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## Interferon- $\beta$ Suppresses Herpes Simplex Virus Type 1 Replication in Trigeminal Ganglion Cells Through a RNase L-dependent Pathway

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### Abstract

The induction of an anti-viral state by type I interferons (IFN) was evaluated in primary trigeminal ganglion cell cultures using herpes simplex virus type 1 (HSV-1). Cells treated with mouse IFN- $\beta$  consistently showed the greatest resistance to HSV-1 infection in comparison to cells treated with IFN- $\alpha$ 1, IFN- $\alpha$ 4, IFN- $\alpha$ 5, IFN- $\alpha$ 6, or IFN- $\alpha$ 9. The anti-viral efficacy was dose-dependent and correlated with the induction of the IFN-inducible, anti-viral genes, 2'-5' oligoadenylate synthetase (OAS) and double-stranded RNA-dependent protein kinase. In trigeminal ganglion cells deficient in the downstream effector molecule of the OAS pathway, RNase L, the anti-viral state induced by IFN- $\beta$  was lost.

### Keywords

IFN- $\alpha$ ; IFN- $\beta$ ; HSV-1; PKR; OAS; neuroimmunology

### 1. Introduction

Herpes simplex virus type 1 (HSV-1) is a highly successful human pathogen establishing a latent infection in sensory ganglion neurons following axoplasmic transport from the initial site of entry (Hay et al., 1998). The prevalence of the virus in the human population is due, in part, to the immune evading mechanisms developed by the virus through co-evolution with the host (Friedman et al., 1984; Jennings et al., 1985; Hill et al., 1995; van Strijp et al., 1988; Jerome et al., 1998; Orange et al., 2002). During latency, evidence suggests the virus continually undergoes spontaneous, incomplete reactivation (Feldman et al., 2002) which may explain the chronic expression of cytokines within the latently-infected ganglion (Shimeld et al., 1995; Liu et al., 1996; Halford et al., 1996a, 1997). Presently, it is not entirely clear what prevents viral reactivation although candidates include neutralizing antibodies (Mikloska et al., 1999) as well as infiltrating leukocytes, interferon (IFN)- $\gamma$  and

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other soluble mediators (Noisakran and Carr, 1999; Liu et al., 2000, 2001). However, the virus can ultimately overcome immune surveillance experimentally shown using hyperthermic stress (Sawtell and Thompson, 1992), an event that eliminates infiltrated T lymphocytes from the trigeminal ganglion (TG) cell cultures (Carr et al., 1998).

Type I IFNs (including IFN- $\alpha$  and IFN- $\beta$ ) are potent cytokines generated in response to viral infections that control HSV-1 replication and spread following ocular infection (Hendricks et al., 1991). While IFNs are effective in blocking HSV-1 replication at the transcriptional level (De Stasio and Taylor, 1990), HSV-1 encodes for a number of proteins including  $\gamma_134.5$  (Chou et al., 1995; Cheng et al., 2001), ICP0 (Mossman et al., 2000; Härle et al., 2002a; Eidson et al., 2002), and US11 (Peters et al., 2002) that counter the activation or activity of IFN-inducible proteins.

In addition, HSV-1 has also been found to antagonize JAK1/STAT1 signaling following IFN receptor stimulation preventing the formation of the IFN-stimulated gene factor 3 (ISGF3) complex and induction of IFN-stimulatory genes (Yokota et al., 2001). The intricate relationship between HSV-1 and one IFN-stimulatory pathway double-stranded RNA-dependent protein kinase (PKR) is exemplified by the restoration of a wild type phenotype using an ICP34.5 deletion mutant in PKR-deficient mice (Leib et al., 2000).

The present study was undertaken to further explore the relationship between type I IFNs and HSV-1 infection focusing on the TG. Specifically, primary TG cell cultures were established and evaluated for resistance to HSV-1 infection in the presence or absence of type I IFNs. Previously, we reported L929 cells transiently transfected with type I IFN transgenes showed various degrees of resistance to HSV-1 infection through a PKR-dependent pathway independent of the OAS pathway (Härle et al., 2002b). Consistent with the previous study, the present investigation found IFN- $\beta$  elicited the greatest anti-viral efficacy in TG cells compared to IFN- $\alpha$  species. However, in the absence of RNase L, the downstream effector molecule of 2'-5' oligoadenylate (OAS), the resistance to viral infection mediated by IFN- $\beta$  was lost suggesting tissue specificity of the IFN activated, anti-viral pathway OAS.

## 2.0 Materials and methods

### 2.1 Virus and cell line

Vero cells and L929 cells originally obtained from the American Type Culture Collection (ATCC, Manassas, VA.) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) or DMEM (for L929 cells, ATCC) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotic/antimycotic solution (AAS, Invitrogen) at 37° C, 5% CO<sub>2</sub>, and 95% humidity. HSV-1 stock (McKrae strain) was prepared as previously described (Halford et al., 1996a). Vesicular stomatitis virus (VSV) was a gift from Dr. Robert Fleishmann (Dept. Microbiology, UTMB).

### 2.2 Plasmid DNA constructs

All murine IFN transgenes were cloned into the eukaryotic expression vector pKCMVintPolylinker (5,087 bp, Vical Inc., San Diego, CA.) containing a simian virus 40

polyadenylation signal and a kanamycin resistance gene (Cull et al., 2002). The IFN genes (575 to 626 bp) are expressed under the control of a human cytomegalovirus immediate-early enhancer/promoter. The plasmid constructs were transformed into *Escherichia coli* strain INV $\alpha$ F' (Invitrogen) and grown in Terrific broth containing 50  $\mu$ g kanamycin/ml followed by purification using a plasmid maxiprep kit (Bio-Rad). After the plasmid isolation, restriction enzyme digestion assays were conducted and the products analyzed by agarose gel electrophoresis.

### 2.3 Establishment of TG cell cultures

TG cell cultures were prepared as described (Halford et al., 1996b) using female ICR outbred mice (25–34 g, Harlan Spague Dawley, Indianapolis, IN.), C57BL/6J wild type mice (The Jackson Laboratory, Bar Harbor, ME.), or mice with a homozygous disruption in the gene encoding RNase L (Zhou et al., 1997) as the source of the TG cells. The cells were cultured in 1.0 ml DMEM containing 0.375% HCO<sub>3</sub> supplemented with 10% FBS and AAS (referred to as complete DMEM). The cultures were maintained at 37° C, 5% CO<sub>2</sub>, and 95% humidity. After 7–12 days in culture, the cells were either transfected with plasmid vector alone or plasmids containing type I IFN transgenes or exposed to various concentrations of type I IFN.

### 2.4 Transfection of TG or L929 cells

Seven to twelve days post initiation of culture, TG cells ( $1.0 \pm 0.3 \times 10^5$  cells/well) in 6-well microtiter plates were transfected with 2  $\mu$ g of plasmid DNA and 10  $\mu$ l of Superfect (Qiagen, Valencia, CA.) in 700  $\mu$ l of complete DMEM for 3 hr. The cells were then washed once with prewarmed (37° C) phosphate buffered saline (PBS, pH 7.4) and 2.0 ml of fresh, prewarmed complete DMEM was added to each well. Twenty-four hr post transfection, supernatants were collected and cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.5. The viral inoculum was removed 1 hr post infection (p.i.) and replaced with fresh complete DMEM. After 24 hr, the cells were freeze-thawed and the clarified supernatants ( $10,000 \times g$ , 1 min) were assayed for HSV-1 titer by plaque assay using Vero cells. In a separate experiment, known concentrations of biologically active IFN (3.2 – 32.0 units) were added to the TG cell cultures 24 hr prior to infection with HSV-1 (MOI = 0.5).

L929 cells were transfected as previously described (Härle et al., 2002b) to generate IFN- $\alpha$ 1, IFN- $\alpha$ 4, IFN- $\alpha$ 5, IFN- $\alpha$ 6, IFN- $\alpha$ 9, and IFN- $\beta$  enriched supernatants. The supernatants were evaluated for the quantity of biologically active IFN by bioassay.

### 2.5 IFN bioassay

To determine the concentration of biologically active IFN secreted at the end of the 24 hr post transfection period, supernatants from the transfected cells were serially diluted and incubated with L929 cells for 24 hr in 96 well plates. Following the incubation period, the supernatant was removed and the cells were infected with VSV (MOI = 0.05). When the cytopathic effect (CPE) in the control wells was maximal (32 hr p.i.), the cells were stained with crystal violet. In each assay, a standard curve using mouse recombinant IFN- $\beta$  (Petska Biomedical Laboratories, New Brunswick, NJ) was used. Fifty percent inhibition of CPE was equivalent to 1.0 unit/ml IFN.

## 2.6 Reverse transcription and real-time PCR

Total TG cell RNA was isolated 12 hr p.i. or 24 hr post IFN-treatment (for OAS and PKR gene expression) in Ultraspect RNA isolation reagent (Biotech Inc., Houston, TX.) according to the manufacturer's protocol. First strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and an oligo (dT) primer (Promega). Real time PCR analysis of targeted gene expression was carried out using the iCycler (Bio-Rad) as previously described (Al-khatib et al., 2002).

## 2.7 Immunohistochemistry

Dissociated TG cells obtained from ICR mice were grown in 24 well microtiter plates coated with laminin and collagen. Seven days following the initiation of the culture, the cells were fixed for 30 min in 3% paraformaldehyde in PBS containing 0.1% Triton X-100 (pH 7.5) and then rinsed three times in 1.0 ml of PBS containing 0.1% Triton X-100 (pH 7.5). Non-specific binding sites were blocked with 10% normal goat serum for 30 min at room temperature. Cells were incubated overnight with rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:200 dilution, DAKO, Carpinteria, CA.), anti-microtubule associated protein (MAP) antibody (1:50 dilution, Accurate Chemical Corp., Westbury, NY) or anti-neuron-specific enolase antibody (1:100 dilution, Chemicon, Temecular, CA) in PBS (pH 7.5) containing 1% normal goat serum at 4° C. Following the overnight incubation, the cells were then rinsed three times with 1.0 ml PBS containing 0.1% Triton X-100 and incubated with Texas red or peroxidase-conjugated goat anti-rabbit IgG (1:200 dilution, Vector Laboratories, Burlingame, CA.) in PBS containing 10% normal goat serum for 1 hr. The cells were then rinsed three times for 5 min each with PBS and substrate (3,3'-diaminobenzidine) was added to peroxidase-labeled cells. Peroxidase- and fluorescently-labeled cells were viewed using a Nikon E800 fluorescent microscope. Cells treated with secondary antibody only served as controls.

## 2.8 Statistics

One-way analysis of variance (ANOVA) and Tukey's t-test were used to determine significant ( $p < .05$ ) differences between the vector and IFN- $\alpha/\beta$  transgene- or protein-treated groups.

## 3.0 Results

### 3.1 Phenotypic characterization of TG cell culture

Dissociated TG cells cultured for 7 days were assessed for the percentage of neurons and Schwann cells using immunohistochemical means. Using either anti-neuron-specific enolase or MAP antibodies, 1.5  $\pm$  0.5% of the cells were defined as neurons whereas 3.1  $\pm$  1.0% of the cells expressed high levels of GFAP (Schwann cells) (Fig. 1). A significant proportion of cells (> 80%) expressed low levels of GFAP and may represent quiescent Schwann cells.

### 3.2 Resistance to HSV-1 infection by transfected TG cells

TG cell cultures were evaluated for resistance to HSV-1 infection following transfection with plasmid DNA containing different type I IFN transgenes. TG cell cultures were highly

susceptible to HSV-1 infection (MOI = 0.5) yielding 698,441 +/- 86,631 plaque forming units (pfu)/culture within 24 hr. There is no biologically active IFN detected in these cultures following HSV-1 infection (limit of detection is 1.0 unit). Cells transfected with plasmid vector or plasmid vector containing type I IFN transgenes were less susceptible to infection with cells transfected with the IFN- $\beta$  showing the greatest degree of resistance to HSV-1 infection (Fig. 2). The degree of anti-viral efficacy comparing the type I IFN transgenes was not due to differences in the amount of biologically active IFN secreted by the cells as all transfected cultures (except vector-transfected cells) secreted similar amounts of IFN (Fig. 2). Since the vector-transfected cells were less susceptible to infection compared to non-transfected cells but did not consistently produce measurable levels of IFN (only 3/7 cultures tested produced at least 1.0 unit of IFN), the process of transfection itself or the plasmid vector alone induced an IFN-independent, anti-viral state in the targeted cells. Consequently, a different approach was necessary in order to compare the anti-viral efficacies of type I IFNs against HSV-1 using primary TG cells.

### 3.3 IFN- $\beta$ antagonizes HSV-1 replication at the transcriptional level

Type I IFN-enriched supernatants obtained from transiently transfected L929 cells were added to TG cell cultures 24 hr prior to HSV-1 infection. In a dose-dependent fashion, IFN- $\beta$  was found to suppress HSV-1 replication compared to cells treated with supernatant from vector-transfected L929 cells as measured by viral titers recovered in the infected TG cell cultures (Fig. 3). Only the highest concentration of IFN- $\alpha$ 4 tested (32 units/ml) was found to antagonize HSV-1 replication in comparison to the other IFN- $\alpha$  species tested (Fig. 3). In a similar fashion, TG cell cultures treated with IFN- $\beta$  were found to show the greatest inhibition of steady state HSV-1 lytic gene expression as measured by real time PCR twelve hr post infection (Fig. 4). In comparison to TG cell cultures incubated with supernatant from plasmid vector-transfected L929 cells, IFN- $\beta$ -enriched supernatant (32 units/ml) suppressed immediate early (infection cell protein 27, ICP27), early (thymidine kinase, TK), and late (virion protein 16, VP16) lytic gene expression. By comparison, the same concentration of the IFN- $\alpha$  species only suppressed the immediate early and late gene but not the early lytic gene TK.

### 3.4 IFN- $\beta$ hinders HSV-1 replication through the OAS/RNase L pathway

Exposure to type I IFNs results in the activation of two prominent anti-viral pathways PKR and OAS. Indeed, when TG cell cultures were incubated with type I IFN-enriched supernatant the expression of both PKR and OAS1a mRNA were significantly ( $p < .05$ ) elevated compared to cultures incubated with supernatant from vector-transfected cells (Fig. 5). Those cultures exposed to the IFN- $\beta$  containing supernatant showed the greatest increase in both OAS and PKR expression by real time PCR. The induction of the OAS pathway results in the activation of latent endoribonucleases RNase L which cleaves mRNA and rRNA ultimately inhibiting protein synthesis (Clemens and Williams, 1978; Floyd-Smith et al., 1981). To further explore the relationship between the activation of the OAS pathway by type I IFNs and the establishment of an anti-viral state, TG cell cultures were prepared from RNase L null mice. TG cells from wild type or RNase L null mice infected with HSV-1 (MOI=0.5) yielded similar levels of HSV-1 24 hr post infection (Fig. 6a). However, in the absence of RNase L, IFN- $\beta$  exposed TG cells exhibited no resistance to HSV-1 infection in

comparison to wild type TG cells (Fig. 6b). We interpret these results to suggest that the OAS pathway is involved in antagonizing HSV-1 but only in IFN-pretreated TG cells.

## 4.0 Discussion

The present study found that in comparison to 5 murine IFN- $\alpha$  species, IFN- $\beta$  showed substantially greater efficacy against HSV-1 infection in primary TG cell cultures as measured by viral titer and lytic gene expression, and this protective effect was mediated through the OAS/RNase L pathway. However, the absence of a functional OAS pathway did not have a significant impact on HSV-1 replication in untreated TG cells. These results imply that naive TG cells infected with HSV-1 do not utilize the OAS/RNase L as a means to antagonize viral replication. Candidate alternative pathways that may function in these cells include PKR (Khabar et al., 2000; Härle et al., 2002b) or pathways independent of OAS or PKR (Zhou et al., 1997, 1999). One intriguing aspect of the present findings is that all type I IFNs tested elevated OAS expression in the TG cells but only IFN- $\beta$  and marginally, IFN- $\alpha$ 4 antagonized HSV-1 replication. We interpret these results to suggest a threshold of OAS must be achieved in order to significantly impact on viral replication (IFN- $\alpha$ 4 and IFN- $\beta$  induced the greatest increase in OAS message). It is likely that the relationship between OAS activation and viral replication is dependent on the infectious dose of HSV-1 as well. Specifically, we have recently found that increasing the viral inoculum used to infect cells overrides any effect that the activation of the OAS pathway might have on viral replication (Al-khatib et al., 2003). Consistent with this observation, HSV-1 encodes for inhibitors of RNase L activation thus eroding the anti-viral state induced by OAS activation (Cayley et al., 1984). Consequently, a reciprocal relationship exists between OAS activation and viral titer. Specifically, as viral protein levels increase, the antiviral effect associated with OAS activation is less effective due to direct hindrance in the formation of active RNase L. One probable outcome of this interaction is the generation of compensatory anti-viral pathways independent of OAS activation that are capable of antagonizing HSV-1 (Zhou et al., 1999).

The reduction in viral yield in the IFN- $\beta$ -treated cultures coincided with suppression of representative genes of all three classes of HSV-1 lytic genes. Paradoxically, all IFN- $\alpha$  species tested also significantly reduced immediate early and late gene expression measuring ICP27 and VP16 respectively. Therefore, the only difference was IFN- $\beta$  suppression of the early lytic gene, thymidine kinase (TK) expression. The three classes of HSV-1 lytic genes are expressed in a coordinated, cascade fashion with the early lytic gene family (which includes TK) responsible for viral DNA replication (Turner and Jenkins, 1997). Consequently, the issue of why IFN- $\beta$  suppresses TK gene expression but does not substantially impact further on late gene expression (i.e., VP16) in comparison with IFN- $\alpha$  (which have no apparent effect on TK mRNA levels) remains unresolved. In no way was this evaluation comprehensive in that only a single gene from each class of HSV-1 lytic genes was measured. Therefore, it is difficult to discern if this pattern of viral gene expression by IFN- $\beta$  is global or specific for the candidate genes evaluated in this study. However, it is intriguing to note that TK is required for reactivation (Coen et al., 1989) making IFN- $\beta$  a candidate molecule to further study in relationship to this event (HSV-1 reactivation).



Collectively, the present study reinforces earlier work showing IFN- $\beta$  confers a greater anti-viral effect against HSV-1 in comparison to multiple IFN- $\alpha$  species (Härle et al., 2002b). The central role of IFN- $\beta$  as an important anti-viral cytokine is substantiated using mice lacking IFN- $\beta$  (Deonarain et al., 2000). These animals are highly susceptible to vaccinia virus infection and cells derived from these mice show attenuation in virus-induced IFN- $\alpha$  and OAS expression. However, IFN- $\alpha$  species appear to afford greater anti-cytomegalovirus efficacy compared to IFN- $\beta$  depending on the viral pathogen under study (Cull et al., 2002) with further synergies in efficacy displayed with combinations of IFN- $\alpha$  and - $\beta$  (Bartlett et al., 2002). Consequently, the future challenge will be to identify those pathways associated with the efficacy of the type I IFN species against the specific viral pathogen and the signaling cascades linked to the activation of the cellular events surrounding the establishment of this anti-viral environment. In so doing, it may be possible to target the induction of selective transcriptional regulatory elements controlling the expression of specific anti-viral genes and thus, circumvent the unwarranted side-effects associated with type I IFN exposure.

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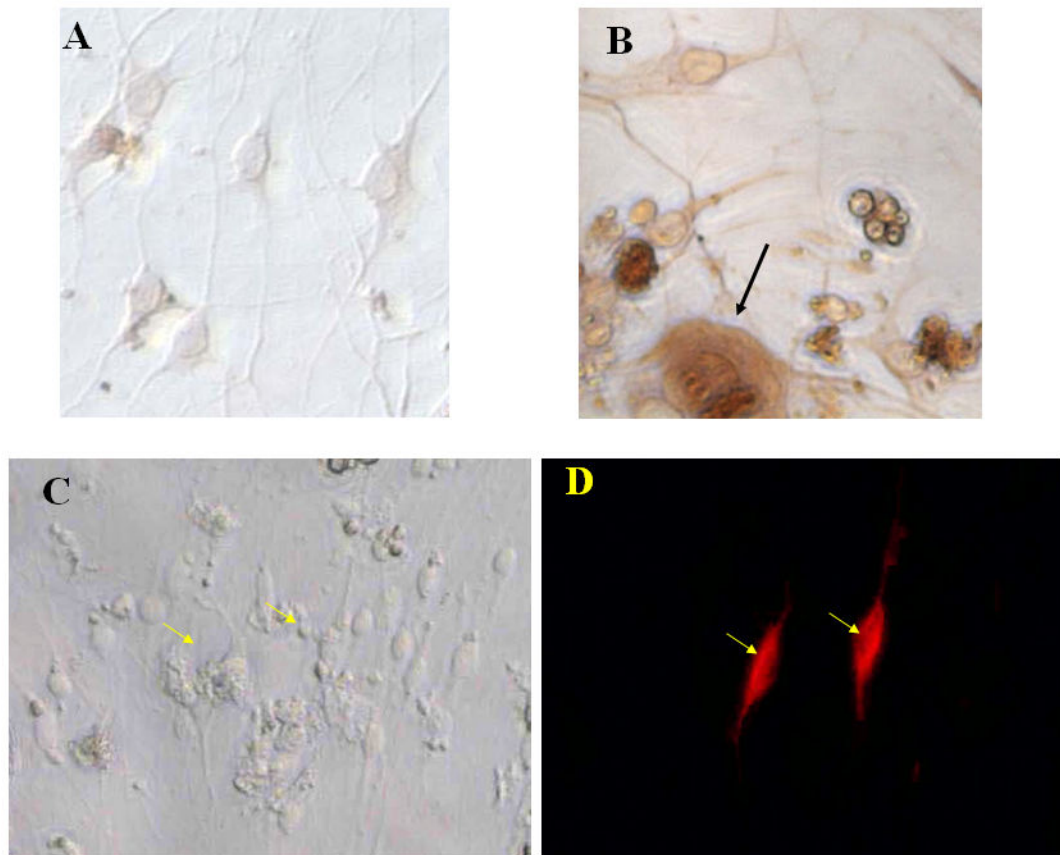
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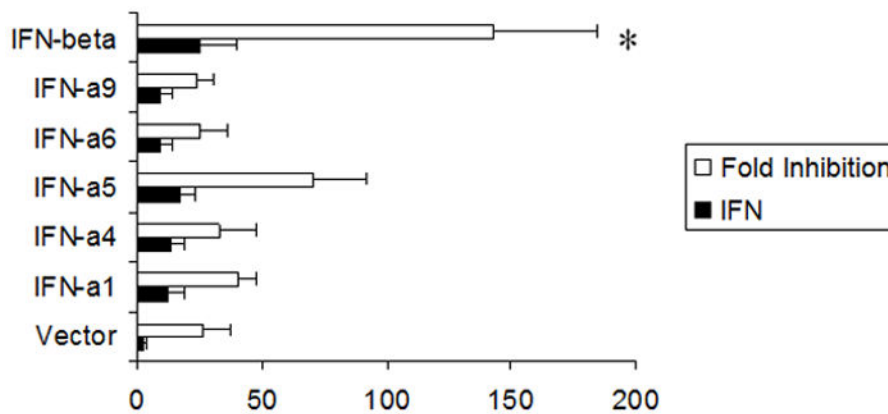


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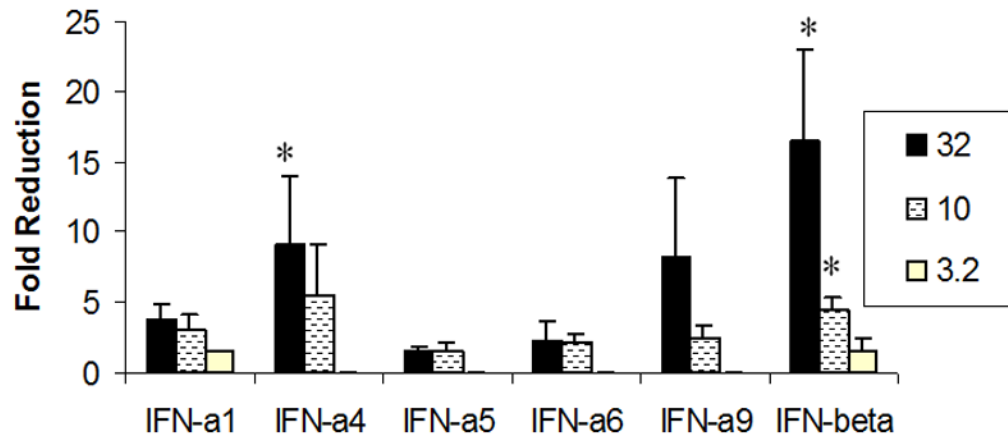


**Figure 1. Phenotypic profile of TG cell cultures**

Primary trigeminal ganglion cells ( $1 \times 10^5$  cells) were cultured for 7 days, fixed, and then assessed for the percentage of neurons and Schwann cells by immunohistochemical means. (A) and (B) show a typical staining pattern for microtubule associated protein staining of neurons (B) and negative control (A). (C) and (D) show a typical staining pattern for GFAP-labeled cells under fluorescent light (D) and light field (C) microscopy. Arrows indicate neuron (black) and Schwann cells (yellow) magnified 200X.

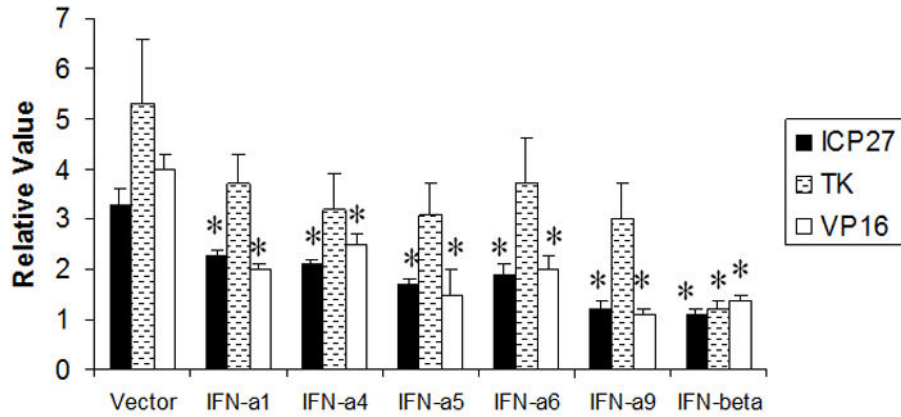


**Figure 2. Transfection of primary TG cells with IFN- $\beta$  transgene suppresses HSV-1 replication**  
 Primary trigeminal ganglion cells ( $1 \times 10^5$  cells) in culture were transfected with 2  $\mu$ g of pkCMV plasmid DNA alone (Vector) or plasmid vector DNA containing the indicated type I IFN transgene. Twenty-four hr post-transfection, the supernatant was removed and assayed for IFN content by bioassay (Units/ml) and the cells were subsequently infected with HSV-1 (MOI=0.5). Twenty-four hr post infection, the cultures were freeze-thawed and viral titers determined. The amount of biologically active IFN is depicted in solid bars whereas the fold inhibition is reflected in the open bars. This figure is a summary of six experiments. \* $p < .05$  comparing the vector- to IFN- $\beta$ -transfected groups as determined by ANOVA and Tukey's t-test. Bars represent mean  $\pm$  SEM.



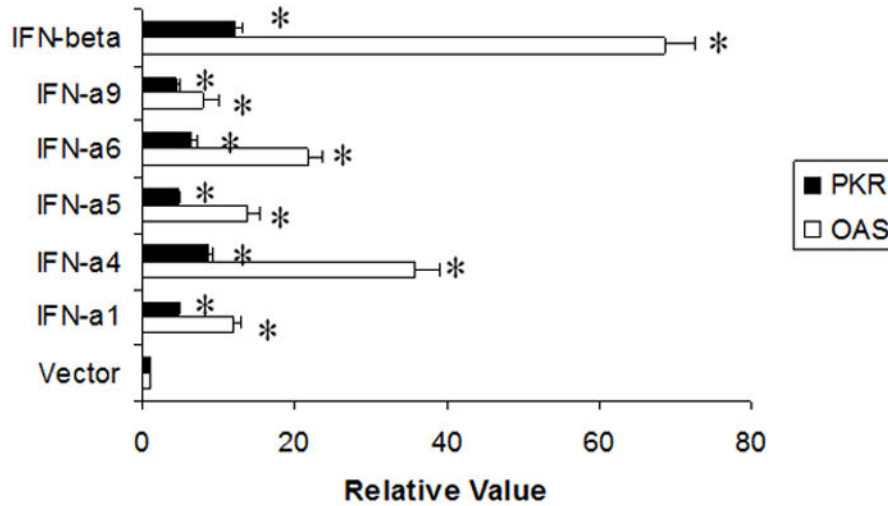
**Figure 3. IFN- $\beta$  antagonizes HSV-1 replication in a dose-dependent fashion in primary TG cell cultures**

TG cell cultures were treated with the indicated concentration of type I IFN and subsequently infected with HSV-1 (MOI=0.5) 24 hr later. Following a 24 hr incubation period, the cultures were freeze-thawed and viral titers were determined by plaque assay. This figure is a summary of four experiments with each treatment conducted in duplicate. \* $p < .05$  comparing the type I IFN-treated group to the vector supernatant-treated control as determined by ANOVA and Tukey's t-test. Bars represent mean  $\pm$  SEM.



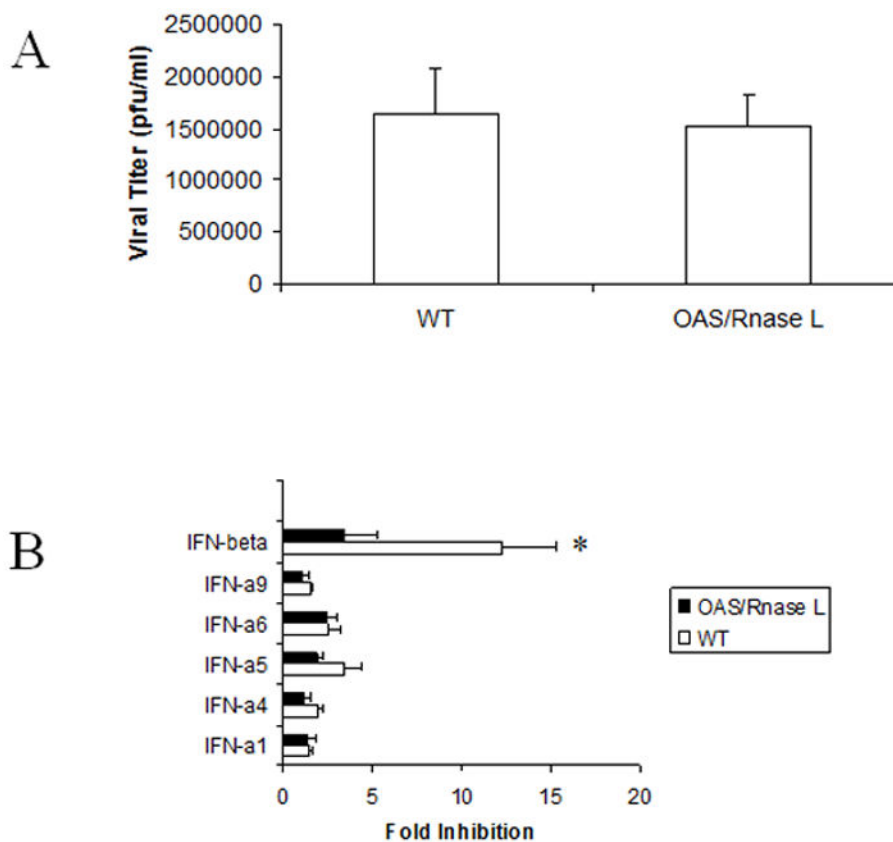
**Figure 4. IFN- $\beta$  suppresses HSV-1 lytic gene expression**

IFN-treated (32 units/culture) primary TG cells were infected 24 hr post-treatment with HSV-1 (MOI=0.5). Twelve hr post-infection, RNA was isolated from each culture and used to generate cDNA. Using oligonucleotide primers specific for the immediate early (ICP27), early (TK), and late (VP16) lytic HSV-1 genes, the relative amounts of each targeted gene was determined by real-time PCR. The relative level for each gene expressed in the TG cells was normalized to the relative value of GAPDH for each group. Undiluted supernatant from vector-transfected cells (Vector) served as the control. This figure is a representative of two experiments each conducted in triplicate. \* $p < .05$  comparing the IFN-treated to vector supernatant-treated group as determined by ANOVA and Tukey's t-test. Bars represent mean  $\pm$  SEM.



**Figure 5. Type I IFN augments the expression of the IFN-stimulatory genes, OAS and PKR**  
TG cells ( $1 \times 10^5$  cells) were stimulated with 32 units of the indicated type I IFN or (control) vector supernatant. Twenty-four hr post treatment, the RNA was isolated from the treated cells and used to generate cDNA. Oligonucleotide primers specific for OAS(1a, 1c) or PKR were used to amplify the targeted gene by real-time PCR. The relative level for each gene expressed in the TG cells was normalized to the relative value of GAPDH. This figure is a representative of two experiments each conducted in triplicate. \*All IFN-treated groups significantly ( $p < .05$ ) elevated OAS and PKR gene expression relative to the control (vector)-treated group as determined by ANOVA and Tukey's t-test. Bars represent mean  $\pm$  SEM.





**Figure 6. The anti-viral efficacy mediated by IFN- $\beta$  is dependent on OAS/RNase L**  
 TG cells from wild type (WT) C57BL/6 or RNase L knockout mice (OAS/RNase L) were cultured and either infected with HSV-1 (MOI=0.5) prior to (A) or (B) after treatment with 32 units of the indicated IFN or control (vector). Twenty-four hr post infection, the cell cultures were freeze-thawed and viral titers determined. This figure is a summary of four experiments with each experiment conducted in duplicate. \* $p < .05$  comparing the IFN- $\beta$  treated, WT TG cells compared to the vector-treated WT controls or the OAS/RNase L TG cells treated with IFN- $\beta$  as determined by ANOVA and Tukey's t-test. Bars represent mean  $\pm$  SEM.