

African swine fever vaccine development: current status and challenges ahead

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Abstract

African swine fever (ASF) is a contagious, hemorrhagic and highly lethal viral disease in swine. Following the initial report of ASF in Kenya in 1921 it spread to East Asia through Western Europe and Russia (including Eastern Europe). Many efforts have been dedicated to controlling ASF but the struggle to eradicate this disease continues. Thus far, the efforts to develop an effective vaccine to control ASF have been unsuccessful. A previous inactivated ASF vaccine, developed by traditional methods, failed to protect pigs from ASF virus (ASFV) infection. Neutralizing antibodies were not effective in inducing protective immunity and it appears that cellular immunity is required. To develop an effective ASF vaccine the identification of protective antigens of ASFV has been explored and subunit vaccines that target these potential protective antigens have induced partial protection. DNA vaccines that induce cellular immunity have been effective in inducing protection against ASFV infection. ASFV live attenuated vaccines (LAVs) can be rationally designed and engineered via comparative and functional genomics. LAVs have a major safety concern despite their high protective efficacy. ASF vaccines are urgently needed to control ASF; however, many obstacles remain to be overcome to develop an effective ASF vaccine.

Keywords: African swine fever (ASF), ASF vaccine, Subunit vaccine, Cellular immunity, Live attenuated vaccines (LAVs)

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Introduction

African swine fever (ASF) is a contagious and hemorrhagic viral disease affecting swine that threatens the swine industry worldwide (Costard *et al.*, 2013; Dixon *et al.*, 2019^a). The causative agent of ASF is the ASF virus (ASFV; the sole member of *Asfarviridae*), a large enveloped, double-stranded DNA virus (Alonso *et al.*, 2018). ASF occurs in several forms, ranging from the highly lethal (up to 100% mortality) to subclinical. The symptoms of the severe form in pigs infected with highly virulent ASFV strains include hemorrhages, edema, ascites, and shock, resulting from leaking blood vessels (Villeda *et al.*, 1993; Gomez-Villamandos *et al.*, 1995). Warthogs (*Phacochoerus aethiopicus*), bushpigs (*Potamochoerus porcus*), and giant forest hogs (*Hylochoerus meinertzhageni*) are natural hosts of ASFV; they do not show symptoms even when infected, transmit ASFV easily, and they are considered a reservoir of ASFV (Blome *et al.*, 2013). Soft ticks belonging to *Ornithodoros*, also act as vectors of ASFV (Pereira de Oliveira *et al.*, 2019).

ASF was first described in Kenya in 1921 (Montgomery, 1921) and remains endemic in sub-Saharan Africa, which includes Kenya, Tanzania, South Africa, and various other countries (Galindo and Alonso, 2017). The first ASF outbreak in Europe occurred in Portugal in 1957 and then spread to Spain, France, Belgium, and other European countries (Galindo and Alonso, 2017). To eradicate ASF, strategies such as culling and the construction of fences were devised, and the disease was eventually eradicated in Europe in the 1990s after 30–40 years of effort.

In 2007, ASF was introduced into Georgia, where it rapidly spread throughout the Caucasus and into Russia, reaching Eastern Europe, including the Baltics, Poland and the Ukraine (Gogin *et al.*, 2013; EFSA Panel on Animal Health and Welfare (AHAW) *et al.*, 2019). From 2007 to 2018, approximately 1300 cases of ASF in domestic and wild pigs were reported by the Russian Federal Agency (Oganessian *et al.*, 2013; Cwynar *et al.*, 2019). It spread to neighboring countries, and outbreaks have been reported in many countries, including Belarus, Poland, Estonia, Latvia, the Czech Republic, Romania, Bulgaria, Slovakia, and northern Greece (Cwynar *et al.*, 2019). Despite strong quarantine policies being applied at great economic costs, the struggle to eradicate the disease continues.

In 2018, an ASF outbreak occurred in China and spread to most provinces of China (Ge *et al.*, 2018). More than four million pigs were killed because of this outbreak, devastating the swine industry in China. ASF spread into East Asia, including Cambodia, Laos, Thailand, and Vietnam (Zhou *et al.*, 2018; 2019). Following the first case of ASF in Vietnam in February 2019, ASF outbreaks were reported across the provinces and more than 5.7 million pigs were culled (Le *et al.*, 2019).

Owing to the fear of outbreaks occurring in neighboring countries, South Korea has made considerable efforts to prevent the importation of the disease (Kim *et al.*, 2020; Yoo *et al.*, 2020). Despite these efforts, the first ASF outbreak occurred in Paju in September 2019, and up to 13 consecutive outbreaks

occurred in Gyeonggi-do and Incheon in October 2019. This resulted in the culling of 145,546 pigs from 89 farms within the containment zone. No further ASF outbreaks in domesticated pigs have been reported in this area since then. Unfortunately, ASF-infected local wild boars have been repeatedly observed moving south near the demilitarized zone (Kim *et al.*, 2020; Jo *et al.*, 2020).

A protective vaccine is important for controlling infectious diseases. For example, in Korea the first outbreak of foot and mouth disease (FMD) occurred in March 2000; it spread nationwide and led to the culling of approximately 3.5 million animals (Yoon *et al.*, 2015). A test and culling policy was initiated to control FMD, but it failed, and the current control policy relies on vaccination. With the FMD vaccine succeeding in immunizing animals, the number of FMD outbreaks has decreased significantly, bringing it under control (Joo *et al.*, 2002; Park *et al.*, 2018).

Conversely, repeated ASF outbreaks occur because of the lack of a commercially available vaccine. It took decades to eradicate ASF from Spain because, in the absence of a vaccine, ASF control relies solely on culling. In addition, it is difficult to control the current ASF outbreak as it has spread across many countries, including Russia, China, and other Asian countries.

In this review, we discuss the status of ASF vaccine development, as well as recent progress and approaches to develop effective ASF vaccines to overcome the obstacles to ASF vaccine development.

Protective immunity for ASF vaccine

Protective immunity against ASFV remains poorly understood. In most viral infections, a humoral immune response is regarded as important and sufficient to provide protection against viral infections (Wang *et al.*, 2018). Neutralizing antibodies are efficient in protecting animals from viral infections (Diaz-San Segundo *et al.*, 2017).

Conversely, in ASF, cellular as well as humoral immunity appears to be important because humoral immunity alone has not been effective in protecting animals from ASFV infection (Revilla *et al.*, 2018). In humoral immunity, passive transfer of ASFV antibodies protects pigs from lethal ASFV infection (Schlafer *et al.*, 1984^{a,b}; Onisk *et al.*, 1994). Anti-ASFV antibodies have shown inhibitory effects on ASFV replication in macrophage cell cultures (Malmquist, 1963). The antibody titer of monocyte infection-inhibition seemed to provide protection against ASFV infection (Ruiz-Gonzalvo *et al.*, 1986^{a,b}; Knudsen *et al.*, 1987; Borca *et al.*, 1994^a). Neutralizing antibodies generated in the antibody-mediated immune response are considered protective against ASFV infection, but appear to show inhibitory effects on the replication of the autologous ASFV strain, but not against heterologous ASFV (Ruiz-Gonzalvo *et al.*, 1986^a; Zsak *et al.*, 1993; Borca *et al.*, 1994^a; Gomez-Puertas *et al.*, 1996; Neilan *et al.*, 2004). In addition, the antibody-mediated cytolytic effect on ASFV was observed *in vitro*, but the effect did not correlate with protective immunity *in vivo* (Norley and Wardley, 1982; 1983). Therefore, it raised doubts about whether the antibody-mediated immune response, including

neutralizing antibodies, could induce protective immunity against ASFV (Zsak *et al.*, 1993; Neilan *et al.*, 2004). Several reports suggest that cellular immunity is important for ASFV protection (Figure 1). Oura *et al.* (2005) reported that cytotoxic CD8⁺ T cells are important for the clearance of ASFV and in protecting animals from ASFV infection. The protective effects were reported to be correlated with ASFV-specific CD8⁺ T cells (Argilaguuet *et al.*, 2012; Lacasta *et al.*, 2015). Additionally, levels of interferon (IFN)-gamma, a major component of the Th1 cellular response, were significantly correlated with protection against heterologous ASF infection. A DNA vaccine induces cellular immunity without generating ASFV-specific

antibodies, thereby providing a degree of protection pigs against ASFV infection (Argilaguuet *et al.*, 2012; Lacasta *et al.*, 2015). Therefore, it appears that cellular immunity, rather than humoral immunity, is a major protective immunity against ASFV infection. Furthermore, to efficiently develop the ASF vaccine, it is necessary to analyze whether the ASF vaccine effectively induces effector and memory cells (Figure 1).

Thus, humoral and cellular immune responses appear to be important for protection, but understanding how they help create anti-ASFV immunity remains a core objective to help develop an effective ASF vaccine.

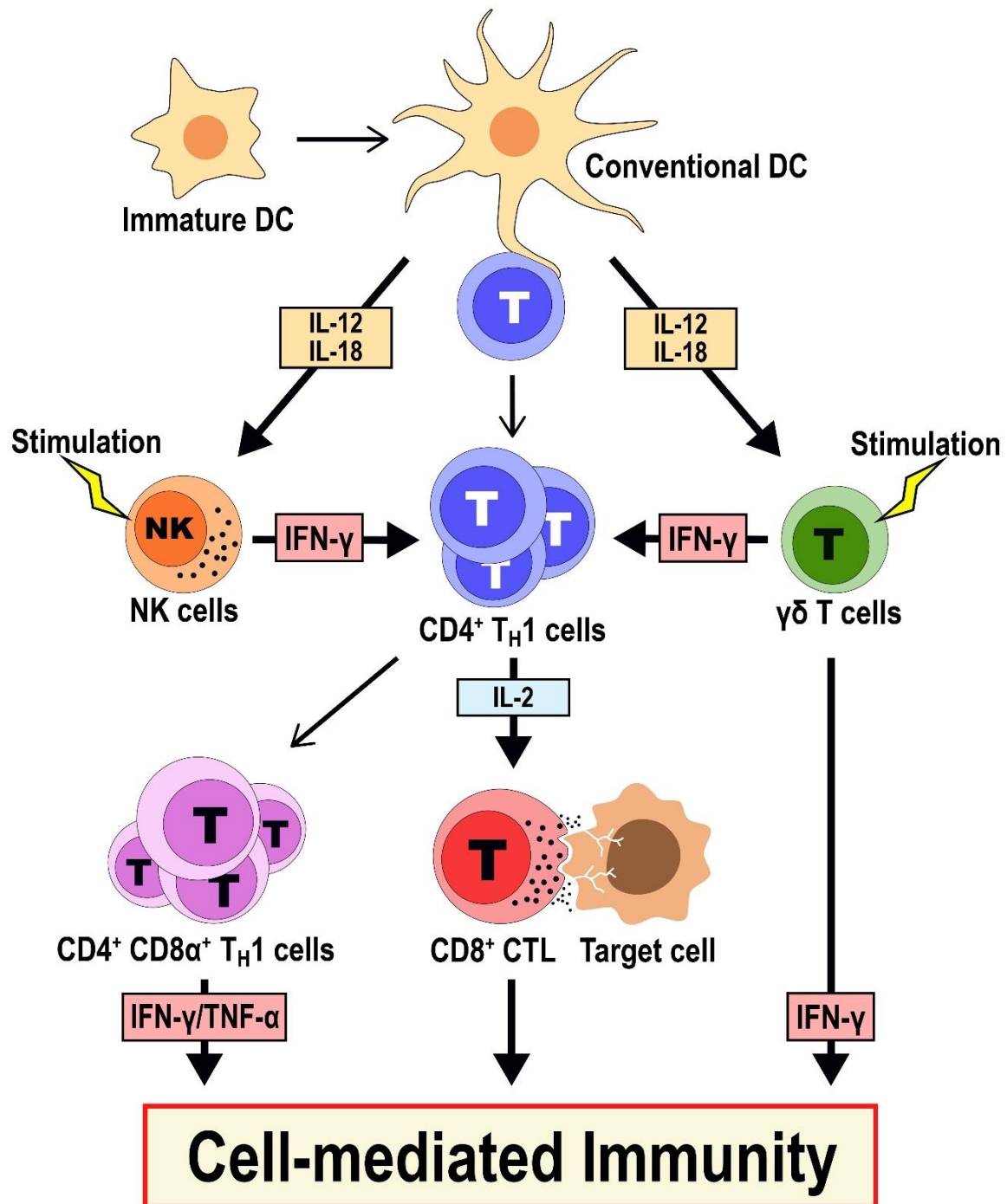


Figure 1 Diagram of the porcine cellular immune response. CTL: cytotoxic T lymphocytes, DC: dendritic cells, IL: interleukin, IFN: interferon, NK cells: natural killer cells, TNF: tumor necrosis factor, T_H1: T helper type 1.

ASF vaccine development

There have been several attempts to develop an effective vaccine against ASF (King *et al.*, 2011; Lacasta *et al.*, 2015; Arias *et al.*, 2017; Revilla *et al.*, 2018).

A traditional inactivated vaccine was attempted to protect against ASFV challenge, but failed to induce protection against the ASFV challenge (Blome *et al.*, 2014^a). Substantial efforts have been made subsequently to develop effective vaccines against ASF. Here, we summarize previous attempts to develop an ASF vaccine and then review approaches to solve the obstacles in producing an effective ASF vaccine.

Inactivated ASF vaccines: An inactivated vaccine is the most commonly used vaccine type, and has been used extensively to control numerous infectious diseases in humans and animals.

Historically, there have been several attempts to protect pigs from ASFV using an inactivated vaccine (Stone and Hess, 1967; Forman *et al.*, 1982; Genovesi *et al.*, 1988). Contrary to expectations, inactivated ASF vaccines failed to induce protection against the ASFV challenge. The attempted formulation of an inactivated vaccine included inactivated ASFV-infected cell extracts, the supernatant of ASFV-infected pig peripheral blood leukocytes, purified and inactivated ASFV virions, glutaraldehyde-fixed ASFV-infected macrophages, and detergent-treated ASFV-infected alveolar macrophage cell cultures (Stone and Hess, 1967; Forman *et al.*, 1982; Genovesi *et al.*, 1988). Moreover, to enhance protective immunity, the novel adjuvants Polygen™ and Emulsigen® were combined with inactivated ASFV but failed to induce protective immunity against ASFV (Blome *et al.*, 2014^b). This is not a feasible strategy for developing an effective ASF vaccine. In this process, researchers found that antibody-mediated immunity by an inactivated ASF vaccine was not sufficient to protect animals from ASFV and that cellular immunity was required to create an effective ASF vaccine (Takamatsu *et al.*, 2013; Revilla *et al.*, 2018). Owing to the ineffectiveness of the inactivated vaccine against ASF, it is necessary to investigate the essential ingredients of ASFV granting protective immunity and adjuvants that induce cellular immunity.

Identification of protective antigens and subunit vaccines

Recombinant protein subunit vaccines: To overcome the limitations of traditional inactivated vaccines, researchers have attempted to identify viral antigens of ASFV that can induce protective immunity. Subunit vaccines comprising specific protective ASFV antigens with delivery systems have been developed to protect animals from ASFV. The identification of protective viral antigens relies on understanding the extensive diversity of ASFV antigens.

The viral proteins of ASFV involved in virus attachment and internalization were investigated for their immunogenic properties. Subsequently, a few ASFV viral proteins, including p30, p54, pp62, p72, pp22, and CD2v, were explored for use as subunit

vaccines. A subunit vaccine using p30, p54, and p72 induced neutralizing antibodies against ASFV (Ruiz-Gonzalvo *et al.*, 1986^a; Zsak *et al.*, 1993; Borca *et al.*, 1994^a; Gomez-Puertas *et al.*, 1996) but provided only partial protection against ASFV. Approximately 50% of the immunized pigs survived a subsequent ASFV infection, and the surviving pigs showed clinical symptoms and high titers of viremia (Gomez-Puertas *et al.*, 1998; Barderas *et al.*, 2001). Neilan *et al.* (2004) also developed a subunit vaccine comprising baculovirus-expressed p30, p54, p72, and p220; however, it failed to protect pigs from a subsequent virulent ASFV infection, despite high titers of ASFV-specific neutralizing antibodies being detected. The CD2v protein (referred to as 8DR or pEP402R) is the viral homolog of the cellular CD2 protein of T cells, and is involved in the co-regulation of cell activation (Dixon *et al.*, 2019^b). CD2v, the hemagglutinin of ASFV, is necessary for mediating hemadsorption by ASFV-infected cells (Rodriguez *et al.*, 1993; Borca *et al.*, 1994^b). Therefore, the CD2v protein of ASFV has been implicated in inducing protective immunity. A subunit vaccine containing CD2v protein-induced hemadsorption-inhibiting antibodies, and monocyte infection-inhibiting antibodies provided partial protection to pigs against the homologous virulent ASFV strain (Ruiz-Gonzalvo and Coll, 1993; Ruiz-Gonzalvo *et al.*, 1996). Argilaguet *et al.* (2012, 2013) revealed that the protective immunity of the CD2v subunit vaccine was mediated by two T-cell epitopes of the CD2v protein. Another study showed that a combined subunit vaccine comprising the CD2v protein and an adjacent C-type lectin protein (EP153R) imparted partial protection (Burmakina *et al.*, 2016; 2019).

Therefore, the investigation of protective antigens of ASFV is one of the most fundamental processes in the development of effective ASF vaccines, and the combination or fusion of protective antigens can enhance the protective efficacy or protect animals from various ASFVs. Additionally, the application of adjuvants or delivery vehicles capable of inducing protective immunity, especially cellular immunity against ASFV, is required to develop an effective ASF vaccine.

DNA vaccine: There is controversy surrounding the protective effects of the humoral immune response induced by the protein subunit vaccine against ASFV because the immunized pigs developed viremia after ASFV infection (Mebus, 1988). Oura *et al.* (2005) demonstrating that CD8⁺ T cells were crucial in protecting against the virulent ASFV OUR/T88/1 strain, while an antibody-mediated immune response was not sufficient. A DNA vaccine was attempted as a platform for a subunit vaccine, in which it was expected that antigens would be expressed intracellularly and presented via MHC Class I, thereby activating CD8⁺ T cells. Argilaguet *et al.* (2012) developed a DNA vaccine encoding p54/E183L and p30/CP204L, but this DNA vaccine failed to produce a protective immune response. To improve the DNA vaccine strategy, a DNA vaccine encoding the extracellular domain of CD2v, p30, and p54 coupled to ubiquitin was developed, and it produce a strong

humoral and cellular immune response, which induced partial protection against the European ASFV E75 strain (Argilaguet *et al.*, 2012; Argilaguet *et al.*, 2013). Furthermore, DNA containing ASFV open reading frames fused to ubiquitin was used to investigate protective ASFV antigens, specifically, how they induced ASFV-specific T cells (Lacasta *et al.*, 2014). This study revealed that a cell-mediated immune response could confer partial protection against ASFV infection.

Screening for immunogenic and protective antigens resulted in more than 50 immunogenic ASFV proteins being identified (Alejo *et al.*, 2018; Jancovich *et al.*, 2018). Unfortunately, among the ASFV proteins, not even one could induce complete protection from infection in domestic pigs. This implies that a subunit vaccine containing multiple viral antigens is required for complete protection and a prime-boost vaccination strategy, such as a DNA-protein vaccination, would be a more appropriate approach to enhance immunogenicity (Kardani *et al.*, 2016). Additionally, to induce protective immunity against ASF proteins, effective adjuvants or delivery vehicles are required to induce effective protective immunity against ASFV.

There are several adjuvants, including mineral salts, emulsions, bacterial-derived components, and saponins. Typical commercial adjuvants for pigs, such as MONTANIDE™ ISA 206 (SEPPIC, France), were efficient in the induction of antibody response (Park *et al.*, 2014), but less effective in inducing cellular immunity. Therefore, it is necessary to discover adjuvants that can stimulate antigen-presenting cells (APCs) and increase cellular immunity by secreting various Th1 cytokines, including IFN- γ , interleukin-2, interleukin-12, and tumor necrosis factor- α . MF59, toll-like receptor agonists such as monophosphoryl lipid A, flagellin, and cytosine phosphate guanine motifs are known as adjuvants that induce cellular immunity. In addition, Bacillus Calmette-Guérin vaccine, a commercial vaccine for human tuberculosis, is highly safe and mediates cellular immunity and it is likely to be an efficient delivery vehicle that induces cellular immunity against ASFV.

Live-attenuated ASF vaccines

Live-attenuated vaccines (LAVs) as ASF vaccines: LAVs have been developed through a serial passage of viruses *in vitro* or through non-host animals and have recently been developed through high technology via the deletion of the specific genes from the virus. LAVs can offer efficient protection against viral diseases (Meeusen *et al.*, 2007; Minor, 2015). LAVs can induce strong immune responses similar to those induced when a virulent virus infects naturally. Naturally or *in vitro* attenuated ASFVs have been investigated as ASF vaccine candidates (Krug *et al.*, 2015). Live-attenuated ASFV has induced long-term resistance in pigs and enabled them to survive a homologous ASFV infection. There were few clinical signs and a reduction in viremia in the LAVs-immunized animals, suggesting that LAVs can control viral replication in the early stage of ASFV infection (Krug *et al.*, 2015). However, LAVs have a limited effect against heterologous ASFV

challenges or under field conditions (Mebus and Dardiri, 1980; Hamdy and Dardiri, 1984; King *et al.*, 2011; Lacasta *et al.*, 2015; Mulumba-Mfumum *et al.*, 2016). The boundaries of homologous cross-protection are not clear (King *et al.*, 2011), which is an obstacle that LAVs must overcome.

Engineered ASF LAVs: Recently, genomic informatics and genetic manipulation technology have been used to develop a next-generation ASF vaccine, a specific virulent factor-deleted ASFV from a virulent wild type, which is safe and able to induce high protective efficacy (Borca *et al.*, 2018). Comparative and functional genomics tools can be used to identify target genes of ASFV, genes associated with virulence, and the host range of ASFV (Tulman and Rock, 2001; Dixon *et al.*, 2004; Chapman *et al.*, 2008; Tulman *et al.*, 2009; Correia *et al.*, 2013). The ASFV genes believed to be associated with virulence and host range are thymidine kinase (TK), 9GL (B119L), NL (DP71L), and multiple members of the multigene families 360 and 530 (MGF 360/530) (Tulman and Rock, 2001). The deletion of these genes from the ASFV strain showed attenuation in the host and induced a protective response against homologous ASFV infection (Moore *et al.*, 1998; Zsak *et al.*, 1998; Lewis *et al.*, 2000; O'Donnell *et al.*, 2015^{a,b}). The TK and 9GL proteins have a function like that of the ICP 34.5 protein of herpes simplex virus, thought to prevent host-cell protein shutoff by directing dephosphorylation 1 α . The TK and 9GL gene-deleted ASFV was defective in replication in the macrophages of pigs (Moore *et al.*, 1998; Lewis *et al.*, 2000), and deletion of the NL gene from the European ASFV E70 strain showed reduced virulence in pigs without changing virus replication *in vitro* (Zsak *et al.*, 1998). The MGF 360/530 genes of ASFV are known to suppress the type I IFN response, and these gene-deleted ASFV showed reduced growth *in vitro* and attenuation in pigs (Zsak *et al.*, 2001; Afonso *et al.*, 2004; Golding *et al.*, 2016). The ASFV BA71 Δ CD2 strain with deletion of the EP402R gene induced strong humoral and cellular responses, and conferred solid protection against a homologous ASFV strain (Monteagudo *et al.*, 2017). Moreover, this ASFV BA71 Δ CD2 strain induced consistent protection against heterologous ASFVs, such as the E75 strain (Genotype I) or the Georgia 2007/1 strain (Genotype II), as well as the homologous strain. This is the first report of an ASFV vaccine candidate conferred against heterologous ASFV strains.

The effect of gene deletion on ASFV varied according to the strain. The NL gene-deleted European ASFV E70 strain showed complete attenuation in pigs, but no attenuation was observed when using the NL gene-deleted African ASFV strains (Afonso *et al.*, 1998; Neilan *et al.*, 2002). Both TK gene-deleted Malawi and Georgia ASFV strains showed attenuation in pigs, but only the TK gene-deleted Malawi ASFV strain induced a protective immune response (Moore *et al.*, 1998; Sanford *et al.*, 2016).

Additionally, multiple gene deletions were attempted to completely remove any virulence from ASFV. Unfortunately, negative effects on the induction of the protective immunogenicity were observed with the multiple gene-deleted ASFV strains. The dual

deletion of virulence-related genes (DP71L and DP96R) from the ASFV OUR T88/3 strain showed an attenuation, but the protective efficacy for immunization against ASFV was only 60% in the immunized pigs, despite the parent ASFV OUR T88/3 showing 100% protection (Abrams *et al.*, 2013). Deletion of the 9GL (B119L) gene and the MGF 360/505 gene cluster from the ASFV Georgia strain showed high attenuation, but this mutant strain could not induce protective immunity against a virulent ASFV challenge in pigs (O'Donnell *et al.*, 2017). Interestingly, the single gene-deleted ASFV Georgia strain (of either the 9GL gene or MGF 360 gene cluster) was not only attenuated but also protected animals against the virulent ASFV Georgia strain (O'Donnell *et al.*, 2015^{a,b}).

Another important consideration is the duration of the protective effect of the ASF vaccine. ASF LAVs have demonstrated protection following a short immunization protocol, when pigs were challenged a few weeks after the first immunization (Sanchez-Cordon *et al.*, 2017; Sanchez-Cordon *et al.*, 2018). In contrast, domestic pigs immunized with the naturally attenuated ASFV OURT88/3 strain and an attenuated ASF BeninΔMGF strain were not protected from the challenge of virulent ASFV Benin 97/1 strain at 130 days post-immunization (Sanchez-Cordon *et al.*, 2020). Considering these facts, the induction of long-term protection in the development of the ASF vaccine is one of the most important challenges to be solved. Furthermore, the mechanism by which immunological memory induces long-term protection against ASF needs to be elucidated.

Safety of ASF LAVs: The safety of LAVs is a major issue because any remaining virulence may prove unhealthy for the recipient, even though their protective efficacy is high (Teklue *et al.*, 2020). In addition, LAVs may recover their virulence. The safety of ASFV LAVs needs to be examined for virulence, especially for viral burden in vaccinated animals, by assessing the characteristics of the inoculated animals. Reports have suggested that LAVs can develop chronic ASF infection in LAVs-inoculated pigs. Manso-Ribeiro *et al.* (1963), reported that an attenuated Portuguese ASFV strain, created via several cell culture passages, induced chronic ASFV infection upon administration. ASFV/NH/P68 (a naturally attenuated ASFV isolate) induced a chronic infection characterized by viremia, late fever, and high levels of anti-ASFV-specific antibodies with a gammaglobulinemia in 25%–47 % of inoculated pigs (Leitao *et al.*, 2001). Moderately attenuated ASFV isolates induced immunopathological lesions, including hypergammaglobulinemia, and caused systemic immune activation involving increased numbers of macrophages, activated B cells, and CD8⁺ T cells (Pan *et al.*, 1970; Takamatsu *et al.*, 1999). Weak side effects of fever and joint swelling were observed in pigs inoculated with a potential ASFV vaccine candidate, OUR T88/3 (King *et al.*, 2011). The side effects varied with the ASFV strain, rather than with the inoculation dose. Moreover, the side effects of LAVs could vary according to the host immune status. Attenuation was observed in pigs inoculated with ASF LAVs, but the virulence of LAVs was retained in specific pathogen-

free pigs (King *et al.*, 2011; Lacasta *et al.*, 2015). This suggests that ASFV LAVs require further safety evaluations before they can be successfully administered to piglets and sows with weak immune status.

Another potential risk is that a virulent ASFV strain could eclipse the attenuated homologous strains *in vivo*. Titov *et al.* (2017), reported that the immunized pigs with the attenuated ASFV Congo strain remained susceptible to the parent virulent strain, and a recrudescence of virulent ASFV strain may occur at a late stage post-infection in the immunized pigs, at least 28 days post challenge. This provides a great incentive for the intensive investigation of the biosafety of ASFV LAVs.

ASF LAVs could induce effective protection against an ASFV challenge, but residual pathogenicity and potential chronic infection due to viral persistence are important issues to overcome. Fortunately, current advanced technologies, such as functional and comparative genomics, have enabled the discovery of specific genes associated with ASFV virulence and host range. This information has contributed to the development of efficacious ASF LAVs by genetic engineering. However, the challenge remains to identify a specific attenuating target in ASFV, which can safely and efficiently induce protective immunity. The immunopathological sequelae of ASF LAVs are important for the safe development of LAVs, but the mechanisms and virus-host interactions associated with ASF LAVs are unclear. Therefore, it is important to evaluate the safety of the ASF LAV candidates. In addition, it is unknown whether persistent ASF LAV infection is associated with long-lasting protection against an ASFV challenge.

Factors to be considered in establishing ASF LAVs using stable cell lines: To use LAVs as commercial ASF vaccines, it is to be considered whether efficient production of LAVs using stable cell lines should be considered. Primary porcine macrophages and monocytes derived from peripheral blood or tissues, such as the lungs and bone marrow, have been used for the isolation and culture of ASFVs, including ASF LAVs. There are several disadvantages to the production of LAVs as vaccines using primary porcine cells. Obtaining primary porcine cells is labor intensive and the standardization of primary porcine cells is difficult because they can be affected by breed, age and other conditions of the animals. In addition, there is a possibility of contamination by other animal pathogens derived from the source animals. Furthermore, concerns regarding animal welfare need to be considered.

Therefore, it is necessary to adapt LAVs to an established cell line for the efficient production of LAVs. There are some factors to be considered in the production of LAVs using cell lines. First, LAVs should be grown in established cell lines and not in swine macrophages. If LAVs are grown in the swine macrophages, LAVs can proliferate and become pathogenic in pigs and also retain the potential of LAVs to recover their virulence. Krug *et al.* (2015), reported that ASFV-G lost its ability to proliferate in swine macrophages after adaptation in Vero cells and

showed complete attenuation in domestic pigs, even though its protective efficacy was not high. ASFV-G- Δ I177L, an attenuated strain of the ASFV Georgia strain, can replicate in primary swine macrophages but its left variable region (LVR) gene-deleted strain, ASFV-G- Δ I177L/ Δ LVR adapted to a stable cell line, Plum Island porcine epithelial cells (PIPEC) and its immunogenicity and attenuation were well maintained even though it could grow in primary swine macrophages (Borca *et al.*, 2021). Therefore, more studies are necessary to develop ASF LAVs that can grow only in stable cell lines and not in primary swine macrophages, while maintaining their protective efficacy and attenuation.

In addition, modification of the ASFV genome may occur during serial passaging of LAVs in the cell lines, and this modification could alter the attenuation of virulence. Approximately 10% to 15% of viral genome deletions occurred during adaptation of the ASFV Georgia strain in Vero cells (Krug *et al.*, 2015). Three MGF110 genes of L60 were deleted during adaptation in Vero cells (Pires *et al.*, 1997), and the Vero cell-adapted ASFV Georgia strain had deletions in multiple MGF genes (Krug *et al.*, 2015). Borca *et al.* (2021) reported that no alteration was observed in the viral genome of ASFV-G- Δ I177L/ Δ LVR even after 30 serial passages in PIPEC. The genome modification of LAVs may change their safety, immunogenicity, and protective efficacy. Therefore, it is necessary to check whether genomic modification of LAVs occurs during the production of LAVs in the stable cell lines.

There are few studies on the productivity of LAVs as ASF vaccines, and there is a need for studies on the efficient and safe production of LAVs as an ASF vaccine.

Parameters affecting vaccine efficacy

The efficacy of a vaccine depends on vaccination parameters, including a specific viral antigen, delivery vehicle, dose, route, and immunization schedule (Zhang *et al.*, 2015). The protective efficacy of a vaccine is also affected by challenging virus factors, such as different strains of the virus, challenge dose, route of inoculation, and host animal factors (Sanchez-Cordon *et al.*, 2017). Low doses of the 9GL-deleted ASFV Georgia strain conferred partial protection at 21 and 28 days post-vaccination against a homologous challenge, whereas high-dose inoculation induced complete protection at 28 days post-vaccination (O'Donnell *et al.*, 2015^b). Inoculation via an intranasal route of low and moderate doses (10^3 and 10^4 tissue culture infectious dose 50% (TCID)₅₀) of the low-virulent ASFV OURL88/3 strain provided complete protection against a homologous challenge, but intramuscular inoculation of this ASFV strain conferred only partial protection (50%–60%) (Sanchez-Cordon *et al.*, 2017).

In addition, vaccine efficacy may vary depending on the status of the host, including the age and weight of the pig, breed, and breeding conditions, including conditional, specific-pathogen free, and inbred animals (Sang *et al.*, 2020). An important issue is the safety of LAVs, with respect to host immune status. Therefore, the safety of ASF LAVs requires a thorough

investigation of vaccinated animals, including piglets and sows.

What mainly affects the efficacy of the ASF vaccine needs to be tested across all ASFV strains, such as those of European origin, African origin, highly virulent, and moderately virulent ASFV.

Subunit vaccines and attenuated ASF LAVs can induce a greater or lesser degree of resistance to a homologous ASFV strain challenge, but rarely to a heterologous ASFV strain challenge (Mebus and Dardiri, 1980; Hamdy and Dardiri, 1984; King *et al.*, 2011; Lacasta *et al.*, 2015; Mulumba-Mfumum *et al.*, 2016). There is strain diversity and variation among the ASFVs. Therefore, it is critical to develop a subunit vaccine using relevant ASFV antigens or ASF LAVs that can overcome the antigenic diversity of ASFV to be a successful ASF vaccine.

The genotyping of ASFV depends on the analysis of the nucleotide sequences of a few distinct genetic loci that can demonstrate variability among ASFV isolates. Typing of ASFV based on the p72 capsid protein gene may provide broad inter-genotypic grouping, and concurrent analysis of the central variable region using tandem repeats of the 9RL/B602L gene could add intra-genotypic grouping (Bastos *et al.*, 2003; Lubisi *et al.*, 2007). However, ASFV genotyping based on the p72 capsid protein gene did not fully correlate with cross-protection against a heterologous ASFV challenge. This result indicates that p72 capsid protein gene-based genotyping may have limited value in providing protection from heterologous ASFV strains (Malogolovkin *et al.*, 2015^a). Further investigation is required to identify a new genotyping method for ASFV that could predict the protective efficacy of ASF vaccine candidates against heterologous ASFV strains.

There is distinct antigenic typing of ASFVs based on hemagglutination inhibition (HAI), which can be determined by the analyzing the amino acid sequences of CD2v and C-type lectin proteins (Detray, 1957; Malogolovkin *et al.*, 2015^b; Vigario *et al.*, 1970; Pan *et al.*, 1974), and eight ASFV HAI serogroups have been identified (Vishnjakov *et al.*, 1991; Balyshhev *et al.*, 1995). This HAI typing subdivides the ASFV p72 genotype 1 into ASFV serogroups 1, 2, and 4 (Malogolovkin *et al.*, 2015^{a,b}). Furthermore, several studies have reported that protective immunity against ASFV infection appears to be HAI serogroup-specific. If ASFVs belong to the same HAI serogroup, they could provide cross-protection against one another (Vishnjakov *et al.*, 1991; Sereda *et al.*, 1992; Balyshhev *et al.*, 1995). In addition, multiple ASFV antigens based on the HAI serogroup might serve to enhance protective immunity. CD2v and C-type lectin proteins are sufficient for the specificity of the ASFV HAI serogroup (Malogolovkin *et al.*, 2015^b).

In conclusion, vaccination is an efficient strategy to control ASF, and much effort has been undertaken to develop an effective ASF vaccine. Early attempts at an ASF vaccine using inactivated ASFV failed to protect pigs from virulent ASFV strains. The neutralizing antibodies in humoral immunity were identified to be ineffective in inducing protective immunity against ASFV infection. Conversely, cellular immunity, especially cytotoxic CD8⁺ T cells, may be important for

immunity in the clearance of ASFV and protection from ASFV infection. To overcome the limitations of the traditional inactivated vaccines, the identification of ASFV protective antigens has been investigated, and an ASF subunit vaccine (including specific protective ASFV antigens with a delivery system) has been developed to protect animals from ASFV infection. Significant research progress in ASFV infection biology has provided opportunities for ASF vaccine development. Comparative and functional genomics of ASFV have provided insight into virulence and host range, which can now be used to rationally design and engineer ASFV LAVs. Although many obstacles remain to be overcome to develop an effective ASF vaccine, advanced biotechnology and genetics will contribute to the development of the next generation of ASF vaccines. When an efficient ASF vaccine is eventually developed, the ASF vaccines may not only control the disease, but also reduce the risk of further spread, which would contribute to reducing the economic losses caused by ASF.

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