Mice with Mutations in Fas and Fas Ligand Demonstrate Increased Herpetic Stromal Keratitis following Corneal Infection with HSV-1

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Mice with Mutations in Fas and Fas Ligand Demonstrate Increased Herpetic Stromal Keratitis following Corneal Infection with HSV-1

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HSV-1 infection of the cornea leads to a potentially blinding immunoinflammatory lesion of the cornea, termed herpetic stromal keratitis. It has also been shown that one of the factors limiting inflammation of the cornea is the presence of Fas ligand (FasL) on corneal epithelium and endothelium. In this study, the role played by FasL expression in the cornea following acute infection with HSV-1 was determined. Both BALB/c and C57BL/6 (B6) mice with HSV-1 infection were compared with their lpr and gld counterparts. Results indicated that mice bearing mutations in the Fas Ag (lpr) displayed the most severe disease, whereas the FasL-defective gld mouse displayed an intermediate phenotype. It was further demonstrated that increased disease was due to lack of Fas expression on bone marrow-derived cells. Of interest, although virus persisted slightly longer in the corneas of mice bearing lpr and gld mutations, the persistence of infectious virus in the trigeminal ganglia was the same for all strains infected. Further, B6 mice bearing lpr and gld mutations were also more resistant to virus-induced mortality than were wild-type B6 mice. Thus, neither disease nor mortality correlated with viral replication in these mice. Collectively, the findings indicate that the presence of FasL on the cornea restricts the entry of Fas+ bone marrow-derived inflammatory cells and thus reduces the severity of HSK. The Journal of Immunology, 2012, 188: 000–000.

Herpetic stromal keratitis (HSK) is a potentially blinding corneal inflammation that accompanies HSV infection of the eye. The disease course in HSK begins with a primary infection by HSV, followed by a period during which the virus enters latency in sensory and autonomic ganglia. Many studies have shown that clinical disease is the result of a mixture of inflammatory cells, consisting of PMNs, macrophages, and T cells (both CD4+ and CD8+) that are recruited to the corneas of patients with HSK (1–4). In the face of this potentially blinding inflammatory attack, the cornea has the ability to reduce inflammation in many ways. These include the presence of immunosuppressive factors such as TGF-β (5), lack of vascularization (6, 7), and the presence of Fas ligand (FasL) (8–14). The presence of FasL is the focus of this article.

Various studies have clearly shown that the presence of FasL in the eye is an important barrier to both inflammatory cells (8–10) and new blood vessels (11–13), both of which are intimately involved in the pathologic process of HSK. Control of inflammation is also known to be a significant component of the immune privilege of the eye (8, 9). FasL expressed on ocular tissues induces apoptosis in Fas+ lymphoid cells that invade the eye in response to viral infection (8) or corneal grafting (10, 11). FasL expressed in the retina and the cornea also controls new vessel growth beneath the retina and in the cornea by inducing apoptosis of Fas-expressing vascular endothelial cells (15–17). These studies clearly indicate that the presence of FasL in ocular tissues restricts inflammatory responses.

An understanding of the cellular interactions between virus-specific immune cells and cells of the cornea and nervous system is crucial in determining the underlying mechanisms of HSK. To more fully examine the role of Fas and FasL during primary HSK, we used mice deficient in Fas (lpr) and FasL (gld). To determine if host genetic background influences the role of T cell subsets in recurrent corneal disease, we performed our experiments in HSV-susceptible (BALB/c) and HSV-resistant (C57BL/6) strains of mice. Our findings indicate that mice defective in either Fas or FasL experience increased HSK disease following infection with HSV-1.

Materials and Methods

Virus and cells

The viruses used in these studies were the McKrae and KOS strains of HSV-1. A plaque-purified stock was grown and assayed on Vero cells in MEM with Earle’s balanced salts containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (18). Virus titers in eye swabs were determined by standard plaque assay (18).

Mice

Investigations with mice conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (B6) and BALB/c mice were purchased...
from the National Cancer Institute. The B6Smn.C3-Tnfsf6lpr and B6. MRL-Tnfsf6lpr mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our colony. For the purposes of this article, we will refer to these mice as B6-gld and B6-lpr, respectively. We also bred the B6-gld and B6-lpr mice to BALB/c mice for a minimum of 12 generations. The resultant strains designation will be C.B6-Tnfsf6lpr and C. B6-Tnfsf6lpr (19, 20) However, we will refer to them as BALB-gld and BALB-lpr, respectively. To ensure that these mice retain their mutations, tail DNA is isolated from individual mice and PCR tested for either the gld or lpr mutation.

**Infection of mice**

Eight- to 12-week-old normal and mutant mice were infected as previously described (21). Briefly, following corneal scarification, we used 10⁷ PFU HSV-1 KOS strain when infecting BALB/c mice or 1 × 10⁶ PFU HSV-1 McKrae strain when infecting C57BL/6. A volume of 5 μl MEM with Earle’s balanced salts containing HSV-1 was placed onto the surface of scarified corneas of BALB/c (HSV-sensitive) or C57BL/6 (HSV-resistant) mice.

**Clinical evaluation**

On the designated days after viral infection or UVB reactivation, a masked observer examined mouse eyes through a binocular-dissecting microscope to score clinical disease. Stromal opacification was rated on a scale of 0–4, where 0 indicates clear stroma, 1 indicates mild stromal opacification, 2 indicates moderate opacity with discernible iris features, 3 indicates dense opacity with loss of defined iris detail except pupil margins, and 4 indicates total opacity with no posterior view. Corneal neovascularization was evaluated as described (18, 21), using a scale of 0–8, where each of four quadrants of the eye is evaluated for the number of vessels that have grown into it. Periocular disease was measured in a masked fashion on a semi-quantitative scale, as previously described (22).

**Viral titering from tissues**

Eye swab material was collected and assayed for virus by standard plaque assay, as previously described (18). Trigeminal ganglia and 6-mm biopsy punches of periocular skin were removed and placed in preweighed tubes containing 1-mm glass beads and 1 ml medium. Trigeminal ganglia and periocular skin homogenates were prepared by freezing and thawing the samples, mechanically disrupting in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, OK), and sonicating. Homogenates were assayed for virus by standard plaque assay, and the amount of virus was expressed as PFU per milliliter of tissue homogenate.

**Construction of bone marrow chimeras**

Radiation bone marrow chimeras between BALB/c and BALB-lpr mice were prepared as follows. Briefly, mice were irradiated with 700 rads from an XRAD 320 irradiator (Precision X-Ray, North Branford, CT) and reconstituted with an equal mixture of 2 × 10⁷ bone marrow and spleen cells. The level of chimerism was determined by PCR genotype analysis of peripheral blood cells 30 d after bone marrow transplantation.

**Viral reactivation assay**

Trigeminal ganglia were removed from infected mice 30–40 d postinfection. To assess reactivation, individual trigeminal ganglia were dissociated (19) and plated on collagen-coated 12-well plates. Supernatants were assayed every 12 h for progeny virus, from 1 to 5 d after plating.

**Assays of Ab titers**

Serum was collected from mice at weekly intervals following infection and examined for HSV-specific Ab content, as previously described (23). Briefly, for ELISA, serial 4-fold dilutions of mouse serum were incubated for 2 h in duplicate wells of a 96-well plate coated with purified HSV-1 glycoprotein. Biotinylated goat anti-mouse IgG was subsequently used in a colorimetric assay to determine specific IgG amounts based on comparison with a standard curve generated as previously described (23).

**Flow cytometric analysis**

Cells were isolated from corneas as previously described (4). Briefly, corneas were excised at 18 and 23 d postinfection and incubated in PBS-EDTA for 15 min at 37°C. Stromas were separated from overlying epithelium and digested in 84 U collagenase type 1 (Sigma-Aldrich, St. Louis, MO) per cornea for 2 h at 37°C and then were triturated to form a single-cell suspension. Suspensions were filtered through a 40-μm cell strainer cap (BD Labware, Bedford, MA) and washed and then stained. Suspensions were stained with the following: PerCP-conjugated anti-CD45 (30-F11) and Alexa Fluor 700-Gr-1 (RB6-8C5) (from BioLegend, San Diego, CA); FITC-conjugated anti-CD4 (RM4-5), PE-conjugated anti-CD8α (53–67), PE-Cy7–conjugated anti-CD11c (HL3) (all from BD Pharmingen, San Diego, CA); and eFluor 450-conjugated CD11b (M1/70) (from eBiosciences, San Diego, CA). Cells were then analyzed on a flow cytometer (FACSAria with FACSDiva data analysis software; BD Biosciences, San Jose, CA).

**Statistical analysis**

All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA). The Student unpaired t-test was used to compare corneal disease scores, virus titer, and Ab titer data. Fisher’s exact χ² tests were used to compare limiting dilution assay data.

**Results**

HSK is a disease that results from the infection of corneal epithelium by HSV-1 (1–4). At the heart of this disease is an immunemediated inflammatory attack on the cornea. As previously described, corneal inflammation is controlled by a number of cell free and surface proteins, including FasL (5–14). Previous studies attempting to determine the role of Fasl in controlling HSK were not able to distinguish differences in corneal disease when the corneas of B6 and their lpr and gld counterparts were infected with the KOS strain of HSV-1 (24). However, because B6 mice are highly resistant to developing HSK when infected with the KOS strain of HSV-1 following corneal infection, we thought this was...
mice were infected with 10^7 PFU of HSV-1, KOS strain, and blepharitis BALB/c wild-type (creased blepharitis following infection with HSV-1, KOS strain. Eyes of Defective expression of Fas, but not FasL, results in in-

FIGURE 2. Defective expression of Fas, but not FasL, results in increased blepharitis following infection with HSV-1, KOS strain. Eyes of BALB/c wild-type (n = 20), BALB-lpr (n = 20), and BALB-gld (n = 20) mice were infected with 10^7 PFU of HSV-1, KOS strain, and blepharitis was measured. Significant virus-induced blepharitis was observed for BALB-lpr (p < 0.01) at days 14 and 21 when compared with both BALB/c controls and BALB-gld mice.

FIGURE 3. Defective expression of both Fas and, to a lesser extent, FasL results in increased HSK following infection of C57BL/6 (B6) mice with HSV-1, McKrae strain. Eyes of B6 wild-type (n = 25), B6-lpr (n = 22), and B6-gld (n = 23) mice were infected with 2 x 10^6 PFU of HSV-1, McKrae strain. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these strains of mice. Significant virus-induced corneal opacity was observed for B6-lpr (p < 0.01–0.001) at days 11–30 when compared with B6 controls. B6-gld mice displayed significantly more opacity than did B6 controls at days 14–30 (p < 0.05–0.01). B6-lpr showed significantly greater neovascularization at days 11–44 (p < 0.05–0.001), and B6-gld mice had greater neovascularization at days 14–44 (p < 0.05–0.01) than did B6 controls.

To confirm what we observed with BALB/c mice, we also tested whether similar results would be seen when B6, B6-lpr, and B6-gld mice were infected with HSV-1. However, we decided to use the McKrae strain of HSV-1, because of previous publications showing that infection with this strain leads to significant disease in B6 mice (21). Infected B6, B6-lpr, and B6-gld mice displayed a disease profile similar to that observed when BALB/c mice were infected with KOS, namely, that B6 mice containing mutations in either Fas or FasL exhibited greater HSK disease than did wild-type B6 mice (Fig. 3).

To better understand the mechanism underlying these observations, we hypothesized that mice expressing either defective Fas (lpr) or FasL (gld) were not able to effectively control inflammatory cell entry into corneas infected with HSV-1. To test whether lack of control of inflammatory cells entering the cornea was the underlying mechanism responsible for increased disease, we constructed bone marrow chimeras between wild-type BALB/c and BALB-lpr mice. BALB-lpr mice were chosen because they displayed the highest HSK disease scores of the strains tested. Thus, we predicted that wild-type BALB/c mice reconstituted with BALB-lpr bone marrow would experience greater disease because their inflammatory cells, which do not express functional Fas, would not be controlled by the Fasl, expressed on corneal endothelium and epithelium (8, 10). In contrast, if a nonlymphoid cell were responsible for this disease, BALB-lpr mice reconstituted with wild-type BALB/c cells would experience greater disease. As shown in Fig. 4, an increased disease phenotype was associated with the genotype of the bone marrow-derived lymphoid cells, as irradiated BALB/c mice reconstituted with BALB-lpr bone marrow had significantly worse corneal disease than did irradiated BALB-lpr mice reconstituted with BALB/c bone marrow. Therefore, we concluded that lack of control of the inflammatory infiltrate leads to increased HSK.

We next isolated cells from the corneas of mice with both severe disease (opacity scores >2) and minimal disease (opacity scores <1) to determine if any qualitative differences existed in the types of cells infiltrating the corneas of BALB/c, BALB-lpr, and BALB-gld mice. Surprisingly, the nature of the inflammatory infiltrate was remarkably similar between these mice. All mice with severe disease consistently exhibited very high percentages of Gr-1+CD11b+ neutrophils (BALB/c, 73.2 ± 6.9%; BALB-lpr, 83 ±
The percentage of T lymphocytes in these severely diseased corneas ranged from 3 to 15%, with the ratio of CD4+ to CD8+ T cells being ∼5:1, but, again, no differences between strains were noted. Similarly, F4/80+ macrophages were between 2 and 5%, with no differences between strains. In addition, no significant differences were observed in inflammatory subsets between strains for those corneas without disease. However, the total number of CD45+ cells was much lower than that in corneas with severe disease, and the percentage of Gr-1+, CD11b+ ranged from 2 to 20% of the total CD45+ cells. The primary cell type in these corneas without disease expressed T lymphocyte markers (range, 40–60%).

Because it is known that mice expressing the lpr and gld mutations do not mount the same degree of specificity toward foreign Ags as wild-type mice, particularly as they age (26), we also wanted to determine how well these mice were able to clear primary infection with HSV-1. No differences could be found in the viral titers of eye swabs from these mice at any of the time points monitored (Fig. 6). Similarly, viral titers from both periocular tissue biopsy specimens and trigeminal ganglia did not reveal any significant differences in viral growth in these tissues (Fig. 7).

It should be noted, however, that even though HSK was worse in mice expressing either the lpr or gld mutations, they had a lower mortality than did wild-type mice when infected with 5 × 10^6 PFU of the McKrae strain of HSV-1 (Fig. 8). In contrast, direct infection of the brain did not result in any differences in mortality, even when <100 PFU was injected intracranially (data not shown). This finding contrasts with what was reported for HSV-2 (27) and suggests that survival of mice with an impaired Fas–FasL interaction is likely due to better control of peripheral infections with a neurovirulent strain of HSV-1, not to an inherently greater susceptibility of brain to infection.

We also performed histologic analysis of trigeminal ganglia to determine if any gross differences in inflammation of these different mouse strains might explain why mice with the lpr mutation exhibit worse disease. These ganglia sections did not reveal any significant differences in inflammation (data not shown). Thus, symptoms possibly indicating a neurologic disease could not be explained by significant differences in viral titers or degree of inflammation between these mouse strains.

**Discussion**

A prime mechanism used by the eye to protect itself from T cell-mediated immunopathologic response is the presence of FasL, which induces apoptosis in Fas+ lymphoid cells (8–11). Our laboratory, as well as several others, has shown that lack of functional Fas–FasL-mediated apoptotic ability in the eye most often leads to greater inflammatory responses (8, 9), increased corneal allograft rejection (10, 28), enhanced neovascularization (12–14), and the inability to develop systemic tolerance following injection of Ag into the anterior chamber (8). In addition to responses specific to the eye, it is also well established that host T cells eliminate virus-
infected cells by either the perforin–granzyme pathway (29) or via apoptosis mediated by the interaction of FasL on effector cells with Fas expressed by virally infected cells (30, 31). Thus, it would seem that mice not able to express either functional FasL or the receptor Fas have the potential for showing a wide variety of abnormalities. These possibly include a propensity for greater inflammatory responses in the eye because of an impaired ability to control entry of inflammatory cells that would normally be subject to apoptosis from engagement of corneal FasL with Fas+ inflammatory cells. One might also hypothesize that these mice would have difficulty clearing virally infected cells because the Fas–FasL pathway of killing virally infected cells is not available to cytotoxic T cells, which could result in the persistence of infectious virus in the cornea.

Histologic analysis of the corneas from lpr and gld mice revealed significantly increased inflammatory infiltrate compared with that seen in wild-type mice. Thus, the entry of inflammatory cells was not well controlled in these mutant mice. This finding

**FIGURE 6.** Defective expression of Fas and FasL does not increase the magnitude of corneal viral shedding but does prolong shedding. Eyes of BALB/c wild-type (n = 30), BALB-lpr (n = 25), and BALB-gld (n = 25) mice were infected with 10^7 PFU of HSV-1, KOS strain, and corneas were swabbed on the indicated days and then titrated. No significant differences were seen in the magnitude of viral shedding. Results displayed are means ± SEM for each group of mice indicated.

**FIGURE 7.** Defective expression of Fas and FasL does not increase the magnitude of viral replication in infected trigeminal ganglia. Eyes of BALB/c wild-type (n = 7 mice/time point), BALB-lpr (n = 5 mice/time point), and BALB-gld (n = 5 mice/time point) mice were infected with 10^7 PFU of HSV-1, KOS strain, and trigeminal ganglia were removed and viral titers determined. No significant differences in viral growth were seen between the mouse strains being compared. Results displayed are means ± SEM for each group of mice indicated.
This observation suggests that the primary reason for increased expression by potentially Fas-expressing resident corneal cells is not due to lack of functional Fas. Increased disease is associated with Fas expression by bone marrow-derived cells and is essentially the same, with neutrophils being the dominant cell found during severe disease. This observation illustrates that HSK in *lpr* and *gld* mice is mediated by the same types of cells that mediate disease in normal mice and is not due to the accumulation or recruitment of some other type of inflammatory cell.

As suggested above, another possible mechanism for increased HSK severity in *lpr* and *gld* mice would be the persistence of HSV-1 infectious virus in infected corneas. Data comparing infectious HSV-1 in tear films from these mouse strains revealed a slight, although insignificant, prolongation of HSV-1 in the corneas of *lpr* and *gld* mice. This prolongation could result from impaired killing of virally infected corneal cells, which has been shown to be mediated by either perforin–granzyme or FasL (29). However, some evidence argues against this hypothesis. First, expression of Fas Ag by corneal cells is below the level of detection by Western blot analysis (19), making it unlikely that these cells would be targets of FasL-mediated killing by CTLs. Second, irradiated BALB-*lpr* chimeric mice that were reconstituted with normal BALB/c bone marrow cells, which would express the *lpr* mutant Fas Ag on resident corneal cells, did not suffer from significant HSK. Furthermore, when viral shedding from these chimeric mice was evaluated, no differences in HSV-1 titers were detected between the chimeric mice we used. Thus, the data do not support the hypothesis that viral persistence plays a significant role in the disease phenotype of mice that have impaired Fas–FasL interactions.

Also possible was that the increased disease seen in *lpr* mice was due to lack of control of neovascularization, as vascular endothelium expresses Fas and corneal expression of FasL has been shown to control neovascularization (14). However, we thought this unlikely, as *lpr* mice express normal Fas on their vascular endothelium (14). Demonstrating that the disease phenotype is associated with lymphoid cells further supports the notion that vascular endothelial expression of Fas is not responsible for increased HSK in BALB-*lpr* mice.

Previous work from us and other investigators has shown that development of an Ab response against HSV-1 can protect mice from severe HSK (18, 32, 33). Consequently, we tested Ab responses in BALB/c, BALB-*lpr*, and BALB-*gld* mice to determine whether *lpr* or *gld* mice had impaired anti–HSV-1 responses. However, no differences between these strains (data not shown) were observed, indicating that the ability to develop an anti–HSV-1 Ab response was not involved.

Taken together, these studies document that mice with impaired Fas–FasL interactions develop significantly increased HSK following infection with HSV-1. Further, the mechanism responsible for increased disease is likely due to increased inflammation of the cornea, which is normally controlled in part by the presence of FasL on resident corneal cells. What do these data suggest concerning therapy to better control HSK? Because the vast majority of people have an intact Fas–FasL system, are there means of potentiating this interaction? Fortunately, a few articles have described methods for potentiating FasL-mediated control of Fas– cells. One such strategy took advantage of the fact that FasL is very sensitive to cleavage by matrix metalloproteases (MMP) (34). Thus stabilization of FasL expression by treatment with MMP inhibitors was demonstrated to significantly increase the success of corneal allografts (35). Similarly, treatment of mice suffering from choroidal neovascularization with either MMP inhibitors or apoptotic-inducing soluble FasL significantly reduced neovascularization (36). These studies present possible strategies using FasL-based therapies to better control unwanted inflammation and neovascularization.

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Disclosures

The authors have no financial conflicts of interest.

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