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Adenoviral vectored porcine interferon omega-5 elicits strong antiviral activity and sterile immunity against foot-and-mouth disease in swine

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Abstract

Foot-and-mouth disease (FMD) is a high economic consequence disease of cloven-hoofed animals, necessitating effective countermeasures for control. Outbreak prevention and control is supported mainly by inactivated vaccines; however, a 7-day period is required for the vaccine to confer protective immunity. In an effort to improve the protective window, biotherapeutic agents such as interferons (IFNs) have been explored for their potent antiviral functions against FMD virus (FMDV). In silico analysis of porcine IFN α1 and porcine IFN ω5 demonstrated distinct biochemical differences that could affect receptor binding and downstream signaling. In this study, we constructed and evaluated an adenovirus vector encoding porcine IFN ω5 (Ad5-pIFN ω5) as a novel biotherapeutic. In porcine cells, Ad5-pIFN ω5 induced antiviral IFN-stimulated genes (ISGs) and demonstrates antiviral activity against FMDV. In vivo, 12 pigs divided into three different groups were inoculated with Ad5-pIFN ω5 (1×10^8, 1×10^9, and 1×10^{10} pfu) and challenged alongside a control group with FMDV O1 Manisa 24 h post-inoculation. The highest dose of Ad5-pIFN ω5 completely protected swine against challenge, suggesting sterile immunity, which is defined as no detection of viral replication in serum or nasal swabs and a lack of neutralizing antibody titers. Further analysis of innate immunity by enzyme-linked immunosorbent assay (ELISA) revealed detectable induction of type I IFN in serum from animals receiving the highest dose, which correlated with protection. These findings demonstrate that pIFN ω5 is a potent type I IFN that can rapidly and effectively protect swine from FMD.

Keywords Foot-and-mouth disease, type I interferon, biotherapeutic, interferon omega, adenovirus, antiviral

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Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease (FMD), a high-impact and economically significant contagious disease that affects cloven-hoofed animals. Due to its rapid rate of infection, spread, and high morbidity, its emergence in FMD-free countries would have a catastrophic impact on national agricultural industries. FMD is largely eradicated in developed countries, but it still threatens livestock-dependent rural regions. Recent outbreaks in Germany, Slovakia, Hungary, Cyprus, and Greece highlight persistent global risk of FMD [1]. Outbreak prevention and control is supported mainly by chemically inactivated vaccines; however, a 7-day period is required for the vaccine to confer protective immunity [2, 3]. This creates a window of vulnerability where rapid viral spread is possible among susceptible animals. Various strategies including the use of interferons (IFNs) and other biotherapeutic agents have been explored in controlled experiments to close this window of susceptibility [4, 5].

During viral infection, pattern recognition receptors (PRRs) recognize viral components, leading to the induction of type I interferon (IFN) genes. These type I IFNs bind to the IFN receptor (IFNAR), causing dimerization and leading to phosphorylation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). This leads to the formation of the IFN-stimulated gene factor 3 (ISGF3), composed of dimerized signal transducer and activator of transcription 1 (STAT1) and STAT2 molecules with interferon regulatory factor 9 (IRF9). These translocate to the nucleus and bind to the IFN-stimulated response elements (ISRE) in the genome, causing the induction of IFN stimulated genes (ISGs) including myxovirus resistance 1 (MX1), 2′5′-oligoadenylate synthetase (OAS), and ISG15 [6]. The myriad of ISGs that are induced have various antiviral activities that restrict viral replication. Many ISGs, such as ISG15, myeloid cell leukemia 1 (MCL1), and DExD/H-box helicase 60 (DDX60), have been shown to reduce FMDV replication [7–9].

IFNs have been constructed and studied as rapid acting biotherapeutics for the efficient control of FMD, particularly type I (alpha [α] and beta [β]), type II (gamma [γ]), and type III (lambda [λ]) IFNs. In studies involving type I IFN, protection in swine extended across multiple FMDV serotypes, including A24 Cruzeiro, O1 Manisa and Asia1, and remained effective even following natural (contact-based) challenge conditions [10]. Mechanistic studies have demonstrated that adenovirus-delivered type I IFN can induce a strong innate antiviral state characterized by the upregulation of certain ISGs along with chemokines in swine [11].

Recent comparative genomic studies have identified an expanded type I IFN locus in swine, with multigene IFN omega (ω) subtypes showing superior antiviral potency in vitro compared with the conventional IFN α subtypes [12, 13]. Similar to other type I IFNs, IFN ω binds to the ubiquitously expressed transmembrane receptor composed of two chains, IFNAR1 and IFNAR2; however, its antigenic structure is distantly related to IFN α, β, and λ and it does not cross-react with antibodies against them. Furthermore, in dose response assays, IFN ω performed as efficiently as IFN α_2 or IFN β in inducing the IRF pathway, indicating that IFN ω binds to IFNARs with high affinity, thus activating the classic IFN/IRF pathway more efficiently. In fact, feline IFN ω displays strong antiviral efficacy and is used clinically as an antiviral in cats in many European countries [14, 15]. Consistent with this, it has been demonstrated that porcine IFN ω7 has potent antiviral activity against FMDV serotype A and O in vitro [16]. However, the in vivo antiviral properties of porcine IFN ω have not been evaluated thus far.

Previously, our group used a replication-defective human adenovirus type 5 (Ad5) as a delivery vector for various IFNs which have provided sterile protection from FMDV infection in natural hosts [17–20]. More recently, efforts to improve the antiviral potency and pharmacokinetics of IFN-based countermeasures have led to the development of purified pegylated porcine IFN α, which demonstrated significantly prolonged serum half-life, enhanced antiviral activity, and effective early protection in swine [21]. In parallel, recombinant glycosylated IFN α produced using a baculovirus mammalian expression system has demonstrated potent early antiviral activity and served as an effective FMD vaccine companion, enhancing both early protection and the magnitude of neutralizing antibody responses in swine [22]. Building on these advances and attempting to identify a more efficient and potent biotherapeutic, we cloned and purified Ad5 porcine IFN ω type 5 (Ad5-pIFN ω5). A total of 12 pigs divided into three different groups were inoculated with Ad5-pIFN ω5 and challenged alongside a control group with FMDV O1 Manisa 24 h post-inoculation. Three doses were tested (1×10^8, 1×10^9, and 1×10^{10} pfu); notably, the highest dose conferred protection and achieved sterile immunity. Measurement of serum levels of different antiviral markers also indicates the induction of an immune response. Our research demonstrates for the first time that pIFN ω5 offers potent antiviral activity against FMDV and represents a promising addition to the IFN-based arsenal for rapid outbreak response.

Materials and methods

Cells and viruses

HEK 293 cells (ATCC CRL-1573) were used for production, amplification, and titration of the human adenovirus type 5 (Ad5) expressing porcine interferon omega

5 (Ad5-pIFN ω5, NCBI RefSeq NC_010443.4), as well as the empty vector Ad5-Blue (AddGene Cat# 174431). LFBKαvβ6 cells [23] were used for titration of the challenge FMDV, for viral detection in nasal swabs and serum samples, and for quantification of neutralizing antibodies in serum. Porcine kidney cells (IBRS-2) were used to confirm the expression and biological activity of pIFN ω5. All cells were maintained at 37 °C and 5% CO_2 in minimum essential medium (MEM) or Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics/antimycotics, and nonessential amino acids.

In addition to Ad5-pIFN ω5 and Ad5-Blue, Ad5-pIFN α1, and Ad5-GFP-mCherry were included in in vitro assays as controls for antiviral activity and for monitoring construct expression and transduction, respectively [24]. FMDV serotype A12 was used as the challenge virus during in vitro testing. FMDV O1 Manisa originally isolated from the vesicular fluid of an infected swine was used as the challenge virus for swine studies as previously described [25].

Protein modeling

Alignment of porcine IFN ω5 (ACV42426.1) and porcine IFN α1 (WEU38382.1) was done with ClustalW [26]. AlphaFold3 was used to predict the structures of each IFN and each IFN associated with the subunits of the IFN receptor (IFNAR1 and IFNAR2) [27]. The figures showed only the extracellular and transmembrane portions of IFN receptors. Visualization of electrostatic and polar contacts was performed using Pymol 2.5.5 [28].

Construction of adenovirus

The Ad5-pIFN ω5 vector was generated following a previously described protocol [25, 29]. The plasmid pAd5-Blue was double digested with XbaI and ClaI which generated 5′ overhangs, and puc57 pIFN ω5 (Genscript) was also enzymatically treated to carry matching compatible ends. The plasmid and insert were ligated together, resulting in the backbone. Correct insertion and orientation was verified by restriction enzyme analysis (ClaI, XbaI, and Hind III) and by Sanger sequencing. The new construct (pAd5-pIFN ω5) was linearized with PacI and transfected into HEK 293 cells through lipid-based transfection (lipofectamine). Transfected monolayers were examined daily until distinct adenoviral cytopathic effect plaques were visible. Individual plaques of infected cells were collected and subjected to three freeze/thaw cycles and used to infect a T150 cell culture flask of HEK 293 cells. After each amplification in cell culture, viral purification was done through cesium chloride gradients, and confirmation of expression of the construct was done through restriction enzyme digests and sequencing.

Biological activity in vitro

IBRS-2 cells were seeded in 6-well plates at a confluency of 1×10^6 cells per well and treated in duplicate, with Ad5-pIFN ω5, Ad5-pIFN α1 as a positive control, and Ad5-GFP-mCherry as a negative control, at a multiplicity of infection (MOI) of 10. Supernatants and cell lysates were collected at 24 h post-infection for further analysis. Cell lysates were used for reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays to confirm the expression of pIFN ω5 or pIFN α1 constructs and other classic interferon-stimulated genes (ISGs), including *OAS*, *MX-1*, *ISG15*, *IRF7*, and *STAT1* using primers described previously [11]. *HPRT1* was used as a housekeeping gene in this assay. RNA was extracted using QIAshredder spin columns for sample homogenization, followed by RNA extraction with a RNeasy Mini kit (Qiagen). A total of 1 µg of RNA was used to synthesize complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers. The cDNA was diluted at 1:8 and qPCR assays were performed with PerfeCTa SYBR Green FastMix Low ROX and the primers mentioned above.

Supernatants were collected and clarified using a 100 kDa centrifugal ultrafiltration unit (Centricon®, MilliporeSigma) for the evaluation of antiviral activity. To remove high molecular weight material, including residual adenoviral virus particles, the supernatants were spun at $3500 \times g$ for 10 min at 4 °C, permitting low molecular weight proteins including secreted IFN to pass through and remain in the filtrate. IBRS-2 cells were seeded at 2.8×10^6 cells per plate in 96-well plates and then were treated with the collected supernatants in tenfold serial dilutions before being left to incubate at 37 °C with 5% CO_2. After 24 h, cells were challenged with FMDV A12 at an MOI of 0.5 and incubated at 37 °C with 5% CO_2. Wells were evaluated for the presentation of cytopathic effect (CPE) at 48 h post-infection. No visible CPE was observed in control uninfected wells.

Animal experiment

The animal experiment was performed in the biosafety level 3 agriculture (BSL-3 Ag) animal facility at the Plum Island Animal Disease Center (PIADC, Greenport, NY, USA). Study protocol and procedures were submitted for prior approval by the Institutional Animal Care and Use Committee of the Plum Island Animal Disease Center (USDA/APHIS/AC certificate number: 21-F-0001; protocol 151.02-22R) and were conducted in compliance with: the Animal Welfare Act (AWA); the Guide for the Care and Use of Laboratory Animals; the 2002 Public Health Service Policy for the Humane Care and Use of Laboratory Animals; and the

US Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (IRAC, 1985).

The animal experiment was performed to evaluate the efficacy of Ad5-pIFN ω5 alone in the prevention of FMD. It was designed with 16 Yorkshire gilts weighing between 40–50 lbs, divided into four rooms with four animals each. After the required acclimation period, three groups were inoculated subcutaneously (SQ) with different doses of Ad5-pIFN ω5: 1×10^{10} pfu/animal, 1×10^{9} pfu/animal, and 1×10^{8} pfu/animal. The last group, a negative control, was inoculated with Ad5-Blue at 1×10^{10} pfu/animal. Inoculation was administered in two 1 ml injections on either side of the neck. At 24 h post-inoculation, all animals were challenged intradermally in the heel bulb (IDHB) in the right rear foot with FMDV O1 Manisa with a 50% tissue culture infectious dose ($TCID_{50}$) of 1×10^{4} per animal, administered as four separate injections of 100 µl each, and were subsequently monitored for 21 days post-challenge (dpc) as described previously [30].

Following Institutional Animal Care and Use Committee (IACUC)-established protocols for blood collection volumes, each treatment group was further divided into two subgroups, which were sampled for blood on alternating days [30]. Nasal swabs were collected daily from all animals. Sampling was performed daily through 7 days post-challenge (dpc). Additional serum samples were collected from all animals at 14 and 21 dpc. Clinical evaluations were performed daily post-challenge through 7 dpc to monitor FMD clinical signs such as fever, lameness, and development of vesicles on the coronary band or snout. Clinical score was calculated based on the total number of toes presenting vesicles (4/foot) along with the snout/mouth, with a maximum score of 17. Lesions restricted to the site of inoculation were not counted [20]. Although FMD is not typically lethal and animals generally recover after virus-induced lesions are healed, humane endpoints were established to prevent extended animal suffering induced by painful vesicular lesions. These endpoints followed defined clinical criteria, including a body temperature exceeding 105.1 °F, pain/lameness, unwillingness to move or bear weight over time due to pain in the limbs, severe depression, and partial or complete claw horn detachment [31].

Viral detection in nasal and serum samples

Sera and nasal swabs from 0 to 7 dpc were processed for detection of FMDV by standard plaque assays using LFBKαvβ6 cells [32]. These samples were also processed for FMDV RNA detection by RT-qPCR as described previously [33].

Humoral immune response

Serum samples collected on 0, 4, and 7 dpc were heat inactivated (30 min, 56 °C) and used to measure neutralizing antibodies by virus neutralization assays. Briefly, serial dilutions of serum were incubated in 96-well plates with 100 median tissue culture infective dose ($TCID_{50}$) of FMDV O1 Manisa for 1 h at 37 °C and 5% CO_2. Afterwards, LFBKαvβ6 cell suspensions were added and incubated for 72 h at 37 °C and 5% CO_2. Titers were measured using the Spearman–Karber method [34].

Innate immune response

Single analyte sandwich ELISA kits were used to determine the serum concentrations of the following cytokines: pIFN α (Sigma, RAB1131), pIFN β (Invitrogen, ES8RB), pIFN γ (Invitrogen, KSC4021), and pISG15 (MyBioSource, MBS9369359). Serum was diluted 1:2 following the manufacturer's protocols. Concentrations of samples with an optical density (OD) below that of background were converted to zeros. Data is presented as percent induction compared with baseline concentration.

Statistical analysis

Data handling, analysis, and graphic representations were performed using Prism version 10.6.1 (GraphPad Software, San Diego, CA) or Microsoft Excel (Microsoft, Redmond, WA, USA). ISG qPCR statistical analysis was done with paired Student's *t*-tests. Antiviral activity statistical analysis was done with one-way analysis of variance (ANOVA) with multiple comparisons done with the Tukey's multiple comparisons test. Survival curves were compared between groups with the log-rank (Mantel–Cox) test. ELISA data was analyzed with mixed effects two-way ANOVA by treatment group and time point, followed by Dunnett's multiple comparisons test.

Results

In silico comparison of pIFN α1 and pIFN ω5 and in vitro characterization of Ad5-pIFN ω5 was performed. In an effort to identify a more efficient type I IFN capable of inducing a robust yet regulated antiviral activity, with less toxicity than previously observed with the Ad5-pFN α, we evaluated porcine IFN ω5. We conducted comparative in silico analysis between the two type I IFN molecules first. The protein sequence alignment was done using ClustalW and revealed substantial conservation between the two IFN molecules, but there were also conservative, semiconservative, and nonconservative substitutions that could result in structural and biochemical differences between the two molecules (Figure 1A). To further explore these distinctions, electrostatic surfaces were examined using AlphaFold3-generated structural

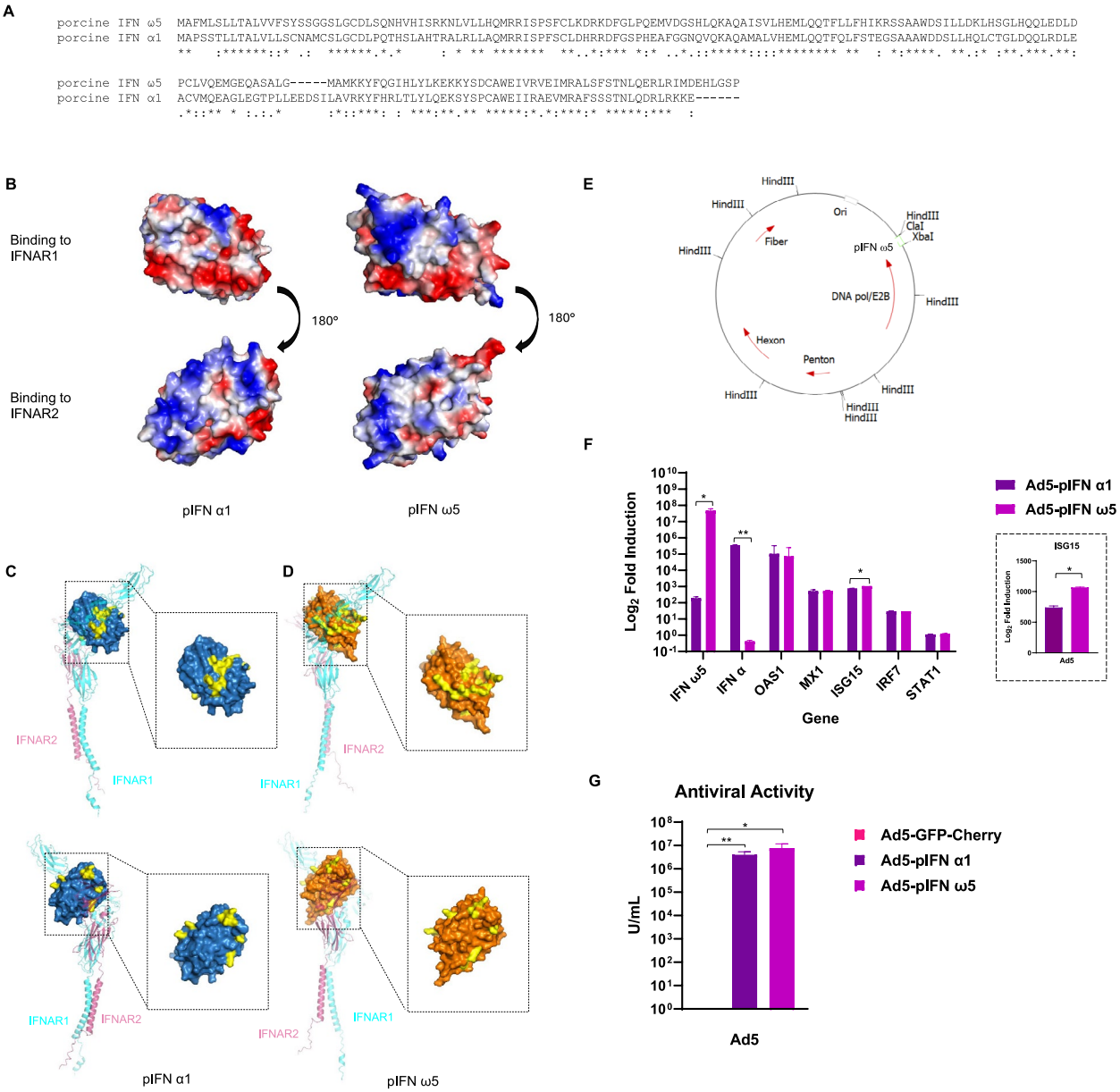

Figure 1 In silico and in vitro characterization of pIFN ω5. A Sequence alignment of the protein sequences of *Sus scrofa* IFN ω5 (ACV42426.1) and *Sus scrofa* IFN α1 (WEU38382.1). The asterisk (*) represents the conserved sequence, the colon (:) represents a conservative amino acid substitution with very similar biochemistry, the period (.) indicates a semiconservative substitution of amino acids with slightly similar biochemistry, and the blank spaces represent no conservation of the sequence at that position. **B** Electrostatic surface representations of pIFN α and pIFN ω. The top of the panel represents the side of the proteins that binds with IFNAR1, and the bottom of the panel represents the side of the proteins that binds with IFNAR2. Blue indicates a positive charge and red indicates a negative charge. **C** and **D** are protein models of pIFN α and pIFN ω, respectively. The IFN receptor is represented as a cartoon, with IFNAR1 colored with cyan and IFNAR2 colored with pink. The IFN molecules are represented with a surface model, with the yellow highlighted regions representing amino acid residues that form polar contacts with the IFN receptor. **E** Schematic of Ad5 porcine interferon omega type 5 (Ad5-pIFN ω5) cloning strategy. Using restriction enzymes XbaI and ClaI, cDNA containing pIFN ω5 was cloned into the Ad5-Blue vector. **F** IBRS-2 cells were transduced at an MOI of 10 (Ad5-pIFN ω5, Ad5-pIFN α, and Ad5-GFP-mCherry), and cell lysates and supernatants were collected at 24 h post-transduction. RNA was extracted from these lysates to prepare cDNA, which was analyzed by qPCR for antiviral genes (*IFN ω5*, *IFN α*, *OAS1*, *Mx1*, *ISG15*, *IRF-7*, and *STAT1*). The graph on the right represents the fold induction for *ISG15* only. Statistical analysis was done with paired Student's *t*-test. **G** Supernatants collected from the transduced cells were diluted 10-fold and used to treat IBRS-2 cells for 24 h. These treated cells were then infected with FMDV A12 at an MOI of 0.5 and monitored for CPE at 48 h post-infection. Statistical analysis was done with one-way ANOVA, with Tukey's multiple comparisons test. *, $p \leq 0.05$, **, $p < 0.01$).

models of each IFN in Pymol. This analysis showed clear differences in electrostatic potential across the receptor binding surface for each of the two IFNs (Figure 1B). There are a few differences between pIFN α1 and pIFN ω5 in the structure, but the major differences are seen in the polar contacts made with both subunits of the IFN receptor (these residues are depicted with yellow in the models), with each type I IFN molecule (Figure 1C and D). Together, the sequence level and biochemical features highlight potential differences in receptor binding that may influence the downstream signaling pathways associated with type I IFNs.

Based on these findings, an adenovirus was engineered to encode pIFN ω5 to evaluate its antiviral potential and its suitability for in vivo protection studies against FMDV (Figure 1E). Following successful vector construction and validation (data not shown), the innate immune responses associated with Ad5-pIFN ω5 in porcine IBRS-2 cells were evaluated. Cells were transduced at an MOI of 10 with either Ad5-pIFN ω5, Ad5-pIFN α, or Ad5-GFP-mCherry, and cell lysates were collected for quantitative qPCR analysis of IFN and ISG transcripts, including *OAS1*, *MX1*, *IRF7*, and *STAT1*. Most of the ISGs had a similar fold induction between the pIFN ω5 and pIFN α constructs, indicating comparable activity, except for *ISG15*, which was upregulated more significantly by pIFN ω5. However, pIFN ω5 did not show an upregulation of *IFN α* production. In a similar fashion, IFN α did not upregulate the expression of *IFN ω5* (Figure 1F). A similar experiment was conducted where the lysates were collected at 48 h and the results showed that the expression of the ISGs were not as strongly upregulated as the 24-h time point. However, treatment with pIFN ω5 induced a significantly higher fold induction of *OAS*, *MX1*, *IRF7*, and *STAT*. In addition, a similar pattern of *IFN α* and *IFN ω* fold induction seen at 24 h was also observed at 48 h (Additional file 1). The antiviral activity of pIFN ω5 against FMDV replication was also investigated. This was accomplished by taking the supernatants from the aforementioned transduced IBRS2 cells and treating newly cultured IBRS-2 cells. The treated IBRS-2 cells were infected with FMDV and monitored for CPE for 24–48 h. The antiviral activity between IFN α and IFN ω5 were similar (Figure 1G). As confirmation of the antiviral activity of the IFN ω5 against FMDV in vitro was obtained, we next evaluated its functionality in vivo.

Inoculation with Ad5-pIFN ω5 protects swine against FMD

To evaluate Ad5-pIFN ω5's in vivo antiviral activity, groups of four pigs were inoculated with the Ad5-pIFN ω5 at high (1×10^{10} pfu), medium (1×10^{9} pfu) and low (1×10^{8} pfu) doses, while the negative control group was inoculated with the empty Ad5-Blue vector. All animals were challenged a day later with FMDV O1 Manisa (Figure 2A). As expected, animals in the control group started developing clinical signs and elevated temperatures at 2–3 dpc, reaching the highest clinical scores between 4–5 dpc, indicative of severe disease progression (Figure 2B). In contrast, swine inoculated with a high dose of Ad5-pIFN ω5 were completely protected against clinical FMD, including the lack of development of FMD

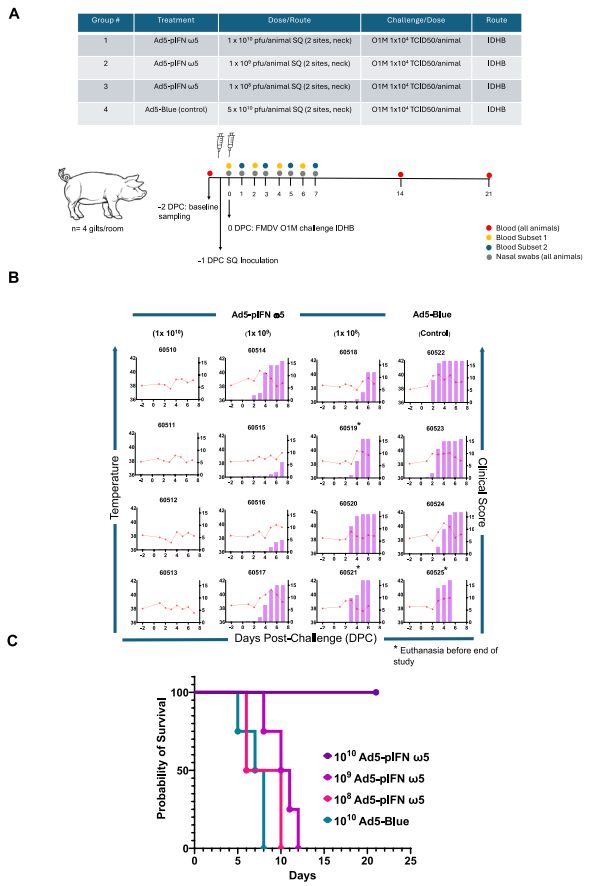

Figure 2 Clinical outcomes of swine treated with Ad5-pIFN ω5. A Experimental design schematic depicting treatment groups and study schedule. Each treatment group contained four Yorkshire gilts, receiving subcutaneously either Ad5-pIFN ω5 (1×10^{10}, 1×10^{9}, and 1×10^{8} pfu per animal) or 1×10^{10} pfu of Ad5-Blue, and were challenged IDHB 1 day later with 1×10^{4} $TCID_{50}$ of FMDV O1 Manisa. The timeline demonstrates the sampling strategy where nasal swabs were collected every day for all animals (gray dots), and blood was drawn on alternating days by subgroup (red and yellow dots). **B** Clinical grading and collection of rectal temperature was performed every day for all animals after challenge. Temperature (°C) is indicated by the red line with the left axis, and clinical score is indicated by the purple bars and the right axis. Each graph represents each individual animal as indicated. The asterisk (*) indicates euthanasia before the end of the study. **C** This graph depicts the survival curve of the challenged pigs and the time it took to their humane euthanasia. Statistical analysis for the survival curve was done with log-rank (Mantel–Cox test).

vesicles or high temperatures during the 7-day observation period (Figure 2B). Furthermore, animals inoculated with medium and low doses of Ad5-pIFN ω5 presented clinical signs of FMD between 2–5 dpc with a mildly delayed progression compared with the control group. To further assess the protective efficacy of Ad5-pIFN ω5 and to determine the threshold protective dose, a survival curve was generated (Figure 2C). In this context, "survival" refers to the proportion of animals in each treatment group that did not reach humane endpoints during the study due to severe disease progression as outlined in the methodology, as FMD is not lethal in swine. The survival curve demonstrated that the high-dose treatment group showed 100% survival throughout the duration of the study (22 days post-challenge), compared with the other treatment groups where all animals reached their humane endpoint and were euthanized prior to the conclusion of observation (6–10 days post-challenge); the difference between these curves was statistically significant ($\chi^2 = 15.65$, df = 3, $p = 0.0013$; Figure 2C). The results indicate that the Ad5-pIFN ω5 high-dose treatment provided complete protection against FMD challenge, identifying 1×10^{10} pfu as a minimum effective dose under the conditions of this study.

Serum and blood samples were collected from animals across subgroups within each treatment group to evaluate the effects of Ad5-pIFN ω5 on viremia and viral shedding (Figure 3). As expected, the control group demonstrated higher levels of viral shedding and viremia, detectable as early as 1 dpc. Viremia levels in this group peaked at around 2–4 dpc, with titers ranging from 4×10^4 to 6.6×10^5 pfu/ml, indicating rapid viral replication in the absence of Ad5-pIFN ω5 treatment. In the low- and medium-dose treatment groups, viremia and shedding were also observed, although the onset was delayed or less amount of virus was detected compared with the control group. Specifically, the low-dose treatments experienced peak viremia levels ranging between 6.5×10^2 to 6.2×10^5 pfu/ml at 3–4 dpc, while the medium-dose treatment showed further reductions in viremia with peak levels ranging between 1.8×10^3 and 2.6×10^4 pfu/ml at 2–6 dpc. Importantly, no presence of virus in blood or viral shedding was observed by either detecting replicating virus or the presence of viral RNA in the highest-dose treatment group at any time point. Overall, these results highlight the efficacy of high-dose Ad5-pIFN ω5 in suppressing both viremia and viral shedding.

In vivo evaluation of innate and humoral responses in animals inoculated with Ad5-pIFN ω5

To investigate the protective effect observed with Ad5-pIFN ω5 as a biotherapeutic in swine, we examined whether animals developed neutralizing antibodies following FMDV challenge. In this study, neutralizing antibodies were induced only when active viral replication occurred. Specifically, neutralizing antibody titers peaked for all three groups except for the highest dose group at 7 dpc (Figure 4A). These results demonstrate that animals receiving the high dose of Ad5-pIFN ω5 (1×10^{10} pfu) effectively blocked FMDV replication in swine upon challenge.

To further investigate the influence of Ad5-pIFN ω5 on the innate immunity in swine, serum levels of cytokines IFN α, ISG15, and IFN γ were measured by ELISA. The data is presented as percent induction, so that levels of each protein are compared with the baseline amount for each animal. ELISA analysis of IFN α indicated an early increase in the group of animals receiving the high and medium dose of Ad5-pIFN ω5. Importantly, the highest prechallenge (0 dpc) IFN α levels were observed in the group inoculated with the highest dose of Ad5-pIFN ω5, and these elevated levels (325%) correlated with full protection after FMDV challenge. Following this early peak, IFN α concentrations in sera declined over time, potentially due to the negative regulation of the type I IFN pathway, limiting the extent and duration of these signals [35]. The control and low-dose groups conversely showed sustained IFN α induction at 3 dpc, likely attributed to ongoing viral replication stimulating an antiviral response. Analysis of IFN β by ELISA, however, showed different trends compared with IFN α, which indicates that IFN ω5 mainly induced IFN α at early time points (Additional file 2).

Secreted ISG15 induction was next analyzed in serum, and the highest-dose group showed minimal induction at early time points (0–2 dpc). Interestingly, at 3 dpc, all groups had detectable and higher levels of ISG15 in serum when compared with 0 dpc. This pattern may reflect stimulation by either the Ad5-pIFN ω5 (high-dose group) protective treatment or by viral replication observed in the control (Ad5-Blue), medium-, and low-dose groups. Because ISG15 stimulation can be triggered by either exogenous IFN or by viral replication, it is difficult to distinguish the definitive cause of the serum ISG15 levels observed.

The next cytokine analyzed in the serum was IFN γ, which as secreted ISG15 was not strongly induced until 3 dpc across all groups. Although ISG15 has been shown to induce IFN γ production in natural killer (NK) and T cells, type I IFNs can also directly stimulate IFN γ production [36, 37]. Notably, the highest-dose group was able to produce similar amounts of IFN γ compared with the other groups without any detectable viral replication taking place (Figure 4B). The results from these experiments indicate that the overall pattern characterized by detectable IFN α in sera prior to challenge suggests that

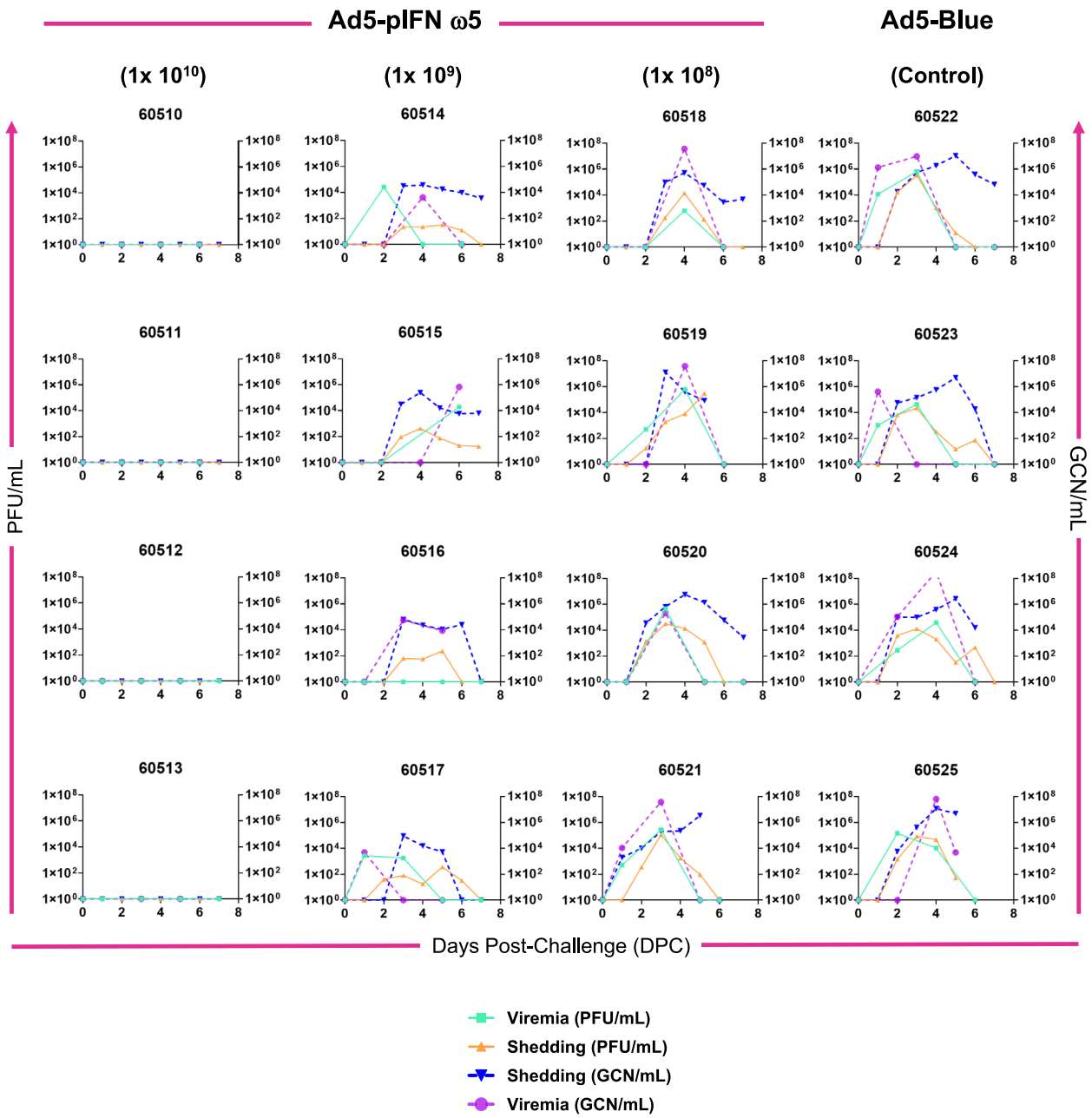

Figure 3 Viremia and virus shedding: serum and nasal secretion samples were collected at the aforementioned time points to assess viremia and virus shedding. Viremia analyzed by virus isolation using a plaque assay is expressed as PFU/mL (green line). Virus shedding in nasal secretion was also quantified by plaque assay and is expressed as PFU/mL (orange line). Virus shedding analyzed by RT-qPCR from nasal secretions is expressed by genome copy number (GCN)/mL (blue line). Viremia analyzed by RT-qPCR is expressed as GCN/mL (purple line).

pIFN ω5 contributes to an antiviral state that can protect against FMDV.

Discussion

This study aimed to expand the scope of research on type I IFNs by exploring the potential of pIFN ω5 as a novel biotherapeutic strategy against FMDV in swine. While pIFN α and β have been extensively studied for their antiviral properties in this context, pIFN ω remains comparatively understudied in vivo.

Phylogenetic studies offer intriguing insights into the evolution of type I IFNs in different species. For instance, prior research on bat species demonstrated contraction or complete loss of IFN α genes, alongside an increase

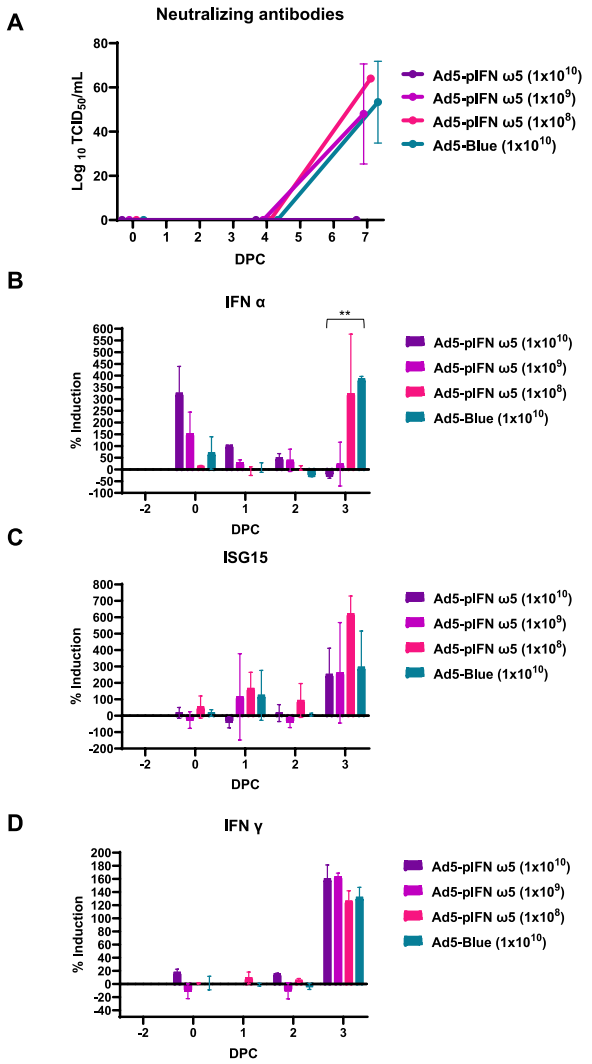

Figure 4 Analysis of immune response with neutralizing antibodies and serum cytokine levels. A Neutralizing antibody titers were measured in heat-inactivated serum against FMDV O1 Manisa at 0, 4, and 7 dpc. **B**, **C**, **D** Serum levels of IFN α, ISG15, and IFN γ were measured by ELISA. The graphs depict the percent induction of the analyzed cytokine for each animal, in comparison with their baseline level of that cytokine at the beginning of the study (−2 dpc). Statistical analysis was done with mixed effects two-way ANOVA by treatment group and time point, followed by Dunnett's multiple comparisons test. **, $p < 0.01$.

in IFN ω genes [13]. The authors postulated a functional shift toward increased tolerance to different viral infections because the potency of IFN ω could control viral replication, leading to the dampening of the proinflammatory response. In livestock species, porcine (and bovine) phylogenetic analysis has shown an expansion of the type I IFN locus, with the presence of seven IFN ω genes. This expansion could indicate the importance of IFN ω in producing a diversified antiviral response. Previous studies have demonstrated that pIFN ω features certain properties, including increased thermostability, broader and higher antiviral activity, and induction of both robust and tunable ISGs [13]. The induction of robust and tunable ISGs allows for more flexibility and regulation in the antiviral response. These findings led to the hypothesis that using this type I IFN would have beneficial effects as compared with previous results shown for pIFN α and pIFN β [5]. Thus, Ad5-pIFN ω5 was developed to determine its potential as a biotherapeutic against FMDV.

In silico analysis of the pIFN α and pIFN ω molecules revealed differences in the biochemical properties of the proteins. Namely, the differences in electrostatics and polar contacts made with IFNAR1 and IFNAR2 could all lead to differences in binding affinity to the IFN receptor. The binding affinity differences may also allow for the substitution of binding partners or the inclusion of additional binding partners. These binding affinity differences could produce changes in kinase activation and signaling, leading to differences in ISG transcription as outlined in the review about IFN structure and signaling [38]. This difference in ISG transcription is evidenced in the previously described study that demonstrated that pIFN ω was able to stimulate some tunable ISGs when compared with pIFN α1 and pIFN β [13]. The differences discovered in protein modeling could potentially result in differences with the antiviral activity associated with each molecule.

Before testing Ad5-pIFN ω5 in vivo, we confirmed and compared its antiviral activity with our established Ad5-pIFN α in vitro. Both constructs demonstrated similar antiviral effects and triggered robust induction of antiviral genes as measured by qPCR, with a declining response at 48 h, possibly indicating that both IFNs can have a rapid expression of ISGs and a rapid shutdown due to negative feedback regulation, which could be mediated by factors such as ubiquitin-specific peptidase 18 (USP18) and suppressors of cytokine signaling (SOCS) [39]. This is expected as both molecules can trigger antiviral signaling pathways mediated by the binding of similar receptors (i.e., IFNAR1/2). A similar study conducted in vitro also demonstrated significant fold increase in the ISGs *Mx1*, *OAS*, and *ISG15* [16]. Interestingly, the Ad5-pIFN ω5 construct did not highly induce IFN α mRNA transcripts compared with the transduction of the Ad5-pIFN α construct and instead was able to induce the expression of more IFN ω. This lack of upregulation could be due to conducting this experiment in vitro, the time point analyzed, and/or the variation in transcriptional regulation of IFN α versus IFN ω [40]. This difference in mRNA transcription could be the result of potential differences in the binding affinity to the IFN receptor, as described in the predicted protein

models, leading to differences in the expression of genes. As mentioned before, IFN ω expression allows for more flexibility and regulation in the antiviral response, so this higher fold induction of IFN ω may be beneficial to the host. The antiviral activities of each construct were evaluated in IBRS-2 cells and both constructs functionally had similar protective capabilities against FMDV in vitro. Importantly, transduction of adenovirus expressing IFNs maintained high levels of antiviral activity, even 48 h post-transduction, while direct treatment of IFN protein (10 ng/mL) only showed high levels of antiviral activity for up to 8 h post-treatment [16]. This demonstrates the benefit of using an adenovirus delivery system, as it will provide longer periods of antiviral activity in vitro.

We next evaluated the Ad5-pIFN ω5 antiviral activity against FMDV in the natural host, observing that swine inoculated with the highest Ad5-pIFN ω5 dose did not display any clinical signs associated with FMD while the medium- and low-dose groups had a delayed onset of clinical signs compared with naive control pigs. In parallel, the highest dose group had a 100% survival rate, while the other groups had to be euthanized at around 6–10 dpc due to FMD progression that required humane euthanasia to reduce suffering (Figure 2). Furthermore, animals treated with the highest dose of Ad5-pIFN ω5 also did not have detectable levels of viremia or shedding and did not have a neutralizing antibody response, indicating sterile immunity in this group (Figures 3 and 4A). In this context, sterile immunity is defined as no observation of FMD clinical signs, no presence of viremia or nasal shedding by our detection methodologies, and no detection of neutralizing antibodies. This sterile immunity was likely generated via a rapid innate immune response, precluding viral replication and thus antigen exposure necessary for a humoral response. These findings are consistent with previous studies involving type I IFNs such as IFN α, which have demonstrated similar antiviral effects [10, 25]. Previous studies using adenovirus delivering IFN α have demonstrated similar characteristics of full protection, no clinical signs, and no neutralizing antibody titers at the dose of 1×10^{11} pfu or in combination with other IFN stimulators such as poly(I:C), whereas in this study, all of this was achieved with 1×10^{10} pfu per animal. On the contrary, the same dosage amount of 1×10^{10} pfu with IFN α demonstrated mild clinical signs in two animals, a detectable neutralizing antibody titer, and even the detection of nonstructural FMDV proteins by ELISA, indicating that there were low levels of FMDV replication [10]. Therefore, the sterile immunity provided by pIFN ω5 is an important facet of this strategy as it may help to minimize FMDV spread during outbreak situations. In addition, in the previous study with IFN α, all animals in the 1×10^{11} pfu dosage group developed jaundice and required 2–3 days to recover. However, in the highest-dosage group in this study, only one pig developed jaundice without fever and the animal recovered within 24 h of inoculation. Therefore, although a head-to-head comparison would be warranted, our results indicated that pIFN ω5 could be more efficacious at providing sterile immunity and could have a better safety profile compared with pIFN α.

The innate immune response was evaluated by measuring serum levels of IFN α, ISG15, and IFN γ. The early detectable induction of IFN α in the highest-dose group was most likely the key factor contributing to the sterilizing immunity observed in this group, consistent with previous studies utilizing adenovirus delivering IFN α [10, 11, 20]. Although the levels of IFN α detected in the sera of fully protected swine were robust, they did not reach statistical significance. This is consistent with IFN's transient nature and limited serum persistence. The waning of the IFN α response in the high-dose group after initial administration may also be beneficial because prolonged proinflammatory signaling can have negative side effects on the host [41]. In contrast, the significant increase in serum of IFN α levels in the lowest dose and control groups at 3 dpc is concurrent with the increase in viremia. In this context, our observation is consistent with previously described studies, where detectable IFN α levels are correlated with high levels of viremia [42].

High induction of serum ISG15 was not observed in earlier sampling days (Figure 4C) for either group inoculated with Ad5 pIFN ω5 or Ad5-Blue. This observation aligns with previous reports demonstrating that ISG15 in sera does not take place immediately after administration of type I IFN and its secretion lags behind IFN induction [36]. The production of extracellular ISG15 is a complex process influenced by various stimuli including pathogen associated molecular patterns (PAMPs) and type I IFNs. In addition, there are various regulated secretion mechanisms involved that can contribute to the delay in production of serum ISG15 [43, 44]. Therefore, it is difficult to ascribe the observed ISG15 levels in this study to any single inducing factor. It is important to note that this serum-based ELISA does not account for intracellular ISG15, which has well-characterized antiviral functions such as ISGylation of viral and host proteins and modulation of immune signaling [45]. These intracellular functions may significantly impact overall antiviral responses and warrant future investigation.

Previous research shows that extracellular ISG15 is secreted by immune cells to activate NK cells and T cells, which in turn secrete IFN γ [46]. Type I IFNs are also known to promote subsequent induction of type II IFNs [37]. In this study, we observed that IFN γ was highly induced at 3 dpc across all treatment groups.

Importantly, the highest dose group showed this IFN γ response without detectable viral replication. Whether IFN γ contributes to protection in this context remains unclear. Previous studies from our group using IFN α (and IFN γ) did not lead to the detection of IFN γ in blood by ELISA. However, one of these studies did demonstrate some detection of iNOS (marker of IFN γ stimulation) in the skin and tonsils of IFN-treated pigs [11, 20]. In this study we see the induction of systemic type II IFNs in the animals treated with pIFN ω5 at 3 dpc; however, the magnitude of IFN γ detected does not allow us to determine whether these levels are associated with a protective outcome in the high-dose group.

IFN γ is known to support antiviral immunity through multiple mechanisms, including antigen presentation for T cell priming, dendritic cell maturation, and major histocompatibility complex (MHC) expression, which are all processes that can influence immunological memory [37]. While our study did not directly evaluate these downstream functions, their potential involvement in the Ad5 pIFN ω5 protective phenotype observed warrants future research. Various studies in FMDV have already evaluated the importance of IFN γ in vaccine-induced protection, suggesting that the innate immune responses seen after pIFN ω5 could potentially be used in conjunction with FMD vaccination, as it produces responses that have an adjuvant effect [47–49]. Indeed, our group has previously shown that IFN α can serve as an effective adjuvant for Ad5 FMD vaccines, raising the possibility that pIFN ω5 may similarly enhance responses to Ad5-vectored or chemically inactivated vaccines [50].

In summary, this study was the first to demonstrate the protective capacity of pIFN ω5 against FMD in swine with the use of the adenovirus delivery system. We have shown the capability of pIFN ω5 at the highest dose to establish early and sterile immunity, as well as the ability of pIFN ω5 to elicit an innate immune response. There are some caveats of this study necessitating future investigation into the use of pIFN ω5 as a biotherapeutic. This includes the sampling strategy used—which only allowed each subgroup of the treatment groups to be sampled on alternate days, restricting temporal resolution of immune responses (i.e., cytokines)—and the limitation to a single serotype of FMDV (O1 Manisa). In addition, the intrinsic variability between each pig and the transient nature of serum cytokine/signaling molecule levels can make it difficult to understand trends in immune responses. Another caveat is the lack of head-to-head comparison of pIFN ω5 with the well-characterized pIFN α in vivo in this study. Further research needs to be done to expand the understanding of the benefits of IFN ω over other type I IFN strategies against various FMDV strains, in the context of swine and cattle experiments. IFN ω remains an understudied molecule as certain aspects such as transcriptional regulation, variation in downstream signaling, and the effects on immune cells are not fully understood. Understanding these knowledge gaps will help to clarify the full potential of IFN ω as a practical biotherapeutic for FMD control. This study used an adenovirus delivery system to transiently express pIFN ω5, but future work should also explore modifications to improve IFN ω pharmacokinetics and potency. For instance, the addition of polyethylene glycol (PEG) chains and fusion with the Fc region of immunoglobulins can help with the stability and circulation of IFN ω to further improve immune responses as it has been shown for others [21, 51, 52]. Overall, this study establishes the foundation for further exploration of IFN ω-based biotherapeutic strategies aimed at enhancing early antiviral immunity and complementing conventional FMD vaccination approaches.

Supplementary information

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Additional File 1. Analysis of transcriptional antiviral activity at 48 h post-treatment. IBRS-2 cells were transduced at an MOI of 10 (Ad5-pIFN ω5, Ad5-pIFN α1, and Ad5-GFP-mCherry) and cell lysates were collected at 48 h post-transduction. RNA was extracted from these lysates to prepare cDNA, which was analyzed by qPCR for antiviral genes (*IFN ω5*, *IFN α*, *OAS1*, *Mx1*, *ISG15*, *IRF-7*, and *STAT1*). Statistical analysis was done with paired Student's *t*-test (*, $p \leq 0.05$, **, $p < 0.01$ ***, $p < 0.001$).

Additional File 2. Analysis of serum IFN β levels by ELISA. The graph depicts the percent induction of IFN β for each animal, in comparison with their baseline level at the beginning of the study. Statistical analysis was done with mixed effects two-way ANOVA by treatment group and time point, followed by Dunnett's multiple comparisons test.

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Authors' contributions

MRC, CS, RDH, SEA, PA, ERM, FDSS, and GM conducted the experiments, validation, and methodology. AM, MRC, CS, RDH, and GM wrote the main manuscript text. MRC, AM, SEA, and CS prepared all the figures and visualization. MRC, CS, RDH, SEA, PA, ERM, FDSS, and GM participated in editing of the manuscript. GM provided supervision, project administration, and funding acquisition.

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Data availability

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Protocol and procedures were submitted for prior approval by the Institutional Animal Care Use Committee of the Plum Island Animal Disease Center (USDA/APHIS/AC certificate number: 21-F-0001; protocol 151.02-22R) and were conducted in compliance with: the AWA; Guide for the Care and Use of Laboratory Animals; the 2002 Public Health Service Policy for the Humane Care and Use of Laboratory Animals; and the US Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (IRAC, 1985).

Competing interests

The authors declare no competing interests.

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