Therapeutic Use of Soluble Fas Ligand Ameliorates Acute and Recurrent Herpetic Stromal Keratitis in Mice

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PURPOSE. The present study was designed to test the therapeutic value of soluble FasL (sFasL) in an acute model of herpetic stromal keratitis (HSK) and, more importantly, a recurrent model of HSK using BALB/c, BALB-*lpr*, and National Institutes of Health (NIH) mice.

METHODS. Mice were infected either acutely with the KOS strain of herpes simplex virus 1 (HSV-1) or latently with the McKrae strain of HSV-1. Acutely infected mice as well as ultraviolet-B (UV-B) reactivated mice (recurrent infection) were treated with sFasL, or soluble TNF-related apoptosis inducing ligand (sTRAIL), or BSA daily or 3 times/wk by using either a combination of subconjunctival injection and topical ointment, or with topical ointment alone. These mice then were evaluated for corneal opacity and neovascularization for 6 weeks.

RESULTS. Following acute and recurrent HSV-1 infection, wild-type BALB/c mice treated with sFasL displayed significantly reduced incidence of corneal opacity and neovascularization compared to the control animals. However, BALB-*lpr* mice, which are deficient in Fas⁺ inflammatory cells, displayed no such differences in ocular disease, as expected. Latently infected NIH mice treated with sFasL displayed similar results. Flow cytometric analysis revealed that the corneal inflammatory infiltrate in those treated with sFasL was significantly less than in sTRAIL- or BSA-treated mice. Furthermore, corneas from sFasL-treated mice displayed relatively more cells undergoing apoptosis.

CONCLUSIONS. This study provides evidence that sFasL treatment has potential therapeutic benefit in reducing inflammatory infiltrate and neovascularization in primary and recurrent forms of HSK, and that it does so by augmenting the restriction of Fas⁺ inflammatory cells mediated by membrane FasL.

Keywords: apoptosis, cornea, neutrophils, herpes simplex virus

Herpetic stromal keratitis (HSK) is a potentially blinding corneal affliction that can accompany herpes simplex virus type 1 (HSV-1) infections. The disease course in HSK begins with a primary infection by HSV-1, typically mucosal surfaces of the mouth or eye. The virus replicates at the site of infection and proceeds via retrograde axonal transport in sensory neurons to sensory (usually trigeminal) and autonomic ganglia.¹ Once inside ganglia, the virus establishes latency, in which the pathogenic virus remains dormant yet the genome persists.² If these latently infected individuals become immunocompromised (as occurs in elderly or malnourished individuals, or with transplant or HIV-infected patients) or the neurons are exposed to a “triggering” immunosuppressive event, such as stress, trauma, sunlight (UV) irradiation, or fever, the virus is reactivated and rapidly replicates. The virus travels via anterograde axonal transport and returns to the original epithelial surface, where another round of replication occurs.^{1,2} A multitude of studies have shown that the resulting clinical disease is not associated with this viral replication in the cornea, but rather is due largely to the host immune response being restimulated by the presence of the virus.¹⁻⁵

As a consequence of these events, active viral replication triggers inflammation and neovascularization of the cornea. Once this inflammation is initiated, the disease continues to

progress even though infectious virus can no longer be detected. This inflammation consists of different leukocyte lineages that are recruited to the cornea of patients with HSK, including polymorphonuclear leukocytes (PMNs; neutrophils), macrophages, and T cells, CD4⁺ and CD8⁺.¹⁻⁵ Animal studies have shown that the predominant cell type in diseased corneas is neutrophils.³ Facing a conceivably blinding inflammatory attack, the cornea possesses several means to reduce inflammation and neovascularization. These include the presence of immunosuppressive factors, such as TGF-β,⁶ IL-1 receptor antagonists, and antiangiogenic factors, such as thrombospondin and pigment epithelium-derived factor (PEDF).^{7,8} Additionally, the presence of Fas ligand (FasL), which is the focus of this study, often is a critical factor in controlling inflammation and neovascularization, thus reducing disease.⁹⁻¹⁷

Fas is a member of the tumor necrosis factor-R family, which is a group of type I transmembrane proteins. FasL is a ligand of Fas and is synthesized as a transmembrane molecule that undergoes cleavage and processing through a metalloprotease to become soluble FasL (sFasL). This sFasL contains a “death domain,” which is essential for the induction of apoptosis.¹⁸ Studies from our laboratory as well as the laboratories of others have demonstrated that the presence of FasL in the eye is an important barrier to inflammatory cells⁹⁻¹¹ and new blood

vessels.^{12–14} In fact, we know that control of inflammation is required for the immune privilege of the eye.^{9,10} FasL expressed on ocular tissues induces apoptosis in Fas⁺ lymphoid cells that invade the eye in response to viral infection⁸ or corneal grafting.^{11,12} FasL expressed in the retina and the cornea also control new vessel growth beneath the retina and in the cornea by inducing apoptosis of Fas⁺ vascular endothelial cells.^{13–15} These studies indicate that the presence of FasL in ocular tissues restricts inflammatory responses.

Recently we published that the interaction of Fas with FasL is an important factor in controlling HSK during acute infection of the cornea.¹⁷ We demonstrated that mice expressing mutations in Fas (*lpr*) or FasL (*gld*) experience significantly worse ocular disease than do wild-type mice, regardless of mouse or viral strain.¹⁷ However, as mentioned, acute infection rarely leads to clinical disease in humans. Instead, the reactivated virus is responsible primarily for the symptoms that define corneal keratitis.^{1–5} Thus, we thought it very important to address the role that Fas and FasL have during recurrent disease when the virus is reactivated from latency. To address the role of Fas-FasL interactions during recurrent HSK, we evaluated this interaction in a mouse model of induced recurrent HSK. We used soluble TNF-related apoptosis inducing ligand (sTRAIL) as our control and standard for comparison, since TRAIL and FasL are members of the TNF family and are known to induce apoptosis in lymphocyte lineages. In this study, we demonstrated that mice treated with sFasL experience a decrease in acute and recurrent models of HSK.

MATERIALS AND METHODS

Virus and Cells

The virus strains used in these studies are the KOS strain of HSV-1 (stock titer of 5×10^9 plaque-forming units [PFU]/mL) and the McKrae strain of HSV-1 (stock titer of 6.8×10^9 PFU/mL). A plaque-purified stock was grown and assayed on Vero cells in minimum essential medium with Earle's balanced salts (MEM-EBS) containing 5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.¹⁹ Virus titers in eye swabs were determined by standard plaque assay.²⁰

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. BALB/c mice were purchased from the National Cancer Institute (NCI; Bethesda, MD, USA). The National Institutes of Health (NIH; Bethesda, MD, USA) inbred strain of mice was originally acquired from Harlan OLAC (Bicester, England) and we maintain a breeding colony of these mice. We initially acquired B6Smn.C3-*Tnfsf6*^{gld}/J and B6.MRL-*Tnfsf6*^{lpr}/J mice from Jackson Laboratories (Bar Harbor, ME, USA) and these mice were backcrossed onto the BALB/c strain for a minimum of 12 generations and are maintained as homozygous in our breeding colony. The resultant strains designations are C.B6-*Tnfsf6*^{gld} and C.B6-*Tnfsf6*^{lpr}.^{20,21} However, we will refer to them as BALB-*gld* and BALB-*lpr*, respectively. To assure 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

For acute infection, 6- to 10-week-old BALB/c mice were infected with 10^7 PFU HSV-1 KOS strain following scarification of the cornea as described previously.²² For recurrent infection, 6- to 10-week-old BALB/c mice or NIH mice were

infected on the scarified cornea with 10^6 PFU HSV-1 McKrae strain as described previously.²³ Each mouse received an intraperitoneal (IP) injection of 0.5 mL pooled human serum (ED50 for virus neutralization = 1:1600; Sigma-Aldrich Corp., St. Louis, MO, USA) concurrent with infection. Administration of anti-HSV antibodies at the time of ocular infection has been shown to protect mice from death and corneal disease during primary infection, while allowing for the establishment of latency and subsequent reactivation of virus after corneal UV-B exposure.^{24–26} These antibodies are undetectable at the time of UV-B irradiation 5 weeks after primary infection. Herpes simplex virus-positive eye swabs obtained 3 days after application of virus confirm primary infection.

UV-B Irradiation and Virus Reactivation

Mice were reactivated from latency as described previously.²³ Briefly, the eyes of all latently infected mice were examined for corneal opacity before irradiation, and only animals with clear corneas were used. At least 5 weeks after primary infection, the eyes of latently-infected and control mock-infected mice were exposed to 250 mJ/cm² of UV-B light using a TM20 Chromato-Vu transilluminator (UVP, Inc., San Gabriel, CA, USA), which emits UV-B at a peak wavelength of 302 nm. Irradiated mice were swabbed with sterile cotton applicators from day 0 to day 7, unless otherwise indicated. The swab material was cultured on VERO cells, as described above, to detect recurrent virus shedding from the cornea. Reactivation was defined as the finding of any HSV positive eye swab on any day post UV-B exposure, with day 0 swabs serving as a control.

Reagents Used

We purchased the human soluble Fas ligand (sFasL) and soluble TRAIL (sTRAIL) from R & D Systems (Minneapolis, MN, USA), and sFasL was quantitated from corneas using the Human Fas Ligand/TNFSF6 Quantikine ELISA Kit from R & D Systems.

Treatment Regimen

Mice were treated with sFasL, sTRAIL, or BSA following either infection with HSV-1 (primary disease) or UV-B reactivation (recurrent disease). Treatment began 1 day following infection or reactivation and consisted of a combination of topical application (10 µg mixed in 3 mL puralube and applied so that cornea was covered) and subconjunctival injection (30 ng in 5 µL), on a daily basis or 3 times per week unless otherwise indicated.

Clinical Evaluation

On the designated days after viral infection or UV-B 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 to 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^{19,22} using a scale of 0 to 8, where each of four quadrants of the eye is evaluated for the amount of vessels that have grown into them. Periocular disease was measured in a masked fashion on a semiquantitative scale as described previously.²⁷

Tissue Viral Titer

Eye swab material was collected daily for 7 days following either primary infection or UV-B-induced reactivation as

described previously.¹⁹ Briefly, the swabs were put into 1 mL of media used to grow the indicator VERO cells and frozen at -80°C until titers determined. Titers were determined by serial dilutions of this swab media, which were plated on VERO cells. A comparison of sFasL treatment to the other treatments did not reveal any significant differences in numbers of animals shedding virus, days shedding virus, or titer of virus.

Hematoxylin and Eosin (H&E) and Immunohistochemical Staining

BALB/c corneas from sFasL-treated and BSA-treated mice were removed at day 15 after reactivation and snap-frozen in OCT with liquid nitrogen and stored at -80°C until sectioned. To evaluate inflammation, these sections were stained by H&E by the Saint Louis University Pathology core facility. Similar sections from these mice also were costained with antibodies against CD45 (rat anti-mouse CD45 IgG2b, clone 30-11; R&D Systems) and active Caspase 3 (polyclonal rabbit IgG; R&D Systems). These sections were costained with Alexa Fluor 594 goat anti-rat IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Grand Island, NY, USA) to determine which cells were undergoing apoptosis.

Flow Cytometric Analysis

Cells were isolated from corneas as described previously.¹⁷ Briefly, corneas were excised at 18 and 23 dpi, and incubated in PBS-EDTA at 37°C for 15 minutes at 37°C . Stromas were separated from overlying epithelium and digested in 84 U collagenase type 1 (Sigma-Aldrich Corp.) per cornea for 2 hours at 37°C and then were triturated to form a single-cell suspension. Suspensions were filtered through a $40\text{-}\mu\text{m}$ cell strainer cap (BD Labware, Bedford, MA, USA) and washed and then stained. The cells then were stained for surface markers with PerCP-conjugated anti-CD45 (clone 30-F11), Alexa Fluor 700-Gr-1 (clone RB6-8C5), and APC-conjugated anti-F4/80 (clone BM8; BioLegend, San Diego, CA, USA), FITC-conjugated anti-CD4 (clone RM4-5), PE-conjugated anti-CD8a (clone 53-6.7), PE-Cy7-conjugated anti-CD11c (clone HL3; BD Biosciences, San Jose, CA, USA) and eFlour 450-conjugated CD11b (clone M1/70; eBioscience, San Diego, CA, USA). Samples were assayed with a BD FACSCalibur flow cytometer, and the data were acquired using FlowJo software (TreeStar, Ashland, OR, USA). The strategy for analysis was to initially gate on live cells and then the CD45⁺ cells. These cells were evaluated further for T cell markers CD4 and CD8, or for macrophage markers F4/80⁺CD11b⁺Gr-1⁻, or neutrophil markers Gr-1⁺CD11b⁺F4/80⁻, or dendritic cell marker CD11c⁺F4/80⁻. Cells then were analyzed on a flow cytometer (FACSARIA with FACSDIVA data analysis software; BD Biosciences).

Statistical Analysis

All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA, USA). The rank sum test was used to compare corneal disease scores. Student's unpaired *t*-test was used to compare virus titer and flow cytometry.

RESULTS

We previously demonstrated that mutations in Fas (*lpr*) and FasL (*gld*) render mice more susceptible to primary HSK.¹⁷ We and others also have shown that corneal expression of FasL is a critical factor in controlling inflammatory cell infiltration and neovascularization of the cornea.^{9–12,14,15} In addition to these

reports, it has been shown that soluble FasL (sFasL) can control unwanted choroidal neovascularization.²⁸ In light of these observations we decided to test the ability of sFasL to reduce corneal disease following acute and recurrent herpetic infections of the cornea.

We infected BALB/c mice with the KOS strain of HSV-1 and treated mice with sFasL, sTRAIL, and BSA by a combination of topical application and subconjunctival injection. The mice then were swabbed for virus for 7 days and disease measured for 5 weeks. Corneal opacity, which is primarily a reflection of inflammatory cell infiltrate, though other factors also contribute to opacity. Neovascularization scores, are an indication of the amount of new blood vessel formation in the cornea. Results indicate that, although there was no difference in viral titers between any of the treatment groups (data not shown), there was significant reduction in corneal disease in those mice treated with sFasL compared to those treated with sTRAIL or with PBS (Fig. 1). Thus, treatment with sFasL does not affect clearance of virus, but does directly impact the development of opacity and neovascularization.

To further show that the reduction in corneal disease was specific to its interaction with Fas, we tested these treatments in BALB-*lpr* mice, which lack functional Fas on inflammatory cells. Accordingly, treatment with sFasL should offer no benefit to the mouse. As expected, treatment with sFasL did not offer any protection from corneal disease (Fig. 2). There was no significant difference in corneal opacity or neovascularization between the mice treated with sFasL and those treated with sTRAIL or with PBS (Fig. 2).

One of the complications that we noted in these experiments was that some mice had an inflammatory reaction to the subconjunctival injection. Consequently, we performed ELISA assays to compare the amount of human sFasL found in the corneas of mice that were given topical treatment and subconjunctival injection with those receiving topical treatment alone. The results of that comparison indicated that the amount of human sFasL was essentially the same between these two treatment protocols (average, 135 vs. 158 pg, respectively). Hence subsequent studies used only topical application of sFasL, sTRAIL, or PBS.

Since human disease primarily is a result of reactivation from latency, we next tested whether treatment with sFasL also could reduce HSK in a mouse model of recurrent disease. Mice were latently infected with the McKrae strain of HSV-1 and then reactivated 8 weeks following primary infection. At this time they were treated daily with sFasL, sTRAIL, and PBS and monitored for corneal disease and viral titers in tear film. In a fashion almost identical to that seen during primary HSK, while there were no significant differences in viral titers or persistence of virus (data not shown), there was a significant reduction in disease in these animals for all time points from 3 weeks after reactivation until the end of the study (Fig. 3). These findings confirmed that sFasL treatment could reduce primary and recurrent forms of HSK (see Figs. 1, 3). To confirm that these observations were again due to the presence of Fas on inflammatory cells, we infected BALB-*lpr* mice, reactivated these mice 8 weeks following their initial infection, and treated them with sFasL, sTRAIL, or PBS. As stated earlier, treatment of BALB-*lpr* mice with sFasL following reactivation from latency should not be of any benefit to these mice. As Figure 4 affirms, there was no significant protection from recurrent corneal opacity or neovascularization in the BALB-*lpr* mice treated with sFasL.

We next wanted to confirm that sFasL reduced corneal disease in varying strains of mice, not just specific to the BALB/c lineage. We chose the NIH inbred strain of mouse, since this particular strain has been used very successfully in recurrent models of HSK.^{24–26} A latent herpetic infection was

FIGURE 1. BALB/c mice treated with sFasL following infection display significantly less corneal disease than mice treated with sTRAIL. Eyes of BALB/c were infected with 10^7 pfu of HSV-1, KOS strain. Following infection, mice were treated with sFasL, sTRAIL, or BSA by a combination of topical application and subconjunctival injection. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these different treatment groups of mice. The incidence of mice experiencing opacity scores greater than 1 was compared between these groups. Results are a combination of two independent studies with a total of 30 mice for each group. Significantly less virus-induced corneal opacity was observed for BALB mice treated with sFasL from 2 to 5 weeks following reactivation (* $P < 0.01$ – 0.001).

established in these mice using the McKrae strain of HSV-1, then these mice were reactivated with UV-B light 6 weeks later. These mice then were treated with sFasL, sTRAIL, or PBS. As shown in Figure 5, sFasL resulted in a significant reduction in disease from weeks 2 through 5 after infection. Thus, treatment with sFasL is not dependent on the strain of mouse used.

Our next objective was to determine whether there were any significant differences in either the quantity or specificity of inflammatory cell infiltrates in the mice tested. Latently infected BALB/c mice were treated with sFasL or BSA following reactivation. These mice then were euthanized 15 days following UV-B exposure and the corneas removed for histologic evaluation by H&E staining. We chose this time

FIGURE 2. BALB-*lpr* mice treated with sFasL following infection do not display differences in corneal disease than mice treated with sTRAIL. Eyes of BALB-*lpr* were infected with 10^7 pfu HSV-1, KOS strain. Following infection, mice were treated with sFasL, sTRAIL, or BSA by a combination of topical application and subconjunctival injection. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these different treatment groups of mice. The incidence of mice experiencing opacity scores greater than 1 was compared between these groups. Results are a combination of two independent studies with a total of 30 to 35 mice for each group. These data did not show any significant differences between the treatment groups with BALB-*lpr* mice.

FIGURE 3. BALB/c mice treated with sFasL following UV-B-induced reactivation displayed significantly less corneal disease than mice treated with sTRAIL. Eyes of BALB/c treated were infected with 10^6 pfu of HSV-1, McKrae strain, and allowed to establish latency. Six weeks following infection, the eyes of BALB/c were irradiated with UV-B to reactivate the latent infection. These mice were separated into three groups, in which they were treated with sFasL ($n = 20$), sTRAIL ($n = 20$), or BSA ($n = 20$) daily beginning on the day following reactivation. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these strains of mice. Significantly less virus-induced corneal opacity and neovascularization was observed for BALB/c mice treated with sFasL from 3 to 5 weeks after infection (* $P < 0.01$ – 0.001).

point as between 2 and 3 weeks corneas display opacity and neovascularization scores that differentiate differences between sFasL treatment and other treatment groups (Fig. 3). As Figure 6 demonstrates, sFasL-treated mice displayed little inflammatory cell infiltration, while corneas from BSA control-treated mice demonstrated significant numbers of inflammatory cells. Separate sections from these mice also were examined immunohistochemically to determine whether the inflammatory infiltrate ($CD45^+$ cells) displayed any evidence of apoptosis following sFasL treatment. Figure 7A demonstrates that there are not many $CD45$ expressing cells, but that several of these also express the Caspase 3 marker for apoptosis. In contrast, there are many more $CD45^+$ cells in the control-treated corneas and the number of these $CD45^+$ cells that coexpressed Caspase 3 are very few (Fig. 7B). It is interesting to note that examination of sections from BALB-*lpr* mice did not display any $CD45^+$ cells that expressed Caspase 3 (data not shown).

FIGURE 4. BALB-*lpr* mice treated with sFasL following UV-B-induced reactivation displayed corneal disease that was indistinguishable from mice treated with sTRAIL. Eyes of BALB-*lpr* were infected with 10^6 pfu of HSV-1, McKrae strain and allowed to establish latency. Six weeks following infection, the eyes of BALB/c were irradiated with UV-B to reactivate the latent infection. These mice were separated into three groups, in which they were treated with sFasL ($n = 20$), sTRAIL ($n = 20$), or BSA ($n = 20$) mice. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these strains of mice. No statistical significance was seen between any of the treatment groups.

To better characterize these inflammatory cells, UV-B reactivated latently infected BALB/c mice were treated with sFasL, sTRAIL, and PBS. Corneas were removed from these mice 17 days following UV-B exposure and single cells were isolated. Then, the cells were characterized for neutrophil, macrophage, and T-cell markers. Consistent with overall reduced opacity, the mice treated with sFasL had significantly fewer total $CD45^+$ cells (2128 ± 919) than did mice treated with sTRAIL ($17,350 \pm 3625$; $P < 0.02$) or PBS ($15,334 \pm 4562$; $P < 0.05$), indicating that cellular inflammation was much better controlled in mice treated with sFasL. When these cells were phenotyped, the most remarkable difference was noted in the percentage of $Gr-1^+CD11b^+F4/80^-$ neutrophils. Mice treated with sFasL had a significantly lower percentage of neutrophils ($P < 0.01$) than their sTRAIL or PBS treated counterparts (Fig. 8B). The percentage of $Gr-1^+CD11b^+F4/80^-$ neutrophils was approximately 55% to 62% in the PBS and sTRAIL-treated mice corneas while the percentage was 23% to 25% in the sFasL-treated mice (Fig. 8B). This significant

FIGURE 5. Treatment of NIH inbred mice with sFasL significantly reduced corneal disease following UV-B-induced reactivation. Eyes of NIH mice were infected with 10^6 pfu of HSV-1, McKrae strain. Six weeks following infection mice were irradiated with UV-B to reactivate the latent infection. Mice were separated into three treatment groups, sFasL ($n = 15$), sTRAIL ($n = 15$), and BSA ($n = 15$). Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these groups of mice. Mice receiving topical treatment of sFasL displayed significantly less virus-induced corneal opacity at 2 to 5 weeks and less neovascularization at 2, 3, and 5 weeks following reactivation ($*P < 0.01$ – 0.001).

decrease in the percentage of Gr-1⁺CD11b⁺F4/80⁺ neutrophils following sFasL treatment was not seen in the other CD45⁺ cells (Fig. 8B). However, as shown in Figure 8A there was a significant decrease in all subsets of CD45⁺ cells, suggesting that these cells likely produce chemokines that attract neutrophils to corneas undergoing HSK. Consequently, this relative increase in T cells and macrophages is not likely important and only reflects a significant reduction in overall inflammation.

FIGURE 6. Histologic sections of corneas indicate that (A) treatment with sFasL results in reduced numbers of inflammatory cells when compared to (B) corneas that were treated with BSA. Hematoxylin and eosin stain of corneal sections are from mice 15 days after reactivation. Original magnification: $\times 20$.

DISCUSSION

Membrane Fas ligand is a critical surface protein that helps to protect the cornea from immunopathologic disease. Our laboratory, along with several others, has demonstrated the wide array of effects that result from dysfunctional Fas-FasL-mediated apoptotic ability. Not only is the eye more prone to an increased inflammatory response^{9,10} and neovascularization,^{13–15} but it is also at risk for increased corneal allograft rejection^{11,29} and the inability to develop systemic tolerance following injection of antigen into the anterior chamber.⁹ Furthermore, with recurrent bouts of inflammation, neovascularization, and scarring of the cornea, those with defects in either Fas or FasL experience worse HSK that can lead to vision loss.¹⁷

In addition to these immune responses that are specific to the eye, it also is well established that host T cells eliminate virally-infected cells by either the perforin-granzyme pathway³⁰ or via apoptosis mediated by the interaction of FasL on effector cells with Fas expressed by virally-infected cells.^{31,32} It is clear that this interaction has a critical role in removing virally infected cells and with controlling the host inflammatory response. As a consequence it is no surprise that mice that are unable to express either functional FasL or their receptor Fas have the potential for expressing a wide range of abnormalities. For instance, mice may experience greater inflammation and the repercussions of such a response (i.e., corneal scarring) due to the impaired ability to control entry of inflammatory cells that normally would be subject to apoptosis from engagement of corneal FasL with Fas⁺ lymphoid cells. In addition, one might hypothesize that mice would have increased difficulty clearing virally-infected cells because the Fas-FasL pathway of killing such cells is not available to cytotoxic T cells. This could result in persistence of infectious virus in the cornea, which has been reported during primary infection with HSV-1.¹⁷ Since mice with mutations in the Fas-FasL pathway are seemingly more vulnerable to worsened corneal inflammation, this finding also suggests that mice treated with sFasL should experience less ocular disease than those lacking such treatment. This is precisely what we demonstrated in this report that treatment with sFasL during

FIGURE 7. Immunohistochemical staining of sections from corneas treated with sFasL display a much higher percentage of CD45+ cells that also express the apoptotic active caspase 3 marker. Corneal sections were removed from mice 15 days following UV-B reactivation that were either (A) sFasL treated (*original magnification*: $\times 40$) or (B) received control treatment (BSA, *original magnification*: $\times 40$). These sections then were stained with rat anti-mouse CD45 + Alexa Fluor 594 goat anti-rat IgG (*red*) and rabbit anti-mouse active caspase 3 + Alexa Fluor 488 goat anti-rabbit IgG (*green*). DAPI (4',6-diamidino-2-phenylindole, *blue*) was used to counterstain the nuclei. *Arrows* refer to cells that coexpress CD45 and caspase 3.

FIGURE 8. As expected, mice treated with sFasL had significantly reduced inflammatory cell infiltrates. Mice treated with sFasL ($n = 10$) or sTRAIL ($n = 10$) were killed 17 days following reactivation and single cells isolated from their corneas. These cells then were characterized for neutrophil, macrophage, and T-cell markers. Cell numbers for each of the subsets of cells (A) and percent of CD45⁺ cells of these subsets of cells (B) were performed. Mice treated with sFasL had significantly fewer cells for all of the subsets evaluated ($^*P < 0.02$ - 0.001) and a lower percentage of neutrophils ($^*P < 0.01$).

primary HSV-1 infection and following reactivation, demonstrated a positive correlation between treatment with sFasL and reduction in corneal disease. It should be noted that we did not crosslink sFasL before use and, thus, it is possible that it could have been more effective had we done so. However, lack of cross-linking did not prevent therapeutic efficacy, nor did it prevent the apoptosis of CD45⁺ inflammatory cells. It is this later point, that corneas treated with sFasL display relatively few CD45⁺ cells by flow cytometry or immunohistochemistry and that a significant number of these cells also demonstrate that they are undergoing apoptosis. Alternatively, corneas undergoing control treatment had numerous CD45⁺ cells and very few of these cells displayed markers of apoptosis. These data support our mechanism that treatment with sFasL reduces disease by better controlling the inflammatory cell infiltrate. This does not, however, discount the effect that sFasL might be playing on restricting neovascularization of the cornea. We have shown that targeting vascular endothelium, which expresses Fas, results in significantly reduced neovascularization.¹⁵ Thus, it is entirely possible that sFasL restricts the Fas expressing inflammatory infiltrate and the Fas expressing vascular endothelium from gaining entry into the cornea.

Cellular analysis of the corneas of BALB/c mice treated with sFasL revealed significantly fewer inflammatory cells than was seen in mice treated with the control sTRAIL. Mice treated with sFasL had significantly fewer total cells ($P < 0.02$) and a lower percentage of neutrophils (Gr-1⁺CD11b⁺ F4/80⁻; $P < 0.01$). Studies conducted by our laboratory and others have indicated that the presence of neutrophils has a fundamental role in the disease pathology of herpetic stromal keratitis.^{3,5,25} Thus, the entry of inflammatory cells was much better managed in sFasL treated mice. These results are quite similar to what was reported when CXCL-1 was neutralized.²⁵ While it has been reported that mice deficient in CXCL1 do not display changes in inflammatory infiltrate shortly after primary infection with HSV-1,³³ we do not measure inflammatory infiltrates until corneal disease is most severe.²⁵ Consequently, when we examined the effects of neutralization of CXCL1 in a recurrent model of HSK we reported that these mice experienced lower clinical scores in opacity and neovascular-

ization following reactivation of HSV-1, as well as a significant reduction in the presence of inflammatory Gr-1⁺ neutrophils in treated animals.²⁵ Similar results also were reported during bacterial^{33,34} and some viral infections.³⁵ Clearly, the importance of controlling neutrophil invasion in the cornea is tightly linked to better disease prognosis. There is precedent for FasL control of neutrophils. In a model of cardiac surgery it was observed that failure to control inflammation via Fas-FasL mediated death led to worse outcomes for cardiac transplants.³⁶ Likewise, it was reported that bacterial endophthalmitis leads to a neutrophilic infiltrate in the vitreous of mice that is more pronounced in mice expressing the *gld* mutation, which compromises their ability to perform Fas-FasL mediated apoptosis.³⁷ Most recently, it has been suggested that FasL controls the survival of neutrophils during lymphocytic choriomeningitis virus infection.³⁸

Furthermore, studies show that T cells, NK cells, and possibly monocytes are the dominant lineages responsible for viral clearance from the cornea, not neutrophils.^{39,40} It is interesting to note that while there are significantly fewer total CD45⁺ cells and Gr-1⁺ neutrophils in sFasL-treated mice, the same mix of T cells, monocytes, and neutrophils are seen in these mice compared to controls, though the percentage of these cells showed some differences. Thus, treatment with sFasL does not result in the recruitment of an unusual or atypical type of inflammatory cell. Seeing as our results with sFasL closely mirror the results of CXCL-1 in clinical disease scores and in minimizing neutrophilic infiltrate, therapeutic use of sFasL could have a role in disease management.

In this study, we used treatment of sTRAIL as a control for sFasL treatment. This was chosen for two reasons, one is that FasL and TRAIL are from the same TNF family, which contains "death domains" that target lymphocytes for destruction.⁴¹ However, when TRAIL was evaluated for its role in T-cell fratricide following HSV-1 infection, it was not involved while FasL was.⁴² Thus, we believed that this would be a good control for treatment with sFasL. Interestingly, it also has been reported that TRAIL is involved in systemic immune tolerance following antigen injection into the anterior chamber,⁴³ thus

illustrating that immunologic responses are dependent on the site of antigen exposure within the eye.

As mentioned above, one may hypothesize that the reason BALB-*lpr* mice, in acute infection and after reactivation of HSV-1, experience no difference in corneal opacity following treatment with sFasL is either due to the impaired ability of T cells to kill virally-infected cells via the FasL-mediated pathway or due to a lack of control of neovascularization. However, evidence refutes this theory. First, HSV-1 viral titers did not differ significantly between BALB/c mice and BALB-*lpr* mice during acute infection.¹⁷ Secondly, expression of Fas antigen by corneal cells is below the level of detection by Western blot analysis,²⁰ which means these infected corneal cells would not be targets of FasL-mediated killing by CTL's. Third, *lpr* mice express normal Fas on their vascular endothelium and, thus, control of noninflammatory corneal neovascularization by FasL is not significantly impaired.¹⁵ Consequently, we concluded that the lack of apoptosis of Fas⁺ lymphoid cells is the primary culprit for the lack of effect of sFasL in these mice.

Taken together, these results and conclusions indicated that treatment with sFasL significantly reduces the severity of corneal disease in acute and recurrent mouse models of HSK. Furthermore, these data suggest that sFasL treatment has the potential to reduce the disease gravity of HSK. Currently, the treatment of choice for HSK is a mixture of antiherpetic drugs aimed to limit viral replication and corticosteroids to reduce inflammation. However, this treatment regime has drawbacks, including unwanted side effects from steroids,⁴⁴⁻⁴⁶ retention of corneal clouding and inflammation,^{47,48} and even tolerance to antiviral treatment.⁴⁹ Since the majority of humans have intact Fas-FasL systems, one must ask whether potentiating this interaction would improve these current therapies. In other studies it has been reported that potentiating FasL-mediated control of Fas⁺ cells by inhibiting matrix metalloproteases (MMP) that cleave FasL leads to increased tumor growth by a FasL-mediated control of Fas⁺ antitumor cells.⁵⁰ It also has been reported that mice treated with MMP inhibitors demonstrate increased FasL stability, which results in increased success of corneal allografts.⁵¹ Furthermore, another study indicated that mice suffering from choroidal neovascularization experienced significantly reduced invasion of new blood vessels when treated with apoptotic-inducing soluble FasL or MMP inhibitors.²⁷ As mentioned, studies also show that neutrophil-neutralizing antibodies, such as anti-CXCL-1, have the potential to reduce corneal disease scores.²⁵ Consequently, it is possible that treatment with a combination of MMP inhibitors, sFasL and neutralizing antibodies to CXCL-1 will provide better control of the infiltration of cells and vessels into the cornea which would result in reduced incidence of corneal disease in HSV-1-infected individuals.

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References

- Pepose JS, Leib DA, Stuart PM, Easty EL. Herpes simplex virus diseases: anterior segment of the eye. In: Pepose JS, Holland GAN, Wilhelmus KR, eds. *Ocular Infection and Immunity*. St. Louis, MO: Mosby; 1996:905-932.
- Farooq AV, Shukla D. Herpes simplex epithelial and stromal keratitis: an epidemiologic Update *Surv Ophthalmol*. 2012; 57:488-462.
- Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol*. 1997;158:1383-1391.
- Maertzdorf J, Verjans GM, Remeijer L, van der Kooi A, Osterhaus AD. Restricted T cell receptor beta-chain variable region protein use by cornea-derived CD4⁺ and CD8⁺ herpes simplex virus-specific T cells in patients with herpetic stromal keratitis. *J Infect Dis*. 2003;187:550-558.
- Divito SJ, Hendricks RL. Activated inflammatory infiltrate in HSV-1-infected corneas without herpes stromal keratitis. *Invest Ophthalmol Vis Sci*. 2008;49:1488-1495.
- Denniston AK, Kottoor SH, Khan I, et al. Endogenous cortisol and TGF-beta in human aqueous humor contribute to ocular immune privilege by regulating dendritic cell function. *J Immunol*. 2011;186:305-311.
- Cursiefen C. Immune privilege and angiogenic privilege of the cornea. *Chem Immunol Allergy*. 2007;92:50-57.
- Koevary SB. Ocular immune privilege: a review. *Clin Eye Vis Care*. 2000;12:97-106.
- Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science*. 1995;270:1189-1192.
- Griffith TS, Yu X, Herndon JM, Green DR, Ferguson TA. CD-95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. *Immunity*. 1996;5:7-16.
- Stuart PM, Griffith TS, Usui N, Pepose JS, Yu X, Ferguson TA. CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival. *J Clin Invest*. 1997;99:396-402.
- Osawa H, Maruyama K, Streilein JW. CD95 ligand expression on corneal epithelium and endothelium influences the fates of orthotopic and heterotopic corneal allografts in mice. *Invest Ophthalmol Vis Sci*. 2004;45:1908-1915.
- Kaplan HJ, Leibole MA, Tezel T, Ferguson TA. Fas ligand (CD95 ligand) controls angiogenesis beneath the retina. *Nature Med*. 1999;5:292-297.
- Volpert OV, Zaichuk T, Zhou W, et al. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic TSP-1 and PEDF. *Nature Med*. 2002;8:349-357.
- Stuart PM, Pan F, Plambeck S, Ferguson TA. Fas/Fas ligand interactions regulate neovascularization in the cornea. *Invest Ophthalmol Vis Sci*. 2003;44:93-98.
- Laurence J, Mitra D, Steiner M, Staiano-Coico L, Jaffe E. Plasma from patients with idiopathic and human immunodeficiency virus-associated thrombotic thrombocytopenic purpura induces apoptosis in microvascular endothelial cells. *Blood*. 1996; 87:3245-3254.
- Morris JE, Zobel S, Yin XT, et al. Mice with mutations in Fas and Fas ligand demonstrate increased herpetic stromal keratitis following corneal infection with HSV-1. *J Immunol*. 2012;188:793-799.
- Huang D, Hahne M, Schroeter M, et al. Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x_L. *Proc Natl Acad Sci U S A*. 1999; 96:14871-14876.
- Stuart PM, Yin XT, Plambeck S, Pan F, Ferguson TA. The role of Fas ligand as an effector molecule in corneal graft rejection. *Eur J Immunol*. 2005;35:2591-2597.
- Keadle TL, Morrison LA, Morris JE, Pepose JS, Stuart PM. Therapeutic immunization with a virion host shutoff-defective, replication-incompetent herpes simplex virus type 1 strain limits recurrent herpetic ocular infection. *J Virol*. 2002;76: 3615-3625.

21. Herndon JM, Stuart PM, Ferguson TA. Peripheral deletion of antigen-specific T cells leads to long-term tolerance mediated by CD8⁺ cytotoxic cells. *J Immunol.* 2005;174:4098-4104.
22. Stuart PM, Summers B, Morris JE, Morrison LA, Leib DA. CD8⁺ T cells control corneal disease following ocular infection with HSV-1. *J Gen Virol.* 2004;85:2055-2063.
23. Keadle TL, Laycock KA, Miller JK, et al. Efficacy of a recombinant glycoprotein D subunit vaccine on the development of primary and recurrent ocular infection with herpes simplex virus type 1 in mice. *J Infect Dis.* 1997;176:331-338.
24. Shimeld C, Hill TJ, Blyth B, Easty D. An improved model of recurrent herpetic eye disease in mice. *Curr Eye Res.* 1989;8:1193-1205.
25. West DM, Del Rosso CR, Yin XT, Stuart PM. CXCL1 but not IL-6 is required for recurrent herpetic stromal keratitis. *J Immunol.* 2014;192:1762-1767.
26. Stuart PM, Keadle TL. Recurrent herpetic stromal keratitis in mice, a model for studying human HSK. *Clin Dev Immunol.* 2012;2012:728480.
27. Smith TJ, Ackland-Berglund CE, Leib DA. Herpes simplex virus virion host shutoff (*vhs*) activity alters periocular disease in mice. *J Virol.* 2000;74:3598-3604.
28. Roychowdhury J, Herndon JM, Yin J, Apte RS, Ferguson TA. Targeting immune privilege to prevent pathogenic neovascularization. *Invest Ophthalmol Vis Sci.* 2010;51:3560-3566.
29. Yamagami S, Kawashima H, Tsuru T, et al. Role of Fas-Fas ligand interactions in the immunorejection of allogeneic mouse corneal transplants. *Transplant.* 1997;64:1107-1111.
30. Dobbs ME, Strasser JE, Chu CF, Chalk C, Milligan GN. Clearance of herpes simplex virus type 2 by CD8⁺ T cells requires gamma interferon and either perforin- or Fas-mediated cytolytic mechanisms. *J Virol.* 2005;79:14546-14554.
31. Rouvier E, Luciani M-F, Golstein P. Fas involvement in Ca²⁺-independent T cell-mediated cytotoxicity. *J Exp Med.* 1993;177:195-200.
32. Liu ZX, Govindarajan S, Okamoto S, Dennert G. Fas-mediated apoptosis causes elimination of virus-specific cytotoxic T cells in the virus-infected liver. *J Immunol.* 2001;166:3035-3041.
33. Bryant-Hudson KM, Carr DJ. CXCL1-deficient mice are highly sensitive to *Pseudomonas aeruginosa* but not herpes simplex virus type 1 corneal infection. *Invest Ophthalmol Vis Sci.* 2012;53:6785-6792.
34. Carlson EC, Lin M, Liu CY, Kao WW, Perez VL, Pearlman E. CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J Leukoc Biol.* 2007;81:786-792.
35. Chintakuntlawar AV, Chodosh J. Chemokine CXCL1/KC and its receptor CXCR2 are responsible for neutrophil chemotaxis in adenoviral keratitis. *J Interferon Cytokine Res.* 2009;29:657-666.
36. Kunes P, Krejsek J, Brtko M, Mandak J, Kolackova M, Trojackova Kudlova M, Andrys C. Neutrophil apoptosis by Fas/FasL: harmful or advantageous in cardiac surgery? *Thorac Cardiovasc Surg.* 2009;57:1-6.
37. Sugi N, Whiston EA, Ksander BR, Gregory MS. Increased resistance to *Staphylococcus aureus* endophthalmitis in BALB/c mice: Fas ligand is required for resolution of inflammation but not for bacterial clearance. *Infect Immun.* 2013;81:2217-2225.
38. O'Donnell JA, Kennedy CL, Pellegrini M, et al. Fas regulates neutrophil lifespan during viral and bacterial infection. *J Leukoc Biol.* 2015;97:321-326.
39. Conrady CD, Zheng M, Stone DU, Carr DJ. CD8⁺ T cells suppress viral replication in the cornea but contribute to VEGF-C-induced lymphatic vessel genesis. *J Immunol.* 2012;189:425-432.
40. Frank GM, Buella KA, Maker DM, Harvey SA, Hendricks RL. Early responding dendritic cells direct the local NK response to control herpes simplex virus 1 infection within the cornea. *J Immunol.* 2012;188:1350-1359.
41. Michowitz Y, Goldstein E, Roth A, et al. The involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in atherosclerosis. *J Am Coll Cardiol.* 2005;45:1018-1024.
42. Raftery MJ, Behrens CK, Müller A, Krammer PH, Walczak H, Schönrich G. Herpes simplex virus type 1 infection of activated cytotoxic T cells: induction of fratricide as a mechanism of viral immune evasion. *J Exp Med.* 1999;190:1103-1114.
43. Griffith TS, Brincks EL, Gurung P, Kucaba TA, Ferguson TA. Systemic immunological tolerance to ocular antigens is mediated by TRAIL-expressing CD8⁺ T cells. *J Immunol.* 2011;186:791-798.
44. Munjal VP, Dhir SP, Jain IS, Gangwar DN, D'souza M. Topical corticosteroids and cataract. *Indian J Ophthalmol.* 1984;32:478-480.
45. Dada T, Nair S, Dhawan M. Steroid-induced glaucoma. *J Curr Glaucoma Prac.* 2009;3:33-38.
46. Renfro L, Snow JS. Ocular effects of topical and systemic steroids. *Dermatol Clin.* 1992;10:505-512.
47. Croxtall JD. Ganciclovir ophthalmic gel 0.15%: in acute herpetic keratitis (dendritic ulcers). *Drugs.* 2011;71:603-610.
48. Wilhelmus KR, Mitchell BM, Jones DB. Photographic monitoring of herpes simplex virus keratitis during anti-inflammatory treatment. *Arch Ophthalmol.* 2011;129:252-253.
49. Duan R, de Vries RD, van Dun JM, et al. Acyclovir susceptibility and genetic characteristics of sequential herpes simplex virus type 1 corneal isolates from patients with recurrent herpetic keratitis. *J Infect Dis.* 2009;200:1402-1414.
50. Mitsiades N, Yu WH, Poulaki V, Tsokos M, Stamenkovic I. Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. *Cancer Res.* 2001;61:577-581.
51. Stuart PM, Pan F, Yin XT, Haskova Z, Plambeck S, Ferguson TA. Effect of metalloprotease inhibitors on corneal allograft survival. *Invest Ophthalmol Vis Sci.* 2004;45:1169-1173.