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A recombinant adenovirus bicistronically expressing porcine interferon-α and interferon-γ enhances antiviral effects against foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is a virulent and economically costly disease in domestic livestock. Since the current vaccine available against FMD provides no protection until 7 days postvaccination, the only alternative method to halt the spread of the FMD virus (FMDV) during outbreaks is by the application of anti-viral agents. The combination of recombinant adenovirus expressing type I interferon (IFN-α) and adenovirus expressing type II IFN (IFN-γ) has been reported to be an effective anti-viral treatment strategy against FMDV. Nevertheless, the recombinant adenovirus mixture may be inefficient because of the low anti-viral efficiency of IFN-γ compared to that of IFN-α. In this study, we generated a recombinant adenovirus co-expressing porcine IFN-α and IFN-γ in tandem using an FMDV 2A sequence to mediate effective cleavage of the two proteins (referred to as Ad-porcine IFN-αγ). We demonstrated that both recombinant porcine IFN-α and IFN-γ were expressed and interferon stimulated gene (ISG)s related with IFN-α and IFN-γ were induced in porcine kidney (IBRS-2) cells infected with Ad-porcine IFN-αγ. Additionally, the anti-viral effects of Ad-porcine IFN-αγ against FMDV were enhanced both in IBRS-2 cells and in CD-1 (ICR) suckling mice compared to that of adenovirus expressing only a single protein. We propose that Ad-porcine IFN-αγ could be a rapid, highly efficient, convenient anti-viral agent against FMDV.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most prevalent and costly diseases affecting livestock globally. It is a highly contagious disease that affects cloven-hoofed animals such as cattle, swine, and sheep (Pereira, 1981).The FMD virus (FMDV) belongs to the genus *Aphthovirus* in the family *Picornaviridae* (Bachrach, 1968).The virus consists of seven serotypes: A, O, Asia1, C, SAT1, SAT2, and SAT3; additionally, numerous subtypes have evolved within each serotype (Knowles and Samuel, 2003).

The use of current FMD vaccines to induce early protection is limited in effectiveness because seroprotection is not effective until a minimum of 7 days after vaccination (Golde et al., 2005). The recombinant adenovirus FMDV subunit vaccine also requires 7 days to induce seroprotection (Diaz-San Segundo et al., 2010). Therefore, anti-viral agents are the only available treatment to induce rapid protection and reduce the spread of FMDV during outbreaks until vaccine-induced protective immunity can work in livestock (Grubman, 2005). In the absence of adequate means of early control, the virus spreads rapidly; pigs excrete high copy numbers of airborne FMDV, and airborne FMDV is spread from pigs to cattle, sheep, and even goats (Alexandersen and Donaldson, 2002; Alexandersen et al., 2003; Donaldson and Alexandersen, 2002). Therefore, it is important to control the aerosol transmission of FMDV from pigs.

Combinatorial treatment strategies have been used to enhance the anti-viral effects against several viruses (Haasbach et al., 2013; Kim et al., 2012; Pariente et al., 2001; Rhoden et al., 2013; Shepherd et al., 2004). The individual components of the combinatorial anti-viral treatments function through distinct mechanisms, which may be advantageous in overcoming viral resistance mechanisms

Abbreviations: CPE, cytopathic effect; DPC, days postchallenge; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; IFN, interferon; IP, intraperitoneal; MOI, multiplicity of infection; SD, standard deviation; RT-PCR, reverse transcriptase polymerase chain reaction; $TCID_{50}$, 50% tissue culture infective dose; PI, postinfection; LD_{50}, 50% lethal dose.

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against anti-viral treatments (Kim et al., 2012). Specifically, synergistic interactions in interferon (IFN) combinatorial therapy between IFN-α and IFN-γ have been reported, resulting in enhanced anti-viral effects against several viruses, including hepatitis C virus, herpes simplex virus, and cytomegalovirus (Sainz and Halford, 2002; Sainz et al., 2005; Vollstedt et al., 2004). Interferons, which induce anti-viral activity against a broad spectrum of mammalian viruses, are commonly classified as type I IFN (IFN-α and IFN-β) or type II IFN (IFN-γ) (Pestka et al., 1987; Samuel, 1991, 2001). Moreover, type III interferon (IFN-λ), which induces similar innate anti-viral responses as type I IFN, has recently been described (Kotenko et al., 2003; Perez-Martin et al., 2012; Sang et al., 2010). Type I IFNs are induced in virus-infected cells, whereas type II IFN is synthesized by cells of the immune system, including natural killer (NK) cells, $CD4^+$ T-helper 1 (Th1) cells, and $CD8^+$ cytotoxic suppressor cells (Samuel, 2001).

FMDV has been reported to be quite sensitive to interferons (Summerfield et al., 2009). IFN-α has significant inhibitory effects against FMDV in cells and pigs, and IFN-γ promotes NK cell and macrophage activation, which can control FMDV replication in the host. Similarly, a mixture of recombinant adenoviruses expressing porcine IFN-α and IFN-γ synergistically enhances the anti-FMDV effects of a single adenovirus alone (Moraes et al., 2007). Moraes and colleagues demonstrated that swine inoculated with a mixture of adenoviruses expressing type I or type II IFN were protected against FMDV challenge, while treatment with a single adenovirus (10^8 plaque-forming units [pfu]) of Ad-IFN-α or 10^9 pfu of Ad-IFN-γ) did not protect against FMDV challenge. However, a major hurdle in the development of an effective recombinant anti-viral therapy is the significantly lower anti-viral efficiency of porcine IFN-γ compared to that of porcine IFN-α (Abbas et al., 2007; Moraes et al., 2007). Thus, preparing a mixture of recombinant adenoviruses expressing porcine IFN-γ and IFN-α may be inefficient both in cost and in labor, since both are necessary for optimal anti-viral activity.

Therefore, development of a novel methodology to co-express the two IFN proteins at comparable expression levels is required. The FMDV 2A sequence, a mediator of primary polyprotein cleavage in FMDV genes, has been successfully used for co-expression of heterologous proteins in various cells (Chan et al., 2011; de Felipe et al., 2006; Furler et al., 2001; Liu et al., 2012). FMDV 2A possesses several advantages over the internal ribosome entry site (IRES), which has been commonly used for mediating the co-expression of two or more genes. The FMDV 2A sequence is short, and protein expression from a bicistronic vector based on FMDV 2A is up to 4 times greater than that from an IRES-based vector (Chinnasamy et al., 2006).

In this study, we generated recombinant adenovirus simultaneously expressing porcine IFN-α and IFN-γ using the FMDV 2A sequence (referred to as Ad-porcine IFN-αγ). We demonstrated that both porcine IFN-α and IFN-γ were co-expressed in porcine kidney (IBRS-2) cells infected with Ad-porcine IFN-αγ. Additionally, the anti-viral effects of Ad-porcine IFN-αγ against FMDV were enhanced in IBRS-2 cells and CD-1 (ICR) suckling mice compared to that of adenovirus expressing a single protein. We propose that Ad-porcine IFN-αγ may be used as a highly efficient, convenient anti-viral agent against FMDV.

2. Materials and methods

2.1. Cells, viruses, and virus titration

Human embryonic kidney cells containing human adenovirus type 5 E1 DNA (293A cells) and porcine kidney cells (IBRS-2) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; pH 7.4) at 37 °C with 5% CO_2. The FMDV strain O/SKR/2002 (Genbank accession numbers AY312589 and AY312588) passaged in IBRS-2 cells was used for viral challenge in cells and suckling mice. Viral titers were determined in IBRS-2 cells for FMDV and 293A cells for recombinant adenovirus. The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the formula of Reed and Muench (Reed and Muench, 1938).

2.2. Design and construction of adenoviral plasmid

To simultaneously express porcine IFN-α1 and IFN-γ, the consensus sequences of porcine IFN-α (Genbank accession number NM214393) with removal of the stop codon, FMDV-2A sequence (CAGCTGTTGAACTTTGACCTGCTCAAGTTGGCAGGAGACGTCGAGCCCAACCCTGGGCCC), and porcine IFN-γ (Genebank accession number AY293733) was synthesized by Bioneer Corp. (Daejeon, Republic of Korea). Porcine IFN-α and IFN-γ genes were codon-optimized for pigs on the basis of original sequences, cloned into a pGEM-T Easy Vector (Promega, Fitchburg, WI, USA), and subcloned into the pShuttle 2 vector (Clontech, Mountain View, CA, USA) by cleaving the restriction sites with the endonucleases *Nhe*I and *Xba*I. To compare with the anti-viral activity of porcine IFN-αγ against FMDV, pShuttle-porcine IFN-α and pShuttle-porcine IFN-γ were prepared. pGEM-T-porcine IFN-αγ was mutated to insert a stop codon downstream of IFN-α for expression of only porcine IFN-α, and IFN-αγ was mutated to delete IFN-α and the FMDV 2A sequence for expression of only porcine IFN-γ using the KOD-Plus Mutagenesis kit (Toyobo, Osaka, Japan). Two pairs of forward and reverse oligonucleotides: (IFN-α-forward, 5′-TCACTCCTT CTTGCGAAGTCTATCCTGCAG-3′; IFN-α-reverse, 5′-CAGCTGTTGAA CTTCGACCTGCTCAAGTTG-3′; IFN-γ-forward, 5′-GGGCCCATGAG TTATACAACTTATTTCTTAG-3′; IFN-γ-reverse, 5′-GCTAGCAATC GAATTCCCGCGGCCGCCATGG-3′) were used for mutagenesis.

2.3. Recombinant adenovirus production

Recombinant human adenoviruses were generated by following the manufacturer's instructions. Briefly, to generate recombinant adenovirus, pShuttle vectors were subcloned with Adeno-X viral DNA (Clontech). The recombinant adenoviral DNAs were linearized and transfected into 293A cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Once an ~80% cytopathic effect (CPE) was observed, the adenovirus was harvested and amplified. The harvested virus was purified using a ViraBind adenovirus purification kit (Cell Biolabs, San Diego, CA, USA). Adenovirus stocks of 10^8–10^9 $TCID_{50}$/mL were used for the experiment.

2.4. Protein detection of porcine IFN-α and porcine IFN-γ by western blot and enzyme-linked immunosorbent assays (ELISAs)

To assay for protein expression, IBRS-2 cells in 75-cm^2 culture flasks were infected with adenovirus at a multiplicity of infection (MOI) of 15. At 48 h postinfection, the supernatant was collected and concentrated using an Amicon Ultra-Centrifugal Filter Unit (Millipore, Billerica, MA, USA) for subsequent western blot. The supernatants were loaded onto NuPAGE 4–12% Bis Tris Precast Gels (Invitrogen) and separated. Proteins were transferred to PVDF membranes and blocked in 5% nonfat dry milk in PBS/Tween 20 (PBST). After washing three times with PBST, membranes were incubated with anti-porcine IFN-α monoclonal antibodies, K9 (Thermo Scientific, Waltham, MA, USA), or anti-porcine IFN-γ polyclonal antibodies (Thermo Scientific). After additional washing steps, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Detection and imaging analyses were performed using Amersham ECL Prime

Western blotting detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and an Imagequant LAS 4000 system (GE Healthcare Life Sciences) according to the manufacturer's instructions. To measure the level of protein expression, IBRS-2 cells in 48-well culture plates were infected with adenovirus at various MOIs. The supernatants of cells were collected at 48 h post FMDV infection, and porcine IFN-α (USCN Life Science Inc., Houston, TX, USA) and porcine IFN-γ (Thermo Scientific) ELISAs were carried out according to the manufacturers' instructions. The concentration of porcine IFN-α or porcine IFN-γ was determined for each protein by extrapolation from standard curves.

2.5. Determination of anti-viral effects in IBRS-2 cells

IBRS-2 cells were plated in each well of a 96-well plate at a density of 3.5×10^4 cells/well. The following day, plated cells were 90% confluent at the time of infection. Cells were inoculated with recombinant adenoviruses with a 10-fold serial dilution. After a 24-h absorption period, the recombinant adenoviruses were removed, and cells were washed twice with DMEM. IBRS-2 cells were immediately infected with 600 $TCID_{50}$ of FMDV. After a 1-h absorption period, the inocula were removed, and 100 μL culture medium with 2% FBS was added. The cells were incubated at 37 °C for 48 h. Supernatants were collected at 48 h postinfection (PI) and analyzed in replication analysis. To assay the effects on FMDV RNA replication, RNA extraction and quantitative real-time RT-PCR were performed. Viral RNA was extracted by MagNa Pure 96 LC system (Roche, Basel, Switzerland). Real-time RT-PCR was conducted as previously described (Kim et al., 2010).

2.6. Determination of anti-viral effects in suckling mice

Animal experiments were approved by the Animal Care and Use Committee of the Animal and Plant Quarantine Agency (QIA). To investigate the anti-viral effects of IFN *in vivo*, 6-day-old CD-1 (ICR) suckling mice weighing 4–5 g (Orient Co. Ltd, Republic of Korea) were used. The dose of FMDV was determined in four 10-fold serial dilutions of the virus. The 50% lethal dose (LD_{50}) of FMDV was estimated by the Reed-Muench method (Reed and Muench, 1938). Suckling mice were inoculated by intraperitoneal (IP) injection with 1×10^7 $TCID_{50}$ of recombinant adenovirus. At 24 h PI of recombinant adenoviruses, the suckling mice were challenged with 125 LD_{50} or 250 LD_{50} of FMDV (O/SKR/2002 strain, 0.05 mL volume) by IP injection. The survival of animals was monitored for 7 days.

2.7. Analysis of IFN-stimulated genes (ISGs) in IBRS-2 cells

To analyze the expression levels of ISGs, quantitative real-time RT-PCR was used to evaluate the levels of indoleamine 2.3-dioxygenase (*INDO*), 10- kDa IFN-γ-inducible protein (*IP-10*), interferon regulatory factor 1 (*IRF-1*), myxovirus resistance (*Mx*), 2′-5′ oligoadenylate synthetase (*OAS*), and double-stranded RNA-dependent protein kinase (*PKR*) mRNAs. IBRS-2 cells were plated in 12-well plates at a density of 3.5×10^5 cells per well and inoculated with adenovirus at an MOI of 5 the following day. After a 2-h incubation period for adsorption, the inocula were removed, and cells were washed twice with DMEM. The cells were collected at 24 h post-adenovirus infection, and RNA extraction, DNase I treatment, and quantitative real-time RT-PCR were conducted as previously described (Kim et al., 2010). Primers and Taqman probes described previously were purchased from Applied Biosystems (Moraes et al., 2007). Porcine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.8. Statistical analysis

T-tests were performed for statistical analysis of the *in vitro* anti-viral assay using GraphPad Instat software (version 3.05; La Jolla, CA, USA). The log-rank test was performed for statistical analysis of the *in vivo* anti-viral assay by GraphPad Prism (Version 4.0). Differences with *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Bicistronic protein expression of Ad-porcine IFN-αγ

Western blot analysis was performed to detect the expression of porcine IFN-α and IFN-γ, and ELISAs were subsequently used to quantify protein expression levels. Both porcine IFN-α and IFN-γ proteins were detected in the supernatants of cells infected with Ad-porcine IFN-αγ, the recombinant adenovirus co-expressing the two IFNs (Fig. 1). The molecular weights of porcine IFN-α and IFN-γ were estimated to be in the range of approximately 19–21 kDa based on SDS–PAGE. Two proteins corresponding to IFN-α and IFN-γ from Ad-porcine IFN-αγ were also detected by porcine IFN-α and IFN-γ ELISAs (Table 1). The IFN-α expression level from Ad-porcine IFN-αγ (288.9 ± 19.2 pg/mL) was similar to that of Ad-porcine IFN-α (309.8 ± 9.0 pg/mL), while the IFN-γ expression level of Ad-porcine IFN-αγ (167.8 ± 35.7 pg/mL) was approximately double that of Ad-porcine IFN-γ (78.2 ± 0.4 pg/mL) following adenovirus infection at 0.2 MOI. IFN-α and IFN-γ expression levels were similar between Ad-porcine IFN-αγ and

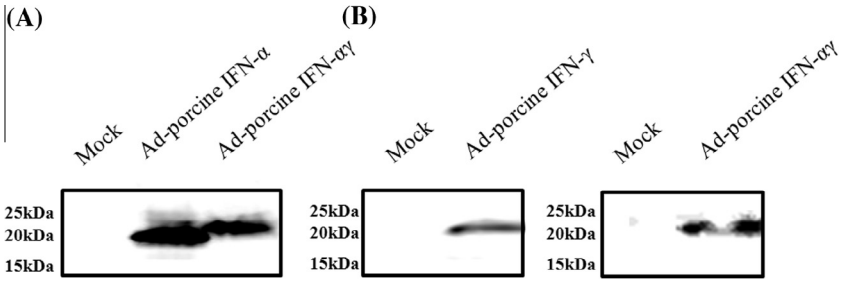

Fig. 1. Western blot analysis of porcine IFN-α and IFN-γ protein expression in IBRS-2 cells infected with recombinant adenoviruses. IBRS-2 cells were infected with adenovirus at a multiplicity of infection (MOI) of 15. The supernatants were collected at 48 h postinfection with recombinant adenoviruses, concentrated, and resolved. Western blot was performed to assess protein expression. Mock lanes are samples from IBRS-2 cells with no treatment, and adenovirus lanes are samples from the cells infected with each respective recombinant adenovirus. (A): lane Mock, Ad-porcine IFN-α, and Ad-porcine IFN-αγ were examined to detect porcine IFN-α protein using the anti-porcine IFN-α monoclonal antibody K9. (B): lane mock, Ad-porcine IFN-γ, mock, and Ad-porcine IFN-αγ were examined to detect porcine IFN-γ protein using anti-porcine IFN-γ polyclonal antibodies. The molecular weights of IFN-α and IFN-γ were estimated at approximately 20 kDa.

Table 1
The quantification of porcine IFN-α and porcine IFN-γ using ELISA.

Treatment	Adenovirus infection (MOI)[a]	Estimated quantity of porcine IFN-α (pg/ml)[b]	Estimated quantity of porcine IFN-γ (pg/ml)[b]
Ad-porcine IFN-α	0.2	309.8 ± 9.0	ND[c]
	0.02	153.5 ± 47.7	ND
	0.002	72.2 ± 32.4	ND
Ad-porcine IFN-γ	0.2	ND	78.2 ± 0.4
	0.02	ND	70.4 ± 13.8
	0.002	ND	ND
Ad-porcine IFN-αγ	0.2	288.9 ± 19.2	167.8 ± 35.7
	0.02	169.4 ± 27.64	67.98 ± 6.6
	0.002	91.5 ± 45.8	ND

[a] MOI: multiplicity of infection.
[b] Data are mean ± standard deviations (SD) from three independent experiments.
[c] ND: no protein detected.

Ad-porcine IFN-α (or Ad-porcine IFN-γ) following adenovirus infection at 0.02 or 0.002 MOI. However, the quantity of porcine IFN-γ was below the detection limit of the ELISA following adenovirus infection at 0.002 MOI.

3.2. Enhancement of anti-viral effects by Ad-porcine IFN-αγ in IBRS-2 cells

To determine whether simultaneous expression of porcine IFN-α and IFN-γ had a greater anti-viral effect than that of individual IFN expression at various recombinant adenovirus titers, FMDV replication in IBRS-2 cells was analyzed after recombinant adenovirus infection (Fig. 2). The anti-viral effects of Ad-porcine IFN-αγ co-expression were enhanced compared to those of either single IFN gene alone under most adenovirus titers. FMDV copy number after infection with adenovirus at 5 MOI was decreased by at least a 3-log titer compared with that of no treatment (negative control); however, the anti-viral effects were not significantly different among Ad-porcine IFN-αγ, Ad-porcine IFN-α, and Ad-porcine IFN-γ ($P > 0.05$, t-test). The inhibitory effects of Ad-porcine IFN-αγ were enhanced compared to those of Ad-porcine IFN-γ after infection with adenovirus at 0.5 MOI ($P < 0.05$, t-test) or 0.05 MOI ($P < 0.005$, t-test). At lower MOIs (i.e., 0.005 or 0.0005 MOI), enhanced anti-viral effects were observed with Ad-porcine IFN-αγ compared with either Ad-porcine IFN-α or Ad-porcine IFN-γ ($P < 0.05$, t-test).

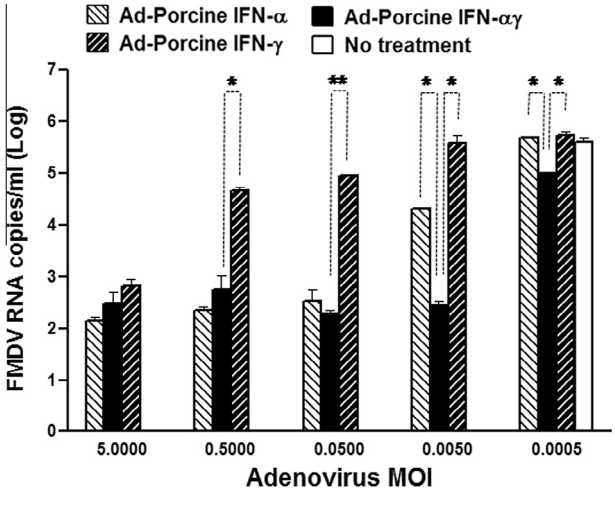

Fig. 2. Anti-viral effects of Ad-Porcine IFN-α, Ad-porcine IFN-γ, or Ad-porcine IFN-αγ at various multiplicities of infection (MOIs) of recombinant adenovirus. IBRS-2 cells were inoculated with recombinant adenoviruses at various MOIs (5, 0.5, 0.05, 0.005, 0.0005) 24 h before FMDV infection. FMDV (O/SKR/2002, 600 $TCID_{50}$) was used to infect the cells after removing the inocula. The supernatants were collected at 48 h post FMDV infection and analyzed using RNA extraction and real-time RT-PCR. The absence of adenovirus treatment was used as a negative control. Error bars indicate standard deviations (SDs) from the mean. A T-test was performed for statistical analysis (*$P < 0.05$ and **$P < 0.005$).

3.3. Enhancement of survival rates by Ad-porcine IFN-αγ in suckling mice

To test the anti-FMDV effects of Ad-porcine IFN-α, Ad-porcine IFN-γ, and Ad-porcine IFN-αγ *in vivo*, we injected the recombinant adenoviruses into suckling mice, followed by a challenge with 125 LD_{50} or 250 LD_{50} FMDV O/SKR/2002 (Fig. 3). Recombinant adenoviruses inhibited the viral replication of FMDV in suckling mice ($P < 0.005$, log rank test). An enhanced survival rate was consistently observed in mice injected with Ad-porcine IFN-αγ compared with that of mice injected with Ad-porcine IFN-α or Ad-porcine IFN-γ from 3 to 7 days postchallenge (DPC; Fig. 3A and B). The survival rate of mice in the Ad-porcine IFN-αγ group (87%) was higher than that of mice in the Ad-porcine IFN-α (76%) or the Ad-porcine IFN-γ (57%) group at 3 DPC following infection with 250 LD_{50}

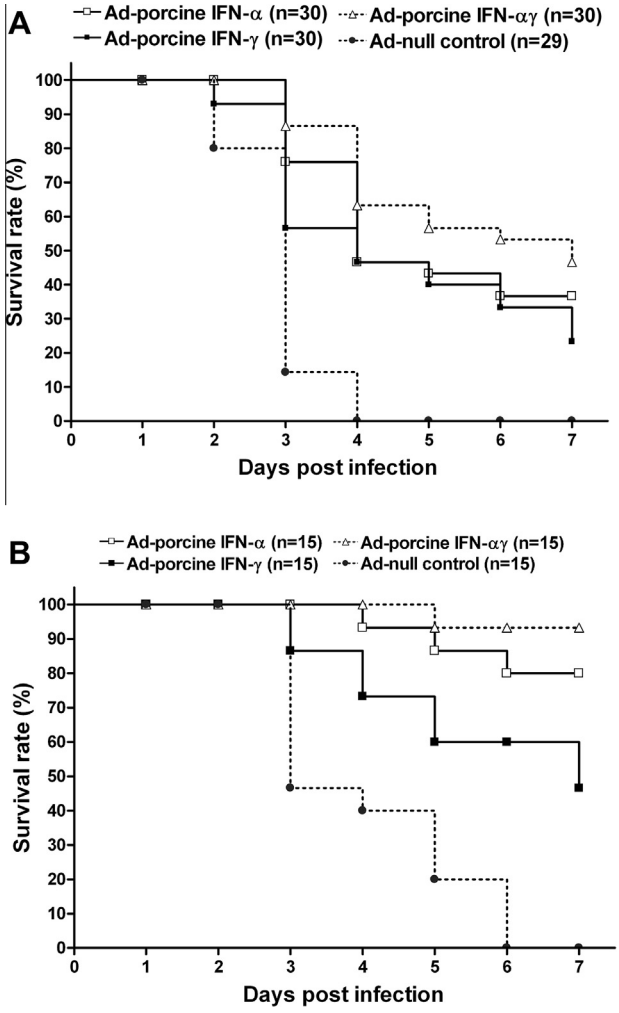

Fig. 3. Enhancement of survival rates after FMDV challenge by injection of a recombinant adenovirus co-expressing porcine IFN-α and IFN-γ. Suckling mice were inoculated with 1×10^7 $TCID_{50}$ of Ad-Porcine IFN-α, Ad-porcine IFN-γ, or Ad-porcine IFN-αγ by intraperitoneal (IP) injection. Recombinant adenoviruses were injected at the same titer 24 h before FMDV infection. Suckling mice were challenged with 250LD_{50} (A) or 125LD_{50} (B) of FMDV(O/SKR/2002). Animals were monitored for a 7-day period.

Table 2
Induction of ISGs by Ad-porcine IFN-α, Ad-porcine IFN-γ and Ad-porcine IFN-αγ.

Treatment[a]	Fold expression[b]					
	INDO	IP-10	IRF1	Mx1	OAS	PKR
Ad-porcine IFN-α	80.8 ± 0.1[c]	198.6 ± 0.5	27.1 ± 0.3	86.3 ± 1.2	108.2 ± 0.7	29.7 ± 0.1
Ad-porcine IFN-γ	39.2 ± 0.2	20.8 ± 0.2	29.1 ± 0.1	2.9 ± 0.01	4.3 ± 0.03	6.4 ± 0.01
Ad-porcine IFN-αγ	92.4 ± 0.9	416.3 ± 1.9	37.1 ± 0.3	135.2 ± 1.2	149.3 ± 1.2	39.8 ± 0.1
Ad-null control	0.03 ± 0.001	0.01 ± 0.001	0.3 ± 0.005	0.2 e± 0.003	0.1 ± 0.003	1.3 ± 0.02

[a] IBRS-2 cells were treated with the adenoviruses at a multiplicity of infection (MOI) of 5.
[b] Fold expression was calculated as the level of expression of each individual gene with respect to the level of expression in mock-IBRS-2 cells. GAPDH was used as an internal control.
[c] Data are mean ± standard deviations (SD) from three independent experiments.

FMDV(Fig. 3A). To a lesser extent, the survival rate of mice in the Ad-porcine IFN-αγ group (47%) was also higher than that of mice in the Ad-porcine IFN-α (37%) or the Ad-porcine IFN-γ (23%) group at 7 DPC. The survival rate of the Ad-porcine IFN-αγ group was significantly higher than that of the Ad-porcine IFN-γ group ($P = 0.037$, log rank test), but was not significantly different from that of the Ad-porcine IFN-α group ($P = 0.074$, log rank test). Additionally, the survival rates of the Ad-porcine IFN-α and Ad-porcine IFN-γ groups were not significantly different ($P > 0.05$, log rank test). The survival rate of mice in the Ad-porcine IFN-αγ group (93%) was also higher than that of mice in the Ad-porcine IFN-α (80%) or the Ad-porcine IFN-γ (46%) group at 7 DPC following infection with 125 LD_{50} FMDV (Fig. 3B). The survival rate of mice in the Ad-porcine IFN-αγ group was significantly higher than that of mice in the Ad-porcine IFN-γ group ($P = 0.0054$, log rank test), but was not significantly different from that of mice in the Ad-porcine IFN-α group ($P = 0.385$, log rank test). The survival rate of mice in the Ad-porcine IFN-α group was also significantly higher than that of mice in the Ad-porcine IFN-γ group ($P = 0.025$, log rank test).

3.4. Enhanced activation of ISG mRNAs by Ad-porcine IFN-αγ in IBRS-2 cells

To examine the intracellular anti-viral effect of Ad-porcine IFN-αγ, we measured the relative mRNA levels of genes related to the IFN pathway after inoculation with adenoviruses at the same MOI as in porcine cell lines (Table 2). *Mx1*, *OAS*, and *PKR* genes, which are known to be induced by IFN-α, exhibited significantly enhanced mRNA levels after treatment with Ad-porcine IFN-αγ or Ad-porcine IFN-α. *INDO*, *IP-10*, and *IRF-1* genes, which are known to be induced by IFN-γ, exhibited enhanced mRNA levels after Ad-porcine IFN-αγ, Ad-porcine IFN-γ, or Ad-porcine IFN-α. The induction of ISG increased following exposure to Ad-porcine IFN-αγ as compared to that after treatment with Ad-porcine IFN-γ (by 1.3–46-fold) or Ad-porcine IFN-α (by 1.1–2-fold). Specially, the level of *IP-10* mRNA induced by Ad-porcine IFN-αγ was 2-fold higher than that induced by Ad-porcine IFN-α and 20-fold higher than that induced by Ad-porcine IFN-γ.

4. Discussion

Interferons are involved in numerous immune interactions during viral infection (Malmgaard, 2004). The combination of type I and type II IFNs represents a promising anti-viral treatment because these IFNs induce early protection within 24 h and then promote cell-mediated immunity (Abbas et al., 2007). In the context of FMD in particular, immunoprotection may be observed when antibody levels are low or undetectable in the natural host following injection with a conventional, high-potency vaccine (Barnett and Carabin, 2002); this is caused by induction of combined innate and early adaptive immune responses (Barnard et al., 2005; Rigden et al., 2003). Furthermore, the interferon combination approach has been shown to synergistically block FMDV replication and protect treated swine from FMDV challenge (Moraes et al., 2007). Therefore, we generated a promising, efficient anti-viral agent against FMDV: a recombinant adenovirus simultaneously expressing porcine IFN-α and IFN-γ using FMDV 2A sequences (Ad-porcine IFN-αγ). Ad-porcine IFN-αγ effectively induced the expression of the two proteins and exhibited enhanced anti-viral effects against FMDV compared with recombinant adenovirus expressing only a single IFN protein.

The FMDV 2A region is a mediator of primary polyprotein cleavage in FMDV genes (Grubman and Baxt, 2004). When used to link multiple genes in one open reading frame under a single promoter, the 2A peptide separates into discrete translation products in eukaryotic cells (de Felipe et al., 2006). In our study, we also observed that the two genes were efficiently expressed, as demonstrated using western blot and ELISAs, consistent with previous studies (Chinnasamy et al., 2006; de Felipe et al., 1999; Furler et al., 2001). We also infected IBRS-2 cells with adenoviruses at MOIs not inducing cell lysis at 48 h postinfection to measure an accurate quantity of IFN in supernatants using ELISA, because we observed that high-level expression of IFN induced cell toxicity (data not shown). The incomplete cleavage of FMDV 2A-mediated fusion products has been reported in several cases (Furler et al., 2001; Milsom et al., 2004). However, we observed that porcine IFN-α protein from Ad-porcine IFN-αγ was expressed at levels similar to that from Ad-porcine IFN-α, while porcine IFN-γ protein from Ad-porcine IFN-αγ was expressed at slightly higher levels than that from Ad-porcine IFN-γ. Thus, our method allowed for the expression of both IFN proteins.

Our *in vitro* and *in vivo* model revealed enhanced anti-viral effects using Ad-porcine IFN-αγ compared to application of an equivalent titer of adenovirus expressing a single type of porcine IFN in IBRS-2 cells and CD-1 (ICR) suckling mice. Interestingly, the anti-viral effects of Ad-porcine IFN-αγ were improved following infection with adenovirus at a low MOI in IBRS-2 cells. Two explanations can be given for this phenomenon. First, the anti-viral effects of IFN-α were significantly higher than those of IFN-γ at the same concentration. This observation was consistent with previous studies reporting that the anti-viral efficiency of type II IFN is significantly lower than that of type I IFN (Abbas et al., 2007; Moraes et al., 2007). Moreover, the anti-viral effects of porcine IFN-α were about 4 times stronger than those of Ad-porcine IFN-γ at the same IFN unit treatment in IBRS-2 cells, and, according to a previous study, the anti-viral effects of Ad-porcine IFN-α and Ad-porcine IFN-γ are titer dependent (Moraes et al., 2007). In our previous study, a superior EC_{50} (effective concentration required to reduce virus CPE by 50% of control value) for Ad-porcine IFN-α ($1.4 \pm 0.5 \times 10^0$ $TCID_{50}$) was observed in IBRS-2 cells (Kim et al., 2012). In addition, the expression level of porcine IFN-γ was lower than that of porcine IFN-α. Thus, it is possible that the lower

expression level of porcine IFN-γ affected these results. Second, the effects of IFN-γ could be reduced in IBRS-2 cells compared to animal models because IFN-γ plays an important role in T cell-mediated immunity (CMI) in animals. Therefore, we also used a mouse model to confirm the enhanced anti-viral effects of Ad-porcine IFN-αγ. We observed an enhanced survival rate in mice in the Ad-porcine IFN-αγ group challenged with FMDV at 7 DPC. Interestingly, the survival rate of mice in the Ad-porcine IFN-αγ group was not significantly different from that of mice in the Ad-porcine IFN-α group, but was markedly higher than that of mice in the Ad-porcine IFN-γ group at 125 and 250 LD_{50}. However, the survival rates of mice in the Ad-porcine IFN-α and Ad-porcine IFN-γ groups were not significantly different at 250 LD_{50}. These results may arise from the differential anti-viral effects of the two IFN proteins; indeed, the anti-viral effects of porcine IFN-γ against FMDV were lower than those of porcine IFN-α. In addition, the anti-viral effects of porcine IFN-γ could be only weakly observed in suckling mice because IFN-γ has a major role in the T-cell immune system, and the immune systems of suckling mice are not entirely mature (Ryman et al., 2000). Therefore, the enhanced anti-viral effects of Ad-porcine IFN-αγ could be enhanced even further in a mature or natural host. Future studies should seek to investigate the effects of combined IFN-α and IFN-γ expression in swine.

We hypothesize that Ad-porcine IFN-αγ may possess advantages as an anti-viral agent in animals. First, we observed an enhanced anti-viral effect against FMDV by using a single recombinant adenovirus (Ad-porcine IFN-αγ), with little additional cost and labor than what was required for Ad-porcine IFN-α or Ad porcine IFN-γ generation alone. Additionally, we used the FMDV 2A sequence, which provided highly efficient protein cleavage compared to the IRES sequence. The replication efficiency of Ad-porcine IFN-αγ was not affected by the insert size in 293A cells because the insert size in the adenovirus construct was approximately 1.1 kb (data not shown). Second, Ad-porcine IFN-αγ may have a synergistic effect for type I and type II IFNs, each with a distinct role in viral inhibition. Previous studies have shown that treatment with IFN-α significantly inhibits FMDV in cells via the ISGs OAS and PKR (Chinsangaram et al., 2001, 1999). This observation suggests that type I IFN induces the cellular synthesis of enzymes that interfere with the transcription of viral RNA or DNA, as well as viral replication (Abbas et al., 2007). This process is reminiscent of the early innate immune response to viral infection. In this study, we also observed induction of ISGs, such as INDO, IP-10, IRF1, Mx, OAS, and PKR following Ad-porcine IFN-αγ expression in IBRS-2 cells (Table 2). Specially, the mRNA levels of the ISGs induced by Ad-porcine IFN-αγ were more highly upregulated than those induced by Ad-porcine IFN-α or Ad-porcine IFN-γ. Thus, while type I IFNs are involved in the regulation of cellular enzyme synthesis, the type II IFN, IFN-γ, increases the efficiency of cytotoxic T lymphocyte (CTL)-mediated immunity and stimulates the Th1 response by increasing expression of the IL-12 receptor. IFN-γ is an important regulator of CMI against viruses (Schroder et al., 2004), activating macrophages and promoting Th1 differentiation for viral inhibition. In addition, IFN-γ has been investigated as an immunomodulator of FMDV vaccination (Shi et al., 2006). Finally, in an important recent study, IFN-mediated FMDV inhibition was shown to be related to both ISG induction and immunomodulatory activity in pigs (Diaz-San Segundo et al., 2010), thereby implicating both type I and type II IFNs. Therefore, we suggest that Ad-porcine IFN-αγ offers a promising method for protection against FMDV because it can stimulate both innate immunity and CMI for inhibition of viruses. Third, we suggest that Ad-porcine IFN-αγ can be used as an anti-viral agent against several viruses. The synergistic interaction between IFN-α and IFN-γ has been reported by interferon combinatorial therapy against a number of viruses (Liu et al., 2004; Ryman et al., 2000; Sainz and Halford, 2002; Sainz et al., 2005; Vollstedt et al., 2004). Therefore, Ad-porcine IFN-αγ may possess anti-viral effects against other viral diseases in swine, in addition to FMD. Furthermore, recombinant adenoviruses have a broad host range and affect a number of species, including pigs. The co-expression of type I and type II IFNs could apply to the inhibition of viral diseases in various hosts. Future animal studies should be performed to investigate the anti-viral effects of Ad-porcine IFN-αγ against FMDV and other viral diseases in swine.

In conclusion, we developed a recombinant adenovirus simultaneously expressing type I and type II IFNs. This model could represent a highly efficient, convenient anti-viral agent to combat various viruses, including FMDV. We propose that Ad-porcine IFN-αγ is a potential candidate for blocking or delaying the transmission of FMDV and it could be used in combination with the FMD vaccine for rapid control of FMD outbreaks. In further studies, we will examine the duration of efficacy, anti-viral effects in combination with other candidates (or vaccines), and optimal injection titer in swine.

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