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IFN-β Provides Immuno-Protection in the Retina by Inhibiting ICAM-1 and CXCL9 in Retinal Pigment Epithelial Cells

John J. Hooks, Chandrasekhararam N. Nagineni, Laura C. Hooper, Kozaburo Hayashi, and Barbara Detrick

The retinal pigment epithelial (RPE) cell is a potent regulatory cell that facilitates normal physiologic processes and plays a critical role in a variety of retinal diseases. We evaluated IFN-β production in human RPE cells through TLR signaling and investigated the effects of IFN-β on RPE cells. RPE cells treated with poly(I:C) or infected with an RNA virus produce IFN-β. Kinetic studies revealed that IFN-β levels continue to increase over a 48-h period and this was associated with the up-regulation of IRF-7 gene expression, a known positive feedback molecule for IFN-β production. Microarray analysis revealed that in IFN-β treated cells, 480 genes of 22,283 genes were up or down-regulated by >2-fold. We hypothesize that IFN-β induction during TLR signaling in the retina is an immunosuppressive factor produced to limit immunopathologic damage. Cytokine activation of RPE cells results in the production of the chemokines, CXCL9 and CXCL10, and the adhesion molecule, ICAM-1. Pretreatment of RPE cells with IFN-β resulted in inhibition of ICAM-1 production and elimination of CXCL9 production. This treatment did not alter CXCL10 production. Anti-IFN-β Ab blocked the inhibitory action of IFN-β. Real time PCR analysis revealed that IFN-β treatment inhibited gene expression of sICAM-1 and CXCL9. The results indicate a critical role for RPE cell derived IFN-β in the down-regulation of CXCL9 and ICAM-1 expression in the retina and suggest that the inhibition of CXCL9 is an immune-suppressive mechanism that protects the retina from excessive inflammation. The Journal of Immunology, 2008, 180: 3789–3796.

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3 Abbreviations used in this paper: RPE, retinal pigment epithelium; VSV, Vesicular stomatitis virus; SFM, serum free medium; EIA, enzyme immunoassay; IRF, IFN regulatory factors; MS, multiple sclerosis.
expressed in RPE cells is IFN-β. Induction of Type 1 IFN gene expression is an essential component of innate immunity (22, 23). Recent studies (24) have also shown that neurons produce high levels of IFN-β through TLR 3 signaling.

TLR signaling provides a rapid, robust, burst of reactivity designed to limit pathogens at the site of infection (21, 23, 24). This burst of reactivity is highlighted by the release of cytokines, chemokines, and adhesion molecules. Within the retina an uncontrolled inflammatory burst can itself lead to cellular damage. Therefore, it is highly probable that a down-regulatory force is also produced to limit immunopathologic damage. We hypothesize that IFN-β produced by RPE cells primarily through TLR signaling and secondarily by autostimulation is a critical component of that limiting force. The general aim of this study is to identify processes by which IFN-β is produced and to characterize mechanisms by which IFN-β can protect the retina through its antiviral actions and immunosuppressive actions. In this study we demonstrate that IFN-β is produced by RPE cells in response to poly(LC) and a RNA virus. The RPE cell can continue to produce IFN-β through a positive feedback system via IRF-7 production. The IFN-β can act as a potent immunosuppressive agent by down-regulating CXCL9 and ICAM-1 gene expression and protein production.

Materials and Methods

Cells

Primary cultures of human RPE cells were prepared from two donor eyes as described earlier (RPE no. 1 and RPE no. 3) and grown in MEM supplemented with 10% FBS, nonessential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (25 ng/ml) (25). In an immunostaining study, all of the cells reacted positively with mAb to cytokeratin indicating that RPE cultures are free of any contamination by other cell types such as fibroblasts, endothelial, and muscle cells present in the retina and the choroid. Moreover, the cells react with monoclonal anti-RPE cell (anti-RPE-65) Ab (6, 26). Stock preparations of RPE cell cultures were frozen and passages 6–12 were used in this study.

Cell stimulation assays

RPE cells were allowed to grow to confluence in 24-well or 6-well plates. On the day of treatment, cells were refed with the appropriate serum-free medium containing 0.1% BSA (Sigma-Aldrich), nonessential amino acids and antibiotic/antimycotic. Cells were then stimulated with either: a cytokine, containing 10 ng/ml TNF-α, 100 U/ml IFN-γ, and 10 ng/ml IL-1β (all from R&D Systems); 100 µg/ml poly(LC) (Sigma-Aldrich) or 10 µg/ml LPS (Sigma-Aldrich). For IFN-β inhibition assays, cells were pretreated with either 1000 or 3000 U/ml IFN-β (BioSource International) for 18 h at 37°C followed by treatment with cytokinin or combinations of specific cytokines. For the Ab inhibition assays, cells were pretreated with 10 µg/ml anti-IFN-β (Santa Cruz Biotechnology) for 1 h at 37°C followed by stimulation with cytokinin for 24 h.

Viral infections

Vesicular stomatitis virus (VSV) Nancy strain and herpes simplex virus (HSV-1) McKrae strain were used for viral infections. Human RPE cultures were grown to confluence in 24-well plates. Cultures were washed with serum free medium (SMF) and left in SMF for 2 h. Then medium was removed and 0.5 ml of virus (multiplicity of infection of ~1.0) was added. After incubation for 2 h at 37°C with occasional shaking, inocula were removed, cells washed with SFM twice. Then 1 ml of SFM was added to each well. Supernatant fluids were removed after incubation of 24 h and the levels of IFN-β were determined by enzyme immunoassay (EIA).

Enzyme immunoassay

The levels of sICAM-1, CXCL9, and CXCL10 were determined using commercially available EIA kits (R&D Systems). EIA was also used to measure IFN-α (PBL Biomedical) and IFN-β (Fujirebio). All samples were tested in duplicate according to the manufacturer’s instructions. The OD of each sample was determined using VERSAmax tunable microplate reader (Molecular Devices). Results were calculated from a standard curve and reported accordingly in picograms, nanograms, or units per milliliter. The minimum detectable dose for each cytokine measured is as follows: sICAM-1 is 0.35 ng/ml, CXCL9 is 3.84 ng/ml, CXCL10 is 1.67 pg/ml. The minimal detectable dose was not established for the IFN-α and IFN-β kits. The value for the lowest standard was: 10 pg/ml for IFN-α, and 2.5 IU/ml for IFN-β. Significance was determined using a two-tailed Student’s t test. Values of p < 0.05 were considered significant.

Microarray analysis of effects of IFN-β on gene expression by RPE cells

Confluent cultures of RPE cells were treated with IFN-β (3000 U/ml) for 8 h in serum free medium. Total RNA prepared was quantitated, and purity and integrity verified by gel electrophoresis. Affymetrix GeneChip arrays (HG U133 plus 2.0) were used. The preparation cRNA, hybridization and posthybridization treatments of microarrays were performed according to the detailed protocol provided by Affymetrix. After hybridization, GeneChip array was washed, stained with streptavidin-PE (Molecular Probes), amplified with biotinylated anti-streptavidin Ab and scanned with an argon ion Confocal Laser at 570 nm (Affymetrix). Affymetrix GeneChip Operating software was used for absolute expression and to normalize the gene expression levels between any two samples. Normalization, filtering, and clustering analysis of the data were performed with the GeneSpring software (Silicon Genetics).

Reverse-transcriptase PCR

Total RNA was isolated from each well of a 6 well plate using RNA Stat and 0.25 µg of this was reverse transcribed to cDNA followed by amplification using a GeneAmp RT-PCR kit (Applied Biosynthesis). The following primers were made by Bio-Synthesis: GAPDH (600 bp) forward, 5’–CCA CCC ATT GCA AAT TAC ATG GCA–3’ and reverse, 5’–TCT ACA AGA CTT CCA AGA ATG TTG AC–3’. For 5’–CCA GCC ACA AGT ACT GTA CTC C–3’ TRL 3 (304 bp) forward, 5’–GAT CGT TCT CAT AAT GGC TTG–3’ and reverse, 5’–GAC GAG TTC CGA ATG CCT GTT–3’. MxA (500 bp) forward, 5’–CAC TGG TAC AGC CCG CTG TGG–3’ and reverse, 5’–CAG GAG CCA GCT GTA GGT GTC C–3’. Following amplification, 10 µl of PCR products were electrophoresed in a 4% agarose gel (BioWhittaker) and hybridized to the following probes labeled with a oligonucleotide tailing kit (Roche): GAPDH, 5’–CTC CTG CAC CAC CAA CTG C; IFN-β, 5’–CTA TTG TTG AGA ACC TCC TGG–3’; IRF-3 (65 bp) forward, 5’–TACCAAGGCCCTGAGGCAC–3’ and reverse, 5’–AGAG GTT ATC TGG AA–3’.

Evaluation of gene expression by real time PCR

Confluent monolayers of RPE cells in 6-well plates were treated with medium alone, with poly(LC) or with cytomix. After 4- or 24-h incubation at 37°C, total cellular RNA was extracted using RNA STAT-60 extraction solution (Tel-Test) according to the manufacturer’s instructions. A total of 2 µg of RNA were reverse transcribed to make cDNAs using TaqMan reverse transcription reagents (Applied Biosystems). Production of cDNA from individual RNA was confirmed by detection of the housekeeping gene, GAPDH, in a 4% agarose gel by electrophoresis after amplification by 35 cycles by conventional RT-PCR. The synthesized cDNA was amplified 40 cycles on an ABI 7700 prism to quantitate expression of IRF-3, Mx, and reverse, 5’–GCA TGG CAG AAG GTC GAG AAG–3’. The density of each band was analyzed on a Macintosh computer using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/NIH-image/).

The following primers were used: GAPDH (209 bp) forward, 5’–AGCT GAACC GGAGA CAGCT CACTG TGG–3’ and reverse, 5’–GGATGG TCTGCTG CTGTGAA GATCC C–3’. IRF-3 (433 bp) forward, 5’–ACCAGG TGGGAGCT TGGTGA GATCC C–3’ and reverse, 5’–TAC CAGGC TTC GAG GAC GCC GCA–3’. IFR-7 (134 bp) forward, 5’–TACCA AGG CGG ACC GGT TCT–3’ and reverse, 5’–CCA AGT CTC CTG TGG–3’.

The primers for CXCL9 were SuperArray PPH00700A-200 obtained from SupperArray.

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LPS (10 g/ml) infected cells did not produce IFN-β expression. To determine whether IFN-β expression was represented as a ratio to the housekeeping gene GAPDH. In this study, we set out to demonstrate that a virus infection could up-regulate IFN-β production in human RPE cells. RPE cells were infected with VSV or HSV-1. Cells were incubated at 37°C for 24 h and supernatant fluids were harvested and assayed for IFN-β by EIA. Results of four separate experiments with duplicate samples have shown that RPE cells infected with VSV produced 63.0 ± 7.6 U of IFN-β. In contrast, HSV-1 infected and mock infected cells did not produce IFN-β. In addition, these RPE cells were treated with poly(I:C) (100 μg/ml), poly d(I:C) (10 μg/ml), or medium. IFN-β was detected in poly(I:C) treated cells and was not detected in cells treated with poly d(I:C) (control for poly(I:C)), or LPS. These studies demonstrate that in addition to poly(I:C) treatment, RPE cells can respond to an ongoing virus infection by producing IFN-β.

RT-PCR assays were performed to evaluate TLR 3 and IFN-β gene expression following poly(I:C) treatment. RPE cells were treated with medium alone or with poly(I:C). Following 24 h incubation period, mRNA was harvested and RT-PCR was performed. As is seen in Fig. 1, there is a significant increase in TLR 3 gene expression, 2.9-fold and a 3.7-fold increase in IFN-β gene expression. To determine whether IFN-β produced by RPE cells is biologically active on these cells, we next used RT-PCR to evaluate MxA gene expression. The MxA gene is specifically up-regulated by type 1 IFN signaling (11.4-fold).

**Results**

**Induction of IFN-β production in RPE cells**

In this study, we set out to demonstrate that a virus infection could up-regulate IFN-β production in human RPE cells. RPE cells were infected with VSV or HSV-1. Cells were incubated at 37°C for 24 h and supernatant fluids were harvested and assayed for IFN-β by EIA. Results of four separate experiments with duplicate samples have shown that RPE cells infected with VSV produced 63.0 ± 7.6 U of IFN-β. In contrast, HSV-1 infected and mock infected cells did not produce IFN-β. In addition, these RPE cells were treated with poly(I:C) (100 μg/ml), poly d(I:C) (10 μg/ml), LPS (10 μg/ml), or medium. IFN-β was detected in poly(I:C) treated cells and was not detected in cells treated with poly d(I:C) (control for poly(I:C)), or LPS. These studies demonstrate that in addition to poly(I:C) treatment, RPE cells can respond to an ongoing virus infection by producing IFN-β.

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**Kinetics of IFN-β production**

We have previously reported that IFN-β was secreted by RPE cells stimulated with poly(I:C) (21). We next evaluated the kinetics of IFN-β production. RPE cells were treated with poly(I:C), LPS, cytokine mix (TNF-α, IL-1β, and IFN-γ), or medium and incubated at 37°C. At 1, 4, 24, and 48 h, supernatant fluids were harvested and assayed by EIA for IFN-β. Poly(I:C) treatment resulted in an increasing production of IFN-β (Fig. 2). In contrast, the other stimulants did not result in IFN-β production. Moreover, IFN-β was not detected in cells treated with Poly d(I:C) (dsDNA), a negative control for poly(I:C) (dsRNA) (data not shown). However, these stimulants (LPS, cytokine mix) capable of inducing sICAM-1 IL-8, MCP-1, MCP-3, RANTES, and TGF-β production in RPE cells (17, 21, 25).

The IFN regulatory factors (IRF), IRF-3 and IRF-7 regulate type 1 IFN induction (27). Moreover, several studies indicate that IRF-7 plays a major role in IFN-β induction and provides a positive feedback for the production of more IFN-β (28, 29). Because IFN-β was continuously up-regulated over the 48 h period, we next wanted to evaluate IRF expression. RPE cells were treated with medium or poly(I:C) and at 4 and 24 h mRNA was harvested and analyzed by real time RT-PCR. As is seen in Fig. 3, at 4 and

**FIGURE 1.** RT-PCR analysis of gene expression in RPE cells following poly(I:C) treatment (A). RPE cells were treated with poly(I:C) (100 μg/ml) or medium alone. After 24-h incubation, cellular RNA was isolated and evaluated for gene expression of GAPDH, IFN-β, TLR, and MxA. B. Densitometry of blots for IFN-β, TLR, and MxA was performed using National Institutes of Health image and expression was represented as a ratio to the housekeeping gene GAPDH.

**FIGURE 2.** Kinetics of IFN-β Production in RPE Cells. RPE cells were treated with medium alone, medium containing poly(I:C) (100 μg/ml), LPS (10 μg/ml), or a cytokine mix consisting of TNF-α (10 ng/ml), IFN-γ (100 U/ml), and IL-1β (10 ng/ml). At 1, 4, 24, and 48 h posttreatment, supernatant fluids were collected and measured for the presence of IFN-β by EIA. The results are derived from one of two representative experiments performed with triplicate samples.

**FIGURE 3.** Real Time PCR analysis of gene expression of IRF-3, IRF-7 in poly(I:C) treated RPE cells. RPE cells were treated with poly I:C (100 μg/ml) or medium alone. After 4- and 24-h incubation RNA was isolated and evaluated for gene expression of IRF-3 and IRF-7. Fold differences compared with medium alone are depicted after normalization with the housekeeping gene GAPDH. The results are derived from one of two representative experiment with triplicate samples.
24 h, poly(I:C) treatment did not up-regulate IRF-3 gene expression. In contrast, IRF-7 gene expression was enhanced 5-fold at 4 h and this was dramatically increased to 50-fold at 24 h. These studies demonstrate that poly(I:C) treatment of RPE cells results in an increasing up-regulation of IRF-7. This may provide a positive feedback message to the cells to continue to produce higher levels of IFN-β.

**Effect of IFN-β on RPE cell gene expression**

To understand the potent modulatory effects of IFN-β on RPE, we used GeneChip Human Genome U133 plus 2.0 (Affymetrix) for the analysis of genome-wide expression. Two RPE cell cultures (RPE no. 1 and RPE no. 3) were treated with IFN-β (3,000 U/ml) for 8 h in serum free medium. Total RNA was isolated from control (untreated) and IFN-β-treated cultures. Genomic expression profiling was used to identify modulated genes by microarray analysis. Initial analysis of a comparison between medium treated cells and IFN-β treated cells showed that 480 genes of 22,283 genes were up or down-regulated by greater than 2-fold and 39 genes are known IFN inducible genes, such as, vipirin, RIG-1, 2’-5’ OAS, and PKR genes. Moreover, MxA, TLR 3, and IRF-7 were significantly up-regulated by IFN-β treatment in RPE cells (Table I). The validity of these results have been confirmed by RT-PCR analysis in poly(I:C) treated cells that produced enhanced quantities of IFN-β in RPE cells (Fig. 2). It is of interest to note that none of the proinflammatory cytokines, such as, IL-1, IL-6, TNF-α (10 ng/ml), IL-1β (10 ng/ml), and IFN-γ (10 U/ml) alone or with various combinations of these cytokines. After 48-h incubation, supernatant fluids were collected and measured for the presence of CXCL9. B, RPE cells were treated with either IFN-β (1000 or 3000 U/ml) or medium alone for 18 h. Supernatant fluids were removed, cells washed and then treated with medium or the cytokinin (IFN-γ, TNF-α, and IL-1-β). After 48-h incubation, supernatant fluids were collected and tested for the presence of CXCL9. C, Effects of anti-IFN-β Ab. Cells were treated with IFN-β or a mixture of IFN-β and anti-IFN-β Ab for 24 h. The IFN-β was removed, cells washed and treated with medium or cytokinin. Supernatant fluids were collected after 24-h incubation and analyzed by EIA. The results are derived from one representative experiment with triplicate samples (A) or the mean ± SE for three experiments with triplicate samples (B and C).
CXCL10 by RPE cells. RPE cells were treated with IFN-β (1000 U/ml), TNF-α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (100 U/ml), alone or with the combinations of TNF-α + IL-1, TNF-α + IL-1 + IFN-γ (cytomix), or cytomix + IFN-β. After 48-h incubation, supernatant fluids were collected and measured for the presence of CXCL10. B, RPE cells were treated with either IFN-β (1000 U/ml or 3000 U/ml) or medium alone for 18 h. Supernatant fluids were removed, cells washed and then treated with medium or the cytomix. After 48-h incubation, supernatant fluids were collected and tested for the presence of CXCL10. The results are from one representative experiment with triplicate samples (A) or the mean ± SE for three experiments with triplicate samples (B).

Production of CXCL10 by RPE cells and inhibition by IFN-β

In addition to being an antiviral molecule, IFN-β can possess immunosuppressive activity and is efficacious in the treatment of chronic relapsing multiple sclerosis. Because RPE cells produce high levels of IFN-β, we next wanted to evaluate the biological activity of IFN-β on these cells. We evaluated the production of the chemokine, CXCL9 by RPE cells and the potential effect of IFN-β on this production. RPE cells were treated with IFN-β, TNF-α, IL-1β, IFN-γ, alone or with the combinations of TNF + IL-1β, TNF + IL-1β + IFN-γ, or TNF-α + IL-1β + IFN-γ + IFN-β. In earlier studies, we have shown that the combination of TNF-α, IL-1β, and IFN-γ (cytomix) is a potent inducer of cytokines in RPE cells (17, 25, 30). As is seen in Fig. 4A, this cytomix was a potent inducer of CXCL9 in RPE cells. Low levels of CXCL9 were also induced by IFN-γ alone but not by IFN-β alone. When IFN-β was added in combination with the cytomix, there was a striking decrease in CXCL9 production (Fig. 4A). To more thoroughly explore this immunomodulation by IFN-β, we incubated RPE cells with medium or IFN-β (1000 and 3000 U/ml). After a 24-h incubation, the IFN-β was removed, cells washed and then treated with medium or the cytomix. As is seen in Fig. 4B, pretreatment of cells with IFN-β dramatically reduced CXCL9 production from 8000 pg/ml to undetectable levels. In a separate experiment, cells were treated with IFN-β or a mixture of IFN-β and anti-IFN-β Ab for 24 h. The IFN-β and the mixture of IFN-β plus anti-IFN-β Ab were removed, cells washed and treated with medium or cytomix. Supernatant fluids were removed after 24-h incubation and analyzed by EIA. As is seen in Fig. 4C, the cytomix resulted in production of 10,000 pg of CXCL9. IFN-β treatment alone resulted in inhibition of production of CXCL9 to 500 pg/ml, whereas the anti-IFN-β treatment reversed the inhibition to 6,000 pg/ml.

Production of CXCL10 by RPE cells

CXCL10 is a second IFN-inducible chemokine. We next evaluated the production of CXCL10 by RPE cells and the potential effect of IFN-β on this production. As is seen in Fig. 5A, TNF-α and IL-1β did not induce CXCL10 production. IFN-β signaling induced 318 pg/ml CXCL10. The cytomix resulted in the production of 68,100 pg/ml CXCL10. Therefore, IFN-β signaling alone in RPE cells did not result in the production of CXCL10 but did induce CXCL9. In contrast to the data obtained with CXCL9, the addition of IFN-β did not lower the induction of CXCL10 (Fig. 5A). Moreover, when RPE cells were pretreated with IFN-β (1000 or 3000 U/ml) for 24 h and then stimulated with the cytomix, there was no inhibition of CXCL10 production (Fig. 5B).

Effect of IFN-β on expression of ICAM-1 and CXCL9

These studies demonstrate that IFN-β can have an immunomodulatory effect on RPE cells by down-regulating the production of
gene expression, a known positive feedback molecule for IFN-

...and this was associated with the up-regulation of IRF-7
gene expression 90-fold, whereas IFN-

...an important immunoregulatory role in the eye, i.e., down-regula-
ducer of ICAM-1 gene expression to 45-fold, whereas IFN-

...CXCL9 gene expression 5-fold.

...RPE cells were pretreated with either IFN-

...The cytomix preparation up-regulated gene expression of

...decreased ICAM-1 gene expression to undetectable levels. Similar

...we have previously shown that TLR 9 gene expression is present but at relatively low levels in RPE cells (21). Therefore, we suggest that TLR 3 signaling in RPE cells infected with RNA viruses is a potent stimulus of IFN-β.

...to human RPE cells, gene expression profiles of IFN-β treated RPE cells were investigated by oligonucleotide microarrays. Following IFN-β treatment, 480 genes were up-regulated and 19 genes were down-regulated compared with their expression in untreated RPE cells. As reported in previous studies, IFN regulated genes, such as, vipirin, MxA, 2′,5′-oligoadenylate synthetase, MDAs, RNA helicase, were strongly up-regulated (34 –37). Although IFN-α and IFN-β share the same type 1 receptor, earlier studies have proven that IFN-β signaling may be different and this may result in unique cellular responses. The first comparative microarray of IFN-α, β, and γ on a human fibrosarcoma cell line (HT1080), clearly showed that IFN-β preferentially induced more genes (34). The studies presented here indicate that IFN-β is a potent trigger of gene expression in unstimulated RPE cells. IFN-β is a multifunctional cytokine that modulates a variety of activities. On the one hand, as is seen in Table I, it can up-regulate caspase-1, MyD88 and TLR 3 which would indicate proinflammatory activity. In contrast, as we have shown in this study, IFN-β may down-regulate specific inflammatory responses. The inhibition of CXCL9 expression has been shown to contribute to decreased T cell inflammation and autoimmune tissue damage (38). Moreover, we found that some additional genes affected by IFN-β that are known to dampen immune reactivity, such as the up-regulation of MHC class 1E and the down-regulation of CXCR4, tissue inhibitor of MMP-3 (TIMP-3), integrin α V (CD51) (Table I), CXCR4 and its specific receptor for SDF-1, known to promote leukocyte trafficking, are reported to be associated with diabetic retinopathy and in choroidal neovascularization (39, 40). TIMP-3, produced by RPE cells and deposited onto Bruch’s membrane, plays critical role in extracellular matrix remodeling. TIMP-3 content is shown to be elevated in Bruch’s membrane in age related macular degeneration patients (41). IFN-β induced down-regulation of CAR, the receptor for coxsackie and many adenoviruses, may reduce the spread of viral infections in the RPE and retina (42). IFN-β also act as an anti-inflammatory molecule by down regulating CAR, which is known to promote neutrophil transmigration across epithelial tight junctions (43). IFN-β also down-regulated CD51 in RPE cells. CD51 plays active role in fibrovascular proliferation and retinal angiogenesis based on the evidence that peptide antagonists block neovascularization in retina (44).

...are key elements that orchestrate immune cell migration into specific tissues, we evaluated the effect of IFN-β on these molecules. In this study, we demonstrate that, indeed, IFN-β treatment of human RPE cells results in the inhibition of cytokine induction of CXCL9 and ICAM-1.

It is of interest to note that the RNA virus, VSV, induced IFN-β production in RPE cells whereas the DNA virus, HSV-1, did not trigger IFN-β production. Similar findings were reported by Pre-haud and associates (24). They evaluated IFN-β production in a human postmitotic neuronal cell line, NT2-N, and found the RNA virus, rabies, induced IFN-β. In contrast, HSV-1 did not increase IFN-β gene transcripts or IFN-β protein production. Both the RPE cell and the neuronal cell have high levels of TLR 3, which has dsRNA as its ligand. HSV-1 is a DNA virus and has been reported to interact with TLR 9 (33). We have previously shown that TLR 9 gene expression is present but at relatively low levels in RPE cells (21). Therefore, we suggest that TLR 3 signaling in RPE cells infected with RNA viruses is a potent stimulus of IFN-β.

To evaluate the magnitude of IFN-β effects on human RPE cells, gene expression profiles of IFN-β treated RPE cells were investigated by oligonucleotide microarrays. Following IFN-β treatment, 480 genes were up-regulated and 19 genes were down-regulated compared with their expression in untreated RPE cells. As reported in previous studies, IFN regulated genes, such as, vipirin, MxA, 2′,5′-oligoadenylate synthetase, MDAs, RNA helicase, were strongly up-regulated (34 –37). Although IFN-α and IFN-β share the same type 1 receptor, earlier studies have proven that IFN-β signaling may be different and this may result in unique cellular responses. The first comparative microarray of IFN-α, β, and γ on a human fibrosarcoma cell line (HT1080), clearly showed that IFN-β preferentially induced more genes (34). The studies presented here indicate that IFN-β is a potent trigger of gene expression in unstimulated RPE cells. IFN-β is a multifunctional cytokine that modulates a variety of activities. On the one hand, as is seen in Table I, it can up-regulate caspase-1, MyD88 and TLR 3 which would indicate proinflammatory activity. In contrast, as we have shown in this study, IFN-β may down-regulate specific inflammatory responses. The inhibition of CXCL9 expression has been shown to contribute to decreased T cell inflammation and autoimmune tissue damage (38). Moreover, we found that some additional genes affected by IFN-β that are known to dampen immune reactivity, such as the up-regulation of MHC class 1E and the down-regulation of CXCR4, tissue inhibitor of MMP-3 (TIMP-3), integrin α V (CD51) (Table I), CXCR4 and its specific receptor for SDF-1, known to promote leukocyte trafficking, are reported to be associated with diabetic retinopathy and in choroidal neovascularization (39, 40). TIMP-3, produced by RPE cells and deposited onto Bruch’s membrane, plays critical role in extracellular matrix remodeling. TIMP-3 content is shown to be elevated in Bruch’s membrane in age related macular degeneration patients (41). IFN-β induced down-regulation of CAR, the receptor for coxsackie and many adenoviruses, may reduce the spread of viral infections in the RPE and retina (42). IFN-β also act as an anti-inflammatory molecule by down regulating CAR, which is known to promote neutrophil transmigration across epithelial tight junctions (43). IFN-β also down-regulated CD51 in RPE cells. CD51 plays active role in fibrovascular proliferation and retinal angiogenesis based on the evidence that peptide antagonists block neovascularization in retina (44).

Inflammatory conditions are frequently restricted to target organs or tissue components (45). This is observed in the skin in psoriasis, in the intestine in Crohn’s disease and ulcerative colitis, in the eye in uveitis, and in the CNS in multiple sclerosis (MS).
These findings exemplify the critical role of selective tissue infiltration by specific leukocytes. Trafficking molecules such as chemokines and adhesion molecules orchestrate immune cell migration into specific tissues. The chemokine, CXCL9 (MIG) is an IFN-γ-inducible chemokine that attracts T cells and NK cells to the site of inflammation. These cells contain the receptor CXCR3 on their surface. Several studies have identified these molecules as key components of host responses in autoimmune diseases, cancer and virus infections (46–51). An example of CXCL9 involvement in autoimmunity was clearly demonstrated by Lang and associates (38). They demonstrated that CXCL9 generation in the liver is required for autoimmune liver disease. In this animal model system the presence of highly activated liver-specific effector CD8 T cells alone were not sufficient to induce immune destruction. However, TLR 3 activation induced IFN-α and TNF-α and the subsequent expression of CXCL9 within the liver, enhanced CD8 T cell infiltration and subsequent immune destruction in the liver. Recent studies have explored the role of CXCL9 in cancer. A critical role for tissue-derived CXCL9 was identified in T cell mediated responses to cutaneous fibrosarcomas (51). CXCL9 production was also shown to be important in infections caused by Thielner’s virus, hepatitis C virus and herpes simplex virus (46, 52, 53). For example, neutralization of CXCL9 in a mouse CNS model system resulted in enhanced Thielner’s virus expression and pathology.

We have established an animal model of retinal degeneration called experimental coronavirus retinopathy (14, 54). Mouse hepatitis C virus and herpes simplex virus (46, 52, 53). For example, neutralization of CXCL9 in a mouse CNS model system resulted in enhanced Thielner’s virus expression and pathology.

Because CXCL9 and ICAM-1 help to traffic T cells and NK cells to target tissues, the down-regulation of these molecules may diminish the inflammatory response in infectious diseases and autoimmunity. Our data demonstrate that IFN-β treatment of cytomegalovirus infected RPE cells inhibit gene expression and protein production of these molecules. It is also of interest to note that the effect of IFN-β was selective, in that CXCL10 was not affected. Earlier studies have shown the IFN inducible chemokines, CXCL9 and CXCL10 can be down-regulated in vitro. The chemokine CXCL9 and CXCL10 gene expression were observed in retinal tissue of retinal degeneration susceptible mice (55). These augmented innate responses observed correlated with the development of autoimmune reactivity and retinal degeneration and thus may contribute to the pathogenic processes.

Because CXCL9 and ICAM-1 help to traffic T cells and NK cells to target tissues, the down-regulation of these molecules may diminish the inflammatory response in infectious diseases and autoimmunity. Our data demonstrate that IFN-β treatment of cytomegalovirus infected RPE cells inhibit gene expression and protein production of these molecules. It is also of interest to note that the effect of IFN-β was selective, in that CXCL10 was not affected. Earlier studies have shown the IFN inducible chemokines, CXCL9 and CXCL10, can have nonredundant activities (46, 51). The exact mechanism by which CXCL9 is inhibited and CXCL10 is not, needs to be elucidated in the future. However, we did observe that IFN-β was not capable of inducing CXCL9 in RPE cells but was able to induce CXCL10 in these cells.

It is known that IFN-β is both labile and has a high cell binding capacity (56). The data presented in Fig. 2 demonstrate that RPE cells produce 200 U/ml IFN-β after 48 h. However, the detection of 200 U/ml in supernatant fluid reflects substantially higher levels produced and subsequently inactivated and/or bound to cells. Because MxA gene expression is observed in these IFN-β treated cells, this confirms that IFN-β binding to the cell surface has occurred. Moreover, the approved dose of IFN-β for MS patients is 8 × 10⁶ IU by s.c. administration. Pharmokinetic studies identified that IFN-β has a high tissue affinity, resulting in prolonged resorption and low serum levels. MS patients receiving the standard dose had measurable IFN-β serum levels up to 475 IU/ml (56). In the studies reported here, we used 1000 and 3000 U/ml IFN-β to inhibit cytokine induction of CXCL9 and sICAM-1. We suggest that this level of IFN-β is justified. Based on the above observations, the administration of 1000 U/ml IFN-β in our system reflects levels produced in vitro. In addition, the 1000 U/ml is <0.1% of the patient administered dose and is only 2-fold higher than levels detected in one ml of patient sera following treatment.

In summary, high levels of IFN-β are produced by RPE cells via TLR 3 signaling and by positive feedback stimulation via IRF-7. TLR 3 signaling in RPE cells is also associated with up-regulation of proinflammatory cytokines and chemokines, such as, IL-6, IL-8, MCP-1, and sICAM-1 (21). Thus, TLR 3 signaling within the retina triggers a proinflammatory burst of activity that may provide protection from invading organisms. However, this innate response must be dampened within the microenvironment of the retina to limit excessive retinal cell damage and loss of vision. We hypothesize that IFN-β is one of the inhibiting components. Herein, we show that IFN-β can indeed inhibit RPE cell gene expression and protein production of the soluble form of an important adhesion molecule, ICAM-1 and the T cell attracting chemokine, CXCL9. Thus, IFN-β can control retina gene expression of molecules that augment inflammation. This mechanism of action is not unique to the RPE cell. We have also seen the inhibition of CXCL9 gene expression and protein production by IFN-β in retinal vascular endothelial cells and in umbilical cord endothelial cells (our unpublished data). We, therefore, suggest that the inhibition of CXCL9 by IFN-β may be a more general phenomena that may also contribute to its efficacy in MS (57).

Disclosures

The authors have no financial conflict of interest.

References


