Infiltrating Cells and IFNγ Production in the Injected Eye after Unilocular Anterior Chamber Inoculation of HSV-1

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PURPOSE. After unilocular anterior chamber (AC) inoculation with HSV-1, the anterior segment of the injected eye becomes inflamed and infected; however, virus does not spread from the anterior segment and infect the retina of the injected eye. The purpose of this study was to identify early infiltrating cells and to determine whether infiltrating cells produce interferon (IFNy).

METHODS. Euthymic, female, BALB/c mice were injected in one AC with 3 x 10^4 PFU of HSV-1 (KOS) in a volume of 2 μL. Mice from each group were killed at 12, 24, 36, 48, and 72 hours post injection (pi), the eyes were enucleated, and frozen sections were stained with antibodies specific for IFNγ, Mac-1 (CD11b), CD49b, F4/80, CD4, CD8, and CD11c. The same antibodies were also used to stain single-cell suspensions of ocular cells for flow cytometry.

RESULTS. In the anterior segment of the injected eye, the ciliary body, and iris were virus infected and inflamed, and infiltrating cells increased throughout the period of observation. Mac-1^+ , CD49b^+ , and F4/80^- cells colocalized with IFNγ in the anterior segment as early as 12 hours pi, and the percentage of Mac-1^+ cells increased in the injected eye beginning at 24 hours pi and continued to 72 hours pi.

CONCLUSIONS. Taken together, these results demonstrate that Mac-1^+ cells are important IFNγ-producing cells in the injected eye before day 3 and suggest that the IFNγ produced by these cells is involved in inhibition of anterior-to-posterior spread of virus in the injected eye. (Invest Ophthalmol Vis Sci. 2009;50: 2269–2275) DOI:10.1167/iovs.08-2874

Acute retinal necrosis (ARN) is a rare but visually devastating disease characterized by circumferential and rapid progression of necrosis, occlusive vasculopathy, vitritis, and inflammation of the anterior segment. In the absence of treatment, ARN usually results in blindness in the affected eye.1,2 In a mouse model of ARN, after inoculation of HSV-1 (KOS) into the AC of one eye of a BALB/c mouse, virus replicates in the anterior segment of the injected eye and then spreads via parasympathetic neurons to the ipsilateral ciliary ganglion at day 2 pi, the ipsilateral Edinger-Westphal nucleus at day 3 pi, and the ipsilateral suprachiasmatic nucleus (SCN) at day 5 pi, finally reaching the contralateral optic nerve and retina at day 7 pi. Despite there being no anatomic barrier between the anterior and posterior segments, virus does not spread directly from the anterior segment to the retina or optic nerve of the injected eye.3–6 Although the retina of the injected eye is spared in immunocompetent BALB/c mice, thymectomized and T-cell-depleted BALB/c mice develop bilateral retinitis.7 In T-cell-depleted mice, virus infects both the ipsilateral and contralateral SCN and both optic nerves, but even in the absence of T cells, there is no evidence of direct anterior-to-posterior spread of virus in the injected eye.8–10 In contrast, direct anterior-to-posterior spread of virus is observed in natural killer (NK) cell-depleted BALB/c mice, resulting in retinitis in the ipsilateral eye.11 These results suggest that NK cells play an important role in protecting the ipsilateral retina by preventing direct anterior-to-posterior spread of the virus early in the course of HSV-1 infection.

Interferons (IFNs) are important antiviral cytokines involved in activation and differentiation of immune cells via intracellular signaling mechanisms. IFNs initiate production of proteins that inhibit translation and cell growth, induce apoptosis, and promote downregulation of mRNA.12,13 IFNγ is known as the immune interferon, because it is specifically produced by cells of the immune system—NK cells, macrophages, neutrophils, and T cells.14–16 whereas, IFN α and β are produced by a variety of cell types.15,17 Several studies have demonstrated that IFNγ plays an important role in viral pathogenesis by overexpressing IFNγ in the eye and by infection of IFNγ-deficient mice. Collectively, these studies indicate that IFNγ plays an important role in clearance of viral infection, including HSV-1.18–22

Although viral infection is necessary for inflammatory response in the injected eye, deciphering the pathogenesis of the infection in this eye necessitates identifying the infiltrating cells, determining the time of their arrival, and understanding their capabilities. Therefore, the overall goal of these studies was to identify the cells that infiltrate the eye after unilocular AC inoculation of HSV-1 and to determine whether infiltrating cells produced IFNγ.

MATERIALS AND METHODS

Animals

Adult female BALB/c mice. 6 to 12 weeks old (Taconic, Germantown, NY), 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 Institutional Animal Care and Use Committee of the Medical College of Georgia. The mice were maintained on a 12-hour light-dark cycle and were given unrestricted access to food and water. They 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. Each group in each experiment had a minimum of five mice, and the 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% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotic. 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**

Mice were anesthetized and inoculated using the AC route as previously described.3 The right eye was propionated and 3 × 10^4 plaque-forming units (PFU) of HSV-1 (KOS) in a volume of 2 mL 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. Mock-injected mice were inoculated with 2 μL of uninfected Vero cell extract diluted in DMEM with antibiotics.

**Immunoelectron Microscopy (IEM)**

Mice were anesthetized and killed by cervical dislocation. Injected eyes were collected at 24, 48, and 72 hours from mock- and virus-injected mice and fixed in 4% formaldehyde and 0.2% glutaraldehyde in 0.1 M Na-cacodylate buffer for at least 24 hours at 4°C. The eyes were then dehydrated in a graded alcohol series and embedded in pure resin (LR White; Electron Microscopy Sciences [EMS], Hatfield, PA). Seventy-five-nanometer sections were collected on polyvinyl butyral resin (Pioloform; Ted Pella, Redding, CA) coated, nickel slot grids (EMS). Grids were incubated in blocking solution (1% BSA, 3% normal goat serum [NGS]; Vector Laboratories, Burlingame, CA), and 0.05% Tween 20 in Tris-buffered saline for 2 hours, followed by rabbit anti-HSV-1 (Accurate Chemical and Scientific Corporation; Westbury, NY)/anti-rabbit colloidal gold, stained in methanolic uranyl acetate and examined using a transmission electron microscope (Jem 1230; JEOL, Tokyo, Japan).

**Flow Cytometry**

Single-cell suspensions were prepared from six pooled, whole, injected eyes of normal control, mock-injected mice at 24 hours pi and from HSV-1 (KOS)-infected mice at 24, 48, 72, and 120 hours pi. The eyes were incubated in 58.5 U/mL of collagenase IV (Sigma-Aldrich, St. Louis, MO) in Hanks’ balanced salt solution (HBSS; Cellgro Mediatech, Manassas, VA) for 1 hour at 37°C, 5% CO₂ and pressed through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA).23 The cells were suspended in HBSS and centrifuged at 250 g for 5 minutes, resuspended in PBS containing 1% BSA, counted and then blocked with 10% mouse serum (Sigma-Aldrich) diluted in staining buffer (1% BSA in PBS) for 2 hours, followed by rabbit anti-HSV-1 (Accurate Chemical and Scientific Corporation; Westbury, NY)/anti-rabbit colloidal gold, stained in methanolic uranyl acetate and examined using a fluorescence microscope (Jem 1230; JEOL, Tokyo, Japan).

**Immunohistochemistry**

Mice were deeply anesthetized and perfused transcardially with PBS for approximately 3 minutes. After perfusion, the injected eye of each mouse was removed, snap-frozen, and embedded (Tissue-Tek OCT Compound; EMS). Eight- to 10-μm sections were prepared on positively charged slides (Fisher Scientific, Pittsburgh, PA) using a cryostat (Microm HM505E; EquipNet, Canton, MA). The frozen sections of inoculated eyes were fixed with 4% paraformaldehyde, washed in PBS, and blocked with 10% NGS (Vector Laboratories), 1% BSA (Fisher Scientific), and 0.5% Triton X-100 (Sigma-Aldrich) diluted in PBS for 2 hours. Sections were incubated with anti-mouse IFNγ (RMMG-1; PBL Biomedical Laboratories; Piscataway, NJ) washed in PBS, incubated with Texas Red-anti rat (Jackson ImmunoResearch Laboratories; West Grove, PA), washed in PBS, and blocked with 3% rat serum (Sigma-Aldrich) diluted in PBS. The sections were then stained with one of the following antibodies: FITC–anti-mouse CD11b (integrin α4 chain, Mac-1 α chain; BD Pharmingen), FITC–anti-mouse CD4/80 (Caltag Laboratories), FITC–anti-mouse CD4 (L3T4, BD Pharmingen), FITC–anti-mouse CD8α (Ly-2, BD Pharmingen), or FITC–anti-mouse CD49b/Pan-NK cells (BD Pharmingen). They were then washed with PBS and mounted (VectorShield containing DAPI; Vector Laboratories). Slides were examined with a fluorescence microscope and images were captured with digital cameras (SPOT Advanced; Diagnostic Instruments, Sterling Heights, MI; or AxioVision 4.6; Carl Zeiss Meditec, Jena, Germany) computer programs.

**Figure 1.** Electron micrographs showing virus capsids (C, F, arrowheads) and virus proteins (identified by colloidal gold particles) in the cytoplasm and nucleus of cells in the anterior segment of the injected eye at 48 and 72 hours pi. (A, B) Enlargement of areas circled in (A) and (D). (C, F) Enlargement of boxed areas in (B) and (E). Original magnification: (A, D) ×50; (B, E) ×5,000; (C) ×25,000; (F) ×20,000.
Hematoxylin and Eosin Staining

The inoculated eye was removed, snap frozen, and embedded (Tissue-Tek OCT Compound; EMS) as described earlier. Frozen sections were fixed in cold acetone for 5 minutes, stained with hematoxylin and eosin (H&E; Fisher Scientific), washed, dehydrated in a graded alcohol series, mounted (Cytoseal; Richard Allan Scientific, Kalamazoo, MI), and allowed to dry overnight.

RESULTS

HSV-1 in the Anterior Segment of the Injected Eye

IEM was used to identify virus and viral proteins in cells of the anterior segment early after infection. HSV-1 capsids were not observed in the anterior segment of virus-infected mice at 24 hours pi (not shown). HSV-1 capsids (Figs. 1C, 1F; arrowhead) and viral proteins (Figs. 1C, 1F; gold particles) were present in the nucleus and cytoplasm (Figs. 1B, 1C, 1E, 1F) of cells in the anterior segment (Figs. 1A, 1D) at 48 and 72 hours pi. HSV-1 capsids were not observed in normal control or mock-injected mice (not shown).

Infiltrating Cells in the Injected Eye

H&E staining was used to examine inflammation and cell infiltration in the anterior segment of the injected eye after HSV-1 infection. H&E staining showed structural disorganization and infiltrating cells in the anterior segment beginning at 36 hours pi and continuing through 72 hours pi (Figs. 2H, 2K, 2N). Cellular debris from necrotic and/or apoptotic cells was observed in the anterior and posterior chambers of virus-infected mice beginning at 36 hours pi (Figs. 2G, 2J, 2M). Moreover, cells with multilobed nuclei, characteristic of neutrophils, were observed beginning 48 hours after infection in the ciliary body, iris (Figs. 2L, 2O), and cornea (not shown).

Flow cytometry was used to quantify cell types activated and/or infiltrating the injected eye after AC injection of HSV-1 (KOS). The number of cells in the eyes of mock (5.24 × 10^6)-inoculated mice was only slightly increased (0.56%) compared with normal, noninjected control eyes (5.21 × 10^6). In HSV-1-infected mice, the total number of cells per virus injected eye increased by 29% at 24 hours pi (7.34 × 10^6), 22% at 48 hours pi (6.64 × 10^6), and 44% at 72 hours pi (9.23 × 10^6). At 120 hours pi, the number of cells had decreased slightly to 39% (8.60 × 10^6).

Detection of cellular markers was used to quantify immune cell types: Mac-1^+, F4/80^−, CD4^+^, CD8^+^, CD49b^+, and CD11c^−. As shown in Table 1, the percent of Mac-1^+^ cells in the eye of normal control mice and of mock injected mice was low (0.80% and 0.71%, respectively). In the injected eye of HSV-1-infected mice, the percent of Mac-1^+^ cells had increased to 3.59% at 24 hours pi and remained elevated at 120 hours pi (16.46%; Table 1 and Fig. 3). No F4/80^−^ cells were observed in normal eyes or mock injected eyes, while a small percentage of the cells were F4/80^−^ at 72 hours pi (0.61% and 1.87%, respectively; Table 1). The percentage of CD49b^+^ cells was increased above mock-injected eyes at 120 hours pi only (5.40%; Table 1). There were no differences in the percentage of CD4^+, CD8^+, or CD11c^−^ cells between normal control or mock-injected mice and virus-infected mice at any time point (Table 1).

In these studies, it would have been ideal to use flow cytometry to quantify the cell types producing IFNγ by double staining for cell type and IFNγ. However, we were unable to double stain eye cell suspensions for cellular markers and IFNγ, perhaps because of the extensive manipulation of the cells required to double stain cells for flow cytometry.

IFNγ Production and Immune Cells

Immunofluorescence was used to identify cells secreting IFNγ and to determine their location in the injected eye after unilateral AC inoculation of HSV-1 (KOS). Mac-1^−^, F4/80^+^, and CD49b^+^ cells were observed in the anterior segment of the injected eye as early as 12 hours pi (not shown) and continuing to 72 hours pi (Fig. 4 and not shown). An occasional Mac-1^−^, F4/80^+^, or CD49b^+^ cell was observed in normal control (not shown).
shown) or mock-injected animals. Single-stained IFNγ+ cells or cells double stained for IFNγ and Mac-1, IFNγ and F4/80, or IFNγ and CD49b were rarely observed in control animals. Beginning at 12 hours pi, Mac-1+ IFNγ+ cells were observed in the limbus of the anterior segment of the injected eye in 56% (5/9) of the mice. As shown in Figure 4, some but not all, Mac-1+ cells were also IFNγ+ and IFNγ+ Mac-1+ cells were observed in the limbus, ciliary body, iris, and cornea at 48 and 72 hours pi.

F4/80+ cells expressing IFNγ were observed beginning at 12 hours pi in and around the limbus of 33% (3/9) of the mice. Some but not all F4/80+ cells, were also IFNγ+, and F4/80+ IFNγ+ cells were observed in the limbus, ciliary body, iris, and cornea of most of the mice beginning at 24 hours and continuing to 72 hours pi (not shown).

CD49b+ cells expressing IFNγ were observed beginning at 12 hours pi in the limbus of the anterior segment in 22% (2/9) of mice, and some but not all, CD49b+ cells were also IFNγ+. CD49b and IFNγ double-positive cells were observed through 72 hours pi in the limbus, ciliary body, iris, and cornea (not shown).

Both CD8+ and CD4+ cells expressing IFNγ were observed at 24 hours pi in the limbus of the anterior segment (not shown). Some, but not all, CD4+ or CD8+ cells were also IFNγ+, and CD4+ IFNγ+ cells as well as CD8+ IFNγ+ cells were observed through 72 hours pi (not shown).

### DISCUSSION

Polymorphonuclear leukocytes (PMNs) and NK cells have been implicated in protecting the ipsilateral retina from the direct anterior-to-posterior spread of HSV-1 (KOS) after unilocular AC inoculation.11,24 but the mechanisms by which these immune cells control virus spread in the injected eye have not been elucidated. IFNγ is an antiviral cytokine produced by cells of the innate immune response (neutrophils, macrophages, myeloid dendritic cells, NK cells, microglia, and B cells).33–37 and the innate immune response is initiated between 0 to 96 hours after infection and HSV-1 (KOS) titers in the inoculated eye peak around day 2 or day 3 pi.27 it is reasonable to expect that IFNγ producing cells arrive and/or are activated on or before day 3 pi.

Unilocular AC inoculation of HSV-1 (KOS) results in acute inflammation of the anterior segment, involving the cornea, iris, and ciliary body, with cell infiltration and loss of structural integrity beginning 24 hours pi.5 Early in infection, virus is present in the ciliary body and iris of the injected eye.6 Virus infection of the anterior segment was confirmed in this study by IEM, and viral antigen–positive cells and viral capsids were observed in the nucleus and cytoplasm of cells in the ciliary body and iris of the injected eye at 48 and 72 hours pi. Although signs of virus infection and virus have been observed in the anterior segment as early as 24 hours pi,5,6 no capsids were seen by EM at this time, a finding that is probably not surprising because of the time required for a cycle of virus replication, 18 to 20 hours in cell culture and somewhat longer in vivo.28

In this study, IFNγ-producing Mac-1+ cells were observed in the anterior segment of the injected eye beginning at 12 hours pi and extending to at least 72 hours pi. These results suggest that Mac-1+ cells, which we believe are largely microglia, migrate to the anterior segment of the injected eye where they proliferate and contribute to early protection of the ipsilateral retina (on or before day 3 pi) by production of IFNγ. The retina develops from the diencephalon of the neural tube and is considered part of the CNS.29 Microglia are the resident macrophages of the CNS and Mac-1+ cells have been previously observed in the anterior segment of the eye after HSV-1 infection and have also been implicated in IFNγ production.26,30 The idea that microglia migrate is not without precedent, since Mac-1+ cells migrate in the retina, proliferate, become more phagocytic and produce cytokines in response to retinal degeneration.31,32

The Mac-1 antigen, also known as the complement receptor (CR3), is expressed on subtypes of neutrophils, macrophages, myeloid dendritic cells, NK cells, microglia, and B cells.33–37 and these cells types may well be represented in the eye at later time points. Since neutrophils have been observed in the HSV-1-injected ipsilateral eye before day 3,24 Mac-1+ cells may represent a subpopulation of Gr-1+ neutrophils.

NK cells are major producers of IFNγ during the innate immune response and they have been shown to play a role in protecting mice from HSV-1 infections.11,38,39 Although CD49b+ staining (and colocalization with IFNγ+ cells) was observed from 24 to 72 hours pi, the largest number of CD49b+ cells was observed at 120 hours pi in virus-injected mice. In NK-depleted mice, evidence of HSV-1 infection of the retina in the injected eye is observed by day 5.11 The results presented in this manuscript suggest that CD49b+ cells, which may be NK cells, contribute to protection of the ipsilateral retina beginning around day 4 pi. Although the reason for the several days’ delay in recruitment of these cells is not known, one possible explanation is that the delay in recruitment of CD49b+ cells to this eye reflects the time until the blood ocular barrier has been compromised which, in turn, allows CD49b+ cells to enter the eye.

Macrophages are important antigen presenting cells in the innate immune response and previous results suggest macrophages are a source of IFNγ in the noninoculated contralateral eye of BALB/c mice on and after day 9 pi.40 Other studies have shown that cultured macrophages from BALB/c mice produce IFNγ after stimulation with IL-12 and -18.41 In the studies presented herein, F4/80+ staining (and colocalization with IFNγ+ cells) was observed from 120 hours pi. However, in the flow cytometry studies, F4/80+ systemic macrophages

### TABLE 1. Types of Infiltrating Cells after Uniocular Anterior Chamber Inoculation of HSV-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Mac1+</th>
<th>F4/80+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD49b+</th>
<th>CD11c+</th>
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</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>0.80*</td>
<td>0.83</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mock injected</td>
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<td>0.32</td>
<td>0.87</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>HSV-1 injected</td>
<td>0.71</td>
<td>0.32</td>
<td>0.87</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 h pi</td>
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<td>0</td>
<td>0.04</td>
<td>0.62</td>
<td>0.04</td>
</tr>
<tr>
<td>48 h pi</td>
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<td>0.23</td>
<td>0.41</td>
<td>0.85</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72 h pi</td>
<td>18.70</td>
<td>0.61</td>
<td>0</td>
<td>0</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>120 h pi</td>
<td>16.46</td>
<td>1.87</td>
<td>0.94</td>
<td>0.61</td>
<td>3.40</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data are percentage of isotype matched control staining subtracted from the percentage of cell marker staining. Data are representative of two independent experiments.
were not detected in the virus-injected eye until 72 hours pi and even at 120 hours pi only 1.87% of the infiltrating cells were F4/80\(^+\). F4/80\(^+\) cells are resident in the AC of normal mice and are important in anterior chamber–associated immune deviation (ACAID), in which delayed-type immunity is suppressed, and pathogens are eliminated in the absence of

**FIGURE 3.** Flow cytometric histograms of infiltrating cells isolated from mock-injected mouse eyes at 24 hours pi and HSV-1 (KOS)-injected eyes at 48, 72, and 120 hours pi. Cells were stained with FITC-conjugated anti-mouse Mac-1 (green trace) or FITC-conjugated Rat IgG2b,κ (solid black). Results were analyzed by flow cytometry. Percentages are the percentage of isotype-matched control staining subtracted from that of cell marker staining. Data are representative of two independent experiments.

**FIGURE 4.** Photomicrographs of the ciliary body of the injected eye showing the location (arrowheads) of IFN\(\gamma^+\) and Mac-1\(^+\) cells in mock-injected animals 24 hours pi and in virus-injected animals 48 and 72 hours pi. Magnification: (A–L) \(\times 200\); (e–l) \(\times 400\).
inflammation by excluding effector immune cells. There are two possible reasons why only a few F4/80⁺ macrophages were identified in the injected eye early after HSV-1 infection of the AC: phagocytic macrophage recruitment and entry to the infected eye is prevented by the blood ocular barrier, or antigen presenting resident F4/80⁺ macrophages migrate from the eye to initiate ACAID.

There was no difference in the number of CD4⁺, CD8⁺, and CD11c⁺ infiltrating cells between normal control mice and virus-infected mice at any time point that suggests that these cells do not contribute to prevention of anterior-to-posterior virus spread in the injected eye. T cells (CD4⁺ or CD8⁺) produce IFNγ, but a significant number of these cells would be expected to be present later (i.e., after 96 hours pi) as part of the adaptive immune response. CD11c⁺ plasmacytoid dendritic cells produce large quantities of IFNα and -β in response to viral infection, and although these cells are resident in the iris and ciliary body of mice, we were unable to quantify CD11c⁺ cells in the injected eye, perhaps because they are immature sentinel antigen capturing dendritic cells that stain weakly for CD11c and only after stimulation by virus do they mature into CD11c⁺ migratory cells that are not found in the eye.

Taken together, the results from these studies suggest that Mac-1⁺ cells are important resident immune cells and involved in early sparing of the ipsilateral retina (before day 3) after AC inoculation of BALB/c mice with HSV-1 (KOS). There was an increase in infiltrating cells in the AC of the injected eye and virus-infected cells were observed at the junction between the ciliary body and retina. IFNγ-producing Mac-1⁺ cells were observed in the anterior segment of the injected eye on or before day 3 pi. Although these studies suggest that Mac-1⁺ cells and other early responders play a role in protecting the retina of the injected eye from infection by preventing direct anterior-to-posterior spread of the virus, they do not define the mechanism by which such protection is accomplished. Now that the infiltrating cells have been described, additional studies will be needed to fully understand the role of each cell type and of the role of IFNγ in the process of virus infection in the injected eye.

Acknowledgments

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References