did not contain the last 68 amino acids as a result of synonymous mutations of 102th and 105th codons into stop codons in carboxyl-terminal TF region (Fig. 5A, mutations in bold and underlined). The Nsp2N construct does not contain the whole TF region therefore representing -1 PRF product. The full-length Nsp2 and Nsp2TF were detected both by IFA (Fig. 5B) and immunoprecipitation with corresponding polyclonal antibodies specific to the carboxyl-terminal epitopes (114kD and 100kD, respectively) (Fig. 5C). Other truncated Nsp2TF proteins were also verified by western blot with anti-flag polyclonal antibody.

The effects of these Nsp2TF-related proteins on the surface expression of SLA class I molecules were evaluated. As expected, the Nsp2TF down-regulated the SLA class I presentation
while the Nsp2 did not (Fig. 5D). Both Nsp2TF-truncated and Nsp2N lost the ability to down-regulate the SLA class I expression, indicating that the TF domain in Nsp2TF, especially the last 68 amino acids, is critically important for downregulating the surface expression of SLA class I.

**DISCUSSION**

PRRSV has a rather restrictive cellular tropism for porcine alveolar macrophages, alveolar septal macrophages, and several subsets of DCs including MoDCs expressing porcine sialoadhesin (pSn), heparan sulfate and CD163. Consistent with previous studies (25, 26), in this study we demonstrated that a highly pneumo-virulent strain of PRRSV VR2385 down-regulates SLA class I surface expression in PAMs (as an example of natural target cells of PRRSV), PK15-CD163 cells (as an example of peripheral tissue cells), and MoDCs (as antigen-presenting cells, APCs). Given the pivotal role of MHC class I molecules in bridging the innate and adaptive immunity (36), inhibition of MHC class I on APCs could hinder the initial priming/generation of PRRSV-specific CD8+ T cells, thus ultimately leading to inadequate numbers of activated T cells. Also, since the effector CTLs destroy virally-infected cells by recognizing surface MHC class I molecules that are complexed with viral peptides, the inhibition of MHC class I on infected tissue cells may affect the effector functions of CD8+ T cells (28). This would result in a failure in elimination and clearance of replicating viruses, and is often employed as a strategy for immune evasion by many viruses that cause persistent infections.

Many potential mechanisms have been proposed to explain how PRRSV employs various strategies to hinder the induction of immune responses and/or evade the effector functions thereafter (37). The negative effects on MHC molecules (SLA) as well as co-stimulatory molecules (38), synergistically with the low level induction of early pro-inflammatory cytokines such as IL-
12 and TNF-α(39, 40), will probably lead to the inadequate activation of CD4+ and CD8+ T cells. Additionally, it was suggested that even after CTLs were activated by homing DCs and then migrated to the site of virus infection, they still have problems in eliminating PRRSV-infected PAMs. This is likely because the infected macrophages fail to present PRRSV peptides on the plasma membrane, which cannot be recognized by virus-specific effector T cells. Consistent with this notion, a previous study (41) showed that re-stimulated PBMCs derived from PRRSV-infected pigs elicited non-detectable CTL activities and lack of IFN-γ production until 49 dpi, despite moderate proliferation of CD3+CD8high cells. In the present study, the observation that PRRSV down-regulated SLA class I expression provides possible explanations: PRRSV-infected macrophages are resistant to CTL-mediated lysis, or/and PRRSV-specific CTLs are functionally impaired.

PRRSV can modulate the expression of innate cytokines at early stage of infection such as by inhibiting IFN responses (18, 42-45), suppressing TNF-α (46), and/or upregulating IL-10 (47), based on in vivo studies of peripheral blood and bronchoalveolar lavage cells (BALCs) (48) as well as in vitro studies of PAMs, MoDCs, and bone marrow-derived DCs (BMDCs). These effects were further demonstrated to be largely resulted from the intervention of cellular mediators along the TLRs and RLRs signaling pathways as well as interferon response pathways (49, 50) by PRRSV proteins, such as nsp1α, 1β, 2 and 11 as well as N (34, 51-55). However, there was no study to identify the viral protein(s) that modulate SLA presentation. For the first time, in the present study we identified three viral proteins, Nsp1α, Nsp2TF and GP3, that were responsible for the virus-induced down-regulation of SLA class I expression.

We demonstrated that the newly-discovered non-structural protein Nsp2TF has the greatest effect on down-regulating SLA class I, whereas Nsp2 does not down-regulate SLA class I. Nsp2
is the largest proteinase released by auto-proteolytic cleavage of polyprotein 1a, and recently reported as a novel structural component of PRRSV particle (56). Compared to Nsp2, the novel Nsp2TF protein has been shown to likely locate in different subcellular compartments, and plays an important role in virus replication. The results from our present study revealed a novel function of Nsp2TF in down-regulating SLA class I expression. Subsequently, we further demonstrated that the last 68 amino acids in Nsp2TF are critically important for the function of Nsp2TF in down-regulating SLA class I. As demonstrated in this study, other viral proteins such as Nsp1α and GP3 may also down-regulate SLA class I expression, and it is also possible that some unknown factor(s) other than viral proteins, such as RNA intermediates and miRNA, may exert effects on the modulation of SLA class I expression. Therefore, future studies are warranted to investigate the role of other factors in down-regulating SLA class I expression and the effect on virus life cycle.

To validate the novel function of Nsp2TF in down-regulating SLA class I expression, we constructed three mutant viruses that do not express Nsp2TF by using PRRSV reverse genetics system. These Nsp2TF-deficient mutant viruses are still viable and infectious but have a lower growth rate, due to the lack of the Nsp2TF protein and the abolishment of -2/-1 PRF per se. In addition to the first mutant VR2385ΔNsp2TF with disrupted frameshifting elements, we generated the latter two mutants with stop codons introduced by mutagenesis in the TF domain, with the aim to reinforce the knockout of the Nsp2TF protein. We showed that infection of cells with the latter two mutants abolished the -2 PRF product, and more importantly restored the SLA class I surface expression in the infected cells. It is possible that the lower replicative property, in addition to the knockout of Nsp2TF expression of the mutant viruses, may also contribute to the restoration of SLA class I expression. However, the mutant VR2385ΔNsp2TF with only the disruption in the shifting elements still possesses the ability to down-regulate SLA class I expression while
displaying comparable replication efficiency with other two mutants containing additional stop
codons in the TF domain. The latter two mutant, by contrast, completely lost their effects on down-
regulating SLA class I. Therefore, based on the results from this study, a more logical explanation
is that PRRSV largely relies on Nsp2TF expression for the negative modulation of SLA class I
surface expression. The fact that even though Nsp1α and GP3 were found to be the other two
negative modulators as well, the knockout of Nsp2TF alone in the virus can fully restore SLA class
I expression, further emphasized the essential role of Nsp2TF in PRRSV-induced downregulation
of SLA class I expression and perhaps in antagonizing antigen presentation. Clearly, additional
studies, which are beyond the scope of the present study, are warranted to further delineate which
intermediate step/subcellular compartment(s) that the Nsp2TF is involved in the MHC class I
assembling pathway.

The PRRSV vaccination program has not been satisfactory, largely due to an impaired
immunity induced by vaccination with MLV as well as by infection of virulent field strains (21,
57), and the problem is further exacerbated by the heterogeneous nature of the constantly-evolving
field strains. Therefore, novel strategies in developing improved vaccines against PRRSV are
needed. The results from this study suggest that it is of value to generate candidate vaccine strains
of PRRSV with altered Nsp2TF expression to enhance immune activation of the virus. Therefore,
the results from this study not only for the first time uncovered an immune-modulatory function
of the PRRSV Nsp2TF in downregulating SLA class I expression, but also provided a promising
approach for improving the existing vaccines towards a better CMI response against PRRSV.
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REFERENCES


23. **Huang YW, Meng XJ.** 2010. Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res **154**:141-149.


27. **Schmidt J, Dojcinovic D, Guillaume P, Luescher I.** 2013. Analysis, Isolation, and Activation of Antigen-Specific CD4(+) and CD8(+) T Cells by Soluble MHC-Peptide Complexes. Front Immunol **4**:218.


Fig. 1. PRRSV down-regulates the surface expression of swine leukocyte antigen class I (SLA-I) in PK15-CD163 cells. PRRSV VR2385-infected (solid line) and mock-infected (dashed line) PK15-CD163 cells were stained with mAbs against intracellular PRRSV N protein (A) as well as the surface SLA class I (B) at 60 hours post-infection (hpi) and analyzed by flow cytometry. (C). A fold change in median fluorescence intensity (MFI) of SLA class I expression was generated by comparing PRRSV VR2385-infected with mock-infected cells. Asterisks indicate statistically significant differences between two groups (*P < 0.05, **P<0.01, ***P<0.001). Error bars represent standard error of the mean. Data were representative of at least three independent experiments. Porcine alveolar macrophages (PAMs) (D) and monocyte-derived dendritic cells (MoDCs) (E) were infected with PRRSV VR2385 (solid line) at 0.2 MOI or mock-infected with medium only (dashed line), and their SLA-class I MFIs were analyzed by flow cytometry at 24 hpi, and the numbers in the FACS panels indicated the MFI of SLA class I.
Fig. 2. Effect of PRRSV structural and non-structural proteins on SLA class I expression in PK15-CD163 cells. The PK15-CD163 cells transfected with each of the 21 PRRSV structural and non-structural protein expression vectors were stained for surface SLA-I expression as well as the intracellular flag-tagged viral proteins, and subsequently analyzed by two-color flow cytometry. Cells were transfected with the empty vector (pIHA-flag) separately, and dual-labeling analysis was shown in (A). The flow cytometry data were analyzed in two ways. First, the MFI of SLA class I was compared between the cells expressing the viral protein in each individual transfection group and the cells transfected with the empty vector (pIHA-flag), in order to generate a fold change in SLA class I for each individual transfection (B). Secondly, as shown in (C), the MFI of SLA-I was also compared between the cells expressing viral protein and the cells not expressing the viral protein in the same transfection group. Similarly, a fold change in SLA class I for each transfection was generated (D). (E). PK15-CD163 cells were transfected with individual viral protein expression vectors and the surface expression of sodium-potassium ATPase in each transfection treatment group was shown as fold change. Asterisks indicated a statistically significant down-regulation of SLA class I expression by the cells expressing the specific exogenous viral proteins (*P < 0.05, **P<0.01, ***P<0.001). Data are representative of at least three independent experiments.
Fig. 3. Schematic diagrams of the construction of the Nsp2TF-deficient PRRSV mutants in the vicinity of the -2 programmed frameshifting (PRF) sites and trans-frame (TF) ORF. (A). Nsp2TF shares the same PLP2 and HVR domains with Nsp2 but differs in carboxyl-terminal TF region using -2 PRF at the site indicated by the arrow. (B). Nucleotide sequences in the vicinity of the -2 frameshifting site of wildtype (WT) PRRSV VR2385 and three different mutant viruses. Highlighted in grey are point mutations introduced into the DNA-launched infectious clone of WT PRRSV VR2385 in order to either abolish the -2 PRF or additionally to incorporate stop codons for the generation of Nsp2TF-deficient mutant viruses. (C). Translation of the corresponding amino acids in the original (ORF1a) and -2 (TF) reading frames. Carboxyl-terminal amino acids of Nsp2TF as the -2 PRF product are underlined with the corresponding codon positions indicated by numbers at the bottom. The asterisks indicate the introduced stop codons in the mutants which are synonymous with the original ORF1a.
Fig. 4. The Nsp2TF-deficient mutant viruses lost the function of down-regulating SLA-I. (A). Growth kinetics of WT PRRSV VR2385 and three mutant viruses in MARC-145 cells. (B). Immunoprecipitation analysis of Nsp2TF expression in the mutant viruses. MARC-145 cells were infected with WT or each of the three mutants or mock-infected with medium only. At 36 hpi, proteins from cell lysates were immuno-precipitated with polyclonal antibodies specific to the carboxyl-terminal epitope of the Nsp2TF and then probed with the same polyclonal antibody by Western blot (left panel). Nsp2TF transient expression by plasmid transfection was used as a positive control, which were immuno-precipitated with a polyclonal antibody against the flag tag and then probed with polyclonal antibodies specific to the Nsp2TF. The unbound portions of virus-infected or mock-infected samples were separated by SDS-PAGE and probed with polyclonal antibody specific to the carboxyl-terminal epitope of the Nsp2 or β-actin (right panel). The expected sizes of Nsp2TF and Nsp2 were indicated by numbers and arrows. (C). PK15-CD163 cells infected with WT PRRSV VR2385 (solid line) or each of mutant viruses (dotted line) or mock infected (dashed line) were stained for both intracellular PRRSV N proteins (Npr) and surface SLA class I. Fold changes in MFI of SLA class I between the cells positively infected by viruses and mock-infected cells were compared (D). Asterisks represented significant differences compared to the mock-infected group (*P < 0.05, **P<0.01, ***P<0.001). Three independent experiments were performed.
Fig. 5

A

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</table>

B

Mock

C

D

SLA class I

Relative level over EV (Flag+/ EV)

IP: α-flag
IB: α-flag

M  Nsp2  Mock  Nsp2TF-Trunc  Nsp2N

β-actin 37 kDa

114kDa  100kDa  88 kDa

114 kDa  88 kDa  77 kDa

** ** ** **
Fig. 5. Effects of Nsp2TF-related proteins on the surface expression of SLA class I. (A). A schematic view of construction of the Nsp2TF-related expression plasmids (pIHA-flag). Nsp2TF was constructed as an in-frame control with point mutations introduced in order to disrupt the -2 PRF and with the insertion of two additional nucleotides UU after the frameshifting site, aiming to mimic the -2 PRF product. The Nsp2TF-truncated protein was constructed by mutating 102th and 105th codons in the TF ORF into stop codons (in bold and underlined). Two stop codons were introduced directly after the frameshifting site in order to construct pIHA-Nsp2N. (B). PK15-CD163 cells were transfected with Nsp2 or Nsp2TF plasmids and immuno-stained after 36 hr post-transfection with polyclonal antibodies specific to either Nsp2 or Nsp2TF. The top panels were mock-transfected cells with lipofectamine reagents whereas the bottom panels were cells transfected either with Nsp2 or Nsp2TF. (C). Western blot analysis of Nsp2TF-related products by transient expression in PK15-CD163 cells (left panel). Proteins were immuno-precipitated with polyclonal antibodies specific to the flag tag, and probed with the same polyclonal antibody by Western blot analysis (middle panel). Mock lanes were from cells transfected with lipofectamine reagents only. The unbound portions of the Nsp2 and Nsp2TF-transfected samples were separated by SDS-PAGE and probed with the same polyclonal antibody by Western blot (right panel). The expected sizes of proteins were indicated by numbers and arrows. (D). PK15-CD163 cells transfected with individual viral protein expression vectors were stained for both surface SLA class I and co-expressing intracellular viral proteins and subsequently analyzed by two-color flow cytometry. The MFI of SLA-I was compared between the cells expressing the individual viral protein and those separately transfected with the empty vector pIHA-flag, in order to generate a fold change in SLA class I intensity for each individual transfection. Asterisks indicate statistically significant differences. Data represented at least three independent experiments.
Fig. S1. Flow cytometry gating strategy after transfection. (A) PK15-CD163 cells were gated for single cells, from which they were gated into flag-positive and flag-negative populations (B) and consistent through all the groups. The flag-negative population was gated according to empty vector transfected group (C). Representative pictures were shown.
Fig. S2. SLA-I expression after PRRSV VR2385 infection. (A) PK15-CD163 cells were gated for single cells as mentioned in Fig. S1, and then were gated into Npr-positive and Npr-negative populations shown in (A). The Npr-negative population was gated according to mock-infected group as shown in (B). SLA-I intensities were compared among Npr-positive and Npr-negative populations of PRRSV VR2385-infected group, as well as the mock-infected group. Representative pictures were shown.
Fig. S3. Confirmation of protein expression of PRRSV structural and non-structural protein expression constructs in PK15-CD163 cells. The PK15-CD163 cells were transfected with individual protein expression constructs, and stained with an anti-Flag antibody, harvested for SDS-PAGE and probed with an anti-Flag antibody in the subsequent Western Blot analysis (A) indicated as *, and the expected sizes were shown at bottom, or IFA (B) at 48h post-transfection with a polyclonal antibody against flag tag. Nsp2TF can be found in Fig. 4 and Fig. 5. Only representative protein expressions are shown here.
Fig. S4. The three viral proteins that are shown to individually down-regulate SLA-I expression do not have synergistic effects in combinations. PK15-CD163 cells were co-transfected with the viral protein expression plasmids in different combinations. SLA class I expression was analyzed as described in Fig. 2 by flow cytometry. At least three independent experiments are indicated.
**Fig. S5**

**Rescue and in-vitro passage of WT-PRRSV VR2385 virus and Nsp2TF-deficient mutant viruses.** BHK-21 cells were transfected with each DNA-launched clone (left panel), and stained with an anti-PRRSV N antibody by IFA at 48 h post-transfection. IFA confirmation (right panel) of the rescue of viruses from MARC-145 cells infected with the supernatants of transfected BHK-21 cells (left panel). There was no fluorescent signal in the BHK-21 cells transfected with lipofectamine reagents or MARC-145 cells infected with the supernatant of mock-transfected BHK-21 cells (bottom panel).
Table S1.

Primers used for construction of PRRSV Nsps, SPs, and Nsp2TF-truncated proteins.

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**Notes:**

a F denotes a forward PCR primer; R denotes a reverse PCR primer.

b Mutations in the frameshifting site and 2 nucleotides insertion are shown in bold and italic for constructing Nsp2TF. Stop codons introduced by PCR mutagenesis are shown in bold for constructing Nsp2TF-truncated and Nsp2N.

c pIHA-SeqF and –SeqR are used for DNA sequencing.
Table S2.

**Primers used for construction and detection of PRRSV mutant viruses.**

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\(^a\) F denotes a forward PCR primer; R denotes a reverse PCR primer.

\(^b\) Numbers refer to nucleotide positions within the genome of pIR-VR2385-CA (VR2385 Genbank accession No. JX044140).

\(^c\) Mutations introduced to disrupt the frameshifting elements and to generate stop codons are shown in bold.

\(^d\) Detect-F was used for DNA sequencing analysis.
CHAPTER IV

GENERAL DISCUSSION

As mentioned throughout the dissertation research, there are substantial barriers for developing an effective PRRSV vaccine that can confer a broad cross-protection including the genetic, pathogenic and antigenic heterogeneity of circulating PRRSV strains, and an impaired and delayed immune response induced by virus exposure (1-3). When new approaches are being investigated in PRRSV vaccine development, it is of great importance to devote efforts to increasing the antigenic diversities in the vaccine, as well as enhancing the immunogenicity with better effector and/or recall immune responses.

The first objective of the dissertation research was to evaluate the immunogenicity of membrane-bound cytokines IL-15 or IL-18 expressed as adjuvants by the recombinant PRRSV MLVs. Both cytokines are successfully expressed onto the cell membrane of porcine alveolar macrophage (PAMs) after infection with the recombinant MLVs in vitro, also expressed in the vaccinated pigs as demonstrated by the increased cytokine expression level in the lung tissues. These results suggested the feasibility of this novel strategy to express cytokines as vaccine adjuvants by utilizing the discontinuous sgRNA synthesis mechanism of PRRSV. In vivo data also demonstrated an improved antiviral response of NK cells and T cells in IFN-γ secretion and/or degranulation against heterologous PRRSV challenge in pigs, indicating that the cytokine-expressing recombinant PRRSV MLVs can serve as potential vaccines with an improved immunogenicity. More importantly, this novel strategy would eliminate the need, and the cost for having to administrate soluble adjuvants along with the vaccines, and by maintaining the cytokine
adjuvants highly localized on cell surface. Local immune cascades are expected to be augmented and excessive systemic inflammation are avoided. As suggested in the study, the cytokines expressed by recombinant PRRSV MLVs on the membrane of infected cells are more likely to stimulate immune cells and facilitate cytotoxic lysis due to its proximity to viral antigen presentation on the same cell. Further evaluations on the adjuvant activities of other appropriate cytokines/chemokines/co-stimulatory molecules are warranted, and the co-expression of two bioactive molecules as “fusokine” are also worth of investigation, as it has been shown to be more effective as adjuvant than a single cytokine (4-8).

One of the concerns in the attempt to modify PRRSV with incorporation of cytokine genes is that the efficiency of cytokine expression by the vaccine virus is generally uncontrollable especially if the recombinant vaccine virus is inoculated into animals, even though the cytokines have been shown to be bioactive and immuno-modulatory in the animal study. Similarly, how effective the cytokines are expressed in the membrane-bound form is inaccessible in vivo, either. On the other hand, due to the constantly-evolving nature of RNA viruses such as PRRSV, the genetic stability of these recombinant MLVs carrying cytokine genes is also of concern if they are being used as commercial vaccines. The same challenge remains when it comes to other investigations in which chimeric viruses contained shuffled envelope genes with improved cross-protection against heterologous infections (9-11). Like most RNA viruses carrying a RNA-dependent RNA polymerase of low fidelity, the selection pressure from the environment for a more fitted virus without editing or modification also contributes to this matter.

On the positive side, despite the various strategies of immune evasion harnessed by PRRSV, it is suggested that once the acquired immune response is properly mounted and shaped in a certain direction (i.e. Th1 response, humoral response, Th2 response, etc.), the memory T or
B cells with specific TCR or BCR repertoires will be activated upon re-infection to combat the invading PRRSVs. Given the fact that the natural elimination of primary PRRSV infection in pigs can be achieved after herd closure (3), we expect that the porcine immune system is able to eventually eradicate PRRSV infection in the long run (3). Therefore it is of great value to develop recombinant MLVs with enhanced immunogenicity and a robust memory response against PRRSV.

PRRSV evolved various mechanisms to either escape from the immune surveillance, or to facilitate its replication in the host. Understanding the viral mechanisms modulating the immune response is an important key to the rational design of an effective PRRSV vaccine. Therefore, another major objective of the dissertation research is to understand the mechanism of immune modulation by PRRSV proteins. In this dissertation research, we identified for the first time a novel function of the newly-discovered Nsp2TF of PRRSV in down-regulating SLA class I expression. Since viral antigen presentation through SLA class I is essential for activation of and antigen recognition by T cells, Nsp2TF may directly play a role in PRRSV-mediated immune modulation.

The finding has important implications for future vaccine development, as disruption of the Nsp2TF’s ability to down-regulate SLA-I expression may improve the existing PRRSV vaccines towards a better CMI response against the virus. For example, a study (12) aiming to impair the function of Nsp1β as a -2 PRF trans-activator thus abolishing Nsp2TF expression in the virus demonstrated that the mutant virus induced increased IFN-α production in vivo, consistently with increased IFN-γ production and enhanced NK cell function. The finding underscores the significance of disrupting -2 PRF as a potential novel strategy to improve the existing PRRSV vaccines towards better immune responses against the virus.
To sum up, the major barriers of PRRS MLV development include the extensive antigenic heterogeneity among circulating strains and the newly emerged strains, as well as the impaired immune response usually induced by either vaccine or field strains. We think one of the solutions is to enhance immune response of MLV, as this dissertation research has presented. We should also put equal emphasis on increasing the antigenic diversities of the MLV strain, possibly by incorporating T cell epitopes (conserved and/or divergent) and neutralizing epitopes from genetically divergent strains. Last but not least, another challenge in the PRRS vaccine development is the instability of genetic modifications that are introduced during the investigation of recombinant MLVs. In light of this, it is also of great importance to devote efforts to increasing the fidelity of PRRSV RdRP of the vaccine strain.
REFERENCE


