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SUPPRESSION OF NK CELL-MEDIATED CYTOTOXICITY AND IMMUNOENHANCEMENT OF CD40 LIGAND AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
Ad	Adenovirus
ADCC	antibody-dependent, cell-mediated cytotoxicity
ADCML	antibody-dependent, complement-mediated cytotoxicity
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
BCR	B-lymphocyte receptor
bmDC	bone-marrow-derived dendritic cell
C-terminal	carboxy-terminal
CD	cluster of differentiation
CHO	Chinese hamster ovary
CMI	cell-mediated immune
CLs	cytotoxic lymphocytes
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
DABCO	1,4-diazobicyclo-2.2.2-octane
DMEM	Dulbecco's Modified Eagle Medium
dpi	days post inoculation
ds	double-stranded
E	small envelope protein
E. coli	Escherichia coli
EAV	equine arteritis virus
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
EU-type	European-type

FACS	Flow cytometry
FCS	fetal calf serum
FITC	fluroscein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony stimulating factor
GP	glycoprotein
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HEK-293A	human embryo kidney-293A cell line
HIV	human immunodeficiency virus
hpi	hours post inoculation
Hsp	heat shock protein
IFA	indirect immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	interleukin
IPMA	immunoperoxidase monolayer assay
kb	kilobases
kDa	kilodalton
KV	killed virus
lDC	lung dendritic cell
LDV	lactate-dehydrogenase elevating virus
LV	Lelystad virus
M	membrane protein
M1	classically activated macrophage
M2	alternatively activated macrophage
mAb	monoclonal antibody

MFI	median fluorescence intensity
MHC	major histocompatibility complex
MLV	modified live virus
moDC	monocyte-derived dendritic cell
m.o.i	multiplicity of infection
N	capsid protein
NK	natural killer
N-glycosylation	asparagine-linked glycan
nsp	non-structural protein
N- terminal	amino-terminal
NA-type	North-American-type
OD450	optical densitiy at 450 nm
ORF	open reading frame
PAM	porcine alveolar macrophages
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
pi	post inoculation
PRRSV	porcine reproductive and respiratory syndrome virus
PrV	pseudorabies virus
RFS	ribosomal frameshift
RIG-I	retinoic acid-inducable protein I
RPMI	roswell park memorial institute medium
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sg mRNA	subgenomic-length mRNA
SHFV	simian hemorrhagic fever virus
Sn	sialoadhesin

ss	single-stranded
SSC	side scatter
TCID50	tissue culture infectious dose with a 50 % end point
TGEV	transmissible gastroenteritis virus
Th	T-helper
TNF	tumor necrosis factor
UV	ultraviolet
WB	western blot

CHAPTER 1

INTRODUCTION

1.1 Porcine reproductive and respiratory syndrome virus

1.1.1 History

Porcine reproductive and respiratory syndrome (PRRS) first emerged in pig herds in the United States in 1987, and was found in Germany 3 years later (Hill, 1990; Keffaber, 1989). It is characterized by reproductive problems in sows including late-term abortions, premature farrowing and increased number of mummified, weak-born piglets, as well as respiratory problems in piglets such as pneumonia and atrophic rhinitis.

Despite several reports describing a number of different agents as the cause of this disease at that time, the causative agent remained unknown leading to the name “mystery swine disease” in the U.S. and either “porcine epidemic abortion and respiratory syndrome” or “blue-ear pig disease” in Europe. Researchers at the Central Veterinary Institute (Lelystad, The Netherlands) were the first group to fulfill Koch’s postulates describing a small, enveloped RNA virus (Lelystad Virus) as the causative agent (Terpstra et al., 1991; Wensvoort et al., 1991). A similar virus (VR-2332) was isolated in the U.S. at the same time (Benfield et al., 1992; Collins et al., 1992) and one-year later in Canada (Dea et al., 1992). Since the first internationally PRRS symposium (May 17-19, 1992, St. Paul, Minnesota), the internationally accepted name to designate the disease is porcine reproductive and respiratory syndrome (PRRS) and the virus is named PRRS virus (PRRSV).

At the beginning of the PRRSV outbreaks, the European genotype was restricted to Europe, and the American genotype was restricted to America (Andreyev et al., 1997) and Asia (Shibata et al., 1996). However, the North American genotype was introduced in Europe due to reversion to virulence of a VR-2332-derived vaccine strain (Botner et al., 1997; van Vugt et al., 2001) and in 2000, a European type PRRSV was isolated from Canadian pigs (Dewey et al., 2000). Consequently, both

genotypes now co-occur in each continent (Ropp et al., 2004). PRRSV appears to be continuously evolving (Chang et al., 2002; Goldberg et al., 2003; Rowland et al., 1999b), which results in the emergence of new PRRSV variants. In 2006, most swine farms in China experienced a “high fever disease of swine” caused by new PRRSV variants (Li et al., 2007; Tian et al., 2007; Zhou et al., 2008). This disease has brought great losses to the Chinese swine production in recent years. It has become an intractable problem for the development of pig industry not only in China but also in the global swine production.

1.1.2 Taxonomy and genetic diversity

The etiological agent of PRRS is an enveloped, single stranded, positive sense RNA virus with properties similar to the *Arteriviridae* family and placed with the *Coronaviridae* in the order *Nidovirales*, based on similarities in genome organization and expression strategy (Cavanagh, 1997).

The order name ***Nidovirales*** is derived from “*nidus*”, Latin for “nest”, and refers to the nested set of 3’co-terminal subgenomic-length mRNAs that is generated for expression of the open reading frames (ORFs) downstream of the replicase gene (Faaberg, 2008; Snijder and Meulenberg, 1998). Based on phylogenetic analysis of the viral RNA-dependent RNA polymerases (RdRps), current nidovirus classification recognizes three distinct families: the *Arteriviridae* (genus *Arterivirus*), the *Coronaviridae* (genus *Coronavirus* and *Torovirus*) and the *Roniviridae* (genus *Okavirus*) (Siddell and Snijder, 2008).

Arteriviruses are small, enveloped, animal viruses with an icosahedral core containing a positive-sense RNA genome, which include PRRSV, lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and equine arteritis virus (EAV). It possess several novel properties related to viral pathogenesis, including cytopathic replication in macrophages, the capacity to establish a persistent infection, as well as cause severe disease. As a group, the

arteriviruses represent the absolute extremes in mammalian pathogenesis. For example, SHFV is nearly 100% fatal in Asian monkeys . In contrast, LDV rapidly reaches levels close to 10^{10} virions per ml in the blood with no apparent clinical signs in mice.

Based on nucleotide sequence comparison as well as biologically distinct, PRRSV is divided into two genotypes: represented by Lelystad virus (LV) of the **European genotype** (EU type or type 1**)** (Wensvoort et al., 1991) and VR-2332 of the **North American genotype** (NA type or type 2) (Benfield et al., 1992; Collins et al., 1992). Even though type 1 and type 2 viruses appeared simultaneously and produce similar clinical signs, the two groups share only about 70% identity at the nucleotide level (Allende et al., 2000; Nelsen et al., 1999; Wootton et al., 2000). Nowadays, both genotypes circulate globally (Kimman et al., 2009; Zimmerman et al., 2006).

Early studies showed that the LV and VR-2332 strains of PRRSV differ by approximately 40% at the genomic level and are also antigenically distinct (Meulenberg et al., 1993; Nelsen et al., 1999; Nelson et al., 1993). Strains within each genotype also vary considerably with sequence differences as high as 20% (Han et al., 2007; Meng, 2000a). EU type appeared to change slowly, but showed greater genetic diversity (~30% maximum difference in pairwise comparisons) than NA type (~21% maximum) that periodically gave rise to more virulent forms (Murtaugh et al., 2010). The highly pathogenic PRRSV emerged in China in 2006 is a NA type virus, which was introduced from North America and followed by local diversification leading to increased virulence (An et al., 2010; Hu et al., 2009; Shi et al., 2010; Zhou et al., 2009a; Zhou and Yang, 2010).

1.1.3 Virion morphology

Briefly, PRRSV virions are spherical or ovallike particles (50-60 nm) that consist of a nucleocapsid core of about 40 nm, surrounded by a lipid bilayer envelope (Dokland, 2010). By cryo-EM, PRRSV virions appear as round or egg-shaped

particles ranging in diameter from 50 nm to 74 nm (Figure 1a.). The virions contain an internal core with an average diameter of about 40 nm, which is separated from the envelope by a 2-3 nm gap. The size and shape of the core generally follows that of the envelope and thus displays considerable variation. The core consists of a 10 nm thick layer of density surrounding a central, lower density area, suggesting that the core is hollow (Dokland, 2010; Spilman et al., 2009).

The lipid envelope contains several envelope proteins: the unglycosylated proteins M and E, and the glycoproteins GP2, GP3, GP4 and GP5 that carry complex-type N-linked glycans (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a). The GP5 and M proteins are considered ‘major’ envelope proteins. They appear in the virion as covalently linked heterodimers, as a result of disulfide-bridge formation between the ectodomains of these proteins (Dokland, 2010; Mardassi et al., 1996). The remaining envelope proteins are most likely present in much lower amounts and are generally designated as ‘minor’ proteins (van Nieuwstadt et al., 1996; Wu et al., 2005). The GP2, GP3 and GP4 appear to associate via non-covalent interactions and there are indications that also the E protein may be associated with the minor glycoprotein trimer (Wissink et al., 2005). Recent data suggested that interactions may exist between major and minor envelope proteins (Das et al., 2010), but further research is needed to confirm this.

The nucleocapsid of PRRSV is built up by nucleocapsid protein (N) and contains the viral genome (Dea et al., 1995; Dokland, 2010; Mardassi et al., 1994a; Spilman et al., 2009). The N proteins are predominant in the PRRSV virion as disulfide-linked homodimers (Mardassi et al., 1996; Wootton and Yoo, 2003). The nucleocapsid was initially supposed to be of icosahedral shape, but recent cryo-electron tomographic analysis indicates that the viral capsid does not show a clear isometric structure (Figure 1b.). More probably, dimers of the N protein are organized in a roughly helical organization around the viral RNA and interact with it via the N-terminal

RNA-binding domain (Dokland, 2010; Spilman et al., 2009).

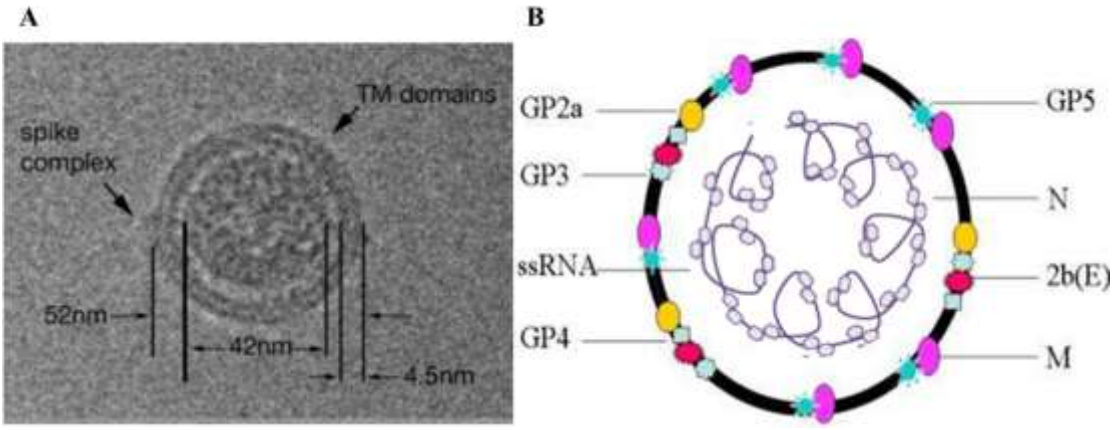


Figure 1. PRRSV virion structure and morphology

(A) Cryo-electron microscopy of a single typical PRRSV particle in vitreous ice with dimensions indicated. A presumed envelope spike complex is indicated, as is the striated appearance most likely corresponding to transmembrane domains (adapted from Spilman et al., 2009). (B) Schematic representation of a PRRSV virion. The PRRSV virion consists of a nucleocapsid, surrounded by a lipid bilayer envelope. The nucleocapsid is composed of nucleocapsid proteins (N) and contains the viral genome. The small envelope protein E, the membrane protein M and the N-glycosylated glycoproteins GP2, GP3, GP4 and GP5 are all embedded in the viral envelope. The major envelope proteins M and GP5 are present as disulfide-linked heterodimers. The minor envelope proteins E, GP2, GP3 and GP4 likely associate via non-covalent interactions. The recently discovered structural ORF5a protein is not depicted.

1.1.4 Genome organization and virus protein

1.1.4.1 Genome organization

The PRRSV genome is a positive-sense, single-stranded RNA molecule of approximately 15 kb which contains 10 open reading frames (ORFs), and is expressed through a 3' co-terminal nested set of polycistronic mRNAs with a common leader sequence at the 5' end (Fig. 2) (Britton and Cavanagh, 2008; Meulenberg et al., 1993; Meulenberg et al., 1997b).

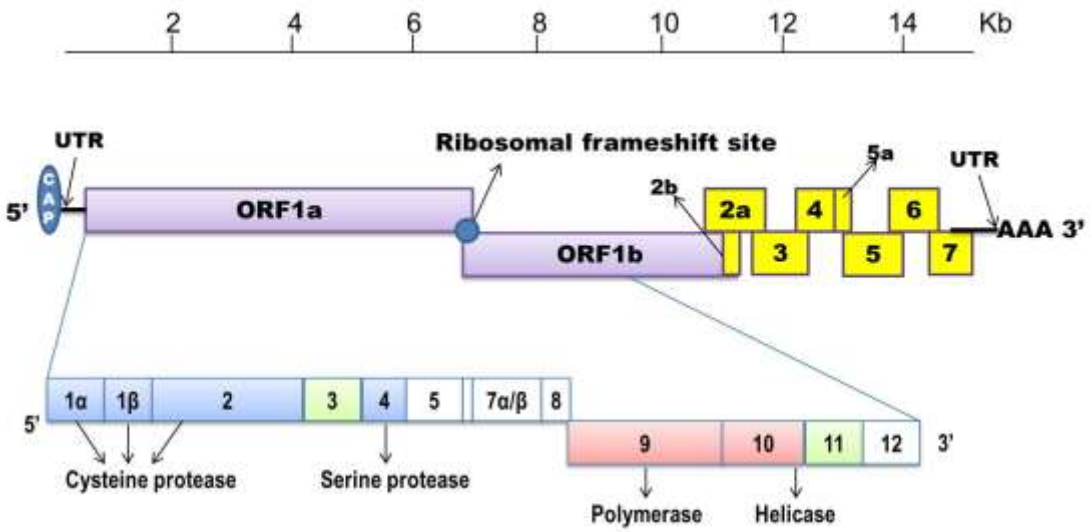


Figure 2. Schematic representation of the PRRSV genome organization.

Blocks indicate open reading frames (ORFS). The top figure depicts the entire PRRSV genome from 5' to 3' direction. The bottom panel depicts the 14 different nsps produced by processing of the pp1a and pp1ab polyprotein translated from ORF1a and ORF1b. The proteases that carries out processing are nsp1α, nsp1β, nsp2 and nsp4 (shown in blue and their protease family is also denoted). The two known proteins that are part of the viral replicase complex are nsp9 (polymerase) and nsp10 (helicase) are shown in red. PRRSV nsps for which no function has been described are in white.

The 5' end of the genome contains a 5' non-coding region which carries a cap at its 5' end and followed by ORF1a and ORF1b which account for almost three quarters of the genome (Britton and Cavanagh, 2008; Faaberg, 2008; Meulenberg et al., 1998a; Sagripanti et al., 1986). These 2 large replicase ORFs encode two long nonstructural polyproteins, pp1a and pp1ab, with expression of the latter depending on a ribosomal frameshift (RFS) signal in the ORF1a/ORF1b overlap region (Brierley, 1995; Fang and Snijder, 2010; Meulenberg et al., 1993; Snijder and Meulenberg, 1998). The pp1a is processed in nine non-structural proteins (nsps): nsp1α, nsp1β, and nsp2 to nsp8. Proteolytic cleavage of pp1ab generates products nsp9 to nsp12. The replicase processing scheme involves the rapid autoproteolytic release of three N-terminal nsps (nsp1α/1β and nsp2) and the subsequent processing of the remaining polyproteins by the "main protease" residing in nsp4 (Snijder et al., 1996), together resulting in a set

of 14 individual nsps.

The sequence downstream of the ORFs1a and 1b contain 8 overlapping ORFs, encoding respectively the structural proteins GP2a, E, GP3, GP4, GP5a, GP5, M and N. These proteins expressed from a nested set of six 3' co-terminal subgenomic-length mRNAs (sg mRNA) through a process of discontinuous RNA transcription (Pasternak et al., 2006; Sawicki et al., 2007). The sg mRNA share a common leader sequence at their 5' end with the genomic RNA, a property unique to both *Coronaviruses* and *Arteriviruses*(Fang and Snijder, 2010; Nedialkova, 2010). Besides the last sg mRNA (sg mRNA7), the sg mRNAs are polycistronic, but only the 5' proximal ORF is translated to generate a structural protein.

1.1.4.2 Virus protein

All PRRSV non-structural and structural proteins are essential for virus replication. Viral non-structural proteins are synthesized in the infected cell and are responsible for processes like replication or host (cell) modulation. They are not structural parts of the virion. In contrast, structural proteins are structural components of the virion. PRRSV infectivity is relatively intolerant to subtle changes within the structural proteins. PRRSV virulence is multigenic and resides in both the non-structural and structural viral proteins

PRRSV non-structural proteins (nsps)

Information regarding most PRRSV nsps is scarce and for several of the nsps, there are no assigned functions. Predictions on their functions are mainly based on sequence homology and studies on EAV and coronaviruses.

As in all *nidoviruses*, *arterivirus* nsps derive from the posttranslational processing of two large precursor polyproteins, pp1a and pp1ab (Ziebuhr et al., 2000). According to the current processing scheme, PRRSV pp1a is encoded by ORF1a, and the synthesis of pp1ab occurs through a RFS at the ORF1a-1b junction (Brierley, 1995;

Snijder and Meulenberg, 1998). The pp1a is processed into 10 end products (including nsp1α/nsp1β and nsp7α/nsp7β) as a result of 9 proteolytic cleavages (den Boon et al., 1995; Snijder et al., 1994; van Aken et al., 2006; Ziebuhr et al., 2000). The same cleavages are presumed to occur in pp1ab, and in addition, the nsp4 main protease cleaves three sites in the ORF1b-encoded portion of pp1ab to produce nsp9 to nsp12 (van Dinten et al., 1996).

The two N-terminal PRRSV replicase cleavage products, **nsp1α** and **nsp1β**, appear to affect Type I IFN synthesis and signaling in different manners. Individually expressed nsp1β has the ability to inhibit both IFN synthesis and signaling, while nsp1α strongly inhibits IFN synthesis only. Nsp1β expression strongly inhibited IFN regulatory factor 3 (IRF3)- dependent gene induction in the signaling pathway leading to IFN-β synthesis (Beura et al., 2010; Chen et al., 2010a). PRRSV **nsp2** is a multi-functional replicase subunit, with different domains being involved in different functions in viral replication and pathogenesis. It is an immunodominant protein with the ability to induce a strong humoral antibody response (Chen et al., 2010b; Han et al., 2007; Oleksiewicz et al., 2001). Its central region is highly variable with deletions and insertions (Fang et al., 2004; Fang et al., 2007; Gao et al., 2004; Kim et al., 2010b; Shen et al., 2000; Zhou et al., 2009b). Recently, nsp2 has been reported as a key factor in counteracting the antiviral function of interferon-stimulated gene 15 (ISG15) (Sun et al., 2012b) and contributes to NF-κB activation (Fang et al., 2012). Based on the analysis of EAV replicase processing, the PRRSV **nsp4** was proposed to mediate 9 cleavages in the nsp3–12 region. While assessing the proteolytic activity of recombinant PRRSV nsp4 in vitro, the nsp3/4, nsp4/5, and nsp11/12 cleavage sites were confirmed as cleavable substrates (Tian et al., 2009; Ziebuhr et al., 2000). The **Nsp9** is the viral RNA-dependent RNA polymerase (RdRp) required for genome replication and transcription and the **Nsp10** encodes for helicase function (Fang and Snijder, 2010).

PRRSV structural proteins

PRRSV structural proteins are encoded by corresponding ORFs: ORF2a-Glycoprotein (GP)2a, ORF2b-E, ORF3-GP3, ORF4-GP4, ORF5-GP5, ORF6-membrane (M) protein and ORF7-Nucleocapsid (N) protein. The four glycosylated proteins GP2a, GP3, GP4 and GP5 along with nonglycosylated proteins E and M are present on the viral envelope. Based on their abundance on the envelope, GP5 and M are considered as the major envelope proteins whereas GP2a, E, GP3 and GP4 are minor envelope proteins. All major and minor proteins are required for infectious virus production (Wissink et al., 2005).

Recently a short ORF (ORF5a) was identified that overlaps with the 5' end of ORF5, but whether the protein product of ORF5a is a structural component of the virion is not known (Firth et al., 2011; Johnson et al., 2011). Since this protein was recently discovered, it was not further considered in this thesis. Table 1 gives an overview of the PRRSV structural proteins (Music and Gagnon, 2010).

Table 1 Overview of the PRRSV structural proteins (adapted from Music and Gagnon, 2010).

Coding sequence	Protein	Number of aa EU/NA	Characteristics and functions
ORF2a	GP2a	249/256	Minor structural protein; contains 2 two highly conserved putative N-linked glycosylation sites; essential for virus infectivity; incorporated into virions as a multimeric complex; viral attachment protein.
ORF2b	E	70/73	Minor unglycosylated and myristoylated structural protein; essential for virus infectivity; incorporated into virions as a multimeric complex; possesses ion-channel like properties and may function as a viroporin in the envelope.
ORF3	GP3	265/254	Minor structural protein, one of the most variable PRRSV proteins; highly glycosylated with potentially seven N-linked oligosaccharides; its membrane topology seems to be strains dependent; highly antigenic and may be involved in viral neutralization; essential for virus infectivity; incorporated into virions as a multimeric complex.
ORF4	GP4	183/178	Minor structural highly glycosylated protein (four N-linked glycosylation sites); essential for virus infectivity; key glycoprotein for formation of the multiprotein complex incorporated into virions; mediates interaction between multiprotein complex of the minor viral glycoproteins and GP5; viral attachment protein and may be involved in viral neutralization.
ORF5	GP5	201/200	Major structural, transmembrane protein with a variable number of potential N-glycosylation sites; the most variable structural proteins in the PRRSV genome with GP3; involved in virus neutralization and protection; the covalent association of GP5 and M is crucial for virus assembly viral attachment protein; involved in the entry of virus into the host cells and in the apoptosis phenomenon.
ORF6	M	173/174	Major unglycosylated structural protein which is the most conserved; play a key role in virus assembly and budding; GP5-M heterodimerization is crucial for virus infectivity.
ORF7	N	128/123	Major unglycosylated, phosphorylated and structural protein; highly immunogenic and a suitable candidate for the detection of virus-specific Abs and diagnosis of the disease; the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions; able to localize in the nucleus/nucleolus and interact with cellular transcription factor.

ORF: open reading frames; EU: European PRRSV strains; NA, North American PRRSV strains.

Minor structural protein

The minor envelope glycoproteins GP2a, GP3, GP4 and the unglycosylated protein E (P2b) interact with each other to form a heterotetrameric complex in infected cells and that formation of this complex is required for the generation of infectious virions (Das et al., 2010; Mardassi et al., 1996; Wissink et al., 2005). The ORF2b gene, which is completely embedded in ORF2a, encodes another non-glycosylated minor protein named E protein (Wu et al., 2001).

GP2a is a 29 to 30 kilodalton (kDa) glycoprotein with an N-terminal signal sequence and a C-terminal membrane anchor domain (Meulenberg et al., 1995a). It contains two distinctive hydrophobic peaks and shares two highly conserved putative N-linked glycosylation sites (Meng et al., 1995; Meulenberg et al., 1995a; Meulenberg et al., 1997b; Morozov et al., 1995; Wissink et al., 2004). The GP2a protein of EU type contains two N-glycosylation sites and it has been shown that glycan addition at these sites is dispensable for infectious virus production (Wissink et al., 2004). The GP2a protein of NA type contains two predicted N-glycosylation sites, at positions N178 and N184. It has been shown that both glycosylation sites are used for glycan addition and glycan addition at position N184 is important for infectious virus recovery. In the meanwhile, glycosylation of at least one site is necessary for efficient interaction with the receptor CD163 (Das et al., 2011).

E (P2b) is an unglycosylated small (10 kDa) hydrophobic protein which is membrane-associated and believed to be present in all arteriviruses (Snijder et al., 1999; Wu et al., 2001). The E protein has a central hydrophobic domain and a hydrophilic C-terminus containing a cluster of basic residues. It possesses a potential N-terminal N-myristoylation site and a potential casein kinase II phosphorylation site (Snijder et al., 1999). Experimental evidence indicates that homo-oligomers of E are formed by non-covalent interactions in the viral envelope that may function as ion channels (Lee and Yoo, 2006; Wu et al., 2005). A recent study suggested that E

protein myristoylation is non-essential for PRRSV infectivity but promotes growth of the virus (Du et al., 2010).

The 45 to 50 kDa molecular weight **GP3** protein is one of the most variable PRRSV proteins, showing approximately 54–60% aa identity between the NA and EU genotypes (Dea et al., 2000). It is highly glycosylated, possesses seven potential N-linked oligosaccharides, and has a single N-terminal hydrophobic domain (Meulenberg et al., 1995a). The most variable region of GP3 is located at the first 35 aa, and the high level of aa sequence variability is mainly caused by the variable amino-terminal putative ER signal peptide which is only 29% identical among EU and NA isolates (Katz et al., 1995; Mardassi et al., 1995a, b; Murtaugh et al., 1995). The structural nature of GP3 is still controversial. The GP3 protein of the prototype EU LV strain was found to be incorporated into virions (Meulenberg et al., 1995a; van Nieuwstadt et al., 1996) and membrane-associated as heterotrimers with GP2a and GP4 (Wissink et al., 2005). However, the GP3 protein of the IAF-Klop reference strain (a typical NA isolate) was non-structural and secreted from PRRSV infected cells (Gonin et al., 1998; Mardassi et al., 1998). GP3 is suggested to contain one or more neutralizing epitope(s) (Cancel-Tirado et al., 2004a; Vu et al., 2011) and has been described to induce protective immunity (Jiang et al., 2007a; Plana-Duran et al., 1997).

GP4 is a 31 to 35 kDa molecular weight protein. It contains a variable amino-terminal ER signal peptide, a carboxy-terminal transmembrane domain and an ectodomain with 4 well-conserved asparagine-linked glycosylation sites, carrying complex type glycans (Meulenberg et al., 1993; Meulenberg et al., 1997a; Meulenberg, 2000; Meulenberg et al., 1995a; Meulenberg et al., 1997b). The GP4 protein of LV possesses neutralizing epitopes which are not conserved among NA and EU strains. Peptide mapping of neutralizing domain of the GP4 of the LV strain reveals a hydrophilic exposed region, adjacent to its N-terminal region (aa position

40–79) which appears to be highly variable among the EU strains (Meulenberg et al., 1997a; Weiland et al., 1999). Some studies implies that ORF4 gene is essential for virus replication and GP4 may utilize or change host cell machinery to transport viral or cellular components to the cell surface (Lee et al., 2004a; Welch et al., 2004).

Major structural protein

A PRRS virion contains three major structural proteins, which represent approximately 90% of the structural protein content: GP5 (25 kDa), M (18 kDa) and N (15 kDa) (Bautista et al., 1996; Mardassi et al., 1996; Meulenberg et al., 1995a; Snijder and Meulenberg, 1998).

GP5 is the most variable structural protein with 50-55% amino acid homology between North American and European isolate (Mardassi et al., 1996; Meng et al., 1995). The N-terminal of approximately 30 aa determine a putative signal peptide which is assumed to be cleaved (Mardassi et al., 1995a; Meng et al., 1995; Meulenberg et al., 1995a; Murtaugh et al., 1995), followed by an ectodomain of approximately 35 residues with a variable number of potential N-glycosylation sites, a hydrophobic region which is presumed to span the membrane 1 to 3 times, and a hydrophilic C-terminus of approximately 70 aa (Meulenberg et al., 1995a; Wissink et al., 2004). GP5 exposed on the surface of the virion and contains epitopes involved in virus neutralization and protection (Ansari et al., 2006; Pirzadeh and Dea, 1997, 1998; Wissink et al., 2003). *In vitro*, mAbs against GP5 are able to neutralize virus infectivity in cell culture (Gonin et al., 1999; Pirzadeh and Dea, 1997; Weiland et al., 1999). *In vivo*, strong correlations have been reported between the appearance of GP5-specific antibodies and virus-neutralizing antibodies (Gonin et al., 1999; Plagemann et al., 2002). However, there are conflicting results exists for NA- and EU-type viruses. For NA-type, antibodies are produced against both the predicted C-terminal endodomain and the N-terminal ectodomain, while studies examining the antibody response against EU-type viruses only found antibodies against the GP5

C-terminus (de Lima et al., 2006; Mulupuri et al., 2008; Rodriguez et al., 2001). Antibodies against the neutralizing epitope in NA-type GP5 do not neutralize EU-type PRRSV, and no evidence exists to date that the corresponding region in EU-type viruses also serves as target for neutralizing antibodies (Pirzadeh and Dea, 1997). A recent study showed that mAbs against the region in GP5 of LV that corresponds to the neutralizing epitope in NA-type GP5 do not neutralize the virus (Van Breedam et al., 2011).

So far, three B-lymphocyte epitopes with one major neutralization epitope located in the middle of the GP5 ectodomain (aa 36–52) (Plagemann et al., 2002), one non-neutralizing epitope (epitope A) and one neutralizing epitope (epitope B) in the ectodomain of the GP5 were identified (Ostrowski et al., 2002). The immunodominant epitope A has been suggested to be a decoy epitope that suppresses the recognition of the neutralizing epitope B. The presence of N-glycans in and around epitope B may also reduce the immunogenicity of this critical neutralization determinant in the GP5 protein. Several studies provide evidence that glycans on GP5 negatively influence the induction of virus neutralizing antibodies as well as virus sensitivity to neutralization (Ansari et al., 2006; Faaberg et al., 2006; Vu et al., 2011). Mutation of N-linked glycosylation sites at aa 30, 33, 40 or 51 results in increased sensitivity to neutralizing antibodies and enhanced immunogenicity of the neutralizing epitope (Ansari et al., 2006; Faaberg et al., 2006).

ORF5a protein was recently discovered to be encoded by ORF5a (overlapping with ORF4 and ORF5) in North American VR2332 PRRSV (Firth et al., 2011; Johnson et al., 2011). Sun *et al* recently indicated that RNA sequences in the overlapping region between ORF5 and ORF5a was essential for virus viability (Sun et al., 2012a). However, the presence of that protein in virions of the European PRRSV genotype has not been confirmed experimentally yet. Thus, ORF5a protein was not further studied in this thesis.

M protein is un-glycosylated and is the most conserved structural protein with 78 to 81 % of aa identity between NA and EU isolates (Mardassi et al., 1995a). Its membrane structure is suggested, by analogy with coronaviruses, consisting a core of three successive membrane-spanning domains preceded by an ectodomain of 13–18 aa and followed by a C-terminal endodomain of 81–87 aa (de Vries et al., 1992; Meulenberg, 2000; Meulenberg et al., 1995a, b). As described for EAV and LDV, M accumulates in the ER of infected cells, and forms disulphide-linked heterodimers with GP5 that are incorporated into virions (Mardassi et al., 1996; Verheije et al., 2002). The covalent association of GP5 and M is crucial for virus assembly (Verheije et al., 2002; Wissink et al., 2005). The M/GP5 complex is involved in attachment to the heparan sulphate receptor and is identified as a ligand for sialoadhesin (Delputte et al., 2002; Van Breedam et al., 2010). M specific mAb revealed the presence of a neutralizing epitope on the M protein (Cancel-Tirado et al., 2004a; Yang et al., 2000). Three replication-defective recombinant adenoviruses were developed as potential vaccines against PRRSV and evaluated in a mouse model (Jiang et al., 2006a). The results showed that the presence of M protein increases the immune response against GP5 by increasing the cellular immune response and the production of neutralizing Abs . Two immunodominant linear B-lymphocyte epitopes have been identified in the M carboxy-terminus (endodomain). It was found that the peptide containing the residues A161VKQGVVNLVKYAK174 can be particularly useful for diagnostic purposes and is an attractive candidate as a negative serological marker in a PRRSV vaccine (de Lima et al., 2006).

N protein possesses 123 to 128 amino acids (aa) depending on the genotype of the strains (NA and EU, respectively) with a molecular weight of 15 kDa. It is a non-glycosylated protein and is abundantly expressed in PRRSV-infected cells and constitutes 20 to 40 % of the virion protein content (Mardassi et al., 1994b; Snijder and Meulenberg, 1998). Like several other RNA viruses, PRRSV replicates in the cytoplasm (Benfield et al., 1992). However, a fraction of the N protein can be found

in the nucleus and nucleolus of PRRSV-infected cells (Rowland et al., 1999a). Two conserved aa stretches, which are similar to sequences that resemble two classical types of nuclear localization signal (NLS), have been identified in the N-terminal region of the N protein (Lee et al., 2006b; Rowland et al., 1999a; Rowland et al., 2003). Since the Golgi apparatus is thought to be the maturation site of arteriviruses, it has been postulated that the PRRSV N protein plays dual roles during virus infection; a virion structural role in the cytoplasm and a non-structural role in the nucleus and/or nucleolus. N protein nuclear localization Modification in the NLS causes attenuation of the virus suggesting that N protein localization in the nucleus is associated with virulence and may play a role in viral pathogenesis (Lee et al., 2006a; Lee et al., 2006b; Pei et al., 2008).

The N protein is highly immunogenic (Loemba et al., 1996; Meulenberg et al., 1995a, b; Rodriguez et al., 1997). The majority of Abs produced during PRRSV infection in pigs are directed against the N protein, for which major antigenic determinants are highly conserved, the N protein has been targeted as a suitable candidate for the detection of virus-specific Abs and diagnosis of the disease. However, none of the N-specific mAbs have been associated with virus neutralization. By using mAb, different B-lymphocyte epitopes, specific to or shared by NA and EU isolates, have been identified on N (Dea et al., 1996; Drew et al., 1995; Meulenberg et al., 1998b; Nelson et al., 1993; Wootton et al., 1998). Although N is not considered to be an important antigen for protective antibody-mediated immunity, it remains to be determined whether N contains protective T-lymphocyte epitopes.

1.1.5 Cell tropism and replication cycle

PRRSV has a very narrow cell tropism. The virus shows a preference for cells of the monocyte/macrophage lineage *in vivo*, and infects specific subsets of differentiated macrophages in lungs, lymphoid tissues and placenta. Some *in vitro* studies showed that the virus is able to replicate in primary porcine alveolar

macrophages (PAM), while bone marrow cells and monocytes are refractory (Duan et al., 1997; Teifke et al., 2001). Monocyte-derived and bone-marrow-derived dendritic cells can be infected *in vitro*, but the primary lung DCs (L-DCs) were not permissive to the virus (Chang et al., 2008; Loving et al., 2007; Wang et al., 2007). Transfection of viral genomic RNA allow PRRSV to replicate in several non-permissive cell lines, which means the restricted cell tropism is determined by the presence or absence of specific entry mediators in the target cell (Kreutz, 1998; Meulenberg et al., 1998a). The most crucial steps in PRRSV entry and replication in the alveolar macrophage are summarized below (Figure 3) (Van Gorp, 2010):

The virus first comes in contact with **heparan sulphate** glycosaminoglycans on the host cell surface.(Vanderheijden et al., 2001). Then this weak interaction is strengthened by a sialic-acid dependent interaction of viral GP5/M heterodimer with macrophage-specific lectin **sialoadhesin** molecule on macrophage (Delputte et al., 2007; Van Breedam et al., 2010; Vanderheijden et al., 2003). Upon binding of PRRSV to sialoadhesin, the virus is internalized via clathrin-mediated endocytosis allowing entry of the virus into the cell (Nauwynck et al., 1999; Vanderheijden et al., 2003). The process of genome release is not yet completely unraveled, but it is known that endosome acidification is required, and that the macrophage molecule **CD163** and certain cellular **proteases** are involved (Misinzo et al., 2008; Van Gorp et al., 2008). CD163, like sialoadhesin, has a restricted expression pattern and is predominantly present on resident tissue macrophages, including alveolar macrophages. But unlike sialoadhesin, CD163 is present on monocytes. Interactions between cellular CD163 and viral GP2 and GP4 have been demonstrated, but the exact role of CD163 in the entry process remains obscure (Das et al., 2010).

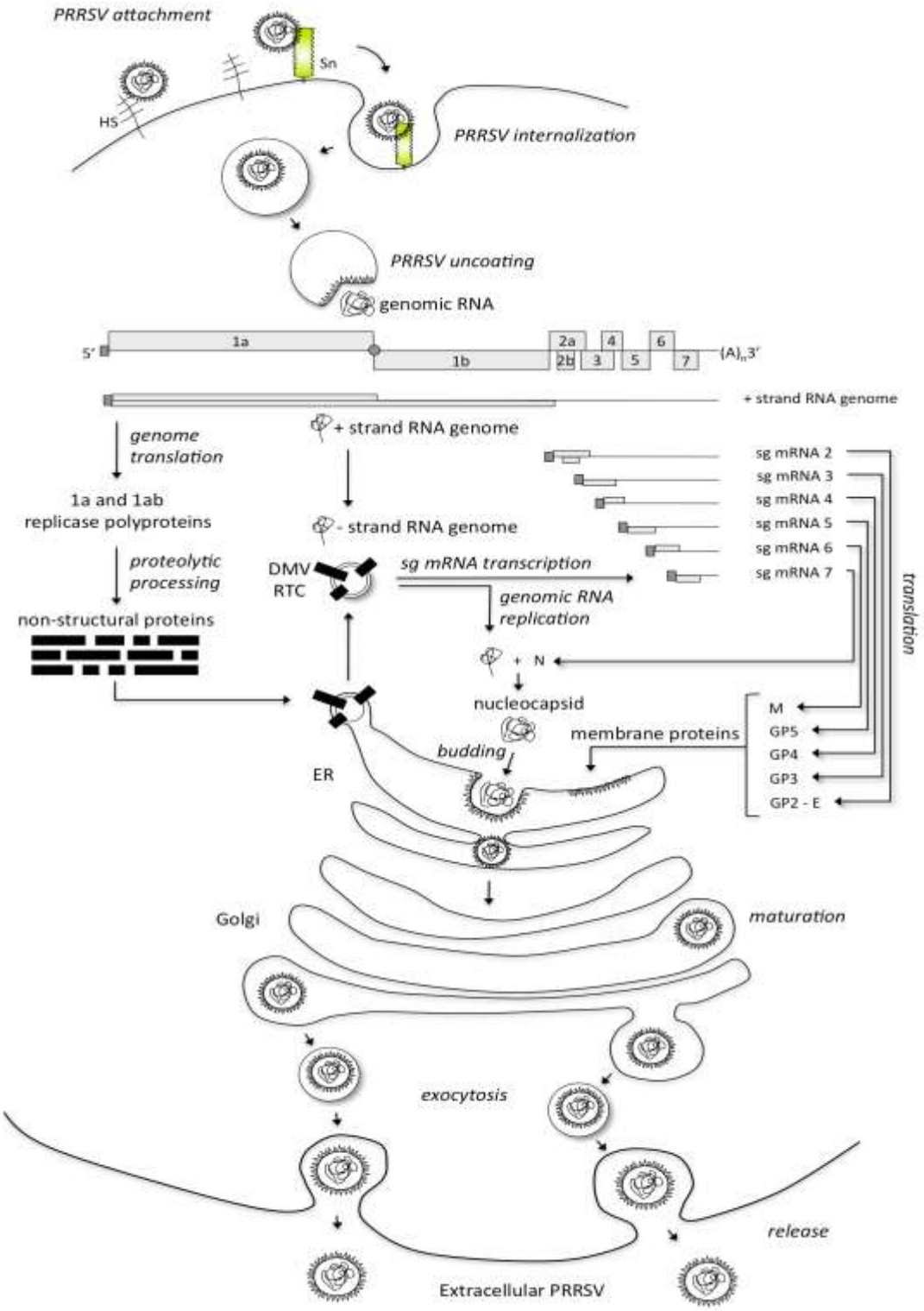
Once the PRRSV genome is released into the cytoplasm of the host cell, the linear viral (+) strand RNA undergoes translation by the host translational machinery This gives rise to pp1a or pp1ab polyprotein which are processed to form the mature

nsps (Fang and Snijder, 2010; Pasternak et al., 2006). These nsps include the viral RNA-dependent RNA polymerase (RdRp; nsp9) and the RNA helicase (nsp10) which are crucial enzymes for viral RNA synthesis. They assemble together with other nsps into the membrane-associated viral replication and transcription complex (RTC), which is responsible for genome replication and the synthesis of the nested set of subgenomic mRNAs (Fang and Snijder, 2010; Pasternak et al., 2006; Snijder and Meulenberg, 1998). The viral replicase complex then synthesizes genome length (-) strand RNA which serve in turn as templates for the synthesis of new genomic RNAs. Apart from the full-length (-) strand RNAs, also subgenomic (sg) (-) strand RNAs are generated via a discontinuous transcription mechanism to produce the templates for sg mRNA synthesis (Fang and Snijder, 2010; Pasternak et al., 2006). The sg RNAs are translated to form various structural proteins (mRNA2-7) (Fang and Snijder, 2010; Meulenberg et al., 1993; Snijder and Meulenberg, 1998). With the exception of the nucleocapsid protein N, the structural proteins are integrated in the ER membrane (Dokland, 2010; Faaberg, 2008; Johnson et al., 2011). The N protein interacts with viral genomic RNA to form nucleocapsid complexes. Preformed nucleocapsid then buds through the smooth ER/Golgi complex and in the process acquires the lipid envelope (Faaberg, 2008; Mardassi et al., 1996; Pol et al., 1997; Wootton et al., 2002; Wootton and Yoo, 2003). The eventual release of newly formed virions occurs via a process of exocytosis (Faaberg, 2008; Pol et al., 1997). Virus-induced apoptosis seems not essential for efficient virus release (Costers et al., 2008).

Figure 3. Overview of the life cycle of PRRSV (adapted from Van Gorp, 2010)

Following entry and uncoating, the arterivirus replication cycle is initiated with translation of the large replicase gene. Upon proteolytic processing, the nonstructural proteins become associated with intracellular membranes to form the RTC that is responsible for sg mRNA transcription and genomic RNA replication. Translation of the sg mRNA transcripts results in production of the structural proteins. The nucleocapsid then buds in the lumen of the ER where the virus acquires its envelope with embedded viral (glyco)proteins. The virion continues its journey through the Golgi complex where it matures before being released in the extracellular space via exocytosis. DMV,

doublemembrane vesicle; RTC, replication/transcription complex; sg, subgenomic.



1.2 PRRSV-specific immunity

PRRSV infection of pigs involves a long period of acute infection (~ 1 month) followed by an extended persistent phase (~ at least 3-4 months or longer). It is clear that PRRSV manipulates and evades protective immunity at the level of both the innate and adaptive immune system. The immune response to PRRSV infection is characterized by slow development of virus-specific interferon-γ (IFN-γ) response (Meier et al., 2003), short duration of cell-mediated immune response (Mateu and Diaz, 2008; Xiao et al., 2004) and delayed appearance of neutralizing antibodies (Lopez and Osorio, 2004). There are various factors that are likely to play multiple roles in delayed clearance of PRRSV from the host. These include interference with innate immune response (Albina et al., 1998a; Flores-Mendoza et al., 2008; Meier et al., 2003), presence of decoy epitopes (Darwich et al., 2010; Ostrowski et al., 2002), and glycan shielding of envelope proteins (Ansari et al., 2006; Faaberg et al., 2006; Vu et al., 2011). This section summarizes the current knowledge on PRRSV-specific immunity and immune evasion.

1.2.1 Innate immune responses to PRRSV

Innate immunity provides frontline antiviral protection and bridges adaptive immunity against virus infections. The innate immune system consists of humoral (cytokines, acute phase proteins, complement, and defensins) and cellular components (natural killer cells, macrophages, dendritic cells etc). However, some viruses can evolved to evade innate immune surveillance. As a result, viruses may persist in host and develop chronic infections. PRRSV is one of examples of animal viruses that has developed diverse mechanisms to evade porcine antiviral immune responses. Several studies showed that PRRSV can interact with primary lung DCs and replicates in differentiated macrophages and some DC subtypes generated *in vitro*, while macrophages and DCs are important innate immune cells (Chang et al., 2008; Duan et al., 1997; Katze et al., 2008; Loving et al., 2007; Sang et al., 2011; Wang et al.,

2007). Therefore, it should not be surprising that PRRSV infection is frequently related to impairment and dysregulation of several innate immune mechanisms.

Interferons (IFN) are multifunctional cytokines that play important roles for antiviral defense and shaping adaptive immunity. IFNs are commonly classified into type I and type II. **Type I IFNs (IFN-α and IFN-β)** are the main cytokines for innate immunity against viral infections (Haller et al., 2006; Kawai and Akira, 2006). Application of exogenous IFN-α could induce significant anti-PRRSV activity: *In vitro*, virus replication in pulmonary alveolar macrophages (PAM) and monocyte-derived DC (moDC) could be strongly reduced by IFN-α (Albina et al., 1998a; Buddaert et al., 1998; Chang et al., 2005; Lee et al., 2004b). *In vivo*, administration of exogenous IFN-α during PRRSV infection improves the development of the adaptive immune response (Buddaert et al., 1998; Loving et al., 2007; Royaee et al., 2004). Stimulation of the IFN-α response prior to PRRSV infection also results in reduced viral replication in lungs. Although IFN-α showing a protective effect, PRRSV-infected pigs display low levels of IFN-α despite the abundant presence of viral RNA in infected cells. IFN-α is not detectable in the lungs of virus-infected pigs where PRRSV actively replicates (Albina et al., 1998a; Van Reeth et al., 1999). Also, only very low level of IFN-α production is observed in virus-infected MARC-145 and PAM *in vitro* (Albina et al., 1998a; Buddaert et al., 1998; Lee et al., 2004b; Miller et al., 2004; Van Reeth et al., 1999). Moreover, IFN-α production in PAM and plasmacytoid DC (pDC) upon stimulation with transmissible gastroenteritis virus (TGEV) is actively inhibited by PRRSV, indicating that the virus not only lacks the potential to induce IFN-α, but also interferes with the production of this cytokine (Calzada-Nova et al., 2011; Loving et al., 2007). Luo et al. postulated that PRRSV-mediated suppression of type I IFN in Marc-145 cells is achieved through interference with the activation of IFN-β promotor stimulator 1 in the ds RNA-RIG-I pathway (Luo et al., 2008). (RIG-I) mediated. Non-structural proteins (Nsps) are known to be involved in PRRSV interference with type I IFN production.

Four of them were identified to contain the suppressive activities: Nsp1, Nsp2, Nsp4, and nsp11 (Han et al., 2013; Kim et al., 2010a). Accordingly, inactivated PRRSV does not abrogate TGEV-induced IFN-α production in macrophages (Chen et al., 2010b; Kim et al., 2009).

As we know, **Type II IFN (IFN-γ)** is predominantly associated with adaptive cell-mediated immune responses. However some innate immune cells, for example, natural killer (NK) cells and γδ T-cells are also able to produce this cytokine which exert innate antiviral effects by stimulating macrophage activity. There were studies showed that by administration of IFN-γ *in vitro*, PRRSV replication in PAM can be inhibited (Bautista and Molitor, 1999). However, PRRSV seems to elicit a weak innate IFN-γ response *in vivo* (Thanawongnuwech et al., 2003; Wesley et al., 2006).

PRRSV infection results in enhanced expression, production and secretion of the immunosuppressive **Interleukin-10 (IL-10)** in peripheral blood mononuclear cells (PBMCs) and broncho-alveolar lavage (BAL) cells starting from 5 dpi (Chung and Chae, 2003; Royaee et al., 2004; Suradhat et al., 2003). IL-10 is an anti-inflammatory cytokine which is involved in suppression of T-helper 1 response and specialized antigen-presenting cells, stimulation of B-cell proliferation and survival. *In vitro* study using PRRSV to stimulate macrophages, PBMC, moDC and bone marrow-derived DC (bmDC) also results in IL-10 production (Park et al., 2008; Peng et al., 2009; Suradhat et al., 2003; Thanawongnuwech et al., 2001). Gimeno et al. found that blocking of IL-10 can partially restore MHC-I and CD80/86 expression (Gimeno et al., 2011). Collectively, these results indicated that PRRSV is directly involved in the induction of IL-10 which is to a certain extent involved in suppression of antigen-presenting cell activity.

The studies on induction of the pro-inflammatory cytokine **tumor necrosis factor-α (TNF-α)** upon PRRSV infection indicated contradictive results . Choi et al. reported that in the lungs of infected pigs, there are TNF-α production by PAMs at

inflammatory sites, while this cytokine was absent or only present in low amounts in other studies (Choi and Chae, 2002; Labarque et al., 2003a; Van Reeth et al., 1999). Besides, PRRSV-stimulated moDC and bmDC can induce TNF-α production, but stimulating PAM with PRRSV during a certain amount of time was shown to inhibit TNF-α production (Chiou et al., 2000; Lopez-Fuertes et al., 2000; Park et al., 2008). Recent study by Gimeno et al. point out that different EU-type PRRSV isolates possess different capacity to induce both TNF-α and IL-10 in PBMC, PAM and bmDC, which may to a certain extent explain these apparent contradictions in literatures (Gimeno et al., 2011).

Mammalian innate immune cells, which are specialized for various functions such as pathogen recognition and killing, immune surveillance and antigen presentation, include granulocytes, NK cells, macrophages and dendritic cells (DCs), as well as epithelial and endothelial cells (Figure 4).

Innate **NK cells** are the lymphocyte subpopulation known for their ability to provide the first line of defense against viral infections (Biron et al., 1999). Early study described that depletion of NK cells by using anti-CD8 mAb prior to and during the first 5 days of PRRSV infection does not cause increased disease and does not influence the ability of the pig to clear virus (Lohse et al., 2004). Moreover, in pigs with satisfactory NK cell cytotoxicity, PRRSV significantly suppresses the NK function by 50-80% from post-infection day 7-24 (Renukaradhya et al., 2010). In addition, an increased frequency in NK cells rich fraction in virus-infected pigs did not result in rescued NK cytotoxicity, suggesting PRRSV-induced modulation in NK cell function (Dwivedi et al., 2012).

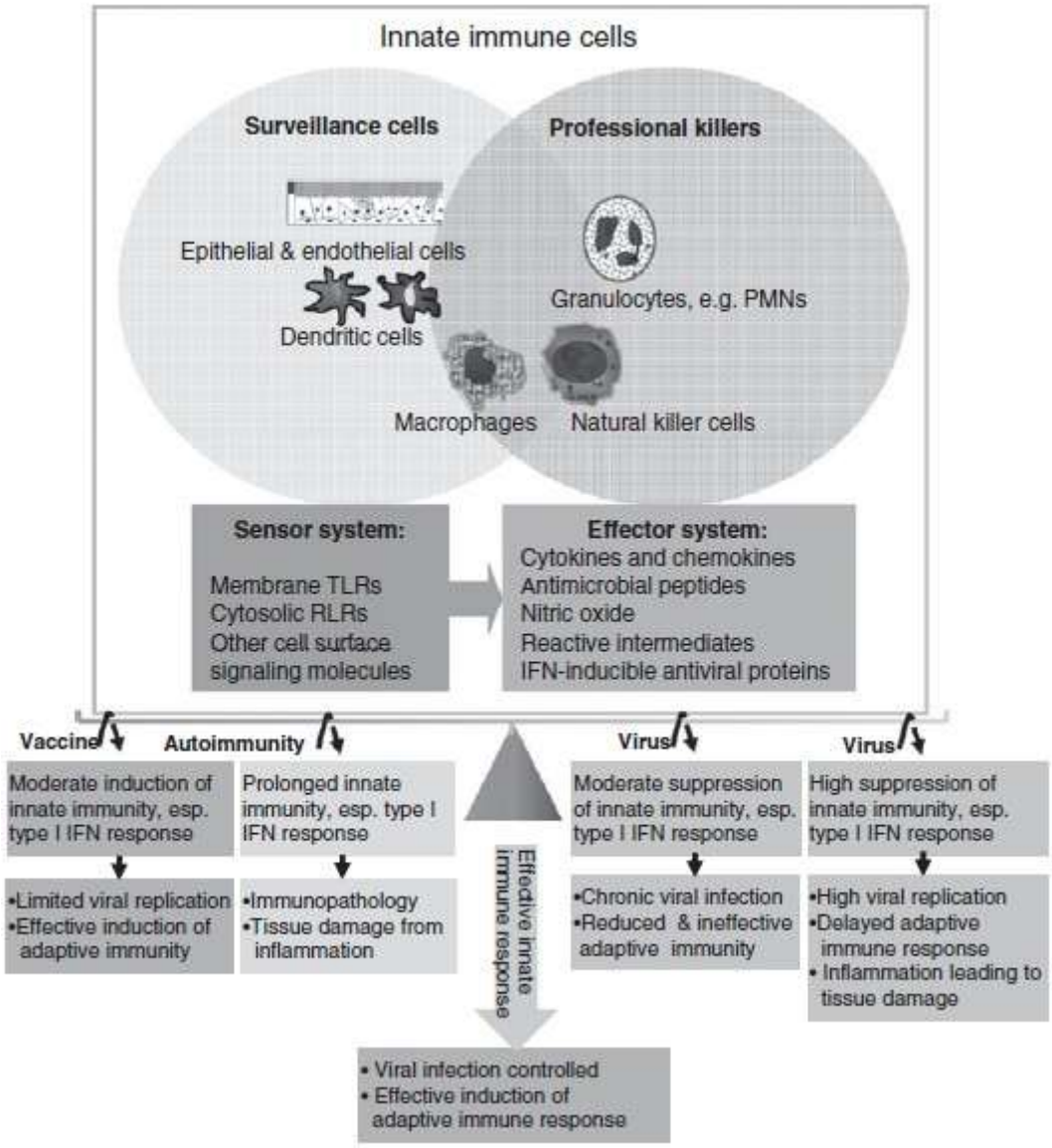


Figure 4. Innate immune homeostasis and interaction with viral infection. Innate immune cells exert antiviral activities of immune surveillance and direct inactivation using various virus-sensing and effector molecules. Whereas most viral attacks are controlled through innate immunity and synergistic induction of adaptive immunity (including vaccine-induced), virus isolates or species that have the capability to divert innate immunity, especially type I IFN responses, alter innate immune balance causing pandemic diseases (adapted from Katze et al., 2008 and Sang et al., 2011).

Activated **macrophages** which produce pro-inflammatory cytokines and possess cytolytic activity, are important phagocytes (Janeway et al., 2005). One of the major functions of macrophages is to assist in initiating and facilitating cell-mediated immune responses against pathogens (Ross et al., 2002), where they are at the interface between the innate and the adaptive immune system. PRRSV shows a preference for cells of the monocyte/macrophage lineage and infects specific subsets of differentiated macrophages in lungs (Duan et al., 1997), which may have consequences on the development of an effective immune response. Several researchers have investigated the consequences of PRRSV infection on phagocytic capacity of PAMs, but the results of these studies are highly ambiguous: decreased, unchanged and increased phagocytic capacities are reported (Chiou et al., 2000; Galina et al., 1994; Oleksiewicz and Nielsen, 1999; Thanawongnuwech et al., 1997). Recently studies from our lab showed that European genotype PRRSV inhibits PAM phagocytosis *in vitro*, through the interaction with its internalization receptor sialoadhesin (Sn). If similar events occur *in vivo*, this interaction may be important in the development of porcine respiratory disease complex (PRDC), as often seen in the field (De Baere et al., 2012).

Dendritic cells (DCs) are immune cells forming part of the mammalian immune system. Their main function is to process antigen material and present it on the surface to other cells of the immune system. That is, DCs function as antigen-presenting cells. They act as messengers between the innate and adaptive immunity. Certain subsets of dendritic cells such as monocyte-derived DCs or bone marrow-derived DCs are susceptible to PRRSV replication *in vitro* (Chang et al., 2008; Flores-Mendoza et al., 2008; Loving et al., 2007; Wang et al., 2007). However, *in vitro* stimulation of moDC, bmDC, plasmacytoid DC (pDC) and primary lung DC (lDC) with PRRSVdoes not result in a significant type-I interferon response. Unlike *in vitro* generated moDC and bmDC, *ex vivo* isolated lDC are refractory to PRRSV infection, even though, *in vitro* stimulation of these cells with PRRSV results in MHC-I

downregulation and abrogation of CD80/86 expression. This indicates that PRRSV probably compromises the activation status of antigen-presenting cells *in vivo*, even without establishing productive infection in these cells (Loving et al., 2007). Infection of DC *in vivo* was never demonstrated, nevertheless, it may have major consequences for the development of an effective innate and virus-specific immune response.

1.2.2 Adaptive immune responses to PRRSV

Adaptive immunity refers to antigen-specific immune response and is more complex than the innate immunity. It provides a second, comprehensive line of defense, capable of specifically recognizing foreign antigens, developing an immunological memory of infection, and rearranging receptor gene segments (Williams and Bevan, 2007). The adaptive response against viruses comprises (i) a cell-mediated immune response consisting of T helper (Th) lymphocytes and CTL in cooperation with macrophages and NK cells; and (ii) a humoral immune response, consisting of specific antibodies produced by plasma cells. Both PRRSV-specific cell-mediated and humoral immune mechanisms have been investigated, and some determinants of protective immunity have been described. PRRSV-infection in naïve pigs induce weak or absent protective immunity. This allows the virus to replicate and spread extensively. However, infected pigs are generally well protected against re-infection with the same virus strain, which indicate that PRRSV can induce a protective adaptive immune response with immunological memory.

Cell-mediated immunity

PRRSV establishes a long-term persistent infection in the host. This suggests that cell-mediated immunity, including IFN-γ production, is not potent or ineffective in curtailing the infection (Batista et al., 2004; Lowe et al., 2005; Murtaugh et al., 2002)., A transient lymphopenia in the peripheral blood of PRRSV-infected pigs has been observed within few days shortly after infection, the absolute numbers and

percentages of total lymphocytes, $CD4^+$ cells and $CD8^+$ cells remarkably decrease (Lamontagne et al., 2003; Nielsen and Botner, 1997; Shibata et al., 2000). Starting from 10 to 24 dpi, a rise of $CD8^+$ T-lymphocytes population with increasing cytotoxic T-lymphocyte (CTL) phenotype is observed in blood, and lungs of PRRSV-infected pigs (Albina et al., 1998b; Lamontagne et al., 2003; Nielsen et al., 2003; Samsom et al., 2000; Shimizu et al., 1996). Remarkably however, no clear increase in $CD8^+$ T-lymphocytes is observed in tonsils and draining lymph nodes of the lungs, which are the sites where virus often persists for a long time (Lamontagne et al., 2003; Xiao et al., 2004). Proliferative T cell responses, mainly characterized by a type I cytokine expression phenotype of IFN-γ and o a lesser extent, IL-2 (Lopez Fuertes et al., 1999). By restimulating PBMC from PRRSV-infected pigs with the virus, virus-specific cell proliferation can be determined by IFN-γ-based lymphocyte proliferation assays, and the exact phenotype of virus-specific proliferating cell subsets can be determined by the use of specific markers. Starting from 4 weeks pi PRRSV-specific T-lymphocyte proliferation responses can be detected in peripheral blood (Bautista and Molitor, 1997). The proliferation mainly involves virus-specific $CD4^+$ MHC-II-dependent and $CD8^+$ MHC-I-dependent T-cells, as well as CD4 and CD8 double-positive cells. Once these cells appear, their number increases only very gradually and reach the peak at 7 weeks pi then disappear after 11 to 14 weeks pi (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999).

The presence of functional $CD8^+$ **CTL** and the balance of $CD4^+$ **Th1 and Th2** lymphocytes during a viral infection are believed to determine the outcome of the infection. Based on the weak and slow IFN-γ (the prototype Th1 cytokine) response which PRRSV induced, it can be stated that PRRSV elicits only a weak Th1 response (Batista et al., 2004) (Diaz et al., 2005; Meier et al., 2003; Xiao et al., 2004)(Batista et al., 2004; Diaz et al., 2005; Meier et al., 2003; Xiao et al., 2004). However, conflicting data exist about the Th1/Th2 balance upon PRRSV infection: In Lopez Fuertes' study, Th1 lymphocyte cytokine expression were observed by

restimulating PBMC derived from PRRSV-infected pigs with PRRSV: IL-2 and IFN-γ expression were induced within 24 h, whereas IL-4 and IL-10 expression were weak to absent (Lopez Fuertes et al., 1999). In contrast, Diaz et al. and other investigators (Feng et al., 2003; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003) detected high levels of IL-10 expression and production instead of IL-2 by PRRSV-stimulated PBMC derived from PRRSV-infected pigs at 1 week pi. The strong IL-10 response that is often observed a shift towards a Th2 response. Study from Costers et al. demonstrated that PRRSV-specific CTL re not able to exert cytolytic activity towards virus-infected PAMs *in vitro* (Costers et al., 2009). This indicates that, even PRRSV-specific CTL develop and infiltrate in the lungs, they are probably not able to efficiently clear infected PAMs *in vivo*. Since the expansion of IFN-γ-secreting cells upon PRRSV infection is positively correlated with IFN-α levels, the absence of a profound IFN-α response has been denoted a major cause of impaired T-cell development. Administration of exogenous IFN-α during PRRSV immunization benefits T-cell development (Meier et al., 2003; Royaee et al., 2004). Meanwhile, inverse correlation between IL-10 levels and the presence of IFN-γ-secreting cells in restimulated PBMC cultures, indicating that IL-10 is involved in T-cell impairment (Diaz et al., 2006).

Regulatory T-cells (Treg) are negative regulators of the immune response: they block lymphocyte proliferation, differentiation and effector functions, thereby preventing excessive immune responses (Bluestone and Abbas, 2003; Chen et al., 2003; Mahic et al., 2008; Roncarolo et al., 2006). Recent studies have demonstrated the potential of PRRSV to stimulate the development of Tregs *in vitro*, although the role of these cells *in vivo* remains to be elucidated (Silva-Campa et al., 2009; Wongyanin et al., 2010).

Humoral immunity

Pigs mount a rapid antibody response to infection by PRRSV which can be

detected from 5 dpi by cell-based immunoassays using infected cells, such as immunoperoxidase monolayer assay (IPMA) or immunofluorescense assay, or by enzyme-linked immunosorbant assay (ELISA). Immunoglobulin M (IgM) is the first antibody isotype produced in response to an antigen. Anti-PRRSV IgM antibodies can be detected in serum and BAL fluid starting from 6 to 8 dpi, and peak around 14-21 dpi then rapidly decrease to undetectable levels (Labarque et al., 2000; Loemba et al., 1996; Mulupuri et al., 2008). Virus-specific IgG (both IgG1 and IgG2) reach maximum values around the third or fourth week pi and remain at high levels for months then decline to low levels. IgG1 and IgG2 antibody titres show a similar time course kinetic, with IgG1 levels always higher than IgG2 levels (Joo et al., 1997; Labarque et al., 2000; Loemba et al., 1996; Nelson et al., 1994). PRRSV-specific IgA antibodies are detected starting from 14 dpi and reach maximum levels at 25 dpi then disappear after 40 dpi (Labarque et al., 2000). These early antibodies are mainly directed to the immunogenic proteins such as N and nsp2. Antibody against M and GP5 proteins and are more variable, but they can generally be detected starting from 14 dpi (Delputte et al., 2004; Loemba et al., 1996; Nelson et al., 1994; Yoon et al., 1995).

Antibodies can protect against viral infections in different ways (Burton, 2002): (i) Antibodies can bind to viral antigens on infected cells, resulting in a lysis by the complement system (antibody-dependent complement-mediated lysis, ADCML), or by killer cells that recognize the antibody-coated cells via Fc-receptors (antibody-dependent cell-mediated cytotoxicity, ADCC). However, viral proteins of PRRSV are not expressed on the cell surface, or at least not in a way rendering them detectable for antibodies. Therefore, PRRSV-infected macrophages are refractory to ADCML and most likely also to ADCC (Costers et al., 2006). (ii) Antibodies can bind to viral proteins on free virus particles and compromising their infectivity which result in neutralization, virus opsonization, followed by phagocytosis, or in complement activation, followed by virolysis. However, PRRSV-specific antibodies which

abundantly appear early in infection are non-neutralizing (Labarque et al., 2000; Loemba et al., 1996). Virus-neutralizing antibodies as measured by seroneutralization (SN) test *in vitro* only appear late in infection (starting from 4 to 6 weeks pi) with low amounts upon PRRSV infection in naïve animal (Figure 5.)(Vanhee, 2011). Viral epitopes that are capable of inducing neutralizing antibodies appear to reside on the M, GP2a, GP3, GP4, and GP5 proteins (Ansari et al., 2006; Cancel-Tirado et al., 2004b; Kim and Yoon, 2008; Plagemann, 2006; van Nieuwstadt et al., 1996; Yang et al., 2000).

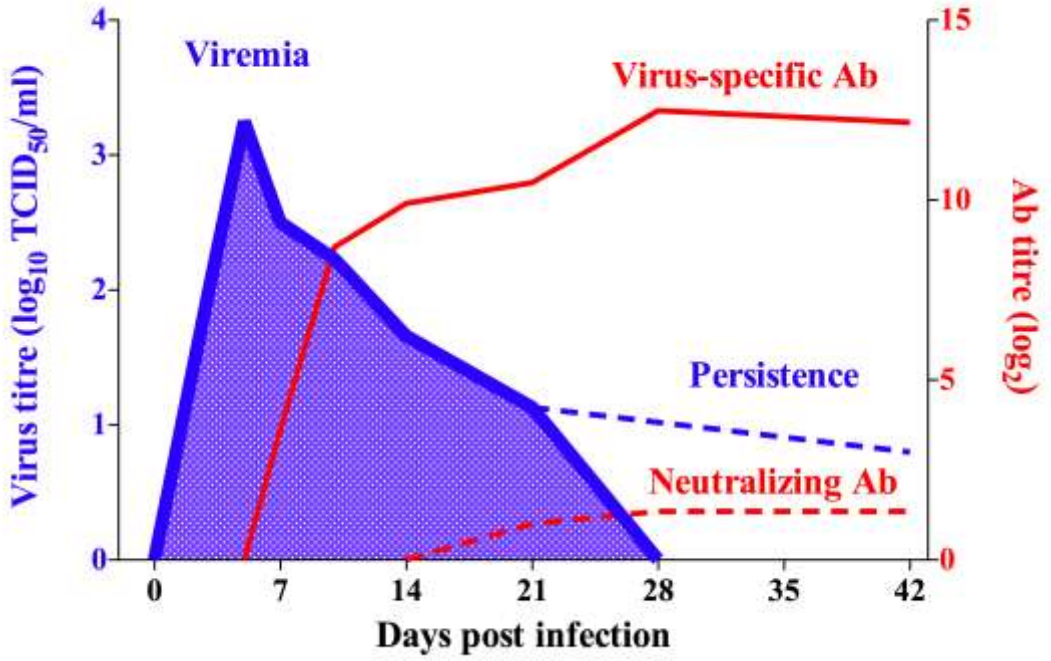


Figure 5. Schematic overview of viremia and the virus-specific and virus-neutralizing antibody response upon PRRSV infection in pigs (adapted from Vanhee, 2011).

Virus-neutralizing antibodies may play a central role in protecting swine against reinfection with PRRSV since passive transfer of antibodies fully protected pregnant sows against challenge with virulent PRRSV and blocked transplacental infection (Osorio et al., 2002). It generally appear within the time frame of viral clearance from lungs and blood, suggesting that they might contribute to resolution of infection. However, viremia may be resolved in the absence of detectable levels of neutralizing

antibodies, and on the other hand, the virus sometimes persists in the presence of virus-neutralizing antibodies, questioning the contribution of neutralizing antibodies in viral clearance upon a first PRRSV infection (Diaz et al., 2006; Labarque et al., 2000; Xiao et al., 2004). Pigs receiving sufficient amounts of virus-neutralizing antibodies can be highly protected (Lopez and Osorio, 2004; Osorio et al., 2002). Confocal analysis showed that neutralizing antibodies may block infection through both a reduction in virus attachment and virus internalization (Delputte et al., 2004).

In conclusion, these findings rather indicate that neutralizing antibodies are not sufficient for PRRSV resolution or complete protection against PRRSV and suggest that other immune mechanism(s) are involved.

1.3 PRRSV vaccines

Since its discovery in the early 1990s, tremendous progresses have been made in developing the vaccines against PRRSV. Although the commercial PRRS vaccines have been available for more than a decade, the disease remains difficult to control. The efficacies of these vaccines especially against heterologous strains remain questionable. Thus, while the currently registered vaccines are still broadly applied, especially in sows, global efforts are made to develop new and better PRRSV vaccines.

1.3.1 Current available vaccines

Two main types of whole virus vaccines are currently used to prevent PRRSV infection: modified live virus (MLV) vaccines and killed virus (KV) vaccines (Dewey et al., 2000; Labarque et al., 2003b; Meng, 2000b; Mengeling et al., 2003a; Misinzo et al., 2006; Nilubol et al., 2004). The former type is generated by *in vitro* cell culture passage of virulent virus until an attenuated phenotype is achieved, while the latter type is generated by chemically or physically inactivating virulent virus. In contrast to the live vaccines, the inactivated vaccines contain adjuvants. Both EU-type and NA-type-based MLV and KV vaccines have been developed and licensed for commercial use.

Modified live virus vaccines

The modified live-attenuated vaccines (MLVs) were generally effective against homologous strains but variable in success against heterologous strains (Huang and Meng, 2010). Vaccinated animals are well protected against clinical disease and severity as well as the duration of viremia and virus shedding (Labarque et al., 2004; Martelli et al., 2007; Scortti et al., 2006; Zuckermann et al., 2007). However, the efficacy of MLV vaccines is largely subject to the genetic background of the challenge virus, and strong to complete protection is only obtained in case the challenge virus is

nearly identical to the vaccine virus. When facing with genetically unrelated PRRSV strains, those vaccines failed to provide sustainable disease control (Murtaugh et al., 2002).

The immune response upon MLV vaccination suffers from the same weaknesses as infection-induced immunity. Virus-neutralizing antibodies induced by MLV vaccination are often only detected in neutralization tests using the vaccine virus as antigen, which explains to a certain extent the strain-specific protective efficacy of these vaccines. MLV vaccination induced cell mediated immunity is characterized by a gradual development of virus-specific IFN-γ-producing cells and a recall of these cells upon infection (Charerntantanakul et al., 2006; Meier et al., 2003; Okuda et al., 2008; Scortti et al., 2006).

Although MLV vaccines can induce a protective immune response against PRRSV infection, there are concerns about safety: The potential of spontaneous spreading should be considered in using MLV (Grosse Beilage et al., 2009). Vaccine virus on itself can cause Viremia and mutant virus variants with increased virulence may emerge during vaccine virus replication. It may spread transplacentally and horizontally with the risk of reverting to virulence. Genetic and phenotypic characterization of isolated field strains suggest that reversion to virulence is not a seldom event and the capacity of the MLV to shape PRRSV evolution by homologous recombination with circulating virus in China have been reported (Li et al., 2009b).

Killed virus vaccines

Killed virus (KV) vaccines is easier to adapt to circulating PRRSV strains than MLV vaccine, and it may be a good alternative for MLV vaccines concerning safety aspects. However, in contrast to PRRS MLV vaccine, vaccination with PRRS KV vaccine does not elicit detectable antibodies and also barely elicits cell-mediated immune response (Bassaganya-Riera et al., 2004; Kim et al., 2011). Generally, KV vaccines do not influence Viremia, virus replication in tissues and shedding, even not

in nearly homologous conditions (Nielsen et al., 1997; Nilubol et al., 2004; Plana-Duran et al., 1997; Scortti et al., 2007; Zuckermann et al., 2007).

The benefit of PRRS KV vaccine is seen more obviously in virus-infected animals. In these cases, the vaccine helps improve reproductive performance, e.g. increased farrowing rate, number of weaned pigs, and health status of piglets born to vaccinated sows (Papatsiros et al., 2006). Upon KV vaccination in naïve animals, virus-neutralizing antibodies are absent or failed to protect pigs against an *in vivo* challenge with the virus. Besides, the anamnestic response is very weak and does not lead to reduction in Viremia (Nielsen et al., 1997; Nilubol et al., 2004; Plana-Duran et al., 1997; Scortti et al., 2007; Zuckermann et al., 2007). This kind of vaccine was not able to stimulate the development of a PRRSV-specific IFN-gamma response, which means it does not stimulate an adaptive cell-mediated immune response. Particular KV vaccine could induce a non-specific IFN-γ response, but the response is not protective and is most likely caused by an adjuvant compound rather than by the inactivated virus itself (Meier et al., 2003; Piras et al., 2005; Zuckermann et al., 2007). While PRRSV-neutralizing antibodies are believed to prevent infection and transplacental infection of pregnant sows, several studies have been performed to find a method for inducing virus neutralizing antibodies, but the vaccines offer only partial protection upon challenge (Delrue et al., 2009; Misinzo et al., 2006; Vanhee et al., 2009). Therefore, More effort should be made to improve the efficacy of inactivated PRRSV vaccines.

1.3.2 New vaccine approaches

Due to immune evasion strategies and the antigenic heterogeneity of the virus, current commercial PRRSV vaccines are of unsatisfactory efficacy, especially against heterologous infection. Continuous efforts have been devoted to develop better PRRSV vaccines, in particular by using new generation vaccine systems (Table 2) (Charerntantanakul, 2012). These efforts reportedly included use of several adjuvants

(Charerntantanakul, 2009; Zhang et al., 2011), use of mixed strains of PRRSV (Mengeling et al., 2003a; Mengeling et al., 2003b), and generation of alternative vaccines, i.e. DNA vaccine (Barfoed et al., 2004; Rompato et al., 2006), subunit vaccine (Pirzadeh and Dea, 1998; Prieto et al., 2011), synthetic peptide vaccine (Charerntantanakul et al., 2006), viral vector vaccines. These experimental PRRSV vaccines indisputably provided valuable insights into PRRSV protective immunity and further paved the way for the development of new vaccines.

Table 2 Alternative PRRS vaccines (adapted from Charerntantanakul, Wasin, 2012)

	Encoded	**Immunogenicity**		**Protection**		**Ref.**
	ORF/GP	Antibody	CMI	Homologous	Heterologous	
DNA vaccine	ORF1-7	+	+	+	ND	(Barfoed et al., 2004; Rompato et al., 2006)
Subunit vaccine	GP5	Poor	Poor	-	ND	(Pirzadeh and Dea, 1998; Prieto et al., 2011)
Synthetic peptide vaccine	GP5	-	-	ND	-	(Charerntantanakul et al., 2006)
Adenovirus vector vaccine	GP3,4,5	+	+	ND	ND	(Cai et al., 2010; Zhou et al., 2010)
PRV vector vaccine	GP5,M	+	+	+	ND	(Jiang et al., 2007b; Qiu et al., 2005)
Poxvirus vector vaccine	GP3,5,M	+	+	+	ND	(Shen et al., 2007; Zheng et al., 2007)
TGEV vector vaccine	GP5,M	+	ND	+	ND	(Cruz et al., 2010)
Alphavirus-derived replicon	GP5,M	+	+	+	+	(Mogler et al., 2009)
Bacterial vector vaccine	GP5,M	+	_	+	ND	(Bastos et al., 2004)
Insect cell-derived vaccine	ORF3,5,7	+	ND	+	ND	(Plana-Duran et al., 1997)
Plant-derived vaccine	GP5	+	+	ND	ND	(Chen and Liu, 2011; Chia et al., 2010)
Gene-deleted MLV (deleted15-mer nsp2 epitope)		+	ND	ND	ND	(de Lima et al., 2008)

CMI: cell-mediated immune; +: Success; -: Failure; ND: Not determined

Adjuvants for Porcine reproductive and respiratory syndrome vaccines

To date, adjuvants including cytokines, chemical reagents and bacterial products are examined to potentiate the immune response conferred by PRRS MLV vaccines, killed vaccines, DNA vaccines, recombinant vector-based vaccines and synthetic peptide vaccines. Some of them do increase the immunogenicity of the adjuvanted PRRS vaccines. The adjuvants for PRRSV vaccine development have been reviewed excellently by Charerntantanakul (2009).

Multistrain vaccines

Previous study showed that a multistrain vaccine containing five attenuated strains of PRRSV is not more effective than a singlestrain vaccine (both vaccines provided partial protection) against a virulent field strain of PRRSV unrelated to any of the strains used for vaccination (Mengeling et al., 2003b). Recently, a graph theory-based method has been established to identify core viruses from a *de novo* constructed virus network derived from virus sequence data (Anderson et al., 2012). This method will be very useful for the selection of representative virus isolates and development of polyvalent vaccines against PRRSV.

Alternative vaccines

DNA vaccines are another strategy for PRRSV vaccine development. Fang and colleagues developed a DNA vaccine containing modified GP5 with enhanced immunogenicity by inserting a Pan Dr T-helper cell epitope (PADRE) ((Fang et al., 2006). Li and co-workers further enhanced the immunogenicity of the GP5-based DNA vaccine by mutating four potential N-glycosylation sites of the protein (Li et al., 2009a). Next to plasmid vectors, also viruses, like pseudorabies virus or bacteria, like mycobacterium bovis can be used as a vector to express recombinant PRRSV proteins. Pseudorabies expressing GP5 of PRRSV failed to induce neutralizing antibodies, but it could partially protect against clinical disease and reduce the duration of the Viremia upon PRRSV challenge (Qiu et al., 2005). Mycobacterium bovis expressing a

truncated form of GP5 and M protein of PRRSV was constructed, and vaccinated pigs showed lower temperature, Viremia and virus load in bronchial lymph nodes than control animals (Bastos et al., 2004). Besides aforementioned vaccines, plant-made PRRSV vaccine has also been reported. Pigs fed with transgenic tobacco leaves could develop specific mucosal and systemic responses against PRRSV, although the neutralization antibody titres in the sera were low (Chia et al., 2010).

1.3.3 Prerequisites for future vaccines

Either current commercial vaccines or experimental vaccines, an ideal vaccine should accomplish following requirements (Mateu and Diaz, 2008):

Safety

The ideal vaccine should be safe. This means that any possibility of reversion to virulence should be eliminated and transmission of the vaccine strain between pigs should be minimal or non-existent. Since PRRSV has capacity to evade the immune system, it can establish persistent infection and influence the immune system in general. Future PRRSV vaccine should not lead to enhanced disease caused by other infections.

Protectivity

A major goal of any PRRSV vaccination is reducing production losses of the disease. It should be able to reduce or eliminate viremia and virus replication in tissues. The efficacy of a vaccine protectvity should be evaluated in a disease model in the end: vaccination-challenge studies in pregnant sows could evaluate the efficacy of vaccination against reproductive failure, while the efficacy against respiratory disease is more difficult to assess, since other pathogens and environmental factors are usually involved.

Adaptability

A lot of studies showed that a major challenge for any PRRSV vaccine is to protect against field PRRSV strains that exhibit considerable genetic diversity (Kimman et al., 2009; Mateu and Diaz, 2008). New vaccines should always aim at inducing broad protection against a wide range of virus variants, and/or should be easily adaptable to emerging strains, no matter how this is achieved. Likewise the degree of plasticity in neutralizing sites is only partially known. Thus it is unknown whether a single "broadly protective" PRRSV vaccine can be formulated, containing for example 2 or 3 prototype strains, as is the case for poliovirus vaccines. Therefore, 'customizing' vaccines for certain farms or regions or regularly adapting vaccines to emerging strains may be valuable alternatives as well.

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CHAPTER 2

AIMS

Porcine reproductive and respiratory syndrome virus (PRRS) is one of the most economically important infectious diseases in the swine industry worldwide. In 2006, most swine farms in China experienced a ‘high fever disease of swine’ caused by new PRRSV variants (Li et al., 2007; Tian et al., 2007; Zhou et al., 2008). PRRSV appears to use several evasion strategies to circumvent both innate and acquired immunity, including interference with antigen presentation, antibody-mediated enhancement of infection, reduced cell surface expression of viral proteins, and shielding of neutralizing epitopes. In particular the downregulation of type I interferon-α production appears to interfere with the induction of acquired immunity. Current vaccines are ineffective because of the immune evasion strategies of the virus and the antigenic heterogeneity of field strains (Kimman et al., 2009).

The general aim of this thesis was to study the immune regulation of innate immune system after PRRSV infection and develop an efficient vaccine against PRRSV.

PRRSV manipulates and escapes from different arms of innate and adaptive immunity. Generally, natural killer cells (NK) are crucial immune components for the resolution of viral infections. Viral evasion from NK cells may consequently contribute to prolonged survival and efficient spread of the virus in the host, and thus to enhanced pathogenicity. To date, little information is available on the specific role of NK cells in controlling a PRRSV infection and on a possible existence of an NK cell evasion mechanism of PRRSV. Therefore, the interaction between NK cells and PRRSV-infected PAMs was studied (Chapter 3) in hope of helping us to better understand why PRRSV is not well controlled by an innate immune response.

Increasing data indicate that PRRSV strains differ in virulence in infected pigs and are biologically, antigenically, and genetically heterogeneous (Meng, 2000). Since current vaccines are not effective in protecting against infections with the genetically diverse field strains of PRRSV, it should be possible to adapt the vaccine virus. Thus,

we use adenovirus vectors which have been considered as an excellent delivery system to express genes of PRRSV structural proteins, for the purpose of generating quickly adapt vaccines. Meanwhile, Due to the multiple effects of CD40 ligand (CD40L) on immunocytes, CD40L has been used as an immunotargeting agent and immunostimulator in this study to up-regulate the immune response of PRRSV (Chapter 4).

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CHAPTER 3

SUPPRESSION OF NK CELL-MEDIATED CYTOTOXICITY AGAINST PRRSV *IN VITRO*

Suppression of NK cell-mediated cytotoxicity against PRRSV-infected porcine alveolar macrophages *in vitro*

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The adaptive immunity against PRRSV has already been studied in depth, but only limited data are available on the innate immune responses against this pathogen. In the present study, we analyzed the interaction between porcine natural killer (NK) cells and PRRSV-infected primary porcine alveolar macrophages (PAMs), since NK cells are one of the most important components of innate immunity and PAMs are primary target cells of PRRSV infection. NK cytotoxicity assays were performed using enriched NK cells as effector cells and virus-infected or mock-inoculated PAMs as target cells. The NK cytotoxicity against PRRSV-infected PAMs was decreased starting from 6 h post inoculation (hpi) till the end of the experiment (12 hpi) and was significantly lower than that against pseudorabies virus (PrV)-infected PAMs. UV-inactivated PRRSV also suppressed NK activity, but much less than infectious PRRSV. Furthermore, co-incubation with PRRSV-infected PAMs inhibited degranulation of NK cells. Finally, using the supernatant of PRRSV-infected PAMs collected at 12 hpi showed that the suppressive effect of PRRSV on NK cytotoxicity was not mediated by soluble factors. In conclusion, PRRSV-infected PAMs showed a reduced susceptibility towards NK cytotoxicity, which may represent one of the multiple evasion strategies of PRRSV.

3.1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases affecting swine industry worldwide. The causative agent, PRRS virus (PRRSV), is a single-stranded and positive sense RNA virus that belongs to the family of the *Arteriviridae* (genus Arterivirus). PRRSV has a restricted host and cell tropism, with porcine alveolar macrophages (PAMs) as important host cells (Duan et al., 1997a, b). It is able to persist in pigs for several weeks to several months after initial infection (Duan et al., 1997b; Labarque et al., 2000). It appears to use several evasion strategies to circumvent both innate and adaptive immunity (Costers et al., 2006; Costers et al., 2008; Costers et al., 2009; Mateu and Diaz, 2008). To date, a great number of studies have been performed to study the adaptive immunity against PRRSV infection, but only incomplete, fragmented, and often contradicting data are available on the innate immune responses evoked by PRRSV.

Innate immunity is a vital part of the overall host immune response controlling primary replication and invasion by pathogens, particularly during virus infection. Generally, NK cells are considered to be crucial immune components for the resolution of viral infections. They are antigen nonspecific lymphocytes which can recognize and eliminate newly malignant cells and cells infected with viruses, bacteria, and protozoa (Biron et al., 1999). NK cells are widely distributed throughout the body and can be found in both lymphoid and non-lymphoid tissues. NK cell activation may be induced by cytokines such as Interleukin-2 (IL-2), IL-12, IL-15, IL-18, Interferon-α (IFN-α) and by the interaction between NK activating receptors and their ligands on target cells (Caligiuri, 2008). NK cell function is controlled by a delicate balance of signals from activating and inhibitory receptors (Lanier, 2005). For example, absence or down-regulation by infection or transformation of self-proteins that are normally expressed by host cells can elicit NK lytic activity (Vivier and Biron, 2002). The role of NK cells has been defined in a lot of virus infections such as influenza virus (Achdout et al., 2010), foot-and-mouth disease virus (Toka et al., 2009), herpes simplex virus

(Thapa et al., 2008), ectromelia virus (Parker et al., 2007), murine and human cytomegalovirus (Arase et al., 2002; Carr et al., 2002), and human immunodeficiency virus (HIV) (Tomescu et al., 2007). In contrast, there is limited information on the NK cell response during PRRSV infection. At present, no studies have been conducted to directly define the activity of NK cells against PRRSV-infected host cells. In the present study, NK cell-mediated cytotoxicity against PRRSV-infected PAMs was examined *in vitro*. The results demonstrated that NK cells are compromised in killing PRRSV-infected PAMs *in vitro*. These data may help to better understand why PRRSV is not well controlled by an innate immune response.

3.2 Materials and methods

3.2.1 Animals

Ethics statement: The experimental procedure was authorized and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

4-week-old pigs originated from hybrid sows (JSR Genepacker 90, English Landrace × Large White) and Piétrain boars from a PRRSV-negative farm were used in this study. The seronegative status of the animals was confirmed by IPMA (Labarque et al., 2000).

3.2.2 Viruses

The European prototype PRRSV strain, Lelystad virus (LV), was propagated in Marc-145 cells. Marc-145-grown LV was semipurified from supernatant by ultracentrifugation at 100,000 ×g for 3 h through a 30% sucrose cushion in a SW41Ti rotor (Beckman Coulter Inc.). Virus pellets were resuspended in phosphate-buffered saline (PBS) in 1:100 of the original volume and the resuspension were kept at -70 ℃. Virus titrations on Marc-145 cells and calculation of the virus titers were performed as previously described (Van Gorp et al., 2008). A recombinant pseudorabies virus (PrV) Becker strain, expressing green fluorescent protein (GFP) fused to the VP26 capsid

protein was a kind gift of Dr. Greg Smith (Northwestern University, Chicago, IL, USA) and Dr. Lynn Enquist (Princeton University, Princeton, NJ, USA). To inactivate PRRSV, virus suspension was irradiated with ultraviolet (UV) light on ice in a CL-1000 UV crosslinker (UVP, Inc.) at a total dose of 1000 mJ / cm^2 during 270 s. In order to confirm if the inactivation was complete, the treated viruses were passaged for 2 generations on both PAMs and Marc-145 cells. The existence of viable virus was detected with an indirect immunoperoxidase staining.

3.2.3 Preparation of peripheral blood mononuclear cells (PBMCs) and primary porcine alveolar macrophages (PAMs)

PBMCs and PAMs were harvested from the same PRRSV-negative pigs. Blood was drawn from pigs into heparin-containing tubes and diluted with PBS (1:2). PBMCs were separated on a Ficoll-paque gradient as described by the manufacturer (Pharmacia Biotech). Cells were finally suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), non-essential amino acids (50 μM each), sodium pyruvate (500 μM), gentamicin (50 μg/mL), penicillin (100 U/mL) and streptomycin (100 μg/mL). Primary PAMs were obtained as described earlier (Wensvoort et al., 1991). PAMs were washed with 1X PBS and seeded ($1x10^6$/ml per well) into HydroCell 24 Multidish (VWR, belgium) in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics.

3.2.4 NK cell enrichment

To obtain the fraction of PBMCs that contains porcine NK cells, $CD21^-/CD172a^-/CD3^-$ cells were enriched by magnetic-activated cell-sorting (MACS) using a cocktail of monoclonal antibodies bound to goat anti-mouse IgG magnetic micro beads (Miltenyi Biotec). Briefly, PBMCs were labeled with a cocktail of the following mAbs on ice for 15min: anti-porcine CD172a (clone 74-22-15, grown in the laboratory), anti-porcine CD3 (Clone FY1H2, grown in the laboratory) and anti-CD21 (Clone B-ly4, BD Biosiences). Subsequently, cells were bound to magnetic beads

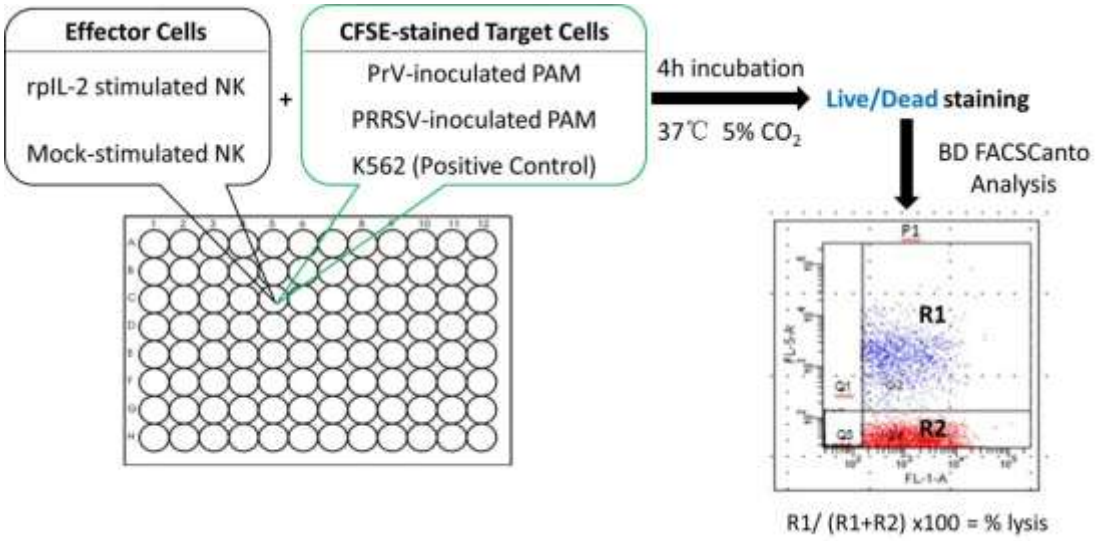
coated with goat anti-mouse IgG by incubating for 15min at 4℃. Finally, cells were washed and passed through a magnetic field for separation. After separation, cells were washed twice and resuspended in RPMI-1640 supplemented with 10% FBS. The purity of the prepared cell subset was examined by FACS analysis before using. The purity of $CD21^-/CD172a^-/CD3^-$ enriched fraction was in the range of 94%-97%.

3.2.5 NK Cell cytotoxicity assay

Target cells: Twenty-four hours cultivated PAMs, inoculated with PRRSV, UV-inactivated PRRSV or PrV at an m.o.i of 10 or mock-inoculated, were used as target cells. As a positive control for NK activity, K562 (human erythroleukemia cell line) tumor cells, which are known to be sensitive target cells for NK cell lysis due to the lack of MHC class I expression, were included in the assays. Carboxy fluorescein succinimidyl ester (CFSE) was used as a tracking dye to label the target cells at a concentration of 5 μM for 15 min on ice. Labeling was stopped by adding RPMI-1640 supplemented with 10% FCS. Cells were washed three times, counted and used in the cytotoxicity assays at appropriate Effector:Target (E:T) ratios.

Effector cells: IL-2 is known to be an activating cytokine of NK cells. Enriched NKs were cultured for 24 h either with or without recombinant porcine IL-2 (100 U/ml) (rpIL-2, ImmunoTools) before using the cells in the NK cell cytotoxicity assay.

Cytotoxicity assay:



A flow cytometry-based assay was used to analyze NK cell cytotoxicity. rpIL-2-stimulated or non-stimulated effector cells were mixed with CFSE-labeled target cells and incubated for 4 h at 37 ℃. Subsequently, all cells were stained with a live/dead discriminating dye (LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit, Invitrogen). The cells were analyzed using a BD FACSCanto flow cytometer (FACS), counting 10,000 target cells per sample. The gate was included all target cells by setting of forward and side scatter parameters (FSC and SSC) (Fig. 1a). After further gating on CFSE-positive target cells (Fig. 1b), the percentages of target cell death then determined in the live/dead versus CFSE histogram (Fig. 1c and d). The percentage of NK cytotoxicity was further calculated as a percentage of target cells in the Q2 region minus the percentage of spontaneous death of target cells. The E:T ratio was fixed at 30:1 when using PAMs as target cells.

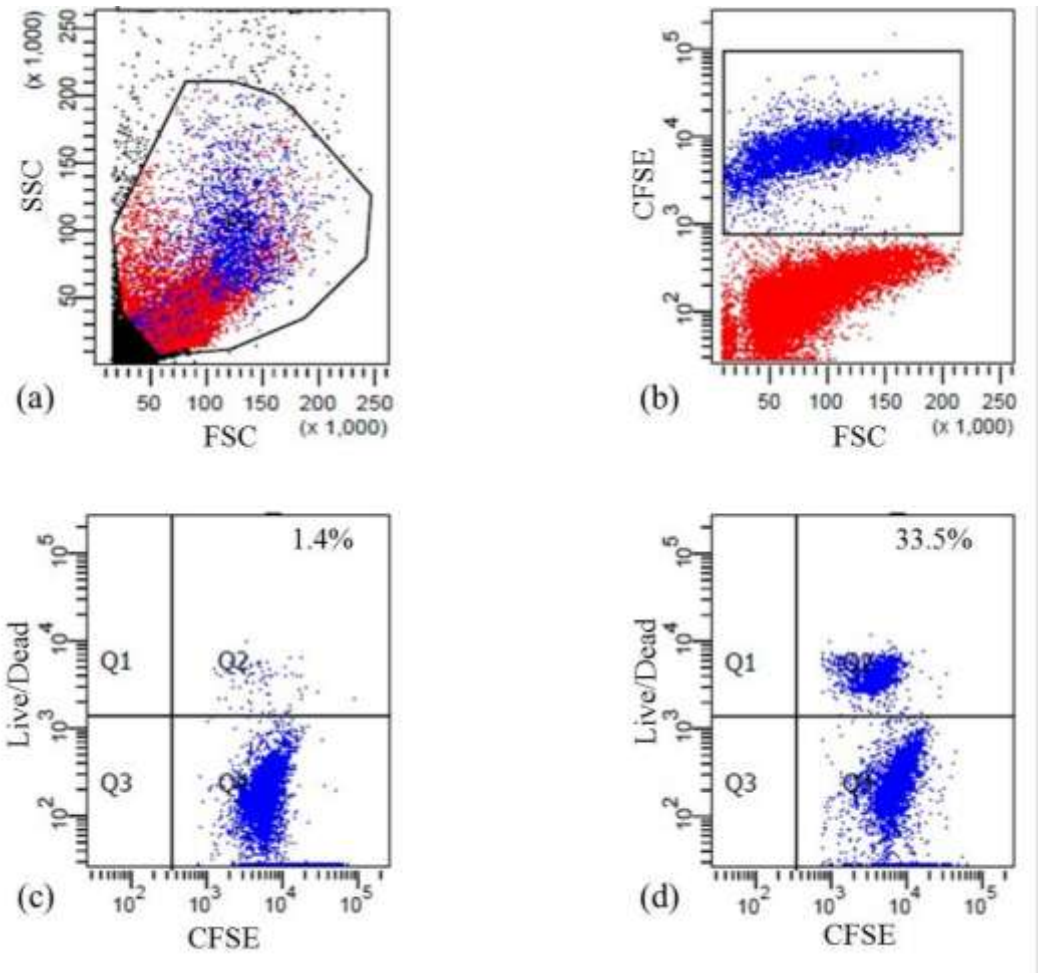


Fig. 1 Gating strategy of natural killer (NK) cytotoxicity assay by two-color flow cytometry. (a) Target cells were gated based on forward and side scatter characteristics. P1 contains all Target cells. (b) Cells contained within P2 are displayed on a plot of carboxyfluorescein diacetate succinimidyl ester (CFSE)-positive expression. (c) Cells positive for Live/Dead and CFSE lying in the upper right quadrant are spontaneously dead target cells (Q2). (d) An example of the measurement of NK cytotoxicity: the percentage of NK cytotoxicity showed in the upper right quadrant was calculated as percentage of target cells in this gating region (Q2) minus the percentage of spontaneous death of target cells.

3.2.6 MHC class I cell surface expression

24 h-cultured, mock-inoculated or PRRSV-inoculated PAMs were stained with primary mAb PT85A (VMRD), followed by secondary goat-anti-mouse FITC (Molecular Probes) for MHC class I staining. Afterwards, cells were analyzed using flow cytometry. The mean fluorescence intensity (MFI) of cells was used as a measure

for the amount of MHC class I expressed. PAMs incubated with the irrelevant, isotype-matched control primary mAb 13H4 (IgG2a; specific for the capsid protein of porcine circovirus 2) showed no staining.

3.2.7 Degranulation assay

CD107a has been described as a marker for CTL and NK cell degranulation (Alter et al., 2004). NK cell degranulation was assessed by FACS using surface mobilization of CD107a. Mouse-anti-porcine CD107a antibody (AbD) was added to the non-stimulated NK cells, rpIL-2 stimulated NK cells and rpIL-2 stimulated NK cells co-cultured with target cells, respectivelly. Cells were incubated for 2 h at 37℃ in 5% CO_2 after which monensin (Golgi-Stop, BD Biosciences) was added and incubated for an additional 2 h at 37℃ in 5% CO_2, goat-anti-mouse R-PE (Molecular Probes) was added during last 1 h. Cells were harvested and then analyzed by flow cytometry. NK cells were first gated as CFSE negative population to be distinguished from PAMs. PE-positive cells, scored by FACS, represented degranulated NK cells. Irrelevant mAb 13D12 (IgG1, specific for the gD protein of the alpha-herpesvirus pseudorabies virus, grown in lab) was used as isotype-matched control and showed no staining.

3.2.8 Soluble factor test

An NK cytotoxicity assay, similar to that described above was performed using co-cultured NK and target cells with supernatants from PRRSV-infected PAMs or non-inoculated PAMs. The supernatants of PRRSV-infected PAMs ($2*10^6$/ml) and non-inoculated PAMs ($2*10^6$/ml) were collected at 12 hpi and centrifuged at 400×g at 4℃ for 5 min in order to discard the cells and debris.

3.2.9 Statistical analysis

Statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Differences of medians between two treatments were assessed by the Mann-Whitney U test, while differences among treatments were assessed by one-way analysis of variances followed by Duncan's multiple range tests. Differences were

considered to be significant when $P \leq 0.05$.

3.3 Results

3.3.1 rpIL-2 activates cell cytotoxicity of porcine NK cells

NK cells were isolated and enriched from PRRSV-negative PBMCs, and the cytotoxicity of the resting NK cells was determined in a cytotoxicity assay (Fig. 2). The resting porcine NK cells killed NK target cell-K562 cells at a very low level. The cytotoxicity of NK cells against K562 cells was significantly increased by prior stimulation with rpIL-2 ($P < 0.05$). Likewise, we used 24 h-cultured PAMs as target cells. The results showed the same tendency. rpIL-2 stimulated enriched NK cells showed a cytotoxicity against both K562 cells and PAMs. The highest E:T ratio resulted in the highest specific killing of target cells. Therefore, NK cells activated by rpIL-2 were used at an E:T ratio of 30:1 in subsequent experiments.

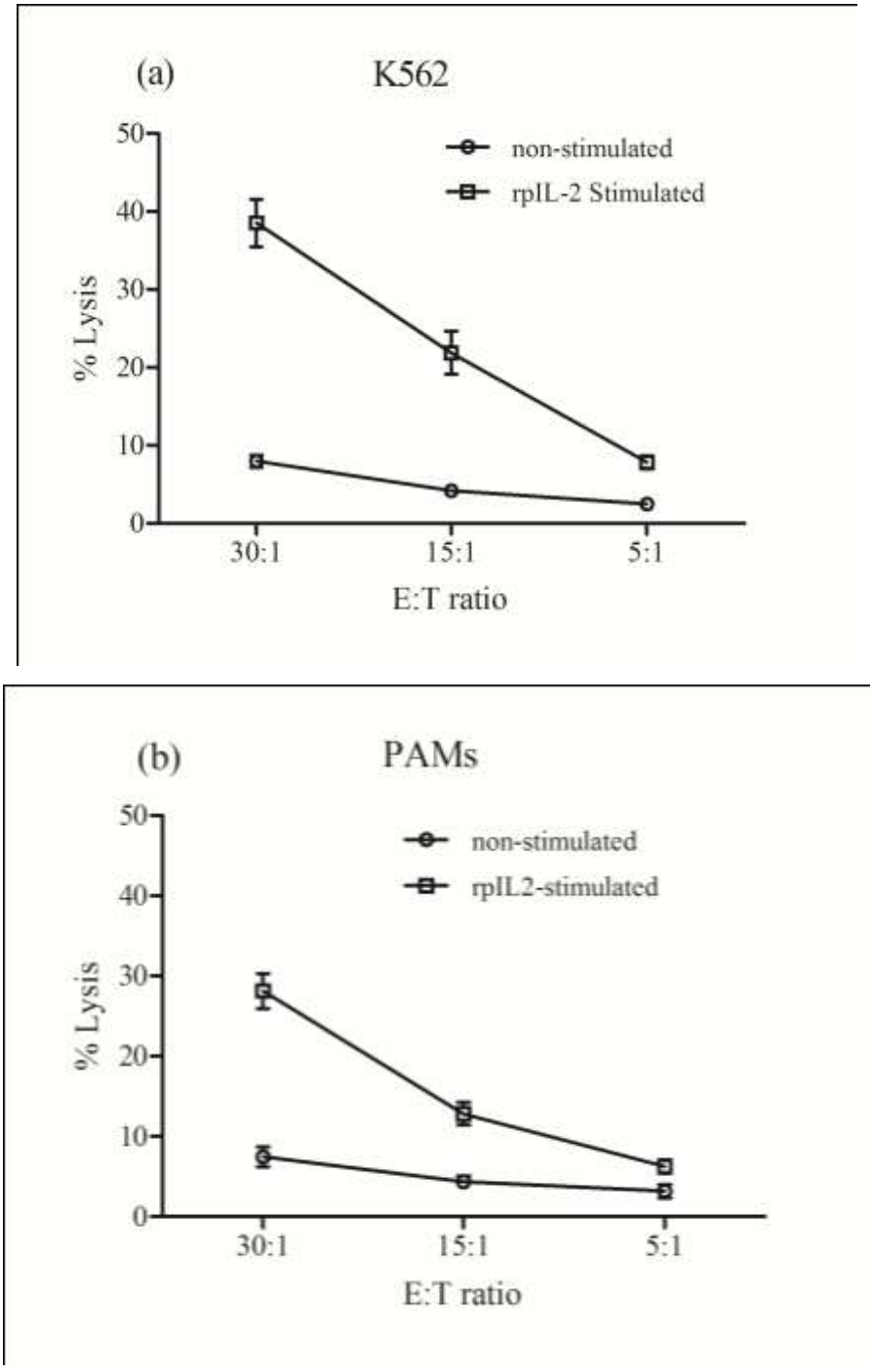


Fig. 2 Either non-stimulated NK cells or NK cells stimulated for 24 h with rpIL-2 (100 U/ml) were used as effectors (E). After additional 4 h incubation with CFSE-labeled target cells (T): (a) K562 cells; (b) 24 h-cultured PAMs, the cytotoxicity was quantified as % Lysis (y-axis) by flow cytometry. The E:T ratio is shown on the x-axis. Data are presented as means ± SD of at least three individual experiments.

3.3.2 Cytotoxicity of rpIL-2 stimulated NK towards PRRSV-infected PAMs at different time points post inoculation

PRRSV has a very restricted host cell tropism both *in vivo* and *in vitro*. *In vivo*, it mainly replicates in sialoadhesin-positive macrophages in lungs and lymphoid tissues. *In vitro*, PRRSV was originally isolated on primary cultures of PAMs (Duan et al., 1997a, b). In PAMs, the replication cycle of PRRSV is completed within 12 hpi. PRRSV prevents the cell from going into apoptosis during the early stages of replication, while apoptosis is induced at the end of the replication cycle (Costers et al., 2008). NK cytotoxicity assays were performed at different time points after inoculation within the first replication cycle. Mock-inoculated and PRRSV-infected PAMs (4, 6, 8, 12 hpi) were used to conduct cytotoxicity assays. As shown in Fig. 3, there was no significant difference in NK-mediated killing between PRRSV-infected PAMs and mock-inoculated PAMs at 4 hpi. Starting from 6 hpi, the NK-mediated lysis of PRRSV-infected PAMs was reduced and was significantly lower than mock-inoculated PAMs ($P < 0.05$).

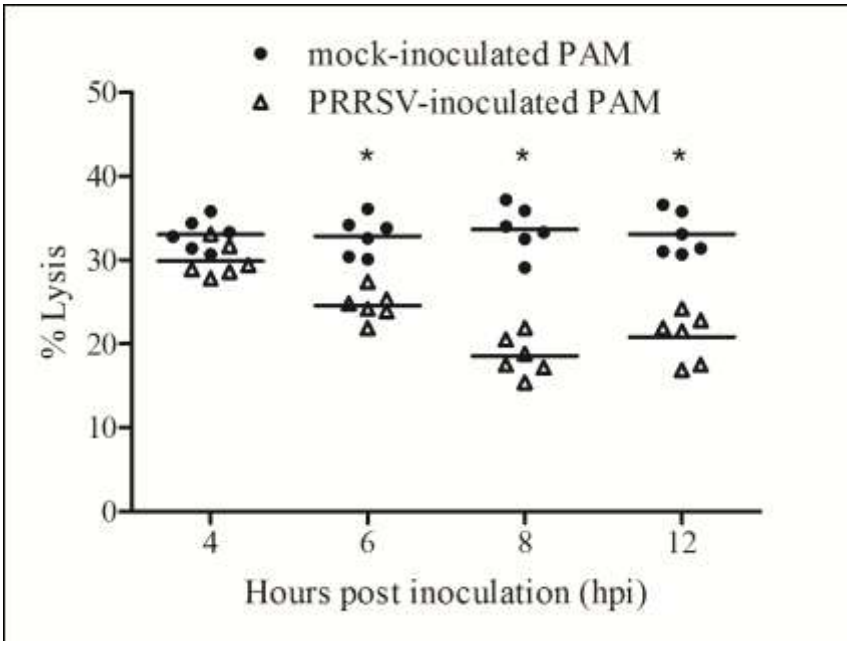


Fig. 3 PAMs were inoculated with PRRSV or mock-inoculated. After different time points post inoculation, PAMs were used as targets in NK cytotoxicity assays at an E:T ratio of 30:1 as described in Materials and Methods. Bars indicate means of percentage of NK lytic level from six animals. Significant differences between mock-inoculated PAMs and PRRSV-infected PAMs ($P \leq 0.05$) are indicated with an asterisk (*).

3.3.3 MHC class I cell surface expression of PAMs

MHC class I can serve as an inhibitory ligand for NK cells. Reduction in the normal levels of surface MHC class I, a mechanism employed by some viruses during immune evasion or in certain tumors, will activate NK cell killing. To check whether the insufficient NK cytotoxicity against PRRSV-infected PAMs is caused by up-regulated expression of MHC class I, staining of MHC class I on 24 h-cultured, mock-inoculated and PRRSV-inoculated PAMs was performed. The results showed that the MHC class I expression is not significantly different among these three groups (Fig. 4). This indicated that the suppression of NK cytotoxicity is not due to the up-regulated expression of MHC class I on cell surface.

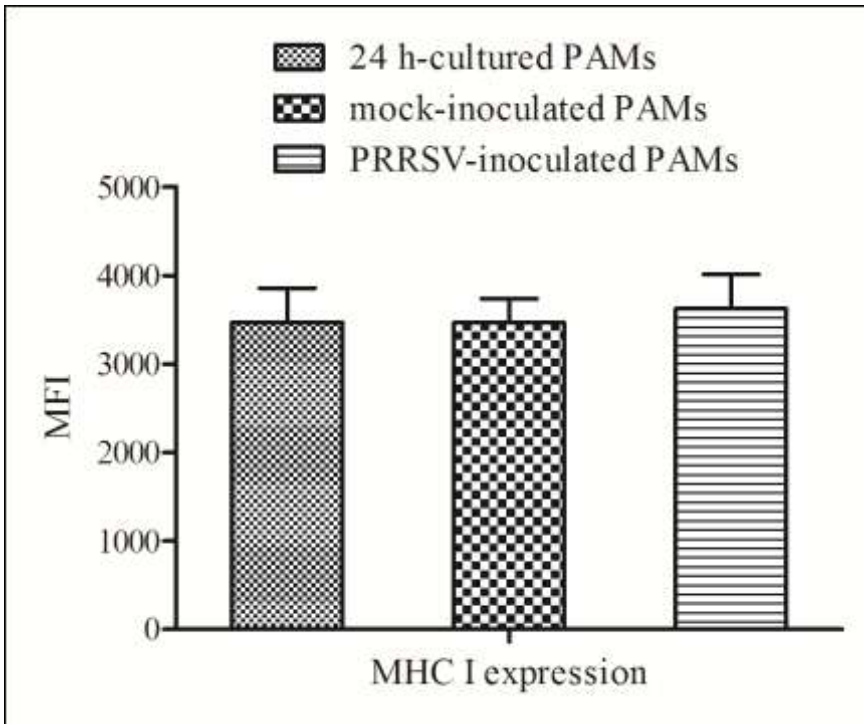


Fig 4 MHC class I expression on cell surfaces. Data are expressed as MFI of viable cells expressing MHC class I molecules on 24 h-cultured PAMs/mock-inoculated PAMs/PRRSV-inoculated PAMs. Unstained and isotype-matched irrelevant stained macrophages are not shown. Data represent the means ± SD of at least 3 independent experiments.

3.3.4 UV-inactivated PRRSV infection causes a low level suppression of NK cytotoxicity

The former results showed that NK cells showed a considerably lower level of cytotoxicity towards PRRSV-infected PAMs compared to the mock-inoculated PAMs. To determine whether a productive infection is required for this suppressed NK cytotoxicity, a cytotoxicity assay was performed using PAMs inoculated with infectious or UV-inactivated PRRSV as target cells. Stimulated NK cells were used as effector cells and the cytotoxicity assay was conducted with an E:T ratio of 30:1. PrV inoculated PAMs and K562 cells were included as positive controls. The results showed that PRRSV-infected PAMs were lysed at a significantly lower level compared to mock-inoculated PAMs (Fig. 5). Lysis of UV-inactivated PRRSV inoculated PAMs was somewhat lower when compared to mock-inoculated PAMs ($P = 0.1$) but still higher

compared to PRRSV-infected PAMs (P = 0.06). In contrast, the lytic level of PrV-infected PAMs was significantly higher than mock-inoculated PAMs. These results suggest that PRRSV infection gives a negative influence on NK cytotoxicity. Binding and internalization of PRRSV particles partially affect the ability of NK cells to lyse PAMs but transcription of viral genes and expression of viral proteins contribute further to the increased resistance of PAMs towards NK-mediated lysis.

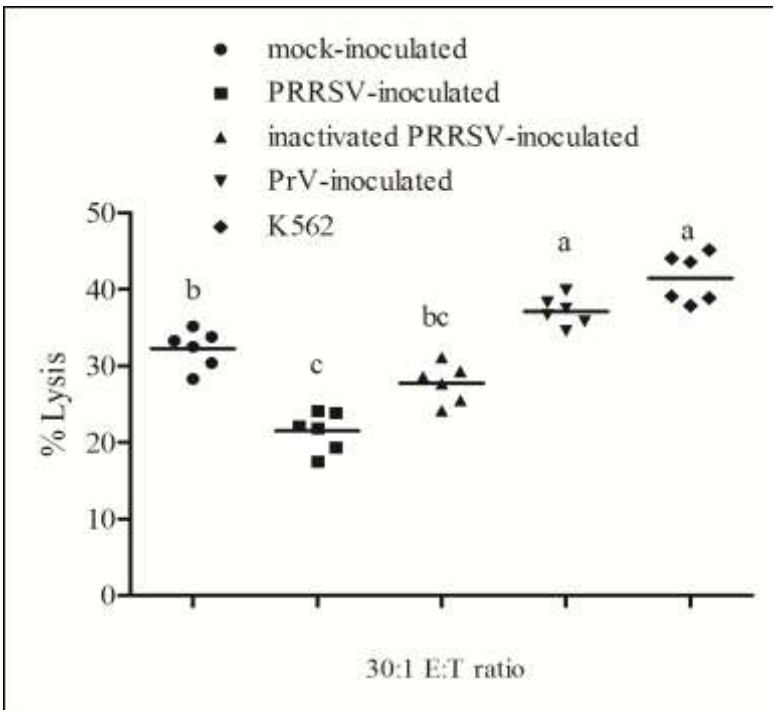


Fig. 5 PAMs were inoculated with PRRSV, UV-inactivated PRRSV, PrV or mock-inoculated. After 12 hpi, cells were used as targets in NK cytotoxicity assays at an E:T ratio of 30:1. K562 cells were included as a positive control at same E:T ratio. Bars indicate means of percentage of NK lytic level from six animals. Different alphabet letters indicate significant difference (P < 0.05).

3.3.5 NK cell degranulation

To further investigate the suppressed cytotoxicity of NK cells against PRRSV-infected PAMs, the expression of CD107a was tested using FACS. CD107a is expressed mainly in endosome-lysosome membranes before degranulation (Alter et al., 2004). When NK cells are involved in cytotoxic activity, they degranulate to release perforin and granzymes, leading to increased CD107a expression on the cell surface. We assessed CD107a expression on non-stimulated NK cells, rpIL-2 stimulated NK

cells and rpIL-2 stimulated NK cells that were co-cultured with different target cells. The results presented in Fig. 4 show that NK cells incubated with K562 cells or with mock-inoculated PAMs displayed higher surface expression of CD107a. In contrast, incubation of NK cells with PRRSV-infected PAMs showed little difference in the expression of CD107a compared to NK cells stimulated with rpIL-2 solely without incubation with target cells (Fig. 6).

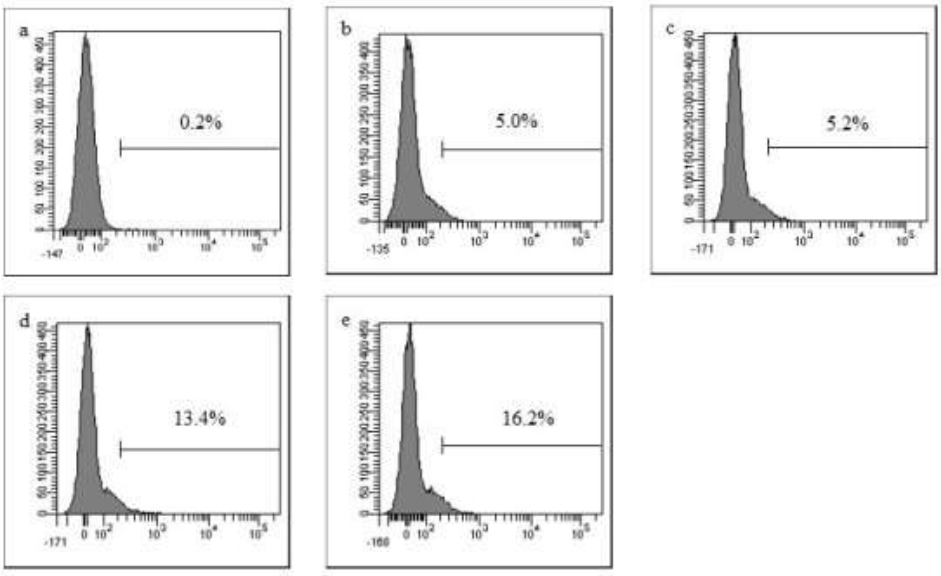


Fig. 6 Degranulation assays. To show evidence of NK cell release of cytotoxic granule contents, cells were stained for the presence of CD107a. (a) non-stimulated NK cells cultured alone; (b) rpIL-2 stimulated NK cells cultured alone; (c) rpIL-2 stimulated NK cells incubated with PRRSV-infected PAMs; (d) rpIL-2 stimulated NK cells incubated with mock-inoculated PAMs; (e) rpIL-2 stimulated NK cells incubated with K562 cells. Histograms are from a single determination from five animals.

3.3.6 Insufficient NK cell cytotoxicity against PRRSV-infected PAMs is not mediated by a soluble factor

NK cytotoxicity assays similar to the ones described above were performed using mock-inoculated PAMs and K562 cells as target cells. The supernatants of both PRRSV-infected and non-inoculated PAMs were collected and added during co-culture

of NK cells and target cells. The supernatant of PRRSV-infected PAMs contains soluble mediators, such as cytokines, produced during infection. The experiment therefore allowed investigating the potential involvement of soluble mediators in the observed inhibition of NK cytotoxicity. In Fig. 7, no significant reduction of NK cytotoxicity was detected in cells cultured with PRRSV-infected supernatant compared to that derived from non-inoculated PAMs. These results show that suppressed NK activity against PRRSV-infected PAMs is not mediated by soluble factors released during infection.

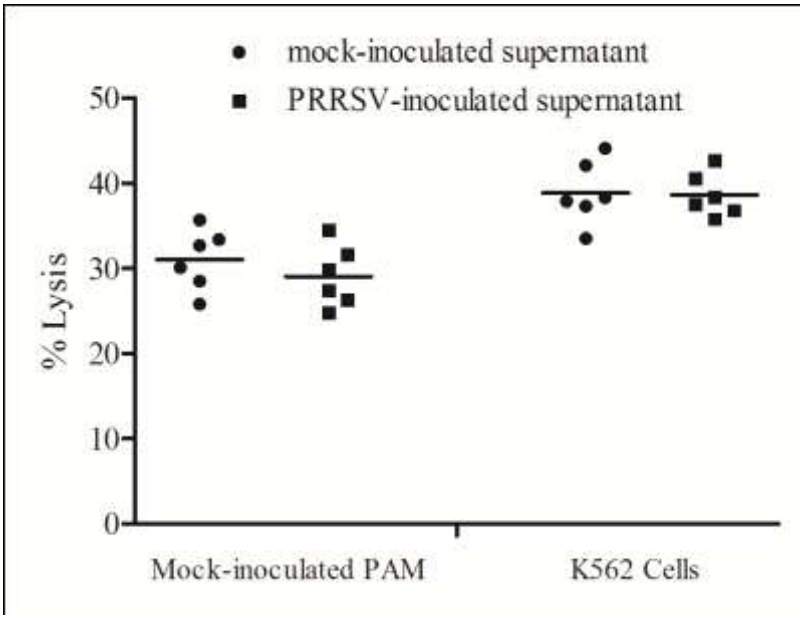


Fig. 7 Stimulated NK cells co-cultured with different targets in the presence of supernatant from PRRSV-infected PAMs at 12 hpi or with medium from non-inoculated PAMs. Lytic levels are presented as % Lysis at an E:T ratio of 30:1. Bars indicate means of percentage of NK lytic level from six animals. No significant differences were observed between non-inoculated PAMs' supernatant treatments and PRRSV-inoculated PAMs' supernatant treatments.

3.4 Discussion

PRRSV manipulates and escapes from different arms of innate and adaptive immunity. Although NK cells represent central players in the innate immunity, little information is available on the specific role of NK cells in controlling a PRRSV infection and on a possible existence of an NK cell evasion mechanism of PRRSV.

Therefore, the interaction between NK cells and PRRSV-infected PAMs was studied in the present paper.

Natural killer cells represent a small fraction of circulating lymphocytes that are neither B nor T cells. In this study, we used magnetic beads to deplete $CD3^+$ cells (T cells), $CD172a^+$ cells (monocytes) and $CD21^+$ cells (B cells) to reduce the interference caused by these cell populations. This enriched NK cell population behaved like resting NK cells, showing only a very low cytotoxicity towards the target cell-K562 cells. Cytokines such as IL-2 and IFN-α are known to be activators of NK cells (Naume et al., 1992; Pintaric et al., 2008). Since PRRSV is known as a poor inducer of IFN-α and the level of IFN-α remains low throughout the course of infection (Albina et al., 1998; Van Reeth and Nauwynck, 2000), recombinant porcine IL-2 was selected to prime the enriched NK cells. Our results showed that exogenous rpIL-2 indeed efficiently activates NK cells *in vitro*.

NK cells are generally known to play a critical role in host defense at early stages of viral infections by killing infected cells and by production of cytokines (Lodoen and Lanier, 2006). However, in the present study, the results from the kinetics experiments indicated that PRRSV-infected PAMs show a reduced susceptibility towards NK cytotoxicity. NK-mediated lysis of infected PAMs decreased from 6 hpi onwards. Reduction of NK cell cytotoxicity starts from 6 hpi (Fig. 2), which coincides with the period of the first observed detectable PRRSV main structural proteins (Costers et al., 2008). Hence, dysfunction of NK cells may be correlated with the appearance of a viral protein or a cellular protein induced or activated by a viral protein. As reported in a previous study of Costers et al. (2008), the PRRSV replication cycle in macrophages is completed within 12 hpi, and PRRSV prevents the cell from going into apoptosis during the early stages of its replication, while apoptosis is induced at the end of the replication cycle (Costers et al., 2008). Induction of apoptosis may be one of the reasons why the lytic level of PRRSV-infected PAMs first decreased and then slightly increased at 12 hpi ($P > 0.05$).

Curiously, we noticed that NK mediated lysis of 24 h-cultured and mock-inoculated PAMs both reached a high level during the experiments (around 30%), which was only 10% lower than in K562 cells. It seems that cultured or mock-inoculated PAMs are targets for rpIL-2 activated NK cells. Some studies performed on human NK cell-mediated lysis of autologous antigen-presenting cells (APCs) indicated that some macrophage subtypes are susceptible to lysis by cytokine conditioned NK cells, for instance, via NKG2D recognition (Bellora et al., 2010; Nedvetzki et al., 2007). Additionally, ligands for NKG2D can be upregulated in response to stress stimuli (Gasser and Raulet, 2006). Hence, the lysis of cultured or mock-inoculated PAMs in our experiment is probably due to stimulation of the NK cells by rpIL-2 and/or stress stimuli generated during *in vitro* cultivation of the PAMs.

MHC class I ligands expressed on target cells behave as inhibitory receptors on NK cells. Absence of MHC class I on target cells often leads to NK cell activation (Elliott et al., 2010). Conversely, up-regulation of MHC Class I on target cell surface may reduce cell susceptibility to killing mediated by activated NK cells (Imboden et al., 2001). Staining of MHC class I expression were performed on 24 h-cultured, mock-inoculated and PRRSV-inoculated PAMs. The results showed that the MHC class I expression on aforementioned groups have no significant differences. This indicated that the suppression of NK cytotoxicity is not due to the change of MHC class I cell surface expression.

To confirm that the insufficient NK activity was PRRSV-induced, PrV-infected PAMs were included as a control. A significantly higher level of NK mediated lysis against PrV-infected PAMs was observed compared to mock-inoculated PAMs and PRRSV-infected PAMs (Fig. 3), which indicated that the NK cytotoxicity against PRRSV-infected PAMs was clearly suppressed. When PAMs were inoculated with UV-inactivated PRRSV, a small but non-significant ($P > 0.05$) reduction of NK mediated lysis compared to mock-inoculated was observed. This indicated that binding and internalization of PRRSV particles may partially contribute to the suppression of NK cytotoxicity against PRRSV-infected cells. However, NK-mediated lysis of

UV-inactivated PRRSV inoculated PAMs was still higher than PRRSV inoculated PAMs (P=0.06). This suggests that transcription of viral genes and expression of viral proteins also contribute to the resistance of PPRSV-infected PAMs towards NK-mediated lysis.

NK cells lyse virus-infected cells by granzyme and perforin degranulation. CD107a, known as a lysosome-associated membrane protein 1 (LAMP-1), is a 110–140 kDa type I membrane glycoprotein. It becomes transiently expressed at the cell surface during degranulation and can therefore be used as a marker of cell degranulation induced by stimulation. The results of degranulation staining showed that NK cells incubated with PRRSV-infected PAMs did not increase the expression of CD107a (Fig. 4). This is an indication that the suppressed NK mediated lysis of PRRSV-infected PAMs is partly due to an inhibition of a process before degranulation. Previously, Sarah Costers et al. showed that proliferating $CD3^{+}CD8^{high}$ cells fail to exert cytolytic T lymphocytes (CTLs) activity towards PRRSV-infected PAMs (Costers et al., 2009). Both NK cells involved in the innate immune response and CTLs involved in the adaptive immune response belong to the group of cytotoxic lymphocytes (CLs), and they can use the same elimination strategy to destroy invading microorganisms. Taken together, it may be hypothesized that PRRSV infection inhibits both NK and CTL activity with a common mechanism.

To investigate the mechanisms underlying the PRRSV-mediated inhibition of NK-cell function, we studied the role of soluble factors in this phenomenon, since it has been reported that some soluble factors may have inhibitory properties against NK activity (Giacomelli et al., 1999). The results suggest that the suppressed NK activity against PRRSV-infected PAMs was not mediated by soluble factors.

Some previous studies reported that during a PRRSV infection *in vivo* a significant reduction in innate NK cell-mediated cytotoxic function in PRRSV-infected pigs was detected (Renukaradhya et al., 2010). These authors speculated that the impaired cytotoxicity function of NK cells was due to insufficient NK activation by a lower level

of activating cytokines, such as IFN-α. However, in the present study it was shown that even upon activation by rpIL-2, NK cytotoxicity against PRRSV-infected PAMs was still suppressed *in vitro*. This observation indicates that the insufficient NK activity post PRRSV infection is not only due to an insufficient activation of NK cells. Apparently, PRRSV-infected PAMs use some strategies to resist NK cytotoxicity. NK cells express both inhibitory and activating receptors, which directly influence the outcome of NK cell activation. For different viruses, it has recently been shown that some virus-encoded gene products engage receptors on NK cells to avoid NK cell-mediated responses (Orange et al., 2002). For example, CD81 ligation by hepatitis C virus HCV-E2 protein potently inhibits NK cell functions through a novel negative signaling mechanism (Crotta et al., 2002). Influenza virus attenuates NK cell lysis by reducing the binding affinity of hemagglutinin (HA) to activating NK receptors (Owen et al., 2007). It could be speculated that during PRRSV infection, the virus modulates the ligands for NK receptors on the surface of PAMs, which may subsequently lead to an insufficient NK cytotoxicity. To investigate which receptor(s) and ligand(s) are potentially involved in the suppression of NK cytotoxicity against PRRSV-infected PAMs, further studies are needed.

In conclusion, our results revealed that rpIL-2 activated porcine NK cells *in vitro*. However, the cytotoxicity of activated NK cells against PRRSV-infected PAMs was insufficient. Co-incubation with PRRSV-infected PAMs inhibited the degranulation of NK cells. The suppressed NK activity was not related to MHC class I expression or soluble factors released during PRRSV infection but was associated with viral protein expression in the PAMs. This study is the first report directly presenting that PRRSV-infected host cells (PAMs) show a reduced susceptibility towards NK cytotoxicity. The insufficient NK cytotoxicity may represent one of the PRRSV evasion strategies, which allow the virus to persist.

Acknowledgments

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CHAPTER 4

IMMUNOENHANCEMENT OF CD40 LIGAND AGAINST PRRSV

CD40 ligand expressed in adenovirus can improve the immunogenicity of the GP3 and GP5 of porcine reproductive and respiratory syndrome virus in swine

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Vaccine (2010)

Porcine reproductive and respiratory syndrome virus (PRRSV) has recently caused heavy economic losses in swine industry worldwide. Current vaccination strategies only provide a limited protective efficacy, thus immune modulators are being considered to enhance the effectiveness of PRRSV vaccines. In this study, the recombinant adenoviruses expressing porcine CD40 ligand (CD40L) and GP3/GP5 of PRRSV were constructed and the immune responses were examined in pigs. The results showed that rAd-CD40L-GP35 (co-expressing CD40L and GP3-GP5) or rAd-GP35 (expressing GP3-GP5) plus rAd-CD40L (expressing CD40L) could provide significant higher specific anti-PRRSV ELISA antibody and neutralizing antibody. And the levels of proliferative responses of peripheral blood mononuclear cells (PBMC), IFN-γ and IL-4 were markedly increased in rAd-CD40L-GP35 and rAd-CD40L plus rAd-GP35 groups than those in rAd-GP35 group. Following homologous challenge with Chinese isolate of the North-American genotype of PRRSV, pigs inoculated with recombinant rAd-CD40L-GP35 and rAd-CD40L plus rAd-GP35 showed lighter clinical signs and lower Viremia, as compared to those in rAd-GP35 group. It indicated that porcine CD40L could effectively increase humoral and cell-mediated immune responses of GP3 and GP5 of PRRSV. Porcine CD40L might be used as an attractive adjuvant or immunotargeting strategies to enhance the PRRSV subunit vaccine responses in swine.

4.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped single-stranded, positive-sense RNA virus. It is a member of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales*. The PRRSV genome with a size of approximately 15 kb contains 9 open reading frames (ORFs). ORF 1a and 1b encoded for non-structural proteins, and ORF 2-7 for structural proteins (Meulenberg et al., 1995; Murtaugh et al., 1995). Among them, GP3, GP4 and GP5 are associated with the development of neutralizing antibodies and protection (Jiang et al., 2008; Yang et al., 2000).

This economically important pandemic disease causes reproductive failure in breeding stock and respiratory tract illness in young pigs all over the world. Especially, since the outbreak of high pathogenic PRRSV in 2006, a great lost has happened in swine industry in China (Li et al., 2008; Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008). Current vaccine strategies have included live attenuated virus (Tian et al., 2009), live vector engineering vaccines (Li et al., 2009b) and DNA vaccines (Jiang et al., 2009). However, they can't effectively induce body immune response. Co-delivery of immunomodulators is being considered as an approach to enhance the effectiveness of PRRSV vaccines.

CD40L belongs to the tumor necrosis factor super family and has a central role in the development and regulation of adaptive immune responses in mammals and avian species (Bodmer et al., 2002; Gares et al., 2006; Locksley et al., 2001). It is a type II integral membrane glycoprotein expressed on activated but not resting T cells (Klaus et al., 1997), activated B cells (Higuchi et al., 2002), and activated platelets (Danese et al., 2003; Henn et al., 1998). Binding of CD40L, to its receptor, CD40, on the surface of B cells stimulates B cell proliferation, adhesion, and differentiation (Armitage et al., 1992), engagement of CD40 leads to B-cell clonal expansion, germinal center formation, isotype switching, affinity maturation, and the generation of long-lived plasma cells (Garside et al., 1998). And CD40 ligand may be solely responsible for

delivering the initial Th-dependent contact signal to B cells. Soluble CD40L generated through proteolytic cleavage of the extracellular domain is biologically active, and multimeric forms, many-trimer forms of soluble CD40L are more active (Fanslow et al., 1994; Graf et al., 1995; Pullen et al., 1999). Because of the multiple effects of CD40L on immunocytes, CD40L has been used in various vaccination strategies (Gomez et al., 2009; Huang et al., 2004; Liu et al., 2008). In this study, we examined the use of CD40L as an immunotargeting agent and immunostimulator to enhance PRRSV immune response in mice and pigs.

4.2 Material and Methods

4.2.1 Viruses and cells

Both recombinant adenovirus and wild-type adenovirus (wtAd, constructed and kept in our Lab) were propagated and tittered in HEK-293A cells (ATCC CRL1573). MARC-145 cells were used to propagate and titer PRRSV SY0608 strain (isolated and kept in our lab, the parental strain was very virulent, 30 passages virus culture was used to challenge in this study) (Li et al., 2007). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. The infected cell lysate was clarified, titrated, diluted to 1×10^4 $TCID_{50}$ and stored at −20 ℃ to be used for animal challenge. A mock-infected cell culture lysate was prepared by the same method.

4.2.2 RT-PCR for amplification of extracellular domain of porcine CD40L gene

The primers were designed for amplifying extracellular domain of CD40L gene as Table 1, based on gene sequence of porcine CD40L gene (GenBank accession number AB040443). Porcine spleen cells from 8-week-old Yorkshire swine were isolated by mechanical disruption and filtration through a 75 μm cell filter followed by hypotonic lysis of erythrocytes, then stimulated with ConA (10μg/ml) for 24h in vitro. Total RNA

was extracted using TRIzol® Reagent (Invitrogen) as the manufacturer's protocol. The cDNA was synthesized using oligo $d(T)_{15}$ primers (Promega). CD40L gene was amplified from cDNA with the primers CD40L-1 and CD40L-2. The amplification was performed in a 50 μl reaction mixture containing 1.5 mM $MgCl_2$, 1× PCR buffer, 0.2mM of each dNTP, 10 pmol of each primer, 1.5 U of TaqDNA polymerase (Promega) and 2μl of cDNA. The reaction was run in a thermocycler (PTC-150) with the following program: initial denaturation at 94 ℃ for 5 min was followed by 33 cycles of denaturation at 94 ℃ for 40 s, annealing at 58 ℃ for 40 s and extension at 72 ℃ for 1 min, and was ended with a final extension step of 10 min at 72 ℃.

4.2.3 Amplification of GP3 and GP5 gene of PRRSV

To amplify GP3 and GP5 genes from PRRSV SY0608, the primers GP3-1 and GP3-2 for GP3 gene, the primers GP5-1 and GP5-2 for GP5 gene, were designed based on the sequence of PPRSV SY0608 isolate (GenBank accession No. EU144079) (Li et al., 2007) as Table 1.

The viral RNA was extracted from PRRSV SY0608 isolate using TRIzol® Reagent as the instruction of manufacture. Reverse transcription was performed at 50 ℃ for 60 min with 13 μl total RNA, 1 μl SuperScript III RT (GIBCOBRL), 1 μl $oligo(dT)_{15}$, 1 μl 0.1 M dithiothreitol, 4μl 5×RT buffer and 1 μl 10 mM dNTPs. The amplification was performed as above.

Table1. Primer sequences for amplification of porcine CD40L gene and GP3/GP5 genes

Name	Sequence	Amplified gene
GP5-1	5’-ACG*AAGCTT*ATGTTGGGGAAGTGCT-3’ (*Hind*III)	GP5
GP5-2	5’-CAG*ATATCC*TAGAGACGACCCCATTG-3’ (*Eco*RV)	
GP3-1	5’-GAC*CTCGAG*ATGGCTAATAGCTGTACATT-3’ (*Xho*I)	GP3
GP3-2	5’-ACA*AAGCTT*TCGCCGTGCGGCACT-3’ (*Hind*III)	
CD40L-1	5’-GCT*GGTACC*ATGATCGAAACGTACA-3’ (*Kpn*I)	CD40L
CD40L-2	5’- GAT*CTCGAG*TTAGAGTTTGAGGAGG-3’ (*XhoI*)	
CD40L-3.1	5’- GACGTCGCCGGCCAACTTGAGAAGGTCAAAGTTGAGTTTGAGGAG-3’ (partial 2A gene)	
CD40L-3.2	5’-GCA*CTCGAG* GGGCCCTGGGTTGGACTCGACGTCGCCGGCCAACTTGAG-3’ (*Xho*I 2A gene)	

4.2.4 Construction of recombinant adenoviruses rAd-GP35, rAd-CD40L, rAd-CD35

PCR amplicons of GP5 and CD40L were respectively digested with *Hin*dIII/*Eco*RV, and *Kpn* I/*Xho* I, and cloned into pShuttle-CMV by which resulting in a recombinant plasmid pShuttle-CMV-GP5 and pShuttle-CMV-CD40L, and then pShuttle-CMV-GP3-GP5 was obtained by cloning GP3 into pShuttle-CMV-GP5 using *Xho* I and *Hind* III (Fig.1). In order to obtain the CD40L-GP3-GP5 fusion protein gene, other PCR primers, CD40L-3.1 and CD40L-3.2, were designed based on 2A of foot and mouth disease virus (FMDV) type A12 strain 119 (GenBank accession no. M10975) which encodes the 17 amino acids self-cleaving 2A protease (NFDLLKLAGDVESNPGP) as reported (Groot Bramel-Verheije et al., 2000; Robertson et al., 1985) (Table 1). And finally the CD40L-2A gene was cloned into pShuttle-CMV-GP3-GP5 vector using *Kpn* I and *Xho* I site (Fig.1). All the recombinant plasmids were sequenced to confirm the correct tandem in frame insertion of CD40L,

GP3 and GP5 gene.

The adenovirus recombinants were produced as described previously (Jiang et al., 2006). The recombinant adenoviruses were propagated in HEK-293A cells and purified with plaque test by three times, and named rAd-CD40L (expressing porcine CD40L), rAd-GP35 (co-expressing GP3 and GP5), rAd-CD40L-GP35 (co-expressing GP3/GP5 fused with CD40L using 2A gene of FMDV as a linker), respectively. Tissue culture infectious dose 50 ($TCID_{50}$) of purified viruses were determined.

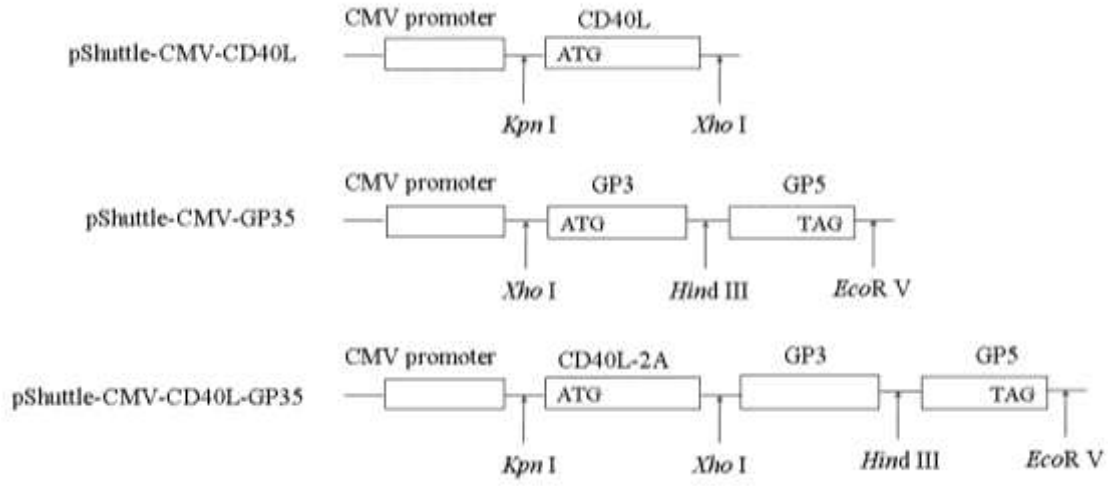

Fig.1. Schematic diagrams of recombinant transfer vectors. The CD40L, GP3 and GP5 gene were cloned into pShuttle-CMV tandem in frame. The linkers between these genes were 5× Glycine or 2A gene of FMDV.

4.2.5 Identification of expression CD40L, GP3 and GP5

Western blot assay

Western blots were used to evaluate proteins expression by infection of 293 cells using recombinant adenoviruses. Large-scale virus preparation was made for electrophoresis and western blotting as previously described (Jiang et al., 2006). Briefly, the lysates of HEK-293A cells infected with recombinant adenoviruses or wtAd were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation). The membrane was incubated with PRRSV specific antiserum (1:100

diluted in PBS containing 0.5% Tween80, PBS-T)or with mouse anti-CD40L (aa48-262) serum (made in our lab by vaccination of mice with purified truncated CD40L (aa48-262) expressed by pET-32a (+) vector in *E.coli* BL21, 1:100 diluted in PBS-T) for 2 h at 37℃ followed by incubation for 1 h at 37℃ with horseradish peroxidase-conjugated Staphylococcal Protein A (SPA-HRP, Boshide, Wuhan, China) or goat-anti-mouse-IgG-HRP (Boshide, Wuhan, China). Detection was performed using chemiluminescence luminol reagents (SuperSignal West Pico Trial kit, PIERCE).

Immunofluorescence assay (IFA)

In order to determine the interest genes expression, HEK-293A cells inoculated with recombinant adenoviruses or wtAd in 96-well culture plate were rinsed with PBS and fixed with cold ethanol for 45 min at 4℃. The cells were washed, and then incubated with PRRSV specific antiserum or with mouse anti-CD40L serum as above for 1 h at 37℃. After washing with PBS-T, the cells were incubated with fluorescein conjugated SPA or goat anti-mouse IgG conjugated with fluorescein (1:50 diluted in PBS-T) for 1 h at 37℃.After rinsing by five times, cells were kept in PBS and observed under a ZEISS fluorescence microscope.

4.2.6 Animal experiments

Immune responses in mice

A total of 90, 6-week-old female BALB/c mice (provided by the Animal Center of Nanjing General Hospital, Nanjing, China) were randomly divided into six groups each with 15. Group 1 was inoculated with $5\times10^{8.0}$ TCID50 of rAd-CD40L and $5\times10^{8.0}$ $TCID_{50}$ of rAd-GP35 in 0.2 ml PBS. Groups 2–5 were individually inoculated with rAd-CD40L-GP35, rAd-GP35, rAd-CD40L and wtAd at the same dose of $5\times10^{8.0}$ $TCID_{50}$ in 0.2 ml PBS. Group 6 was inoculated with 0.2 ml PBS. All groups were inoculated subcutaneously twice at 3-week intervals. At 21, 35 and 49 days post primary immunization (dpi), five mice from each group were euthanized and the sera were harvested for the detection of antibodies against PRRSV and the lymphocytes

were separated from the spleen of each mouse individually for the detection of PRRSV-specific cell mediate immune (CMI) responses. Meanwhile, the supernatants of the lymphocytes stimulated with PRRSV SY0608 isolate (M.O.I. = 1.0) at 35 dpi and 49 dpi were obtained to detect the levels of the Th1-type cytokine of IFN-γand Th2-type cytokine of IL-4.

Immune responses in pigs

Twenty-five 21-day-old crossbreed (Landrace×local stock) swine free of PRRSV were randomly divided into five groups and housed in separate rooms. Four groups were immunized intramuscularly at day 0 and boosted at day 14 with $10^{10.0}$ $TCID_{50}$ rAd-CD40L+rAd-GP35, rAd-CD35, rAd-GP35 and wtAd in 2ml PBS, respectively. One control group received 2ml PBS following the same immunization protocol. The sera were collected from each pig at 14, 28 and 35 dpi to detect antibody to PRRSV using indirect enzyme-linked immunosorbent assay (iELISA) and virus neutralization assay. At the mean time heparinized blood was used to isolate peripheral blood mononuclear cells (PBMCs) for T-lymphocyte proliferation assay. Meanwhile, the supernatant of the lymphocytes stimulated with PRRSV SY0608 isolate at 14 dpi and 35 dpi were obtained to detect the levels of IFN-γ and IL-4. All groups were challenged intranasally at 35 dpi with $2\times10^{4.0}$ $TCID_{50}$ PRRSV SY0608 strain. Animals were monitored for 21 days after challenge. Rectal temperatures and clinical signs were observed daily. The blood samples were collected from all animals at 0, 7, 14 and 21 days post challenge (dpc) for detection of PRRSV.

Indirect ELISA (iELISA)

For the titration of the antibodies present in both the pigs and mice sera, the SY0608 PRRSV antigen was purified by ultracentrifugation and quantitated by optical density (OD) measurement as described previously (Jiang et al., 2006). It was used as iELISA antigen and coated in 96-well plates at the concentration of 1.6 μg/ml. The plates were blocked with 0.15% BSA in PBS. The sera of mouse or pigs were diluted in

PBS-T and added into the plates. After incubation for 60 min at 37 ℃, the wells were washed three times and incubated with Goat anti-mouse IgG-HRP or SPA-HRP for 60 min at 37 ℃. The plates were incubated with substrate solution Tetramethyl benzidine (TMB) at 37 ℃ for 15 min and the reaction was stopped with 2M H_2SO_4. The optical density (OD) was read at 450 nm in an ELISA reader. Meanwhile, the uninfected MARC-145 cells antigen coated in 96-well plates following the same protocol were used to describe the level of the background response. The results were expressed as the ratio of OD450 nm produced by the serum samples minus the background response.

Serum neutralization assays

SN assays were performed as previously described (Jiang et al., 2006). All serum samples from mice and swine were heat inactivated (56 ℃, 30 min) and serially diluted. Then the serial dilutions of serum were mixed with equal volume of PRRSV isolate SY0608 containing 100 $TCID_{50}$. After incubation at 37 ℃ for 1h, the mixtures were transferred to MARC-145 monolayers in 96-well tissue culture plates. After incubation for 96h at 37 ℃ in a humidified atmosphere containing 5% CO_2, cells were examined for cytopathic effects (CPE). CPE was used to determine the end-point titers that were calculated as the reciprocal of the highest serum dilution to neutralize 100 $TCID_{50}$ of PRRSV in 50% of the wells.

T-lymphocyte proliferation assay

Lymphocytes were isolated individually from the spleen of each mouse or heparinized blood of swine with lymphocyte separation medium (Boshide, Wuhan,China), and suspended in RPMI-1640 complete medium (RPMI 1640 containing 10% FCS). Subsequently, these cells were cultured in triplicate at 5×10^5 per well in 96-well flat-bottomed tissue culture plates in 100 μl volumes of RPMI-1640 complete medium. The culture was stimulated with PRRSV isolate SY0608 (M.O.I. = 1.0). After incubation for 72 h at 37 ℃ with 5% CO_2, the proliferation responses were detected by the standard MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium

bromide) (1 mg/ml, Sigma) method. T-lymphocyte proliferation was expressed as stimulation index (SI), which is the average ratio of OD570 value of stimulated wells to the average OD570 value of wells containing mock antigen.

Cytokine assays

The secretion of IFN-γ and IL-4 were detected by commercially cytokines quantitative ELISA kit. The lymphocytes (5×10^6/ml, 100 μl/well) were isolated from the spleen of mice and PBMC from the pigs and stimulated with purified PRRSV antigen at the final concentration of 20 μg/ml. After 72 h, the cells were centrifuged and the supernatant was collected to examine the level of IFN-γ and IL-4 following the manufacturer's instructions (mouse IFN-γ/IL-4 kits, ADL USA; porcine IFN-γ/IL-4 kits, Biosource).

Real-time PCR

Total RNA was extracted from 300μl serum collected at 0, 3, 7, 14 and 21 dpc using TRIzol® Reagent. cDNA was synthesized using oligo$(dT)_{15}$ and was performed as mentioned above. SYBR Green real-time PCR was performed to evaluate PRRSV ORF7 mRNA level, using the sequence of sense primer: 5'-AAT AAC AAC GGC AAG CAG CAG-3'and antisense primer: 5'-CCT CTG GAC TGG TTT TGT TGG-3'. The cDNA was used as the template. The reaction was performed at 95℃ for 2min, followed by 40 cycles of 95℃ for 15 s and 61 ℃ for 1min using the ABI 7300 detection system. For quantification, cDNA of known $TCID_{50}$ of virus containing the ORF7 gene were 10-fold serially diluted and were used to generate standard curve. Virus quantity of the unknown samples was determined by linear extrapolation of the Ct value plotted against the standard curve.

4.2.7 Statistics

The differences in the level of humoral responses, cytokine production and viremia between different groups were determined by One-way repeated measurement

ANOVA and Least significance difference (LSD). Differences were considered statistically significant when $P < 0.05$.

4.3 Results

4.3.1 Construction and characterization of recombinant viruses

The shuttle vectors individually encoding CD40L, GP3/GP5 and CD40L-GP3/GP5 were constructed and DNA sequencing confirmed that the nucleotide sequence of the insert genes in recombinant plasmids had the same sequence as designed, as well as in the proper open reading frame. After homologous recombination between the shuttle plasmids and vector backbone pAdEasy-1 in *BJ5183* cells, three adenoviral plasmids pAd-CD40L, pAd-GP3-GP5 and pAd-CD40L-GP3-GP5 were obtained. Subsequently, by transfection of HEK-293A cells with these plasmids, three recombinant adenoviruses, rAd-CD40L, rAd-GP35 and rAd-CD40L-GP35, were produced, respectively. The titers of the recombinant adenoviruses were $10^{9.25}$ $TCID_{50}$ /ml (rAd-CD40L-GP35) and $10^{10.0}$ $TCID_{50}$/ml (rAd-CD40L and rAd-GP35).

Expressions of the foreign proteins were detected by Western Blotting and IFA with antibodies to CD40L and GP3/GP5 of PRRSV. The results of Western Blotting showed that specific protein bands were consistent with the predicted size of the GP3/GP5 and CD40L-GP35, whereas no specific protein band was found in wtAd-infected HEK-293A cells (Fig.2a.). Meanwhile, the results of IFA showed that recombinant adenoviruses infected HEK-293A cells could be stained with PRRSV-specific or/and CD40L-specific antibodies and SPA-FITC or goat anti-mouse IgG-FITC, but wtAd-infected cells could not be stained (Fig. 2b).

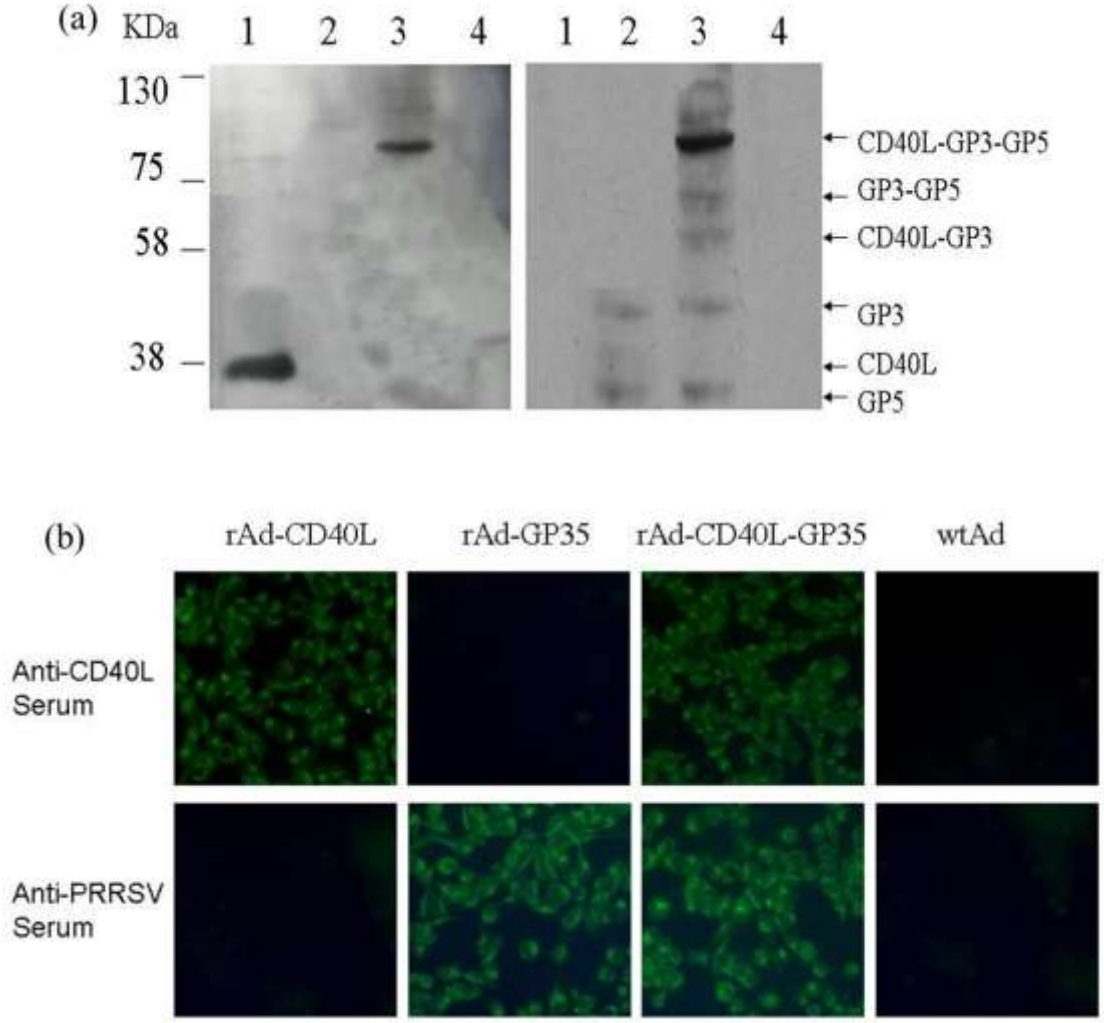

Fig.2. Identification of expression of CD40L, GP3 and GP5 in vitro. (a)Western blot analysis of cell lysates infected with, rAd-CD40L (lane 1), rAd-GP35 (lane 2), rAd-CD40L-GP35 (lane 3), and wild type adenovirus (wtAd, lane 4), respectively, by using mouse anti-CD40L (aa48-262) serum (left) or pig anti-PRRSV serum (right). Proteins standards are showed on left side of panel. (b) IFA analysis of 293 cell monolayers infected with the recombinant adenoviruses by using mouse anti-tCD40L (aa48-262) serum or pig anti-PRRSV serum.

4.3.2 Immune responses in mice

Humoral immune responses of rAd-CD+35, rAd-CD35 and rAd-GP35, rAd-CD40L

The serum took at 21, 35, 49 dpi were used to detect the PRRSV specific antibody

level. As shown in Fig.3a, rAd-CD40L plus rAd-GP35 group induced significantly higher level of PRRSV specific antibody at 21, 35 and 49 dpi comparing with rAd-GP35 group ($P < 0.05$). The rAd-CD40L-GP35 group could also induce high level of PRRSV specific antibody but lower than rAd-CD40L plus rAd-GP35 group. And comparing with rAd-GP35, it only showed significantly higher level at 49 dpi. Meanwhile, the antibody against adenovirus antigen was also measured with ELISA. There were no significant differences among groups immunized with recombinant adenoviruses and wtAd ($P > 0.05$) (data not shown).

The ability of sera from rAds-inoculated mice to neutralize PRRSV infectivity in vitro was also investigated. The results indicated that the levels of neutralizing antibodies in rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 were also markedly higher than that in with rAd-GP35 at 35 dpi (Fig.3b). No neutralizing antibodies against PRRSV could be detected in mice immunized with wtAd or PBS.

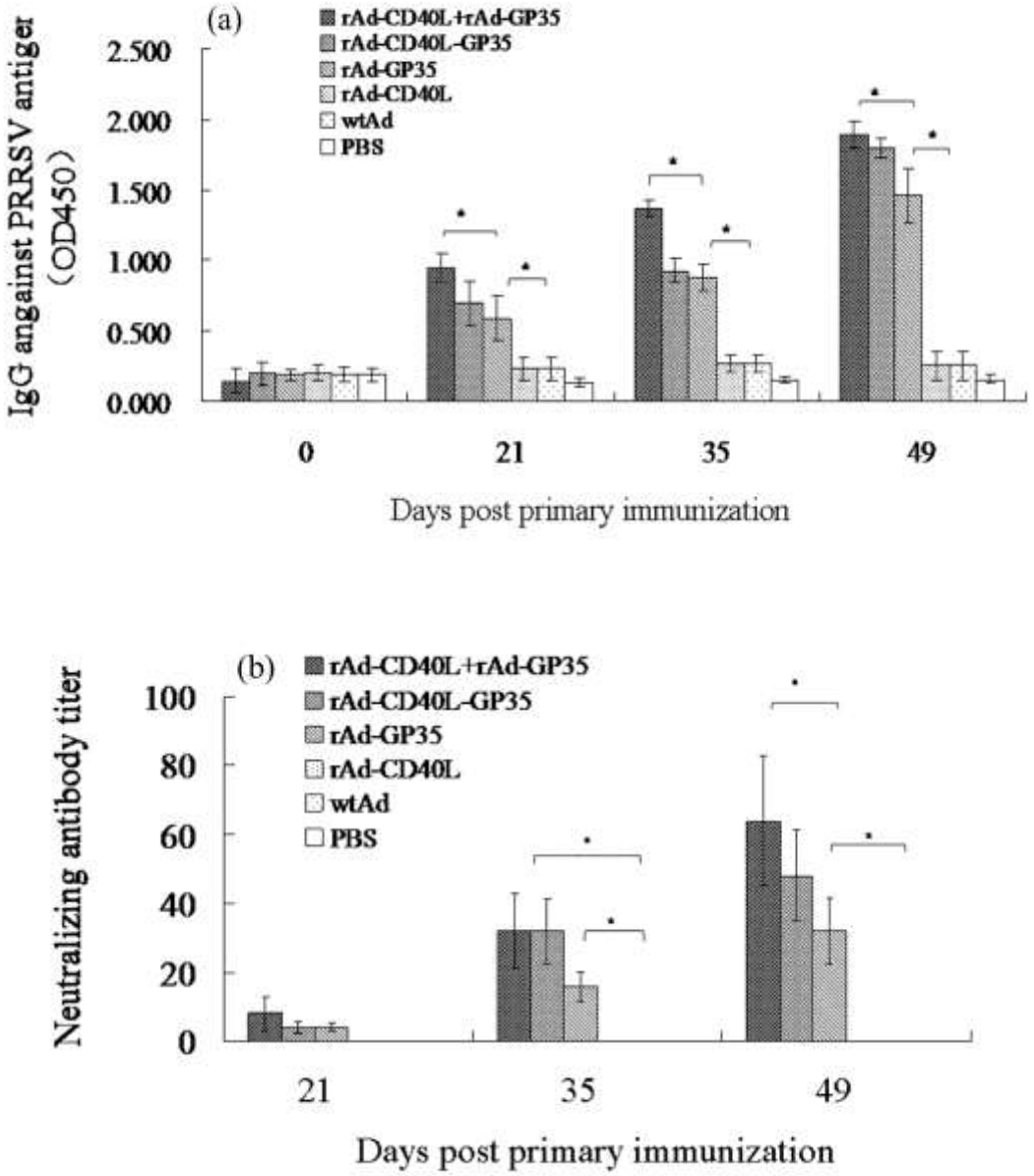

Fig.3. Humoral immune responses in mice vaccinated with the recombinant adenoviruses. Serum samples (n = 5) were collected at various time-points and antibodies to PRRSV were detected using iELISA (a) and neutralizing assay (b). The titers of neutralizing antibodies were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Data were shown as mean ±S.D.

T-lymphocyte proliferation responses

At days 35 and 49 dpi, spleen cells were pooled and the PRRSV-specific

lymphocyte proliferation responses were detected. The results showed that the recombinant adenovirus rAd-CD40L plus rAd-GP35 could induce significant higher levels of the proliferation at 35 and 49 dpi, comparing with rAd-GP35 ($P < 0.05$) (Fig.4). This result revealed that CD40L improved the immune response of the GP3/GP5 in mice.

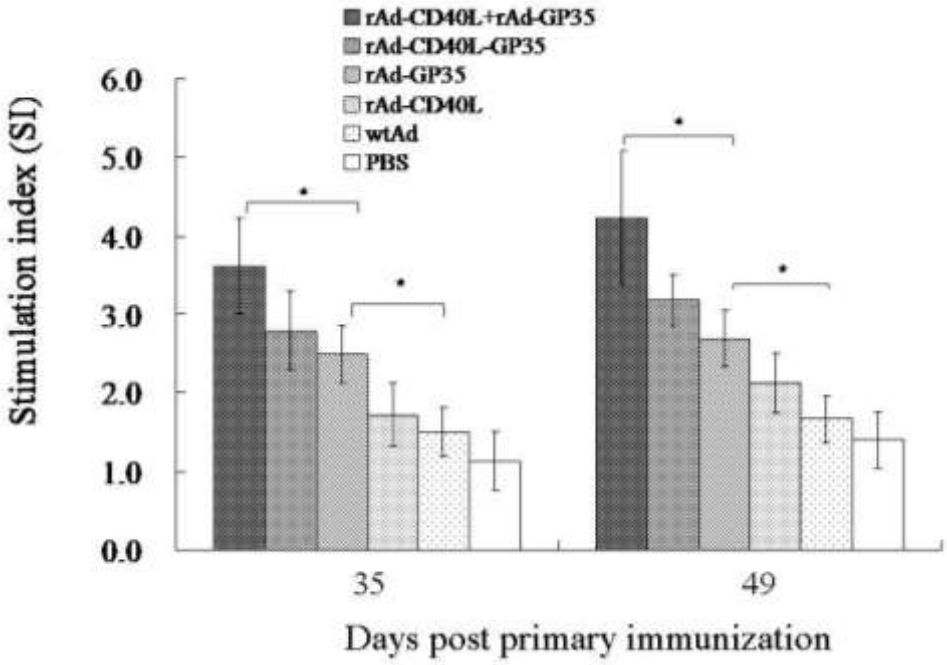

Fig.4. Lymphocyte proliferative responses in mice immunized with recombinant adenoviruses. Splenocytes samples (n =5) were collected at days 35 and 49 post primary immunization (dpi) and were stimulated with purified PRRSV antigen in triplicate. After 72 h of stimulation, the proliferation responses were detected by a standard MTT method. Data were shown as mean ±S.D.

Th1-type and Th2-type cytokine responses

The production of IFN-γ and IL-4 were analyzed at 49 dpi by quantitative ELISA kits. As shown in Fig.5, The groups of rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 had markedly higher levels of IFN-γ production than rAd-GP35 ($P < 0.05$). But they did not provide significant higher levels of IL-4 than rAd-GP35 ($P > 0.05$). It indicated that Th1-type immune response was enhanced by the CD40L.

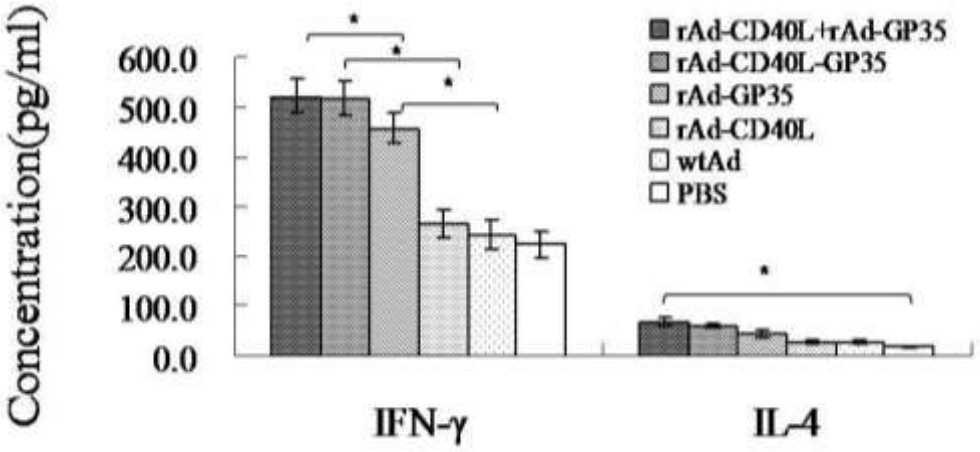

Fig.5. Concentration (pg/ml) of Th1-type cytokine of IFN-γ and Th2-type cytokine of IL-4 in the supernatants of stimulated splenocytes of mice. Lymphocytes isolated from the spleen of mice (n = 5) at 35 dpi were stimulated with purified PRRSV antigen. After 72 h, the supernatant were collected to examine the levels of IFN-γ and IL-4 using commercially available mice cytokine ELISA kits. Data were shown as mean ±S.D.

4.3.3 Immune responses of recombinant adenoviruses in pigs

Humoral immune responses

The immunogenicity of recombinant adenoviruses was further investigated in pigs. As shown in Fig.6a, Anti-PRRSV antibody in pigs vaccinated with rAd-CD40L plus rAd-GP35, rAd-CD40L-GP35 and rAd-GP35 could be detected by ELISA at 14 dpi, and increased after the booster. At 35 dpi, the levels of IgG from the group of rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 were significantly higher than that from the group of rAd-GP35 ($P < 0.05$). But no significant difference was observed between rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 groups ($P > 0.05$). Meanwhile, the antibody against adenovirus antigen was also measured with ELISA. There were no significant differences among groups immunized with rAds and wtAd ($P > 0.05$) (data not shown).

The results of neutralization assay indicated that the rAd-CD40L plus rAd-GP35 group provided significant higher levels of neutralizing antibodies than the rAd-GP35, wtAD and PBS groups ($P < 0.05$). There was no significant difference between the

group of rAd-CD40L-GP35 and rAd-GP35 (P > 0.05). Meanwhile, no neutralizing antibody was detected in wtAd and PBS group (Fig. 6b).

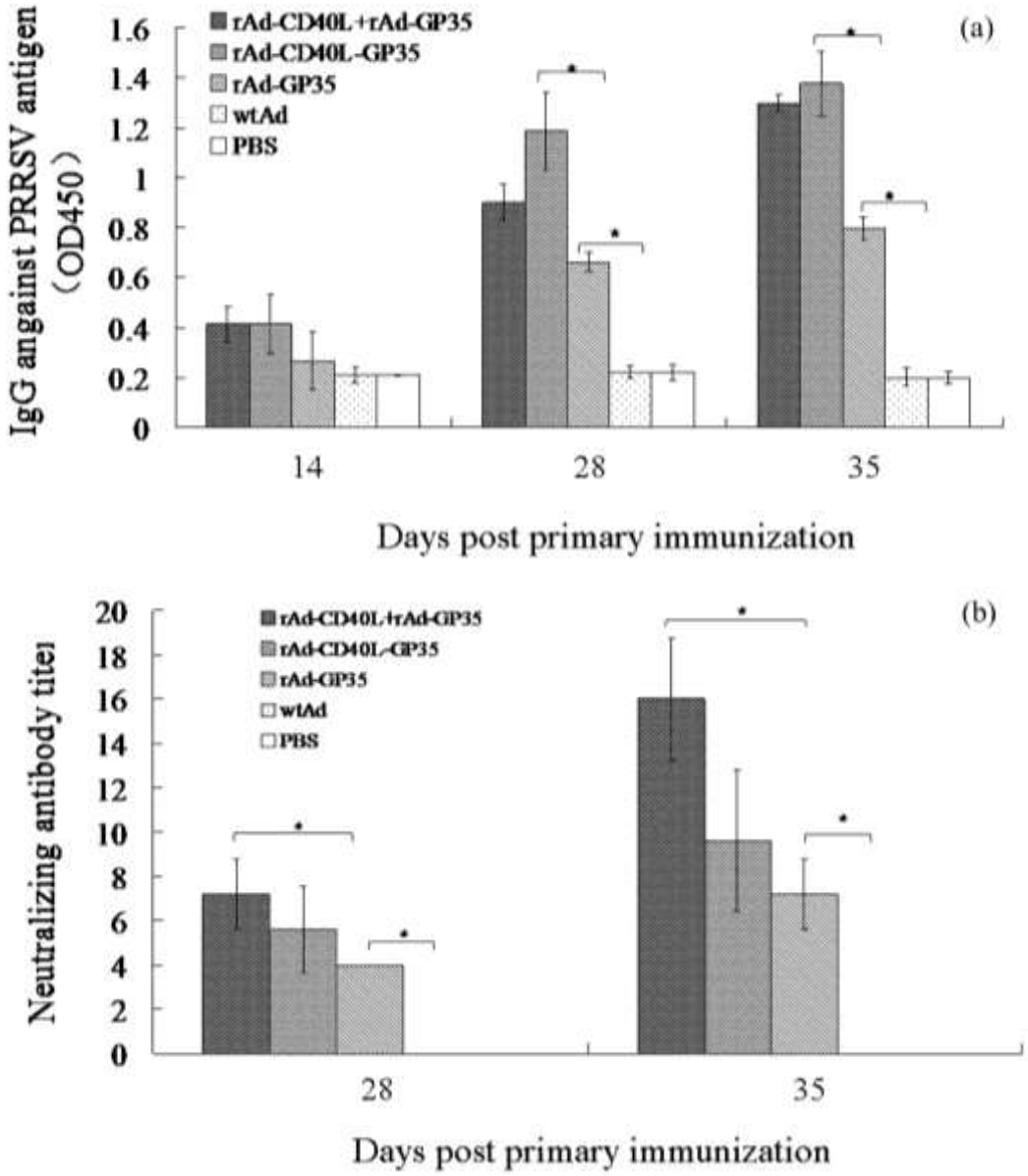

Fig.6. Humoral immune responses in pigs inoculated with the recombinant adenoviruses. Serum samples (n = 5) were collected at various time-points and antibodies to PRRSV antigen were detected using indirect ELISA (a) and neutralizing assay (b). The titers neutralizing antibodies were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Data were shown as mean ±S.D.

T-lymphocyte proliferation responses

As shown in Fig.7, recombinant adenovirus rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 could induce high level of T-lymphocyte proliferation. The difference between rAd-CD40L-GP35 and rAd-GP35 was significant at 35 dpi ($P < 0.05$). But there was no significant difference between the group of rAd-CD40L-GP35 and rAd-CD40L plus rAd-GP35 ($P > 0.05$).

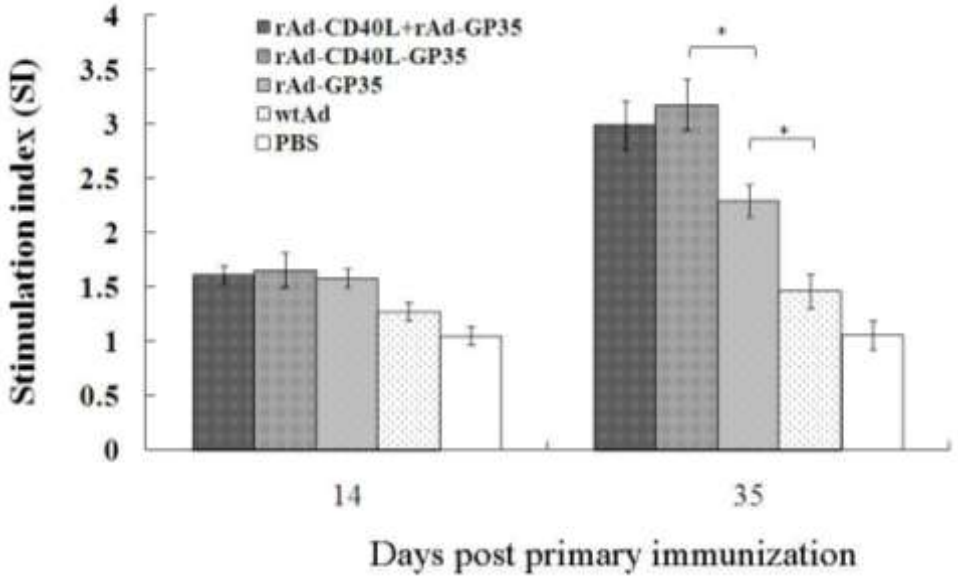

Fig.7. Lymphocyte proliferative responses in pigs immunized with recombinant adenoviruses. Splenocytes samples (n = 5) were collected at days 14 and 35 post primary immunization (dpi) and were stimulated with purified PRRSV antigen in triplicate. After 72 h of stimulation, the proliferation responses were detected by a standard MTT method. Data were shown as mean ±S.D.

Cytokine responses

Swine cytokines detection kits were employed to detect the production of IFN-γ and IL-4 at 35 dpi before challenge. The results showed that the mean level of serum IFN-γ was significant higher in groups that received rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35, comparing with that of rAd-GP35 ($P < 0.05$) (Fig.8a). For IL-4, significant difference was also observed between groups that received rAd-CD40L-GP35 and rAd-GP35 ($P < 0.05$) (Fig.8b). The results indicated that rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 could potentiate Th1-type and

Th2-type cytokine responses. And Th2-type cytokine responses were higher than the Th1-type cytokine responses.

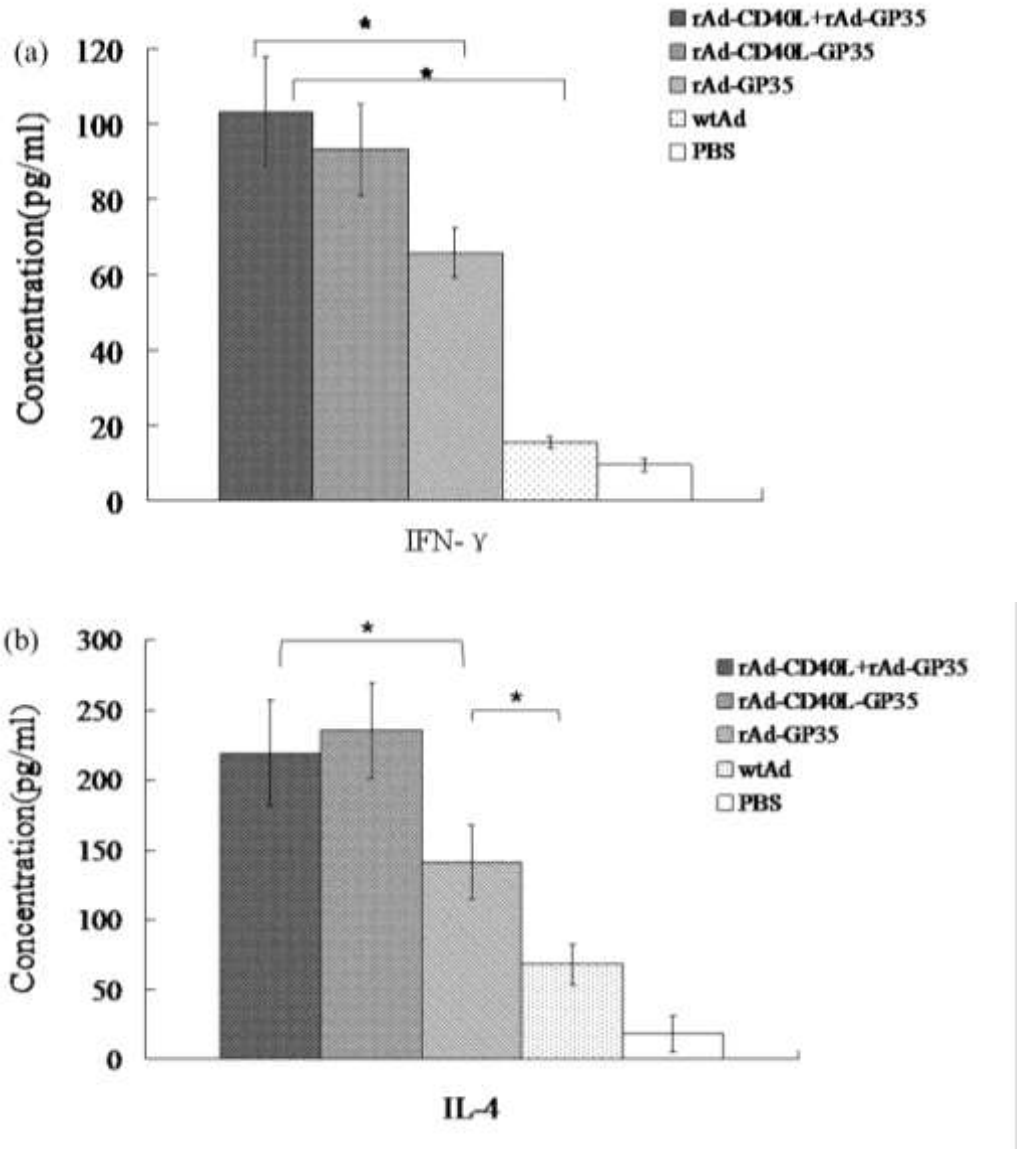

Fig.8. Concentration (pg/ml) of Th1-type cytokine of IFN-γ (a) and Th2-type cytokine of IL-4 (b) in the supernatants of stimulated PBMC of pigs. Lymphocytes isolated from the PBMC of pigs at 35 dpi were stimulated with purified PRRSV SY0608 virus antigen. After 72 h, the supernatant were collected to examine the levels of IFN-γ and IL-4 using commercially available pigs cytokine ELISA kits. Data were shown as mean ±S.D.

4.3.4 Protective efficiency against PRRSV challenge

Clinical signs and body temperature change

After challenge with virulent PRRSV, all pigs in wtAd control group had high fever (≥40 ℃) and displayed a range of clinical signs, including inappetence, lethargy, rough hair coats, dyspnoea, periocular oedema, eyelid oedema and lightly diarrhea. And similar light clinical signs were observed in group of rAd-GP35. But pigs immunized with rAd-CD40L plus rAd-GP35 and rAd-CD40L-GP35 only appeared low fever or a little fluctuation of rectal temperatures during 18 days post-challenge (Fig.9).

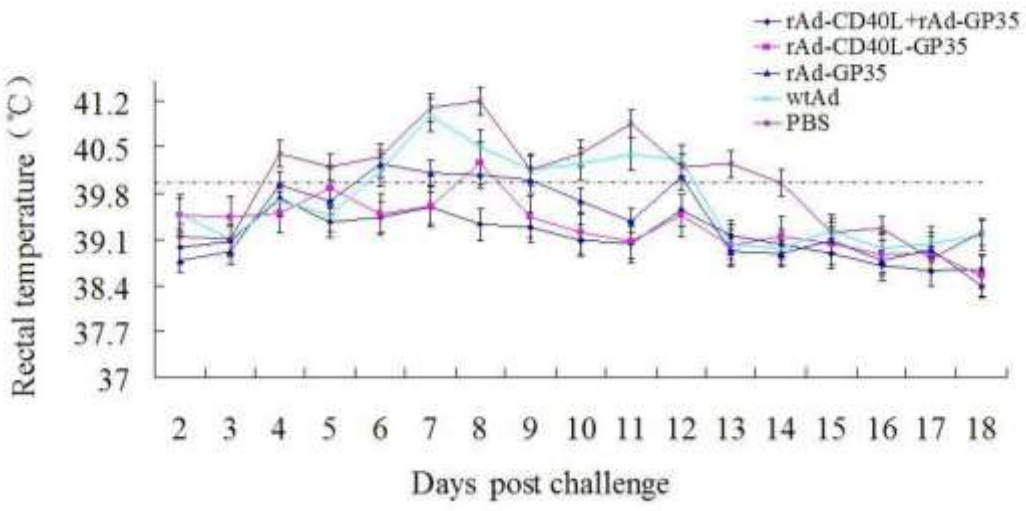

Fig.9. Mean rectal temperature of five pigs in each group inoculated with recombinant adenoviruses or wtAd/PBS and challenged with PRRSV SY0608 strain. Data were shown as mean±S.D.

Viremia after challenge

The blood samples of the pigs were collected at 0, 7, 14 and 21 days post-challenge. And the Viremia was monitored by Real-time PCR. At 7 dpc, pigs inoculated with rAd-CD40L plus rAd-GP35 showed a significantly lower Viremia in blood than that of wtAd ($P < 0.01$) and rAd-GP35 ($P < 0.05$). Meanwhile, pigs inoculated with wtAd had the highest viremia at both 7 and 14 dpc (Fig.10).

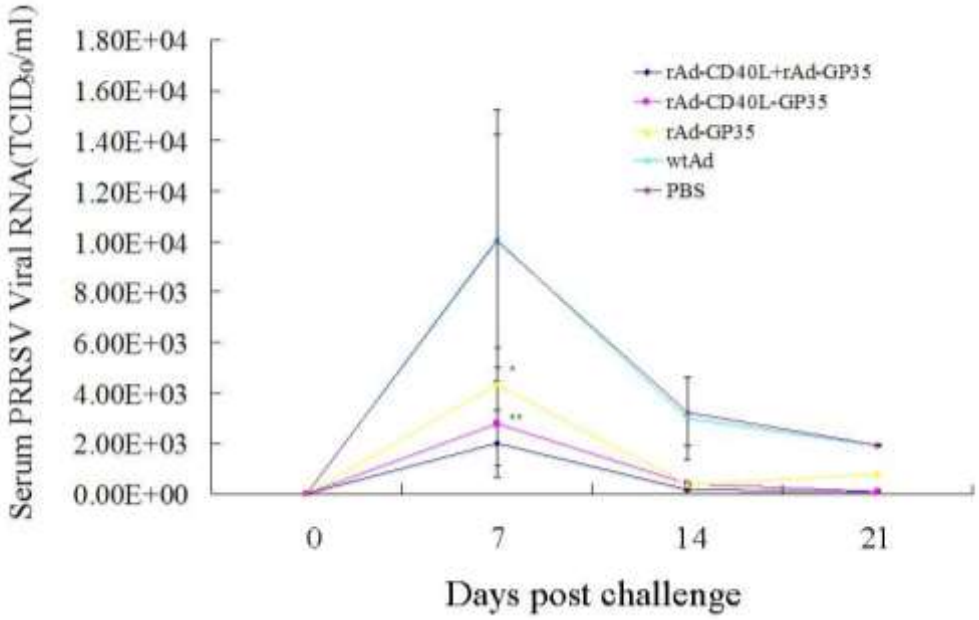

Fig.10. Viremia of pigs inoculated with recombinant adenovirus or wtAd/PBS and challenged with a PRRSV SY0608 strain. Viral RNA of serum was detected at 0 (before challenge), 7, 14 and 21 dpc. Data were shown as mean ± S.D. for five pigs per group.

4.4 Discussion

Porcine reproductive and respiratory syndrome, frustrating challenge to the global swine industry, is one of the most economically significant viral diseases of swine. Since the limited protection of current commercial vaccination strategies, PRRSV genetic engineered vaccines have recently been reported, including pseudorabies virus expressing GP5 (Qiu et al., 2005), recombinant fowlpox virus co-expressing GP5/GP3 and swine IL-18 (Shen et al., 2007), recombinant adenoviruses expressing GP5/GP4/GP3 (Jiang et al., 2008), and mycobacterium bovis BCG expressing GP5 and M (Bastos et al., 2002; Bastos et al., 2004). In order to increase the efficiency of the vaccine, an alternative approach is to co-deliver cytokines to up-regulate the immune response of PRRSV, including HSP70 (Li et al., 2009a), IL-18 (Shen et al., 2007), GM-CSF (Wang et al., 2009). In this study, porcine CD40L was amplified from Con A stimulated PBMC and recombinant adenoviruses expressing CD40L and GP3-GP5 of PRRSV were constructed. It was found that the porcine CD40L could effectively increase the humoral and cellular immune responses of GP3 and GP5 of PRRSV in

mice and pigs. Both fusing and mixing strategies of CD40L and GP3-GP5 could provide partly protective efficacy against PRRSV challenge in swine.

GP5 protein is a structural PRRSV protein with the size of 25 KDa. Both in *vitro* and in *vivo*, PRRSV neutralization is associated with antibodies against GP5 (Plagemann, 2004). The GP3 is a glycoprotein with high antigenic with the size of 45-50 KDa. It could provide protection for piglets against PRRSV infection in the absence of a noticeable neutralizing antibody response (Jiang et al., 2007). The GP3 and GP5, as well as GP3/GP5 or GP5/GP3 together has been widely used in inducing PRRSV protective immune response. In our previous research, GP3-GP5 fusing protein could induce higher level of immune response against PRRSV (Jiang et al., 2008). In this study, fusing protein CD40L -GP3-GP5 was expressed in adenovirus with the linker of 2A of FMDV. The results of Western-bolt in Figure 2b showed that there were some bands in rAd-GP35 and rAd-CD40L -GP35 lines, the size of which were similar to the GP5, GP3, CD40L-GP3, GP3-GP5 or CD40L -GP3-GP5 fusion protein. It might be relate to the decomposing of the fusion protein.

Usually, neutralizing antibodies play an important role in the immune protection. It is known that GP5 and M proteins have neutralizing epitopes [3], but only weak neutralizing antibodies could be induced by GP5 alone (Pirzadeh and Dea, 1998). In this study, the animal experiment results indicated that rAd-GP35 could provide neutralizing antibodies against PRRSV. But we could not ascertain whether these neutralizing antibodies were raised against both or just one of the GP5 and GP3 in the fusion protein, because of the lack of groups of animals vaccinated solely with either GP3 or GP5 (with and without CD40L), even thought GP3 could not induce neutralizing antibodies in mice [38] . Moreover, previous reports indicated that M protein might play an important role in enhanced neutralizing antibodies, and it was suggested that M-GP5 fusion protein might also boost the transport of GP5 from ER to the Golgi complex, making authentic post-translational modifications, resulting in more immunogenic GP5, or some neutralizing conformation-depended epitopes of GP5 or M alone might become available to cells involved in immune responses by

interaction between GP5 and M protein in M-GP5 fusion protein (Jiang et al., 2006). Here, it was interesting that CD40L probably enhanced the amount of neutralizing antibodies but did not accelerate the kinetics of the response. It might be related to the fact that the structures or the transport of GP5 in the cells has not been changed by CD40L as M protein of PRRSV. Its mechanism should be studied in the future.

CD40L is a co-stimulatory molecule that is expressed primarily by activated T cells, and is essential in both cellular and humoral immunity. It plays an important role in the development of Th1 type responses (Balasa et al., 1997; Cella et al., 1996), antibody isotype switching and antibody production (Borrow et al., 1996; Oxenius et al., 1996), productive T cell-dendritic cell and T cell-macrophage interactions, and the anti-viral immune response (Yang et al., 1996). CD40L ligation of CD40 on B cells influences various stages in B cell development (Banchereau et al., 1994; Foy et al., 1994; Van Kooten and Banchereau, 1996), including secretion of cytokines and Ig isotype switchingm (Burdin et al., 1996). And the immunostimulatory functions of CD40L have been exploited to enhance B- and T-cell responses in various vaccination strategies in animals, such as bovine herpesvirus-1 (Huang et al., 2004; Manoj et al., 2003, 2004), HIV (Harcourt et al., 2003; Liu et al., 2008; Stone et al., 2006) , and tumors (Huang et al., 2004; Manoj et al., 2003, 2004). In this study, both CD40L fusion and mixing strategies were used to improve the immune response of PRRSV major protective antigens. The results revealed that the two strategies could both increase the immune responses of PRRSV. But the results from CD40L fusion strategies were not exactly the same as those from the mixing strategies. The mice experiment results showed that rAd-CD40L plus rAd-GP35 group provided higher humoral and cellar immune responses than rAd-CD40L-GP35. It indicated that the immunostimulatory functions of the recombinant CD40L protein expressed alone might be higher than those co-expressed as fusion protein CD40L-GP3-GP5. But the following experiment on pigs revealed that the fusion protein CD40L-GP3-GP5 was more effective in accelerating the humoral response and T-cell response, compared with rAd-CD40L plus rAd-GP35. More interestingly, the amount of IFN-γ was lower than that of IL-4.

This is in contrast to the IFN-γ/IL-4 ratio observed in mice and is an unexpected finding, as CD40L usually has strong Th1 stimulating properties. The amount of IFN-γ observed in our previous experiments using the same adenovirus vector GP3/GP5 construct in combination with GM-CSF or HSP70 (as another CD40 agonist) was considerably higher(Li et al., 2009a; Wang et al., 2009). It suggested that antigen presenting of GP3/GP5 fused with different adjuvant molecules might be different. IFNγ responses were thought to be important in PRRSV clearance. IL-4 represented Th2 type immune response and the high level of IL-4 secreting might represent the characters of bioactivity of CD40L in pigs. Obviously, the reasons of the different immune responses enhanced by CD40L should be studied in the future.

To confirm the protective immune response induced by the recombinant adenoviruses, the pigs were challenged with virulent PRRSV isolate SY0608. Temperature and Viremia were examined to evaluate the protective efficiency. The results showed that rAd-CD40L plus rAd-GP35 could provide higher protective efficiency than rAd-GP35, although PRRSV infection was not fully prevented after homogenous challenge. Here, it was noted that the levels of Viremias of the pigs were also very different from each other in the same group (Fig 8). However, due to the lacking of the detection of PRRSV-specific neutralizing antibodies and IFN-γ after challenge, we did not know the correlation between the viral load and the levels of neutralizing antibodies or IFN-γ. PRRSV SY0608 strain was a highly pathogenic isolate and could cause severe disease which could lead to more than 25% mortality in young pigs [20]. However, in this study no severe disease was observed in challenge control group. It might be related to lower dose of inoculation or the high passage cultures of the isolate SY0608, or the breed variety of the pigs (local stock ×Landrace) [53, 54]. Of course, as a new vaccine, immune comparison between the constructs and commercial modified live virus and the protection efficiency against heterologous challenge should be further examined.

To our knowledge, this study is the first demonstration that swine CD40L fused or mixed with GP3/GP5 of PRRSV could markedly enhance the immune responses and

provide partial protection against homologous virulent PRRSV challenge in pigs. CD40L might be a useful molecular adjuvant in improving PRRSV immune response and maybe it will be further used in PRRSV vaccine.

Acknowledgements

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CHAPTER 5

GENERAL DISCUSSION

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows and is associated with the respiratory disease complex affecting pigs of all ages (Collins et al., 1992; Wensvoort et al., 1991). The syndrome has a major impact on the worldwide pig industry, not only at the economical level, but also at the level of animal welfare (Bilodeau et al., 1991) (Christianson et al., 1992; Neumann et al., 2005; Pejsak et al., 1997). The causative agent of PRRS, PRRS virus (PRRSV), belongs to the family Arteriviridae, order Nidovirales (Cavanagh, 1997). *In vivo*, this virus infects sialoadhesin-positive macrophages that are mainly present in lungs and secondary lymphoid tissues (Duan et al., 1997).

PRRSV infections are difficult to control in the field. The pathogenesis of PRRSV infection in naive pigs is characterized by an extended period of viremia, sometimes followed by persistence of PRRSV in lymphoid tissue for several weeks or months (Allende et al., 2000; Labarque et al., 2003; Wills et al., 1997). Viruses have developed a wide range of strategies to evade the host's immune system to promote their persistence in the host. The prolonged presence of PRRSV in pigs is an important problem in PRRSV control programs, since those pigs may appear clinically normal, but are still able to transmit virus to non-infected susceptible pigs (Bilodeau et al., 1994; Terpstra et al., 1991). Elucidation of the mechanisms that cause the prolonged presence of PRRSV in the pigs offers opportunities to fight PRRSV infection and to control PRRSV in the field.

The immune system is divided into two major branches: the innate immune system and the adaptive immune system. The innate immune system is non-specific as to the type of organism it fights and is ready to be mobilized upon the first signs of infection. The adaptive immune system launches attacks specific to the invading pathogen and requires some time to tailor its custom-made response. The adaptive system “remembers” antigens it has encountered and reacts more quickly and efficiently the next time that antigen is found, yet more slowly than the innate system.

Innate NK cells are the lymphocyte subpopulation known for their ability to provide the first line of defense against viral infections (Biron et al., 1999). Normally, NK cells are activated to mediate innate antiviral cytotoxic activity following infection. However, many researches described that PRRSV does not elicit significant innate interferon and cytokine responses (Chiou et al., 2000; Lopez-Fuertes et al., 2000; Van Reeth et al., 1999), while innate interferons and cytokines are described to be crucial for the activation of cellular innate immune components and consequently also for the induction of an efficient adaptive immune response (Janeway et al., 2005). PRRSV suppresses production of IFN-α and other important cytokines which may contribute to innate cell activation at the site of infection (Murtaugh et al., 2002). Therefore, it could be suspected that PRRSV may poorly activates cellular components of the innate immunity including NK cells. Whereas, it is interesting to know if NK cells activated by exogenous cytokines or some products can fully develop their functions and help to eliminate PRRSV during early stage of infection.

In this thesis, a study focusing on the interaction between porcine NK cells and PRRSV-infected cells has been performed to better understand the innate immune response upon PRRSV infection (Chapter 3). Since PAMs are primary target cells of PRRSV infection, we use PAMs as NK target cells for evaluating NK cytotoxicity after exogenous cytokines stimulation. The NK cells used in our study was activated by recombinant porcine IL-2 (rpIL-2) and the IFN-γ secreting level was tested by ELSA kit. However, cytotoxicity and cytokine production, both features of activated NK cells, do not necessarily coincide. Differential regulation of these functions has been demonstrated in virus-infected animals (Cousens et al., 1997; Orange and Biron, 1996; Une et al., 2000). The results from our study indicated that PRRSV-infected PAMs showed a reduced susceptibility towards NK cytotoxicity *in vitro*.

NK cell function is controlled by a delicate balance of signals from activating and inhibitory receptors (Lanier, 2005). MHC class I ligands expressed on target cells behave as inhibitory receptors on NK cells. Absence of MHC class I on target cells often leads to NK cell activation (Elliott et al., 2010). Reduction in the normal levels

of surface I MHC class, will activate NK cell killing. Alternatively, cytotoxic T cells (CTL) recognize virus-infected cells via surface MHC class I molecules carrying viral peptides. In order to escape CTL-mediated lysis, many viruses have evolved strategies to interfere with the MHC class I pathway (Hewitt, 2003). Previously, Costers et al. showed that proliferating $CD3^{+}CD8^{high}$ cells fail to exert cytolytic T lymphocytes (CTLs) activity towards PRRSV-infected PAMs (Costers et al., 2009). However, staining of MHC class I expression in our study showed that the MHC class I expression on mock-inoculated and PRRSV-inoculated PAMs have no significant differences and downregulation of surface MHC class I molecules was not observed in PRRSV-positive macrophages in Coster's study either. These results indicated that the MHC class I cell surface expression is not the most important reason of the functional impairment of either NK cytotoxicity or CTL activity during PRRSV infection.

Both NK cells involved in the innate immune response and CTLs involved in the adaptive immune response belong to the group of cytotoxic lymphocytes (CLs), and they can use the same elimination strategy to destroy invading microorganisms. CLs lyse virus-infected cells by granzyme and perforin degranulation. The results of degranulation staining indicated that the suppressed NK mediated lysis of PRRSV-infected PAMs is partly due to an inhibition of a process before degranulation. It may be hypothesized that PRRSV infection inhibits both NK and CTL activity with a common mechanism.

In our study, NK cells were first activated by rpIL-2 indicating that the insufficient NK activity post PRRSV infection is not only due to an insufficient activation by a lower level of activating cytokines, such as IFN-α expression etc. The NK cytotoxicity against PRRSV-infected PAMs was decreased starting from 6 h post inoculation (hpi) till the end of the experiment (12 hpi) and was significantly lower than that against pseudorabies virus (PrV)-infected PAMs. UV-inactivated PRRSV also suppressed NK activity, but much less than infectious PRRSV. This suggests that transcription of viral genes and expression of viral proteins also contribute to the

resistance of PRRSV-infected PAMs towards NK-mediated lysis. In line with this, study from another group in USA evaluating immune responses during early stage of PRRSV infection showed that increased frequency in NK cells rich fraction in virus-infected pigs did not result in rescued NK cytotoxicity *in vivo*. All pigs with reduced NK cell cytotoxicity were viremic with titers greater than 2 logs (Dwivedi et al., 2012). The results from both study all suggested PRRSV may induce a modulation in NK cell function and replicating PRRSV mediated suppression of NK cell function. It could be speculated that during PRRSV infection, the virus modulates the ligands for NK receptors on the surface of PAMs, which may subsequently lead to an insufficient NK cytotoxicity. To investigate which receptor(s) and ligand(s) are potentially involved in the suppression of NK cytotoxicity against PRRSV-infected PAMs, further studies are needed.

PRRSV manipulates and evades protective immunity at the level of both the innate and adaptive immune system using different strategies (Costers et al., 2008; Costers et al., 2009; Mateu and Diaz, 2008). The suppression of NK cytotoxicity could be one of the innate immune evasion mechanisms of PRRSV. In the meantime, it is also well known in modulating host's adaptive immunity such as slow development of virus-specific interferon-γ (IFN-γ) response (Meier et al., 2003), short duration of cell-mediated immune response (Mateu and Diaz, 2008; Xiao et al., 2004) Xiao et al., 2004) and delayed appearance of neutralizing antibodies (Lopez and Osorio, 2004). The initial step for a complete eradication of PRRSV starts with the prevention of infection and its control. One major approach to PRRSV prevention resides in the use of more efficient vaccines that would improve the ones available now.

An ideal vaccine should be safe, protective and adaptable. Adenovirus vectors, particularly those constructed from human adenovirus serotype 5 (Ad5), have been shown to be an excellent delivery system to express genes of interest for vaccine development. The Ad5 recombinant virus is often replication-defective due to a large deletion in the early transcription region 1 (E1) of the genome. These

replication-defective Ad5 viruses can grow only in cells, like HEK-293 cells, that complement the E1 region of the adenovirus genome (Graham et al., 1977). For bio-safety demanding, a replication-defective Ad5 is considered to be more suitable for vaccine development. Moreover, high levels of expression are achieved in the Ad5 vector system when foreign genes are under the control of constitutive promoters like the CMV promoter (Ambriovic et al., 1997). Other advantages of the human Ad5 viruses are their broad host range and, in particular for livestock, the lack of pre-existing maternally derived antibodies which can interfere with vaccine efficacy (in young and growing pigs).

PRRSV structure proteins GP3, GP4 and GP5 are associated with the development of neutralizing antibodies and protection (Costers et al., 2010; Jiang et al., 2008; Plagemann, 2004), making these proteins possible candidates for subunit vaccines. Due to the high flexibility of PRRSV, subunit vaccines should focus on inducing high titres of antibodies to multiple functionally important domains on PRRSV proteins in a short time. Fusion protein strategy is simple, feasible, and steady, which potentially affects the structure and bioactivity of the two proteins individually (Wang et al., 2009). Thus, the co-expression of GP3/GP5 or GP5/GP3 together has been widely used in inducing PRRSV protective immune response. In our previous research, GP3-GP5 fusing protein could induce higher level of immune response against PRRSV (Jiang et al., 2008).

An ideal vaccine against PRRSV is not only involved in the production of high level of virus neutralizing antibodies but also involved in the induction of an effective CMI response. It should be able to evoke a massive response by several immune components against several PRRSV targets in a very short time upon challenge, making it difficult for PRRSV to escape. In order to induce stronger immune response, adjuvants are often used to modify or augment the effects of a vaccine by stimulating the immune system to respond to the vaccine more vigorously, and thus providing increased immunity to a particular disease.

CD40L belongs to the tumor necrosis factor super family and has a central role in the development and regulation of adaptive immune responses in mammals and avian species (Bodmer et al., 2002; Gares et al., 2006; Locksley et al., 2001). Several studies have demonstrated the efficacy of exogenous CD40L as a vaccine adjuvant to promote increased T cell proliferation and effector functions, including T cell polyfunctionality and cytokine production (Gomez et al., 2009; Lin et al., 2009; Manoj et al., 2004). Therefore, we performed a study to examine the use of CD40L as an immunotargeting agent and immunostimulator in enhancing PRRSV immune response (Chapter 4). Porcine CD40L was amplified from Con A stimulated PBMC and recombinant adenoviruses expressing CD40L and GP3-GP5 of PRRSV were constructed and used to vaccinate pigs. Since NAs play an important role in clearing PRRSV and preventing infection (Meier et al., 2003; Yoon et al., 1994), while cell-mediated immunity (CMI) is also extremely important in PRRSV infection. Pigs recovering from experimental PRRSV infection had strong lymphocyte proliferative responses (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). These two indicators are used to assess humoral and cellular immune responses induced by the recombinant adenoviruses after vaccination. Protective efficacy was assessed by evaluating the clinical signs and body temperature change together with viremia upon challenge – a factor directly linked with viral pathogenesis and spread. It was found that the porcine CD40L could effectively increase the humoral and cellular immune responses of GP3 and GP5. Both fusing and mixing strategies of CD40L and GP3-GP5 could provide partly protective efficacy against PRRSV challenge in pigs. CD40L might be a useful molecular adjuvant in improving PRRSV immune response and it may be further used in PRRSV vaccine.

To sum up, the work described in this thesis (i) showed an insufficient NK cytotoxicity against PRRSV infection which may help us to better understand why PRRSV is not well controlled by an innate immune response. (ii) The immonoenhancing property of CD40L indicated that co-delivery of immunomodulators should be useful for developing new vaccine against PRRSV.

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SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important pathogenics affecting swine industry worldwide. PRRSV is a single-stranded and positive sense RNA virus that belongs to the family of the *Arteriviridae* (genus Arterivirus). PRRSV has a restricted host and cell tropism, with porcine alveolar macrophages (PAMs) as important host cells. In 2006, most swine farms in China experienced a “high fever disease of swine” caused by new PRRSV variants, which led to great economic losses to Chinese swine industry, bring new challenge to the prevention and control of the disease. PRRSV is able to persist in pigs for several weeks to several months after initial infection. It appears to use several evasion strategies to circumvent both innate and adaptive immunity and the currently registered vaccines can not provide good protective efficiency in practice. Global efforts are made to better understanding host's immune response to PRRSV infection and develop better PRRSV vaccines.

In this dissertation, **Chapter 1** is an introduction on PRRSV. The first section of chapter 1 focuses on PRRSV history, taxonomy, genetic diversity, virion morphology, genome organization, virus proteins and virus replication. The second section of chapter 1 reviews the innate and adaptive immune response evoked by PRRSV. The third section of chapter 1 gives a general overview of PRRSV vaccines.

In **Chapter 2**, the aims of this thesis are stated.

Chapter 3 is dedicated to study PRRSV innate immunity. The NK cytotoxicity assays were performed to directly investigate the interaction between NK cells and PRRSV-infected host cells. IL-2 stimulated enriched NK cells were used as effector cells and virus-infected or mock-inoculated PAMs were used as target cells. The NK cytotoxicity against PRRSV-infected PAMs at different time points post inoculation were examed. The killing towards different target cells including pseudorabies virus (PrV), UV-inactivated PRRSV and infectious PRRSV were compared. After co-incubation, the degranulation level of NK cells were checked to investigate the influence of PRRSV-infected PAMs on NK cells activity. Supernatant of

PRRSV-infected PAMs were collected at 12 hpi to test the effect of soluble factors. Taken together, the results from this study revealed that PRRSV-infected PAMs showed a reduced susceptibility towards NK cytotoxicity, which may represent one of the multiple evasion strategies of PRRSV.

In **Chapter 4**, focus is shifted to study the immunoenhancement of CD40 ligand in PRRSV adaptive immunity in order to develop an efficient vaccine . Recombinant adenoviruses expressing structural protein of PRRSV GP3/GP5 and porcine CD40L were constructed. The immonoenhancing property of CD40L was studied in both mouse and pigs model by inoculation with constructed recombinant adenoviruses. Protective efficacy of these recombinant adenoviruses against highly pathogenic PRRSV infection was examined in pigs. The results showed that porcine CD40L could effectively increase humoral and cell-mediated immune responses of PRRSV GP3-GP5. Porcine CD40L might be used as an attractive adjuvant or immunotargeting strategies to enhance the PRRSV subunit vaccine responses in swine.

Finally, **Chapter 5** provides a general discussion on the research data generated in this thesis.

CURRICULUM VITAE

CURRICULUM VITAE

Jun Cao was born in Jiangsu, China, on August 10th, 1984. From September 2002 to June 2006, she has been majoring in the specialty of Bio-technology at Nanjing Agricultural University and graduated with the degree of Bachelor of Science in 2006. Afterwards, she began the continuous academic program that involves postgraduate and doctoral in Laboratory of Animal Diseases Diagnostic and Immunology, College of Veterinary Medicine, Nanjing Agricultural University.

In May 2009, she obtained the scholarship from Chinese council for studying abroad. In Oct 2009 she joined the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University as a joint PhD, and continue her PhD studies on PRRSV in pigs. Her PhD studies were funded by the «Chinese scholarship council» (CSC), grants from the national natural science foundation to Dr. Ping Jiang (31230071) and the «Special Research Fund» (BOF) Ghent University.

Research publications

1. **Jun Cao,** Korneel Grauwet, Ben Vermeulen, Bert Devriendt , Ping Jiang, Herman Favoreel, Hans Nauwynck (2013). Suppression of NK cell-mediated 1 cytotoxicity against PRRSV-infected porcine alveolar macrophages in *vitro*. Veterinary Microbiology.

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8. Yijun Du, Yufeng Li, Hairong He, Jing Qi, Wenming Jiang, Xinglong Wang, Bo Tang, **Jun Cao**, Xianwei Wang, Ping Jiang (2008). Enhanced immunogenicity of multiple-epitopes of foot-and-mouth disease virus fused with porcine interferon α in mice and protective efficacy in guinea pigs and swine. Journal of Virological Methods, 2008 Apr; 149(1):144-52. (IF:2.07)

Poster presentations

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