RESEARCHREPORT

SWINE HEALTH

Title: Construct and Test Recombinant Adenoviruses Vectoring African Swine Fever Virus Genes in Inducing Immune Protection in Pigs - **NPB #15-128**

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Industry Summary:

African swine fever (ASF) is a highly lethal swine viral disease caused by African swine fever virus (ASFV). Despite extensive research, there are currently no commercial vaccines available due to the biological complexity of the virus. Some experimental vaccines using live attenuated ASFV can induce full protection against the challenge of the same or similar wildtype strains, indicating there are antigen-specific immunity involved in the protection; however, these experimental vaccines cannot be immediately commercialized due to some technical issues in terms of vaccine production and/or safety. To develop an alternative vaccine platform that avoids these drawbacks, we adapted a computational approach to select eight ASFV genes as vaccine antigens according to the expression profiles and biological functions of virus and host genes in infected pig macrophages and used a replication-defective adenovirus to vector these selected antigens. Additionally, we applied knowledge learned from published scientific literatures to design the vaccine to specifically induce immunity that is most likely to be protective based on the nature of ASFV, protein structures and known immune mechanisms. Two porcine genes were included in the vaccine design to enhance the immune responses to the vaccination. Four replication defective recombinant adenoviruses inserted with ASFV and/or pig genes were produced according to the design. These viruses have been validated by DNA sequencing and titrated. A vaccine candidate consisting of the four recombinant adenoviruses has been produced for animal testing; however, the animal experiment could not conducted due to the shutdown of animal facility at PIADC for repair of waste water treatment system. We tried to use the animal facility in Kansas State University but could not find the space for the experiment in the funding period. Currently, the animal facility at PIADC remains close. We plan to conduct the animal experiment in the future when the facility is available.

Keywords: African swine fever, African swine fever virus, ASF, ASFV, recombinant adenovirus, vaccine, bioinformatics

Scientific Abstract:

African swine fever (ASF) is a highly lethal swine viral disease caused by African swine fever virus (ASFV). Despite extensive research, there are currently no commercial vaccines available due to the biological complexity of this virus. Some experimental vaccines using live attenuated ASFV can induce full protection against the challenge of the same or similar wildtype strains, indicating that antigen-specific immunity is involved in the protection. However, the attenuated ASFV cannot be immediately commercialized mostly due to the safety issues. To develop an alternative vaccine platform that avoids these drawbacks, we adapted a genomic and bioinformatic approach to select eight ASFV genes as vaccine antigens according to the expression profiles and biological functions of virus and host genes in pig macrophages infected with ASFV Georgia strain and used a replication-defective adenovirus to vector the selected antigens. Additionally, we applied knowledge learned from published scientific literatures to design the vaccine to specifically induce immunities that are most likely to be protective based on the nature of ASFV, protein structures, and known immune mechanisms. Two porcine genes were also included in the vaccine design in order to enhance the immune responses to the vaccination. Four replication defective recombinant adenoviruses inserted with ASFV and/or porcine genes were produced according to the design. A vaccine candidate consisting of the four recombinant adenoviruses has been produced for animal testing; however, the animal experiment could not conducted due to the shutdown of animal facility at PIADC for repair of waste water treatment system. We tried to use the animal facility in Kansas State University but could not find the space for the experiment in the funding period.

Introduction:

African swine fever (ASF) is a highly lethal swine viral disease caused by African swine fever virus (ASFV). Despite extensive research, there are currently no commercial vaccines available. Like classical swine fever (CSF), ASF displays very similar clinical symptoms including the susceptible hosts and infected cells, but it is caused by a very large DNA virus instead of an RNA virus. CSF can be effectively prevented by several platforms of vaccines and commercial vaccines are available. In contrast, only attenuated ASFV can induce full immune protection, but the safety issues prevent the viruses from commercialization. The technical difficulties due to the biological complexity of the virion and its large DNA genome hinder the development of ASF vaccines. ASFV contains 54 different viral proteins in a virus particle compared to only 4 different proteins in a CSF virus (CSFV) particle. ASFV has a DNA genome approximately 180-kbp coding ~190 open reading frames (protein coding genes) in contrast to about a 12-kbp CSFV RNA genome containing a single polyprotein that is processed into 10 proteins. After infecting cells, ASFV genes are transcribed at different time, which are classified into immediate early, early, intermediate and late genes according to the temporal transcription (Rodríguez and Salas, 2013). ASFV is equipped with sophisticated immune evading mechanisms by expressing a large number of proteins that can interfere with the immune response (Alonso et al., 2013; Sánchez et al., 2013). The biological complexity of ASFV probably is the reason why it is a challenge for the host to mount a protective immunity after infection.

Experimental vectored vaccines based on a limited number of mostly virus structural genes or an expression library containing random virus genome restriction fragments provided only partial protection (Argilaguet et al., 2012; Argilaguet et al., 2013; Lacasta et al., 2014). Currently, live attenuated ASFV strains (obtained by genetic manipulation or by serial passages in different cells) are the only effective experimental vaccines that induce full protection against the challenge (King et al., 2011; Oura et al., 2005). However, it is difficult to use these attenuated strains as commercial vaccines. First, these live attenuated strains produce protection only against the homologous parental virus, and most of the protective strains resulted in permanent infections and significant adverse effects (a significant safety issue). Second, deletion of the same gene, such as DP71L, attenuated certain strains but not others. Third, it has been reported that additional deletion of virulent genes in an attenuated strain reduced the adverse effects on pigs but also reduced the protective efficacy (Abrams et al., 2013), and the infection of swine with a fully attenuated ASFV did not confer protection against the challenge of the virulent parental ASFV (Krug et al., 2015).

Research showed that both humoral and cell-mediated immunity contributed to the immune protection against ASFV infection (Escribano et al., 2013; Takamatsu et al., 2013). However, it appears that cell mediated immunity plays a more important role in protecting pigs against ASFV than the humoral immunity. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection (Neilan et al., 2004), whereas the depletion of CD8+ T lymphocytes in pigs abrogates protective immunity to ASFV induced by an attenuated virus (Oura et al., 2005). Based on reported research results, it has been proposed that identifying antigens with the main protective CD8+ T cell epitopes is a critical step to develop an ASF vaccine (Dixon et al., 2013).

To understand the complexity of ASFV infection, we have used functional genomics approaches to measure virus and host gene expression in infected pig macrophages. Our results show that ASFV changes expression profiles at different times post infection (unpublished). The expression percentages of individual viral genes decreased with the increase of time post infection, which could be troublesome for the immune system not only to mount effective immune response against a specific virus protein but also to eliminate the infected cells. On the other hand, the expression of three ASFV proteins, A179L, A224L, and EP153R that can suppress apoptosis (Alonso et al., 2013), increased more than 5 folds after 9 hours of infection. Because cytotoxic T cells kill infected cells via inducing apoptosis, it may be hypothesized that the infected cells may be killed more effectively by cytotoxic T cells during the early infection. Therefore, vaccines containing genes expressed in the early hours of infection may be more effective in inducing protective immunity against ASFV.

On the other hand, the expression profiles of host genes after ASFV infection also suggest that the immune response to ASFV early proteins could be more effective in protecting pigs than the immunity against late proteins. CD8+ cytotoxic T cells use specific chemokine receptors (CXCR1, CXCR2, and CXCR3) to find infected cells by sensing the concentration gradients of CXCL1, 2, 3, 6, 8 and 10 released by infected cells (Griffith et al., 2014). The results of our microarray analysis show that the expression of CXCL1, 2, 6, and 8 in the infected cells were induced mostly at 3 hours post infection but suppressed afterwards, whereas the expression of CXCL3 was

down-regulated at all tested times compared to non-infected cells. In contrast, the expression of CXCL10 was several folds higher in the infected cells than those in non-infected cells from 3 to 18 hours post infection. The expression of CXCL1, 2, 3, 6 and 8 was reported to be regulated by pro-inflammatory cytokines and virus infection (Imaizumi et al., 2014; Koffmann et al., 2002; Maeda et al., 2015; Zhu et al., 2006), whereas CXCL10 expression is mostly under the regulation of interferons (Oslund et al., 2014; Yeruva et al., 2008); therefore, the recruitment of cytotoxic T cells by CXCL10 may be mainly to infected tissues rather than to infected cells.

Interestingly, our result of interferon expression also support the inference. Both the expression of type I and II interferon receptors was down-regulated starting at 6 hours post infection, whereas the interferon expression was induced after infection. In contrast, the expression of these chemokines in CSFV infected macrophages was induced throughout the infection period tested and the expression of the receptors was not down-regulated (data from Gladue et al., 2010). Additionally, the expression of genes involved in antigen processing and MHC antigen presentation in ASFV-infected macrophages decreased as increase of infection times. These results suggest that ASFV-infected macrophages did not express enough of the chemokines to recruit cytotoxic T cells thus their antigen processing and presentation were compromised in the late stage of infection. Therefore, we hypothesize that vaccines containing viral proteins expressed at 3 hours are more likely to induce protective cytotoxic immunity.

In summary, published research suggest that cell-mediated immunity plays a more important role in protecting pig against ASFV than humoral immunity, and our gene expression profiles of ASFV-infected macrophages suggest that vaccines targeting at early ASFV infection would be more likely to induce protective cell mediated immunity. Traditional approaches using virus structural proteins as vaccine antigens might not be as effective as early proteins because ASFV structural proteins are mostly late proteins. Therefore, we designed a vaccine candidate mainly aiming to induce protective cell-mediated immunity based on the nature of ASFV, protein structures, temporal expression of virus and host genes, and known immune mechanisms to be tested in this study.

Objectives:

The objective of the proposed research is to conduct a proof of principle study by constructing and testing adenovirus-vectored ASF vaccine candidates in pigs. The objective will be achieved in four milestones: (1) constructing recombinant adenovirus vectors containing ASFV genes based on the vaccine design, (2) producing and testing recombinant adenoviruses, (3) scale-up production of recombinant adenoviruses for animal trials, and (4) testing recombinant adenoviruses in pigs for protection against ASFV.

Materials & Methods:

1. Selection of ASFV antigens and vaccine design

Six ASFV open reading frames [A151R, CP204L, CP312R and 3 MGF110 (4L, 5L-6L and 7L)] with the highest expression at 3 hours post infection and two structural

membrane ASFV proteins (EP153R and EP402R) were selected as the antigens for the vaccine design. The expression of six highly expressed ASFV genes ranges from 9 to 36 percent of the total ASFV gene expression based on the signal intensity, whereas the expression of the two ASFV membrane protein was less than 0.5% (Table 1). The coding sequence of A151R, CP204L and CP312R were concatenated together and the four MGF110 (3L, 4L, 5L-6L and 7L) open reading frames were connected using linker peptides as single open reading frames. Extracellular domain sequences of EP153R (1-206aa) and EP402R (50-158aa) were identified using SMART program (Letunic et al., 2015) and linked together as a single polypeptide. Porcine XCL1 coding sequence with a peptide linker was inserted into the 5'-end of the concatenated A151R, CP204L and CP312R sequence to be expressed as one polypeptide. To enhance the vaccination response, IRF2 gene was selected as an adjuvant for the vaccine candidate. The DNA fragments of these three open reading frames as shown in Figure 1 and the IRF2 coding sequence were synthesized by Genscript Inc.

Table 1. The percentages of signal intensity of selected ASFV open reading frames in the total signal intensity of all ASFV genes at different time points post infection

ASFV gene	3 hours	6 hours	9 hours	12 hours	15 hours	18 hours	Average
A151R	5.76	2.98	1.86	2.40	2.07	1.93	3.53
CP204L	5.61	2.54	0.98	2.49	2.15	1.71	3.05
CP312R	5.25	3.37	1.92	2.19	2.04	1.94	3.51
MGF_110-4L	7.14	3.19	1.20	2.34	1.94	1.92	2.95
MGF_110-5L-6L	5.73	3.22	1.71	2.33	1.90	1.83	2.79
MGF_110-7L	7.18	3.57	1.43	2.41	2.07	1.85	3.08
EP153R	0.07	0.04	0.04	0.11	0.12	0.10	0.08
EP402R	0.05	0.07	0.24	0.17	0.21	0.29	0.17
Total %	36.80	18.97	9.39	14.44	12.49	11.56	19.17

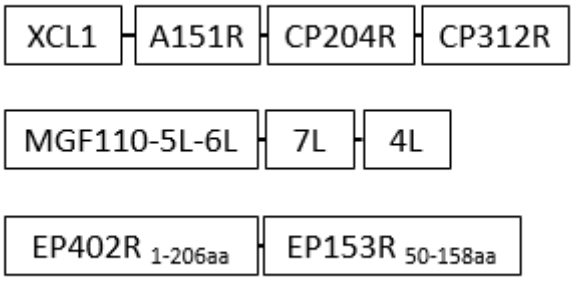

Figure 1. The design of three fused ASFV polypeptides connected with peptide linkers

2. Construction of recombinant adenoviruses

The four synthesized DNA fragments were inserted into an adenovirus plasmid vector, Ad5-blue (Moraes et al., 2001), individually. The cloned plasmids with the correct inserts were used to transfect HEK293 cells with Lipofectamine 2000 in Opti-MEM after digestion with PacI restriction enzyme (New England Biolabs, Ipswich, MA). The recombinant adenoviruses were plaque-isolated and grown in 6-well tissue culture

plates. Virus DNA samples were purified using a genomic DNA isolation kit. The DNA was used as PCR templates to amplify the sequences inserted in the Ad5 genomes. The amplified DNA fragments were purified with a Qiagen PCR reaction purification kit and sequenced to confirm the virus clones. The validated recombinant adenoviruses were sent to Welgen Inc (Worcester, MA) for scale-up virus production.

3. Titration of adenoviruses

Titers of recombinant adenoviruses were determined based on tissue culture infectious dose (TCID50) using HEK293 cell monolayer in 96 well plates according to Moraes et al. (2002). Briefly, the cells were plated at a density of $1x10^4$ cells per well, incubated at 37°C with 5% CO_2 for 3 days or 95-100% confluency. Tenfold serial dilutions starting at 10-5 to 10-12 in Minimum Essential Medium (MEM, GIBCO) were prepared in 1.7 ml sterile micro-centrifuge tubes. Prior to inoculation, the cell culture media was removed and 100 µl per well of the diluted samples was added. Sixteen replicates per dilution per titration and two independent titrations were performed. The plates were incubated at 37°C, 5% CO_2 and checked for the presence of CPE daily for 10 days. Spearman-Karber 50% endpoint viral titers were calculated as TCID50 and then the titers were converted to plaque forming unit (pfu).

4. Animal challenge

A composited vaccine consisting of equal amount of the recombinant adenoviruses will be injected s.c. to vaccinate pigs. Four pigs (50-60 lb.) per treatment group will be used in this experiment. After the primary vaccination, a booster injection will be given two weeks after the primary vaccination. Each pig of Group 1 will be injected s.c. with 10^9 pfu of Ad5-IRF2, Ad5-XCL1-ASFV3P, Ad5-3MGF110 and Ad5-ASFV2EP viruses, and Group 2 will be injected with 10^9 pfu of Ad5-XCL1-ASFV3P, Ad5-3MGF110 and Ad5-ASFV2EP viruses and 300 µg of poly(I:C). One week after the secondary vaccination, these pigs will be challenged by inoculating intramuscularly with either 10^2 or 10^4 HAD50 of wildtype Georgian strain of ASFV as reported by Krug et al. (2015). A control group of pigs will be injected with the same amount ($4X10^9$ pfu) of a recombinant adenovirus without DNA insertion and challenged with the same ASFV as the treated groups. Blood samples will be collected to isolate white blood cells for IFNγ ELISPOT assay before and after the challenge. Clinical signs will be recorded for the evaluation of vaccine efficacy.

Results:

1. Construction of recombinant adenovirus vectors containing ASFV genes

The synthesized DNA fragments were inserted into the Ad5-blue plasmid vector. The cloned plasmids were sequenced to validate the inserted open reading frames. After sequence validation, approximately 300 µg of each plasmid were produced with Qiagen Plasmid MidiPrep kits for DNA transfection.

2. Production and testing of recombinant adenoviruses

More than 3 clones of recombinant adenoviruses were produced from each DNA transfection of HEK293 cells using the Ad5 plasmids inserted with the ASFV protein

genes. These recombinant adenoviruses were validated with DNA sequencing. The sequencing results show that amplified PCR fragments contain expected sequences. Four clones of the recombinant adenoviruses named Ad5-IRF2, Ad5-XCL1-ASFV3P, Ad5-3MGF110 and Ad5-ASFV2EP were sent to Welgen Inc. for scale-up production.

3. Scale-up production of recombinant adenoviruses

We have received 3 ml of each recombinant Adenoviruses from Welgen Inc. The aliquots of the recombinant adenoviruses have been validated by DNA sequences and titrated. The sequencing results confirmed the recombinant adenoviruses expected. A recombinant adenovirus made from an empty vector has been purchased from Welgen to be used as a negative control. All titers of the recombinant adenoviruses are higher than $3.5X10^{10}$ TCID50 per ml.

4. Vaccine trials in pigs for protection against ASFV

A vaccine candidate consisting of the four recombinant adenoviruses has been produced for animal testing; however, the animal experiment could not be conducted due to the shutdown of our animal facility for emergency repair of the waste water treatment system. We tried to use the animal facility in Kansas State University but could not find the space for the experiment in the funding period.

Discussion:

Published researches suggest that cell-mediated immunity plays a more important role in protecting pigs against ASFV than humoral immunity. Therefore, we designed this vaccine candidate with a mind to induce adapted immunity that can kill ASFV-infected cells. To kill infected cells, cytotoxic CD8+ T cells must recognize antigen epitopes presented by the MHC class I molecules of infected cells. Additionally, the activation of CD8+ T cells by dendritic cells and the killing of infected cells by cytotoxic T cells depend on the density of peptide-MHC recognized by individual T cell receptor cluster in the immunosynapse (Manz et al., 2011). Currently, it is unknown which major antigen epitopes are presented on ASF-infected cells. We selected a few ASFV genes with the highest expression hoping to induce cytotoxic T cells recognizing the ASFV peptide-MHC with the highest density on ASFV-infected cells. Because (1) the percent of expression of individual ASFV gene decreases, (2) the expression of ASFV genes with anti-apoptotic effect increases, and (3) the ability of antigen presentation of ASFV-infected macrophages decreases (inferred from our results of macrophage gene expression after ASFV infection) as the time post infection increases, selecting genes highly expressed during the early infection might induce immunity that can more effectively kill ASFV infected cells. On the other hand, killing infected cells in the early infection will probably reduce or prevent the production of infectious virus.

After the antigens were selected, we designed how these antigens will be expressed from the adenovirus vector for immune induction. Ad5-XCL1-ASFV3P was designed to produce secreted ASFV proteins and to induce cytotoxic T cell immunity via antigen cross-presentation according to a mechanism described by Hartung et al. (2015). We hypothesize that antigens targeted to secretory pathways could avoid cell death of Ad5

infected cells caused by over-expression of ASFV proteins in order to prolong antigen production. Longer duration of antigen could induce better immune responses. On the other hand, Ad5-3MGF110 is expected to induce cytotoxic T cell immunity via an alternative TAP-independent MHC class I antigen presentation because of their signal peptide sequences and endoplasmic reticulum retention motifs (Netherton et al., 2004) according to a mechanisms described by Oliveira and van Hall (2015). We also included two lowly expressed ASFV membrane proteins (EP153R and EP402R) in the vaccine design. These two ASFV membrane proteins were reported to be expressed on the surface of ASFV infected cell (Galindo et al., 2000; Rodriguez et al., 1993). Based on the predicted protein structures, we selected the extracellular domains to design a fused protein that is more likely to induce antibodies capable of killing ASFV-infected cells via antibody-directed cell cytotoxicity and compliment-mediated cell lysis.

Replication defective recombinant adenovirus has been approved to be used as a vaccine vector by USDA. Ad5 based FMDV vaccine has been approved for commercial production. Besides DIVA capability, this vaccine platform could be safer than attenuated virus vaccines. We selected six highly expressed ASFV genes in order to increase the chance of including the major protective ASFV antigens in this vaccine design. However, the animal experiment could not conducted due to the shutdown of the animal facility at PIADC for repair of waste water treatment system. The effect of this vaccine candidate remains to be tested in pigs.

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