

Article

Vaccination with an African Swine Fever Virus Multiepitope Protein Chitosan Nanoparticle-Based Subunit Vaccine Elicits Robust Immune Responses In Vivo

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Abstract

Background/Objectives: African swine fever virus (ASFV), the causative agent of African swine fever (ASF), is a highly contagious virus affecting both domestic and feral pig populations with mortality rates approaching 100% within one week of infection. Currently, there are limited treatments or vaccines available to control the disease. Although ASF is endemic in sub-Saharan Africa, the virus has also spread widely, reaching regions of the European Union, Russia, China, Southeast Asia, and, more recently, to the Dominican Republic and Haiti, bringing the threat closer to the United States (U.S.). ASF introduction to the U.S. would have severe consequences for swine producers and the national pork industry. Consequently, there is an urgent need to develop effective vaccine strategies to manage ongoing outbreaks abroad and mitigate the risk of future ASF incursions. Recent efforts have identified several ASFV epitopes and evaluated them in experimental vaccine trials. However, these vaccine candidates have elicited limited protective immune responses and have not demonstrated full protective efficacy. **Methods:** In this study, we employed in silico modeling and epitope prediction tools to design a synthetic multiepitope ASF protein incorporating key immunogenic regions of ASFV. The goal was to generate a single-antigen construct capable of inducing broad and robust immune responses when formulated with an established nanoparticle-based vaccine platform. The multiepitope ASF protein was subsequently expressed and entrapped into mannose-conjugated chitosan (M-CS) nanoparticles for vaccine formulation. The candidate vaccine, formulated with M-CS nanoparticle-entrapped adjuvant (ADU S100), was administered intramuscularly to pigs, and both T- and B-cell responses were assessed following the primary (DPV 22) and booster (DPV 42) doses. **Results:** Our M-CS ASF protein vaccine elicited antigen-specific T- and B-cell responses, both of which are recognized as central correlates of protection against ASFV. **Conclusions:** These promising preliminary immunological findings suggest that this nanoparticle vaccine has the potential to confer protection against ASFV challenge, a hypothesis that will be examined in future studies.

Keywords: African swine fever; subunit vaccine; nanoparticle; immunogenicity



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1. Introduction

First reported in Kenya in 1921, African swine fever virus (ASFV), the causative agent of ASF, is a deadly, highly contagious viral hemorrhagic disease affecting domestic and feral pigs and suids of all ages [1]. Over 100 years since its emergence, ASF has spread around the world, making its way to Western Europe, Latin America, Eastern Europe, China, Southeast Asia, and, more recently, the Dominican Republic and Haiti, marking the first time ASF was detected in North America in 40 years [2,3]. ASF poses a significant economic threat to the U.S. livestock and swine industry, prompting an arms race in preparedness for combating its introduction and spread. To date, Vietnam is the first country to approve the use of only two commercially available ASF vaccines. One of these vaccines, AVAC ASF LIVE, has been exported to the Philippines, as well as Nigeria and Indonesia. However, its widespread use remains restricted outside of Vietnam while the vaccine undergoes registration and efficacy evaluation. Consequently, ASF control in much of the world relies on the mass depopulation of infected animal herds as an eradication method, as widely approved vaccines remain unavailable.

ASFV, the only member of the *Asfarviridae* family, is a double-stranded, icosahedral DNA virus whose large genome encodes over 150 proteins [4,5]. The dominant structural component, major capsid protein p72, comprises the outermost shell of the virion and encompasses ~30% of the total mass of the virion [6]. The C-terminus of the p72 protein has been historically used as the predominant genotyping method due to its stability and conservation among ASF genotypes [7]. However, recently, researchers have reclassified and reduced the number of ASFV genotypes from twenty-five to only six unique genotypes based on a rigorous reassessment of all the publicly available nucleotide sequences of p72, after concluding that many of the deposited sequences were duplicated in honest scientific error [7]. The size and complexity of this virus have historically rendered it difficult to target with subunit vaccines.

Genotype I and II strains of ASFV are typically the most virulent and result in high fever, loss of appetite, visible hemorrhages on the skin and internal organs, splenomegaly, and death within seven days of infection. However, low virulent strains, originating from mutations in highly virulent strains, have been identified and cause chronic or subclinical disease [8]. In Africa, where ASF is endemic, low virulent strains continue to circulate, and the disease often presents as subclinical and/or asymptomatic infections [9]. In fact, pigs that survive initial infection with low virulent strains may become chronically infected and serve as viral reservoirs, further complicating disease eradication programs [10].

Vaccination is likely the best option for the prevention and control of ASF worldwide. However, the continued emergence of novel ASF variants poses a significant threat to vaccine-based control strategies, especially when compounded by the use of substandard vaccines and the lack of adherence to vaccine administration guidelines. Insufficient vaccine protection, improper dosing, and the use of inadequately evaluated vaccines increases the risk of vaccine-escape mutants and viral recombination with circulating field strains. In regions where ASF is endemic, sustained transmission between wildlife reservoirs and domestic pigs promotes long-term environmental persistence of the virus, intensifies evolutionary pressure, and facilitates the emergence of new strains. Although live-attenuated vaccines (LAVs) are thought to be the most effective candidates for ASF control [11], their widespread application remains limited by (1) ongoing concerns with vaccine strain reversion to virulence, (2) vaccine strain recombination with field strains, and (3) the inability to differentiate between vaccinated and infected animals [12,13].

Subunit vaccines, containing a cocktail of ASFV structural proteins, have the potential to circumvent the concerns surrounding LAVs due to their high safety and stability profile, increased effectiveness by direct targeting of the immune system, minimal side effects, and

scalable production [14]. Traditionally, subunit vaccines have been viewed as less effective than LAVs at targeting ASFV due to the size and complexity of the virus, resulting in insufficient vaccine protection [15]. However, nanoparticle vaccine delivery systems can greatly improve the performance of protein-based vaccines, especially for complex pathogens, such as ASFV, where subunit antigens alone have been proven to be weakly immunogenic and unable to provide full protection from disease [14,16–18]. Therefore, combining a subunit vaccine antigen in a nanoparticle delivery system could result in better immune targeting, thereby substantially increasing the efficacy of the ASFV subunit vaccine.

The rational design of highly effective vaccines depends on the identification and incorporation of critical immune response stimulators. However, for ASFV, the factors determining protective immunity are complex, posing a significant challenge to vaccine development. Many proposed immune correlates of protection are derived from live-attenuated vaccine models, making them difficult to translate to subunit vaccine approaches, while host variability further complicates their standardization and evaluation across studies [19]. However, the role of CD8⁺ cytotoxic T-cells in ASFV infection has been extensively reviewed [20] and they have been shown to play a crucial role in immune protection and memory against ASFV infection [20,21]. The activation of humoral immunity, particularly the induction of virus-neutralizing antibodies following ASFV infection, also remains a subject of debate. Although original reports indicated sera collected from ASFV-infected animals lacked neutralizing activity, more recent evidence suggests that neutralizing antibodies may play an important role in protection. Importantly, the detection of neutralizing antibodies in vitro is technically challenging and may be underestimated. Studies suggest that the susceptibility of ASFV to in vitro neutralization is influenced by additional factors that hinder the reliable detection of neutralizing activity, such as the passage number of ASFV variants, the cell line, and the virus purification method used [22].

Here, we sought to improve the ASFV subunit vaccine platform by encapsulating a synthetic ASFV multiepitope protein with a well-established mannose-conjugated chitosan (M-CS) nanoparticle delivery system [23–25]. Chitosan-based drug delivery systems are biocompatible and shield the antigen from degradation while allowing for controlled, sustained release [26]. The use of multiepitope vaccines is emerging as a promising strategy against viral infections, combining rationally chosen epitopes to induce a targeted immune response [27]. Using in silico modeling and prediction tools to engineer a synthetic, multi-epitope ASF protein containing key immunogenic ASFV sites, we sought to induce robust, cross-protective immune responses utilizing a single-protein vaccine antigen when coupled with existing chitosan nanoparticle vaccine technology.

2. Materials and Methods

2.1. Protein Selection and Pickpocket Analysis of ASFV Peptides

A search of the National Library of Medicine's PubMed performed in 2018, including search terms "African swine fever protective immune response" and "ASFV neutralizing immune response", resulted in 16 publications. These publications implicated p54, p30, p72, p22, CD2v, p12, p15, p35, and EP153R as partially protective against ASFV challenge [14,28–35]. Available protein sequences were downloaded from the protein database of the National Library of Medicine for all available strains and aligned using the Nucleotide Blast software version 2.8.1. Six proteins were chosen based on levels of protection in the previous literature and the highest levels of sequence conservation. Protein sequences were analyzed via the Pickpocket 1.1 server using settings NetMHCcon, 8-11mer peptides, Pig (SLA), SLA-1*0401, and SLA-2*1002. Average SLA-1 and SLA-2 binding affinities were manually compared to areas with the highest sequence conservation

across protein sequences derived from 23 different full-length ASFV sequences deposited in the NCBI nucleotide database.

2.2. Synthesis of the Multiepitope ASF Protein

Synthetic nucleotides encoding the multiepitope protein (~24 kDa) were codon optimized for human expression and ordered as a gBlock gene fragment (IDT DNA). Fragments were cloned into the pRSET A bacterial expression plasmid (Invitrogen) utilizing the BamHI/EcoRI restriction sites. The plasmid clones were verified using restriction enzyme digestion and Sanger sequencing. One confirmed positive insertion plasmid clone (clone #8) was used to transform BL21 (DE3) *E. coli* (NEB), according to the manufacturer's instructions. Protein expression was induced by autoinduction, as previously described [36]. Bacteria were lysed for 30 min using bacterial protein extraction reagent (BPER) (Thermo Fisher, Waltham, MA, USA) supplemented with HALT protease inhibitors (Thermo Fisher, Waltham, MA, USA). Insoluble material containing the multiepitope protein was collected by centrifugation at $10,000\times g$ for 10 min. The resulting insoluble pellets were washed twice with inclusion body wash buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100), then resolubilized using 50 mM CAPS, pH 11.0, 1% N-lauroylsarcosine, and 1 mM DTT with end-over-end mixing for 2 h. Supernatant containing soluble protein was collected by centrifugation at $10,000\times g$ for 10 min at room temperature. Solubilized protein was purified by nickel affinity chromatography (Pierce High-Capacity Ni-IMAC Resin, EDTA Compatible, Invitrogen, Carlsbad, CA, USA). Purified proteins were confirmed by denaturing them by boiling in $1\times$ Laemmli buffer, separating by SDS polyacrylamide gel electrophoresis, followed by Coomassie blue staining. Protein concentrations were quantified using the Bradford assay and stored at $-80\text{ }^{\circ}\text{C}$ until lyophilization. Lyophilized proteins were used for nanoparticle encapsulation.

2.3. M-CS Nanoparticle Encapsulation of ASF Protein and ADU S100

The mannose-conjugated chitosan (M-CS) nanoparticle ASF protein vaccine (M-CS-ASF protein) was prepared by the ionic gelation method using methods described previously, with some modifications [24,37]. Briefly, 1.0% (*w/v*) low-molecular-weight chitosan polymeric (Millipore Sigma, St. Louis, MO, USA) solution was prepared in an aqueous solution of pH 4.3 under magnetic stirring. A total of 10 mg of lyophilized ASF protein was added to 10 mL of 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer at pH 7.4 (or 4 mg of ADU-S100 adjuvant (InvivoGen, San Diego, CA, USA)) in 4 mL of MOPS buffer) and was added dropwise to the M-CS protein solution. The cross-linker, tripolyphosphate (TPP) (Millipore Sigma, MO) 1% (*w/v*) stock solution was prepared in deionized water and added dropwise to the M-CS ASF protein solution/M-CS ADU S100 solution to give the nanoparticle encapsulated form of either the ASF protein antigen or the ADU S100 adjuvant. The M-CS-ASF protein solution was concentrated by centrifugation at $10,976\times g$ for 30 min, washed twice, and dispersed in deionized water. The mixture was sonicated for 5–6 min using a water bath sonicator and lyophilized (adding 5% sucrose as cryoprotectant). The average M-CS-ASF protein and M-CS-ADU S100 size and zeta potential surface charge distributions were individually measured using a Zetasizer Nano ZS90 (Malvern Panalytical, Westborough, MA, USA) analyzer.

2.4. Experimental Animals

Mixed sex large White-Duroc crossbred 4–6-week-old piglets ($n = 18$) were obtained from the Ohio State (OSU) breeding facility for use in this study. Piglets were confirmed PCR-negative for PRRSV, *M. hyopneumoniae*, PEDV, PDCoV, and TGEV. Piglets were porcine rotavirus A, B, and C positive. However, by vaccination day, there were no signs of diarrhea associated with rotavirus, so the experiment continued as planned. All piglets were housed

(3 groups, 6 pigs per group) at the OSU BSL-2 animal facility with a 12 h light/dark cycle and fed 2× per day with water access ad libitum. All animal studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at OSU. Animals were allowed to acclimate for 12 days prior to vaccination.

2.5. Vaccination

Prior to vaccination, blood was collected from the jugular vein of all animals. Piglets (6 per group) were intramuscularly (IM) vaccinated on the right side of the neck with (i) mock saline, (ii) M-CS ASF protein + M-CS ADU S100, and (iii) ASF protein + M-CS ADU S100. Three weeks after the initial vaccine, blood was collected from the jugular vein of all piglets, and piglets received an IM booster on the left side of the neck. Three weeks after the booster vaccine was administered, pigs were anesthetized with Tiletamine-Zolazepam-Ketamine-Xylazine (TKX) injectable anesthesia and euthanized with Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA). The spleen, superficial cervical lymph node, and superficial inguinal lymph node were collected in RNAlater to use for gene expression analysis. Serum was collected from blood for ELISA and antibody avidity assays. Peripheral blood mononuclear cells (PBMCs) were isolated from blood and used for T-cell proliferation assays and flow cytometry.

2.6. Peripheral Blood Mononuclear Cells (PBMC) Extraction from Pig Blood

Whole blood was collected in 2% EDTA and kept at room temperature. Blood was then diluted with an equal volume of 1× PBS + 2% fetal bovine serum (FBS) and mixed gently. The diluted blood was added to a SepMate 50 (STEMCELL Technologies, Cambridge, MA, USA) tube containing Lymphoprep Density Gradient Medium (STEMCELL Technologies Cambridge, MA, USA) and centrifuged at 1200× g for 20 min at room temperature. The buffy coat layer was collected via serological pipette and put into a new 50 mL tube and washed twice with 1× PBS + FBS. The cells were pelleted by centrifugation at 1200× g for 10 min. The pelleted cells were resuspended in enriched Roswell Park Memorial Institute (RPMI) media (Thermo Fisher, Waltham, MA, USA), and stored at 4 °C until future use.

2.7. T-Cell Proliferation Assay

1×10^7 PBMCs were prepared in 1 mL of RPMI. A total of 100 µL of cells (1×10^6 cells/well) were added to a 96-well plate in triplicate. Then, 100 µL of RPMI was added to the unstimulated (control) wells, while 100 µL of stimulation media (RPMI + 50 µg ASF multiepitope protein/well) was added to the stimulation wells. The cells were incubated for 48 h at 37 °C with 5% CO₂. Additionally, 20 µL/well of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and Phenazine Ethosulfate/Methosulfate (PMS) solution was added to each well. The plate was covered with foil and incubated at 37 °C for 4 h. After incubation, the absorbance was read on the spectrophotometer at 490 nm.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

ASF multiepitope protein-specific IgG titers were determined by ELISA. Briefly, 96-well flat-bottom high-binding affinity plates were coated with pre-titrated (20 µg/mL) ASF multiepitope protein in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were washed five times with PBS containing Tween-20 (0.05%) (PBST) and subsequently blocked at 37 °C for 2 h with 4% dry milk powder prepared in PBST. Test samples were serially diluted in 2% nonfat dry milk powder in PBST at a starting dilution of 1:50 for serum samples, and 50 µL per well was added to the plates and incubated at 37 °C for 2 h. Following another PBST wash step, plates were incubated for 2 h at 37 °C with goat anti-swine IgG(H+L) conjugated to HRP (Novusbio.com) diluted 1:500 in 2% nonfat dry

milk in PBST. After washing the plates, 50 μ L/well of a 1:1 mixture of peroxidase solution B and TMB (KPL, MD) was added and incubated for 10–20 min at RT to develop. The reaction was stopped with 0.3 M phosphoric acid (50 μ L/well), and the optical density (OD) was measured by a Spectramax (Molecular Devices, San Jose, CA, USA) microplate reader at 450 nm. The corrected OD values were obtained by subtracting the average value of the blank from the test samples.

2.9. Antibody Avidity ELISA

To measure the overall stability of the antibody–antigen complex, we performed an avidity ELISA. Briefly, 96-well flat-bottom high-binding affinity plates were coated with pre-titrated (20 μ g/mL) ASF multiepitope protein prepared in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were then washed five times with PBST and blocked at 37 °C for 2 h using 4% dry milk in PBST. Test samples were serially diluted in 2% nonfat dry milk in PBST at a starting dilution of 1:50 for serum samples, and 50 μ L/well was added to the plates and incubated for 2 h at 37 °C. While the ELISA plates were being incubated with samples, 2-fold dilutions of NH_4SCN (5.0 M, 2.5 M, 1.25 M, 0.625 M, 0.312 M and 0 M) were made in a dilution plate (using 1 $\times$ PBS-T as diluent). This step was followed by washing with PBST, followed by the addition of Ammonium Thiocyanate (NH_4SCN). A 10 M solution of NH_4SCN in 1 $\times$ PBS-T. 50 μ L of the diluted NH_4SCN was transferred to the ELISA plate and incubated at room temperature for 15 min. After washing, HRP conjugated goat anti-swine IgG(H+L) (Novusbio.com) was added at a 1:500 dilution in 2% nonfat dry milk in PBST and incubated for 2 h at 37 °C. Following a subsequent wash step, 50 μ L/well of a 1:1 mixture of peroxidase solution B and TMB (KPL, MD) was added and incubated for 10–20 min at RT. The reaction was stopped by adding 0.3 M phosphoric acid (50 μ L/well), and the optical density (OD) was measured by a Spectramax microplate reader at 450 nm. The corrected OD values were obtained by subtracting the average blank value reading from each test sample.

2.10. Flow Cytometry

A total of 5 million PBMCs/well/animal were seeded in duplicate in 1 mL of enriched RPMI medium in a 48-well plate. Cells were supplemented with 2 ng/ μ L of recombinant porcine IL-2 and stimulated with 10 μ g/mL of ASF multiepitope protein (stimulated plate) for 48 h. One plate was left unstimulated (IL-2 + RPMI only). After 42 h, protein transport inhibitor Brefeldin A was added for the last 6 h of incubation. After 48 h, cell culture supernatants were harvested for cytokine ELISA.

The staining procedure was followed as described previously [38]. Briefly, 1 million cells (subsampling from the original 5 million) were transferred to a 96-well plate for surface and intracellular staining. The antibody panels and their corresponding isotype controls used in the study are provided in Table 1. Appropriate isotype-matched controls were included as a negative control for the flow cytometry analysis. The cells were fixed using 1% paraformaldehyde at 4 °C for 30 min and resuspended in FACS buffer (bovine serum albumin, sodium bicarbonate, 10 $\times$ Hank's Balanced Salt Solution (HBSS) with phenol red, MilliQ water, and sodium azide). Data acquisition was performed on a BD FACS Aria II flow cytometer, and the analyses were carried out using FlowJo software (FlowJo v10, Becton, Dickinson & Company; BD, Franklin Lakes, NJ, USA). Graphs were generated using GraphPad Prism v10. The gating strategy followed to analyze Cytotoxic T cells and T-helper cell populations is given in the Supplementary Figure S1.

Table 1. Flow cytometry antibody panels.

	Name	Isotype
1	Mouse Anti-porcine CD3 AF488 (Southern biotech, Birmingham, AL, USA)	Mouse IgG1 κ
2	Mouse Anti-porcine CD8 α -biotin (Southern biotech, Birmingham, AL, USA)	Mouse IgG2a κ
3	Mouse Anti-porcine CD8 β -unlabeled (Monoclonal Antibody Center-Washington State University) Secondary Antibody: Goat Anti-Mouse IgG2a APC/CY7 (Southern Biotech, Birmingham, AL, USA)	Mouse IgG2 α
4	Mouse Anti-porcine CD4 PE (Southern Biotech, Birmingham, AL, USA)	Mouse IgG2b κ
Live/ Dead	Fixable AF700 (Invitrogen, Carlsbad, CA, USA)	

2.11. qPCR to Detect Immune Gene Expression

Total RNA was extracted from homogenized spleen and superficial cervical lymph node tissues using the QIAmp Viral RNA extraction kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. cDNA was synthesized from 2 μ g cDNA/sample using the iScript cDNA Synthesis kit (BioRad BioRad, Hercules, CA, USA). Quantitative PCR was performed using the SYBR Green Supermix kit (BioRad, Hercules, CA, USA), and the RNA copy numbers were quantified relative to the housekeeping gene (GAPDH) control. Primers were designed in-house using the PrimerQuest tool (IL-6 F: CCAGGAACCCAGCTATGAAC, Rev: CTTCTCATCTTCATCGTCA) (IL-10 F: AG-GAGGTGAAGAGTGCCTTTAG, Rev: TCGTCATGTAGGCTTCTATGTAGTT) (GAPDH F: GACATCAAGAAGGTGGTGAAGC, Rev: CACTGTTGAAGTCACAGGACAC). Values of mock vaccinates were subtracted from the vaccinated group values. Standard double delta Ct values were normalized with the housekeeping gene, GAPDH.

2.12. Statistical Analysis

Data plotting, interpolation, and statistical analysis were performed using GraphPad Prism 10. Statistical details of experiments are described in the figure legends. A *p*-value of less than 0.05 is considered statistically significant.

3. Results

3.1. Sequence Analysis of Multiple ASFV Genotypes Reveals Conserved Epitopes in Several Immunogenic Proteins

Subunit vaccines are considered a safer alternative to live-attenuated vaccines; however, subunit vaccines targeting ASFV have shown to be inconsistently protective with limited cross-protective efficacy. Therefore, we sought to improve the ASFV subunit vaccine platform by engineering a synthetic protein antigen containing multiple different immunogenic ASFV epitopes. Although similar multiepitope vaccine designs have been proposed for ASFV, they have yet to be evaluated in vivo [39–41]. To do this, a literature search was performed, which identified six ASFV proteins that have been shown to be partially protective in previous subunit vaccine studies (Table 2) [14]. To identify immunogenic epitopes, we first performed a BLAST search with each of our proteins of interest across the six different ASF genotypes. We then performed a multiple-sequence alignment to identify areas of high sequence homology across most or all genotypes. Using a comparative

sequence analysis approach including BLAST, multiple sequence alignment, and epitope mapping, we were seeking to identify epitopes that could provide cross-protection against heterologous challenge strains.

Table 2. ASFV Multiepitope Protein Immunogenic Epitopes.

ASF Gene	Protein Product (Protein ID)	Function	Antigen-Specific Antibody Response	T Cell Response	Epitope AA Sequence Binding Affinity (nM)
EP402R	CD2v AJB28398.1	Involved in hemadsorption to infected cells	Yes	Yes	TYQLVYSRNR (35,176.57 nM)
					SPPPKPCPPPKPCPP (30,560.95 nM)
E119L	j18L AXZ95901.1	Virion protein, involved in viral entry	N/A	Yes	LTICQNIK (20,149.14 nM)
B646L	p72 CAD2068444.1	Viral capsid protein	Yes	Yes	AGKQDITPITDATY (41,825.10 nM)
					EGAVYTLVDPFGRPI (15,457.23 nM)
CP530R	pp62, p15 CAD2068456.1	Involved in assembly and maturation of viral particle core	Yes	Yes	FLNHNLSKL (4335.14 nM)
					DLLEIINN (32,787.64 nM)
EP153R	C-type lectin AAC28424.1	Modulates expression of MHC I antigens	Yes	Yes	SFLNLTCLYHHHSY (28,485.48 nM)
					NRKKSHYTDLLFICS (33,324.12 nM)
					SVDSPTITY (8.38 nM)
K78R	p10 CAD2068411.1	Structural protein that binds ss/dsDNA, viral assembly	No	N/A	YQNGSRLTK (20,258.44 nM)

N/A—Not Applicable.

We chose epitopes from each of the six target ASFV genes, ranging from 9 to 15 amino acids in length (Table 2). The PickPocket 1.1 software program was used to predict binding affinity of each epitope, choosing epitopes with the highest average binding affinity to common swine leukocyte antigen (SLAs) alleles, as previously described [42]. The chosen epitopes were combined into one multiepitope protein using two types of linkers (1), a glycine-rich linker, GPGLP, which has the capability to improve sequence flexibility without affecting the function of the proteins they attach to and (2) a three amino acid linker, AAY, that is a cleavage site of proteasomes in mammalian cells, which will help to process the multiepitope polypeptide to release natural epitopes to enhance epitope presentation and immunogenicity [43]. The graphical representation predicted 3-dimensional structure, and expression confirmation of the multiepitope construct is provided (Figure 1).

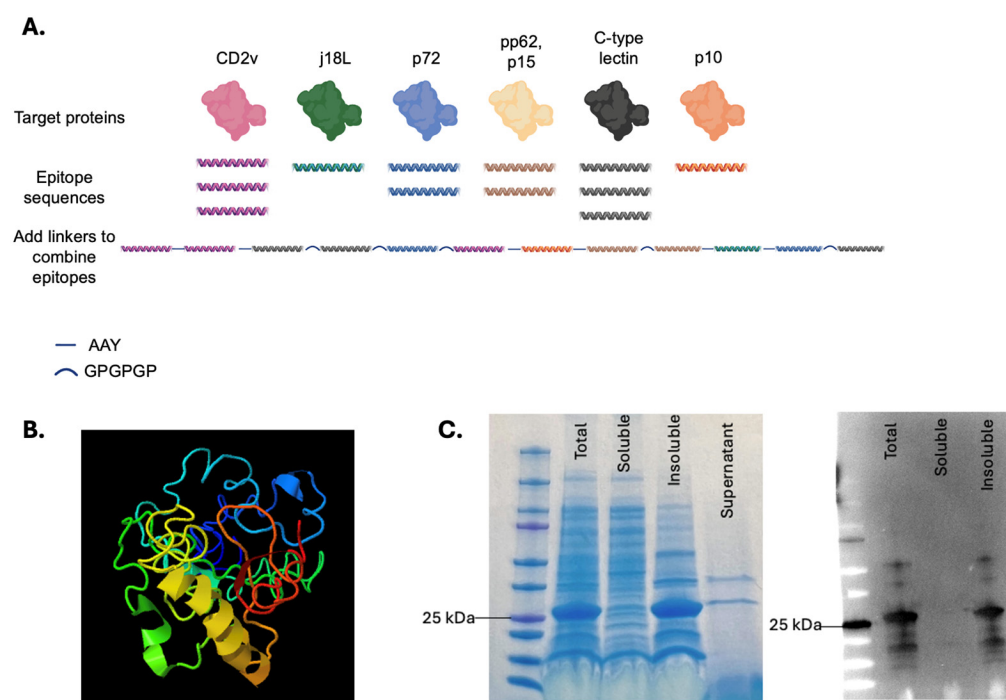


Figure 1. Graphical representation and predicted 3-dimensional structure of the multiepitope construct. **(A)** Graphical representation of the epitopes chosen from ASFV target proteins. Epitopes were combined into one synthetic ‘multiepitope protein’ using two types of linkers. **(B)** The 3-dimensional structure of the multiepitope construct, as predicted by I-TASSER. **(C)** Expression of the multiepitope protein (expected size 24 kDa) was confirmed by SDS-PAGE stained with Coomassie blue and further validated using Western blot using an anti-Xpress antibody.

3.2. Characterization of M-CS ASF Protein + M-CS ADU S100

Multiepitope ASF protein was entrapped in mannose-conjugated chitosan nanoparticles (M-CS ASF protein) [25,44,45]. The Stimulator of Interferon Genes (STING) agonist, ADU-S100, has shown promising results in multiple clinical trials for the treatment of cancers and in swine Influenza A vaccine trials [37,46–51]. In this study, ADU-S100 was used as an adjuvant and was entrapped separately in mannose-conjugated chitosan nanoparticles (M-CS ADU S100). To evaluate the effect of antigen encapsulation, an additional group was included, ASF protein + M-CS ADU S100, in which the ASF protein antigen was administered in a free, non-encapsulated form. The protein-entrapped nanoparticles were analyzed for their loading efficiency, size and polydispersity index (Table 3).

Table 3. Characterization of M-CS NP formulations.

Nanoparticle Type	Loading Efficiency (%)	Mean Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
Empty M-CS	-	261.4	0.2602	42.47
M-CS ASF Protein	70.2%	120.4	0.3665	29.61
M-CS ADU S100	79.75%	256.9	0.2468	39.49

3.3. Safety of M-CS-ASF Protein in Swine

A schematic of the experimental design in weaned piglets is provided (Figure 2).

Liveweight of each pig was estimated on day post-vaccination (DPV) 42 using a measuring tape and the heart girth method [52]. There was no statistical difference found between mock vaccinated and any experimental vaccination groups, suggesting that our vaccine did not cause any systemic adverse effects (Figure 3). Furthermore, the intramuscu-

larly (IM) injected M-CS ASF protein + M-CS ADU S100 formulation did not induce any local lesions at the injection site in the vaccinated pigs.

Group #	# of Pigs	Vaccine Group	Vaccine Composition (per Pig, per Dose)
1	6	Mock	2 mL PBS
2	6	M-CS ASF protein + M-CS ADU S100	1.5 mg ASF protein + 50 µg adjuvant
3	6	ASF protein + M-CS ADU S100	1.5 mg ASF protein + 50 µg adjuvant

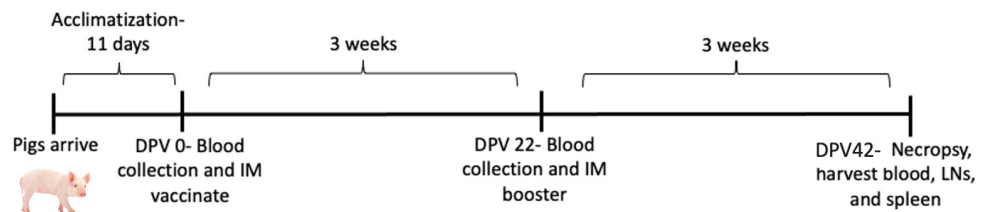


Figure 2. Experimental design of vaccine study in weaned piglets. The 4-week-old, weaned piglets ($n = 18$) were acquired from a high-health herd. Piglets were randomly divided into 3 groups as per the table and vaccinated on day post-vaccination (DPV) 0. A booster vaccine was given 3 weeks later (DPV 22). Then, 3 weeks later (DPV 42), piglets were humanely euthanized and tissues were collected at necropsy.

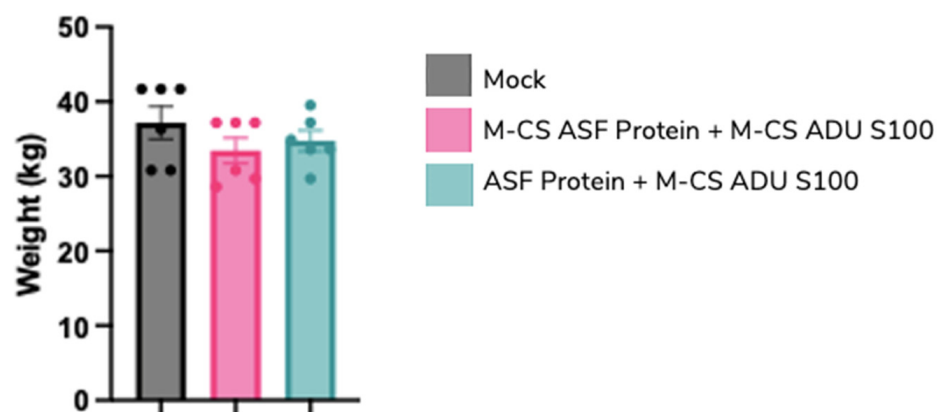


Figure 3. Liveweight estimation of pig vaccinates. Liveweight of each pig was estimated at DPV 42 using a measuring tape. Individual pig weights are plotted as dots. One-way ANOVA ($p < 0.05$) indicated there was no statistical difference in weights between the vaccinates. Mean $\pm$ SEM is plotted.

3.4. T-Cells Harvested from Vaccinated Animals Proliferate in Response to Vaccine Antigen Stimulation Ex Vivo

T-cells play an important role in cell-mediated immunity and can mediate adaptive immunity. Peripheral blood mononuclear cells (PBMCs) can be used to assess cell-mediated immunity via antigen-specific re-stimulation ex vivo. PBMCs were harvested from vaccinated pigs at DPV 22 and 42 and stimulated with 10 µg/mL of multiepitope protein for 4 h. Even though it is not statistically significant, there is an increase in the stimulation index between stimulated mock and vaccinated groups at DPV 42 (Figure 4), suggesting that T-cells harvested from vaccinated animals are proliferating in response to vaccine antigen stimulation.

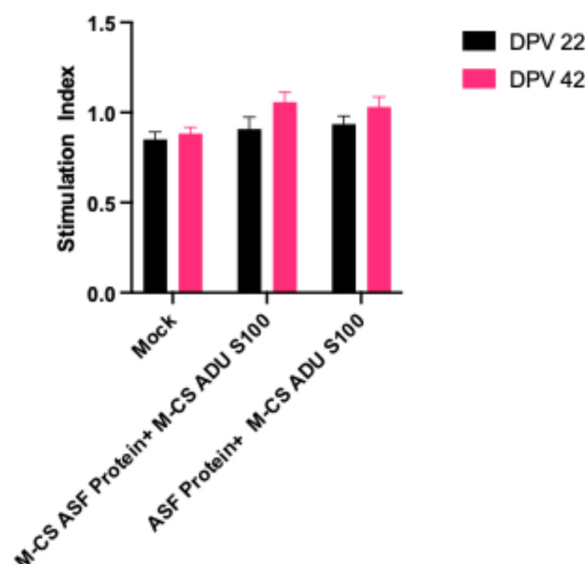


Figure 4. T-cell proliferation assay. PBMCs were harvested from vaccinates at DPV 22 and 42 and stimulated with 10 µg/mL of multiepitope protein for 4 h. Stimulation index is interpreted as the average of stimulated vs. unstimulated absorbance read at 490 nm. Significance was analyzed using Two-way ANOVA with Tukey's post hoc test ($p < 0.05$). Mean $\pm$ SEM is plotted.

3.5. M-CS ASF Protein Vaccine Induces a Detectable Antibody Response After One Vaccination

To evaluate the induction of humoral immune responses, sera from vaccinates were analyzed for multiepitope protein-specific antibody titers by ELISA. Both the M-CS ASF vaccine and soluble ASF protein alone vaccinated groups had a significantly higher antibody response at DPV 42 than mock vaccinated groups (Figure 5). Additionally, the M-CS ASF protein vaccinated group had a detectable antibody response after DPV 22, which was not observed in the ASF protein alone group, demonstrating that the M-CS nanoparticle formulation is promoting humoral immunity better than the ASF protein alone.

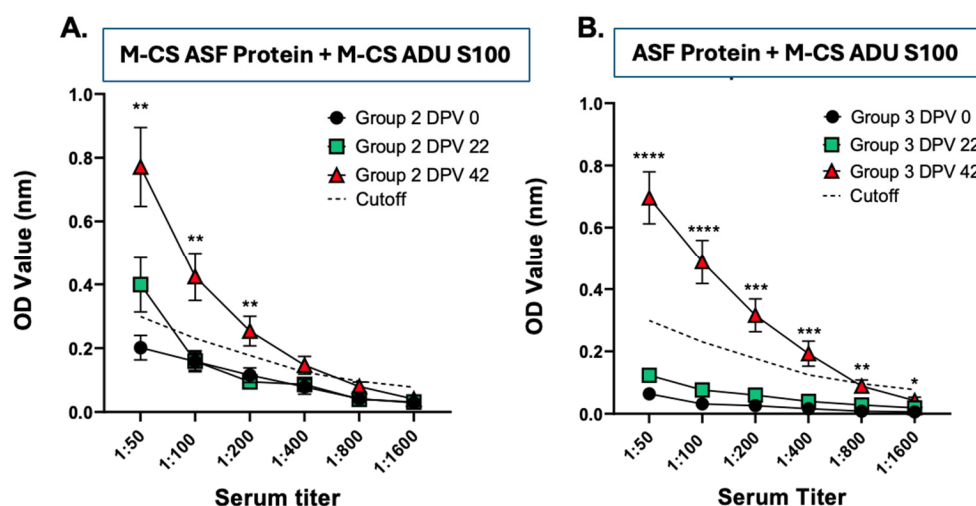


Figure 5. ASF-protein-specific antibody detection by ELISA. Antibody (IgG) responses to ASF-protein-specific vaccine antigens were tested by ELISA using sera from (A) M-CS ASF protein or (B) ASF protein vaccinated animals at DPV 22 and 42. Mean OD values $\pm$ SEM are plotted for each group. Significance was assessed using One-way ANOVA with multiple comparisons test ($* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, $**** = p < 0.0001$). Mean $\pm$ SEM is plotted.

3.6. The M-CS Entrapped Multiepitope Protein Vaccine Induces a Stronger Antibody Avidity After One Vaccine Dose

Antibody avidity can be defined as the strength of antibody binding to its target antigen, and the level of antigen–antibody binding avidity has been shown to correlate with vaccine protection [36,53–55]. Antibody elution from the antigen in the presence of ammonium thiocyanate, a chaotropic agent, is used as a measure of antibody binding strength. The avidity of ASF-protein-specific IgG antibodies was measured using an avidity ELISA. The M-CS ASF protein vaccine induced a stronger antibody avidity, compared to the ASF protein alone formulation, at DPV 22 in the presence of the strongest concentration of ammonium thiocyanate (Figure 6). While in sera of DPV, 42 pigs showed consistently higher avidity at all the concentrations of the chaotropic agent treatment compared to the control protein vaccinated group. Taken together with the previous ELISA results, this suggests that the M-CS ASF protein vaccine formulation is inducing a strong functional humoral immune response, compared to the ASF protein alone.

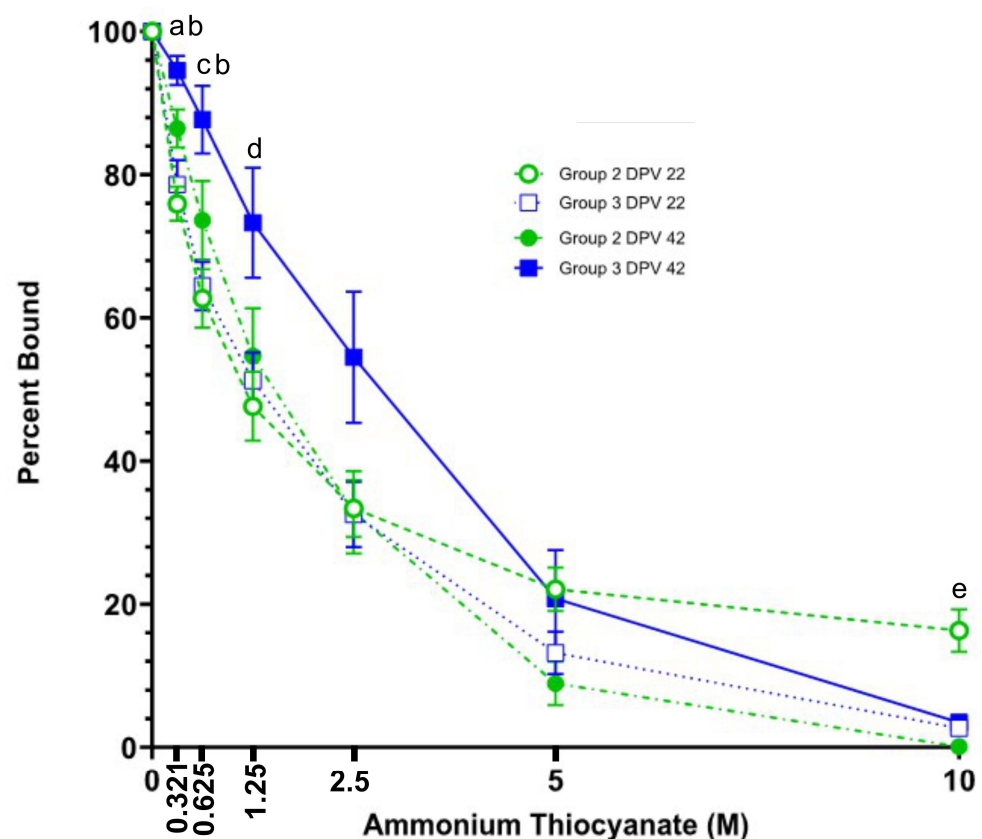


Figure 6. Antibody avidity ELISA. Antibody (IgG) avidity responses to ASF-protein-specific vaccine antigens were analyzed using an avidity ELISA using sera from vaccinated animals at DPV 22 and 42. Resistance to thiocyanate elution is used as a measure of avidity, where the x-axis represents the percent of antibodies bound at a particular concentration of ammonium thiocyanate. Significance was assessed using One-way ANOVA with multiple comparisons test, “a” = DPV 22 Gr 2 vs. DPV 42 Gr 3; $p < 0.001$, “b” = DPV 22 Gr 3 vs. DPV 42 Gr 3; $p < 0.01$, “c” = DPV 22 Gr 2 vs. DPV 42 Gr 3; $p < 0.01$, “d” = DPV 22 Gr 2 vs. DPV 42 Gr 3; $p < 0.05$, “e” = DPV 22 Gr 2 vs. all; $p < 0.0001$. Mean $\pm$ SEM is plotted.

3.7. Increased T-Helper Cell Response Was Observed in the M-CS ASF Protein Vaccinated Group

The induction of T-helper cells is vital to the promotion of long-lasting immunity via their role in the proliferation of memory T-cells and effector T-cells. PBMCs were collected and used to analyze the proportion of T-helper cells in vaccinates at DPV 42. We detected

an increase in the mean frequency of T-helper cells between mock and M-CS ASF protein vaccinated groups (Figure 7A), signifying that the M-CS ASF protein vaccine induces a beneficial T-helper cell response in vaccinates.

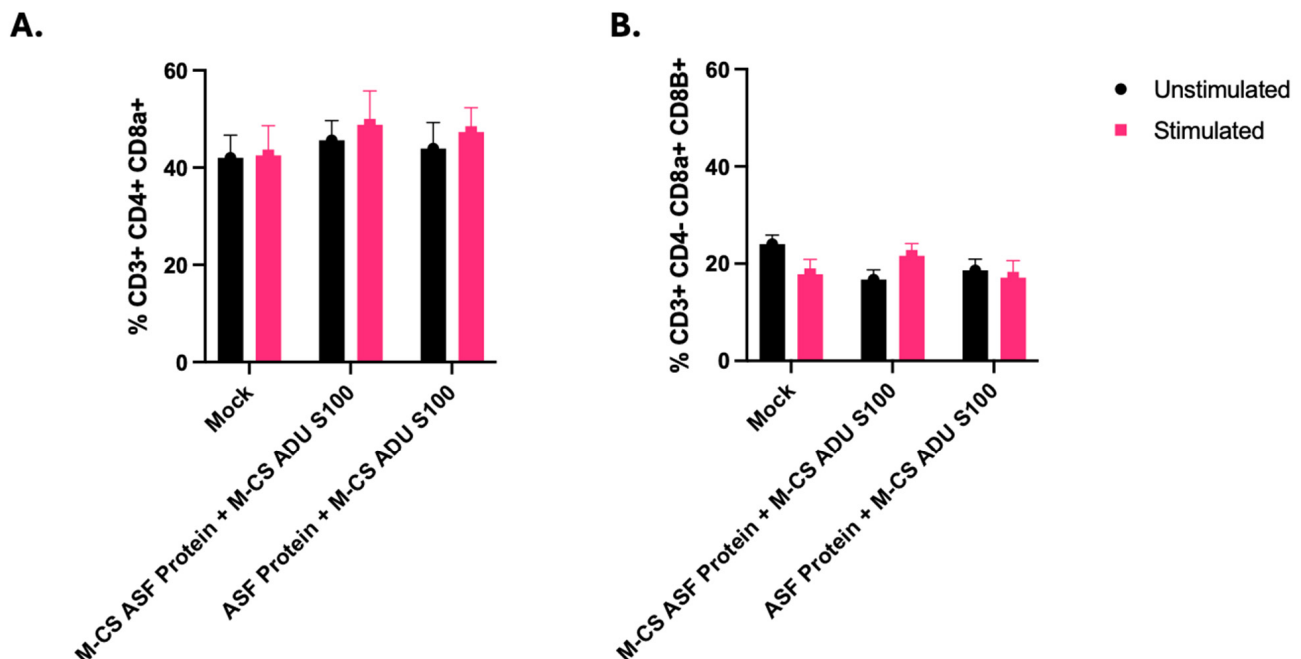


Figure 7. Induction of T-cell responses in vaccinates. PBMCs were analyzed using flow cytometry for their proportion of (A) T-helper cells (CD3+ CD4+ CD8α+) or (B) CD8+ T-cells (CD3+ CD4− CD8α+ CD8β+) in vaccinates at DPV 42. No significant differences ($p < 0.05$) were observed between groups using Two-way ANOVA with multiple comparisons. Mean $\pm$ SEM is plotted.

3.8. The M-CS ASF Protein Vaccine Induces a Robust Cytotoxic CD8+ T-Cell Response

CD8+ T-cells, also known as cytotoxic T-cells, play a vital role in the recognition and killing of virus-infected cells, and therefore, the activation of these cells is a desirable trait in a vaccine. PBMCs were collected and used to analyze the proportion of antigen-specific activation of CD8+ T-cells in vaccinates at DPV 42. Although no significant differences were observed between the vaccinated groups, there appears to be an increase in the mean frequency of specific CD8+ T-cells between the mock vaccinated and M-CS ASF protein vaccinated group (Figure 7B), suggesting that the nanoparticle formulation is promoting a CD8+ T-cell response better than the ASF protein alone.

3.9. Immune Gene Expression in M-CS-ASF Protein Vaccinates

Immunological responses of IL-6 and IL-10 were evaluated in the spleen and mandibular lymph nodes tissue of vaccinates, providing insights into the balance of the immune response following vaccination. IL-6 functions as a pro-inflammatory cytokine, whereas IL-10 serves as an anti-inflammatory regulator that counteracts the IL-6 driven inflammation. Compared to the unentrapped ASF protein vaccinates, the M-CS ASF protein vaccinates, anti-inflammatory cytokine, IL-10, was downregulated in the lymph nodes (Figure 8A) and significantly downregulated in the spleen (Figure 8B), while pro-inflammatory cytokine, IL-6, was downregulated in both the lymph nodes (Figure 8C) and the spleen (Figure 8D).

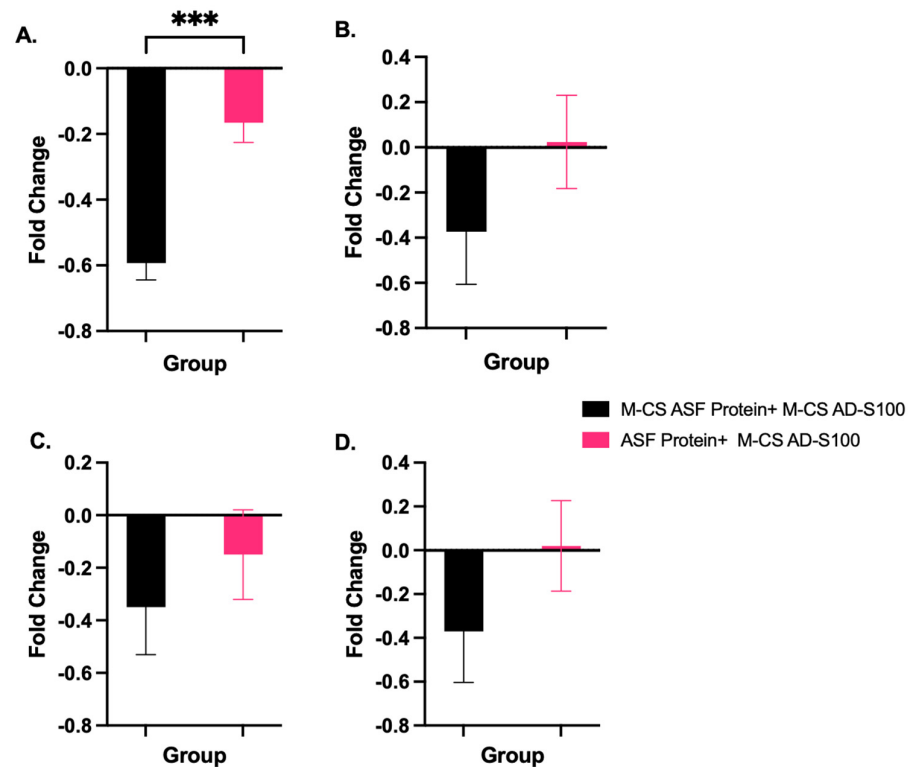


Figure 8. Detection of immune gene expression in the spleen and lymph nodes of vaccinates. Spleen tissue was analyzed for its expression of (A) IL-10 and (C) IL-6 in vaccinates at DPV 42. Mandibular lymph node tissue was analyzed for its expression of (B) IL-10 and (D) IL-6. A significant difference (***) = $p < 0.0001$) was observed between groups using unpaired t -test. Mean $\pm$ SEM is plotted.

4. Discussion

ASFV continues to be one of the most devastating foreign animal disease threats to both wild and domestic swine in the U.S., Europe, and Asia. To our knowledge, this is the first ASFV subunit vaccine platform consisting of a rationally designed multiepitope protein antigen, delivered via M-CS nanoparticles, and evaluated in vivo. Our preliminary findings demonstrate that our nanoparticle formulation is safe in pigs and is capable of inducing ASFV-specific cellular and humoral immune responses earlier than the vaccine antigen alone, supporting the feasibility of nanoparticle-based subunit vaccines as a promising control strategy for ASF control.

Here, a major objective was to overcome the limited cross-protective efficacy, which has historically hindered ASFV subunit vaccines. By selecting conserved epitopes from six immunogenic ASFV proteins and validating sequence homology across the chosen epitopes, we developed a vaccine antigen designed to target shared viral proteins across strains. Through this rational, sequence-based vaccine design strategy, we aimed to address the challenge posed by ASFV genetic diversity, including the emergence of novel field strains and the strain-restricted homology characteristic of LAV candidates [56]. The multiepitope construct, therefore, offers a logical mechanistic approach for the design of vaccine antigens to support future cross-protection studies.

A key strength of this platform is the use of M-CS nanoparticles, an optimal delivery platform that improved immunogenicity in this study. Compared to the protein alone, the nanoparticle formulation enhanced seroconversion and resulted in higher levels of antigen-specific IgG titers after a single vaccine dose. Furthermore, after a single M-CS ASF protein vaccine dose, we observed significant antibody avidity compared to the ASF protein alone, indicating enhanced antibody affinity maturation. While high-avidity antibodies have been

associated with neutralizing capacity in SARS-CoV-2 studies, neutralization assays are required to determine the functional activity of the antibodies in this study [57,58]. Here, higher antibody avidity supports the possibility of generating more functionally effective antibody responses following vaccination or viral challenge.

The M-CS platform was also effective at enhancing cellular immune responses. We observed increased specific T-helper cell frequency and a trend of elevated CD8⁺ T-cell responses, consistent with the well-established role of cytotoxic T-cells in the protection against ASF [59,60]. Because the presence of neutralizing antibodies generally declines more quickly than cell-mediated immune responses, the induction of cellular immune responses is vital in driving long-lasting immunity [61–64]. Antigen-specific T-cell proliferation further illustrates that the synthetic multiepitope ASF protein vaccine antigen is efficiently processed and recognized by immune cells. The inclusion of the cGAS-STING pathway agonist, ADU-S100, as a vaccine adjuvant likely contributed to this enhancement, as STING activation is known to strengthen antigen presentation and promote robust T-cell priming [65]; however, our limited number of experimental groups and lack of M-CS and ADU-S100-only groups complicates direct attribution to single vaccine components.

An additional layer of insight into the immunological effects of the M-CS ASF multiepitope protein formulation comes from the cytokine gene expression analysis. We observed downregulation of both IL-6 and IL-10 in the lymphoid tissues of vaccinates, with significantly lower IL-10 levels detected in the spleen. IL-6 is a pro-inflammatory cytokine involved in early innate immune activation, while IL-10 is a key anti-inflammatory regulator that suppresses T-cell activation [66–68]. The coordinated reduction of these cytokines suggests that the M-CS ASF protein vaccine formulation does not induce excessive inflammatory signaling, consistent with a more favorable clinical safety profile. Furthermore, a reduction in IL-10 expression may promote the activation of adaptive immunity, enabling stronger T-cell activity and improved antigen presentation, which is an advantageous outcome given the critical role of CD8⁺ T-cells in protection against ASFV infection [67,68].

Interestingly, both IL-6 and IL-10 were downregulated in our study despite measurable levels of humoral and cellular immune response activation, suggesting that the nanoparticle is promoting immunogenicity without an unnecessarily heightened systemic inflammatory response. This may reflect the controlled, targeted uptake of M-CS nanoparticles by antigen-presenting cells, enabling efficient antigen processing with minimal systemic inflammatory responses. The cytokine profile after vaccination observed here may shift in the context of viral challenge, when innate immune signals and antigen load are expected to be substantially increased. We anticipate that upon ASFV challenge, IL-6 responses will increase as part of the anti-viral response, thereby amplifying downstream T-cell expansion and antibody maturation. Therefore, the gene expression results presented here support the notion that our vaccine can initiate adaptive immunity, without excessive immune activation, and may act synergistically with the innate immune signals induced during true infection.

Additionally, the modular nature of our multiepitope antigen design provides a highly adaptable vaccine platform that can be rapidly modified to incorporate additional epitopes, epitopes from emerging ASFV strains, or immunogens from other economically important swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), foot and mouth disease (FMD), or influenza A virus (IAV) [69]. As the swine industry faces increasing threats from co-circulating and newly emerging viruses, scalable and flexible vaccine platforms are urgently needed, and the platform described here has the potential to simultaneously target multiple viral diseases.

Several limitations to this study should be acknowledged. First, the study described here did not include ASFV challenge, which we recognize remains essential to determine

protection, in addition to evaluating true cross-protective capability. Second, long-term immunological evaluation was not performed, and future investigations will include extended timepoints to assess the persistence and durability of the immune response induced by this multiepitope nanoparticle vaccine. Third, immune profiling was limited to the analysis of a restricted cytokine panel; as such, future studies will expand the cytokine analysis to better define the Th1/Th2 polarization and functional antiviral responses. Although early immune responses were detectable, we would expect these responses to become more robust in the presence of viral challenge, as challenge-driven antigen load typically amplifies T-cell expansion, thereby driving antibody titers and affinity maturation [70]. Therefore, the immune responses reported here likely represent a conservative baseline following vaccination. Lastly, sample size limitations also may have contributed to immune response variability, and longer-term studies will be needed to assess the durability of immunity and the safety of the vaccine.

5. Conclusions

In summary, this study demonstrates that a conserved multiepitope ASF protein delivered via M-CS nanoparticles is safe and capable of inducing early humoral and cellular immune responses associated with protective immunity against ASFV. The enhanced immunogenicity observed in the M-CS nanoparticle formulation, combined with rational epitope-driven design, supports progression to viral challenge studies and further vaccine optimization. Beyond ASFV, the modularity and translational versatility of this delivery platform make it a valuable candidate for rapid vaccine development against a wide range of swine viral diseases.

6. Patents

A provisional patent is filed for the vaccine design reported in this manuscript.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/vaccines14020187/s1>, Figure S1: Flow Cytometry Gating Strategy.

Author Contributions: Conceptualization, C.M.L., S.P.K. and R.J.G.; methodology, C.M.L., S.P.K. and R.J.G.; data curation, R.S., P.A.B., O.C.S., J.S., S.D., M.S., S.K., K.K.Y., D.B., J.H., R.J.G. and S.P.K.; writing—original draft preparation, C.M.L.; writing—review and editing, R.S., O.C.S., S.D., S.K., R.J.G., J.S., P.A.B., J.H. and S.P.K.; visualization and analysis, C.M.L., P.A.B. and R.S.; supervision, R.J.G., S.P.K. and J.H.; funding acquisition, R.J.G. and S.P.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was carried out in strict accordance with the recommendations by the Public Health Service Policy, USDA Regulations, National Research Council's Guide for the Care and Use of Laboratory Animals and the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. All the pigs were maintained, samples collected and euthanized, and all efforts were made to minimize the suffering of pigs as per the approved institutional, state and federal regulations and policies regarding animal care and use at The Ohio State University on the Ethics for Animal Experiments (Protocol Number: 2022A00000049, Date of Approval: 1 August 2022).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

Abbreviations

The following abbreviations are used in this manuscript:

U.S.	United States
M-CS	Mannose-conjugated chitosan
DPV	Days post vaccination
ASFV	African swine fever virus
ASF	African swine fever
LAV	Live-attenuated vaccine
SLA	Swine leukocyte antigen
NP	Nanoparticle
PBMC	Peripheral blood mononuclear cell
PRRSV	Porcine reproductive and respiratory syndrome virus
FMD	Foot and mouth disease
IAV	Influenza A virus

References

1. Penrith, M.L.; Kivaria, F.M. One hundred years of African swine fever in Africa: Where have we been, where are we now, where are we going? *Transbound. Emerg. Dis.* **2022**, *69*, e1179–e1200. [\[CrossRef\]](#)
2. Yang, S.; Miao, C.; Liu, W.; Zhang, G.; Shao, J.; Chang, H. Structure and function of African swine fever virus proteins: Current understanding. *Front. Microbiol.* **2023**, *14*, 1043129. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Arcega Castillo, G.; Schultze, M.L.; Schulte, R.; Schambow, R.A.; Hervé-Claude, L.P.; León, E.A.; Perez, A.M. African swine fever incursion risks in Latin America and the Caribbean: Informal and legal import pathways. *Front. Vet. Sci.* **2025**, *12*, 1587131. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Hakobyan, A.; Galindo, I.; Nañez, A.; Arabyan, E.; Karalyan, Z.; Chistov, A.A.; Streshnev, P.P.; Korshun, V.A.; Alonso, C.; Zakaryan, H. Rigid amphipathic fusion inhibitors demonstrate antiviral activity against African swine fever virus. *J. Gen. Virol.* **2018**, *99*, 148–156. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Revilla, Y.; Pérez-Núñez, D.; Richt, J.A. African Swine Fever Virus Biology and Vaccine Approaches. *Adv. Virus Res.* **2018**, *100*, 41–74. [\[CrossRef\]](#)
6. Andrés, G.; Charro, D.; Matamoros, T.; Dillard, R.S.; Abrescia, N.G.A. The cryo-EM structure of African swine fever virus unravels a unique architecture comprising two icosahedral protein capsids and two lipoprotein membranes. *J. Biol. Chem.* **2020**, *295*, 1–12. [\[CrossRef\]](#)
7. Spinard, E.; Dinhl, M.; Tesler, N.; Birtley, H.; Signore, A.V.; Ambagala, A.; Masembe, C.; Borca, M.V.; Gladue, D.P. A Re-Evaluation of African Swine Fever Genotypes Based on p72 Sequences Reveals the Existence of Only Six Distinct p72 Groups. *Viruses* **2023**, *15*, 2246. [\[CrossRef\]](#)
8. Sun, E.; Zhang, Z.; Wang, Z.; He, X.; Zhang, X.; Wang, L.; Wang, W.; Huang, L.; Xi, F.; Huangfu, H.; et al. Emergence and prevalence of naturally occurring lower virulent African swine fever viruses in domestic pigs in China in 2020. *Sci. China Life Sci.* **2021**, *64*, 752–765. [\[CrossRef\]](#)
9. Avagyan, H.; Hakobyan, S.; Baghdasaryan, B.; Arzumanyan, H.; Poghosyan, A.; Bayramyan, N.; Semerjyan, A.; Sargsyan, M.; Voskanyan, H.; Vardanyan, T.; et al. Pathology and Clinics of Naturally Occurring Low-Virulence Variants of African Swine Fever Emerged in Domestic Pigs in the South Caucasus. *Pathogens* **2024**, *13*, 130. [\[CrossRef\]](#)
10. Lopera-Madrid, J.; Osorio, J.E.; He, Y.; Xiang, Z.; Adams, L.G.; Laughlin, R.C.; Mwangi, W.; Subramanya, S.; Neilan, J.; Brake, D.; et al. Safety and immunogenicity of mammalian cell derived and Modified Vaccinia Ankara vectored African swine fever subunit antigens in swine. *Vet. Immunol. Immunopathol.* **2017**, *185*, 20–33. [\[CrossRef\]](#)

11. Choi, S.A.; Kim, Y.; Lee, S.J.; Moon, S.C.; Ahn, K.S.; Zheng, X.; Kim, D.S.; Lee, S.Y.; Shin, S.P.; Tark, D.; et al. African Swine Fever Vaccine Candidate ASFV-G-ΔI177L/ΔLVR Protects Against Homologous Virulent Challenge and Exhibits Long-Term Maintenance of Antibodies. *Animals* **2025**, *15*, 473. [\[CrossRef\]](#)
12. Gladue, D.P.; Borca, M.V. Recombinant ASF Live Attenuated Virus Strains as Experimental Vaccine Candidates. *Viruses* **2022**, *14*, 878. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Urbano, A.C.; Ferreira, F. African swine fever control and prevention: An update on vaccine development. *Emerg. Microbes Infect.* **2022**, *11*, 2021–2033. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Gaudreault, N.N.; Richt, J.A. Subunit Vaccine Approaches for African Swine Fever Virus. *Vaccines* **2019**, *7*, 56. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Ntakiyisumba, E.; Tanveer, M.; Hirwa, F.; Won, G. Quantitative evaluation of the efficacy of non-replicating vaccines for controlling African swine fever in domestic pigs: A systematic review and meta-analysis. *Front. Vet. Sci.* **2025**, *12*, 1614479. [\[CrossRef\]](#)
16. Gong, X.; Gao, Y.; Shu, J.; Zhang, C.; Zhao, K. Chitosan-Based Nanomaterial as Immune Adjuvant and Delivery Carrier for Vaccines. *Vaccines* **2022**, *10*, 1906. [\[CrossRef\]](#)
17. Zhang, Y.; Mei, X.; Zhang, C.; Wang, H.; Xie, X.; Zhang, Z.; Feng, Z. ASFV subunit vaccines: Strategies and prospects for future development. *Microb. Pathog.* **2024**, *197*, 107063. [\[CrossRef\]](#)
18. Zhang, G.; Liu, W.; Gao, Z.; Yang, S.; Zhou, G.; Chang, Y.; Ma, Y.; Liang, X.; Shao, J.; Chang, H. Antigenicity and immunogenicity of recombinant proteins comprising African swine fever virus proteins p30 and p54 fused to a cell-penetrating peptide. *Int. Immunopharmacol.* **2021**, *101*, 108251. [\[CrossRef\]](#)
19. Lotonin, K.; Brito, F.; Mehinagic, K.; García-Nicolás, O.; Liniger, M.; Donzé, N.; Python, S.; Talker, S.; Ploegaert, T.; Ruggli, N.; et al. Correlates of protection against African swine fever virus identified by a systems immunology approach. *eLife* **2025**, *14*, RP107579. [\[CrossRef\]](#)
20. Orosco, F.L. Host immune responses against African swine fever virus: Insights and challenges for vaccine development. *Open Vet. J.* **2023**, *13*, 1517–1535. [\[CrossRef\]](#)
21. Sánchez-Cordón, P.J.; Montoya, M.; Reis, A.L.; Dixon, L.K. African swine fever: A re-emerging viral disease threatening the global pig industry. *Vet. J.* **2018**, *233*, 41–48. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Escribano, J.M.; Galindo, I.; Alonso, C. Antibody-mediated neutralization of African swine fever virus: Myths and facts. *Virus Res.* **2013**, *173*, 101–109. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Dhakal, S.; Renu, S.; Ghimire, S.; Lakshmanappa, Y.S.; Hogshead, B.T.; Feliciano-Ruiz, N.; Lu, F.; HogenEsch, H.; Krakowka, S.; Lee, C.W.; et al. Mucosal Immunity and Protective Efficacy of Intranasal Inactivated Influenza Vaccine Is Improved by Chitosan Nanoparticle Delivery in Pigs. *Front. Immunol.* **2018**, *9*, 934. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Dolatyabi, S.; Renu, S.; Schrock, J.; Renukaradhya, G.J. Chitosan-nanoparticle-based oral *Salmonella enteritidis* subunit vaccine elicits cross-protection against *Salmonella typhimurium* in broilers. *Poult. Sci.* **2024**, *103*, 103569. [\[CrossRef\]](#)
25. Renu, S.; Feliciano-Ruiz, N.; Patil, V.; Schrock, J.; Han, Y.; Ramesh, A.; Dhakal, S.; Hanson, J.; Krakowka, S.; Renukaradhya, G.J. Immunity and Protective Efficacy of Mannose Conjugated Chitosan-Based Influenza Nanovaccine in Maternal Antibody Positive Pigs. *Front. Immunol.* **2021**, *12*, 584299. [\[CrossRef\]](#)
26. Stefanache, A.; Lungu, I.I.; Anton, N.; Damir, D.; Gutu, C.; Olaru, I.; Plesea Condratovici, A.; Duceac, M.; Constantin, M.; Calin, G.; et al. Chitosan Nanoparticle-Based Drug Delivery Systems: Advances, Challenges, and Future Perspectives. *Polymers* **2025**, *17*, 1453. [\[CrossRef\]](#)
27. Li, J.; Ju, Y.; Jiang, M.; Li, S.; Yang, X.-Y. Epitope-Based Vaccines: The Next Generation of Promising Vaccines Against Bacterial Infection. *Vaccines* **2025**, *13*, 248. [\[CrossRef\]](#)
28. Gómez-Puertas, P.; Rodríguez, F.; Oviedo, J.M.; Brun, A.; Alonso, C.; Escribano, J.M. 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* **1998**, *243*, 461–471. [\[CrossRef\]](#)
29. Neilan, J.G.; Zsak, L.; Lu, Z.; Burrage, T.G.; Kutish, G.F.; Rock, D.L. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology* **2004**, *319*, 337–342. [\[CrossRef\]](#)
30. Oviedo, J.M.; Rodríguez, F.; Gómez-Puertas, P.; Brun, A.; Gómez, N.; Alonso, C.; Escribano, J.M. High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *J. Virol. Methods* **1997**, *64*, 27–35. [\[CrossRef\]](#)
31. Malogolovkin, A.; Burmakina, G.; Tulman, E.R.; Delhon, G.; Diel, D.G.; Salnikov, N.; Kutish, G.F.; Kolbasov, D.; Rock, D.L. African swine fever virus CD2v and C-type lectin gene loci mediate serological specificity. *J. Gen. Virol.* **2015**, *96*, 866–873. [\[CrossRef\]](#) [\[PubMed\]](#)
32. 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.; et al. African swine fever virus serotype-specific proteins are significant protective antigens for African swine fever. *J. Gen. Virol.* **2016**, *97*, 1670–1675. [\[CrossRef\]](#) [\[PubMed\]](#)

33. Simón-Mateo, C.; Andrés, G.; Almazán, F.; Viñuela, E. Proteolytic processing in African swine fever virus: Evidence for a new structural polyprotein, pp62. *J. Virol.* **1997**, *71*, 5799–5804. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Carrascosa, A.L.; Saastre, I.; González, P.; Viñuela, E. Localization of the African swine fever virus attachment protein P12 in the virus particle by immunoelectron microscopy. *Virology* **1993**, *193*, 460–465. [\[CrossRef\]](#)
35. Argilagué, J.M.; Pérez-Martín, E.; Gallardo, C.; Salguero, F.J.; Borrego, B.; Lacasta, A.; Accensi, F.; Díaz, I.; Nofrarias, M.; Pujols, J.; et al. Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. *Vaccine* **2011**, *29*, 5379–5385. [\[CrossRef\]](#)
36. Studier, F.W. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **2005**, *41*, 207–234. [\[CrossRef\]](#)
37. Renu, S.; Feliciano-Ruiz, N.; Ghimire, S.; Han, Y.; Schrock, J.; Dhakal, S.; Patil, V.; Krakowka, S.; Renukaradhya, G.J. Poly(I:C) augments inactivated influenza virus-chitosan nanovaccine induced cell mediated immune response in pigs vaccinated intranasally. *Vet. Microbiol.* **2020**, *242*, 108611. [\[CrossRef\]](#)
38. Patil, V.; Renu, S.; Feliciano-Ruiz, N.; Han, Y.; Ramesh, A.; Schrock, J.; Dhakal, S.; HogenEsch, H.; Renukaradhya, G.J. Intranasal Delivery of Inactivated Influenza Virus and Poly(I:C) Adsorbed Corn-Based Nanoparticle Vaccine Elicited Robust Antigen-Specific Cell-Mediated Immune Responses in Maternal Antibody Positive Nursery Pigs. *Front. Immunol.* **2020**, *11*, 596964. [\[CrossRef\]](#)
39. Sira, E.M.J.S.; Fajardo, L.E.; Banico, E.C.; Odchimar, N.M.O.; Orosco, F.L. Design of a Multiepitope Pan-Proteomic mRNA Vaccine Construct Against African Swine Fever Virus: A Reverse Vaccinology Approach. *Vet. Med. Int.* **2025**, *2025*, 2638167. [\[CrossRef\]](#)
40. Fajardo, L.E.; Banico, E.C.; Sira, E.M.J.S.; Odchimar, N.M.O.; Orosco, F.L. Computational multi-epitope based design of a multivalent subunit vaccine against co-infecting African swine fever virus and porcine circovirus type 2. *Vet. Integr. Sci.* **2025**, *23*, 949–968. [\[CrossRef\]](#)
41. Simbulan, A.M.; Banico, E.C.; Sira, E.M.J.S.; Odchimar, N.M.O.; Orosco, F.L. Immunoinformatics-guided approach for designing a pan-proteome multi-epitope subunit vaccine against African swine fever virus. *Sci. Rep.* **2024**, *14*, 1354. [\[CrossRef\]](#)
42. Zhang, H.; Lund, O.; Nielsen, M. The PickPocket method for predicting binding specificities for receptors based on receptor pocket similarities: Application to MHC-peptide binding. *Bioinformatics* **2009**, *25*, 1293–1299. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Salahlou, R.; Farajnia, S.; Bargahi, N.; Bakhtiyari, N.; Elmi, F.; Shahgolzari, M.; Fiering, S.; Venkataraman, S. Development of a novel multi-epitope vaccine against the pathogenic human polyomavirus V6/7 using reverse vaccinology. *BMC Infect. Dis.* **2024**, *24*, 177. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Chaubey, P.; Mishra, B. Mannose-conjugated chitosan nanoparticles loaded with rifampicin for the treatment of visceral leishmaniasis. *Carbohydr. Polym.* **2014**, *101*, 1101–1108. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Bugybayeva, D.; Dumkhang, E.; Patil, V.; Yadagiri, G.; Suresh, R.; Singh, M.; Schrock, J.; Dolatyabi, S.; Shekoni, O.C.; Yassine, H.M.; et al. Evaluation of Efficacy of Surface Coated versus Encapsulated Influenza Antigens in Mannose–Chitosan Nanoparticle-Based Intranasal Vaccine in Swine. *Vaccines* **2024**, *12*, 647. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Corrales, L.; Glickman, L.H.; McWhirter, S.M.; Kanne, D.B.; Sivick, K.E.; Katibah, G.E.; Woo, S.-R.; Lemmens, E.; Banda, T.; Leong, J.J.; et al. Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Rep.* **2015**, *11*, 1018–1030. [\[CrossRef\]](#)
47. Foote, J.B.; Kok, M.; Leatherman, J.M.; Armstrong, T.D.; Marcinkowski, B.C.; Ojalvo, L.S.; Kanne, D.B.; Jaffee, E.M.; Dubensky, T.W.; Emens, L.A. A STING Agonist Given with OX40 Receptor and PD-L1 Modulators Primes Immunity and Reduces Tumor Growth in Tolerized Mice. *Cancer Immunol. Res.* **2017**, *5*, 468–479. [\[CrossRef\]](#)
48. Jin, L.; Hill, K.K.; Filak, H.; Mogan, J.; Knowles, H.; Zhang, B.; Perraud, A.-L.; Cambier, J.C.; Lenz, L.L. MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP. *J. Immunol.* **2011**, *187*, 2595–2601. [\[CrossRef\]](#)
49. Patil, V.; Hernandez-Franco, J.F.; Yadagiri, G.; Bugybayeva, D.; Dolatyabi, S.; Feliciano-Ruiz, N.; Schrock, J.; Suresh, R.; Hanson, J.; Yassine, H.; et al. Characterization of the Efficacy of a Split Swine Influenza A Virus Nasal Vaccine Formulated with a Nanoparticle/STING Agonist Combination Adjuvant in Conventional Pigs. *Vaccines* **2023**, *11*, 1707. [\[CrossRef\]](#)
50. Woodward, J.J.; Iavarone, A.T.; Portnoy, D.A. c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I interferon response. *Science* **2010**, *328*, 1703–1705. [\[CrossRef\]](#)
51. Yan, H.; Wang, X.; KuoLee, R.; Chen, W. Synthesis and immunostimulatory properties of the phosphorothioate analogues of cdiGMP. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5631–5634. [\[CrossRef\]](#)
52. Groesbeck, C.; Lawrence, K.; Young, M.; Goodband, R.; DeRouchey, J.; Tokach, M.; Nelssen, J.; Dritz, S. Using heart girth to determine weight in finishing pigs. *Kans. Agric. Exp. Stn. Res. Rep.* **2002**, 166–168. [\[CrossRef\]](#)
53. Bauer, G. The potential significance of high avidity immunoglobulin G (IgG) for protective immunity towards SARS-CoV-2. *Int. J. Infect. Dis.* **2021**, *106*, 61–64. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Dobaño, C.; Sanz, H.; Sorgho, H.; Dosoo, D.; Mpina, M.; Ubillos, I.; Aguilar, R.; Ford, T.; Díez-Padriza, N.; Williams, N.A.; et al. Concentration and avidity of antibodies to different circumsporozoite epitopes correlate with RTS,S/AS01E malaria vaccine efficacy. *Nat. Commun.* **2019**, *10*, 2174. [\[CrossRef\]](#) [\[PubMed\]](#)

55. Singh, S.; Kumar, N.K.; Dwiwedi, P.; Charan, J.; Kaur, R.; Sidhu, P.; Chugh, V.K. Monoclonal Antibodies: A Review. *Curr. Clin. Pharmacol.* **2018**, *13*, 85–99. [[CrossRef](#)] [[PubMed](#)]
56. Chen, S.; Wang, T.; Luo, R.; Lu, Z.; Lan, J.; Sun, Y.; Fu, Q.; Qiu, H.-J. Genetic Variations of African Swine Fever Virus: Major Challenges and Prospects. *Viruses* **2024**, *16*, 913. [[CrossRef](#)]
57. Muecksch, F.; Weisblum, Y.; Barnes, C.O.; Schmidt, F.; Schaefer-Babajew, D.; Wang, Z.; Lorenzi, J.C.C.; Flyak, A.I.; DeLaitch, A.T.; Huey-Tubman, K.E.; et al. Affinity maturation of SARS-CoV-2 neutralizing antibodies confers potency, breadth, and resilience to viral escape mutations. *Immunity* **2021**, *54*, 1853–1868.e7. [[CrossRef](#)]
58. Oostindie, S.C.; Lazar, G.A.; Schuurman, J.; Parren, P.W.H.I. Avidity in antibody effector functions and biotherapeutic drug design. *Nat. Rev. Drug Discov.* **2022**, *21*, 715–735. [[CrossRef](#)]
59. Oura, C.A.L.; Denyer, M.S.; Takamatsu, H.; Parkhouse, R.M.E. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J. Gen. Virol.* **2005**, *86*, 2445–2450. [[CrossRef](#)]
60. Schäfer, A.; Franzoni, G.; Netherton, C.L.; Hartmann, L.; Blome, S.; Blohm, U. Adaptive Cellular Immunity against African Swine Fever Virus Infections. *Pathogens* **2022**, *11*, 274. [[CrossRef](#)]
61. Mosmann, T.R.; McMichael, A.J.; LeVert, A.; McCauley, J.W.; Almond, J.W. Opportunities and challenges for T cell-based influenza vaccines. *Nat. Rev. Immunol.* **2024**, *24*, 736–752. [[CrossRef](#)] [[PubMed](#)]
62. Pollard, A.J.; Bijker, E.M. A guide to vaccinology: From basic principles to new developments. *Nat. Rev. Immunol.* **2021**, *21*, 83–100. [[CrossRef](#)] [[PubMed](#)]
63. Song, X.; Li, Y.; Wu, H.; Qiu, H.-J.; Sun, Y. T-Cell Epitope-Based Vaccines: A Promising Strategy for Prevention of Infectious Diseases. *Vaccines* **2024**, *12*, 1181. [[CrossRef](#)] [[PubMed](#)]
64. Tarke, A.; Coelho, C.H.; Zhang, Z.; Dan, J.M.; Yu, E.D.; Methot, N.; Bloom, N.I.; Goodwin, B.; Phillips, E.; Mallal, S.; et al. SARS-CoV-2 vaccination induces immunological T cell memory able to cross-recognize variants from Alpha to Omicron. *Cell* **2022**, *185*, 847–859.e11. [[CrossRef](#)]
65. Shen, M.; Jiang, X.; Peng, Q.; Oyang, L.; Ren, Z.; Wang, J.; Peng, M.; Zhou, Y.; Deng, X.; Liao, Q. The cGAS–STING pathway in cancer immunity: Mechanisms, challenges, and therapeutic implications. *J. Hematol. Oncol.* **2025**, *18*, 40. [[CrossRef](#)]
66. Aliyu, M.; Zohora, F.T.; Anka, A.U.; Ali, K.; Maleknia, S.; Saffarioun, M.; Azizi, G. Interleukin-6 cytokine: An overview of the immune regulation, immune dysregulation, and therapeutic approach. *Int. Immunopharmacol.* **2022**, *111*, 109130. [[CrossRef](#)]
67. Carlini, V.; Noonan, D.M.; Abdalalem, E.; Goletti, D.; Sansone, C.; Calabrone, L.; Albin, A. The multifaceted nature of IL-10: Regulation, role in immunological homeostasis and its relevance to cancer, COVID-19 and post-COVID conditions. *Front. Immunol.* **2023**, *14*, 1161067. [[CrossRef](#)]
68. Saraiva, M.; Vieira, P.; O’Garra, A. Biology and therapeutic potential of interleukin-10. *J. Exp. Med.* **2020**, *217*, e20190418. [[CrossRef](#)]
69. Kamboj, A.; Dumka, S.; Saxena, M.K.; Singh, Y.; Kaur, B.P.; da Silva, S.J.R.; Kumar, S. A Comprehensive Review of Our Understanding and Challenges of Viral Vaccines Against Swine Pathogens. *Viruses* **2024**, *16*, 833. [[CrossRef](#)]
70. Johansen, P.; Storni, T.; Rettig, L.; Qiu, Z.; Der-Sarkissian, A.; Smith, K.A.; Manolova, V.; Lang, K.S.; Senti, G.; Müllhaupt, B.; et al. Antigen kinetics determines immune reactivity. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5189–5194. [[CrossRef](#)]

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