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Classical swine fever virus replicon particles: A versatile and robust system for vaccine and gene expression applications

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Abstract

Replicons are replication-competent viral genomes that cannot generate progeny particles due to the lack of genome sequences encoding structural proteins. By means of a complementing cell line that provides the missing structural proteins, replicons can be packaged to form virus replicon particles (VRP). The replicative nature of the replicon offer the advantage of prolonged protein expression. This was exploited to develop a vaccine against classical swine fever (CSF), a highly contagious disease of pigs. VRP of the classical swine fever virus (CSFV) were constructed by deleting the E^{rns} gene, coding for one of the four structural proteins. CSF-VRP are safe and allow differentiation of infected from vaccinated animals. Immunization with CSF-VRP protects from lethal disease but does not completely prevent replication of the virus. Therefore the present study is aimed at exploring possibilities of improving the immunogenicity of CSF-VRP. CSFV counteracts the innate immune activation by abrogating the induction of interferon-α/β (IFN-α/β), a phenotype mediated by the viral nonstructural protein N^{pro}. A single amino acid substitution in N^{pro} can abrogate the capacity of CSFV to interfere with IFN-α/β induction. As expected, CSF-VRP with mutated N^{pro} induced IFN-α/β in cell culture. This mutation did not impair the long term replication of the replicon. Treatment with IFN-β reduced the initial number of VRP-infected cells if applied prior to infection but was unable to cure infected cells. In order to further improve the VRP, we constructed bicistronic VRP that expressed granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that possesses adjuvant activity. We compared the immunogenicity of the IFN-α/β–inducing and the GM-CSF–expressing VRP with the parent VRP in vivo. To this end, we vaccinated pigs intradermally and challenged them with a lethal dose of a highly virulent CSFV strain. After vaccination, the pigs remained healthy. The IFN-α/β–inducing and the GM-CSF–expressing VRP were similar to unmodified VRP in terms of CSFV-specific antibody response and induction of IFN-γ–secreting cells. They were slightly superior in their capacity to reduce the blood levels of challenge virus RNA. Using ex vivo restimulation assays with peripheral blood mononuclear cells derived from CSFV-immune pigs we assessed the potential of the VRP to stimulate recall immune responses. Restimulation with the IFN-α/β–inducing VRP resulted in a stronger CSFV-specific antibody response and in more IFN-γ–secreting cells than with the parent VRP. This indicates that IFN-α/β–inducing VRP possess enhanced immunostimulatory capacity. In addition our data show that CSFV replicons can serve for gene expression applications.

Contents

Introduction

Vaccines against viral diseases

The most effective way to combat viral diseases is prevention through vaccination. Different strategies such as live attenuated viruses, inactivated whole viruses and subunit vaccines have been developed for vaccination against viral infections [1–4].

Live attenuated viruses

The use of live attenuated viruses for disease prevention is the oldest vaccination approach. Live attenuated viruses replicate in the vaccinee and induce a protective immune response against disease. The initial vaccines were based on live attenuated viruses that were obtained by using related viruses from other host species [2]. In the vaccinee as a non-natural host, these vaccine viruses typically induce a protective immunity but no disease. A famous example is the small pox vaccine [2, 4]. Alternatively, live attenuated virus vaccines are obtained by extensive passage of the original virus in an unrelated animal species or in cell culture. Attenuation can also be achieved by applying modern gene technology such as reverse genetics to obtain reassortant viruses, chimeric viruses, deletion mutants, codon deoptimized viruses and viruses with altered replication fidelity [3]. Live attenuated viruses are the most efficacious vaccines, resulting in the highest degree of protection with effective humoral and cellular immune responses. However, with live attenuated vaccines there is a constant risk of reversion to virulence and severe disease, due to genetic instability and residual virulence of the vaccine virus.

Inactivated viruses

To increase safety, vaccines consisting of completely inactivated viruses were developed. Inactivation of the virus is achieved typically by chemicals such as formalin, formaldehyde and β-propiolactone or heat. Inactivated viruses are safer than live attenuated viruses but less efficacious. In addition, long lasting immunity requires often repetitive immunization. Inactivated whole virus vaccines function essentially by stimulating humoral rather than cellular immune responses.

Subunit vaccines

Subunit vaccines are based on purified viral proteins isolated from virus particles or produced by recombinant gene expression technology. Such vaccines offer excellent safety, since they do not contain infectious material. However, strong adjuvants are required to induce protective immune responses that are directed only against the specific protein present in the vaccine. Like inactivated whole virus preparations, subunit vaccines elicit mainly humoral immune responses and only poor cellular immunity.

New vaccination strategies

There are still numerous pathogens for which an effective vaccine is missing, mainly because the traditional approaches remained unsuccessful. Therefore, there is a high demand for new safe and efficacious vaccine strategies that elicit potent humoral and cellular immune responses. Many new strategies have been proposed with different success rates. Novel approaches consist for instance of live recombinant viruses carrying foreign antigens, virosomes, virus replicon particles (VRP) and naked DNA plasmids [1–3, 5]. The present study is dedicated to the development of a VRP-based vaccine.

Replicons

From replicons to VRP

Replicons are replication-competent viral genomes that are unable to generate progeny virus due to a functional defect of at least one structural gene [6–11]. Typically, replicons are derived from viruses with a RNA genome. While the structural genes may be completely deleted from the original viral genome, all genes required for genome replication are maintained in the replicon. These essential genes code for the viral RNA-dependent RNA polymerase and all the viral proteins of the replication complex. Like viruses, replicons utilize the translation and metabolic machinery of their host cell for replication. The functional defect or lack of structural coding sequences within the replicon results in the absence of functional proteins necessary to package the genome into the virion. Therefore, no infectious particles are secreted from replicon infected cells. However, if the host cell is engineered to express and transcomplement the lacking structural proteins, virions are formed (Figure 1). Such virus particles harbouring a replicon are termed VRP. Because the surface structure of the VRP is indistinguishable from the original virus, VRP have the same tropism as their parent virus and infect cells with the same efficiency. After VRP have infected a cell, they replicate their genome without generating new virions, and thus represent single-cycle infectious particles (Figure 2). Horizontal cell-to-cell spreading is excluded, while vertical transmission can occur during cell division. These features make VRP novel vaccine candidates that combine the safety of nontransmissible inactivated vaccines with the efficacy of replicating vaccines.

Delivery of replicons into cells

Replicons can be delivered to the host cells in three different ways:

(i) Cells can be transfected or injected directly with naked RNA molecules. To this end, the genomic RNA is transcribed in vitro with bacteriophage RNA polymerase T7, T3 or SP6 from a plasmid template containing the corresponding promoter inserted upstream of the complementary DNA (cDNA) sequence of the replicon. This delivery mode works only with replicons derived from positive-strand RNA viruses, because the sense RNA genome functions as messenger RNA (mRNA). In contrast, the genomes of negative-strand RNA viruses cannot be translated directly. They must first be transcribed to an antigenome of positive polarity by the viral RNA polymerase complex, as exemplified below with vesicular stomatitis virus (VSV) replicons.

(ii) Alternatively, replicons can be delivered by DNA launch. To this end, the replicon cDNA sequence is cloned downstream of a eukaryotic promoter sequence such as the cytomegalovirus promoter. The plasmid is introduced into the host cells by transfection or injection, and the replicon genome is transcribed in the nucleus by the cellular RNA polymerase II.

(iii) Finally, cells can be infected with VRP containing the positive-sense or negative-sense replicon RNA as discussed above.

Alphavirus replicons

Alphaviruses are enveloped viruses with a single-strand RNA genome of positive polarity. The genomic RNA molecule is approximately 12 kb in length and contains a CAP structure at its 5′ terminus and a polyA tract at the 3′ end [12]. The

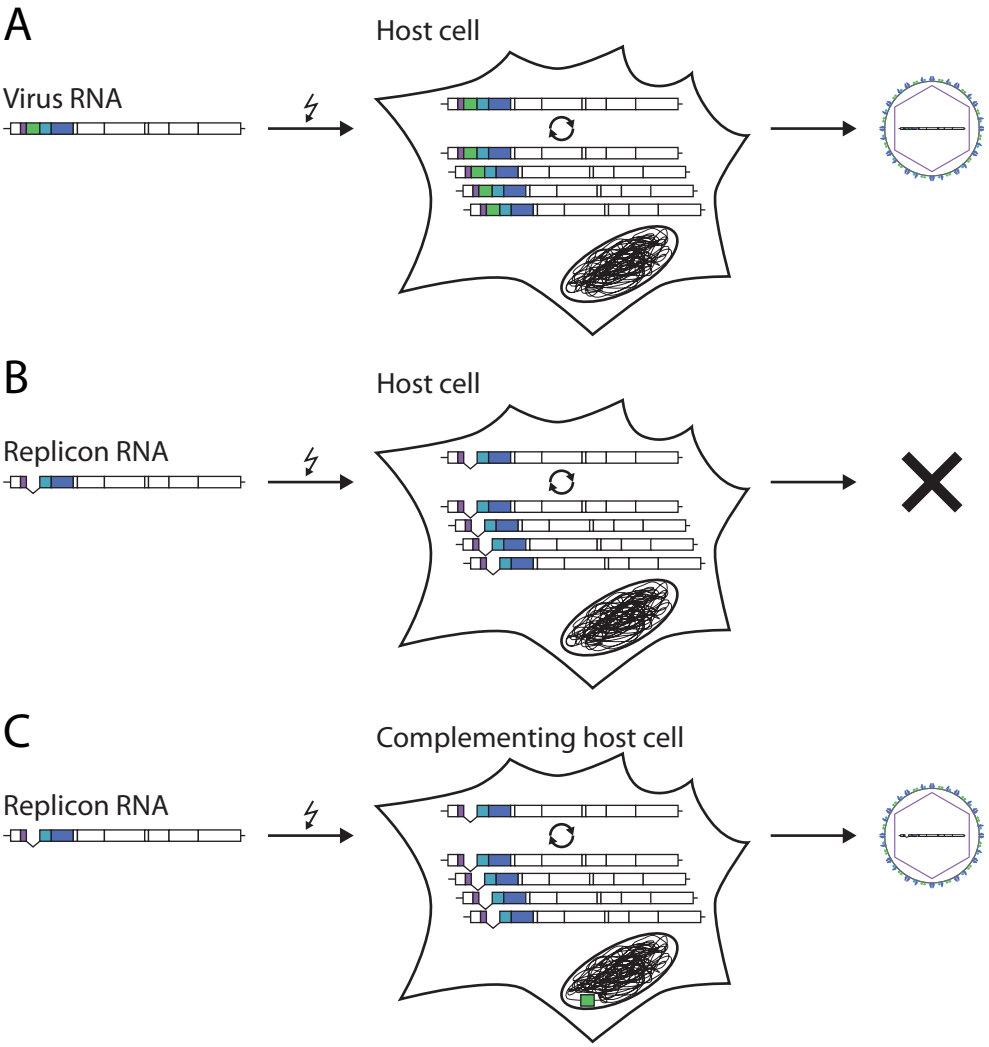

Figure 1: Rescue of virus and VRP. (A) Transfection of host cells with full-length in vitro transcribed viral RNA results in the production of progeny viruses. (B) In contrast, transfection of host cells with replicon RNA results in intracellular RNA replication without particle formation due to the lack of structural gene elements. (C) VRP can be rescued by transfection of cells expressing the structural gene that is lacking in the replicon.

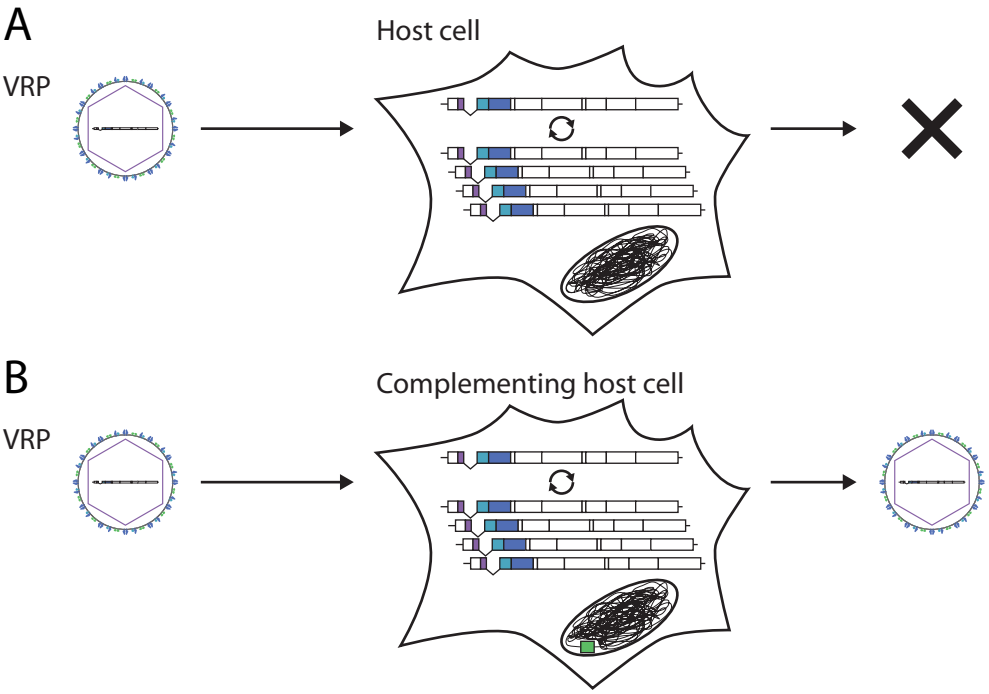

Figure 2: VRP are single-round infectious particles. (A) Infection of cells with VRP results in RNA replication without formation of progeny virus. (B) VRP can be propagated by infection of complementing cells.

first two third of the genome contains a large open reading frame (ORF) coding for the nonstructural proteins. This nonstructural region is separated from the downstream structural genes by a translation termination codon followed by a subgenomic 26S promoter. During alphavirus replication, two different types of positive-sense RNA are generated, the full-length genomic RNA and a smaller subgenomic RNA of approximately 4 kb transcribed from the 26S promoter and produced in higher amounts than the genomic RNA. This subgenomic mRNA encodes the structural proteins and possesses a CAP structure and a polyA tail like the full-length genome. Alphaviruses can serve as efficient gene expression systems by replacement of the entire structural region with foreign genes. Infection of cells with alphaviruses results in a cytopathic effect leading to cell death due to inhibition of host cell protein synthesis. The cytopathogenicity of alphaviruses is independent of the presence of structural genes. Therefore, the alphavirus replicons are cytopathogenic like the parent virus [13]. Alphavirus replicon systems for Semliki Forest virus [14], Sindbis virus [15, 16] and Venezuelan equine encephalitis virus [17] are well established (reviewed in [7, 9, 10]). Several strategies are used to package alphavirus replicons into VRP. Cells can be cotransfected with the replicon RNA and with a defective helper RNA lacking most of the nonstructural genes and the packaging signal. Since recombination of the helper RNA and the replicon RNA was frequently observed, the packaging system was improved by splitting the structural genes on two separated defective helper RNA molecules [17]. Alternatively, packaging cell lines producing defective helper RNA are successfully employed [18].

Picornavirus replicons

Picornaviruses are nonenveloped viruses with a single positive-strand RNA genome of 7.5 kb in length [19]. A small protein called VPg (virion protein, genome linked) is covalently linked to the 5′ terminus of the genome. This protein is however not required for infectivity of in vitro transcribed viral RNA. The 3′ end of the genome contains a polyA tail. A single ORF codes for the structural genes required for capsid formation in the N-terminal third and for the nonstructural genes on the rest of the genome. The polyprotein is cleaved into the individual proteins by viral proteases. Poliovirus replicons were established by removing the structural genes [20]. Foreign proteins can be expressed by replacing the structural genes with the corresponding genes of interest. Cleavage by the viral protease 2A allows the release of the translated foreign gene product [21, 22]. Alternatively, a stop codon can be introduced downsream of the foreign gene, followed by an internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) allowing reinitiation of translation of the poliovirus ORF [23]. Polivirus replicons can be successfully packaged by transcomplementation with recombinant vaccinia virus expressing the structural proteins of poliovirus [24].

Flavivirus replicons

Flaviviruses are enveloped viruses with a single-strand RNA genome of positive polarity [25]. The genomic RNA is approximately 11 kb in length. Flavivirus RNA is capped at the 5′ end but does not contain a 3′-terminal polyA tract. The genomic RNA molecule acts as mRNA, with a single ORF encoding the structural genes in the first third of the genome followed by the nonstructural genes. The viral polyprotein is cleaved co- and posttranslationally into the individual proteins by cellular and viral proteases. Unlike alphaviruses, flaviviruses are typically noncythopathogenic, which results in prolonged replication in the host cells. Replicons expressing foreign genes were established for a number of different flaviviruses including West Nile virus [26–29], yellow fever virus [30, 31], Dengue virus [32, 33], tick-borne encephalitis virus [34–36], Japanese encephalitis virus [37] and Kunjin virus (KUN) [8, 38]. The KUN replicon is the best characterized system. Replication of KUN replicons requires the first 60 nucleotides (20 codons) of the core gene C and the last 66 nucleotides (23 codons) of the envelope protein E gene [38]. Several strategies for gene insertion have been developed using ubiquitin-mediated protein processing or foot-and-mouth disease virus 2A autoprotease cleavage to release the foreign protein from the polyprotein (reviewed in [7]). In analogy to the poliovirus replicons, the EMCV IRES was used for the generation of a second transcriptional unit, also called cistron. To package the KUN replicons, a Semliki Forest virus replicon that expresses the KUN structural proteins has been employed [39, 40]. A tetracycline-inducible packaging cell line that expresses the KUN structural proteins represents an alternative approach for production of KUN VRP [41].

VSV replicons

VSV is an enveloped virus of the *Rhabdoviridae* family [42]. The virus contains a nonsegemented negative-strand RNA genome that codes for five major proteins in the order of nucleocapsid protein (N), phosphoprotein (P), matrix protein (M),

glycoprotein (G), and the large protein (L). The genomic RNA is not infectious per se. For virus and replicon rescue from cDNA, the RNA polymerase complex must be present in the cell to initiate RNA replication and protein translation. To package VSV replicons lacking the gene for the glycoprotein G, cells are first infected with recombinant vaccinia virus expressing the T7 phage RNA polymerase [11]. Subsequently, cells are transfected with a plasmid encoding the RNA genome in antigenomic orientation, three helper plasmids to express the viral RNA polymerase complex and an additional plasmid coding for the missing glycoprotein G. All these plasmids harbour the T7 promoter for RNA transcription by the vaccinia virus-encoded T7 phage RNA polymerase. Once VRP are generated, they can be passaged on helper cell lines expressing the deleted structural protein in trans. So far, VSV replicons with a deletion of the glycoprotein G were established [43, 44]. In addition, VSV replicons were applied for the expression of heterologous genes by replacement of the glycoprotein G gene with a foreign gene [43–45].

Pestivirus replicons

Pestiviruses are enveloped, typically noncytopathogenic viruses with a genome consisting of a single-stranded RNA molecule of positive polarity. The pestiviruses belong to the family *Flaviviridae* together with the flaviviruses and the hepaciviruses [25, 46]. For the pestivirus bovine viral diarrhea virus (BVDV) naturally occurring cytopathogenic subgenomic replicons were described [47]. These were also found with classical swine fever virus (CSFV) after multiple passages in cell culture [48]. Studies with systemic sequential deletions demonstrated that the nonstructural genes NS3 to NS5B flanked by a 5′ and 3′ nontranslated region (NTR) represent the minimal elements for RNA replication [47, 49]. So far, pestivirus replicons were developed for vaccination against BVDV and CSFV [50–54]. Replicons lacking a single structural gene were packaged to form VRP using complementing cell lines expressing the missing structural proteins in trans. Replicons were also used to study the viral life cycle of CSFV [55] and BVDV [56–60]. In these studies, reporter genes encoding firefly luciferase, neomycin phosphotransferase, green fluorescent protein (GFP) and β-glucuronidase were applied. However, no generic gene expression system based on pestivirus replicons has been developed so far.

Applications of replicons

One of the main applications of replicons is the usage as a vaccine platform against viral diseases. In a homologous system, replicons that have only a partial deletion of the structural genes induce an immune response against the remaining structural genes. Packaged in VRP, such replicons are a useful tool for immunization against the virus they originate from (see above and reference [61]). As heterologous vaccines, replicons encoding foreign antigens are employed to generate an immune response against a target pathogen of interest (reviewed in [7–11]). In contrast to subunit vaccines, replicons can efficiently induce cellular immune responses [8]. The highest efficacy is usually achieved with replicons packaged in VRP. But replicons delivered by DNA launch also induce an efficient immune response. Delivery of naked RNA molecules has not yet been established, essentially because RNA is susceptible to RNases,

resulting in inefficient transfer to cells. The most elaborated replicon systems are derived from alphaviruses [9, 10] and KUN virus [8]. They are used immunize against antigens of influenza virus, human immunodeficiency virus (HIV), hepatitis C virus (HCV), Ebola virus, to mention only a few examples.

Replicons can also be used in anticancer therapy [7, 8]. Modified replicons that induce interferon-α/β (IFN-α/β) or express immunostimulatory cytokines are used for intratumoral injection or infection if packed in VRP. Cytokines and IFN-α/β secreted by replicon-infected cells will attract immune cells and thereby promote an immune response against the tumor [7, 8, 62].

Classical swine fever virus

Classical swine fever (CSF) is a highly contagious disease of pigs and wild boars. CSF is caused by CSFV and has an important economical impact. The virus is highly contagious, and infection results in severe haemorrhagic symptoms with a high mortality rate, especially in piglets. Therefore, CSF is a notifiable disease according to the Office International des Epizooties (OIE). This implicates that every confirmed case is registered by the OIE that defines the measures to be taken to control and eradicate the outbreak.

Virus classification

CSFV belongs to the genus *Pestivirus* within the family *Flaviviridae*, along with the genera *Flavivirus* and *Hepacivirus* and the GB viruses, a group of yet unassigned agents [25, 46]. The *Flaviviridae* are enveloped viruses with a positive sense single-strand RNA genome.

The genus *Flavivirus* (from Latin *flavus*, yellow) comprises more than 50 species, with yellow fever virus, dengue virus, West Nile virus, Japanese encephalits virus and tick-borne encephalitis virus being the most common species. Most flaviviruses are important human pathogens that cause severe disease. They are transmitted by arthropod vectors such as mosquitos and ticks.

The genus *Pestivirus* (from Latin *pestis*, plague) encompasses viruses with a specific tropism for pigs and ruminants including cattle, sheep, goats and wild ruminants. While border disease virus (BDV) and bovine viral diarrhoea virus 1 and 2 (BVDV-1 and BVDV-2) affect sheep and cattle, respectively, CSFV infects pigs. Atypical pestiviruses have been isolated from giraffe [63], from pronghorn antelope [64], and from foetal calf serum ('HoBi' virus) [65]. Recently, Bungowannah virus was isolated from pigs [66]. In contrast to the flaviviruses, there is no intermediate invertebrate host for pestiviruses.

HCV, the sole member the genus *Hepacivirus* (from Greek *hepar*, *hepatos*, liver), is an important human pathogen. HCV is restricted to humans and transmission occurs almost exclusively by parenteral exposure to contaminated blood and blood products. Note that CSFV was formerly called hog cholera virus and was renamed to avoid confusion with hepatitis C virus (HCV).

Molecular biology of CSFV

Genome

The CSFV genome consists of a single-strand RNA molecule of positive polarity of 12.3 kb in length [67, 68]. The viral genome functions as viral mRNA. Consequentially, transfection of cells with the in vitro generated RNA

genome is sufficient to generate infectious CSFV [69–71]. The genome carries a single ORF, flanked by a 5′ and a 3′ NTR (Figure 3A). The ORF encodes a large polyprotein composed of four structural and eight nonstructural proteins in the order N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. The polyprotein is co- and posttranslationally cleaved into the single viral proteins by cellular and viral proteases (Figure 3B). The 5′ NTR comprises 373 nucleotides and is folded in a complex secondary structure with several stem-loop domains [72]. It functions as an IRES for CAP-independent translation initiation. The 3′ NTR is 231 nucleotides long with some length differences between strains. It does not carry a polyA tail [67, 73] but appears to end with a short cytosine stretch instead. The 3′ NTR contains a variable region followed by a conserved 3′-terminal region that forms two hairpins separated by a single-stranded region [72, 74]. The terminal hairpin and the single-stranded region function most probably in cis to direct the synthesis of the minus-strand [74].

Virion and structural proteins

The enveloped spherical virus particles are 40–60 nm in diameter [75, 76]. They are difficult to purify because of limited amount of particles released by infected cells and association with cellular debris [77]. Therefore, the structure and chemical composition of the virion has not yet been determined. The virion is composed of the genome RNA surrounded by the core protein C and a lipid bilayer carrying the envelope glycoproteins E^{rns}, E1 and E2 (Figure 4A) [78, 79]. CSFV is sensitive to heat, organic solvents and detergents, and can be inactivated by UV light [75, 80]. It is stable at pH between 5 and 10 [80].

C The core protein C is a small, highly basic polypeptide of 14 kD in size that complexes the genomic RNA. It is an intrinsically disordered protein with a high plasticity that binds RNA nonspecifically [81, 82]. The N terminus of the core protein is formed through autoproteolytic cleavage of N^{pro} (see below). At the

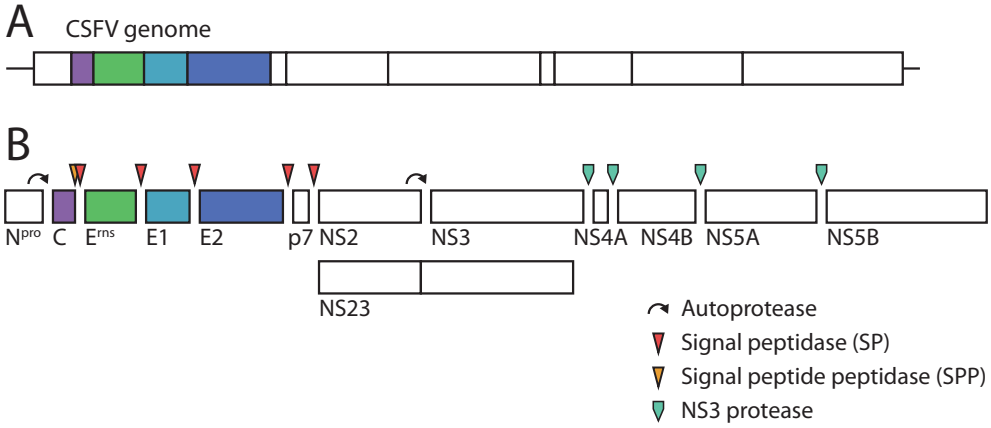

Figure 3: The CSFV genome and processing of the viral polyprotein. Schematic representation of the CSFV genome RNA (A) and of the encoded polyprotein (B). The coloured boxes represent the structural genes (A) and the corresponding proteins (B). The polyprotein is processed co- and posttranslationally into the individual proteins by cellular and viral proteases as indicated.

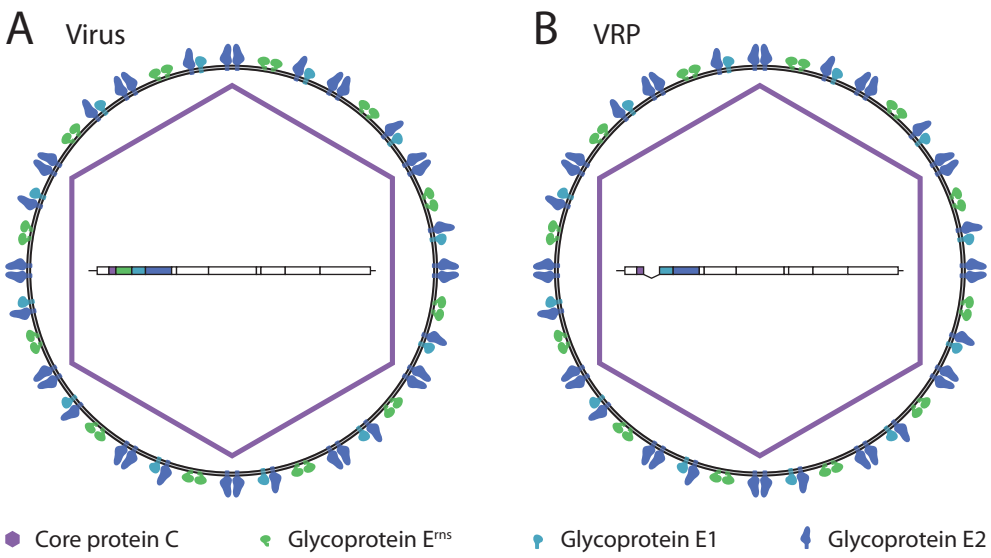

Figure 4: CSFV virion and VRP. The CSFV virion (A) contains a full-length viral RNA genome while the VRP (B) harbours a replicon RNA lacking coding sequences of structural genes. The envelope of the VRP is indistinguishable from the envelope of a CSFV virion. The VRP therefore exhibit the same cell tropism as parent CSFV.

C terminus, the core protein is first cleaved by signal peptidase (signalase or SP), which generates the N terminus of the E^{rns} glycoprotein [78]. C is then further processed by a signal peptide peptidase (SPP) [83]. The SPP cleaves within a hydrophobic membrane-spanning domain [83] that acts as a signal sequence for translocation of the downstream glycoprotein E^{rns} into the endoplasmatic reticulum (ER) [78]. The three-dimensional structure of the core protein C has not yet been defined.

E^{rns} The glycoprotein E^{rns} (rns stands for ribonuclease, soluble) is unique to pestiviruses. It is highly N-glycosylated and forms disulfide-linked homodimers [79]. In contrast to the other envelope glycoproteins, E^{rns} lacks a typical transmembrane domain [78]. Nevertheless, E^{rns} binds membranes, although with less affinity than a classical membrane-anchored protein. Binding occurs through an atypical C-terminal anchor sequence that is predicted to form an amphipathic α-helix [84, 85]. Due to this weaker interaction with membranes, considerable amounts of E^{rns} are secreted from infected cells [78, 86, 87]. The C-terminal sequence of E^{rns} can mediate translocation of E^{rns} from the outside through the plasma membrane into a target cell [85].

Interestingly, E^{rns} possesses RNase activity [88–90] that preferentially cleaves 5′ of uridine residues [91]. In vitro, this RNase activity is required for E^{rns} of BVDV to target extracellular or endosomal double-stranded RNA and thereby to interfere with the induction of IFN-α/β in host cells [92–94].

E^{rns} is essential for the formation of infectious CSFV particles [50, 54]. Genomic deletion of the E^{rns} gene can be complemented by E^{rns} expression in trans. Interestingly, pseudotyped retroviral particles carrying the CSFV glycoproteins

E1 and E2 but lacking E^{rns} are able to infect cells [95]. This suggests that E^{rns} is dispensable for viral entry but may take part in the attachment of the virion to the cell and may also play a role in post-entry stages. This is supported by the finding that mutation of E^{rns} to abolish dimerization only slightly affects virus growth but decreases the affinity for heperansulfate [96]. In addition, the RNase activity of E^{rns} can be knocked out by single amino acid substitutions without affecting virus replication [97, 98]. However, such a mutation attenuates the virus in vivo [98]. It was reported that a CSFV vaccine strain with an RNase inactive E^{rns} was cytopathogenic [97]. This was not reproducible with other strains [98].

E1 and E2 The glycoproteins E1 and E2 are type I transmembrane proteins with a C-terminal hydrophobic anchor and an N-terminal ectodomain [78]. E1 carries two to three N-linked glycosylation sites, while E2 has four to six sites, depending on the strain [25, 99, 100]. While removal of all putative glycosylation sites in either E1 or E2 completely impairs generation of viable virus, mutation of individual glycosylation sites in E1 or E2 can result in attenuated viruses [99, 100]. Therefore glycosylation of E1 and E2 plays an important role in CSFV virulence. In the virions E1 and E2 are present as disulfide-linked E1-E2 heterodimers and as E2 homodimers [79]. For BVDV it was shown that charged amino acid residues in the transmembrane domains of E1 and E2 are important for heterodimer formation, which is crucial for virus entry into host cells [101]. The glycoproteins E2, E^{rns} [102] and E1 [95], altogether mediate attachment and binding of the virion to the host cell. Thereby, E2 plays a major role in determining the cell tropism [103, 104]. Proteomics computational analysis suggested that E2 is a truncated class II fusion protein that mediates fusion between the viral and the cellular membrane [105]. E2 is essential for infectious particle formation [53]. Virus mutants with partial or complete deletion of E2 are nonviable but can be rescued by complementation of E2 in trans [51, 53].

Nonstructural proteins

N^{pro} The first protein encoded by the pestivirus genome is the nonstructural protein N^{pro}. There is no corresponding protein in HCV and the flaviviruses. N^{pro} is an atypical cysteine autoprotease that cleaves itself cotranslationally from the nascent polypeptide at its C terminus between a cysteine and a serine amino acid residue [106–108]. Interestingly, besides being an autoprotease, N^{pro} interferes with the induction of IFN-α/β. N^{pro} downregulates interferon regulatory factor 3 (IRF3) via induction of polyubiquitination and subsequent proteasomal degradation of IRF3 [109–113]. IRF3 is the main transcription factor for the induction of IFN-α/β. The function of N^{pro} to targed IRF3 for proteasomal degradation is independent of the protease activity [112, 114]. Mutation of N^{pro} by substitution of the aspartic acid residue 136 with asparagine ($D_{136}N$) or of the cysteine residue 112 with arginine ($C_{112}R$) interferes with binding of a zinc atom required for protein stability. These mutations abolish the N^{pro}-mediated degradation of IRF3 but do not affect proteolytic cleavage of the nascent polyprotein [114]. Neither the knock-out of the IRF3-degrading function of N^{pro} nor the deletion of the entire N^{pro} affect virus replication [112, 115]. N^{pro} itself is an unstable protein that is degraded by the cellular proteasome machinery

in an ubiquitin-dependent manner. The degradation of N^{pro} is not a direct consequence of its ability to target IRF3 for degradation [116].

p7 The small hydrophobic peptide p7 links the glycoprotein E2 and the nonstructural protein NS2-3. It consists of a short charged sequence facing the cytosol flanked by two hydrophobic transmembrane domains [117]. The function of p7 has not been determined yet. The p7 protein can be supplemented in trans and is dispensable for RNA replication [47, 118]. It is however required for virus particle formation but is not incorporated into the virion [119]. Cleavage between E2 and p7 by signal peptidase is inefficient. The precursor E2-p7 is not incorporated into the virions either [117] and has no essential role in the viral life-cycle [119].

NS2 The nonstructural protein NS2 is an autoprotease that cleaves the NS2-3 precursor protein between NS2 and NS3. The cleavage depends on the cellular cofactor Jiv (J-domain protein interacting with viral protein) [120, 121]. While the precursor protein NS2-3 is essential for the production of infectious particles [118], cleaved NS2 per se has no additional essential function in the virus replication cycle [55].

NS3 The nonstructural protein NS3 and its precursor NS2-3 are multifunctional proteins harbouring serine protease, RNA binding, NTPase and helicase activity [122–125]. The serine protease located in the N-terminal region of NS3 catalyzes the release of the downstream proteins NS4A, NS4B, NS5A and NS5B from the polyprotein. The NS3 cleavage sites of BVDV are characterized by a strictly conserved leucine residue at the P1 position and a serine, alanine or asparagine residue at the P1' position [126, 127]. Processing at the NS3/4A site seems to occur exclusively in cis, while all other cleavages can occur in trans [125]. The cleavage of the NS4B/5A and NS5A/5B sites by NS3 depend strictly on the presence of NS4A as a cofactor [127]. The helicase and NTPase domains are located in the C-terminal part of NS3 [123, 124]. NS3 binds to the 3' NTR of the plus-strand and minus-strand through its helicase domain [128], which results in unwinding of the secondary structure in the 3' NTR and in initiation of RNA genome replication. Both, the helicase and the NTPase domains of BVDV NS3 are required for minus-strand synthesis [129]. The NTPase activity of CSFV NS3 is stimulated by the viral polymerase NS5B [130].

NS2-3 The cleavage of the precursor protein NS2-3 is tightly regulated by the cellular cofactor Jiv [131]. The cytopathic effect observed with accumulation of large amounts of cleaved NS3 and with increased viral RNA replication demonstrates the important role of a regulated cleavage [25, 132, 133]. Uncleaved NS2-3 is required for virus morphogenesis [55, 118]. NS3 however is essential for RNA replication and cannot be replaced functionally in the replication complex by the uncleaved precursor NS2-3 [120]. Early after infection, cellular Jiv acts as a cofactor of the NS2 protease and allows cleavage of the majority of NS2-3 [131]. After cleavage, Jiv remains bound to NS2 and is not available for further reactions. Because of limited amounts of Jiv and the restriction of the NS2 protease to cis cleavage, NS2-3 cleavage occurs only early after infection. For noncytopathogenic BVDV, NS2-3 autoprocessing decreases to nearly undetectable levels at six to nine hours postinfection, while only

slightly reduced cleavage activity is observed with the cytopathogenic BVDV [120]. This limits RNA replication in noncytopathogenic BVDV at later times after infection, which is crucial for virus persistence.

NS4A The nonstructural protein NS4A is a small 64 amino acid protein with a hydrophobic N-terminus followed by a highly charged and acidic C-terminal domain. NS4A acts as cofactor of the NS3 serine protease, which involves the interaction of a central domain of NS4A with the N-terminal region of NS3 [134].

NS4B Pestivirus NS4B is an integral membrane protein with six membrane-associated hydrophobic domains [135, 136]. For BVDV, NS4B was shown to colocalize with Golgi markers [136]. In addition, BVDV infection induces membrane rearrangement in the host cell in association with NS4B [136]. Since BVDV NS4B is essential for RNA replication [137] and can be cross-linked with NS5A and NS3 [135], a role in the replication complex was suggested. Accordingly, NS4B colocalizes with NS5A and NS5B [136]. A single amino acid mutation in NS4B can render the BVDV strain NADL noncytopathogenic despite the presence of high levels of NS3 and viral RNA accumulation [135].

NS5A The pestivirus NS5A is a large hydrophilic protein involved in virus replication. It is predicted to interact with cellular membranes through a N-terminal amphipathic α-helix [138]. Cellular serine/threonine kinases phosphorylate NS5A preferentially on serine residues [139]. This phosphorylation is conserved among all members of the *Flaviviridae* family, suggesting an important role in their life cycle. In addition, BVDV NS5A interacts with the α subunit of the bovine translation elongation factor-1 [140]. NS5A is the only protein required for RNA replication that can be complemented in trans [137]. Its exact function remains however largely unknown.

NS5B NS5B represents the viral RNA-dependent RNA polymerase [141–144]. Its structure has been determined at 2.9 Å resolution [145, 146]. NS5B contains a sequence motif of one glycine and two aspartic acid residues (GDD), which is a motif common to all viral RNA-dependent RNA polymerases of the family *Flaviviridae* [147]. The two aspartic acids coordinate metal ions that catalyze the addition of NTP to the nascent polynucleotide chain. Primer-independent initiation of RNA synthesis requires a high concentration of GTP, but no other nucleotide [148–150]. GTP binds close to the catalytic GDD-containing motif inside the RNA template-binding channel and thereby mimics a nascent RNA strand [146]. The polymerase activity of NS5B is stimulated by NS3 in a dose-dependent manner by binding to NS5B [151].

Replication cycle

The first steps of virus infection are binding to the cell surface and entry into the cell. The specific cell surface interaction partners and receptors of CSFV are not yet characterized. For BVDV, a viral receptor is bovine CD46 [152, 153]. The viral glycoproteins E1 and E2 are sufficient and essential to mediate entry of CSFV and BVDV [95, 101]. Interestingly, passage of CSFV in cell culture can lead to heparan sulfate dependent strains, a phenomenon attributed to a single amino acid change in the E^{rns} glycoprotein [154]. Several studies using metabolic inhibitors demonstrate that BVDV infection occurs by clathrin-mediated endocytosis

[155–158]. Further, BVDV infection depends on endosomal acidification that allows low pH-dependent membrane fusion.

After release into the cytosol, the pestivirus genome RNA functions as mRNA. Translation is initiated by an IRES located in the 5′ NTR of the genome, independently of a CAP structure. The translated polyprotein is then cleaved co- and posttranslationally into the individual proteins (Figure 3B).

Pestivirus RNA replication requires the nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B [47, 49]. The corresponding nonstructural proteins of HCV are also components of the replication complex that associates with altered cellular membranes of the ER [159–161]. The proteins translated from the viral RNA released in the cytoplasm assemble to a replication complex that binds to the 3′ end of the viral RNA and synthesizes full-length complementary minus-strand RNA. This generates an intermediate double-stranded RNA molecule. Subsequently, plus-strand RNA is produced by using the minus-strand RNA as a template. The newly synthesized plus-strand RNA serves as genome for progeny virus and as mRNA. Minus- and plus-strand RNA can be detected 4 to 6 hours post infection. This is followed by an asymmetric accumulation of minus-strand RNA and with an excess plus-strand RNA molecules [162].

Virus assembly occurs in intracellular vesicles and the particles are released by exocytosis [163]. Accordingly, no structural proteins can be detected on the outer surface of the plasma membrane [87, 164, 165]. The flavivirus C protein associates with membranes of the ER [166]. It was suggested that the flavivirus C protein interacts with the viral genomic RNA to form a nucleocapsid precursor [25]. The pestivirus C protein is an intrinsically disordered RNA-binding protein [81]. It interacts with ER membranes, presumably through a C-terminal hydrophobic domain [82]. Flavivirus nucleocapsids then bud into the ER and thereby become enveloped with a lipid membrane carrying the viral glycoproteins [25]. The immature flavivirus particles are transported through the secretory pathway where the glycoproteins are processed and the particles mature [167]. Interestingly, the viral nonstructural proteins p7, NS2-3, NS4A and NS5B are essential for assembly of pestivirus particles, although they are not part of the infectious virions [55, 56, 58, 118, 119].

Disease and pathogenesis

CSFV infects its host typically via the oronasal route after direct or indirect contact with infected pigs. Other sources of infection are swill feeding [168] and insemination with contaminated semen [169]. The tonsils are the primary site of virus replication and entry into the host. The virus then spreads through lymphatic vessels to lymph nodes where it can be detected within hours after infection. Thereafter, the virus enters the blood stream giving rise to a first viremia after 16 to 24 hours. At that time, the virus can be detected in the spleen and other lymphoid organs. Further virus replication sites are the bone marrow, the gut-associated lymphoid tissue and the thymus [170, 171]. The highest virus titres are observed in the blood, spleen and lymph nodes. CSFV is also found in other organs such as the pancreas, liver, kidney, urinary bladder, lung and the central nervous system [171–173]. Depending on the virulence of a CSFV strain,

the course of the disease can be acute, subacute or chronic, and can remain unnoticed with low virulent strains.

Acute CSF

In the acute form of the disease, affected pigs develop high fever (41 °C), depression and inappetence within 2 to 6 days after infection. They often show central nervous system disorders characterized by ataxia, paralysis of the hind legs, teeth grinding and convulsions. Cyanosis, particularly of the ears, and haemorrhages in the skin and mucosa are occasionally observed. Other signs of CSF are lacrimation, nasal discharge and mild constipation followed by diarrhoea [171]. The severity of the disease depends on the virus strain and on the age of the infected pig. Young pigs are more severely affected than older animals [174]. The mortality rate in piglets may reach up 90 % and most animals die 10–14 days after the onset of disease.

Typical lesions consist of swollen haemorrhagic lymph nodes and petechial haemorrhages in the kidneys, spleen and urinary bladder, infarction of the margin of the spleen, petechiae in the larynx and epiglottis, lesions in the stomach and large intestine, and atrophy of the thymus [171, 175, 176] (Figure 5).

Chronic CSF

Chronic CSF is defined as a lethal clinical form of CSF that lasts at least 30 days [171, 177]. This form of CSF develops if the infected animals do not generate an effective immune response [175]. Usually, the disease develops slowly, with a single organ system (lung, gastro-intestinal tract, central nervous system) being predominantly affected [171]. The initial signs resemble those of acute CSF but at later stages, the clinical picture is often uncharacteristic. Predominantly nonspecific signs like intermittent fever, chronic enteritis and wasting are observed. Petechial haemorrhages are less prominent than in the acute form. Ulcers in the large intestine, associated with an enteritis, are occasionally observed [171].

Prenatal CSF

CSFV can cross the placenta of pregnant pigs and thereby infect the foetuses [171, 174–176]. The outcome of the transplacental infection depends on the day of gestation and on the virulence of the virus. Infection during early pregnancy results in abortion and stillbirth, mummification or malformation. If the infection occurs between day 50 and 70 of gestation, persistently infected piglets may be born. Initially, these are apparently healthy although they are viraemic. They rapidly become growth retarded and have a low survival rate. Infection after 85 days of gestation results in abortion or may yield normal, nonviraemic piglets [171, 176].

Interaction of CSFV with the host cells

The main target cells for virus replication are endothelial cells, lymphoreticular cells, macrophages and dendritic cells [171, 178, 179]. CSFV replicates without altering the morphology and the viability of the infected cells. In monocytes and macrophages, CSFV induces secretion of inflammatory cytokines, including interleukin-1 and prostaglandin E_2 [179]. Importantly, in these cells, the virus subverts the immune system by blocking the induction of IFN-α/β through

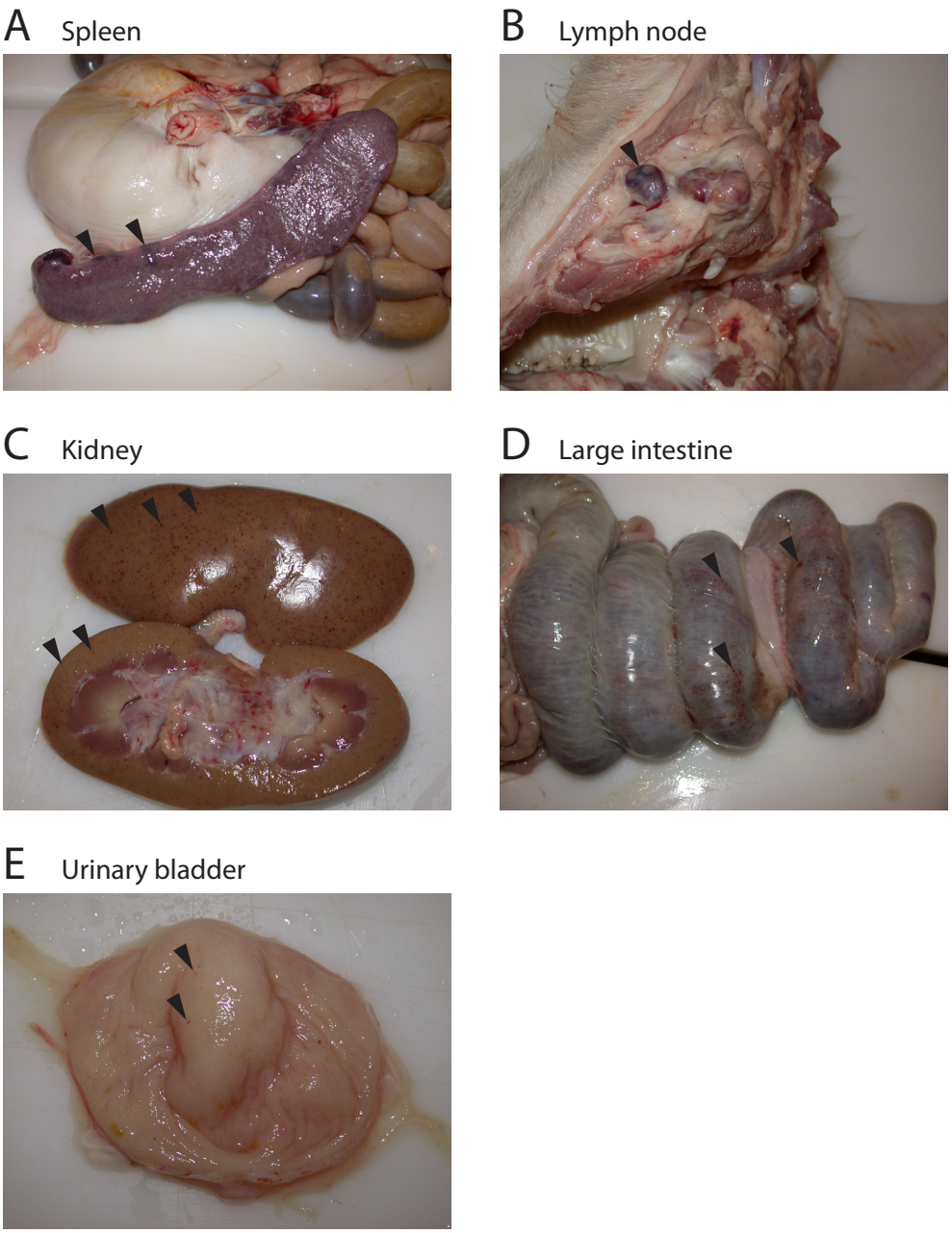

Figure 5: CSFV causes a haemorrhagic disease. Marginal infarcts in the spleen (A), haemorrhagic lymph nodes (B) and petechial bleedings in the kidney (C), large intestine (D) and urinary bladder (E) of a pig infected with the moderately virulent CSFV vA187-1 (photographs by Nicolas Ruggli, Institute of Virology and Immunoprophylaxis, Mittelhäusern).

N^{pro}-mediated proteasomal degradation of IRF3 [109, 180, 181]. This allows the virus to replicate efficiently, spread and induce viraemia. According to the current model [112, 182], CSFV then infect plasmacytoid dendritic cells (pDC) present in the blood and lymphatic organs such as spleen, thymus and lymph nodes. In these latter cells, also known as natural interferon producing cells, the virus induces large amounts of IFN-α independently of IRF3, by signalling via

interferon regulatory factor 7 (IRF7) [182, 183]. The secretion of large amounts of IFN-α and pro-inflammatory cytokines in turn leads to a cytokine storm and depletion of T and B lymphocytes [184].

Epidemiology

CSF occurs worldwide. Currently, it is endemic in Central and South America, South-East Asia and some countries of Eastern Europe (Figure 6) [185]. For the latest information on the disease situation, see http://www.oie.int/wahid . After implementation of strict control measures, Australia, Canada, New Zealand and the United States of America (USA) eradicated the disease. Also most countries of the European Union (EU) are free of CSF in domestic pigs. In Switzerland, the last outbreaks occurred in 1993 in domestic pigs [186] and in 1998 in wild boars [187]. For economical reasons, Europe applies a non-vaccination policy in order to maintain the pig population seronegative and to allow free trade of pigs [175].

Wild boars represent the natural reservoir of CSFV. Approximately 60 % of the primary CSF outbreaks in domestic pigs between 1993 and 1998 were due to direct or indirect contact with wild boars [168]. As long as CSFV circulates in the wild boar reservoir, it represents a constant threat for the pig farming. The latest severe epidemic of CSF occurred in 1997–1998 in the Netherlands [188, 189]. This epidemic spread to Spain, Italy and Belgium. In the Netherlands, 429 infected herds with a total of 700 000 pigs were detected and culled. An additional 11 Mio pigs were preventively slaughtered. A breeding ban was implemented because it was considered unethical to breed piglets that would almost certainly have to be destroyed. In 2000, CSFV was introduced to the United Kingdom (UK) [175, 190, 191]. CSFV has not yet completely disappeared from Europe. It is still present in Eastern Europe in domestic pigs and in Germany in wild boars [175, 192, 193].

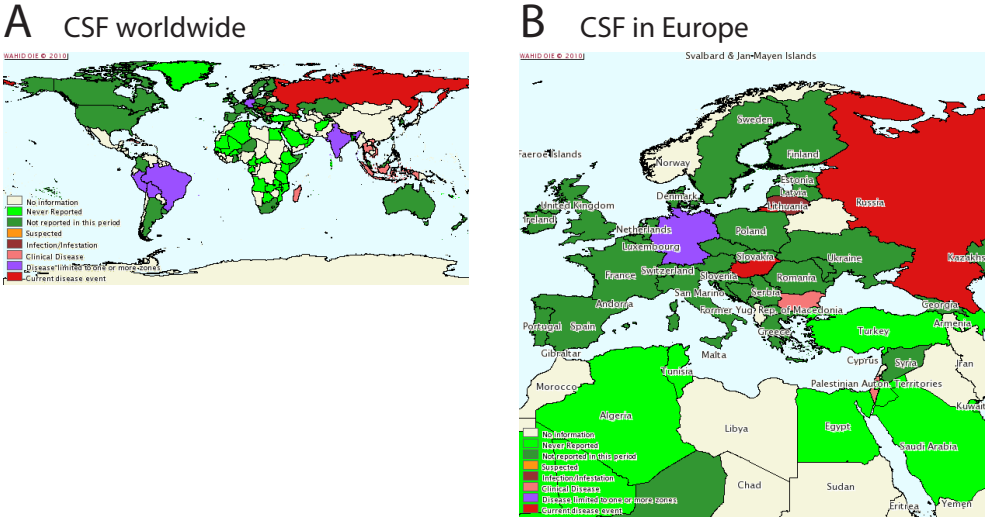

Figure 6: Current geographic distribution of CSF. Distribution of CSF worldwide (A) and in Europe (B) from July to December 2009 (maps from http://www.oie.int/wahid).

Vaccines against CSFV

Europe has implemented a non-vaccination policy because the available live attenuated vaccine strains of CSFV do not allow serological differentiation of immunized pigs from pigs that were infected with a CSFV field strain [175]. To control a CSF outbreak, mass killing is required, which causes high economical losses. Therefore, there is a need for new strategies to control CSFV. One aspect is the development of potent marker vaccines that allow differentiation of infected from vaccinated animals (DIVA) [194].

Immune responses against CSFV

Humoral immune response

Humoral immunity plays an important role in the protection from CSFV infection. Newborn piglets are protected by colostral antibodies, and protection can be transferred to pigs of all ages by the application of hyperimmune serum [176]. However, passive immunization does not completely prevent virus replication in piglets [195]. In addition, maternal antibodies have a limited lifetime with a half-life of approximately 14 days [174].

Pigs infected with CSFV develop antibodies against the glycoproteins E^{rns} and E2 and against the nonstructural protein NS3. The neutralizing antibodies are directed against epitopes of the E2 and E^{rns} glycoproteins. The major protective epitopes are located on the E2 glycoprotein [86, 196–198]. Antibodies directed against the nonstructural protein NS3 are incapable of neutralizing the virus because NS3 is not present in the virion.

After infection with CSFV, neutralizing antibodies are detectable 2 weeks after infection [174]. In the case of chronic CSF, neutralizing antibodies appear approximately one month after the infection and disappear after a few days [174, 199]. If pigs recover from CSF, they are protected against CSF for several years or even lifelong [174]. Due to the importance of humoral immunity in protection against CSF, an efficacious CSFV vaccine must induce a long-lasting virus-neutralizing antibody response.

Cell-mediated immune response

Interestingly, pigs vaccinated with the CSFV C-strain are protected as early as 1 weak after vaccination, before antibodies are detected [194]. Protection from CSFV in the absence of measurable neutralizing antibodies was reported by several groups [194, 200, 201]. These findings indicate an important contribution of cell-mediated immune responses in the defence against CSFV. Accordingly, CSFV-specific cytotoxic T cells can be detected in the blood of animals following CSFV infection or vaccination [202–205].

A hallmark of cell-mediated immunity is IFN-γ production. IFN-γ has several immunoregulatory effects involved in the induction of anti-viral immunity including the activation of cytotoxic T lymphocytes, natural killer cells and phagocytes. The production of IFN-γ in response to viruses is believed to reflect directly the virus-specific lymphocyte activies [206, 207]. Therefore, enzyme-linked immunosorbent spot (ELISPOT) assays were developed to detect CSFV-specific IFN-γ secreting cells [202, 208, 209]. With this approach, CSFV-specific IFN-γ secreting cells were detected in peripheral blood mononuclear cells (PBMC) of pigs vaccinated with the CSFV C-strain as early as 6 days following

vaccination [209]. Classical swine fever virus replicon particles (CSF-VRP) lacking the E^{rns} gene were also shown to induce CSFV-specific IFN-γ secreting cells [50].

PBMC of a CSFV-infected pig proliferate specifically in response to 15-mer peptides of the E^{rns}, E1, E2, NS2, NS3, NS4A, NS4B and NS5A regions [202]. Viral epitopes eliciting MHC class I-restricted cytotoxic T lymphocyte responses were identified in the nonstructural proteins NS2, NS3 and NS4A [202, 204, 208]. MHC class II-restricted helper T cell epitopes were found in the nonstructural proteins NS2 and NS3 [202, 208].

Inactivated virus vaccines

The first vaccines that were prepared against CSFV were inactivated whole virus vaccines [176, 210]. To generate these vaccines, pigs were infected with virulent CSFV, and virus was isolated from spleen, lymph nodes and blood. The virus was then inactivated by treatment with formalin, crystal violet or other inactivation reagents. Alternatively, cell culture-derived CSFV was used for vaccine production [210]. These vaccines were safe but generated a poor immune response. In addition, they had no efficacy in presence of maternal antibodies. Breeding pigs were protected from disease when challenged with virulent CSFV. Their foetuses however were often infected, resulting in abortion, malformations and persistently infected piglets.

Live attenuated vaccines

To improve efficacy, live attenuated vaccines were developed. Attenuation of CSFV was achieved by serial passages in rabbits [174, 176, 210–212]. These lapinized vaccines were efficacious and induced a long-lasting and stable immunity. They were widely used as live vaccines for immunization of pigs in the United States and other countries [176, 210]. However, the degree of attenuation was not sufficient. Vaccination of pregnant sows was repeatedly associated with reproductive disorders due to transplacental infection of the foetuses. The residual virulence was particularly obvious in young piglets that showed clinical reactions, haemorrhagic lesions and died occasionally. In addition, the attenuated virus was excreted and spread to unvaccinated pigs. After a few serial passages in pigs, the attenuated virus could regain virulence [176, 210]. Therefore, the safety of the live attenuated vaccines had to be improved to minimize the adverse effects. Two different approaches were followed. First, the passage number in rabbits was increased. Second, temperature sensitive virus mutants were selected. This led to the most efficacious CSFV vaccines currently available [176, 210, 213]. Among these vaccines are the Chinese strain (C-strain), GPE^- strain and the Thiverval strain that have been widely applied in the field. They protect pigs as early as 3 days after vaccination even though no neutralizing antibodies can be detected at that time. The lapinized C-strain was obtained through extensive passage in rabbits, in embryonated eggs and in ovine and porcine cells [176, 210, 213]. The C-strain is safe for pregnant pigs and possess excellent efficacy. The protective immunity against CSFV lasts for at least 18 months. Occasionally, the vaccine virus spreads to contact pigs or crosses the placenta without causing adverse effects. The attenuated GPE^- strain was generated in Japan by multiple passages of the original virulent CSFV strain ALD in cell culture and selection for temperature sensitive mutants [176, 210]. Unlike

the C-strain, the GPE$^-$ strain can be easily propagated in cell culture. Due to cold adaptation, the GPE$^-$ strain grows better at 30 °C than at 40 °C. During the attenuation process, the GPE$^-$ strain lost the capacity to block IFN-α/β induction as demonstrated by the interference with growth of the IFN-sensitive VSV [214–216]. This is due to a substitution of aspartic acid by asparagine at position 136 in N^{pro} ($D_{136}N$) of the GPE$^-$ strain [112]. The Thiverval vaccine strain was obtained by serial passages of the virulent strain Alfort in cell culture at 30 °C, resulting in a cold-adapted virus with optimal growth at 33–34 °C. Replication of this vaccine strain is drastically reduced at the normal porcine body temperature of 39–39.5 °C. [176, 210].

Taken together, live attenuated vaccines are efficacious, with two major drawbacks. First, there is the constant risk of reversion to virulence through mutation or recombination. Second, live attenuated vaccines do not allow DIVA [174, 175]. To fulfil the DIVA principle, marker vaccines are required. Marker vaccines induce an antibody pattern that differs from the antibody pattern found in infected animals.

Subunit vaccines

The first marker vaccines consisted of a subunit vaccine based on the viral E2 glycoprotein that was expressed in insect cells using a baculovirus vector [197, 200, 217, 218]. Since this vaccine contains the E2 protein as sole antigen, only E2-specific antibodies are generated. The antibodies against E^{rns} and NS3 produced during a CSFV infection serve for DIVA. This implies an accompanying serological test specific for E^{rns} antibodies [218]. The E2 subunit vaccines provide good protection against CSF [219, 220]. However, they do not prevent horizontal and vertical transmission of the challenge virus [220–222]. In addition, the discriminatory enzyme-linked immunosorbent assays (ELISA) developed so far are less sensitive than conventional CSF antibody ELISAs [223].

Novel vaccine approaches

Modern vaccines must be safe, highly efficacious and fulfil the DIVA principle. Since E2 is the most important immunogen of CSFV in terms of induction of neutralizing antibodies, the marker vaccines developed recently all contain E2 and rely on E^{rns} for DIVA [223]. Antibodies directed against NS3 are not a suitable marker because of the high prevalence of non-CSFV pestiviruses in pigs and the high conservation of the NS3 protein among pestiviruses.

Novel CSFV marker vaccines comprise immunogenic CSFV peptides [224, 225], DNA vectors [226–229], viral vectors expressing CSFV proteins [230–232], chimeric pestiviruses [104, 233] and VRP [50, 51, 53, 54] (for review see [234, 235]). Although peptide vaccines and the DNA vaccines can induce a protective immunity, their efficacy is limited. They require adjuvants or booster vaccination to be fully protective. Therefore, replicating vectors, chimeric viruses and VRP are more promising.

Chimeric pestiviruses

Chimeric pestivirus vaccines are live attenuated viruses containing structural coding sequences from two different pestiviruses. Efficacious chimeric CSFV vaccines were developed on the basis of BVDV strain CP7 by replacement of the E2 glycoprotein (CP7_E2alf) or E1 and E2 glycoproteins [233, 236] with the

corresponding sequences of the CSFV strain Alfort. Other approaches were the substitution of the E^{rns} or E2 genes of the CSFV C-strain with the corresponding genes of BVDV-2 strains [104]. Alternatively, parts of the E2 gene of the CSFV C-strain were replaced with corresponding sequences from the BDV strain Gifhorn [237]. Such chimeric pestiviruses are safe and potent vaccines, as tested under experimental conditions. The risk of reversion is lower compared to live attenuated vaccines. Recently, the CP7_E2alf chimera was selected for a field trial bait vaccination in wild boars [238].

CSF-VRP

VRP contain a mutated RNA genome capable of replication and expression viral proteins (Figure 4B). They cannot form particles due to the lack of structural coding sequences. Therefore, VRP are safe non-transmissible vaccines. They fulfil the requirements of a marker vaccine because no antibodies are generated against the missing gene. CSF-VRP with deletions in the E^{rns} or E2 gene were constructed and packaged efficiently with complementing cell lines [50, 51, 53, 54].

The first CSF-VRP developed for vaccine applications were based on the CSFV C-strain, with a complete or partial deletion of the E^{rns} gene [54]. Trans-complementation in a cell line that expresses the E^{rns} glycoprotein produced VRP capable of infecting and replicating in swine kidney cells (SK-6) but devoid of infectious virus production. In a pilot experiment, the pigs were vaccinated simultaneously via the intramuscular, the intravenous, the intradermal and the intratracheal route. After challenge with highly virulent CSFV, the pigs were protected from severe disease, developing only a mild fever. In this latter study however, no CSFV-specific antibodies were detected before challenge.

In a subsequent study, the different application routes were assessed individually [53]. Complete protection was achieved by intradermal vaccination with 10^5 50 % tissue culture infectious doses ($TCID_{50}$) of a C-strain–derived CSF-VRP lacking the E^{rns} gene. The intramuscular vaccination with the same VRP and dose resulted only in partial protection, while the intranasal immunization was unsuccessful. Therefore, the intradermal route was chosen for a subsequent vaccination experiment in which CSF-VRP with a complete or partial deletion of the E2 gene were tested. Pigs vaccinated with these VRP were only partially protected. The best results where obtained when the antigenic domain A of the E2 gene was maintained and the B/C domain deleted.

VRP based on CSFV strain Alfort with complete or partial deletion of E2 were also evaluated [51]. Oronasal vaccination with 10^7 $TCID_{50}$ of either of the two CSF-VRP induced partial protection against challenge with a lethal dose of highly virulent CSFV strain Eystrup. Intradermal vaccination with CSF-VRP with partially deleted E2 gene resulted in a protective immune response while CSF-VRP lacking the complete E2 gene did not. With these latter VRP, the antibodies against E^{rns} were below detection limit. Although the CSF-VRP with a partial deletion of the E2 gene are protective to some extent, they cannot serve as marker vaccine because they elicit antibodies that are detected by all E2-based ELISA currently available.

The most recent vaccination study with CSF-VRP uses strain Alfort/187-derived particles lacking the entire E^{rns} gene [50]. While oral application again resulted in partial protection only, a single intradermal vaccination with 10^7 $TCID_{50}$ CSF-VRP protected pigs from lethal challenge with highly virulent CSFV strain Eystrup. Intradermal vaccination elicited neutralizing antibodies and E2-specific antibodies as measured by ELISA. A cellular immune responses was also observed, as measured by an increase of IFN-γ secreting cells.

Taken together, best protection with CSF-VRP vaccines is currently achieved with intradermal application of particles lacking the E^{rns} gene.

CSFV as a vector for gene expression

Several full-length CSFV cDNA clones from which infectious RNA can be transcribed in vitro were constructed [69–71, 154, 239, 240]. The possibilities of using CSFV as gene expression system were explored. Since the CSFV genome contains a single ORF, foreign genes must be inserted in frame with the ORF of the monocistronic RNA. Alternatively, a second translation unit or cistron must be included under the control of an additional IRES, resulting in a bicistronic construct. Insertion of a foreign gene in the CSFV ORF results in a chimeric protein consisting of viral and the foreign polypeptides. With the bicistronic vector, the foreign protein can be released without any viral polypeptide attached.

Because the viral protein N^{pro} is not required for virus replication, it was chosen as insertion site for foreign genes [115]. The N terminus of N^{pro} was considered most suitable for accommodating gene insertions because the 19 N-terminal amino acids of N^{pro} are dispensable for the autoproteolytic activity of N^{pro} and for the block of IFN-α/β induction [112]. The bacterial chloramphenicol acetyltransferase (CAT) gene was inserted at the 5′ end of the N^{pro} gene in the full-length vA187-1 virus [241]. The growth characteristics of the virus carrying the CAT gene were indistinguishable from the parent virus. Importantly, the chimeric CAT-N^{pro} protein maintained the enzymatic activity of CAT and of the N^{pro} protease [241]. In order to insert a foreign gene in the 3′ NTR, a second cistron with an EMCV IRES was inserted at the 3′ end of the CSFV genome [242]. With the green fluorescent protein (GFP) as a model, this construct resulted only in weak expression.

An interesting gene expression system involving bicistronic replicons for the expression of foreign genes was established for BVDV [56, 57]. The first cistron contains the firefly luciferase gene inserted directly at the 3′ terminus of the N^{pro} gene. The luciferase gene is followed by a sequence coding for ubiquitin, the neomycin phosphotransferase and a stop codon. Downstream of the stop codon, an EMCV IRES initiates translation of the nonstructural proteins required for RNA replication. All structural genes were deleted. Translation of the first cistron results in a chimeric precursor protein that is subsequently cleaved by N^{pro} and by the cellular ubiquitin C-terminal hydrolase downstream of ubiquitin, to release the proteins of interest.

Aim of this project

Generation of CSF-VRP with improved immunogenicity

CSF-VRP induce a protective immune response against CSF in pigs [50, 53, 54]. Due to the lack of structural coding sequences, VRP are non-transmissible and therefore fulfil the requirements of a safe marker vaccine. However, vaccination with CSF-VRP does not completely prevent replication of CSFV in pigs, as opposed to live attenuated virus vaccines. The first aims of this study are therefore (i) to investigate the characteristics of the CSF-VRP–mediated immune responses and (ii) to explore the possibilities of enhancing the vaccine efficacy of CSF-VRP. To this end, we analysed the contribution of the proteins expressed from the replicating RNA to the development of an immune response in vivo. We also explored the possibilities of providing the VRP with adjuvant functions. It is known that the N^{pro} protein blocks induction of IFN-α/β [180, 181]. Because IFN-α/β has adjuvant activities [243–245], we analysed the effect of knocking out the N^{pro}-mediated block of IFN-α/β–induction in the context of CSF-VRP. We also analysed the potential of including granulocyte macrophage colony-stimulating factor (GM-CSF) as a genetic adjuvant to increase the immunogenicity of the CSF-VRP. For this work, we based our constructs on E^{rns} gene-deleted CSF-VRP developed earlier in our laboratory [50, 51].

Establishment of a generic gene expression system based on CSF-VRP

In order to establish a vector system based on CSFV replicons, we followed two strategies: (i) a monocystronic replicon with an insertion site for foreign genes in the 5′-terminal region of the N^{pro} gene, resulting in the expression of a chimeric protein and (ii) a bicistronic replicon carrying an insertion site at the 3′ end of the N^{pro} gene, resulting in expression and release of the authentic protein after proteolytic cleavage by N^{pro}. In the latter construct, an EMCV IRES inserted between the foreign gene and the C gene directs reinitiation of translation of the remaining viral proteins.

Results

Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN-α/β and carry foreign genes

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Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN-α/β and carry foreign genes

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ABSTRACT

Virus replicon particles (VRP) are genetically engineered infectious virions incapable of generating progeny virus due to partial or complete deletion of at least one structural gene. VRP fulfil the criteria of a safe vaccine and gene delivery system. With VRP derived from classical swine fever virus (CSF-VRP), a single intradermal vaccination protects from disease. Spreading of the challenge virus in the host is however not completely abolished. Parameters that are critical for immunogenicity of CSF-VRP are not well characterized. Considering the importance of type I interferon (IFN-α/β) to immune defence development, we generated IFN-α/β–inducing VRP to determine how this would influence vaccine efficacy. We also evaluated the effect of co-expressing granulocyte macrophage colony-stimulating factor (GM-CSF) in the vaccine context. The VRP were capable of long-term replication in cell culture despite the presence of IFN-α/β. In vivo, RNA replication was essential for the induction of an immune response. IFN-α/β–inducing and GM-CSF–expressing CSF-VRP were similar to unmodified VRP in terms of antibody and peripheral T-cell responses, and in reducing the blood levels of challenge virus RNA. Importantly, the IFN-α/β–inducing VRP did show increased efficacy over the unmodified VRP in terms of B-cell and T-cell responses, when tested with secondary immune responses by in vitro restimulation assay.

1. Introduction

Replicons are replication-competent viral genomes incapable of generating infectious progeny virus due to a functional defect of at least one structural gene that is partially or completely deleted (for review see [1–3]). Replicons can be packaged into the viral envelope to form virus replicon particles (VRP) using a complementing cell line expressing the missing structural protein [1,2]. Transcomplemented VRP infect cells with the same efficiency as the parent virus, because their virion shell is indistinguishable from the envelope of the original virus. Horizontal cell-to-cell spreading of the VRP is excluded due to the absence of newly synthesized infectious particles [2,4]. VRP can serve as safe vaccine and gene delivery vectors, since they are non-transmissible and cannot revert to fully competent virus, as opposed to live-attenuated virus [4,5]. Compared to subunit vaccines, VRP have the advantage of prolonged antigen production and of induction of cytotoxic T-cell responses [2]. Furthermore, they do not require adjuvants as the replicating nature of the replicon generates RNA molecules that trigger innate immune defences providing the necessary signal for induction of adaptive immune responses [6,7].

Classical swine fever virus (CSFV) is a highly contagious virus that can cause a severe haemorrhagic disease in pigs, resulting in important economic losses worldwide. CSFV is a member of the genus *Pestivirus*, closely related to the genera *Flavivirus* and *Hepacivirus* within the family *Flaviviridae* [8]. The CSFV genome consists of a positive sense single-stranded RNA of 12.3 kb with one single open reading frame (ORF). The encoded polyprotein is co- and post-translationally cleaved into the four structural proteins C, E^{rns}, E1 and E2, and the eight non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The genes NS3 to NS5B flanked by the 5′ and 3′ non-translated regions are the minimal elements required for autonomous pestivirus RNA replication [9,10]. N^{pro}, the first protein of the polyprotein, is not essential for virus replication [11]. It is unique to the members of the genus *Pestivirus* and has an autoprotease activity responsible for co-translational cleavage at its C-terminus [12,13]. An important function of N^{pro} is to block interferon (IFN)-α/β induction in infected cells [14–17], by promoting the proteasomal degradation of interferon regulatory factor 3 (IRF3) [18–20]. This function can be knocked out by single amino acid substitutions disrupting the zinc binding domain of N^{pro} [21,22]. In infected animals, the major protective antibodies are directed against the glycoprotein E2 [23–25]. Interestingly, classical swine fever virus replicon particles (CSF-VRP) lacking the entire E2 gene can nevertheless confer partial protection against infection with virulent CSFV [26]. This is consistent with earlier

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observations that neutralizing antibodies are also elicited against the glycoprotein E^{rns} [27]. The E^{rns}-specific antibodies are of minor importance for protection, since CSF-VRP lacking all the immunogenic epitopes of the E^{rns} gene function as efficacious vaccines [28–30]. The level of protection with these VRP can vary with different application routes [28,31]. Best results are obtained with intradermal vaccination resulting in protection from severe classical swine fever [28,29]. However, vaccination with CSF-VRP is not able to completely abolish spreading of the challenge virus in the host.

The present work aims to study the parameters that are critical for the immunogenicity of CSF-VRP. Both the humoral and cellular immune responses play an important role in the protection against CSFV [32]. To explore the possibilities of improving VRP-mediated immune activation, the effect of IFN-α/β induction was evaluated in terms of the humoral and cellular immune responses. We reasoned that VRP able to induce IFN-α/β due to a dysfunctional N^{pro} [21,22] would stimulate higher innate and thereby also adaptive immune responses. This was considered to be advantageous due to the inability of VRP to spread within the host resulting in a lower overall antigen content and lower immunogenicity when compared to a live virus vaccine. Consequently, VRP with N^{pro} lacking the ability to promote degradation of IRF3 were constructed and characterized in vitro and in vivo. In addition, based on previous studies demonstrating that CSFV can serve as a vector for foreign gene expression [33,34], we evaluated the effect of co-expressing bioactive intracellular and secreted proteins in the vaccine context.

2. Materials and methods

2.1. Cells

The porcine kidney cell line SK-6 [35] was maintained in Earle's minimal essential medium (MEM) supplemented with 7% horse serum (SVA, Håtunaholm, Sweden). The replicon packaging cell line SK-6(E^{rns}) [28] was propagated in MEM containing 7% horse serum and G418 (Calbiochem). The porcine kidney cell line PK-15 (American Type Culture Collection) was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% horse serum, non-essential amino acids and 100 mM sodium pyruvate. MDBK-t2 cells were kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom) and cultured in MEM supplemented with 7% fetal bovine serum (FBS) and 10 μg/ml blasticidine (Invitrogen). The human erythroleukemic cell line TF-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ-No ACC 334) was maintained in RPMI medium 1640 supplemented with 10% FBS, GlutaMAX-I and 5 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF). Porcine peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Paque PLUS (GE Healthcare) in 50 ml Leucosep tubes at 800 × *g* for 25 min as described before [36]. Contaminating erythrocytes were lysed with hypotonic ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.25) for 5 min on ice. Finally, PBMC were washed with PBS supplemented with 1 mM EDTA.

2.2. Plasmid constructs

Plasmid pA187-1, used for the rescue of infectious CSFV vA187-1, carries a full-length cDNA clone of the CSFV strain Alfort/187 (GenBank accession number X87939) [37]. This plasmid served as basis for all replicon and virus cDNA constructs that were established using standard DNA cloning techniques. The nucleotide and amino acid numbers mentioned in the text refer to the CSFV strain Alfort/187. The features of the different replicon constructs are depicted in Fig. 1A. For all replicons, the corresponding viruses were constructed. Details of the cloning procedures can be obtained on request. The unique NotI endonuclease restriction site (indicated by an asterisk) was inserted at nucleotide positions 35–42 of the N^{pro} gene of the monocistronic replicon A187-delE^{rns} [28] using two annealed oligonucleotides carrying the corresponding nucleotide substitutions. N^{pro} carrying a $D_{136}N$ mutation was subcloned into the replicon cDNA from the plasmid described earlier for the rescue of vA187-$D_{136}N$ [21]. The luciferase gene from the plasmid pGL-Control (Promega) was inserted in frame with N^{pro} in the NotI restriction site of the monocistronic replicon, resulting in a cDNA clone to rescue the replicon A187-Luc-delE^{rns} expressing a chimeric luciferase-N^{pro} polypeptide. The cDNA clone of the bicistronic replicon A187-N^{pro}-IRES-C-delE^{rns} carries a unique NotI restriction site inserted immediately downstream of the N^{pro} gene, followed by a stop codon and an internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) to reinitiate translation from the core gene. The luciferase gene and the porcine GM-CSF gene [38] were inserted in frame with N^{pro} in the NotI restriction site of the bicistronic replicon, resulting in cDNA clones to rescue the replicons A187-N^{pro}-Luc-IRES-C-delE^{rns} and A187-N^{pro}-GMCSF-IRES-C-delE^{rns}, respectively.

2.3. Recombinant VRP and viruses

In vitro transcription of RNA and transfection of SK-6 and SK-6(E^{rns}) cells was performed essentially as described before [10,37]. Briefly, plasmids were linearized with the SrfI endonuclease and RNA run-off transcription was performed using the MEGAscript T7 kit (Ambion). SK-6(E^{rns}) and SK-6 cells were transfected by electroporation with 1 μg replicon RNA and 1 μg virus RNA, respectively. Two or three days after transfection, VRP or virus was released by two cycles of freezing and thawing and the lysate clarified by centrifugation at 1000 × *g* for 10 min. To generate working stocks of VRP and virus, 8×10^6 SK-6(E^{rns}) and SK-6 cells were seeded in a 150 cm^2 flask and infected, respectively, with virus and VRP at a multiplicity of infection (MOI) of 10^{-3} 50% tissue culture infectious doses ($TCID_{50}$) per cell. After 4 days of incubation, VRP and virus were released as described above and titrated. For the preparation of mock material, 8×10^6 SK-6(E^{rns}) and SK-6 cells were seeded in 150 cm^2 flasks and incubated for 4 days followed by lysis by two cycles of freezing and thawing and clarification through centrifugation.

2.4. Titration of VRP and virus

The virus titre, expressed in $TCID_{50}$/ml, was determined by end point dilution on SK-6 cells and immunoperoxidase staining as described earlier [39], using the monoclonal antibody HC/TC26 [40] kindly provided by Irene Greiser-Wilke, Hannover Veterinary School, Hannover, Germany.

2.5. Bioassay for IFN-α/β

The bioactivity of IFN-α/β was quantified as described before [16,17] using the Mx/CAT reporter gene assay developed for the quantification of bovine IFN-α/β [41] and kindly provided by Martin D. Fray, Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom. Briefly, cell supernatants were treated with a CSFV-neutralizing pig serum, diluted 1:4 and added to MDBK-t2 cells that express chloramphenicol acetyltransferase (CAT) under the control of the Mx promoter. Expression of CAT was measured using a CAT enzyme-linked immunosorbent assay (ELISA) (Roche).

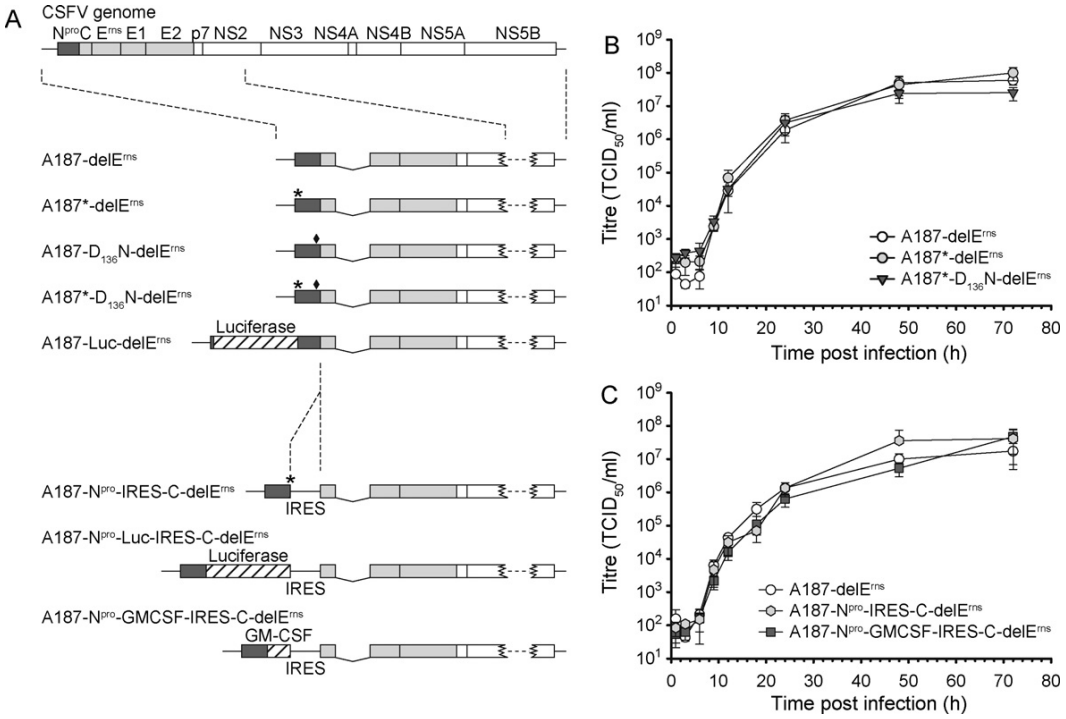

Fig. 1. Construction and replication characteristics of CSF-VRP. (A) The replicon constructs used in this study are represented schematically, with the N^{pro} gene shown in dark grey and the structural proteins in light grey. The foreign genes are shown with hatched boxes. The NotI endonuclease restriction site is indicated with the '*' symbol and the $D_{136}N$ mutation in N^{pro} is represented with the '♦' symbol. (B and C) Complementing SK-6(E^{rns}) cells were infected at a MOI of 0.1 $TCID_{50}$/cell with VRP carrying monocistronic (B) and bicistronic (C) replicons. After 1 h of incubation, cells were washed und cultured in fresh medium. VRP accumulation in the supernatant was determined at the indicated times by titration on SK-6 cells. Each curve represents the mean of three infections, with error bars showing the standard deviation.

2.6. Luciferase assay

To assess the expression levels of luciferase, VRP-infected cells were washed once with PBS, lysed with Firefly Luciferase Assay Lysis Buffer (Biotium) at room temperature for 15 min and stored at −70 °C. Luciferase activity was quantified with a Centro LB 960 luminometer (Berthold Technologies) using a Firefly Luciferase Assay Kit (Biotium) containing D-luciferin as a substrate.

2.7. Bioassay for GM-CSF

Bioactivity of GM-CSF was determined essentially as described before [38] by using a proliferation assay based on the human erythroleukemic cell line TF-1 that depends on GM-CSF [42]. To this end, 1×10^4 TF-1 cells were incubated with several dilutions of cell supernatant in RPMI medium 1640 supplemented with 10% FBS and GlutaMAX I for 3 days. Cytokine-dependent proliferation was quantified by measuring ^{3}H-thymidine incorporation during the last 18 h of the culture. Recombinant porcine GM-CSF [38] was used as a positive control and internal standard. One TF-1 unit was defined as the cytokine concentration giving half maximum proliferation.

2.8. Flow cytometry

Flow cytometry (FCM) was performed as described elsewhere [43,44]. Briefly, cells were detached from culture dishes by trypsin treatment, fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin in PBS. Viral NS3 expression was detected with the monoclonal antibody C16 [45] kindly provided by Irene Greiser-Wilke, Hannover Veterinary School, Hannover. To this end, the fixed and permeabilized cells were incubated in presence of C16 diluted with PBS/0.3% saponin, followed by a washing step with PBS/0.1% saponin and incubation with secondary rabbit $F(ab')_2$ anti-mouse immunoglobulins conjugated with R-phycoerythrin (Dako, R0439) diluted in PBS/0.3% saponin. Finally cells were washed with Cell Wash (BD Biosciences) and subjected to FCM (FACSCalibur, Becton Dickinson).

2.9. Qualitative analysis of replicon RNA by reverse transcription (RT) and PCR

Total RNA was extracted from cells using the NucleoSpin RNA II kit (Macherey-Nagel). First-strand cDNA was generated with the SuperScript III First-Strand Synthesis System (Invitrogen) and PCR was performed with Taq DNA polymerase (Sigma). PCR products were analysed by agarose gel electrophoresis.

2.10. UV-inactivation of VRP

VRP A187-delE^{rns} were UV inactivated with a GS Gene Linker UV Chamber (Bio-Rad). A volume of 750 μl of VRP stock was placed into a well of a 6-well plate and UV-irradiated for 8 min corresponding to a total energy of 1.5 J.

2.11. Anti-E2 antibody ELISA

The CSFi anti-E2 antibody ELISA to detect antibodies against the viral glycoprotein E2 was performed as described before [46].

2.12. Immunization of pigs and challenge infection

Ten-week-old specific pathogen free (SPF) pigs obtained from the breeding unit of the Institute of Virology and Immunoprophylaxis were housed in separate stables for immunization. The

pigs were immunized by intradermal injection of 0.5 ml of cell culture extract of VRP applied in five different spots of 0.1 ml each in the dermis of the neck. Negative control pigs were mock immunized with clarified lysates of SK-6(E^{rns}) cells used for VRP production. The live-attenuated CSFV strain Riems (collected from baits used for vaccination of wild boars, Riemser Arzneimittel AG) was used as positive control immunization and was applied with the same inoculation procedure as the VRP. On the day of challenge, pigs were relocated to bring VRP-immunized, CSFV strain Riems-immunized, and mock-immunized animals together. On day 26 after immunization, all pigs were challenged via the oronasal route with 10^6 $TCID_{50}$/animal of the highly virulent CSFV strain Koslov obtained from the National Reference Laboratory of CSF, Friedrich-Loeffler-Institut Insel Riems, Germany, and prepared as described before [47]. The challenge virus was diluted in 10 ml PBS. One half of the dose was administered dropwise intranasally and the other half orally. Body temperature and clinical score were monitored daily according to a defined scoring system [48] with the modifications described previously [28]. The animal experiments were performed in compliance with the Swiss animal protection law and approved by the animal welfare committee of the canton of Berne (authorization number 58/07).

2.13. IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay

The IFN-γ ELISPOT assay was performed as described elsewhere [49] with some minor modifications [50]. Briefly, 96-well MultiScreen-IP filter plates (Millipore, MAIPS4510) were coated with 0.5 μg/ml mouse anti-porcine IFN-γ monoclonal antibody (BD Biosciences, P2G10) in PBS at 4 °C overnight. The plates were washed at least twice with DMEM/10% FBS and blocked with DMEM/10% FBS at 37 °C for at least 2 h. VRP or virus (MOI = 0.1 $TCID_{50}$/cell, based on SK-6 titres) was added to the PBMC that were then seeded in triplicate in two-fold serial dilutions, with the highest concentration being 0.5×10^6 cells/well. PBMC stimulated with the mitogen concanavalin A (Con A, Amersham Pharma Biotech) served as positive control. After 2 days, the medium was removed and the cells were lysed with cold deionised water for 5 min. The wells were washed three times with washing buffer (PBS/0.5% Tween 20) and incubated with 0.17 μg/ml biotinylated anti-porcine IFN-γ monoclonal antibody (BD Biosciences, P2C11) at 4 °C overnight. The plates were washed five times and incubated with 0.4 μg/ml streptavidin-HRP (Dako, P0397) diluted in PBS at 37 °C for 1 h. The plates were then washed three times with washing buffer and revealed with SIGMAFAST 3,3′-Diaminobenzidine tablets (Sigma) dissolved in deionised water. After 1 h incubation at room temperature in the dark, the plates were washed with water and dried. The spots were counted with a computer-assisted video image analyser (ELISPOT reader, AID Diagnostika GmbH).

2.14. Neutralizing peroxidase-linked assay

CSFV-specific neutralizing antibodies in sera of immunized pigs were assessed with a neutralizing peroxidase-linked assay as described previously [26,51]. Briefly, serial two-fold dilutions of heat-inactivated serum were mixed with an equal volume of MEM containing 200 $TCID_{50}$/0.1 ml of CSFV strain Alfort/187. After incubation for 1 h at 37 °C, 0.1 ml portions of each sample were distributed into four wells of a 96-well plate containing 2×10^5 SK-6 cells/well. After 2 days, CSFV infection was visualized by immunoperoxidase staining using the monoclonal antibody HC/TC26 as described earlier [39]. The titre was expressed as the reciprocal of the highest serum dilution resulting in complete neutralization.

2.15. Analysis of lymphocyte numbers in the blood

Lymphocytes were counted from 100 μl blood samples containing Alsever's solution. Erythrocytes were lysed by addition of 900 μl ammonium chloride lysis buffer as described above and incubation on ice for 10 min. After erythrocyte lysis, 100 μl of the lysis buffer-treated blood was added to 20 μl CountBright absolute counting beads (Invitrogen) containing a defined concentration of beads, and the number of cells was determined by FCM.

2.16. Relative quantification of viral RNA

Total cellular RNA was extracted from the serum samples using the NucleoSpin Multi 96 Virus kit (Macherey-Nagel) on a Freedom EVO robot (Tecan). Real-time RT-PCR was performed as described elsewhere [52]. The results were expressed as the total number of cycles minus Ct-value.

2.17. In vitro restimulation assays

For the generation of immune pigs, SPF animals were immunized against CSFV essentially as described before [53]. The pigs were vaccinated by intramuscular injection of 40 μg of recombinant baculovirus-expressed E2 glycoprotein of CSFV [54]. The vaccine was formulated by emulsifying the E2 glycoprotein in a double water–oil–water emulsion (Montanide ISA 206 (w/v), kindly provided by Dr Laurent Dupois (Seppic, France), and administered at two injection sites in the neck. Four weeks after vaccination, pigs were booster vaccinated oronasally with 5×10^6 $TCID_{50}$/pig of virulent CSFV strain Alfort/187. For the in vitro restimulation assays, PBMC were purified from CSFV immune pigs and cultured in DMEM supplemented with 10% FBS, 100 U penicillin, 100 μg streptomycin (Gibco, 15140-122) and 20 μM 2-mercaptoethanol at 39 °C. For the B-cell restimulation assay, 5×10^6 PBMC were seeded into each well of 24-well plates and stimulated with VRP or virus at a MOI of 0.1 $TCID_{50}$/cell based on titres obtained on SK-6 cells. Aliquots of the supernatants were harvested after 6 and 9 days of incubation and tested for antibody secretion using the anti-E2 antibody ELISA (see above). To assess the restimulation of IFN-γ–secreting cells, 3.5×10^6 PBMC were expanded in 6-well plates in the presence of VRP or virus (MOI = 0.1 $TCID_{50}$/cell, based on SK-6 titres) for 5 days. Cells were then collected and an IFN-γ ELISPOT assay was performed as described above.

3. Results

3.1. Construction of CSF-VRP to accommodate foreign gene insertions and induce IFN-α/β

We constructed replicons lacking the entire E^{rns} gene, with either a monocistronic (A187*-delE^{rns}) or a bicistronic genome (A187-N^{pro}-IRES-C-delE^{rns}) to accommodate the foreign gene insertion (Fig. 1A). The bicistronic replicons carry an IRES upstream of the C gene, for internal initiation of translation. In order to facilitate foreign gene insertion, a unique NotI endonuclease restriction site (asterisk in Fig. 1A) was included near the 5′ end or at the 3′ end of the N^{pro} gene of the monocistronic and bicistronic replicon, respectively. In the monocistronic replicon, the insertion of the NotI restriction site generated the amino acid substitutions $T_{12}S$, $N_{13}G$ and $K_{14}R$ at positions 12, 13 and 14 of N^{pro}. A gene insertion at this position resulted in a chimeric protein with the foreign protein expressed between amino acids 13 and 14 of N^{pro}. With this strategy, we constructed a monocistronic and bicistronic replicon encoding the luciferase protein in fusion with N^{pro} (Fig. 1A). For the expression of a secreted bioactive protein, we cloned the porcine

GM-CSF gene including the signal sequence in frame with the N^{pro} ORF into the NotI restriction site of the bicistronic replicon (Fig. 1A). Autoproteolytic cleavage of the encoded protein by the N^{pro} autoprotease was expected to release the GM-CSF for translocation into endoplasmatic reticulum and secretion. In order to modify the inherent capacity of the CSF-VRP to prevent IFN-α/β induction, we constructed replicons with N^{pro} containing a aspartic acid to asparagine substitution at amino acid position 136 ($D_{136}N$), known to abrogate the CSFV-mediated counteraction of IFN-α/β induction, without impairing the autoprotease activity [21]. All replicons were packaged into VRP by transfection of E^{rns} complementing SK-6(E^{rns}) cells with replicon RNA. VRP stocks with a titre of up to 5×10^7 $TCID_{50}$/ml were obtained after one passage on SK-6(E^{rns}) cells. Analyses of replication kinetics in SK-6(E^{rns}) cells showed that neither the amino acid substitutions for modifying the IFN-α/β regulation nor the NotI or GM-CSF insertions adversely affected replication and VRP formation; this was seen for both the monocistronic (Fig. 1B) and bicistronic (Fig. 1C) replicons. Infection of the non-complementing parent SK-6 cells with the VRP at a MOI below 1 $TCID_{50}$/ml resulted in single infected cells, demonstrating the inability of the VRP to produce infectious progeny. After cell division, foci of two to four antigen positive cells were observed, as opposed to virus-infected cells typically showing large, fast growing, antigen positive foci (data not shown). This suggests that the replicons can be transmitted vertically to daughter cells during cell division as expected. Multiple passages of VRP-infected SK-6 cells demonstrate the absence of any infectious virus or VRP production in non-complementing cells (data not shown).

3.2. Analysis of CSF-VRP–mediated IFN-α/β induction and foreign gene expression

In order to verify that the $D_{136}N$ substitution of N^{pro} permits IFN-α/β induction, PK-15 cells were infected with VRP or infectious virus as control, and IFN-α/β bioactivity in the supernatant was quantified. Only VRP and virus carrying the $D_{136}N$-mutated N^{pro} induced IFN-α/β (Fig. 2A). We then tested the functionality of the foreign proteins expressed by the monocistronic and bicistronic constructs. Lysates of SK-6 cells infected with VRP and viruses carrying the luciferase gene contained luciferase activity as early as 6 h after infection (Fig. 2B). No difference in luciferase expression levels was detected between the VRP and the viruses 6 h and 18 h after infection, irrespectively of the MOI. After 48 h, the VRP resulted in lower luciferase activity than virus when SK-6 cells were infected at a low MOI of 0.01 $TCID_{50}$/cell (Fig. 2B). This is consistent with the absence of infectious progeny with the VRP as opposed to the viruses. In order to determine GM-CSF expression and secretion by the bicistronic replicons, cell lysates and supernatants from VRP-infected SK-6 cells were analysed by Western blot. No GM-CSF protein could be detected in either cell lysates or supernatants, although N^{pro} was correctly cleaved from the downstream GM-CSF according to its molecular weight (data not shown). Considering that the N^{pro} was cleaved from the polyprotein, we tested the supernatant of VRP-infected SK-6 cells for GM-CSF bioactivity. To this end we employed a proliferation assay based on TF-1 cells, with recombinant porcine GM-CSF as standard. Supernatants from SK-6 cells infected with the GM-CSF–expressing replicon stimulated TF-1 cell proliferation, in contrast to supernatants from SK-6 cells infected with the parent VRP (Fig. 2C). The concentration of GM-CSF in the supernatant was calculated to be 1–3 U/ml (2–15 ng/ml). This demonstrates that the bicistronic replicon encoding GM-CSF expresses the inserted foreign gene and that the protein is secreted in a bioactive form.

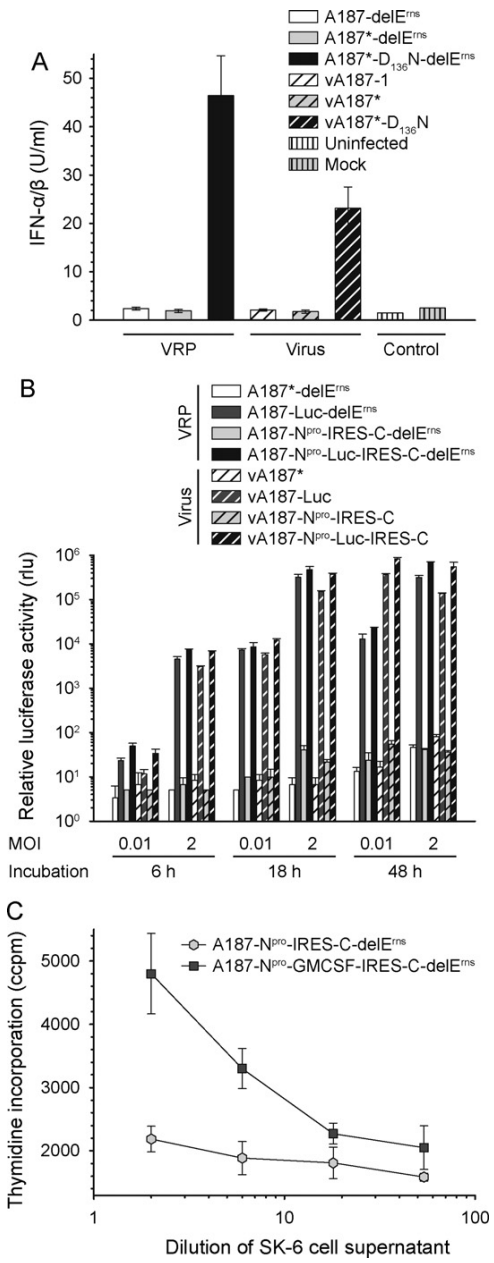

Fig. 2. CSF-VRP can be modified to induce IFN-α/β and express foreign genes. (A) VRP and viruses carrying the mutated N^{pro}($D_{136}N$) induce IFN-α/β. PK-15 cells were infected with VRP or virus at a MOI of 10 $TCID_{50}$/cell as indicated, and IFN-α/β bioactivity was assessed in the cell culture supernatant 70 h after infection using the Mx/CAT reporter gene assay. The mean of three measurements is shown, with error bars representing the standard deviation. (B) Monocistronic and bicistronic VRP and viruses carrying the luciferase gene express functional luciferase. SK-6 cells were infected at a MOI of 0.01 or 2 $TCID_{50}$/cell and washed after incubation for 1 h. At the indicated times, the cells were assayed for luciferase activity. The results are mean values of three replicates, with the error bars representing the standard deviation. (C) GM-CSF expressed by bicistronic replicons is bioactive. SK-6 cells were infected at a MOI of 10 $TCID_{50}$/cell and incubated for 3 days. The supernatants were then analysed at several dilutions with a bioassay based on GM-CSF–dependent proliferation of TF-1 cells. Cell proliferation was quantified by ^{3}H-thymidine incorporation expressed in corrected counts per minute (ccpm). The results are shown as mean values of five replicates, with the error bars indicating the standard deviation.

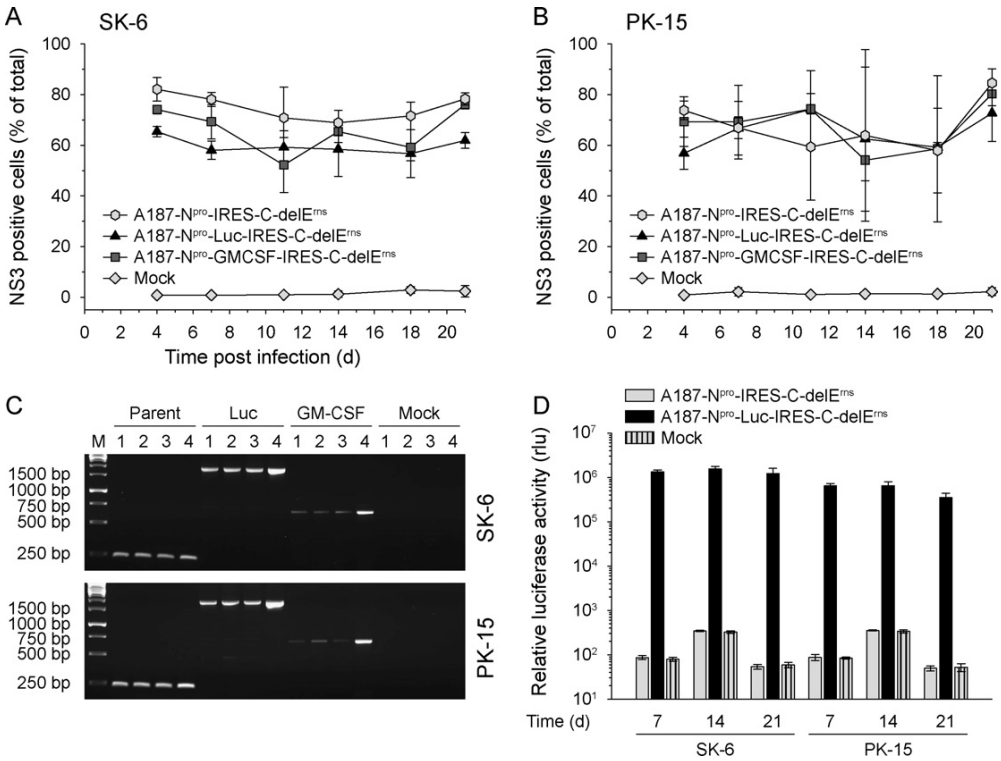

Fig. 3. Foreign gene expression by CSFV replicons is stable in cell culture. SK-6 and PK-15 cells were infected in triplicates with the indicated VRP at a MOI of 1 $TCID_{50}$/cell or with mock SK-6 cell lysate, and passaged twice a week. (A and B) At the indicated times after infection, the percentage of NS3 positive cells was quantified by FCM. The results are shown as mean values of three replicates with error bars representing the standard deviation. (C) At day 21 after infection, total RNA was extracted from infected cells and analysed by RT-PCR using a sense primer in the N^{pro} gene and an antisense primer in the EMCV IRES. For each replicon, lanes 1 to 3 are the RT-PCR products from the three independent replicates and lane 4 is the PCR product obtained with the plasmid from which the VRP was derived. The expected fragment lengths are 237 bp for A187-N^{pro}-IRES-C-delE^{rns} (Parent), 1896 bp for A187-N^{pro}-Luc-IRES-C-delE^{rns} (Luc), and 681 bp for A187-N^{pro}-GMCSF-IRES-C-delE^{rns} (GM-CSF). (D) Luciferase activity was assayed 7, 14 and 21 days after infection. The results are mean values of the three replicates, with the error bars representing the standard deviation.

3.3. Stability of foreign gene expression by bicistronic CSFV replicons

There is no selective pressure to maintain the expression of the foreign gene in the bicistronic CSFV replicons. Therefore, we analysed the long-term replication of the bicistronic replicons and the stability of the GM-CSF and the firefly luciferase gene insertions. After VRP infection, 60% to 80% of SK-6 and PK-15 cells expressed NS3 as measured by FCM (Fig. 3A and B). The percentage of infected cells remained unchanged during 6 cell culture passages for a period of 21 days. After 21 days of replication in cell culture, the full-length firefly luciferase and GM-CSF gene insertions were maintained in the replicon, as demonstrated by RT-PCR analysis (Fig. 3C). Accordingly, the level of firefly luciferase activity was stable at 7, 14 and 21 days after infection, showing that CSFV replicons are capable of long-term stable foreign gene expression (Fig. 3D).

3.4. CSFV replicons can persist in cell cultures in presence of IFN-α/β

Replicon vaccines have the advantage over inactivated and subunit vaccines in offering cycles of replication providing more antigen and therefore a prolonged protein expression. As shown in Fig. 3, CSFV replicons are capable of long-term replication in cell culture. In order to analyse how the time-line for the duration of active replicons in cells is influenced by IFN-α/β, we compared the replication of the $N^{pro}(D_{136}N)$-mutated VRP in the IFN-α/β–competent PK-15 cells with their replication in SK-6 cells defective in IFN-α/β production. VRP-infected PK-15 and SK-6 cells were maintained in culture for six passages over a period of 20 days. At each passage, the proportion of NS3-expressing cells was determined by FCM. The percentage of NS3-expressing SK-6 cells varied between 60% and 90% with all replicons analysed (Fig. 4A). A higher variability in the percentage of infected cells was observed in PK-15 cells (Fig. 4B). These data demonstrated long-term replication with viral protein synthesis of CSFV replicons irrespectively of the $D_{136}N$ mutation in N^{pro}, in both IFN-incompetent and IFN-competent cells. To elaborate on this, we analysed the effect of IFN-β treatment of SK-6 and PK-15 cells on VRP infectivity and replication. The IFN-β–mediated induction of an antiviral state in these cells was monitored with the highly IFN-sensitive vesicular stomatitis virus replicon particles expressing GFP instead of the structural glycoprotein G [55] (data not shown). IFN-β pretreatment induced a dose-dependent reduction of NS3 positive SK-6 and PK-15 cells infected with VRP. Again, no significant difference was noted when VRP with intact N^{pro} were compared with replicons expressing $D_{136}N$-mutated N^{pro} (Fig. 4C and D). Moreover, a long-term resistance of the replicons to IFN-α/β was observed in cell cultures kept for 20 days and treated with IFN-β at each passage (Fig. 4E and F). IFN-β pretreatment reduced the number of infected cells (Fig. 4C–E) which then remained constant during five passages under IFN-β treatment (Fig. 4E). When the cells were treated 24 h after infection, the percentage of NS3 positive cells was notably higher (Fig. 4F). These results were confirmed with immunoperoxidase staining for E2 expression in cells cultured in the presence of IFN-β (data not shown). Taken together, the data demonstrate that the CSFV replicons with wild type and with $D_{136}N$-mutated N^{pro} can persist in cell culture, independently of IFN-α/β pressure.

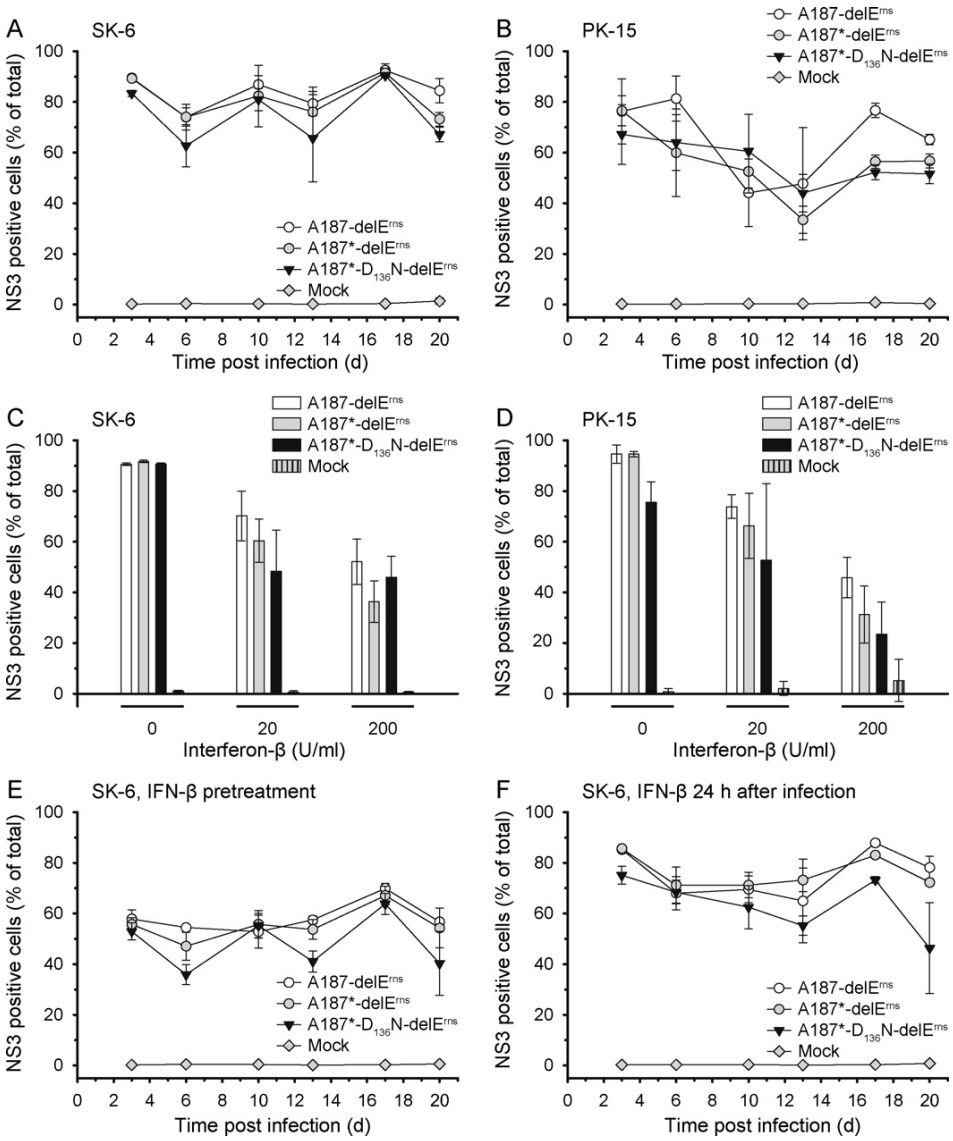

Fig. 4. CSFV replicons persist in cell culture, independently of the antiviral activity of IFN-α/β. In (A) and (B), SK-6 cells and PK-15 cells were infected at a MOI of 1 $TCID_{50}$/cell and passaged twice a week. At each passage the percentage of infected cells was determined by quantification of the NS3 positive cells by FCM. In (C) and (D), SK-6 cells and PK-15 cells were primed with 0, 20 or 200 U recombinant porcine (rpo) IFN-β/ml for 24 h, and then infected with VRP at a MOI of 1 $TCID_{50}$/cell. After 3 days, the proportion of NS3 positive cells was measured by FCM. In (E) and (F), SK-6 cells were treated with 20 U rpoIFN-β/ml either 24 h before (E) or after (F) infection with VRP at a MOI of 1 $TCID_{50}$/cell. The cells were then passaged twice a week and each time supplemented with fresh IFN-β (20 U/ml). At the indicated times NS3 positive cells were quantified by FCM. All results are presented as mean values of three replicates with error bars showing the standard deviation.

3.5. Replication of replicon RNA in vivo is essential for efficient induction of humoral immune responses

In order to understand the contribution of the proteins of the VRP envelope and of the proteins synthesized de novo by the VRP to the induction of an immune response, we compared antibody responses induced by live and UV-inactivated VRP. Complete inactivation of the VRP by UV light was controlled by the absence of NS3 antigen production in cells treated with the highest possible dose of the inactivated VRP (data not shown). Pigs were immunized intradermally with either 10^7 $TCID_{50}$ of the live VRP A187-delE^{rns}, or an equivalent dose of the UV-inactivated VRP. A booster immunization was performed 25 days after the first immunization with the same dose and route. Only pigs immunized with live VRP seroconverted (Fig. 5, empty symbols), whereas animals immunized with UV-inactivated VRP remained negative for E2-specific antibodies even after the second immunization (Fig. 5, solid symbols). This demonstrates that replication of the replicon RNA is essential for the VRP to induce a specific B-cell immune response, with the dose of VRP employed.

3.6. B-cell and T-cell responses after vaccination with the IFN-α/β–inducing and the GM-CSF–expressing CSF-VRP

Based on the known adjuvant properties of IFN-α/β [56,57] and GM-CSF [58–60], we investigated the primary immune responses in vivo with VRP carrying the $D_{136}N$ mutation to induce IFN-α/β secretion and with VRP expressing GM-CSF. Groups of five pigs were immunized by intradermal injection of 10^7 $TCID_{50}$/pig of VRP A187-delE^{rns}, VRP A187*-D_{136}N-delE^{rns} and VRP A187-N^{pro}-

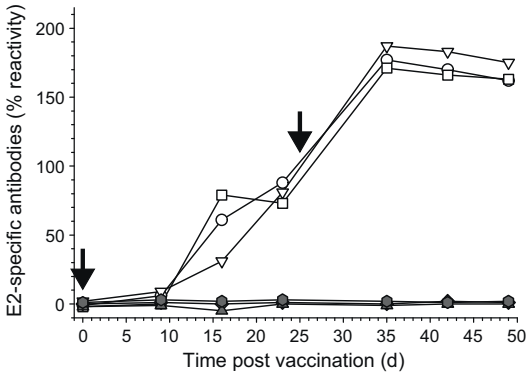

Fig. 5. RNA replication is essential for the generation of a humoral immune response. Three pigs per group were vaccinated by intradermal injection (arrow, day 0) of 10^7 $TCID_{50}$/pig of VRP A187-delE^{rns} that had been either UV-inactivated (solid symbols) or not (empty symbols). Each pig received an intradermal booster vaccination on day 25 with the same VRP (UV-inactivated or not; arrow, day 25) as before. Serum samples were analysed with the anti-E2 antibody ELISA, and the titres expressed in percentage reactivity compared to a reference serum.

GMCSF-IRES-C-delE^{rns}. As reference, two pigs were vaccinated with the standard live-attenuated CSFV vaccine strain Riems, with 2×10^4 $TCID_{50}$/pig injected by the intradermal route. Two pigs were mock immunized with SK-6(E^{rns}) cell lysate as negative control. An E2-specific antibody response was detected by ELISA 12 days after immunization, without any significant difference between the different VRP constructs (Fig. 6A). Significantly higher E2-specific antibody levels were observed with the live CSFV strain Riems. At day 22 after immunization, the CSFV-specific neutralizing antibody titres varied between 10 and 20 for all VRP-vaccinated pigs, without any significant difference between the different groups, while the neutralizing antibody titres of the pigs vaccinated with CSFV Riems were between 80 and 160 (data not shown). The pigs were challenged 26 days after immunization with the virulent CSFV strain Koslov. After challenge, the E2-specific ELISA antibody titres increased for all animals, the strongest increase being observed with the VRP-vaccinated pigs (Fig. 6A). This contrasted with the mock-immunized animals that never developed antibodies against E2, even after challenge infection (Fig. 6A). The T-cell immune response was assessed using IFN-γ ELISPOT assays with PBMC. At day 8 after immunization, both, VRP and CSFV strain Riems induced enhanced IFN-γ responses, but no significant differences between the groups were observed (Fig. 6B). In conclusion, the modifications of the VRP to induce IFN-α/β and to express GM-CSF did not alter the B-cell and T-cell responses in vivo.

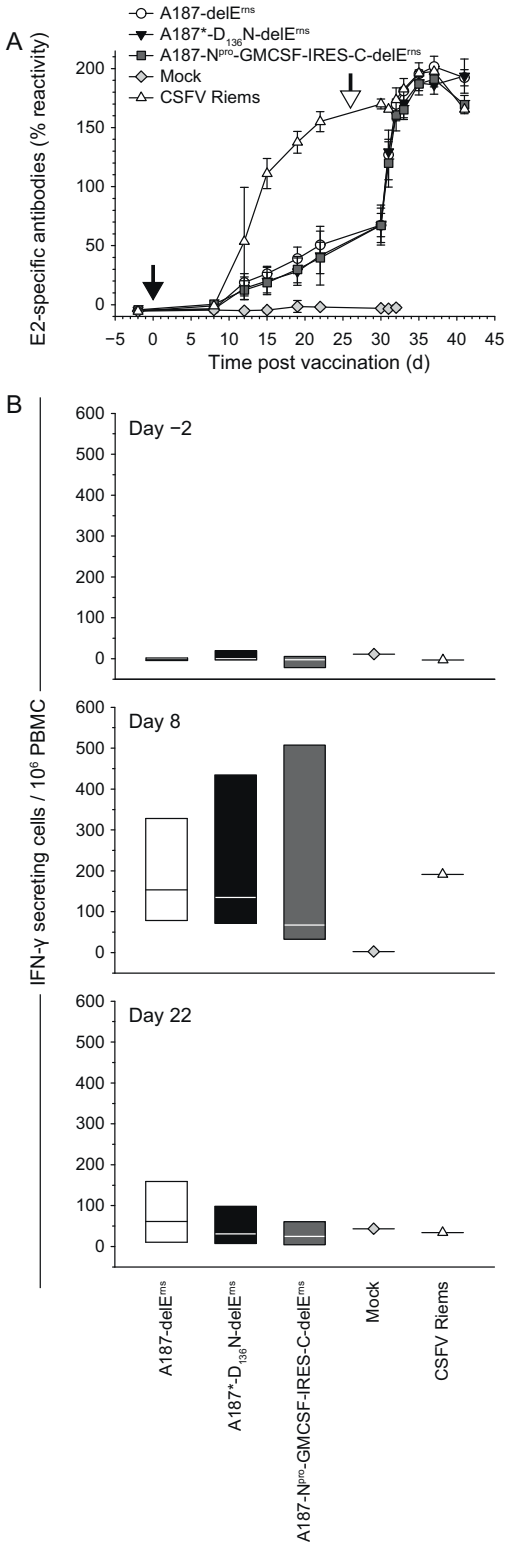

Fig. 6. B-cell and T-cell responses after intradermal vaccination with CSF-VRP. Pigs were vaccinated by intradermal injection of either 10^7 $TCID_{50}$/animal of VRP, 2×10^4 $TCID_{50}$/animal of CSFV strain Riems, or of an equivalent volume of mock SK-6(E^{rns}) lysate (solid arrow). All pigs were challenged by oronasal application of the highly virulent CSFV strain Koslov (10^6 $TCID_{50}$/animal) on day 26 after vaccination (empty arrow). The mock-vaccinated animals were sacrificed 6 days after challenge due to severe classical swine fever. (A) Serum samples were analysed with the anti-E2 antibody ELISA, and the titres expressed in percentage reactivity compared to a reference serum. Each curve shows mean values of different groups of pigs vaccinated with either one of the VRP, the CSFV strain Riems or mock. Error bars represent the standard deviation. (B) The cell-mediated immune response was assessed by IFN-γ ELISPOT assay on day −2, 8, and 22 after vaccination. PBMC of pigs vaccinated with VRP or virus as indicated were stimulated in triplicate with virus vA187-1 or SK-6 cell lysate (Mock), and the number of IFN-γ–secreting cells per 10^6 PBMC was determined. The mean numbers of IFN-γ–secreting cells from PBMC stimulated with SK-6 lysate were subtracted from the mean numbers of IFN-γ–secreting cells from PBMC stimulated with virus vA187-1. The results are shown as box plots. The lower boundary of each box indicates the 25th percentile, the line within the box marks the median and the upper boundary of the box indicates the 75th percentile.

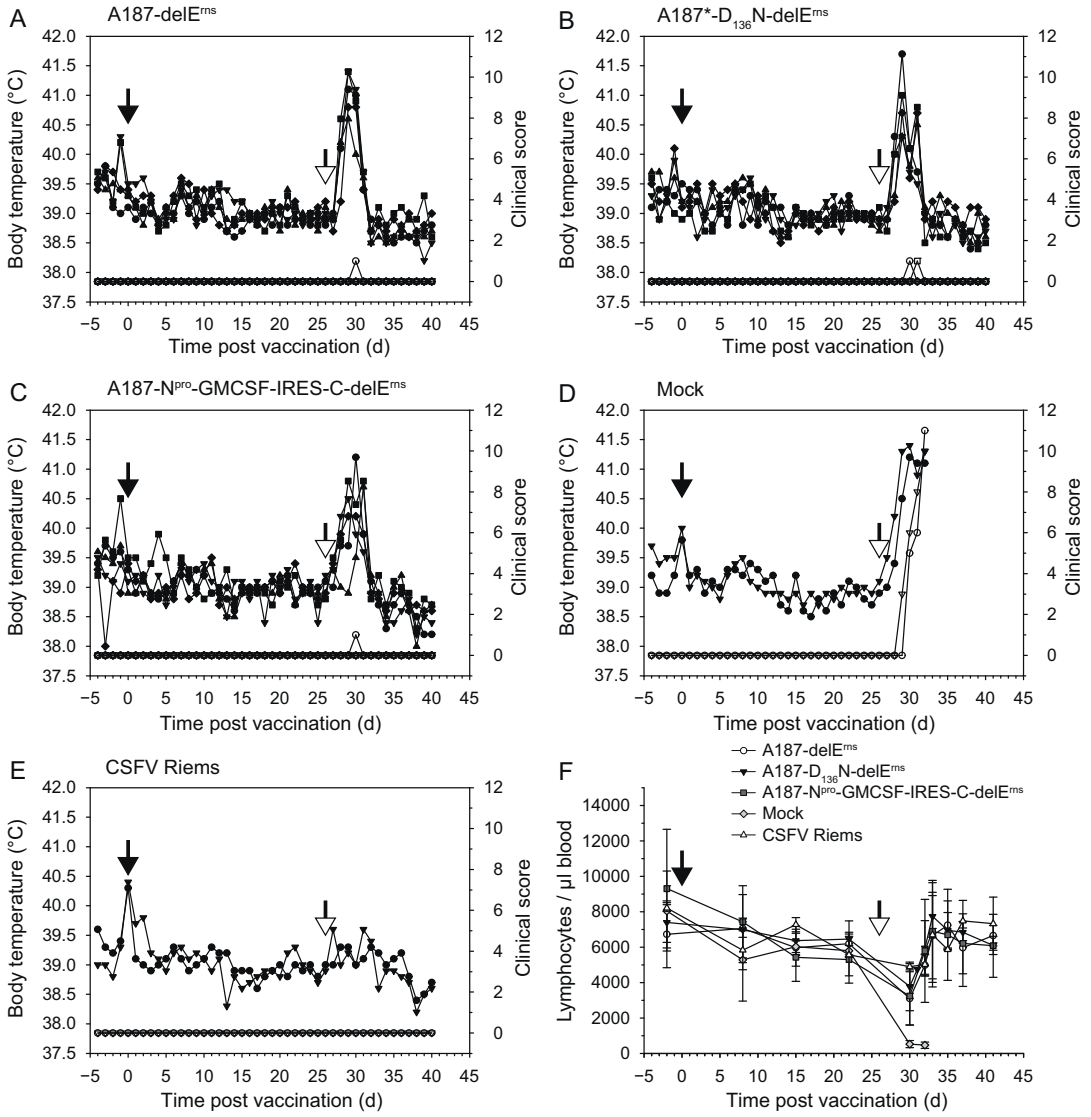

Fig. 7. CSF-VRP vaccines protect pigs from lethal CSF. Pigs vaccinated with 10^7 $TCID_{50}$/animal of VRP (A–C) or 2×10^4 $TCID_{50}$/animal of CSFV strain Riems (E) or mock SK-6(E^{rns}) lysate (D) on day 0 (black arrow) were challenged with CSFV strain Koslov (10^6 $TCID_{50}$/animal) 26 days after vaccination (empty arrow). The mock-vaccinated animals were sacrificed 6 days after challenge due to severe classical swine fever. (A–E) The body temperature (solid symbols) and the clinical score (empty symbols) of each animal are indicated for each group in a different panel. (F) Lymphocyte numbers were measured by FCM. Each curve represents mean values of individual groups, with error bars showing the standard deviation.

3.7. Protective capacity of the IFN-α/β–inducing and the GM-CSF–expressing CSF-VRP against challenge infection

After challenge infection, all pigs immunized with VRP remained healthy without any clinical symptoms apart from a transient increase of the body temperature (Fig. 7A–C, solid symbols). One animal of each group immunized with VRP had reduced appetite, resulting in a clinical score of 1 on day 4 after challenge (Fig. 7A–C, empty symbols). One animal immunized with the D_{136}N-mutated VRP had a clinical score of 1 on day 5 after challenge due to fever for 4 consecutive days (Fig. 7B). The mock-immunized pigs developed high fever and high clinical scores (Fig. 7D), with severe lameness, neurological disorders and diarrhoea. These animals were euthanized on day 6 after challenge. The pigs immunized with CSFV strain Riems did not show any elevated body temperature or other clinical symptoms (Fig. 7E). A drop in the number of circulating lymphocytes was observed in all animals 4 days after challenge. Except for the mock-immunized pigs, all vaccinated pigs recovered a normal lymphocyte count within 6 days of the challenge infection (Fig. 7F).

No challenge virus was isolated from the serum of any of the VRP-vaccinated and the CSFV strain Riems-vaccinated animals, as opposed to the mock-immunized pigs (data not shown). This contrasted with real-time RT-PCR analysis of viral RNA in the serum. On day 4 after challenge, all pigs immunized with VRP, except for one animal immunized with the GM-CSF–expressing VRP, were clearly positive for challenge virus RNA in the blood, but the levels were lower than with the mock-immunized animals (Fig. 8). On day 5, two pigs in each group immunized with the IFN-α/β–inducing and GM-CSF–expressing CSF-VRP were negative for challenge virus RNA. In contrast, all pigs immunized with the parent VRP A187-delE^{rns} were positive. At day 6 after challenge, 4 out of the 5 pigs immunized with the IFN-α/β–inducing and

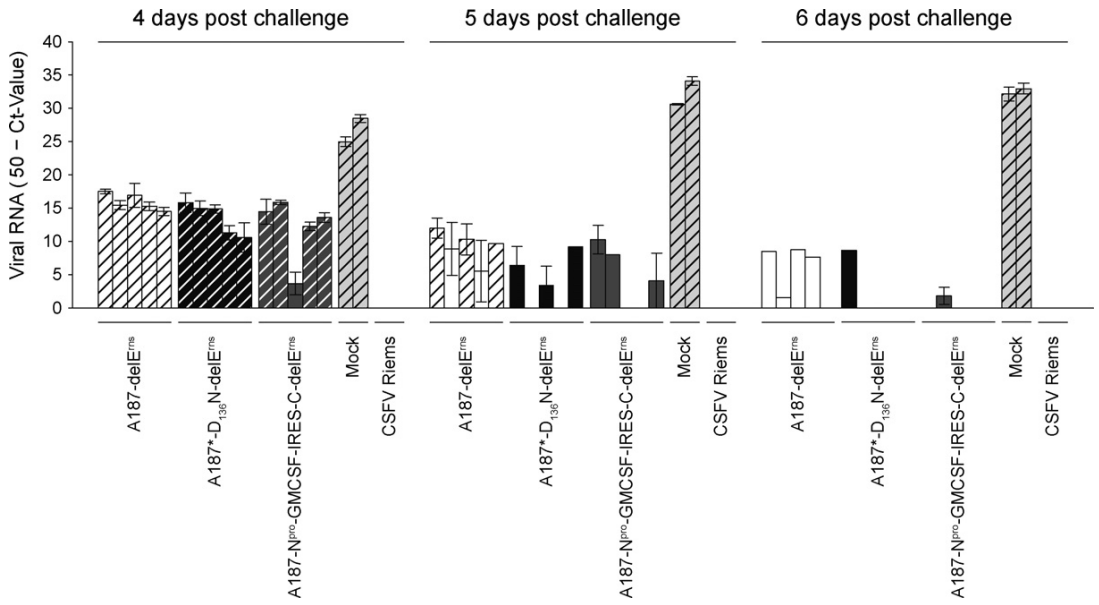

Fig. 8. CSF-VRP vaccines reduce the load of challenge virus RNA. For all pigs vaccinated with VRP, virus or mock as indicated, two independent RNA extractions were performed from serum samples of days 4, 5 and 6 post challenge. Each extraction was tested in triplicate, resulting in a total of six tests per sample. Samples with a Ct value below 50 were considered positive. Hatched bars represent mean values of samples that were positive in all tests. Solid bars show mean values of samples for which one to five tests were positive. In this case, negative samples were omitted for calculation. No bar is shown if all tests were negative. Error bars represent standard deviations.

with the GM-CSF–expressing VRP were negative for challenge virus RNA. This contrasted again with the pigs immunized with the parent VRP A187-delE^{rns}, for which 4 of the 5 animals were still positive for viral RNA. The mock-immunized animals showed strong signals for challenge virus RNA at all time points tested, while the animals immunized with CSFV strain Riems remained negative. At day 7 after challenge and later on, all immunized animals were negative for CSFV RNA (data not shown). Taken together, these data show that the modifications of the replicon did not alter their capacity to induce a protective immune response against challenge virus infection. Interestingly, the D_{136}N mutation and GM-CSF gene insertion appeared to provide an added value to the induction of this immune response, seemingly by inducing a more efficient clearance of the challenge virus.

3.8. VRP inducing IFN-α/β display enhanced immune activation in vitro

To further elaborate on the immunogenicity of IFN-α/β-inducing VRP, we assessed their potential to restimulate immune responses. To this end, we employed ex vivo PBMC-based assays to measure the humoral and cellular immune responses after antigen-specific stimulation. PBMC obtained from CSFV-immune SPF pigs were incubated in vitro with VRP or virus at a MOI of 0.1 $TCID_{50}$/cell. After 6 and 9 days of incubation, the cell supernatants were assayed for E2-specific antibodies. Fig. 9A shows that the D_{136}N mutation in N^{pro} of the VRP and viruses enhanced the induction of E2-specific antibodies, when compared with VRP and viruses carrying wild type N^{pro}. T-lymphocyte activity was assessed in terms of IFN-γ–secreting cells quantified by ELISPOT assay after a five-day restimulation period. Again, more IFN-γ–secreting cells were observed with VRP and virus expressing mutated N^{pro} to induce IFN-α/β (Fig. 9B). The data shown in Fig. 9 are representative of nine independent experiments with PBMC from three different pigs. Taken together, these results show that N^{pro}(D_{136}N)-mutated VRP mediate enhanced B- and T-lymphocyte secondary responses in vitro.

4. Discussion

CSF-VRP lacking the E^{rns} gene can induce protective immunity, with both humoral and cell-mediated immune responses being activated upon a single intradermal vaccination of pigs [28,29]. Nevertheless, CSF-VRP vaccines are not as efficacious as a standard live-attenuated CSFV vaccine [28]. Consequently, to evaluate whether CSF-VRP–mediated immune activation can be improved, we studied different parameters that may be critical for their immunogenicity.

CSFV is known for its capacity to regulate IFN-α/β induction by promoting proteasomal degradation of IRF3 through the leader protein N^{pro} [18,19,21]. Here we show that the CSFV replicon has a similar capacity for controlling IFN-α/β induction, and that a D_{136}N mutation in the N^{pro} gene abrogates this property of the VRP, as was shown previously for the virus [21]. Considering the known ability of IFN-α/β to promote adaptive immune defences, we sought to determine a potential improvement of VRP immunogenicity by creating IFN-α/β–inducing VRP. Remarkably, the replicon with the D_{136}N mutation abrogating the N^{pro}-mediated counteraction of IFN-α/β induction had similar long-term replication characteristics as the parental replicon. Moreover, induction of an antiviral state by pretreatment with IFN-β did not clear the replicon infection, even after repeated treatment over the 20-day period of observation. These results suggest that CSF-VRP have the potential of long-term replication as it was shown recently for live-attenuated and chimeric CSFV vaccines in the tonsils of immunized animals [61]. The observed resistance of CSFV against IFN-α/β–mediated clearance is consistent with data obtained with BVDV showing that once infection is established, BVDV is largely resistant to the antiviral activity of IFN-α/β [62]. Altogether, these characteristics of long-term pestivirus replication in immune-competent systems represent an element of major importance for the potency of CSF-VRP as vaccines and gene delivery systems.

We next assessed the relative capacity of the proteins of the VRP itself to induce immune responses in vivo, compared with the de novo synthesized antigens expressed from the replicon

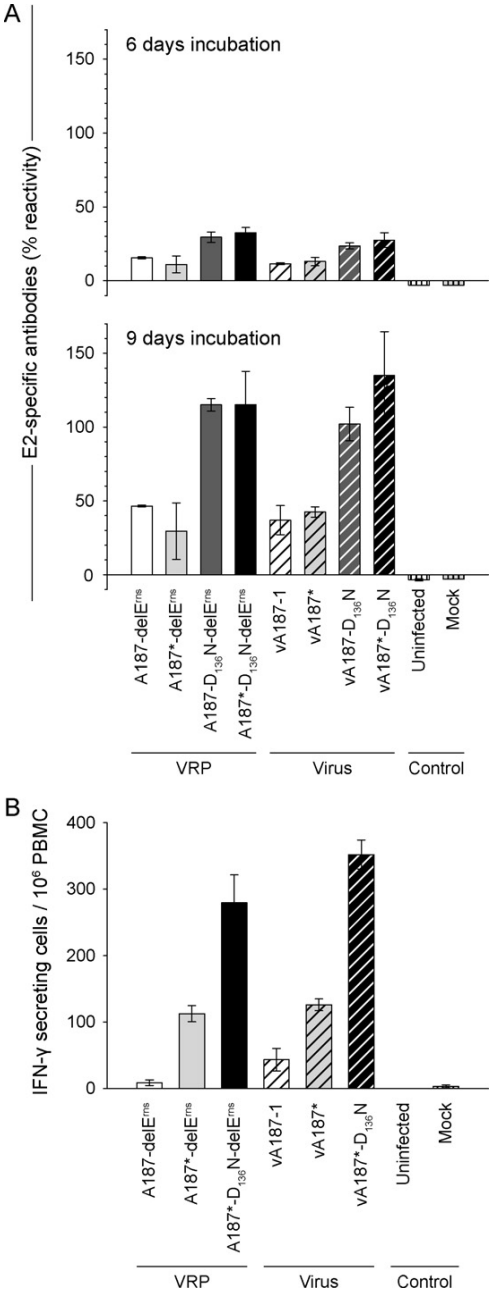

Fig. 9. CSF-VRP inducing IFN-α/β display enhanced immune activation in vitro. (A) PBMC from CSFV-immune pigs were restimulated with VRP or virus at a MOI of 0.1 $TCID_{50}$/cell. After 6 and 9 days of incubation, specific antibodies in the supernatants were measured with the anti-E2 antibody ELISA. The antibody titre was expressed as percentage reactivity compared to a reference serum. (B) PBMC were restimulated with VRP or virus at a MOI of 0.1 $TCID_{50}$/cell and expanded for 5 days. Thereafter, the PBMC were subjected to an IFN-γ ELISPOT assay by restimulating with the same VRP or virus and the same MOI as during the expansion phase. The results are displayed as mean values with error bars representing the standard deviation.

RNA. Pigs immunized with UV-inactivated VRP did not develop any detectable anti-E2 antibodies, even after a booster immunization, demonstrating that the E2 antigen present in the VRP shell did not reach immunogenic levels in the animals. A specific antibody response was only obtained with the active VRP carrying the self-replicating replicon, showing that RNA replication and de novo synthesis of antigen were the critical elements required for inducing the immune response. These findings are consistent with previous studies showing that pigs immunized with CSF-VRP lacking the entire E2 gene do not generate anti-E2 antibodies, despite the presence of E2 antigen in the VRP shell [26,29]. In addition, it was reported that intradermal vaccination of pigs with CSF-VRP lacking the E^{rns} gene induced anti-E2 antibodies without any detectable E^{rns}-specific response [28]. With these characteristics, replicon-based vaccines can be designed to enable differentiation of vaccinated from infected animals. Furthermore, when employed as vector systems, replicons can be designed that do not induce antibodies against the proteins of the VRP, enabling efficient multiple vaccinations.

In vivo, the D_{136}N-mutated IFN-α/β–inducing VRP and the GM-CSF–expressing VRP were indistinguishable from the parent VRP A187-delE^{rns} in terms of B-cell and T-cell responses and protection from clinical disease after challenge infection. We did not analyse the local IFN-α/β and GM-CSF secretion levels after vaccination. Given the low levels of cytokines measured in vitro, it was unlikely to obtain detectable local cytokine levels in vivo following vaccination. In vitro however, the restimulation assays demonstrated that $N^{pro}(D_{136}N)$-mutated IFN-α/β–inducing VRP were the most efficient at stimulating the PBMC, suggesting that induction of IFN-α/β was influential in enhancing both B-cell and T-cell responses. These data are consistent with previous reports on IFN-α/β acting as an adjuvant (reviewed in [57]). The present work also elaborated on the potential of CSFV replicons to accommodate foreign genes with the aim of improving their immunogenicity or employing them as vectors. Both, monocistronic and bicistronic replicons expressed similar levels of firefly luciferase. Furthermore, a bicistronic replicon mediated secretion of bioactive GM-CSF at concentrations comparable to those obtained with Kunjin virus replicons [63]. The concentration of GM-CSF was calculated to be in the range of 2–15 ng/ml, while the Kunjin virus expressed GM-CSF was approximately 15 ng/ml [63]. GM-CSF represents a well-known beneficial cytokine as vaccine adjuvant [58–60,64] and was therefore also tested for its ability to improve the immunogenicity of CSF-VRP vaccines. While the IFN-α/β–inducing VRP and the GM-CSF–expressing VRP were indistinguishable from the parent VRP A187-delE^{rns} in terms of induced B-cell and T-cell responses and clinical protection, a positive effect on viral clearance was observed as measured by real-time RT-PCR analysis for viral RNA in the serum. Importantly, no infectious virus was detectable after challenge in any of the VRP- and virus-vaccinated animals, suggesting that the VRP-induced neutralizing antibodies were sufficient to completely prevent infectivity. However, this effect requires future investigations and confirmation by employing different doses of vaccine.

Taken together, we demonstrate that CSF-VRP represent a robust and versatile system for gene expression and vaccination in pigs. We show that their non-cytopathogenic nature allows long-term intracellular replication and protein expression, a property shared by flavivirus replicons such as West Nile virus and Kunjin virus replicons [2,65], and a major difference to alphavirus replicon systems [66,67]. Moreover, the long-term expression is not altered in IFN-α/β–inducing replicons, an observation related to the intrinsic IFN resistance of the CSFV replicons (this study), similar to BVDV [62]. The induction of IFN-α/β however resulted only in little beneficial effect in vivo by slightly reducing the level of detectable viral RNA, although in vitro B-cell and T-cell restimulation were significantly enhanced. Co-expression of GM-CSF did not significantly enhance immunogenicity either. Clearly, the CSF-VRP were less efficacious than the CSFV vaccine strain Riems. This is certainly a consequence of the single infection cycle of the VRP in vivo, as opposed to virus amplification occurring with replication-competent live-attenuated CSFV. Whether the efficacy of CSF-VRP

may further be improved by optimizing the immunization protocol (dose, route, booster vaccination) and co-expressing alternative cytokines with adjuvant effect remains to be explored. It is important also to consider the added value of the replicon beyond the field of classical swine fever, in terms of its ability to carry and translate foreign genes, functioning therefore as a self-replicating, biosafe vaccine vector.

Acknowledgments

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Unpublished data

CSFV replicons do not recombine in SK-6(E^{rns}) cells to form infectious virus

With VRP vaccines, a major concern is the risk of recombination of the replicon RNA with the gene transcribed by the complementing cell line, which may result in reversion to an infectious virus. Therefore we searched thoroughly for potential revertant viruses during long-term culture of VRP-infected cells. To this end, we infected complementing SK-6(E^{rns}) cells in triplicate with different VRP at a MOI of 1 $TCID_{50}$/cell (Table 1), and passaged the infected cells twice a week. After 10 passages we tested the supernatants on non-complementing SK-6 cells for the potential emergence of any infectious virus, using immunoperoxidase staining for the E2 protein. Nearly all non-complementing cells were infected after the first transfer. However, after a subsequent transfer on SK-6 cells, only a few foci of two to five antigen positive cells were detected with all samples. In the third transfer, all samples were negative except for a few samples showing single infected cells each. In contrast, the transfer of the supernatants on SK-6(E^{rns}) cells resulted in nearly 100 % infected cells in all three passages. This demonstrates the presence of VRP and absence of infectious virus. Interestingly, in one sample (A187*-delE^{rns} replica #2), a severe cytopathic effect was observed after 8 passages on SK-6(E^{rns}) cells. The cell supernatant of this sample was transferred on SK-6 and SK-6(E^{rns}) cells, and analysed for viral E2 protein expression. Foci of two to four antigen positive cells were observed in the SK-6 cells while all SK-6(E^{rns}) cells were antigen positive. This confirmed the presence of VRP in absence of infectious virus. The cytopathic effect was observed in all subsequent passages of this sample. We did not further investigate the cause of the cytopathic effect. In all other samples, the cells showed no changes in cell morphology nor any cytopathic effect throughout the whole duration of the experiment.

Table 1: Replicons tested for genomic stability in SK-6(E^{rns}) cells

Sample #	Replicon	Delivery mode
1	Mock	SK-6(E^{rns}) lysate
2	A187-delE^{rns}	VRP
3	A187*-delE^{rns}	VRP
4	A187-D_{136}N-delE^{rns}	VRP
5	A187*-D_{136}N-delE^{rns}	VRP
6	A187-N^{pro}-IRES-C-delE^{rns}	VRP
7	A187-N^{pro}(D_{136}N)-IRES-C-delE^{rns}	VRP
8	A187-N^{pro}-GMCSF-IRES-C-delE^{rns}	VRP
9	A187-N^{pro}(D_{136}N)-GMCSF-IRES-C-delE^{rns}	VRP
10	A187-N^{pro}-GMCSF-His-IRES-C-delE^{rns}	VRP
11	A187-N^{pro}(D_{136}N)-GMCSF-His-IRES-C-delE^{rns}	VRP
12	A187-N^{pro}-Luc-IRES-C-delE^{rns}	VRP

Replication of the virus vA187-Luc is impaired in monocyte-derived dendritic cells (MoDC) but not in SK-6 cells

We have demonstrated that foreign gene insertion in a CSFV replicon does not affect RNA replication in cell culture and packaging of the replicon into VRP. Insertion of a foreign gene might however have other effects on the CSFV life cycle that remained unnoticed in permanent cell lines and may only be detected by using live virus but not VRP. In order to address this, we constructed a monocistronic virus vA187-Luc that carries the firefly luciferase gene in the 5'-terminal region of the N^{pro} gene, in frame with the CSFV ORF, resulting in expression of a chimeric luciferase-N^{pro} protein (Figure 7). In vivo, dendritic cells and macrophages are the main target cells of CSFV. Therefore we compared the vA187-Luc and the parent vA187-1 viruses in primary MoDC and in the SK-6 cell line in terms of virus replication and protein expression. We infected the cells at a MOI of 10 $TCID_{50}$/cell and analysed virus and protein yield after one and two days of replication. The two viruses yielded similar virus titres in SK-6 cells at day 1 and 2 (Figure 8A). Accordingly, the luciferase activity was high in SK-6 cells infected with vA187-Luc (Figure 8B). In MoDC however, the vA187-Luc titre was significantly lower than the vA187-1 titre at day 2 after infection, and the luciferase activity decreased markedly between day 1 and day 2 (Figure 8B). As expected, no luciferase activity was detected in cells infected with vA187-1 virus or mock. Finally, we quantified viral NS3 expression in MoDC and SK-6 cells by flow cytometry (Figure 8C and D). The percentage of NS3 positive cells was significantly lower in MoDC infected with the vA187-Luc virus when compared to MoDC infected with the parent virus vA187-1 (Figure 8C). In contrast, more than 70 % of the SK-6 cells infected with vA187-1 and more than 49 % SK-6 cells infected with vA187-Luc were positive for NS3 at day 1 and 2 after infection (Figure 8D). Replication of the virus vA187-Luc in the porcine kidney cell line PK-15 was only slightly reduced when compared with the SK-6 cell line, as determined by particle production, luciferase activity and percentage of NS3 expressing cells (data not shown). Virus titres and percentage of NS3 positive cells were similar when vA187-Luc and vA187-1 were compared in PK-15 cells. In conclusion, replication of the virus vA187-Luc is severely impaired in primary MoDC when compared with the SK-6 and PK-15 cell lines.

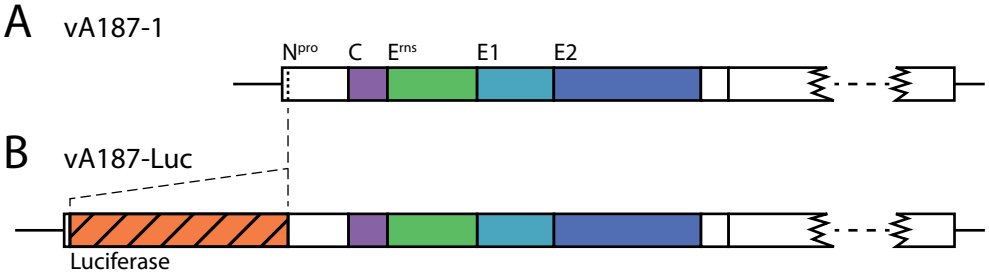

Figure 7: Schematic representation of the virus vA187-1 and vA187-Luc genomes. The luciferase gene was inserted at the 5' end of the parent virus vA187-1 (A) resulting in the luciferase expressing virus vA187-Luc (B). The luciferase gene is shown with a hatched, orange box. The other coloured boxes represent the structural genes.

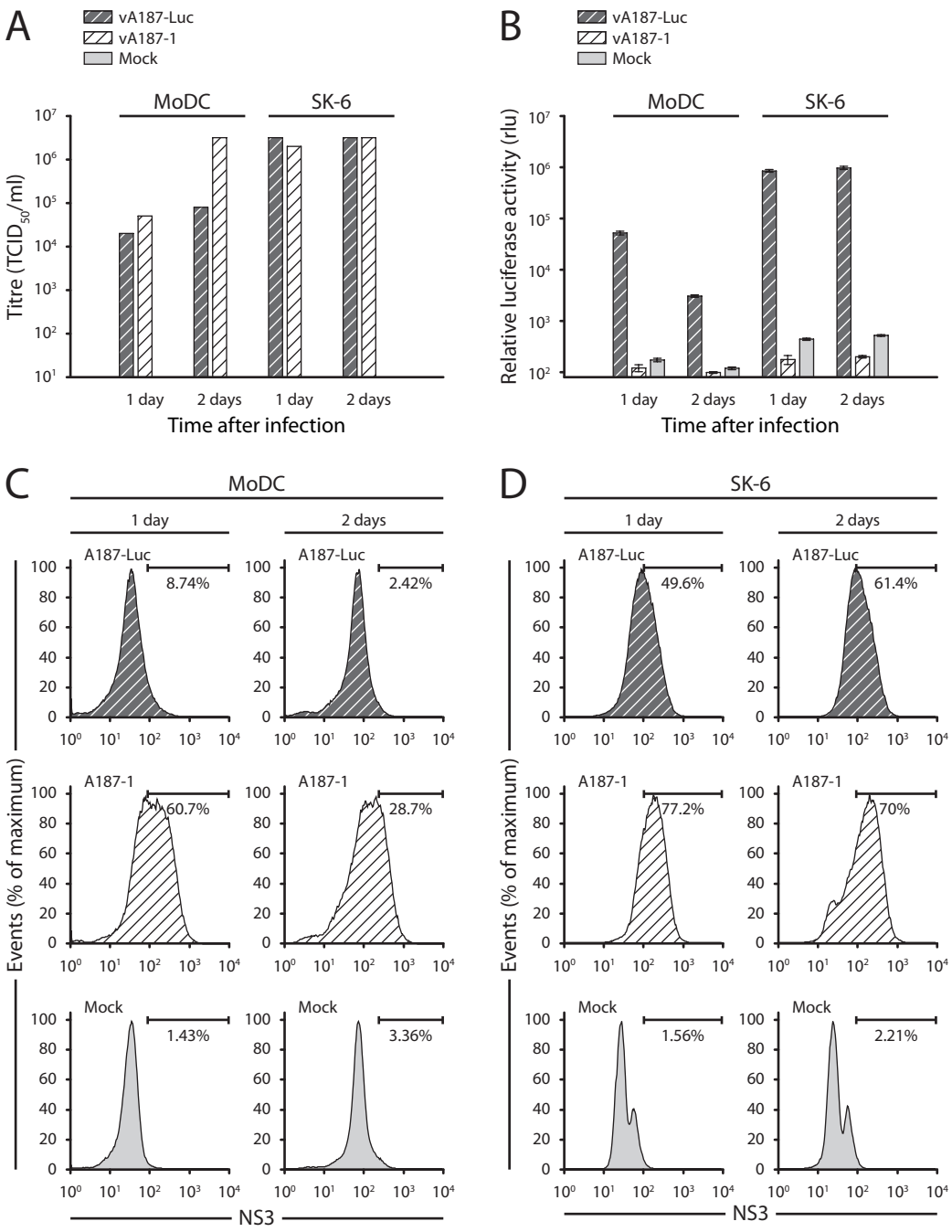

Figure 8: Replication of the virus vA187-Luc is impaired in MoDC but not in SK-6 cells. MoDC and SK-6 cell were infected with virus at a MOI of 10 $TCID_{50}$/cell. After 1 and 2 days virus titres in the supernatant were determined on SK-6 cells (A). Luciferase activity was measured by luciferase assay (B). The percentage of NS3 expressing MoDC (C) and SK-6 cells (D) was analyzed by flow cytometry.

Discussion

VRP carry a replication competent viral RNA genome but cannot generate infectious progeny due to the lack of at least one structural gene element. This makes VRP a safe gene delivery system with protein expression and vaccine applications. Compared to live attenuated virus however, VRP are less efficacious. Therefore, the present study was aimed at characterizing the VRP in terms of replication characteristics and immunogenicity.

With CSF-VRP as a model, we demonstrated the requirement of genome replication and protein translation for the induction of an immune response. Mutation of the VRP to induce IFN-α/β enhanced the VRP-mediated restimulation of humoral and cellular immune responses in vitro. However, in a vaccination-challenge experiment in pigs, the VRP that were mutated to induce IFN-α/β were indistinguishable from the parent VRP in terms of B-cell and T-cell responses. Nevertheless, the viral RNA levels after a challenge virus infection were reduced in the pigs vaccinated with the modified VRP. The discrepancy between the in vitro data obtained with restimulation assays and the in vivo data of the vaccination-challenge experiment emphasize the limitations of the ex vivo restimulation assays for the prediction of immunogenicity in vivo.

All the CSF-VRP tested induced a protective immune response in pigs without differences between the different groups, except for the reduced viral RNA levels observed after challenge infection. As expected, immunization with CSFV Riems resulted in a strong virus-specific antibody response with only a minor booster effect after challenge infection. The body temperature of CSFV Riems-vaccinated pigs was normal throughout the experiment, while the pigs vaccinated with the CSF-VRP developed a transient fever after challenge infection. In contrast to pigs vaccinated with CSF-VRP, no RNA of the challenge virus was detected in the serum of pigs vaccinated with CSFV Riems. Whether VRP can be further optimized to become as efficacious as live attenuated virus remains an open question. Because live attenuated viruses propagate in their host, they produce higher amounts of antigen and have a higher chance of encountering antigen presenting cells. This might explain the stronger immune reaction induced by live attenuated virus compared to VRP.

In theory, VRP are supposed to be safe. Nevertheless, one has to consider the possibility that replicons recombine in the complementing cell line to form infectious virus. In our hands, no recombination event leading to infectious virus occurred despite extensive passaging in the complementing cell line. For the pestiviruses BVDV and BDV however, the integration of cellular sequences in the genome is a frequent observation [133, 246–249]. These insertions are typically associated with cytopathogenicity due to increased NS3 production. Flaviviruses are also prone to recombination, as demonstrated for Japanese encephalitis virus with a reciprocal packaging system consisting of a pair of replicons [250]. In addition, recombination of RNA molecules is well documented for alphaviruses

[10, 251, 252]. To minimize the risk of recombination in the alphavirus replicon system, the replicons were complemented by two different helper plasmids expressing the structural proteins [10, 17, 253, 254].

Another issue is the genetic stability of the replicons. For vaccine and gene expression purposes, it is crucial that the heterologous gene is maintained and expressed. We used the luciferase gene to show stability in absence of selective pressure. Interestingly, in one sample we observed a cytopathic effect. Spontaneous occurrence of cytopathogenic replicons from CSFV-infected cells was described previously [48, 255]. The cytopathic effect is typically associated with a deletion of the genomic region coding for N^{pro} to E2, which results in increased RNA replication and NS3 expression. A similar deletion might have occurred in our experiments. But this was not further analyzed.

In conclusion, VRP fulfil the criteria of a safe marker vaccine. Compared to subunit vaccines, VRP have the advantages of increased antigen production over a prolonged period of time. They prime both, B- and T-cell immune responses. However, VRP do not yet achieve the efficacy of live attenuated viruses.

Outlook

Many questions on the potency of the CSF-VRP remain open. It is still unclear to which extent the modulation of the CSF-VRP to induce IFN-α/β and to express GM-CSF may enhance the immunogenicity of the VRP. A vaccination-challenge experiment in pigs using less VRP than in our vaccination experiment described herein might be more informative. Live attenuated CSFV C-strain protects against CSF as early as 3 to 4 days after vaccination. Therefore it is of interest, to elucidate the time necessary for the VRP to establish a protective immunity. Challenge infection of pigs at different times after vaccination with VRP would certainly answer this question. Because no challenge virus could be isolated from serum of vaccinated pigs, while low levels of viral RNA were detectable, it remains unclear whether the signal comes from infectious virus particles able to disseminate in the circulation or from viral RNA fragments. Whether VRP-vaccinated pigs secrete infectious virus after the CSFV challenge infection has not been investigated either.

To further improve the immunogenicity of VRP one could consider the insertion of other genetic adjuvants such as macrophage inflammatory protein-1α (MIP-1α), fms-like tyrosine kinase 3 ligand (Flt3L), interleukin 12 (IL-12) or interleukin 2 (IL-2) [256, 257]. The use of cytopathogenic replicons might be another possibility to improve the immunogenicity of the VRP. This can be achieved for instance by inserting sequences coding for proteins that are known to induce a cytopathic effect. One example would be the M protein of VSV [258–260].

We have demonstrated the potential of the CSFV replicons to serve as gene expression system. However, there is a size limit for sequences to be inserted. Genetic information representing up to approximately 25 % of the standard genome can be inserted in the pestiviral genome [133, 242]. Deletion of all structural genes might allow to accommodate larger foreign sequences. In this case however, the replicons need to be packaged with a cell line complementing for all structural proteins. And the CSF-VRP lacking all structural genes can of course not be used as a vaccine against CSF. Simultaneous expression of multiple foreign genes would be desirable for a generic gene expression system. For this, the 2A protease of foot-and-mouth disease virus was applied to express and process two different proteins in a single open reading frame [261–263]. This small protease is only 16 amino acids long and cleaves between its C-terminal glycine residue and a downstream proline [264, 265]. Finally, CSFV replicons might also be applied as naked RNA molecules, without being packaged in virion particles. Naked RNA can be injected or formulated with an appropriate transfection reagent. This allows to circumvent the restricted tropism of CSFV for porcine cells.

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Abbreviations

BDV	Border disease virus
BVDV	Bovine viral diarroea virus
CD46	cluster of differentiation 46 (CD46 is an inhibitory complement receptor)
cDNA	complementary DNA
CSF	classical swine fever
CSFV	classical swine fever virus
CSF-VRP	classical swine fever virus replicon particle
DIVA	differentiation of infected from vaccinated animals
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EMCV	encephalomyocarditis virus
ER	endoplasmatic reticulum
GDD	glycine-aspartic acid-aspartic acid motif in NS5B
GTP	guanosine triphosphate
HCV	hepatitis C virus
IFN	interferon
IRES	internal ribosome entry site
kD	kilodalton
KUN	Kunjin virus
MoDC	monocyte-derived dendritic cell
mRNA	messenger RNA
NTP	nucleoside triphosphate
NTPase	nucleoside triphosphatase
NTR	nontranslated region
OIE	Office International des Epizooties
PBMC	peripheral blood mononuclear cells
PK-15	porcine kidney cell line
RNA	ribonucleic acid
SK-6	swine kidney cell line
SK-6(E^{rns})	SK-6 cells stably expressing E^{rns}
$TCID_{50}$	50 % tissue culture infective dose
VRP	virus replicon particle
VSV	vesicular stomatitis virus

Declaration of originality

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I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

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