Expression of porcine fusion protein IRF7/3(5D) efficiently controls foot-and-mouth disease virus replication

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Several studies have demonstrated that administration of type I, II, or III interferons (IFNs) delivered using a replication-defective human adenovirus 5 (Ad5) vector can effectively control foot-and-mouth disease (FMD) in cattle and swine during experimental infections. However, relatively high doses are required to achieve protection. In this study, we identified the functional properties of a porcine fusion protein, poIRF7/3(5D), as a biotherapeutic and enhancer of IFN activity against FMD virus (FMDV). We showed that poIRF7/3(5D) is a potent inducer of type I IFNs including IFNα, β, and ω but not type III IFN (IL28B), without inducing cytotoxicity. Expression of poIRF7/3(5D) significantly and steadily reduced FMDV viral titers by up to 6 log10 in swine and bovine cell lines. Treatment with an IFN receptor inhibitor (B18R) combined with an anti-IFNα antibody neutralized the antiviral activity in the supernatants of Ad5-poIRF7/3(5D) transduced cells. However, several transcripts with known antiviral function, and including type I IFNs, were still highly up-regulated (ranging from 8 to over 500 fold increase) by poIRF7/3(5D) in the presence of B18R. Furthermore, mice treated with Ad5-poIRF7/3(5D) showed antiviral activity in sera that was associated with high induction of IFNα and resulted in complete protection against FMDV challenge at 6, 24 or 48 hours post-treatment. This study, highlights for the first time, the antiviral potential of Ad5-poIRF7/3(5D) in vitro and in vivo against FMDV.

**Importance:** FMD remains one of the most devastating diseases that affect livestock worldwide. Effective vaccine formulations are available but are serotype specific and require approximately 7 days for protective immunity. We have shown that vector-delivered IFN is an option to protect animals against many FMDV serotypes as soon as 24 h and for about 4 days post administration. Here we demonstrate that delivery of a constitutively active transcription factor that induces the
production of endogenous IFNs and potentially other antiviral genes is a viable strategy to protect against FMD.
INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most contagious viral diseases that affect cloven-hoofed livestock worldwide. The disease is enzootic in many regions of Africa, South America, and Asia, causing an enormous economic and social impact (1,2). The causative agent, FMD virus (FMDV), is a non-enveloped virus that belongs to the Picornaviridae family (1, 3, 4).

FMDV is an antigenically variable virus comprising seven serotypes and multiple subtypes. Serotypes A, O, C, were first isolated in Europe and occur worldwide, while serotypes SAT 1-3 and Asia-1 have traditionally been restricted to Africa and Asia, respectively (1,4,5).

Infection of animals with FMDV results in rapid replication, spread, and shedding of large amounts of virus, resulting in high morbidity. Therefore, in case of an outbreak, FMD is controlled by restriction of animal movement, slaughter of in-contact susceptible animals, and in some instances, vaccination with an inactivated vaccine followed by slaughter. Although in some countries where the disease is enzootic, preventive vaccination is commonly used (1), FMD-free countries tend to avoid vaccination due to the more restrictive trading policies imposed by the World Organization for Animal health (OIE) (1). The current inactivated whole virus vaccine is effective, but a number of limitations such as difficulty in distinguishing infected from vaccinated animals (DIVA), and requirement of an expensive high-containment facility for vaccine production has led investigators to develop alternative vaccine approaches (2,6,7).

Although vaccination is largely utilized worldwide to protect against FMD in enzootic countries, current vaccines do not always prevent infection, but rather limit or block clinical signs and require at least 5-7 days to elicit a protective immune response which results in some animals becoming long-term carriers. Therefore, in case of FMD outbreaks in disease-free countries is necessary to limit disease spread and thus potentially reduce the number of animals that have to
be slaughtered by inducing rapid protection prior to the development of vaccine-induced adaptive immunity.

Biotherapeutics represent an option to induce very early protection against FMDV infection (8). The interferon (IFN) response is one of the first antiviral mechanisms naturally induced in an infected host cell (9), (10), (11). IFNs are produced upon viral infection and play a crucial role in early innate as well as in subsequent adaptive immunity (12). The expression of type I IFN is regulated by the activation of transcription factors, such as members of the nuclear factor-kappaB (NF-κB) family (13), activating transcription factor 2 (ATF-2)/c-Jun complex (14), interferon regulatory factor 7 (IRF-7) (12, 15) and IRF-3 (11) that bind to specific sequences present at IFN promoter regions. IFN transcription, followed by translation, secretion and binding to specific receptors trigger the induction of IFN stimulated genes (ISGs) which code for antiviral products that affect viruses at different stages of their replication cycle and different viruses are susceptible to different ISG products (10, 16).

FMDV has developed several mechanisms to evade the host immune response including inhibition of cap-dependent host translation, inhibition of IFN expression and/or IFN-signaling, presumably by virus dependent degradation of NF-κB, suppression of IRF-3 and IRF-7 activation, and deubiquitination of retinoic acid-inducible gene I (RIG-I), TANK-binding kinase 1 (TBK1), tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3), and TRAF6 (17), (18-23). However, IFN proteins are still detected in serum and tissues of animals infected with FMDV, suggesting that inhibition of translation induced by the virus might be temporal and tissue or even cell specific (24). Animals that over-express IFN delivered by inoculation of a replication-defective human adenovirus type 5-based vector (Ad5) are protected against clinical manifestations of disease and in some cases protected from primary infection in a dose
dependent manner (25-28), suggesting that strength, timing and location of virus-host interactions are critical determinants for the outcome of the disease. In any case, high doses of Ad5-IFNs are required to achieve protection resulting in an expensive approach to control FMD; therefore there is a need to enhance the potency of this approach.

A construct IRF-7/3(5D) was previously described using human sequences (29) and contains 246 amino acids from IRF-7 (DNA binding and constitutive activation domains) and 295 amino acids from IRF-3(5D) (transactivation and signal response domains). Expression of this construct in cultured human cells induced activation of IFN promoters *in vitro* (29). Adjuvant properties of plasmids expressing IRF-3(5D) or IRF-7/3(5D) have also been described in mice, but IFN expression was not detected after intramuscular injection presumably as a consequence of the low efficacy of plasmid-derived gene transfer in muscular tissue (30). In this manuscript, we describe the functional characterization of a constitutively active porcine (po) IRF7/3(5D) synthetic construct as an antiviral against FMDV. We found that this fusion protein is a potent inducer of several type I IFNs (but not type III IFNs) in cells from several species. Expression of poIRF7/3(5D) enhances the antiviral activity of Ad5-poIFNβ against FMDV. Furthermore, mice inoculated with an Ad5 vector expressing poIRF7/3(5D) developed no viremia after FMDV A24 challenge and had high antiviral activity correlating with increased systemic levels of muIFNα/β. This antiviral strategy can contribute to the development of improved biotherapeutics to control FMDV infection in animals.

**MATERIAL AND METHODS**

**Cell and reagents**
Swine kidney cells (SK6 and IBRS-2) were obtained from the Foreign Animal Disease Diagnostic Laboratory (APHIS) at Plum Island Animal Disease Center (PIADC), Greenport, NY. Madin-Darby bovine kidney (MDBK, ATCC CCL-22), baby hamster kidney (BHK-21, clone 13, ATCC CCL-10), and mouse L929 (ATCC CCL-1) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were used for plasmid transfection or Ad5 vector transduction. Human 293 cells (ATCC CRL-1573) were also purchased from ATCC and were used to propagate recombinant Ad5 vectors (31). Mouse embryonic fibroblasts (MEFs) were propagated from original clones kindly provided by Dr David E. Levy (New York University) (26). Cells were cultured under standard tissue culture conditions, maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and non-essential amino acids. Ten percent tryptose phosphate broth was included in the media of BHK-21 cells.

B18R inhibitor (eBioscience, San Jose, CA) was used to block the IFN type I receptor signaling. Prior to transfection or infection, cells were incubated for 1 h at room temperature in complete media containing B18R inhibitor at a concentration of 200ng/ml. B18R inhibitor was maintained in the media during transfection and replenished after viral infection. Anti-pig IFNα, clone K9 antibody (Ab) (PBL Interferon Source, Piscataway, NJ) was used to neutralize IFNα activity present in supernatants (3 ug of antibody/ml of supernatant) of treated cells. In some transfections, poly I:C (Invivogen, San Diego, CA) was used at the specified concentration as an inducer of the IFN expression.

Viral infections

A laboratory-adapted vesicular stomatitis virus (VSV) serotype Indiana (VSIV) was kindly provided by Judith Ball (Texas A&M University). VSV serotype New Jersey (VSNJV)
field strain (95COB) and FMDV serotype A12 was generated from full-length virus infectious
cloned (32). FMDV A24 isolated from the field and passed once in BHK-21 cells was used for
mouse experiments. All experimental infections using VSNJV or FMDV were conducted at the
USDA-ARS Plum Island Animal Disease Center under biosafety level (BSL)-3Ag conditions.
Infections with VSIV were performed at Texas A&M under BSL-2 conditions.

Cells were infected at specified times post transfection or Ad5-transduction at the
indicated MOIs. In all cases FMDV or VSV were adsorbed for 1 h at 37°C. For FMDV,
unabsorbed virus was removed by washing the cells with 150 mM NaCl–20 mM
morpholineethanesulfonic acid (MES) (pH 6.0). Incubation continued for 24 h unless otherwise
specified. Virus was released by one freeze-thaw cycle. Viral titers were determined by a
standard TCID₅₀ method using IBRS-2 cells and results were expressed as log₁₀ of the
TCID₅₀/ml. Viral titers in FMDV infected mice sera were determined by plaque assay, using
standard procedures (33) and expressed as plaque forming units (PFU)/ml of serum.

Cell toxicity assay

Cell toxicity after transfection or transduction of plasmids or Ad5 vectors expressing
poIRF7/3(5D) was determined by using the XTT based in vitro toxicology assay kit (Sigma, St.
Louis, MO) following the manufacturer’s recommendations. Optical density was read at 450 nm
and filtered at 650 nm after 4 h incubation. Microscopic examination of cell morphology in the
monolayer after transfection/transduction was also used as an indicator of cell toxicity.

Plasmid construction

Partial DNA sequences of porcine IRF-7 (GenBank AB287430, nucleotides 212-964) and
IRF-3 (GenBank AB116563.1, nucleotides 400-1259) were used to synthesize a fusion construct
poIRF7/3(5D). This fusion construct was then cloned at XbaI/EcoRV sites of the pcDNA 3.1
A plasmid expressing the green fluorescent protein (GFP) in the pcDNA 3.1 zeo background (pGFP) was kindly provided by Dr. Michael Golding (Texas A&M University) and was used as a control.

**Ad5 vector construction**

PcDNA 3.1 zeo+ poIRF7/3(5D) was digested with ClaI and XbaI, and the resulting DNA fragment was ligated into a pAd5-Blue vector (34) digested with the same enzymes to create recombinant Ad5-poIRF7/3(5D). Replication-defective human adenovirus type 5 (Ad5) expressing poIRF7/3(5D) was produced by transfection of 293 cells with the Pac I-linearized recombinant pAd5-poIRF7/3(5D). Viruses were isolated, propagated, and purified by CsCl gradient centrifugation (34). Ad5-Blue, and Ad5-poIFNβ vectors were constructed previously (35), (26).

**Analysis of mRNA expression by quantitative real time RT-qPCR**

Total RNA was isolated from cell lysates using a commercially available extraction kit (Qiagen, Valencia, CA). Two hundred to thousand ng of RNA were used to synthesize cDNA using random hexamers with qScript kit mix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer’s instructions. Copied DNA was diluted tenfold and used as template for qPCR with PerfeCTa® SYBR® Green FastMix, ROX (Quanta Biosciences, Gaithersburg, MD). Samples were run in an Applied Biosystems® 7500 or StepOne Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA).

The expression of the genes of interest was normalized using GAPDH and β-actin. Relative quantification was performed for IRF-7 and a panel of previously described ISGs (10, 27). Standard curves were run to standardize a SyBr-green based PCR array of the subtypes of porcine type I IFN. Sequences for detecting subtypes of porcine IFN type I (IFNα, β, κ, ε, ω, δ)
were published previously (36-39).

RT-qPCR analysis was performed following MIQE Guidelines (40). Data was analyzed using the comparative ΔΔCt method (41).

**IFN bioassay**

Antiviral activity induced by IFN expression was tested with a VSV infection inhibition assay as previously described (20). Supernatants from cell cultures previously transduced with Ad5 vectors were filtered through centricon 100 columns (Millipore, Billerica, MA) to remove adenovirus particles.

Samples were two-fold diluted and incubated on IBRS-2 cells for approximately 24 h. Supernatants were then removed, and cells were infected with VSV NJ (MOI=2). Twenty four or 48 h later, cytopathic effect (CPE) was determined by microscopic examination, followed by staining with 1% crystal violet. Antiviral activity (IFN units/ml) was expressed as the reciprocal of the highest dilution of supernatant able to suppress VSV-induced cytopathic effect on the 50% of assayed wells.

Antiviral activity in serum samples of mice inoculated with Ad5 vectors was tested on L929 cells as previously described (42). Briefly, 2-fold dilutions of serum samples were applied to confluent monolayers of L929 cells. At 24 h cells were infected with VSV (MOI= 20) followed by 48-72 h incubation at 37°C. CPEs were scored by microscopic examination followed by staining with 1% crystal violet.

**Murine IFN ELISA**

Serum samples from mice infected with Ad5-poIRF7/3(5D) or Ad5-Blue control vector were tested for the presence of muIFN-α and muIFN-β using VeriKine mouse ELISAs (PBL Interferon Source, Piscataway, NJ) as per manufacturer’s directions. The absorbance at 450 nm
was measured in an ELISA plate reader (VersaMax, Molecular Devices, Sunnyvale, CA).

Cytokine concentrations were calculated based on the optical densities obtained with standard curves.

**Mouse challenge studies**

All animal work was conducted in compliance with the Animal Welfare Act (AWA), the 2011 Guide for Care and Use of Laboratory Animals, 2002 PHS Policy for the Humane Care and Use of Laboratory Animals, and U.S. Government Principles for Utilization and Care of Vertebrates Animal Used in Testing, Research and Training (IRAC 1985), as well as a specific animal protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Animal Disease Center (PIADC).

C57BL/6, 6 to 7-week-old female mice were purchased from Jackson Laboratory (Bar Harbor, ME) and acclimated for one week. In the first experiment, two groups of five mice each were inoculated with Ad5-poIRF7/3(5D) (3 x 10^7 or 3 x 10^8 PFU/mouse) or Ad5-Blue (3 x 10^8 PFU/mouse) subcutaneously (s.c.) in the dorsal flank. One day after Ad5 treatment, serum samples were collected for IFNs ELISA and total antiviral activity, and mice were euthanized.

In the second experiment, groups of five mice were inoculated s.c with 3 x 10^8 PFU/mouse of Ad5-poIRF7/3(5D) or Ad5-Blue. A control group was inoculated with PBS. At 6, 24 or 48h after Ad5 treatment, mice were infected s.c. in the right rear footpad with 10^5 PFU of FMDV A24 in 50 ul of PBS as previously described (43). Animals were monitored for 7 days, and blood was collected at 1, 3, 5, and 7 days post-challenge (dpc). Viremia was determined by plaque assay on BHK-21 cells (33).

Similarly, in a third experiment, groups of five mice were inoculated s.c with PBS, Ad5-poIRF7/3(5D) (3 x 10^7 PFU/mouse), Ad5-Blue (3 x 10^7 PFU/mouse) or combinations of these...
treatments with 100 IU of murine IFNa (muIFNa) [(Ad5-poIRF7/3(5D) 3 x 10^7 PFU/mouse +100 IU muIFNa, Ad5-poIRF7/3(5D) 3 x 10^6 PFU/mouse +100 IU muIFNa, Ad5-Blue 3 x 10^7 PFU/mouse +100 IU muIFNa, Ad5-Blue 3 x 10^6 PFU/mouse +100 IU muIFNa, or 100 IU muIFNa]. Ad5 inoculations were performed 48h prior to FMDV challenge while recombinant muIFNa was applied 24h before FMDV challenge. Two days after Ad5 treatment (or 24h after IFN treatment), mice were infected s.c. in the right rear footpad with 10^5 PFU of FMDV A24. Animals were monitored for 7 days, and blood was collected at 1, 3, 5, and 7 days post-challenge (dpc). Viremia was determined by plaque assay on BHK-21 cells (33).

Statistics and data analysis

Treatment differences were determined using, Student’s t-test, Dunnett’s method, or Wilcoxon-rank sum test as indicated in each figure legend. Data show representative results of three independent replicates except for mice experiments. Statistical analyses were performed using JMP software, version 8.0.2. Values are expressed as mean ± standard error (SEM), and statistical significance is indicated in figure legends.

RESULTS

PoIRF7/3(5D) induces high levels of ISGs expression

The IRF-7 transcription factor shares structural features with IRF-3, and includes a conserved DNA binding domain (DBD) and a serine-rich C-terminal region that is the target of virus-inducible phosphorylation (44). Here, using analogous domains to human IRF7/3A, we generated a chimeric construct of porcine IRF-7 and IRF-3, poIRF7/3(5D). The construct contains the DBD and constitutive activation domain (CAD) from porcine IRF-7, but lacks the inhibitory domain (ID) (Fig 1A). It also has the proline-rich domain (Pro), transactivation
domain (TAD) and SRD signal response domain from porcine IRF-3. Analogous to the human construct described previously (29), poIRF7/3(5D) contains 5 mutations in the C-terminal IRF-3 domain that mimic phosphorylation and therefore results in constitutive activation (Fig 1A).

Swine cells were transfected with the plasmid expressing the fusion protein. Since pro-apoptotic and cytotoxic effects of a human IRF7/3(5D) construct have been previously described (29), (45), we evaluated the possible effects resulting from overexpression poIRF7/3(5D) in swine cells. No cytotoxic effects of poIRF7/3(5D) were detected when 25 to 100 ng of plasmid were transfected in 2.5 x 10^5 cultured porcine cells (Fig 1B). At higher concentrations, i.e. 500 ng, a significant increase in cytotoxicity was observed (p< 0.05).

Expression of poIRF7/3(5D) was analyzed by RT-qPCR using primers and probes that detected IRF-7 and IRF-3 transcribed regions. An IRF-7 primer set detected both, poIRF7/3(5D) derived and cellular IRF-7 mRNAs. However, two sets of primer were required to detect either plasmid derived (IRF-3) or cellular (eIRF-3) transcripts. Total IRF-7 and specific IRF-3 transcribed from poIRF7/3(5D) plasmid were significantly up-regulated (~40-fold or 10-fold, respectively) in cells transfected with poIRF7/3(5D) plasmid as compared to control groups, mock or p-GFP transfected (p<0.05) (Fig 1C). Basal levels of IRF-7 were detected in mock or pGFP transfected cells similarly to endogenous IRF-3 which also remained unchanged in the poIRF7/3(5D) transfected cells.

To determine if the overexpression of poIRF7/3(5D) was able to induce changes in host gene profiles, three known ISGs, 2’,5’-oligoadenylate synthetase (OAS-1), IFN-stimulated gene 54 (ISG-54) and myxovirus resistance 1 (Mx-1) as well as IFN β were analyzed by RT-qPCR. While little or no induction was detected in SK6 cells mock treated or transfected with p-GFP, a significant up-regulation was detected in cells transfected with 25 ng of the poIRF7/3(5D) fusion
protein (Fig 1D). Inductions varying from 100 to 250 fold were detected for all analyzed genes indicating that the fusion poIRF7/3(5D) protein was active.

PoIRF7/3(5D) expressed in porcine cells has antiviral properties against FMDV and VSV

To test the biological functions of the fusion protein poIRF7/3(5D) in the context of a viral infection, SK6 cells were mock transfected or transfected with 25 ng of poIRF7/3(5D), or pGFP and later infected with either FMDV or VSV. A striking reduction (5-6 log10) in virus yield of FMDV (Fig 2A) or VSV (Fig 2B) ($p<0.0001$, in all cases) was observed in cells transfected with poIRF7/3(5D), while no effect was detected in cells transfected with the control pGFP. Consistently, antiviral activity was only detected in supernatants from poIRF7/3(5D) transfected cells and this activity was greatly decreased, but not completely neutralized, when cells were treated with an anti-poIFNα antibody (Table 1).

Antiviral activity against FMDV and VSV was also evaluated in supernatants of IBRS-2 cells transfected with plasmids expressing the poIRF7/3(5D) construct (Fig 2C-D). Similarly to SK6 cells, there was a substantial reduction of viral titers (FMDV and VSV) varying from 4-6 log10 after transfection with plasmids expressing the poIRF7/3(5D) with no inhibition detected in the p-GFP transfected cells. However, significantly less (6x) antiviral activity was detected in the supernatants of transfected IBRS-2 as compared to SK6 cells (Table 1). Most of the detected antiviral activity was neutralized by addition of an anti-porcine IFNα antibody, suggesting that poIRF7/3(5D) mainly induced this type of IFN in swine cells (Table 1).

PoIRF7/3(5D) steadily reduces viral yield and enhances the activity of Ad5-poIFNβ

To determine if poIRF7/3(5D) could induce a sustained reduction of viral titers, IBRS-2 cells were transfected with poIRF7/3(5D) and infected with FMDV A12 at different hours post-transfection (hpt). No differences in viral yields were detected earlier that 6 hpt with
poIRF7/3(5D), pGFP, or mock transfected (Fig 3A). However, by 24 hpt a 3 log₁₀ reduction in virus yield was detected and was sustained for up to 120 h only in the cells transfected with poIRF7/3(5D).

In order to deliver poIRF7/3(5D) more efficiently, we cloned its coding sequence in a replication defective human Ad5 vector (Ad5-Blue) previously developed in our lab (34).

Infection of IBRS-2 cells with Ad5-poIRF7/3(5D) at MOI 2 resulted in a reduction of approximately 5 log₁₀ in FMDV TCID₅₀/ml (Table 2). Addition of IFN neutralizing reagents (B18R inhibitor and anti-IFNα) neutralized most of the antiviral activity although some residual activity (~1 log₁₀) was still detected (Table 2).

Next, we evaluated whether Ad5-poIRF7/3(5D) could enhance Ad5-poIFNβ antiviral activity (28). SK6 cells were infected with Ad5-poIRF7/3(5D), or combinations of Ad5-poIFNβ and Ad5-poIRF7/3(5D) or Ad5-Blue (empty vector) 24 h prior to FMDV challenge. A reduction of approximately 3 log₁₀ was detected when cells were infected with Ad5-poIRF7/3(5D) at MOI of 0.2. Interestingly, a dose dependent decrease in viral yield, 2 to 3.5 log₁₀, was observed when cells were co-infected with Ad5-poIRF7/3(5D) at MOIs (0.02, 0.1, and 0.2) combined with very low amounts of Ad5-poIFNβ (MOI=10⁻⁴) as compared to mock treatment (Fig 3B). Combinations of Ad5-poIRF7/3(5D) (MOI=0.2) and Ad5-poIFNβ (MOI=10⁻⁴) resulted in significant reduction ~2 log₁₀ (p<0.01), relative to cells treated with our control vector Ad5-Blue combined with Ad5-poIFNβ at similar MOIs. At the highest MOI used (0.2), Ad5-blue did not significantly contribute to the reduction in virus titer induced by Ad5-poIFNβ (p>0.1). These results suggest that treatment with Ad5-poIRF7/3(5D) enhances the antiviral activity of Ad5-poIFNβ against FMDV.

**PoIRF7/3(5D) induces antiviral responses in vitro in species other than swine**
Since IRFs family members share some homology, we studied the effect of poIRF7/3(5D) expression in vitro across several species. Phylogenetic relationships among several IRF-3 protein sequences from some species available in public databases were deduced by maximum likelihood (ML) analysis (Fig 4A) and verified by Bayesian inference. We confirmed that species from more closely related taxonomical groups such as bovine, swine, and sheep form a monophyletic group based on IRF-3 sequences while primates, carnivores and rodents are more distantly related.

Interestingly, poIRF7/3(5D) induced a functional antiviral response in vitro in cell lines from several species including MDBK (bovine), BHK-21 (hamster), or L929 (mouse) (Fig 4). After infection with Ad5 poIRF7/3(5D) or transfection with poIRF7/3(5D) and subsequent challenge with FMDV or VSV these cell lines exhibited a drastic reduction in viral yield as compared with mock treated cells. The antiviral effect in mouse cells, L929 (Fig 4B) or MEF (not shown), was lower as compared to porcine (Fig 2A-D) and bovine cell lines (Fig 4C). Consistent with our previous data (42), transduction with Ad5-poIFNβ did not protect murine cell lines from FMDV infection (Fig 4B). Reduction in FMDV yield in BHK-21 cells treated with the fusion protein (Fig 4D) was similar to the effect observed for SK6 cells (Fig 3A) but BHK-21 cells did not develop an antiviral response after poly I:C stimulation.

Characterization of antiviral response induced by poIRF7/3(5D) in swine cells

The antiviral activity elicited by transfection of the plasmid expressing poIRF7/3(5D) in SK6 or IBRS-2 cells was not fully neutralized by addition of an anti-IFNα antibody (Table 1). To determine whether the residual antiviral activity could be attributed to the expression of other subtypes of porcine type I IFN, we quantitated relative transcript levels of the IFN type I subtypes (IFNα, β, κ, ε, ω, δ) in cells treated with the Ad5-poIRF7/3(5D). Infection with Ad5-
poIRF7/3(5D) induced expression of IFNα, β, and ω in IBRS-2 (Fig 5) and in SK6 cells (data not shown). However IFNκ, ε, δ or IL28B (IFN λ3) mRNAs were not up-regulated in any of these two cell lines.

We also questioned if an antiviral effect independent of type I IFN might be involved in the strong antiviral response of IBRS-2 cells even when these cells showed lower ability to induce antiviral responses as compared to SK6 cells (Table 1). To neutralize the IFN induced response we used B18R inhibitor, a vaccinia virus encoded product that competes with IFN for binding to the type I IFN receptor. Based on the production of antiviral activity induced by poIRF7/3(5D) in SK6 or IBRS-2 cells (Table 1), we used a dose of B18R that was sufficient to neutralize up to 500 IFN units of IFNα without causing toxicity. Transduction of IBRS-2 with Ad5-poIRF7/3(5D) completely blocked FMDV replication, and this effect was only partially reversed by the addition of the B18R inhibitor (Table 2). Although, antiviral activity in the supernatants of the cells was fully neutralized by B18R (Table 3), inhibition of FMDV replication was partially reversed (Table 2) and up-regulation of 46-, 448-fold, and 8-fold for IFNα, β and ω transcripts, respectively (Fig 5) was observed even in the presence of the B18R inhibitor combined with an anti-IFNα. Even though treatment with B18R inhibitor in combination with anti-IFNα markedly reduced the expression of all the ISGs tested, several ISGs including BST2, IP10, ISG56, ISG54, GBP4, MDA5, and OAS1 were up-regulated to relatively high levels in cells maintained with the IFN neutralizing treatment (Fig 5). Fold change in transcripts level of genes such as IP10, OAS1 and ISG56 dropped from 8026 to 2235, 5793 to 296 and from 3464 to 565, respectively. These results suggested that poIRF7/3(5D) may stimulate genes with antiviral function even when type I IFNs are neutralized. However, the
identity of antiviral genes induced by poIRF7/3(5D) fully independent of IFN stimuli in a porcine system remains to be determined.

**Ad5-poIRF7/3(5D) protects mice against FMDV challenge.**

It has been previously shown that high doses of FMDV cause fatal disease in adult (6 to 7 weeks old) C57Bl/6 mice (43) that can be prevented by treatment with IFN or IFN inducing agents (42). To determine if Ad5-poIRF7/3(5D) could induce the production of systemic IFN and protect against FMDV challenge, we inoculated mice with two doses 3x10^7 or 3x10^8 PFU/mouse) of Ad5-poIRF7/3(5D) or Ad5-Blue control. We found that mice inoculated at the high dose (3x10^8 PFU/mouse) of Ad5-poIRF7/3(5D) had statistically significantly higher levels of IFN-α (p<0.001), -β (p<0.05), and total induced antiviral activity (p<0.001) as compared to an equivalent dose of Ad5-Blue control (Fig 6A, B, C). Notably, Ad5-Blue induced some antiviral activity and production of IFN-α or -β, when it was used at a high dose (3x10^8 PFU/mouse).

Mice treated with the high dose of Ad5-poIRF7/3(5D) produced on average 21,195 pg/ml of IFN-α as compared to 210 pg/ml produced by mice treated with an Ad5-Blue control. Also mice treated with the high dose of Ad-poIRF7/3(5D) produced on average 171 pg/ml of IFN-β as compared to 85 pg/ml produced by mice treated with an Ad5-Blue control.

In a second experiment, groups of mice were treated with Ad5-poIRF7/3(5D) (3x10^8 PFU/mouse), Ad5-Blue (3x10^8 PFU/mouse) or PBS followed by challenge with FMDV at 6h, 24h or 48h after treatment. All mice inoculated with Ad5-poIRF7/3(5D) were protected from FMDV challenge (Fig 7A), developed no viremia (Fig 7B) and had high levels of antiviral activity in serum (Fig 7C). In contrast, partial protection was observed in the groups treated with Ad5-Blue at 6h or 24h prior to FMDV challenge [40% survival (Fig 7A)], but all animals died in the group treated with Ad5-Blue 48h before challenge. Consistently with these results, viremia
was detected in the three Ad5-Blue inoculated groups with highest levels in the group inoculated 48h prior to challenge (Fig 7B), however no systemic antiviral activity was detected. All animals treated with PBS died, developed high levels of viremia, and displayed no antiviral activity in serum.

In a third experiment, groups of mice were treated with 2 lower doses of Ad5-Blue or Ad5-poIRF7/3(5D) (3x10^6 or 3x10^7 PFU/mouse) in combination with 100 IU of muIFNα followed by FMDV challenge (Fig 8). We found that 100% of mice treated with 3x10^7 PFU of Ad5-poIRF7/3(5D), 3x10^7 PFU of Ad5-poIRF7/3(5D) +100 IU muIFNα, or 80% of mice treated with 3x10^6 PFU of Ad5-poIRF7/3(5D) +100 IU muIFNα survived FMDV challenge, developed viremia (~10^3-5 PFU/ml) and had high antiviral activity in serum (Fig 8A-C). In contrast, 100% mortality, high viremia (~10^7 PFU/ml), and antiviral activity below detection levels were found in all groups treated with Ad5-Blue alone or in combination with 100 IU muIFNα (Fig 8A-B). Unexpectedly, treatment with Ad5-poIRF7/3(5D) by itself or in combination with 100 IU muIFNα induced similar protection. However, treatment with 100 IU of muIFNα alone did not protect against FMDV replication and no animals survived the challenge.

DISCUSSION

In order to prevent or limit the spread of FMDV during outbreaks, it is imperative to develop methods that rapidly enhance innate immune responses in susceptible animals. It has been shown that over-expression of IFNs delivered with an Ad5 vector is effective in protecting swine and cattle against different serotypes of FMDV (25, 26, 28, 35). However, high amounts of Ad5-IFNs are required to fully protect swine and partially protect bovine. Thus, this strategy can be very expensive to protect large animals. To circumvent this limitation, a number of
strategies have been examined, including use of a combination of type I and II IFN which results in enhanced activity at lower doses (35), use of type III IFN (46), (24), use of adjuvants/modulators of innate immunity such as polyICLC (8) or use of Venezuelan equine encephalitis replicon particles (VRPs) (42). Here, we report an additional strategy that involves a fusion construct generated from porcine sequences of IRF-7 and IRF-3, namely poIRF7/3(5D). The resulting protein induced activation of type I IFNs and consequently ISGs. We demonstrated that poIRF7/3(5D) is a powerful inducer of antiviral activity against FMDV and VSV.

Even though a low amount (25 ng) of the poIRF7/3(5D) construct was transfected into porcine cells, IRF-7 transcripts were significantly increased (Fig 1C) and there was a significant induction of ISGs (Fig 1D) and antiviral activity. These results demonstrated that even a low expression of this fusion protein is sufficient to induce innate responses in porcine cells. Importantly, the expression of this construct in vitro or in vivo did not result in cytotoxicity at doses that drastically reduced viral replication (~6 log10).

Administration of inactivated FMD vaccine or an Ad5 vector expressing the FMDV capsid requires approximately 7 days to induce protective immunity (47), (48). As a result, vaccinated animals exposed to virus within the first 7 days after vaccination are still susceptible to the disease (42, 49, 50). Here, we show that administration of sufficient amounts of Ad5-poIRF7/3(5D) can completely protect mice against FMD as early as 6h and for at least 48h after treatment. Furthermore, studies in vitro indicated that inhibition of viral replication was sustained for at least 5 days post treatment. These results suggested that Ad5-poIRF7/3(5D) may not only induce rapid innate immunity, but also a relatively long lasting response that is needed for protecting animals until the vaccine induced adaptive immunity is effective. Moreover our results suggest that co-administration of Ad5-poIRF7/3(5D) might enhance the antiviral activity
of a particular IFN such as poIFNβ. This is expected as Ad5-poIRF7/3(5D) stimulates several
subtypes of type I IFN such as α, β, and ω that could enhance the antiviral properties of a single
type/subtype of porcine IFN. This information is instrumental in supporting future experiments
to explore if Ad5-poIRF7/3(5D) will allow for Ad5-poIFN dose-sparing to protect animals from
FMD.

IBRS-2 cells have been traditionally used to grow FMDV in cell culture. Apparently,
high levels of viral replication are achieved in this cell line because no induction of IFN-α/β
mRNA is detected (51). In this study we confirmed that IBRS-2 cells are somewhat impaired in
their ability to induce IFNα/β as compared to another porcine cell line, SK6 cells. Unexpectedly,
we detected antiviral activity in the supernatants of IBRS-2 cells transfected with poIRF7/3(5D)
suggesting that other IFNs or IFN-independent genes with direct antiviral activity might have
been induced by the fusion protein.

Previous reports found that expression of IRF-3 alone does not induce the synthesis of
endogenous IFN-α1 and IFN-β (45, 52). However, a subset of genes were activated in cells
expressing the constitutively active form IRF3(5D) combined with neutralizing antibodies
against IFNα/β (52). This result led us to investigate whether poIRF7/3(5D) could induce genes
with antiviral function independently of IFN. We neutralized the antiviral activity in the
supernatants of IBRS-2 cells by combining an anti-swine IFNα antibody (clone K9) and B18R
inhibitor, a vaccinia virus encoded product prevents the binding of type I IFN to its natural
receptor (IFN α/β receptor) (53). The B18R protein has broad activity across species (54), is
soluble outside the cell and present on the cell surface thus blocking type I IFN autocrine and
paracrine functions (53). In the presence of B18R and anti-swine IFNα at doses that fully
neutralized antiviral activity in supernatants of cells infected with Ad5-poIRF7/3(5D), induction
of IFN α, -β, and -ω transcripts was still highly up-regulated. In accordance with findings from Grandvaux et al. (2002) we found that ISG54 and ISG56 were still highly up-regulated in the presence of IFN neutralization treatment. Other ISGs including GBP4, (but not GBP2), IP10, MDA-5, Mx-1, and OAS-1 genes were also highly up-regulated in the presence of the IFN neutralization treatment after transduction with Ad5-poIRF7/3(5D). This is consistent with a predominantly positive transcription signature described for IRF-7 (55) or a STAT1-independent mechanism of induction as previously reported for IP10 during HIV infection of astrocytes (56).

A possible explanation to the high up-regulation of IFN transcription in the presence of B18R inhibitor could be the two-step positive feedback loop that IFNs α/β employ to amplify their own expression (57, 58). While B18R inhibits signaling through the IFN α/β receptor (second wave), earlier expression of IFNβ and IFNα-4 (57) remains unaffected by the use of B18R inhibitor. Alternatively, our treatments with B18R and anti IFNα might have not been sufficient to neutralize all subtypes of type I IFN induced by the fusion protein. The high induction of IFN transcripts in the presence of IFN neutralizing treatment does not necessarily imply that induction of genes with antiviral function by poIRF7/3(5D) is fully independent of IFN.

Another study using B18R to block the IFN response has shown induction of a lipid raft associated protein BST-2 (also known as tetherin or CD317) independently of IFN. Tetherin inhibits viral infection by preventing the diffusion of virus particles after budding from infected cells (59). Here, we confirmed that transcript levels of BST-2 were induced by 16-fold in the presence of Ad5-poIRF7/3(5D) and IFN neutralizing treatment. This antiviral protein inhibits the release of diverse enveloped virus particles and it plays a role in neutralizing VSV (60). However, a role in controlling infection of non-enveloped viruses such as FMDV remains to be elucidated.
Similarly, we have also demonstrated that poIRF7/3(5D) induces potent antiviral effects in multiple species including bovine, hamster and murine cell lines. In the case of murine cells (L929), the Ad5-poIRF7/3(5D) seemed to be less efficient to reduce viral yield, a result that was also observed when the Ad5-poIRF7/3(5D) was tested in mouse embryonic fibroblasts (data not shown). This observation is consistent with a more distant phylogenetic relationship between mouse and porcine sequences. However, contrary to the case in murine cells, reduction in antiviral properties of Ad5-poIRF7/3(5D) in hamster cells was not observed. In fact, it was surprising to detect a strong antiviral activity induced by Ad5-poIRF7/3(5D) in cell lines previously reported as defective in IFNα/β sensing or signaling (61,62) that are used routinely for viral expansion and production of FMD vaccine (63). BHK-21 cells did not respond to poly I:C stimulation (or viral infection), but responded to treatment with the poIRF7/3(5D) protein suggesting that expression of this protein bypasses certain limitations in antiviral pathways. Treatment with poIRF7/3(5D) in BHK-21 or IBRS-2 cells might bypass a defect in pathogen associated molecular pattern (PAMP) sensing or transduction pathways and directly induce strong transcription of IFN or other genes with antiviral activities. Further studies are required to characterize the plethora of responses that could be induced by the fusion protein in these cell lines.

Characterization of the antiviral activity induced by poIRF7/3(5D) in porcine cells led us to identify type I but not type III IFNs (IFNλ3 or IL28B) as major players in the induced antiviral effect. Type III IFN includes three IFN-λ molecules (IFN-λ1, -λ2 and -λ3, which are also known as interleukin-29, IL-28A and IL-28B, respectively) (39). These cytokines induce similar innate antiviral responses as type I IFN, but they have a different structure and bind a different cell-surface receptor (9, 24). Contrary to our findings using poIRF7/3(5D) in porcine cells, it has
been reported that human IFN-λ2/3 gene expression is mainly controlled by IRF-7 (64). In accordance with our results, another study suggested that type III IFNs are induced through independent actions of IRFs and nuclear factor-kappa B (NF-κB) (65). Another report also suggested c-REL/p65 NF-κB heterodimer and IRF-1 are the main transcriptional regulators of type III IFNs (66). Further studies are needed to study the regulation of type III IFN in porcine cells.

We found that expression of poIRF7/3(5D) induced the expression of various but not all type I IFN mRNAs including IFNα, β, or ω. We are currently working to identify the subtypes of IFN type I with higher antiviral activity during FMDV infection in the presence and absence of the Ad5-poIRF7/3(5D) stimulation. These results are consistent with previous reports in which IFNε was mainly associated with cells of reproductive function and IFNκ expressed in epidermal keratinocytes (67). IFNδ has been shown to have high antiviral activity in porcine cells and a relevant biological role during early pregnancy when it is secreted by the trophoderm of the pig conceptus (68).

Finally, the results in vitro prompted us to evaluate the effectiveness of poIRF7/3(5D) in protecting mice from FMDV infection. We found that mice challenged with FMDV A24 at 6, 24, or 48 h post inoculation with Ad5-poIRF7/3(5D) fully survived viral challenge and developed no viremia. To evaluate if expression of the chimeric protein poIRF7/3(5D) could potentially allow dose sparing in the amount of Ad5-IFN required to induce protection, we also performed experiment in mice. Since poIFNα is not active in the murine system we combined recombinant muIFNα, at a dose known not to be protective by itself (100 IU/animal), with Ad5-poIRF7/3(5D)

Unfortunately, we could not detect an enhancement of the antiviral activity in the sera of the treated animals nor an increase in the rate of survival after inoculation with the combination
treatment. Presumably the antiviral activity of muIFNα protein decayed rapidly after administration. Even though further standardization of the dose of type I IFN is required to demonstrate potentiation between IFN and Ad5-poIRF7/3(5D), here we report that low doses (3x10^6 PFU/mouse) of Ad5-poIRF7/3(5D) fully or partially protected mice from FMDV challenge. These findings support future work in which the potentiation ability of Ad5-poIFNs and Ad5-poIRF7/3(5D) will be assessed using swine.

Confirming the results from our in vitro studies, mice treated with Ad5-poIRF7/3(5D) at high dose (3x10^8 PFU/mouse) produced on average 100 times more IFNα than the control group. Ad5-poIRF7/3(5D) at high dose also induced up regulation of IFNβ to a lesser extent than the induction of IFNα. These results were consistent with the rapid antiviral activity detected in mice sera 24 hpt. In contrast, IFNβ transcripts were induced at approximately 10-fold higher levels than IFNα in cultured epithelial porcine cells infected with Ad5-poIRF7/3(5D). Nevertheless, when IFNβ transcripts were highly up-regulated in vitro, antiviral activity accounted for only less than 10% after neutralization with IFNα specific antibodies. Further analysis of IFN transcripts and protein from in vivo experiments using porcine tissues and serum is necessary to make a more relevant comparison.

Altogether, our results demonstrated that poIRF7/3(5D) is a robust inducer of innate immunity in porcine cells. Furthermore, poIRF7/3(5D) inhibits viral replication in cell lines from several species including porcine, murine, and bovine, suggesting that a single poIRF7/3(5D) construct might hold biotherapeutic properties across species of interest thus potentially inducing protection against FMDV, a virus that affects a wide range of livestock and more than 70 species of wildlife. A more precise understanding of the role of the poIRF7/3(5D) in blocking FMDV replication in vivo will come from future studies in the natural host.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Expression of poIRF3/7(5D) induces the expression of IFN and ISGs mRNA.

(A) Schematics of porcine fusion [poIRF-7/3(5D)] and parental [poIRF-7 and poIRF-3(5D)] proteins used in this work. Asterisks represent 5 phosphomimetic aminoacid substitutions (5D) at the C-terminus of the IRF-3 DNA binding domain (DBD); proline-rich domain (pro); transactivation domain (TAD); signal response domain (SRD); constitutive activation domain (CAD); inhibitory domain (ID).

(B) Cytotoxicity assays (XTT) on IBRS-2 cells 24h after transfection with 25-500 ng of plasmids poIRF7/3(5D) or p-GFP.

(C) Expression of plasmid-derived IRF-3/IRF-7 or endogenous IRF-3 (eIRF-3) mRNA was measured by RT-qPCR in SK6 cells at 24h post transfection with plasmids poIRF7/3(5D) and p-GFP (green fluorescent protein). Mock transfection was used as control.

(D) Expression of OAS-1, ISG-54 and Mx-1 or IFNβ mRNAs was measured by RT-qPCR in SK6 cells at 24h post plasmid- or mock-transfection. Statistical analysis was performed using Dunnett’s method (*p<0.05) in panel B and Wilcoxon-rank sum test (* p<0.05) in panel C and D.
Figure 2. PoIRF7/3(5D) has significant antiviral activity against FMDV and VSV in porcine cell lines. (A-B) SK-6 or (C-D) IBRS-2 cells were transfected with 25 ng of plasmids poIRF7/3(5D), p-GFP, or mock. Twenty-four hours post transfection cells were infected with FMDV A12 or VSV Indiana at a MOI=0.1. Viral titers were determined by TCID<sub>50</sub> at 24h post-infection. Statistical analysis was performed using Student’s t-test (** p<0.01).

Figure 3. PoIRF7/3(5D) induces sustained antiviral activity and potentiates Ad5-IFNβ effects. (A) IBRS-2 cells were transfected with 25 ng of plasmids poIRF7/3(5D), p-GFP, or mock transfected. At specified times post-transfection, cells were infected with FMDV A12 at MOI=1. Twenty-four hours post-infection, supernatants were collected for viral titration by TCID<sub>50</sub>. (B) SK6 cells were co-infected with Ad5-poIFNβ (MOI=10e-4) and Ad5-poIRF7/3(5D) at three different MOIs (0.2; 0.1 or 0.02); or Ad5-poIFNβ (MOI=10e-4), Ad5-poIRF7/3(5D) (MOI=0.2), Ad5-Blue (MOI=0.2), or mock infected. At 24h, cells were challenged with FMDV MOI=0.1 for 24h followed by determination of viral titers by TCID<sub>50</sub>. Statistical analysis was performed using student’s t-test (** p<0.01).

Figure 4. PoIRF7/3(5D) induces antiviral response in vitro in cells from several species. (A) Consensus tree generated using ML. Bootstrapping values are displayed in each branch. Boxes represent four different taxonomical groups represented in the tree. (B) L929 or (C) MDBK cells were infected with Ad5-poIRF7/3(5D), Ad5-poIFNβ or Ad5-Blue at MOI= 20, or mock treated (media). Twenty four hours post treatment, cells were infected with VSV new Jersey MOI= 1 for 24h. Viral titers were determined by TCID<sub>50</sub>. (D) BHK-21 cells were transfected with plasmids poIRF7/3(5D), p-GFP, mock transfected (media), or treated with poly I:C (100ng/ml). Twenty four hours post treatment cells were infected with FMDV A12 at a
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**Figure 5. Characterization of several type I IFNs and other genes with antiviral functions induced by Ad5-poIRF7/3(5D) in porcine cells.** Analysis of gene expression in IBRS-2 cells infected with Ad5-poIRF7/3(5D) or Ad5-Blue was performed by RT-qPCR. Relative gene expression was analyzed in cells infected with Ad5-poIRF7/3(5D) or Ad5-Blue in the presence (+) or absence (-) of B18R inhibitor and an anti-IFN$\alpha$ antibody. Mock treated cells were used as reference to calculate relative gene expression using the $\Delta\Delta$ Ct method.

**Figure 6. Inoculation with Ad5-poIRF7/3(5D) induces high levels of IFN$\alpha/\beta$ and total antiviral activity in mice serum.** Groups of mice (n=5) were inoculated with Ad5-poIRF7/3(5D) or Ad5-Blue at indicated doses. (A) muIFN-$\alpha$ or (B) muIFN-$\beta$ protein levels in serum were tested by ELISA. Values are represented as natural logarithm [ln] of IFN concentration in pg/ml. (C) Antiviral activity was measured in serum using a VSV bioassay. Values are represented as natural logarithm [ln] of IFN U/ml. Statistical analyses were performed using Student’s t-test (*** p<0.001).

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activity below detection levels.

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(A) Consensus tree generated using ML. Bootstrapping values are displayed in each branch. Boxes represent four different taxonomical groups represented in the tree. (B) L929 or (C) MDBK cells were infected with Ad5-polRF7/3(5D), Ad5-polIFNβ or Ad5-Blue at MOI= 20, or mock treated (media). Twenty four hours post treatment, cells were infected with VSV New Jersey MOI= 1 for 24h. Viral titers were determined by TCID50. (D) BHK-21 cells were transfected with plasmids polRF7/3(5D), p-GFP, mock transfected (media), or treated with poly I:C (100ng/ml). Twenty four hours post treatment cells were infected with FMDV A12 at a MOI= 0.1 for 24h. Viral titers were determined by TCID50. Statistical analysis was performed using student’s t-test (* p<0.05).
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Figure 6. Inoculation with Ad5-polIRF7/3(5D) induces high levels of IFNα/β and total antiviral activity in mice serum.

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Table 1. Antiviral activity in the supernatants of porcine SK6 and IBRS-2 cells after transfection with poIRF7/3(5D)⁹

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<th>SEM</th>
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</table>

⁹ Antiviral activity bioassay was performed in supernatants of SK-6 or IBRS-2 collected 24 h post transfection of 25 ng of plasmid DNA.

b In some supernatants, a neutralizing mouse anti-porcine IFNα antibody was added to the cell supernatants prior to testing of VSV antiviral activity.
Table 2. Antiviral activity induced by Ad5-poIRF7/3(5D)\(^a\).

<table>
<thead>
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<th>B18R +</th>
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<td>Anti-IFNα +</td>
</tr>
<tr>
<td>Ad5 poIRF7/3(5D)</td>
<td>&lt; 0.1</td>
<td>0.0</td>
<td>4.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Media</td>
<td>5.5</td>
<td>0.0</td>
<td>6.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Ad5 Blue</td>
<td>5.1</td>
<td>0.4</td>
<td>5.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

\(^a\) FMDV viral titers (log TCID\(_{50}/\text{ml}) recovered from supernatants of IBRS-2 cells 24 h after infection with Ad5-poIRF7/3(5D) in presence (+) or absence (-) of IFN neutralizing agents (B18R and anti-porcine IFNα antibody).
Table 3. Antiviral activity from IBRS-2 cells filtered supernatants after treatment with Ad5-poIRF7/3(5D) in presence (+) or absence (-) of IFN neutralizing treatment.

<table>
<thead>
<tr>
<th></th>
<th>B18R-</th>
<th>B18R+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-IFNα -</strong></td>
<td>IFN U/ml</td>
<td>IFN U/ml</td>
</tr>
<tr>
<td>Ad5 poIRF7/3(5D)</td>
<td>11.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Media</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ad5 Blue</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Antiviral activity bioassay was performed in supernatants of IBRS-2 collected 24 h after infection with Ad5-poIRF7/3(5D) in presence (+) or absence (-) of IFN neutralizing agents (B18R and anti-porcine IFNα antibody).*