Virus Research 289 (2020) 198151

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Classical swine fever virus: the past, present and future

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ARTICLE INFO

Keywords:
Vaccine development
CSF
Virulence determinants
Disease control
Immunity
Taxonomy
Disease pathogenesis
Diagnostic tools
CSFV

ABSTRACT

Classical swine fever (CSF) is among the most relevant viral epizootic diseases of swine. Due to its severe economic impact, CSF is notifiable to the world organisation for animal health. Strict control policies, including systematic stamping out of infected herds with and without vaccination, have permitted regional virus eradication. Nevertheless, CSF virus (CSFV) persists in certain areas of the world and has re-emerged regularly. This review summarizes the basic established knowledge in the field and provides a comprehensive and updated overview of the recent advances in fundamental CSFV research, diagnostics and vaccine development. It covers the latest discoveries on the genetic diversity of pestiviruses, with implications for taxonomy, the progress in understanding disease pathogenesis, immunity against acute and persistent infections, and the recent findings in virus-host interactions and virulence determinants. We also review the progress and pitfalls in the improvement of diagnostic tools and the challenges in the development of modern and efficacious marker vaccines compatible with serological tests for disease surveillance. Finally, we highlight the gaps that require research efforts in the future.

1. Introduction

Classical swine fever (CSF) is considered one of the most relevant re-emergent viral diseases in swine (Postel et al., 2019; Xing et al., 2019). Considering its severe repercussions from an economic and sanitary standpoint, the disease is notifiable to the world organisation for animal health (OIE) (OIE, 2019a). The only natural reservoir of the aetiological agent, CSF virus (CSFV), are members of the *Suidae* family and the disease affects both domestic and wild pigs (Blacksell et al., 2006; Depner et al., 1995; Everett et al., 2011). CSFV (previously called Hog cholera virus) belongs to the *Pestivirus* genus within the *Flaviviridae* family, which also comprises the *Flavivirus*, *Hepacivirus* and *Pegivirus* genera (Simmonds et al., 2017, 2012). Other members of the *Pestivirus* genus, causing important diseases in animal health, include bovine viral diarrhoea virus-1 (BVDV-1), BVDV-2, and border disease virus (BDV).

CSFV is an enveloped virus of icosahedral symmetry and viral particles measure between 40–60 nm in diameter. The viral genome is a single-stranded positive-sense RNA of approximately 12.3 kb in length with a single open reading frame (ORF) surrounded by two untranslated regions (UTRs), the uncapped 5′-UTR carrying an internal ribosome entry site (IRES), and the uridine-rich 3′-UTR. The ORF encodes a polyprotein that is cleaved into four structural (capsid protein C, envelope glycoproteins E^{rns}, E1 and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Lamp et al., 2011; Meyers et al., 1989; Moormann et al., 1990; Rijnbrand et al., 1997; Rümenapf et al., 1993, 1991).

The first reports of CSF were from the Midwestern and Southern regions of the United States (US) and date back to 1810 in Tennessee. During the first half of the 19th century, outbreaks of CSF were reported in 10 different states in the US and, after 1860, the disease spread rapidly throughout the US territory, possibly related to the development of railways during the mid-century (Birch, 1922; Edwards et al., 2000).

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https://doi.org/10.1016/j.virusres.2020.198151
Received 29 May 2020; Received in revised form 24 August 2020; Accepted 28 August 2020
Available online 6 September 2020

The first reliable reports of CSF in Europe date back to 1862 in England. From there, the disease spread to Sweden, France and Denmark (Birch, 1922), and from the 1960s on, it was present worldwide (Cole et al., 1962).

Recently, a reconstruction of the viral-host co-evolutionary history of the *Pestivirus* genus showed that, based on the time for the most recent common ancestor, CSFV emerged towards the end of the 18th century (Rios et al., 2017). It was hypothesized that this was likely due to a jump of Tunisian sheep virus (TSV) from the host *Ovis aries* to the new host, *Sus scrofa*. Coincidentally, the first import of Tunisian sheep to the US dates back to 1799 in Pennsylvania (Brier, 2013). The breed became very popular throughout the US, including some of the regions where CSF was reported for the first time (Carman et al., 1892; Peters, 1810). At that time, it was a common practice to keep animals of different species together in the same herds, favouring the hypothetical cross-species transmission of TSV to swine. The close genetic relationship of CSFV with TSV and with other ovine pestiviruses described recently, supports that CSFV may have originated by a spillover of pestiviruses from sheep to pig (Postel et al., 2015; Rios et al., 2017; Sozzi et al., 2019; Wang et al., 2020).

Since its emergence, CSF has always generated important economic losses in the swine industry. These are due to the high morbidity and mortality of the virus and to the severe restrictions imposed on pig and pork-derived products and trade (Meuwissen et al., 1999; Saatkamp et al., 2000). In addition, for an affected country or region, it is a complex and costly process to recover a CSF-free status recognized by the OIE. A progressive eradication programme has been implemented in Europe since the 1990s (Paton and Greiser-Wilke, 2003), which included strict serological surveillance accompanied by stamping out of infected and contact animals under a non-vaccination policy for domestic pigs (Anonymous, 2001). The costs resulting from the last two CSF outbreaks in Spain (1997 and 2001) were around 108 million Euros combined (Fernández-Carrión et al., 2015). The outbreak in 1997/98 in the Netherlands had a total estimated cost of over 2 billion US$. The majority of these costs were related to "welfare sacrifice", in which healthy animals outside of the control zones had to be euthanized due to the movement restrictions (Elbers, 2002; Meuwissen et al., 1999). Notably, the ethical and animal welfare implications of such practices were controversial.

Currently, the OIE list of CSF-free territories consists of 38 countries, including all of North America, Oceania, as well as a large part of the European Union (EU) (OIE, 2019b). CSF remains endemic in Asia, South and Central America and the Caribbean, while the OIE has declared specific regions of Brazil, Colombia and Ecuador free of the disease, even if the countries remain endemic (OIE, 2019b). The CSF situation in Africa is largely unknown, even though outbreaks have been reported in Madagascar and South Africa (Ji et al., 2015; Sandvik et al., 2005). According to the world animal health information system (WAHIS), the most recent CSF outbreaks have been reported in Korea, Colombia, Russia, Brazil and Japan (OIE, 2019c). In the case of Japan, after 26 years of CSF-free status, the disease has re-emerged in both domestic pigs and wild boar (OIE, 2019c; Postel et al., 2019). In contrast to the policy adopted by the majority of EU countries for the eradication of CSF in the past, the Japanese government has implemented preventive vaccination in domestic pigs for the control of the recent CSFV outbreak (Isoda et al., 2020). However, similarly to Europe, in Japan, the infected wild boar populations represent a risk for domestic pigs (Fritzemeier et al., 2000; Ito et al., 2019). Additionally, the endemic countries pose a constant challenge for surveillance programs in CSF-free territories. Thus, CSFV remains an endemic and re-emerging virus in pigs and continues to threaten pork production worldwide and the food security of populations in developing countries. Considering the ongoing importance of CSF to the global pig industry, the present review aims to provide a comprehensive and updated overview of; CSFV taxonomy and evolution, CSF pathogenesis and host immunity, virus-host interactions and viral virulence, the state-of-the-art in CSFV diagnostics and, finally, progress and perspectives in vaccine development.

2. Taxonomy of CSFV and related pestiviruses

In contrast to BVDV-1, BVDV-2 and BDV, which infect a wide range of ruminant species and pigs, CSFV has a narrow host range restricted to domestic pigs, wild boar as well as other members of the family *Suidae* (Everett et al., 2011; Postel et al., 2018). Due to a growing number of novel pestiviruses detected in pigs, in domestic and wild ruminants, and in other mammalian host species not belonging to the order *Artiodactyla*, the taxonomy of the genus *Pestivirus,* including the nomenclature of the different *Pestivirus* species, has been revised recently by the *Flaviviridae* Study Group of the International Committee on Taxonomy of Viruses (Simmonds et al., 2017; Smith et al., 2017). Seven novel species were added to the four approved members (BVDV-1, BVDV-2, CSFV, and BDV) of the genus, and today *Pestivirus* species are named in a host-independent manner using the format *Pestivirus* X, which results in the eleven recognized species *Pestivirus* A to K (Smith et al., 2017). These changes refer to the nomenclature of virus species names, while the original virus names for the established pestiviruses and virus isolates are maintained. In addition to these eleven species, several novel tentative *Pestivirus* species were found recently in pigs, ruminants, rodents, bats, whales, and pangolins (Becher et al., 2020; Firth et al., 2014; Gao et al., 2020; Jo et al., 2019; Lamp et al., 2017; Sozzi et al., 2019; Wu et al., 2018, 2012). Under natural conditions, pigs can be infected with CSFV (*Pestivirus C*), BVDV-1 and -2 (*Pestivirus A* and *B*), BDV (*Pestivirus D*), and atypical porcine pestivirus (APPV, *Pestivirus K*). Moreover, genetically distinct pestiviruses like Bungowannah virus (*Pestivirus F*) and Linda virus can infect pigs and have caused unique disease episodes in single farms characterized by myocarditis and congenital tremor, respectively (Kirkland et al., 2007; Lamp et al., 2017). So far, attempts to confirm the presence of similar viruses in domestic pig and wild boar failed, and the natural hosts of Bungowannah virus and Linda virus remain unknown (Cagatay et al., 2018). The most widely distributed *Pestivirus* in domestic pigs and wild boar is APPV followed by CSFV, while infections of pigs and wild boar with ruminant pestiviruses are rare events (Becher et al., 2020; Postel et al., 2018, 2017b).

3. Genetic variability of CSFV

Replication of viral RNA genomes by CSFV and other pestiviruses is mediated by viral RNA-dependent RNA polymerases (RdRP) lacking proofreading activity (Becher and Tautz, 2011). The accumulation of point mutations under selective pressure represents the major driving force for CSFV evolution resulting in the emergence of genetically highly variable CSF viruses. Three major CSFV genotypes (1, 2, and 3) have been described. Genotype 1 can be further divided into seven subgenotypes (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7), while three subgenotypes (2.1, 2.2, 2.3) have been reported for genotype 2 (Garrido Haro et al., 2018; Postel et al., 2013b, 2012; Silva et al., 2017). More recently, it has been proposed to rename previously identified subgroups within subgenotypes 2.1 and 2.2, resulting in the segregation of genotype 2 CSFV strains into seven subgenotypes (2.1–2.7) and the establishment of two additional genotypes (4 and 5) (Rios et al., 2018). These latter two genotypes comprise the distantly related CSFV strain "congenital tremor" (Great Britain/1964) and two strains from Korea (KR/1998, KR/1999) (Fig. 1).

Molecular typing of CSFV isolates allows discrimination of virus isolates and can help trace the source of an outbreak. Historically, genetic typing of CSFV isolates was based on phylogenetic comparison of rather small viral genomic regions including a 150 nucleotide (nt) sequence of the 5′-UTR, a partial E2 encoding sequence of 190 nt, or a 409 nt fragment from the polymerase gene (Lowings et al., 1996; Paton et al., 2000; Vilček et al., 1996). Comparison of such short sequences provided usually the allocation of virus isolates to one of the three major genotypes and in many cases to a defined subgenotype. Determination of

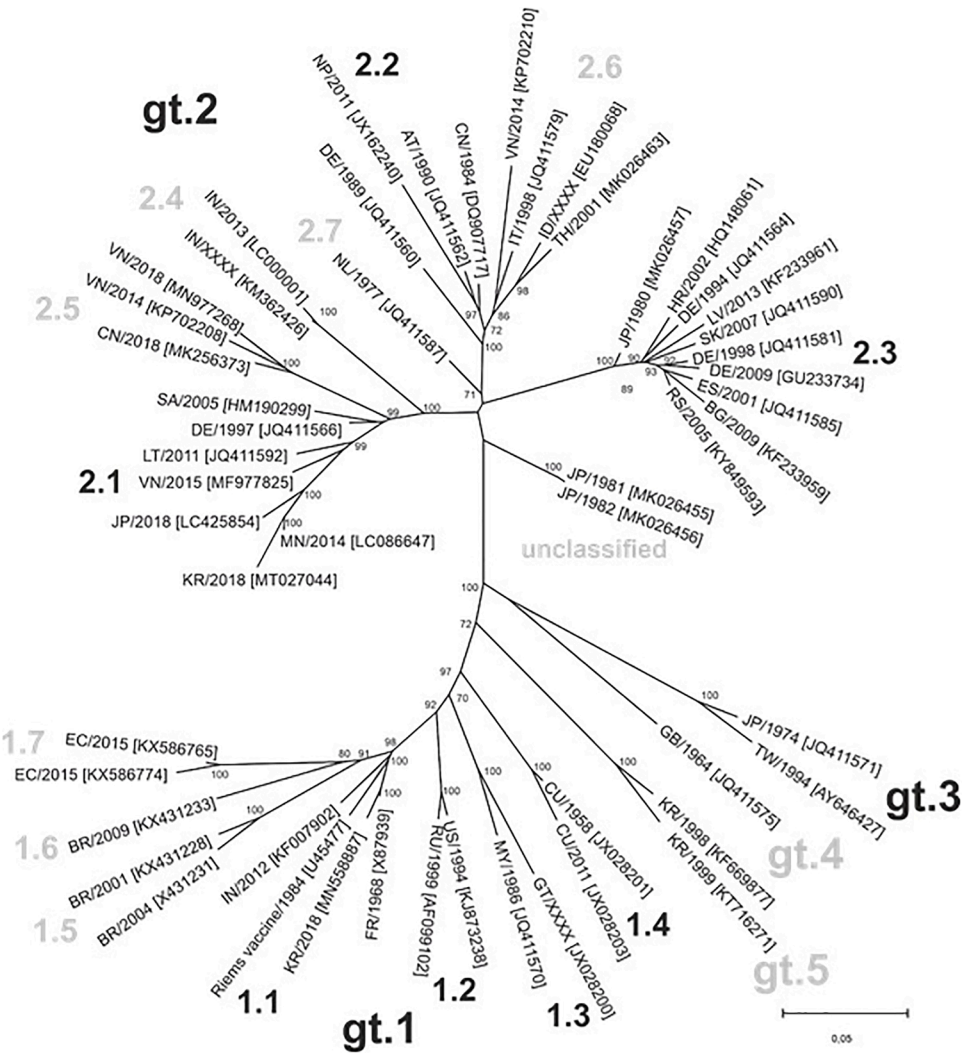

Fig. 1. Genetic variability of CSFV. For phylogenetic analysis, 53 complete E2 encoding sequences (1119 nt) were aligned by Clustal W and genetic distances were calculated with the Kimura 2-parameter substitution model using the Mega X software. Maximum likelihood construction of the tree included 100 repetitions for bootstrap analysis (values of 70 or higher are indicated). For each sequence, country of origin, year of isolation and GenBank number are given. Indicated are genotypes (gt.) 1-5 and corresponding subgenotypes (1.1-1.7, 2.1-2.7); gt.4, gt.5 as well as subgenotypes 1.5-1.7 and 2.4-2.7 recently proposed by Rios et al. (2018) are shown in grey.

longer sequences has become more feasible, and the use of complete E2 encoding sequences was recommended for more detailed and statistically sound phylogenetic analyses, resulting in more reliable discrimination of closely related CSFV isolates obtained during epidemiologically linked outbreaks (Postel et al., 2012). Today, phylogenetic analysis of this genomic region represents the commonly accepted standard for genetic typing of CSFV isolates (Garrido Haro et al., 2018; Jiang et al., 2013; Postel et al., 2019, 2013a, 2013b, 2012, Rios et al., 2018, 2017; Silva et al., 2017; H. Zhang et al., 2015a, 2015b). A large number of CSFV sequences, including more than 400 complete E2 encoding sequences, is deposited in the CSF database at the EU & OIE Reference Laboratory for CSF at the University of Veterinary Medicine in Hannover, Germany and can be accessed upon request (Postel et al., 2016). Moreover, high-throughput sequencing has facilitated the establishment of complete viral genomic sequences, and subsequent analyses can even provide insights into the role of viral quasispecies (Fahnøe et al., 2014; Töpfer et al., 2013).

4. Pathogenesis and immunity

4.1. CSF pathogenesis

CSFV is transmitted through the oronasal route, by direct or indirect contact with infected pigs, as well as through consumption of contaminated feed. Vertical transmission from infected sows to their offspring can also occur (Moennig et al., 2003). Considering that CSFV is shed from all mucosal surfaces and has also been found in semen, the virus may also be transmitted through insemination (Floegel et al., 2000). CSFV has also been reported to survive over long periods in cooled and frozen meat and pork derivate products, which can be reservoirs for the virus (Edwards, 2000).

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

CSFV has a particular affinity for endothelial cells and the mononuclear phagocyte system, *i.e.* macrophages and dendritic cells (DCs) (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 a vehicle for virus spread in the organism (Jamin et al.,

2008). Likewise, the virus exploits the migratory ability of granulocytes and macrophages, using these cells as a "Trojan horse" for its dissemination to other organs in the body (Carrasco et al., 2004; Muñoz-González et al., 2015b).

CSFV infection leads to a breakdown of the immune system which, accompanied by an aberrant pro-inflammatory response (referred to as a "cytokine storm"), is unable to control disease progression (Knoetig et al., 1999; Sánchez-Cordón et al., 2005b, 2002; Summerfield et al., 2006; Tarradas et al., 2010). 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., 2003; 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 (Gómez-Villamandos et al., 2003), with an altered population of T cells and depletion of lymphocytes (Pauly et al., 1998; Van Oirschot et al., 1983), mainly CD4+ and CD8+ T cells (Summerfield et al., 2001). In this regard, CSFV infection promotes marked bystander apoptosis of the surrounding uninfected B and T cells, by mechanisms that are still not completely understood, contributing to the strong immunosuppression and high mortality rates (Carrasco et al., 2004; Ganges et al., 2008; Summerfield et al., 2001, 1998b; Susa et al., 1992). Depending on the virulence of the CSFV strain, pigs can have up to 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 viremia has been established (Summerfield et al., 1998b), 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., 2008; Pauly et al., 1998; Summerfield et al., 2000, 1998b). CSFV infection has been related with interleukin (IL)-10 production by CD4-/CD8+ T cells, which might be implicated too in the immunosuppression observed after infection (Muñoz-González et al., 2015b; Suradhat et al., 2005).

In advanced phases of the disease granulocytopenia is observed, which is followed by the circulation of immature precursors in peripheral blood (Bohórquez et al., 2019a; Ganges et al., 2005; Muñoz-González et al., 2015b; Nielsen et al., 2010; Summerfield et al., 1998a). In the bone marrow, the main targets of infection are immature myeloid cells SWC3+/SWC8-, as well as the less-differentiated SWC3low/SWC8- myeloid precursor cells, which differentiate towards SWC8+ granulocytic and 6D10+ cells (Muñoz-González et al., 2015b; Summerfield et al., 2001, 2000). This finding explains the occurrence of infected peripheral blood granulocytes during CSF (Bohórquez et al., 2019a; Summerfield et al., 2001, 1998a).

4.2. Humoral and cellular immunity against CSFV

After infection, the increase of interferon (IFN)-γ-secreting T lymphocytes, consisting mainly of CD4-/CD8+ effector cytotoxic T lymphocytes (CTLs), contributes to the early control of viral replication and protects against leukopenia before the onset of neutralizing antibodies (Franzoni et al., 2013; Graham et al., 2012a; Piriou et al., 2003; Tarradas et al., 2010). In addition, activated CD4+/CD8+/CD25+ memory T cells contribute to the early control of viral replication. The CD4+ T cells and the CTLs are induced from one to three weeks after CSFV infection (Ganges et al., 2008; Piriou et al., 2003). The E2 and NS3 viral proteins are the main inducers of the specific CTL response (Ceppi et al., 2005; Ganges et al., 2008; Graham et al., 2012b).

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 (Sánchez-Cordón et al., 2005a). Despite the strong activation of T cell immunity, this response has only been related to partial protection against CSFV infection (Franzoni et al., 2013; Graham et al., 2012b; Summerfield and Ruggli, 2015; Tarradas et al., 2014, 2011).

It is worth noting that the neutralizing antibody (Ab) response achieves sterilizing protection against CSFV (Blome et al., 2017a; Ganges et al., 2008, 2005; van Oirschot, 2003v). CSFV neutralizing Ab titres higher than 1/32 can be protective, preventing viral excretion and transmission (Terpstra and Wensvoort, 1988). The antibodies induced in infected pigs recognize E^{rns}, E2 and NS3 proteins (Graham et al., 2012b; Greiser-Wilke et al., 1992; König et al., 1995; Weiland et al., 1992). Anti-NS3 antibodies cross-react with NS3 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 (König et al., 1995; Terpstra and Wensvoort, 1988; Van Rijn et al., 1996) and are the only antibodies able to confer clinical protection against CSFV challenge (Rümenapf et al., 1991). Therefore, this protein is the main target for the development of recombinant vaccines against CSFV and other pestiviruses.

Maternal-derived antibodies (MDA) can be transmitted by sows to their offspring *via* colostrum, which can protect piglets against disease during the first weeks of life, although MDA do not prevent CSFV replication and excretion completely (van Oirschot, 2003v). As piglets grow older, the protection by MDA decreases (van Oirschot, 2003v; Vandeputte et al., 2001). However, MDA can interfere with the development of vaccine-induced immunity, an aspect to keep in mind for immunisation schedules (Blome et al., 2017a).

5. The clinical forms of CSF

The clinical signs of CSF are highly variable and determined by the virulence of the viral strain, the host's immune responses, the age, breed, genetic background, the general health condition of the pigs, as well as by concomitant infections (Belák et al., 2008; Borca et al., 2019; Cao et al., 2018; Ganges et al., 2008; Tarradas et al., 2014; Trautwein, 1988; von Rosen et al., 2013v). The clinical forms of CSF can follow peracute, acute, subacute, chronic or unapparent subclinical courses (Ganges et al., 2008; van Oirschot and Terpstra, 1989v). The majority of experimental studies focus on the characterization of acute disease after inoculation with virulent strains, characterized by a short incubation period, accompanied by scarce signs, and rapid mortality within a few days after exposure (Belák et al., 2008; Dune, 1973; Hüsser et al., 2012; Knoetig et al., 1999; Tarradas et al., 2014). The chronic and unapparent forms of disease are observed mostly in endemic areas, where they may be misdiagnosed and become a source of new CSF outbreaks (Coronado et al., 2019a; Floegel-Niesmann et al., 2003; Ji et al., 2014; Pérez et al., 2012; Shen et al., 2011; Wensvoort and Terpstra, 1985).

5.1. The acute form of CSF

Acute CSF is caused typically by highly virulent strains, although strains of moderate virulence can also trigger this form of infection. It is more frequent in piglets up to 12 weeks of age, while milder forms are usually observed in older pigs (Moennig et al., 2003; Muñoz-González et al., 2017). After a short incubation period (two to six days after exposure), pigs develop a sustained pyrexia (>40 °C) (Belák et al., 2008; Mittelholzer et al., 2000; Moennig et al., 2003; Tarradas et al., 2014). In peracute cases, no gross changes are found at necropsy. Highly virulent CSFV strains cause marked immunosuppression and high mortality (Gómez-Villamandos et al., 2003; Lee et al., 1999; Susa et al., 1992). Leukopenia (less than 8000 cells/μl in blood) appears rapidly, even before the animals show fever or viremia (Stegeman et al., 2000). In addition, thymus atrophy has also been described in acute forms of CSF, wherein massive lymphoid depletion was also found due to lymphocyte apoptosis (Sánchez-Cordón et al., 2002).

As a result of central nervous system infection, pigs show progressive depression and uncoordinated movements (Ganges et al., 2005; Moennig, 2000). The most characteristic feature is the haemorrhagic syndrome, including petechiae of the skin, mucosae, and cyanosis of the

abdomen, ears, snout and medial side of the extremities. Clinical findings include conjunctivitis, constipation followed by severe diarrhoea ("cholera"), tremor, locomotive disturbance, reluctant walking, swaying movement of the hindquarter, posterior paresis, mild to severe convulsions and prostration in the terminal phase (Mittelholzer et al., 2000; Tarradas et al., 2014; van Oirschot, 2004v). The lethality is close to 100 %, with death occurring between 10–20 days post-infection (Dune, 1975; Moennig et al., 2003). In general, the typical pathologic lesions consist of swollen or haemorrhagic lymph nodes and petechial bleedings of the tonsils and the inner organs and serosa, especially the gastro-intestinal tract, kidneys, spleen and lymph nodes (Belák et al., 2008; Gómez-Villamandos et al., 2003, 2000). In peracute/acute cases however, these pathologic lesions can often be inconspicuous or inexistent. The subacute form manifests also with pyrexia, diarrhoea, central nervous disorders, but the symptoms are less severe and lethality is lower (Floegel-Niesmann et al., 2009a, 2003). The surviving animals generate long-lasting and robust humoral immunity, characterized by the presence of neutralizing antibodies appearing after two weeks post-infection (Chander et al., 2014; Moennig et al., 2003).

5.2. The chronic form of CSF

In general terms, an infection is considered as chronic when the survival of infected animals exceeds 30 days (Dune, 1975; Liess, 1984; Muñoz-González et al., 2015b). The chronic form of CSF occurs usually when the pigs are not able to develop an effective immune response against the infection (Petrov et al., 2014a; Tarradas et al., 2014). Considering that, chronic infections can be established in the presence of neutralizing antibodies (Mengeling and Packer, 1969). The chronic form of the disease manifests with stunting, anorexia, and intermittent pyrexia and diarrhoea. In a first phase, the clinical picture can be similar to the acute form of CSF. After overcoming the initial phase, clinical signs can disappear and animals can appear apparently healthy (Mengeling and Cheville, 1968). Over time, however, the disease progresses with nonspecific signs, with the re-occurrence of intermittent fever, diarrhoea and wasting, which are not always easy to identify in the farm (Moennig et al., 2003). CSFV is shed from the onset of clinical signs until death. The affected animals can survive up to 2–3 months after exposure (Weesendorp et al., 2011).

In the chronic form, the Ab response is insufficient to eliminate the virus. The antibodies are not always detectable because they are bound by the virions. These immune complexes can deposit in the kidney and cause a characteristic glomerulonephritis (Choi and Chae, 2003a, 2003b; Gómez-Villamandos et al., 2000). The post-mortem changes are not very characteristic of the disease and haemorrhagic lesions may not always be present. However, it is quite common to find thymus atrophy (Cheville and Mengeling, 1969), ulcerative and necrotic lesions ("button ulcers") in the ileocecal valve in animals with chronic diarrhoea, as well as along the ileum and colon (Blome et al., 2017b). Necrotic ulcers are also common in epiglottis and larynx. Secondary bacterial infections are frequent (Cheville and Mengeling, 1969; Choi and Chae, 2003a, 2003b; Coronado et al., 2019a). Overall, the rather unspecific clinical signs and pathologic findings in this form may be confused with other diseases, that should be included in the differential diagnosis (Elbers et al., 2003; Moennig et al., 2003; Rout and Saikumar, 2012).

5.3. Congenital persistent CSFV infection

It is well established that transplacental transmission of CSFV can lead to the generation of persistently infected offspring, especially if the infection takes place during mid-gestation, a phenomenon known as "carrier sow syndrome" (Aynaud et al., 1977; Carbrey et al., 1977; Liess, 1984; Van Oirschot, 1979a, 1979b; Van Oirschot and Terpstra, 1977). Moreover, depending on viral virulence and on the time of infection during gestation, transplacental infection may result in abortion, stillbirth, mummification, malformations, or in the birth of weak, or apparently healthy, piglets persistently infected with CSFV (Bohórquez et al., 2020; Trautwein, 1988).

Persistently infected piglets are not able to induce Ab responses against CSFV and they have lifelong viremia (Bohórquez et al., 2020; Coronado et al., 2019a; de Smit et al., 2000d; Van Oirschot, 1979b). Even though they may appear clinically healthy at birth, or may show unspecific clinical signs, such as poor growth, wasting or occasionally congenital tremor, they invariably die from CSF. The development of mild anorexia, depression, conjunctivitis, dermatitis, diarrhoea, runting, and locomotive disturbance leading to paresis and eventually death, may take several months (Trautwein, 1988). Survival for up to 11 months after birth has been reported (Coronado et al., 2019a; Van Oirschot and Terpstra, 1977). This course of infection is referred to as "late-onset CSF" (van Oirschot and Terpstra, 1989). Persistently infected piglets act as a viral reservoir and spread the virus while remaining undetected in serological tests (Coronado et al., 2019a; van Oirschot, 2004v; Van Oirschot and Terpstra, 1977). These animals are refractory to vaccination and contribute to virus circulation in regions where the disease is endemic (Coronado et al., 2019a). The occurrence of persistently infected animals is favoured by the prevalence of lower virulence strains in these regions as a result of a positive selection pressure exerted by inefficient vaccination (Coronado et al., 2019b; Ji et al., 2014; Rios et al., 2017).

The mechanisms underlying the establishment of congenital CSFV persistent infection have been related to a specific immunotolerance to the virus (Carbrey et al., 1977; Trautwein, 1988; Van Oirschot and Terpstra, 1977; Vannier et al., 1981). This immunotolerance was attributed to a lack of pathogen recognition due to the immature state of the fetal immune system. However, it was demonstrated recently that fetuses are able to generate an innate immune response against CSFV after transplacental transmission, in terms of IFN-α induction, indicating that the virus can be recognized as a pathogen by the fetal innate immune system (Bohórquez et al., 2020). This suggests that the immunological phenomena involved in the establishment of congenital persistent CSFV infection is more complex than previously thought and warrants further study (Van Oirschot, 1979b; Van Oirschot and Terpstra, 1977).

Notably, immunosuppressive cell populations, known as myeloid-derived suppressor cells (MDSC), were found to be increased in cord blood and neonatal peripheral blood, playing a homeostatic role in maternal-fetal tolerance (Rieber et al., 2013). These cell populations may favour the establishment of CSFV persistent infection in fetuses (Bohórquez et al., 2019a).

5.4. Postnatal persistent CSFV infection

The capacity of CSFV to induce viral persistence following postnatal infection has also been demonstrated in both domestic pigs and wild boars (Cabezón et al., 2015; Muñoz-González et al., 2015b). This form of disease has been described after infection of newborn piglets, and animals up to three weeks of age, with a moderately virulent CSFV strain (Bohórquez et al., 2019b; Muñoz-González et al., 2015b). Piglets that develop persistent CSF after postnatal infection will appear clinically healthy or have unspecific clinical signs while showing high viral replication and shedding in the absence of adaptive immune response against the virus. By contrast, the innate immune response to the virus, in terms of IFN-α in serum, is not affected in these persistently infected piglets at seven days post-infection. However, the IFN-α pathway is subsequently impaired in these animals as they fail to mount an IFN-α response six weeks after infection, even against pathogens such as African swine fever virus that induce exacerbated IFN-α responses (Cabezón et al., 2017). The lack of immune responses in persistently infected pigs has been related to immunosuppression, since these animals were not able to elicit any immune response, neither against CSFV nor other antigens (Muñoz-González et al., 2015b).

Age plays an important role for the pathogenesis of postnatal

persistent CSFV infection: a lower proportion of offspring were persistently infected when the infection was carried out at three weeks of age, compared with infection a few hours after birth (Bohórquez et al., 2019b; Muñoz-González et al., 2015b). In CSFV postnatal persistently infected piglets a low CD4/CD8 ratio was detected (Bohórquez et al., 2019b). This suggests that these animals may be in a state of immune exhaustion, a phenomenon in which chronic stimulation of the immune system by a pathogen leads to its premature aging and to differentiation of cells to terminal states (Yao and Moorman, 2013). In addition, it has been reported that precursor myeloid cell populations were increased in the bone marrow and peripheral blood of piglets with postnatal persistent CSFV infection (Bohórquez et al., 2019a; Muñoz-González et al., 2015b). These cells were found to be phenotypically and functionally similar to immunosuppressive MDSC populations found in humans (Bohórquez et al., 2019a). In particular, the phenotype of the 6D10+/CD11b+/CD33+ MDSC found in persistently infected piglets indicated that these cells belong to the polymorphonuclear-MDSC (PMN-MDSC) subset and not the monocytic-MDSC (M-MDSC) (Fig. 2). MDSC subsets have been found to play a role during persistent infection in humans and recently have been implicated in impairing immune checkpoint therapy in cancer (Serrano-Villar et al., 2014; Tacke et al., 2012; Weber et al., 2018). As previously mentioned, MDSC are increased in cord blood and during neonatal stages, which may explain the higher proportion of persistently infected piglets when the animals are infected with CSFV a few hours after birth compared to infection of 3 week-old animals. Moreover, the immunomodulatory capabilities of MDSC may block the onset of exacerbated immune responses, despite the high viral replication in persistently infected animals, favouring the survival of these animals for long periods without any clinical signs associated with CSF. Nevertheless, further studies are needed to gain an in-depth

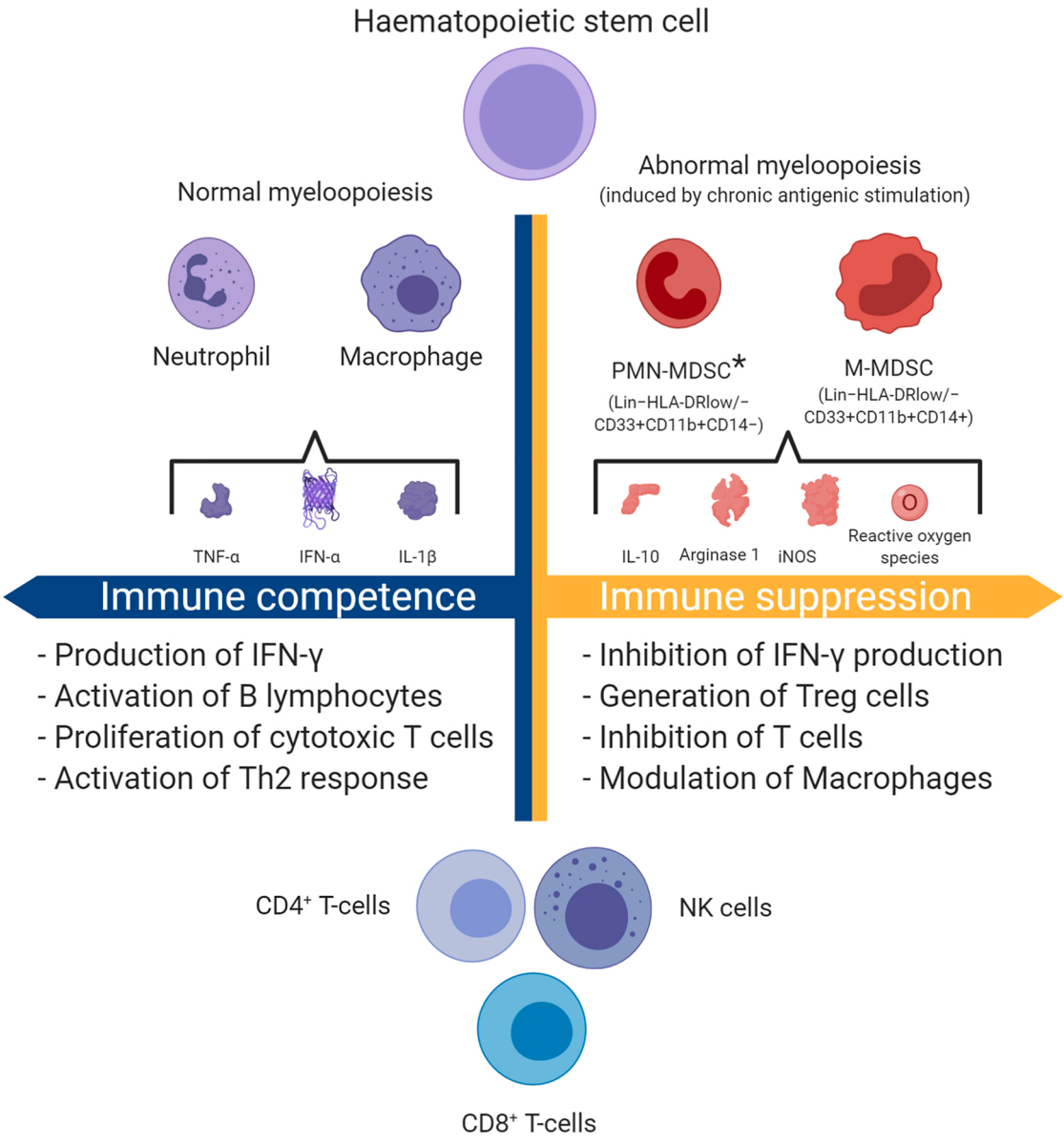

Fig. 2. Schematic representation of the generation and mechanisms of immune suppression of MDSC populations. Haematopoietic stem cells can be differentiated into cell populations promoting a pro-inflammatory status in the animal following stimulation by an antigen (left side, purple cells and cytokines). When the immune response does not lead to the elimination of the pathogen, the chronic antigenic stimulation may lead to abnormal myelopoiesis, resulting in the generation of MDSC populations with immunomodulatory properties (right side, red cells and cytokines). The phenotype indicated in the graphic corresponds to the cellular markers attributed to MDSC populations in humans. The specific mechanisms of pro-inflammatory and immunomodulatory cell populations are detailed in the lower part of the graphic. The asterisk indicates cells that have been characterized in swine during postnatal persistent CSFV infection (phenotype 6D10+ CD33+ CD11b+) according to (Bohórquez et al., 2019a). Image created with Biorender.com.

understanding of the role of immune exhaustion and MDSC in the pathogenesis of postnatal persistent CSFV infection, as well as their possible therapeutic roles against viral infections in animals and humans.

5.5. CSFV and the superinfection exclusion (SIE) phenomenon

The SIE phenomenon, or homologous interference, is defined as the capacity of a primary viral infection to interfere with a secondary infection by the same or a closely related virus (Folimonova, 2012; Karpf et al., 1997; Y.-M. Lee et al., 2005a, 2005b; Ramírez et al., 2010). This phenomenon fulfils a conservative strategy, from an evolutionary point of view, reducing the likelihood of recombination events between related strains, favouring the stability of viral sequences within the same cell (Formella et al., 2000; Huang et al., 2008; Lee et al., 2005a, 2005b). SIE has been applied in the treatment and prevention of viral infections. A widely accepted practice is the cross-protection of crops by purposeful infection with milder virus isolates, which is an effective and economical antiviral management strategy (Gal-On and Shiboleth, 2006). It has also been proposed that transplantation of hepatitis C virus (HCV)-infected liver grafts may be useful for the treatment of HCV-infected patients, considering that SIE would prevent re-infection of the transplanted organs, whereas healthy organ transplants would be damaged rapidly (Y.-M. Lee et al., 2005a, 2005b; Webster et al., 2013). With pestiviruses, acute infection of cells with BVDV protected them from a second infection by a homologous BVDV strain (Y.-M. Lee et al., 2005a, 2005b). Also, cells persistently infected with CSFV were resistant to superinfection and protected from infection by a cytopathic strain (Mittelholzer et al., 1998).

Even though multiple studies have demonstrated the SIE phenomenon *in vitro*, very few reports have proven the occurrence of SIE at the organism level (Bergua et al., 2014; Campbell et al., 2014; Walters et al., 2004). An interesting finding was that piglets persistently infected with CSFV after postnatal infection were refractory to vaccination with a lapinised CSFV C-strain vaccine (Muñoz-González et al., 2015a). The fact that vaccine RNA could not be detected by vaccine-specific RT-qPCR in these animals suggested a phenomenon of SIE, in which the high viral load of the primary persistent infection was able to block a secondary infection by the CSFV vaccine strain. A separate study found that such persistently infected piglets were protected from acute disease after inoculation with the highly virulent CSFV Margarita strain, indicating an efficient suppression of the secondary infection in the persistently infected animals (Muñoz-González et al., 2016). The absence of IFN-α and the lack of IFN-γ producing cells in the persistently infected animals after the secondary infection with the Margarita strain indicated a state of immunosuppression, which suggests SIE in these animals (Cabezón et al., 2017, 2015, Muñoz-González et al., 2015a, 2015b). This reduction of type I and II IFN responses may promote the maintenance of a high and constant CSFV load, preventing the secondary viral entry.

Even though the SIE mechanism needs to be studied in greater depth, it cannot be ruled out that it is playing a role in CSFV evolution, likely favouring the prevalence of strains with high replication capacity (Huang et al., 2020; Mittelholzer et al., 1998; Muñoz-González et al., 2016). Likewise, it may also have an impact on disease control, particularly by affecting the efficacy of vaccination with live attenuated vaccines. The model of persistent CSF after early postnatal infection constitutes the first demonstration of the SIE mechanism at a systemic level in a mammalian host. A better understanding of the mechanisms behind this phenomenon and its potential future applications require further studies.

6. Virus-host interaction

6.1. The virus life cycle

Among the three *Pestivirus* surface glycoproteins, E2 and E^{rns} mediate attachment and interaction with the cellular receptors, while E1 is involved in fusion and entry (S. Li et al., 2017a, 2017b; Tautz et al., 2015; F.-I. Wang et al., 2015a, 2015b). As for many viruses, initial attachment of pestiviruses with the host cell occurs *via* heparan sulphate (HS)-containing glycosaminoglycans, which is a rather unspecific mechanism mediated essentially by E^{rns} (Cheng et al., 2019; Eymann-Häni et al., 2011; Hulst et al., 2000; Iqbal and McCauley, 2002; van Gennip et al., 2004v). In addition to HS binding, E^{rns} attaches also to the laminin receptor (Chen et al., 2015). CD46 plays also a role in CSFV entry but its receptor function through interaction with E2 is not as clear as for BVDV (Dräger et al., 2015; Maurer et al., 2004). While antibodies against CSFV E2 can prevent cell entry completely, antibodies against porcine CD46 can only partially inhibit infection, even when combined with HS blockers, suggesting a contribution of additional co-receptors (Dräger et al., 2015). Integrin β3 is also required for efficient infection and replication, and CSFV induces its upregulation in vascular endothelial cells (W. Li et al., 2014a, 2014b; Tang et al., 2010).

After binding to the cell surface receptors, CSFV enters *via* caveolin-1 and clathrin-mediated endocytosis, a pH-dependent process that involves dynamin and cholesterol and depends on the small GTPases Rab5, 7 and 11 (Ning et al., 2016; Shi et al., 2016; Y.-N. Zhang et al., 2018a, 2018b, 2018c). E2 plays a central role in CSFV entry and membrane fusion since two fusion peptides were identified on E2 (Fernández-Sainz et al., 2014; Holinka et al., 2016). It acts probably as a heterodimer with E1, as suggested by a study with BVDV (Ronecker et al., 2008) and reviewed elsewhere (F.-I. Wang et al., 2015a, 2015b). Recently, the membrane protein MERTK, a member of the TAM (Tyro3, Axl and MerTK) receptor tyrosine-protein kinases, was found to promote virus entry through interaction with E2 (Zheng et al., 2020). E2 interacts also with β-actin, which may further assist virus entry and early replication (F. He et al., 2014a, 2014b). There is also increasing evidence for the importance of cellular cholesterol in CSFV entry. Disruption of lysosomal cholesterol trafficking inhibits CSFV replication upstream of membrane fusion and RNA replication, suggesting virion trafficking through endolysosomal pathways after endocytosis (Liang et al., 2019). Accordingly, depletion of cellular cholesterol with methyl--β-cyclodextrin does significantly inhibit CSFV infection, which can be reverted by exogenous addition of cholesterol, supporting the engagement of cellular cholesterol in the CSFV life cycle (Yu et al., 2019).

Following entry and uncoating, a defined sequence of events in the biosynthesis of the CSFV nonstructural proteins orchestrates *Pestivirus* replication (Lamp et al., 2011; Tautz et al., 2015). Self-replicating genomes (replicons) with gene deletions and reporter gene insertions were used in *trans*-complementation approaches to determine the minimal viral genome and *cis*-requirements of the viral proteins for genome replication (Behrens et al., 1998; Liang et al., 2009; Moser et al., 1999; Risager et al., 2013). This defined the 5-' and 3′-UTRs and the replicase proteins NS3 to NS5B as the minimal *Pestivirus* replicon. The crystal structure of NS5B, the RdRP with the canonical GDD motif was solved for BVDV and more recently for CSFV (Choi et al., 2006; W. Li et al., 2018a, 2018b). This latter study identified a novel fold in the N-terminal domain of CSFV NS5B, whose N-terminal and palm domain interact and modulate RdRP fidelity (W. Li et al., 2018a, 2018b; W. Liu et al., 2018a, 2018b). The complex virus-host interactions that regulate CSFV replication were reviewed in (S. Li et al., 2017a, 2017b). Recently, it was shown that the ribosomal protein L13 is a critical regulator of IRES-driven translation, which is not specific to CSFV, as it was also observed for picornaviruses (Han et al., 2019). Also, for CSFV and BDV, the IIId2 sub-domains of the IRES are required for 80S ribosomes assembly and IRES activity (Willcocks et al., 2017). The association of the eukaryotic translation initiation factor 3 subunit E with NS5A facilitates CSFV replication (X. Liu et al., 2018a, 2018b). The Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1, as well as Rab2 and Rab5 were also associated with CSFV proliferation (Liang et al., 2017; J. Lin et al., 2017a, 2017b).

Finally, apart from N^{pro} and NS4B, all structural and nonstructural

proteins contribute to virion morphogenesis of pestiviruses (Dubrau et al., 2019; Klemens et al., 2015; Sheng et al., 2014), reviewed in (Fukuhara and Matsuura, 2019; S. Li et al., 2017a, 2017b; Tautz et al., 2015; F.-I. Wang et al., 2015a, 2015b). However, the precise mechanisms of virus assembly and budding remain poorly characterized. Little is known yet on the host's proteins and organelles involved in morphogenesis. It was found that Annexin A2 is required for infectious particle formation (Sheng et al., 2015). Rab1A binds NS5A and is also involved in virion assembly (Lin et al., 2018).

6.2. Host responses to CSFV infection

Cells respond to invading pathogens by dysregulation of the cellular homeostasis, involving autophagy, apoptosis, necroptosis and pyroptosis, and by expression of antiviral proteins (reviewed in (Ma et al., 2019)). These cellular responses are not necessarily antiviral. They can also be proviral. Autophagy is essential for sustained CSFV replication (Fan et al., 2020; Pei et al., 2014; Zhu et al., 2019). CSFV induces autophagy through the inhibition of mammalian target of rapamycin complex 1 (mTORC1) phosphorylation, which favours replication (Luo et al., 2018; Xie et al., 2020). As a negative feedback to the CSFV-induced mTORC1 hypophosphorylation, the phosphatidylinositol-3-kinase-/protein kinase B (Akt)/mTORC1 pathway is activated, thereby maintaining an equilibrium between cell survival and viral replication (Luo et al., 2018). Autophagy induction through the inhibition of mTOR phosphorylation occurs in conjunction with NS5A-mediated induction of the calcium/calmodulin-dependent protein kinase kinase 2 - protein kinase AMP-activated catalytic subunit alpha axis (Xie et al., 2020). Moreover, the induction of autophagy inhibits IFN-β induction through interaction between mitochondrial antiviral-signalling protein (MAVS) and beclin-1 (BECN1), which again favours CSFV replication (Xie et al., 2020). By means of NS5A, CSFV does also induce the canonical arms of the unfolded protein response involving the activating transcription factor 6, the inositol-requiring endonuclease 1 and the protein kinase R-like ER kinase, which is proviral (Chengcheng et al., 2020; Zhu et al., 2019). This is consistent with the opposite observation that type I IFN-induced interferon-stimulated gene (ISG)15 upregulation promoted BECN1 ISGylation, which in turn inhibited autophagy and CSFV replication (C. Li et al., 2020a, 2020b, 2020c). CSFV utilizes also mitochondrial fission and mitophagy to inhibit apoptosis, thus supporting cell survival and viral replication (Gou et al., 2017b; L. Zhang et al., 2018a, 2018b, 2018c). A recent study indicates that CSFV induces pyroptosis in monocytes, which may also play a role in supporting CSFV replication (Fan et al., 2018). CSFV infection activates caspase-1 thereby inducing the formation of the inflammatory complexes apoptosis-associated speck-like protein containing a CARD and NOD-, LRR- and pyrin domain-containing protein 3 to activate and release of IL-1β. *In vivo*, the situation is more complex and less studied. Autophagy, apoptosis, pyroptosis and T cell death following CSFV infection were also described in peripheral lymphoid organs (Gou et al., 2017a; J. Yuan et al., 2018a, 2018b). There, it may be associated with the characteristic lymphocyte depletion and immunosuppression associated with CSFV infection.

The up-regulation of pro-inflammatory and IFN responses in a virulence-dependent manner is a hallmark of the host response to CSFV infection and is observed both, *in vivo* and in primary cells (reviewed in (Goraya et al., 2018; Summerfield and Ruggli, 2015)). This was described initially with RT-qPCR-based mRNA analysis of selected genes involved in the modulation of the host immune response in macrophages infected *ex vivo* with a virulent strain (Borca et al., 2008). The dysregulated genes included pro-inflammatory cytokines, cytokine receptors, chemokines, IFNs, and toll-like receptors (TLR). Later, microarray analysis of PBMC collected before and at three consecutive days post-infection with either a highly or moderately virulent CSFV strain provided insights into the different virulence-dependent kinetics of cytokine and cell death/apoptosis-related ISG expression profiles (Renson et al., 2010). Studies of changes in the transcriptome in the tonsils of vaccinated and infected animals also highlight the involvement of the ISG15 pathway in the host's response to vaccination and infection (McCarthy et al., 2019). A recent field study confirmed pro-inflammatory cytokine expression after CSFV infection under natural conditions, while vaccination resulted essentially in elevated IFN-γ (Khatoon et al., 2019). Recently, porcine blood-derived conventional DC (cDC) 1, cDC2, pDC and monocyte subsets were identified by transcriptional profiling using RNA sequencing of FACS-sorted subsets (Auray et al., 2016). Based on this, the same DC subsets were isolated from lymph nodes and tonsils from pigs at 18 and 42 h after infection with a high- and low-virulent CSFV and their activation was characterized by unbiased transcriptomic analyses (Auray et al., 2020). High-virulent CSFV induced a stronger inflammatory and antiviral response but a weaker cell cycle response than the low-virulent virus. The high-virulent virus reduced also the antigen presentation functions of DC. These data provide high-resolution information on DC activation in pigs and information on how DC modulation may be linked to CSFV immunopathology. Several factors such as the pro-inflammatory allograft inflammatory factor 1 modulate the cytokine responses (Gong et al., 2020). The potential role of pro-inflammatory responses in tissue damage and haemorrhages is not well understood. Recently, the upregulation of the chemokine IL-8 in umbilical vein endothelial cells through NS4A-induced MAVS signalling was proposed to regulate the permeability of the endothelium (Dong et al., 2018).

The cellular antiviral response is mediated essentially by ISGs. A recent RNAi screen revealed several ISGs with CSFV antiviral activities (Wang et al., 2016). The IFN-induced guanylate-binding protein 1 inhibits CSFV replication in a GTPase-dependent manner (Li et al., 2016). The IFN regulatory factor (IRF)1-mediated ISG15 induction following polyIC stimulation of porcine cell lines was also found to inhibit CSFV replication (X. Q. Li et al., 2018a, 2018b). There is also good evidence of antiviral activity of Mx proteins against CSFV in cell culture and *ex vivo* in cells from MxA transgenic pigs (D. N. He et al., 2014a, 2014b; X. Zhang et al., 2015a, 2015b; Zhao et al., 2016, 2011). It was shown recently that porcine Mx proteins exert their antiviral activity against CSFV by interacting with NS5B (Zhou et al., 2018). The antiviral activity of mouse Mx1 against CSFV relied on a mutation that favoured the cytoplasmic localization of Mx1 and interaction with the capsid protein C (Chen et al., 2020). Another recent study suggested that interferon-induced transmembrane protein (IFITM)1, 2 and 3 interfere with CSFV entry, and cell surface expression of IFITM1 decreased following infection (Li et al., 2019). The ISG Rsad2 (viperin) is upregulated in response to CSFV infection and interacts with NS5A (C. Xu et al., 2020a, 2020b, 2020c). Rsad2 is also found in close vicinity of E2 and NS5B and inhibits CSFV replication with a yet unknown mode of action (W. Li et al., 2017a, 2017b). CSFV can also trigger the innate antiviral responses and inhibit virus replication through activation of type III IFNs (Cai et al., 2017). Tumor necrosis factor (TNF) does also restrict CSFV replication. Pigs infected with CSFV induce significant levels of TNF (Choi et al., 2004; Khatoon et al., 2019; von Rosen et al., 2013v; Wang et al., 2018), and TNF was reported to inhibit the replication of CSFV *in vitro* (Chen et al., 2019; Li et al., 2015).

Cells also respond to invading pathogens with microRNA (miRNA) upregulation, which does typically modulate the cytokine responses at the mRNA level (Trobaugh and Klimstra, 2017). A genome differential miRNA and mRNA expression analysis in PBMC of pigs infected with a CSFV vaccine *versus* mock-vaccinated pigs identified miR-22–5p and miR-27b-5p upregulation (Sailo et al., 2019). Selected mRNA targets of these miRNAs, *i.e.* CD40, SWAP70, TLR4 and Lyn were modulated accordingly.

6.3. Viral modulation of the antiviral host responses

Viral proteins that are not essential for the basic life cycle of the virus have typically accessory functions such as modulation of the host innate

immune responses. However, modulation of host responses is not necessarily restricted to non-essential viral elements. CSFV controls the innate immune responses with the non-essential protein N^{pro} and with the soluble form of the E^{rns} protein (reviewed in (Summerfield and Ruggli, 2015), and possibly also through the sequestration of miR-17 (Scheel et al., 2016).

N^{pro} is a two-domain protein with an N-terminal protease domain and a C-terminal domain carrying a Zn-binding TRASH motif, and the two domains contribute to its stability (Gottipati et al., 2016, 2014; Mine et al., 2015; Szymanski et al., 2009). The deletion of N^{pro} does not abolish infectious particle formation but impairs virus replication in cells capable of producing type I IFN (Ruggli et al., 2003). Cells infected with CSFV are protected to some extent against dsRNA-induced apoptosis and necroptosis by inhibition of caspases activation, of mitochondrial apoptotic pathways and of type I IFN induction, which depends on the presence of N^{pro} (Itakura et al., 2020; Johns et al., 2010; Ruggli et al., 2003). N^{pro} interacts directly with IRF3 monomers and dimers and mediates the proteasomal degradation of IRF3 independently of its protease activity by yet unknown mechanisms, blocking efficiently type I IFN induction in non-pDC (Bauhofer et al., 2007; Gottipati et al., 2016; La Rocca et al., 2005; Ruggli et al., 2005). Interestingly, a recent study showed that restoration of the N^{pro} function in an N^{pro}-defective conditionally cytopathogenic strain (GPE^{-}) prevented the induction of necroptosis but not of apoptosis, while an N^{pro}-defective vALD-A76 mutant incapable of inhibiting type-I IFN production induced necroptosis and CPE (Itakura et al., 2020). Although N^{pro} was characterized essentially in cell culture, it is perfectly functional *in vivo* where it was shown to downregulate the local IFN-α mRNA expression in the infected tissues thereby contributing to viral pathogenicity (Tamura et al., 2014). In pDC, which are the main type I IFN producing cells *in vivo* and require IRF7 rather than IRF3 for type I IFN induction, N^{pro} only partially dampens type I IFN induction by interacting with IRF7 without inducing its degradation (Fiebach et al., 2011). This may explain why CSFV induces IFN-α *in vivo* despite functional N^{pro}, with levels that are proportional to the virulence of the isolate (Summerfield et al., 2006). Recently, N^{pro} was also found to suppress IRF1-mediated IFN-λ production by inhibiting IRF1 expression and its nuclear translocation (T. Cao et al., 2019a).

The second CSFV protein known to date to impair host type I IFN induction is the soluble form of the structural E^{rns} protein, a function that depends on its RNase activity (Python et al., 2013), reviewed in (Summerfield and Ruggli, 2015). The biochemical mechanisms by which E^{rns} interferes with type I IFN induction through dsRNA degradation were dissected *in vitro* for BVDV E^{rns} (Lussi et al., 2018; Magkouras et al., 2008; Mätzener et al., 2009; Zurcher et al., 2014). E^{rns} is supposed to function in a similar way for the two pestiviruses, with, however, different consequences in the pathogenesis of the associated disease, *i.e.* CSF *versus* persistent BVD/Mucosal disease (for extensive reviews, see (Lussi and Schweizer, 2016; Summerfield and Ruggli, 2015)). Besides its membrane-anchored and virion-associated form, a minor fraction of the protein is secreted from infected cells (Burrack et al., 2012; Fetzer et al., 2005). E^{rns}-mediated inhibition of type I IFN induction through extracellular targeting and degradation of dsRNA by E^{rns} was shown *in vitro* (Iqbal et al., 2004; Luo et al., 2009; Magkouras et al., 2008; Mätzener et al., 2009), but evidence for E^{rns} activity in extracellular compartments *in vivo* is lacking. E^{rns} is more likely to function in endocytic compartments (reviewed in (Lussi and Schweizer, 2016)). Indeed, E^{rns} can be rapidly endocytosed and degrades endosomal RNA in cell lines and porcine pDC (Python et al., 2013; Zurcher et al., 2014). Interestingly, similar to observations with HCV (Takahashi et al., 2010), CSFV-infected porcine macrophages or cell lines stimulated pDC for IFN-α production (Python et al., 2013). Importantly, cells transduced with CSFV replicons lacking E^{rns} expression were significantly more potent at stimulating pDC than virus-infected cells, suggesting a virion-free transfer of a viral RNA trigger to pDC as for HCV, but in addition, an inhibitory role of E^{rns}. This was eventually demonstrated to be dependent on RNase-active E^{rns} by reconstitution of functional E^{rns} *in trans*, which significantly reduced the replicon-mediated pDC activation as opposed to the RNase-inactive E^{rns} (Python et al., 2013). The relevance of these mechanisms remains to be demonstrated *in vivo*.

In addition to N^{pro} and E^{rns}, there is evidence from lentiviral expression experiments in monocyte-derived macrophages (MDMs) that the nonstructural proteins NS2 to NS5A may also modulate the TLR pathways (Z. Cao et al., 2019). However, these data need further validation in the virus context. Also, unbiased differential transcriptome analyses in porcine alveolar macrophages infected *ex vivo* with CSFV showed an increase of p53 and p21 expression and a decrease of cyclin E1 and CDK2, leading to cell cycle arrest at the G1 phase (Ning et al., 2017). How CSFV modulates the host cell and whether this supports replication requires further investigation.

Pestivirus replication depends strongly on miR-17 and let-7 binding (Scheel et al., 2016). Consequently, viral sequestration of miR-17 results in dysregulation of cellular transcripts harbouring miR-17 binding sites, which was confirmed at the protein level. Unbiased transcriptomic analyses of MDMs infected *ex vivo* with CSFV revealed a significant time-dependent upregulation of cellular transcripts harbouring a miR-17 binding sequence in the 3′-UTR (Scheel et al., 2016). There are also several lines of evidence that miRNA modulates CSFV replication indirectly through the regulation of host factor expression. CSFV downregulates miR-140, a negative regulator of Rab25 that supports CSFV replication (P. Xu et al., 2020a, 2020b, 2020c). It can be debated whether this is a countermeasure of the virus to counteract the host-mediated miR-140-dependent downregulation of the pro-viral Rab25.

7. Viral determinants of virulence

CSFV isolates cover a broad range of virulence, as witnessed for example by analysis of the virulence of CSFV isolated in Europe between 1996 and 2007 (Floegel-Niesmann et al., 2009a, 2003). Other examples are a comparative study of CSFV of different virulence in terms of pathogenicity, tissue distribution and immunohistochemistry (Belák et al., 2008), and virulence studies with genotype 2.3 isolates in wild boar (Kaden et al., 2004).

Here we define virulence of CSFV as the ability of a given virus – field isolate, laboratory strain or cDNA derived-virus – to cause clinical signs and pathological damage in pigs, which is often also be referred to as pathogenicity. Strictly taken, however, the definition of the degree of virulence of a given CSFV is associated with defined host conditions. A virus for which virulence was defined under specific conditions may manifest with different degrees of pathogenicity depending on host factors such as age, breed, genetic background, hygiene and immunological status. Therefore, in this review, we distinguish between viral determinants of virulence and viral elements and host factors that modulate virulence. The numerous mechanisms by which virus-host interactions modulate virulence and influence the pathogenicity of a virus in the field or in experimental infections were described in the previous chapters. Different experimental approaches to define the viral genetic basis of virulence were reviewed extensively in (Leifer et al., 2013).

7.1. The non-essential protein N^{pro}

In the sense of what we defined above, the main viral element that modulates virulence without being considered a virulence determinant *per se* is the non-essential protein N^{pro}. This can be illustrated with the following studies. When the N^{pro} gene of the highly virulent Eystrup strain was replaced with that of the live attenuated vaccine strain Riems, the virulence of the chimeric Eystrup virus remained unchanged, while the deletion of N^{pro} from the high-virulent Eystrup or the moderately Alfort/187 backbone attenuated the viruses (Mayer et al., 2004).

Second, with highly virulent isolates, mutations that abrogate the IRF3 degrading function of N^{pro} did not reduce the virulence of the Eystrup strain and partially reduced virulence of the less virulent Alfort/187 virus (Ruggli et al., 2009). Thus, the same mutation of N^{pro} modulates virulence to a different degree, depending on the viral virulence determinants that differ between Eystrup and Alfort/187. A follow-up study showed that N^{pro} contributes to virulence in a sense that it is essential for the full functionality of the virus to inhibit type I IFN induction at local replication sites in different organs *in vivo* (Tamura et al., 2014). The localization of N^{pro} in the nucleus was also related to virulence with yet unknown mechanisms (Y. Li et al., 2014a, 2014b).

7.2. Loss- versus gain-of-function approaches to identify virulence determinants

In analogy to N^{pro}, the different E^{rns} features, *i.e.* its integration in the virion membrane and its RNase activity in soluble form as well as its dimerization are essential for the full functionality of the virus *in vivo*, and therefore knocking out these functions modulates virulence without strictly representing virulence determinants. Mutations inactivating the RNase activity or preventing E^{rns} homodimer formation by disruption of the intermolecular cysteine bond (Cys-171) attenuate the virus in pigs (Meyers et al., 1999; Tews et al., 2009). Reversion of E^{rns} dimerization by a spontaneous compensatory mutation *in vivo* restored virulence, confirming the importance of E^{rns} dimer formation for CSFV virulence (Tucakov et al., 2018). The report showing that functional N^{pro} and E^{rns} are essential for persistent transplacental infection with BVDV support the importance of these two proteins for pestiviruses to express their full virulence and pathogenic potential (Meyers et al., 2007). Interestingly, the repeated amplification of CSFV in permanent cell lines by which the virus acquires increased affinity to cellular HS is associated with mutations in E^{rns} and loss of virulence *in vivo* (Eymann-Häni et al., 2011; Hulst et al., 2001; van Gennip et al., 2004v). For all three structural glycoproteins, numerous reports demonstrate that knocking out glycosylation sites affects virulence (for selected references, see (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008)). Other mutations that modify virulence were described in the envelope proteins (Risatti et al., 2006, 2005a, 2005b; van Gennip et al., 2004v) or in combination with changes in the nonstructural proteins (Tamura et al., 2012; van Gennip et al., 2004v). Alteration of the E1-E2 heterodimerization and mutations in NS4B did also affect virulence (Fernandez-Sainz et al., 2011, 2010). Cytopathogenicity of CSFV correlated also with attenuation (Gallei et al., 2008). Recently, it was suggested that E2 and the 3′-UTR affect virulence synergistically (Wu et al., 2017). All these latter reports rely on targeted loss-of-function studies using reverse genetics. A different approach consisting of the analysis of CSFV evolution under different conditions revealed positive selection-driven alterations of the glycoprotein structures and glycosylation or of the haplotype composition of the virus, which was in part correlated with virulence (Fahnøe et al., 2019; Tang et al., 2008; Wu et al., 2010). Cloning and analysis of full-length genetic haplotypes revealed interesting features of viral genome requirements for virulence (Fahnøe et al., 2015, 2014). As opposed to previous studies, we used reverse genetics with a gain-of-function approach to demonstrate a synergistic effect of the envelope glycoprotein E2 and the nonstructural protein NS4B in determining virulence (Tamura et al., 2012). This latter study built on forward genetics data from vaccine virus mutants that had recovered virulence upon artificial serial passages in pigs. By applying reverse genetics, the mutations acquired were reintroduced in the vaccine backbone and the resulting phenotype was determined in pigs. A combination of amino acid mutations in the envelope protein E2 and in the N-terminus of NS4B enhanced virulence. The virulence of this latter mutant was further increased by substitution of the N-terminal domain of NS4B with that of the highly virulent Eystrup strain, which resulted in changes in ER membrane association of NS4B and increased replicase activity (Tamura et al., 2015). Forward genetic studies with field isolates from Cuba that evolved under positive selection pressure resulting from suboptimal vaccination did also suggest a role of E2 for virulence (Pérez et al., 2012). The loss of virulence of these field isolates was associated with an antigenic drift in the major immunogenic epitopes of E2 (Coronado et al., 2019b). However, gain-of-function experiments using reverse genetics are required to determine formally the role of these E2 mutations for virulence. Very recently, with such an approach we have demonstrated the role for virulence in newborn piglets of a novel poly-uridine sequence in the 3′-UTR acquired by one of these latter field isolates in Cuba (Coronado et al., 2017; Wang et al., 2019).

8. Diagnosis

Rapid clinical diagnosis is of particular importance as the first line of defence for the detection and control of primary CSF outbreaks (Moennig et al., 2003; Moennig and Becher, 2015). The evolution towards moderately or low virulent strains that do not cause typical signs of disease and spread slowly within a herd has resulted in longer high-risk periods facilitating unnoticed spread of CSF between farms. Several other viral diseases such as African swine fever, porcine respiratory and reproductive syndrome, porcine dermatitis and nephropathy syndrome, post-weaning multisystemic wasting syndrome as well as infections with various bacteria causing septicaemia may be confused with CSF; these bacterial infections include erysipelas, salmonellosis, pasteurellosis, actinobacillosis, eperythrozoonosis, and infections with *Haemophilus parasuis*. Therefore, the variability of clinical signs and *post mortem* lesions does not allow unequivocal diagnosis of CSF (Blome et al., 2017b; OIE, 2019d; Postel et al., 2018). Even typical clinical signs or *post mortem* lesions can only lead to a suspicion of CSF and any tentative diagnosis must be confirmed by laboratory diagnosis. Methods for detection of CSFV antigen and nucleic acid as well as CSF-specific antibodies are described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2019 and in the CSF Diagnostic Manual 2002/106/EC Technical Annex which has been recently updated (Anonymous, 2020; OIE, 2019d). A high-quality laboratory diagnosis is a prerequisite for rapid detection and efficient control of CSF. For each method used in different laboratories for CSF diagnosis, specificity, sensitivity and other quality parameters are evaluated on a regular basis to ensure a high-quality standard. For the National Reference Laboratories for CSF in the European Union, participation in an inter-laboratory comparison test is obligatory and represents an efficient and important tool to maintain a high standard of CSF diagnosis (Floegel-Niesmann et al., 2009b).

8.1. Detection of virus, viral antigen, and viral nucleic acid

For virus isolation and detection of viral antigen or viral genomic RNA, anticoagulated blood samples and body fluids from live animals, as well as tonsils, spleen, lymph nodes, thymus, intestine, kidney, lung, and other organ samples from dead animals, can be used. Swab samples from both, live and dead domestic pigs and wild boar, are also suited for detection of CSFV (Petrov et al., 2014b; Postel et al., 2018). CSFV can be easily grown in various porcine tissue cultures. Therefore, virus isolation represents a classical method for CSFV diagnosis, which is used routinely in many laboratories. The porcine kidney cell lines PK15 and SK6, as well as a swine testis endothelial (STE) cell line, are particularly suited for CSF diagnosis (Anonymous, 2020). PK15 cells can be contaminated with porcine circovirus-1 (Tischer et al., 1982). For CSF diagnosis, it is recommended to use circovirus-free PK15 cells. Porcine cells are, at least to some extent, susceptible to BVDV and other ruminant pestiviruses whose presence can significantly interfere with CSF diagnosis. Accordingly, it is important to demonstrate the absence of BVDV and other ruminant pestiviruses in all cell lines used for CSF diagnosis. STE, PK15, and SK6 cells can be adapted to grow in medium supplemented with horse serum instead of fetal calf serum, thereby minimizing the risk of a contamination with BVDV and other ruminant pestiviruses

(Anonymous, 2020). With a very few exceptions, the vast majority of CSFV isolates are noncytopathogenic and do not cause visible effects in cell culture (Gallei et al., 2008, 2005; Kosmidou et al., 1998). Consequently, after infection of cells with CSFV, the presence of viral antigen has to be visualized by immunological reactions including immunoperoxidase or immunofluorescence staining. Monoclonal antibodies directed against CSFV-specific epitopes have replaced antisera obtained after immunisation of animals with the infectious virus or with genetically engineered CSFV antigens. In addition to the detection of viral antigen after virus isolation and propagation in tissue culture cells, CSFV antigen can be detected directly in cryo-sections of organs from infected animals by fluorescence Ab test (FAT). In many laboratories, the FAT was replaced by more sensitive molecular diagnostics, *e.g.* reverse transcription and PCR (RT-PCR).

In the late 1980s, the first prototype CSFV antigen enzyme-linked immunosorbent assays (ELISAs) were developed and used for the diagnosis of CSF. The sensitivity and in particular the specificity can vary among different ELISA setups and have to be thoroughly evaluated. The CSFV antigen ELISAs detect typically the viral glycoprotein E^{rns}. In general, antigen ELISAs are suited for monitoring CSF at farm or population level but are not suited to demonstrate absence of CSFV in individual animals. Moreover, in case of a primary CSF outbreak, it is recommended to confirm a positive ELISA result by virus isolation in cell culture or detection of viral nucleic acid by RT-PCR (see below); such a confirmation is obligatory in the European Union and many other parts of the world.

In the 1990s, detection of viral genomic RNA by RT-PCR was added to the repertoire of methods routinely used in CSF diagnosis (Díaz de Arce et al., 1998; Katz et al., 1993; Vilček et al., 1994). Both, CSFV-specific and pan-pestivirus RT-PCRs – the latter detecting a broad range of pestiviruses including CSFV – have been established. For broadly reactive RT-PCRs, subsequent nucleotide sequencing of the amplified nucleic acid is essential for CSF diagnosis. The conventional gel-based RT-PCRs were replaced by various highly sensitive and rapid real-time quantitative RT-PCR (qRT-PCR) methods, which no longer need gel electrophoresis to visualize amplified nucleic acids (Anonymous, 2020; Hoffmann et al., 2005; Leifer et al., 2011; McGoldrick et al., 1999; OIE, 2019d; Postel et al., 2012). Today, qRT-PCR is the method of choice for rapid and highly sensitive CSF diagnosis. Several in-house methods and commercial kits are available which target different regions in the CSFV genome. In an ideal world, the available RT-PCR kits detect all CSFV isolates but do not detect any other *Pestivirus*. For reliable and solid diagnosis of CSF by qRT-PCR, the increasing genetic variability of CSFV has to be taken into account and the individual test methods must be evaluated thoroughly by investigating recent CSFV isolates on a regular basis. In case of a test failure, the sequence(s) of the primer(s) used for amplification in the PCR reaction must be adapted to cover the increased spectrum of CSFV variants. One example for such a test failure was the lack of detection of a CSFV 2.1 isolate from Israel by a widely used commercial qRT-PCR kit (David et al., 2011); subsequent analysis of the nucleotide sequence targeted by the primers and adaptation of primer sequences solved the problem. This example illustrates the necessity to monitor continuously the genetic variability of re-emerging CSFV isolates and to re-evaluate the reliability of qRT-PCR and other available test methods by analysis of recently evolved virus variants.

8.2. Detection of CSFV-specific antibodies

Approximately 10–14 days after infection with CSFV, the first antibodies can be detected in most animals (Moennig et al., 2013). Infections with BVDV, BDV and other closely related ruminant pestiviruses may induce cross-reacting antibodies interfering with CSF diagnosis (Avalos-Ramirez et al., 2001; Becher et al., 2003; Darbyshire, 1960; Dekker et al., 1995; Postel et al., 2015). A recent study demonstrated that the distantly related APPV does not interfere with CSF diagnosis (Postel et al., 2017a). Nevertheless, cross-reactive antibodies, in particular those induced by infection with BDV, are a major challenge in serological diagnosis of CSF.

For Ab detection, serum samples as well as body fluids, for instance from shot wild boar, can be investigated by virus neutralization test (VNT) and CSFV Ab ELISA (Moennig and Becher, 2015). Due to its high sensitivity and high specificity, the VNT represents the gold standard for Ab detection. It is based on the ability of neutralizing antibodies to inhibit virus infection. Accordingly, the VNT requires appropriate tissue culture cells to propagate infectious CSFV and a laboratory infrastructure with permission to handle the virus. This method is laborious and not suited for analysis of a large number of samples. Assays that allow serological multiplexing (*e.g.* by microsphere technology) or that use genetically modified, fluorescence-labelled test viruses may facilitate the serological differentiation of *Pestivirus*-induced antibodies, but are not widely established yet (Li et al., 2013; Tetsuo et al., 2020; Xia et al., 2015). An advantage of the VNT compared to conventional ELISA is its potential to discriminate CSFV-specific antibodies from antibodies resulting from infections with BVDV, BDV and other ruminant pestiviruses. Thus, VNT is the most important tool to follow up questionable or positive results obtained in Ab ELISAs and to determine whether these antibodies are induced by CSFV or non-CSFV pestivirus infection. Importantly, with the VNT, the capacity to determine the specificity of serum antibodies is highly dependent on the selection of an appropriate panel of test strains routinely used in the laboratory as well as the knowledge of which pestiviruses are circulating in the field.

CSFV Ab ELISAs detect antibodies against the glycoproteins E^{rns} and E2. ELISAs have the advantage that a large number of samples can be investigated in a short time, underlining their suitability for CSF surveillance, in particular to confirm a CSF-free status at the herd level (Moennig and Becher, 2015). In addition to E2-based Ab ELISAs, several E^{rns} Ab detection assays have been developed (Aebischer et al., 2013; Lin et al., 2005; Luo et al., 2015; Meyer et al., 2017). The E^{rns} double-antigen ELISA (dELISA) is particularly suited for highly sensitive and specific Ab detection thanks to fewer problems with cross-reactive Abs than most indirect ELISAs (iELISA) and a sensitivity comparable to competitive ELISAs (cELISA) without requiring a competing monoclonal Ab. The first E^{rns} dELISA developed allowed detection of Abs already at early time points after infection and showed high specificity even with samples derived from wild boar that are often problematic in serological tests (Meyer et al., 2017). Design and optimization of tests targeting E^{rns}-specific Abs is of particular interest in the context of CSF marker vaccines that are able to induce neutralizing E2-specific Abs in absence of a CSFV E^{rns}-specific Ab response (Meyer et al., 2018; Pannhorst et al., 2015; Schroeder et al., 2012). The reliable discrimination between infected and vaccinated animals (DIVA) remains a major challenge in serological diagnosis of CSF. A robust DIVA test is an important prerequisite for the implementation of control strategies based on emergency vaccination with marker vaccines.

9. Recent progress on CSF vaccines

Vaccination strategies against CSF depend on the epidemiology of the disease, on the animals affected – domestic pigs and/or wild boar – and on the economic situation, resulting in different requirements for the vaccines to be applied. For example, in endemic regions with domestic pigs and with little international trade, the priority is to protect against losses due to clinical disease. Such regions require safe, effective vaccines that are economical, easy to apply and give long-lasting immunity. In CSFV-free areas and where international trade in domestic pigs is important, emergency vaccination strategies that rapidly contain disease spread are important. This requires fast-acting vaccines, ideally with DIVA capacity, to minimise the impact on trade. Such DIVA vaccines are also desirable to assist in eradication programmes. The situation is different when vaccines are needed to control the virus in wild *suidae* populations. This requires a vaccine for oral application that

provides long-lasting immunity with a single dose, which ideally has DIVA capacity to monitor the progress of vaccination campaigns.

Live attenuated vaccines are widely used to control CSF disease in many areas and have paved the way to successful eradications, but these vaccines have the disadvantage that they lack DIVA. Two marker subunit DIVA vaccines, Porcilis Pesti (MSD Animal Health) and BayoVac (BAYER AG), both based on the immunogenic E2 protein expressed in baculovirus systems were developed (van Oirschot, 2003v). These vaccines, for which the DIVA principle relies on the detection of antibodies directed against the CSFV E^{rns} protein to identify infection with field virus, protect against disease. However, their efficacy is lower than the live attenuated vaccines, with a later onset of immunity, incomplete protection against vertical transmission, and the requirement for a two-dose inoculation regime. In addition, the early accompanying DIVA tests lacked sensitivity and these vaccines are also not compatible with oral delivery to wildlife (van Oirschot, 2003v). Due to these limitations research strove towards a second generation of marker vaccines. Extensive efforts within a multi-partner collaboration lead to the licencing by the European medicine agency of a novel CSF marker vaccine, Suvaxyn, in 2014 (Blome et al., 2017c). This vaccine, also referred to as CP7_E2Alf, is a chimeric pestivirus constructed in a BVDV virus backbone in which the E2 gene is replaced by the E2 gene from the CSFV strain Alfort/187. This vaccine is safe and as efficacious as the classical live attenuated vaccines but with the added benefit of DIVA capability. Also, it is possible to formulate this vaccine for oral delivery to be applied in wild boar. A drawback with this vaccine is the cross-reactivity observed with sera from BDV- and BVDV-infected animals in the E^{rns} based differential ELISAs (Blome et al., 2017c). Reduced specificity was also observed in animals with maternally-derived antibodies and whilst DIVA differentiation is possible these factors complicate serological surveillance (Meyer et al., 2018). In addition, there was only partial protection against vertical transmission after early challenge with highly virulent strains. The licencing of this vaccine on the European market came at a time when CSF was no longer reported in the region, thanks to the long efforts to eliminate the disease from domestic and wild pig populations. Therefore, there has been limited need to apply this vaccine, and hence the benefits of its use to contain disease with minimal impact on trade with international partners, compared to a stamping-out policy, is yet to be demonstrated by extensive application in the field (Blome et al., 2017b).

9.1. Improving live attenuated (non-DIVA) vaccines and their application

Among the many live attenuated CSF vaccines developed, the C-strain is the most widely used and can prevent 100 % of CSF clinical signs, regardless of the genotype of the challenge strain (Aynaud and Launais, 1978; Graham et al., 2012a, 2012b, Suradhat and Damrongwatanapokin, 2003; Vandeputte et al., 2001). However, the virus can persist even in areas where vaccination with these highly effective vaccines is mandatory (Coronado et al., 2019a; Zhou, 2019). Various reasons contribute to the ongoing circulation of the virus in vaccinated populations, one of which is the ineffective application of vaccines in the field. Both, the cost of vaccines and ease of application are barriers that can contribute to lowering effective vaccine coverage, particularly in resource-poor settings (Coronado et al., 2017). Additionally, the inability to maintain the cold chain can affect the conservation of the vaccine in field situations. To understand the continued circulation of CSFV in endemic areas under vaccination, viral evolution studies have been conducted. Several reports described the possible emergence of new CSFV strains because of a positive selection pressure (positively selected strains). This evolutionary force resulted from a “bottleneck” effect due to the indiscriminate and mishandled use of conventional vaccines (Coronado et al., 2017; Hu et al., 2016; Jang et al., 2020; Ji et al., 2014; Luo et al., 2017; Pérez et al., 2012; Rios et al., 2017; Shen et al., 2011). Several authors have reported that prolonged suboptimal vaccination programs may have caused changes in the pathogenicity and antigenicity of the new emerging strains that could potentially escape vaccination (Coronado et al., 2019b, 2017; Hu et al., 2016; Jang et al., 2020; Ji et al., 2014; Shen et al., 2011). This may favour the high prevalence of chronic and persistent CSF, leading to unsuccessful control of the disease. Efforts that facilitate the implementation of optimal vaccination programs with live attenuated vaccines are therefore beneficial.

Many C-strain variants are produced in different countries. Unfortunately, some countries still produce the vaccines in rabbits and efforts to produce vaccines in cell culture are encouraged. Good cell culture-adapted vaccines, such as the Riems strain used in the EU for wild boar vaccinations, exist but some lapinised versions of C-strain grow poorly in cell culture leading to poor productivity. Sequencing of cell culture passaged C-strain, identified eight amino acid mutations which, when re-introduced into the parental virus by genetic modification, resulted in a genetically stable virus with enhanced growth. This virus retained the ability to protect pigs from challenge at 4 weeks post-vaccination (T. Cao et al., 2019b). Knowledge on such changes that can increase virus replication and enhance efficient production, and hence reduce costs of high-quality live vaccines, has the potential to benefit effective vaccine application. The current commercial oral bait vaccine contains liquid C-strain vaccine within corn-covered blister packs and requires storage at −20 °C, which limits its use in remote regions with warmer climates. In addition, the cost of the commercially formulated baits could be a factor against effective deployment in some sectors. Vaccine baits, prepared by absorption of C-strain onto bread and subsequent lyophilisation, were stable for 18 months at 4 °C and pigs consuming these vaccine baits seroconverted after 14 days (Kunu et al., 2019). Whilst still requiring some form of cold storage, 4 °C is more feasible to achieve than −20 °C, and this could provide a more cost-effective method to improve vaccination in these hard to reach pig populations.

9.2. Developments in live attenuated DIVA vaccines

During development of the CP7_E2Alf vaccine, studies to assess protection from vertical transmission used early challenge with a highly virulent virus (Henke et al., 2018). Under these stringent conditions, which were aimed at testing the early protective capacity of this vaccine in an emergency situation, some animals were not protected from vertical transmission. This led to the inclusion of a warning in the summary of product characteristics, stating that sows should not be vaccinated due to the risk of birth of persistently-infected offspring (EMA, 2019). Nevertheless, the CP7_E2 Alf vaccine protected against experimental challenge with a moderately virulent strain representative of currently circulating viruses, 3 weeks post-vaccination, indicating a low risk of undetected persistently infected offspring arising (Henke et al., 2018).

A promising vaccine candidate has been developed by genetic elimination of a highly conserved CSFV-specific epitope of the E2 glycoprotein and the inclusion of a Flag epitope in E1 as a positive marker (Holinka et al., 2014). This virus induces effective immunity against challenge as early as 3 days after vaccination (Holinka et al., 2017). DIVA tests relying on the positive and negative antigenic markers of this vaccine candidate remain to be developed.

Another live attenuated chimeric DIVA vaccine, Flc-LOM-BE^{rns}, has been described recently and is applied in South Korea (Lim et al., 2019). The Flc-LOM-BE^{rns} vaccine is based on an infectious clone of the LOM (low virulence of Miyagi) vaccine strain in which the 3′-end of the capsid gene and the full E^{rns} gene were replaced with the equivalent sequences from a BVDV-1 virus. DIVA capacity is therefore possible with the Flc-LOM-BE^{rns} vaccine, with detection of antibodies against CSFV E^{rns} being indicative of a field virus infection. This vaccine protects against vertical transmission: no virus was detected in fetuses from sows vaccinated 3 weeks before insemination and challenged at three different stages during pregnancy. This vaccine promises to provide an improved vaccination option in South Korea compared to the currently

used parental LOM vaccine strain, which can cause abortion and stillbirth in pregnant sows (Choe et al., 2019). Vaccination with the LOM-BE^{rns} vaccine started in South Korea in 2020 and use under field conditions will provide valuable data on the safety of the vaccine and the robustness of the DIVA concept.

9.3. Recent developments in viral vector CSFV vaccines

Like chimeric pestiviruses, the use of viral vectors to deliver CSFV antigens can retain the advantages that live vaccines have over subunit vaccines by targeting multiple aspects of the immune response to provide greater efficacy. A number of recent studies have continued investigations into the use of viral vectors as candidate DIVA vaccines.

Constructs expressing the CSFV E2 and E^{rns} proteins in a Newcastle disease vaccine (NDV) strain have been produced (Kumar et al., 2019). NDV has advantages as a delivery vector in that it can grow to high titres in embryonated eggs and in cell cultures, which allows cost-effective vaccine production. The vector is also able to infect *via* the intranasal route, thereby targeting induction of responses at the primary site of CSFV entry and replication. Another interesting study has investigated the use of recombinant baculovirus vectors as a vehicle to deliver DNA encoding CSFV E2 in pigs (Liu et al., 2017).

A recombinant swine pox virus construct rSPV-E2, expressing the E2 protein, has also been produced (H. Lin et al., 2017a, 2017b). Like other poxviruses, the swinepox virus can encode large amounts of recombinant proteins and is a potent stimulator of both, cellular and humoral immunity. Pigs immunised intramuscularly with two doses of the rSPV-E2 candidate were clinically protected against CSFV challenge and only very low levels of CSF viral RNA were detected in serum shortly after challenge, indicating the potential for this construct to also protect against transmission, although further work would be needed to demonstrate this. Advantages of this system include the potential to construct a multivalent vaccine incorporating antigens against other porcine viruses, and the potential for skin prick subcutaneous delivery (X. Yuan et al., 2018a, 2018b). As with all novel viral vectors for vaccines based on viruses that infect and cause even mild disease in the porcine host, adequate attenuation and safety of the viral vector, as well as lack of interference due to pre-existing immunity to the vector, needs to be demonstrated.

One of the most promising viral vector/replicon CSF vaccine candidates is the adenovirus/alphavirus replicon based vaccine, rAdV-SFV-E2, which uses the replication-defective Ad5 vector to deliver a Semliki Forest virus replicon to express the E2 gene. Comprehensive studies with this candidate were reviewed previously, and this vaccine is under investigation for use in eradication campaigns in China (Blome et al., 2017a). Since that review, the efficacy of using a 2-dose regime of this vaccine in animals with MDA, derived either from vaccination with C-strain or rAdV-SFV-E2, was confirmed (Xia et al., 2016): Piglets with MDA vaccinated at 4 weeks of age, and boosted after 3 weeks, were completely protected against subsequent challenge with virulent CSFV. The lack of expression of the E2 protein until after delivery of the vector to cells, and lack of induction of antibodies to the Ad5 vector, may account for this absence of MDA interference and is a significant benefit of this rAdV-SFV-E2 vaccine candidate.

With the aim of developing vaccines protective against multiple pig diseases, various studies have reported the construction and testing of existing porcine vaccine strains manipulated to express antigens from additional pathogens (Abid et al., 2019; Gao et al., 2018; Lei et al., 2016; Tong et al., 2020; Y. Wang et al., 2015a, 2015b; Zhang et al., 2017).

9.4. Improved E2 subunit vaccines

A baculovirus-expressed E2 vaccine, similar to the previously licenced subunit E2 vaccines but based on a local genotype 1.1 CSFV isolate VN91 from Vietnam, protected against clinical disease, using a 2-dose regime. The challenge viruses were local genotype 1.1, 2.1 and 2.2 CSFV isolates. Neither challenge virus nor viral RNA were detected in the spleens of vaccinated animals, supporting the suitability of the vaccine for further development (Tran et al., 2020). To improve cost-effective production of secreted protein from baculovirus-infected insect cells, a signal sequence from the E2 protein of the CSFV isolate ZJ from Zhejiang was used (H. Xu et al., 2020a, 2020b, 2020c). The E2 from this ZJ strain also appeared to have some enhanced immunogenicity and clinical protection was observed after a single immunisation of pigs with a low dose of only 5 μg of protein. CSFV antigen was not detected by immunohistochemistry in animals vaccinated with this single low dose but no PCR nor virus isolation data were presented. A further baculovirus-expressed E2 subunit vaccine, based on the E2 from the C strain, has been developed and commercialised (Gong et al., 2019). A prime-boost vaccination with this Tian Wen Jing (TWJ-E2®) vaccine, which was licenced for use in China in 2018, protects against challenge with virulent genotype 2 strains currently circulating in China. Only background levels of viral RNA were detected in blood, tissues or swabs indicating a good potential to protect against transmission, but the level of protection in pregnant sows against transplacental transmission remains to be established.

The use of E2 expressed in plants has been examined as a method to provide cost-effective protein production. Plants don't require specialized facilities and production can be easily scaled up. E2 produced in transgenic *Arabidopsis thaliana* (cress) plants was immunogenic in mice and could be recognized by CSFV-specific antibodies (Sohn et al., 2018). *Nicotiana benthamiana* plants produce higher amounts of biomass than Arabidopsis. Dimeric E2, produced in *N. benthamiana* and formulated with different adjuvants, has been assessed for protection against CSFV challenge 35 days after either a single or a double dose (Laughlin et al., 2019; Park et al., 2019). Whilst the efficacy of a single dose regime with plant-derived E2 to provide adequate protection against horizontal and transplacental transmission is yet to be demonstrated, the lower production costs offered are encouraging and offer the potential for a cost-effective increase in the antigen concentration in a single dose, which may enhance vaccine efficacy. Recent studies have investigated the dose required, and the onset and duration of protection conferred by an E2 candidate that uses the novel KNB oil-in-water adjuvant, in combination with baculovirus and plant expressed E2, to assess if this adjuvant provides sufficient enhancement to protect with a single dose (Madera et al., 2018). The incorporation of food-grade saponin into this novel oil-in-water-based adjuvant has also been investigated (Burakova et al., 2018). A recent study has investigated the use of Gram-positive enhancer matrix (GEM) particles for surface display of glycosylated E2, expressed in the yeast *Pichia pastoris* (D. Li et al., 2020a, 2020b, 2020c). Higher anti-E2 and neutralizing Ab responses were obtained in mice immunised with the GEM loaded particles, compared to the E2 protein alone. Attachment of E2 to gold nanoparticles has also produced interesting preliminary results in mice (Li et al., 2020a, 2020b, 2020c) and it will be interesting to see if these systems enhance protection in pigs.

Promising enhancement of immunity to E2 subunit vaccine has been achieved in the past by inclusion of immunomodulatory proteins, such as IFN-α, together with E2 (Toledo et al., 2010). This concept has been extended recently to investigate the impact of the inclusion of IFN-γ as an immunoadjuvant (H. Zhang et al., 2018a, 2018b). It will be interesting to see if this approach can provide robust protection against vertical transmission in pregnant sows. A very recent study has investigated a system to provide both economic production and enhanced immunogenicity by using a transgenic *N. benthamiana* plant-based expression of E2 fused to porcine Fc (Park et al., 2020).

One of the furthest developed improved E2 subunit vaccines is the Porvac vaccine, which is being used in a CSF eradication programme in Cuba. This vaccine exploits the porcine CD154 protein, which is the ligand of the CD40 molecule, as a novel molecular adjuvant. CD154 is expressed transiently on CD4+ T helper cells and engagement with CD40 on antigen presenting cells results in their increased activation

and maturation. A system for the production of this chimeric E2-CD154 protein by secretion from a stable HEK293 cell line adapted to suspension culture was generated (Suárez et al., 2017). Both, humoral and cellular immune responses in mice were enhanced compared to immunisation of E2 without CD154 in the same adjuvant formulation (Sordo et al., 2018). Immunisation with the E2-CD154 fusion protein protected pigs from challenge infection as early as 7 days after a single dose vaccination. No clinical signs were observed, and no virus was detected by virus isolation and by PCR, neither in the blood nor in the tonsils and spleens of vaccinated animals (Suárez et al., 2017). Immunisation of pregnant sows with two doses of this vaccine also protected against vertical transmission after intramuscular challenge with a high dose of a highly virulent CSFV (Muñoz-González et al., 2017). Together with good surveillance and biosecurity, this vaccine candidate is promising for CSF control and eradication in endemic situations.

10. Final remarks and conclusions

Despite the extensive efforts in CSFV research, diagnostics and eradication, the virus continues to persist and re-emerge, posing a threat for food supply in affected regions. There are still numerous gaps in understanding the disease immunopathogenesis, the complex virus-host interactions and the viral determinants of virulence. In addition, the reasons behind the narrow host range of CSFV and the genetic determinants of host susceptibility to CSFV infection also need further attention. Understanding of these knowledge gaps will support the development of efficient approaches to diagnose and control subclinical and persistent infections in domestic pigs and wild boar. Studies aimed at understanding CSFV evolution and vaccine escape variants in the field are of utmost importance to support eradication efforts. Finally, despite significant advances in the development of new DIVA prototype vaccines, appropriate DIVA serological tests remain a challenge to be resolved in the near future to guarantee disease control supported by emergency vaccination.

Authors' contributions

All authors contributed equally to this review and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests

Acknowledgements

We would like to thank the funding sponsored by the Ministerio de Ciencia, Innovación y Universidades from Spanish governmentRTI2018-100887-B-I00 and pre-doctoral fellowshipFPI 2016. IRTA is supported by CERCA Programme / Generalitat de Catalunya. HC was financially supported by the UK Department for Environment, Food and Rural Affairs (Defra), the Scottish Government and the Welsh Government (SV3000)

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