Universitat Autònoma de Barcelona

Study of molecular determinants involved in the Classical swine fever virus attenuation and the persistence in the host

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PhD Thesis

Bellaterra, 2022

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Tesis doctoral presentada por **Miaomiao Wang** para acceder al grado de doctor en el marco del programa de Doctorado en Medicina y Sanidad Animales de la Facultad de Veterinaria de la Universidad Autonoma de Barcelona, bajo la dirección de Dra. **Llilianne Ganges** y la tutoría de Dr. **Mariano Domingo Álvarez**.

Bellaterra, 2022

IRTA CReSA

OIE Reference Laboratory for Classical swine fever

This work has been financially supported by grants RTI2018-100887-B-I00 from the Ministerio de Ciencia e Innovación of the Spanish government.

Miaomiao Wang received a predoctoral fellowship CSC scholarship (201708110186) from the Chinese government.

Cover design: Alexandra Ruiz Piñero

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Que la memoria titulada "**Study of molecular determinants involved in the Classical swine fever virus attenuation and the persistence in the host**" presentada por **Miaomiao Wang**, para la obtención del grado de Doctor en Medicina y sanidad animales, se ha realizado bajo su dirección y tutoría, y autorizan su presentación para que sea valorada por la comisión establecida.

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List of abbreviations

ASFV	African swine fever virus
BDV	Border disease virus
BSL3	Biosafety level 3
BVDV	Bovine viral diarrhea virus
CSF	Classical swine fever
CSFV	Classical swine fever virus
Ct	Cycle threshold
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
DIVA	Differentiability of infected from vaccinated animals
DMEM	Dulbecco's modified Eagle medium
dpi	days post infection
dsRNA	double-stranded RNA
EDC	Endothelial cell
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's Minimal Essential Medium
ER	Endoplasmic reticulum
EU	European Union
EURL	European Union Reference Laboratory

FAT	Fluorescence antibody test
FBS	Fetal bovine serum
GPE$^-$	Guinea-pig exaltation-negative
h.p.i	hours postinfection
HCLV	Hog cholera lapinized virus
HCV	Hepatitis C virus
HRP	Horseradish peroxidase
HS	Heparan sulfate
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
IRF3	Interferon regulatory factor 3
ISGs	Interferon-stimulated genes
Kb	kilobase
mAb	monoclonal antibody
MACS	Magnetic cell sorting system
MDA	Maternal-derived antibodies
MDA-5	Melanoma differentiation-associated protein 5
MDM	Monocyte-derived macrophages
MDSC	Myeloid-derived suppressor cell

MEM	Minimum essential medium
MOI	Multiplicity of infection
МΦ	Macrophage
NPLA	Neutralization peroxidase-linked assay
NSPs	Nonstructural proteins
NTPase	Nucleoside triphosphatase
OIE	World organization for animal health
ORF	Open reading frame
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
pDC	plasmacytoid dendritic cell
PdR	Pinar de Rio
PI	Persistently infected
PLA	Peroxidase linked assay
poly-U	poly-uridines
PRRs	Pattern recognition receptors
RdRp	RNA-dependent RNA polymerase
RE	Restriction enzyme
RIG-I	Retinoic acid-inducible gene I
RNase	Ribonuclease

RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative PCR
SIE	Superinfection exclusion
SL	Stem-loop
SLA	Swine leukocyte antigen
ssRNA	single-stranded RNA
$TCID_{50}$	50% tissue culture infective doses
TIR	Toll/interleukin-I receptor
TLR	Toll-like receptor
TSV	Tunisian Sheep Virus
UK	United Kingdom
US	United States
UTR	Untranslated region
WAHIS	World animal health information syste

Abstract

Classical swine fever virus (CSFV) remains a re-emerging virus that affects swine health and the pork industry worldwide. The prevalence of low and moderate virulence CSFV strains under vaccination programs could induce unapparent disease forms and facilitate virus persistence in the field, posing a challenge for CSF eradication. Therefore, the study of CSFV molecular determinants in virulence is highly relevant for the development of solid diagnostic tools and vaccines to ensure effective disease control.

The present thesis has investigated the role of a unique, uninterrupted 36 poly-uridines (poly-U) sequence found in the 3' untranslated region (3'UTR) of the low virulence CSFV isolate Pinar de Rio (PdR) for CSFV virulence and transmissibility in 5-day-old pigs. The reverse genetics approach was used to construct a pair of cDNA-derived viruses based on the PdR backbone, one carrying the long poly-U insertion in the 3'UTR (vPdR-36U) and the other harbouring the standard 5 uridines at this position (vPdR-5U). The infection with both viruses showed that the long poly-U insertion in the PdR strain can control viral replication, reduce disease severity and activate immunity in the infected piglets without affecting viral transmission, being a CSFV virulence factor.

In addition, the unique Ribonuclease (RNase) activity from the exclusive E^{rns} glycoprotein of *Pestivrius* has been associated with the high virulence pestiviruses attenuation *in vivo*, however, the role of this function in low virulence CSFV strains pathogenesis remains unknown. Therefore, this thesis evaluated the role of the E^{rns} RNase activity from the low virulence CSFV PdR strain in pathogenesis, viral replication and transmissibility in 5-day-old pigs. The E^{rns} RNase inactivated clone, vPdR-H_{30}K-36U, was

constructed based on the vPdR-36U backbone. The vPdR-H_{30}K-36U virus was attenuated in piglets compared to the parental vPdR-36U, showing for the first time that E^{rns} RNase inactivation in a low virulence CSFV PdR strain could reduce viral replication capacity and CSFV persistence and transmissibility in the infected animals. The vPdR-H_{30}K-36U mutant activated the innate immune response in the infected piglets. Likewise, infection with vPdR-H_{30}K-36U resulted in higher antibody levels against the E2 and E^{rns} glycoproteins and enhanced neutralizing antibody response when compared with vPdR-36U. This dampening effect on E^{rns}-specific immunity related to the RNase activity of E^{rns} may bring difficulties in the development of differentiability of infected from vaccinated animals (DIVA) assays based on seroconversion against E^{rns}.

Moreover, the potential synergistic role of the E^{rns} RNase activity and the poly-U insertion in the 3'UTR from PdR strain in the innate and adaptive immunity regulation and its relationship with the disease pathogenesis in 5-day-old and 3-week-old pigs was assessed. A double mutant vPdR-H_{30}K-5U with E^{rns} RNase inactivation and with 5 uridines instead of the 36 in the 3'UTR was generated. The lack of both, the poly-U insertion and the E^{rns} RNase function could induce severe clinical manifestations in two infected groups, higher mortality rate was shown in 5-day-old piglets (89.5%) than in 3-week-old pigs (33.3%). The pigs infected with vPdR-H_{30}K-5U showed high viral replication in tonsils but mild viremia and low viral excretion. Among nine cytokines quantified, only IFN-α and IL-12 were highly elevated in the two groups. Additionally, high IL-8 levels were found in the newborn but not in the older pigs, indicating the important role of these cytokines for the CSFV disease severity. Additionally, infection with vPdR-H_{30}K-5U resulted in the reduced adaptive immune response from the pigs.

These results from the present thesis provide new potential targets for the development of novel vaccines and diagnostic tools for efficient CSF control. Moreover, it gives a better understanding for CSF pathogenesis and provides new directions for the study of the natural CSFV attenuation molecular determinants.

Resumen

La peste porcina clásica (PPC) es una de las enfermedades más importantes que afecta la industria porcina. Su agente etiológico, el virus de la PPC (VPPC), continúa siendo prevalente en diversas zonas del mundo, principalmente las cepas de baja y moderada virulencia. Este tipo de cepas virales están asociadas con la generación de formas crónicas y persistentes, las que complican los programas de control en los países endémicos, representando un desafío para la erradicación de la PPC. Teniendo en cuenta, el estudio de los determinantes moleculares asociados a la virulencia del VPPC, son de gran relevancia para el desarrollo de vacunas y herramientas diagnósticas eficaces que garanticen el control.

La presente tesis ha investigado la implicación en virulencia de la secuencia única e ininterrumpida de 36 poli-uridinas (poli-U) dentro de la región 3´ no codificante (3'UTR) presentes en el aislado de VPPC de baja virulencia Pinar de Río (PdR). La estrategia de genética reversa se utilizó para generar un par de virus derivados del ADNc de la cepa de baja virulencia PdR. Uno de los virus llevó el poli-U de 36 nucleótidos en el 3'UTR (vPdR-36U), mientras que el segundo, contenía solamente las 5 uridinas estándar para VPPC en esta posición (vPdR-5U). Se infectaron diferentes grupos de cerditos de cinco días de nacidos y se demostró, que la secuencia de 36 poli-U del 3'UTR de la cepa PdR, reduce la capacidad de replicación del virus *in vivo*, su capacidad de transmisión, además de activar la respuesta innata del hospedador. Por lo tanto, el poli-U en el fragmento 3'UTR, es un factor de virulencia del VPPC.

Por otro lado, la glicoproteína E^{rns}, presente exclusivamente en los pestiviruses, tiene una característica única de tener actividad ribonucleasa (RNasa). No obstante, su papel en la patogénesis y la respuesta inmune,

así como su relación con la capacidad de generar infecciones crónicas y persistentes, ha sido poco estudiado. El segundo estudio de esta tesis evaluó el rol de la actividad RNasa de la cepa PdR, en la patogénesis, la replicación viral y la transmisibilidad en cerdos de cinco días. Partiendo del clon vPdR-36U, se construyó el vPdR-H_{30}K-36U, al cual se le inactivó la función RNasa de la E^{rns}. Este virus resultó ser atenuado en replicación y en su capacidad de transmisión. No obstante, activó de forma eficaz la respuesta inmune innata y adaptativa en los lechones infectados. El mutante generó niveles más altos de anticuerpos contra las glicoproteínas E2 y E^{rns} y de anticuerpos neutralizantes, en comparación con la capacidad de generar respuesta humoral del vPdR-36U. Evidenciando que la E^{rns} está involucrada en mecanismos de evasión de la respuesta inmune del hospedador, siendo problemática su utilización en sistemas de diagnóstico.

En el tercer estudio, se demostró el papel sinérgico de la actividad RNasa de la E^{rns} y la inserción del poli-U descrito en 3'UTR. Tras la inoculación en animales con el nuevo virus generado, vPdR-H_{30}K-5U, se demostró su rol regulador en la respuesta innata del hospedador; así como en la capacidad de replicación del virus *in vivo*. La infección con este virus generó una alta mortalidad en cerditos, con un incremento en la replicación del virus en tonsila. Del mismo modo, se detectó un drástico incremento en la respuesta de citoquinas proinflamatorias a nivel sistémico, asociándose con la mortalidad y severidad de la enfermedad. Esta tesis marca una nueva dirección en el camino de los diseños de estrategias DIVA frente al VPPC. Además, proporciona una nueva metodología para el estudio de los factores de virulencia del virus y una mejor comprensión de la patogénesis de la PPC.

Resum

La pesta porcina clàssica (PPC) és una de les malalties més importants que afecta la indústria porcina. El seu agent etiològic, el virus de la PPC (VPPC), segueix essent prevalent a diverses zones del món, sobretot les soques de baixa i moderada virulència. Aquests tipus de soques virals estan associades a la generació de formes cròniques i persistents, que dificulten els programes de control en països endèmics, i fan de l'eradicació de la PPC tot un repte. Tenint en compte l'estudi dels determinants moleculars associats a la virulència del VPPC, esdevenen molt rellevants per al desenvolupament de les vacunes i d'eines diagnòstiques eficaces que en garanteixin el control.

La present tesi ha investigat la implicació en virulència de la seqüència única i ininterrompuda de 36 poliuridines (poli-U) dins la regió 3' no codificant (3'UTR), presents en l'aïllat de VPPC de baixa virulència Pinar del Río (PdR). Per fer-ho, es va utilitzar l'estratègia de genètica reversa per a generar dos virus derivats de l'ADNc de la soca PdR. Un dels virus duia el poli-U de 36 nucleòtids en el 3'UTR (vPdR-36U), mentre que l'altre, contenia només les 5 uridines estàndard pel VPPC en aquesta posició (vPdR-5U). Es van infectar diferents grups de garrins de cinc dies de vida i es va demostrar que, la seqüència de 36 poli-U del 3'UTR de la soca PdR, redueix la capacitat de replicació del virus *in vivo* i la seva capacitat de transmissió, a més d'activar la resposta innata de l'hoste. Per tant, el poli-U en el fragment 3'UTR, és un factor de virulència del VPPC.

D'altra banda, la glicoproteïna E^{rns}, present exclusivament en els pestivirus, té la característica única de tenir activitat ribonucleasa (RNasa). No obstant, el seu paper en la patogènesi i la resposta immunològica, així com la seva relació amb la capacitat de generar infeccions cròniques i

persistents, ha estat poc estudiada. El segon estudi d'aquesta tesi va avaluar el rol de l'activitat RNasa de la soca PdR en la patogènesi, la replicació viral i la transmissibilitat en porcs de 5 dies. Partint del clon vPdR-36U, es va construir el vPdR-H_{30}K-36U, al qual se li va inactivar la funció RNasa de l'E^{rns}. Aquest virus va resultar ser atenuat en replicació i en la seva capacitat de transmissió. No obstant, va activar de forma eficaç la resposta immune innata i adaptativa en els garrins infectats. El mutant va generar nivells més elevats d'anticossos contra les glicoproteïnes E2 i E^{rns} i d'anticossos neutralitzants, en comparació amb la capacitat de generar resposta humoral del vPdR-36U; evidenciant així que la E^{rns} està involucrada en mecanismes d'evasió de la resposta immune de l'hoste, resultant problemàtica la seva utilització en sistemes de diagnòstic.

En el tercer estudi, es va demostrar el paper sinèrgic de l'activitat RNasa de la E^{rns} i la inserció del poli-U descrit en 3'UTR. Un cop duta a terme la inoculació en animals amb el nou virus generat, vPdR-H_{30}K-5U, es va demostrar el seu rol regulador en la resposta innata de l'hoste, així com en la capacitat de replicació del virus *in vivo*. La infecció amb aquest virus va generar una alta mortalitat en garrins, amb un increment de replicació del virus a la tonsil·la. De la mateixa manera, es va detectar un dràstic increment en la resposta de citoquines proinflamatòries a nivell sistèmic, associant-se amb la mortalitat i severitat de la malaltia. Aquesta tesi marca una nova direcció en el camí dels dissenys d'estratègies DIVA front al VPPC. A més proporciona una nova metodologia per a l'estudi dels factors de virulència del virus i una millor comprensió de la patogènesi de la PPC.

Part I

General introduction and objectives

Chapter 1

General introduction

1.1 Classical Swine Fever: History and Origin

The first reports of classical swine fever (CSF), initially named Hog cholera, were in 1810 in Tennessee based on the description from farmworkers. During the first half of the 19th century, CSF outbreaks were reported in 10 different states from the Midwestern and Southern regions of the United States (US) (Anonymous, 1889; Hanson, 1957). Afterwards, under the facilitation by the railway's development, CSF was widespread in Europe and America by the 1860s (Birch, 1922), and worldwide by the 1960s (Choori et al., 2015; Cole et al., 1962; Díaz de Arce et al., 2005; Tu et al., 2001).

Considering the severe impact of the disease on the pig industry and animal health, CSF eradication programs have been implemented for several decades, with multiple successful outcomes. Under the efficient vaccination strategy and comprehensive culling program, North America and Australia achieved CSF-free status during the 1960s. Since the early 1990s, European Union (EU) has carried out strict stamping-out policies (Garner et al., 2001; Paton and Greiser-Wilke, 2003). The costs for free status remain to be totally evaluated, while the last two CSF outbreaks in Spain (1997 and 2001) were around 108 million Euros combined (Fernández-Carrión et al., 2016). The enormous expense was not only for the direct economic losses but also due to the welfare sacrifice under the strict movement restrictions (Elbers, 2002).

Nevertheless, CSF remains one of the most important viral diseases affecting swine (Ganges et al., 2020; Hanson, 1957). It is a notifiable disease by the world organization for animal health (OIE), due to its severe social and economic impacts (OIE, 2019).

1.2 Classical Swine Fever: Current situation

Nowadays, 38 countries are recognized as CSF-free territories by OIE, including all of North America, Oceania, and a large part of the EU (OIE, 2021). CSF remains endemic in South and Central America, Asia, and the Caribbean, while some specific regions of Brazil, Colombia, and Ecuador have been also declared free of the disease, although those countries remain endemic (OIE, 2021). Little is known about the African situation, even though outbreaks have been reported in Madagascar and South Africa (Ji et al., 2015; Sandvik et al., 2005). The most recent CSF outbreaks have been reported in Korea, Colombia, Russia, Brazil, and Japan (OIE, 2021). Notably, the reemergence of the disease in both domestic pigs and wild boar in Japan after 26 years of CSF-free status further shows the complexity of CSF outbreaks, and the preventive vaccination is being carried out in domestic pigs and wild boars for the control of the outbreak (Ito et al., 2019; Postel et al., 2019). Hence, CSF remains an endemic and re-emerging disease in pigs and continues to threaten pork production worldwide and the food security of populations in developing countries.

1.3 Etiology and Taxonomy

The CSF etiologic agent was clarified as the CSF virus (CSFV) in 1904, since CSF could be transmitted by the filtered serum from infected animals (De Schweinitz and Dorset, 1903; Schwenitiz and Dorset, 1904). Recently, it was hypothesized under a cophylogenetic analysis, that CSFV emerged around the end of the 18th century when the Tunisian Sheep Virus (TSV) jumped from its natural host *Ovis aries* to swine (Rios et al., 2017). This is supported by the fact that, at that moment, animals from different species were allowed to be kept together, and the Tunisian sheep were also imported in some regions where the first reports of CSF originated (Brier, 2013; Carman et al., 1892; Peters, 1810).

CSFV is an enveloped virus of icosahedral symmetry and 40-60 nm in diameter. The viral genome is a positive single-stranded RNA of approximately 12.3-kilo bases (kb) (Figure 1). It belongs to the *Pestivirus* genus within *Flaviviridae* family. *Pestivirus* genus consists of 11 different officially recognized viral species based on the revised taxonomy of the genus *Pestivirus* in 2017. CSFV is known as "*Pestivirus* C" (ICTV, 2020; Smith et al., 2017). Other members of *Pestivirus* genus, Bovine Viral Diarrhoea virus type 1 (BVDV-1), BVDV type 2 (BVDV-2), Border disease virus (BDV), Pronghorn antelope pestivirus, Bungowannah virus, giraffe pestivirus, Hobi-like pestivirus, Aydin-like pestivirus, rat pestivirus and atypical porcine pestivirus were renamed as *Pestivirus* A, B and D to K respectively. Due to the increasing members from the *Pestivirus* genus, an updated study recommended the inclusion of eight tentative species recently found in pigs (Linda virus), ruminants (Tunisian sheep-like pestivirus; ovine/IT pestivirus), rodents (rodent pestivirus), bats (bat pestivirus), whales (Phocoena pestivirus), and pangolins (pangolin pestivirus), named *Pestivirus* L to S (Firth et al., 2014; W. H. Gao et al., 2020; Jo et al., 2019; Lamp et al., 2017; Meyer et al., 2021; Postel et al., 2021; Sozzi et al., 2019; Wu et al., 2018). The *Pestivirus* genus is a member of the *Flaviviridae* family, which also included the other three important genera, *Flavivirus*, *Hepacivirus*, and *Pegivirus* (Simmonds et al., 2017).

1.3.1 CSFV Genome Organization

CSFV contains a single open reading frame (ORF) surrounded by two untranslated regions (UTRs), the uncapped 5'UTR carrying an internal ribosome entry site (IRES), and the uridine-rich 3'UTR lacking Poly-A. The ORF encodes a polyprotein that is cleaved into four structural proteins

(capsid protein C, envelope glycoproteins E^{rns}, E1 and E2) and eight nonstructural proteins (NSPs) (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Figure 1) (Lamp et al., 2011; Meyers and Thiel, 1996; Stark et al., 1990; Tautz et al., 2015).

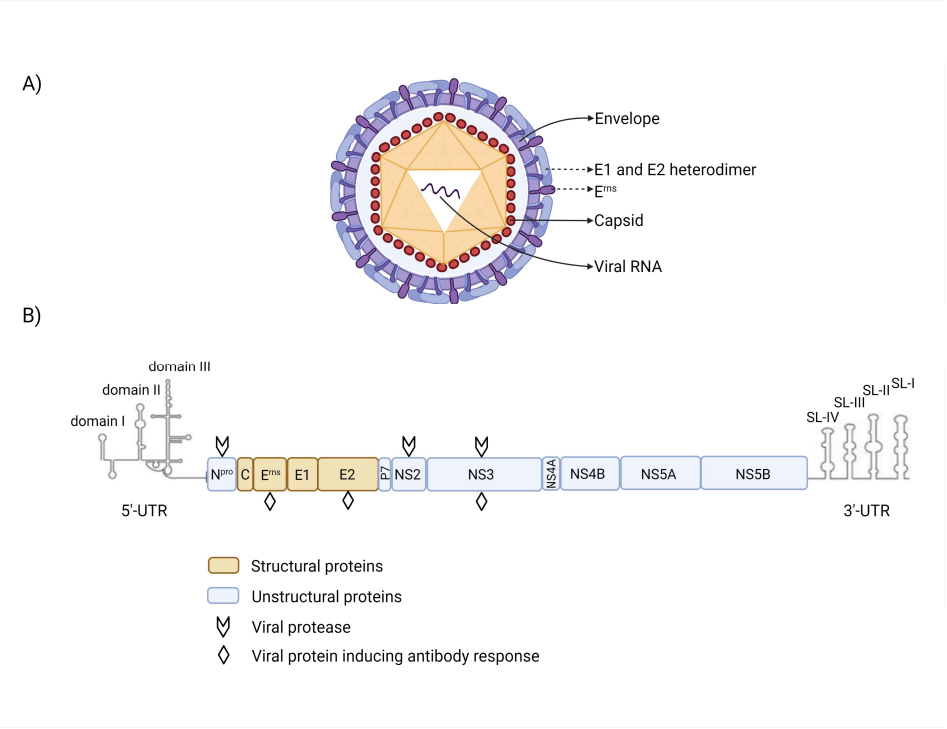

Figure 1. (A) Graphic representation of the CSFV virion structure. (B) The CSFV genome organization and the encoding proteins. CSFV contains a single ORF surrounded by 5'UTR and 3'UTR. The ORF encodes a polyprotein that is processed into four structural proteins and eight nonstructural proteins. Adapted from (Li et al., 2017). Image created with BioRender.com.

The CSFV infection cycle begins with the attachment and entry process, which is mediated by the three surface glycoproteins. E^{rns} could mediate the attachment with the host cells via heparan sulfate (HS)-containing glycosaminoglycans and laminin receptor, though the mechanism behind this is still under study (Chen et al., 2015; Hulst and Moormann, 2001). E1

and E2, containing transmembrane domains, form either E1-E2 heterodimer, or E1 and E2 homodimers, involved in fusion and entry (Li et al., 2017; Mu et al., 2021b; Tautz et al., 2015; F. I. Wang et al., 2015). Recently, a study reported that a disintegrin and metalloproteinase-17 (ADAM17) is essential for CSFV entry by binding directly to E2 protein in a zinc-dependent manner in the metalloproteinase domain (Yuan et al., 2021). Moreover, the membrane-bound signal mediator, integrin β3, is required in CSFV infection and proliferation (W. Li et al., 2014). After CSFV entry, the membrane fusion is mediated by glycoproteins via caveolin-1 and clathrin-mediated endocytosis in a pH-dependent process (Shi et al., 2016; Y.-N. Zhang et al., 2018). Two peptides identified on E2 may be involved in membrane fusion (Fernández-Sainz et al., 2014; Holinka et al., 2016). Furthermore, CSFV is trafficked to the early endosome after endocytosis helped by activated and rearranged microfilaments. Afterwards, CSFV recruits microtubules to assist membrane fusion of the virions from the late endosome to the lysosome under the help of a molecular motor, dynein (Cheng et al., 2021). The viral core releases the viral RNA into the cytosol of the infected cells for the initiation of translation and virus replication. The 5'UTR forms three structural domains (domains I, II, and III) and a pseudoknot with the sequence downstream of the initiation codon (Baird et al., 2006; Fletcher and Jackson, 2002). The IRES located in domain III allows the initiation of cap-independent translation by recruiting the 40 S ribosomal subunit (Hashem et al., 2013). Recently it was shown that ribosomal protein L13 and nucleolin, a stress-responsive RNA-binding protein, are substantial regulators of IRES-driven translation (Han et al., 2021, 2020).

The IRES mediates translation of a polyprotein of approximately 3900 amino acids, which is co-and post-translationally processed by cellular and

viral proteases into mature viral proteins (Meyers and Thiel, 1996; Tautz et al., 2015). The first mature protein, N^{pro}, a cysteine autoprotease, undergoes autocatalytic cleavage at the N^{pro}/C site (Stark et al., 1993). This is followed by the structural proteins, including a capsid protein C and three envelope glycoproteins (E^{rns}, E1, and E2). Those proteins are processed by the host signal peptidase, which is initiated by a cleavage between the capsid protein and the precursor E^{rns}E1E2, followed by cleavage between E^{rns}E1 and E2, and then into E^{rns} and E1 (Rümenapf et al., 1993). The NSPs (p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) are processed by the viral proteases. NS2 acts as an autoprotease activated by a cellular chaperone Jiv, which is responsible for the *cis*-cleavage of NS2-3 (Lackner et al., 2006). And NS3 harbors a serine protease involved in the *cis*-cleavage of NS3-4A and *trans*-cleavage of NS4A-4B-5A-5B (Tautz et al., 2000).

The CSFV replication started by the synthesis of the RNA (-), which serves as a template for the further synthesis of progeny RNA (+). The minimal *Pestivirus* replicon includes 5'and 3'UTR and replicase proteins NS3 to NS5B (Moulin et al., 2007). As the initial part of the replication, 3'UTR consists of four consecutive stem-loop structures (SL-I, SL-II, SL-III, and SL-IV), which contain *cis*-active elements that are indispensable for viral replication and translation (Fan et al., 2008; C. Li et al., 2014). Previous studies revealed that the SL-I and the region between SL-I and SL-II are indispensable for pesitiviral replication and lacking both SL-II and SL-III will lead to un-infectious particles (Pankraz et al., 2005). The conserved sequence between SL-I and SL-II could bind cellular microRNA-17, which is critical for virus replication and could enhance translation and RNA stability (Scheel et al., 2016). The precursor NS2-3 is essential for the high productivity of CSFV *in vivo*, while it was proven that the

uncleaved NS2-3 is not required for the virion morphogenesis of pestiviruses (Dubrau et al., 2019; Lattwein et al., 2012). The mature NS2 localizes in endoplasmic reticulum (ER), induces ER stress and modulates the cellular growth and cell cycle progression (Tang et al., 2010). NS3 is a multifunctional protein, its N-terminus has a serine protease activity, while the C-terminus acts as a helicase, and nucleoside triphosphatase (NTPase) (Tamura et al., 1993). NS4A works as cofactor of NS3 to process all downstream cleavage sites of viral NSPs (Xu et al., 1997). NS4B contains two conserved domains, the Walkers A and B, that exhibit NTPase activity and are essential for RNA replication (Gladue et al., 2011). The CSFV NS5A is highly conserved and possesses a zinc finger domain that is required for viral RNA synthesis and viral replication. NS5A regulated the synthesis of viral RNA by interacting with both the 3′UTR and with NS5B (Chen et al., 2012). It has been reported that cellular Hsp27 interact with the NS5A protein, negatively regulating viral replication by the NF-κB signaling pathway (Ling et al., 2018). It has also been suggested that viperin could inhibit viral replication by interacting with NS5A (Xu et al., 2020). NS5B, as an RNA-dependent RNA polymerase (RdRp), interacts with NS3 and NS5A, which is essential for viral replication (Sheng et al., 2010; Wang et al., 2010). The crystal structure analysis revealed that the NS5B contains the typical polymerase finger, palm, thumb domains and a unique N-terminal domain which is very important for RdRp activity (Li et al., 2018).

1.3.2 Molecular Determinants of CSFV Virulence

CSFV virulence is defined as the ability to cause clinical and pathological signs in pigs. The knowledge of the molecular determinants of CSFV virulence could contribute to effective disease control and provide targets

for the development of safe and effective marker vaccines. Importantly, the application of reverse genetic technology in CSFV facilitates the study of the molecular basis of virulence (Meyers et al., 1996; Moormann et al., 1996). Mainly seven proteins (N^{pro}, C, E^{rns}, E1, E2, p7 and NS4B) have been reported to be relevant for CSFV virulence via reverse genetic modification.

N^{pro}, one of the unique proteins of *Pestivirus*, is involved in antagonism of type I interferon (IFN) and double-stranded RNA (dsRNA)-mediated mitochondrial apoptosis by interacting with interferon regulatory factor 3 (IRF3) and inducing its ubiquitination and proteasome-dependent degradation (Gottipati et al., 2016; Hardy et al., 2021). Several studies showed that N^{pro} acts as a virulence factor in different aspects, with the high virulence Eystrup or the moderate virulence Alfort/187 without N^{pro} gene being attenuated in pigs (Mayer et al., 2004). Mutations abrogating the IRF3 degrading function of N^{pro} partially reduced virulence of the Alfort/187 virus while it showed no reduction in the virulence of the Eystrup strain, which is restricted to strains (Ruggli et al., 2009). Preventing type I IFN induction at local replication via N^{pro} was also shown to contribute to pathogenicity in pigs (Tamura et al., 2014). Moreover, the nuclear localization of N^{pro} could also play a significant role in the CSFV replication and virulence, considering that a CSFV mutant expressing IRF3-N^{pro} fusion protein that was only localized in the cytoplasm could markedly attenuate *in vitro* and *in vivo* (Y. Li et al., 2014).

The C protein possesses RNA chaperone activity and forms the viral capsid (Murray et al., 2008). It initiates particle morphogenesis, partially relying on overall protein charge. The C protein is not essential for particle assembly but is important for CSFV virulence (Riedel et al., 2012, 2010).

Previous studies show that core deficient CSFV particles were fully attenuated in the natural host. Interestingly, the poor growing of CSFV induced by modification of the core gene could be compensated by the single amino acid substitutions in the helicase domain of NS3 (Riedel et al., 2012).

The E1 glycoprotein contains its own ER retention signal, determined by six fully conserved polar residues in the middle part of the transmembrane domain which could serve as a functional group that intensely affects the generation of infectious viral particles (Mu et al., 2021a). However, among the three *Pestivirus* surface glycoproteins, the functions of E1 are the least well understood. The E2 and E^{rns} glycoproteins mediate attachment and interaction with the cellular receptors and are the main targets for neutralizing antibodies and induce protective immunity in infected animals (Li et al., 2017; Tautz et al., 2015). E^{rns} forms disulfide-linked homodimers in infected cells and secreted virions and is heavily glycosylated except for its C-terminal region. The C-terminal amphipathic α-helices are important for the anchoring of viral particles to the membrane. E^{rns} has a unique feature of RNase activity among other RNA virus protein, which is involved in blocking the host's type I IFN (Mätzener et al., 2009; Schneider et al., 1993). E^{rns} could modulate virulence through various mechanisms including its RNase activity, homodimer formation and glycosylation. This is supported by the fact that inactivating the RNase activity by catalytic site-directed mutagenesis and preventing E^{rns} homodimer formation by disruption of the intermolecular cysteine bond (Cys-171) attenuate the virus in pigs (Meyers et al., 1999; Tews et al., 2009). Reversion of E^{rns} dimerization by a spontaneous compensatory mutation *in vivo* restored virulence, confirming the importance of E^{rns} dimer formation for CSFV virulence (Tucakov et al., 2018). Likewise, the

removal of an N-linked glycosylation site of CSFV Brescia E^{rns} reduced its virulence in swine (Sainz et al., 2008).

The E2 consists of four domains followed in order of B/C/D/A, constituting two independent structural units, B/C and D/A (van Rijn et al., 1994). Recently several host binding partners have been found to promote virus entry and modulate viral replication through interaction with E2, such as membrane protein MERTK, a member of the TAM (Tyro3, Axl and MerTK) receptor tyrosine-protein kinases, Torsin-1A, Coiled-Coil Domain-Containing 115 (CCDC115), SERTA Domain Containing Protein 1 (SERTAD1) and Dynactin Subunit 6 (DCTN6) (Borca et al., 2019; Vuono et al., 2021, 2020a, 2020b). Alteration of the E1-E2 heterodimerization and the N-linked glycosylation condition of CSFV could modulate its virulence in swine (Fernandez-Sainz et al., 2011, 2009; Li et al., 2021; Sainz et al., 2008). The interaction between host cell factor SERTAD1 and E2 protein plays a critical role in CSFV virulence (Vuono et al., 2020a).

CSFV p7, a short-lived viroporin, is the small hydrophobic transmembrane protein (Lin et al., 2014). It could induce the secretion of interleukin-1β (IL-1β) in macrophages and interact with host protein CAMLG, an integral ER transmembrane protein involved in intracellular calcium release regulation and signal response generation, and could efficiently mediate calcium permeability in the ER (Gladue et al., 2018; Lin et al., 2014). It was reported that the presence of p7 is essential for CSFV replication *in vitro*, and partial to complete attenuation of virulence *in vivo* was observed when pigs were inoculated with alanine scanning mutagenesis within the p7 amino acid sequence (Gladue et al., 2012).

The NS4B protein was found to interact with ferritin heavy chain, which enhances CSFV replication and has a positive role in viral anti-apoptosis (Qian et al., 2018). There is a putative Toll/interleukin-I receptor (TIR)-like domain in NS4B that consists of two conserved domains, mutations in this domain in the highly virulent Brescia strain could result in an attenuated phenotype along with enhanced activation of toll-like receptor (TLR)-7-induced genes (Fernandez-Sainz et al., 2010). Studies also showed that two amino acid residues within the predicted transmembrane domains of NS4B are involved in viral genome replication and could contribute to the pathogenicity of CSFV in pigs (Tamura et al., 2012). In addition, the N-terminal domain of NS4B has been associated with viral replication in cell culture and viral pathogenicity in pigs (Tamura et al., 2015). Furthermore, the synergistic virulence determinants like E2 and NS4B or E2 and 3'UTR were also determined in the attenuated strains in recent years (Tamura et al., 2012; Wu et al., 2017).

Thus far, several different approaches have been used to identify the molecular determinants of CSFV virulence (Ganges et al., 2020; Leifer et al., 2013), such as the attenuation of CSFV by modifying glycosylation or homodimer sites of the envelope glycoproteins, the attenuation through serial passages in non-natural hosts (Wu et al., 2017), the gain of pathogenicity by passaging the CSFV in pigs (Tamura et al., 2012) and loss- or gain-of-function via selected individual viral proteins from certain CSFV strains (Fernandez-Sainz et al., 2009; Ruggli et al., 2009; Tews et al., 2009; Vuono et al., 2021). Little information is available about the attenuation mechanisms that underlie the low virulence CSFV strains prevalent in the endemic areas. Interestingly, there are some speculations about this, like the U-rich insertions in the 3'UTR of the attenuated vaccine strains and low virulence isolate from the endemic regions, which has been

speculated to modulate CSFV virulence (Coronado et al., 2017; Fan et al., 2008; Wu et al., 2001). Recently, a study also showed the unique insertion-deletion mutations in the 3'UTR of the LOM-derived field CSFV strains may possibly trigger a reversion to low virulence and allow the virus to adapt to improve its persistence in the field (Jang et al., 2020).

1.4 Pathogenesis and Immunity

1.4.1. CSF Pathogenesis

CSFV is usually transmitted by the oronasal route through direct or indirect contact with infected pigs, the consumption of contaminated feed, or via vertical transmission from infected sows to their offspring (Moennig et al., 2003). In addition, as CSFV is shed from all mucosal surfaces, sexual transmission is also a possibility (Floegel et al., 2000).

CSFV primarily passes through the epithelial cells of the tonsillar crypts, replicating in the tonsils and local oropharyngeal lymph nodes (Trautwein, 1988). Thereafter, the reticuloendothelial cell system, such as macrophages (MΦ), dendritic cells (DC), and endothelial cells (EDC) will be infected as the primary targets for CSFV (Knoetig et al., 1999; Summerfield and Ruggli, 2015). Considering that, CSFV could induce the proliferation of DCs and promote the migratory ability of MΦ, which opens the way for spreading the virus to other organs. The virus could invade the regional lymph nodes after entering the lymphatic capillaries, providing the opportunity for entering the efferent blood capillaries. After viremia, the virus could reach other target organs, such as the spleen, bone marrow, lymph nodes and lymphoid structures associated with the small intestine and finally the parenchymatous organs (Belák et al., 2008). Furthermore, wider tissue distribution and cell tropism have been found

during longer durations of infection, which also confirms the complexity of CSFV pathogenesis in different clinical forms (Liu et al., 2011).

Highly virulent strains infection leads to a breakdown of the immune system, this is reflected with an aberrant pro-inflammatory response and exaggerated cytokine production, known as cytokine storm (Figure 2). As the DCs are primarily responsible for the initial recognition of pathogens associated with Swine leukocyte antigen (SLA) class I and class II, and also important for the regulation of the innate immunity (Carrasco et al., 2004). High induction of IFN-α and pro-inflammatory cytokines were produced from these cells, which leads to the direct damage of the organism, associated with severe lymphopenia and lymphocyte apoptosis instead of disease control (Sánchez-Cordón et al., 2005b; Summerfield et al., 2006). On the other hand, the moderate or low virulence CSFV infection will induce lower levels of IFN-α or pro-inflammatory cytokines, leading to control the disease process (Figure 2) (Summerfield et al., 2006; Tarradas et al., 2014; von Rosen et al., 2013). Mild or transient lymphopenia also occurred during the infection with these types of strains (Nielsen et al., 2010). Additionally, the production of IL-10 and granulocytopenia has been observed, which might be related with the immunosuppression state after infection (Bohorquez et al., 2019; Muñoz-González et al., 2015b; Summerfield et al., 2006).

Moreover, CSFV could evade the host immune response by intervening in the IFN signal pathways by the two unique proteins, N^{pro} and E^{rns}, to facilitate the CSFV persistence in the host (Figure 2) (Summerfield and Ruggli, 2015). The N^{pro} possesses a zinc-binding domain that is required for interaction with IRF3, which induces efficient proteasomal degradation of IRF-3 and being potent antagonism of IFN type I induction by CSFV.

Likewise, pDCs can express IRF-7 uniquely, and N^{pro} could help CSFV replicate efficiently in pDCs by inhibiting IRF-7 dependent induction of type I IFN. (Bauhofer et al., 2007; Fiebach et al., 2011; Gottipati et al., 2016; Ruggli et al., 2005). Interestingly, the N^{pro} interaction with IRF3 could also inhibit dsRNA-mediated apoptosis, mediated by an inhibition of the IRF3-dependent mitochondrial translocation of a proapoptotic Bcl-2 family protein (Hardy et al., 2021). A recent study also demonstrate that type-I IFN-dependent necroptosis related to non-functional N^{pro} is the main mechanism for cytopathic effect induction by cytopathogenic strain ($vGPE^-$) (Itakura et al., 2020). Additionally, N^{pro} was also found to suppress IRF1-mediated IFN-λ production by inhibiting IRF1 expression and its nuclear translocation (Cao et al., 2019).

The other unique essential envelope protein E^{rns}, which possesses RNase activity, is attached to membranes by a long amphipathic helix and is partially secreted from infected cells (Aberle et al., 2014; Schneider et al., 1993). E^{rns} could inhibit concanavalin A-induced proliferation of porcine, bovine, ovine, and human lymphocytes, and the protein synthesis of lymphocytes without cell membrane damage which induces apoptosis of lymphocytes (Bruschke et al., 1997). Studies have shown that E^{rns} can degrade viral single-stranded RNA(ssRNA) and dsRNA both in the extracellular space and in endocytic compartments (Iqbal et al., 2004; Mätzener et al., 2009). E^{rns} could inhibit TLR-7-dependent IFN-α induction in plasmacytoid DCs (pDC) after direct CSFV infection or by cell contact manner with CSFV-infected cells (Python et al., 2013). Interestingly, a recent study revealed that the C-terminal amphipathic helix of E^{rns} containing the GAG-binding site determines the efficiency of cell entry, RNase activity and uptake into cells are both required for E^{rns} to act

as an IFN antagonist (Carmela et al., 2021). All those mechanisms are revealed *in vitro* level and still need to be demonstrated *in vivo*.

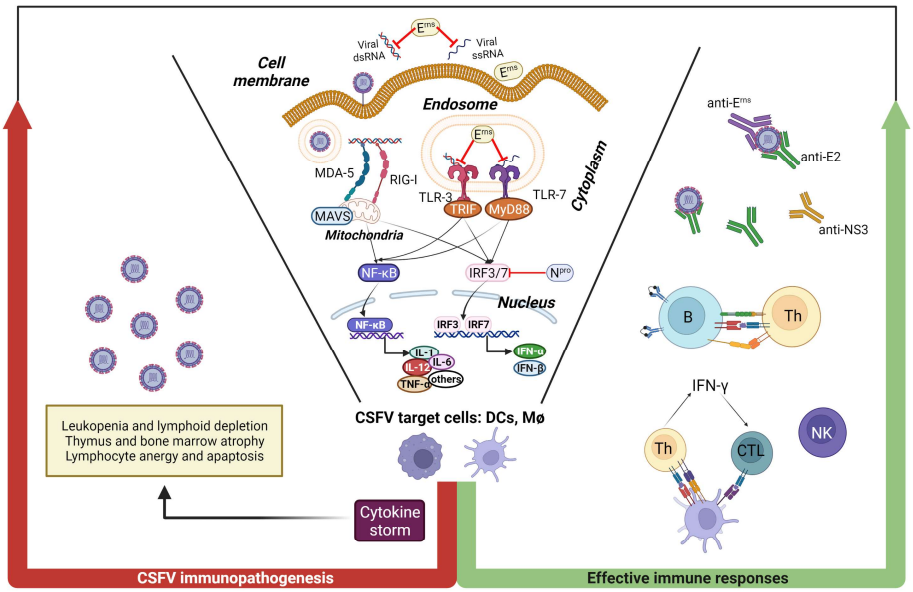

Figure 2. The main CSFV virus-host interaction mechanisms. Immunological pathways for effective immune response (green) and immunopathogenic (red) are shown. Adapted from (Summerfield and Ruggli, 2015). Image created with BioRender.com.

1.4.2. The Innate and Acquired Immune Response

The innate immune system of the host acts as a first line of defence to control the viral infection. The initial innate immune response is triggered by the viral RNA via the interaction between the host cell receptors and the pathogens, like pattern recognition receptors (PRRs). These are the essential sensors that can detect different pathogen associated molecular patterns (PAMPs), which activate innate immune signals that induce the secretion of IFNs, cytokines, chemokines and several co-stimulatory molecules that restrict the virus infection (Thompson et al., 2011). During the CSFV infection, ssRNA and dsRNA serve as the two important

PAMPs that interact with TLR-3, 7 and retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5), leading to the robust expression of IFNs (Figure 2) (Dong et al., 2013; Summerfield and Ruggli, 2015). Besides these, the E2 and NS3 viral proteins are the main inducers of the specific cytotoxic T lymphocytes (CTLs) response which could contribute to the early control of viral replication and protect against leukopenia (Franzoni et al., 2013; Graham et al., 2012; Tarradas et al., 2010). The expression of interferon-stimulated genes (ISGs) encodes proteins such as ISG15, viperin and Mx, have been shown to have anti-CSFV effects (Li et al., 2020; Xu et al., 2020; Zhou et al., 2018).

On the other hand, the neutralizing antibody response could confer sterilizing protection against CSFV, the differentiation of B cells to plasma cells facilitated by Th2 response (IL-4, IL-10), will produce the antibody response against CSFV (Figure 2) (Ganges et al., 2005; Sánchez-Cordón et al., 2005a; Tarradas et al., 2010). The proteins E^{rns}, E2 and NS3 have been known to induce neutralizing antibody production in pigs, though NS3 was not able to confer partial protection in vaccination challenge studies. The E2 is the only glycoprotein commercialized as subunit vaccine, known to confer protection against challenge. The neutralizing antibodies could be detected between 10 to 20 days post infection (Blome et al., 2017b; Ganges et al., 2008; Summerfield and Ruggli, 2015). In addition, offspring could obtain maternal-derived antibodies (MDA) from the colostrum of vaccinated sows, which can protect piglets against disease during the first weeks of life, even if they do not prevent CSFV replication and excretion (Bironf et al., 1987; Terpstra, 1977; Van Oirschot, 2003). The protective capacity by MDA are

variable due to the vaccination period and could decrease over time (Van Oirschot, 2003).

1.5 The Clinical Forms of CSF

A wide range of clinical signs can be observed from peracute deaths to unapparent courses, depending on both virus and host factors, such as the virulence of the CSFV strain, the age, the general health condition of the pigs and secondary infections, among others. CSF may follow peracute, acute, subacute, chronic, or subclinical courses (Belák et al., 2008; Blome et al., 2017b; Ganges et al., 2008; Petrov et al., 2014; Tarradas et al., 2014; Zhou, 2019). Nowadays, the growing numbers of CSFV isolates from endemic regions make the CSF forms more complex, the chronic or subclinical forms of CSF induced by low or moderate virulence strains may be currently prevalent in the field. Recently, a new CSF manifestation was characterized, that is the postnatal persistent infection, in which the infected pigs show life-long viremia while lacking antibody response (Bohórquez et al., 2019; O. Cabezón et al., 2017; Muñoz-González et al., 2015b). Those types of forms may facilitate the concealment and spread of viruses and pose the risk for future outbreaks (Coronado et al., 2017, 2019a; Ganges et al., 2008; Kameyama et al., 2019).

1.5.1 CSF Acute Form

In acute-lethal courses, mortality can reach up to 100%, death usually occurs 2 to 4 weeks after CSFV infection (Floegel-Niesmann et al., 2003). As reported, high virulence strains used for vaccine testing can induce this form of disease in all age ranges of the infected animals (Tarradas et al., 2014). The typical acute clinical signs could be generated in young pigs after infection with moderate or high virulence CSFV strains. In this case, infected animals will develop atypical clinical signs during the first two

weeks post infection, such as high fever, anorexia, weight loss, diarrhea, generally apathy, and conjunctivitis (Belák et al., 2008; Floegel-Niesmann et al., 2003; Laevens et al., 1999; Moennig et al., 2003; Tarradas et al., 2014). In the next two weeks post infection, the typical clinical symptoms will be observed, like skin hemorrhages, cyanosis in the ears, limbs, and abdomen as well as neurological signs, like ataxia, paralysis and convulsions (Blome et al., 2017b; Ganges et al., 2005; Moennig et al., 2003; Tarradas et al., 2014). Additionally, the infected animals could suffer severe secondary infections due to the immunosuppressive status, which can overlay the CSF signs and further complicate this disease course (Depner et al., 1999). Gross pathology reveals that enlarged lymph nodes, tonsillitis, hemorrhages and petechiae in lungs, kidneys, intestines and urinary bladder, splenic infarctions, necrotic ulcers in the intestines, non-purulent encephalitis and thymus atrophy can be observed in the infected pigs (Belák et al., 2008; Depner et al., 1994; Gómez-Villamandos et al., 2000).

Moreover, pigs could suffer the peracute CSF course with high mortality or less severe subacute CSF form with less mortality. In the peracute cases, there are no clear lesions found at necropsy (MacLachlan et al., 2017). In the subacute cases, pigs developed fever, diarrhea and neurological clinical signs. The surviving animals will develop long-lasting humoral immunity against CSFV (Chander et al., 2014).

1.5.2 CSF Chronic Form

The chronic form of CSF is usually generated when pigs are infected by low or moderated virulence strains and infected animals are not able to elicit an effective immune response against CSFV. An important indicator of this case is that the survival of infected animals exceeds 30 days

(Coronado et al., 2019a; Jenckel et al., 2017; Moennig et al., 2003; Muñoz-González et al., 2015b; Tarradas et al., 2014).

A similar disease process with acute form will be observed in the pigs suffering the first phase of the chronic course, followed by a period in which animals will be apparently healthy (Mengeling and Packer, 1969). Nevertheless, the pigs will develop unspecific clinical signs over time, like intermittent fever, anorexia, stunting, chronic enteritis, wasting, and diffuse dermatitis. As the outcome of this form, the pigs will die after two or three months suffering (Blome et al., 2017b; Moennig et al., 2003). The pathological findings include thymus atrophy and necrotic ulcerative lesions in the ileocecal valve, ileum and rectum (Moennig et al., 2003). It has been described previously that the development of glomerulonephritis could occur in the animals suffering the chronic form due to the inefficient and intermittent antibody response (Choi and Chae, 2003). Antibodies might not always be detectable, as they are consumed by the CSFV, which is still an important point to differentiate with persistent infection (Depner et al., 1996). In addition, secondary infections are frequent, which may exacerbate the clinical signs and complicate the differential diagnosis (Coronado et al., 2019a).

1.5.3 CSF Congenital Persistent Form

The congenital persistent form can be established based on the transplacental transmission capacity of CSFV. It is well established that the low virulence CSFV infection of the pregnant sow during mid-gestation will lead to persistently infected (PI) offspring, a phenomenon known as “carrier sow syndrome”. (Liess, 1984; Van Oirschot, 1979; Van Oirschot and Terpstra, 1977). The different outcomes of transplacental infection of fetus will depend on the infection time during gestation or the

virulence of the strains. Those infections may result in abortion, stillbirth, absorption, mummification, malformation or weak, PI, apparently heathy fetuses (Blome et al., 2017b; Van Oirschot and Terpstra, 1977).

Congenitally PI piglets may show poor growth, wasting or occasionally congenital tremor although they were clinically healthy at birth. Those piglets will survive for up to 11 months. During the "late onset" process, PI pigs will develop more severe clinical signs such as anorexia, depression, conjunctivitis, dermatitis, diarrhea and neurological disorders (Coronado et al., 2019a; Moennig et al., 2003; Trautwein, 1988; Van Oirschot and Terpstra, 1977). The PI piglets may show lifelong spread of the virus, due to the inability for antibody response development and may evade current CSF surveillance, posing a risk for CSFV circulation and outbreaks as the reservoirs and disseminators of the virus (Blome et al., 2017b; Kaden et al., 2005; Moennig et al., 2003; OIE, 2019).

The immunotolerance phenomenon due to a lack of pathogen recognition in the immature state of the fetal immune system has been studied as one mechanism underlying the establishment of the congenital persistent infection (Peterhans and Schweizer, 2010; Schweizer and Peterhans, 2014; Van Oirschot, 1979). However, a recent study suggests that this mechanism may be more complicated, as IFN-α induction, related with the innate immune response against the CSFV after transplacental transmission, was detected in the fetus, indicating that the fetal immune system could recognize the CSFV (Bohórquez et al., 2020). Moreover, as an increased myeloid-derived suppressor cell (MDSC) population were found in the cord blood and neonatal peripheral blood, immunosuppressive cell populations may play an important role in maternal-fetal tolerance, while further studies are needed (Rieber et al., 2013).

1.5.4 CSF Postnatal Persistent Form

The postnatal persistent infection was generated recently in both newborn piglets or wild boars, following infection within the first 24 hours after birth with a moderate virulence CSFV isolate (O. Cabezón et al., 2017; Muñoz-González et al., 2015b). The postnatal PI piglets were monitored up to nine weeks post infection, they were clinically healthy or developed some unspecific clinical signs, like mild diarrhea or polyarthritis. The absence of humoral immune response and high level of virus replication and virus secretion were shown in this form of disease, which is similar to the congenital persistent infection (Blome et al., 2017b; Muñoz-González et al., 2015b; Van Oirschot, 1979). Notably, the postnatal PI piglets should induce an initial innate immune response in terms of IFN-α, which is contrasted to the immunotolerance mechanism that explained in the congenital PI cases (Muñoz-González et al., 2015b; Van Oirschot, 1979). Nevertheless, this IFN-α pathway was blocked six weeks later in the PI animals as they were not able to induce IFN-α against African swine fever virus (ASFV), a virus with a proven capacity to induce high IFN-α response (Oscar Cabezón et al., 2017; Muñoz-González et al., 2015a). Thus, the mechanism of this clinical form is more related to immunosuppression, which is different from the immunotolerance against CSFV since the congenital PI pigs could induce an immune response to other pathogens besides CSFV (Carbrey et al., 1977; Trautwein, 1988; Van Oirschot, 1979; Vannier et al., 1981).

Likewise, the postnatal persistent infection was also generated in 3-week-old piglets infected with a moderate virulence CSFV strain, although a lower population of the PI pigs were obtained in this case compared with the pigs infected within 24 hours after birth (Bohórquez et al., 2019).

Further study suggests that the PI pigs may go through the immune exhaustion state indicated by the low CD4/CD8 ratio. In addition, an increased number of precursor myeloid cell population were detected in bone marrow and peripheral blood of PI piglets (Bohorquez et al., 2019). These cells were found to be similar with immunosuppressive MDSC populations that have been related with persistent infection in humans (Bohorquez et al., 2019; Tacke et al., 2012).

Moreover, the CSFV superinfection exclusion (SIE) phenomenon was demonstrated in those postnatal PI pigs. They were resistant to not only a CSFV C-strain vaccine, but also a high virulence CSFV strain infection (Muñoz-González et al., 2016, 2015a). The SIE phenomenon or homologous interference, is defined as the capacity of primary viral infection to inhibit a second infection with the same or related virus, favoring the stability of viral genome within the same cells (Formella et al., 2000; Huang et al., 2008; Lee et al., 2005). Interestingly, it has been applied for pathogen control in crops and proposed for the treatment of hepatitis C virus (HCV)-infected patients by transplantation of HCV infected liver (Shoresh et al., 2006; Webster et al., 2013). Although further studies are required to reveal the mechanism of the SIE phenomenon, it should be noted that those pigs were refractory to vaccination. While as the main PI pigs inducer, low and moderate CSFV strains are currently circulated in the endemic regions, which poses a big obstacle to CSF control (Muñoz-González et al., 2015a). Nevertheless, a recent study showed the urgency of prevention and control this form of the disease, considering that an ineffective vaccination program could induce a high prevalence of chronic and persistent CSF forms in the field (Coronado et al., 2019a).

1.6 CSF Diagnosis

Traditional diagnostic for CSF includes clinical signs, pathological lesions and antigen and antibody detection. The rapid clinical diagnosis of CSF is difficult due to the current complicated signs of CSF disease and the similarity between CSF forms and multiple swine diseases (Moennig et al., 2003; Moennig and Becher, 2015). Subclinical disease induced by low or moderate CSFV strains make the spread of the disease more concealed and harder to be detected. Likewise, CSF can be masked with other viral diseases, such as ASF that currently is endemic in some CSF affected countries (Cabezón et al., 2017; J et al., 2021; Schulz et al., 2017). Otherwise, secondary infection with bacterial agents causing septicemia can take place after CSFV infection and those isolated pathogens may mask the real cause of the disease. Hence, accurate and fast CSF diagnosis by the laboratory methods is essential to expose the real pathogen CSFV due to the possibility for misleading by clinical diagnosis (van Oirschot, 2004; L. Wang et al., 2020).

The well-established laboratory diagnostic methods, including antigen and antibody detection, have been well described in the OIE manual (OIE, 2019). In the case of the antigen detection, four types of methods are listed. The oldest and *gold standard* laboratory technique is virus isolation from sera, plasma, whole blood in EDTA, organs and leukocytes preparations in susceptible cell lines (PK-15, SK-6 commonly used) (de Smit et al., 1994). Moreover, the other CSFV permissive cell lines from porcine, cattle, goat or swine primary cells could be also employed to enhance the chances for CSFV isolation (Roehe and Edwards, 1994). Considering that most CSFV strains are non-cytopathic, the virus visualization in cell culture relies on peroxidase linked assay (PLA) by using fluorescent or

horseradish peroxidase (HRP)-conjugated antibodies (Wensvoort et al., 1986). Besides virus isolation, CSFV antigen can be detected directly in cryosection of tissues or fixed cells by fluorescence antibody test (FAT) and also by the CSFV antigen-capture enzyme linked immunosorbent assay (ELISA) based on the double-antibody-sandwich principle for rapid diagnosis of CSF by detecting CSFV E2 or E^{rns} proteins in sera, blood, plasma or tissue extracts (Moennig and Becher, 2015; Shannon et al., 1993). However, both antigen detection tests showed low sensitivity and specificity, which limits their wide use for CSF diagnosis. The reverse transcription polymerase chain reaction (RT-PCR) tests were developed in 1990s and included for the routine diagnosis of CSFV, however, the applied RT-PCR protocol must prove to be at least as sensitive as virus isolation (OIE, 2019; Vilček et al., 1994). Notably, quantitative RT-PCR (RT-qPCR) has now replaced the gel-based conventional RT-PCR, leading to rapid and highly sensitive CSF diagnosis. Several commercial kits and in-house methods were used by targeting different fragment of CSFV (Hoffmann et al., 2005; Postel et al., 2012). Considering the growing members of the *Pestivirus* genus, including the new discovered ovine Pestivirus, the specific CSFV RT-qPCR needs to be improved (Bohórquez et al., 2021; M. Wang et al., 2020b).

For the detection of CSFV-specific antibodies, virus neutralizing test (VNT) and CSFV Antibody ELISAs are listed in the OIE manual. VNT is the gold standard for antibody detection from sera and body fluids samples due to its high sensitivity and specificity. The VNT assay is based on the whole virus antigen, and allows for the differentiation the neutralizing antibody titers against different CSFV strains and other pestiviruses (Greiser-Wilke et al., 2007). However, VNT is also a time-consuming procedure for cell culture preparation and visualization of results by PLA,

thus it is not suitable for mass analysis of samples (Terpstra et al., 1984). The CSFV antibody ELISAs against glycoproteins E2 or E^{rns} have been developed to overcome the disadvantage of VNT, which makes it possible to evaluate a larger number of samples in a short time. Thus, it is recommended for application in CSF surveillance at the herd level (OIE, 2019). The CSFV E2 ELISA has been commonly used considering its higher sensitivity than CSFV E^{rns} ELISAs (Moennig and Becher, 2015). While CSFV ELISA against E^{rns} could be applied as a test to differentiate infected from vaccinated animals, combined with CSF marker vaccines (Meyer et al., 2017; Pannhorst et al., 2015; Schroeder et al., 2012).

1.7 Phylogenetic Analysis and Molecular Epidemiology of CSFV

The identification of CSFV subtypes could improve our understanding of CSFV evolution and epidemiology and might provide markers for biological differences, such as virulence. The application of molecular genetic methods has significantly improved the laboratory detection and characterization of CSFV and makes classification of CSFV genotypes possible (Hofmann et al., 1994). The 5'UTR region was first used to characterize and group the pestiviruses, then a 190 bp region of E2 and a 210 bp region of NS5B were selected for the construction of CSFV phylogenetic trees (Lowings et al., 1996). Which three sequencing-based methods were confirmed by monoclonal antibody (mAb) typing and limited restriction enzyme (RE) mapping, although both mAb and RE methods showed poor resolution (Lowings et al., 1996; Vilček et al., 1996). To date, the conserved sequences of 5' and 3'UTR, N^{pro}, NS5B and E2 have been used for CSFV phylogenetic analysis (Liu et al., 2009). The implementation of next-generation sequencing techniques allows for the rapid determination of complete genome sequences, which further

improve the accuracy and speed for pathogens genetic typing (Barzon et al., 2013). Against this background, the EU and OIE Reference Laboratory for classical swine fever (EURL CSF) maintains a sequence database of classical swine fever virus (CSFV) genomes as an easy-to-use sequencing tool for molecular epidemiology (Postel et al., 2016). CSFV is a stable RNA virus with only one serogroup and strains have been divided into three genotypes and 11 subgenotypes (1.1-1.4, 2.1-2.3, and 3.1-3.4) (Postel et al., 2016, 2013). With growing numbers of new CSFV isolates, it seems the accumulation of point mutations under selective pressure played a role as the major driving force for CSFV evolution resulting in the emergence of genetically highly variable CSF viruses. Recently, a phylogenetic analysis including the full E2 sequence from 517 CSFV isolates, led to a proposal to reclassify previously identified subgroups. Under this study, Genotypes 1 and 2 can be further divided into seven subgenotypes each (1.1-1.7 and 2.1-2.7, respectively). While it also established two additional genotypes, genotype 4 included a United Kingdom (UK) strain causing congenital tremor (Great Britain/1964) and genotype 5 included two South Korean isolates (KR/1998, KR/1999) (Garrido Haro et al., 2018; Rios et al., 2018; Silva et al., 2017).

According to recent CSF outbreaks reported by the world animal health information system (WAHIS), CSFV Subgenotypes 2.1 and 2.3 could be responsible for the more recent outbreaks in Europe (Postel et al., 2018). Subgenotypes 1.1, 2.1, 2.2, and 2.3 are prevalent in Asia (Malik et al., 2020; Postel et al., 2019; Zhou, 2019). Meanwhile, the subgenotypes 1.1 and 1.4 have been circulating for Caribbean and South American (de Oliveira et al., 2020; Postel et al., 2013).

1.8 Vaccine Strategies Against CSFV

Due to the enormous economic and animal welfare disadvantages of stamping-out policies, systematic vaccination as the main CSF control strategy has been implemented for decades. Several highly efficacious live attenuated CSF vaccines have been widely used and supported the successful eradication in many areas (Blome et al., 2017b; Van Oirschot, 2003). Nevertheless, the application of those vaccines lacks the differentiability of infected from vaccinated animals (DIVA), which interferes with the serological diagnosis and poses a thorny problem for CSF eradication. Considering this, only emergent vaccination was permitted to limit the disease spread in the CSF-free zone or where eradication is in progress, however, the vaccinated animals were slaughtered after the outbreak to regain CSF-free status with the non-vaccination policy (Bazarragchaa et al., 2021; Postel et al., 2018). In addition, considering wild boars are also the natural reservoirs of CSF, strict controls, like oral vaccination with C-strain and population and movement restriction, could avoid CSF outbreaks in domestic pigs that originated from wild boars (Brauer et al., 2006; Kaden et al., 2002). For example, a CSF epidemic study from September 2018 to the end of August 2019 showed that the re-emergence of CSF in Gifu Prefecture (Japan) was mainly driven by wild boar cases with sporadic outbreaks on domestic pig farms (Ito et al., 2019). Afterwards, oral vaccination of wild boar was applied to control the CSF spread, starting at the end of March 2019, through continuous vaccination periods, which partially reduced the risk of disease transmission (Bazarragchaa et al., 2021).

The conventional live attenuated vaccines could elicit both cellular and humoral immune response, which provides sterilizing protection in pigs against CSFV from all three genotypes (Yuzi Luo et al., 2017). The widely used C-strain, also known as the hog cholera lapinized virus (HCLV) was

developed jointly by the China institute of Veterinary Drug Control and the Harbin Veterinary Research Institute in 1956. It was attenuated by at least 480 passages of a high virulent strain in rabbits (Wei et al., 2021). Afterwards, more methods were studied for CSFV attenuation, such as low-temperature adapted Japanese guinea-pig exaltation-negative (GPE^-) strain (Ishikawa et al., 1995), the French cell culture adapted Thiverval strain (Lamothe-Reyes et al., 2021), and Mexican PAV strain (Mendoza et al., 2007). They are safe in young piglets or pregnant sows, even in immunosuppressed individuals (Bironf and Leunen, 1988). The vaccinated pigs could be clinically protected as early as 3 days after a single vaccination and the provided immunity can last at least 6 to 18 months or even be lifelong (Blome et al., 2017a; Kaden et al., 2004). In addition, the oral immunization of these vaccine could also provide the solid protection even for vertical protection in the pregnant wild sows (Kaden et al., 2008). Although this type of vaccine almost meets all the requirements of the ideal vaccine (safety, efficacy, and marketability), they are banned in CSF-free zones or where eradication is in progress due to the main drawback of failing to comply with the DIVA concept. Thus, the application of these vaccines is followed by severe trade restrictions (Ganges et al., 2020, 2008; Postel et al., 2018).

Aiming to fulfill the DIVA concept, multiple vaccine strategies have been proposed to obtain a marker vaccine since the early 1990s, however only two of them have been licensed, both chimeric pestivirus vaccines. The first one is the “CP7_E2alf” DIVA vaccine licensed in Europe in 2014, which was constructed using the infectious cDNA clone of the cytopathogenic BVDV strain CP7 expressing E2 gene of CSFV strain Alfort/187 (Blome et al., 2017c). This vaccine has been demonstrated to be as safe and efficacious as the conventional live attenuated vaccine by

multiple studies. Moreover, the combined DIVA diagnostic kits based on CSFV E^{rns} detection have been developed and commercialized (Leifer et al., 2009; Pannhorst et al., 2015). However, aspects regarding the vertical protection of this vaccine has restricted its use in sows (Blome et al., 2017a). Also, the late onset of E^{rns} antibody and its cross-reaction with BVDV and BDV reduces the specificity of its DIVA diagnosis (Meyer et al., 2017). The other vaccine is "Flc-LOM-BE^{rns}" which was licensed in 2017 in South Korea. In this chimera, the 3'end capsid gene and the complete E^{rns} gene of the live attenuated CSFV vaccine strain LOM were replaced by the corresponding sequences of KD26 BVDV strain. There is little data available about this vaccine application due to its short time being used in the field. As it shares the same DIVA diagnosis idea with "CP7_E2alf", it also has the same drawback regarding the E^{rns} antibody detection (Lim et al., 2019). Another promising strategy for the generation of live attenuated marker vaccines, the recombinant deletion or mutation of DIVA vaccine viruses, has led to the development of candidates such as FlagT4Gv, with a Flag epitope insertion and elimination of an epitope of the E2 glycoprotein recognized by monoclonal antibodies (Holinka et al., 2014). This vaccine could protect pigs against challenge as early as 3 dpv, while its combined DIVA test remains to be developed (Holinka et al., 2017).

Viral vector vaccine candidates have recently been developed, using several viral vectors, such as swinepox virus, pseudorabies virus, and adenovirus. The use of viral vectors to deliver CSFV antigens, mainly E2 glycoprotein, could maintain the advantage of live vaccines to activate the immune response for greater efficacy (Hahn et al., 2001; Lin et al., 2017; Sun et al., 2013; Y. Wang et al., 2015). Notably, this strategy could offer the chance for developing vaccines protective against multiple pig diseases

(Abid et al., 2019; F. Gao et al., 2020). Another DIVA-development strategy is the subunit vaccines based on recombinant CSFV immunodominant glycoprotein E2 expressed in different systems, like in insect cells or cost-effective plants. (Blome et al., 2006; Gong et al., 2019; Park et al., 2021).

At present, two E2 subunit DIVA vaccines have been licensed in Europe, the BAYOVAC® (Bayer AG) and the Porcilis® Pesti (MSD Animal Health), and one was officially licensed and commercialized in China, the Tian Wen Jing (TWJ-E2®) (Blome et al., 2006; Gong et al., 2019; Schroeder et al., 2012). However, its delayed onset of immunity, lack of protection against trans-placental infection, failure for oral immunization and extra doses required, are still major points for future study (OIE, 2019). Several strategies have been tried to improve the E2 subunit vaccine in recent years, such as in combination with molecular adjuvant. In this regard, a subunit vaccine candidate has been developed in which the CSFV E2 antigen was fused to the porcine CD154, a glycoprotein that belongs to the tumor necrosis factor superfamily. A single-dose vaccination with E2-CD154 could protect pigs from the challenge after 7 days post-infection and a two-dose vaccination could protect the fetuses from transplacental transmission after the challenge of pregnant sows (Madera et al., 2016; Muñoz-González et al., 2017; Suárez-Pedroso et al., 2021).

It should be noted that the intensive vaccination process implemented under poor measures, for example, low quality and availability of the vaccine and gaps in the cold chain between vaccine production and application, among others, may induce a failure in the induction of sterilizing immunity in pig herds (Díaz de Arce et al., 2005; Ganges et al., 2008). This, in turn, may provide opportunities for the emergence of

escaping variants of CSFV strains, which would lead to complex situation, including different forms of disease severity from acute hemorrhagic to chronic and subclinical disease (Coronado et al., 2019b, 2019a; Fatima et al., 2021; Muñoz-González et al., 2015b). Nevertheless, successful CSF eradication still calls for early and accurate detection of CSF and the development of potent DIVA vaccines along with its respective DIVA diagnostic test.

Chapter 2

Objectives

CSFV remains a re-emerging virus that affects swine health and the pork industry worldwide. In endemic countries, after several decades of vaccination programs, the low and moderate virulence CSFV strains, associated with a broad range of clinical manifestations, including subclinical forms, are prevalent. The infected pigs with unapparent forms become the blind spot for the CSF diagnostic and serve as the virus carrier to maintain the persistence in endemic regions and the possible re-emergence in the CSF-free countries.

The level of CSF severity is determined by the virus-host interaction mechanisms, dependent on the virulence level of the infecting strain, the age and the host's immune status, among others. Despite the importance of the CSFV virulence determinants for the outcome of CSF, the molecular basis behind the natural CSFV attenuation has not been studied. Among the viral proteins, the E^{rns} glycoprotein has been known to contribute in the establishment of Pestivirus persistent infection in ruminant. Besides the E^{rns} glycoprotein, the 3'-UTR also has been associated with the viral determinants that can be involved in the attenuation of some CSFV live attenuated vaccines. In this regard, a low virulence CSFV strain, isolated from an endemic situation under vaccination, was proven to generate subclinical and persistent infection in pigs. Notably, an uninterrupted poly-U insertion in the 3'-UTR with an average length of 36 nucleotides, unique for CSFV genome, was found in this low virulence CSFV isolate.

The present thesis focuses on the study of molecular basis involved in the CSFV attenuation and their implications in the development of CSF subclinical and persistent forms. To this end, the role of the E^{rns} glycoprotein and 3'UTR in the CSFV natural attenuation and their relationship with the level of viral replication *in vitro* and *in vivo* was

studied. In addition, the role of these two viral determinants in pathogenesis, immunity, and transmission capacity in pigs was assessed using the reverse genetics approach. The specific objectives were defined as follows:

- To investigate the potential role of the poly-U sequence found in the low virulence CSFV strain previously recovered from an endemic country (PdR strain) in attenuation and disease pathogenesis. Two functional cDNA clones were generated, one with the sequence of the original PdR isolate carrying the 36-uridine sequence (pPdR-36U) and a mutant with the standard 5 uridines (pPdR-5U) at this position. The viruses were rescued from the respective cDNA clones and used to assess the role of the poly-U sequence for replication in cell culture and for virulence and transmission in new-born piglets (Study I).

- To assess the role of the RNase activity of the E^{rns} glycoprotein *in vivo*. To this end, the E^{rns} RNase-negative mutant CSFV vPdR-H_{30}K-36U virus, using the functional cDNA backbone pPdR-36U described previously was constructed. The virus replication and type I IFN responses *in vitro* and *in vivo* and the role of the E^{rns} RNase activity for viral pathogenicity, persistence, and transmission in pigs were evaluated (Study II).

- To study the potential synergistic effect of the E^{rns} RNase activity and the poly-U insertion in the 3'UTR in the innate and adaptive immunity regulation and its relationship with the CSF pathogenesis in pigs. To this end, a reverse genetics approach was used to generate a double mutant with the inactivation of the RNase

function from the E^{rns} glycoprotein and with 5 uridines instead of the aforementioned 36 in the 3'UTR of the low virulence PdR strain. The relationship between viral replication and the IFN-α activation in pDCs and in pigs of two different age ranges was also assessed (Study III).

Part II

Studies

Chapter 3

Study I. A Polyuridine Insertion in the 3'Untranslated Region of Classical Swine Fever Virus Activates Immunity and Reduces Viral Virulence in Piglets *(J Virol. 2020 Jan 6;94(2): e01214-19)*

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3.1 Abstract

Low-virulence classical swine fever virus (CSFV) strains make CSF eradication particularly difficult. Few data are available on the molecular determinants of CSFV virulence. The aim of the present study was to assess a possible role for CSFV virulence of a unique, uninterrupted 36-uridine (poly-U) sequence found in the 3' untranslated region (3'UTR) of the low-virulence CSFV isolate Pinar de Rio (PdR). To this end, a pair of cDNA-derived viruses based on the PdR backbone were generated, one carrying the long poly-U insertion in the 3'UTR (vPdR-36U) and the other harboring the standard 5 uridines at this position (vPdR-5U). Two groups of 20 5-day-old piglets were infected with vPdR-36U and vPdR-5U. Ten contact piglets were added to each group. Disease progression, virus replication, and immune responses were monitored for 5 weeks. The vPdR-5U virus was significantly more virulent than the vPdR-36U virus, with more severe disease, higher mortality, and significantly higher viral loads in serum and body secretions, despite similar replication characteristics in cell culture. The two viruses were transmitted to all contact piglets. Ninety percent of the piglets infected with vPdR-36U seroconverted, while only one vPdR-5U-infected piglet developed antibodies. The vPdR-5U-infected piglets showed only transient alpha interferon (IFN-α) responses in serum after 1 week of infection, while the vPdR-36U-infected piglets showed sustained IFN-α levels during the first 2 weeks. Taken together, these data show that the 3' UTR poly-U insertion acquired by the PdR isolate reduces viral virulence and activates the innate and humoral immune responses without affecting viral transmission.

3.2 Importance

Classical swine fever (CSF), a highly contagious viral disease of pigs, is still endemic in some countries of Asia and Central and South America. Considering that the 3' untranslated region (3'UTR) plays an important role in flavivirus replication, the present study showed for the first time that a long polyuridine sequence acquired in the 3'UTR by an endemic CSFV isolate can activate immunity, control viral replication, and modulate disease in piglets. Our findings provide new avenues for the development of novel vaccines against infections with CSF virus and other flaviviruses. Knowledge of molecular virulence determinants is also relevant for future development of rapid and efficient diagnostic tools for the prediction of the virulence of field isolates and for efficient CSF control.

3.3 Keywords

3'UTR; CSFV; polyuridine insertion; viral replication; virulence

3.4 Introduction

Classical swine fever (CSF) is a highly contagious viral disease that affects domestic and wild pigs. Due to its socioeconomic importance in domestic pigs, CSF is notifiable to the World Organisation for Animal Health (OIE). The disease is characterized by a broad spectrum of clinical signs. The individual outcome depends on several factors, such as the age of the infected animal and the virulence of the virus (Moennig et al., 2013). Currently, CSF is endemic in several countries of South and Central America and some parts of Asia (Blome et al., 2017b).

The disease is caused by CSF virus (CSFV), which belongs to the *Pestivirus* genus within the *Flaviviridae* family (Smith et al., 2017).

CSFV is composed of a lipid envelope, a capsid, and a single-stranded, positive-sense RNA genome of 12.3 kb. The genome carries a single long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR) that are important for genome replication and initiation of viral protein translation. The ORF encodes four structural and eight nonstructural proteins (Tautz et al., 2015). The envelope glycoprotein E2 represents the major immunogenic protein of pestiviruses, plays a central role in virus entry, and is associated with virulence (Leifer et al., 2013). CSFV strains can be classified as of high, moderate, or low virulence. While infections with highly virulent strains induce acute disease, strains with a lower degree of virulence can result in chronic, persistent, and subclinical forms of the disease (Ganges et al., 2008; Muñoz-González et al., 2015b). For these reasons, low-virulence CSFV strains are particularly challenging for CSF eradication. Little is known on the molecular determinants of CSFV attenuation and of disease pathogenesis. Recently, a unique uninterrupted poly-uridine (poly-U) sequence of an average length of 36 nucleotides was found in the 3'UTR of the low-virulence CSFV strain Pinar del Rio (PdR), isolated from a country of endemicity under a vaccination program (Coronado et al., 2017; Postel et al., 2015). According to GenBank, all other CSFV genomes known to date harbor 4 or 5 uridines at this position of the 3'UTR (Coronado et al., 2017). The highly virulent CSFV strain Margarita, likely the parental strain of the PdR virus (Coronado et al., 2017), also carries the standard 5 uridines at this position. However, a similar 6- to 32-nucleotide-long uridine-rich insertion was found in the 3'UTR of several attenuated CSF vaccine virus strains, but this sequence was located approximately 90 nucleotides upstream of the PdR poly-U insertion (Fan et al., 2008; Wu et al., 2001). Previous studies showed that

the 3'UTR of flaviviruses are implicated in virus replication (Chang et al., 2013; Schnell et al., 2012). Accordingly, it was speculated that the uridine-rich sequences of these vaccine strains and the poly-U insertion of PdR may contribute to viral attenuation (Coronado et al., 2017; Wu et al., 2001).

In the present study, we investigated the potential role of the poly-U sequence of the PdR strain in attenuation and disease pathogenesis. To this end, we generated two functional cDNA clones, one with the sequence of the original PdR isolate carrying the 36-uridine sequence (pPdR-36U) and a mutant with the standard 5 uridines (pPdR-5U) at this position. The viruses were rescued from the respective cDNA clones and used to assess the role of the poly-U sequence for replication in cell culture and for virulence and transmission in newborn piglets.

3.5 Results

3.5.1 CSFV PdR recombinants with 36 and 5 uridines in the 3'UTR do not differ in replication in cell culture.

In order to study the role of the poly-U sequence found in the 3'UTR of CSFV PdR, a functional cDNA clone of the PdR strain (pPdR-36U) and a mutant with the standard 5 uridines at this position (pPdR-5U) were constructed as described in Materials and Methods. The vPdR-36U and vPdR-5U viruses were rescued from the respective full-length cDNA clones by transfection of the porcine aortic endothelial cell line PEDSV.15 with *in vitro*-transcribed RNA (see Materials and Methods). For the two constructs, the specific infectivity of the RNA transcripts and the virus titer in PEDSV.15 cells 65 h after transfection were higher than 105 focus-forming units/μg of RNA and 5×10^6 50% tissue culture

infective doses ($TCID_{50}$)/ml, respectively, which demonstrates the functionality of the two cDNA clones. After one additional passage in PEDSV.15 cells, the complete nucleotide sequence of the two viruses was determined, which confirmed the presence of the 36- and 5-uridine sequences in the 3'UTR of the respective viruses and excluded any other difference or accidental mutation. Thus, vPdR-36U and the parental PdR virus (GenBank accession number KX576461) have identical consensus sequences. The growth characteristics of vPdR-36U, vPdR-5U, and the PdR isolate were analysed in PEDSV.15 cells and in porcine monocyte-derived macrophages. The parental and the two cDNA-derived viruses did not differ in their replication kinetics (Fig. 1). This validates vPdR-36U as a functional cDNA-derived PdR strain. It demonstrates also that the poly-U sequence does not influence the basic replication characteristics of the virus in cell culture.

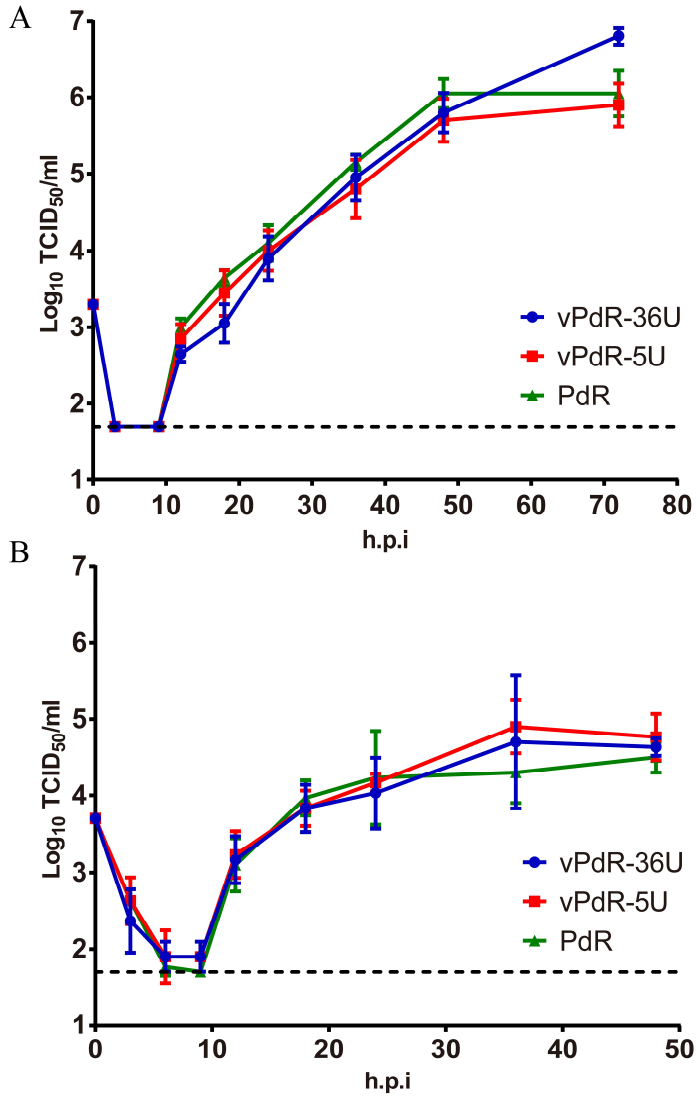

FIG 1. Virus replication kinetics in PEDSV.15 cells and porcine macrophages. PEDSV.15 cells (A) and porcine monocyte-derived macrophages (B) were infected in quadruplicate and in triplicate, respectively, with CSFV PdR and with cDNA-derived vPdR-36U and vPdR-5U at an MOI of 0.02 $TCID_{50}$/cell based on titers obtained in the homologous cell type (plotted on day 0). At the indicated hours postinfection (h.p.i), virus was harvested by one freeze-thaw cycle and the infectious titer was determined in PEDSV.15 (A) or in SK-6 cells (B) by endpoint dilution. The limit of detection of the titration (1.7 $\log_{10}$ $TCID_{50}$/ml) is represented with a dashed line. Each point

represents the mean titer from four parallel infections in panel A and three in panel B, with error bars showing the SDs.

3.5.2 The 36-uridine sequence in the 3'UTR of PdR is associated with reduced disease severity in piglets.

In order to determine whether the long poly-U sequence of the PdR virus has an effect on virulence *in vivo*, two groups of 5-day-old piglets (20 piglets each) were inoculated with vPdR-36U (group 1) and vPdR-5U (group 2). Notably, the swine is the natural host for CSFV, and piglets are highly susceptible to infection. Virus transmission was assessed by addition of 10 naive piglets to each group 24 h later (contact groups). The clinical signs were monitored daily for each individual animal in a blinded manner (Fig. 2), and a clinical score value was assigned accordingly (Data S1).

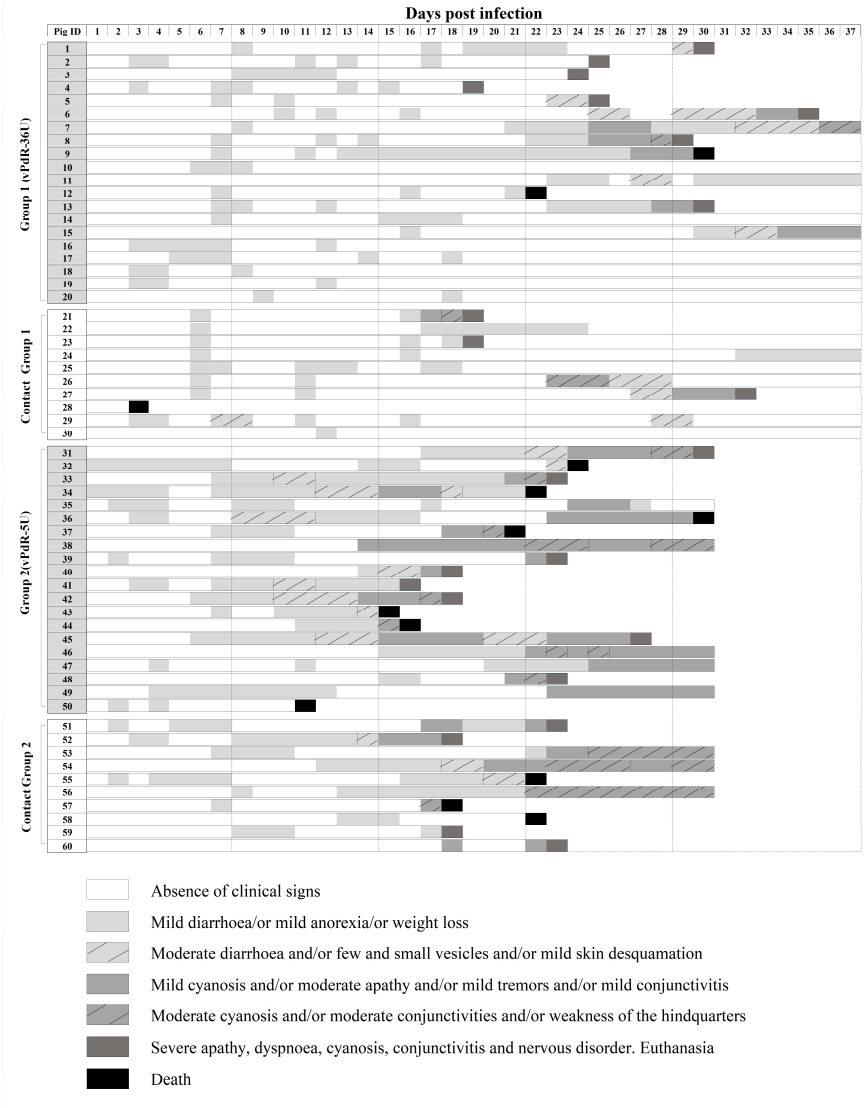

FIG 2. Individual clinical signs monitored after CSFV infection. Animals 1 to 20 (group 1) were infected with vPdR-36U and animals 21 to 30 were added to group 1 as contact animals after 24 h. Animals 31 to 50 were infected with vPdR-5U (group 2) and animals 51 to 60 were added to group 2 as contact animals after 24 h. The piglets were monitored clinically on a daily basis during the 37 days of the study. The severity of the clinical signs is represented by different shades of gray with or without hatches as shown in the key.

During the first week after infection, very mild diarrhea and apathy were observed with both viruses, resulting in mean clinical scores close to zero (Fig. 3), with the exception of one of the vPdR-36U contact animals that died on the third day of the trial (Fig. 2 and Data S1). No significant difference was found between the groups during that time, except for the vPdR-36U contact animals compared with the other groups at 6 days post infection (dpi) ($P = 0.046$) (Fig. 3).

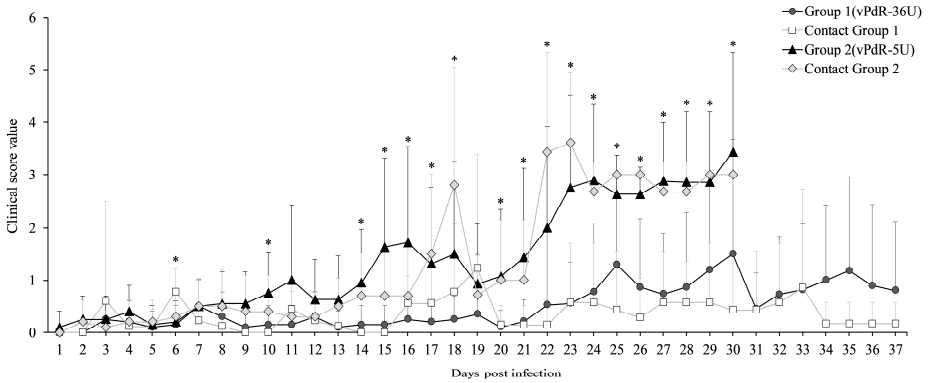

FIG 3. Mean clinical score values. The mean daily clinical score value (from zero to six) was calculated for group 1 (vPdR-36U-inoculated) and group 2 (vPdR-5U-inoculated) and for their respective contact groups. Asterisks indicate statistically significant differences of the mean clinical scores between the groups ($P < 0.05$).

Significant differences in disease manifestation between the groups appeared in the second week of infection. During this time, only mild sporadic diarrhea was observed in vPdR-36U-infected piglets, including the contact animals, which had clinical scores between 0 and 1. Meanwhile, the majority of vPdR-5U-infected piglets (inoculated and contacts) developed diarrhea, with some of them also showing other clinical signs, resulting in clinical scores between 1 and 2 (Fig. 2 and

Data S1). One animal from group 2 died suddenly at 11 dpi (piglet 50 [Fig. 2]). Accordingly, during the second week, the vPdR-5U-infected piglets showed significantly higher clinical scores than the vPdR-36U-infected piglets at 10 and 14 dpi ($P = 0.047$ and 0.018, respectively) (Fig. 3).

During the third week postinfection, the disease was consistently more severe in vPdR-5U- than in vPdR-36U-infected piglets, with significantly higher clinical scores between the two groups on 5 out of 7 days ($P \leq 0.042$) (Fig. 3). Specifically, during this week, nearly all vPdR-36U-inoculated and remaining contact piglets were either clinically healthy or developed only mild sporadic diarrhea (clinical scores between 0 and 1). Nevertheless, one vPdR-36U-inoculated animal (number 4) and two contact animals had to be euthanized at 19 dpi due to high clinical score (Fig. 2 and Data S1). At the same time, most of the surviving vPdR-5U-inoculated animals developed clinical signs with scores of >3. Three piglets had to be euthanized and three others died suddenly (Fig. 2 and Data S1). Similarly, in the vPdR-5U contact group, the piglets developed progressive clinical signs, most of them with scores of >3; one died, and two had to be euthanized at 18 dpi (Fig. 2 and Data S1).

From the fourth week on, 7 of the 19 remaining vPdR-36U-infected piglets (group 1) were healthy, while 2 died suddenly. Eventually, seven piglets of this group were euthanized before the end of the experiment with clinical scores of 5 (Fig. 2). Among the contact animals of this group, five were clinically healthy until the end of the study and the remaining two showed clinical signs; one of them recovered during the fifth week, whereas the other had to be euthanized due to high clinical

score (Fig. 2). In contrast, at the same time, all but one of the remaining vPdR-5U-inoculated and contact animals were severely ill. Considering that, the experiment was terminated by euthanasia of all remaining animals at 30 dpi after some animals had reached the endpoint criteria or were found dead (Fig. 2 and Data S1). Overall, from 22 dpi until 30 dpi, significantly higher clinical scores were observed in vPdR-5U-infected piglets than in vPdR-36U-infected piglets ($P \leq 0.032$), especially at 23 dpi, with $P < 0.001$ (Fig. 3).

3.5.3 The long poly-U sequence of PdR is associated with a prolonged IFN-α response.

No significant differences in the mean IFN-α levels were detected in the sera of the two groups after 1 week of infection; however, the IFN-α responses were more variable among the vPdR-5U-infected piglets (Fig. 4). Interestingly, a statistically significant difference between the IFN-α values of the two groups was observed in the second week postinfection ($P < 0.001$). All vPdR-36U-infected animals had detectable serum IFN-α levels at this time, while IFN-α was undetectable in most of the animals infected with vPdR-5U (Fig. 4).

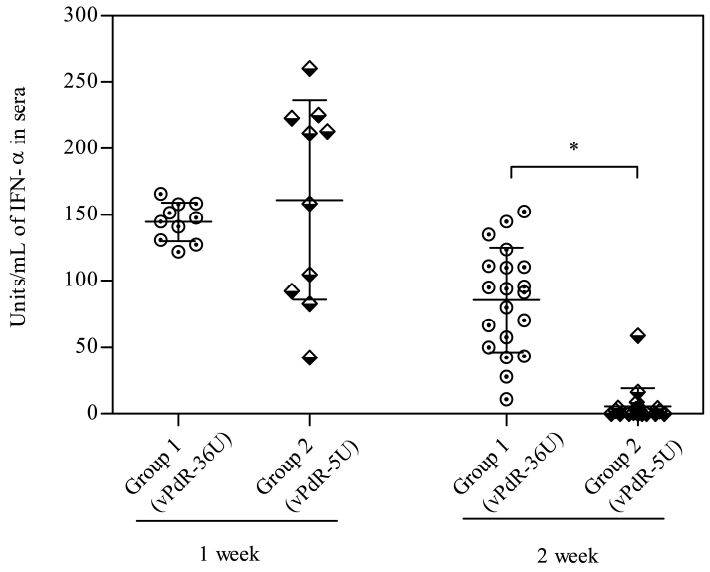

FIG 4. IFN-α levels in sera of CSFV-infected animals during the first 2 weeks postinfection. IFN-α levels were determined at 1 and 2 weeks postinfection in group 1 (vPdR-36U inoculated) and group 2 (vPdR-5U inoculated). The asterisk indicates a statistically significant difference between group 1 and group 2 at 2 weeks postinfection ($P < 0.001$).

3.5.4 The poly-U insertion is related to lower viral replication *in vivo*.

During the first 3 weeks postinfection, the cycle threshold (C_T) values for viral RNA in the sera of the piglets inoculated with vPdR-36U were significantly higher than the C_T values measured in the sera of the vPdR-5U-inoculated piglets ($P < 0.001$) (Fig. 5). The same was true for the corresponding contact animals during the second and third weeks postinfection (Fig. 5). At these times, the contact piglets of the vPdR-36U-infected animals had the lowest viral RNA loads overall. Importantly, the highest viral RNA levels in the serum (C_T values below 23) were found at 2 and 3 weeks postinfection in most of the vPdR-5U-inoculated piglets (group 2) and in their contact animals on week 3; these values were significantly higher ($P < 0.001$) than the levels found in

vPdR-36U-inoculated (group 1) and contact animals (Fig. 5). At 4 weeks postinfection, 38% and 43% of the vPdR-36U-inoculated and contact piglets, respectively, had cleared the virus, i.e., were negative for CSFV RNA by quantitative reverse transcription-PCR (RT-qPCR), while 15 and 28%, respectively, had still high viral RNA levels. In contrast, only 12% of the vPdR-5U-inoculated piglets and none of their contact animals had cleared the virus (Fig. 5), with still high CSFV RNA levels in 50% and 33% of the surviving piglets, respectively. At 5 weeks after infection, the proportion of CSFV RNA-negative sera increased with respect to the previous week in the groups of vPdR-36U-inoculated and contact piglets, reaching 55 and 57%, respectively (Fig. 5). As mentioned above, none of the vPdR-5U-inoculated and contact animals survived at this time of the trial.

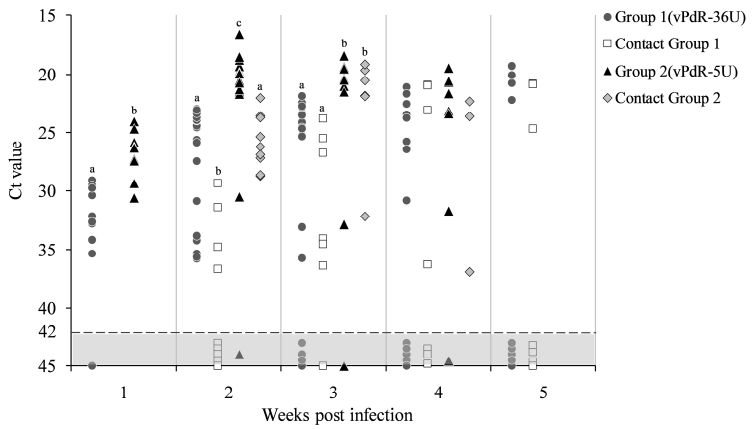

FIG 5. Detection of CSFV RNA in sera at weekly intervals. The sera were analyzed by RT-qPCR for the CSFV RNA content at weekly intervals during the whole experiment. C_T values above 42 (light gray area with dotted line) were considered negative. For each week, the same letters represent no significant differences and different letters represent statistically significant differences between groups ($P < 0.001$).

In terms of virus isolation at 3 weeks postinfection, virus was recovered from all sera that were positive by RT-qPCR with values below 32.22 (Table 1). Interestingly, virus was isolated from serum of 11 out of the 13 (85%) surviving vPdR-5U-inoculated piglets and from all their remaining contact animals. This contrasted with the vPdR-36U-inoculated piglets and their contact animals, for which virus could be recovered from only 12 out of 18 (66%) and 3 out of 7 (43%) remaining animals, respectively (Table 1). Finally, the titers of the virus-positive sera were in agreement with the RT-qPCR data (Table 1). From this we conclude that the modified PdR virus with 5 instead of 36 uridines in the 3'UTR replicates more efficiently in piglets than the vPdR-36U virus.

Table 1. CSFV isolation and titration in the serum samples at 3 weeks post infection[a]

Groups of pigs		C_T value	CSFV isolation	Titer[b]	Titer[b]	CSFV isolation	C_T value		Groups of pigs
Group 1 (vPdR-36U)	1	24.49	+	$10^{4.1}$	$10^{5.5}$	+	20.40	31	Group 2 (vPdR-5U)
	2	22.45	+	$10^{4.5}$	$10^{5.4}$	+	20.99	32	
	3	25.23	+	$10^{3.7}$	10^{6}	+	19.54	33	
	4	†			$10^{6.7}$	+	18.41	34	
	5	23.40	+	$10^{4.6}$		-	Undet.	35	
	6	24.11	+	$10^{4.2}$		-	32.90	36	
	7	22.81	+	$10^{4.5}$			†	37	
	8	23.51	+	$10^{4.6}$	$10^{5.6}$	+	20.34	38	
	9	25.33	+	$10^{3.5}$	$10^{5.3}$	+	20.47	39	
	10	Undet.	-				†	40	
	11	22.73	+	$10^{4.5}$			†	41	
	12	†					†	42	
	13	24.56	+	$10^{4.1}$			†	43	
	14	Undet.	-				†	44	
	15	33.13	-		10^{6}	+	19.41	45	
	16	24.68	+	$10^{4.1}$	$10^{6.2}$	+	19.37	46	
	17	35.72	-		$10^{6.1}$	+	19.33	47	
	18	Undet.	-		$10^{5.8}$	+	19.50	48	
	19	21.85	+	$10^{5.3}$	$10^{5.4}$	+	21.48	49	
	20	Undet.	-				†	50	
Contact Group 1	21	†			10^{6}	+	19.64	51	Contact Group 2
	22	34.1	-				†	52	
	23	†			10^{1}	+	32.22	53	
	24	25.50	+	$10^{3.7}$	$10^{5.2}$	+	21.82	54	
	25	Undet.	-		$10^{6.8}$	+	19.14	55	
	26	34.51	-		$10^{5.4}$	+	21.84	56	
	27	26.76	+	$10^{3.5}$			†	57	
	28	†					†	58	
	29	23.82	+	$10^{4.8}$			†	59	
	30	36.35	-		$10^{5.5}$	+	20.51	60	

[a]†, the animal died or was euthanized before 3 weeks postinfection; +, the sera were positive by CSFV isolation; –, the sera were negative by CSFV isolation. Undet., undetermined.
[b]Virus titration in $TCID_{50}$ per milliliter.

3.5.5 The long poly-U sequence is associated with reduced viral shedding from infected animals.

All the rectal and nasal swabs from the vPdR-5U-infected piglets (group 2), including the contact animals, were positive for viral RNA by RT-qPCR throughout the study, unlike for the vPdR-36U-infected piglets (group 1 and contact group 1), for which a considerable number of negative samples were detected (Fig. 6). The differences in viral RNA levels in rectal and nasal swabs between the vPdR-5U- and vPdR-36U-infected piglets were in perfect agreement with the RT-qPCR data obtained from serum (see above and Fig. 5). During the first 3 weeks postinfection, the vPdR-5U-infected animals showed significantly lower C_T values, corresponding to higher CSFV RNA loads, than the vPdR-36U-infected animals ($P < 0.001$) (Fig. 6). At 4 weeks postinfection, the rectal and nasal swabs of all the surviving vPdR-5U-infected animals-including contacts-remained RT-qPCR positive, while they were negative for 4 out of 14 and 2 out of 7 vPdR-36U-inoculated and contact piglets, respectively. In the fifth week after infection, only the vPdR-36U-infected piglets survived, showing similar viral RNA profiles in the nasal and rectal swabs (Fig. 6).

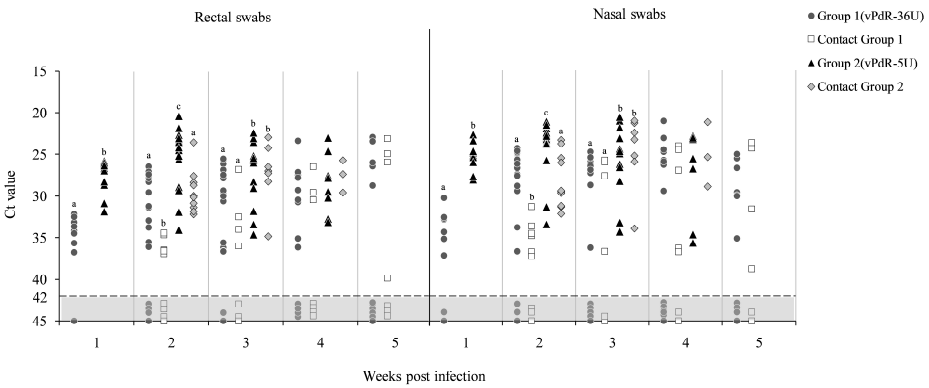

FIG 6. Detection of CSFV RNA in rectal and nasal swabs at weekly intervals. The CSFV RNA present in nasal and rectal swabs was evaluated weekly during the whole experiment. C_T values above 42 (light gray area with dotted line) were considered negative. For each week, the same letters represent no significant differences and different letters represent significant differences between groups ($P < 0.001$).

3.5.6 The poly-U sequence is associated with stronger antibody responses to infection.

Interestingly, there was a striking difference in terms of antibody responses between the vPdR-36U-infected (group 1) and vPdR-5U-infected (group 2) piglets (Fig. 7). One of the vPdR-36U-infected animals (number 18) seroconverted against E2 during the second week postinfection, and by the third week 13 out of 18 piglets (72%) had developed E2-specific antibody responses as detected by a blocking enzyme-linked immunosorbent assay (ELISA). At 4 weeks after infection, 10 out of 13 surviving piglets from this group were positive by ELISA, while 2 piglets remained negative until the end. The vPdR-36U contact piglets started to seroconvert during the 4th week, with 3 positive animals out of 7. One week later, 5 out of the 6 remaining contact piglets were seropositive. In contrast, only one of the vPdR-5U-inoculated piglets responded with E2-specific antibodies at 3 and 4 weeks postinfection, and all contact piglets remained seronegative throughout the trial (Fig. 7).

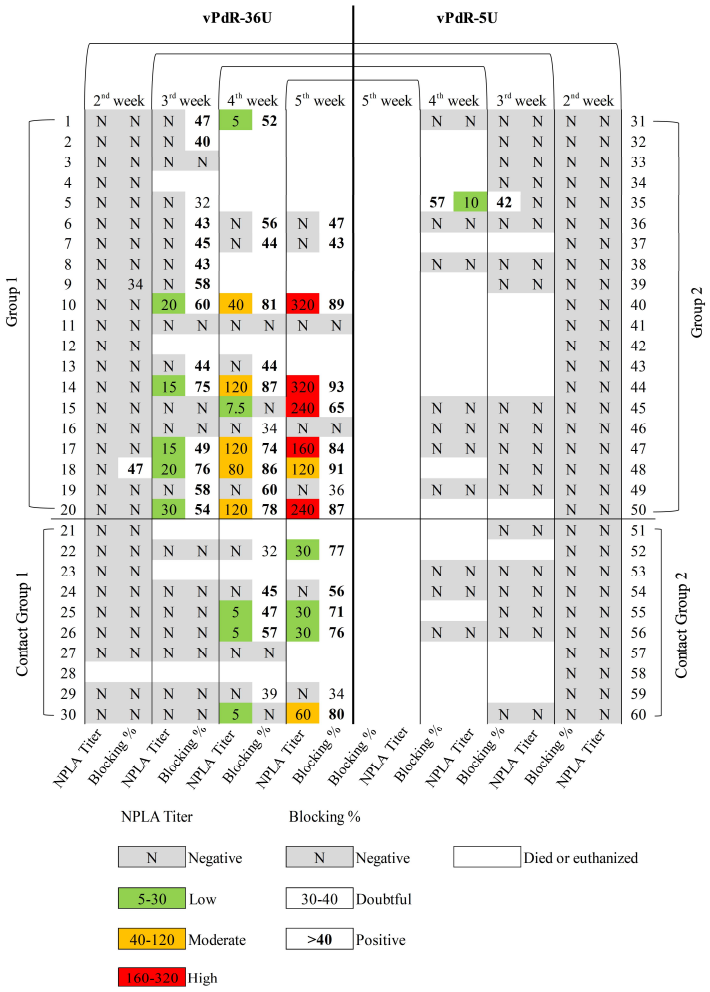

FIG 7. Assessment of the humoral immune responses after vPdR-36U versus vPdR-5U infection at weekly intervals. The antibody response against the E2 glycoprotein was detected by ELISA and is represented as blocking percentage. The neutralizing antibody titers after infection were evaluated by the NPLA.

Accordingly, all the piglets were negative for neutralizing antibodies during the first 2 weeks of the experiment as measured with the neutralization peroxidase-linked assay (NPLA). By the third week postinfection, 5 out of 18 vPdR-36U-inoculated animals had developed neutralizing antibodies, with titers between 1:15 and 1:30. At 4 weeks

after infection, seven vPdR-36U-inoculated and three contact piglets were positive in the NPLA, with titers up to 1:120 and 1:5, respectively. The neutralizing antibody responses increased during the fifth week postinfection, with titers of 1:120 to 1:320 in 6 out of 11 surviving vPdR-36U-infected piglets and of 1:30 to 1:60 in 4 out of the 6 remaining contact animals (Fig. 7). For the vPdR-5U-infected piglets, the single animal that had seroconverted had also a 1:10 neutralizing antibody titer 1 week later.

3.5.7 The poly-U sequence in vPdR-36U was conserved between inoculated and contact animals, despite not being completely stable.

Conventional RT-PCR and nucleotide sequencing were used to amplify and analyze the CSFV 3'UTR from the sera of all inoculated and contact piglets 3 weeks postinfection. RT-PCR fragments were obtained only with the samples that had C_T values below 32.9. Consequently, there was a smaller proportion of RT-PCR-positive samples with vPdR-36U- than with vPdR-5U-infected piglets (Table 1). From vPdR-36U-inoculated piglets, two fragments were obtained by RT-PCR in 5 out of 12 positive samples. After sequencing, these fragments showed two profiles, one with 36 uridines and the other with 4 or 5 uridines (data not shown). For the corresponding contact animals, one out of three RT-PCR positive samples showed one specific fragment with the 36 uridines while the other two samples revealed two fragments with 36 and 5 uridines. Finally, the 3'UTR amplified from the sera of vPdR-5U-inoculated and contact animals showed the same fragment in all the samples analyzed with the expected 5 uridines (data not shown).

3.6 Discussion

Long-term prevalence and evolution of CSFV in regions of endemicity have resulted in viruses with a broad range of clinical manifestations, from severe to mild disease (Pérez et al., 2012; H. Zhang et al., 2018). In this context, several studies have emphasized the relevance of moderate- and low-virulence CSFV strains for virus persistence in countries of endemicity under vaccination programs, representing a challenge for CSFV eradication (Blome et al., 2017b; Coronado et al., 2017). Despite this, little is known on the genetic changes that are selected in the CSFV genomes in an endemic situation, leading to virus attenuation and persistence in the field. Therefore, the present study tackled these aspects by building on the PdR field isolate that resulted from natural CSFV evolution under endemic conditions for over 3 decades (Coronado et al., 2017; Pérez et al., 2012). During circulation in the field, this strain has acquired a unique uninterrupted poly-U sequence of an average length of 36 nucleotides in the 3'UTR that is absent in the related highly virulent Margarita virus (Coronado et al., 2017). It should be noted that the Margarita strain was proposed to be the most probable origin of the Cuban outbreak in the 1990s and also is likely the parental strain for the PdR isolate (Díaz de Arce et al., 2005, 1999).

We used reverse genetics to characterize the role of the poly-U sequence for virulence and transmission in piglets by comparing cDNA-derived PdR with 36 and 5 uridines in the 3'UTR, i.e., vPdR-36U and vPdR-5U, respectively. In accordance with previous data reported for the CSFV PdR strain isolated from the field (Pérez et al., 2012), the clinical signs observed after infection with the vPdR-36U virus were mild and unspecific in newborn piglets (Muñoz-González et al., 2015b). In contrast, piglets infected with vPdR-5U virus showed severe clinical signs, ranging from diarrhea, cyanosis, and hemorrhagic lesions to

nervous disorders, consistent with previous studies using high-virulence CSFV strains, such as the Margarita strain (Blome et al., 2017b; Tarradas et al., 2014).

Interestingly, different replication levels were observed with vPdR-36U and vPdR-5U *in vivo*, despite similar replication kinetics observed in PEDSV.15 cells and in primary porcine macrophages. The PEDSV.15 cells were used to rescue and amplify the viruses for subsequent *in vivo* experiments because we had shown previously that these cells do not select for heparan sulfate-adapted mutants as opposed to other cell lines (Eymann-Häni et al., 2011). Infection of piglets with vPdR-5U resulted in significantly higher viral RNA loads in sera and in rectal and nasal swabs than with vPdR-36U. In agreement with previous studies that identified a critical role of the 3'UTR in replication of pestiviruses and flaviviruses (Brinton and Miller, 2015; de Borba et al., 2019; Kellman et al., 2018; Pankraz et al., 2005), our data confirm the relevance of the 3'UTR in CSFV replication *in vivo* and demonstrate for the first time that a poly-U insertion acquired naturally in the 3'UTR of CSFV can reduce replication and virulence in young piglets. The RNA secondary structure of the CSFV 3'UTR was predicted previously, and four consecutive stem-loop (SL) structures were identified (Fan et al., 2008; Huang et al., 2012). Previous studies also revealed that SL-I and the single-stranded intervening sequence (SS) region between SL-I and SL-II are essential for viral replication (Huang et al., 2012; Pankraz et al., 2005). The predicted RNA secondary structure of the poly-U insertion in the genome of the PdR isolate leads to a long single-stranded intervening sequence between SL-I and SL-II (Coronado et al., 2017). The change in the RNA secondary structure due to this insertion may

interfere with the normal function of the 3'UTR, which influences replication *in vivo* by a yet-unknown mechanism.

Notably, both the vPdR-36U and vPdR-5U viruses generated in the present study were capable of infecting contact piglets by the natural route. For each of the two viruses, the clinical signs, replication rates, and antibody responses were comparable between the inoculated and the respective contact animals, which is supported statistically. Additionally, sequence analysis of the virus recovered from serum at 3 weeks postinfection showed also similar profiles between the inoculated animals and their respective contact groups. Surprisingly however, the 36-uridine sequence of vPdR-36U was not completely stable *in vivo*. Nevertheless, there was no selection of a specific variant (36 or 5 uridines) during natural virus transmission to contact animals.

Type I interferons (IFN), such as IFN-α, induce antiviral and immunomodulatory effects, being a relevant part of the innate immune responses against viruses and hindering viral dissemination (Summerfield and Ruggli, 2015). For CSFV, transient elevated IFN-α levels are related to disease severity after infection with highly virulent strains (Muñoz-González et al., 2015a; Pérez et al., 2012). In the present study, the vPdR-36U-infected piglets had more consistent and prolonged serum IFN-α levels than the vPdR-5U-infected piglets during the first 2 weeks of infection, suggesting a role for the poly-U sequence in the activation of innate antiviral immune responses. This may be one reason for the lower replication of vPdR-36U versus vPdR-5U in infected piglets, contrasting with the similar replication kinetics of the two viruses in cell culture (Fig. 1), in which CSFV prevents efficiently type I IFN induction by means of N^{pro}-mediated IRF3 degradation (Bauhofer et al.,

2007). This is not surprising since functional N^{pro} is present in both vPdR-36U and vPdR-5U. Notably, the highest IFN-α levels overall were found during the first week in some of the vPdR-5U-infected piglets. These were similar to the levels found previously in piglets infected with the highly virulent Margarita strain (Tarradas et al., 2014). Interestingly also, the vPdR-36U-infected piglets had serum IFN-α levels that were comparable to those observed previously in piglets infected with the low-virulence PdR strain, considering that this cytokine was detected over a longer period for these animals (Muñoz-González et al., 2015b). It should be noted here that the 3'UTR in *Flaviviridae* has been implicated in the modulation of type I IFN responses and virus replication in different ways. For instance, flaviviruses can inhibit type IFN responses by means of a short noncoding RNA issued from the 3'UTR (Chang et al., 2013; Clarke et al., 2015). Conversely, a long poly-U/UC tract found in the 3'UTR of hepatitis C virus was shown to activate innate immune responses, including the type I IFN pathway, and control infection through the engagement of RIG-I (Schnell et al., 2012). This points toward a potential role for the long poly-U sequences in the 3'UTR of CSFV in the activation of the innate immune response, which requires further investigation.

In line with a more sustained innate immune activation by vPdR-36U than by vPdR-5U, the former also induced adaptive immune responses as measured by total and neutralizing antibodies. This contrasted with vPdR-5U, which did not induce any seroconversion during the 4 weeks the piglets survived. The CSFV-specific humoral immune responses were particularly high in animals showing a milder disease and lower viral load. Thus, our results strongly suggest a role for the long poly-U sequence of the PdR isolate in immune activation, control of viral

replication, and eventually modulation of disease severity. This is supported by the severe clinical signs observed following vPdR-5U infection, associated with the high replication rate and the incapacity to induce antibody responses. The findings of the present study, i.e., the attenuation of a CSFV strain by the poly-U insertion in the 3'UTR, provide a potential target for an efficient control of CSFV infection *in vivo* that could be considered in the development of new vaccines against infections with CSFV and other flaviviruses. From a general point of view, our data contribute to understanding of the molecular determinants of CSFV virulence, which supports vaccine development and the implementation of efficient CSF surveillance and control measures.

3.7 Materials and methods

3.7.1 Cells and viruses.

The porcine kidney cell line PK-15 was obtained from the ATCC (CCL-33) and the SK-6 cell line (Kasza et al., 1972) was provided by M. Pensaert, Faculty of Veterinary Medicine, Ghent, Belgium. The porcine aortic endothelial cell line PEDSV.15 (Seebach et al., 2001) was kindly provided by J. Seebach, University of Geneva, Switzerland. The PK-15 cells were grown in minimum essential medium (MEM) supplemented with 10% pestivirus-free fetal bovine serum (FBS). The SK-6 and PEDSV.15 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with sodium pyruvate, nonessential amino acids, 7% horse serum, and 2% porcine serum. Porcine monocyte-derived macrophages were prepared as described previously (Ogno et al., 2019). Briefly, peripheral blood mononuclear cells were isolated from the blood of 6- to 12-month-old specific-pathogen-free Large

White pigs from the breeding facility of the Institute of Virology and Immunology (IVI) in Switzerland (in compliance with the Swiss animal protection law, under license number BE131/17, approved by the animal welfare committee of the canton of Bern, Switzerland) using Ficoll-Paque Plus density centrifugation (GE Healthcare). Monocytes were then enriched by positive selection for CD172a with the monoclonal antibody clone 74-22-15A (hybridoma kindly provided by A. Saalmüller, Veterinary University of Vienna, Austria) using a magnetic cell sorting system (MACS) with LS columns (Miltenyi Biotec GmbH). The enriched monocytes were seeded at a density of 5×10^5 cells per milliliter in DMEM without phenol red, supplemented with Glutamax (Life Technologies), 10% pestivirus-free FBS, and recombinant porcine colony-stimulating factor 1 (20 U/ml), and cultured at 39°C with 5% CO2 for 72 h for differentiation to macrophages.

For virus amplification, PEDSV.15 cells were infected with 0.1 50% tissue culture infective dose ($TCID_{50}$)/cell in the presence of 2% serum, and the virus was harvested 72 h later. Viral titers were determined by endpoint dilution in PEDSV.15, PK-15, and SK-6 cells using the peroxidase-linked assay (PLA) (Wensvoort et al., 1986), and the $TCID_{50}$ per milliliter was calculated using standard statistical methods (Reed and Muench, 1938). The CSFV PdR isolate (CSF1058) originated from the Cuban CSF epizootic in 2010 (Coronado et al., 2017). The CSFV reference strain Alfort/187 was kindly provided by the CSFV EU Reference Laboratory (EURL), Hannover, Germany, and was used for virus neutralization assays.

3.7.2 Assembly of full‑length cDNA clones of the PdR isolate.

The strategy for the construction of the full-length functional cDNA clone of the PdR isolate was based on the backbone vector pACNR1180, described previously (Ruggli et al., 1996). The polylinker of the pACNR1180 vector was replaced between SalI and XhoI with a short DNA cassette carrying the SalI and PspXI restriction sites. The complete genome of the PdR isolate was amplified from serum of an infected piglet by reverse transcription (RT) and PCR as previously described (Coronado et al., 2017). Briefly, the 5' and 3' halves of the genome were assembled in 2 separate pACNR1180-derived constructs termed pPdR-5'h and pPdR-36U-3'h using large overlapping RT-PCR fragments, according to the sequence information (GenBank accession number KX576461) reported earlier (Coronado et al., 2017). A forward oligonucleotide carrying a SalI restriction endonuclease site for insertion in pACNR1180, a bacteriophage T7 polymerase promoter sequence with the single guanosine of the PdR genome at the transcription start site, and the 5'-terminal 22 nucleotides of PdR served to place the 5' end of the genome at the proper position in pPdR-5'h for precise viral genome transcription. An SrfI restriction endonuclease site for runoff transcription was placed at the 3' end of the genome in pPdR-36U-3'h using a reverse oligonucleotide complementary to the 20 last nucleotides of PdR and carrying the *Srf*I site and a *PspX*I restriction endonuclease site for insertion into the modified pACNR1180 plasmid. The unique *Spe*I site with cleavage after nucleotide 8273 in the NS4B gene of PdR served as junction site to assemble the full-length cDNA clone pPdR-36U from the pPdR-5'h and pPdR-36U-3'h constructs. The pPdR-36U plasmid was then modified by deletion of 31 uridines from the poly-U sequence of the 3'UTR to obtain the standard 5 uridines at this position in a plasmid termed pPdR-5U. To this end, a PCR-based method was

applied to generate a fragment encoding 5 uridines instead of the 36 uridines, which was used to replace the BamHI-PspXI cassette in pPdR-36U-3'h, resulting in pPdR-5U-3'h. Plasmid pPdR-5U was then assembled from pPdR-5'h and pPdR-5U-3'h using the *Spe*I junction site.

3.7.3 Virus rescue from cDNA.

The viruses vPdR-36U and vPdR-5U were rescued from the respective cDNA clones as described previously (Moser et al., 1999). Briefly, plasmids pPdR-36U and pPdR-5U were linearized with the SrfI restriction endonuclease and served as templates for runoff RNA transcription with the MEGAscript T7 kit (Invitrogen by Thermo Fisher Scientific) according to the manufacturer's protocol. The *in vitro*-transcribed RNAs were treated with 1 U of DNase I at 37°C for 15 min and purified on S-400 HR columns (GE Healthcare Life Sciences). For electroporation, PEDSV.15 cells were washed twice with ice-cold phosphate-buffered saline (PBS). A total of 8 × 106 cells in 0.4 ml of PBS were mixed with 1 μg of purified RNA transcript, transferred to a 0.2-cm electroporation cuvette (Bio-Rad), and electroporated immediately with a Gene Pulser electroporation device (Bio-Rad) by applying two pulses at 200 V and 500 μF. The electroporated cells were seeded in 75-cm^2 flasks and incubated at 37°C and 5% CO2. After 65 h, the supernatants were harvested and used to infect fresh PEDSV.15 cells. The specific infectivity of the RNA transcripts was determined with an infectious-center assay on PEDSV.15 cells as described earlier to ensure functionality of the constructs (Mendez et al., 1998). The entire genomes of the rescued viruses were verified by nucleotide sequencing to exclude any accidental mutation. The virus titers were determined in PEDSV.15

and PK-15 cells and on porcine monocyte-derived macrophages as described above.

3.7.4 Virus replication kinetics in cell culture.

PEDSV.15 cells (150,000/well) and porcine macrophages (500,000/well) were infected with the viruses of interest at a multiplicity of infection (MOI) of 0.02 $TCID_{50}$/cell (based on titers obtained in the homologous cell line) in the corresponding serum-free medium in 24-well plates. After 1 h at 37°C, the inoculum was removed and the cells were washed once with serum-free medium and then incubated in complete culture medium at 37°C. At 3, 6, 9, 12, 18, 24, 36, 48, and 72 h (PEDSV.15 only) after infection, the 24-well plates were frozen at -70°C. After thawing, the supernatant and cells debris were harvested, cleared by centrifugation at 3,000 × g for 10 min at 4°C, and then stored at –70°C. The virus titers were determined in PEDSV.15 and SK-6 cells as described above.

3.7.5 Experimental infection.

Sixty 5-day-old piglets from pestivirus-free sows were housed in two boxes of the biosafety level 3 (BSL3) animal facilities at IRTA-CReSA (Barcelona, Spain). Heating panels and lamps as well as chipped-wood bedding were provided in each box. The animals were fed with commercial pig milk substitute with lyophilized bovine colostrum (Patavie; Celtilait Ltd., Ploudaniel, France) until 14 days of age and subsequently with a conventional piglet starter diet and pellets until the end of the trial (Neopigg, Kwikstart; Cargill, Zaragoza, Spain) (O. Cabezón et al., 2017). The piglets were distributed randomly between the two groups. The piglets of the first experimental group (group 1,

n = 20, numbered 1 to 20) were inoculated intranasally with 2.5×10^4 $TCID_{50}$ of vPdR-36U (according to the titer in PEDSV.15 cells). At 24 h after infection, 10 contact piglets were added to group 1 (contact group 1, numbered 21 to 30). The piglets of the second experimental group (group 2, n = 20, numbered 31 to 50) were inoculated intranasally with 2.5×10^4 $TCID_{50}$ of vPdR-5U. Finally, 10 piglets were added to group 2 as contact animals 24 h after infection (contact group 2, numbered 51 to 60). At 7 dpi, nasal and rectal swabs and serum were collected from 10 randomly selected animals/group from groups 1 and 2. Subsequently, these samples were collected from all animals at 7-day intervals until 5 weeks after infection (i.e., the end of the trial).

The animals were monitored daily by a trained veterinarian in a blinded manner. The clinical status of the animals was scored from 0 to 6 as previously described (Muñoz-González et al., 2016): 0, no signs; 1, mild diarrhea; 2, mild clinical signs; 3, mild to moderate clinical signs; 4, moderate clinical signs; 5, moderate to severe clinical signs; and 6, death. For ethical reasons, the animals were euthanized when the clinical score reached 5, when exhibiting a fall of the hindquarters, when there was inability to drink or feed, when prostration occurred, or when exhibiting moderate nervous disorders. The procedure for the euthanasia of the animals was based on an accepted method included in European Directive 2010/63/EU, using an overdose of 60 to 100 mg of pentobarbital per kilogram of body weight, administered via the vena cava cranialis. The experiment was approved by the Ethics Committee from the Generalitat of Catalonia, according to Spanish and European regulations.

3.7.6 Detection of CSFV RNA.

RNA was extracted using the MagAttract 96 cador pathogen kit (Qiagen), following the manufacturer's instructions. In all cases, extraction was performed from an initial sample volume of 200 µl to obtain a final volume of 100 µl of RNA, which was stored at -80°C. The presence of CSFV RNA in the serum and in nasal and rectal swabs was analyzed by RT-qPCR (Hoffmann et al., 2005). C_T values equal to or lower than 42 were considered positive. Samples in which fluorescence was undetectable were considered negative. As described previously, C_T values from 10 to 22 were considered high, from 23 to 28 moderate, and between 29 and 42 low RNA viral loads (Tarradas et al., 2012).

3.7.7 Assessment of the humoral immune responses by ELISA and virus neutralization test.

E2-specific antibodies after infection were detected by ELISA using a commercial CSFV antibody test (IDEXX Laboratories, Liebfeld, Switzerland) according to the manufacturer's recommendations. The samples were considered positive when the blocking percentage was ≥40%. In order to evaluate the neutralizing antibody response after infection, serum samples were also tested by neutralization peroxidase-linked assay (NPLA) (Terpstra et al., 1984) against the Alfort/187 strain. The titers were expressed as the reciprocal dilution of serum that neutralized 100 $TCID_{50}$ in the 50% of the culture replicates.

3.7.8 ELISA for IFN-α detection in serum samples.

An in-house ELISA was performed in order to quantify IFN-α levels in sera at 8 and 15 dpi as previously described (Díaz de Arce et al., 1992; Tarradas et al., 2010). Anti-IFN-α monoclonal antibodies (K9 and K17) were used for detection and serial dilutions of IFN-α recombinant protein

(PBL Biomedical Laboratories, Piscataway, NJ) was employed as a standard. A regression curve based on the optical densities of the cytokine standard used in the test was used to determine the cytokine concentrations (units/milliliter) in sera.

3.7.9 CSFV rescue in serum samples from infected piglets.

Serum samples from all animals of the two experimental groups were subjected to virus isolation at 3 weeks postinfection. To this end, PK-15 cells were seeded in a 96-well plate and incubated at 37°C and 5% CO_2. After 24 h, 100 µl of 10- and 100-fold dilutions of each sample were added. After 72 h of incubation, the virus was detected by PLA test (Wensvoort et al., 1986). In addition, the virus titers were determined as described above.

3.7.10 Analysis of the 3'UTR nucleotide sequence in serum from infected piglets.

The following two primers were designed for the specific detection of the 3'UTR sequence: forward primer (12023 to 12049), 5'-AAGGAGGCTGAGAGTCATGATGATGAC-3', and reverse primer (12304 to 12329), 5'-GGGCCGTTAGGAAATTACCTTAGTCC-3'. RNAs from serum samples from all animals of the two experimental groups were tested at 3 weeks postinfection. The RT-PCRs, consisting of 30 min at 50°C (reverse transcription) and 10 min at 95°C (inactivation of the reverse transcriptase and activation of the Taq polymerase) followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 45 s at 72°C, were performed using a one-step RT-PCR kit (Qiagen) in a final volume of 25 µl. The amplification products were analyzed by electrophoresis on a 2% agarose gel and were purified with a GeneJET

gel extraction and DNA cleanup microkit (Thermo Fisher Scientific). The sequencing reactions were performed using the BigDye terminator cycling technique and an ABI 3130xl genetic analyzer. The sequences were assembled using the Contig BioEdit software (Hall, 1999).

3.7.11 Statistical analysis.

NCSS software (Hintze. J 2004 NCSS and PASS Number Cruncher Statistical Systems. Kaysville, UT) served for statistical analyses using "piglet" as the experimental unit. The nonparametric Kruskal-Wallis test was used to compare the clinical scores and the virological (viral loads in serum, rectal swabs, and nasal swabs) and immunological (IFN-α levels) parameters among the four experimental groups. The significance level (α) was set at a P value of $\leqslant 0.05$, whereas statistical tendencies were reported when P values were $\leqslant 0.10$.

3.8 Supplemental material

Supplemental file 1:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6955259/. (19K, xlsx)

3.9 Acknowledgments

This research was supported by grant RTI2018-100887-B-I00 from the Ministerio de Ciencia, Innovación y Universidades of the Spanish government. M.W. has a predoctoral fellowship from the China Scholarship Council (2017) of the Chinese government. J.A.B. has a predoctoral fellowship, FPI 2016, from the Ministerio de Ciencia, Innovación y Universidades of the Spanish government.

We thank Ivan Cordón and the CReSA animal facility staff for their help during the *in vivo* part of the experiment.

Chapter 4

Study II. Abrogation of the RNase activity of E^{rns} in a low virulence classical swine fever virus enhances the humoral immune response and reduces virulence, transmissibility, and persistence in pigs

(Virulence. 2021 Dec;12(1):2037-2049)

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4.1 Abstract

The prevalence of low virulence classical swine fever virus (CSFV) strains makes viral eradication difficult in endemic countries. However, the determinants for natural CSFV attenuation and persistence in the field remain unidentified. The aim of the present study was to assess the role of the RNase activity of CSFV E^{rns} in pathogenesis, immune response, persistent infection, and viral transmission in pigs. To this end, a functional cDNA clone pPdR-H_{30}K-36U with an E^{rns} lacking RNase activity was constructed based on the low virulence CSFV field isolate Pinar de Rio (PdR). Eighteen 5-day-old piglets were infected with vPdR-H_{30}K-36U. Nine piglets were introduced as contacts. The vPdR-H_{30}K-36U virus was attenuated in piglets compared to the parental vPdR-36U. Only RNA traces were detected in sera and body secretions and no virus was isolated from tonsils, showing that RNase inactivation may reduce CSFV persistence and transmissibility. The vPdR-H_{30}K-36U mutant strongly activated the interferon-α (IFN-α) production in plasmacytoid dendritic cells, while *in vivo*, the IFN-α response was variable, from moderate to undetectable depending on the animal. This suggests a role of the CSFV E^{rns} RNase activity in the regulation of innate immune responses. Infection with vPdR-H_{30}K-36U resulted in higher antibody levels against the E2 and E^{rns} glycoproteins and in enhanced neutralizing antibody responses when compared with vPdR-36U. These results pave the way toward a better understanding of viral attenuation mechanisms of CSFV in pigs. In addition, they provide novel insights relevant for the development of DIVA vaccines in combination with diagnostic assays for efficient CSF control.

4.2 Keywords

Classical swine fever virus (CSFV), E^{rns} RNase activity, viral replication, type I IFN, viral attenuation, viral persistence, viral transmission, humoral response, *Pestivirus*

4.3 Introduction

Classical swine fever (CSF) is one of the oldest and most studied diseases in animal health and continues to be a threat in the porcine sector worldwide (Ganges et al., 2020). The causative agent, classical swine fever virus (CSFV), is an enveloped RNA virus that belongs to the *Pestivirus* genus within the *Flaviviridae* family. The CSFV genome is a single-stranded, positive-sense RNA of 12.3 kb carrying one long open reading frame (ORF). The ORF is flanked by a 5'- and a 3'-untranslated region (UTR) and encodes a polyprotein that is cleaved into four structural (C, E^{rns}, E1, and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Meyers and Thiel, 1996; Tautz et al., 2015). Among these proteins, E2 and E^{rns} are the main targets for neutralizing antibodies and induce protective immunity in infected animals (Langedijk et al., 2001; Van Rijn et al., 1996).

The E^{rns} glycoprotein, exclusively present in pestiviruses, has a unique feature of intrinsic ribonuclease (RNase) activity among all viral envelope proteins (Schneider et al., 1993). This function is not essential for virus replication in tissue culture (Hulst et al., 1998; Meyer et al., 2002; Meyers et al., 1999). E^{rns} was reported to induce apoptosis in lymphocytes *in vitro*, and its RNase activity is required to prevent type I interferon (IFN) induction (Lussi and Schweizer, 2016; Python et al., 2013). Studies in cell culture have shown that E^{rns} can degrade viral single- and double-stranded RNA both in the extracellular space and in endocytic compartments (Lussi et al., 2018; Mätzener et al., 2009;

Zurcher et al., 2014). Interestingly, E^{rns} inhibits Toll-like receptor (TLR)7-dependent IFN-α induction in plasmacytoid dendritic cells (pDC) after direct CSFV infection or when activated through contact with CSFV-infected cells (Python et al., 2013). Nevertheless, the *in vivo* implications of these mechanisms has not been demonstrated yet. In the case of bovine viral diarrhea virus (BVDV), another important member of the *Pestivirus* genus, this function may help to establish and maintain persistent infections in cattle (Lussi and Schweizer, 2016; Meyers et al., 2007). Previous studies showed that abrogation of the E^{rns} RNase activity in virulent pestiviruses including CSFV may reduce replication and clinical signs *in vivo* (Meyer et al., 2002; Meyers et al., 1999). However, the role of the E^{rns} RNase in CSFV pathogenesis, immune response, and its relationship with the viral ability to generate chronic and persistent infections, have been scarcely studied.

It was shown previously that the low virulence CSFV field isolate Pinar de Rio (PdR) that resulted from natural CSFV evolution under endemic conditions (Coronado et al., 2017) could lead to chronic and persistently infected piglets (Coronado et al., 2019a; Muñoz-González et al., 2015b). In addition, the unique uninterrupted poly-uridine (poly-U) sequence found in the 3'UTR of this isolate was identified as a new virulence factor that could activate immunity and attenuate virulence in piglets (M. Wang et al., 2020a). This previous study pointed towards a possible role played by the long poly-U sequence in the activation of innate immunity, which may be controlled by the RNase activity of E^{rns}. Therefore, the present study focused on investigating the role of the RNase activity of E^{rns} *in vivo*. To this end, we generated the RNase-negative E^{rns} mutant CSFV vPdR-H_{30}K-36U using the functional cDNA backbone pPdR-36U described previously (M. Wang et al., 2020a). We assessed virus

replication and type I IFN responses *in vitro* and *in vivo* and the role of the E^{rns} RNase activity for viral pathogenicity, persistence, and transmission in pigs.

4.4 Materials and methods

4.4.1 Cells and viruses

The PK-15 cell line (ATCC CCL-33), the porcine aortic endothelial cell line PEDSV.15 (Seebach et al., 2001) (obtained from Jörg Seebach, University of Geneva, Switzerland) and the SK-6 cell line (Kasza et al., 1972) (obtained from M. Pensaert, Faculty of Veterinary Medicine, Ghent, Belgium) were tested for absence of pestiviruses. The PK-15 cells were cultivated in minimum essential medium (MEM) containing 10% pestivirus-free fetal bovine serum (FBS) and the two other cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) containing sodium pyruvate, non-essential amino acids, and 7% horse serum. The PEDSV.15 were supplemented with an additional 2% porcine serum. Porcine monocyte-derived macrophages (MDM) and $CD172a^+$ enriched pDC were prepared as described previously (Ogno et al., 2019; Python et al., 2013; M. Wang et al., 2020a).

The CSFV vPdR-36U was rescued from the cDNA clone pPdR-36U (M. Wang et al., 2020a). This clone corresponds to the isolate Pinar del Rio (PR-11/10-3) from the Cuban CSF epizootic in 2010 (Coronado et al., 2017; Pérez et al., 2012). PR-11/10-3 is also referred to as CSF1058 according to the nomenclature of the European Union Reference Laboratory for Classical Swine Fever (EURL-CSF), Hannover, Germany. The CSFV Alfort/187 strain, also provided by the EURL-CSF was used in the virus neutralization assay. The cDNA-derived viruses were rescued as described below. All viruses were amplified by infecting

cells with 0.1 tissue culture infectious dose ($TCID_{50}$)/cell and were harvested after 72 hours. End-point dilution was used to determine the virus titers in SK-6, PEDSV.15, and porcine MDM cells using 96-well tissue culture plates and the peroxidase-linked assay (PLA) (Wensvoort et al., 1986) with the monoclonal antibody (mAb) HC/TC-26 (Greiser-Wilke et al., 1990) against E2. The virus titer was expressed in $TCID_{50}$/ml (Reed and Muench, 1938).

4.4.2 Construction of the infectious clone pPdR-H_{30}K-36U

The construction of the functional cDNA clone pPdR-H_{30}K-36U encoding RNase-inactive E^{rns} was based on the plasmid backbone pPdR-36U cDNA described previously (M. Wang et al., 2020a). The pPdR-36U plasmid was modified by substitution of the histidine codon (cac) at amino acid position 30 of E^{rns} (amino acid 297 of the ORF, GenBank accession number KX576461) with a lysine codon (aaa). To this end, standard PCR-based mutagenesis was applied, and the PCR fragment carrying the mutation was used to replace the ClaI-KasI cassette in pPdR-36U-5′h described previously (M. Wang et al., 2020a), resulting in pPdR-H_{30}K-5′h. The plasmid pPdR-H_{30}K-36U was then obtained by insertion of the *Spe*I to *PspX*I cassette of pPdR-36U (from nucleotide position 8274 to the end of the genome including the *Srf*I run-off site) in the corresponding sites of pPdR-H_{30}K-5′h. The construct was verified by Sanger sequencing. A trained veterinarian recorded the temperature and clinical signs daily in a blinded manner. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB) under number 8642, according to existing national and European regulations.

4.4.3 Virus rescue from cDNA

The vPdR-H_{30}K-36U virus was rescued from cDNA as described previously for vPdR-36U (M. Wang et al., 2020a). Briefly, the plasmid was linearized with SrfI and RNA was transcribed with the MEGAscript T7 kit (ThermoFisher Scientific). The RNA was treated with DNase I and purified on S-400HR columns (GE Healthcare Life Sciences). 1 µg RNA was then mixed with 8×10^6 PEDSV.15 cells in 0.4 ml ice-cold phosphate-buffered saline (PBS). The mix was transferred to a 0.2 cm electroporation cuvette, and electroporation was performed using a Gene Pulser (Bio-Rad) by applying 2 pulses at 200 V and 500 µF. The cells were then cultured in 75 cm^2 flasks at 37°C and 5% CO_2 during 65 hours. The rescued virus was passaged once into PEDSV.15 cells. In order to control the functionality of the constructs, the infectivity of the RNA transcripts was determined with an infectious center assay as described previously (Mendez et al., 1998). The complete genome sequences of the rescued viruses were verified by nucleotide sequencing to exclude any accidental mutation. Finally, viral titers were determined in PEDSV.15 and porcine MDM (M. Wang et al., 2020a).

4.4.4 Virus replication in cell culture

For the analysis of virus replication kinetics in cell culture, PEDSV.15 (150,000/well), and porcine MDM (500,000/well) were infected, respectively, in quadruplicate and in triplicate with vPdR-H_{30}K-36U or vPdR-36U at a multiplicity of infection (MOI) of 0.02 $TCID_{50}$/cell (according to titers from the homologous cell type) in 24-well plates using serum-free medium. The inoculum was removed after 1 h at 37°C and the cells were washed once with a serum-free medium followed by incubation in a complete culture medium at 37°C. Virus was harvested

at the indicated hours post infection (h.p.i.) after one freeze/thaw cycle. The virus titer was determined by endpoint dilution in SK-6 cells.

4.4.5 RNase activity assay

The RNase activity assay was performed as described previously (Itakura et al., 2020; Python et al., 2013). Briefly, SK-6 cells were infected in 6-well plates (10 cm^2 wells) with vPdR-36U or vPdR-H_{30}K-36U or were mock infected or left uninfected. After 24 hours of incubation in complete medium, the cells were washed 5 times with PBS and cultured for additional 20 hours in serum-free MEM. Cell extracts were obtained by hypotonic lysis with H_2O (400 µl per well). A 50-mer RNA probe (Dy-781-O1-RNA) labeled with Dyomics 781 at the 5′end (prepared by Dr. Fabian Axthelm, Microsynth AG, Balgach, Switzerland) was mixed at 40 nM with the cell extracts to be tested for RNase activity. RNase A (3×10^{-3} U/ml in MEM) was included as digestion control, and 50 mM TrisHCl pH 7.4 served as negative control. The reactions were incubated for 1 h at 37°C. Subsequently, two volumes of 97% formamide (Sigma) were added to the treated probes and a 10% polyacrylamide and 35% urea gel in 133 mM TrisHCl, 45.5 mM boric acid, and 3.2 mM EDTA were used for separation. The Odyssey Infrared Imaging System (LI-COR) was employed for image acquisition.

4.4.6 Stimulation of pDC by infected cells

Enriched porcine pDC were stimulated with infected cells for IFN-α production as described earlier (Python et al., 2013). Briefly, porcine MDM were infected at a MOI of 5 $TCID_{50}$/cell in 24-well plates (200′000 cells/well) and cultured for 24 hours. CpG D32 at 5 µg/ml was used as a positive control for pDC stimulation. The cells were then washed four times to remove the inoculum, and 10^6 freshly isolated $CD172a^+$-

enriched pDC were added to each well. After another 22 hours, the supernatants were harvested and analyzed for IFN-α by enzyme-linked immunosorbent assay (ELISA). The infection of the MDM was verified and quantified by optical density at 450 nm (OD450) using a CSFV E2-specific immunoperoxidase assay with mAb HC/TC-26. Means and standard deviations were calculated from six parallel cultures.

4.4.7 IFN-α detection by ELISA

IFN-α in cell supernatants and sera samples was quantified by ELISA as described previously, using the anti-pig IFN-α K9 and the F17 mAbs (provided by Dr. B. Charley, INRA, Jouy-en-Josas, France) (Díaz de Arce et al., 1992; Guzylack-Piriou et al., 2004). Serial dilutions of recombinant IFN-α protein (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) were employed as a standard. Optical densities obtained with the cytokine standard were used in a regression curve for the determination of the cytokine concentrations (units/ml) in samples.

4.4.8 Experimental infection

The experimental infection was performed following the protocol applied for the parental RNase-positive vPdR-36U virus used as control in the present study and described previously (M. Wang et al., 2020a). Twenty-seven piglets at 5 days of age, born from pestivirus-free sows from the same farm as in the previous study, were allocated in a box of the animal biosafety level 3 (aBSL3) facilities at IRTA-CReSA (Barcelona, Spain). The piglets (n=18, numbered 1-18) were inoculated intranasally with 2.5×10^4 $TCID_{50}$ of vPdR-H_{30}K-36U (according to the titer in PEDSV.15 cells). At 24 hours after infection, nine piglets (numbered from 19 to 27) were introduced as contact animals. Serum as well as nasal and rectal swabs were collected from the inoculated group

at 7 days post infection (dpi). Subsequently, these samples were collected from all animals weekly until the end of the trial (5 weeks after infection). In addition, tonsil samples were obtained after euthanasia.

As described previously, a veterinarian monitored the animals daily in a blinded manner (Muñoz-González et al., 2016). For ethical reasons, the animals were euthanized when they developed moderate to high clinical signs, including fall of the hindquarters, inability to drink or feed, prostration, or moderate to severe nervous disorders. Euthanasia was carried out according to the European Directive 2010/63/EU, with 60-100 mg/kilogram of bodyweight of pentobarbital injected into the vena cava cranialis. The Ethics Committee of the Generalitat of Catalonia approved the experiment in accordance with the Spanish and European regulations, under the animal experimentation project number 10514.

4.4.9 Detection of CSFV RNA

CSFV RNA was extracted from sera, nasal, and rectal swabs, and tonsils. The tonsil tissue was homogenized in Eagles MEM (1 g tissue + 9 ml medium) supplemented with penicillin 10,000 U/ml, streptomycin 10,000 µg/ml, and used for CSFV RNA detection. The MagAttract 96 cador Pathogen Kit (Qiagen) was used for RNA extraction following the manufacturer's instructions, and the RNA was stored at −80°C until CSFV RNA analysis by quantitative reverse transcription-PCR (RT-qPCR) (Hoffmann et al., 2005). Cycle threshold (Ct) values of 40 and below were considered as positive. Ct values between 29 and 40 were considered as low, from 23 to 28 as moderate and from 10 to 22 as high viral RNA load as previously (Tarradas et al., 2012).

4.4.10 CSFV rescue in tonsil samples from infected pigs

Tonsil samples were subjected to virus isolation in PK-15 cells. Briefly, the cells were seeded in 96-well plates for 24 hours before addition of 100 µL of 10- and 100-fold diluted samples. The virus was detected by PLA test after 72 hours of incubation (Wensvoort et al., 1986). Viral load was determined in the positive samples (M. Wang et al., 2020a).

4.4.11 Antibody detection by ELISA and virus neutralization assay

The serological analyses included the sera from the vPdR-H_{30}K-36U-infected pigs of the present study and sera from pigs infected with vPdR-36U under the same conditions in a previous study (M. Wang et al., 2020a). ELISA was used to analyze the E2- and E^{rns}-specific antibodies using the CSFV Ab test (IDEXX Laboratories, Liebefeld, Switzerland), and the pigtype CSFV E^{rns} Ab test (INDICAL), respectively, according to the manufacturer's recommendations. For CSFV E2-specific antibodies, samples were considered positive when the blocking percentage was ≥40%. Likewise, CSFV E^{rns}-specific Sample/Positive (S/P) values ≥0.5 were considered to be positive. Virus neutralization was determined using the neutralization peroxidase linked assay (NPLA) against the Alfort/187 strain with the sera samples that had scored positive by ELISA. The neutralization titers were expressed as the reciprocal dilution of serum that neutralized 100 $TCID_{50}$ in the 50% of the culture replicates.

4.5 Results

4.5.1 The RNase activity of E^{rns} has no effect on the replication kinetics of the virus in cell culture

The specific infectivity of the RNA transcripts and the titers of the rescued viruses were higher than 10^5 focus forming units/µg RNA and 5

× 10^6 $TCID_{50}$/ml, respectively, in PEDSV.15 cells, which showed that the two cDNA clones were functional. The complete nucleotide sequence of the two viruses, vPdR-36U, and vPdR-H_{30}K-36U, was determined after one additional passage in PEDSV.15 cells, confirming the expected E^{rns} sequence in the respective virus and excluding any other difference or accidental mutation. The growth characteristics of vPdR-H_{30}K-36U and vPdR-36U were analyzed in PEDSV.15 cells (Figure 1(a)) and in porcine MDM (Figure 1(b)). No difference in the replication kinetics was found for the two viruses. This demonstrates that the RNase activity of E^{rns} does not influence the basic replication characteristics of the virus, neither in a porcine endothelial cell line nor in porcine MDM.

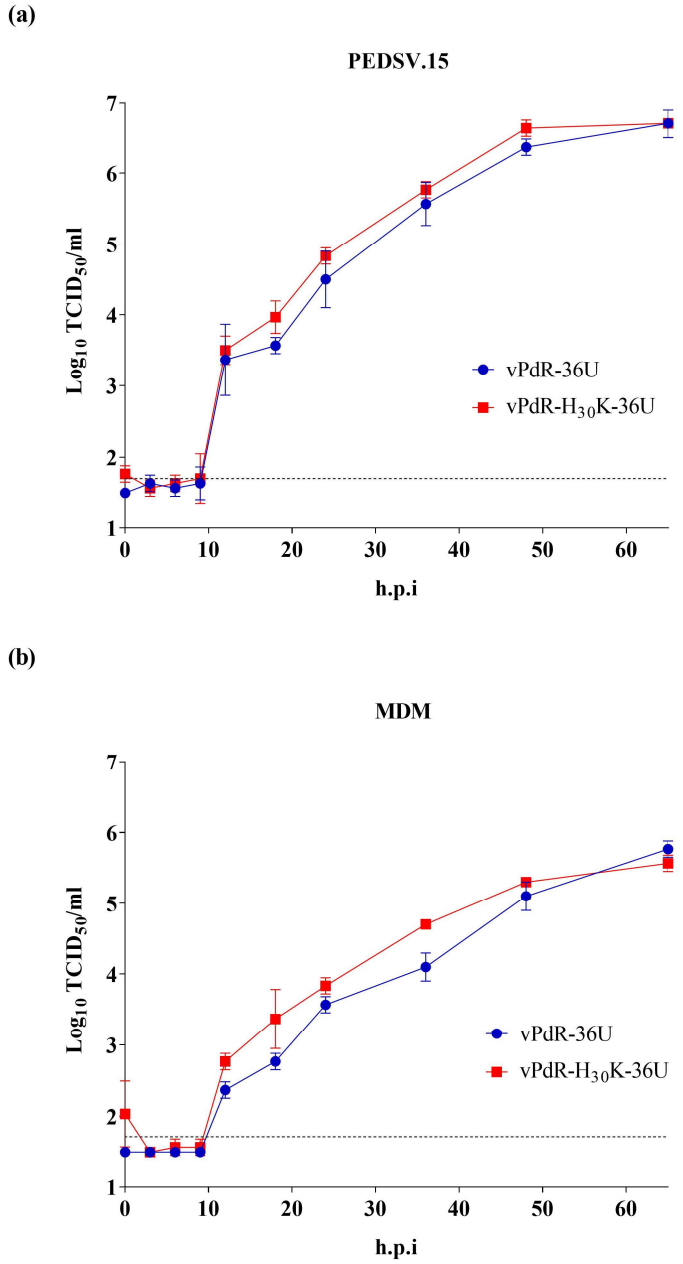

Figure 1. Kinetics of virus replication in PEDSV.15 cells and porcine MDM. PEDSV.15 cells and porcine MDM were infected in quadruplicate or in triplicate with vPdR-36U (in blue) or vPdR-H_{30}K-36U (in red), respectively. The mean virus titer was determined in SK-6 cells at 3, 6, 9, 12, 18, 24, 36, 48 and 72 h.p.i and error bars show the standard deviation.

4.5.2 The E^{rns} of vPdR-H_{30}K-36U lacks RNase activity

The histidine at amino acid position 30 of E^{rns} lies within the first catalytic domain of the RNase active site of E^{rns} (Meyers et al., 1999). We analyzed the E^{rns}-specific RNase activity in cells infected with vPdR-36U and vPdR-H_{30}K-36U and showed that the H_{30}K mutation of E^{rns} abolished the CSFV-mediated degradation of an IRDye-labeled RNA probe completely (Figure 2). In contrast, the parent vPdR-36U virus digested the RNA probe at least as efficiently as the RNase A used as control (Figure 2). This confirms that substitution of the histidine codon 30 of E^{rns} with a lysine codon abolishes the RNase activity of the virus in infected cells as expected.

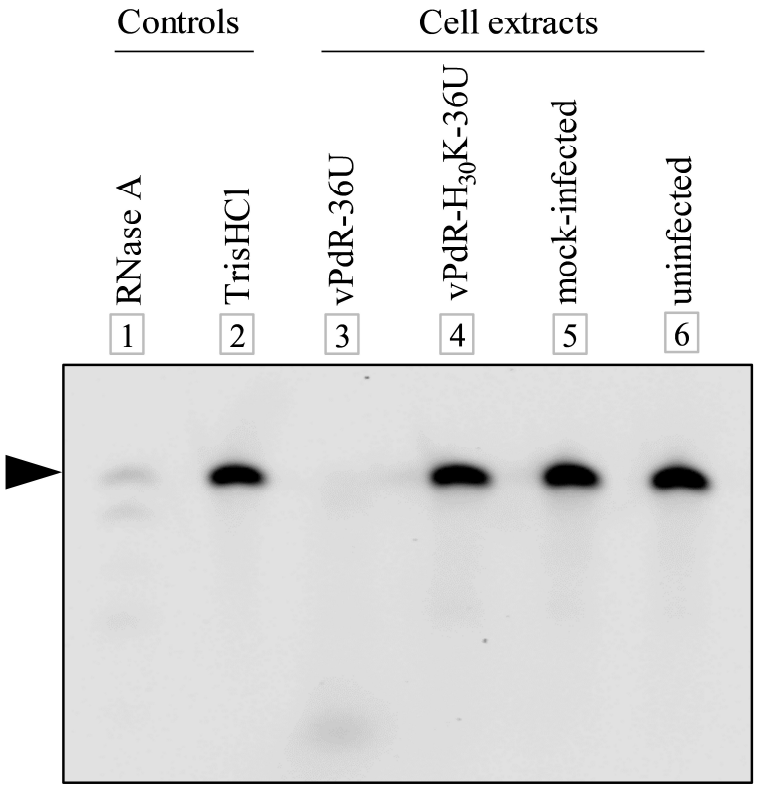

Figure 2. RNase activity assay with vPdR-36U and vPdR-H_{30}K-36U. SK-6 cells were infected with vPdR-36U or vPdR-H_{30}K-36U or mock or left uninfected. The cells were lysed after 44 hours and the extracts were incubated with the Dy-781-O1-RNA probe. The probe (black arrowhead) treated with the different cell extracts as indicated was separated by urea/polyacrylamide gel electrophoresis. Positive and negative controls were included in lanes 1 and 2, respectively.

4.5.3 The RNase-inactive vPdR-H_{30}K-36U has lost the capacity to prevent IFN-α induction in porcine pDC

It was shown previously that E^{rns} prevents pDC from producing IFN-α upon contact with CSFV-infected cells, which is dependent on the RNase activity E^{rns} (Python et al., 2013). Here we showed that MDM infected with vPdR-36U did not induce any detectable IFN-α in pDC brought in

contact with them (Figure 3(a)). This contrasted with the vPdR-H_{30}K-36U mutant that activated pDC efficiently for IFN-α production (Figure 3(a)). Importantly, the two viruses infected the MDM in contact with the pDC with the same efficiency (Figure 3(b)). Of note, CpG stimulation of parallel cultures of pDC resulted in 1420 IFN-α U/ml (±75), which confirms the functionality of the pDC for IFN-α production. This shows that the vPdR-H_{30}K-36U as opposed to vPdR-36U activates the pDC for IFN-α production directly and in a cell-contact-dependent manner *in vitro*.

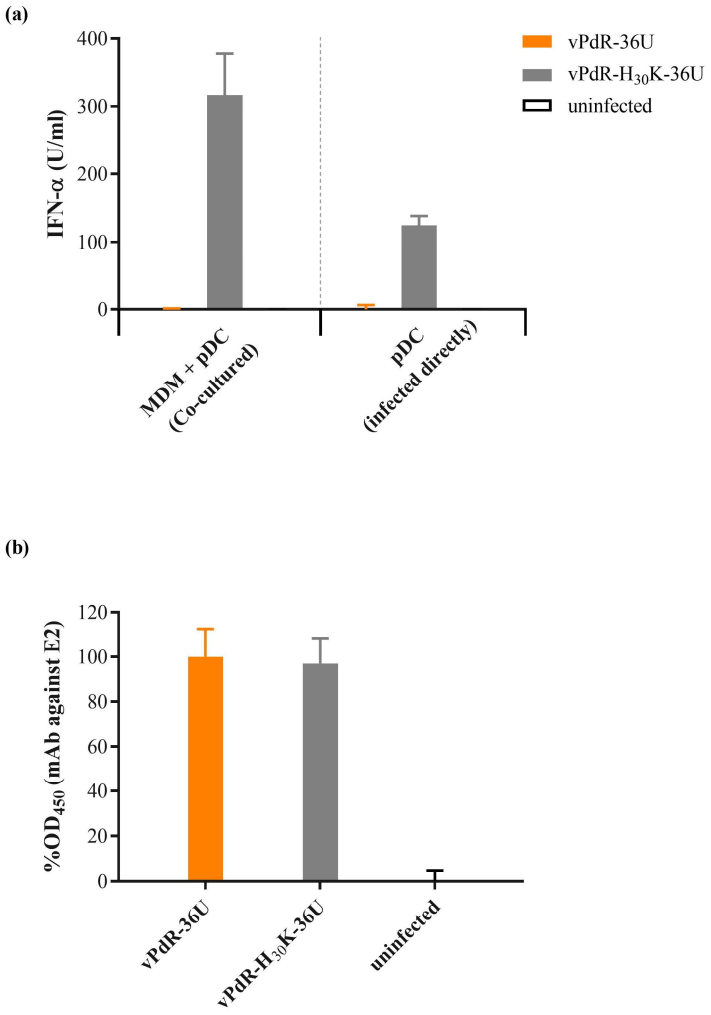

Figure 3. IFN-α induction in pDC by co-culture with infected MDM or by direct infection with vPdR-36U or vPdR-H_{30}K-36U. (a) Mean IFN-α concentration in cell culture supernatants from enriched pDC co-cultured with uninfected MDM (white bars) or MDM infected with the vPdR-36U (orange bars) or vPdR-H_{30}K-36U (gray bars, left panel) or from pDC infected directly with the indicated respective viruses (right panel). (b) The level of infection of the MDM by the respective viruses is shown as percentage of optical density (% OD) of the vPdR-36U signal obtained after immunoperoxidase staining of E2. Means and standard deviations (error bars) were calculated from six parallel cultures.

4.5.4 Very mild clinical signs were observed in pigs infected with the E^{rns} RNase-inactive CSFV mutant

Mild diarrhea was the main clinical sign in the vPdR-H_{30}K-36U-inoculated group (Figure 4). In general, diarrhea was detected during the second week of the study and did not last longer than 5 days in any case. From the third week on until the end of the trial, the animals were clinically healthy. However, one animal from the inoculated group had to be euthanized at 9 dpi after it had reached the endpoint criteria, despite showing only mild diarrhea the previous day. The contact pigs were clinically healthy during the entire trial, except for 3 out of 9 animals that showed sporadic mild diarrhea during 1 day in the second week of the study (Figure 4).

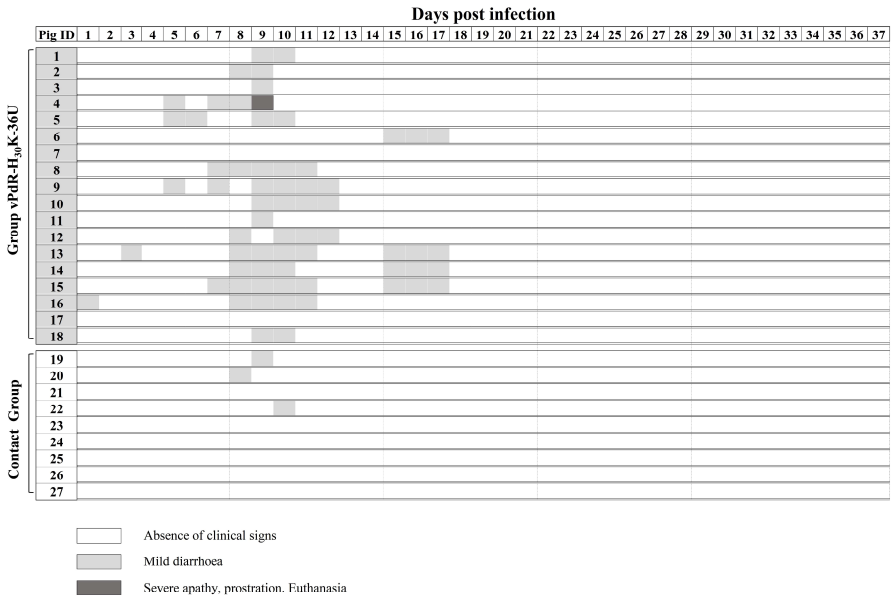

Figure 4. Individual clinical signs evaluated after vPdR-H_{30}K-36U infection. Pigs 1 to 18 were inoculated with vPdR-H_{30}K-36U and pigs 19 to 27 served as contact pigs introduced 24 hours post infection. The clinical signs were monitored daily during the complete study. Different shades of gray represent the severity of the clinical signs according to the legend.

4.5.5 The E^{rns} RNase-inactive CSFV mutant showed a very low replication capacity in pigs

Low levels of CSFV RNA were detected in the sera from less than half of vPdR-H_{30}K-36U inoculated piglets only, mostly during the first two weeks post infection, while the other piglets of the group remained negative (Figure 5(a)). Interestingly, from the third week onwards, nearly all infected animals were negative for viral RNA in the serum, with the exception of two samples, one at four (pig number 6) and another at five (pig number 3) weeks post infection (Ct value above 31.9). At necropsy, five out of 18 infected animals were negative for CSFV RNA in the tonsils. Low viral RNA load was found in 12 tonsil samples, the majority of them with Ct value above 30. Only one animal, i.e. pig number 4 that was euthanized at 9 dpi (Figure 4), showed moderate CSFV RNA levels in the tonsil with a Ct value of 28 (Figure 5(b)). However, no live virus could be isolated from any of the RT-qPCR positive tonsil samples.

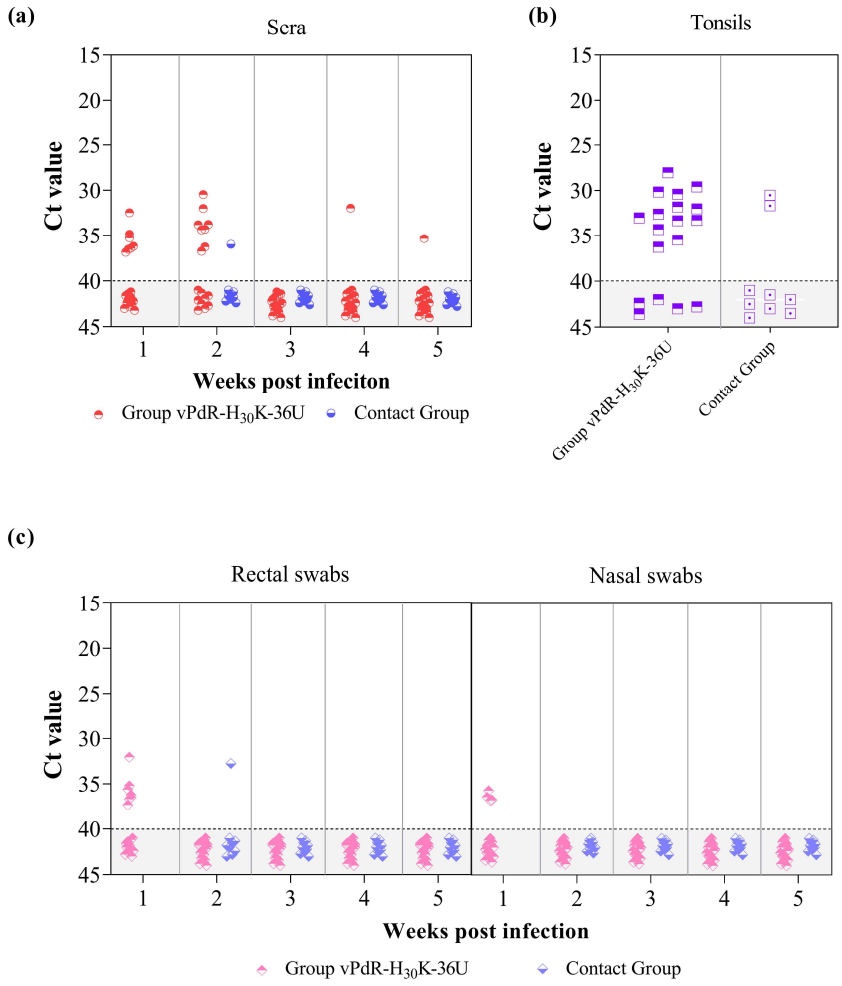

Figure 5. Detection of CSFV RNA in sera, tonsils and swabs. The CSFV RNA content was determined by RT-qPCR in the sera at weekly intervals (a) the tonsils obtained at necropsy (b) and the rectal and nasal swabs (c) collected on a weekly basis. Ct values higher than 40 (shaded area under the dotted line) were considered as negative.

4.5.6 The E^{rns} RNase-inactive virus showed a very low capacity for excretion and transmission

Low virus shedding was observed in half of the infected pigs in the first week post infection only, as shown by Ct values above 30 in the nasal or rectal swabs. By contrast, the other half of infected animals remained

negative during the whole trial (Figure 5(c)). Accordingly, virus transmission was observed only in one of the nine contact piglets (pig number 19) the second week of the study with a low RNA load in both, the serum and the rectal swab (Ct values higher than 32) (Figure 5(a,c)). In addition, two contact pigs were weakly positive for CSFV RNA in the tonsils but were again tested negative for virus isolation (Figure 5(b)).

4.5.7 The CSFV E^{rns} RNase-negative mutant activates the IFN-α response in pigs

The vPdR-H_{30}K-36U-infected piglets had variable levels of IFN-α in the serum during the first two weeks post-infection while almost all the contact piglets remained negative. In the first week, 15 out of the 18 infected piglets were positive for IFN-α, five of which had serum IFN-α levels higher than 100 units/mL (Figure 6). The IFN-α levels were overall lower during the second week post infection. Six piglets had serum IFN-α below 25 units/mL or remained negative during the whole trial. Notably, in the contact group, only pig number 22 (which was also positive for CSFV RNA in the serum) had a high serum IFN-α level (153 units/mL) the second week of the study (data not shown).

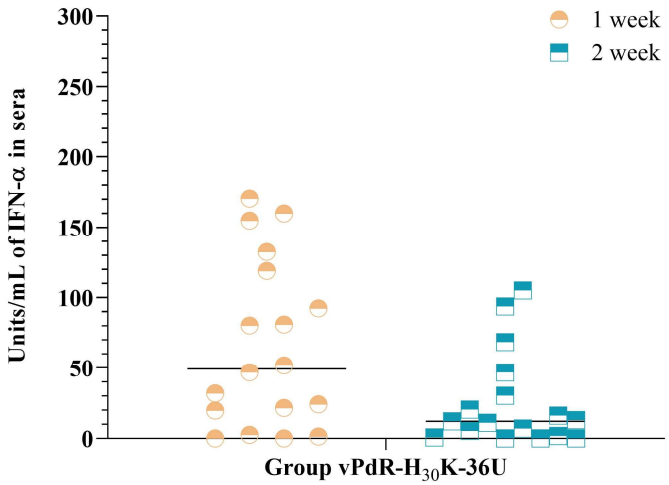

Figure 6. IFN-α levels in sera during the first two weeks post infection. Serum IFN-α levels were determined on the first (yellow symbols) and second (blue symbols) week after infection with vPdR-H_{30}K-36U. The black lines represent mean values.

4.5.8 The absence of RNase activity of E^{rns} resulted in a more consistent E2-specific antibody response in pigs

For the analysis of immune responses, we included serum samples from age-matched piglets infected with the parent RNase-positive vPdR-36U virus from our previous study using the same setup (M. Wang et al., 2020a) and compared them with the sera from the vPdR-H_{30}K-36U-infected piglets of the present study using ELISA and NPLA (Figure 7). In the second week post infection, only one vPdR-36U-infected piglet seroconverted against E2, while all other piglets from the two groups were still negative (Figure 7(a)). In the third and fourth weeks post infection, the E2 antibody responses of the two groups were similar, with only two E2-doubtful pigs for the vPdR-H_{30}K-36U group and four negative or doubtful E2-specific responses for the vPdR-36U group on week 4. In the last week of the experiment, three of the surviving vPdR-36U-infected pigs were still E2-negative or doubtful by ELISA, while

the rest were positive, especially for all piglets from the vPdR-H_{30}K-36U group. The contact pigs of the vPdR-36U group seroconverted during the fourth week, with three positive animals out of seven. A week later, five of the seven remaining contact pigs were seropositive. In accordance with the lack of vPdR-H_{30}K-36U transmission, the contact pigs from this group remained E2-negative, except for the single animal (number 22) that was infected by contact.

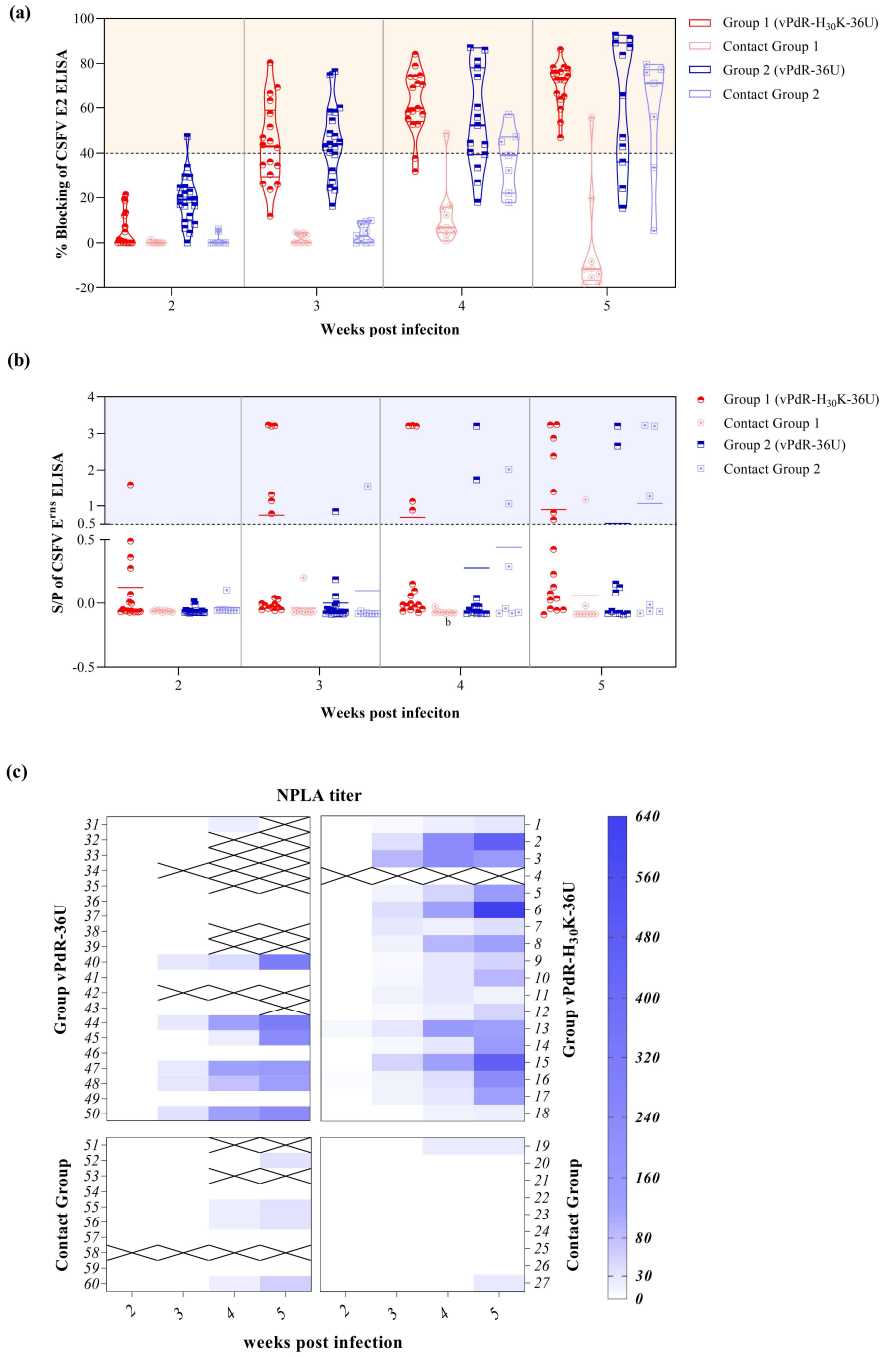

Figure 7. Evaluation of the humoral immune response after vPdR-H_{30}K-36U and vPdR-36U infection. (a) The E2-specific antibody responses after infection with vPdR-H_{30}K-36U and vPdR-36U are shown as percentage of blocking as measured by ELISA.

Values between 30 and 40% of blocking were considered doubtful, and values equal or greater than 40% were considered positive. (b) The E^{rns}-specific antibody responses after vPdR-H_{30}K-36U and vPdR-36U infection are shown as S/P ratios as determined by ELISA. S/P ratios between 0.3 and 0.5 were considered doubtful, S/P ratios equal or greater than 0.5 were considered as positive. (c) The neutralizing antibody titers after vPdR-H_{30}K-36U and vPdR-36U infection were determined by the NPLA at weekly intervals. Neutralizing antibody titers from low to high are represented on a scale from light to deep blue. Negative samples are shown in white. A cross shows that the animal was euthanized or dead.

4.5.9 The E^{rns} RNase-negative mutant induces enhanced E^{rns}-specific antibody responses in pigs

In terms of E^{rns}-specific antibody responses, only two of the pigs inoculated with vPdR-36U expressing functional E^{rns} RNase seroconverted against E^{rns}, one on week 3, and the second on week 4. Consistent with the poor seroconversion against E^{rns} after vPdR-36U infection, only three out of 10 contact animals in this group were E^{rns} antibody-positive by the end of the study. Meanwhile, with the RNase-defective virus, seroconversion against E^{rns} started on the second week, and six animals had seroconverted against E^{rns} on the third week (Figure 7(b)). In accordance with the poor transmission of the vPdR-H_{30}K-36U virus, only one contact pig became E^{rns} antibody-positive on the fifth week of the study (Figure 7(b)). Together, the RNase-negative PdR induced a more robust antibody response against E^{rns} while being less transmissible to contact animals, when compared with the parent PdR-36U virus carrying functional E^{rns}.

4.5.10 The RNase-defective vPdR-H_{30}K-36U virus induced earlier and stronger neutralizing antibody responses than the parent virus

The neutralizing antibody responses induced by vPdR-36U versus vPdR-H_{30}K-36U were compared by the NPLA test. During the first 2 weeks following the infection, none of the animals had neutralizing antibodies (Figure 7(c)). In the third week post infection, 16 out of the 17 vPdR-H_{30}K-36U-infected pigs developed neutralizing antibodies, compared to only 5 out of the 18 survivors of the vPdR-36U group (Figure 7(c), top panels). At this time, in the latter group all neutralizing titers were below 1:30 (Figure 7(c), top left panel). In the fourth week, all vPdR-H_{30}K-36U-infected piglets were positive, with neutralizing titers by up to 1:240. This contrasted with the vPdR-36U group in which only half of the survivors had neutralizing antibodies at this time, with maximum titers of 1:120. In the last week of the study, the neutralizing antibody titers further increased in all pigs of the vPdR-H_{30}K-36U group, reaching 1:640. The neutralizing titers ranged from 1:120 to 1:320 in the survivors of the vPdR-36U group, with still half of the pigs lacking detectable neutralizing antibodies. In the contact animals of this latter group, titers ranged from 1:30 to 1:60. Low levels of neutralizing antibodies were found also during the fourth and fifth weeks of the trial in the two vPdR-H_{30}K-36U animals that were infected by contact.

4.6 Discussion

Nowadays, CSFV strains of low and moderate virulence continue to be prevalent in some endemic countries, making the CSF control particularly difficult (Coronado et al., 2017; Izzati et al., 2021). However, data on the viral factors that determine attenuation and persistence remain scarce. The present study shows for the first time the effect of knocking out the RNase activity of E^{rns} in a low virulence CSFV strain, on viral replication, pathogenesis and transmission capacity in

pigs. The results obtained here suggest a role of functional RNase activity of E^{rns} in dampening immune response activation and in the establishment of viral persistence in pigs. In accordance with previous studies showing that substitution or deletion of histidine residues within the catalytic domain of the E^{rns} RNase abolished RNase activity (Meyers et al., 1999; Schneider et al., 1993), the vPdR-H_{30}K-36U mutant did not show any detectable E^{rns} RNase activity when comparted with its vPdR-36U parent. However, the RNase inactivation did not affect the viral replication in cell cultures, as vPdR-36U and vPdR-H_{30}K-36U had similar replication characteristics in PEDSV.15 cells and in MDM. This is consistent with the fact that neither of the two viruses can induce any type I IFN in these latter cells as in any non-pDC, due to functional N^{pro} that mediates degradation of IFN regulatory factor 3 (IRF3) (Python et al., 2013; Ruggli et al., 2005). In direct contrast with this, when compared with vPdR-36U from a previous study (M. Wang et al., 2020a), the E^{rns} RNase-negative vPdR-H_{30}K-36U showed reduced or undetectable viral replication in pigs, as measured in sera, body secretions, and tonsils, in accordance with the very mild or even absence of clinical signs observed. Thus, the abrogation of the RNase activity in the low virulence PdR isolate had a strong attenuating effect *in vivo*, in agreement with previous studies with virulent CSFV or BVDV (Meyer et al., 2002; Meyers et al., 1999).

In addition, the low replication capacity *in vivo* of the E^{rns} RNase-negative virus resulted in a nearly complete loss of viral transmission of the virus to contact piglets. This was consistent with the absence of live virus in the nasal and rectal swabs, although traces of viral RNA were detectable by RT-qPCR with Ct values above 30. A previous study using the parental vPdR-36U showed that no live CSFV could be isolated from

samples with Ct values above 32.2 (M. Wang et al., 2020a). In fact, only two contact piglets were infected with vPdR-H_{30}K-36U, likely caused by infection via fomites rather than by pig-to-pig transmission, although the latter cannot be excluded. Notably, infection with vPdR-H_{30}K-36U resulted in very low levels or absence of CSFV RNA in the tonsil, despite the general high capacity of CSFV to persist in the tonsils from infected or vaccinated animals for over 30 days (Kaden et al., 2004). This suggests that the lack of E^{rns} RNase activity may also reduce the capacity of CSFV to persist in the infected host. It should be noted here that the wild-type CSFV PdR isolate (Coronado et al., 2017), with functional E^{rns} RNase activity is capable of generating persistently infected piglets, with absence of seroconversion as a hallmark (Muñoz-González et al., 2015b). In contrast, such persistence did not occur with the RNase-negative mutant under the same conditions in the present study. The vPdR-H_{30}K-36U virus had reduced viral replication, with absence of infectious CSFV in the tonsils. These results are in line with the observation that the abrogation of the E^{rns} RNase activity in BVDV reduced the capacity to establish persistent infections in bovine fetuses (Meyers et al., 2007).

Type I IFNs are key cytokines to establish an antiviral state and to potentiate the adaptive immune response (Rojas et al., 2021). In agreement with a previous study (Python et al., 2013), the E^{rns} RNase-negative PdR mutant strongly activated the IFN-α production in pDC *in vitro* (Figure 3). This mutant also showed a clear activation of the IFN-α response in the infected pigs, however, with substantial differences between the animals. The serum IFN-α levels were lower when compared with those detected in a previous study, in pigs infected with the parent vPdR-36U virus expressing functional E^{rns} RNase (M. Wang

et al., 2020a). Thus, the activation of IFN-α *in vivo* showed an inverse correlation with respect to the response observed in cell cultures (Python et al., 2013), which could be explained by the different replication levels achieved in each system. The viral RNA-mediated trigger of IFN-α induction in the infected animals may be lower with the vPdR-H_{30}K-36U due to the significantly lower replication than that reported previously for the parent vPdR-36U, which may not be compensated by the lack of the IFN antagonistic function of E^{rns} in pDC with the mutant (Rojas et al., 2021; M. Wang et al., 2020a). These results support the role of CSFV E^{rns} RNase activity in the regulation of viral replication and in the modulation of innate immune activation in pigs.

Interestingly, the E^{rns} RNase-negative PdR mutant enhanced the adaptive immune response. High antibody levels against E2 and E^{rns}, as well as an efficient neutralizing antibody response was detected in the vPdR-H_{30}K-36U infected pigs. Notably, from four weeks post-infection onwards, the neutralizing antibody titers against non-homologous virus were above the threshold established for protection against CSFV infection (Terpstra et al., 1984) in nearly all the vPdR-H_{30}K-36U-infected piglets. It was shown that CSFV-infected pigs can develop anti-E^{rns} antibodies as early as 10 dpi (Meyer et al., 2017). This has raised interest in E^{rns} as a potential diagnostic target for the differentiation of vaccinated from infected animals (DIVA) (Meyer et al., 2017). In the present study, we also evaluated the humoral response generated in pigs after infection with the vPdR-36U virus, using the sera collected previously (M. Wang et al., 2020a). The vPdR-36U is a cDNA-derived version of the field isolate Pinar del Rio (PR-11/10-3) from Cuba (Coronado et al., 2017; Pérez et al., 2012). Remarkably, the vPdR-36U virus, failed to induce detectable E^{rns}-specific antibody responses in

piglets during five weeks of infection. In this regard, the abrogation of the E^{rns} RNase activity enhanced E^{rns}-specific antibody response significantly, suggesting a better E^{rns} protein immunogenicity following the loss of the RNase activity. Therefore, the difficulties in the development of diagnostics and DIVA assays based on seroconversion against E^{rns} (Pannhorst et al., 2015; Postel and Becher, 2020) may be attributable to a dampening effect on E^{rns}-specific immunity related to the RNase activity of E^{rns}. Further studies are required to clarify the role of the RNase activity of E^{rns} in the host immune modulation (Python et al., 2013) by CSFV strains of different virulence.

These results pave the way towards a better understanding of CSFV attenuation related to the RNase function of E^{rns} through regulation of viral replication and immune responses in pigs. Our findings also provide new insights relevant for the development of DIVA vaccines against CSFV with appropriate accompanying diagnostic tests for efficient control and eradication of CSF and for the distinction from infections with other pestiviruses.

4.7 Acknowledgments

This research was supported by Ministerio de Ciencia, Innovación y Universidades from the Spanish government under the grant RTI2018-100887-B-I00. M.W. has a pre-doctoral fellowship CSC scholarship (2017) from the Chinese government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from Spanish government. IRTA is supported by CERCA Programme/Generalitat de Catalunya. YH received a Swiss Government Excellence Research Scholarship from the Federal Commission for Scholarships for Foreign Students (ESKAS). The authors thank Ivan

Cordón and the CReSA animal facility staff for their help during the *in vivo* part of the experiment.

4.8 Funding Statement

This work was supported by the China Scholarship Council [CSC scholarship (2017) from the Chinese government]; Swiss government excellence research scholarship from the federal commission for scholarships for foreign students; Ministerio de Ciencia, Innovación y Universidades [RTI2018-100887-B-I00]; Ministerio de Ciencia, Innovación y Universidades [Pre-doctoral fellowship FPI 2016].

4.9 Disclosure statement

The author(s) declare no competing interests.

4.10 Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Chapter 5

Study III. The E^{rns} RNase and the 3´UTR poly-uridine insertion in a low virulence classical swine fever virus strain prevent IFN-α, IL-12 and IL-8 upregulation and disease severity

(Manuscript)

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5.1 Abstract

The molecular mechanisms behind the evolution of classical swine fever virus (CSFV) towards low virulence in endemic areas remain poorly understood. In the present study, we assessed the potential role of the RNase activity of E^{rns} and of the poly-uridine insertion in the 3'UTR of the low virulence CSFV isolate Pinar de Rio (PdR) in attenuation and innate and adaptive immune regulation. We knocked out the E^{rns} RNase activity of PdR and replaced the long poly-uridine sequence of the 3'UTR with 5 uridines found typically at this position, resulting in a double-mutant called vPdR-H_{30}K-5U. The vPdR-H_{30}K-5U induced severe CSF in 5-day-old piglets and six 3-week-old pigs, with higher lethality in the newborn piglets (89.5%) than in the older pigs (33.3%). This, together with our previous studies with PdR mutants, suggest a cooperative effect of the 3´UTR and the E^{rns} RNase activity in controlling the virulence of this virus. Contrasting with disease severity, the viremia and viral excretion were surprisingly low, while the virus load was high in the tonsils. Among nine cytokines quantified, only IFN-α and IL-12 were highly and consistently elevated in the two groups. In addition, high IL-8 levels were found in the newborn but not in the older pigs. This points towards a role of these cytokines in the outcome of disease, with age-related differences. Infection with vPdR-H_{30}K-5U resulted in poor neutralizing antibody responses when compared with results obtained previously with the parent and RNase knockout PdR. Together, these results provide a better understanding of the relationship between CSFV virulence, viral replication and host innate and adaptive immunity, revealing new insights into the molecular determinants of natural CSFV attenuation.

5.2 Introduction

Classical swine fever (CSF) is one of the most highly contagious viral disease that affects swine worldwide (Ganges et al., 2020). The disease is caused by the CSF virus (CSFV), a member of the *Pestivirus* genus within the *Flaviviridae* family. CSF has been eradicated in central Europe but remains endemic in some regions of South and Central America, Asia and in the Caribbean (OIE, 2021). Long-term prevalence of CSFV in endemic regions has led to a broad range of clinical outcomes, from acute to chronic and subclinical, including persistent infection (Coronado et al., 2019a; Ganges et al., 2020).

CSFV is an enveloped virus with a single-stranded, positive-sense RNA genome of approximately 12.3 kb. The RNA carries a single open reading frame (ORF) that encodes a polyprotein flanked by a 5′ and a 3′ untranslated region (UTR). The translated polyprotein is processed into four structural (C, E^{rns}, E1, and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Meyers and Thiel, 1996; Tautz et al., 2015).

The pestivirus proteins N^{pro} and the E^{rns} interfere with the host's innate immune responses by counteracting type-I interferon (IFN) synthesis with implications in pathogenesis (Mätzener et al., 2009; Meyers et al., 2007; Ruggli et al., 2005; Summerfield and Ruggli, 2015). Functional E^{rns} possesses a unique intrinsic ribonuclease (RNase) activity (Schneider et al., 1993) which acts as an antagonist for IFN induction by degrading viral single- and double-stranded RNA conditionally in the extracellular space and in endocytic compartments (Lussi and Schweizer, 2016; Magkouras et al., 2008).

Previous studies showed that the low virulence CSFV field isolate Pinar de Rio (PdR) which can cause chronic and persistent forms of CSF,

contains a unique and uninterrupted 36-uridine (poly-U) insertion in the 3´UTR (Coronado et al., 2019a, 2017; Muñoz-González et al., 2015b). This poly-U insertion has been associated with activation of immunity and reduction of virulence in piglets (M. Wang et al., 2020a). Likewise, a previous study also revealed loss of pathogenicity and replication capacity in pigs when the E^{rns} RNase function was abrogated in the low virulence CSFV PdR strain carrying the poly-U (M. Wang et al., 2021). Therefore, in the present study, we investigated the potential synergistic effect of the E^{rns} RNase activity and the poly-U insertion in the 3'UTR in the regulation of innate and adaptive immunity and its relationship with the CSF pathogenesis in pigs. To this end, a reverse genetics approach was used to analyze the phenotype of a double-mutant of the low virulence PdR strain in which the RNase activity of E^{rns} was knocked out and the aforementioned 36 uridines of the 3'UTR were replaced with 5 uridines common to most CSFV isolates at this position. We analyzed the replication of this virus in monocyte-derived macrophages (MDM) and the IFN-α induction in plasmacytoid dendritic cells (pDC) and determined pathogenicity, replication, cytokine induction and immune responses in 5-day- versus 3-week-old pigs.

5.3 Materials and Methods

5.3.1 Cells and Viruses

The three cell lines used in this study, *i.e.* the porcine kidney cell line PK-15, the porcine aortic endothelial cell line PEDSV.15 (Seebach et al., 2001) and the swine kidney cell line SK-6 (Kasza et al., 1972) were tested free of pestiviruses. The PK-15 cells, obtained from ATCC (CCL-33), were grown in minimum essential medium (MEM) supplemented with 10% *Pestivirus*-free fetal bovine serum (FBS). The PEDSV.15 cells,

provided by Jörg Seebach, University of Geneva, Switzerland, were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with sodium pyruvate, non-essential amino acids, 7% horse serum and 2% porcine serum. The SK-6 cells were obtained from M. Pensaert, Faculty of Veterinary Medicine, Ghent, Belgium and were cultured with the same medium as PEDSV.15 cells but without porcine serum. In addition, $CD172a^+$ enriched porcine pDC and porcine MDM were prepared from specific pathogen-free (SPF) pigs as described previously (Ogno et al., 2019; Python et al., 2013; M. Wang et al., 2020a). For this, blood was collected from SPF pigs bred at the Institute of Virology and Immunology IVI in Mittelhäusern, Switzerland, in compliance with the animal welfare regulation of Switzerland under the cantonal license BE127/2020.

The CSFV strain Alfort/187, provided by the CSFV EU Reference Laboratory, Hannover, Germany, was used for virus neutralization assays. The cDNA-derived vPdR-36U corresponding to the wild-type CSFV strain PdR, and the vPdR-5U virus lacking the poly-U sequence were from previous studies (M. Wang et al., 2020a). The vPdR-H_{30}K-36U virus devoid of E^{rns} RNase activity was also included in the present work (M. Wang et al., 2021). The cDNA-derived virus vPdR-H_{30}K-5U was rescued from the respective cDNA as described below. All viruses were amplified by infecting cells with 0.1 tissue culture infectious dose ($TCID_{50}$)/cell and harvested after 72 h. The virus titers were determined by end-point dilution in SK-6 and PEDSV.15 cells and in porcine MDM using the peroxidase-linked assay (PLA) (Wensvoort et al., 1986). The virus titers expressed in $TCID_{50}$/ml were calculated using standard statistical methods (Reed and Muench, 1938).

5.3.2 Construction of the infectious clone pPdR-H_{30}K-5U

The functional cDNA clone pPdR-H_{30}K-5U encoding RNase-inactive E^{rns} and 5 uridines in the 3'UTR was obtained by combining the plasmids pPdR-5U and pPdR-H_{30}K-36U described previously (M. Wang et al., 2021, 2020a). To this end, the *Spe*I to *PspX*I cassette of pPdR-H30K-36U was replaced with the corresponding cassette released from pPdR-5U, resulting in pPdR-H_{30}K-5U. This construct was verified by Sanger sequencing.

5.3.3 Virus rescue from cDNA

The vPdR-H_{30}K-5U virus was rescued from cDNA as described previously (M. Wang et al., 2020a). Briefly, the pPdR-H_{30}K-5U was linearized with *Srf*I, and RNA was transcribed and purified. Then, 8 x 10^6 PEDSV.15 cells were mixed with 1 µg of RNA in 0.4 ml of ice-cold phosphate-buffered saline (PBS) and electroporated with two pulses at 200 V and 500 µF in a 0.2 cm electroporation cuvette (Bio-Rad) using a Gene Pulser (Bio-Rad). The cells were then incubated in 75 cm^2 flasks during 65 h at 37°C and 5% CO_2. The rescued virus was passaged once in PEDSV.15 cells. In order to control the functionality of the constructs, the infectivity of the RNA transcripts was determined with an infectious center assay as described previously (Mendez et al., 1998). The complete genomes of the rescued viruses were determined by nucleotide sequencing to exclude any accidental mutation. Viral titers were determined in PEDSV.15 cells.

5.3.4 Virus replication kinetics in cell culture

Porcine MDM (500,000/well) were seeded in 24-well plates using serum-free medium. The cells were infected with vPdR-H_{30}K-5U, vPdR-H_{30}K-36U and with the parent vPdR-36U at a multiplicity of infection (MOI) of 0.005 $TCID_{50}$/cell. After one hour incubation at 37°C, the inoculum was removed and the cells were washed once with serum-free medium

followed by cultivation in complete culture medium at 37°C. At different times after infection, the plates were frozen at -70°C. After thawing, the supernatant was cleared from cell debris by low-speed centrifugation and the virus titers were determined by endpoint dilution in SK-6 cells and immunoperoxidase staining of CSFV E2 using the monoclonal antibody (mAb) HC/TC-26 (Greiser-Wilke et al., 1990).

5.3.5 RNase activity assay

Extracts of CSFV-infected SK-6 cells were analyzed for RNase activity as described previously (Itakura et al., 2020; Python et al., 2013; M. Wang et al., 2021). A 50-mer RNA probe (Dy-781-O1-RNA) (prepared by Dr. Fabian Axthelm, Microsynth AG, Balgach, Switzerland) was mixed with the samples to be tested at a final concentration of 40 nM. RNase A (3×10^{-3} U /ml in MEM) was used as digestion control, and 50 mM TrisHCl pH 7.4 served as the negative control. After 45 min incubation of the reactions at 37°C, 2 volumes of 97% formamide (Sigma) were added and a 10% polyacrylamide and 35% urea gel in 133 mM TrisHCl, 45.5 mM boric acid and 3.2 mM EDTA was used for separation. Finally, the image was acquired with an Odyssey Infrared Imaging System (LI-COR).

5.3.6 Western blot analyses

Cells were lysed with a denaturing buffer (62.5 mM Tris HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol and 0.05% bromophenol blue) and the proteins were separated by 10% SDS–polyacrylamide gel electrophoresis under non-reducing conditions. The proteins were then transferred to PVDF membranes (Immobilon-FL, Merck-Millipore) using standard protocols. The membranes were blocked with Odyssey blocking buffer (LI-COR) and incubated with the HC/TC-26 primary antibody and

goat anti-mouse IRDye680 secondary antibody (LI-COR). An Odyssey Infrared Imaging System (LI-COR) was used for data acquisition.

5.3.7 Stimulation of pDC for IFN-α production

Enriched porcine pDC were stimulated with infected porcine MDM cells for IFN-α production as described previously (Python et al., 2013). Briefly, porcine MDM were seeded in 24-well plates (200'000 cells/well) and infected at a MOI of 5 $TCID_{50}$/cell for 24 hours. The cells were washed four times, followed by addition of 10^6 freshly isolated $CD172a^+$-enriched pDC per well. The supernatants were harvested after another 22 hours and analyzed for IFN-α production by enzyme-linked immunosorbent assay (ELISA). The infection of the MDM was verified by immunodetection of E2 using the mAb HC/TC-26 and quantification of the immunoperoxidase signal at 450 nm.

5.3.8 Experimental infection of 5-day- and 3-week-old pigs

The experimental infection of pigs was designed following the protocol described previously, using animals from the same origin (M. Wang et al., 2021, 2020a). Nineteen newborn (5-day-old) piglets, born from Pestivirus-free sows, were allocated in the Biosafety level 3 (BSL3) animal facilities at IRTA-CReSA (Barcelona, Spain). The piglets, numbered from 1 to 19, were inoculated intranasally with 2.5×10^4 $TCID_{50}$ of vPdR-H_{30}K-5U (based on titers in PEDSV.15 cells). Nasal and rectal swabs and serum samples were collected from all animals at 8, 15, 22, 29 and 37 days post infection (dpi). Tonsil samples were collected after euthanasia.

In addition, the virulence of the double-mutant virus was also evaluated in six 3-week-old pigs from *Pestivirus*-free sows (numbered 20-25), as in previous studies (Bohórquez et al., 2021; Lamothe-Reyes et al., 2021).

The six pigs were inoculated intranasally with 2.5×10^4 $TCID_{50}$ of vPdR-H_{30}K-5U. Nasal and rectal swabs and serum samples were collected at 5, 11, 18 and 21 dpi. Finally, tonsil samples were obtained after euthanasia.

The pigs were monitored daily in a blinded manner by a trained veterinarian as described before (Muñoz-González et al., 2016). When the animals reached the discontinuation criteria, euthanasia was performed with 60-100 mg pentobarbital per kg bodyweight injected in the *vena cava cranialis*, according to the European Directive 2010/63/EU. The end point criteria were moderate to severe clinical signs, including inability to drink or feed, fall of the hindquarters, prostration or moderate to severe nervous disorders. The experiment was approved by the Ethics Committee from the Generalitat of Catalonia under the animal experimentation project number 10514, in accordance with the Spanish and European regulations, June 21, 2019.

5.3.9 IFN-α quantification by ELISA

IFN-α from pDC supernatants was quantified by ELISA as describe elsewhere (Díaz de Arce et al., 1992; Guzylack-Piriou et al., 2004). The anti-pig IFN-α K9 and the F17 mAbs were provided by Dr. B. Charley, INRA, Jouy-en-Josas, France. Serial dilutions of recombinant IFN-α protein (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) served as a standard. The IFN-α concentrations (units/ml) in samples were determined by a regression curve based on optical densities of an IFN-α standard with known bioactivity.

5.3.10 Detection of CSFV in the infected pigs

The CSFV RNA were extracted from sera, nasal and rectal swabs, and tissues from the vPdR-H_{30}K-5U infected pigs by the MagAttract 96 *cador*

Pathogen Kit (Qiagen), according to the manufacturer's instructions. Tissue samples were ground in sterile Eagle's Minimal Essential Medium (EMEM; 1 g of tissue + 9 ml EMEM) supplemented with 2% of penicillin 10,000 U/ml and streptomycin 10,000 U/ml. RT-qPCR (Hoffmann et al., 2005) was used to quantify the CSFV RNA in the collected samples. Ct values equal or lower than 40 were considered as positive. Samples that did not show any detectable fluorescence were considered negative. As previously quantified, Ct values from 10 to 23 were defined as high, from 23 to 29 as moderate and between 29 and 40 as low RNA viral load (Tarradas et al., 2012; M. Wang et al., 2020a).

5.3.11 Detection of antibody responses by ELISA and virus neutralization test

The specific antibodies against CSFV E2 and E^{rns} in serum samples were analyzed using the CSFV Ab test (IDEXX Laboratories, Liebfeld, Switzerland) and pigtype CSFV E^{rns} Ab test (INDICAL BIOSCIENCE GmbH, Leipzig, Germany), respectively. For CSFV E2-specific antibodies, the results were represented as blocking percentage value, and samples ≥40% were considered positive. Likewise, CSFV E^{rns}-specific sample/positive (S/P) values ≥0.5 were considered as positive. Neutralizing antibodies were quantified with the neutralization peroxidase linked assay (NPLA) against the Alfort/187 strain. The titers were expressed as the reciprocal dilution of serum that neutralized 100 $TCID_{50}$ in the 50% of the culture replicates.

5.3.12 Detection of cytokines by Luminex assay

The IFN-α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFN-γ and TNFα cytokines in serum samples were quantified with the Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel 1 (Invitrogen, Bender

MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions. Results were acquired on a Luminex® 200™ and cytokine concentrations were expressed as pg/ml, calculated according to a standard curve. A panel of serum samples from previous studies were included as control in the Luminex assay (M. Wang et al., 2021, 2020a). The panel included ten serum samples from pigs infected with the vPdR-36U and the vPdR-5U, respectively (M. Wang et al., 2020a), in addition to 18 serum samples from piglets infected with the vPdR-H_{30}K-36U (M. Wang et al., 2021), all collected at the end of the first week of infection.

5.3.13 Statistical analysis

The statistical analyses were performed using SPSS software, version 26.0 (IBM Corp., Armonk, NY, USA), using "pig" as the experimental unit. Independent-samples T test was chosen to compare cytokine levels among all experimental groups. The significance level (α) was set at $P<0.05$.

5.4 Results

5.4.1 The inactivation of the E^{rns} RNase and the deletion of the poly-U sequence in PdR did not impair virus replication in cell culture when compared with the parent virus.

The high specific infectivity of the RNA transcripts at rescue and the nucleotide sequence analysis of the vPdR-H_{30}K-5U virus passaged twice in PEDSV.15 cells confirmed the functionality and identity of the double-mutant. The replication kinetics of the vPdR-H_{30}K-5U virus did not differ from the replication of the parent vPdR-36U and the RNase knock-out vPdR-H_{30}K-36U mutant in freshly prepared porcine monocyte-derived macrophages (Fig 1a). The absence of any detectable E^{rns} RNase activity of the vPdR-H_{30}K-5U virus and of the vPdR-H_{30}K-36U described

previously was verified in SK-6 cells and compared with the parent vPdR-36U and vPdR-5U control viruses (Fig 1b), while viral protein expression was comparable for the 4 viruses (Fig 1c).

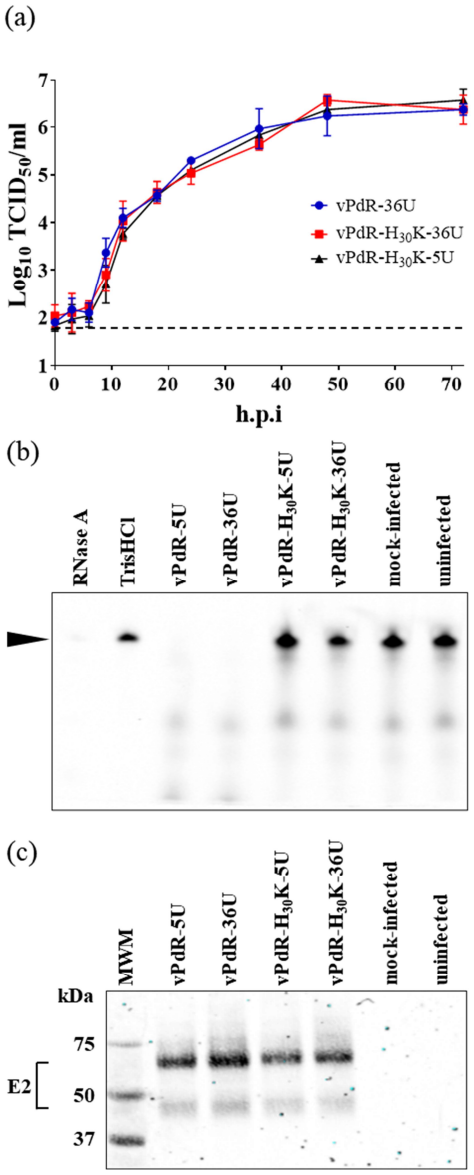


Fig 1. Kinetics of replication and RNase activity of the different mutant vPdR-derived viruses. (a) Porcine MDM were infected parallel multi-well plates (one per time point) with either vPdR-36U (in blue), vPdR-H_{30}K-36U (in red) or vPdR-H_{30}K-5U (in black)

and incubated for the indicated times. The mean virus titer from triplicate infections was determined in SK-6 cells and error bars represent the standard deviation. (b) In order to determine the E^{rns} RNase activity of the different mutant viruses, SK-6 cells were infected, mock-treated or left untreated for 40 hours. After total cell protein harvest by hypotonic lysis, the Dy-781-O1-RNA probe (black arrowhead) was incubated with EMEM containing RNase A or with the cell extracts for and finally separated by urea PAGE. (c) Protein extracts from parallel infected cell cultures were separated by SDS-PAGE under non-reducing conditions and analyzed by Western blotting and immunodetection with the anti-E2 (HC/TC26) and anti-β-actin mAbs and with IRDye680-labeled as secondary antibody.

5.4.2 The vPdR-H_{30}K-5U double-mutant strongly activated the IFN-α response in pDC.

The lack of E^{rns} RNase activity resulted in strong activation of pDC, and a significantly stronger IFN-α production was observed when the pDC were stimulated by contact with infected MDM compared with direct infection. Remarkably, the length of the poly-U sequence of the 3'UTR had no effect on the level of IFN-α induction in pDC (Fig 2). This was observed both, in the MDM-pDC co-culture setup and after direct infection of pDC. The two viruses with functional E^{rns}, vPdR-36U and vPdR-5U, did not induce any detectable IFN-α levels in pDC, neither by direct infection nor by contact with infected MDM (Fig 2).

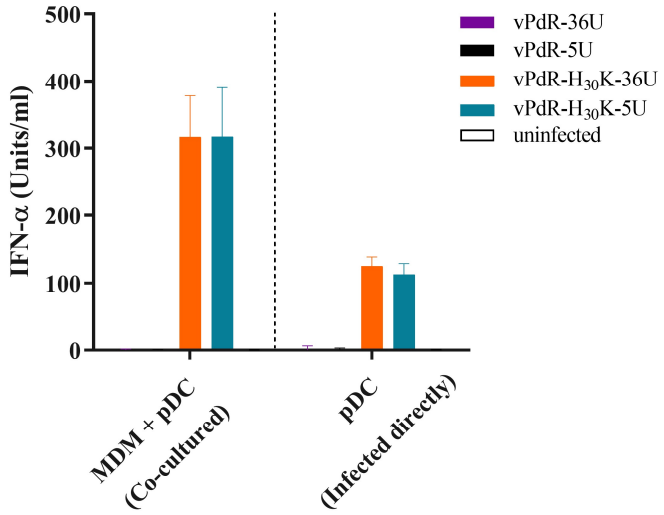


Fig 2. IFN-α induction in pDC by direct infection with virus or by co-culture with pre-infected MDM. Mean IFN-α concentration in cell culture supernatants from enriched pDC activated with MDM pre-infected with vPdR-36U, vPdR-5U, vPdR-H_{30}K-36U or vPdR-H_{30}K-5U or left uninfected (Co-cultured), or from enriched pDC infected directly with the indicated viruses. IFN-α was analyzed by ELISA and quantified based on porcine IFN-α of known bioactivity. The values represent the mean of 3 replica experiments, with the error bars showing the standard deviation.

5.4.3 The double-mutant vPdR-H30K-5U induced acute CSF with high mortality in 5-day-old piglets.

We analyzed the phenotype of the double-mutant vPdR-H_{30}K-5U in 5-day-old piglets. Unexpectedly, seven out of 19 infected piglets were found dead in the pen between day 5 and 9 after vPdR-H_{30}K-5U infection. Ten additional piglets were euthanized between day 6 to 11 post-infection due to the severity of clinical signs including prostration, weakness of hindquarters, moderate to severe conjunctivitis, cyanosis, tremor and severe nervous disorders (Fig 3). The two last piglets of the group (number 11 and 19) survived with moderate to mild clinical signs, including light

diarrhea, weight loss and in some cases conjunctivitis, from 8 dpi until the end of the study (Fig 3). Thus, the overall lethality of vPdR-H_{30}K-5U in newborn piglets was 89.5%.

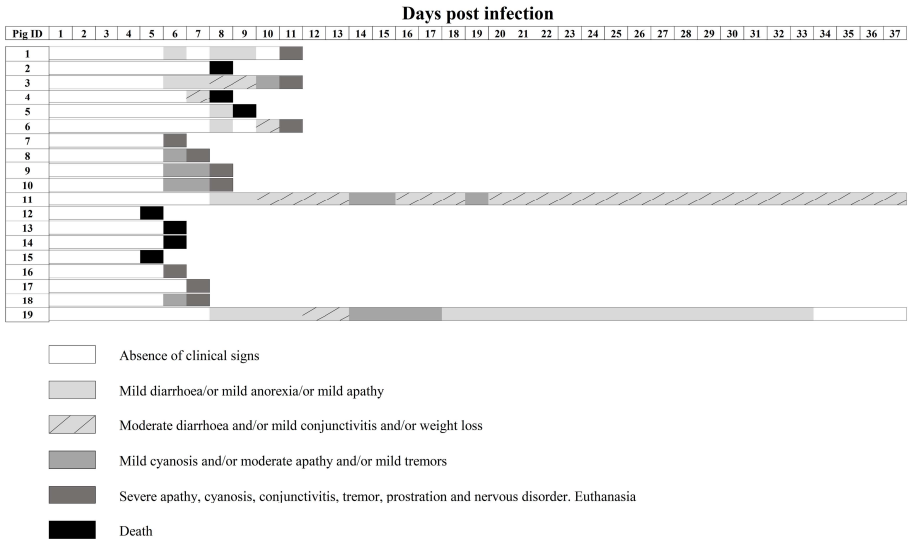


Fig 3. Individual clinical signs observed in piglets infected at the age of five days with vPdR-H_{30}K-5U. Pigs 1 to 19 were inoculated intranasally with vPdR-H_{30}K-5U, and the clinical signs were monitored daily during the whole study. Different shades of gray represent the severity of the clinical signs according to the legend.

5.4.4 Infection of 5-day-old piglets with vPdR-H_{30}K-5U resulted in high virus load in tonsils despite low viremia and limited virus secretion.

Serum samples could be collected from seven out of the eleven piglets that died or had to be euthanized before 8 dpi as described above. Five of them were CSFV positive, showing Ct values between 30.61 and 34.58. At 8 dpi, seven out of the eight surviving pigs had a low serum CSFV load, with Ct values ranging from 29.42 to 35.91 (Fig 4). After this time point, four animals either died or were euthanized from 9 to 11 dpi, showing Ct values between 27.63 and 34.62 in the serum samples. Interestingly, one of these

8 piglets (number 6) had a weak RT-qPCR signal (Ct of 34.62) in the serum on day 11 only, despite severe clinical sign requiring euthanasia. Only two animals survived until the end of the trial (piglets 11 and 19), with low to undetectable CSFV RNA in the serum. Pig 11 had a weak RT-qPCR signal in the serum on day 8 only while pig 19 was weakly positive on days 8, 15 and 29 (Fig 4). Accordingly, the piglets did not secrete the virus in the feces nor through the nose, except for two pigs with severe clinical signs (number 1 and 9) that were positive for CSFV in nasal swabs on day 11 and 8, respectively (Fig 4). In addition, one of the two survivor pig (number 11) was positive in the nasal swab on day 15.

Interestingly and in contrast with the low CSFV load in the serum, all the tonsil samples collected from the 17 piglets who were euthanized due to severe CSF or were found dead, were RT-qPCR positive for CSFV RNA, with moderate to high viral load (Fig 4). The two surviving piglets euthanized at 37 dpi were weakly positive in the tonsil, showing Ct value of 37.18 at that time (piglet 11), while piglet 19 was negative (Fig 4). Importantly, sequence analyses of the virus recovered from tonsils confirmed the expected 3'UTR sequence and RNase knockout sequence in Erns, without any accidental mutation in these two genome regions (data not shown).

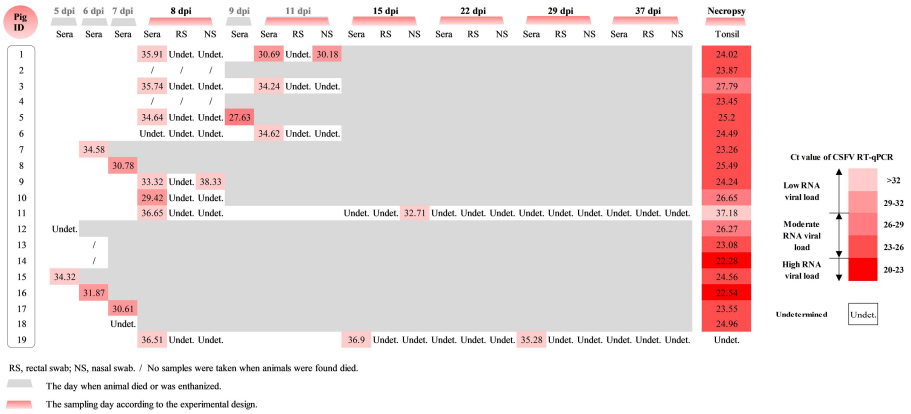


Fig 4. CSFV RNA detection in sera, swabs and tonsils from the piglets infected with vPdR-H_{30}K-5U. The CSFV RNA levels were determined by RT-qPCR in the sera and swabs at the indicated sampling time points and in the tonsils obtained at necropsy.

5.4.5 The piglets that survived the vPdR-H_{30}K-5U infection seroconverted after 3 weeks.

The two out of 19 piglets that survived the vPdR-H_{30}K-5U virus infection developed CSFV E2-specific antibodies after 15 dpi with the first positive values measured on day 22 (Fig 5). One of them was also positive for E^{rns} antibodies from 22 dpi onwards, while the other was anti-E^{rns}-positive for the first time on day 37, *i.e.* at the end of the study (Fig 5). Low CSFV neutralizing antibody titers were detected in the two animals on day 22, reaching values of 1:20. Likewise, a slight increase in the neutralizing antibody titers was noted for both animals at the end of the study, with values of 1:80 and 1:40 (Fig 5).

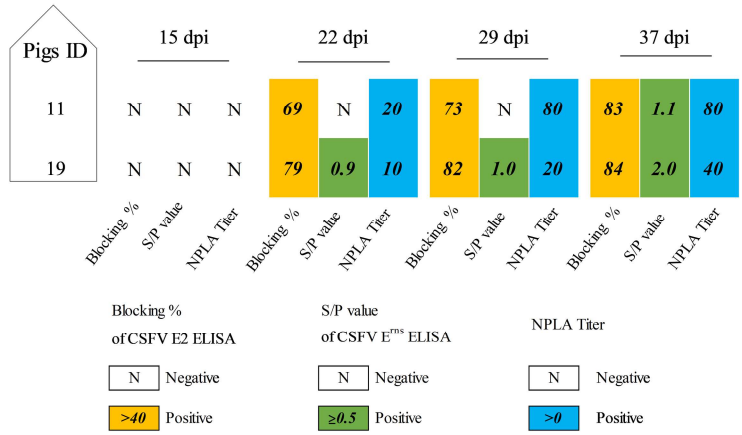


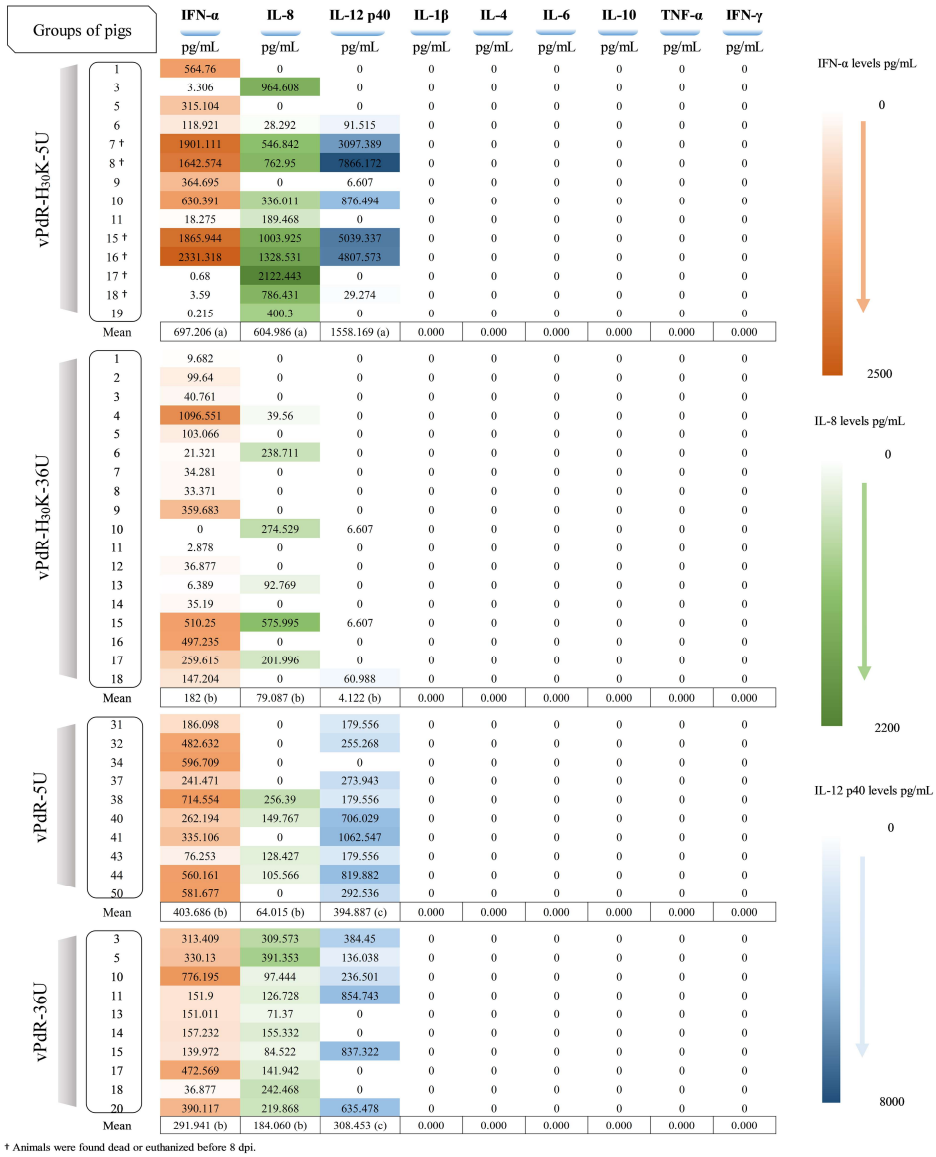
Fig 5. Evaluation of the humoral immune response in piglets after vPdR-H_{30}K-5U infection. The E2 and E^{rns}-specific antibody responses were measured by specific ELISAs and shown as a percentage of blocking or as S/P ratios, respectively. The neutralizing antibody titers were determined by the NPLA at weekly intervals. The cut-off values for positive samples are indicated for the respective test.

5.4.6 The vPdR-H_{30}K-5U double-mutant induced stronger IFN-α, IL-8, and IL-12 than its parent viruses.

To assess the role of the RNase activity and the 3´UTR poly-U insertion in immune activation, the cytokine profile of 14 serum samples from 5-day-old piglets infected with the vPdR-H_{30}K-5U double-mutant were evaluated using the Luminex system. Twelve samples were from animals that died or developed a severe disease, collected from 5 to 8 dpi and the other two samples were from the surviving pigs at 8 dpi. These sera were compared with samples from age-matched piglets infected with cDNA-derived wild-type or mutant PdR virus from previous studies.

Remarkably, the highest IFN-α levels were detected in the serum samples of animals infected with vPdR-H_{30}K-5U that were found dead or had to be euthanized from 5 to 11 dpi (Fig 6), with IFN-α concentrations ranging from 315 to 2331 pg/ml. These values were significantly higher than in the

other three groups ($p \leq 0.001$). For IL-8, the serum concentrations were also significantly higher in the piglets infected with the vPdR-H_{30}K-5U compared with the other groups ($p \leq 0.003$), with values between 28.292 and 2122.44 pg/ml. Notably, the four piglets with the highest serum IFN-α levels after infection with vPdR-H_{30}K-5U (numbers 7, 8, 15 and 16) had also IL-12 levels exceeding 3000 pg/mL. This contrasted with the absence of any IL-12 response in the vPdR-H_{30}K-36U group (three background signals, $p \leq 0.001$) (Fig 6). Interestingly, the two piglets that survived the vPdR-H_{30}K-5U infection showed background levels of IFN-α and IL-12 and low IL-8 levels. Of note is the complete absence of any detectable TNFα, IL-1β, IL-4, IL-6, IL-10 and IFN-γ signal in any of the serum samples analyzed, which was validated with the positive controls.



Groups of pigs		IFN-α pg/mL	IL-8 pg/mL	IL-12 p40 pg/mL	IL-1β pg/mL	IL-4 pg/mL	IL-6 pg/mL	IL-10 pg/mL	TNF-α pg/mL	IFN-γ pg/mL
vPdR-H30K-5U	1	564.76	0	0	0	0	0	0	0	0
	3	3.506	964.608	0	0	0	0	0	0	0
	5	315.104	0	0	0	0	0	0	0	0
	6	118.921	28.292	91.515	0	0	0	0	0	0
	7 †	1901.111	546.842	3097.389	0	0	0	0	0	0
	8 †	1642.574	762.95	7866.172	0	0	0	0	0	0
	9	364.695	0	6.607	0	0	0	0	0	0
	10	630.391	336.011	876.494	0	0	0	0	0	0
	11	18.275	189.468	0	0	0	0	0	0	0
	15 †	1865.944	1003.925	5039.337	0	0	0	0	0	0
	16 †	2331.318	1328.531	4807.573	0	0	0	0	0	0
	17 †	0.68	2122.443	0	0	0	0	0	0	0
	18 †	3.59	786.431	29.274	0	0	0	0	0	0
	19	0.215	400.3	0	0	0	0	0	0	0
	Mean	697.206 (a)	604.986 (a)	1558.169 (a)	0.000	0.000	0.000	0.000	0.000	0.000
vPdR-H30K-36U	1	9.682	0	0	0	0	0	0	0	0
	2	99.64	0	0	0	0	0	0	0	0
	3	40.761	0	0	0	0	0	0	0	0
	4	1096.551	39.56	0	0	0	0	0	0	0
	5	103.066	0	0	0	0	0	0	0	0
	6	21.321	238.711	0	0	0	0	0	0	0
	7	34.281	0	0	0	0	0	0	0	0
	8	33.371	0	0	0	0	0	0	0	0
	9	359.683	0	0	0	0	0	0	0	0
	10	0	274.529	6.607	0	0	0	0	0	0
	11	2.878	0	0	0	0	0	0	0	0
	12	36.877	0	0	0	0	0	0	0	0
	13	6.389	92.769	0	0	0	0	0	0	0
	14	35.19	0	0	0	0	0	0	0	0
	15	510.25	575.995	6.607	0	0	0	0	0	0
	16	497.235	0	0	0	0	0	0	0	0
	17	259.615	201.996	0	0	0	0	0	0	0
	18	147.204	0	60.988	0	0	0	0	0	0
	Mean	182 (b)	79.087 (b)	4.122 (b)	0.000	0.000	0.000	0.000	0.000	0.000
vPdR-5U	31	186.098	0	179.556	0	0	0	0	0	0
	32	482.632	0	255.268	0	0	0	0	0	0
	34	596.709	0	0	0	0	0	0	0	0
	37	241.471	0	273.943	0	0	0	0	0	0
	38	714.554	256.39	179.556	0	0	0	0	0	0
	40	262.194	149.767	706.029	0	0	0	0	0	0
	41	335.106	0	1062.547	0	0	0	0	0	0
	43	76.253	128.427	179.556	0	0	0	0	0	0
	44	560.161	105.566	819.882	0	0	0	0	0	0
	50	581.677	0	292.536	0	0	0	0	0	0
	Mean	403.686 (b)	64.015 (b)	394.887 (c)	0.000	0.000	0.000	0.000	0.000	0.000
vPdR-36U	3	313.409	309.573	384.45	0	0	0	0	0	0
	5	330.13	391.353	136.038	0	0	0	0	0	0
	10	776.195	97.444	236.501	0	0	0	0	0	0
	11	151.9	126.728	854.743	0	0	0	0	0	0
	13	151.011	71.37	0	0	0	0	0	0	0
	14	157.232	155.332	0	0	0	0	0	0	0
	15	139.972	84.522	837.322	0	0	0	0	0	0
	17	472.569	141.942	0	0	0	0	0	0	0
	18	36.877	242.468	0	0	0	0	0	0	0
	20	390.117	219.868	635.478	0	0	0	0	0	0
	Mean	291.941 (b)	184.060 (b)	308.453 (c)	0.000	0.000	0.000	0.000	0.000	0.000

† Animals were found dead or euthanized before 8 dpi.

Fig 6. Cytokine levels in serum samples from vPdR-36U-, vPdR-5U-, vPdR-H_{30}K-36U- and vPdR-H_{30}K-5U-infected piglets between day 5 and 8 post infection. Cytokine concentrations were expressed as pg/ml, calculated according to a standard curve. IFN-α, IL-8 and IL-12 levels (pg/mL) from low to high were represented on a scale from light to deep orange, green and blue, respectively. The mean value was determined for each cytokine per group. For each cytokine, the results from statistical comparison of the means between the groups is shown with letters in brackets, with different letters meaning

significant differences between the groups (P≤0.003) while same letters show that there is no significant difference.

5.4.7 In 3-week-old pigs, the double-mutant vPdR-H$_{30}$K-5U induced a milder disease with lower lethality than in 5-day-old piglets.

We repeated the experiments by infecting 3-week-old weaned pigs with vPdR-H$_{30}$K-5U, to clarify whether the phenotypes observed above related to RNase-inactive E^{rns} in absence of the poly-U sequence in the 3'UTR of PdR are restricted to newborn piglets. The pigs were monitored during 21 days after infection and developed disease starting with apathy from day 4 after infection onwards. The clinical picture worsened for two of the animals that had to be euthanized on day 7 (pigs number 22 and 24) after they had constantly elevated body temperature and developed severe apathy, dehydration, and prostration (Fig 7). The remaining pigs showed reduced liveliness and suffered from conjunctivitis, diarrhea and weight loss but recovered eventually from 10 to 12 dpi until the end of the trial (Fig 7). This corresponds to a 33% lethality. This is significantly different when compared to the 89.5% lethality of the same virus in newborn piglets.

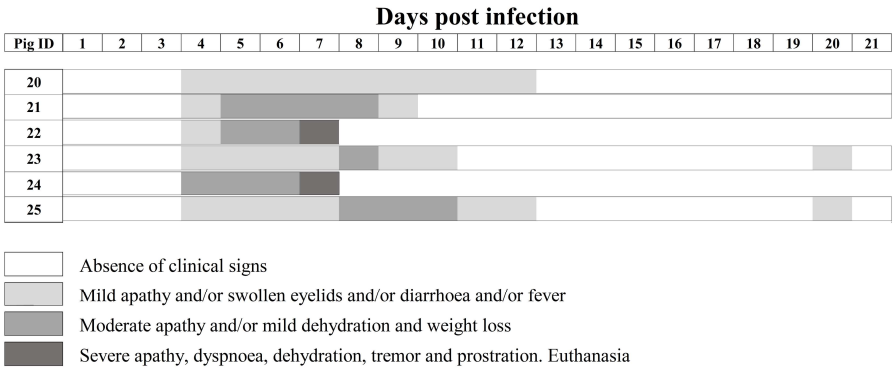


Fig 7. Individual clinical signs observed in pigs infected at the age of three weeks with vPdR-H$_{30}$K-5U. Pigs 20 to 25 were inoculated intranasally with vPdR-H$_{30}$K-5U, and the

clinical signs were monitored daily through the whole trial. Different shades of gray represent the severity of the clinical signs according to the legend.

5.4.8 In 3-week-old pigs, the vPdR-H$_{30}$K-5U mutant resulted in high virus load in tonsils with limited viremia and secretion as in 5-day-old piglets.

As for the piglets, we analyzed the course of viremia, nasal and rectal virus secretion and the viral RNA in the tonsils of the 3-week-old pigs (Fig 8). On day 5 after infection, two out of the six pigs were positive for CSFV RNA in serum with Ct values of 34.71 and 36.31, suggesting a low viral load (pigs number 20 and 22). On this same day, three of the four pigs (number 21, 23 and 25) that had undetectable viremia were positive in the nasal swabs only, while the last pig was still completely negative in all samples. At 7 dpi, the two pigs that had to be euthanized were also positive in sera and swabs. Subsequently, among the remaining 4 pigs that survived, only one animal tested weakly positive for CSFV in the serum on day 11, while all remaining pigs were negative in the serum and swab samples until the end of the study (Fig 8).

In the tonsils, the two pigs that had to be euthanized 7dpi showed moderate to high viral RNA load (Fig 8). The four remaining animals showed low to moderate CSFV RNA loads in the tonsils at the end of the study 21 dpi. In addition, as in the previous experiment with the newborn piglets, sequence analyses of the virus recovered from the tonsils confirmed the integrity of the 3'UTR sequence and the RNase knockout sequence in E^{rns} (data not shown).

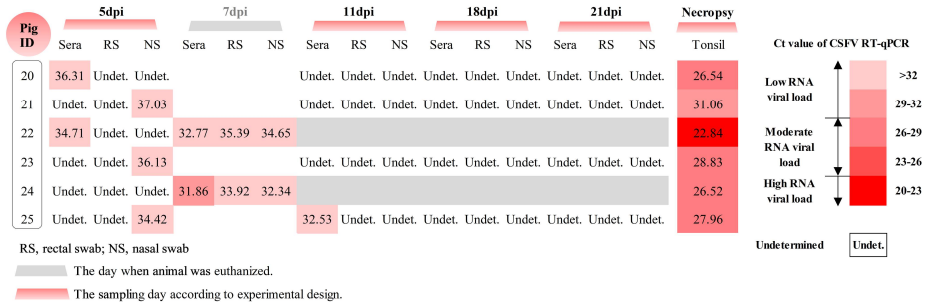
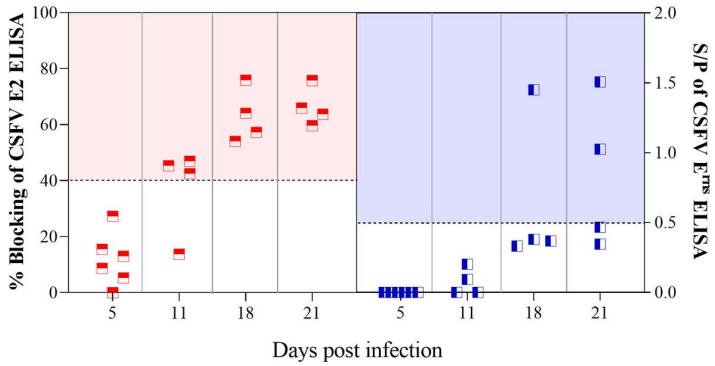


Fig 8. Determination of CSFV RNA in sera, swabs and tonsils from pigs infected at the age of three weeks with vPdR-H_{30}K-5U. CSFV RNA was determined by RT-qPCR in the sera and swabs at different sampling time points, and from the tonsil samples obtained at necropsy.

5.4.9 The 3-week-old pigs that survived the vPdR-H_{30}K-5U infection seroconverted from day 11 post infection.

CSFV-specific antibodies against the E2 glycoprotein were detected on day 11 after infection in three out of the four pigs that survived the vPdR-H_{30}K-5U infection (Fig 9a). At 18 dpi, the four pigs had seroconverted. Meanwhile, the anti-E^{rns} antibody response could not be detected on day 11, and only one pig seroconverted against E^{rns} at 18 dpi, while a second pig became positive on day 21 (Fig 9a). Notably, neutralizing antibody responses were detected in the surviving animals mainly after 18 dpi. However, the neutralizing titers never reached values over 1:20 (Fig 9b). As expected, the two animals that were euthanized at 7 dpi (pigs 22 and 24) were negative for CSFV antibody responses (data not shown).

(a)



(b)

NPLA titers

Pigs ID	5 dpi	11 dpi	18 dpi	21 dpi
20	0	0	***10***	0
21	0	***10***	***20***	***20***
22*	0			
23	0	0	***10***	***20***
24*	0			
25	0	0	***20***	***20***

* Pigs were euthanized at 7 dpi

(c)

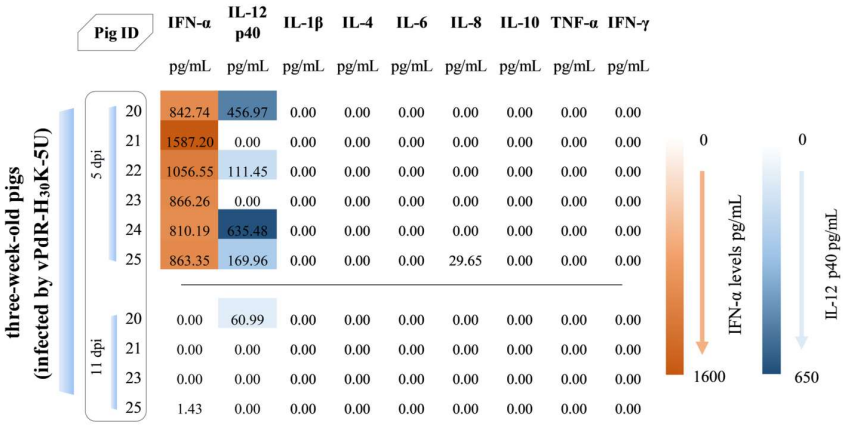


Fig 9. Evaluation of the humoral and innate immune responses in pigs infected at the age of three weeks with vPdR-H_{30}K-5U. (a) Anti E2- and E^{rns}-specific antibody responses are shown as percentage of blocking (left Y-axis, red squares) and S/P retios (right Y-axis, blue squares) respectively, as measured by specific ELISAs. For E2 antibodies, values between 30 and 40% of blocking were considered doubtful, and values equal or greater than 40% were considered positive (red shaded area in left-side panel). For E^{rns} antibodies, S/P ratios between 0.3 and 0.5 were considered doubtful while S/P ratios equal or greater than 0.5 were considered as positive (blue shaded area in right-side panel). (b) Neutralizing antibody titers determined by the NPLA. (c) Cytokine levels in serum samples at 5 and 11 dpi. IFN-α, and IL-12 levels from low to high are represented on a scale from light to deep orange, and blue, respectively.

5.4.10 In 3-week-old pigs, the vPdR-H_{30}K-5U double-mutant induced drastic elevation of IFN-α and IL-12, without IL-8 induction.

The cytokine profile on day 5 after infection of the 3-week-old pigs with the vPdR-H_{30}K-5U double-mutant was like the profile observed 5 to 8 dpi in 5-day-old piglets, except for IL-8 that remained mostly undetectable in the older pigs. All six pigs showed high concentrations of serum IFN-α at 5 dpi, with values ranging from 810.19 to 1587.20 pg/mL. Interestingly, a steep decrease in the IFN-α levels was registered at 11 dpi, and all the serum samples became negative in the surviving pigs (Fig 9c). For IL-12, the concentrations were lower than in newborns, with values between 111.45 and 635.48 pg/mL detected in four out of six pigs at 5 dpi (Fig 9c). Like the IFN-α, IL-12 decreased at 11 dpi, being undetectable in three out of the four surviving animals (Fig 9c). As for the newborn piglets, TNFα, IL-1β, IL-4, IL-6, IL-10, and IFN-γ remained undetectable at the times analyzed (Fig 9c).

5.5 Discussion

The present study shows for the first time, a synergistic effect of the E^{rns} RNase activity and the long 3´UTR poly-U sequence of the CSFV PdR strain on attenuation, since the lack of these two features resulted in enhancement of virulence and CSF severity. The infection with the vPdR-H_{30}K-5U double-mutant induced an acute disease with a high lethality in 5-day-old piglets as opposed to the parent low virulence PdR strain characterized in previous studies. The low virulence of PdR was observed with wild-type parent virus in the field and demonstrated with clone-derived virus (vPdR-36U) (Coronado et al., 2017; Pérez et al., 2012; M. Wang et al., 2020a). Of note, deletion of the long 3´UTR poly-U sequence alone (vPdR-5U) partially enhanced virulence while abrogation of the E^{rns} RNase activity alone (vPdR-H_{30}K-36U) reduced virulence and *in vivo* replication (M. Wang et al., 2021, 2020a). Despite these differences, similar replication kinetics were observed in MDM infected with these four viruses (see also ref (M. Wang et al., 2021; Wang et al., 2020)), which altogether points on relevant virus-host interactions during CSFV replication *in vivo* that are not reflected in cell culture.

A high lethality of up to 89.5% was observed in the vPdR-H_{30}K-5U-infected 5-day-old piglets between 5 and 11 dpi. Likewise, the clinical signs recorded in the infected piglets were typical for the acute form of CSF, with rapid onset of disease and a lethal outcome within a few days (Ganges et al., 2020). In 3-week-old pigs, clinical signs of CSF were observed earlier (4 dpi) but were overall milder. Nevertheless, two of six pigs were euthanized for animal welfare reasons at 7 dpi. The rest of the infected animals (66%) recovered. These results support (i) age-dependent difference in CSFV susceptibility and disease severity (Bohórquez et al., 2019; Muñoz-González et al., 2015; Nielsen et al., 2010) and (ii) the role

of the E^{rns} RNase activity and the 3'UTR in modulating the CSFV pathogenesis in pigs (M. Wang et al., 2021; Wang et al., 2020).

We observed a synergistic effect of knocking out the E^{rns} RNase and deleting the long 3'UTR poly-U sequence in the PdR strain on CSF exacerbation and on increased CSFV replication in the tonsils, independently of the age of the pigs. The epithelial cells of the tonsillar crypts are described as primary sites of CSFV replication, preceding the viremic phase (Ganges et al., 2020). For Pestiviruses and Flaviviruses, the 3'UTR is a critical element influencing viral RNA replication (Brinton and Miller, 2015; Kellman et al., 2018; Pankraz et al., 2005). It is likely that the loss of the long poly-U in the 3'UTR improves the capacity of the virus to replicate in the target cells of the tonsils (Wang et al., 2020). Contrasting with high replication in the tonsils, vPdR-H_{30}K-5U infection resulted in mild viremia and low virus secretion. This may be a consequence of immune activation due to the lack of the E^{rns} RNase activity in the vPdR-H_{30}K-5U mutant (M. Wang et al., 2021), favoring IFN-α responses at the systemic level, in the tissues where virus replicates before viremic stage (Summerfield et al., 2006). This disproportional activation of innate immunity might limit systemic viral spread from the tonsils and increase virus clearance, and at the same time induce strong cytokine-mediated symptoms. In addition, an IFN-α over expression may induce severe lymphopenia and lead to hematologic disruption (Summerfield et al., 2006; Summerfield and Ruggli, 2015), which could also increase the CSF severity.

The double-mutant vPdR-H_{30}K-5U resulted in significantly higher IFN-α induction in piglets than the single mutants or the parent vPdR-36U characterized previously (Fig 6). High levels of systemic IFN-α were

observed repeatedly in pigs infected with high virulence CSFV (Ruggli et al., 2009; Tarradas et al., 2014), which appears to be a hallmark of acute sever CSF (Ganges et al., 2020; Summerfield et al., 2006; Summerfield and Ruggli, 2015). Contrasting with the differences in IFN-α induction by vPdR-H_{30}K-5U and vPdR-H_{30}K-36U *in vivo* (Fig 6), similar levels of IFN-α were found in infected pDCs (Fig 2). This confirms the role of CSFV E^{rns} in the inhibition of IFN-α induction *in vivo* (M. Wang et al., 2021) and in pDC cultures (Python et al., 2013), and suggests a contribution of the 3'UTR to innate immune activation *in vivo* but not in MDM-pDC co-cultures. Of note, none of the mutations in PdR affected replication in MDM (Fig 1a). Thus, the differences in IFN-α induction observed *in vivo* with vPdR-H_{30}K-5U and vPdR-H_{30}K-36U are more complex than simply a consequence to the basic replicative capacity of the virus.

Besides IFN-α, IL-12 was also drastically elevated in pigs that died or were euthanized after the vPdR-H_{30}K-5U infection, both in newborn and older pigs. Interestingly, IL-8 was elevated in the infected newborn piglets only (Fig 6 *versus* 9c). These results, together with the fact that IL-1β, IL-4, IL-6, IL-10; TNF-α and IFN-γ could not be detected at the systemic level after vPdR-H_{30}K-5U infection suggest that IFN-α, IL-12 and to some extent IL-8 are the major cytokines contributing to CSF pathogenesis. A cytokine storm has been associated previously with lethal forms of CSF due to high virulence CSFV, but this phenomenon has not been extensively studied (Ganges et al., 2020). It should be noted that high levels of IL-8 and IL-12 were described in humans with a fatal outcome of influenza A (H5N1) or with a severe SARS-CoV-2 infection (Costela-Ruiz et al., 2020; De Jong et al., 2006; Guan et al., 2020; Kesmez Can et al., 2021). Likewise, high levels of IL-8 have been also detected in pigs suffering from severe African swine fever (S. Wang et al., 2021).

Neutralizing antibodies play and essential role in protection against CSFV infection (Ganges et al., 2020). The surviving animals of both ages developed specific anti-E2 and -E^{rns} antibodies after infection with vPdR-H_{30}K-5U. However, the neutralizing antibody titers were low, especially in the pigs inoculated at the age of three weeks, in which the neutralizing antibody titers were even lower than the threshold established for protection against CSFV infection (Terpstra et al., 1984). This may be related to the high levels of virus detected in the tonsils, suggesting reduced virus clearance with a possible chronic outcome (Rout and Saikumar, 2012). The high amount of IFN-α levels detected in these animals at 5 dpi may also explain the low antibody responses due to the B cell toxicity (McNab et al., 2015). Thus, while the abrogation of the E^{rns} RNase activity in the vPdR-36U backbone enhanced the antibody responses (M. Wang et al., 2021), additional removal of the long poly-U sequence of the 3'UTR resulted in the opposite effect with reduced adaptive immune response in terms of neutralizing antibodies.

Taken together, the present study highlights a cooperative effect the 3' UTR poly-U insertion and the E^{rns} RNase activity in controlling the host's immune response to the virus and thus contributing to virus attenuation. In addition, these results provide new insights for CSFV vaccine development.

5.6 Acknowledgments

This research was supported by Ministerio de Ciencia, Innovación y Universidades from the Spanish government under the grant RTI2018-100887-B-I00. M.W. has a pre-doctoral fellowship CSC scholarship (2017) from the Chinese government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from

Spanish government. IRTA is supported by CERCA Programme / Generalitat de Catalunya. The authors thank Ivan Cordón and the CReSA animal facility staff for their help during the *in vivo* part of the experiment.

5.7 Declaration of Interest Statement

The authors declare no competing interests.

Part III

General discussion and conclusions

Chapter 6

General discussion

Despite the great efforts made so far in diagnosis, research and control strategies, CSFV continues to circulate in certain areas of the world (Ganges et al., 2020; Wei et al., 2021). The prevalence of low and moderate virulence CSFV strains under the vaccination programs could induce unapparent disease forms and facilitate the virus persistence in the field, making CSFV eradication particularly difficult (Belák et al., 2008; Coronado et al., 2019a; Y Luo et al., 2017). Therefore, the study of CSFV molecular determinants in virulence is highly relevant for the development of solid diagnostic tools and vaccines to ensure effective disease control in endemic areas.

The study of genetic basis for CSFV virulence has been approached from different perspectives, such as the attenuation through serial passages in non-natural hosts (Wu et al., 2017) or the gain of pathogenicity by passaging the low virulence CSFV in pigs (Tamura et al., 2012). Moreover, the role of quasispecies (Töpfer et al., 2013), loss- or gain-of-function via selected individual viral proteins from certain CSFV strains (Fernandez-Sainz et al., 2009; Ruggli et al., 2009; Tews et al., 2009; Vuono et al., 2021), among others, have also been evaluated (Ganges et al., 2020; Leifer et al., 2013). Despite its potential for the development of control strategies, the virulence factors that underlie the low virulence strains isolated from endemic areas remains poorly studied.

Recently, the low virulence CSFV PdR strain was isolated from an endemic region under vaccination program. Previous studies have demonstrated that this strain could induce chronic and persistent infection in pigs (Muñoz-González et al., 2015b; Coronado et al., 2017). Notably, a poly-U insertion of an average length of 36 nucleotides in the 3'UTR of the PdR strain was discovered, being unique among all CSFV genomes

(Coronado et al., 2017). Interestingly, the likely parental strain of PdR isolate, the high virulence Margarita strain (Pérez et al., 2012), holds 5 uridines in the same position.

Considering this background, the role of the poly-U insertion of the PdR strain in CSFV attenuation and disease pathogenesis has been investigated in the study I of the present thesis. To achieve this objective, the reverse genetic approach was used to construct the infectious clones based on the low virulence CSFV PdR isolate backbone. Two infectious clones vPdR-36U and the vPdR-5U, harbouring 36- or 5-uridines in the 3'UTR, were used to infect 20 piglets, respectively, and ten piglets were added per each group as a contact.

The clinical picture of the piglets after infection with vPdR-36U was consistent with the wild-type PdR strain, that induces mild and unapparent CSF manifestations (Muñoz-González et al., 2015b). By contrast, the infection with the vPdR-5U caused more severe and specific CSF clinical manifestations in piglets, similar with the clinical picture developed after infection with high virulence CSFV strains, such as the CSFV Margarita strain (Blome et al., 2017b; Tarradas et al., 2014). The reduced clinical signs associated with the poly-U insertion in the 3'UTR of the PdR strain correlated with the lower replication capacity showed by the vPdR-36U in the sera and swab samples recovered in the infected piglets. According with previous studies that identified a critical role of the 3'UTR in pestiviruses and flaviviruses replication (Brinton and Miller, 2015; de Borba et al., 2019; Kellman et al., 2018; Pankraz et al., 2005), this result (in Study I), confirms the relevance of the 3'UTR in CSFV replication *in vivo*. In addition, it demonstrates for the first time that a poly-U insertion acquired naturally in the 3'UTR of CSFV can reduce replication and

virulence in young piglets. The predicted RNA secondary structure of CSFV 3'UTR includes four consecutive stem-loop (SL) structures (Fan et al., 2008; Huang et al., 2012). In this regard, the poly-U insertion in PdR strain leads to a long intervening sequence between SL-I and SL-II (Coronado et al., 2017). This intervening sequence has been shown to be essential for Pestivirus replication (Pankraz et al., 2005).Taking that into account, , the change in the RNA secondary structure due to the poly-U insertion may interfere with the normal function of the 3'UTR, conferring the reduction of the viral replication capacity.

Interestingly, study I also showed that the poly-U insertion did not affect the transmission capacity of PdR strain by the natural route. The contact groups showed similar clinical pictures, replication rates, and antibody responses to their respective inoculated group. Notably, the 36-uridine sequence of vPdR-36U was not completely stable *in vivo* according to the sequence analysis of the virus recovered from serum at 3 weeks post infection. Nevertheless, there was no selection of a specific variant (36 or 5 uridines) during natural virus transmission to contact animals according to the similar sequence profiles between the inoculated animals and their respective contact groups.

The type I IFNs, like IFN-α, constitute the first line of innate host defence against virus infection (Summerfield and Ruggli, 2015). In the present study, the vPdR-5U induced the highest IFN-α levels in the infected pigs, though only during a short period of time, being similar to the animals infected with high virulence CSFV, like the Margarita strain (Tarradas et al., 2014). According to previous studies, transient and elevated IFN-α systemic levels has been related with CSF severity after infection with highly virulent strains (Ganges et al., 2020; Muñoz-González et al., 2015b).

Interestingly, the pigs infected with the vPdR-36U generated more consistent and prolonged serum IFN-α levels during the first two weeks post infection, pointing out a potential role of poly-U insertion for the activation of the innate immune response. Notably, this was supported by previous studies showing that the 3'UTR poly-U tract in the hepatitis C virus, belonging to the *Flaviviridae* family, could activate the innate immune response via RIG-I signalling or the TLR7 (Schnell et al., 2012; Zhang et al., 2009). This may be one reasons for the lower replication of vPdR-36U versus vPdR-5U in infected piglets, contrasting with the similar replication kinetics of the two viruses in cell culture (Figure 1, Study I), in which CSFV prevents efficiently type I IFN induction by means of N^{pro}-mediated IRF3 degradation (Bauhofer et al., 2007).

In line with a more sustained innate immune activation, the vPdR-36U also induced strong adaptive immunity. This contrasted with the antibody response generated by the vPdR-5U, which did not induce seroconversion during the 4 weeks the piglets survived. The CSFV-specific humoral immune response was particularly high in animals showing a milder disease and lower viral load. Thus, study I showed the important role of the 3'UTR poly-U insertion in the PdR virus to activate the host immunity, reduce viral replication and disease severity in pigs.

It is worth highlighting that the E^{rns} glycoprotein, an exclusive protein of pestiviruses, has a unusual RNase activity which has not been found in any other viral envelope protein so far (Schneider et al., 1993). This function of E^{rns} is not only required to block the type I IFN induction (Magkouras et al., 2008; Mätzener et al., 2009; Python et al., 2013) but also played an important role for pestiviruses virulence *in vivo*. Previous studies showed that abrogation of the E^{rns} RNase activity in highly virulent pestiviruses

including CSFV could reduce virulence in natural host (Meyer et al., 2002; Meyers et al., 1999). In addition, the RNase function has also been associated with the establishment of BVDV persistent infections (Meyers et al., 2007). However, the role of the E^{rns} RNase activity in the low virulence CSFV strains in pathogenesis, and its relationship with the viral ability to generate chronic and persistent infections remains unknown.

To better understand the natural attenuation mechanism and considering the relevance of the E^{rns} RNase function in pestiviruses, the second objective of this thesis was focused on the study of the role of the E^{rns} RNase activity in a low virulence CSFV strain in viral pathogenicity, persistence, and transmission in pigs. To this end, the E^{rns} RNase inactivated clone vPdR-H_{30}K-36U was constructed based on the vPdR-36U backbone, previously generated in the study I by reverse genetic approach (M. Wang et al., 2020a). In line with previous studies, the RNase activity of the PdR strain was abolished by substitution of the histidine at amino acid position 30 of E^{rns} (Schneider et al., 1993; Meyers et al., 1999). Therefore, this study supports that the substitution or deletion of histidine residues within the catalytic domain of the E^{rns}, abolishing the RNase activity. In addition, following the methodology described in study I, a similar experimental design was carried out. Eighteen piglets were infected with the vPdR-H_{30}K-36U and other 9 piglets were included as a contact group.

Despite showing similar replication rates *in vitro*, the vPdR-H_{30}K-36U showed reduced or undetectable viral replication in pigs when compared with the infection generated *in vivo* with the vPdR-36U (Study I). The low replication capacity was directly correlated with the very mild or even absence of clinical signs observed in the vPdR-H_{30}K-36U infected piglets.

This demonstrated a strong attenuating effect of the RNase inactivation in the low virulence CSFV PdR isolate *in vivo*. This result was in agreement with the attenuating role of the E^{rns} RNase inactivation in high virulence strains of CSFV or BVDV (Meyer et al., 2002; Meyers et al., 1999). Likewise, reduced transmission capacity of the vPdR-H_{30}K-36U to contact piglets was also detected, being consistent with the decreased viral load detected in the nasal and rectal swabs from the infected animals. Notably, only two contact piglets were positive for the CSFV detection, however, a very low RNA viral load was found in their tonsil sample. The infection carried out with the vPdR-36U in study I also showed that no CSFV could be isolated from samples detected by RT-qPCR with Ct values above 32.2 (M. Wang et al., 2020a). In this regard, two vPdR-H_{30}K-36U contact piglets were detected CSFV positive, which was likely caused by infection via fomites rather than by pig-to-pig transmission, considering that only traces of viral RNA were detectable from the swab samples of infected piglets with Ct values above 30.

Previous studies showed that either virulent CSFV or vaccine strains had a high capacity to persist in the tonsils of pigs, the primary target of the virus, for over 30 days (Kaden et al., 2004). Thus, the lack of the E^{rns} RNase activity may result in a very low levels or absence of CSFV RNA detection in the tonsil, suggesting a reduced capacity of the vPdR-H_{30}K-36U to persist in the infected host. It should be noted here that the wild-type CSFV PdR isolate, with functional E^{rns} RNase activity was capable of generating persistently infected piglets, with absence of seroconversion as a hallmark (Muñoz-González et al., 2015b). By contrast, the persistent infection did not occur with the RNase-negative single mutant vPdR-H_{30}K-36U under the same conditions. Hence, in addition to the important role of E^{rns} RNase activity played for the establishment of BVDV

persistent infection in bovine fetuses (Meyers et al., 2007), this result proves for the first time that functional E^{rns} RNase can help a low virulence CSFV to maintain persistent infection in piglets.

The E^{rns} RNase-negative PdR mutant also showed a high capacity to activate the IFN-α production in pDC *in vitro* (Figure 3, study II) (Python et al., 2013). Interestingly, a clear activation of the IFN-α response in the vPdR-H_{30}K-36U infected pigs was also detected. However, the serum IFN-α levels were lower when compared with the those registered in pigs' sera after the infection with the parental vPdR-36U virus (M. Wang et al., 2020a). Thus, the activation of the IFN-α response in the infected pigs showed an inverse correlation with respect to the response observed in cell cultures, which could be explained by the different replication levels achieved in each system. The viral RNA-mediated trigger of IFN-α induction in the infected animals may be lower with the vPdR-H_{30}K-36U due to the significantly lower replication than that reported previously for the parental vPdR-36U (Study I), which may not be fully compensated by the lack of the IFN antagonistic function of E^{rns} in pDC infected with the mutant (Rojas et al., 2020). This finding supports the role of CSFV E^{rns} RNase activity to modulate the innate immunity activation in pigs.

On the other hand, the E^{rns} RNase-negative PdR mutant showed the capability to enhance the adaptive immune response in pigs, as the vPdR-H_{30}K-36U infected pigs generated high antibody levels against the E2 and the E^{rns} glycoproteins, as well as an efficient neutralizing antibody response. It was shown that CSFV-infected pigs can develop anti-E^{rns} antibodies as early as 10 dpi (Meyer et al., 2017), this has raised interest in E^{rns} as a potential DIVA diagnostic target. Notably, the vPdR-36U, failed to induce detectable specific anti-E^{rns} response in piglets during five

weeks of infection. In this regard, the infection with vPdR-H_{30}K-36U enhanced E^{rns}-specific antibody response in pigs, suggesting a better E^{rns} protein immunogenicity following the loss of the RNase activity. Therefore, the difficulties in the development of DIVA diagnosis based on seroconversion against E^{rns} might be due to the negative effect on E^{rns}-specific immunity by the RNase activity (Pannhorst et al., 2015; Postel and Becher, 2020). Further studies will be carried out to clarify the role of the E^{rns} RNase function in CSFV strains with different virulence on the host immunity regulation. These results showed for the first time the important role of E^{rns} RNase inactivation in a low virulence CSFV PdR strain in viral virulence reduction and immune response activation and the suppression of viral persistence in pigs.

Considering the results from studies I and II, the relevant role of the 3'UTR poly-U insertion and the E^{rns} RNase activity for CSFV replication, pathogenesis and immunity modulation was demonstrated. Taking this into account, study III was focused on the study of the synergistic effect of lacking both, the E^{rns} RNase activity and the 3'UTR poly-U insertion in the low virulence CSFV PdR strain. In addition, the levels of innate and adaptive immunity and its relationship with the CSF pathogenesis in pigs were also evaluated. To this end, the double mutant vPdR-H_{30}K-5U was constructed based on the vPdR-5U backbone used in study I (M. Wang et al., 2020a). Nineteen 5-day-old piglets from the same origin that the pigs used in the study I and II were infected with the vPdR-H_{30}K-5U. Bear in mind that CSFV persistent infection could be also generated in pigs at three weeks-old (Bohórquez et al., 2019), an extra group of six pig of this age were also infected with the vPdR-H_{30}K-5U double mutant.

It is worth noting that the infection with the vPdR-H_{30}K-5U in 5-day-old piglets resulted in high mortality, nearly 90% before 11 dpi. The clinical signs observed in these infected animals were comparable with the CSF acute form with the onset of rapid and high mortality rate a few days after infection, sometimes accompanied by scarce clinical signs (Ganges et al., 2020). Moreover, severe to moderate CSF manifestations with a 33% of mortality were recorded in the 3-week-old infected pigs in the first week after infection. Most of these infected animals were recovered at the end of the study (21 dpi), showing greater resistance to the virus than the 5-day-old piglets. In this regard, these results point out the relevant role of the host age as a predisposing factor in CSFV susceptibility (Bohórquez et al., 2019; Muñoz-González et al., 2015b; Nielsen et al., 2010). Likewise, they also suggest the relevance of the E^{rns} RNase activity and the 3'UTR in modulating the CSFV pathogenesis in pigs (M. Wang et al., 2021, 2020a).

In addition to the clinical severity, the double mutant also increased the CSFV replication capacity in the tonsils of the two experimental groups. Considering that the 3'UTR from CSFV is a critical factor to trigger viral replication (Study I), it is likely that the loss of the long poly-U in the 3'UTR could improve the CSFV ability to replicate first in the target tonsil cells (M. Wang et al., 2020a). This is in line with the CSFV pathogenesis, in which the virus infects primarily the epithelial cells of tonsillar crypts regardless of the entry route, before the viremia phase (Ganges et al., 2020). However, the vPdR-H_{30}K-5U infected animals showed mild viremia and reduced virus excretion. A possible explanation can be that the loss of E^{rns} RNase function in the vPdR-H_{30}K-5U could activate the innate immune system of the host, in terms of the IFN-α response, according with the study II (M. Wang et al., 2021). This disproportional activation of the

innate immunity, might limit viral spread from the tonsil target cells, being able to clear the virus in sera, preventing the viremia phase, although with drastic repercussions for the host. This may be supported by the fact that high IFN-α secretion could occur in the tissues with more virus replication levels, before the viremia stage (Summerfield et al., 2006).

High levels of serum IFN-α are a hallmark of the acute disease phase induced by virulent CSFV (Tarradas et al., 2014). In this sense, the double mutant infection in both experimental groups induced drastically higher levels of sera IFN-α than those detected with previously characterized infectious clones, supporting the implication that IFN-α plays in the CSF severity and progression (Ganges et al., 2020; Summerfield and Ruggli, 2015). However, despite the differences generated *in vivo*, similar levels of IFN-α in infected pDCs were found in both the E^{rns} RNase inactivated vial mutants (vPdR-H_{30}K-5U or vPdR-H_{30}K-36U) which may be due to the different replication capacity presented in each system (M. Wang et al., 2021).

Besides the dramatic elevation of IFN-α response, a drastic increase of IL-8 and IL-12 levels in sera was detected from pigs that were found dead or were euthanized in two experimental groups. It should be noted that high levels of IL-8 and IL-12 have been associated with a fatal outcome of influenza A (H5N1) or with a severe SARS-CoV-2 infection in humans (Costela-Ruiz et al., 2020; De Jong et al., 2006; Guan et al., 2020; Kesmez Can et al., 2021). Likewise, high levels of IL-8 have been also reported in pigs suffering cytokine storm during the severe ASF disease phase (Wang et al., 2021). In addition, the present study also showed the complete absence of IL-1β, IL-4, IL-6, IL-10, TNF-α and IFN-γ at the systemic level in pigs after vPdR-H_{30}K-5U infection. In this regard, study III

demonstrates for the first time the role played by the IFN-α, the IL-8, and the IL-12 in the cytokine storm generated in pigs against CSFV and their pathogenic role during CSF disease.

Interestingly, only the surviving animals from both infected groups developed specific antibodies against the CSFV E2 and E^{rns} glycoproteins. These animals also developed neutralizing antibodies although with low titers, being especially lower in the 3-week-old pigs. The high levels of CSFV detected in the tonsil suggested the chronicity status that could be developed in these infected animals in the presence of a low neutralizing antibody titers (Rout and Saikumar, 2012). On the other hand, the high amount of IFN-α levels detected in the sera from the infected animals could also promote leukopenia, affecting the generation of an effective antibody-mediated response (McNab et al., 2015). Unlike the Study II, where the abrogation of the E^{rns} RNase activity in the single mutant vPdR-H_{30}K-36U was carried out, the double mutant vPdR-H_{30}K-5U has a reduced ability to activate neutralizing antibodies. Hence, the capacity of the 3' UTR poly-U insertion and the E^{rns} RNase activity to modulate and even exacerbate the host immune response to the virus has been proven, supporting their role as CSFV virulent factors.

Taken together, the present thesis points out the direction for the research in order to determine the natural attenuation CSFV molecular determinants. The 3'UTR long poly-U insertion in a low virulence CSFV strain could reduce the viral virulence in pigs, providing a potential target for efficient CSFV diagnostic and control. In addition, this result could contribute to the development of new vaccines against infections with CSFV and even provide relevant information in the antiviral strategies against other viruses in the *Flaviviridae* family.

The present thesis also showed for the first time the role of the E^{rns} RNase activity inactivation in a low virulence CSFV isolate to reduce the CSFV virulence, activate the immune response and therefore to prevent the viral persistence in pigs. Notably, the synergistic effect of lacking the 3' UTR poly-U insertion and the E^{rns} RNase activity in a low virulence CSFV strain revealed the important role of the innate immune system to modulate the CSFV pathogenesis. These findings also pointed out the important advantages of the low virulence CSFV strains as the backbone for the study of molecular determinants for CSFV virulence pathogenesis and host immunity modulation.

This thesis brings new insights relevant for the development of DIVA vaccines against CSFV with the appropriate accompanying diagnostic tests. Finally, the present thesis paves the way for better understanding of the CSFV pathogenesis and the importance of the natural attenuated virulence factors, which could help to establish effective disease control in endemic areas and eradicate CSF.

Chapter 7

Conclusions

1. The long polyuridine insertion of 36 nucleotides, naturally acquired in the 3′ UTR region of the low virulence CSFV PdR strain, can control viral replication, reduce disease severity and activate immunity in the infected piglets, being a CSFV virulence factor.

2. The CSFV vPdR-H_{30}K-36U, lacking the E^{rns} RNase activity, was attenuated in piglets compared to the parental vPdR-36U. Only RNA traces were detected in sera and body secretions and CSFV could not be isolated from tonsils, showing that the E^{rns} RNase inactivation may reduce CSFV persistence and transmissibility in the infected animals.

3. The vPdR-H_{30}K-36U mutant strongly activated the IFN-α production in pDC, while *in vivo*, the IFN-α response was variable, from moderate to undetectable depending on the animal. This suggests a role of the CSFV E^{rns} RNase activity in the innate immune response regulation in the infected host.

4. Infection with vPdR-H_{30}K-36U resulted in higher antibody levels against the E2 and E^{rns} glycoproteins and enhanced neutralizing antibody response when compared with vPdR-36U. Therefore, the difficulties in the development of diagnostics and DIVA assays based on seroconversion against the E^{rns} glycoprotein may be attributable to a dampening effect on E^{rns}-specific immunity related to the RNase activity of E^{rns}.

5. The vPdR-H_{30}K-5U double mutant, with the E^{rns} RNase inactivation and with 5 uridines instead of the 36 in the 3'UTR, induced severe clinical manifestations, with a higher mortality rate in 5-day-old piglets than in 3-week-old pigs. These indicate the important role of pig's age for CSFV susceptibility and the pathogenesis modulation by the synergistic effect of the 3'UTR and the E^{rns} RNase inactivation.

6. Among nine cytokines quantified (IFN-α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFN-γ and TNFα), only IFN-α and IL-12 were highly and consistently elevated in the vPdR-H_{30}K-5U infected 5-day-old and 3-week-old pigs. In addition, high IL-8 levels were found in the newborn but not in the older pigs. This points towards a role of these cytokines in the outcome of disease, with age-related differences.

7. Contrasting with disease severity, the viremia and viral excretion were surprisingly low from all the vPdR-H_{30}K-5U infected animals, while the virus load was high in the tonsils, independently of the age of the pigs. This supports the relevant role of the tonsil as a primary site for CSFV replication, preceding the viremic phase.

8. The highly elevated cytokines during the vPdR-H_{30}K-5U infection, including the IFN-α response, might limit systemic viral spread from the tonsils and increase virus clearance, and at the same time induce strong cytokine-mediated symptoms.

9. The surviving animals of both ages developed specific anti-E2 and -E^{rns} antibodies after infection with vPdR-H_{30}K-5U. However, the neutralizing antibody titers were low, especially in the pigs inoculated at the age of three weeks, in which the neutralizing antibody titers were even lower than the threshold established for protection against CSFV infection. This may be related to the high levels of virus detected in the tonsils, suggesting reduced virus clearance with a possible chronic outcome.

10. The 3' UTR poly-U insertion and the E^{rns} RNase activity of the PdR strain have a synergistic effect in controlling the host's immune response to CSFV and contributing to virus attenuation.

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