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Primate Neural Retina Upregulates IL-6 and IL-10 in Response to a Herpes Simplex Vector Suggesting the Presence of a Pro-/Anti-inflammatory Axis

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Abstract

Injection of herpes simplex virus vectors into the vitreous of primate eyes induces an acute, transient uveitis. The purpose of this study was to characterize innate immune responses of macaque neural retina tissue to the herpes simplex virus type 1-based gene delivery vector hrR3. PCR array analysis demonstrated the induction of the pro-inflammatory cytokine IL-6, as well as the anti-inflammatory cytokine IL-10, following hrR3 exposure. Secretion of IL-6 was detected by ELISA and cone photoreceptors and Muller cells were the predominant IL-6 positive cell types. RNA *in situ* hybridization confirmed that IL-6 was expressed in photoreceptor and Muller cells. The IL-10 positive cells in the inner nuclear layer were identified as amacrine cells by immunofluorescence staining with calretinin antibody. hrR3 challenge resulted in activation of NFκB (p65) in Muller glial cells, but not in cone photoreceptors, suggesting a novel regulatory mechanism for IL-6 expression in cone cells. hrR3 replication was not required for IL-6 induction or NFκB (p65) activation. These data suggest a pro-inflammatory (IL-6)/anti-inflammatory (IL-10) axis exists in neural retina and the severity of acute posterior uveitis may be determined by this interaction. Further studies are needed to identify the trigger for IL-6 and IL-10 induction and the mechanism of IL-6 induction in cone cells.

Keywords

HSV-1; viral vectors; gene therapy; retina; inflammation; IL-6; IL-10

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1. Introduction

Retinal degenerative diseases such as retinitis pigmentosa and macular degeneration are significant causes of blindness in the United States affecting an estimated two million people each year. To date, there are few therapies for these diseases, but a number of approaches are under investigation, including biologics such as ranibizumab, retinal transplantation, stem cells, and gene therapy (Al-Saikh, 2013; Brandt, 2013; Lipinski et al., 2012; Rosenfeld et al., 2006). Gene therapy offers the potential advantage of a permanent therapy.

Several different viruses have been utilized as ocular gene delivery vectors including herpes simplex virus (HSV) (Liu et al., 1999), adenovirus (AdV) (Sweigard et al., 2010), adeno-associated virus (AAV) (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008), and lentiviruses (Balaggan and Ali, 2012). Many factors must be considered when designing a vector for ocular gene delivery including selection of viral vector, delivery route, cellular target, and choice of promoter (Brandt, 2013). The large size of the HSV genome, compared to the genome of adeno or lentiviruses, makes it possible to insert larger genes, or multiple genes, when used as a gene delivery vector (Spencer et al., 2001).

Our work in rodents showed that gene delivery with HSV vectors did not induce inflammation of the eye (Brandt et al., 1997; Spencer et al., 2000). In contrast, we found that HSV, AdV, and lentiviral vectors induced a transient inflammatory response in non-human primate (NHP) eyes (Liu et al., 1999; unpublished data). The pro-inflammatory cytokine IL-6 has been detected in inflammatory eye diseases and HSV-1 ocular infections, suggesting a role in virus-induced inflammation (Biswas et al., 2006; De Vos et al., 1992; Feghali and Wright, 1997). Transient inflammation (Ikeda et al., 2009), or the induction of Th2 cytokines including IL-10 (Bennett, 2003), following subretinal injection of lentiviral and AAV vectors has also been reported. Studies using a murine cytomegalovirus retinitis model and experimental autoimmune uveitis suggest that retinal cells may suppress intraocular inflammation via stimulation of suppressor cytokines such as IL-10, IL-27, and SOCS (Blalock et al., 2013; Lee et al., 2011). The findings that both IL-6 and IL-10 have been associated with various forms of inflammation in the neural retina raises the possibility that a pro-/anti-inflammatory axis exists and the severity of uveitis could depend on which of the axes is predominant.

In this study, we found increased expression of both IL-6 and IL-10 in macaque neural retina tissue exposed to the HSV-1 gene delivery vector hrR3. We chose to study neural retina *ex vivo* because it is the tissue contacted directly following intra-vitreous injection of vectors and we wished to study early innate response of the retina in the absence of infiltrating cells. Cone and Muller cells were the predominant IL-6 positive cell type in the neural retina, while IL-10 staining was detected in amacrine cells. hrR3 activated NF κ B (p65) in Muller cells, but not in cone photoreceptors, even though they expressed IL-6. The induction of IL-6 and IL-10 did not require viral replication. These results suggest that combined effects of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 may determine the extent of ocular inflammation following viral gene delivery in the primate eye.

2. Material and Methods

2.1 Virus

High titer stocks of wild type HSV-1 strain KOS, and HSV-1 hrR3, were prepared in Vero cells (Grau et al., 1989), and purified on sucrose gradients as previously described (Visalli and Brandt, 1993). The hrR3 vector contains an insertion of the *E. coli* β -galactosidase gene into the large subunit of HSV-1 KOS ribonucleotide reductase (UL39, ICP6) (Cai and Brandt, 2008; Goldstein and Weller, 1988). Precautions were taken during virus preparation and all experiments to minimize endotoxin levels. High titer viral stocks were tested for endotoxin levels with the ToxinSensor Chromagenic LAL Endotoxin Assay Kit (Genscript, L00350, Piscataway, NJ). Vector preparations contributed 0.5 endotoxin units (EU)/ml in all experiments. Complete media (DMEM/F-12 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine) contained similar levels of endotoxins (0.5 EU/ml).

2.2 Macaque retina tissue

Eyes from euthanized rhesus macaques (*Macaca mulatta*), or cynomolgus macaques (*Macaca fascicularis*), were obtained as they became available from the UW Primate Center or Covance (Madison, WI). No monkeys were deliberately sacrificed for these studies. Macaque eyes were kept on ice and dissected immediately after sacrifice. Posterior eye cups were incubated with PBS/1 mM EDTA for 30 minutes at 37°C to loosen neural retina tissue and separate the retina from RPE cells. Neural retina tissue was rinsed in PBS and utilized in *ex-vivo* experiments as indicated.

2.3 RNA Isolation

Cynomolgus macaque neural retina tissue was incubated overnight in complete media or complete media containing 1.8×10^8 plaque forming units (pfu) of hrR3 at 37°C in 5% CO₂. Neural retina tissue from 3 different cynomolgus macaques for each condition was used to provide biological triplicates. Tissues were rinsed in PBS prior to homogenization in TRIzol reagent (Ambion/Life Technologies, Grand Island, NY, #15596-026). RNA isolation was performed following the TRIzol Reagent protocol. DNase digestion (Qiagen, Valencia, CA, RNase-Free DNase Set, #79254) was completed prior to RNA cleanup on RNeasy spin columns (Qiagen, RNeasy Mini Kit, #74104). RNA was eluted in RNase-free H₂O and quantitated on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, #ND-1000).

2.4 PCR

500 ng of purified neural retina RNA per sample was converted into cDNA (Qiagen, RT² First Strand Kit, 330401). Each cDNA synthesis reaction was then run on a Rhesus Macaque Innate and Adaptive Immune Response RT² Profiler PCR Array (Qiagen, #PAQQ-052ZA) using RT² SYBR Green ROX qPCR Mastermix (Qiagen, #330520) and an ABI 7300 cycler. Results from biological triplicates were grouped and compared using the Qiagen RT² Profiler PCR Array Data Analysis v3.5. β -actin, β -2-microglobulin, and glyceraldehyde 3-phosphate dehydrogenase were utilized as housekeeping genes for data normalization. RT²

qPCR primer assays were performed for each cDNA with IL-6 (Qiagen, #PPQ09482B) or IL-10 (Qiagen, #PPQ01623B) and β -actin primers (Qiagen, #PPQ08986B) following the standard protocol. Primer assay data were analyzed by the C_T method and results expressed as fold-change in gene expression

2.5 Viral replication

Cynomolgus macaque neural retina tissue from three animals was incubated with 6.8×10^7 pfu of hrR3 or KOS in complete media for 1 hr at 37°C in 5% CO₂. A sample of the supernatant was removed and labeled as “input virus.” Neural retina tissue was rinsed twice with PBS to remove unbound virus, complete media was added, and tissue was incubated for 24 hours at 37°C. Culture media was removed and labeled as “24 hour virus.” Samples were titrated on Vero cells (Grau et al., 1989), and mean titers plotted as pfu/ml.

2.6 ELISA

Neural retina tissue, obtained from two cynomolgus and one rhesus macaque, was cultured for 24 hours at 37°C in complete media or complete media containing 6.6×10^7 pfu hrR3. Supernatants were collected and analyzed in duplicate with a Monkey IL-6 Instant ELISA Kit (eBioscience, San Diego, CA, #BMS641INST). Duplicate OD values were averaged, and pg/ml IL-6 determined from the standard curve. A two sample t-test was performed on mean pg/ml values assuming equal variance ($n=3$, $p=0.0002$). Cleared supernatants from media or hrR3 treated macaque neural retina tissue were assayed in duplicate for IL-10 with a Monkey IL-10 Instant ELISA kit (eBioscience, #BMS642INST).

2.7 Immunofluorescence

Macaque neural retina tissue was cultured for 24 hours at 37°C in complete media or complete media containing 6.6×10^7 pfu of hrR3. Tissue pieces were washed in PBS, fixed in 4% paraformaldehyde/PBS, paraffin embedded, and sectioned. Tissue sections were de-paraffinized and antigen retrieval was performed via heat treatment (10mM Tris/1mM EDTA, pH 9, 20 minutes 92°C). Where noted, tissue sections were treated with Autofluorescence Eliminator Reagent (Millipore, Billerica, MA, #2160) prior to staining following the manufacturer's protocol. Slides were blocked with 5% FBS before incubation with the primary antibody in 2.5% FBS/PBS for 1 hour at RT. Note that in Fig. 5, sections were stained ON at 4°C with the primary antibodies against IL-10 and calretinin. BSA was substituted for FBS in the staining protocol for sections stained with FITC-PNA. The secondary antibodies, diluted 1:400 in PBS containing 2.5% FBS, were applied for 1 hour at RT. Sections were stained with Hoechst (1 μ g/ml) to visualize nuclei before mounting with Immu-mount (Thermo Scientific, Kalamazoo, MI, #9990402). Fluorescence was observed with a Zeiss Axioplan 2 microscope with an Axiocam HRm camera and Axiovision LE 4.8 software (Zeiss Microimaging, Oberkochen, Germany).

The primary antibodies and dilutions utilized were: rabbit anti-IL-6 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, #sc-7920), mouse anti-IL-6 (1:250, Abcam, Cambridge, MA #ab9324), mouse anti-IL-10 (1:100, Abcam # ab134742), goat anti-NF κ B (p65) (1:50, Santa Cruz, #sc-372-G), mouse anti-c-Rel (1:50, Santa Cruz #sc-6955), rabbit anti-RelB (1:50, Santa Cruz, #sc-226), rabbit anti-NF κ B (p100/p52) (1:50, Abcam, #ab86090), rabbit

anti-NF κ B (p105/p50) (1:250, Abcam, #ab32360), rabbit anti-Calretinin (1:100, Santa Cruz, #sc-50453), goat anti-Arrestin-C (1:100, Santa Cruz, #sc-54355), rabbit anti-Vimentin (1:200, Santa Cruz, #sc-5565), mouse anti-gD (1:1000, Virusys, Taneytown, MD, #HA025), mouse anti-ICP4 (1:1000, Virusys H1A021), mouse anti-ICP8 (1:50, Santa Cruz, #sc-53329), mouse anti-VP5 (1:50, Santa Cruz, #sc-13525), mouse anti-VP16 (1:50, Santa Cruz, #sc-7545), rabbit anti-IL-6R (1:200, Abcam, #1280008), and mouse anti-Nectin 1 (1:50, Santa Cruz, #sc-69718).

The secondary antibodies purchased from Life Technologies included: goat anti-rabbit Alexa Fluor 594 (#A11012), goat anti-mouse Alexa Fluor 594 (#A11032), donkey anti-rabbit Alexa Fluor 594 (#A21207) donkey anti-goat Alexa Fluor 594 (#A11058), donkey anti-goat Alexa Fluor 488 (#A11055), goat anti-mouse Alexa Fluor 488 (#A11029), donkey anti-mouse Alexa Fluor 488 (#21202), goat anti-rabbit Alexa Fluor 488 (A11008) and donkey anti-rabbit Alexa Fluor 488 (#A21206). Sections stained with FITC-PNA (Sigma, St. Louis, MO, #L7381) were blocked with 5% BSA and incubated with 25 μ g/ml FITC-PNA in 2.5% BSA/PBS for 1 hour at RT during the secondary antibody incubation step.

2.8 RNA *in situ* hybridization

Cynomolgus macaque neural retina tissue was incubated overnight in complete media containing 1.8×10^8 pfu hrR3 at 37°C in 5% CO₂. Tissue was fixed for 24 hours at room temperature in 10% neutral buffered formalin (Thermo Fisher, Kalamazoo, MI, #305-510) before embedding and sectioning. Sections were prepared and pretreated according to Advanced Cell Diagnostics Pretreatment Guide for FFPE Tissue with pretreatment 2 and 3 times adjusted to 7 and 20 minutes, respectively. RNA *in situ* hybridization was performed using the Advanced Cell Diagnostics RNAscope 2.0 HD Detection Kit (BROWN) (Advanced Cell Diagnostics - ACD, Hayward, CA, #310035) with the following probes: negative control probe DapB (ACD, #310043), positive control probe POLR2A (ACD, #310451), and human IL-6 probe Hs-IL-6 (ACD, #310371). Hematoxylin staining time was reduced to 30 seconds.

3. Results

3.1 Gene expression profile of neural retina tissue

RNA isolated from neural retina tissue incubated with media or the HSV-1 vector hrR3 was analyzed on a Rhesus Macaque Innate and Adaptive Immune Response RT² Profiler PCR Array, which profiles the expression of 84 genes involved in the host inflammatory and innate immune response. Fig. 1A shows a heat map of the changes in gene expression for hrR3 treated retina tissue versus media treated control tissue. Fig. 1B highlights the genes whose expression was most altered by hrR3 exposure. The expression of several interleukins (IL) including IL-10, IL-1 α , IL-1 β , and IL-6 and the cluster of differentiation molecules CD80 and CD86 were increased following hrR3 treatment (Fig. 1B). Caspase 1, a key enzyme involved in the inflammasome pathway, and two Toll-Like Receptors (TLR), TLR6 and TLR7, also showed increased expression in response to hrR3 exposure (Fig. 1B). The expression of some genes, including CCL2, CCR4, IL-15, IRF1, JAK2, JUN, LYZ, MIF, and NF κ B1A decreased following hrR3 treatment, but these changes were less than

2.6-fold and may not be significant (Fig. 1B). No changes in gene expression of IFN α receptor 1, IFN β 1, IFN γ , or IFN γ receptor 1 were detected following hrR3 challenge of macaque neural retina tissue.

The cytokine IL-6 is a significant pro-inflammatory mediator (Wolf et al., 2014) whereas IL-10 suppresses immune responses (Ghasemi et al., 2012). Our observation that both IL-6 and IL-10 were up-regulated raises the possibility that a pro-/anti-inflammatory axis might exist in the NHP retina, so we focused on the role of IL-6 and IL-10 in the response of macaque neural retina to HSV-1 vector challenge.

To confirm the PCR array data for IL-6 and IL-10 we performed RT² qPCR primer assays with primers to IL-6 or IL-10 and β -actin. We compared cDNAs from 3 media treated neural retina samples with cDNAs from 4 hrR3 treated neural retina samples, using β -actin to normalize. The average increase in IL-6 or IL-10 expression for hrR3 treated tissue versus control tissue was 68-fold and 28-fold, respectively. These data support the conclusion that treatment of macaque neural retina tissue with hrR3 increased expression of both the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10.

3.2 IL-6 is secreted following vector challenge

To determine whether IL-6 was secreted from retina tissue in response to virus challenge, we examined culture supernatants by ELISA. Neural retina tissue from 3 macaques was incubated overnight at 37°C with media or media containing hrR3. A low level of IL-6 (15 pg/ml) was secreted by neural retina exposed to media only. In contrast, exposure of neural retina tissue to hrR3 increased IL-6 secretion approximately 5-fold to 80 pg/ml (n=3, $p=0.0002$). These ELISA results indicated that exposure of macaque neural retina tissue to hrR3 induced secretion of the pro-inflammatory cytokine IL-6 and corroborate the quantitative PCR data. ELISA analysis of supernatants from media or hrR3-treated neural retina tissue failed to detect IL-10 in any of the samples. This suggests that IL-10 was either not secreted by hrR3 challenged neural retina tissue, or produced in amounts below the level of detection.

3.3 IL-6 is expressed in Muller cells and cone photoreceptors

To determine which cells in the retina were expressing IL-6, we performed immunofluorescence (IF) on cynomolgus macaque retina tissue. Macaque neural retina tissue was cultured overnight at 37°C in media or media plus hrR3. Sections were treated with autofluorescence eliminator reagent and stained with a rabbit polyclonal antibody to IL-6. Very bright IL-6 staining was detected in ovoid-shaped cells in the photoreceptor layer of hrR3-treated retina tissue (Fig. 2D) compared to media-treated retina, in which little IL-6 staining was evident (Fig. 2B). Similar IF results were obtained on neural retina tissue from a total of 10 macaques (6 rhesus and 4 cynomolgus).

IF with a monoclonal antibody to IL-6 antibody also stained filamentous cells that extended through the retina of cynomolgus macaque tissue (Figs. 3B, D). Similar results were obtained when tissue from two rhesus macaques was stained with the same mouse anti-IL-6 antibody. These results differ from those obtained with the rabbit anti-IL-6 antibody (Fig. 2). Interestingly, both the rabbit and mouse antibodies detected IL-6 positive cells in the

photoreceptor layer and filamentous cells that extended through the retina when utilized in a standard immunohistochemistry protocol on cynomolgus macaque neural retina tissue (data not shown).

Based on the location and morphology of IL-6 positive cells in the photoreceptor layer of the neural retina, we suspected they might be cone cells. We therefore performed IF with rabbit anti-IL-6 and an antibody to the cone marker protein Arrestin-C (ARR3) (Wilden et al., 1986). The Arrestin-C antibody stained the inner and outer segments of cone cells as expected (Figs. 2C, E). Panel E demonstrates co-localization of IL-6 and Arrestin-C positive cells in hrR3 treated retina, thus identifying the IL-6 positive cells as cone photoreceptors. Similar results were obtained when rhesus macaque neural retina tissue or retina tissue from a different cynomolgus macaque were stained with rabbit anti-IL-6 and the Arrestin C antibody.

To further confirm the identity of IL-6 positive photoreceptor cells, we performed IF with rabbit anti-IL-6 and FITC conjugated *Arachis hypogaea* lectin (FITC-PNA), which binds specifically to cone cells (Blanks and Johnson, 1984). IL-6 staining was evident in the photoreceptor layer of hrR3 treated cynomolgus macaque retina (Fig. 2F), and this staining co-localized with FITC-PNA (Fig. 2G), notably in the outer segments of cone cells. Similar results were obtained with rabbit anti-IL-6 antibody and FITC-PNA on neural retina tissue from a different cynomolgus macaque.

To identify the IL-6 positive filamentous cells, we performed double label IF with mouse anti-IL-6 and an antibody to the intermediate filament protein Vimentin, which is a marker for Muller retinal cells (Bhatia et al., 2009). Initial attempts to utilize an antibody to GFAP, a more traditional marker for Muller cells, failed as we could not find an antibody that reacted with paraffin embedded macaque tissue. Media or hrR3 treated cynomolgus macaque retina sections stained with mouse anti-IL-6 demonstrated a filamentous staining pattern (Figs. 3B, D). Merging of IL-6 (rhodamine) and vimentin (FITC) signals indicated partial co-localization of IL-6 positive cells with Muller cell bodies and processes (Figs. 3C, E). These results demonstrated that Muller retinal cells expressed IL-6. Co-localization of IL-6 positive cells with vimentin staining was also observed in separate experiments performed on neural retina sections from two rhesus macaques.

3.4 IL-6 RNA is detected in the INL and ONL of neural retina

To confirm that IL-6 was expressed in Muller and cone cells, we performed RNA *in situ* hybridization on cynomolgus macaque neural retina tissue exposed to hrR3. Retina tissue hybridized with the negative control probe showed only background signal (Figs. 4A, B, C), whereas the positive control probe POLR2A hybridized to cells within the outer nuclear layer (ONL) and inner nuclear layer (INL) (Figs. 4D, E, F). The hybridization signal of the positive control probe was less than ideal, despite extensive trouble shooting, for reasons that are not clear. Hybridization with the IL-6 probe detected positive signal in cells within the ONL, where the nuclei of photoreceptor cells are located (Fig. 4H), and within cells in the INL, where the nuclei of Muller cells are located (Fig. 4I). This hybridization pattern indicated that IL-6 was expressed in both photoreceptor and Muller cells, and supports the

earlier data identifying cone and Muller cells as IL-6 positive by IF. RNA in situ experiments were repeated three times to verify IL-6 expression.

3.5 IL-10 is expressed in amacrine cells

To determine which cells in the retina were expressing IL-10, we performed IF on retina tissue. Macaque neural retina tissue was challenged overnight at 37°C with hrR3. Tissue was processed for IF and stained with a primary antibody to IL-10. Small, round cells within the INL, as well as larger round cells in the ganglion cell layer (GCL) of the retina stained positive for IL-10 (Fig. 5B). The location and shape of the IL-10 positive cells in the INL suggested they might be amacrine cells, of which more than 20 subtypes exist (Cheng et al., 2013). Sections were stained with an antibody to the amacrine marker calretinin (Kolb et al., 2002) and IL-10 to determine whether the IL-10 expressing cells were calretinin positive. Fig. 5C demonstrates calretinin staining of round amacrine cells within the INL. Co-localization of IL-10 positive cells (Fig. 5D) with some of the calretinin stained cells, indicates that IL-10 is produced by at least a subset of amacrine cells in macaque neural retina tissue.

3.6 NFκB is activated in Muller cells by hrR3

The nuclear factor kappa beta (NFκB) family of transcription factors are key regulators of inflammatory cytokines including IL-6 (Matsusaka et al., 1993). To determine whether NFκB (p65) was activated in retina tissue in response to HSV-1 vector challenge, cynomolgus macaque neural retina was cultured overnight at 37°C in media or media plus hrR3 before embedding and processing for IF. NFκB (p65) in media treated retina was localized predominantly in the cytoplasm of cells located on the border of the photoreceptor and ONL, with no nuclear staining evident in the INL (Fig. 6A). Retina tissue challenged with hrR3 exhibited bright NFκB (p65) staining of nuclei, which co-localized with DAPI staining, within cells of the INL (Fig. 6B). Similar results were obtained on neural retina tissue from eight other macaques (2 cynomolgus and 6 rhesus). These data indicated that NFκB (p65) was activated in cells within the INL of macaque neural retina tissue following hrR3 challenge.

We performed IF with antibodies to other members of the NFκB family of transcription factors, including c-Rel, Rel-B, NFκB p100/p52, and NFκB p105/p50, to determine if these transcription factors were also activated in response to hrR3 challenge. IF on uninfected neural retina demonstrated a cytoplasmic staining pattern for c-Rel, Rel-B, NFκB p100/p52, and NFκB p105/p50 (data not shown). Treatment with hrR3 did not alter the cytoplasmic distribution of c-Rel, Rel-B, NFκB p100/p52, or NFκB p105/p50, suggesting that these NFκB subunits are not activated following hrR3 challenge of macaque neural retina (data not shown).

To identify the NFκB (p65) positive retinal cells, we utilized marker antibodies to various cell types within the retina. For amacrine cells, we performed double label IF with an anti-NFκB (p65) antibody and an antibody to the calcium binding protein calretinin (Kolb et al., 2002). Calretinin stained cells did not co-localize with NFκB (p65) positive nuclei in the INL, suggesting that the NFκB (p65) positive cells were not amacrine cells (data not

shown). For Muller cells, we performed double label IF with anti-NF κ B (p65) and anti-vimentin antibodies. Retina sections stained with anti-vimentin demonstrated a filamentous staining pattern extending throughout the retinal layers as expected for Muller cells (Fig. 6C). Fig. 6D highlights NF κ B (p65) positive nuclei in the INL post hrR3 challenge. Merging of vimentin (FITC) and NF κ B (p65) (Rhodamine) signals indicated that the NF κ B (p65) positive nuclei co-localized with vimentin positive Muller cell bodies and processes (Fig. 6E). Similar staining patterns were observed on neural retina sections from three rhesus macaques. These images indicated that challenge of macaque neural retina tissue with hrR3 resulted in NF κ B (p65) activation in Muller retinal cells.

3.7 hrR3 binds to and enters, but does not replicate in, neural retina tissue

HSV-1 infection occurs rapidly and replication is usually complete within 15–18 hours post infection (Roizman et al., 2013). To determine whether hrR3 replicated in retina tissue, neural retina tissue from three cynomolgus macaques was exposed to hrR3 for 1 hour at 37°C. Retina tissue was rinsed with PBS to remove unbound virus and incubated overnight at 37°C. After 24 hours, mean hrR3 titers decreased from 3.2×10^8 to 5.7×10^6 plaque forming units per milliliter (pfu/ml), corresponding to a 98% reduction in viral titer. This experiment suggested that hrR3 did not replicate in cultured macaque neural retina tissue.

To confirm a lack of viral replication and to determine if hrR3 virions were entering retinal cells, we cultured cynomolgus macaque neural retina tissue with hrR3 for 24 hours. IF was then performed with HSV-1 antibodies specific for α , β , and γ genes. The α protein ICP4 and the single stranded DNA binding β protein ICP8 were not detected indicating that α and β genes were not expressed (Figure 7 B, C). Signal detected in the photoreceptor layer of neural retina tissue stained with ICP4 or ICP8 antibodies was similar to signal observed in the no primary antibody control, indicating that autofluorescence was responsible (Figure 7 A, B, C). IF with anti-gD antibody demonstrated bright staining at the internal limiting membrane (ILM), but not in retinal cells, indicating late genes were not expressed (Fig. 7D). An antibody to the major capsid protein VP5 detected VP5 within the ganglion cell layer (Figure 7E). The tegument protein VP16 was detected along the ILM and extending into filaments within the GCL which morphologically resemble Muller cells (Figure 7F). These results indicated the structural proteins VP5 and VP16 were internalized and transported into retinal cells after viral and cell membrane fusion. Based on these experiments, we propose that HSV-1 virions are binding to the ILM of macaque neural retina and are entering cells, notably Muller cells, but fail to initiate the replication cycle due to lack of essential early protein production.

4.0 Discussion

The eye is considered an immunologically privileged organ, but infection with pathogens or other stimuli can break this status (Bennett, 2003; Ikeda et al., 2009). Previous studies have shown that delivery of HSV, lentivirus, and adenovirus-based gene delivery vectors induced a transient uveitis in the eyes of NHPs ((Ikeda et al., 2009; Liu et al., 1999), unpublished data). Since NHPs are closely related to humans, there is a high probability that these vectors will induce uveitis in humans and this could negatively affect their potential use in

ocular gene therapy. The transient uveitis occurs within the first 24 to 48 hours of vector administration, strongly suggesting that innate sensing of pathogen associated molecular patterns (PAMPs) is involved (Pandey et al., 2013). The signaling pathways for PAMP receptors culminate in the induction of pro-inflammatory cytokines and interferons that mediate acute inflammation (Bowie and Haga, 2005). The pro-inflammatory cytokine IL-6, expression of which is increased following PAMP receptor activation, is an important mediator of inflammation and has been detected in a variety of ocular inflammatory conditions including HSV-1 infection (Biswas et al., 2006; De Vos et al., 1992; Feghali and Wright, 1997). The role of anti-inflammatory cytokines, such as IL-10, in the control of ocular inflammation is becoming increasingly apparent (Ghasemi et al., 2012).

IL-6 is a multifunctional cytokine whose production is tightly regulated and linked to the expression pattern of its two receptors, the signal-transducing β -subunit glycoprotein 130 (gp130) and the IL-6 α -receptor (IL-6R) (Wolf et al., 2014). Most pro-inflammatory roles of IL-6 can be attributed to the trans-signaling pathway, in which IL-6 binds to a soluble form of the IL-6R (sIL-6R), and this complex can then activate any cell type due to the ubiquitous expression of gp130 (Wolf et al., 2014). In the healthy retina, gp130 is located primarily in astrocytes, Muller cells and ganglion cells, and its expression can be elevated with glaucoma-related stressors (Echevarria et al., 2013). Classic IL-6 signaling is mediated by IL-6 binding directly to IL-6R which is expressed on hepatocytes and some leukocytes (Wolf et al., 2014). IF staining of macaque neural retina tissue indicates that IL-6R is expressed along the ILM and by cells within blood vessels (data not shown). The expression of gp130 and IL-6R in neural retina implies that both signaling pathways could influence the ability of IL-6 to induce intracellular signaling pathways and ocular inflammation.

ELISAs detected IL-6 but not IL-10 in neural retina culture supernatants following hrR3 challenge. This difference may be attributed to the low copy number of IL-10 RNA, as evident by weak qPCR signals compared to IL-6, or the weak IF signal and smaller subset of cells expressing IL-10 compared to IL-6. Alternately, differences in extracellular release of IL-6 versus IL-10 may explain our ELISA results. Additional experiments with a human Muller cell line are ongoing to address this issue.

We detected increased IL-10 expression following hrR3 challenge of macaque neural retina tissue and identified the primary cell type expressing IL-10 as amacrine cells. The most important biological activities of IL-10 include down-modulation of the inflammatory response, antiviral activity, and inhibition of cell growth (Ghasemi et al., 2012). IL-10 potentially inhibits the synthesis of many inflammatory cytokines including IL-1 (Rosenbaum and Angell, 1995), IL-2 (Tumpey et al., 1994), IL-6 (Tumpey et al., 1998), IL-8 (Yoshida et al., 2001), IL-17 (Blalock et al., 2013; Sarangi et al., 2008) and TNF- α (Rosenbaum and Angell, 1995). IL-10 has been identified as a key component involved in the control of HSV-1 induced stromal keratitis (Sarangi et al., 2008). In fact, treatment with IL-10 resulted in the reduction of ocular inflammation in herpes stromal keratitis human corneal buttons (Boorstein et al., 1994) and decreased corneal disease in murine HSV-1 keratitis (Tumpey et al., 1998; Tumpey et al., 1994). Lentiviral delivery of IL-10 also reduced the severity of murine endotoxin-induced uveitis (Trittibach et al., 2008). In the mouse retina, increased IL-10 expression was detected in photoreceptor and Muller cells and

is thought to protect the neuroretinal cells from the toxic effects of pro-inflammatory cytokines during EAU (Lee et al., 2011). IL-10 production is stimulated in murine HSV-1 and mCMV retinitis and is believed to play a role in down regulation of pro-inflammatory cytokines and viral clearance (Blalock et al., 2013; Jost et al., 2014; Saitoh-Ishibashi et al., 2003). Since hrR3 induces uveitis in NHP but not in mice, it is not clear that data in mice are translatable to NHPs. Recently, however, increased intraocular levels of IL-10 have been linked to viral uveitis in human subjects, and proposed as a diagnostic indicator (Sauer et al., 2015). These data, and our current study which identifies amacrine cells as the main IL-10 positive cell type in NHP retina post hrR3 challenge, suggest a role for IL-10 in modulating HSV-1 induced ocular uveitis.

Our data indicate that Muller retinal cells produce IL-6 in response to HSV-1 vector challenge. Muller cells, the most abundant glial cell in the retina, participate in retinal innate immunity through several mechanisms including phagocytic activity, secretion of cytokines and chemokines, and recognition of PAMPs by Toll-Like receptors (TLRs) (Kumar et al., 2013; Singh et al., 2014). The activation of TLRs on the human Muller cell line MIO-M1, which expresses TLR1-10, has been linked to NF κ B up-regulation and the production of inflammatory mediators including TNF-alpha, IL-1 β , IL-6, and IL-8 (Kumar and Shamsuddin, 2012). Herpes viruses contain several pathogen associated molecular patterns (PAMPs) that are recognized by TLRs including glycoproteins (TLR2), double-stranded RNA (TLR3), single-stranded RNA (TLR7, TLR8), and unmethylated CpG (TLR9) (Boehme and Compton, 2004; Bowie and Haga, 2005; Cervantes et al., 2012). TLR2, which is located at the plasma membrane, recognizes HSV-1 glycoproteins gH/gL and gB during the initial phases of HSV-1 infection (Cai et al., 2013; Leoni et al., 2012). HSV-1 virions that could attach to cells, but were defective in fusion/entry, were still able to activate NF κ B (Cai et al., 2013; Leoni et al., 2012). We postulate that HSV-1 glycoprotein interaction with TLR2 expressed on Muller cells at the ILM, is responsible for NF κ B (p65) activation, and subsequent IL-6 induction, in macaque neural retina tissue. Interestingly, TLR2 signaling was required for production of pro-inflammatory cytokines including IL-6, during HSV-1 infection of murine microglial cells (Aravalli et al., 2005). TLR2/TLR4 signaling was also necessary for IL-10 production and immunosuppression in an animal model of subretinal fibrosis (Yang et al., 2013). It is less likely that TLR3, TLR7, TLR8, or TLR9 are involved in activation of NF κ B (p65) in the neural retina, as interaction of these endosomal TLRs with their PAMPs would require viral replication, which was not observed in cultured neural retina. The cytoplasmic helicase proteins MDA5 and RIG-1, which interact with viral dsRNA to promote a host antiviral response, are also unlikely to be involved as HSV-1 dsRNA intermediates would only be generated during viral infection (Kato et al., 2006; Roizman et al., 2013).

NF κ B is a family of transcription factors that activates many inflammatory cytokines including IL-6 (Matsusaka et al., 1993; Medzhitov and Horng, 2009). In retina pigment epithelial cells, the induction of IL-6 was dependent on hrR3 replication and activation of the transcription factor NF κ B (p65) (Cai and Brandt, 2008). Previous studies showed that inflammation in the adenoviral infected cornea was triggered by NF κ B subunit activation (Rajaiya et al., 2009). These results suggest a link between NF κ B (p65) activation following virus challenge and an inflammatory response in the eye.

One surprising finding in our study was that, although IL-6 was induced in cone photoreceptors and Muller glial cells, NF κ B (p65) was not translocated to the nucleus of cone cells. Recent studies with a mouse photoreceptor cell line have demonstrated IL-6 secretion and NF κ B (p65) activation following TLR ligand and bacterial challenge (Singh and Kumar, 2015). This discrepancy could be explained by a species difference, the ex-vivo culture model we are using, or the tumorigenic nature of the mouse cell line (Tan et al., 2004). Alternately, IL-6 induction could have been caused by a different NF κ B family member, however, we found no evidence of nuclear translocation of c-Rel, Rel-B, NF κ B p100/p52, or NF κ B p105/p50 in cone or Muller cells after hrR3 challenge of macaque neural retina. Interestingly, adeno-associated virus has been shown to activate an alternate NF κ B pathway in HeLa cells, leading to the production of pro-inflammatory cytokines and chemokines including IL-6 (Jayandharan et al., 2011). These results raise the possibility that a non-canonical pathway may be inducing IL-6 in photoreceptor cells. Another possibility is that Muller glial cells are generating the activating signal and inducing IL-6 indirectly in cone cells. Further studies are needed to identify the mechanism of IL-6 induction in cone photoreceptors.

Our data indicated that the HSV-1 vector hrR3 entered neural retina tissue, as demonstrated by IF with anti-VP5 and VP16 antibodies, but did not replicate. HSV-1 virion binding along the ILM of the retina may be the result of an interaction between the viral glycoprotein gD and one of its receptors (Spear, 2004). Nectin-1, whose expression in macaque neural retina has been confirmed by IF (data not shown), might be responsible for gD binding. Alternately, HSV-1 virions could be binding to the ILM by another gD receptor, such as HVEM or 3-*O*-sulphate modified heparan sulfate (Spear, 2004). Intravitreal injection of hrR3 virions would allow interactions with Muller end feet at the ILM and interactions between viral glycoproteins and Muller cell surface TLR2 could be responsible for the NF κ B activation we observed in this cell type. Previous studies have indicated that TLR2:HSV-1 viral glycoprotein interaction, and subsequent NF κ B activation, could occur in the absence of viral replication (Leoni et al., 2012). Thus, viral replication is not needed to initiate signaling mechanisms that could lead to ocular inflammation.

5.0 Conclusions

The innate immune response, following viral gene delivery vector injection into the primate eye, must be considered when exploring ocular gene delivery methods (Bennett, 2003; Ikeda et al., 2009; Liu et al., 1999). In this study we have demonstrated that the expression of several genes related to innate immunity and inflammation was increased following hrR3 challenge of macaque neural retina tissue. IL-6 was produced by cone photoreceptors and Muller cells, and NF κ B (p65) activation was detected in Muller cells, despite the lack of viral replication. Expression of the anti-inflammatory cytokine IL-10, localized to amacrine cells, was also increased after vector challenge. These data imply the existence of a pro/anti-inflammatory axis in the retina, whose balance may determine the extent of ocular inflammation and disease. The secretion of, and the number of cell types expressing IL-6, compared to IL-10, may skew this axis toward inflammation in the macaque neural retina and explain our previous findings (Liu et al., 1999). Further research involving retinal organ culture and animal studies will be needed to explore the roles that IL-6 and IL-10 play in

transient uveitis, and to determine the contribution of infiltrating cells to the amplification of the retinal inflammatory response.

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Abbreviations

HSV	herpes simplex virus
AdV	adenovirus
AAV	adeno-associated virus
NHP	non-human primate
IL	interleukin
TLR	Toll-Like Receptors
pfu	plaque forming units
PR	photoreceptors
ONL	outer nuclear layer
INL	inner nuclear layer
GCL	ganglion cell layer
ILM	internal limiting membrane
FITC-PNA	FITC conjugated <i>Arachis hypogea</i> lectin
NFκB	nuclear factor kappa-beta
gp130	signal-transducing β subunit glycoprotein 130
IL-6R	IL-6 α -receptor
IF	immunofluorescence
ON	overnight
RT	room temperature

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Highlights

- The HSV-1 gene delivery vector hrR3 induced IL-6 in Muller and cone cells
- hrR3 induced the anti-inflammatory cytokine IL-10 in amacrine cells
- NF κ B (p65) was activated in Muller cells but not in cone photoreceptors
- Vector replication was not required for cytokine induction or NF κ B (p65) activation
- A pro-/anti-inflammatory axis may determine the extent of vector induced uveitis

A.

B.

<u>Position</u>	<u>Gene</u>	<u>Fold-Increase</u>	<u>Position</u>	<u>Gene</u>	<u>Fold-Decrease</u>
A2	CASP1	4.0	A3	CCL2	-2.6
B1	CD80	2.7	A5	CCR4	-2.3
B2	CD86	2.7	C7	IL15	-1.6
C5	IL10	3.1	D7	IRF1	-1.8
C10	IL1A	3.4	D11	JAK2	-1.4
C11	IL1B	3.3	E1	JUN	-2.0
D5	IL6	5.9	E5	LYZ	-1.5
G7	TLR6	3.6	E9	MIF	-1.5
G8	TLR7	3.1	F2	NFKBIA	-2.1

Figure 1. Gene expression of HSV-1 hrR3 treated macaque neural retina tissue
 Results from Rhesus Macaque Innate and Adaptive Immune Response RT² Profiler PCR Array. A) Heat map represents fold-increase (red) or fold-decrease (green) of gene expression in hrR3 treated neural retina tissue versus media treated control tissue. B) Chart highlights the nine genes with greatest increase or decrease in gene expression.

Figure 2. IL-6 is expressed in photoreceptors

After overnight incubation with media (A, B, C) or hrR3 (D, E, F, G), cynomolgus macaque neural retina tissue was fixed, paraffin embedded and processed for immunofluorescence. Sections were treated with autofluorescence eliminator reagent prior to staining with no primary antibody (A) rabbit anti-IL-6 (B, D, F) rabbit anti-IL-6 plus goat anti-arrestin C (C, E) or rabbit anti-IL-6 plus FITC-PNA (G). The secondary antibodies included donkey anti-rabbit Alexa Fluor 594 and donkey anti-goat Alexa Fluor 488. Nuclei were visualized by Hoechst staining. Rhodamine (red) = IL-6; FITC (green) = Arrestin-C (panel C, E) or PNA (panel G); DAPI (blue) = nuclei. Photoreceptors (PR), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL). Arrows indicate IL-6 staining (B, D, F), co-localization of IL-6 and arrestin C (E), or co-localization of IL-6 and FITC-PNA (G).

Figure 3. Retinal Muller cells express IL-6

Cynomolgus macaque neural retina tissue treated overnight at 37°C with media or hrR3 was fixed, paraffin embedded, and processed for immunofluorescence. Sections were stained with no primary antibody (A) mouse anti-IL-6 (B, D), or mouse anti-IL-6 plus rabbit anti-vimentin (C, E). The secondary antibody mixture contained goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488. Nuclei were visualized by Hoechst staining. Three color photos represent as follows: Rhodamine (red) =IL-6; FITC (green) = vimentin; DAPI (blue) = nuclei. Photoreceptor layer (PR), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL). Arrows in panels B and D indicate IL-6 staining. Arrows in panels c and e indicate co-localization of IL-6 and vimentin.

Figure 4. IL-6 RNA is detected in the INL and ONL of macaque neural retina tissue

Cynomolgus macaque neural retina tissue was incubated overnight with hrR3. Tissue was fixed, embedded, sectioned, and pretreated as indicated. RNA in situ hybridization was performed using a negative control probe DapB (A, B, C), a positive control probe POLR2A (D, E, F), or a human IL-6 probe Hs-IL-6 (G, H, I). Arrows indicate binding of probe to target RNA, as visualized by chromogen precipitation.

Figure 5. IL-10 is expressed in amacrine cells

After overnight incubation with hrR3, cynomolgus macaque neural retina tissue was fixed, paraffin embedded, and processed for immunofluorescence. Sections were treated with autofluorescence eliminator reagent and stained overnight at 4°C with no primary antibody (A) mouse anti-IL-10 (B), rabbit anti-calretinin (C) or mouse anti-IL-10 and rabbit anti-calretinin (D) primary antibodies. The secondary antibodies included goat anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594. Nuclei were visualized by Hoechst staining. FITC (green) = IL-10, Rhodamine (red) = calretinin, DAPI (blue) = nuclei. Photoreceptors (PR), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL). Arrows in panel A indicate IL-10 staining. Arrows in panel B indicate calretinin staining. Arrows in panel C indicate co-localization of IL-10 and calretinin staining.

Figure 6. hrR3 activates NF B (p65) in Muller glial cells

Cynomolgus macaque neural retina tissue was incubated overnight with media (A) or hrR3 (B, C, D, E). Tissue was fixed, paraffin embedded, and processed for immunofluorescence. Sections were stained with goat anti-NF κ B (p65) (A, B, D), rabbit anti-vimentin (C), or both antibodies (E). Secondary antibodies included donkey anti-goat Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 488. Nuclei were visualized by Hoechst staining. Rhodamine (red) = NF κ B (p65), FITC (green) = vimentin, DAPI (blue) = nuclei. Arrows indicate nuclei of INL (A), double labeling of Hoechst stained nuclei with NF κ B (p65) (B, D), vimentin stained Muller cells (C), or vimentin-stained Muller cells with NF κ B (p65) positive nuclei (E). Photoreceptors (PR), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL).

Figure 7. hrR3 binds to and enters cultured macaque neural retina tissue but does not replicate Cynomolgus macaque neural retina tissue was challenged overnight at 37°C with hrR3. Tissue was fixed, paraffin embedded, and processed for immunofluorescence as described. Sections were stained with no primary antibody (A), anti-ICP4 (B), anti-ICP8 (C), anti-gD (D), anti-VP5 (E), or VP16 (F) followed by donkey anti-mouse AlexaFluor 488. Nuclei were visualized by Hoechst staining. Arrows indicate gD staining (D), VP5 staining (E), or VP16 staining (F). Photoreceptors (PR), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL), internal limiting membrane (ILM).