Characterization of recombinant Modified Vaccinia virus Ankara delivering African swine fever virus proteins

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Für Smilla

Freiheit ist für dich durch nichts ersetzbar,
Widerspruch ist dein kostbarstes Gut.
Liebe macht dich unverletzbar
Wie ein Bad in Drachenblut.

Du bist ein Riese, Max Lied von Reinhard Mey

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I. ABBREVIATIONS

°C	Celsius
AHSV	African horse sickness virus
AmpR	ampicillin-resistence gene
ASF	African swine fever
ASFV	African swine fever virus
BHK cell	baby hamster kidney cell
BRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
BSL	biosafety level
CAM	chorioallantois membrane
CEF	chicken embryo fibroblast
CO_2	carbon dioxide
CSF	classical swine fever
CVA	chorioallantois vaccinia virus Ankara
DF-1	chicken embryo fibroblast cell line DF-1
DIVA	differentiating infected from vaccinated animals
D-MEM	Dulbecco`s modified Eagle`s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpi	days post infection
EDTA	ethylene-diamine tetraacetatic acid
EFSA	European Food Safety Authority
ELISpot	enzyme-linked immunosorbent spot assay
Endo-H	endoglycosidase H
ER	endoplasmic reticulum
EURL	European Reference Laboratory
FBS	fetal bovine serum
FCS	fetal bovine serum
FLI	Friedrich-Loeffler-Institute
GOI	gene of interest
HaCat	human keratinocyte

HAD	haemadsorption phenomenon
HEK cell	Human embryonic kidney cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
hpi	hours post infection
i.m.	intra muscular
IEV	intracellular enveloped virion
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
kb	kilo base
kDa	kilodalton
l	liter
LAV	Live attenuated vaccines
LB	lysogeny broth
M	Mol
MCS	multiple cloning site
MEM	minimal essential medium
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimol
MOI	multiplicity of infection
mpi	minutes post infection
MV	mature virion
MVA	Modified Vaccinia virus Ankara
NCLDV	nucleoplasmic large DNA viruses
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nM	nanometer
NYVAC	New York attenuated vaccinia virus

o/n	over night
OIE	The World Organization for Animal Health
ORF	open reading frame
PBMC	Peripheral Blood Mononuclear Cell
PBS(T)	phosphate buffered saline (tween20)
PCR	polymerase chain reaction
PFU	plaque-forming units
PK15	porcine kidney-15
PNGase F	peptide-N-glycosidase F
PPRV	Peste des petits ruminants virus
RABV	Rabies lyssavirus
rAd	replication-deficient human adenovirus
rMVA	recombinant Modified Vaccinia virus Ankara
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SchwPestV	Schweinepest-Verordnung
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TNF	tumor necrosis factor
UV	ultraviolet
VACV	Vaccinia virus
VARV	Variola virus
WSL	wild boar lung derived cells

II. INTRODUCTION

African swine fever is one of the most feared infectious diseases of pigs. The continued spread of the devasting disease throughout Europe, China and the Russian Federation has raised awareness of the threat to the global swine industry and food security. The disease is caused in pigs and wild boars by African swine fever virus (ASFV) infection and often leads to rapid death of almost all infected animals. The supply of pork products to the human population is at risk, especially since the disease broke out in China in 2018 where half of the world's pigs are farmed. The lack of an effective vaccine complicates the prevailing situation. The only current effective methods to control the disease, are quarantine of the affected area and slaughter of infected animals. The World Organization for Animal Health (OIE) has classified African swine fever as a notifiable disease. International cooperation is needed to control the disease and its spread. The race to develop an effective vaccine has begun (Dixon, Sun, and Roberts 2019; Galindo and Alonso 2017; OIE WORLD ORGANISATION FOR ANIMAL HEALTH 2020).

To date, there are no licensed vaccines against ASFV, and the development of those, faces challenges such as the high complexity of the virus paired with the lack of a licensed, effective continuous cell line for virus isolation. Live attenuated vaccines (LAV) bear the risk of phenotypic changes up to reversion to virulence following genetic modifications of ASFV. The ability to immunize wild boar through oral administration would be desirable, as well as the ability to distinguish infected from vaccinated animals (DIVA) (Turlewicz-Podbielska et al. 2021).

Modified vaccinia virus Ankara (MVA) is a highly attenuated and replication-deficient vaccina virus offering an outstanding safety profile and a strong immunogenicity. This makes MVA one of the safest and most advanced platforms for the development of effective vaccines against infectious diseases. The aims of this study were the generation and characterization of new recombinant MVA viruses, expressing four selected ASFV proteins, which are promising to overcome some previous hurdles in vaccine development.

III. LITERATURE REVIEW

1. African Swine Fever Virus

The African swine fever virus is the only member of the genus *Asfivirus* of the family *Asfarviridae*, which belongs to the group of the order Megavirales. A group of eukaryotic viruses, each composed of a double-stranded DNA genome ranging in size from 100 kb to over 2.5 megabases (Alonso et al. 2018; Iyer, Aravind, and Koonin 2001; Iyer et al. 2006; Koonin and Yutin 2019; Colson et al. 2013). ASFV is the only known DNA arbovirus (anthropod borne virus) that circulates in a sylvatic cycle between natural reservoirs (Warthogs *Phacochoerus africanus*, bush pigs *Potamochoerus larvatussoft* and ticks of the genus *Ornithodoros*) (Gaudreault et al. 2020). The virus genome contains a covalently closed double-stranded DNA molecule of 170 to 193 kb. Depending on the virus strain it includes between 150 and 167 open reading frames (ORFs) (Yáñez et al. 1995; Dixon et al. 2013). Like other nucleoplasmic large DNA viruses (NCLDV), ASFV encodes for around 150-200 viral proteins, including 68 structural and more than 100 nonstructural proteins (Alejo et al. 2018; Wang et al. 2021). The multi-layered structure of the ASFV particle, with a diameter of about 200 nM, appears in an overall icosahedral morphology. On the inside, an internal core constituted by the central genome-containing nucleotide surrounded by a thick protein layer called the nuclear envelope. An inner lipid membrane enveloping the core and the capsid, which represents the outermost layer of the intracellular virions. Finally, an outer envelope generated by cleavage from the plasma membrane constitutes the extracellular virions (Salas and Andrés 2013).

ASFV mainly infects swine macrophages and monocytes. The replication cycle starts with the entry in the host cell most likely through macropinocytosis and clathrin-mediated endocytosis which is primarily mediated through an unknown receptor. Phagocytosis, receptor-mediated endocytosis and Fc-receptor mediated entry have also been suggested entry mechanisms (Sánchez, Pérez-Núñez, and Revilla 2017; Galindo and Alonso 2017; Gaudreault et al. 2020). The virus is then transported by the endosomal pathway, through macropinosomes or early endosomes (1-30 mpi) to late endosomes (30-90 mpi). Increasing acidification in the endosomal environment is essential for the

desheathing of the ASFV outer membrane and the capsid. With a pH below 5, the ASFV inner envelope and the endosomal membrane ultimately fuse and the viral core is released into the cytoplasm (Hernáez et al. 2016; Gaudreault et al. 2020). ASFV uses a temporal gene expression strategy like other large DNA viruses (Dixon et al. 2013). Early, intermediate, and late genes are activated by the viral RNA polymerase to initiate the various cycles of replication. The early gene expression takes place 4-6 hpi and 6-8 hpi the viral DNA polymerase (G1211R) initiates the ASFV genome replication. ASFV replication occurs with an initial brief replication phase in the cell nucleus and then mainly takes place in viral factories in perinuclear region of the cytoplasm (Gaudreault et al. 2020; Simões et al. 2019). At 8-16 hpi the expression of intermediate and late genes leads to the production of structural proteins that are assembled into the virion (Karger et al. 2019). Virions are transported to the cell membrane for budding. Microtubules and kinesin play an essential role in cellular transport and support this process. While budding through the cell membrane, the virus acquires its host derived outer envelope (Jouvenet et al. 2004).Overall, the entire infection cycle, from attachment and entry to budding of mature ASFV particles, is finished within 24 hpi (Muñoz-Moreno et al. 2015).

1.1. African Swine Fever

African swine fever (ASF) has its roots in Africa but was transmitted to Georgia in 2007. From there, the disease spread to Russia in 2007 in the Transcaucasian region. As of 2014, the virus entered the European Union and affected a few countries, including, Latvia, Poland, the Czech Republic and Belgium. In 2018, the disease reached China, the world's largest pig producer, and affected also several other Asian countries. In September 2020, the first ASF cases were found in wild boars in Germany, along the Polish border of Brandenburg. The first German domestic pigs infected with ASFV were found in Germany 2021 (Dixon, Sun, and Roberts 2019; Sauter-Louis et al. 2021; FLI Bundesforschungsinstitut für Tiergesundheit 2022).

The virus is transmitted through both direct animal contact and indirect contact. Transmission via vectors such as the African leather tick (*Ornithodorus. moubata*) in Africa does not play a role in Central European countries. In particular, transmission through animal products, by-products and food waste

has contributed to the spread of the disease (Ravaomanana et al. 2010; Guinat et al. 2016; Olesen et al. 2017; Jori et al. 2013; Sánchez-Vizcaíno et al. 2015; Dixon et al. 2019; Jori and Bastos 2009; Gaudreault et al. 2020).

Depending on the virus isolate, severe symptoms occur after an incubation period of approximately 4 days and usually lead to death within a week. Symptoms include high fever, anorexia, gastrointestinal and respiratory symptoms, skin lesions, petechial haemorrhages on serous skins and haemorrhagic lymphadenitis. Temporary and chronic courses of disease can be caused by less virulent isolates. A distinction between classical swine fever (CSF) and African swine fever is not possible based solely on clinical symptoms (Sánchez-Vizcaíno et al. 2015; Dixon, Sun, and Roberts 2019).

Control of African swine fever in Germany is carried out according to the "Verordnung zum Schutz gegen die Schweinepest und die Afrikanische Schweinepest, Verordnung über hygienische Anforderungen beim Halten von Schweinen and VO EU 2021/605". Stamping out strategies are based on extensive veterinary police measures on suspected and outbreak scenarios including far-reaching authorizations of the authorities such as investigations, traffic restrictions for animals and people, cleaning and disinfection or disinfestation and blocking and culling measures. There are also extensive regulations for prophylactic hygiene measures in pig farms. A major disadvantage in the control of ASFV, compared with the control of classical swine fever, is that there is no approved vaccine available. (FLI Bundesforschungsinstitut für Tiergesundheit 2022).

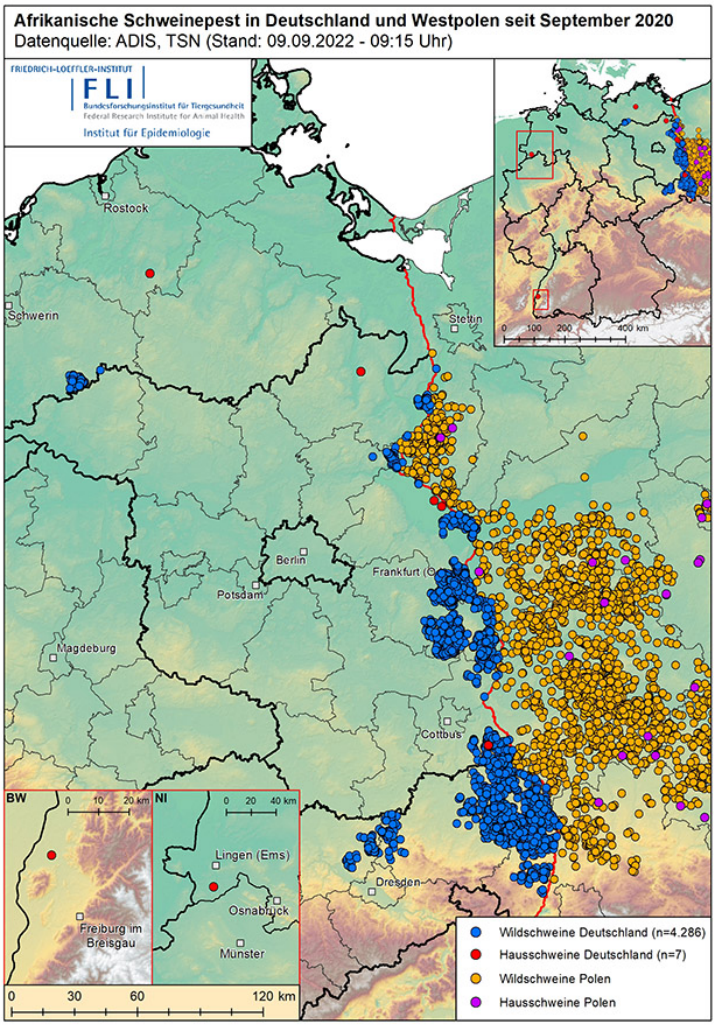

Figure 1: ASF cases in Germany and west Poland from September 2022. Blue dots on the map mark outbreaks of African swine fever disease in wild boars in Germany, while the red dots represent domestic pigs infected with ASFV. (Reference: Friedrich-Loeffler-Institut)

1.2. CD2v (EP402R)

ASFV CD2v is a type I transmembrane protein encoded by ORF EP402R, it consists of 360-407 amino acids and has a predicted molecular weight of approximately 42-46 kDa. CD2v is located on the external envelope of the virus particle and is expressed on the surface of infected macrophages. In literature various terms have been used for its identity: pEP402R, CD2v, CD2-like or 8-DR (Borca et al. 1998; Goatley and Dixon 2011; Kay-Jackson et al. 2004; Rodríguez et al. 1993). The viral glycoprotein CD2v has a homologous sequence to the host adhesion molecule CD2, expressed on T-lymphocytes and natural killer cells. CD2v possesses an extracellular N-terminal and a cytosolic C-terminal region. The N-terminal region contains two immunoglobulin like domains, while the c-terminal region shares no clear sequence homology with the cytosolic domain of the cellular CD2 (Rodríguez et al. 1993). CD2v plays an important role in immune evasion and virus dissemination. It is required to bind extracellular virus particles to erythrocytes and induce the hemadsorption phenomenon (HAD), a rosette-like binding of erythrocytes around the infected cell. Both effects are most likely caused by the extracellular N-terminal domain of CD2v (Borca et al. 1998; Rodríguez et al. 1993). Interestingly, while requiring CD2v for successful induction of HAD, the ASFV-expressed EP153R gene expresses a C-type lectin, which induces and stabilizes the interaction between CD2v and its corresponding host cell receptor (Galindo et al. 2000). ASFV infection of cultured peripheral blood mononuclear cells (PBMC) has been shown to inhibit mitogen-dependent proliferation of infected lymphocytes. This effect could not be observed when ASFV deletion mutants, lacking the gene encoding CD2v, were used. This indicates immunomodulatory properties of CD2v (Borca et al. 1998). Burmakiner et al. 2016 showed that antibodies from infected pigs have the ability to inhibit the HAD of red blood cells around infected cells and correlate with cross-protection. CD2v along with the C-type lectin (EP153R) play a key role, suggesting a possible role of CD2v protein in protection (Malogolovkin et al. 2015; Burmakina et al. 2016).

As mentioned above, the cytoplasmic domain of CD2v differs from that of the mammalian CD2. It contains variable numbers of proline-rich repeats and binds to the SH3 domain of the host protein SH3P7. This mediates binding to actin, which plays a role in intracellular vesicle transport. During intracellular transport

from the endoplasmic reticulum via the Golgi apparatus to the cell surface, CD2v is glycosylated and shows an increase in predicted molecular mass from about 42 kDa to about 100 kDa. Digestion experiments with endoglycosidase-H and endoglycosidase-F confirmed this process (Kay-Jackson et al. 2004). Related to vesicular transport, CD2v also interacts with the AP-1 factor of the Golgi apparatus (Pérez-Núñez et al. 2015).

The full effects of CD2v on virulence throughout an ASFV infection cycle have not yet been conclusively elucidated. Deletion of EP402R from European isolate BA71 resulted in total virus attenuation *in vivo* and was as deletion mutant LAV capable of protecting pigs against the homologous BA71 virus (Monteagudo et al. 2017), while deletion of CD2v from more virulent strains such as Malawi and Georgia 2007 did not significantly affect virulence (Borca et al. 1998; Borca, O'Donnell, et al. 2020). Interestingly, deletion mutants (lacking CD2v) resulted in different infection phenotypes after infection of pigs, depending on the parental viral strain (Chaulagain et al. 2021). CD2v has been shown to be essential for replication in the tick midgut as well as for generalization of infection in the tick (Kleiboeker et al. 1998; Rowlands et al. 2009).

Recent data indicate that CD2v plays an important role in interferon activation. CD2v induces activation of NF-kB in infected macrophages and lymphocytes, resulting in transcription of IFN-β. IFN-signaling induces apoptosis in swine lymphocytes and macrophages (Chaulagain et al. 2021).

1.3. pp220

The structural protein pp220 encoded by ORF CP2475L, is one of two polyprotein precursors whose mature products co-assemble into the core shell. The ORF consists 8148 bp and the protein has a molecular weight of about 220 kDa (Simon-Mateo, Andres, and Vinuela 1993). The core shell consists of an approximately 30 nM thick protein layer, encompassing the central nucleotide and is defined as an independent domain of the virus core (Andrés, Alejo, et al. 2002; Andrés, Simón-Mateo, and Viñuela 1997). It is essentially composed of the mature products of the polyproteins pp220 and pp62. Both polyproteins are processed by the viral cysteine proteinase pS273R in an ordered cascade of cleavages which occurs at the consensus sequence “GGX” (Simón-Mateo et al. 1997; Simon-Mateo, Andres, and Vinuela 1993). The eight main components of

the nuclear envelope include the following proteins: p15, p35 and p8, derived from the polyprotein precursor pp62, and p5, p34, p14, p37 and p150 from the polyprotein precursor pp220 (Alejo et al. 2018). These components are present in equimolecular amounts in the mature virus particle and collectively account for over a third of the total mass of the virion (Salas and Andrés 2013). The viral protease is encoded by the S273R gene and belongs to the SUMO-1 specific and related protease family, which also includes the vaccinia I7 and adenovirus proteases (Andrés et al. 2001; Li and Hochstrasser 1999). It has been shown to process the polyproteins reliably, as recombinant purified pS273R enzyme, in assays using transfected cells and extracts from transfected cells (Andrés et al. 2001; Rubio et al. 2003). Among DNA viruses is this mechanism of gene expression unique (Salas and Andrés 2013).

Interestingly, repression of the polyprotein pp220 leads to empty icosahedral particles in infected cells. The virus core and nucleotide, including viral DNA, are absent, while the outer layers from the inner envelope and icosahedral capsid remain intact. Budding of coreless particles occurs. Remarkably, the products of polyprotein pp62 and the protease pS273R, both of which are normally expressed, are not incorporated into the virion, because their processing is blocked (Andrés, García-Escudero, et al. 2002; Salas and Andrés 2013). The described study indicates an independent process between core maturation and the construction of the icosahedral capsid on the inner envelope. Finally this suggests that polyprotein pp220 plays a central role in core assembly, as well as genome encapsulation and condensation. (Salas and Andrés 2013).

1.4. pK145R

PK145R is a mostly uncharacterized virion protein encoded by the ORF K145R, with a predicted molecular weight of 17,2 kDa (Yáñez et al. 1995; Alejo et al. 2018). Various studies have shown that the K145R gene is highly expressed in ASFV infected pigs or cell lines. It has been shown to be among the top 5 intracellularly expressed gene products of two ASFV-infected primate cell lines, Vero and HEK-293 cells. The product of K145R was observed to be the most prominent viral protein expressed in ASFV-infected wild boar lung cells (WSL) (Kessler et al. 2018). Using a transcriptome analysis of infected pigs whole

blood samples, K145R showed an abundant expression at the mRNA-level (Jaing et al. 2017). In contrast, the protein derived from K145R ranked only at position 21 among the ASFV-encoded proteins in a proteome analysis of a virion (Alejo et al. 2018).

PK145R is a late expressed non-structural protein (Yáñez et al. 1995) with a diffuse distribution in the cytoplasm of the infected cell after about 7 hpi and is not detectable in the purified virion (Hübner 2018). As demonstrated with a deletion mutant, pK145R is not essential for ASFV replication *in vitro* (Hübner 2018). Additionally, the protein product of K145R is immunogenic. It was identified by screening viral cDNA expression library in convalescent pigs (Kollnberger et al. 2002). Therefore, pK145R might be used as a potential negative serological marker in diagnostic tests for DIVA (Rathakrishnan et al. 2021). In a vaccine study conducted by Rathakrishnan and colleagues, a double deletion mutant of genotype II isolate Georgia 207/1 with the deleted genes DP148R and K145R was examined. In combination with the deletion of K145R, a delay in the onset of clinical signs of ASF could be observed. However, the severity of the symptoms did not decrease and the pigs were culled after 10-12 days post infection (Rathakrishnan et al. 2021).

1.5. pI73R

pI73R is an uncharacterized immunogen encoded by the ORF I73R, with a predicted molecular weight of 8,5 kDa (Alejo et al. 2018). ORF I73R is expressed during the early phase of viral infection at high levels, detected with northern hybridization analysis (Rodriguez, Salas, and Viñuela 1992). In 2018, pI73R was detected by mass spectrometry and ranked among the top 20 most abundant expressed proteins in WSL and HEK-293 cells (Kessler et al. 2018).

I73R was part of an experiment in which immunogenic ASFV proteins in recovered pigs were identified using a gamma interferon ELIspot assay and then incorporated into adenovirus and MVA vectors for subsequent immunization and challenge experiments in pigs. Despite the observation that I73R protein induced the secretion of IFNγ in all three inbred pig lines, a cellular immune response could not be detected following immunization of pigs with viral vectors (Netherton et al. 2019).

1.6. Treatment and Prevention Strategies

The African swine fever pandemic has already spread across several continents. Worldwide, disease control is based on early detection of infected animals through rapid diagnostic measures and subsequent culling of entire herds (Busch et al. 2021). The World Organization for Animal Health (OIE) and the European Reference Laboratory (EURL) provide strict recommendations for laboratory diagnostics of ASF (Wozniakowski et al. 2017). In addition, biosecurity protocols are in place through national disease control regulations to prevent disease transmission to disease-free farms (Busch et al. 2021). Nevertheless, the virus is spreading because these strategies are apparently insufficient and are not feasible in some countries due to lack of resources (Bosch-Camos, Lopez, and Rodriguez 2020; European Food Safety et al. 2021; Mauroy et al. 2021). In addition, measures such as preventive culling of entire animal herds are met with big incomprehension by livestock farmers (Busch et al. 2021). The availability of an effective and licensed vaccine seems essential to control the ASF pandemic, and work is underway worldwide to develop an ASFV vaccine by various research groups.

However, there are several concerns when developing a functional vaccine against ASFV. The complex structure of the virus complicates the construction of an effective vaccine. ASFV encodes more than 80 structural proteins (Alejo et al. 2018) that play a role in host cell attachment, entry, and replication (Sanchez et al. 2012). It is able to replicate in the highly unfavorable environment within macrophages (Redrejo-Rodríguez and Salas 2014), allowing it to spread rapidly and cause clinical symptoms in pigs. It accomplishes this by avoiding detection and inhibiting host defense mechanisms. An important point here is the inhibition of the induction of IFN type I in response to infection (Reis et al. 2020). In addition, ASFV encodes anti-apoptotic proteins (e.g., A197L, EP153R), preventing destruction of the infected host cell (Brun et al. 1996; Galindo et al. 2008). To evade the host immune response, ASFV takes over control of important host cell mechanisms. Activation of proinflammatory factors and cytokines, as well as cellular transcription factors, are among those taken over by the virus, and are controlled by expression of ASFV-encoded genes such as A238L (Granja et al. 2006; Turlewicz-Podbielska et al. 2021).

The lack of a licensed and established macrophage cell line poses a problem in development and especially commercial production for some vaccine platforms. Live attenuated vaccines are among the vaccine candidates whose development is hampered by the fact that primary macrophages are difficult to produce, prone to contamination, and at the same time expensive. When virulent field isolates are adapted to another cell line (MS or Vero cells), there is a risk of point mutations in the viral genome, which can lead to phenotypic changes in the virus (Portugal et al. 2020; Dixon et al. 2019; Krug et al. 2015).

Developing a vaccination strategy for wild boar could significantly reduce the spread of the ASF. Warthogs in Africa and wild boars in Europe are very susceptible to ASFV and, particularly in Europe responsible for the spread of the disease and its introduction into domestic pig populations through direct or indirect transmission (Turlewicz-Podbielska et al. 2021). According to the European Food Safety Authority (EFSA), pure hunting or hygienic culling does not lead to a sufficient reduction in wild boar populations (Bieber and Ruf 2005). In the past, classical swine fever could be controlled by oral administration of a functional vaccine to wild boars (Iacolina et al. 2021). A vaccine against ASFV for wild boar would be desirable. In addition, the vaccine should be compatible with the DIVA strategy. This principle relies on distinguishing the antibody response elicited by the vaccine from that of the wild-type pathogen (Uttenthal et al. 2010). High hopes for such a vaccine lie on vector and subunit vaccine platforms. Viral vector vaccines in particular offer an optimal option for vaccine markers due to their viral encoded immunogens (Gaudreault and Richt 2019).

Various vaccine platforms (subunit, virus-vectored, DNA, inactivated and live-attenuated ASFV vaccines) are being researched for the development of a functional ASFV vaccine. The most promising vaccine platforms appear to be live attenuated vaccines, as well as subunit and virus-vector vaccines (Gaudreault and Richt 2019).

Vector vaccines include one or more genes from a pathogenic microorganism in their viral genome that encode protective antigens against a particular disease (Turlewicz-Podbielska et al. 2021). A replication deficit or removed virulence genes of the vector genome ensure its safety. Vector vaccines elicit cell mediated as well as humoral immune responses and are compatible with

the DIVA strategy (Gaudreault and Richt 2019). Feng et al. 2020 (Feng et al. 2020) showed that with a PRV-attenuated strain expression of ASFV-CD2v-protein (PRV-ΔgE/ΔgI/ΔTK-(CD2v)) in mouse models, a specific humoral and cellular immune response against CD2v could be induced (Feng et al. 2020). Vectorized antigen cocktails were tested in pig models. Strong antigen-specific humoral and cellular immune responses were induced by adenovirus with the ASFV antigens p30 + p54 + p72 + pp62, and ASFV genes A151R + B119L + B602L + EP402RΔPRR + B438L + K205R + A104R. Another study was based on the MVA vector with the following ASFV antigens: p72, CD2v, and C-type lectin. Antigen-specific antibodies were not induced, but a T-cell response could be detected in immunized pigs for each individual antigen. None of these vectorized immunization studies have yet been tested against virulent virus challenge (Lokhandwala et al. 2016; Murgia et al. 2019; Lopera-Madrid et al. 2017). Goatley and colleagues (Goatley et al. 2020) performed a promising study using a pool of ASFV genes vectorized from a replication-deficient human adenovirus 5 (rAd) prime and a modified Vaccinia-Ankara (MVA) Boost system. The study tested viral vector pools included antigens, which were selected based on their immunogenicity in pigs. Induction of cellular immune responses and ASFV-specific antibodies were found. 100 % of pigs were free of fatal disease following immunization with one of these pools and challenged with a usually lethal dose of virulent ASFV. The vaccination comprised eight virally vectored ASFV genes (B602L, B646L/p72, CP204L/p30, E183L/p54, E199L, EP153R, F317L, and MGF505-5R) (Goatley et al. 2020).

Most vaccines in current trials are based on live attenuated technologies. Live attenuated vaccines (LAVs) can be based on virulent strains that have been attenuated by active deletion of virulence factors, or on naturally occurring ASFV strains with reduced virulence (Turlewicz-Podbielska et al. 2021). A promising new LAV vaccine candidate was proposed by Borca et al. 2020 (Borca, Ramirez-Medina, et al. 2020). The previously unknown ASFV I177L protein with unknown function has been shown to confer sterile immunity against Eurasia strain when this gene is deleted from ASFV. The highly pathogenic strain ASFV Georgia (ASFV-G) was used to develop this LAV and resulted in the virus (ASFV-G ΔI177L), which was completely attenuated in pigs. According to this study, all intramuscularly (i.m). vaccinated animals produced

a virus-specific antibody response and were protected after challenge with the parental ASFV-G strain over an observation period of 28 days (Borca, Ramirez-Medina, et al. 2020). One year later, Borca et al. (Borca et al. 2021) conducted another study in which the described vaccine was administered via the oronasal route and resulted in similar efficacy to i.m application. All challenged pigs survived and showed no clinical signs over an observation period of 21 days. Additionally, the infected animals did not shed enough virus to infect naive pigs (Borca et al. 2021).

2. Modified Vaccinia virus Ankara (MVA)

2.1. History of MVA

Modified Vaccinia virus Ankara (MVA) is a highly attenuated vector virus which is derived from the Vaccinia virus strain (VACV) Ankara. MVA was developed through continuous passages on primary chicken embryonic fibroblasts (CEF) and finally ensured a safer vaccine than its predecessor VACV, during the WHO smallpox eradication campaign. VACV Ankara was developed in Turkey and served as a vaccine against human smallpox. However, various side effects, ranging from local to generalized reactions, were known to occur with vaccination of replicating VACV. The most severe side effects, which could lead to death, were caused by post vaccine encephalitis (Mayr 2003; Gilbert 2013; Volz and Sutter 2017). In the 1950s, VACV Ankara was cultivated on chorioallantoic membranes (CAM) from embryonated chicken eggs and renamed the Chorioallantoic Vaccinia Virus Ankara (CVA) in Munich (Herrlich and Mayr 1954). As a result of about 500 serial passages on CEF cells, the ancestor virus became a highly attenuated virus with reduced virulence and an inability to replicate in human and most other mammalian cells. This new virus variant was named Modified Vaccina virus Ankara (MVA) in 1968 (Stickl and Hochstein-Mintzel 1971; Mayr and Munz 1964). MVA was given to the Bavarian State Institute for Vaccines where its suitability for smallpox vaccine production was tested (Stickl and Hochstein-Mintzel 1971). After no serious side effects were found in large vaccination trials with MVA vaccine preparations, it obtained MVA's first marketing authorization as a primary prevaccination in Germany in 1977 (Stickl et al. 1974). Overall, the MVA smallpox vaccine was given to more than 120,000 humans by 1980, remarkably, without any documentation of severe adverse events (Mahnel and Mayr 1994). With the end of the smallpox vaccination program, immunizations with the first licensed MVA vaccine were stopped (Volz and Sutter 2017).

2.2. Taxonomy, Morphology & Viral Life Cycle

The family of poxviridae is divided into the two subfamilies: *chordopoxvirinae* and *entomopoxvirinae.* The subfamily of *chordopoxvirinae* contains the genus of orthopoxviruses, to which the species vaccinia virus (VACV) belongs and MVA is an attenuated strain (variant) of vaccinia virus (Gubser et al. 2004).

Poxviruses are among the largest known DNA viruses. Orthopoxviruses have the appearance of brick-shaped virions under the electron microscope with a size of 250-350 nM x 200 nM (Gelderblom and Madeley 2018). The two-layer lipid envelope consists of an outer and an inner membrane, which enclose a biconcave capsid, as well as two embedded side bodies (Bidgood 2019; Moss 1996a). Inside the capsid is the double-stranded DNA genome with 130-300 kb, which is folded into an s-shaped structure. The nucleoprotein complex with viral enzymes is closely linked to the genome (Susanne Modrow 2022; Knipe et al. 2013; Westwood et al. 1964).

The most distinctive feature of the poxviruses is their ability to replicate entirely in the cytoplasm of an infected cell, despite their DNA genome. To accomplish this, they use their own transcription machinery (Moss 1996b). During replication, two different forms of infectious particles are formed: intracellular mature virions (MVs), surrounded by a single lipid membrane, and extracellular enveloped virions (EVs), which are surrounded by an additional lipid membrane (Smith, Vanderplasschen, and Law 2002; Howley and Knipe 2020) As soon as one of the virions (MV / EV) attaches to the host cell and penetrates the membrane through endocytosis or fusion, the viral core is released to the cytoplasm for uncoating, viral DNA is activated and early genes are transcribed with help of the virus-encoded enzymes located in the virus core. These enzymes include a DNA-dependent RNA polymerase with several subunits, a transcription factor, capping and methylation enzymes and a poly (A) polymerase, all of which make it possible to synthesize translatable mRNAs with typical eukaryotic features (Moss 1996b; Moss and Earl 2001). The early genes encode proteins and transcription factors which are necessary for viral replication and transcription of intermediate genes (Broyles 2003; Moss 1996b). In so-called virus factories, viral progeny DNA is used as a template to transcribe intermediate and late genes (Katsafanas and Moss 2007). Products of these genes are essential for virion morphogenesis and assembly, including structural proteins (Broyles 2003). During morphogenesis and assembly of infectious particles (MVs), newly synthesized early transcription factors, encoded by late genes, are packaged in the virions together with the viral genome (Moss and Earl 2001). The MVs are transported and packaged by two membranes at the Golgi-apparatus to form intracellular enveloped virions (IEVs)

(Hiller and Weber 1985; Schmelz et al. 1994). Finally, IEVs are transported to the periphery and released by fusion with the plasma membrane as EVs (Blasco and Moss 1992; Cudmore et al. 1995; Ward and Moss 2001).

2.3. Advantages and properties of MVA

Poxviruses and especially the prototypical Othopoxvirus VACV have been established as recombinant expression systems in vaccine development after the eradication of human smallpox in 1980. They have many properties that are advantageous for use as vaccine backbones (Fenner 1993; Moss 1996b; Mackett, Smith, and Moss 1982; Panicali and Paoletti 1982). They are able to integrate large amounts of DNA and even foreign genes into their huge genomes (Smith and Moss 1983). Since their complete replication cycle is confined to the host cell cytoplasm, gene expression takes place without any integration of viral DNA into the host genome. This is made possible by tight control of virus-specific transcription and RNA modification systems. Moreover, poxviruses are able to induce strong adaptive immunity by humoral as well as cellular response (Smith and Moss 1983; Perkus et al. 1985; Moss 1996b; Draper, Cottingham, and Gilbert 2013; Kreijtz, Gilbert, and Sutter 2013).

Nevertheless, the use of poxviruses as vector vaccines has repeatedly been associated with serious side effects, especially in immunocompromised individuals (Lane et al. 1969; Redfield et al. 1987). Thus, the recombinant MVA virus was able to establish itself as a viral vector system due to its replication deficiency in cells of mammalian origin and a resulting exceptional safety profile (Sutter and Moss 1992; Volz and Sutter 2017).

The high safety profile of MVA, is due in large part to the fact that the MVA genome is reduced by 15% when compared to its ancestral virus, CVA's, genome (Meyer et al. 1991). In addition, it contains six large deletion sites and several point mutations in the genome, which arose via long-term passaging on CEF cells. (Meyer, Sutter, and Mayr 1991; Antoine et al. 1998; Meisinger-Henschel et al. 2007).

As a result, MVA also lost a significant amount of virulence factors and host-range genes, which are responsible for the host tropism. After infection of a host cell with mammalian origin, the viral replication cycle stops after the formation of immature virions. Cell lines are classified into three groups, according to MVA's ability to replicate within them: permissive cells (e.g. CEF, BHK-21, DF-1), semi-permissive cells (e.g. Vero, MA104, BS-C1, CV-1) and non-permissive cells (e.g. HeLa, HaCaT) (Sutter and Moss 1992; Carroll and Moss 1997; Garber et al. 2009; Okeke, Nilssen, and Traavik 2006; Drexler et al. 1998).The Robert Koch Institute classified MVA as risk group 1 (des Robert Koch-Instituts 2002). MVA, despite its reduced genome, exhibits high genetic stability as evidenced by several independent genomic sequence analyses (Antoine et al. 1998; Esposito).

Due to the six deletion sites and its 178kb long genome, MVA is able to integrate a large amount and even several different sequences of heterologous DNA. This allows a high variability of many foreign proteins and ensures their expression (Antoine et al. 1998; Sutter and Moss 1995; Moss et al. 1996; Drexler et al. 2000). Both early and late proteins are expressed without processing mature virions. MVA infects the host cell and starts its molecular life cycle, which is blocked late at the stage of virion assembly (Sutter and Moss 1992; Volz and Sutter 2017; Sutter 2020). Because replication occurs independently of the host genome and gene expression is controlled by multiple virus-specific promoters, the viral transcription complex can be activated throughout the viral life cycle. MVA promoters can be distinguished in early, intermediate and late promoters, with specific early-late promoters being able to maintain the transcription and translation of gene products for the longest period of time. MVA is thus able to produce almost unimpaired viral or recombinant proteins, distinguishing it from other replication-deficient poxvirus vectors, like the New York attenuated vaccinia virus (NYVAC) (Davison and Moss 1989a, 1989b; Sutter and Moss 1992). Here the viral life cycle is blocked at an earlier stage, resulting in a non-efficient expression of intermediate and late genes (Paoletti 1996; Tartaglia et al. 1992).

For the generation of recombinant MVA vectors, well established protocols are available. These are based on the principle of homologous recombination

between viral DNA and a plasmid transfer vector carrying the gene sequence to be integrated. (Kremer et al. 2012). Overall, MVA is characterized by its high safety profile, genetic stability, and the availability of established protocols for virus production on an industrial scale.

2.4. Immunmodulation of MVA

MVA is able to induce strong immune responses (Sutter et al. 1994) in not only the adaptive but also the innate immune pathways (Forster, Wolf, and Mayr 1994; Waibler et al. 2007) This can be explained by the absence of many viral immune evasion factors in MVA, which distinguishes it from other orthopoxviruses. The lack of viral immune evasion factors is the result of continuous passaging on CEF cells, resulting in a fragmentation and deactivation of some gene sequences of the MVA genome (Antoine et al. 1998). The induction of significant amounts of interferon type I, the expression of pro-inflammatory chemokines and the activation of the complement system by MVA, stimulates the migration of leukocytes in the region of the inoculated tissue (Buttner et al. 1995; Blanchard et al. 1998; Lehmann et al. 2009; Price et al. 2015). This in turn stimulates the adaptive immune response in which both the induction of the T cell response and the antigen-specific antibody formation are contributors (Halle et al. 2009; Drexler et al. 2003; Wyatt et al. 2004). Sutter and colleagues demonstrated a highly efficient stimulation of cytotoxic CD8+ T cells, as well as influenza antigen-specific antibodies. For this purpose, mice were immunized with the recombinant MVA vector vaccine against the influenza A virus H1N1 (Sutter et al. 1994). In fact, protective immunity is also induced when only low doses of the MVA vaccine are administered (Volz et al. 2014; Kreijtz et al. 2009). Ultimately, the induction of the immune response by MVA is so strong that it can be used as a pure adjuvant without serving as an antigen vector itself (Price et al. 2013; Norder et al. 2010; Mayr et al. 1985).

2.5. MVA as a successful vaccine platform

Currently, the world is kept in suspense by the circulating COVID-19 pandemic. A zoonosis caused by SARS-CoV-2 infection, with drastic consequences, not only for health, but also for the global economy. This pandemic highlights the need for safe, effective and rapidly generated vaccine platforms.

Historically, zoonotic diseases have played a major role in the emergence of global diseases and are the link between animal health and the health of the worldwide human population. Rabies, a fatal disease in humans and animals known since ancient times, still circulates in many countries today (Fooks et al. 2017). Life-threatening hemorrhagic fever disease, caused by Ebola virus, was responsible for the West African epidemic between 2013 and 2016 (Feldmann, Sprecher, and Geisbert 2020). Highly pathogenic influenza A viruses have been circulating since 2003, causing severe respiratory diseases (de Jong and Hien 2006), and MERS-CoV, as a zoonotic pathogen with a reservoir in dromedaries, first appeared in 2012 and has had pandemic potential since then (Haagmans et al. 2016).

MVA combines the prerequisites needed for a successful vaccine platform. During the Smallpox Eradication Program in Germany in the 1970s, its safety was proven in more than 100,000 individuals (Pittman et al. 2019). Furthermore, initial clinical trials with the recombinant MVA vaccines against Malaria, HIV (Cosma et al. 2003; Webster et al. 2006) and MUC1-presenting tumors (Rochlitz et al. 2003), showed no serious side effects. MVA-based vaccines, including those against *Mycobacterium tuberculosis* and Ebola virus have been tested in preclinical and clinical trials (Anywaine et al. 2019; Manjaly Thomas et al. 2019). Recent studies have demonstrated the safety and immunogenicity of recombinant MVA expressing Middle East respiratory syndrome coronavirus spike protein (MVA-MERS-S) (Koch et al. 2020; Weskamm et al. 2022; Fathi et al. 2022). When administered simultaneously by intramuscular and intranasal vaccination of dromedary camels, the same vector vaccine was able to confer solid protection against respiratory virus replication following infection with MERS-CoV (Haagmans et al. 2016).

Also of interest in veterinary medicine, recombinant MVA vaccines have been tested against virus or bacterial diseases of veterinary importance such as Schmallenberg virus (SBV) (Wernike et al. 2018), bovine respiratory syncytial virus (BRSV) (Antonis et al. 2007), Peste des petits ruminants virus (PPRV) (Chandran et al. 2010), African horse sickness virus (AHSV) (Castillo-Olivares et al. 2011; Alberca et al. 2014; Calvo-Pinilla et al. 2018), Bluetongue virus (Utrilla-Trigo et al. 2022), Protozoa Leishmania (Ramos et al. 2008), or Rabies lyssavirus (RABV) (Weyer et al. 2007), and could provide promising results.

Since July 2020, a recombinant MVA virus serves as an approved heterologous Ebola vaccination regimen Ad26.ZEBOV/MVA-BN-Filo in the European Union (Volkmann et al. 2021). Consequently, MVA has great potential as a safe vaccine platform against highly pathogenic viruses in human and veterinary medicine.

IV. OBJECTIVES

Due to the currently circulating and spreading African swine fever disease and the lack of a licensed vaccine or antiviral agent, this work describes the following:

1. the generation of four recombinant MVA viruses expressing the ASFV proteins:

- pp220 (MVA-pp220)
- EP402R (MVA-EP402R)
- K145R (MVA-K145R)
- I73R (MVA-I73R)

2. *in vitro* characterization of recombinant MVA constructs

- genetic analysis at the DNA level
- analysis of recombinant protein synthesis
- analysis of virus replication

V. MATERIAL AND METHODS

1. Cells

1.1. Cell lines

Cell line	Origin	Experiment
CEF	chicken embryo fibroblast	virus, amplification, protein expression, growth kinetics
DF-1	chicken embryo fibroblast cell line	virus, amplification, protein expression, plaque passage
HaCaT	human keratinocyte	growth kinetics
PK15	porcine kidney-15	growth kinetics

Tab. 1: Cell lines used in this study

1.2. Cell culture

CEF cells (chicken embryo fibroblast) were freshly isolated from eleven day old specific pathogen free chicken eggs. They were kept in Minimum Essential Medium Eagle (MEM), supplemented with 1% non-essential amino acids and 10% FCS (fetal calf serum).

DF-1 cells (chicken embryonic fibroblast cell line) were maintained in D-MEM (VLE Dulbecco´s Modified Eagle Medium) supplemented with 1% non-essential amino acids and 5% FCS.

HaCaT cells (human keratinocyte cell line) were maintained in D-MEM supplemented with 7% FCS and 2% Hepes solution.

PK15 cells (Porcine Kidney-15 cell line) were maintained in D-MEM, supplemented with 5% FCS and 1% non-essential amino acids.

All cells were cultivated in a humidified incubator at 37°C under 5% CO2 and split twice a week. To detach the cells, Trypsin-EDTA was used. All cells were kept in their specific medium with 2% FCS during infection.

1.3. Cell Counting

Cells were trypsinized and diluted (1:4) with PBS, before counting using a Neubauer chamber.

2. Viruses

In this study, four recombinant MVA viruses were constructed as potential vaccine candidates against the African Swine Fever Virus (ASFV). All of them contain different genes of interest (GOI) of the African Swine Fever Virus. These four genes code for different ASFV proteins. The proteins pp220 and EP402R are structural proteins, while K145R and I73R code for uncharacterized proteins. For each of these ASFV proteins we designed a recombinant MVA with the early/late promoter PmH5. To insert the GOIs, MVA_P11GFP was used as a non-recombinant backbone.

Virus	Feature	Supplier
MVA_P11GFP	non-recombinant MVA backbone virus	Prof. Dr. Sutter, LMU
MVA_F6	Non-recombinant-wild-type-MVA	Prof. Dr. Sutter, LMU
MVA_pp220	Promoter PmH5, Deletion III	This study, LMU
MVA_ EP402R	Promoter PmH5, Deletion III	This study, LMU
MVA_K145R	Promoter PmH5, Deletion III	This study, LMU
MVA_I73R	Promoter PmH5, Deletion III	This study, LMU

Tab. 2: Viruses used in this study

3. Generation of a vector plasmid

To construct a recombinant MVA virus, a vector plasmid which includes the GOI is needed. For that purpose, cDNA pUC57 plasmids, containing the chosen ASFV sequences were synthesized (by Genewiz, Leipzig, Germany) and then cloned into transfer plasmids PIIIH5red.

The synthesized DNA sequences of pp220 and EP402R contain a c-terminal Flag-tag sequence as well as a *Bam*HI and *Not*I restriction site, while the synthesized sequences of K145R and I73R only contain a *Bam*HI and *Not*I restriction site, without Flag-tag sequence.

The synthesized plasmids pUC-57-x were amplified before use (see 3.2). After purifying the DNA from competent bacteria (see 3.3), the plasmid DNA was digested using restriction endonucleases to separate the gene of interest from the rest of the plasmid (see 3.4). The GOI K145R. and I73R were additionally generated from pUC57 by means of PCR to add on the Flag-tag sequence (see 3.5). All four different GOI were then digested with endonucleases. The vector plasmid pIIIH5red was digested by the same restriction endonucleases as the GOI. After separation via gel electrophoresis (see 3.7) followed by removal and purification of the DNA, the GOI could be cloned into the vector plasmid by ligation (see 3.9).

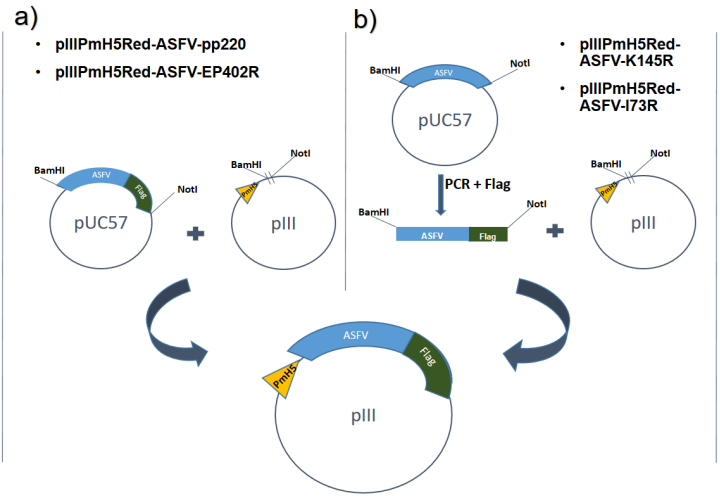

Fig. 2: Generation of vector plasmids: a) Insertion sequences of pp220 and EP402R were synthesized in pUC57 containing a C-terminal Flag-tag sequence and cloned into transfer plasmids pIIIH5red, including PmH5 promoters. b) Insertion sequences of K145R and I73R were synthesized in pUC57, a c-terminal Flag-Tag was added via PCR amplification and resulting DNA was cloned into transfer plasmids pIIIH5red, including PmH5 promoters.

3.1. Plasmids

Plasmid	Supplier	feature
pUC57-ASFV-pp220	Genewiz, Leipzig, Germany	Synthesized Plasmid, Flag-Tag, *Bam*H1, *Not*I
pUC57-ASFV-EP402R	Genewiz, Leipzig, Germany	Synthesized Plasmid, Flag-Tag, *Bam*H1, *Not*I
pUC57-ASFV-K145R	Genewiz, Leipzig, Germany	Synthesized Plasmid, *Bam*H1, *Not*I
pUC57-ASFV-I73R	Genewiz, Leipzig, Germany	Synthesized Plasmid, *Bam*H1, *Not*I
pIIIH5- ASFV-pp220	this study,	pIIIPmH5Red, Flag-Tag, pp220 GOI
pIIIH5-ASFV-EP402R	this study,	pIIIPmH5Red, Flag-Tag, EP402R GOI
pIIIH5-ASFV-K145R	this study,	pIIIPmH5Red, Flag-Tag, K145R GOI
pIIIH5-ASFV-I73R	this study,	pIIIPmH5Red, Flag-Tag, I73R GOI
pIIIH5red	this study,	mCherry marker, PmH5 promoter

Tab. 3: Plasmids used in this study

3.2. Transformation

For amplifying plasmids, competent cells were thawed on ice. By careful pipetting, the cooled down plasmid working stock was added and left for 20 min on ice. After that, the mixture was heat-shocked at 42°C for 2 min and cooled down again on ice. S.O.C-Medium (NEB) was added to the tubes, which were then incubated at 37°C for 1 h at 400 rpm. Next, the bacteria were plated on LB-agar plates (with ampicillin) and incubated at 37°C for 16 h. Some colonies that had grown were then harvested and incubated overnight in 3 ml LB-Medium (with ampicillin).

3.2.1. Organism

For heat-shock transformation and amplification of plasmid DNA, One Shot™ TOP 10 Chemically Competent Cells (Invitrogen) were used.

3.3. Isolation of DNA

For the extraction of plasmid DNA from a bacterial liquid culture, the commercial kit “PureYield Plasmid Miniprep System” (Promega) was used, according to the manufacturer´s instructions. For purifying viral DNA from virus the “QIAamp DNA Mini Kit” (Qiagen) was used.

3.4. Digestion with restriction enzymes

All restriction enzymes (*Bam*HI, *Not*I, *Nco*I (HF)) used in this study were obtained from New England Biolabs, Frankfurt, Germany. The NEBuffer™ 3.1 is compatible with all these enzymes. For DNA digestion the following protocol was used:

3 µg in 10 µl total volume	plasmid DNA
2 µl	NEBuffer™ (10xconc 3.1)
0,3 µl	enzyme 1
0,3 µl	enzyme 2
7,4 µl	ddH_2O
digest at 37°C for one h	

Tab. 4: Restriction digest protocol

3.5. Polymerase chain reaction

In this study, PCR was used to detect and amplify different genes within plasmids or to confirm the presence of a specific viral DNA. It was used to add a C-terminal Flag-Tag-Sequence to the sequences of the GOI K145R and I73R. in addition, PCR was performed as fingerprint PCR, amplifying the six major deletion sites (Del I-VI) of wildtype MVA as well as constructed MVA-ASFVs (Fig 3). The oligonucleotide primers are summarized in Tab. 9. The following protocols were used for polymerase chain reaction (Tab. 5-8), always including between 10 ng-30 ng DNA (1 µl).

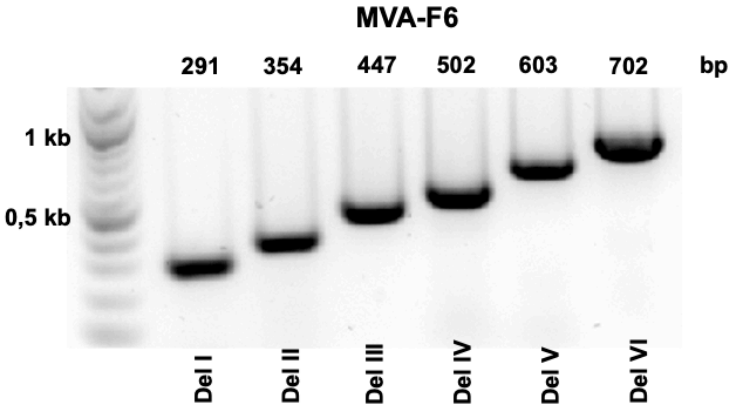

Fig. 3: Fingerprint PCR of wildtype MVA_F6 amplifying the six major deletion sites (Del I-VI) of the virus.

Q5-High-Fidelity PCR, used for Flag-Tag-PCR	
7 µl	RNase free water
12.5 µl	Q5 High-Fidelity Master-Mix
1.25 µl	forward oligonucleotide primer
1.25 µl	reverse oligonucleotide primer
1 µl	template DNA

Tab. 5: Protocol Q5-High-Fidelity PCR

Circle	Step	Time	Temperature
Step1 1x	polymerase activation	5 min	98°C
Step2 30x	a) denaturation b) annealing c) extension	30 sec 30 sec 1min	98°C 48°-60°C 72°C
Step3 1x	extension	5 min	72°C

Tab. 6: Temperature profile of Q5-High-Fidelity PCR, used for Flag-Tag-PCR

SIGMA Taq PCR with $MgCl_2$, used for plasmid or viral DNA control	
22 µl	RNase free water
25 µl	SIGMA ReadyMix™ reaction mix
1 µl	forward oligonucleotide primer
1 µl	reverse oligonucleotide primer
1 µl	template DNA

Tab. 7: Protocol SIGMA Taq PCR with MgCl

Circle	Step	Time	Temperature
Step1 1x	polymerase activation	3 min	95°C
Step2 35x	a) denaturation b) annealing c) extension	30 sec 30 sec 1 min	95°C 48°-60°C 72°C

Tab. 8: Temperature profile of SIGMA Taq PCR with $MgCl_2$, used for plasmid or viral DNA control

3.6. Oligonucleotide primers

Oligonucleotide primers	Sequence 5´→ 3´	Product Size bp
MVA-Del 1 f	CTT TCG CAG CAT AAG TAG TAT GTC	291
MVA-Del 1 r	CAT TAC CGC TTC ATT CTT ATA TTC	
MVA-Del 2 f	GGG TAA AAT TGT AGC ATC ATA TAC C	354
MVA-Del 2 r	AAA GCT TTC TCT CTA GCA AAG ATG	
MVA-Del 3 f	GAT GAG TGT AGA TGC TGT TAT TTT G	446
MVA-Del 3 r	GCA GCT AAA AGA ATA ATG GAA TTG	
MVA-Del 4 f	AGA TAG TGG AAG ATA CAA CTG TTA CG	502
MVA-Del 4 r	TCT CTA TCG GTG AGA TAC AAA TAC C	
MVA-Del 5 f	CGT GTA TAA CAT CTT TGA TAG AAT CAG	603
MVA-Del 5 r	AAC ATA GCG GTG TAC TAA TTG ATT T	
MVA-Del 6 f	CGT CAT CGA TAA CTG TAG TCT TG	702
MVA-Del 6 r	TAC CCT TCG AAT AAA TAA AGA CG	
Insert-1-pp220 f	AAA TAA TAC GAG CGA TGT TGT G	674
Insert-1-pp220 r	TAT GAT TGA TTT GCT CCT CTT G	
Insert-2-pp220 f	TTA ACA AAA TGG TTC AAG TTC G	299
Insert-2-pp220 r	AAT GAT TTG GTT AAC ACC TTC C	
Kombi-1-pp220 f	GAT GGC CAT GTT ATC CTC CTC	725
Kombi-1-pp220 r	GTG CTG ACC TAT ATC ACG GAA C	
Kombi-2-pp220 f	GGT TAA TGG CGT CTG CAA TC	716
Kombi-2-pp220 r	GGG CTC CTT ATA CCA AGC ACT C	
Insert-1-EP402R f	GAC ACC ACT TCC ATA CAT GAA C	247
Insert-1-EP402R r	GTA GCG GGA TAC TAG GTA GTG G	
Insert-2- EP402R f	CTA GCT ACA TGT GGA AAA GCA G	634
Insert-2- EP402R r	GTT CAT GTA TGG AAG TGG TGT C	
Insert-1-K145R f	AGG GTC ATC CCT TTC TTT TTA G	390
Insert-1-K145R r	AGG ATT CTT CTC CTC CTT CTT C	
Insert-2-K145R f	GTC GAA AAC CAA TGA AAA AGA G	118

Insert-2-K145R r	CCC AAT GGG ATC TAG CTT ATA C	
Flag-K145R f	CGG GAT CCA CCA TGG ATC ATT ATC TTA AAA AAT TAC AAG ATA TTT ATA CGA AGC TC	455
Flag-K145R r	TTC CGC GGC CGC TAT GGC CGA CGT CGA CGC GGC CGC TTA TTA CTT GTC GTC ATC GTC TTT GTA GTC GCC GCC GGA TTC TTC TCC TCC TTC TTC AAC AAT CTT TGC CCA AGT TTT TT	
Insert-1-I73R f	AAG TTG ATT TCC ATG GTT AAG G	207
Insert-1-I73R r	TTA GTT TTT CCG TAT CCA AAG C	
Insert-2-I73R f	TTG ATT TCC ATG GTT AAG GAA G	108
Insert-2-I73R r	TCC TGT AGG TAA GAC GAC ATT G	
Flag.I73R f	CGG GAT CCA CCA TGG AGA CTC AGA AGT TGA TTT CCA TG	236
Flag.I73R r	TTCCGC GGC CGC TAT GGC CGA CGT CGA CGC GGC CGC TTA TTA CTT GTC GTC ATC GTC TTT GTA GTC GCC GCC GTT TTT CCG TAT CCA AAG CGG TGG ATA AAT GGT ATT TGT CTT ATC AAT	

Tab.9: Oligonucleotides used in this study

3.7. Gel electrophoresis

Agarose gel electrophoresis was used to separate PCR products or digested PCR products according to size. Gels with 1%-1.5% agarose, GelRed and 1xTAE running buffer were used. Before loading into the gel, the DNA was mixed with loading buffer (GEL Loading Dye, Purple (6x), NEB). Two different molecular weight markers (100 bp Ladder and 1 kb Ladder (New England Biolabs)) were applied. Subsequently, nucleic acid was detected with a CHemiDoc™ MP Imaging system (Bio-Rad), using UV light.

3.8. Purification of DNA fragments from Agarose Gels

DNA fragments were precisely separated with a scalpel, under UV light control. DNA was purified from the gel using the “Wizard® SV Gel and PCR Clean-Up System” (Promega), according to manufacturer’s specifications.

3.9. Ligation

The basic protocol for ligation is shown in table 10. The vector : insert ratio was 1:5. The ingredients were mixed and incubated at 16°C overnight.

vector	50 ng
insert	200-500 ng
10x T4 DNA Ligase Reaction Buffer (NEB)	1 µl
T4 DNA Ligase (NEB)	1 µl
H_2O	filled up to 10

Tab.10: Ligation protocol used in this study

4. Generation of recombinant MVA

For generation of recombinant MVAs, standard methodologies were used, as described previously (Kremer et al. 2012). DF-1 cells grown in 6-well tissue culture plates until 90-95% confluence, were infected with MVAp11GFP at MOI 0.1, as a non-recombinant backbone. 15 min post infection, cells were transfected with 1 µg PIII_ASFV Plasmid, according to manufacturer´s instructions of X-tremeGENE HP DNA Transfection Reagent. Cells were collected after 24 h incubation at 37°C. By screening for transient co-expression of red fluorescent marker protein mCherry, recombinant MVA was clonally isolated in serial rounds of plaque purification on DF-1 cell monolayers. Recombinant MVA was amplified on CEF and serum free CEF cells in T175 tissue culture flasks. The virus stocks were frozen, thawed and sonicated for three rounds and centrifuged for 5 min at 1500 rpm.

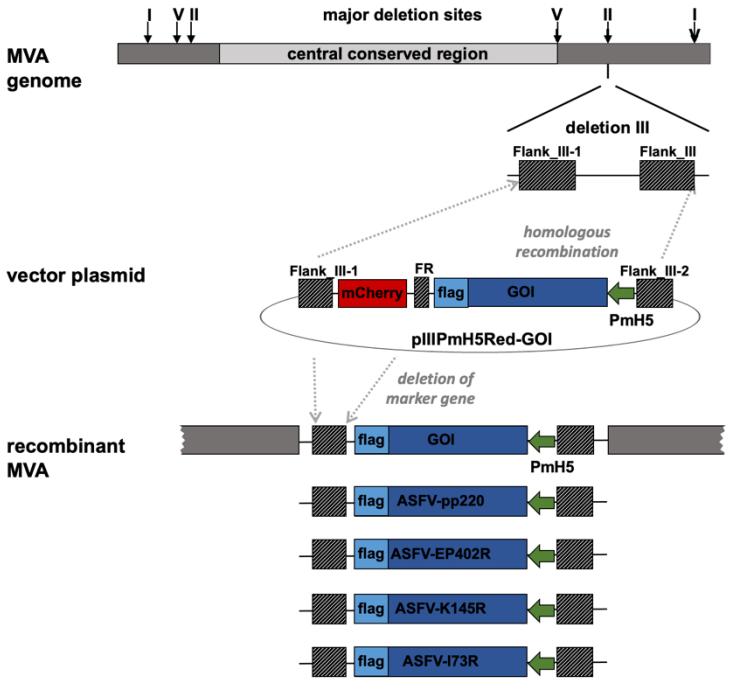

Fig. 4: Construction of recombinant MVA: Schematic diagram of the MVA genome indicating the six major deletion sites I-VI. Deletion III was used to insert ASFV DNA sequences by homologous recombination. To remove the marker gene mCherry by intragenomic homologous recombination, the repetitive sequence (FR) was designed.

4.1. Virus titration in plaque-forming units (PFU)

The sonicated virus stock was diluted in 10-fold serial steps $1:10^3$-$1:10^8$ and plated in duplicate on confluent CEF cell monolayers in 6-well plates. After 48h incubation, the cell monolayers were fixed with a 1:1 mixture of ice-cold acetone/methanol for 5 min. They were blocked with PBS+3%FCS over night at 4°C. After blocking, the primary antibody (anti-Vaccinia virus) was diluted 1:2000, added to each well, and incubated at room temperature for 1 h. Cells were washed three times with PBS+3%FCS and incubated with the secondary antibody (goat anti-mouse) with a dilution of 1:5000, for 1 h. Plates were again washed three times with PBS+3%FCS. True-blue substrate was added for 10

min and monitored for color development. By counting the plaques in each dilution, the viral titer was determined, in plaque forming units per milliliter (PFU/ml). All virus stocks were titrated three times in duplicate.

4.2. Virus growth kinetics

The characterization of growth kinetics on permissive and non-permissive cell lines was used to confirm the viruses' replication deficiency on non-permissive cells, as well as the genetic stability of recombinant MVA with the inserted GOI in permissive cells. With a confluence of 90-95%, CEF, HaCaT and PK15 cells were seeded on 6-well tissue culture plates. Each cell line was subsequently infected with recombinant MVA at a low multiplicity of infection (MOI) of 0.01, in duplicate. Cells were harvested and frozen at -20°C after different time points (0h, 4h, 8h, 16h, 24h, 48h). Each sample went through three cycles of freezing, thawing, and sonicating, before they were back titrated on CEF cells (4.1).

4.3. Western Blot Analysis of Recombinant Proteins

CEF cell monolayers with a confluence of 90-95% were infected at an MOI of 5-10 with recombinant MVA expressing the ASFV protein. Non-infected (mock) or wild type MVA infected CEF cells served as controls. 24 h after infection, cell lysates were prepared and harvested with lysis buffer (Triton-X100 (Sigma-Aldrich)) kept on ice. Cell lysates of the construct MVA-EP402R, were additionally treated with the endoglycosidases EndoH (NEB) to remove high mannose oligosaccharides from N-linked glycoproteins or PNGase F (NEB), which removes almost all N-linked oligosaccharides from glycoproteins, according to the manufacturer's instructions. All samples were diluted with 4 x loading buffer (Laemli buffer (Bio-Rad) and loaded onto a pre-cast Gel (Mini-PROTEIN TGX Gels (BIO-Rad). Proteins were separated by electrophoresis performed in 1x Tris/Glycin/SDS running buffer (Bio-Rad) at 70-100 volts for approximately 45-120 min. As a molecular weight marker, Precision Dual Color Protein Ladder (Bio-Rad), was used. Subsequently, samples were transferred onto a 0.2 mm nitrocellulose blotting membrane (Bio-Rad), using the Trans Blot Turbo System (Bio-Rad). Afterwards the membrane was blocked in blocking buffer (5%-nonfat milk-PBS-Tween) overnight at 4°C.

The primary antibody (anti Flag) was diluted (1:2000) in blocking buffer and the membrane was incubated for 1 h at RT. Before and after incubation with the

secondary antibody (goat anti-mouse HRP), diluted in blocking buffer (1:5000), the membrane was washed three times in 0.1% PBS-Tween for 10 min per wash. For visualizing positive signals, Clarity™ Western ECL Substrate (Bio-Rad) was added at the membrane and proteins were detected by using the ChemiDocTMMP Imaging System.

4.3.1. Antibodies

Antibodies used for Western blot		
Specificity	**Dilution**	**Company**
FLAG-tag Antibody	1:2000	SIGMA-ALDRICH, Saint Louis, USA
Vaccinia Virus (Lister Strain) rabbit polyclonal antibody	1:2000	OriGene Technologies GmbH, Herford, Germany
Anti-rabbit IgG Peroxidase conjugated AffiniPure Goat	1:5000	Jackson Immuno Research, Suffolk, United Kingdom

Tab. 11: Antibodies used in this study

VI. RESULTS

1. Construction of recombinant MVAs expressing the African swine fever virus proteins pp220, EP402R, K145R and I73R

Four recombinant MVA viruses were constructed expressing different proteins of the ASFV: pp220, EP402R, K145R and I72R. These specific sequences were inserted into the site of deletion III within the MVA genome, using the constructed vector plasmids pIIIH5Red-ASFV-pp220, pIIIH5Red-ASFV-EP402R, pIIIH5Red-ASFV-K145R and pIIIH5Red-ASFV-I73R. Transcription of these genes was under the control of a strong synthetic early/late promoter PmH5. To facilitate quality control, the different ASFV genes contained a Flag-tag (DYKDDDDK-tag) (Fig. 4) sequence for protein analysis, since no specific antibodies for the detection of the ASFV genes were available at the time of generation.

1.1. Construction of vector plasmids

All target sequences were obtained from the ASFV isolate Pig/Heilongjiang/2018 (Pig/HLJ/18, MK33180.1) and were planed and optimized using the SeqBuilder software (DNASTAR Inc.). While codon optimization, TTTTTNT regions and G/C-runs were changed at the genomic level of the amino acid sequences. All plasmids were designed with *Bam*HI and *Not*I restriction enzyme sites. Withing two of the planned plasmids (pUC57-ASFV-pp220, pUC57-ASFV-EP402R) a c-terminal Flag-tag sequence was also added. The plasmids were synthesized by Genewiz and were subsequently controlled by restriction digestion.

Plasmid pIIIH5Red was used as the initial vector for the shuttle plasmids. It contains MVA gene sequences, which are required for the insertion of the GOI into the Del III, a fluorescence marker mCherry, a resistance gene (AmpR) and the early/late promoter PmH5, which is connected in front of the multiple cloning site (MCS) (Fig 5).

The target sequences pp220 and EP402R were cloned into the shuttle plasmids by restriction digestion and ligation. To add flag-tag- sequence to the sequences of K145R and I73R, which were obtained by commercial gene synthesis, specific oligonucleotide primers were constructed (Flag-K145R f, Flag-K145R r, Flag.I73R f, Flag.I73R r, see Tab. 7) and the GOI was amplified by PCR. Subsequently, the specific DNA was extracted from agarose gel and cloned into the shuttle plasmid pIIIH5Red by restriction digestion and ligation (Fig 2).

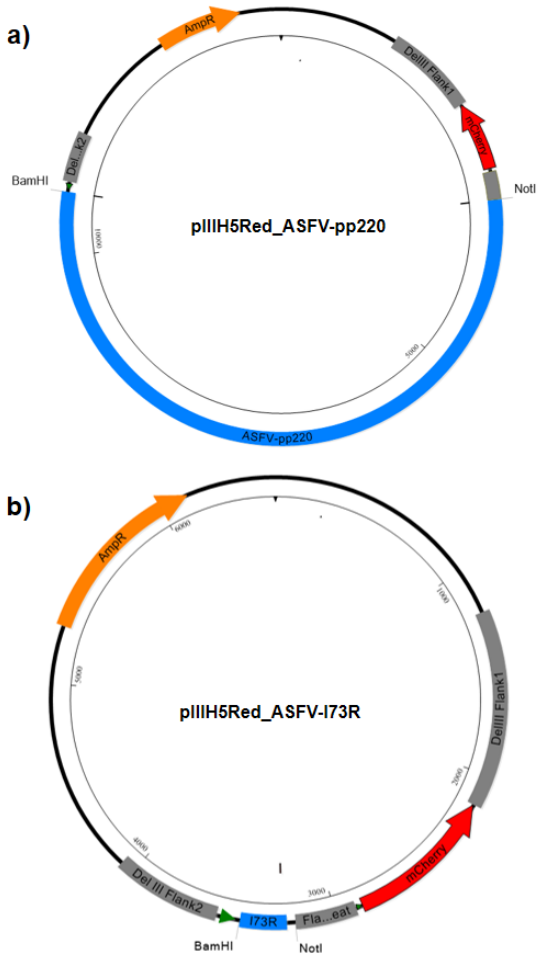

Fig. 5: Vector plasmid pIIIH5Red_ASFV-GOI. ASFV-GOI sequences (blue; e.g. a) ASFV-pp220, b) ASFV-73R) were cloned into the multiple cloning site between the flanking regions (grey). under control of promoter PmH5 (green). The fluorescence marker gene mCherry (red) was placed between flanking regions (flank_III-1 and Flank_III-1 repeat, grey) for intragenomic homologous recombination during plaque purification. The resistance gene AmpR (orange) was used for selection of competent cells during Transformation after integration of the ASFV sequence into the shuttle plasmid.

1.2. Characterization of vector plasmides

All vector plasmids were checked for correct insertion of the GOI using multiple PCRs and restriction analyses. PCR control was performed with specific oligonucleotide primers (Tab. 7) amplifying the insertion site between the flanking regions as well as short segments of the GOI, respectively (Fig 7). Control digestions were performed at two levels. First, the plasmids were digested with the same restriction enzymes (*Bam*HI and *Not*I) used for cloning, so that the GOI was cut out and visible after agarose gel electrophoresis (data not shown). On the other hand, the plasmids were digested with the restriction enzyme *Nco*I, which cuts the plasmids at different locations and thus the correct reading direction of the GOI in the plasmid could be verified (Fig 6).

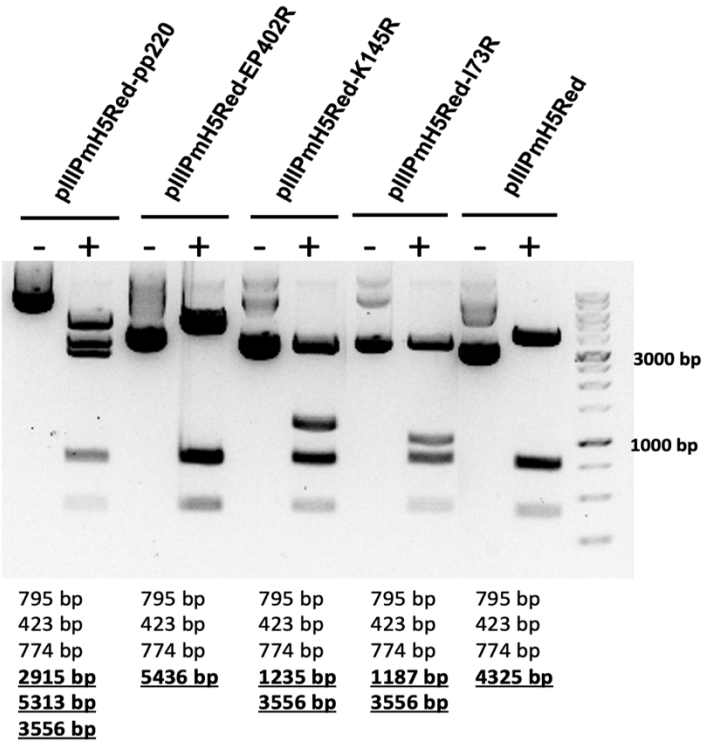

Fig.6: Digestion control of vector plasmids: Vector plasmids were digested with restriction enzyme *NCO*I. (+) demonstrating the expected sizes of restriction digestion. (-) control of non-digested vector plasmid.

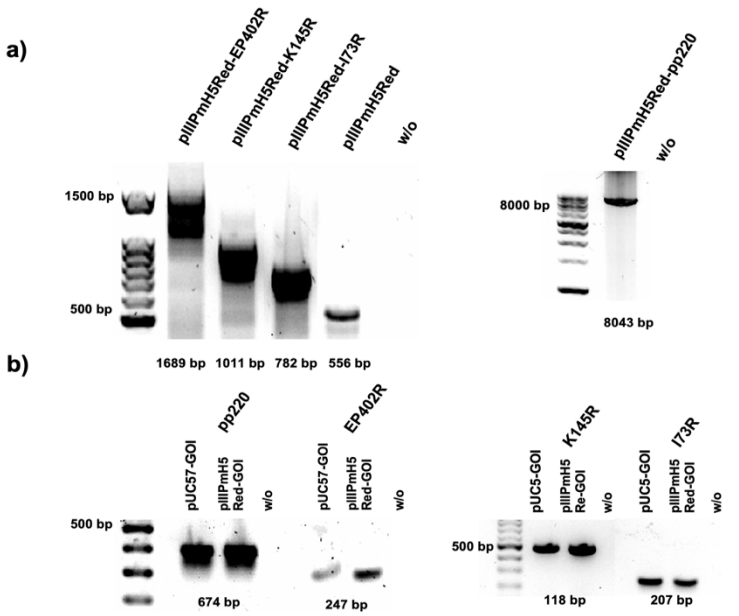

Fig.7: Characterization of vector plasmids by PCR: Amplification of the deletion site III (a) and GOI specific DNA fragments (b). Expected sizes are shown for every PCR product. w/o = negative/water control, pUC57-GOI = plasmid pUC57 including the GOI. pIIIH5Red-GOI = shuttle plasmid pIIIH5Red including the GOI.

1.3. Generation and clonal isolation of recombinant MVA

The ASFV target sequences were integrated into deletion III of MVA genome by homologous recombination between the adjacent DNA sequences of deletion site III in the MVA genome and the DNA cassette between flank 1 and flank 2 of the described vector plasmids. MVA_p11GFP, which contains a green fluorescent reporter GFP sequence in Del III, was used as a recombinant MVA backbone. Upon plaque purification, co-expression of the red fluorescent reporter protein mCherry facilitates detection of MVA-ASFV and is readily distinguishable from the green fluorescent reporter protein GFP of the wild type variant (Fig. 8). Later in the course of plaque purification the incorporation of the short repetitive MVA-DNA sequence (Flank_III-1 repeat) facilitates the removal of mCherry from the genome of the final recombinant MVA by intragenomic homologous recombination (Fig. 4).

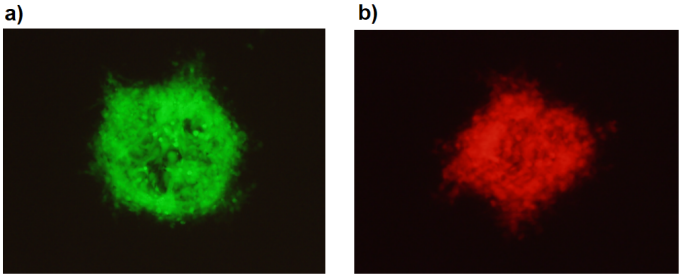

Fig. 8: Expression of marker proteins during generation and clonal isolation of recombinant MVA: (a) Green fluorescence of GFP indicates replication of MVA_p11GFP within DF-1 cells. After homologous recombination with the vector plasmid and several rounds of plaque picking, recombinant MVA-ASFV is expressing the red fluorescent marker protein mCherry (b).

2. *In vitro* characterization of recombinant MVA constructs

2.1. Verification of correct insertion of ASFV genes

Standard experiments previously described were used for quality control of the generated recombinant MVA constructs. (Kremer et al. 2012). MVA plaques free of the red fluorescent marker protein mCherry were harvested and grown into a large viral stock. To control for genetic integrity, correct insertion of heterologous gene sequences, and stability of recombinant vector viruses, viral DNA of virus stocks was purified. Polymerase chain reaction (PCR) was used to confirm genomic viral DNA for genetic identity. A specific fingerprint PCR, analyzing the six major deletion sites of MVA (Fig 3) was performed with all MVA-ASFVs (Fig 9). Additionally, all recombinant viruses were tested with PCRs, amplifying short segments of the GOI (Fig: 10).

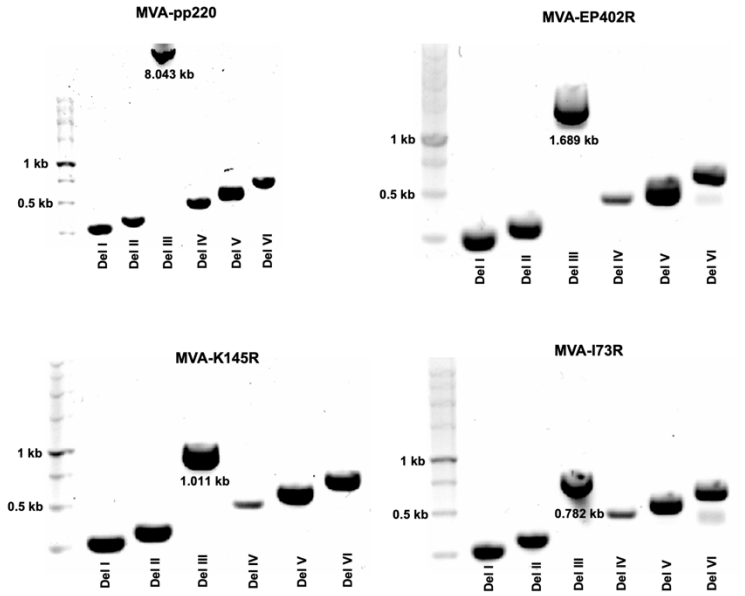

Fig. 9: Fingerprint PCR demonstrating the correct insertion of the GOI in deletion III of MVA-ASFV: Expected size of the Del III-PCR product is indicated.

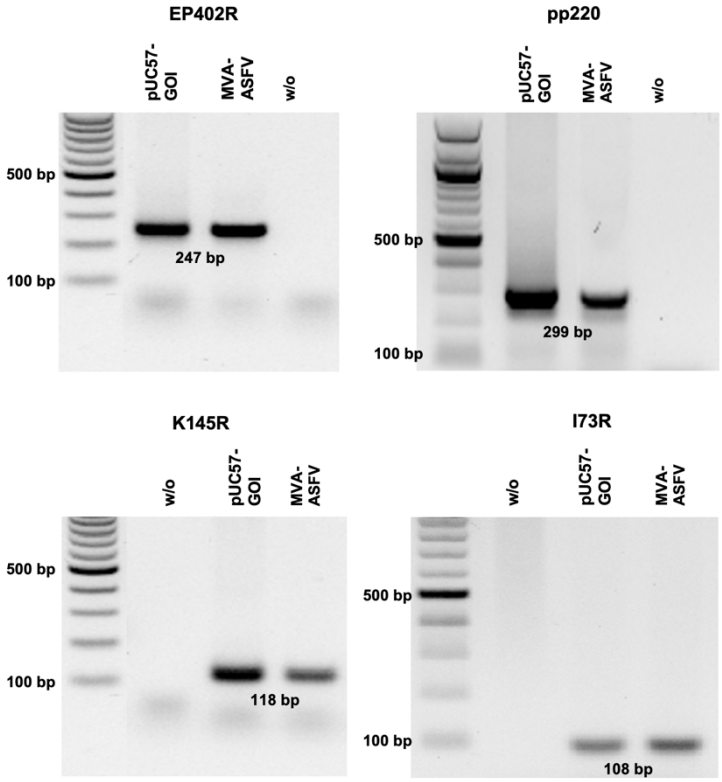

Fig. 10: GOI-PCR demonstrating the correctness of the insert within MVA-ASFV.

2.2. Multi-step growth kinetic of recombinant MVA constructs

Another important step in quality control of recombinant MVA was to assess the growth behavior of newly constructed recombinant MVAs on permissive and non-permissive cell lines by multi-step growth kinetics. The following cell lines were used for the study: CEF cells, which are routinely used in the vaccine development of recombinant MVA vaccine platforms, the human cell line HaCaT and the mammalian cell line PK15, which is based on porcine kidney cells. As expected, the human cell line HaCaT, as well as the mammalian cell line PK15 were not permissive for productive virus growth of the constructed recombinant MVAs (Fig. 12). This confirmed the characteristic replication deficiency of MVA in cells of mammalian origin, including the new recombinant MVAs. In addition,

all recombinant MVA-ASFVs showed efficient replication in CEF cells (Fig. 12), which was comparable with wild-type MVA (Fig. 11).

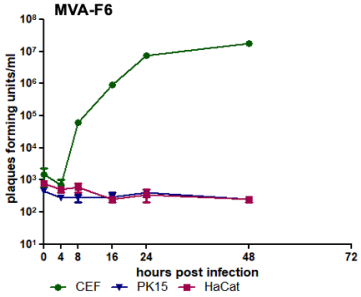

Fig. 11: Multiple-step growth analysis of wildtype MVA-F6. CEF, PK15 and HaCat cells were infected with MOI of 0.01. Samples were taken at different time points post infection as indicated.

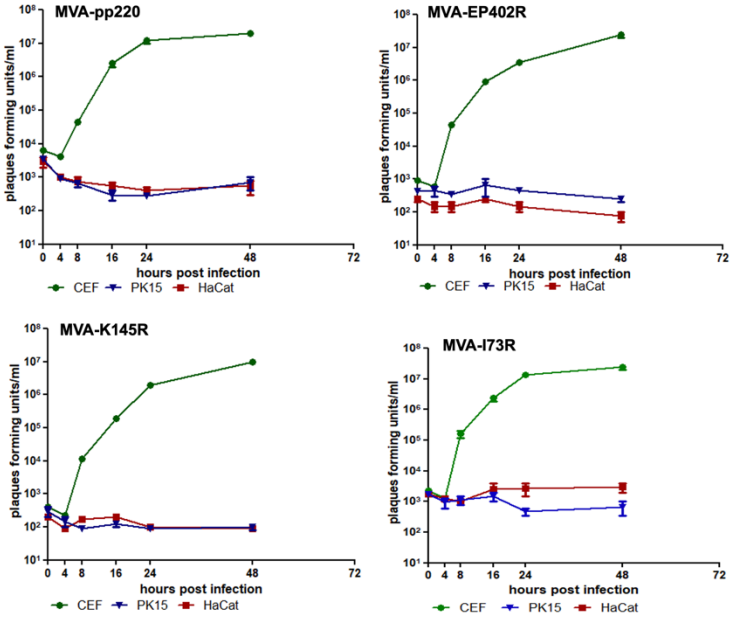

Fig. 12: Multiple-step growth analysis of recombinant MVA-ASFV constructs. CEF, PK15 and HaCat cells were infected with MOI of 0.01. Samples were taken at different time points post infection as indicated

2.3. Recombinant MVA constructs express ASFV proteins

Successful determination of growth kinetics was followed by analysis of the ability of the recombinant MVA-ASFV to produce ASFV proteins by immunoblotting. For this purpose, CEF cells were infected with the recombinant MVAs (MVA-pp220, MVA-EP402R, MVA-K145R, MVA-I73R) and protein lysates were prepared 24 hpi which were subsequently separated by size using SDS-PAGE. Using a FLAG-tag specific monoclonal antibody, for MVA-pp220, MVA-K145R and MVA-I73R, we were able to confirm the production of proteins with molecular masses matching the predicted sizes of the ASFV proteins (MVA-pp220 280 kDa, MVA-K145R 17.2 kDa and MVA-I73R 8.5 kDa (Fig. 13), respectively. Immunoblotting of MVA-EP402R samples revealed a protein with a molecular mass of 70-100 kDa. We hypothesized the glycosylation of this protein, since the expected size of EP402R protein was about 42 kDa. To confirm this hypothesis, we incubated the cell lysates with PNGase F, which removes all N-linked oligosaccharide chains, or and Endo-H, which cleaves the N-linked glycans between two N-acetylglucosamine residues in the core of mannose-rich sugars. The glycosidase treatment reduced the molecular mass of the protein EP402R from 70-100 kDa to 50-55 kDa (Fig. 13d).

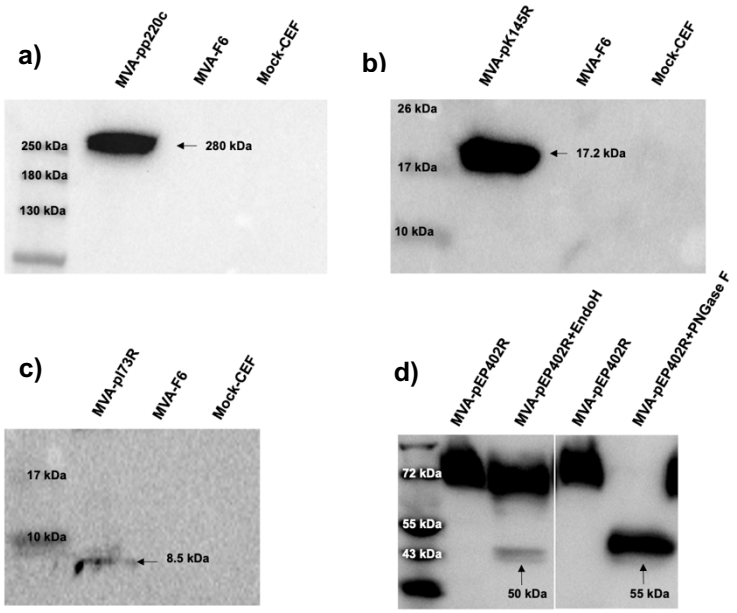

Fig. 13: Immunoblot analysis of recombinant ASFV virus proteins. CEF-cells were infected with recombinant a) MVA-pp220, b) MVA-K145R, c) MVA-I73R, d) MVA-EP402R at a MOI 5-10 and collected 24 hpi. Wild-type MVA-F6 served as a control. Cell lysates of d) MVA-EP402R were additionally deglycosylated using glycosidases EndoH and PNGase F, respectively. Using a Flag-tag antibody all cell lysates were analyzed by immunoblotting and showed the expected molecular weights of the recombinant proteins.

VII. DISCUSSION

The African swine fever pandemic has spread across three continents and, as a fatal hemorrhagic disease, threatens not only the pig industry through economic losses, but also the food security of large swaths of the world's population, who depend on pork for animal protein. These factors underline the urgent need to develop a safe vaccine against African swine fever virus (ASFV).

The modified vaccinia virus Ankara (MVA) is an established and promising vector platform for the development of vaccines against several bacterial and viral infections. Due to the lack of virulence factors and immune evasion proteins, MVA offers high safety with improved immunogenicity and is therefore superior to other vaccinia virus expression vectors.

In this study, four recombinant MVAs were generated, each expressing a full-length ASFV protein: pp220, CD2v, K145R and I73R. They have undergone characterization on a molecular-biological level as well as investigations into growth behavior and protein expression. Finally, their potential for use as a vaccine against African swine fever virus will be evaluated.

1. Requirements and challenges for vaccines against ASFV

One of the most important requirements for the development of an ASFV vaccine is the inclusion of the DIVA strategy, which is a necessary condition for the official registration of a potential ASF vaccine (Turlewicz-Podbielska et al. 2021). Distinguishing between vaccinated and diseased animals is a useful tool in combating animal diseases. Another prerequisite for the vaccine to be developed is a functioning immunization strategy for wild boar. These animals are the main factor driving the spread and introduction of the disease into domestic pig herds, particularly in Europe, through direct and indirect transmission (Tarasiuk and GiŻEjewski 2021). For their immunization, oral administration of the vaccine is more convenient than intramuscular injection and is recommended for its proven effectiveness in eradicating classical swine fever in wild boar (Iacolina et al. 2021).

The lack of a licensed and established macrophage cell line complicates research in vaccine development and poses a problem for some vaccine platforms, such as live attenuated vaccines, especially in large-scale production. The adaptation of virulent field isolates to other cell lines highlights the risk of a phenotypic change due to point mutations in the genome (Krug et al. 2015; Dixon et al. 2019; Portugal et al. 2020).

Due to the high complexity of ASFV and its ability to evade the host's immune system, several previous studies have suggested that the ASFV vaccine must be able to induce both specific antibody and CD8+ T cell responses to provide adequate protection (Turlewicz-Podbielska et al. 2021).

The safety of the ASFV vaccine used in pigs must also be assured. Live attenuated vaccines in particular harbor a potential for the vaccine virus to revert to a virulent strain, when it replicates in the pig. This was observed in Portugal and Spain in the 1960s, when LAVs turned virulent in breeding pigs, causing 10-50% mortality (Coggins, Moulton, and Colgrove 1968; Turlewicz-Podbielska et al. 2021).

In summary, the ASF virus poses several challenges for vaccine development. Despite decades of research, no suitable vaccine has been developed. Vaccine approaches such as inactivated viruses did not provide protection and proved to be ineffective. Modified live viruses and attenuated isolates have shown greater success in some studies. Nevertheless, protection in surviving pigs has so far only been observed against homologous viral variants and not against heterologous viral variants. In addition, side effects such as joint swelling and skin lesions occur (Gaudreault and Richt 2019). Chronic infections or even a return to the virulent form of the isolate can occur after vaccination with a live attenuated virus. The production of these vaccines is complicated by the lack of a primary macrophage cell line (Turlewicz-Podbielska et al. 2021). These are some reasons for the use of MVA as a suitable vaccine platform which could solve the mentioned challenges.

2. Advantages of MVA as a suitable vector platform for ASFV vaccines

MVA is a reliable vector vaccine platform because it is safe, efficacious and cost-effective, and therefore combines all the properties needed for the development of an ASFV vaccine. As described previously (VI.1.), the safety profile of an ASFV vaccine plays a crucial role and other vaccine platforms have struggled with vaccination side effects, chronic infections and even the return to virulence isolates. MVA was proven early on to have a high level of safety in pigs (Moss 1993) as well as *in vivo* in various animal models (Stittelaar et al. 2001). MVA's replication deficiency in mammalian cells is a decisive advantage here. Complete clearance of the recombinant virus and the expressed target antigen occurs just a few days after vaccination (Altenburg et al. 2014). MVA also has a high safety profile in humans, which it has been able to prove in numerous phase I-III studies with HIV patients, in a phase I study with MERS-CoV or in registration studies as an MVA smallpox vaccine (Vollmar et al. 2006; Harrer et al. 2005; Koch et al. 2020).

The classification of MVA in risk group 1 by the Robert Koch Institute because of its greatly reduced ability to replicate in human cell cultures, (des Robert Koch-Instituts 2002) enables easy handling in research and at vaccine production-scale. In the case of live attenuated ASFV vaccines, the lack of an approved macrophage cell line complicates vaccine research and renders large-scale production very cost-intensive (Turlewicz-Podbielska et al. 2021). Established protocols are available for the research and manufacture of recombinant MVA vectors (Altenburg et al. 2014; Kremer et al. 2012). The effective growth of MVA on permissive cells like CEF cells or DF-1 cell line supports easy and efficient handling during vaccine development. The existence of an approved MVA vector vaccine for immunization against Ebola proves that there are sufficiently large-scale production capabilities available at an economical production level (Ewer et al. 2016).

A strong immune response is essential for a vaccine to be effective. Due to the high complexity of ASFV and its distinctive host defense mechanism abilities, the induced immune response of a vaccine should be based on both a humoral and a cellular immune response. The vector virus MVA, as a highly attenuated

poxvirus, is able to infect host cells and induce intracellular expression of one or more specific antigens (Draper and Heeney 2010; Moss 1993). Crucial for the activation of a T-cell response is the fact that antigen-presenting cells are the primary cells involved in MVA infection (Altenburg et al. 2017). During the viral replication cycle, antigenic proteins are processed within the host cell by the proteasome via de novo synthesis and subsequently presented as peptides on MHC-I molecules on the cell surface (Brewoo et al. 2010). As a result, a strong and efficient antigen specific CD8+ T cell response is activated via this MHC class I pathway (Altenburg et al. 2017; de Vries and Rimmelzwaan 2016; Altenburg et al. 2014), a desirable property required in the development of vaccine candidates against ASFV. Another advantage of MVA is the ability to synthesize stable proteins. *In vivo*, these proteins are superior *in vivo* for rapid activation of immune responses, since the T cell response is activated more efficiently when stably produced MVA antigens are cross presented by dendritic cells (Gasteiger et al. 2007). The expression of foreign antigens in their native form enables the activation of a humoral immune response in the form of antigen-specific antibodies (de Vries and Rimmelzwaan 2016). In addition, MVA lacks several immunomodulatory proteins compared to wild-type VACV, resulting in the activation of several intracellular host cell detection mechanisms during the infection cycle and release of chemokines, interferons and inflammatory cytokines. MVA further lacks IFN-α/β receptors that activate the type I IFN response during host cell infection, and also lacks the functional receptor for INF-γ, which is a crucial factor for cytotoxic T cell activation (Blanchard et al. 1998; Altenburg et al. 2014). Thus, MVA provides a balanced immune response on both the humoral and cellular side, justifying our decision to use MVA as a viral vector system against ASFV.

For a successful disease control of African swine fever, a two-pronged vaccination strategy would beneficial, which on the one hand avoids the spread of ASF through wild boars and on the other hand offers a sensible vaccination strategy for the domestic pig population. A feasible option for domestic pigs would be a DIVA vaccination strategy. The classical swine fever disease was, among other factors eradicated through a DIVA vaccine for domestic pigs, based on the E2 subunit maker vaccine (Porcilis® Pesti, MSD Animal Health, Unterschleißheim, Germany), which proved to be safe and provided clinical

protection against CSF (Blome et al. 2017). MVA would be a suitable candidate for a subunit DIVA vaccine strategy. To this point, Goatley et. al. published a DIVA-compatible subunit vaccine, based on a rAd -prime/MVA boost system comparing a pool of eight ASFV antigens, which protected pigs from fatal disease after challenge with a virulent ASFV (Goatley et al. 2020). These results were further validated by other published studies on the suitability of MVA as a compatible vaccine platform for DIVA (Calvo-Pinilla et al. 2018; Utrilla-Trigo et al. 2022). For example, MVA expressing AHSV-VP2 was shown to be a suitable approach in developing an efficacious, and safe vaccine including DIVA against African horse sickness (Calvo-Pinilla et al. 2018). Another factor in eradication of CSF disease was the oral immunization of wild boars via a bait format adapted vaccine, based on live-attenuated CSF vaccine (Blome et al. 2017). Based on the proven effectiveness of this strategy, it would also be recommended for the control of ASFV. MVA could be a suitable option, as a vaccination platform for oral immunization of wild boars but this needs to be proven in future studies. Early in its rise to prominence as a vector vaccine, MVA demonstrated the ability to induce mucosal immunity after oral immunization and showed a partial protection against influenza virus in mice (Bender et al. 1996). An MVA related recombinant vaccina virus (VACV) which is based on oral vaccination with VACV expressing the RVGP gene, has proven to be a successful vaccine to immunize wildlife against rabies. This vector vaccine system successfully immunized red foxes, raccoons and coyotes for nearly 20 years and played a crucial role for the elimination of rabies in many European countries (Astray, Jorge, and Pereira 2017). A major advantage when aiming at a vaccine baiting strategy would be the great stability of MVA, since MVA can be freeze-dried and stored without special refrigeration and additionally loses little of its original titer at room temperature (Chen et al. 2021).

In conclusion these arguments underline the suitability of MVA as a vector platform to develop an effective vaccine against ASFV. In particular, the highly advantageous characteristics of MVA to induce a specific antibody response, as well as cellular immune response emphasize its potential as a strong immunization vector. Easy handling, construction with well-established protocols and production capabilities on a large-scale level are some benefits of this backbone platform. Finally, MVAs excellent safety profile paired with the

great opportunities for a successful disease control in the domestic pig population support the decision to use MVA as vaccine platform.

3. Generation of recombinant MVA constructs to produce ASFV- antigens

For generation of four MVA-ASFV constructs, gene sequences of pp220, EP402R, K145R and I73R were chosen from ASFV isolate Pig/Heilongjiang/2018 (Pig/HLJ/18). Pig/HLJ/18 is a highly virulent and transmissible isolate in domestic pigs, which was the first African swine fever virus isolated in China in 2018. The disease entered China in August 2018 with a rapid spread across the whole country (Zhao et al. 2019). With a domestic pig population including more than 50% of the world´s pig population, the epidemic outbreak in China threatens the global swine industry and food security (Dixon, Sun, and Roberts 2019).

The chosen gene sequences encode different proteins that serve as highly expressed virion-associated candidate antigens of ASFV. EP402R (CD2v) is an immunogenic protein, which provided partial protection in different studies, correlated with induction of specific CD8+ T-cell responses in absence of detectable ASFV antibodies (Lopera-Madrid et al. 2017; Argilaguet et al. 2013; Lacasta et al. 2014). Furthermore it induces IFN-β signal pathways resulting in apoptosis in swine lymphocytes/macrophages (Chaulagain et al. 2021). Polyprotein pp220, with a molecular mass of about 220 kDa, is a huge protein (Simon-Mateo, Andres, and Vinuela 1993) whose mature products together with those of pp64, co-assemble into the core shell, which accounts for more than a third of the total mass of the ASFV virion (Salas and Andrés 2013). The genes K145R and pI73R both code for mostly uncharacterized but immunogenic and highly expressed proteins of ASFV (Keßler 2019; Kollnberger et al. 2002; Netherton et al. 2019). Moreover K145R proved to be a potential negative serological marker in diagnostic tests for DIVA-strategy (Rathakrishnan et al. 2021). For these reasons, Ep402R, pp220, K145R and I73R were selected for construction of promising MVA-ASFV candidates in this study.

For strong expression of recombinant MVA-ASFV constructs, the modified VACV early/late promoter H5 (PmH5) was used. This synthetic promoter

modified by Wyatt et al. is able to induce a strong gene expression throughout the whole replication cycle, containing a specifically strong early component (Wyatt et al. 1996), which is beneficial for induction of cellular immune response. In addition, it improves the genetic stability of the integrated foreign gene and the immunogenicity recombinant MVA retains even after multiple cell passages (Wang et al. 2010).

The chosen ASFV genes, were integrated through homologous recombination in deletion site III of MVA. Deletion III is one out of six deletion sites in the MVA genome and has been successfully tested for stability as well as precise insertion of foreign genes (Volz and Sutter 2017; Meyer, Sutter, and Mayr 1991). Homologous recombination is a naturally occurring phenomenon of vaccina viruses and was used early on to integrate foreign genes (Mackett, Smith, and Moss 1982). With the aid of selection markers in the initial virus and vector plasmid, recombinant MVAs were visually selected using a fluorescence microscope. This is a simple method with pronounced effectiveness, as the use of selective substances such as mycophenolic acid (MPA) can be dispensed with and the risk of reduced replication capacity of viruses and cells is minimized (Kremer et al. 2012; Falkner and Moss 1988).

4. *In vitro* characterization of recombinant MVA constructs

To validate the correct insertion of the foreign ASFV genes and to demonstrate the genetic stability as well as identity of the MVA genome, a specific fingerprint PCR was performed on all recombinant MVAs generated (Fig.11). This PCR analyzed all six major deletion sites of the recombinant MVA, excluding the presence of wild type virus in the virus preparation. A short sequence product, in this case the empty deletion site III (447 bp) would be preferred in amplification in contrast of the longer sequence product of the ASFV genes (0.782 kb-8.043 kb). Since there was no short band of 447 bp detected in any constructed MVAs, the lack of wild type virus can be assumed. Further the GOI-PCR mapping a part of the inserted ASFV gene with specific oligonucleotide primers confirms the correct insertion of the foreign gene into MVA. Interestingly, the large variance in product size of the selected ASFV target

sequences (0.782 kb-8.043 kb) confirms one of MVAs advantages as a vector platform, to have the capacity to insert large amounts of foreign DNA into its genome (Antoine et al. 1998; de Vries and Rimmelzwaan 2016).

The multi-step growth kinetic of recombinant MVA constructs on permissive CEF cells, consists of no deficits in replication levels compared to the non-recombinant MVA-F6. Analyzing the growth curves of all constructs, a titer loss appears after 4 hpi. This phenomenon is based on the “uncoating” during the infection cycle of poxviruses, what takes place after penetration of the host cell membrane (Joklik and Becker 1964) and depends on the less detectible number of infectious virions during the virus´ DNA-replication cycle (Garber et al. 2009). All multi-step growth kinetics performed showed the same growth behavior and effective replication, including the nonrecombinant MVA-F6 on permissive cells. The titer level appears to be 10^3 times higher than the initial infection titer level after 48 hpi. The human cell line HaCaT and the mammalian cell line PK15, which is based on porcine kidney cells were not permissive for productive virus growth of the constructed recombinant MVAs. This confirmed the characteristic replication deficiency of MVA in cells of mammalian origin (Volz and Sutter 2017), including for the recombinant MVAs, and validates their handling at safety level one. Also important to note, whole growth curve on HaCaT cells does not reach effective titer-levels higher than 10^4 pfu.

Successful investigation of growth kinetics was followed by analysis of the ability of recombinant MVA constructs to produce ASFV protein, determined by immunoblotting. As demonstrated in western blot analyses, MVA is able to express high levels of ASFV proteins. The protein CD2v encoded by gene EP402R, has a predicted molecular weight of 42-46 kDa. It consists of an N-terminal extracellular and a C-terminal cytosolic region (Rodríguez et al. 1993). Immunoblotting of MVA-EP402R revealed a band of size 70-100 kDa, which led to the hypothesis of glycosylation of this protein. Kay-Jackson et al. showed that CD2v is intracellularly transported, by the host protein SH3P7, from the endoplasmic reticulum via the Golgi apparatus to the cell surface and gets glycosylated on its way. On the one hand they showed in absence and presence of tunicamycin (inhibitor of N-linked glycosylation) a reduction of the molecular weight from 100 kDa to the predicted molecular mass of ~42 kDa. On the other hand, they underlined the hypothesis that CD2v is transported and glycosylated

on the way to the host cell surfaces with digestion through endo-H and endo-F. Endo H only cleaves the glycosyl groups added within the endoplasmic reticulum, while endo-F is able to cleave complex glycosyl groups added from the trans-Golgi network. The result showed a reduction of the molecular mass after the digestion with endo-H, but part of the molecular mass was resistant to digestion with endo-H. This can be compared to the digestion with endo-F, which resulted in a disappearance of the molecular mass which remained while digesting with endo-H. (Kay-Jackson et al. 2004). The results of our study showing the expression of CD2v protein by MVA-EP402R digested with Endo-H and PNGase F are in agreement with the results described. This confirms, first, that the protein expressed by MVA behaves in the same way as when infected with the original ASFV virus, and second, that it is capable of expressing ASFV proteins at high levels.

The polyprotein pp220 is encoded by a huge gene with 8148 bp and encompasses a described molecular weight of 220 kDa. Our calculation predicted a molecular mass of 280 kDa and was confirmed by the apparent 280 kDa band in SDS-Page analyses expressed by MVA-pp220. Simon-Mateo and Andres, also assumed a predicted molecular mass of 280 kDa of the polyprotein 220 and explained the difference between the expected 280 kDa and the observed 220 kDa by either deviations in the molecular mass evaluation by SDS-Page in this size range or by abnormal migration (Simon-Mateo, Andres, and Vinuela 1993). Therefore we assume that our expected size of the polyprotein pp220 was correctly confirmed by the result of the SDS page analysis expressed by MVA-pp220.

The SDS-Page analyses of the proteins K145R an I73R expressed by MVA-K145R and MVA-I73R, resulted a band for K145R of 17.2 kDa and a band for I73R of 8.5 kDa. These data correspond with the literature results (Yáñez et al. 1995; Alejo et al. 2018) and validate the correct expression of ASFV-genes through recombinant MVAs. In summary, all MVA constructs show the expected properties with regard to insertion of the ASFV genes, replication and expression behavior.

5. Future Perspectives

In numerous preclinical and clinical studies, MVA has proven to be an effective vaccine platform with strong immunogenic capacity and a high safety level through its replication deficit in mammalian cells with the simultaneous property to fully express recombinant proteins in non-permissive cells. A great advantage of MVA, which is particularly useful in the fight against the ASF disease, is the capability to induce a specific antibody response and a cellular immune response. The selected ASFV genes are highly interesting due to their different advantages, such as a high protein expression level, high immunogenicity of proteins or the potential to serve as negative serological marker for DIVA. Therefore, the MVA-ASFVs described in this study might be promising vaccine candidates, which need to be further tested in future studies, especially with regard to their efficacy and safety in animal models.

VIII. SUMMARY

African swine fever is a fatal hemorrhagic disease of wild boars and domestic pigs. The disease has spread continuously across three continents and has threatened global food security since the 2018 outbreak in the domestic pig population in China, resulting in a reduction of the global pig population by about a quarter. African swine fever virus (ASFV) is the causative agent of this disease and, as a large double-stranded DNA virus the only member of the family *Asfarviridae.* Despite many years of research, there is still no licensed vaccine against ASFV in Europe and most development strategies focus on live attenuated vaccines (LAV). Vaccine development currently faces the following challenges: The high complexity of ASFV coupled with the lack of a licensed continuous cell line for isolation, an unacceptable safety level due to the risk of phenotypic changes and reversion to virulence after genetic modification of LAVs, and finally the inability to distinguish vaccinated from infected animals. The current pandemic control measures supported and controlled by the OIE and EURL are based on the earliest possible diagnosis paired with preventive culling of entire herds containing ASFV infected pigs. The objective of this work was to generate new candidate ASFV vector vaccines based on the vaccinia virus MVA backbone, thus occupying a special position among vaccine technology platforms with regard to their immunogenicity as well as their high safety profile. Several previous problems in ASF vaccine development could thus be avoided. In this work, four recombinant MVA viruses expressing promising ASFV proteins were generated and subsequently characterized at the molecular and biological level. The correct insertion of the foreign genes as well as their genetic stability was demonstrated, the precise protein synthesis was shown by Western blot analysis and the growth behavior of the recombinant MVA constructs was examined. This confirmed the unrestricted ability of all recombinant MVA viruses to multiply in embryonic chicken fibroblast cells, which would enable biotechnological vaccine production on a large-scale. Finally, biological safety of the candidate MVA vectors is supported by their maintained and confirmed replication deficiencies in human and porcine cell lines. These results could form the basis for the development of a safe and effective vector vaccine against African swine fever virus.

IX. ZUSAMMENFASSUNG

Die Afrikanische Schweinepest, ist eine tödlich verlaufende hämorrhagische Erkrankung von Wildschweinen und Hausschweinen. Die Seuche hat sich kontinuierlich über drei Kontinente ausgebreitet und bedroht die globale Ernährungssicherheit seit dem Ausbruch der Seuche in domestizierten Schweinen in China 2018, was zu einer Reduktion der weltweiten Schweinepopulation um etwa ein Viertel geführt hat. Das Afrikanische Schweinepestvirus (ASPV) ist der ursächliche Erreger dieser Erkrankung und gehört als großes doppelsträngiges DNA-Virus als alleiniges Mitglied der Familie der *Asfarviridae* an. Trotz langjähriger Forschung gibt es bis heute keinen in Europa zugelassenen Impfstoff gegen das ASP-Virus und die meisten Entwicklungsstrategien konzentrieren sich auf abgeschwächte Lebendimpfstoffe (LAV). Die Impfstoffentwicklung steht aktuell vor folgenden Herausforderungen: Die hohe Komplexität von ASFV, gepaart mit dem Fehlen einer lizensierten kontinuierlichen Zelllinie zur Isolierung, ein mangelhaftes Sicherheitslevel mit der Gefahr phänotypischer Veränderungen und einer Reversion zur Virulenz nach genetischer Modifikation von LAVs, und schließlich die Unfähigkeit geimpfte von infizierten Tieren unterscheiden zu können. Die von der OIE und EURL unterstützten und kontrollierten Maßnahmen zur Einschränkung der Pandemie, beruhen auf frühestmöglicher Diagnostik, gepaart mit präventiver Keulung gesamter Schweinebestände mit ASPV infizierten Tieren. Ziel dieser Arbeit war die Generierung neuer ASPV-Vektorvakzine-Kandidaten, die auf der Grundlage des Vacciniavirus MVA-Vektorsystems basieren und damit im Hinblick auf ihre Immunogenität sowie ihre Sicherheit eine besondere Stellung unter Impfstoffplattformen einnehmen. So könnten einige vorhergegangene Probleme in der Impfstoffentwicklung gegen ASP vermieden werden. Im Rahmen dieser Arbeit wurden vier rekombinante MVA-Konstrukte generiert, die vielversprechende ASPV-Proteine exprimieren und anschließend auf molekularbiologischer Ebene charakterisiert. Es wurde die korrekte Insertion der Fremdgene, sowie deren genetische Stabilität nachgewiesen, die korrekte Proteinsynthese mittels Western-Blot-Analyse aufgezeigt und das Wachstumsverhalten der rekombinanten MVA-Viren überprüft. Dabei bestätigte sich die uneingeschränkte Vermehrungsfähigkeit aller rekombinanten MVA-Konstrukte

in embryonalen Hühnerfibroblastenzellen, wodurch eine biotechnologische Impfstoffproduktion im großen Maßstab möglich wäre. Schließlich konnte auch die biologische Sicherheit der hergestellten Vektorviren garantiert werden, da die für MVA charakteristische Replikationsdefizienz in humanen und porzinen Zelllinien erhalten blieb. Diese Ergebnisse könnten die Grundlage für die Entwicklung eines sicheren sowie effektiven Vektorimpfstoffes gegen das Afrikanische Schweinepest Virus bilden.

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XI. APPENDIX

1.1. Chemicals

Chemical	Supplier
2-Propanol ≥ 99.8 %	Carl Roth, Karlsruhe, Germany
4x Laemmli Sample Buffer	New England Biolabs, Ipswich USA
Acetone ≥ 99.5 %	Carl Roth, Karlsruhe, Germany
Biozym LE Agarose	Biozym Scientific, Hessisch Oldendorf, Germany
Clarity Western ECL Substrate	New England Biolabs, Ipswich USA
DMSO	Sigma-Aldrich, Taufkirchen, Germany
Ethanol 96 %,	Carl Roth, Karlsruhe, Germany
GelRed Nucleic Acid Gel Stain, 10 000x	Biozol GmbH, Eching, Germany
Glycin	PanReac AppliChem, Darmstadt, Germany
KPL TrueBlue™ Peroxidase Substrate	HiSS Diagnostics GmbH, Freiburg im Breigau, Germany
MACSQuant Running Buffer	Milenyi Biotec, Bergisch Gladbach, Germany
Methanol ≥ 99 %	Carl Roth, Karlsruhe, Germany
Mini-PROTEAN® TGX™ Precast Gels	New England Biolabs, Ipswich USA
Natriumchlorid	Carl Roth, Karlsruhe
Nonfat dried milk powder	PanReac AppliChem, Darmstadt, Germany
Proteinase K	Merck, Darmstadt
Q5® High-Fidality DNA Polymerase	New England Biolabs, Ipswich USA
Q5® Reaction Buffer	New England Biolabs, Ipswich USA
ReadyMix™ Taq PCR-Reaktionmix with MgCl2	Sigma-Aldrich, Taufkirchen, Germany

SOC Outgrowth Medium	New England Biolabs, Ipswich USA
Tris/Glycine Buffer	Bio-Rad, München, Germany
Triton-X100	Sigma-Aldrich, Taufkirchen, Germany
Tween20	Sigma-Aldrich, Taufkirchen, Germany
X-tremeGENE HP DNA Transfection Reagent	Roche, Basel Schweiz

1.2. Consumables

Material	**Supplier**
24-well tissue culture plates	Sarstedt, Nümbrecht, Germany
6- well tissue culture plates	Sarstedt, Nümbrecht, Germany
CryoPure tube	Sarstedt, Nümbrecht, Germany
Disposal bag	Sarstedt, Nümbrecht, Germany
Ep T.I.P.S Standard 20-300 µl	Eppendorf AG, Hamburg, Germany
Filter tips (100 µl)	Sarstedt, Nümbrecht, Germany
Filter tips (20 µl)	Sarstedt, Nümbrecht, Germany
Filter tips (200 µl)	Sarstedt, Nümbrecht, Germany
Filtopur S0.45	Sarstedt, Nümbrecht, Germany
MiniCollect vials	Greiner Bio-One, Frickenhausen, Germany
Nitrocelluose Blotting Membrane	GE Healthcare Europe, Freiburg, Germany
SafeSeal reaction tube 1.5 ml	Sarstedt, Nümbrecht, Germany
SafeSeal reaction tube 2 ml	Sarstedt, Nümbrecht, Germany
Serological pipette 10 ml	Sarstedt, Nümbrecht, Germany
Serological pipette 25 ml	Sarstedt, Nümbrecht, Germany
Serological pipette 5 ml	Sarstedt, Nümbrecht, Germany
TC flask 175	Sarstedt, Nümbrecht, Germany
TC flask 25	Sarstedt, Nümbrecht, Germany

TC flask 75	Sarstedt, Nümbrecht, Germany
Tube 15 ml	Sarstedt, Nümbrecht, Germany
Tube 50 ml	Sarstedt, Nümbrecht, Germany

1.3. Laboratory equipment

Laboratory equipment	**Supplier**
Avanti® J-26 XP Centrifuge	Beckmann Coulter, Krefeld, Germany
Biofuge fresco	Heraeus, Hanau, Germany
Biostep UV Transilluminator UST-30M-8R	Biostep GmbH, Burkhardtsdorf, Germany
Centrifuge 5424	Eppendorf AG, Hamburg, Germany
ChemiDocTMMP, Imaging System	Bio-Rad, Munich, Germany
Galaxy 170S Incubator	New Brunswick (Eppendorf), Hamburg, Germany
KEYENCE BZ-X710 All-in one Fluorescence Microscope	KEYENCE Deutschland GmbH, Neulsenburg, Germany
MJ Research PTC-200 Peltier Thermal Cycler	GMI, Ramsey, USA
Olympus CKX41	Olympus Life Sciences, Hamburg, Germany
OptimaTMLE-80K Ultracentrifuge	Beckman Coulter, Krefeld, Germany
Sonoplus	Bandelin electronic, Berlin, Germany

1.4. DNA and protein marker

Material	Supplier
1 kb DNA ladder	New England Biolabs, Frankfurt, Germany
100 bp DNA ladder	New England Biolabs, Frankfurt, Germany
Precision Dual Color Protein Ladder	New England Biolabs, Frankfurt, Germany

1.5. Commercial Kits

Material	Supplier
NucleoBond Xtra Midi Kit	MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany
PureYield Plasmid Miniprep System	Promega, Madison USA
QIAamp DNA Mini Kit (250)	Quiagen, Venlo Niederlande
Wizard® SV Gel and PCR Clean-Up System	Promega, Madison USA

1.6. Media and supplements for cell culture

Material	Supplier
DMEM	Sigma-Aldrich, Taufkirchen, Germany
DMEM (high glucose)	Sigma-Aldrich, Taufkirchen, Germany
DPBS	Thermo Fisher Scientific, Planegg, Germany

FBS	Thermo Fisher Scientific, Planegg, Germany Fisher Scientific
HEPES solution	Sigma-Aldrich, Taufkirchen, Germany
L-Glutamine	Thermo Fisher Scientific, Planegg, Germany
MEM	Sigma-Aldrich, Taufkirchen, Germany
MEM non-essential amino acid solution	Sigma-Aldrich, Taufkirchen, Germany
Penicillin-Streptomycin	Sigma-Aldrich, Taufkirchen, Germany
SFP eggs	VALO BioMedia GmbH, Cuxhaven, Germany

1.7. Buffer

Lysis buffer 1 % Triton X-100 25 mM Tris 1 M NaCl	**Transfer buffer (conc.)** 24 g Tris 114,6 g Glycin
5x Running buffer 72.5 g Glycin 15,2 g Tris 25 ml 20 % SDS	**Transfer (working solution)** 80 ml Towbin buffer (conc.) 200 ml Methanol Ad 1 l ddH2O
LB-Medium (pH= 7.5) 5 g/l NaCl 5 g/l Yeast extract 10 g/l Trypton	**50x TAE buffer (pH= 7.4)** 242 g Tris 57.1 ml acetic acid glacial 18.6 g EDTA
10x PBS 2 g/l KCl 2 g/l KH_2PO_4 80 g/l NaCl 11.5 g/l Na_2HPO_4	**LB-agar** 1.5 % Agar-Agar in LB-Medium

1.8. Software

Adobe Reader	Adobe Systems, San Jose, USA
BioRender	BioRender, Toronto, USA
DNASTAR Lasergene	DNASTAR, Inc., Madison, Wisconsin, USA
FlowJo LLC	BD Life Sciences, Ashland, USA
GraphPad prism	GraphPad Software, San Diego, USA
Microsoft Office 2016	Microsoft Corp., Redmond, USA
NetNGlyc 1.0 Server	http://www.cbs.dtu.dk/services/NetNGlyc/
Image Lab 5.0 Software	Bio-Rad, Feldkirchen, Germany
A.EL.VIS V6.1	A.EL.VIS GmbH, Hannover, Germany

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