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Antiviral activity of bovine type III interferon against foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is one of the most serious threats to the livestock industry. Despite the availability of a vaccine, recent outbreaks in disease-free countries have demonstrated that development of novel FMD control strategies is imperative. Here we report the identification and characterization of bovine (bo) interferon lambda 3 (IFN-λ3), a member of the type III IFN family. Expression of boIFN-λ3 using a replication-defective human adenovirus type 5 vector (Ad5-boIFN-λ3) yielded a glycosylated secreted protein with antiviral activity against FMD virus (FMDV) and vesicular stomatitis virus in bovine cell culture. Inoculation of cattle with Ad5-boIFN-λ3 induced systemic antiviral activity and up-regulation of IFN stimulated gene expression in multiple tissues susceptible to FMDV infection. Our results demonstrate that the type III IFN family is conserved in bovines and boIFN-λ3 has potential for further development as a biotherapeutic candidate to inhibit FMDV or other viruses in cattle.

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Introduction

Foot-and-mouth disease virus (FMDV) is the etiologic agent of one of the most devastating diseases that can affect cloven-hoofed livestock. Infection with FMDV causes an acute disease that spreads very rapidly and is characterized by fever, lameness and vesicular lesions on the feet, tongue, snout and teats, with high morbidity but low mortality (Grubman and Baxt, 2004). With the exception of North America, Western Europe, New Zealand and Australia, FMD is enzootic in the rest of the world and disease control is achieved by inhibition of animal movement, slaughter of infected and in contact animals, disinfection of contaminated premises and vaccination with an inactivated whole virus antigen. However, use of this vaccine is not recommended in FMD free-countries due to technical limitations in differentiating vaccinated from infected animals, the need of expensive high containment facilities to produce the antigen and the more severe trade restrictions established by the international organization of animal health for animals or animal products derived from countries that use this vaccine (OIE, 2007). Furthermore, since the current vaccine does not confer protection until at least five to seven days post-vaccination, vaccinated animals are susceptible to FMD prior to this time. Thus, in recent years the OIE has recognized that to be successful, FMD control programs should include the use of antivirals and/or immunomodulatory molecules that, in addition to newly developed marker vaccines would rapidly control the disease before the onset of the vaccine-induced adaptive immune response (Scudamore and Harris, 2002).

In all vertebrates, expression of interferons (IFN) constitute the first step in the immune response against viral infection and, indeed, administration of IFNs as biotherapeutics has been effective in controlling several viral infections (Basler and García-Sastre, 2002; Stark et al., 1998; Fensterl and Sen, 2009). In the case of FMDV, we have previously demonstrated that treatment of bovine, porcine and ovine cells with type I or type II IFNs dramatically inhibits viral replication (Chinsangaram et al., 1999, 2001; Moraes et al., 2007). Furthermore, swine inoculated with a replication-defective human adenovirus 5 vector (Ad5) that delivers porcine (po) IFN-α were sterilely protected when challenged with FMDV 24 h and up to 3–5 days post-inoculation (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003). Studies to understand the mechanism by which type I and II IFNs protect swine against FMD have shown that at least some IFN stimulated genes (ISGs) and migration of immune cells to the sites of FMDV infection play a significant role in controlling viral replication (Chinsangaram et al., 1999; de los Santos et al., 2006; Diaz-San Segundo et al., 2010; Moraes et al., 2007). However, a similar approach in cattle has only shown delayed disease and reduced clinical signs (Wu et al., 2003).

Recently, a new family of IFNs has been described, type III IFN or IFN-λ (Kotenko et al., 2003; Sheppard et al., 2003). These IFNs are related to the type I IFN gene family and also to the interleukin (IL) 10

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family of ligands. Within the type III IFN family, three structurally related members have been identified in humans, mice and chickens: IFN-λ1 (IL29), IFN-λ2 (IL28A) and IFN-λ3 (IL28B) (Karpala et al., 2008; Kotenko et al., 2003; Sheppard et al., 2003) and two members, IFN-λ1 (IL29) and IFN-λ3 (IL28B) have been recently identified in swine (Sang et al., 2010a, 2010b). Similar to type I IFN, expression of type III IFN is induced in response to recognition of pathogen associated molecular patterns (PAMPs) and activation of transcription factors, such as nuclear factor κB (NF-κB) and interferon regulatory factors (IRF)-3 and -7 (Iversen et al., 2010). Type III IFN signals through a heterodimeric cellular receptor that is composed of IL28-Rα, a type III IFN specific subunit, and IL10-Rβ, a subunit shared by other IL10 related cytokines. Despite the fact that type I and type III IFNs act on different receptors, they trigger strikingly similar responses through the activation of multiple members of the signal transducer and activator of transcription (STAT) family (Zhou et al., 2007). However, expression of the type III IFN receptor in a tissue specific manner, mainly in epithelia, has been proposed as one of the mechanisms evolved by different organisms to possibly prevent and protect themselves from viral invasion through the skin and mucosal surfaces (Sommereyns et al., 2008) especially with pathogens like FMDV, whose first site of replication is the mucosa (Arzt et al., 2010). Although not strictly robust, IFN-λ has been shown to induce protection against several viruses in cell culture, as well as in animal models, including herpes simplex virus type 2 (HSV-2) (Ank et al., 2006), hepatitis B and hepatitis C viruses (Marcello et al., 2006; Robek et al., 2005), influenza virus and other human pneumotropic viruses (Mordstein et al., 2010). IFN-λ has also been described as an inducer of antiproliferative activity by triggering host antitumor mechanisms (Maher et al., 2008). Furthermore, a role in the adaptive immune response, as well as modulation of the balance of Th1/Th2 immune responses has been recently proposed for IFN-λ1 biasing toward a stronger block of Th2 responses (Jordan et al., 2007; Morrow et al., 2010).

So far, no member of the type III IFN family has been described in bovines and sequencing of the bovine genome has not provided evidence of predictive sequences for this type of IFN (The Bovine Genome Sequencing and analysis consortium et al., 2009). Very recently a predictive sequence of an IL28B-like mRNA has been deposited in GenBank but no related literature is available.

Here we report the identification, cloning and characterization of a member of the bovine (bo) type III IFN family, boIFN-λ3 or boIL28B. Nucleotide and protein sequence analyses indicated that the cloned boIFN-λ3 displays significant homology with respect to previously identified porcine, human and mouse IFN-λ3 sequences, and to the predicted dog, chicken, rat and monkey sequences. Using an Ad5 vector we have expressed boIFN-λ3 protein in tissue culture and tested for biological antiviral activity against FMDV and vesicular stomatitis virus (VSV). Consistently, ISG expression was up-regulated in bovine cells after treatment with boIFN-λ3. Furthermore, inoculation of cattle with Ad5-boIFN-λ3 resulted in systemic antiviral activity and induction of ISG mRNA and protein expression in several tissues, including those of the upper respiratory tract and skin.

These results indicate that bovine type III IFN behaves similarly to other species orthologs and has the potential of being developed as a biotherapeutic to limit FMDV infection, replication and/or spreading.

Results

Identification of boIFN-λ3

In previous studies we used DNA microarrays to evaluate transcription profiles of bovine cells infected with FMDV (Zhu et al., 2010). Although bovine type III IFNs sequences had not been identified when we initiated this project, we included in the microarray several probes with sequence homology to human type III IFNs. Interestingly, we observed that upon FMDV infection there was significant up-regulation of the mRNA detected by the human IFN-λ3 probes. Therefore to better understand if type III IFN plays any role in controlling FMDV infection, we amplified the full length coding sequence of boIFN-λ3 by RT-PCR of mRNA extracted from primary embryonic bovine kidney (EBK) cells. Analyses of 3 independent clones allowed us to identify a fragment of 585 nucleotides within an 818 nucleotide region, corresponding to a full length open reading frame of 195 amino acids that displayed significant sequence homology to the previously identified IFN-λ3 (IL28B) sequences in *Sus scrofa* (po) (NM_001166490), *Homo sapiens* (hu) (NM_172139) *Mus musculus* (mu) (NM_177396) and an IFN-λ3 predicted *Bos taurus* (bo) sequence recently deposited in GenBank (XM_002695050) (Fig. 1). The closest homology was observed with respect to the *S. scrofa* counterpart with values of 85% for the DNA and 76% for the protein sequences. Comparison of protein sequences across species and localization of the putative signal sequence, suggested that a previously deposited boIFN-λ3 sequence (predictive) likely contains an aberrant N-terminal extension. To this end the identified boIFN-λ3 sequence encodes for a protein of MW = 21587.6 with a pI of 8.20, a predicted signal peptide between amino acids 1 and 23 (Bendtsen et al., 2004) and a putative N-linked glycosylation sequence between amino acids 112 and 115 (NetNGlyc 1.0 Server, Gupta et al., 2004) (Fig. 1B).

boIFN-λ3 displays antiviral activity in vitro

Expression of boIFN-λ3

To corroborate the predicted biochemical properties and determine if the identified boIFN-λ3 sequence encoded for a biologically active IFN product, we expressed the protein using a replication-defective human Ad5 vector containing the cytomegalovirus (CMV) immediate early promoter to drive transcription of the gene (Moraes et al., 2001). Porcine IBRS2 cells, which do not express endogenous type I IFNs (Chinsangaram et al., 2001), were infected with the Ad5-boIFN-λ3 vector and recombinant protein expression was analyzed in cell extracts and supernatants. A protein with multiple bands between 21 and 34 kDa was detected by western blot analysis in both fractions using a specific rabbit polyclonal antibody produced in our laboratory (Fig. 2A). Infection of IBRS2 cells with a control Ad5 vector did not result in the induction of a comparable protein. Addition of tunicamycin, an inhibitor of N-linked glycosylation, to the Ad5-boIFN-λ3 infected IBRS2 cells, resulted in a protein with a discrete MW of approximately 21 kDa in both, the cell extract and the supernatant, indicating that boIFN-λ3 protein was glycosylated at Asn residues.

Detection of antiviral activity in vitro

The biological activity of the expressed boIFN-λ3 protein was tested in EBK and Madin–Darby bovine kidney (MDBK) cells which were pretreated for 24 h with filtered supernatants from Ad5-boIFN-λ3 infected IBRS2 cells and challenged with FMDV and VSV respectively. Fig. 2B shows that the supernatants from Ad5-boIFN-λ3 infected IBRS2 cells contained between 32,000 and 64,000 U/ml of antiviral activity against FMDV. A stronger response 51,200 to 102,400 U/ml was observed when the same supernatant was tested in MDBK cells challenged with VSV (Fig. 2C). The specificity of the response was determined by incubating the same supernatants with rabbit anti boIFN-λ3 serum prior to the assay. Most of the antiviral activity, 80 to 90%, was neutralized by addition of the specific polyclonal antibody (Fig. 2D). No background antiviral activity was detected in supernatants from uninfected or control Ad5-Blue infected porcine IBRS2 cells and no neutralization of antiviral activity was observed by addition of preimmune rabbit serum (Fig. 2D, arrow), or anti-bovine IFN-α antibody (Fig. 2D, arrowhead). These results indicated that the

Fig. 1. DNA and amino acid sequence analysis of boIFN-λ3 (boIL28B). Nucleotide (A) and deduced amino acid (B) sequences were aligned to known homologous sequences. pred.: predicted; hu: human; mu: murine; po: porcine; aa: amino acid; cds: coding sequence. Predicted signal peptide: amino acids 1–23 (boxed in red). Putative N-glycosylation site: amino acids 112–115 (boxed in blue).

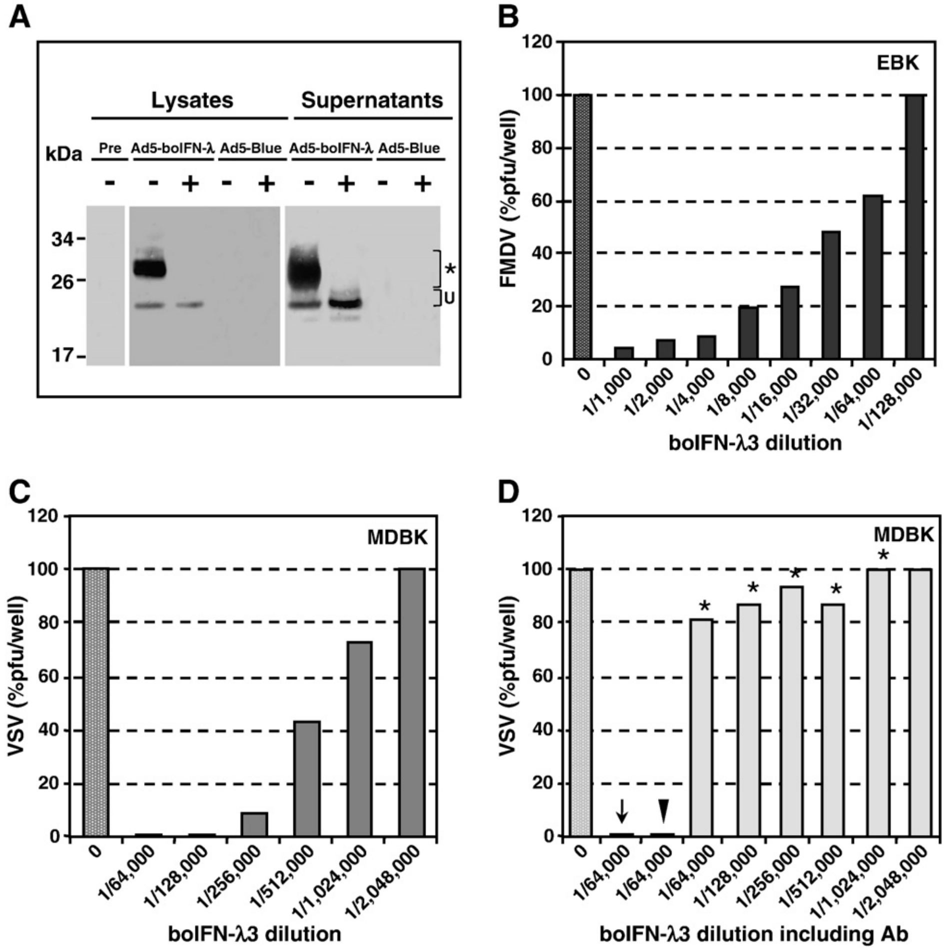

Fig. 2. Characterization of boIFN-λ3 biochemical activity. IBRS2 cells were infected with Ad5-boIFN-λ3 or Ad5-Blue for 24 h in the presence (+) or absence (−) of tunicamycin. Cell lysates and supernatants were evaluated for the presence of recombinant boIFN-λ3 protein by western blot analysis using a polyclonal rabbit antibody. Pre: Cell lysate of Ad5-boIFN-λ3 infected cells probed with rabbit pre-immune serum. *: Glycosylated species of boIFN-λ3. U: Un-glycosylated form (A). Antiviral activity of the recombinant boIFN-λ3 protein expressed in IBRS2 cells was evaluated in primary EBK cells challenged with FMDV (B) and in MDBK cells challenged with VSV (C). Neutralization of antiviral activity with anti-boIFN-λ3 polyclonal antibody in MDBK cells challenged with VSV. Arrow and arrowhead represent neutralization in the presence of rabbit pre-immune serum and polyclonal anti-bovine IFN-α, respectively. Asterisk (*) denotes neutralized antiviral activity (D).

expressed boIFN-λ3 displays antiviral activity in bovine epithelial cells.

It has been reported that although type I and type III IFN bind distinct receptors, they induce similar cellular responses (Kotenko et al., 2003; Sheppard et al., 2003; Zhou et al., 2007; Dumoutier et al., 2004). We therefore assayed the antiviral activity in MDBK or EBK cells treated with boIFN-α and boIFN-λ3 or a combination of both IFNs against VSV and FMDV, respectively. We also incubated the cells with poIFN-α, which we have demonstrated to exhibit antiviral activity in bovines (Wu et al., 2003). Overall, treatment with different

Table 1
Bovine oligonucleotide primer and probe sequences for real-time RT-PCR.

Gene	Accession #	Forward primer	TaqMan probe	Reverse primer
CCL2	EU276059	GCTACTCACAGTAGCTGCCTTCAG-30	CCGAGGTGCTCGCTCAGCCAG-56	GCGACTTGGGAGTTAATTGCA-98
CCL3	AY077840	AGCCAGGTCTTCTCGGCAC-124	ATTTGGCGCTGACACC-144	AGAAGCAGCAGGCCGTTG-178
CCL20	NM_174263	CCAGTATTCTTGTGGGCTTCACA-166	AGCAGCTGGCCAATGAAGCCTGTG-190	GGTGTAAAAGACAACTGCATTGATG-239
CXCL10	EU276062	GTCATTCCTGCAAGTCAATCCTG-142	CCACGTGTCGAGATTA-166	CCCATTCTTTTTCATTGTGGC-204
GAPDH	NM_001034034	GCATCGTGGAGGGACTTATGA-572	CACTGTCCACGCCATCACTGCCA-594	GGGCCATCCACAGTCTTCTG-638
IFN-α	XM_001250455	AGCACACCTTCCAGCTTTTCA-319	CAGAGGGCTCGGCC-341	GGAGGCTCTTGTCCCACACA-376
IFN-β	M15477	CTACAGCTTGCTTCGATTCCAA-278	ACGTCAGAGCCTTAAA-302	CTGCCC AGAG TT TGAC-341
IL10-Rβ	NM_001076975	TTTGACAAACTGAGCGTCATCA-913	AAGTGTCTGAAAGCTGCAA-938	CGGCCCCAGGGTTCA-973
IL28-Rα	XM_868941	CCAGCTGCCGCATTGTCT-435	CCCCAACCAGATATG-454	TCCTTCCAGAAATTCACCTCATAGT-494
IFN-λ3	HQ317919	ACTCATCCCTGGGCCACA-335	CCTGGAGCAGCCCCTTCTCACG-354	GCTTGGAGTGGATGTTCTGCA-397
IRF7	BC151518	GGACTGTGACACGCCCATCT-1535	ACTTCGGCACCTTCT-1558	CCCGGAACTCCAGCAGTTC-1596
ISG15	BC102318	GCGTGTACAAGCGGACCAGT-409	CTGGCTGTCTTTTGAAGGGAGGCCC-430	AGCGGGTGCTCATCATCC-474
MDA5	XM_615590	AGGAGTCAAAGCCCACCATCT-2541	ATTGGCGCTGGACACA-2563	TTCTGTGTCATGGGCTTGAACT-2606
MX1	AY251193S10	CGTCCGGAGCACGAAGAA-595	CGTCCGGAGCACGAAGAA-614	CGTCCGGAGCACGAAGAA-650
OAS1	AY243505	CCAAAGTTGTGAAGGGTGGC-161	CTCAGGCAAAGGC-183	TGATCGTCCCCTGAGGGTC-216
PKR	BC126646	TGCCAAACTGGCTTATGAAAAG-545	CAGAAACAATGAGAGATGG-574	TCACCACACGCAGCACTGA-613
RIGI	XM_580928	GACTCATTGCCCCAGGTCATT-1192	CTGACTGCCTCAGTTGGCGTTGGA-1216	TCGGCTGTGTTTTTGGCATT-1259

doses of type I and type III IFNs in combination resulted in enhanced antiviral activity, as expected and previously reported for other species (Supp. Fig. 1).

Analysis of gene expression in bovine cells treated with boIFN-λ3

To better understand the mechanism of antiviral activity elicited by boIFN-λ3 in bovine cells, we measured the levels of RNA by quantitative real time reverse transcription PCR (qRT-PCR) of IFNs and several ISGs whose expression has been shown to be up-regulated by type III IFN in other species (Ank et al., 2006). For the analysis of gene expression we focused on IFN-α, IFN-β, IFN-λ3 and ISGs, including chemokines CCL2, CCL3, CCL20, CXCL10, activators of the IFN pathway such as IRF-7, RIG I and MDA5 and some genes with known antiviral activity, ISG15, Mx1, OAS1 and PKR (Table 1). We also measured the expression of type III IFN receptors, IL28-Rα and IL10-Rβ. Little or no induction of type I or type III IFN mRNA was observed in these cells after treatment with boIFN-λ3, boIFN-α or poIFN-α. However, a dose response effect was observed for several ISGs when the cells were incubated with each IFN independently (Fig. 3) and 50 U of each IFN were sufficient to reach the saturation level. We did not detect any enhanced effect when boIFN-λ3 was used in combination with either poIFN-α or boIFN-α. Analysis of the expression of the type III IFN receptors (IL28B-Rα and IL10-Rβ) showed considerable basal levels of RNA with ct values of 24–28 as compared to ct values of 20–21 for the housekeeping gene GAPDH, in samples containing 5 ng of total RNA. Expression of such levels of IL28B-Rα and IL10-Rβ is consistent with the sensitivity of EBK cells to type III IFN.

Ad5-boIFN-λ3 induces antiviral activity and ISG up-regulation in cattle

In order to test the response of cattle to inoculation of Ad5-boIFN-λ3, a pilot experiment was performed with 4 animals that were inoculated with Ad5 vectors expressing IFNs as transgenes or empty vector control Ad5-Blue (Moraes et al., 2001). We chose a dose of 10^{11} pfu of Ad5-IFN/animal based on previous studies with type I IFN, in which 10^{10} pfu/animal was required to induce any measurable antiviral activity in serum, and treatment with Ad5-poIFN-α was evaluated at the same time because this vector displayed the highest antiviral activity despite the species difference (Wu et al., 2003). The systemic IFN biological antiviral activity one day after administration of the vectors was 25 U/ml for the animal inoculated with Ad5-Blue, 50 U/ml for the animals receiving each IFN alone and 160 U/ml for the animal receiving the combination of IFNs, indicating that all Ad5 IFN constructs were expressing the recombinant proteins.

Expression of ISGs in tissues

Twenty-four hours after Ad5 inoculation, animals were euthanized, necropsies were performed and tissues of the respiratory tract, skin and lymphoid organs were sampled based on previously reported studies about the pathogenesis of FMDV (Arzt et al., 2010). Peripheral blood mononuclear cells (PBMCs) were also extracted before and after treatment. The expression of several genes including IFN and ISGs was measured by qRT-PCR and an induction of 2 fold or more was considered as a "hit" and was represented in red. While treatment with either Ad5-IFN had 95 and 98 hits respectively, treatment with the combination of Ad5-IFNs displayed 139 hits in comparison to the Ad5-Blue control animal (Fig. 4). Approximately 48% of these hits

Treatment	IFNα	IFNβ	IFNλ3	CCL2	CCL3	CCL20	CXCL10	IRF7	RIGI	MDA5	ISG15	MX1	OAS1	PKR	IL28Rα	IL10Rβ
5U boIFN-λ3	2.5	0.5	1.0	1.1	1.0	0.8	4.7	4.6	19.8	18.2	735.1	53.6	334.3	8.4	1.1	1.0
10U boIFN-λ3	1.3	0.6	0.1	1.3	0.9	1.1	6.6	5.8	31.1	26.2	1114.0	67.4	394.8	10.4	0.8	0.9
50U boIFN-λ3	3.2	2.5	1.7	1.7	0.8	1.5	8.5	8.5	56.4	61.2	2073.5	103.1	758.6	16.1	0.9	1.2
100U boIFN-λ3	0.9	0.4	1.5	1.9	1.0	2.6	8.6	9.8	64.8	68.6	2513.7	111.6	761.4	16.9	1.2	1.2
5U poIFN-α	3.0	1.0	1.6	2.0	1.1	1.7	5.8	8.4	20.7	53.2	2189.6	111.4	735.0	16.2	0.9	1.2
10U poIFN-α	4.2	0.7	0.4	2.0	1.1	1.2	8.1	9.7	32.7	79.3	3114.6	126.0	860.9	18.8	1.0	1.2
50U poIFN-α	4.9	0.8	1.6	4.4	1.2	2.8	121.5	14.7	54.8	132.7	4469.5	145.2	1090.4	23.0	1.2	1.2
100U poIFN-α	1.2	1.1	0.8	4.6	1.2	5.2	178.7	15.1	56.9	127.9	5081.6	137.9	1022.8	23.1	1.1	1.4
5U boIFN-α	1.6	1.1	0.8	0.9	0.8	0.8	4.9	3.6	49.8	13.3	516.2	43.2	308.8	6.7	0.8	0.7
10U boIFN-α	0.9	0.7	0.8	1.1	0.8	1.9	10.0	4.8	73.7	24.1	881.1	56.6	430.4	9.5	0.9	0.7
50U boIFN-α	1.5	1.7	1.1	1.9	0.8	2.0	9.3	7.2	96.7	48.8	1721.1	80.2	638.4	12.3	0.9	0.9
100U boIFN-α	1.9	1.7	0.8	1.7	0.8	2.3	27.8	7.2	99.3	53.6	1740.1	69.6	675.7	13.6	0.9	0.9
5U boIFN-λ3 + 5U poIFN-α	3.4	0.5	0.9	1.4	0.8	1.5	2.8	5.9	46.3	33.4	1237.3	73.0	519.2	9.7	0.9	0.8
5U boIFN-λ3 + 5U boIFN-α	3.6	0.9	0.5	1.1	0.8	1.8	2.9	3.9	26.5	16.0	656.6	39.9	328.4	7.5	0.7	0.8
50U boIFN-λ3 + 50U poIFN-α	1.6	0.8	0.6	3.0	0.8	4.0	53.8	11.0	96.4	94.0	3031.4	99.1	905.1	18.3	0.9	1.0
50U boIFN-λ3 + 50U boIFN-α	2.9	0.2	0.7	1.4	0.6	0.8	8.3	8.4	69.5	44.6	1906.5	64.7	532.0	11.4	0.6	0.7

Fold induction ≥ 2 with boIFN-λ3 treatment
Fold induction ≥ 2 with poIFN-α treatment
Fold induction ≥ 2 with boIFN-α treatment
Fold induction ≥ 2 with boIFN-λ3 and boIFN-α treatment

Fig. 3. Examination of gene expression in bovine cells treated with IFNs. EBK cells were treated for 24 h with varying concentrations of boIFN-λ3, poIFN-α or boIFN-α alone or in combination, as indicated. RNA was isolated and gene expression was analyzed by qRT-PCR. Primers and probes are described in Table 1. Results are expressed as relative fold induction of cells treated with IFN with respect to cells treated with medium (mock). Shaded colored areas represent induction relative to mock treated cells. Color coding indicates gene induction according to each treatment.

(marked with an E) displayed enhanced expression (equivalent to the addition of each independent effect or higher) suggesting that combination treatment results in a stronger IFN response. The expression of IRF-7 was highly up-regulated by the combination treatment in all examined tissues. It was also noticeable that in PBMCs, Ad5-poIFN-α induced twice the number of up-regulated genes as compared to Ad5-boIFN-λ3. In parallel, the levels of mRNA for type III IFN receptor subunits were also measured to evaluate the sensitivity of each analyzed tissue to this type of IFN. Constitutive basal expression of type III IFN receptors (IL28-Rα and IL10-Rβ) was detected in all analyzed tissues indicating that these tissues could be susceptible to type III IFN (Fig. 5). Interestingly, expression of both receptor subunits, and particularly IL28-Rα, was up-regulated after IFN treatment in most of the analyzed tissues including the naso- and oropharynx (Fig. 4), tissues recently reported as the initial site of virus replication after aerosolization of FMDV (Arzt et al., 2010).

Detection of Mx1 protein

Visualization of Mx1 protein by immunohistochemistry (IHC) is a common technique to evaluate *in situ*, the effects of type I and type III IFNs (Jung and Chae, 2006; Sommereyns et al., 2008). One day after inoculation, animals treated with Ad5-boIFN-λ3 showed increased Mx1 protein signal in the epithelium of the dorsal soft palate, other mucosal epithelia of the upper airways and different locations of skin including coronary band or interdigital skin as compared to Ad5-Blue inoculated animals (Fig. 6A, compare panels c and g with a and e and Table 2). With the exception of the proximal lung, the Mx1 signal in epithelial tissues was always weaker in the animal treated with Ad5-poIFN-α than in the animal treated with Ad5-boIFN-λ3 (Fig. 6A, panels b and f, and Table 2). In contrast, the animal treated with the combination of Ad5-IFNs showed the highest Mx1 signal in most of the tissues (Fig. 6A, panels d and h and Table 2). No or almost undetectable positive signal was seen in the control animal (Fig. 6A, panels a and e). The levels of Mx1 protein

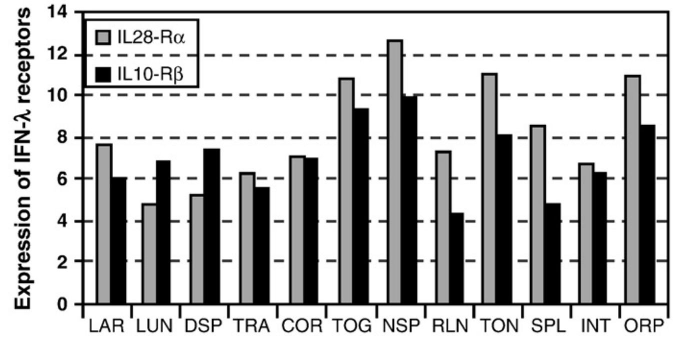

Fig. 5. Expression of type III IFN receptors, IL28-Rα and IL10-Rβ, in bovine tissues. Gene expression was analyzed by qRT-PCR in RNA samples extracted from the bovine tissue of the control animal (larynx [LAR], proximal lung [LUN], dorsal soft palate [DSP], trachea [TRA], coronary band skin [COR], tongue [TOG], nasopharynx [NSP], retropharyngeal lymph node [RLN], palatine tonsil [TON], spleen [SPL], interdigital skin [INT] and oropharynx [ORP]) and normalized against GAPDH.

detected by IHC correlated with the up-regulation in the expression of mRNA analyzed by qRT-PCR (Fig. 6B and Table 2). Interestingly, the protein signal in lymphoid tissue, *i.e.*, palatine tonsil, retropharyngeal lymph node or spleen in the animal inoculated with Ad5-poIFN-α was stronger than the signal observed in the same organs of the animal treated with Ad5-boIFN-λ3 (Table 2).

Taken together, our results indicated that boIFN-λ3 has antiviral activity in cattle *in vivo* and induces the expression of several ISGs when used alone or in combination with IFN-α.

Discussion

Members of the type III IFN family, also known as IL28A, IL28B and IL29, have been recently identified in several species including

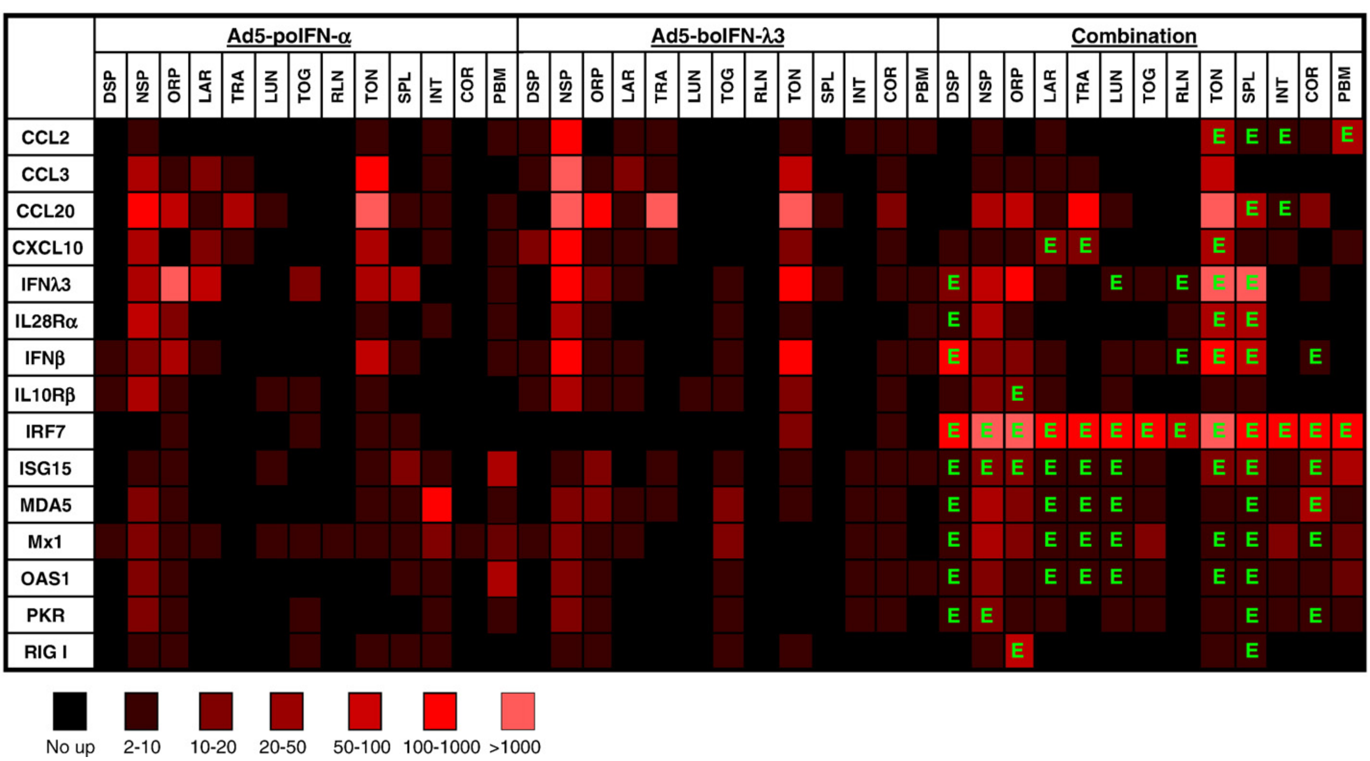

Fig. 4. Examination of gene expression in tissues and PBMCs isolated from bovines treated with Ad5-boIFN-λ3, Ad5-poIFN-α, or a combination of both IFNs. Gene expression was measured by qRT-PCR in RNA samples extracted from the listed bovine tissues (dorsal soft palate [DSP], nasopharynx [NSP], oropharynx [ORP], larynx [LAR], trachea [TRA], proximal lung [LUN], tongue [TOG], retropharyngeal lymph node [RLN], palatine tonsil [TON], spleen [SPL], interdigital skin [INT], coronary band skin [COR] and PBMCs [PBM]). Results are expressed as relative fold induction values of Ad5-IFN treated with respect to Ad5-Blue-control treated animals. Relative gene induction is represented by red color indexing [brightest red (greater than 1000 fold) to black (lower than 2 fold)]. E denotes enhanced up-regulation with the combination treatment.

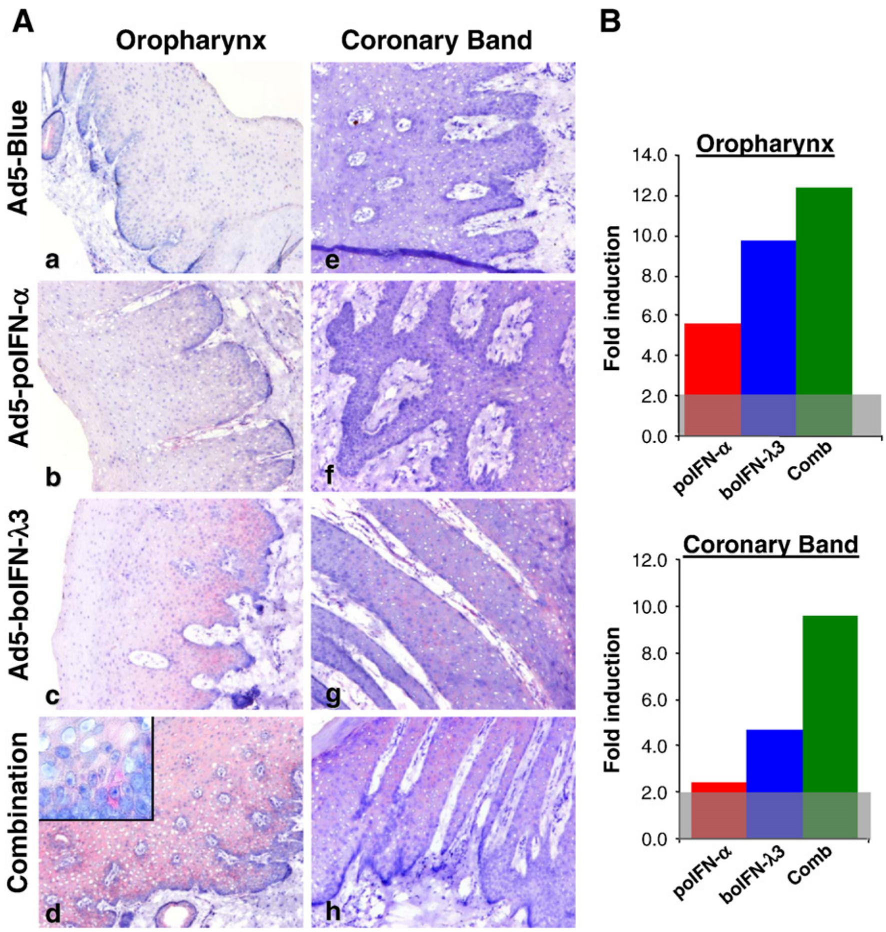

Fig. 6. Detection of Mx1 expression by immunohistochemistry (IHC) and real time RT-PCR. A. Tissues from oropharynx (panels a to d) and from coronary band skin (panels e to h) were harvested from bovine necropsies 24 hpi with Ad5-Blue (control), Ad5-poIFN-α, Ad5-boIFN-λ3 or a combination of Ad5-boIFN-λ3 and Ad5-poIFN-α (combination). Mx1 protein was detected by staining with mouse mAb against human Mx1, and a biotinylated anti mouse/avidin–peroxidase complex developed with Fast Red TR/Naphthol. Positivity is shown in bright purple. Sections were counterstained with Harry's hematoxylin (blue). Magnification, ×10. Insert in panel d shows a higher magnification (×60) of the oropharynx basal epithelium layer in the indicated region. B. Relative expression of Mx1 mRNA analyzed by real time RT-PCR in oropharynx and skin from coronary band. Primers and probes are described in Table 1. Results are expressed as relative fold induction of mRNA in tissue from cows treated with Ad5-IFNs with respect to mRNA in tissue from a cow treated with Ad5-Blue (control). Relative mRNA levels were determined by comparative cycle threshold analysis and a relative fold induction of 2 or less was considered as background which is depicted as a light gray area across the graphs. Expression of GAPDH mRNA was used as normalizer.

human, mouse and swine (Kotenko et al., 2003; Sang et al., 2010a, 2010b; Sheppard et al., 2003). These IFNs are expressed in response to virus infections and mediate the induction of antiviral activity (Ank et al., 2006; Kotenko et al., 2003; Meager et al., 2005; Robek et al., 2005). However, no sequences for type III IFN are available in the published bovine genome (The Bovine Genome Sequencing and Analysis Consortium et al., 2009). By using PCR primers homologous to human sequences, we amplified the mRNA coding for one member of the bovine type III IFN family, boIFN-λ3 (boIL28B). Although the same sequence was obtained in several independent clones, the presence of allelic polymorphism, a common feature within IFN families, cannot be ruled out (van Pesch et al., 2004; Sang et al., 2010a, 2010b). More studies are required to demonstrate this hypothesis. Cloning of the identified boIFN-λ3 gene and expression in mammalian cells resulted in the synthesis of an N-linked glycosylated protein of approximately 21–34 kDa that displayed specific antiviral activity against FMDV and VSV as examined by plaque or virus titer reduction assays in bovine tissue cultures. Analysis of gene expression in cells treated with boIFN-λ3 showed patterns similar to those displayed by treatment with bovine or porcine IFN-α. Induced genes included PKR and OAS1, which have antiviral activity against FMDV (Chinsangaram et al., 1999; de los Santos et al., 2006), and CXCL10 which has been proposed to play a role in immune cell infiltration to the sites of FMDV replication (Diaz-San Segundo et al., 2010). Expression of ISG15 was also significantly up-regulated in bovine cells treated with IFNs. Recently we have shown that in bovine cells infected with FMDV there is an up-regulation of ISG15 (Zhu et al., 2010). Interestingly, the levels of ISG15 are higher when the infection is carried out with an attenuated strain of FMDV, leaderless virus, as compared to wild-type

Table 2
Expression of Mx1 in different tissues in animals treated with Ad5-poIFNα, Ad5-boIFNλ or the combination of the two.

	Ad5-poIFNα	Ad5-boIFNλ3	Combination
Dorsal soft palate	+[a]/2.9[b]	++/2.1	+++/6.9
Oropharynx	±/5.7	++/9.7	+++/12.4
Nasopharynx	+/20.5	++/15.0	+++/28.6
Proximal lung	++/2.3	±/1.0	+++/4.2
Tongue	±/3.1	++/13.4	+++/11.2
Retro LN	++/2.3	+/1.1	++/1.7
Palatine tonsil	+++/2.4	+/1.7	+++/4.9
Spleen	++/3.9	±/0.7	++/5.9
Skin interdigital	±/15.9	+/7.2	++/11.9
Skin coronary band	±/2.4	+/4.7	++/9.6

[a] Semi-quantitative analysis of Mx1 protein signal in tissue sections detected by IHC: − is negative and +++ maximum signal (±<+<++<+++).
[b] Level of fold induction of mRNA detected in each tissue by real time RT-PCR, compared with the same tissue in a non-treated animal. Only animals with an expression ≥2 are considered up-regulated.

FMDV, however further work is required to demonstrate if this gene has a role in controlling FMD.

To determine if boIFN-λ3 had activity *in vivo*, we performed an initial experiment inoculating one cow each with Ad5 vectors delivering poIFN-α, boIFN-λ3, a combination of both, or an empty control (Ad5-Blue). We chose poIFN-α instead of boIFN-α because in previous experiments we had observed a good antiviral response of this IFN despite the species difference (Wu et al., 2003). We inoculated the animals with a relatively high dose of Ad5-vector (10^{11} pfu/animal) to have a measurable response without FMDV challenge. Despite these high Ad5 doses, the levels of antiviral activity in serum were rather low, however significant variation had been previously observed in cattle and swine inoculated with type I IFN alone (Wu et al., 2003; Dias et al., 2011) and protection against FMD has still been observed (Dias et al., 2011). Inoculation of cattle with Ad5-boIFN-λ3 alone or in combination with Ad5-poIFN-α induced a systemic antiviral activity and up-regulation of specific gene expression in multiple tissues, particularly in the upper respiratory tract, the initial site of virus infection, and in the skin, the main site of FMDV replication (Arzt et al., 2010). FMDV is highly sensitive to the actions of type I and type II IFNs *in vitro* and *in vivo* (Chinsangaram et al., 1999, 2001; Dias et al., 2011; Moraes et al., 2007), however, since treatment with IFN-α only conferred partial protection in cattle (Wu et al., 2003), there is an active interest in developing and testing new antivirals with proven efficacy in this species.

Similar to the results *in vitro*, expression of several genes was induced in the tissues and PBMCs of cows treated with type III IFN. Although most of the analyzed genes were also induced by IFN-α, there is evidence in other species suggesting that selective expression of the type III IFN receptor in epithelial cells contributes to a better response to this type of IFN and prevents undesired side effects (Ank et al., 2006; Sommereyns et al., 2008). In support of this hypothesis, Ank et al. (2006) have shown that type III IFN treatment is effective in controlling herpes simplex virus 2 infection in epithelial tissue in vaginal mucosa. It has been reported that upon aerosolization of FMDV there is a pre-viremic phase when the virus mainly targets epithelial cells of the upper respiratory tract; once viremia is established, the virus preferentially infects epithelial cells of the skin and mouth (Arzt et al., 2010; Pacheco et al., 2010). Therefore, targeting antivirals to epithelial cells of the upper respiratory tract could potentially help control FMDV amplification at the initial site of infection. Indeed, expression of type III IFN receptors, IL28-Rα and IL10-Rβ was detected in most of the bovine tissues analyzed, but up-regulation, especially for IL28-Rα, was highest in the oro-, nasopharynx, tongue and palatine tonsil, all tissues present in the upper airways. The strongest and broadest up-regulation in gene expression in response to IFN treatment was observed for IRF-7, with highly enhanced up-regulation for the combination treatment of type I and type III IFNs, particularly in the oro-, nasopharynx and palatine tonsil. Expression of IRF-7 is induced by IFNs and mediates a positive feedback loop that controls the expression of most subtypes of IFN-α and other immunomodulatory molecules (Honda et al., 2005; Marie et al., 1998). Although we did measure, by qRT-PCR, the levels of boIFN-α we did not detect its induction with any treatment (data not shown). However, since there are at least 13 predicted IFN-α gene sequences within the bovine genome (The Bovine Genome Sequencing and Analysis Consortium et al., 2009) it is possible that other subtypes were induced but not detected with the available reagents. Interestingly, in PBMCs, the number of up-regulated genes induced by Ad5-poIFN-α was almost double as compared to Ad5-boIFN-λ3 treatment, indicating that blood cells are not so susceptible to type III IFN. In support of this observation, Lasfar et al. have reported that bone marrow-derived macrophages and splenocytes showed a very weak activation in response to type III IFN treatment (Lasfar et al., 2006). Overall, up-regulation of gene expression indicated a systemic response to type I and type III IFN treatment.

Our results suggest that, as previously reported in other species, boIFN-λ3 is involved in establishing an antiviral state in specific bovine tissues such as those present in the upper airways and the skin which are the preferred sites for FMDV replication. Therefore, use of boIFNλ3 has potential for development as an antiviral strategy to control FMD or other bovine diseases that target similar organs.

Material and methods

Cell lines and viruses

Human 293 cells (ATCC CRL-1573) were used to generate and propagate recombinant adenovirus. EBK cells were obtained from the Animal Plant and Health Inspection Service (APHIS), Ames, Iowa and porcine kidney cells (IBRS2) were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center (PIADC), Greenport, NY. EBK cells were used for determination of IFN bioactivity against FMDV and IBRS2 cells were used to express IFN proteins from Ad5-IFN constructs. MDBK cells (ATCC CCL-22) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and used for determination of IFN bioactivity against VSV. All cells were maintained in Eagle's minimal essential medium (EMEM, GIBCO BRL, Invitrogen, Carlsbad, CA) containing either 10% calf serum or 10% fetal bovine serum (FBS) supplemented with antibiotics. Baby hamster kidney cells (BHK-21, ATCC CCL-10) were used to propagate and titer FMDV. These cells were maintained in EMEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with antibiotics. FMDV subtype A12 was generated from the full-length infectious clone pRMC35 (Rieder et al., 1993) and used for the biologic assay of IFN. VSV serotype New Jersey (VSVNJ) was provided by FADDL at PIADC. Recombinant Ad5 viruses including Ad5-boIFN-λ3, Ad5-poIFN-α and Ad5-Blue were obtained as previously described (Moraes et al., 2003).

Identification, cloning and expression of boIFN-λ3

RNA was isolated from EBK cells infected, at a multiplicity of infection (MOI) of 1 for 6 h, with an attenuated FMDV serotype A12 lacking the leader protein coding region (leaderless virus), and using an RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's directions. Approximately 1 μg of RNA was treated with DNase I (Sigma, St. Louis, MO) and was used to synthesize cDNA with M-MLV reverse transcriptase (Invitrogen) and random hexamers following the manufacturer's directions. Two oligonucleotides: 5′ ***ATCGAT***ATGGCCCCGGGCT GCACGCT 3′ (FW) and 5′ ***TCTAGA***TTAGACACACTGGTCTCCGCTGGC 3′ (RW) containing ClaI and XbaI restriction sites, respectively, were designed to amplify cDNA containing the putative IFN-λ3 (IL28B) sequence using bovine cDNA as template and PfuUltra™ high-fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA). The amplified PCR fragment was cloned in pPCR4 blunt TOPO (Invitrogen) and the sequence of 3 independent clones was determined by standard DNA sequencing in an ABI 3730 XL system (Applied Biosystems, Foster City, CA). The sequence of boIFN-λ3 was identical in all 3 clones and it was submitted to GenBank, and assigned accession number HQ317919. One of the clones was digested with ClaI/XbaI and the resulting 588 bp boIFN-λ3 DNA fragment was ligated to ClaI/XbaI digested pAd5-Blue (Moraes et al., 2001) for expression in mammalian systems. Recombinant Ad5 viruses, including Ad5-boIFN-λ3, Ad5-poIFN-α and Ad5-Blue, were produced by transfection of 293 cells with previously PacI-digested pAd5 plasmids. Viruses were plaque-isolated, propagated in 293 cells and purified by CsCl gradient centrifugation. Viral titer was determined by the method of tissue culture infectious dose 50 ($TCID_{50}$) and converted to plaque-forming units (pfu)/ml. To analyze the protein expressed by Ad5-boIFN-λ3, IBRS2 cells were infected with the Ad5-boIFN-λ3 vector and cellular extracts and supernatants were collected

24 h post-infection and subjected to SDS-PAGE followed by Western blot analysis. A polyclonal antibody was obtained by inoculation of rabbits with the same Ad5-boIFN-λ3 vector. When indicated tunicamycin (10 μg/ml) was added during the infection to examine for the presence of N-linked glycosylation.

Determination of IFN biological activity

Plaque reduction assay

IBRS2 cells were infected with the Ad5 vectors and 24 hpi the supernatants were removed, centrifuged to pellet cell debris, and filtered through a Centricon 100 at 4000 rpm for 10 min to remove Ad5 vector. EBK and MDBK cells were pre-treated with 2-fold serial dilutions of the filtered supernatants. When required, a neutralizing polyclonal antibody was used (1 μl rabbit immune serum per ml of cell supernatant). Twenty-four hours later, supernatants were removed, and cells were washed and challenged with approximately 100 pfu of FMDV (EBK cells) or VSV (MDBK cells) for 1 h followed by overlay with gum tragacanth and incubation for 24–48 h. Plaques were visualized by staining with crystal violet. Antiviral activity was determined 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 untreated cells. Results were expressed as units of antiviral activity/ml of sample. A similar technique was used to measure antiviral activity in plasma derived from inoculated cattle.

Virus yield reduction assay

EBK cells were treated as previously described with 2-fold serial dilutions of filtered IBRS2 supernatants infected with Ad5-IFNs. Twenty-four hours later, treated cells were washed with PBS followed by infection with FMDV at a MOI of 1. One hour after the infection, cells were washed with 2-(N-morpholino) ethanesulfonic acid buffer (MES) pH = 6 followed by incubation in cell culture media for an additional 7 h (total incubation period is 8 h). Supernatants were then collected and virus yield was measured on BHK-21 cells by standard plaque assay (Chinsangaram et al., 1999). Results were expressed as $\log_{10}$/ml of sample.

Animal experiment

An animal experiment was performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Animal Disease Center and animals were kept under strict controlled conditions in the PIADC biosafety level 3 animal facilities. Four male Holstein cows, of about 400 lb each, were inoculated intramuscularly (IM) with 2×10^{11} pfu of Ad5 vectors encoding different IFNs or Ad5-Blue control. One animal was inoculated with 2×10^{11} pfu of Ad5-Blue (control), one animal with 10^{11} pfu of Ad5-poIFN-α and 10^{11} pfu of Ad5-Blue, one animal with 10^{11} pfu of Ad5-boIFN-λ3 and 10^{11} pfu of Ad5-Blue and one animal was inoculated with a combination of 10^{11} pfu of Ad5-poIFN-α and 10^{11} pfu of Ad5-boIFN-λ3. Twenty-four hours after Ad5 inoculation, animals were euthanized and necropsies were performed to isolate multiple tissues and analyze the expression of several genes by qRT-PCR. Animals were bled before and after the treatment for measurement of IFN biological antiviral activity in plasma, as described above and PBMCs were extracted from heparinized blood to obtain mRNA.

Analysis of mRNA

A qRT-PCR assay was standardized and used to evaluate the mRNA levels of multiple genes in monolayers of EBK cells or bovine tissues and PBMCs exposed to different IFNs and control Ad5-Blue vector. RNA and cDNA were prepared as described above. An aliquot (1/40) of the cDNA was used as template for qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers and TaqMan minor groove binding (MGB) probes were designed with Primer Express™ software v.1.5 (Applied Biosystems). Forward and reverse primers were purchased from Invitrogen and the FAM-labeled TaqMan MGB probes from Applied Biosystems. Bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize the values for each sample. The sequences for the primers and probes are listed in Table 1. Reactions were performed in an ABI Prism 7900 Sequence detection system (Applied Biosystems).

IHC for detection of Mx1 protein in bovine tissues

Frozen 4 μm-sections of multiple bovine tissues obtained after necropsy were mounted onto electrostatically charged glass slides (SuperFrost Plus, Fisher Scientific, Worcester, MA) and fixed for 10 min in acetone at −20 °C. Thereafter, the slides were kept at −70 °C for up to 8 wk, until they were stained. For immunostaining, the slides were incubated with the primary antibody mouse mAb anti-human Mx1, kindly provided by Dr. Otto Haller, University of Freiburg. The bound primary antibodies was detected by the avidin–biotin–peroxidase complex technique (Vectastain ABC Kit Elite; Vector, Burlingame, CA) according to the manufacturer's instructions and developed with Fast Red TR/naphthol (Sigma, St. Louis, MO). Slides were counterstained with Harry's hematoxylin and coverslipped by using routine methods. To control the specificity of antibody binding, a duplicate negative control serial section treated with nonspecific primary antibody was used.

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