CHAPTER THREE

African Swine Fever Virus Biology and Vaccine Approaches

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Abstract

African swine fever (ASF) is an acute and often fatal disease affecting domestic pigs and wild boar, with severe economic consequences for affected countries. ASF is endemic in sub-Saharan Africa and the island of Sardinia, Italy. Since 2007, the virus emerged in the republic of Georgia, and since then spread throughout the Caucasus region and Russia. Outbreaks have also been reported in Belarus, Ukraine, Lithuania, Latvia, Estonia, Romania, Moldova, Czech Republic, and Poland, threatening neighboring West European countries. The causative agent, the African swine fever virus (ASFV), is a large, enveloped, double-stranded DNA virus that enters the cell by macropinocytosis and a clathrin-dependent mechanism. African Swine Fever Virus is able to interfere with various cellular signaling pathways resulting in immunomodulation, thus making the development of an efficacious vaccine very challenging. Inactivated preparations of African Swine Fever Virus do not confer protection, and the role of antibodies in protection remains unclear. The use of live-attenuated vaccines, although rendering suitable levels

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of protection, presents difficulties due to safety and side effects in the vaccinated animals. Several African Swine Fever Virus proteins have been reported to induce neutralizing antibodies in immunized pigs, and vaccination strategies based on DNA vaccines and recombinant proteins have also been explored, however, without being very successful. The complexity of the virus particle and the ability of the virus to modulate host immune responses are most likely the reason for this failure. Furthermore, no permanent cell lines able to sustain productive virus infection by both virulent and naturally attenuated African Swine Fever Virus strains exist so far, thus impairing basic research and the commercial production of attenuated vaccine candidates.

1. EMERGENCE AND HOST RANGE OF AFRICAN SWINE FEVER VIRUS

African swine fever virus (ASFV), believed to be one of the most dangerous infectious diseases of pigs, causes hemorrhagic fever in domestic and feral pigs (Costard et al., 2013; Vinuela, 1985). Depending on the virulence of the 23 characterized genotypes of the virus, the course of ASF infection causes a range of clinical syndromes (OIE, 2012), ranging from highly acute disease with 100% mortality, to long-term persistent infection. Upon infection with highly virulent strains, clinical signs involve pulmonary oedema, serious depression, high fever, anorexia, spotty skin, cyanosis, thrombocytopenia, lymphopenia, and hemorrhagic lesions (Blome et al., 2013; Karalyan et al., 2012a; Zakaryan et al., 2014). Conversely, African wild pigs (bushpigs, warthogs) are usually asymptomatically infected and constitute the reservoir hosts of African Swine Fever Virus in Africa (Penrith et al., 2013).

In the past, African Swine Fever Virus affected domestic pigs in European countries, including Spain, Portugal, Italy, and France. Nevertheless, the disease was eradicated from these countries by the mid-1990s, with the exception of Sardinia, where it is still endemic (Iglesias et al., 2017; Martinez-Lopez et al., 2015; Sanchez-Vizcaino et al., 2013).

ASF was reintroduced in 2007 to Eastern Europe via the Transcaucasus, especially Georgia, from which it spread to Armenia, Belarus, Ukraine, the Russian Federation, and in very recent times, to Estonia, Lithuania, Latvia, Romania, Moldova, Czech Republic, and Poland (ESFA AHAW Panel, 2015; Gallardo et al., 2014; Sanchez-Vizcaino et al., 2013; Wozniakowski et al., 2016) causing numerous outbreaks within domestic pigs and/or wild boar populations (Gogin et al., 2013; Sanchez-Vizcaino et al., 2013). A considerable risk of African Swine Fever Virus introduction into central Europe exists, since this region offers a growing population of wild boar (Massei et al., 2015), abundant road transportation of pigs (Eurostat, 2013), and transient traffic and shipments through large seaports and airports

(Costard et al., 2013; De la Torre et al., 2015; Mur et al., 2012a,b). Furthermore, an ASF outbreak indeed occurred in Belgium in 1985 after the illegal introduction of infected pork products, which was rapidly eradicated (Bironet al., 1987).

2. VIRUS TRANSMISSION AND SPREAD

Transmission of African Swine Fever Virus occurs via contact among infected animals, intake of infected material, and/or soft tick vectors (Ornithodoros) (Boinas et al., 2004; European Food Safety Authority Panel on Animal Health and Welfare, 2010; European Food Safety Authority, 2014; Sanchez-Vizcaino et al., 2012). Three transmission cycles have been reported in endemic areas: (i) a domestic pig/pig cycle, which does not involve other vertebrate or invertebrate hosts, (ii) a domestic pig/tick/wild pig cycle, and (iii) a domestic pig/tick cycle without warthog involvement (Gallardo et al., 2011). Persistence of the virus may occur for many months and some of the infected animals may not show clinical signs, and may even mimic other diseases (Botija, 1982; Penrith and Vosloo, 2009; Thomson et al., 1979). Persistently infected pigs seem to be involved in the spread of African Swine Fever Virus, producing sporadic outbreaks in formerly African Swine Fever Virus-free zones. This hypothesis has been supported by the fact that—under experimental conditions—pigs which survive with subacute infections shed virus for at least 70 days (de Carvalho Ferreira et al., 2013a,b). The disease, whose notification to the World Organization for Animal Health (OIE) is mandatory, produces important socioeconomic consequences (De la Torre et al., 2015; European Food Safety Authority, 2014) and presents a severe risk of spread to many countries worldwide.

3. MOLECULAR BIOLOGY OF THE VIRUS

African Swine Fever Virus is a large, cytoplasmic, double-stranded DNA virus that replicates in cells of the mononuclear phagocyte system, mainly monocytes and macrophages, although other cell types can be infected, especially later during the infection; the activation of target cells by African Swine Fever Virus replication plays a key role during African Swine Fever Virus infection, being a modulating element of virus pathogenesis (Carrasco et al., 1996; Carrascosa et al., 1999; Gomez-Villamandos et al., 2013; Vinuela, 1985).

African Swine Fever Virus virions are icosahedral structures of approximately 200 nm, which are formed by concentric layers (Fig. 1): the internal

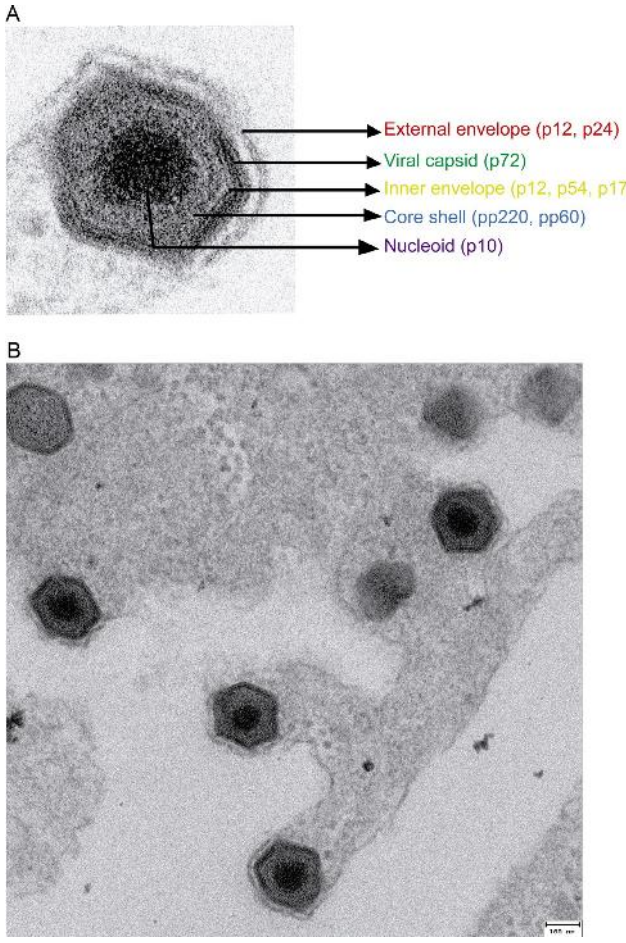

Fig. 1 Structure of the extracellular African Swine Fever Virus and virion egress from cells. (A) Electron microscopy image of the extracellular African Swine Fever Virus particle. The African Swine Fever Virus virion is composed of different concentric layers: the external envelope (red), the viral capsid (green), the inner envelope (yellow), the core shell (blue), and the nucleoid (purple). Examples of proteins present in each layer are shown in brackets (reviewed in Salas and Andres, 2013). (B) Electron microscopy image of African Swine Fever Virus virions emerging from infected cells.

core, the core shell, the inner membrane, the capsid, and, in the extracellular virions, the external envelope (Andres et al., 1997; Breese and DeBoer, 1967; Carrascosa et al., 1984).

The internal core is formed by the nucleoid, containing the viral genome and some nucleoproteins such as the DNA-binding protein p10 (Munoz et al., 1993). Furthermore, this layer organizes all the elements necessary for the synthesis of the early messenger RNAs (mRNAs): the transcriptional machinery,

the poly(A) polymerase, and the capping enzyme (Salas and Andres, 2013). The core shell is a wide protein layer surrounding the core, mostly composed by the polyproteins pp220 and pp62 (Andres et al., 1997, 2002a,b). Both polyproteins are successively processed by a viral protease (S273R gene) (Alejo et al., 2003). The inner envelope is next to the core shell and appears as a single lipid membrane by electron microscopy (Salas and Andres, 2013). This layer is derived from the endoplasmic reticulum (ER) (Andres et al., 1998) and contains the viral proteins p54, p17, and p12 (Rodriguez et al., 2004; Salas and Andres, 2013; Suarez et al., 2010). The capsid is the furthest layer of the intracellular virions and is composed of about 2000 hexagonal capsomers. The p72 protein is the major component of the capsomers and constitutes 33% of the total mass of the virion (Carrascosa et al., 1984; Garcia-Escudero et al., 1998). The outer envelope of the extracellular viral particles is taken from the cellular plasma membrane during the budding process by which African Swine Fever Virus egresses from the cell (Breese and DeBoer, 1967; Carrascosa et al., 1984). The outer envelope contains one of the viral proteins with a molecular weight of about 12 kDa, which has been identified as the viral factor needed for the attachment of the virus to host cells (Carrascosa et al., 1991). Other proteins described to be localized within the virus particle are the homologue of the cellular CD2 protein, called CD2v (D. Pérez-Núñez, M.L. Nogal, and Y. Revilla, personal communication), which mediates the hemadsorption to infected cells (Borca et al., 1998; Rodriguez et al., 1993). African Swine Fever Virus morphogenesis occurs in specialized areas of the cytoplasm, named viral factories, which develop in infected cells near the nucleus and the microtubule organization centre. Viral factories essentially exclude host proteins but are surrounded by ER membranes and vimentin boxes (Heath et al., 2001). In addition to ER membranes, mitochondria are also recruited to the periphery of viral factories (Rojo et al., 1998).

The African Swine Fever Virus genome is a linear double-stranded DNA molecule which varies in length from 170 to 190 kbp among different African Swine Fever Virus strains (Chapman et al., 2008; Portugal et al., 2015; Yanez et al., 1995). This is due to the size variability of several ORFs, especially in the multigene families, and to the variation of short tandem repeats within genes and intergenic regions (Lubisi et al., 2007). African Swine Fever Virus genes are closely distributed, codified in both DNA strands without introns. Genome termini are hairpin loops covalently cross-linked, being present in two possible forms, inverted and complementary to each other (Almazan et al., 1992; Almendral et al., 1990; Dixon et al., 2013).

African Swine Fever Virus mRNAs are structurally similar to the corresponding mRNA molecules of the cellular machinery. In vitro

transcribed viral mRNAs possess a cap structure in its 5′ UTR and a poly(A) tail of 33 nucleotides in its 3′ UTR (Salas et al., 1981). The cap structure is mostly of the m7G (5′) pppAm type, suggesting that an enzymatic activity for viral RNA capping is required. In this regard, African Swine Fever Virus encodes a guanylyltransferase (ORF NP868R) able to exert triphosphatase and guanylyltransferase activities (Pena et al., 1993; Yanez et al., 1995). In addition to the capping enzyme, the African Swine Fever Virus genome encodes a gene (ORF C475L) with similarity to other poly(A) polymerases, suggesting that the virus also possesses its own viral poly(A) polymerase (Rodriguez and Salas, 2013; Yutin et al., 2009).

African Swine Fever Virus is independent from the host cell machinery to carry out transcriptional processes and encodes about 20 genes considered to be important for mRNA modification and translation (Rodriguez and Salas, 2013). Indeed, the African Swine Fever Virus virion contains a DNA-dependent RNA polymerase consisting of several subunits, which is able to control the expression of viral genes (Rodriguez and Salas, 2013) in a time-dependent manner (Fig. 2). Early transcription uses virus-encoded transcription enzymes enclosed in the core (Kuznar et al., 1980; Salas et al., 1983). Most of immediate early and early genes belong to the multigene families, which are believed to be involved in the control of host responses to African Swine Fever Virus infection (Afonso et al., 2004) and in virus DNA replication (Rodriguez and Salas, 2013). The mechanism used by African Swine Fever Virus to temporally express its genes has been reported to be similar to the mechanism displayed by poxviruses (Broyles, 2003; Rodriguez and Salas, 2013).

4. VIRUS ENTRY, INTRACELLULAR TRAFFIC, AND VIRAL MACHINERY OF TRANSLATION

The mechanism by which African Swine Fever Virus enters host cells has been a controversial matter. Viral entry into host cells is also a key target for inhibiting African Swine Fever Virus infection and for potential vaccine development. Early studies described African Swine Fever Virus cell entry as a temperature, energy, cholesterol, and low-pH-dependent procedure, which involves receptor-mediated endocytosis (Alcami et al., 1989a,b; Carrascosa et al., 1999; Valdeira et al., 1998). Other, more recent studies determined that viral entry requires dynamin and is a clathrin-dependent process (Galindo et al., 2015; Hernaez and Alonso, 2010). Through the application of various pharmacological inhibitors and specific protein constructions inducing a dominant negative effect against key protein players in virus entry, it has been demonstrated that African Swine Fever Virus entry in

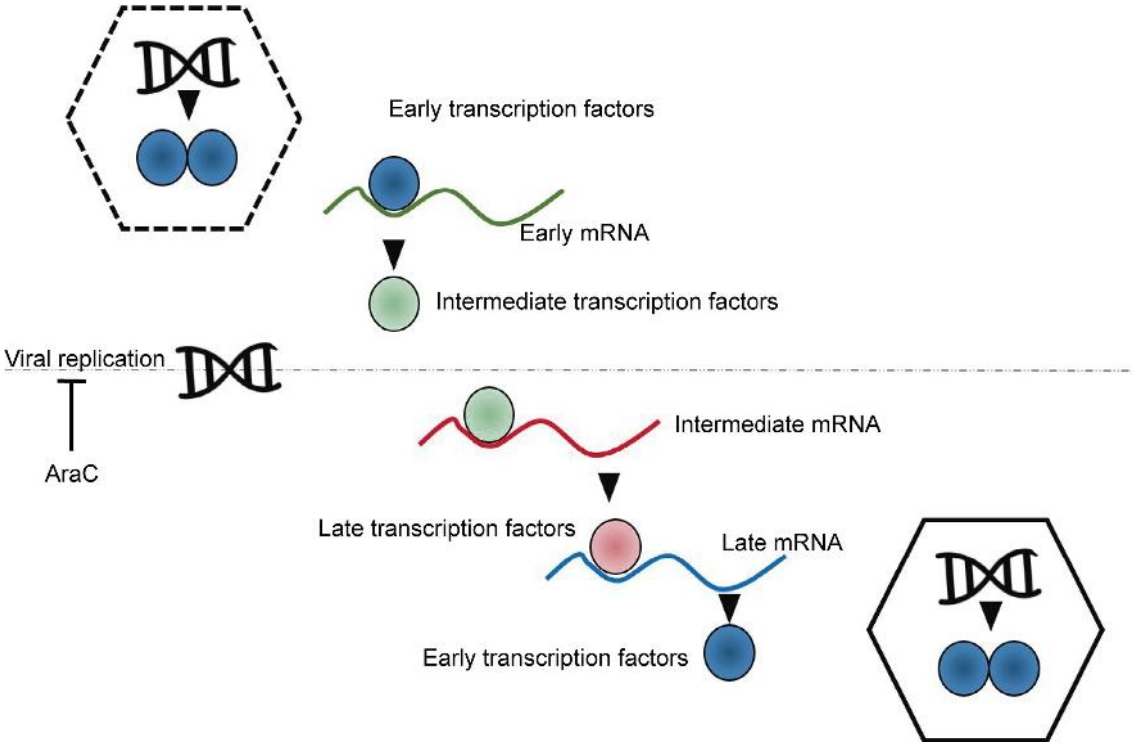

Fig. 2 Schematic representation of the transcriptional cascade mechanism proposed for African Swine Fever Virus. After viral entry, immediate early and early genes are expressed, while after viral replication intermediate- and late gene synthesis takes place. AraC is an inhibitor of viral replication.

Vero and porcine IPAM cells is mainly achieved by macropinocytosis, in a process that requires sodium/proton exchangers (Na^+/H^+), activation of EGFR, phosphorylation of PI3K and Pak1 kinases, together with the activation of the small Rho-GTPase Rac1, resulting in actin-dependent blebbing/ruffling perturbations of the cell membrane, allowing virions to get access to the host cell (Fig. 3). Inhibition of macropinocytosis regulators, as well as treatment with the drug EIPA (5-(N-ethyl-N-isopropyl) amiloride), results in a marked decrease in African Swine Fever Virus entry and infectious virus (Sanchez et al., 2012).

4.1 African Swine Fever Virus Entry and Traffic

It has been recently described that the mechanism of African Swine Fever Virus entry includes outer envelope disruption, capsid disassembly, and inner envelope fusion before the viral core can be released from endosomes (Andres, 2017; Hernaez et al., 2016). Linking of African Swine Fever Virus entry with the endosomal pathway (Alcami et al., 1989a,b, 1990; Valdeira and Geraldes, 1985), virus progression throughout the endocytic pathway and the role of Rab GTPases have been also described (Cuesta-Geijo et al., 2012).

Many viruses alter the cellular trafficking machinery by encoding specific viral proteins able to interact with key functional cytoplasm proteins, in order to induce novel membrane structures that constitute centers where viruses replicate and viral morphogenesis is achieved (Cruz and Buchkovich, 2017). These structures, named viral factories (or virosomes), consist of replicase proteins, virus genomes, and host proteins and are able to recruit host factors associated with cellular stress and defense mechanisms, suggesting that

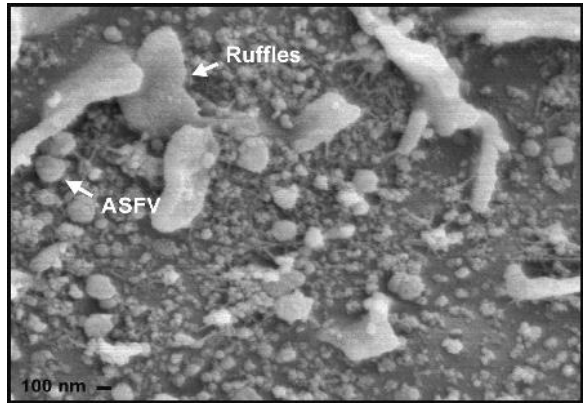

Fig. 3 Ruffles and Blebs formation at the cellular surface after 10 min of African Swine Fever Virus addition to cells. Scanning electronic microscopy image showing African Swine Fever Virus-Ba71V strain after 10 min of virus addition to Vero cells.

cell defense routes are subverted by pathogens to create sites of replication (Netherton et al., 2007).

African Swine Fever Virus has also been shown to induce the collapse of endoplasmic reticulum cisternae, and this collapse is dependent on the viral protein p54, an inner envelope protein targeting the ER (Rodriguez et al., 2004; Windsor et al., 2012; reviewed in Munoz-Moreno et al., 2015).

The adaptor protein 1 (AP-1) is a heterotetramer involved in the transport of proteins from the trans-Golgi network (TGN) to endosomes (Nakatsu and Ohno, 2003). AP-1 engages clathrin to form clathrin-coated vesicles that select their cargo by recognizing sorting signals in the cytoplasmic tail of integral membrane proteins. Two sorting signals, selectively recognized by AP-1, have been identified so far: the tyrosine (YXXF) and the dileucine ([D/E]XXXL[L/I]) motifs (Canagarajah et al., 2013). Several viruses are able to bind AP-1; for instance, HIV-Nef binds to AP-1 through a well-characterized dileucine (di-Leu) motif (Bresnahan et al., 1998; Madrid et al., 2005) leading to the stabilization of AP-1 and resulting in an alteration of the endocytic pathway and increase in HIV virulence. The E6 protein from bovine papillomavirus type 1 (BPV-1) also binds AP-1 (Tong et al., 1998), and AP-1 has been associated with viral spread in human herpes virus 6 infection (Mori et al., 2008).

It has been proposed by Perez-Nunez et al. (2015), that binding of ASF viral proteins to AP-1 might induce reorganization of the Golgi and reorganization of cellular traffic during African Swine Fever Virus infection, with the aim of facilitating viral replication, encapsulation and/or viral progression. The CD2v African Swine Fever Virus-protein shares significant similarity to that of the CD2 protein in T cells and is responsible for hemadsorption. It was identified as the viral protein binding to AP-1, and localizing around the viral factory during African Swine Fever Virus infection (see Fig. 4). Furthermore, it was shown that after

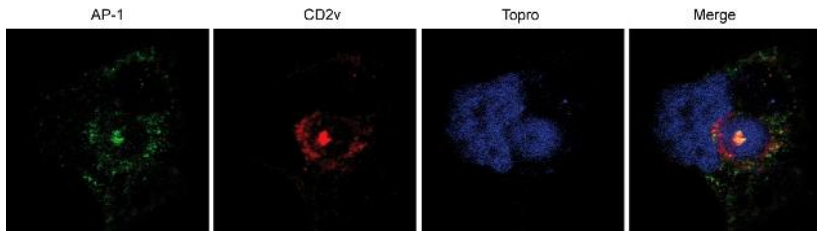

Fig. 4 Subcellular localization of CD2v during African Swine Fever Virus infection. COS cells infected with African Swine Fever Virus-E70 strain after 16 h were stained with specific antibodies against the adaptor protein 1 (AP-1) (in green) and CD2v protein (red). Nucleic acids were stained with Topro3.

BFA treatment, a drug that targets AP-1 delocalization from membranes to cytoplasm (Stamnes and Rothman, 1993), AP-1 was dispersed into the cytoplasm in African Swine Fever Virus-infected cells; in contrast, CD2v localization was not affected by BFA and remained attached to the membranes surrounding the viral factories (Perez-Nunez et al., 2015). Interestingly, sequence analysis of CD2v identified a di-Leu motif predicted to mediate binding to AP-1, but Perez-Nunez et al. (2015) demonstrated that this di-Leu motif in CD2v was neither involved in colocalization nor in interaction between CD2v and AP-1. These results are in contrast to the above described mechanism for HIV-Nef. In addition, a region within the cytoplasmic tail of CD2v was identified, which does not contain the di-Leu motif, but interacts with AP-1, suggesting that this region could represent an as yet uncharacterized novel viral AP-1-binding motif with vital consequences for cellular traffic and virus infectivity (Perez-Nunez et al., 2015).

4.2 African Swine Fever Virus Control of Cellular Protein Synthesis

It is well known that viruses depend on the cellular machinery to replicate and synthesize viral proteins, as they display limited genomes that cannot encode the whole functions required to accomplish these complex processes. Therefore, several viruses have developed complicated mechanisms to hijack the cellular translation machinery in order to use it for their own protein synthesis. The majority of these strategies are based on switching on/off the activity of the eukaryotic initiation factors (eIFs), which play key roles in protein synthesis. Frequently, viruses target two important steps in eukaryotic translation initiation: (i) the phosphorylation of eIF2 and (ii) the recruitment of ribosomal subunits to the mRNA by targeting eIF4F and eukaryotic translation factor 4E-binding proteins (4E-BPs). Phosphorylation of eIF2α (P-eIF2α) by PKR is an important host defense mechanisms against viral infections. Many viruses have developed mechanisms to evade PKR activation. Among them, Epstein–Barr virus encodes double-stranded (ds) RNAs that bind PKR but do not trigger the activation of the kinase (Schneider and Mohr, 2003; Walsh and Mohr, 2011). Vaccinia Virus (VV), herpes simplex virus-1 (HSV-1), influenza, and reovirus encode dsRNA-binding proteins that mask or sequester dsRNA, preventing the activation of PKR (Beattie et al., 1995; Khoo et al., 2002; Lloyd and Shatkin, 1992; Mulvey et al., 1999; Salvatore et al., 2002). Frequently, viruses use redundant mechanisms to guarantee the inhibition of antiviral pathways. For instance HSV-1, in addition to inhibiting PKR activation, encodes for proteins that avoid P-eIF2α by activating specific

phosphatases (Mulvey et al., 2003). Another regulation mechanism is the recruitment of ribosomes and eIF4F formation. eIF4F is a complex formed by three proteins: eIF4A, eIF4E, and eIF4G (Prevot et al., 2003) and, as central part of the cap-dependent translation machinery, is tightly regulated to protect against stress and viral infections. Complex DNA viruses, in contrast to nearly all other viruses studied so far, allow the formation of eIF4F but still are able to inhibit cellular protein synthesis (Walsh and Mohr, 2004; Walsh et al., 2005, 2008).

African Swine Fever Virus, in analogy to other complex DNA viruses, has developed a sophisticated mechanism to hijack the cellular translation machinery by not only altering the activity of the translation initiation factors but also their cellular localization. It has been described that, similar to VV infections, P-eIF2α level diminished at early time points post-African Swine Fever Virus infection (Castello et al., 2009b). African Swine Fever Virus encodes a protein, called DP71L (also called l14L or 23NL, depending on the viral strain), which possesses a characteristic binding protein phosphatase 1 (PP1) motif (VxF) (Rivera et al., 2007). Furthermore, it possesses an N-terminal sequence of basic residues and its C-terminal domain is similar to the HSV-1-encoded neurovirulence factor ICP34.5 (Goatley et al., 1999). Importantly, DP71L interacts with PP1 in vitro (Rivera et al., 2007) and in vivo (Zhang et al., 2010). Transfection of DP71L induces a decrease of phosphorylated eIF2α and enhances the expression of cotransfected reporter molecules, suggesting that DP71L plays a role in keeping the cellular translation machinery active in order to allow viral protein synthesis (Zhang et al., 2010). Deletion of the l14L gene (the DP71L gene of African Swine Fever Virus strain E70) from the genome of the virulent Spanish African Swine Fever Virus isolate E70, reduced virulence of the E70 virus for domestic pigs (Zsak et al., 1996), whereas deletion of the gene from the virulent Malawi LIL 20/1 isolate did not reduce its virulence, suggesting that the Malawi isolate may encode another viral gene, which can compensate for the loss of l14L/DP71L (Afonso et al., 1998).

eIF4G is a substrate for caspase-3 (Bushell et al., 1999; Prevot et al., 2003), during apoptosis activation (Marissen and Lloyd, 1998). However, it was reported that eIF4G was resistant to caspase-3 cleavage in African Swine Fever Virus-infected cells (Castello et al., 2009b). In addition, it was previously described that African Swine Fever Virus encodes for an inhibitor of apoptosis -like protein (IAP) called A224L, which functions as an inhibitor of caspase 3 (Nogal et al., 2001). The possibility that the African Swine Fever Virus protein A224L protects the cellular translation machinery from caspase 3-mediated degradation needs to be explored in

the future. Furthermore, ASVF induces mTOR-mediated phosphorylation of eIF4G at Ser1108 (Castello et al., 2009b), which has been associated with "translational activation" (Kimball et al., 2000; Raught et al., 2000), and triggers the phosphorylation of eIF4E at Ser209 by Mnk-1. eIF4E phosphorylation reaches its maximum levels at 16-h postinfection and is suppressed in the presence of Mnk-1 inhibitors, suggesting that Mnk-1 activation is critical during African Swine Fever Virus infection, as reported before in other systems (Pyronnet, 2000; Pyronnet et al., 1999). Similar to VV and other DNA viruses (Buchkovich et al., 2008), African Swine Fever Virus infection promotes 4E-BP1-hyperphosphorylation at early time points postinfection allowing activation of translation, whereas it is hypophosphorylated at later time points postinfection; this step results in cap-dependent translation inhibition. The differential phosphorylation stages of 4E-BP1 probably reflect an African Swine Fever Virus-mediated mechanism to support viral protein synthesis during early African Swine Fever Virus replication stages and stop viral protein synthesis during the late morphogenesis, a time at which viral proteins have been already synthesized, and cell energy should be used for virion particle assembly and egress. Furthermore, many of the components of the translation machinery (eIF4G, eIF4E, eIF2, eIF3b, and the eukaryotic elongation factor 2), ribosomes, and mitochondria are relocated from a diffused distribution throughout the cytoplasm to the viral factories (Castello et al., 2009b). Since viral RNAs localize at the periphery of the viral factories, it is likely that active translation of viral mRNAs is limited to these foci in African Swine Fever Virus-infected cells.

During infection, many viruses modify the distribution and quantity of cellular mRNAs by using different strategies, for example, by interfering with the efficiency of translation. In this regard, HSV-1 accumulates cellular mRNAs at the nucleus of infected cells (Sandri-Goldin, 2011), whereas adenovirus (Yatherajam et al., 2011), vesicular stomatitis virus (von Kobbe et al., 2000), poliovirus (Castello et al., 2009a; Park et al., 2008), and influenza virus (Satterly et al., 2007) impair host mRNAs export from the nucleus to the cytoplasm. HSV-1 (Cheng and Deutscher, 2005) and gammaherpesviruses (Covarrubias et al., 2011; Richner et al., 2011) stimulate cellular mRNA degradation. VV also induces mRNA degradation, probably via viral-encoded decapping enzymes (Parrish and Moss, 2007; Parrish et al., 2007). A decrease in the amount of cytoplasmically located polyadenylated mRNAs, together with an increase of nuclear RNAs, was described during African Swine Fever Virus infection (Castello et al., 2009b), indicating that poly(A) mRNA stability and nuclear RNA export are targeted during African Swine Fever Virus infection. Finally, the African

Swine Fever Virus genome encodes for a protein called g5R/African Swine Fever Virus-DP, which displays the Nudix motif, which is present in the host decapping enzyme 2 (Dcp2) or the VV viral proteins D9 and D10 (McLennan, 2007). g5R has in vitro decapping activity (Parrish et al., 2009), localizes around viral factories, and is able to bind mRNA during African Swine Fever Virus infection, among other functions (Quintas et al., 2017). Therefore, this viral enzyme represents a candidate to be the viral factor involved in the degradation of cellular mRNAs, probably contributing to the cellular shutoff.

In summary, African Swine Fever Virus cell entry is a temperature, energy, cholesterol, and low-pH-dependent procedure, requiring dynamin and clathrin (Galindo et al., 2015; Hernaez and Alonso, 2010). African Swine Fever Virus entry in Vero and porcine IPAM cells is mainly achieved by macropinocytosis, in a process that requires Na^+/H^+, activation of EGFR, phosphorylation of PI3K and Pak1 kinases, together with the activation of the small Rho-GTPase Rac1, resulting in actin-dependent blebbing/ruffling perturbations of the cell membrane, allowing virions to get access to the host cell. African Swine Fever Virus, in analogy to other complex DNA viruses, has developed a sophisticated mechanism to hijack the cellular translation machinery in order to use it for its own protein synthesis. The majority of these strategies are based on African Swine Fever Virus-induced switching on/off the activity of the eIFs, together with alterations of their subcellular distribution during African Swine Fever Virus infection.

5. AFRICAN SWINE FEVER VIRUS GENES MODULATING HOST RESPONSES

When the complete 170–190 kbp DNA sequence of African Swine Fever Virus (Boinas et al., 2004; Chapman et al., 2008; de Villiers et al., 2010; Granberg et al., 2016; Rodriguez et al., 2015; Yanez et al., 1995) was analyzed, genes coding for structural proteins, for enzymes with functions related to DNA replication, for DNA repair, gene transcription, and protein modification (Yanez et al., 1995) as well as for proteins involved in virus–host interactions were identified (Borca et al., 1998; Miskin et al., 1998; Nogal et al., 2001; Rodriguez et al., 2002). Among them, gene products involved in DNA replication and repair, including a DNA polymerase (G1211R) and a proliferating cell nuclear antigen-like protein (E301R) (Yanez et al., 1995), have been reported. African Swine Fever Virus also encodes enzymes involved in DNA repair, such as a DNA polymerase (O174L), an ATP-dependent DNA ligase (NP419L), and an

endonuclease (E296R) (Garcia-Escudero et al., 2003; Jezewska et al., 2006; Redrejo-Rodriguez et al., 2006). It is also assumed that African Swine Fever Virus encodes enzymes and factors required to transcribe and process mRNAs, since ASF viral transcription does not require the host RNA polymerase II and virions are transcriptionally active (Salas et al., 1981, 1986; Santaren and Vinuela, 1986).

African Swine Fever Virus manipulates cellular mechanisms and the host defense by encoding a variety of immunomodulatory proteins that efficiently interfere with host immune responses (reviewed in Munoz-Moreno et al., 2015). Actually, the ability of African Swine Fever Virus to evade immune surveillance is via the modulation of the expression of proinflammatory molecules and cytokines. In this regard, it has been shown that ORF A238L contains ankyrin repeats which are homologous to those described in the IκB family, and binds the transcription factor p65-NFκB to act as a bona fide IκB-α viral homologue (Revilla et al., 1998); this results in the modulation of genes depending on NFκB in infected cells. This was first demonstrated by ectopic expression of A238L and by analysis of the expression of genes under the control of NFκB, showing that the observed NFκB inhibition was specific (Powell et al., 1996; Revilla et al., 1998). Interestingly, the A238L sequence does not contain motifs phosphorylated by IκB kinase, suggesting that the viral protein A238L is a natural, constitutive, and irreversible suppressor of NFκB activity (Tait et al., 2000). It is largely known that monocytes and macrophages play a central role in antigen presentation and secretion of bioactive molecules, e.g. prostaglandin E2 (PGE2), whose synthesis is firmly controlled by cyclooxigenase-2 (COX-2) (Janelle et al., 2002). Several viruses regulate COX-2 expression in order to modulate, among other things, the synthesis of PGE2 (Fang et al., 2012; Janelle et al., 2002; Murono et al., 2001; Pollara et al., 2012; Steer et al., 2003; Tung et al., 2011). African Swine Fever Virus has been shown to modulate not only the expression of COX-2 but also other two important inflammatory factors, such as TNF-α and iNOS (Granja et al., 2006a,b), through the regulation of the transcriptional coactivator proteins CBP and p300 (Granja et al., 2008, 2009). CBP/p300 interacts with numerous transcription factors to coordinate the expression of specific sets of genes in response to diverse physiological stimuli (Goodman and Smolik, 2000; Vo and Goodman, 2001; reviewed in Sanchez et al., 2013).

In addition, viral proteins homologous to host apoptosis inhibitory proteins have been described: (i) ORF A179L, structurally and functionally similar to Bcl2 (Banjara et al., 2017; Revilla et al., 1997) and ORF A224L, an

IAP homologue that inhibits caspase activation and also promotes survival through NFκB activation (Nogal et al., 2001; Rodriguez et al., 2002). Other reported African Swine Fever Virus proteins interfering with host immune responses include the DP71L protein, which putatively prevents the shutoff of protein synthesis induced by phosphorylation of PKR (Rivera et al., 2007; Zhang et al., 2010), the I329L protein, which acts as an inhibitor of Toll-like receptor pathways, and several members of the multigene families 360 and 530, which have been implicated in inhibiting type I interferon induction (Afonso et al., 2004; de Oliveira et al., 2011). Finally, it is important to note that African Swine Fever Virus also encodes adhesion proteins, such as EP153R (a lectin-like protein) which is involved in apoptosis and MHC presentation (Hurtado et al., 2004, 2011), and the ORF EP402R/CD2v, whose N-terminal domain shares high homology with the host CD2 protein. This viral protein is essential for the binding of erythrocytes to infected macrophages, a phenomenon called hemadsorption (Rodriguez et al., 1993), whose role in viral pathogenesis is not clearly understood so far (Borca et al., 1998). The CD2v intracellular domain has a role in binding to cytoplasmic adaptor protein SH3P7 (Kay-Jackson et al., 2004) and to AP-1, a cellular factor involved in cellular traffic (see earlier) and regulated by CD2v (Perez-Nunez et al., 2015).

6. AFRICAN SWINE FEVER VIRUS VACCINES

Pigs surviving African Swine Fever Virus infection develop a strong and protective immunity, indicating that an effective vaccine against African Swine Fever Virus might be possible. Attenuated African Swine Fever Virus strains induce immune responses which afford long-term resistance to homologous but not to heterologous African Swine Fever Virus challenge (Leitao et al., 2001; Mulumba-Mfumu et al., 2016), and the correlate of protection seem to be virus-specific T-cell responses (King et al., 2011; Revilla et al., 1992). Nevertheless, attenuated African Swine Fever Virus vaccine candidates produce several side effects such as chronic lesions, fever, viremia, hypergammaglobulemia, and/or joint swelling (King et al., 2011; Leitao et al., 2001; Revilla et al., 1992). Despite the fact that several research groups during the past few years have developed novel vaccine technologies, ranging from inactivated viruses, recombinant proteins/peptides, and DNA vaccines to live-attenuated vaccine (LAV) candidates, an efficacious, safe African Swine Fever Virus vaccine does currently not exist.

6.1 Modulation of the Host Immune System by African Swine Fever Virus

Animals surviving African Swine Fever Virus infection develop a protective immune response. Therefore, naturally or genetically attenuated or moderately virulent African Swine Fever Virus strains have been used as LAV candidates. These attenuated African Swine Fever Virus strains induced a long-term resistance to homologous but not to heterologous virus challenge (King et al., 2011; Leitao et al., 2001; Mulumba-Mfumu et al., 2016), and the protection was found to correlate with virus-specific T-cell responses and cytokine production (King et al., 2011; Revilla et al., 1992). However, the correlates of protective immunity to African Swine Fever Virus infection are weakly characterized yet, although questions about viral antigen presentation and MHC modulation in the context of African Swine Fever Virus-infected porcine macrophages have been partially answered (Gonzalez Juarrero et al., 1992; reviewed in Alvarez et al., 2013).

There are conflicting reports on the role of cytokines and IFN during African Swine Fever Virus infection. Replication of virulent African Swine Fever Virus strains in porcine macrophages pretreated with bovine IFNα (Esparza et al., 1998) and of cell culture-adapted African Swine Fever Virus-Ba71V in Vero cells pretreated with human IFNα was reduced (Paez et al., 1990). In contrast, induction of IFN in porcine macrophages by polyI:C did not affect the replication of either the virulent Kirawira or the attenuated Uganda strain of African Swine Fever Virus (Wardley et al., 1979). Older studies from Revilla and coworkers described that African Swine Fever Virus infection induced the modulation of IFNγ production by T lymphocytes (Revilla et al., 1992). More recently, it has been reported that nonvirulent African Swine Fever Virus strains, which do not encode genes from multigene family (MGF)360 and MGF505 (Chapman et al., 2008; Portugal et al., 2015), induce moderate amounts of IFN after in vitro infection of pig macrophages, in comparison to virulent African Swine Fever Virus strains, which produce lower amounts of IFNs and other regulatory cytokines (Afonso et al., 2004; Gil et al., 2008; Zhang et al., 2006). In this context, it is noteworthy to mention that IFNα and IFNβ expression occurred in animals during infection with African Swine Fever Virus Georgia2007/1 (Karalyan et al., 2012b) and other virulent African Swine Fever Virus strains. The most relevant source of IFN production in vivo after African Swine Fever Virus virulent infection are most likely dendritic cells (DCs) (Golding et al., 2016), which had been previously demonstrated to produce significant amounts of IFN in response to other types of viral infections (O'keeffe et al., 2012). However, African Swine Fever Virus infection of DCs has not been clearly demonstrated so far, neither

in vitro nor in vivo, posing the interesting question whether IFN production by DCs is indirect due to factors secreted by African Swine Fever Virus-infected macrophages or by direct infection of this subset of cells.

6.2 Cells Susceptible to African Swine Fever Virus Infection

In infected animals, pig monocytes and alveolar macrophages are the major target cells for African Swine Fever Virus replication (Gomez-Villamandos et al., 2013); this has important consequences for the pathogenesis of African Swine Fever Virus, as these cells play key roles in the immune response through phagocytosis, antigen presentation, or cytokine secretion (Gordon et al., 1995; Van Furth et al., 1972). Porcine alveolar macrophages (PAM) express CD14, SLAII, CD163, CD169, CD203, and CD16 receptors (Ezquerra et al., 2009). CD163 is a scavenger receptor whose expression is restricted to the monocyte/macrophage lineage and is normally used as a marker for monocytic differentiation and maturation (Law et al., 1993; Sanchez et al., 1999); it acts as the high-affinity scavenger receptor for the hemoglobin/haptoglobin complex and activates a signaling pathway that induces pro- and antiinflammatory cytokines (Poderoso et al., 2011).

The susceptibility to African Swine Fever Virus is linked to the maturation status of monocyte–macrophage cells that correlates with an upregulation of CD163 (Mccullough et al., 1999; Sanchez-Torres et al., 2003). Yet, the role of CD163 as an African Swine Fever Virus receptor is controversial, as its presence is not enough to increase susceptibility of nonpermissive cells to the virus (Lithgow et al., 2014). Furthermore, pigs lacking CD163 are not resistant to Georgia 2007/1 African Swine Fever Virus strain (Popescu et al., 2017).

Due to the difficulty to obtain reproducible results, the lot-to-lot variations, the costly cell preparations, and animal welfare reasons, freshly made PAM are not an ideal cell population to produce African Swine Fever Virus. These problems were partially overcome several years ago by the adaptation of the Ba71 (virulent) African Swine Fever Virus strain to Vero cells (Ba71V) (Enjuanes et al., 1976) and recently, by the finding that COS-7 cells (an SV40 T-antigen-transformed epithelial green monkey cell line), allow productive African Swine Fever Virus infection (Granja et al., 2006a; Hurtado et al., 2010). However, the generation of LAV still requires porcine cell lines able to sustain a productive African Swine Fever Virus infection. We have recently characterized four different permanent porcine cell lines—IPAM WT (Weingartl et al., 2002) (CRL-2845), IPAM-CD163 (Lee and Lee, 2010), WSL (Keil et al., 2014), and CΔ2+ (Chitko-Mckown et al., 2013)—regarding their ability to sustain productive infections with virulent

African Swine Fever Virus isolates Armenia/07 and E70, and the attenuated NH/P68 African Swine Fever Virus isolate; we also determined their virus production capacity (Sanchez et al., 2017). Our results indicate that these porcine cell lines do not show a mature macrophage phenotype and that the level of infection and viral production in IPAM-WT, IPAM-CD163, and CΔ2+ are much lower than those obtained in PAM, with the exception of WSL; the latter cell line is able to sustain the growth of the attenuated, but not virulent, African Swine Fever Virus strains. Thus, a permanent porcine cell line able to allow the study of African Swine Fever Virus–macrophage interaction and to sustain the productive infection both of attenuated and virulent African Swine Fever Virus strains is not available so far. This fact seriously compromises the production of putative LAVs for ASF, since such cells are the basis for the commercial production of these vaccines.

6.3 Live-Attenuated African Swine Fever Virus Vaccines

During the period of ASF outbreaks in Spain and Portugal which lasted from 1962 to 1995, natural African Swine Fever Virus isolates were obtained and attenuated by cell passages (Ribeiro, 1982); cell-attenuated African Swine Fever Virus strains were used to vaccinate an indeterminate number of pigs in the field. It has been reported that attenuated African Swine Fever Virus strains OURT88/3 and NH/P68 protect pigs against challenge with homologous virulent strains (Leitao et al., 2001; Malogolovkin et al., 2015; Mulumba-Mfumu et al., 2016), although only partial crossprotection was shown against heterologous viruses. Furthermore, these naturally attenuated strains induced various side effects, depending on the dose, and post-vaccination reactions including petechiae, necrotic foci, joint swelling, and pneumonia were noted. Partial protection against a heterologous African Swine Fever Virus strain, apparently related to virus-specific IFNγ production, was observed with the attenuated African Swine Fever Virus strain OURT88/3 (King et al., 2011). Strategies focused on reducing the side effects of LAVs have been unsuccessful so far, since the deletion of virulence genes such as DP71L and DP96R reduced the ability of the attenuated virus to protect against homologous challenge (Abrams et al., 2013). The generation of genetically modified variants derived from naturally attenuated African Swine Fever Virus strains has been approached (see Fig. 5). The recombinant NH/P68 variants, lacking several genes involved in virus–cell interaction (e.g., A224L, A238L, A276R), showed various degree of protection (60%–100%), both against homologous and heterologous (Armenia 2007) African Swine Fever Virus strains (C. Gallardo and Y. Revilla, personal communication). However, the NH/P68-based vaccine candidate's

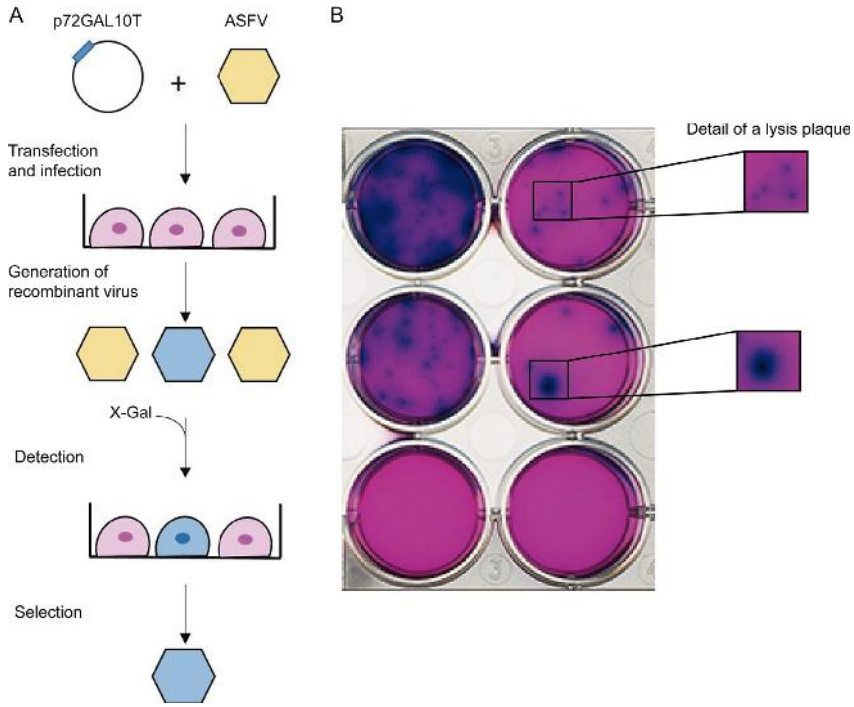

Fig. 5 Method to construct recombinant ASF viruses lacking specific genes. (A) Schematic representation of the experimental procedures. The flanking sequences of the gene of interest are cloned into the p72-BetaGAL10T vector. Cells are transfected with these constructions and African Swine Fever Virus-infected, to allow homologous recombination. Positive recombinant viruses carry a copy of beta-GAL instead of the targeted gene, allowing the selection of recombinant virus by titration in the presence of X-GAL. (B) Examples of blue plaques representing recombinant ASF viruses.

still induced (low) viremia and side effects such as arthritis and necrotic foci in most of the vaccinated pigs which most likely prevents their commercial use. We believe that some degree of virus replication is needed to induce protective immunity, and it might be difficult to eliminate some of the observed side effects. The genetic manipulation of the virulent Georgia 2007 isolate by deleting the 9GL and the MGF360/505 genes has been shown to attenuate the virus, but vaccination with the attenuated deletion mutant did not confer protection against parental virus challenge in pigs (O'donnell et al., 2016).

Lately, several groups have develop attenuated African Swine Fever Virus vaccines by using either virulent strains (e.g., Benin, Georgia, OUR/88/1, and Ba71), which, after genetic manipulation and deletion of genes regulating type I IFN responses, or deletion of the CD2v gene, are attenuated and confer

protection against homologous and heterologous African Swine Fever Virus strains (López-Monteagudo et al., 2016; Reis et al., 2016). Interestingly, vaccination experiments using recombinant viruses revealed several key characteristics of the immune response against African Swine Fever Virus, namely, that deletion of genes downregulating IFNβ is a key factor in the mechanism of attenuation and that deletion of genes such as DP148R or A276R, both of them involved in IFNβ modulation, produces attenuated viruses which are efficacious against African Swine Fever Virus challenge (Reis et al., 2016; Revilla et al., manuscript in preparation). As discussed earlier, it is clear that novel innovative technology and new approaches are needed to produce an efficacious and safe African Swine Fever Virus vaccine. It is pertinent to state that the identification of virulence genes and the correlate of protective immune responses to African Swine Fever Virus infection can only be elucidated through detailed studies on the molecular mechanisms used by the virus to modulate host immune responses and the contribution of the innate and adaptive immune systems to protection; only after this critical information is obtained, rationally designed vaccines against ASF can be constructed.

6.4 Subunit Vaccines

Studies carried out many years ago showed that vaccination of pigs with different amounts of percoll-purified, UV-inactivated African Swine Fever Virus-Ba71V did not confer protection against live virus, either homologous or heterologous (Forman et al., 1982; Mebus and Dardiri, 1980; Revilla, Y., personal communication; Stone and Hess, 1967). Recently, these results have been confirmed, showing that inactivated African Swine Fever Virus does not confer protection, even in the presence of various adjuvants (Blome et al., 2014), suggesting that antibodies induced by African Swine Fever Virus structural protein might not be sufficient to protect pigs against virulent African Swine Fever Virus challenge. In contrast, transfer of antibodies derived from African Swine Fever Virus-infected pigs was reported to protect pigs against a lethal African Swine Fever Virus challenge (Onisk et al., 1994). Several African Swine Fever Virus proteins are able to induce neutralizing antibodies in immunized pigs. Among these proteins, p54 and p30 (Gomez-Puertas et al., 1996, 1998) were shown to be involved in various steps of virus attachment and internalization. Nevertheless, immunization of pigs with recombinant p54 and p30 proteins expressed in baculovirus did not protect against virulent African Swine Fever Virus challenge. Similarly, a vaccine based on African Swine Fever Virus proteins p30, p54, p22, and p72 produced in baculovirus failed to protect pigs against a virulent

African Swine Fever Virus challenge, despite producing neutralizing antibodies (Neilan et al., 2004); the above discussed results added to the controversy about the role of antibody-mediated neutralization of African Swine Fever Virus in protection against ASF (Escribano et al., 2013). On the other hand, vaccination with baculovirus-expressed African Swine Fever Virus proteins EP402R/CD2v revealed a considerable degree of protection against homologous African Swine Fever Virus challenge (Ruiz-Gonzalvo et al., 1996).

Several protocols using specific African Swine Fever Virus DNA vaccines have been also developed. In one of those attempts, the African Swine Fever Virus genes p30 and p54 were cloned in-frame with a fragment encoding for the single variable chain of a specific antibody recognizing swine leukocyte antigen II, in order to target the viral proteins to cells bearing SLA-II. Following this strategy, specific T cells against African Swine Fever Virus proteins could be detected; however, neither neutralizing antibodies nor protection against a virulent challenge was achieved (Argilaguet et al., 2011). Others generated constructs expressing the extracellular domain of HA fused to viral p30 and p54 proteins, showing that this modification significantly enhanced both humoral and cellular immune responses in pigs, however, without conferring protection against virulent African Swine Fever Virus challenge. In another attempt, cDNA constructs encoding various African Swine Fever Virus genes, further fused to ubiquitin, conferred partial protection against challenge in the absence of African Swine Fever Virus-specific antibodies; protection correlated with robust CTL response, mainly with antigen-specific CD8+ T cells (Argilaguet et al., 2012; Lacasta et al., 2014). These results reinforce the role of virus-specific T-cell responses in African Swine Fever Virus protection and suggest the existence of multiple African Swine Fever Virus antigens with potential protective capacity.

Our recent approach using a combination of a variety of African Swine Fever Virus-specific proteins and cDNAs (heterologous prime-boost vaccine) was able to induce a robust immune response in terms of neutralizing antibodies and IFNγ production; however, vaccinated pigs were not protected against virulent challenge with the Armenia 2007 strain (Y. Revilla and J.A. Richt, personal communication).

In conclusion, DNA- and peptide-based vaccines have been shown to induce specific antiviral immune responses based on either neutralizing antibodies or virus-specific T-cells, but either no protection or only partial protection was afforded when vaccinated pigs were challenged with virulent African Swine Fever Virus.

6.5 The Future of African Swine Fever Virus Vaccinology

While an effective vaccine for ASF is not currently available, several vaccine approaches—as discussed in detail earlier—have demonstrated varying levels of protection. LAVs are the best hope for a protective ASF vaccine but some of them still do not offer good heterologous protection and suffer from side effects and safety issues (reversal to virulence). Detailed studies on the molecular mechanisms used by African Swine Fever Virus to modulate host immune responses and deleting respective virulence genes from the viral genome will most likely allow scientists in the near future to make safer LAVs without the above described negative effects. Producing LAVs, which afford heterologous protection against a variety of African Swine Fever Virus genotypes, will be much harder. In order to do this, crossprotective epitopes on African Swine Fever Virus-specific proteins have to be identified, and the role of cellular and humoral immune responses in protection against ASF needs to be elucidated. In contrast to LAVs, subunit protein/DNA ASF vaccines are safe and do not cause serious side effects, but only offer partial protection. This might be due to the delivery method or the requirement of different protective African Swine Fever Virus antigens in the vaccine mix. Since the role of the humoral and cellular arm of the adaptive immune system in protection against ASF is still not clear and the protective African Swine Fever Virus antigens are not defined, this approach at this time seems more a trial-and-error than a rationally designed approach. In addition to the open questions regarding the protective African Swine Fever Virus antigens, some of the viral proteins might induce antibodies which might enhance uptake of the virus into cells. Such an antibody-dependent enhancement could occur when virus-specific antibodies facilitate virus entry into host cells which carry Fc receptors on their surface. This could lead to a more rapid and severe disease after African Swine Fever Virus challenge of vaccinated animals. There is some evidence that immunopathological mechanisms are part of the ASF pathogenesis. Therefore, in order to rationally design future efficacious and safe LAVs and subunit vaccines, the detailed identification of virulence genes and the correlate of protective immune responses to African Swine Fever Virus infection need to be achieved. Molecular mechanisms used by the virus to modulate host immune responses, the role of African Swine Fever Virus protein in protection, and immunopathology, as well as the contribution of the innate and adaptive immune systems to protection need to be studied in detail. Only a significant investment into basic African Swine Fever Virus research will translate into a mitigation tool, i.e., vaccine, which can be safely used to avoid severe economic hardship for pig farmers around the world.

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