

Sensory Nerve Retraction and Sympathetic Nerve Innervation Contribute to Immunopathology of Murine Recurrent Herpes Stromal Keratitis

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Received: October 16, 2021

Accepted: January 3, 2022

Published: February 1, 2022

Citation: Yun H, Yin XT, Stuart PM, St. Leger AJ. Sensory nerve retraction and sympathetic nerve innervation contribute to immunopathology of murine recurrent herpes stromal keratitis. *Invest Ophthalmol Vis Sci.* 2022;63(2):4. <https://doi.org/10.1167/iovs.63.2.4>

PURPOSE. Herpes stromal keratitis (HSK) represents a spectrum of pathologies which is caused by herpes simplex virus type 1 (HSV-1) infection and is considered a leading cause of infectious blindness. HSV-1 infects corneal sensory nerves and establishes latency in the trigeminal ganglion (TG). Recently, retraction of sensory nerves and replacement with “unsensing” sympathetic nerves was identified as a critical contributor of HSK in a mouse model where corneal pathology is caused by primary infection. This resulted in the loss of blink reflex, corneal desiccation, and exacerbation of inflammation leading to corneal opacity. Despite this, it was unclear whether inflammation associated with viral reactivation was sufficient to initiate this cascade of events.

METHODS. We examined viral reactivation and corneal pathology in a mouse model with recurrent HSK by infecting the cornea with HSV-1 (McKrae) and transferring (intravenous [IV]) human sera to establish primary infection without discernible disease and then exposed the cornea to UV-B light to induce viral reactivation.

RESULTS. UV-B light induced viral reactivation from latency in 100% of mice as measured by HSV-1 antigen deposition in the cornea. Further, unlike conventional HSK models, viral reactivation resulted in focal retraction of sensory nerves and corneal opacity. Dependent on CD4⁺ T cells, inflammation foci were innervated by sympathetic nerves.

CONCLUSIONS. Collectively, our data reveal that sectoral corneal sensory nerve retraction and replacement of sympathetic nerves were involved in the progressive pathology that is dependent on CD4⁺ T cells after viral reactivation from HSV-1 latency in the UV-B induced recurrent HSK mouse model.

Keywords: HSV-1, corneal nerves, anti-viral immunity

Herpes stromal keratitis (HSK) is a potentially blinding disease caused by herpes simplex virus type 1 (HSV-1) infection of the cornea. The incidence of HSV keratitis is approximately 1.5 million worldwide, including 40,000 new cases of related blindness each year.^{1,2} After primary infection, the virus gains access to corneal nerves and invades the trigeminal ganglion (TG) where it establishes a lifelong latency. During times of stress, exposure to UV-B light, and increased levels of corticosteroids, the virus can reactivate,^{3–7} travel down sensory afferents to the cornea where an inflammatory immune response can occur.⁸ HSK does not happen in all individuals after primary infection; however, when a pathological reactivation stimulus does occur the chances of subsequent reactivation events increases.⁹

Mouse models have proven to be critical to understanding the nature and progression of disease during HSK. Early investigations of the disease identified myeloid-derived cells as the vital immune contributors of corneal pathology in that inflammatory cytokines and chemokines, such as interferon gamma (IFN- γ) tumor necrosis factor alpha (TNF- α),

CXCL-1, IL-8, metalloproteases, and other factors, such as nitric oxide, led to the corneal opacification and necrosis.^{10–12} Furthermore, CD4⁺ T cells were explicitly linked to HSK in that they acted as an additional source of proinflammatory cytokines and chemokines to perpetuate inflammation and disease^{13–15}; however, whether the antigen is a viral or host peptide remains unknown. More recently, corneal anesthesia was recognized as a hallmark of HSK, and this is characterized by a replacement of corneal sensory nerves with unsensing sympathetic nerves. The loss of the blink reflex associated with corneal anesthesia leads to a dry eye phenotype that exacerbates viral-mediated disease.^{16–18} Since then, vascular endothelial growth factor-A (VEGF-A), which is produced by corneal CD4⁺ T cells and myeloid-derived cells, was identified as a critical factor mediating the disruption of corneal nerves.¹² Despite the current mechanistic understanding of HSK, there are limitations to the conventional model of murine HSK in that disease is established after primary infection and blinding pathology does not rely on overt viral reactivation from latency which is different from human HSK.

Primary models of HSK develops symptoms by 10 dpi^{17,19,20}; the human manifestation of the disease is progressive and, usually, depends on numerous rounds of viral reactivation from latency over the span of years.²¹ To better recapitulate progressive corneal pathology, we used a modified version of the Shimeld mouse model where viral latency is established non-pathogenically by simultaneously infecting the cornea with the McKrae strain of HSV-1 and injecting human IgG.²² The McKrae strain of HSV-1 is considered highly pathogenic due to its ability to induce encephalitis in a high frequency of mice,²³ but the co-administration of human IgG provides protection from encephalitis and allows the virus to enter latency in the TG without ocular or systemic pathology. This method allowed us to assess how HSK and the corneal nerve landscape were affected by a UV-B light-induced viral reactivation event. Furthermore, we were also able to test the role CD4⁺ T cells played in this recurrent HSK (rHSK) model.

METHODS

Animals

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Male and female NIH inbred mice were obtained from Harlan Olac (Oxford, UK). All the mice were housed in the Animal Resource Facility at Saint Louis University (St. Louis, MO, USA) and used at 8 to 12 weeks of age in all experiments. The use of animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus

The human isolated HSV-1 McKrae strain was used in this study. 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. The intact virions were purified and stored at -70°C. The concentration of HSV-1 was determined in a standard virus plaque assay.

Mouse Infection

Mice were anesthetized by intraperitoneal (IP) injection of ketamine hydrochloride (60 mg/kg body weight) and xylazine (5 mg/kg body weight) in Hanks' balanced salt solution (HBSS). Topical corneal infection was performed by scarification of the cornea with a sterile 30-gauge needle under a dissecting microscope and applying 5 µL HBSS containing 1×10^6 pfu of HSV-1. At the same time as infection, each mouse received an IP injection of 1 mL of pooled human serum (Sigma, Temecula, CA, USA; anti-HSV reactivity with an effective dose for 50% viral neutralization of 1:800) to protect the mice during primary infection.

UV Irradiation

Mice with primary infection were observed for at least 5 weeks before UV irradiation, only the ones with clear corneas as determined by slit lamp analysis from a trained ophthalmologist were included in the study. In addition, mice with signs of encephalitis were excluded from the experiments. The eyes of both C57BL/6J or NIH inbred 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.

Virus Reactivation

The mouse eyes were swabbed with surgical spears (Weckel, Xomed-Treace, Jacksonville, FL, USA) before UV irradiation and once a day for 7 days after. The swabbed material was then co-cultured with VERO cells to detect the virus. If the HSV culture is negative before UV irradiation but turns into positive on any day after irradiation, then the mouse is categorized as with virus reactivation. We then examined the virus antigen deposition in the corneas by immunohistochemistry (IHC) to confirm the virus reactivation.

Monitor HSK Severity

HSK severity was monitored using a dissecting microscope by a masked and trained ophthalmologist. As the corneal lesion of recurrent HSK is usually focal and regional, we divided the cornea into four quadrants to be evaluated individually and added up all the scores as the final score. The corneal opacity of each quadrant was scored on a 4-point scale protocol as follows: 0.5 = any corneal imperfection; 1 = mild corneal haze; 2 = moderate opacity; 2.5 = moderate opacity with dense regional opacity; 3 = diffuse dense opacity obscuring the iris; 3.5 = diffuse dense opacity with corneal ulcer; or 4 = corneal perforation. The corneal neovascularization was scored by dividing each cornea into 4 quadrants, and each quadrant was scored as 0 = no vessels visible; 2 = vessels extending into the paracentral cornea; or 4 = vessels extending to the central cornea. The total score of the 4 quadrants was then divided by 16 (360 degrees vascularization to the central cornea) and multiplying by 100, which represents the percentage of each cornea that was vascularized. We include the CD31 staining of corneas to illustrate the degree of vascularization in Supplementary Figure S1.

Corneal blink reflex was tested by loosely holding the mouse and touching the cornea with the blunt tips of surgical forceps without touching the eyelashes and whiskers. The cornea was divided into 5 areas (4 quadrants and center area), loss of blink reflex referred to the inability of the mouse to blink when an area was touched and was recorded as 0. Positive blink reflex referred to the ability to blink when an area of the cornea was touched and was recorded as 1. The total score of the five regions would be the final score of the corneal blink reflex for a mouse. A score of 0 indicated a complete loss of corneal sensation such that the mouse failed to blink when any area of the cornea was touched. A score of five indicated retention of some degree of sensation such that the mouse blinked when any area of the cornea was touched.

CD4 Depletion

Mice were depleted of CD4⁺ T cells with an IP injection of 0.15 mg rat anti-mouse CD4 Ab clone GK1.5 (InVivoMab anti-mouse CD4, BioXcell, West Lebanon, NH, USA) or mock depleted with an IP injection of 0.15 mg anti-keyhole limpet hemocyanin (LTF, InVivoMab rat IgG2b isotype control). Injections were given 1 day before UV irradiation, the day of UV irradiation, and 7 days post-UV irradiation. The efficacy of CD4 depletion was confirmed by a lack of CD4⁺ T cells

in the blood of depleted mice as assessed by flow cytometry (not shown) 1 week after the final depletion treatment.

Immunohistochemistry

Corneas were dissected and fixed at room temperature for 1 hour in 1.3% paraformaldehyde in PBS, and radial incisions were made to facilitate flat-mounting of the corneal tissues. Corneas were washed in PBS 5 times, permeabilized in 1% Triton X-100 in PBS at room temperature for 60 minutes and blocked with 20% goat serum (Cedarlane, Burlington, NC, USA) in blocking buffer (0.3% Triton X-100/0.1% Tween-20 in PBS) for 1 hour. The corneas were then incubated in a 100 μ L cocktail of primary antibodies at room temperature for 2 hours, followed by an additional incubation overnight at 4°C. After five 5-minute washes in wash buffer (0.1% Tween-20 in PBS), the corneas were incubated in a 100 μ L cocktail of secondary antibodies in blocking buffer at room temperature for 2 hours. Following five 10-minute washes with wash buffer, the corneas were mounted on slides and dried at 4°C for at least 12 hours before imaging.

Primary antibodies were: rabbit polyclonal anti-Beta III tubulin (1:1000, cat #ab18207), chicken polyclonal anti-tyrosine hydroxylase (TH, 1:200, cat #76442), all from Abcam, Cambridge, MA, USA; or rat anti-substance P (anti-SP, 1:300, cat #4311672, BD Bioscience, San Jose, CA, USA), HSV type-1 antibody, biotin conjugate (Thermo Scientific Prod #PA1-26169 Lot #RB2160088). Secondary antibodies included: Alex Fluor 488 goat anti-rabbit IgG (H + L) (1:500, cat #GR233725-3, Abcam, Cambridge, MA, USA); Alex Fluor 546 goat anti-chicken IgG (H + L) (1:500, cat #1618409, Life Technologies, Grand Island, NY, USA); Alex Fluor 633 goat anti-rat IgG (H + L) (1:500, cat #73B1-1) from Molecular Probes, Eugene, OR, USA; and BV421 Streptavidin and 4'-6-diamidino-2-phenylindole (DAPI 1:5000, Sigma, St. Louis, MO, USA).

Confocal Microscopy

Stitched Z-stacks spanning entire corneal whole mounts were acquired with an OLYMPUS BX61 motorized upright Fluoview 1200 laser scanning confocal microscope equipped with a x20 (numerical aperture, 0.85) objective lens and an automated stage. The Z-stack images were saved in the native Olympus Image Binary (OIB) format and stitched together using FV10-ASW 2.0 software (Olympus Life Science, Tokyo, Japan). Brightness levels in the figures were adjusted for display.

Representative corneal regions in the epithelial-stroma interphase were selected for the subbasal nerve density counting. Briefly, five regions (300 μ m \times 300 μ m) were picked from each cornea: one from the central cornea and the other 4 regions from 4 quadrants located 500 μ m away from the center. The evaluation of nerves in each cornea was processed using Simple Neurite Tracer (Longair et al., 2011³⁸) in the segmentation package in FIJI programs and then analyzed by the 3D Skeletonize (Arganda-Carreras et al., 2010³⁹) FIJI plugin. The total length of nerves in each area was calculated from the data provided.

Statistical Analysis

All values are presented as mean \pm SEM. The significance of differences between groups was determined by unpaired parametric *t*-test. Chi-square test was used to analyze the

severity of HSK of C57BL/6J and NIH mice. The statistical significance of overall group differences was determined by 1-way ANOVA, followed by the Tukey posttest to assess the significance of differences between individual subgroups or determined by unpaired *t*-test. The statistical significance between the two treatments in the CD4 depletion experiment was determined by 2-way ANOVA and the statistical differences between timepoints of disease scoring was determined by Multiple *t*-test (unpaired non-parametric test) in which the Mann-Whitney test and Holm-Sidak's multiple comparisons tests were performed. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

UV-B Light Induces Viral Reactivation in all Mice Latently Infected With HSV-1

In humans, HSV-1 reactivation is characterized by the delivery of infectious virions to the ocular surface from the TG, which coincides with an inflammatory response at the cornea.⁸ This can temporarily and eventually permanently damage the optically clear collagen network within the cornea. Under normal conditions, HSV-1 does not reliably reactivate from latency in the wild-type C57BL/6J strain of mice.²⁴ Stimuli, such as psychological stress, can lead to an increase in viral genome copies within the TG, which has been characterized as a viral reactivation event. However, under these conditions, live virus is not readily detected at the ocular surface. Here, we used a model of HSV-1 reactivation that relies on UV-B light to reactivate HSV-1 McKrae from latency.²⁵⁻²⁷ At 28 days post primary infection (dpi), we exposed NIH and C57BL/6J mice to UV-B light to induce viral reactivation from latency. Beginning at 2 days post UV-B (dpu) through 7 dpu, we swabbed eyes every day and performed plaque assays to assess live virus. In both strains of mice, we detected live virus in about 30% of the sample mice (Fig. 1A). It was possible that neutralizing antibodies against HSV-1 produced after primary infection were reducing the detectability of infectious virus. Therefore, we next measured whether viral antigens could be detectable in the cornea after UV-B light exposure. Indeed, 100% of mice exposed to UV-B light had HSV-1 antigens deposited in the cornea by 7 dpu, which were gradually lost from the cornea by 28 dpu (see Fig. 1B, Supplementary Fig. S2). When 3D images were made of corneas, it was clearly discernible that HSV-1 antigens were deposited in the epithelial layer of the cornea with limited detectability near the endothelial portion of the cornea (see Fig. 1C), and this pattern of staining was consistent between 7 dpu and 28 dpu. Importantly, corneas from mice that were not exposed to UV-B light after primary infection (pHSK; see Fig. 1B), showed no detectable viral antigens, which supports the notion that viral reactivation is, indeed, occurring in this model of UV-B exposure. These data suggested that transient viral reactivation does, indeed, occur in all mice exposed to UV-B light in this rHSK model.

HSV-1 Reactivation Results in Corneal Disease and Loss of Blink Reflex

Knowing that UV-B light caused a detectable viral reactivation event in all experimental mice, we next asked whether viral reactivation events can lead to corneal disease. Indeed, mice exposed to UV-B light developed mild to severe corneal

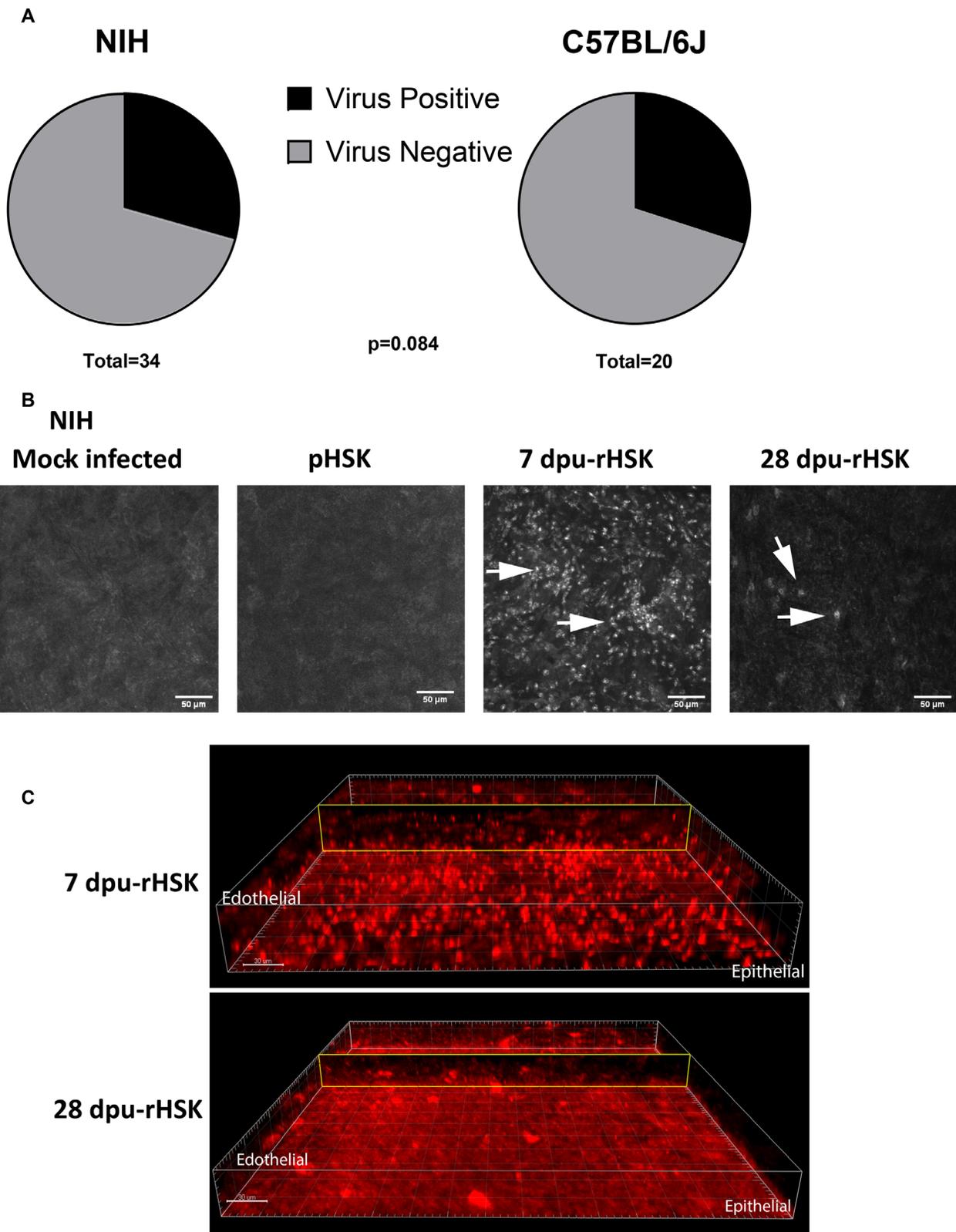


FIGURE 1. UV-B light induces viral reactivation in all mice latently infected with HSV-1. NIH or C57BL/6J mice (28 dpi, male and female) were exposed to UV-B light. Beginning at 2 days post UV-B (dpu) through 7 dpu, eyes were swabbed and plaque assays were performed to assess live virus. **(A)** Live virus was detected in about 30% of both strains of mice chi-square (1, $N = 49$) = 0.055, $P = 0.814$, by chi-square test. **(B, C)** At 7 dpu and 28 dpu, NIH mice were euthanized and corneas were excised, flat mounted, and labeled with antibody to HSV-1 antigen. NIH mouse corneas with primary infection but without UV-B irradiation were also examined for HSV-1 antigen. The arrows point to the cells with positive staining of anti-HSV-1 antigen. **(C)** Images show 3D construction of a cornea to illustrate the epithelial localization of HSV-1 antigen. Results in all panels were from two to three independent experiments.

opacity and vascularization (Figs. 2A–C). In addition, we observed a significant reduction in blink reflex among mice after exposure to UV-B light (see Figs. 2A, 2B). Notably, there were statistical differences in disease scores between the NIH and C57BL/6J strains. Specifically, 38.4% (16 of 42) of NIH mice developed moderate to severe corneal opacity, whereas only 11.5% (3 of 26) of C57BL/6J mice developed moderate to severe corneal opacity (see Fig. 2D). Furthermore, 35.7% (15 of 42) of NIH mice had a complete loss of blink reflex compared to 15.4% (4 of 26) of C57BL/6J mice completely losing blink reflex (see Fig. 2E). We also separated the combined data according to the sex of the mice, and we observed no statistical difference between male and female mice (Supplementary Fig. S3). Together, these data suggested that HSK severity after UV-B light-induced viral reactivation is largely dependent on the genetic strain differences, and there is no sexual dimorphism in this model of disease.

UV-B Induced Virus Reactivation Causes More Severe Corneal Nerve Damage in HSV-1-Infected Mice

The loss of sensory nerves from the cornea contributes to the severity of HSV-induced disease,^{12,17} and our analyses revealed that exposure to UV-B light after HSV-1 infection leads to a decrease in corneal sensitivity. Therefore, we wanted to assess the corneal nerve landscape after UV-B mediated viral reactivation from latency. Cornea whole-mount staining of the subbasal nerve plexus showed that there were no changes in the corneal nerve landscape 28 days after UV-B irradiation of eyes that were not previously infected with HSV-1. The UV-B irradiation does, indeed, cause retraction of the corneal sensory nerves; however, those sensory nerves are re-established to densities similar to control mice by the time of euthanization or 28 dpu. Although HSV-1 infection alone resulted in a significant reduction in the subbasal nerve plexus within the corneal epithelia in NIH mice, C57BL/6J mice maintained a subbasal nerve plexus similar to uninfected controls. Furthermore, the total corneal nerve plexus lengths per field (300 μm \times 300 μm) in both strains of mice were significantly reduced when infected and exposed to UV-B light compared to the UV-B alone control and all uninfected controls (Figs. 3A, 3B).

In the primary HSK model where symptoms manifest around 10 dpi and maintained indefinitely, sympathetic nerves dominate the cornea after sensory nerve retraction.^{17,28} We next wanted to define the class of nerves that remain in the cornea with rHSK. Due to the loss of nerves within the corneal epithelia in the HSV-infected groups that we described in this and other studies, we analyzed the innervation of the corneal stroma. Compared to uninfected eyes, infected eyes that were exposed to UV-B light and developed severe HSK were more abundantly innervated with unsensing sympathetic nerves (see Fig. 3C). Notably, despite their absence in the cornea epithelia, sympathetic nerves hyperinnervate the cornea stroma during HSK, which is why there appears to be an abundance of β III tubulin staining in HSK cornea stromas (see Fig. 3C) compared to the epithelia (see Fig. 3A). Moreover, in an area of the cornea where blink reflex is maintained, the cornea is primarily innervated with substance P (SP)⁺ sensory nerves (see Fig. 3D, left). Conversely, in an area of the cornea where the blink reflex was lost, we observed an increased prevalence of

nerves that expressed tyrosine hydroxylase (TH)—a marker of sympathetic nerves (see Fig. 3D, right). In the model of HSK where pathology develops after primary infection, sympathetic nerves overtake the cornea during peak disease with no detection of sensory nerves. Here, we show that this does not occur in the rHSK model because pathology begins after reactivation with UV-B light and the replacement of sensory nerves with sympathetic nerves is less uniform. These results illustrate that the progressive disease observed in this model of rHSK is due to the partial disruption of corneal nerves after the reactivation event. Further, it is likely that repeated reactivation events, as is normally observed in human HSK, would lead to continual disruption of the corneal nerve landscape and worsening disease.

We also stained the corneal whole-mounts with anti-CD45 antibody, and we found that CD45⁺ inflammatory cells formed cell clusters in the rHSK corneal stroma. These clusters were not present in the corneas of non-infected mice that were exposed or not exposed to UV light (Supplementary Fig. S4). The observation that sympathetic nerves innervated the centers of CD45⁺ cell clusters (Fig. 4A) suggested that the cross-talk between sympathetic nerves and inflammatory cells played a role in the pathogenesis of rHSK. Although the cell identities within the clusters were varied, CD4⁺ T cells made up a substantial percentage of the CD45⁺ cells in each cluster (see Fig. 4B).

CD4⁺ T Cells Mediate Corneal Nerve Disruption After Viral Reactivation From Latency

In conventional models of ocular HSV-1 infection, CD4⁺ T cells are pathogenic and infiltrate the cornea as early as 2pi.^{14,28} These cells perpetuate disease indefinitely by producing inflammatory cytokines and VEGF-A, which can lead to corneal necrosis and disruption of corneal nerve homeostasis.¹² We next examined if the observed pathology in the rHSK model was also mediated by CD4⁺ T cells. To do this, we IP) administered a CD4 depletion antibody one day prior to reactivation (27 dpi) and 7 days after reactivation. By 7 dpu, we observed significant improvements in disease within the CD4-depleted group compared to the mock-depleted group. Specifically, corneas were less opaque, harbored fewer blood vessels, and maintained blink reflex in more areas (Figs. 5A–C). Furthermore, the depletion of CD4⁺ T cells also resulted in less dense sympathetic innervation of the cornea (see Fig. 5D). We did, however, observe re-establishment of corneal nerves within the cornea epithelia. Specifically, using β III tubulin staining, we observed that there was a more prominent subbasal nerve plexus and fine sensory endings in CD4-depleted mice compared to mock-depleted mice (see Fig. 5E). This pattern of nerve morphology together with the recovery of the blink reflex are indicative that sensation and sensory nerves are at least partially restored in corneas that have experience viral reactivation from latency and were depleted of CD4⁺ cells. Additionally, CD4⁺ T cell depletion almost completely eliminated CD45⁺ cell clusters from the rHSK corneal stroma (see Fig. 5F), suggesting that CD4⁺ T cells play a central role in the cross-talk between the sympathetic nerves and inflammatory cells. These results indicate that CD4⁺ T cells, similar to in the conventional HSK model, are critical for the development of corneal pathology and the disruption of the corneal nerve architecture.

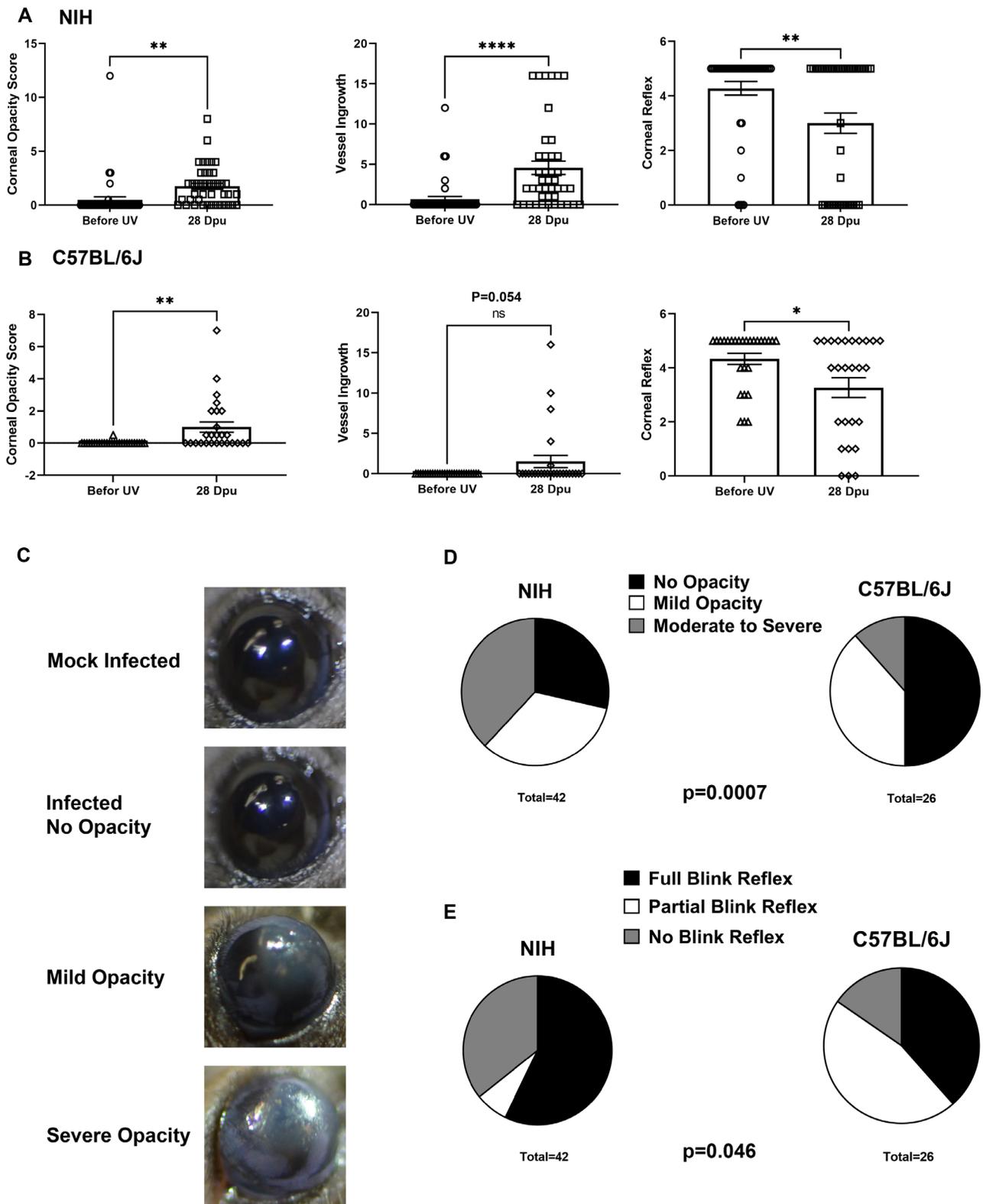


FIGURE 2. HSV-1 reactivation results in corneal disease and loss of blink reflex. NIH or C57BL/6J mice (28 dpi, male and female) were exposed to UV-B light. (A, B) The mice were examined before UV-B irradiation and at 28 dpi for the severity of HSK, and the corneal opacity, vascularization and blink reflex were scored and recorded. (C, D) HSK progression was then classified by corneal opacity as mild (opacity of all quadrants, 0.5 to 1), moderate (opacity of any quadrants, 1.5 to 2), or severe (opacity of any quadrants 2.5 or above). There were 38.4% (16 of 42) of NIH mice that developed moderate to severe corneal opacity, whereas 11.5% (3 of 26) of C57BL/6J mice developed moderate to severe corneal opacity. Chi-square test (2, $N = 66$) = 6.18, $P = 0.046$. (E) Blink reflex was classified as full blink reflex (with blink reflex in 5 areas), partial blink reflex (with blink reflex in less than 5 areas), and no blink reflex (no blink reflex in all 5 areas). There were 35.7% (15 of 42) of NIH mice that had a complete loss of blink reflex, whereas 15.4% (4 of 26) of C57BL/6J mice completely lost blink reflex, chi-square test (2, $N = 66$) = 14.57, $P = 0.0007$, by chi-square test. Results in all panels are presented as mean \pm SEM pooled from four to five independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

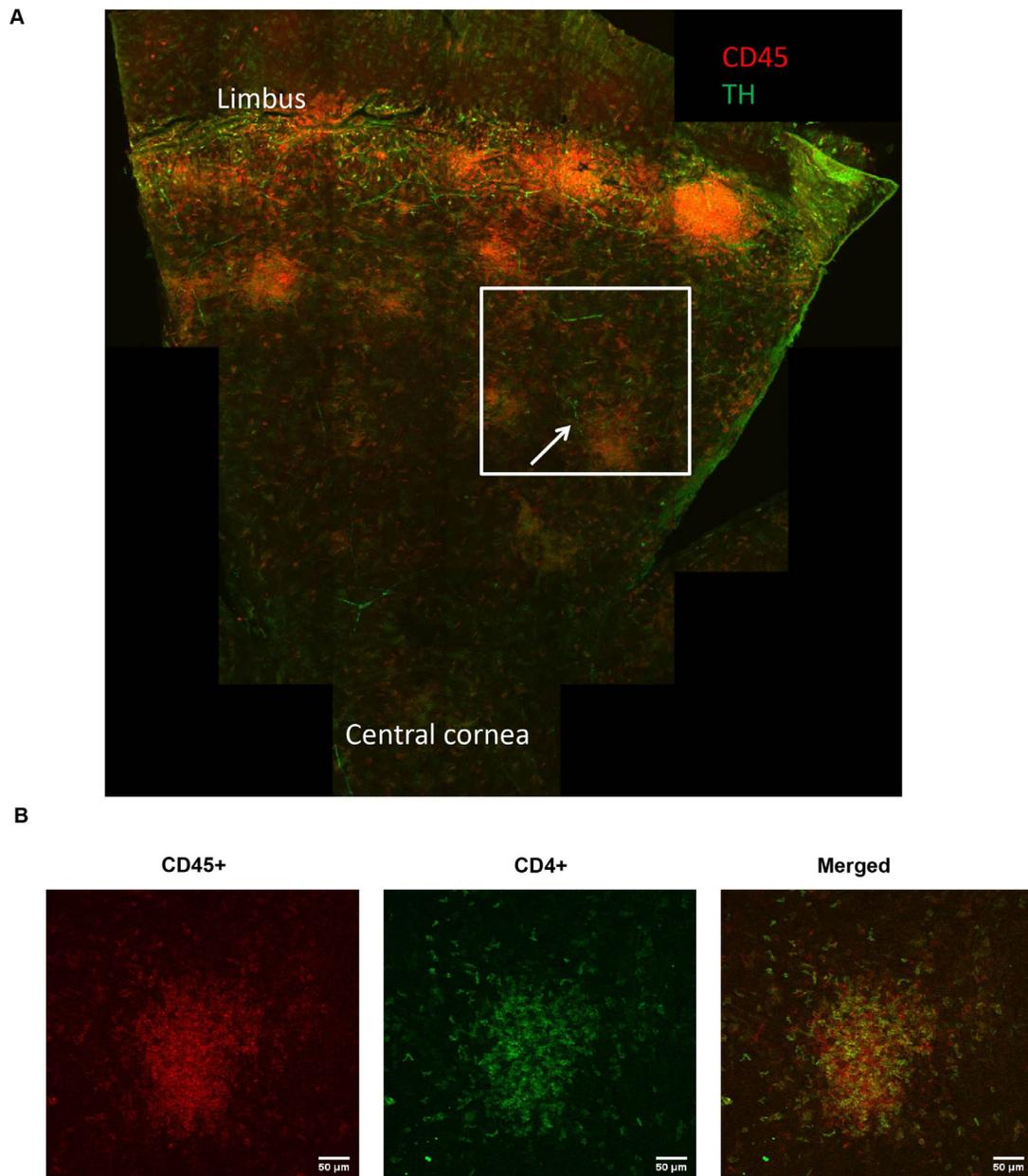


FIGURE 4. Clusters of CD45⁺ cells form around sympathetic nerves within the cornea. At 27 dpu, both strains of mice were euthanized and the corneas were excised, flat-mounted, and labeled with anti-TH antibody (green) and anti-CD45⁺ antibody (red). **(A)** The arrows points to a CD45⁺ cell cluster. The area in the frame was then examined in a 3D close look. **(B)** Corneal whole mount staining also showed that CD4⁺ T cells (green) constituted a substantial percentage in a CD45⁺ (red) cell cluster.

Historically, pathology observed in the murine primary HSK model resembles the leukocytic infiltration, chemokine production, and vascularization observed during peak disease in humans.²⁹ However, in humans, HSK is progressive and normally takes years and multiple rounds of viral reactivation from latency. The rHSK model used here better resembles human disease in that pathology is progressive and begins to develop only after UV-B light-induced viral reactivation.^{22,25–27} Similar to previous studies, we observed the progressive development of HSK. Our study differs from previous studies in that we also assessed the partial, rather than complete, loss of sensory nerves and blink

reflex after viral reactivation, which was not observed in uninfected control mice that were only exposed to UV-B light. Notably, sympathetic nerves invaded the cornea where sensory nerves retracted. Further, rather than diffuse stromal opacity observed in the primary HSK model, the UV-B light-induced viral reactivation caused focal stromal opacities. In sum, our data build off from previous work in the rHSK field by establishing a correlation between neurologic changes and physiological corneal pathologies in recurrent HSK.

Methods to induce HSV-1 viral reactivation from latency in mice, especially in the C57BL/6J strain, have proven to

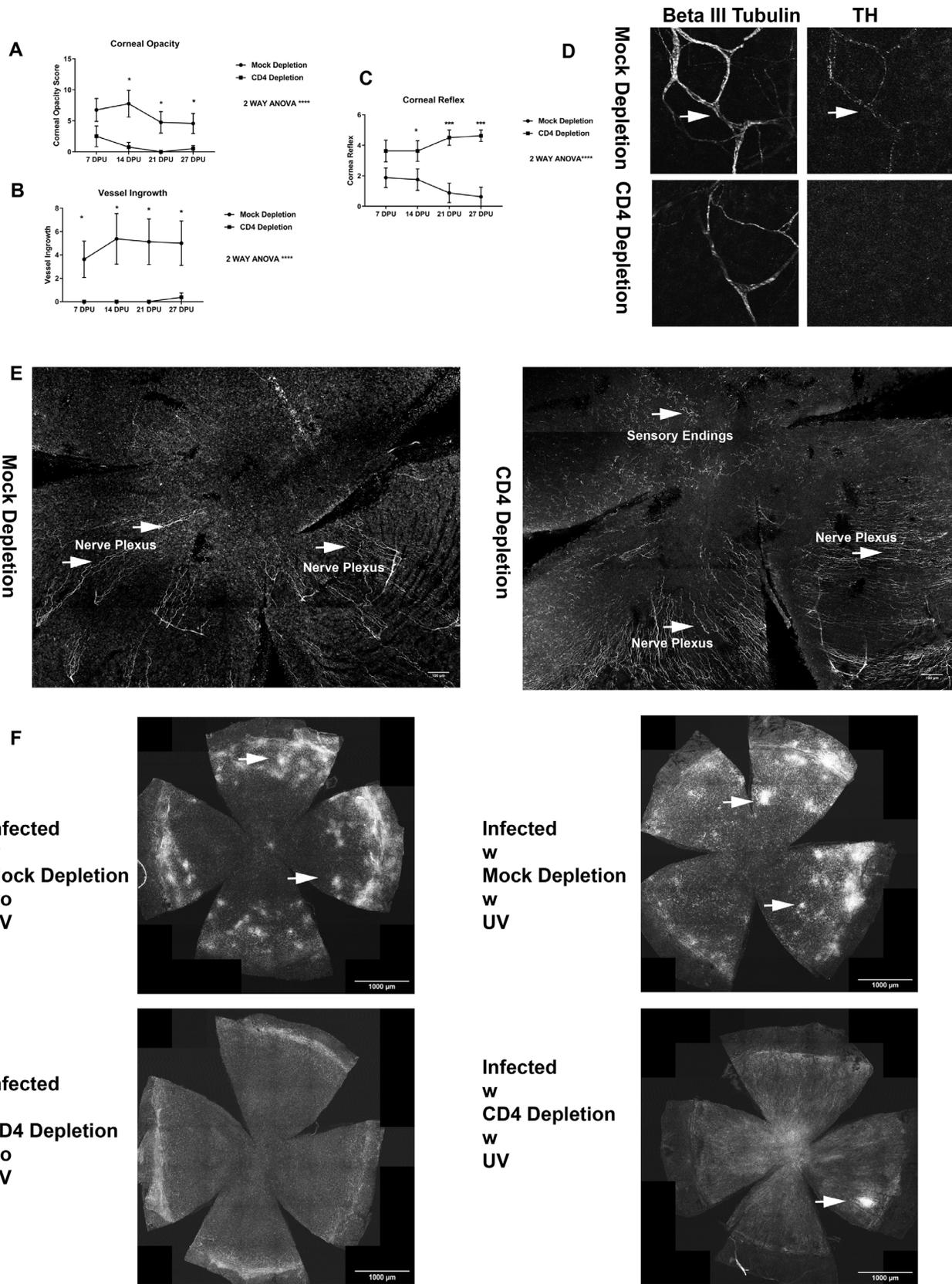


FIGURE 5. CD4⁺ T cells mediate corneal nerve disruption after viral reactivation from latency. C57BL/6J mice were mock depleted or depleted of CD4⁺ T cells by intraperitoneal administered α CD4 antibody or control antibody. The mice were monitored at 7 dpu, 14 dpu, 21 dpu, and 27 dpu for the severity of HSK, and the (A) corneal opacity, (B) vascularization, (C) and blink reflex were scored and recorded. The differences between CD4⁺ T cells depleted mice and the mice with mock depletion were examined by 2-way ANOVA. In addition, we used unpaired Mann-Whitney tests with Holm-Sidak's multiple comparisons to evaluate the significance at each time point.

Significance for each time point is noted above the graph. (D) At 27 dpu, mice were euthanized and corneas were excised, flat-mounted, and labeled with antibodies to β III tubulin and TH. The arrow points to the β III tubulin⁺/TH⁺ sympathetic nerves in the mock depleted cornea. (E) Representative images of cornea to show the corneal nerve morphology comparing mock depleted and CD4 depleted mice. Arrows either point to the nerve plexus or fine sensory endings. (F) At 27 dpu, both strains of mice were euthanized and corneas were excised, flat-mounted, and labeled with anti-CD45⁺ antibody. Arrows point to CD45⁺ cell clusters. Results in all panels are represented as mean \pm SEM from two independent experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

be inconsistent.³⁰ These methods have focused on restraint stress,^{4,6} chemical interventions,³¹ and other means,³² and resulted in increases in viral genome copies within the TG and in some instances, the brainstem; however, viral shedding at the periphery has not been reliably detected. These data cast doubt to the conclusions that link increases in viral genome copies to a true reactivation event that delivers live virus to the cornea. The UV-B model of reactivation used in this study causes viral reactivation in the TG and the cornea.²⁷ Notably, our model of viral reactivation relies on scarification of the cornea prior to infection. Whereas other studies from other groups have shown that ocular infection and pathology can take place without scarification, we chose to scarify the cornea to ensure a more robust phenotype. In addition, although other groups have shown that simple scarification of the cornea disrupted the corneal nerve landscape, here, we showed that corneal nerves—by 3 days post scarification—are re-established in the cornea similar to unscarified eyes.^{25,26} Therefore, we concluded that the scarification model was more appropriate for our studies.

Although the focus of these studies centered on the nature of corneal nerve disruption after HSV-1 viral reactivation, our studies allowed us to reach conclusions about the detectability of virus after UV-B light exposure. Specifically, our studies reveal that measuring live virus may underestimate true reactivation events due to our observation that UV-B light exposure leads to the detection of HSV-1 antigens near the epithelial layer of the cornea in 100% of eyes. In a previous investigation, 34% of mice that did not shed live virus after UV-B exposure developed stromal disease, suggesting that the conventional methods, like live virus quantification through plaque assay counting, may not be sufficient to detect all viral reactivation events.³³ In a follow-up set of studies, we have been able to highlight and describe that UV-B exposure also leads to the deposition of viral RNA into the cornea (data not shown). We did not include these data as they are beyond the scope of this work and are included in a manuscript that is in preparation with a different group of investigators. Together, these data suggest that viral antigen detection can be a supplementary method to assist conventional methods in the measurement of HSV-1 reactivation events.

The level of viral gene expression and translation in the TG that is required for the deposition of HSV-1 antigens in the cornea is unclear. Further, we are uncertain whether full virions are produced and immediately neutralized by antibodies, or if viral proteins/incomplete and non-infectious virions are transported to the cornea from the TG and/or the peripheral conjunctiva. Regardless of this, we observed that UV-B light exposure and subsequent HSV-1 viral reactivation from latency led to the disruption of corneal nerves and corneal pathology. Although CD4⁺ T cells are essential for pathology, there is some uncertainty as to whether those CD4⁺ T cells are directly responding to HSV-1 antigens or if HSV-1 infected nerves produce a host factor that directly or indirectly stimulates CD4⁺ T cells to cause pathology after

UV-B exposure. If CD4⁺ T cells were directly responding to HSV-1 antigens, one would expect disease to gradually decrease due to our data revealing that HSV-1 antigens are gradually lost after UV-B exposure. Our results, however, reveal that disease peaks at 14 dpu, which is after HSV-1 antigens begin progressively losing prominence in the cornea. These data would suggest that alterations in corneal nerves and/or the corneal inflammatory status dictate CD4⁺ T cell functionality and subsequent disease. Due to the similarities in corneal nerves between uninfected mice exposed or not to UV-B light, we are confident that UV-B light alone does not cause the CD4⁺ T cell-dependent pathology we observed in our model of viral reactivation. Conversely, infection alone was not sufficient to cause disease. Therefore, we reached the conclusion that UV-B induced viral reactivation from latency is necessary to cause the observed disruptions in corneal nerves that lead to pathology. Whether HSV-1 antigens are directly responsible for this process is yet to be determined and is the subject of a future study.

One would expect that the level of viral shedding would correlate with the severity of disease; however, our analyses do not reveal a correlation between those parameters. Because the studies described here are largely longitudinal, it was not possible to correlate the level of HSV antigen staining with disease because disease (peaks at 28 dpu) clearly lagged behind the peak detection of viral antigens within the cornea (7 dpu). These results highlight the need to determine the criteria required to define a true reactivation event.

Similar to the primary HSK model, our results highlighted CD4⁺ T cell as a critical player in pathology after a viral reactivation event. Without CD4⁺ T cells, CD45⁺ cells did not form clusters which were innervated by sympathetic nerves, suggesting the crosstalk between CD4⁺ T cells and sympathetic nerves is critical to the pathogenesis of rHSK. Specifically, CD4⁺ T cells disrupt the corneal nerve landscape by producing VEGF-A, which results in the replacement of sensory nerves with sympathetic nerves in the cornea.¹² Another mechanism in which CD4⁺ T cells disrupt corneal nerve architecture is through the production of the complement component, C3.³⁴ In tandem, these mechanisms contribute to pathological loss of sensation at the cornea that leads to disease. Indeed, in this study, CD4⁺ T cells contributed to an increase in sympathetic innervation of the cornea after viral reactivation from latency; however, innervation was less diffuse and affected only regions of the cornea rather than the entire tissue. These data implicate that either VEGF-A and/or the complement cascade influences disease in this model; however, studies are ongoing. Moreover, the factor that stimulates CD4⁺ T cells to produce VEGF-A remains elusive.

NIH mice were more vulnerable to corneal sensory nerve plexus loss before and after UV-B light-induced viral reactivation compared with the C57BL/6J strain of mice, which is consistent with previous studies.^{19,35,36} Similarly, NIH mice developed more severe corneal opacification after UV-B irradiation. Although genetic differences in the host immune response as well as microbiome differences cannot be elim-

inated, it is possible that the lack of pigmentation producing cells in NIH mice may be responsible for the increased susceptibility to disease. In humans, sunlight is a potent stimulus for the reactivation of HSV-1 as skin pigmentation is known to affect the absorbance of UV light.³⁷

Here, we have highlighted how a UV-B light-induced recurrent HSK model may be a more physiological substitute for the conventional model of HSK where rapid inflammation causes blinding pathogenesis independent of detectable live viral reactivation in the cornea from latency. Similar to the conventional model, CD4⁺ T cells continue to play a major role in disease through the disruption of corneal nerves and subsequent loss of tactile sensation. Our studies continue to point toward a dynamic relationship between the neurological and immunological systems in the development of blinding ocular surface disease. Whereas our studies deepen our understanding of the underlying mechanisms responsible for the development of HSK, it is clear that these same mechanisms likely function in other corneal neuropathic conditions.

Acknowledgments

The authors thank Robert Hendricks for beginning this work and contributing to the early development of this project.

Supported by NIH grants R01 EY026891 (A.J.S.), R01 EY026071 (P.M.S.), P30 EY08098. In addition, we thank the support from the Eye & Ear Foundation of Pittsburgh and unrestricted grants from Research to Prevent Blindness Inc, New York, NY.

Disclosure: **H. Yun**, None; **X.-T. Yin**, None; **P.M. Stuart**, None; **A.J. St. Leger**, None

References

1. Farooq AV, Shukla D. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. *Surv Ophthalmol*. 2012;57:448–462.
2. Pepose JS, Keadle TL, Morrison LA. Ocular herpes simplex: changing epidemiology, emerging disease patterns, and the potential of vaccine prevention and therapy. *Am J Ophthalmol*. 2006;141:547–557.
3. Padgett DA, Sheridan JF, Dorne J, Berntson GG, Candelora J, Glaser R. Social stress and the reactivation of latent herpes simplex virus type 1. *Proc Natl Acad Sci USA*. 1998;95:7231–7235.
4. Freeman ML, Sheridan BS, Bonneau RH, Hendricks RL. Psychological stress compromises CD8⁺ T cell control of latent herpes simplex virus type 1 infections. *J Immunol*. 2007;179:322–328.
5. Cherpes TL, Busch JL, Sheridan BS, Harvey SA, Hendricks RL. Medroxyprogesterone acetate inhibits CD8⁺ T cell viral-specific effector function and induces herpes simplex virus type 1 reactivation. *J Immunol*. 2008;181:969–975.
6. Himmelein S, St Leger AJ, Knickelbein JE, Rowe A, Freeman ML, Hendricks RL. Circulating herpes simplex type 1 (HSV-1)-specific CD8⁺ T cells do not access HSV-1 latently infected trigeminal ganglia. *Herpesviridae*. 2011;2:5.
7. Yu W, Geng S, Suo Y, et al. Critical Role of Regulatory T Cells in the Latency and Stress-Induced Reactivation of HSV-1. *Cell Rep*. 2018;25:2379–2389.e2373.
8. Al-Dujaili IJ, Clerkin PP, Clement C, et al. Ocular herpes simplex virus: how are latency, reactivation, recurrent disease and therapy interrelated? *Future Microbiol*. 2011;6:877–907.
9. Liesegang TJ, Melton LJ, 3rd, Daly PJ, Ilstrup DM. Epidemiology of ocular herpes simplex. Incidence in Rochester, Minn, 1950 through 1982. *Arch Ophthalmol*. 1989;107:1155–1159.
10. Chucair-Elliott AJ, Gurung HR, Carr MM, Carr DJJ. Colony Stimulating Factor-1 Receptor Expressing Cells Infiltrating the Cornea Control Corneal Nerve Degeneration in Response to HSV-1 Infection. *Invest Ophthalmol Vis Sci*. 2017;58:4670–4682.
11. Kvanta A, Sarman S, Fagerholm P, Seregard S, Steen B. Expression of matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) in inflammation-associated corneal neovascularization. *Exp Eye Res*. 2000;70:419–428.
12. Yun H, Yee MB, Lathrop KL, Kinchington PR, Hendricks RL, St Leger AJ. Production of the Cytokine VEGF-A by CD4(+) T and Myeloid Cells Disrupts the Corneal Nerve Landscape and Promotes Herpes Stromal Keratitis. *Immunity*. 2020;53:1050–1062.e1055.
13. Keadle TL, Morris JL, Pepose JS, Stuart PM. CD4(+) and CD8(+) cells are key participants in the development of recurrent herpetic stromal keratitis in mice. *Microb Pathog*. 2002;32:255–262.
14. Lepisto AJ, Frank GM, Xu M, Stuart PM, Hendricks RL. CD8 T cells mediate transient herpes stromal keratitis in CD4-deficient mice. *Invest Ophthalmol Vis Sci*. 2006;47:3400–3409.
15. Rajasagi NK, Rouse BT. The Role of T Cells in Herpes Stromal Keratitis. *Front Immunol*. 2019;10:512.
16. Hamrah P, Cruzat A, Dastjerdi MH, et al. Corneal sensation and subbasal nerve alterations in patients with herpes simplex keratitis: an in vivo confocal microscopy study. *Ophthalmology*. 2010;117:1930–1936.
17. Yun H, Rowe AM, Lathrop KL, Harvey SA, Hendricks RL. Reversible nerve damage and corneal pathology in murine herpes simplex stromal keratitis. *J Virol*. 2014;88:7870–7880.
18. Rowe AM, Yun H, Hendricks RL. Exposure Stress Induces Reversible Corneal Graft Opacity in Recipients With Herpes Simplex Virus-1 Infections. *Invest Ophthalmol Vis Sci*. 2017;58:35–41.
19. 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.
20. 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.
21. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. *Cornea*. 2001;20:1–13.
22. Shimeld C, Hill T, Blyth B, Easty D. An improved model of recurrent herpetic eye disease in mice. *Curr Eye Res*. 1989;8:1193–1205.
23. Wang H, Davido DJ, Morrison LA. HSV-1 strain McKrae is more neuroinvasive than HSV-1 KOS after corneal or vaginal inoculation in mice. *Virus Res*. 2013;173:436–440.
24. Halford WP, Balliet JW, Gebhardt BM. Re-evaluating natural resistance to herpes simplex virus type 1. *J Virol*. 2004;78:10086–10095.
25. BenMohamed L, Osorio N, Khan AA, et al. Prior Corneal Scarification and Injection of Immune Serum are Not Required Before Ocular HSV-1 Infection for UV-B-Induced Virus Reactivation and Recurrent Herpetic Corneal Disease in Latently Infected Mice. *Curr Eye Res*. 2016;41:747–756.
26. Jiang X, Brown D, Osorio N, Hsiang C, BenMohamed L, Wechsler SL. Increased neurovirulence and reactivation of the herpes simplex virus type 1 latency-associated transcript (LAT)-negative mutant dLAT2903 with a disrupted LAT miR-H2. *J Neurovirol*. 2016;22:38–49.

27. Shimeld C, Easty DL, Hill TJ. Reactivation of herpes simplex virus type 1 in the mouse trigeminal ganglion: an in vivo study of virus antigen and cytokines. *J Virol.* 1999;73:1767–1773.
28. Suvas S, Azkur AK, Kim BS, Kumaraguru U, Rouse BT. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol.* 2004;172:4123–4132.
29. Stuart PM, Keadle TL. Recurrent herpetic stromal keratitis in mice: a model for studying human HSK. *Clin Dev Immunol.* 2012;2012:728480.
30. Webre JM, Hill JM, Nolan NM, et al. Rabbit and mouse models of HSV-1 latency, reactivation, and recurrent eye diseases. *J Biomed Biotechnol.* 2012;2012:612316.
31. Neumann DM, Bhattacharjee PS, Hill JM. Sodium butyrate: a chemical inducer of in vivo reactivation of herpes simplex virus type 1 in the ocular mouse model. *J Virol.* 2007;81:6106–6110.
32. Doll JR, Thompson RL, Sawtell NM. Infectious Herpes Simplex Virus in the Brain Stem Is Correlated with Reactivation in the Trigeminal Ganglia. *J Virol.* 2019;93:e02209–18.
33. Stumpf TH, Shimeld C, Easty DL, Hill TJ. Cytokine production in a murine model of recurrent herpetic stromal keratitis. *Invest Ophthalmol Vis Sci.* 2001;42:372–378.
34. Royer DJ, Echegaray-Mendez J, Lin L, et al. Complement and CD4(+) T cells drive context-specific corneal sensory neuropathy. *Elife.* 2019;8:e48378.
35. 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.
36. Laycock KA, Lee SF, Brady RH, Pepose JS. Characterization of a murine model of recurrent herpes simplex viral keratitis induced by ultraviolet B radiation. *Invest Ophthalmol Vis Sci.* 1991;32:2741–2746.
37. Mazzarello V, Ferrari M, Decandia S, Sotgiu MA. Sunlight and Herpes Virus, Human Herpesvirus Infection - Biological Features, Transmission, Symptoms, Diagnosis and Treatment. *IntechOpen.* 2018. Available at: <https://www.intechopen.com/chapters/64844>.
38. Longair MH, Baker DA, Armstrong JD. Simple Neurite Tracer: Open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics.* 2011;27:2453–2454.
39. Arganda-Carreras I, Fernandez-Gonzalez R, Munoz-Barrutia A, Ortiz-De-Solorzano C. 3D reconstruction of histological sections: application to mammary gland tissue. *Microsc Res Tech.* 2010;73:1019–1029.