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WASHINGTON UNIVERSITY

University College Department of Biology

THE ROLE OF FAS WITH FAS LIGAND INTERACTION IN HERPETIC STROMAL KERATITIS (HSK)

by

Hamideh Zakeri

A thesis presented to the
Graduate School of University College
of Washington University in
partial fulfillment of the
requirements for the
degree of Master of Arts in Biology

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2011

THE ROLE OF FAS WITH FAS LIGAND INTERACTION IN HERPETIC STROMAL KERATITIS (HSK)

Abstract:

Herpes Simplex Virus (HSV)-induced herpetic stromal keratitis (HSK) is a leading cause of infectious blindness world wide. The pathogenesis of this disease involves an inflammatory attack in the cornea following infection or reactivation of HSV-1. Previous studies have shown that the apoptotic molecule Fas ligand (FasL) is very important in controlling inflammatory responses that occur in the cornea. Consequently we decided to investigate the role that Fas, the receptor for FasL, and FasL play during HSK. To that end, previous studies done by others in the lab have shown that mice which do not express the Fas antigen (*lpr*) have particularly enhanced HSK. The focus of this project was to determine whether the lack of functional Fas expression by lymphoid cells was responsible for the disease phenotype seen in *lpr* mice. In order to investigate this we created bone marrow chimeras between BALB/c and BALB-*lpr* mice which were subsequently infected with HSV-1. Our results indicate that 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 as evident by increased opacity, neovascularization and blepharitis scores in these mice.

Thus our data confirm the hypothesis that FasL controls the entry of Fas-expressing inflammatory cells during acute HSK.

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Introduction:

Herpes Simplex Virus is a double stranded DNA virus. It belongs to the Simplexvirus genus with two species, HSV-1 and HSV-2. The prevalence of HSV infection varies among different regions and populations in the world. HSV-1 prevalence is high in United States while more cases of HSV-2 are reported for sub-Saharan African women and to a lesser degree in men (34). HSV-1 infection mostly occurs during childhood and adolescence and is more common than HSV-2 infection. Herpes Simplex Virus (HSV)-induced herpetic stromal keratitis (HSK) is usually caused by HSV1 and is a leading cause of infectious blindness in humans worldwide (1, 2), and probably the leading cause in the United States (3). It is estimated that there are 500,000 cases of ocular HSV infections annually in the United States alone (4). Of these cases, approximately 20% will develop stromal disease, which can lead to permanent scarring of the cornea and loss of vision (1, 5). The disease is believed to be immune-mediated in which several different subsets of lymphoid cells have been implicated, including T cells, neutrophils, and macrophages (6-10). The roles of each of these cell types have not been fully determined, nor has the mechanism of tissue damage been clearly delineated. Although primary HSV-1 infection in mice included multiple symptoms and phenotypes, it is the recurrent infection that presents clinical features such as microdendrites, focal stromal opacities, disciform endothelitis and cornea neovascularization which are similar to those observed in humans (11). Loss of vision typically occurs following several episodes of re-activation of latent HSV from the trigeminal ganglia (11). Thus an understanding of the cellular interactions between viral specific immune cells and the cornea and nervous system are crucial in determining the underlying mechanisms of this disease. Such an

understanding is critical in designing effective vaccines and therapies that will prove useful in treating HSK.

In the face of this potentially blinding inflammatory attack, the cornea has the ability to reduce inflammation. These include the presence of immunosuppressive factors such as TGF-β, reduced MHC expression on dendritic cells (33) and the presence of Fas ligand (FasL) (12-17). It is the presence of FasL that is the focus of this project. FasL is a type-II transmembrane protein and Fas is a transmembrane receptor in the tumor necrosis (TNF) receptor gene family. FasL binding with the Fas receptor results in apoptosis or cell death of inflammatory cells upon infection. When these two genes are defective, the presence of inflammatory cells could lead to disease progression and manifestation. The interaction of FasL with Fas receptor plays an important role in the regulation of the immune system and cancer progression.

Various studies have clearly demonstrated that the presence of FasL in the eye is an important barrier to both inflammatory cells (12-14) and new blood vessels (15-17), both of which are intimately involved in the pathology of HSK. Control of inflammation is also known to be a significant component of the immune privilege of the eye (12, 13). FasL expressed on ocular tissues induces apoptosis in Fas^+ lymphoid cells that invade the eye in response to viral infection (12) or corneal grafting (14). 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 (18-20). These studies clearly indicate that the presence of FasL in ocular tissues restricts inflammatory responses. In fact preliminary data from this laboratory indicates that mice expressing

mutations in either Fas (*lpr*) or FasL (*gld*) display significantly greater ocular disease than do wild-type mice during both primary and recurrent HSK.

In this study, we intended to more fully investigate the role that Fas-FasL interaction plays during HSK in *lpr* (having mutation in Fas gene*)* mice. Since the mutation is expressed by both lymphoid and non-lymphoid cells, the relevant cell expressing the *lpr* mutation that gives rise to the increased phenotype was not identified. We predicted that mice with a lymphoid system expressing the *lpr* mutation will show greater HSK than mice with wild type lymphoid cells regardless of the Fas phenotype of all other cells within the mouse. We tested this hypothesis by constructing bone marrow chimeras between BALB/c (control) and BALB-*lpr* (with mutation in Fas gene) mice and infecting them with the KOS strain of HSV-1. Clinical disease (stromal opacification, corneal neovascularization and blepharitis (disease of the eye lid)) was scored every week for four weeks post infection. Results confirmed our hypothesis that increased HSK observed in *lpr* mice was due to lack of functional Fas on lymphoid cells.

Methods and materials:

Mice:

We purchased 6 week old BALB/c (control) mice from Jackson Laboratory. The BALB-lpr (BALB/c mice with mutation in Fas gene) mice were produced in our laboratory by backcrossing C57BL/6-lpr (C57BL/6 mice with mutation in Fas gene) mice to BALB/c mice for 12 generations and maintained by brother sister mating. These mice are maintained in an ALARA accredited facility at Saint Louis University. All investigations

with these mice conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Bone Marrow Chimeras:

Radiation bone marrow chimeras between BALB/c and BALB-lpr mice were prepared as described (11). Briefly, bone marrow and spleen cells were extracted from a pool of wild type (BALB/c) mice. Bone marrow and spleen cells were extracted from a pool of lpr (Fas negative or having mutation in Fas gene) mice. A group of wild type and a group of lpr mice were irradiated with 700 Rads from an XRAD 320 irradiator (Precision X-Ray, North Branford, CT) to kill all their bone marrow cells. Subsequently, the wild type mice were given an equal mixture of $2x10^7$ bone marrow and spleen cells from lpr mice and the lpr mice were given an equal mixture of $2x10^7$ bone marrow and spleen cells from BALB/c mice. The level of chimerism was determined by PCR genotype analysis of peripheral blood cells 30 days after bone marrow transplantation. These mice were then infected 50 days following bone marrow reconstitution and phenotypic differences were observed over time.

Infection with HSV-1:

The viruses used in this study were of the KOS strain of HSV-1. 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 penicillin ml^{-1} and 100ug Streptomycin ml^{-1} (21). Chimera mice were infected as described previously (22). Briefly, following corneal scarification, 2×10^7 pfu of HSV-1 KOS strain in 5ul MEM-EBS was placed onto the surface of both corneas of each mouse.

Quantitation of virus in tear film swabs:

Vero cells were seeded in 48 well plates containing growth media. Cell growth media was prepared by using 1000ml DMEM media (Sigma D6546) supplemented with 5% FBS (Atlanta biological S11150), 10ml 100x pen/strep, 1ml 1000x fungizon, and 10ml L-Glutamine (Sigma G7513). Briefly 100ul of eye swab material was used to infect 90% confluent Vero cells in 48 well plates. Eye swabs were assayed for the presence of virus by standard plaque assay (22) 48 hours following addition of eye swab material.

Clinical evaluation

On the designated days after viral infection, a masked observer examined mouse eyes through a binocular-dissecting microscope in order 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 (21) using a scale of 0-8, 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 previously described (23).

Statistical analysis:

The descriptive statistics were used to draw mean and standard error of mean values. The unpaired t test was used for comparing means between groups of mice. The t test for two sample assuming unequal variances were used to compare data for different time points within the same group.

Results:

Previous work from this laboratory, as well as others, has indicated that the expression of FasL in the eye is a critical factor in limiting inflammatory responses there (24, 12, 25, and 26). To that end recent work has demonstrated that mice carrying mutations in either Fas (*lpr*) or FasL (*gld*) exhibited increased HSK, as determined by opacity and neovascularization than seen in wild-type BALB/c mice following infection with HSV-1, KOS strain (Fig. 1). Interestingly, only BALB-*lpr* mice demonstrated significant blepharitis scores following infection with HSV-1, KOS strain (Fig. 2).

Figure 1A

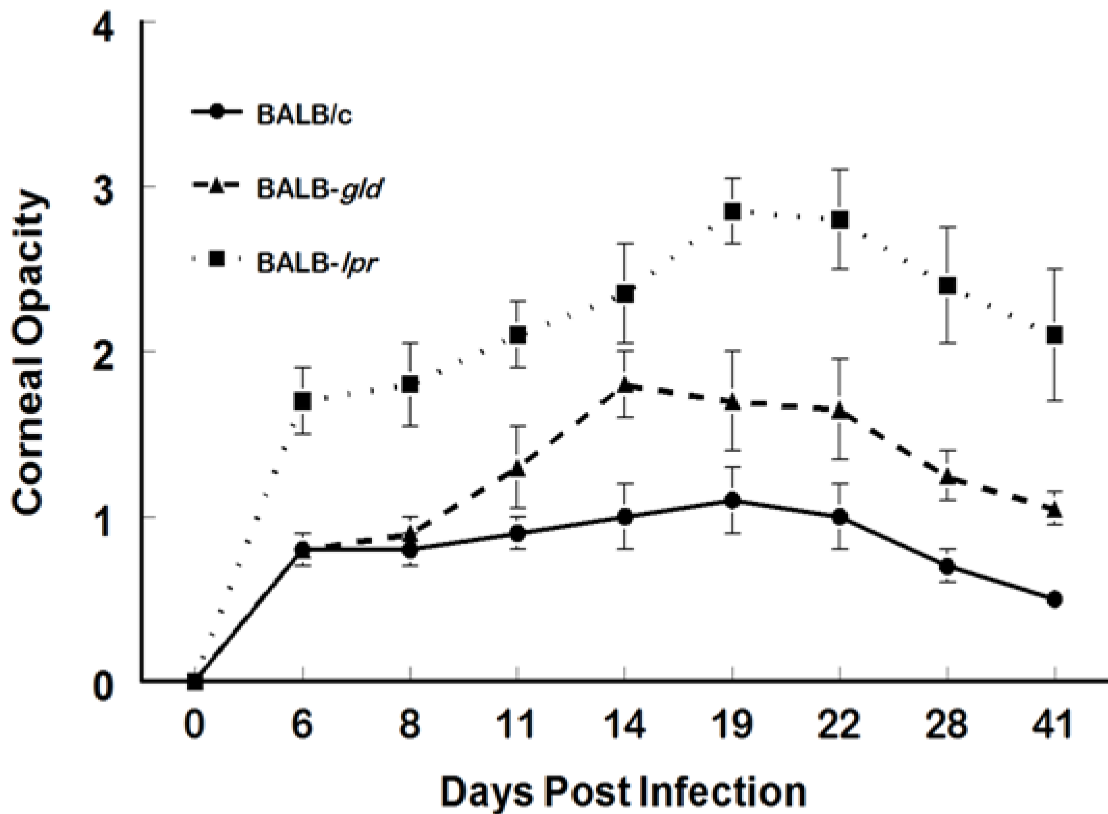

Figure 1B

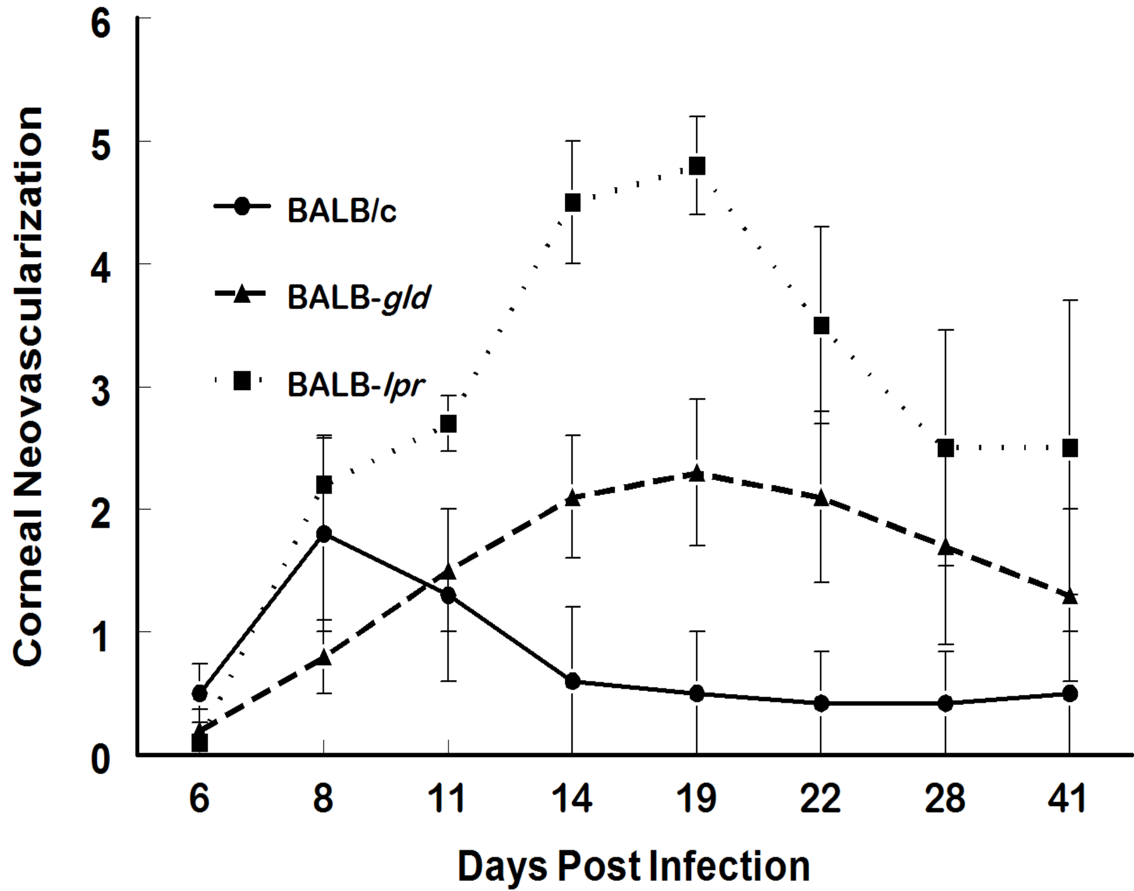

Figure 1: Defective expression of both Fas and to a lesser extent FasL, results in increased HSK following infection with HSV-1, KOS strain. Eyes of BALB/c wild-type (n=30), BALB-*lpr* (n=25) and BALB-*gld* (n=25) mice were infected with 10^7 pfu of HSV-1, KOS strain. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these strains of mice. Significant virus-induced corneal opacity was observed for BALB-*lpr* ($P<0.01$) at all time points when compared to BALB/c controls. BALB-*gld* mice displayed significantly more opacity than did BALB/c controls at days 14-35 (P<0.05-0.01). BALB-*lpr* displayed significantly greater neovascularization at days 11-41 (P<0.05-0.01) and BALB-*gld* mice had greater neovascularization at days 14-22 (P<0.05-0.01) than did BALB/c controls. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated. Student t test was used for statistical analysis. This is an unpublished observation by Jessica E. Morris etal.

Figure 2

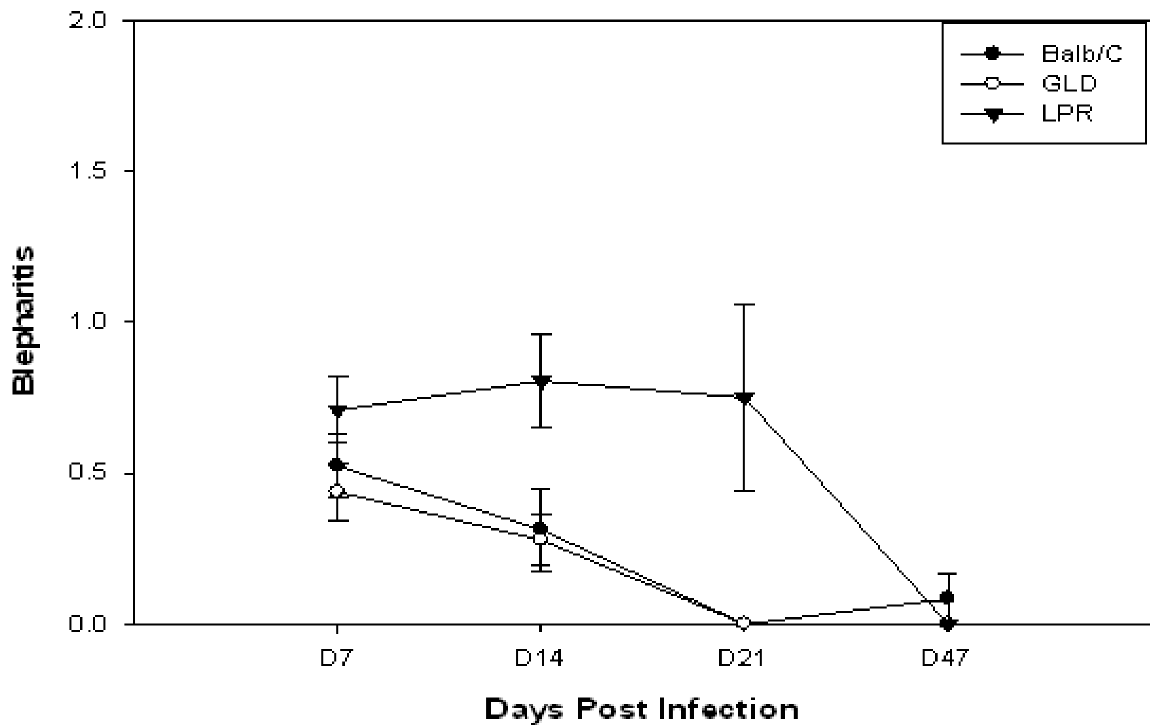

Figure 2: Defective expression of Fas but not FasL, results in increased blepharitis following infection with HSV-1, KOS strain. Eyes of BALB/c wild-type (n=20), BALB-*lpr* (n=20) and BALB-*gld* (n=20) mice were infected with 10^7 pfu of HSV-1, KOS strain and blepharitis measured. Significant virus-induced blepharitis was observed for BALB-*lpr* ($P<0.01$) at days 14 and 21 when compared to both BALB/c controls and BALB-*gld* mice. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated. Student t test was used for statistical analysis. This is an unpublished observation by Jessica E. Morris etal.

Since both lymphoid (inflammatory cells (12-14)), and non-lymphoid cells (vascular endothelium (16,17)) can express Fas, it is possible that the disease seen in *lpr* mice following corneal infection with HSV-1 is due to the lack of Fas expression on either one or both of these general types of cells. We have shown that resident cells of the cornea do not express Fas (27) and thus should not play a role in corneal disease. Consequently, we hypothesized that the cells responsible for the severity of HSK phenotype in *lpr* mice are bone marrow derived. We believed that increased disease is due to uncontrolled

infiltration of the corneas by lymphoid cells which are not susceptible to FasL-mediated lysis. To test this hypothesis, we decided to produce bone marrow chimeric mice between BALB/c and BALB-*lpr* mice to identify the relevant cell population for the *lpr* phenotype. Two months following bone marrow reconstitution of these mice, we infected their corneas with HSV-1, KOS stain. Our data indicates that BALB/c mice that are reconstituted with BM cells from *lpr* mice display significantly greater corneal disease than do BALB-*lpr* mice that are reconstituted with BALB/c BM cells at 2 and 3 weeks following infection (Fig. 3-5). It should also be pointed out that mice possessing BALB-*lpr* BM also presented other indications of increased disease as evidenced by hunched posture and weight loss, which were not seen in BALB-*lpr* mice receiving BALB/c BM cells.

Figure 3

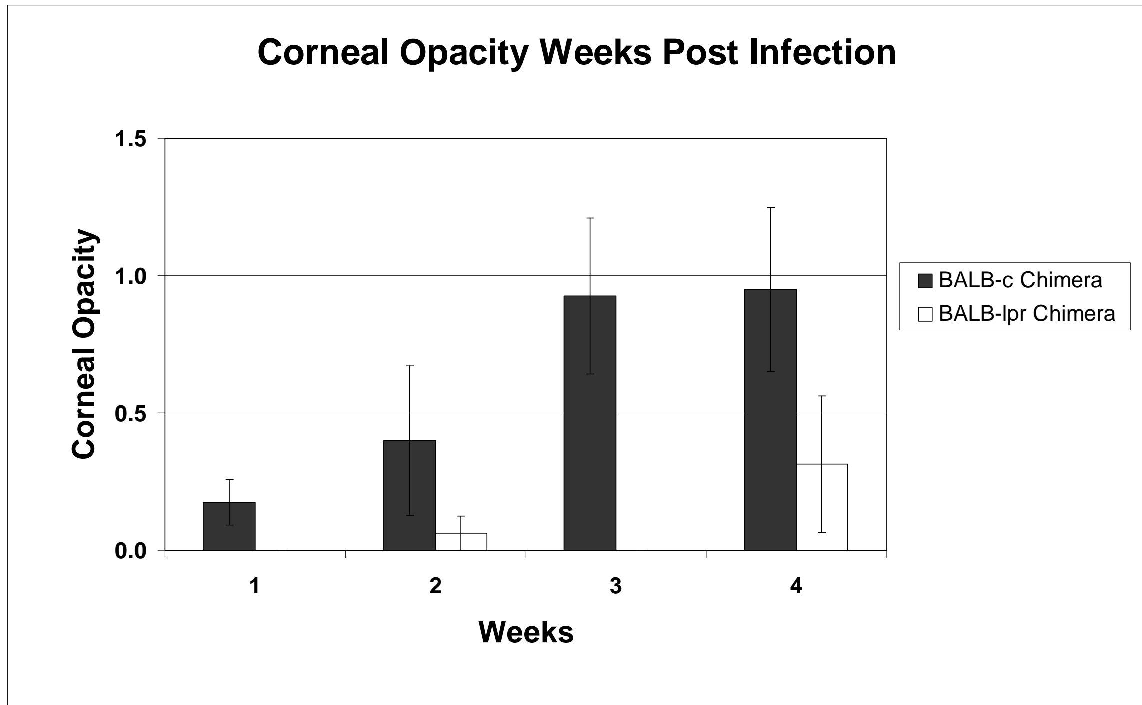

Figure 3: Defective expression of Fas, results in increased HSK following infection with HSV-1, KOS strain. Eyes of BALB/c chimera (n=20) and BALB-*lpr* chimera (n=8) mice were infected with 10^7 pfu of HSV-1, KOS strain. Corneal opacity was measured and compared between these two groups of mice. Significant virus-induced corneal opacity was observed for BALB/c chimera mice at week 1 ($P<0.05$) and week 3 ($P<0.01$) post infection when compared to BALB-*lpr* chimera mice. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated. The mean corneal opacity for each of the four weeks observed for Balb/c chimera mice differ significantly ($p<0.05$) from that of BALB-*lpr* chimera mice. Student t test was used for statistical analysis.

Figure 4

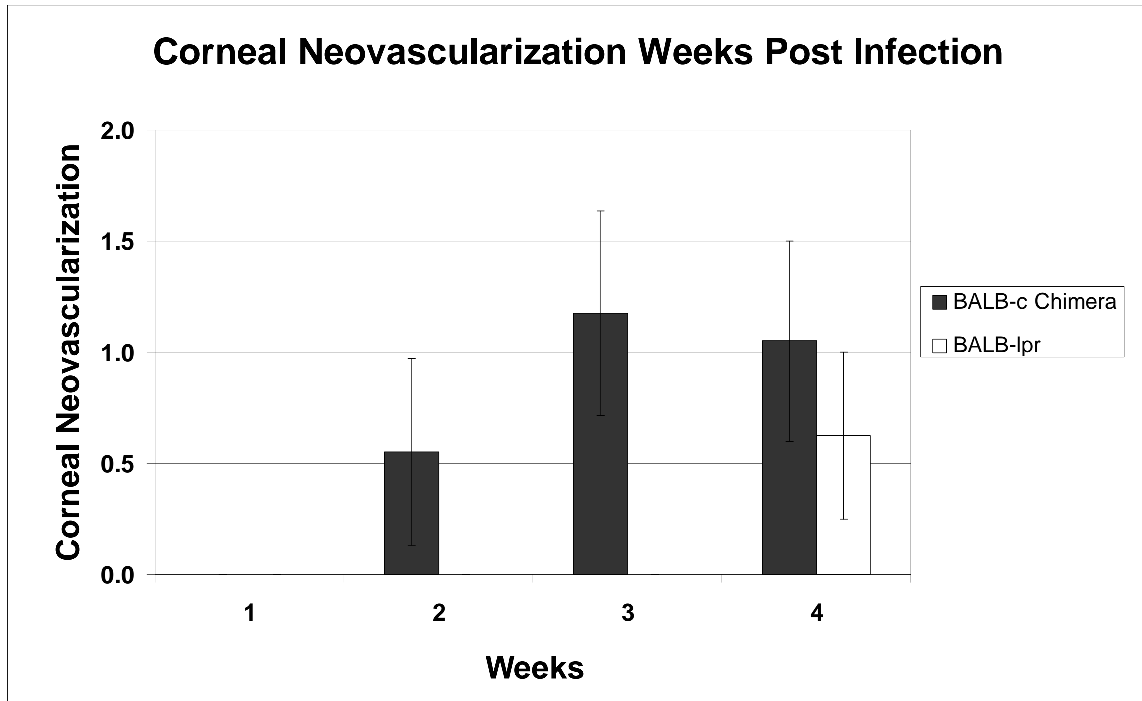

Figure 4: Defective expression of Fas, results in increased HSK following infection with HSV-1, KOS strain. Eyes of BALB/c chimera (n=20) and BALB-*lpr* chimera (n=8) mice were infected with 10^7 pfu of HSV-1, KOS strain. Corneal neovascularization was measured and compared between these two groups of mice. Significant ($p<0.05$) virus-induced corneal neovascularization was observed for Balb/c chimera mice at three weeks post infection when compared to BALB-*lpr* chimera mice. Student t test was used for statistical analysis. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated.

Figure 5

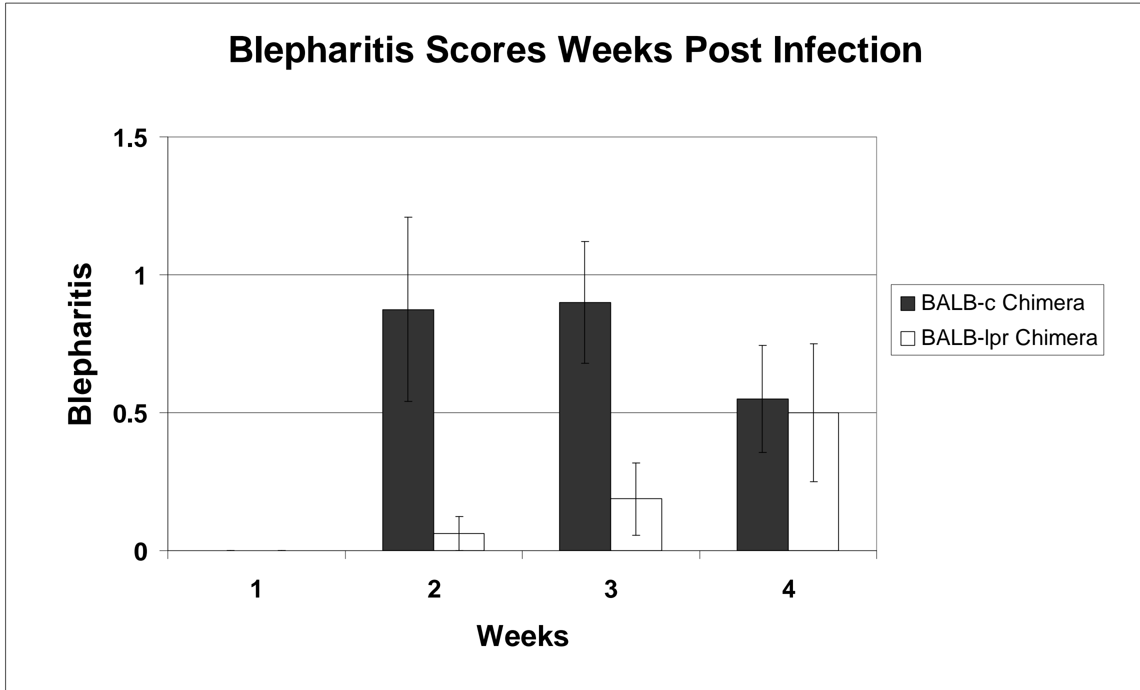

Figure 5: Defective expression of Fas, results in increased HSK following infection with HSV-1, KOS strain. Eyes of BALB/c chimera (n=20) and BALB-*lpr* chimera (n=8) mice were infected with 10^7 pfu of HSV-1, KOS strain. Significant virus-induced blepharitis was observed for BALB/c chimera mice at week 2 ($P<0.05$) and week 3 ($P<0.01$) post infection when compared to BALB-*lpr* chimera mice. Student t test was used for statistical analysis. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated.

Another possible reason for increased disease could be that following infection with HSV-1 the inflammatory cells entering the corneas of BALB-*lpr* do not clear virus as quickly as do BALB/c mice. Thus, when HSV-1 virus persists in the cornea this could lead to potentially stronger inflammatory responses due to impaired clearance of virus. Previous studies have indicated that virus does persist in the corneas of parental BALB-*lpr* mice for 2-4 days longer than in BALB/c mice. To assess the viral persistence in chimeric mice we took eye swabs from mice for the first 9 days following infection. The

presence of virus was then assayed by plating on the HSV-1 susceptible VERO cells. Results indicated that both types of chimeric mice cleared virus by day 9 following infection and that the number of mice displaying positive eye swabs at both day 6 and day 7 were not significantly different (31% for BALB/c mice with BALB-*lpr* BM and 28% for BALB-*lpr* mice with BALB/c BM. Consequently, our results do not support the thesis that persistence of virus in chimeric mice is responsible for increased disease.

Figure 6

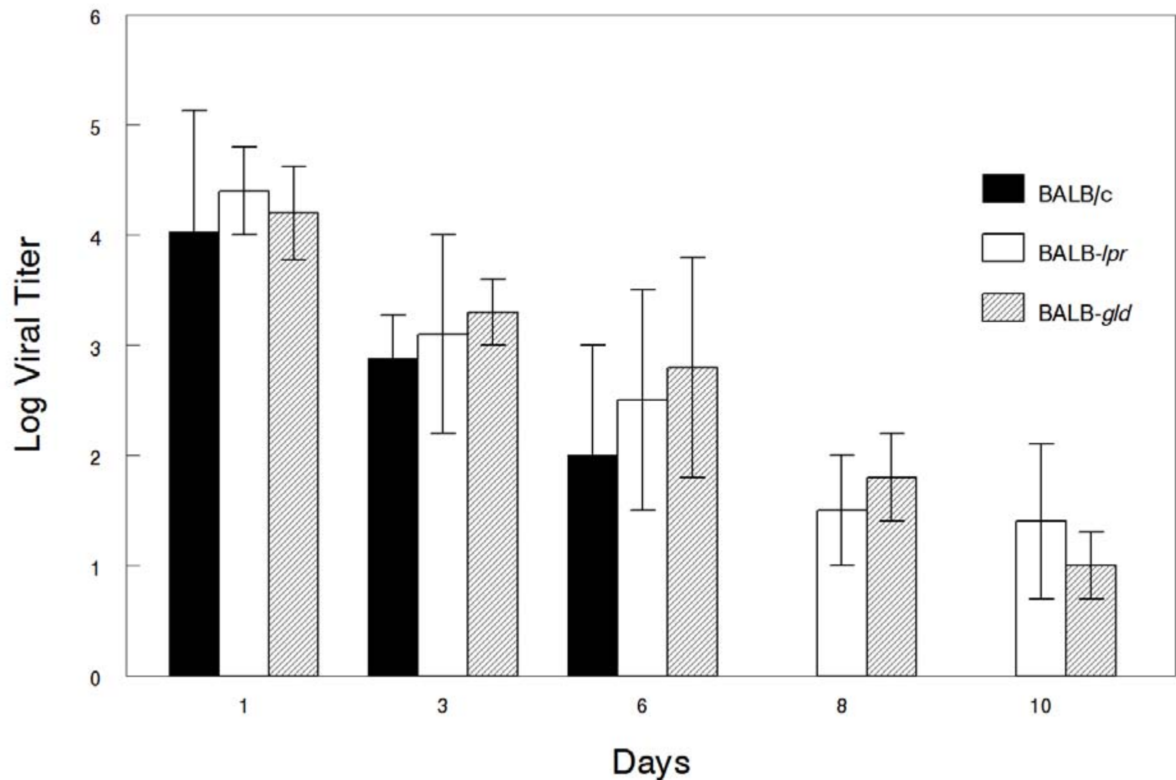

Figure 6: Defective expression of Fas and FasL, does not increase the magnitude of corneal viral shedding but does prolong shedding. Eyes of BALB/c wild-type (n=30), BALB-*lpr* (n=25) and BALB-*gld* (n=25) mice were infected with 10^7 pfu of HSV-1, KOS strain and corneas were swabbed on the indicated days and then tittered. No significant differences were seen in the magnitude of viral shedding. Student t test was used for statistical analysis. Results displayed are means $\pm$ S.E.M. for each of the groups of mice indicated. This is an unpublished observation by Jessica E. Morris etal.

Discussion:

One of the prime mechanisms the eye uses to protect itself from T cell–mediated immunopathologic response is the presence of FasL which induces apoptosis in Fas^+ lymphoid cells (28). Our laboratory, as well as several others have shown that lack of functional Fas-FasL-mediated apoptotic ability in the eye most often leads to increased inflammatory responses (12), increased corneal allograft rejection (13,29), increased neovascularization (17) and the inability to develop systemic tolerance following injection of antigen into the anterior chamber (13). In addition to these responses that are specific to the eye, it is also well established that host T cells eliminate viral infected cells by either the perforin-granzyme pathway (30) or via apoptosis mediated by the interaction of FasL on effector cells with Fas expressed by virally infected cells (31,32). Thus it would seem that mice that are not able to express either functional FasL or their receptor Fas have the potential of expressing a wide variety of abnormalities. These might include being more prone to greater inflammatory responses in tissues such as the eye where FasL plays such a large role in controlling inflammation. One might also hypothesize that they would have difficulty clearing virally infected cells because that pathway of killing is not available to them.

We decided to investigate the role that Fas, the receptor for FasL, and FasL play during HSK. Previous studies done by others in the lab have shown that mice which do not express the Fas antigen (*lpr*) have particularly enhanced HSK (see Figures 1 and 2). The focus of this project was to determine whether the lack of functional Fas expression by lymphoid cells was responsible for the disease phenotype seen in *lpr* mice. In order to investigate this we created bone marrow chimeras between BALB/c and BALB-*lpr* mice

which were subsequently infected with HSV-1. Our results indicate that 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 as evident by increased opacity, neovascularization and blepharitis scores in these mice (see Figures 3-5).

We also examined these chimeric mice to determine if viral persistence was a factor in increased disease phenotype. However, there were no differences in the persistence of HSV-1 in the corneas of the chimeric mice, as both cleared virus with the same kinetics. Thus viral persistence cannot be a mechanism responsible for increased HSK seen in chimeric BALB/c mice with BALB-*lpr* lymphoid cells.

Prior to these studies, it had been possible to agree that the increased disease seen in *lpr* mice could have been due to lack of control of neovascularization as vascular endothelium expresses Fas and neovascularization of the cornea has been shown to be controlled by corneal expression of FasL (17). However, we thought that was unlikely as *lpr* mice express normal Fas on their vascular endothelium (17). By demonstrating that the disease phenotype is associated with lymphoid cells, this further supports the notion that vascular endothelial expression of Fas alone is not responsible for increased HSK in BALB-*lpr* mice. This however does not rule out the possibility that lack of Fas expression by vascular endothelium plays some role in ocular disease, but it is not the sole or even a major contributor to the disease phenotype seen in BALB-*lpr* mice.

Taken together, our data confirm the hypothesis that lack of FasL-mediated control of Fas-expressing inflammatory cells is responsible for the increased disease phenotype seen in BALB-*lpr* mice.

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