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Antiviral activity of type I interferons and interleukins 29 and 28a (type III interferons) against Apeu virus

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ABSTRACT

Interferons (IFNs) are cytokines with important immunomodulatory activity in vertebrates. Although type I IFNs and interleukins (IL) 29 and 28a (type III IFNs) bind to different cellular receptors and have distinct structures, most of their biological activities are redundant. Apeu virus (APEUV) is a member of the Bunyaviridae family isolated from the Brazilian rain forest. In this paper we evaluated the antiviral activity of type I and type III IFNs against APEUV. All tested IFNs were able to induce an antiviral state against the virus in a dose-dependent way. The activity of type III IFNs did not need the presence of type I IFNs. Mixing both types of IFNs did not improve the biological activity of each type alone. The tested IFNs were also able to protect human peripheral blood mononuclear cells from infection. IFN alpha2, IFN beta, IL-29 and IL-28a induced the expression of 2′,5′-oligoadenylate synthetase (2′5′OAS) and 6–16 genes. Although MxA gene was related to antiviral activity against Bunyaviruses, there was no induction of MxA in our model. We were able to show activity of type I and type III IFNs against a RNA virus, and that this activity is not dependent on MxA gene.

1. Introduction

Interferons (IFNs) were first described as molecules capable of interfering with the multiplication of Influenza virus in vitro (Isaacs and Lindenmann, 1957). Now it is known that besides their antiviral activity, IFNs also possess important immunomodulatory and physiologic activities. The first IFN gene probably evolved from an interleukin gene around 600–500 million years ago in chordates. IFNs belong to the class 2 cytokine receptor family, and are divided into three groups (Krause and Pestka, 2005).

Type I IFNs are divided into nine subtypes in mammals, and each order contains at least one IFN alpha and one IFN beta. All type I IFNs binds to a common cellular receptor formed by two chains. The binding of a type I IFN to the receptor initiates a signaling pathway that culminates in the activation of transcriptional factors that regulate the expression of interferon-stimulated genes (ISGs). Virtually all cell types are able to produce and respond to type I IFNs. These IFNs are important in the antiviral response and act as a link between innate and adaptive immune responses (Pestka et al., 2004).

IFN gamma is the only type II IFN known. It binds to a distinct receptor and its main function is immunomodulatory. It is produced mainly by immune cells and is important in modulating the adaptive immune response (Kontsek et al., 2003; Alcami and Smith, 2002).

The type III IFNs were described through in silico analysis of the human genome, and are represented by three genes with introns in the human chromosome 19. They were named interleukins 29, 28a and 28b (also noted as IFNs lambda1, lambda2 and lambda3, respectively). Similar to type I IFNs, type III IFNs are able to phosphorylate signal transducers and activators of transcription (STATs) 1 and 2, activate transcriptional factors gamma activated sequence (GAS) and IFN stimulated response element (ISRE), induce classical ISGs such as 2′,5′-oligoadenylate synthetase (2′5′OAS) and 6–16, upregulate class I major histocompatibility complex (MHC) and are produced after poly I:C stimulation or in response to viral infections. Type III IFNs binds to a distinct receptor complex, composed of one exclusive chain and a second chain that is shared with the receptors for IL10, 22 and 26 (Sheppard et al., 2003; Kotenko et al., 2003).

The biological activity of type III IFNs is redundant with the biological activity of type I IFNs, but is generally less intense and more restricted (Meager et al., 2005). Dendritic cells infected with Influenza virus or Sendai virus or treated with poly I:C, lipopolysaccharide or CpG produce type I and type III IFNs (Coccia et al., 2004).

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Similar to type I IFNs, type III IFNs also shows antiproliferative activity (Dumoutier et al., 2004; Zitzmann et al., 2006; Sato et al., 2006) and their activated ISGs are inhibited by suppressor of cytokine signaling (SOCS) 1 and 3 activity (Brand et al., 2005b; Zitzmann et al., 2007).

Type III IFNs also have immunomodulatory activities. Treatment of immature dendritic cells with human IL-29 results in a distinct maturation process, which results in mature dendritic cells with high levels of class I and class II MHC that are able to migrate but retain phagocytic capacity (Mennechet and Uzé, 2006). IL-29 also modulates human cytokine response (Jordan et al., 2007a,b).

Type III IFNs are capable of interfering with the multiplication of several human and murine viruses in vitro, such as encephalomyocarditis virus, Sinbdis virus (Sheppard et al., 2003; Kotenko et al., 2003), hepatitis B and C viruses (Robek et al., 2005), human and murine cytomegalovirus (Brand et al., 2005a) and murine herpes simplex virus 2 (Ank et al., 2006). It was also shown that type III IFNs can interfere with the multiplication of murine herpes simplex virus 2 in vivo (Ank et al., 2006). Recombinant vaccinia virus expressing murine IL-28a is attenuated when used to infect BALB/c mice (Bartlett et al., 2005). Recently, a viral evasion mechanism that directly targets type III IFNs was described, suggesting that these IFNs are important during viral infections (Huang et al., 2007).

The Bunyaviridae family is composed of a large group of more than 300 viruses distributed into five genera: Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus. Mainly transmitted by arthropods, they are maintained in nature by an alternative cycle involving blood-feeding arthropods and susceptible small mammals (Calisher, 1996; Schmaljohn and Hooper, 2001). Members of the Bunyaviridae family are involved in producing mild to severe conditions varying from non-specific fever and encephalitis to hemorrhagic fever in humans (e.g. Hantaan, Rift Valley fever, La Crosse, and Crimean-Congo hemorrhagic fever viruses) and are recognized as posing an increasing threat to human health, an example of the so-called "emerging infections" (Elliott, 1997; Gerrard et al., 2004).

Earlier studies have demonstrated that type I IFNs have an antiviral effect against members of the Bunyaviridae family (Tamura et al., 1987; Morrill et al., 1989; Pinto et al., 1990; Weber and Elliott, 2002; Livonesi et al., 2007). Anti-bunyaviral activity has been investigated for the Mx proteins which are large GTPases (Frese et al., 1996). Growth of several members of the family, including La Crosse, Crimean-Congo hemorrhagic fever, Hantavirus and Dugbe viruses, is strongly inhibited if human MxA protein is expressed in cells (Kochs et al., 2002; Andersson et al., 2004; Kanerva et al., 1996; Bridgen et al., 2004).

Apeu virus (APEUV) (BeAn 848) is a member of group C from the genus Orthobunyavirus, family Bunyaviridae and was isolated in 1955 from a sentinel female monkey (Cebus apella) in the region of Apeu, Belém, Brazilian Amazon (Causey et al., 1961). In humans, the APEUV infection causes an illness characterized by high fever, myalgia and photophobia, usually with a favorable outcome (Gibbs et al., 1964). In other vertebrates, the APEUV can cause encephalitis and hepatitis post-infection (De Mucha Macias et al., 1969; Vasconcelos et al., 1991). Previous studies showed that members of group C, including APEUV, were sensitive to a mixture of IFNs produced in human amnioniotic membrane cells and their sensitivity was dependent on the type of assay used (Petrillo-Peixoto et al., 1980).

However, to date, the antiviral activity of type III IFNs against viruses from the Bunyaviridae family remains unknown. In this paper, we compared the antiviral activity of type I and type III IFNs against APEUV.

2. Materials and methods

2.1. Viruses

APEUV (BeAn 848) was obtained from the American Type Culture Collection (ATCC) and propagated in Vero cells at a multiplicity of infection (MOI) of 0.1. Viruses present in the supernatant were harvested 3 days after infection, titrated and kept frozen at −70 °C.

2.2. Cells

Vero cells (African green monkey kidney cell line) were obtained from the ATCC and grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 5% fetal calf serum (Cultilab, Campinas, SP, Brazil) and antibiotics.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were purified using the Ficoll Hypaque purification technique (Böyum, 1968). Fresh blood collected in vacuum tubes containing heparin was diluted in an equal volume of 1× phosphate buffered saline (PBS). Twenty mililiters of the diluted blood were carefully added over 10 ml of Ficoll, and centrifuged at 400 × *g* for 40 min. The PBMC layer was collected, washed in 1× PBS and cultured in RPMI media supplemented with 1% fetal calf serum (Cultilab, Campinas, Sao Paulo, Brazil) and antibiotics.

2.3. Interferons

Recombinant human IFN alpha2A, produced in *Escherichia coli*, was purchased from Bergamo, Sao Paulo, Brazil. Human IFN beta, purified from human fibroblasts, was purchased from Rentschier Arzneimittel GmbH & Co, Germany. Recombinant IL-29 and IL-28a were produced in 293T cells. Plasmids pSPXIIneo containing cloned human IL-29 and IL-28a were kindly donated by Dr. Gilles Uzé (Universitè de Montpellier II, France) and used to transfect 293T cells. The supernatant was collected and titrated, using an IFN alpha2 sample as reference (Ferreira et al., 1979). Supernatants from mock-transfected cells were used as control.

2.4. Titration

Viral titer was determined by plaque assay (Dulbecco and Vogt, 1953). Frozen infected cells were submitted to two freeze–thaw cycles, resuspended and serially diluted in DMEM. The 10-fold dilutions were used to infect Vero cells seeded in six-well plates. After 1 h of adsorption at 37 °C, the cells were covered with 3 ml of carboxymethylcellulose diluted in DMEM supplemented with 2% fetal calf serum (Cultilab, Campinas, Sao Paulo, Brazil) and antibiotics. The cells were monitored daily to determine the formation of viral plaques. Five days after the infection the cells were fixed with 3.7% formaldehyde and stained with 1% crystal violet. Plaque forming units were counted and the viral titer was determined as plaque forming units per ml (pfu/ml).

2.5. Antiviral activity

Vero cells treated with IFNs for 18 h were infected with APEUV to evaluate the induction of an antiviral state (Ferreira et al., 1979). To evaluate the antiviral activity of type I and type III IFNs against APEUV, Vero cells were seeded in 96 wells plates for 24 h and then treated with 1000 U/ml of human IFNs alpha2, beta, IL-29, IL-28a diluted in DMEM or left untreated. Eighteen hours after treatment the cells were infected with APEUV (MOI 1). Then, at 18, 24 or 48 h after infection, the plates were frozen at −70 °C. The plates were submitted to two freeze–thaw cycles and titrated as described above.

2.6. Dose–response curve

To further characterize the action of IFNs, Vero cells were seeded in 96-well plates for 24 h and then treated with 1000, 100 or 10 U/ml of human IFNs alpha2, beta, IL-29, IL-28a or left untreated. Eighteen hours after treatment the cells were infected with APEUV (MOI 1). Twenty-four hours after infection the plates were frozen at −70 °C. The plates were submitted to two freeze–thaw cycles and titrated as described above.

2.7. Influence of type I in type III IFNs activity

To make sure that the antiviral activity of type III IFNs was not due to the activity of induced type I IFNs, we used Vaccinia virus recombinant B18R protein as a type I IFN inhibitor. Vero cells were seeded in 96-well plates. After 24 h the cells were treated with 100 U/ml of human IFNs alpha2, beta, IL-29, IL-28a or culture media previously incubated with 20 μl of B18R purified protein for 1 h at 37 °C. Eighteen hours after treatment the cells were infected with APEUV (MOI 1). Twenty-four hours after infection the plates were frozen at −70 °C. The plates were submitted to two freeze–thaw cycles and titrated as described above.

2.8. Co-treatment with type I and type III IFNs

To determine if the antiviral activity of type I and type III IFNs could be improved by co-treatment, Vero cells were seeded in 96 wells plates. After 24 h the cells were treated with a combination of type I and type III IFNs at a high dose (IFN beta at 1000 U/ml plus IFN alpha2 or IL-28a at 100 U/ml and IL-28a at 1000 U/ml plus IFN beta or IL-29 at 100 U/ml) or low dose (IFN beta 100 U/ml plus IFN alpha2 or IL-28a at 10 U/ml and IL-28a 100 U/ml plus IFN beta or IL-29 at 10 U/ml). Eighteen hours after treatment the cells were infected with APEUV (MOI 1). Twenty-four hours after infection the plates were frozen at −70 °C. The plates were submitted to two freeze–thaw cycles and titrated as described above.

2.9. Activity in human PBMCs

To verify the antiviral activity of IFNs against APEUV in human primary cells, freshly purified PBMCs were seeded in 96-well plates. Twenty-four hours later the cells were treated with 1000 U/ml of human IFNs alpha2, beta, IL-29, IL-28a or left untreated. Eighteen hours after treatment the cells were infected with APEUV (MOI 1). Twenty-four hours after infection the plates were frozen at −70 °C. The plates were submitted to two freeze–thaw cycles and titrated as described above.

2.10. RNA extraction, reverse transcription and quantitative PCR

To measure the levels of IFN stimulated genes related to antiviral activity, Vero cells were seeded in six-well plates for 24 h and then treated with 1000 U/ml of human IFNs alpha2, beta, IL-29, IL-28a or left untreated. Total cellular RNA was extracted 18 h after treatment using the RNeasy mini kit (QIAGEN) as described by the Manufacturer.

Total RNA obtained after extraction were used as templates in reverse transcriptions carried out using MMLV reverse transcriptase (PROMEGA) as described by the Manufacturer.

Quantitative PCRs were done using a Lightcycler Real Time PCR Machine (Applied Biosystems). The results were analyzed using SDS 2 software, and all data are expressed as a ratio relative to the beta-actin level.

PCR primers for 6–16, 2′5′OAS, MxA and beta-actin were as follows: 6–16 forward 5′-CATGCGGCAGAAGGCGGTAT-3′ and reverse 5′-CGACGGCCATGAAGGTCAGG-3′; 2′5′OAS forward 5′-AACTGCTTCCGACAATCAAC-3′ and reverse 5′-CCTCCTTCTCCCTCCAAAA-3′ M × A forward 5′-ATCCTGGGATTTTGGGGCTT-3′ and reverse 5′-CCGCTTGTCGCTGGTGTCG-3′ and beta-actin forward 5′-TGACGGGGTCACCCACACTGTGCCC-3′ and reverse 5′-CTAGAAGCATTTGCGGTGGACGATGG-3′.

2.11. Statistical analysis

The Student's *t*-test and ANOVA were used to analyze the results. Differences with $p < 0.05$ were considered to be statistically significant. Analyses were made using the GraphPad Software (La Jolla, CA).

3. Results

3.1. Antiviral activity

Treatment of Vero cells with IFNs alpha2, beta, IL-29 or IL-28a 18 h before infection was able to inhibit the multiplication of APEUV (Fig. 1). When all IFNs were compared, the percentage of inhibition was similar at 18 and 24 h, but at 48 h IFN beta was more potent at inhibiting APEUV multiplication then the other IFNs.

3.2. Dose–response curve

Vero cells treated with 10 U/ml of IFNs alpha2, beta, IL-29 or IL-28a were not protected from APEUV infection. Treatment with 100 and 1000 U/ml doses of all IFNs protected cells from infection when compared to the untreated cells (Fig. 2). IFN beta was the most potent IFN at the 100 U/ml dose when compared to the other IFNs.

3.3. Influence of type I in type III IFNs activity

B18R is a viroceptor produced by Poxviruses that binds to type I IFNs and abolishes their activity, but does not influence type III IFNs activity (Huang et al., 2007). Therefore, it can be used as an inhibitor of type I IFNs. As expected, when IFNs alpha2 or beta were incubated with B18R, their activity was abolished (Fig. 3). When type III IFNs were incubated with B18R, their activity was not altered, demonstrating that the antiviral activity seen with these IFNs directly resulted from them and not from type I IFNs that could have been produced after the first treatment. Cells treated with B18R alone and infected were also not protected.

3.4. Co-treatment with type I and type III IFNs

When a type I IFN (beta) or type III IFN (IL-28a) were mixed with another type I IFN or type III IFN, we did not see an improvement over the antiviral activity of each one alone (Fig. 4). Improvement was not detected when high doses (1000 U/ml of the first and 100 U/ml of the second IFN) or low doses (100 U/ml of the first and 10 U/ml of the second IFN) were used. There was also no additive effect when two IFNs of the same type were used together.

3.5. Activity in human PBMCs

To verify whether the antiviral activity described was restricted to Vero cells or if it could also be detected in other cell types,

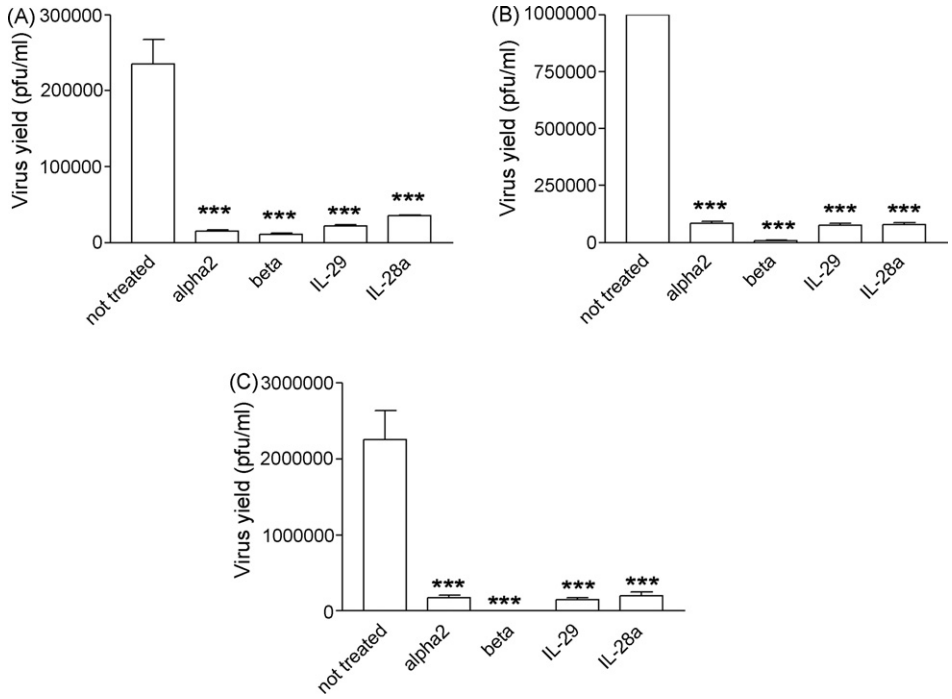

Fig. 1. Type I and type III IFNs have antiviral activity against APEUV. Vero cells were treated with IFNs at 1000 U/ml 18 h before infection with APEUV at a MOI of 1. Infected cells were frozen 18, 24 and 48 h after infection and titrated. Viral titer at 18 h (A), 24 h (B) and 48 h (C) after infection.

fresh human cells were used. These cells were treated with IFNs and infected with APEUV 18 h later. Twenty-four hours after infection the viral load in each sample was determined by titration. When compared to untreated PBMCs, human PBMCs treated with 1000 U/ml IFNs alpha2, beta, IL-29 or IL-28a 18 h before infection were protected from APEUV infection (Fig. 5). The amount of inhibition observed was similar for all IFNs.

3.6. Real time PCR analysis

All tested IFNs induced the expression of 2′5′OAS and 6–16, the ISGs chosen as markers of IFN antiviral activity (Fig. 6). Both genes were induced at higher levels by IFN beta. We could not detect the induction of the MxA gene (data not shown), since this gene is not induced by IFNs in Vero cells (Kanerva et al., 1996).

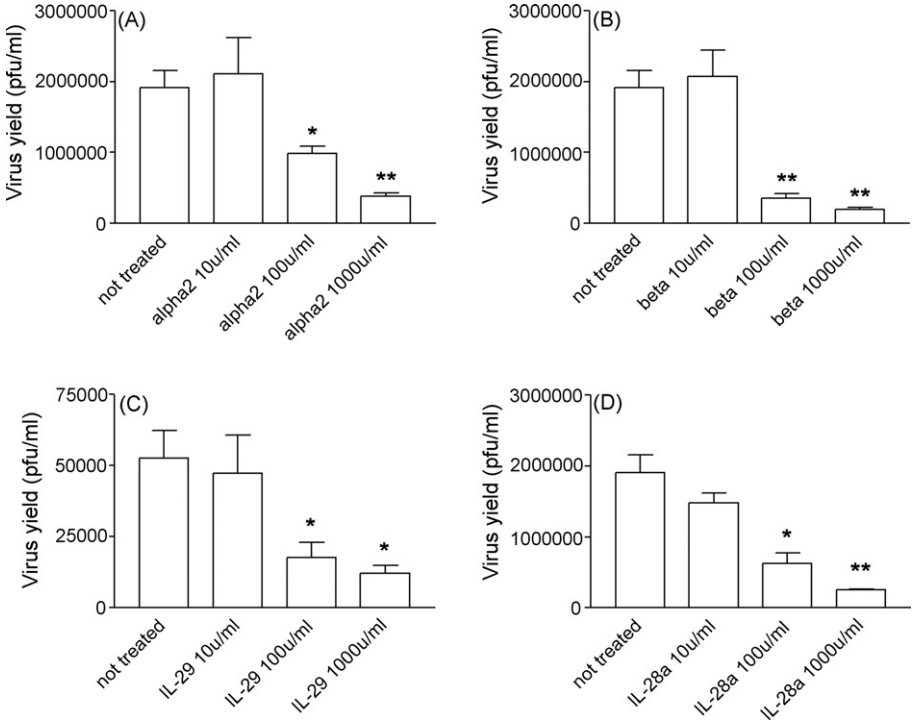

Fig. 2. The antiviral activity of type I and type III against APEUV is dose-dependent. Vero cells were treated with different doses of IFNs 18 h before infection with APEUV at a MOI of 1. Infected cells were frozen 24 h after infection and titrated. Viral titer at cells treated with IFN alpha2 (A), IFN beta (B), IL-29 (C) and IL-28a (D).

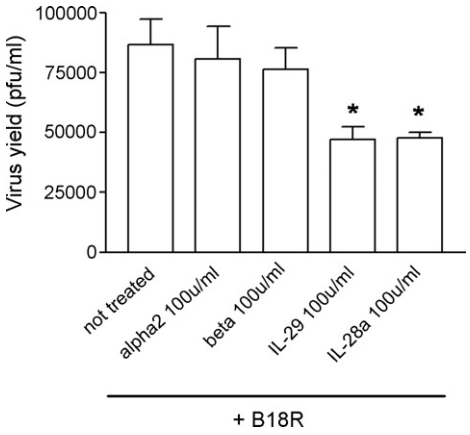

Fig. 3. Type III IFNs antiviral activity does not require type I IFNs activity. IFNs were incubated with B18R protein 1 h before being used to treat Vero cells. Eighteen hours later the cells were infected with APEUV at a MOI of 1. Infected cells were frozen at 24 h after infection and titrated.

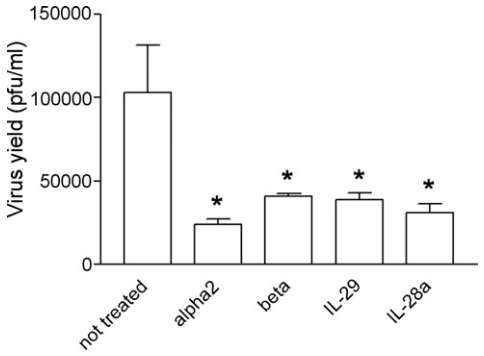

Fig. 5. Type I and type III IFNs have antiviral activity against APEUV in human peripheral blood mononuclear cells. Human PBMCs were treated with IFNs at 1000 U/ml 18 h before infection with APEUV at a MOI of 1. Infected cells were frozen 24 h after infection and titrated.

4. Discussion

Bunyaviruses are a large group of viruses that can cause disease in humans and other mammals, and thus far are recognized as an emerging infection. Aspects from the biology of several viruses from this family and their interaction with their hosts remain unknown. APEUV belongs to the Orthobunyavirus genera, and was isolated in the Brazilian rain forest in 1955 but has not yet been fully characterized. There is some evidence that IFNs influence the multiplication of APEUV, but this has not been described in detail.

It is known that type I and type III IFNs have several immunomodulatory activities, including the establishment of an antiviral state in treated cells. Although type III IFNs bind to a distinct receptor complex, most of their biological activities are redundant with the activities of type I IFNs. In this paper, we show that both type I and type III IFNs possess antiviral activity against APEUV, another redundant activity of type I and type III IFNs against an RNA virus.

In addition, IFNs antiviral activity against APEUV is dose-dependent. While IFNs alpha2, IL-29 and IL-28a have similar activity, it was demonstrated that IFN beta is more efficient in protecting cells against the virus either at a lower dose or at 48 h after treatment. By using the B18R protein, a type I IFN inhibitor, we have shown that the antiviral activity of type III IFNs is independent of

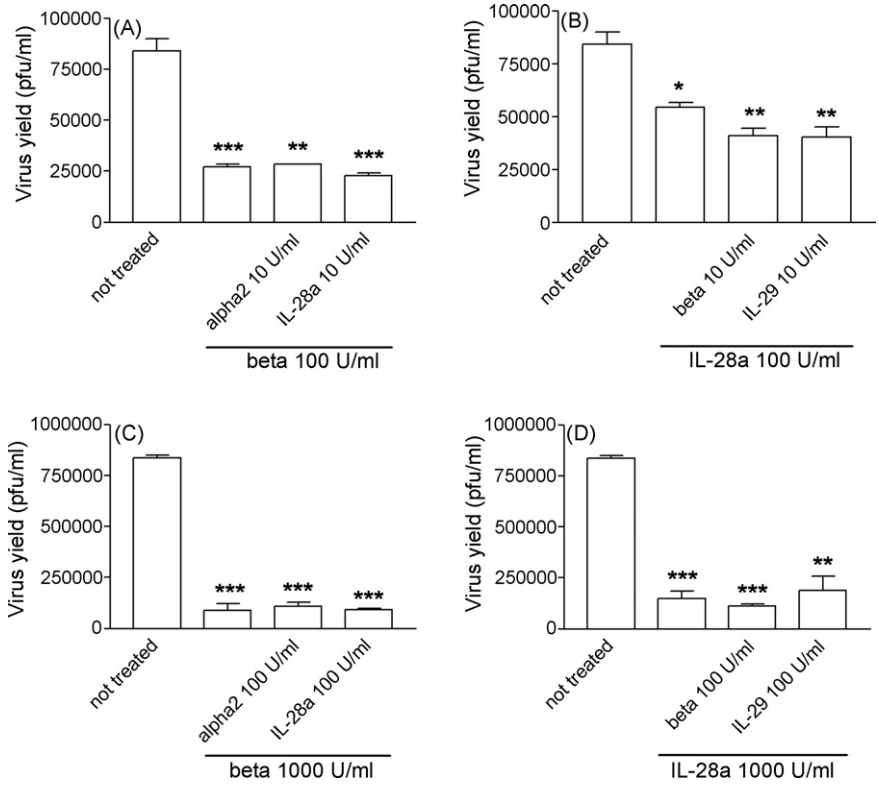

Fig. 4. There is no antiviral activity enhancement after co-treatment with type I and type III IFNs. Vero cells were treated with a combination of type I and type III IFNs 18 h before infection with APEUV at a MOI of 1. Infected cells were frozen 24 h after infection and titrated. Viral titer of cells treated with 100 U/ml of IFN beta alone or mixed with 10 U/ml of IFN alpha2 or 10 U/ml of IL-28a (A), with 100 U/ml of IL-28a alone or mixed with 10 U/ml of IFN beta or 10 U/ml of IL-29 (B), with 1000 U/ml of IFN beta alone or mixed with 100 U/ml of IFN alpha2 or 100 U/ml of IL-28a (C), with 1000 U/ml of IL-28a alone or mixed with 100 U/ml of IFN beta or 100 U/ml of IL-29 (D).

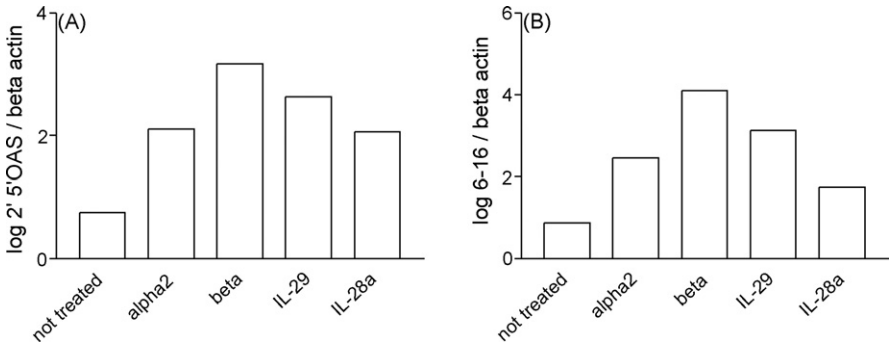

Fig. 6. 2′5′OAS and 6–16 genes are induced after IFN treatment. Vero cells were treated with IFNs at 1000 U/ml and total cellular RNA was extracted 18 h later. The levels of 2′5′OAS (A) and 6–16 (B) mRNAs were measured by quantitative PCR, and the results were normalized by the beta-actin levels of each sample.

type I IFN activity, which is consistent with the fact that VERO cells are not able to produce type I IFNs due to deletions in their genome (Diaz et al., 1988).

The anti-APEUV activity was verified not only in Vero cells, but also in fresh primary human cells. The antiviral activity detected in human PBMCs suggests that IFN activity has an important effect against APEUV during systemic infections. Further studies in vivo are necessary to make this clear.

The results from quantitative PCRs showed that two classical ISGs related to the antiviral response, 2′5′OAS and 6–16, were induced at high levels at the moment cells were infected in all experiments. IFN beta induced higher levels of both genes, and happened to be the strongest inhibitor of APEUV replication. Although it is tempting to associate the antiviral activity of IFNs against APEUV to those restricted genes, further studies are necessary to clarify this matter.

There are several reports describing the activity of the MxA gene against Bunyaviruses, and of viral evasion mechanisms against this gene. We could not detect the induction of MxA mRNA in Vero cells treated with IFNs, as described previously (Kanerva et al., 1996). Since we could see IFN antiviral activity even in the absence of MxA, we can speculate that this gene is not essential for IFN protection against APEUV. The importance of other ISGs against the virus, however, remains to be elucidated.

This is the first time that Vero cells were shown to respond to human type III IFNs. These cells are not able to produce type I IFNs, but can respond to them as well as to type III IFNs. This makes these cells a useful system to study the biological activities of type III IFNs without the interference of type I IFNs.

We can conclude that the antiviral activity against APEUV is another redundant activity of type I and type III IFNs. IFN beta was the strongest inducer of an antiviral state. All other IFNs tested had similar activities.

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