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# Venezuelan Equine Encephalitis Replicon Particles Can Induce Rapid Protection against Foot-and-Mouth Disease Virus

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**We have previously shown that delivery of the porcine type I interferon gene (poIFN- $\alpha/\beta$ ) with a replication-defective human adenovirus vector (adenovirus 5 [Ad5]) can sterilely protect swine challenged with foot-and-mouth disease virus (FMDV) 1 day later. However, the need of relatively high doses of Ad5 limits the applicability of such a control strategy in the livestock industry. Venezuelan equine encephalitis virus (VEE) empty replicon particles (VRPs) can induce rapid protection of mice against either homologous or, in some cases, heterologous virus challenge. As an alternative approach to induce rapid protection against FMDV, we have examined the ability of VRPs containing either the gene for green fluorescent protein (VRP-GFP) or poIFN- $\alpha$  (VRP-poIFN- $\alpha$ ) to block FMDV replication *in vitro* and *in vivo*. Pretreatment of swine or bovine cell lines with either VRP significantly inhibited subsequent infection with FMDV as early as 6 h after treatment and for at least 120 h posttreatment. Furthermore, mice pretreated with either 10<sup>7</sup> or 10<sup>8</sup> infectious units of VRP-GFP and challenged with a lethal dose of FMDV 24 h later were protected from death. Protection was induced as early as 6 h after treatment and lasted for at least 48 h and correlated with induction of an antiviral response and production of IFN- $\alpha$ . By 6 h after treatment several genes were upregulated, and the number of genes and the level of induction increased at 24 h. Finally, we demonstrated that the chemokine IP-10, which is induced by IFN- $\alpha$  and VRP-GFP, is directly involved in protection against FMDV.**

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that has significant economic consequences in affected countries. The infectious agent, FMD virus (FMDV), is a member of the *Aphthovirus* genus of the *Picornaviridae* family and contains a single-stranded positive-sense RNA genome of about 8,500 nucleotides encapsidated by 60 copies each of four structural proteins (1). FMDV is an antigenically variable virus consisting of seven serotypes (A, O, C, Asia, and South African Territories 1, 2, and 3 [SAT 1-3]) and multiple subtypes (1, 2). In the event of an outbreak in a previously disease-free country, FMD is controlled by restriction of animal movement, slaughter of infected and in-contact susceptible animals, and in some cases vaccination with an inactivated whole virus vaccine followed by slaughter of these animals (1). However, in countries in which the disease is enzootic, vaccination is used, and in general these animals are not slaughtered.

Infection of animals with FMDV results in rapid replication and spread and subsequent shedding of virus into the environment. Administration of the inactivated vaccine or an experimental vaccine based on a replication-defective human adenovirus 5 (Ad5) vector containing the FMDV capsid and 3C proteinase coding regions requires approximately 7 days to induce protective immunity in animals (3–5). As a result, vaccinated animals exposed to virus within the first 7 days after vaccination are still susceptible to the disease. To address the need of protecting vaccinated animals during this window of susceptibility, we have used an approach that induces the innate immune response. We have shown that FMDV replication is inhibited by prior treatment of cells with interferon type I (IFN- $\alpha/\beta$ ), II, or III (6–8). Based on this information, we constructed Ad5 vectors containing porcine type I IFN genes (Ad5-poIFN- $\alpha/\beta$ ) and demonstrated that swine inoculated with Ad5-poIFN- $\alpha/\beta$  are protected from challenge

with FMDV A24 Cruzeiro as early as 1 day after Ad5-poIFN- $\alpha/\beta$  administration (4, 9, 10). Protection can last for 3 to 5 days (4). We further showed that this approach is also protective against several other FMDV serotypes, e.g., O1, Manisa, and Asia-1 (10). However, when this approach was used to rapidly protect cattle, we found that only one of six treated animals did not develop vesicular lesions; although all of the other treated animals developed disease, it was delayed and less severe than that in control animals (11). More recently, we found that cattle respond best to type III IFN delivered by an Ad5 vector; in a group of three animals treated with an Ad5 vector containing bovine IFN- $\lambda$ 3 (Ad5-boIFN- $\lambda$ 3), one animal did not develop disease after aerosol challenge with FMDV O1 Manisa while the other two animals developed disease 6 or 9 days later than the control challenged animals (12).

It is known that animals initially detect pathogens through pattern recognition receptors (PRRs), molecules that recognize specific nonhost molecules, e.g., pathogen-associated molecular patterns (PAMPs), such as single- and double-stranded viral RNA (13–15). Host PRRs include Toll-like receptors (TLRs), present

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on the plasma membrane or on endosomal membranes, as well as cytoplasmic soluble receptors. Recognition of PAMPs by PRRs triggers a series of events, which may differ for each PRR engaged, and results in the induction of a protective IFN-dependent antiviral response. The events involved in the induction of the antiviral IFN response include the activation of a series of transcription factors, i.e., IFN regulatory factors (IRFs), nuclear factor  $\kappa$ B (NF- $\kappa$ B), etc. Activated IRFs and NF- $\kappa$ B are required for IFN induction as well as upregulation of additional antiviral genes, some of which are induced by mechanisms independent of type I IFN (15). So far, our strategy has been to directly utilize type I, II, or III IFNs to induce rapid protection through upregulation of various IFN-stimulated genes (ISGs). However, this approach bypasses the natural pathway of pathogen induction of the host innate immune response, including the activation of constitutively expressed and induced transcription factors. We hypothesize that treatment of animals with both IFN and various PAMPs may result in a broader, enhanced, and prolonged antiviral response than with Ad5-IFN treatment alone.

Venezuelan equine encephalitis virus (VEE) is a positive-sense RNA virus belonging to the *Alphavirus* genus, *Togaviridae* family. It is an arthropod-borne virus that has been associated with epidemics and equine epizootics. Pushko et al. (16) have constructed VEE replicon particles (VRPs) that contain a defective VEE genome lacking the genes for the structural proteins. As a result, while the viral genome is replicated, it is not packaged, and only a single round of infection occurs. VRPs have been used as vaccine vectors for various foreign genes (16–18) including FMDV (unpublished data). Recently, Konopka et al. (19, 20) demonstrated that null VRPs, VRPs lacking any foreign gene, induce an early innate immune response in mice within 1 to 3 h postinfection (hpi), resulting in the upregulation of a number of ISGs and the production of type I IFN protein. Furthermore, null-VRP-inoculated mice are protected from lethal challenge with VEE as early as 6 h after VRP administration as well as 24 h later, and this pretreatment induced protection against heterologous challenge with influenza virus but not vesicular stomatitis virus (VSV) (20).

In this study, we demonstrated that pretreatment of cells with VRPs containing green fluorescent protein (VRP-GFP) as well as porcine IFN- $\alpha$  (VRP-poIFN- $\alpha$ ) significantly reduced FMDV replication in infected porcine or bovine cells, and inhibition lasted for at least 5 days. A number of genes were upregulated after treatment of swine cells with either VRP-GFP or VRP-poIFN- $\alpha$ . We also examined the effectiveness of VRP treatment in a small-animal model. Adult C57BL/6 mice, which can be lethally infected with FMDV, survived challenge when pretreated with VRP-GFP. The chemokine 10-kDa IFN- $\gamma$ -inducible protein 10 (IP-10) was significantly upregulated in IFN- or VRP-treated cells and in mice at early times after VRP treatment, as well as in swine or cattle after IFN treatment. While 100% of wild-type (WT) mice pretreated with murine IFN- $\alpha$  (muIFN- $\alpha$ ) survived FMDV challenge, only 30% of IP-10 knockout (KO) mice pretreated with muIFN- $\alpha$  were protected, demonstrating a role for IP-10 in protection against FMDV.

These results suggest that VRP treatment is an effective approach to rapidly protect against FMD. In addition, we found that VRP-induced protection against FMDV requires a functional type I IFN system that is directly dependent on IP-10.

## MATERIALS AND METHODS

**Cells and viruses.** Porcine kidney cell lines (IB-RS-2 and SK6) and bovine kidney cells (LF-BK) (21) were used for the experiments. These cells were maintained in minimal essential medium (MEM; Gibco-BRL/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) and supplemented with 1% antibiotics and nonessential amino acids. BHK-21 cells (baby hamster kidney cells strain 21, clone 13; ATCC CL10) obtained from the American Type Culture Collection (Rockville, MD) were used to propagate virus stocks and to measure virus titers. BHK-21 cells were maintained in MEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with 1% antibiotics and nonessential amino acids (Gibco-BRL/Invitrogen). Murine L929 fibroblasts were maintained in MEM containing 10% horse serum supplemented with 1% antibiotics and nonessential amino acids and used to test antiviral activity of mouse serum after encephalomyocarditis virus (EMCV) infection. Cell cultures were incubated at 37°C in 5% CO<sub>2</sub>. FMDV serotypes A12 and A24 Cruzeiro were used in the experiments. Human 293 cells (ATCC CRL-1573) were used to generate and propagate recombinant adenoviruses and determine virus titer (22). A certified Vero cell line derived from a master cell bank prepared from cells obtained from the World Health Organization was used to generate VRPs. Vero cells were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The cells were grown in MEM supplemented with 5% fetal bovine serum, nonessential amino acids, and antibiotic-antimycotic solution (Gibco-BRL/Invitrogen).

**Replicon construction.** The pVEK replicon vector is based on the current investigational new drug (IND) VEE virus vaccine (TC-83) (18, 23). The poIFN- $\alpha$  and GFP genes were PCR amplified from existing DNA plasmids (9, 24). Each PCR product coded for XbaI restriction sites at the 5' and 3' end. The PCR products were then cloned into the XbaI site of the transfer vector pcDNA3.3 (25). The orientations of the poIFN- $\alpha$  and GFP genes in pcDNA3.3 were determined by restriction analysis, and positive clones were sequenced to ensure that no errors were introduced into the gene during PCR amplification. Each of the genes was then subcloned as an AscI fragment into the AscI site of the pVEK replicon plasmid. The orientation of the gene was determined by restriction analysis, and clones in the sense orientation were selected.

**RNA transcription, electroporation, and VRP production.** The methods used to *in vitro* transcribe replicon RNA, electroporate RNA into Vero cells, and produce and purify VRP vaccines were described previously (25). The infectious titer (in infectious units [IU]) of VRP-poIFN- $\alpha$  was determined by immunofluorescence assay (IFA) using goat anti-VEE nsP2-specific polyclonal antiserum as the primary antibody and donkey anti-goat Alexa Fluor 488 (Invitrogen) as the secondary antibody on methanol-fixed cells using a Nikon Eclipse TE300 fluorescence microscope. The infectious titer of VRP-GFP was determined directly from infected cells without the use of antibody reagents. The VRPs were tested for the presence of contaminating replication-competent VRP (RCV) using two blind passages on Vero cells, as described previously (25).

**Ad5 vector construction.** The Ad5-Blue, Ad5-VSVG (containing the glycoprotein gene of vesicular stomatitis virus-NJ), and Ad5-poIFN- $\alpha$  vectors were constructed as previously described (9, 26). Ad5-GFP and an Ad5 vector containing a small interfering RNA (siRNA) directed against GFP (Ad5-siGFP) were produced using the pAd5-Blue direct ligation system (26). All vectors were purified as previously described and tested for foreign gene expression in IB-RS-2 cells (27).

**Expression of poIFN- $\alpha$ .** Vero cells were infected with VRP-poIFN- $\alpha$ , and 24 h later supernatants were obtained, centrifuged at 14,000 rpm to remove cellular debris, and filtered through a Centricon-100 filter to remove VRPs. Concentrated supernatants were examined for the presence of IFN protein by Western blot analysis.

**Virus infection.** Cells were infected with VRPs at a multiplicity of infection (MOI) of 1 for 1 h washed with MEM, and 1 ml of MEM was added per well. At various times (see figure legends) the medium was removed, centrifuged, and filtered through a Centricon-100 filter at 4,000 rpm for 10 min in a Sorvall centrifuge. Samples were stored at -70°C until

assayed for antiviral activity or the presence of poIFN- $\alpha$  by enzyme-linked immunosorbent assay (ELISA). The cells were then infected with FMDV A12 at an MOI of 1 for 1 h, and unabsorbed virus was inactivated by washing the cells with 150 mM NaCl–20 mM morpholineethanesulfonic acid (MES) (pH 6.0). MEM was added, and incubation continued for 24 h. Virus was released by one freeze-thaw cycle. As a control, to measure infectious FMDV remaining after the acid wash, infected cells were frozen and thawed at 1 h postinfection. FMDV yields were determined by plaque assay on BHK-21 cells as previously described (5) and expressed by subtracting the titers of virus in cells infected for 1 h from the 24-h titers. The detection level of this assay is 5 PFU per ml (PFU/ml).

**Analysis of mRNA.** Total RNA was isolated from SK6 or IB-RS-2 cells, infected with VRPs or treated with poly(I · C), using an RNeasy isolation kit (Qiagen, Valencia, CA) following the manufacturer's directions. RNA yield and quality were determined in a NanoDrop 1000 spectrophotometer (Thermo Fisher, Waltham, MA) and in a Bioanalyzer (Agilent Technologies, Santa Clara, CA). A quantitative real-time reverse transcription-PCR (qRT-PCR) assay was used to evaluate the mRNA levels of a number of porcine genes as previously described (28). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the values of each sample. Primer and probe sequences were previously described (29). Reactions were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by comparative cycle threshold analysis (30) utilizing as a reference the samples at 0 dpi from the control groups. We considered genes upregulated only if there was a 2-fold or greater induction.

RNA was also extracted from spleen cells of mice inoculated for 3, 6, and 24 h with VRP-GFP. Spleens were incubated with RPMI 1640 complete medium containing 10% fetal bovine serum (FBS), L-glutamine, 0.05 M  $\beta$ -mercaptoethanol, and antibiotic/antimycotic solutions. Single-cell suspensions were obtained by mechanical disruption pressing the whole spleen through a plastic grid (BD Bioscience, Franklin Lakes, NJ). Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Single-cell suspensions were washed twice in phosphate-buffered saline (PBS) and once with complete RPMI 1640 medium, and cells were counted. A total of  $1 \times 10^7$  cells were lysed with 700  $\mu$ l of RLT buffer (Qiagen) containing  $\beta$ -mercaptoethanol and stored at  $-70^\circ\text{C}$  until RNA extraction using an RNeasy isolation kit (Qiagen). qRT-PCR was performed using the RT<sup>2</sup> Profiler PCR modified antiviral response pathway from SA Bioscience-Super Array (Qiagen), following the manufacturer's instructions from cDNA synthesis to data analysis. Reactions were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). We analyzed a total of 84 genes (Table 1), and only those that showed changes were reported.

**Interferon biological assay.** Antiviral activity was evaluated in supernatants as previously described (4). Samples were diluted and incubated on IB-RS-2 cells for approximately 24 h. Supernatants were removed, and the cells were infected for 1 h with approximately 100 PFU of FMDV serotype A12 and overlaid with gum tragacanth. Plaques were visualized 24 h later by staining with 1% crystal violet. Antiviral activity (U/ml) was reported as the reciprocal of the highest supernatant dilution that resulted in a 50% reduction in the number of plaques relative to the number of plaques in the mock-treated infected cells.

Serum samples of mice inoculated with VRP-GFP at different time points (3, 6, and 24 h) were collected, and antiviral activity was tested in L929 cells as previously described (20). Briefly, serum samples were diluted 1:10 in medium and acidified to pH 2.0 for 24 h. Following neutralization to pH 7.4, the samples were titrated by 2-fold dilutions and added to confluent monolayers of L929 cells. Twenty-four hours after the addition of the serum, IFN-sensitive EMCV ( $2 \times 10^5$  PFU) was added to each well and incubated at  $37^\circ\text{C}$ . At 18 to 24 h postinfection, the remaining cells were then stained with 1% crystal violet. The percent cytopathic effect (CPE) in each well was scored by direct observation. The antiviral titers (IU/ml) were calculated based on the standard curves generated with commercial IFN- $\alpha/\beta$ ; the endpoint titer was calculated from the dilution

of IFN- $\alpha/\beta$  required to protect 50% of the cell monolayer from EMCV-induced CPE.

**IFN- $\alpha$  ELISA.** An ELISA was performed as previously described (4). Porcine IFN- $\alpha$  concentrations in SK6 cell supernatants were expressed in picograms per milliliter and calculated by linear regression analysis of a standard curve generated with serial 2-fold dilutions of recombinant poIFN- $\alpha$  (PBL Biomedical Laboratories, NJ). All samples were assayed in duplicate. Levels of poIFN- $\alpha$  protein of  $<200$  pg/ml were not considered meaningful.

Serum from mice infected with VRP-GFP at different time points (6 and 24 h) and control mice inoculated with PBS were tested for the presence of IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\lambda$  with VeriKine mouse IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\lambda$  2/3 ELISAs (PBL Interferon Source, Piscataway, NJ) following the manufacturer's directions. All ELISAs were developed with 3,3',5,5'-tetramethylbenzidine (TMB) from KPL (Gaithersburg, MD). The absorbance at 450 nm was measured in an ELISA reader (VersaMax, Molecular Devices, Sunnyvale, CA). Cytokine concentrations were calculated based on the optical densities obtained with the standards and are expressed in relative levels with respect to the levels observed at day 0 postinfection.

**Mouse challenge studies.** C57BL/6 WT or IP-10 KO (C57BL/6 background) 6- to 7-week-old female mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were acclimated for 1 week. 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 (protocol number 204-09-R) reviewed and approved by the Institutional Animals Care and Use Committee (IACUC) of Plum Island Animal Disease Center (PIADC) (USDA/APHIS/AC certificate number 21-F-0001).

For virus infection experiments, groups of 5 C57BL/6 mice were anesthetized with isoflurane (Webster Veterinary, Devens, MA) and immediately infected subcutaneously (s.c.) in the left/right rear footpad with  $10^4$ ,  $10^5$ , or  $10^6$  PFU of FMDV A24 or O1 Campos or PBS in 50  $\mu$ l. Animals were monitored for 7 days, and blood was taken at day 0 and on alternate days until the end of the experiment. Viremia was determined by plaque assay on BHK-21 cells as described above.

To test the *in vivo* effect of VRP-GFP, groups of five mice were anesthetized as described above and immediately infected s.c. in the right rear footpad with  $10^6$ ,  $10^7$ , or  $10^8$  IU of VRP-GFP or PBS in 50  $\mu$ l. One day after VRP-GFP treatment, mice were anesthetized and challenged with  $5 \times 10^4$  PFU/mouse FMDV A24 in 50  $\mu$ l in the left rear footpad.

In a second experiment, groups of five mice were inoculated with  $10^7$  or  $10^8$  IU of VRP-GFP and challenged at 6, 24, or 48 h with  $5 \times 10^4$  PFU/mouse FMDV A24 as above. A PBS-inoculated control group was challenged with FMDV 24 h later. In this experiment groups of mice were also inoculated with  $10^8$  IU of VRP-GFP and euthanized at 3, 6, or 24 h later. A control group was inoculated with PBS and euthanized 24 h later. Serum was obtained, and the spleen was isolated from each animal; red blood cells were lysed, and the cells were washed. The cells were resuspended in RLT buffer (Qiagen). RNA was extracted using an RNeasy miniprep kit following the manufacturer's instructions (Qiagen). The serum was examined for the production of a number of proteins by ELISA (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and IFN- $\lambda$ 3/2, [all from PBL]), following the manufacturer's instructions, and RNA was examined for the induction of various genes as described above.

Finally, groups of 10 C57BL/6 IP-10 KO mice and WT mice were treated with  $10^4$  U of murine IFN- $\alpha$  (muIFN- $\alpha$ ) by intraperitoneal (i.p.) inoculation and challenged s.c. with  $5 \times 10^4$  PFU of FMDV A24 at 4 or 18 h after treatment. Ten untreated C57BL/6 WT or IP-10 KO mice were included as a control group. Animals were monitored for 7 days, and blood taken at day 0 and on alternate days until the end of the experiment.

TABLE 1 Complete list of analyzed genes in spleen lymphocytes after treatment of mice with VRP-GFP for different times

Symbol	Gene name	Other name(s)	Description	GenBank
Aim2	Gm1313	Ifi210	Absent in melanoma 2	NM_001013779
Atg12	4931423H11Rik	A330058M13Rik, Apg12l, Atg12l	Autophagy-related 12 (yeast)	NM_026217
Atg5	2010107M05Rik	3110067M24Rik, AW319544, Apg5l, Atg5l, C88337, Paddy	Autophagy-related 5 (yeast)	NM_053069
Azi2	AA410145	AZ2	5-Azacytidine-induced gene 2	NM_013727
Card9	Gm782		Caspase recruitment domain family, member 9	NM_001037747
Casp1	ICE	Il1bc	Caspase 1	NM_009807
Casp8	CASP-8	FLICE, MACH, Mch5	Caspase 8	NM_009812
Ccl3	MIP-1 $\alpha$	AI323804, G0S19-1, LD78 $\alpha$ , MIP1-(a), MIP1- $\alpha$ , Mip1a, Scya3	Chemokine (C-C motif) ligand 3	NM_011337
Ccl4	MIP-1B	AT744.1, Act-2, Mip1b, Scya4	Chemokine (C-C motif) ligand 4	NM_013652
Ccl5	MuRantes	RANTES, SISd, Scya5, TCP228	Chemokine (C-C motif) ligand 5	NM_013653
Cd40	AI326936	Bp50, GP39, HIGM1, IGM, IMD3, T-BAM, TRAP, Tnfrsf5, p50	CD40 antigen	NM_011611
Cd80	B71	Cd28l, Ly-53, Ly53, MIC17, TSA1	CD80 antigen	NM_009855
Cd86	B7	B7-2, B7.2, B70, CLS1, Cd28l2, ETC-1, Ly-58, Ly58, MB7, MB7-2, TS, A-2	CD86 antigen	NM_019388
Chuk	AI256658	Chuk1, Fbx24, Fbxo24, IKBKA, IKK1, Ikka, MGC25325, NFKBIKA	Conserved helix-loop-helix ubiquitous kinase	NM_007700
Cnpy3	1600025D17Rik	2410050O22Rik, AI413153, CAG4A, ERDA5, PRAT4A, Tnrc5	Canopy 3 homolog (zebrafish)	NM_028065
Ctsb	CB		Cathepsin B	NM_007798
Ctsl	1190035F06Rik	Ctsl1, MEP, fs, nkt	Cathepsin L	NM_009984
Ctss			Cathepsin S	NM_021281
Cxcl10	IP-10	IP10, Ifi10, Scyb10, gIP-10, mob-1, C7, CRG-2, INP10	Chemokine (C-X-C motif) ligand 10	NM_021274
Cxcl11	Cxcl11	H174, I-tac, Ip9, Itac, Scyb11, Scyb9b, b-R1, betaR1	Chemokine (C-X-C motif) ligand 11	NM_019494
Cxcl9	BB139920	CMK, Mig, MuMIG, Scyb9, crg-10	Chemokine (C-X-C motif) ligand 9	NM_008599
Cyld	2010013M14Rik	2900009M21Rik, C130039D01Rik, CDMT, CYLD1, EAC, mKIAA0849	Cylindromatosis (turban tumor syndrome)	NM_173369
Dak	BC021917	MGC28742	Dihydroxyacetone kinase 2 homolog (yeast)	NM_145496
Ddx3x	D1Pas1-rs2	Ddx3, Fin14	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	NM_010028
Ddx58	RIG-I	6430573D20Rik, C330021E21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	NM_172689
Dhx58	Lgp2	B430001I08Rik, D11Lgp2e, LPG2	DEXH (Asp-Glu-X-His) box polypeptide 58	NM_030150
Fadd	FADD	Mort1	Fas (TNFRSF6)-associated via death domain	NM_010175
Fos	cFos	D12Rfj1, c-fos	FBJ osteosarcoma oncogene	NM_010234
Hsp90aa1	86kDa	89kDa, AL024080, AL024147, Hsp86-1, Hsp89, Hsp90, Hspca, hsp4	Heat shock protein 90, alpha (cytosolic), class A member 1	NM_010480
Ifih1	MDA5	9130009C22Rik, Helicard, Hlcl, MGC90959	Interferon induced with helicase C domain 1	NM_027835
Ifna2	Ifa2		Interferon alpha 2	NM_010503
Ifnar1	Infar	CD118, Ifar, Ifrc	Interferon (alpha and beta) receptor 1	NM_010508
Ifnb1	IFN-beta	IFNB, Ifb	Interferon beta 1, fibroblast	NM_010510
Ikbkb	IKKbeta	AI132552, IKK-2, IKK $\beta$ , IKK2, IKK $\beta$	Inhibitor of kappaB kinase beta	NM_010546
Il12a	IL-12p35	Il-12a, Ll12a, MGC151228, MGC151232, p35	Interleukin-12A	NM_008351
Il12b	IL-12b	Il-12p40, Il12p40, p40	Interleukin-12B	NM_008352
Il15	AI503618		Interleukin-15	NM_008357
Il18	IL-18	Igif	Interleukin-18	NM_008360
Il1b	IL-1beta	Il-1b	Interleukin-1 beta	NM_008361
Il6	IL-6		Interleukin-6	NM_031168
Irak1	IRAK	AA408924, IRAK-1, IRAK1-S, Il1rak, Plpk, mPLK	Interleukin-1 receptor-associated kinase 1	NM_008363
Irf3	IRF-3	C920001K05Rik, MGC91046	Interferon regulatory factor 3	NM_016849
Irf5	mirf5	AW491843	Interferon regulatory factor 5	NM_012057
Irf7			Interferon regulatory factor 7	NM_016850
Isg15	IGI15	G1p2, IP17, Irfp, MGC103144, MGC130321, MGC18616, UCRP	ISG15 ubiquitin-like modifier	NM_015783
Jun	AP-1	JunC, c-jun	Jun oncogene	NM_010591
Map2k1	MAPKK1	MEKK1, Mek1, Prkmk1	Mitogen-activated protein kinase kinase 1	NM_008927
Map2k3	AW212142	MEK3, MKK3, Prkmk3, mMKK3b	Mitogen-activated protein kinase kinase 3	NM_008928
Map3k1	MAPKKK1	MEKK1, Mekk	Mitogen-activated protein kinase kinase kinase 1	NM_011945
Map3k7	B430101B05	C87327, Tak1	Mitogen-activated protein kinase kinase kinase 7	NM_172688
Mapk1	9030612K14Rik	AA407128, AU018647, C78273, ERK, Erk2, MAPK2, PRKM2, Prkm1, p41mapk, p42mapk	Mitogen-activated protein kinase 1	NM_011949
Mapk14	CSBP2	Crk1, Csbp1, MGC102436, Mxi2, PRKM14, PRKM15, p38, p38-alpha, p38MAPK, p38a, p38alpha	Mitogen-activated protein kinase 14	NM_011951
Mapk3	Erk-1	Erk1, Ert2, Esrk1, Mnk1, Mtap2k, Prkm3, p44, p44erk1, p44mapk	Mitogen-activated protein kinase 3	NM_011952
Mapk8	AI849689	JNK, JNK1, Prkm8, SAPK1	Mitogen-activated protein kinase 8	NM_016700
Mavs	IPS-1	D430028G21Rik, MGC25836, Visa, cardif	Mitochondrial antiviral signaling protein	NM_144888
Mefv	FMF	MGC124344, MGC124345, TRIM20, pyrin	Mediterranean fever	NM_019453
Mx1	Mx-1	AI893580, Mx	Myxovirus (influenza virus) resistance 1	NM_010846
Myd88			Myeloid differentiation primary response gene 88	NM_010851
Nfkb1	NF-KB1	NF- $\kappa$ B, NF- $\kappa$ B1, p105, p50, p50, p105	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	NM_008689
Nfkbia	Nfkb1	AI462015	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_010907
Nlrp3	AGTAVPRL	AII, AVP, Cias1, FCAS, FCU, MGC129375, MWS, Mmig1, NALP3, Pypaf1	NLR family, pyrin domain containing 3	NM_145827
Nod2	ACUG	BLAU, CD, Card15, F830032C23Rik, IBD1, Nlrc2	Nucleotide-binding oligomerization domain containing 2	NM_145857

(Continued on following page)

TABLE 1 (Continued)

Symbol	Gene name	Other name(s)	Description	GenBank
Oas2	Oasl11		2',5'-oligoadenylate synthetase 2	NM_145227
Pin1	0610025L01Rik	D9Bwg1161e	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1	NM_023371
Pstpip1	CD2BP1	def-2	Proline-serine-threonine phosphatase- interacting protein 1	NM_011193
Pycard	9130417A21Rik	Asc, CARD5, TMS-1, TNS1, masc	PYD and CARD domain containing	NM_023258
Rela	p65		V-rel reticuloendotheliosis viral oncogene homolog A (avian)	NM_009045
Ripk1	D330015H01Rik	RIP, Rinp, Rip1	Receptor (TNFRSF)-interacting serine- threonine kinase 1	NM_009068
Spp1	2AR	Apl-1, BNSP, BSPI, Bsp, ETA-1, Eta, OP, Opn, Opnl, Ric, Spp-1	Secreted phosphoprotein 1	NM_009263
Stat1	2010005J02Rik	AA408197	Signal transducer and activator of transcription 1	NM_009283
Sugt1	2410174K12Rik	SGT1	SGT1, suppressor of G2 allele of SKP1 ( <i>Saccharomyces cerevisiae</i> )	NM_026474
Tank	C86182	E430026L09Rik, I-TRAF	TRAF family member-associated Nf-kappa B activator	NM_011529
Tbk1	1200008B05Rik	AI462036, AW048562, MGC150301, MGC150302	TANK-binding kinase 1	NM_019786
Tbkbp1	3110043L15Rik	ProSAPiP2, SINTBAD	TBK1 binding protein 1	NM_198100
Ticam1	AW046014	AW547018, TICAM-1, TRIF	Toll-like receptor adaptor molecule 1	NM_174989
Tlr3	AI957183		Toll-like receptor 3	NM_126166
Tlr7			Toll-like receptor 7	NM_133211
Tlr8			Toll-like receptor 8	NM_133212
Tlr9			Toll-like receptor 9	NM_031178
Tnf	TNF- $\alpha$	DIF, MGC151434, TNFSF2, TNF- $\alpha$ , Tnfa, Tnfsf1a	Tumor necrosis factor	NM_013693
Tradd	9130005N23Rik	AA930854	TNFRSF1A-associated via death domain	NM_001033161
Traf3	AI528849	CAP-1, CD40bp, CRAF1, LAP1, T-BAM, amn	Tnf receptor-associated factor 3	NM_011632
Traf6	2310003F17Rik	AI851288, C630032O20Rik	Tnf receptor-associated factor 6	NM_009424
Trim25	AA960166	AL022677, EFP, Zfp147	Tripartite motif-containing 25	NM_009546
Gusb	AI747421	Gur, Gus, Gus-r, Gus-s, Gus-t, Gus-u, Gut, asd, g	Glucuronidase, beta	NM_010368
Hprt	C81579	HPGRT, Hprt1, MGC103149	Hypoxanthine guanine phosphoribosyl transferase	NM_013556
Hsp90ab1	90kDa	AL022974, C81438, Hsp84, Hsp84-1, Hsp90, Hspcb, MGC115780	Heat shock protein 90 alpha (cytosolic), class B member 1	NM_008302
Gapdh	Gapd	MGC102544, MGC102546, MGC103190, MGC103191, MGC105239	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084
Actb	Actx	E430023M04Rik, beta-actin	Actin, beta	NM_007393
MGDC	MIGX1B		Mouse Genomic DNA Contamination	SA_00106
RTC	RTC		Reverse Transcription Control	SA_00104
Ifng	IFN-g	Ifg	Interferon gamma	NM_008337
Ifn7				
Ifna4	Ifa4	MGC143607	Interferon alpha 4	NM_010504
Il28ra				
PPC	PPC		Positive PCR Control	SA_00103
Aim2	Gm1313	Ifi210	Absent in melanoma 2	NM_001013779

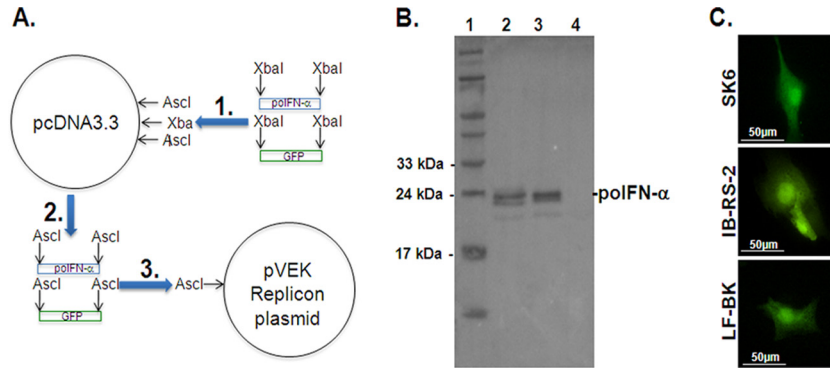
**Statistical analyses.** Data handling, analysis, and graphic representation were performed by using Prism, version 5.0 (GraphPad Software, San Diego, CA), or Microsoft Excel. Statistical differences were determined using a Student *t* test.

## RESULTS

**Synthesis of IFN by VRP-poIFN- $\alpha$ .** Previous work has shown that alphavirus replicon vectors efficiently deliver significant amounts of foreign proteins into mammalian cells (25). We therefore constructed VRPs that express poIFN- $\alpha$  under the control of the alphavirus 26S promoter element (Fig. 1A). Vero cells were infected with VRP-poIFN- $\alpha$ , and 24 h later supernatants were assayed for the presence of active IFN protein. Western blot analysis revealed high levels of IFN expression (Fig. 1B) which correlated with the detection of significant antiviral activity against FMDV as measured by a plaque reduction neutralization assay (unpublished data). To determine if VRP replicons could infect cells derived from the FMDV natural host, swine (SK6 and IB-RS-2) and bovine (LF-BK) cell lines were treated with 50 IU of a reporter VRP-GFP replicon. A similar level of GFP fluorescence was detected in all three cell lines (Fig. 1C), indicating that they were equally susceptible to infection with VRPs.

**Pretreatment with VRPs inhibits FMDV replication.** SK6 cells were infected with either VRP-GFP or VRP-poIFN- $\alpha$ , and at 6, 24, or 48 h posttreatment (hpt) supernatants were removed, and cells were washed and infected with FMDV A12. After 24 h, virus yields were determined by plaque assay. The FMDV yield was reduced greater than 1,000-fold as early as 6 h posttreatment with VRP-poIFN- $\alpha$  relative to cells treated with medium (Fig. 2A). A 5-fold reduction was detected in the cells pretreated with VRP-GFP. Inhibition was significantly increased after 24 h of VRP-GFP treatment (1,000-fold) and was maintained for at least 48 h. To examine the duration of protection provided by a 24-h treatment with VRPs, we infected SK6 cells with FMDV at 0, 24, 48, 96, and 120 h posttreatment (Fig. 2B). The level of inhibition of FMDV remained essentially the same throughout the experiment. Furthermore, similar results were obtained in bovine LF-BK cells (unpublished data). These results indicated that treatment with either VRP-poIFN- $\alpha$  or VRP-GFP can significantly inhibit FMDV replication for a considerable length of time.

The presence of poIFN- $\alpha$  protein was measured by ELISA, and FMDV antiviral activity was measured by a biological assay. Su-

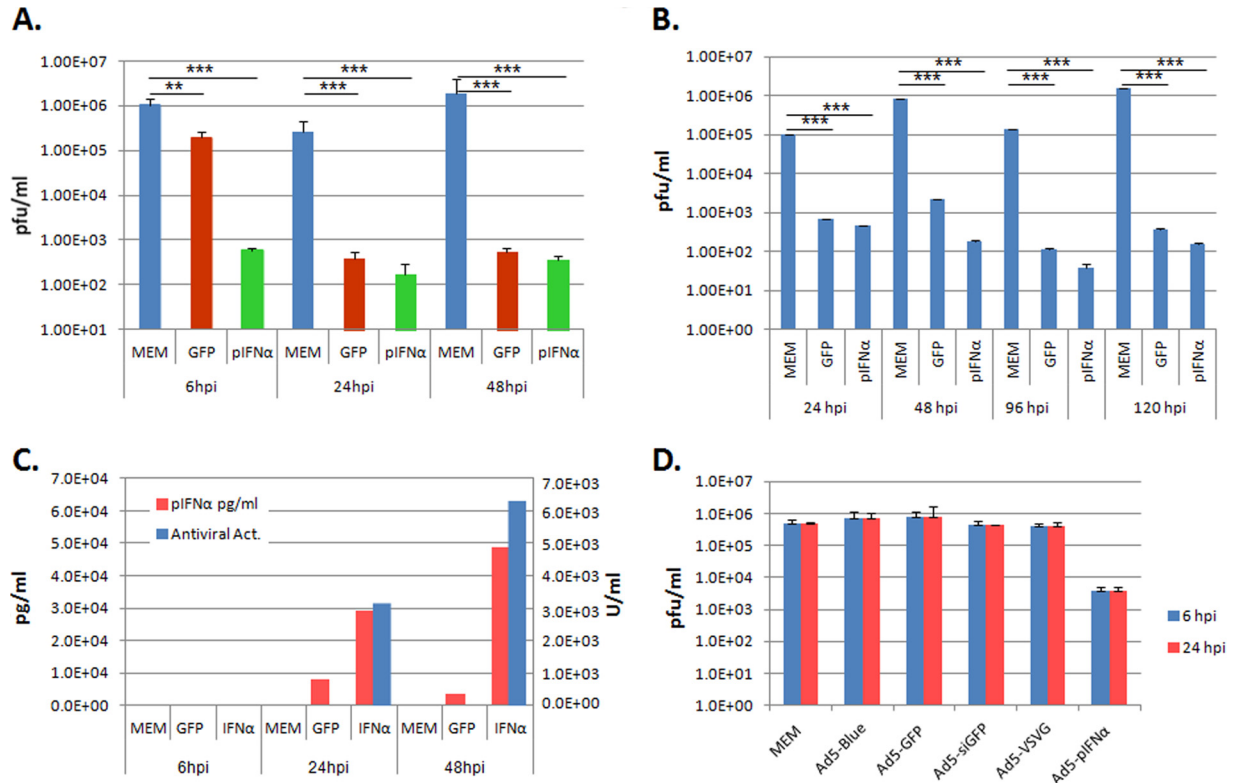


**FIG 1** (A) Schematic diagram of construction of VRP replicon vectors. Genes were PCR amplified with XbaI restriction enzyme recognition sites engineered at the 5' and 3' ends (step 1). The genes were cloned into the unique XbaI site in pcDNA3.3. The genes were removed from the intermediate plasmid by digestion with the AsclI restriction enzyme (step 2). The fragments were cloned into the unique AsclI restriction site in the pVEK replicon vector (step 3). (B) Expression of poIFN-α in cells infected with VRP-poIFN-α. Vero cells were infected with 50 IU of VRP-poIFN-α, and 24 h later the supernatant was centrifuged to remove cell debris, filtered through a Centricon-100 filter, treated at pH 2 overnight, and neutralized to ~pH 7. IB-RS-2 cells were infected at an MOI of 20 with Ad5-poIFN-α, and supernatants were collected at ~24 h and treated as above. The supernatants were run on a 12% SDS-PAGE gel, transferred to a membrane, probed with a 1:250 dilution of rabbit anti-poIFN-α, and detected with a 1:2,000 dilution of goat anti-rabbit alkaline phosphatase. Lane 1, molecular size markers; lane 2, Ad5-poIFN-α-infected IB-RS-2 cell supernatant; lane 3, VRP-poIFN-α-infected Vero cell supernatant; lane 4, supernatant from mock-infected cells. (C) Expression of GFP in cells infected with VRP-GFP. Different porcine (SK-6 and IB-RS-2) or bovine (LF-BK) cell lines were infected with VRP-GFP for 24 h, and GFP expression was examined.

pernatants from VRP-poIFN-α-treated cells had high levels of poIFN-α protein (28,000 to 48,000 pg/ml) at both 24 and 48 h, consistent with significant levels of antiviral activity (3,000 to 6,000 U/ml) (Fig. 2C). Low levels of poIFN-α protein and no

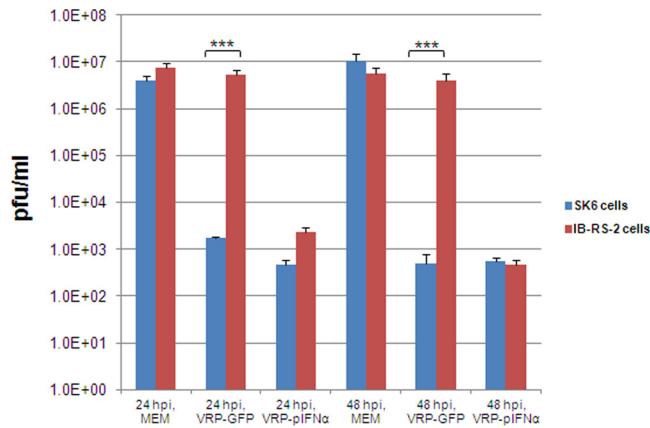
antiviral activity were detected in the supernatants of VRP-GFP-treated cells at 24 and 48 h.

We have previously reported that Ad5 vectors containing the gene for poIFN-α/β can sterily protect swine when challenged 1



**FIG 2** Effect of VRP or Ad5 infection on FMDV replication in SK6 cells. SK6 cells were infected at an MOI of 1 with either VRP-GFP or VRP-poIFN-α and at 6, 24, or 48 h (A) or 24, 48, 96, or 120 h (B) after VRP infection, supernatants were removed, and cells were washed and subsequently infected at an MOI of 1 with FMDV A12 for 24 h. Virus yields were determined by plaque assay and are expressed in PFU/ml. (C) Supernatants of SK6 cells infected with VRP-GFP or VRP-poIFN-α at an MOI of 1 for at 6, 24, or 48 h were examined for the presence of poIFN-α by ELISA (expressed in pg/ml) or antiviral activity by plaque reduction assay (expressed in U/ml). (D) SK6 cells were treated with various Ad5 vectors including Ad5-Blue, Ad5-GFP, Ad5-siGFP, Ad5-VSVG, and Ad5-poIFN-α; 6 or 24 h later supernatants were removed, and cells were washed and infected with FMDV A12 at an MOI of 1 for 24 h. Virus yields were determined by plaque assay and are expressed in PFU/ml. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .





**FIG 3** Effect of VRP infection on FMDV replication in SK6 or IB-RS-2 cells. SK6 or IB-RS-2 cells were infected at an MOI of 1 with either VRP-GFP or VRP-poIFN- $\alpha$ ; 24 h later supernatants were removed, and cells washed and subsequently infected with FMDV A12 at an MOI of 1 for 24 h. Virus yields were determined by plaque assay and are expressed in PFU/ml. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

day later with FMDV (4, 9, 10). To determine if, similar to VRP-GFP, Ad5 vectors in addition to Ad5-poIFN- $\alpha$  could inhibit FMDV replication, we tested various vectors including Ad5-Blue (an Ad5 vector containing the  $\beta$ -galactosidase  $\alpha$  gene fragment [26]), Ad5-GFP, Ad5-siGFP (containing a GFP siRNA), Ad5-VSVG (containing the glycoprotein gene of vesicular stomatitis virus New Jersey which is a known ligand of TLR4) (31), and Ad5-poIFN- $\alpha$ . SK6 cells were transduced with these Ad5 vectors followed by infection with FMDV 6 or 24 h later. Only cells pretreated with Ad5-poIFN- $\alpha$  reduced the yield of FMDV, with an approximately 100- to 200-fold decrease at each time point (Fig. 2D), consistent with the detection of high levels of antiviral activity (unpublished data). Similar results were obtained in Ad5-transduced bovine LF-BK cells (unpublished data).

**IB-RS-2 cells do not respond to VRP-GFP infection.** Konopka et al. (20) have shown that signaling through the IFN- $\alpha/\beta$  receptor is required for both antiviral gene induction by VRP replicons and protection against VEE and influenza virus challenge. We have previously shown that the porcine cell line IB-RS-2 is sensitive to type I IFN protein but does not express type I IFN mRNA (6), and thus presumably treatment of these cells with VRP-GFP would not induce protection from subsequent FMDV challenge. IB-RS-2 cells were pretreated with VRP-GFP or VRP-poIFN- $\alpha$  for 24 h and then infected with FMDV immediately or 24 h later. A control experiment was run in parallel in SK6 cells. As previously shown, FMDV yields in either VRP-GFP- or VRP-poIFN- $\alpha$ -pretreated SK6 cells were reduced approximately 10,000-fold at both 24 and 48 h compared to yields in an untreated control (Fig. 3). In IB-RS-2 cells pretreated with VRP-poIFN- $\alpha$ , FMDV yield was reduced by approximately 2,000-fold at 24 h and 10,000-fold at 48 h. However, in VRP-GFP-pretreated IB-RS-2 cells, there was no reduction in FMDV yield compared to mock-pretreated cells at either time. These results support the data of Konopka et al. (20) indicating that a functional type I IFN system is required for the antiviral response induced by VRPs.

**Effect of VRP treatment on gene induction.** SK6 and IB-RS-2 cells were treated with VRP-poIFN- $\alpha$  or VRP-GFP for 24 h, and RNA was extracted and assayed by qRT-PCR for induction of a

number of genes including ISGs and chemokines (Table 2). Treatment of SK6 cells with either VRP resulted in the induction of the same genes although VRP-poIFN- $\alpha$  generally induced higher levels. However, significant differences between the two VRPs were detected in the number and levels of genes induced in IB-RS-2 cells. All genes, except for IFN- $\lambda$ 3, were more upregulated in VRP-poIFN- $\alpha$ -treated than in VRP-GFP-treated cells. These included a number of ISGs (ISG15, Mx1, and OAS), chemokines (IP-10, CCL2, and CCL5) as well as cytoplasmic PRRs (RIG-I and MDA-5). As a control of gene induction, we treated each cell line with 10  $\mu$ g/ml of synthetic double-stranded RNA (dsRNA) [poly(I · C)]. In SK6 cells the number of genes upregulated was the same after either VRP treatment but for most genes to a lower extent than gene induction after poly(I · C) treatment. In IB-RS-2 cells poly(I · C) gene induction mimicked VRP-poIFN- $\alpha$  treatment although the level of gene induction varied for individual genes (Table 2).

**Treatment with VRP-GFP protects C57BL/6 mice against FMDV infection.** To examine the ability of VRP-GFP to induce an innate protective response against FMDV *in vivo*, we used an FMDV mouse model system. Salguero et al. (32) demonstrated that certain strains of adult mice, including C57BL/6, are susceptible to FMDV when infected subcutaneously in the footpad. Infected animals develop a significant viremia and die within a few days of infection. To confirm that C57BL/6 mice are susceptible to the strains of FMDV that we use in our lab, we infected groups of five mice with 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> PFU of FMDV A24 Cruzeiro or O1 Campos per mouse. Mice infected with 10<sup>4</sup> or 10<sup>5</sup> PFU of A24 developed very high levels of viremia, with levels of 10<sup>7</sup> to 10<sup>8</sup> PFU/ml (unpublished data), and 80% died by 2 days postchallenge. All animals in the group infected with 10<sup>6</sup> PFU of FMDV A24 died by 2 days postchallenge (Fig. 4A). Infection with FMDV O1 Campos resulted in a more severe phenotype. All mice infected with 10<sup>4</sup> PFU of FMDV O1 Campos had high levels of viremia (unpublished data) and died by 2 days postchallenge, while 100% of the animals in groups infected with 10<sup>5</sup> or 10<sup>6</sup> PFU FMDV died

**TABLE 2** Gene expression in IB-RS-2 or SK6 cells after treatment with poly(I · C), VRP-GFP, or VRP-poIFN- $\alpha$ <sup>a</sup>

Genes	IB-RS-2 cells			SK6 cells		
	Poly IC	VRP-GFP	VRP-poIFN $\alpha$	Poly IC	VRP-GFP	VRP-poIFN $\alpha$
IFN $\beta$	243.8	49.2	58.6	5329.7	1043.8	2555.9
IFN $\lambda$ 3	8661.3	244.3	127.0	251572.6	587.6	1848.5
IFN $\lambda$ 2R	8.1	0.6	1.9	1.4	0.0	0.0
IFN $\alpha$ 8	1.1	0.8	1.9	1.5	0.8	0.5
ISG15	25.6	1.3	7.5	667.4	817.6	896.9
IRF1	73.1	9.5	37.3	15.3	7.0	8.3
IRF3	1.3	0.6	1.5	5.6	2.1	1.5
IRF7	2.3	1.0	7.8	16.0	18.5	26.4
MX-1	10.5	1.0	5.4	1532.5	1361.4	962.4
OAS	111.1	1.0	123.9	116.1	244.7	259.8
CCL2	501.7	16.0	1884.3	1997.3	106.4	314.7
CCL5	13.9	8.1	140.0	7027.3	1437.4	2881.4
IP-10	531.0	3.2	23889.6	3598.9	67.8	605.7
IL1	7.5	51.5	112.8	17.9	8.0	3.7
IL6	114.6	8.6	40.5	724.3	14.4	27.6
IL10	3625.9	27.5	68.3	4082.1	8.4	17.0
RIG-I	17.5	1.5	14.1	277.8	1389.5	1938.4
MDA-5	27.4	0.9	4.0	82.9	36.7	46.2
TBK1	0.8	0.9	2.0	0.9	0.8	1.0
TRAF-6	2.4	0.9	2.1	3.3	0.7	0.6
TNF- $\alpha$	1352.7	23.4	80.6	5492.5	7.4	14.5

<sup>a</sup> Numbers in red indicate upregulation ( $\geq 2$ ) compared with the value for mock-infected cells.

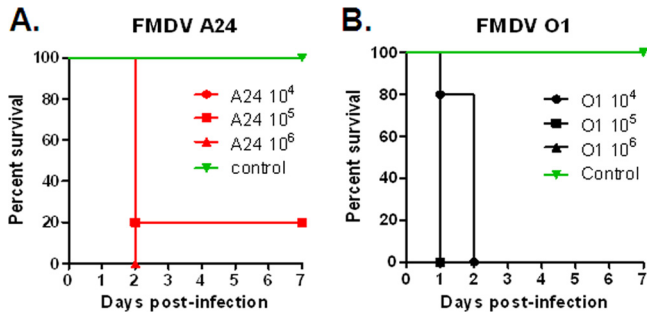


FIG 4 Survival curve of C57BL/6 mice infected with different serotypes of FMDV. Groups ( $n = 5$ ) of 7-week-old mice were infected with different doses ( $10^4$ ,  $10^5$ , or  $10^6$  PFU) of FMDV serotype A24 (A) or O1 Campos (B) in the rear footpad. Percent survival was evaluated at the indicated times.

at 1 day postchallenge (Fig. 4B). Based on these results we decided to use FMDV A24 in subsequent experiments.

Groups of five mice were inoculated with  $10^6$ ,  $10^7$ , or  $10^8$  infectious units (IU) of VRP-GFP or PBS (control group) followed by challenge 1 day later with  $5 \times 10^4$  PFU of FMDV A24. All animals in the control group died between 2 and 3 days after challenge (Fig. 5A), displaying high levels of viremia ( $4 \times 10^7$  to  $7 \times 10^8$  PFU/ml) (Fig. 5B). In the group inoculated with  $10^6$  IU of VRP-GFP, 20% died by day 2, and 100% were dead by 3 days postchallenge (Fig. 5A). In this group, all the animals developed viremia, but the levels were slightly lower than in the animals of the control group (Fig. 5B). Interestingly, 100% survival was observed in the groups inoculated with  $10^7$  or  $10^8$  IU of VRP-GFP. Furthermore, three of five animals in the  $10^7$ -IU VRP-GFP group and only one of five animals in the  $10^8$ -IU VRP-GFP group developed viremia at levels 2 to 3 logs lower ( $10^4$  to  $10^6$  PFU/ml) than the control group (Fig. 5B; also unpublished data). In fact, the levels of viremia at 2 days postchallenge of the different VRP-GFP-treated groups were statistically significantly different than the levels of the control group ( $10^6$  IU of VRP-GFP,  $P \leq 0.05$ ;  $10^7$  and  $10^8$  IU of VRP-GFP,  $P \leq 0.01$ ).

In the next experiment we examined how rapidly and for how long VRP-GFP-inoculated mice would be protected from subsequent FMDV challenge (Fig. 6). Groups of five mice were inoculated with either  $10^7$  or  $10^8$  IU of VRP-GFP and challenged 6, 24, or 48 h later with FMDV A24. A control group was inoculated with PBS and challenged with FMDV 24 h later. As expected 100% of the animals in the control group died by 3 days postchallenge

(Fig. 6A), and all animals in this group developed levels of viremia from  $1 \times 10^6$  to  $2.5 \times 10^7$  PFU/ml (Fig. 6B). Two out of five mice in the two groups inoculated with  $10^7$  IU of VRP-GFP and challenged 6 and 48 h later survived (Fig. 6A). In these groups, the levels of viremia were slightly lower than in the control group (Fig. 6B) although there was not a statistically significant difference, and one animal in each group never developed viremia (data not shown). In the group challenged at 24 h, only the animal that developed viremia ( $1.3 \times 10^6$  PFU/ml) died (Fig. 6B; also unpublished data), and the levels of viremia at 2 days postchallenge were different from those of the control group ( $P \leq 0.05$ ). In contrast, all groups inoculated with  $10^8$  IU of VRP-GFP survived the challenge (Fig. 6A). Although 6 out of the 15 animals in these groups developed viremia, the levels were considerably lower than in the animals that died in the other groups ( $P \leq 0.05$  to  $P \leq 0.01$ ), with viremia levels, e.g., of  $1 \times 10^4$  to  $3.7 \times 10^5$  PFU/ml.

**Inoculation of C57BL/6 mice with VRP-GFP induces an IFN- $\alpha$ -mediated antiviral state.** The experiments described above demonstrated the ability of VRP-GFP to rapidly induce a protective response against FMDV. Konopka et al. (20) have previously shown that BALB/c mice infected with null VRPs (replicons that do not express any foreign gene) display high systemic levels of biologically active IFN as early as 3 to 6 h postinfection. Furthermore, a robust upregulation of four host antiviral genes was detected in several organs. To determine if VRP-GFP treatment induces a similar response in C57BL/6 mice, groups of animals were inoculated with  $10^8$  IU of VRP-GFP and sacrificed at 3, 6, or 24 h after treatment. A control group was inoculated with PBS and euthanized at 24 h. Animals were not challenged with FMDV. Sera were assayed for antiviral activity and for the production of muIFNs by ELISA, and gene analysis was performed on RNA extracted from purified spleen lymphocytes (Fig. 6C and D; Table 3).

Mice inoculated with VRP-GFP developed significant antiviral activity by 3 h postinfection, reaching a peak at 6 h followed by a decline at 24 h (Fig. 6C). A similar kinetics was observed for induction of muIFN- $\alpha$  protein (Fig. 6D). However, we did not detect any significant levels of muIFN- $\beta$ , - $\gamma$ , and - $\lambda$  in the sera of VRP-treated animals (data not shown), suggesting that all the observed antiviral activity was presumably due to IFN- $\alpha$ .

**Effect of VRP-GFP treatment on systemic gene induction in mice.** Pathogens express various PAMPs that are detected by the host through different PRRs, potentially inducing a number of pathways that are involved in the host antiviral response. As demonstrated above, VRP inoculation induced a strong IFN response.

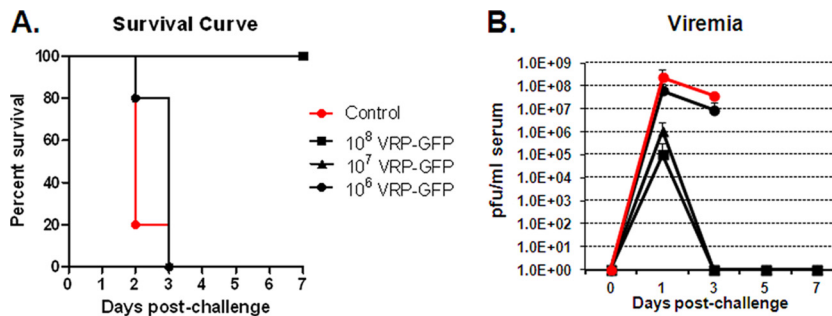
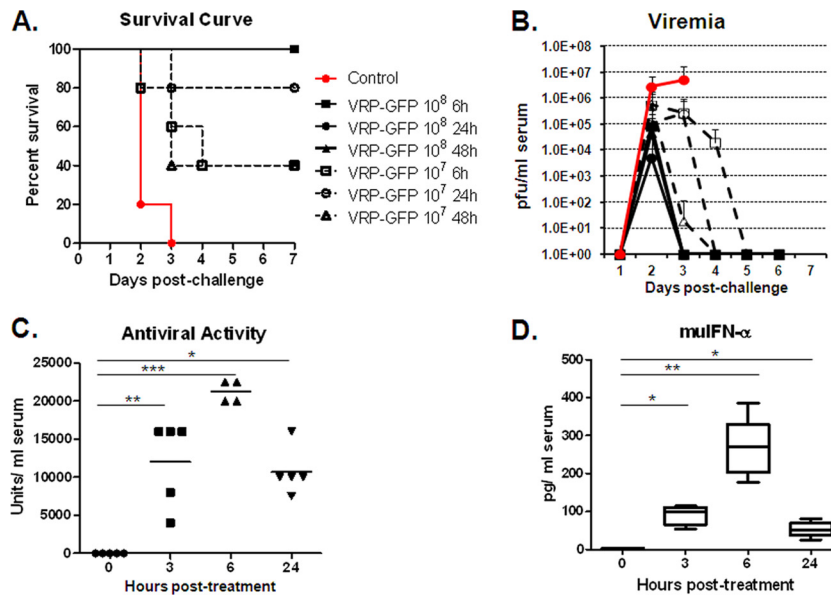


FIG 5 Effect of VRP-GFP treatment on FMDV infection in mice. C57BL/6 mice ( $n = 5$ /group) were inoculated with different doses ( $10^6$ ,  $10^7$  or  $10^8$  PFU) of VRP-GFP in the right rear footpad and challenged 24 h later in the left rear footpad with  $5 \times 10^4$  PFU of FMDV A24. Disease was followed (A), and serum samples were collected for 7 days after challenge to assay for viremia (B).



**FIG 6** Duration of effect of VRP-GFP treatment on FMDV infection in mice. C57BL/6 mice ( $n = 5/\text{group}$ ) were inoculated with different doses ( $10^7$  or  $10^8$  PFU) of VRP-GFP in the right rear footpad and challenged 6, 24, and 48 h later in the left rear footpad with  $5 \times 10^4$  PFU of FMDV A24. Disease was followed (A), and serum samples were collected for 7 days after challenge to assay for viremia (B). Serum of mice was tested for antiviral activity (C) and presence of IFN- $\alpha$  (D) at different time points (3, 6, and 24 h) after VRP-GFP treatment before FMDV challenge. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

To identify the genes induced by VRP treatment that may lead to the protective antiviral response, splenic lymphocytes were isolated from treated animals, RNA was purified, and the expression of 84 genes was analyzed by real-time RT-PCR using an RT<sup>2</sup> Profiler PCR custom-modified antiviral response pathway kit (Table 1). RNA from a PBS-inoculated group euthanized at the beginning of the experiment was used as a control. At 3 h only a few genes were induced, including the three IFN- $\alpha$  subtypes in our array and IFN- $\beta$ , although induction was not statistically significant (Table 3). However, by 6 h, a number of genes were statistically significantly upregulated (fold induction of  $\geq 2$ ;  $P \leq 0.05$ ). These included the following: (i) PRRs TLR3, TLR7, TLR8, TLR9, RIG-I, MDA-5, and LPG2; (ii) proinflammatory cytokines interleukin-15 (IL-15), IL-18 and IL-6; (iii) costimulatory molecule CD86; (iv) transcription factors involved in the IFN pathway including IRF7, MYD88, and Stat1; (v) ISGs ISG15, Mx1, and OAS2; and (vi) chemokines MIP-1 $\alpha$ , IP-10, CXCL-9, and CXCL-11 (Table 2). No IFNs were upregulated at 6 h. The most upregulated gene at 6 h was IP-10. Twenty-four hours after treatment, most of these genes remained upregulated but at reduced levels compared to levels at 6 h (Table 3). Furthermore, a number of other genes were induced at this time, including the three IFN- $\alpha$  subtypes and IFN- $\beta$ , which were all significantly upregulated, and the type III IFN receptor subunit present in our array.

**IP-10 is directly involved in the type I IFN-induced protective response.** Thus far, we have demonstrated that inoculation of mice with VRP-GFP induced a strong protective antiviral response against FMDV which correlated with increased levels of IFN- $\alpha$  in serum. This is in accordance with the demonstrated antiviral and protective effect of type I IFN against FMDV in the natural host (4, 9, 10). To confirm the antiviral effect of IFN- $\alpha$  against FMDV in the mouse model, we treated groups of five mice with  $10^4$  U of muIFN- $\alpha$  followed by challenge with  $5 \times 10^4$  PFU of FMDV A24 at 4 or 18 h after treatment. Five untreated mice were

included as a control group. As expected, the presence of IFN protein was detected in serum, with the level peaking at 4 h after IFN treatment (Fig. 7A) and correlated with detectable levels of antiviral activity (Fig. 7B). All (100%) of the animals treated with IFN- $\alpha$  survived FMDV challenge (Fig. 7C). Interestingly, none of the animals treated 4 h prior to challenge developed viremia, and only two of five animals in the group treated with IFN- $\alpha$  for 18 h developed viremia, which was 100-fold lower than that in the control animals ( $P \leq 0.01$ ) (Fig. 7D). These results demonstrated that recombinant muIFN- $\alpha$  is able to inhibit FMDV replication in C57BL/6 mice.

In the previous experiment we demonstrated that mice inoculated with VRP-GFP had high levels of IFN- $\alpha$  in sera and induction of numerous ISGs in splenocytes. Some of these genes might be involved in the IFN protection conferred against FMDV. IP-10 was the most upregulated gene at 6 h after treatment. This chemokine is of particular interest because we have previously demonstrated that swine or cattle inoculated with Ad5-poIFN- $\alpha$  or Ad5-boIFN- $\lambda 3$ , respectively, and protected against FMD display selective upregulation of IP-10 (7, 8, 12, 33). To determine if IP-10 has a role in the IFN-induced protection against FMDV, we treated IP-10 KO mice (10 per group) with muIFN- $\alpha$  or PBS 4 h prior to challenge. As controls, we included WT C57BL/6 mice that were treated similarly. The IFN-treated IP-10 KO mice had detectable but lower levels of IFN- $\alpha$  and antiviral activity than the WT IFN-treated mice (Fig. 8A and B, compare with 7A and B). Ninety percent of PBS-treated WT mice died 2 to 4 days after challenge (Fig. 8C) and developed high levels of viremia (Fig. 8D). All WT muIFN- $\alpha$ -treated mice survived FMDV A24 challenge and did not develop viremia (Fig. 8C and D). In contrast, IP-10 KO mice did not respond to muIFN- $\alpha$  treatment, displaying a survival rate of 30%, similar to the survival rate of the IP-10 KO or WT mice treated with PBS (20% and 10%, respectively). Overall, the levels of viremia were the same for muIFN- $\alpha$ -treated IP-10 KO

TABLE 3 Gene expression in spleen lymphocytes after treatment of mice with VRP-GFP for different times

Genes		3 h		6 h		24 h	
		<i>Fold induction</i>	<i>P value</i>	<i>Fold induction</i>	<i>P value</i>	<i>Fold induction</i>	<i>P value</i>
<u>IFN &amp; IFN pathway</u>	IFN $\alpha$ 2	2.63 <sup>a</sup>	0.246191	0.86	0.466226	22.50	0.071424
	IFN $\alpha$ 4	5.6	0.206939	1.22	0.608305	62.95	0.092891
	IFN $\alpha$ 7	4.27	0.213146	0.57	0.343659	35.32	0.084141
	IFN $\beta$ 1	3.91	0.250578	0.48 <sup>b</sup>	0.351841	25.03	0.059114
	IFN $\gamma$	1.35	0.237723	1.97	0.053467	3.97	0.000751
	IL28ra	2.13	0.234084	0.55	0.226972	9.66	0.049797
	IRF3	1.75	0.253831	0.62	0.191625	5.16	0.050329
	IRF7	0.83	0.140633	15.81	0.000117	8.69	0.005479
	Stat1	0.75	0.044758	4.92	0.000000	1.78	0.006905
	<u>ISGs</u>	ISG15	0.97	0.943103	32.59	0.000011	10.07
Mx1		1.44	0.314996	34.62	0.000311	17.4	0.000029
OAS2		0.67	0.271944	18.33	0.000054	11.23	0.000009
<u>Pro-inflammatory cytokines &amp; proteins</u>	IL12b	1.52	0.246191	0.79	0.156999	6.71	0.031207
	IL15	1.53	0.175364	7.23	0.001103	5.51	0.000004
	IL18	1.53	0.004976	2.75	0.005214	1.99	0.000117
	IL1b	2.78	0.002358	1.45	0.206729	2.18	0.020273
	IL6	2.38	0.198424	3.52	0.008270	12.91	0.057188
	Mefv	0.99	0.797361	1.53	0.203894	4.03	0.007090
	Nlrp3	1.69	0.113898	1.44	0.176663	3.67	0.001961
	Spp1	1.56	0.254452	1.26	0.272122	9.85	0.026040
<u>Chemokines</u>	CCL3	1.2	0.317019	1.82	0.147049	2.41	0.008493
	MIP-1b	1.23	0.347210	2.55	0.029841	2.76	0.010919
	IP-10	1.46	0.119504	124.73	0.000117	14.73	0.000918
	Cxcl11	5.32	0.207501	2.67	0.003416	53.22	0.060117
	Cxcl9	1.18	0.094395	4.52	0.000033	2.40	0.004760
<u>Cell prolifer<sup>c</sup></u>	CD80	1.26	0.323187	0.97	0.915370	2.51	0.040226
	CD86	1.23	0.098300	2.29	0.005785	1.29	0.062576
	Aim2	1.4	0.334853	1.76	0.086456	4.01	0.049203
<u>PRRs</u>	RIG-I	0.93	0.241102	6.47	0.000129	2.35	0.001207
	mda-5	0.98	0.246191	28.41	0.000396	9.05	0.000119
	Lpg2	1.01	0.51479	9.37	0.000032	3.82	0.000297
	Nod2	1.12	0.368251	1.89	0.067574	5.51	0.006049
	TLR3	0.78	0.284456	4.55	0.000125	3.18	0.001224
	TLR7	0.91	0.451904	2.7	0.000245	2.19	0.000104
	TLR8	0.98	0.925725	2.54	0.050990	3.77	0.000669
	TLR9	0.59	0.000059	2.3	0.000619	1.23	0.070618
<u>Adap.<sup>d</sup></u>	Trim25	0.66	0.067851	4.52	0.000334	1.45	0.090908
	Myd88	0.97	0.699230	3.77	0.000195	2	0.000738
	Tbkbp1	0.88	0.528735	1.12	0.634106	3.04	0.013323
<u>Apoptosis</u>	Card9	1.1	0.474987	1.03	0.786197	3.46	0.005801
	Casp1	1.49	0.127514	2.35	0.006339	1.26	0.096922
	Casp8	1.2	0.173247	1.15	0.386998	2.92	0.063306
	Fadd	1.93	0.225227	0.94	0.598978	6.65	0.082939

<sup>a</sup> Numbers in red indicate upregulation ( $\geq 2$ ) compared with the level in mock-infected animals.

<sup>b</sup> Numbers in blue indicate downregulation ( $< 0.5$ ) compared with the level in mock-infected animals.

<sup>c</sup> Cell prolifer, cell proliferation.

<sup>d</sup> Adap, adaptor proteins.

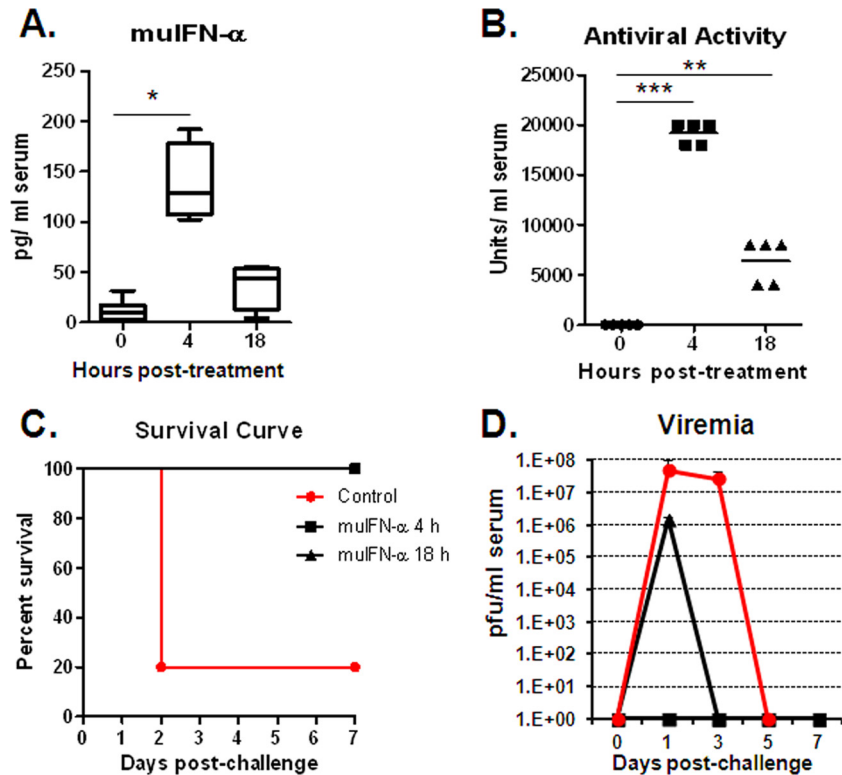


FIG 7 Effect of IFN- $\alpha$  treatment on FMDV infection in mice. C57BL/6 mice ( $n = 5/\text{group}$ ) were treated with  $10^4$  U of muIFN- $\alpha$  and challenged with  $5 \times 10^4$  PFU of FMDV A24 in the rear left footpad at different times (4 or 18 h) after treatment. Serum of the mice was tested for the presence of IFN- $\alpha$  (A) and antiviral activity (B). Disease was followed (C) and serum samples were collected for 7 days after challenge to assay for viremia (D). \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

mice and PBS-treated IP-10 KO or WT mice (Fig. 8D). These results indicated that in our C57BL/6 mouse model, IP-10 mediates at least one of the IFN- $\alpha$ -induced mechanisms of protection against FMD.

## DISCUSSION

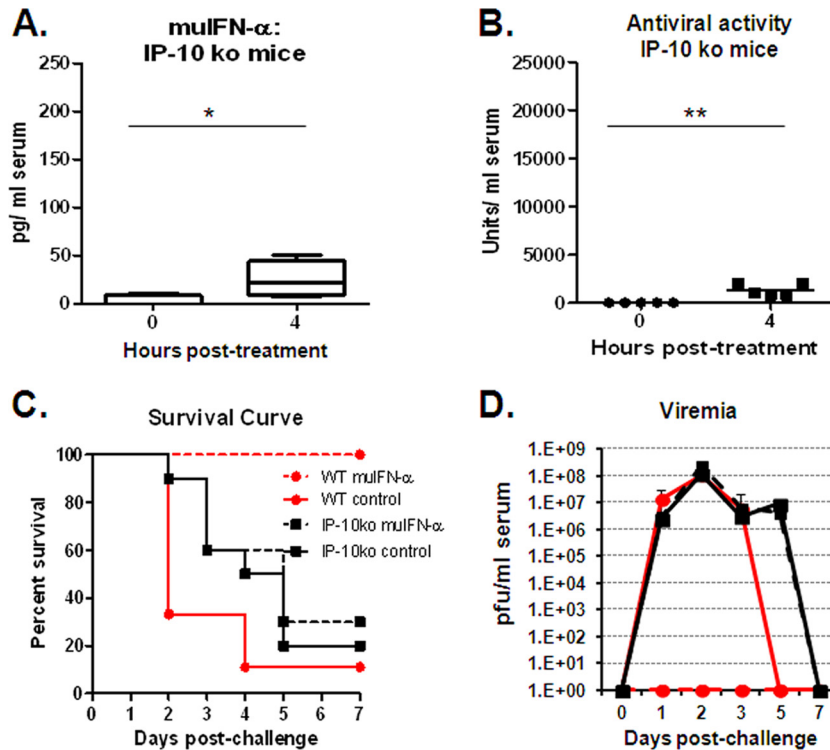
We have previously demonstrated that swine inoculated with Ad5-poIFN- $\alpha$  are rapidly protected when challenged 1 day later with FMDV (4, 9, 10). More recently we also showed that treatment of bovines with Ad5-boIFN- $\lambda 3$  can significantly delay and reduce clinical disease (12). However, in each case the protective dose is relatively high, limiting the applicability of such a strategy in the livestock industry. To overcome these limitations, we hypothesized that a broader and more robust innate immune response could be obtained by adding to IFN treatment a mimic of natural viral infection. We found that treatment of cell cultures with poly(I · C) or swine with poly(IC) stabilized with poly-L-lysine and carboxymethyl cellulose [poly(ICLC)], both mimics of dsRNA produced during virus infection, inhibits FMDV replication and induces a rapid protective response (29). Others have shown that the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) can induce a rapid innate immune response in animals (34). Recently, Molinari et al. (35) demonstrated that mice pretreated with AcNPV as early as 3 h and up to 3 days prior to challenge with FMDV survived. The authors speculated that protection was presumably a result of induction of IFN but did not directly assay for the initiation of an antiviral response.

In this article we examined the potential of VEE replicons as an alternative approach to induce rapid protection against FMDV.

Empty VRPs have recently been shown to induce a rapid innate response in mice and protect against challenge with homologous VEE virus as well as the heterologous influenza virus but did not protect against VSV (20). We demonstrated that treatment of the SK6 swine cell line with either VRP-poIFN- $\alpha$  or VRP-GFP rapidly inhibited subsequent FMDV replication and that the duration of inhibition lasted for at least 5 days. In agreement with Konopka et al. (20), we found that VRP-induced protection requires a functional type I IFN system. Swine IB-RS-2 cells, which do not produce type I IFN, are not protected from FMDV infection by VRP-GFP treatment but are protected after VRP-poIFN- $\alpha$  treatment.

Treatment of SK6 cells with either VRP induced a broad array of genes (17 to 18 of 21 genes examined), but in VRP-poIFN- $\alpha$ -treated cells the majority of these genes were induced to higher levels than in VRP-GFP-treated cells (Table 2). It is important to note that in SK6 cells VRP-GFP as well as poly(I · C) induces the upregulation of both type I and III IFNs as well as various ISGs. We previously obtained similar results in bovine cells treated with poly IC, but treatment of these cells with boIFN- $\alpha$  or boIFN- $\lambda 3$  only upregulated ISGs and not IFNs (8; also unpublished data), indicating that this new approach is inducing a broader response.

While both VRP-GFP- and VRP-poIFN- $\alpha$ -treated SK6 cells were protected from subsequent FMDV infection, only supernatants from VRP-poIFN- $\alpha$ -treated cells contained significant levels of poIFN- $\alpha$  protein and antiviral activity at 24 and 48 hpi (Fig. 2C). However, even supernatants from VRP-GFP- or VRP-poIFN- $\alpha$ -treated cells containing undetectable or low levels of poIFN- $\alpha$  protein or antiviral activity could still significantly re-



**FIG 8** Effect of IFN- $\alpha$  treatment on FMDV infection in IP-10 KO mice. WT and IP-10 KO mice were treated with  $10^4$  U of muIFN- $\alpha$  and challenged with  $5 \times 10^4$  PFU of FMDV A24 in the rear left footpad at 4 h after treatment. Serum of the mice was tested for the presence of IFN- $\alpha$  (A) and antiviral activity (B). Disease was followed (C), and serum samples were collected for 7 days after challenge to assay for viremia (D). \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ .

duce virus yield (as much as 1,000-fold) (Fig. 2A and C). These results suggest that the observed inhibition of viral replication could be due to a cytokine other than poIFN- $\alpha$  or that the IFN- $\alpha$  ELISA or our method of measuring antiviral activity is much less sensitive than the evaluation of a reduction in viral replication. The gene induction data in Table 2 indicated that poIFN- $\beta$  and poIFN- $\lambda 3$  were both upregulated in cells treated with the VRPs. It should be pointed out, however, that our antiviral assays were performed in IB-RS-2 cells which we know lack a functional IFN- $\lambda$  receptor (E. Perez-Martin et al., unpublished data). Therefore, the absence of antiviral activity at 6, 24, and 48 h in the supernatants of VRP-GFP-treated SK6 cells and at 6 h in the supernatants of VRP-poIFN- $\alpha$ -treated cells may as well be due to the presence of low levels of poIFN- $\alpha/\beta$  proteins undetectable by our relatively low sensitivity assay or to poIFN- $\lambda$  to which IB-RS-2 cells are insensitive. In any case, all the antiviral activity observed *in vivo* in mice was consistent with the detected serum levels of IFN- $\alpha$ , suggesting that this cytokine primarily mediates the VRP-GFP-induced effect. However, it is possible that other cytokines may also be involved.

It has been reported by numerous investigators that adenovirus vectors can induce a very rapid innate immune response both in cell culture and in mice (36–39). We have also demonstrated that inoculation of swine with an Ad5-VSVG vector induces a very rapid systemic antiviral response and high levels of poIFN- $\alpha$  as early as 4 hpi; but the antiviral response essentially is not detectable by 24 hpi, and animals inoculated with this vector are not protected from FMD when challenged 1 day after administration (4). Therefore, we examined the ability of various Ad5 vectors,

including Ad5-GFP, to induce an FMDV-protective response in SK6 and LF-BK cells. Surprisingly, only the Ad5-poIFN- $\alpha$  vector inhibited FMDV replication (Fig. 2D). Although we currently do not understand the molecular basis of this difference, the results in the cell lines used in this study suggest that VRPs have the ability to induce a more potent protective innate response than Ad5 vectors.

The cell culture results prompted us to examine the effectiveness of VRP-GFP in protecting adult mice from FMDV infection. Although it would have been preferable to directly compare the two constructs used *in vitro*, we did not use VRP-poIFN- $\alpha$  in these studies because our cell culture data indicated that poIFN- $\alpha$  was not effective in mouse cells (unpublished data). Nevertheless, the main purpose of these experiments was to determine if VRPs are able to activate a protective innate immune response against FMDV. Confirming our hypothesis, we observed that mice treated with VRP-GFP survived FMDV challenge as early as 6 h posttreatment (hpt) and for at least 48 h. As shown by Konopka et al. (20) and confirmed in our study, the effectiveness of treatment is dependent on the dose of the VRP. After VRP treatment there is a very rapid rise in antiviral activity, which appears to be attributed to the induction of muIFN- $\alpha$  since we were unable to detect the presence of IFN- $\beta$ , IFN- $\gamma$ , or IFN- $\lambda$ . To confirm this data, we demonstrated that animals treated with recombinant muIFN- $\alpha$  and challenged at different times after treatment were completely protected from FMDV. Similarly to the gene induction data in cell culture, a number of genes were upregulated in mice treated with VRP-GFP. As early as 3 hpt several of the 84 genes analyzed were induced, including the three subtypes of muIFN- $\alpha$  present in our array as well as IFN- $\beta$ . Marie et al. (40) have found that muIFN- $\alpha 4$

is the most rapidly induced IFN in mouse fibroblasts infected with Newcastle disease virus, and our results indicate that this subtype along with muIFN subtypes 2 and 7 were all detectable by 3 hpt. By 6 hpt many genes were induced at a statistically significant level, including genes with direct antiviral activity, transcription factors, chemokines, and PRRs. In particular, IP-10 was upregulated over 124-fold. By 24 hpt more genes were upregulated, but the level of induction of most of the genes first detectable at 6 hpt was reduced. Furthermore, type I IFN genes were significantly induced.

In our previous studies in swine and cattle, as well as in the current mouse study, we demonstrated a significant induction of IP-10 by type I, II, or III IFN or VRP treatment (12, 33). IP-10 is a chemokine involved in the recruitment, proliferation, and activation of natural killer (NK) cells (41). In the swine study we demonstrated that in animals treated with Ad5-poIFN- $\alpha$ , protection against FMDV challenge correlated with recruitment of dendritic cells (DCs) and NK cells to the skin and lymph nodes, respectively (33). Furthermore, 1 day after Ad5-poIFN- $\alpha$  administration, there was a significant upregulation of IP-10 in the skin and lymph nodes. Using a mouse model, we confirmed that IP-10 plays a role *in vivo* in protecting animals from subsequent FMDV infection. As expected, less than 20% survival was observed in WT and IP-10 KO mice after FMDV challenge. However, while preadministration of muIFN- $\alpha$  protected 100% of WT mice against virus challenge, only 30% survival was observed when IP-10 KO mice were treated similarly. These results demonstrated that IP-10 plays a critical role in the IFN-induced protection against FMDV although presumably other ISGs may also be involved in inhibition of FMDV replication. As mentioned before, we have previously demonstrated in cell culture that PKR and the OAS/RNase L system control FMDV replication (6, 28). It will be useful to examine the role of additional ISG products in this process. Testing other KO mice, performing ISG-overexpression or -downregulation screens, etc., will help elucidate the IFN-dependent mechanisms of FMDV inhibition.

As we previously demonstrated, administration of Ad5-poIFN- $\alpha$  sterilely protected swine from FMDV infection (10). Since our results in cell culture indicate that VRPs containing an IFN gene induce an enhanced protective response and higher levels of ISGs than VRP-GFP treatment, we plan to initiate studies in naturally susceptible animals with different doses of VRP-poIFN- $\alpha/\beta$  and examine their efficacy against FMDV challenge.

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