DEVELOPMENT OF ADENOVIRUS-VECTORED PROTOTYPE VACCINES FOR AFRICAN SWINE FEVER VIRUS AND BOVINE VIRAL DIARRHEA VIRUS

A Dissertation

by

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ABSTRACT

The objective of this work was to develop adenovirus-vectored prototype vaccines against two pathogens, African Swine Fever Virus (ASFV) and Bovine Viral Diarrhea Virus (BVDV), which cause disease in two major livestock species, swine and cattle respectively.

The African Swine Fever Virus is a transboundary animal pathogen that causes a lethal hemorrhagic fever in domestic pigs. Attempts to develop a vaccine for ASFV have failed thus far. This manuscript describes the use of recombinant adenovirus to deliver two unique formulations of ASFV antigens in swine (in two separate *in-vivo* studies) and the subsequent evaluation of the antigen-specific antibody and cellular responses induced. The robust antigen-specific immune responses observed in both studies are promising and their protective potential will be evaluated in future efficacy studies

The Bovine Viral Diarrhea Virus is a globally prevalent pathogen that can cause severe diarrhea, respiratory disease, abortions and sometimes death in calves. Killed and modified live vaccines (MLV) for BVDV have been in use since the 1960s but are not effective due to lack of cross-protection and retention of immunosuppressive characteristics. This thesis also describes the use of the recombinant adenovirus vector to deliver a cocktail of multiple mosaic BVDV antigens in calves followed by the evaluation of protection conferred upon challenge. The prototype vaccine was more immunogenic and cross-protective (based on neutralizing antibodies) than a commercial MLV BVDV vaccine. Regarding protective efficacy, all calves immunized with

prototype vaccine cleared the virus within a week post-challenge, whereas one calf that received the MLV vaccine still remained viremic. Future efficacy studies with diverse BVDV strains are required to validate the cross-protective potential of this prototype vaccine.

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Contributors

This work was supervised by a dissertation committee consisting of Professors Waithaka Mwangi (advisor), Michael Criscitiello, Sanjay Reddy and Suryakant Waghela of the Department of Veterinary Pathobiology, Professor Luc Berghman of the Department of Poultry Science and Professor Jane Welsh of the Department of Veterinary Integrative Biosciences.

The Indirect Fluorescence Antibody assays and western blot experiments (described in Chapters II and III) involving the use of live African Swine Fever Virus were performed at Plum Island Animal Disease Center by Dr. Thomas Burrage and Ms. Lindsay Gabbert, respectively. The Bovine Viral Diarrhea Virus serum neutralization assays (described in Chapter IV) were performed at Dr. Christopher Chase's laboratory at South Dakota State University by Karim W. Abdelsalam.

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CHAPTER I

INTRODUCTION

Traditional approaches for developing vaccines, such as inactivated or live-attenuated vaccines have proved successful against several veterinary viral pathogens. Examples would be the attenuated rinderpest virus vaccine (Plowright vaccine) which is responsible for the global eradication of rinderpest or the recently licensed inactivated porcine circovirus Type 2 (PCV2) vaccine for prevention of postweaning-multisystemic wasting syndrome in pigs [1]. However, these approaches have certain limitations. The biggest concern with live attenuated vaccines is safety. These vaccines pose a risk of introduction of carrier states due to incomplete viral clearance and the possibility of reversion to virulent strain. Two examples of use of live-attenuated vaccines in the past serve as reminders to proceed with caution when trying to deploy these vaccines in the field. The first example is of the use of live attenuated North-American strain of Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) to vaccinate against the European strain prevalent in Denmark in 1996. The vaccine strain reverted to virulence and ultimately led to the introduction of a new strain in Denmark [1, 2]. Another example is the use of attenuated African Swine Fever Virus (ASFV) strain in Portugal in 1970 which resulted in severe immune-mediated reactions in the vaccinated pigs. Inactivated vaccines, though safer, are generally not as immunogenic and require strong adjuvants. In addition, since they are not actively replicating, the viral antigens are not processed by the MHC-I presentation pathway (cross-presentation may occur) and hence

these vaccines are not efficient at priming $CD8^+$ T-cells. This lack of induction of cytotoxic T-lymphocytes (CTLs) results in inefficient clearance of virus-infected cells. Furthermore, the use of strong adjuvants poses a greater risk of inducing auto-immune or allergic disorders and inoculation-site sarcomas [3].

An alternative approach for developing vaccines is use of live viral vectors. Recent advances in the fields of viral molecular biology and genetics have enabled the development of recombinant viral vectors for vaccine and immunotherapeutic applications. Use of recombinant viral vectors for *in-vivo* delivery of antigens is relatively safer than attenuated virus vaccines because the viral particles produced are replication deficient/incompetent and hence do not pose a threat to the host. This approach is better than using recombinant proteins because live vectored vaccines allow for intracellular expression of the antigen in the cytoplasm and thus making it amenable for MHC-I presentation and subsequent priming of $CD8^+$ T-lymphocytes. Antigen released from the infected cells is also taken up via the endocytic pathway for MHC-II presentation to prime $CD4^+$ T-lymphocytes. Another advantage of vectored vaccines is that the viral vector backbone is capable of stimulating innate immune responses due to the presence of Pathogen Associated Molecular Patterns (PAMPS). One limitation of using this approach, however, is the requirement for identification of protective vaccine candidate antigen(s) such that the immune response generated against the antigen(s) is sufficient for conferring protection against the pathogen.

The major focus of this work was to develop adenovirus-vectored prototype vaccines for the African Swine Fever Virus. This virus is a large double-stranded DNA

virus with a 170-190 kb long genome that encodes for >150 proteins. The aim was to use adenovirus as a delivery vector to evaluate the immunogenicity of several ASFV antigens in swine with an overarching goal of identifying promising candidates for inclusion in a multi-antigen prototype vaccine. We selected the replication deficient human adenovirus as a delivery vector due to several reasons such as its safety track record, large capacity for transgenes, high transgene expression, replicates at high titers in complementing cell lines and thus production is scalable and reproducible. A single dose of an adenovirus-vectored vaccine induces robust immune responses in neonates [4-9]. Importantly, adenovirus-vectored vaccines can be administered via multiple routes such as intradermal and intranasal for induction of systemic and mucosal immunity [7, 10-12]. More importantly, an adenovirus vector induces both antibody as well as T-cell responses and it has been shown that a single dose immunization with an adenovirus-vectored vaccine induces stronger CTL responses than recombinant vaccinia virus vector, plasmid DNA, or a combination of these two [13]. In addition, an adenovirus vector induce both innate and adaptive immune responses in mammalian hosts, in part, by dendritic cell (DC) modulation through Toll-Like Receptor (TLR)-dependent and -independent pathways [4-6]. Furthermore, adenovirus transduces Langerhans cells efficiently and immunization of neonates at birth with a single dose of an adenovirus-vectored vaccine induces robust immune responses [11, 14]. Relevant to this project, it is important to note that adenovirus vectors have been used successfully in swine immunization studies and have been shown to be safe and efficacious [15-19].

Chapters II and III of this dissertation describe the results of two separate in-*vivo* studies where the immunogenicity and safety of two cocktails of recombinant adenoviruses expressing rationally selected ASFV antigens were evaluated in swine. The first study evaluated a cocktail of well characterized and previously evaluated ASFV antigens at two doses and formulated in two novel adjuvants. The second study evaluated a cocktail of novel ASFV antigens that haven't been previously evaluated for their immunogenic potential. Past efforts on ASFV vaccine development, justification for using this delivery platform along with rationale for selection of the antigens have been elaborated upon in the introductions of these two chapters. In addition to these efforts of developing prototype vaccines for ASFV, the recombinant adenovirus delivery platform was also used to evaluate the protective efficacy of a multi-antigen cross-protective Bovine Viral Diarrhea Virus (BVDV) prototype vaccine in calves. A similar immunogen-adjuvant formulation of recombinant adenoviruses expressing mosaic BVDV antigens was inoculated into calves which were then subsequently challenged by a BVDV type 2a strain. The results of this study are described in Chapter IV. A brief background on the virus, its pathogenesis, problems associated with the currently available vaccines and the rationale for developing this prototype vaccine are discussed in the introduction of the chapter.

CHAPTER II

INDUCTION OF ROBUST IMMUNE RESPONSES IN SWINE USING A COCKTAIL OF ADENOVIRUS-VECTORED AFRICAN SWINE FEVER VIRUS ANTIGENS*

2.1. Overview

The African Swine Fever Virus (ASFV) causes a fatal hemorrhagic disease in domestic swine and, at present, no treatment or vaccine is available. Natural and gene-deleted, live attenuated strains protect against closely related virulent strains, however, they are yet to be deployed and evaluated in the field to rule out chronic persistence and potential for reversion to virulence. Previous studies suggest that antibodies play a role in protection, but induction of cytotoxic T-lymphocytes (CTLs) could be the key to complete protection. Hence, generation of an efficacious subunit vaccine depends on identification of CTL targets along with a suitable delivery method that will elicit effector CTLs capable of eliminating ASFV-infected host cells and confer long-term protection. To this end, we evaluated the safety and immunogenicity of an adenovirus-vectored ASFV multi-antigen cocktail formulated in two different adjuvants and at two immunizing doses in swine. Immunization with the cocktail rapidly induced unprecedented ASFV antigen-specific antibody and cellular immune responses against

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all the antigens. The robust antibody responses underwent rapid isotype-switching within one-week post-priming, steadily increased over a two-month period and underwent rapid recall upon boost. Importantly, the primed antibodies strongly recognized the parental ASFV (Georgia 2007/1) by IFA and western blot. Significant antigen-specific IFN-γ+ responses were detected post-priming and post-boosting. Furthermore, this study is the first to demonstrate induction of ASFV antigen-specific CTL responses in commercial swine using Ad-ASFV multi-antigens. The relevance of the induced immune responses in regards to protection need to be evaluated in a challenge study.

2.2. Introduction

African Swine Fever (ASF) is a highly contagious and fatal hemorrhagic swine disease. It has case morbidity and mortality rates that approach 100% [20]. Swine that recover become carriers and shed the virus for up to 70 days [21]. There is no treatment or vaccine available, and the only control strategy in case of an outbreak is quarantine, and removal of infected and in-contact animals. The ASF causes economic losses worldwide and severely affects the pork industry in sub-Saharan Africa where it is endemic [22].

The pathogen, African Swine Fever Virus (ASFV), is a double-stranded DNA enveloped icosahedral arbovirus belonging to the genus *Asfivirus* and the only member in the family *Asfarviridae* [23]. ASFV has a 170-190 kb non-segmented genome containing 150-167 ORFs [24, 25]. The ASFV has a natural sylvatic transmission cycle between *Ornithodoros* tick species and wild suids such as warthogs [22]. Infections in wild suids are asymptomatic and persistent, leading to a carrier state and transmission to

domestic pigs, which hinders eradication. Although an effective ASFV vaccine has not yet been developed, the fact that swine exposed to less virulent isolates (naturally or experimentally attenuated) are protected when challenged with homologous or closely related virulent isolates, suggests that vaccine development is possible [26-28]. Published data suggests that antibodies and T-cells play critical roles in virus control [28-35]. ASFV-infected convalescent swine serum can neutralize the infectivity of homologous and some heterologous strains *in vitro* and *in vivo*, possibly by inhibiting virus attachment and internalization [35-38]. Generally, anti-ASFV antibodies are detectable from about 6 days post-infection, and if the animal survives, antibodies may persist for long periods. However, despite the presence of antibodies, virus neutralization may not occur. Thus, the specific role of antibodies in ASFV protection is not yet fully understood [39]. The importance of cytotoxic T-lymphocytes (CTLs) in protection against ASFV has been demonstrated in a number of studies. Importantly, *in vivo* depletion of $CD8^+$ T-cells decreases protection against ASFV in swine and *in vitro* studies indicate that there is preferential proliferation of $CD8^+$ T-cells in the presence of live virus, whereas both $CD4^+$ and $CD8^+$ T-cells are stimulated by UV-inactivated virus [33, 40]. In addition, ASFV-specific CTL activity is detected in swine infected with non-lethal ASFV isolates [28, 29, 31, 32]. The requirement for CTLs in protection is further supported by the observation that adjuvant-formulated killed ASFV and recombinant vaccine candidate antigens that induce high antibody responses do not confer solid protection, and these outcomes strongly suggest that MHC class I presentation of ASFV antigens is critical [34, 41-44]. In addition, it has been observed

that swine that generate high antibody titers, but low cellular responses following immunization with a live, attenuated virus, develop chronic disease [45].

Although live attenuated ASFV can protect swine against the disease, it is not an ideal vaccine due to the potential risk of vaccine virus persistence and reversion to virulence. Additionally, a live, naturally-attenuated ASFV vaccine used in Portugal in the 1960s resulted in severe immune-mediated post-vaccination reactions in immunized animals, precluding any further use during outbreaks [46]. Subunit vaccines based on some of the most extensively studied ASFV antigens such as p32, p54, and p72 envelope protein, have shown some promise. These antigens, among others, have been tested as vaccine candidates either as baculovirus-expressed recombinant proteins or via DNA plasmid delivery [34, 41-44]. Delayed onset of viremia, delayed mortality and partial protection have been observed in most of these studies, which suggest that these antigens do play a role in protection but are not capable of conferring complete protection when used singly or in combination. Thus, it is envisaged that development of an efficacious vaccine requires empirical identification of multiple ASFV antigens formulated in a suitable delivery system that can successfully induce robust immunity.

Given that one or a combination of a few subunit antigens have not been able to confer complete protection so far, we set out to test the ability of a live-vectored ASFV multi-antigen cocktail to elicit strong CTL, IFN-γ-secreting T-cell, and B-cell responses. We selected replication-deficient human adenovirus (Ad5) vector as the antigen delivery platform for several reasons such as safety, high transgene expression and scalability [4-7, 9]. Additionally, adenovirus-vectored vaccines have been shown to induce stronger

CTL responses than *vaccinia* virus, plasmid DNA or a combination of these two [13]. To test our approach, we used p32, p54, and p72 antigens since they are well characterized. Furthermore, antigens p32 and p72 have been previously identified as CTL targets [29, 31]. We also included polyprotein pp62, which is a major component of the core shell, essential for viral core development and is very strongly recognized by ASFV-specific convalescent serum [47]. We tested this multi-antigen (4-way) cocktail in a prime-boost regimen using two different adjuvant formulations and at two different immunizing doses.

2.3. Material and Methods

2.3.1. Generation of plasmid constructs encoding ASFV antigens

The ASFV p32, p54, pp62 polyprotein (pp62), and p72 amino acid sequences based on the epidemiologically relevant Georgia 2007/1 isolate (GenBank accession FR682468) were modified to add, in-frame, a FLAG- and HA-tag at the N- and C-termini, respectively. This allowed for the use of one primer pair to move the expression cassettes of all antigens across multiple expression vectors, in addition to using the tags for tracking protein expression and affinity purification of recombinant proteins. The resultant amino acid sequences of the ASFV antigens were used to design synthetic genes codon-optimized for protein expression in the swine host. Codon optimization, gene synthesis, cloning into pUC57, and gene sequence validation was outsourced (GenScript). Each gene was then subcloned into pcDNA™3.3-TOPO® TA, pAd/CMV/V5-DEST™Gateway®, and pFastBac™ HBM TOPO vectors (Invitrogen) to

generate DNA plasmid constructs for protein expression in mammalian cells, generation of recombinant adenoviruses, and recombinant baculoviruses, respectively, using manufacturer's protocols. The constructs generated were validated by DNA sequencing.

2.3.2. Generation of virus constructs encoding ASFV antigens

The pAd constructs mentioned above were used to generate recombinant replication-incompetent adenoviruses using the ViraPower Adenoviral Expression System (Invitrogen). Following validation of protein expression by immunocytometric analysis, the recombinant adenoviruses were scaled up to generate virus for immunizations. Virus titers (infectious focus units, IFU) were determined using the QuickTiter™ Adenovirus Titer Immunoassay Kit (Cell Biolabs, VPK-109) with a minor modification. We used purified rabbit anti-adenovirus polyclonal IgGs (1:500 dilution) (made in-house by immunizing rabbits with heat killed Ad-Luciferase) as the primary antibody, followed by an alkaline-phosphatase-conjugated anti-Rabbit IgG (1:1,000) (Jackson ImmunoResearch, Cat #711-055-152) as the secondary antibody and Fast Red TR–Naphthol AS-MX as the substrate (Sigma, F4523). A recombinant adenovirus expressing luciferase (Ad-Luc) was similarly scaled up and titrated to serve as the negative control immunogen. To generate recombinant baculoviruses, the pFastBac constructs were used to generate Bacmids which were subsequently transfected into Sf-9 cells. One clone of each baculovirus was scaled up, titered and then used to infect High-Five cells (Invitrogen) to generate FLAG-tagged recombinant proteins which were affinity purified with anti-FLAG M2 affinity gel (Sigma, A2220). Recombinant pp62 was generated using the HEK 293 Freestyle Expression system (Invitrogen).

2.3.3. Evaluation of protein expression

Protein expression by the plasmid constructs, and by the recombinant viruses encoding the ASFV antigens was validated by immunocytometric analysis as previously described [48]. Briefly, HEK-293A cell monolayers transfected with the plasmid constructs or infected with the recombinant adenoviruses, were probed with mouse anti-FLAG M2–alkaline phosphatase conjugate (Sigma, St. Louis, MO) diluted 1:1,000 in Blocking buffer (PBS with 5% fetal bovine serum). Duplicate transfected or infected HEK-293A cell monolayers were first incubated with a gamma-irradiated convalescent swine serum (1:500) (Several ASFV isolates were used to produce the convalescent sera from a donor pig that was sequentially infected with a series of tissue culture adapted and wild-type viruses from p72 genotypes I (DR11, Haiti 81, Lisbon 60, Malawi 83and UG-61], VIII and X. The serum was a kind gift from E. J. Kramer, Plum Island Animal Disease Center) and then probed with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-porcine IgG (Southern Biotech, Cat# 6050-04). The alkaline phosphatase activity was then detected using Fast Red TR–Naphthol AS-MX substrate (Sigma, F4523). Mock transfected/infected cells served as negative controls. Protein expression by the generated recombinant baculoviruses was similarly evaluated by probing infected Sf-9 cells.

2.3.4. Immunization of swine

Twenty weaned piglets (barrows; ~30lbs) were acquired and during the quarantine period, commercial vaccines against defined pathogens were administered to meet institutional requirements. Three groups of piglets (n=5), were immunized with a

cocktail of the recombinant adenoviruses expressing ASFV antigens formulated in defined adjuvants (Table 1). Sham-control piglets (n=5) were inoculated with an equivalent amount of the Ad-Luc virus (Table 1). The inoculum was administered intramuscularly in the neck region behind the ears. Fourteen weeks post-priming, the animals were boosted with the cognate priming dose and adjuvant. The pigs were terminated, one group a week starting at 8 weeks post-boost.

Table 1: Swine Immunization Protocol

Group	No. of pigs	Immunogen	Dose/pig	Adjuvant
T1	5	Ad5-ASFV 4-way cocktail	4×10^{10} IFU*	ENABL$^{\delta}$
T2	5	Ad5-ASFV 4-way cocktail	4×10^{11} IFU**	ENABL
T3	5	Ad5-ASFV 4-way cocktail	4×10^{11} IFU**	Zoetis$^{\phi}$
T4	5	Ad5-Luc	4×10^{11} IFU***	ENABL

* ASFV 4-way cocktail: pool of 4 Ad5-ASFV constructs each at 1×10^{10} IFU (Infectious Focus Units)
** ASFV 4-way cocktail: pool of 4 Ad5-ASFV constructs each at 1×10^{11} IFU
*** Ad5-Luc sham inoculated control
δENABL adjuvant (Cat. # 7010106-C6)
ϕExperimental adjuvant (proprietary formulation)

2.3.5. ELISA

Direct ELISA was used to evaluate antigen-specific antibody responses as previously described [48]. Briefly, microplates coated with 100 µl of 1µg/ml of affinity-purified antigen in Bicarbonate coating buffer were first incubated with 100 ul of sera (diluted at

1:100) in triplicates, followed by incubation with 100µl of Peroxidase-conjugated anti-swine IgG (Jackson ImmunoResearch, Cat. #114-035-003) (1:5,000 dilution). The plates were developed using Sure Blue Reserve TMB substrate (KPL, Cat.# 53-00-02) and the reaction stopped using 1 N Hydrochloric acid. The optical density at 450 nm was then determined using a microplate reader. To determine antigen-specific IgM responses in sera from blood collected week 1 post-priming, HRP conjugated anti-swine IgM (1:10,000) (Bethyl Laboratories, A100-100P) was used as the secondary antibody. Antigen-specific IgG end point titers were determined for sera from blood collected week 1 post-boost by making a range of two-fold serum dilutions starting at 1: 1.6 X 10^4 to 1: 1.6 X 10^7. Similarly diluted pre-immunization sera served as cognate controls. The titer was then determined to be the dilution of the post-boost sera for which the mean of the OD was higher than the mean +3 times the standard deviation of the cognate pre-immunization sera. The significance of the difference in antigen-specific IgG titers among the groups was determined by ANOVA, followed by Tukey's multiple-comparison test, and a P value of ≤ 0.05 was considered significant.

2.3.6. Indirect Fluorescence Antibody assay (IFA)

Teflon-coated slide wells (Electron Microscopy Sciences, Cat. No. 63425-05) were pre-treated by incubating with 300 µg/ml of rat-tail collagen (Corning, Cat. No. 354249) in D-PBS (Invitrogen 14190-144) for 1hr at 37°C, oven-drying for 30 mins and incubating overnight in a bio-safety cabinet (15 cm from UV light). Primary monocytes/macrophages were isolated from whole swine blood as previously described [49] and infected with ASFV (Georgia 2007/1) at 1 MOI for 1 hr. at 37°C.

Approximately 4 x 10^5 infected cells and also mock-infected cells were then added to the wells of the pre-treated Teflon slides (25 µl/ well). The slides were incubated overnight at 37°C with 5% CO2, followed by fixing with a chilled (-20°C) solution containing acetone and methanol (1:1) for 10 min and stored at -70°C until required. To carry out the IFA, the slides were incubated with blocking solution (5% non-fat dry milk, 2% horse serum, 2% calf serum, 2% fetal calf serum and 5% BSA in D-PBS) for 30 min in a humidified chamber at 37°C. After blocking, the infected and mock-infected wells were incubated with a 1:20, 1:100 and 1:200 dilution of sera (week 1 post-boost) in blocking buffer for 1hr. at 37°C. ASF-specific convalescent serum (1:500) was used as a positive control and normal swine serum (GIBCO) was used as a negative control. Following three rinses with D-PBS, the wells were then incubated with FITC-conjugated goat anti-swine sera (Kirkegaard and Perry Cat No. 02-14-02) for 45 minutes at 37°C. The wells were rinsed similarly again and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Cat. No. PT389868). The results observed at 1:20 serum dilution are represented in Fig. 5A and the results of 1:200 serum dilutions are summarized in Table 2. The IFAs were conducted at Plum Island Animal Disease Center.

Table 2: Summary of IFA Data

T1: Swine No.	Reactivity		T3: Swine No.	Reactivity	
	ASFV-infected Macrophage	Mock-infected Macrophage		ASFV-infected Macrophage	Mock-infected Macrophage
33	++	-	31	+++	-
35	+++	-	37	++	-
36	+++	-	93	++++ Best	-
40	++	-	94	+++	-
42	++	-	96	+++	-
T2: Swine No.			**T4: Swine No.**		
34	+++	-	32	-	-
41	++	-	38	-	-
43	++++	-	39	-	-
46	+++	-	44	-	-
48	+++	-	45	-	-
Anti-ASFV convalescent serum	++++	-	Normal serum	-	-

The reactivity of the serum from each animal was compared to the reactivity of the positive control serum i.e. ASFV-specific convalescent serum. The number of + signs represents the intensity of the reaction, with ++++: as strong as positive control serum; and ++: weak but positive signal.

2.3.7. Western blot

Swine sera from week 1 post-boost were blotted against cell lysates prepared from Georgia 2007/1 ASFV (Vero cells adapted)-infected Vero cells (ATCC CCL-81). Briefly, ASF-infected Vero cells exhibiting cytopathic effect (CPE) at 72 hr. post-infection were harvested by centrifugation, lysed in M-PER Reagent (Thermo Scientific #78501), mixed 1:1 with 2X NuPAGE® LDS Sample Buffer, boiled, electrophoresed on a NuPAGE 4-12% Bis-Tris Gel for 35 minutes and transferred to 0.2um PVDF membranes (Invitrogen #LC2002). Following blocking in PBST containing 5% non-fat dry milk, membranes were transferred to the Protean II Slot-Blotter and incubated with sera diluted 1:50 in blocking buffer in individual wells. Following washes, the membranes were then removed from the blotting apparatus and incubated with Goat anti-swine-HRP (KPL #14-14-06) diluted 1:2,000 in blocking buffer for 1 hr. The blots were washed and then developed by exposure to DAB substrate (Sigma #D4293). ASFV-specific convalescent serum (1:10,000) was used as a positive control and normal swine serum was used as a negative control (1:200). A similar blot was carried out using mock-infected cell lysates to gauge background reactivity to host cell antigens. These western blots were performed at Plum Island Animal Disease Center.

2.3.8. IFN-γ ELISPOT assays

The frequencies of antigen-specific IFN-γ-secreting T-cells were determined by an enzyme-linked immunospot (ELISPOT) assay bi-weekly post-priming and weekly post-boost. The assay was conducted in triplicate wells of MultiScreen-HA plates (Millipore) using the Mabtech kit (Cat. # 3130-2A), as per manufacturer's instructions and as

described previously [48]. Briefly, 0.25 X 10^6 whole blood derived PBMCs or splenocytes were incubated with affinity purified antigens (2.5 μg/ml) in 100 μl per well of complete RPMI-1640 media. PHA mitogen (5 μg/ml) was used as a positive control and media alone served as a negative control. The spots were quantified with an ELISPOT reader and AID software (AutoImmun Diagnostica V3.4, Strassberg, Germany). The results were presented as the mean number of antigen-specific IFN-γ spot-forming cells per 10^6 PBMCs after subtracting background media counts. The significance of the differences in IFN-γ^+ PBMC responses between each vaccinated group (T1, T2, T3) and control group (T4) was analyzed by ANOVA, followed by Bonferroni's multiple-comparison test, and a P value of ≤ 0.05 was considered significant.

2.3.9. CTL assays

A standard chromium (^{51}Cr) release assay was used to measure antigen-specific T-cell cytotoxicity as previously described [50].

2.3.9.1. Generation of effector cells

PBMCs isolated from blood collected four weeks post-boost were resuspended in RPMI 1640 media (Lonza) containing 45% Click's Media (Irvine Scientific, Cat# 9195), 10% FBS, 1X β-Mercaptoethanol, 1X Glutamax, 50 μg/ml Gentamycin, 1X Pen/Strep (GIBCO) at a cell density of 4 X 10^6 /ml and distributed in aliquots of 1ml/well of a 24-well culture plate. The PBMCs were infected with each of the recombinant adenoviruses at a 1000 MOI for *in-vitro* stimulation of the T-cells. After 10 days, the

cells were harvested and centrifuged on a Ficoll gradient to remove dead cells. The live cells were then washed with PBS, resuspended in complete RPMI 1640 and counted to serve as effectors for the CTL assay.

2.3.9.2. Generation of target cells

Prior to immunization, skin biopsies were taken from each piglet using 4mm biopsy punches (American Screening Corp., Cat # 3785707). Primary skin fibroblast cultures were established from these skin tissues as previously described [51]. Briefly, the skin tissues were cut into small pieces under sterile conditions and cultured in 12-well plates containing 1 ml DMEM supplemented with 10% FBS, 1X Glutamax, 50 μg/ml Gentamycin, 1X Pen/Strep (GIBCO). When the fibroblasts reached confluency, they were passaged and frozen regularly till sufficient stocks were generated. Target cells were generated 24 hr. prior to conducting the CTL assay by transfecting autologous fibroblasts with the pCDNA plasmid constructs expressing the ASFV antigens using Gene-In transfection reagent (MTI-Global Stem, GST-1000) as per manufacturer's instructions. Transfection efficiencies of about 20-30% were achieved (pre-determined by immunocytometric analysis). On the day of conducting the assay, the cells were detached using Accutase, washed with DMEM containing 10% FBS, labelled with 100 μCi per 10^6 cells of $Na_2{}^{51}CrO_4$ (Perkin Elmer) for 1hr. at 37°C, 5% CO_2 washed three times and resuspended in complete RPMI 1640 for use as targets in the assay.

2.3.9.3. Chromium release assay

The effectors and targets were added at effector: target ratios of 50:1 and 25:1 in duplicate wells of a 96-well round bottom microtiter plates in final volumes of 100 µl per well and incubated at 37°C, 5% CO_2 for 6h. The plate was then centrifuged at 1000 rpm for 4 min and supernatants harvested to measure chromium release in a Micro-beta counter (Perkin Elmer, 1450 LSC & Luminescence counter). Spontaneous release of the label was measured from supernatants of targets incubated without effectors and maximum release was measured from targets lysed with 5% Triton-X. Percent specific lysis was calculated as described previously [50]. Fibroblasts transfected with a construct expressing a chimera of VP1 and 3D polymerase antigens of the Foot and Mouth Disease Virus (FMDV) served as a negative control to assess background lytic activity.

2.3.10. Statistical analysis

All analyses were performed with GraphPad Prism Version 6.05 using a significance level of $P<0.05$. The antigen-specific IgG titers amongst the treatment groups were compared using One-way ANOVA followed by Tukey's multiple comparison test. For all the IFN-γ ELISPOT assays, the mean IFN-γ response of treatment groups (T1-T3) was compared to the mean response of the sham treated control group (T4) using One-Way ANOVA followed by Bonferroni's multiple comparison test.

2.3.11. Ethics statement

All animal procedures were conducted in accordance with the Animal Use Protocol 2012-59, reviewed and approved by the Texas A&M University Institutional Animal

Care and Use Committee (IACUC) (Permit 2009067) which adheres to the regulations, policies and guidelines outlined in the Animal Welfare Act (AWA), USDA Animal Care Resource Guide and the PHS Policy on Humane Care and Use of Laboratory Animals. Pigs were monitored twice daily for any clinical signs and to document any localized and or systemic adverse effects. The animals were euthanized with an overdose of sodium pentobarbital. A lack of heartbeat was then confirmed by a stethoscope.

2.4. Results and Discussion

2.4.1. Protein expression by constructs encoding ASFV antigens

Codon-optimized synthetic genes encoding p32, p54, pp62, and p72 ASFV antigens fused in-frame to FLAG tag were used to generate pcDNA3 constructs, recombinant adenoviruses, and recombinant baculoviruses. Immunocytometric analysis of HEK-293A cells transfected with the pcDNA3 constructs and probed with anti-FLAG mAb confirmed expression of each antigen (Fig. 1A). Similarly, HEK-293A cells infected with the recombinant adenoviruses and probed with anti-FLAG mAb confirmed protein expression (Fig. 1B) and in addition, infected cells probed with the ASFV-specific convalescent serum validated that the expressed antigens are authentic (Fig. 1C). The recombinant baculoviruses were used to generate affinity-purified recombinant ASFV proteins, which were used for ELISA and IFN-γ ELISPOT. We did not generate recombinant baculovirus for antigen pp62 since transfection of 293 Freestyle cells with the pCDNA construct and subsequent purification yielded sufficient protein for *in-vitro* analyses. The affinity-purified proteins were shown to be authentic by western blot

analysis using ASFV-specific convalescent serum (see appendix Fig. A1.)

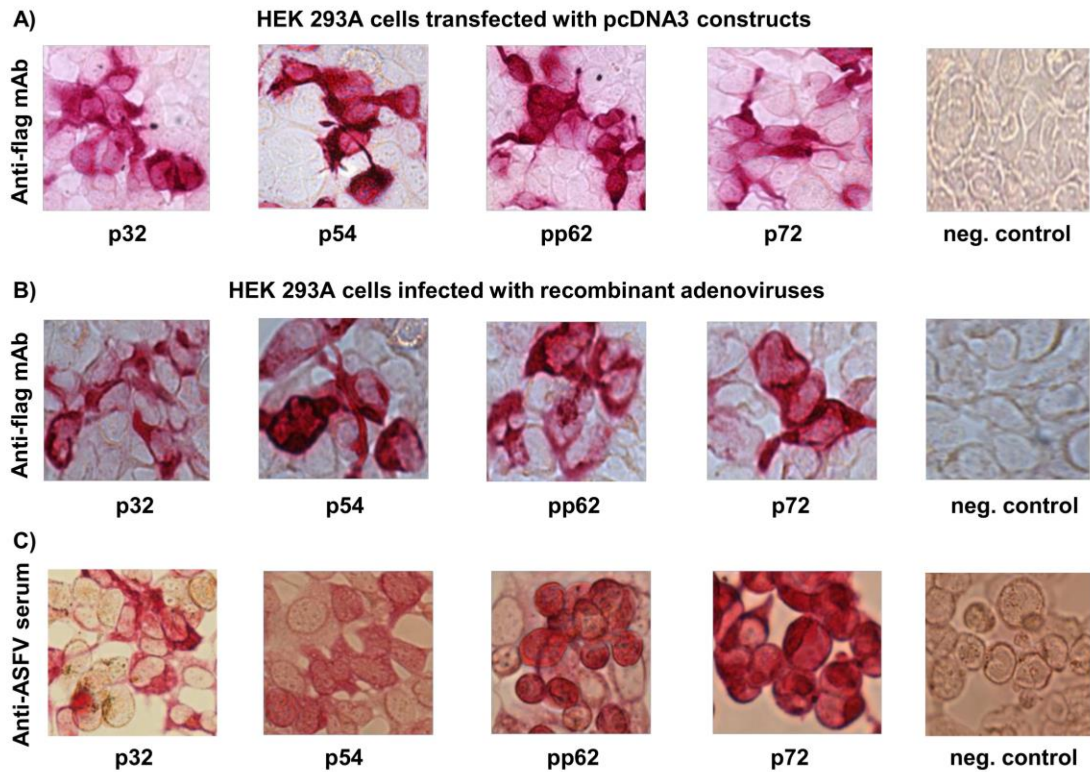

Figure 1. Protein expression by ASFV constructs.
Protein expression by the constructs encoding ASFV antigens was evaluated by immunocytometric analysis of HEK-293A cells. Panels: A) Cells transfected with pCDNA3 constructs and probed with anti-FLAG mAb; B) Cells infected with recombinant adenoviruses and probed with anti-FLAG mAb; and C) Cells infected with recombinant adenoviruses and probed with gamma-irradiated ASFV-specific convalescent serum. Negative controls are mock transfected (A) or mock infected (B & C) HEK-293A cells.

2.4.2. Ad-ASFV multi-antigen cocktail rapidly induced robust antibody responses

2.4.2.1. Post-prime response

ELISA evaluation of antigen-specific IgM and IgG antibody responses in sera from blood collected one week post-priming showed that, all the pigs inoculated with either

the 10^{10} (T1) or 10^{11} (T2, T3) Ad-ASFV multivalent cocktail dose, but not the negative controls (T4), had sero-converted and mounted robust ASFV antigen-specific antibodies (Fig. 2). More importantly, most pigs underwent isotype switching within one week based on relatively higher antigen-specific IgG than IgM antibody responses (Fig. 2). The IgM and IgG profiles were similar for p32, p54, and pp62 antigens, with no notable difference between the 3 treatment groups. However, compared to the pigs immunized with the ENABL adjuvant (T1 and T2), the pigs immunized using the Zoetis adjuvant (T3) clearly had higher p72 antibody responses, which were IgM dominant. Bi-weekly monitoring of antigen-specific IgG responses in each animal showed that the post-prime antibody responses peaked anytime between weeks 2 to 8 and gradually declined by week 10 in most animals for all antigens (Fig. 3). Not much difference was detected in the p32-, p54-, and pp62-specific IgG responses among treatment groups (Fig. 3A-C). In contrast, p72-specific IgG responses were highest in T3 animals, slightly lower in T2 animals and the lowest in T1 vaccinees (Fig. 3D). One animal in the control group, T4 had a high anti-p72 IgM and IgG response at week 1 but this response was not detected in the subsequent weeks, suggesting that the response at week 1 was non-specific and not necessarily primed by the immunization (Fig. 2D and 3D). In addition, adenovirus vector-specific IgG responses were generally consistent with the ASFV antigen-specific IgG responses (see appendix Fig. A2). Overall, post-prime antibody response data clearly showed the ability of the vaccine cocktail to rapidly induce ASFV-specific IgM and IgG responses in all vaccinees following single dose inoculation (Figs. 2 and 3).

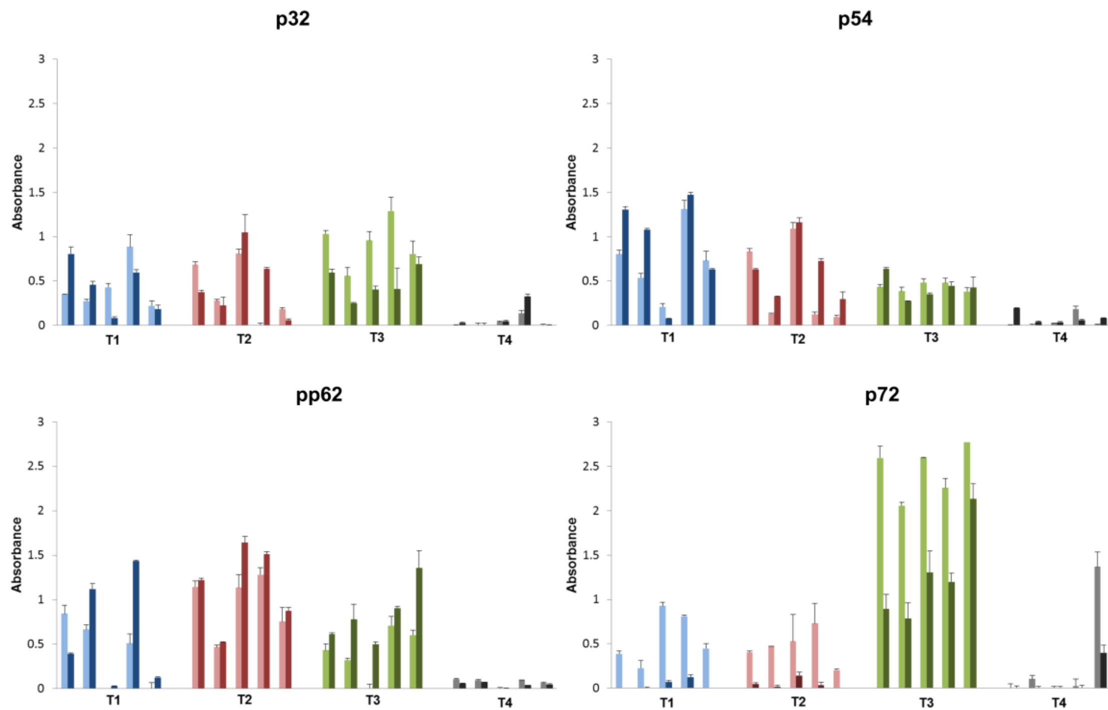

Figure 2. Ad5-ASFV multi-antigen cocktail rapidly primed antibody responses. Antigen specific IgMs (lighter shades) and IgGs (darker shades) in sera from week 1 post-prime were evaluated by ELISA. Color scheme used, T1: Blue; T2: Maroon; T3: Green; and T4 (Negative control): Gray. Individual animal response to each antigen was evaluated in triplicate and is depicted as the mean of the absorbance values at 450 nm minus the mean absorbance of cognate pre-immune sera. The error bars represent the standard deviation between triplicates.

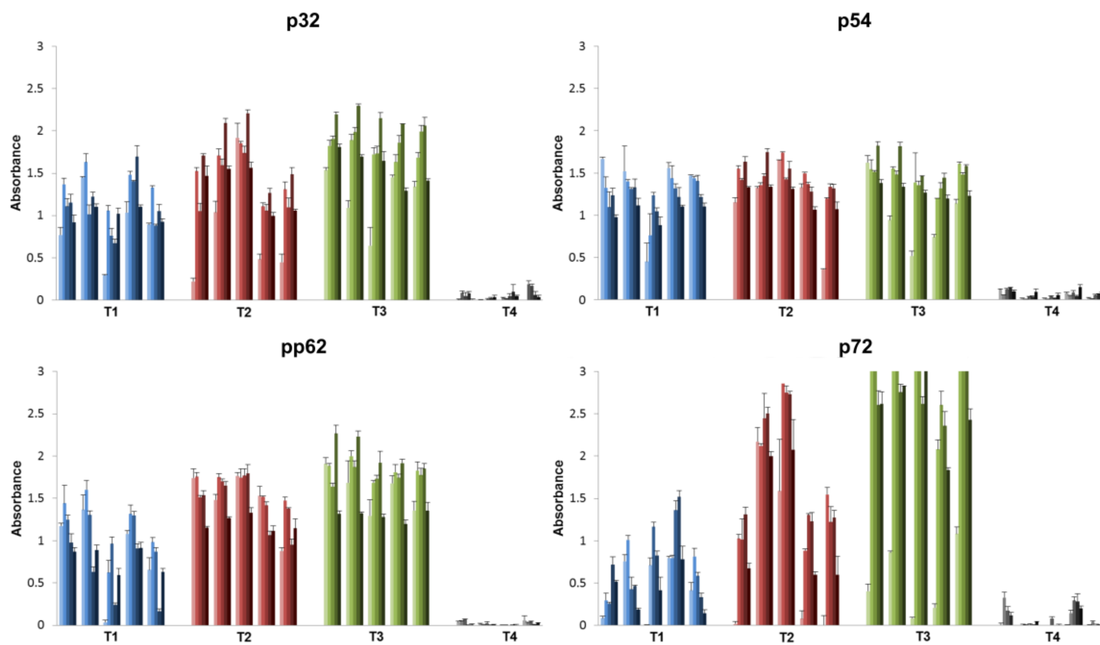

Figure 3. Antigen-specific serum IgG profiles post-priming.
Antigen-specific IgG were monitored biweekly post-prime up to week 10 by ELISA. Color scheme used, T1: Blue; T2: Maroon; T3: Green; and T4 (Negative control): Gray. The absorbance values at 450 nm across weeks 2, 4, 6, 8 & 10 post-prime for each animal are depicted using a color gradient where the lightest shade (first bar) represents week 2 and the darkest shade (last bar) represents week 10. Error bars show standard deviation among triplicate absorbance values.

2.4.2.2 Post-boost response

The gradual increase in antigen- and vector-specific antibody titers up to 8 weeks post-priming was an unexpected result and was a critical determinant with respect to the administration of the booster dose at week 14 in order to reduce impact due to existing adenovirus-specific antibodies. Following boosting, robust antigen-specific recall IgG responses against all four antigens were detected in sera collected at weeks 1 - 4 post-boost (see appendix Fig. A3). Evaluation of antigen-specific end-point titers by ELISA in sera collected at week 1 post-boost showed that all vaccinees (T1-T3), but none of the

sham treated controls (T4) had high antibody titers against each antigen (Fig. 4). Amongst the four ASFV antigens, pp62-specific titers were the highest and the p72-specific titers were the lowest (approximately 10^3 times lower) (Fig. 4). Convalescent serum ASFV-antigen specific titers were also evaluated. The majority of vaccinees had p32- and p54-specific titers that were equivalent or higher compared to those in convalescent serum (Fig. 4A and 4B), whereas for pp62 only T3 animals had titers equal or higher than the convalescent serum (Fig. 4C). However, in contrast, p72-specific titers in convalescent serum were higher compared to T1-T3 vaccinees (Fig. 4D). A multiple comparison of antigen-specific titers between the three treatment groups showed a significant difference only for pp62, with T3 titers significantly higher than both T1 ($p< 0.01$) and T2 ($p<0.001$) (Fig. 4C). A comparison of pre-boost and post-boost ASFV antigen-specific antibody responses showed that boosting with the cognate priming dose and adjuvant effectively amplified the primary response resulting in high antibody titers post-boost (Fig. 4 and A3). Importantly, given that two dose immunization with the Ad-ASFV multi-antigen cocktail induced titers comparable to the ASFV-specific convalescent serum, clearly demonstrates the ability of the multi-antigen cocktail formulations to elicit very strong immune responses.

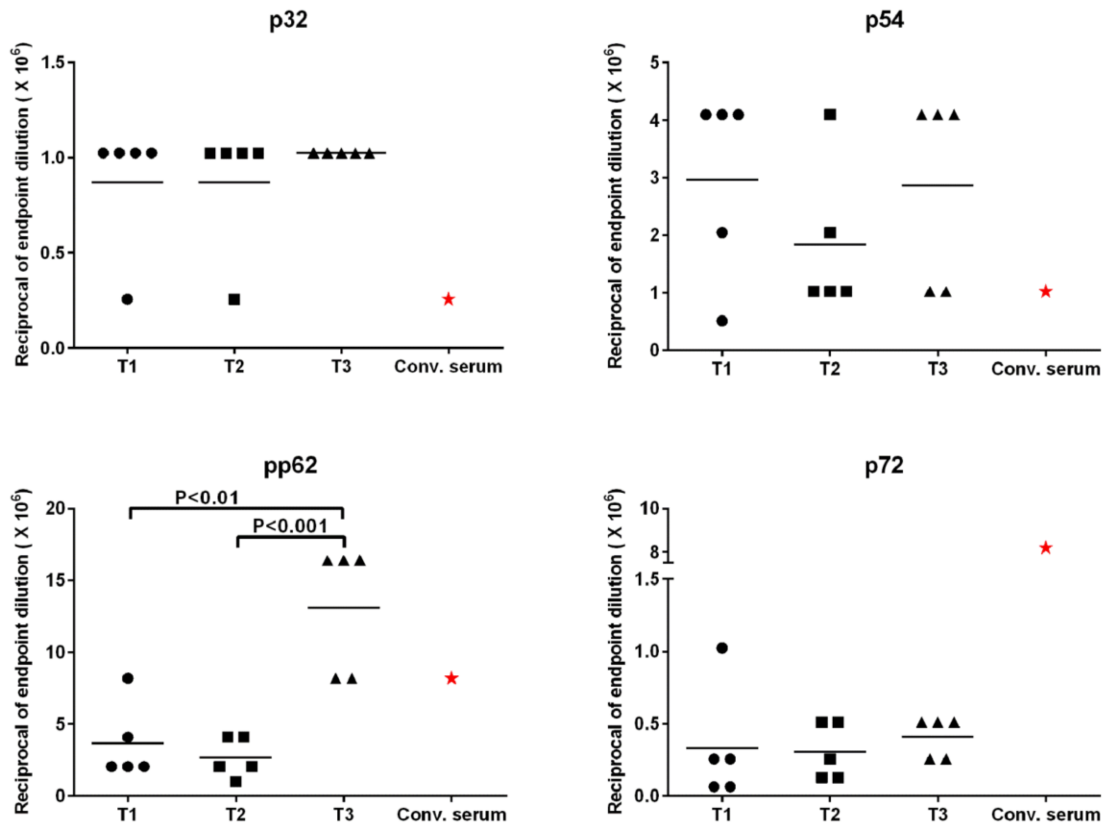

Figure 4. ASFV-specific end-point antibody titers.

Antigen specific end-point titers of sera collected week 1 post-boost were determined by ELISA. The endpoint dilution was determined to be the dilution at which the sample OD was higher than the OD of cognate pre-bleed +3 standard deviations. Data is represented as the reciprocal of the endpoint sera dilution x 10^6. The lowest titers were 1:64 x 10^3 against p72 and some of the highest titers were as high as 1:16 x 10^6 against pp62. The ASF-specific convalescent serum was also titrated and the titer against each antigen is depicted by the red star symbol. Sera from T4 animals showed no reactivity above background to any of the antigens (see Fig. A3). The antigen-specific titers amongst the treatment groups were compared using ANOVA followed by Tukey's multiple comparison test.

A critical role for antibodies in protection against ASFV has not been clearly established. Partial to complete protection has been reported following immunization of pigs with a combination of recombinant, subunit p30 and p54 antigens [42]. In addition, complete protection was reported in another study in which swine were immunized with recombinant CD2v (HA) and then challenged with wildtype ASFV [52]. However, in a separate study, antibodies induced following a combination of recombinant, subunit p22, p30, p54, and p72 antigens did not provide sufficient protection [43]. Furthermore, an immunization strategy to avoid ASFV-specific antibody responses by genetic fusion of recombinant, subunit p30, p54, and CD2v antigens to ubiquitin conferred protection against lethal challenge in a proportion of vaccinees [34]. These disparate findings have not allowed the protective role of host antibodies, if any, to be clearly defined during virulent ASF infection. Protective role for antibodies in ASFV is strongly supported by the observation that passive immunization with anti-ASFV serum confers complete protection against a subsequent lethal challenge [53]. Results in the present study using an Ad-ASFV multivalent cocktail formulated in two different adjuvants and administered in a prime-boost regimen induced detectable antibody titers in 100% of immunized pigs and against each of the four ASFV antigens.

2.4.3. Ad-ASFV multi-antigen cocktail primed antibodies recognized ASF virus

Indirect Fluorescence Antibody Assay (IFA) and western blot analysis using sera from week 1 post-boost confirmed that antibodies induced by the experimental Ad-ASFV multi-antigen cocktail recognized intact, native ASFV virus. All Ad-ASFV multi-antigen cocktail immunized swine, but none of the sham treated controls, had strong IFA signal

against primary swine macrophages infected with the ASFV Georgia 2007/1 isolate (Fig. 5A). Overall IFA results strongly demonstrated that the Ad-ASFV multi-antigen cocktail induced authentic ASFV-specific antibody responses (Table 2). This outcome was also confirmed by western blot using lysates from Vero cells infected with the ASFV Georgia 2007/1 isolate. Sera from all the three treatment groups (T1-T3), but not the control group (T4), strongly recognized the ASFV antigens (Fig. 5B). A control western blot conducted using mock-infected Vero cell lysate showed no background reactivity against host cell antigens (see appendix Fig. A4). It is important to note that these results do not suggest that the primed antibodies can neutralize ASFV virus, however they do confirm that the synthetic genes used to generate the Ad-ASFV constructs expressed authentic antigens.

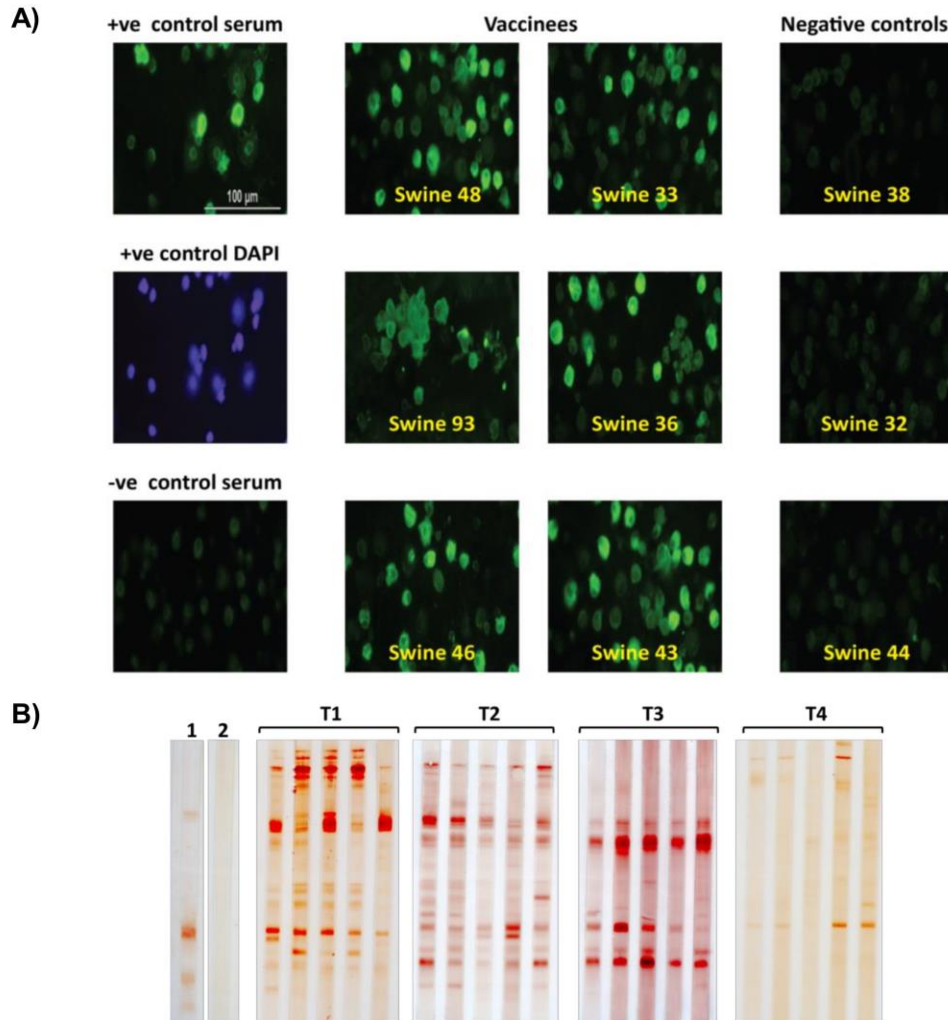

Figure 5. The Ad-ASFV multi-antigen cocktail induced authentic ASFV-specific antibody responses.

Indirect Immunofluorescence Antibody assay (IFA) and western blotting using sera from week 1 post-boost were used to confirm whether antibodies induced by the experimental Ad-ASFV multi-antigen cocktail recognized ASFV infected cells and ASFV-derived antigens. Panel A) Primary swine macrophages infected with the ASFV Georgia 2007/1 isolate and probed with individual, representative sera from six vaccinated and three control animals. ASFV-specific convalescent serum was used as the positive control, whereas normal pig serum was used as the negative control. The overall results are summarized in Table 2. Panel B) Western blot of lysates from Vero cells infected with the ASFV Georgia 2007/1 isolates, probed with sera from all animals. Lane: 1) ASFV specific convalescent serum; 2) Normal swine serum. The sera were also tested on mock-infected Vero cell lysates to check for background reactivity against host cell antigens (Fig. A4)

2.4.4. Ad5-ASFV multi-antigen cocktail primed antigen specific IFN-γ-secreting cells

ASFV antigen-specific IFN-γ-secreting cells were detected in whole peripheral blood mononuclear cells (PBMCs) post-prime and post-boost by IFN-γ ELISPOT assays (Fig. 6). Post-prime, majority of pigs in the three treatment groups (T1-T3) had higher IFN-γ responses against p54 and pp62 antigens compared to p32 and p72 antigens. Notably, the p54-specific IFN-γ responses were significantly higher in T1 pigs ($p<0.05$) and in T2 pigs ($p<0.01$) compared to the T4 sham treated controls (Fig. 6A). In addition, pp62-specific IFN-γ responses were significantly higher in T2 pigs ($p<0.001$) compared to the T4 controls (Fig. 6A). Antigen-specific IFN-γ recall responses observed in PBMCs three weeks post-boost, most notably the p32 and p72-specific responses, highlighted the booster dose effect (Fig. 6B). Compared to the T4 sham treated controls, significantly higher ($p<0.05$) IFN-γ responses against p32, pp62, and p72, but not p54, were observed in T1, but not in T2 and T3 animals (Fig. 6B). Post-boost, T1 pigs had higher, detectable IFN-γ responses against all four antigens tested compared to the other two treatment groups. This result differs from the post-prime results in which T2 immunized pigs were overall the best responders (Fig. 6A and 6B). One possibility for the discordant post-prime and post-boost results may be due to the relatively higher anti-adenovirus titers in T2 versus T1 at the time of boost which reduced the overall effectiveness of the booster dose in T2.

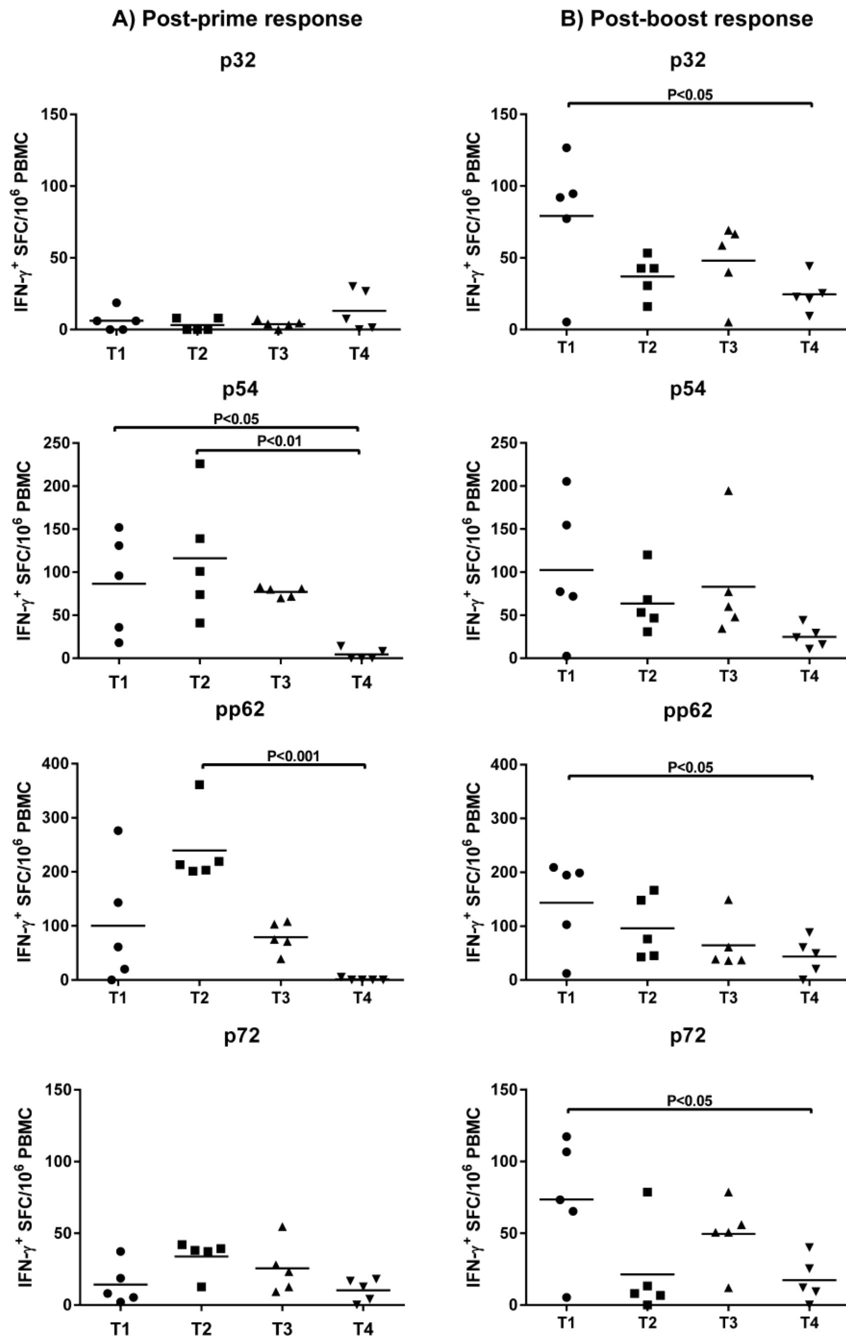

Figure 6. ASFV antigen-specific IFN-γ responses post-prime and post-boost.
The frequency of antigen-specific IFN-γ-secreting cells in PBMCs induced A) two weeks post-prime B) three weeks post-boost was evaluated by IFN-γ ELISPOT. The response is presented as IFN-γ Spot Forming Cells (SFC)/10^6 PBMCs. The mean response of treatment groups (T1-T3) was compared to the mean response of the sham treated control group (T4) using ANOVA followed by Bonferroni's multiple comparison test.

ELISPOT assays performed at study termination (week 8 post-boost) using isolated splenocytes confirmed the presence of antigen-specific IFN-γ-secreting memory cells. Notably, strong IFN-γ^+ responses against the four ASFV antigens were detected (Fig. 7). Compared to the T4 controls, significantly higher p32-specific IFN-γ+ responses were detected in T1 ($p<0.01$) and T2 ($p<0.05$) swine (Fig. 7). Significant ($p<0.01$) p54-specific IFN-γ+ responses were only detected in T1 animals, whereas, significant ($p<0.01$) pp62-specific IFN-γ^+ responses and ($p<0.05$) p72-specific IFN-γ^+ responses were only detected in T2 animals (Fig. 7).

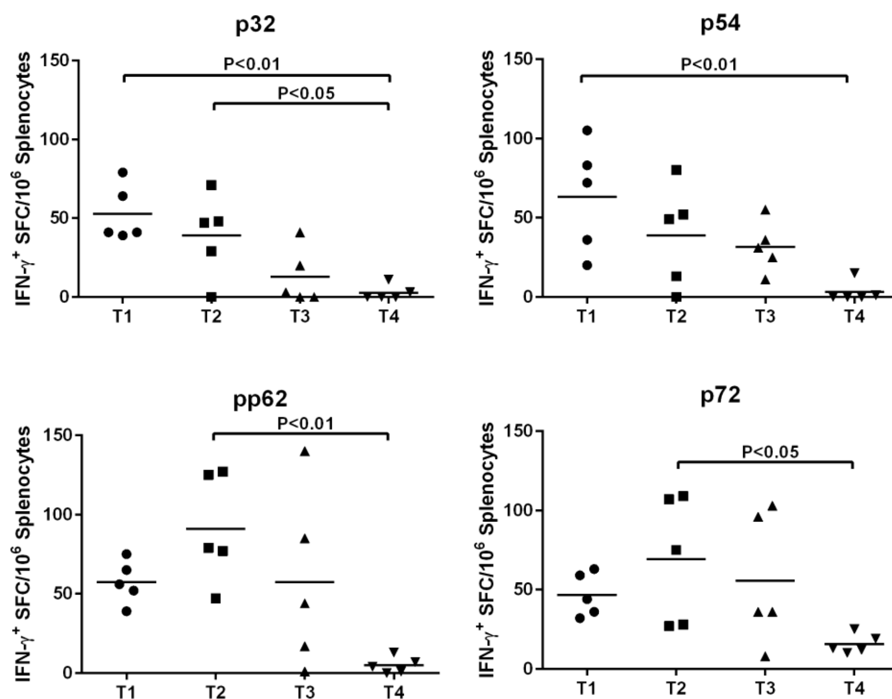

Figure 7. ASFV antigen-specific IFN-γ recall responses in spleen.
Presence of antigen-specific IFN-γ secreting memory T-cells in the spleen at study termination (week 8 post-boost) was evaluated by IFN-γ ELISPOT assay as above. The response is presented as IFN-γ Spot Forming Cells (SFC)/10^6 Splenocytes. Statistical analysis was done as described in Fig. 6.

A recent study using BacMams expressing a p30-p54-CD2v chimera reported partial protection upon sub-lethal challenge and a direct correlation between protection and induction of ASFV-specific IFN-γ^+ T-cells [54]. In this study, strong IFN-γ^+ peripheral and splenic tissue responses were elicited against each antigen in the Ad-ASFV multi-antigen cocktail in the majority of immunized swine (T1-T3) following prime-boost. Taken together, the ASFV antigen-specific IFN-γ responses observed in this immunogenicity study are promising and support the need to evaluate their potential to confer protection in a challenge study.

2.4.5. Ad5-ASFV cocktail primed antigen-specific Cytotoxic T Lymphocytes (CTLs)

One round of *in vitro* re-stimulated PBMCs (T1-T3) isolated 4 weeks post-boost were shown to effectively lyse autologous skin fibroblast transfectants in an ASFV antigen-specific manner at defined effector-target (E:T) ratios in ^{51}Cr-release assays (Fig. 8). Lytic activity against autologous skin fibroblast transfected with a construct expressing an FMDV negative control antigen remained at 20% or less for all animals, thus validating that the lytic activity observed against ASFV antigens can be attributed to ASFV antigen-specific CTLs and not non-specific NK cells (Fig. 8). Stimulation of the PBMCs for an additional round to further enrich CTLs failed to increase CTL activity, possibly due to activation-induced death of effectors. Among the T1-T3 immunized swine, the level of antigen-specific lysis was equivalent in T1 and T3 and lower in T2. This result is consistent with the post-boost observation discussed earlier in which relatively higher anti-adenovirus titers in T2 versus T1 at the time of boost may have reduced the overall effectiveness to amplify the primed CD8$^+$ T-cell responses.

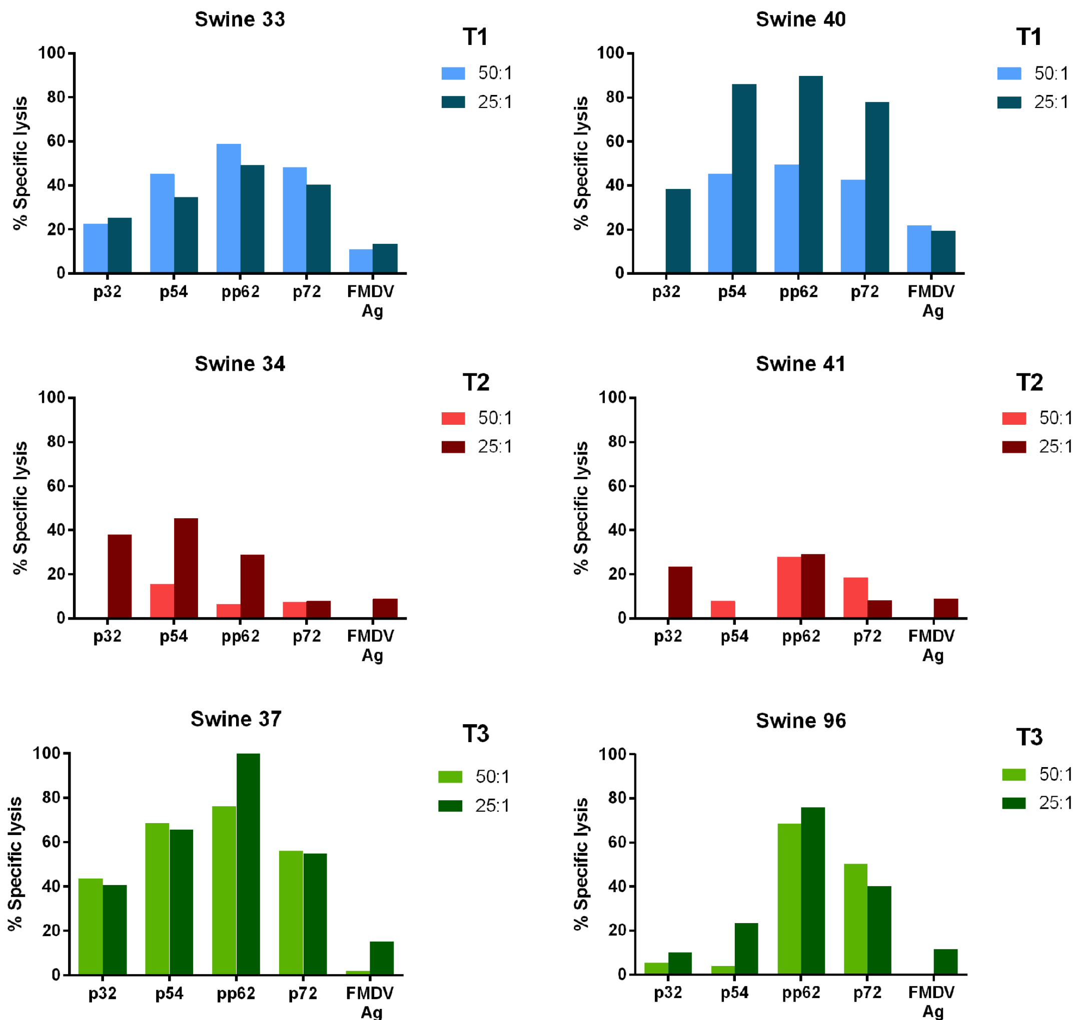

Figure 8. Ad5-ASFV multi-antigen cocktail primed ASFV antigen-specific Cytotoxic T Lymphocytes (CTL) responses.

Antigen specific CTL responses in PBMCs collected post-boost (week 4) were evaluated at effector to target ratios of 50:1 and 25:1 using the standard ^{51}Cr release assay. Data is represented as % specific lysis against each ASFV antigen and a FMDV negative control antigen. Representative data for 2 animals from treatment groups T1, T2 and T3 is shown. Assays were not conducted for animals from the control group, T4.

The heterogeneous CTL responses observed within the context of the study and assay designs are consistent with expected outcomes from the outbred commercial pigs used. This study is the first to demonstrate induction of ASFV antigen-specific CTL responses in commercial swine using Ad-ASFV multi-antigen cocktail, but will require further evaluation in subsequent studies using ASFV-infected target cells.

Substantial evidence in published literature emphasizes the importance of CTLs in protection against ASFV. Studies have shown that ASFV-specific CTLs can be induced in swine infected with live, attenuated ASFV and the primed CTLs were shown to be responsible for clearance of infected cells [32]. Significant proportions of ASFV-specific $CD4^{+}CD8^{high+}$ CTLs have been detected in immune swine that are protected from clinical disease than from immune but clinically diseased pigs, suggesting that these CTLs are required for disease control [28]. Furthermore, depletion of $CD8^{+}$ T-cells in swine results in loss of protective immunity to ASFV infections [33]. Within this context, the CTL results reported herein are noteworthy and clearly demonstrate the ability of a replication-deficient viral vectored ASFV multi-antigen cocktail to induce antigen-specific CTLs that are capable of recognizing and lysing autologous ASFV-antigen presenting fibroblasts.

2.4.6. Ad5-ASFV cocktail was well tolerated

Following inoculation of the Ad-ASFV multi-antigen cocktail, both the 10^{10} and the 10^{11} doses and adjuvant formulations (Table 1) were reasonably well tolerated in all the swine. Although no adverse systemic effects or injection site reactions were observed, some pigs in T1, T2 and T4 that received the ENABL formulation had mild injection site

swelling, were transiently depressed (lethargic) and had reduced appetite for two days following the booster dose. T3 vaccinees (Zoetis adjuvant) were active but all had a pink discoloration at the injection site. However, by the 3rd day post-boost, all the swine were active, healthy and with good appetite, and remained so for the rest of the study period. Thus, overall, the Ad-ASFV multi-antigen cocktails formulated at both doses and with both adjuvants were safe and well tolerated by all the swine.

2.5. Conclusions

The ASFV is a large complex DNA virus encoding >150 proteins. Experimental, subunit vaccines based on a few of these antigens have generated different protective outcomes, demonstrating that these antigens do play some role in host protection. Immunization of animals with an expression library of restriction enzyme digested ASFV genome fragments protected 60% of the animals [55]. This outcome suggests that protection through subunit vaccines is feasible, but is unlikely to be highly efficacious using a single or only a few antigens. Empirical identification of antigens necessary for inducing a protective response, along with a suitable antigen delivery system that elicits strong cellular as well as humoral responses may be a reasonable strategy to develop an efficacious, prototype ASFV vaccine. The immunogenicity data generated from this proof-of-concept study showed that the replication-deficient adenovirus vector, dose, adjuvant formulation and the immunization regimen effectively induced strong antibody (with unprecedented rapid isotype-switching) and cellular responses against four ASFV antigens. An analysis of the overall differences in antibody and T-cell immune response observed across the three differential treatment groups revealed some interesting

outcomes. In case of the humoral responses, the T3 animals (immunized with the Zoetis adjuvant) had a slightly higher antibody response, however the end-point titration data (Fig. 4) failed to demonstrate any significant differences in the titers amongst the three treatment groups for three of the four ASFV antigens tested (pp62 was the only exception). With respect to T-cell mediated immune responses, the post-prime antigen-specific IFN-γ response clearly showed that T2 (high dose, ENABL adjuvant) animals were the best overall responders. However, the post-boost data suggests that the low dose prime and low dose boost (T1) had the highest recall response. Based on this outcome, it may be useful in future immunogenicity and efficacy studies to test whether priming with the low dose (10^{10} IFU/Ad-ASFV construct) and boosting with the high dose (10^{11} IFU/Ad-ASFV construct) will elicit better immune responses. In addition, there is merit to testing both adjuvants in future efficacy studies to better understand the relevance of the varied immune responses induced in context of the protection conferred.

In conclusion, an Ad-ASFV multi-antigen cocktail two dose formulation was immunogenic and safe when administered in a prime-boost regimen. Results showed evidence of rapid post-prime antibody class switching, induction of robust antibody responses which recognize ASFV-infected cells, and the generation of antigen-specific IFN-γ and antigen-specific CTL responses to all four ASFV antigens. The immunogenicity data from this study validates our approach of using an adenovirus-vectored cocktail of ASFV antigens and sets the stage for conducting future challenge studies using a cocktail of the above antigens as well as other novel ASFV antigens. Collectively, these data validate a synthetic gene-based approach to generate ASFV

antigen delivery constructs and provide a rational strategy for further screening of ASFV antigen targets toward development of a DIVA compatible, multi-antigen, efficacious ASF vaccine.

CHAPTER III

ADENOVIRUS-VECTORED NOVEL AFRICAN SWINE FEVER VIRUS ANTIGENS ELICIT ROBUST IMMUNE RESPONSES IN SWINE

3.1. Overview

African Swine Fever Virus (ASFV) is a high-consequence Transboundary Animal pathogen that often causes hemorrhagic disease in swine with a case fatality rate close to 100%. Lack of treatment or vaccine for the disease makes it imperative that safe and efficacious vaccines are developed to safeguard the swine industry. In this study, we evaluated the immunogenicity of seven adenovirus-vectored novel ASFV antigens, namely A151R, B119L, B602L, EP402RΔPRR, B438L, K205R and A104R. Immunization of commercial swine with a cocktail of the recombinant adenoviruses formulated in adjuvant primed strong ASFV antigen-specific IgG responses that underwent rapid recall upon boost. Notably, most vaccinees mounted robust IgG responses against all the antigens in the cocktail. Most importantly and relevant to vaccine development, the induced antibodies strongly recognized Georgia 2007/1 ASFV-infected cells by IFA and the actual ASF viral proteins by western blot analysis. The recombinant adenovirus cocktail also induced ASFV-specific IFN-γ-secreting cells that were recalled upon boosting. Evaluation of local and systemic effects of the recombinant adenovirus cocktail post-priming and post-boosting in the immunized animals showed that the immunogen was well tolerated and no serious negative effects were observed. Taken together, these outcomes showed that the adenovirus-vectored

novel ASFV antigen cocktail was capable of safely inducing strong antibody and IFN-γ^+ cell responses in commercial swine. The data will be used for selection of antigens for inclusion in a multi-antigen prototype vaccine to be evaluated for protective efficacy.

3.2. Introduction

The African Swine Fever Virus (ASFV) is a high-consequence Transboundary Animal Disease (TAD) pathogen that causes hemorrhagic fever in swine and has mortality rates approaching 100% [20]. There is no vaccine or treatment available for this disease. The ASFV causes major economic losses in endemic areas and poses a high risk to swine production in non-affected areas as it continues to spread globally [22]. Therefore, it is imperative that appropriate counter-measures are developed to reduce the prevalence of this disease in endemic areas, prevent further outbreaks in affected countries and safeguard the swine industries in non-affected areas.

Development of an efficacious vaccine for ASFV is still a challenge. There is strong evidence to suggest that protection against ASFV can be induced since attenuated virus has been shown to protect against parental or closely related virulent isolates [26, 27, 56]. Attenuated vaccines, however, are yet to be rigorously tested in the field in readiness for deployment. Development of an affordable DIVA (Differentiating Infected from Vaccinated Animals) ASFV subunit vaccine is a more attractive option, especially for use in non-endemic areas, in case of an outbreak.

Subunit vaccines based on one or two ASFV antigens have so far failed to induce immunity strong enough to confer significant protection among vaccinees, [34, 42, 43, 54] but, immunizing swine with DNA plasmids expressing a library of restriction

enzyme digested ASFV-genome fragments conferred protection in a majority (60%) of the vaccinees against lethal challenge [55]. This result, though in favor of developing subunit based vaccines for ASFV, also highlights the main challenges associated with developing subunit vaccines: identification of protective antigens as well as a suitable delivery vector to induce strong protective responses. It is envisaged that successful development of an effective subunit vaccine will require empirical identification and validation of multiple suitable antigens that will induce significant protection in majority of the vaccinees.

We have previously shown that immunizing swine using a cocktail of replication deficient adenoviruses expressing ASFV antigens p32, p54, pp62, and p72 elicited robust antigen-specific antibody, IFN-γ^+ cellular and cytotoxic T-lymphocyte (CTL) responses [57]. We used replication-incompetent human adenovirus (Ad5) vector since it is safe, gives high protein expression levels and replicates at high titers in completing cells making production scalable and reproducible [4, 5]. In addition, efficacy of adenoviruses in swine immunizations has previously been demonstrated in the successful development of a recently USDA-licensed recombinant Foot and Mouth Disease vaccine [16, 58]. In this study, we evaluated immunogenicity of seven ASFV vaccine candidates selected based on published literature (Table 3).

Table 3: Antigens Selected for Evaluation of Immunogenicity

Gene/Antigen	Functional Characteristics /Immune Relevance	Reference
A151R	Essential for the virus replication and morphogenesis. May play a role in viral transcription.	[59]
B119L	Critical for virus assembly. 90% of deletion mutants are crippled and fail to generate viable viral particles	[27], [60]
B602L	Chaperone for p72 (major capsid protein), repression leads to decrease of p72 expression, inhibition of pp220 and pp62 processing. Deletion severely alters viral assembly. Recognized by domestic pig and bush pig hyper-immune sera	[61] , [62], [63], [64].
EP402R	ASFV Hemagglutinin. Extracellular domain contains protective T-cell epitopes. Protective immunity against homologous infection maybe haemadsorption inhibition (HAI) serotype-specific.	[65], [34], [66], [67]
B438L	Required for formation of vertices in icosahedral capsid	[68]
K205R	Induces strong antibody responses, but ability to elicit T-cell responses has not been tested. Recognized by domestic pig and bush pig hyper-immune sera	[63], [64]
A104R	Histone-like protein. Primes strong antibody responses, mainly detected in asymptomatic than chronically infected pigs. Presence of T-cell determinants has not been evaluated.	[69], [64]

The ability of these antigens to induce antibody and T-cell responses in commercial swine has not been evaluated so far. The antigen, EP402R, has been previously evaluated, however, only the extracellular domain was included and expressed as a fusion chimera along with other ASFV antigens, p32 and p54. In this study, we altered the EP402R protein sequence to delete the proline-rich repeats in the

cytoplasmic tail and the resultant protein was designated EP402RΔPRR. The proline-rich repeats have been shown to interact with the adaptor protein SH3P7 in host cells and it is theorized that this interaction could, in part, be responsible for the immunomodulatory role of the EP402R protein [70]. Thus, deletion of the proline-rich repeats is expected to abrogate immunomodulatory effects when the EP402R protein is included in a multi-antigen subunit vaccine.

The focus of this work was to evaluate the immunogenicity of seven novel ASFV antigens, in commercial swine using replication-deficient adenovirus as a delivery platform with an end goal of identifying candidates for rationally designing a prototype multi-antigen ASFV subunit vaccine.

3.3. Materials and Methods

3.3.1. Generation of recombinant adenoviruses expressing ASFV antigens

The amino acid sequences of the ASFV antigens (Georgia 2007/1 isolate) were obtained from Genbank (Accession FR682468). The EP402RΔPRR sequence was generated by deleting the proline-rich repeats from the EP402R cytoplasmic domain [70]. Since K205R and A104R polypeptides are short, they were fused in frame to generate a chimeric sequence, designated K205R-A104R. The sequences of the target antigens (A151R, B119L, B602L, EP402RΔPRR, B438L, and K205R-A104R) were then modified to add, in-frame, a FLAG- and HA- tag at the N- and C-termini, respectively, and the resultant protein sequences were used to generate synthetic genes which were codon-optimized for protein expression in swine. Synthesis, codon-optimization, cloning

in pUC57 vector, and sequence-verification of these genes was outsourced (GenScript, NJ, USA). Each gene was then amplified by PCR using *attB1*-FLAG specific forward and *attB2*-HA specific reverse primers and subcloned into Gateway pDonR221 vector (Invitrogen) as per manufacturer's protocols. Positive pDonR clones were validated by sequencing and used to transfer the gene cassette into the adenovirus backbone vector, pAd/CMV/V5-DEST (Invitrogen) by homologous recombination. Validated positive pAd clones were then used to generate recombinant adenoviruses, designated AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R, using the ViraPower Adenoviral Expression System (Invitrogen). Antigen expression by the adenoviruses was confirmed by immunocytometric analysis of infected Human Embryonic Kidney (HEK)-293A cells. The viruses were scaled up to generate bulk stocks for immunization and titrated (infectious focus units, IFU) in HEK-293A cells as previously described [57]. A recombinant adenovirus expressing luciferase, designated Ad-Luc, was similarly prepared and served as a negative control immunogen.

3.3.2. Generation of recombinant ASFV antigens

The genes encoding the A151R, B602L, EP402RΔPRR, B438L and K205R-A104R antigens were PCR amplified from the respective pDonR clones using FLAG-specific forward and HA-specific reverse primers. The resultant PCR products were cloned into the pFastBac™ HBM TOPO shuttle vector (Invitrogen). Positive clones were identified by PCR screening and used to generate recombinant baculoviruses using the Bac-to-Bac HBM TOPO Secreted Expression System (Invitrogen). Protein expression by the generated viruses was confirmed by immunocytometric analysis of infected Sf-9 cells.

One clone of each baculovirus was then scaled up and used to infect High Five cells (Invitrogen) to generate recombinant proteins. These proteins were affinity-purified using the anti-FLAG M2 affinity gel (Sigma, A2220). Recombinant B119L was affinity purified similarly, but from AdB119L-infected HEK-293A cell lysates.

3.3.3. Validation of protein expression

3.3.3.1. Immunocytometric analysis

Protein expression by the recombinant adenoviruses was evaluated by immunocytometric analysis as described previously [48]. Briefly, HEK-293A cell monolayers infected with the adenoviruses were incubated with a gamma-irradiated ASFV-specific convalescent serum (1:250 dilution) [57]. Following 3X washes, the cells were further incubated with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-porcine IgG (Southern Biotech, Cat# 6050-04) for 1 hr. Following washes as above, Fast Red TR–Naphthol AS-MX substrate (Sigma, F4523) was added to the cells to detect the alkaline-phosphatase activity. Protein expression by the recombinant baculoviruses was similarly evaluated by infecting Sf-9 cells. Mock infected cells served as negative controls.

3.3.3.2. Western blot

Affinity-purified recombinant proteins (A151R, B119L, B602L, EP402RΔPRR, B438L, and K205R-A104R) were resolved by SDS-PAGE and transferred to Immobolin-P PVDF Membrane (Fisher Scientific). Following an overnight incubation at 4°C with blocking buffer (10% non-fat dry milk TBST), the membrane was incubated with

ASFV-specific convalescent serum (1: 2,500 dilution in blocking buffer) for 1hr. The membrane was then washed 3X with TBST and incubated with peroxidase-conjugated Goat anti-swine IgG (1:5,000) (Jackson ImmunoResearch, Cat #114-035-003). Chemiluminescence was detected by the SuperSignal West Pico PLUS substrate (Thermo Scientific, Prod #34577).

3.3.4. Swine immunizations

Twenty weaned swine were randomly distributed into the treatment and control groups (n=10). The treatment group was immunized with the Ad-ASFV cocktail (1 X 10^{11} IFU) of each construct (formulated in ENABL adjuvant (Benchmark Biolabs, Cat# 7010106-C6)). The control group received Ad-Luc formulated as above. The inoculum was injected intramuscularly in the neck area behind the ears. The animals were then boosted similarly after 8 weeks. Blood was collected for sera and PBMC isolation once pre-immunization and then biweekly post-prime, and then weekly post-boost for 3 weeks to run ELISAs and IFN-γ ELISPOTs. The animals were euthanized at 4 weeks post-boost.

3.3.5. ELISA

Antigen-specific antibody responses were evaluated by a direct ELISA as previously described [57]. Briefly, microplates coated overnight at 4°C with 100 μl of 1μg/ml of affinity-purified antigen in bicarbonate coating buffer were washed and blocked with 10% non-fat dry milk in PBS with 0.1% Tween 20 for 1 hr. Sera were diluted at 1:100 (week 4 post-prime) or 1: 8,000 (week 2 post-boost) in blocking buffer and added at 100 μl per well in triplicates. After incubation for 1 hr. at 37°C, the plates were washed and

incubated for another hr. with 100 μl/well of a 1: 5,000 dilution of peroxidase-conjugated anti-swine IgG (Jackson ImmunoResearch, Cat# 114-035-003). Following washes, the plates were developed with Sure Blue Reserve TMB substrate (KPL, Cat# 53-00-02) and reaction was stopped using 1N Hydrochloric acid. The IgG response by each animal to each antigen was calculated as mean absorbance of test sera minus the mean absorbance of the cognate pre-immunization sera. To determine antigen-specific IgG end-point titers, sera from blood collected two weeks post-boost was serially diluted two-fold starting at 1: 4,000 up to 1: 4 X 10^6. The pre-immunization serum was similarly diluted. The end-point titer was calculated as described previously [57].

3.3.6. Indirect Fluorescence Antibody Assay (IFA)

Pretreated Teflon coated slides with fixed ASFV (Georgia 2007/1)-infected and mock-infected Vero cells (ATCC CCL-81) were used to perform the IFA as previously described [11]. Briefly, the slides were incubated with sera from two weeks post-boost diluted 1:250 for 1 hr. at 37°C. ASFV-specific convalescent serum (1:10,000) was used as a positive control and normal swine serum (1:250) (GIBCO) was used as a negative control. Following extensive washes with D-PBS, the wells were incubated with FITC-conjugated goat anti-swine sera (Kirkegaard and Perry Cat No. 02-14-02) for 45 minutes at 37°C, washed again and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Cat. No. PT389868). The cells were visualized using an Olympus immuno-fluorescent microscope (model BX-40) and photographed by an Olympus digital camera (model DP 70). The IFAs were conducted at Plum Island Animal Disease Center.

3.3.7. Western blot with ASFV-infected cell lysates

Lysates from ASFV Georgia 2007/1 (Vero cell-adapted)-infected Vero cells were used to perform a western blot as previously described [57]. Briefly, the prepared cell lysates were electrophoresed on a NuPAGE 4-12% Bis-Tris Gel (1.0mm X 2D well) for 35 mins, followed by transfer to 0.2um PVDF membranes (Invitrogen #LC2002) for 1 hour. The membranes were then blocked for 1 hr. in blocking buffer (PBST+5% non-fat dry milk) and transferred to the Protean II Slot-Blotter. Sera from week 2 post-boost were diluted 1:50 in blocking buffer and added to individual wells for 1 hr. at room temperature with shaking. After washing the wells 3X with PBST, the membranes were removed from the blotting apparatus and incubated for 1 hr. with a 1: 2,000 dilution of Goat anti-swine-HRP (KPL #14-14-06). Following washes, the membranes were developed using DAB (Sigma #D4293). ASFV-specific convalescent serum (1:10,000) was used as a positive control and normal swine serum (GIBCO) was used as a negative control. Background reactivity to host-cell antigens was gauged similarly using mock-infected lysates. The western blot analysis was carried out at Plum Island Animal Disease Center.

3.3.8. IFN-γ ELISPOT assays

Antigen-specific IFN-γ^+ cell response was evaluated by an enzyme-linked immunospot (ELISPOT) assay using the Mabtech kit (Cat# 3130-2A), as per manufacturer's instructions and as previously described [57]. Briefly, whole blood-derived PBMCs resuspended in complete RPMI-1640 media were added to wells of MultiScreen-HA plates (Millipore) at a density of 250,000 cells/well. Affinity-purified antigens were

added to the cells at a final concentration of 2.5 μg/ml in triplicates. Phytohemagglutinin (PHA) mitogen (5 μg/ml) was used as a positive control, whereas media served as the negative control. The spots were counted by an ELISPOT reader and AID software (AutoImmun Diagnostica V3.4, Strassberg, Germany). The mean number of IFN-γ^+ Spot-Forming Cells (SFC) for each sample was calculated by subtracting the mean number of spots in the negative control wells from the mean number of spots in the sample wells. The data is presented as mean number of SFC per 10^6 PBMCs.

3.3.9. Statistical analysis

The differences in the mean antigen-specific antibody and IFN-γ^+ responses between the treatment and the control group were analyzed by an unpaired t-test with Welch's correction, and a P value of ≤ 0.05 was considered significant. The analysis was performed with GraphPad Prism Version 6.05 using a significance level of $P<0.05$.

3.3.10. Ethics statement

All animal procedures were conducted as per the Animal Use Protocol 2014-0020, reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). The Texas A&M IACUC follows the regulations, policies and guidelines outlined in the Animal Welfare Act (AWA), USDA Animal Care Resource Guide and the PHS Policy on Humane Care and Use of Laboratory Animals. At the termination of the study, the animals were euthanized with an overdose of sodium pentobarbital.

3.4. Results and Discussion

3.4.1. Recombinant constructs encoding ASFV antigens

Codon-optimized synthetic genes encoding antigens, A151R, B119L, B646L, EP402RΔPRR, B438L, and K205R-A104R fused in-frame to FLAG and HA tags were used to generate recombinant adenoviruses designated AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, and AdK205R-A104R. The immunogenicity of K205R and A104R was evaluated as a chimera since both proteins are relatively small (~20kDa and ~10kDa) and delivering them *in vivo* as a chimera would reduce the number of adenoviruses to be inoculated. Evaluation of protein expression by immunocytometric analysis of adenovirus-infected HEK-293A cells using ASFV-specific convalescent serum showed that the assembled recombinant adenoviruses expressed the encoded antigens (Fig. 9A). The synthetic ASFV genes were also used to generate recombinant baculoviruses for generation of affinity-purified recombinant proteins needed for *in vitro* evaluation of antigen-specific antibody and cell responses. However, despite several attempts, we were unsuccessful in generating a recombinant baculovirus expressing B119L and thus we used affinity-purified antigen from AdB119L-infected HEK-293A cells for *in-vitro* readouts. The authenticity of the affinity-purified recombinant proteins was validated by western blot using ASFV-specific convalescent serum (Fig. 9B). A very faint band (depicted by an arrow) was observed for antigen B438L at the expected molecular weight (~50 KDa). The antigen loads were optimized for signal detection. However, for antigen B438L the signal intensity remained weak despite increasing antigen load to microgram quantities. This could be due to low B438L-specific antibodies in the ASFV-specific convalescent serum,

also evidenced by the low anti-B438L end point titer of 1:4000 observed by ELISA (see Fig. 11).

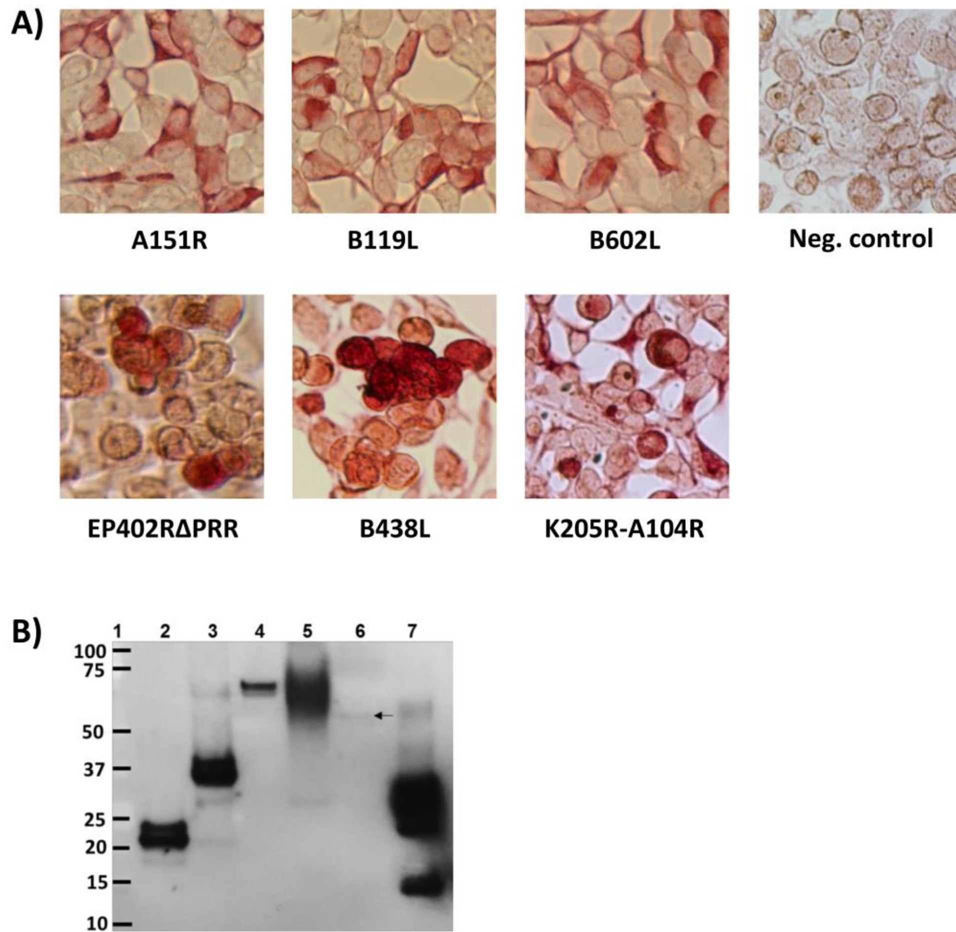

Figure 9. Validation of protein expression by ASFV constructs.
The authenticity of the ASFV antigens expressed by the constructs was confirmed by immunocytometric and western blot analysis using gamma-irradiated ASFV-specific convalescent serum. Panels: A) HEK-293A cells infected with recombinant adenoviruses; and B) A western blot of the affinity purified ASFV proteins probed with the convalescent serum. Lane: 1) Mwt marker in kDa; 2) A151R; 3) B119L; 4) B602L; 5) EP402RΔPRR; 6) B438L and 7) K205R-A104R chimera. The arrow points to the faint band detected for B438L (the signal intensity of the band increased with longer exposure times).

3.4.2. Ad5-ASFV cocktail primed ASFV antigen-specific antibodies

Twenty commercial swine were randomly divided into two groups (n=10). The treatment group was immunized with a cocktail of six recombinant adenoviruses expressing the A151R, B119L, B602L, EP402RΔPRR, B438L, and K205R-A104R ASFV antigens, whereas the negative control group received Ad-Luc sham treatment. After priming, antigen-specific IgG responses were detected in a majority of swine in the treatment group, but not the control group. Data from sera analyzed four weeks post-priming is shown (Fig. 10A).

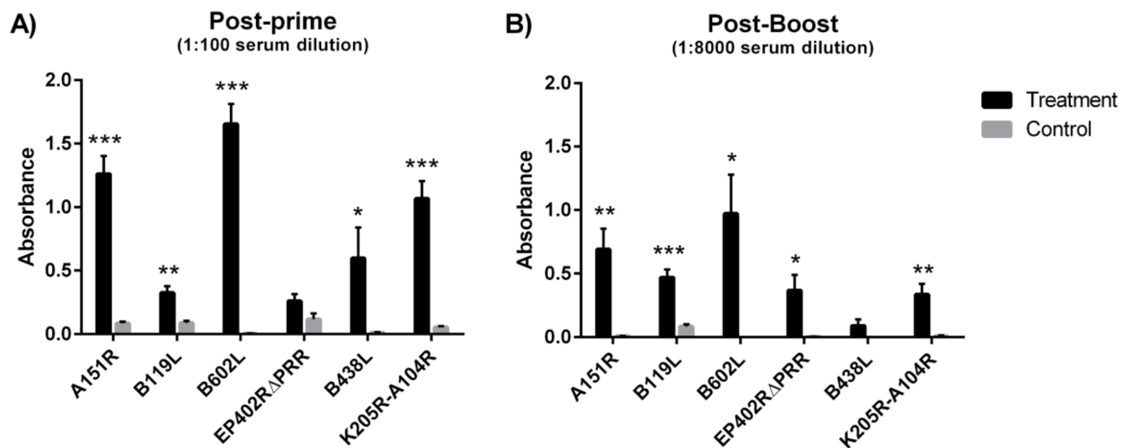

Figure 10. Mean antigen-specific IgG responses post-priming and post-boost. Antigen-specific IgG response was evaluated post-prime and post-boost by ELISA. A) Sera from week 4 post-prime were evaluated at a 1:100 dilution. B) Sera from week 2 post-boost were evaluated at a 1:8,000 dilution (to prevent the absorbance values from going out of range). The error bars represent the SEM. The asterisks denote a significant difference between the mean response of the treatment and control animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The mean response of the treatment group was significantly higher than the control group for antigens A151R ($p<0.001$), B119L ($p<0.01$), B602L ($p<0.001$), B438L

($p<0.05$) and K205R-A104R ($p<0.001$). The mean antibody response against the EP402RΔPRR antigen by the swine in the treatment group was slightly higher than the controls but not significant. The strong mean responses observed against antigens B602L and K205R-A104R is consistent with previous studies where these antigens have been shown to be strongly recognized by domestic pig and bush pig hyper-immune sera [63, 64]. Following boosting at 8 weeks post-priming, antigen-specific recall IgG responses against all antigens were detected in the animals in the treatment group (Fig. 10B). The mean response of the treatment group was significantly higher than the control group for antigens A151R ($p<0.01$), B119L ($p<0.001$), B602L ($p<0.05$), EP402RΔPRR ($p<0.05$), and K205R-A104R ($p<0.01$), but not for antigen B438L. It is important to note that the responses at week 2 post-boost were evaluated at 1:8,000 sera dilution, whereas the responses post-prime were evaluated at a 1:100 sera dilution (Fig. 10). This eliminated the background responses observed against some antigens post-prime in the control group. However, for antigen B119L, the control group still had a low-level of background reactivity after boosting. This background response could be attributed to vector-specific antibodies since the affinity-purified B119L antigen was derived from lysates of AdB119L-infected HEK-293A cells. Also the response seen in the treatment group is likely to be inclusive of a low level of vector-specific antibodies. Evaluation of antigen-specific end-point titers post-boost in the immunized pigs showed that a majority of the vaccinees had titers $\geq 1:256 \times 10^3$ against antigens A151R, B119L, B602L, and K205R-A104R (Fig. 11). The highest titer was $1:2 \times 10^6$ against B602L in one of the vaccinees (Fig. 11). A comparison of the antigen-specific titers in sera from

the vaccinees with the titer of the ASFV-specific convalescent serum revealed that Ad-ASFV cocktail was able to induce titers higher or equivalent to the convalescent serum in a majority of animals for antigens B119L (90% of vaccinees), B438L (90% of vaccinees), B602L (80% of vaccinees), and EP402RΔPRR (80% of vaccinees). This is a noteworthy result, since these animals received only two immunizations of the Ad-ASFV cocktail, whereas the positive control convalescent serum came from an animal that received multiple inoculations of live ASFV [57]. However, for antigen, K205R-A104R only 3 of 10 vaccinees had titers that matched up to the convalescent serum, whereas for antigen, A151R the titers induced in the vaccinees did not match up to the convalescent serum.

The role of antibodies in ASFV protection is not yet completely understood [35]. Protection reported by passively acquired anti-ASFV antibodies, however, is strong evidence in favor of antibodies and supports the evaluation of humoral responses in immunogenicity studies focused on identification of novel targets for subunit vaccine development [53, 71]. In the current study, a cocktail of replication-incompetent adenovirus constructs expressing multiple ASFV antigens primed strong antibody responses against all antigens in a majority of the animals.

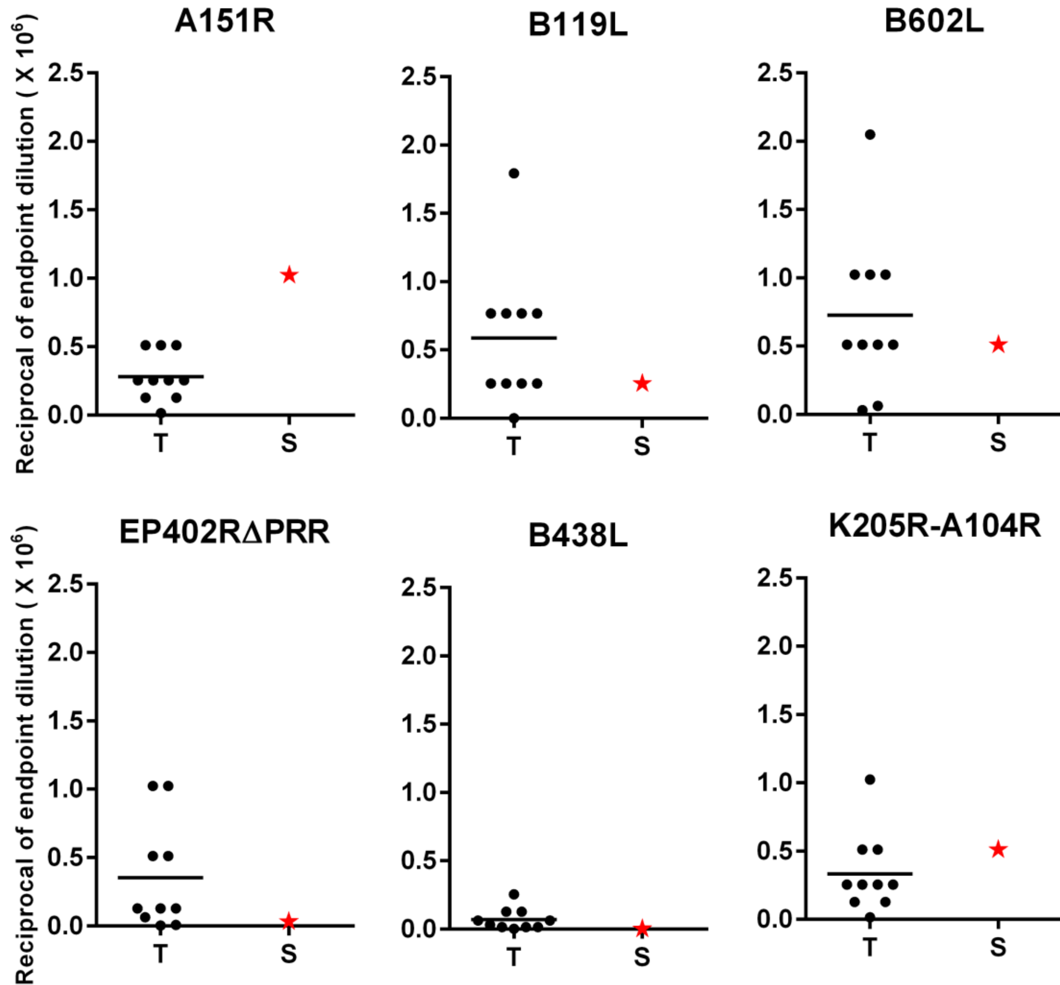

Figure 11. Antigen-specific end-point IgG titers.
Antigen specific antibody titers, determined by ELISA, in sera from treatment group animals (T) collected two weeks post-boost. The dilution of the sera at which the absorbance reading was higher than that of the cognate pre-bleed +3 standard deviations is reported as the end-point titer. The ASFV-specific convalescent serum was titrated similarly and is represented by the red star symbol (S). Data is represented as the reciprocal of the end-point sera dilution x 10^6. For antigen B119L, the sera from control group animals was also titrated to gauge background reactivity to host-cell and vector-derived antigens. An average of the titers of the control group animals was then subtracted from the titer of each treatment group animal to give B119L-specific titers. For the remaining antigens, the post-boost sera from the control group animals showed no reactivity as seen in Fig. 10B.

3.4.3. Antibodies primed by the Ad5-ASFV cocktail recognized ASF virus

Indirect Immunofluorescence Antibody Assay (IFA) performed with sera from blood collected from the vaccinees two weeks post-boost, confirmed that the antibodies primed by the Ad5-ASFV cocktail recognized Vero cells infected with the actual ASF virus (Georgia 2007/1 isolate) but not mock-infected cells (Fig. 12A). Sera from 8 out of 10 swine in the treatment group, but none from the controls, recognized the ASFV-infected cells (Table 4). Sera from 2 animals (swine 89 and swine 91) were most reactive and reacted with the plasma membrane, a virus factory like structure and general cytoplasm. western blot analysis of ASFV-infected Vero cell lysates probed with the post-boost sera also validated the above results (Fig. 12B). This outcome showed that synthetic genes encoding antigens of ASFV (a Risk Group 3 pathogen) that requires BSL3 biocontainment can safely be used at BSL2 level to develop and test immunogenicity and tolerability of prototype ASFV vaccines. These results, however do not directly demonstrate that the ASFV antigen-specific antibodies have functional activity. In case of ASFV, it is generally acceptable in the scientific community, that conventional plaque reduction assay to measure ASFV antibody neutralization activity is technically difficult since low-passage (virulent) ASFV strains show no or a significant delay in plaque formation, and is especially difficult to conduct the assay in primary swine macrophage cells. A highly attenuated ASFV Georgia strain that is adapted to a suitable cell line (e.g., Vero cells), or a genetically modified ASF virus expressing a chromogenic marker gene, for use in testing study samples for virus neutralization activity was not available at the time the study was conducted.

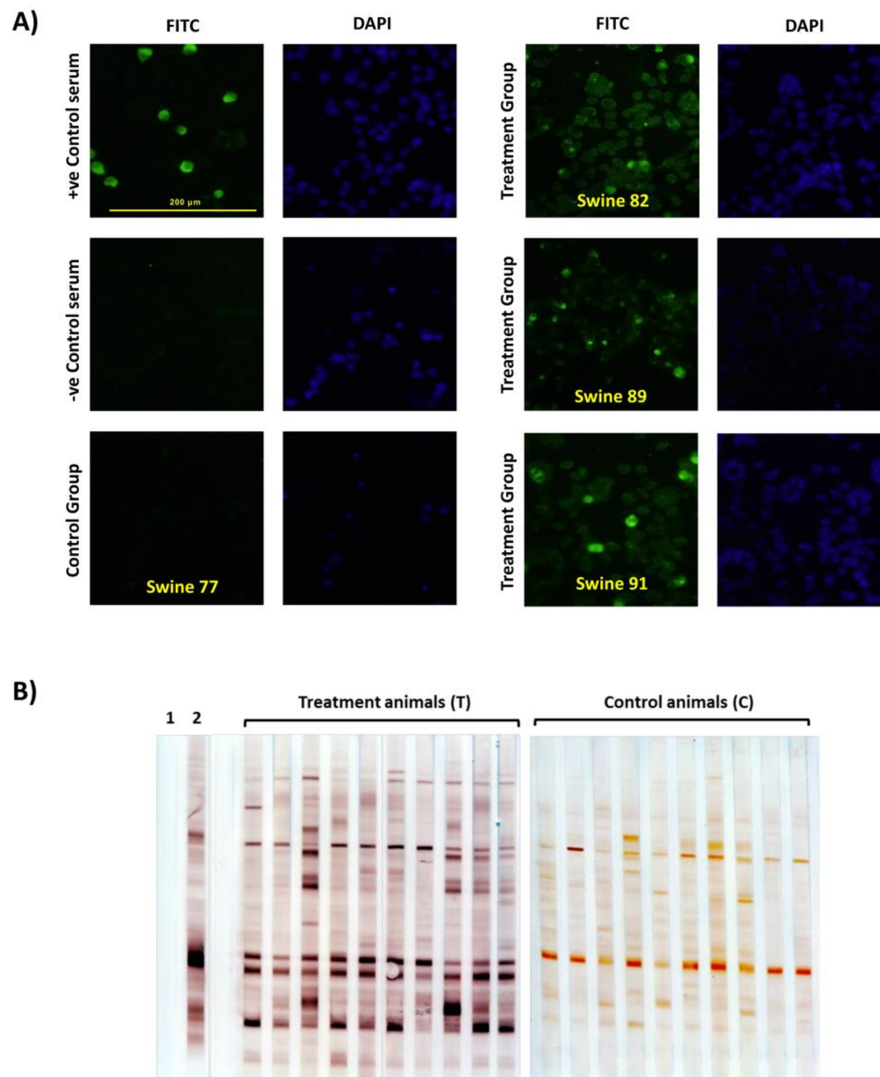

Figure 12. Antibodies primed by the Ad-ASFV cocktail recognized ASF virus. Analysis of sera from two weeks post-boost by Indirect Immunofluorescence Antibody assay (IFA) and western Blot showed that the antibodies primed by the Ad-ASFV cocktail recognized the parental ASFV infected cells and ASFV-derived antigens. Panel A) Vero cells infected with ASFV Georgia 2007/1 probed with sera from treatment and negative control animals. ASFV specific convalescent serum was used as the positive control and normal swine serum served as the negative control. Data for three animals (that gave the strongest reaction) from the treatment group and one animal from the control group is shown. A summary of IFA results for all animals is presented in Table 4; B) Lysates from Vero cells infected with ASFV Georgia 2007/1 isolate were blotted and probed with sera from all animals; Lane: 1) Normal swine serum (negative control); 2) ASFV-specific convalescent serum (positive control). Differences in coloration are due to actual band intensities; darker color is higher concentration of antibody bound to antigen (antigen concentration is constant).

Table 4: IFA Results

Treatment Group: Swine No.	Reactivity		Control Group: Swine No.	Reactivity	
	ASFV-infected Vero cells	Mock-infected Vero cells		ASFV-infected Vero cells	Mock-infected Vero cells
76	+++	-	77	-	-
78	-	-	79	-	-
81	++	-	80	-	-
82	+++	-	84	-	-
83	-	-	85	-	-
89	+++	-	87	-	-
90	+	-	88	-	-
91	++++	-	93	-	-
92	+	-	95	-	-
96	+++	-	99	-	-
ASFV convalescent serum	++++	-	Normal serum	-	-

The number of '+' signs represents the comparison between the intensity of a positive signal from the sera of the animals and that from the ASFV-specific convalescent serum (positive control). '++++': signal as strong as positive control; '+': weakest but positive signal; '-': No signal detected.

3.4.4. Ad5-ASFV cocktail primed IFN-γ-secreting cells

Low frequencies of antigen-specific IFN-γ responses were detected in a few animals by IFN-γ ELISPOT analysis of PBMCs collected one-week post-priming (Fig. 13A).

Specifically, a significant difference ($p<0.05$) between the mean response of the treatment group and negative control group animals was detected only for antigen A151R (Fig. 13A). However, after boosting, strong recall IFN-γ^+ responses were detected in a majority of animals for all the antigens (Fig. 13B). The mean response of the treatment group was significantly higher than the control group for all antigens ($p<0.05$ for antigens B119L, B602L, EP402RΔPRR; and $p<0.01$ for antigens A151R, B438L, and K205R-A104R). The IFN-γ ELISPOT data clearly showed that the homologous booster dose was able to sufficiently amplify the primary response to give strong recall responses against all antigens in a majority of the vaccinees (Fig. 13). The high frequencies of antigen-specific IFN-γ^+ cellular responses induced are promising in light of the results reported from other subunit vaccine studies. Notably, immunization with an ubiquitin tagged chimera of antigens p30, p54, and CD2v using DNA plasmids conferred protection against lethal challenge in some of the vaccinees [34]. In addition, in another study, by the same authors, immunizing animals with BacMams expressing the same antigen chimera (p30, p54, and CD2V) conferred partial protection upon a sub-lethal challenge, and a direct correlation between protection and ASFV-specific IFN-γ^+ response was observed [54]. Interestingly, in both studies the IFN-γ response against the extracellular domain of EP402R was negligible. We have shown that the adenovirus-vectored EP402RΔPRR induced strong antigen-specific IFN-γ^+ responses in 70% of the vaccinees post-boost.

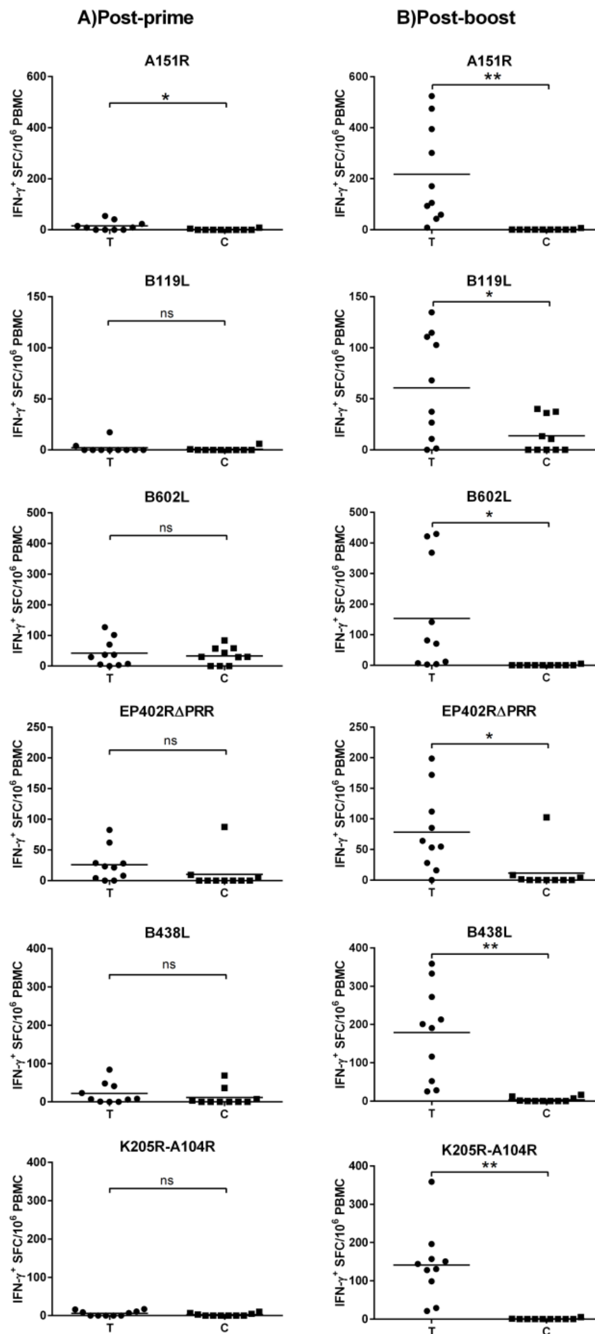

Figure 13. Antigen-specific IFN-γ response post-prime and post-boost.
The frequency of antigen-specific IFN-γ-secreting cells in PBMCs collected post-prime and post-boost was evaluated by IFN-γ ELISPOT assays. Data for A) One week post-prime; and B) three weeks post-boost is shown. The response is presented as IFN-γ Spot Forming Cells (SFC)/106 PBMCs. The mean response of the treatment group (T) was compared to the mean response of the control group (C) using an unpaired t-test with Welch's correction. ** represents $p<0.01$, * represents $p<0.05$ and 'ns' stands for a non-significant difference.

3.4.5. Ad5-ASFV cocktail was well tolerated

Following inoculation of the Ad-ASFV cocktail, three swine in the treatment group were observed to be depressed and one had mild fever on the first day. However, all the swine were normal on all subsequent days. After boosting, one pig in the treatment group was observed to be depressed and had fever that required treatment with Banamine. All the swine in the negative control group were normal post-priming and post-boosting. Overall, the Ad-ASFV cocktail was well tolerated with no adverse effects.

The overall outcome is evidence that a vaccine formulated using a cocktail of replication-incompetent adenovirus expressing protective ASFV antigens is likely to be well tolerated by commercial swine at doses as high as 10^{11} IFU used in a homologous prime-boost immunization regimen. This scenario is anticipated since effective ASFV subunit vaccines will likely require delivery of multiple antigens given that studies conducted so far have shown that a combination of one or a few antigens does not confer complete protection.

3.5. Conclusions

The African Swine Fever Virus (ASFV) continues to pose a high risk to the swine industry and it is still causing economic losses in endemic areas. Since there is no vaccine or treatment available yet, it is important to identify viral proteins that can elicit strong immune responses and therefore be considered viable candidates for subunit vaccine development. We have optimized an adenovirus-vector based ASFV antigen delivery system which allows for immunization of swine with multiple ASFV antigens

and the subsequent evaluation of their immunogenicity. The robust antigen-specific IFN-γ^+ responses induced by the adenovirus vector against all the antigens tested in this study as well as other ASFV antigens evaluated in our previous study make it a promising delivery platform for testing vaccine candidates for protection against ASFV [57]. An interesting observation is the relatively low B438L-specific humoral responses in contrast to the strong B438L-specific IFN-γ^+ responses induced (Fig. 11 and Fig. 13). The inability of this antigen to induce strong antibody responses was corroborated by the fact that the ASFV-specific convalescent serum also had a comparatively low B438L-specific titer (1:4,000). Thus, even though B438L does not induce a high antibody response, it still is an attractive candidate for future efficacy studies based on its ability to induce strong IFN-γ^+ cell responses. This study also showed that an adenovirus-based ASFV vaccine can be used successfully for homologous prime-boost vaccination. If this approach is shown to confer protection, it will cut costs incurred by use of a heterologous prime-boost immunization strategy. Thus, these findings support use of the replication-incompetent adenovirus as a vector for the development of a commercial vaccine for protection of pigs against African swine fever virus. The next logical step is to test whether these multiple ASFV antigens delivered in a cocktail format can confer protection in a challenge study.

CHAPTER IV

PRIMING CROSS-PROTECTIVE BOVINE VIRAL DIARRHEA VIRUS-SPECIFIC IMMUNITY USING LIVE-VECTORED MOSAIC ANTIGENS[†]

4.1. Overview

Bovine viral diarrhea virus (BVDV) plays a key role in bovine respiratory disease complex, which can lead to pneumonia, diarrhea and death of calves. Current vaccines are not very effective due, in part, to immunosuppressive traits and failure to induce broad protection. There are diverse BVDV strains and thus, current vaccines contain representative genotype 1 and 2 viruses (BVDV-1 & 2) to broaden coverage. BVDV modified live virus (MLV) vaccines are superior to killed virus vaccines, but they are susceptible to neutralization and complement-mediated destruction triggered by passively acquired antibodies, thus limiting their efficacy. We generated three novel mosaic polypeptide chimeras, designated $N^{pro}E2^{123}$; NS^{231}; and NS^{232}, which incorporate protective determinants that are highly conserved among BVDV-1a, 1b, and BVDV-2 genotypes. In addition, strain-specific protective antigens from disparate BVDV strains were included to broaden coverage. We confirmed that adenovirus constructs expressing these antigens were strongly recognized by monoclonal antibodies, polyclonal sera, and

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[†] Reprinted from "Lokhandwala S, Fang X, Waghela SD, Bray J, Njongmeta LM, Herring A, et al. Priming Cross-Protective Bovine Viral Diarrhea Virus-Specific Immunity Using Live-Vectored Mosaic Antigens. PLoS ONE 2017;12(1): e0170425. doi:10.1371/journal.pone.0170425." Copyright 2017 Shehnaz Lokhandwala, Xin Fang, Suryakant D. Waghela, Jocelyn Bray, Leo M. Njongmeta, Andy Herring, Karim W. Abdelsalam, Christopher Chase and Waithaka Mwangi.

IFN-γ-secreting T cells generated against diverse BVDV strains. In a proof-of-concept efficacy study, the multi-antigen proto-type vaccine induced higher, but not significantly different, IFN-γ spot forming cells and T cell proliferation compared to a commercial MLV vaccine. In regards to the humoral response, the prototype vaccine induced higher BVDV-1 specific neutralizing antibody titers, whereas the MLV vaccine induced higher BVDV-2 specific neutralizing antibody titers. Following BVDV type 2a (1373) challenge, calves immunized with the proto-type or the MLV vaccine had lower clinical scores compared to naïve controls. These results support the hypothesis that a broadly protective subunit vaccine can be generated using mosaic polypeptides that incorporate rationally selected and validated protective determinants from diverse BVDV strains. Furthermore, regarding biosafety of using a live vector in cattle, we also showed that recombinant human adenovirus-5 was cleared within one week following intradermal inoculation.

4.2. Introduction

Bovine viral diarrhea virus (BVDV), an infectious pathogen that is prevalent in cattle herds globally, is a key agent responsible for causing Bovine Respiratory Disease Complex (BRDC) [72]. Infection with BVDV can cause severe diarrhea, respiratory disease, immunosuppression, abortion, congenital malformations, and birth of persistently infected (PI) calves, which play a major role in virus transmission in herds [73]. Immunosuppression caused by acute infection of unprotected calves allows secondary infections to establish and cause pneumonia or enteritis [74]. The secondary infections are responsible for high rates of morbidity and mortality, and it is estimated that the U.S.

livestock industry loses >$1billion annually due to BRDC [75, 76].

This virus is classified as a member of the genus Pestivirus within the family *Flaviviridae* [77]. Two BVDV genotypes (type 1 and 2) are recognized according to serological and genetic relatedness [78]. The BVDV isolates circulating in the world are heterogeneous: BVDV genotype 1 (BVDV-1) is subdivided into a minimum of 12 sub-genotypes (BVDV1a, b, c….l), whereas BVDV genotype 2 (BVDV-2) is classified into 4 subtypes, 2a-2d [79, 80]. The BVDV can also be divided into cytopathic and non-cytopathic biotypes (cpBVDV and ncpBVDV, respectively), based on their lytic effects on infected cells. The BVDV isolates cause a wide range of disease manifestations, which include sub-clinical and persistent infections, fetal infections, and host immunosuppression [81]. Infected cattle begin to shed the virus into the environment for about ten continuous days starting as early as four days after subclinical infection, whereas PI animals shed the virus for their entire lifetime [82, 83]. The prevalence of PI animals in the United States is estimated at 1.7% of the cattle population, and these animals are considered to be the primary source of infection of susceptible animals [84].

BVDV infection in cattle induces high titers of neutralizing antibodies that prevent reinfections especially with the same genotype/sub-genotype [85, 86]. Some studies have demonstrated prevention of clinical signs, but not viral shedding, in cattle upon challenge with BVDV-2 following immunization with BVDV-1 [87, 88]. Failure of vaccination has been attributed to infection with variant genotype(s) as well as development of antigenically distinct viruses in exposed animals [89, 90]. Individual PI cattle may also be a source of genetic variants that amplify following infection of

susceptible cattle [91, 92]. However, in the absence of neutralizing antibodies, mutations occur faster and more frequently in BVDV following infection of pregnant animals [93] . Many of the virus genome mutations result in amino acid changes in E2 glycoprotein, a key target of the neutralizing antibodies [92, 94]. The E2 glycoprotein is highly immunogenic and at least nine epitopes have been mapped within three antigenic domains [95-99]. One of these antigenic determinants is immunodominant in BVDV-1 and there are three in BVDV-2 that induce neutralizing antibodies in animals [96]. However, it is also reported that viremia can occur despite the presence of neutralizing antibodies in infected animals, and some animals can be protected against BVDV infection in the absence of E2-specific neutralizing antibodies, suggesting a role for neutralizing epitopes from other antigens and/or T cells in protection [100, 101]. Clearance of BVDV infections has also been associated with strain-specific MHC-restricted $CD4^+$ and $CD8^+$ T cell responses [86, 102]. Cell mediated response to infection is initially provided by E2 and NS2-3 antigen-specific helper $CD4^+$ T cells [103-105].

Despite availability of vaccines, BVDV prevalence has not markedly reduced due, in part, to failure of the vaccines to confer broad protection [106, 107]. Currently, both killed and modified live virus (MLV) vaccines are commercially available [108]. The killed vaccine elicits primarily a humoral response with minimal cell mediated response, whereas MLV vaccines are better at inducing $CD4^+$ and $CD8^+$ T cells responses in addition to antibody responses [109]. Since the presence of BVDV-specific maternal antibodies interferes with efficacy of BVDV vaccines, especially MLV, immunization is

usually delayed until most of the maternal BVDV antibodies have waned [90, 110]. However, BVDV-specific antibodies in each animal decline at different rates and thus, antibody titers in some calves fall below protective levels much earlier than expected, and in the presence of PI calves in the herd, there is a high risk of infection [111]. MLV vaccines are currently the most effective, but genotype-specific vaccines are not effective at conferring cross-protection and thus, protection against BVDV-1 and 2 requires a vaccine formulation that contains a representative of genotype type 1 and 2 viruses. The MLV vaccines are not considered to be safe since the attenuated virus can revert to wild type virus, cause in-utero infections and mucosal disease, carry the risk of vaccine contamination with adventitious viruses, and are immunosuppressive [112, 113]. Furthermore, MLV strains may cause ovarian lesions leading to infertility in cows [114]. Both killed and MLV vaccine virus are traditionally grown in MDBK cells and recent findings show that calves fed colostrum from some dams vaccinated with killed BVDV vaccine formulated with adjuvant have a high incidence of a syndrome characterized by spontaneous bleeding, severe anemia with heavy bone marrow damage. There is evidence to show that the damage is due to maternal alloantibodies induced by the vaccines against bovine cell antigens, including MHC-I molecules, and the syndrome has been named bovine neonatal pancytopenia [115-117].

Given the limitations of the current vaccines, there is a need to develop improved vaccines for safe, robust, and broad protection against diverse BVDV genotypes. Empirical selection and validation of protective immune targets that are conserved among diverse BVDV strains can be used to generate novel mosaic antigens for

development of a contemporary vaccine. Similar strategies have been used to develop broadly protective vaccines to overcome a wide Influenza and HIV-1 genetic diversity [118-120]. The BVDV envelop (E2) and non-structural (NS2-3) antigens are immunodominant, and neutralizing antibody as well as T cell responses directed against these antigens can confer protection [121, 122]. Importantly, evaluation of BVDV-specific immune responses following resolution of acute infection has revealed that the E2, NS2-3, and N-terminal protease fragment (N^{pro}) antigens contain $CD4^+$ T-cell epitopes [102]. In addition, MHC *DR*-restricted T cell epitopes have been identified from conserved regions of E2 and NS2-3 [102, 104, 105, 122, 123].

In this study, we generated a prototype vaccine composed of recombinant adenoviruses expressing three novel mosaic polypeptide chimeras, designated $N^{pro}E2^{123}$; NS^{231}; and NS^{232}. These antigens incorporated neutralizing epitopes, defined and predicted IFN-γ-inducing $CD4^+$ T cell as well as cytotoxic T lymphocyte determinants that are highly conserved among BVDV-1a, b, and BVDV-2 genotypes [95, 96, 102, 122, 123]. In addition, strain-specific protective antigens from disparate BVDV strains whose genome sequences are available were included to broaden coverage. We compared the immunogenicity and protective efficacy of this prototype adenovirus-vectored vaccine to a commercial MLV vaccine in calves.

Adenovirus-vectored subunit vaccines are undergoing clinical trials in readiness for deployment [124, 125]; there is concern that persistence of the construct in host tissues may increase chances of generating replication-competent progenies if recombination with closely related viruses occurs. Thus, we set out to determine replication-incompetent

recombinant human adenovirus-5 persistence at the skin injection site, the draining lymph node, and the spleen of calves following intradermal inoculation.

4.3. Materials and Methods

4.3.1. Design of genes encoding novel mosaic BVDV antigens

Published reports on protective BVDV antibody and T-cell epitopes, sequenced genomes, and bioinformatics tools were used to design novel mosaic polypeptides, which incorporated consensus and strain-specific key antigenic determinants from BVDV-1 and 2 strains [95, 96, 102, 118, 119, 123, 126, 127]. Analysis of sequenced genomes showed that the N^{pro} antigen is highly conserved, but the E2 and the NS2-3 antigens have conserved and variable domains. Amino acid sequences of the E2 proteins from currently defined BVDV-1 or BVDV-2 genotypes were aligned and three novel mosaic E2 polypeptides, designated $E2^{1,2,3}$ ($E2^{1-3}$), each containing consensus E2 determinants plus defined strain-specific neutralization epitopes were selected, and wherever there was no consensus at a specific amino acid position for the BVDV-1 genotypes, amino acid from the BVDV-1b sequence was selected since this is the most prevalent sub-type in North America. The $E2^{1-3}$ polypeptide sequence was fused in-frame to the C-termini of the N^{pro} polypeptide and the resultant chimeric polypeptide, designated $N^{pro}E2^{1-3}$, was used to generate a codon-optimized synthetic gene, designated n_{pro}-$e2_{1a}$-$e2_{1b}$-$e2_{2}$ ($n_{pro}e2_{1-3}$,), that also included *flag* tag sequence at the 3' end. Two additional mosaic polypeptides that incorporated consensus amino acids from diverse NS2-3 proteins, designated NS^{2-31} (from BVDV-1 genotypes) and NS^{2-32} (from BVDV-2

genotypes) were similarly designed and used to generate two synthetic gene sequences, designated $ns2\text{-}3_{1}$, and $ns2\text{-}3_{2}$, respectively, that also included the *flag* tag sequence at the 3' end. Synthetic genes were codon-optimized, custom-made, cloned into pUC57 vector, and sequence-verified by GenScript Inc., NJ, USA.

4.3.2 Generation of recombinant adenovirus plasmid expression constructs

The three synthetic genes ($n_{pro}e2_{1\text{-}3}$, $ns2\text{-}3_{1}$, and $ns2\text{-}3_{2}$) were subcloned into pDonR vector using the Gateway Technology (Life Technologies, NY, USA) to generate shuttle constructs. Positive clones were identified by PCR screening of plasmid DNA in bacteria colonies using vector-specific forward primer and gene-specific reverse primer. Authentic entry constructs, designated pDonRN^{pro}E2$^{1\text{-}3}$, pDonRNS$^{2\text{-}31}$, and pDonRNS$^{2\text{-}32}$, respectively were selected by DNA sequencing. The selected constructs were used to transfer each gene into pAd adenovirus plasmid backbone by homologous recombination (Gateway Technology, Life Technologies, NY, USA) and recombinant constructs were identified by PCR screening as above. Authentic recombinant plasmid constructs, designated pAdN^{pro}E2$^{1\text{-}3}$, pAdNS$^{2\text{-}31}$, and pAdNS$^{2\text{-}32}$, respectively were selected after DNA sequencing.

4.3.3 Protein expression by plasmid constructs and generation of recombinant adenoviruses

Protein expression was evaluated by immunocytometric analysis of human embryonic kidney (HEK)-293A cells grown in 12-well tissue culture plates and transfected with 1 μg of the selected clones of the pAd DNA constructs, and then probed with anti-FLAG

mAb at 48 hr. post-transfection as previously described [128]. Five clones of each pAd construct were selected based on efficiency of protein expression as judged by the immunocytometric analysis, and 2 μg DNA of each construct was digested with Pac-I restriction enzyme. The digested DNA was transfected into HEK-293A cells grown in 6-well plates to generate recombinant adenoviruses that were designated $AdN^{pro}E2^{1-3}$, $AdNS^{2-31}$, and $AdNS^{2-32}$, respectively. In addition, adenovirus expressing luciferase (AdLuc) was generated to serve as a negative control. Protein expression by the $AdN^{pro}E2^{1-3}$, $AdNS^{2-31}$, and $AdNS^{2-32}$ adenoviruses was tested and validated by immunocytometric analysis of infected HEK-293A cells as above, whereas fluorescence was used to confirm luciferase expression.

One clone of each recombinant adenovirus was selected for amplification based on protein expression. The bulk viruses were tested for protein expression as above and following titer determination, replication competence of the recombinant adenoviruses was determined by immunocytometric analysis of HEK-293A (which supports adenovirus replication) and MDBK cells (susceptible to adenovirus infection, but do not support replication of replication-incompetent adenovirus) infected overnight with one MOI of each virus construct and then probed with an in-house generated rabbit anti-adenovirus polyclonal IgG (1:500 dilution) followed by an alkaline-phosphatase-conjugated anti-Rabbit IgG (1:1000) (Jackson ImmunoResearch, Cat #711-055-152) secondary antibody and Fast Red TR–Naphthol AS-MX as the substrate (Sigma, F4523) to evaluate infectivity.

4.3.4. Validation of the mosaic antigens using BVDV-specific antibodies and T cells

Authenticity of the mosaic $N^{pro}E2^{1-3}$, NS^{2-31}, and NS^{2-32} antigens was confirmed by immunocytometric analysis using E2-specific neutralizing monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) generated against diverse BVDV strains. Briefly, HEK-293A cells grown in 12-well plates were infected for 48 hr. with $AdN^{pro}E2^{1-3}$, $AdNS^{2-31}$, $AdNS^{2-32}$, or AdLuc and probed with anti-BVDV E2 mAbs 348 and 26A (VMRD, Inc., Pullman, WA), goat anti-BVDV polyclonal sera (VMRD), and bovine anti-BVDV hyperimmune sera from steers immunized and challenged with multiple BVDV-1 and 2 strains [129]. Antigen authenticity was further confirmed by ELISA and western blot analysis using the above mentioned antibodies.

The authenticity of the T-cell epitopes in the mosaic antigens was validated by proliferation assays using peripheral blood mononuclear cells (PBMCs) isolated from the BVDV-immunized steers [129]. Recombinant $N^{pro}E2^{1-3}$, NS^{2-31} and NS^{2-32} antigens were expressed by using recombinant baculoviruses in High Five cells (Thermo Fisher Scientific) generated using the Bac-to-Bac HBM TOPO Secreted Expression System (Thermo Fisher Scientific) as per manufacturer's instructions and validated as above. These antigens were then affinity purified using Anti-FLAG M2 Affinity Gel (Sigma) and used at 5µg/ml to conduct ^{3}H-Thymidine incorporation assays to quantify antigen-specific T cell responses as previously described [128]. Heat killed BVDV-1b (CA0401186a) and BVDV-2 (A125) at 5µg/ml served as positive control antigens, whereas medium alone was the negative control. The outcome of the cell proliferation was presented as counts per minute (cpm).

4.3.5. Immunization and challenge of calves

Three groups (A, B, and C), of age-matched BVDV sero-negative and virus-free weaned Holstein calves (n=5) were identified as previously described [130] and used in this study as shown in Table 5. Each calf in group A was inoculated subcutaneously (SQ) with a cocktail, designated AdBVDV, containing the AdN^{pro}E2$^{1-3}$, AdNS$^{2-31}$, and AdNS$^{2-32}$ recombinant adenoviruses (5 x 10^{10} $TCID_{50}$/construct) formulated in adjuvant E (BenchMark-Vaxliant). Each calf in group B was similarly inoculated, but with a commercial MLV BVDV-1 and 2 vaccine (Bovi-Shield Gold™, Zoetis Inc., Kalamazoo, MI), whereas each calf in group C was inoculated with the recombinant AdLuc. Seventy-nine days post-priming, the AdBVDV vaccinees and the negative controls received inoculation of the respective priming immunogen and dose as above. One hundred and forty-nine days post-boosting, all the calves were challenged by intranasal administration of 2 x 10^6 $TCID_{50}$ of BVDV-1373 using a human nasal atomizer. (http://www.teleflexarcatalog.com/anesthesia-respiratory/airway/categories/552).

Table 5: Calf Immunization Protocol

Calf ID	Vaccine-Prime	Vaccine-Boost
4	AdBVDV	AdBVDV
12	AdBVDV	AdBVDV
13	AdBVDV	AdBVDV
22	AdBVDV	AdBVDV
23	AdBVDV	AdBVDV
3	BVDV MLV	-
14	BVDV MLV	-
19	BVDV MLV	-
24	BVDV MLV	-
27	BVDV MLV	-
10	Ad Luciferase	Ad Luciferase
18	Ad Luciferase	Ad Luciferase
25	Ad Luciferase	Ad Luciferase
28	Ad Luciferase	Ad Luciferase
29	Ad Luciferase	Ad Luciferase

Calves in the treatment group were inoculated subcutaneously with a cocktail of the $AdN^{pro}E2^{1\text{-}3}$, $AdNS^{2\text{-}31}$, and $AdNS^{2\text{-}32}$ recombinant adenoviruses (AdBVDV) expressing the BVDV antigens, whereas calves in the positive control group received a commercial BVDV MLV vaccine. Calves in the negative control group were inoculated with the recombinant AdLuc. The calves were boosted with the respective priming inoculum and dose.

4.3.6. Cellular and humoral immune responses

Two weeks post-priming and bi-weekly thereafter, PBMCs were isolated to evaluate and quantify proliferation of BVDV-specific T-cell responses as previously described [128]. The PBMCs (2.5×10^5 cells/well) were cultured for 72 hr. at 37°C in triplicate wells of

round-bottom 96-well plates in a total volume of 100 µl of complete RPMI-1640 (cRPMI) medium containing 2.5 µg/ml defined BVDV $CD4^+$ T cell epitope peptides [103]. The positive control was cRPMI medium containing 1.3 µg/ml concanavalin A (ConA), whereas medium alone served as a negative control. Cells were labeled with 0.25 µCi of ^{3}H-thymidine for 6 hr., harvested using a semi-automatic cell harvester (Tomtec Life Sciences, Hamden, CT), and the incorporated ^{3}H-thymidine was counted with a Micro-Beta liquid scintillation counter (Perkin Elmer, Waltham, MA). The incorporation of ^{3}H-thymidine by the proliferating PBMCs was presented as mean counts per minute (cpm) of triplicate wells (±1 SD).

The PBMCs were also used to quantify BVDV-specific IFN-γ-secreting cells by EliSpot assay as previously described [128]. The PBMCs (2.5×10^5 cells/well) were seeded into triplicate wells of MultiScreen-HA plates (EMD Millipore, Billerica, MA) in a final volume of 100 µl cRPMI medium containing 2.5 µg/ml BVDV $CD4^+$ T cell epitope peptides. The positive control was 1.3 µg/ml ConA, whereas medium alone served as a negative control. The plates were incubated for 36 hr. at 37°C, washed, developed, and dried overnight as previously described [128]. Following quantification of the spots using an EliSpot reader (AID, Diagnostika GmbH, Germany), the mean number of spots in the negative control wells was subtracted from the mean number of spots in the cognate test wells to determine the mean number of BVDV-specific IFN-γ-secreting PBMCs and the results were presented as the mean number of spot-forming cells/10^6 PBMCs. Sera from blood collected two weeks post-boost and one week pre-challenge were tested to determine BVDV-1 and BVDV-2 neutralizing antibody titers

using BVDV-1 (Singer, NADL, BJ, TGAC, CA0401186a) and BVDV-2 (890, 1373, A125) strains as previously described [131, 132]. Briefly, serum was heat inactivated at 56°C for 30 min, and 25 µl of each serum was serially diluted (2-fold) in cell culture media without FBS in 96-well microtiter plates. Stock BVDV virus containing 100 $TCID_{50}$/25µl was added to each test well. In each test, a positive control serum was also included. This serum/virus mixture was incubated for 1 hr., at 37°C, MDBK cells added, and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 hr. The cells were observed daily for CPE for cytopathic strains, whereas the non-cytopathic strains were detected by Immuno-peroxidase assay [133]. The results were presented as serum neutralization titers (SNT) [134].

4.3.7. Clinical parameters: viremia/WBC-platelet counts

Calves were observed daily pre-immunization, post-immunization and post-challenge for coughing, nasal discharge and diarrhea. Rectal temperature post-challenge was taken daily [135]. To determine virus titers post-challenge, blood was collected in vacutainer tubes (containing Sodium-EDTA) by jugular venipuncture, freeze-thawed to lyse cells, centrifuged and supernatants were used to determine BVDV titers as previously described [136]. Whole blood was used to determine CBC using Cell-Dyn 3700 analyzer (Abbott Diagnostics, Lake Forest, IL 60045, USA) with veterinary package as bovines for automated counts (WBC, RBC, Hgb, MCV, PLT). Thin blood smears were stained with Giemsa for differential white blood cell counts [137]. Platelet count verification, WBC count verification, RBC and WBC morphology was evaluated microscopically.

4.3.8. Persistence of recombinant adenovirus in cattle

Presence of recombinant replication-incompetent adenovirus in cattle was tracked for three weeks post-inoculation by rescue of virus from tissue biopsies taken from the intradermal inoculation site. Briefly, recombinant adenovirus (5 x 10^9 ifu) was inoculated (I.D) into nine marked sites on the neck of four steers. One skin biopsy was taken from each site using a 4mm Biopsy Punch (American Screening, Shreveport, LA) on days 1-7, 14, and 21. In addition, skin biopsies were concurrently collected from the flank region of each steer to serve as negative controls. The steers were euthanized three weeks post-inoculation and samples of draining lymph node and spleen were collected. The fresh tissue samples collected were snap frozen in liquid nitrogen, ground and then resuspended in 1 ml DMEM (Invitrogen). Following centrifugation, supernatants were filtered through 0.22 µm pore membrane, and 0.5 ml was added to one well of HEK-293A cells (which supports adenovirus replication) grown in 12-well plates. Supernatant from HEK-293A cells infected overnight with the recombinant adenovirus, and subjected to the same treatment as above was used as a positive control. Three days post-infection, presence of adenovirus in the HEK-293A cells was evaluated by immunocytometric analysis using the rabbit anti-adenovirus polyclonal IgGs as above. Medium from the above HEK-293A cells was used to infect fresh cells, and seven days later the above process was repeated to confirm presence or absence of adenovirus.

4.3.9. Statistical analysis

Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test was used to analyze the significance of the differences in BVDV-specific immune responses and disease indices between the treatments (groups A and B) and the negative control (group C) using GraphPad Prism 6 (Version 6.07, GraphPad Software, Inc. La Jolla, USA). Statistical significance was considered when $P < 0.05$.

4.3.10. Ethics statement

The study was conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals as specified in the Health Research and Extension Act of 1985 (Public Law 99-158) or in accordance with the U.S Department of Agriculture policies as required by the Animal Welfare Act of 1966 (7.USC.2131 et seq) as amended in 1970, 1976, and 1985. The research protocol: AUP21010-65 was reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee to ensure compliance with PHS standards. All animal care facilities are inspected twice per year. The facilities and procedures for maintenance and care of animals are accredited by the American Association for Accreditation of Laboratory Animal Care. Efforts were made to minimize suffering, and at the completion of the study, the calves were euthanized with an overdose of sodium pentobarbital. This method is approved by the Panel on Euthanasia of the American Veterinary Medical Association

4.4. Results and Discussion

4.4.1. Expression constructs encoding novel mosaic BVDV antigens

Three synthetic genes (designated $n_{pro}e2_{1\text{-}3}$, $ns2\text{-}3_1$, and $ns2\text{-}3_2$) encoding novel BVDV mosaic antigens were designed as depicted in Fig. 14A. The $n_{pro}e2_{1\text{-}3}$ chimeric gene encodes the N-terminal protease fragment (N^{pro}), a consensus BVDV-1a envelope glycoprotein E2 mosaic gene ($e2_1$), a consensus BVDV-1b envelope glycoprotein E2 mosaic gene ($e2_2$), and a consensus BVDV-2 envelope glycoprotein E2 mosaic gene ($e2_3$) fused in-frame to *flag*-tag. The $ns2\text{-}3_1$ DNA fragment encodes a consensus BVDV-1 Nonstructural protein 2-3 fused in-frame to *flag*-tag, whereas the $ns2\text{-}3_2$ DNA fragment encodes a consensus BVDV-2 Nonstructural protein 2-3 fused in-frame to *flag*-tag (Fig. 14A).

4.4.2 Expression of the mosaic BVDV antigens

Immunocytometric analysis of HEK-293A cells transfected with the $pAdN^{pro}E2^{1\text{-}3}$, $pAdNS^{2\text{-}31}$, or $pAdNS^{2\text{-}32}$ constructs probed with anti-FLAG mAb confirmed that each construct expressed the encoded antigen (Fig. 14B). Similarly, immunocytometric analysis of HEK-293A cells infected with the $AdN^{pro}E2^{1\text{-}3}$, $AdNS^{2\text{-}31}$, or $AdNS^{2\text{-}32}$ recombinant adenoviruses probed with anti-FLAG mAb confirmed protein expression (Fig. 14C). Analysis of replication competency confirmed that the $AdN^{pro}E2^{1\text{-}3}$, $AdNS^{2\text{-}31}$, and $AdNS^{2\text{-}32}$ recombinant adenoviruses were replication-incompetent.

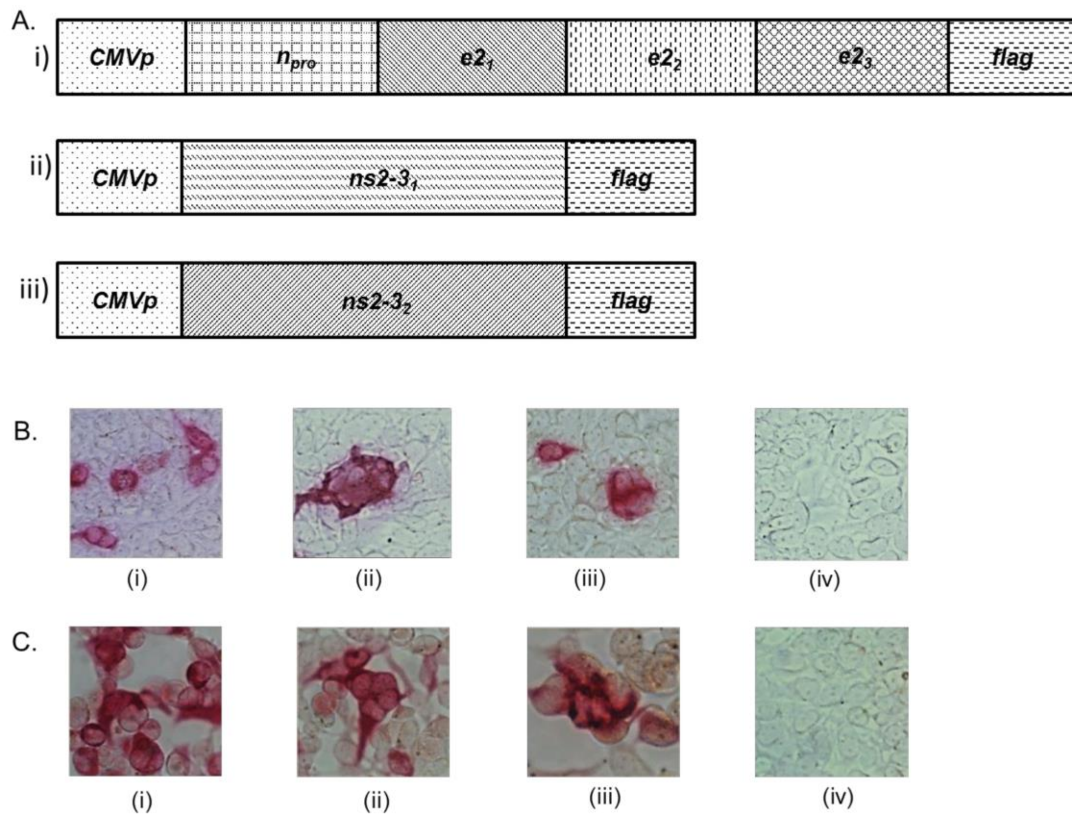

Figure 14. BVDV antigen expression constructs.

A) Schematic diagram of expression cassettes encoding BVDV mosaic antigens: i) Composition of the *nproe2*$_{1\text{-}3}$, chimeric gene: *npro* encodes Npro antigen; *e2*$_1$ encodes a mosaic BVDV-1a E2 envelope glycoprotein; *e2*$_2$ encodes a mosaic BVDV-1b E2 envelope glycoprotein; whereas *e2*$_3$ encodes a mosaic BVDV-2 E2 envelope glycoprotein. ii) *ns*$_{2\text{-}31}$ encodes a mosaic BVDV-1 nonstructural protein 2-3. iii) *ns*$_{2\text{-}33}$ encodes a mosaic BVDV-2 nonstructural protein 2-3. A gene (*flag*) encoding the FLAG tag was fused in-frame at the 3' end of each chimeric gene for tracking protein expression and transcription was under the direction of the CMV promoter (CMVp). The genes were cloned into adenovirus backbone plasmid vector and the resultant constructs were designated pAdNproE21-3, pAdNS2-31, and pAdNS2-32, respectively.

B) Protein expression by recombinant plasmid constructs: The plasmid DNA constructs encoding the three genes described in (A) above were transfected into HEK-293A cell monolayers and protein expression was evaluated by immunocytometric analysis using anti-FLAG M2-AP Conjugate as follows: HEK-293A cells monolayers were transfected with the following constructs: i) pAdNproE21-3; ii) pAdNS2-31; iii) pAdNS2-32; and iv) pAd vector (negative control).

C) Protein expression by recombinant adenovirus constructs: HEK-293A cells monolayers were infected with the following recombinant adenovirus: i) AdNproE21-3; ii) AdNS2-31; iii) AdNS2-32; and iv) Ad-Luciferase. Protein expression was evaluated by immunocytometric analysis as above.

4.4.3. Novel mosaic BVDV antigens are recognized by multiple BVDV-specific antibodies

Authenticity of the mosaic antigens ($N^{pro}E2^{1-3}$, NS^{2-31}, and NS^{2-32}) expressed by the recombinant adenoviruses was confirmed by immunocytometric analysis of infected HEK-293A cells probed with BVDV neutralizing monoclonal antibodies and polyclonal sera raised against diverse BVDV strains (Fig. 15). Anti-BVDV polyclonal sera from immunized goat and cattle reacted with all three recombinant antigens ($N^{pro}E2^{1-3}$, NS^{2-31}, and NS^{2-32}), whereas monoclonal antibodies 26A and 348, specific for the glycoprotein E2, reacted with $N^{pro}E2^{1-3}$ antigen only (Fig. 15A). The outcome confirmed that neutralization epitopes in the mosaic $N^{pro}E2^{1-3}$ antigen were correctly expressed, and that the NS^{2-31} and NS^{2-32} mosaic antigens were specifically recognized by anti-BVDV polyclonal sera. Thus, these antigens were expected to induce authentic BVDV-specific immune responses in cattle. This expected outcome was consistent with previous demonstration that multicomponent mosaic antigens generated using this strategy elicit broadly protective pathogen-specific immune responses [126, 138, 139].

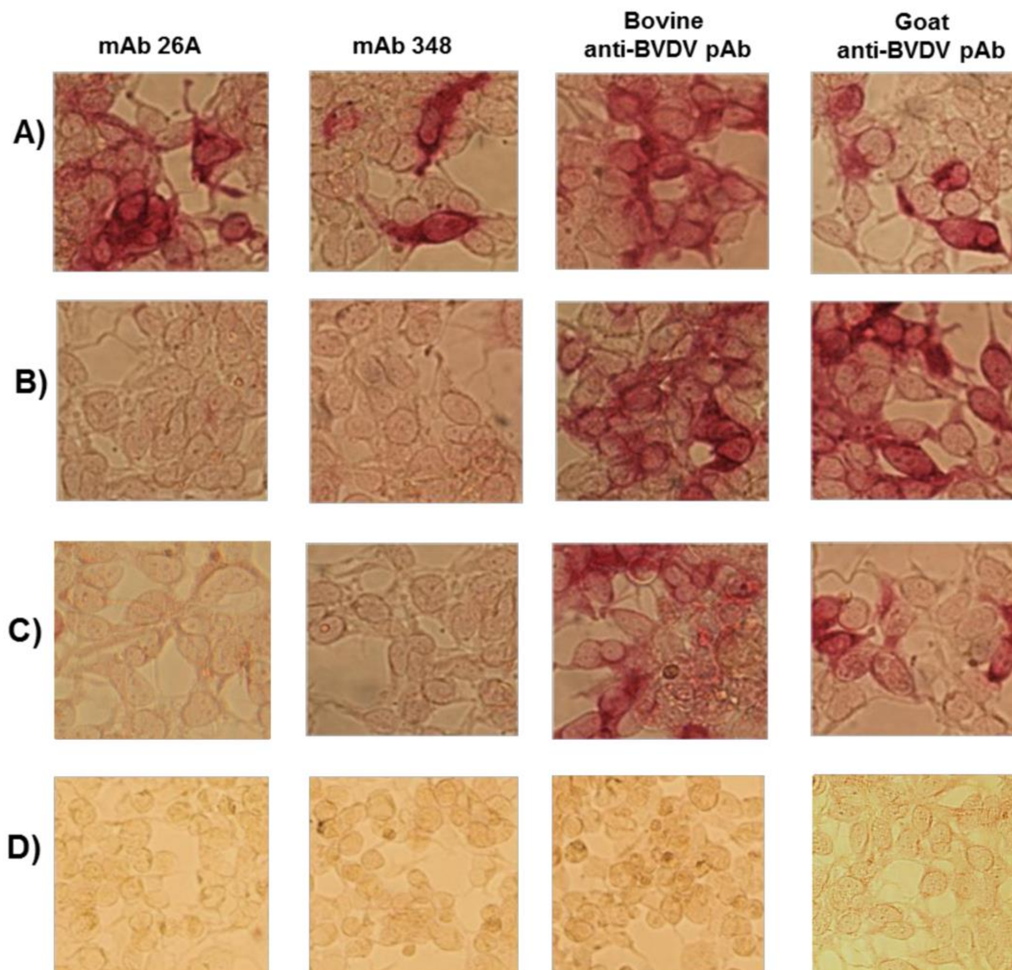

Figure 15. Validation of B-cell epitopes in the mosaic BVDV antigens.
Authenticity of the adenovirus-expressed novel BVDV mosaic antigens was confirmed by immunocytometric analysis using E2-specific neutralizing monoclonal antibodies 26A and 348 (both neutralize BVDV-1 & 2); bovine anti-BVDV hyper-immune serum (generated by immunizing steers multiple times with BVDV-1 & 2 vaccines followed by boosting with killed diverse BVDV-1 & 2 strains and then challenged with wild type BVDV-1 & 2 strains (The sera have high BVDV-1 & 2 neutralizing titers [129]); and goat anti-BVDV polyclonal serum generated against multiple wild-type BVDV-1 & 2 strains. A) HEK-293A cells expressing NproE21-3; B) HEK-293A cells expressing NS2-31; C) HEK-293A cells expressing NS2-32; and D) HEK-293A cells expressing luciferase.

4.4.4. Novel mosaic BVDV antigens are recognized by BVDV-specific T lymphocytes

The $N^{pro}E2^{1\text{-}3}$, $NS^{2\text{-}31}$, and $NS^{2\text{-}32}$ antigens stimulated robust proliferation of PBMCs from BVDV-immunized steers (Fig. 16). The recall responses stimulated by the mosaic antigens were significantly ($P<0.01$) higher than the responses elicited by whole killed BVDV-1b or BVDV-2, suggesting that the mosaic antigens are likely to prime and amplify robust antigen-specific immune responses *in vivo* (Fig. 16). These outcomes showed that the mosaic antigens were properly processed to generate peptides that were presented by MHC molecules to cognate BVDV-specific memory T-cells. Previous studies have shown that mosaic antigens are processed by host APCs to generate relevant peptides for MHC presentation to elicit protective T-cell responses [140, 141].

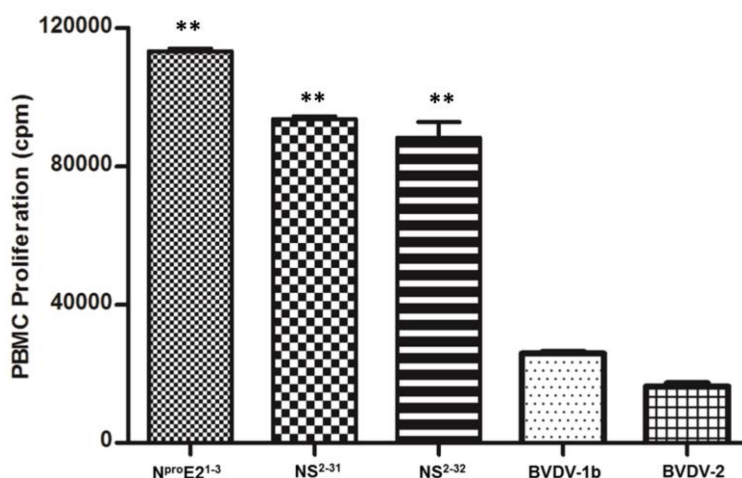

Figure 16. Validation of mosaic antigens using BVDV-specific T-cells.
Authenticity of T-cell epitopes in the mosaic BVDV antigens was validated by proliferation assay using PBMCs from a BVDV-1 & 2 hyper-immune steer [129]. The data shown is minus background counts from negative control (media alone) treatment. The asterisks denote a statistically significant difference ($P<0.01$) between the proliferation induced by the $N^{pro}E2^{1\text{-}3}$, $NS^{2\text{-}31}$ and the $NS^{2\text{-}32}$ antigens and both whole killed viruses BVDV-1b and BVDV-2. This outcome is representative of assays conducted using PBMCs from other BVDV immune steers.

4.4.5. Mosaic antigens elicited stronger BVDV-specific T-cell immune responses

Immunogenicity and protective efficacy of the AdN^{pro}E2$^{1-3}$, AdNS$^{2-31}$, and AdNS$^{2-32}$ recombinant adenovirus cocktail, designated AdBVDV, was evaluated in steers using a homologous prime-boost immunization regimen (Table 5 and Fig. 17).

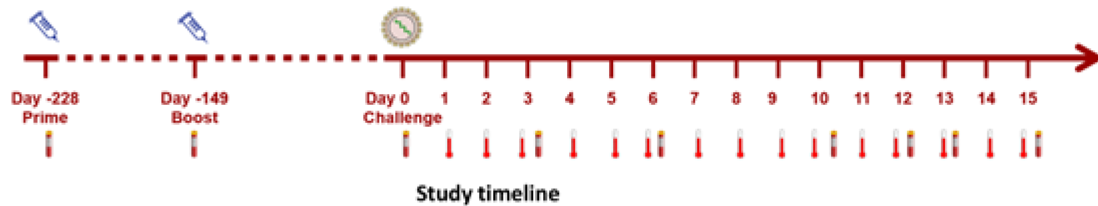

Figure 17. Immunization timeline.
On day -228 pre-challenge, cattle in the treatment group were vaccinated with a cocktail of the recombinant adenoviruses expressing mosaic BVDV antigens (AdBVDV), whereas positive control cattle received a commercial MLV BVDV vaccine. Negative control cattle were inoculated with the recombinant Ad-luciferase. On day -149 pre-challenge, the cattle were boosted with the respective priming inoculum and dose (Table I). On day 0, all the cattle were challenged by intranasal delivery of a BVDV-1373 using an atomizer. Blood samples were collected on selected days (0, 3, 6, 10, 12, 13 and 15), whereas clinical observations and rectal temperatures were monitored and recorded daily from days 1-15 post-challenge.

One week after the AdBVDV vaccinees were boosted, the cocktail elicited higher, but not significantly different, BVDV-specific IFN-γ-secreting PBMCs as well as BVDV-specific PBMC proliferation compared to the vaccinees that received the commercial MLV BVDV vaccine (Fig. 18A & C). The mean responses mounted by the AdBVDV vaccinees, but not the MLV vaccinees, were significantly higher ($P<0.05$) than the negative controls. Before challenge (five months after the AdBVDV vaccinees were boosted) the AdBVDV-induced mean IFN-γ^+ response had increased and was

significant (P<0.05) compared to the negative controls, whereas the mean IFN-γ$^+$ response in the MLV vaccinees had already declined (Fig. 18B). This decrease in the mean IFN-γ response in the MLV BVDV vaccine treatment group one week before challenge, might have had an impact on clearance of the challenge virus.

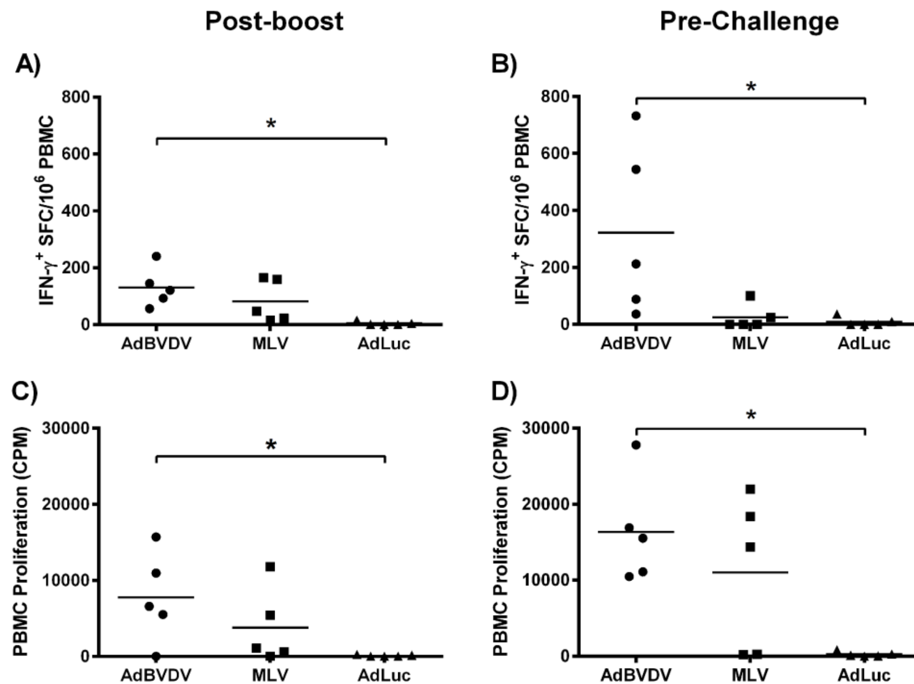

Figure 18. Mosaic BVDV vaccine elicited strong T-cell responses.
Immunization of cattle with adenovirus-vectored mosaic BVDV vaccine primed and expanded BVDV-specific T-cells. A commercial MLV BVDV vaccine served as a positive control, whereas Ad-Luciferase served as a negative control. EliSpot assays were used to evaluate BVDV-specific IFN-γ-secreting PBMC responses against defined BVDV T-cell epitopes and data is shown for A) One-week post-boost; and B) Day 0 of challenge. Outcome is presented as IFN-γ+ SFC/106 PBMC. Cell proliferation assays were used to evaluate BVDV-specific PBMC responses and data is shown for C) One-week post-boost; and D) one-week pre-challenge. Proliferation of the PBMCs in response to defined BVDV T-cell epitopes is presented as the means ± standard deviations of ^{3}H-thymidine incorporation by the cells from triplicate wells. In both assays, medium alone served as the negative control and the data shown is minus media background counts. The group mean is represented by a bar. Asterisks denote statistically significant differences, *P<0.05.

The mean BVDV-specific PBMC proliferation increased in both the vaccinated groups, but only the AdBVDV- and not the MLV-induced response was significantly different ($P<0.05$) from the AdLuc control group (Fig. 18D). The increase in mean IFN-γ response and PBMC proliferation in the AdBVDV treatment group at five months post-boost, were not significantly different from the responses recorded at one week post-boost (Fig. 18).

4.4.6. Mosaic antigens elicited cross-neutralizing BVDV-specific antibody responses

Following boosting of the AdBVDV vaccinees, the levels of BVDV neutralizing serum antibodies against five BVDV-1 strains and three BVDV-2 strains were evaluated at one-week post-boost and one-week pre-challenge (Fig. 19 and 20). The adenovirus cocktail induced higher mean neutralizing antibody titers post-boost against all BVDV-1 strains compared to the responses stimulated by the commercial MLV BVDV vaccine and the AdLuc controls. The difference between the mean titers however, was significant only for the non-cytopathic BVDV-1b BJ ($P<0.05$) and BVDV CA0401186a strains (AdBVDV vs MLV, $P<0.05$; AdBVDV vs AdLuc, $P<0.01$) (Fig. 19A). Furthermore, the mean AdBVDV titers increased up to five months post-boost (one-week pre-challenge) against 4 of 5 BVDV-1 strains whereas, the mean MLV titers either remained the same or declined. These mean AdBVDV titers remained significantly higher ($P<0.05$) than the MLV vaccinees and the AdLuc controls for the BJ strain, and only the AdLuc controls for the cytopathic BVDV-1a NADL strain. Interestingly, for all three BVDV-2 strains, the mean titers of the MLV vaccinees were higher (in contrast to BVDV-1) than the AdBVDV vaccinees post-boost (Fig. 20A). These mean MLV titers were

significantly higher (P<0.05) than the AdBVDV vaccinees only for strain A125 and significantly higher than the AdLuc controls for all three strains (P<0.05 for strain 890; P<0.01 for strains 1373 and A125). The mean BVDV-2-specific titers in both the AdBVDV vaccinees as well as the MLV vaccinees increased before challenge. Thus overall, the AdBVDV vaccine cocktail was able to induce high titers against all 8 BVDV strains tested in 3 out of 5 calves, whereas the MLV vaccine was able to induce high titers against only BVDV-2 strains. It is also noteworthy that the 3 AdBVDV vaccinees had substantially higher neutralizing titers (1:1,024 - 1: 2,048) when compared to the MLV vaccinees (1:32 - 1:256) against the NADL strain which is a component of the commercial MLV vaccine they received.

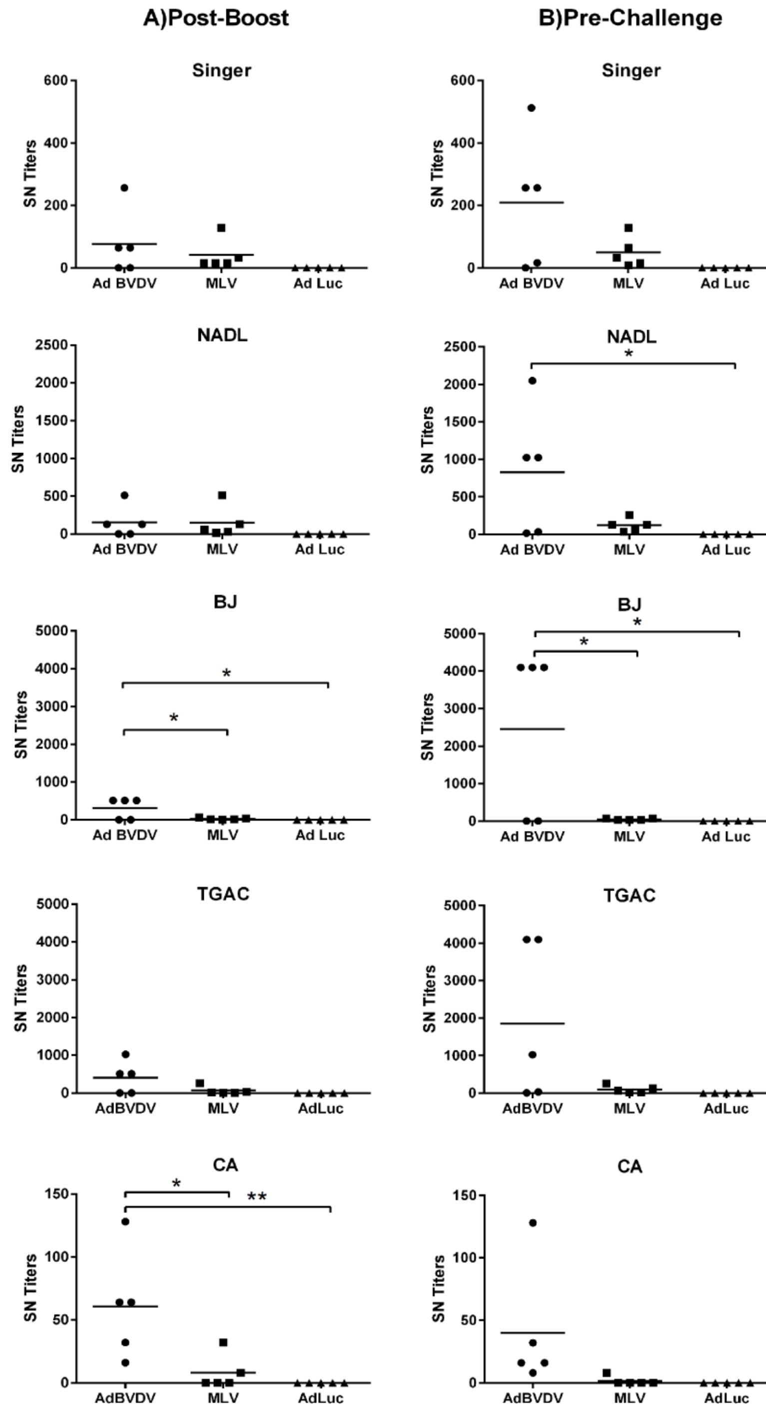

Figure 19. Mosaic BVDV vaccine induced BVDV-1 specific neutralizing antibodies. Serum neutralization assays were used to evaluate BVDV-1-specific neutralization titers at A) One-week post-boost; and B) one-week pre-challenge against five BVDV type 1 strains. Mean group titers are represented by the bars. Statistically significant differences between the groups are denoted by asterisks. *P<0.05; **P<0.01.

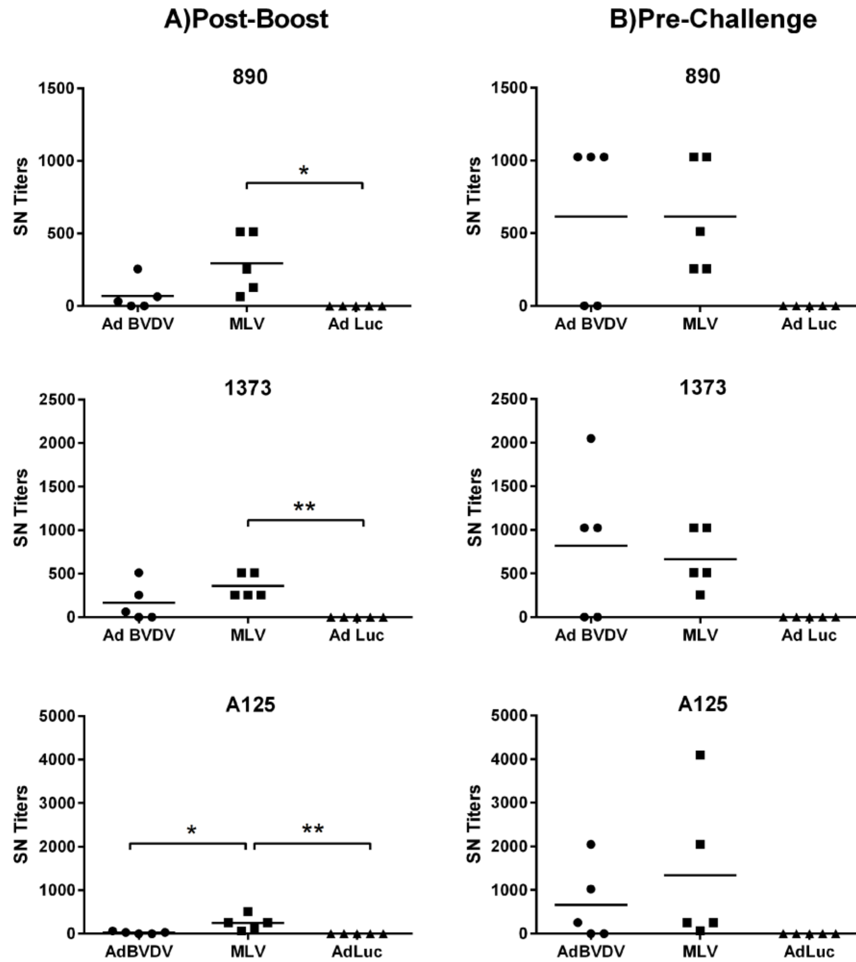

Figure 20. Mosaic BVDV vaccine induced BVDV-2 specific neutralizing antibodies. BVDV-2-specific neutralization titers against three BVDV type 2 strains were evaluated at A) two weeks post-boost; and B) one week pre-challenge. Mean group titers are represented by the bars. Statistically significant differences between the groups are denoted by asterisks *P<0.05; **P<0.01.

4.4.7. Clinical observations, hematology and viremia

Following the BVDV challenge, there were no obvious differences in clinical score among all the animals, however, characteristic biphasic pyrexia was observed for the negative controls but not for the AdBVDV or the MLV vaccinees (Fig. 21A). On day 5 post-challenge, the transient rise in mean rectal temperatures of the negative controls was significantly higher (P<0.001) than the MLV vaccinees but not the AdBVDV vaccinees. The mean rectal temperatures for the negative controls rose again on day 9, peaked at day 10 and normalized by day 11 post-challenge. The mean temperatures of the controls were significantly higher than AdBVDV vaccinees on days 9 (P<0.05) and 10 (P<0.001) post challenge, and the MLV vaccinees on days 8 (P<0.01), 9 (P<0.001) and 10 (P<0.001) post-challenge (Fig. 21A). The negative control animals also exhibited transient leucopenia from days 6 to 9 post-challenge with a 32-40% reduction against baseline (day 0) white blood cell (WBC) counts. This reduction of WBCs in the negative controls was significant compared to the AdBVDV vaccinees on days 6 (P<0.05) and 9 (P<0.01) post-challenge, and the MLV vaccinees on days 6 (P<0.01), 7 (P<0.01) and 9 (P<0.001) post-challenge (Fig. 21B). There was no significant difference in platelet counts among the treatment groups post-challenge. On days 7 and 10 post-challenge, no virus was detected in all the AdBVDV vaccinees (Table 6). However, BVD virus was detected from the blood of one of the steers that received the commercial MLV BVDV vaccine on day 7 but not on day 10 post-challenge, and from the blood of all the negative controls up to day 15 post-challenge (Table 6).

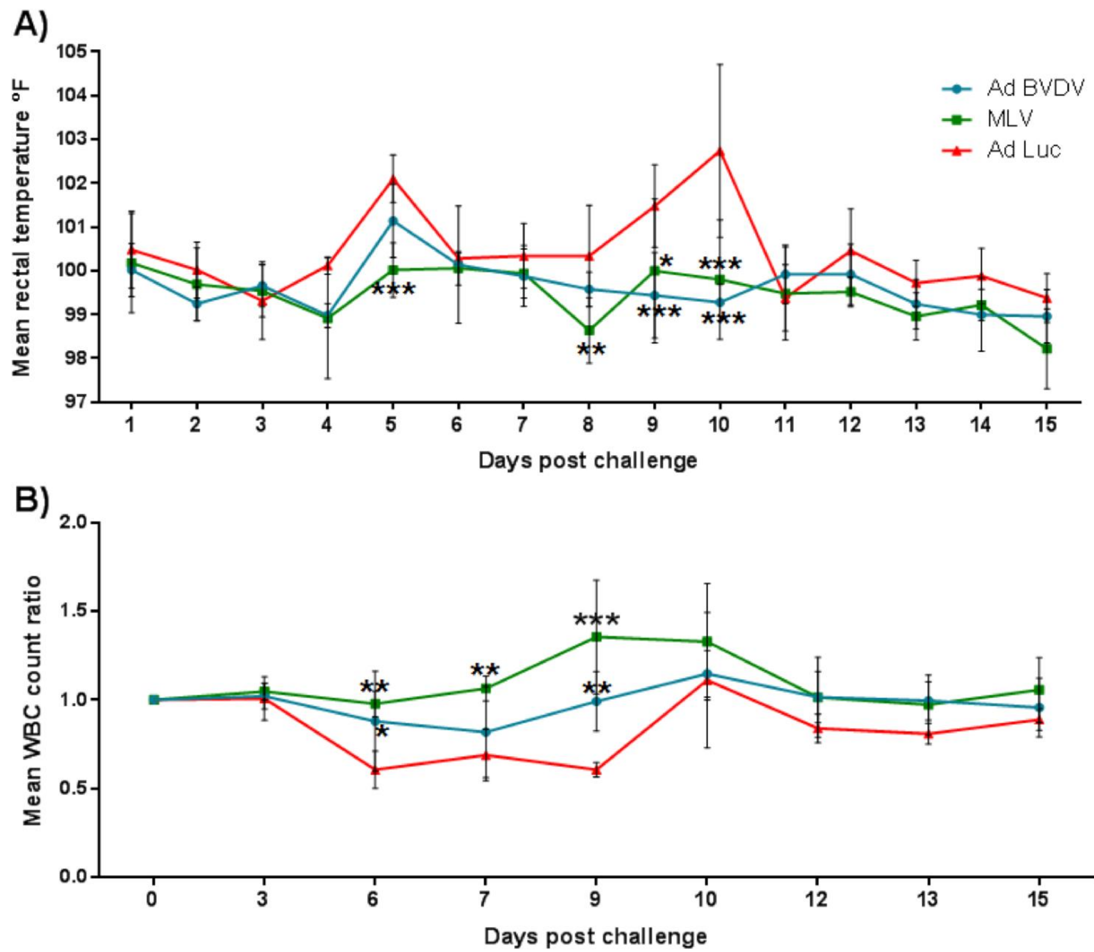

Figure 21. Clinical manifestations post-challenge.
A) Mean rectal temperature fluctuation; and B) Mean change ratios of white blood cell counts in the vaccinated and negative control groups post-challenge. Asterisks denote statistically significant differences as compared to the negative controls. *P<0.05; **P<0.01 and ***P<0.001.

Table 6: Virus Isolation from Calves on Day 7 and Day 10 Post-Challenge

Cattle ID	Vaccine	Viremia	
		Day 7 post-challenge	Day 10 post-challenge
4	AdBVDV	-	-
12	AdBVDV	-	-
13	AdBVDV	-	-
22	AdBVDV	-	-
23	AdBVDV	-	-
3	BVDV MLV	-	-
14	BVDV MLV	-	-
19	BVDV MLV	-	-
24	BVDV MLV	-	-
27	BVDV MLV	+ (10^{-2})	-
10	Ad Luciferase	+ (10^{-3})	+ (10^{-3})
18	Ad Luciferase	+ (10^{-3})	+ (10^{-3})
25	Ad Luciferase	+ (10^{-3})	+ (10^{-3})
28	Ad Luciferase	+ (10^{-3})	+ (10^{-3})
29	Ad Luciferase	+ (10^{-3})	+ (10^{-3})

Viremia in blood samples taken on days 7 and 10 post-challenge was evaluated by immunocytometric analysis of MBDK cells probed with goat anti-BVDV polyclonal serum. The dilution at which the samples were positive is specified. Sample dilutions further than 10^{-3} were not tested.

4.4.8. Recombinant adenovirus inoculated intradermally is short lived

Persistence of recombinant replication-incompetent adenovirus at the intradermal inoculation site, the draining lymph node, and the spleen was monitored by HEK-293A cell-dependent virus rescue followed by immunocytometric analysis using adenovirus-specific polyclonal antibody. One-day post-inoculation, adenovirus was readily

recovered from the skin biopsies collected from the inoculation sites, but not from the control sites (Fig. 22B and C). Virus recovery decreased drastically by day two post-inoculation and very few viral particles were recovered at day three (Fig. 22 E and H). No virus was recoverable from all skin biopsies collected on days 4-7 post-inoculation (Fig. 22 K). Skin biopsies collected on days 14 and 21, and draining lymph node and spleen samples collected on day 21 were all negative (Fig. 22 N and O). The medium from the HEK-293A cells used to test the samples collected on days 4-7, 14, and 21, was negative after a second round of screening. These outcomes are consistent with previous findings in rodents [142]. Given that ABSL2 biocontainment is required for *in vivo* studies using the replication-incompetent adenovirus, data from this pilot study suggest that it is safe to downgrade biocontainment after seven days post-inoculation. However, the fate of the vector genome in cattle and environmental risk assessment will need to be determined.

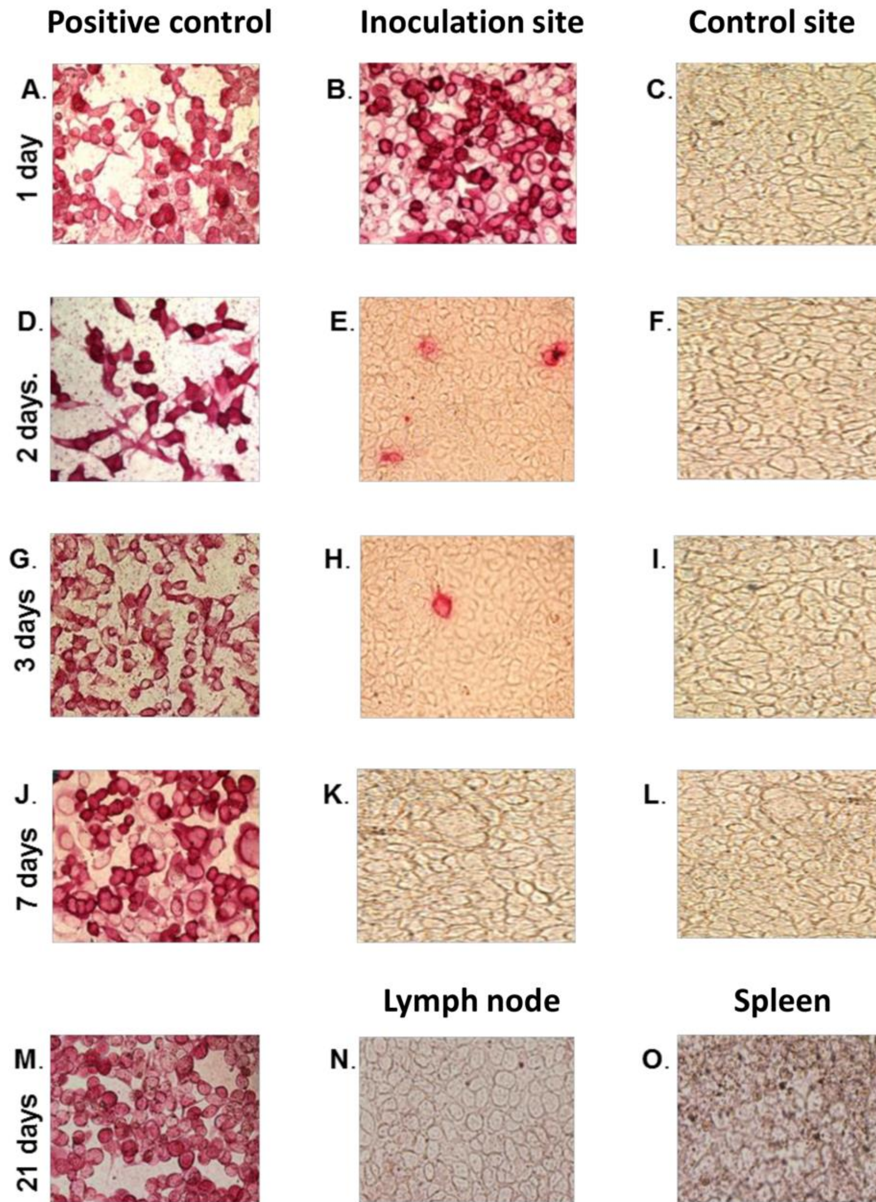

Figure 22. Persistence of replication-incompetent adenovirus in cattle.
Viable recombinant adenovirus inoculated intradermally is only recoverable within three days. Presence of adenovirus rescued from tissue samples of four steers at defined time points was tracked by immunocytometric analysis of HEK-293A cells. Representative data from one steer is shown: A, D, G, J, and M are positive controls at 24 hr., 48 hr., 72 hr., day 7, and day 21, respectively. B, E, H, and K, are skin biopsies taken from the inoculation sites on the neck of the steers at 24 hr., 48 hr., 72 hr., and day 7, respectively, whereas C, F, I, and L, are cognate control skin biopsies taken concurrently from the flank. N and O are draining lymph node and spleen samples, respectively, collected three weeks post-inoculation.

4.5. Conclusions

The purpose of this study was to develop an efficacious BVDV vaccine which a) overcomes the several disadvantages associated with the MLV vaccine mentioned previously and b) provides broad protection against multiple BVDV genotypes. To this end, we designed mosaic polypeptide consensus sequences of highly immunogenic BVDV antigens such as N^{pro}, E2 glycoprotein and the Nonstructural protein 2-3 based on multiple genotypes. We selected live replication deficient adenovirus as a vector for delivery of these antigens to prime strong humoral as well as cell mediated immune responses. Polyclonal anti-BVDV sera and monoclonal anti-E2 antibodies strongly recognized these mosaic antigens by immunocytometric analysis. Furthermore, PBMCs from BVDV immune steers proliferated strongly upon stimulation by these mosaic antigens. The above outcomes confirmed the authenticity of both B-cell and T-cell epitopes in all the mosaic antigens.

Calves immunized with a cocktail of recombinant adenoviruses expressing these antigens had stronger IFN-γ^+ and proliferation responses to defined BVDV $CD4^+$ T-cell epitopes as compared to calves vaccinated with the commercial BVDV MLV vaccine. In addition, the AdBVDV vaccinees had higher serum neutralizing titers against BVDV-1 than the MLV vaccinees. In case of BVDV-2, the MLV vaccinees had higher mean titers one-week post-boost, but the AdBVDV mean titers increased over time and before challenge were equivalent or higher than the MLV vaccinees for 2 of 3 strains tested. Importantly, both BVDV-1 and BVDV-2 neutralizing antibody titers along with the cellular IFN-γ^+ and proliferation immune responses considerably increased for up to five

months post-boost (one week before challenge) in most AdBVDV vaccinees, whereas only the BVDV-2 specific titers and the mean proliferation responses amplified in the MLV vaccinees. Upon challenge with a BVDV-2a strain, both vaccinated groups showed no clinical signs of infection. The negative controls, however, had a mild fever on day 5 post-challenge followed by more severe pyrexia on day 10 post-challenge. Moreover, the negative controls also had significantly lower WBC counts than both vaccinated groups. Rapid clearance of virus is an attractive trait in a BVDV vaccine. All the AdBVDV vaccinees had cleared the virus as early as 7 days post-challenge, whereas one MLV vaccinee was still viremic on day 7 but not on day 10 post-challenge. All negative controls remained viremic up to day 15 post-challenge. With regards to the safety concern and ABSL2 biocontainment when using human Ad5 as a delivery vector, we showed that the replication-incompetent Ad5 virus is cleared from the inoculation site within four days post-injection and is not recovered from either the draining lymph node or the spleen after 21 days post-inoculation.

Overall, data from this study showed that the AdBVDV prototype vaccine is more immunogenic and offers better cross-protection than the commercial MLV vaccine in terms of cell mediated and neutralizing antibody responses. As far as protective efficacy is concerned, the AdBVDV vaccine performed at par if not better than the MLV vaccine upon challenge by BVDV-2a strain. Notably, this study is the first to report heterologous protection using subunit BVDV vaccines. Future studies with larger animal sample sizes, different vaccine doses and challenge with diverse BVDV strains need to be conducted to further optimize the AdBVDV prototype vaccine.

The protective potential of the BVDV E2 antigen has been successfully demonstrated in the past using various delivery platforms like live-vectors, DNA immunizations or as a recombinant protein produced in different expression systems [9, 143-145]. Current efforts are now focused on enhancing this potential using modern adjuvants and antigen carriers such as PRR activators, APC targeting molecules and silica nanoparticles [146-149]. This study highlights the cross-protective potential of the novel mosaic polypeptides and is the first to report heterologous protection using subunit BVDV vaccines. Thus, future studies using these mosaic polypeptide sequences in conjunction with modern immune-response enhancing strategies may lead to a very effective and cross-protective BVDV vaccine.

CHAPTER V

SUMMARY

The goal of the work described in this dissertation was to rationally develop prototype vaccines against two pathogens, ASFV and BVDV. The major conclusions drawn from the *in-vivo* studies reported here are as follows:

5.1. African Swine Fever Virus

- The multivalent Ad-ASFV cocktails induced strong antibody responses and IFN-γ^+ responses against each antigen in the cocktail.
- The Ad-ASFV cocktail was able to prime CTLs (evaluated only in the first study) capable of recognizing and killing target cells presenting each antigen in the cocktail. This is the first demonstration of induction of ASFV antigen-specific CTL responses in commercial pigs using an Ad-ASFV multivalent cocktail.
- Both Ad-ASFV cocktails were well tolerated with no adverse effects.
- The protective potential of the responses induced need to be evaluated in efficacy studies

5.2. Bovine Viral Diarrhea Virus

- The AdBVDV prototype vaccine was more immunogenic and cross-protective than the commercial MLV vaccine

- This is the first demonstration of heterologous protection in case of BVDV using a subunit vaccine.
- Further studies involving large sample sizes, vaccine dose optimization and challenge with various BVDV strains are required before the possible commercialization of this prototype vaccine.

Overall, these *in-vivo* studies demonstrate that using live-adenovirus vector to deliver rationally selected/designed antigens can be a promising and safe approach for developing veterinary vaccines.

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APPENDIX

Supplementary Data

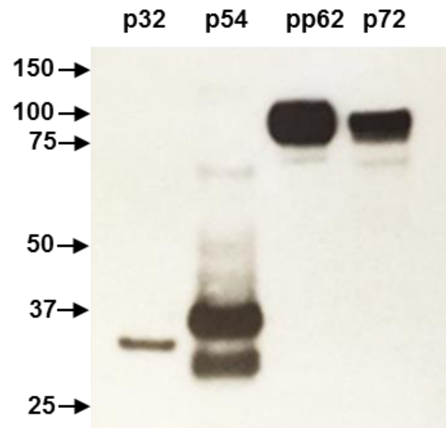

Figure A1. Validation of affinity purified ASFV antigens.
Recombinant ASFV antigens were affinity purified using anti-FLAG agarose and their authenticity was confirmed by western blotting using ASF-specific convalescent serum.

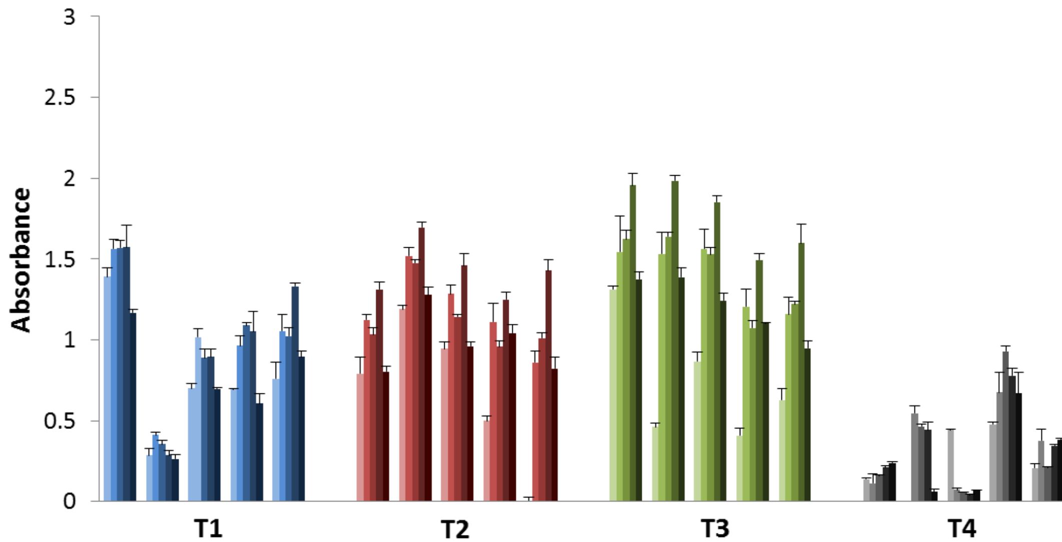

Figure A2. Adenovirus vector-specific serum IgG profiles post-priming. Adenovirus vector-specific IgG response was monitored bi-weekly post-prime up to week 10 by ELISA (sera were diluted at 1:1000). Color scheme used, T1: Blue; T2: Maroon; T3: Green; and T4: Gray. The absorbance values at 450 nm across weeks 2, 4 ,6, 8 & 10 post-prime for each animal are depicted using a color gradient where the lightest shade (first bar) represents week 2 and the darkest shade (last bar) represents week 10. Error bars show standard deviation among triplicate absorbance values. The profile is similar to that observed for ASFV antigen-specific antibodies, specifically the decline seen at week 10.

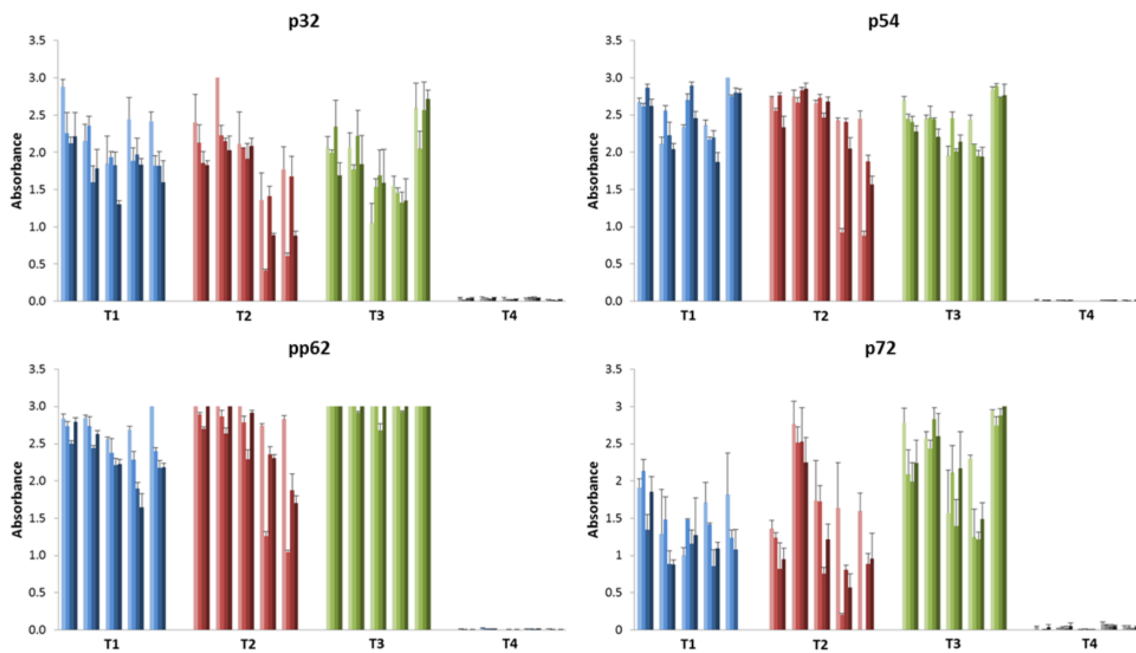

Figure A3. Recall antigen-specific serum IgG profiles post-boost.
Antigen-specific IgG responses were monitored weekly post-boost up to week 4 by ELISA (sera were diluted at 1:1000). The color scheme for the treatment groups is same as shown in Figure 3. The absorbance values at 450 nm across weeks 1, 2, 3 & 4 post-boost for each animal are depicted using a color gradient where the lightest shade (first bar) represents week 1 and the darkest shade (last bar) represents week 4. The absorbance for some animals exceeded the upper limit of detection (greater than 3.0) and is shown in the profiles at a maximum value of 3.0. Error bars show standard deviation among triplicate absorbance values.

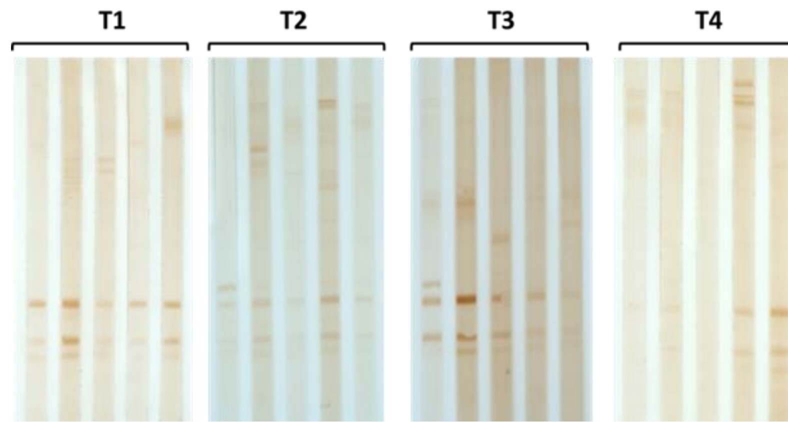

Figure A4. Western blot of lysates from mock-infected Vero cells.
Blots were probed with individual serum for each animal in the study to assess background reactivity to host cell antigens.