Psychological Stress Compromises CD8+ T Cell Control of Latent Herpes Simplex Virus Type 1 Infections

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Recurrent HSV-1 ocular disease results from reactivation of latent virus in trigeminal ganglia, often following immunosuppression or exposure to a variety of psychological or physical stressors. HSV-specific CD8\(^+\) T cells can block HSV-1 reactivation from latency in ex vivo trigeminal ganglia cultures through production of IFN-γ. In this study, we establish that either CD8\(^+\) T cell depletion or exposure to restraint stress permit HSV-1 to transiently escape from latency in vivo. Restraint stress caused a reduction of TG-resident HSV-specific CD8\(^+\) T cells and a functional compromise of those cells that survive. Together, these effects of stress resulted in an approximate 65% reduction of cells capable of producing IFN-γ in response to reactivating virus. Our findings demonstrate persistent in vivo regulation of latent HSV-1 by CD8\(^+\) T cells, and strongly support the concept that stress induces HSV-1 reactivation from latency at least in part by compromising CD8\(^+\) T cell surveillance of latently infected neurons. 

experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh.

In vivo CD8 depletion

Latently infected mice received a single i.p. injection of 0.5 mg of anti-CD8α mAb (clone 2.43, rat IgG2b) 3 days before TG excision.

Restrain stress

On 4 consecutive nights beginning 30 days after infection, mice were physically restrained in a 50-ml conical tube containing approximately one hundred 0.4-cm diameter holes. The 12-h restraint sessions in their home cages were begun 2 h into the dark cycle. Each restraint session was followed by a 12-h period without restraint. Because the stressed mice lacked access to food and water during the restraint sessions, nonstressed control mice were similarly food and water deprived, but not restrained.

Serum corticosterone assay

Serum corticosterone levels were assayed using a standard radioimmunoassay (MP Biomedicals) according to the manufacturer’s protocol. Levels were determined using a standard curve and expressed in nanograms per milliliter.

Single-cell suspensions of TG

At various times after infection, mice were euthanized by exsanguination. TG were excised, pooled, digested with 100 µl of DMEM (BioWhittaker) containing 10% FCS (HyClone) and 400 U/ml collagenase type I (Sigma-Aldrich) per TG for 1 h at 37°C, and dissociated into a single-cell suspension by trituration. Proliferation was assayed by labeling TG suspensions with 2.5 µM CFSE (Molecular Probes) and incubating for 72 h with or without HSV-1 glycoprotein B (gB)498–505 peptide (Invitrogen Life Technologies).

Flow cytometry

TG cell suspensions were passed through a 40-µm filter-cap tube to remove debris. Ab surface staining was performed as described previously (7), with minor modifications. Fluorochrome-conjugated Abs against CD8α (53-6.7), CD8β (53-5.8), CD45 (30-F11), and CD45.1 (A20) were purchased from BD Pharmingen. Proper isotype control Abs were used. PE-conjugated H-2Kb/gB498–505(SSIEFARL) tetramers (National Institute of Allergy and Infectious Diseases Tetramer Core Facility) were used to identify the H-2Kb-restricted HSV-1 gB498–505-specific CD8+ T cell population. Intracellular IFN-γ (XMG1.2; BD Pharmingen) assays were performed using the Cytofix/Cytoperm kit with GolgiPlug (BD Pharmingen) according to the manufacturer’s instructions. Preliminary studies established that TG-resident gB498–505-CD8+ T cells were optimally stimulated for IFN-γ production by adding 10−12 M gB498–505 peptide-pulsed B6WT3 cells (17).

Quantitative real-time PCR

Total DNA was isolated from single-cell TG suspensions using DNeasy columns (Qiagen), diluted to 1 ng/μl in nuclease-free dH2O, and 25 ng of DNA or water control was mixed in duplicate with 25 µl of TaqMan primers and probes for the HSV-1 glycoprotein H gene.
incubation, were stained for surface CD8 and intracellular IFN-γ. Cells that underwent one or more rounds of proliferation, and MFI of proliferating cells in cultures without peptide (A8/group) were analyzed by flow cytometry. Data are presented as the mean (±SEM) percentage of CD8+ T cells.

Results

CD8+ T cells are required in vivo to prevent viral DNA synthesis

Although HSV-specific CD8+ T cells block HSV-1 reactivation from latency in ex vivo TG cultures (4, 7), a similar protective function has not been established in vivo. In this study, we show that within 3 days of a single injection of anti-CD8 mAb, CD8+ T cells were effectively depleted from mouse TG that harbored latent HSV-1 (Fig. 1A), and the HSV-1 genome copy number in the TG was significantly increased (Fig. 1B). Thus, CD8+ T cells actively monitor latent HSV-1 in sensory neurons and inhibit replication of the viral genome.

Restraint stress during latency increases viral DNA copy number and protein expression

Because stress inhibits CD8+ T cell function and induces HSV-1 reactivation, we hypothesized that reactivation results at least in part from a stress-induced transient compromise in the immunosurveillance capability of CD8+ T cells within latently infected TG. To test this hypothesis, latently infected mice were subjected

**FIGURE 3.** Stress reduces CD8+ T cells in latently infected TG. Latently infected mice were subjected to restraint stress as described in Fig. 2. A. The mean absolute number of CD8+ T cells/TG (±SEM) in stressed (n = 23) and nonstressed (n = 16) mice. *p = 0.03, Student’s t test. B. Percentage of CD8+ T cells that recognize the gB498–505 epitope. Group differences were not significant (p = 0.1436, Student’s t test).

**FIGURE 4.** Stress compromises the function of HSV-specific CD8+ T cells in latently infected TG. Latently infected mice were subjected to restraint stress as described in Fig. 2. After the fourth restraint stress session (34 days after infection), TG were excised from stressed and nonstressed mice, dispersed into single-cell suspensions, and incubated for 72 h. TG cells were stained with CFSE and cultured with or without added HSV-1 gB498–505 peptide. After incubation, the cells were stained with anti-CD8α mAb and CFSE staining intensity of CD8+ T cells was analyzed by flow cytometry. A. Representative dot plots show extent of CFSE dilution in stressed and nonstressed cultures gated on CD45+CD8+ T cells. The box designates cells that have undergone one or more rounds of cell proliferation, and the MFI of these cells is shown. B and C. Data are presented as the mean (±SEM) percentage of CD8+ T cells. D. Single-cell suspensions of TG from stressed and nonstressed mice were incubated directly ex vivo for 6 h without stimulation in the presence of GolgiPlug (directly ex vivo, n = 3–4/group) or were cultured for 72 h and received GolgiPlug (TG culture, n = 13–14/group). E. Cultures received HSV-1 gB498–505 Peptide (n = 4/group) and GolgiPlug for the last 6 h of the 72-h incubation, were stained for surface CD8α and intracellular IFN-γ, and analyzed by flow cytometry. Data are presented as the mean (±SEM) percentage of IFN-γ-positive CD8+ T cells. *p < 0.05, **p < 0.01, all data were analyzed with Student’s t test.

**Immunohistochemistry**

Acetone-fixed 6-μm frozen TG sections were stained for HSV Ags using a polyclonal rabbit anti-HSV Ab (Accurate Chemical) followed by goat anti-rabbit Ig, ABC reagent (Vectastain ABC Kit; Vector Laboratories), and detection with diaminobenzidine substrate (Vector Laboratories) as previously described (6). Sections were then dehydrated, mounted in Immuno-mount (Thermo Electron), and covered with a coverslip.
FIGURE 5. CD8⁺ T cells in TG of stressed mice exhibit an intrinsic compromise of IFN-γ production. A, The preparation of mixed TG cultures from stressed (CD45.1) and nonstressed (CD45.2) latently infected congenic mice. Congenic mice were infected with HSV-1 and 30 days later were stressed or not, TG were obtained and dispersed into single-cell suspensions, and mixed cultures of TG from stressed and nonstressed mice were prepared as depicted. After 90 h, GolgiPlug was added to cultures without (B) or with (C) HSV-1 gB₄₉₈–₅₀₅ peptide for the last 6 h of incubation. Cells were recovered from cultures and surface stained with anti-CD8α and anti-CD45.1 mAb followed by intracellular stain for IFN-γ. Representative dot plots show recovery of CD8⁺ T cells that originated from stressed (CD45.1) and nonstressed (CD45.2) TG from the mixed cultures and IFN-γ expression in each population. Percents in lower right corner indicate the percentage of CD8⁺ T cells in the gated population. The graph shows the mean (±SEM) percentage of CD8⁺ T cells from stressed and nonstressed TG that expressed IFN-γ in the mixed TG cultures (n = 18). Data were analyzed with a Student’s paired t test.

to four 12-h restraint sessions as illustrated in Fig. 2A. The effectiveness of the stress protocol was established by uniformly elevated serum levels of the hypothalamic-pituitary-adrenal-derived effector molecule corticosterone following treatment (Fig. 2B). That this stress protocol influenced the state of viral latency was established by an ~2-fold increase in the viral genome copy number in DNA from TG of stressed relative to nonstressed mice (Fig. 2C), similar to the increased copy number observed following CD8⁺ T cell depletion (Fig. 1B).

Stress-induced reactivation was further established by direct ex vivo detection of infectious HSV-1 in one of eight stressed TG and detection of HSV-1 proteins in serial sections of one of four stressed TG (Fig. 2D and data not shown). The inconsistent detection of replicating virus in the presence of uniformly elevated viral genome copy number probably reflects the enhanced sensitivity of viral DNA detection by real-time PCR relative to detection of infectious virions on monolayers of susceptible cells (18).

Stress reduces CD8⁺ T cells in latently infected TG

The increased viral copy number was associated with a 30% reduction in the number of CD8⁺ T cells within the TG of stressed mice (Fig. 3A). This reduction was transient and by 2 days after stress cessation CD8⁺ T cell numbers returned to nonstressed levels (data not shown). A similar reduction was observed in the spleen (data not shown), indicating that the effects of stress were systemic and not limited to the TG. Most, if not all, HSV-specific CD8⁺ T cells in latently infected TG of C57BL/6 mice are specific for the gB₄₉₈–₅₀₅ epitope and can be quantified by staining dispersed TG-derived immune cells with tetramers containing this epitope (gB₄₉₈–₅₀₅/H-2KⅡ) followed by flow cytometric analysis (7). Tetramer staining of CD8⁺ T cells from stressed and nonstressed TG revealed a similar frequency of gB₄₉₈–₅₀₅-specific CD8⁺ T cells demonstrating that stress did not selectively deplete HSV-specific CD8⁺ T cells (Fig. 3B). Nonetheless, the absolute number of TG-resident HSV-specific CD8⁺ T cells was reduced by 30% following stress.

HSV-specific CD8⁺ T cells that are retained in the TG following stress are functionally compromised

In our model, HSV-1 begins to reactivate from latency in ex vivo TG cultures within the first 72 h of incubation, and reactivation can be inhibited by gB₄₉₈–₅₀₅-specific CD8⁺ T cells (7). Therefore, we tested the effects of in vivo stress on the functional capabilities of TG-resident CD8⁺ T cells during a 72-h incubation of latently infected TG alone (latently infected neurons are the only source of viral Ags) or when an optimal stimulatory dose of gB₄₉₈–₅₀₅ peptide was added to the TG cultures. Latently infected TG obtained from stressed and nonstressed mice were dispersed into single-cell suspensions, stained with CFSE, and cultured for 72 h with or without gB₄₉₈–₅₀₅ peptide. The cells were then removed from culture, stained with anti-CD8α mAb, and CD8⁺ T cells were analyzed by flow cytometry for proliferation as assessed by CFSE dilution.

Fig. 4A shows a representative dot plot in which cells that underwent one or more rounds of proliferation are gated and the mean fluorescence intensity (MFI) of proliferated cells is indicated (7). Tetramer staining of CD8⁺ T cells from stressed and nonstressed TG within the first 72 h of incubation, and reactivation can be inhibited by gB₄₉₈–₅₀₅-specific CD8⁺ T cells (7). Therefore, we tested the effects of in vivo stress on the functional capabilities of TG-resident CD8⁺ T cells during a 72-h incubation of latently infected TG alone (latently infected neurons are the only source of viral Ags) or when an optimal stimulatory dose of gB₄₉₈–₅₀₅ peptide was added to the TG cultures. Latently infected TG obtained from stressed and nonstressed mice were dispersed into single-cell suspensions, stained with CFSE, and cultured for 72 h with or without gB₄₉₈–₅₀₅ peptide. The cells were then removed from culture, stained with anti-CD8α mAb, and CD8⁺ T cells were analyzed by flow cytometry for proliferation as assessed by CFSE dilution.
these cells underwent in the various groups. In cultures of non-stressed TG, ~50% of CD8+ T cells proliferated during the 72-h incubation in the presence or absence of gB498–505 peptide. In contrast, significantly fewer CD8+ T cells in stressed TG proliferated in response to reactivating virus (Fig. 4B). The addition of gB498–505 peptide to stressed TG cultures increased CD8+ T cell proliferation slightly (28% no peptide, 41% with peptide), but even in the presence of added peptide fewer CD8+ T cells in stressed TG proliferated when compared with similarly stimulated CD8+ T cells in nonstressed TG (Fig. 4C). Moreover, those CD8+ T cells in stressed TG that did proliferate in response to peptide underwent fewer rounds of proliferation than their counterparts in nonstressed TG as indicated by higher MFI (Fig. 4C).

A very low frequency (<2%) of TG-resident CD8+ T cells expressed detectable intracellular IFN-γ when tested directly ex vivo. However, when dispersed cells from latently infected TG were incubated for 72 h, IFN-γ production was induced in ~10% of CD8+ T cells in nonstressed TG. In contrast, IFN-γ production by CD8+ T cells in stressed TG was not significantly increased during the 72-h incubation and was significantly lower than that of their counterparts in nonstressed TG (Fig. 4D). The addition of gB498–505 peptide to cultures greatly augmented IFN-γ production such that 40% of CD8+ T cells in both stressed and nonstressed TG were IFN-γ positive (Fig. 4E). These findings are consistent with the tetramer staining results showing a similar frequency of HSV-1 gB498–505-specific CD8+ T cells in the TG of stressed and nonstressed mice, but demonstrate that CD8+ T cells in stressed TG are compromised in their ability to produce IFN-γ in response to viral reactivation in neurons.

CD8+ T cells in TG of stressed mice exhibit an intrinsic compromise of IFN-γ production

The reduced IFN-γ production in response to latently infected neurons alone could reflect either an intrinsic compromise in the function of the CD8+ T cells in stressed TG or an extrinsic effect such as less Ag production and presentation by neurons in stressed TG. To eliminate possible extrinsic effects, mixed cultures of latently infected TG from stressed (CD45.1) and nonstressed (CD45.2) congenic mice were used (illustrated in Fig. 5A). In these mixed TG cultures, CD8+ T cells from stressed and nonstressed mice experienced identical antigenic exposure and could be identified based on expression of different CD45 alleles. As illustrated in Fig. 5B, 40% fewer CD8+ T cells from stressed than nonstressed TG produced IFN-γ in response to identical exposure to reactivating virus. However, when an optimal stimulatory dose of the gB498–505 peptide was added to the mixed cultures, the percentage of IFN-γ-producing CD8+ T cells was increased and the effect of stress on IFN-γ production was lost (Fig. 5C). Thus, the reduced response of CD8+ T cells from stressed mice does not reflect a reduced frequency of HSV-specific CD8+ T cells or altered antigenic expression by stressed neurons, but rather appears to result from a stress-induced functional compromise that can be overcome by addition of gB498–505 peptide to cultures.

Discussion

The association of stress and HSV-1 reactivation from latency is well established in mice, but a specific mechanism by which stress facilitates HSV-1 reactivation has not been elucidated. The potential involvement of CD8+ T cells in maintaining HSV-1 in a latent state has only recently been appreciated (3, 5–8). CD8+ T cells surround HSV-1-infected neurons in latently infected TG of both mice and humans (4, 7), maintain an activation phenotype (7), form an apparent immunological synapse with neurons (7), and can block HSV-1 reactivation from latency in ex vivo cultures of latently infected TG (7, 8). These findings are consistent with a dynamic form of latency in which HSV-specific CD8+ T cells constantly monitor and repress viral reactivation in at least a portion of latently infected neurons. This study provides the first direct evidence that constant monitoring by CD8+ T cells is required to uniformly maintain HSV-1 latency. The rapid rise in HSV-1 genome copy number following CD8+ T cell depletion (>2-fold increase in genome copy number within 3 days) demonstrates that some latently infected neurons are reactivation competent at any given time, and prevented from progressing to full reactivation with virion formation only through the constant vigilance of CD8+ T cells.

A similar increase in viral DNA was observed in mice that were subjected to restraint stress. The increase in viral DNA was apparent by the end of the 4-day stress protocol and reached statistical significance by 4 days after stress cessation. Although all stressed TG showed elevated viral DNA, the degree of increase varied substantially in individual stressed TG. Only one of eight tested TG exhibited replicating HSV-1 directly ex vivo (data not shown). The detection of replicating virus in even one stressed TG is highly significant as we and others have uniformly failed to detect infectious virions in any latently infected nonstressed TG (19, 20). A recent study showed only 30% of samples containing $1 \times 10^5$ genome copies were culture positive (18). Thus, it is likely that only those stressed TG with the greatest increase in viral DNA achieved a necessary threshold for detection in culture.

The similar rise in viral genome copy number following CD8+ T cell depletion and exposure to restraint stress is consistent with the possibility that stress-induced HSV-1 reactivation results from a transient compromise in CD8+ T cell surveillance. In support of this concept is the observed 30% reduction of CD8+ T cells in stressed TG. The reduced numbers of CD8+ T cells might reflect in part the impaired proliferative capacity of CD8+ T cells in stressed TG when responding to reactivating virus. The latter finding is in agreement with the previous observation that restraint stress can impair mitogen-stimulated T cell proliferation (21). We demonstrated that the basal rate of proliferation of HSV-specific memory CD8+ T cells in latently infected TG is regulated at least in part by Ag stimulation, whereas their counterparts in noninfected tissue are maintained by homeostatic proliferation in response to cytokines including IL-15 (17). Thus, a reduced capacity to proliferate in response to reactivating virus would likely result in a gradual reduction in the TG-resident CD8+ T cell pool. However, it is unlikely that impaired proliferation alone would account for the 30% reduction in CD8+ T cell numbers in stressed TG because the rate of turnover of these cells is quite low, <20% of CD8+ T cells incorporate BrdU during a 1-wk treatment period (17). Moreover, we demonstrated a higher rate of apoptosis in CD8+ T cells in stressed as compared with those in nonstressed TG (data not shown). Thus, proliferative impairment and elevated apoptosis likely contribute to the reduced CD8+ T cell pool in stressed TG.

In addition to the reduced numbers of HSV-specific CD8+ T cells in stressed TG is the observed functional impairment of the surviving cells. When tested directly ex vivo, <2% of CD8+ T cells in stressed or nonstressed TG exhibited detectable IFN-γ production. However, when nonstressed TG were incubated for 72 h, ~10% of the endogenous CD8+ T cells were stimulated to produce IFN-γ. Our studies have established that TG-resident CD8+ T cells in part through IFN-γ production prevent HSV-1 reactivation from latency that is first apparent by 72 h of culture. Because only neurons harbor latent virus in latently infected ganglia (8, 22–24), and CD8+ T cells prevent full HSV-1 reactivation with...
virus formation in TG cultures (7, 8), we conclude that TG-resident HSV-specific CD8⁺ T cells produce IFN-γ in ex vivo TG cultures in response to reactivating HSV-1 in sensory neurons. Therefore, it is important to note that TG-resident CD8⁺ T cells in nonstressed TG did not significantly increase IFN-γ production during a 72-h ex vivo TG culture. The stressed TG contained a similar frequency of HSV-specific CD8⁺ T cells, and the addition of an optimal stimulatory dose of gB498–505 peptide induced a similar frequency of IFN-γ-producing CD8⁺ T cells in stressed and nonstressed TG cultures. Together these findings suggest that the stress-induced functional impairment of CD8⁺ T cells is not absolute, but rather appears to specifically inhibit the capacity of HSV-specific CD8⁺ T cells to respond to reactivating HSV-1 in sensory neurons.

Several factors point to a low epitope density as a likely explanation for the selective inability to respond to latently infected neurons. First, HSV-1 gB is a yl gene that is expressed at low levels before the initiation of viral DNA synthesis. Therefore, gB gene expression would be very low during the early stages of HSV-1 reactivation from latency in neurons. Moreover, neurons typically express very low levels of MHC class I, but appear to up-regulate expression during the HSV-1 lytic cycle (25, 26). Thus, it is likely that neurons express a low density of the gB498–505 epitope early in the reactivation process.

It appears that the impaired response of CD8⁺ T cells to reactivating virus primarily reflects an intrinsic effect of stress on the functional program of the CD8⁺ T cells. Mixed cultures of stressed and nonstressed TG in which the origin of the CD8⁺ T cells could be determined by expression of different CD45 alleles showed less IFN-γ production by CD8⁺ T cells from stressed TG, even when exposed to an identical stimulatory environment. These studies ruled out the possibility that stress-induced differences in Ag presentation or cytokine milieu accounted for the reduced IFN-γ production by CD8⁺ T cells in stressed TG. Moreover, the frequency of IFN-γ⁺ CD8⁺ T cells in nonstressed TG was similar when nonstressed TG were incubated alone or combined with stressed TG, suggesting that the function of CD8⁺ T cells in nonstressed TG is not influenced by exposure to cells from stressed TG.

Our studies demonstrate that the function of TG resident CD8⁺ T cells is very significantly compromised when mice are exposed to restraint stress. The combination of a 30% reduction in the number of HSV-specific CD8⁺ T cells and a 40% reduction in the frequency of CD8⁺ T cells capable of producing IFN-γ in response to reactivating neurons would translate into a 65% reduction in the capacity of CD8⁺ T cells to react to a reactivation event. This might represent an underestimation of the actual CD8⁺ T cell functional impairment because the IFN-γ response was measured after the CD8⁺ T cells were removed from the inhibitory microenvironment within the stressed animal. In addition to direct effects on the T cells, there may also be stress-induced effects on the ability of target cells to respond to cytokines. The synthetic glucocorticoid dexamethasone has been demonstrated to inhibit IL-2, IL-4, IL-7, IL-15, and IFN-γ signaling in part by disrupting expression of Jak-STAT pathway components (27, 28). Glucocorticoids have also been shown to repress activation of the transcription factor NF-κB (29, 30), as well as impair transcriptional activation by AP-1 (31). Whether signaling by these and other cytokines are disrupted in the latently infected TG and, in particular, within latently infected neurons, is the subject of current ongoing research in our laboratory.

Viewed in the context of these findings, the “spontaneous” HSV-1 reactivation observed in humans but not in mice might reflect periodic exposure of humans to a more stressful environment. Moreover, human neurons might express a lower density of HSV-1 epitopes than mouse neurons during HSV-1 reactivation from latency due to the selective capacity of the HSV-1 immediate-early protein ICP47 to block human TAP transport of viral peptides for loading on MHC class I for presentation to CD8⁺ T cells (32). In conjunction with a stress-induced elevation in the threshold of epitope density required to activate CD8⁺ T cells, this would render humans more susceptible to HSV-1 reactivation than mice.

Our findings provide a broader conceptual framework in which to consider HSV-1 latency and reactivation. The fact that latency appears to be uniquely established in neurons underscores the importance of the neuronal microenvironment in the establishment and maintenance of the latent state. However, a growing body of data including this report supports the notion that maintenance of latency in some latently infected neurons requires ancillary support from contiguous CD8⁺ T cells. Moreover, our findings provide a link between stress, a known inducer of HSV-1 reactivation, and transient functional compromise of TG-resident CD8⁺ T cells. We propose that the incidence of recurrent herpetic disease might be reduced by strategies that augment the numbers or function of HSV-specific CD8⁺ T cells within the latently infected sensory ganglia, or by reducing the effects of stress on the function of these cells.

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Disclosures
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References


