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PhD Thesis

Bellaterra, 2017

Universitat Autònoma de Barcelona

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Tesis doctoral presentada por **Sara Muñoz González** para acceder al grado de doctor en el marco del programa de Doctorado en Medicina y Sanidad Animal de la Facultat de Veterinaria de la Universitat Autònoma de Barcelona, bajo la dirección de la Dra. **Llilianne Ganges Espinosa** y del Dr. **Mariano Domingo Álvarez.**

Bellaterra, 2017

CReSA
Centre de Recerca en Sanitat Animal

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

Generalitat de Catalunya
Government of Catalonia

This work has been financially supported by grant AGL2012-38343 from Spanish government

Sara Muñoz-González received predoctoral fellowship FI-DGR 2014 from AGAUR, Generalitat de Catalunya.

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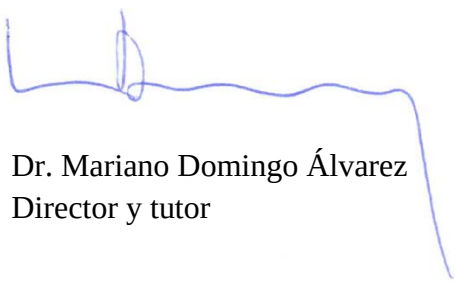

Dr. Mariano Domingo Álvarez
Director y tutor

Sara Muñoz González
Doctoranda

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LIST OF ABBREVIATIONS

APPV	Atypical porcine pestivirus
ASF	African swine fever
BDV	Border disease virus
BMHC	Bone marrow hematopoietic cells
BSL3	Biosecurity level 3
BVDV	Bovine viral diarrhea virus
CD	Cluster differentiation
CNS	Central nervous system
Cp	Cytopathogenic
CSF	Classical swine fever
CSFV	Classical swine fever virus
CTL	Cytotoxic T lymphocyte
DAS	Double-antibody-sandwich
DC	Dendritic cell
DIC	Disseminated intravascular coagulation
DIVA	Differentiation of infected from vaccinated animals
DPI	Days post infection
DPV	Days post vaccination
EDC	Endothelial cells
ELISA	Enzyme-linked immunosorbent assays
EU	European Union
EURL	European Union Reference Laboratory

FAT	Fluorescent antibody test
HC	Hog cholera (now classical swine fever)
IFN	Interferon
IL	Interleukin
IRES	Internal Ribosome Entry Site
ISG	IFN stimulated genes
MΦ	Macrophage
NPLA	Neutralizing peroxidase–linked assay
NrPV	Norwegian rat pestivirus
OIE	World Organisation for Animal Health
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
pDC	Plasmocytoid dendritic cell
PDNS	Porcine Dermatitis and Nephropathy Syndrome
PHA	Phytohaemagglutinin
PI	Persistently infected
PLA	Peroxidase-linked assay
RaPestV	*Rhinolophus affinis bats*
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SIE	Superinfection exclusion
SLA	Swine Leucocyte Antigens or histocompatibility complex (MHC) proteins
TSV	Tunisian sheep virus

UTR	Untranslated regions

ABSTRACT

Classical swine fever (CSF) is a highly contagious viral disease of domestic and wild pigs, included in the list of diseases notifiable to the World Organisation for Animal Health. The causative agent, CSF virus (CSFV), belongs to the *Pestivirus* genus, *Flaviviridae* family. Over the last century, great efforts have been directed towards the control and eradication of CSF, which today remains one of the most important diseases for animal health and in the pig industry worldwide. The circulation and intrinsic importance of low and moderate virulence strains in the endemic regions has been extensively described. Also, it has been shown the role of these type of viral strains in the generation of "the pregnant carrier sow syndrome" and persistent infection of piglets after trans-placental transmission. However, the mechanisms involved in this form of CSFV infections are not well known, and the existing studies date over 40 years ago. By contrast, the possible generation of viral persistence after postnatal infection of pigs is still a question to resolve.

Against this background, this thesis has demonstrated the capacity of low and moderate virulence CSFV strains to produce postnatal persistent infection early after birth. To this end, newborn piglets were inoculated intranasally with CSFV. During six weeks, these piglets remained apparently healthy, although they were not able to generate detectable CSFV specific humoral nor cellular immune responses, maintaining high virus load in blood, organs and body secretions and excretions. In addition, it has been demonstrated the ineffectiveness of vaccination of six-week-old PI pigs, which were unable to elicit a detectable innate immune response, in terms of IFN type-I production, as well as acquired immune responses (i.e. IFN type-II and antibodies) following vaccination with a CSFV live attenuated vaccine (C-strain). The RNA of the vaccine could not be detected by a specific RT-qPCR in any of the samples analysed after vaccination, including the tonsil, suggesting a superinfection exclusion (SIE) phenomenon between the persistently infecting virus (primary infection) and the CSFV

vaccine strain (secondary infection). Finally, six-week-old wild boars with CSFV persistent infection (first infection) were inoculated with a CSFV strain of high virulence (second infection or superinfection). PI wild boars did not develop clinical signs and showed a high load of the primary virus, causing persistence, in all samples analysed. In contrast, the secondary virus was not detected by either RT-qPCR or sequence analyses, thus demonstrating the phenomenon of superinfection exclusion (SIE). The lack of innate and acquired immune responses supported the previous studies from this thesis. In addition, *in vitro* assays with the PBMCs isolated *ex vivo* from persistently infected animals, a well-known target for the CSFV viral replication, further corroborated the CSFV SIE phenomenon. These findings demonstrated for the first time the ability of CSFV to induce SIE *in vivo,* in swine with persistent infection. The SIE phenomenon would likely explain the unresponsiveness of animals with persistent infection after vaccination with the live attenuated vaccine. Considering the existence of CSFV strains of low and moderate virulence and their ability to produce persistent infection forms, the results showed here may have potential epidemiological repercussions, especially in the endemic context. Importantly, persistently infected animals would remain unnoticed under serological control, given the absence of specific antibody response. This doctoral thesis has contributed to the understanding of the pathogenesis of CSFV infection, which depends not only on the virulence of the strain but also on the virus-host interaction, and opens new lines of research to understand the immunological bases and of viral pathogenesis for the generation of immunotolerance and persistence, hitherto unknown.

RESUMEN

La peste porcina clásica (PPC) es una enfermedad viral altamente contagiosa de cerdos domésticos y salvajes, incluida en la lista de enfermedades de declaración obligatoria a la Organización Mundial de Sanidad Animal. El agente causal, el virus PPC (VPPC), pertenece al género *Pestivirus*, familia *Flaviviridae*. A pesar de los grandes esfuerzos dirigidos a controlar y erradicar la PPC, continúa siendo una de las enfermedades más importantes para la sanidad animal y la industria porcina en todo el mundo. La circulación y la importancia intrínseca de cepas de VPPC de baja y moderada virulencia en las regiones endémicas ha sido objeto de discusión en los últimos años. Varios trabajos han demostrado el papel de cepas de baja virulencia en el nacimiento de lechones persistentemente infectados (PI) con el VPPC. No obstante, los mecanismos involucrados en este tipo de infecciones por VPPC no son bien conocidos, y los estudios existentes datan de hace 40 años. Por otro lado, la posible generación de persistencia viral después de la infección postnatal con VPPC era todavía una cuestión a resolver.

Esta tesis demuestra la capacidad de cepas de VPPC de baja y moderada virulencia de inducir persistencia vírica de forma postnatal en cerdo doméstico y salvaje. Para ello, cerditos recién nacidos fueron inoculados de forma intranasal con VPPC. Durante seis semanas, estos lechones permanecieron aparentemente sanos, a pesar de no generar respuesta inmunológica celular ni humoral específica para VPPC. Se demostró viremia permanente y alta carga de virus en todas las muestras de tejido, secreciones y excreciones de los animales persistentemente infectados (PI), hasta el fin del estudio. Además, se demostró la ineficacia de la vacunación de cerdos PI, de seis semanas de edad, los cuales no generaron respuesta de IFN de tipo I ni de tipo II, ni de anticuerpos durante 21 días tras la vacunación con una vacuna viva atenuada de VPPC (la C-strain). La falta de detección del ARN vacunal mediante RT-qPCR específica en sangre, excreciones y tejidos, incluyendo la tonsila, sugirió la ausencia de replicación del

virus vacunal en los animales PI y un posible fenómeno de interferencia viral. Finalmente, jabalíes de seis semanas de vida con infección persistente de VPPC (primera infección) fueron inoculados con una cepa de alta virulencia de VPPC (segunda infección o superinfección). Los jabalíes PI no desarrollaron signos clínicos y mostraron alta carga del virus primario, causante de la persistencia, en todas las muestras analizadas. Por el contrario, el virus secundario no fue detectado ni por RT-qPCR ni por secuenciación, demostrando así el fenómeno de exclusión de la superinfección (ESI). La ausencia de una respuesta de IFN de tipo I y II y de anticuerpos respaldó los resultados previos de los estudios de esta tesis. Estos hallazgos demostraron por primera vez la capacidad del VPPC para inducir ESI *in vivo*, en cerdos con infección persistente. El fenómeno ESI probablemente explicaría la falta de respuesta de los animales con infección persistente después de la vacunación con la vacuna viva atenuada. Considerando la existencia de cepas de virulencia baja a moderada y su capacidad para producir formas de infección persistente, los resultados aquí presentados pueden tener potenciales repercusiones epidemiológicas, especialmente en el contexto endémico. Es importante destacar que los animales persistentemente infectados pasarían desapercibidos bajo control serológico, dada la ausencia de respuesta de anticuerpos específicos. Esta tesis doctoral ha contribuido a la comprensión de la patogénesis producida por el VPPC, que depende no sólo de la virulencia de la cepa sino también de la interacción virus-huésped, y abre nuevas líneas de investigación para comprender los mecanismos subyacentes que conducen a la generación de inmunotolerancia y persistencia del VPPC.

PART I

General introduction and objectives

Chapter 1

General introduction

1.1 Classical swine fever: distribution and economic impact along the history

Classical swine fever (CSF) is a highly contagious viral disease of domestic and wild pigs (Moennig, 2015; Moennig and Becher, 2015) included in the list of diseases notifiable to the World Organisation for Animal Health (OIE, www.oie.int). Despite CSF constitutes one of the oldest diseases in animal health and, indeed, has been one of the most studied, it still causes major losses in stock farming, and is considered to be one of the most devastating diseases for the pig industry throughout the world from both the economic and sanitary point of view (Moennig and Becher, 2015).

The causative agent, CSF virus (CSFV), was discovered in 1904 (Schweinitz and Dorset, 1904), but CSF disease was first recognized in 1810 in Tennessee and in 1833 in Ohio, USA (Hanson, 1957). CSF was widespread in Europe and America by the 1860s (Cole et al., 1962). The spread of the infection may have been facilitated by the development of railways during the mid-19th century (Birch, 1992; Cole et al., 1962).

The global distribution of CSF has been extensively reviewed (Beer et al., 2015; Dong and Chen, 2007; Frías-Lepoureau and Greiser-Wilke, 2002; Greiser-Wilke and Moennig, 2004). The disease is endemic in Asia and is prevalent in many countries of central and South America (de Arce et al., 2005; Beer et al., 2015; Frías-Lepoureau and Greiser-Wilke, 2002; Pereda et al., 2005; Pérez et al., 2012; Postel et al., 2013b). The situation in Africa is unclear due to limited or no surveillance; CSF has been reported in Madagascar, Equatorial Guinea and in South Africa (Sandvik et al., 2005). In Spain, the disease was first recorded in 1875 (Cole et al., 1962).

While North America and Australia were declared CSF-free several decades ago, it took longer time to achieve the free-status in the European Union, where a progressive eradication program since the early 1990s was implemented (Paton and Greiser-Wilke, 2003). However, given the huge porcine population density,

and despite strict animal importation regulations, a high risk of re-emerging still exists. A non-vaccination policy, the culling of infected animals or those in contact with infected herds (*stamping out*) and the restriction of animal movements or their products, are the main strategies intended to avoid new entrances. In the EU, only emergency vaccination is allowed (European Community, 2001). The virus has been introduced periodically into the EU from wild boars or pig trade, as occurred during the 1990s in Belgium, Germany, The Netherlands, Spain and Italy (Beer et al., 2015; Greiser-Wilke et al., 2000). The cost of these outbreaks remains to be entirely calculated, but it has been estimated that the Netherlands outbreak required the destruction of 11 million pigs and caused economic losses as high as € 2.3 billion (Meuwissen et al., 1999). Afterwards, small outbreaks have occurred recently in many European countries e.g. in the UK, Spain (2001), France (2005-2007), Germany (2005-2007 and 2009), Hungary (2008-2009), Latvia (2014-2015) and Lithuania (2009, 2015) (Beer et al., 2015; Dong and Chen, 2007; Paton and Greiser-Wilke, 2003; Postel et al., 2013a). In 2015 the OIE identified as endemic countries Bolivia, Cambodia, Cuba, Ecuador, Haiti, Laos, Madagascar, Nepal, Serbia and Montenegro, Vietnam, China, Dominican Republic, India, Indonesia, Mongolia, Peru and Philippines. In Bhutan, Colombia, Russia and Thailand the disease is restricted to one or more zones. The last outbreaks in 2016 were recorded in Cambodia, Cuba, Mongolia, South Korea, Nepal, Serbia and Vietnam (OIE-WAHIS interface, 2016).

Many countries have implemented successful control and eradication programs. Nevertheless, CSF disease is still in continuous expansion, and will remain an important animals health and economic threat worldwide, because of the globalization and intensification of pig trade and transport, the increase in pig density in many areas, increased numbers of wild boar, which act as reservoir of CSFV, and the feeding of improperly sterilized swill (Edwards et al., 2000; Moennig and Becher, 2015; Monger et al., 2014).

1.2 Classical swine fever virus

The aetilogical agent, CSF virus (CSFV), is a member of the genus *Pestivirus* within the family Flaviviridae, together with the genera *Flavivirus, Hepacivirus* and *Pegivirus* (Beer et al., 2015; Horzinek, 1991; ICTV, 2015; Thiel, 2005). CSFV has an icosahedric simetry and 40-60 nm of diameter. It is composed of a lipid envelope, a capsid and a single plus-stranded RNA genome of approximatey 12000 pb (Rümenapf and Thiel, 2008; Tautz et al., 2015).

1.2.1 Physicochemical properties and taxonomy

As an enveloped virus, CSFV is rapidly inactivated by organic solvents and detergents. CSFV is also sensitive to a wide range of chemicals, including chlorine-based disinfectants, detergents, phenolics, quaternary ammonium compounds, and aldehydes (formaldehyde, glutaraldehyde). The virus is inactivated with 2% hypochlorite; 6% cresol, 5% phenol and sodium hydroxide 2% (Moennig and Plagemann, 1992). CSFV is stable at neutral to slightly alkaline pH values (range pH 5-10), but is sensitive to pH less than 3 or greater than 11 (Terpstra, 1991). CSFV can remain almost unchanged with minimal loss of titre in lyophilized state or deep frozen at -70 °C for months or years. In addition, it is sensitive to ultraviolet radiation, heat (100 ºC less than a minute or one hour at 56 °C) and drying (Edwards, 2000).

Other members of the genus *Pestivirus* are bovine viral diarrhea virus 1 (BVDV 1), bovine viral diarrhea virus 2 (BVDV 2), and border disease virus (BDV) of sheep (ICTV, 2015; Thiel, 2005). Additional putative pestivirus species have been identified, which encode for proteins being unique for members of the genus *Pestivirus*, namely the N-terminal protease N^{pro} and the secreted glycoprotein E^{rns} containing also the conserved motif required for RNAse activity. The putative pestivirus species, listed in chronological order of published reports, are Giraffe (isolated from giraffes in the Nanyuki District of Kenya suffering from mucosal disease-like symptoms) (Avalos-Ramirez et al., 2001; Thiel, 2005), a new putative BVDV-3 species, also called HoBi-like virus

(first isolated from fetal bovine serum originating in Brazil and later from samples originating in Southeast Asia) (Bauermann et al., 2013; Liu et al., 2009; Schirrmeier et al., 2004; Stalder et al., 2005), Pronghorn Antelope pestivirus (isolated from an emaciated blind pronghorn antelope in U.S.) (Vilcek et al., 2005), Bungowannah virus (isolated following an outbreak in pigs, resulting in still birth and neonatal death, in Australia) (Kirkland et al., 2007, 2015) and Tunisian sheep virus (TSV; previously termed "Tunisian isolates") (Liu et al., 2009; Thabti et al., 2005). Based on the last phylogenetic analysis comprising the 5′UTR (untranslated region), complete N^{pro} (N-terminal protease) and E2 (envelope protein 2) gene regions, it has been proposed to classify pestiviruses into nine species: BVDV-1, BVDV-2, HoBi-like virus, Pestivirus of giraffe, CSFV, BDV, TSV, Pronghorn Antelope pestivirus and Bungowannah virus (Liu et al., 2009). More recently, new putative pestiviruses have been identified, namely Norwegian rat pestivirus (NrPV) (Firth et al., 2014) and a putative pestivirus (RaPestV-1) in *Rhinolophus affinis bats* (Wu et al., 2012). Finally, another pig pestivirus, named atypical porcine pestivirus (APPV) has been described in USA and Germany (Hause et al., 2015; Postel et al., 2016).

1.2.2. Genome organization and expression

CSFV has a single plus-stranded non-segmented RNA genome, with a large open reading frame (ORF) flanked by two untranslated regions (UTRs), a non-capped 5'UTR and a uracil-rich 3'UTR lacking a poli A region (Moennig, 1992; Tautz et al., 2015; Thiel et al., 1991). The RNA genome serves three distinc roles in the viral cycle, namely as messenger RNA for translation of viral proteins, as template during RNA replication and as genetic material packaged within viral particles (Rümenapf and Thiel, 2008). The translation is iniciated after entering the cell by endocytosis, into a low-pH vesicle, which triggers conformational changes in the virion, fusion of the viral and cell membranes, particle disassembly, and the release of the viral genome in the cytosol as a ribonucleoprotein complex (Ji et al., 2015). Cap-independent initiation of the genome translation is mediated by an Internal Ribosome Entry Site (IRES)

included into the 5'UTR (Poole et al., 1995; Zhu et al., 2010), binding the cellular ribosomal 40S subunits in association with the endoplasmic reticulum membranes (Hashem et al., 2013). The ORF encodes a single large polyprotein of approximately 3900 amino acids that is cleaved by a combination of host and viral proteases, processed in the four structural proteins Core (C), E^{rns}, E1, E2 and in the 8 non-structural proteins N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Figure 1) (Rümenapf and Thiel, 2008).

The structural proteins are nonessential for replication of the genome, but they are necessary for the viral infectious cycle (Frey et al., 2006). Once the necessary viral proteins for replication are produced, a negative genomic RNA strand serves as template for synthesis of progeny RNA (Donis, 1995). The 3'UTR is involved in the initiation of virus replication (Ji et al., 2015; Tautz et al., 2015). RNA packaging and virion assembly occurs in the endoplasmic reticulum or in the Golgi complex, where they acquire their lipid envelope. Finally, extracellular mature virions reach the compartment by exocytosis (David and Knipe, 2013; Donis, 1995). The order of the cleavage products in the CSFV polyprotein is NH_2-N^{pro}-C-Erns-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Figure 1) (Rümenapf and Thiel, 2008).

The first mature protein encoded in the long ORF is unique to pestiviruses, the non-structural and highly conserved N^{pro}, an autoprotease responsible for cleavage at the N^{pro}/C site (Tratschin et al., 1998). N^{pro} also acts as an inhibitor of interferon type I (-α/-β) induction either by blocking transcription or by direct interaction with Interferon Regulatory Factor (IRF)3 and IRF7, avoiding the early innate immune response (Bauhofer et al., 2007; Fiebach et al., 2011; Gottipati et al., 2013, 2016; Ruggli et al., 2003; Tamura et al., 2014).

The C protein is a conserved small polypeptide of about 86 amino acids, which N-terminus is generated by N^{pro}, and the C-terminus is processed by two host proteases, a signal peptidase and a signal peptide peptidase (SPP) (Heimann et al., 2006). As its name indicates, this protein forms the viral capsid; it also

initiates the viral morphogenesis binding the genomic RNA (Lindenbach et al., 2007; Liu et al., 1998).

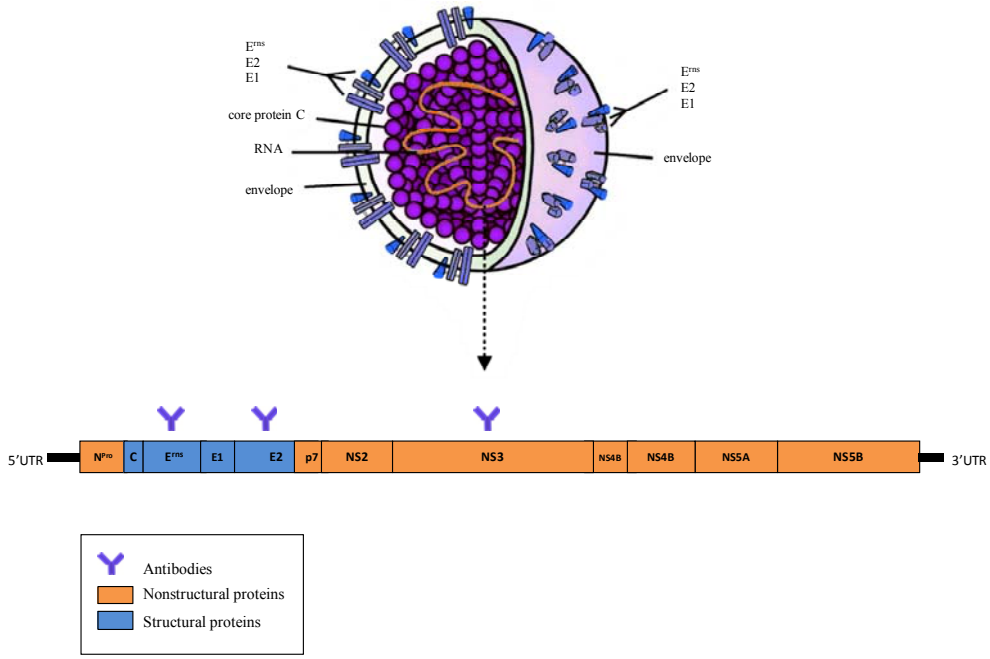

Figure 1. Schematic description of the genome organisation and virion structure of CSFV. Antibodies are directed against the envelope proteins E^{rns}, E2 and the non-structural protein NS3. E2 is the major immunogenic protein inducing high levels of neutralizing antibodies. Functions of selected proteins: N^{pro}, N-terminal protease; C, capsid protein; E, envelope glycoprotein; E^{rns}, RNAse (secreted); p7, ion channel; NS2-NS3, virus particle assembly; NS3, NTPase/RNA helicase/protease; NS4, NS3-cofactor; NS5B, RNA-dependent RNA polymerase. (modified from Beer et al., 2007)

The three structural proteins comprising the viral envelope, E^{rns}, E1 and E2, are associated with the cell membrane by the C-terminus and their expression is required for the release of virus by budding from the extracellular membrane (Weiland et al., 1999). The E^{rns}, exclusive to pestiviruses, is a highly glycosylated

protein with endoribonuclease activity, that can anchor the cell membrane by a non-hydrophobic C-terminus (Fetzer et al., 2005; Lussi and Schweizer, 2016) or either been excreted by the infected cells (Rümenapf et al., 1993; Weiland et al., 1992, 1999). Pestiviral replicons with a deletion of the E^{rns}-coding sequence are unable to produce infectious virus (Frey et al., 2006; Reimann et al., 2007; Widjojoatmodjo et al., 2000). This RNAse is also related with the CSFV virulence by preventing the induction of type I interferon (IFN) (Luo et al., 2009; Mätzener et al., 2009). E^{rns} protein can induce neutralizing antibodies, which are protective against infection (Weiland et al., 1992).

The infection cycle begins with the attachment and entry process to the host cell, mediated by E1 and E2 (Wang et al., 2004) and an still undefined cell surface receptor (Gladue et al., 2014). However, integrin β3 has been demonstrated to be required in CSFV infection and proliferation, since CSFV proliferation was dramatically reduced, up to 99%, in integrin β3 constantly-defected cells (Li et al., 2014b).

The E1 and the E2 glycoproteins are integral membrane glycoproteins that can associate as disulphide-linked heterodimers or even homodimers in the case of E2 (Rümenapf et al., 1993; Thiel et al., 1991). The glycosylation of these proteins has been associated to CSFV virulence; when the glycosilations are removed it provokes viral attenuation (Fernández-Sainz et al., 2009, 2014; Holinka et al., 2016; Risatti et al., 2007a, 2007b). Nevertheless, these results would be expected since glycosylation is required for viral replication (Ansari et al., 2006; Hulse et al., 2004; Leifer et al., 2013).

The E1 protein seems to function as a chaperone for E2. The E2 glycoprotein is essential for virus attachment and invasion, determining the cellular tropism (Gladue et al., 2014; Wang et al., 2004; Weiland et al., 1999).

The E^{rns} and the E2 are the only proteins able to induce neutralizing antibodies, but E2 is considered the most immunogenic protein of CSFV, and the main

responsible of neutralizing antibodies induction; furthermore, the E2 alone can confer protection by itself (König et al., 1995; Rau et al., 2006; Terpstra and Wensvoort, 1988). By using monoclonal antibodies (moAb) to determine the antigenic structure of the E2, four domains have been defined (A, B, C, D) that constitute two independent subunits, the B/C subunit (consisting on B, C domains and the A1 subdomain, disulphide-linked) and the A/D subunit (formed by A2, A3 subdomains and the D domain, disulphide-linked). The epitopes within the subdomain A1 and A2 are highly conserved among the CSFV strains (van Rijn et al., 1993). The subunit B/C is the most immunogenic and can confer protection against CSFV infection (van Rijn et al., 1996). The C-terminus is an hydrophobic transmembrane domain (Garry and Dash, 2003), whose deletion does not affect moAb reactivity, that recognize the N-terminus (van Rijn et al., 1993). The N-terminus is more variable (Paton et al., 2000; van Rijn et al., 1994), suggesting that CSFV antigenic regions could be under the constant selective pressure of the immune response (Chen et al., 2010). On this subject, positive selection pressure analysis conducted in endemic areas that were subjected to a regular vaccination policy, estimated new sites on E2 partial gene analysed (de Arce et al., 2005; Hu et al., 2016; Ji et al., 2014; Pérez et al., 2012).

The smallest non-structural protein is p7, with a molecular mass of about 7 kDa, an integral membrane protein that can form ion channels in the endoplasmic reticulum membrane, suggesting that it serves in virus assembly and/or entry (Gladue et al., 2012; Largo et al., 2014, 2016).

NS2 and NS3 are derived from maturation of its precursor NS2-NS3 (Lamp et al., 2013). The NS2-NS3 is the most conserved among the pestiviruses. NS2 is a cysteine protease responsible for processing the NS2-NS3 (Lackner et al., 2006) and it is also necessary for optimal virus particle assembly (Jirasko et al., 2008). NS3 induces specific but non-neutralizing antibodies against CSFV (Greiser-Wilke et al., 1992), and potentially promotes T cell responses in vitro (Rau et al., 2006). NS3 is responsible for cleavage of the polyproteins that follow (NS3 /

NS4; NS4A / NS4A, NS4B / NS5A and NS5A / NS5B) by using NS4A as a cofactor (Lamp et al., 2011). NS3 also has helicase and nucleosid-tryphosphate (NTPase) activity, playing a role in virus replication (Sheng et al., 2007; Tamura et al., 1993; Warrener and Collett, 1995). NS2-3 recruits NS4A to form a complex involved in the formation of viral particles (Lamp et al., 2013; Moulin et al., 2007). The NS4B protein is also necessary to form the replication complex (Blight, 2011). NS5A and NS5B are involved in replication (Collett et al., 1988). NS5A is needed for viral replication and regulation, when it is present in high concentrations inhibits viral RNA replication by binding to NS5B and 3'UTR (Chen et al., 2012; Sheng et al., 2012). The NS5B is a RNA-dependent RNA polymerase, assembling with NS3 in the replication complex in the 3'UTR (Liu et al., 2009; Sheng et al., 2007, 2012, 2013). The 3' UTR is the recognition site of the viral replicase to initiate minus-strand RNA synthesis. Particularly, the presence of adenosine-uridine rich elements (ARE) in this structure is conserved in all known CSFV strains (Nadar et al., 2011; Vilcek et al., 1999; Vilcek and Belák, 1997). Also, the 3´-UTR of pestiviruses contains cis-acting elements that are indispensable for viral replication and translation (Austermann-Busch and Becher, 2012; Huang et al., 2012; Li et al., 2014a; Pankraz et al., 2005). Changes in its nucleotide sequence may lead to significant changes in viral RNA synthesis (Pankraz et al., 2005; Xiao et al., 2004). Furthermore, it base pair with the sequence in the IRES IIId1, the 40 S ribosomal subunit binding site for the translational initiation, located at the 5'UTR, thus playing a regulatory role in IRES-mediated translation (Huang et al., 2012).

1.3 Pathogenesis

CSFV is typically transmitted oronasally (Weesendorp et al., 2009a). It has a particular affinity for the mononuclear phagocyte system, being primary targets endothelial cells (EDC), macrophages (MΦ) and dendritic cells (DCs) (Carrasco et al., 2001; Knoetig et al., 1999; Ressang, 1973; Summerfield and Ruggli, 2015;

Trautwein, 1988), which are central in orchestrating innate and adaptive immune responses (McCullough et al., 2009; Pulendran et al., 2001). DCs are primarily responsible for the initial recognition of pathogens and regulation of the early phases of the induced immune response, as well as the presentation of antigen associated with SLA class I and class II. After infection, CSFV induces proliferation of DCs without interfering with their maturation and antigen presentation capacity, thus, serving as vehicle for virus spread in the organism (Jamin et al., 2008); likewise, the virus exploits the migratory ability of macrophages to disseminate all over the body (Carrasco et al., 2004).

The spread of the virus is characterized by a lymphatic, viraemic and visceral phase. The virus infects primarily the epithelial cells of tonsillar crypts, regardless of the entry route (Ressang, 1973; Trautwein, 1988). Afterwards, it invades the lymphoid tissues. After entering the lymphatic capillaries, the virus is carried to the regional lymph nodes and enters the efferent blood capillaries giving rise to viraemia. Thereafter, the virus reach bone marrow and secondary lymphoid organs, such as spleen, lymph nodes and lymphoid structures associated with the small intestine, wherein it replicates. Late in the viraemic phase, the parenchymatous organs are invaded (Belák et al., 2008; Liu et al., 2011a; Ressang, 1973).

1.3.1. Virus-host interaction

The course of infection varies according to host parameters (age, breed, health condition) but it is also largely dependent on virulence of the CSFV strain (Donahue et al., 2012; Floegel-Niesmann et al., 2003; Moennig et al., 2003). Virulence of a strain can be defined as the ability to produce clinical and pathological signs in the host, ranging from lowly, moderately and highly virulent strains. The virus-host interaction determines the outcome of the disease (Figure 2), with different clinical forms of CSF that are described in section 1.4. Sequence comparison and reverse genetics of related virulent and avirulent strains sought to determine virulence factors (van Gennip et al., 2004; Gladue et

al., 2010, 2011, Risatti et al., 2005b, 2005c, 2006, 2007a, 2007b; Sainz et al., 2008; Tamura et al., 2012, 2014), but those determinants are still far from being understood. The different points of view in the context of virulence have been previously discussed (Leifer et al., 2013). Also, there is a black box for the knowledge of viral interactions with target cells and with regulatory and control systems on the natural hosts. Thus, the virulence of a particular virus strain is best determined by experimental infection of pigs under standardized conditions (Belák et al., 2008; Floegel-Niesmann et al., 2003; Mittelholzer et al., 2000; Petrov et al., 2014). Highly virulent CSFV induces strong pathologic responses that are accompanied by immediate and strong overexpression of the IFN stimulated genes (ISG) involved in cell death or apoptosis processes, while the kinetics of host response to the infection is more progressive and delayed with the moderately virulent CSFV, giving pigs a chance to recover (Graham et al., 2012b; Petrov et al., 2014; Renson et al., 2010; von Rosen et al., 2013; Tarradas et al., 2014).

CSFV infection leads to a breakdown of the immune system which, accompanied by an aberrant pro-inflammatory response (known as a "cytokine storm"), is unable to control disease progression (Knoetig et al., 1999; Sánchez-Cordón et al., 2002, 2005a; Summerfield et al., 2006). To overcome the disease, an adequate immune response which leads to viral clearance is needed. However, CSFV has developed strategies to circumvent the innate immune system and to prolong infection (Figure 2). On the one hand, N^{pro} ease the virus evasion at the primary local sites of infection by blocking the IFN type I, allowing its spread to the secondary sites of replication (Fiebach et al., 2011; Tamura et al., 2014). Also, N^{pro} plays an important role in the process of escaping RNA-induced apoptosis through interactions with HAX-1 (Johns et al., 2010a, 2010b) promoting cell survival (Han et al., 2006). On the other hand, E^{rns} further contribute to prevention of type I interferon (IFN) induction through degradation of extracellular ssRNA and dsRNA (Luo et al., 2009; Mätzener et al., 2009) and

by preventing the stimulation of plasmacytoid dendritic cells (pDC) by infected cells (Python et al., 2013).

The disease is associated with severe lymphopenia and lymphocyte apoptosis (Summerfield et al., 1998b; Susa et al., 1992), thrombocytopenia, platelet aggregation (Bautista et al., 2002), bone marrow depletion affecting myelopoiesis and megakaryocytopoiesis (Gómez-Villamandos et al., 2003b; Summerfield et al., 2000), and thymus atrophy as well as thymocyte apoptosis (Pauly et al., 1998; Sánchez-Cordón et al., 2002). Lymphoid depletion is generalized, not only affecting peripheral blood and lymph nodes but also the mucosal tissue (Sánchez-Cordón et al., 2003), with an altered population and depletion of lymphocytes (van Oirschot et al., 1983b; Pauly et al., 1998), mainly $CD4^+$ and $CD8^+$ T cells (Ganges et al., 2005; Summerfield et al., 2001). In this regard, CSFV infection promotes marked bystander apoptosis of the surrounding uninfected B and T cells, by mechanisms still not completely understood, contributing to the strong immunosuppression and high mortality rates (Carrasco et al., 2004; Ganges et al., 2008; Summerfield et al., 1998b, 2001; Susa et al., 1992). In addition, some studies demonstrated the induction of *in vitro* lymphocyte apoptosis by E^{rns}, suggesting its possible implication in the leucopoenia associated to CSF (Bruschke et al., 1997; Pauly et al., 1998; Summerfield et al., 1998b). Depending on the virulence of the CSFV strain, pigs can have as much as 90% of their total T cells depleted in the final stages of the disease (Pauly et al., 1998). This effect can be observed as early as one day after infection, even before viraemia has been established (Summerfield et al., 1998a), much earlier than seroconversion and clinical signs of disease, which is relevant both for early diagnosis and for the study of viral pathogenesis (Ganges et al., 2005; Pauly et al., 1998; Summerfield et al., 1998a, 2000).

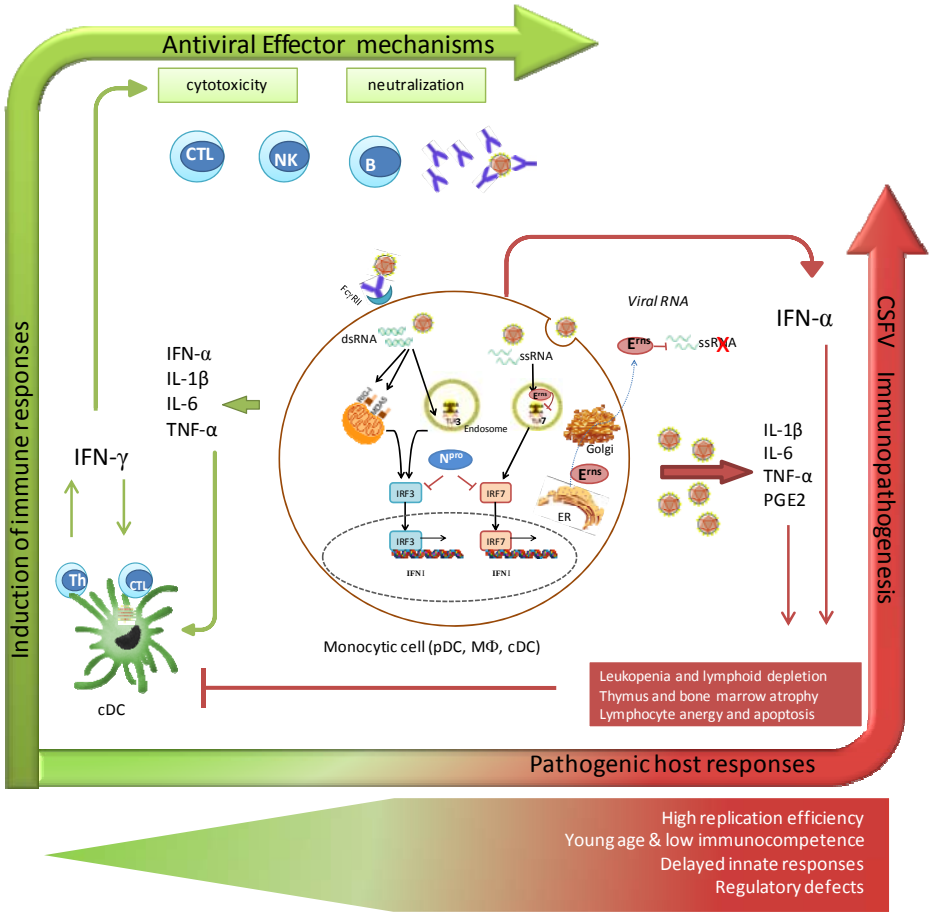

Figure 2. Schematic representation of the main virus-host interaction mechanisms modified from (Summerfield and Ruggli, 2015). CSFV targets both monocytic cells, including MΦ and conventional and plasmacytoid DC (cDC and pDC), which are mainly responsible for the typical pro-inflammatory responses. CSFV has developed strategies to circumvent the innate immune system at the primary local sites of infection, avoiding type I IFN response by means of N^{pro} and E^{rns} proteins. N^{pro} induces proteasomal degradation, namely IRF3, the main antagonist mechanism of IFN Type I induction. In pDC, which constitutively express IRF7, a partial inhibition trough N^{pro} has also been described. Also E^{rns} contribute as an IFN antagonist by degrading ssRNA and thereby prevent TLR7 activation. E^{rns} can also be secreted to the extracellular compartment where it can also degrade RNA, preferentially ssRNA, or alternatively can be endocytosed to degrade endosomal viral RNA in neighbouring cells. Large systemic levels of IFN-α, produced mostly by pDC, play a central role in cytokine storm induced by CSFV. Prolonged systemic responses are associated with pathogenic host responses while time-limited production appears to promote protective adaptive Th1 effector responses. The critical immunopathological pathways are represented in green for protective responses, and in red for pathogenic processes during acute CSFV infection.

CSFV activates infected endothelial cells towards a pro-inflammatory response and avoid apoptosis and interferon type I synthesis (Bensaude et al., 2004; Campos et al., 2004). Therefore vascular endothelial cells, and also macrophages are mainly responsible for the typical pro-inflammatory responses, including IL-1α, IL-1β, IL-6 and TNF-α and vasoactive mediators (Knoetig et al., 1999; Núñez et al., 2005; Sánchez-Cordón et al., 2002, 2005a). Conventional and plasmacytoid DCs further contribute to this response, mainly with IFN-α production (Fiebach et al., 2011; Hüsser et al., 2012). Regardless the antiviral role of type I interferons (Nan et al., 2014; Pestka, 2007), a strong IFN-α response is a hallmark of disease severity and intensity of viral replication, and it is believed to trigger the onset of the observed lymphopenia (Hüsser et al., 2012; Summerfield et al., 2006; Tarradas et al., 2010, 2014). In such scenario, the high levels of IFN-α mediate aberrant responses leading to immunopathology, rather than to control of viral infection. The pro-inflammatory and antiviral factors would also explain the changes in haemostatic balance (Knoetig et al., 1999), such as disseminated intravascular coagulation (DIC) and fibrinolysis, drawn in the characteristic hemorrhagic picture of CSFV (van Oirschot, 1988; Summerfield et al., 2000).

CSFV infection has been related with IL-10 production by $CD4^-/CD8^+$ T cells, which might be implicated too in the immunosuppression observed after infection (Suradhat et al., 2005). IL-10 is a well-known immunosuppressive cytokine with a pleiotropic function, including suppression of T-cell proliferation and B-cell responses (Sabat et al., 2010). In the final stage of acute and severe CSF, the complement system is activated (Sánchez-Cordón et al., 2002, 2005a). Overall, it results in a circulation failure, hypotension, and death (van Oirschot, 2004). Conversely no or lower levels of IFN-α and pro-inflammatory cytokines correlates with milder infection forms (von Rosen et al., 2013; Summerfield et al., 2006; Tarradas et al., 2014); if controlled, such infections result in life-long immunity against CSFV (van Oirschot et al., 1983a).

In advanced phases of the disease granulocytopenia is observed, which is followed by the circulation of immature precursors in peripheral blood (Ganges et al., 2005; Nielsen et al., 2010; Summerfield et al., 1998a). The leading cause of granulocytopenia is necrosis and apoptosis of bone marrow hematopoietic cells (BMHC) (Summerfield et al., 2000) which are uninfected. The fundamental mechanism for apoptosis is not clear, but contact between infected and non-infected BMHC seems to be critical (Summerfield et al., 2001). In the bone marrow, the main targets of infection are immature myeloid cells $SWC3^{+}/SWC8^{-}$, as well as myeloid precursors, less-differentiated cells, $SWC3^{low}/SWC8^{-}$, which continue to differentiate into $SWC8^{+}$ granulocytic cells (Summerfield et al., 2000, 2001). This finding explains the occurrence of infected peripheral blood granulocytes during CSF (Summerfield et al., 1998a, 2001). At later stages, signs of peripheral lymphocyte activation and proliferation are also found (Sánchez-Cordón et al., 2006; Suradhat et al., 2001, 2005).

1.3.2. Humoral and cellular responses in CSFV infection

$CD4^{+}$ T cells and specific killer cell activity by cytotoxic $CD8^{+}$ T lymphocytes (CTL's) are induced from one to three weeks after infection, and are as well important for an effective early immune response (Ganges et al., 2005, 2008; Piriou et al., 2003). The specific CTL's mainly recognize the E2 and NS3 proteins of CSFV (Ceppi et al., 2005; Ganges et al., 2005, 2008; Rau et al., 2006). The increase of IFN-γ -secreting T lymphocytes during the disease, mainly effector CTL's $CD4^{-}/CD8^{+}$ T cell subset (but also activated memory T cells $CD4^{+}/CD8^{+}/CD25^{+}($, contributes to early control of viral replication and protects against leukopenia before the onset of neutralizing antibodies (Franzoni et al., 2013; Graham et al., 2012b; Piriou et al., 2003; Tarradas et al., 2010, 2011a, 2014).

However, T cell-mediated response has been related only to a partial protection (Franzoni et al., 2013; Ganges et al., 2005; Graham et al., 2012a; Summerfield and Ruggli, 2015; Tarradas et al., 2010, 2014). The initial Th1 cytokine

expression (IL-2 and IFN-γ) switches later on to a Th2 response (IL-4, IL-10), helping the differentiation of B cells to immunoglobulin-producing plasma cells (Ganges et al., 2005; Sánchez-Cordón et al., 2005a; Tarradas et al., 2010). Altogether with the lymphopenia, this Th1-Th2 switch may influence the delayed humoral response characteristic of CSFV infection (Sánchez-Cordón et al., 2005a). Meanwhile the role of neutralizing antibodies is crucial to getting a sterilizing protection (Ganges et al., 2005, 2008, Tarradas et al., 2010, 2011b). In this regard, it has been found that CSFV neutralizing antibodies titres over 1/32 after challenge can be protective, with neither viral excretion nor transmission (Terpstra and Wensvoort, 1988). The antibodies induced in infected pigs recognize E^{rns}, E2 and NS3 proteins (Greiser-Wilke et al., 1992; König et al., 1995; Rau et al., 2006; Weiland et al., 1992). Anti-NS3 antibodies recognize the proteins of different pestiviruses and are not neutralizing (Weiland et al., 1992). Neutralizing antibodies against E2, the most immunogenic CSFV protein, are produced between 10 and 20 days after natural infection (Bouma et al., 1999; Ganges et al., 2005; König et al., 1995; van Rijn et al., 1996; Terpstra and Wensvoort, 1988) and are the only ones capable to confer clinical protection against CSFV challenge by itself (Rau et al., 2006). Also, the E2 presents high antigenic cross-reactivity with other pestiviruses and between different CSFV isolates; however differentiation between strains is possible by using MoAb against different epitopes within the E2 (Greiser-Wilke et al., 2007).

Sows can transmit passive immunity to the litter via colostrum. Maternal derived antibodies (MDA) protect piglets against disease during their firsts weeks of life, although not against CSFV replication and excretion (van Oirschot, 2003b). This protection declines as piglets grow older and maternal antibody titres decrease (van Oirschot, 2003b; Vandeputte et al., 2001). However, MDA can interfere with the development of vaccinal immunity, an aspect to keep in mind when preparing immunization schedules (Huang et al., 2014).

1.4 Clinical forms of CSF

The clinical signs of CSF are highly variable and strongly determined by the virulence of the strain, age of the pigs and to a lesser extent by the breed and condition of the animals (Belák et al., 2008; Ganges et al., 2008; von Rosen et al., 2013; Tarradas et al., 2014). Postnatal and congenital infections have been described, for which different clinical forms are recognised, including peracute, acute to chronic courses (van Oirschot and Terpstra, 1989). Most studies focus on the characterization of acute disease after inoculation with virulent strains, characterized by a short incubation period, accompanied by scarce signs, and a rapid mortality within a few days after exposure (Belák et al., 2008; Dune, 1973; Ganges et al., 2005; Hüsser et al., 2012; Knoetig et al., 1999; Sánchez-Cordón et al., 2005b). In addition, congenital CSFV infection by virulent strains will likely result in abortions or weak born pigs that will die shortly after birth (Terpstra, 1991). On the other hand, strains of low virulence can induce high proportion of chronic cases that may be unapparent or atypical (Ganges et al., 2008; van Oirschot, 1988; von Rosen et al., 2013), or that may result in the reproductive form after trans-placental transmission (Carbrey et al., 1977; Trautwein, 1988). Notably, some of the congenital infected animals with low virulent strains may result in congenitally persistently infected pigs (van Oirschot, 2004).

Chronic and unapparent courses are currently present in endemic countries (Floegel-Niesmann et al., 2003; Ji et al., 2014; Pérez et al., 2012; Shen et al., 2011; Wensvoort and Terpstra, 1985). Those mild infections may be misdiagnosed and become a possible source of new CSF outbreaks (Vannier et al., 1981), and albeit their potential impact, especially in endemic countries, the understanding of related pathogenesis and disease progression after infection with lowly or moderately virulent CSFV isolates is still puzzling (Pérez et al., 2012; Tarradas et al., 2014).

1.4.1. CSF acute form

Acute CSF is typically caused by highly virulent strains, and is more frequent in piglets until 12 weeks of age; in older breeding pigs the course of the infection is often mild or subclinical (Moennig et al., 2003). After a short period of incubation (two to six days after exposure) pigs develop a maintained pyrexia (>40 °C) accompanied by anorexia (Belák et al., 2008; Mittelholzer et al., 2000; Moennig et al., 2003; Tarradas et al., 2014). Highly virulent CSFV strains cause marked immunosuppression and high mortality (Gómez-Villamandos et al., 2003b; Lee et al., 1999; Susa et al., 1992). Leukopenia (less than 8000 cells/mm^3 in blood) is rapidly established, even before showing fever or the viraemia is demonstrable (Stegeman et al., 2000).

The most characteristic feature is the haemorrhagic syndrome, with petechiae of the skin, mucosae, and cyanosis of the abdomen, ears, snout and medial side of the extremities. As a result of central nervous system (CNS) involvement, infected pigs become progressively depressed and uncoordinated (Gómez-Villamandos et al., 2006; Moennig, 2000). Frequent clinical findings are also conjunctivitis, constipation followed by severe diarrhoea ("cholera"), tremors, locomotive disturbance, reluctant walking, swaying movement of the hindquarter, posterior paresis, convulsions from mild to severe and prostration in the terminal phase (Mittelholzer et al., 2000; van Oirschot, 2004; Tarradas et al., 2014). The mortality is near 100%, dying between 10 to 20 days post infection (Dune, 1975; Moennig et al., 2003).

In peracute cases, no gross changes are found at necropsy. In acute cases, severe pathologic lesions can often be inconspicuous, or can be inexistent. In general, swollen or hemorrhagic lymph nodes and petechial bleeding of tonsil and inner organs such as kidneys, spleen and lymph nodes is commonly observed (Belák et al., 2008; Gómez-Villamandos et al., 2000, 2003a). In addition, thymus atrophy has also been described in acute CSF forms, wherein massive lymphoid depletion

was also found due to lymphocyte apoptosis in atrophied thymuses (Sánchez-Cordón et al., 2002).

Subacute form runs with pyrexia, diarrhoea, central nervous disease and low mortality, but it is less severe than the acute form (Floegel-Niesmann et al., 2003, 2009). The surviving animals usually mount a long-lasting humoral immunity with neutralizing antibodies after 10-12 days of infection (Chander et al., 2014; Moennig et al., 2003).

1.4.2. CSF chronic form

In general terms, an infection is considered as persistent when survival of infected animals exceeds 30 days (Dune, 1975). CSFV persistent infections include chronic and "late onset" infections, always fatal (Floegel-Niesmann et al., 2003; Moennig et al., 2003). It develops when pigs are not able to mount an effective immune response against the infection (Petrov et al., 2014; Tarradas et al., 2014).

It is known that chronic infections can be established in the presence of neutralizing antibodies (Mengeling and Packer, 1969), but the mechanisms by which CSFV overcome the pig's first line of defence is still under study. Chronic disease elapses with stunting, anorexia, pyrexia and intermittent diarrhoea. In a first phase, the clinical picture is similar to acute illness. After overcoming the initial phase signs can disappear and animals are even apparently healthy (Mengeling and Cheville, 1968). However, over time the disease progresses with nonspecific signs, with occurrence of intermittent fever, chronic enteritis and wasting, which are not always easy to identify in the farm (Moennig et al., 2003). CSFV is constantly shed from the onset of clinical signs until death (Weesendorp et al., 2011a); survival of affected animals ranges from 2 to 3 months after exposure (Moennig et al., 2003). The immune system triggers an antibody response that is never enough to eliminate the virus. These antibodies are not always detectable because they are consumed by CSFV and therefore is very characteristic the presence of immune complexes, especially at kidney, causing

glomerulonephritis (Choi and Chae, 2003a; Gómez-Villamandos et al., 2000). The post-mortem changes are not very characteristic of the disease and bleeding may not be present neither in organs nor in seroses. However, it is quite common to find thymic atrophy (Cheville and Mengeling, 1969), ulcerative and necrotic lesions ("button ulcers") in the ileocecal valve in animals with chronic diarrhoea, as well as along ileum and colon (Rümenapf and Thiel, 2008). Necrotic ulcers are also common in epiglottis and larynx. Secondary bacterial infections are frequent (Cheville and Mengeling, 1969; Choi and Chae, 2003b). Essentially, clinical signs and pathologic findings are not characteristic of the disease, which should include other compatible diseases in the differential diagnosis (Elbers et al., 2003; Moennig et al., 2003; Rout and Saikumar, 2012).

1.4.3. CSF congenital persistent form

It is well established that trans-placental transmission of classical swine fever virus (CSFV) can lead to persistently infected (PI) offspring (Aynaud et al., 1977; Carbrey et al., 1977; van Oirschot, 1979a, 1979b), especially during mid-gestation (Frey et al., 1980; Liess, 1984; van Oirschot, 1977, 1979b). The outcome of trans-placental infection of foetuses depends largely on the time of gestation and viral virulence, respectively (van Oirschot, 1977), and may result in abortion, stillbirth, mummification, malformations, or the birth of weak or apparently healthy piglets, but persistently infected with CSFV (Trautwein, 1988). This is so-called 'carrier sow syndrome'.

Congenitally persistently infected piglets do not produce neutralizing antibodies to CSFV and have a lifelong viremia (Liess, 1984; van Oirschot, 1977, 1979b). Although persistently infected offspring may be clinically normal at birth, they invariably die from CSF. They may show poor growth, wasting or occasionally congenital tremor; however it may take several months (>6) before those pigs develop mild anorexia, depression, conjunctivitis, dermatitis, diarrhoea, runting, and locomotive disturbance leading to paresis and death (Trautwein, 1988). Survival periods of 11 months after birth have been observed (van Oirschot,

1977). This course of infection is referred to as `late onset CSF' (van Oirschot and Terpstra, 1989). These piglets are a dangerous virus reservoir, spreading and maintaining the infection within the pig population, being undetectable through traditional serological methods (van Oirschot, 1977, 2004). This situation is comparable to cattle persistently infected with BVDV (Peterhans and Schweizer, 2010).

The pathogenesis of CSF congenital persistent infection has been related to a specific immunotolerance to CSFV, meaning the ability to respond against unrelated antigens (e.g pig parvovirus or phytohaemagglutinin, PHA) in terms of antibody response and lymphocyte reactivity, but not specifically to CSFV (Carbrey et al., 1977; Ehrensperger, 1988; van Oirschot, 1977; Trautwein, 1988; Vannier et al., 1981). Although probably directly related to the ontogeny of the immune response, this immunotolerance has also been induced following experimental congenital infection at 90 days of pregnancy (van Oirschot, 1979b), after the onset of immunocompetence (between 60 and 75 days of foetal life in pigs) (Sinkora and Butler, 2009). Accordingly, it has been described the birth of persistently infected piglets is greater when infection occurs between 70 and 90 days of gestation, whereas incidence of prenatal and postnatal mortality is higher at 40 and 65 days of pregnancy, respectively (Liess, 1984; van Oirschot, 1977). However, the specific mechanisms to induce CSFV immunotolerance are not quite understood and need further study (Moennig and Plagemann, 1992; van Oirschot, 1977, 1979a). Besides the congenital persistent form, there have been few reports only suggesting a possible occasional occurrence of virus persistence after postnatal infection (Baker and Sheffy, 1960; Ehrensperger, 1988; Vannier et al., 1981).

1.5 Epidemiology

Swine is the only natural reservoir of CSFV (Moennig, 2000). Wild Boar and other wild swine are equally susceptible to infection (Everett et al., 2011; Gers et al., 2011; Laddomada, 2000). The virus does not infect humans, however, it can be transmitted experimentally to ruminants and rabbits (Moennig, 2015).

1.5.1. Transmission and spread

Blood, tissues, semen, secretions and excretions contain CSFV (Floegel et al., 2000; de Smit et al., 1999). The disease is mainly transmitted by the faecal-oral route (Klinkenberg et al., 2002), but infection can occur through the conjunctiva, mucous membranes, skin abrasions, insemination, and percutaneous blood transfer (e.g. iatrogenic) (Floegel et al., 2000; Hennecken et al., 2000; de Smit et al., 1999). Shedding can begin before onset of clinical signs (van Oirschot, 2004; Terpstra, 1991). Thus, infection can be done by either direct or indirect contact between pigs (Weesendorp et al., 2009a, 2011b). Transmission and spread of the disease in a pig herd depends on the clinical course of infection, which is associated with high and low levels of viral excretion (Durand et al., 2009; Weesendorp et al., 2009b, 2011a). The excretion of highly virulent CSFV in oronasal discharges and faeces is high, but transmission period is short until animals die (Durand et al., 2009; Weesendorp et al., 2009b, 2011a). The moderate or the low virulence variants of CSFV are accompanied by a restricted *in vivo* viral replication and invasion (Dahle and Liess, 1992; Mengeling and Packer, 1969), but high titres of virus are secreted for longer, generating up to 40,000 times more virus in the chronic than in the acute form (Belák et al., 2008; Weesendorp et al., 2009b). These high-excreting pigs play a crucial role in spreading CSFV during outbreaks (Rout and Saikumar, 2012). Finally, the low virulent strains cause infections with low oronasal virus excretion over a short infection period (Terpstra, 1991; Weesendorp et al., 2009a, 2009b).

Persistence in the farm environment is influenced by the initial concentration of the virus and the presence of organic matter, e.g. saliva, blood or feces of

infected pigs (Edwards, 2000), surviving for long periods, especially in manure (Bøtner and Belsham, 2012; Edwards, 2000; Turner et al., 2000).

Aerosol transmission is possible over short distances, but is not considered to play a major role (Dewulf et al., 2000; Weesendorp et al., 2009a, 2009b). Also insects can act as mechanical vectors over short distances (van Oirschot, 2004; Stewart et al., 1975), however, there is no evidence that CSFV replicates in invertebrate vectors. Working personal and husbandry practices also play an important role in CSF transmission (vehicles, equipment, clothing, contaminated needles, artificial insemination) (Moennig et al., 2003; Ribbens et al., 2004, 2007; Stegeman et al., 2002). Transmission by rodents, birds or pets has been shown to be unlikely (Dewulf et al., 2001).

Uncooked swill or contaminated food (access of CSFV infected wild boars to food supply) are recognized sources of introduction of CSFV in uninfected herds (Edwards, 2000; Fritzemeier et al., 2000; Sharpe et al., 2001). In a protein-rich environment, CSFV is very stable and can survive for months in refrigerated meat and for years in frozen meat; in moist environments, e.g., ham, fresh pork and excretions of infected pigs it can survive for weeks or even months (Edwards, 2000). In recognition of this danger, swill feeding is officially banned in many countries. The major outbreaks that spread through European Union (EU) countries in 1997–1998 were caused by a virus that is believed to has originated in Asia (Moennig, 2000) and to have been introduced into European domestic pigs via swill illegally fed to pigs in a single area in Germany towards the end of 1996 (Elber et al., 1999; Moennig, 2000).

Outbreaks in areas with a high density of pigs often led to extensive epidemics (Fritzemeier et al., 2000). In parallel, long distance animal trade highly contribute to the problem. Despite the practical eradication from member states of the EU by the 90s, the high density of pigs accompanied by a non-vaccination policy, and the geographical proximity to Eastern European countries where CSF remains enzootic, represented a high risk of re-introduction and subsequent

spread of the disease (Postel et al., 2013a). Finding examples of such outbreaks were in Netherlands in 1997/1998 plus the UK in 2000 (Edwards et al., 2000; Moennig et al., 2003). Animal trade from Germany was the probable source of the 1997 CSF outbreak in the Netherlands (Elber et al., 1999). These outbreaks have generated large financial losses due to the mass slaughtering and the ban of animal exportation (Blome et al., 2010; Ganges et al., 2008; Moennig et al., 2003).

The latest reports from the OIE in Eastern Europe confirmed the existence of subclinical forms of CSF in wild boars (OIE report, 2015). In this regard, the role of the wild boar as a CSFV reservoir and possible source of infection for the domestic pig is well known (Laddomada et al., 1994; Moennig, 2015; Rossi et al., 2005; Ruiz-Fons et al., 2008). Hence, the high density and increasing population of wild boar (Keuling et al., 2013) complicates the control of CSF, which can be maintained for long periods of time, as in the case of Germany in the decade of the 90s (Penrith et al., 2011; Postel et al., 2013a). However, the implication of these animals for the epidemiology of CSF is still under study (Moennig, 2015).

During the last decades the characterization and trace of circulating strains of low and moderate virulence has been of concern (Floegel-Niesmann et al., 2003; Pérez et al., 2012; Shen et al., 2011; Tarradas et al., 2014; Wensvoort and Terpstra, 1985), related to the appearance of outbreaks of the disease that can be misdiagnosed or late recognized. These isolates may produce mild or unapparent forms of disease that confuse the diagnosis and prevent its early detection. By being unnoticed for a time, such mild infections may spread to large populations of pigs, causing serious epizootiological and economic consequences. As an example, the epizootic of 2001-2002 in Spain and especially in Catalonia, with mild and non-specific clinical signs, complicated and appeared a delay for the diagnosis, favouring the initial dissemination of the virus and consequent economical losses (Allepuz et al., 2007).

Finally, trans-placental infection with viral strains of low virulence can generate persistently infected animals, reservoirs of the virus that complicate the diagnosis and control of disease (van Oirschot, 2004; van Oirschot and Terpstra, 1989). Over the last four decades the congenital persistent infection has been recognized as one source for the maintenance of CSF in domestic pigs (de Arce et al., 2005; Moennig et al., 2003; Paton and Greiser-Wilke, 2003; Pérez et al., 2012).

1.5.2 Molecular epidemiology

One characteristic of the pestiviruses is their both antigenically and structurally close relation, and their ability to share host (van Rijn, 2007). Polyclonal antibodies cross-react with other pestiviruses, failing to distinguish neither species, strains nor isolates (Becher et al., 2003; Ganges et al., 2008). The comparison among viral sequences allows differentiation between pestiviruses (Liu et al., 2009; Postel et al., 2016). Generally these studies are based on the comparison of the 5' UTR, N^{pro}, the 5 'end of the E2, NS5B protein and the 3 'UTR (Liu et al., 2009; Rümenapf and Thiel, 2008).

Molecular genetic studies of the CSFV provide information to understand disease spread and outbreak dynamics (Depner et al., 2006; Paton et al., 2000; Postel et al., 2013a). To simplify this task, a CSFV database is maintained at the European Union Reference Laboratory (EURL) for CSF in Hannover, Germany (Greiser-Wilke et al., 2006). Based on the nucleotide sequences of fragments of the 5'UTR, and of the region encoding the glycoprotein E2 and the NS5B, isolates of CSFV can be subdivided into genetic groups (I to III) and subgroups (1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3 and 3.4) (Leifer et al., 2010; Paton et al., 2000; Postel et al., 2013b). Those genotypes can be assigned to distinct geographical regions (Beer et al., 2015), that are represented in figure 3.

Furthermore, phylogenetic analysis aid to understand the patterns of viral evolution in the field (de Arce et al., 2005; Lowings et al., 1994; Paton et al., 2000; Pérez et al., 2012). Particularly, the E2 protein-based studies have provided the further discrimination and characterization of the field isolates (Beer et al.,

2015; Lowings et al., 1996; Paton et al., 2000; Pérez et al., 2012; Sun et al., 2013; Titov et al., 2015; Vilcek et al., 1996). Recently, full-length E2 encoding sequences were best recommended for reliable in detail phylogenetic analyses when highly similar CSFV strains are compared (Leifer et al., 2010; Postel et al., 2012). Finally, there are no recognized serotypes among CSFV strains, which show a high degree of genomic homology (Moennig, 1992; Moennig et al., 2003).

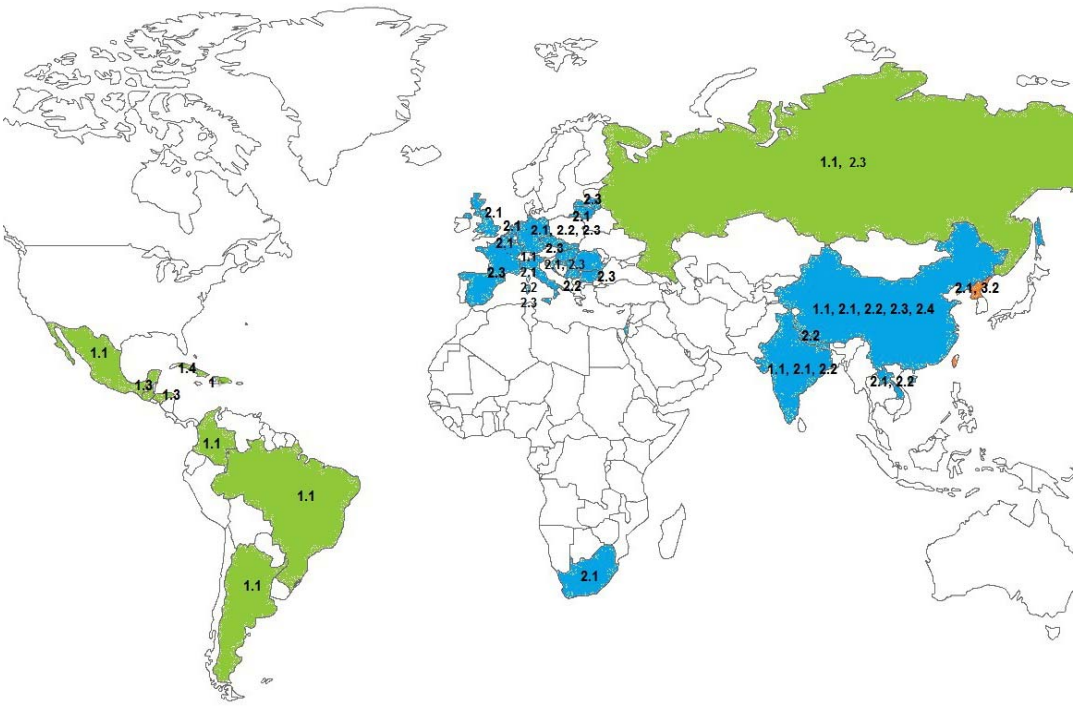

Figure 3. Worldwide distribution of genogroups over the past two decades. The most prevalent genotype over the past two decades has been genotype 2 (in blue), especially sub-genotypes 2.1 and 2.3, most particularly in Europe. However, field isolates from America, mostly in South and Central America, were all placed into genotype 1 (in green). The situation in the African continent is unclear, and there is only information about the sub-genotype 2.1 Russian CSFV strains are mostly clustered within sub-genotype 1.1 In India and China, sub-genotype 1.1, 2.1 and 2.2, and 2.3 also in China. In Taiwan, genotype 3 (in orange). (Image modified from Beer et al., 2015)

1.5.3. Control and surveillance

The two main strategies to control CSF epidemic are systematic prophylactic vaccination and a non-vaccination *stamping out* policy. Nevertheless, the most successful policy for the eradication of CSFV in a region has been *stamping out* of infected and in-contact pig herds with destruction of the carcasses (Elber et al., 1999). There are reasonable arguments against this option in terms of feasibility, animal welfare reasons, costs and public acceptance (van Oirschot, 2003b). This measure should be accompanied by rigorous serological monitoring of the porcine farms for detection of specific CSF antibodies, but the impracticability of differentiating vaccinated from infected animals based on positive serologies leads to the implementation of non-vaccination policies (Moennig, 2000). In this respect, emergency vaccination around the outbreak focus can limit disease spread, but vaccinated animals are usually slaughtered after the outbreak has been controlled to regain status of freedom without vaccination. However, population reduction and oral vaccination of wild boars are permitted to control and avoid new CSF outbreaks in the wild population within the European union (Kaden et al., 2000; Kaden and Lange, 2001; Moennig, 2015). For this purpose, the modified live C-strain virus is used, which must be safe, effective and appropriate for all ages (Brauer et al., 2006; Chenut et al., 1999; Kaden et al., 2000, 2009).

A monitoring and surveillance system is essential to demonstrate the successful eradication, designed to ensure early detection of CSF. Guidelines for surveillance are provided in the Terrestrial Animal Health Code of the World Organisation for Animal Health (Office International des Epizooties (http://www.oie.int). Additionally, the importance of on-farm biosecurity cannot be overestimated to prevent the entry.

On the other hand, the endemic countries implement vaccination programs against classical swine fever virus (CSFV) in their health policies for prevention and control. However, even with intensive vaccination programs in endemic

areas for more than 30 years, CSF has not been eradicated yet, and a trend to appear and re-appear in a clinically very mild or in a completely unapparent form has been described (Pérez et al., 2012; Shen et al., 2011; Tarradas et al., 2014; Wensvoort and Terpstra, 1985). Recent works suggest that CSFV evolution towards lowly virulent viruses in these regions was driven in part by a positive selection pressure related to inefficient vaccination programs (de Arce et al., 2005; Ji et al., 2014; Pérez et al., 2012; Shen et al., 2011; Suradhat and Damrongwatanapokin, 2003). In this context, persistent and chronic CSF may go unnoticed under serological tests, putting at risk the surveillance and control programs. Though, to avoid the reproductive form of CSF, the vaccination programs provide the vaccination of sows before pregnancy (Moennig et al., 2003).

1.6 Diagnosis and related diagnostic tools

Disease caused by CSFV does not result in specific clinical signs nor lesions and not a single sign is characteristic for CSF (Elbers et al., 2001, 2002, 2003; Floegel-Niesmann et al., 2003). Furthermore, infections by ruminant pestiviruses in pigs sometimes lead to clinical disease that is indistinguishable from the mild CSF, whereby it is essential to make a differential diagnosis with other pestiviruses considering the high antigenic reactivity and genomic homology (Pérez et al., 2011; Terpstra and Wensvoort, 1988). Importantly, acute CSF can be confused with African swine fever (ASF) on clinical and pathological grounds (Haines et al., 2013; van Oirschot, 2004). Other viral diseases can also be confused with CSF, such as porcine dermatitis and nephropathy syndrome (PDNS), post-weaning multisystemic wasting syndrome, or thrombocytopenic purpura. Also, septicemic conditions in which pigs have high fever should be carefully investigated. Approximately 75 % of pigs with acute CSF have microscopic lesions of a viral meningoencephalitis and various septicemic disorders, such as salmonellosis (especially that caused by *Salmonella*

choleraesuis), erysipelas, pasteurellosis, actinobacillosis and *Haemophilus parasuis*, among others (van Oirschot 2004). In fact, these bacteria often cause concurrent infections, and isolating these pathogens may mask presence of CSFV, which is the real cause of the disease. Similarly, PDNS may mask an underlying CSFV infection (van Oirschot, 2004). Hence, laboratory methods are essential for a prompt and clear diagnosis, even when clinical signs and lesions are highly suggestive of CSF (Moennig, 2015).

Laboratory diagnostic methods are based on 3 pillars, that are (i) virus isolation (ii) detection of viral antigen in tissues and (iii) detection of specific antibodies, and should be validated according to World Organisation for Animal Health (OIE) standards (OIE terrestrial manual, 2015). The *gold standard* technique is CSFV virus isolation in cell lines such as permissive pig kidney (PK-15, SK- 6 are the most employed in the laboratory), or other CSFV permissive cell lines from porcine, cattle, goat and primate origin (Moennig, 1988; Moennig et al., 1990; Roehe and Edwards, 1994). Cell cultures are inoculated with serum, plasma, whole blood in EDTA, macerated organs or leukocytes from animals suspected of CSF (Greiser-Wilke et al., 2007). Some authors uphold that nictitating membrane (third eyelid) provides a useful source of virus in pigs which have undergone autolysis, much less affected by autolysis than the internal organs (Teifke et al., 2005). CSFV is not cytopathogenic, so the infection must be visualized using indirect methods, e.g., fixing cells and staining viral antigen using mono- or polyclonal antibodies conjugated with enzymes or fluorescent dyes (Moennig, 2015). Peroxidase-linked assay (PLA) (Wensvoort et al., 1986) is commonly used for viral titration following the statistical methods described by Reed and Muench (Reed and Muench, 1938).

For quick results, direct antigen detection on fixed cryosections of organ material can be carried out by using fluorescent antibody test (FAT) or immunoperoxidase staining against CSFV (van Oirschot, 2004). The most used tissue is the tonsil, as it is the primary organ infected, regardless of the infection route (de Smit,

2000). However, the interpretation of test results requires experienced laboratory personnel and the sensitivity of this method is limited, then a negative result does not rule out CSF in case of a clinical suspicion (Greiser-Wilke et al., 2007). The commercial antigen-capture enzyme-linked immunosorbent assays (ELISAs), based on the double-antibody-sandwich (DAS) principle, can be used for rapid diagnosis of CSF, but the low sensitivity of the assay limits its use on a herd bases (Moennig and Becher, 2015; OIE terrestrial manual, 2015; Penrith et al., 2011).

However, virus isolation is time consuming and antigen detection techniques are not fully sensitive or specific. Therefore, they are currently being displaced by the technique of reverse transcription-polymerase chain reaction (RT-PCR) (Hoffmann et al., 2005; Liu et al., 2007; Moennig and Becher, 2015; Wen et al., 2010; Zhao et al., 2008). RT-PCR is the method of choice for rapid diagnostic (Moennig and Becher, 2015) since it is highly specific and gives immediate results, allowing early detection even at very low viral load rates (Dewulf et al., 2004; Greiser-Wilke et al., 2007; Hoffmann et al., 2005).

The real-time RT-PCR assays (RT-qPCR) allow quantitatively determining the viral load of the sample and reduces the risk of contamination compared to PCR on gel (Depner et al., 2007; Hoffmann et al., 2005; Pérez et al., 2011). Generally these RT-qPCR systems use the highly conserved 5'UTR of the CSFV genome as template (Hoffmann et al., 2005; Risatti et al., 2003, 2005a; Zhao et al., 2008), but there are also some based in the N^{pro} region (Liu et al., 2011b), the NS5A (Leifer et al., 2010) or the E2 (Titov et al., 2015). In practice only two fluorogenic mechanisms are used for RT-qPCR: Hydrolysis probes (Taqman®) (Eberling et al., 2011; Hoffmann et al., 2005; Huang et al., 2009; Liu et al., 2009, 2011b) or SYBR Green (Greiser-Wilke et al., 2007; Pérez et al., 2011; Zhang et al., 2011). Taqman probes are desirable over direct SYBR green labelling, as specificity of the latter reaction depends on the presence of contaminating

products, mispriming, primer–dimers, or some other artefacts (Greiser-Wilke et al., 2007).

Over recent years the design of multiplex conventional and real time RT-PCR techniques allow the differentiation against other pestiviruses (Díaz de Arce et al., 2009) and between CSFV field and vaccine strains (Blome et al., 2011; Leifer et al., 2009a; Liu et al., 2011b), as well as the differentiation of other infections fitted in the differential diagnosis, such as ASF (Belák, 2005; Haines et al., 2013). Additionally, the RT-PCR allows further analysis of the isolate, e.g., genotyping or analysis of viral virulence in animal experiments (Belák et al., 2008; Ji et al., 2015; Leifer et al., 2013; Weesendorp et al., 2011a).

Finally, serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination. Only assays that use monoclonal antibodies can distinguish antibodies against CSFV from responses to ruminant pestiviruses (Edwards et al., 1991). The commercial available antibody ELISAs are mostly blocking assays coated with the E2 glycoprotein or the whole particle, but because their lower specificity they are only useful on a herd basis screening (Loeffen, 2005; Schroeder et al., 2012). The definitive test for confirmation is neutralizing peroxidase–linked assay [NPLA] (Terpstra et al., 1984), which, by the use of specific antibodies, allow discrimination between the neutralizing titre of antibodies to different CSFV strains and related pestiviruses (Greiser-Wilke et al., 2007). It is important to note that neutralizing antibodies are at the earliest detectable two-three weeks following onset of disease (Greiser-Wilke et al., 2007). NPLA is useful in studies assessing the specific CSFV immune response, and is of application in the development of new vaccination strategies, as well as in *in vivo* vaccine potency tests.

1.7 Vaccine strategies against CSFV

The complexity of virus/host interaction, a subject not quite well understood yet, places obvious limitations on the development of new vaccines and antiviral strategies (Ganges et al., 2008; Leclerc, 2003). The first immunization strategy was CSFV hyper immune serum inoculation, derived from infected surviving pigs (Dorset et al., 1908). Due to its high production cost and the ongoing spread of the disease, crystal violet or formalin inactivated vaccines were experimented, but the inactivation was not complete and nor was effective in protection (Saulmon, 1973). By the 40s, the first attenuated vaccine after serial passage in rabbit (lapinised vaccines) was used (Baker, 1946; Koprowski et al., 1946). A stable lapinised strain by adapting the virulent strain Shimen was selected as the standard strain for commercial use, and has become the most commonly used until now (Dong and Chen, 2007; Kaden et al., 2000, 2004; Kaden and Lange, 2001). It was called Chinese vaccine strain (C-strain) or hog cholera lapinised virus (HCLV). Other commercial strains were derived from C-strain, such as Pestiffa (French), SUVAC (Hungary), Lapest (Poland), Suiferin C (former East Germany), JIK (former USSR), VADIMUN (USA) and Riems (Germany). Live attenuated strains were also obtained after adaptation to cell cultures at 30 °C, such as Japanese guinea-pig exaltation-negative strain (GPE^-) derived from virulent strain ALD and French cell culture adapted strain Thiverval derived from virulent strain Alfort (Dong and Chen, 2007).

Live attenuated vaccines are safe (Aynaud, 1988), induce no disease in young piglets or sows, even whether they have been immunosuppressed with corticosteroids or anti-thymus sera (Dong and Chen, 2007; van Oirschot, 2003b). They promote sterilizing immunity (neither viraemia nor excretion after challenge) (de Smit et al., 2001), with high titters of neutralizing antibodies, as well as early protection associated to cell immunity (Ganges et al., 2005; van Oirschot, 2003b; Suradhat et al., 2001; Tarradas et al., 2011b). Finally, they confer clinical protection against highly virulent strains, irrespective of the challenge strains used (Aynaud, 1988; Graham et al., 2012a, 2012b; Suradhat et

al., 2007; Vandeputte et al., 2001). The bestowed protection is long-lasting, at least 6-18 months or even lifelong (Ganges et al., 2008; van Oirschot, 2003b).

Although guaranteeing high protection rates, the live attenuated vaccines against CSF have some disadvantages: this type of vaccines elicit similar antibody patterns to those observed in naturally infected animals, and also, their ability to replicate in the host, even at very low rates, makes it extremely difficult to distinguish vaccinated from infected animals, favouring a straight policy regarding vaccination against CSFV in non-endemic countries (Ganges et al., 2008; Kaden et al., 2004; Moennig, 2000). Furthermore, the ability to prevent congenital infection has not been reported (van Oirschot, 2003b). On the other hand, incorrect handling (errors in the conservation and/or manipulation) and incomplete covering of the overall susceptible population might lead to failure in protection, to the appearance of carrier animals that are indistinguishable with the currently used diagnostic tools (Blome et al., 2017; Ganges et al., 2008; Pérez et al., 2012) and to the existence of viral vaccine escape variants of mild virulence that further hamper the diagnostic and control of CSF (Ji et al., 2014; Pérez et al., 2012).

With these premises, actual efforts in research are attempting to create efficient and safer marker or DIVA (differentiation of infected from vaccinated animals) vaccines, accompanied by a proper and potent test-system, to assist in the control of future CSF outbreaks (Blome et al., 2017; Dong and Chen, 2007; Ganges et al., 2008; de Smit, 2000). The possible use of an emergency vaccination with marker vaccines is expected to avoid the ethically questionable and expensive stamping out strategy, increasing the public acceptance of the eradication policy and lowering costs. A new EU Council Directive 2001/89/EC included for the first time marker vaccines and corresponding discriminatory tests (Anonimous, 2001). On the other hand, these vaccines can also be useful for basic investigations of the mechanisms behind the induction and control of immunity. Much of the current understanding about protective immune response is thanks to

the use of genetically engineered marker vaccines, which have led to the further characterization of the mechanisms involved in the induction and control of immunity, and also have enabled the detection of antigenic T and B epitopes along the genome (Dong and Chen, 2006a, 2006b; Monsó et al., 2011; Tarradas et al., 2010, 2011a, 2011b, 2012). Especially, B epitopes within the B/C domain of the E2, being the residues 693-712 which confers the best response by induction of CSFV-neutralizing antibodies (Dong et al., 2006). Research of DIVA vaccines mainly covers four strategies based on genetic engineering: subunit vaccines, viral vectors (chimera vaccines and replicons), immunogenic CSFV peptides and DNA vaccines (Beer et al., 2007; Blome et al., 2017; Ganges et al., 2008).

As the major target for neutralizing CSFV antibodies, the existing marker vaccines mainly include the E2 envelope glycoprotein or peptides within the antigenic domain of E2 in their design (Beer et al., 2007; Dong and Chen, 2007; Ganges et al., 2008). The discrimination of infected from vaccinated animals can be performed by an ELISA for detection of antibodies against E^{rns} protein, that are only developed on infection (Floegel-Niesmann, 2001; de Smit et al., 2001). However, specific antibodies against E^{rns} are not developed until 3–6 weeks post-infection, so diagnosis at early stages of infection is difficult (de Smit, 2000). The available E^{rns} based ELISA (PrioCHECK CSFV Erns, Thermofisher) has high sensitivity, but cross-react with BVDV and BDV strains, and multiple vaccination or reduced quality of the sample reduces its specificity (Blome et al., 2017; Pannhorst et al., 2015). At the moment, this test should be applied only on a herd bases screening, but not for individual samples.

The information available on different vaccine formulations against CSFV, different inoculation routes and different vaccination and challenge schedules is too diverse to allow the objective comparison of vaccine candidates (Ganges et al., 2008). Currently, only a E2 subunit vaccine (Porcilis Pesti, Intervet) and a live recombinant E2 gene deleted BVDV containing CSFV E2 glycoprotein

(Suvaxyn CSF marker, Zoetis) have been authorised for the market (Felberbaum, 2015; Reimann et al., 2016) The commercialization of E2 subunit vaccine (Porcilis Pesti, Intervet) has been limited due to the incomplete vertical transmission protection and to the need of two immunization doses, being only approved for emergency vaccination within restricted control zone (EMA, 2006). Very recently, another baculovirus-expressed E2 subunit candidate has proved total protection after a single vaccination dose (Madera et al., 2016). On the other hand, the Suvaxyn is a promising marker vaccine, supported by the existence of a specific RT-qPCR for vaccine detection, in addition of the CSFV E^{rns} commercial ELISA (Leifer et al., 2009a; Pannhorst et al., 2015). In this context, Suvaxyn has demonstrated good results in the clinical and virological protection after challenge, even in the presence of maternal derived antibodies; however, little information is available regarding the protection against CSFV trans-placental transmission (Blome et al., 2017), being only approved for emergency vaccination within restricted control zone (EMA, 2014). Meanwhile, live attenuated vaccines remain the most used in endemic countries although they does not meet the DIVA concept. For this reason, research in the vaccine and diagnostic tools development against CSFV remains as one of the strategic targets in the field of animal health. Overall, the implementation of an efficient and safe vaccination program together with a solid control policy will guarantee the future CSF eradication.

Chapter 2

Objectives

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In endemic countries, where vaccination in the control programs is in force, a trend towards CSFV less virulent strains has been described. Pigs infected with lowly or moderately virulent strains that develop mild or unapparent forms can eliminate the virus continuously or intermittently for long periods of time, and constitute a powerful source of re-infection for endemic countries as well as a threat to disease-free countries. Despite the prevalence and intrinsic importance of these strains in endemic countries, the associated pathogenesis and immune response are poorly understood.

On the other hand, the occurrence of low-virulence CSFV strains in the field and their role in the "pregnant carrier sow syndrome" and in congenital infection of the foetus by trans-placental transmission have been extensively described. The pathogenesis of CSF congenital persistent infection has been related to a specific immunotolerance against CSFV due to the immaturity of the foetal immune system. This fact leads to the birth of viremic animals incapable of generating a specific immune response, and which often have no symptoms, making detection on the farm difficult. However, the mechanisms involved are not known, and the existing studies date over 40 years ago. On the other hand, the generation of postnatal persistence has not yet been documented in the scientific literature.

Considering the above premises, this thesis aims to evaluate the ability of CSFV to generate persistent infection in newborn piglets, to characterize the associated immune response, pathogenesis, as well as to elucidate the possible virological and epidemiological implications of this type of infection. The specific objectives to be addressed were as follows:

2.1. To evaluate the ability of two CSFV field isolates of low (Pinar del Río strain, 2011, from the Cuba epidemic, genotype 1.4) and moderate virulence (Catalonia 01 strain, from the epizootic of 2001 in Spain, genotype 2.3), respectively, to induce viral persistence after early postnatal infection in swine, as well as to study the characteristics of the immunological response related to viral persistence. Specifically, the implication of cytokines involved in innate and

acquired immunity such as IFN-α, IL-10 and IFN-γ, and the ability to induce specific humoral immune response (Study I).

2.2 To study the immune response against a live attenuated CSFV vaccine (C-strain) in six-week-old CSFV postnatally persistently infected pigs, and the impact of the vaccination on the kinetics of CSFV replication in the persistent infection (Study II).

2.3. To assess the superinfection of a virulent CSFV strain (Margarita strain from the Cuba epidemic, genotype 1.4) in six-week-old wild boars, rendered CSFV-persistently infected after birth with the Catalonia 01 strain. Specifically, to study the replication capacity of the secondary virus as well as its effect on the replication of the persistently infecting virus (primary infection). In addition, the development of diagnostic tools to evaluate the viral replication of CSFV strains from different genogroups, in order to differentiate both infections (Study III).

PART II

Studies

Chapter 3

Study I: *Postnatal persistent infection with classical swine fever virus and its immunological implications*

PLoS One (2015); 10: e0125692.

PLOS | ONE

RESEARCH ARTICLE

Postnatal Persistent Infection with Classical Swine Fever Virus and Its Immunological Implications

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Citation: Muñoz-González S, Ruggli N, Rosell R, Pérez LJ, Frías-Leuporeau MT, Fraile L, et al. (2015) Postnatal Persistent Infection with Classical Swine Fever Virus and Its Immunological Implications. PLoS ONE 10(5): e0125692. doi:10.1371/journal.pone.0125692

Academic Editor: Gourapura J Renukaradhya, The Ohio State University, UNITED STATES

Received: October 22, 2014

Accepted: March 23, 2015

Published: May 4, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This research was supported by grant AGL2012-38343 from Spanish government. Author who received the funding: Llilianne Ganges (LG). S. Muñoz-González received predoctoral fellowship FI-DGR 2014 from AGAUR, Generalitat de Catalunya. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

It is well established that trans-placental transmission of classical swine fever virus (CSFV) during mid-gestation can lead to persistently infected offspring. The aim of the present study was to evaluate the ability of CSFV to induce viral persistence upon early postnatal infection. Two litters of 10 piglets each were infected intranasally on the day of birth with low and moderate virulence CSFV isolates, respectively. During six weeks after postnatal infection, most of the piglets remained clinically healthy, despite persistent high virus titres in the serum. Importantly, these animals were unable to mount any detectable humoral and cellular immune response. At necropsy, the most prominent gross pathological lesion was a severe thymus atrophy. Four weeks after infection, PBMCs from the persistently infected seronegative piglets were unresponsive to both, specific CSFV and non-specific PHA stimulation in terms of IFN-γ-producing cells. These results suggested the development of a state of immunosuppression in these postnatally persistently infected pigs. However, IL-10 was undetectable in the sera of the persistently infected animals. Interestingly, CSFV-stimulated PBMCs from the persistently infected piglets produced IL-10. Nevertheless, despite the addition of the anti-IL-10 antibody in the PBMC culture from persistently infected piglets, the response of the IFN-γ producing cells was not restored. Therefore, other factors than IL-10 may be involved in the general suppression of the T-cell responses upon CSFV and mitogen activation. Interestingly, bone marrow immature granulocytes were increased and targeted by the virus in persistently infected piglets. Taken together, we provided the first data demonstrating the feasibility of CSFV in generating a postnatal persistent disease, which has not been shown for other members of the Pestivirus genus yet. Since serological methods are routinely used in CSFV surveillance, persistently infected pigs might go unnoticed.

Competing Interests: The authors have no competing interests.

In addition to the epidemiological and economic significance of persistent CSFV infection, this model could be useful for understanding the mechanisms of viral persistence.

Introduction

Classical swine fever (CSF) is a highly contagious viral disease of domestic pigs and wild boars [1], which has caused major losses in stock farming [2]. The causative agent, CSF virus (CSFV), is a member of the genus *Pestivirus* within the family *Flaviviridae* [1]. CSFV is composed of a lipid envelope, a capsid and a single plus-strand RNA genome carrying a single, large open reading frame (ORF) flanked by two untranslated regions (UTRs). The ORF encodes a polyprotein of approximately 3900 amino acids, which are processed by cellular and viral proteases in the four structural proteins C, E^{rns}, E1, E2 and in the 8 non-structural proteins N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [3].

Although CSF has been widely eradicated, it remains endemic in certain areas of Asia, Europe, Central and South America, and parts of Africa [4–10], representing a constant threat to the pig industry. Depending on the virulence of the strain, varying degrees of disease severity have been observed, ranging from acute or chronic to subclinical forms [7,11,12]. In general, while infections with virulent strains result in acute haemorrhagic disease, the infection caused by less virulent isolates can become chronic or subclinical [11,13]. Pigs infected with low virulent strains can shed the virus continuously or intermittently for months, representing a constant source of reinfection in endemic areas and a threat to virus-free countries [4,14]. Interestingly, in endemic areas, such as Cuba and China, a trend towards milder, chronic clinical manifestations of CSF has been observed [4,5,15]. It was suggested that CSFV evolution towards low virulent viruses in these regions was driven in part by a positive selection pressure linked to inefficient vaccination programs, leading to mostly unapparent clinical manifestations. Therefore, these viral strains are of great significance in endemic countries [4,5,16,17]. However, the pathogenesis and disease progression after infection with low virulent CSFV isolates are poorly understood.

The occurrence of low virulence CSFV strains in the field and their role in the "pregnant carrier sow syndrome" and in congenital infection of the foetus by trans-placental transmission have been extensively described [18–21]. There has been, however, some controversy over the importance of such congenital persistent infections in virus dissemination [22,23]. Numerous reports on experimental congenital infections have shown that congenitally persistently infected piglets result mostly from infection during mid-gestation [13,20,21,24,25]. However, the pathogenesis of this persistence is not completely understood and has been related to a specific immunotolerance to CSFV [19,25–27]. At birth, congenitally persistently infected piglets are often not recognised as infected animals, appearing healthy and developing a runting-like syndrome only later, with lesions that are not characteristic of CSF. As opposed to congenital infections, however, there have been few reports only suggesting a possible occasional occurrence of virus persistence after postnatal infection of newborns [28] and after infection of 6-week-old weaned pigs [27,29].

Considering the above premises, the aim of this work was to evaluate the ability of two CSFV field isolates of low and moderate virulence, respectively, of different origins and genotypes to induce viral persistence after early postnatal infection, as well as to study the characteristics of the immunological response related to viral persistence.

Materials and Methods

Cells and viruses

PK-15 cells (ATCC CCL 33) were cultured in DMEM medium, supplemented with 10% foetal bovine serum (FBS) pestivirus-free at 37°C in 5% CO_2. The cells were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 48 h later. Peroxidase-linked assay (PLA) [30] was used for viral titration following the statistical methods described by Reed and Muench [31]. The Catalonia 01 (Cat01) strain used in this study was isolated from the CSF Spanish epizootic in 2000–2001 [32]. This isolate belongs to the CSFV 2.3 genogroup [4]. The course of the infection by this strain was found to be mild [32,33]. The Pinar del Rio (PR) strain is a prototype low virulence CSFV isolate circulating currently in Cuba [4]. It was isolated after more than 18.5 years of endemic CSF in Cuba, during which CSFV evolved under constant immunological pressure exerted by suboptimal vaccination [4,34]. Finally, the Thiverval vaccine strain (provided by Pasteur Institute, Romania) was used as stimulus in the Elispot assays for CSFV-specific IFN-γ-producing cells detection. This strain belongs to the CSFV 1.1 genogroup [35].

Experimental design

Two pregnant, pestivirus-free sows (landrace) of 108 days into gestation were housed in the BSL3 animal facility at CReSA (Barcelona, Spain). Each sow was housed in a separate box with standard facilities to allocate pregnant and lactating sows. Their deliveries were synchronised with d-cloprostenol 75 pg/sow at 114 days of gestation. After 24 hours, the deliveries were initiated, and 10 piglets were inoculated intranasally during the first 8 hours after birth with 2.5 mL of 2.5 x 10^4 TCID of the PR strain or Cat01 strain. The inoculation of the piglets was conducted separately from their mothers. Additionally, 2 piglets at 6 weeks of age, from a sow of the same origin, served as control, non-inoculated pigs (numbered 32 and 33). The piglets were kept with their mothers during the 6 weeks of the experiment, and they received feed (StartRite, Cargill, Spain) from week 5 onwards. After infection, serum samples were collected every week over the 6 weeks post-infection, and nasal and rectal swabs were obtained at 2, 3, 4, 5 and 6 weeks post-infection. Blood samples for the isolation of PBMCs were obtained at 4 and 6 weeks post-infection, and tissues from the tonsils and thymus were obtained after euthanasia. 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 clinical signs daily in a blinded manner. To reduce handling to the litters during the first three days of life, the rectal temperature was recorded from 3 days after infection (after birth) until the end of the trial. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB) under number 5796, according to existing national and European regulations.

Detection of CSFV RNA

RNA was extracted from all the samples using the NucleoSpin RNA isolation kit (Macherey-Nagel), according to the manufacturer's instructions. In all cases, RNA was extracted from an initial sample volume of 150 μL to obtain a final volume of 50 μL of RNA, which was stored at -80°C. The presence of CSFV RNA in the serum and in nasal and rectal swabs, as well as tonsil, thymus and bone marrow samples, was analysed by real time (RT)-PCR [36]. This test was used in our laboratory for inter-laboratory comparisons of CSFV diagnoses, organised by the EU Reference Laboratory. Positive results were considered for threshold cycle values (CT)

equal to or less than 42. Samples in which fluorescence was undetectable were considered negative.

Detection of E2-specific and neutralising antibodies

The serum samples were tested with neutralisation peroxidase-linked assay (NPLA) [37], and the titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the Cat01 or PR strain in 50% of the culture replicates. The detection of E2-specific antibodies was performed using a commercial ELISA kit (IDEXX); the samples were considered positive when the blocking percentage was ≥40%, following the manufacturer's recommendations.

ELISA for IFN-α detection in serum samples

Anti-IFN-α monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) were used in an ELISA assay to detect IFN-α in serum samples [38–41]. The cut-off value was calculated as the average of the optical density of negative controls (blank and negative serums before CSFV infection) plus three standard deviations. Cytokine concentrations in serum were determined using a regression line built with the optical densities of the cytokine standards used in the test.

PBMCs and ELISPOT assay for CSFV-specific IFN-γ-producing cells

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood collected at 4 and 6 weeks post-infection, and these cells were separated by density-gradient centrifugation with Histopaque 1077 (Sigma). The number and viability of the PBMCs were determined by staining with Trypan Blue [42]. ELISPOT assay to detect CSFV-specific IFN-γ cells was performed as previously described [41]. Briefly, plates (Costar 3590, Corning) were coated overnight with 5 μg/ml of capture antibody (P2G10, Pharmigen). Detection was performed using a biotinylated antibody (P2C11, Pharmigin). A total of $5x10^5$ PBMCs/well were plated in triplicate at 0.1 multiplicity of infection (MOI) of the Cat01 and PR CSFV strains. Moreover, the same samples were incubated in the presence of Thiverval strain at 0.01 MOI and phytohaemagglutinin (PHA) (10 μg/ml). The controls were incubated in the presence of mock-stimulated wells. The numbers of spots in the media for mock-stimulated wells were considered to be the baseline for the calculation of antigen-specific frequencies of IFN-γ-producing cells.

BMHC collection and phenotype analysis

Bone marrow haematopoietic cells (BMHCs) were obtained from the femurs of selected pigs (pigs 1 and 3: infected with the PR strain; pigs 19 and 23: infected with the Cat01 strain; and two non-infected pigs) at 6 weeks of age, following the protocol previously described [43,44]. To phenotype these cells, flow cytometry was performed using the corresponding hybridoma supernatants in the indirect labelling for SLA-I (74-11-10, IgG2b), SLA-II (1F12, IgG2b), CD163 (2A10/11, IgG1), CD172a (BA1C11, IgG1), granulocyte precursors (6D10, IgG2a), and c-kit or CD117 (2B8/BM IgG1); all of the hybridoma supernatants were kindly donated by Dr. J. Dominguez (INIA, Madrid, Spain). For the detection of CSFV-infected cells, a polyclonal FITC-labelled anti-CSFV conjugate (PrioCON FITC conjugate PAb-CSF, Prionics, Switzerland) was used. For the cellular markers, the secondary antibody was R-phycoerythrin goat anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK). Briefly, 2.5×10^5 cells/50 μl/well were labelled for 1 h at 4°C. The anti-CSFV conjugate was diluted 1:100 in cold PBS with 2% FBS. After 1 h of incubation at 4°C, the cells were washed with cold PBS with 2% FBS by centrifugation at 450 × *g*, at 4°C for 5 min. Then, the secondary antibody conjugated with R-

phycoerythrin diluted 1:200 was added for SLA-I, SLA-II, CD163, CD172a, 6D10 and CD117 markers. The cells were incubated for a further 45 min at 4°C and then were washed as before and resuspended in PBS with 2% FBS. SLA-I, SLA-II, CD163, CD172a, 6D10, CD117, and CSFV-positive cells and unstained cells were counted using FACSaria I (Becton Dickinson), and the data were analysed by FACSDiva software, version 6.1.2. Irrelevant isotype-matched mAbs, unlabelled or labelled with the different fluorochromes, were used as negative controls. The gate strategy was applied in 90% of living cells using the forward and side scatter (FS/SS) characteristics. For two colour immunolabelling, the same procedure described above for incubation and washing was followed. To 2.5×10^5 cells/50 μl/well, 50 μl of SLA-II marker was added, followed by the secondary antibody conjugated with R-phycoerythrin diluted 1:200. Mab CD172a was biotinylated using standard protocols (CD172a_b). After the third wash, the cells were incubated with CD172a_b for 1 h at 4°C. Finally streptavidin-allophycocyanin (APC) was added at a 1:100 dilution. For $6D10^+/CSFV^+$ labelling, the polyclonal FITC-labelled anti-CSFV conjugate and 6D10 hybridoma supernatants revealed with R-phycoerythrin conjugate were used. $CD172a^+/SLA\text{-}II^+$ or $6D10^+/CSFV^+$ was acquired using FACSAria I (Becton Dickinson, San Jose, California, USA), and the positive percentages were analysed by FACS-Diva software, version 6.1.2.

Sorting of $6D10^+$ cells

The $6D10^+$ cell subsets were sorted using a live sterile cell sorting system (FACSAria, Beckton Dickinson, San Jose, California, USA). To obtain $6D10^+$ cells, 24 $x10^6$ BMHCs were incubated with 6D10 hybridoma supernatant for 1 h on ice, washed with PBS containing 2% FBS, and incubated with R-phycoerythrin conjugate goat $(Fab')_2$ anti-mouse Ig (Dako, Denmark). Single cell sorting was performed in using purity precision mode, with a 70 μm nozzle. The fluorescence reading was performed upon excitation with a 488 nm argon laser. The $6D10^+$ and $6D10^-$ cells were more than 95% pure by flow cytometry, and a total of 6701444 $6D\text{-}10^+$ cells were recovered with 97% efficiency. The presence of CSFV RNA in both types of recovered cells was analysed by RT-PCR [36].

Stimulated IL-10 production by PBMCs from CSFV-infected pigs

To elucidate the role of IL-10 in postnatal persistence, the levels of IL-10 were firstly determined in the sera from the piglets and sows. These samples were analysed by ELISA (IL-10 Swine ELISA Kit, Life-Technologies, USA) at 7, 14, 21 and 42 days post infection (dpi). In contrast, to measure IL-10 production, 2.5 x 10^6 PBMCs/mL were cultivated for duplication in the presence or absence of neutralising IL-10 clone (148801, R&D System, USA) at 6 μg/10^6 cells in RPMI medium with 10% FBS. A total of 2.5x10^5 PBMCs/well were stimulated at 37°C in 96-well plates with mock, CSFV (Catalonia strain) at 0.1 MOI or PHA (10 μg/ mL). The supernatants were removed after 96 h, and the concentrations of IL-10 were determined by ELISA.

Moreover, to investigate the effect of IL-10 on IFN-γ production, PBMCs collected at 6 weeks p.i. from pigs 23, 25, 27 (CSFV persistently infected pigs), 32 and 33 (non-infected pigs) were cultivated in one ELISPOT assay in the presence or absence of neutralising IL-10 clone (148801, R&D System, USA) at 6 μg/10^6 cells. These cells were cultivated for duplication and were stimulated with mock, CSFV (Catalonia strain) at 0.1 MOI and PHA (10 μg/mL). These experiments were repeated twice under the same conditions.

Statistical analysis

All of the statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, Illinois, USA), using "piglet or sow" as the experimental unit. The significance level (α)

was set at $P<0.05$. Throughout the trial, a non-parametric test (Mann-Whitney) was chosen to compare values obtained from the immunological parameters between groups (Cat01 or PR strain). This non-parametric analysis was chosen due to the small number of animals used in each experimental group. Finally, Fisher's exact test was used to test the associations of the percentage of CSFV-positive piglets in serum, nasal and rectal swabs, and the tonsils and thymus among the experimental groups throughout the trial.

Results

Clinical signs developed in the newborn infected piglets

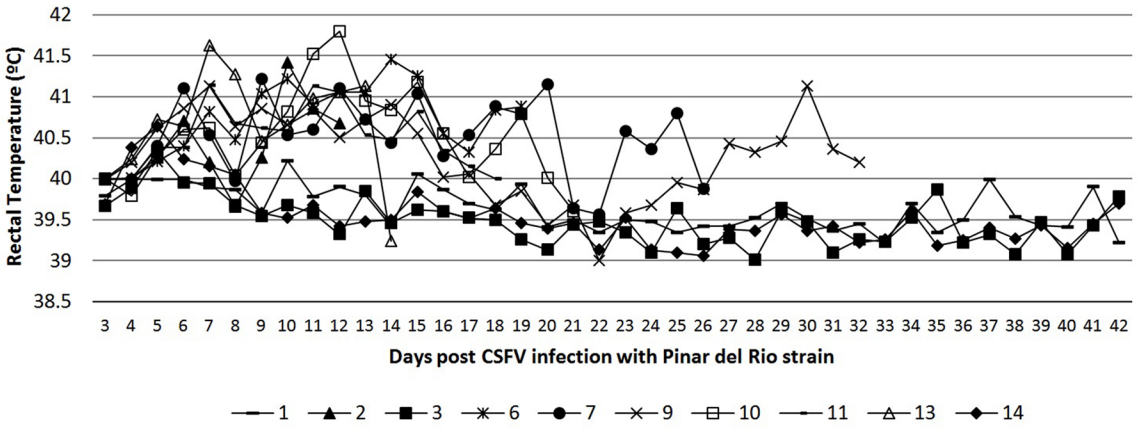
The pathogenicity of the two CSFV field isolates was studied in newborn piglets. To this end, two litters of 10 piglets each were inoculated intranasally on the day of their birth with 2.5 x 10^4 $TCID_{50}$ of CSFV strains PR and Cat01, respectively. The infected piglets were kept with the dam for 6 weeks and were monitored for clinical symptoms, virus replication and immune responses against CSFV. In the PR and Cat01 group, three and five animals, respectively, did not show visible clinical signs over the 6 week duration of the experiment. Fever peaks during the first 15 days post-infection were detected in the infected animals, especially in PR infected group. In addition, similar profiles in the rectal temperature values were found for both groups during the trial. Only 5 days (3, 6, 7, 13 and 15 dpi) were significantly different between the groups (Figs 1 and 2).

Six of PR and three of Cat01 animals died or were euthanised between the 3rd and 6th weeks after CSFV inoculation after they suddenly become apathic and developed severe secondary infections, resulting in omphalophlebitis, mild diarrhoea, polyarthritis or prostration (Fig 2). Finally, one apparent healthy piglet from the PR group and two from the Cat01 group were lost during the second, first and third weeks, respectively, killed by their dams (Figs 1 and 2). Interestingly, irrespective of the outcome of CSFV infection, the thymus was very small at the time of necropsy in all of the piglets except for pigs 1, 3 and 14 from the PR group (data not shown).

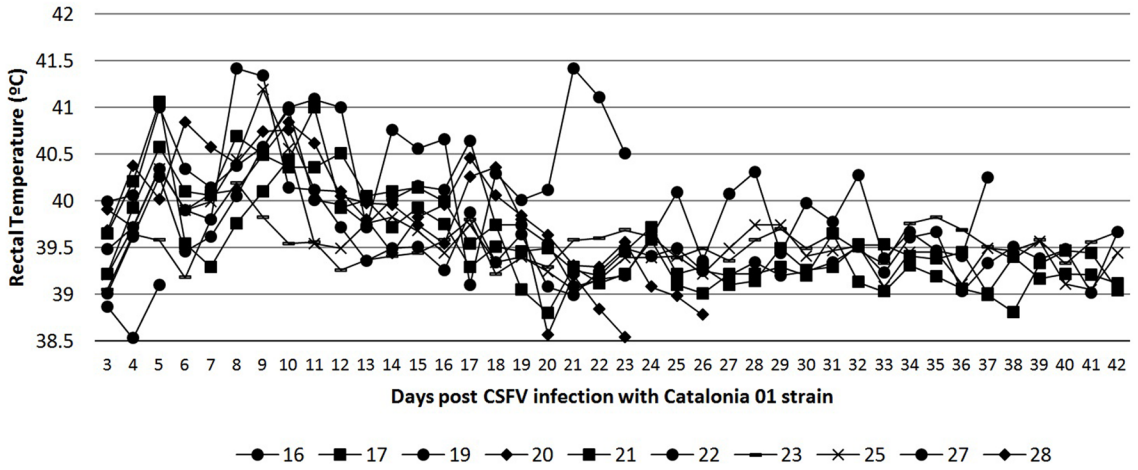
The majority of the newborn CSFV-inoculated piglets did not clear the virus

CSFV replication after perinatal inoculation of the piglets with the PR and Cat01 strains was monitored by qRT-PCR for viral RNA at weekly intervals in serum, nasal and rectal swabs and in the tonsils, thymus and bone marrow at necropsy (Fig 3). Despite the absence of clinical signs, all of the piglets had strong viral RNA signals in their serum 7 days after inoculation. At later time points, the viral RNA load further increased and remained high until the end of the experiment or until death or euthanasia in all of the piglets infected with Cat01 and in 7 of 10 piglets infected with PR. The three remaining piglets in the PR group (pigs 1, 3 and 14) cleared the virus from their circulation by day 28 post-inoculation, as determined by real time RT-PCR (Fig 3A). Interestingly, the Cat01 virus-infected piglets had significantly higher CSFV RNA levels in their serum than the PR virus-infected piglets ($P = 0.0002$), which might indicate a lower virulence of the PR strain. Virus titration at 21 and 28 days after inoculation confirmed the qRT-PCR data, with overall higher virus titres in the serum of the Cat01-infected *versus* the PR-infected piglets ($P = 0.006$, Table 1). Additionally, the three PR-infected piglets that had undetectable viral RNA at day 28 post-inoculation were also negative for virus isolation already at day 21 (Table 1 and Fig 3). However, in the two sows, the virus could not be detected in the serum, either by qRT-PCR or by virus isolation at any time during the experiment, confirming the low virulence of the two strains. Nevertheless, the dam of the Cat01-infected piglets had low levels of viral RNA detectable in the nasal and rectal swabs until the end of the experiment,

(a)


Rectal Temperature (ºC)
42
41.5
41
40.5
40
39.5
39
38.5
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Days post CSFV infection with Pinar del Rio strain
1 2 3 6 7 9 10 11 13 14

(b)


Rectal Temperature (ºC)
42
41.5
41
40.5
40
39.5
39
38.5
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Days post CSFV infection with Catalonia 01 strain
16 17 19 20 21 22 23 25 27 28

(c)

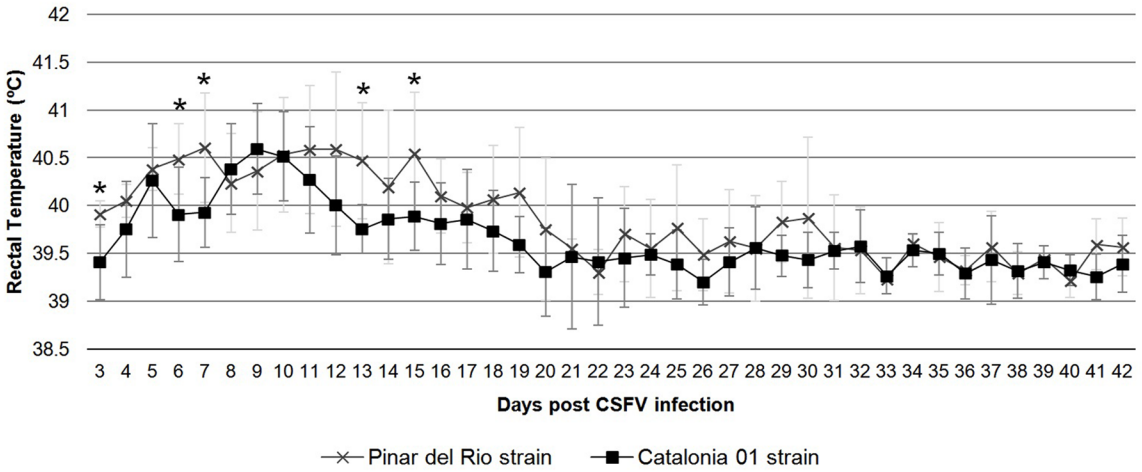

Rectal Temperature (ºC)
42
41.5
41
40.5
40
39.5
39
38.5
*
*
*
*
*
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Days post CSFV infection
Pinar del Rio strain
Catalonia 01 strain

Fig 1. Rectal temperature values (°C) at 3 days post-infection with PR or Cat01 CSFV strains daily during the trial. (a) Individual rectal temperature values (°C) in pigs inoculated with the PR strain (pigs 1 to 14). (b) Individual rectal temperature values (°C) in pigs inoculated with the Cat01 strain (pig 16 to 28). (d) Mean and standard deviation values of the rectal temperature (°C) per group (PR and Cat01) per day. Cross symbol shows the mean value for PR group. The square symbol shows the mean value for Cat01 group. The light grey bars indicate the standard deviation value for PR group. Dark grey bars indicate the standard deviation value for Cat01 group. Values greater than 40°C were considered to indicate fever. An asterisk indicates a statistically significantly higher rectal temperatures in piglets infected with the PR strain, compared with the levels found in piglets infected with the Cat01 strain ($P<0.05$).

doi:10.1371/journal.pone.0125692.g001

while the PR-infected sow remained negative from the swabs (Fig 3B and 3C). CSFV RNA was detected in the nasal and rectal swabs of all of the inoculated piglets until the end of the experiment or the day on which they were euthanised, except for the 3 PR-infected piglets that recovered from infection and stopped shedding the virus by day 28 (Fig 3B and 3C). The viral RNA load in the nasal and rectal swabs from the pigs infected with the Cat01 strain was higher than for the PR-infected piglets at 14 days p.i. only. At necropsy, CSFV RNA was detected in the tonsils, thymus and bone marrow of all of the piglets infected with Cat01 strain and from the 7

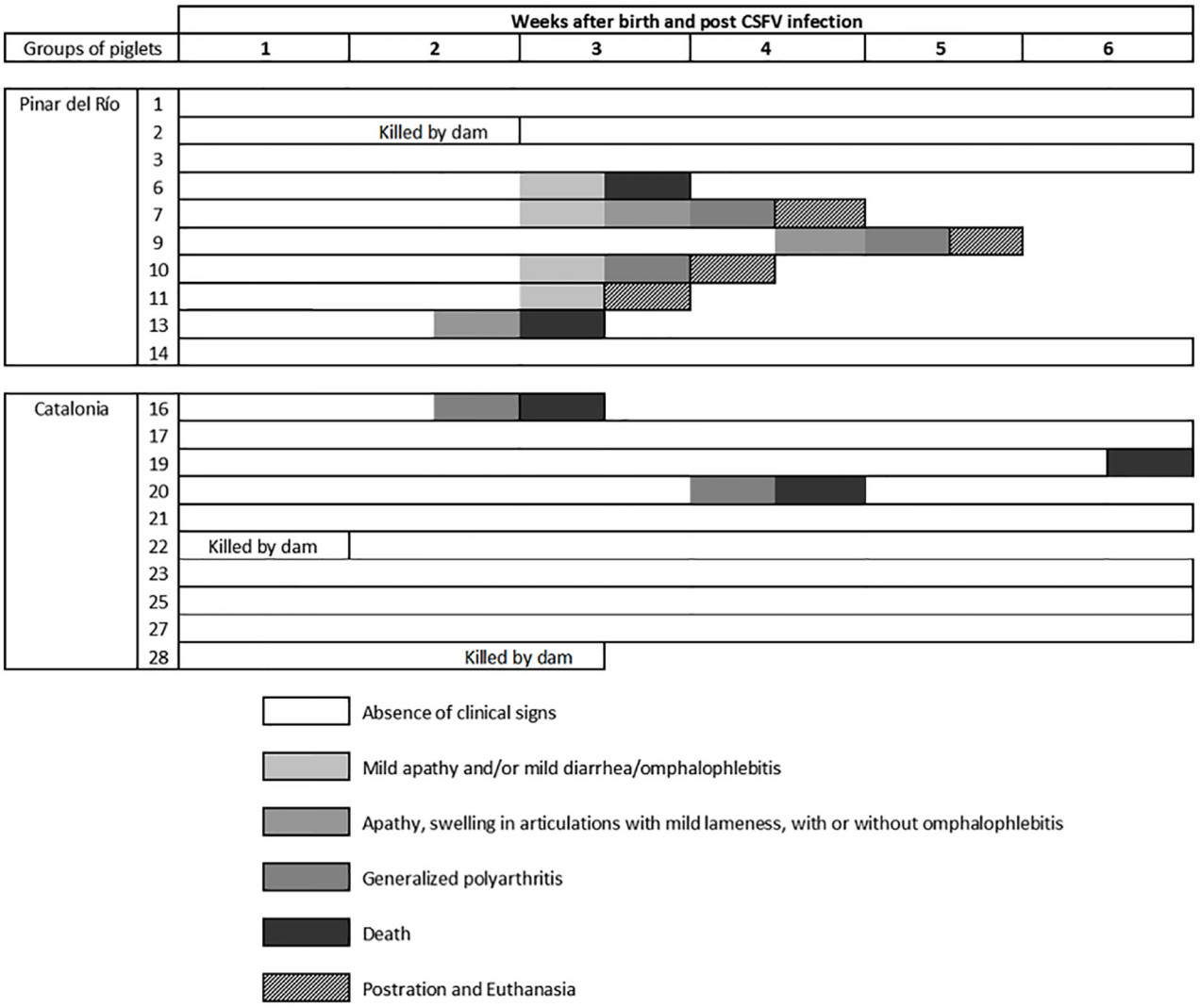


Fig 2. Individual clinical signs in piglets after early postnatal infections with CSFV PR or Cat01 field isolates. The piglets were monitored daily over the 6 weeks of the study. The evolution in the development of clinical signs is represented by an intensity colour scale (from low to high).

doi:10.1371/journal.pone.0125692.g002

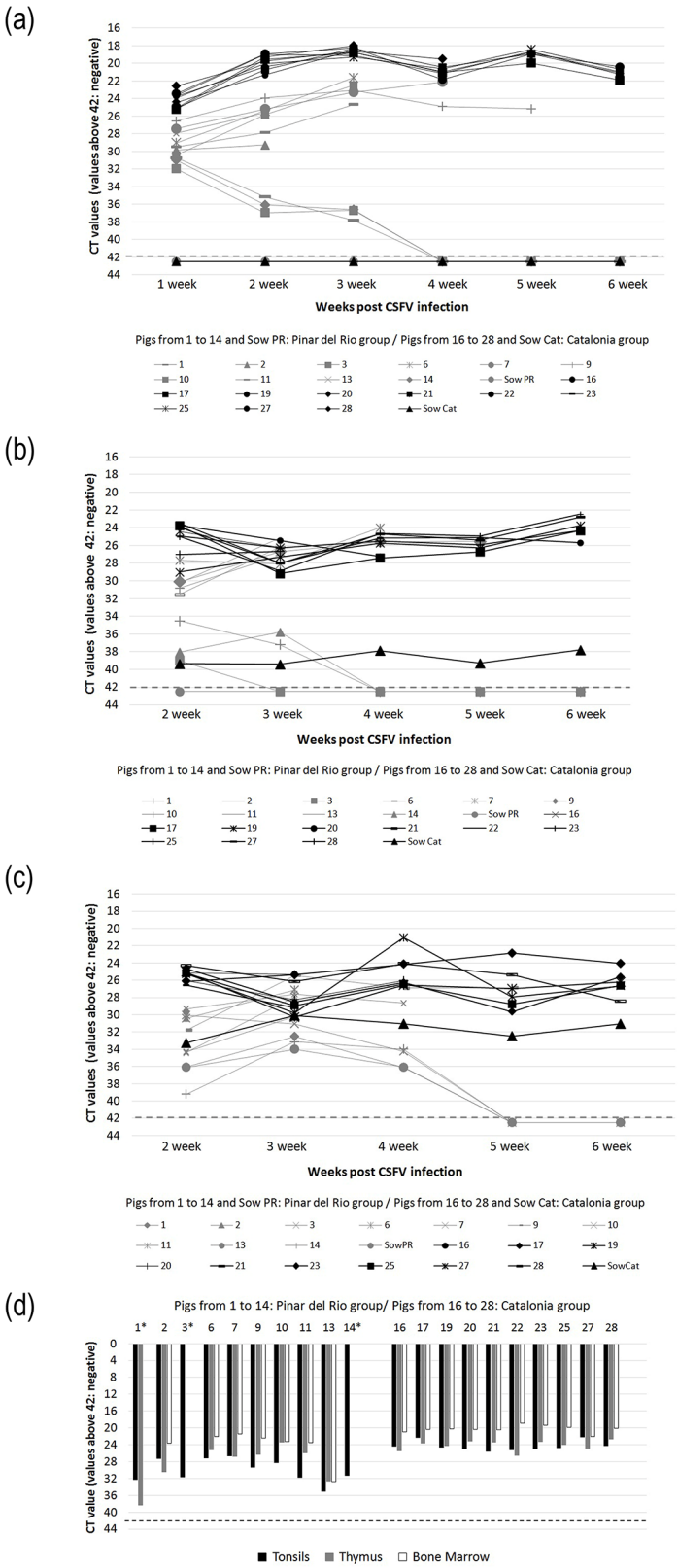


Fig 3. Detection of CSFV RNA through real-time PCR in serum and nasal and rectal swabs. Detection of CSFV RNA through real-time PCR in serum (a) and nasal (b) and rectal (c) swabs. The piglets and the

sows infected with the PR and the Cat01 strains are represented in grey and black colours, respectively. Positive results were considered for CT values equal to or less than 42. (d) Detection of CSFV RNA through RT-PCR in the tonsils, thymus and bone marrow are represented in black, grey and white colours, respectively. Positive results were considered for CT values equal or less than 42. An asterisk indicates negative results in pigs 1, 3 and 14 in some of the tissues analysed.

doi:10.1371/journal.pone.0125692.g003

PR virus-infected piglets that did not clear the virus (Fig 3D). It is worth noting that the CSFV RNA levels detected in the tonsils, thymus and bone marrow were significantly higher in the Cat01 group than in the PR group (tonsils, $P = 0.0002$; thymus, $P = 0.009$; bone marrow, $P = 0.001$). Interestingly, the two sows were also qRT-PCR-positive in the tonsils (data not shown). Altogether, 85% of the piglets inoculated on the day of their birth were not capable of clearing the virus within the 4 to 6 weeks of their lifespans.

The newborn piglets incapable of clearing CSFV did not seroconvert

To determine whether the inability to clear CSFV was related to a deficient humoral immune response, serum samples from the infected and control pigs were analysed weekly for CSFV-specific antibodies. No detectable antibody response were found in the control pigs (data not shown). Likewise, there were no detectable antibody responses after infection in any of the piglets infected with the Cat01 strain or in the 7 piglets infected with the PR strain that did not clear the virus during the six weeks of the experiment or until death or euthanasia (Fig 4A and 4B). In contrast,

Table 1. Virus isolation and virus titration in PK-15 cells with serums samples at 3 and 4 weeks post-infection.

		3 week post-infection		4 week post-infection	
Inoculum	**Pig number**	**Virus isolation**	**Virus titration[a]**	**Virus isolation**	**Virus titration[a]**
Pinar del Rio strain	1	Negative	Negative	Negative	Negative
	3	Negative	Negative	Negative	Negative
	6	+	$10^{5.5}$	†	
	7	+	$10^{6.35}$	+	$10^{7.00}$
	9	+	$10^{5.66}$	+	$10^{5.57}$
	10	+	$10^{6.35}$	†	
	14	Negative	Negative	Negative	Negative
	Sow PR	Negative	Negative	Negative	Negative
Catalonia strain	16	+	$10^{6.66}$	†	
	17	+	$10^{6.57}$	+	$10^{6.20}$
	19	+	$10^{7.12}$	+	$10^{6.80}$
	20	+	$10^{6.66}$	+	$10^{7.16}$
	21	+	$10^{6.57}$	+	$10^{6.49}$
	23	+	$10^{6.66}$	+	$10^{6.66}$
	25	+	$10^{6.75}$	+	$10^{6.71}$
	27	+	$10^{6.57}$	+	$10^{7.00}$
	28[b]	+	$10^{7.00}$	†	
	Sow Cat	Negative	Negative	Negative	Negative

Negative: The virus isolation was negative.

Symbol +: The virus isolation was positive.

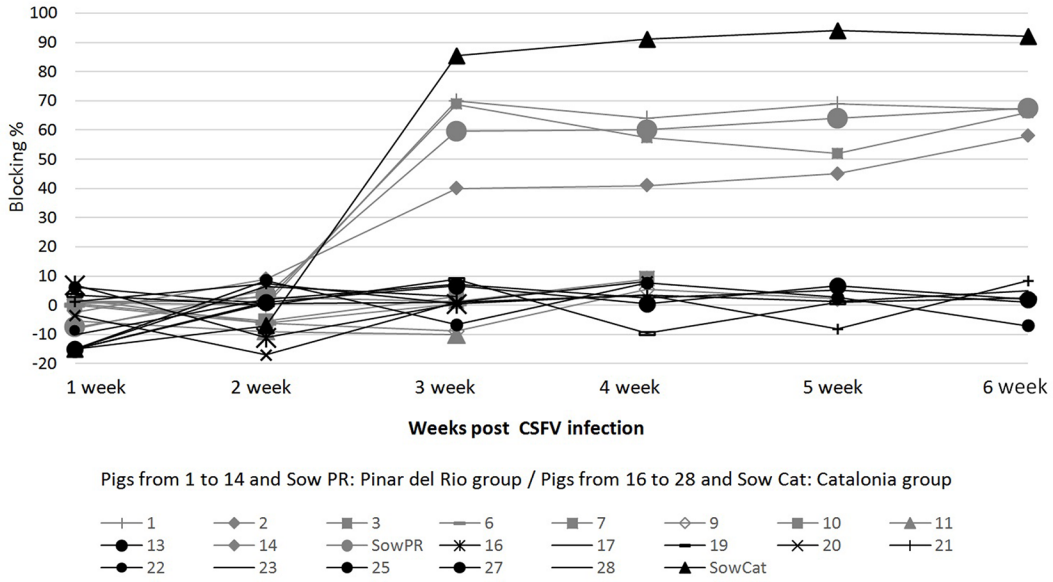
Symbol †: Dead

[a]: Virus Titration in TCID 50/mL

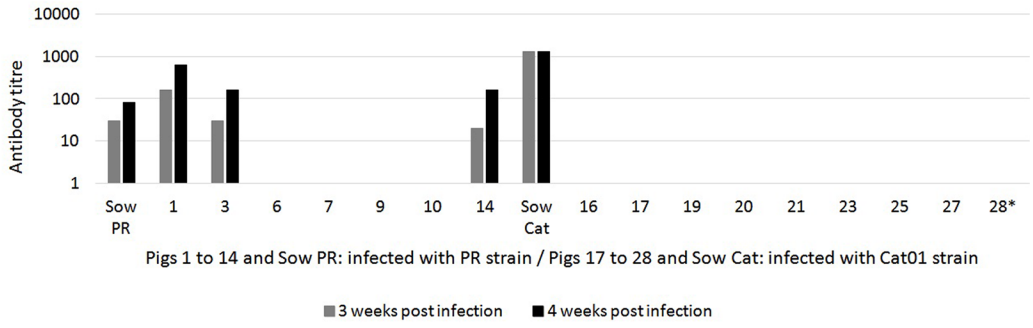
[b]: sample from pig 28 analysed at 19 dpi

doi:10.1371/journal.pone.0125692.t001

(a)


Blocking %
100
90
80
70
60
50
40
30
20
10
0
-10
-20
1 week
2 week
3 week
4 week
5 week
6 week
Weeks post CSFV infection
Pigs from 1 to 14 and Sow PR: Pinar del Rio group / Pigs from 16 to 28 and Sow Cat: Catalonia group
1
2
3
6
7
9
10
11
13
14
SowPR
16
17
19
20
21
22
23
25
27
28
SowCat

(b)


Antibody titre
10000
1000
100
10
1
Sow PR
1
3
6
7
9
10
14
Sow Cat
16
17
19
20
21
23
25
27
28*
Pigs 1 to 14 and Sow PR: infected with PR strain / Pigs 17 to 28 and Sow Cat: infected with Cat01 strain
3 weeks post infection
4 weeks post infection

(c)

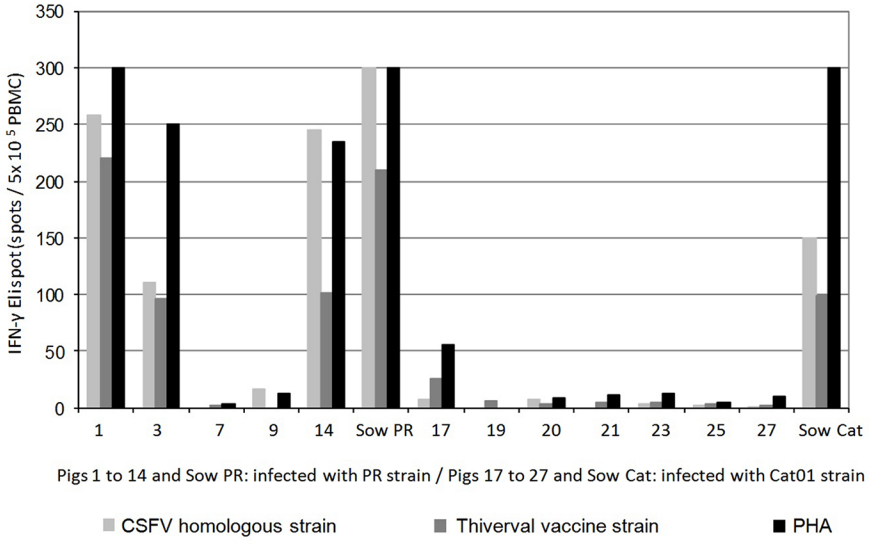

IFN-γ Elispot (spots / 5x 10 5 PBMC)
350
300
250
200
150
100
50
0
1
3
7
9
14
Sow PR
17
19
20
21
23
25
27
Sow Cat
Pigs 1 to 14 and Sow PR: infected with PR strain / Pigs 17 to 27 and Sow Cat: infected with Cat01 strain
CSFV homologous strain
Thiverval vaccine strain
PHA

Fig 4. Humoral and cellular immune response against CSFV infection. (a) Antibody response to E2 glycoprotein detected by ELISA (IDEXX) after infection (in blocking %). Values greater than 40% blocking were considered positive. (b) Neutralising antibody titres against PR (pigs 1 to 14 and sow PR) and Cat01 (pigs 16 to 28 and sow Cat) CSFV strains at 3 and 4 weeks p.i. (c) Lack of IFN- γ response by ELISPOT assay in CSFV postnatally persistently infected piglets and detection of effective response in immunocompetent pigs from the PR group at 4 weeks p.i. A total of $5x10^5$ PBMCs/well were plated in triplicates at 0.1 multiplicity of infection (MOI) of CSFV homologous strains: PR strain (samples from pigs 1 to 14 and sow PR) and Cat01 strain (samples from pigs 17 to 27 and sow Cat). Moreover, the samples were incubated in the presence of Thiverval strain at 0.01 MOI and phytohaemagglutinin (PHA) (10 μg/ml). Asterisk symbol indicates the sample from pig 28 analysed at 19 dpi.

doi:10.1371/journal.pone.0125692.g004

the 3 piglets (#1, 3 and 14) that cleared the PR virus were positive for E2-specific antibodies and virus-neutralising antibodies from 21 days p.i. onwards. The sows were also positive for both binding and neutralising antibodies (Fig 4A and 4B). Interestingly, the Cat01 virus induced a stronger antibody response than the PR virus in the sows, which again might be related to the higher replication rate of the Cat01 virus.

CSFV-specific IFN-γ-producing cells were lacking in the piglets incapable of clearing CSFV

PBMCs from all of the piglets and from the two sows were analysed for virus-specific and-non-specific IFN-γ responses by ELISPOT assay at four weeks p.i. Very few IFN-γ-producing cells were found upon CSFV and PHA stimulation of PBMCs from all 7 of the surviving piglets in the Cat01-infected group and from the two surviving piglets from the PR-infected group that had not cleared the virus (Fig 4C). In contrast, a large proportion of CSFV-specific and PHA-responsive IFN-γ-producing cells were detected in the PBMCs from the three PR-infected piglets that had seroconverted and from the two sows (Fig 4C). In conclusion, these data altogether showed that early postnatal infection of piglets with low virulence CSFV could result in virus persistence due to a lack of B- and T-cell responses. The lack of responsiveness of PBMCs to PHA suggested that the persistently infected piglets were immunosuppressed.

All of the piglets responded to CSFV infection with IFN-α production

After having shown that the adaptive immune response of the persistently infected piglets was clearly impaired, we wondered whether their innate immune responses were also affected. Thus, serum IFN-α levels in the persistent and immunocompetent piglets and the sows were analysed by ELISA at 7, 14, 21 and 42 dpi. Considerable but variable levels of serum IFN-α were found in all of the animals mainly at day 7 p.i. and in some of the PR virus persistently infected piglets at 14 and 21 d.p.i. (Fig 5). The mean IFN-α content was significantly higher in the piglets infected with the PR strain than in the piglets infected with the Cat01 strain ($P = 0.0046$), although viraemia was lower. Interestingly, the three immunocompetent piglets responded with IFN-α similar to that of the persistently infected animals. Interestingly, the two sows exhibited different IFN-α patterns than the piglets, with the Cat01-infected sow having a higher concentration of IFN-α in the serum than the sow infected with the PR strain (Fig 5). These results showed that the IFN-α response was not affected in the persistently infected piglets, as opposed to the adaptive humoral and cellular immune responses.

The bone marrow $6D10^+$ immature granulocytes were increased and targeted by CSFV in persistently infected piglets

The data suggested that immunosuppression occurred at a certain point in the adaptive immune response. Therefore, we wondered whether myeloid bone marrow cells were affected by viral persistence. The fate and infection of the myeloid BMHCs were determined at necropsy in 6-week-old (i) non-infected piglets, (ii) seropositive piglets infected with the PR virus (pigs 1

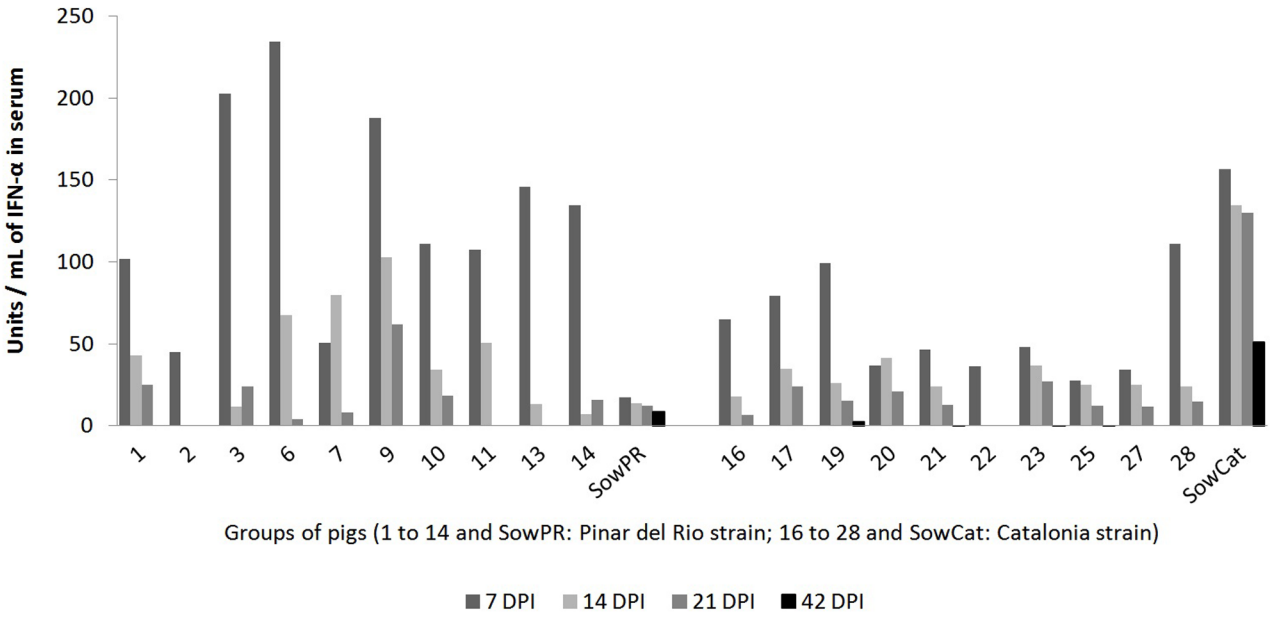


Fig 5. Serum IFN-α levels in the piglets infected with the CSFV strains PR or Cat01. Serum IFN-α levels in the piglets infected with the CSFV strains PR or Cat01 at four different times p.i. (7, 14, 21 and 42 days post-infection (DPI)). IFN-α levels were statistically significantly higher in piglets infected with the PR strain, compared with the levels in the piglets infected with the Cat01 strain ($P<0.05$).

doi:10.1371/journal.pone.0125692.g005

and 3) and (iii) seronegative piglets infected with the Cat01 virus (pigs 19 and 23). The percentages of myeloid ($CD172a^+$, $CD163^+$) cells were clearly increased in both the PR and Cat01 virus-infected piglets (Fig 6). In contrast, the increases in $SLAII^+$ and $CD172a^+/SLAII^+$ cells were more prominent in the immunocompetent piglets infected with the PR strain. Consistent with viral persistence, the percentage of CSFV-positive BMHCs overall was three times higher in the pigs infected with Cat01, compared with the PR virus-infected piglets that had seroconverted to the infection (Fig 6A and 6B). Accordingly, a higher percentage of immature granulocytes ($6D10^+$) and $CSFV^+/6D10^+$ cells was found in the BMHCs from the Cat01 virus-infected piglets (Fig 6C and 6D). This finding was confirmed by qRT-PCR with $6D10^+$ sorted cells (Fig 6E).

CSFV induced high IL-10 production in PBMCs from persistently infected piglets

To determine the IL-10 levels in the sera of the persistent and immunocompetent piglets and of the dams, serum samples were analysed by ELISA at 7, 14, 21 and 42 dpi. IL-10 was not detected in any of the serum samples analysed (data not shown). In parallel, the IL-10 production of stimulated PBMCs from persistently infected piglets was analysed. PBMCs were collected from 6-week-old piglets that were either persistently infected with CSFV Cat01 (pigs 17, 19, 23, 25 and 27) or that were left uninfected as control (pigs 32 and 33). PBMCs from persistently infected piglets produced IL-10, while PBMCs from non-infected piglets did not (Fig 7). Importantly, PBMCs from persistently infected and from uninfected pigs responded to IL-10 production with PHA mitogen stimulation. None of the pigs had detectable levels of IL-10 in the supernatant of sham-stimulated cells (Fig 7).

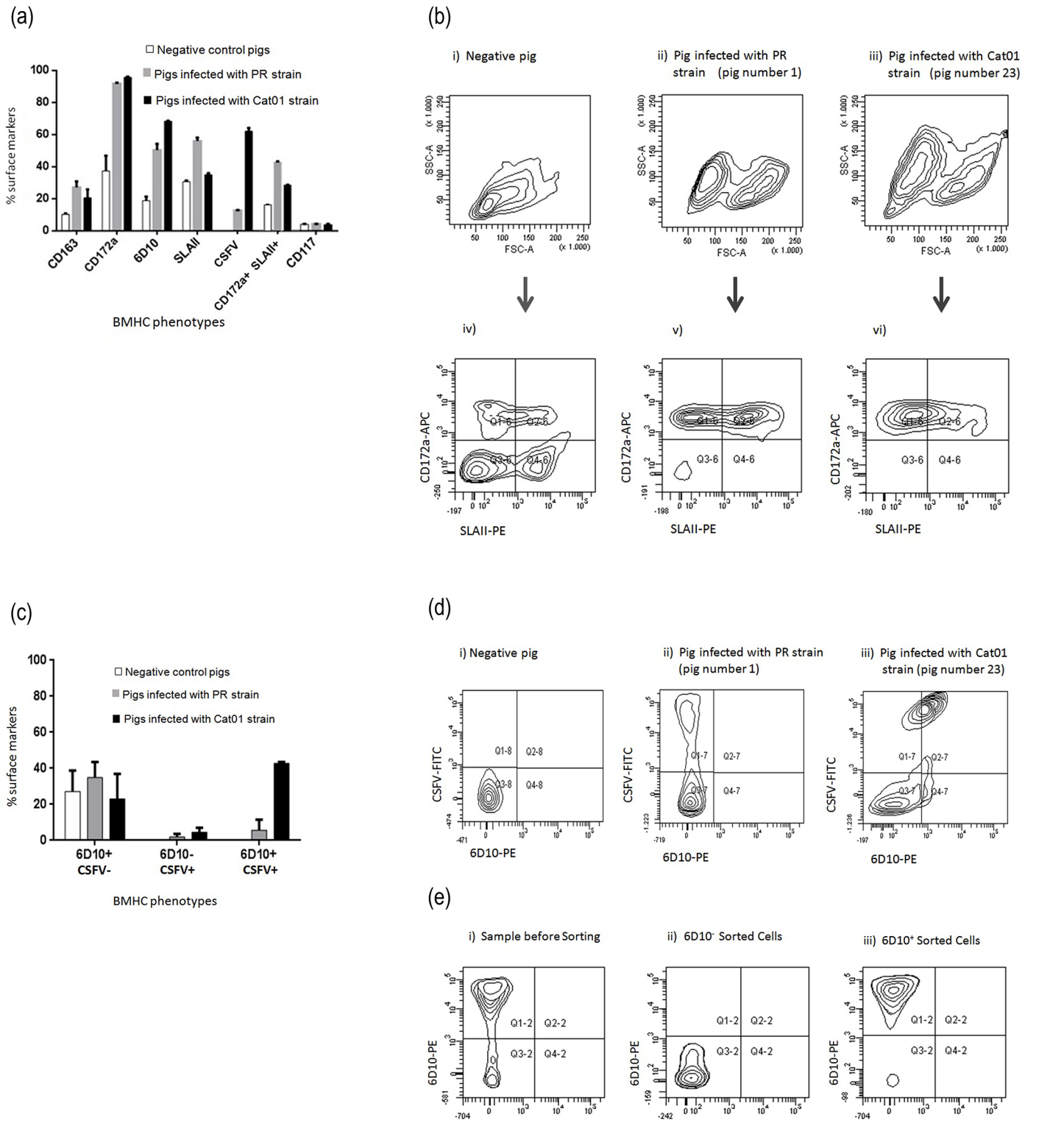


Fig 6. Expression of cellular markers and comparative phenotypes of BMHCs from PR, Cat01 and non-infected pigs. (a) Expression of 5 different surface markers and CSFV in BMHCs from infected and non-infected pigs. (b) Comparative phenotypes of BMHCs obtained from non-infected pigs (i, iv) and pigs infected with the PR (ii, v) and Cat01 (iii, vi) strains. (i, ii and iii): forward scatter (relative cell size, x-axis) and side scatter (relative granularity, y-axis). (iv, v and vi): double labelling immunofluorescence image of the BMHCs from (i, ii and iii), in terms of CD172a common myeloid marker (y-axis) and SLA-II (x-axis). (c) Percentage of granulocyte 6D10^{+} and 6D10^{+}_CSFV^{+} double-positive cells in the BMHCs. (d) Double labelling immunofluorescence image of the BMHCs from one non-infected and two infected pigs, in terms of 6D10, immature granulocytes marker (x-axis) and CSFV (y-axis). (e) Sorting of 6D10^{+} BMHCs. These experiments were repeated twice under the same conditions.

doi:10.1371/journal.pone.0125692.g006

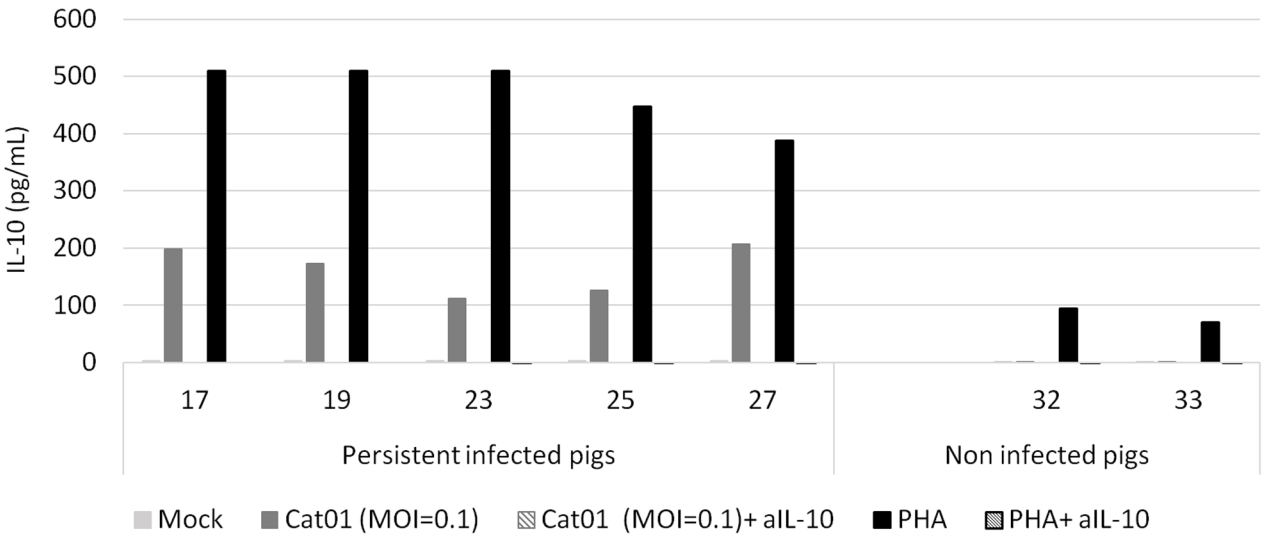


Fig 7. IL-10 levels detected by ELISA in the supernatants of PBMCs from CSFV-infected piglets developing the persistent form of CSF. PBMCs stimulated with Mock; CSFV Cat01 strain (MOI = 0.1) or PHA (1μg/ml) in the presence or absence of the anti-IL-10 neutralising Ab (aIL-10). PBMCs from pigs developing persistent CSF disease at 6 weeks post-infection (pigs 17, 19, 23, 25 and 27) and pigs 32 and 33 (non-infected pigs).

doi:10.1371/journal.pone.0125692.g007

Defective CSFV-specific IFN-γ production by PBMCs from persistently infected piglets is not solely due to IL-10

Because IL-10 production could be stimulated with virus only in the PBMCs from the persistently infected piglets (Fig 7) and because there were no IFN-γ producing cells in the stimulated

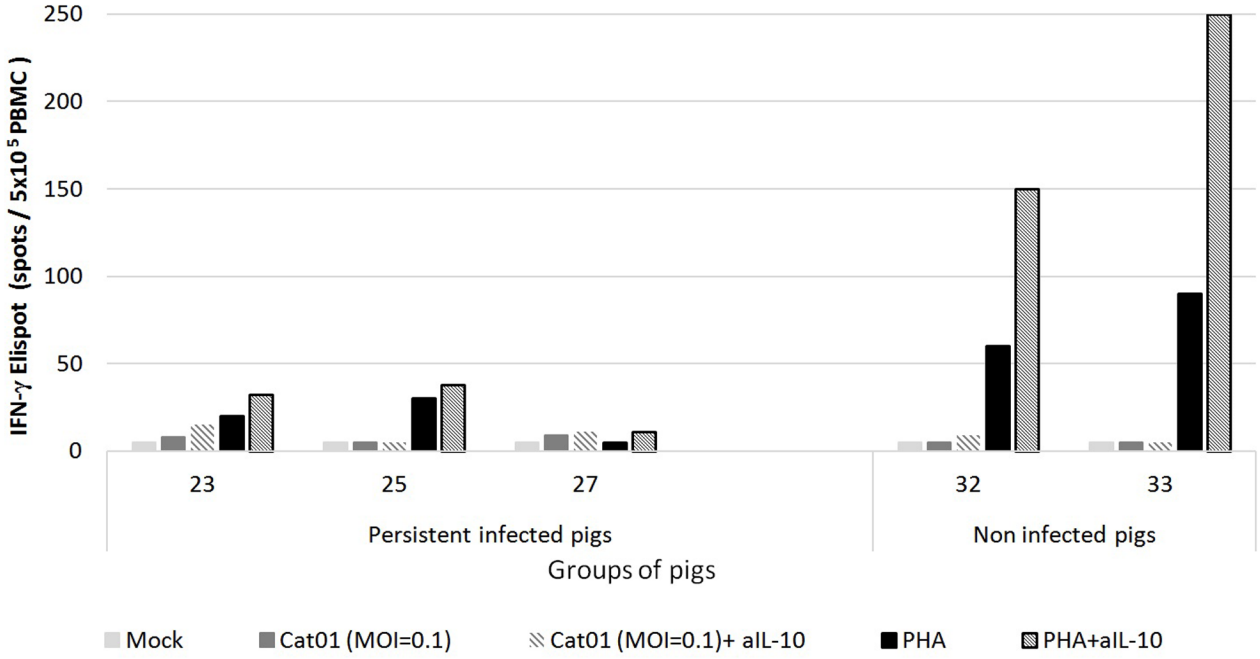


Fig 8. IFN-γ production in the presence or absence of the anti-IL-10 neutralising Ab in PBMCs from piglets persistently infected with CSFV. PBMCs from pigs developing persistent CSF at 6 weeks post-infection (pigs 23, 25 and 27) and pigs 32 and 33 (non-infected pigs) were stimulated with mock, with CSFV strain Cat01 (MOI = 0.1) or with PHA (10μg/ml) in the presence or absence of anti-IL-10 neutralising Ab (aIL-10).

doi:10.1371/journal.pone.0125692.g008

PBMCs from these animals (Fig 4C), the number of IFN-γ-producing cells upon CSFV stimulation was determined with or without the presence of the anti-IL-10 neutralising antibody (Ab). Additionally, the addition of anti-IL-10 Ab alone to PBMC cultures in the absence of antigen had no effect (data not shown). However, despite the anti-IL-10 Ab in the PBMC culture from persistently infected piglets, the response of the IFN-γ-producing cells was not restored (Fig 8). Nevertheless, IFN-γ production by PBMCs from non-infected piglets was significantly increased in the presence of anti-IL-10 neutralising Ab.

Discussion

Despite intensive vaccination programs in some endemic countries, CSF has not been eradicated, due to failures in the responses to vaccination associated with poor handling of the vaccine, among other issues [4,5,7,16,17]. Moreover, the virus tends to evolve towards low virulence variants that circulate and persist in the pig population in association with common porcine infectious diseases [4,5,15]. CSFV persistence in offspring after trans-placental infection during mid-gestation has been well documented, contrary to postnatal infection [reviewed in [26]] and vaccination with CSFV live attenuated vaccine before the ingestion of colostrum, which conferred good protection against CSF in newborn pigs [17,45]. Here, we show that persistently infected piglets could be generated following infection with two different CSFV field isolates (of low and moderate virulence) on the day of their birth. These piglets remained healthy for several weeks, without any specific immunological response to CSFV and with high virus loads in the blood, organs and body secretions. In this context, persistently infected pigs might play an important role in virus dissemination. Some of these piglets developed fever peaks during the first 15 days post-infection and non-specific clinical signs and lesions, mostly associated with omphalophlebitis and secondary bacterial infections (confirmed at necropsy) which resulted in death or required euthanasia. On the other hand, severe thymus atrophy was the main gross pathological lesion (data not shown). Interestingly, all of the persistently infected pigs were CSFV RNA positive in the thymus. In contrast, a reduction in the number of PBMCs from these animals was also observed (data not shown). Thymus atrophy has been described in previous studies after CSFV congenital persistent infection with the Bergen strain [20,25]. However, thymus atrophy is not an exclusive finding of this form of the disease because it has also been described in the CSF acute form, wherein massive lymphoid depletion was also found due to lymphocyte apoptosis in atrophied thymuses [46]. In addition, previous studies have shown B-lymphocyte, helper T-cell and cytotoxic T-cell depletion during CSF acute disease [47]. Finally, thymus atrophy can also be caused by other viral infections, such as porcine circovirus-2 [48], porcine reproductive and respiratory syndrome virus [49,50] and influenza A virus [51], all of which are related to lymphoid depletion.

Owing to omphalophlebitis (a clinical sign not related to CSFV infection) developing in the pigs inoculated with the PR strain, the mortality was higher in this group. Nevertheless, the viral load in these piglets was consistently lower than the viral load detected after Cat01 strain infection. Considering as one virulence criterion the CSFV replication levels [12,32,52,53], our results might indicate lower virulence of the PR strain. Indeed, the three piglets inoculated with the PR virus became immunocompetent, clearing the virus from sera after three weeks post-infection, whereas none of the Cat01-infected piglets seroconverted. Furthermore, the sows from both groups were infected when in contact with their offspring. However, only the sow in the Cat01 group was CSFV-positive from rectal and nasal swabs during the last four weeks of the trial, being an asymptomatic carrier of the virus despite the neutralising antibody response.

Viral detection in some organs from the immunocompetent piglets was remarkable, and it might have been due to the low levels in the neutralising antibody titres detected from the

fourth to the sixth weeks post-infection. Previous studies have shown that vaccine-challenged pigs that were not fully protected were CSFV positive in the tonsils after viral challenge, despite having some neutralising antibody response [7,54–56]. In contrast, the constant exposure of the immunocompetent piglets to the virus from the remainder of the infected litter likely influenced the neutralising antibodies' consumption, thus avoiding the anamnestic effect of the humoral response [57].

Thus, the virulence of CSFV might be a critical factor in determining the outcome of early post-natal infection, considering the capacity of the Cat01 moderate virulence strain to induce persistent disease. Nevertheless, it is not known whether this outcome could also apply to trans-placental infections at mid-gestation. The proportion of persistently infected piglets could likely vary between reports with different viruses [13,20,21].

Compared to the adaptive immune response, the innate immune response to the virus, as measured by type I IFN-α in the serum, was not impaired in the persistently infected piglets. At seven days post-infection, when the viral loads were similar in all of the piglets, the IFN-α levels were comparable in the pigs that had seroconverted and in the persistently infected piglets (Fig 5). Similarly, serum IFN-α levels were measured in Cat01 virus-infected 10-week-old immunocompetent pigs in a separate study [32]. Surprisingly, the persistently infected piglets inoculated with the PR strain had overall significantly higher serum IFN-α levels than the Cat01-infected pigs, although their virus load was lower. This situation was different from acute CSFV infections, in which the serum IFN-α levels were consistently higher with higher virus titres in the circulation [58,59]. Additionally, it was recently demonstrated that E^{rns} impaired pDC-mediated IFN-α secretion in response to CSFV infection [60]. Different efficiencies of the E^{rns} of PR *versus* Cat01 in terms of pDC inhibition might account for the discrepancy observed here.

Four weeks after infection, the PBMCs from the persistently infected and seronegative piglets were unresponsive to both specific CSFV and non-specific PHA stimulation, in terms of IFN-γ producing cells. On the contrary, the three piglets that cleared the virus and seroconverted responded equally well to CSFV and PHA stimulation. PHA and Concanavalin A have been used previously to characterise the general functionality of the cellular immune response in the context of acute and persistent CSFV infections. After acute CSFV infections, the responses of PBMC to mitogens were also partly or completely impaired, indicating general transient immunosuppression [32,42,61]. In contrast, with trans-placental infections, immunotolerant pigs showed a normal lymphocyte response to PHA, indeed suggesting specific immunotolerance, rather than general immunosuppression [21,62,63].

IL-10 is a well-characterised immunosuppressive cytokine that inhibits a broad spectrum of immune responses, including the suppression of stimulatory cytokine production, T-cell proliferation, and B-cell responses [64–68]. A previous study provided the ability of a highly virulent CSFV strain to induce detectable levels of IL-10 in the serum of pigs developing the acute form at 7 days post-infection [69]. However, IL-10 was not detected in any of the serum samples analysed from the persistently infected piglets. This finding could suggest that different roles are played by this cytokine in the two CSF forms. Nevertheless, the CSFV- and PHA-stimulated PBMCs from persistently infected piglets produced high levels of IL-10. In this regard, hepatitis C virus (HCV), which is also a member of the *Flaviviridae* family, induces the production of IL-10 by cells of the innate immune system, principally by monocytes. This response has been associated with the suppression of the adaptive immune response in HCV persistently infected patients [66,70]. In this context, CSFV behaves in a similar manner to HCV, avoiding clearance by the immune system of the host. Moreover, the addition of neutralising IL-10 Ab did not restore the number of IFN-γ-producing cells in PBMCs from persistently infected piglets. Therefore, other mechanisms might also be involved in the general suppression of the T-cell

response upon CSFV and mitogen activation. Previous studies have shown a drastic decrease in the T cell populations due to lymphocyte apoptosis during the acute form [59], and it could be another factor preventing the activation of the adaptive immune response in persistently infected animals, which also showed PBMC depletion.

Interestingly, the percentage of CD172a+/SLAII+ cells found in the BMHCs from persistent infected piglets was increased by 20% over the value found in naive pigs. Previous studies with the CSF acute form have shown that, after severe immunosuppression, SLAII+ cell populations decreased considerably [71]. Similarly, immature granulocytes, specifically 6D10+ cells [72], were the predominant cell population in these pigs, similar to the cellular profiles found after the CSF acute form [46,71]. These cells were infected with a high concentration of viral RNA, promoting virus spread in the persistently infected animals.

To the best of our knowledge, this is the first comprehensive study showing the ability of CSFV to generate viral persistence after early postnatal infection, which has not been described with other members of the *Pestivirus* genus either. In endemic areas where serological methods are used without virus detection in CSFV surveillance, postnatally persistently infected piglets would remain unnoticed. In addition to the epidemiological and economic significance of persistent CSFV infections, this model will be useful for understanding the mechanisms of viral persistence.

Acknowledgments

We thank Marta Muñoz, Valentí Rosell and Iván Cordón for their help in the laboratory and in the animal facilities.

Author Contributions

Conceived and designed the experiments: FE AS NR LG. Performed the experiments: SM RR LC MD LG. Analyzed the data: LF MD NR AS LG. Contributed reagents/materials/analysis tools: LG LP MF MM. Wrote the paper: SM NR LG.

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Chapter 4

Study II: *Efficacy of a live attenuated vaccine in classical swine fever virus postnatally persistently infected pigs.*

Vet Res. (2015); 46:78-87

Muñoz-González et al. Veterinary Research
DOI 10.1186/s13567-015-0209-9

VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Efficacy of a live attenuated vaccine in classical swine fever virus postnatally persistently infected pigs

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Abstract

Classical swine fever (CSF) causes major losses in pig farming, with various degrees of disease severity. Efficient live attenuated vaccines against classical swine fever virus (CSFV) are used routinely in endemic countries. However, despite intensive vaccination programs in these areas for more than 20 years, CSF has not been eradicated. Molecular epidemiology studies in these regions suggests that the virus circulating in the field has evolved under the positive selection pressure exerted by the immune response to the vaccine, leading to new attenuated viral variants. Recent work by our group demonstrated that a high proportion of persistently infected piglets can be generated by early postnatal infection with low and moderately virulent CSFV strains. Here, we studied the immune response to a hog cholera lapinised virus vaccine (HCLV), C-strain, in six-week-old persistently infected pigs following post-natal infection. CSFV-negative pigs were vaccinated as controls. The humoral and interferon gamma responses as well as the CSFV RNA loads were monitored for 21 days post-vaccination. No vaccine viral RNA was detected in the serum samples and tonsils from CSFV postnatally persistently infected pigs for 21 days post-vaccination. Furthermore, no E2-specific antibody response or neutralising antibody titres were shown in CSFV persistently infected vaccinated animals. Likewise, no of IFN-gamma producing cell response against CSFV or PHA was observed. To our knowledge, this is the first report demonstrating the absence of a response to vaccination in CSFV persistently infected pigs.

Introduction

Classical swine fever (CSF) is one of the most devastating diseases for the pig industry throughout the world affecting both domestic pigs and wild boars [1,2]. It is endemic in Asia, areas of Central and South America and in many Eastern European countries [3,4] with sporadic occurrence in Western Europe. The CSF virus (CSFV), the etiological agent of CSF, is an icosahedral and enveloped positive stranded RNA virus that, together with bovine viral diarrhoea virus (BVDV) and border diseases virus, belongs to the *Pestivirus* genus of the *Flaviviridae* family [5].

As with many other diseases affecting livestock, the most efficient vaccines currently available against CSFV are live attenuated and were developed over 50 years ago [4,6]. The HCLV vaccine was developed in China, by passage in rabbits. Because of its high efficacy and safety, the HCLV vaccine was introduced into many other countries and became known as the Chinese vaccine strain (C-strain) [7]. Immune responses elicited by these vaccines do not allow differentiating infected from vaccinated animals (DIVA). However, live attenuated vaccines are still used in endemic countries. Furthermore, although intensive control programs have been implemented for over 20 years, the virus is still circulating in these regions; therefore, the disease has not been eradicated. Various degrees of CSF severity can be observed, ranging from acute to chronic or subclinical forms.

Recent molecular epidemiology studies from some endemic countries suggest that the virus circulating in the field has evolved under the positive selection pressure exerted by the immune response to the vaccine, leading to new attenuated viral variants that reproduce milder forms of CSF disease [3,8]. On the other hand, moderate virulence strains were found throughout Europe,

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as in the case of the Catalonia strain responsible for the 2001–2002 CSFV outbreak in Spain that caused mild and nonspecific clinical signs of CSF, an outbreak that was controlled using a non-vaccination policy by stamping-out strategy [9,10].

Recent work by our group demonstrated that persistently infected piglets can be generated by early postnatal infection with CSFV of low and moderate virulence [11]. For six weeks after postnatal infection, most of the piglets remained clinically healthy, despite persistent high virus titres in the serum, tissues, nasal and rectal swabs. Notably, these animals were unable to mount any detectable humoral and cellular immune response. At necropsy, the most prominent gross pathological lesion was severe thymus atrophy. Contrary to persistent infection, animals developing the chronic form of CSF are able to generate a specific immune response against the virus, mainly an antibody response [4,12,13].

Considering the CSF epidemiological situation in endemic areas, where low virulence strains are prevalent [3,4,8,14,15] and the epidemiological implications that persistently infected animals can exert in the eradication of the disease (revised in [16,17]) we studied the immune response to a live attenuated CSFV vaccine in six-week-old CSFV postnatally persistently infected pigs. Interestingly, none of the vaccinated persistently infected piglets developed a detectable immune response after vaccination. In addition, a complete lack of viral RNA was detected in the serum samples and tonsils from CSFV postnatally persistently infected pigs during the 21 days post-vaccination (dpv). These results have important implications for vaccine control programs in the endemic context.

Materials and methods

Cells and viruses

PK-15 cells (ATCC CCL 33) were cultured in DMEM medium, supplemented with 10% pestivirus-free foetal bovine serum (FBS) at 37 °C in 5% CO2. The cells were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 48 h later. Peroxidase-linked assay (PLA) [18] was used for viral titration following the statistical methods described by Reed and Muench [19]. The Catalonia 01 strain belongs to the CSFV 2.3 genogroup [3], was isolated from CSF Spanish epizootic in 2000–2001 [9,10] and was the strain that originated the persistently infected pigs used in this study [11]. The HCLV vaccine (C-strain) belongs to CSFV 1.1 genogroup and was used in Spain in the 1980s for CSF control. This vaccine has 100% homology with the Z46258 strain into the N^{pro} region [7]. Finally, the Thiverval vaccine strain (provided by Pasteur Institute, Romania) was used as the stimulus in the Elispot assay for detecting CSFV-specific interferon-gamma (IFN-γ) producing cells. This strain belongs to the CSFV 1.1 genogroup [20].

Experimental design

To elucidate the immune response induced by the HCLV vaccine (C-strain) in postnatally CSFV persistently infected pigs, two groups with four domestic pigs each at six weeks old were vaccinated with a pig dose (equivalent with 100 Protective Doses (PD) by intramuscular injection in the neck. Group 1 included four CSFV postnatally persistently infected pigs born in a biosafety level 3 (BSL3) animal facility (CReSA, Barcelona, Spain) [11], numbered from 1 to 4. These pigs, which had been intranasally infected in the first 8 h after birth with the CSFV Catalonia 01 strain, were viraemic and apparently healthy at six weeks old (study time), although they lacked a humoral response [11].

The second group (Group 2), housed in an independent isolation unit at the BSL-3 facility of CReSA, consisted of four pigs (numbered 5–8) from a sow of the same origin as Group 1. Group 2 was free from *Pestivirus*, porcine circovirus type 2 and porcine reproductive respiratory syndrome virus. Both groups had an average weight of 12.6 kg per pig.

Serum, whole blood samples, nasal and rectal swabs were taken at 0, 4, 8, 13, 15 and 21 dpv. The tonsils were collected at the time of necropsy (21 dpv). The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB) according to existing national and European regulations.

Clinical signs evaluation after vaccination

A trained veterinarian recorded rectal temperature and clinical signs daily in a blinded manner. The pigs were scored daily as follows: one point: pyrexia; two points: pyrexia + mild clinical signs; three points: severe clinical signs; and four points: death. After euthanasia (with intravenous pentobarbital sodium injection), animals were subjected to an exhaustive necropsy in which pathological signs in different organs and tissues were evaluated.

PBMCs collection and performing the ELISPOT assay for the detection of CSFV-specific IFN-γ producing cells

Blood collected in 5 mM EDTA at 15 dpv was used to obtain peripheral blood mononuclear cells (PBMCs) by density-gradient centrifugation with Histopaque 1077 (Sigma). The total number of recovered live PBMCs was obtained by staining with trypan blue [21]. The Elispot assay to detect CSFV-specific IFN-γ cells was performed as previously described by Tarradas et al. [22]. Briefly, 5×10^5 live PBMC/well were plated in duplicate at 0.1 multiplicity of infection (MOI) of CSFV Catalonia or Thiverval strain at 0.01 MOI. As controls, duplicate of cells were incubated in the presence of mock-stimulated wells and Phytohaemagglutinin (PHA) (10 μg/mL). The counts of spots in the media for mock-stimulated wells

were considered as the baseline for the calculation of antigen-specific frequencies of IFN-γ producing cells.

CSFV neutralising and E2 specific antibodies detection

Serum samples taken at 0, 4, 8, 13, 15 and 21 dpv were tested by performing a neutralisation peroxidase-linked assay (NPLA) [23], and titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the Catalonia strain in 50% of the culture replicates. The sera were also tested in the CSFV specific E2 ELISA (HerdChek CSFV Ab, IDEXX); when the blocking percentage ≥40%, the samples were considered positive, following the manufacturer's recommendations.

Detection of CSFV RNA

The RNA was extracted from all of the samples using the viral RNA isolation kit Nucleospin II according to the manufacturer's instructions (Macherey-Nagel). In all cases, an initial volume of 150 μL was used to obtain a final volume of 50 μL of RNA, which was stored at −80 °C. The presence of CSFV Catalonia strain RNA was analysed by RT-qPCR [24]. Positive results were considered for threshold cycle values (CT) equal or less than 42. Samples in which fluorescence was undetectable were considered negative. Furthermore, the presence of vaccine virus (C-strain) RNA was detected by RT-qPCR [7].

ELISA for IFN-α detection in serum samples from the persistently infected-vaccinated group

To assess the innate immune response, serum IFN-α levels in the persistently infected-vaccinated pigs were evaluated in serum samples at 0, 4, 8, 13 and 21 dpv. Anti-IFN-α monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) were used in an ELISA assay to detect IFN-α in serum samples [22,25-27]. The cut-off value was calculated as the average optical density of negative controls (blank and negative serums before CSFV infection) plus three standard deviations. Cytokine concentrations in the serum were determined using a regression line built with the optical densities of the cytokine standards used in the test.

Results

Clinical signs after vaccination

After 21 dpv, no clinical signs were detected in vaccinated pigs from Group 2, and rectal temperatures remained within the established normal range until the end of the experiment, (Figure 1). Conversely, the vaccinated CSFV persistently infected pigs showed varying rectal temperature values; one of the pigs showed fever from day 2 until day 15 post-vaccination (pig #4), and had to be euthanized at 16 dpv after developing hypothermia, as well as severe clinical signs (diarrhoea, mild tremors, polyarthritis). Two pigs (#2 and #3) developed fevers starting at day 17 and 21 post-vaccination, respectively, in the absence of other clinical signs. Finally, pig #1 did not have an increase in rectal temperature at any point in the study with a healthy clinical status during the trial (Figures 1 and 2).

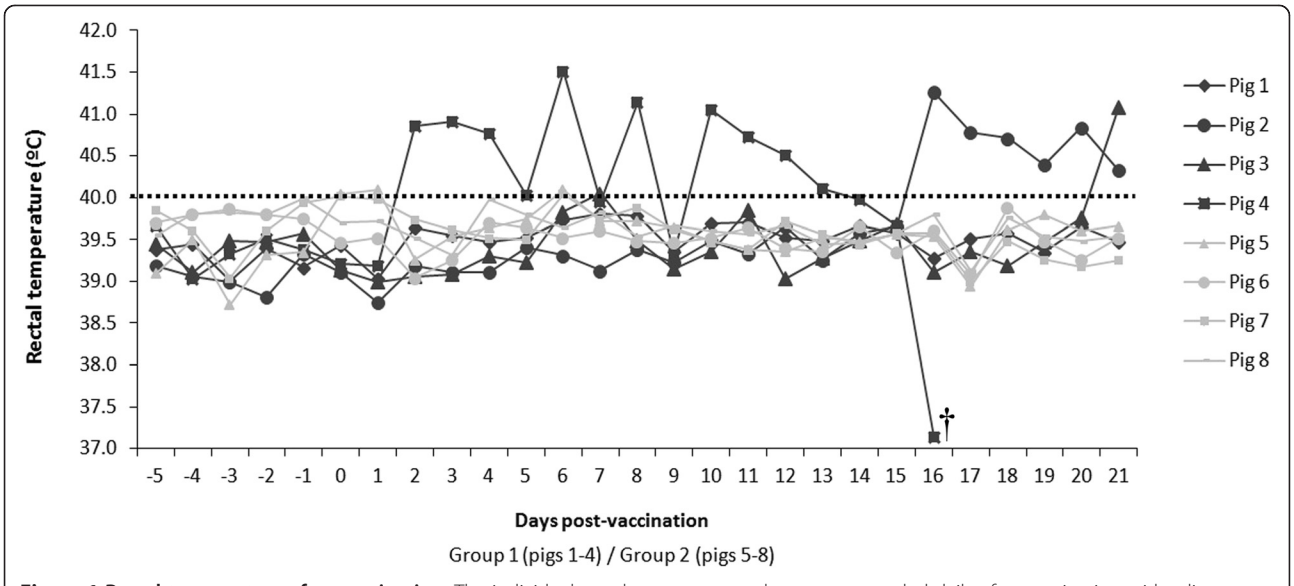

Figure 1 Rectal temperature after vaccination. The individual rectal temperature values were recorded daily after vaccination with a live attenuated vaccine (C-strain). Temperatures greater than 40 °C were considered fevers (indicated with a black dotted bar). CSFV persistently infected-vaccinated pigs (Group 1: numbers 1 to 4) and Pestivirus-Free vaccinated pigs (Group 2: numbers 5 to 8). † The pig was euthanized at 16 dpv.

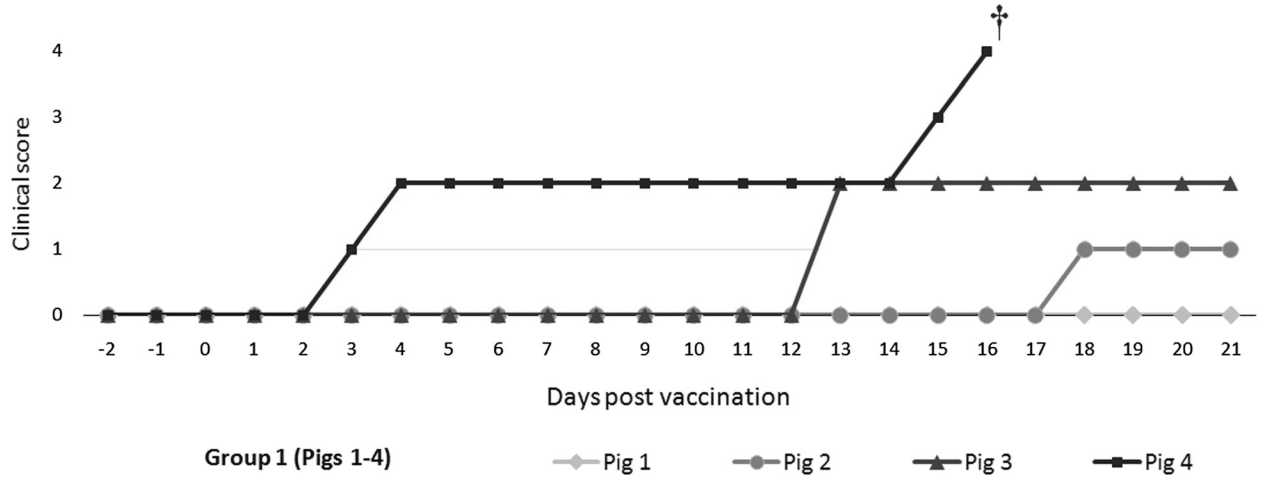

Figure 2 Clinical score values in CSFV persistently infected-vaccinated pigs. The individual clinical signs were recorded daily after vaccination until 21 days post-vaccination. The scores are defined in Section 2. † The pig was euthanized at 16 dpv.

Complete lack of response of the CSFV-specific IFN-γ producing cells from the persistently infected-vaccinated group

The ELISPOT assay results for the detection of IFN-γ in PBMC from persistently infected pigs (Group 1) showed a complete lack of response to stimulation against CSFV (MOI = 0.1 and 0.01) and PHA after 15 dpv (Figure 3). On the contrary, PBMC from vaccinated pigs in Group 2 showed a specific IFN-γ-producing cell response against CSFV and (PHA) stimuli (Figure 3).

Absence of E2-specific antibodies and neutralising activity after vaccination of the persistently infected pigs

To evaluate the induction of CSFV-specific antibodies, serum samples were analysed at different times after vaccination. All vaccinated pigs from Group 2 showed E2-specific antibodies response detected by ELISA from 15 to 21 dpv (Figure 4A). Likewise, neutralising antibody titres were detected at 15 and 21 dpv (Figure 4B). In contrast, an absence of antibody response, in terms of E2-specific antibodies and neutralising titres, was found in all CSFV persistently infected-vaccinated pigs (Group 1) during the entire experiment (Figures 4A and B).

CSFV RNA detection in serum, nasal and rectal swabs samples after vaccination

CSFV Catalonia strain-RNA was detectable in all of the samples analysed from postnatally persistently infected animals (Group 1) before vaccination until the end of the trial. A high level of Catalonia strain RNA was detected in

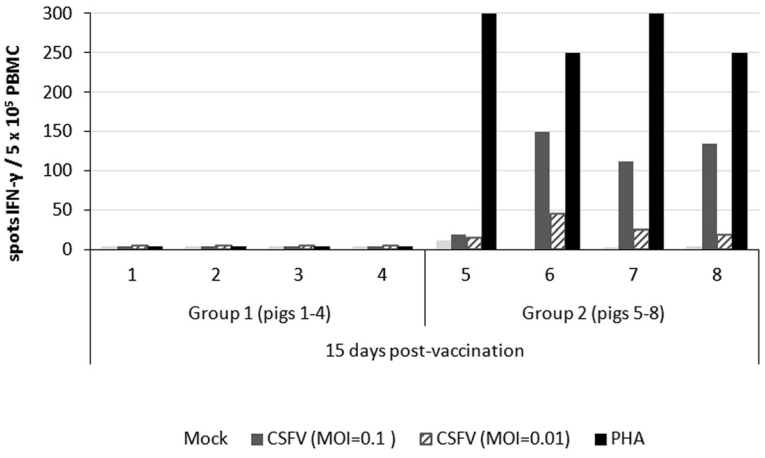

Figure 3 CSFV-specific IFN-γ producing cells at 15 dpv. CSFV persistently infected-vaccinated pigs (Group 1: numbers 1 to 4) and Pestivirus-Free vaccinated pigs (Group 2: numbers 5 to 8). Induction of CSFV-specific IFN-γ producing cells against different stimuli: mock, CSFV (MOI = 0.1 and MOI = 0.01, respectively) and PHA.

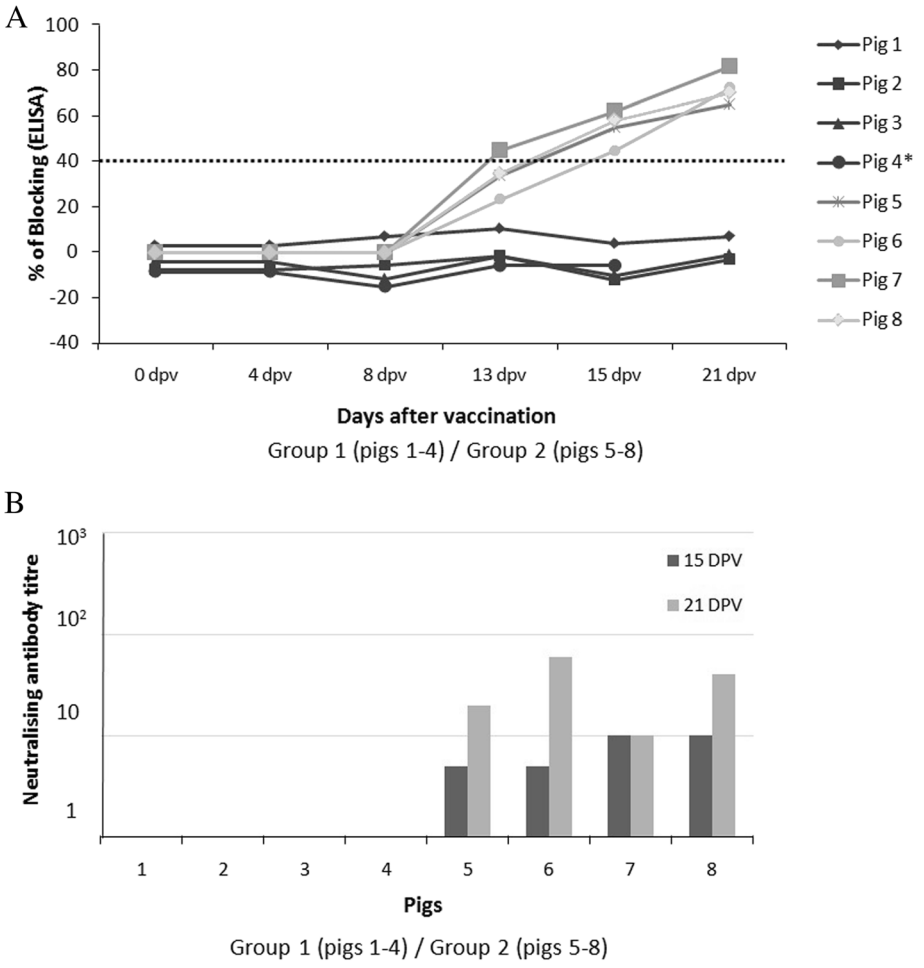

Figure 4 E2-specific antibody detection and neutralising activity during 21 days post-vaccination. CSFV persistently infected-vaccinated pigs (Group 1: numbers 1 to 4) and Pestivirus-Free vaccinated pigs (Group 2: numbers 5 to 8). **A**) Antibody response against the E2 glycoprotein detected by ELISA (in blocking %) at 0, 4, 8, 13, 15 and 21 days post-vaccination. Values greater than 40% were considered positive (indicated by a black dotted bar). **B**) Neutralising antibodies titres at 15 and 21 dpv. * This animal was euthanized at 16 dpv.

serum samples throughout the study. Additionally, high amounts of Catalonia strain-RNA were detected in nasal and rectal excretions, as well as in the tonsils. There was a mostly constant amount of Catalonia strain RNA in serum samples and nasal swabs after one week post-vaccination (Figures 5A, B and C). In contrast, all vaccinated pigs from Group 2 were CSFV Catalonia strain-RNA negative throughout the experiment (data not shown).

Lack of vaccine virus RNA detection in postnatally persistently infected pigs

CSFV vaccine virus RNA was detected in all of the sera samples analysed at 4 and 8 dpv and also in the tonsils from vaccinated pigs in Group 2. By contrast, a lack of vaccine virus (C-strain) RNA was detected in all of the samples tested from postnatally persistently infected-vaccinated pigs, including in the tonsils (Group 1) (Table 1).

Lack of IFN-α detection in serum samples from persistently infected-vaccinated group

In general, an absence of IFN-α was found in all of the serum samples analysed both before (day 0) and after vaccination from persistently infected-vaccinated pigs (data not shown). In the case of vaccinated pigs from Group 2, positive values were found only at 4 dpv ranged up to 40 U/mL (Data not shown).

Discussion

It was shown over 40 years ago that congenital persistent infection is the most important cause by which CSFV is perpetuated in the domestic pig population [12]; however, little is known about the mechanisms involved. Currently, CSF causes significant losses in the pig farming industry worldwide, and despite the intensive control programs implemented in endemic countries for more than 20 years, the disease has not been eradicated in the world.

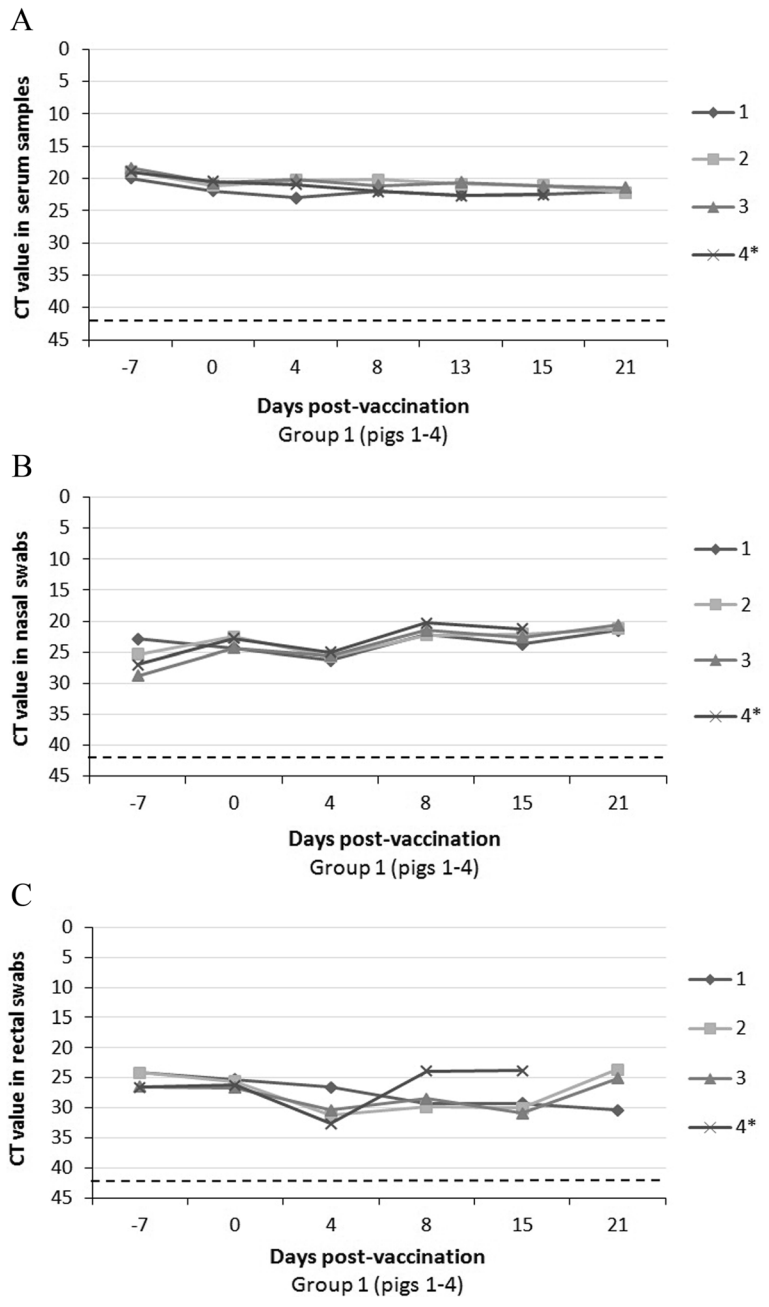

Figure 5 Catalonia strain RNA detection in serum, nasal and rectal swabs at different times after vaccination with Hoffman assay. CSFV persistently infected-vaccinated pigs (Group 1: numbers 1 to 4). **A**) Catalonia strain RNA detection in serum samples from CSFV persistently infected-vaccinated pigs at 0, 4, 8, 13, 15 and 21 dpv. **B**) Catalonia strain RNA detection in nasal swabs at 0, 4, 8, 15 and 21 dpv. **C**) Catalonia strain RNA detection in rectal swabs at 0, 4, 8, 15 and 21 dpv. A dotted bar indicates the detection limit of the technique above 42 CT. * This animal was euthanized at 16 dpv.

Considering the complex epidemiology in endemic areas, viral evolution studies conducted in some of these zones that suggest the origin and circulation of low to moderate virulence strains, and the role that these types of strains may play as one important risk factor for the development of CSFV persistence in pigs [3,8,14,15,28-31], the existence of CSFV persistently infected pigs in endemic countries cannot be ruled out [16,17].

Recent work by our group demonstrated that CSFV persistently infected piglets can be generated by early postnatal infection either with a low or a moderate virulence CSFV strains [11].

Interestingly, the postnatally persistently infected animals became viraemic, excreting high viral loads during the six weeks of the study, but were unable to generate either humoral or cellular immune responses against

Table 1 CSFV-vaccine RNA detection (C-strain) in serum at different times post-vaccination and in the tonsil samples

Group of pigs	N° of pig	CT value in serum samples (Days post-vaccination)						CT value in tonsil samples[a]
		0 dpv	4 dpv	8 dpv	13 dpv	15 dpv	21 dpv	
Persistently infected-vaccinated pigs (Group 1)	1	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
	2	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
	3	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
	4[b]	Undetected	Undetected	Undetected	Undetected	Undetected	-	Undetected
Pestivirus-Free vaccinated pigs (Group 2)	5	Undetected	39,34	39,68	40,41	Undetected	Undetected	26,27
	6	Undetected	40,10	40,20	40,05	Undetected	Undetected	26,07
	7	Undetected	39,11	39,12	Undetected	Undetected	Undetected	26,52
	8	Undetected	39,06	40,13	Undetected	Undetected	Undetected	28,32

[a] Tonsil samples collected after the necropsy (21 dpv).
[b] This animal was euthanized for ethical reasons at 16 dpv.

CSFV. In the present work, we studied the immune response to a live attenuated vaccine (HCLV C-strain) in six-week-old CSFV persistently infected pigs, considering that this type of vaccine is routinely used in many of the endemic countries [3,7,8,32]. A complete lack of the vaccine viral RNA was detected in the serum samples and tonsils from CSFV postnatally persistently infected pigs during 21 days post-vaccination. Previous studies have shown that the tonsil is considered a target for the vaccine virus replication and wherein the vaccine virus persists for more than 30 days post-vaccination [4,33]. Furthermore, a lack of response to E2-specific antibodies and an absence of neutralising antibody titres were shown in CSFV persistently infected-vaccinated animals. Likewise, an absence of IFN-γ-producing cell response against CSFV or PHA was also observed. Considering the role played by IFN-γ in the control of CSFV infection [22,34], persistently infected-vaccinated animals maintained an immunosuppressive state. Herein lies one of the main differences between persistently and chronic infected pigs; in animals suffering from chronic infection, a CSFV-specific immune response is generated. Furthermore, the immunological anergy developed in postnatally persistently infected pigs supported the previously results described [11].

On the other hand, CSFV exacerbates the IFN-α response, which is detected in the serum of infected pigs; this response has been hypothesised to be related to disease severity rather than to protective immune responses [22,35]. Notwithstanding, IFN-α values were undetectable in the sera from postnatally persistently infected pigs after vaccination.

The absence of a CSFV-specific immune response generated following immunisation could be related to the apparent absence of replication of the vaccine virus in the samples analysed from these animals. Immunological tolerance has been described for CSF when the virus is transmitted in utero, leading to a persistently infected farrow [13]. Understandably, the pigs of this study (postnatally persistently infected), besides their immunological anergy [11], also might be immunotolerant. The blocking of a CSFV-specific immune response generated by the host should be beneficial for virus replication [36], as is the case for the CSFV Catalonia strain that induced the persistence in these animals. Paradoxically, the vaccine virus did not follow this logic, given the lack of viral detection in samples analysed after the vaccination of postnatally persistently infected pigs. Perhaps, the high viral load generated by the strain that induced the persistent infection (Catalonia strain) may be preventing the vaccine virus replication in the target tissues, changing or avoiding its replication capacity. Previous studies conducted in cell cultures with BVDV demonstrated that cells acutely infected with this virus were protected from the second infection by a homologous BVDV [36]. Probably, this interference phenomenon previously described in BVDV and CSFV in in vitro assays, would explain the lack of vaccine virus (C-strain) RNA detection in the samples analysed after the vaccination of postnatally persistently infected pigs, since the RNA from the vaccine strain could not enter into the host cells [37,38]. On the contrary and as expected, all vaccinated pigs in Group 2 were able to mount efficient humoral and cellular responses between 15 and 21 dpv [4,9,16,22,34], which can be associated with the RNA vaccine virus detection in the tonsil from these pigs. The efficacy of the C-strain vaccine in preventing clinical CSF seems to approach 100%. Most data from previous studies indicate a very high level of protection against the development of clinical signs after challenge, irrespective of the challenge strains used, even if the strains are from different CSFV genotypes [34,39-43]. Additionally, the C-strain vaccine (1.1 genotype) induced a detectable humoral response to CSFV [4,16].

The persistently infected-vaccinated pigs maintained high viral loads only for the CSFV Catalonia strain in serum throughout the study, and also had high amounts of viral RNA of this strain in nasal and rectal excretions. There was a constant amount of viral RNA in serum

samples during the trial. The constant viraemia, as well as the high amount of viral excretion and the inability to induce a specific immune response, are common characteristics of *Pestivirus*-persistently infected animals [44]. However, contrary to CSFV postnatally persistently infected pigs, calves persistently infected with BVDV are able to develop immune responses against different pathogens, as well as respond to vaccination [45,46]. This fact suggests a different relationship between CSFV and its host despite both viruses being classified in the *Pestivirus* genus. The underlying factors for the development of clinical signs after a long period of incubation in persistently infected animals are unknown [47]. The late onset of disease has been described in this form, coursing with depression, anorexia, elevated temperature, conjunctivitis, dermatitis and locomotion disturbances [17]. Perhaps the vaccination could be a trigger for the disease progression, as would be the case for pig #4 (Figures 1 and 2). Before vaccination, this pig remained apparently healthy, but developed clinical signs (principally, fever peaks) two days post-vaccination (Figure 1). Our findings pose a better understanding of persistent infection with CSFV and also emphasise the need for diagnostic tools that can detect the existence of this CSF form in the field. Furthermore, our work supports once again that the vaccination strategies alone are not sufficient to eradicate the disease [4].

Considering their high levels of viral excretion, these animals can promote transmission to other healthy pigs in the herd, especially in situations where vaccination is not practiced or where the vaccination program is inefficient. Then, they can cause the short-cycle type of infection, which produces an acute fatal disease with high mortality [12]. Epidemiologically, it is not known how these pigs behave in the field, or the role they play in maintaining the infection in endemic countries, particularly important considering a population of 1% of persistently infected calves can maintain infection with BVDV in a farm [45]. There is still much more to know about CSFV postnatal persistent infection. Immunologically, we are only beginning to discover the mechanisms underlying the establishment of this form of disease; on a molecular level, it is known to be associated only with low-moderate virulence strains, but we still do not understand the reason why.

To our knowledge, this is the first report demonstrating the absence of a response to vaccination in pigs persistently infected with CSFV for 21 days post-vaccination. These results may have relevant implications for CSF control by vaccination. Likewise, these results might be of great value to understand the response to other persistent viral infections in humans and animals.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LG conceived and designed this study. LG, SM, MP, MM, MD and JAB performed the experiments. LG, SM, AS, and NR analysed data. LG, RR, MD contributed reagents/material/analysis tools. SM and LG wrote the paper. LG, AS, NR and MD critically read the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Valentí Rosell, Iván Cordón and David Solanes for their help in the animal facilities. This research was supported by grant AGL2012-38343 from Spanish government. S. M. had a predoctoral fellowship FI-DGR 2014 from AGAUR, Generalitat de Catalunya.

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Received: 28 January 2015 Accepted: 21 May 2015
Published online: 09 July 2015

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Chapter 5

Study III: *Classical swine fever virus vs. classical swine fever virus: The superinfection exclusion phenomenon in experimentally infected wild boar.*

PLoS One (2016); 11: e0149469

PLOS ONE

RESEARCH ARTICLE

Classical Swine Fever Virus vs. Classical Swine Fever Virus: The Superinfection Exclusion Phenomenon in Experimentally Infected Wild Boar

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Citation: Muñoz-González S, Pérez-Simó M, Colom-Cadena A, Cabezón O, Bohórquez JA, Rosell R, et al. (2016) Classical Swine Fever Virus vs. Classical Swine Fever Virus: The Superinfection Exclusion Phenomenon in Experimentally Infected Wild Boar. PLoS ONE 11(2): e0149469. doi:10.1371/journal.pone.0149469

Editor: Matthias Johannes Schnell, Thomas Jefferson University, UNITED STATES

Received: October 28, 2015

Accepted: January 31, 2016

Published: February 26, 2016

Data Availability Statement: All relevant data are within the paper.

Funding: This research was supported by grant AGL2012-38343 from the Spanish government. S.M. and A.C. had a predoctoral fellowship FI-DGR 2014 from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), Generalitat de Catalunya. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Abstract

Two groups with three wild boars each were used: Group A (animals 1 to 3) served as the control, and Group B (animals 4 to 6) was postnatally persistently infected with the Cat01 strain of CSFV (primary virus). The animals, six weeks old and clinically healthy, were inoculated with the virulent strain Margarita (secondary virus). For exclusive detection of the Margarita strain, a specific qRT-PCR assay was designed, which proved not to have cross-reactivity with the Cat01 strain. The wild boars persistently infected with CSFV were protected from superinfection by the virulent CSFV Margarita strain, as evidenced by the absence of clinical signs and the absence of Margarita RNA detection in serum, swabs and tissue samples. Additionally, in PBMCs, a well-known target for CSFV viral replication, only the primary infecting virus RNA (Cat01 strain) could be detected, even after the isolation in ST cells, demonstrating SIE at the tissue level *in vivo*. Furthermore, the data analysis of the Margarita qRT-PCR, by means of calculated ΔCt values, supported that PBMCs from persistently infected animals were substantially protected from superinfection after *in vitro* inoculation with the Margarita virus strain, while this virus was able to infect naive PBMCs efficiently. In parallel, IFN-α values were undetectable in the sera from animals in Group B after inoculation with the CSFV Margarita strain. Furthermore, these animals were unable to elicit adaptive humoral (no E2-specific or neutralising antibodies) or cellular immune responses (in terms of IFN-γ-producing cells) after inoculation with the second virus. Finally, a sequence analysis could not detect CSFV Margarita RNA in the samples tested from Group B. Our results suggested that the SIE phenomenon might be involved in the evolution and phylogeny of the virus, as well as in CSFV control by vaccination. To the best of our knowledge, this study was one of the first showing efficient suppression of superinfection in

Competing Interests: The authors have declared that no competing interests exist.

animals, especially in the absence of IFN-α, which might be associated with the lack of innate immune mechanisms.

1. Introduction

Members of the Pestivirus genus, within the *Flaviviridae family*, account for a variety of diseases in farm animals, the most economically important of which are bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV). Classical swine fever virus (CSFV) is the etiological agent of a highly contagious viral disease of swine affecting domestic pigs and wild boars [1], which has caused major losses in stock farming [2, 3]. CSFV is composed of a lipid envelope, a capsid and a single plus-strand RNA genome carrying a single, large open reading frame (ORF) flanked by two untranslated regions (UTRs). The ORF encodes a polyprotein of approximately 3900 amino acids, which are processed by cellular and viral proteases in the four structural proteins—C, E^{rns}, E1, E2—and in the 8 non-structural proteins—N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [4].

Recently, it was proved that CSFV can generate postnatal persistence by infecting both newborn piglets and wild boars with either low- and/or moderate-virulence strains, respectively. Over the six weeks after postnatal infection, most of the infected animals remained clinically healthy, despite persistent high virus titres in the blood, organs and body secretions. Importantly, these animals were unable to mount any detectable humoral or cellular immune responses. At necropsy, the most prominent gross pathological lesion was severe thymus atrophy. Four weeks after infection, PBMCs from persistently infected seronegative piglets were unresponsive to both specific CSFV and non-specific PHA stimulation in terms of IFN-γ-producing cells. These results suggested the development of an immunosuppression state in these postnatally persistently infected pigs [5, 6]. In addition, it was shown that six-week-old, persistently CSFV-infected pigs were unable to elicit specific immune responses following vaccination with a CSFV lapinised C-strain vaccine (HCLV) [7]. Interestingly, the RNA of the vaccinal C-strain was undetectable by specific RT-PCR [8] in any of the samples analysed after vaccination, including blood, nasal and rectal swabs, or organs throughout the experiment, suggesting a phenomenon of homologous interference, also known as superinfection exclusion (SIE), between the high viral load generated by the primary persistent infection and the CSFV vaccine strain.

The SIE phenomenon, defined as the ability of a primary virus infection to interfere with a secondary infection by the same or a closely related virus, has been described in a broad range of virus-host systems, including bacteria, plants, and animals, and in important pathogens of humans, such as rubella virus, human immunodeficiency virus (HIV), and hepatitis C virus (HCV), among others [9–20]. From an evolutionary standpoint, SIE might be a conservative strategy, reducing the likelihood of recombination events between related strains [17, 21, 22], thus determining the stability of viral sequences within the same cell. From a practical standpoint, SIE has significant implications for the treatment or prevention of viral infections. In this regard, cross-protection of crops by purposeful infection with milder virus isolates is a widely accepted practice, and it is viewed as an effective and economical antiviral management strategy [23]. Additionally, transplantation of HCV-infected liver grafts has been suggested as a treatment for already infected patients, given that the transplantation of a healthy organ would lead to rapid damage to the newly transplanted liver by the virus of the recipient patient [15, 24].

Previous studies conducted in cell cultures with BVDV demonstrated that cells acutely infected with this virus were protected from a second infection by a homologous BVDV strain

[17]. Additionally, it was shown that CSFV is generally noncytopathic, and it readily establishes persistent infections in cell culture. Nevertheless, when persistently infected cultures were serially passaged more than 100 times, spontaneous generation of cytopathogenic (cp) CSFV variants could occur. The few surviving cells of the cytopathic effect (CPE), although still infected, were also protected from the CPE after superinfection with cp CSFV [25]. Both studies supported the ability of pestiviruses to generate SIE in cell cultures. Thus, along with the availability of a persistent infection model of CSFV, in the present study, we sought to assess SIE against a highly virulent CSFV strain at the organism level in six-week-old wild boars, rendered persistently CSFV-infected at birth. Our results showed that SIE could occur at the systemic level in CSFV-infected swine.

2. Materials and Methods

2.1. Cells and viruses

PK-15 cells (ATCC CCL 33) and SK6 cells [26] 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 cells were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 48 h later. Additionally, ST cells (ATCC CRL 1746) were cultured in DMEM, supplemented with L-glutamine (2%) and 10% foetal bovine serum (FBS), Pestivirus-free at 37°C in 5% CO_2. Peroxidase-linked assay (PLA) [27] was used for viral titration following the statistical methods described by Reed and Muench [28].

The Catalonia 01 (Cat01) strain used in this study was isolated from the Spanish CSF epizootic in 2000–2001 [29]. This isolate belongs to the CSFV 2.3 genogroup [30]. The course of infection by this strain was found to be mild [29, 31]. Finally, the virulent Margarita strain, which belongs to the CSFV 1.4 genogroup [29, 32, 33], was used.

2.2. Experimental design

To elucidate the capacity of CSFV to generate SIE, two groups (A and B), with three male, six-week-old wild boars in each, were used. These animals were acquired from Gestion Cinegetica Integral SL farm (Segovia, Spain) and were housed in the experimental isolation facilities in the biosecurity level 3 laboratory of the Centre de Recerca en Sanitat Animal (CReSA); they were fed a conventional piglet starter diet and pellets until the end of the trial (Startrite 100, Kwik-start, and Prestarter; SCA Iberica S.A., Zaragoza, Spain) and were handled according to previous studies conducted in CReSA [6]. Group A (animals 1 to 3) was used as controls, and they tested Pestivirus-free at the beginning of the study. The second group (Group B), housed in an independent isolation unit at the BSL-3 facility of CReSA, (animals 4 to 6), were postnatally persistently CSFV-infected animals. These animals, which had been intranasally infected in the first 24 h after birth with the CSFV Cat01 strain, were viraemic and apparently healthy at six weeks old, although being immunosuppressed, they lacked CSFV-specific cellular and humoral responses [5, 6]. Both groups had an average weight of 6 kg per animal. After a five-day acclimation period, all of the animals were experimentally infected by i.m. injection in the neck [33–35] with 10^5 $TCID_{50}$ CSFV Margarita strain. In previous studies, this viral dose caused acute CSF and often induced death at 10–15 days post-infection (dpi) [36]. Sera and nasal and rectal swabs were collected at 0, 3, 7, 10 and 13 dpi. Blood samples for the isolation of PBMCs were obtained at day 0 and at the time of euthanasia.

A trained veterinarian recorded the clinical signs daily in a blinded manner [36]. The clinical signs compatible with CSFV infection were anorexia, fever, conjunctivitis, diarrhoea, constipation, cyanosis of the skin, abdominal petechiae, dyspnoea, tremors, locomotive disturbances, reluctant walking, swaying movement of the hindquarters, posterior paresis, convulsions from

mild to severe and prostration. Particular stress was placed upon the registration of nervous symptoms [29, 33, 34, 36]. The clinical status of the animals was scored from 0 to 6 [29, 33, 34, 36] as follows: 0: no signs; 1: mild pyrexia; 2: pyrexia plus mild clinical signs; 3: mild-to-moderate clinical signs; 4: moderate clinical signs; 5: moderate-to-severe clinical signs; and 6: death. For ethical reasons, the animals were euthanised when the clinical score reached 5, when exhibiting a fall of the hindquarters, when there was inability to drink or feed, when prostration occurred or when exhibiting moderate nervous disorders. After euthanasia, an exhaustive necropsy was conducted, in which the presence of pathological symptoms in different organs and tissues was evaluated. Surviving wild boars were euthanised at 13 dpi, and urine and tissues (spleen, liver, intestine, mesenteric lymph node, prescapular lymph node, bone marrow, medulla oblongata, lung, kidney, thymus and tonsil) were obtained at necropsy. Euthanasia was performed according to European Directive 2010/63/EU, using a pentobarbital overdose of 60–100 mg/kg administered via the anterior vena cava. The animal care and procedures were in accordance with the guidelines of the Good Experimental Practices (GEP), under the supervision of the Ethical and Animal Welfare Committee of the Autonomous University of Barcelona (UAB), and they were approved under number 8804, according to the existing national and European regulations. Additionally, the biosafety level of the viruses used in this study was stated as biosecurity level 3, as approved by the Biosafety Committee of the UAB, with registration assignment AR-296-15.

2.3. Design and validation of a new qRT-PCR for the detection of specific CSFV Margarita strain RNA

Fifteen representative sequences of the three CSFV genogroups were retrieved from GenBank and aligned using BioEdit [37]. Two primers and probes were designed for specific detection of the Margarita strain sequence (1.4 CSFV genogroup) by targeting the 5′ end of the E2 gene, as follows: forward primer (2333–2356), 5′-AAGATTACGACCACAATTTACAAC-3′; reverse primer (2411–2431), 5′-TCC TACTGACCACATTAAGCG-3′ and probe (2369–2389), 5′-CCATCAAGGCTATCTGCACGG-3′. The nucleotide positions were based on the genome sequence of the Margarita strain (GenBank accession number AJ704817). The probe was labelled with 6-FAM at the 5′ end and with BHQ1 at the 3′ end. The primers and probe were purified by reverse phase HPLC. The one-step RT-PCR protocol was undertaken using the commercially available TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems Roche). The real-time RT-PCR assay was optimised using a total volume of 25 μl. Real-time qRT-PCR was performed using an Applied Biosystems® 7500 Fast Real-Time PCR System. The temperature profile was 30 min at 50°C (reverse transcription), 15 min at 95°C (inactivation reverse transcriptase/activation Taq polymerase), followed by 42 cycles of 15 s at 94°C (denaturation), 30 s at 57°C (annealing) and 30 s at 68°C (elongation). Identical temperature profiles were used for all of the real-time RT-PCR runs, and fluorescence values were collected during the annealing step. Twenty CSFV RNA preparations strains were used to determine the specificity and sensitivity of the assay (Table 1) [30, 38]. To exclude the possibility of presence of CSFV Cat01 strain RNA interfering with the assay sensitivity for the CSFV Margarita strain RNA detection, mixtures from serial RNA dilutions from both viral strains were analysed. In addition, mixtures from RNA serum samples of group B (prior to the Margarita strain inoculation), with samples from group A at 7 days post-infection with the Margarita strain, were analysed.

2.4. Detection of CSFV RNA

RNA was extracted from all of the samples using the NucleoSpin RNA isolation kit (Macherey-Nagel), according to the manufacturer's instructions. In all cases, RNA was extracted from an

initial sample volume of 150 μL to obtain a final volume of 50 μL of RNA, which was stored at -80°C. The presence of CSFV RNA in the serum and in nasal and rectal swabs, as well as in tissue samples, was analysed by a generic CSFV qRT-PCR [39]. This test was used in our laboratory for inter-laboratory comparisons of CSFV diagnoses, organised by the EU Reference Laboratory. Positive results were considered for threshold cycle values (Ct) equal to or less than 42. Samples in which fluorescence was undetectable were considered negative. Additionally, the qRT-PCR specific for the Margarita strain, designed in this work (described above), was used to distinguish those samples infected with the Margarita strain.

2.5. Detection of E2-specific and neutralising antibodies

Serum samples were tested with neutralisation peroxidase-linked assay (NPLA) [40], and the titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the Cat01 or Margarita strain in 50% of the culture replicates. The detection of E2-specific antibodies was performed using a commercial ELISA kit (IDEXX); the samples were considered positive when the blocking percentage was $\geq$40%, following the manufacturer's recommendations.

2.6. Detection of IFN-α in serum samples

Anti-IFN-α monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) were used in ELISA to detect IFN-α in serum samples at 0, 3, 7 and 10 dpi [34, 41–43]. The cut-off value of the assay was calculated as the average of the optical density of negative controls (blank and negative sera before CSFV infection) plus three standard deviations. Cytokine concentrations in serum were determined using a regression line built with the optical densities of the cytokine standards used in the tests.

2.7. PBMCs and ELISPOT assay for CSFV-specific IFN-γ-producing cells

ELISPOT assay to detect CSFV-specific IFN-γ cells was performed as previously described [34], using PBMCs that were obtained at day 0 and at the time of euthanasia. Briefly, plates (Costar 3590, Corning) were coated overnight with 5 μg/ml capture antibody (P2G10, Pharmigen). Detection was performed using a biotinylated antibody (P2C11, Pharmigen). A total of $5x10^5$

Table 1. Viruses used in the standardisation of Margarita strain real-time TaqMan assay.

CSFV Genotype/ subtype	References strain/isolate	Source
Genotype 1.1	HCLV vaccine (C-strain) (Muñoz-Gonzalez et al., 2015)	CReSA, Sapin
Genotype 1.4	Margarita	CReSA, Spain
Genotype 2.1	Paderborn (CSFV277 reference strain)	CReSA, Spain
Genotype 2.2	Clinical samples from experimentally infected pigs with CSF0018 reference strain (5 samples)	EU Reference Laboratory for CSF, Germany
	CSF573 reference strain (*Italy Parna'98*)	CReSA, Spain
Genotype 2.3	Clinical samples from experimentally infected pigs with CSF0864 reference strain (4 samples)	EU Reference Laboratory for CSF, Germany
	Clinical samples from experimentally infected pigs with CSF0634 reference strain (5 samples)	EU Reference Laboratory for CSF, Germany
	Uelzen (CFS639 reference strain)	CReSA, Spain
	Catalonia 01 (Pérez et al., 2012)	CReSA, Spain
	Spreda (CSF123 reference strain)	CReSA, Spain

doi:10.1371/journal.pone.0149469.t001

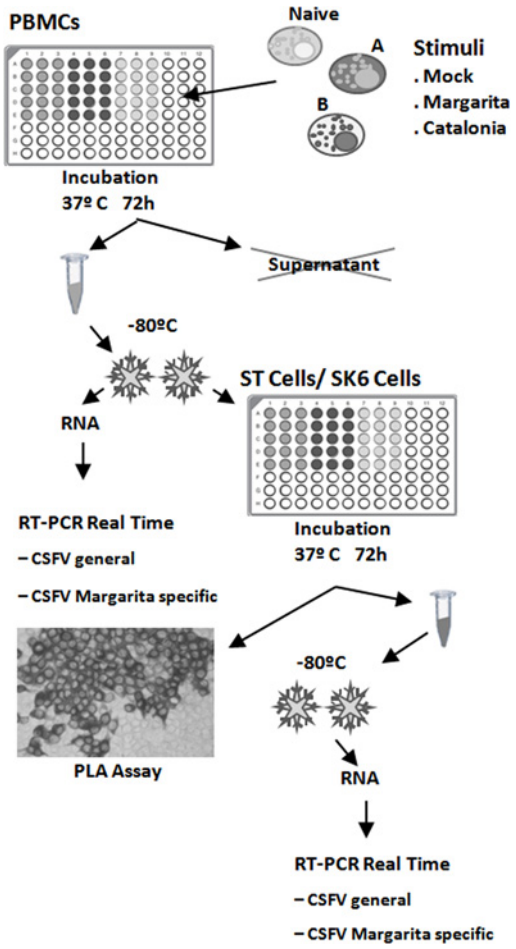

Fig 1. Experimental procedures to examine superinfection exclusion in PBMCs and ST cells.

doi:10.1371/journal.pone.0149469.g001

PBMCs/well were plated in triplicate at 0.1 multiplicity of infection (MOI) of the Cat01 and Margarita CSFV strains. Moreover, the same samples were incubated in the presence of phytohaemagglutinin (PHA) (10 μg/ml). The controls were incubated in the presence of mock-stimulated wells. The numbers of spots in the media for mock-stimulated wells were considered to constitute the baseline for the calculation of antigen-specific frequencies of IFN-γ-producing cells.

2.8. Cell culture assay

Samples from animal 1 (Group A: Margarita acutely infected wild boar; 10 dpi), animal 5 (Group B: Cat01 persistently infected wild boar and superinfected with CSFV Margarita strain; 13 dpi), and a Pestivirus-free wild boar (animal 1 before infection), were used to assess SIE in PBMCs (Fig 1). The PBMCs were isolated from whole blood by centrifugation on Ficoll gradients (Histopaque-1077; Sigma). The number and viability of the PBMCs were determined by staining with Trypan blue [33]. A total of $4x10^5$ PBMCs/well from each animal were plated in quintuplicate at 37°C in 96-well plates with: (i) vehicle; (ii) the Cat01 strain at a 0.1 multiplicity of infection (MOI); and (iii) the Margarita strain (0.1 MOI). After 72 h, the PBMCs were accurately washed twice and were resuspended in a final volume of 200 μl of PBS per well. To release the virus from the cells, two freeze-thaw cycles at -80°C were undertaken, and the quintupled samples were harvested in a single aliquot. The presence of virus RNA in PBMC samples

was analysed by generic CSFV qRT-PCR [39] and for the specific Margarita strain by qRT-PCR detection assay (see above, section 2.3). For virus isolation, an established cell line sensitive for specific CSFV proliferation, ST cells, were cultured at 37°C in 96-well plates in triplicate in the presence of each of the collected cell suspensions. After 72 h, the supernatants were removed, and the collected ST cells were washed twice and resuspended in 200 μl of sterile PBS. After two cycles of freeze-thaw at -80°C, the presence of CSFV RNA in the ST cell samples was analysed by qRT-PCR for CSFV [39] and the Margarita strain (see above). In parallel, a ST plate similarly inoculated with cell suspensions was used for confirmation by PLA [27]. A delta Ct (ΔCt) for Margarita strain RNA detection was calculated as the differences between (i) the Margarita Ct value detected from the isolation of ST from groups A or B and (ii) the Ct value in ST inoculated with Margarita-infected naïve PBMC extract, being $\Delta Ct = Ct_{(a)}\text{-}Ct_{(b)}$. The whole protocol was repeated twice, in ST and also in SK6 cells using PBMCs from animals 1 (Group A), 4 and 5 (group B) and cells from the naïve animal (number 1, Group A), collected before Margarita infection.

2.9. Sequence analysis

The E2-gene fragment reported by Lowings et al. [44] was amplified by end point RT-PCR [45] in sera, tonsil, lung and spleen from animals 1, 3 (Group A), 4 and 5 (Group B), collected at necropsy. Additionally, the viral inoculums used in the experimental infections (Cat01 and Margarita strains) were evaluated. The amplification products were checked by electrophoresis on 2% agarose gel and were directly cleaned with a Wizard® PCR Preps DNA Purification System (Promega, Madison, Wisconsin, USA). Sequencing reactions were conducted under BigDye™ terminator-cycling conditions using an ABI 3130XL. Forward and reverse sequences obtained from each amplicon were assembled using the Contig Express application in Vector NTI software, version 11 (Invitrogen). The sequences from the E2-gene fragment obtained were aligned to analyse the sequence found in each sample.

3. Results

3.1. Specificity and sensitivity of Margarita strain real-time TaqMan assay

Of the 20 CSFV RNA strains analysed, the assay detected only the CSFV RNA from the Margarita strain (1.4 genogroup), while the other 19 CSFV RNA extractions were negative (Table 1). This result indicated that the newly developed assay was highly specific for the detection of the CSFV Margarita strain, and there was no cross-reactivity with the other tested CSFV strains from genogroup 2 (including the Cat01 strain). The specificity of the assay was based primarily on mismatches in the probe-binding region but also to some extent on mismatches in primer-binding regions. The sensitivity of the assay was evaluated by testing 10-fold dilutions of the Margarita strain RNA. The analytical sensitivity was estimated to be as high as 0.4 $TCID_{50}$. The assay had a reaction coefficient (R^2) of 0.994 (data not shown). Positive results were considered for threshold cycle values (Ct) equal to or less than 38. Finally, the presence of Cat01 RNA strain in the sample containing the Margarita strain RNA did not affect the assay sensitivity (Data not shown).

3.2. Wild boars persistently infected with CSFV were clinically protected after infection with a CSFV Margarita virulent strain

Animals persistently infected with the Cat01 strain and inoculated with the virulent Margarita strain (Group B) showed neither clinical signs of disease nor fever at any time throughout the study, maintaining good health status (Fig 2). In contrast, animals from group A, infected with the Margarita strain, presented mild clinical signs at 2 dpi that progressed to moderate within

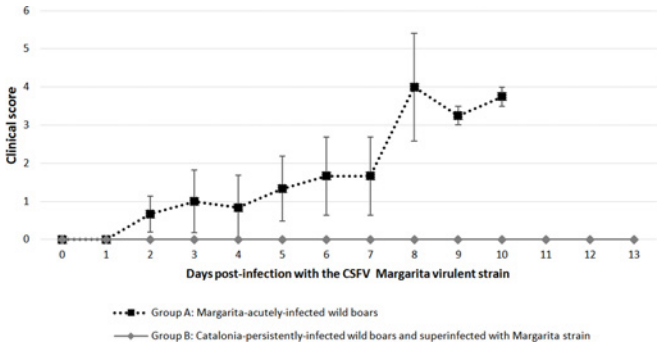

Fig 2. The animals persistently infected with Cat01 were clinically protected after infection with the virulent Margarita strain. Means and standard deviations of the daily individual clinical score values after CSFV virulent Margarita strain infection are represented. Dark grey bars indicate the standard deviation values for group A. The clinical score values are defined in the Materials and Methods section.

doi:10.1371/journal.pone.0149469.g002

48–72 h. At 7 dpi, animal 2 showed a clinical score value of 4; however, it was found dead at 8 dpi, with lesions of haemorrhagic diathesis. Animals 1 and 3 progressed to dyspnoea, weight loss, swaying movement of the hindquarters, posterior paresis and high fever until 10 dpi, when euthanasia was performed.

3.3. Absence of detectable Margarita strain RNA in CSFV-superinfected wild boars

Margarita RNA was undetected in the sera from animals in group B, except for animal 4 at 13 dpi with a high Ct value (Ct 36.84), considered a low RNA viral load (36, 39) (Fig 3). Additionally, CSFV Margarita strain RNA could not be detected in any of the nasal or rectal swabs collected from group B (data not shown). Furthermore, in group B, CSFV Margarita RNA was found only in the liver of animal 4 and also in the spleen of animals 4 and 5, with a low RNA viral load. In contrast, all wild boars from group B (CSFV persistently infected with Cat01 strain) maintained during the whole trial a high and constant CSFV RNA load in serum, swabs and organs, when examined by generic CSFV q-RT-PCR (Table 2).

In contrast, both qRT-PCRs (generic and specific for Margarita strain) were positive in organs and samples collected from animals in group A (Table 2). The Ct values were positive by the CSFV generic qRT-PCR [39], in both serum and swab samples, from 3 dpi onwards. Ct values for the specific Margarita assay were similar to those obtained by the CSFV generic qRT-PCR.

3.4. Absence of humoral response in terms of E2-specific and neutralising antibodies in CSFV-superinfected animals

To evaluate the induction of CSFV-specific antibodies, serum samples were analysed at different times after CSFV Margarita strain infection. The absence of antibody response, in terms of E2-specific antibodies and neutralising antibody titres, was found in both CSFV acutely and persistently superinfected groups during the entire experiment (Data not shown).

3.5. Levels of endogenous IFN-α increased with progression of acute disease but remained undetectable in CSFV-superinfected animals

Previously, it was shown that CSFV PI animals were unable to elicit an innate immune response, in terms of IFN-α production, against a CSFV life-attenuated vaccine [7]. However,

we wondered whether superinfection with a CSFV virulent strain would trigger detectable levels of IFN-α in the CSFV-superinfected wild boars (Group B), given that IFN-α has been largely related to disease severity, as a hallmark of CSFV acute infection [34, 46]. In the present work, we observed that progression of disease in group A was correlated with an increase in the levels of endogenous IFN-α after infection, as measured by ELISA, with values that reached more than 240 U/ml in two of three animals at 7 dpi and 10 dpi (data not shown). In contrast, IFN-α was undetectable in all of the serum samples analysed both before (day 0) and after Margarita inoculation of CSFV Catalonia persistently infected pigs (Group B) (data not shown).

3.6. CSFV-specific IFN-γ-producing cells were lacking in CSFV-superinfected animals

PBMCs from all of the animals were analysed for virus-specific and non-specific IFN-γ responses by ELISPOT assay at 0 and 13 dpi post-Margarita strain inoculation. Very few IFN-γ-producing cells were found upon CSFV and PHA stimulation of PBMCs from all 3 of the CSFV-superinfected animals (Group B). These results supported our previous results showing that postnatal infection of piglets with CSFV could result in virus persistence due to a lack of B- and T-cell responses (data not shown).

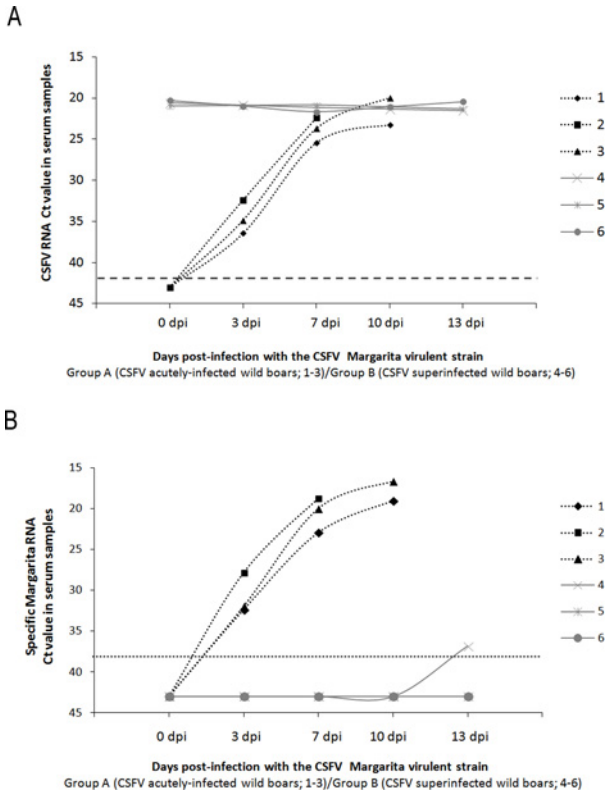

Fig 3. Swine persistently infected with the CSFV Cat01 strain were protected from the typical viraemia generated by the CSFV Margarita strain. (A) Daily detection of CSFV RNA through generic qRT-PCR in sera [39]. The Ct values from group A (CSFV acutely Margarita-infected wild boars; 1–3) and group B (CSFV-superinfected wild boars; 4–6) are represented in black and grey colours, respectively. (B) Daily detection of CSFV RNA Margarita strain through specific qRT-PCR in serum. The Ct mean values from group A (CSFV acutely infected wild boars; 1–3) and group B (CSFV-superinfected wild boars; 4–6) are represented in black and grey colours, respectively. Positive results for the CSFV RNA detection [39] were considered for Ct values equal to or less than 42, indicated with a dashed line. Positive results for the specific CSFV RNA Margarita strain detection were considered for Ct values equal to or less than 38, indicated with a dotted line

doi:10.1371/journal.pone.0149469.g003

Table 2. Swine persistently infected with the CSFV Catalonia strain are protected from CSFV Margarita strain infection in tissue samples.

	Group A (CSFV acutely infected wild boars; 1–3)						Group B (CSFV-superinfected wild boars; 4–6)					
	1		2		3		4		5		6	
Tissues	**CSFV RNA [a]**	**Margarita RNA [b]**	**CSFV RNA**	**Margarita RNA**	**CSFV RNA**	**Margarita RNA**	**CSFV RNA**	**Margarita RNA**	**CSFV RNA**	**Margarita RNA**	**CSFV RNA**	**Margarita RNA**
Spleen	18,57	17,70	17,2	17,90	17,09	16,23	21,4	32,91	20,35	36,15	20,85	*Undet.*
Liver	22,3	21,36	21,05	21,94	19,35	19,50	23,96	32,24	24,01	*Undet.*	25,05	*Undet.*
Mes. Ln.[c]	21,78	21,04	21,4	20,32	18,05	16,43	Not det.[g]	Not det.	22,31	*Undet.*	22,76	*Undet.*
Pres. Ln.[d]	19,27	18,12	18,91	18,02	17,88	16,16	22,92	*Undet.*[h]	23,81	Undet.	25,15	*Undet.*
B.M.[e]	19,9	17,92	19,68	16,77	19,49	18,34	19,54	*Undet.*	22,75	*Undet.*	20,72	*Undet.*
M. oblongata[f]	26,44	25,94	26,99	24,86	24,41	25,27	Not det.	Not. Det.	24,4	*Undet.*	24,64	*Undet.*
Urine	31,08	28,49	17,95	18,94	31,76	28,77	20,05	*Undet.*	Not. Det	Not. Det.	21,77	*Undet.*
Lung	23,57	22,70	22,66	21,01	19,14	17,78	20,21	*Undet.*	19,84	*Undet.*	19,97	*Undet.*
Kidney	25,08	23,32	25,24	23,25	21,98	20,57	21,62	*Undet.*	22,55	*Undet.*	21,77	*Undet.*
Thymus	27,46	25,18	23,64	21,31	21,02	19,36	20,5	*Undet.*	21,23	*Undet.*	21,15	*Undet.*
Tonsil	20,33	19,91	21,36	19,06	18,4	16,36	22,87	*Undet.*	25,03	*Undet.*	21,49	*Undet.*

[a] Ct value detected with the generic CSFV q RT-PCR assay (37).

[b] Ct value detected with the specific CSFV RNA Margarita strain qRT-PCR assay.

[c] Mes. Ln = Mesenteric lymph node.

[d] Pres. Ln. = Prescapular lymph node.

[e] B.M. = Bone marrow.

[f] M. oblongata = Medulla oblongata.

[g] Not Det. = Not determined.

[h] Undet. = Undetected.

doi:10.1371/journal.pone.0149469.t002

3.7. CSFV interference in the PBMCs from CSFV-superinfected hosts

It is well known that white blood cells, including the PBMCs, are targets for CSFV replication [47,48]. Consequently, to examine whether the PBMCs collected from the CSFV-superinfected animals (group B) and the acutely infected animals (group A), were permissive (or not) to CSFV superinfection, we assayed *in vitro* inoculation of such samples, with either Cat01 or Margarita CSFV strains. Similarly, PBMC samples were mock-infected. Additionally, PBMCs from a naïve animal were used as controls. As was expected, CSFV-specific Margarita RNA was detected in the PBMCs from animals developing the CSF acute disease (group A) in both mock and Margarita-infected samples. Furthermore, PBMCs from group B *in vitro* inoculated with Margarita were also positive for CSFV-specific Margarita RNA detection, but with a high Ct value correlated with a lower RNA load (Table 3). Otherwise, PBMCs from group B *in vitro* mock-infected were negative for CSFV-specific Margarita RNA detection (Table 3). Following these findings, to decipher whether the detected RNA load in group B might correspond to RNA traces from the inocula or to the infecting virus, the previously analysed PBMC extracts were inoculated into a ST cell line. Consistently, the detected RNA load notably increased in ST after inoculation with the extract from Margarita *in vitro* inoculated-naïve PBMCs; the obtained 7.76Δ Ct positive value confirmed the infectivity of the virus recovered from the PBMC samples. In contrast, Margarita RNA in group B *in vitro* mock-infected PBMCs remained undetectable even after ST inoculation. Furthermore, Margarita RNA load detection

Table 3. CSFV interference in the PBMCs from superinfected hosts.

	Experimental groups					
	Naive		CSFV acutely infected wild boar 1 (10 dpi; group A)		CSFV-superinfected wild boar 5 (13 dpi; group B)	
	CSFV Ct [a]	Specific Margarita Ct[b]	CSFV Ct	Specific Margarita Ct	CSFV Ct	Specific Margarita Ct
PBMC extracts						
Mock-infected PBMC	*Undetected*	*Undetected*	24.99	23.10	26.38	*Undetected*
CSFV Catalonia-infected PBMCs (MOI 0.1)	34.90	*Undetected*	25.86	23.61	25.60	*Undetected*
CSFV Margarita-infected PBMCs (MOI 0.1)	32.50	31.47	25.06	22.91	25.14	31.05
ST cell extracts						
Mock	*Undetected*	*Undetected*	29.70	27.81	25.57	*Undetected*
CSFV Catalonia (MOI 0.1)	25.93	*Undetected*	29.37	27.95	28.67	*Undetected*
CSFV Margarita (MOI 0.1)	26.80	23.71	28.14	25.83	28.92	35.31
CSFV Margarita Δ CT value determination						
PBMC extracts—ST cell extracts		7.76[c]		-2.92[c]		-4.26[c]
ST cell extracts—ST cell extracts from naïve animals				2.12[c]		11.6[c]

[a] Ct value detected with the generic CSFV qRT-PCR assay (37).

[b]Ct value detected with the specific CSFV RNA Margarita strain q RT-PCR assay.

[c]Δ Ct value.

doi:10.1371/journal.pone.0149469.t003

in group B *in vitro* Margarita-infected PBMC samples decreased after inoculation of ST cells, corresponding to higher Ct values than those previously detected directly from PBMC extracts. Remarkably, an 11.6 ΔCt value was found in the ST cells with Margarita *in vitro* inoculated PBMCs from group B, relative to the value obtained in the ST cell extracts from Margarita-inoculated naïve PBMCs (Table 3). The whole protocol was repeated twice for animals 1 (group A), 4 and 5 (group B) in both SK6 and ST cells, supporting the results with similar Ct values (data not shown). Similarly, the cells' positive infection was confirmed by PLA testing, although this test cannot differentiate between Cat01 and Margarita CSFV strains.

3.8. Sequence analysis could not detect CSFV Margarita RNA in tissues from CSFV-superinfected animals

To detect the presence of CSFV RNA of both viral strains (Cat01 and/or Margarita) in the sera, tonsil, and spleen of animals 1 and 3 (Group A) and 4 and 5 (Group B), the E2-gene fragment reported by Lowings et al. [44] as a phylogenetic marker was amplified by end point RT-PCR [45]. In all of the samples analysed from animals that developed the CSF acute form (Group A), the sequence corresponding to the Margarita strain (AJ704817) used as the inoculum was detected. Furthermore, the samples analysed from superinfected animals (Group B: CSFV Catalonia 01 persistently infected inoculated with CSFV Margarita strain) only showed the sequence corresponding to the Cat01 strain [30] (Fig 4).

4. Discussion

Despite its significance, the mechanisms of mutual exclusion by viral variants are far from being completely understood, and the actual knowledge is basically derived from studies at the cellular level in established cell lines [14, 16, 19, 49]. Very few reports have demonstrated the

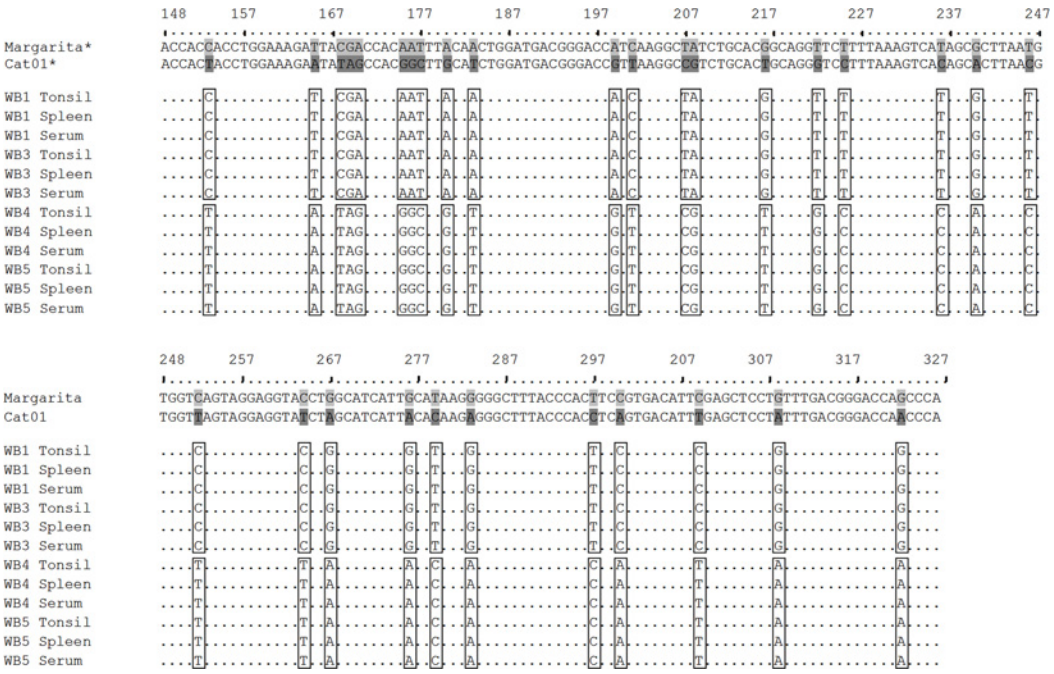

Fig 4. Sequence analysis of the partial E2 sequence does not detect the CSFV Margarita RNA in the tissues from superinfected animals. The Margarita and Cat01 viral strain sequences used as viral inocula in the animal infection experiments were considered as references. Sequences from sera, tonsil and spleen samples from group A (CSFV acutely infected wild boars; 1, 3) and group B (CSF-superinfected wild boars; 4, 5) are shown. Differences in the nucleotide sequences between the CSFV Margarita and Catalonia 01 strains are shown in grey and dark grey, respectively.

doi:10.1371/journal.pone.0149469.g004

phenomenon of SIE at the organism level, and, to our knowledge, these models have been limited to plant viruses, West Nile virus (WNV) in mosquitoes, and Peking duck hepatitis B virus (DHBV) [50–52]. In addition, it has not yet been demonstrated in a mammalian host at the systemic level.

Previous works have reported the capability of CSFV to generate postnatally persistent infection in both domestic pigs and wild boars [5, 6]. Subsequently, it was also shown that postnatally persistently infected pigs were unable to elicit a specific immune response to a CSFV live attenuated vaccine and that the viral vaccine RNA was undetectable in any of the samples analysed [7]. Against this background, we assessed the capacity of CSFV to generate SIE in CSFV persistently infected swine. For that purpose, CSFV persistently infected wild boars were inoculated with a CSFV strain that induce acute disease with a higher replication rate [29, 36].

Because pestiviruses are immunologically and genetically closely related, accurate serological characterisation of CSFV isolates is impeded by the extensive cross-reactions observed among Pestivirus members and the limited availability of MAbs capable of differentiating among different CSFV isolates [27, 45, 53]. To differentiate the CSFV Margarita strain RNA from the CSFV Cat01 strain RNA in the samples from the present study, a specific qRT-PCR for Margarita strain RNA detection was developed. Thus, alongside the model of infection with the Margarita strain, the qRT-PCR assay developed allowed for clear discernment of whether there was actually a blockage that prevented susceptibility to infection by the second virus in both the absence of clinical signs and the absence of molecular detection of the superinfecting virus.

Notwithstanding the high infection rate of the Cat01 strain in persistently infected animals from group B (primary virus infection), good health status was maintained after inoculation with the Margarita CSFV virulent strain (secondary infection) in the absence of viral detection

in sera throughout the study, except in one animal at 13 dpi with a low Margarita strain RNA load (animal 4). Despite the important role that neutralising antibodies play in CSFV protection [29, 54], complete absence of neutralising antibodies response was found after Margarita strain infection in these animals. Similarly, absence of an IFN-γ-producing cell response against CSFV or PHA was also observed. Considering the role played by IFN-γ in the control of CSFV infection [34, 55] and the lack of responsiveness to IFN-γ-producing cells after PHA stimulation, the CSFV-superinfected animals maintained a immunosuppression state similar to that previously described in postnatal persistent infection [5, 6]. Previous work has proved how the failure to induce optimal levels of the humoral and cellular responses after CSFV infection promoted the spread of the virus and its relationship with disease progression [29, 54]. In this regard, the implications of the cellular and neutralising antibody response in clinical protection against the acute form in the CSFV-superinfected animals from this study are excluded.

Furthermore, no superinfecting virus excretion was detected in any of the animals from Group B, whilst the high viral load generated by the strain that induced the persistent infection (Cat01 strain or primary infection) was maintained until the end of the trial, supporting our previous results [7]. In contrast, the CSFV Margarita strain generated the acute form of the disease in animals from group A, with high Margarita RNA loads in all of the samples analysed. In addition, the failure of the humoral response in the pigs that developed acute CSF was previously described [29].

In addition to the adaptive immune response, the innate immune response to the virus, as measured by type I IFN-α in the serum, also seemed to be impaired, in terms of IFN-α detection because IFN-α values were undetectable in the sera from postnatally persistently infected wild boars after CSFV Margarita strain inoculation. At the same time, the progression of the acute disease in group A was correlated with an increase in levels of endogenous IFN-α, as has been previously described [29, 46, 56, 57]. The absence of an IFN-α response in the Cat01 persistently infected animals after Margarita strain inoculation (secondary infection) probably was due to the almost complete lack of Margarita strain replication in these animals. Otherwise, specific CSFV-blockade phenomena for IFN-α might be occurring. Efficient viral strategies to escape the type I IFN-induced antiviral mechanisms have been described within Pestivirus. In this regard, the viral RNA triggers IFN synthesis, and the viral RNase E^{rns} inhibits IFN expression induced by extracellular viral RNA [58]. In addition, the viral protein N^{pro} suppresses type I IFN (IFN-α/β) induction by mediating proteasomal degradation of IFN regulatory factor 3 (IRF-3) [58–60]. For instance, in persistent infection, BVDV maintains "self-tolerance" by avoiding the induction of IFN, without compromising the IFN action against unrelated viruses ("nonself") [58]. In the case of CSFV-infected pigs, it has been recently demonstrated that functional N^{pro} significantly reduced local IFN-α mRNA expression responses at local sites of virus replication [61]. These highly selective "self" models of evasion of the interferon defence system might be key elements in the success of persistent infections and could promote, in addition, the generation of SIE phenomena.

Previous reports have suggested that the availability of mammalian models for SIE *in vivo* is hampered by the interferon response generated against the infecting virus in these species [11, 24]. It is noteworthy that CSFV postnatally persistently infected swine have shown an immunosuppression state comprising a reduction in interferon responses (Types I and II) [5–7]. This immunological status might promote the maintenance of a high and constant CSFV load, as already described, preventing second viral entry [5]. Nevertheless, further studies would be needed to clarify the molecular mechanisms involved in this phenomenon.

At 13 dpi, low levels of Margarita RNA were detected only in some collected tissues from persistently and superinfected wild boars (Group B), principally in animal 4, in which Margarita RNA was detected from the spleen and liver, as well as in the serum. However, the level of

Margarita RNA detection was approximately fifteen times less than the acutely infected animals from group A (Table 2). The Margarita RNA levels found in the superinfected animals might be correlated with the low Margarita strain viral loads in some macrophages in these tissues [62].

In contrast, despite PBMCs being a well-known target for CSFV viral replication [62], after *in vitro* assay, the presence of CSFV Margarita RNA could not be detected in either the PBMCs or ST cell extracts from Group B. Additionally, the *in vitro* superinfection of isolated PBMCs failed when they were derived from persistently infected piglets but were clearly positive for assays with cells from naïve animals, as demonstrated by means of calculated ΔCt values, supporting that PBMCs from persistently infected animals were substantially protected from superinfection after *in vitro* inoculation with the Margarita virus strain. These results suggest that SIE still occurs at the tissue level (Table 3). In contrast, the Margarita strain RNA could not be detected after the sequence analyses of the samples from persistently infected Margarita-inoculated animals (Group B) nor even in the tonsil, one of the main targets for CSFV replication [3, 63]. Nevertheless, next-generation sequence analyses would be of great interest to analyse these samples in detail, emphasising the spleen and liver tissues that were also positive for RNA Margarita strain detection after superinfection.

Altogether, although it is a very complex mechanism, if compared with the acutely infected group A, these results showed that a phenomenon of CSFV SIE occurred at the systemic level. Nevertheless, the colonisation of a multi-cellular host is a complex process during which the viral load can dramatically change in different organs and at different stages of the infection, and not all of the potential target cells are infected in persistently infected animals despite the high viral load generated by the Cat01 CSFV strain in persistently infected animals [5, 6]. Illustrative examples include some of the works performed to demonstrate SIE at the cellular level because some cells uninfected by one viral primary infection are subsequently infected by the second viral infection [11, 50]. In contrast, the implications of other mechanisms in the host cannot be excluded, and it remains unclear whether the observed phenomenon is really due to a blockage at the level of infection of cells. This was precisely in the case of a citrus tristeza virus (CTV) SIE model, wherein a CTV protein (p33) was required to mediate SIE at the organism level but that did not appear to be implicated in exclusion at the cellular level [50].

Overall, our results suggested efficient suppression of viral superinfection in a mammalian host, especially in the absence of IFN-α, indicating a lack of innate immune mechanisms. Considering the role of this phenomenon from an evolutionary standpoint, their implications within an epidemic situation might be relevant to the evolution and phylogeny of CSFV. Although this phenomenon must be studied in greater depth, the possible outcome for the generation of new CSFV strains circulating in an endemic situation and the impact on disease control, including vaccination with live attenuated vaccines, cannot be underrated.

Acknowledgments

We thank Valentí Rosell, Iván Cordón and David Solanes for their help in the animal facilities.

Author Contributions

Conceived and designed the experiments: LG. Performed the experiments: SM MP LG MD JAB OC AC. Analyzed the data: LG SM MD RR OC. Contributed reagents/materials/analysis tools: LG RR MD SL IM LJP. Wrote the paper: SM LG. Critically read the manuscript: LG OC MD. Read and approved the final manuscript: SM MP AC OC JAB RR LJP IM SL MD LG.

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PART III

General discussion and conclusions

Chapter 6

General discussion

Over the last century, great efforts have been directed towards the control and eradication of CSF, which today remains one of the most important diseases for animal health and in the pig industry worldwide. By applying costly control and surveillance measures, affected first world countries have been able to eradicate the disease successfully, including non-vaccination policies and the mass culling of the susceptible pig population. In contrast, the disease still generates high economic impact on developing countries, contributing to the deterioration of their socio-economic situation and, consequently, putting food security at risk in some cases. The lack of financial resources impedes to display massive stamping out policies and therefore they have to include the intensive vaccination for disease control. In addition, failures in the vaccination strategies, along with poor capacity of the veterinary services and the inefficient diagnostic tools favour the circulation of low and moderate CSFV strains which may generate mild clinical disease or unapparent CSF form. There is a huge need to comprehend the aspects that underlay this complex epidemiological situation to better address the control and surveillance campaigns of each region.

The problematical of the disease has been extensively reviewed; the importance of chronic and subclinical forms is a topic of currently discussion, but over the last decades several authors have been claimed the existence of the CSF persistent form as an important source for the perpetuation of the virus in the field. Congenital persistent CSFV infection is defined as a maintained viraemia and permanent virus excretion, in the absence of specific antibodies production. Since the surveillance strategies are basically based on clinical and serological diagnosis, this course can remain unnoticed, especially when there are no manifested clinical signs. Persistence after congenital infection has been revised in the past, both under natural and experimental infections, demonstrating the ability of the virus to circulate silently in the pig population. Despite its importance and the wide endemism that is present in the affected countries, these studies date back to the 70's and 80's, and since then no one has deepened in the immunopathogenesis of such forms. Furthermore, these works were limited by

the need of more advanced immunological tools. Likewise, the postnatal subclinical forms are not well understood, and the generation of postnatal persistence has not yet been documented in the scientific literature. The words of Professor van Oirschot already claimed:

" [...] numerous gaps exist in our present knowledge on various aspects of Hog Cholera. Much complicated, but fascinating research will be required to gain some insight into viral and host determinants involved in the expression of virulence, and in the pathogenesis of this multifarious virus disease"(van Oirschot, 1988).

Unlike persistent congenital infection, previously described for both CSFV and other pestiviruses, this thesis is the first work demonstrating CSF persistent form after postnatal infection, and for the first time within the genus *Pestivirus* (Study I). Simulating the natural infection route, ten newborn piglets per group were inoculated with the CSFV Pinar del Río (PdR) strain (genotype 1.4) (Pérez et al., 2012; Postel et al., 2013b), of low virulence, and CSFV Catalonia (Cat01) strain (genotype 2.3), characterized by Tarradas *et al*. as moderately virulent (Tarradas et al., 2014). The key points of this research are discussed below.

Both strains, of different degree of virulence, and different genogroup, generated persistently infected (PI) piglets with high and constant viral titters in blood, nasal and rectal excretions throughout their entire life. In spite of persistent infection, many of the PI animals were apparently healthy during the experiment, mostly animals infected with the CSFV moderately virulent Cat01 strain. On the other hand, the clinical presentation, if present, was non-specific for CSF, and was associated with secondary infections causing omphaloblebitis, whereas no pathological findings were observed in persistent animals showing no clinical signs in the study. Unless the higher mortality and clinical presentation, the obtained results supported the lower virulence of Pinar del Río virus in comparison with the Cat01 strain, as indicated by the lower viral load in blood and tissues, and the viral clearance after seroconversion of three animals (piglets

#1,#3, #14 and sows PdR and Cat01) (Fig 3a, 4a and 4b, Table 1, Study I). A recent report from our group further confirmed the low virulence of PdR strain, that caused no clinical signs in weaner pigs, which were able to overcome the infection in the presence of neutralizing antibodies (Coronado et al., 2017).

Previous reports showed that vaccination of newborn piglets before the ingestion of colostrum, with the CSFV live attenuated vaccine, conferred good protection against CSF (Suradhat and Damrongwatanapokin, 2003; Vandeputte et al., 2001). These works point to the immunocompetence capacity of newborn piglets. However, in this study (Study I), piglets that were postnatally persistently infected developed leukopenia and were not able to generate an acquired T-cell or B-cell response, as demonstrated by the absence of CSFV IFN-γ specific response by ELISPOT and the total absence of specific antibodies (Fig. 4, Study I). While congenital infection is related to the phenomenon of immunotolerance, the lack of cellular response to stimulation with mitogen PHA in the PI piglets of this study suggested that it was a state of immunosuppression. By contrast, three piglets (piglets #1,#3, #14) infected with the CSFV low virulent strain (PdR group) showed neutralizing antibody response from the third week post infection onwards. Therefore, analysing the antibody response, we were able to establish that pigs that had not developed a CSFV specific humoral immune response after the third week post infection (and continued with CSFV load in serum, secretions and tissues), remained persistently infected until the end of the study. In addition, the immunocompetent pigs (piglets #1,#3, #14) were also able to elicit an acquired CSFV-specific response in terms of IFN-γ producing cells.

On the other hand, PI piglets showed an innate response, in terms of systemic IFN-α production, comparable to immunocompetent animals (piglets #1,#3, #14 and sows PdR and Cat01) (Fig. 5, Study I). This finding suggested that the immunosuppression observed after postnatal infection occurred at some point in the adaptive immune response. However, immunotolerance of the CSFV postnatally PI pigs cannot be excluded.

It is well established the need of an acquired immunity to achieve CSFV disease protection (Franzoni et al., 2013; Ganges et al., 2008; Summerfield and Ruggli, 2015; Tarradas et al., 2014). It is possible that thymus atrophy might have led to lower numbers of mature $CD8^+$ and $CD4^+$ T cells (Sánchez-Cordón et al., 2002). Interestingly, the thymus samples from all of the PI pigs were CSFV RNA positive (Fig. 3a, Study I). Also, after CSFV acute infection the drastic depletion of circulating T cell populations by bystander apoptosis is very characteristic (Carrasco et al., 2004; Ganges et al., 2008; Summerfield et al., 2001). Several authors defend the role of cellular immunity to combat CSF infection (Franzoni et al., 2013; Ganges et al., 2005; Graham et al., 2012b; Tarradas et al., 2010, 2014), so both circumstances might compromise the adequate activation of adaptive immune responses in persistently infected animals. Furthermore, a phenotype analysis of the BMHC showed that percentages of myeloid cells lineage ($CD172a^+$) was clearly increased in contrast to the samples from naive piglets (Fig. 6, Study I). This finding might reflects a homeostatic imbalance in response to the leukopenia observed in the infected animals. On the other hand, the percentage of CSFV-positive BMHCs overall was near 80% for the Cat01 PI pigs, in contrast with the 15% for the PdR infected seropositive piglets. This fact is supported by the levels of SLAII expression of the myeloid lineage, which were much lower in the Cat01 PI pigs, in comparison to the samples from animals that overcame the viremia (PdR group). Additionally, bone marrow $6D10^+$ immature granulocytes were also increased and targeted by CSFV in PI animals. Probably, the releasing of these immature and infected forms to circulation would help to perpetuate the infection. More studies are needed to analyse in detail those populations, including flow cytometry assays, in both lymphoid tissues and blood.

Contrary to latency used by herpesviruses, or the antigenic variation used by lentiviruses, such as Visna-Maedi in sheeps and Caprine arthritis encephalitis in goats (Minguijón et al., 2015), the evasion of the adaptive immune response is an unique strategy among persistent viral infections. The modification of the host

cytokine production and signalling is one the most commonly theme in viral subversion (Kane and Golovkina, 2010), as seen for BVDV self-tolerance to the IFN-α production (Schweizer et al., 2006). Interestingly, in vitro assays showed that PBMC from these CSFV postnatally PI animals specifically produced large levels of soluble interleukin-10 (IL-10) against CSFV and after PHA stimuli, in the total absence of IFN-γ response. The IL-10 is a well-known immunosuppressive cytokine with a pleiotropic function, including suppression of T-cell proliferation and B-cell responses (Sabat et al., 2010). It is widely known the involvement of IL-10 in persistent viral diseases by stimulating its production to suppress the antiviral immune response (Brady et al., 2003; Clerici et al., 1996; Huang et al., 2007; Shin et al., 2000). The up-regulation of regulatory T-cell functions, that also produce IL-10, is a similar strategy involved in various persistent viral infections (Li et al., 2008). On the other hand, previous reports have associated CSFV with activation of IL-10 secreting $CD4^-/CD8^+$ T cells in vivo, suggesting their possible involvement in the immunosupression observed after infection (Suradhat et al., 2005).

Finally, in spite of the humoral response, the bristles of Cat01 and of PdR infected litters were positive for RNA detection in tonsil and in nasal and/or rectal swabs. In addition, the tonsils of the three immunocompetent pigs (PdR group) were also CSFV RNA positive. This finding support that the tonsils, besides being the primary target organ for CSFV replication, also may constitute the site for viral persistence in the infected host, generating thus, non-viraemic asymptomatic carriers (Bironf et al., 1987; Eblé et al., 2014; Rout and Saikumar, 2012; Terpstra, 1991). Furthermore, protection against CSFV is largely dependent on the levels of the neutralizing antibody titres (van Oirshot et al., 2003; Ganges et al., 2005; Tarradas et al., 2014). Considering that, our data illustrates the incapacity of some non-viraemic asymptomatic carriers for the complete virus elimination. CSFV asymptomatic carrier animals have high epidemiological relevance, as they can remain completely unnoticed under viral detection diagnosis if blood is the unique sample chosen. Our results highlight

the suitability of RT-qPCR technique to detect viral antigen as a part of a solid diagnostic framework and support the feasibility for CSFV detection in rectal swabs, being an easy to acquire and very sensitive sample for the viral detection in CSFV non-viraemic asymptomatic carriers and in CSFV PI animals. In addition, these results also support the capacity of CSFV strains of low-moderate virulence used to be horizontally transmitted.

Also remarkably, this is one of the first reports demonstrating the feasibility of a CSFV moderately virulent strain, which often causes fever and clinical signs in weaner pigs (Tarradas et al., 2014), to produce the CSF persistent form in the absence of specific clinical signs. In parallel, a later published study with wild boars showed their same susceptibility to the persistent infection after CSFV Cat01 strain inoculation at birth, and support these results (Cabezón et al., 2015). These findings show the importance of the host-virus interaction to weave the outcome of an infection, which depends not only on the virulence of the strain.

Noteworthy, lowly and moderately virulent CSFV strains are prevalent in the current endemic zones (Hu et al., 2016; Ji et al., 2014; Pérez et al., 2012) where intensive vaccination programs with the live attenuated CSFV vaccine (lapinised C-strain) are applied. Although there is no data from epidemiological studies, once demonstrated the capacity of low and moderately virulent CSFV strains to generate CSF persistent forms (Muñoz-González et al., 2015b; van Oirschot, 1979a), the real implication of PI animals in the endemic situation should not be despised, as for BVDV, in that only 1% of PI incidence is able to maintain the infection in a herd (Peterhans and Schweizer, 2013). CSFV PI clinically healthy pigs can be moved between farrowing and finishing, spreading the disease widely, and would remain unnoticed under serological control, despite excreting large amounts of virus steadily throughout their lives. In addition, when clinical disease is present, as we also observed, is very non-specific and often due to secondary bacterial infections, confusing the diagnostic. Poor growth, delay in fattening and high incidence of bacterial infections may be overlooked or

attributed to other causes (van Oirschot, 1988). Taking into account these precedents, in the study II we assessed the immune response to a live attenuated CSFV vaccine (lapinised C-strain), of 6-week-old domestic piglets persistently infected with the CSFV Cat01 strain, following the procedures previously established in the study I. Former studies show the protective ability of the live attenuated vaccine even when the challenge occurs after two days of immunization (Franzoni et al., 2014; van Oirschot, 2003a). Thus, we might hypothesize that vaccination could act as a boost effect creating an anamnestic response in the PI pigs, that would lead to virus clearance or at least a reduction of the viral load.

After vaccination, one animal remained in a good health status over the three weeks post vaccination, and only fever peaks were observed at the end of the study in other two PI pigs (Fig. 1 and 2, Study II). However, immediately after two dpv onwards, fever and unspecific clinical signs appeared and progressed in one of the PI animals, until required euthanasia. The vaccine may have been a trigger for the worsening of the clinical status of PI animals. For CSFV PI congenital infection, it is always fatal and survival has not been described beyond 11 months of life (van Oirschot, 1988; Van Oirschot and Terpstra, 1977). Therefore, the clinic observed may instead be the natural progression of CSFV persistent postnatal infection (observed up to at least 9 weeks of life, when euthanasia was performed in three out of four animals at the end of the study II).

Surprisingly, the vaccination did not trigger any change in the Cat01 CSFV RNA load in the blood, swabs nor tissues of the PI animals, remaining constant and similar to values founds in the study I. Also in accordance with the study I (Muñoz-González et al., 2015b) neither cell nor humoral adaptive responses could be detected at any point of the trial after vaccination (Fig. 3 and 4, study II). However, contrary to CSFV postnatally persistently infected pigs, calves persistently infected with BVDV are able to develop immune responses against different pathogens, as well as respond to vaccination (Bolin et al., 1985;

Peterhans and Schweizer, 2013). Moreover, in contrast with the first study of this thesis, no response of IFN-α was observed after vaccination. This suggested that once the persistence status is already established, a blockade occurs in the IFN type I system, at least for IFN-α production, as seen for BVDV persistent infection (Lussi and Schweizer, 2016; Schweizer et al., 2006; Schweizer and Peterhans, 2014). The immunological anergy developed in the CSFV PI pigs entails one of the main differences with CSF chronic disease, in which a specific immune response against the virus is generated, albeit it is not enough to get rid of the infection (Petrov et al., 2014; Summerfield and Ruggli, 2015; Tarradas et al., 2014).

Considering the immunological anergy previously described in the PI animals (Study I), it could be expected that this fact may be an advantage for the replication of the virus that generate the persistence in the host. Paradoxically, there was an absence of vaccine virus RNA in samples from PI animals, including tonsil. The lack of vaccine virus RNA replication could explain the immune unresponsiveness following vaccination. The tonsil is a known replication target for the vaccine virus, being detectable up to two months after vaccination (Kaden et al., 2004). The vaccine virus RNA presence and the detection of CSFV-specific antibodies after immunization of *Pestivirus*-free control pigs (Table 1, study II) ruled out a defective batch or a vaccine dose failure. Given the nature of the vaccine (live attenuated virus), we suggested that it could be due to an interference phenomenon between the persistent Cat01 virus and the vaccine virus. It has been demonstrated the ability of BVDV (*Pestivirus* genus) to produce viral interference at the cell level in vitro (Lee et al., 2005). In the case of CSFV, Mittelholzer *et al.* had previously tried to asses viral interference by passaging CSFV persistent infections in cell culture over 100 times; after generation of spontaneous cytopathogenic (cp) CSFV variants, the still infected surviving cells were protected towards cytopathic effect after superinfection with cp CSFV (Mittelholzer et al., 1998). However, the lack of biomolecular tools to differentiate between the infecting and the super infecting

virus make difficult to drawn accurate conclusions from that work (Mittelholzer et al., 1998).

The interference phenomenon is best known as superinfection exclusion (SIE), and it is defined by the ability of a primary virus infection to interfere with a secondary infection by the same or a closely related virus, either by preventing entry to the cell, or by interfering at the level of translation and / or replication. Apparently, SIE has been developed as a conservative strategy to maintain strains, reducing the likelihood of recombination events (Formella et al., 2000; Huang et al., 2008; Lee et al., 2005). Although a broad range of virus-host systems use this strategy, including bacteria, plants and animals, the mechanisms of mutual viral exclusion are still not well understood, not only at the level of cell-virus interaction, but also at the organism level where other host factors may be involved, such as interferon system in mammal hosts (Folimonova, 2012; Webster et al., 2013).

To discard the possibility that the lack of detection of vaccine virus RNA was due to its lower replication fitness compared to the moderate virulence Cat01 strain, we evaluated the CSFV capacity to interfere against infection by superinfecting with another CSFV strain. Thus, in the Study III we assessed the infection with the virulent CSFV Margarita strain (genotype 1.4) (Ganges et al., 2005; Pérez et al., 2012; Postel et al., 2013b; Tarradas et al., 2014) in the CSFV Cat01 strain -persistently infected six-week-old wild boars (Cabezón et al., 2015). The CSFV Margarita strain usually causes the death or generates severe clinical signs between 7 and 14 dpi (Ganges et al., 2005; Tarradas et al., 2014).

Due to the immunologically and genetically close relation, accurate serological characterisation of CSFV isolates is impeded by the extensive cross-reactions observed among *Pestivirus*. Consequently, the differentiation of CSFV strains through the use of antibodies is limited (Greiser-Wilke et al., 2007). For that reason, it was necessary to design a new RT-qPCR assay for detection of CSFV genotype 1.4, based on the 5´end of E2 gene, in order to discriminate from the

virus Cat01 genotype 2.3. The test was proven to be sensitive (up to 0.4 $TCID_{50}$) and specific for genotype 1.4 after analysing twenty CSFV RNA preparations of strains from different genotypes and origins (Table 1, study III). This tool can also be very useful for endemic countries in the Caribbean where this genogroup is currently circulating.

After infection with the virulent Margarita strain, the absence of clinical signs as well as the practically absence of Margarita superinfecting virus RNA detection (Table 2, study III) evidenced the SIE phenomenon in the PI pigs, also supported by the *in vitro* assays with PBMCs (table 3, Study III). Detection of low Margarita RNA load in some tissues like liver and spleen at 13 days post infection may correspond with non-infective viral particles (Leifer et al., 2009b) and would be explained by the presence of not infected macrophages with the primary virus (Dräger et al., 2015). However, given the low RNA load detection of CSFV Margarita strain in these tissues, only the RNA of Cat01 strain could be identified by sequence analysis, using the Sanger Method (Figure 4, Study III). More studies including next sequence analysis would be interesting to accurate better conclusions. Altogether, a plausible resistance to superinfection has been demonstrated in this experiment, supported by the total absence of disease in PI-superinfected animals, while the control group succumbed between 7 and 10 dpi with the Margarita strain, showing severe signs of CSFV and needed euthanasia.

The lack of innate and acquired immune responses, including lack of type I IFN detection, was also in accordance with the previous study II (Muñoz-González et al., 2015a). The absence of IFN-α detection probably was due to a specific CSFV- blockade of type I IFN, appearing as a mechanism of the persisting primary virus to evade the innate immune system. It is known the role of the pestiviral N^{pro} and E^{rns} proteins to prevent the induction of type I IFN (Lussi and Schweizer, 2016). For CSFV it has been demonstrated *in vitro* and *in vivo*, respectively (Hüsser et al., 2012; Tamura et al., 2014). In addition, BVDV block the induction but also is resistant to the antiviral role of IFN type I, without

compromise the antiviral activity against unrelated viruses (Peterhans and Schweizer, 2010). It would be interesting to know if the CSFV PI animals are also capable to generate an innate response against unrelated infections, or if we are facing a different mechanism of persistence within the genus *Pestivirus.*

To our understanding, no mammalian model had been able to demonstrate SIE so far. Thus, these findings demonstrate for the first time the ability of CSFV to induce SIE *in vivo*, in swine with persistent infection, and for the first time in a mammalian host. The SIE phenomenon also may explain the unresponsiveness of animals with persistent infection after vaccination with the live attenuated vaccine (Study II), commonly used in endemic countries to control the disease.

Following the BVDV example (Ståhl and Alenius, 2012), detection and elimination of chronically and unapparently CSFV infected pigs, and specially PI animals, should be one of the priorities for the success of CSF control programmes in endemic regions. This doctoral thesis opens new lines of research to understand the immunological bases and of viral pathogenesis for the generation of immunotolerance and persistence, hitherto unknown. Further studies will be required in order to confirm the existence of this form of disease in the field, as well as, to elucidate the virological and immunological mechanisms that trigger and maintain the postnatal persistent infection. In addition, future studies will be essential in order to comprehend the particular processes involved in the generation of SIE at both the cell and the systemic level.

Chapter 7

Conclusions

1. CSFV strains of low and moderate virulence are able to generate postnatal persistent infection in swine early after birth.

2. Persistently infected animals can remain in a good health status at least for six to nine weeks post infection. However, the clinical presentation of CSFV postnatally PI pigs, if present, is unspecific of CSF.

3. Postnatally PI piglets eliminate high and maintained constant viral load during the study, even in the absence of clinical signs. This fact may favour the prevalence of CSFV in the field. Considering the circulation of strains of low and moderate virulence, and the proven capacity they have to generate CSFV persistent infection, the real implication of PI animals in the endemic situation should be addressed.

4. Piglets that are not able to develop a specific humoral response after three weeks post CSFV infection are unable to generate antibodies against the virus, being CSFV persistently infected during the study. The PI piglets are not able to generate neither an acquired cellular nor humoral immune response against CSFV; therefore, these animals would remain undiagnosed under serological test.

5. CSFV strains of low and moderate virulence are also able to generate carrier animals in which the virus can persist in lymphoid tissue and can be excreted in the body secretions mainly by the rectal route, despite the presence of some levels of neutralizing antibodies titres.

6. Immature granulocytes, specifically $6D10^{+}$ cells, are the predominant cell population in the bone marrow and are targeted by CSFV in persistently infected piglets.

7. Rectal swab is a good candidate for CSFV RNA detection by RT-qPCR in order to detect PI piglets and CSF carriers animals, being a non-invasive method, easy to collect and feasible to use at the herd level.

8. After vaccination with a live attenuated vaccine, commonly used in endemic countries, the failure of PI animals to induce an innate or specific immune responses, and the absence of detectable CSFV live attenuated vaccine RNA by RT-qPCR in blood and tissues was likely due to the viral interference or SIE phenomenon.

9. After superinfection of PI animals with the CSFV Margarita strain, a highly pathogenic strain in naïve pigs, the absence of clinical signs, coupled with the lack of innate response, mainly in terms of Type I interferon, and the inability to induce CSFV specific immune response, suggested the ability of CSFV to generate SIE in vivo.

10. After superinfection of PI animals with the CSFV Margarita strain, the lack of secondary CSFV RNA detection in blood, rectal and nasal swabs and tissue samples, confirmed the SIE generation capacity of CSFV at systemic level in swine. This is the first demonstration of SIE in a mammalian host.

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