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Safety and immunogenicity of mammalian cell derived and Modified Vaccinia Ankara vectored African swine fever subunit antigens in swine

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ABSTRACT

A reverse vaccinology system, Vaxign, was used to identify and select a subset of five African Swine Fever (ASF) antigens that were successfully purified from human embryonic kidney 293 (HEK) cells and produced in Modified vaccinia virus Ankara (MVA) viral vectors. Three HEK-purified antigens [B646L (p72), E183L (p54), and O61R (p12)], and three MVA-vectored antigens [B646L, EP153R, and EP402R (CD2v)] were evaluated using a prime-boost immunization regimen swine safety and immunogenicity study. Antibody responses were detected in pigs following prime-boost immunization four weeks apart with the HEK-293-purified p72, p54, and p12 antigens. Notably, sera from the vaccinees were positive by immunofluorescence on ASFV (Georgia 2007/1)-infected primary macrophages. Although MVA-vectored p72, CD2v, and EP153R failed to induce antibody responses, interferon-gamma (IFN-γ^+) spot forming cell responses against all three antigens were detected one week post-boost. The highest IFN-γ^+ spot forming cell responses were detected against p72 in pigs primed with MVA-p72 and boosted with the recombinant p72. Antigen-specific (p12, p72, CD2v, and EP153R) T-cell proliferative responses were also detected post-boost. Collectively, these results are the first demonstration that ASFV subunit antigens purified from mammalian cells or expressed in MVA vectors are safe and can induce ASFV-specific antibody and T-cell responses following a prime-boost immunization regimen in swine.

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1. Introduction

African swine fever (ASF) is one of the most important disease of domestic pigs (Sánchez-Vizcaíno et al., 2015). The etiology agent, ASF virus (ASFV), is a large, complex DNA arbovirus and only member of the *Asfarviridae* family (Tulman et al., 2009). ASFV shares some features with poxviruses, including cytoplasmic genome organization and gene expression (Tulman et al., 2009). Although ASFV infection is generally asymptomatic in African wild suids, ASFV infection of domestic pigs usually results in a highly contagious hemorrhagic disease (Costard et al., 2013). Pigs that survive and recover initial infection may become persistently infected and

serve as virus reservoirs in areas where the disease is endemic (Oie, 2012). Outside the African continent, the disease is endemic to Madagascar and Sardinia, and intensive eradication programs following ASFV outbreaks in Portugal, Spain, South America, and the Caribbean were successful. Since its introduction into Georgia in 2007, ASFV has spread rapidly into vast areas of Western and Southern Russia, Ukraine, and the Republic of Belarus (Costard et al., 2009). There is no effective treatment or vaccine against ASF, thus current control measures rely mainly on detection and elimination of infected animals (Sánchez-Vizcaíno et al., 2013). Studies in domestic pigs using conventional vaccine approaches such as inactivated, avirulent or live attenuated ASF viruses (tissue culture adapted, rational gene deleted) have reported varying levels of homologous protection, however, some surviving animals have been shown to develop subclinical disease, thus raising the possibility of vaccinated animals becoming carriers (Sanchez-Vizcaino et al., 2009). Although protective immune mechanisms are poorly understood, cellular immune responses (particularly CD8+ T-cells) (Oura et al., 2005; Takamatsu et al., 2013), and humoral responses (Escribano et al., 2013; Onisk et al., 1994; Wardley et al., 1985) are thought to have important roles in host protection. Early vaccination studies that tested several, B-cell immunodominant ASFV subunit, recombinant proteins (i.e., p30, p54 and p72) produced using either baculovirus or DNA-based vaccines yielded variable success (Argilaguet et al., 2012, 2011; Barderas et al., 2001; Gómez-Puertas et al., 1998). Interestingly, immune responses elicited by DNA vaccines were variable and dependent on the fusion tag (i.e., soluble HA or ubiquitin) selected for ASFV recombinant antigen expression (Argilaguet et al., 2012). A recent study using an ASFV E75 expression library containing approximately 4000 individual plasmid clones (excluding p30, p54, and CD2v) demonstrated a correlation between protection and CD8+ T-cell response (Lacasta et al., 2014). Results from this study showed that the ASFV genome (~170–190 kb) contains additional antigens with protective potential, and implied that identification of such determinants would enable advances in the development of protective subunit vaccine candidates. To this end, we applied an *in silico* bioinformatic tool to identify and rank ASFV open reading frames (ORFs) that possess attributes desirable in selecting vaccine targets. Vaxign is the first web-based vaccine design program that predicts vaccine targets based on genomic sequences utilizing the reverse vaccinology (RV) strategy (He et al., 2010b). Using the entire annotated protein sequences from genome(s), Vaxign identifies open reading frames that possess a high probability of being good vaccine candidates based on (i) protein subcellular location, (ii) transmembrane helices, (iii) adhesin probability, (iv) sequence ortholog analysis among pathogenic strains, (v) sequence exclusion from genome(s) of nonpathogenic strain(s), and (vi) epitope binding to MHC (major histocompatibility complex) class I and class II. Vaxign has been used for rational design of experimental vaccines against several intracellular pathogens including Brucella and *Rickettsia prowazekii* (Caro-Gomez et al., 2014; Gomez et al., 2013), and has been successfully used for prediction of potential vaccine targets in uropathogenic *Escherichia coli* (He et al., 2010b), *Streptococcus agalactiae* (Pereira et al., 2013), and human herpes simplex viruses (Xiang and He, 2013).

In the current study, the Vaxign tool was used to analyze 12 ASFV annotated genomes (using Georgia 2007/1 strain as the reference genome), and identify and rank open reading frames (antigens) for subsequent recombinant expression. Five ASFV genes were successfully purified from mammalian cell HEK 293 (Human embryonic kidney cells 293) and/or MVA (Modified Vaccinia virus Ankara) recombinant antigen expression systems and were subsequently evaluated for safety and immunogenicity in swine using different prime-boost immunization regimens.

2. Materials and methods

2.1. Ethics statement

Yorkshire barrows, weighing 18–20 kg, were used in accordance with USDA policies under the supervision of the Texas A&M University Institutional Animal Care and Use Committee (IACUC). The pigs were obtained from a local, commercial source (State of Texas Department of Criminal Justice, Wynne Unit, 810 FM Road West, Huntsville, TX 77349). All experimental animal work was approved under Animal Use Protocol 2013-009, and reviewed and approved by the Texas A&M University IACUC Permit 2009067. The experiments were performed under BSL-2 conditions for pigs receiving MVA-vectored ASFV antigens and under BLS-1 conditions for pigs receiving HEK 293-purified recombinant ASFV antigens through the termination of the experiment on Day 42 post-immunization. Pigs were monitored at least twice daily for any clinical signs and to document any localized and or systemic adverse effects throughout the post-immunization phase. Animal care to alleviate any animal suffering was provided by the attending veterinarian. The pigs were fed an antibiotic-free commercial pig ration twice daily and water *ad libitum*. At the end of the study, the pigs were euthanatized with an intravenous overdose of a commercial euthanasia solution (Beuthanasia-D Special, sodium pentobarbital 390 mg/ml and sodium phenytoin 50 mg/ml, Intervet/Merck Animal Health, Madison, NJ) and osculated with a stethoscope to confirm lack of heartbeat.

2.2. Vaxign ranking and recombinant antigen selection

A total of 12 ASFV genomes were used for Vaxign analysis. Five genomes were downloaded from the National Center for Biotechnology Information RefSeq database: BA71 V (Accession #: NC_001659.1), Benin 97/1 (AM712239.1), E75 (FN557520.1), Georgia 2007/1 (FR682468.1), and OURT 88/3 (avirulent field isolate) (AM712240.1). Seven genome sequences were kindly provided by Dr. John Neilan: Kenya 1950 (AY261360.1), Malawi Lil-20/1(1983) (AY261361.1), Mkuzi 1979 (AY261362.1), Pretoris-uskop/96/4 (AY261363.1), Tengani 62 (AY261364.1), Warmbaths (AY261365.1), and Warthog (AY261366.1). Since annotations of individual genes from these seven genomes were unavailable at the time of the study, the gene coding sequences of these genomes were determined by using Glimmer software (Delcher et al., 2007). Annotated protein sequences were used as input for the Vaxign pipeline analysis using the Georgia 2007/1 strain genome (Chapman et al., 2011) as the seed genome. For each protein sequence of the Georgia 2007/1 genome, Vaxign calculated and predicted the following five features: i) transmembrane domains, ii) adhesin probability, iii) sequence conservation among other ASFV strains, and epitopes binding to iv) MHC class I and v) MHC class II molecules (He et al., 2010b; Xiang and He, 2013). These five features were selected for analysis based on the following rationale: 1) the presence of transmembrane domain(s) suggested a possible virion surface location; 2) adhesin proteins suggested to be critical for virus attachment and entry; 3) expansive protection against diverse strains requires the use of conserved genes among different pathogenic ASFV strains as vaccine candidates; and 4) MHC class I/II epitope predictions for immunity determinants.

The testing of Vaxign Vaxitope method in epitope prediction has been described in previous studies (He et al., 2010b; Xiang and He, 2013). Since limited swine leukocyte antigens (SLA) specific epitopes were available for training of the epitope prediction function of Vaxitope, the human leukocyte antigen (HLA) epitope prediction method in Vaxitope was used to predict ASFV epitopes for binding to swine MHC. For the epitope screening for HLA alleles, we used six "Supertype" HLA Class I alleles and eight "supertype"

HLA Class II alleles as reported previously (De Groot et al., 2009). This approach was rationalized based on reports that swine and human share many similarities in terms of genetics and physiology (Meurens et al., 2012; Suenderhauf and Parrott, 2012) and human MHC and swine SLA complexes share multiple paralogous regions (Chardon et al., 2000). The key feature of the MHC gene clusters is the encoding of class I and class II membrane-anchored glycoproteins that differ in structure, cellular and tissue expression specificity, origin of the peptides presented to T-cells, and T-cell subset activation (Chardon et al., 2000). The SLA class I antigens are constitutively expressed on the surface of nucleated cells and present peptides derived from nuclear and cytosolic proteins to CD8+ cytotoxic T-cells. They also interact with natural killer cells to prevent NK-mediated cytotoxicity (Lunney et al., 2009). The SLA class II antigens are expressed primarily on the surface of antigen presenting cells such as macrophages, B-cells, T-cells, and dendritic cells and present peptides derived from exogenous proteins to CD4+ helper T-cells (Lunney et al., 2009).

The Vaxign scores for each feature were combined using weighted ranking strategy conceived based on the project team's knowledge of the factors contributing to potential ASF protection. Specifically, the following ASFV weighted ranking was used: 35% adhesion probability, 20% MHC Class I epitope binding, 20% MHC Class II epitope binding, 15% transmembrane domain, and 10% sequence conservation among the 12 ASFV genomes analyzed. The highest weight of 40% was assigned to MHC class epitopes I and II epitope prediction scores since MHC class I epitopes may stimulate cytotoxic CD8+ T-lymphocyte activity while MHC class II epitopes may stimulate CD4+ helper T−cell and antibody activity. Adhesion probability and transmembrane prediction scores assigned weights were based on their potential contribution to protein topography and virulence. This weighted ranking strategy was applied to calculate a single score for each of the ORFs in the Georgia 2007/1 genome.

2.3. Recombinant protein expression in human embryonic kidney 293 cells

The coding sequences for the 14 selected candidates (see Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004) were submitted for mammalian codon optimization using Blue Heron Biotech's codon optimization tool and chemically synthesized (Seattle, USA). Sequences (without ATG of each gene) were cloned into the KpnI and BamHI sites of pcDNA4/HisMax A vector (Invitrogen catalog # V864-20, Carlsbad, USA) to obtain pcDNA4/HisMax A-ASFV constructs. Sequence-verified plasmids were transfected into HEK 293 Freestyle cells (Invitrogen catalog # R790-07) using $42\,\mu g$ of plasmid per 30×10^6 cells in an orbital shaker at $37\,°C$. Cells were harvested, lysed, and proteins purified using sequential affinity tag and ion-exchange chromatography. Proteins were analyzed by Western blot using ASF convalescent serum to confirm recombinant protein expression.

2.4. Recombinant protein expression in baculovirus

The pcDNA4/HisMax A-ASFV constructs were used as template for PCR. New fragments were cloned as secretion competent MBP fusions in previously described baculovirus shuttle vectors (Brown et al., 2011). High five cells (seeded in 1 l at 2×10^6 cells/ml in 2.8 l Fernbach flasks) were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 2, and grown at $20\,°C$ for 72 h. The recombinant proteins were purified by chromatography using amylose resin, metal-affinity, or size exclusion columns; and subsequently solubilized in 50 mM sodium phosphate (pH 7.4), 300 mM sodium chloride and 10% glycerol with 0.2% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (final

storage buffer). Recombinant proteins were analyzed by Western blot using ASF convalescent serum to confirm protein expression. Five recombinant proteins (B646L (p72), E183L (p54), EP153R (C-type lectin), EP402R (CD2 v), and O61R (p12)) were used for evaluating T-cell responses.

2.5. Generation of Modified Vaccinia Ankara (MVA) recombinant viruses

Individual pcDNA4/HisMax A-ASFV constructs bearing the ORF of B646L, EP153R, and EP402R were used as templates for PCR amplification. PCR products contained, at the 5′ end, a BamHI cloning site prior to an ATG codon, and an EcoRI cloning site at the 3'end followed by the a TAA codon and the vaccinia transcription terminator sequence TTTTTCT. The PCR-amplified fragments were digested with BamHI and EcoRI restriction enzymes and cloned into pI2-RED plasmid, which encodes the DsRed fluorescent protein flanked by sequences of the TK locus of MVA genome for homologous recombination.

All ORFs were expressed by the strong SE/L (early/late) vaccinia promoter. Virus recombination and amplification were performed in CEF (chicken embryo fibroblast) cells according to published procedures (Staib et al., 2004). ASFV recombinant protein expression and sequence of inserted DNA were confirmed for all recombinant MVA-ASF constructs.

2.6. Swine safety and immunogenicity study

Twenty-two pigs (Yorkshire, barrows) were sourced and vaccinated intramuscularly (IM) (Streptococcus suis autogenous vaccine, MVP Labs, Omaha, NE; Porcine circovirus, Circumvent pcv Type 2 M^{TM}, Intervet Inc./Merck Animal Health, Omaha, NE) at 28 days of age. Additionally, at 57 days of age, the pigs received intramuscular vaccinations (Actinobacillus pleuropneumoniae serotypes 1, 5 and 7 bacterin, Emulsivac^{TM}, MVP Labs, Omaha, NE; Porcine circovirus, Circumvent pcv Type 2 M^{TM}, Intervet Inc./Merck Animal Health, Omaha, NE; Streptococcus suis autogenous vaccine, MVP Labs, Omaha, NE; Erysipelothrix rhusiopathiae Ery-Shield^{TM}, Novartis Animal Health, Inc., Larchwood, IA). At 70 days of age, the pigs were vaccinated IM (Swine influenza H1N1, H1N2, H3N2, FluSure XP^{TM}, Zoetis, Inc., Kalamazoo, MI; Porcine reproductive and respiratory syndrome virus, Fostera PRRS^{TM}, Zoetis, Inc., Kalamazoo, MI; Actinobaccillus pleuropneumoniae, Bordatella brochiseptica, Erysipelothrix rhusiopathiae, Hemophilus parasuis, Pasteurella multocida bacterin, Parapleuro Shield P+ BE^{TM}, Elanco, Inc., Larchwood, IA; Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, L. pomona bacterin, Lepto Shield 5^{TM}, Elanco, Inc., Larchwood, IA). These vaccinations were administered in accordance with guidelines for intrastate swine movement.

Pigs were immunized IM with antigens as outlined in Table 1. On day 0 of the experiment when the pigs were at 77 days of age, weighing 18–22 kg, recombinant MVA-ASFV constructs were administered IM at 10^7 TCID$_{50}$ (tissue culture infective dose) in 1 ml dose of Tris 1 mM (pH 9.0), and HEK-purified recombinant ASFV antigens were administered in a 2 ml cocktail comprised of $200\,\mu g$/antigen (PBS [phosphate-buffered saline], pH 7.4) and TS6 adjuvant (kindly provided by Merial, Duluth, GA). TS6 is a proprietary exclusive oil in water, animal lipids free adjuvant, and able to stimulate cell mediated immunity and antibodies; details may be found in US patent number US7371395 B2, Merial Limited, Duluth, GA. Injection sites were tattooed and clinically examined daily throughout the 42-day experiment. Peripheral blood samples were collected at days −14 (pre-bleed), 14, 28 (pre-boost), 35 and 42 (last day of in-life phase) for immunological readouts.

Table 1
Swine study design.

Treatment Group	Number of pigs	Pig ID[a]	Prime (Day 0)	Boost (Day 28)	IM[b] site[c]
T1	5	1–5	B646L-HEK	B646L-HEK	3
			E183L-HEK	E183L-HEK	5
			O61R-HEK	O61R-HEK	4
T2	3	6–8	TS6 adjuvant	TS6 adjuvant	1
T3	6	9–14	MVA- B646L	B646L-HEK	3
			MVA-EP153R	PBS[d]	2
			MVA-EP402R	EP402R-HEK	1
T4	6	15–20	MVA- B646L	MVA- B646L	3
			MVA-EP153R	MVA-EP153R	2
			MVA-EP402R	MVA-EP402R	1
T5	2	21–22	MVA vector	MVA vector	1

Pigs received two intramuscular immunizations at 28 day intervals, boosted with matching antigens at same site and euthanized 14 days post-boost.

[a] ID (identification number of each pig in the study).
[b] IM: intramuscular.
[c] Immunization sites (1: left atlantal draining lymph node, 2: left prescapular draining lymph node, 3: left popliteal draining lymph node, 4: right atlantal draining lymph node, 5: right prescapular draining lymph node).
[d] PBS was used for this site injection since the required amount of EP153R-HEK recombinant antigen was unavailable.

2.7. ASFV-specific antibody detection − ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate ASFV antigen-specific antibodies in pig sera. Briefly, ELISA plates were coated with 100 μl B646L-HEK (3 ng/μl), E183L-HEK (5 ng/μl), and O61R-HEK (5 ng/μl) diluted in 50 mM Carbonate-Bicarbonate Buffer (pH 9.6) and incubated overnight. Plates were washed thrice using PBST (phosphate-buffered saline with Tween 20) (Sigma, St. Louis, USA), blocked with 200 μl Blocking buffer (10% Non-fat milk in 1X PBS, pH 7.6) for 1 h, and removed. Subsequently, 100 μl of diluted serum (1:500 in blocking buffer) from each immunized animal was added to the plates, and incubated at room temperature for 2 h. Plates were washed thrice with PBST and 100 μl (1:5000 in blocking buffer) of horseradish peroxidase conjugated goat anti-porcine IgG (6050-05; Southern Biotech, Birmingham, USA) was added, incubated at room temperature for 1 h. Plates were washed thrice with PBST and 100 μl peroxidase substrate (SureBlue Reserve TMB [tetramethylbenzidine], KPL, Gaithersburg, USA) was added. After incubation at 30 min at room temperature, 100 μl TMB Stop Solution (KPL) was added and spectrophotometric absorbance (450 nm) measured. The results were analyzed using analysis of variance (ANOVA); a significance level of $P < 0.05$ was used for all analyses.

2.8. ASFV-specific antibody detection −immunofluorescence assay (IFA)

Teflon-coated slide wells (Electron Microscopy Sciences, Hatfield, USA) were incubated with 0.3 mg/ml of rat-tail collagen (Corning, New York, USA) diluted in Dulbecco's Phosphate Buffered Saline (D-PBS [Dulbeccos's phosphate-buffered saline], Invitrogen) for 60 min at 37 °C and then oven-dried for 30 min. Coated slides were incubated overnight in a biological safety cabinet (15 cm from the UV light). Fresh peripheral blood mononuclear cells (PBMCs) were enriched for macrophages (Genovesi et al., 1990) and rinsed with PBS three day post-enrichment. Macrophages were detached from flasks with 10 mM EDTA (Ethylenediaminetetraacetic acid), and centrifuged for 10 min at 180xg. The pelleted cells were suspended in complete culture media and infected at a multiplicity of infection of 1 with ASFV (Georgia 2007/1) for 1 h at 37 °C. Then, 25 μl of infected or mock-infected cells (approximately 4×10^5 cells) were placed into wells of the pre-coated Teflon slides, and incubated overnight at 37 °C with 5% CO2. The slides were fixed

with a chilled (−20 °C) solution comprised of reagent grade acetone and methanol (1:1) for 10 min and stored at −70 °C until use.

For IFA evaluation, a D-PBS based blocking solution [2% equine sera (HyClone Lab, Logan, USA), 2% calf sera (Invitrogen), 2% fetal bovine sera (HyClone), 5% non-fat dry milk (Carnation, Markham, Canada) and 5% bovine serum albumin (Sigma Aldrich A9418)] was added for 30 min in a humidified chamber at 37 °C. Serial dilutions (1:50, 1:250, and 1:500 in blocking solution) of serum samples from immunized or control pigs were incubated with infected and mock-infected wells for 1 h at 37 °C. Commercial swine sera (1:100; Jackson ImmunoResearch, West Grove, USA) was used as a negative control and swine serum (1:500) from a convalescent animal inoculated with multiple ASFV isolates (kind gift from E. J. Kramer, Plum Island Animal Disease Center) was used as a positive control. Slides were rinsed three times with D-PBS and incubated with goat anti-swine sera conjugated with fluorescein isothiocyanate (1:100) (FITC; KPL) for 45 min at 37 °C. The slides were washed three times with D-PBS and mounted with Prolong® Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). An Olympus immuno-fluorescent microscope (Model BX-40) and an Olympus digital camera (Model DP 70) were used to record images. IFA was performed at Plum Island Animal Disease Center.

2.9. IFN-γ ELISpot and ³H-Thymidine incorporation

On days 7, 15, 21, 28, 35, and 42, PBMCs were isolated and used to evaluate interferon gamma (IFN − γ) responses against recombinant ASFV antigens (10 μg/ml) by enzyme-linked immunospot assays (ELISpot) using Porcine IFN-γ ELISpot kit (MABTECH, Inc., Cat.# 3130-2A, Cincinnati, USA). ELISpot readouts are reported as Spot Forming Cells (SFC)/million PBMCs. ³H-Thymidine incorporation was used to quantify T-cell proliferation against the recombinant ASFV antigens pre- and post-immunization using PBMCs as previously described (Njongmeta et al., 2012). Briefly proliferation assay was conducted using 2.5×10^5 PBMCs/well in triplicate-wells of 96-well plates in a total volume of 100 μl of complete medium containing recombinant ASFV antigens (10 μg/ml). ConA mitogen (1.3 μg/ml) was used as the positive control, whereas culture media served as the negative control. Recombinant maltose binding protein (MBP) was used (10 μg/ml) as a control for the recombinant ASFV proteins expressed as MBP-fusions. The cells were cultured for 72 h at 37 °C with 5% CO2, labeled with 0.25 μCi of ³H-thymidine for 6 h, collected using an automated cell harvester (Tomtec, Hamden, USA), and the incorporated ³H-

thymidine was counted with a liquid scintillation counter. The incorporation of ^{3}H-thymidine by the proliferating B-cells was presented as mean counts per minute (cpm) of triplicate wells. ELISpot and proliferation assay readouts were analyzed using analysis of variance (ANOVA) followed by determining Fishers Least Significant Difference if distributional assumptions for ANOVA were met or a Kruskal-Wallis test (non-parametric equivalent of ANOVA) if ANOVA was not appropriate. A significance level of $P < 0.05$ was used for all analyses.

3. Results

3.1. Vaxign ranking results

All ASFV ORFs were weighted and ranked using Vaxign methods as described in the Methods section. The Vaxign top 30 ranked ORFs are provided in Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004. Although the Vaxign ranking was used as the basis for selection of vaccine candidate antigens, the final candidates selected for immunogenicity evaluation in swine considered additional factors, such as gene function, and previously reported antigenic and immunogenic properties of particular genes. Based on these considerations, twelve of the top 30 Vaxign ranked ORFs and two previously well characterized antigens with reported induction of neutralizing antibodies which may facilitate protective immunity, B646L (p72) and CP204L (Neilan et al., 2004), were also selected for attempted expression in both HEK 293 cells and in MVA viral vectors (see Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004).

3.2. HEK and MVA recombinant proteins

An attempt was made to generate fourteen recombinant ASFV antigens using the HEK-293 cells transfected with plasmid constructs encoding sequence-verified codon-optimized synthetic genes, but only seven antigens were successfully expressed: E146L, E183L, E199L, E248R, EP402R, O61R and B646L (see Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004). Troubleshooting did not yield different outcomes and the factor(s) responsible for the lack of expression of some of the antigens in HEK-293 cells are unknown. Among the seven ASFV constructs that were expressed, four proteins (O61R, E183L, B646L, and EP402R) were successfully purified, as demonstrated by Western blot (see Supplemental Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004) in sufficient quantity for target immunizing doses required for the swine immunogenicity study. Three proteins, E146L, E199L, and E248R, which contain putative transmembrane domains, were insoluble in Triton X-114. Due to limited funding and time constraint, further optimization of protein solubilization and purification were not pursued.

Ten candidates (see Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004) were selected for MVA construction and three recombinant MVA-ASFV constructs (B646L, EP153R, and EP402R) were scaled up successfully, as demonstrated by Western blot (Supplemental Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004), in sufficient quantity for the swine immunogenicity study. Further optimization of the MVA constructs was required for the remaining seven candidates, E146L, I10L, E183L, E199L, E248R, O61R, and CP204L (see Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetimm.2017.01.004), since the resulting recombinant MVAs expressing these proteins were unstable and did not grow in cell culture. The genetic instability observed in these recombinants might be due to toxicity of the gene products to the MVA-infected fibroblasts, since the addition of the secretory signal tPA (human tissue plasminogen activator) was required to successfully generate these recombinant MVA-ASFV viruses. Since extended time and optimization were required for successful generation of these MVA constructs, which occurred after the completion of the swine immunogenicity study, these constructs were not evaluated in swine immunogenicity study.

All fourteen candidates were also selected for expression in the baculovirus protein expression system. All but one (I329L) of the fourteen candidates were successfully expressed and purified from baculovirus. The five recombinant antigens which matched the HEK 293 (B646L, E183L, O61R,) and MVA produced (B646L, EP153R, EP402R) antigens used for swine immunizations were used as reagents for the T-cell response assays.

A final subset of five recombinant antigens (E183L, EP402R, EP153R, O61R, and B646L) that were successfully purified in sufficient quantity were utilized for testing and evaluation in the swine safety and immunogenicity study (Table 2) (Borca et al., 1994a,b; Galindo et al., 2000; Gómez-Puertas et al., 1998). Four antigens exhibited optimal Vaxign ranking in the range of 4–20 and one antigen exhibited a low ranking of 107; the range of Vaxign ranking is 1–192, 1 is maximum and 192 is minimum.

3.3. Safety of HEK-ASFV recombinant proteins and MVA-ASFV recombinant viruses in swine

None of the intramuscularly injected test articles or adjuvant control preparations induced local or systemic lesions in the pigs. One injection site (HEK-ASFV) had a 2.5 cm encapsulated abscess containing white inspissated pus and necrotic debris detected at post-mortem examination on day 42. Abscesses, culture positive for Trueperella pyogenes, Streptococcus equisimilis, or Streptococcus porcinus, unrelated to the injection sites were observed in 10 of 22 pigs during the first two weeks of the experiment. Since no adverse tissue reactions or granulomas in the injection sites of subunit proteins, adjuvants, or MVA preparations after the primary and booster injections, nor febrile response in any of the vaccinated pigs after either vaccinations were detected, these results indicate that the preparations were not toxic within the limits of these clinical parameters.

3.4. ASFV antigen-specific antibody responses

To evaluate the induction of humoral responses, sera from all test animals (Treatment Groups 1–5) were analyzed for antigen-specific antibodies titers by ELISA. In T1, B646L-specific IgG responses were detectable in four of five pigs at 35 and 42 days post-immunization (1 and 2 weeks post boost) (Fig. 1A). All five pigs immunized with HEK-derived E183L and O61R showed statistically significant IgG responses at 35 and 42 days post-immunization compared to pre-immune and pre-boost sera (Fig. 1B). No specific IgG response was detected in sera from immunized pigs that received a MVA-ASFV prime dose (T3-T5).

To confirm these ELISA results, sera from day 42 (2 weeks post-boost) were tested for reactivity to Georgia 2007/1-infected macrophages by immunofluorescence assay (IFA) (Fig. 2). In T1, diluted (1:50, 1:250, and 1:500) sera from 4 of 5 pigs showed ASFV-specific IgG responses, whereas sera from T2 pigs, TS6 adjuvant injected negative control groups, diluted 1:50 showed no IgG responses (Fig. 2). Consistent with ELISA results, sera from T3 and T4 animals immunized with recombinant MVA-ASFV demonstrated no detectable IFA reactivity, no IgG responses.

Table 2
ASFV recombinant antigens used for the swine immunogenicity study.

Gene Feature	Gene Name				
	E183L	EP402R	EP153R	O61R	B646L
Vaxign Ranking	4	15	17	20	107
Protein ID	CBW46791.1	CBW46724.1	CBW46723.1	CBW46764.1	CBW46748.1
Length (aa)	184	360	158	61	646
Predicted size (kDa)	19.9	41.0	18.4	6.7	73.2
Adhesin Probability score	0.566	0.457	0.372	0.41	0.248
Transmembrane helices	1	1	1	1	0
hMHC-I epitopes score	0.668	0.685	0.730	0.636	0.653
hMHC-II epitopes score	0.729	0.659	0.781	0.632	0.739
Present in all 12 ASFV genomes (Y/N)	Y	Y	Y	Y	Y
Protein localization on viral particle	Viral capsid	Viral capsid	Localized to host cell	Viral capsid	Viral capsid
Gene function	Virus entry (Gómez-Puertas et al., 1998)	RBC hemadsorption (Borca et al., 1994b)	Enhances RBC hemadsorption (Galindo et al., 2000)	Viral attachment protein (Gómez-Puertas et al., 1998)	Viral capsid protein (Borca et al., 1994a)

The Georgia 2007/1 strain genome was used as the seed genome. Vaxign ranked each protein based on predictions for the following features: the number of transmembrane helices, adhesin likelihood score, MHC-I and II epitopes score, and sequence conservation among 12 ASFV strains. The range of the feature score unit is 0–1; 0 is minimum and 1 is maximum. The range of the Vaxign rank is 1–192; 1 is maximum and 192 is minimum; 192 corresponds to the total number of identified ORFs within the ASFV genome.

3.5. Measurement of ASFV antigen-specific T-cell responses in immunized pigs

Baseline (pre-immunization) T-cell reactivity against baculovirus-expressed purified ASFV antigens was tested using PBMCs prior to vaccination. Background reactivity (media alone) was relatively low and the mitogen positive control (ConA) worked as expected, although the negative control (Maltose Binding Protein, MBP) used for some ASFV-MBP recombinant antigens yielded high background counts, which may be due to prior exposure of study animals to E. coli.

PBMCs from immunized swine were collected at days 35 and 42 and used for IFN-γ ELISpot and T-cell proliferation assays. B646L-specific IFN-γ T-cell responses in pigs primed with recombinant MVA-ASFV and boosted with HEK-ASFV antigens were significantly higher ($P < 0.05$) (T3) compared to pigs primed and boosted with the MVA-ASFV constructs (T4) or the MVA-sham treated pigs (T5) (Fig. 3). One pig in T4 exhibited a strong response one week post-boost, however this response waned at two weeks post-boost (Fig. 3). There were no significant B646L-specific IFN-γ T-cell responses at either one or two weeks post-boost in pigs primed and boosted with HEK-ASFV cocktails (T1) (Fig. 3). This was also observed for each of the HEK-ASFV antigens tested. Overall, MBP negative control IFN-γ T-cell response were relatively high and may have masked any ASFV antigen-specific responses.

Antigen-specific T-cell proliferation responses were detected in all antigen treatment groups compared to the negative controls. Among the pigs primed and boosted with the HEK-ASFV antigens (T1), PBMCs from one pig (#5) exhibited a strong response against O61R at day 42 (Fig. 4A). However, the T1 mean PBMC response was not statistically different from the mean of the negative control treatment (T2) (Fig. 4). The T1 treatment group exhibited a statistically significant ($P < 0.05$) response to B646L at two weeks post-boost (Fig. 4B). No response was observed against E183L at two weeks post-boosting (Fig. 4C).

Among pigs primed with the recombinant MVA-ASFV and boosted with the HEK-ASFV antigens (T3), lymphocytes from 4 and 6 pigs responded to B646L at days 35 and 42, respectively; 3 and 1 pig (s) responded to EP153R at days 35 and 42 respectively; and 5 pigs responded to EP402R at days 35 and 42 (Fig. 5A–C). Among the pigs primed and boosted with the recombinant MVA-ASFV constructs (T4), 3 and 4 pigs responded to B646L at days 35 and 42, respectively; 5 pigs responded to EP153R at days 35 and 42; and 6 and 4 pigs responded to EP402R at days 35 and 42, respectively

(Fig. 5A–C). In addition, PBMCs from two pigs immunized with MVA-B646L (p72) responded against adenovirus-expressing B646L (Fig. 5D), demonstrating responses to two different expression formats of the B646L antigen (baculovirus and adenovirus).

4. Discussion

Since its accidental introduction almost a decade ago in the Caucasus region and subsequent spread into eastern Russia, ASFV continues to pose a significant risk to wild and domestic swine populations in Europe and Asia (Sánchez-Vizcaíno et al., 2015). Vaccine development progress has been hindered by the gap in knowledge of antigens that can be effectively used for protective prophylactic vaccination. ASFV has a relatively large genome and encodes for some proteins that play a role in host immune response evasion (Dixon et al., 2004). To date, inactivated ASFV preparations, live, attenuated ASFV viruses, bacteria expressed recombinant subunit antigens, and DNA vaccine approaches have all been met with limited success in providing consistently high efficacy against homologous or heterologous challenge with virulent ASFV (Argilaguet et al., 2013; Barderas et al., 2001; Jenson et al., 2000; Ruiz-Gonzalvo et al., 1996). In order to help identify potential new ASFV antigen targets and recombinant antigen delivery systems, in the present study an *in silico* antigen prediction program, Vaxign, was used in combination with two different ASFV recombinant antigen delivery systems - mammalian cells and modified Vaccinia Ankara.

Vaxign predicts antigenic protein candidates *in silico* from genome sequences using immunoinformatics algorithms to predict T- and B-cell epitopes (He et al., 2010a) and its performance and comparison to other informatics tools have been reported (He et al., 2010b; Xiang and He, 2013). Vaxign predicts relevant features by utilizing publicly available and in house/internally developed bioinformatics tools. For example, Vaxign utilizes HMMTOP for transmembrane helix topology analysis (Kall et al., 2007), SPAAN for adhesion probability prediction (Sachdeva et al., 2005), and OrthoMCL (Li et al., 2003a) for sequence conservation analysis. For MHC class I and class II binding epitope prediction, an internally developed bioinformatics tool, Vaxitope, was used (He et al., 2010b; Xiang and He, 2013). The Vaxitope program is based on the position specific scoring matrice (PSSM) (Stormo et al., 1982) and refinements to the cutoff scoring. Instead of a percentage or top number, Vaxitope utilizes the statistical P value of 0.05 to determine the
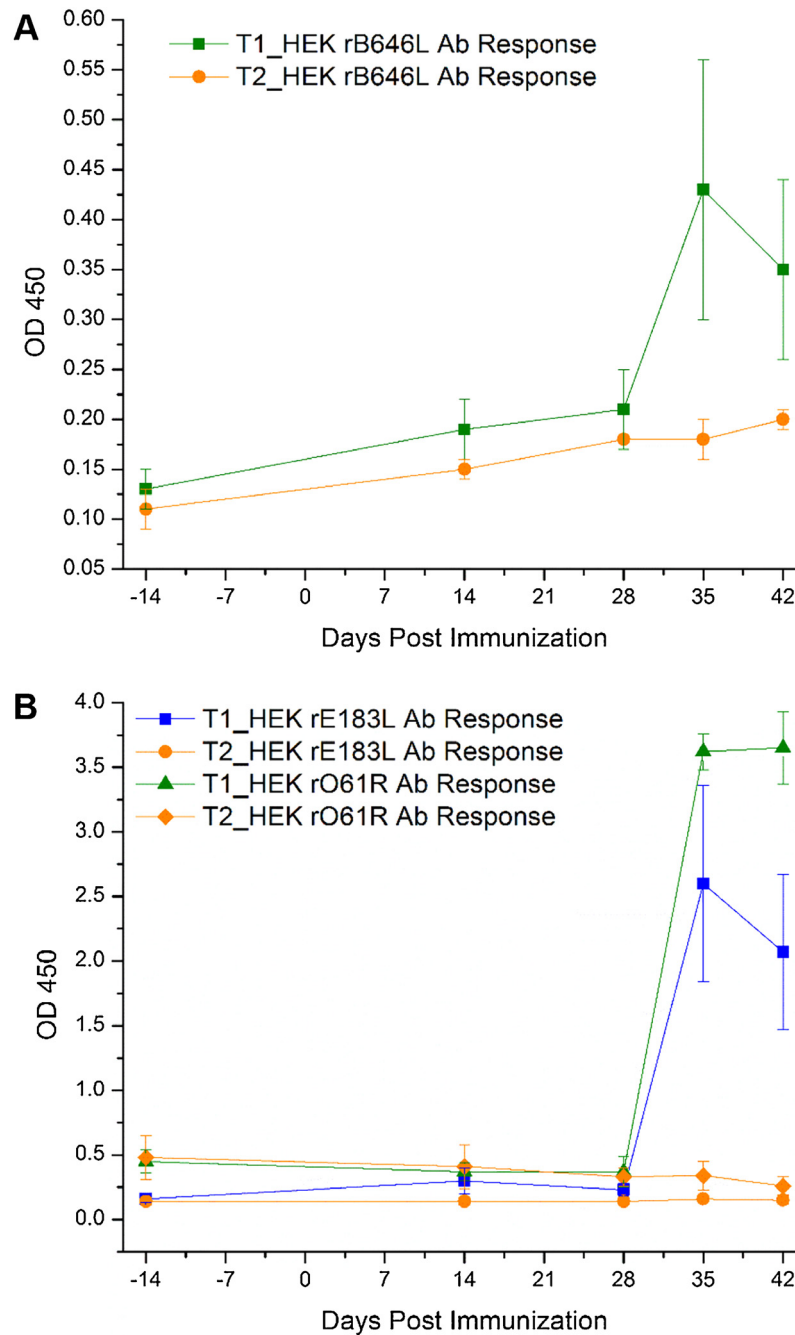
Fig. 1. ASFV-specific antibody response detection by ELISA. Antibody responses (IgG) to recombinant ASFV antigens were assessed by ELISA using sera from experimental (T1) and adjuvant only (T2) groups. Average OD values and standard deviations for sera collected at indicated days post-immunization (dpi) are provided. (A) rB646L specific antibody responses; and (B) rE183L and rO61R specific antibody responses. ANOVA and Turkey-Kramer analysis indicate statistically significant difference ($p < 0.05$) for day 35 and 42 sera from day −14, 14, and 28 sera.

cutoff value that provides high sensitivity and specificity (He et al., 2010b; Xiang and He, 2013).

Since SLA epitopes knowledge was limited, the HLA epitope prediction method for predicting ASFV epitopes for swine MHC classes was employed as an approximate approach. The employed approach was justified by 1) the high degree of structural similarity and identification of cross-species influenza virus cytotoxic T lymphocytes epitopes between HLA and SLA class I molecules (Zhang et al., 2011), 2) phylogenetic analyses that demonstrated strong sequence homology between SLA and HLA class II genes (Smith et al., 2005), and 3) previously reported swine epitope prediction studies (Burgara-Estrella et al., 2013; Díaz et al., 2009; Zimic

et al., 2011). Recent advancements associated with swine epitope prediction have been reported (Fan et al., 2016; Gutiérrez et al., 2016, 2015) and these expanded immunoinformatics knowledge and improved tools may result in better prediction of ASFV SLA epitopes for future analysis.

The Vaxign ranking is this study was based on a method of assigning different weights for each analysis feature. The weighting was based on parameters related to potential immunogenicity (e.g., 40% weight to MHC class epitopes, 35% weight to adhesion probability, 15% to transmembrane domain presence, and 10% to sequence conservation). A 35% weight was assigned to adhesion probability since adhesion proteins may be surface attachment
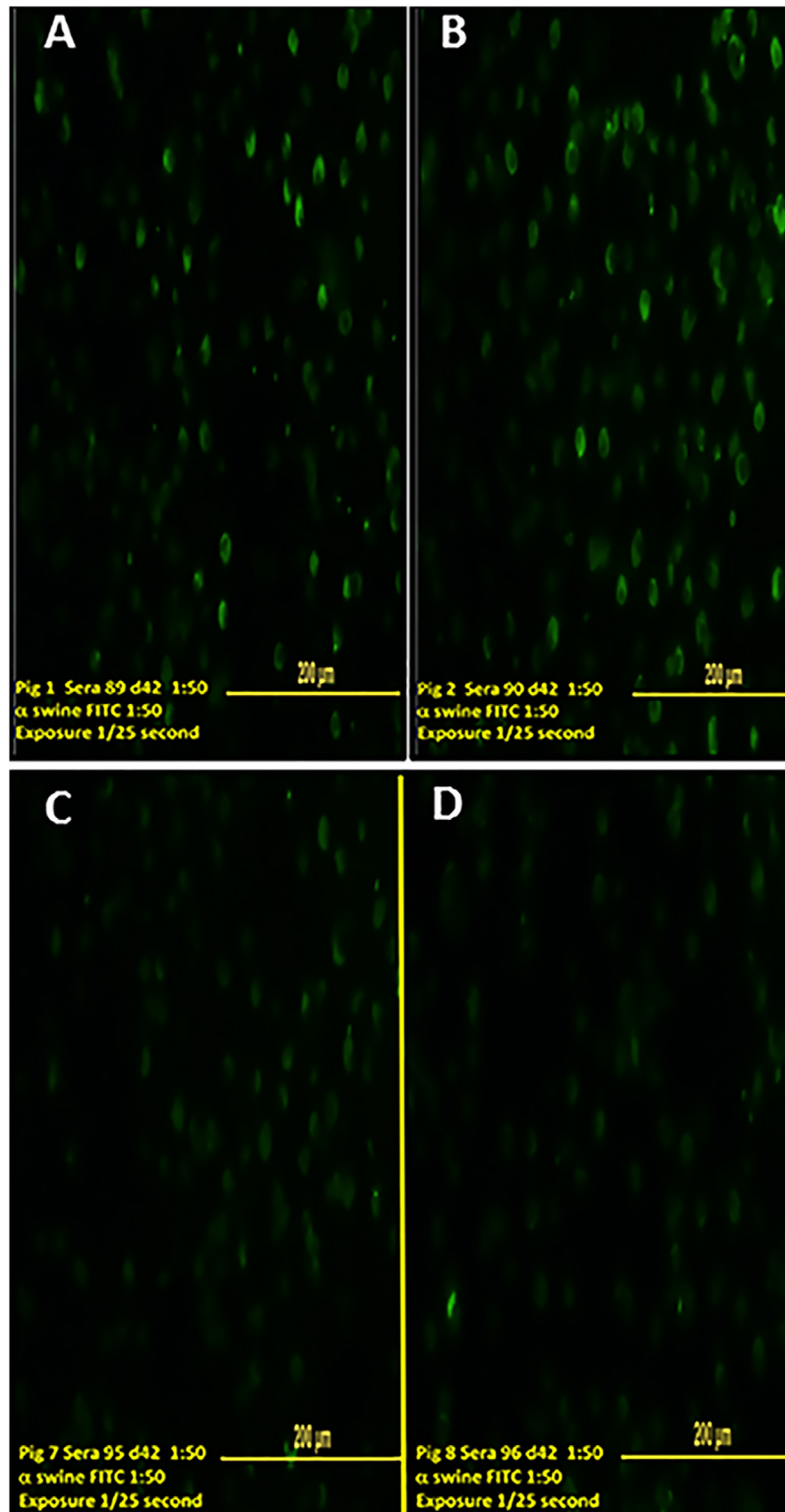
Fig. 2. ASFV-specific antibody response detection by Immunofluorescent Assay (IFA). ASFV-specific IgG responses in serum from HEK-ASFV immunized pigs. (A, B) Positive staining of ASFV Georgia 2007/1- infected macrophages using sera (1:50 dilution) from HEK-ASFV immunized pigs (T1) at 2 weeks post-boost (d42). Sera from four of five immunized pigs were reactive; data for two pigs, pig 1 (A) and pig 2 (B) are shown. (C, D) Negative staining of ASFV Georgia 2007/1-infected macrophages using sera (1:50 dilution) from T2 (adjuvant injected pigs) at 2 weeks post-boost. Data for two pigs, pig 7 (C) and pig 8 (D) are shown.

and cell fusion proteins that are important for cell invasion and virulence (Sachdeva et al., 2005). For example, SPAAN (used in Vaxign) identified many spike glycoproteins from Human coronavirus as adhesins (Sachdeva et al., 2005) and the spike protein (S), a membrane component of Severe acute respiratory syndrome coronavirus (SARS-CoV), was identified to be critical for viral pathogenesis (Li et al., 2003b) and experimentally proven to be effective as a vaccine candidate (Bisht et al., 2004). The identified adhesion probability scores for EP402R (CD2v) (score 0.457) and EP153R (score 0.372) correlate with previous reports that have shown that
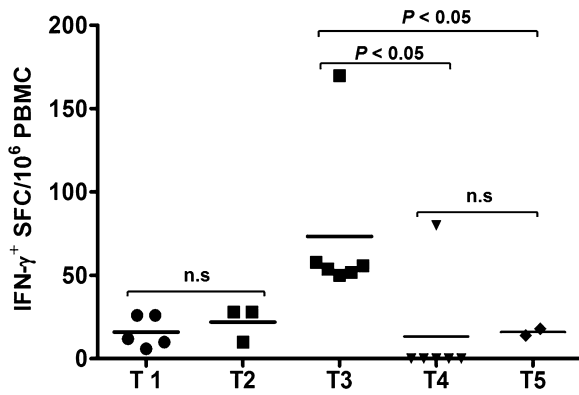
Fig. 3. ASFV antigen (B646L)-specific IFN-γ T-cell responses post-boost. ASFV antigen-specific IFN-γ T-cell responses were evaluated by ELISpot assay one week post-boost. Data are adjusted to IFN-γ+ spot forming cells (SFC)/10^6 PBMC after subtracting background media counts. Data are shown for B646L-specific responses. T2 and T5 are negative controls; T2, TS6 adjuvant; T5, MVA Vector. Statistics (GraphPad Prism, La Jolla, USA): One-way ANOVA, followed by Bonferroni post-test analysis α = 0.05 (95% confidence intervals).

these proteins are involved in ASFV hemadsorption (HA) *in vitro* (Borca et al., 1998, 1994b; Galindo et al., 2000; Neilan et al., 1999; Rodríguez et al., 1993; Ruiz-Gonzalvo et al., 1996). Adhesion probability scores were identified for B646L/p72 (score 0.25), CP204L/p30 (score 0.363), and E183L/p54 (score 0.566), all previously reported to be potential protective antigens (Argilaguet et al., 2012; Gómez-Puertas et al., 1998; Lokhandwala et al., 2016; Neilan et al., 2004). A 20% weight for each MHC class epitopes was chosen to select both classes of epitopes equally to maximize immunogenicity potential. A recent report correlated two SLA I-restricted 9-mer peptides within CD2v capable of *in vitro* stimulating the specific secretion of IFN-γ from PBMCs from challenged survivor pigs (Argilaguet et al., 2012). MHC class I and II scores that range from 0.60-0.78 were identified for previously reported potential protective antigens B646L, CP204L, E183L, EP153R, and EP402R. A 15% weight was assigned to transmembrane domain presence based on the assumption that transmembrane proteins would be on the surface of the virion when ASFV has the capsid outer layer embedded

onto inner membrane. Overall, the Vaxign ranking for previously reported potential protective antigens are as follows: B646L (rank 107), CP204L/p30 (rank 79), E183L (rank 4), EP153R (rank 17), and EP402R (rank 15). Three of the previously reported potential protective antigens are ranked within the top 20. The lower ranking for B646L and CP204L is likely due to the lack of predicted transmembrane domain, which was assigned 15% weight to the overall ranking. Many of the top 30 Vaxign ranked antigens, including the rank 1 antigen, E146L, have not previously been evaluated in immunogenicity studies. To our knowledge, this study represents the first report on a strategy for weighting analysis features for antigen ranking by Vaxign. The swine immunogenicity results obtained in the present study support that a ranking approach can be subsequently used to down select ASFV recombinant antigens that induce a detectable immune response.

Twelve of the top 30 Vaxign ranked candidates and two previously characterized antigens (CP204L and B646L) were chosen for attempted recombinant protein expression in HEK 293 mammalian cells and MVA vector system. The mammalian HEK 293 expression system was selected based on the hypothesis that authentic soluble post-translationally modified, recombinant ASFV proteins would induce both humoral and cellular immune responses. The poxvirus MVA vector system was selected based on its demonstrated safety in swine and its ability to induce *in vivo* expression of one or more specific antigens in the administered host (Moss, 1993). Following *in vivo* administration, MVA infected cells will express the recombinant antigen(s) de novo for subsequent processing by the proteasome to generate peptides that are presented on the cell surface by MHC-I molecules for T-cell recognition (Brewoo et al., 2010). The presentation of antigenic peptides by professional antigen presenting cells via this MHC class I pathway induces the activation of CD8+ T-cells, a feature postulated to be desirable for ASFV protection. In addition, the MVA vector system also enables the synthesis of long-lived antigens (stable proteins) which is advantageous since these proteins, as immunogens, are superior for efficient activation of immune responses *in vivo*. Furthermore, it has been demonstrated that T-cell responses are more efficiently activated when dendritic cells cross-present stable MVA-produced antigens (Gasteiger et al., 2007). For these reasons, the MVA system was
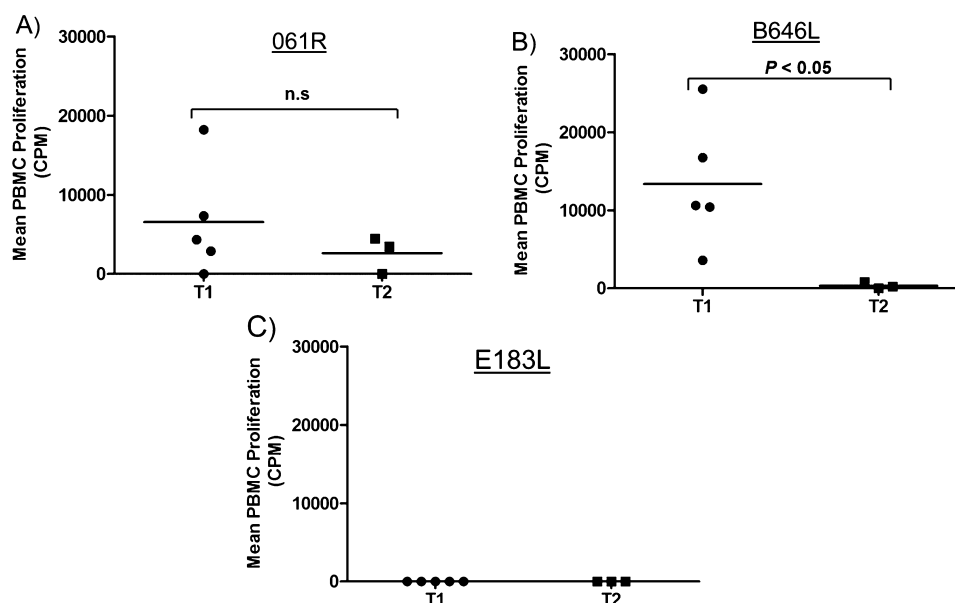

Fig. 4. ASFV antigen-specific PBMC proliferation following homologous HEK-ASFV antigen post-boost. Antigen-specific PBMC proliferation responses two weeks post-boosting were evaluated by proliferation assay. A) PBMC proliferation against O61R; B) PBMC proliferation against B646L; and C) No responses against E183L were detected. T2, TS6 adjuvant, negative control.
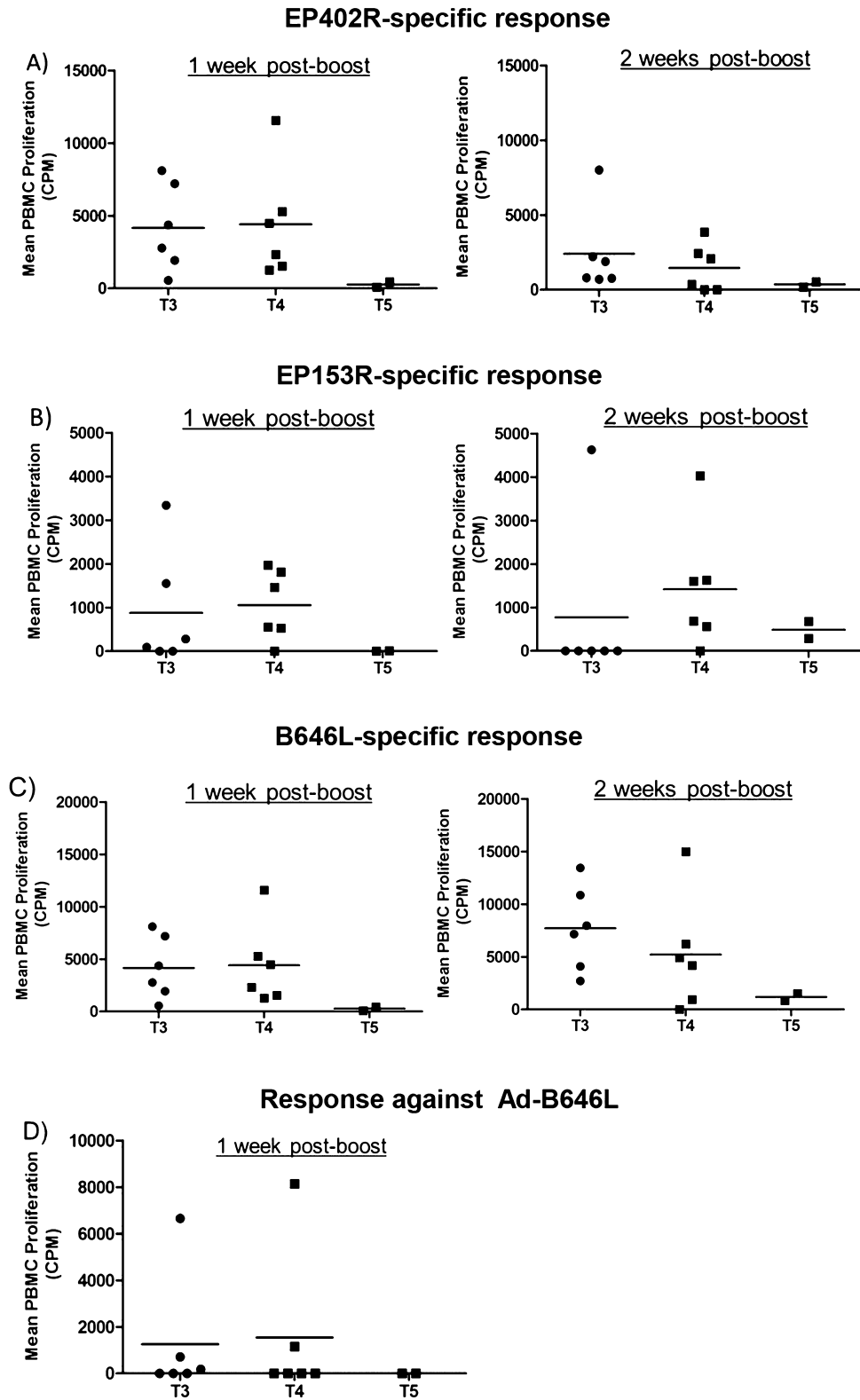
EP402R-specific response

EP153R-specific response

B646L-specific response

Response against Ad-B646L

Fig. 5. ASFV antigen-specific PBMC proliferation following MVA-ASFV homologous and heterologous prime-boost. PBMC proliferation against ASFV antigens was evaluated one- and two-weeks following homologous and heterologous prime-boost. (A) PBMC proliferation against EP402R at one- and two- weeks post-boost. (B) PBMC proliferation against EP153R at one- and two- weeks post-boost. (C) PBMC proliferation against B646L at one- and two- weeks post-boost. and (D), PBMC proliferation against adenovirus expressing B646L at two- weeks post-boost. There was no significant difference between any of the treatments and negative controls (T5, MVA vector). Statistics (GraphPad Prism, La Jolla, USA): One-way ANOVA, followed by Bonferroni post-test analysis α=0.05 (95% confidence intervals).

selected for its potential induction of ASFV-specific swine immune responses.

Expression and purification of the fourteen selected antigens (many antigens exhibiting the highest Vaxign scores) proved to be challenging using the HEK and MVA systems, thus limiting the antigens evaluated in this study to those that have been previously evaluated. Although troubleshooting was performed, the factors contributing to lack of protein expression in the HEK system are unknown. The various physicochemical and structure features of and post-translational modifications associated with each target protein as well as transcript stability and proteolysis may have contributed to the unsuccessful protein expression. Three proteins, E146L, E199L, and E248R, that were expressed contain putative transmembrane domains and were insoluble in Triton X-114. The technical challenges initially observed with the MVA system - genetic instability and lack of cell growth for the recombinants - was resolved through the addition of the secretory signal tPA (human tissue plasminogen activator) which enabled successful generation of these recombinant MVA-ASV viruses. The inclusion of the secretory tPA signal likely reduced the toxicity of the gene products, allowing for stable recombinants expressing E146L (Vaxign rank 1), I10L (rank 3), E183L (rank 4), E199L (rank 6), E248R (rank 9), O61R (rank 20), and CP204L (rank 79). Unfortunately, these recombinants were not successfully constructed and purified in time for the current swine immunogenicity study, but could be evaluated in future swine immunogenicity studies.

The five antigens that were successfully purified in sufficient quantity for swine safety and immunogenicity study were E183L, EP402R, EP153R, O61R, and B646L (Table 2). E183L, EP402R, EP153R, and O61R exhibited optimal Vaxign ranking in the range of 4–20 and B646L exhibited a low ranking of 107. Among the 12 ASFV genomes analyzed, all five genes are highly conserved, have MHC HLA Class I and Class II scores greater than 0.60, and adhesion probability scores ranging from 0.25 to 0.57. With the exception of B646L, four of these genes encode for at least one transmembrane domains and one of these (EP402R) encodes for a CD2 homologue adhesion protein (CD2v). B646L had a relatively low 107 Vaxign ranking likely due to the absence of any transmembrane domains and a low adhesion probability. However, p72 is a major component of the virus capsomer, accounting for approximately one-third of the virus protein mass (García-Escudero et al., 1998). Moreover, p72 is known to be associated with membranes in infected cells and p72-specific antibodies block ASFV entry in a way that suggests that p72 may play some role in host target cell attachment (Gómez-Puertas et al., 1996). Interestingly, E183L had the highest adhesion probability of the five antigens used in the swine immunogenicity study, and has been demonstrated to possess neutralizing activity to block virus attachment to host cell targets (Gómez-Puertas et al., 1996).

Despite the observed protein expression and purification challenge, the antigens ranking generated by Vaxign based on adhesion probability, MHC Class I and II epitope binding, transmembrane domains, and sequence conservation among ASFV strains, may be valuable in future ASF vaccine studies that aim to evaluate additional antigenic ASFV candidates. Furthermore, the difficulties encountered during the expression of highly Vaxign ranked antigens, including the addition of secretory signals, may provide additional knowledge for future ASF studies attempting to evaluate and implement novel antigen delivery systems.

Following homologous HEK-ASFV antigens prime-boost administration, evidence of ASFV antigen-specific antibody responses was clearly demonstrated by the detection of IgGs against O61R, E183L, and B646L in sera collected one and two weeks post-boost. Isotype switching post-boost was also demonstrated by the ELISA and IFA data given that the recall responses are ASFV antigen-specific IgG responses. These antibody responses do not imply that the induced antibodies can neutralize ASFV but confirms that the administered antigens were authentic.

In contrast, pigs that were administered MVA-ASFV construct cocktails failed to generate detectable ASFV antibody responses. This outcome was somewhat unexpected since this expression system elicited antibodies when used with other antigens (Brewoo et al., 2010) and another viral-vector system (human adenovirus 5) elicited antibodies when used with some of the same (E183L, B646L) antigens (Lokhandwala et al., 2016). The lack of antibody response may be related to a defect in antigen presentation, e.g., the retention of the MVA expressed ASFV antigens inside infected cells may have limited their presentation to B-cells (Borrego et al., 2006; Ganges et al., 2005). The use of molecular elements such as tPA to enhance intracellularly expressed antigen secretion for B-cell recognition and MHC II presentation for priming of helper CD4 T-cells (Brewoo et al., 2010; Embry et al., 2011) has been previously reported. Since the administered MVA-ASFV constructs lacked the secretory tPA signal, the absence of observed antibody response may be due to limited B-cell presentation. Subsequent to the results reported herein, we have been successful in the construction of seven, stable MVA-tPA-ASFV virus recombinants that could be evaluated in future swine immunogenicity studies for their ability to induce antibody and T-cell response (Lopera-Madrid, unpublished data).

Onset of ASFV antigen specific T-cell responses was demonstrated by antigen-specific cell proliferation and IFN-γ-secreting cells. Antigen-specific T-cell proliferation responses to B646L were detected in PBMCs from homologous HEK-ASFV antigens prime-boost treatment group (T1). Although no statistically significant difference in mean antigen-specific T-cell proliferation responses were detected in PMBCs from MVA-ASFV homologous and heterologous prime-boost treatment groups (T3 and T4) and negative controls (T5), antigen-specific T-cell proliferation responses to all immunizing antigens, EP153R, EP402R, and B646L, were observed in PBMCs from individual pigs in both treatment groups (Fig. 5). Furthermore, ASFV antigen specific IFN-γ^+ T-cell responses were detected in PBMCs from MVA-ASFV primed and HEK-ASFV antigens boosted pigs (T3). These results are in congruence with previous reports that emphasize the importance of heterologous prime/boost regimes (Boyd et al., 2013; Draper et al., 2013; Pattacini et al., 2012; Ratto-Kim et al., 2012) and correlate with recent relevant reports. Interestingly, antigen-specific IFN-γ^+ T-cell responses to B646L were also observed following an adenoviral-vector homologous prime/boost immunization strategy (Lokhandwala et al., 2016). However it should be noted that the correlation between IFN-γ^+ T-cell responses and host protection against ASFV remains unclear, at least in the context of using live, attenuated ASFV models (Carlson et al., 2016). The current study results also correlate with the recent study using ASFV interserotypic CD2 v (EP402R)/C-type lectin (EP153R) recombinant chimeric viruses and vaccination/challenge experiments in swine, which demonstrated partial protection as defined by delayed time-to death (3 days), onset of fever, and onset of viraemia. The partial protection was likely due to host response to homologous CD2 v and C-type lectin antigens and provide evidence that these antigens are important for homologous protective immunity (Burmakina et al., 2016). The Burmakina et al. report and the data and Vaxign ranking reported herein support the potential role of CD2 v (rank 15) and C-type lectin (rank 17) as protective antigens.

Despite the absence of a humoral response following homologous or heterologous MVA-ASFV antigens prime-boost administrations, T-cell proliferative and IFN-γ^+ T-cell responses were detected following HEK-ASFV antigen boost. These results are not surprising given that infection of host target cells by recombinant viruses results in endogenous antigen expression and antigen presentation in the context of MHC molecules resulting in activation of T lym-

phocytes (Rocha et al., 2004). Although detected T-cell responses do not imply a role in ASF protection, these results are encouraging and suggest that the MVA vector may be a suitable platform for evaluation of its ability to confer protection.

Recently, an adenovirus-vectored ASFV multiantigen cocktail expressing p32 (CP204L), p54 (E183L), pp62 (polyprotein) and p72 (B646L) antigens was demonstrated to induce antigen-specific antibodies and T-cell responses in the absence of ASFV challenge (Lokhandwala et al., 2016). The antibody and T-cell activity reported with this adenovirus-vectored ASFV multiantigen cocktail appears to be more robust and broader compared to the MVA-ASFV prime/HEK-ASF antigen boost strategy used in the present study. One possible explanation for this difference is that the time interval between the prime and boost doses may be critical to the generation of optimal immune responses. In the adenovirus-vectored ASFV study a 14-week time interval was used compared to the 4 week interval in the current study. A protracted time period may allow for the induction of higher numbers of antigen-specific memory cells. Alternatively, the two different adjuvant systems used in the adenovirus-vectored study may be more potent than the TS6 adjuvant used in the current study.

The Lokhandwala and current studies, using different antigens and delivery systems have demonstrated T-cell mediated responses which may contribute to ASF protection. Previous studies have demonstrated the important role of T-cell mediated immunity in host protection against ASFV infection. In one study, pigs immunized with the live, attenuated OUR/T88/3 isolate were protected from subsequent challenge with the virulent, parental OUR/T88/1 isolate. Subsequent depletion of CD8$^+$ cells in live, attenuated OUR/T88/3 immunized swine resulted in disease susceptibility following virulent OUR/T88/1 challenge thus demonstrating the importance of cell-mediated immunity in protection from ASF (Oura et al., 2005). Another study demonstrated that an experimental DNA vaccine comprised of an ubiquitin tagged p30-p54-soluble CD2v hemagglutinin induced antigen-specific CD8$^+$ T-cell responses and conferred partial protection in the absence of detectable ASFV antibodies (Argilaguet et al., 2012). Similarly, another study that used a baculovirus-based mammalian cell expressed fusion p30-p54-soluble CD2v hemagglutinin protein also demonstrated partial protection that correlated with virus-specific IFN-γ-secreting T-cells in the absence of antibodies (Argilaguet et al., 2013). A follow-up study to identify additional protective T-cell determinants in which an entire ASFV genomic expression library lacking CD2v, p54, and p30 was used for vaccination resulted in 60% of the vaccinated pigs being protected when challenged with E75. Protection was correlated with detection of specific CD8+ T-cell responses in the absence of detectable specific antibodies prior to challenge (Lacasta et al., 2014).

The reported partial protection results described above in previous publications suggest that a network of multiple antigens as well as multiple immune mechanisms are involved in induction of ASF protection. It is likely that previously identified potential protective antigens and those yet to be identified are required for complete protection. The combination of multiple protective antigens, optimal antigen delivery and adjuvant systems, linked to different immunization strategies may be necessary to successfully induce humoral and cell-mediated immunity and confer complete protection.

5. Conclusions

ASF vaccine development has been significantly hindered by knowledge gaps in the ASFV antigens and associated immune mechanisms responsible for host protection. This report demonstrates how Vaxign, an *in silico* antigen prediction program, can be used as a tool to help identify and rank potential antigenic features of novel ASFV proteins. This is the first reported study to apply the Vaxign tool, express a down selected subset of five ASFV antigens in both a mammalian (HEK 293) as well as MVA viral vector system, and demonstrate safety and antigenicity in swine. Vaccination with HEK-purified ASFV proteins promoted humoral immune responses and detectable but less intense cellular immunity. Even though humoral immune responses were not induced following MVA-ASFV vaccination, cellular immunity characterized by T-cell proliferation and IFN-γ producing cells was observed. The induced T-cell responses using a heterologous MVA-ASFV prime/HEK-ASFV antigen boost immunization regimen support the rationale for future studies to better define these responses and to determine a correlation, if any, to protection against subsequent challenge with ASFV Georgia 2007/1. If robust T-cell and B-cell ASFV immune responses can be consistently obtained in immunized swine using this strategy and approach, then the prime-boost regimen comprised of the best ASFV antigens can be used in future studies to evaluate ASFV vaccine candidates for their ability to induce a durable, protective immunity against virulent homologous and heterologous ASFV challenge.

Conflicts of interest

"The authors declare no conflict of interest.Ädditionally, 'the founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results'.

Author contributions

J.E.O., Y.H., Z.X., L.G.A., W.M., S.S., J.N., D.B., A.C. and M.A.B. conceived and designed the experiments; J.L.M., Y.H., Z.X., L.G.A., R.C.L., T.B., W.M., M.A.B. performed the experiments; J.L.M., Y.H., Z.X., L.G.A., R.C.L., T.B., W.M., M.A.B. analyzed the data; J.L.M., S.S., W.C.B., W.M., M.A.B. contributed reagents/materials/analysis tools; J.L.M., Y.H., T.B., L.G.A., W.M., M.A.B., D.B. wrote the paper.

Acknowledgments

References

Argilaguet, J.M., Pérez-Martín, E., Gallardo, C., Salguero, F.J., Borrego, B., Lacasta, a., Accensi, F., Díaz, I., Nofrarías, M., Pujols, J., Blanco, E., Pérez-Filgueira, M., Escribano, J.M., Rodríguez, F., 2011. Enhancing dna immunization by targeting asfv antigens to sla-ii bearing cells. Vaccine 29, 5379–5385, http://dx.doi.org/10.1016/j.vaccine.2011.05.084.

Argilaguet, J.M., Pérez-Martín, E., Nofrarías, M., Gallardo, C., Accensi, F., Lacasta, A., Mora, M., Ballester, M., Galindo-Cardiel, I., López-Soria, S., Escribano, J.M., Reche, P.a., Rodríguez, F., 2012. DNA vaccination partially protects against African swine fever virus lethal challenge in the absence of antibodies. PLoS One 7, e40942, http://dx.doi.org/10.1371/journal.pone.0040942.

Argilaguet, J.M., Pérez-Martín, E., López, S., Goethe, M., Escribano, J.M., Giesow, K., Keil, G.M., Rodríguez, F., 2013. BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus. Antiviral Res. 98, 61–65, http://dx.doi.org/10.1016/j.antiviral.2013.02.005.

Barderas, M.G., Rodríguez, F., Gómez-Puertas, P., Avilés, M., Beitia, F., Alonso, C., Escribano, J.M., 2001. Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins. Arch. Virol 146, 1681–1691, http://dx.doi.org/10.1007/s007050170056.

Bisht, H., Roberts, A., Vogel, L., Bukreyev, A., Collins, P.L., Murphy, B.R., Subbarao, K., Moss, B., 2004. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc. Natl. Acad. Sci. U. S. A. 101, 6641–6646, http://dx.doi.org/10.1073/pnas.0401939101.

Borca, M.V., Irusta, P., Carrillo, C., Afonso, C.L., Burrage, T., Rock, D.L., 1994a. African swine fever virus structural protein p72 contains a conformational neutralizing epitope. Virology 201 (2), 413–418, http://dx.doi.org/10.1006/viro.1994.1311.

Borca, M.V., Kutish, G.F., Afonso, C.L., Irusta, P., Carrillo, C., Brun, A., Sussman, M., Rock, D.L., 1994b. An African swine fever virus gene with similarity to the T-lymphocyte surface antigen CD2 mediates hemadsorption. Virology 199 (2), 463–468, http://dx.doi.org/10.1006/viro.1994.1146.

Borca, M.V., Carrillo, C., Zsak, L., Laegreid, W.W., Kutish, G.F., Neilan, J.G., Burrage, T.G., Rock, D.L., 1998. Deletion of a CD2-like gene, 8-DR, from African swine fever virus affects viral infection in domestic swine. J. Virol. 72, 2881–2889.

Borrego, B., Fernandez-Pacheco, P., Ganges, L., Domenech, N., Fernandez-Borges, N., Sobrino, F., Rodriguez, F., 2006. DNA vaccines expressing B and T cell epitopes can protect mice from FMDV infection in the absence of specific humoral responses. Vaccine 24, 3889–3899, http://dx.doi.org/10.1016/j.vaccine.2006.02.028.

Boyd, A.C., Ruiz-Hernandez, R., Peroval, M.Y., Carson, C., Balkissoon, D., Staines, K., Turner, A.V., Hill, A.V.S., Gilbert, S.C., Butter, C., 2013. Towards a universal vaccine for avian influenza: protective efficacy of modified Vaccinia virus Ankara and Adenovirus vaccines expressing conserved influenza antigens in chickens challenged with low pathogenic avian influenza virus. Vaccine 31, 670–675, http://dx.doi.org/10.1016/j.vaccine.2012.11.047.

Brewoo, J.N., Powell, T.D., Stinchcomb, D.T., Osorio, J.E., 2010. Efficacy and safety of a modified vaccinia Ankara (MVA) vectored plague vaccine in mice. Vaccine 28, 5891–5899, http://dx.doi.org/10.1016/j.vaccine.2010.06.054.

Brown, W.C., DelProposto, J., Rubin, J.R., Lamiman, K., Carless, J., Smith, J.L., 2011. New ligation-independent cloning vectors compatible with a high-throughput platform for parallel construct expression evaluation using baculovirus-infected insect cells. Protein Expr. Purif. 77, 34–45, http://dx.doi.org/10.1016/j.pep.2011.01.007.

Burgara-Estrella, A., Díaz, I., Rodríguez-Gómez, I.M., Essler, S.E., Hernández, J., Mateu, E., 2013. Predicted peptides from non-structural proteins of porcine reproductive and respiratory syndrome virus are able to induce IFN-γ and IL-10. Viruses 5, 663–677, http://dx.doi.org/10.3390/v5020663.

Burmakina, G., Malogolovkin, A., Tulman, E.R., Zsak, L., Delhon, G., Diel, D.G., Shobogorov, N.M., Morgunov, Y.P., Morgunov, S.Y., Kutish, G.F., Kolbasov, D., Rock, D.L., 2016. African swine fever virus serotype-specific proteins are significant protective antigens for African swine fever. J. Gen. Virol. 97, 1670–1675, http://dx.doi.org/10.1099/jgv.0.000490.

Carlson, J., O'Donnell, V., Alfano, M., Velazquez Salinas, L., Holinka, L., Krug, P., Gladue, D., Higgs, S., Borca, M., 2016. Association of the host immune response with protection using a live attenuated African swine fever virus model. Viruses 8, 291, http://dx.doi.org/10.3390/v8100291.

Caro-Gomez, E., Gazi, M., Goez, Y., Valbuena, G., 2014. Discovery of novel cross-protective Rickettsia prowazekii T-cell antigens using a combined reverse vaccinology and in vivo screening approach. Vaccine 32, 4968–4976, http://dx.doi.org/10.1016/j.vaccine.2014.06.089.

Chapman, D.A.G., Darby, A.C., da Silva, M., Upton, C., Radford, A.D., Dixon, L.K., 2011. Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. Emerg. Infect. Dis. 17, 599–605, http://dx.doi.org/10.3201/eid1704.101283.

Chardon, P., Renard, C., Rogel Gaillard, C., Vaiman, M., 2000. The porcine Major Histocompatibility Complex and related paralogous regions: a review. Genet. Sel. Evol. 32, 109–128, http://dx.doi.org/10.1051/gse:2000101.

Costard, S., Wieland, B., de Glanville, W., Jori, F., Rowlands, R., Vosloo, W., Roger, F., Pfeiffer, D.U., Dixon, L.K., 2009. African swine fever: how can global spread be prevented? Philos. Trans. R Soc. Lond. B Biol. Sci. 364, 2683–2696, http://dx.doi.org/10.1098/rstb.2009.0098.

Costard, S., Mur, L., Lubroth, J., Sanchez-Vizcaino, J.M., Pfeiffer, D.U., 2013. Epidemiology of African swine fever virus. Virus Res. 173, 191–197, http://dx.doi.org/10.1016/j.virusres.2012.10.030.

Díaz, I., Pujols, J., Ganges, L., Gimeno, M., Darwich, L., Domingo, M., Mateu, E., 2009. In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. Vaccine 27, 5603–5611, http://dx.doi.org/10.1016/j.vaccine.2009.07.029.

De Groot, A.S., Ardito, M., McClaine, E.M., Moise, L., Martin, W.D., 2009. Immunoinformatic comparison of T-cell epitopes contained in novel swine-origin influenza A (H1N1) virus with epitopes in 2008–2009 conventional influenza vaccine. Vaccine 27, 5740–5747, http://dx.doi.org/10.1016/j.vaccine.2009.07.040.

Delcher, A.L., Bratke, K.A., Powers, E.C., Salzberg, S.L., 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23, 673–679, http://dx.doi.org/10.1093/bioinformatics/btm009.

Dixon, L.K., Abrams, C.C., Bowick, G., Goatley, L.C., Kay-Jackson, P.C., Chapman, D., Liverani, E., Nix, R., Silk, R., Zhang, F., 2004. African swine fever virus proteins involved in evading host defence systems. Vet. Immunol. Immunopathol. 100, 117–134, http://dx.doi.org/10.1016/j.vetimm.2004.04.002.

Draper, S.J., Cottingham, M.G., Gilbert, S.C., 2013. Utilizing poxviral vectored vaccines for antibody induction-progress and prospects. Vaccine 31, 4223–4230, http://dx.doi.org/10.1016/j.vaccine.2013.05.091.

Embry, A., Meng, X., Cantwell, A., Dube, P.H., Xiang, Y., 2011. Enhancement of immune response to an antigen delivered by vaccinia virus by displaying the antigen on the surface of intracellular mature virion. Vaccine 29, 5331–5339, http://dx.doi.org/10.1016/j.vaccine.2011.05.088.

Escribano, J.M., Galindo, I., Alonso, C., 2013. Antibody-mediated neutralization of African swine fever virus: myths and facts. Virus Res. 173, 101–109, http://dx.org/10.1016/j.virusres.2012.10.012.

Fan, S., Wu, Y., Wang, S., Wang, Z., Jiang, B., Liu, Y., Liang, R., Zhou, W., Zhang, N., Xia, C., 2016. Structural and biochemical analyses of swine major histocompatibility complex class I complexes and prediction of the epitope map of important influenza a virus strains. J. Virol. 90, 6625–6641, http://dx.doi.org/10.1128/JVI.00119-16.

Gómez-Puertas, P., Rodríguez, F., Oviedo, J.M., Ramiro-Ibáñez, F., Ruiz-Gonzalvo, F., Alonso, C., Escribano, J.M., 1996. Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. J. Virol. 70, 5689–5694.

Gómez-Puertas, P., Rodríguez, F., Oviedo, J.M., Brun, A., Alonso, C., Escribano, J.M., 1998. The african swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. Virology 243, 461–471, http://dx.doi.org/10.1006/viro.1998.9068.

Galindo, I., Almazán, F., Bustos, M.J., Viñuela, E., Carrascosa, A.L., 2000. African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells. Virology 266, 340–351, http://dx.doi.org/10.1006/viro.1999.0080.

Ganges, L., Barrera, M., Núñez, J.I., Blanco, I., Frias, M.T., Rodríguez, F., Sobrino, F., 2005. A DNA vaccine expressing the E2 protein of classical swine fever virus elicits T cell responses that can prime for rapid antibody production and confer total protection upon viral challenge. Vaccine 23, 3741–3752, http://dx.doi.org/10.1016/j.vaccine.2005.01.153.

García-Escudero, R., Andrés, G., Almazán, F., Viñuela, E., 1998. Inducible gene expression from African swine fever virus recombinants: analysis of the major capsid protein p72. J. Virol. 72, 3185–3195.

Gasteiger, G., Kastenmuller, W., Ljapoci, R., Sutter, G., Drexler, I., 2007. Cross-priming of cytotoxic T cells dictates antigen requisites for modified vaccinia virus Ankara vector vaccines. J. Virol. 81, 11925–11936, http://dx.doi.org/10.1128/JVI.00903-07.

Genovesi, E.V., Villinger, F., Gerstner, D.J., Whyard, T.C., Knudsen, R.C., 1990. Effect of macrophage-specific colony-stimulating factor (CSF-1) on swine monocyte/macrophage susceptibility to in vitro infection by African swine fever virus. Vet. Microbiol. 25, 153–176, http://dx.doi.org/10.1016/0378-1135(90)90074-6.

Gomez, G., Pei, J., Mwangi, W., Adams, L.G., Rice-Ficht, A., Ficht, T.A., 2013. Immunogenic and invasive properties of Brucella melitensis 16 M outer membrane protein vaccine candidates identified via a reverse vaccinology approach. PLoS One 8, e59751, http://dx.doi.org/10.1371/journal.pone.0059751.

Gutiérrez, A.H., Martin, W.D., Bailey-Kellogg, C., Terry, F., Moise, L., De Groot, A.S., 2015. Development and validation of an epitope prediction tool for swine (PigMatrix) based on the pocket profile method. BMC Bioinf. 16, 290, http://dx.doi.org/10.1186/s12859-015-0724-8.

Gutiérrez, A.H., Loving, C., Moise, L., Terry, F.E., Brockmeier, S.L., Hughes, H.R., Martin, W.D., De Groot, A.S., 2016. In vivo validation of predicted and conserved T cell epitopes in a swine influenza model. PLoS One 11, e0159237, http://dx.doi.org/10.1371/journal.pone.0159237.

He, Y., Rappuoli, R., De Groot, A.S., Chen, R.T., 2010a. Emerging vaccine informatics. J. Biomed. Biotechnol. 2010, 218590, http://dx.doi.org/10.1155/2010/218590.

He, Y., Xiang, Z., Mobley, H.L.T., 2010b. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. J. Biomed. Biotechnol. 2010, http://dx.doi.org/10.1155/2010/297505.

Jenson, J.S., Childerstone, A., Takamatsu, H., Dixon, L.K., Parkhouse, R.M., 2000. The cellular immune recognition of proteins expressed by an African swine fever virus random genomic library. J. Immunol. Methods 242, 33–42.

Kall, L., Krogh, A., Sonnhammer, E.L.L., 2007. Advantages of combined transmembrane topology and signal peptide prediction-the Phobius web server. Nucleic Acids Res. 35, W429–W432, http://dx.doi.org/10.1093/nar/gkm256.

Lacasta, A., Ballester, M., Monteagudo, P.L., Rodriguez, J.M., Salas, M.L., Accensi, F., Pina-Pedrero, S., Bensaid, A., Argilaguet, J., Lopez-Soria, S., Hutet, E., Le Potier, M.F., Rodriguez, F., 2014. Expression library immunization can confer protection against lethal challenge with african swine fever virus. J. Virol. 88, 13322–13332, http://dx.doi.org/10.1128/JVI.01893-14.

Li, L., Stoeckert, C.J., Roos, D.S., 2003a. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13, 2178–2189, http://dx.doi.org/10.1101/gr.1224503.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003b. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450–454, http://dx.doi.org/10.1038/nature02145.

Lokhandwala, S., Waghela, S.D., Bray, J., Martin, C.L., Sangewar, N., Charendoff, C., Shetti, R., Ashley, C., Chen, C.-H., Berghman, L.R., Mwangi, D., Dominowski, P.J., Foss, D.L., Rai, S., Vora, S., Gabbert, L., Burrage, T.G., Brake, D., Neilan, J., Mwangi, W., 2016. Induction of robust immune responses in swine by using a cocktail of adenovirus-vectored african swine fever virus antigens. Clin. Vaccine Immunol. 23, 888–900, http://dx.doi.org/10.1128/CVI.00395-16.

Lunney, J.K., Ho, C.-S., Wysocki, M., Smith, D.M., 2009. Molecular genetics of the swine major histocompatibility complex, the SLA complex. Dev. Comp. Immunol. 33, 362–374, http://dx.doi.org/10.1016/j.dci.2008.07.002.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V., 2012. The pig: a model for human infectious diseases. Trends Microbiol., http://dx.doi.org/10.1016/j.tim.2011.11.002.

Moss, B., 1993. Poxvirus vectors: cytoplasmic expression of transferred genes. Curr. Opin. Genet. Dev. 3, 86–90, http://dx.doi.org/10.1016/S0959-437X(05)80346-6.

Neilan, J.G., Borca, M.V., Lu, Z., Kutish, G.F., Kleiboeker, S.B., Carrillo, C., Zsak, L., Rock, D.L., 1999. An African swine fever virus ORF with similarity to C-type lectins is non-essential for growth in swine macrophages in vitro and for virus virulence in domestic swine. J. Gen. Virol. 80, 2693–2697.

Neilan, J.G., Zsak, L., Lu, Z., Burrage, T.G., Kutish, G.F., Rock, D.L., 2004. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. Virology 319, 337–342, http://dx.doi.org/10.1016/j.virol.2003.11.011.

Njongmeta, L.M., Bray, J., Davies, C.J., Davis, W.C., Howard, C.J., Hope, J.C., Palmer, G.H., Brown, W.C., Mwangi, W., 2012. CD205 antigen targeting combined with dendritic cell recruitment factors and antigen-linked CD40L activation primes and expands significant antigen-specific antibody and CD4(+) T cell responses following DNA vaccination of outbred animals. Vaccine 30, 1624–1635, http://dx.doi.org/10.1016/j.vaccine.2011.12.110.

Oie, 2012. African swine fever. Terr Anim Heal Code 1-6.

Onisk, D.V., Borca, M.V., Kutish, S., Kramer, E., Irusta, P., Rock, D.L., 1994. Passively transferred African swine fever virus antibodies protect swine against lethal infection. Virology 198, 350–354, http://dx.doi.org/10.1006/viro.1994.1040.

Oura, C., a, L., Denyer, M.S., Takamatsu, H., Parkhouse, R.M.E., 2005. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. J. Gen. Virol. 86, 2445–2450, http://dx.doi.org/10.1099/vir.0.81038-0.

Pattacini, L., Mize, G.J., Graham, J.B., Fluharty, T.R., Graham, T.M., Lingnau, K., Wizel, B., Perdiguero, B., Esteban, M., Pantaleo, G., Shen, M., Spies, G.A., McElrath, M.J., Lund, J.M., 2012. A novel HIV vaccine adjuvanted by IC31 induces robust and persistent humoral and cellular immunity. PLoS One 7, e42163, http://dx.doi.org/10.1371/journal.pone.0042163.

Pereira, U.P., Soares, S.C., Blom, J., Leal, C.A.G., Ramos, R.T.J., Guimarães, L.C., Oliveira, L.C., Almeida, S.S., Hassan, S.S., Santos, A.R., Miyoshi, A., Silva, A., Tauch, A., Barh, D., Azevedo, V., Figueiredo, H.C.P., 2013. In silico prediction of conserved vaccine targets in Streptococcus agalactiae strains isolated from fish, cattle, and human samples. Genet. Mol. Res. 12, 2902–2912, http://dx.doi.org/10.4238/2013.August.12.6.

Ratto-Kim, S., Currier, J.R., Cox, J.H., Excler, J.-L., Valencia-Micolta, A., Thelian, D., Lo, V., Sayeed, E., Polonis, V.R., Earl, P.L., Moss, B., Robb, M.L., Michael, N.L., Kim, J.H., Marovich, M.A., 2012. Heterologous prime-Boost regimens using rAd35 and rMVA vectors elicit stronger cellular immune responses to HIV proteins than homologous regimens. PLoS One 7, e45840, http://dx.doi.org/10.1371/journal.pone.0045840.

Rocha, C.D., Caetano, B.C., Machado, A.V., Bruna-Romero, O., 2004. Recombinant viruses as tools to induce protective cellular immunity against infectious diseases. Int. Microbiol. 7, 83–94.

Rodríguez, J.M., Yáñez, R.J., Almazán, F., Viñuela, E., Rodriguez, J.F., 1993. African swine fever virus encodes a CD2 homolog responsible for the adhesion of erythrocytes to infected cells. J. Virol. 67, 5312–5320.

Ruiz-Gonzalvo, F., Rodríguez, F., Escribano, J.M., 1996. Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. Virology 218, 285–289, http://dx.doi.org/10.1006/viro.1996.0193.

Sánchez-Vizcaíno, J.M., Mur, L., Martínez-López, B., 2013. African swine fever (ASF): five years around Europe. Vet. Microbiol. 165, 45–50, http://dx.doi.org/10.1016/j.vetmic.2012.11.030.

Sánchez-Vizcaíno, J.M., Mur, L., Gomez-Villamandos, J.C., Carrasco, L., 2015. An update on the epidemiology and pathology of African swine fever. J. Comp. Pathol. 152, 9–21, http://dx.doi.org/10.1016/j.jcpa.2014.09.003.

Sachdeva, G., Kumar, K., Jain, P., Ramachandran, S., 2005. SPAAN: a software program for prediction of adhesins and adhesin-like proteins using neural networks. Bioinformatics 21, 483–491, http://dx.doi.org/10.1093/bioinformatics/bti028.

Sanchez-Vizcaino, J.M., Martinez-Lopez, B., Martinez-Aviles, M., Martins, C., Boinas, F., Vial, L., Michaud, V., Jori, F., Etter, E., Albina, E. and Roger, F., 2009. SCIENTIFIC REPORT submitted to EFSA Prepared by Sanchez-Vizcaino, J.M., Martinez-Lopez, B., Martinez-Aviles, M., Martins, C., Boinas, F., Vial, L., Michaud, V., Jori, F., Etter, E., Albina, E. and Roger, F., 1–141.

Smith, D.M., Lunney, J.K., Ho, C.-S., Martens, G.W., Ando, A., Lee, J.-H., Schook, L., Renard, C., Chardon, P., 2005. Nomenclature for factors of the swine leukocyte antigen class II system. Tissue Antigens 66, 623–639, http://dx.doi.org/10.1111/j.1399-0039.2005.00492.x.

Staib, C., Drexler, I., Sutter, G., 2004. Construction and isolation of recombinant MVA. Methods Mol. Biol. 269, 77–100, http://dx.doi.org/10.1385/1-59259-789-0:077.

Stormo, G.D., Schneider, T.D., Gold, L., Ehrenfeucht, A., 1982. Use of the Perceptron algorithm to distinguish translational initiation sites in E. coli. Nucleic Acids Res. 10, 2997–3011, http://dx.doi.org/10.1093/nar/10.9.2997.

Suenderhauf, C., Parrott, N., 2012. A physiologically based pharmacokinetic model of the minipig: data compilation and model implementation. Pharm. Res., 1–15, http://dx.doi.org/10.1007/s11095-012-0911-5.

Takamatsu, H.-H.H., Denyer, M.S., Lacasta, A., Stirling, C.M., Argilaguet, J.M., Netherton, C., Oura, C.A., Martins, C., Rodr'iguez, F., 2013. Cellular immunity in ASFV responses. Virus Res. 173, 110–121, http://dx.doi.org/10.1016/j.virusres.2012.11.009.

Tulman, E.R., Delhon, G.A., Ku, B.K., Rock, D.L., 2009. African swine fever virus. Curr. Top. Microbiol. Immunol., http://dx.doi.org/10.1007/978-3-540-68618-7_2.

Wardley, R.C., Norley, S.G., Wilkinson, P.J., Williams, S., 1985. The role of antibody in protection against African swine fever virus. Vet. Immunol. Immunopathol. 9, 201–212, http://dx.doi.org/10.1016/0165-2427(85)90071-6.

Xiang, Z., He, Y., 2013. Genome-wide prediction of vaccine targets for human herpes simplex viruses using Vaxign reverse vaccinology. BMC Bioinf. 14 (Suppl. 4), http://dx.doi.org/10.1186/1471-2105-14-s4-s2.

Zhang, N., Qi, J., Feng, S., Gao, F., Liu, J., Pan, X., Chen, R., Li, Q., Chen, Z., Li, X., Xia, C., Gao, G.F., 2011. Crystal structure of swine major histocompatibility complex class I SLA-1 0401 and identification of 2009 pandemic swine-origin influenza A H1N1 virus cytotoxic T lymphocyte epitope peptides. J. Virol. 85, 11709–11724, http://dx.doi.org/10.1128/JVI.05040-11.

Zimic, M., Gutiérrez, A.H., Gilman, R.H., López, C., Quiliano, M., Evangelista, W., Gonzales, A., García, H.H., Sheen, P., 2011. Immunoinformatics prediction of linear epitopes from Taenia solium TSOL18. Bioinformation 6, 271–274.