Overexpression of Herpes Simplex Virus Glycoprotein K (gK) Alters Expression of HSV Receptors in Ocularly-Infected Mice

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Purpose. We have shown previously that HSV-1 glycoprotein K (gK) exacerbates corneal scarring (CS) in mice and rabbits. Here, we investigated the relative impact of gK overexpression on host responses during primary corneal infection and latency in trigeminal ganglia (TG) of infected mice.

Methods. Mice were infected ocularly with HSV-gK^3 (expressing two extra copies of gK replacing latency associated transcript [LAT]), HSV-gK^3 revertant (HSV-gK^{3}R), or wild-type HSV-1 strain McKrae. Individual corneas on day 5 post infection (PI) and TG on day 28 PI were isolated and used for detection of gB DNA in the TG, HSV-1 receptors in the cornea and TG, and inflammatory infiltrates in TG.

Results. During primary HSV-1 infection, gK overexpression resulted in altered expression of herpesvirus entry mediator (HVEM), 3-O-sulfated heparin sulfate (3-OS-HS), paired immunoglobulin-like type 2 receptor-α (PILR-α), nectin-1, and nectin-2 in cornea of BALB/c, but not C57BL/6 mice. However, gK overexpression did have an effect on 3-OS-HS, PILR-α, nectin-1, and nectin-2 expression (but not HVEM expression) in TG of C57BL/6 mice during latency. These differences did not affect the level of latency, but instead were correlated with the presence of CS. The presence of LAT increased HVEM expression and this effect was enhanced further by the presence of CS in latently-infected mice. Finally, the presence of LAT, but not overexpression of gK, affected CD4, CD8, TNF-α, Tim-3, PD-1, IL-21, IL-2, and IFN-γ expression in TG.

Conclusions. We demonstrate a novel link between gK exacerbation of CS and HSV-1 receptors, suggesting a gK-induced molecular route for the pathogenesis as well as selective advantage of these entry routes for the pathogen during latency-reactivation cycle.

Keywords: virus replication, latency, corneal scarring, exhaustion, cytokines, T cells

Glycoprotein K (gK) is one of more than 12 known HSV-1 glycoproteins.[1–7] Studies using insertion/deletion mutants have shown the importance of gK in virion morphogenesis and egress.[8–11] Likewise, deletion of gK results in the formation of extremely rare microscopic plaques, indicating that gK is required for virus replication,[8,9] a concept that is supported by the observation that gK-deficient virus can be propagated only on complementing cells that express gK.[8,9] We demonstrated previously that immunization of mice with gK, but not with any of the other HSV-1 glycoproteins, resulted in exacerbation of corneal scarring (CS) and herpetic dermatitis following ocular HSV-1 infection.[4,6] We also showed that transfer of whole serum or purified IgG from gK-immunized mice to naive mice resulted in the same severe exacerbation of CS following ocular HSV-1 infection as seen in gK-immunized mice.[12] More recently, we have shown that depletion of CD8$^+$ T cells in gK-immunized mice reduced exacerbation of gK-induced CS in ocularly-infected mice,[13] and have now shown that these CD8$^+$ T cells are CD8$^+$CD25$^+$.[14]

It has been shown that overexpression of gK in gK-transformed cells collapses the Golgi apparatus into the ER; thus, inhibiting virion egress, glycoprotein transport, and virus-induced cell fusion.[15] Similarly, recently we have shown physiological signs of ER stress, such as ER aggregation, in cell lines overexpressing gK.[16] The implications of ER stress are well documented in human diseases, such as diabetes mellitus atherosclerosis, hypoxia, neoplasia, neurodegeneration, and retinal degeneration.[17–19] We also have shown that gK binds to signal peptide peptidase (SPP), and this binding is required for HSV-1 infectivity in vitro and in vivo.[16] Blocking of this interaction by SPP shRNA reduced ER stress on infected cells.[16]

We had analyzed previously the contribution of gK to CS using a recombinant HSV-1 (rather than deleting the essential *gK* gene) with two extra copies of *gK*, and found that similar to gK immunization, this recombinant virus caused elevated levels of CS in mice and rabbits.[20] We also determined that the pathogenic region of gK is located within the signal sequence of gK.[21,22] Similarly, it was shown that the amino terminus of gK is essential for neuroinvasiveness and acute herpes keratitis in the mouse eye model.[11] In addition, we have shown that sera from HSK individuals had higher anti-gD and -gK antibody titers than sera from seropositive individuals with no history of HSK despite having similar levels of neutralizing antibody titers and HSV-1 IgG.[23] Moreover, recently it was shown that the population size, specificity, and function of HSV-specific CD8$^+$

T cells epitopes from asymptomatic versus symptomatic patients were different and, while asymptomatic epitopes protected HLA-A*02:01 transgenic against ocular HSV-1 infection, symptomatic epitopes did not protect immunized mice.[24,25] Overall, our published studies suggest that HSV-1 gK has a major role in viral immunopathogenesis.

Although gK is not involved in the processes of virus attachment or penetration, it is involved in virus replication and we have shown that these functions are dependent on SPP.[16] Previously, we have shown a strong correlation between severity of eye disease, the load of latent virus in the trigeminal ganglia (TG), and the cellular infiltrates in cornea and TG of BALB/c mice ocularly-infected with wild-type (wt) HSV-1 strain McKrae.[26] However, very little is known if viral entry receptors are regulated by gK overexpression. Therefore, in this study, we sought to determine if gK overexpression is associated with: increase of HSV-1 receptor expression in the corneas of susceptible (BALB/c) and refractory (C57BL/6) to infection strains of mouse during primary infection and in the TG during latent infection, the load of latent virus in mice TG with and without CS, and changes in the levels of various immune-related mRNAs in TG during latency. Our results suggested a strong correlation between gK overexpression and 3-O-sulfated heparin sulfate (3-OS-HS), paired immunoglobulin-like type 2 receptor-α (PILR-α), nectin-1, and nectin-2 expression levels, but not herpesvirus entry mediator (HVEM) expression level, in TG of latently-infected C57BL/6 mice.

Materials and Methods

Virus, Cells, and Mice

Plaque purified wt McKrae (wild-type, latently associated transcript [LAT]-plus), HSV-gK^3 (LAT-minus), and HSV-gK^{3}R (LAT-minus) viruses were used in this study. The HSV-gK^3 was derived from dLAT2903 in which two copies of the open reading frame (ORF) encoding HSV-1 glycoprotein K (gK) was inserted into the LAT regions of dLAT2903, resulting in the virus containing 3 copies of gK.[20] The HSV-gK^{3}R is a rescued virus, in which the inserted *gK* gene was removed and the original deletion of the *LAT* gene was restored. This virus behaves similarly to dLAT2903.[20,27] Rabbit skin (RS) cells (used for the preparation of virus stocks and the culturing of mouse tear films) were grown in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal calf serum (FCS). Female BALB/c and C57BL/6 mice 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animal procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and according to institutional animal care and use guidelines.

Ocular Infection and Eye Swab

Mice were infected ocularly with 2 μL of tissue culture media containing 2×10^5 plaque-forming units (PFU)/eye of the HSV-1 strains McKrae, HSV-gK^{3}R, or HSV-gK^3 without corneal scarification. Tear films were collected from 10 eyes on day 5 post infection (PI) for each group as described previously.[6] Each swab was placed in 1 mL of tissue culture media, and the amount of virus in the media was determined using a standard plaque assay on RS cells.

Detection of HSV-1 gB in Cornea and TG of Infected Mice

On day 5 PI, corneas of ocularly-infected mice were excised and total RNA was isolated as we described previously.[14] All isolated corneas used for RT-PCR described below were free of contamination from other parts of the mouse eye, vitreous fluid, and tears. On day 5 PI, corneal cells were infected with HSV-1,[28,29] and they showed some signs of geographical ulcer as we described previously.[6] Similarly, on day 28 PI, TG from infected mice were harvested for RNA and DNA extraction. RNA or DNA was isolated from homogenized individual cornea or TG using the commercially available Kit (Qiagen, Stanford, CA, USA) according to the manufacturer's instructions. The PCR or RT-PCR analyses were done using gB specific primers (forward, 5′-AACGCGACGCACATCAAG-3′; reverse, 5′-CTGGTACGCGATCAGAAAGC-3′; and probe, 5′-FAM-CAGCCG CAGTACTACC-3′). The amplicon length for this primer set is 72 base pairs (bp). Relative copy numbers for gB DNA were calculated using standard curves generated from the plasmid pAc-gB1.

Gene Expression Analyses

Cornea and TG from ocularly-infected mice were collected on specified date PI and immersed in RNAlater RNA stabilization reagent, and stored at –80°C until processing as we describe previously.[27,30] The expression levels of the various transcripts (nectin-1, nectin-2, HVEM, PILR-α, 3-OS-HS, CD4, CD8, IL-2, IFN-γ, TNF-α, IL-21, Tim-3, and PD-1) were evaluated using commercially available TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) with optimized primer and probe concentrations. Primer probe sets consisted of two unlabeled PCR primers and the FAM dye-labeled TaqMan MGB probe formulated into a single mixture. Additionally, all cellular amplicons included an intron–exon junction to eliminate signal from genomic DNA contamination. The assays used in this study were as follows: (1) HVEM, Mm00619239_m1 – amplicon length = 65 bp; (2) Nectin-1, ABI Mm00445392_m1 – amplicon length = 71 bp; (3) Nectin-2, ABI Mm00436144_m1 – amplicon length = 65 bp; (4) PILR-α, ABI Mm00463324_m1 – amplicon length = 77 bp; (5) heparin sulfate-3-O-sulfotransferase, ABI Mm00479621_m1 – amplicon length = 65 bp; (6) CD4, ABI assay ID Mm00442754_m1 – amplicon length = 72 bp; (7) CD8 (α chain), ABI assay ID Mn01182108_m1 – amplicon length = 67 bp; (8) IL-2, ABI assay ID Mm00434256_m1 – amplicon length = 82 bp; (9) IFN-γ, ABI assay ID Mm00801778_m1 – amplicon length = 101 bp; (10) TNF-α, ABI Mm00443258_m1 – amplicon length = 81 bp; (11) IL-21, ABI Mm00517640_m1 – amplicon length = 67 bp; (12) PD-1 (programmed death 1; also known as CD279), ABI Mm00435532_m1 – amplicon length = 65 bp; and (13) GAPDH, ABI assay ID Mm999999.15_G1 – amplicon length = 107 bp. Additionally, custom-made primers and probe set were used for LAT as follows: forward primer, 5′-GGGTGGGCTCGTGTTACAG-3′; reverse primer, 5′-GGACGGG TAAGTAACAGAGTCTCTA-3′; and probe, 5′-FAM-ACAC CAGCCCGTTCTTT-3′– amplicon length = 81 bp. In all experiments GAPDH was used for normalization of transcripts and HSV-1 infection had no effect on levels of GAPDH mRNA as determined by RT-PCR.

The qRT-PCR was performed using an ABI ViiA7 sequence detection system (Applied Biosystems) in 384-well plates. The threshold cycle (CT) values, which represent the PCR cycles at which there is a noticeable increase in the reporter fluorescence above baseline, were determined using ViiA7 RUO software (Applied Biosystems).

Statistical Analysis

Fisher's exact tests were performed using the computer program Instat (GraphPad, San Diego, CA, USA). Results were considered statistically significant when the P value was <0.05.

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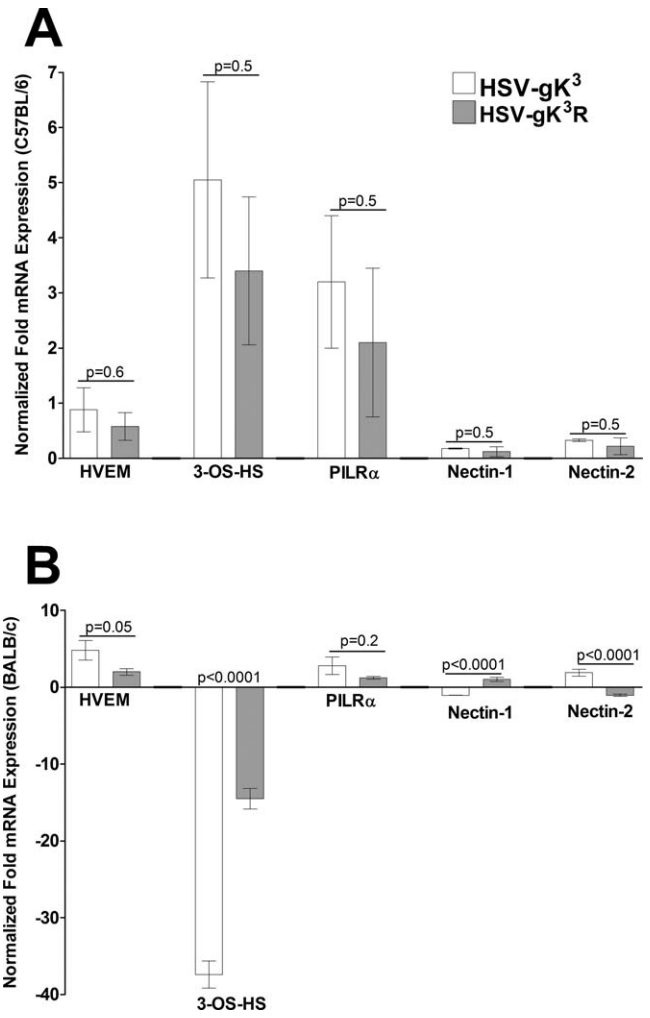

Figure 1. Effect of gK overexpression on HSV-1 receptors in cornea during primary infection. The C57BL/6 or BALB/c mice were ocularly-infected with HSV-gK^3 or HSV-gK^{3}R, and the corneas from infected mice were isolated individually on day 5 PI and quantitative RT-PCR was performed using total RNA. The HVEM, 3-OS-HS, PILR-α, nectin-1, and nectin-2 expression in naive mice was used to estimate the relative expression of each transcript in cornea of infected mice. The GAPDH expression was used to normalize the relative expression of each transcript in TG of latently-infected mice. Each *status bar* represents the mean ± SEM from 10 corneas. (**A**) C57BL/6 mice and (**B**) BALB/c mice.

Results

Effect of Overexpression of gK on HSV-1 Receptors in Cornea of Infected Mice

Previously, we have shown that gK overexpression exacerbates corneal disease in ocularly-infected mice.[20] The gK is thought to be an important determinant of virus-induced cell fusion, since single amino acid changes within gK cause extensive virus-induced cell fusion.[31–34] Also, gK is an important determinant of cytoplasmic virion envelopment, since viruses lacking gK fail to acquire efficiently a cytoplasmic envelope, which leads to a drastic defect in virion egress and spread.[8–10] The membrane-fusing activity of HSV depends in part on the binding of HSV-1 to one of its many major receptors, such as HVEM, 3-OS-HS, PILRα, nectin-1, or nectin-2.[2,35–39] To investigate the role of these receptors in gK-induced eye disease, we examined mRNA levels of HSV-1 receptors in C57BL/6 mice infected with HSV-gK^3 or HSV-gK^{3}R. Quantitative RT-PCR analysis of mRNA levels in cornea at 5 days PI showed no significant differences in the corneal expression levels of HSV receptors between HSV-gK^3 and HSV-gK^{3}R–infected mice (Fig. 1A, $P > 0.05$). However, 3-OS-HS and PILR-α expression levels showed more enhancement than that of HVEM, nectin-1, or nectin-2 in HSV-gK^3– and HSV-gK^{3}R–infected mice. The above results suggested that overexpression of gK had no significant effect on expression of HSV-1 receptors in cornea of C57BL/6 mice during primary HSV-1 infection.

In contrast to C57BL/6 mice that are refractory to the viruses that we used in this study, BALB/c mice are highly susceptible to the above viruses. Therefore, we subsequently looked at the effect of gK overexpression on the levels of HSV-1 receptor transcripts in BALB/c mice. Mice were ocularly infected as above and RT-PCR was performed on RNA isolated from the cornea on day 5 PI. Significant differences were detected in the expression levels of all HSV-1 receptors in mice infected with HSV-gK^3 compared to mice infected with HSV-gK^{3}R (Fig. 1B). The HVEM and PILR-α expressions were higher in HSV-gK^3–infected mice compared to HSV-gK^{3}R infected mice but the differences were not statistically significant (Fig. 1B). In contrast, 3-OS-HS expression was significantly suppressed in groups, however this reduction was significantly higher in cornea of HSV-gK^3 infected mice than in HSV-gK^{3}R infected mice (Fig. 1B). Similarly the level of nectin-1 was also decreased in the HSV-gK^3 infected mice compared to HSV-gK^{3}R, while the pattern was reversed for nectin-2 (Fig. 1B). This result suggested that the pattern of some of the receptor expression was affected by gK overexpression in BALB/c mice, but not in C57BL/6 mice.

Virus Replication in Eyes of Infected Mice

To determine if the differences in the expression patterns of HSV-1 receptors in BALB/c mice are due to gK overexpression or whether they are associated with differences in virus replication between HSV-gK^3 and HSV-gK^{3}R viruses, tear films were collected from the above ocularly-infected mice before isolating corneas for RT-PCR and the amount of virus in each eye was determined by standard plaque assay. The BALB/c mice infected with HSV-gK^3 had a lower viral titer than mice infected with HSV-gK^{3}R (Fig. 2, $P < 0.05$, BALB/c), while the pattern of virus titers in C57BL/6 mice infected with HSV-gK^3 or HSV-gK^{3}R virus was the same (Fig. 2, $P > 0.05$, C57BL/6). Thus, gK overexpression did not affect HSV-1 replication in C57BL/6 mice, but it reduced viral titer in eyes of BLAB/c-infected mice. Consequently, the differences between HSV-gK^3 and HSV-gK^{3}R in BALB/c mice may be due to the differences in virus replication in the eye and possibly the cornea of infected mice.

Lack of Correlation Between Severity of CS and Latency in TG of Latently-Infected Mice

Next, we sought to determine whether gK overexpression in the presence or absence of CS was correlated with a higher load of latent virus in the TG as judged by gB expression levels. Since BALB/c mice are highly susceptible to ocular infection with 2×10^5 PFU/eye of viruses that we used in this study, we instead used C57BL/6 mice that survived the challenge dose of virus, but still developed CS in approximately 40% of infected mice. To obtain an adequate number of mice with no CS or CS in both eyes, 20 mice from two separate experiments were infected ocularly with 2×10^5 PFU/eye of HSV-gK^3, HSV-gK^{3}R, or wt HSV-1 strain McKrae. On day 28 PI, the eyes of surviving mice were evaluated for presence or absence of CS. Based on

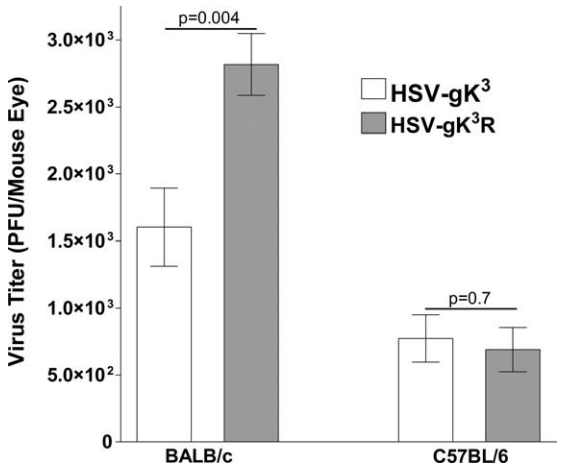

Figure 2. Virus replication in eye of infected mice. Tear films were collected from mice infected in Figure 1 above on day 5 PI and before euthanasia. The virus titers in the eye swabs were determined by standard plaque assays. Each *status bar* represents the average of the titers from 10 eye swabs ± SEM.

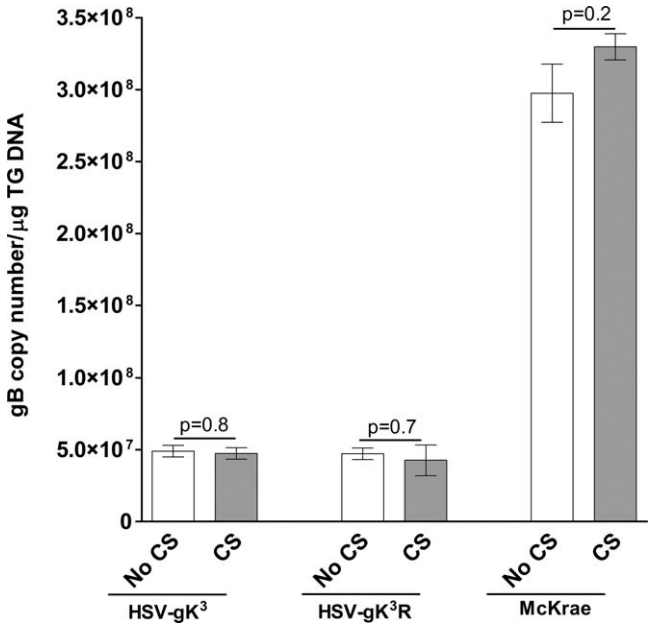

Figure 3. Effect of gK overexpression on HSV-1 latency in TG of latently-infected mice. The C57BL/6 mice were ocularly-infected with HSV-gK^3, HSV-gK^{3}R, or wt HSV-1 strain McKrae. On day 28 PI the surviving mice were evaluated for presence or absence of CS. Based on CS, the infected mice were separated into two groups: those with no CS and those with CS in eyes (mice with CS in one eye were excluded from the study). Quantitative PCR was performed on individual mouse TG harvested from the two groups for HSV-1 gB DNA. In each experiment, an estimated relative copy number of gB was calculated using standard curves generated from pGem-gB1. Briefly, DNA template was serially diluted 10-fold, such that 5 μL contained from 10^3 to 10^{11} copies of gB, then subjected to TaqMan PCR with the same set of primers. By comparing the normalized threshold cycle of each sample to the threshold cycle of the standard, the copy number for each reaction was determined. The GAPDH expression was used to normalize the relative expression of gB DNA in the TG. Each *point* represents the mean ± SEM from 10 TG.

CS, we grouped mice into mice with no CS in both eyes and mice with CS in both eyes (mice with CS in one eye were excluded from the study). After separation of mice into the above two groups on day 28, TG from latently-infected mice were isolated and the level of gB DNA was determined by TaqMan PCR performed using total DNA isolated from each TG. The amount of gB DNA detected in the TG of mice infected with each virus with no CS was similar to the amount detected in the TG of mice that had CS in both eyes (Fig. 3, $P > 0.05$). As expected, the level of latency was higher in mice infected with wt McKrae (LAT-plus) versus mice infected with HSV-gK^3 (LAT-minus) or HSV-gK^{3}R (LAT-minus, Fig. 3). This result showed no correlation between the severity of CS and the level of gB DNA in TG of latently-infected C57BL/6 mice and is in contrast to the correlation observed in BALB/c mice infected with McKrae.[26]

Effect of gK Overexpression on HSV-1 Receptors During Latency

A major cause of CS is the scarring induced by HSV-1 following reactivation from latency[40,41] as CS is more likely to occur following recurrent, rather than primary infection.[42–44] Although our results described in Figure 1 above suggested that gK overexpression had no effect on HSV-1 receptors in C57BL/6 mice during primary HSV-1 infection, this does not rule out their potential involvement during latent infection. Consequently, to determine if gK overexpression affects the level of mRNA encoded by these HSV-1 receptors during latency, C57BL/6 mice were infected ocularly with 2×10^5 PFU/eye of HSV-gK^3, WT HSV-1 strain McKrae, or HSV-gK^{3}R as above. On day 28 PI, infected mice were divided into two groups: mice with no CS in both eyes; and mice with CS in both eyes (mice with CS in one eye were again excluded from the study). Total RNA then was isolated from individual TG and analyzed by RT-PCR as described in Materials and Methods. The level of HVEM mRNA in TG of HSV-gK^3– and HSV-gK^{3}R–infected mice with and without CS decreased, and this decline in HVEM expression was significantly higher in mice showing CS than in mice with no CS (Fig. 4A, $P < 0.0001$). In contrast, HVEM expression in McKrae-infected mice was elevated compared to HSV-gK^3 and HSV-gK^{3}R viruses, and this increase was significantly higher in mice showing CS than in mice with no CS (Fig. 4A, $P < 0.05$, McKrae).

Expression of 3-OS-HS (Fig. 4B, $P < 0.05$) and nectin-1 (Fig. 4D, $P < 0.05$) was significantly different between TG from mice with and without CS in only the HSV-gK^3 group, while the expression of 3-OS-HS was the same in the CS and no CS mice infected with either HSV-gK^{3}R or McKrae. Furthermore, the levels of 3-OS-HS (Fig. 4B, $P < 0.05$) and nectin-1 (Fig. 4D, $P < 0.05$) were significantly higher in mice infected with one copy of gK (HSV-gK^{3}R or McKrae) than mice infected with the HSV-gK^3 virus. Expressions of PILRα (Fig. 4C, $P < 0.05$) and nectin-2 (Fig. 4E, $P < 0.05$) were significantly suppressed in the presence of additional copies of gK irrespective of scarring when compared to HSV-gK^{3}R and McKrae-infected mice (Figs. 4C, 4E). Finally, the levels of PILRα and nectin-2 expressions were the same in HSV-gK^{3}R and McKrae-infected mice with or without CS (Figs. 4C, 4E). Overall, gK overexpression and the presence of CS appeared to alter receptor mRNA levels in TG of latently-infected mice with the trend appearing to be that the addition of extra copies of gK leads to decreased expression of all receptors, except HVEM, which appears to be regulated by the presence of LAT and not the amount of gK. These differences in receptor expression prompted us to investigate if there would be differences in inflammatory responses in TG of latently-infected mice.

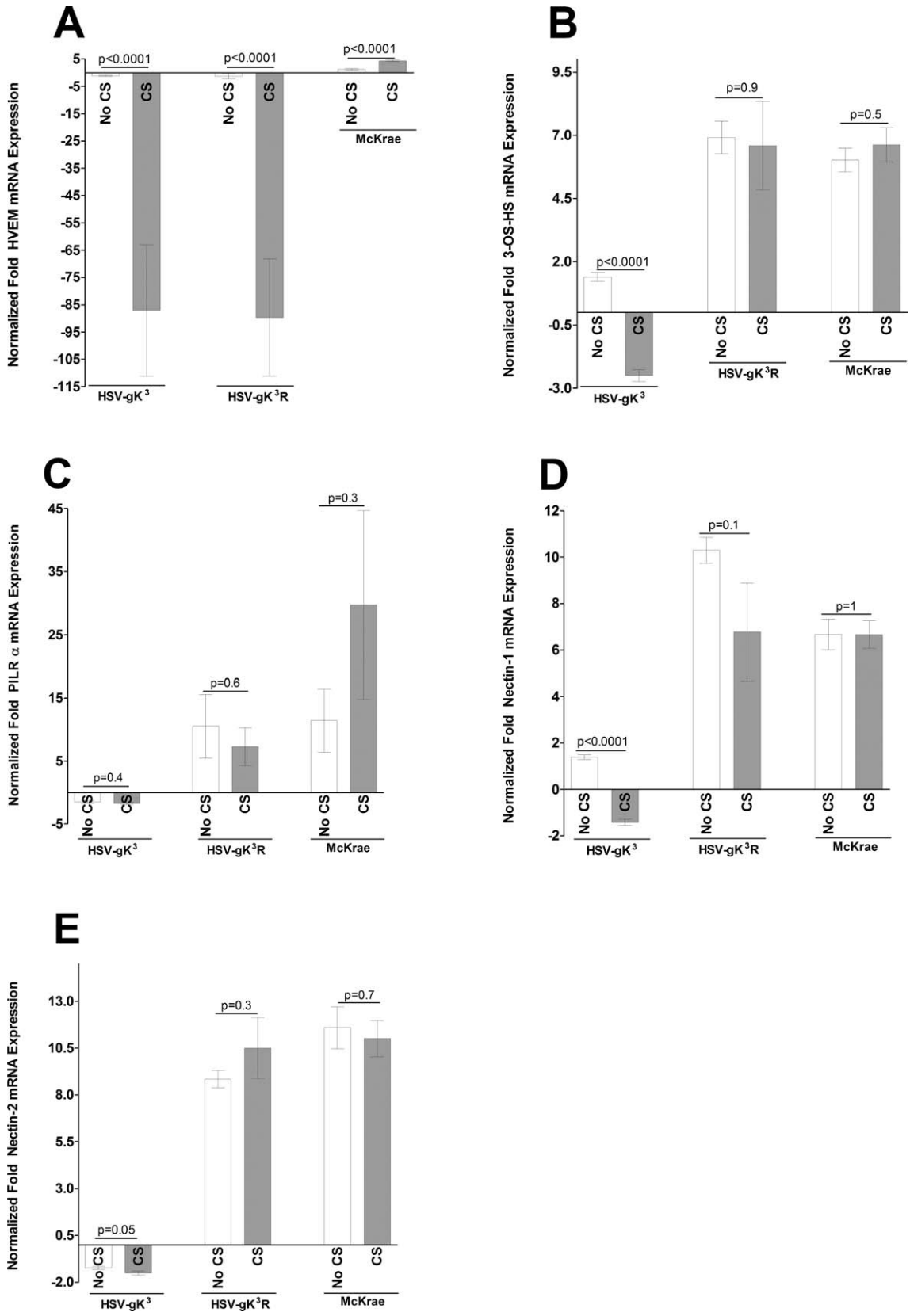

Figure 4. Effect of gK overexpression on HSV-1 receptors in TG of latently-infected mice. The C57BL/6 mice were ocularly-infected with HSV-gK^3, HSV-gK^{3}R, or wt HSV-1 strain McKrae as in Figure 3 above. On day 28 PI, infected mice were separated into mice with no CS or mice with CS in both eyes. Individual TG was harvested and qRT-PCR was performed on each individual mouse TG as in Figure 1 above. The HVEM, 3-OS-HS, PILR-α, nectin-1, and nectin-2 expression in naive mice was used to estimate the relative expression of each transcript in TG of infected mice. The GAPDH expression was used to normalize the relative expression of each transcript in TG of latently-infected mice. Each *status bar* represents the mean ± SEM from 10 TG. (**A**) HVEM, (**B**) 3-OS-HS, (**C**) PILR-α, (**D**) Nectin-1, and (**E**) Nectin-2.

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Inflammatory Responses in TG of Latently-Infected Mice Correlated With CS and Not gK Overexpression

To investigate potential differences in TG infiltrates of mice with no CS compared to mice with CS in eyes in the presence of gK overexpression, we performed RT-PCR in the same TG samples used in Figure 4 above to determine expression levels of mRNAs characteristic of T cells (CD4, CD8), inflammatory cytokines (IL-2, TNF-α, IFN-γ), and T-cell exhaustion markers (IL-21, PD-1, Tim-3). The levels of CD4 (Fig. 5A), CD8 (Fig. 5B), and TNF-α (Fig. 5C) transcripts were significantly lower in the TG from mice with CS in eyes compared to mice with no CS in HSV-gK^3 and HSV-gK^{3}R groups ($P < 0.05$). In contrast, in McKrae-infected mice the levels of CD4 (Fig. 5A), CD8 (Fig. 5B), and TNF-α (Fig. 5C) transcripts were significantly higher in TG of mice with CS compared to mice without CS ($P < 0.05$). Overall, the levels of CD4 (Fig. 5A), CD8 (Fig. 5B), and TNF-α (Fig. 5C) transcripts were the same in TG of mice without CS that were infected with HSV-gK^3, HSV-gK^{3}R, or McKrae (Figs. 5A–C, No CS).

Upregulation of PD-1,[45–47] T-cell immunoglobulin and mucin domain–containing protein-3 (Tim-3),[48–50] and IL-21[51–53] are associated with T-cell exhaustion. Previously, we have shown that the presence of LAT is correlated with increased mRNA levels of PD-1 and Tim-3,[30] but we did not look at this relationship in the context of gK expression or CS. Mice with CS in eyes in HSV-gK^3 and HSV-gK^{3}R groups appeared to have significantly lower mRNA levels of Tim-3 (Fig. 5D), PD-1 (Fig. 5E), and IL-21 (Fig. 5F) than their counterparts with no CS ($P < 0.05$), while in McKrae-infected mice the levels of all three transcripts were higher in mice with CS than mice with no CS (Figs. 5D–F, McKrae; $P < 0.05$) suggesting that the differences are attributed to the presence of LAT and not the level of gK. Finally, the levels of Tim-3 (Fig. 5D, No CS), PD-1 (Fig. 5E, No CS), and IL-21 (Fig. 5F, No CS) transcripts were the same in TG of mice without CS that were infected with HSV-gK^3, HSV-gK^{3}R, or McKrae.

Both IL-2 and IFN-γ have been implicated in protection or increase of CS in ocularly-infected mice.[28,29,54] The level of IL-2 transcript in TG of mice with no CS was significantly higher than the level in mice with CS (Fig. 5G, $P < 0.0001$). In addition, the decrease in expression level of IL-2 in mice with CS was significantly higher in the HSV-gK3 and HSV-gK^{3}R groups than the McKrae group (Fig. 5G, $P < 0.05$, CS). Finally, the level of IFN-γ transcript was higher in the TG of mice with no CS than mice with CS (Fig. 5H, $P < 0.05$). Although the level of IFN-γ transcript was decreased in HSV-gK^3 and HSV-gK^{3}R groups, it was significantly increased in the McKrae group (Fig. 5H).

Discussion

The HSV-1 glycoproteins are the major inducers and targets of humoral and cell-mediated immune responses following HSV-1 infection.[4,55–57] Previously, we constructed and characterized recombinant baculoviruses expressing high levels of each of the 11 HSV-1 glycoproteins.[3–6,12,58–66] Using these recombinant viruses, we have shown that immunization with gB, gC, gD, gE, or gI completely protects mice against lethal challenge.[58–62] However, eye disease and the establishment of latency were not eliminated.[4,6] No significant protection was seen with any of the other 6 glycoproteins,[5,6,63–65] and immunization with gK leads to severe exacerbation of eye disease.[3,12,66]

Previously, we evaluated the effect of gK overexpression on HSV-1 infection using a recombinant HSV-1 virus that expresses two additional copies of the gK gene in place of the LAT.[20] This mutant virus (HSV-gK^3) expressed higher levels of gK than either the wt-type McKrae virus or the parental dLAT2903 virus in vitro and in vivo. The HSV-gK^3-infected BALB/c and C57BL/6 mice had significantly higher CS than did McKrae-infected mice. The T-cell depletion studies suggested that this CS enhancement in the HSV-gK^3–infected mice was mediated by a CD8$^+$ T-cell response. More recently, we have shown that the presence of LAT is correlated with higher levels of latency and T-cell exhaustion,[30] as well as higher expression of HVEM[27] in TG of latently-infected mice. In addition, our recent study has shown that HSV-1 gK binds to SPP, and is required for HSV-1 infectivity in vitro and in vivo.[16]

The HSV-1 has several major entry routes to infect cells productively, including HVEM, nectin-1, nectin-2, 3-OS-HS, and PILR-α).[2,35,67–69] This redundancy in HSV-1 receptors is likely responsible for the ability of HSV-1 to infect most cell types.[35,39,67,68,70–73] The role of HSV-1 receptors have been studied in detail in vitro,[70,71,73] but very little is known if gK overexpression regulates viral entry receptors during primary and latent cycles of HSV-1 infection. This study was designed to determine the effect of gK overexpression on HSV-1 receptors during primary and latent infection, as well as to correlate severity of CS with expression of HSV-1 receptors and immune infiltrates in TG of latently-infected mice. In this study, during primary ocular infection of C57BL/6 mice, no differences were detected in the expression levels of HVEM, 3-OS-HS, PILRα, nectin-1, and nectin-2 in HSV-gK^3–infected mice versus HSV-gK^{3}R–infected mice. In contrast to C57BL/6 mice, we detected significant differences for expression of the above receptors in BALB/c mice infected with HSV-gK^3 compared to mice that were infected with HSV-gK^{3}R virus. These differences in expression of HSV-1 receptors during primary infection could be due to strain differences or differences in virus replication in the eye of BALB/c mice in the presence of three copies of gK (HSV-gK^3) versus one copy of gK (HSV-gK^{3}R).

In contrast to the lack of any correlation between gK overexpression and HSV-1 receptors during primary infection in C57BL/6, but not BALB/c mice, gK overexpression altered expression of HSV-1 receptors during latency. The gK overexpression affected 3-OS-HS, PILRα, nectin-1, and nectin-2 expression in HSV-gK^3–infected mice compared to HSV-gK^{3}R and wt McKrae. Previously, we have shown that HSV-gK^3–infected BALB/c and C57BL/6 mice had significantly higher CS than did McKrae-infected mice.[20] Therefore, it is possible that continuous upregulation of 3-OS-HS, PILRα, nectin-1, and nectin-2 by gK may lead to more efficient binding of HSV-1 envelope glycoproteins and, thus, increased subclinical reactivation. Thus, this low level reactivation may contribute to higher CS despite no differences in the level of latency between mice with CS compared to mice with no CS. Therefore, one of the mechanisms by which gK enhances CS appears to be through modulation of 3-OS-HS, PILRα, nectin-1, and nectin-2 expression, while higher subclinical reactivation appears to be through modulation of HVEM expression. In contrast to 3-OS-HS, PILRα, nectin-1, and nectin-2 expression during latency, gK overexpression had no effect on HVEM expression. In HSV-gK^3– and HSV-gK^{3}R–infected mice, the level of HVEM expression decreased and this decline was significantly higher in TG of mice with CS than mice with no CS. Similar to our results here with HVEM, recently we have shown that LAT upregulates HVEM expression in vivo and in vitro.[27] Thus, the lower HVEM expression in HSV-gK^3 and HSV-gK^{3}R mice is due to the absence of LAT in these viruses.

Previously we found a direct correlation between the severity of CS and higher levels of latency in the TG as judged by the level of LAT RNA in BALB/c mice infected with wt McKrae.[26] In contrast, in C57BL/6 mice we did not detect any

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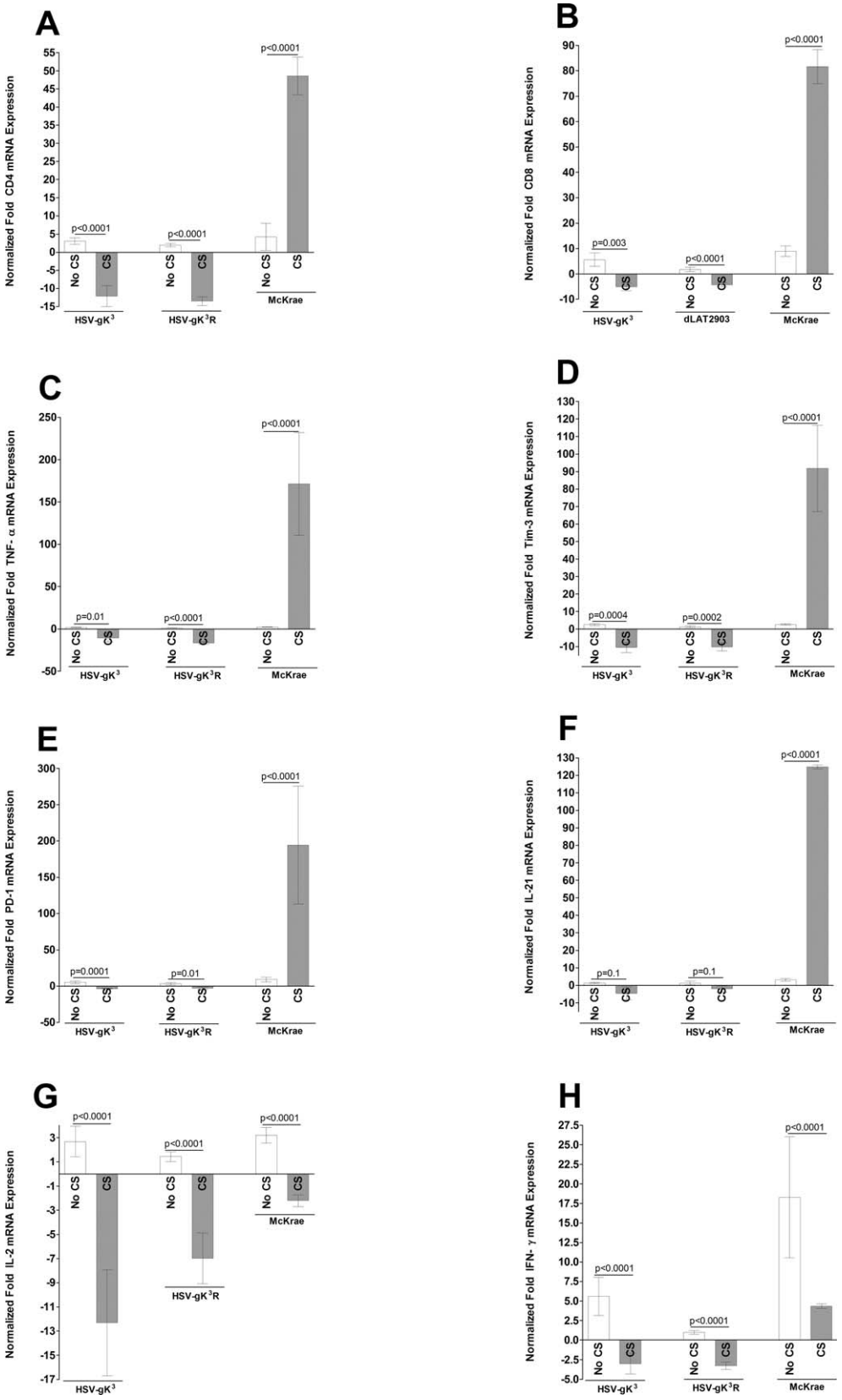

Figure 5. Association of gK overexpression with the level of inflammatory infiltrates in TG of latently-infected mice. The qRT-PCR was performed using total RNA isolated from TG of mice from the experiment described in Figure 4 above. The CD4, CD8, TNF-α, Tim-3, PD-1, IL-21, IL-2, and IFN-γ expressions in naive mice were used to estimate the relative expression of each transcript in the TG of mice with no CS versus mice with CS in eyes. The GAPDH expression was used to normalize the relative expression of each transcript in TG of latently-infected mice. Each *point* represents the mean ± SEM from 10 TG. (**A**) CD4 transcript, (**B**) CD8 transcript, (**C**) TNF-α, (**D**) Tim-3 transcript, (**E**) PD-1 transcript, (**F**) IL-21 transcript, (**G**) IL-2 transcript, and (**H**) IFN-γ transcript.

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correlation between presence or absence of CS and higher levels of latency in the TG of mice infected with any of the three viruses we used here as judged by the level of gB DNA. As expected and similar to our previous study,[30] the level of latency was higher in McKrae (LAT-plus) compared to the level of latency in HSV-gK[3] (LAT-minus) and HSV-gK[3]R (LAT-minus) viruses.

We also found a correlation between increased levels of CS, and higher levels of CD4, CD8, TNF-α, Tim-3, PD-1, and IL-21 in TG of mice infected with wt McKrae virus. However, the changes in the infiltrates in presence or absence of CS were not correlated with level of latency in McKrae-infected mice. These differences are in contrast with our previous study using BALB/c mice in which we detected a strong correlation between latent virus in the TG, level of CS and presence of infiltrates in the TG of mice with CS in eyes compared to mice with no CS.[26] In contrast to the presence of infiltrates in McKrae-infected mice, the levels of CD4, CD8, TNF-α, Tim-3, PD-1, and IL-21 infiltrates were lower in HSV-gK[3]– and HSV-gK[3]R–infected mice with CS compared to their counterparts with no CS. These results suggested that the presence of LAT rather than gK is contributing to higher infiltrates in TG of latently-infected mice. We also have shown that increased IL-2 and IFN-γ levels are correlated with no CS. The PD-1, Tim-3, and IL-21 are markers for T-cell exhaustion and a primary factor leading to exhaustion. Thus, our results indicated that there were higher levels of inflammatory infiltrates in the TG in the presence of LAT (McKrae virus) than in the absence of LAT, and although this does not indicate any direct cause and effect relationship between infiltrates in mouse TG and CS, it is possible that the latent virus in mouse TG is involved in the initiation of HSK, as might be expected in humans and rabbits where HSK is known to be related to reactivation of virus in the TG.

In this report, we found that overexpression of gK has the potential to alter the level of many of the transcripts we examined, which may, in turn, result in the differences observed in CS in the TG of latently-infected mice. These included the mRNA levels of 3-OS-HS, PILR-α, nectin-1, and nectin-2. In contrast, HVEM expression was gK-independent, but LAT-dependent.

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References

1. McGeoch DJ, Dalrymple MA, Davison AJ, et al. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol*. 1988;69:1531–1574.
2. Spear PG, Eisenberg RJ, Cohen GH. Three classes of cell surface receptors for alphaherpesvirus entry. *Virology*. 2000; 275:1–8.
3. Ghiasi H, Slanina S, Nesburn AB, Wechsler SL. Characterization of baculovirus-expressed herpes simplex virus type 1 glycoprotein K. *J Virol*. 1994;68:2347–2354.
4. Ghiasi H, Kaiwar R, Nesburn AB, Slanina S, Wechsler SL. Expression of seven herpes simplex virus type 1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI): comparative protection against lethal challenge in mice. *J Virol*. 1994;68:2118–2126.
5. Ghiasi H, Kaiwar R, Slanina S, Nesburn AB, Wechsler SL. Expression and characterization of baculovirus expressed herpes simplex virus type 1 glycoprotein L. *Arch Virol*. 1994;138:199–212.
6. Ghiasi H, Bahri S, Nesburn AB, Wechsler SL. Protection against herpes simplex virus-induced eye disease after vaccination with seven individually expressed herpes simplex virus 1 glycoproteins. *Invest Ophthalmol Vis Sci*. 1995;36:1352–1360.
7. Hutchinson L, Goldsmith K, Snoddy D, Ghosh H, Graham FL, Johnson DC. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J Virol*. 1992;66:5603–5609.
8. Foster TP, Kousoulas KG. Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress. *J Virol*. 1999;73:8457–8468.
9. Hutchinson L, Johnson DC. Herpes simplex virus glycoprotein K promotes egress of virus particles. *J Virol*. 1995;69:5401–5413.
10. Hutchinson L, Roop-Beauchamp C, Johnson DC. Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. *J Virol*. 1995;69: 4556–4563.
11. Saied AA, Chouljenko VN, Subramanian R, Kousoulas KG. A replication competent HSV-1(McKrae) with a mutation in the amino-terminus of glycoprotein K (gK) is unable to infect mouse trigeminal ganglia after cornea infection [published online ahead of print January 8, 2014]. *Curr Eye Res*.
12. Ghiasi H, Cai S, Slanina S, Nesburn AB, Wechsler SL. Nonneutralizing antibody against the glycoprotein K of herpes simplex virus type-1 exacerbates herpes simplex virus type-1-induced corneal scarring in various virus-mouse strain combinations. *Invest Ophthalmol Vis Sci*. 1997;38:1213–1221.
13. Osorio Y, Cai S, Hofman FM, Brown DJ, Ghiasi H. Involvement of CD8+ T cells in exacerbation of corneal scarring in mice. *Curr Eye Res*. 2004;29:145–151.
14. Allen SJ, Mott KR, Ljubimov AV, Ghiasi H. Exacerbation of corneal scarring in HSV-1 gK-immunized mice correlates with elevation of CD8+CD25+ T cells in corneas of ocularly-infected mice. *Virology*. 2010;399:11–22.
15. Foster TP, Rybachuk GV, Alvarez X, Borkhsenious O, Kousoulas KG. Overexpression of gK in gK-transformed cells collapses the Golgi apparatus into the endoplasmic reticulum inhibiting virion egress, glycoprotein transport, and virus-induced cell fusion. *Virology*. 2003;317:237–252.
16. Allen SJ, Mott KR, Matsuura Y, Moriishi K, Kousoulas KG, Ghiasi H. Binding of HSV-1 Glycoprotein K (gK) to signal peptide peptidase (SPP) is required for virus infectivity. *PLoS One*. 2014;9:e85360.
17. Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. *Physiol Rev*. 2006;86:1133–1149.
18. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest*. 2005;115:2656–2664.
19. Kroeger H, Messah C, Ahern K, et al. Induction of endoplasmic reticulum stress genes, BiP and Chop, in genetic and environmental models of retinal degeneration. *Invest Ophthalmol Vis Sci*. 2012;53:7590–7599.
20. Mott KR, Perng GC, Osorio Y, Kousoulas KG, Ghiasi H. A recombinant herpes simplex virus type 1 expressing two additional copies of gK is more pathogenic than wild-type virus in two different strains of mice. *J Virol*. 2007;81:12962–12972.
21. Osorio Y, Mott KR, Jabbar AM, et al. Epitope mapping of HSV-1 glycoprotein K (gK) reveals a T cell epitope located within the signal domain of gK. *Virus Res*. 2007;128:71–80.
22. Mott KR, Chentoufi AA, Carpenter D, Benmohamed L, Wechsler SL, Ghiasi H. The role of a glycoprotein K (gK) CD8+ T-cell epitope of herpes simplex virus on virus replication and pathogenicity. *Invest Ophthalmol Vis Sci*. 2009;50:2903–2912.

23. Mott KR, Osorio Y, Maguen E, et al. Role of anti-glycoproteins D (anti-gD) and K (anti-gK) IgGs in pathology of herpes stromal keratitis in humans. *Invest Ophthalmol Vis Sci*. 2007; 48:2185–2193.
24. Dervillez X, Qureshi H, Chentoufi AA, et al. Asymptomatic HLA-A*02:01-restricted epitopes from herpes simplex virus glycoprotein B preferentially recall polyfunctional CD8+ T cells from seropositive asymptomatic individuals and protect HLA transgenic mice against ocular herpes. *J Immunol*. 2013; 191:5124–5138.
25. Khan AA, Srivastava R, Lopes PP, et al. Asymptomatic memory CD8 T cells: from development and regulation to consideration for human vaccines and immunotherapeutics [published online ahead of print February 5, 2014]. *Hum Vaccin Immunother*. 2014;10.
26. Mott KR, Bresee CJ, Allen SJ, BenMohamed L, Wechsler SL, Ghiasi H. Level of herpes simplex virus type 1 latency correlates with severity of corneal scarring and exhaustion of CD8+ T cells in trigeminal ganglia of latently-infected mice. *J Virol*. 2009;83:2246–2254.
27. Allen SJ, Rhode-Kurnow A, Mott KR, et al. Regulatory interactions between herpesvirus entry mediator (TNFRSF14) and latency associated transcript (LAT) during HSV-1 latency. *J Virol*. 2014;88:1961–1971.
28. Ghiasi H, Wechsler SL, Cai S, Nesburn AB, Hofman FM. The role of neutralizing antibody and T-helper subtypes in protection and pathogenesis of vaccinated mice following ocular HSV-1 challenge. *Immunology*. 1998;95:352–359.
29. Ghiasi H, Wechsler SL, Kaiwar R, Nesburn AB, Hofman FM. Local expression of tumor necrosis factor alpha and interleukin-2 correlates with protection against corneal scarring after ocular challenge of vaccinated mice with herpes simplex virus type 1. *J Virol*. 1995;69:334–340.
30. Allen SJ, Hamrah P, Gate DM, et al. The role of LAT in increased CD8+ T cell exhaustion in trigeminal ganglia of mice latently-infected with herpes simplex virus type 1. *J Virol*. 2011;85: 4184–4197.
31. Debroy C, Pederson N, Person S. Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology*. 1985;145:36–48.
32. Bond VC, Person S. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. *Virology*. 1984;132:368–376.
33. Little SP, Schaffer PA. Expression of the syncytial (syn) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two syn loci. *Virology*. 1981;112:686–702.
34. Pogue-Geile KL, Spear PG. The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP. *Virology*. 1987;157:67–74.
35. Satoh T, Arii J, Suenaga T, et al. PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. *Cell*. 2008;132:935–944.
36. Shukla ND, Tiwari V, Valyi-Nagy T. Nectin-1-specific entry of herpes simplex virus 1 is sufficient for infection of the cornea and viral spread to the trigeminal ganglia. *Mol Vis*. 2012;18: 2711–2716.
37. Karaba AH, Kopp SJ, Longnecker R. Herpesvirus entry mediator is a serotype specific determinant of pathogenesis in ocular herpes. *Proc Natl Acad Sci U S A*. 2012;109:20649–20654.
38. Tiwari V, Liu J, Valyi-Nagy T, Shukla D. Anti-heparan sulfate peptides that block herpes simplex virus infection in vivo. *J Biol Chem*. 2011;286:25406–25415.
39. O'Donnell CD, Kovacs M, Akhtar J, Valyi-Nagy T, Shukla D. Expanding the role of 3-O sulfated heparan sulfate in herpes simplex virus type-1 entry. *Virology*. 2010;397:389–398.
40. Dawson CR. Ocular herpes simplex virus infections. *Clin Dermatol*. 1984;2:56–66.
41. Barron BA, Gee L, Hauck WW, et al. Herpetic Eye Disease Study. A controlled trial of oral acyclovir for herpes simplex stromal keratitis. *Ophthalmology*. 1994;101:1871–1882.
42. Brown DC, Nesburn AB, Nauheim JS, Pavan-Langston D, Kaufman HE. Recurrent herpes simplex conjunctivitis. *Arch Ophthalmol*. 1968;79:733–735.
43. Dix RD. Prospects for a vaccine against herpes simplex virus types 1 and 2. *Prog Med Virol*. 1987;34:89–128.
44. Koelle DM, Ghiasi H. Prospects for developing an effective vaccine against ocular herpes simplex virus infection. *Curr Eye Res*. 2005;30:929–942.
45. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682–687.
46. Wherry EJ, Ha SJ, Kaech SM, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity*. 2007;27:670–684.
47. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677–704.
48. Jones RB, Ndhlovu LC, Barbour JD, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med*. 2008;205:2763–2779.
49. Golden-Mason L, Palmer BE, Kassam N, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol*. 2009;83:9122–9130.
50. Jin HT, Anderson AC, Tan WG, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A*. 2010;107:14733–14738.
51. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science*. 2009;324:1569–1572.
52. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science*. 2009;324:1572–1576.
53. Frohlich A, Kisielow J, Schmitz I, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science*. 2009;324:1576–1580.
54. Ghiasi H, Hofman FM, Cai S, Perng GC, Nesburn AB, Wechsler SL. Vaccination with different HSV-1 glycoproteins induces different patterns of ocular cytokine responses following HSV-1 challenge of vaccinated mice. *Vaccine*. 1999;17:2576–2582.
55. Burke RL. Development of a herpes simplex virus subunit glycoprotein vaccine for prophylactic and therapeutic use. *Rev Infect Dis*. 1991;13:S906–S911.
56. Burke RL. Contemporary approaches to vaccination against herpes simplex virus. *Curr Top Microbiol Immunol*. 1992; 179:137–158.
57. Dix RD, Mills J. Acute and latent herpes simplex virus neurological disease in mice immunized with purified virus-specific glycoproteins gB or gD. *J Med Virol*. 1985;17:9–18.
58. Ghiasi H, Nesburn AB, Kaiwar R, Wechsler SL. Immunoselection of recombinant baculoviruses expressing high levels of biologically active herpes simplex virus type 1 glycoprotein D. *Arch Virol*. 1991;121:163–178.
59. Ghiasi H, Kaiwar R, Nesburn AB, Slanina S, Wechsler SL. Baculovirus-expressed glycoprotein E (gE) of herpes simplex virus type-1 (HSV-1) protects mice against lethal intraperitoneal and lethal ocular HSV-1 challenge. *Virology*. 1992;188: 469–476.
60. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus expressed herpes simplex virus type 1 glycoprotein C protects mice from lethal HSV-1 infection. *Antiviral Res*. 1992;18:291–302.
61. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Expression of herpes simplex virus type 1 glycoprotein I in baculovirus:

Investigative Ophthalmology & Visual Science

preliminary biochemical characterization and protection studies. *J Virol*. 1992;66:2505–2509.

62. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Expression of herpes simplex virus type 1 glycoprotein B in insect cells. Initial analysis of its biochemical and immunological properties. *Virus Res*. 1992;22:25–39.
63. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus-expressed glycoprotein G of herpes simplex virus type 1 partially protects vaccinated mice against lethal HSV-1 challenge. *Virology*. 1992;190:233–239.
64. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus-expressed glycoprotein H of herpes simplex virus type 1 (HSV-1) induces neutralizing antibody and delayed type hypersensitivity responses, but does not protect immunized mice against lethal HSV-1 challenge. *J Gen Virol*. 1992;73:719–722.
65. Ghiasi H, Nesburn AB, Cai S, Wechsler SL. The US5 open reading frame of herpes simplex virus type 1 does encode a glycoprotein (gJ). *Intervirology*. 1998;41:91–97.
66. Ghiasi H, Cai S, Nesburn AB, Wechsler SL. Vaccination with herpes simplex virus type 1 glycoprotein K impairs clearance of virus from the trigeminal ganglia resulting in chronic infection. *Virology*. 1996;224:330–333.
67. Hadigal S, Shukla D. Exploiting herpes simplex virus entry for novel therapeutics. *Viruses*. 2013;5:1447–1465.
68. Taylor JM, Lin E, Susmarski N, et al. Alternative entry receptors for herpes simplex virus and their roles in disease. *Cell Host Microbe*. 2007;2:19–28.
69. Spear PG. Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol*. 2004;6:401–410.
70. Yoon M, Zago A, Shukla D, Spear PG. Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. *J Virol*. 2003;77:9221–9231.
71. Montgomery RI, Warner MS, Lum BJ, Spear PG. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell*. 1996;87:427–436.
72. Shukla D, Liu J, Blaiklock P, et al. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell*. 1999;99:13–22.
73. Shah A, Farooq AV, Tiwari V, Kim MJ, Shukla D. HSV-1 infection of human corneal epithelial cells: receptor-mediated entry and trends of re-infection. *Mol Vis*. 2010;16:2476–2486.

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