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DISTRIBUTION OF CELLULAR INTERFERON BETA (IFN-β) IN MURINE FIBROBLAST CELL LINES UPON INFECTION OF HSV-1

A Thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

Rachael Elizabeth Curtis
B.S., Wilmington College, 1999

2011
Wright State University

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WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

December 12, 2011

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Rachael Elizabeth Curtis ENTITLED Distribution of cellular Interferon beta (IFN-β)in fibroblasts upon infection of HSV-1 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Curtis, Rachael Elizabeth. M.S., Department of Microbiology and Immunology,
Wright State University, 2011. Distribution of cellular Interferon beta (IFN-β in fibroblasts upon infection of HSV-1.

The hypothesis for this study was: IFN-β expression differs between murine fibroblasts (A2R1 and L929) of different mouse strains upon infection of HSV-1. Fluorescent microscopy was used to examine localization of IFN-β in the different murine fibroblast and keratinocyte cell lines after 6 hours of infection with herpes simplex virus type-1 (HSV-1). Because keratinocytes cell lines HEL-30 and PAM-212 grew in clusters, staining patterns in individual cells could not be determined. A notable difference in localization of IFN-β immune staining was seen between the two cell lines when infected with HSV-1. The A2R1 cell line showed perinuclear (PN) localization, while the L929 cell line showed nuclear localization suggesting a distinction in response to HSV by these fibroblast lines. These responses were compared with those stimulated by an inducer of Type I interferons, polyriboinosinic:polyribocytidylic acid (poly I:C). In

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Poly I:C-treated fibroblasts, cytoplasmic staining was observed at 6 hours after treatment.

Image J Fire program provided by National Institute of Health (NIH) was used to determine fluorescent intensities in both the keratinocyte cell lines and the fibroblast cell lines. The fluorescent intensity was significantly greater in the L929 fibroblast cell line when treated with poly IC than the untreated L929 fibroblast. There was no difference in fluorescent intensities in any of the other cell lines following poly IC treatment.

I attempted to measure IFN-β in the culture supernatants from the infected cells at different time points: 0, 6, 12 and 24 hours, from all cell lines (A2R1, L929, Hel-30 and Pam-212) using a Mouse Interferon Beta ELISA kit (range 15.6 – 1000pg/ml), but none was detected, except for an insignificant amount in the L929 cells stimulated with Poly IC at 24 hours. The control standards that were supplied with the kit gave the expected results.

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LIST OF ABBREVIATIONS

EIF2A = Eukaryotic translation initiation factor 2A

ELISA = Enzyme-linked immunosorbent assay

HSV-1 = Herpes simplex virus type – 1

ICP0 = Infected cell protein 0

ICP34.5 = Infected cell protein 34.5

IFN = Interferon

IFN –α = interferon alpha

IFN-β = Interferon beta

IFNAR1 = interferon (alpha, beta and omega) receptor 1

IFNAR2 = interferon (alpha, beta and omega) receptor 2

IkB = inhibitor of kappaB

IL1 = interleukin 1

IRF = interferon regulatory factor

ISGF3 = interferon stimulated gene factor 3

ISRE = interferon stimulated response elements

JAK = Janus-family tyrosine kinases

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MOI = Multiplicity of infection

mRNA = messenger ribonucleic acid

NFkB = nuclear factor kappaB

NLS = nuclear localization sequence

PFU = plaque forming units

PKR = Protein Kinase RNA-activated

PN = perinuclear

Poly IC = polyriboinosinic:Polyribocytidylic acid

RT = room temperature

STAT1 = signal transducer and activator of transcription 1

TNF = tumor necrosis factors

Tyk2 = tyrosine kinase 2

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ACKNOWLEDGEMENT

I would like to thank my advisor Dr. Nancy Bigley for all of her guidance and support that she has given to me throughout my program of study. I would also like to thank Dr. Paula Bubulya for her kind gift of the secondary fluorescent antibody used in this study. I would also like to acknowledge and thank Dr. Valerie Neff and Jackie Sisco for their time and assistance in the microscopy lab and Rick Salisbury for his assistance with the ELISA. A special thank you to Dr. Barbara Hull and Dr. Cheryl Conley for their contributions and suggestions they have given to me.

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DEDICATION

I would like to dedicate this thesis to my husband Christopher Curtis and my children Riley and Cameron who have given me inspiration and encouragement and are truly a gift in my life.

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INTRODUCTION

On initial infection, a cell responds to a virus by producing Type 1 interferon. There are two subtypes of type 1 interferons: interferon alpha (IFN- α) and interferon beta (IFN-β). These interferons are released from infected cells and protect neighboring cells from viral invasion and virus replication by binding to the Type 1 interferon receptors (IFNARs) located on the plasma membrane of neighboring cells (Perry et al, 2005).

The amount of interferon produced depends on the cell type and on the type of pathogen. HSV-1 has been shown to inhibit type 1 interferon production in certain cell types Perry et al, 2005). In this study, IFN-β responses produced by several murine fibroblast and keratinocyte cell lines were investigated. However, with the keratinocyte cell lines, the localization of IFN-β could not be studied because these cell lines grow in clumps so accurate cell counts and fluorescent visualization were not possible and therefore these data sets were omitted from this analysis (figure 1). There was also not a difference in the mean fluorescent intensity in the Hel-30 and Pam-212 keratinocyte cell lines (figure 2).

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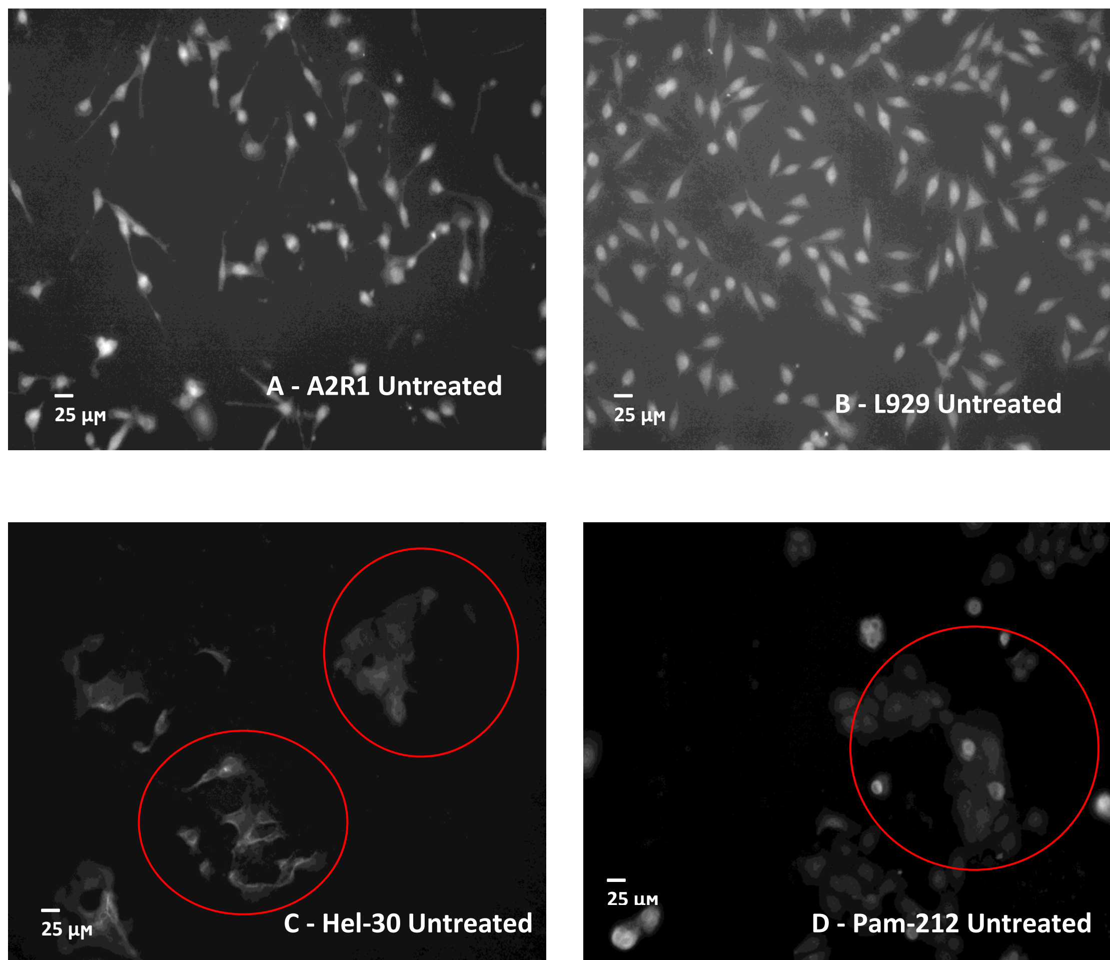

Figure 1: Immunostaining of IFN-β in four different cell lines untreated at a 6 hour time point. Original magnification: 20X. Notice how the fibroblasts morphology is well defined and separate where with the keratinocytes, the morphology is not well defined and clumping occurs. (a) A2R1 fibroblasts; (b) L929 fibroblast; (c) Pam-212keratinocyte; (d) Hel-30 keratinocyte. Red circles denote where clumping is seen and individual cells are very difficult to discern.

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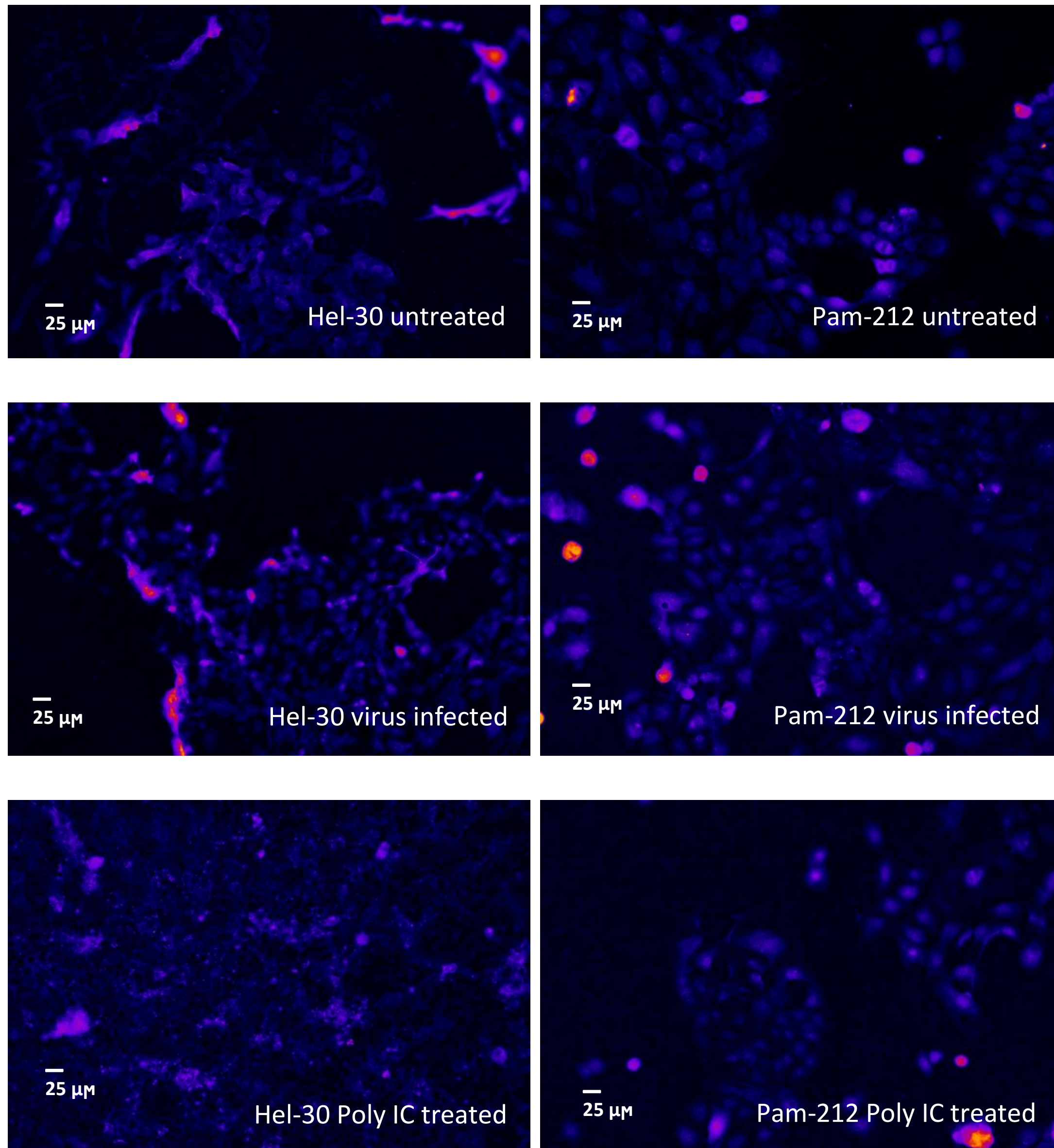

Figure 2. Mean Fluorescent Intensity using image J Fire program. Hel-30 and Pam-212 keratinocytes untreated, Poly IC treated and virus infected. There is no significant difference with the virus infected cells and the poly IC treated cells compared to the untreated cells.

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The hypothesis for this study was: IFN-β expression is different in murine fibroblasts (A2R1 and L929) derived from different mouse strains and different from that of keratinocyte cell lines (Hel-30 and Pam-212) upon infection of HSV-1. To demonstrate potential differences, these murine cell lines were infected with HSV-1 and then the expression and/or intracellular localization of interferon-β was determined by immunofluorescent microscopy. To determine if there were varying amounts of IFN-β in the culture supernatant fluids, ELISA was used to quantify IFN-β at 0, 6, 12 and 24 hour time points post infection.

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LITERATURE REVIEW (BACKGROUND)

HSV-1 Infection

HSV-1 is a double stranded DNA virus that can establish both productive and latent infections and persists in the body for the life of the carrier by becoming latent in the cell bodies of nerves, where it hides from the immune system. Reactivated virus can be transported by the nerve axon to the skin where the virus replicates and causes new sores (Norkin, 2010). HSV-1 infects approximately 60%-80% of the world population and can lead to ocular herpes, which is one of the major causes of blindness in the Western world. (Cunningham et al, 2006). HSV-1 promotes a strong cytokine response in cells that are infected with the virus, stimulates gene products that interfere with the signaling pathways of type 1 interferons, and inhibits the synthesis of type 1 interferons. Viruses have developed successful strategies to overcome the protective effects of interferons (Peng et al, 2008).

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Epithelial Anatomy

Because the initial site of HSV-1 infection as well as the sites for lesion formation involves epithelia (skin and mucous membranes) (figures 3 & 4), it is important to understand the structure of the epithelial surfaces of the body. The skin offers a barrier of protection against many environmental factors and from pathogens. The outermost layer of the skin is the epidermis and is made up of Merkel cells, keratinocytes, and also contains melanocytes and Langerhans cells. The epidermis contains five layers: Stratum corneum, Stratum lucidum, Stratum granulosum, Stratum spinosum and Stratum germinativum. There are two layers below the epidermis: the dermis and the hypodermis (figure 5). The dermis is made up of two regions: the papillary region and the reticular region. The papillary regions contain loose connective tissue that resembles fingerlike projections which interact with the epidermis. The reticular region is composed of dense connective tissue and is also the area that contains the hair roots, sweat glands, sebaceous glands and blood vessels (Tortora, 2006).

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The hypodermis is not part of the skin and its function is to connect the skin with the bone and muscle. The dermis and hypodermis both contain fibroblasts. Fibroblasts are found everywhere within the body and have long been considered to be essential structural elements of tissue integrity. Tissue-specific fibroblasts play an important role as immunoregulatory cells that can mount and influence immune responses by being able to secrete chemokines and cytokines (Ibelgauft, 2011).

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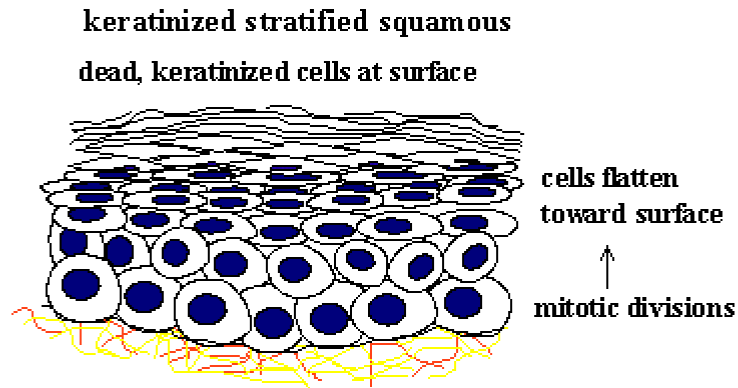

Figure 3. Illustration of the top layer of the epithelium which is keratinized and protects against friction and invasion from pathogens. Adapted from McGraw Hill, 1999.

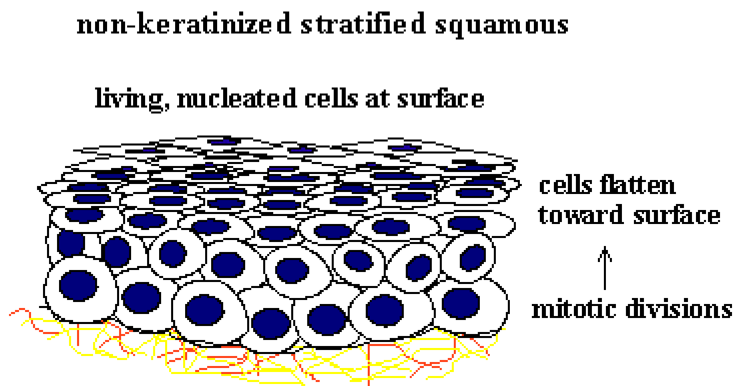

Figure 4. Illustration of the top layer of the epithelium which is the non-keratinized type (mucous membrane) found in the oral cavity. Adapted from McGraw Hill, 1999.

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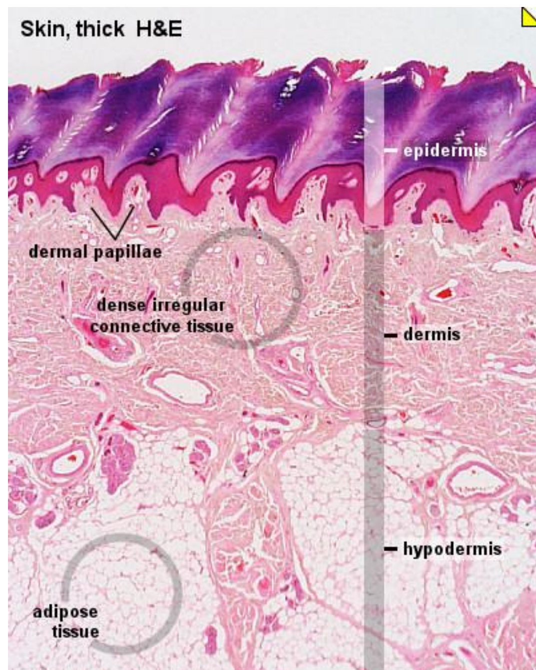

Figure 5. Illustration of the Layers of skin adapted from Slomianka, 2009.

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Interferon Beta (IFN-β)

Interferons are proteins that are made and released by cells upon invasion of a pathogen. In humans, there are many different forms (isotypes) of type I interferon and all of them bind to the same receptor on the surface of the plasma membrane and are similar in structure. The only one form of beta interferon in humans also binds to the same IFNAR as the IFN-α molecules. In contrast to IFN-α, IFN-β of other animal species is inactive in human cells (Davidson College, 2000).

IFN-β can act in a paracrine or autocrine fashion. The receptor that is found on the plasma membrane initiates the signal transduction pathway after binding of IFN-β (figure 6). Once IFN-β has bound to its receptor, the intracellular domains of IFNAR1 and IFNAR2 chains associate with two Janus-family tyrosine kinases, JAK1 and Tyk2, which transphosphorylate each other and phosphorylate the receptors. IFNAR1 and IFNAR2 bind to STAT1 and STAT2 (signal transducer and activator of transcription1). The STAT proteins dimerize and move to the nucleus where they activate specific genes (Peng, 2007).

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Interferon-β is generally produced by fibroblasts and some epithelial cell types. (Norkin, Virology p. 85) Interferon-β can be induced by viruses, double stranded RNA and micro-organisms. It can also be induced by cytokines such as TNF and IL1. (Ibelgauft, 2011)

Mouse Interferon beta (mu IFN-β) is very rapidly internalized by receptor-mediated endocytosis and binds to the nuclei of mouse fibroblasts. The receptors on the nucleus of the fibroblasts are not only present in a higher affinity than on the plasma membrane, but the receptors are also more dense on the nuclear membrane than on the plasma membrane (Kushnaryov et al, 1985 & 1986). Kushnaryov and collegues also showed that after binding of mu IFN-β to the receptor on the plasma membrane of mouse L cells, it only took 3 minutes for most of the IFN-β molecules to reach the nucleus.

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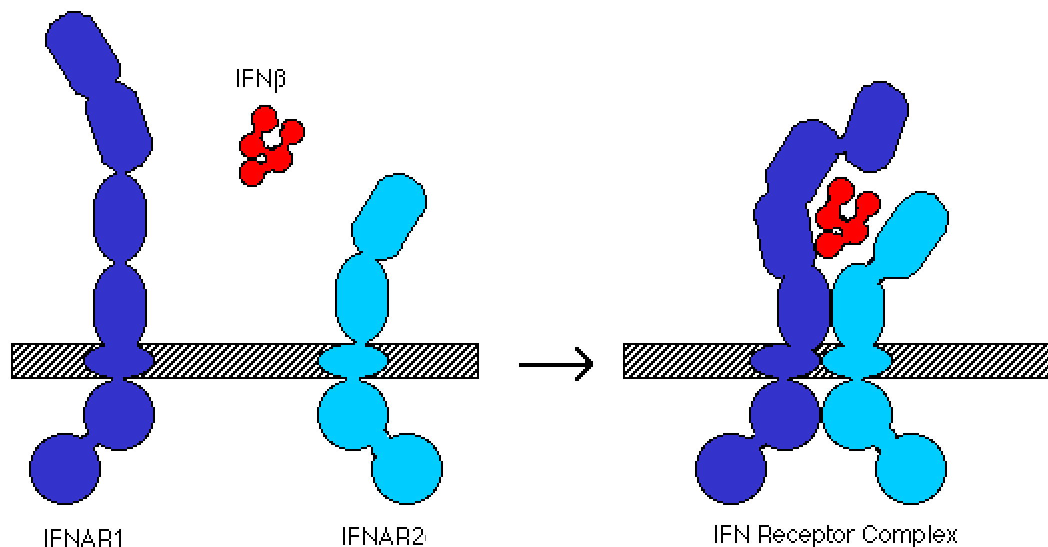

Figure 6. Illustrations of Type 1 interferon receptors (IFNAR1 and IFNAR2) binding to Interferon beta in the plasma membrane of a cell. Adapted from Davidson College Department of Biology, Davidson, NC http://www.bio.davidson.edu/courses/immunology/Students/spring2000/white/ifnbeta.html

Mechanisms of inducing Interferon Beta

Type 1 interferons are transcriptionally regulated and IRF3 (interferon regulatory factor) primarily regulates production of IFN-β (Figure 7). (Perry et al, 2005). IRF3 is continually expressed in all cells and is found in an inactivated state in the cytoplasm of unstimulated cells. (Perry et al, 2005). Upon stimulation, IRF3 becomes phosphorylated, dimerizes and translocates into the nucleus and then activates the expression of IFN-β. When IFN-β is produced, it starts a positive feedback loop and acts in an

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autocrine and paracrine fashion by binding to IFNAR. Once this occurs, the JAK/STAT pathway is activated and a transcription factor complex (ISGF3)(IFN-stimulated gene factor 3) translocates into the nucleus and binds to IFN-stimulated response elements (ISRE) to induce expression of a large group of IFN-inducible genes. (Perry et al, 2005).

Protein Kinase RNA-activated (PKR) is another signaling pathway in which interferon is induced. PKR is activated by interferon or double stranded RNA which is synthesized from infection by a virus. PKR becomes phosphorylated, which then phosphorylates EIF2A. This phosphorylated EIF2A then inhibits cellular mRNA translation, and then prevents viral protein synthesis. Active PKR also induces NFkB by phosphorylating IkB, which upregulates Interferon beta. L929 cells utilize PKR as a predominant antiviral, IFN-mediated pathway when infected with HSV-1. (Harle, 2002).

Mechanisms HSV-1 has for evading Induction of Interferon Beta

HSV-1 inhibits the production of IFN-β by the immediate early viral protein ICP0 and ICP34.5. ICP0 and ICP34.5 inhibit the nuclear accumulation of IRF-3 and enhance the degradation of activated IRF-3

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which interferes with the pathway of IFN-beta production.(Melroe et al, 2004) and (Johnson et al, 2008).

HSV-1 blocks the signaling effects of type 1 interferons by reversing the effects of the double-stranded RNA-activated protein kinase (PKR). HSV-1 also blocks the phosphorylation of STAT1 and STAT2. (Melroe et al, 2004).

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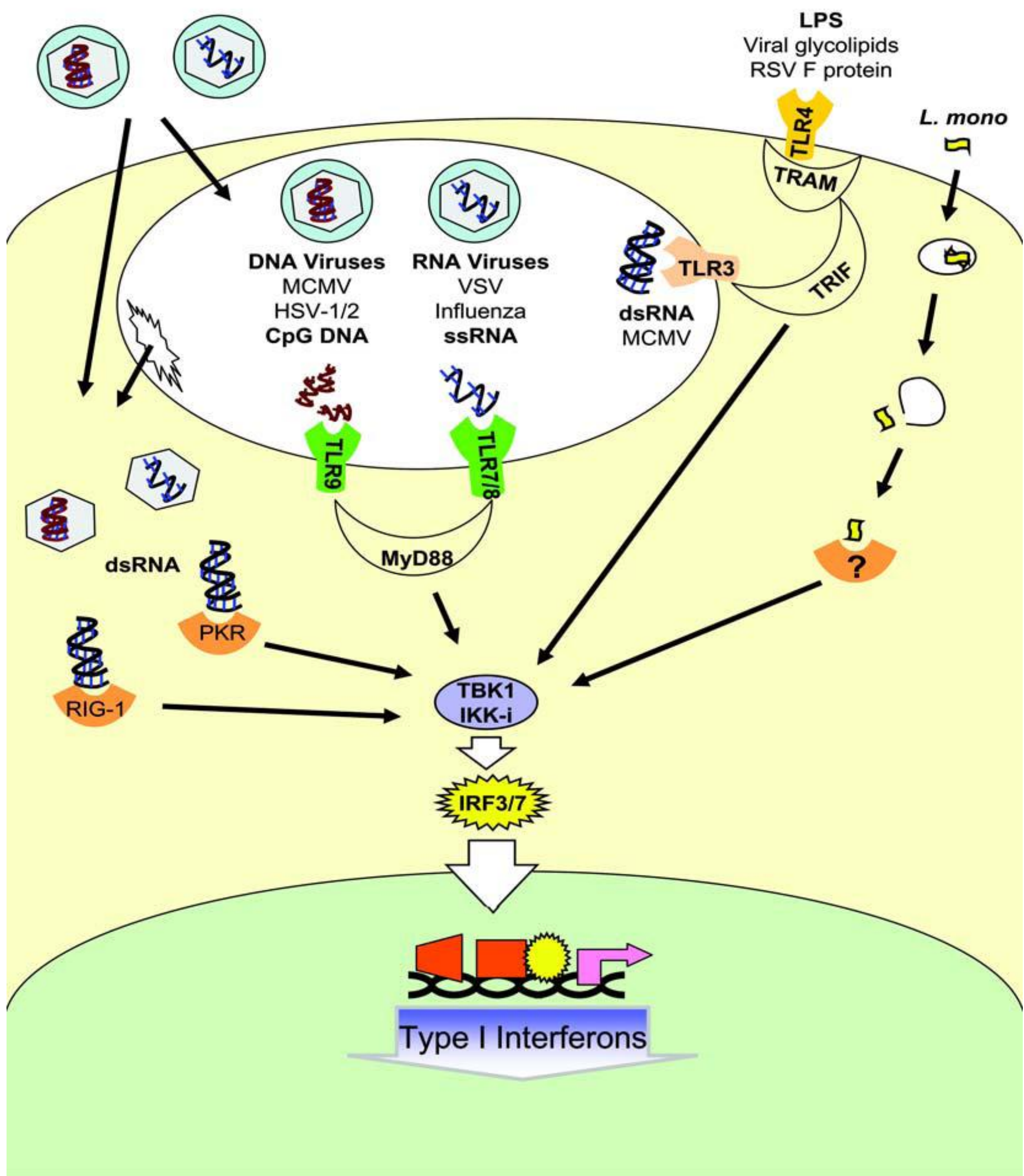

Figure 7. The different pathways that are involved in the activation of Type 1 interferon production. Adapted from Perry et al, 2005.

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MATERIALS AND METHODS

Cell Culture

L929 cells from C3H mice and A2R1 cells from Balb/C mice are fibroblasts that populate the dermal layer of the skin. These cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal calf serum, and 1% gentamicin solution (50mg/ml). The cells were grown in $100mm^2$ tissue culture dishes and incubated in a humidified incubator at a temperature of 37° C with 5% CO2.

Virus and Cell Infection

HSV-1 ($syn\text{-}17^+$) (initially provided by Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH) was titrated using Vero Cells (CCL-8, American Type Culture Collection). When the cytopathic effect was evident, the cells and supernatant were harvested and stored in 200ul aliquots at -80 degrees C. The number of plaque forming units (PFU) were calculated after performing a plaque assay on Vero cells. All four cells lines

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were plated either on sterile glass coverslips placed in 24 well plates or directly on 24 well culture plates, depending upon the experiment. The virus was dispensed at 0.1 MOI in DMEM containing 2% calf serum on the cell monolayer in the 24 well culture plates when they were about 80% confluent and then placed in an incubator at 37° C. The virus was left on the cells for 2 hours and then the culture medium was removed and replaced with fresh culture medium. Mock infected cells were treated the same way, except 2% culture medium was used. For the immunostaining, each cell line was dispensed on glass coverslips that were in 24 well plates and infected as previously described. The coverslips were removed at the 6 hour time point, which has been shown to be a significant time to see IFN-β production and immunostaining was performed (Rosztoczy et al, 1986); (Fujisawa et al, 1997); (Li et al, 2011a).

Immunostaining for IFN-β

At the end of the 6 hour time point, the cells were rinsed once with room temperature (RT) phosphate buffered saline (PBS) and then the PBS was removed. 10% Formalin fix which is used to preserve cell morphology was added and incubated at room temperature (RT) for fifteen minutes.

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The fix was removed and quenched with sodium borohydride that was prepared just prior to use in PBS. The cells were incubated in this solution for 15 minutes at RT. The cells were then washed 3 times with PBS. The cells were then permeabilized with 0.2% Triton-X 100 in PBS for 10 minutes at RT and then washed again in PBS three times. 3% BSA (Bovine Serum Albumin) blocking solution was added to the cells and incubated at RT for 30 minutes. The primary mouse IFN-β polyclonal antibody was prepared in the blocking buffer diluted 1:100 and then it was applied to each coverslip. The coverslips were placed in a humidified chamber cell side down and allowed to incubate at 4° C overnight. The coverslips were transferred back to into their dishes and washed three times with PBS. The secondary fluorescent-labeled (cyanine 5) anti-mouse IgG, was prepared and applied to each coverslip as described above and allowed to incubate for 2 hours at RT in the dark. The coverslips were placed back into their dishes and washed 3 times with PBS. The coverslips were then mounted onto glass slides (two on each slide) using liquid mounting media Vectashield (Vector Labs) and then sealed with clear nail varnish. The slides were then analyzed by light microscopy. Calibration bars were added to the pictures and were

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saved onto the picture by merging the calibration bar with the picture before saving them to the computer.

ELISA

Cells were plated onto 24 well culture dishes and infected with virus as described above. Supernatant was removed from separate wells at specified time points of 0, 6, 12 and 24 hours post infection and placed at -20° C until samples could be analyzed. Untreated cells were used as the negative control and cells treated with Poly-IC were used as the positive control. All four cell lines (Hel-30, PAM-212, L929, A2R1) were used in 3 separate experiments. The assay quantitates mouse interferon beta in media using a sandwich enzyme immunoassay. The microtiter plate is pre-coated with IFN-β capture monoclonal antibody to which the samples and standards are added. The supernatant was then prepared according to the manufacturers specifications for the ELISA (PBL Biomedical Laboratories, Piscataway, NJ). The substrate supplied with the kit was TMB which is a colorimetric substrate for horseradish peroxidase (HRP). The color intensity is proportional to the amount of HRP activity which is correlated to the amount of the target protein, in this case, IFN-β. The results were obtained

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by reading the plate on an ELISA Plate Reader at a wavelength of 450 nm. This ELISA kit has an assay range of 15.6 – 1000pg/ml.

Statistical Analysis

Statistical Analysis was performed using Sigma Plot 12.0. The values that are expressed are the Standard Error of the Mean (SEM). The Holm-Sidak method was used to determine the significance between the treated groups and the control groups.

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RESULTS AND DISCUSSION

Selection of the MOI for transfection of virus in the different cell lines

Different MOI's were used to determine the appropriate dilution to use on the cell lines. Cytopathic effects assays were used to determine the MOI (data not shown) 0.1 MOI was chosen because it had cytopathic effects on the cells. Data from Frey et al, 2009 were also used as reference and those results were similar as well.

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IFN-β localization in A2R1 fibroblast in response to HSV-1

A minimum of 100 cells per experiment was counted, all cells per field were counted with a minimum of 3 fields per experiment and observed for cytoplasmic fluorescence, nuclear fluorescence, or perinuclear fluorescence. At least three replicates of each experiment were performed.

The untreated A2R1 cells have nuclear localization of IFN-β. When infected with the virus, both perinuclear (PN) and cytoplasmic localizations of IFN-β in response to the virus was observed (figures 8 & 9). These results suggest that HSV-1 stimulates IFN-β expression/localization. This analysis supports Frey's findings that fibroblasts were better protected from HSV-1 than the keratinocytes following treatment with IFN-γ which synergizes with the innate IFN-β response. When treated with Poly IC, IFN-β becomes localized primarily in the cytoplasm compared with the nuclear localization of the untreated cells (figure 8C & 10). This observation indicates that A2R1 fibroblast cell line was capable of producing IFN-β.

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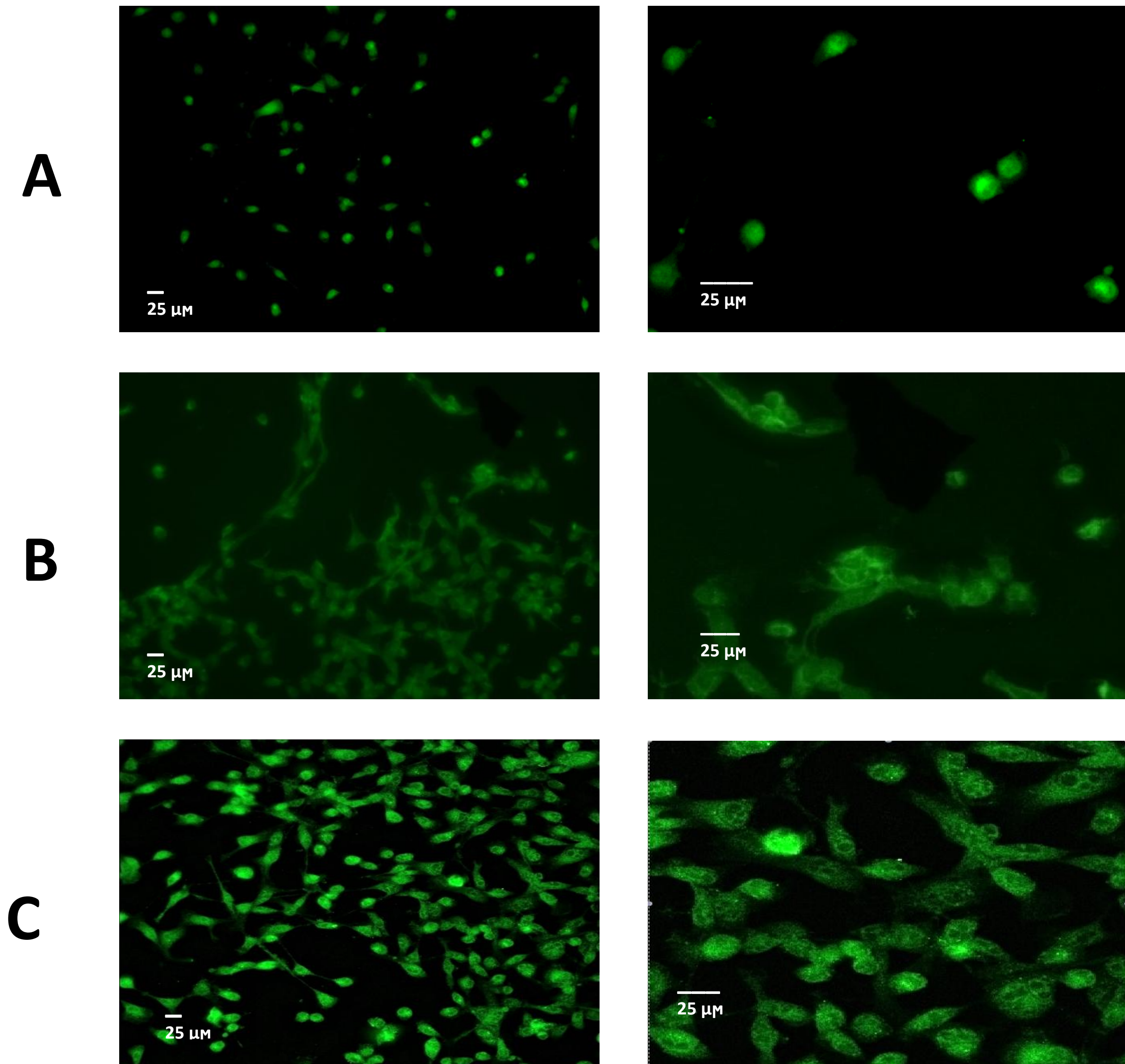

Figure 8. Fluorescent microscopy of A2R1 fibroblast cells presenting IFN-β localization. a. Untreated A2R1 fibroblast with nuclear localization of IFN-β. b. Virus infected A2R1 fibroblast with perinuclear localization of IFN-β, 6 hr PI. c. Poly IC treated A2R1 fibroblast with cytoplasmic localization of IFN-β, 6 hr PI.

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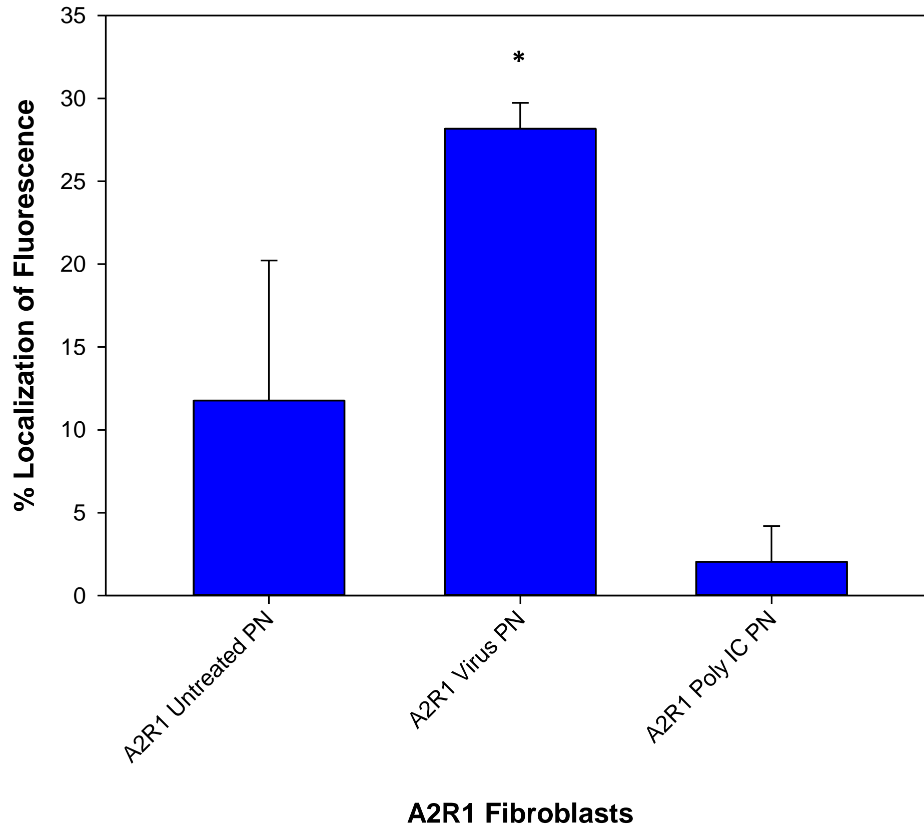

Figure 9. Percent localization of fluorescence of interferon beta in the perinuclear region of A2R1 fibroblasts. The untreated cells were used as a control. In The virus infected cells, the localization was measured 6 hours after infection with HSV-1. Difference from control: *p=0.042.

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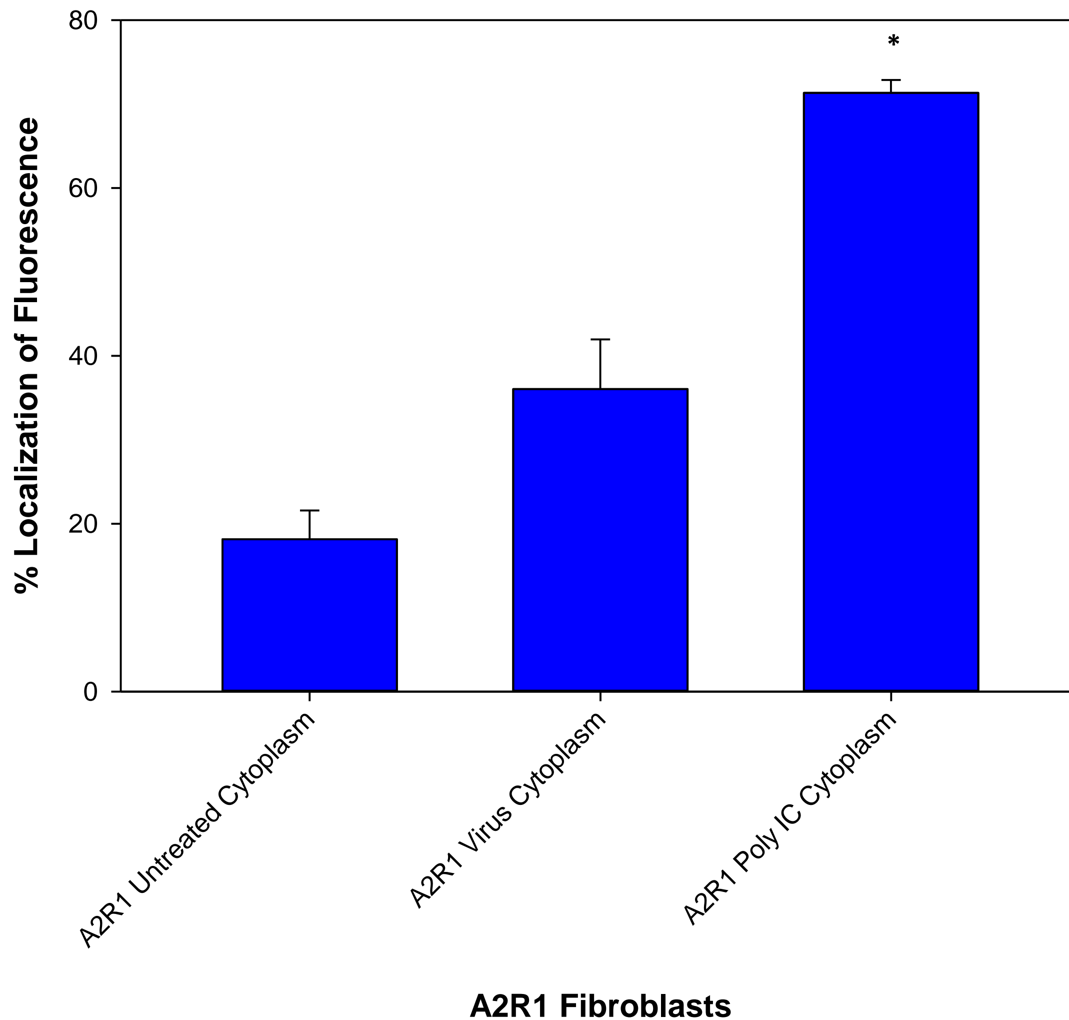

Figure 10. Percent Localization of fluorescence of Interferon Beta in the cytoplasm of A2R1 Fibroblasts.

The Untreated cells were used as control. In the virus infected cells, the localization was measured 6 hours after infection with HSV-1. Difference from controls: *P<0.001.

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IFN-β localization in L929 fibroblasts in response to HSV-1

After six hours post infection (PI) there was nuclear localization of IFN-β which was the same as the untreated cells which also showed nuclear localization of IFN-β. However, upon stimulation with Poly IC, the L929 cells displayed significant levels of cytoplasmic fluorescence compared with the untreated cells (figure 11A, 11C & 12); like the A2R1 fibroblasts, this staining pattern shows that the L929 cells are capable of producing IFN-β.

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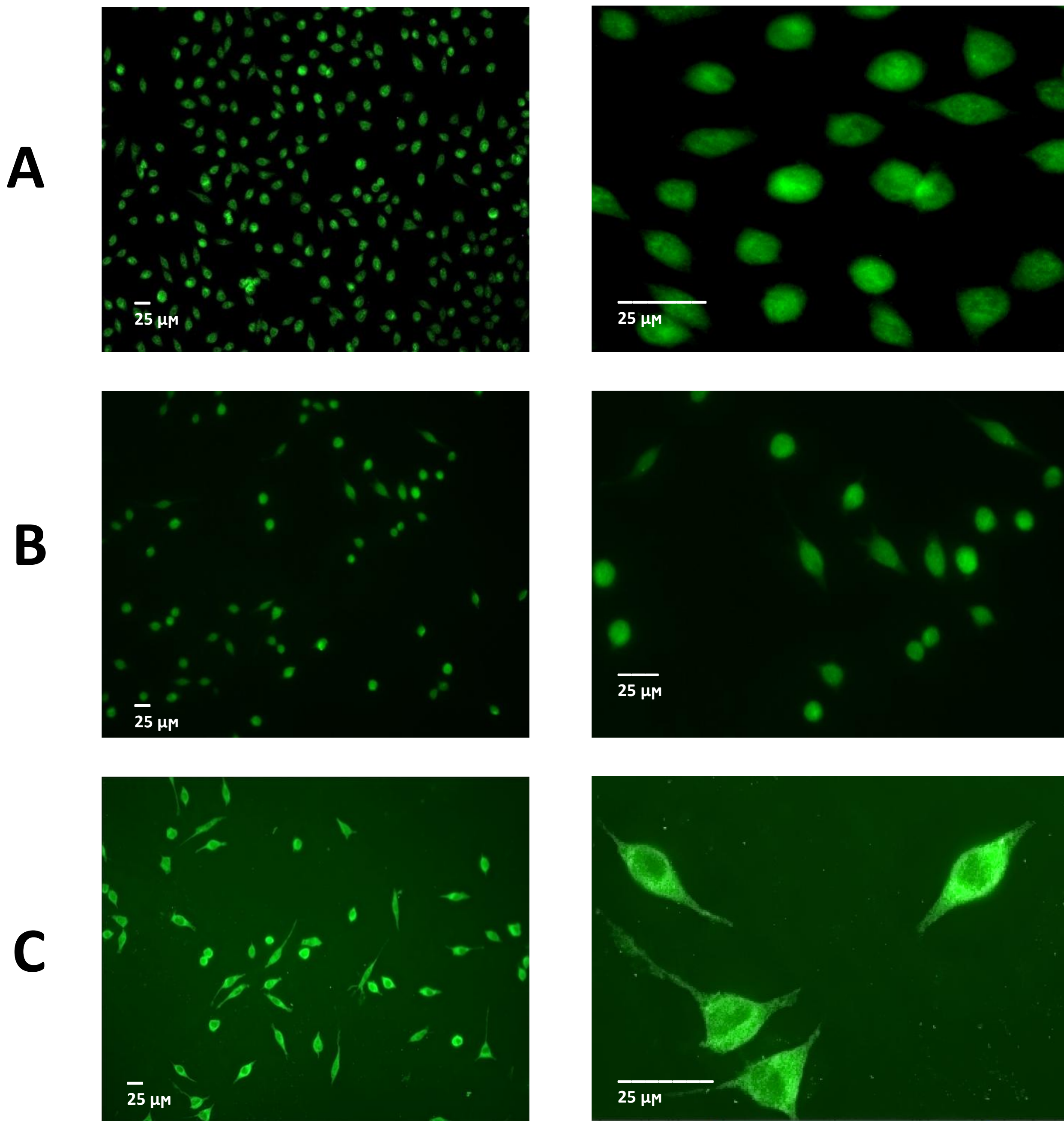

Figure 11. Fluorescent microscopy of L929 fibroblast cells presenting IFN-β localization. a. Untreated L929 fibroblast with nuclear localization of IFN-β. b. Virus infected L929 fibroblast with nuclear localization of IFN-β, 6 hr PI. c. Poly IC treated L929 fibroblast with cytoplasmic localization of IFN-β, 6 hr PI.

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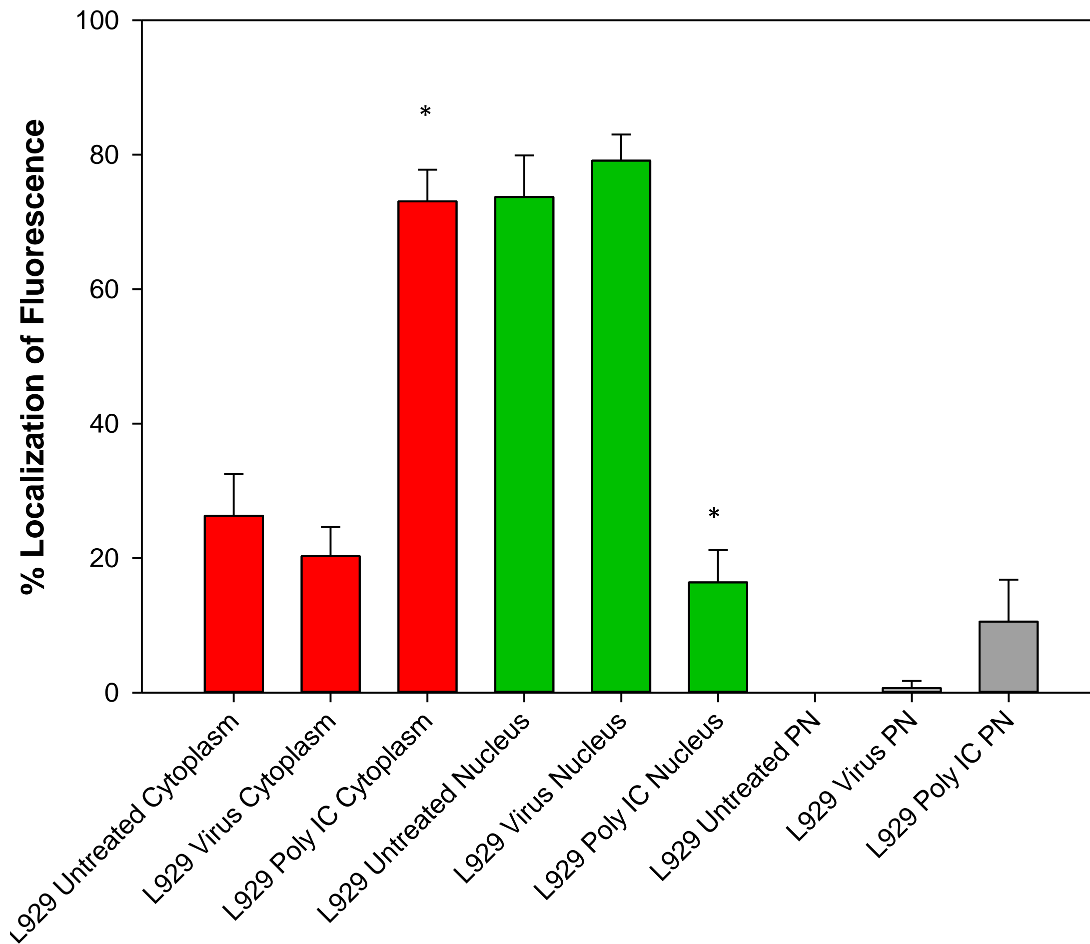

Figure 12. Percent localization of fluorescence of IFN-β 6 in L929 fibroblasts. The Untreated cells were used as control. In the virus infected cells, the localization was measured 6 hours after infection with HSV-1. Difference from controls: *P<0.001.

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Comparison of IFN-β localization in A2R1 fibroblast with L929 fibroblast in response to HSV-1

Following infection when infected with virus, the A2R1 showed perinuclear localization while the L929 had nuclear localization (figures 13 & 14). Since L929 cells utilize the PKR as a predominant antiviral pathway (Harle et al, 2002) and HSV-1 disrupts this pathway by dephosphorylating PKR (Harle et al, 2002), IFN-β production and secretion is inhibited in the virus-infected L929 fibroblast.

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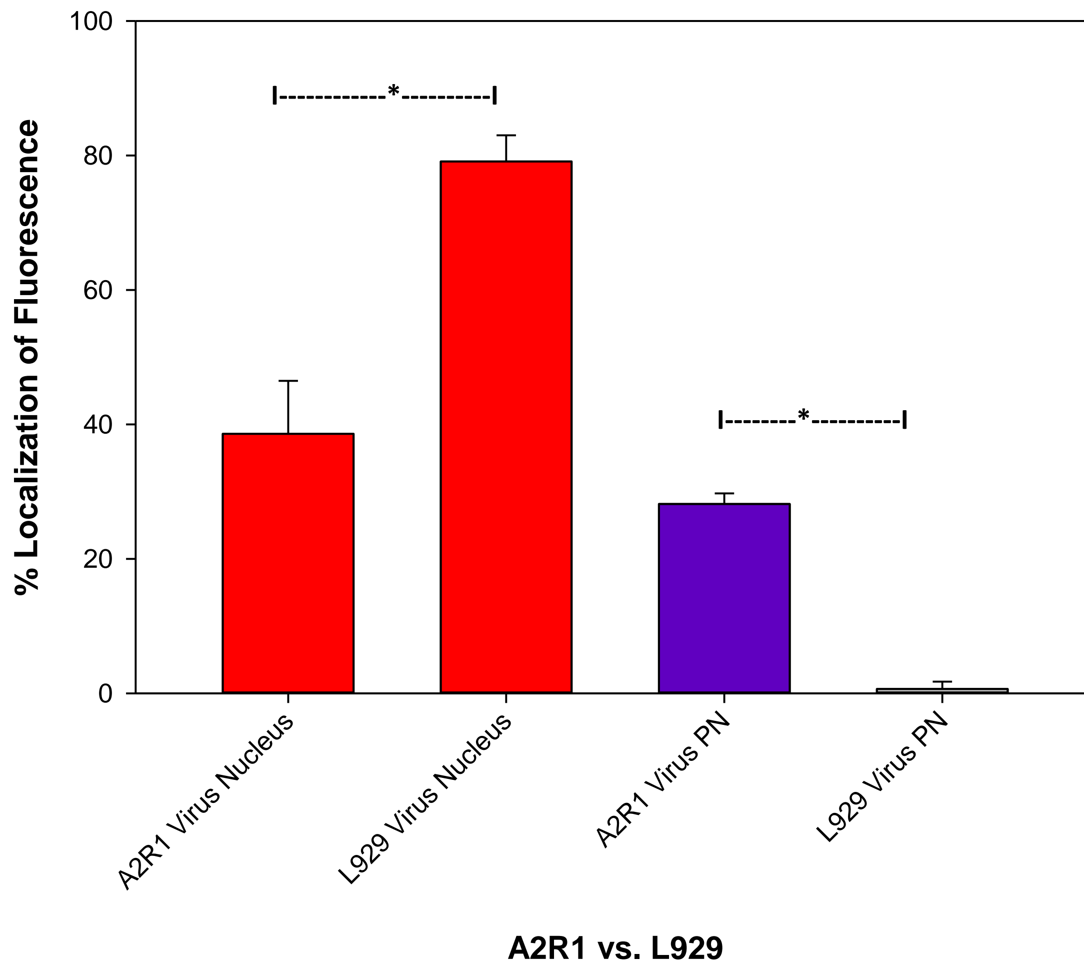

Figure 13. Percent localization of fluorescence of interferon beta in the nucleus and perinucleus (PN) of virus infected A2R1 and L929 fibroblast cell lines. The difference in % localization is a comparison between the two cell lines: *P=<0.001.

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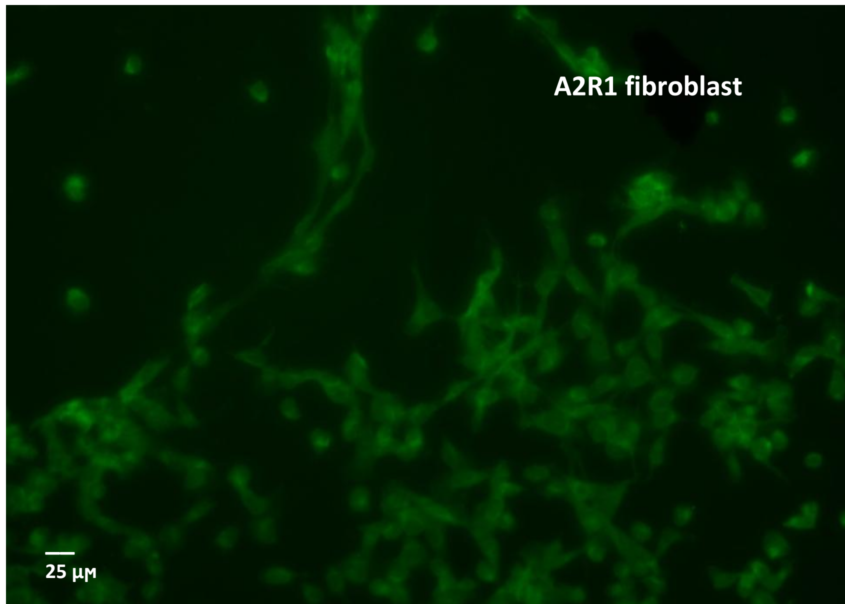

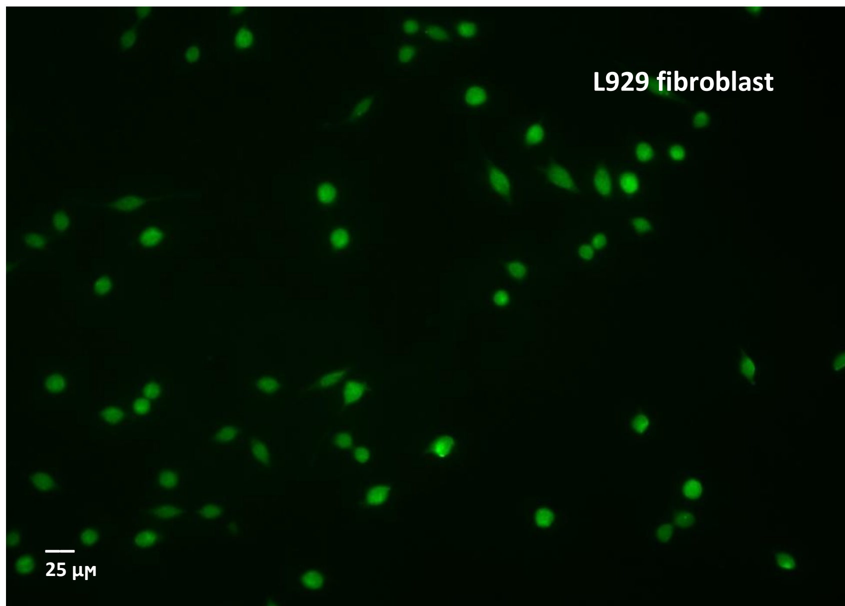

Figure 14. Comparison of A2R1 fibroblast with perinuclear localization and L929 fibroblast with nuclear localization at 6 hours PI.

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Comparing mean fluorescence between A2R1 fibroblast the L929 fibroblast

The mean fluorescence intensity was measured using Image J Fire Program to quantify IFN-β expression. At least three fields per group and a minimum of three experiments performed. The same fields were assessed using Image J that were used in determining the localization of IFN-β. The pixel intensity was measured and the mean fluorescent intensity was calculated using this program.

When the mean fluorescence was measured, there was a significant difference in the L929 cells treated with poly IC compared with the untreated L929 cells (figures 15 & 16). However, in the A2R1 fibroblast cells, the mean fluorescence was about the same as the untreated A2R1 (figures 15 & 16).

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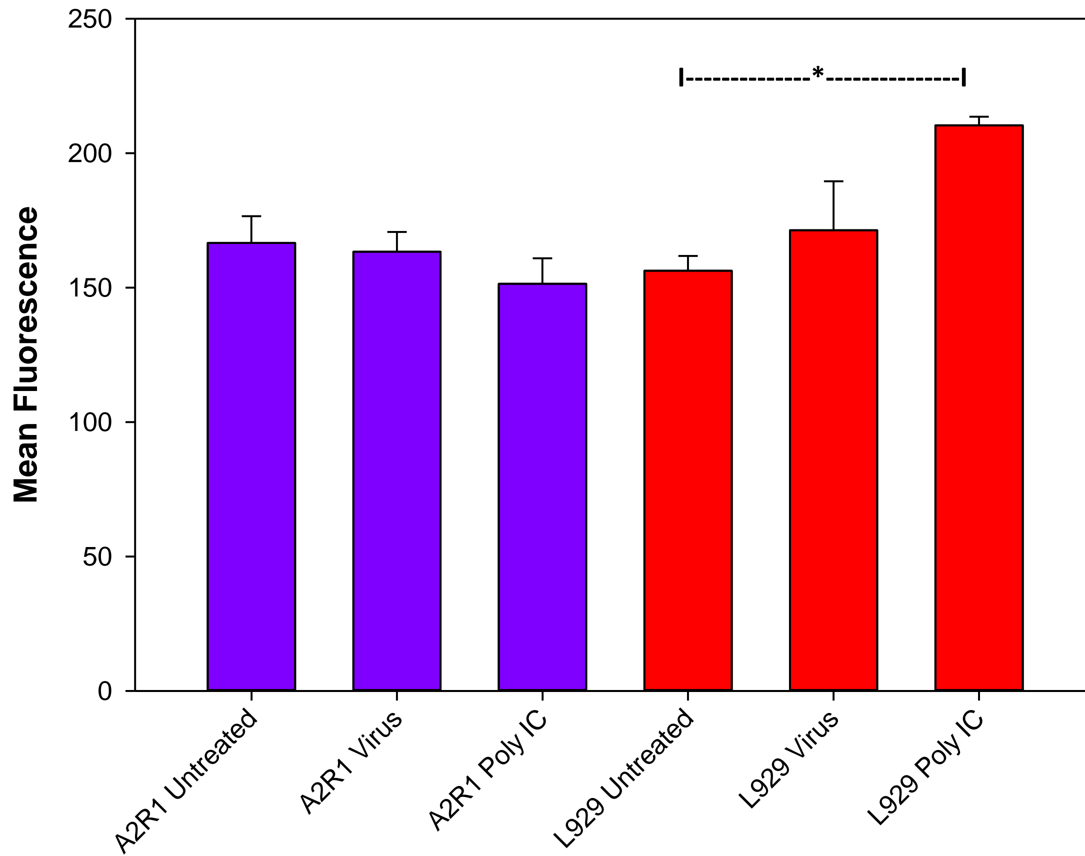

Figure 15. Poly IC treated L929 has significantly more mean fluorescence than the L929 untreated cell. There is no significant difference in the mean fluorescence of the A2R1 fibroblast cell line. Difference from untreated: *p=0.036.

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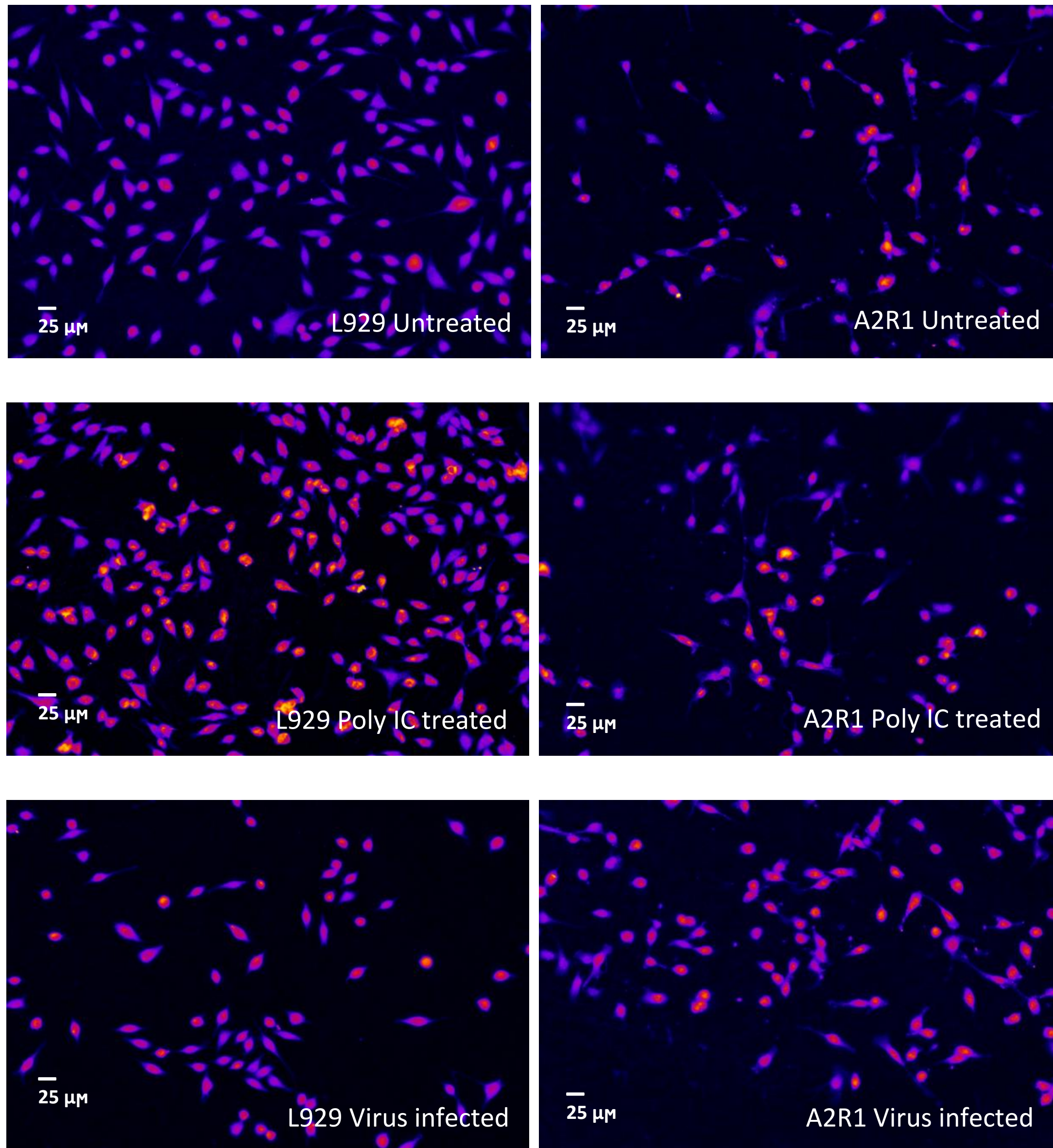

Figure 16. Mean Fluorescent Intensity using image J Fire program. L929 and A2R1 fibroblasts untreated, Poly IC treated and virus infected. Note the difference in the untreated L929 cells with the Poly IC treated L929 cells. There is no significant difference between the virus infected cells and the untreated cells.

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ELISA

I attempted to measure IFN-beta in the culture supernatants from the infected cells from all four cell lines at different time points: 0, 6, 12 and 24 hours, using a Mouse Interferon Beta ELISA kit (range 15.6 – 1000pg/ml), but could not detect any IFN-β within this range. A detectable, but insignificant amount was released by the L929 cells stimulated with Poly IC at 24 hours. The control standards that were supplied with the kit gave the expected results. The samples were stored at -80°C with no protease inhibitors added.

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CONCLUSION AND FUTURE STUDIES

HSV-1 has evolved several successful strategies to overcome the antiviral state produced by the cell upon infection. A difference in localization of IFN-β was noted between the A2R1 fibroblast and the L929 fibroblast at 6 hours after infection with HSV-1. A2R1 cells showed perinuclear fluorescence while the L929 fibroblasts exhibited nuclear localization. HSV-1 infected L929 cells appeared unresponsive to IFN-β production in comparison with uninfected L929 cells which also showed nuclear localization. It is difficult to interpret the nuclear staining for IFN-β in the infected cells. Since the culture medium contained 10% calf serum, mitogens in the serum could have stimulated expression of IFN-β. Culturing the fibroblast cell lines in serum-free medium would address this concern. Removing serum-induced mitogenicity should reduce or eliminate IFN-β staining at six hours after infection if the staining was due to serum mitogens.

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Another aspect of the nuclear staining pattern to be investigated involves the observation of Kushnaryov et al. (1985 and 1986) that the nuclear envelope contains a denser distribution as well as higher affinity receptors for IFN-β than does the plasma membrane of murine fibroblasts. If the nuclear envelope of mitogen-stimulated fibroblasts were saturated with IFN-β-receptor interactions, the entire nucleus would appear fluorescent when the whole cell is observed microscopically. Using confocal microscopy to optically section the cell should differentiate between staining of the nuclear envelope and the nucleoplasm. In addition, results of confocal co-localization experiments using different fluorescent tags for antibodies to nuclear pore proteins and IFN-β would indicate whether IFN-β was exiting the nucleus or being synthesized at the nuclear envelope.

Upon stimulation with Poly IC, both fibroblast cell lines display cytoplasmic fluorescent localization, both of which are significant when compared to the untreated cells. Perinuclear staining for IFN-β would suggest successful egress or synthesis and cytoplasmic staining of IFN-β, as seen in the poly-IC-stimulated cells. This would indicate successful synthesis of IFN-β and accumulation in the cytoplasm.

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The use of phase-contrast microscopy in conjunction with fluorescent microscopy would show differences in the numbers of intact cells stained as opposed to unstained cells. For example, in the Image J Fire analysis many fewer cells showed fluorescent staining in the virus-infected samples at six hours after infection, a time at which viral lysis would not have occurred. The use of phase-contrast microscopy would determine whether some of the cells had not stained positive for IFN-β.

Frey (2009) noted that L929 fibroblasts were better protected from HSV-1 than keratinocytes following treatment with IFN-γ and suggested that the IFN-γ synergizes with the innate IFN-β response. He also noted that IFN-β mRNA transcripts were not enhanced in HSV-1-infected L929 cells over those seen in uninfected cells (Frey, 2009). In a future study, it would be of interest to determine whether IFN-γ-treated L929 fibroblasts are able to produce both IFN-β transcripts (as detected by PCR) and protein (as detected by perinuclear and cytoplasmic localization) as well as secreted IFN-β (as detected in an ELISA assay). The synergistic or additive anti-viral relationship between IFN-γ and IFN-β in eliciting a greater IFN-β response in the A2R1 fibroblast can be measured in a virus plaque assay.

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Greater cell survival and fewer virus plaques should be detected in HSV-1 infected cells pretreated with both IFN-β and IFN-γ than with either IFN.

Examining the IFN-β response in IFN-γ-treated keratinocyte cell lines would also be of interest. Frey et al (2009) showed that keratinocytes (HEL-30) were refractory to IFN-γ induction of an antiviral state upon HSV-1 infection. In contrast, IFN-γ induced an antiviral state in fibroblasts (L929). This effect in keratinocytes was attributed to the production of suppressor of cytokine signaling-1 (SOCS-1) which played an important role in the inhibition of the antiviral effect of IFN-γ in HSV-1-infected keratinocytes. Both IFN-γ and HSV-1 were found to upregulate production of SOCS-1 in keratinocytes. By comparison, HSV-1 infection exerted minimal to no effect on induction of SOCS-1 protein in L929 fibroblasts (Frey et al, 2009). It would be of interest, in future experiments, to determine whether an inhibitor of SOCS-1 would permit the keratinocyte to display an IFN-β response and protect the cells from lytic HSV-1 infection similar to that seen by Frey et al (2009). Ahmed et al (2010) has recently found that SOCS-1 inhibitors enhanced the IFN-β levels of L929 fibroblasts following infection with vaccinia virus or encephalomyocarditis virus . Keratinocytes treated with an inhibitor of SOCS-1 could be protected by IFN-γ from HSV-1

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lytic infection (Frey et al, 2009). It might also be worthy to investigate the role of IFN-α similar to that of IFN-β in these four cell lines using fluorescently labeled anti-murine IFN-α antibody in immune localization experiments.

Enhancement of the detection of secreted IFN-β by ELISA could be improved by: (1) concentrating the tissue culture supernatant fluids before testing; (2) inclusion of a protease inhibitor in the collected supernatant fluids to prevent proteolysis of any interferon in the sample;(3) examining treated cells at an earlier times, e.g. 2 and 4 hours post-treatment or post-infection; and (4) selection of less dilute monoclonal antibody pairs in the ELISA assays. Ahmed et al (2010) recently noted that L929 fibroblasts treated with an inhibitor of SOCS-1 protein for 30 and 60 minutes exhibited increased basal levels of IFN-β which were below 10 pg/ml as measured by ELISA. Obviously, the ELISA kit used in this study was not sensitive enough to detect the basal IFN-β levels seen in the immunofluorescence experiments.

In the innate immune responses to HSV-1 infection, Lopez (1975) assumed that difference in type I interferon responses (IFN-α/β) augmented by type II IFN-γ produced by NK cells in initial infection

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accounted for the differences seen in response by several mouse strains to intraperitoneal infection with HSV-1. Others have not confirmed these observations. More recently, Halford and colleagues (2004) re-evaluated the natural resistance to HSV-1 by some of the same mouse strains and noted that the individual murine species do not appear to differ in their innate resistance to HSV-1 infection when considering differences in virus strains, transmission sites, and conditions. Consequently, little can be attributed to the differences seen in this study in the IFN-β response to HSV-1 infection by A2R1 (Balb/c) and L929 (C3H) fibroblasts. The early interferon responses can only account for host resistance to virus for the first five to seven days of infection after which the immune response contributes to viral pathogenesis and resistance (Mercalf and Michaelis, 1984).

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