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Live-vectored antigen cocktail confers protection against African swine fever virus (ASFV) Georgia 2007/1 challenge



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African swine fever (ASF) is a lethal disease of swine caused by the ASF virus (ASFV), for which no licensed vaccine is currently available in non-endemic countries. Here, replication-competent adenovirus-vectored ASFV multi-antigen constructs were shown to express ASFV antigens in primary swine cells. Pigs immunized with a cocktail of these constructs, with or without Quil-A adjuvant, tolerated the formulation well. The constructs elicited robust ASFV-specific IgG responses ($p < 0.0001$), which were boosted upon reimmunization ($p < 0.05$). Following challenge with the virulent ASFV Georgia 2007/1 strain using a natural transmission model, five of six pigs vaccinated without Quil-A survived, while all pigs receiving adjuvanted constructs succumbed to ASF. Survivors cleared the virus, exhibited only mild clinical signs, gained weight, and remained healthy for the remainder of the study. Histopathological analysis revealed an absence of ASFV-associated lesions in survivors, whereas severe lesions were observed in pigs vaccinated with adjuvanted constructs and in negative controls. Although neutralizing antibodies were undetectable, granzyme B-producing CD8 α^+ T cell responses were observed in survivors, indicating a likely correlation for cellular immunity in protection. These findings highlight the protective potential of ASFV antigen expression constructs and inform subunit vaccine design.

African swine fever (ASF) is a highly contagious and often fatal hemorrhagic disease of suids caused by African swine fever virus (ASFV), a large enveloped double-stranded DNA virus and the sole member of the genus *Asfivirus* (family *Asfarviridae*). The ASFV genome (~170–193 kb) encodes over 150 proteins, many of which remain poorly characterized¹. Of the 24 known ASFV genotypes, only genotypes I and II have been detected outside sub-Saharan Africa^{2,3}. Over the past decade, ASFV has spread rapidly with confirmed outbreaks in 63 countries and territories by the end of 2024, resulting in over 1.87 million animal losses^{4–6}.

ASFV is maintained in a sylvatic cycle involving warthogs (*Phacochoerus africanus*) and *Ornithodoros* ticks in sub-Saharan Africa. However, infection of domestic pigs and wild boars causes peracute to acute disease or chronic disease with case fatality rates associated with highly virulent ASFV strains such as Georgia 2007/1 approaching 100%⁴. The clinical presentation and associated gross and histopathological findings of

ASFV infection in domestic pigs vary with viral virulence, route and dose of infection, and host immune status⁷. Transmission occurs primarily through direct contact with infected animals or ingestion of contaminated materials, including carcasses and fomites^{8,9}. Viral persistence in frozen tissues and uncooked pork products further facilitates spread. Survivors of infection may become chronic carriers, complicating eradication efforts¹⁰. Currently, there is no safe vaccine in non-endemic countries or antiviral treatment for ASF. Management of outbreaks relies on rapid diagnosis, quarantine, mass depopulation, carcass disposal, and strict biosecurity measures¹¹. Despite recent progress, the development of a safe and effective ASFV vaccine remains a global priority^{1,12}.

Although some attenuated ASFV strains have shown promising protection, the efficacy of gene-deletion mutants is often strain-specific, and incomplete attenuation and reversion to virulence raise safety concerns. Furthermore, production of live attenuated vaccines (LAVs) requires

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high-level biocontainment¹³, and ASFV susceptible primary or permanent cell lines¹⁴. Vaccine development is further hindered by a limited understanding of ASFV pathogenesis and immune evasion mechanisms, undefined correlates of protection, and incomplete knowledge of protective antigens^{6,15}. Notably, the ASFV-G-Δ1177L and ASFV-G-ΔMGF LAVs recently approved for field use in Vietnam have demonstrated poor efficacy against newly emerged recombinant ASFV strains generated through field recombination between genotypes I and II^{16–18}. Challenges in deploying LAVs in non-endemic regions include potential reversion to virulence, lack of Differentiating Infected from Vaccinated Animals (DIVA) strategies, and the risk of chronic infection^{16,19}.

Subunit vaccines offer a safe alternative, enabling targeted immune responses and DIVA compatibility²⁰. However, progress in subunit vaccine development is hampered by limited knowledge of ASFV immunopathogenesis and protective antigens. The protective role of ASFV-specific antibodies remains unclear, and recent evidence suggests that antibody responses may exacerbate disease severity^{21–23}. Instead, cellular immunity, particularly cytotoxic T lymphocytes (CTLs), is considered critical for viral clearance^{24–26}. CD8⁺ T cells have been detected in convalescent pigs and in those immunized with attenuated ASFV^{25,26}. Despite promising immunogenicity, most subunit vaccine candidates, including those employing replication-deficient adenoviral vectors encoding multiple ASFV antigens, have failed to confer protection against challenge with virulent genotype II strain^{23,27–30}. Previous homologous and heterologous prime-boost immunization strategies using adenovirus and modified vaccinia Ankara vectors conferred partial protection against genotype I but not genotype II ASFV, underscoring the need for improved antigen selection and delivery platforms^{29,30}.

We hypothesized that expressing the ASFV proteome in vivo via self-replicating adenoviral constructs would better mimic the immunostimulatory properties of attenuated ASFV and enhance the breadth and magnitude of protective T-cell responses³¹. To test this, we developed a cocktail of 43 attenuated replication-competent adenovirus type 5 (RC-Ad5) constructs collectively referred to as RC-Ad5-ASFV encoding multicistronic cassettes that span the entire ASFV Georgia 2007/1 (genotype II) proteome. The RC-Ad5-ASFV cocktail, formulated with or without the Quil-A adjuvant, was evaluated in piglets for safety, immunogenicity, and protective efficacy following challenge with virulent ASFV Georgia 2007/1-infected shedders. Additionally, a Quil-A-*adjuvanted* cocktail excluding previously well-studied antigens was tested²⁰. Protective efficacy is defined as a vaccine's ability to prevent the development of clinical or pathological evidence of acute or chronic ASF. Quil-A is a potent saponin commercial adjuvant commonly used in veterinary and research settings to boost antibody and T cell responses^{32,33}. This strategy aims to guide empirical identification of protective antigens to support the rational design of subunit vaccines capable of conferring broad protection across ASFV genotypes.

Results

Expression of ASFV antigens by adenovirus-5 constructs

Forty-three recombinant RC-Ad5 constructs (RC-Ad5-01 to RC-Ad5-43) were generated, each containing multicistronic cassettes (3.8–4.5 kb) encoding up to nine codon-optimized ASFV antigens. Antigens were separated by self-cleaving P2A motifs and included in-frame hemagglutinin (HA) and FLAG epitope tags, along with adenoviral E1 subunits to ensure replication competence (Fig. 1a; Supplementary Table 1). Immunocytometric analysis of infected HEK293A cells confirmed antigen expression by the RC-Ad5 constructs using tag-specific monoclonal antibodies (mAbs), with antigen identity further validated using ASFV convalescent swine serum (Fig. 1b). However, protein expression from each of the genes contained in the multicistronic cassettes was not individually verified. Expression of green fluorescent protein (GFP) from the negative control RC-Ad5-GFP construct was also confirmed (Fig. 1b). RC-Ad5 constructs, but not replication-deficient adenovirus type 5 (RD-Ad5) constructs expressing ASFV antigens, were replicated in primary swine alveolar epithelial cells, as demonstrated by immunofluorescence assay (IFA) using ASFV

convalescent serum (Fig. 1c). Expression of ASFV antigens by all 43 RC-Ad5 constructs in primary swine alveolar epithelial cells was confirmed and quantified by flow cytometry using ASFV convalescent serum (Fig. 1d; Supplementary Table 2). All constructs, except RC-Ad5-43 (24.9%), showed >50% antigen-positive cells via flow cytometry (Supplementary Table 2). Sequencing of cesium chloride-purified virus stocks confirmed the integrity of each expression cassette. These validated constructs were then used to evaluate safety, immunogenicity, and protective efficacy in pigs.

RC-Ad5-ASFV virus cocktail elicited strong antibody responses

Piglets received three intramuscular doses of 43 RC-Ad5-ASFV constructs, with Quil-A adjuvant (groups 1 and 2, G1–G2) or without (group 3, G3) (Fig. 2a; Supplementary Table 3). Both formulations were well-tolerated by the immunized pigs, as evidenced by body weight gain and stable temperature throughout weekly monitoring (Supplementary Fig. 1). No viral shedding was detected in nasal swabs collected from piglets immunized with either the RC-Ad5-ASFV cocktail with or without adjuvant or the RC-Ad5-GFP control, as assessed by virus rescue assays using either primary swine alveolar epithelial cells or HEK293A cells (Supplementary Table 4). Cells infected with RC-Ad5-ASFV served as positive controls (Supplementary Table 4). Nasal samples collected one week post-boost remained negative as judged by adenovirus rescue using HEK293A cells probed with anti-adenovirus type 5 antibodies or ASFV convalescent serum; GFP expression was absent in samples collected from RC-Ad5-GFP-inoculated animals.

The RC-Ad5-ASFV cocktail elicited strong ASFV-specific immunoglobulin G (IgG) responses after the prime immunization ($p < 0.0001$), which were further enhanced one week after both boost 1 and boost 2 ($p < 0.05$) (Fig. 2b). The induced antibody responses were predominantly IgG, with balanced IgG1 and IgG2 subclasses, minimal IgM, and no detectable IgA (Fig. 2b). At all-time points, antibody levels in all vaccinated groups were significantly higher than those in the RC-Ad5-GFP control group, with no significant differences among the three RC-Ad5-ASFV-vaccinated groups (G1–G3) (Fig. 2b). Sera from pigs immunized with the RC-Ad5-ASFV cocktail whether or not formulated with Quil-A recognized ASFV (Georgia 2007/1)-infected primary swine macrophages by IFA, but did not react with uninfected macrophages (Fig. 2c; Supplementary Table 5). This demonstrates that the RC-Ad5-ASFV cocktail elicited antibodies capable of recognizing wild-type ASFV-infected cells.

RC-Ad5-ASFV virus cocktail conferred protection

Naïve ASFV-infected shedders were co-housed with treatment and control pigs to model a natural contact challenge. Notably, five of six pigs immunized with the RC-Ad5-ASFV cocktail without Quil-A adjuvant (G3) survived challenge with the virulent ASFV Georgia 2007/1 strain ($p < 0.0001$; Fig. 3a). In contrast, all pigs in Group 1 (G1, cocktail with Quil-A) and Group 2 (G2, cocktail lacking constructs RC-Ad5-03 to -05 with Quil-A) succumbed to infection, except for one G1 pig (#47) that survived until day 18 post-challenge (Fig. 3a). All mock-immunized controls (Group 4, G4; RC-Ad5-GFP + Quil-A) also succumbed (Fig. 3a) by day 15 post co-housing with naïve contact challenge (CC) spreader pigs, which developed severe clinical signs and were euthanized during days 5–7 post-inoculation (Fig. 3b). With the exception of pig #30 in G4, CC spreader pigs exhibited comparable viremia in nasal swabs, and all animals showed similar viral loads in fecal samples at the time of euthanasia (Fig. 3d and Supplementary Fig. 2). Similarly, all pigs in G1, G2, and G4 exhibited elevated clinical scores and were humanely euthanized at various time points post-challenge (Fig. 3a). Most animals in these groups experienced weight loss and abrupt temperature fluctuations preceding euthanasia (Supplementary Fig. 3). Remarkably, five of six G3 pigs remained clinically healthy through day 39 post-challenge, displaying mild or no clinical signs, stable body temperatures, and steady weight gain (~0.91 kg/day) (Supplementary Fig. 3). The exception in G3 was pig #37, which showed clinical decline on days 10–11 and was humanely euthanized on day 11 due to anorexia and recumbency (Fig. 3b). As expected, CC spreaders developed high viremia by day 5

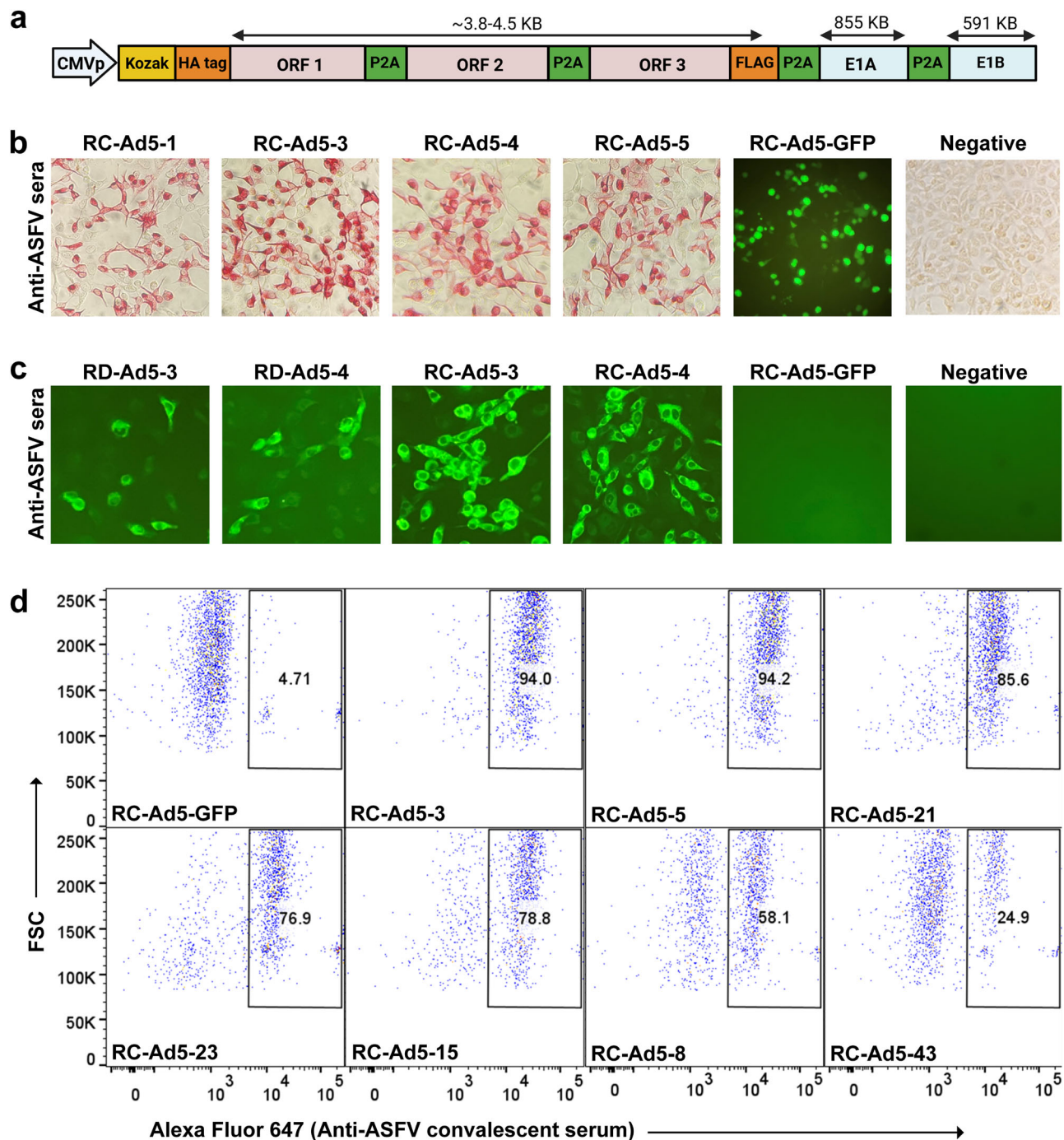


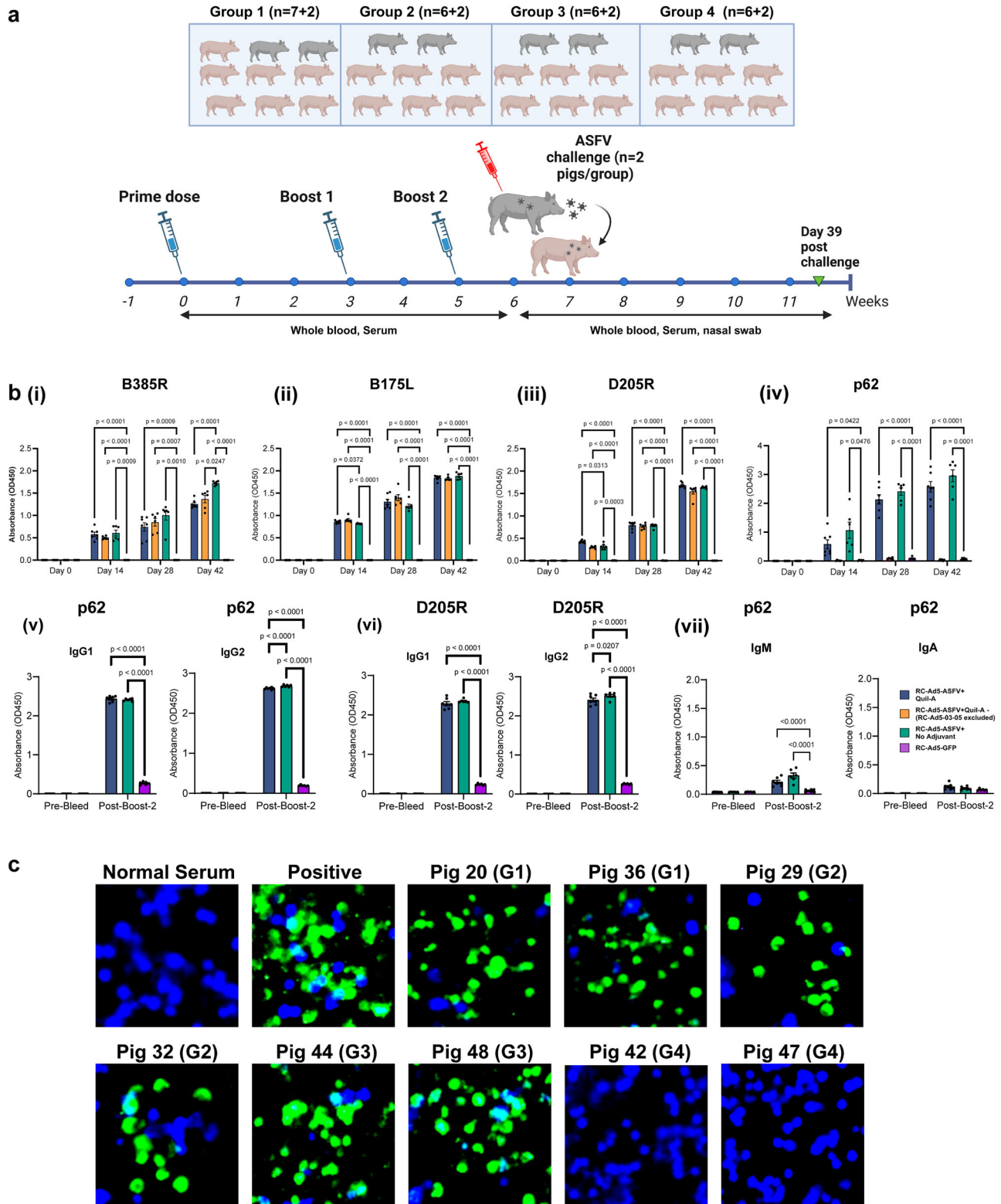
Fig. 1 | Design, expression, and replication of multicistronic RC-Ad5-ASFV constructs. **a** Schematic of multicistronic gene cassettes encoding up to nine codon-optimized ASFV antigens separated by P2A motifs and fused, in-frame with adenoviral E1 subunits (Supplementary Table 1). **b** Representative immunocytometric validation of ASFV antigen expression in HEK293A cells infected with RC-Ad5-1, RC-Ad5-3, and RC-Ad5-5, or negative control using tag-specific mAbs, ASFV convalescent serum, or GFP fluorescence. **c** Immunofluorescence assay of virus replication in primary swine alveolar epithelial cells infected with replication-defective adenovirus-5 expressing p72-p15-B602L or p62-p32-p54-EP153R-p10 (designated RD-Ad5-3 and RD-Ad5-4, respectively), or replication-competent

(RC-Ad5-3 and RC-Ad5-4) adenoviruses. RD-Ad5-Luc and RC-Ad5-GFP served as negative controls. Cells were probed with ASFV convalescent serum. **d** Flow cytometric analysis of ASFV antigen expression in epithelial cells infected with selected RC-Ad5 constructs showing high (RC-Ad5-3, -5), medium (RC-Ad5-10, -13), or low (RC-Ad5-8, -43) expression levels. RC-Ad5-GFP served as a negative control. Expression data for all constructs are summarized in Supplementary Table S2. ASFV African swine fever virus, RC-Ad5 replication-competent adenovirus serotype 5, RD-Ad5 replication-defective adenovirus serotype 5, GFP green fluorescent protein, IFA immunofluorescence assay, mAb monoclonal antibody.

post-challenge and efficiently transmitted ASFV to all G1–G4 contact pigs, resulting in ~50% of pigs in G1, G2, and G4 being euthanized by days 11 and 12 (Fig. 3c; Supplementary Fig. 4). The last surviving G1 pig (#49) was euthanized on day 18. In G3, pig #37 became viremic on day 10 and was euthanized on day 11, while the remaining five G3 pigs developed detectable

viremia starting on day 12 of co-housing with the CC spreaders but progressively cleared the virus by day 26 (Fig. 3c; Supplementary Fig. 4).

Quantitative PCR (qPCR) revealed high ASFV DNA loads in lymphoid tissues, non-lymphoid organs, gastrointestinal (GI) tract, and fecal samples from CC spreaders exhibiting comparable viral loads,



vaccinated pigs in G1 and G2, and control pigs in G4 (Fig. 3d; Supplementary Fig. 5). Comparable viral loads were observed in the non-survivor G3 pig (#37). In contrast, no ASFV DNA was detected in any tissue samples from the five surviving G3 pigs at the time of necropsy on day 39 post co-housing with CC spreader pigs. Virus isolation from spleen and pericardial fluid confirmed the absence of infectious virus in these survivors, whereas infectious virus was recoverable from

the same tissues in pig #37 and the two CC spreaders co-housed with the G3 pigs (Fig. 3e).

Comparative gross and histopathology of survivors and non-survivors

Postmortem analysis revealed gross lesions consistent with acute ASF in naïve CC spreaders and pigs from G1 and G2 (Fig. 4a). Gross pathology was

Fig. 2 | RC-Ad5-ASFV immunization elicits ASFV-specific antibody responses in pigs. **a** Experimental design and timeline. Piglets were divided into four treatment groups: (1) RC-Ad5-ASFV cocktail (43 constructs) with Quil-A adjuvant; (2) RC-Ad5-ASFV cocktail (excluding RC-Ad5-03 to -05) with Quil-A; (3) RC-Ad5-ASFV cocktail without adjuvant; and (4) RC-Ad5-GFP with Quil-A (negative control). After a 1-week acclimation, pigs received intramuscular priming on Day 0, followed by boosts at weeks 3 and 5. Two-weeks post-boost, contact-challenge pigs were intramuscularly inoculated with ASFV (Georgia 2007/1) to simulate field-like transmission. Clinical monitoring and sampling continued until day 39 post-challenge or euthanasia. **b** ASFV-specific antibody responses. ELISA revealed IgG responses against the B385R, B175L, D205R, and p62 (not present in Group 2) antigens in vaccinated pigs. Panels (i–iv) show individual OD450 values with group

means $\pm$ SEM. The IgG responses in each treatment group increased significantly ($p < 0.05$) after each boost. Panels (v–vii) indicate isotype responses: both IgG1 and IgG2 were induced, while IgM levels were minimal and IgA was negligible. **c** Vaccine-induced antibodies recognize ASFV-infected cells. Post-boost sera from vaccinated pigs, but not controls, stained ASFV-infected primary swine macrophages by indirect immunofluorescence assay. Representative results from two pigs per group are shown. Full IFA dataset is summarized in Supplementary Table S5. RC-Ad5 replication-competent adenovirus serotype 5, ASFV African swine fever virus, ELISA enzyme-linked immunosorbent assay, IFA immunofluorescence assay, IgG immunoglobulin G, IgM immunoglobulin M, IgA immunoglobulin A, OD optical density, SEM standard error of the mean.

somewhat less severe in mock-vaccinated controls (G4), while only minor lesions were observed in G3 vaccinees including progressor pig #37 (Fig. 4a), with significantly lower gross pathology scores than all other groups ($p < 0.0001$; Fig. 4a). Marked splenomegaly and hemorrhagic or edematous lymphadenopathy, particularly affecting the gastrohepatic, mandibular, and renal lymph nodes, were frequently observed in pigs with acute ASF (Fig. 4b; Supplementary Fig. 6d; Supplementary Table 9). Additional gross lesions included hyperemia of ear tips and extremities, skeletal muscle atrophy, and polyserositis with fibrinous and blood-tinged effusions in the peritoneal, pleural, and pericardial cavities (Supplementary Fig. 7a). Pericardial effusion was accompanied by fibrin deposition and epicardial edema (Supplementary Fig. 7A3). Moderate to severe pulmonary congestion was common in pigs with acute ASF, along with edema and hemorrhage in the gallbladder and biliary tract (Supplementary Fig. 7A1, A4). Petechial to ecchymotic hemorrhages were evident on the renal capsule and extended into the parenchyma upon sectioning (Supplementary Fig. 7A2). Severe acute ASF was also associated with GI involvement, including hemorrhagic diarrhea (Supplementary Fig. 6a), gastric mucosal edema, irritation, or ulceration, and commonly contained partially digested blood. Mucosal edema, hemorrhage occurred segmentally in the small and large intestine, frequently accompanied by cecal tonsil swelling and ulceration. Gross lesions consistent with acute or chronic ASF-induced disease were not observed in G3 vaccinees.

Histopathological findings in pigs with acute ASF disease were predominantly characterized by: (1) histiocytolysis, lympholysis, and lymphoid depletion and (2) varying degrees of vasculopathy, coagulopathy, and accompanying ischemic necrosis⁷. Standardized scoring of total histopathological changes and categorically by organ systems; lymphoid tissue (spleen and lymph nodes), and visceral organs (lung, liver, kidney) are summarized in Fig. 5a, b and Supplementary Fig. 6b. Pigs vaccinated with the two different Quil-A-adjuvanted RC-Ad5-ASFV cocktails (G1 and G2) and the CC spreaders exhibited moderate to severe lesions, with significantly elevated mean histopathology scores of 72, 68, and 82, respectively ($p < 0.0001$; Fig. 5a). Mock-vaccinated pigs (G4) showed modestly elevated histopathology scores (mean = 40), whereas G3 pigs vaccinated without Quil-A exhibited the lowest histopathology scores (mean = 14), which approached statistical significance compared to the G4 pigs ($p = 0.057$; Fig. 5a). The single non-survivor in G3 (pig #37), which failed to control viremia and was euthanized at 11 days post-challenge (DPC) of the CC spreaders, showed minimal lympholysis and moderate visceral pathology (score = 25), similar to the survivors in this group (Fig. 5a). Scores of 20 or less in the surviving G3 pigs and the two negative control animals are minimal to mild inflammatory changes with the absence of vascular and necrotic lesions attributed to ASF. Representative histology of spleen and gastrohepatic lymph nodes from G3 survivors showed intact lymphoid architecture (e.g., pigs #25, #27, #44) or moderate hyperplasia (e.g., pigs #35, #48) (Fig. 5c; Supplementary Figs. 8, 9).

Pigs with histopathology scores >49 exhibited moderate to severe lesions consistent with acute ASF that include lympholysis and lymphoid depletion in the spleen and lymph nodes, accompanied by ischemic necrosis and vasculopathy. In advanced cases, nearly complete loss of splenic lymphocytes in both follicles and periarteriolar lymphoid sheaths (e.g., pigs #46/

G1, #33/G2, #42/G4, #28 and #21/CC spreader) was accompanied by hemorrhage, fibrin deposition in splenic cords and sinuses, and septal and vascular necrosis (e.g., pigs #45/G1, #32/G2, #41/G4, and most CC spreaders) (Fig. 5c). Lymph nodes displayed similar findings, including hemorrhage, fibrin deposition, and multifocal necrosis (Supplementary Fig. 9). Vascular injury included hyalinization and necrosis of small- to medium-sized arteries, moderate erythrocyte extravasation to frank hemorrhage, with fibrin and edema accumulation (Supplementary Fig. 9; e.g., pigs #29, #45, #41, #43). Histiocytolysis was observed in more intact lymph nodes as swollen, pale histiocytes, or fragmented debris within germinal centers (Supplementary Fig. 9; e.g., pigs #23, #42, #42, #23).

Visceral pathology associated with acute ASF and coagulopathy is shown in Supplementary Fig. 6h. In the skin (Supplementary Fig. 7), numerous fibrin thrombi were identified in dermal capillaries and arterioles, with evidence of early epithelial degeneration (pig #46). Liver lesions (Supplementary Fig. 7) included mild to moderate cholangiohepatitis with necrotic inflammatory infiltrates extending beyond the limiting plate and scattered lobular hepatocyte necrosis (Supplementary Fig. 7, panels C, C1–C2). The kidneys showed multifocal to coalescing hemorrhagic infarcts, with fibrin thrombi in glomerular and interstitial vessels, accompanied by glomerular necrosis (Supplementary Fig. 7, panels D, D1–D2). In the colon, hemorrhagic mucosal infarcts were observed, secondary to thrombi in submucosal and mucosal vessels (Supplementary Fig. 7, panels E, E1–E2). Pulmonary lesions included fibrinocellular thrombi in alveolar capillaries and veins (panel F, F1), mild to moderate pulmonary edema, intra-alveolar hemorrhage, and necrotizing alveolitis.

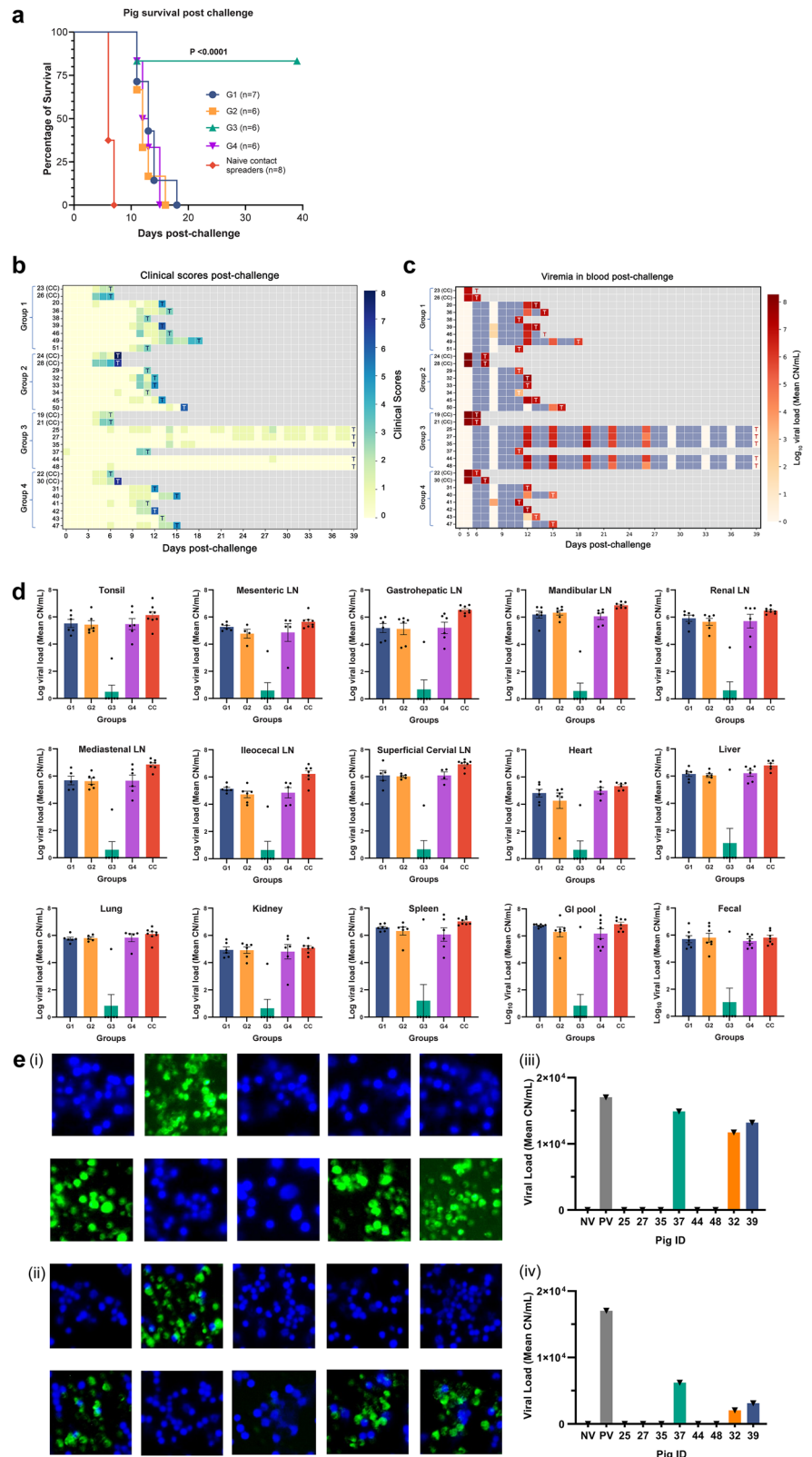
Histological changes consistent with chronic ASFV disease were not observed in G3 survivors. In contrast, severe lesions characteristic of acute ASFV infection were evident in other groups. Representative histopathology from a severely affected tonsil of pig #36/G1 is shown in Supplementary Fig. 7, panel G, with prominent fibrin thrombi in submucosal arteries (panels G and G1; arrowhead), extensive necrosis of septa and lymphoid cords between crypts and follicles (panels G, C–F, and G2, F), and abscessed crypts distended with necrotic debris and degenerate inflammatory cells.

Characterization of the immune response induced by RC-Ad5-ASFV

The neutralizing capacity of antibodies elicited by the different RC-Ad5-ASFV cocktails was assessed using primary swine macrophages. Despite robust antibody binding to wild-type ASFV-infected cells (Fig. 2c), none of the sera, including those from five of six protected pigs, blocked infection or neutralized the virus, even at a 1:1 dilution of the sera [Fig. 6a(i)]. qPCR analysis confirmed equivalent levels of viral replication in macrophages regardless of whether sera were heat-inactivated or not [Fig. 6a(ii–iii); Supplementary Table 10], suggesting that antibody-mediated virus neutralization was likely not a contributing mechanism of protection.

Cytotoxic T lymphocytes (CTLs) are important for eliminating ASFV-infected cells^{24–26}. A flow cytometric assay for granzyme B (GrB), a central CTL effector molecule³⁴, would enable quantification of GrB⁺ T cells. To evaluate the role of T cell-mediated immunity, a porcine-specific monoclonal antibody against GrB (clone 13D12-2) was developed and

Fig. 3 | Post-challenge survival, clinical outcomes, and viremia profiles. **a** Kaplan–Meier survival analysis. Survival curves of pigs in different treatment groups following contact challenge with ASFV Georgia 2007/1. Five of six pigs in Group 3 (RC-Ad5-ASFV without adjuvant) survived, whereas all pigs in Groups 1, 2, and 4 succumbed. Contact-challenge (CC) pigs (in red) were intramuscularly inoculated with ASFV and euthanized on days 5–7 post-infection due to severe symptoms. **b** Clinical scores. Heatmap of daily clinical scores post-challenge. CC pigs developed ASF symptoms by day 5 and were euthanized by day 7. All immunized pigs in Groups 1, 2, and the controls in Group 4 showed progressive disease and were terminated by day 18. One pig (#37) in Group 3 was euthanized on day 11; the remaining five showed only mild symptoms and survived until day 39. Termination days are marked with “T”. **c** Viral load in blood. qPCR detection of ASFV DNA (p72) in whole blood post-challenge. Viremia was detected by day 5 in CC pigs and by day 12 in most pigs from Groups 1–4. The five survivors from Group 3 cleared the virus by day 26. Heatmap indicates log₁₀ viral loads (mean CN/mL), with yellow indicating low and red indicating high viremia. Blue squares denote non-sampling days; “T” indicates termination day when pigs were euthanized. **d** Tissue viral load. Log₁₀-transformed ASFV DNA levels in necropsied tissues were assessed using qPCR. High viral loads were detected in all tissues from deceased pigs. No detectable virus was observed in any tissue from the five surviving pigs in Group 3. **e** Virus rescue assay. Primary swine macrophages incubated with splenic (i) or pericardial fluid (ii) samples from Group 3 survivors showed no virus replication by IFA. Samples from the deceased pig (#37) and CC pigs (#32 and #39) showed rescuable virus. Virus absence in survivor samples was confirmed by qPCR (iii, iv). RC-Ad5 replication-competent adenovirus serotype 5, ASFV African swine fever virus, IFA immunofluorescence assay, qPCR quantitative polymerase chain reaction, CC contact challenge, CN copy number.



employed in intracellular cytokine staining assays (Supplementary Fig. 10). Peripheral blood mononuclear cells (PBMCs) from vaccinated pigs restimulated in vitro with recombinant adenovirus expressing ASFV antigens or ASFV, exhibited GrB⁺CD8⁺ and interferon gamma-producing (IFN- γ) CD8⁺ T cell recall responses against predicted swine leukocyte antigen class I (SLA-I)-restricted peptides (Fig. 6b). Epitope mapping identified

three immunodominant peptides derived from ASFV protein A859L^{156–164} (STDETRTAI), A859L^{428–436} (LVDQGVYAL), and A859L^{573–581} (QLARHAIRY) that consistently recalled both GrB⁺CD8⁺ and IFN- γ CD8⁺ T cells (Fig. 6c; Supplementary Table 11). Post-challenge, direct stimulation of PBMCs from the five surviving (G3) pigs with ASFV exhibited GrB⁺CD8⁺ T cell responses on days 26 and 33 [Fig. 6d(i)]. At necropsy on

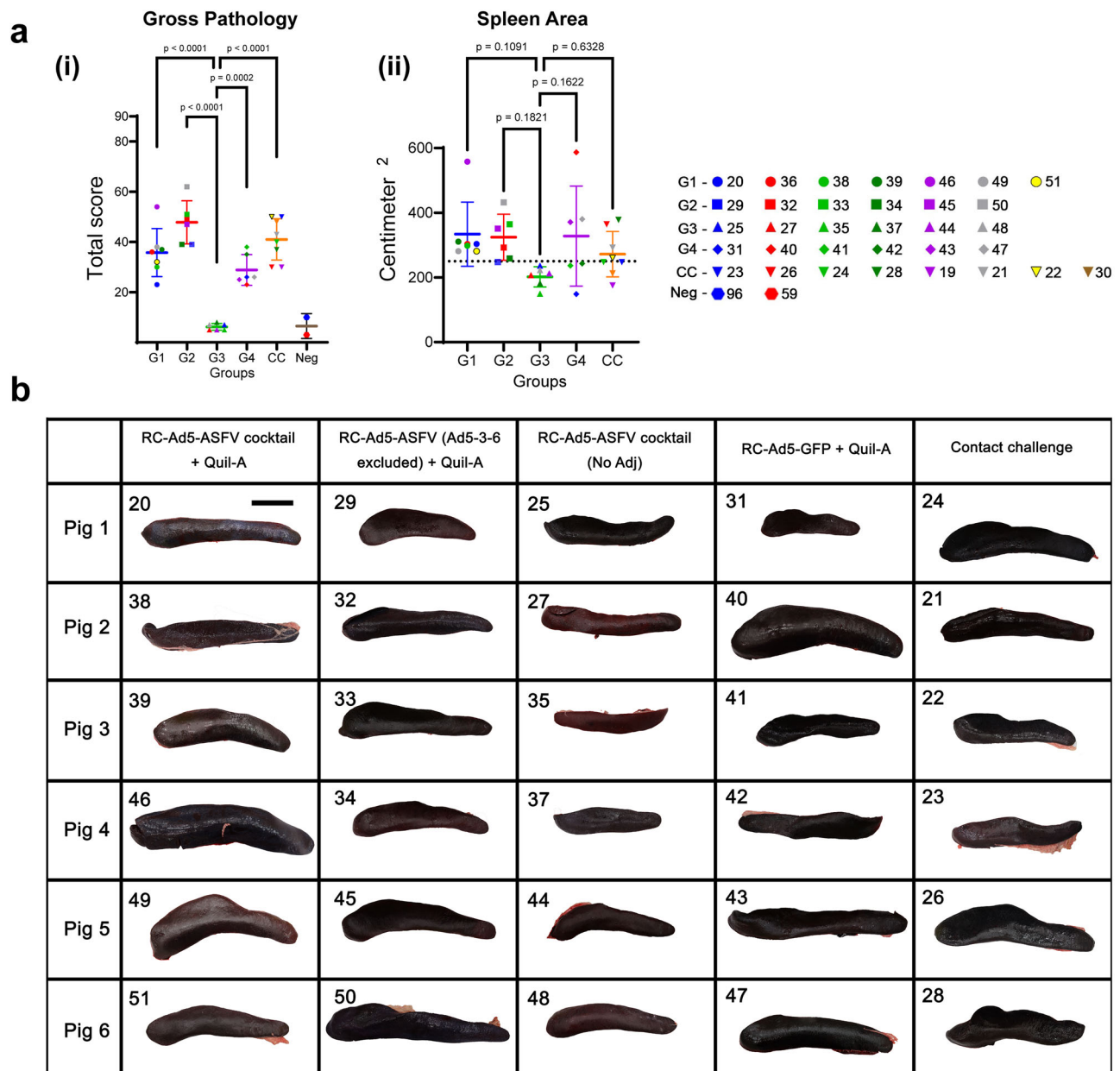


Fig. 4 | Gross pathological lesions in ASFV-challenged pigs. a i Gross pathology scores. Organ systems were scored at necropsy using established ASFV gross pathology criteria (Supplementary Table 6). Pigs in Group 3 (non-adjuvanted RC-Ad5-ASFV) showed no gross lesions, while Groups 1, 2, 4, and contact challenge (CC) pigs developed moderate-to-severe pathology ($p < 0.001$). Notably, pig #37 from Group 3, which failed to clear viremia and progressed to clinical ASF, also lacked overt gross pathology attributed to ASF. Mean spleen area, a marker of splenomegaly, was not significantly different among groups, but Group 3 spleens

tended to be smaller (see Supplementary Table 8). **a ii, b** Representative gross spleen images. Spleens from Group 3 pigs appeared grossly normal with areas below the 250 cm² cutoff (indicated by the dotted line). Groups 1, 2, 4, and CC exhibited hallmark ASF lesions: splenic enlargement, dark discoloration, and friability. Nearly all pigs in the CC group had moderate to marked enlarged dark red and friable spleens, with spleen areas >250 cm² and gross spleen scores >4 (Supplementary Table 8), consistent with ASFV splenomegaly and necrosis. Scale bar = 5 cm.

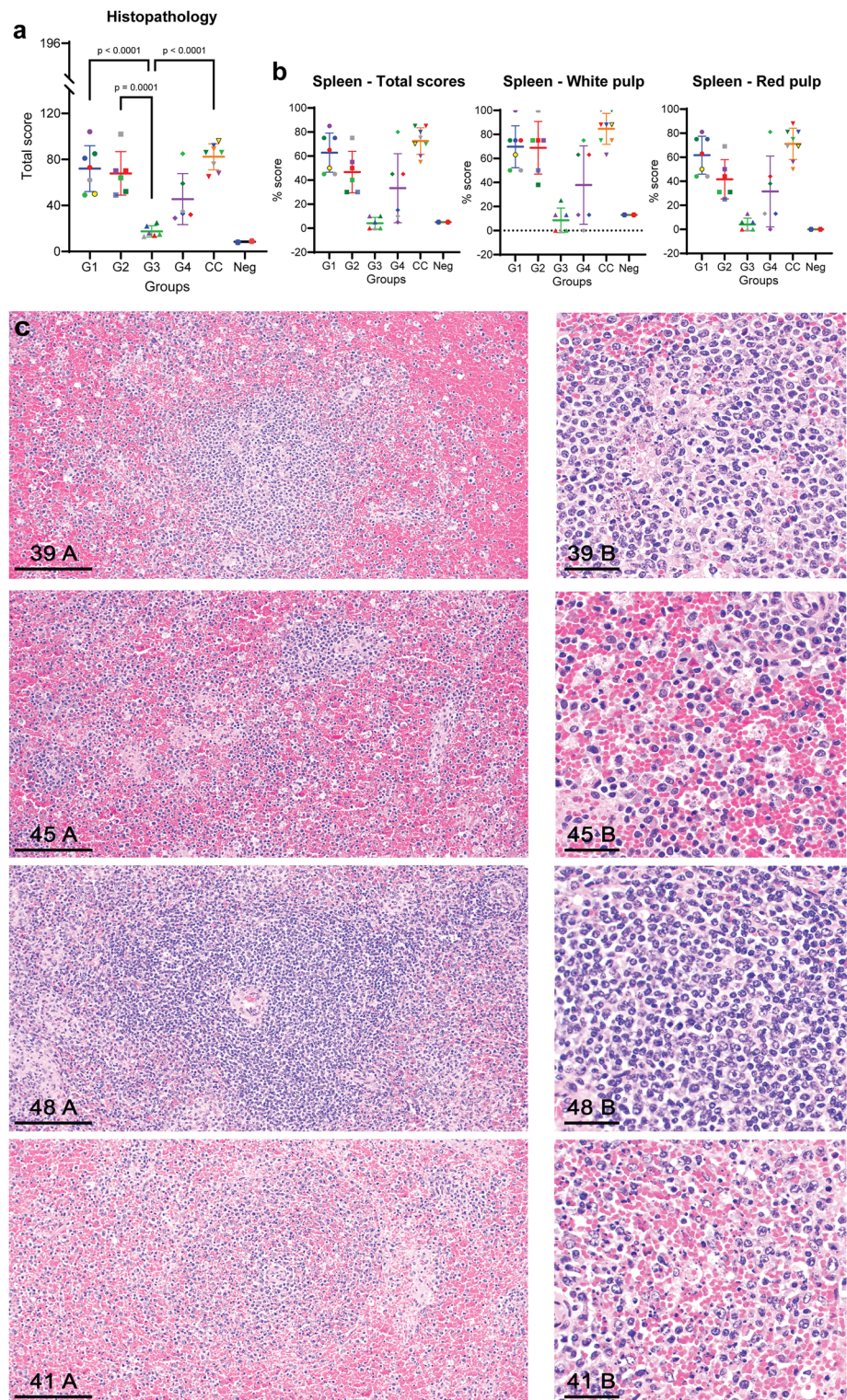
day 39, a higher number of ASFV-specific GrB⁺CD8 α ⁺ T cells were detected in splenocytes from four survivors [Fig. 6d(ii)]. Moreover, recall epitope-specific GrB⁺CD8 α ⁺ and perforin-positive (Perf⁺) CD8 α ⁺ T cells targeting A859L¹⁵⁶⁻¹⁶⁴ and A859L⁴²⁸⁻⁴³⁶ were detected in ASFV-stimulated PBMCs and splenocytes at the study endpoint (Fig. 6e).

SLA class I and class II genotyping of pigs from protected (G3) and non-protected (G1) groups revealed no definitive correlation between haplotype and survival. Both groups shared several SLA-I and SLA-II alleles (Table 1). The 12 pigs analyzed (25% Landrace, 25% Yorkshire, 50% Duroc) represented 11 distinct SLA-I and 11 SLA-II haplotypes (Supplementary

Table 13). Two SLA-I haplotypes (Hp-04.0 and Hp-35.0) accounted for 54.2% of class I diversity, while three SLA-II haplotypes (Hp-0.05, Hp-0.02, and Hp-0.15b) encompassed 50% of class II diversity. Notably, the three immunodominant peptides A859L¹⁵⁶⁻¹⁶⁴ (STDETRTAI), A859L⁴²⁸⁻⁴³⁶ (LVDQGVYAL), and A859L⁵⁷³⁻⁵⁸¹ (QLARHAIRY) were predicted to be strong binders of SLA-1*04:01 (Supplementary Table 12), an allele expressed by all G3 survivors but not by the single G3 non-survivor. However, SLA-1*04:01 was also found in some non-survivors in G1, suggesting that while SLA-1*04:01 may contribute to protective T cell responses, it is not solely predictive of protection.

Fig. 5 | Histopathology in ASFV-challenged pigs.

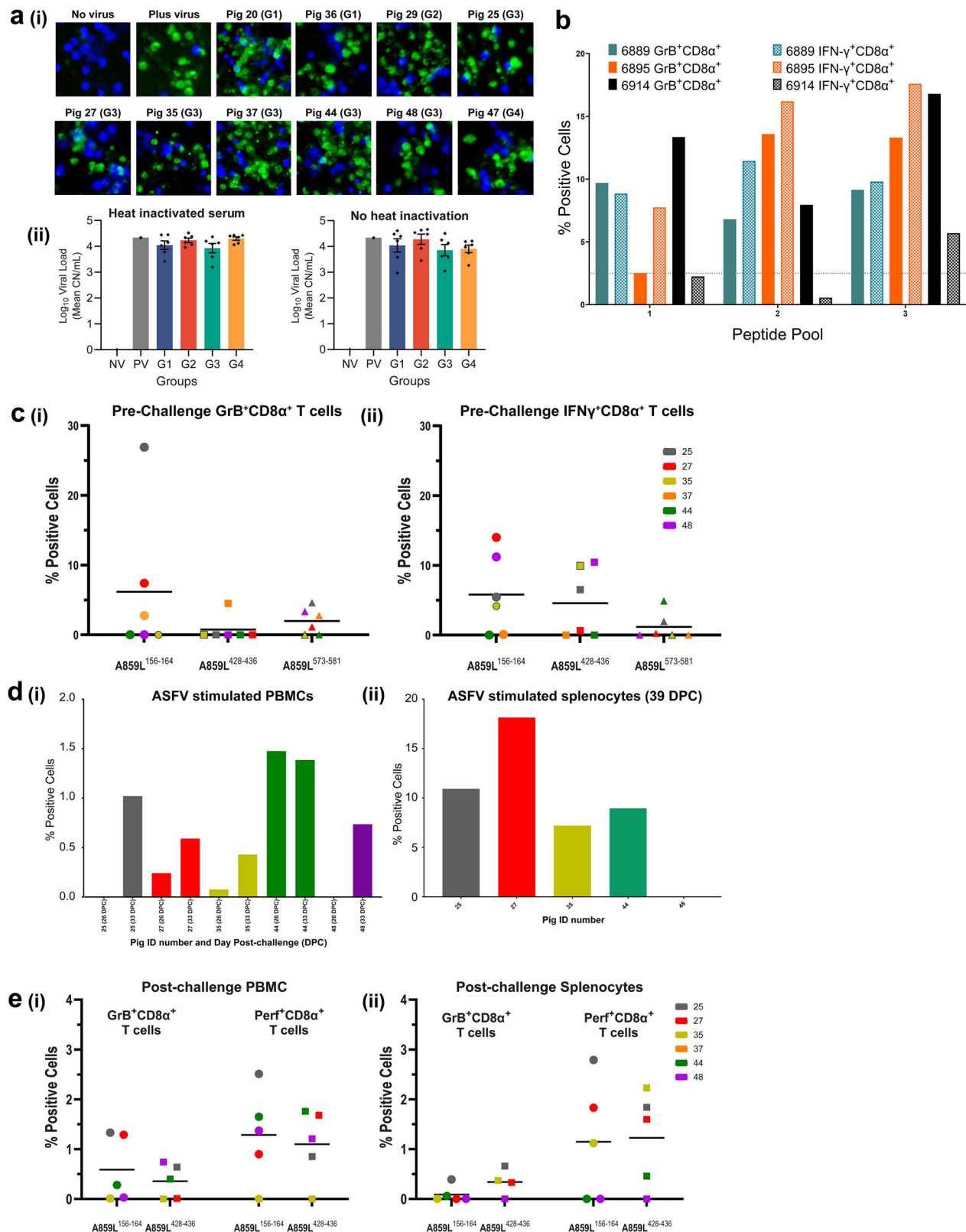
a Histopathological scores. Relevant organ systems were scored for ASFV histological changes according to criteria in Supplementary Table 7. Total ASFV histopathological scores were significantly lower in Group 3 (mean = 15) than in the other groups ($p < 0.001$). Scores of 20 or less consist of minimal to mild inflammatory changes with the absence of vascular and necrotic changes attributed to ASF. Pig #37 from Group 3 also lacked significant histological lesions despite clinical progression. **b** Spleen histopathology scores and white (lymphoid) and red pulp (vascular) compartments. Mean spleen scores in Group 3 were 1/20, compared to 13 (G1), 9 (G2), 7 (G4), and 14 (CC), reflecting minimal lymphoid and red pulp changes in vaccinated survivors. **c** Representative spleen histology. Five of six Group 3 pigs showed minimal histologic lesions. Pig #37 had mild germinal center necrosis (37A–B) (Supplementary Fig. 8). Pigs #35 and #48 showed moderate lymphoid hyperplasia without typical ASF lesions. In contrast, pigs from other groups showed severe ASF pathological changes, lympholysis and white pulp depletion, fibrin deposition, necrosis, and hemorrhage. Severe cases showed near-complete obliteration of the white pulp and fibrinoid necrosis of the red pulp (e.g., pigs #46 G1, #33 G2, #42 G4, #22/24 CC). Mild-to-moderate lesions were observed in pigs #45 G1, #32 G2, #40 G4, and #24 CC. Additional spleen histology is provided in Supplementary Fig. 8. Hematoxylin and eosin stain, Scale bar = 20 μ m. ASF African swine fever, ASFV African swine fever virus, RC-Ad5 replication-competent adenovirus 5, CC contact-challenged, G1–G4 Groups 1–4, H&E hematoxylin and eosin.



Discussion

Effective and safe vaccines are urgently needed to combat ASFV, a persistent and significant threat to global swine production. Although some gene-deleted attenuated ASFV mutants have demonstrated promising protective efficacy, safety concerns of reversion to virulence and the potential for recombination in the field leading to more virulent strains persist^{16–19}. Subunit vaccines offer a safer alternative to live attenuated ASFV vaccines. While several virus-vectored subunit candidates have conferred some protection against ASFV genotype I, they have consistently failed to protect

against the more virulent genotype II strain^{29,30}. Despite their favorable safety profile, subunit vaccine development remains challenging due to limited understanding of protective immune correlates, the complex nature of ASFV, and the absence of well-defined protective antigens^{14,20}. Given these challenges and the failure of replication-deficient adenoviral vectors encoding multiple ASFV antigens to protect against virulent genotype II strains^{29,30}, we developed a panel of attenuated RC-Ad5 constructs encoding the ASFV Georgia 2007/1 proteome. Each construct included a multicistronic cassette encoding up to nine codon-optimized ASFV antigens,



along with co-expression of the adenoviral early region 1A (E1A) and 1B (E1B) genes, separated by a self-cleaving P2A peptide. This approach restored replication competence in primary swine cells.

The P2A self-cleaving peptide enables efficient cleavage between adjacent protein products and is particularly well-suited for multicistronic expression, allowing efficient and coordinated expression of multiple

full-length antigens from a single transcript^{35,36}. This strategy provides a rational approach to broaden immune coverage against a complex virus like ASFV, which likely requires targeting of multiple viral proteins to achieve effective protection. All 43 RC-Ad5 constructs successfully expressed ASFV antigens in HEK293A cells, as validated by immunocytochemistry using tag-specific mAbs and convalescent swine serum from a hyperimmunized

Fig. 6 | Protective role of immune responses induced by the RC-Ad5-ASFV cocktail. **a** Lack of virus neutralization by vaccine-induced antibodies. (i) IFA of ASFV-infected primary swine macrophages revealed no in vitro neutralizing activity in sera from either protected (G3) or non-protected (G1, G4) pigs, even at a 1:1 dilution. (ii–iii) ASFV p72 DNA was detected by qPCR in cultures treated with heat-inactivated (ii) or non-heat-inactivated (iii) sera, confirming absence of neutralization and complement activation. **b** Identification of candidate T-cell epitopes. PBMCs from Ad5-vaccinated pigs (6889, 6895, 6914)²⁸ were stimulated with ASFV (1 MOI) and screened for recall CD8 α ⁺ T-cell responses to peptide pools predicted to bind SLA-I as previously described²⁸ (Pools 1–3; see Supplementary Table 7). Responses were measured via flow cytometry for intracellular Granzyme B (GrB) and IFN- γ . **c** Peptide-specific recall CD8 α ⁺ T-cell responses post-immunization. PBMCs from survivor pigs (1 week after the second boost) were stimulated with RC-Ad5-26 (A859L, B318L, B169L) and tested for reactivity to peptides from Pool 3. Three epitopes elicited robust GrB/IFN- γ ⁺ CD8 α ⁺ T-cell responses:

A859L^{156–164} (STDETRTAI), A859L^{428–436} (LVDQGVYAL), and A859L^{573–581} (QLAR-HAIRY). Data are presented as percentage positive cells after subtracting baseline mean response from RC-Ad5-GFP-immunized animals. **d** GrB⁺CD8 α ⁺ T-cell responses post-challenge. Robust recall GrB⁺CD8 α ⁺ responses were detected in PBMCs at days 26 and 33 post-challenge (i) and in splenocytes at study termination (ii), following ASFV stimulation in vitro (1 MOI). **e** Peptide-specific GrB⁺ and Perforin⁺ T-cell responses post-challenge. Terminal PBMCs (i) and splenocytes (ii) from survivors stimulated with ASFV or specific peptides (A859L^{156–164}; STDETRTAI; and A859L^{428–436}; LVDQGVYAL) showed antigen-specific GrB⁺ and Perforin⁺CD8 α ⁺ T-cell responses, confirming cellular immune memory. ASFV African swine fever virus, RC-Ad5 replication-competent adenovirus 5, NV no virus, PV plus virus, PBMCs peripheral blood mononuclear cells, IFA immunofluorescence assay, GrB granzyme B, Perf perforin, IFN- γ interferon gamma, SLA swine leukocyte antigen, MOI multiplicity of infection, ICS intracellular staining, qPCR quantitative polymerase chain reaction.

Table 1 | Lack of correlation between SLA haplotypes and survival following RC-Ad5-ASFV immunization

Pig ID	SLA Class I					SLA Class II				Group	Protection
	SLA-1	SLA-3	SLA-2	Hp	SLA-6	DRB1	DQB1	DQA	Hp		
36	04:01	04:01	04:01	04:0	ksu01	04:02	02:02	02:02:02	0.15b	1	No
	08:03 or 08:10	06:01	12:01–02	22.0		04:02	02:02	02:02:02	0.15b		
38	12:01, 13:01	05:02	10:01	35.0	ksu01	10:06	06:03	01:01–02	0.23		
	08:01	07:01	08:01	45.0		09:01	04:02	03:01	0.11		
39	04:01	04:01	04:01	04:0	ksu01	Blank	09:01	04:01	0.29		
	08:06 or 08:08	05:02–04	Blank	49.0		05:01	02:01	02:02:02	0.05		
46	04:01	04:01	04:01	04:0	ksu01	05:01	02:01	02:02:02	0.05		
	04:01:02	04:03	11:05	75.0		05:01	02:01	02:02:02	0.05		
49	04:01	04:01	04:01	04:0	ksu02	02:01	02:01	02:01	0.02		
	08:06 or 08:08	05:02–04	09:01–02	29.0mod		01:04	04:01:01–02	02:06	0.35		
51	08:09–10	04:03	09:01–02	CH-3.0	ksu03	01:04	05:01–02	04:01	0.21		
	12:01	04:04	06:01–02	46.0		Blank	05:03	02:05	30.0mod		
37	12:01	04:04	06:01–02	46.0	ksu03	04:03	03:03	02:04:02	0.13	3	Yes
	15:01–3	07:01:02	16:02	31.0/63.0		01:04	05:01–02	04:01	0.21		
25	12:01, 13:01	05:02	10:01	35.0	ksu01	10:06	06:03	01:01–02	0.23		
	04:01	04:01	04:01	04:0		08:01 or 09:06	02:02–03	02:03	0.8 or NN		
27	04:01	04:01	04:01	04:0	ksu01	05:01	02:01	02:02:02	0.05		
	04:01	04:01	04:01	04:0		02:01	02:01	02:01	0.02		
35	04:01	04:01	04:01	04:0	ksu02	02:01	02:01	02:01	0.02		
	15:01–03	07:01:02	16:02	31.0/63.0		04:02	02:01	02:02:02	0.15b		
44	04:01	04:01	04:01	04:0	ksu01	05:01	02:01	02:02:02	0.05		
	12:01, 13:01	05:02	10:01	35.0		02:01	02:01	02:01	0.02		
48	04:01	04:01	04:01	04:0	ksu01	Blank	09:01	04:01	0.29		
	11:03	05:03	16:02	59.0		08:01 or 09:06	02:02–03	02:03	0.8 or NN		

SLA class I and II haplotypes were compared between six non-survivors in Group 1 and six pigs in Group 3 (five survivors) to assess any association with vaccine-mediated protection. Both groups received the same RC-Ad5-ASFV constructs, with Group 1 also receiving Quil-A adjuvant. LRYSD: 25% Landrace, 25% Yorkshire, 50% Duroc; SLA: Swine Leukocyte Antigen; Hp: Haplotype; ksu: Kansas State University; Blank: Undetermined alleles.

donor²⁷. Moreover, all constructs replicated efficiently in primary swine alveolar epithelial cells and expressed ASFV antigens, as confirmed via immunofluorescence and flow cytometry. The RC-Ad5 constructs were further attenuated through deletion of the E3 adenoviral transcriptional

unit, which encodes proteins involved in immune evasion and host modulation^{37,38}. Replication-competent adenoviral vectors have been shown to elicit more robust humoral and cellular immune responses than replication-incompetent platforms^{31,39}. Deletion of the E3 gene enhances the

safety profile of these vectors while maintaining their capacity for in vivo replication and antigen amplification. This approach may simulate aspects of attenuated ASFV infection, enhancing the priming of virus-specific cytotoxic and helper T lymphocytes. Notably, E3-deleted adenoviral vectors have demonstrated favorable safety and immunogenicity in multiple pre-clinical models and clinical trials^{31,38}.

The second objective was to determine whether immunization of commercial piglets with a cocktail of replication-competent RC-Ad5 constructs (RC-Ad5-ASFV) could safely elicit ASFV-specific immune responses and protect against acute or chronic disease following challenge with virulent genotype II ASFV (Georgia 2007/1). Three doses of the RC-Ad5-ASFV constructs, with or without the Quil-A adjuvant, were well tolerated, and no viral shedding was detected in nasal secretions, suggesting that E3 deletion rendered the recombinant viruses susceptible to immune clearance, consistent with previous reports on this vector platform's in vivo safety^{31,37,38}. Significant ($p < 0.0001$) ASFV-specific IgG responses were elicited within 2 weeks post-priming and boosting further increased IgG titers ($p < 0.05$), indicating effective B cell activation and class switching. Although antigen-specific antibody responses to each encoded protein were not individually quantified, sera from vaccinated pigs strongly recognized representative antigens B385R, B175L, D205R, and p62, as well as ASFV-infected primary swine macrophages, confirming effective in vivo expression by the adenoviral constructs. For a complex pathogen like ASFV with numerous proteins and undefined protective determinants, multicistronic antigen delivery offers a rational strategy for identification of subunit vaccine candidates. This is consistent with previous demonstrations that adenoviral vectors encoding multiple antigens have been successfully employed to induce broad immune responses for vaccine development for both infectious and non-infectious diseases⁴⁰.

Histopathological analysis ruled out the development of chronic ASFV disease in surviving animals. In contrast, characteristic lesions associated with acute ASFV infection were prominent in pigs immunized with adjuvanted RC-Ad5-ASFV constructs (G1 and G2), which exhibited higher gross and histopathological scores than mock-vaccinated controls (G4). The severity and distribution of lesions in these groups closely resembled those observed in intramuscularly challenged CC spreader pigs receiving high-dose ASFV, underscoring the failure of these adjuvanted formulations to mitigate acute disease. While not statistically significant, this trend suggests that the Quil-A adjuvant may have exacerbated disease severity following natural transmission, a finding consistent with prior reports of vaccine-enhanced disease in vaccine trials^{22,41}.

This study employed a natural contact challenge model using ASFV-infected shedders, mimicking the dominant mode of transmission in both endemic and non-endemic settings, where pig-to-pig spread is common in the absence of tick vectors^{8,42–44}. Previous studies using this transmission model and ASFV strain demonstrated a rapid onset of severe, lethal acute ASFV disease within 10–14 days post-exposure²⁸. Under these conditions, five of six pigs immunized with the non-adjuvanted RC-Ad5-ASFV cocktail cleared viremia and showed no gross or histological lesions consistent with acute or chronic ASF. A major challenge in developing safe and effective ASFV vaccines, including modified live and live-vectored subunit vaccines, is the risk of inducing chronic ASF disease, which can lead to chronic inflammatory or lymphoproliferative pathology, severe secondary bacterial infections, and viral persistence within the host and population^{45,46}. To our knowledge, this is the first report demonstrating that a live-vectored vaccine cocktail encoding the ASFV proteome can confer protection against the highly virulent genotype II Georgia 2007/1 strain, an outcome not achieved in previous studies using similar platforms^{28–30,47}. Prior failures may have resulted from inadequate immune priming, suboptimal antigen coverage, or vector systems incapable of efficiently inducing effective protective immunity.

Gross and histopathological analyses at study termination revealed marked differences between survivors (resolved ASF) and non-survivors (succumbed to ASF and were humanely euthanized) following ASFV challenge. Pigs that succumbed, including CC spreaders, RC-Ad5-GFP

controls, and Groups 1 and 2 immunized with Quil-A-adjuvanted RC-Ad5-ASFV, exhibited classic peracute-to-acute ASF lesions, including severe splenomegaly, hemorrhagic lymphadenopathy, and multifocal hepatic and renal necrosis^{28,48,49}. Marked splenomegaly was observed in all pigs from G1 and G2 and in over half of G4 and CC spreaders. Histologically, spleens showed diffuse congestion, severe lymphoid depletion, and loss of follicular architecture, consistent with ASFV's tropism for mononuclear phagocytes and its immunosuppressive pathology^{48,49}. Lymph nodes exhibited extensive hemorrhage, necrosis, and fibrin deposition, indicative of vascular injury and consistent with severe ASFV-induced vasculopathy⁴⁸. In contrast, the five surviving pigs immunized with the RC-Ad5-ASFV cocktail without Quil-A displayed intact lymphoid architecture, minimal histological damage, and only mild to moderate lymphoid hyperplasia, supportive of immune activation. The absence of lesions, ASFV DNA, and recoverable virus confirmed effective viral clearance and protection conferred by the non-adjuvanted vaccine constructs. Direct comparison of viral DNA in tissues is limited by differing necropsy time points between survivors and non-survivors. Among lymphoid tissues, the gastrohepatic, mandibular, and renal lymph nodes were most severely affected in non-survivors, consistent with prior reports on genotype II ASFV pathology⁴³. Further pathological analyses may help elucidate viral distribution and host responses.

Despite eliciting ASFV-specific IgG responses, similar antibody profiles were observed in sera from survivors, non-survivor pig #37, and vaccinees in Groups 1 and 2. However, none of the sera, regardless of dilution or heat inactivation, neutralized ASFV or inhibited macrophage infection in vitro. Viral genome copies in macrophages remained unchanged, suggesting that antibody responses did not mediate virus neutralization. Although antibody-dependent cellular cytotoxicity was not assessed, the lack of correlation between IgG levels and survival suggests that antibodies were unlikely to be the primary protective mechanism. Anamnestic responses were not evaluated during early post-exposure viremia, but survivors in Group 3 appeared to mount a rapid, effective immune response that limited disease progression, cleared viremia, and prevented pathology. These results align with prior studies suggesting minimal involvement of antibodies in ASFV^{21,23,27,28,30,41,50,51}, though they contrast with others reporting a protective role for ASFV-specific antibodies^{52–54}. One explanation may be that in vivo expression of ASFV antigens via the RC-Ad5-ASFV platform did not fully replicate conformational epitopes presented by the whole attenuated virus. Notably, studies supporting antibody-mediated protection via passive serum transfer often lack conclusive evidence excluding residual live virus in donor serum^{52–54}. Thus, while our data support a limited role for antibodies, their contribution cannot be definitively excluded, and further studies are warranted to clarify their involvement in ASFV immunity.

The absence of neutralizing activity in sera from survivors suggests that protection was primarily mediated by cellular immunity, particularly cytotoxic T cells targeting ASFV-infected cells. This supports current models proposing T cell-mediated mechanisms as central to protection against ASFV^{21,24–26,30,44,47,51,55}. Three conserved epitopes from ASFV antigen A859L (STDERTAI, LVDQGVYAL, QLARHAIRY) elicited GrB⁺CD8α⁺ and IFN-γ⁺CD8α⁺ responses in ASFV-stimulated PBMCs from G3 vaccinees prior to challenge. These epitopes showed strong predicted binding to multiple SLA-I alleles and were conserved across ASFV strains, supporting their relevance for broadly protective subunit vaccines. Robust GrB⁺CD8α⁺ responses were detected in ASFV-stimulated PBMCs from all five survivors on days 26 and 33 post-challenge, and GrB⁺CD8α⁺ T cells were enriched in splenocytes from four survivors at necropsy (day 39). These three conserved A859L-derived peptides also induced GrB⁺CD8α⁺ and PerF⁺CD8α⁺ responses in both PBMCs and splenocytes. ASFV-specific responses were recalled after 5-day stimulation with live virus, whereas peptide-specific responses were selectively observed in ASFV-stimulated lymphocytes. This is expected, as total virus-specific memory typically outnumbers individual epitope-specific memory cells. Although cytotoxic activity was not directly measured, the presence of GrB⁺ and PerF⁺ CD8α⁺ T cells is consistent with a CTL

phenotype. These responses were absent in RC-Ad5-GFP-vaccinated controls, supporting vaccine-primed T cell immunity. We have previously shown that adenoviral vectors encoding ASFV antigens can elicit CTLs in pigs, albeit at a low frequency^{50,56}. Given the complexity of the ASFV proteome, flow cytometric detection of GrB⁺Perf⁺ CD8 α ⁺ T cells offers a practical first step in identifying antigens associated with protective responses. Protection did not correlate with SLA haplotypes, as both survivors in G3 and non-survivors in G1 immunized with the same antigen cocktail shared identical SLA-I and SLA-II alleles. This aligns with previous findings showing that protection conferred by a live-vectored subunit vaccine did not correlate with SLA genotypes following ASFV genotype I challenge³⁰. While the A859L-derived epitopes were predicted to bind strongly to SLA-I*04:01, present in all G3 survivors but absent in the lone non-survivor from G3, this allele was also detected in non-survivors from G1. Thus, while SLA-I*4:01 may contribute to antigen presentation and T cell activation, it is not solely predictive of protection. It is also possible that the Quil-A-formulated RC-Ad5-ASFV cocktail elicited antibody responses but failed to induce protective T-cell responses due to viral inactivation⁵⁷.

Protection observed in five of six pigs immunized with the RC-Ad5-ASFV cocktail without Quil-A, but not in those receiving the adjuvanted formulation, was unexpected. Despite comparable ASFV-specific IgG responses across groups, full cellular immune profiling was not performed in all vaccinees, leaving group-specific T cell differences unresolved. In pigs, IgG2 is typically associated with T helper 1 (Th1)-biased responses and IgG1 with Th2 responses^{58,59}. Quil-A, a saponin-based adjuvant approved for veterinary use, promotes IgG2 production and enhances cell-mediated immunity, including macrophage and CTL activation^{32,33}. However, the lack of protection in Quil-A-adjuvanted groups suggests that the adjuvant may have interfered with RC-Ad5-ASFV viral entry or replication, potentially through virion inactivation, as reported previously⁵⁷.

While the observed efficacy of the RC-Ad5-ASFV cocktail is promising, several limitations must be acknowledged. The formulation was tested only against the ASFV Georgia 2007/1 strain; further studies are needed to assess cross-protection against other circulating genotypes to determine broader protective potential. The small sample size limits generalizability, highlighting the need for larger-scale evaluation. More importantly, the formulation contained 43 antigen constructs, but the contribution of individual antigens to protection was not dissected. Identifying key protective antigens will aid in streamlining future vaccine development. Although GrB⁺CD8⁺ T-cell responses were detected in survivors, the mechanistic insights remained incomplete, and comprehensive T-cell profiling was limited by the loss of key frozen samples.

Protective immunity against ASFV in pigs has been reported using live-vectored subunit vaccine approaches, although protection has been variable and context dependent^{29,30,60}. Heterologous rAd5/MVA and homologous rAd5 prime-boost intramuscular immunization strategies targeting multiple ASFV antigens protected pigs against genotype I challenge; however, the homologous rAd5 prime-boost regimen failed to confer protection against genotype II, underscoring the challenges of achieving cross-genotype immunity²⁹. These observations are consistent with our previous findings that homologous prime-boost intramuscular immunization with a replication-deficient rAd5 antigen cocktail, despite overlapping antigen content, was insufficient to confer protection against genotype II²⁸. In contrast, the robust protection against genotype II observed with the replication-competent Ad5-ASFV cocktail in the present study suggests that vector replication may be an important determinant of protective efficacy. Notably, a homologous prime-boost intramuscular/intranasal regimen using replication-deficient rAd2 encoding five ASFV antigens conferred protection against genotype II challenge, despite these antigens being included in our non-protective rAd5 cocktail²⁸, suggesting that vector-specific properties and immunization route, rather than antigen composition alone, likely contribute to protective efficacy⁶⁰.

In conclusion, this study demonstrates that immunization with a cocktail of replication-competent adenovirus type 5 (RC-Ad5) constructs encoding the ASFV Georgia 2007/1 proteome can confer protection against

lethal challenge in pigs. Notably, five of six animals vaccinated with the non-adjuvanted RC-Ad5-ASFV formulation survived virulent ASFV exposure, cleared viremia, and remained free of clinical disease and pathological lesions. Protection was not associated with neutralizing antibody activity but was associated with the induction of ASFV-specific GrB⁺CD8 α ⁺ and Perf⁺CD8 α ⁺ T cell responses, supporting a role for cellular immunity. The observed lack of protection in pigs receiving the Quil-A-adjuvanted formulation suggests that adjuvant interactions with live viral constructs may impair vaccine efficacy. These findings underscore the potential of live-vectored, multicistronic antigen delivery to elicit protective T cell responses and provide a rational framework for identifying and prioritizing protective antigens. Future studies are warranted to evaluate cross-protection against diverse ASFV strains, refine antigen selection, and optimize vaccine formulations for safe and broad field deployment.

Methods

Generation of recombinant adenoviruses encoding ASFV antigens

Recombinant attenuated RC-Ad5 constructs encoding ASFV Georgia 2007/1 antigens (GenBank: FR682468) were generated as previously described, with minor modifications⁶¹. Briefly, the ASFV proteome was used to design multicistronic cassettes separated by self-cleaving P2A linkers to enable co-delivery of multiple antigens within fewer viral constructs³⁶. Each cassette included an N-terminal HA-tag, a C-terminal FLAG tag, followed by P2A-E1a–P2A–E1b sequence to support replication in primary cells. Forty-three codon-optimized gene cassettes were synthesized for swine expression and cloned into the pcDNA3.1 vector (GenScript, Piscataway, NJ, USA). Protein expression was validated in HEK293A cells via immunocytochemistry using anti-HA and anti-FLAG mAbs (5 μ g/mL; Sigma-Aldrich, St. Louis, MO, USA), followed by confirmation with ASFV convalescent swine serum as previously described⁵⁰. Expression cassettes were transferred into the pDONR221 vector and then recombined into the pAd/CMV/V5-DEST adenoviral backbone using Gateway[®] cloning technology (Thermo Fisher Scientific, Waltham, MA, USA) as previously described⁶¹. Positive clones were selected based on protein expression and used to transfect HEK293A cells, resulting in the generation of 43 RC-Ad5 viruses (designated RC-Ad5-01 to RC-Ad5-43), as previously described^{50,61}. A control adenovirus expressing GFP (RC-Ad5-GFP) was generated using the same procedure. All viruses were amplified, purified by cesium chloride gradient centrifugation, titered, and stored at -80°C in 10% glycerol, following standard protocols^{50,61,62}.

Protein expression

HEK293A cells transfected with the pcDNA3.1 or pAd constructs, or infected with RC-Ad5-01 to RC-Ad5-43 viruses, were fixed in cold methanol for 10 min and blocked with 5% fetal bovine serum in phosphate-buffered saline containing 0.1% Tween-20 (PBST), as described previously^{28,61}. Cells were incubated with 5 μ g/mL of mouse anti-HA (Sigma-Aldrich, St. Louis, MO, USA) or anti-FLAG[®] M2-alkaline phosphatase (AP) antibody (Sigma-Aldrich, St. Louis, MO, USA), followed by PBST washes. In parallel, duplicate samples were probed with ASFV convalescent swine serum (1:250 dilution)⁵⁰. For detection, AP-conjugated goat anti-mouse IgG or goat anti-swine IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used, and AP activity was visualized with Fast Red TR/Naphthol AS-MX substrate (Sigma-Aldrich, St. Louis, MO, USA). GFP expression from the RC-Ad5-GFP virus was visualized using a CKX53 fluorescence microscope (Olympus Corporation, Tokyo, Japan), and images were captured with a MU1803-HS digital camera (AmScope, Irvine, CA, USA).

Virus replication in primary swine cells

Two RC-Ad5 constructs, RC-Ad5-03 (encoding p72-p15-B602L) and RC-Ad5-04 (encoding p62–p32–p54–EP153R–p10), were selected to evaluate replication in primary swine alveolar epithelial cells (Cell Biologics, IL, USA) via IFA. Replication-deficient (RD)-Ad5 constructs expressing the same

antigens (RD-Ad5-03 and RD-Ad5-04) were used as controls. Cells were infected at a multiplicity of infection (MOI) of 0.01 for 48 h and then probed with ASFV convalescent swine serum⁵⁰. After washing, cells were incubated with goat anti-swine IgG-fluorescein isothiocyanate (FITC) secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h, washed again, and visualized via fluorescence microscopy.

Flow cytometric enumeration of ASFV antigen expression in primary cells

ASFV antigen expression from each RC-Ad5 construct (RC-Ad5-01 to RC-Ad5-43) in primary swine cells was quantified by flow cytometry as previously described²⁸. Primary swine alveolar epithelial cells (3×10^6 ; Cell Biologics, Chicago, IL, USA) were infected at an MOI of 0.01 and harvested after 72 h using 0.25% Trypsin-EDTA (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were permeabilized using the Cyto-Fast[®] Fix/Perm Buffer Set (BioLegend, San Diego, CA, USA), incubated with ASFV convalescent swine serum (1:200 dilution) for 20 min at 4 °C, washed, and then incubated with goat anti-porcine IgG-FITC antibody (1:250 dilution; Jackson ImmunoResearch, West Grove, PA, USA) for another 20 min. After final washes, cells were resuspended in PBS with 2% goat serum. Data acquisition was performed using a BD LSRFortessa[®] flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (BD Biosciences, Ashland, OR, USA), as described previously²⁸.

Immunization of pigs

Thirty-three healthy crossbred piglets (25% Landrace, 25% Yorkshire, 50% Duroc; 15–18 kg, 49–56 days old) were sourced from Oak Hill Genetics (Altona, IL, USA) and randomly assigned by weight into four groups: three treatment groups (G1–G3) and one negative control group (G4). Each group consisted of 6–7 immunized piglets, along with two non-immunized piglets designated as ASFV-infected contact spreaders for challenge studies. Group 1 piglets received intramuscular (IM) vaccination with the full RC-Ad5-ASFV cocktail (RC-Ad5-01 to RC-Ad5-43; 10^9 infection units [IFU]/construct), mixed with Quil-A adjuvant (300 µg/pig; InvivoGen, San Diego, CA, USA). Group 2 received the same formulation with Quil-A adjuvant but excluded constructs RC-Ad5-03 to -05, which encoded antigens previously studied extensively²⁰. Group 3 received the RC-Ad5-ASFV cocktail without adjuvant. Group 4 received a Quil-A-adjuvanted control cocktail containing RC-Ad5-GFP at an equivalent dose. Two booster immunizations were administered using the same formulation but with a 10-fold higher viral concentration. Weekly body weight and rectal temperature were recorded. Whole blood was collected for PBMC and serum isolation. Nasal swabs were obtained to monitor viral shedding. Filtered nasal swab supernatants were used to inoculate swine alveolar epithelial and HEK293A cells. After 48 h, infected cells were probed with either anti-Ad5 polyclonal antibody or ASFV convalescent serum, followed by detection via immunocytochemistry as previously described⁵⁰.

Evaluation of RC-Ad5-ASFV-induced antibodies

Antibody responses were measured via indirect enzyme-linked immunosorbent assay (ELISA). Microplates were coated with 100 µL of 1 µg/mL purified ASFV antigens (B385R, B175L, D205R, or p62) in bicarbonate coating buffer and incubated overnight at 4 °C. After washing with PBST (PBS + 0.1% Tween-20), plates were blocked with 2% bovine serum albumin in PBS for 1 h at 37 °C and then incubated with pig sera (1:250 dilution) in triplicate for 1 h at 22 °C. Plates were washed again and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-swine IgG (1:5000; Jackson ImmunoResearch, West Grove, PA, USA). Colorimetric detection was performed using 3,3',5,5'-tetramethylbenzidine substrate (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min in the dark. Reactions were terminated with 1 M HCl, and absorbance was measured at 450 nm using a BioTek Epoch spectrophotometer (Agilent Technologies, Winooski, VT, USA). Isotype-specific responses were assessed using HRP-conjugated anti-swine IgM and IgA antibodies (1:10,000; Bio-Rad Laboratories, Hercules, CA, USA). For IgG subclass analysis, HRP-

conjugated anti-IgG1 or anti-IgG2 secondary antibodies (1:10,000; Bio-Rad Laboratories, Hercules, CA, USA) were used to evaluate responses against p62 and D205R.

Pig challenge

Two weeks after the final booster immunization, two non-immunized pigs in each group, designated as contact challenge (CC) spreaders, were intramuscularly inoculated with 50 hemadsorbing doses (50 HAD₅₀) of ASFV Georgia 2007/1 (kindly provided by Dr. Linda Dixon, Pirbright Institute, UK). Inoculated CC pigs were then reintroduced into their respective housing groups to cohabitate with immunized animals, simulating natural virus transmission via direct contact and contaminated environment, as previously described^{27,28}.

Post-challenge sampling and clinical assessment

Following the challenge, pigs were monitored daily for rectal temperature and clinical signs of ASF using a validated scoring system²⁸. Body weight, EDTA-anticoagulated blood (for viremia and PBMC isolation), and nasal swabs were collected on DPC 0, 5, 8, 12, 15, 19, 22, 29, 36, and 39, or at the time of euthanasia if animals reached humane endpoints. At necropsy, comprehensive tissue collection was performed, including lymph nodes (tonsil, gastrohepatic, mandibular, cranial mediastinal, mesenteric, and renal), kidney, liver, lung, spleen, heart, and pericardial fluid (if effusion was present). Tissues were stored for virological and histopathological analyses.

Pigs reaching predefined humane endpoints were sedated by intramuscular administration of xylazine (2.2–4.4 mg/kg) and Telazol (4.4–8.8 mg/kg). Following confirmation of a deep surgical plane of anesthesia, as indicated by loss of corneal reflexes and absence of response to external stimuli, animals were euthanized by exsanguination via transection of the major cervical vasculature (carotid arteries and jugular veins). Death was confirmed by cessation of cardiac and respiratory activity prior to necropsy. This approach was used to facilitate recovery of high-quality viable cells and to preserve tissue integrity for subsequent histopathological analyses.

ASFV viremia quantification

Genomic DNA was extracted from EDTA-treated blood and 10% tissue homogenates using the Taco[™] Automatic Nucleic Acid Extraction System with the Taco DNA/RNA Kit (GeneReach USA, Lexington, MA, USA) with minor modifications²⁸. For blood samples, 250 µL of whole blood was mixed with 250 µL of AL buffer (Qiagen, Hilden, Germany), vortexed, and incubated at 70 °C for 10 min. A 200 µL of lysate was extracted and eluted into 100 µL. 200 µL of clarified PBS tissue homogenate (TissueLysar LT, Qiagen) was mixed with 300 µL of ATL buffer and 40 µL of Proteinase K (Qiagen) and incubated at 56 °C for ≥2 h at 550 rpm using a ThermoMixer F2.0 (Eppendorf, Hamburg, Germany). The lysates were diluted 1:1 in AL buffer and processed on Taco[™]. ASFV DNA was quantified via qPCR targeting the p72 gene using PerfeCTa[®] FastMix[®] II (Quantabio, Beverly, MA, USA)²³. Reactions (20 µL total) contained 10 µL of master mix, 0.2 µL each of forward and reverse primers, 0.4 µL probe, 5 µL DNA template, and nuclease-free water. Amplification was performed on a QuantStudio[™] 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Samples with Ct < 38 in both replicates were considered positive. Viral genome copy numbers were calculated using a standard curve methodology generated with a quantitated p72 plasmid.

Virus rescue from tissue and fluid samples

ASFV recovery was assessed by culturing primary porcine macrophages with spleen lysates or pericardial fluid in duplicate wells at 37 °C and 5% CO₂ for 48 h. Viral replication was evaluated via IFA using ASFV convalescent serum, followed by nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Newark, CA, USA), as previously described⁵⁰. DNA was extracted from duplicate wells using the Taco[™] system, and viral DNA was quantified using qPCR as outlined above.

Gross and histopathology

All pigs were humanely euthanized post-challenge and underwent complete necropsy. Gross pathology was evaluated across multiple organ systems, including body condition, integument, cardiovascular system, liver, lungs, kidneys, spleen, tonsils, and lymph nodes (mandibular, cranial mediastinal, mesenteric, renal, gastrohepatic, and prescapular). Lesions were scored using the established criteria^{23,49}, with minor modifications to include additional parameters for GI involvement (Supplementary Table 7). GI pathology was assessed for the presence of mucosal ulcers, edema, diarrhea, hemorrhage, abdominal effusion, and fibrinous peritonitis.

Formalin-fixed tissues were processed using standard histological protocols performed at the Kansas State Veterinary Diagnostic Laboratory. Blinded histopathological scoring was performed using modified criteria to assess the spleen, tonsil, lungs, liver, kidneys, and the aforementioned lymph nodes, as previously described^{23,28,49} (Supplementary Table 8). Histological findings were categorized into total organ, lymphoid, visceral, and GI scores to facilitate interpretation of disease severity and treatment effects. The spleen score (maximum 20) was subdivided into white pulp (8 points) and red pulp (12 points). Individual scores were also recorded for gastrohepatic and mandibular lymph nodes. Tissues from two uninfected, untreated pigs housed under the same environmental conditions were included as negative controls for baseline lesion scoring and comparative analysis.

Virus neutralization assay

Heat-inactivated serum samples (56 °C for 30 min) were diluted 1:1 and 1:2 in RPMI-1640 medium and incubated with ASFV (MOI 0.1 HAD₅₀) at 37 °C for 8 h in duplicate. The virus–serum mixtures were then added to PBMCs isolated from naïve pigs and incubated overnight. Following three PBS washes, fresh culture medium was added, and cells were incubated for an additional 48 h to monitor cytopathic effects.

To assess ASFV infection, one set of cells was fixed in cold methanol, incubated with ASFV convalescent swine serum (1:500 dilution), and stained with FITC-conjugated goat anti-porcine IgG (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature in the dark. After PBS washes, cells were visualized under an EVOS FL AMF4300 fluorescence microscope (Life Technologies, Carlsbad, CA, USA).

For qPCR, DNA was extracted from the second set of ASFV-infected PBMCs using the GeneReach Taco™ extraction system. ASFV genome levels were quantified using the p72-targeting assay described above on the QuantStudio™ 5 Real-Time PCR System. ASFV-infected PBMCs without serum served as positive controls, and uninfected PBMCs were used as negative controls.

Granzyme B monoclonal antibody generation

A gene encoding porcine granzyme B (GrB; GenBank: NP_001137182.1) with a C-terminal FLAG tag was synthesized and cloned into the pcDNA3.1+ expression vector (GenScript, Piscataway, NJ, USA). Recombinant protein was expressed using the Expi293 expression system (Thermo Fisher Scientific, Waltham, MA, USA) and used to immunize BALB/c mice. Spleens from antibody-producing mice were fused with SP2/0 myeloma cells to generate hybridomas. Positive clones were identified via indirect ELISA, isotyped, subcloned, and used for the production and purification of IgG mAbs (GenScript, Piscataway, NJ, USA).

Validation of anti-granzyme B mAbs

Candidate anti-GrB mAbs (13D12-2, 13E2-2, 14D11-1, 14G9-2, 17B6-1, and 29C12-2) were validated via indirect ELISA and western blotting for binding to recombinant porcine GrB. Specificity was confirmed via intracellular staining and flow cytometry using Expi293 cells transfected with the pcDNA3-GrB construct and porcine PBMCs stimulated *in vitro*. Expi293 cells (3×10^7) were transfected with 30 µg pcDNA3-GrB, and harvested 48 h post-transfection. Cells were aliquoted at 1×10^6 cells per tube. PBMCs from RC-Ad5-immunized pigs were stimulated with RC-Ad5-03 and RC-Ad5-25 (MOI 1) for 5 days, followed by a 4-h re-

stimulation in the presence of 10 µg/mL Brefeldin A (BioLegend, San Diego, CA, USA) as described previously²⁸. Cells were fixed and permeabilized using Cyto-Fast™ Fix/Perm Buffer (BioLegend, San Diego, CA, USA), stained with anti-GrB mAbs (2.5–10 µg/mL) or matched isotype controls, and incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (1:100, BioLegend). Data were acquired on a BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (BD Biosciences, Ashland, OR, USA) as above. Unstained and secondary-only controls were used for gating.

Epitope prediction

Nonamer peptides from selected ASFV antigens (p72, p15, B602L, NP868R, H339R, M1249L, 173R, G1211R, B962, E75, H171R, C962, MGF505-3R, C147L, A859L, B318L, and B169L) were predicted *in silico* using NetMHCpan v4.1 (<https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/>), targeting peptides with a percentile rank < 0.5 for strong binding to well-characterized swine major histocompatibility complex (MHC) class I (SLA-I) alleles, as previously described⁵⁶. Epitope conservation across ASFV genotypes was assessed using multiple sequence alignment. Top-ranked strong binders were synthesized (Peptide 2.0 Inc., Chantilly, VA, USA), reconstituted in sterile water with 25% dimethyl sulfoxide at 10 mg/mL, aliquoted, and stored at –80 °C for downstream immunological assays.

T-cell epitope mapping

Synthetic peptides were screened for their ability to recall CD8α⁺ T-cell responses defined by GrB⁺, Perf⁺, and IFN-γ⁺ expression in cells from pigs immunized with adenovirus constructs expressing ASFV antigens. Briefly, memory T cells were expanded *in vitro* by restimulating PBMCs for five days with ASFV (Georgia 2007/1) at an MOI of 1, followed by screening peptide pools. Pools of 20 peptides (0.4 µg/mL each) were screened via intracellular cytokine staining, followed by flow cytometric analysis for GrB⁺, Perf⁺, and IFN-γ⁺ CD8α⁺ T-cell responses as described below. Reactive peptide pools were deconvoluted to identify individual epitope peptides. Positive peptides were used to enumerate recall GrB⁺, Perf⁺, and IFN-γ⁺ CD8α⁺ T-cell responses in PBMCs and splenocytes isolated from the pigs immunized with the RC-Ad5-ASFV virus cocktail. Briefly, PBMCs and splenocytes (1×10^6 /well) were stimulated with ASFV (MOI 1) for 3 days, followed by incubation for 4 h with individual peptides (0.4 µg/mL) in the presence of autologous monocytes as antigen-presenting cells (APCs) and 10 µg/mL Brefeldin A (BioLegend, San Diego, CA, USA). Monocytes were enriched from pre-immunization PBMCs via CD14 antibody labeling and positive selection with Miltenyi magnetic beads, and their identity confirmed by flow cytometry as previously described^{63,64}. The peptide-restimulated PBMCs and splenocytes were washed twice with PBS and stained for viability using LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (1:1000; Life Technologies, Waltham, MA, USA) at room temperature for 30 min. Cells were washed twice with PBS containing 2% FBS, and Fc receptors were blocked using 1 mg/mL of rat IgG at 4 °C for 10 min. Surface staining was performed using PE-Cy5-conjugated anti-porcine CD3e mAb PPT3 (1:125; SouthernBiotech, 4510-13, AL, USA) and FITC-conjugated anti-porcine CD8α mAb 76-2-11 (1:62; SouthernBiotech, 4520-02, AL, USA) for 30 min at 4 °C in the dark, followed by two PBS washes. Cells were then fixed and permeabilized for 45 min at 4 °C using 200 µL of the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Intracellular staining was performed with either mouse anti-porcine GrB mAb 13D12-2 (10 µg/mL), mouse anti-Perf mAb dG9 (1:31; Thermo Fisher Scientific), or mouse anti-IFN-γ mAb P2G10 (1:31; BD Biosciences, Ashland, OR, USA) for 30 min at 4 °C in the dark. After washing, cells were incubated with phycoerythrin (PE)-labeled goat anti-mouse poly4053 (1:1000; BioLegend, San Diego, CA, USA) for GrB and Perf, or APC-Cy-7-labeled goat anti-mouse poly4053 (1:1000; BioLegend, San Diego, CA, USA) for IFN-γ. Cells were washed twice with

permeabilization buffer and twice with PBS, and resuspended in 200 μ L PBS. Data were acquired using an Attune™ NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using FlowJo software (BD Biosciences). Intracellular cytokine–positive CD8 α ⁺ T cells were defined as CD3 ϵ ⁺CD8 α ⁺IFN- γ ⁺, CD3 ϵ ⁺CD8 α ⁺GrB⁺, or CD3 ϵ ⁺CD8 α ⁺Perf⁺ populations and reported as positive cells per 10⁶ PBMCs or splenocytes with background from mock-RC-Ad5-GFP immunized animal responses subtracted as baseline. Unstained cells and singly stained controls were used for gating and compensation to define background and validate response specificity.

SLA typing

To investigate the association between SLA haplotype and vaccine-mediated protection, SLA class I and II genotyping was performed on 28 pigs using low-resolution PCR with sequence-specific primers (PCR-SSP). Genomic DNA was extracted from 5 $\times$ 10⁶ PBMCs using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). PCR assays were assembled using HotStarTaq™ Plus Master Mix (Qiagen), Coral-Load buffer, 0.2 pmol/ μ L allele-specific and α -actin control primers (Eurofins Genomics, Louisville, KY, USA), and 20 ng of DNA in a total reaction volume of 10 μ L. Amplification was performed on a Biometra T Gradient thermal cycler (Analytik Jena, Jena, Germany) under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. PCR products were resolved on 2.5% agarose gels (Biozym, Hessisch Oldendorf, Germany) in 1 $\times$ Tris–acetate–EDTA (TAE) buffer at 150 V for 5 min using the Micro SSP Gel System (One Lambda, West Hills, CA, USA). Gels were stained with GelStar™ nucleic acid stain (Lonza, Basel, Switzerland), and amplicons were visualized under UV illumination. SLA allele assignment was based on the expected amplicon size. Haplotype determination followed established SLA nomenclature and was compared with both published SLA datasets^{65–71} and farm-specific reference datasets^{65,72} (unpublished).

To further elucidate the contribution of specific SLA alleles to ASFV-specific adaptive immune responses, high-resolution sequence-based typing was performed for SLA class I and II loci using validated primers and protocols from previous studies^{73–75}. Additionally, exons 2 and 3 of SLA-6 were PCR-amplified using the primers SLA-6/1-3fw (5'-CCT CCC TGT TAT AGG ATC CCA CTC G-3') and SLA-6/1-3rev (5'-CAG CAC GTC CTT CCC CTT CT-3'). Amplified locus-specific PCR products from genomic DNA were cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each locus and animal, at least 16 clones were bidirectionally sequenced (Eurofins Genomics, Louisville, KY, USA), and the resulting sequences were aligned against the Immuno Polymorphism Database for Major Histocompatibility Complex SLA reference database (Release 3.11.0.0, 2023-07, build 211) using BLAST for allele identification⁷⁶.

Statistical analyses

Statistical analyses were performed using GraphPad Prism v10.1.2 (GraphPad Software, San Diego, CA, USA). Survival data were analyzed using the Mantel–Cox log-rank test. Antibody responses were compared using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Viral titers, gross pathology scores, and histopathology scores were analyzed by two-way ANOVA with Tukey's multiple comparisons test. Comparisons were conducted between each treatment group and the RC-Ad5-GFP mock control group, as well as among the immunized groups. Flow cytometry data were analyzed using FlowJo software v10.8.1 (BD Biosciences, Ashland, OR, USA) to gate cell populations and quantify antigen expression.

Ethics statement

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC; Protocols #3871 and #4411) and Institutional Biosafety Committee (IBC; Registration

#1713), in accordance with The Institute for Laboratory Animal Research, Guide for the Care and Use of Laboratory Animals, Eighth edition. Protocols included daily clinical scoring, as well as weight and temperature monitoring, to assess animal health.

Data availability

All data supporting the findings of this study are available from the corresponding author upon request.

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Author contributions

W.M. designed and oversaw the development and characterization of the experimental vaccine. The vaccine was generated by R.K. and T.K. Animal immunization, animal monitoring, sample collection, and challenge experiments were done by R.K., T.K., M.Z., H.S., E.H., D.K., J.M., S.O., and W.M. Evaluation of antibody responses, clinical outcomes, viremia, and neutralization assays was conducted by R.K., T.K., M.Z., and E.H. Generation and characterization of Granzyme B mAbs was conducted by N.S., L.B., K.M. and M.Z. Evaluation of T cell responses was conducted by M.Z., B.A., and J.M. Necropsy was conducted by J.T., S.A., R.K., T.K., M.Z., H.S., E.H., D.K., J.M., and J.A.R. Gross and histopathology were performed by R.K. and J.T. SLA typing was conducted by S.H. R.K., T.K., S.H., M.Z., and W.M. were involved in data analysis and result interpretation. R.K., T.K., J.T., S.H., and W.M. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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