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Rapid Communication

Interferon-beta and interferon-gamma synergistically inhibit the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV)

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Abstract

Recent studies have shown that interferon-gamma (IFN-γ) synergizes with IFN-α/β to inhibit the replication of both RNA and DNA viruses. We investigated the effects of IFNs on the replication of two strains of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). While treatment of Vero E6 cells with 100 U/ml of either IFN-β or IFN-γ marginally reduced viral replication, treatment with both IFN-β and IFN-γ inhibited SARS-CoV plaque formation by 30-fold and replication by 3000-fold at 24 h and by $> 1 \times 10^5$-fold at 48 and 72 h post-infection. These studies suggest that combination IFN treatment warrants further investigation as a treatment for SARS.

Keywords: Coronavirus; Interferon-gamma; Interferon-beta

Introduction

Severe acute respiratory syndrome (SARS) is a newly recognized illness that spread from southern China in late 2002/early 2003 to several countries in Asia, Europe and North America (Guan et al., 2003). SARS usually begins with a fever greater than 38 °C. Initial symptoms can also include headache, malaise, and mild respiratory symptoms. Within 2 days to a week, SARS patients may develop a dry cough and have trouble breathing. Patients in more advanced stages of SARS develop either pneumonia or respiratory distress syndrome. In the initial outbreak, there were 8098 cases worldwide, with an overall mortality of 9.6% (http://www.who.int/csr/sars/en/). A previously unrecognized coronavirus (CoV) has been demonstrated to be the cause of the new disease (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). In a remarkably short period of time, the entire genetic sequences of several strains of the novel SARS-CoV were determined and the virion receptor identified (Li et al., 2003). Similar CoV have been isolated from civets and other animals that are trapped for food or medicine at live animal markets in Guangdong province China, the presumed epicenter of the outbreak (Guan et al., 2003). SARS-CoV or a closely related CoV also infects animals in the wild and appears to have entered the human population in the past (Zheng et al., 2004).

Current strategies for the treatment of SARS patients have included broad-spectrum antibiotics, glucocorticoids and ribavirin (Fujii et al., 2004); however, the efficacy of these treatments is still unclear. Therefore, to develop better treatment strategies for future outbreaks, it is imperative to understand the relationship between the

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virus and the host immune system. Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) are important components of the host immune response to viral infections. IFN-α and IFN-β are produced by most cells as a direct response to viral infection, while IFN-γ is synthesized almost exclusively by activated natural killer (NK) cells and activated T cells in response to virus-infected cells (Pfeffer et al., 1998). Both types of IFNs achieve their antiviral effects by inducing the synthesis of several proteins that interfere with viral replication (Goodbourn et al., 2000). Several studies have examined the anti-viral effects of IFNs against SARS-CoV. Although results from clinical studies using IFN-α are inconclusive (Haagmans et al., 2004; Loutfy et al., 2003), in vitro studies strongly suggest that IFN-β at concentrations greater than 1000 U/ml can marginally inhibit the replication of SARS-CoV (Cinatl et al., 2003; Hensley et al., 2004; Spiegel et al., 2004; Stroher et al., 2004). Likewise, analysis of cytokine levels in SARS patients suggests that the presence of IFN-γ at the early stages of disease onset correlates with resolution of the viral infection (D. Kelvin and M. Cameron, personal communication); however, IFN-γ has little antiviral effect against SARS-CoV in vitro (Cinatl et al., 2003; Spiegel et al., 2004). Recent studies examining the anti-viral effects of IFNs against both RNA and DNA viruses have shown that when used in combination, IFN-γ synergizes with the innate IFNs (IFN-α and IFN-β) to inhibit the replication of viruses such as herpes simplex virus type-1 (Sainz and Halford, 2002), hepatitis C virus (Larkin et al., 2003), Lassa virus (Asper et al., 2004), and cytomegalovirus (Sainz et al., unpublished data). To further test this principle, we examined the antiviral effects of recombinant IFN-β and/or IFN-γ against SARS-CoV replication in vitro.

Results

IFN-β and IFN-γ synergistically inhibit SARS-CoV plaque formation

The capacity of human IFN-β and/or IFN-γ to inhibit the replication of SARS-CoV (strain Urbani) was initially compared in a plaque reduction assay on Vero E6 cells. In cultures pre-treated with 100 U/ml of IFN-β or IFN-γ alone, SARS-CoV formed an average of 14 and 15 plaques, respectively (Table 1). The level of inhibition achieved with either type I or type II IFN treatment as compared to vehicle-treated cultures was ≤3-fold. In contrast, the level of inhibition achieved in cultures pre-treated with a combination of type I and type II IFNs was significantly greater ($P < 0.001$). In cultures treated with 100 U/ml of both IFN-β and IFN-γ, SARS-CoV plaque formation was inhibited by 30-fold, yielding an average of approximately 2 plaques. The level of inhibition achieved with combination IFN-β and IFN-γ treatment was not a consequence of doubling the amount of IFN per culture, as increasing the concentration of IFN in individually treated IFN groups to 200 U/ml did not achieve a similar inhibitory effect (Table 1).

We also tested the relevance of this phenomenon by comparing the antiviral effect of IFN-β and IFN-γ treatment against another strain of SARS-CoV (strain Hong Kong; HK) and against the murine hepatitis virus (MHV) strain A59, an unrelated member of the *Coronaviridae* family. Consistent with the result obtained for the Urbani strain (Table 1), combination IFN-β and IFN-γ treatment inhibited HK plaque formation by greater than 40-fold (data not shown). Interestingly, the effect of IFNs on MHV-A59 plaque formation was significantly different. While the level of inhibition achieved with either IFN-β or IFN-γ treatment alone was approximately 2-fold, combination IFN-β and IFN-γ treatment

Table 1
IFN-β and IFN-γ inhibit CoV plaque formation

CoV	Treatment (U/ml)[a]	Mean no. plaques[b] ± SEM	Fold-reduction[c]
Urbani	Vehicle	40.3 ± 5.9	–
	IFN-β (100)	14 ± 4.8*	3
	IFN-β (200)	15 ± 1.0*	2
	IFN-γ (100)	19.3 ± 5.8*	2
	IFN-γ (200)	11.5 ± 1.0*	3
	IFN-β (100) + IFN-γ (100)	**1.8 ± 0.5***	**30**
MHV-A59	Vehicle	60.3 ± 1.8	–
	IFN-β (100)	24.7 ± 1.1*	2
	IFN-β (200)	21.0 ± 0.7*	2
	IFN-γ (100)	32.0 ± 0.8*	2
	IFN-γ (200)	27.7 ± 1.1*	2
	IFN-β (100) + IFN-γ (100)	9.7 ± 1.1*	6

[a] Vero E6 cells were treated with vehicle, hu IFN-β, hu IFN-γ or hu IFN-β and hu IFN-γ for 12 h before infection with approximately 40 PFU of SARS-CoV strain Urbani. L2 cells received identical treatments with murine IFNs and were infected with approximately 50 PFU of MHV-A59.
[b] Average number of plaques per well of a 10^5 diluted stock determined 3–4 days post-infection (p.i.). Values represent (mean ± SEM) from three independent experiments.
[c] Fold-reduction in each group was calculated as 'plaques in vehicle/plaques in treatment.' Values represent mean from three independent experiments. Boldface type indicates a greater than 30-fold reduction in viral plaque formation.
* $P < 0.05$, as determined by one-way ANOVA and Tukey's post hoc t test comparison of this treatment to vehicle.

inhibited MHV-A59 plaque formation on L2 cells by only 6-fold (Table 1).

Fig. 1 shows a representative photograph of SARS-CoV plaque formation on IFN-treated Vero E6 cells 4 days post-infection (p.i.). Consistent with the experimental results summarized in Table 1, SARS-CoV plaque efficiency was inhibited in cultures treated with both IFN-β and IFN-γ (Fig. 1D). In addition, while plaque morphology in vehicle-, IFN-β- or IFN-γ-treated cells averaged 2 to 4 mm in size, plaques observed in cultures treated with both IFN-β and IFN-γ were consistently smaller, averaging ≤ 1 mm in size (Fig. 1A vs. 1D).

IFN-β and IFN-γ synergistically inhibit SARS-CoV replication

To further characterize the inhibitory effect of IFN-β and IFN-γ treatment on SARS-CoV replication, three-day viral growth assays were performed. Vero E6 cells were pre-treated for 12 h with 100 U/ml of IFNs separately or in combination, infected with SARS-CoV (Urbani or HK strain) at a MOI of 0.01 PFU per cell, and culture supernatants were titered for infectious virus at 24, 48 and 72 h p.i. In cultures treated with 100 U/ml of IFN-β or IFN-γ, Urbani and HK replication was significantly inhibited ($P < 0.001$) at 24 and 48 h p.i., with the greatest level of inhibition observed in IFN-β treated cultures (Figs. 2A, B). At 72 h p.i. however, viral titers in IFN-β- or IFN-γ-treated cultures approached levels of that detected in vehicle-treated groups (Figs. 2A, B). Relative to vehicle control cultures, viral titers recovered at 72 h p.i. from cultures treated with either IFN-β or IFN-γ were reduced by 3-fold in Urbani-infected cultures (Fig. 2D), and 5- and 2-fold in HK-infected cultures, respectively (Fig. 2E). As with our plaque reduction assays, we observed a potent inhibitory effect when Vero cultures were treated with both IFN-β and IFN-γ. Compared to viral titers of greater than 1×10^5 PFU/ml in vehicle-treated cells, in cultures treated with 100 U/ml each of IFN-β and IFN-γ, Urbani replicated to titers of 55, 11 and 6 PFU/ml at 24, 48 and 72 h p.i., respectively (Fig. 2A), and HK replicated to titers 324, 79 and 17 PFU/ml at 24, 48 and 72 h p.i., respectively (Fig. 2B). The inhibitory effect achieved with combination IFN-β and IFN-γ treatment was consistently greater than 3000-fold at all time points tested and reached levels of greater than 1×10^5-fold at 72 h p.i. relative to vehicle treated Vero E6 cells (Figs. 2D, E). In contrast, when tested against MHV-A59, combination IFN-β and IFN-γ treatment showed only a 8-10-fold increased antiviral effect when compared to cultures treated with IFN-β or IFN-γ separately (Figs. 2C, F). Similar results were obtained on 17CL-1 cells (data not shown). Although this result would suggest that the synergistic antiviral effect observed in cultures treated with both IFN-β and IFN-γ is specific to SARS-CoV, we cannot exclude the possibility that the effect observed with regards to MHV plaque formation and replication is cell type-specific.

The degree of cytopathic effect (CPE) in cultures treated with IFNs was also examined. CPE was extensive in vehicle-treated groups infected with either Urbani or HK at 120 h p.i. (Figs. 3A, E), as evident by the reduced number of cells present following staining with crystal violet. However, varying degrees of CPE were observed in IFN-treated cultures. For example, the extent of CPE observed in IFN-γ-treated cultures at 120 h p.i. (Figs. 3C, G) was considerably less than the extent of CPE observed in IFN-β-treated cultures at 120 h p.i. (Figs. 3B, F). This observation is surprising as the levels of viral titers recovered from both IFN-β- and IFN-γ-treated cultures at 72 h (Figs. 2A, B) and 120 h (data not shown) p.i. were similar. Moreover, as compared to vehicle-treated and individually IFN-treated cultures, the degree of CPE observed in cells treated with both IFN-β and IFN-γ is less evident at 120 h p.i., and monolayers appeared evenly stained with little to no visible CPE (Figs. 3D, H). This observation is consistent with the level of viral titers recovered from these cultures at 120 h p.i. (data not shown).

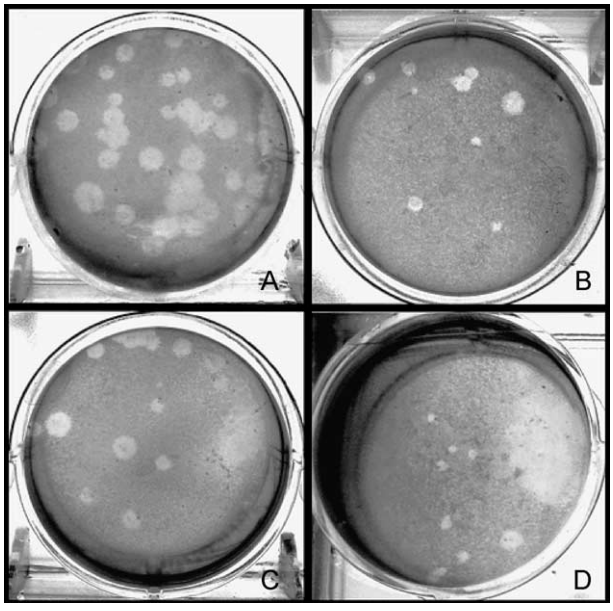

Fig. 1. IFN-β and/or IFN-γ inhibit SARS-CoV plaque formation on Vero E6 cells. Cultures were pre-treated for 12 h with (A) vehicle or 100 U/ml each of (B) IFN-β, (C) IFN-γ or (D) IFN-β and IFN-γ before infection. Monolayers were inoculated with variable titers SARS-CoV to produce numerous visible plaques. Plaque numbers in this figure do not correspond to quantitative data presented in Table 1. Cells were stained with neutral red 3 days p.i. and cultures were photographed 24 h later.

Discussion

In previous studies examining the antiviral effects of IFNs against SARS-CoV replication in vitro, the antiviral effect of IFN treatment varied based on the concentration and type of IFN. Studies examining the antiviral efficacy of IFN-β alone against SARS-CoV replication showed levels

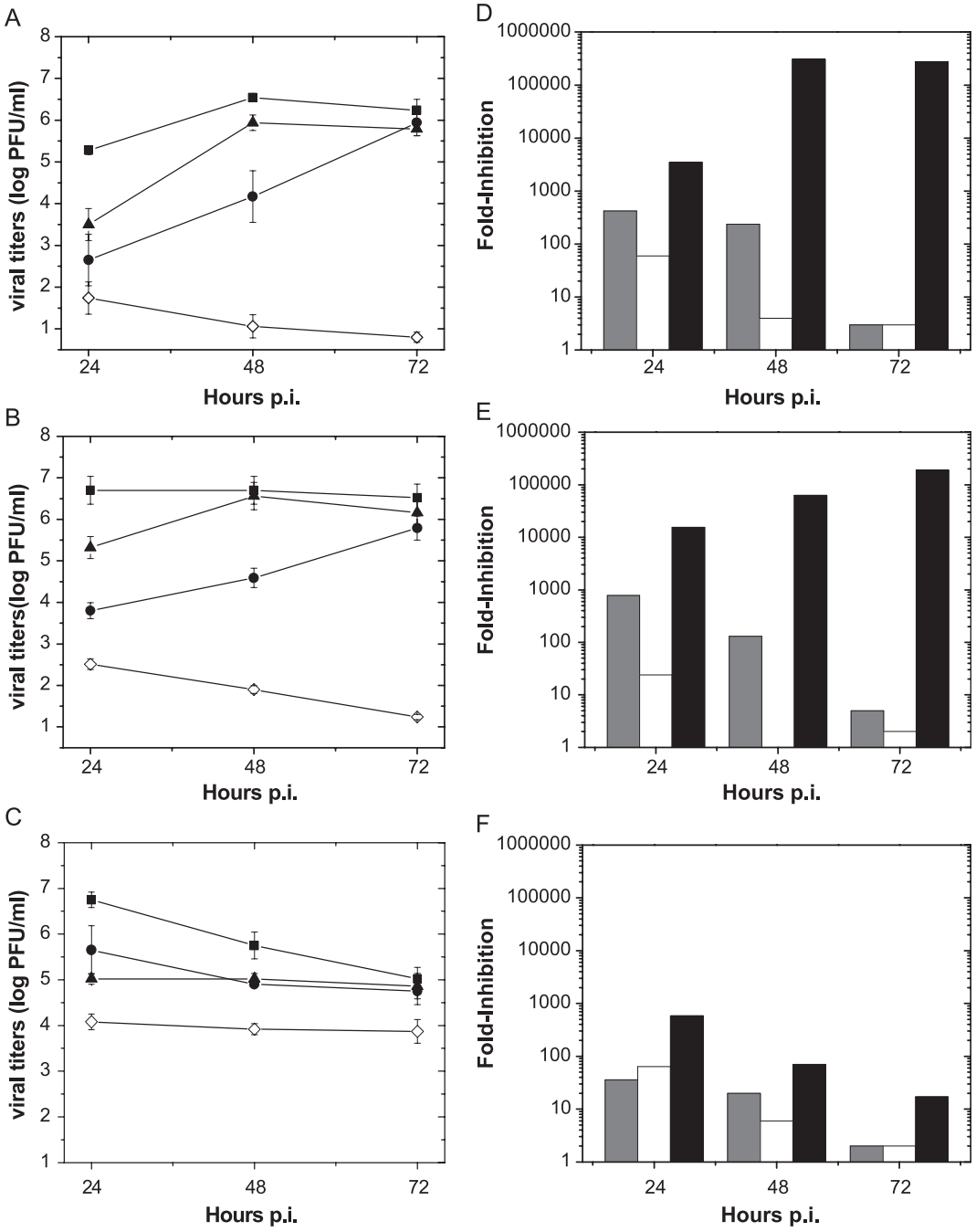

Fig. 2. IFN-β and/or IFN-γ inhibit SARS-CoV replication in Vero E6 Cells. Vero E6 or L2 cells were treated with (■) vehicle or 100 U/ml each of (●) IFN-β, (▲) IFN-γ or (◇) IFN-β and IFN-γ 12 h before infection with SARS-CoV strain (A, D) Urbani, SARS-CoV strain (B, E) HK or (C, F) MHV-A59 at a MOI of 0.01 PFU per cell. Supernatants were harvested on the indicated days p.i., and viral titers were determined by plaque assay as described in Materials and methods. Significant differences in viral titers in Vero E6 cells treated with IFNs relative to cells treated with vehicle are denoted by a single asterisk ($P < 0.001$, one-way ANOVA and Tukey's post hoc t test). (D–F) Average fold inhibition in viral replication observed in cells treated 100 U/ml each of (■) IFN-β, (□) IFN-γ or (■) IFN-β and IFN-γ was calculated as (average viral titers in vehicle-treated/average viral titers in IFN-treated). One-way ANOVA followed by Tukey's post hoc t test confirmed that the fold-inhibition of SARS-CoV by combination IFN-β and IFN-γ was highly significant ($P < 0.001$) at all time points tested.

of inhibition averaging ≤ 1000-fold at concentration of 1000 U/ml or greater (Cinatl et al., 2003; Hensley et al., 2004; Spiegel et al., 2004). Likewise, similar effects were seen with IFN-α treatment (Stroher et al., 2004); however, IFN-γ was shown to be an ineffective inhibitor of SARS-CoV replication (Cinatl et al., 2003; Spiegel et al., 2004). The results of the present study, however, demonstrate that as little as 100 U/ml each of IFN-β and IFN-γ can potently inhibit SARS-CoV replication by 1×10^5-fold. The inhibitory effect observed was measured at the level of

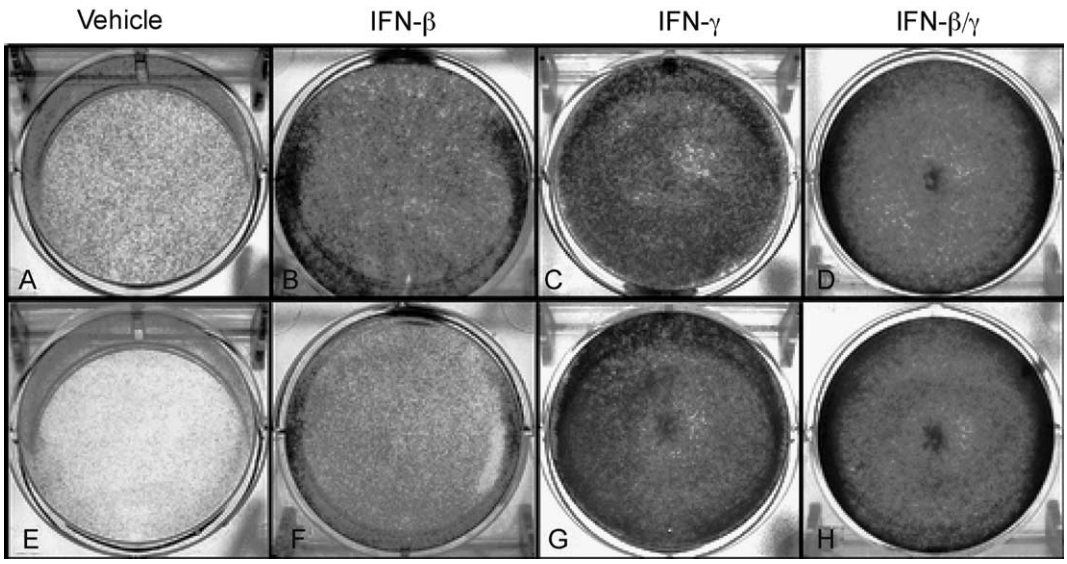

Fig. 3. IFN-β and/or IFN-γ inhibit SARS-CoV replication in Vero E6 cells. Cultures were pre-treated for 12 h with (A, E) vehicle or 100 U/ml each of (B, F) IFN-β, (C, G) IFN-γ or (D, H) IFN-β and IFN-γ before infection with SARS-CoV strains Urbani (A-D) or HK (E-H) at a MOI of 0.01 PFU/cell. Monolayers were fixed, stained with crystal violet and photographed 120 h p.i.

viral plaque formation (Table 1) and replication (Fig. 2), and the results presented herein indicate that: (1) the effect is far greater than additive, (2) maintainable up to 120 h p.i. (maximum length of time tested) and (3) effective against two strains of SARS-CoV and not against MHV.

If the potent inhibitory effect observed in cultures treated with both IFN-β and IFN-γ were synergistic in nature, the data would fit the inequalities of synergism described Berenbaum (Berenbaum, 1989). For example, a synergistic relationship would exist if the level of inhibition achieved with 100 U/ml of IFN-β plus 100 U/ml of IFN-γ were significantly greater than the level of inhibition achieved with 200 U/ml of either IFN-β or IFN-γ separately. The plaque reduction data presented in Table 1 support this inequality and suggest a synergistic antiviral relationship between type I and type II IFNs with regards to SARS-CoV plaque formation. Likewise, the fold-inhibition data presented in Fig. 2 strongly supports the hypothesis that IFN-β and IFN-γ synergistically inhibit the replication of SARS-CoV. Specifically, if the observed effect were additive, then the level of inhibition in viral replication predicted for cultures treated with 100 U/ml of both IFN-β and IFN-γ would be equal to the sum of the level of inhibition achieved in culture treated with 100 U/ml of IFN-β and IFN-γ separately. For both Urbani and HK (Figs. 2D, E), the level of inhibition achieved in cultures treated with 100 U/ml of both IFN-β and IFN-γ was approximately 1000 times greater than the sum of the fold-inhibition achieved in cultures treated with 100 U/ml of IFN-β and IFN-γ separately ($P<0.001$). More extensive synergistic analyses will need to be conducted to formally prove synergy; however, the data presented herein strongly argue in favor of this hypothesis.

Interestingly, the degree of CPE observed in cultures treated with either IFN-β or IFN-γ differed, although the level of viral replication in both treatment groups was similar (Fig. 3 vs. Figs. 2A, B). We hypothesize that the difference in CPE may be reflective of the different IFN pathways used by each IFN respectively, and may be an essential factor when considering the mechanism by which IFN-β and IFN-γ synergistically inhibit SARS-CoV replication. Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) activate distinct but related Jak/STAT signal cascades resulting in the transcription of several hundred IFN-stimulated genes (Goodbourn et al., 2000). Although similar genes are activated by all three IFNs, Der, *et al.* have identified numerous genes differentially regulated by IFN-α, IFN-β or IFN-γ using oligonucleotide arrays (Der et al., 1998). In particular, IFN-β stimulation resulted in the identification of twice as many genes as compared to IFN-γ. This differential regulation of IFN-induced genes may explain in part the synergistic effect achieved with both IFN-β and IFN-γ. It remains to be determined, however, the profile of different IFN-stimulated genes present in cells treated with both type I and type II IFNs.

Public health interventions, such as surveillance, travel restrictions and quarantines, contained the original spread of SARS-CoV in 2003 and again appear to have stopped the spread of SARS after the appearance of a few new cases in 2004. It is unknown, however, whether these draconian containment measures can be sustained with each appearance of the SARS-CoV in humans. The immune response to SARS-CoV infection appears capable of clearing the infection in most individuals. By reducing SARS-CoV load it may be possible to extend the window of time during which an effective immune response could arise. Thus, treatments that reduce SARS-CoV load by several logs in infected individuals could enable more individuals to control, eliminate and survive SARS-CoV infections. Combination

IFN treatment therefore warrants further consideration as a treatment for SARS.

Materials and methods

Cells, viruses and interferons

Vero E6 and L2 cells (American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium (MEM) or Dulbecco modified Eagle medium (DMEM), respectively, and supplemented with 5% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 mg/ml) and 2 mM L-glutamine, at 37 °C in 5% CO_2. SARS-CoV strains Urbani and Hong Kong 3pm 02-7029, hereafter called HK, were propagated in Vero E6 cells. MHV strain A59 (ATCC, VR764) was propagated in L2 cells.

Recombinant human (hu) IFN-β, hu IFN-γ, murine (mu) IFN-β and mu IFN-γ (PBL Biomedical Laboratories, New Brunswick, NJ) were added to cell cultures 12 hs (h) before infection and maintained after viral infection. For all experiments described herein, hu IFNs were used exclusively on Vero E6 cells while mu IFNs were used to treat L2 cells. In addition, concentrations of 100 U/ml were used for all experiments unless stated otherwise.

Viral plaque reduction assays

For plaque reduction assays, Vero E6 cells or L2 cells were seeded at a density of 1×10^6 cells in each well of a 6-well plate, and 24 h later, various doses of IFN-β and/or IFN-γ were added to the culture medium. After 12 h of IFN treatment, medium was removed and monolayers were infected with a fixed inoculum of SARS-CoV (strain Urbani or HK) or MHV-A59. After 1 h adsorption, the inoculum was removed, cells were washed twice with 1× phosphate buffered saline, and then overlaid with 10% FBS/DMEM containing 0.5% SeaPlaque Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and the same IFN type and concentration used during pre-treatment. Cells were stained with neutral red 2 days p.i. (MHV) or 3 days p.i. (SARS-CoV), and plaque numbers were determined 24 h later.

Viral replication assays

For virus replication assays, Vero E6 cells or L2 cells were seeded at a density of 1×10^6 cells in each well of a 6-well plate, and 24 h later, 100 U/ml of IFN-β and/or IFN-γ were added to the culture medium. After 12 h of IFN treatment, cell monolayers were inoculated with SARS-CoV (strain Urbani or HK) or MHV-A59 at a multiplicity of infection (MOI) of 0.01 PFU per cell. After 1 h adsorption, the inoculum was removed, monolayers were washed twice with 1× phosphate buffered saline, and fresh IFN-containing culture medium was returned to each well. Twenty-four, 48 or 72 h p.i., titers of infectious virus in cell supernatants was determined by a serial dilution plaque assay on Vero E6 cells for SARS-CoV or L2 cells for MHV.

Statistics

Data are presented as the means ± standard error of the means (sem). Data from IFN-treated groups were compared to vehicle-treated groups and significant difference were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc *t* test (GraphPad Prism Home, San Diego, CA).

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