NK Cell Modulation of Murine Cytomegalovirus Retinitis

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*J Immunol* 1998; 160:5826-5831; ; http://www.jimmunol.org/content/160/12/5826
NK Cell Modulation of Murine Cytomegalovirus Retinitis

John E. Bigger,* Charles A. Thomas III,* and Sally S. Atherton2*†

CMV retinitis, the most common ophthalmic infection of AIDS patients, causes blindness if left untreated. To study the role of NK cells in the modulation of CMV ocular infection, 9.0 × 102 plaque-forming units of the Smith strain of murine CMV (MCMV) was injected into the supraciliary space of the left eyes of BALB/c mice. Lysis of NK-sensitive target cells (YAC-1) by effectors from the draining lymph nodes peaked at day 5 postinfection, while the splenic cytolytic response was biphasic, with peaks at days 2 and 7 postinfection. Flow cytometry showed that NK cells (DX-5+) increased in spleens and eyes 5 days after supraciliary infection with MCMV compared with uninfected or mock-infected controls. Eight days after supraciliary injection with 9.0 × 102 plaque-forming units of MCMV, 7 of 10 NK-depleted mice developed retinitis compared with only 2 of 10 non-NK-depleted control mice. Poly(I-C) activation of NK cells in T cell-depleted animals protected mice from MCMV retinitis; only 2 of 10 mice in the poly(I-C)-treated group developed retinitis compared with 8 of 10 T cell-depleted, non-poly(I-C)-treated control mice. These results show the importance of NK cells in preventing MCMV retinitis and suggest that NK cells may also be involved in modulation of cytomegalovirus retinitis in human patients. The Journal of Immunology, 1998, 160: 5826–5831.

From 70 to 90% of human adults are infected with CMV, a member of the betaherpes virus family (1). Primary infection with cytomegalovirus generally causes only mild or subclinical disease, which is followed by lifelong, latent infection in immunocompetent humans (2). However, a primary infection or reactivation of CMV infection in immunocompromised patients may result in significant morbidity and mortality (1–6).

CMV retinitis is the most common ophthalmic infection in AIDS patients; up to 40% of AIDS patients develop CMV retinitis during the course of the disease (5, 6). CMV retinitis is a focal, but progressive, necrotizing infection of the retina, which causes reduction in visual acuity and, if left untreated, blindness (5, 6). Classically, CMV retinitis was a complication of end-stage AIDS, but as treatments for AIDS-associated infections have allowed patients to live longer, CMV retinitis has become more challenging to control therapeutically. Antiviral therapies for ocular CMV infections are initially effective, but disease progression occurs frequently due to the emergence of drug-resistant virus. Additionally, therapies are limited because of patient compliance and drug toxicity (7, 8).

Since members of the cytomegalovirus family are extremely species restricted, the pathogenesis of CMV infection of the retina has been studied using murine cytomegalovirus (MCMV)-infected BALB/c mice (9–14). Injection of a low dose (5.0 × 102–1.0 × 103 plaque-forming units (pfu)) of MCMV into the supraciliary space of one eye of an immunosuppressed BALB/c mouse causes a focal, necrotizing retinal infection that resembles CMV retinitis in human (10, 11). This mouse model has been used to study the pathogenesis of ocular CMV infections (10, 11).

One component of the primary immune response to most virus infections is NK cells. NK cells, a subset of large, granular lymphocytes, are activated by the cytokines IFN-α, IFN-β, IFN-γ, and IL-12 and can, upon activation, secrete antiviral cytokines such as IFN-γ and TNF-α (13, 14). Additionally, NK cells lyse virus-infected cells upon conjugate recognition of those cells by an “MHC-unrestricted” mechanism (15, reviewed in Ref. 16). However, the eye constitutively expresses NK-suppressing cytokines, including TGF-β (17–19) and a 15-kDa protein (20), both of which have been shown to diminish NK cytotoxicity in vitro. In mice, NK cells have been shown to modulate MCMV replication in many tissues, including lung, liver, spleen, and salivary gland (9, 13, 14, 21, 22). In humans, NK responses to ocular CMV infections have not been reported; however, NK activity has been shown to be elevated during active CMV disease in kidney transplant patients (23). Importantly, stage IV AIDS patients have been reported to have lost NK cytolytic activity in vitro (24, 25). The purpose of the studies reported herein was to examine the role of NK cells in MCMV retinal infection. The results of these studies show that NK cells are critical for preventing MCMV retinitis in immunocompetent mice and that NK cells can also protect T cell-depleted mice from retinitis.

Materials and Methods

Animals

Female euthymic BALB/c mice, 6–8 wk old, were obtained from Tacconic (Germantown, NY). Animals were housed on a 12-h light/dark cycle and given unrestricted access to food and water. Animals were housed in accordance with National Institutes of Health guidelines, and all procedures in this study conformed to the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus and virus titrations

Stocks of MCMV (Smith strain) were prepared from salivary gland homogenates from MCMV-infected BALB/c mice as previously described (10). Virus stocks were titered in duplicate on Swiss Brown mouse embryonic fibroblasts.
Thymectomy and T cell depletion

Thymectomies were performed using a modification of a protocol by Chin (26). Thymectomized mice were rested for 1 wk before T cell depletion. T cell depletion was accomplished by i.v. injection of 500 μg of anti-CD4 (GKL1.5) and 150 μg of anti-CD8 (2.43) (American Type Culture Collection, Manassas, VA). The non-cross-reactive Abs PE-anti-Ly-3.2 (clone 53-5.8; PharMingen, San Diego, CA) and FITC-anti-L3T4 (clone RM4-4; PharMingen) recognizing CD8 and CD4, respectively, were used to determine the efficiency of T cell depletion.

Ocular inoculation

Mice were anesthetized by i.m. injection of a mixture containing 0.02 ml of Rompun and 0.03 ml of Ketamine per 25 g of body mass. The left eyes of mice were injected with 9.0 × 10^5 pfu of MCMV in a volume of 2 μl via the supraciliary route as previously described (10). Briefly, a superficial trans-scleral entry wound was made parallel and just posterior to the limbus by introducing the bevel of a sharp 30-gauge needle into the supraciliary space. Two micrometers of virus (or diluted salivary gland homogenate) followed by 3 μl of air were then injected. The injection was judged successful if indirect ophthalmic observation showed a chorioretinal detachment associated with the appearance of air in the supraciliary space immediately after injection.

Flow cytometry

Animals were deeply anesthetized, perfused with PBS, and spleens and eyes were harvested. Eyes were cleaned of all muscle and connective tissue, leaving only the globe with some conjunctival tissue and approximately 1 mm of optic nerve. The cornea and lens were removed, and a single cell suspension of the remaining ocular tissue was made by pressing it through a 70-μm nylon mesh. Cells were washed three times in HBSS, RBC were lysed by ammonium chloride lysis buffer (ACK) treatment, and cells were washed three times in HBSS and resuspended in FACS buffer (PBS with 3% FBS). Samples were incubated with anti-CD32/CD16 (Fc receptor block) (PharMingen) according to the manufacturer's recommendations for 15 min. Cells were then resuspended in FITC-anti-L3T4 (CD4) (PharMingen); PE-anti-Ly-3.2 (CD8) (PharMingen); or FITC-anti-mouse pan-NK (DX-5) (PharMingen). After 15 min, cells were washed three times in FACS buffer and resuspended in FACS buffer for analysis. Large granular cells (predominately lymphocytes and macrophages) were included in the gate, and cell debris and small cells were excluded. By this method, 77.6% of the cells from a spleen, 8.9% of the cells from a normal eye, and 14.3% of the cells from an infected eye were included in the gate. Percent positive cells = % positive stained cells minus % positive cells from the same uninfected sample.

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-quarters of the retinal section; 1 = moderate atypical retinopathy; absence of cytomegaly plus retinal folds involving more than three-quarters of the retinal section but not involving 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-quarter retinal section from the ciliary body; 3 = necrotizing retinitis: cytomegaly plus full-thickness retinal necrosis existing further than one-quarter 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. Differences between groups were determined using the Mann-Whitney U test (27).

Chromium release assay

One million NK-sensitive YAC-1 target cells were labeled with 100 μCi of ^51^Cr for 1 h in 500 μl of RPMI 1640 with 5% FBS (RPMI-5). The target cells were then washed three times and seeded into 96-well plates at 10^5 cells/100 μl/well. Effector cells were prepared by grinding the spleens or lymph nodes between frosted slides, followed by aspiration through a 22-gauge needle to make a single cell suspension. Cells were washed three times in HBSS, RBC were lysed by ACK treatment, and cells were washed three times in HBSS and resuspended RPMI-5. Cells were counted and 100 μl/well of the dilution to give the appropriate effector:target (E:T) ratio was plated in triplicate. For spontaneous or maximum release, 100 μl of media or 100 μl of 10% Triton X-100 (Sigma, St. Louis, MO) was added, respectively, instead of effector cells. Plates were centrifuged for 30 s at 11,000 g and incubated for 4 h at 37°C. One hundred microliters of each supernatant were counted in a Wizard 1470 gamma counter (Wallac, Turku, Finland). Each set of triplicates was averaged, and specific lysis was determined. Percent specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Spontaneous release in all experiments was less than 10% of the maximum release.

Depletion of NK cytotoxic activity

The protocol for in vivo depletion of NK cytotoxic activity was optimized using the chromium release assay. A single i.v. injection of 10 μg of anti-asialo GM1 (Wako Chemicals, Richmond, VA) reduced poly(C)-activated splenic NK activity to undetectable levels through day 3. Since NK activity was detectable again by day 5 (not shown), mice were injected on day −1 and day +4 with anti-asialo GM1 (Wako Chemicals) to maintain NK cell depletion. This regimen had no effect on the splenic T cell population as determined by flow cytometry. Control animals were mock-depleted with i.v. injections of the same volume of PBS. Later experiments showed no difference in retinal pathology 8 days after supraciliary infection with 9.0 × 10^5 pfu of MCMV between mice injected with PBS or 10 μg of normal rabbit serum.

Poly(I-C) enhancement of NK activity

Two hundred microliters of poly(I-C) enhanced splenic NK cell activity, which peaked on day 3 as shown by chromium release (not shown). Therefore, for experiments involving enhancement of NK activity in T cell-depleted animals, 200 μg of poly(I-C) in 200 μl of PBS was injected i.p. on days −1, +2, and +5. Control animals were injected i.p. with PBS.

Results

Supraciliary MCMV infection elicits an NK response

Supraciliary injection of MCMV into immunocompromised mice causes, in addition to retinitis, a marked infection of the iris, which forms part of the posterior border with the anterior chamber (10, 11). Because anterior chamber infections of the eye may depress delayed type hypersensitivity responses, resulting in anterior chamber-associated immune deviation (19, 28), and because the aqueous humor and vitreous humor of the eye contain multiple substances that depress NK activity (17–20), it was necessary to determine whether supraciliary infection with MCMV resulted in suppression of NK activity. To define the extent of NK cell response, mice were injected via the supraciliary route with 9.0 × 10^5 pfu of MCMV 1, 2, 3, 5, or 7 days before assay. The superficial cervical and mandibular lymph nodes ipsilateral to the site of injection were harvested, pooled, and lymph node cells were assayed for cytolytic activity against NK-sensitive targets (YAC-1). In the chromium release assay, mice were injected via the supraciliary route with 9.0 × 10^5 pfu of MCMV and analyzed by flow cytometry for the presence of NK cells (Fig. 2). Mock-infected times in HBSS, RBC were lysed by ACK treatment, and cells were washed three times in HBSS and resuspended RPMI-5. Cells were counted and 100 μl/well of the dilution to give the appropriate effector:target (E:T) ratio was plated in triplicate. For spontaneous or maximum release, 100 μl of media or 100 μl of 10% Triton X-100 (Sigma, St. Louis, MO) was added, respectively, instead of effector cells. Plates were centrifuged for 30 s at 11,000 g and incubated for 4 h at 37°C. One hundred microliters of each supernatant were counted in a Wizard 1470 gamma counter (Wallac, Turku, Finland). Each set of triplicates was averaged, and specific lysis was determined. Percent specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Spontaneous release in all experiments was less than 10% of the maximum release.
and uninfected animals were used as controls. Spleens from uninfected and mock-infected animals contained an average percent (±SEM) of 5.8 ± 0.4 and 5.7 ± 0.1 NK cells (DX-5<sup>1</sup>), respectively, while the average percent of NK cells in spleens from infected mice was 8.1 ± 0.3. There was a small population of DX-5<sup>1</sup> cells (12.5 ± 0.4) in uninjected eyes and in mock-infected eyes (14.9 ± 4.6), which increased in MCMV-infected eyes to 19.4 ± 2.3. A small percentage of DX-5<sup>1</sup> splenocytes are CD3<sup>1</sup> (information provided by PharMingen). In normal spleens, a population of CD4<sup>+</sup>, DX-5<sup>+</sup> cells (0.5 ± 0.2 above background) was detected; however, this population did not increase in infected animals and was not detected in infected or uninfected eyes. Additionally, on day 5 p.i., CD8<sup>+</sup> T cells were not detected in infected eyes by flow cytometry (not shown).

**Infection of NK-depleted animals causes retinitis**

To determine whether the NK response following supraciliary inoculation of MCMV mediates protection from MCMV retinitis, mice were treated with rabbit anti-asialo GM<sub>1</sub> serum to deplete NK cells (see Materials and Methods). The supraciliary space of the left eye of each mouse was infected with 9.0 × 10<sup>7</sup> pfu of MCMV on day 0; mice were harvested on day 8. Eyes were fixed, sectioned, stained, and scored for retinitis.

As shown in Figure 3A, the retinas of NK-depleted animals had retinal necrosis with loss of retinal layer integrity. Cytomegalic cells were present in the outer and inner nuclear layers of the retina as well as in the retinal pigment epithelium. Additionally, the eyes of NK-depleted mice had marked choroiditis as well as shortening of the photoreceptors and atypical folding of the retinal layers. The choroid of infected eyes from mock-depleted, MCMV-infected mice (as well as mock-infected eyes from NK-depleted mice, not shown) appeared to be normal, and only minor atypical retinal folding resulting from the injection was observed (Fig. 3B). Eight days following supraciliary injection of MCMV, 70% of the NK-depleted mice developed retinitis compared with only 20% of the control mice (Table I). The average retinal score as well as the average section score of eyes of NK-depleted mice were significantly higher than those of control mice (Table I).

**Poly(I-C) enhancement of NK cells protects mice from retinitis**

Previous studies have shown that T cell-depleted mice infected with MCMV via the supraciliary route develop retinitis (10, 11). Therefore, to determine whether NK cells can prevent or modulate MCMV retinitis in the absence of T cells, mice were thymectomized and T cell depleted by injection of mAbs to CD4 and CD8. Mice were rested for 2 wk following T cell depletion and then injected with the NK cell activator poly(I-C) or PBS (control mice) on day −1, −2, and −5. All mice were injected with 9.0 × 10<sup>7</sup> pfu of MCMV on day 0. On day 8 p.i., mice were sacrificed; eyes were sectioned for histopathologic examination, and spleens were removed to verify T cell depletion. Flow cytometric analysis of

![FIGURE 1. Cytotoxicity of NK-sensitive targets (YAC-1) by draining lymph node cells (A) or splenocytes (B) following supraciliary infection with MCMV. Mice were infected via the supraciliary route with 9.0 × 10<sup>2</sup> pfu of MCMV 1, 2, 3, 5, or 7 days before assay. Draining lymph nodes (A) or spleens (B) were harvested and single cell suspensions were prepared and used in chromium release assays against labeled YAC-1 targets. Results from one of three experiments are shown.](http://www.jimmunol.org/)

![FIGURE 2. Flow cytometry histograms of spleens and eyes from uninjected mice, and from mice 5 days after supraciliary injection with 9 × 10<sup>3</sup> pfu of MCMV. Cells were stained with anti-CD4-phycoerythrin and DX-5-FITC. UNST., unstained. Histograms represent one mouse from each group; n = 3/group.](http://www.jimmunol.org/)
biphasic with peaks on days 2 and 7 p.i. This type of biphasic node response peaked at day 5 p.i., while the splenic response was cytolytic response against NK-sensitive YAC-1 targets. The lymph caused both a local (draining lymph node) and a systemic (splenic) model, since infection of mouse eyes via the supraciliary route does not appear to prevent induction of an NK response in this system. However, these immunosuppressive ocular cytokines, such as TGF-β, have been shown to modulate splenic immune responses following ocular infection (18, 19, 28, 32).

Important flow cytometry of DX-5-FITC-stained cells detected NK cells in ocular tissues 5 days following MCMV infection. The percentage of ocular cells falling into the gated forward and side scatter light parameters increased in MCMV-infected eyes, reflecting the infiltration of inflammatory cells. A higher percentage of gated cells recovered from MCMV-infected ocular tissues were DX5+ compared with mock-infected and uninjected controls. Surprisingly, DX-5+ cells were detected in normal eyes of perfused mice, a finding that suggests that there is a resident population of DX-5+ cells in the eye. It remains to be determined whether this DX-5+ population is truly an NK population and if so, whether NK cells are continually cycling in and out of the eye or are strictly local. The increase of NK activity (and of DX5+ cells, Bigger and Atherton, unpublished observation) in the draining lymph nodes following infection suggests that the increase in the DX-5+ population in ocular tissues results, at least in part, from migration of these cells into the eyes, and not just from an expansion of resident cell populations.

However, merely determining that supraciliary inoculation of MCMV induces an NK response does not provide information about the role of such a response during ocular infection. Depletion of NK cells allowed MCMV to infect and subsequently destroy the retina in a significantly higher percentage of mice injected with MCMV via the supraciliary route. Results from the NK depletion studies show the importance of NK cells in protection of the retina in this model and are further supported by the finding that poly(I-C)-induced NK cells protect the retina following MCMV infection in susceptible mice. Although Ikeda et al. (31) showed that delayed mortality in SCID mice infected with MCMV via the i.p. route and treated with poly(I-C) was independent of NK cells, our studies show that NK cells are required for poly(I-C)-mediated protection in the eye. Although high levels of IFNs have been detected in the peritoneal cavity following i.p. injection of poly(I-C), it is unlikely

Table I. NK depletion increases the incidence of retinitis following supraciliary inoculation of 9.0 × 103 pfu of MCMV

<table>
<thead>
<tr>
<th>Group</th>
<th>Retinal Score</th>
<th>Mean Retinal Score ± SEM</th>
<th>Av. Score of All Eye Sections</th>
<th>% Retinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-depleted</td>
<td>3,3,3,3,3</td>
<td>2.6 ± 0.22</td>
<td>1.78 ± 0.11</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>3,3,2,1,0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock-depleted</td>
<td>3,3,1,1,1</td>
<td>1.5 ± 0.28</td>
<td>1.03 ± 0.09</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1,1,1,0,5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Maximum posterior segment score for each injected eye.

Significantly different from mock-depleted, p < 0.02, Mann-Whitney, n = 10.
that these IFNs play a significant role in virus replication in the eye, especially since serum levels of such IFNs decrease within 18 h following i.p. injection of poly(I-C) (31).

The observation that poly(I-C) enhancement of NK cells protected the retina in MCMV-infected, T cell-depleted mice suggests that NK cells may modulate CMV retinal disease in humans and that therapeutic augmentation of NK activity might be used to treat CMV retinitis. NK cells may modulate ocular CMV infection in AIDS patients, since approximately 90% of AIDS patients are seropositive for CMV but only about half of these develop CMV retinitis (5, 6). Interestingly, kidney transplant patients with active CMV disease have elevated NK activity compared with patients without active CMV infection, even during posttransplant immunosuppression (23) and, while viral pneumonia is common in these patients, retinitis is not (5–6).

In humans, CMV retinitis correlates with low CD4+ lymphocyte counts (33), and in BALB/c mice, T cell depletion predisposes to MCMV retinitis following ocular infection (10, 11). While there is a correlation between low CD4+ cell counts and CMV retinitis (33) and a correlation between AIDS progression and loss of NK function (24, 25), studies have not been done to determine whether there is a correlation between NK cell activity and development of CMV retinitis. AIDS patients have been reported to have very low T cell responses to CMV and yet to live for 5 yr or longer before the onset of CMV retinitis (34). There are also reports of patients with high CD4+ lymphocyte counts who have CMV retinitis (5, 6, 33). Loss of NK function, which has been reported in human AIDS patients as they progress to stage IV AIDS (24, 25), could contribute to or be sufficient for retinal infection by CMV. Similarly, a functional NK response could be responsible for protecting T cell-depleted patients from CMV retinitis. The results of these animal studies suggest that further investigation of NK activity in AIDS patients may be warranted.

In conclusion, the results of these studies show that depletion of NK cells in otherwise immunocompetent BALB/c mice predisposes the retina to MCMV infection. Enhancement of NK activity in T cell-depleted mice protected the retina from destructive retinitis. By extrapolation, these results suggest that NK activity may provide protection against CMV retinitis in seropositive human patients and that strategies to boost NK activity may be efficacious in modulating CMV retinitis, even in patients with low T cell counts.

Table II. Poly(I-C) enhancement of NK activity protects T-cell-depleted mice from retinitis following supraciliary injection with 9.0 × 10^7 pfu of MCMV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Retinal Score*</th>
<th>Mean Retinal Score ± S.E.M.</th>
<th>Av. Score of All Eye Sections</th>
<th>% Retinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I-C)</td>
<td>3,3,3,2,2</td>
<td>2.0 ± 0.21e</td>
<td>1.36 ± 0.12c</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2,3,3,3,1</td>
<td>2.5 ± 0.87d</td>
<td>2.08 ± 0.13c</td>
<td>80</td>
</tr>
<tr>
<td>PBS</td>
<td>4,3,3,3,3</td>
<td>2.8 ± 0.25</td>
<td>2.08 ± 0.13c</td>
<td>20</td>
</tr>
<tr>
<td>Poly (I-C), anti-asialo GM₁</td>
<td>3,3,3,2</td>
<td>2.75 ± 0.25f</td>
<td>1.75 ± 0.19f</td>
<td>75</td>
</tr>
</tbody>
</table>

* Mice were T cell-depleted and treated with poly(IC) and/or anti-asialo-GM₁ before MCMV infection. Control mice were T cell-depleted and injected i.p. with an equivalent volume of PBS. Statistical comparisons by Mann-Whitney test.

**S.E.M.** Maximum posterior segment score for each injected eye.

† Significant difference from PBS-treated group, p < 0.02, n = 10 per group.

‡ Significantly different from PBS-treated group, p < 0.0001, n = 60 (10 injected eyes × 6 sections/eye).

§ Not different from PBS-treated group, p < 0.79.

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<th>References</th>
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