A Central Role for Sympathetic Nerves in Herpes Stromal Keratitis in Mice

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PURPOSE. Herpes simplex virus type 1 (HSV-1) is a neurotrophic virus that can cause herpes stromal keratitis (HSK), a severe corneal inflammation that can lead to corneal scarring and blindness. This study identified neurologic changes that occur in HSV-1–infected corneas and related them to HSV-1–induced immunopathology.

METHODS. Corneas of BALB/c and C57BL/6 mice were infected with HSV-1 strains that induce HSK. Changes in sensory nerves were identified by immunofluorescence staining of sensory and sympathetic nerves for substance P (SP) and tyrosine hydroxylase (TH), respectively, and confocal microscopic examination. Some mice received superior cervical ganglionectomy (SCGx) to eliminate sympathetic nerves from the cornea.

RESULTS. Normal corneas exclusively expressed sensory nerves that entered the stroma as large nerve stalks, branched to form a plexus at the epithelial/stromal interface, and extended termini into the epithelium. These nerves completely retracted from the infected cornea and were replaced by sympathetic nerves that sprouted extensively to hyperinnervate the corneal stroma but failed to form a plexus or extend termini into the epithelium. The hyper-innervating nerves expressed the sympathetic nerve marker TH and their invasion was blocked by performing SCGx. Moreover, the corneal opacity and neovascularization that normally characterizes HSK in this mouse model were largely abrogated by SCGx. Sensory nerves reinnervated infected corneas following SCGx, reformed a nerve plexus, and extended termini into the epithelium resulting in recovery of corneal sensitivity.

CONCLUSIONS. Sympathetic nerves have a central role in HSK in mice, preventing reinnervation by sensory nerves and promoting severe and persistent corneal inflammation.

Keywords: sympathetic nerves, sensory nerves, herpes stromal keratitis (HSK), herpes simplex virus type 1 (HSV-1), nerve degeneration, cornea, hyperinnervation

Herpes stromal keratitis (HSK) resulting from corneal infection with herpes simplex virus type 1 (HSV-1) causes more cases of monocular blindness than any other infectious eye disease in the United States and other developed countries.[1] HSK in mice is often characterized by a severe and often persistent inflammation involving a mainly neutrophilic infiltration that is largely restricted to the corneal stroma.[2] Previous studies have established that this inflammation is predominantly regulated by CD4+ T cells that produce the T helper (Th)1 cytokines IL-2 and IFN-γ and the Th17 cytokine IL-17. Importantly, the inflammation persists long after HSV-1 is cleared and viral antigens are no longer detectable in the cornea.[3–6] Similarly, human HSK often develops in the absence of detectable virus. After the initial infection of peripheral tissue, the virus invades and establishes a latent infection in sensory nerves of the trigeminal ganglion (TG). The latent HSV-1 can subsequently reactivate forming new virions that traverse the neuronal axons in an anterograde direction to potentially cause recurrent lesions in the innervated tissue. In mice, primary corneal infection also results in establishment of latency in sympathetic nerves of the superior cervical ganglion

(SCG),[7] but a possible involvement of SCG neurons in HSK has not to our knowledge been investigated.

Loss of corneal sensitivity, one of the hallmarks of human HSK,[8,9] is associated with reductions of the sensory nerve plexus at the corneal epithelial/stromal interface[10] and tends to progress in severity with HSK recurrences.[11] It appears that these corneal sensory nerves do not regenerate, leaving the cornea increasingly susceptible to desiccation and neurotrophic damage due, respectively, to loss of blink reflex and a reduced contribution of sensory nerves to normal corneal physiology.

Primary infection of corneas of BALB/c or C57BL/6 (B6) mice with a relatively large dose (1×10^5 plaque forming units [pfu]) of the RE strain of HSV-1 results in severe HSK that can persist for several months.[12] The KOS strain of HSV-1 causes similar disease in BALB/c but not in B6 mice. Furthermore, HSK development in mice coincides with complete loss of corneal sensory nerves and can be partially prevented or reversed by protecting the infected cornea from exposure and desiccation through tarsorrhaphy (stitching the eyelid closed). This observation established a causal relationship between corneal desiccation due to loss of corneal sensory nerves and severity of HSK pathology. The nerve

loss in HSV-1–infected corneas included the nerve endings in the epithelium, the nerve plexus at the epithelial/stromal interface, and the large nerve stalks that enter the corneal stroma at the limbus. A subsequent study established that following loss of sensory nerves the corneal stroma gradually reinnervates, although the cornea remains insensitive to touch.[13] The innervating nerves sprout extensively to hyperinnervate the corneal stroma but do not form the nerve plexus at the epithelial/stromal interface or extend neurites into the epithelium. The nervous and immune systems, the major adaptive systems of the body, are known to cross-regulate each other's functions. Therefore, understanding the immunologic consequences of neuronal changes in HSV-1–infected corneas is critical to understanding the pathogenesis of HSK.

Here we report that (1) the nerve fibers that hyperinnervate the corneal stroma after loss of sensory nerves result from ingrowth and sprouting of sympathetic nerves derived from the SCG; (2) sympathetic hyperinnervation can be inhibited by excising the SCG; and (3) preventing sympathetic hyperinnervation of the corneal stroma dramatically reduces the severity of HSK, permits regeneration of the sensory nerve plexus and epithelial nerve endings, and re-establishes corneal sensitivity.

METHODS

Mice and Virus Infection

Female BALB/c and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and used at 6–8 weeks of age in all experiments. Mice were anesthetized by intraperitoneal injection of 100 mg/kg body weight ketamine hydrochloride and 0.1 mg/ kg body weight xylazine (Phoenix Scientific, San Marcos, CA, USA) in 0.2 mL Hanks' balanced salt solution (BioWhittaker, Walkersville, MD, USA). Topical corneal infection was performed by scarification of the central cornea with a sterile 30-gauge needle in a crisscross pattern and applying 3 μL RPMI (BioWhittaker) containing 1×10^5 pfu of HSV-1. BALB/c mice were infected with the KOS strain of HSV-1, whereas B6 mice were infected with RE HSV-1. These viral/mouse strain combinations resulted in HSK in 80%–100% of mice. The HSV-1 was grown in Vero cells, and intact virions were purified on OptiPrep density gradients (Accurate Chemical and Scientific Corp., Westbury, NY, USA) and stored at −80°C. Concentration of HSV-1 was determined in a standard virus plaque assay. Experimental procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Monitoring HSK Severity and Corneal Blink Reflex

Herpes stromal keratitis severity was monitored by microscopic (Olympus SZX16; Olympus Life Science, Tokyo, Japan) examination of infected corneas on alternate days after HSV-1 corneal infection and scored on a four-point scale based on opacity. Scores were assigned as follows: 0.5, any corneal imperfection; 1, mild corneal haze; 2, moderate opacity; 2.5, moderate opacity with regional dense opacity; 3, diffuse dense opacity obscuring the iris; 3.5, diffuse dense opacity with corneal ulcer; or 4, corneal perforation. Photomicrographs of the corneas were obtained, and opacity scores were independently confirmed. Vessel ingrowth of each cornea was also recorded based on the photomicrographs.

Corneal blink reflex was tested by loosely holding the mouse and touching four quadrants and the central area of the cornea with a surgical forceps with blunted tips, being careful to avoid touching the eyelashes and whiskers as described previously.[14] Loss of blink reflex refers to the inability of mice to blink when any area of the cornea was touched. Recovery of blink reflex refers to the ability to blink when any area of the cornea is touched.

Superior Cervical Ganglionectomy

Complete superior cervical ganglionectomy (SCGx) from mice was accomplished using a standard surgical procedure that is widely used for rat SCGx.[15] The protocol includes methodology to demonstrate complete removal of the SCG. Under ketamine/xylazine anesthesia, a 1-cm incision was made in the shaved and disinfected skin of the ventral neck region. The salivary glands were exposed and retracted to expose the underlying muscles. After sectioning the omohyoid muscles and dissecting the common carotid artery, the SCG was identified behind the carotid bifurcations and then gently pulled until their avulsion. Mock SCGx was performed by exposing but not removing the SCG. Skin incisions were closed with nylon sutures.

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 five times, permeabilized in 1% Triton-X-100 in PBS at room temperature for 60 minutes, and blocked with 20% goat serum (Ceradlan, 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 125-μL cocktail of primary antibodies or in 20% normal rabbit serum (Ceradlan) 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 125-μL cocktail of secondary antibodies and 4′-6-diamidino-2-phenylindole (DAPI, 1:5000; Sigma, St. Louis, MO, USA) 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 included the following: rabbit polyclonal anti-βIII tubulin (1:1000, cat #ab18207) and 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 #556312; BD Bioscience, San Jose, CA, USA). Secondary antibodies included the followoing: Alex Fluor 488 goat anti-rabbit IgG (H+L) (1:500, cat #GR233725-3; Abcam); Alexa Fluor 546 goat anti-chicken IgG (H+L) (1:500, cat #1618409; Life Technologies, Grand Island, NY, USA); Alexa Fluor 633 goat anti-rat IgG (H+L) (1:500, cat #73B1-1; Molecular Probes, Eugene, OR, USA); and DAPI.

Confocal Microscopy and Image Analysis

Z-stacks spanning entire corneal whole mounts were acquired with an inverted Olympus IX81 Fluoview 1000 laser scanning confocal microscope equipped with a ×20 oil (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 were selected from each stitched volume in MetaMorph 7.7.8 (Molecular Devices, LLC, Sunnyvale, California, USA). Five regions (500 × 500 μm) were picked from each cornea: one from the central cornea and the other four regions from four quadrants located 500 μm away from the center (Supplementary Fig. S1). For each cornea, epithelial depth was determined using orthogonal views, and
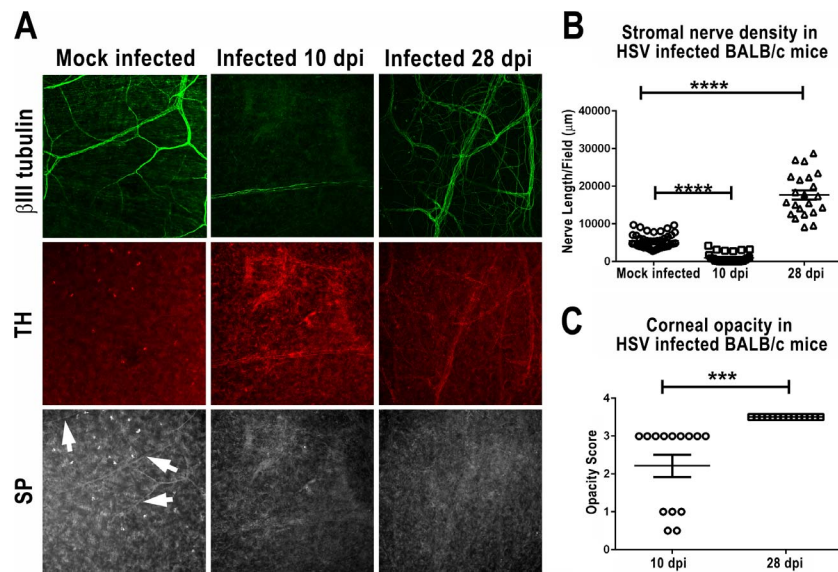
FIGURE 1. BALB/c mice developed sympathetic nerve hyperinnervation associated with severe HSK at 28 dpi. BALB/c mice were mock infected or infected with 1×10^5 pfu of HSV-1 KOS strain. At 10 or 28 dpi, the corneal opacity was recorded, and then the mice were killed, infected corneas were excised and fixed, and whole mounts were stained for the neuronal marker βIII tubulin (*green*), the sympathetic nerve marker TH (*red*), and the sensory nerve marker SP (*gray*). Confocal images were acquired and analyzed as described in Methods. (**A**) Changes in nerve innervation of the stroma at 10 and 28 dpi. The corneal stroma of mock infected mice show a low density of nerves that express SP but not TH. In contrast, corneas obtained at 10 dpi exhibit an almost complete lack of corneal nerves, whereas corneas obtained at 28 dpi show a corneal stroma that is hyperinnervated by nerve fibers that express the sympathetic marker TH but not the sensory marker SP. (**B**) Stromal nerve density measured by cumulative length of nerve fiber at 10 or 28 dpi. (**C**) Corneal opacity in HSV-infected mice recorded prior to death at 10 or 28 dpi. No opacity was observed in mock infected corneas (data not shown). ***$P < 0.001$, ****$P < 0.0001$.

the epithelial layer was removed with FIJI software.[16] The image was then processed using Simple Neurite Tracer[17] in the segmentation package and then analyzed by the 3D Skeletonize[18] FIJI plugin. The total length of nerves in each region was calculated from the data provided. Data are reported as nerve density defined as the total length of nerve fibers within each 500×500-μm region of corneal stroma.

Video Acquisition

Video recordings were obtained using an Olympus SZX16 stereo dissecting microscope and DP80 Monochrome/Color Camera (Olympus Corp.) and CellSens software (Olympus Life Science, Tokyo, Japan). Displayed images were adjusted for brightness and color balance.

Statistical Analysis

All values are presented as mean ± SEM. 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. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Persistent HSK Is Associated With Sympathetic Nerve Hyperinnervation of the Infected Corneal Stroma

Previous studies demonstrated that BALB/c mice develop HSK concurrent with a loss of corneal sensory nerves and corneal sensation[14] after which the corneal stroma becomes hyperinnervated without recovery of corneal sensation.[13] To confirm

these findings and extend them to another mouse/HSV-1 strain combination, BALB/c mouse corneas were infected with HSV-1 KOS or B6 corneas were infected with HSV-1 RE, corneas were excised, and corneal whole mounts were prepared at 10 or 28 dpi. Noninfected corneas showed the expected nerve plexus at the epithelial/stromal interface and nerve trunks in the stroma (based on βIII tubulin expression), with the majority of nerve fibers staining positive for sensory nerve-specific neuropeptide SP, but not for sympathetic nerve-specific enzyme TH (Figs. 1A, 2A). This contrasts sharply with the virtually complete absence of nerves in HSV-1–infected corneas observed at 10 dpi (Figs. 1A, 1B, 2A, 2B). By 28 dpi, βIII tubulin staining of infected corneas demonstrated hyperinnervation that was restricted to the corneal stroma (Figs. 1A, 1B, 2A, 2B). These corneas exhibited a complete loss of corneal sensitivity as demonstrated by failure of the mice to blink when their corneas were touched in any of the four quadrants or in the central cornea with a surgical forceps (Supplementary Videos S1 and S2A). The sprouting nerve fibers in the infected corneal stroma at 28 dpi lacked SP staining, but most expressed TH (Figs. 1A, 2A). Sympathetic hyperinnervation of the corneal stroma between 10 and 28 dpi was associated with development of severe HSK as measured by corneal opacity (Figs. 1C, 2C; Supplementary Fig. S3B) and vessel ingrowth (Supplementary Fig. S3B). These findings demonstrate neurologic changes in HSV-1–infected BALB/c and B6 mouse corneas characterized by an initial loss of sensory nerves and corneal sensation (10 dpi) followed by hyperinnervation of the corneal stroma with sympathetic nerves in association with development of severe HSK (10–28 dpi).

Early SCGx (10–14 dpi) Prevents Corneal Hyperinnervation by TH⁺ Nerve Fibers and Development of Severe Persistent HSK

The soma of sympathetic nerves that innervate the eye are located in the SCG, and SCGx eliminates sympathetic nerve fibers from
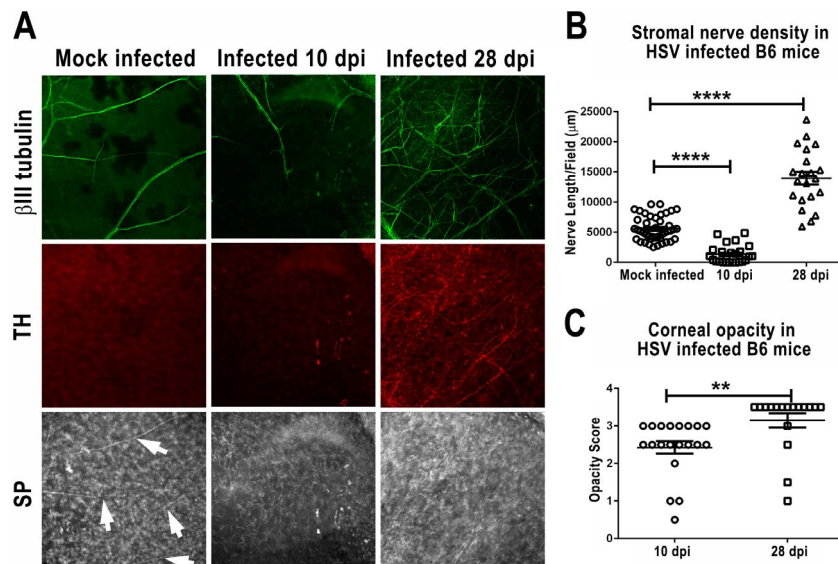
FIGURE 2. B6 mice developed sympathetic nerve hyperinnervation associated with severe HSK at 28 dpi. B6 mice were mock infected or infected with 1×10^5 pfu of the HSV-1 RE strain. At 10 or 28 dpi, the corneal opacity was recorded, and then the mice were killed, infected corneas were excised and fixed, and whole mounts were stained for the neuronal marker βIII tubulin (*green*), the sympathetic nerve marker TH (*red*), and the sensory nerve marker SP (*gray*). Confocal images were acquired and analyzed. (**A**) Changes in nerve innervation of the stroma at 10 and 28 dpi. Mock infected mice show a low density of nerves that express SP, but not TH, whereas corneas obtained at 10 dpi exhibit an almost complete lack of corneal nerves, yet corneas obtained at 28 dpi show a corneal stroma hyperinnervated by nerve fibers that express the sympathetic marker TH but not the sensory marker SP. (**B**) Stromal nerve density measured by cumulative length of nerve fiber at 10 or 28 dpi. (**C**) Corneal opacity in HSV-infected mice recorded prior to death at 10 or 28 dpi. Mock infected corneas remained clear (data not shown). $^{**}P < 0.01$, $^{****}P < 0.0001$.

the eye without affecting corneal sensory nerves.[19] The presence of sympathetic nerves in normal corneas is controversial, with one study reporting nerve fibers within the normal mouse cornea,[20] and another group reported sympathetic nerve presence primarily in the limbal region of the peripheral cornea.[21] Our findings agree with the latter study, showing TH+ nerve fibers only sparsely present in the normal corneal limbus of both BALB/c and B6 mice (data not shown). We proposed that the TH+ nerve fibers that hyperinnervate the corneal stroma following HSV-1 infection derived from the SCG and that hyperinnervation could be prevented by performing SCGx. Accordingly, groups of HSV-1 infected BALB/c and B6 mice received SCGx or mock surgery prior to the onset of HSK and hyperinnervation (10 dpi). At various times after surgery HSK was scored, corneal sensitivity was tested based on corneal blink reflex, and corneal whole mounts were evaluated for sympathetic nerve hyperinnervation and sensory nerve reinnervation.

Corneas of BALB/c and B6 mice that received mock SCGx at 10 dpi developed corneal hyperinnervation with TH+ nerve fibers and severe corneal opacity (Figs. 3, 4; Supplementary Fig. S3B) and corneal vessel ingrowth (Supplementary Fig. S3B), similar to that seen in infected corneas of nontreated mice. In contrast, mice that received SCGx at 10 dpi had nerve densities in the corneal stroma that were similar to those seen in mock-infected corneas (Figs. 5A, 5B), and no TH+ nerve fibers were observed (Figs. 3A, 3B, 4A, 4B) when re-examined at 28 dpi. These corneas did exhibit TH-negative nerves, some of which were beginning to express the sensory neuropeptide SP (Figs. 3A, 4A). Most of the B6 mice and some of the BALB/c mice recovered corneal sensitivity as indicated by blink reflex (Supplementary Videos S2A, S2B). Moreover, performing SCGx at 10 dpi reduced clinical signs of HSK (corneal opacity and vascularization) in both BALB/c and B6 mice at 28 dpi (Figs. 3C, 4C; Supplementary Fig. S3A).

Additional B6 mice received SCGx at 14 dpi and were followed through 54 dpi. At 54 dpi, corneas were excised and corneal nerves evaluated in corneal whole mounts. These corneas lacked sympathetic hyperinnervation of the corneal stroma (Figs. 4A, 4B) and exhibited stromal nerve densities that were not significantly different from those in noninfected corneas (Fig. 5B). Furthermore, corneal opacity was dramatically improved in these mice (Fig. 4C).

Late SCGx (38–54 dpi) Decreases Corneal Hyperinnervation, Abrogates TH Expression, and Significantly Reduces HSK Severity

When SCGx was performed after sympathetic hyperinnervation and opacity were established (38 dpi for B6 mice and 54 dpi for BALB/c mice), the corneal stroma remained hyperinnervated with βIII tubulin–positive nerve fibers up to 56 days after surgery (Figs. 3A, 3B, 4A, 4B, 5A, 5B). However, the nerve fibers no longer expressed TH. Moreover, by 94 dpi, these corneas exhibited SP+ sensory nerves that began to form a nerve plexus at the stroma/epithelial interface (Supplementary Fig. S2). Late SCGx also resulted in a dramatic reduction in opacity and vessel ingrowth compared with the mock SCGx controls (Figs. 3C, 4C; Supplementary Figs. S3C, S3D), even though no recovery of corneal blink reflex was observed (Supplementary Videos S3A, S3B).

Overall, our data demonstrate that sensory nerves almost completely retract from HSV-1–infected corneas of both BALB/c and B6 mice around 10 days of infection, that the corneal stroma then becomes hyperinnervated by sympathetic nerves derived from the SCG that fail to form a nerve plexus or extend termini into the epithelium, and that sympathetic nerve hyperinnervation of the corneal stroma is closely associated with failure of sensory nerve reinnervation and with severe HSK.

DISCUSSION

Our findings document significant neurologic changes in mouse corneas that develop HSK. Normal corneas are heavily
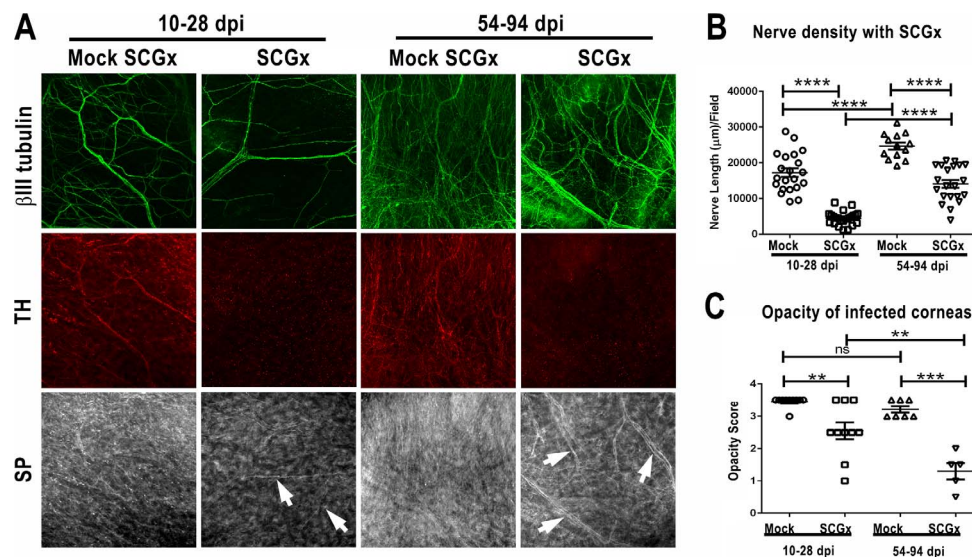
FIGURE 3. Superior cervical ganglionectomy eliminates sympathetic nerves, allows regrowth of sensory nerves, and prevents development of severe persistent HSK in BALB/c mice. BALB/c mice were infected with 1×10^5 pfu of the HSV-1 KOS strain. Groups of infected mice received mock SCGx or SCGx at 10 dpi and were followed to 28 dpi, whereas other groups received mock SCGx or SCGx at 54 dpi and were followed to 94 dpi. Opacity scores were recorded on the day mice were killed. Whole mounted corneas were stained for βIII tubulin (*green*), TH (*red*), and SP (*gray*). (**A**) Superior cervical ganglionectomy administered at 10 dpi effectively eliminated the hyperinnervation at 28 dpi and permitted regrowth of a low density of SP-positive sensory nerves. Administering SCGx after hyperinnervation when sympathetic nerves had established (54 dpi) did not eliminate the hyperinnervating fibers, but the nerves did not express the sympathetic nerve marker TH. Note the hyperinnervation of the corneal stroma of mice that received mock SCGx at 10 or 54 dpi, with nerve fibers that expressed the sympathetic nerve marker TH but not the sensory nerve marker SP. (**B**) Nerve density in corneal stromas of mock or SCGx-treated mice at 28 and 94 dpi was quantitated as the cumulative length of nerve fibers in 500×500-μm areas of the corneal stroma. (**C**) Opacity of infected corneas was recorded just prior to death at 28 and 94 dpi. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

innervated by nerve fibers that enter the stroma at the limbus, branch to form a swirling plexus at the epithelial/stromal interface, and extend termini that interdigitate among epithelial cells.[22] Most of these nerves express the neuropeptide SP, identifying them as a subpopulation of sensory neurons. Our previous study demonstrated that the severe and persistent inflammation in corneas with HSK develops when corneal sensory nerves retract and can be reduced by protecting the cornea from desiccation by tarsorrhaphy.[14] This suggested that most of the inflammation associated with HSK in mice is directly or indirectly caused by the loss of corneal sensation and blink reflex, leading to corneal desiccation. However, our current findings reveal a more complex mechanism.

Following loss of corneal sensory nerves (10 dpi), TH-positive sympathetic nerve fibers invade the cornea and undergo massive sprouting to hyperinnervate the corneal stroma. Unlike the sensory nerves, these nerves do not form a plexus at the epithelial/stromal interface and do not extend termini into the corneal epithelium, suggesting that the source of the neurotrophic factor(s) that attracts sympathetic nerve axon extension and sprouting is focused in the corneal stroma. The sympathetic nerves that hyperinnervate the corneal stroma are SCG derived, and their invasion of the corneal stroma is prevented by performing SCGx at 10 dpi. These corneas develop a transient intermediate severity of HSK that largely resolves by 28 dpi. Recovery from HSK is accompanied by regrowth of sensory nerves into the cornea which form a nerve plexus at the epithelial/stromal interface and extend nerve endings into the corneal epithelium. Reduced HSK severity occurred concurrently with recovery of corneal blink reflex. Taken together with our previous observation that tarsorrhaphy can prevent or resolve severe inflammation in infected corneas that have lost corneal blink reflex, these findings suggest an important contribution for corneal

desiccation to HSK severity. However, HSK also resolved in infected corneas of mice that received SCGx but had not yet recovered observable corneal blink reflex at 28 dpi. The latter observation suggested that corneal desiccation might be necessary, but not sufficient for development of severe HSK.

An interesting observation is that hyperinnervation is reduced, but not eliminated, when SCGx is performed after sympathetic hyperinnervation of the corneal stroma is established. These hyperinnervating nerve fibers lack detectable TH expression. It is possible that the original hyperinnervating SCG-derived sympathetic nerve fibers can persist in the corneal stroma for at least 40 days after SCGx is performed but do not maintain TH expression. Alternatively, TH- and SP-negative nerves of unknown origin might hyperinnervate the corneal stroma following elimination of hyperinnervating sympathetic nerves by SCGx.

We do observe innervation by some SP-positive sensory nerves following both early and late SCGx, but these nerve fibers do not show the extensive sprouting pattern of sympathetic nerves and instead form a plexus and extend termini into the epithelium. Thus, SCG-derived sympathetic nerves and sensory nerves exhibit a markedly different pattern of innervation in the cornea, suggesting that sensory nerves may respond to different neurotrophic factors or guidance molecules, or that the source and perhaps location of these factors is altered by SCGx. Although partial reinnervation by sensory nerves occurred following both early and late SCGx, we note that corneal sensitivity as assessed by blink reflex was recovered within 18 days of early SCGx, but failed to recover more than 50 days after late SCGx. It is unclear if the hyperinnervating nerve fibers that remained in the corneal stroma following late SCGx influenced the function of the reinnervating sensory nerves or if the nerves and muscles that control eyelid blinking continue to be compromised following
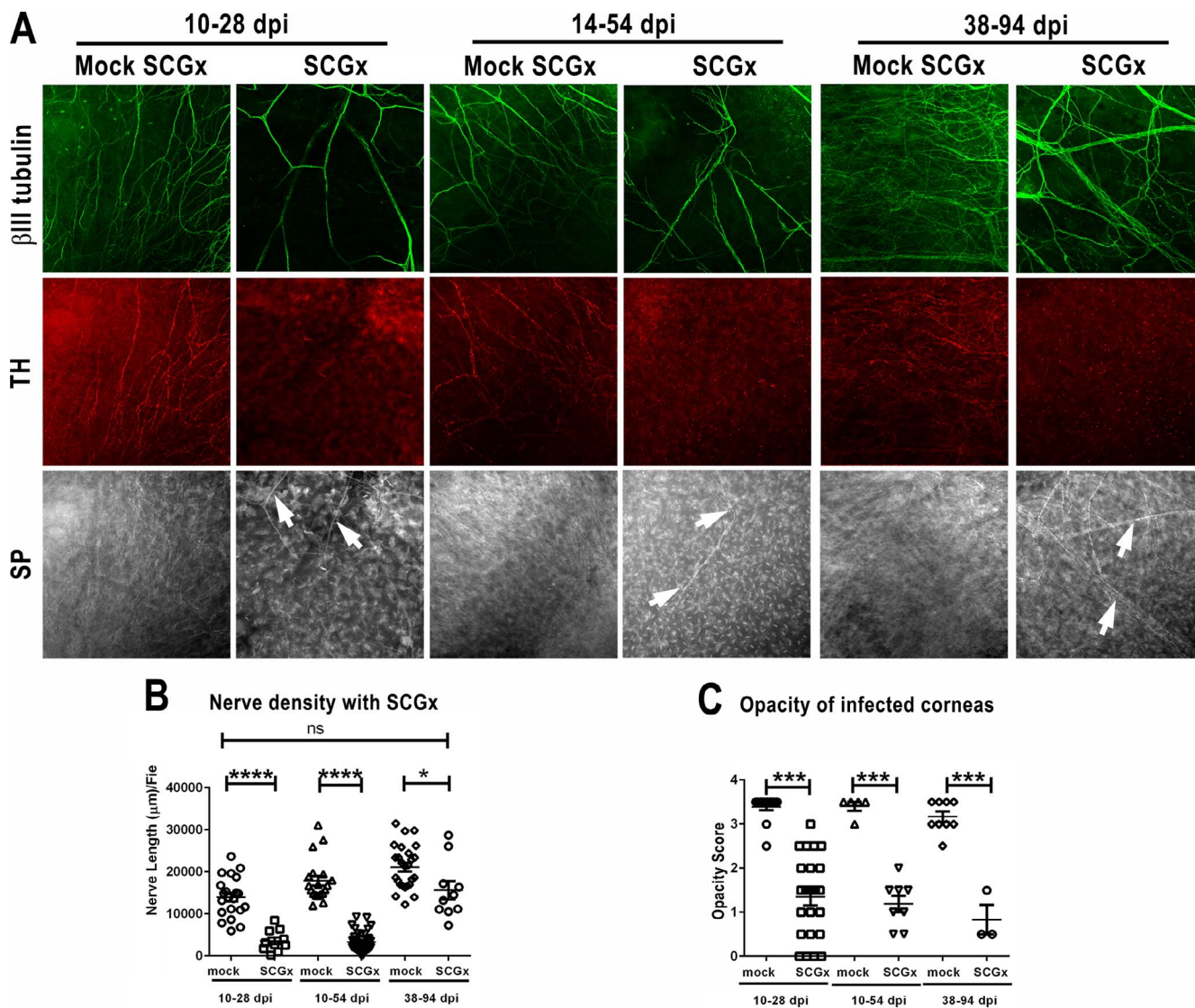
FIGURE 4. Superior cervical ganglionectomy eliminates sympathetic nerves, allows regrowth of sensory nerves, and prevents development of severe persistent HSK in B6 mice. B6 mice were infected with 1×10^5 pfu of the HSV-1 RE strain. Groups of infected mice received mock SCGx or SCGx at 10, 14, or 38 dpi and were followed to 28, 54, and 94 dpi, respectively. Opacity scores were recorded on the day the mice was killed. Corneas were excised and fixed, and whole mounts were stained for βIII tubulin (*green*), TH (*red*), and SP (*gray*). Confocal images were acquired and analyzed as described in the Methods. (**A**) Superior cervical ganglionectomy before hyperinnervation was established (10 and 14 dpi) effectively eliminated the hyperinnervation with TH-positive sympathetic nerve fibers and permitted regrowth of a low density of SP-positive sensory nerves. Performing SCGx after hyperinnervation with sympathetic nerves was established (38 dpi) did not eliminate the hyperinnervating fibers, but the nerves failed to express the sympathetic nerve marker TH. Note the hyperinnervation of the corneal stroma of mice that received mock SCGx at 10, 14, and 54 dpi, with nerve fibers that expressed the sympathetic nerve marker TH but not the sensory nerve marker SP. (**B**) Nerve density in corneal stromas of mock or SCGx treated mice at 28, 54, and 94 dpi quantitated as the cumulative length of nerve fibers in 500×500-µm areas of the corneal stroma. (**C**) Opacity of infected corneas was recorded just prior to death at 28, 54, and 94 dpi. **$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

late SCGx. Further studies will be required to distinguish these possibilities.

Although late SCGx did not result in recovery of corneal blink reflex, it did result in a dramatic reduction in the severity of HSK, suggesting that the late phase of HSK can significantly resolve even in the face of continued corneal exposure and desiccation. We propose that the early HSK development results primarily from loss of sensory nerves and corneal desiccation, because it develops before sympathetic nerves hyperinnervate the corneal stroma. Corneal exposure and desiccation also contribute to the severe, persistent inflammation that develops during the later stages of HSK in mice (15–

>30 dpi) because tarsorrhaphy prevents and resolves this phase of HSK as well. However, our current findings demonstrate that the persistent, severe inflammation that characterizes the later stages of HSK in mice also requires a function of SCG-derived sympathetic nerve fibers that hyperinnervate the stroma of infected corneas.

Given that the focus of both HSK-associated inflammation and sympathetic hyperinnervation is in the corneal stroma, we propose that the sympathetic nerve fibers that hyperinnervate the corneal stroma regulate the severe and persistent inflammation that characterizes HSK in our mouse model. The specific action of the sympathetic nerve fibers that
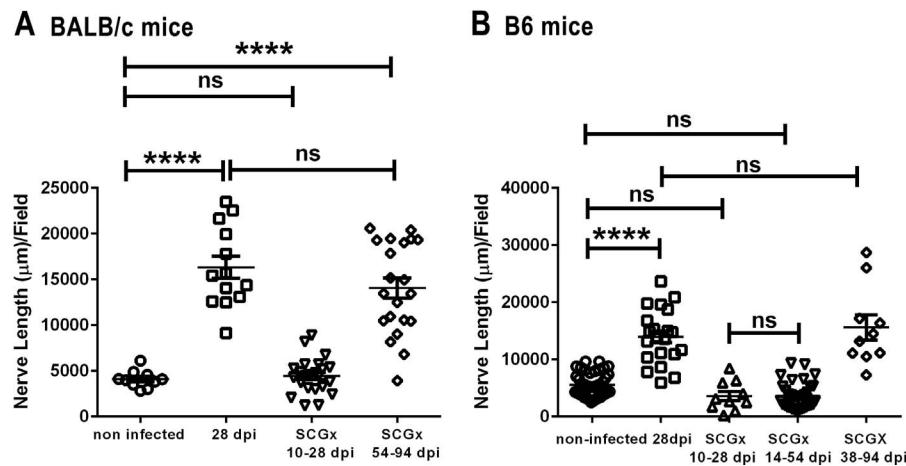
A BALB/c mice

B B6 mice

FIGURE 5. Early SCGx allows regeneration of normal density sensory nerves in BALB/c and B6 mice with HSK. (**A**) BALB/c and (**B**) B6 mice were mock infected or infected with 1×10^5 pfu of the HSV-1 KOS or RE strain, respectively. BALB/c mice received mock SCGx at 10 dpi, and corneas were excised at 28 dpi, or mice received SCGx at 10 or 54 dpi and corneas were excised at 28 and 94 dpi, respectively. B6 mice received mock SCGX at 10 dpi, and corneas were excised at 28 dpi, or received SCGx at 10, 14, or 38 dpi and corneas were excised at 28, 54, and 94 dpi, respectively. Corneal whole mounts were stained for βIII tubulin. Nerve density in various areas of the corneal stroma was imaged with confocal microscopy and quantified with FIJI. ****$P < 0.0001$.

maintains inflammation remains to be identified, but we note that resolution of HSK correlates with loss of TH staining in the hyperinnervating nerve fibers following late SCGx. The TH enzyme is the rate-limiting step in the production of the catecholamines epinephrine and norepinephrine,[23] suggesting a possible role for these catecholamines in promoting inflammation in the cornea. Although norepinephrine is often associated with inhibition of inflammation, several recent studies demonstrate that it can also promote inflammation, particularly inflammation involving neutrophilic infiltration of tissue as is seen in HSK in mice.[24] Although ingrowth of sympathetic nerves in human corneas with HSK and in mouse corneas with recurrent HSK is currently under investigation, our findings are consistent with an important general role for sympathetic nerves in regulating inflammation in corneas with HSK.

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