SWINE HEALTH

Title: Identification of protective antigens of African swine fever virus – **NPB #19-157**

Investigator: D. L Rock

Institution: University of Illinois, Urbana-Champaign

Date Submitted: September 26, 2022

Industry Summary:

African swine fever (ASF) is an acute viral disease of domestic swine with mortality rates approaching 100%. Currently endemic in extensive regions of Eurasia, ASF is “out of Africa” forever, a situation that poses a grave threat to the US swine industry. While our current concern is ASFV Georgia-07, other emerging ASFV strains from African reservoirs threaten swine populations for the indefinite future. Economic analysis indicates an ASF outbreak in the U.S. would result in approximately $15 billion USD in losses, assuming the disease is rapidly controlled (1).

Despite the continual African swine fever (ASF) threat and the fact that the disease was first described over a century ago, it is surprising that no ASF vaccine is available. Current data indicate that ASF vaccines could indeed be developed, as pigs surviving ASFV infection develop long-term resistance to homologous virus challenge but rarely to heterologous virus challenge. Significant progress has been made on engineering attenuated ASF viruses as potential vaccines and promising vaccine candidates are available and at various stages of evaluation (2, 3). However, none have been properly examined for safety and efficacy under field conditions and none have been licensed for use in the US or Europe. Likely, a first-generation Georgia-07 vaccine will be a live-attenuated vaccine (LAV) and its availability will positively impact disease control in endemic regions. However, it is hard to imagine a scenario where they would be used in nonendemic countries with highly developed swine industries; issues of efficacy, residual pathogenicity with immunopathologic sequelae, and potential for long-term viral persistence raise significant questions about their suitability for use as an emergency response tool in the US (2). ASF outbreaks in China linked to use of unlicensed ASF gene-deleted vaccine viruses and the recent safety concerns raised following use of an ASF LAV in Vietnam highlight potential problems with ASF LAV vaccine use in the field (Reuters 1-21-2, Pig Progress 9-6-22 and multiple personal communications). Efforts to design and develop safe and efficacious DIVA-compatible subunit or vectored ASF vaccines suitable for use in the US are urgently needed.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

To date, identification of **ASFV protective antigens (PA)**, the viral proteins capable of stimulating immunity in the pig and the critical first step in developing a subunit vaccine, has proven challenging. The ASF virus (ASFV) is complex (>170 proteins), virus infection-biology is poorly understood and, in most cases, the experimental strategies used to identify ASFV PA have employed unfocused "discovery-based" approaches. In the work described here, we have used comparative ASF genomic analysis together with a novel inter-serogroup ASFV chimeric virus strategy and vaccination challenge experiments in pigs to identify ASFV PA.
The ASFV chimeric virus approach successfully identified four putative serotype-associated PA *(CD2v* (pEP402R)*, C-type lectin* (EP153R) *and ASF locus tags: 789 and 289).* Protection observed in protected animals was solid; only transient fever/clinical responses were observed accompanied by significantly delayed and markedly reduced viremias of approximately 100 to1000-fold (Fig. 4, Burmakina and Khatiwada et al., unpublished data). One or possibly two additional putative viral PA likely will be identified from ongoing studies and improved overall protection results for vaccinated animals may result.

This work provides critical foundational information regarding potential ASFV PA necessary for design/development of safe and efficacious DIVA compatible subunit or vectored ASF vaccines that would be of considerable value for use in the US should ASF be introduced.

Key Findings:

- A novel inter-serogroup ASFV chimeric virus strategy and vaccination challenge experiments in pigs proved successful for identifying putative ASFV PA
- Immunization with four ASFV serotype-associated PA, (CD2v (pEP402R), C-type lectin (EP153R) and ASF locus tags: 789 and 289) protected animals following ASFV challenge.
- Identified ASFV PA should be further evaluated as components of DIVA-compatible subunit or vectored ASF vaccines.

Keywords:

African swine fever (ASF), African swine fever virus (ASFV), subunit vaccine, vector vaccine protective antigens (PA), ASFV chimeric viruses, host protective responses.

Scientific Abstract:

Efforts to design and develop safe and efficacious DIVA-compatible subunit or vectored ASF vaccines suitable for use in the US are urgently needed. To date, identification of ASFV **protective antigens (PA)**, the first critical step in developing a subunit vaccine, has proven challenging. As ASF protective immunity may be serotype-specific, we have focused on ASFV serotype-associated proteins (SAP) as potential protective antigens (PA) for inducing immunity in the pig.

Here, we have used comparative ASF genomic analysis together with a novel inter-serogroup ASFV chimeric virus strategy (ACV) and vaccination /challenge experiments in pigs to identify ASFV PA. This approach successfully identified four putative serotype-associated PA *(CD2v* (pEP402R)*, C-type lectin* (EP153R) *and ASF locus tags: 789 and 289).* Protection observed in immunized/protected animals was solid; only transient fever/clinical responses were observed accompanied by significantly delayed and markedly reduced viremias of approximately 100 to1000-fold (Fig. 3 and 4; Burmakina and Khatiwada et al., unpublished data). This work provides critical foundational information regarding ASFV PA necessary for design/development of safe and efficacious DIVA compatible subunit or vectored ASF vaccines that will be of considerable value for use in the US should an ASF threat emerge.

Introduction:

ASF subunit vaccines where only specific protective viral antigens and optimized delivery/vector systems are used to vaccinate the host will remove the significant safety concerns associated with ASF LAV use. Before subunit vaccine strategies can be designed, relevant ASFV PA and the breadth of their natural antigenic diversity need to be known. Knowledge of ASFV protective antigens and viral protein(s) responsible for serotype specific cross-protective immunity is the first step toward development of an ASF subunit vaccine that would be DIVA (Differentiate Infected from Vaccinated Animal) compatible and suitable for use under emergency conditions in the United States. Additionally, the eventual availability of any effective ASF vaccine will significantly reduce the risk to US agriculture and swine producers, by controlling the disease where it is endemic thus reducing the possibility of disease introduction into this country.

To date, identification of ASFV PA has proven challenging. The ASF virus is complex (>170 proteins), virus infection-biology is poorly understood and, in most cases, the experimental strategies used to identify ASFV PA have employed unfocused "discovery-based" approaches and unvalidated predictive algorithms for humans and mice. Often, studies have focused only on the most antigenic ASFV proteins to the exclusion of all others. Problematically, research strategies have relied on using two unknowns to study each other; ASFV PA are not known, nor are ways to optimally present the antigens to the host to induce protective host responses. Together, these issues account for the rather disappointing progress to date for this critically important area. We previously have shown: **1)** ASF protective immunity may be serotype-specific, as viruses within a haemadsorption-inhibition (HAI) serogroup cross-protect against one another while viruses outside the serogroup do not [4-9]); **2)** two ASFV-encoded proteins, CD2v (EP402R) and/or C-type lectin (EP153R), are sufficient for mediating HAI serologic specificity [10]) and, they are important viral antigens for inducing protective immune responses in the pig [8, 11] **3)** neutralization of CD2v-induced IFN signaling by anti-CD2v antibodies may represent a novel antibody-mediated protective immune mechanism that prevents extensive bystander lymphocyte apoptosis observed in lymphoid tissue during acute ASFV infection in the pig [12] and **4)** CD2v and C-type lectin alone are not sufficient for providing solid protection for all immunized animals [8, 11], suggesting additional viral PA, very likely serogroup-associated, are necessary. We hypothesized that additional ASFV serotype-specific PA are necessary for full protection in the pig and, we have used comparative ASF genomic analysis together with a novel inter-serogroup ASFV chimeric virus strategy and vaccination challenge experiments in pigs to identify them.

Objective: Identification of ASFV protective antigens (PA)

Materials & Methods:

Identification of ASF serotype-associated proteins (SAP) using comparative ASF genomic analysis. Comparative ASFV genomic analysis of multiple ASFV genomes representing all known HAI serogroups was performed to select additional ASFV proteins demonstrating significant serotype-association (Tulman et al., unpublished data). Nucleotide and conceptually translated gene datasets were subjected to standard comparative analyses to assess genetic variability and predicted protein features as previously described [13-16]. SAP gene candidates, ORF locus tags: 183, 789, 764, 145, 782, 707, 761, and 289 were identified. Serogroup-specific antigenicity, phylogenetic analyses were conducted to further assess candidate PA for discrete genetic distance between serogroups. Candidate translated gene sequences were aligned with standalone MAFFT [17], or CLUSTALW and/or MUSCLE [18] as implemented in SEAVIEW4 [29], which was also used for manual review and refinement of alignments. Phylogenetic analyses were conducted as implemented in PHYLIP [19] and Phyml [20] in SEAVIEW4. These analyses are not necessarily the most correct model of evolution that is sought, but rather provide a tree which indicates genetic distance and potential antigenic divergence between serogroups.

Construction of chimeric ASF viruses for animal immunization. Our novel experimental approach to define ASFV PA involved immunization with ASFV inter-serogroup chimeric viruses and challenge infection of pigs as previously described [8, 11]. The approach used an ASFV virus of one serogroup as an expression vector to express SAP genes from a second ASFV serogroup. Here, inter-serogroup chimeric ASFV viruses were constructed; ASFV Mozambique strain (MOZa), a **serogroup** (**SG**) 3 virus, containing SAP genes from the ASFV Congo strain, a SG-2 virus, were examined using vaccination/challenge experiments in pigs for their ability to protect against challenge infection with ASFV Congo strain. We anticipated that serotype specific cross-protective immunity would be shifted from SG3 to SG2 by exchanging a

Figure 1: Rationale of Identification of ASFV Protective Antigens using Inter-Serotypic Recombinant (Chimeric) Viruses

	Immunization *Virus*	Challenge Infection *Virus*	Protection Result
A	SG2-Congo$_a$	SG2$_V$-Congo	++++
B	SG3-Moz$_a$		-
C	Moz$_a$$^{+1\text{-Congo protein}}$		+/-
D	Moz$_a$$^{+2\text{-Congo proteins}}$		+/++
E	Moz$_a$$^{+3\text{-Congo proteins}}$		++++

Fig. 1. *Rationale for identification of ASFV Congo protective antigens using inter-serotypic recombinant (chimeric) viruses. Immunization with attenuated ASFV (e.g. serogroup 2, SG2a) protects pigs against challenge with virulent virus of the same serogroup (SG2v;* ***A****). In contrast, pigs immunized with attenuated virus of a different serogroup (SG3a) are not protected when challenged with SG2v virus (****B****). Immunization with a chimeric SG3a virus containing putative serotype-associated ASFV genes from SG2a, however, will result in partial protection against SG2v virus challenge (****C****). And, additional exchange of viral genes will completely shift immunity from one serogroup to the other (****D, E****).*

subset of critical viral genes between the viruses (Fig. 1). We found this to be a robust experimental approach as it alleviates many of the confounding issues that have characterized other approaches aimed at identifying specific ASFV PA [8]. Specifically, the chimeric ASFV strategy ensures proper expression and post-translational modification of the putative viral PA in macrophages, delivery of the appropriate antigen dose and proper antigen presentation to the pig in the context of natural infection. A MOZa-strain chimeric virus expressing CD2v/lectin proteins from the ASFV Congo strain ($MOZa^{+ Congo\ CD2/Lectin}$) was used as a parental virus for construction of other chimeric viruses to assess additional candidate PA. Congo SAP genes and upstream promoter regions were PCR-amplified or synthesized (GenScript,Inc.) with appropriate flanking restriction sites and cloned directly into the right-variable-region (RVR) recombination plasmid of the $MOZa^{+ Congo\ CD2/Lectin}$ genome creating chimeric viruses diploid for the SAP gene of interest.. Through this process an HA-tag is cloned in frame to the C-terminus of the SAP to be expressed. Chimeric ASFVs were obtained by homologous recombination following infection with parental virus and transfection with a right variable region recombination plasmid containing the additional Congo gene and a GFP reporter protein in macrophage cell cultures (see Fig. 2). Recombinant chimeric ASF viruses ($MOZ_a^{+ Congo\ CD2/Lectin + 1\ SAP}$) were selected by limiting dilution and reporter gene expression (GFP) under a fluorescent microscope. Viral purity and recombinant sequence fidelity was assessed by PCR and DNA sequencing of the recombinant viruses. Growth characteristics of ASFV chimeric viruses was evaluated in both macrophage and bone marrow cell cultures as previously described (8, 21, 22). Chimeric viruses containing Congo genes ORF, locus tags: 183, 789, 764, 145, 782, 707, 761, and 289 were constructed and evaluated for expression of the cloned Congo SAP (HA-tagged) by IFA and/or Western blot using anti-HA antibodies conjugated with fluorescein or HRP [23].

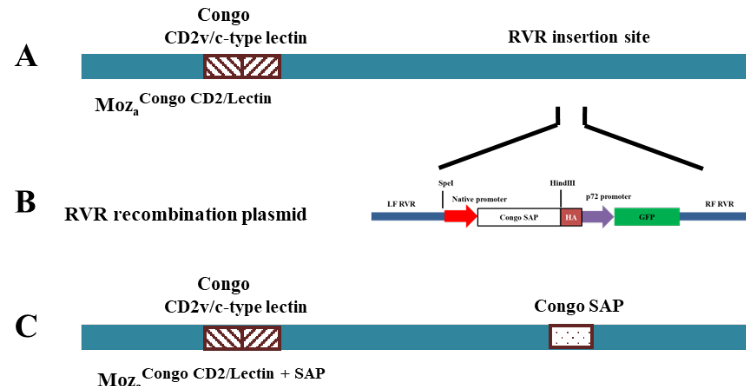

***Fig. 2. A**. Parental ASF SG-3 attenuated Mozambique virus expressing SG-2 Congo CD2v/c-type lectin. **B**. RVR recombination plasmid containing sequences for (from left to right) left flanking region (LF RVR), SAP native promoter, HA-tagged Congo SAP ORF, p72 promoter, green fluorescence protein (GFP), and right flanking region (RF RVR). **C**. Chimeric virus* $\boldsymbol{Moz_a}^{+Congo\ CD2/Lectin + 1SAP}$ *after insertion of Congo SAP into the RVR.*

B) Animal immunization and challenge infection. - Landrace and large white pigs (30–35 kg) were divided into groups (*n* = 8) and housed separately throughout the experiment. Animals were mock-vaccinated or vaccinated by the intramuscular route with 10^6 haemadsorbing units (HAU) of each ASFV MOZa/Congo chimera(s) being evaluated and boosted with the same virus dose at 21 days post-vaccination. Three weeks later, animals were challenged intramuscularly with 10^3 HAU of virulent ASFV Congo stain as we have previously described [8, 24] and monitored for 35 days. Control groups included animals immunized with attenuated MOZa and, an unimmunized control group. Following challenge infection, clinical signs (rectal temperature greater than or equal to 40° C, anorexia, lethargy, shivering, cyanosis, and recumbency), survival rate and time-

to-death were recorded daily as described previously [8]. Blood/serum samples were collected at regular intervals post-vaccination and for 35 days post-challenge (DPC). Quantitative PCR of ASFV DNA in blood samples was performed as previously described [8].

Challenge data with multiple data points per group was assessed by one- way ANOVA, or Kruskal–Wallis one-way ANOVA on ranks if data failed Shapiro–Wilk normality testing, and by Tukey's or Dunn's multiple comparison tests between significant groups as implemented in SigmaPlot v11 (Systat Software).

Results:

Immunization with serotype-specific ASFV CD2/Lectin provides protection from ASF

To determine if serotype-specific CD2v and C-type lectin proteins mediate ASFV protective immunity, pigs were vaccinated with MOZa$^{+\text{ Congo CD2/Lectin}}$, an attenuated Mozambique strain-based chimeric virus in which native CD2v/C-type lectin genes were deleted and replaced by those from the heterologous Congo virus (K-49) strain, and then challenged with virulent Congo K49 virus. With the substitution of MOZa CD2v and C-type lectin with Congo proteins, approximately 25-40% survival rate was observed for the MOZa$^{+\text{ Congo CD2/Lectin}}$ immunized animals (Fig. 3A and Fig. 4A). As expected, control animals and animals immunized with an attenuated Mozambique strain (MOZa) virus were not protected from challenge with virulent

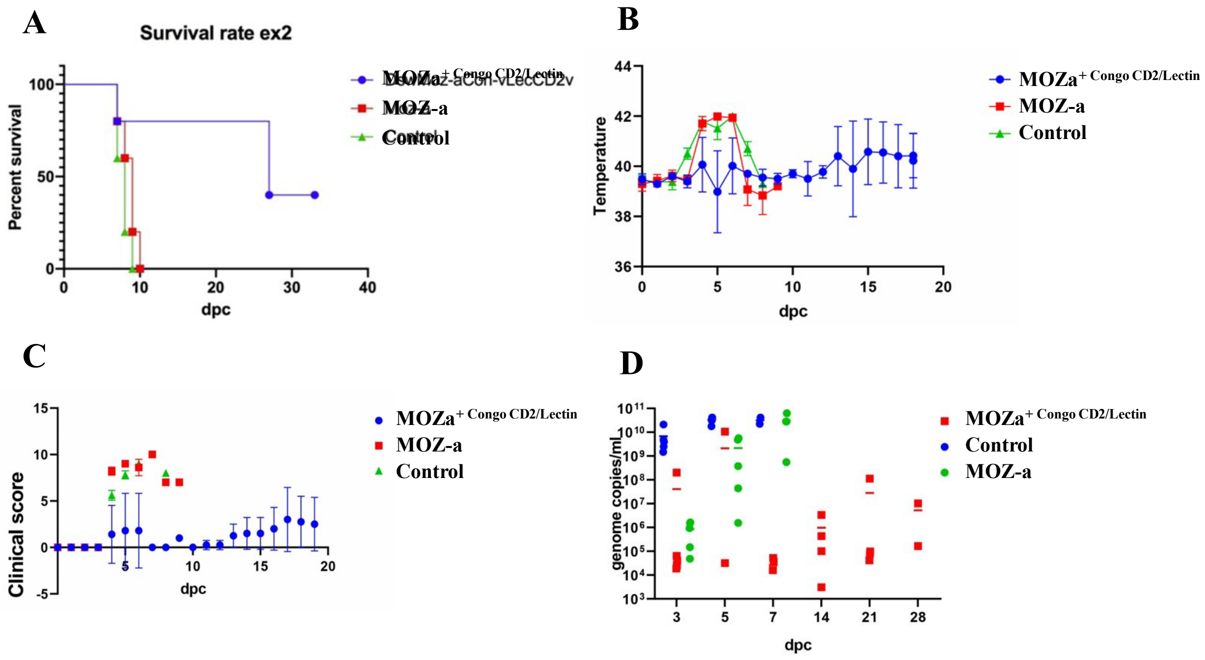

Fig.3. *Pigs (n=5) were mock-vaccinated or vaccinated by the IM route with 10^6 HAU of MOZ-a, MOZa$^{+\text{ Congo CD2/Lectin}}$ and boosted six times with the same virus dose at 14-day intervals. Three weeks after the final boost, animals were challenged IM with 10^3 HAU of virulent Congo virus. Survival analysis (A), temperature (B), clinical score (C) and PCR in blood (D) of challenged animals are shown.*

Congo K49. Lack of heterologous protection included rapid onset of fever and other ASF clinical signs and high-titered viremic loads compared to animals immunized with MOZa$^{+\text{ Congo CD2/Lectin}}$

(Fig. 3B-D). Results indicate that immunization with serotype -specific ASFV CD2/Lectin proteins protects some pigs from ASF and further suggest that additional serotype specific protective antigens may be required.

Immunization with serotype-specific ASFV CD2/Lectin and ASF locus tags: 789 and 289 proteins enhances protection to ASF

A MOZa-strain chimeric virus expressing CD2v/lectin proteins from the ASFV Congo strain, $\text{MOZa}^{+\text{ Congo CD2/Lectin}}$ was used as a parental virus for construction of chimeric viruses to assess additional candidate PA. Additional Congo-SAP were cloned into a site located in the right variable (RVR) region of the $\text{MOZa}^{+\text{ Congo CD2/Lectin}}$ genome creating chimeric viruses diploid for two additional SAP gene of interest ORF locus tags: 789 and 289, (Fig 2). To determine if inclusion of these two additional Congo SAP (ORF locus tags: 789 and 289) enhanced ASFV protective immunity, pigs were vaccinated with ASFV MOZ/Congo chimera(s), and then challenged with virulent Congo K49 virus.

Inclusion of these two additional Congo SAP (ORF locus tags: 789 and 289) increased the survival rate in immunized animals, improving upon protection results obtained following immunization with Congo CD2v and C-type lectin proteins alone (Fig. 4A). Notably, the protection observed in these animals was solid; only transient fever/clinical responses were observed accompanied by significantly delayed and markedly reduced viremias of approximately 100 to1000-fold (Fig. 4B-D). Importantly, these data indicate that four ASFV serotype-associated PA, (CD2v (pEP402R), Lectin (EP153R), ORF locus tags: 789 and 289) are associated with protection in immunized animals.

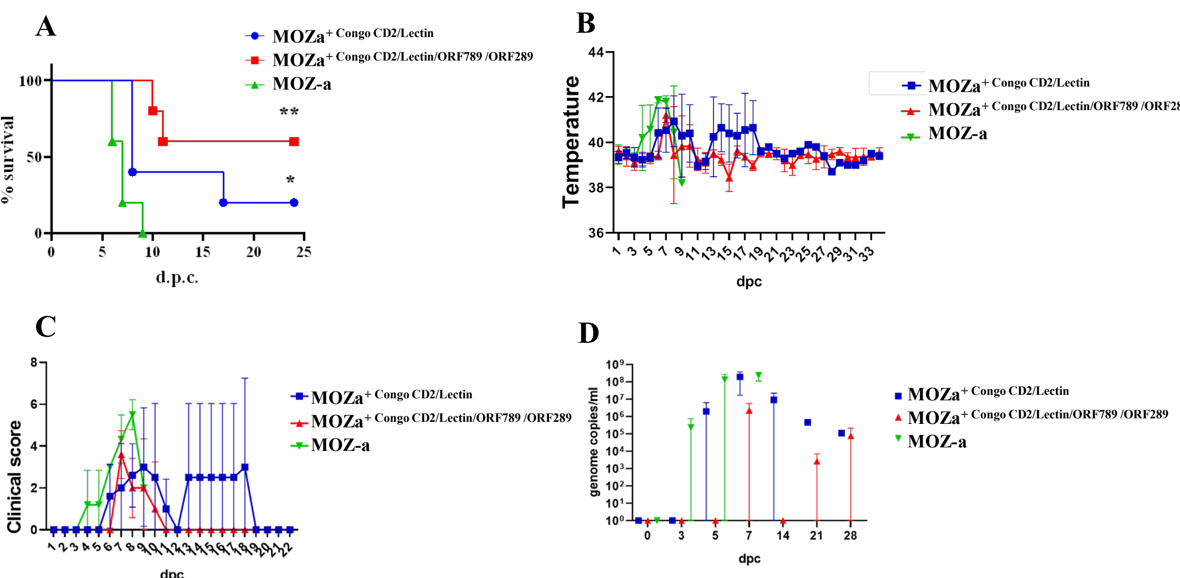

Fig. 4. *Pigs (n=8) were mock-vaccinated or vaccinated by the IM) route with 10^6 HAU of MOZ-a or the respective ASFV MOZ/Congo chimera(s) and boosted with the same virus dose at 21 days post-vaccination. Three weeks later, animals were challenged IM with 10^3 HAU of virulent Congo virus. Survival analysis (A), temperature (B), clinical score (C) and PCR in blood (D) of challenged animals are shown.*

Discussion:

Results obtained provide additional support for the role of ASFV serotype-associated proteins in protective immunity. Here, protection was observed in pigs immunized with Congo CD2v and C-type lectin proteins expressed from a MOZa-based expression virus construct (Fig. 3 and 4). The protection observed in these experiments was markedly enhanced over our prior results where a France-Congo CD2v/Lectin expression vector was used to immunize pigs [8]. Immunization schedule or enhanced replication of the MOZa-based expression vector observed following immunization may account for the increased vaccine efficacy observed in these experiments.

Further, results indicate that immunization including two additional Congo serotype-associated proteins, ORF locus tags: 789 and 289, increased the survival rate in immunized animals, improving upon protection results obtained following immunization with Congo CD2v and C-type lectin proteins alone (Fig. 3 and 4). Notably, the protection observed in these animals was solid; only transient fever/clinical responses were observed accompanied by significantly delayed and markedly reduced viremias of approximately 100 to 1000-fold (Burmakina and Khatiwada et al., unpublished data). Importantly, data indicate that a degree of protection can be induced in animals following immunization with four ASFV serotype-associated PA, (CD2v (pEP402R), Lectin (EP153R) and ORF locus tags: 789 and 289).

Notably, following immunization with inter-serogroup chimeric ASF viruses, we observed both protected and unprotected animals after challenge (see Fig. 3 and Fig. 4), which leads us to hypothesize that host immune responses to these ASFV PA in unprotected animals differed qualitatively or quantitively from those observed in the protected animals. It is known that ASFV CD2v antibodies appear late and at relatively low titer during infection and that ASFV C-type lectin induces weak serologic responses in animals [8, 11, 25, 26, 27, 28 and unpublished results]. Conceivably, increased vaccine dose and/or an altered immunization schedule may be required to obtain protective immune responses (humoral and cellular) in all immunized animals.

Given the encouraging results obtained here, it will be important to properly evaluate these ASFV PA as vaccine candidates for ASFV Georgia-07 using a suitable subunit or vaccine-vectored platform/strategy. A better understanding of diversity among PA and mapping protective epitopes on the proteins will facilitate design and development of synthetic, consensus and/or multivalent antigens capable of immunizing animals against multiple viral strains; this capability would be invaluable for emergency response use in the US.

Establishing correlates of ASF protective immunity have proven challenging [2]. However, now that only a smaller subset of viral PA are being examined, may simplify analysis permitting insight not readily obtainable working with the complexity of the complete virus. Now, it may be possible to establish a protective response signature and measurable correlates of protection in the pig: a tool of considerable value for evaluating/benchmarking ASFV vaccine candidates.

The ASFV inter-serogroup chimeric virus strategy together with vaccination/challenge experiment in pigs outlined here proved highly successful for identification ASFV protective antigens. We found this a robust experimental approach alleviating many of the confounding issues that have characterized other approaches aimed at identifying ASFV PA [8, 11]. Specifically, the chimeric ASFV strategy ensures proper expression and post-translational

modification of the putative viral PA in macrophages, delivery of the appropriate antigen dose and proper antigen presentation to the pig in the context of natural infection. It is reasonable to suggest other ASFV proteins, other than those identified here, also may serve as ASFV PA. The inter-serogroup chimeric virus strategy may be both an efficient and reliable approach for identifying other relevant ASFV PA. Additional vaccination/challenge experiments evaluating other MOZ/Congo chimeric viruses individually and in pools to identify additional PA are ongoing by our research team and we anticipate other viral PA will be identified and that the overall protection rate may be enhanced.

References

1- Carriquiry, M., Elobeid, A., Swenson, D.; Hayes, D. 2020. Impacts of African Swine Fever in Iowa and the United States. CARD, Iowa State University, Ames, www.card.iastate.edu.

2- Rock, D.L. 2021. Thoughts on African Swine Fever Vaccines. Viruses 13, 943. https://doi.org/10.3390/ v13050943.

3- Gladue, D.P; Borca, M.V. 2022. Recombinant ASF Live Attenuated Virus Strains as Experimental Vaccine Candidate. Viruses 14, 878. https://doi.org/10.3390/v14050878.

4 Vishnjakov, I.; Mitin, N.; Karpov, G.; Kurinnov, V.; Jashin, A. 1991. Differentiation African and classical swine fever viruses. Veterinariya 4, 28–31. (In Russian).

5- Sereda, A.D.; Solovkin, S.L.; Fugina, L.G.; Makarov, V.V. 1992. Immune reactions to the African swine fever virus. Vopr. Virusol. 37, 168–170. (In Russian).

6- Balyshev, V.M.; Fedorishhev, I.V.; Salina, M.V.1995. Study of serotype interactions of ASF virus strains both in vitro and in vivo. In Viral Diseases of Animals; FGBI ARRIAH: Vladimir, Russia, p. 230.

7- Sereda, A.D., Balyshev, V.M., 2011.[Antigenic diversity of African swine fever viruses]. Vopr Virusol, 56, 38-42. (In Russian).

8- Burmakina, G.; Malogolovkin, A.; Tulman, E.R.; Zsak, L.; Delhon, G.; Diel, D.G.; Shobogorov, N.; Morgunov, Y.; Morgunov, S.; Kutish, G.F.; Kolbasov, D. 2016. African swine fever virus serotype-specific proteins are significant protective antigens for African swine fever. J. Gen. Virol. 96, 866–873.

9- Sereda, A.E.; Balyshev, V.M.; Kazakova, A.S.; Imatdinov, A.R.; Kolbasov, D.V. 2020. Protective properties of attenuated strains of African swine fever virus belonging to seroimmunotypes I–VIII. Pathogens 9, 274. doi:10.3390/pathogens9040274.

10- Malogolovkin, A.; Burmakina, G.; Tulman, E.R.; Delhon, G.; Diel, D.G.; Salnikov, N.; Kutish, G.F.; Kolbasov, D.; Rock, D.L. 2015. African swine fever virus CD2 v and C-type lectin gene loci mediate serological specificity. J. Gen. Virol. 96, 866–873.

11- Burmakina, G.; Malogolovkin, A.; Tulman, E.; Delhon, G.; Kolbasov, D.; Rock, D.L. 2019. Identification of T-Cell epitopes in African swine fever virus CD2v and C-type lectin proteins. J. Gen. Virol. 100, 259–265.

12 - Chaulagain, S., Delhon, G., Khatiwada, S., and Rock, D.L. 2021.African Swine Fever Virus CD2v protein induces β-Interferon expression and apoptosis in swine peripheral blood mononuclear cells. *Viruses* 13, 1480. https://doi.org/10.3390/v13081480.

13 - Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F., Rock, D.L., 2001. Genome of lumpy skin disease virus. J Virol. 75, 7122-30.

14 - Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Sur, J.H., Sandybaev, N.T., Kerembekova, U.Z., Zaitsev, V.L., Kutish, G.F., Rock, D.L., 2002. The genomes of sheeppox and goatpox viruses. J Virol. 76, 6054-61.

15 - Delhon, G., Tulman, E.R., Afonso, C.L., Lu, Z., de la Concha-Bermejillo, A., Lehmkuhl, H.D., Piccone, M.E., Kutish, G..F, Rock, D.L., 2004. Genomes of the parapoxviruses Orf virus and bovine papular stomatitis virus. J Virol. 78, 168-77.

16 - Tulman, E.R., Liao, X., Szczepanek, S.M., Ley, D.H., Kutish, G.F., Geary, S.J. 2012. Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of *Mycoplasma gallisepticum.* Microbiology 158, 2073-88.

17 - Katoh, K., Misawa, K., Kuma, K., Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30: 3059-3066.

18 - Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32, 1792-97.

19 - Felsenstein, J. 1989. Mathematics vs evolution: Mathematical evolutionary theory. Science 246, 941-942.

20 - Guindon, S., Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 52, 696-704.

21- Zsak, L., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L. 1996. An African swine fever virus virulence-associated gene NL-S with similarity to the herpes simplex virus ICP34. 5 gene. J. Virol. 70, 8865–8871.

22- Zsak, L.; Onisk, D.V.; Afonso, C.L.; Rock, D.L. 1993. Virulent African swine fever virus isolates are neutralized by swine immune serum and by monoclonal antibodies recognizing a 72-kDa viral protein. Virology 196, 596–602.

23- Khatiwada, S., Delhon, G., Ponnuraj, N., Luo, S., Diel, D.G., Flores, E.F., Rock, D.L. A parapoxviral virion protein inhibits NF-κB signaling early in infection., 2017. Plos Pathogens, 13(8):e1006561. doi: 10.1371/journal.ppat.1006561

24 - Zsak, L., Borca, M. V., Risatti, G. R., French, R. A., Lu, Z., Kutish, G. F., Neilan, J. G.,Callahan, W. M., Rock, D. L., 2005. Preclinical Diagnosis of Africa Swine Fever in Contact Exposed Swine by a Portable Real-Time PCR Assay. J. Clin. Micro. 43, 112-119.

25 - Malmguist, W., 1963. Serologic and immunologic studies with African swine fever virus. Am. J. Vet. Res. 24, 450–459.

26 -Ruiz-Gonzalvo, F., Carnero, M.E., Caballero, C., Martinez, J., 1986. Inhibition of African swine fever infection in the presence of immune sera in vivo and in vitro. Am. J. Vet. Res. 47, 1249–1252.

27 - Ruiz-Gonzalvo, F., Coll, J.M., 1993. Characterization of a soluble hemagglutinin induced in African swine fever virus-infected cells. Virology 196, 769–777.

28 - 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.

29 - Gouy, M., Guindon, S., Gascuel, O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221-224.

.