Universitat Autònoma de Barcelona

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PhD Thesis

Bellaterra, 2021

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Tesis doctoral presentada por **José Alejandro Bohórquez Garzón** 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 la Dra. **Llilianne Ganges Espinosa** y el Dr. **Mariano Domingo Álvarez**.

Bellaterra, 2021

IRTA CReSA

· OIE Reference Center for Classical Swine Fever.
· Collaborating Center of the OIE for Research and control of emerging and re-emerging pig diseases in Europe.

This work has been financially supported by grants AGL2015–66907 and RTI2018-100887-B-I00 from the Ministerio de Ciencia e Innovación of the Spanish government.

José Alejandro Bohórquez Garzón received a predoctoral fellowship FPI 2016 from the Ministerio de Ciencia e Innovación of the Spanish government.

Cover design: Joulie Rojas (behance.net/joulie)

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Certifican:

Que la memoria titulada "**Immunopathogenesis of persistent and subclinical infections generated by Classical swine fever virus**" presentada por **José Alejandro Bohórquez Garzón**, 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.

Para que conste a los efectos oportunos, firman el presente certificado en Bellaterra, Barcelona, el 08 de Julio de 2021

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José Alejandro Bohórquez Garzón
Doctorando

List of abbreviations

ASF	African swine fever
ASFV	African swine fever virus
BDV	Border disease virus
BMHC	Bone marrow hematopoietic cells
BSL3	Biosafety level 3
BVDV	Bovine viral diarrhoea virus
Cat01	Catalonia strain
CD	Cluster of differentiation
CSF	Classical swine fever
CSFV	Classical swine fever virus
Ct	Cycle threshold
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DIVA	Differentiation of vaccinated and infected animals
DNA	Deoxyribonucleic acid
dpi	Days post infection
dpb	Days post birth
dsRNA	Double stranded RNA
EDC	Endothelial cells
ELISA	Enzyme linked immunosorbent assay
EMA	European medicines agency
ENC	Equivalent number of copies
ER	Endoplasmic reticulum
EU	European Union
EURL	European Union reference laboratory
FAT	Fluorescent antibody test
FITC	Fluorescein isothiocyanate
HBV	Hepatitis B virus
HCLV	Hog cholera lapinized virus
HCV	Hepatitis C virus
HIV	Human immune-deficiency virus
HRP	Horse radish peroxidase

ICTV	International committee on taxonomy of viruses
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	induced Nitric oxide synthases
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
Kb	Kilobase
Kg	Kilogram
µL	microliter
MΦ	Macrophage
mAbs	Monoclonal antibodies
MDA	Maternally derived antibodies
MDSC	Myeloid derived suppressor cells
mg	milligram
M-MDSC	Monocytic MDSC
mL	millilitre
MLV	Modified live vaccines
N	Normal
nm	nanometre
NPLA	Neutralisation peroxidase linked assay
nt	Nucleotide
OIE	World organisation for animal health
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDC	plasmacytoid dendritic cells
PdR	Pinar del Rio strain
pg	picogram
PGE2	Prostaglandin E2
PHA	Phyto-haemagglutinin
PLA	Peroxidase linked assay
PMN-MDSC	Polymorphonuclear MDSC
polyA	Poly adenine
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR

RT-qPCR	Reverse transcription quantitative PCR
sCD163	Soluble CD163
SIE	Super infection exclusion
SL	Stem loop
SLA	Swine leukocyte antigen
ssRNA	Single stranded RNA
TCID	Tissue culture infective dose
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethyl benzidine
TNF-α	Tumour necrosis factor alpha
TSV	Tunisian sheep virus
UAB	Universitat Autonoma de Barcelona
UK	United Kingdom
US	United States of America
UTR	Untranslated region
WAHIS	World animal health information system

Abstract

Classical swine fever (CSF) remains one of the most important diseases in animal health, being included into the list of notifiable diseases for the World Organisation for Animal Health (OIE). It is caused by the CSF virus (CSFV), a member of the *Pestivirus* genus within the *Flaviviridae* family. Due to the major consequences of CSF, in socio-economical terms, the control of the disease in endemic countries is considered a task of great importance. One of the challenges for disease control is the presence of persistent forms of CSF in the field, which can be congenital or postnatal. Animals that develop persistent CSF forms will be clinically healthy, while excreting high viral loads. Furthermore, they will not develop antibody response, which will make them undetectable by the currently recommended serological methods for diagnosis. Moreover, CSFV persistently infected animals will be refractory to vaccination. In the case of congenital CSF persistence, this has been linked to immunotolerance, due to a failure in pathogen recognition by the foetus after CSFV infection. The understanding of the mechanisms underlying these forms of CSF will be crucial for the design of new diagnostic tools or antiviral strategies to achieve CSF control.

The present thesis focused in the elucidation of the virological and immunological mechanisms involved in the establishment and maintenance of congenital and postnatal persistent CSFV infection. To this end, pregnant sows were infected with CSFV strains of varying degrees of virulence at a time point in which congenital persistent infection has been reported to be induced. The virulence of the infecting strain was found to play a major role for trans-placental transmission, as high and moderate virulence strains showed more efficient trans-placental transmission than low virulence CSFV. Moreover, the moderate virulence strain was able to

generate congenital viral persistence in piglets. In addition, evidence of pathogen recognition and induction of innate immune response, in terms of Interferon alpha (IFN-α) by the foetuses and piglets was found. This was in contrast with the generally acknowledged definition of the immunotolerance phenomenon and CSFV congenital persistent infection.

The role of age in the establishment of postnatal persistent infection with CSFV was also assessed by infecting piglets at weaning age with a moderately virulent CSFV strain. Postnatal persistent infection was generated in some of these animals, however, the proportion or persistently infected pigs was found to be lower than previous experiments in which infection was carried out in the first hours after birth. This strongly suggests the role of age for the induction of CSF postnatal persistent form. Markers of altered immune states were detected in the persistently infected animals. Finally, precursor cell populations found to be increased in CSFV persistently infected animals were determined to be similar, in phenotype and functionality, to immunosuppressive cell populations previously reported in humans. The results of the present thesis provide insight into the virological and immunological basis of viral persistence in CSFV. It is possible that CSFV postnatal persistently infected pigs could be used as a model which can aid in the study of immune evasion pathways implicated in human and animal diseases.

Resumen

La peste porcina clásica (PPC) sigue siendo una de las enfermedades más importantes en sanidad animal, siendo incluida en la lista de enfermedades de declaración obligatoria de la Organización Mundial para la Sanidad Animal (OIE). La enfermedad es causada por el virus de la PPC (VPPC), un miembro del género *Pestivirus* en la familia *Flaviviridae*. Debido a las tremendas consecuencias de la PPC, en términos socioeconómicos, el control de la enfermedad en países endémicos es considerado como una labor de gran importancia. Uno de los desafíos para el control de la enfermedad es la presencia de formas persistentes de PPC en el campo, las cuales pueden ser congénitas o postnatales. A pesar de estar excretando altas cargas virales, los animales que padecen formas persistentes de PPC son aparentemente sanos. Estos animales tampoco desarrollan respuesta de anticuerpos, siendo indetectables por los métodos serológicos recomendados para diagnóstico. Los animales persistentemente infectados con VPPC también son refractarios a la vacunación. En la forma persistente congénita de PPC, esto ha sido asociado a inmunotolerancia, debido al fallo del sistema inmune fetal en el reconocimiento del patógeno tras la infección. El conocimiento de los mecanismos que subyacen estas formas de PPC será crucial para el diseño de nuevas herramientas diagnosticas o estrategias antivirales para lograr el control de la PPC.

La presente tesis se enfocó en elucidar los mecanismos virológicos e inmunológicos involucrados en el establecimiento y mantenimiento de infección persistente congénita y postnatal por el VPPC. Con este fin, cerdas preñadas fueron infectadas con cepas de VPPC de diversos grados de virulencia, en un momento de la gestación en el que se ha reportado la inducción de persistencia congénita. Se determinó que la virulencia de la cepa infectante juega un papel importante para la transmisión trans-

placentaria, ya que las cepas de alta y moderada virulencia fueron transmitidas más eficientemente a través de esta ruta que la cepa de baja virulencia de VPPC. Además, la cepa de moderada virulencia fue capaz de generar persistencia congénita con VPPC en lechones. Adicionalmente, se encontró evidencia de reconocimiento del patógeno e inducción de respuesta inmune innata, en términos de interferón alfa (IFN- α) por los fetos y lechones. Este resultado contrastó con la definición generalmente reconocida del fenómeno de inmunotolerancia y la infección persistente con VPPC.

El papel de la edad en el establecimiento de infección persistente postnatal con VPPC fue estudiado infectando cerdos a edad de destete con una cepa de moderada virulencia. Infección persistente postnatal fue inducida en algunos de estos animales, sin embargo, la proporción de cerdos persistentemente infectados fue más baja que estudios anteriores en los cuales la infección fue llevada a cabo durante las primeras horas después del nacimiento. Esto sugiere fuertemente que la edad a la que se infecta el animal juega un papel en la inducción de la forma persistente postnatal de PPC. Marcadores de estados inmunes alterados fueron detectados en los animales persistentemente infectados. Finalmente, se encontró que poblaciones celulares precursoras, las cuales se encuentran aumentadas en animales persistentemente infectados con VPPC, son similares en fenotipo y funcionalidad a poblaciones celulares inmunosupresoras reportadas anteriormente en humanos. Los resultados de la presente tesis ayudan a entender las bases virológicas e inmunológicas de la persistencia viral causada por VPPC. Es posible que los animales que padecen las formas postnatales persistentes puedan ser usados como un modelo para ayudar en el estudio de los mecanismos de evasión de la respuesta inmune implicados en enfermedades de sanidad animal y humana.

Resum

La pesta porcina clàssica (PPC) continua sent una de les malalties més importants en sanitat animal, inclosa a la llista de malalties de declaració obligatòria de l'Organització Mundial de Sanitat Animal (OIE). És causat pel virus de la PPC (VPPC), membre del gènere *Pestivirus* de la família *Flaviviridae*. A causa de les greus conseqüències de la PPC, en termes socioeconòmics, el control de la malaltia als països endèmics es considera una tasca de gran importància. Un dels reptes per al control de la malaltia és la presència de formes persistents de PPC al camp, que poden ser congènites o postnatals. Els animals que desenvolupen formes persistents de PPC són clínicament sans, mentre que excreten càrregues virals elevades. A més, no desenvoluparan resposta d'anticossos, cosa que els farà indetectables mitjançant els mètodes serològics per diagnòstic recomanats actualment. Addicionalment, els animals infectats persistentment seran refractaris a la vacunació. En el cas de la persistència congènita de la PPC, aquesta s'ha relacionat amb la immunotolerància, a causa de la incapacitat del fetus de reconèixer patògens després de la infecció pel VPPC. La comprensió dels mecanismes subjacents a aquestes formes de PPC serà crucial per al disseny de noves eines de diagnòstic o estratègies antivirals per aconseguir el control de la PPC.

La present tesi es va centrar en l'elucidació dels mecanismes virològics i immunològics implicats en l'establiment i el manteniment de la infecció persistent congènita o postnatal pel VPPC. Amb aquesta finalitat, truges embarassades es van infectar amb soques del VPPC amb diversos graus de virulència en un moment en què s'ha establert que és possible induir una infecció congènita persistent. Es va detectar que la virulència de la soca infectant té un paper important en la transmissió transplacentària, ja que

les soques de virulència alta i moderada van mostrar una transmissió més eficient per aquesta ruta que el VPPC de baixa virulència. A més, la soca de virulència moderada va ser capaç de generar persistència viral congènita en garrins. Addicionalment, es va trobar evidència de reconeixement del patogen i d'inducció de resposta immunitària innata, en termes d'interferó alfa (IFN-α) per part dels fetus i els garrins. Això contrastava amb la definició generalment reconeguda del fenomen d'immunotolerància i de la infecció congènita persistent pel VPPC.

El paper de l'edat en l'establiment d'una infecció persistent postnatal pel VPPC també es va avaluar mitjançant la infecció de garrins a l'edat de deslletament amb una soca del VPPC moderadament virulenta. En alguns d'aquests animals es va generar infecció persistent postnatal. Tanmateix, es va trobar que la proporció de porcs infectats persistentment era inferior a la dels experiments anteriors en què es va dur a terme la infecció en les primeres hores després del naixement. Aquest resultat suggereix un paper molt important de l'edat en la inducció de la infecció persistent postnatal pel VPPC. En els animals infectats persistentment es van detectar marcadors d'estats immunitaris alterats. Finalment, es va determinar que les poblacions de cèl·lules precursores augmentades en animals persistentment infectats amb VPPC eren similars, en fenotip i funcionalitat, a poblacions de cèl·lules immunosupressores descobertes anteriorment en humans. Els resultats de la present tesi proporcionen informació sobre les bases virològiques i immunològiques de la persistència viral en VPPC. És possible que els porcs amb infecció postnatal persistent pel VPPC es puguin utilitzar com a model per a l'estudi de les vies d'evasió de la resposta immunitària implicades en malalties humanes i animals.

Part I

General introduction and objectives

Chapter 1

General introduction

1.1 Classical swine fever: History and economic impact

Within the realm of animal health, classical swine fever (CSF) remains one of the most relevant viral diseases, affecting both domestic and wild swine (Blome, Staubach, et al., 2017). The disease is notifiable to the world organisation for animal health (OIE), given that it is highly contagious and has severe repercussions from an economic and sanitary standpoint (OIE, 2019a). The only natural reservoir of the CSF virus (CSFV) are members of the *Suidae* family and the virus is able to infect domestic and wild swine (Blacksell et al., 2006; Depner et al., 1995; Everett et al., 2011). CSFV does not infect humans, but experimental infection of ruminants and rabbits has been demonstrated (Rovid Spickler, 2003).

The first reports of CSF date as back as 1810 in Tennessee. However, they rely on observations from farm workers based on a very unspecific description of the symptomatology of the disease (Anonymous, 1889; Hanson, 1957). Nevertheless, outbreaks of CSF were reported in 10 different states from the Midwestern and Southern regions of the United States (US) during the first half of the 19th century, being more prevalent in areas with high concentration of pigs (Hanson, 1957). After 1860, the disease spread rapidly throughout the US territory, a fact that has been related to the development of railways during the mid-century (Birch, 1922). In Europe, the first concrete reports of CSF date from 1862 in England and five years later it spread to Sweden, France and Denmark (Birch, 1922), and by the 1960s the disease had spread worldwide (Cole et al., 1962a). Initially named "Hog cholera", CSF was thought to be a bacterial disease caused by *Bacterium suispestifer* until 1903, when it was discovered that the disease was viral, given that it could be transmitted by filtered serum from infected animals (De Schweinitz & Dorset, 1903).

Recently, a cophylogenetic analysis of CSFV hypothesized that, based on the time for the most recent common ancestor, the virus emerged by the end of the 18th century. This was likely due to a jump of Tunisian sheep virus (TSV) from the host *Ovis aries* to the new host, *Sus scrofa* (Rios et al., 2017). Coincidentally, the first import of Tunisian sheep to the US has been reported to have taken place in the year 1799 in Pennsylvania (Brier, 2013). The breed became very popular and spread rapidly throughout the US, including some of the regions where the first reports of CSF originated (Carman et al., 1892; Peters, 1810). In accordance with the costume at the time, animals from different species were allowed to be in close contact, favouring the hypothetical cross-species transmission of TSV to swine.

Historically, CSF has been one of the most devastating diseases for the swine industry, due to the economic losses generated after an outbreak. These are related to the high morbidity of the virus, as well as to the fact that a CSF outbreak also leads to severe restrictions in pigs and pork-derived products trade (Meuwissen et al., 1999; Saatkamp et al., 2000). In Europe, a progressive eradication program has been implemented since the 1990s (Paton & Greiser-Wilke, 2003). Under this program, a non-vaccination policy was adopted and strict serological surveillance was introduced accompanied by a stamping out strategy, in which the infected animals and those in contact with the infected herds were to be eliminated (European Union, 2016). As a result of this policy, the last two outbreaks of CSF in Spain (1997 and 2001) were worth around 108 million Euros combined (Fernández-Carrión et al., 2015). The outbreak in 1997/98 in the Netherlands had a total estimated cost of over 2 billion US$. The majority of these costs were related with "welfare sacrifice", in which healthy animals outside of the control zones had to be euthanized due to the movement restrictions (Elbers, 2002; Meuwissen et al., 1999). Hence,

besides the economic losses, this control strategy also has implications from an animal welfare standpoint (Elbers, 2002; Greiser-Wilke & Moennig, 2004; Koenen et al., 1996). Moreover, the recovery of the OIE CSF-free status by an affected country is a complex and costly process in itself.

1.2 CSF distribution

Currently the OIE list of CSF free territories consists of 35 countries (OIE, 2019d), including all of North America and Oceania, as well as a large part of the European Union (EU), which has been free of the disease for decades (OIE, 2019d). CSF remains endemic in large parts of Asia, South and Central America and the Caribbean, while the OIE has declared specific regions within Brazil, Colombia and Ecuador as free of the disease, even if the countries remain endemic (OIE, 2019d). The CSF situation in Africa is mostly unknown, though outbreaks have historically been reported in Madagascar and South Africa (W. Ji et al., 2015; Sandvik et al., 2005). According to the world animal health information system (WAHIS), setup by the OIE, in recent years outbreaks of CSF have taken place in Korea, Colombia, Russia, Brazil and Japan (OIE, 2019c). The case of Japan is of particular interest, since the disease has re-emerged after 26 years, in both domestic pigs and wild boar (OIE, 2019c; Postel et al., 2019).

Nevertheless, the virus has been periodically reintroduced into the EU through wild boars and pig trade and a large outbreak took place in 1997, with cases in Belgium, Germany, The Netherlands, Spain and Italy (Greiser-Wilke et al., 2000). In the 2000s, several small outbreaks were reported in many European countries, such as the United Kingdom (UK), Spain, France, Germany, Hungary and Lithuania (Beer et al., 2015; Paton

& Greiser-Wilke, 2003; Postel, Moennig, et al., 2013), while in the 2010s, outbreaks in the EU remained limited to eastern Europe (OIE, 2019c). Infected wild boar populations represent a risk for domestic pigs, as it was reported that 60% of the CSF outbreaks in Germany between 1993 and 1998 could be traced back to wild boar populations (Fritzemeier et al., 2000).

1.3 Aetiology

CSFV is an enveloped virus of icosahedral symmetry. Viral particles measure between 40-60 nm in diameter and contain a 12.3-kilo bases (kb), positive-polarity single-stranded RNA ((+)ssRNA) genome (Figure 1). It is a member of the *Pestivirus* genus, an ever-growing viral genus encompassing 11 different officially recognized viral species, even though more members have been proposed. The *Pestivirus* genus together with the *Flavivirus*, *Hepacivirus* and *Pegivirus* genera, form the *Flaviviridae* family (Simmonds et al., 2012, 2017). Recently, the international committee on taxonomy of viruses (ICTV) adopted a new nomenclature scheme for members of the *Pestivirus* genus (ICTV, 2020; Simmonds et al., 2017; Smith et al., 2017). Under this nomenclature, CSFV is known as "*Pestivirus C*". Other members of the *Pestivirus* genus with major relevance in animal health include Bovine Viral Diarrhoea virus type 1 (BVDV-I, Pestivirus A), BVDV type 2 (BVDV-II, Pestivirus B) and Border disease virus (BDV, Pestivirus D).

1.3.1 Physicochemical properties and stability

As an enveloped virus, CSFV can be inactivated by organic solvents, detergents, chlorine-based disinfectants, phenolics, highly concentrated alcohol solutions and quaternary ammoniums aldehydes (Edwards, 2000; Kramera et al., 2009). The virus is stable at a pH from 5 to 10, although

stability at lower pH has been reported in low temperature and protein-rich environments, conditions usually found during meat storage (Edwards, 2000; Kramera et al., 2009). Likewise, survival in meat products has been reported between months and years, depending on the conditions for processing and storage (Edgar et al., 1949; Edwards, 2000). CSFV can be inactivated at 100 °C in less than 1 minute, 2 minutes at 90 °C, 3 minutes at 80 °C and 5 minutes at 70 °C (Edwards, 2000). Recently, it was reported that inactivation of serum samples using phosphate buffered saline-$Tween_{20}$ followed by incubation at 56 °C for 30 min was also feasible without affecting the detection of antibodies (Meyer et al., 2019).

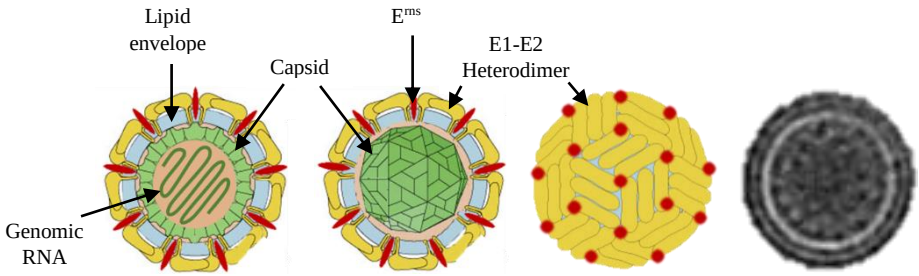
1.3.2 Genome organisation and expression

CSFV has a positive-polarity single-stranded RNA ((+) ssRNA) of approximately 12.3 kb, which contains a single open reading frame (ORF) surrounded by two untranslated regions (UTRs, Figure 1B): the uncapped 5'-UTR and the uridine-rich 3'-UTR (Tautz et al., 2015). The ORF encodes for a polyprotein that is cleaved into four structural (Core, E1, E2 and E^{rns}) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, Figure 1C) (Blome, Staubach, et al., 2017; Tautz et al., 2015).

The E^{rns} protein mediates the initial contact with the cell and the E2-E1 heterodimer is essential for receptor binding and subsequent endocytosis (Blome, Staubach, et al., 2017; Hulst et al., 2001; Iqbal et al., 2000; Z. Wang et al., 2004). The CSFV cellular receptor has not been defined, however the complement regulatory protein cluster of differentiation (CD) 46 has been identified as the receptor for BVDV in cattle, and has been shown to play a major role in attachment of CSFV *in vitro* (Dräger et al.,

2015; Krey et al., 2006). Moreover, integrin β3 has been demonstrated to be required for CSFV infection (W. Li et al., 2014).

A)



B)

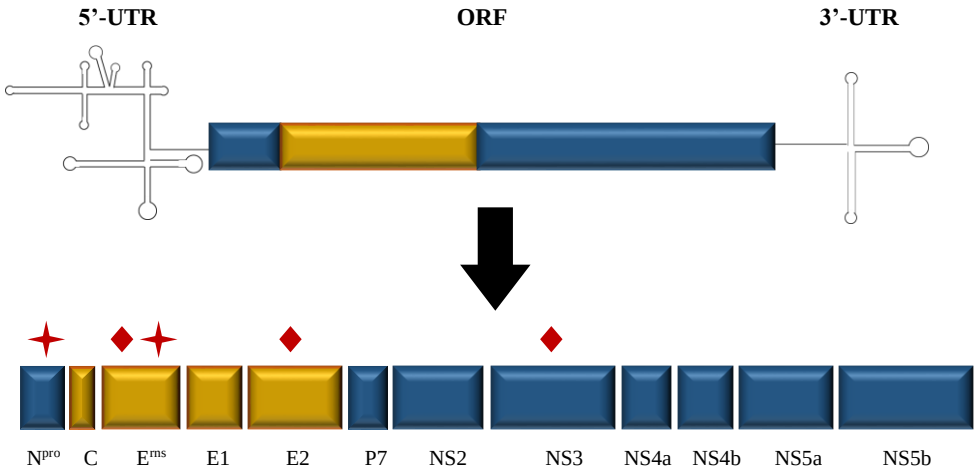


Figure 1. (A) Graphic representation of the elements included in a *Pestivirus* virion and electron microscopy photograph of a viral particle. Adapted from (Hulo et al., 2011), electron microscopy obtained from (Callens et al., 2016). (B) Schematization of the CSFV genome showing the single CSFV ORF, flanked by the 5' and 3' UTR regions. Structural proteins are represented in yellow and non-structural proteins in blue. Red star indicates proteins unique to the *Pestivirus* genus and red rhombus shows proteins inducing antibody response. Adapted from (Tautz et al., 2015).

It is possible that multiple factors are needed for CSFV entry, something that has been reported for other flaviviruses such as Hepatitis C virus (HCV) (Ding et al., 2014; Dräger et al., 2015). Upon entry of the virus, fusion of the envelope with the endosomal membrane takes place and the viral core releases the viral RNA into the cytoplasm for the initiation of viral translation.

The structure of the 5'-UTR is of high importance for the initiation of translation. It contains an internal ribosome entry site (IRES) domain that binds to the 40s small ribosomal subunit, leading to the recruitment of the 60s ribosomal unit and the translation of a viral polyprotein (H. Ji et al., 2004). This will be approximately 3900 amino acids in length, processed into mature viral proteins by viral and host cellular proteases in a co-translational manner (Stark et al., 1993; Tautz et al., 2015).

The first protein encoded by the ORF is the N^{pro}, an autoprotease that cleaves its own carboxy-terminus during translation. This is one of two proteins unique to the *Pestivirus* genus, the other being the E^{rns}, and its function is to protect the cell from apoptosis by blocking the type I interferon (IFN) pathway (Bauhofer et al., 2007; Z. Chen et al., 2007; Fiebach et al., 2011; Gottipati et al., 2016). Cleavage of the carboxy-terminus of the N^{pro} generates the amino-terminal of the capsid protein, which contains a signal sequence for translocation to the endoplasmic reticulum (ER), where the protein is cleaved by cellular peptidases (Tautz et al., 2015). It has been suggested that the capsid protein binds to the RNA genome in a histone-like manner (Murray et al., 2008).

Downstream from the capsid protein, the structural proteins are encoded, all of which are processed by cellular peptidases. The first of these is the E^{rns} protein, which is heavily glycosylated and can also be secreted. This

is important to its function as an RNase with a predilection for ssRNA (Schneider et al., 1993). E^{rns} appears to play a role in preventing the induction of type I IFN response in plasmacytoid dendritic cells (pDCs) (Mätzener et al., 2009; Python et al., 2013)

The next protein to be translated is the E1, for which no structure or specific function has been discovered yet (Tautz et al., 2015). It has been proposed that E1 contains the fusion peptide of CSFV, since the crystal structure of E2 did not reveal any obvious fusion peptide. However, this seems unlikely due to the size of the E1 and E2 proteins and the distance to the target membrane (El Omari et al., 2013).

The E2 glycoprotein, on the other hand, has been thoroughly characterized, since it is the most immunogenic protein of the virus. The E2 protein is responsible for receptor-binding and viral species tropism in pestiviruses, being also the main target for neutralizing antibodies (Gladue et al., 2014; Liang et al., 2003; Z. Wang et al., 2004; Weiland et al., 1990). The C-terminus of E2 contains a hydrophobic sequence that acts as transmembrane anchor (Garry & Dash, 2003), while the protein itself is glycosylated and contains 17 cysteines that form eight intra-molecular and one extra-molecular disulphide bonds (El Omari et al., 2013; Y. Li et al., 2013). These are important for the formation of the protein structure, as well as for dimerization either with other E2 proteins or with E1. The structure of the E2 protein contains four structural domains (A, B, C and D) (Van Rijn et al., 1993). These structural domains form two independent subunits, A/D and B/C, the latter of which is highly immunogenic, being able to confer protection against CSFV infection (Van Rijn et al., 1996). Based on bioinformatics analyses, this subunit and the N-terminus of E2 have been identified as a site for positive selection pressure, exerted by

vaccination, in endemic countries (Díaz De Arce et al., 2005; Hu et al., 2016; W. Ji et al., 2014; L. J. Pérez et al., 2012). A cell-binding peptide is also present in the B domain, indicating that this is the likely binding region with the cellular receptor (X. Li et al., 2011). Recently, the crystal structure of BVDV E2 glycoprotein has been solved, and it appears to confirm the findings of the mAbs studies regarding the four domains of the E2 glycoprotein (El Omari et al., 2013; Y. Li et al., 2013).

Following the structural proteins, the first non-structural protein to be translated is p7, which is also the smallest protein of the virus. Due to its biochemical characteristics, the p7 has been proposed to be a viroporin (Harada et al., 2000; Largo et al., 2014), functioning as an ion channel and reducing intracellular vesicle acidification required for assembly of infectious progeny virus (Gladue et al., 2018; Steinmann et al., 2007; Wozniak et al., 2010). The next proteins to be translated are the NS2 and NS3. These are first expressed as a single NS2-3 protein, which is necessary for virus particle assembly before being processed by the autoprotease activity of NS2 (Lackner et al., 2004). All the translated proteins downstream of the NS2-3 site are processed by the NS3 protease (Harada et al., 2000; Lamp et al., 2011, 2013).

The NS3 protein has two domains: The N-terminus has a protease activity (Lamp et al., 2013), while the C-terminus works as a helicase and NTPase, necessary for viral replication (C. Sheng et al., 2007; Chun Sheng et al., 2013). It is also able to induce humoral and cellular immune response against CSFV (Greiser-Wilke et al., 1992; Rau et al., 2006; Tarradas et al., 2012). The protease function of NS3 is directly linked with the NS4A protein, which is translated directly after it, given that NS4A works as a cofactor of NS3 (Xu et al., 1997). It has also been reported that NS4A

plays a role in viral morphogenesis (Lamp et al., 2013). Meanwhile, the exact function of NS4B remains unknown, even though it is necessary for viral replication (Blight, 2011)

The NS5A and NS5B are the last proteins to be translated and processed and both have functions in viral replication. NS5A regulates viral replication either by binding with the 3'-UTR or by direct interaction with the NS5B, since it is necessary for the binding of NS5B to the RNA (Y. Chen et al., 2012; C. Sheng et al., 2012). On the other hand, NS5B is a multifunctional protein, which has been identified to play a role in viral morphogenesis (Ansari et al., 2004). However, its main role is as a RNA-dependent RNA polymerase (RdRp), a function that is heavily dependent on NS3 and NS5A (L. Liu et al., 2009; C. Sheng et al., 2007, 2012).

Immediately after translation termination at the 3'-end, the replication complex assembles to start the synthesis of the first negative polarity strand of the genome, with the 3'-UTR serving as the leading sequence for replication. Since there is not a poly-adenylated (polyA) sequence in the 3'-UTR of CSFV, recognition by the replication complex relies on its secondary structure (Tautz et al., 2015).

The 3'-UTR of CSFV contains three stem-loops (SL) named SLI, SLII and SLIII (Pankraz et al., 2005). The SLI is the closest to the 3' end of the sequence and it is recognized by the replication complex, making it the only SL strictly necessary for viral replication. Recently, a 36-uridine insertion within the 3'-UTR of a low virulence strain from an endemic situation was determined to control viral replication and modulate the immune response and disease severity. This demonstrated the role of the 3'-UTR as a virulence factor in CSFV (M. Wang et al., 2019). Viral particle assembly occurs intracellularly and the maturation of the

carbohydrate chains of the envelope proteins takes place during transport of the particles to the cell surface (Burrack et al., 2012; Macovei et al., 2006). The ER serves as the site for packaging of the capsid-RNA complex and viral particles are then exported from the cell via the secretory pathway without damaging the cell (Schmeiser et al., 2014).

1.4 Pathogenesis

The outcome of CSFV infection is dependent on multiple factors, some related to the host (species, age, breed, immunity status, etc.) and others to the pathogen, mainly the virulence of the infecting strain (Floegel-Niesmann et al., 2003; Nielsen et al., 2010; Petrov et al., 2014). Multiple virulence factors have been identified in CSFV by various methods, even though their mechanisms have not been fully understood (Leifer et al., 2013; M. Wang et al., 2019). Furthermore, the interaction of the virus with its target cells and their regulation of the immune response in the host are aspects of the virus, which are still under study. Hence, the evaluation of the virulence of a CSFV strain still relies on the experimental infection of pigs under standardized conditions (Coronado et al., 2017; Floegel-Niesmann et al., 2003; Kameyama et al., 2019; Petrov et al., 2014; Tarradas et al., 2014).

CSFV is typically transmitted by the oro-nasal route (Ressang, 1973a; Weesendorp et al., 2009b). After infection, primary replication takes place in the tonsils, where it probably infects epithelial cells and M-cells in the tonsillar crypts, and spread to the surrounding lymphoid tissue (Liess, 1987). Once there, the virus will infect its primary targets, such as endothelial cells (EDC), Macrophages (MΦ) and dendritic cells (DC) (Knoetig et al., 1999; Ressang, 1973b; Summerfield, Hofmann, et al., 1998; Summerfield & Ruggli, 2015; Susa et al., 1992). By promoting the

proliferation and maturation of DC and using the migratory capabilities of MΦ, CSFV is able to spread to multiple organs (Carrasco et al., 2004; Jamin et al., 2008).

The cellular targets of CSFV, particularly DC, are important for pathogen recognition, presentation of antigens by the swine leukocyte antigen (SLA) I and II and regulation of the early immune response (Carrasco et al., 2004). Infection with high virulence CSFV strains will induce an exaggerated activation and cytokine production of these cells, particularly high induction of IFN-α and pro-inflammatory cytokines (Figure 2). It is highly likely that this massive pro-inflammatory response is what leads to the development of disease, as direct damage by the virus is not linked with the lesions found in CSF (Lange et al., 2011; Summerfield & Ruggli, 2015). The production of these cytokines is a normal part of the innate immune response. However, rather than control the viral infection, the augmented inflammatory response will promote apoptosis and cell death, as well as tissue damage (Sánchez-Cordón, Núñez, Salguero, Pedrera, et al., 2005; Summerfield et al., 2006). This phenomenon is known as cytokine storm and will have consequences like severe lymphopenia, thrombocytopenia and thymus atrophy (Pauly et al., 1998; Sánchez-Cordón et al., 2002).

After infection of the local lymphoid tissues, CSFV will infect the regional lymph nodes through the lymphatic capillaries and from there the virus will enter the bloodstream. Viremia leads to the spread of CSFV to secondary replication sites including the spleen, bone marrow and lymph nodes and, eventually, the virus will reach the parenchymatous organs (Belák et al., 2008; J. Liu et al., 2011; Ressang, 1973b). It has been established that longer infection courses will lead to wider organ

distribution and cell tropism (Kaden et al., 2007; J. Liu et al., 2011; Summerfield et al., 2000). On the other hand, damage to the endothelial cells, thrombocytopenia and capillary vasodilation related with the cytokine storm are all involved in the development of haemorrhagic lesions in the infected animals (Gómez-Villamandos et al., 2000; Heene et al., 1971)

Infection with strains of moderate or low virulence, by contrast, induce lower levels of IFN-α or pro-inflammatory cytokines (Figure 2) (Summerfield et al., 2006; Tarradas et al., 2014; von Rosen et al., 2013). However, transient lymphopenia has been detected in animals infected with these type of strains (Nielsen et al., 2010). Infection with CSFV will lead to life-long immunity, if controlled by the immune system. Notwithstanding, if it is not controlled, infection with low or moderate virulence strains might also lead to chronic forms of disease, highly relevant for disease control (Moennig et al., 2003).

1.4.1 V**irus-host interaction**

CSFV is able to evade the immune response in the primary sites of replication thanks to the N^{pro} and E^{rns} proteins (Summerfield & Ruggli, 2015). N^{pro} has a role in shutting down the IFN pathway by interaction with IFN regulatory factor (IRF) 3 and IRF-7, as well as avoiding cellular apoptosis (Bauhofer et al., 2007; Fiebach et al., 2011; Gottipati et al., 2016; Ruggli et al., 2005). Meanwhile, E^{rns} is responsible for degrading extracellular ssRNA or double stranded RNA (dsRNA), important stimulators of type I IFN response, especially in pDCs (Figure 2) (Iqbal et al., 2000; Magkouras et al., 2008; Mätzener et al., 2009).

Highly virulent CSFV strains will induce massive levels of IFN-α by pDCs (Jamin et al., 2008). Additionally, synthesis of pro-inflammatory

cytokines, such as interleukin (IL) 1α, IL-1β, IL-6, TNFα, IFN-α and vasodilators will take place after infection of EDC and activation of MΦ and DC (Knoetig et al., 1999; Núñez et al., 2005; Sánchez-Cordón et al., 2002; Sánchez-Cordón, Núñez, Salguero, Pedrera, et al., 2005). Moreover, high IFN-α levels have been associated with bone marrow depletion, which will affect myelopoiesis and megakariopoiesis (Gómez-Villamandos, Salguero, et al., 2003; Summerfield et al., 2000).

CSFV causes depletion of lymphocytes, mainly $CD4^+$ and $CD8^+$ (Ganges et al., 2005; Summerfield et al., 2001). This, combined with the abhorrent pro-inflammatory response, will eventually lead to a breakdown in the immune response. It has been reported that in the late stages of CSF, animals can have as much as 90% of their T-cell populations depleted (Pauly et al., 1998). However, depletion of B and T lymphocytes can begin as early as one day after infection, even before the establishment of viremia (Summerfield et al., 2001; Summerfield, Knötig, et al., 1998). B and T-cell depletion is not associated with CSFV infection in these cell subsets (Summerfield, Knötig, et al., 1998), but rather with bystander apoptosis promoted by the cytokine storm (Summerfield et al., 2006). By contrast, anti-apoptotic pathways are activated in infected cells to guarantee the survival of the virus (Bensaude et al., 2004). In addition, other mechanisms of immune suppression have been reported for high and moderate virulence CSFV strains, such as production of IL-10 (Khatoon et al., 2019; Muñoz-González, Ruggli, et al., 2015; Suradhat et al., 2005). This pleiotropic cytokine is known to suppress T-Cell proliferation and B-cell responses (Rojas et al., 2017).

During later stages of the disease, B and T lymphocytes are activated as indicated by their cytokine production, despite being anergic at the

beginning of infection (Sánchez-Cordón et al., 2006; Suradhat et al., 2001, 2005). Additionally, due to the bone marrow depletion caused by the cytokine storm, granulocytopenia will also take place and immature granulocyte precursors will be found in peripheral blood (Blome, Staubach, et al., 2017; Ganges et al., 2005; Summerfield, Hofmann, et al., 1998). These cells are targets of infection and their contact with other naive bone marrow haematopoietic cells (BMHC) will lead to apoptosis of the uninfected cells (Zingle et al., 2001).

1.4.2 Effective immune response against CSFV

Early protection against CSFV has been associated with cellular immunity. This is supported by the fact that pigs vaccinated with DNA or subunit vaccines were protected against CSF before the onset of neutralising antibody response (Ganges et al., 2005; Kaden & Lange, 2001; van Oirschot, 2003). Afterwards, it was proven that a modified live vaccine (MLV) was able to confer protection as early as 3-5 days after vaccination (Graham, Everett, et al., 2012; Graham, Haines, et al., 2012)

In terms of cellular immune response, $CD4^+$ and cytotoxic $CD8^+$ lymphocytes (CTL) are highly relevant in protection against CSFV (Ganges et al., 2005, 2008). The CTL have been found to produce high levels of IFN-γ (Franzoni et al., 2013; Graham, Haines, et al., 2012), a well-known antiviral cytokine that has been associated with early control of CSFV replication and protection against leukopenia (Franzoni et al., 2013; Graham, Haines, et al., 2012; Lin & Young, 2013; Tarradas et al., 2010, 2012). Furthermore, the E2 and E^{rns} proteins have epitopes recognized by CTL, however, these epitopes do not induce a protective immune response (Ganges et al., 2005, 2008; Rau et al., 2006). Considering that, the contribution of T-cell response and IFN-γ to

protection against CSFV remains unclear and it is likely that the cellular immune response confers only partial protection against CSFV (Franzoni et al., 2013; Ganges et al., 2005, 2008; Graham, Haines, et al., 2012; Tarradas et al., 2010, 2014).

The initial cellular immune response is accompanied by Th1 cytokine production, mainly IL-2 and IFN-γ. Eventually, the cytokine production switches to a Th2 response, with an increase in IL-4 and IL-10, which facilitates the differentiation of B-cells into plasma cells and thus the production of an antibody response (Ganges et al., 2005; Sánchez-Cordón, Núñez, Salguero, Carrasco, et al., 2005; Tarradas et al., 2010). The delay in this switch, as well as the lymphopenia, contribute to the delay in humoral response. The antibody response will be detectable between 10 to 14 days after infection (Blome, Staubach, et al., 2017; Summerfield & Ruggli, 2015). Neutralising antibodies against CSFV target the E^{rns} and E2 proteins, although antibodies against the NS3 protein are also induced (Ganges et al., 2005; König et al., 1995; Terpstra & Wensvoort, 1988; Van Rijn et al., 1996). Neutralising antibody response has been shown to be crucial for sterilizing immunity and neutralising antibody titres over 1:32 can be protective against CSFV, avoiding viral excretion and transmission (Ganges et al., 2008; Tarradas, Monsó, et al., 2011; Terpstra & Wensvoort, 1988).

The induction of an antibody response in sows, even months before pregnancy, will lead to passive transfer of immunity to their offspring through maternal derived antibodies (MDA) in the colostrum (Terpstra, 1977). This is particularly important considering that placentation in swine is epitheliochorial and thus, immunity cannot be transferred through the placenta during gestation (Chavatte-Palmer & Tarrade, 2016; Suradhat et

al., 2007). The level of immunity transferred to the piglets can vary, depending on the interval between vaccination and farrowing, which plays a role in the protective capacity of antibodies in the colostrum (Precausta et al., 1983). MDA are able to protect piglets against CSFV during the first weeks of life, however, they do not avoid viral replication after infection (Bironet al., 1987; van Oirschot, 2003). The duration of MDA in piglets can also vary, being approximately between 14 days and two months (Terpstra, 1977) in domestic pigs and as high as three months in wild boar (Kaden & Lange, 2004). This is highly important as MDA can have a negative effect in the induction of an immune response through vaccination (Suradhat et al., 2007; van Oirschot, 2003).

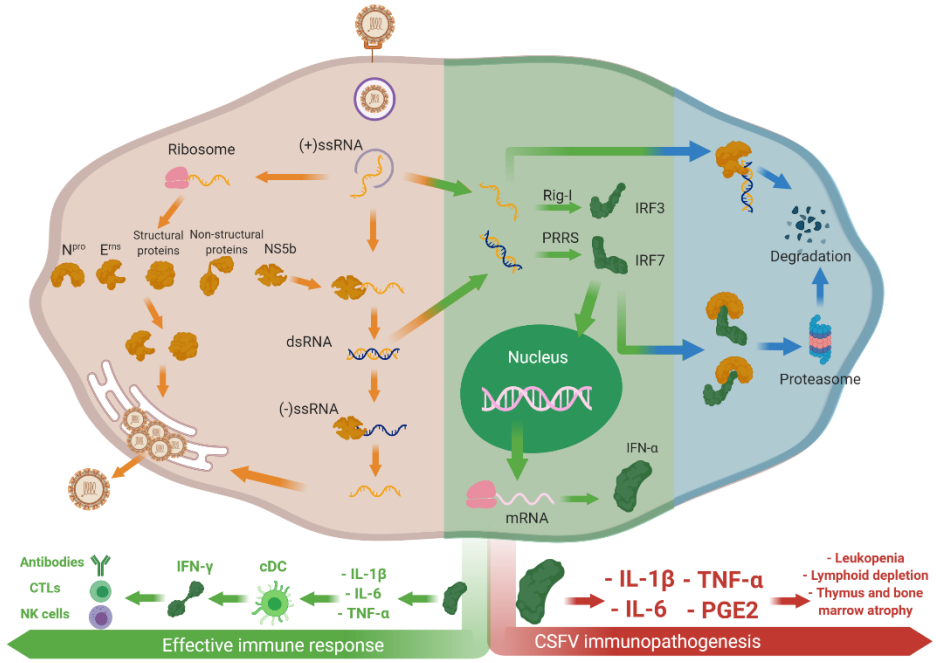


Figure 2. Replication and immunopathogenesis of CSFV. Pathways of viral replication (orange), induction of immune response (green) and immune evasion (blue) are shown. Adapted from (Summerfield & Ruggli, 2015) Image created using Biorender.com.

1.5 Clinical forms of CSF

The clinical manifestations and outcome of CSF are highly variable and depend on multiple factors, mainly the virulence of the infecting CSFV strain and the age of the host, although other host factors also play a role (Belák et al., 2008; Blome, Staubach, et al., 2017; Ganges et al., 2008; Petrov et al., 2014; Tarradas et al., 2014). According to the moment of the infection, CSFV is able to cause congenital or postnatal infection and each of these infection forms can be subdivided according to the clinical profile (Blome, Staubach, et al., 2017; Moennig et al., 2003). Congenital infections with high virulence strains can lead to foetal death and abortions or to the birth of weak piglets that will die soon after birth (Terpstra, 1991).

By contrast, if trans-placental transmission of low virulence strains takes place, it is likely that the CSF congenital persistent form will be developed. In this case, piglets may be clinically healthy, despite being infected and shedding CSFV (Moennig et al., 2003; Van Oirschot & Terpstra, 1977).

On the other hand, postnatal infection with CSFV will lead to either acute or chronic CSF (Blome, Staubach, et al., 2017). The majority of works have focused on the study of acute disease, caused by highly virulent strains. This form of CSF is characterized by a short incubation period and high mortality within a few days after infection, accompanied by severe clinical signs (Belák et al., 2008; Ganges et al., 2005; Petrov et al., 2014). Conversely, low and moderate virulence strains are more likely to cause the chronic form of CSF, which may be unapparent in the field and is currently prevalent in endemic countries (Coronado et al., 2017; Coronado, Bohórquez, et al., 2019; Ganges et al., 2008; Kameyama et al., 2019). The misdiagnosis of this disease form might lead to outbreaks in the future. In spite of its high epidemiological importance, the pathogenesis of this CSF form is not yet fully understood (Coronado, Bohórquez, et al., 2019; L. J. Pérez et al., 2012; Tarradas et al., 2014). The most recently described manifestation of CSF is the postnatal persistent infection, generated when new-born piglets or wild boar are infected with moderate CSFV moderate virulence strains within the first 24 hours after birth (Cabezón et al., 2015; Muñoz-González, Ruggli, et al., 2015). Similar to congenital CSF persistence, these animals are healthy or show unspecific clinical signs, while being disseminators of the virus. Multiple aspects regarding the timeframe or immunological phenomena underlying this type of infection remain to be elucidated.

1.5.1 CSF acute form

The acute form of CSF is mainly associated with infection of highly virulent CSFV strains in young pigs (Blome, Staubach, et al., 2017; Moennig et al., 2003). However, it has been described that high virulence strains used for vaccine potency tests can induce this form of disease, with a 100% mortality rate, regardless of the age of the infected animals (Tarradas et al., 2014).

Following a short incubation period (less than 10 days after exposure), infected pigs will show unspecific clinical signs during the first two weeks. These may include fever, anorexia, apathy, conjunctivitis and constipation followed by diarrhoea (Belák et al., 2008; Floegel-Niesmann et al., 2003; Laevens et al., 1999; Moennig et al., 2003; Tarradas et al., 2014). Leukopenia is rapidly established, before the onset of viremia (Stegeman et al., 2000). Afterwards, between the second and fourth week post infection, the more typical clinical signs associated with CSF are developed. Haemorrhagic lesions and cyanosis can be observed in the ears, limbs and abdomen and these can be accompanied by neurological clinical signs such as ataxia, paralysis and convulsions (Blome, Staubach, et al., 2017; Ganges et al., 2005; Moennig et al., 2003; Tarradas et al., 2014).

Due to the immunosuppression in the infected animals, secondary infections can mask or overlap with the disease course and complicate the clinical diagnosis (Depner et al., 1999). Mortality is near 100% with animals dying between 10 and 20 days after infection (Floegel-Niesmann et al., 2003, 2009). Pathological findings in pigs suffering the acute form of CSF may include enlarged lymph nodes, petechial haemorrhages in lungs, kidneys, intestine and urinary bladder, tonsillitis, necrotic ulceration in the intestine, splenic infarctions, thymus atrophy and non-purulent encephalitis (Belák et al., 2008; Gómez-Villamandos et al., 2000; Gruber

et al., 1995). None of the clinical signs or pathological findings observed can be considered pathognomonic for CSF.

A peracute clinical courses of CSF with high mortality and no evident gross changes at necropsy, has also been described (MacLachlan et al., 2017). In addition, a subacute CSF form, characterized by fever, diarrhoea and neurological signs, has been reported. The mortality and severity of clinical signs is lower in subacute CSF and the surviving animals will develop lifelong immunity against CSFV (Floegel-Niesmann et al., 2003, 2009).

1.5.2 CSF chronic form

An infection with CSFV can be defined as chronic if it lasts over 28 days (Coronado, Bohórquez, et al., 2019; Moennig et al., 2003). The chronic form of CSF is mostly caused by moderate and low virulence strains, although it appears that host factors play a more prominent role in the establishment of this disease form (Jenckel et al., 2017). Chronic CSF is developed when animals are not able to induce an effective immune response against the virus (Coronado, Bohórquez, et al., 2019; Petrov et al., 2014; Tarradas et al., 2014).

Initially, this form of disease will resemble acute CSF followed by a period in which animals will be apparently healthy (Mengeling & Packer, 1969). Nevertheless, over time the pigs will develop unspecific clinical signs, such as stunting, anorexia, intermittent fever, chronic enteritis, wasting and diffuse dermatitis (Blome, Staubach, et al., 2017; Moennig et al., 2003). It is generally acknowledged that these animals will eventually die 2-3 months after infection (Blome, Staubach, et al., 2017; 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).

Animals suffering the chronic form of CSF will develop an inefficient and intermittent antibody response, which is not able to clear the virus (Blome, Staubach, et al., 2017). However, it might not always be detectable, as the antibodies are consumed by the large amounts of virus (Depner et al., 1996). These immune complexes have been reported to accumulate in the kidneys and lead to the development of glomerulonephritis (Choi & Chae, 2003). The generation of an immune response, albeit inefficient, in this form of CSF is what distinguishes the chronic from the persistent forms of CSF. Secondary infections are also common and may exacerbate the clinical signs (Coronado, Bohórquez, et al., 2019).

1.5.3 CSF congenital persistent form

CSFV has the capacity to cross the placental barrier and infect the foetus. Nevertheless, the outcome of trans-placental transmission will depend on the moment of infection and the virulence of the CSFV strain. When viral infection takes place during early gestation (<50 days), it leads to absorption or mummification of the foetuses (Blome, Staubach, et al., 2017; Van Oirschot & Terpstra, 1977). Later in gestation, if foetuses are infected with CSFV high virulence strains, the most likely outcome is foetal death. These piglets will exhibit haemorrhagic lesions in multiple organs, likely caused by the cytokine storm and high viral replication (Muñoz-González et al., 2017; Stewart et al., 1973). More importantly, when low virulence strains infect the foetus between days 50 and 90 of gestation, congenital CSF persistence may be established (Liess, 1984; Van Oirschot, 1979a, 1979b; Van Oirschot & Terpstra, 1977). This is known as the “carrier sow syndrome”.

Congenitally persistent infected piglets may be clinically healthy at birth and will survive for months, before dying of the so-called "late onset" CSF (Moennig et al., 2003; Van Oirschot & Terpstra, 1977). These piglets may show growth retardation, wasting and in some cases even congenital tremor, but the development of more severe clinical signs will take months. After this time, pigs will show anorexia, depression, conjunctivitis, dermatitis and diarrhoea, followed by neurological disorders, leading to paresis and, eventually, death (Moennig et al., 2003; Trautwein, 1988). The survival of these animals has been reported as long as 11 months after birth (Van Oirschot & Terpstra, 1977).

The CSF persistent forms are very important from the epidemiological standpoint, since high viral replication and shedding are hallmarks of this form of disease for both domestic and wild swine(Carbrey et al., 1977; Depner et al., 1995; Kaden et al., 2005; Van Oirschot, 1979a). All of this will be taking place in absence of an immune response, which has been related to the immunotolerance phenomenon, described in section 1.6.1 (Peterhans & Schweizer, 2010; Schweizer & Peterhans, 2014; Van Oirschot, 1979a). The lack of antibodies against CSFV makes these animals undetectable by the currently used techniques for surveillance (OIE, 2019b). Therefore, these animals are acting as reservoirs and disseminators of the virus, maintaining the circulation of CSFV in the farms (Blome, Staubach, et al., 2017; Kaden et al., 2005; Moennig et al., 2003).

1.5.4 CSF postnatal persistent form

Postnatal CSFV persistence was first reported in 2015 (Muñoz-González, Ruggli, et al., 2015). This form of disease was generated after infection of new-born piglets or wild boar within the first hours after birth with a

moderately virulent CSFV strain (Cabezón et al., 2015; Muñoz-González, Ruggli, et al., 2015). These animals were clinically healthy or showed unspecific clinical signs, such as mild diarrhoea or polyarthritis, during a period of up to nine weeks post infection.

The postnatal and congenital CSF persistence forms show a similar profile, in terms of virological and immunological parameters. High viral replication and shedding in absence of adaptive immune response has been detected in both forms of disease (Blome, Staubach, et al., 2017; Muñoz-González, Ruggli, et al., 2015; Van Oirschot, 1979a). Nevertheless, in postnatal persistent infection, an initial innate immune response has been found in the piglets after infection, in terms of IFN-α. Therefore, the pathogen is being recognized by the immune system, in contrast with the immunotolerance mechanism generally acknowledged for the congenital CSF persistent form (Van Oirschot, 1979a). Another difference between congenital and postnatal CSF persistent forms is the apparent blockade of the IFN pathway in the latter. Piglets suffering the congenital persistence form are able to recognize other pathogens and induce an immune response, despite being immunotolerant to CSFV (Carbrey et al., 1977; Trautwein, 1988; Van Oirschot, 1979a; Vannier et al., 1981). By contrast, postnatal persistently infected pigs were not able to induce an IFN-α response against other pathogens once persistent infection was established, even against high IFN-α inducers such as African swine fever virus (ASFV) (Cabezón et al., 2017; Muñoz-González, Pérez-Simó, et al., 2015).

Considering that low and moderate virulence strains are currently circulating in the field, it is highly likely that postnatal and congenital CSF persistence are playing a role in the maintenance of the virus in the field

(Beer et al., 2015; Coronado et al., 2017; Kameyama et al., 2019; Y. Luo et al., 2017; L. J. Pérez et al., 2012). In this regard, a high prevalence of chronic and persistent CSF forms has been recently reported in an endemic situation under an ineffective vaccination program (Coronado, Bohórquez, et al., 2019). These pigs were refractory to vaccination, in line with previous reports for CSFV persistently infected animals (Muñoz-González, Pérez-Simó, et al., 2015).

1.5.5 Superinfection exclusion

The superinfection exclusion (SIE) phenomenon, or homologous interference, is defined as the capacity of a primary viral infection to inhibit a second infection with the same or a closely related virus (Folimonova, 2012; Muñoz-González et al., 2016). The SIE mechanism has been described for multiple viral infections in mammals, bacteria, plants and insects and it has even been applied for pathogen control in crops (Julve et al., 2013; Karpf et al., 1997; Lee et al., 2005; Michel et al., 2005; Ramírez et al., 2010; Soller & Epstein, 1965). Furthermore, it appears to fulfil an evolutionary role, reducing the probability of recombination events and determining the stability of viral sequences within the cell (Formella et al., 2000; Huang et al., 2008; Lee et al., 2005).

Recently, it was demonstrated that once a CSFV postnatal persistently infection was established by a moderate virulence strain, the animals were resistant to infection with a highly virulent CSFV strain (Muñoz-González et al., 2016). Thus, even though not all of the potential target cells are infected in persistently infected animals, the SIE mechanism might be taking place in them at a systemic level. This has been proposed as an explanation for their unresponsiveness to vaccination.

1.6 Mechanisms for CSFV persistence

1.6.1 The immunotolerance phenomenon

One of the defining features of members of the *Pestivirus* genus is their ability to generate viral persistence through the immunotolerance mechanism (Schweizer & Peterhans, 2014). In persistently infected animals, viral replication goes unchecked, as they do not show an immune response against the virus, while being able to develop normal immunity against other pathogens (Schweizer et al., 2006).

In the case of CSFV, infection of pregnant sows with low virulence strains between days 50 and 90 of gestation has been found to generate immunotolerance in the piglets (Blome, Staubach, et al., 2017; Van Oirschot, 1979a). The establishment of immunotolerance hinges on avoiding the innate immune response following trans-placental infection. This is likely achieved through a blockade of the type I IFN pathway by the N^{pro} and E^{rns} proteins (Lussi & Schweizer, 2016; Peterhans & Schweizer, 2010; Schweizer et al., 2006). As previously mentioned, N^{pro} is able to impair the type I IFN response by promoting the degradation of IRF-3 and IRF-7 (Tautz et al., 2015). On the other hand, E^{rns} inhibits the activation of the IFN pathway by Toll-like receptors (TLR) through degradation of ssRNA and dsRNA (Lussi & Schweizer, 2016; Mätzener et al., 2009). This strategy of nucleic acid degradation in order to avoid immune response is similar to the natural mechanisms for "self" and "non-self" recognition in animals and plays a role in the avoidance of autoimmunity (Deane & Bolland, 2006; Karikó et al., 2005). Hence, by interrupting the innate immune response, pestiviruses are also able to bypass the adaptive immunity and continue to replicate in the host.

Some aspects regarding the establishment of immunotolerance remain to be understood. It is still not fully known why this phenomenon has only

been associated with the non-cytopathic ncp biotype, in the case of BVDV, and low virulence strains, in the case of CSFV, despite the fact that all the pestiviruses express the same proteins (Brodersen, 2014; Van Oirschot, 1979a; Van Oirschot & Terpstra, 1977). Furthermore, this phenomenon has been attributed to a lack of pathogen recognition by the immune system, due to its immature status (Blome, Staubach, et al., 2017). This aspect has to be clarified, since mediators of innate and adaptive immunity are developed as early as 26 and 45 days of gestation, respectively (Šinkora & Butler, 2009).

1.6.2 Immunosuppressive mechanisms in CSFV persistence

Taking into account that the immunotolerance phenomenon is directly linked to the immaturity of the foetal immune system (Lussi & Schweizer, 2016; Van Oirschot, 1979a), different mechanisms must be necessary for the establishment of CSFV postnatal persistent infection. However, considering the novelty of the reports for this disease form, these are only now beginning to be elucidated. In this regard, peripheral blood mononuclear cells (PBMC) from CSFV postnatal persistently infected animals have been found to produce abnormally high levels of IL-10 when stimulated with CSFV (Muñoz-González, Ruggli, et al., 2015). Considering that the main function of this cytokine is as a down-regulator of the immune response, IL-10 is likely to be playing an immunosuppressive role during CSFV postnatal persistent infection (Muñoz-González, Ruggli, et al., 2015; Rojas et al., 2017). Nevertheless, multiple capabilities have been attributed to this cytokine during viral infection and its role during CSFV persistence is not entirely understood. Moreover, immunosuppression during the postnatal persistent CSF form is not solely dependent on IL-10, since a blockage of this cytokine was not

able to restore the IFN-γ secreting capabilities of PBMC from persistently infected pigs (Muñoz-González, Ruggli, et al., 2015).

Besides production of immunosuppressive cytokines, CSFV postnatal persistently infected pigs have also been found to have altered cellular populations. An increase in the granulocyte precursor population was detected in the BMHC from these pigs (Muñoz-González, Ruggli, et al., 2015). Remarkably, immunosuppressive capabilities have been reported for some immature myeloid cell populations found in humans and mice (Bronte et al., 2016; Goh et al., 2013). These populations, named myeloid derived suppressor cells (MDSC), were first discovered in 1987, in association with tumour microenvironments (Young et al., 1987). More recently, MDSC have been found to be implicated in viral persistent infection caused by HCV, hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (Lv et al., 2018; Qin et al., 2013; Tacke et al., 2012). These cells favour immune suppression by multiple mechanisms, such as arginase 1, induced Nitric oxide synthases (iNOS), reactive oxygen species and induction of IL-10 synthesis (Beury et al., 2014; Bronte et al., 2003). Nevertheless, the relation between these cell subsets and CSFV persistent infection is yet to be determined.

1.7 Epidemiology, control and surveillance

As previously mentioned in section 1.4, the main route of horizontal CSFV transmission is faecal-oral (Klinkenberg et al., 2002). However, body secretions, blood, tissues and semen can transmit CSFV (De Smit et al., 1999; Floegel et al., 2000; OIE, 2020; Rovid Spickler, 2003). Besides the oral route, direct contact infection can occur through the conjunctiva, mucous membranes, skin abrasions, insemination and percutaneously (Blome, Staubach, et al., 2017; De Smit et al., 1999; Floegel et al., 2000).

In addition, indirect transmission can also take place through contaminated garbage or swill, considering that the virus can remain stable for long periods in protein-rich environments (Edwards, 2000; Fritzemeier et al., 2000). This has led to the prohibition of swill feeding in many countries. Mechanical transmission through contaminated equipment or insects has also been demonstrated, even though there is no evidence of viral replication in invertebrates (van Oirschot, 1999, 2004). Infection through aerosols has been experimentally proven, although it is not believed to play a major role in the field (Dewulf et al., 2000). Vertical transmission via trans-placental infection has been determined at all stages of pregnancy, playing a major role in the generation of persistently infected animals (see section 1.5.3).

The course of a CSFV outbreak can be determined by multiple factors, such as the form of infection or the swine population density (Durand et al., 2009; Koenen et al., 1996; Weesendorp et al., 2009b, 2011). Thus, in acute CSF, excretion of highly virulent CSFV strains in faecal or oro-nasal discharges is high, but these animals will only be a source of infection during a short period before dying. By contrast, in chronic and persistent CSF, infection with low or moderate virulence strains will lead to prolonged excretion, generating up to 40.000 times more virus than in the acute form and playing a major role during outbreaks (Belák et al., 2008; Durand et al., 2009; Weesendorp et al., 2009b, 2011). It is also possible that infection with low virulence strains leads to low excretion over a short period (Terpstra, 1991; Weesendorp et al., 2009b, 2009a).

On the other hand, since the first reports of CSF it has been acknowledged that outbreaks in areas of high animal density lead to extensive epidemics (Fritzemeier et al., 2000; Hanson, 1957). This has been evidenced in

outbreaks such as the one in the Netherlands in 1997/98. In this country, eight outbreaks had taken place in the previous five years in areas of low-pig density. These were managed without major problems, according with the control strategies used in the country (Elbers et al., 1999). However, the 1997 outbreak started in an area with one of the highest pig and herd densities in Europe, which contributed to its impact (Elbers et al., 1999; Pluimers et al., 1999).

Another source of concern is the fact that the CSFV strains currently circulating in endemic countries have been characterized as having low or moderate virulence (Coronado et al., 2017; Floegel-Niesmann et al., 2003; Kameyama et al., 2019; L. J. Pérez et al., 2012; Shen et al., 2011; Tarradas et al., 2014). This will lead to erroneous or delayed diagnosis in the field. These strains may go unnoticed during long periods and spread to larger populations, causing serious damage from an economic and epidemiological standpoint. This was the case in the 2001-2002 CSFV outbreak in Spain (Allepuz et al., 2007).

1.7.1 Molecular epidemiology

CSFV strains are closely related in structural and antigenic terms and, in general, CSFV is highly stable, especially for an RNA virus. This is reflected in the fact that there is only one serogroup of CSFV as antibodies cross-react between strains and even with other pestiviruses (Blome, Staubach, et al., 2017; van Rijn, 2007). Thus, molecular epidemiology studies rely on genetic studies, which in the case of CSFV have been carried out using sequences from the 5'-UTR, N^{pro}, NS5b, 3'-UTR and a 190 nucleotide fragment from the E2 (L. Liu et al., 2009). To facilitate the study of CSFV outbreaks from an epidemiological standpoint, the European Union Reference Laboratory (EURL) for CSF in Hannover

maintains a database of sequences combined with tools for phylogenetic analysis (Postel et al., 2016).

Recently, it was demonstrated that the most reliable results for molecular epidemiology studies could be obtained by analysing the full E2 sequence (Postel et al., 2012; Rios et al., 2017). Using this methodology, CSFV isolates can be divided in three genotypes (named from 1 to 3). These genotypes were further subdivided, with genotypes 1 and 3 having four subgenotypes and genotype 2 having only three (Leifer et al., 2010; Paton et al., 2000; Postel et al., 2012; Postel, Schmeiser, et al., 2013). The genotypes obtained from this analysis can be assigned to different geographical regions. Broadly speaking, according to this approach, genotype 1 is mostly prevalent in Central and South America, although it can also be found in parts of Asia (Díaz De Arce et al., 1999, 2005; Postel, Schmeiser, et al., 2013). On the other hand, all European CSFV isolates after 1990 belong to genotype 2, which can also be found in China, India and Vietnam (Beer et al., 2015; Greiser-Wilke et al., 2000; Leifer et al., 2010). Isolates of genotype 3 appear to occur only in Asia (Figure 3).

In a recent study, an exhaustive characterization comprising the full E2 sequences from 517 CSFV isolates was performed using the maximum likelihood method. The parameters used in this study for genotype and subgenotype differentiation were set at 86% and 91% of identity, respectively. This analysis resulted in the differentiation of CSFV into five genotypes (named from 1 to 5). Genotypes 1 and 2 were the only ones subdivided, in seven subgenotypes each. The methodology used in this study allowed for the correct characterization of certain CSFV strains, which had been assigned to wrong genogroups, based on flawed analysis. Thus, it was found that the Asian isolates within genotype 2 form different

subgenotypes than those previously established. Furthermore, Brazilian and Ecuadorian strains were also assigned to novel subgenotypes within genotype 1. Genotype 3 was restricted to isolates from Taiwan, whereas the novel genotypes 4 and 5 corresponded with a UK strain causing congenital tremor and two South Korean isolates, respectively (Rios et al., 2018).

1.7.2 Control and surveillance of CSF

The strategies for CSFV control can be divided depending on whether or not they contemplate vaccination. So far, the most successful eradication policy has been the so-called "stamping-out", which has been employed in the EU for decades (Elbers et al., 1999; Postel et al., 2018). This strategy requires the elimination of all infected animals, as well as pig herds in contact with them, accompanied by destruction of the carcasses. It also requires rigorous serological monitoring of antibodies against the virus, and therefore is not compatible with vaccination with MLVs, considering the limitations for differentiation of infected and vaccinated animals (DIVA) diagnosis (Moennig, 2000; Postel et al., 2018).

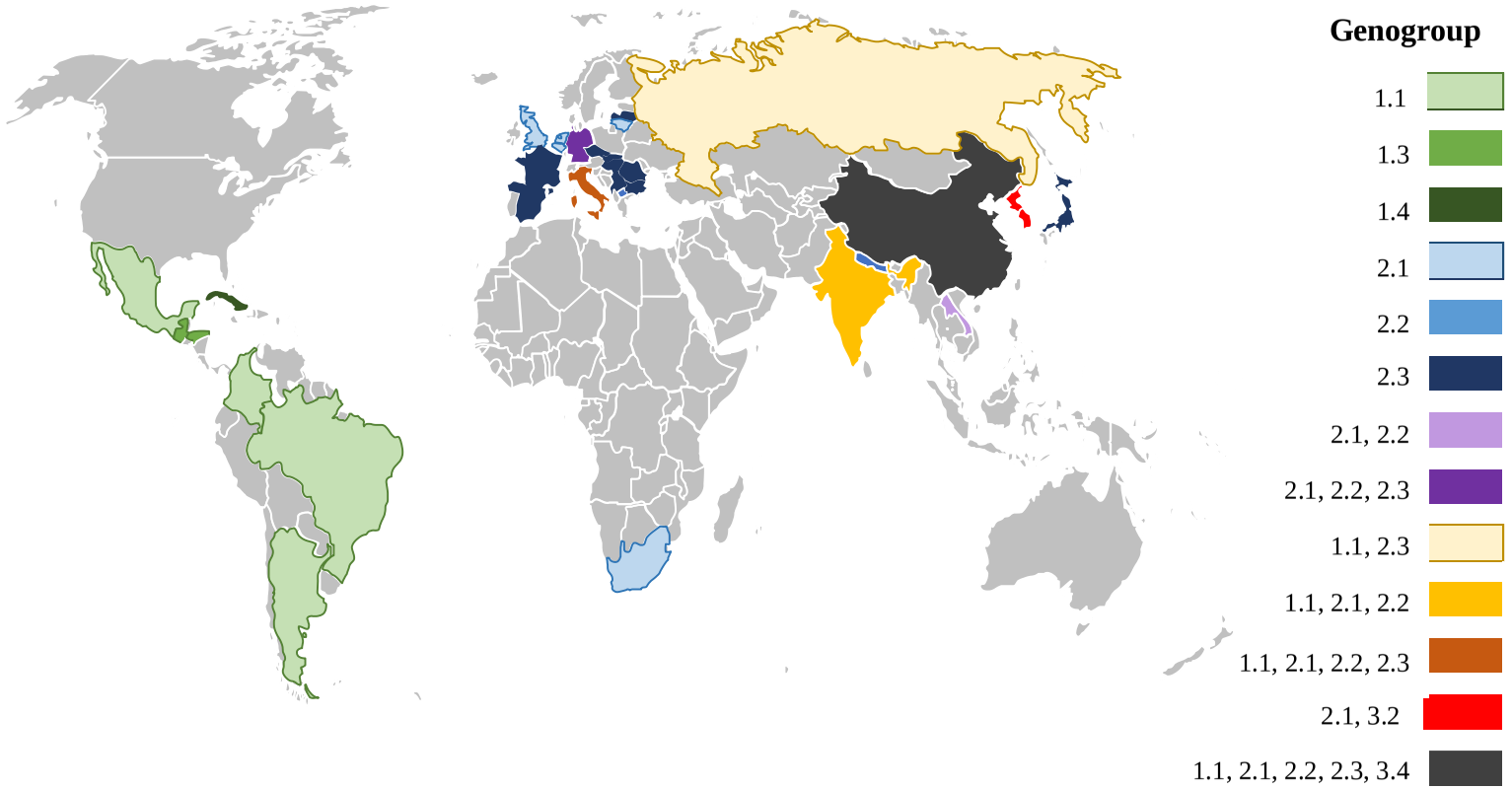


Figure 3. Distribution of CSFV by genogroup over the past two decades. Adapted from (Blome, Staubach, et al. 2017)

Currently, vaccination is permitted in emergency situations around the area of an outbreak in order to limit the spread of the disease (European Union, 2016). However, the vaccinated animals have to be eliminated after the outbreak to regain the status of freedom without vaccination. Moreover, oral vaccination with the C-strain and population control of wild boars is permitted to avoid outbreaks in domestic animals that may originate from wild boar (Brauer et al., 2006; Kaden et al., 2002). In addition, farm biosecurity and strict trade regulation is of the utmost importance in the application of this type of strategy. There are major drawbacks to the implementation of this kind of policy, from an animal welfare and economic standpoint (van Oirschot, 2003). In the Netherlands outbreak of 1997/98, only 8% of all the sacrificed animals were actually CSFV infected. The rest of the sacrificed pigs were pre-emptively slaughtered either for depopulation around the area of the outbreak or due to "welfare slaughter", in which healthy animals outside of the outbreak area have to be sacrificed due to movement and trade restrictions. Incidentally, welfare slaughter represented the highest percentage of the economic losses from the outbreak (Meuwissen et al., 1999).

Vaccination strategies have been successful in the past when implemented correctly and in combination with strict hygiene measures (Greiser-Wilke & Moennig, 2004). Nevertheless, in currently endemic countries, CSFV vaccination programs have been implemented in control programs for over 30 years. Despite these efforts, CSF has not been eradicated yet and lower virulence strains have emerged in these countries (Coronado et al., 2017; Kameyama et al., 2019; Tarradas et al., 2014). Recent studies have shown that the positive selection pressure exerted by ineffective vaccination, has led to the aforementioned viral attenuation (Coronado, Rios, et al., 2019; L. J. Pérez et al., 2012; Shen et al., 2011). Considering this, the currently

circulating strains of lower virulence may favour the generation of persistently infected animals that go unnoticed in surveillance schemes (Coronado, Bohórquez, et al., 2019). As previously mentioned, these animals will be important disseminators of the virus.

1.7.3 CSF diagnosis

None of the clinical signs or pathological lesions found in CSFV infected animals is pathognomonic (Elbers et al., 2003; Moennig et al., 2003). Furthermore, CSF forms can resemble multiple swine diseases. One of the most important examples, due to the current situation worldwide, is African Swine Fever (ASF). The clinical signs and pathological findings for both diseases are strikingly similar and each should always be in the list of differential diagnosis for the other (Gómez-Villamandos, Carrasco, et al., 2003; Schulz et al., 2017). Other viral and bacterial infections (such as Salmonellosis, Erysipelas, Pasteurellosis and Actinobacillosis, among others) can resemble infection with CSFV. Furthermore, secondary infection with bacterial agents can take place after CSFV infection and may mask CSF clinical signs. Thus, laboratory methods are essential for accurate and fast CSF diagnosis, since clinical diagnosis can be highly misleading (van Oirschot, 2004).

The OIE recognizes two types of laboratory tests for CSFV: Agent identification or detection of the immune response (OIE, 2019b). In this regard, four types of agent identification tests are approved. Virus isolation in susceptible cell lines (PK-15 is suggested) is the recommended technique for confirmation of clinical cases. Organ preparations, leukocyte preparations, or whole blood samples can be used (de Smit et al., 1994). Because CSFV is mostly a non-cytopathic virus, the interpretation of the technique relies on peroxidase linked assay (PLA) (Wensvoort et al.,

1986). The tonsil is the most suitable organ for virus isolation, however, spleen, kidney, ileum or lymph nodes can also be used (OIE, 2019b). Considering that viral isolation is a time consuming diagnostic method (taking between 3 and 4 days), the OIE also allows reverse transcription polymerase chain reaction (RT-PCR) tests for the confirmation of clinical cases. Nevertheless, any RT-PCR protocol used must prove to be at least as sensitive as virus isolation (OIE, 2019b). The quantitative RT-PCR (RT-qPCR) protocol described by Hoffmann (Hoffmann et al., 2005) is widely used and it is recommended by the OIE for international interlaboratory comparison testing. Other agent identification methods, like antigen detecting enzyme-linked immunosorbent assay (ELISA) or fluorescent antibody test (FAT), are also recognized by the OIE. However, they are not the recommended methods, considering they show limitations regarding their sensitivity and practicality (Greiser-Wilke et al., 2007).

For the detection of immune responses to the virus, two techniques are approved by the OIE. The ELISA test for the specific detection of antibodies against CSFV is recommended for a variety of applications, ranging from contribution to eradication policies to analysis of the immune status in individuals after vaccination (OIE, 2019b). Nevertheless, ELISA tests can only be validated by serum neutralisation assays. In this regard, ELISA tests should sensitive enough to score positive any serum from infected animals, at least at 21 days post-inoculation, that reacts in the neutralisation test (OIE, 2019b). The most common target for antibody ELISA tests is the E2 glycoprotein, even though there are currently alternatives in the market which detect antibodies against E^{rns} (Pannhorst et al., 2015; Schroeder et al., 2012). These tests, however, are not as sensitive or specific as the anti-E2 antibody ELISAs (OIE, 2019b). Finally, the neutralisation assays are performed in cell cultures using a constant-

virus/varying-serum method. These assays rely on the use of PLA for their interpretation, being named neutralisation peroxidase linked assay (NPLA) (Terpstra et al., 1984). Importantly, NPLA assays allow for the differentiation between the neutralising antibody titres against different CSFV strains and other pestiviruses (Greiser-Wilke et al., 2007). In contrast with antibody ELISA tests, neutralisation assays can be accepted by the OIE for the confirmation of clinical cases, even though they are not the most recommended technique (OIE, 2019b).

1.7.4 Vaccine strategies against CSFV

The first immunization strategy against CSFV was inoculation with hyper-immune sera derived from surviving animals that were repeatedly inoculated with de-fibrinated blood from sick pigs (Dorset, 1906; Dorset et al., 1908). Afterwards, phenolized and crystal violet vaccines were developed and were even used in multiple countries (Cole et al., 1962b). These vaccines were replaced during the first half of the 20th century by MLV, which were quickly found to be more effective (Baker, 1946; Blome, Moß, et al., 2017). The most commonly used MLVs are derived from the lapinized Chinese strain (C-strain), also known as the hog cholera lapinized virus (HCLV). This vaccine was developed in the Harbin Veterinary Research Institute in 1956, by over 480 serial passages of a virulent strain in rabbit (Yuzi Luo et al., 2014). Different attenuation methods, such as cell culture passage at low temperatures, have led to the development of other MLVs, such as the Thiverval, GPE- and PAV strains, produced in France, Japan and Mexico, respectively (Blome, Moß, et al., 2017; Dong & Chen, 2007).

Due to their high efficacy and safety, MLVs are currently used for control programs in endemic countries and have aided in eradication efforts in

some regions (Greiser-Wilke & Moennig, 2004). MLVs are able to induce protective immunity as early as three days after infection, as well as protect against trans-placental transmission (Ganges et al., 2008; Graham, Everett, et al., 2012; Kaden & Lange, 2001; van Oirschot, 2003). Additionally, they have no side effects and are effective even in immunosuppressed individuals (Bironte & Leunen, 1988). The immunity conferred by MLVs is sterilizing, meaning that there is neither viremia nor shedding after challenge and it provides broad protection against all the genotypes of CSFV (de Smit et al., 2001; Graham, Everett, et al., 2012). Furthermore, animals vaccinated with MLVs are protected for over 6 months, probably lifelong (Kaden & Lange, 2001; van Oirschot, 2003). Notwithstanding their tremendous efficacy, MLVs have one major drawback: the immune response elicited by these vaccines does not comply with the DIVA concept (Blome, Moß, et al., 2017; Ganges et al., 2008; van Oirschot, 2003). This rules out their implementation in non-endemic countries and favours restrictive vaccination policies in control programs (Blome, Moß, et al., 2017; Ganges et al., 2008; Kaden et al., 2004).

The current standards established by the OIE require that vaccines against CSFV provide short- and long-term safety for target and non-target species (especially for oral vaccines), stability, rapid induction of a stable, preferably life-long immunity, efficacy against all strains and genotypes of field viruses, full clinical protection and protection against carrier states and prevention of horizontal and vertical transmission (OIE, 2019b). Furthermore, marker vaccines will have to be accompanied by reliable discriminatory tests. Manufacture should provide for consistency of production and validation. In light of this, multiple vaccine strategies have been proposed, aiming to obtain a marker vaccine that complies with the DIVA concept and affords solid and fast protection (Blome, Moß, et al.,

2017; Ganges et al., 2008). Early CSF marker vaccines were based on recombinant CSFV glycoprotein E2 expressed in insect cells (Beer et al., 2007; Hulst et al., 1993; OIE, 2019b). DIVA testing would rely on the detection of antibodies against E^{rns}. However, the delayed onset of immunity, lack of protection against trans-placental infection and increased number of immunizations required are major disadvantages for these subunit vaccines compared with MLV (EMA, 2011; OIE, 2019b).

On the other hand, the most promising marker vaccine candidate developed is based in a chimeric BVDV virus expressing the E2 glycoprotein of the Alfort-187 CSFV strain. This chimera has been named "Cp7_E2alf" and it has been thoroughly evaluated, culminating in its licensing by the European medicines agency (EMA) and authorisation for use in a USA vaccine bank (Blome, Moß, et al., 2017; Blome, Wernike, et al., 2017; EMA, 2019). This vaccine has shown good clinical and virological protection, even in animals with MDA (Blome, Wernike, et al., 2017). This marker vaccine has been authorized to be used only on pigs older than 7 weeks and under emergency vaccination in restricted control zones (EMA, 2019). DIVA testing for these vaccine relies on the detection of antibodies against E^{rns} and accompanying diagnostic kits have been developed and commercialized (Leifer et al., 2009; Pannhorst et al., 2015). The drawback of this strategy is the late onset of antibodies against E^{rns} (3-6 weeks post infection) as well as its cross-reaction with BVDV and BDV, which reduces its specificity. Additionally, Aspects regarding their protection against trans-placental transmission remain to be elucidated and hence, the use of these vaccines in sows is not yet permitted (Blome, Moß, et al., 2017; EMA, 2019; Henke et al., 2018).

Chapter 2

Objectives

Understanding the virus-host interaction in persistent infection forms is the cornerstone for the design of diagnostic tools and immunization strategies that can be effective against all forms of CSF. This is particularly important in endemic countries, where the alimentary security is affected by the sanitary status of sources of animal protein, such as pork.

Taking into account the epidemiological importance of the persistent CSF forms, their control is highly relevant in eradication efforts. This task poses a challenge from a diagnostic and vaccination standpoint. Notably, these animals are refractory to vaccination and do not develop antibody response against the virus, making them undetectable by the serological methods currently used in surveillance programs.

The insight obtained from the study of CSFV persistently infected animals might be of great value for the generation of antiviral strategies in other models of viral persistent infection. Taking into account that viral persistence occurs across multiple species, it is possible that the cytokines and cell populations playing a role in CSFV persistent infection are similar to those used by other viruses causing viral persistence in other species, such as humans. In this scenario, CSFV persistently infected pigs could provide a model for the study of the immunological and virological basis for persistent infection.

Considering this background, the present thesis aimed to study the immunological mechanisms involved in the establishment and maintenance of CSFV persistent infection, the role of cellular subsets and markers of altered immune states in these forms of infection and the virus-host interaction after CSFV foetal and postnatal infection. The specific objectives were as follows:

- To evaluate the capacity for vertical transmission by trans-placental infection in CSFV strains with high, moderate and low virulence degree and to determine the relationship between the replication rate and vertical transmission in CSFV. Additionally, to analyse the immune response induced by the different strains in sows and their litters (Study I).
- To determine the capability of the CSFV Catalonia strain of moderate virulence to generate congenital CSF persistence. Also, to assess the viral replication in sows and piglets, as well as the capability of these animals to generate immune response (Study I).
- To evaluate the capacity of the moderately virulent CSFV Catalonia strain, previously proven to induce the postnatal persistent CSF form following infection during the first hours after birth, to generate this form of disease in animals at weaning age (21 days). Moreover, to determine the relation between markers related with altered immune states and CSF persistently infected animals (Study II).
- To study the presence of granulocyte precursor cell populations, previously identified to be increased in BMHC postnatal persistently infected animals, in the bloodstream of these animals. Likewise, to gain insight into their phenotypical and functional profile in terms of inhibition of the immune response against specific and unspecific antigens, as well as to determine their immunosuppressive role as a candidate MDSC population Study III).

Part II

Studies

Chapter 3

Study I. Foetal immune response activation and high replication rate during generation of classical swine fever congenital infection *(Pathogens. 2020;9(4):285)*

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3.1 Abstract

Classical swine fever virus (CSFV) induces trans-placental transmission and congenital viral persistence; however, the available information is not updated. Three groups of sows were infected at mid-gestation with either a high, moderate or low virulence CSFV strains. Foetuses from sows infected with high or low virulence strain were obtained before delivery and piglets from sows infected with the moderate virulence strain were studied for 32 days after birth. The low virulence strain generated lower CSFV RNA load and the lowest proportion of trans-placental transmission. Severe lesions and mummifications were observed in foetuses infected with the high virulence strain. Sows infected with the moderately virulence strain showed stillbirths and mummifications, one of them delivered live piglets, all CSFV persistently infected. Efficient trans-placental transmission was detected in sows infected with the high and moderate virulence strain. The trans-placental transmission occurred before the onset of antibody response, which started at 14 days after infection in these sows and was influenced by replication efficacy of the infecting strain. Fast and solid immunity after sow vaccination is required for prevention of congenital viral persistence. An increase in the $CD8^+$ T-cell subset and IFN-alpha response was found in viremic foetuses, or in those that showed higher viral replication in tissue, showing the CSFV recognition capacity by the foetal immune system after trans-placental infection.

3.2 Keywords

classical swine fever; virulence; trans-placental transmission; persistent congenital infection; foetal immune response; classical swine fever virus; replication; sows

3.3 Introduction

Classical swine fever virus (CSFV) is one of the most relevant viruses in the *Pestivirus* genus, being the causative agent of classical swine fever (CSF), a highly impactful disease for the porcine industry worldwide (Blome, Staubach, et al., 2017). The capacity of pestiviruses to generate persistent infection by trans-placental transmission has already been described (Aynaud et al., 1977; Carbrey et al., 1977; Liess, 1984; Van Oirschot, 1979a; Van Oirschot & Terpstra, 1977). Particularly, low virulence CSFV strains have been related to the development of congenital viral persistence in their offspring when infection of the sows occurs between 50 and 90 days of gestation (Aynaud et al., 1977; Blome, Staubach, et al., 2017; Carbrey et al., 1977; Van Oirschot, 1979a; Van Oirschot & Terpstra, 1977). Piglets that develop this form of infection are born infected, showing high viral replication and shedding in the absence of specific antibody response (Carbrey et al., 1977; Van Oirschot & Terpstra, 1977; Vannier et al., 1981). This type of viral persistence has been explained by the immunotolerance mechanism, due to a lack of CSFV recognition by the immature immune system of the foetus (Van Oirschot, 1979a).

CSF still remains endemic in countries in Asia, the Caribbean, and Central and South America (Blome, Staubach, et al., 2017). Previous studies have demonstrated the evolutionary capacity of CSFV towards less virulent strains in endemic situations under inefficient vaccination programs (L. J. Pérez et al., 2012; Rios et al., 2017). In this type of

scenario, a recent study showed that CSF persistence was the predominant form, favouring virus prevalence and hampering the control tools (Coronado, Bohórquez, et al., 2019).

CSFV also has the ability to generate viral persistence after postnatal infection, although unlike the congenital persistence forms, the generation of postnatal persistence has been associated with the CSFV moderate virulence strains (Bohórquez, Wang, et al., 2019; Muñoz-González, Ruggli, et al., 2015). Previous studies have also shown that moderate virulence strains are widely distributed (Beer et al., 2015; Henke et al., 2018; Y. Luo et al., 2017). In this regard, the strain of CSFV that recently caused an epidemic in Japan after 26 years has been characterised to be of moderate virulence (Kameyama et al., 2019; Postel et al., 2019).

Despite the known capacity of CSFV to be transmitted by the trans-placental route and to induce persistent congenital infection, few scientific works have dealt with the immunopathogenesis of this form of the disease, especially from a virus–host interaction standpoint. Considering this background, the aim of this work is to evaluate the capacity of CSFV strains with different virulence degrees to infect pregnant sows and its relation with the vertical transmission by trans-placental infection of fetuses. Likewise, the implication of the virulence degree in the generation of CSFV congenital persistent infection is also assessed. The levels of viral replication, as well as the immune response, in terms of cytokine production and changes in immune system cell populations were evaluated in foetuses and piglets from the infected sows.

3.4 Materials and Methods

3.4.1 Cells and viruses

Production of the viral strains was carried out by infecting susceptible cells with viral suspensions in 2% *Pestivirus-free* foetal bovine serum using the porcine kidney cell line PK-15 (ATCC CCL 33, Middlesex, England), cultured in Eagle's minimum essential medium supplemented with 5% foetal calf serum. Following the infection, cells were incubated at 37 ºC in 5% CO_2, and after 72 h, the virus was harvested. Peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral titration following the statistical methods described by Reed and Muench (Reed & Muench, 1938). The CSFV PdR and the Margarita strains, both belonging to the 1.4 subgenotype (Díaz De Arce et al., 1999; Postel, Schmeiser, et al., 2013), have been characterised as low and high virulence strains, respectively (Coronado et al., 2017; Ganges et al., 2005). The Cat01 strain, which belongs to subgenotype 2.3, was selected as a moderate virulence prototype (Tarradas et al., 2014).

3.4.2 Experimental design

Six pregnant sows (Landrace) of 68 days of gestation, from a commercial farm, were housed in the biosafety level 3 (BSL3) animal facility at CReSA (Barcelona, Spain). The animals were purchased from *Pestivirus-free* farms, and they were also checked for antibodies against CSFV before arriving at the CReSA facilities. Animals were numbered from one to six and distributed in three groups (from A to C), each group in a separate box with standard facilities for pregnant sows. In accordance with the previously established methodology to evaluate the capacity of CSFV for trans-placental transmission, two sows were included in each experimental group (Muñoz-González et al., 2017; OIE, 2019b). After five days of acclimatisation period (74 days of gestation),

Sows 1 and 2 (Group A) were inoculated with the CSFV Margarita strain, Sows 3 and 4 (Group B) with the PdR strain and Sows 5 and 6 (Group C) with the Cat01 strain. The viral dose for all the inocula was 10^5 $TCID_{50}$ per animal, and the inoculation was carried out by intramuscular injection in the neck (Leifer et al., 2012; Muñoz-González et al., 2017; Tarradas et al., 2014). After infection, a trained veterinarian recorded clinical signs daily in a blinded manner. Two experiments were carried out, Experiment 1 included Groups A and B, while Experiment 2 included the Group C sows.

In Experiment 1 of the trial, serum and nasal and rectal swab samples were collected on the day of infection and at 4, 8, 14, and 22 dpi, which corresponded with days 74, 78, 82, 88 and 96 of gestation, respectively. At this time, the sows were euthanized, following the accepted procedures accordingly with the European Directive 2010/63/EU. Whole blood in EDTA was obtained in the day of infection and before euthanasia for ex vivo collection of PBMCs. After necropsy, tissue samples from tonsil and Peyer's patch were collected (Muñoz-González et al., 2017). In parallel, the foetuses from all gilts were obtained, following procedures previously described to avoid foetal distress (Mellor et al., 2005; Muñoz-González et al., 2017). All foetuses were subjected to an exhaustive necropsy in which the presence of macroscopic lesions in different organs was evaluated (Ahrens et al., 2000). Sera and whole blood samples and tissues (tonsil, spleen, and thymus) were collected from 13 foetuses per each sow.

In Experiment 2, sera samples were collected on the day of infection and at 7, 14, 21, and 28 dpi (days 81, 87, 95, and 102 of pregnancy, respectively). At farrowing, rectal swabs were collected from all piglets.

Sows were kept with their litters for 21 days, and, after removal of the sow, piglets were fed an age-appropriate diet (StartRite, Cargill, Spain) until the end of the trial. The handling of the piglets was performed following previously described protocols (Muñoz-González, Ruggli, et al., 2015).

Serum and nasal and rectal swabs were collected from piglets at 7, 15, 23, 27, and 32 dpb. At this time, whole blood samples were collected and piglets were euthanized following procedures according to the European Directive, using a pentobarbital overdose of 60–100 mg/kg of weight, administered via the jugular vein. In addition, sows and piglets were euthanized before the end of the trial if they presented clinical signs compatible with severe CSF or exhibited prostration behaviour, in accordance with previous studies (Tarradas et al., 2014). The experiment was approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB), according to existing Spanish and European regulations.

3.4.3 Detection of CSFV RNA

The NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) was used in order to extract RNA from sera and nasal and rectal swab samples, as well as from organ samples, following the protocol provided by the manufacturer. In all cases, a final volume of 50 µL of RNA was extracted from an initial sample volume of 150 µL. The detection of viral RNA was carried out by a previously described RT-qPCR assay (Hoffmann et al., 2005), validated in our laboratory for the detection of CSFV RNA in sera, nasal, and rectal swabs and tissue samples (Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2014). Samples were considered positive when the Ct values were equal to or less than 42. In

addition, using the Ct value, samples were determined to have either high (Ct value below 23), moderate (between 23 and 28), or low (Ct value above 28) CSFV RNA load, as previously described (Tarradas et al., 2012; M. Wang et al., 2019). Samples in which fluorescence was undetectable (Undet) were considered negative.

3.4.4 Determination of E2-specific and neutralising antibodies

CSFV E2-specific antibodies were evaluated in sera from sows, foetuses, and piglets, using a commercial ELISA kit (IDEXX Laboratories, Liebfeld, Switzerland). Positive results were considered when the blocking percentage was ≥40%, following the manufacturer's recommendations. Additionally, neutralising antibodies against the respective infecting strain were determined using an NPLA assay (Terpstra et al., 1984); thus, animals from Groups A, B, and C were evaluated for neutralising antibodies against the Margarita, PdR, and Cat01 strain, respectively. The neutralising antibody titres were expressed as the reciprocal dilution of serum that neutralised 100 TCID of 50% of the culture replicates.

3.4.5 IFN-α ELISA test in serum samples

IFN-α concentration was determined in sera from foetuses and sows from Groups A and B, as well as piglets from Group C at 7 and 15 dpb, using a previously described in-house ELISA test (Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2010). Briefly, plates were coated overnight with an anti-IFN-α monoclonal antibody (K9 clone, PBL Biomedical Laboratories, Piscataway, New Jersey, USA). After washing, 50 µl of serum samples and serial dilutions of IFN-α recombinant protein (PBL Biomedical Laboratories) were plated by duplicate and incubated for 1 hour at 37 ºC. Afterwards, plates were

washed, and a biotinylated anti-IFN-α antibody was added (F17 clone, PBL Biomedical Laboratories). Following an incubation of 1 hour at 37 ◦C, the plates were washed, and streptavidin-HRP was added. Finally, after a 30-minute incubation, 3,3′,5,5′-tetramethylbenzidine (TMB) was used for revealing the technique, using H_2SO_4 1N as a stop solution. Plates were read at 450 nm, and cytokine concentrations (units/ml) were determined using a regression line built with the optical densities of the cytokine standards used in the test.

3.4.6 ELISA detection of IFN-γ and sCD163

IFN-γ and sCD163 were analysed in sera from foetuses and sows from Groups A and B. Commercial ELISA test was used for detection of IFN-γ (IFN-γ ELISA Kit, Porcine, Life Technologies), following the manufacturer's instructions and the results were expressed as picograms per millilitre (pg/ml). Finally, a formerly described ELISA using lysates from CD163 transfected CHO cells as standard was used to quantify sCD163 (Cabezón et al., 2017; C. Pérez et al., 2008). Results were expressed as the equivalent numbers of CD163-transfected CHO cells (ENC).

3.4.7 PBMCs collection and flow cytometry assay

PBMCs were obtained from whole blood collected at the time of necropsy from three animals of each group in Experiments 1 and 2 of the trial, previously characterised by RT-qPCR. Cells were separated by density-gradient centrifugation with Histopaque 1077 (Sigma-Aldrich St. Louis, MO, USA), followed by osmotic shock in order to eliminate the remaining red blood cells. The number and viability of the PBMCs were determined by staining with Trypan Blue (Ganges et al., 2005). Additionally, thymocytes were obtained from three uninfected foetuses,

and whole blood samples were also collected from three uninfected foetuses and piglets at the same time of gestation/days after birth as the foetuses from Experiment 1 or the piglets from Experiment 2, respectively.

The phenotypic profile of PBMCs from foetuses and piglets was evaluated by flow cytometry. Single staining was performed using the mAbs to porcine CD4 (74-12-4, IgG2b) Alexa Fluor 647 conjugate (BD Biosciences), and CD8-α (76-2-11, IgG2a) FITC-labelled (BD Biosciences, Franklin Lakes, NJ, USA).

The staining protocols were performed as previously described (Bohórquez, Wang, et al., 2019; Muñoz-González, Ruggli, et al., 2015). After staining, cells were filtered and passed in the cytometer (FACSAria IIu, BD Biosciences), with 10,000 cell events being recorded for each sample. The cells were analysed by FACSDiva software, version 6.1.2 and the results were expressed as the percentage of positive cells obtained for each staining, using irrelevant isotype-matched mAbs as staining controls.

3.5 Results

3.5.1 Clinical Evaluation of Sows Infected with Pinar del Rio (PdR) vs. Margarita CSFV Strains

In the first experiment, aiming to determine the capacity of CSFV strains of different virulence levels to induce trans-placental infection, two groups of pregnant sows were inoculated with CSFV at 74 days of gestation. Group A (Sows 1 and 2) was infected with the highly virulent CSFV Margarita strain, while Group B (Sows 3 and 4) were inoculated with the low virulence PdR strain. Clinical signs were recorded daily by a trained veterinarian in a blinded manner.

After inoculation, both CSFV Margarita-infected sows (Group A) showed anorexia and apathy between 6 and 11 days post-infection (dpi). Subsequently, Sow 2 started to eat normally, whereas the clinical condition of Sow 1 deteriorated progressively, showing constipation/diarrhoea, some peaks of fever, evident weight loss, and, eventually, weakness of the hindquarters. This animal was euthanized at 17 dpi (91 days of gestation) for animal welfare reasons, while the remaining sows were euthanized at 22 dpi (96 days of gestation). Both Margarita infected sows showed similar lesions at necropsy, consisting of petechiae in the kidneys, stomach, and intestine, and, in the case of Sow 1, also in the urinary bladder. Conversely, Sows 3 and 4, inoculated with the PdR strain, remained healthy throughout the study, and no lesions related to CSFV infection were found at necropsy.

3.5.2 CSFV RNA level detected in sows after infection with Margarita or PdR strains

CSFV RNA was evaluated by reverse transcription-quantitative PCR (RT-qPCR) (Hoffmann et al., 2005) in serum samples collected weekly and tissue samples collected at necropsy. The RNA load was characterised as high, moderate or low in accordance with the cycle threshold (Ct) value, as described in the materials and methods section. The CSFV RNA load detected in sera oscillated from moderate to low load (Ct value from 28 to 35) regardless of the virulence degree of the strain used to infect the sows. The RNA was detected at 8 dpi in all the animals in Group A and B (infected with Margarita or PdR strains, respectively). However, at 14 dpi, and until the end of the experiment, samples from the two animals in Group B and from Sow 2 (Group A) were negative (Figure 1A). Notably, only Sow 1 infected with the CSFV

Margarita strain was positive at 14 dpi and at the time of euthanasia (17 dpi), although with low RNA load (Ct value 34).

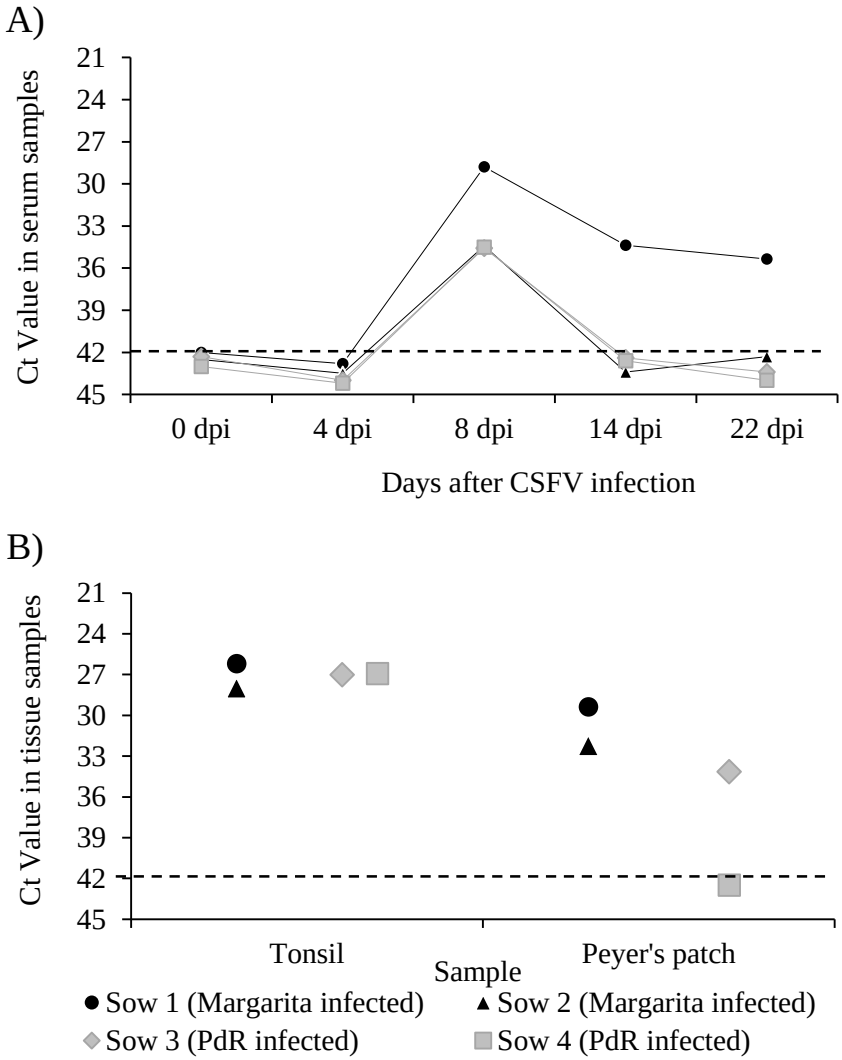

Figure 1. Classical swine fever virus (CSFV) RNA detection by RT-qPCR in sow samples. (A) RNA levels detected in sera at different times post-infection. (B) RNA levels detected in tissues from sows infected with either the CSFV Margarita (black symbols) or PdR strain (grey symbols). Cycle threshold (Ct) values over 42 (dotted line) were considered as negative. Asterisk indicates the animal that was euthanized at 17 dpi.

In the tissue samples, the CSFV RNA load detected in the tonsils samples was similar in both experimental groups, with Ct value around 27 (moderate RNA load). The viral RNA load in Peyer's patch samples was also similar for both groups, with the exception of Sow 4 (PdR infected), which was negative (Figure 1B).

3.5.3 The high virulence CSFV strain Margarita elicited faster and higher humoral response than PdR strain in the infected sows

Specific anti-E2 and neutralising antibodies were evaluated weekly in sera by ELISA and neutralisation peroxidase linked assay (NPLA) [19], respectively. Anti-E2 antibodies were detected in both of the CSFV Margarita-infected sows (Group A) at 14 dpi and at the time of euthanasia. In Group B, infected with the PdR strain, only one animal showed anti-E2 antibodies at 22 dpi (Figure 2A).

Similarly, both of the Margarita-infected sows showed neutralising antibody titers by NPLA assay starting at 14 dpi, which increased at 17 and 22 dpi for Sows 1 and 2, respectively. In the case of PdR infected sows, neutralising antibody response was only detected in Sow 3 at 22dpi, while sow 4 did not show neutralising antibodies throughout the whole trial (Table 1).

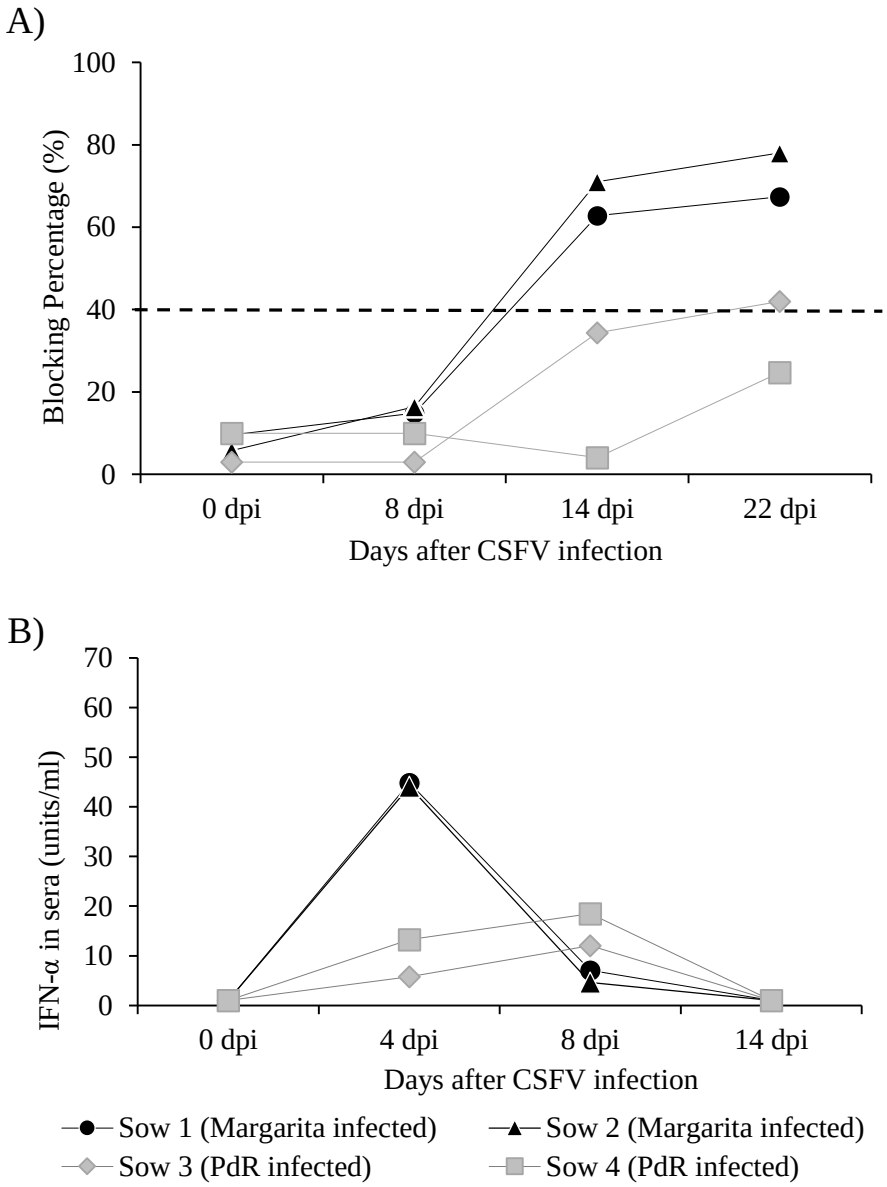

Figure 2. Immune response of sows after CSFV infection. (A) CSFV specific anti-E2 antibody response against the E2 glycoprotein detected by ELISA (in blocking %), values above 40% (dotted line) being considered as positive. (B) Interferon alpha (IFN-α) response in serum determined by ELISA test from sows infected with either the CSFV Margarita (black symbols) or PdR strain (grey symbols). The IFN-α concentration in sera is expressed as units/mL. Asterisk indicates the animal that was euthanized at 17 dpi.

Table 1. Neutralising antibody response in sows after CSFV infection.

		Time after CSFV infection			
Sow ID		0 dpi	8 dpi	14 dpi	22 dpi
Margarita	1	0	0	1 : 640	1 : 1280
infection group	2	0	0	1 : 320	1 : 960
PdR infection	3	0	0	0	1 : 60
group	4	0	0	0	0

3.5.4 IFN-α and IFN-γ response in sows infected with high or low virulence CSFV strains

Interferon alpha (IFN-α) and interferon gamma (IFN-γ) were evaluated by ELISA test in sera from sows at different time-points after infection. IFN-α was detected in the sera of all the sows from Groups A and B at 4 and 8 dpi. Notably, the highest levels were registered in sows from Group A (Sows 1 and 2) at 4 dpi (Figure 2B). For IFN-γ, no detectable levels in sera were found in either experimental group after infection.

3.5.5 Evaluation of the foetuses from CSFV infected sows at necropsy

Foetuses from the CSFV Margarita-infected sows showed internal haemorrhages in tonsil, intestine, kidneys, lymph nodes and spleen. Furthermore, four of the foetuses from Sow 1 and three foetuses from Sow 2 had generalised haemorrhagic lesions in the skin (data not shown). Additionally, one mummified foetus was found in both of them. Conversely, foetuses from Sows 3 and 4, inoculated with the PdR low virulence strain, showed no lesions at necropsy.

3.5.6 Vertical transmission and CSFV replication in the foetuses

Following hysterectomy, serum and tissue samples were collected from all the foetuses, about two weeks before the expected delivery day, in order to determine CSFV transmission from sows to their foetuses. All the foetuses from Group A were RT-qPCR positive with high CSFV RNA load (Ct values between 15.67 and 23) in the majority of sera, tonsil, spleen and thymus samples (see Table 2). In the different organs, the mean Ct value ranged from 17.28 to 20.33 for foetuses from Sows 1 and 2, respectively. By contrast, only 3 out of 13 (23%) foetuses from each of the PdR-infected sows were positive in sera by RT-qPCR, ranging from high to low RNA load (Table 2). However, after analysis by RT-qPCR of tonsil, spleen and thymus samples, the number CSFV positive foetuses increased to 11 out of 13 (Sow 3) and 9 out of 13 (Sow 4), respectively, with high to low CSFV RNA load in the positive tissues.

Table 2. Detection of CSFV RNA in foetuses from sows infected with CSFV Margarita or PdR strain

Margarita infection Group A	CSFV RT-qPCR (Ct value)				PdR infection Group B	CSFV RT-qPCR (Ct value)			
Fetus ID	Serum	Tonsil	Spleen	Thymus	Fetus ID	Serum	Tonsil	Spleen	Thymus
Foetus from Sow 1					*Foetus from Sow 3*				
1	19,65	18,52	17,91	16,54	1	Undet.	Undet.	34,65	Undet.
2	23,13	20,61	19,56	25,86	2	Undet.	Undet.	Undet.	Undet.
3	20,40	21,96	19,11	15,70	3	Undet.	32,73	29,94	35,45
4	21,35	26,58	18,95	16,85	4	Undet.	Undet.	32,44	Undet.
5	18,48	19,69	17,00	19,18	5	Undet.	Undet.	37,43	Undet.
6	17,65	19,59	16,14	15,92	6	Undet.	35,32	33,82	Undet.
7	18,71	20,53	16,59	16,46	7	22,42	22,75	15,86	17,69
8	23,23	19,92	17,75	15,56	8	Undet.	36,67	Undet.	Undet.
9	18,73	16,13	16,24	14,70	9	Undet.	Undet.	Undet.	Undet.
10	19,55	18,78	16,85	17,31	10	Undet.	36,60	34,56	Undet.
11	20,56	23,52	18,45	17,18	11	24,76	23,18	18,30	21,15
12	17,58	18,05	16,62	16,31	12	32,36	23,37	23,68	24,85
13	17,27	18,61	16,62	16,37	13	Undet.	33,91	31,51	36,33
Mean	**19,72**	**20,33**	**17,49**	**17,28**	**Mean**	**38,50**	**37,19**	**33,28**	**36,33**
Desvest	**2,05**	**2,72**	**1,22**	**2,92**	**Desvest**	**7,16**	**5,88**	**9,89**	**9,02**
Foetus from Sow 2					*Foetus from Sow 4*				
1	20,30	25,16	17,84	20,28	1	Undet.	Undet.	Undet.	Undet.
2	18,68	17,92	17,44	15,79	2	28,26	28,36	18,37	21,79
3	18,50	19,28	17,43	16,95	3	Undet.	35,01	28,32	28,24
4	27,06	28,74	25,01	24,94	4	Undet.	34,59	36,29	Undet.
5	18,94	16,87	17,36	15,70	5	Undet.	Undet.	Undet.	36,54
6	19,51	18,93	17,44	16,78	6	Undet.	Undet.	Undet.	Undet.
7	16,81	18,01	16,93	16,78	7	Undet.	Undet.	Undet.	Undet.
8	16,60	17,25	23,57	17,51	8	Undet.	Undet.	Undet.	36,58
9	18,65	18,36	16,47	16,85	9	Undet.	Undet.	Undet.	Undet.
10	20,67	22,67	17,22	16,97	10	Undet.	Undet.	34,36	Undet.
11	16,91	18,37	17,26	16,92	11	34,54	29,63	26,51	25,84
12	16,94	18,37	18,96	17,66	12	Undet.	36,52	Undet.	Undet.
13	15,67	16,88	16,41	16,26	13	21,52	20,59	18,58	18,32
Mean	**18,75**	**19,30**	**18,46**	**17,43**	**Mean**	**38,87**	**36,88**	**35,16**	**36,70**
Desvest	**2,99**	**3,34**	**2,81**	**2,44**	**Desvest**	**6,69**	**7,01**	**9,21**	**8,55**

Undet.: Undetectable

3.5.7 Immune response in the foetuses from CSFV infected sows

Absence of CSFV specific humoral response was found in sera from all the foetuses in the study. However, IFN-γ levels were detected only in serum sample of five out of the 13 foetuses from the Sow 1 (infected with Margarita strain), in values ranging between 23.2 and 130.9 pg/ml. In addition, detectable levels of IFN-α were also registered in 11 samples in foetuses from both Margarita-infected sows (Table 3). Interestingly, foetuses from sows infected with the low virulence strain (PdR) showed higher levels of IFN-α (between 100 to 200 units/ml). Notably, the positive values were found in the foetuses that were CSFV RNA positive for the four samples analysed or in those that showed the higher CSFV RNA load in the tissue samples (Table 2 and Table 3). Finally, detectable levels of soluble CD163 (sCD163) were found in foetal sera samples from both experimental groups, being about 10 times higher the concentration in samples from Group A (Figure 3).

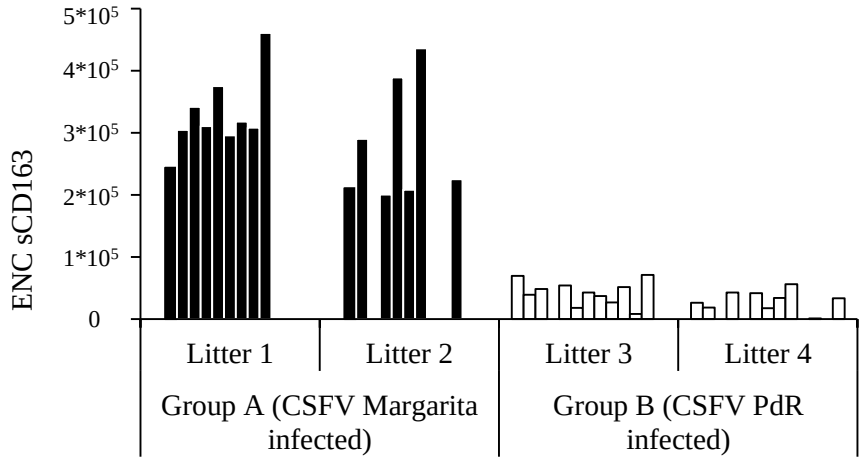

Figure 3. sCD163 levels in foetal sera. Foetuses from sows infected with either the Margarita (black bars) or PdR (white bars) CSFV strains are represented. Results are expressed as the equivalent number of copies of CD163 transfected cells.

Table 3. IFN-α levels in foetal serum

Margarita infection group A		PdR infection group B	
Foetus ID	IFN-α	Foetus ID	IFN-α
Foetus from Sow 1		*Foetus from Sow 3*	
1	48,5	1	0,0
2	0,0	2	0,0
3	0,0	3	60,3
4	237,7	4	0,0
5	0,0	5	0,0
6	18,7	6	0,0
7	0,0	7	116,7
8	0,0	8	0,0
9	0,0	9	0,0
10	31,3	10	0,0
11	0,0	11	102,7
12	8,0	12	209,6
13	0,0	13	0,0
Foetus from Sow 2		*Foetus from Sow 4*	
1	0,0	1	0,0
2	25,5	2	227,8
3	26,7	3	239,5
4	49,3	4	0,0
5	0,0	5	0,0
6	0,0	6	0,0
7	0,0	7	0,0
8	14,3	8	0,0
9	0,0	9	0,0
10	12,2	10	0,0
11	43,2	11	173,4
12	0,0	12	0,0
13	0,0	13	126,9

3.5.8 Phenotypical profile in foetal PBMCs after CSFV infection

Samples from whole blood were obtained from three foetuses in each infected group, and peripheral blood mononuclear cells (PBMCs) were isolated. Flow cytometry analysis was performed to study the phenotypical profile in these cells. The PBMCs analysed corresponded with foetuses that showed CSFV RNA levels in serum samples.

Additionally, PBMCs of three foetuses from uninfected sows, from the same farm of origin, were also analysed to use as reference, uninfected controls. The $CD4^+$ T-cell subset ranged from 4% to 16% of PBMC from the Margarita infected foetuses (Group A), showing a reduction in two out of three samples analysed with values below 5% (Figure 4). This cell population ranged from 14% to 17% in the three foetal PBMC tested from Group B, while a wider range was detected in the PBMC from naïve samples (from 11% to 38%). By contrast, the $CD8^+$ T-cells were increased in the CSFV infected foetuses, with percentages between 29% and 56% in Group A, and 20% to 27% in Group B (infected with PdR strain), whereas it was always below 15% for the naïve samples (Figure 4).

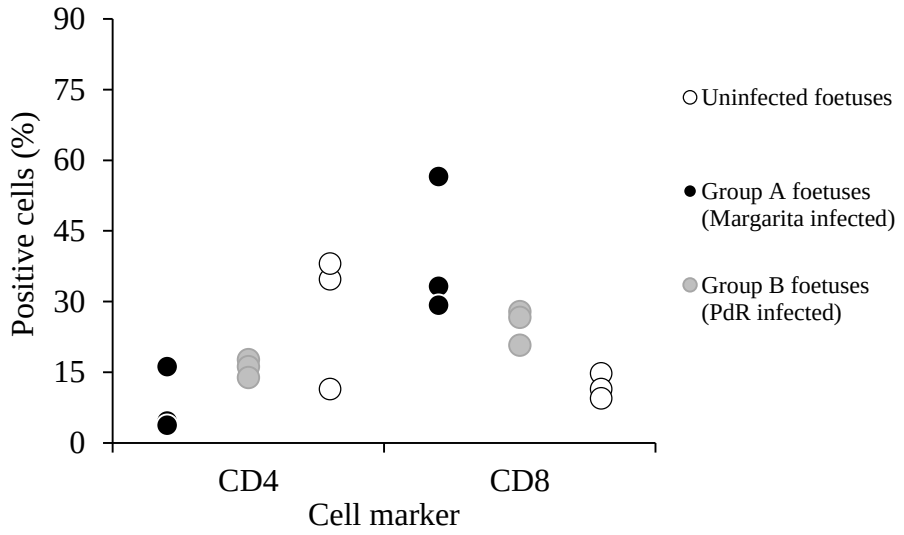

Figure 4. Comparative expression of the T-$CD4^+$ and T-$CD8^+$ cell subsets in PBMCs from uninfected foetuses (white dots), foetuses infected with the Margarita (black dots) or the PdR (grey dots) CSFV strain are shown.

3.5.9 Infection with the CSFV moderately virulent strain: Clinical signs and CSFV replication in sows

In the second experiment, in order to evaluate the capacity of a CSFV moderately virulent strain to induce trans-placental infection and congenital viral persistence, two pregnant sows (Sows 5 and 6) were inoculated with the Catalonia 01 (Cat01) strain. As in Experiment 1, the infection was carried out at 74 days of gestation, and a trained veterinarian recorded clinical signs daily.

The Cat01 infected sows did not show any clinical signs after inoculation. However, at 34 dpi (108 days of gestation), Sow 6 went into early labour and gave birth to eight stillbirths and two live piglets. All the stillbirths showed haemorrhagic lesions, whereas the live piglets were very weak and had to be euthanized on the same day for ethical reasons. The sow was also euthanized at this time. Both Cat01 infected sows were CSFV RNA positive in sera, and rectal and nasal swabs at 7dpi. The CSFV RNA load was low in all the samples, with Ct values ranging from 31 to 37. Afterwards, both sows cleared the virus, only Sow 6 was positive in rectal swab at 28 dpi, although at low RNA concentration (Ct 35.59) (data not shown).

3.5.10 Vertical transmission and congenital viral persistence generated by the moderate virulence CSFV strain

At 114 days of gestation, Sow 5 gave birth to eight live piglets and six stillbirths, the live animals were active and fed normally from the mother immediately after birth. During the seven days after farrowing, two piglets were found dead in the pen, having being crushed by the sow, whereas no clinical signs were registered in the remaining six animals (Piglets 1 to 6). Piglets 1, 3, and 5 remained clinically healthy during the

32 days of the trial. Meanwhile, the other three piglets (Piglets 2, 4, and 6) developed sporadic fever peaks (below 41 ºC) from day 10 until the end of the study. Piglet 6 developed mild polyarthritis from day 10, and Piglets 2 and 4 at days 30 and 23, respectively. Notably, at the time of euthanasia, the piglets weighed around 8.5 kg and continued to show normal feeding behaviour.

On the day of birth, the piglets were positive by RT-qPCR with high CSFV RNA load (Ct values about 23) in the rectal swab samples (Table 4). Despite the absence of CSF specific clinical signs, a high CSFV RNA load (Ct value about 20) was detected in all the serum samples during the study, indicating a permanent viremia in the piglets during the trial (Table 4). In parallel, high and permanent excretion in nasal and rectal swabs was found in all the sampling time points during the 32 days after birth. The Ct values increased in the majority of animals throughout the trial, reaching Ct values around 22 and 24 in nasal and rectal swabs (Table 4).

3.5.11 Immune response generated by the moderately CSFV strain in sows and their litters

After infection, Sow 6 developed CSFV specific humoral response at 14 dpi, while Sow 5 was positive at 21 dpi, with blocking percentage values of 42% and 60%, respectively, which increased throughout the study. Neutralising antibody response appeared at 21 dpi on Sow 5 (titre 1:120), and at 14 dpi in Sow 6 (titre 1:20), and increased, reaching titres of 1:160 in both sows by 28 dpi. Nevertheless, none of the piglets showed an antibody response either by ELISA or NPLA during the 32 days after birth. Interestingly, IFN-α was detected in the sera from 4 piglets at 8 and 15 days post-birth (dpb) (Figure 5A).

Table 4. Detection of CSFV RNA in piglets from sows infected with the CSFV Cat01 strain

Piglet ID	CSFV RT-qPCR (Ct value)															
	Day of birth	7 dpb			15 dpb			23 dpb			27 dpb			32 dpb		
	Rectal swab	Serum	Nasal swab	Rectal swab	Serum	Nasal swab	Rectal swab	Serum	Nasal swab	Rectal swab	Serum	Nasal swab	Rectal swab	Serum	Nasal swab	Rectal swab
1	25	17,06	24,77	23,77	16,88	16,87	24,52	16,55	23,03	22,38	16,51	24,70	23,62	17,30	18,80	22,47
2	23,94	16,10	26,09	20,42	16,34	25,76	23,91	16,06	20,82	25,53	15,96	21,16	23,52	17,28	20,59	27,20
3	24,01	16,01	23,15	21,46	16,36	25,88	23,36	16,80	27,16	24,49	17,29	21,66	23,74	19,27	19,25	24,42
4	22,63	16,31	22,30	20,86	16,74	19,26	26,91	17,01	18,02	25,36	16,73	22,20	25,44	16,36	23,53	22,86
5	23,44	15,71	27,94	22,81	16,68	27,00	24,38	16,61	20,42	24,77	16,89	20,41	25,83	17,64	19,30	25,99
6	24,55	15,39	23,20	23,79	25,52	22,65	26,56	16,56	21,28	23,19	16,66	20,57	22,70	16,74	19,86	22,42
Mean	23,93	16,10	24,58	22,19	18,09	22,90	24,94	16,60	21,79	24,29	16,67	21,78	24,14	17,43	20,22	24,23
Desvest	0,83	0,57	2,13	1,47	3,65	4,09	1,45	0,32	3,09	1,25	0,44	1,58	1,22	1,01	1,73	2,01

Dpb: Days post birth

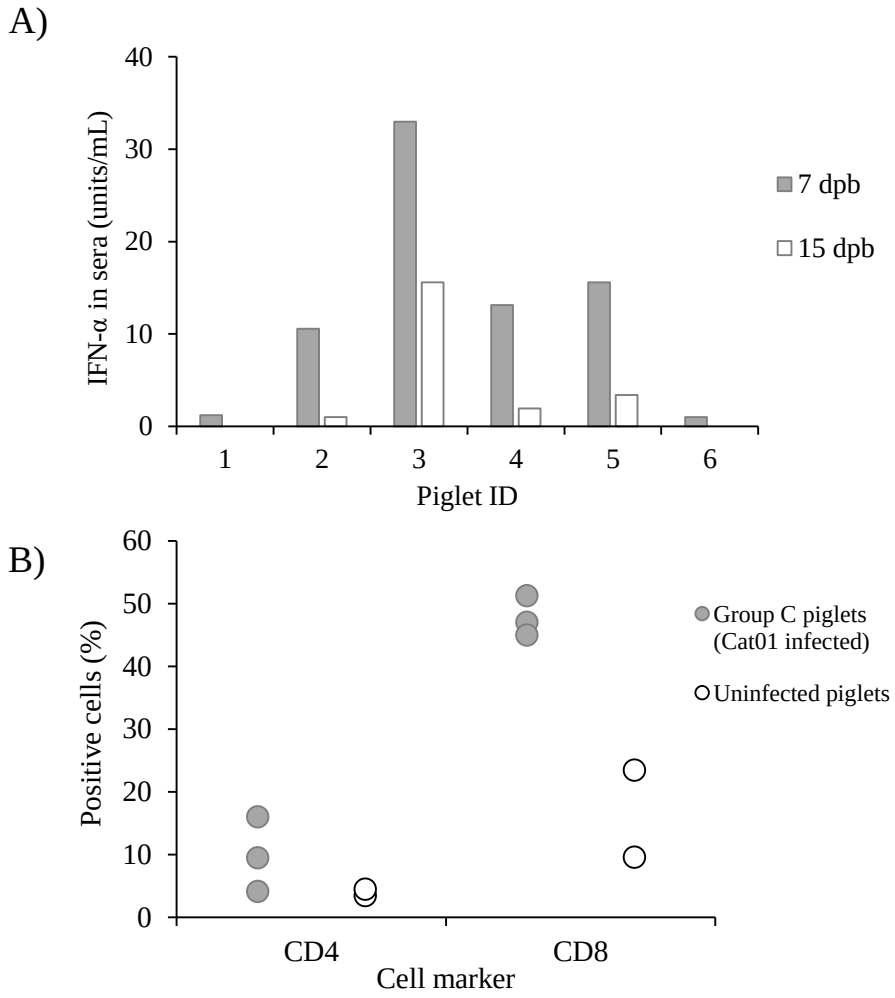

Figure 5. IFN-α levels and phenotypic profile in PBMCs from CSFV congenital persistently infected piglets. (A) Concentration of IFN-α in sera expressed as units/mL from CSFV persistently infected piglets at 7 dpb (grey bars) and 15 dpb (white bars). (B) Comparative expression of the T-CD4$^+$ and T-CD8$^+$ cell subsets in PBMCs from uninfected piglets (white dots) and piglets infected with the Cat01strain (grey dots).

On the other hand, alterations in the $CD4^+$ and $CD8^+$ T-cell subsets from Cat01-infected piglets were found in the analysed PBMC from persistently infected piglets. While the T-$CD4^+$ population did not exceed 5% in the uninfected, age-matched piglets, these cells ranged from 4% to 16% in persistently infected animals. On the other hand, the $CD8^+$ cell subset was increased (about 50%) in the infected animals, being between 9.6 and 23.5% in the uninfected animals (Figure 5B).

3.6 Discussion

CSF congenital persistent infection was described several decades ago; however, some aspects regarding the generation of this form of the disease remain to be elucidated, and the available information is not up to date (Liess, 1984; Van Oirschot & Terpstra, 1977). In the present work, three groups of sows were infected with either the Margarita, Cat01, or PdR CSFV strains. Each of these strains has been previously characterised as of high, moderate, and low virulence, respectively (Coronado et al., 2017; Ganges et al., 2005; Tarradas et al., 2014). In accordance with previous studies, the infection was carried out at 74 days of gestation, a time-point in which persistent congenital infection can be generated (Van Oirschot & Terpstra, 1977). The capacity for trans-placental transmission and induction of foetal immune response was compared side by side between the high and low virulence strains (Figure 1 and Figure 2, Table 1 and Table 2). In accordance with previous data found in piglets, the highly virulent CSFV Margarita strain induced high serum IFN-α levels in sows over a short period of time (M. Wang et al., 2019). By contrast, the IFN-α response induced by the low virulence PdR strain was lower, although it lasted one week longer. This supports the role of high replication rates for the previously described exacerbated

innate immune response in the host after infection with highly virulent CSFV strains. This may explain the differences in pathogenesis between the sows from these two groups, with more severe lesions and an inability to clear the virus in the Margarita-infected sows, compared with the clinically healthy status and low replication of the PdR-infected ones. Trans-placental transmission was more efficient with the highly virulent Margarita strain, and high viral RNA load was detected in sera and tissues from the foetuses in this group. Conversely, a small proportion of the foetuses from the PdR infected sows were viraemic with high viral replication in organs, while the majority of them were either non-infected or only showed low viral RNA in tissues. Despite the immune response developed, mainly in Margarita infected sows, CSFV crossed the trans-placental barrier from the sows to their foetuses (Table 1 and Table 2, Figure 2). In agreement with previously described data, the high replication rate found in sows infected with a highly virulent CSFV strain may explain the activation of neutralising antibody response in these animals. However, taking into account that the onset of the antibody response in the sows was after two weeks, it is likely that the generation of trans-placental transmission took place during the first week after infection. Considering the previously described data, in order to avoid trans-placental transmission, it is necessary that effective neutralising antibody response be already present at the moment of infection, with titres of at least 1/320 (Muñoz-González et al., 2017).

Mummifications and haemorrhagic lesions were found in the Margarita infected foetuses. Probably, these animals would have died during the perinatal period. On the contrary, neither mummifications nor macroscopic lesions were observed in the PdR infected foetuses, even in those that showed viremia and high levels of viral replication in organs.

It is very well known that sows transmit passive immunity to CSFV to the litters via colostrum (Suradhat et al., 2007; van Oirschot, 2003). These maternally derived antibodies (MDA) protect piglets against disease, including CSF, during their firsts weeks of life (Henke et al., 2018; Suradhat et al., 2007; van Oirschot, 2003). Considering that, in the case that the piglets had been born, the low immunity generated in the sows after infection with the low virulence PdR strain would result in an inefficient transmission of MDA to these litters. There might be major consequences to this situation since the suboptimal level of MDA would favour the infection of the non-infected piglets by their congenital persistently infected littermates and lead to chronic or postnatal persistent infection (Coronado, Bohórquez, et al., 2019; Muñoz-González, Ruggli, et al., 2015). Recently, it was reported that the lack of maternal immunity led to a high prevalence of CSFV persistently infected piglets in an endemic scenario (Coronado, Bohórquez, et al., 2019). Notably, the CSFV persistently infected piglets have been proven to be refractory to vaccination (Coronado, Bohórquez, et al., 2019; Muñoz-González, Pérez-Simó, et al., 2015). This complex situation may lead to a vicious circle, which greatly impairs control programs of regions where CSF persistent infections are occurring.

In the case of infection carried out with the moderately virulent CSFV Cat01 strain, early labour in one of the infected sows and mummification and stillbirths in both of them were detected. Interestingly, both Cat01 infected sows developed a CSFV neutralising antibody response. However, the viral trans-placental transmission was not impaired, and all the piglets that were born alive in one of the Cat01 infected litters developed persistent congenital infection. These piglets showed normal weight gain, according to standards (Collins et al., 2017), despite being

infected and excreting high viral load with a lack of CSFV specific antibody response (Blome, Staubach, et al., 2017; Liess, 1984; Van Oirschot, 1979a). Interestingly, the level of viral replication was comparable, or even higher than those found in the foetuses from sows infected with the high virulence strain (Table 2 and Table 4). This finding suggests an immunomodulatory capacity of the moderate virulence CSFV strains in the interaction with the host. Previous data showed the efficacy of this type of CSFV strain to also generate persistent postnatal infection (Muñoz-González, Pérez-Simó, et al., 2015; Muñoz-González, Ruggli, et al., 2015). Similar to persistent postnatal infection, low levels of IFN-α were found during congenital viral persistence, despite the high viral replication, pointing towards immunosuppressive regulation. Similar mechanisms might be taking place during the establishment of congenital or postnatal viral persistence. Recently, myeloid-derived suppressor cell populations have been determined to play a relevant role in the generation of CSFV postnatal persistence infection (Bohórquez, Muñoz-González, et al., 2019). It cannot be discarded that these cell subsets are playing a role during the establishment of CSFV congenital persistent infection, considering that they have been found in cord blood and during neonatal stages in humans (Rieber et al., 2013; Schwarz et al., 2018). On the other hand, a low CD4/CD8 ratio has been reported as a marker for dysregulation of the immune response (Dustin, 2017; Gandhi et al., 2017; Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014). An increase in the $CD8^+$ T-cell population, resulting in a low CD4/CD8 ratio, has been reported in CSFV postnatal persistently infected animals (Bohórquez, Wang, et al., 2019). In the present study, an increase in the $CD8^+$ T-cell subset was observed in the PBMC of infected foetuses and piglets from all the experimental groups.

This finding may indicate that immunosuppressive mechanisms are also taking place in animals after trans-placental infection by CSFV.

Activation of innate immunity, evidenced by the IFN-α and IFN-γ levels detected in sera, was found in the foetuses and piglets regardless of the infecting strain and the maturity level of the immune system (Table 3 and Figure 5). Type I interferon response activates the innate immunity after viral infection by playing an antiviral and immunomodulatory role. CSFV has the capacity to induce high levels of IFN-α response in pigs, being associated with disease severity and viral replication in the infected animals (Summerfield & Ruggli, 2015). The highest IFN-α response was found in the viraemic foetuses or in those that showed higher viral replication in organs from the group infected with the low virulence PdR strain. Notably, the capacity of the PdR strain for high and prolonged IFN-α activation in piglets has been associated with an uninterrupted 36-uridine sequence found in the 3′ untranslated region of the CSFV genome(M. Wang et al., 2019). Activation of IFN-α response in ruminant and human foetuses, following infection with bovine viral diarrhoea and Zika virus, respectively, has been described, and it may support the results obtained in this study (J. Chen et al., 2017; Smirnova et al., 2014). Thus, the immunotolerance mechanism that was previously associated with the development of CSF congenital persistent form (Blome, Staubach, et al., 2017; Van Oirschot, 1979a) is a complex immunologic phenomenon, and further studies may explain this mechanism and its relation with the establishment of viral persistence.

Previous reports have shown that the levels of sCD163 can be increased as a result of tissue damage during acute infection with highly pathogenic viruses, such as the African swine fever virus (ASFV) (Cabezón et al.,

2017; Lacasta et al., 2015). In addition, increased IFN-γ levels have also been found as part of the cytokine storm phenomenon responsible for the pathogenesis of ASFV (Alfonso et al., 2004; Cabezón et al., 2017; Lacasta et al., 2015). In agreement with the haemorrhagic lesions and levels of viral replication found in foetuses infected with the high virulence CSFV Margarita strain, it is likely that the increase of IFN-γ and sCD163 may be associated with the exacerbated immune response in the host after infection, leading to cellular homeostasis imbalance and tissue damage.

Taken together, our results show that the infecting CSFV strain capacity for viral replication influences its efficacy for trans-placental transmission and the establishment of persistent infection. Likewise, the CSFV strain with a moderate virulence degree proved to be very efficient in generating CSFV congenital persistent infection following trans-placental transmission. Our results indicate that trans-placental infection took place very fast before the neutralising antibody response could be generated in sows. Therefore, vaccines against CSFV indicated for pregnant sows must induce fast and strong immunity to guarantee the viral protection of their offspring against this type of infection.

On the other hand, the foetal immune system is able to recognise the virus and generate immune response after trans-placental infection. Further studies are needed to elucidate the mechanisms by which the specific immune response against CSFV is being impaired, following the initial recognition of the pathogen. To the best of our knowledge, this is the first report showing the foetal immune response after CSFV infection.

3.7 Acknowledgments

This research was supported by grant RTI2018-100887-B-I00, Ministerio de Ciencia, e Innovación from the Spanish government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia e Innovación from Spanish government. The authors thank Ivan Cordón and the CReSA animal facility staff for their help during the *in vivo* part of the experiment. IRTA is supported by CERCA Progamme/Generalitat de Catalunya.

3.8 Author Contributions

Conceptualization, L.G.; Investigation, J.A.B., S.M.G. and L.G.; Methodology, L.G., S.M.G., J.A.B., M.P.S., I.M., R.R., L.C. and M.D.; Formal Analysis, J.A.B., S.M.G. and L.G.; Resources, L.G., R.R.; Writing—Original Draft Preparation, J.A.B. S.M.G. and L.G.; Writing—Review & Editing, J.A.B., S.M.G, M.D. and L.G.; Supervision, L.G.; Project Administration, L.G.; Funding Acquisition, L.G.

3.9 Conflicts of Interest

The authors declare no conflicts of interest.

Chapter 4

Study II. Low CD4/CD8 ratio in classical swine fever postnatal persistent infection generated at 3 weeks after birth

(Transboundary and Emerging Diseases. 2019. 66 (2): 752-762)

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4.1 Abstract

Classical swine fever virus (CSFV) is one of the most important pathogens affecting swine. After infection with a moderate virulence strain at 8 hours after birth, CSFV is able to induce viral persistence. These animals may appear clinically healthy or showed unspecific clinical signs despite the permanent viremia and high viral shedding, in absence of immune response to the virus. Given the role played by this infection in disease control, we aimed to evaluate the capacity of CSFV to induce postnatal persistent infection at 3 weeks after birth. Nine pigs were CSFV infected and sampled weekly during 6 weeks and viral, clinical, pathological and immunological tests were carried out. Also, the CD4/CD8 ratio was calculated with the purpose to relate this marker with the CSFV persistent infection. The IFN-α response was detected mainly 1 week after infection, being similar in all the infected animals. However, 44.4% of animals were CSFV persistently infected, 33.3% died and 22.2% developed specific antibody response. Interestingly, in persistently infected pigs, the T-CD8 population was increased, the T-CD4 subset was decreased and lower CD4/CD8 ratios were detected. This is the first report of CSFV capacity to confer postnatal persistent infection in pigs infected at 3 weeks after birth, an age in which the weaning could be carried out in some swine production systems. This type of infected animals shed high amounts of virus and are difficult to evaluate from the clinical and anatomopathological point of view. Therefore, the detection of this type of infection and its elimination in endemic areas will be relevant for global CSF eradication. Finally, the low CD4/CD8 ratios found in persistently infected animals may be implicated in maintaining high CSFV replication during persistence and

further studies will be performed to decipher the role of these cells in CSFV immunopathogenesis.

4.2 Keywords

CD4/CD8 ratio, CSFV, CSFV RNA load, pathogenesis, viral persistence

4.3 Introduction

Classical swine fever (CSF) continues to be one of the most important diseases affecting swine. Due to its high social impact in some affected regions, the disease has been considered as a problem for the food security of the population. Currently, CSF is endemic in some countries of Asia, Eastern Europe and Latin America (Ganges et al., 2008; L. J. Pérez et al., 2012).

The aetiological agent, CSF virus (CSFV), belongs to the *Pestivirus* genus, within the *Flaviviridae* family (Schweizer & Peterhans, 2014). The virus targets different cell types of the immune system, such as those that are derived from the monocyte-macrophage lineage (Summerfield, Hofmann, et al., 1998; Zingle et al., 2001). Therefore, the infection in this type of cells plays an important role in viral pathogenesis, viral persistence and spread. It has been established that the balance between the nature of the virus and different host conditions, for instance age or immunological status, plays a role in the severity of disease, which can vary from acute to subclinical or chronic forms (Blome, Staubach, et al., 2017). However, these underlying interactions are not completely known.

A significant trait of the *Pestivirus* genus is its ability to induce congenital persistent infection of the foetus by viral trans-placental transmission, being known for over 40 years that congenital persistent

infection is the most important cause by which CSFV is perpetuated in swine population (Liess, 1984). Nevertheless, little is known about the mechanisms involved. Persistently infected animals may appear to be healthy for some weeks, and develop runting-like signs later in life, despite replicating and excreting significantly high viral load (Liess, 1984; Van Oirschot, 1979a, 1979b).

Recently, it has been established that persistent infection can also occur after early postnatal infection, in pigs infected within the first 24 hr after birth, with a moderately virulent CSFV strain (Muñoz-González, Ruggli, et al., 2015). These animals remained apparently healthy for several weeks, or showed clinical signs different from those previously described for CSF (Liess, 1984; Muñoz-González, Ruggli, et al., 2015). Notably, these infected animals showed a high and persistent viral load in blood and body secretions for several weeks, as well as inability to generate specific cellular and humoral response to the virus. In addition to the adaptive immune response, recent studies have also shown that the innate immune response to the virus, as measured by type I IFN-α in the serum, was impaired in pigs with a CSFV postnatal persistent infection, therefore, an immunological anergy has been observed in these animals (Cabezón et al., 2017; Muñoz-González, Ruggli, et al., 2015).

Previous studies have shown that a low CD4/CD8 ratio can be interpreted as a measure of dysregulation of a patient's immune system (Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014), which has proven to be very useful marker in human immunodeficiency virus (HIV) therapy. Lower CD4/CD8 ratios have been detected in humans suffering persistent and chronic infections with HIV and hepatitis C virus (HCV) (Dustin, 2017). In this regard, a lower CD4/CD8

ratio has also been found in patients with persistently higher HIV-1 viral load (Gandhi et al., 2017).

Bearing in mind the proven CSFV ability to generate postnatal persistent infection in new-born piglets, the aim of the present study was to evaluate the capacity of CSFV to induce postnatal persistent infection at a later time point than previously reported. To this end, pigs were CSFV infected at 21 days after birth, and the characteristics of the immunological and virological response related to viral persistence were studied during a 6-week period. In addition, the phenotypic profile of peripheral blood mononuclear cells (PBMC) was evaluated for lymphocytic and myeloid lineages. Finally, the CD4/CD8 ratio was determined with the purpose to relate this marker with the CSFV persistent infection.

4.4 Materials and methods

4.4.1 Cells and viruses

PK-15 cells (ATCC CCL 33) and SK6 cells (Kasza et al., 1972) were cultured in DMEM medium, supplemented with 10% foetal bovine serum (FBS) *Pestivirus-free* at 37°C in 5% CO2. The Catalonia 01 (Cat01) strain used in this study belongs to the CSFV 2.3 genogroup (L. J. Pérez et al., 2012). This strain has been characterized as a moderately virulent strain (Tarradas et al., 2014). Alfort 187 strain was used in the neutralisation peroxidase-linked assay (NPLA). Viral stocks were produced using PK-15 cells that were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 72 hr later. For virus isolation, both cell lines (PK-15 and SK6) were used. Peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral titration following the

statistical methods described by Reed and Muench (Reed & Muench, 1938).

4.4.2 Experimental design

One pregnant sow (Landrace), *Pestivirus-free*, of 108 days into gestation was housed in the BSL3 animal facility at IRTA-CReSA (Barcelona, Spain). Delivery was at 115 days of gestation and nine piglets were born. Twenty-one days after birth, piglets were numbered from 1 to 9 and were inoculated intranasally with 2.5×10^4 TCID of Cat01 strain. The inoculation of the piglets was conducted separately from their mother and the sow was kept with the piglets until 9 days after inoculation (30 days after birth). The piglets were fed (StartRite, Cargill, Spain) from week 4 onwards. Serum and rectal swab samples were collected every week after infection during the 6 weeks of the trial. Whole blood samples for the isolation of PBMCs were obtained at 4 weeks post infection. The tonsils were collected at necropsy and were used to quantify CSFV RNA and in virus titration assay. After macroscopic examination, a portion of the tonsil from pigs numbers 3, 5, 6 and 7 was placed into 10% phosphate buffered formalin in order to conduct histopathological evaluation. Fixed samples were sliced and embedded in paraffin wax after dehydration through increasing alcohol concentrations and xylene. Four micrometre sections were mounted on glass microscope slides which were stained with haematoxylin and eosin for routine morphological evaluation. Whole blood and tonsil samples from two non-infected pigs from the same origin as the sow and with the same age of the infected animals (7 and 9 weeks respectively) were also collected (numbered 10 and 11). These samples were used as negative controls.

The procedure for the euthanasia of the animals was based on an accepted method included in European Directive 2010/63/EU, using an anaesthetic overdose of 60–100 mg of pentobarbital per kilogram of weight, administered via the vena cava.

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 CSFV RNA detection

RNA extraction was performed using the NucleoSpin RNA isolation kit (Macherey-Nagel). For all the analysed samples, an initial volume of 150 µl was used in order to obtain 50 µl of RNA, which was stored at −80°C. The presence of CSFV RNA in sera, rectal swabs and tonsil was analysed by real time (RT-qPCR) (Hoffmann et al., 2005). Cycle threshold (Ct) values equal or less than 42 were considered as positive. Samples in which fluorescence was undetectable were considered negative.

4.4.4 Humoral response detection by ELISA and NPLA

The presence of E2-specific antibodies in serum was evaluated using a commercial ELISA kit (IDEXX), the samples were considered as positive when the blocking percentage value was ≥40%. Serum samples were also tested by NPLA (Terpstra et al., 1984) against homologous and heterologous CSFV strains, Cat01 and Alfort-187 respectively. Neutralising antibody titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the CSFV strains in 50% of the culture replicates.

4.4.5 ELISA for IFN-α detection in serum samples

IFN-α concentration in serum was evaluated by ELISA at 0, 7 and 14 days post infection (dpi) (Muñoz-González, Pérez-Simó, et al., 2015; Muñoz-González, Ruggli, et al., 2015) using monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, NJ, USA). Cytokine concentrations (units/ml) in sera were determined using a regression line built with the optical densities of the cytokine standards used in the test

4.4.6 PBMC collection and flow cytometry analysis

Considering the virological and immunological profile previously evaluated, whole blood sample was collected from animals 3, 5, 6 and 7 at 4 weeks post infection in order to obtain PBMC. Cells were separated by density-gradient centrifugation with Histopaque 1077 (Sigma) and afterwards were subjected to osmotic shock in order to eliminate the remaining erythrocytes. The number and viability of the PBMCs were determined by staining with Trypan Blue. In addition, PBMCs from non-infected pigs, served as control (samples from pigs 10 and 11).

Flow cytometry was used in order to phenotype the PBMCs from infected and naïve pigs at 4 weeks after infection (7 weeks of age). Hybridoma supernatant (kindly provided by Dr. J. Dominguez (INIA, Madrid, Spain)) was used for staining CD172a (BA1C11, IgG1), with an anti-Mouse IgG1 antibody labelled with Alexa Fluor 647 (Thermofisher scientific, produced in goat) used as a secondary antibody to detect the primary anti-CD172a antibody. Moreover, conjugated mAbs detecting porcine T-CD4 (Alexa Fluor® 647 Mouse Anti-Pig CD4a 74-12-4,

IgG2b, BD Pharmingen), and T-CD8-α (FITC Mouse Anti-Pig CD8a 76-2-11, IgG2a, BD Pharmingen) were also used.

Briefly, 5×10^5 cells were plated in each well and the cell culture medium was removed after centrifugation. For single-colour staining, cells were incubated with either hybridoma supernatant or conjugated mAbs for 20 min at 4°C. After washing with PBS + 2% FBS, the secondary antibody was added to cells that had been incubated with hybridoma supernatant. Meanwhile, for two-colour staining, cells were incubated simultaneously with both primary mAbs (mouse anti-pig T-CD4 and T-CD8) for 20 min at 4°C. Finally, a viability control (propidium iodide, 1 μg/ml) was added and twenty thousand live cell events were recorded for each sample in the cytometer (FACSAria IIu, BD Biosciences).

The cells were analysed by FACSDiva software, version 6.1.2 and the results were expressed as the percentage of positive cells obtained for each staining, using irrelevant isotype-matched mAbs as staining controls. In order to corroborate the reproducibility of the results, flow cytometry assays were performed twice for the infected animals and three times for the naïve animals. Afterwards, the mean value and standard deviation of each staining were calculated for every animal. Following the flow cytometry analysis, the CD4/CD8 ratio in PBMCs from pigs numbers 3, 5, 6, 7, 10 and 11 was determined using the mean value obtained from each marker.

4.4.7 Sorting of T-CD8$^+$ cells

The T-CD8$^+$ cell subsets were sorted using a live sterile cell sorting system (FACSAria, Beckton Dickinson; San Jose, California, USA). 20

$\times 10^6$ PBMC from pig number 5 were incubated with T-CD8 conjugated monoclonal antibody (FITC Mouse Anti-Pig CD8a 76-2-11, IgG2a, BD Pharmingen) for 30 min on ice and washed with PBS containing 2% FBS. Finally, the viability control was added and single cell sorting was performed in using the yield mode, with a 70 μm nozzle. The fluorescence reading was performed upon excitation with a 488 nm argon laser. Recovered cells after sorting were resuspended in RPMI-1640 Medium (Lonza) at final concentration of 2×10^6 cells/ml. The presence of CSFV RNA in T-CD8$^+$ and T-CD8$^-$ sorted cells was quantified by qRT-PCR (Hoffmann et al., 2005) and viral isolation in PK-15 cells was performed as explained above.

4.5 Results

4.5.1 Clinical manifestations during 6 weeks of CSFV infection

Twenty-four hours post infection, an increase in the rectal temperature value was registered in all the infected pigs. Likewise, from 4 to 9 dpi, the temperature values increased above 40°C in all the infected animals, with five of nine pigs showing temperatures above 41°C reaching 42°C in some of them. Subsequently, at 10 dpi, a decrease in rectal temperature was recorded in six of nine infected animals (Figure 1a). Values of rectal temperature generally below 40°C were recorded again in six animals from the study after this time. These values were maintained until the end of the experiment and in few cases individual peaks of fever were registered in some of them during the trial, never surpassing the 40.5°C. On the other hand, two animals (numbers 8 and 9) showed high fever peaks from 11 to 18 dpi, which maintained high temperatures values until 17 and 21 dpi (Figure 1a).

Besides an increase in body temperature, the majority of the pigs did not show any clinical signs during the first week post infection, with only one animal showing mild apathy starting at 5 dpi. At the beginning of the second week of the trial, four of nine animals developed mild diarrhoea and/or mild apathy during 3 days. At 10 dpi, two animals, pigs 2 and 8 showed moderate apathy and weakness of the hindquarters respectively. Pig 2 was found dead in the pen at 11 dpi, whereas, pig 8 developed diarrhoea, tremors, cyanosis in the ears and severe apathy and was euthanized at 17 dpi (Figure 1b).

Entering into the third week post infection, five pigs remained with a clinically healthy status, meanwhile the other three showed from mild to severe clinical manifestations, such as, moderate skin lesions (mainly vesicles and desquamation in the skin) which began to appear in pigs 1 and 9 at 19 and 16 dpi respectively. Pig number 9 went on to develop ulcerations in the abdomen, as well as severe dyspnoea at 20 dpi and thus had to be euthanized at 21 dpi (Figure 1b). Thus, during the second and third weeks post infection, the most severe clinical outcome was observed, with three animals that were either euthanized or found dead (33.3%).

From week 4 to the end of the trial (week six), six pigs were maintained, two of them showed mild diarrhoea, some vesicles and skin desquamation (pigs 1 and 5). Finally, the four remaining animals were apparently clinical healthy during this period (Figure 1b).

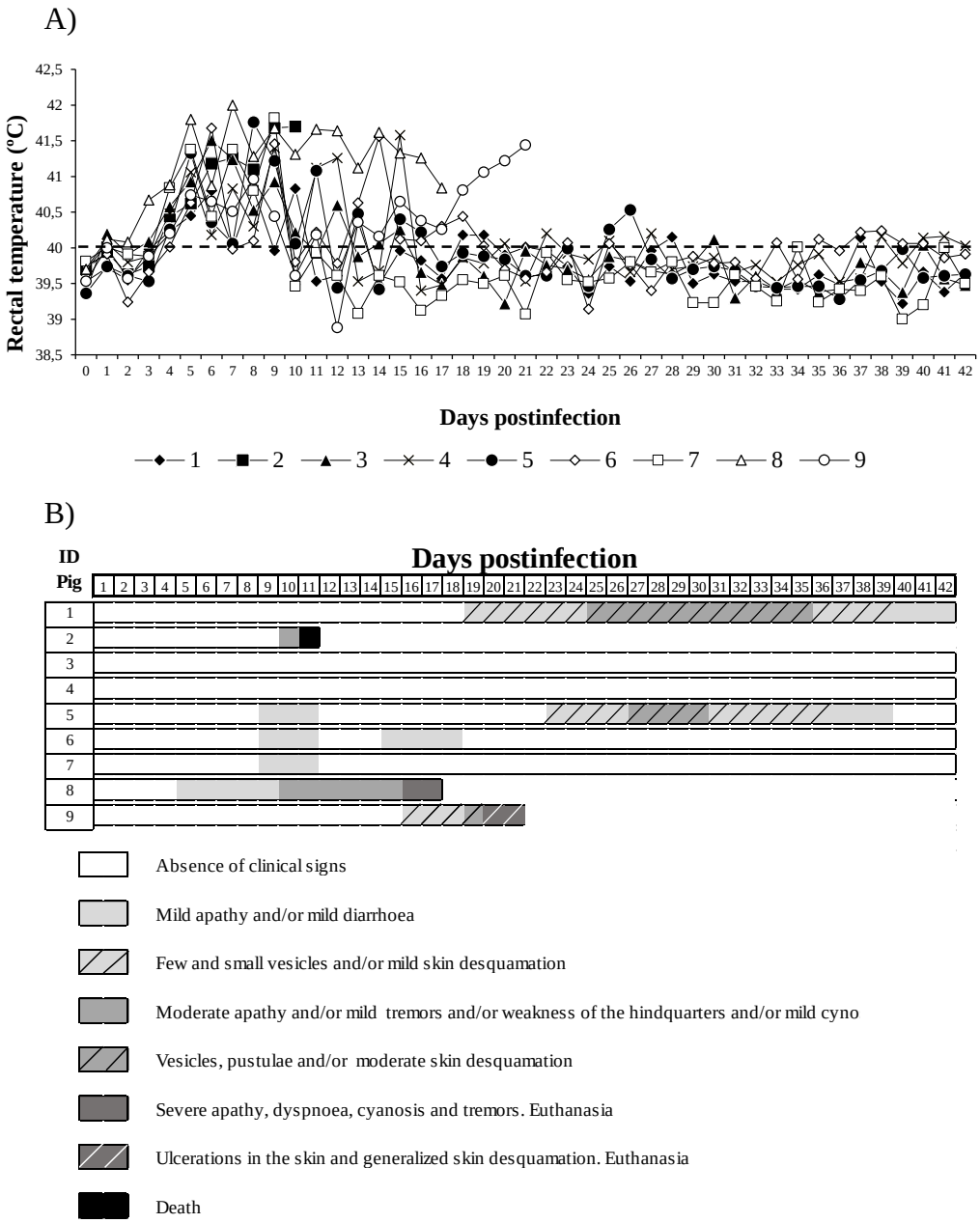


Figure 1. Clinical signs developed after CSFV postnatal infection at 21 days of age. (A) Daily rectal temperature measured in infected animals. Values greater than 40°C (dotted line) were considered to indicate fever. (B) Individual clinical signs in piglets after postnatal infections with CSFV Cat01 strain at 21 days of age. The piglets were monitored daily during the 6 weeks of the study. The severity of the clinical signs is represented by the colour scale, from white (absence of clinical signs) to black (death).

4.5.2 Macroscopic and histopathological findings in tonsils after 6 weeks post CSFV infection

No macroscopic changes were found in the tonsils from the analysed animals after infection, when compared to the control pigs. In addition, after histopathological examination, no significant abnormalities were observed in the tonsils from the infected pigs. A mild increase in the amount of tingible body macrophages in the lymphoid follicles was evidenced only in pigs numbers 3 and 7, when compared to the tonsils from pigs 5 and 6 as well as, the non-infected (Figure 2).

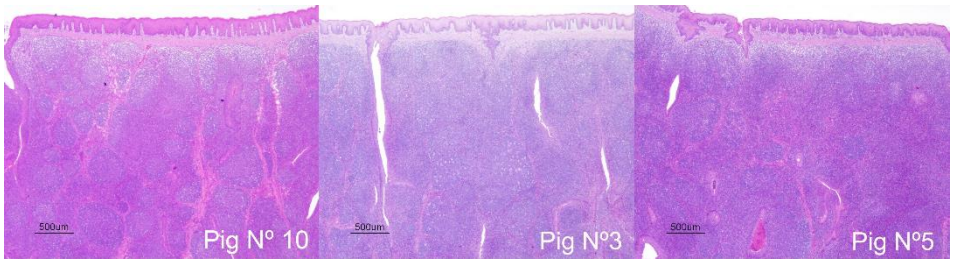


Figure 2. Histopathological evaluation of tonsil samples from CSFV infected pigs at 6 weeks post infection. Haematoxylin and eosin staining of tonsil microdissections from an uninfected animal (pig 10), one CSFV infected pig that seroconverted (pig 3) and a CSFV infected pig that showed permanent viremia (pig 5).

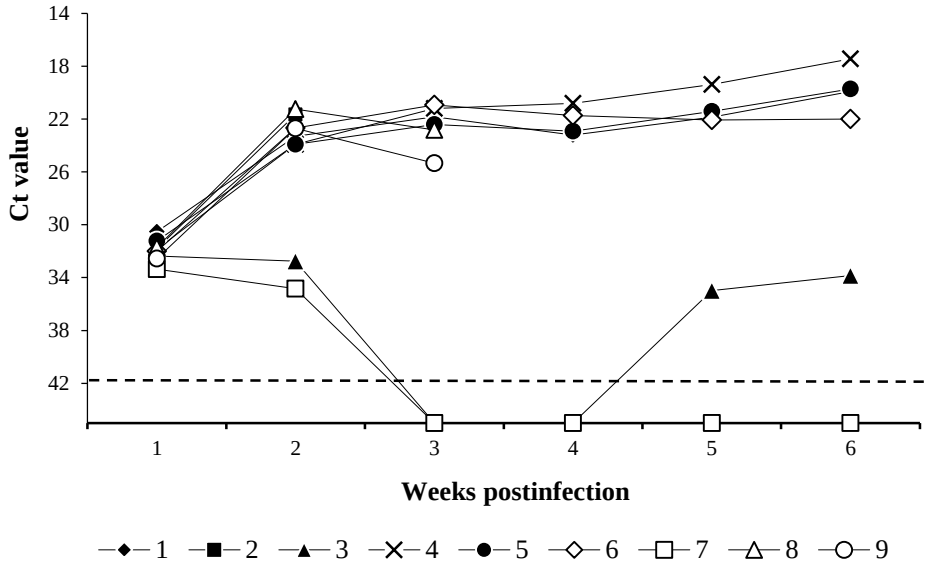
4.5.3 Most of the infected animals showed permanent CSFV load in sera and rectal swabs

During the first week of the study, similar viral RNA load was detected in all the CSFV infected animals, with Ct values between 30.5 and 33.3. In the second week of the study, two profiles of CSFV RNA load in sera were detected, with six animals, numbers 1, 4, 5, 6, 8 and 9, showing low Ct values between 21.6 and 23.9, and two animals (numbers 3 and 7) with high Ct values (32.7 and 34.8). Subsequently, no viral RNA was detected in sera from pigs 3 and 7 at 3 and 4 weeks post infection. On

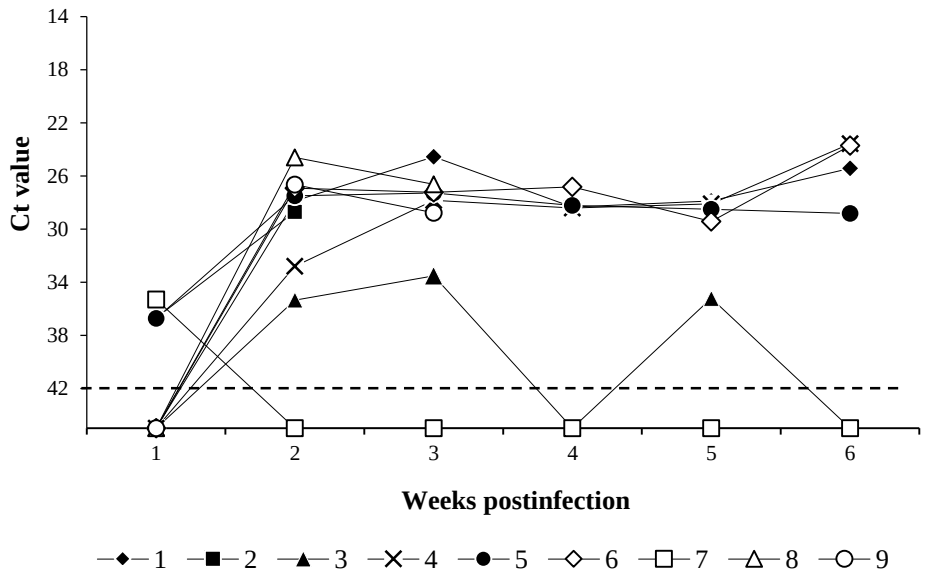
the other hand, CSFV RNA was detected in the remaining animals (44.4%) with similar Ct values to those found in the second week of the trial, being the RNA levels maintained or even increased during weeks 5 and 6 (Ct values between 17.4 and 22). Alternatively, during these 2 weeks, one pig was negative (pig 7) and the other showed high Ct values in the fifth and sixth weeks (Figure 3a).

In rectal swabs, CSFV RNA was detected with similar Ct values in three of nine infected pigs (numbers 2, 5 and 7) in the first week after infection. At week 2 post infection, most of the animals were positive with moderate Ct values (between 27 and 35.3), with the exception of pig 7 that was negative until the end of the study (Figure 3b). During the third week post infection, pig 3 showed a Ct value of 33.5. Whereas in the remaining animals (numbers 1, 4, 5, 6, 8 and 9), lower Ct values (between 24.5 and 28.7) were observed. These Ct values were similar for the surviving animals (1, 4, 5 and 6) during the fourth and fifth weeks and in some of them were decreased at week 6 (from 23.5 to 25). Meanwhile, pig number 3 was negative at weeks 4 and 6 after infection, however, it was positive with high Ct value (35.2) at week 5 of the study (Figure 3b).

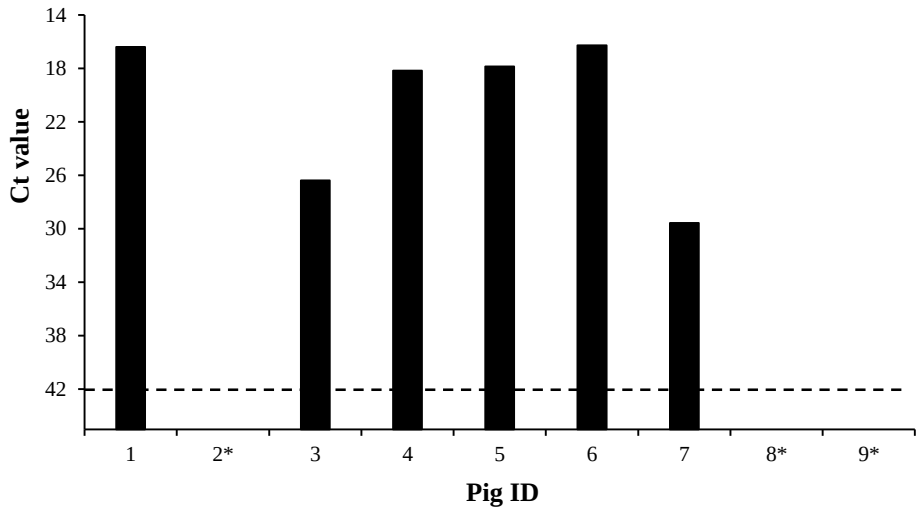
A)


14
18
22
26
30
34
38
42
Ct value
1
2
3
4
5
6
Weeks postinfection
1
2
3
4
5
6
7
8
9

B)


14
18
22
26
30
34
38
42
Ct value
1
2
3
4
5
6
Weeks postinfection
1
2
3
4
5
6
7
8
9

C)



D)

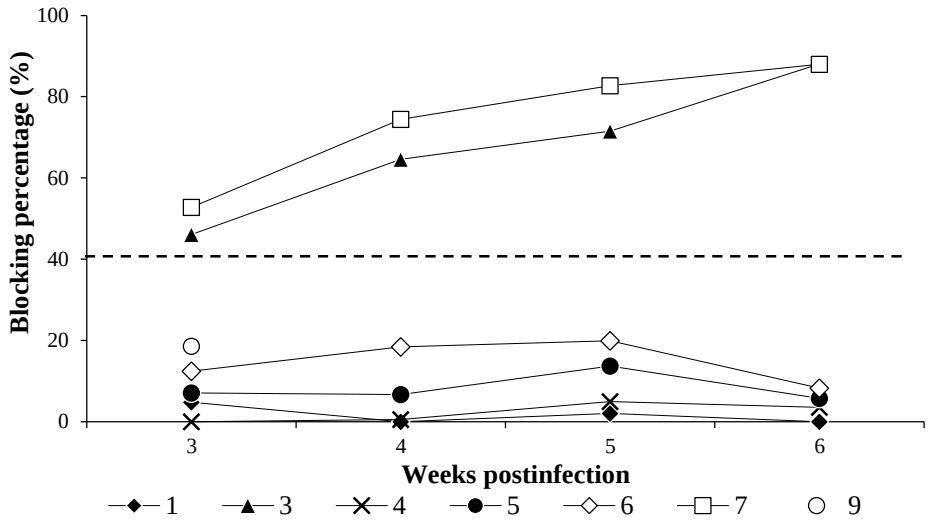


Figure 3. Detection of CSFV RNA and specific humoral response during 6 weeks of infection in pigs infected at 21 days of age. CSFV RNA was evaluated weekly in sera (a) and rectal swab (b) samples as well as in tonsil at time of euthanasia (c). Ct values above 42 (dotted line) were considered as negative. Antibodies against the E2 glycoprotein of CSFV were evaluated weekly (d). Blocking percentage values above 40% (dotted line) were considered as positive

All of the analysed tonsil samples were positive, animals 1, 4, 5 and 6 showed low Ct values, while animals 3 and 7 showed higher Ct values (from 26.3 to 29.5 respectively) (Figure 3c). Only tonsil samples from pigs numbers 1, 4, 5 and 6 were positive by viral isolation test, the viral load in samples from pigs 1, 4 and 5 was about $10^{6.6}$ $TCID_{50}$/ml, whereas the viral load in the tonsil from pig number 6 was $10^{5.6}$ $TCID_{50}$/ml.

4.5.4 Only two infected pigs were able to seroconvert

Seven of the nine infected animals did not develop a humoral response detectable neither by ELISA nor neutralization test during the 6 weeks of the trial. By contrast, the remaining two animals, numbers 3 and 7 (22.2%) developed specific anti-E2 antibody response, detected by ELISA test, starting at 3 weeks post infection (Figure 3d). The results were confirmed by NPLA test and the neutralizing antibody response against the heterologous CSFV strain (Alfort-187) was detected also at 3 week post infection with a low antibody titre (1:10). The titres were increased between weeks 5 and 6 post infection with a titre of 1:60 in both pigs. Meanwhile, neutralizing antibody titres against the homologous CSFV strain (Cat01) were detected 1 week earlier in animal 7 with higher antibody titres (1:120) to those found against the heterologous strain. The titres were increased at 3 weeks post infection until the end of the trial in both animals (from 1:80 to 1:1280).

4.5.5 IFN-α levels detected mainly 1 week after infection

At time of viral infection, all the pigs showed low baseline levels (between 10 and 20 units/ml) of IFN-α in serum sample. An increase in IFN-α values was recorded for all the animals at 1 week after infection with the CSFV Cat01 strain, with values ranging from 20 to 42 units/ml. During the second week post infection, IFN-α concentration had severely

decreased in all the animals, showing concentrations between 4.44 and 8.65 units/ml (Figure 4).

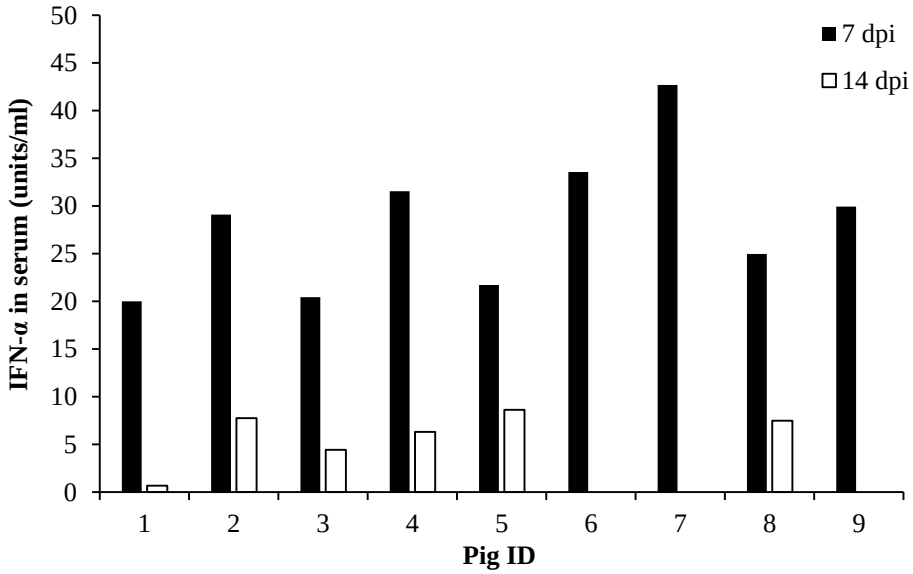


Figure 4. IFN-α levels in serum from CSFV infected pigs. IFN-α was evaluated at 7 (black bars) and 14 dpi (white bars) in CSFV infected pigs

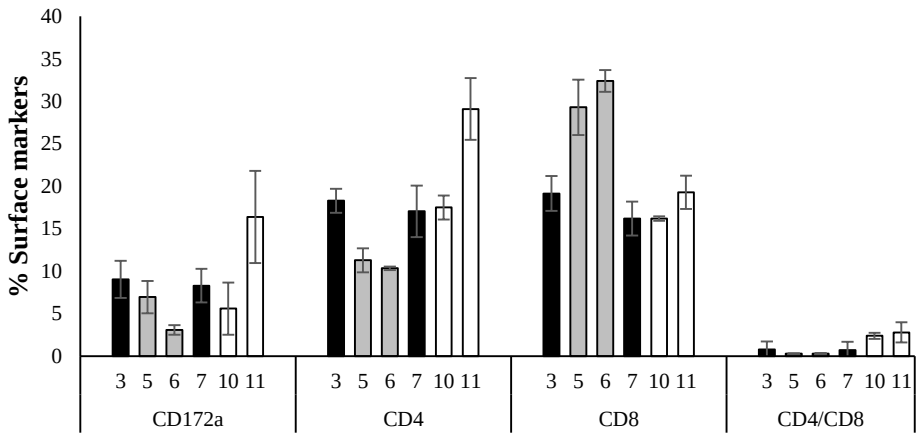
4.5.6 Increased T-CD8$^+$ subset targeted by CSFV in pigs showing permanent viremia

The percentage of T-CD4$^+$ cells oscillated between 17.05% and 18.3% in samples from pigs 3 and 7, while for pigs 5 and 6 the values were lower, ranging from 10.3% to 11.3%, being similar in the control pigs (10.8% and 12.3%). Additionally, CD4$^+$/CD8$^+$ double positive cells were found to be 0.8% in pigs 3 and 7 and lower values (0.3%) were detected in pigs 5 and 6. In control animals, percentage values of 0.4% and 3.2% were also detected (Figure 5). The CD172a cell population ranged between 3% and 9% in PBMCs from infected pigs, reaching in animals 3 and 7 from 8.3% to 9.05% and in samples from pigs 5 and 6 from 3.1% to 6.9%. This cell population ranged from 6% to 12% in control animals

(Figure 5). The T-CD8^{+} subset oscillated from 16.2% to 32.4% in infected animals. Notably, samples from animals 3 and 7 were between 19.15% and 16.2%. Higher values of 29.3% and 32.4% were found in pigs 5 and 6 respectively. Meanwhile, T-CD8 marker from control samples ranged from 8.2% to 14% (Figure 5).

The T-CD8^{+} cells were sorted from PBMC of pig number 5 (29.3% of T-CD8^{+} cells). With a purity over 91%, a total of 1373002 cells were recovered. While, 3350150 T-CD8^{-} cells were obtained (purity over 98%). CSFV RNA was detected in the T-CD8^{+} cells with a Ct value of 25.82. In the case of T-CD8^{-} cells, the Ct value was 24.41. The presence of CSFV was confirmed by viral isolation test in PK-15 cells.

A)



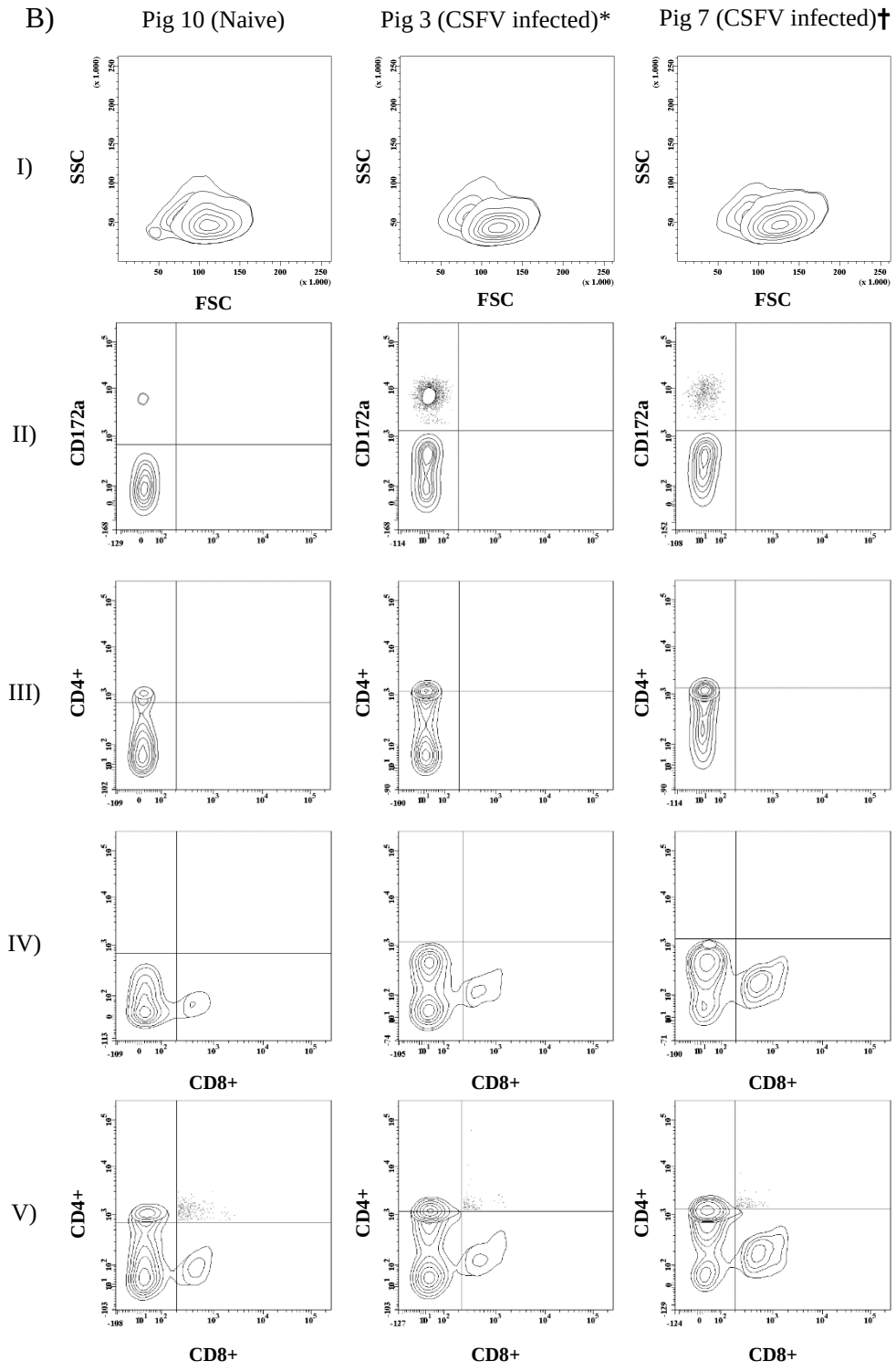

B)
Pig 10 (Naive)
Pig 3 (CSFV infected)*
Pig 7 (CSFV infected)†
I)
II)
III)
IV)
V)
SSC
FSC
CD172a
CD4+
CD8+

Figure 5. Expression of cell surface markers in PBMCs from naïve and CSFV infected pigs at 4 weeks post infection. (a) Phenotypic profile in PBMCs from CSFV seropositive (black bars) seronegative (grey bars) and naïve (white bars) animals. (b) Comparative phenotypes in PBMCs from an animal representative of naïve and CSFV infected pigs. *Indicates an infected pig that seroconverted, †Indicates an infected pig that did not seroconvert. Parameters evaluated included (I) forward scatter (relative cell size, x-axis) and side scatter (relative granularity, y-axis) and expression of cellular markers CD172a (II, y-axis), CD4 (III and V, y-axis), CD8 (IV and V, x-axis) and CD4/CD8 double positive cells (V). The experiments were repeated twice under the same conditions.

4.5.7 Low CD4/CD8 ratio in pigs showing permanent viremia

Finally, the CD4/CD8 ratio was calculated, values ranging from 0.31 to 1.05 resulted in samples for infected animals. The lower ratio was found in pigs 5 and 6 with value about 0.3 in both of them. On the contrary, the CD4/CD8 ratio resulted in values about 1 for pigs 3 and 7. Likewise, the values obtained in PBMC from naïve pigs were 0.95 and 0.88 (Figure 6).

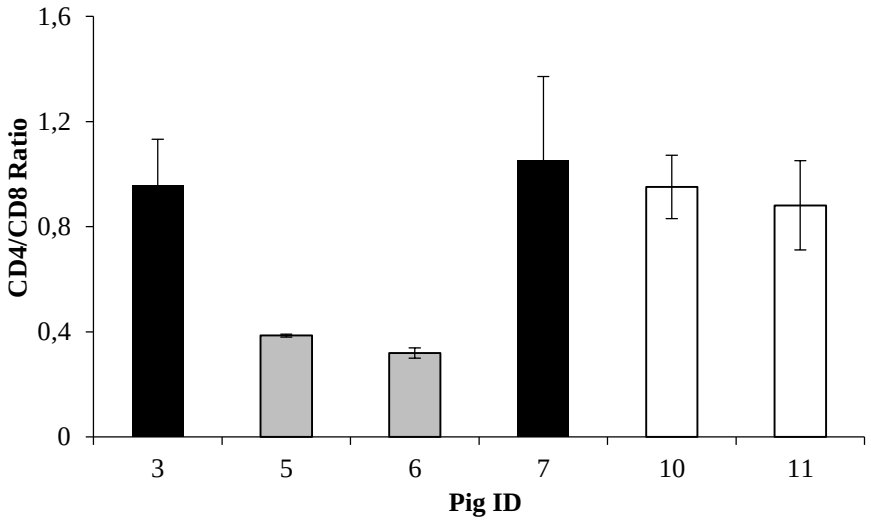


Figure 6. CD4/CD8 ratio in naïve and CSFV infected animals at 28 days post infection. The CD4/CD8 ratio was calculated for pigs that seroconverted (black bars), pigs that did not seroconvert (grey bars) and naïve animals (white bars).

4.6 Discussion

Here, we show that persistently infected piglets could also be generated following infection using a previously characterized CSFV strain (Muñoz-González, Ruggli, et al., 2015) at 3 weeks after birth, an age in which the weaning could be carried out in some swine production systems (Mahan et al., 1998; B.-C. Park et al., 2014). After the infection that was monitored during 6 weeks, besides the increase in rectal temperature, very mild or even no clinical signs were observed in some of the infected pigs. Notably, a significant number of these animals (44.4%) showed permanent viremia with high viral load in sera, rectal swabs and tonsils samples in the absence of CSFV specific humoral immune response for the 6-week duration of the experiment. These findings are in agreement with the profile previously described for persistently infected pigs that were infected during the first hours after birth (Muñoz-González, Ruggli, et al., 2015). Considering that, the proportion of animals that may develop CSFV persistent infection decreases with age, being 60% when the infection is carried out during the first 24 hr after birth (Muñoz-González, Ruggli, et al., 2015) to 44.4% when it is performed at 3 weeks of age. Nevertheless, taking into account the important role of persistently infected animals in the CSF control (van Oirschot, 2003; Van Oirschot & Terpstra, 1977), the proportion of persistently infected animals remains significant when the infection takes place at 3 weeks of age.

A previous report also showed that humoral response is not generated after new-born piglets infection with the CSFV Cat01 strain during 6 weeks (Muñoz-González, Ruggli, et al., 2015). By contrast, when animals are infected at 3 weeks old, this viral strain was capable to

generate humoral response starting the second and the third weeks post infection, although in a small proportion of infected animals (22.2%). Hence, the age of infection also plays a relevant role in the establishment of an immune response against CSFV. Furthermore, these two pigs were capable to clear the virus or reduce the viral load in serum samples, rectal swabs and tonsils considerably compared to persistently infected animals. However, both animals became CSFV asymptomatic carriers. On the other hand, similar to previous work, some of the infected animals from this trial (33.3%) either died or had to be euthanized due to the severity in the clinical manifestation. Likewise, all of infected animals showed high rectal temperature values during the first 2 weeks after infection, being in agreement with a previous report (Muñoz-González, Ruggli, et al., 2015).

It is noteworthy that some infected pigs in the present work, including some of persistently infected animals, developed vesicular lesions, ulcerations and skin desquamation after 3 weeks post infection. This fact highlights the ability of CSFV in its interaction with the host to generate a wide variety of clinical signs (Blome, Staubach, et al., 2017; Ganges et al., 2008; Tarradas et al., 2014). Therefore, the CSF persistent forms may manifest clinical signs so far never described. It is likely that, given the level of immune suppression (Muñoz-González, Ruggli, et al., 2015), the clinical manifestations that may develop in these animals could be related to the endemic pathogens circulating in each environment in coinfection with CSFV.

One of the most common anatomopathological findings associated after CSFV infection is found in the tonsil, a target tissue for CSFV replication and wherein the virus persists for more than 30 days post infection

(Koenig et al., 2007; Vrancken et al., 2009). The most relevant histopathological finding in the tonsil are congestion of the blood vessels along with focal to diffuse areas of haemorrhage (Malswamkima et al., 2015). Necrosis and depletion of lymphocytes, haemorrhages and tonsillitis have also been described (Blome, Staubach, et al., 2017; Quezada et al., 2000). Notably, despite the high viral load detected in the tonsils from persistently infected pigs, neither macroscopic nor histopathological lesions were found, a fact that may hinder the detection of this type of infected animals.

IFN-α levels in sera after CSFV infection are indicative of the activation of innate immunity to the virus (Summerfield et al., 2006; Tarradas et al., 2014). Remarkably, the levels of IFN-α detected after 7 days post infection were similar to those observed in new-born piglets (Muñoz-González, Ruggli, et al., 2015) and in 10-week-old pigs infected with the same CSFV strain (Cat01) (Tarradas et al., 2014). Thus, we consider that the innate immune response, in terms of IFN-α levels in sera, is similarly induced in the above mentioned ages. Therefore, even though an immunotolerance related with the age of the host might be taking place in the pathogenesis of CSFV persistent infection, further studies will clarify the role of this mechanism.

Likewise, it should be noted that previous studies have shown that a hallmark of acute CSF form is the high IFN-α levels found in the serum early after infection with strains of high degree of virulence, being this cytokine related to the cytokine storm phenomenon previously described in this disease form (Tarradas et al., 2014). So, unlike pigs that suffer from the CSF acute form and in agreement with previous studies, CSFV persistently infected animals from the present study also showed lower

IFN-α levels (Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2014). Hence, the impact of this phenomenon may be decreased or has not been detected thus far, explained in part the difference in the clinical outcome between both types of CSF forms.

To understand the effect of infection in the different immunological profiles found in CSFV infected pigs, we focussed on the main T lymphocyte populations as well as a myelomonocytic marker. An increase in the T-CD8 subset was observed in persistently infected pigs in comparison with pigs that seroconverted and the naive animals. After the evaluation of the viral load in this sorted cell population, we proved that these cells were CSFV infected. Thus, the CSFV infected T-CD8 cells subset may promote the virus dissemination into the host and may aid the virus persistence.

The T-CD4 cell population was found decreased in the two persistently infected animals analysed. Previous reports have underlined the role of porcine T-CD4 cells in activation of an effective anamnestic response against CSFV (Ganges et al., 2005). Considering that, the effect of CSFV infection in T-CD4 cells from persistently infected pigs may aid in understanding the lack of humoral response in this form of infection and subsequently the immunological anergy state. The decrease in the T-CD4 cell population may impair the development of an effective adaptive immune response and also favour the viral persistence (Agrati et al., 2016).

As we explained above, the CD4/CD8 ratio correlates with the effectivity of the immune response against different infection models (Agrati et al., 2016; Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014). The low ratio values have been associated with an immune

exhausted state with a strong immunosuppression in infected hosts (Ferrando-Martinez et al., 2011; Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014). Immune exhaustion is a phenomenon in which the constant activation by viral antigens or other mechanisms causes some effector or activated T cells to persist but become functionally unresponsive to further antigen stimulation (Yao & Moorman, 2013). This phenomenon is associated with chronic and persistent infections, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection (Dustin, 2017; Ferrando-Martinez et al., 2011; Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014). Previous reports assert that CD4/CD8 ratio values around 1 correlated with the generation of an effective immune response in swine (Appleyard et al., 2002; Cordes et al., 2012). Accordingly, the CD4/CD8 ratio values in the CSFV seropositive pigs were around 1. By contrast, in CSFV persistently infected pigs, that showed high and constant viral load, we found a lower CD4/CD8 ratio with respect to naïve or CSFV antibody positive pigs. This finding is in agreement with studies in persistent infection in humans, where the low CD4/CD8 ratio correlated with high viral load and an inability of the immune system to clear the virus (Gandhi et al., 2017). Also, it has been established that during HIV persistent infection, the T-cell functions including cytokine secretion and proliferative capacity appear to decrease gradually, being associated with the immune exhaustion phenomenon (Khaitan & Unutmaz, 2011).

Consequently, considering the low CD4/CD8 ratio and the reduced impact of the cytokine storm phenomenon, at least in terms of IFN-α, evidenced in CSFV persistently infected animals after super-infection with CSFV or African swine fever virus (Cabezón et al., 2017; Muñoz-

González et al., 2016); we suggest the possible implication of the immune exhaustion mechanism that may favour the constant and high CSFV replication levels during CSFV persistence (Cabezón et al., 2017; Muñoz-González et al., 2016). Nevertheless, further studies will be performed to clarify the role of the immune exhaustion phenomenon and other immunosuppressive disorders in the CSFV pathogenesis.

Taken together, our findings provide the first report of CSFV postnatal persistent infection in pigs at 21 days after birth. This type of infected animals shed high amounts of virus and are difficult to evaluate from the clinical and anatomopathological point of view. Considering that, persistent infected pigs may go unnoticed in the serological methods used for surveillance, as well as being unresponsive to vaccination (Muñoz-González, Pérez-Simó, et al., 2015). Therefore, the detection of this type of infection and its elimination in endemic areas is relevant for global CSF control. Finally, our findings support the use of the CSFV postnatal persistent infection as a model in the study of immunological phenomena associated with viral persistence.

4.7 Acknowledgements

This research was supported by grant AGL2015–66907, Ministerio de Ciencia, Innovación y Universidades from the Spanish government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from Spanish government. M.W. has a pre-doctoral fellowship CSC scholarship (2017) from Chinese government. The authors thank Ivan Cordón and the animal facility staff for their help during the in vivo part of the experiment as well as Maria Jesus Navas for her assistance in the flow cytometry assay.

Chapter 5

Study III. Identification of an Immunosuppressive Cell Population during Classical Swine Fever Virus Infection and Its Role in Viral Persistence in the Host

(Viruses. 2019. 11 (9). pii: E822)

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5.1 Abstract

Classical swine fever virus (CSFV) remains a highly important pathogen, causing major losses in the swine industry. Persistent infection is highly relevant for CSFV maintenance in the field; however, this form of infection is not fully understood. An increase in the granulocyte population has been detected in CSFV persistently infected animals. The aim of this work was to evaluate the possible immunosuppressive role of these cells in CSFV persistent infection. The phenotype of peripheral blood and bone marrow cells from persistently infected and naïve animals was evaluated by flow cytometry, and the capacity of specific cell subsets to reduce the interferon gamma (IFN-γ) response against unspecific and specific antigen was determined using co-culture assays. The frequency of granulocytic cells was increased in cells from CSFV persistently infected pigs and they showed a phenotype similar to immunosuppressive cell populations found in persistent infection in humans. These cells from persistently infected animals were able to reduce the IFN-γ response against unspecific and specific antigen. Our results suggest that immature immunosuppressive cell populations play a role in CSFV persistent infection in swine. The information obtained by studying the role of myeloid derived suppressor cells (MDSC) during CSFV persistent infection may extrapolate to other viral persistent infections in mammals.

5.2 Keywords

CSFV; viral persistence; immunosuppression; peripheral blood; bone marrow cells; 6D10 cells; MDSC; interferon gamma

5.3 Introduction

Classical swine fever (CSF) remains a highly relevant disease in swine, causing major losses to the industry which are related to various forms of disease (Blome, Staubach, et al., 2017). CSF has been eradicated in the US and Western Europe and remains endemic in several countries, including Asia, Central and South America, and Eastern Europe, with a recent outbreak being reported in Japan (Postel et al., 2019). The disease is caused by the CSF virus (CSFV), a positive stranded RNA virus that belongs to the *Pestivirus* genus within the *Flaviviridae* family (Simmonds et al., 2012).

Several viruses have developed mechanisms to induce persistent infection and evade the immune response in the host, favouring viral prevalence. Examples of viral persistence can be found in humans and animals after infections with different viruses such as *Adenovirus*, *Herpesvirus*, *Retrovirus*, *Flavivirus*, *Hepadnavirus*, *Papillomavirus*, and *Pestivirus* among others (Carossino et al., 2019; Gomez-Lucia et al., 2018; Lutz et al., 2009; Lv et al., 2018; Qin et al., 2013; Tacke et al., 2012; Taniwaki et al., 2013; Van Oirschot, 1979b). In this regard, after infection in utero, CSFV can generate congenital persistent infection (Blome, Staubach, et al., 2017; Van Oirschot, 1979a, 1979b) similarly to other pestiviruses affecting ruminants, such as bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) (Johnson & Muscoplat, 1973; Terpstra, 1981). Likewise, it has also been reported that CSFV is able to cause postnatal persistent infection following infection with a moderate virulence strain in piglets from the day of birth until 21 days after (Bohórquez, Wang, et al., 2019; Muñoz-González, Ruggli, et al., 2015), demonstrating the viral capacity to induce persistent infection in the host. Both congenitally and

postnatally persistently infected animals are clinically healthy or develop clinical signs that are not commonly associated with CSF. These pigs develop high viraemia and shed a high virus level for a long period in the absence of a specific humoral response to the virus (Bohórquez, Wang, et al., 2019; Muñoz-González et al., 2016; Muñoz-González, Ruggli, et al., 2015). Interestingly, an immunosuppressed status in the peripheral blood mononuclear cells (PBMCs) from postnatal persistently infected animals has been demonstrated (Muñoz-González, Ruggli, et al., 2015), which were measured for their inability to generate IFN-γ response against CSFV or a mitogen/polyclonal activator like phytohaemagglutinin (PHA) (Muñoz-González, Ruggli, et al., 2015). Additionally, high quantities of interleukin 10 (IL-10), a well-characterised immunosuppressive cytokine that inhibits a broad spectrum of immune responses (Rojas et al., 2017), was detected after CSFV stimulation in PBMCs of persistently infected animals (Muñoz-González, Ruggli, et al., 2015). Notably, during the persistent infection phase with CSFV, the type I IFN response was impaired in the infected animals (Cabezón et al., 2017; Muñoz-González et al., 2016). Moreover, the phenotypical profile in the bone marrow haematopoietic cells (BMHCs) from these animals showed an increase in the granulocytic ($6D10^{+}$) cell population (C. Pérez et al., 2007), being identified as a target for CSFV replication (Muñoz-González, Ruggli, et al., 2015).

Myeloid derived suppressor cells (MDSC) are immature myeloid cell populations that are able to induce immune suppression (Goh et al., 2013). First described in 1987 (Young et al., 1987), MDSC were initially associated with immunosuppression in tumour microenvironments through multiple mechanisms, mainly IL-10, arginase 1, induced Nitric oxide synthases (iNOS), and reactive oxygen species (Bronte et al., 2016;

Goh et al., 2013). More recently, MDSC have been implicated with viral infections such as Hepatitis C virus (HCV), Hepatitis B virus (HBV), and human immunodeficiency virus (HIV) (Lv et al., 2018; Qin et al., 2013; Tacke et al., 2012). In these infection models, the immunosuppression caused by MDSC populations has been suggested to facilitate the viral persistence and is also related to the worst clinical outcomes. So far, these cells have only been characterized in human and murine models, and in both species, there appears to be mainly two phenotypes, the monocytic-MDSC (M-MDSC) and the polymorphonuclear-MDSC (PMN-MDSC), which are morphologically and phenotypically similar to monocytes and neutrophils, respectively (Bronte et al., 2016; Goh et al., 2013; Ostrand-Rosenberg & Fenselau, 2018).

The characterization of the mechanisms used by MDSC to cause immunosuppression after viral infection has been one of the areas of interest in research as of late. However, the understanding of the interaction of these cells with the infected host in an in vivo model and their relation with the viral persistence in mammalian hosts remains a poorly studied area (Goh et al., 2013; Ostrand-Rosenberg & Fenselau, 2018; Stegelmeier et al., 2019). Considering this background, we hypothesized that the increase in the granulocyte precursor cell population during CSFV persistent infection might be an underlying factor in the impaired innate and adaptive immune response against CSFV in the infected host. Thus, we studied the role of the granulocyte precursor cell population as a MDSC population and its association with immunosuppression during CSFV persistent infection. The phenotypic profile of BMHC and PBMC from CSFV persistently infected animals was evaluated and multiple functional assays were performed in order to

characterize the population associated with the immunosuppressive capacity that may promote viral persistence.

5.4 Materials and Methods

5.4.1 Cells and Viruses

The porcine kidney cell line PK-15 was obtained from ATCC (CCL-33, Middlesex, England) and were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), *Pestivirus-free*, at 37 °C in 5% CO_2.

The CSFV moderately virulent Catalonia 01 (Cat01) strain, which belongs to the 2.3 subgenotype and has been proven to induce postnatal persistent infection in new-born piglets, was used (Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2014). The Alfort 187 strain, genogroup 1.1, was used in the neutralisation peroxidase-linked assay (NPLA). Viruses were grown in the PK-15 cell line that were infected with 0.1 $TCID_{50}$/cell in 2% FBS during 72 h of incubation. Peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral isolation and titration following the statistical methods previously described (Reed & Muench, 1938). The attenuated vaccine (C-strain) belongs to the CSFV 1.1 genogroup and was used in Spain in the 1980s for CSF control. This vaccine has 100% homology with the Z46258 strain. This vaccine strain was employed to immunize animals in order to obtain IFN-γ producing PBMCs for the co-culture assays (Blome, Moß, et al., 2017; Muñoz-González, Pérez-Simó, et al., 2015).

5.4.2 Experimental Infection

To reproduce the CSF persistent infection, an experimental infection in piglets was carried out according to previous studies (Muñoz-González,

Ruggli, et al., 2015). Seven piglets (Group 1, numbered 1 to 7), born to a *Pestivirus-free* sow in the biosecurity level 3 animal facility (BSL3) of the Centre de Recerca en Sanitat Animal (CReSA), were inoculated intranasally at 5 days of age with 2.5×10^4 TCID of CSFV Cat01 strain. The inoculation of the piglets was conducted separately from the sow and the animals were housed with their mother until 28 days after birth. Afterwards, the piglets were fed a conventional piglet starter diet (Neopig, Kwikstart, CARGILL, Zaragoza, Spain) until their time of euthanasia according to previous studies conducted in CReSA (Aragon et al., 2010; Cabezón et al., 2015).

A trained veterinarian recorded the rectal temperature and clinical signs daily in a blinded manner. Serum and rectal swab samples were collected every week after infection for five weeks. At this time, the piglets were euthanized and whole blood samples were collected in order to isolate peripheral blood mononuclear cells (PBMCs). During necropsy, the femurs and humeri of selected piglets (pigs 1, 2, 4, 5, and 6) were obtained in order to harvest bone marrow haematopoietic cells (BMHCs). The procedure for the euthanasia of the animals was based on an accepted method included in European Directive 2010/63/EU, using an anaesthetic overdose of 60–100 mg of pentobarbital per kilogram of weight, administered via the vena cava.

In addition, a whole blood sample was obtained from two donor animals (Group 2, numbered 8 and 9) from a vaccine trial (Muñoz-González, Pérez-Simó, et al., 2015); these animals were 6 weeks of age and had been vaccinated with one pig dose (equivalent to 100 protective doses) of the C-strain vaccine. They were proven to have an efficient specific cellular immune response against CSFV, which was measured by ELISPOT assay

at the time of sampling (Muñoz-González, Pérez-Simó, et al., 2015). Finally, three naïve animals (Group 3, numbered 10 to 12) of the same origin and age were used as controls. Whole blood samples, as well as the femurs and humeri from these animals were collected at necropsy. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB), under number 8646, (31st July 2017) according to existing national and European regulations.

5.4.3 CSFV RNA and Antibody Detection

Sera and rectal swab samples from these animals were used for RNA extraction with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The presence of CSFV RNA in the samples was detected by real time RT-qPCR (Hoffmann et al., 2005). Positive results were considered for threshold cycle values (CT) equal to or less than 42. Samples in which fluorescence was undetectable were considered negative. As previously described, Ct values from 10 to 22 were considered as high, from 23 to 28 were considered as moderate, and between 29 and 42 were considered as low RNA viral load (Tarradas, Álvarez, et al., 2011). The specific humoral immune response against CSFV was evaluated using a commercial ELISA kit (IDEXX, Liebfeld, Switzerland) detecting the presence of E2-specific antibodies in serum. According to the manufacturer's instructions, the samples were considered as negative when the blocking percentage value was below 30% and positive when it was above 40%. Serum samples were also tested (Terpstra et al., 1984) against the Alfort-187 strain. Neutralising antibody titres were expressed as the reciprocal dilution of serum that

neutralised 100 $TCID_{50}$ of the CSFV strains in 50% of the culture replicates.

5.4.4 PBMC and BMHC Collection and Phenotypical Analysis

PBMCs were obtained from whole blood of all the animals. Cells were separated by density-gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) followed by osmotic shock in order to eliminate the remaining erythrocytes. At this time, bone marrow haematopoietic cells (BMHCs) were also obtained from the femurs and humeri of selected pigs (pigs 1, 2, 4, 5, and 6) following the protocol previously described (Carrasco et al., 2004; Mussá et al., 2011). The number and viability of the cells were determined by staining with Trypan Blue (Ganges et al., 2005) and the cells were frozen until time of use at −80 °C in FBS with 10% dimethyl sulfoxide (DMSO).

Flow cytometry was used in order to evaluate the surface markers from PBMCs or BMHCs. Hybridoma supernatants were used for staining the swine cell surface markers CD172a (BA1C11, IgG1) (Alvarez et al., 2000), 6D10 (IgG2a) (C. Pérez et al., 2007), and CD33 (5D5, IgG1) (Álvarez et al., 2015). Secondary anti-Mouse IgG1 antibodies labelled with Alexa Fluor 647 or eFluor 450 (Thermofisher scientific, produced in goats and rats, respectively), as well as secondary goat Anti-Mouse IgG2a, labelled with R-Phycoerythrin (Jackson immunoresearch, West Grove, PA, USA) or Alexa Fluor 488 (Thermofisher scientific, Waltham, MA, USA), were used. Moreover, a conjugated mAb detecting CD11b from multiple species (Anti-CD11b antibody [M1/70] PE/Cy5, rat IgG2b, abcam) was also used.

For single-colour staining, cells (5×10^5/well) were plated and, after removal of the medium, 50 µL of either the hybridoma supernatant or the

conjugated anti-CD11b antibody were added. Following incubation at 4 °C for 20 min, the cells were washed with PBS + 2% FBS and the corresponding isotype-specific secondary antibody was added to the cells that had been incubated with hybridoma supernatant.

For two-colour staining, cells were first incubated with anti-6D10 hybridoma supernatant for 20 min at 4 °C. After washing, the second labelling was carried out either with anti-CD33 (hybridoma supernatant) or anti-CD11b (conjugated antibody). Afterwards, cells were washed with PBS + 2% FBS, followed by incubation with the isotype-specific secondary antibodies. A similar protocol was also used for three-colour staining, first labelling with the anti-6D10, followed by the anti-CD33. The anti-CD11b conjugated antibody was added when staining with the isotype-specific secondary antibodies. Finally, a viability control (propidium iodide, 1 µg/mL) was added and 30,000 live-cell events were recorded for each sample in the cytometer (FACSAria IIu, BD Biosciences, Franklin Lakes, NJ, USA).

The cells were analysed using the FACSDiva software, version 6.1.2, and the results were expressed as the percentage of positive cells obtained for each staining with respect to the live cells, using irrelevant isotype-matched mAbs as staining controls.

5.4.5 Sorting of 6D10^{+} Cells

The granulocyte precursor cell subset (6D10^{+}) was sorted using a sterile magnetic sorting mechanism (Miltenyi scientific, Bergisch Gladbach, Germany). Briefly, BMHC from three Group 1 animals (CSFV Cat01 infected pigs 1, 3, and 4) and from both Group 3 animals (naïve pigs), as well as PBMC from one Group 1 animal (pig 4), were defrosted and resuspended in RPMI Medium, supplemented with 10% FBS, *Pestivirus*-

free. Between 6–8 × 10^7 total cells were incubated at 4 °C for 20 min with anti-6D10 at a ratio of 500 µL of hybridoma supernatant for every 1 × 10^7 cells. The antibody was removed after centrifugation and the cells were resuspended in sorting buffer (PBS + 0.5% bovine serum albumin) containing 10% anti-mouse IgG labelled magnetic beads (Miltenyi scientific, Bergisch Gladbach, Germany) at a ratio of 100 µL of solution for every 1 × 10^7 cells. The incubation was carried out at 4 °C for 20 min in a dark environment. After washing, the cells were resuspended in 1 mL of sorting buffer and filtered using a 70 nm filter in order to pass through the magnetic column. Afterwards, the sorted cell subsets were washed and resuspended in RPMI medium supplemented with 10% FBS.

After sorting, the cells were counted in order to determine the yield, as well as analysed by flow cytometry in order to determine the purity of the eluted fraction. In addition, the presence of CSFV RNA, as well as the viral load, in 1,000,000 cells (either pre-sorted or $6D10^+$ cell subsets) were determined by qRT-PCR (Hoffmann et al., 2005) and by viral titration using the PLA test (Wensvoort et al., 1986), respectively.

5.4.6 Co-Culture Experiments and Determination of IFN-γ Production by ELISPOT

The number of IFN-γ secreting cells were determined using an ELISPOT assay which was previously described (Muñoz-González, Pérez-Simó, et al., 2015; Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2014). Plates were coated with 5 µg/mL capture antibody (P2G10, Pharmigen, BD Biosciences, Franklin Lakes, NJ, USA). Different cell combinations were seeded in the plates and stimulated for 72 h. Thereafter IFN-γ secreting cells were revealed by sequential incubations with a biotinylated

anti- IFN-γ mAb (P2C11, Pharmigen, BD Biosciences, Franklin Lakes, NJ, USA).

The functional assay comprised three experiments (Figure 1). For the first experiment, in step one, PBMCs from two naïve animals (pigs 10 and 11) were seeded by triplicate at a ratio of 3×10^5 cells/well in a previously coated ELISPOT plate. In step two, the PBMCs were co-cultured with either unsorted BMHCs or $6D10^+$ BMHC cell subset. These cells were added at a rate of 1×10^5 cells/well. The BMHCs had been collected from either naïve (pigs 10 to 12) or CSFV persistently infected animals (pigs 1 to 3). During step 3, the samples were either stimulated with phytohaemagglutinin (PHA) (10 µg/mL) or incubated in RPMI medium supplemented with 10% FBS (mock stimulated) for 72 h (Figure 1). Control wells containing only PBMCs, which were also mock or PHA stimulated, were included in the study.

For the second experiment, in step one, PBMCs, at a rate of 3×10^5 cells/well, were seeded and, in step two, co-culture with the same BMHC subsets (either unsorted BMHCs or $6D10^+$ cells) from Group 3 (pigs 10 and 11) or group 1 (pigs 1, 3, and 4) at a ratio of 1×10^5 cells/well was carried out. However, unlike in the previous experiment, the PBMCs used for this assay had been collected from animals showing specific CSFV cellular immune response in terms of IFN-γ production (pigs 8 and 9). The cells were either mock stimulated or stimulated with the CSFV Cat01 strain (MOI = 0.1) and were incubated for 72 h (step three). Control wells containing the PBMCs without co-culture, incubated in the presence or absence of CSFV Cat01 strain, were also included in the study (Figure 1).

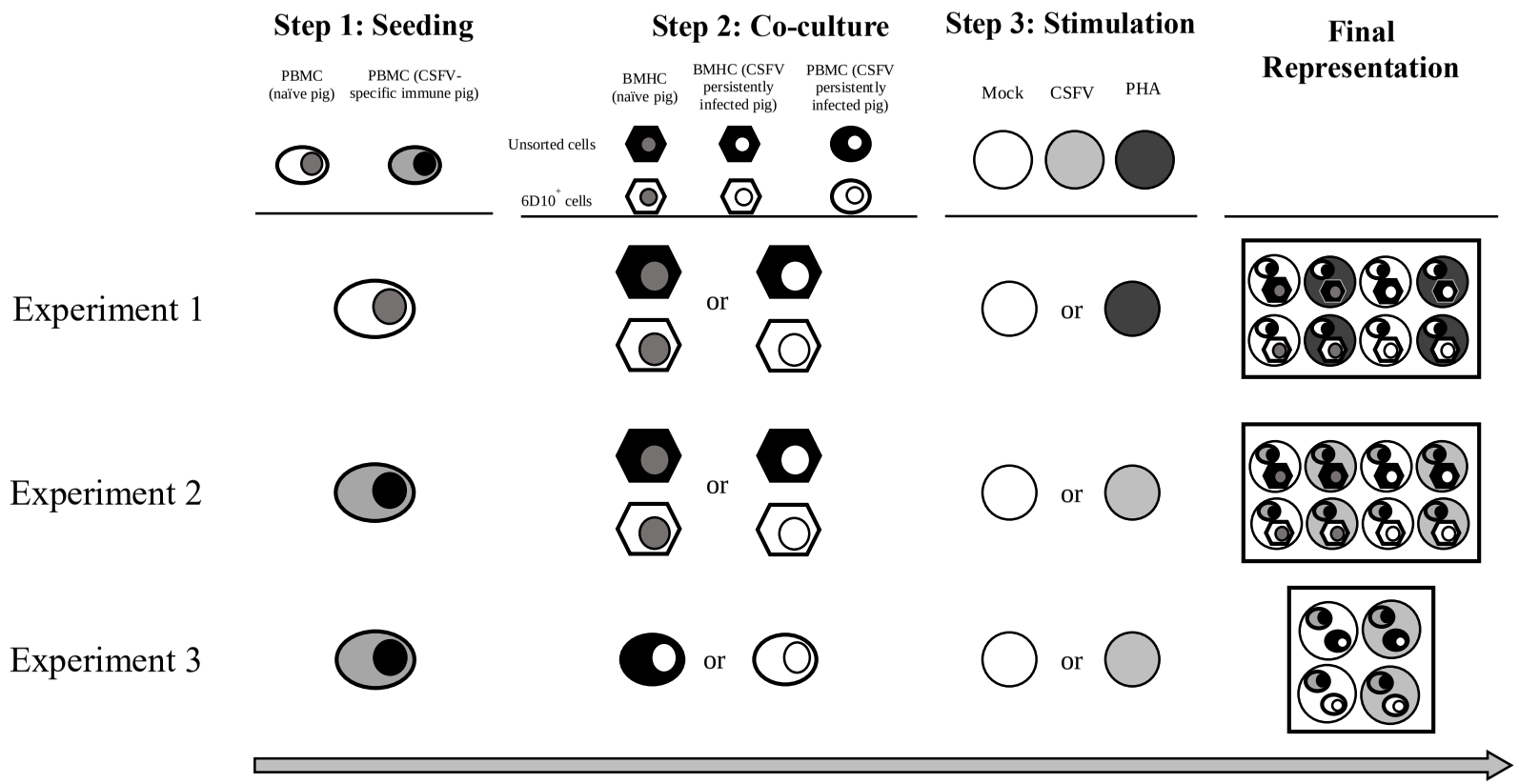


Figure 1. Illustration of the co-culture experiment layout. The figure shows an overview of the different cell combinations and experimental conditions tested. Three experiments were carried out, each of them consisting of three steps. Step 1: seeding, step 2: co-culture, and step 3: stimulation. A final representation of the cells and stimuli included in the wells for each experiment is also shown.

For the second experiment, in step one, PBMCs, at a rate of 3×10^5 cells/well, were seeded and, in step two, co-culture with the same BMHC subsets (either unsorted BMHCs or $6D10^+$ cells) from Group 3 (pigs 10 and 11) or group 1 (pigs 1, 3, and 4) at a ratio of 1×10^5 cells/well was carried out. However, unlike in the previous experiment, the PBMCs used for this assay had been collected from animals showing specific CSFV cellular immune response in terms of IFN-γ production (pigs 8 and 9). The cells were either mock stimulated or stimulated with the CSFV Cat01 strain (MOI = 0.1) and were incubated for 72 h (step three). Control wells containing the PBMCs without co-culture, incubated in the presence or absence of CSFV Cat01 strain, were also included in the study (Figure 1).

For the third experiment, in the first step, PBMCs collected from an animal showing specific CSFV cellular immune response (Pig 9) were seeded at a ratio of 3×10^5 cells/well. During the second step, these cells were co-cultured either with unsorted or $6D10^+$ cell subsets from pig 4 at a rate of 1×10^5 cells/well, which was the same rate as the previous experiments. However, instead of BMHCs, the cell subsets used for co-culture in this experiment were sorted from PBMCs of a Group 1 animal (Pig 4). For the third step, the same stimulations (Mock or CSFV Cat01) as in the second experiment were carried out (Figure 1).

All the assays were performed three times in order to evaluate the reproducibility. After detection with the biotinylated antibody, the percentage of difference in IFN-γ production between PBMCs co-cultured with the unsorted cell population and PBMCs co-cultured with the $6D10^+$ cell subset was calculated (Rieber et al., 2013).

5.5 Results

5.5.1 Establishment of CSFV Postnatal Persistent Infection

The piglets inoculated with the CSFV Cat01 strain showed fever during the first two weeks of the trial, however, the fever subsided after this time. Besides fever, these piglets did not develop other clinical signs during the first four weeks of the trial. In the last week of the trial, a variety of clinical signs were observed, such as mild and sporadic diarrhoea (pigs 2, 3, and 7), conjunctivitis (pigs 4 and 6), mild weakness of the hindquarters (pig 1), and for a few days, mild cyanosis in the ears (animals 4 and 5). Also, skin desquamation (pigs 6) and pustules were registered (pigs 4, 5, and 7).

CSFV RNA was detected in sera from all the pigs inoculated with the CSFV Cat01 strain during the first week after infection. The animals showed Ct values corresponding with high (three out of seven piglets), moderate (two out of seven), and low (two out of seven) CSFV RNA load. After the second week and until the time of euthanasia, the viral RNA load detected for all infected animals was high, with the exception of pig 5, which showed moderate CSFV RNA load on the last day of the trial (Figure 2).

For the rectal swab sample, CSFV RNA load was detected in all the inoculated piglets in the first week after infection and ranged from high (one of seven piglets) to low Ct values (four out of seven animals). During the second week after infection, the CSFV RNA load increased and was high for all the animals. Thereafter, the viral RNA load in the rectal swab sample was between high and moderate for all the animals until the end of the trial (Figure 2). None of the CSFV Cat01 infected animals showed an antibody response against CSFV during the five weeks of the trial, by either the ELISA or the NPLA test.

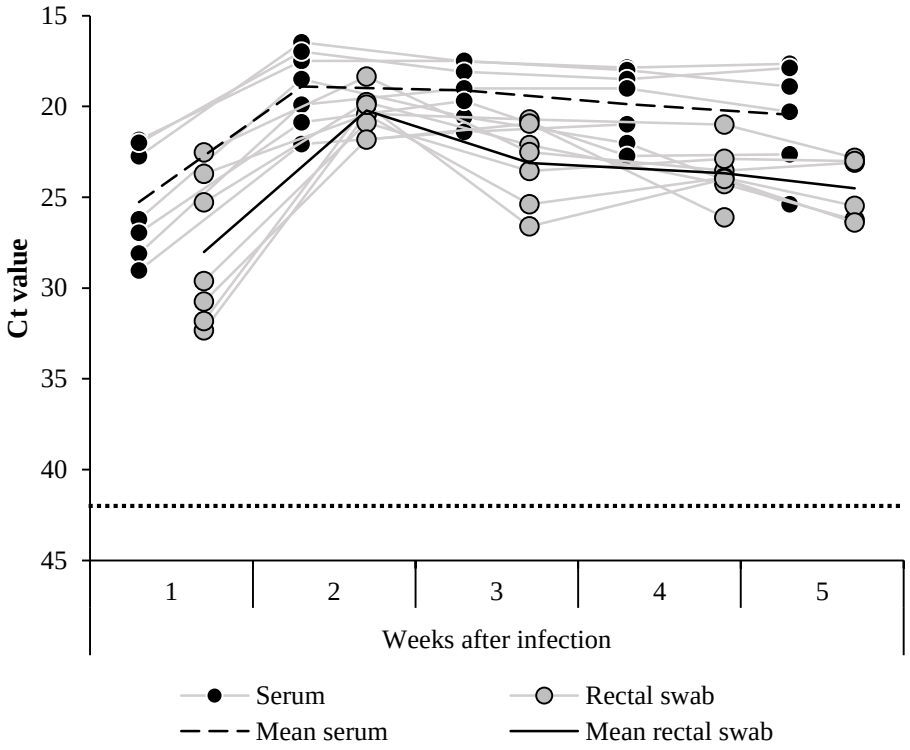


Figure 2. RNA detection in the sera and rectal swabs from the Cat01 infected pigs. Classical swine fever virus (CSFV) RNA was evaluated weekly in the sera (black dots) and rectal swab (gray dots) samples for 5 weeks after infection. Ct values above 42 (dotted line) were considered negative. Mean values for the serum and rectal swab samples are also indicated.

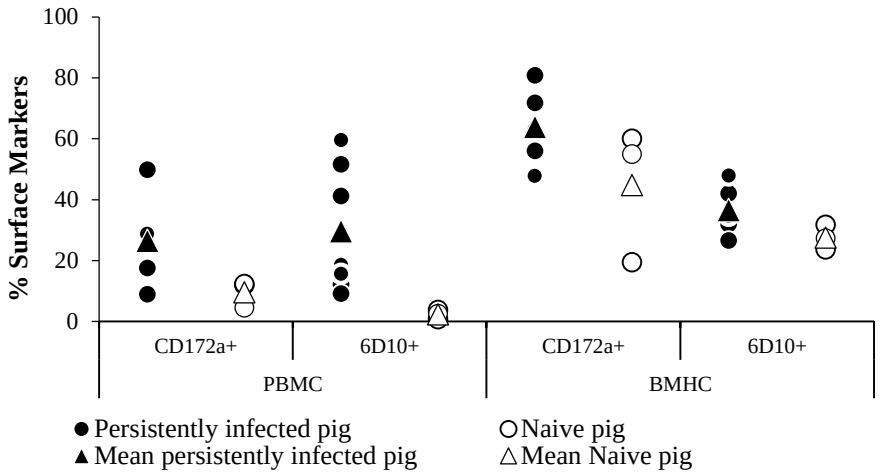
5.5.2 Granulocyte Precursor Cells Are Increased in CSFV Persistently Infected Animals and Show a Similar Phenotype to MDSC Populations

In cells collected from CSFV persistently infected animals, between 9.17% and 59.5% of cells in the PBMC fraction were found to be $6D10^{+}$. In the naïve animals, by contrast, the percentage of $6D10^{+}$ cells in PBMC was between 0.75% and 3.85%. In the BMHC samples, the $6D10^{+}$ cells ranged from 26.58% to 47.9% in CSFV persistently infected piglets and from 23.7% to 31.8% in naïve animals (Figure 3A).

In the case of the CD172a cell surface marker, the percentage of $CD172a^+$ cells was found to be between 8.93% and 49.84% in PBMC from CSFV persistently infected pigs. This cell subset ranged from 4.5% to 12.3% in PBMCs from naïve animals. Finally, in the BMHC samples of CSFV persistently infected pigs, between 47.8% and 80.8% of cells were $CD172a^+$, whereas in the same samples from naïve animals, the percentage of $CD172a^+$ cells ranged from 19.5% to 60% (Figure 3A).

Regarding the double and triple staining, in the BMHC samples from naïve animals, $6D10^+$, $CD33^+$, and $CD11b^+$ populations were found, however, only a low percentage of $6D10^+/CD33^+$ and no $6D10^+/CD11b^+$ double positive cells were detected (between 2.5% and 13%). In the PBMC from naïve animals, neither $6D10^+/CD33^+$ nor $6D10^+/CD11b^+$ were observed and, therefore, no $6D10^+/CD33^+/CD11b^+$ were observed. Whereas, in the BMHC samples, only between 2% and 5% of triple positive cells were found. Conversely, in BMHC and PBMC from CSFV Cat01 infected pigs, both $6D10^+/CD33^+$ and $6D10^+/CD11b^+$ cell populations were present, although in a lower proportion in the BMHC sample. Triple positive $6D10^+/CD33^+/CD11b^+$ cells were found in BMHC from persistently infected animals (representing between 6% and 12% of the $6D10^+$ cell population). Finally, the highest proportion of $6D10^+/CD33^+/CD11b^+$ cells was observed in PBMC samples from these animals, in which the percentage of positive cells ranged between 11.4% and 46.7% of the $6D10^+$ cell subset (Figure 3B).

A)


100
80
60
40
20
0
% Surface Markers
CD172a+
6D10+
CD172a+
6D10+
PBMC
BMHC
● Persistently infected pig
▲ Mean persistently infected pig
○ Naive pig
△ Mean Naive pig

B)

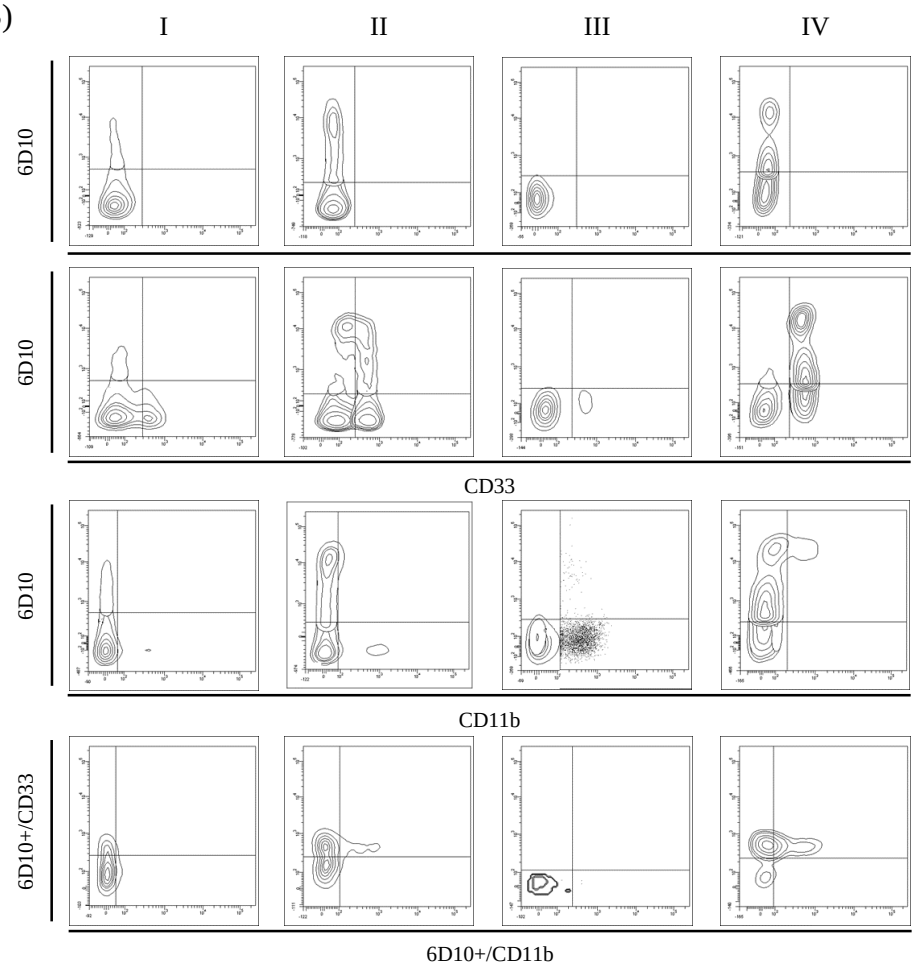

I
II
III
IV
6D10
6D10
CD33
6D10
CD11b
6D10+/CD33
6D10+/CD11b

Figure 3. Illustration of cell surface markers in peripheral blood mononuclear cells (PBMC) and bone marrow haematopoietic cells (BMHC) from CSFV persistently infected and naïve animals at 6 weeks of age. (A) Comparative expression of CD172a (myelomonocytic cells) and 6D10 (granulocyte lineage) cell surface markers in PBMCs and BMHCs from CSFV persistently infected (black dots) and naïve (white dots) animals. Mean values for each group are indicated (triangle symbol). (B) Comparative expression of MDSC surface markers labelled by single (6D10), double (6D10/CD33 and 6D10/CD11b), and triple (6D10/CD33/CD11b) staining in different cell populations (in the bottom panels, $6D10^+$ cells were gated and the expression of CD33vs CD11b analysed). The cell populations evaluated were: I) BMHC from a naïve animal, II) BMHC from a CSFV persistently infected animal, III) PBMC from a naïve animal, and IV) PBMC from a CSFV persistently infected animal. The experiments were repeated twice under the same conditions.

5.5.3 Granulocyte Precursor Cells Are a Target for CSFV

The $6D10^+$ cell subset was sorted from the BMHC of two naïve animals (pigs 10 and 11) and three CSFV persistently infected pigs (pigs 1, 3, and 4), as well as from the PBMC of pig number 4. The purity of the $6D10^+$ sorted cells was between 70% and 96% and the amount of cells recovered for each sorting ranged between 1–2 × 10^6 cells (Figure 4). Moreover, large and small cells were observed for both the cell populations (Forward scatter (FSC), Figure 4). However, in the sorted $6D10^+$ population, the cells with the higher FSC also showed the high side scatter (SSC) values, in contrast with the unsorted cells, which showed both high and low SSC (Figure 4).

CSFV RNA was evaluated from the cell populations and the Ct value in the samples from CSFV persistently infected animals was between 22.67 and 23.5 per million cells for the whole BMHC population and between 21.84 and 22.69 per million cells for the $6D10^+$ cell subset. The viral titre was determined for these cell subsets, with both the whole BMHC cell

population and the 6D10^{+} cells showing viral titres between $10^{3.4}$ and $10^{3.95}$ TCID/10^{6} cells. Finally, the viral titre was also determined for the 6D10^{-} fraction in the BMHC, ranging between $10^{1.6}$ and $10^{2,4}$ TCID/10^{6} cells. Cells from the naïve animals were negative for CSFV RNA.

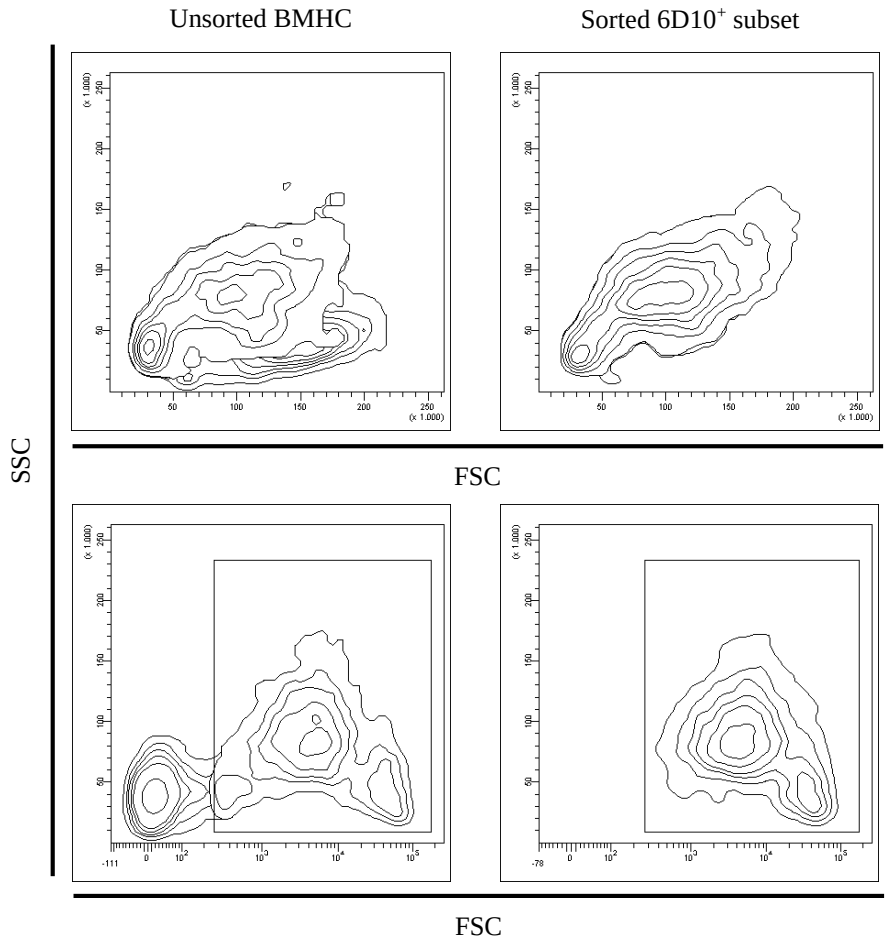


Figure 4. Evaluation of the unsorted and granulocytic (6D10+) cell populations in BMHC. The size and complexity (FSC versus SSC, upper part) as well as the expression of the 6D10 surface cell marker (lower part) was evaluated before and after magnetic sorting in order to detect differences in the phenotypic profile of BMHC and to ensure the correct performance of the magnetic sorting, respectively. The figure is representative of CSFV persistently infected animals (pig 3).

5.5.4 Granulocyte Precursor Cells from CSFV Persistently Infected Animals Are Able to Induce Immunosuppression

PBMCs from naïve animals showed a normal response in terms of IFN-γ when stimulated with PHA in the absence of any co-culture (200–250 IFN-γ spots/ 3×10^5 cells). The mean IFN-γ production after PHA stimulation in PBMCs from naïve animals was 150 and 90 IFN-γ spots/ 3×10^5 cells when these cells were co-cultured in the presence of the whole BMHC or $6D10^+$ cell populations from naïve animals, respectively (reduction of 38%, Figure 5A). A slightly higher reduction in the IFN-γ response was observed after co-culture of the same PBMC from naïve animals with the different BMHC cell subsets from CSFV PI animals. In this regard, the IFN-γ response was reduced by 46% when the cells were co-cultured with the $6D10^+$ subset compared to the co-culture and stimulation with the whole BMHC population. Specifically, around 130 and 70 IFN-γ spots/ 3×10^5 cells were evidenced for PBMCs co-cultured with the unsorted BMHC and the $6D10^+$ cell subsets, respectively (Figure 5A).

With regard to the second functional experiment, no differences in the CSFV specific IFN-γ response were observed between the co-culture of PBMCs from vaccinated animals with either the unsorted or $6D10^+$ cell subsets of BMHC from naïve animals. When the PBMCs from vaccinated animals were co-cultured with the unsorted BMHC population from CSFV persistently infected pigs, the IFN-γ response ranged between 39 and 155 spots/ 3×10^5 PBMC (Figure 5B). However, the IFN-γ levels observed when PBMCs were co-cultured with the $6D10^+$ cell population and stimulated with CSFV were between 24 and 30 spots/ 3×10^5 cells (Figure 5B), thus showing a reduction in the IFN-γ response between 40.9% and 84.9%.

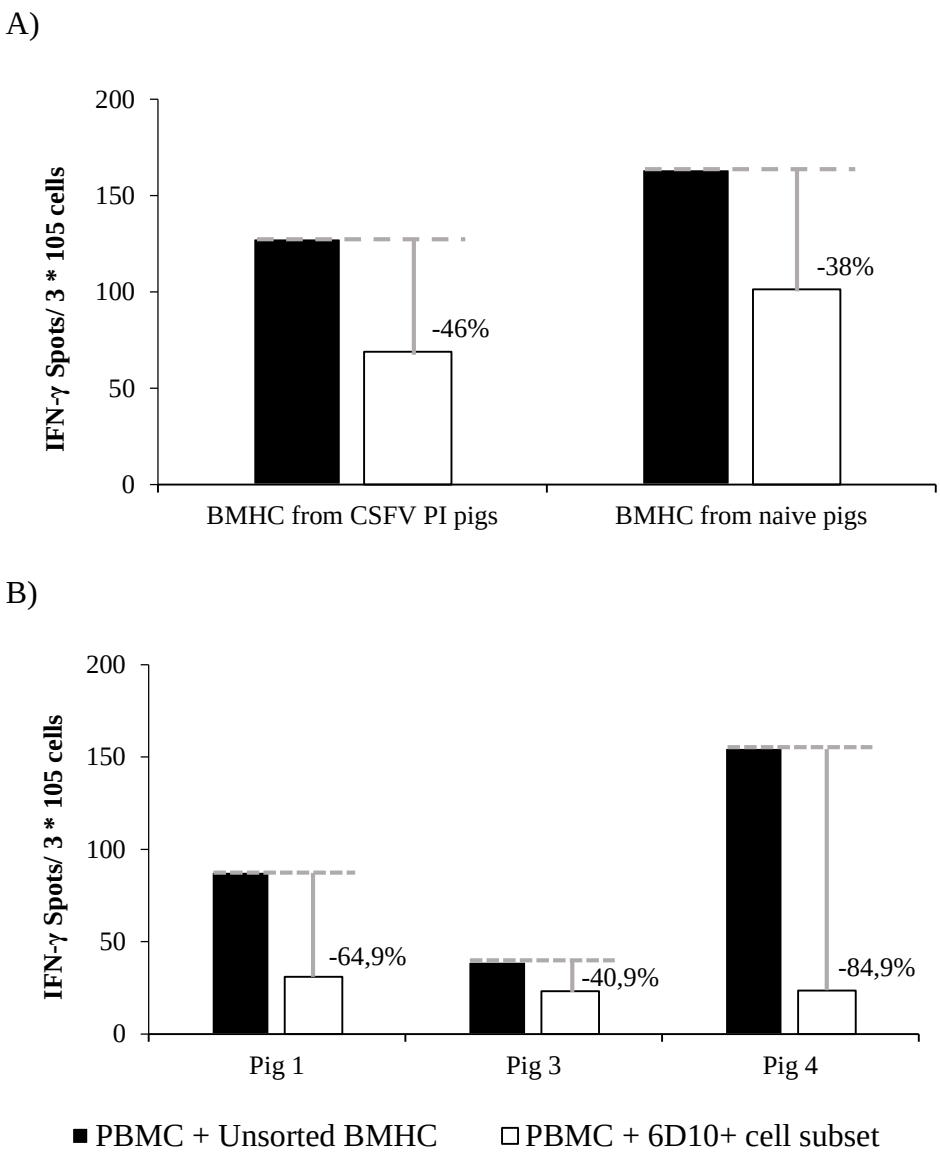


Figure 5. Reduction of the IFN-γ response by the granulocytic cell subset from BMHC. (A) PBMCs from a naïve animal were co-cultured with unsorted or $6D10^{+}$ cells from the BMHC of naïve or CSFV persistently infected pigs in a 1:3 ratio and stimulated with phytohaemagglutinin (PHA). (B) PBMCs from a CSFV-specific immune animal were co-cultured with unsorted or $6D10^{+}$ cells from BMHC of naïve or CSFV persistently infected pigs in a 1:3 ratio and stimulated with CSFV Cat01 (MOI = 0.1). The x axis indicates the origin of the BMHCs used for co-culture. IFN-γ production was measured by ELISPOT assay. All the assays were performed by triplicate to corroborate the results.

Finally, the cellular immune response, in terms of IFN-γ, in PBMC from a vaccinated animal that had previously shown specific IFN-γ response against CSFV (Pig 9) was altered when these cells were stimulated in the presence of different PBMC populations from animal 4 (CSFV persistently infected animal). In this regard, IFN-γ production after CSFV stimulation was determined to be 156 spots/ 3×10^5 cells when PBMCs from pig 9 were co-cultured in the presence of the full PBMC population from pig 4. However, when PBMCs from pig 9 were stimulated with CSFV in the presence of the $6D10^+$ cell subset from pig 4, IFN-γ production was around 27 spots/ 3×10^5 cells, showing a strong reduction of 82.9% (Figure 6).

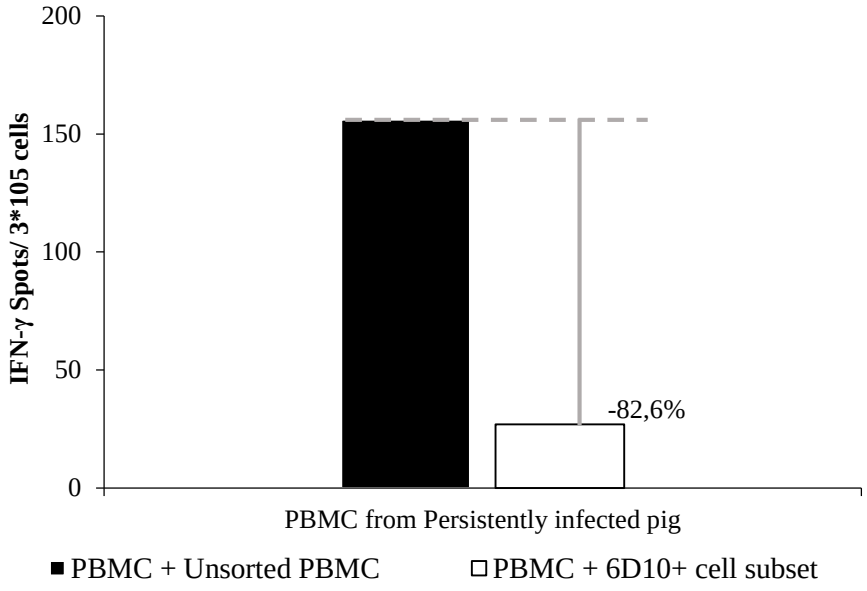


Figure 6. Reduction of the IFN-γ response by the granulocytic cell subset from PBMC of CSFV persistently infected animals. PBMCs from a CSFV-specific immune animal were co-cultured with unsorted (black bars) or sorted $6D10^+$ (white bars) cells from PBMC of a CSFV persistently infected pig in a 1:3 ratio and stimulated with CSFV Cat01 (MOI = 0.1). The x axis indicates the origin of the PBMCs used for co-culture. IFN-γ production was measured by ELISPOT assay. All the assays were performed by triplicate to corroborate the results.

5.6 Discussion

The capacity of the viruses from the *Pestivirus* genus to induce persistent infection is one of their most striking features, being fundamental for the maintenance of these viruses in farms. In this regard, the CSFV ability to induce persistence after a congenital infection has been documented since the late 1970s (Van Oirschot, 1979a, 1979b). However, more recent studies have also proven the establishment of CSFV persistent infection following postnatal infection in both domestic pigs and wild boar (Cabezón et al., 2015; Muñoz-González, Ruggli, et al., 2015). Nevertheless, the role of viral factors, such as the virulence or genogroup of the infecting strain, in the establishment of this form of disease remains to be studied. Additionally, the immunological phenomena taking place, as well as the alterations in the phenotypic profile of cells from the immune system in persistently infected pigs have not been fully elucidated. The animals infected with the Cat01 strain in the present study did not develop any humoral response against the virus and were found to be highly viraemic and shedding high amounts of the virus throughout the study. Therefore, these animals complied with the criteria for persistently infected animals previously stablished (Muñoz-González, Ruggli, et al., 2015; Van Oirschot, 1979a, 1979b).

Regarding the changes in cell populations, the increase in the percentage of $6D10^+$ cells in CSFV persistently infected animals after five weeks post infection is remarkable. This surface marker is present in cells of the granulocytic lineage and is highly expressed in precursor forms (C. Pérez et al., 2007). Additionally, $6D10^+/CD33^+/CD11b^+$ cells were evidenced in BMHC and particularly PBMC from persistently infected animals, whereas they were not found in the naïve pigs. Taking into account that

MDSC are immature cell populations, as well as their characterization in other species as $CD33^+/CD11b^+$ (Bronte et al., 2016; Cassetta et al., 2019; Ostrand-Rosenberg & Fenselau, 2018), our findings strongly suggested the role of an MDSC population in CSFV persistently infected animals. This cell population most likely represents PMN-MDSC, considering their granulocytic lineage, as well as their high size and complexity. Notably, it has been reported that, in the majority of cancer models where MDSC have been characterized, PMN-MDSC represent more than 80% of all MDSC (Gabrilovich, 2017; Kumar et al., 2016). The variability in the percentage of $6D10^+$ cells in PBMC and BMHC between the persistently infected animals from the present study might be attributed to factors such as the natural variability of each individual or even the fact that PMN-MDSC have been reported as being particularly sensitive to cryopreservation and/or thawing (Mandruzzato et al., 2016).

It should be noted that the surface markers evaluated in the present work could also be present in mature neutrophils. However, PMN-MDSC are low density cells and thus would remain in the upper fraction during the density gradient separation by which PBMCs are collected, whereas neutrophils would likely be lost during this process (Dumitru et al., 2012). In addition, the $6D10^+$ cell subset from CSFV persistently infected pigs was found to have immunosuppressive capabilities, reducing the IFN-γ production by PBMC after PHA stimulation, an important feature of MDSC characterization (Bronte et al., 2016; Goh et al., 2013). Most notably, this cell subset also induced suppression in terms of IFN-γ production of antigen-specific PBMC responses to CSFV. This finding is particularly significant given that this type of assay has been described as more relevant to in vivo conditions (Bronte et al., 2016). The largest reduction in the IFN-γ production was observed in the antigen-specific

assays, which is in agreement with previous studies, which have shown that a suppression in antigen-specific immune response is a primary feature of PMN-MDSC, in contrast to M-MDSC (Koehn et al., 2015; Marigo et al., 2010).

It is worth highlighting that the increase of the $6D10^+$ cell population was found in both BMHC and PBMC from persistently infected pigs. This implies that these cells are actively circulating in the bloodstream of these animals. Studies of persistent infection in humans in which MDSC have been implicated, such as HIV and HCV, have reported a similar increase of MDSC in PBMC from persistently infected patients (Qin et al., 2013; Tacke et al., 2012). The generation of MDSC populations during persistent infection has been associated with abnormal myelopoiesis and activation of neutrophils and monocytes. In normal conditions, this activation does not last long, as the stimulus is eliminated from the organism. Conversely, a prolonged stimulation might lead to pathological activation of myelopoiesis, which will produce cells with altered functions such as poor phagocytosis and production of anti-inflammatory cytokines (Bronte et al., 2016; Ostrand-Rosenberg & Fenselau, 2018; Youn et al., 2012). MDSC use multiple mechanisms to induce immunosuppression in the host, mainly IL-10, iNOS, and reactive oxygen species. The production of high levels of IL-10 by PBMC from CSFV persistently infected pigs has been previously reported (Muñoz-González, Ruggli, et al., 2015), further suggesting the role of MDSC populations in this form of infection. However, the blocking of IL-10 did not restore the production of IFN-γ in these cells, implying that there are other mechanisms involved in the immune suppression detected in these animals. Hence, the specific biochemical pathways used by the $6D10^+$ cells from CSFV persistently infected animals to induce immune suppression remain to be elucidated

and should be the subject of future works. Additionally, the presence of MDSC populations in organs from CSFV persistently infected animals cannot be discarded and warrants further studies considering that they have also been found in tumour microenvironments as well as in other lymphoid organs in cancer patients (Gabrilovich, 2017; Kumar et al., 2016).

Previous results have shown that CSFV persistent infection can be established when animals are infected as late as 21 days after birth (Bohórquez, Wang, et al., 2019), although at a lower rate than when pigs are infected a few hours after birth. This finding suggests a role of age in the establishment of CSFV persistent infection, which is likely related to the presence of MDSC. Previous studies showed that this cell population has been found in cord blood and around the neonatal period in humans and it has been implied that their presence might contribute to an increased susceptibility to infection (Rieber et al., 2013). So far, other aspects such as gender or swine leukocyte antigen type have not been related to MDSC.

Taken together, our results suggest that MDSC are playing a role in the establishment and maintenance of CSFV persistent infection in swine. The cells characterized in the present work showed a similar phenotype to previously described human MDSC populations, although no equivalent has been described for the $6D10^{+}$ surface marker in humans (C. Pérez et al., 2007). The fact that only a single marker ($6D10^{+}$) was used for the sorting of a cell subset, which was found to have immunosuppressive capabilities, may facilitate the study of MDSC in swine. Nevertheless, a thorough characterization of the mechanisms used by MDSC to generate persistent infection requires further studies. The information obtained by studying the role of MDSC during CSFV persistent infection may extrapolate to other viral persistent infections in mammals where similar

immunosuppressive mechanisms are taking place such as HIV, HCV, or HBV among others. In this manner, useful insight into the functionality of MDSC could be achieved, which is relevant for a thorough understanding of viral persistence, and may have repercussions in viral therapy and disease control.

5.7 Author Contributions

Conceptualization, J.D. and L.G.; Methodology, J.A.B., S.M.G., M.P.S. and C.R.; Formal Analysis, J.A.B., J.D., and L.G.; Resources, J.D. and L.G.; Writing—Original Draft Preparation, J.A.B. and L.G.; Writing—Review & Editing, J.D., J.A.B., and L.G.; Supervision, L.G.; Project Administration, L.G.; Funding Acquisition, L.G.

5.8 Funding

This research was funded by grants AGL2015–66907 and RTI2018-100887-B-100 Ministerio de Ciencia, Innovación y Universidades from the Spanish government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from Spanish government.

5.9 Acknowledgments

The authors thank Ivan Cordón and the CReSA animal facility staff for their help during the in vivo part of the experiment as well as Raquel Maeso and María Jesús Navas for her assistance in the flow cytometry assay. Raquel, you will be dearly missed, rest in peace.

5.10 Conflicts of Interest

The authors declare no conflicts of interest.

Part III

General discussion and conclusions

Chapter 6

General discussion

Considering the multidimensional impact that CSF can have in an affected country, from an economic, humanitarian and even animal welfare standpoint, the control of this disease is of great importance (Blome, Staubach, et al., 2017; Meuwissen et al., 1999). Historically, CSF control strategies have been built around serological surveillance and stamping out policies or vaccination schemes (Ganges et al., 2008; van Oirschot, 2003). Despite exhaustive efforts, CSF remains a problem in large areas of the world, threatening the food security and economic stability of the population. Moreover, as the virus has evolved towards lower virulence strains, mild or unapparent forms of disease have taken on a larger role in the epidemiology of CSF (Coronado et al., 2017; Coronado, Rios, et al., 2019; W. Ji et al., 2014; Kameyama et al., 2019). Some of these forms of disease, such as congenital persistent CSF, have been known for decades but had not been studied for a long time. By contrast, the discovery of the postnatal persistent CSF form is fairly recent and there are still aspects regarding the establishment and maintenance of this disease form that remain to be elucidated. Understanding of the virus-host interaction taking place in the persistent forms of CSF might help to develop novel strategies for their control, therefore contributing to a better global control of CSF. In this regard, it should be taken into account that these disease forms may have a severely negative impact for the eradication efforts on multiple fronts. On the one hand, animals suffering these disease forms will be able to spread the virus in farms for a long time, as they appear clinically healthy and are unresponsive to vaccination (Muñoz-González, Pérez-Simó, et al., 2015; Muñoz-González, Ruggli, et al., 2015; Van Oirschot, 1979a). On the other, due to their inability to induce an antibody response against CSFV, the will not be detectable by the currently used serological methods for surveillance.

The present thesis provides novel insight into the congenital persistence form of CSF. This disease form has been known for decades to be caused by low virulence CSFV strains, following trans-placental transmission (Van Oirschot & Terpstra, 1977). Study I offers evidence regarding the importance of the virulence degree of the infecting strain to induce trans-placental transmission, as well as congenital CSFV persistence. Three groups of sows were infected with either the Margarita, Cat01 or PdR CSFV strains, which had been previously characterized as high, moderate and low virulence, respectively. The highest percentage of trans-placental transmission was induced by the high and moderate virulence CSFV strains, despite the fact that these strains also induced better immune response in the sows. Conversely, the immune response in the PdR infected sows was low or non-existent. Nevertheless, a lower percentage of CSFV infection was detected in the litters from these sows. This strongly suggests that the replication efficacy of the infecting strain favours the generation of trans-placental infection.

As mentioned above, trans-placental transmission was highly efficient in litters from sows that eventually developed a strong immune response. It is likely that foetal infection in these litters may be taking place before the onset of protective immunity in the sows. This highlights the need for vaccines that guarantee the rapid induction of a protective immune response in sows, within the first week after immunization, in order to prevent this type of scenarios. Furthermore, the use of these vaccines needs to be in accordance with the guidelines established by the OIE with regard to the timing of vaccination, aiming to generate strong maternal immunity in piglets. It is also important that these vaccines are handled in an appropriate manner, avoiding problems like over dilution of the vaccine or breaking of the cold chain, as these issues may lead to poor immune

response in sows. It has recently been reported in an endemic situation that suboptimal vaccination in sows led to poor immune response in these animals and high prevalence of persistent CSF forms in their litters (Coronado, Bohórquez, et al., 2019).

Notwithstanding the tremendous capacity of the highly virulent Margarita strain to induce CSFV trans-placental transmission, it is highly unlikely that this would have resulted in the establishment of congenital persistence. This is due to the fact that foetuses from these litters showed severe lesions and the most likely outcome for them would have been death during the perinatal period. By contrast, foetuses from the PdR infected sows did not show any lesions and would have probably been born clinically healthy, despite some of them being persistently infected with CSFV. Even though not all of these animals would have developed the congenital persistent CSF form, the naïve piglets would have been infected by their littermates during the early postnatal period. This is particularly important, as the PdR strain has been previously reported to induce postnatal persistent infection (Muñoz-González, Ruggli, et al., 2015). Furthermore, high prevalence of CSF persistent forms has been reported in an endemic country under an ineffective vaccination program (Coronado, Bohórquez, et al., 2019), suggesting that this situation might already be taking place.

In addition, the results from Study I also show the capacity of a moderately virulent CSFV strain to induce congenital persistent infection in a very efficient manner, even though this form of disease has been mainly linked to low virulence strains. Interestingly, the congenital persistently infected piglets showed comparable or higher levels of viral replication than the foetuses from sows infected with the high virulence strain (Tables 2 and 4,

Study I). This may be due to the modulation of the immune response in persistently infected animals, which allows for uncontrolled viral replication in the host.

Remarkably, innate immune response was detected in some of the foetuses after infection with either high or low virulence CSFV (Table 3, study I). This suggests that the foetal immune system, despite not being fully mature, is able to recognize CSFV as a foreign pathogen and generate immune response. Moreover, IFN-α levels were also found in the piglets congenitally persistently infected with the moderately virulent CSFV strain. These findings appear to be at odds with the traditional definition of immunotolerance, which entails a total lack of recognition of the pathogen by the foetal immune system. Thus, it is probable that the immunotolerance phenomenon is more complex than previously thought, likely relying on the finely tuned regulation between viral replication and the modulation of the immune response. Nevertheless, the specific virus-host interaction mechanisms by which CSFV is able to establish viral persistence, warrant further studies. To the best of our knowledge, this is the first report of foetal immune response against CSFV.

Recently, it was reported that an increase in the type I and II IFN response takes place in the establishment of persistent infection with BVDV in bovine (Smirnova et al., 2014). However, in the case of BVDV, the activation of the IFN pathway coincided with a slight decrease in viral load, which allowed the virus to avoid the immune response and establish persistent infection. This did not appear to be the case in CSFV, where the highest levels of IFN-α coincided with the foetuses infected with the low virulence strain, showing the highest viral replication level (Tables 2 and 3, study I). In this regard, it is worth noting that study I strongly suggested

the involvement of cellular mechanisms of immune suppression in the foetuses and piglets. It is possible that due to the involvement of these cellular mechanisms, the establishment of CSF congenital persistence may not require a decrease in viral replication to avoid the immune response.

It should be highlighted that an increase in the percentage of $CD8^+$ T-cells was detected in the infected foetuses and piglets from study I, as well as in the postnatal persistently infected animals from study II (Figure 4 and 5, study I; Figure 5, study II). In both cases, the increase in this cell population resulted in a low CD4/CD8 ratio for these animals. A low CD4/CD8 ratio has been linked with an impaired immune response in some models of viral persistence in humans (Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014). In this scenario, the prolonged activation of T cells by antigens, will promote the proliferation and differentiation of $CD8^+$ T-cells into terminal states. This will eventually lead to exhaustion of the immune system, rendering it unresponsive against further stimuli (Yao & Moorman, 2013). Considering the results of studies I and II, it is likely that the immune system of the CSFV persistently infected animals is exhausted due to the constant pro-inflammatory stimulus of the virus. Nevertheless, other markers for immune exhaustion need to be studied in order to assert if this mechanism is playing a role in both congenital and postnatal CSF persistent forms (Khaitan & Unutmaz, 2011; Yao & Moorman, 2013).

Different clinical manifestations and immune response after CSFV infection were observed in animals of study II. This was in spite of the fact that the nine pigs in this study were the same age (21 days) and had been inoculated with the same viral load of the CSFV Cat01 strain. Specifically, two animals were able to generate antibody response against CSFV,

however, the antibody response was ineffective and these animals did not clear the virus, showing the chronic form of CSF. On the other hand, four pigs developed postnatal persistent CSF (Figure 1, Study II). Notably, the CD4/CD8 ratio in the pigs that were able to develop antibody response was similar to naïve animals and higher than the persistently infected pigs (Figure 6, study II). This suggests that, despite the immune system being subjected to chronic stimulation in both persistent and chronic CSF, the immunological modulation might differ between both forms of disease. Moreover, the remaining three animals showed severe clinical signs and either died or had to be euthanized. The clinical signs observed in these animals, as well as some of the persistently infected pigs, do not correspond with those typically associated with CSF. If these clinical signs are directly caused by CSFV, it would likely be associated with viral replication in abnormal target cells, due to the prolonged infection, rather than the exaggerated immune response. This is supported by the low IFN-α levels detected after infection. If this were the case, CSF might be manifesting in atypical forms in the field, hindering diagnosis. Secondary opportunistic infections cannot be ruled out either in these animals, considering the immunosuppressive capabilities of CSFV.

Besides the cellular immunological phenomena taking place during CSFV postnatal persistence, the role of age in the establishment of this disease form was evaluated in study II. It is widely acknowledged that the age of the host plays a significant role in the pathogenesis and clinical manifestations of CSF (Blome, Staubach, et al., 2017; Moennig et al., 2003). Still, given the novelty of the reports of postnatal persistent CSF, the timeframe for the establishment of this disease form has not been entirely determined. CSFV was found to generate postnatal persistent infection in animals after infection with a moderately virulent strain at 21

days after birth (Figures 1 and 3, study II). This is a point of concern, taking into account that piglets might be weaned at this time and mixed with animals from other litters (Mahan et al., 1998). Thus, weaned CSFV persistently infected pigs might be able to infect naïve pen-mates in the pre-fattening period and generate postnatal persistence in some of these animals. The occurrence of this scenario in field conditions and its importance in the epidemiology of CSF remain to be assessed. Interestingly, the proportion of CSFV postnatal persistently infected animals was decreased in study II compared with the previous reports of this disease form, in which infection was carried out hours after birth (Muñoz-González, Ruggli, et al., 2015). It is likely then, that the probability of an animal developing CSF postnatal persistence might decrease with age, indicating that this might be a determining factor for the establishment of the postnatal persistent CSF form.

One explanation for this relies on immunosuppressive cellular populations, such as MDSC, which might be present in younger animals but decrease with age. MDSC populations are present in cord blood as well as during the perinatal period in humans and they have been found to play a role in the maternal-foetal tolerance (Rieber et al., 2013; Schwarz et al., 2018). In study III, the presence of MDSC populations in pigs suffering the postnatal persistent CSF form was assessed. The evaluation of these cell populations comprised the phenotypical and functional characterization of the MDSC candidate cell subset in BMHC and PBMC from persistently infected pigs.

Flow cytometry analysis showed that granulocyte precursor cells ($6D10^+$) were increased in both BMHC and PBMC from the persistently infected animals (Figure 3, study III). In addition, these cells were found to have a phenotype matching the previously described for MDSC ($CD33^+$ $CD11b^+$)

(Goh et al., 2013). These cells ($6D10^+/CD33^+/CD11b^+$) appeared to be unique for CSFV persistently infected pigs (Figure 3, study III). Considering that one of the markers used in the characterization of the MDSC cell population was specific to granulocytes ($6D10^+$), the MDSC populations present during CSF postnatal persistence were identified as PMN-MDSC. Nevertheless, phenotypical characterization is not sufficient for the determination of MDSC (Bronte et al., 2016; Mandruzzato et al., 2016) and functional analysis was carried out in order to determine the immunosuppressive capabilities of the MDSC candidate subset. The $6D10^+/CD33^+/CD11b^+$ cells from the postnatal persistently infected pigs were able to reduce IFN-γ secretion against unspecific and specific antigen (Figures 5 and 6, study III). The detected decrease in the immune response was found to be more drastic against specific antigen stimulation. This is in line with previous reports showing that PMN-MDSC favour this form of immunosuppression, contrary to the M-MDSC, which mainly affect immune suppression against unspecific antigens (Kumar et al., 2016).

Multiple factors had suggested the implication of MDSC populations during postnatal persistent CSF, such as the reported increase in granulocyte precursors in BMHC from these animals. Likewise, some of the mechanisms known to be used by MDSC had previously been reported to occur in this form of disease, i.e. the increased production of IL-10 (Muñoz-González, Ruggli, et al., 2015). Nevertheless, the implication of these cell populations during other forms of CSF cannot be discarded and warrants further analysis.

Taken together, the results from the present thesis provide insight into the intricate virus-host interaction taking place during viral persistence caused by CSFV. The results obtained in study I underline the importance of fast

and solid immunity against CSFV in sows and shed light into the generation of an immune response during the foetal period. These results may have important repercussions for disease control in endemic countries and open the way for the reconsideration of the dogmatic view regarding the immuntolerance phenomenon. On the other hand, the results of studies II and III show that CSFV uses multiple pathways for modulation of the immune response during postnatal persistent infection. Interestingly, immune exhaustion and MDSC are mechanisms that have been described to play important roles during chronic and persistent infections in humans (Serrano-Villar, Sainz, et al., 2014; Tacke et al., 2012). Furthermore, they have also been found to impair immune checkpoint inhibitor therapy and are associated with poor outcomes in cancer (S. M. Park & Youn, 2019; Saito et al., 2019; Weber et al., 2018). The present thesis provides the first reports of these mechanisms in swine, however, further studies are needed in order to fully assess the role of these immune modulation pathways in the postnatal persistent CSF form. Specifically, the evaluation of other important markers for immune exhaustion, such as the programed cell death protein 1 (PD-1), remains to be carried out (Saito et al., 2019). In addition, a biochemical study of the specific mechanisms used by PMN-MDSC during CSF postnatal persistence will aid in the characterization of these cell populations (Mandruzzato et al., 2016). Hence, further studies are needed in order to expand on the knowledge regarding the implication of these mechanisms in persistent forms of CSF. The insight obtained from these studies could potentially lead to the determination of the CSF persistent forms as a model for the study of these immunological phenomena. This might be helpful in overcoming current challenges in CSF control, as well as problems in antiviral and cancer therapy.

Chapter 7

Conclusions

1. The highly and moderately virulent CSFV strains with higher replication capacity are more efficient for the induction of trans-placental transmission than low virulence strains. Moreover, moderate virulence CSFV strains are also able to induce CSFV congenital persistent infection, showing that this form of disease is not solely associated with low virulence strains.

2. The trans-placental transmission of CSFV takes place before the onset of immune response in naïve sows. In order to avoid trans-placental transmission, sows should be immunized with vaccines that are able to induce fast and strong immune response within the first days after vaccination.

3. Following infection of the sows at 74 days of gestation with high and low virulence CSFV strains, the porcine foetal immune system is able to recognize CSFV as a foreign pathogen and generate innate immune response, in terms of IFN-α production. The innate immune response in foetuses appeared to directly correlate with the level of viral replication after infection.

4. CSFV induces alterations in the phenotypical profile of PBMCs from infected foetuses and congenital persistently infected piglets, mainly an increase in the $CD8^+$ cell subset.

5. The postnatal persistent form of CSF can be established after infection of piglets at 21 days of age after infection with a moderately virulent CSFV strain. However, the proportion of persistently infected animals at this time point is lower than when

infection is carried out within the first hours after birth. This supports the role of age as a host factor relevant for the establishment of CSFV postnatal persistent infection.

6. A low CD4/CD8 ratio was found in CSFV postnatal persistently infected animals, which has been used as a marker of immune exhaustion in other species. It is possible that, during CSFV postnatal persistent infection, immune exhaustion is reducing the capacity of the host to generate specific immune response against the virus, favouring viral persistence in the infected animals.

7. An increase in the granulocyte precursor cell subset ($6D10^+$) was detected in BMHC and PBMC from CSFV postnatal persistently infected animals. These cells were found to have a phenotype previously described for MDSC populations ($CD33^+/CD11b^+$), making them a PMN-MDSC candidate subset.

8. The immunosuppressive capabilities of the MDSC candidate cell subset ($6D10^+/CD33^+/CD11b^+$) from the BMHC and PBMC of postnatal persistently infected animals was determined ex vivo. These cells were able to decrease the IFN-γ production by PBMCs against unspecific and specific antigen. Therefore, they comply with the currently accepted requirements for characterization as MDSC.

9. Immune suppression during CSFV postnatal persistent infection appears to be dependent on multiple mechanisms, such as immune exhaustion and MDSC. Other viral persistent infections in mammal species, including humans, appear to use similar mechanisms for

evasion of the immune response. Therefore, the CSFV postnatal persistent infection model could be used for the study of these phenomena and the development of antiviral strategies. Moreover, considering the immunomodulatory capabilities of MDSCs, these cell populations might be of use for conditions in which disease is caused by exacerbated immune responses.

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