RESEARCH REPORT
SWINE HEALTH

Title: Protective efficacy of adenovirus-vectored ASFV multi-antigen cocktail – **NPB #16-007**

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Industry Summary:

Introduction: The African Swine Fever Virus (ASFV) causes hemorrhagic fever in pigs, but there is no vaccine or treatment available. The ASFV continues to spread globally and the USA swine industry is at risk. Therefore, it is important that appropriate solutions are developed to safeguard the industry. The overall goal of this project was to test whether immunization of commercial pigs with an experimental vaccine can confer protection against ASFV. The experimental vaccine was previously developed and tested for its safety and ability to induce immune responses in commercial pigs [NPB Project #13-176]. The experimental vaccine is a cocktail of attenuated adenoviruses expressing multiple ASFV antigens. In this study, we immunized commercial pigs with the experimental vaccine and then challenged them with wild type ASFV.

Results from previous studies show that protection against ASFV can in principal be induced with a vaccine since pigs which recover from infection with less virulent isolates are protected from challenge by related virulent isolates. However, due to safety concerns, a subunit vaccine, but not an attenuated ASFV vaccine, is likely to be approved for use in the USA. The main challenges facing development of a subunit vaccine are the identification of protective antigens and development of a suitable method for inducing protective immunity in pigs. Previous studies have shown that immunization of pigs with one or two ASFV vaccine candidate antigens failed to induce immunity strong enough to confer significant protection. Successful development of an effective subunit vaccine will more likely require identification and validation of multiple suitable antigens that will induce significant protection in the majority of vaccinated pigs.

In the previous NPB-funded study [NPB Project #13-176], we showed that an experimental adenovirus-vectored ASFV vaccine formulated in an adjuvant was safe and well tolerated by commercial pigs. Importantly, the vaccine induced strong immune responses that were amplified after boosting. The objective of the current project was to evaluate the ability of the experimental vaccine to induce protection against ASFV.

Materials and Methods

Generation of prototype vaccine: Briefly, the recombinant replication-incompetent adenoviruses [AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R] developed in the previous study mentioned above were scaled up. Protein expression by these recombinant viruses was validated using ASFV-specific convalescent serum from a pig that survived exposure to ASF virus. In addition, purified recombinant proteins were generated for evaluating immune responses after immunization of pigs.

Pig study: Twenty four weaned piglets (~30lbs) were acquired, allowed to acclimatize and then randomly divided into two groups of 12 piglets each. Each piglet in group 1 (vaccinated group) was immunized with a cocktail containing AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R adenoviruses, whereas each piglet in group 2 (control group) was inoculated with an equivalent amount of an adenovirus, designated AdLuc, expressing an irrelevant protein (Luciferase). Four weeks post-immunization, the piglets were boosted with the respective formulation used above. Antibody responses were evaluated weekly post-immunization. Four weeks post-boost, the pigs were challenged with wild type ASFV and clinical outcomes post-challenge were evaluated to assess the protective efficacy of the vaccine.

***Study Outcomes and significance*:**

Immunization of commercial piglets with the cocktail of the recombinant adenoviruses expressing the ASFV A151R, B119L, B602L, EP402RΔPRR, B438L, K205R and A104R proteins induced strong antibody responses in all the vaccinated piglets. Notably, all the piglets responded to each antigen in the cocktail. Overall, the experimental vaccine was well tolerated and no serious negative effects were observed. However, when challenged with wild type ASFV, the vaccinated pigs had higher mean body temperatures and clinical scores than the negative controls. The vaccinated pigs also had a sharper decrease in white blood cells after challenge as compared to the negative controls. Taken together, the outcomes from this study suggests that the antibody responses induced in the vaccinated pigs proved to be detrimental and enhanced virus infection rather than providing the expected protection. These findings advocate for the development of alternate vaccine delivery strategies that would focus on inducing stronger T-cell immune responses while limiting antibody responses.

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Scientific Abstract:

African Swine Fever Virus (ASFV) is a high-consequence transboundary animal pathogen that places a huge economic burden on affected countries. The pathogen causes a hemorrhagic disease in swine with a case fatality rate close to 100%. Lack of treatment or vaccine for the disease makes it critical that safe and efficacious vaccines be developed to safeguard the swine industry. Previously, we evaluated the immunogenicity of seven adenovirus-vectored

novel ASFV antigens, namely A151R, B119L, B602L, EP402RΔPRR, B438L, K205R and A104R by immunizing commercial swine with a cocktail of recombinant adenoviruses formulated in an adjuvant. The cocktail primed strong ASFV antigen-specific IgG responses as well as ASFV-specific IFN-gamma responses that were recalled upon boosting. To evaluate protective efficacy of the antigen cocktail, we replicated the experiment above and subsequently challenged the pigs with 10^4 $TCID_{50}$ of ASFV-Georgia 2007/1 isolate. The cocktail induced very strong ASFV antigen-specific IgG responses against each antigen in all vaccinees as previously observed. These responses underwent rapid recall upon homologous boost four weeks post-priming. However, upon challenge, the pigs in the treatment group had higher mean clinical scores, mean body temperatures, and decreased WBC counts as compared to the controls. Notably, the mean body temperatures of the pigs in the treatment group was significantly ($p<0.05$) higher than the controls on day four post-challenge. In addition, six of the pigs in the treatment group and only three of the control pigs had to be euthanized on day five post-challenge for animal welfare reasons. Overall, the data suggests that the ASFV-antigen specific antibodies induced in the pig's enhanced ASF virus uptake by macrophages following challenge. The outcome also suggests that the IgG responses induced by these antigens are non-protective and that development of protective ASFV subunit vaccine will likely require an immunization strategy that will elicit strong cytotoxic T lymphocyte response while limiting humoral response.

Introduction:
The African Swine Fever Virus (ASFV) is a macrophage-tropic double-stranded DNA arbovirus with a genome size of 170-190 kb. Infection of domestic pigs with virulent isolates can cause 100% morbidity and nearly 100% mortality. Pigs that do recover become carriers and shed the virus for a long period of time and this makes eradication of the virus difficult. The virus is endemic in sub-Saharan Africa, but has spread to Iberian Peninsula, Mediterranean region, South America, the Caribbean, and more recently, the Caucasus region. The global threat posed by ASFV is due, in part, to its ease of transmission by humans and or fomites since it can withstand extreme environmental conditions, survive long periods of storage, persist in tissues of dead animals for months and can survive for 30-100 days in pork products. These factors have partly been responsible for the recent spread from Western Russia to Eastern Europe, and this has led to heightened awareness of a threat of the disease spread to other countries since there is unprecedented global trafficking of humans and goods. Although there hasn't been an outbreak in the U.S, outbreaks in the Caribbean islands were close. Given that there is a large population of feral pigs and four tick species capable of transmitting ASFV in North America, and they can become reservoirs if infected, there is an urgent need for the development of vaccines to safeguard the U.S swine industry.

Development of an ASFV vaccine is feasible given that pigs that recover from infection by attenuated virus are protected upon challenge with related virulent isolates. However, attenuated ASF virus is not an attractive option for use as a vaccine in U.S because it results in carrier pigs with a mutant virus which is likely to become virulent as reported in Portugal in 1970. Another problem with attenuated virus is the severe immune-mediated reactivity in immunized pigs because of virus persistence in lymphoid organs. Therefore, development of a DIVA compatible subunit vaccine based on rationally selected ASFV antigens is more desirable for use in U.S.A. However, among the current challenges are an identification of protective ASFV vaccine antigens and optimization of an effective antigen delivery platform for immunizing pigs.

Published data suggest that antibodies play a role in protection by probably blocking virus attachment and internalization into susceptible cells, but the key immune component most likely required for complete protection could be induction of T-cells, especially cytotoxic T lymphocytes (CTLs) capable of killing infected cells. Importantly, ASFV specific CTL activity has been detected in pigs infected with non-lethal ASFV isolates. In addition, lymphocytes

from convalescent pigs have been shown to kill macrophages pulsed with a defined p72 peptide in the context of *SLA* class I. Furthermore, macrophages infected with a vaccinia virus expressing p32 were shown to be targets for T-cells from convalescent pigs. A recent study showed that immunization of pigs with BacMam expressing ASFV CD2v, p32, and p54 conferred partial protection against a sub-lethal challenge. An important outcome from this study was the direct correlation between protection and induction of ASFV-specific IFN-γ^+ T-cells. It has also been shown that immunization of pigs with a DNA-encoded chimera of p32 and p54 antigens conferred some level of protection.

Taken together, published data suggests that the antigens tested could have a role in inducing protection, but they are not sufficient since vaccine candidates developed so far using one or a few antigens have failed to induce solid protection in vaccinees. We envisage that development of an efficacious subunit vaccine will require empirical identification of multiple protective antigens and a suitable delivery system that will effectively induce CTLs and confer long-term memory in a majority of vaccinees. The objective of this study was to evaluate the ability of a live-vectored ASFV multivalent cocktail to confer protection in commercial pigs.

Objectives: Evaluate protective efficacy of an adenovirus-encoded ASFV multi-antigen cocktail.

Materials & Methods:
Generation of prototype vaccine: Recombinant adenoviruses expressing ASFV antigens A151R, B119L, B602L, EP402RΔPRR, B438L, K205R and A104R were generated as previously described (*Lokhandwala S. et. al., 2017*). Briefly, the amino acid sequences of the above listed antigens (Georgia 2007/1 isolate) were obtained from Genbank (Accession FR682468) and modified to add in-frame a FLAG- and HA- tags at the N- and C-termini, respectively. The resultant amino acid sequences were used to design synthetic genes optimized for protein expression in swine cells and the genes were synthesized commercially. The synthetic genes were used to generate recombinant replication-incompetent adenoviruses designated AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R. Protein expression by these recombinant viruses was tested using the anti-HA tag antibody and authenticity was validated using ASFV-specific immune serum from a pig that had been immunized with the ASF virus (*Lokhandwala S. et. al., 2017*). In addition, the synthetic genes were used to generate recombinant Baculoviruses which were used to express recombinant proteins needed for evaluating immune responses after pig immunization.

Pig study: Twenty four weaned commercial piglets were acquired and randomly divided into two groups of 12 piglets each. The immunization protocol and a summary of the *in-vivo* study timeline is shown in Table 1 and Fig. 1, respectively. Briefly, each piglet in group 1 (treatment group) was immunized with a cocktail containing 10^{10} IFU of AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R adenoviruses, whereas each piglet in group 2 (negative control group) was inoculated with an equivalent amount (6 x 10^{10} IFU) of an adenovirus, designated AdLuc, expressing an irrelevant protein (Luciferase). Four weeks post-immunization, the piglets were boosted with a higher dose of the same cocktail (10^{11} IFU of each adenovirus or 6 x 10^{11} IFU of Ad-Luc) as above. Antibody responses to the ASFV antigens were evaluated weekly by ELISA. Four weeks post-boost, the pigs were challenged with 10^4 $TCID_{50}$ of ASFV-Georgia 2007/1 isolate. Clinical scores and body temperatures were recorded daily post-challenge. White Blood Cell (WBC) counts were evaluated on day 3 post-challenge and on the day of termination (day 5 or 6 post-challenge). Viremia levels in blood samples collected on the day of termination were analyzed by qPCR using a standard curve generated with serial log dilutions of a known quantity of ASFV Georgia 2007/1 isolate.

Table 1: Immunization protocol

Groups	No. of pigs	Immunogen	Dose/pig
Treatment group	12	Ad5-ASFV 6-way cocktail*	Prime: 6 X 10^{10} IFU Boost: 6 X 10^{11} IFU
Control group	12	Ad5-Luc	Prime: 6 X 10^{10} IFU Boost: 6 X 10^{11} IFU

* Ad5-ASFv 6-way cocktail = AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R

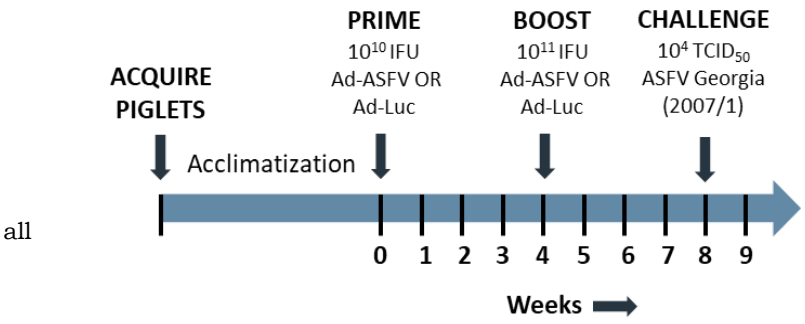

Figure 1: **Summary of study time line**. After acclimatization, the piglets were primed with either Ad5-ASFV cocktail (treatment) or Ad-Luc (control). Four weeks post-priming, the animals were boosted (with a one log higher dose) of the Ad-ASFV cocktail or Ad-Luc. Four weeks post-boost, animals were challenged with the wild type ASFV Georgia (2007/1) isolate.

all

Evaluation of antibody responses: Antigen-specific antibody responses were monitored starting on day 7 post-priming and tracked weekly up to challenge. Antibody responses were evaluated by ELISA using plates coated with the affinity purified recombinant ASFV antigens generated as mentioned above. ELISA was also used to determine antigen-specific endpoint antibody titers of sera collected two weeks post-boost. Pre-immunization sera served as the reference normal swine control, whereas ASFV-specific convalescent swine sera served as the positive control. The outcomes were presented as mean OD_{450nm} of triplicate wells of serial sera dilution and an endpoint titer was considered positive if it was 3 standard deviations above the cognate dilution of normal swine control serum.

Results:

Recombinant Adenovirus expressed encoded antigens: The replication-deficient recombinant adenoviruses designated AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R generated previously (*Lokhandwala S. et. al., 2017*) were scaled up in HEK-293A cells to generate sufficient bulk virus for immunization. Evaluation of protein expression by immunocytochemistry of adenovirus-infected HEK-293A cells using anti-HA mAb and the ASFV-specific convalescent serum showed that the scaled up recombinant adenoviruses expressed authentic ASFV antigens.

Antigen-specific IgG endpoint titers post-boost: Sera from blood drawn two weeks post-boost were evaluated by ELISA to determine antigen-specific antibody titers. Evaluation of antigen-specific end-point titers post-boost in the immunized pigs showed that a majority of the vaccinees had titers ≥ $1:10^6$ against antigens A151R, B119L, B602L, EP402RΔPRR and K205R-A104R (Fig. 2). The highest titer was $1:4x10^6$ against A151R in three of the vaccinees. A comparison of the antigen-specific titers in sera from the vaccinees with the titer of the ASFV-specific convalescent serum revealed that Ad-ASFV cocktail induced titers higher or equivalent to the convalescent serum in all vaccinees for antigens B602L, EP402RΔPRR, and B438L. Regarding antigen A151R, and antigens B119L and K205R-A104R, 83% and 91% of the vaccinees, respectively, had antigen-specific titers higher or equivalent to the convalescent serum. This result is in agreement with the observation made in our previous immunogenicity study (*Lokhandwala S. et. al., 2017*) and confirms that two doses of the Ad-

ASFV cocktail can induce higher antigen-specific titers than the positive control convalescent serum from an animal that received multiple inoculations of live ASFV.

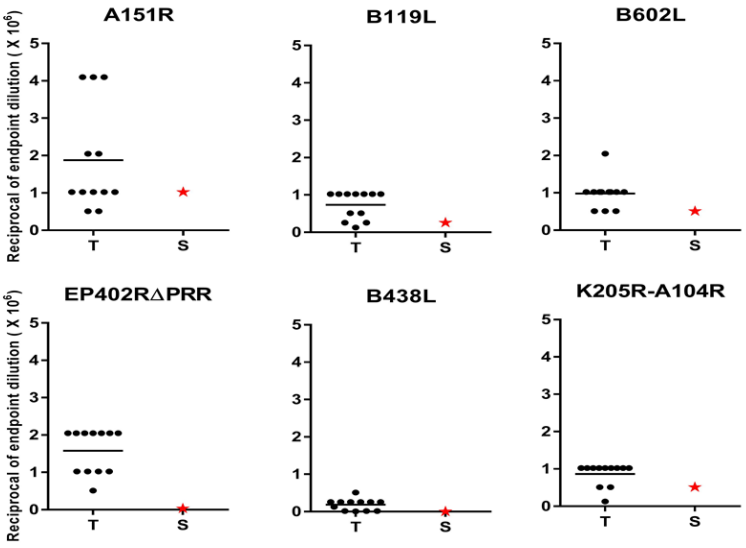

Figure 2. ASFV-specific end-point antibody titers. Antigen specific end-point titers of sera collected 2 weeks post-boost were determined by ELISA. The endpoint dilution was determined to be the dilution at which the sample OD was higher than the OD of cognate pre-bleed +3 standard deviations. Data are presented as the reciprocal of the endpoint sera dilution x 10^6. The ASF-specific convalescent serum was also titrated and the titer against each antigen is depicted by the red star symbol. Sera from control animals showed no reactivity to any of the antigens.

Clinical outcomes post-challenge: Clinical scores, body temperatures, WBC counts and viremia were evaluated following challenge with 10^4 $TCID_{50}$ of ASFV Georgia 2007/1 isolate. The animals began exhibit clinical symptoms of disease 3 days post-challenge. Notably, the treatment group had higher mean clinical scores and body temperatures than the control group on days 4, 5, and 6 post-challenge (Fig. 3A and B). The mean body temperatures of the treatment groups was significantly higher ($p<0.05$) than the controls on day 4 post-challenge (Fig. 3A). In addition, the animals in the treatment groups exhibited a sharper decline in WBC counts compared to the controls (Fig. 4A). On day 5 post-challenge, 50% of the animals in the treatment group (6/12) and only 27% (3/11) of animals in the control group had to be euthanized for animal welfare reasons. All remaining animals had to be euthanized by day 6 post-challenge. No significant difference was observed in viremia in blood collected on the day of termination between the treatment and control groups (Fig. 4B).

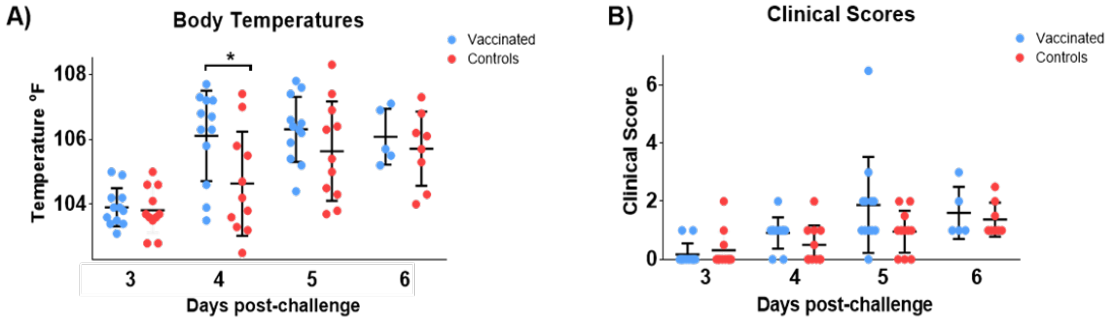

Figure 3. **Clinical manifestations post-challenge**. A) Individual and mean body temperatures; and B) Individual and mean clinical scores of the vaccinated and control groups on days 3, 4, 5, and 6 post-challenge. Asterisk denotes statistically significant differences as compared to the negative controls. *P<0.05.

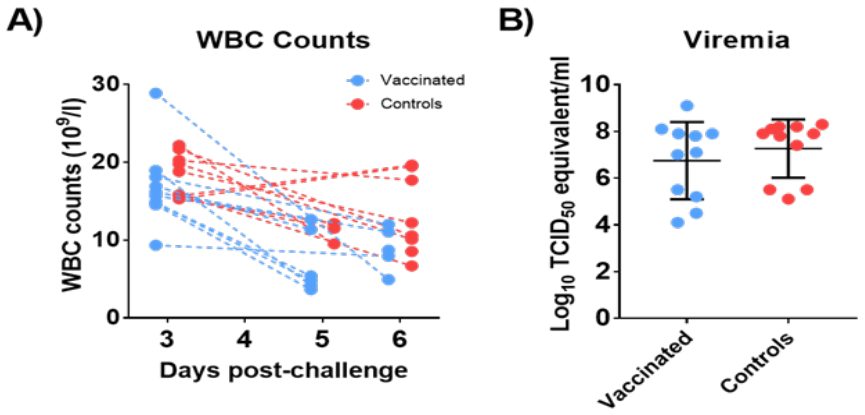

Figure 4. WBC counts and Viremia post-challenge. A) WBC counts in the vaccinated and control groups on day 3 post-challenge and on the day of termination (day 5 or day 6 post-challenge) and B) Viremia (detected by qPCR) in blood samples of vaccinated and control animals on day of termination (day 5 or day 6 post-challenge).

Discussion:

The African Swine Fever Virus (ASFV) poses a high risk to the USA swine industry as it continues to spread globally and since there is no vaccine or treatment available, we generated a rationally designed live-vectored novel prototype ASFV multi-antigen vaccine and evaluated its protective efficacy in commercial pigs. Synthetic genes were used to generate recombinant replication-incompetent adenoviruses designated AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R. Protein expression by these recombinant viruses and the authenticity of the expressed antigens was validated using ASFV-specific immune serum from a pig that had been immunized with the ASF virus (*Lokhandwala S. et. al., 2017*). In addition, these genes were used to generate affinity purified recombinant antigens for use in *in vitro* tests to evaluate and quantify antibody and IFN-γ-secreting T-cell responses as readouts for vaccine immunogenicity in pigs. The purified antigens were also shown to be authentic as judged by Western Blot probed with the ASFV-convalescent serum (*Lokhandwala S.* et. al., 2017).

In our previous study, (*Lokhandwala S. et. al., 2017*), we showed that immunization of piglets with a cocktail containing the above mentioned recombinant adenoviruses (1 x 10^{11} IFU/each) induced strong ASFV antigen-specific antibody responses. A majority of animals had end-point titers (at 2 weeks post-boost) ≥1:256x10^3 against antigens A151R, B119L, B602L, and K205R-A104R. Most importantly and relevant to ASFV vaccine development, the induced antibodies strongly recognized the actual ASF viral proteins and ASFV-infected cells as judged by Western Blot and IFA analysis, respectively. Also post-boosting, strong recall IFN-γ$^+$ responses were detected in a majority of animals for all the antigens. Based on these promising outcomes, in the present study we immunized 24 piglets (treatment groups: n=12, control groups: n=12) with the Ad5-ASFV cocktail and evaluated protective efficacy upon challenge with the ASFV Georgia 2007/1 isolate. Post immunization, evaluation of antigen-specific end-point antibody titers at 2 weeks post-boost revealed that a majority of the vaccinees had titers ≥ 1:1x10^6 against antigens A151R, B119L, B602L, EP402RΔPRR and K205R-A104R (Fig. 2). Overall, the antibody titers induced in the present study were higher than those induced in our previous immunogenicity study (*Lokhandwala S. et. al., 2017*). When challenged with the wild type ASFV Georgia (2007/1) isolate, contrary to our expectations, the vaccinees had higher mean body temperatures and clinical scores, and decreased WBC counts than the control animals. On day 5 post-challenge, due to development of severe disease, 6 of the 12 vaccinees had to be euthanized. In contrast, only 3 of the 11 control animals were euthanized on day 5 post-challenge. The remaining animals were euthanized (and the study was terminated) on day 6 post-challenge.

Taken together, the outcomes from this study showed that the adenovirus-vectored ASFV multi-antigen vaccine cocktail is capable of inducing strong antibody responses in commercial piglets. However, these responses were not protective when the piglets were challenged with wild type ASFV. Furthermore, the disease progression was faster and the symptoms more

severe in the vaccinated pigs compared to the controls. This suggests that the ASFV antigen-specific antibodies induced in the vaccinated pigs contributed towards enhancement of infection. These results imply that for successful development of an ASFV sub-unit vaccine, we not only need to keep searching for protective novel ASFV antigens, but also need to develop an immunization strategy that will primarily induce strong ASFV antigen-specific cytotoxic T-lymphocyte responses while limiting the induction of antibody responses.