Interferon-gamma, Macrophages, and Virus Spread after HSV-1 Injection

Heather M. Cathcart,¹ Mei Zheng,¹ Jason J. Covar,¹ Yi Liu,¹ Robert Podolsky,² and Sally S. Atherton¹

PURPOSE. After unioocular anterior chamber (AC) injection of HSV-1, the anterior segment of BALB/c mice becomes inflamed and infected; however, virus does not spread from the anterior segment to cause retinitis in the injected eye. The purpose of these studies was to determine whether interferon (IFN)- γ and Mac-1⁺ cells play a role in preventing direct anterior-to-posterior spread of HSV-1 in the injected eye.

METHODS. One AC of adult female BALB/c mice was injected with HSV-1 (KOS). The location of IFN- α , IFN- β , and IFN- γ in the injected eye was determined by immunofluorescence, and mRNA expression was quantified by qPCR. Injected eyes of IFN- γ knockout or clodronate-treated macrophage-depleted mice were examined to determine whether the absence of IFN- γ or Mac-1⁺ macrophages affected the sites or timing of virus spread.

RESULTS. IFN- α , IFN- β , and IFN- γ were observed in the anterior segment of injected eyes through 72 hours and mRNA levels of IFN- β and IFN- γ were increased in virus-infected eyes 48 to 120 hours after infection. However, the absence of IFN- γ or macrophages did not affect either the sites or the timing of HSV-1 infection in injected eyes.

CONCLUSIONS. Protection of the retina of the injected eye does not depend on a single cell type or cytokine. In addition, in the eye, as in other sites of the body, there are redundancies in the innate response to virus infection. (*Invest Ophthalmol Vis Sci*. 2011;52:3984–3993) DOI:10.1167/iovs.10-6449

During the innate and adaptive immune response to virus infection, major histocompatibility complex class I and II induction and activation, as well as the differentiation of T cells and the activation of macrophages, are influenced by interferons (IFN-s).¹ IFN-s are produced by the cells of the innate immune system: macrophages, neutrophils, plasmacytoid dendritic cells, and natural killer (NK) cells.^{2–11} Type 1 (IFN- α and IFN- β) and type 2 (IFN- γ) IFN-s impede viral replication through similar cell signaling mechanisms, inducing the production of proteins that inhibit translation and cell growth, induce apoptosis, and promote the downregulation of mRNA in virus-infected cells.^{9,12} Several studies in which IFN- γ was overexpressed or in which IFN- γ -deficient mice were infected with HSV-1^{13–19} established that IFN- γ plays an important role

in viral pathogenesis. For example, when compared with wild-type littermates that developed bilateral disease, transgenic mice that overexpressed IFN- γ in the eye were protected from HSV-1 infection in the uninfected contralateral eye after unioocular intravitreal injection of HSV-1.¹³ In a model of herpes stromal keratitis (HSK), IFN- γ -deficient mice were more susceptible to encephalitis, and virus persisted longer in their corneas than it did in control mice.¹⁴ IFN- γ also has been shown to play an important role in the clearance of HSV-1.^{13–19}

Macrophages participate actively in host resistance to HSV-1 and produce cytokines that play a major role in the recruitment of other immune cells. Furthermore, macrophages have been identified as the major cell type infiltrating the eye after HSV-1 inoculation.^{6,20,21} Inflammation induced by macrophages can accelerate corneal destruction; however, macrophages also play an important role in restricting HSV-1 growth after corneal infection and are important for enhancing the T-cell-mediated adaptive immune response.^{22–24}

Acute retinal necrosis (ARN), first described as Kirisawa uveitis, is a potentially blinding disease caused by herpes family viruses.²⁵ ARN is characterized in humans by occlusive retinal vasculitis, prominent inflammation of the anterior segment and vitreous, and localized retinal necrosis that are often followed by optic neuritis and retinal detachment.^{26–28} Innate immune cell types have been implicated in the pathogenesis of ARN.^{29–32} In the mouse model of ARN, inoculation of HSV-1 (KOS) through the AC route results in acute inflammation of the anterior segment, involving the cornea, iris, and ciliary body, with cell infiltration beginning 24 hours postinoculation (p.i.). Subsequently, the virus spreads through the CNS to the optic nerve and retina of the uninoculated contralateral eye, causing retinal necrosis.^{33–35} Curiously, the retina of the injected eye is spared despite there being no anatomic barrier to limit HSV-1 transmission directly from the anterior segment to the retina. Studies from our laboratory suggest NK cells, macrophages, and PMNs respond to and modulate virus infection in the injected eye, but the roles of the cytokines produced by these immune cell types are not well defined.^{6,29,30} Therefore, the goal of these studies was to determine whether IFN- γ and Mac-1⁺ macrophages play a role in preventing the spread of the virus from the anterior segment to the posterior segment (retina) in the injected eye early after AC inoculation of HSV-1.

METHODS

Animals

Adult female BALB/c mice, 6 to 12 weeks old (Taconic, Germantown, NY) or C.129S7 (B6)-IFN- γ ^{tm1Ts/J} (IFN- γ ^{-/-} mice on a BALB/c background) and BALB/cJ (IFN- γ ^{+/+}) control mice (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. The mice were housed in accordance with National Institutes of Health guidelines. All study procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the

From the ¹Department of Cellular Biology and Anatomy and the ²Center for Biotechnology and Genomic Medicine, Medical College of Georgia, Augusta, Georgia.

Supported by National Institutes of Health Grant EY006012 (SSA). Submitted for publication August 20, 2010; revised January 3, 2011; accepted February 3, 2011.

Disclosure: H.M. Cathcart, None; M. Zheng, None; J.J. Covar, None; Y. Liu, None; R. Podolsky, None; S.S. Atherton, None

Corresponding author: Sally S. Atherton, Department of Cellular Biology and Anatomy, Medical College of Georgia, 1459 Laney Walker Boulevard, Augusta, GA 30912; satherton@georgiahealth.edu.

Institutional Animal Care and Use Committee of the Medical College of Georgia. The mice were maintained on a 12-hour light/12-hour-dark cycle and were given unrestricted access to food and water. Animals were anesthetized with 0.5 to 0.7 mL/kg of a mixture of 42.9 mg/mL ketamine, 8.57 mg/mL xylazine, and 1.43 mg/mL acepromazine before all experimental manipulations. Experiments were repeated at least once.

Virus

The KOS strain of HSV-1 was used in all experiments. Stock virus was prepared by low multiplicity of infection (0.1 PFU/cell) passage of Vero cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS (Hyclone, Logan, UT) and antibiotics. The titer of the virus stock was determined by plaque assay on Vero cells. Aliquots of stock virus were stored at -80°C , and a fresh aliquot was thawed and diluted for each experiment.

AC Inoculation of HSV-1

Mice were anesthetized and inoculated through the AC route, as previously described.³³ The right eye was proptosed, and 3×10^4 to 6×10^4 PFU of HSV-1 (KOS) in a volume of 2 μL were injected into the AC with a 30-gauge needle attached to a 100- μL microsyringe (Hamilton, Reno, NV). The inoculum was prepared by diluting virus stock in DMEM with antibiotics. The AC of mock-infected mice was injected with 2 μL uninfected Vero cell extract diluted in DMEM with antibiotics.

Macrophage Depletion

For macrophage depletion, BALB/c mice were injected intravenously with 150 μL and subconjunctivally ipsilateral to the side of virus injection with 30 μL dichloromethylene-bisphosphonate liposomes (Cl_2MBP , clodronate; Encapsula, Nashville, TN) on days -1 and 2 , a

regimen that has been shown to deplete macrophages *in vivo*.^{23,24,36,37} Mock-depleted mice were injected intravenously with 150 μL and subconjunctivally ipsilateral to the side of virus injection with 30 μL liposomes containing PBS on days -1 and 2 . On day 0, all mice were inoculated with 3×10^4 to 6×10^4 PFU of HSV-1 (KOS) in the AC of one eye. Macrophage-depleted and mock-depleted mice were anesthetized and killed by cervical dislocation, and the injected eye was enucleated 48 or 72 hours p.i.

Flow Cytometric Analysis of Mac-1⁺ cells

Single-cell suspensions were prepared from the eyes of HSV-1-infected mice, HSV-1-infected mice treated with Cl_2MBP liposomes, HSV-1-infected mice treated with PBS liposomes, and uninfected untreated mice at different days p.i. Three whole eyes for each group were pooled and incubated in 58.5 U/mL of collagenase IV (Sigma-Aldrich, St. Louis, MO) in HBSS (Cellgro Mediatech, Herndon, VA) for 1 hour at 37°C in an atmosphere of 5% CO_2 , and ocular cells were collected by extrusion through a 70- μm nylon cell strainer (BD Falcon, Bedford, MA).³⁸ Cells were suspended in HBSS, pelleted by centrifugation at 250g at 4°C for 5 minutes, and resuspended in PBS containing 1% FBS before counting.

Eye cell suspensions were blocked with 10% mouse serum (Sigma-Aldrich) diluted in staining buffer (PBS containing 1% FBS) for 15 minutes at 4°C . The antibodies FITC-anti-mouse CD11b (clone M1/70, integrin α_M chain, Mac-1 α chain; BD PharMingen, San Diego, CA) and FITC-rat IgG_{2b}, κ isotype control (BD PharMingen), were used to determine the extent of Mac-1⁺ cell depletion. Flow cytometry of stained cell samples was performed (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ), and flow cytometry data were collected and analyzed using CellQuest software (Becton Dickinson).

Flow cytometry results were analyzed by comparing acquisition dot plots of total events (20,000 counts), initially sorting cells based on size

FIGURE 1. Representative photomicrographs of the ciliary body (CB) of the injected eye showing the location (arrowheads) of IFN- α^+ cells in normal control animals (A–C) and in virus-injected animals 24 (D–F), 48 (G–I), and 72 (J–L) hours p.i. The cornea (C), iris (I), and retina (R) are indicated. Original magnification (A–L), $\times 200$.

and granularity. Small cells with little granularity (i.e., dead or dying cells) were gated out. Histogram plots that included relatively large granular cells were used for subsequent analysis of single-color flow cytometry. FITC-Mac-1⁺ cells were identified, and percentages were calculated by comparing plots of unstained cells, isotype control antibody-stained cells, and Mac-1⁺-stained cells for each time point in all groups. The percentage of Mac-1⁺ cells was calculated by subtracting isotype-matched control staining from the percentage of cell marker staining. The number of Mac-1⁺ cells was determined from adjusted percentages of cell marker staining and based on the total number of cells. The total number of cells per three eyes was counted immediately after collagenase treatment but before cell marker staining. Percentage depletion was calculated by comparing the CL₂MBP-treated HSV-1-infected group with the PBS liposome-treated HSV-1-infected group as follows: $[100 - (\text{total Mac-1}^+ \text{ cells CL}_2\text{MBP treated} / \text{total Mac-1}^+ \text{ cells mock treated} \times 100)]$.

Immunohistochemistry

Mice were deeply anesthetized and perfused transcardially with PBS for approximately 3 minutes. After perfusion, the injected eye of each mouse was removed, embedded in optimal cutting temperature compound (Tissue-Tek; EMS, Hatfield, PA), and immediately stored at -80°C . Sections measuring 8 μm to 10 μm were prepared on positively charged slides (Fisher Scientific, Pittsburgh, PA) using a cryostat (Microm HM505E; EquipNet, Canton, MA). Frozen eye sections were fixed with 4% paraformaldehyde, washed in PBS, and blocked in a solution of 10% NGS (Vector Laboratories, Burlingame, CA), 1% BSA (Fisher Scientific) and 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 2 hours. For the detection of IFN- α , IFN- β , or IFN- γ , sections were incubated with rat anti-mouse IFN- α , rabbit anti-mouse IFN- β (Chemicon International, Billerica, MA), or rat anti-mouse IFN- γ (RMMG-1; PBL Biomedical Laboratories, Piscataway, NJ), washed in PBS, and incu-

bated with Texas Red anti-rat (Jackson ImmunoResearch, West Grove, PA) or Texas Red anti-rabbit (Vector Laboratories), washed with PBS again, and mounted (VectaShield containing DAPI; Vector Laboratories). Slides were examined using a fluorescence microscope and images were captured using SPOT Advanced (Diagnostic Instruments, Sterling Heights, MI) or AxioVision 4.6 (Carl Zeiss Meditec, Jena, Germany) computer programs. A total of 10, 11, and 11 eyes (one eye per mouse) were examined for IFN⁺ staining at 24, 48 and 72 hours p.i., respectively.

Virus Staining in the Ciliary Body and Central Retina

For semi-quantitative measurement of the area of HSV-1⁺ staining in CL₂MBP-treated and IFN- γ ^{-/-} mice, frozen sections were fixed with 4% paraformaldehyde, washed with PBS, and blocked with 5% donkey serum (Sigma-Aldrich) for 2 hours. Sections were then incubated for 1.5 hours at room temperature with unconjugated goat anti-HSV-1 (Accurate Chemical and Scientific Corporation, Westbury, NY), washed in PBS, incubated with FITC-anti-goat (Jackson ImmunoResearch), and mounted (VectaShield-containing DAPI). The ciliary body and central retina of the injected eye were examined using a digital monochromatic camera (Cool Snap; Roper Scientific, Trenton NJ), and MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Images captured at 200 $\times$ magnification were used to quantify the area of virus staining around the ciliary body and the central retina. One representative section from each slide was captured for the ciliary body and for the central retina adapted from McCluskey³⁹. Stained pixels were digitally marked on each image, and the percentage of stained pixels/standard area was determined. A mixed-model ANOVA was used to compare percentage area of virus staining in the ciliary body or central retina in IFN- γ ^{+/+} versus IFN- γ ^{-/-} mice and in CL₂MBP-treated versus PBS liposome (mock)-treated mice. Statistical

FIGURE 2. Representative photomicrographs of the ciliary body (CB) of the injected eye showing the location (arrowheads) of IFN- β ⁺ cells in normal control animals (A–C) and in virus-injected animals 24 (D–F), 48 (G–I), and 72 (J–L) hours p.i. The cornea (C), iris (I), and retina (R) are indicated. Original magnification, (A–L), $\times 200$.

analysis was performed (SAS 9.1; SAS Institute, Cary, NC), and data were plotted graphically. A total of 18 eyes from IFN- $\gamma^{+/-}$ mice and 16 eyes from IFN- $\gamma^{-/-}$ mice were examined at 72 and 120 hours p.i. Twenty-one eyes from mock-treated mice and 19 eyes from Cl₂MBP-treated mice were examined at 48 and 72 hours p.i.

RNA Isolation and qPCR

Whole infected eyes were collected, frozen, and stored in liquid nitrogen. Five eyes (one eye per mouse) were pooled and pulverized (Bessman Tissue Pulverizer; Fisher Scientific), and RNA was isolated from the pulverized tissue. Relative mRNA levels of IFN- α (subtypes 2, 4, 9, and 11), IFN- β , and IFN- γ were measured by real-time RT-PCR using total RNA of healthy control noninjected eyes, mock-injected eyes 48 hours p.i., and HSV-1-injected eyes at 24, 48, 72, and 120 hours p.i. cDNA was reverse transcribed and amplified from 0.2 μ g total RNA for each sample (RT² First-Strand Kit; SA Biosciences, Frederick, MD). Before cDNA synthesis, each sample was subjected to a genomic DNA elimination step. The amplified cDNA was mixed with distilled deionized water and quantitative PCR (qPCR) master mix (2 $\times$ RT²; SA Biosciences) and was aliquotted (10 μ L per well) into a 384-well PCR array plate with prehybridized primers (RT² profiler mouse custom plate; SA Biosciences); each sample was analyzed in triplicate. Real-time PCR reaction and detection were carried out using an real-time thermal cycler (ABI 7900HT; Applied Biosystems, Foster City, CA) with 1 cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold value for each gene from various samples was standardized against the average of three housekeeping genes: *GAPDH*, *HPRT1*, and *PGK1*. Additionally, for each sample tested, there was a mouse genomic DNA control well that detected any genomic DNA contamination. Data were analyzed using SDS software (Applied Biosystems) and the PCR array data analysis Web portal (<http://www.SABiosciences.com/pcrarraydataanalysis.php>).

A mixed-model ANOVA was used to compare the average ΔC_t value from two independent experiments for each gene between time points after injection. Pairwise comparisons were made using Tukey's test and statistical analysis software (SAS 9.1; SAS Institute), and results were plotted graphically.

RESULTS

IFN- α^{+} , IFN- β^{+} , and IFN- γ^{+} Cells in the Anterior Segment

At 24 hours p.i., IFN- α^{+} (Fig. 1), IFN- β^{+} (Fig. 2), and IFN- γ^{+} (Fig. 3) cells were observed in the anterior segment by immunofluorescence in 80% (8 of 10), 82% (9 of 11), and 82% (9 of 11) of the mice, respectively. IFN⁺ cells were observed initially in the limbus and then in the ciliary body, iris, and cornea, through 72 hours p.i. in 100% of the animals observed (Figs. 1–3). IFN- α^{+} , IFN- β^{+} , and IFN- γ^{+} cells were not observed in normal control mice (Figs. 1–3) and were only rarely observed in mock-injected mice (not shown).

Quantification of IFN in the Injected Eye by qPCR

qPCR was used to determine whether the expression of IFN- α (subtypes 2, 4, 9, and 11), IFN- β , or IFN- γ genes was increased in the eye early after AC inoculation with HSV-1 compared with the eyes of normal control and mock-injected mice. There was no significant difference in ΔC_t values between normal control mice and mock-injected mice for any of the genes tested (including the housekeeping genes *GAPDH*, *HPRT1*, and *PGK1*) ($P > 0.04$ for all). IFN- β gene expression was increased in the eyes of virus-infected mice at 48, 72, and 120 hours p.i. compared with normal control mice. The average ΔC_t value of normal control mice was 10.14, but there was a significant

FIGURE 3. Representative photomicrographs of the ciliary body (CB) of the injected eye showing the location (arrowheads) of IFN- γ^{+} cells in normal control animals (A–C) and in virus-injected animals 24 (D–F), 48 (G–I), and 72 (J–L) hours p.i. The cornea (C), iris (I), and retina (R) are indicated. Original magnification, (A–L), $\times 200$.

FIGURE 4. IFN gene expression (fold upregulation) in mock-injected eyes 48 hours p.i. and virus-injected eyes 24, 48, 72, and 120 hours p.i. compared with normal control eyes (average of two independent experiments shown). To determine the fold change in gene expression, the normalized expression of the gene of interest (GOI) in the experimental sample was divided by the normalized expression of the same GOI in the control sample (normal control): $2^{-\Delta\Delta C_t} = \frac{2^{-\Delta C_t(\text{experimental})}}{2^{-\Delta C_t(\text{control})}}$.

decrease in ΔC_t values, in HSV-1-infected mice at 48, 72, and 120 hours p.i. (4.92 [$P = 0.02$], 5.35 [$P = 0.04$], and 4.49 [$P = 0.02$]), respectively, corresponding to a 37.16-, 27.65-, and 50.05-fold upregulation of IFN- β 1 in the eyes of HSV-1-infected mice (Fig. 4). IFN- γ gene expression was also increased in the eyes of virus-infected mice. The average ΔC_t value of normal control mice was 10.28, but there was a significant decrease in ΔC_t values in HSV-1-infected mice at 48, 72, and 120 hours p.i. (3.21 [$P = 0.001$], 3.77 [$P = 0.002$], and 3.65 [$P = 0.002$]),

corresponding to a 133.99-, 91.21-, and 99.30-fold upregulation of IFN- γ in the eyes of HSV-1-infected mice (Fig. 4). IFN- α gene expression was not increased in the eyes of virus-infected mice at any time point for any of the IFN- α subtypes tested compared with normal control animals ($P > 0.12$; Fig. 4).

HSV-1⁺ Staining in IFN- γ ^{-/-} and IFN- γ ^{+/+} Mice

Virus staining and area of virus staining were used to evaluate the spread of HSV-1 from the anterior-to-posterior segment and

FIGURE 5. Representative photomicrographs of the ciliary body (CB) in the injected eye showing HSV-1⁺ staining in IFN- γ ^{+/+} (A–C) or IFN- γ ^{-/-} (D–F) normal control (A, D) and virus-infected mice at 72 (B, E) and 120 (C, F) hours p.i. Average percentage area of HSV-1⁺ staining in the CB (IFN- γ ^{+/+}, $n = 18$; IFN- γ ^{-/-}, $n = 16$). The cornea (C), and iris (I) are also shown. * $P = 0.03$, significantly different from IFN- γ ^{+/+} mice (G). Statistical significance was evaluated using a mixed-model ANOVA. Data represent the average of two independent experiments. Error bars represent 95% confidence intervals. Original magnification, $\times 200$.

to determine whether the lack of IFN- γ affected either the pattern of HSV-1 spread or the area infected in the eyes of IFN- $\gamma^{-/-}$ mice compared with IFN- $\gamma^{+/+}$ mice. Previous studies failed to show a difference in virus titer in sites of HSV-1 infection early after corneal inoculation in IFN- $\gamma^{-/-}$ mice⁴⁰⁻⁴²; therefore, semiquantitative staining measurements were used to assess the extent of virus infection in the anterior and posterior segments. In 100% of both IFN- $\gamma^{-/-}$ and IFN- $\gamma^{+/+}$ mice at 48, 72, and 120 hours p.i., HSV-1⁺ staining was observed in the anterior segment (ciliary body, iris, and cornea).⁴³ The pattern of HSV-1 staining in the ciliary body of IFN- $\gamma^{-/-}$ mice was similar to that seen in IFN- $\gamma^{+/+}$ mice; however, more of the ciliary body was HSV-1⁺ in IFN- $\gamma^{-/-}$ mice (Figs. 5A-F). As shown in Figure 5G, analysis of the overall pattern of virus staining through the course of the experiment (at 72- and 120-hour time points) revealed that the extent of virus infection in the area of the ciliary body was significantly different between IFN- $\gamma^{-/-}$ mice and IFN- $\gamma^{+/+}$ mice (IFN- $\gamma^{-/-}$ mice, 9.59%; IFN- $\gamma^{+/+}$ mice, 5.59%; $P = 0.03$).

Although destructive retinitis is rarely observed in the injected eye of either mouse strain and although the RPE and ganglion cells of the central retina are more often HSV-1⁺ in IFN- $\gamma^{-/-}$ mice than in IFN- $\gamma^{+/+}$ mice,⁴³ in these studies HSV-1⁺ staining was observed in the central retina of both IFN- $\gamma^{-/-}$ and IFN- $\gamma^{+/+}$ mice at 72 and 120 hours p.i. However, there was no significant difference in virus staining at 72 and 120 hours p.i. in the central retina between IFN- $\gamma^{-/-}$ mice and IFN- $\gamma^{+/+}$ mice (IFN- $\gamma^{-/-}$ mice, 2.51%; IFN- $\gamma^{+/+}$ mice, 2.08%; $P = 0.80$). As expected, virus-positive cells were not observed in the eyes of uninjected control IFN- $\gamma^{-/-}$ mice (0 of 4) or control IFN- $\gamma^{+/+}$ mice (0 of 4; Fig. 5) or in the eyes of mock-injected IFN- $\gamma^{-/-}$ mice (0 of 5) or mock-injected IFN- $\gamma^{+/+}$ mice (0 of 5; not shown).

Depletion of Mac-1⁺ Cells

To investigate the role played by Mac-1⁺ cells in HSV-1 infection of the injected eye, Mac-1⁺ cells were depleted by intravenous and subconjunctival injection of clodronate-containing liposomes. The extent of Mac-1⁺ depletion was assessed by flow cytometry of the injected eye of HSV-1-infected BALB/c mice, PBS liposome-treated HSV-1-infected mice, Cl₂MBP liposome-treated HSV-1-infected mice, and uninfected BALB/c mice at 48 and 72 hours p.i. (Figs. 6, 7). After treatment with Cl₂MBP liposomes, approximately 87% of Mac-1⁺ cells in the injected eye were depleted at 48 hours p.i. (Table 1). After continued treatment with Cl₂MBP liposomes, approximately 52% Mac-1⁺ cells in the injected eye were depleted at 72 hours p.i. (Table 1).

HSV-1⁺ Staining in the Eyes of Cl₂MBP-Treated versus Mock-Treated Mice

To determine whether the depletion of Mac-1⁺ cells affected either the pattern of HSV-1 spread or the extent of infection in the injected eye, injected eyes of Cl₂MBP-treated and mock-depleted mice were stained for virus. HSV-1⁺ staining was observed in the anterior segment (ciliary body, iris, and cornea) in 90% (9 of 10) and 91% (9 of 11) of mock-treated mice and in 100% (9 of 9 and 11 of 11) of Cl₂MBP-treated mice at 48 and 72 hours p.i., respectively. The pattern of HSV-1 staining in the ciliary body of Cl₂MBP-treated mice was similar to that seen in mock-treated mice (Figs. 8A-D); however, more of the ciliary body was HSV-1⁺ in Cl₂MBP-treated mice (15.36%) compared with mock-treated mice (10.39%). There was a significant difference between the area of the ciliary body that was HSV-1⁺ when treatments were compared by combining the 48- and 72-hour time points ($P = 0.005$; Fig. 8E).

FIGURE 6. Flow cytometric histograms of Mac-1⁺ cells isolated from the eyes of normal control mice, HSV-1-injected mice, mock-treated/HSV-1-injected mice, and Cl₂MBP-treated/HSV-1-injected mice ($n = 3$) 48 hours p.i. Cells were stained with FITC-conjugated anti-mouse Mac-1 (green line) or FITC-conjugated rat IgG2b,κ (solid black). Percentages are those of isotype-matched control staining subtracted from those of cell marker staining. Data are representative of two independent experiments.

FIGURE 7. Flow cytometric histograms of Mac-1⁺ cells isolated from the eyes of normal control mice, HSV-1-injected mice, mock-treated/HSV-1-injected mice, and CI₂MBP-treated/HSV-1-injected mice ($n = 3$) 72 hours p.i. Cells were stained with FITC-conjugated anti-mouse Mac-1 (green line) or FITC-conjugated rat IgG2b,κ (solid black). Percentages are those of isotype-matched control staining subtracted from those of cell marker staining. Data are representative of two independent experiments.

HSV-1⁺ staining was observed in the central retina in 22% (2 of 9) and 55% (6 of 11) of CI₂MBP-treated mice at 48 and 72 hours p.i., respectively. HSV-1 staining was observed in the central retina in 11% (1 of 9) and 55% (6 of 11) of mock-treated mice at 48 and 72 hours p.i. The amount of HSV-1 staining in the central retina was similar between CI₂MBP-treated mice (0.64%) and mock-treated mice (0.56%) (not shown). Although destructive retinitis was not observed, HSV-1⁺ RPE and ganglion cells were seen in the central retina in both CI₂MBP-treated mice and mock-treated mice. There was no significant

difference between area of the central retina that was HSV-1⁺ when the combined 48- and 72-hour time points were compared between the two groups ($P = 0.96$; not shown).

DISCUSSION

Interferons are soluble antiviral cytokines that upregulate proteins such as protein kinase R (PKR), adenosine deaminase (ADAR), and guanylate-binding proteins (GBP), which inhibit viral protein synthesis and viral replication and induce apoptosis in virus-infected cells through intracellular JAK-STAT signaling pathways.^{1,12} Virtually all cell types produce IFN-α and IFN-β; however, plasmacytoid dendritic cells are the predominant producers of these IFNs during viral infection.^{2,9–11} Macrophages, NK cells, and neutrophils produce IFN-γ and play a role in innate immune resistance to HSV ocular infections.^{3–8,20,29,30,44} Studies using a mouse model of ARN have established the route and timing of virus spread from the ipsilateral AC through the CNS to the contralateral (uninjected) eye where retinitis occurs.^{33–35} However, the cells and immunomodulators that protect the retina of the injected eye are not well understood. This study investigated whether IFN-γ and Mac-1⁺ macrophages play a role in the protection of the ipsilateral retina after unioocular AC inoculation of HSV-1.

IFN-α⁺, IFN-β⁺, and IFN-γ⁺ cells were observed in the anterior segment of HSV-1-injected eyes through 72 hours p.i. IFN-γ is observed in the cornea of HSV-1-infected mice beginning at 3 days p.i. and colocalizes with Mac-1⁺ cells in the anterior segment early after AC HSV-1 inoculation.^{6,45} In our studies, qPCR showed that both IFN-β and IFN-γ were upregulated in virus-injected eyes between 48 and 120 hours p.i., and these results correlated well with the immunofluorescence studies. However, it is probably not surprising that the upregu-

TABLE 1. Macrophage Depletion at 48 and 72 Hours after Infection in the Injected Eyes of HSV-1-Infected BALB/c Mice

Hours	Group	Eye Cells	Mac-1 ⁺	Mac-1 ⁺ (%) [*]	Depletion (%)
48	1	1.71×10^7	0.00×10^0	0.00 [*]	87.23 [†]
	2	1.33×10^7	2.58×10^6	19.37	
	3	2.84×10^7	7.32×10^6	25.75	
	4	2.29×10^7	9.35×10^5	4.08	
72	1	2.03×10^7	3.93×10^5	1.93	52.39
	2	4.25×10^7	1.85×10^7	43.51	
	3	3.40×10^7	1.40×10^7	41.08	
	4	2.38×10^7	6.66×10^6	28.02	

Data are representative of two independent experiments. Group 1, normal control BALB/c mice; Group 2, HSV-1-infected BALB/c mice; Group 3, PBS liposome-treated/HSV-1-infected BALB/c mice; Group 4, CI₂MBP liposome-treated/HSV-1-infected BALB/c mice.

^{*} % Mac-1 = Isotype-matched control staining subtracted from the percentage of cell marker staining.

[†] Compared with mock liposome-treated HSV-1-infected (group 3) [100-(total Mac-1⁺ cells CI₂MBP-treated/total Mac-1⁺ cells mock treated $\times 100$)].

FIGURE 8. Representative photomicrographs of the ciliary body in the injected eye showing HSV-1⁺ staining in mock-treated/HSV-1-infected (A, B) or Cl_2 MBP-treated/HSV-1-infected mice (C, D) at 48 and 72 hours p.i. Average percentage area of HSV-1⁺ staining in the ciliary body (mock treated, $n = 21$; Cl_2 MBP treated, $n = 19$) (E). * $P = 0.005$, significantly different from mock treated. Statistical significance was evaluated using a mixed-model ANOVA. Data represent the average of two independent experiments. Error bars represent 95% confidence intervals. Original magnification, $\times 200$.

lation of IFN- α was not detected because only 4 of the 14 known subtypes of mouse IFN- α were examined. In addition, because of the extensive homology among the IFN- α subtypes, analysis of individual gene expression is problematic.⁴⁶

IFN- γ plays an important role in viral clearance during active infection, as demonstrated by studies involving the overexpression of IFN- γ and the virus infection of IFN- γ -deficient mice.^{13–16,18,19,42} However, previous studies did not show a difference in virus titer in sites of HSV-1 infection early after corneal inoculation of IFN- $\gamma^{-/-}$ mice.^{40–42} In the studies reported herein, we measured the area of virus staining to semiquantitatively assess the extent of infection in the eye. Although the absence of IFN- γ did not affect the spread of HSV-1 from the anterior segment to the posterior segment (central retina) in the injected eye, there was more virus staining in the ciliary body and the RPE, and ganglion cells of the central retina were more often HSV-1⁺ in IFN- $\gamma^{-/-}$ mice than in wild-type mice after unocular AC inoculation. It has been suggested that IFN- γ synergizes with IFN- α and IFN- β to inhibit HSV-1 replication in vivo and that this interaction inhibits virus more effectively than activation of a single signaling pathway.^{47,48} In addition, the absence of IFN- γ alone may have a greater effect on persistent infection because of a delay in activation of adaptive immune T cells in IFN- $\gamma^{-/-}$ mice than on acute infection, which can still be modulated by IFN- α and IFN- β .⁴⁹

We previously observed IFN- γ -producing Mac-1⁺ cells in the anterior segment of the injected eye through 72 hours p.i. and proposed that the Mac-1⁺ cells are microglia that proliferate and contribute to early protection of the ipsilateral ret-

ina.^{6,43} To investigate the role of Mac-1⁺ cells in HSV-1 infection of the injected eye, depletion experiments were performed using both intravenous and subconjunctival injections of clodronate-containing liposomes, which results in the depletion of Mac-1⁺ cells in the eye. Depletion studies have shown that macrophages attenuate HSV-1 infection in in vivo models of HSK and in the contralateral eyes of mice with ARN.^{22–24,32} Mac-1⁺ cells were depleted in the eye; however, we noted that depletion was less effective after the second injection on day +2 (Table 1), possibly because of continued stimulation of the mouse immune response by repeated injections of Cl_2 MBP liposomes and ocular injection of virus within a 4-day period. This may also reflect the fact that total cells were counted immediately after collagenase treatment but before cell marker staining, and the number of Mac-1⁺ cells was based on original total cell counts. Subsets of neutrophils, macrophages, myeloid dendritic cells, NK cells, microglia, and B cells express the complement receptor (CR3) Mac-1⁺ antigen,^{50–54} and these cell types may be present in the eye. However, the Mac-1⁺ cells observed in the eye are not likely to be F4/80⁺, CD11c⁺, CD49b⁺, CD8⁺ or CD4⁺ as well because previous studies from our laboratory demonstrated that few, if any, of these cells are detected in the eye before day 4 p.i.⁶ Neutrophils have been observed in the HSV-1-injected ipsilateral eye before day 3,³⁰ and it is possible that Mac-1⁺ cells represent a subpopulation of these Gr-1⁺ cells.

Although the pattern of HSV-1 staining in the ciliary body of Cl_2 MBP-treated and mock-treated mice was similar in these studies, the area of HSV-1⁺ staining was increased in the ciliary body of the Cl_2 MBP-treated mice compared with mock-treated

mice. HSV-1⁺ staining was observed in the central retina of both CL₂MBP-treated mice and mock-treated mice but, in contrast to the observations in the ciliary body, there was no difference in the area of HSV-1⁺ staining, and destructive retinitis was not observed in either CL₂MBP-treated mice or mock-treated mice. Studies suggest that macrophages in the eye are important for initiating cell-mediated immunity.^{23,24} Although macrophages are important phagocytic cells during the innate immune response, it is likely that macrophage depletion impacts antigen presentation, which, in turn, reduces the production of cytokines and eventually interferes with or prevents the activation of T cells.^{23,24,55}

IFN- γ and Mac-1⁺ cells in the anterior segment of the injected eye are well positioned to mediate protection of the retina of this eye.^{6,21,45,56,57} However, in these studies, depletion of a single cytokine or cell type did not result in panretinal HSV-1 infection, suggesting, perhaps not surprisingly, that protection of the retina of the injected eye is not dependent on single cell types or on a single cytokine. Many biological systems have some measure of redundancy, and it is likely that the innate (early) response of the eye to intraocular HSV-1 infection is no different. In the eye, microglia, macrophages, dendritic cells, and neutrophils and their products likely mediate early protection in the absence of one cell type or immunomodulator. The role of Mac-1⁺ cells and IFN- γ in preventing intraocular virus spread in human patients is not known, but, similar to what has been observed in the mouse, there are multiple immunomodulators in humans that contribute to protection against virus infection and that may explain why few patients with anterior HSV uveitis develop a concurrent retinal infection.

Acknowledgments

The authors thank Jeanene Pihkala and Brendan Marshall for assistance with flow cytometry.

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