Lack of iNOS Facilitates MCMV Spread in the Retina

Ming Zbang, Jun Zbou, Brendan Marshall, Hua Xin, and Sally S. Atherton

PURPOSE. The purposes of this study were to identify iNOS-producing retinal cells and to determine whether lack of iNOS facilitates MCMV spread and replication in the retina.

METHODS. Immunosuppressed (IS) iNOS−/− mice or C57BL/6 (wild-type) mice were inoculated with 5 × 10⁴ PFU of MCMV K181 strain (K181) via the supraciliary route. Injected eyes were collected at several times after inoculation and examined by plaque assay for replicating virus, RT-PCR for iNOS RNA, Western blot for iNOS protein and by staining for MCMV early antigen (EA), iNOS, and retinal cell antigens.

RESULTS. iNOS mRNA and iNOS proteins were expressed in the MCMV-infected eye of wild-type mice. Most iNOS-producing cells were F4/80-positive, including macrophages, RPE-derived macrophages, and resident microglia. Significantly higher titers of virus were recovered from the injected eyes, and more infected cells were detected in the retina of IS iNOS−/− mice than in IS wild-type mice. Retinal necrosis and loss of retinal architecture throughout the retina were noted in IS iNOS−/− mice, whereas cytomegalic cells and retinitis were present only in the peripheral retina of IS wild-type mice.

CONCLUSIONS. iNOS produced by macrophages, especially resident macrophages including microglia and RPE derived macrophages, plays an important role in limiting spread of MCMV in the retina. (Invest Ophthalmol Vis Sci. 2007;48:285–292) DOI:10.1167/iovs.06-0792

Cytomegalovirus (CMV) retinitis is the most common sight-threatening opportunistic infection observed in adult and pediatric patients who are immunosuppressed (IS) as a result of chemotherapy, malignancy, or the acquired immunodeficiency syndrome. Although CMV retinitis is also observed in newborn infants with congenital cytomegalovirus infection, the pathogenic features of CMV retinitis have been well characterized, the pathogenesis of ocular CMV infection has not been fully determined.

Nitric oxide (NO) is a hydrophilic molecule and a highly diffusible free radical, generated through oxidation of l-arginine to l-citrulline by a family of constitutive or cytokine-inducible isoenzymes, the NO synthases (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed in selected tissues and are transiently activated by an increase in intracellular concentration of free Ca²⁺. Although the pathogenic features of CMV retinitis have been well characterized, the mechanism of virus infection has not been fully determined.

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Monoclonal antibody to an MCMV early gene product was labeled with FITC (Sigma-Aldrich, St. Louis, MO) or biotinylated with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer’s instructions. Rabbit anti-iNOS was purchased from BD Transduction (San Diego, CA). Rabbit anti-RPE 65 (kindly provided by Michael Redmond, National Eye Institute, National Institutes of Health) was used to stain the cells of the retinal pigment epithelium (RPE). Retinal glial cells were stained with mouse anti-glial fibrillary acidic protein (GFAP; BD PharMingen, San Diego, CA). Rat anti-F4/80, which was used to identify macrophages/microglia in some experiments. Mouse anti-neurofilament (NF; Sigma-Aldrich) was used to stain ganglion cells as well as some horizontal cells. Mouse anti-Goα (Chemicon) was used to identify both rod and cone bipolar cells. Mouse anti-calbindin-28 K (Sigma-Aldrich) was used to stain horizontal cells.

The injected eyes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 minutes, immersed in 25% sucrose overnight, snap frozen, and sectioned on a cryostat. For staining using mouse-derived primary antibodies (anti-GFAP, anti-Goα, anti-calbindin, and anti-NF), each antibody was mixed with biotinylated anti-mouse antibody (Dako ARK; Dako Corp., Carpinteria, CA); after 15 minutes, the blocking reagent (Dako ARK), containing normal mouse serum, was added to the mixture. The mouse immunoglobulin in the blocking reagent bound to the biotinylated anti-mouse antibody that was not bound to primary antibody, thus minimizing reactivity with endogenous immunoglobulin in the specimen. The biotin-labeled primary antibody was then applied to the section. Positive staining was detected using FITC-Avidin (Vector Laboratories, Burlingame, CA). For cell identification using rabbit-derived antibodies (anti-iNOS or anti-RPE-65), the slides were blocked in PBS containing 10% normal goat serum, 1% BSA, and 0.5% Triton X-100, then incubated overnight at 4°C in the primary antibody. After they were washed, the sections were reacted with Texas Red-labeled anti-rabbit antibody (Vector Laboratories). For cell identification using the rat-derived anti-Mac-1 antibody,
the slides were incubated with anti-Mac-1 first and then with FITC labeled anti-rat IgG (mouse serum absorbed; Vector Laboratories).

For double staining of MCMV EA and iNOS or RPE-65, the sections were stained first with anti-iNOS or anti-RPE-65 and then reacted with Texas Red-labeled anti-rabbit IgG. The slides were then incubated with FITC-anti-MCMV EA, mounted (Vectashield mounting medium with DAPI; Vector Laboratories), and examined microscopically. For double staining of iNOS and retinal antigens, including GFAP, F4/80, Mac-1, calbindin, Goa, and NF, the sections were stained first with anti-iNOS and then reacted with Texas Red-labeled anti-rabbit IgG. The slides were then stained with antibodies against one of the retinal antigens, the reaction was developed using FITC-avidin as described earlier, and the slides were examined microscopically.

**Retinitis Scoring**

Eyes were fixed in buffered formalin, embedded in paraffin, and sectioned at six levels, 200 μm apart. The sections were then stained with hematoxylin and eosin. Changes in the posterior segment of each section were evaluated microscopically as follows: 0, normal or injection artifact; 1/2, mild atypical retinopathy: absence of cytomegaly plus retinal folds involving less than three fourths of the retinal section; 1, moderate atypical retinopathy: absence of cytomegaly plus retinal folds involving more than three fourths of the retinal section plus photoreceptor atrophy or retinal infiltration by leukocytes involving more than one-fourth of the retina; 2, retinal infection: cytomegaly of retinal cells plus partial-thickness retinal necrosis or full-thickness necrosis extending from the ciliary body, but not beyond a one-fourth retinal section from the ciliary body; 3, necrotizing retinitis: cytomegaly plus full-thickness retinal necrosis existing further than one fourth of a retinal section from the ciliary body or full-thickness retinal necrosis extending from the ciliary body through one quarter of the section; 4, severe necrotizing retinitis: cytomegaly with full-thickness necrosis involving the entire retinal section.

A score of 3 or higher was considered positive for retinitis. The highest posterior segment score for each eye was the retinal score and was used to determine the mean retinal score. The average score for all retinal sections of each group was also determined. Significant differences between groups were determined using the Mann-Whitney test.

**RNA-PCR for iNOS mRNA**

At days 3, 7, 10, and 14 after MCMV inoculation, the injected eyes were collected and RNA was extracted (TRizol; Invitrogen-Gibco, Grand Island, NY) according to the manufacturer’s instructions. RNA from eyes of normal uninfected iNOS−/− mice and wild-type mice was also extracted as control. Three eyes were pooled at each time point. RNA-PCR was performed (Access RT-PCR System; Promega Corp., Madison, WI), according to the manufacturer’s manual. The primers for iNOS and β-actin were as follows: iNOS: primer 5′-CTTGCCCCTG-GAAGTTTCTCTT(189-210) and 5′-primer GCCTGGTAGGTCTCTGTTGTTC-3′ (686-707). β-actin: 5′-TCTTCCGTGCCGTCACAC-3′ (44-65) and 5′-CGTCTCAGGATCCATCACA-3′ (534-552).

**Western Blot Analysis**

At days 3, 7, and 10 after MCMV inoculation, the injected eyes were removed and immediately frozen in liquid nitrogen. After pulverization (Bessman Tissue Pulverizer; Fisher Scientific, Pittsburgh, PA), the tissue was lysed in modified radioimmunoprecipitation (RIPA) buffer with protease inhibitor cocktail (Roche, Basel, Switzerland) for 10 minutes at 4°C. Crude lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C. The protein concentration in the cleared lysates was measured by the Bradford method. Proteins were extracted from the eyes of normal uninfected iNOS−−/ mice and wild-type mice as control. Three eyes were pooled at each time point, and 80 μg of protein from each sample was loaded onto a 7.5% SDS-polyacrylamide gel (Bio-Rad, Her-
cules, CA). The separated proteins were transferred onto a nitrocellu-
lose membrane (GE Healthcare, Piscataway, NJ) which was blocked
with 5% nonfat dry milk for 1 hour at room temperature and incubated
with anti-iNOS antibody (1:1000 dilution; Transduction Laboratories,
Lexington, KY) overnight at 4°C. The membrane was washed and
incubated with anti-rabbit IgG antibody coupled to horseradish perox-
idase (BD PharMingen) at a dilution of 1:5000 for another hour. After
another extensive washing, the corresponding iNOS protein (130 kDa)
was detected using a chemiluminescence detection kit (GE Health-
care). Prestained protein markers (Bio-Rad) were used for molecular
mass determinations. To verify equal loading among lanes, the mem-
branes were stained with the intrinsic protein actin (mouse monoclo-
nal anti-β-actin antibody; Sigma) after staining for iNOS.

RESULTS

Replication and Spread of MCMV in iNOS−/− Mice and Wild-Type Mice

After immnosuppression with methylprednisolone to deplete T cells as well as macrophages29,30 and inoculation of 5 × 10^4 PFU MCMV into the suprachiliary space, virus infected cells were observed in the ciliary body and choroid of IS iNOS−/− mice and IS wild-type mice at day 3 PI (Fig. 1). By day 5 PI, MCMV-infected cells were observed in the retina of both groups of mice, but more infected cells were observed in the retina of IS iNOS−/− mice (Figs. 1, 2A). By day 14 PI, MCMV-infected cells were rarely observed in the retina of IS wild-type mice. In contrast, MCMV-infected cells were still observed in the retina of IS iNOS−/− mice, and the retinal architecture was focally disrupted (Fig. 1). Throughout the course of infection, the amount of replicating virus was higher in the injected eyes of IS iNOS−/− mice than in the eyes of IS wild-type mice (Fig. 2B).

iNOS Expression in the Injected Eyes of Wild-Type Mice after MCMV Infections

To determine whether iNOS was expressed in the injected eyes of wild-type mice after MCMV infection, iNOS mRNA and protein were detected by RT-PCR and Western blot, respectively. No iNOS mRNA was detected in the eyes injected with

![Figure 5. Photomicrographs of staining of F4/80 and iNOS in the injected eye of an IS wild type mouse at day 10 PI iNOS−/− cells were observed in all layers of the retina and were F4/80+ macrophages/microglia (arrows and arrowheads). Some iNOS− and F4/80− macrophages were pigmented RPE cells (arrows, iNOS+, F4/80+, RPE+) and some were non-RPE derived macrophages/microglia (arrowheads, iNOS+, F4/80+, RPE-). (F4/80, A; iNOS, B; DAPI, C; pigmented cells, D; merged image, E).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/ on 06/20/2017)
medium alone or in the uninjected eyes of wild-type mice (Fig. 3). However, iNOS mRNA was consistently detected in the injected eyes of IS mice from day 3 PI to day 14 PI (Fig. 3). As expected, no iNOS mRNA was expressed in either uninfected or MCMV-infected iNOS−/− mice (Fig. 3). Detection of iNOS protein was similar to that of iNOS mRNA and iNOS protein was detected by Western blot in the injected eyes of IS wild-type mice (Fig. 4).

Identification of iNOS Producing Cells in the Retina after MCMV Infection
To identify which retinal cells express iNOS during viral infection, frozen sections of injected eyes of IS wild-type mice were double stained with antibody to iNOS and with antibodies to different types of retinal cells or MCMV early antigen. As shown in Figure 5, most of the iNOS-producing cells in the injected eyes of wild-type mice were F4/80+ macrophages/microglia which were observed in all layers of retina of IS mice. Some of the F4/80+ macrophages appeared to have originated from RPE cells since they were pigmented and located mainly in the RPE and photoreceptor layers (Fig. 5, arrows). Both MCMV infected and noninfected RPE cells produced iNOS (Fig. 6). In IS wild-type mice, some iNOS+ cells were GFAP+ glia (Fig. 7). Double staining of cells for iNOS and neuronal antigens including Gox, calbindin and NF was not observed, suggesting that neurons do not produce iNOS (data not shown).

Retinitis in Immunosuppressed iNOS−/− and Wild-Type Mice
To determine whether the presence of iNOS influenced the course of retinitis, IS iNOS−/− and IS wild-type mice were infected with 5 × 10⁷ PFU of MCMV on day 0. Mice were euthanatized on day 10. Eyes were removed, fixed, sectioned, stained, and scored for retinitis. As shown in Figure 8, virus-infected cytomegalic cells were observed in the retinas of both IS iNOS−/− and IS wild-type animals. However, retinal necrosis and loss of retinal architecture throughout the retina were noted in IS iNOS−/− mice (Fig. 8A), whereas cytomegalic cells and retinitis were present only in the peripheral retina of IS
wild-type mice (Fig. 8B). Ten days after supraciliary injection of MCMV, 100% of IS iNOS⁻/⁻ mice developed retinitis compared with 50% of wild-type mice (Table 1). The average retinal score as well as the average score of sections of eyes of IS iNOS⁻/⁻ mice were both significantly higher than that of IS wild-type mice (Table 1).

Figure 7. Photomicrographs of staining of GFAP and iNOS in the injected eye of a IS wild-type mouse at day 10 PI. Some of iNOS⁺ cells were GFAP⁺ glia (arrow) (GFAP, A; iNOS, B; DAPI, C; pigmented cells, D; merged image, E).

Figure 8. Photomicrographs of hematoxylin and eosin stained sections of MCMV injected eyes of an IS iNOS⁻/⁻ mouse (A) and of an IS wild-type mouse (B) at day 10 PI. More severe retinitis and retinal destruction were observed in immunosuppressed iNOS⁻/⁻ mice than in IS wild-type mice (iNOS⁺/⁺).
The results presented herein demonstrate that iNOS mRNA and iNOS proteins were expressed in the injected eyes after supraciliary inoculation of MCMV into IS C57BL/6 wild-type mice. Although most iNOS-producing cells were Mac-1⁺ macrophages/microglia, a few iNOS⁺ cells were GFAP⁺ glia. Although most systemic macrophages are depleted after methylprednisolone treatment,29,30 in this study, many F4/80⁺ cells were still observed in the retina during MCMV infection. Although some of these F4/80⁺ macrophages might be residual systemic macrophages or resident microglia, many of them appeared to be derived from pigmented RPE cells. A previous report from Bodaghi et al.20 showed that CMV infection inhibited iNOS in cultured human RPE cells. However, in these studies of MCMV infection, both MCMV infected and noninfected RPE cells produced iNOS, suggesting that human and mouse RPE cells may differ in the response to cytomegalovirus infection.

To test the hypothesis that iNOS derived NO produced by macrophages plays a role in MCMV infection of the retina, the same amount of MCMV was injected into the supraciliary space of IS iNOS⁻/⁻ mice. More replicating virus was recovered from eyes of IS C57BL/6 wild-type mice. More replicating virus was recovered from eyes of IS iNOS⁻/⁻ mice than in the retina of IS wild-type mice.


**NOTE:** The table below shows the incidence of retinitis after supraciliary inoculation of MCMV.

<table>
<thead>
<tr>
<th>Retinal Score*</th>
<th>Mean ± SEM†</th>
<th>Average Score, All Eye Sections‡</th>
<th>% Retinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>3, 3, 3, 3, 2, 2</td>
<td>2.5 ± 0.55</td>
<td>2.17 ± 0.70</td>
</tr>
<tr>
<td>iNOS⁻/⁻</td>
<td>4, 4, 4, 4, 4, 3</td>
<td>3.7 ± 0.52</td>
<td>5.71 ± 0.46</td>
</tr>
</tbody>
</table>

Measurements were obtained after inoculation of 5 × 10⁴ PFU of MCMV into IS iNOS⁻/⁻ and IS wild-type mice.

* Maximum posterior segment score for each injected eye.
† Significantly different from iNOS⁻/⁻: P < 0.05, Mann-Whitney, n = 6.
‡ Significantly different from iNOS⁻/⁻: P < 0.001, Mann-Whitney, n = 36 (six injected eyes × six sections/eye).

**DISCUSSION**

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When comparing the protective roles of T cells, NK cells, and various cytokines,28–33 the role of macrophages in immunity against CMV infection of retina has yet to be elucidated. Because most iNOS-expressing cells in the retina were Mac-1⁺ macrophages/microglia, the results presented herein provide indirect evidence to support the idea that macrophages, especially resident macrophages including microglia and RPE-derived macrophages, are important effector cells against MCMV infection in the retina. In the future, use of in vitro and in vivo models such as retinal cultures will define the mechanism by which such resident macrophages interact with virus in the retina and may provide information about how the function of these cells could be exploited to modulate cytomegalovirus infection of the retina.

**References**


35. Dix RD, Podack ER, Cousins SW. Murine cytomegalovirus retinitis during retrovirus-induced immunodeficiency (MAIDS) in mice: interleukin-2 immunotherapy correlates with increased intraocular levels of perforin mRNA. Antiviral Res. 2003;59:111–119.
