Stress Compromises HSV-1-Specific Immunity in Latently Infected Sensory Ganglia

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Stress Compromises HSV-1-Specific Immunity in Latently Infected Sensory Ganglia

Michael Lawrence Freeman, Ph.D.

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Recurrent HSV-1 ocular disease results from reactivation of latent virus in trigeminal ganglia (TG), 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 TG cultures in part through production of IFN-γ. Here we establish that either $CD8^+$ T cell depletion or exposure to restraint stress permits HSV-1 to transiently escape from latency in vivo. Restraint stress caused a glucocorticoid-associated reduction of TG-resident HSV-specific $CD8^+$ T cells, and a functional compromise of those cells that survive, at least partly mediated by catecholamines. Together, these effects of stress resulted in an approximate 65% reduction of cells capable of producing IFN-γ, and impairment in the ability of those cells to release lytic granules, in response to reactivating virus. We also establish that restraint stress during the primary infection results in a 54% reduction of virus-specific IL-7Rα^+ memory precursor cells in the TG at the peak of expansion. When mice stressed early were stressed again during latency, their T cell response may be further compromised. 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.

PREFACE

"Whence Reason o'er the world presides,
And man from brute, and man from man, divides;
Compares and measures by imagined lines
Ellipses, circles, tangents, angles, sines;
Repeats with nice libration, and decrees
In what each differs, and in what agrees;
With quick volitions unfatigued selects
Means for some end, and causes of effects;
All human science worth the name imparts,
And builds on Nature's base the works of arts."

Erasmus Darwin, from *The Temple of Nature*
published posthumously in 1803

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1.0 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Control of latent herpesvirus infections is a multifaceted process that involves the virus, the infected cell, and the host immune system. Upon ocular herpes simplex virus type 1 infection, the virus establishes a latent state in the trigeminal ganglia, where it remains for the life of the host. Viral latency is controlled in part by surveillance by activated memory T cells of the host immune system. Various stimuli result in the reactivation of the latent virus, leading to recurrent disease at the site of primary infection and potentially to more severe consequences. Psychological stress is a well-known inhibitor of the T cell response to herpes simplex virus and has been associated with reactivation of latent virus. **We hypothesized that psychological stress could induce viral reactivation by compromising the efficacy of ganglion-resident $CD8^+$ memory T cells.** We tested this hypothesis using a mouse model of herpes simplex virus type 1 corneal infection and latency and restraint, a well-established model of psychological stress.

1.2 HERPES SIMPLEX VIRUS TYPE 1

1.2.1 Epidemiology and Disease

Herpes simplex virus type 1 (HSV-1, also known as human herpesvirus 1 [HHV-1]) is a ubiquitous human pathogen that infects a majority of people worldwide. In the US, seroprevalence of HSV-1 increases from 44% in young adults (aged 12-19 years) to 90% in persons aged >70 years, and overall HSV-1 seroprevalence is 68% (1). These trends are similar to those observed in populations worldwide, although HSV-1 prevalence may actually vary widely by country, region within a country, population subgroup, socioeconomic status, and risk behavior (1,2).

Primary HSV-1 infection is often asymptomatic but may manifest as a cold sore or fever blister on the mouth or lip. Most HSV-1 infections occur early in life via close contact with infectious mucosal secretions (3). The virus can infect the sensory nerves that innervate the site of primary infection, travel up the axon to the neuron cell body, and establish latency, a lifelong association of nonreplicative viral genomes within neurons. Host and viral genetic determinants and the initial inoculum size influence the amount of latent viral genomes that are retained, as well as the ability of the virus to reactivate from latency, the process by which latent genomes are replicated and produce infectious progeny (4-7). Up to 5% of the normal adult population asymptomatically sheds HSV-1 at any given time (3). Clinical HSV-1 disease can lead to a spectrum of consequences including herpes labialis, gingivostomatitis, herpetic stromal keratitis, endophthalmitis, and potentially lethal encephalitis. Pregnant woman can transmit virus to their child in utero, intrapartum, or postnatally. These infections can prove fatal to the fetus or newborn (3).

HSV-1 is genetically very similar to HSV-2, a sexually transmissible pathogen that often leads to genital lesions. HSV-1 can also infect the genital area and establish latency in dorsal root ganglia. However, genital HSV-1 infections are usually less severe clinically and reactivate less frequently than HSV-2 (3). These phenomena may be attributable in part to a difference in the neuron subtype in which HSV-1 and HSV-2 preferentially establish latency (8).

There are a wide variety of strategies for designing vaccines against HSV-1 and HSV-2, both prophylactic and therapeutic. One major hindrance to vaccine development is the inability of circulating antibody to effectively limit recrudescent disease. At the present time, there are no vaccines that prevent either HSV infection or reactivation from latency (3,9). Therefore, HSV disease management is mainly limited to the use of antiviral drugs such as acyclovir. As these drugs target viral DNA synthesis, they are only effective during productive infection and neither prevent the virus from establishing latency and may not prevent latent virus from reactivating (3,10,11).

1.2.1.1 Herpes Stromal Keratitis

In the US, HSK is the leading cause of infectious disease-related blindness and a leading indication for corneal transplantation (11). In humans, HSK is an immunopathology that results from periodic reactivation and recurrent viral antigen presentation in the cornea. Conversely, most animal models of HSK derive from primary disease (12). The immune infiltrate in the cornea that follows ocular HSV-1 infection produces inflammatory cytokines, such as IFN-γ and IL-2, which, although protective against skin disease, are immunopathologic in the cornea (13-15). Despite the requirement for viral replication in the induction of HSK in mice (16), HSK in humans does not usually respond to anti-herpetic drugs and is treated clinically with corticosteroids, to reduce inflammation (17,18). For the studies presented in this dissertation, we

used the C57BL/6 mouse strain to model HSV-1 infection and latency: the abundance of immunologic reagents available, existence of a defined immunodominant H-2K^b-restricted viral epitope, and the development of HSK make this mouse model an excellent choice (19,20).

1.2.2 Morphology and Genome Structure

HSV-1 is a linear double-stranded DNA (dsDNA) virus and the prototypical member of the alphaherpesvirus sub-family of the family *Herpesviridae*. The HSV-1 virion (Figure 1) is approximately 200 nm wide and is comprised of four basic units: (i) a core containing the approximately 152 kbps dsDNA viral genome, (ii) an icosadeltahedral capsid comprised of viral proteins, (iii) a tegument layer containing viral proteins necessary for efficient initiation of viral transcription, and (iv) a lipid bilayer envelope derived from the host membrane with viral glycoproteins embedded within (21).

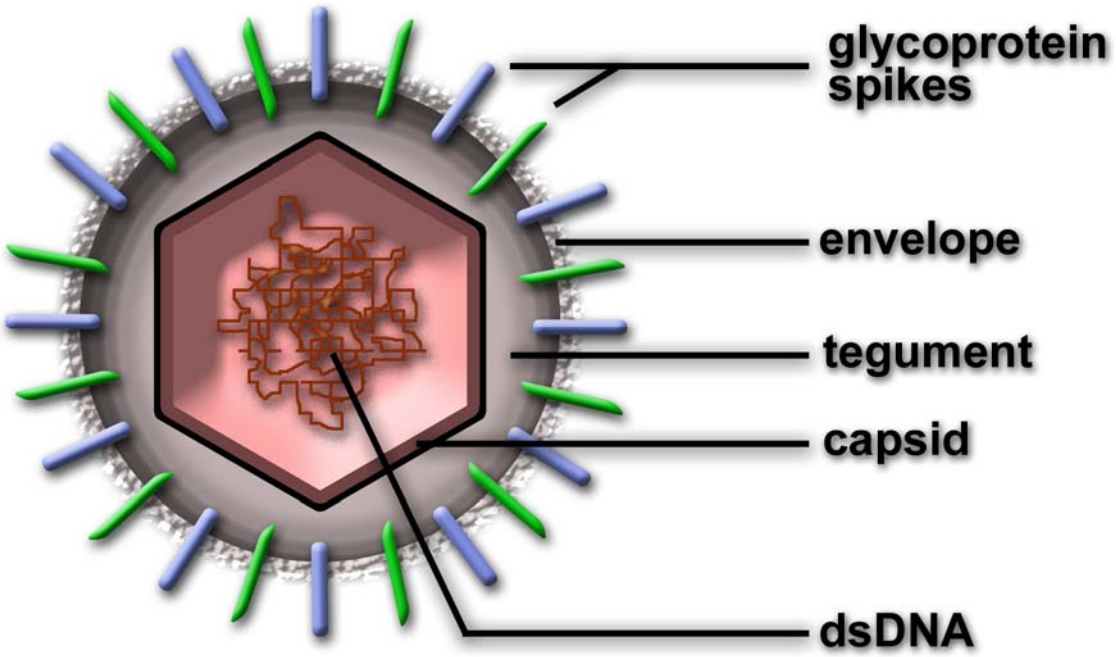

Figure 1. The HSV-1 virion.

Viral double-stranded DNA (dsDNA) is contained within the capsid. The capsid is surrounded by a tegument layer and an outer envelope from which various viral glycoprotein spikes extend. Figure not to scale.

The HSV-1 genome is segmented into two unique DNA segments, called the unique long (U_L) and unique short (U_S) regions, flanked by inverted repeats (Figure 2). The repeats of the U_L segment are designated *ab* and *b'a'*, and the U_S segment repeats are designated *a'c'* and *ca*. The genome contains approximately 84 open reading frames located on both DNA segments; some genes are present in two copies and located in the repeat regions. The genome size is not absolute, however, and may contain multiple copies of inverted repeats of variable size (21). The U_L and U_S segments can be found inverted relative to one another, and within a population of HSV-1, the viral genomes are present in equimolar concentrations of four isomers, designated P (prototype), I_L (inversion of U_L), I_S (inversion of U_S), and I_{SL} (inversion of U_L and U_S). All four isomers give rise to infectious virus (21).

Figure 2. Illustration of the HSV-1 genome.

The genome is divided into unique long (U_L) and unique short (U_S) portions partitioned by inverted repeat sequences at the termini (a, b, c) and internally (a', b', c'). Figure not to scale.

1.2.3 Life Cycle

The HSV-1 life cycle is divided into three basic categories: (i) productive, or lytic, infection, which results in the production of infectious progeny virus and lysis of the host cell; (ii) non-productive, or latent, infection, in which viral gene transcription is limited and no infectious progeny are produced; and (iii) reactivation from latency, the process by which viral gene transcription during latency leads to DNA replication and de novo production of infectious progeny virus in the absence of any tegument or virion components. Figure 3 shows a simplified illustration of lytic and latent infections.

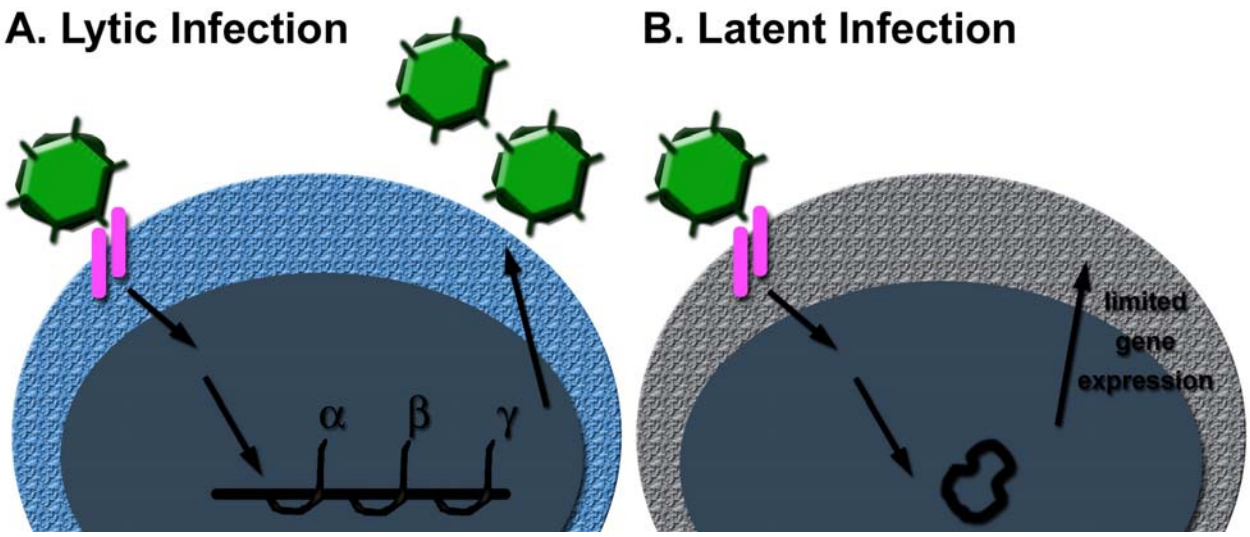

Figure 3. Lytic and latent infections.

(A) In a lytic infection, virus enters the cell via interactions with cell surface receptors (see text) and virus DNA is transcribed and translated in a tightly regulated, temporal manner. Viral DNA is synthesized, packaged into newly formed virions, and exported out of the cell. **(B)** In a latent infection, the virus enters the target neuron where the DNA is contained in a histone-bound extrachromasomal episome. During latency there is limited viral gene expression, but a major gene product produced is the stable mRNA LAT (see text). Figure not to scale.

1.2.3.1 Lytic Infection

Lytic infection is initiated upon infection of a susceptible target cell. Infection begins as viral glycoproteins on the surface of the HSV-1 envelope bind to target cell surface receptors. Glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM have all been shown to be expressed on the HSV-1 surface, but not all are critical for virus attachment and entry.

Attachment of HSV-1 to its target cell is mediated by binding of gC or gB to glycosaminoglycan moieties, such as heparan sulfate, on the cell surface. Entry of HSV-1 to its target requires binding of gD to one of several receptors on the cell surface, including the herpesvirus entry mediator (HVEM, or HveA), a member of the tumor necrosis factor (TNF) receptor superfamily; nectin-1 and nectin-2, members of the immunoglobulin (Ig) superfamily;

and 3-O-sulfated heparan sulfate. Virus entry into the target cell is complete upon fusion of the viral envelope to the target cell plasma membrane, a process that requires gB, gD, and the gH/gL complex. Following viral fusion, the nucleocapsid is transported to the nucleus with some of the tegument proteins. Other tegument proteins remain in the cytoplasm, such as virus host shutoff (vhs), a protein that immediately begins to degrade host mRNA. When the nucleocapsid reaches the nuclear pore, the viral DNA is released into the nucleus. There the viral tegument VP16 associates with host cell factor (HCF) and Oct-1, a transcription factor that binds to the TAATGARAT sequence in viral immediately early (IE, or α) promoters.

HSV-1 gene transcription is organized in a tightly regulated, sequential manner (Table 1). The α genes are the first to be transcribed; their transcription does not require de novo viral protein synthesis. IE genes encode proteins that are important for turning on the transcription of the other two classes of genes: early (E, or β) genes, which are important for viral DNA synthesis, and late (L, or γ) genes, which are important for capsid assembly and include the glycoproteins. The γ genes are split into two subclasses, γ1, which can be transcribed prior to DNA replication but peak after DNA synthesis, and γ2, which require DNA replication for their transcription.

Table 1. Classes of HSV-1 genes.

Class	Kinetics of transcription	Functions
Immediate Early (α)	Prior to de novo protein synthesis	Transactivate β and γ gene transcription; repress host and α gene transcription; immune evasion
Early (β)	After de novo protein synthesis, prior to DNA replication	DNA replication machinery
Leaky-Late (γ1)	Initiated prior to DNA replication, majority transcribed after	Virus attachment and assembly; tegument proteins
True-Late (γ2)	Requires DNA replication for transcription	Capsid assembly; virus attachment; tegument proteins

IE (α) genes are transcribed as early as 2 to 4 h post infection (p.i.). There are six α genes, infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, ICP47, and $U_S1.5$, a protein encoded in a region overlapping with ICP22. Although all α genes except ICP47 can stimulate gene expression, the two most important α proteins are ICP0 and ICP4. ICP0, an E3 ubiquitin ligase, is a promiscuous transactivator of transcription, but does not itself bind DNA. ICP0 is not essential for viral replication, yet it is important for optimal infection, gene expression, and reactivation from latency (21-23). ICP0 may also be important in the fate decision between lytic and latent infections, although its role is still contentious (24-27). Additionally, ICP0 has a role in repressing interferon (IFN)-stimulated genes, a collection of genes that are central to the host defense against HSV-1 infection (28-31). The other major α gene is ICP4, a potent transactivator of transcription for β and γ genes, and a repressor of α gene expression (21). ICP4 can bind DNA at both consensus and nonconsensus sites. Indeed, most β and γ promoter regions do not contain putative ICP4 binding domains. ICP4 also binds host transcription factors and modulates their activity as a method for indirectly activating viral genes (32-34). It should be noted that the promoter regions for α, β, and γ genes are structurally quite different. As depicted in Figure 4, α promoters contain a TAATGARAT element and binding sites for various transcription factors, often including a putative ICP4 binding site. β promoters contain upstream binding sites for transcription factors, but do not have a consensus ICP4 binding sequence. γ promoters contain minimal upstream transcription factor binding sites, but do have an initiator element (Inr) and often contain downstream activation sites (21,35).

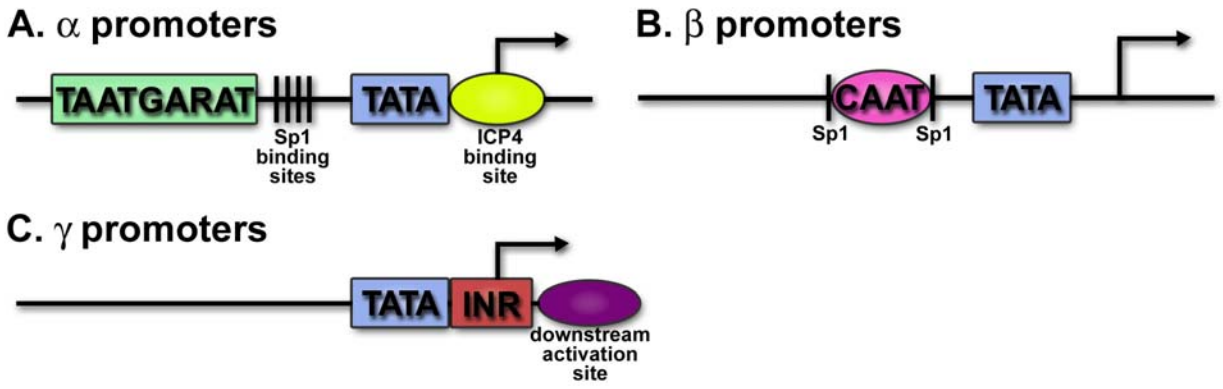

Figure 4. Schematic illustration of HSV-1 gene promoters.

General structure of α, β, and γ promoters is shown. The number and arrangement of *cis*-acting regulatory elements varies among promoters of each class. **(A)** α promoters contain a TATA box and upstream TAATGARAT elements, as well as binding sites for transcription factors (i.e. Sp1) and, in some α promoters, ICP4, which may repress gene transcription. **(B)** In addition to a TATA box, β promoters contain upstream binding sites for transcription factors, here denoted as Sp1 and a CAAT box. **(C)** γ promoters contain a TATA box, and an initiator element (INR). Some γ promoters also contain a downstream activation site. Adapted from (35). Figure not to scale.

The β genes reach peak rates of expression between 4 and 8 h p.i. This gene class contains the viral DNA replication machinery, including the origin binding protein, single-stranded DNA binding protein, the DNA helicase-primase complex, the DNA polymerase, and various protein kinases. HSV-1 DNA has traditionally been thought to replicate first by theta replication, and then later, by rolling circle replication (21,36), although recent data has called this process into question (24). Rolling circle replication results in the production of viral DNA concatemers that are then cleaved as the DNA is packaged into new virions.

Generally, the late (γ) genes do not reach peak gene expression until after DNA synthesis. The leaky-late (γ1) genes are expressed in low amounts early in infection, but increase several-fold after DNA replication has occurred. The true-late (γ2) genes are absolutely dependent on viral DNA synthesis for their expression; these differences may be due in part to variations in the

structures of their promoters. γ genes include the capsid assembly proteins, the glycoproteins important for virus attachment and entry into target cells, antiapoptotic proteins, and various tegument proteins, including VP16, the activator of α gene expression upon infection (21,35).

1.2.3.2 Latent Infection

A hallmark of herpesvirus infections is latency, a quiescent state in which viral gene expression is limited, replication of viral DNA does not occur, and viral progeny are not synthesized. HSV-1 establishes latency in the peripheral neurons that innervate the site of primary infection. In our corneal infection model, latency is established in the trigeminal ganglion (TG), the sensory ganglion that innervates the eye, nose, and mouth. Here the viral DNA exists as an extra-chromosomal episome tightly associated with histones (37). The mechanism by which the virus enters latency is not fully characterized, but may involve circularization induced by ICP0 downregulation (24). As opposed to the well-characterized regulation of viral genes observed during lytic infection (Section 1.2.3.1), in latency there is minimum expression of viral genes, and viral proteins are difficult to detect (38). One gene product that is abundantly expressed during latency is the latency associated transcript (LAT), a lariat-shaped intronic RNA species, in part antisense to ICP0, that may act in neurons to help prevent apoptosis (39,40). LAT may also be important for both the entry into, and escape from, neurons, but is not required for establishment of latency (41-43). A newly discovered micro RNA species encoded by the LAT gene (miR-LAT) has been shown to prevent apoptosis in cell culture, and may work in vivo by enhancing survival of latently infected neurons by inhibiting transforming growth factor (TGF)-β signaling (44). It should also be noted that the sensory neurons within the TG are a heterogeneous population, phenotypically defined by at least two

populations based on expression of the A5 and KH10 surface markers (4). Both neuron subtypes are infected proportionally in acute infection, but latency is preferentially established by HSV-1 in the A5 neuronal population and by HSV-2 in the KH10 neuronal population (8). This difference could be reversed using recombinant viruses that expressed the opposite LAT molecules (8,45). Thus, expression of LAT, and, perhaps, miR-LAT expression, may promote survival of latently infected neurons by inhibiting either virus-induced, or immune-mediated, apoptosis.

1.2.3.3 Reactivation from Latency

The exact point where latency ends and reactivation begins is still rather contentious. HSV-1 latency is classically defined as the retention of a functional viral genome in the extended absence of infectious virus particles (46). Clinically, this definition makes sense since recurrent disease can only occur in the presence of infectious virus. Therefore, having reactivation begin at virion formation allows for the transient or extended expression of a limited array of viral lytic genes during latency in the absence of DNA replication, as is seen with other herpesviruses. Figure 5 shows a simplified cartoon overview of the establishment of, and reactivation from, latency in our corneal infection model.

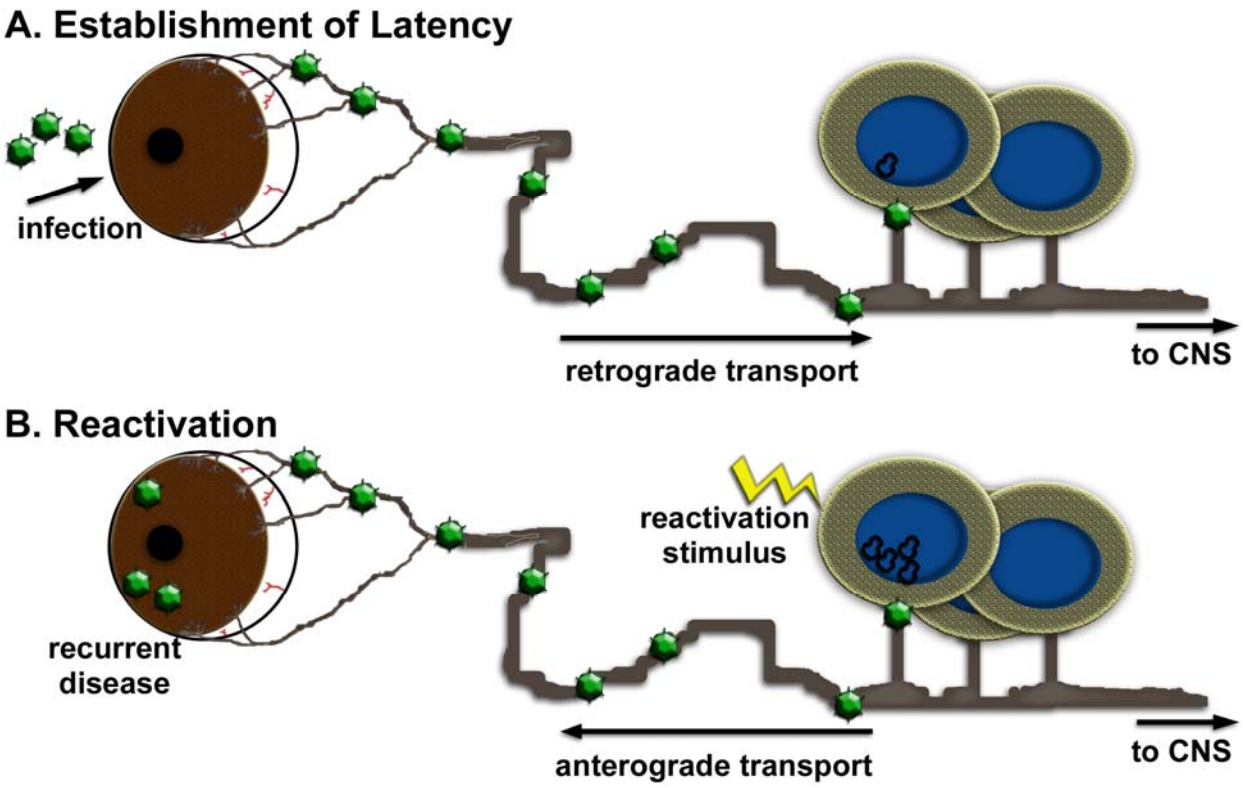

Figure 5. Latency and reactivation.

A cartoon depiction of the two main phases of the virus life cycle in the trigeminal ganglion in our model. **(A)** Establishment of latency: virus infects the cornea where it gains access to sensory nerve termini. Virus then travels up the axon by retrograde transport to the trigeminal ganglia, where it has restricted gene expression and does not produce infectious virions (see text). **(B)** Reactivation: one of any number of stimuli initiate viral DNA replication in the TG, leading to production of infectious virus particles which travel down the axon by anterograde transport back to the site of initial infection, leading to recurrent disease (see text). Figure not to scale. CNS, central nervous system.

Exactly what viral genes are required for reactivation from latency is currently unknown. Limited gene expression has been observed in the absence of virion formation, including the γ2 gene gC (38,47,48). However, recombinant viruses lacking proteins such as ICP4, ICP8 and TK are capable of establishing latency (evidenced by LAT expression), but fail to reactivate (21,49). In cell culture, recombinant viruses missing all or some α genes establish quiescent infections, but do not replicate (50), but addition of ICP0 to quiescently infected fibroblasts or latently

infected neurons in culture can induce reactivation (51,52). Spontaneous HSV-1 reactivation is extremely rare in the mouse (38,53), but cellular stresses such as hyperthermia, UV irradiation, and TG explant can all induce reactivation of latent virus (54,55). Of these, UV irradiation and hyperthermia have been shown to induce ICP0 promoter activity (56). In some models, reactivation is accompanied by changes in cytokines, chemokines, or upregulation of the intracellular cyclin-dependent kinase (cdk)-2 and cdk-4 (57,58).

Recent work in our lab and others has begun to delineate a role for the immune system in actively maintaining latency by preventing viral reactivation (reviewed in (46,59), and discussed below). Reactivation can be inhibited by the antiviral cytokines gamma-interferon (IFN-γ) and tumor necrosis factor (TNF)-α (52,60), both of which are expressed in latently infected ganglia (61,62). Hyperthermia-induced reactivation is increased in IFN-γ receptor knockout mice versus controls, suggesting IFN-γ plays a protective role in vivo (63). Indeed, addition of IFN-γ to TG cultures can prevent reactivation by inhibiting ICP0 promoter activity as well as events downstream of the gC promoter (52). $CD8^+$ T cells, which are present in latently infected ganglia, can prevent reactivation ex vivo (64-66), and have been shown to produce IFN-γ and TNF-α in response to antigenic stimulation (65,67). Thus, a combination of host immune, neuron intrinsic and viral factors may be required for full induction of reactivation of latent HSV-1.

1.3 IMMUNITY TO HSV-1

The pathogenesis of HSV-1 is tempered by the actions of the host immune system. There are two main mechanisms of mammalian immunity: the innate and adaptive immune responses. The

innate immune response is not restricted by pathogen specificity, but instead relies on pattern recognition receptors to distinguish self from non-self. Adaptive immunity, on the other hand, is generated toward specific defined antigens and results in the formation of a memory response that allows for a quicker, more robust secondary response in the event of re-infection or reactivation of a latent infection. An innate immune response does not culminate in memory generation; each subsequent response is neither stronger nor weaker than the previous response.

1.3.1 Innate immune response to HSV-1

In our ocular infection model, HSV-1 first infects corneal epithelial cells at the site of inoculation. In response, these cells produce type I IFN antiviral cytokines, such as IFN-α and IFN-β. The presence of IFN-α induces the migration of macrophages and polymorphonuclear (PMN) cells such as neutrophils into the central cornea. This primary immune infiltrate, consisting of macrophages, neutrophils, natural killer (NK) cells, and γδ TCR^+ cells (68-71), is most important in initial clearance of HSV-1 from the cornea. Despite the innate immunity in the cornea, virus undoubtedly gains access to sensory nerve termini and reaches the TG. Although unique intrinsic properties of neurons may contribute to the control of HSV-1 replication in the TG, the host innate and adaptive immune systems provide a second level of protection. Indeed, the initial control of HSV-1 replication within the TG clearly involves components of innate immunity. HSV-1 begins to replicate in the TG within 2-3 days of corneal infection (72), although the majority of genomes enter latency immediately upon accessing the TG Concurrently, the TG is infiltrated by macrophages and γδ TCR^+ T cells (73,74). The macrophages produce antiviral molecules such as nitric oxide (NO) and TNF-α (75). Depleting

macrophages, blocking NO production, or neutralizing TNF-α during the first 5 days after HSV-1 corneal infection significantly increases viral titers and the number of infected neurons in the TG (75). These findings suggest that macrophages function within the TG to limit virus replication and lateral spread.

Depletion of γδ TCR^+ T cells from mice during the first 7 days after corneal infection also increased virus replication and spread and eliminated most of the early type II interferon (IFN-γ) production within the TG (75,76). IFN-γ not only possesses antiviral activity, but is also a potent activator of macrophages, inducing their production of NO and TNF-α (77). Thus, early control of HSV-1 replication and spread in the TG appears to involve an orchestrated response in which γδ TCR^+ T cells activate macrophages to produce antiviral compounds. Type I IFN also appear important in the early control of HSV-1 replication in the TG; they are produced after corneal infection concurrent with virus transport to the ganglion (78). In addition, over-expression of IFN-α1 in astrocytes inhibited HSV-1 replication and establishment of a latent infection in the TG (78). Another study showed that in primary TG cultures transduced with an adenoviral vector expressing IFN-β, HSV-1 replication was repressed in a dose–dependent fashion (79). Together, these findings demonstrate that innate immunity is primarily responsible for controlling early HSV-1 replication in the TG following primary infection, which is not surprising in light of the fact that the bulk of viral replication occurs during the first 5 days after infection, a time when the adaptive immune response is developing in the lymphoid organs.

1.3.2 Adaptive Immunity to HSV-1

In addition to the innate immune response, the body defends itself by the adaptive immune response, a system that can respond to specific pathogens and develops memory, the ability to respond faster and more vigorously upon reinfection. The main players in the adaptive immune response are professional antigen presenting cells, antibody-producing B cells, and T lymphocytes. T cells come in many flavors with many functions, but the three main classes of T lymphocytes are $CD4^+FoxP3^+$ T regulatory cells (Treg), $CD4^+$ T helper cells (Th), and $CD8^+$ cytotoxic T lymphocytes (CTL). For simplicity and relevance, the bulk of background information presented here will focus on the T cell response, particularly $CD8^+$ T cells.

T cells originate in the bone marrow from hematopoietic stem cells and, in the thymus, undergo several linear phases that differentiate pluripotent progenitors into either $CD4^+$ or $CD8^+$ T cells. These two coreceptors are instrumental in the recognition of host major histocompatibility complexes (MHC) by T cells. In the thymus, T cells undergo gene rearrangements that result in the expression of cell surface T cell receptors (TCR). The T cells are then selected first by positive selection, in which cells expressing TCR that do not recognize MHC molecules expressed by cells in the thymus are eliminated, and then by negative selection, in which cells that react to MHC molecules and ubiquitous self ligands are eliminated. These processes ensure that the cells that migrate out of the thymus are specific for non-self antigens that can be presented by specific MHC types. The coreceptors CD8 and CD4 are important for reaction to MHC class I and MHC class II, respectively, although in the absence of $CD4^+$ T cells and in other cases, MHC class II-restricted $CD8^+$ T cells may develop (80-82). Once T cells have left the thymus, they migrate to lymph nodes (LN) and other regions of lymph tissue such as the Peyer's patches and spleen. There, these naïve, antigen-inexperienced cells wait for the proper

antigen stimulation to proliferate and form a memory response. It has been estimated that the frequency of naïve T cells specific for any given antigen specificity is remarkably low (83-86).

The T cell response to acute pathogens in vivo can be divided into four main phases, i) activation and expansion, ii) contraction, iii) long-lived memory,and iv) vigorous recall (87). Before a T cell response can be initiated, however, professional antigen presenting cells (APC) such as Langerhans cells or dendritic cells (DC) must pick up antigen at the initial site of infection. MHC class I-restricted peptides are generated by the immune proteasome, and are generally 8-10 amino acids long. The DC that is expressing the antigen travels to the draining lymph node (LN) and there presents the antigen itself or potentially passes the antigen off to non-migratory, LN-resident APC (88).

T cell priming in the LN requires three distinct signals that combine to regulate the potency of the primary T cell-mediated immune response as well as secondary responses. The first signal is cognate MHC and antigen complexes presented by professional APC. The second signal is costimulation. Mature, activated DC express costimulatory ligands on their cell surface that react with receptors on the surface of the responding T cell. For $CD8^+$ T cell stimulation, important costimulatory DC-expressed molecules appear to be B7.1 and B7.2, which bind to CD28 on the T cell (89,90), and may be most important early in priming (91), and 4-1BBL, which binds to 4-1BB (92,93), and may be more important later in the primary response and for secondary responses (91). These molecules, among others, help ensure that the $CD8^+$ T cell responds by expansion and development of memory instead of a state of functional anergy, or tolerance (94). The third signal is inflammation, which can be provided by IL-12, type I IFN, or signals from toll-like receptor (TLR) ligands. In some models, IL-12 promotes proliferation or cell survival during the primary response or plays a role in the development of cytotoxicity (95-

98). Type I IFN can also promote activated T cell survival and differentiation into effector cells (99-101). The effects of these stimuli may overlap and vary greatly depending on the model of antigen stimulation or infection (102,103). Interestingly, IL-12 signals have recently been shown to influence the expression of the transcription factor T-bet, which may be important in the commitment decision between becoming a short-lived effector cell or a long-lived memory cell (104,105).

It is not certain exactly what length of interaction is required for APC to present antigen to a T cell to initiate expansion, the window of presentation has been experimentally measured to be within the first 3 d of infection (106,107) and antigen presentation in the LN has been observed in as little as 2 h following HSV-1 infection in a transgenic mouse model, even though expansion of the activated cells did not begin until 24 h post infection (108,109). The term expansion refers to the proliferation of cells (up to 50,000-fold over naïve numbers) following activation and the migration out of the lymph nodes and into the periphery (110,111). Migration out of the lymph nodes is mediated by various chemokines and their receptor expression on the newly activated $CD8^+$ T cells, as well as downregulation of adhesion molecules such as CD62 ligand (CD62L) on the T cells. In ocular HSV-1 infections, DC in the cornea take up HSV antigens and migrate out of the eye to draining LN, where the antigens are presented to naïve T cells with proper costimulation. During expansion, T cells migrate out of the LN to the cornea (the site of initial inflammation), the TG (the site of viral latency), and numerous other tissues and organs, where, following contraction (a dramatic loss of effector cells), persistent memory pools remain for the life of the host (65,67,68,112-115). These results are in accordance with other studies showing promiscuous localization of $CD8^+$ memory T cell responses (116-118). Virus-specific effector $CD8^+$ T cells at sites of infection with a variety of viruses have been

shown to express the receptors CCR2, CCR5, and CXCR3, matching the typical chemotactic cytokine (chemokine) profile of RANTES, MIP-1α and β, IP-10, and MCP-1 (119). RANTES and MIP-1 bind to CCR5, IP-10 binds to CXCR3, and MCP-1 binds to CCR2 (120). IP-10 and RANTES have been demonstrated to be in the TG of mice latently infected with HSV-1 (62,121). These chemokines are not only important for the migration of effector cells to target tissues, but also for maintaining T cell numbers at peripheral sites.

Since latency is uniformly established by 10 d in our corneal infection model, and contraction leaves a stable memory population in the TG and elsewhere to monitor the latent virus, the factors that regulate the generation and maintenance of the memory pool are of considerable importance.

It is not yet known exactly when in an immune response memory precursors are programmed to become memory cells. Expression of the IL-7Rα subunit (CD127) during the effector phase has been shown to be correlated with generation of T cell memory, and IL-7Rα^+ effector T cells have come to be known as memory precursor cells (122). These memory precursor cells somehow survive the contraction phase and develop into long-lasting memory cells, which, following an acute infection, may not require TCR-mediated signals for their continued survival (123,124). However, expression of IL-7Rα is not sufficient for survival following acute viral infections or peptide-pulsed DC immunizations (125-127), and IL-7 signals are not required for the generation of a memory pool (128,129). The surface molecule killer cell lectin-like receptor G1 (KLRG1) has recently been associated with development of short-lived effector cells (104,125). KLRG1 expression appears to influence cell senescence, and upregulation of KLRG1 may signal a commitment to a shortened lifespan (104,130) and reduced functionality (131). Additionally, expression of the transcription factors T-bet and eomesodermin

may influence the lineage decision of newly activated T cells (105,132). Inflammation may play a role in the generation of memory cells, as IL-12 has recently been reported to regulate expression of T-bet and eomesodermin (104,133,134).

Recently, Chang, et al. have shown that when a $CD8^+$ T cell is primed by an APC expressing its cognate peptide/MHC complex and proper costimulatory molecules, the T cell divides in an asymmetric manner, sequestering certain proteins (CD3, CD8, LFA-1) to the proximal pole and other proteins (PKC-ζ) to the distal pole. In a TCR transgenic system, distal daughter cells protect better than proximal daughters following bacterial infection 30 d after transfer into a naïve host (135). These data suggest that T cell lineage commitment may occur as early as the first division during priming.

It is intriguing that the fate of a T cell could be determined as early as priming, but environmental factors certainly play a role in the establishment and survival of the memory pool. Inflammation and antigen load, in particular, appear to mediate the rate and extent of the contraction phase, and the rate at which memory cells develop (127,136-138). Chemokine expression may also influence memory cell generation. A recent report identified downregulation of the chemokines CCL19, CCL21, and CXCL13 in the spleen during acute infections, and during immunizations with virus-like particles (139). The transient downregulation occurred even while chemokines such as MCP-1 and RANTES were increased, and it was dependent on the presence of $CD4^+$ T cells and expression of IFN-γ (139). The downregulation may also influence the expression of sphingosine-1-phosphate (S1P)-1, a lipid molecule that is required for lymphocyte egress from lymphoid organs (140). Type I IFN induce CD69 upregulation and binding to S1P1, mediating S1P1 downregulation, and retaining the activated cells within lymphoid tissues (141).

Mature $CD8^+$ T cells appear to exist in at least four stages of differentiation: naïve, effector, effector memory, and central memory. These stages have been defined phenotypically based on differential expression of various receptors and molecules, outlined in Table 2. Naïve cells become effectors upon priming, and the majority of effector cells are eliminated during the contraction phase, leaving populations of the effector memory and central memory phenotypes.

Table 2. Phenotype of mature $CD8^+$ T cells.

	CD62L	CD18	CD44	CD69	Ly6C	CCR7
Naïve	hi	low	low	-	low	hi
Effector	low	hi	hi	+	hi	low
Effector Memory	low	hi	hi	+/-	hi	low
Central Memory	hi	hi	hi	-	hi	hi

In systemic acute infection models, the majority of memory cells are of the central memory phenotype, and their numbers are proportional to the initial burst size during expansion (142). Persistent or latent infections, however, result in memory cells that persist as effector memory cells (143). $CD8^+$ effector memory T cells are functionally distinct from central memory cells, and the size of the effector memory $CD8^+$ T cell pool is less dependent on the initial burst size (144). Perhaps the most striking difference between effector and central memory cells are their localization within the immune host. While central memory cells reside mainly in lymphoid organs, effector memory cells reside primarily in non-lymphoid tissues, and may form the first line of defense against reinfection (144). Functionally, $CD8^+$ effector memory T cells are heterogeneous. In some systems they appear very similar to effector cells in that they produce IFN-γ and are cytotoxic directly ex vivo. These cells are functionally distinct from central memory cells that produce little IFN-γ, lack granzyme B expression, and are not cytotoxic, but they maintain perforin expression. In other systems, $CD8^+$ effector memory cells produced large

amounts of IFN-γ but lacked lytic function (143). The reason for these differences has not been determined. A recent study by Woodland and colleagues investigated memory function closely and determined that it was the activation phenotype of the memory cell (i.e., CD27, CD43, and CXCR3 expression) that determined the functional hierarchy of memory, as opposed to the localization phenotype (i.e., CD62L and CCR7 expression) that has been traditionally used to distinguish memory populations (145). In their analysis of $CD44^{+}CD8^{+}$ T cells, they defined populations, $CXCR3^{hi}CD27^{hi}CD43^{lo}$ cells were the best, $CXCR3^{hi}CD27^{hi}CD43^{hi}$ cells were intermediate, and $CXCR3^{lo}CD27^{lo}CD43^{lo}$ cells were the poorest responders, and each population contained cells of both effector memory and central memory phenotypes (145). These data confirm a previous study examining activation status and function of memory cells to chronic infections in humans (146).

In our model, virus-specific $CD8^{+}$ T cells enter the TG beginning at 6 d p.i., and their numbers peak around 8 d p.i. (65). Whether there is a secondary expansion within the TG is currently under investigation. Expansion in the LN is followed by a severe contraction of $CD8^{+}$ T cell number. How much of contraction is due to programmed cell death and how much is due to migration out of the LN is unknown. The contraction phase is observed throughout the mouse, however, and in the TG, there is approximately a 80-90% reduction in $CD8^{+}$ T cell number by 34 d p.i. compared to the peak at 8 d p.i. (65). A sizable population of $CD8^{+}$ T cells remains in the host for the life of the animal (65,68). This retention of $CD8^{+}$ T cells is also observed in humans, where latently infected neurons remain surrounded by $CD8^{+}$ T cells throughout life (114,115). In humans and mice, the population of virus-specific T cells that reside in the TG throughout latency are in an activated state (65,115), whereas virus-specific memory $CD8^{+}$ T cells in non-infected organs are not activated (67). The ganglion-resident $CD8^{+}$ memory T cell pool is best

defined as activated effector memory cells, as they express high levels of CD44, CD69 and IL-7Rα, produce IFN-γ and TNF-α upon ex vivo stimulation, are cytotoxic directly ex vivo, and are positive for granzyme B and perforin (65,67,147,148). Initial migration into the TG appears to require extensive cell division and upregulation of the activated isoform of CD43, but CD43 is soon downregulated at peripheral sites (149).

The factors that govern the maintenance of a memory population are still under intense investigation. Exposure to antigen may be required for maintenance of effector memory populations (which often reside in tissues infected with viruses that cause persistent or latent infections) (150), whereas central memory populations may be maintained through homeostatic proliferation (123,151-153). In acute infections, $CD8^+$ T cell memory forms following the elimination of the pathogen, and is maintained by homeostatic proliferation regulated by the cytokines IL-2, IL-7, and *trans*presentation of IL-15 (154-160). Chronic infections, on the other hand, appear to generate an "exhausted" $CD8^+$ T cell memory population that is incapable of responding to homeostatic signals from these cytokines (161-164). Maintenance of this population requires constant antigenic exposure, resulting in reduced expression of the IL-7 and IL-15 receptors, and upregulation of inhibitory receptors such as programmed death 1 (PD-1). The resulting memory population is functionally impaired, lacking cytotoxic function and incapable of producing IFN-γ and TNF-α (165-168). Function can be transiently restored by systemic treatment with antibody to the PD-1 ligands PD-L1, expressed promiscuously in tissues, and PD-L2, expressed only on APC (165,169-171). Recently, interaction of PD-L1 with B7-1 on T cells was shown to repress T cell activation and cytokine production (172).

Latent HSV-1 infections represent a situation where $CD8^+$ T cells are intermittently exposed to low levels of antigen exclusively in the sensory ganglia. This compartmentalized

persistent expression of viral antigens allows for comparison of the requirements for maintaining $CD8^+$ memory T cell populations in infected and non-infected tissues of the same animal (67). Here, neither IL-15 nor IL-2 are required for the maintenance of $CD8^+$ memory T cell numbers in the TG, presumably due to persistent low level antigen stimulation. In contrast, the HSV-specific memory population decays over time in the absence of IL-15 in the lungs, where there is no latent virus or viral antigen expression. IL-15 deficiency was also associated with a functional compromise in IFN-γ synthesis and degranulation, but not TNF-α production (67), suggesting that even though the numbers of memory cells remained constant in the TG, their ability to monitor latently infected neurons may have been impaired. The results of this study confirm a previous report in which IL-15 administration protected mice from a lethal systemic HSV-2 infection (173). Another rather intriguing property of the TG is the potential for regulation of the $CD8^+$ memory T cell response by the neurons themselves. TG neurons have been shown to express the nonclassical MHC-Ib molecule Qa-1^b, which binds to the NK cell receptor G2A family (NKG2A) inhibitory receptor and CD94 heterodimer complex expressed on $CD8^+$ T cells in the TG (148,174). Blocking this interaction results in cytolysis of neurons ex vivo (148), which presumably does not normally occur, as $CD8^+$ T cells have been shown to be able to prevent reactivation in an IFN-γ-dependent, non-cytolytic mechanism (52,60,64). MHC-I expression is also required for the maintenance of the HSV-specific $CD8^+$ memory T cell pool. Although specific antigen presentation by cells within the ganglion is not required for the infiltration of virus-specific cells, expression of granzyme B decreases in virus-specific $CD8^+$ memory T cells when the immunodominant H-$2K^b$-restricted $gB_{498-505}$ epitope cannot be presented in the ganglion by parenchymal cells (147). Thus, it appears that antigen presentation by MHC-I molecules within the ganglion during latent HSV-1 infection is instrumental for

maintenance of a proper memory T cell response, at least in part by overriding the requirement for IL-15 (67,147). Whether the MHC signals are provided by the latently infected neurons themselves is still unknown, but neurons have been shown to upregulate MHC-I during acute HSV-1 infection (175) and IFN-γ can induce MHC-I expression in neurons (176), leaving the possibility open that latently infected neurons can positively regulate the $CD8^+$ memory pool via presentation of MHC-I. Indeed, a recent study demonstated that virus-mediated downregulation of MHC-I increased reactivation from latency (177). Additonally, $gB_{498-505}$-specific $CD8^+$ T cells in latently infected ganglia polarize their TCR toward select neurons, suggesting the establishment of an immunologic synapse between the T cell and the neuron (65), and thus, another indication of neuronal MHC-I expression during latency.

Virus-specific $CD8^+$ T cells can prevent HSV-1 reactivation from latency in ex vivo TG cultures in an MHC-restricted manner (64,65,145,177). This protection is mediated at least in part by $CD8^+$ T cell synthesis and secretion of IFN-γ, a potent antiviral cytokine that can directly inhibit HSV-1 gene expression and prevent reactivation in ex vivo TG cultures (52,60,65).

Taken together, the above information all points to a complex system that involves antigen duration, inflammatory cytokines, chemokines, and $CD4^+$ T cell help that program, and then guide, a naïve cell to a memory cell fate. Once established, however, $CD8^+$ T cell memory populations face many additional mechanisms by which their numbers and functionality can be altered.

1.4 THE STRESS RESPONSE

The stress response is a simple term used to describe a complex and integrated series of events that connects the nervous, immune, and endocrine systems. In humans, ultraviolet (UV) radiation, psychological and physical stress, and immune compromise have all been shown to induce HSV-1 reactivation from latency. In animal models, stress, UV radiation, hyperthermia, hypothermia, shaking, and immunosuppression by dexamethasone treatment are all associated with inducing reactivation from latency. The ability of many of these stimuli to enhance reactivation may stem from the activation of the stress response. Stress has long been associated with HSV-1 reactivation, but currently stress has not been definitively shown to cause reactivation through any direct mechanism (178).

Figure 6 shows a cartoon depiction of the two main functional arms of the stress response: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS).

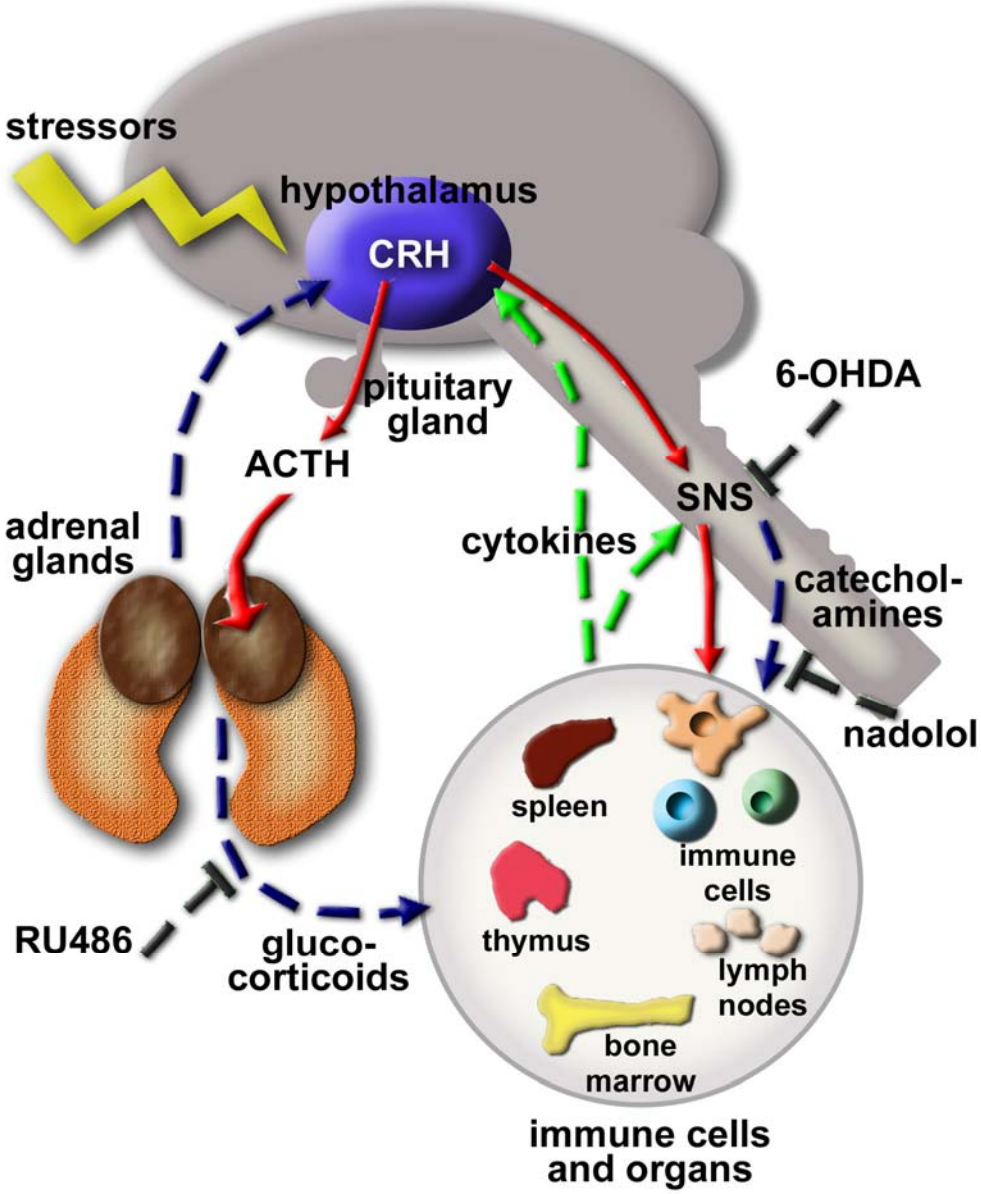

Figure 6. The stress response.

Blue dashed lines represent negative regulatory pathways, solid red lines represent positive regulatory pathways. Green dashed lines represent bimodal regulatory pathways. Black lines represent action of inhibitors. CRH, corticotropin releasing hormone; ACTH, adrenocorticotropin hormone; SNS, sympathetic nervous system; 6-OHDA, 6-hydroxydopamine; RU486, mifepristone. Adapted from (179,180).

1.4.1 The Hypothalamic-Pituitary-Adrenal Axis

The HPA axis is a series of signaling events that originate in the paraventricular nucleus of the hypothalamus upon physical, psychosocial, or inflammatory stimulation. Three important molecules of the human HPA axis are corticotropin releasing hormone (expressed in the hypothalamus), adrenocorticotropin-releasing hormone (released into the bloodstream by the anterior pituitary gland), and cortisol (synthesized and released from the adrenal glands) (180,181). These molecules act in a sequential manner resulting in the production and release of cortisol, the main glucocorticoid effector molecule of the human stress response. In mice, the adrenal steroid corticosterone serves this role. Glucocorticoids can then signal back to the hypothalamus and pituitary to downregulate the HPA axis (Figure 6 and (179)).

Adrenal steroids function through both type I (mineralcorticoid) and type II (glucocorticoid) receptors. Both receptor subtypes are expressed in immune cells, albeit at different levels in different tissues. Although type I receptors have a much higher binding efficiency for glucocorticoids, it is expression of type II receptors that determines the susceptibility of a cell to glucocorticoids. The type II receptors bind glucocorticoids, become activated through a conformational change, localize to the nucleus, dimerize, and then bind DNA (182). Corticosteroid binding globulin (CBG) binds free corticosteroids thus preventing an interaction with glucocorticoid receptors. An increase in corticosteroid production down-regulates CBG production, establishing a positive feedback loop of corticosteroid action.

In reference to a latent HSV infection, the effects of stress hormones and glucocorticoids, in particular, are not well understood. Changes in T cell localization or proliferation due to glucocorticoids may be sufficient to allow HSV to reactivate from latency. In addition to these effects, however, glucocorticoids may also directly act upon the latently-infected neurons,

establishing an environment within the cell favorable for HSV reactivation from latency. The effects of glucocorticoids in the brain may lead to either enhancement of memory, as is the case with acute stress, or damage of the hippocampus, cognition impairment, and general neurodegeneration, as with chronic stress (183,184). Glucocorticoids may also cause dendritic atrophy, but low levels can protect against neuronal apoptosis during development. On a molecular level, glucocorticoids may influence gene expression within latently-infected neurons, providing an environment favorable for HSV reactivation. Activated glucocorticoid receptor (GR) binding to CREB binding protein (CBP) may enhance an endogenous histone acetylation activity of CBP (182). This could allow for previously inaccessible DNA sequences of the latent viral episome to become accessible to transcription factors. Although this effect of glucocorticoids has not been conclusively shown to occur in neurons, a similar effect in neurons might be expected. Glucocorticoids may also repress gene expression by binding to, and interfering with, transcription factors such as AP-1, NF-κB, Sp1, Ets, NF-AT, and STATs, some of which are involved in transcription of HSV-1 genes (182,185). Glucocorticoids may also act by blocking downstream targets of these transcription factors.

There are many actions of glucocorticoids on immune function. It is generally accepted that glucocorticoids can reduce inflammation and, in many models, prevent lethality, in response to infection and other inflammatory stimuli. Conversely, blockade of the HPA axis has proven to increase mortality and inflammation in these same models (180). It is important to note that acute stress (generally less than an hour in animal models) may enhance the immune response whereas chronic stress (hours to days) often leads to immune suppression (181,186). Indeed, acute stress at the time of vaccination may even act as an adjuvant, inducing a better immune response (187).

Currently, however, the mechanism by which duration of stress exposure leads to this dichotomy is unknown.

Corticosterone in mice and rats has been shown to induce lymphoid cell migration out of peripheral blood and into tissues. Reduction of lymphocyte numbers from the blood is a common readout of stress. Neutrophils react oppositely, however, and neutrophil numbers in the blood are increased due to glucocorticoid treatment. These shifts in immune cell distribution are probably due to glucocorticoid-induced changes in adhesion molecule expression, either on immune cells or epithelial cells, or both. However, change in adhesion molecule expression may not be directly due to the glucocorticoid action, but as a consequence of an indirect effect of glucocorticoid downregulation of cytokine expression. Glucocorticoids have been shown to affect the expression of cytokines such as IFN-α, -β, and -γ, IL-1, IL-2, IL-4, IL-6, IL-8, and TNF-α (181,188). Cytokine receptor expression may also be regulated, as IL-2 receptor β and γc chains are inhibited, but IL-1 receptor and IL-6 receptor expression are enhanced by glucocorticoids (189,190). Interestingly, glucocorticoids induce a shift in immune responses from Th1 (IL-2, IFN-γ) to Th2 (IL-4, IL-6). Glucocorticoids also regulate T cell function by inducing apoptosis. Although this property of glucocorticoids has been conclusively shown during T cell development, many questions remain as to whether they can induce apoptosis in peripheral T cells (181). Glucocorticoids are also known to inhibit T cell proliferation and may regulate T cell function in part by blocking the cell cycle at G_0/G_1 (191). Additionally, glucocorticoids can inhibit antigen processing by impairing the generation of peptides, leading to reduced function of APC (192).

1.4.2 The Sympathetic Nervous System

The main effector molecules of the SNS are catecholamines, including the tyrosine-derived compounds epinephrine and norepinephrine (193). Catecholamines function via α- or β-adrenergic receptors on cells. SNS nerve fibers innervate the thymus, spleen, bone marrow, and lymph nodes (194-196). Upon SNS activation, norepinephrine (NE) can be released by sympathetic neurons, diffuse to surrounding areas, and affect immune cells. Lymphocyte responsiveness to NE depends on β-adrenergic receptor expression, which can be either positively or negatively regulated by activation (196). β-adrenergic stimulation leads to increase in intracellular cyclic AMP (cAMP). T cell proliferation has been shown to be impaired by β-adrenergic receptor signaling and other inducers of cAMP (197-199). In NK cells, specific lysis of target cells is compromised by catecholamines at certain concentrations, and the effect is reversible by a β-adrenergic receptor antagonist (200). Similar observations, including the inhibition of lytic activity, have been made in regard to T cells (201-203).

It is important to note that NE has been detected in lymphocytes, both by *de novo* synthesis and uptake from extracellular sources. This storage of NE could be an additional source of the catecholamine released during stress (204). Catecholamines have also been shown to be crucial for the establishment of optimal T cell responses to primary HSV-1 infection, suggesting their roles in regulating the immune system are varied (205,206).

The mechanisms by which catecholamines affect the HSV-specific immune response have been studied in some detail. When mice were restrained during a primary footpad infection, treatment with the β-adrenergic receptor antagonist nadolol restored cytotoxic ability of cells in the draining LN. Blocking Type II corticosteroid (glucocorticoid) receptors with RU486 did not

restore cytotoxic ability, suggesting that the stress-induced compromise in cytotoxicity is mediated by catecholamines and not glucocorticoids (203). It should be noted that in the same study, the stress-induced reduction in draining LN cell number was restored by RU486 treatment but not nadolol treatment. These data present a dichotomy of mechanisms induced by restraint stress.

The effects of catecholamines can also be measured by treatment of mice with 6-hydroxypodamine (6-OHDA), a neurotoxin that selectively destroys the termini of sympathetic noradrenergic fibers, but does not cross the blood-brain barrier (207). One caveat of 6-OHDA treatment, however, is its activation of HPA axis, resulting in an increase of serum glucocorticoid levels (205). Another caveat is that sympathetic denervation by 6-OHDA results in a bolus of norepinephrine being released, that may elevate serum and tissue levels of norepinephrine (207). In any case, 6-OHDA treatment 1 d p.i. or during latency results in a downmodulation of specific lysis of HSV-infected targets. When mice treated with 6-OHDA during latency were also treated with nadolol, cytotoxic function was restored (206).

In addition to affecting virus-specific T cells, catecholamines could potentially directly influence viral reactivation by binding to adrenergic receptors on the neuronal cell surface and stimulating cAMP production via G-protein coupled receptors (196). The cAMP could then act through cAMP response element (CRE) binding protein (CREB) to bind CREs on the latent viral episome, initiating transcription of viral genes (208). Whether catecholamines can induce HSV-1 reactivation by this mechanism is currently unknown.

1.4.3 Restraint Stress Model

Our studies employ a restraint model of psychological stress in which mice are placed in a 50 ml conical tube for 12 h, on four consecutive nights (see Section 3.9.3). Several restraint paradigms have been shown to upregulate serum levels of corticosterone, the hallmark of stressful stimuli (209-213). Additonally, the susceptibility to viral and bacterial diseases is increased following the restraint treatment, suggesting that stress, in the form of restraint, can impair immune responses (211,214,215), perhaps in part by a delay in the kinetics of humoral and adaptive immune responses (216,217). Restraint stress appears to affect both the HPA axis and the SNS, as blocking treatments with RU486 or nadolol appear to have differential effects (203).

2.0 SPECIFIC AIMS

The specific goals that were set forth in my thesis proposal and the progress made during my studies are summarized below.

Specific Aim I: Define whether and how stress dysregulates $CD8^+$ and $CD4^+$ T cells within the TG of C57BL/6 mice latently infected with HSV-1.

Psychological and physical stressors, and direct application of stress-associated hormones, have been shown to impair immune function. We hypothesized that psychological stress could induce an observable compromise of T cell efficacy within the latently infected TG. To address this aim, we subjected latently infected mice to repeated sessions of restraint and observed a $CD8^+$ T cell intrinsic deficiency in IFN-γ in TG culture and at low, physiological antigenic epitope densities. Portions of this aim were published in the Journal of Immunology (218).

Specific Aim II: Determine if disruption of immune function by stress leads to HSV-1 reactivation in latently infected neurons.

Following HSV-1 infection, an activated immune infiltrate enters the TG and remains for the life of the animal. We hypothesized that disrupting the function of these activated T cells would result in reactivation of HSV-1 from latency. To address this aim, $CD8^+$ T cells were

depleted in vivo or mice were restrained or treated with corticosterone to impair T cell function. In each case, we measured a significant increase in viral genome copy per TG, and we were able to detect viral proteins by immunohistochemistry in stressed TG, indicating that compromising $CD8^+$ T cell function in vivo results in reactivation of latent HSV-1. Portions of this aim were published in the Journal of Immunology (218).

Specific Aim III: Examine how stress affects the establishment, maintenance, and responsiveness of $CD8^+$ memory T cells in the TG of mice latently infected with HSV-1.

Studies have shown that stress early in viral infection delays the immune infiltrate and changes the nature of the primary immune response (209,216,217,219-225). We hypothesized that stress during the establishment of the HSV-1 specific memory response influenced not only the progression of disease but also the nature of immunological memory upon establishment of latency. To address this aim, we corticosterone-treated or stressed mice during acute infection and measured $CD8^+$ T cell number, viral genome copy number, and ability to synthesize IFN-γ 34 d p.i.

3.0 MATERIALS AND METHODS

3.1 MICE AND HSV-1 INFECTION

Six- to 8-wk-old male C57BL/6J (B6; H-2K^b, CD45.2) or B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (B6.SJL; H-2K^b, CD45.1) mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized by i.p. injection of 66.7 mg/kg ketamine hydrochloride and 1.33 mg/kg xylazine (Phoenix Scientific, St. Joseph, MO) in 0.2 ml HBSS (BioWhittaker, Walkersville, MD). Corneas of anesthetized mice were scarified 15-30 times in a crisscross fashion using a sterile 30-gauge needle and eyes were topically infected with 3 μl RPMI (BioWhittaker) containing 10^5 PFU purified wild-type or recombinant RE strain HSV-1. Infection efficiency was monitored by topical application of fluorescein (Akorn, Abita Springs, LA) 2 days post infection (p.i.) and observed under a slit-lamp microscope. All animal studies experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.2 VIRUSES

Wild-type and recombinant HSV-1 RE strain were grown in Vero cells and intact virions were purified using OptiPrep™ gradient columns (Accurate Chemical and Scientific Corporation, Westbury, NY) according to the manufacturer's instructions. Plaque forming units (PFU) of

HSV-1 was determined by standard virus plaque assay on Vero cells. Recominant reporter HSV-1 RE (pgB-EGFP [EGFP driven by the gB promoter], pgC-EGFP [EGFP driven by the gC promoter], and pgB-EGFP-pgC-mRed [EGFP driven by the gB promoter and mRed driven by the gC promoter]) were produced in collaboration with Dr. Paul R. Kinchington, University of Pittsburgh, and their construction is described in detail elsewhere (52,226).

3.3 REAGENTS

HSV-1 glycoprotein B (gB) peptide SSIEFARL ($gB_{498-505}$) was purchased from Research Genetics (Invitrogen, Carlsbad, CA). Peptide purity was determined to be greater than 95% by reverse phase high performance liquid chromatography analysis. Phycoerythrin (PE)-conjugated H-$2K^b$/$gB_{498-505}$ tetramers were provided by the NIAID Tetramer Core Facility (Emory Vaccine Center, Atlanta, GA). PE-conjugated H-$2K^b$:Ig dimers (BD Pharmingen, San Diego, CA) were complexed to $gB_{498-505}$ according to manufacturer's protocol.

3.4 SINGLE-CELL SUSPENSIONS

3.4.1 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, Logan, UT or Atlanta Biologicals, Lawrenceville, GA) and 400 U/ml collagenase type I (Sigma-Aldrich,

St. Louis, MO) per TG for 1 h at 37ºC, and dissociated into a single-cell suspension by trituration.

3.4.1.1 Preparation of TG cultures

TG were dispersed into a single cell suspension as above. TG were washed and resuspended in 2 ml/TG DMEM with 10% FCS, 50 μM 2-mercaptoethanol (2-ME; Fisher Scientific, Fair Lawn, NJ), and 500 U/ml rIL-2 (R & D Systems, Minneapolis, MN). TG cells were dispersed at the rate of 200 μl/well (equivalent to 0.1 TG/culture) in a 96-well flat bottom plate (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ) or 400 μl/well (equivalent to 0.2 TG/culture) in a 48-well plate (Falcon). Cultures were incubated up to 10 d at 37ºC/5% CO_2.

3.4.1.2 Measuring ex vivo cytokine response

66- or 90-h after culture initiation, 1 μl per TG of Golgiplug™ (BD Pharmingen) was added, with or without $gB_{498\text{-}505}$ at 10^{-12} M (1 μl of the 2 mg/ml working stock per ml culture), and, in some cases, with fluorescently-labeled anti-CD107a (BD Pharmingen) or isotype control. 6 h later, TG cultures were harvested with versene (8 g NaCl, 0.2 g KCl, 1.15 g dibasic Na_2HPO_4, 0.2 g monobasic KH_2PO_4, 0.2 g Na_4EDTA; pH 7.5 in 1 L dH_2O), washed, and stained for surface markers and intracellular cytokines as described below.

3.4.1.3 Measuring reactivation of latent virus

Direct fluorescence microscopy

TG cultures were examined daily for HSV-1 reactivation by monitoring reporter virus-infected cultures for spread of fluorescence to the surrounding fibroblast monolayer (52) by

imaging on an Olympus BX60 upright microscope with digital camera attachment. Confocal imaging was performed on a Nikon TS100F inverted microscope or with an Olympus IX70 microscope with a Bio-Rad laser-scanning confocal microscope attachment, using LaserSharp2000 software (Bio-Rad, Hercules, CA).

Plaque Assay

Every other day of TG culture, 30 μl (for 0.1 TG/well cultures) or 60 μl (for 0.2 TG/well cultures) of culture supernatant were removed from each well, replaced with fresh culture media, and stored at -80ºC. Samples were then thawed and plated on monolayers of Vero cells for 1 h at 37ºC. Samples were then overlayed with media containing 0.5% methylcellulose for 48 h. Detection of virus from latent TG cultures indicates HSV-1 reactivation.

Ab-mediated depletion of cells from culture

TG suspension were depleted of either $CD8^+$ (Ly-2) or $CD45^+$ cells by positive selection using antibody-conjugated DynaBeads (Dynal Biotech, Invitrogen) per manufacturer's instructions. In short, TG were incubated with beads at a 6:1 bead to cell of interest ratio for 2 min, 4-7 times, at room temperature on a magnet (Advanced Magnetics, Cambridge, MA) then washed between incubations with buffer (PBS/2% FCS). Flowthrough was collected and resuspended in TG culture media for plating. Depletion efficiency was determined by flow cytometry or hemacytometer count.

3.4.2 Spleens

Splenocytes were collected by grinding spleens through a 40 μm cell strainer and rinsing with media. Preparations were washed, incubated 5 min at room temperature in red blood cell lysis buffer (0.16 M NH_4Cl, 0.17 M Tris, in dH_2O, pH 7.2), and then washed again. A 20 μl aliquot was then diluted in 10 ml azide-free isotonic diluent (Val Tech Diagnostics, Breckenridge, PA) and counted on a Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA).

3.5 FLOW CYTOMETRY

3.5.1 Determination of surface phenotype

Purified or fluorochrome-conjugated Abs against CD4 (clone: RM4-5), CD8α (53-6.7), CD8β.2 (53-5.8), CD16/CD32 (2.4G2), CD25 (7D4 or PC61), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD69 (H1.2F3), CD107a (1D4B), NKG2A/C/E (20d5), IFN-γ (XMG1.2), TNF-α (MP6-XT22), and 5-bromo-2-deoxyuridine (BrdU, 3D4), were purchased from BD Pharmingen. Fluorochrome-conjugated Abs against CTLA-4 (UC10-4B9), FoxP3 (FJK-16s), and NKG2D (CX5) were purchased from eBioscience (San Diego, CA); allophycocyanin (APC)-conjugated anti-granzyme B Ab (GB11) was purchased from Caltag (Carlsbad, CA). When appropriate, matching isotype Abs were used as controls. TG single-cell suspensions were passed through a 40 μm filter-cap tube (BD Pharmingen) to remove debris. Ab surface staining was performed according to manufacturer's instructions and as described previously (65). Flow cytometry was

performed on a BD FACSAria Cell Sorter using FACSDiva 4.1 or 5.0.2 software (BD Pharmingen).

3.5.2 Flow-based T cell function assay

For intracellular cytokine and granzyme B staining and lytic granule release determination, the BD Cytofix/Cytoperm kit with GolgiPlug™ was used following manufacturer's protocol. GolgiPlug™ contains brefeldin-A, an inhibitor of protein trafficking through the trans-Golgi network. Thus, cells treated with brefeldin-A can synthesize, but not secrete, cytokines, allowing for the identification of cytokine-producing cells by intracellular flow cytometry. In short, TG suspensions were stimulated with B6WT3 fibroblasts pulsed with varying concentrations of $gB_{498\text{-}505}$ peptide for varying lengths of time in the presence of GolgiPlug™ (65,227) and, in some instances, anti-CD107a Ab (218). Following stimulation, suspensions were labeled with appropriate surface antibodies as above. Samples were then washed in FACS buffer (1% FCS, 0.1% NaN_3 in PBS) and permeabilized with Cytofix/Cytoperm solution for 20 min on ice. Intracellular Abs were diluted in 1X BD Perm/Wash solution and incubated with samples for 30 min on ice. After labeling, suspensions were washed with 1X Perm/Wash and resuspended in FACS buffer for analysis by flow cytometry. CD107a, a molecule on lysosomal and lytic granule membranes, is a marker of degranulation; it is briefly expressed on the cell surface following lytic granule membrane fusion to the cell membrane during release of lytic granules (228).

3.5.3 CFSE ex vivo proliferation assay

Proliferation ex vivo was assayed by labeling TG suspensions with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and incubating for 72 h with or without HSV-1 $gB_{498-505}$. Samples were then labeled with surface antibodies as above and analyzed by flow cytometry. As each cell division reduces cytoplasmic CFSE labeling by half, CFSE dilution can be used to quantify cell proliferation and division.

3.6 MEASURING APOPTOSIS

3.6.1 Annexin-V labeling

Mice were euthanized by perfusion with 1% paraformaldehyde (Sigma-Aldrich) in PBS. TG were excised, washed with PBS, and placed in one TG per well in a round-bottom 96-well plate, then incubated with PE-labeled anti-CD8α mAb (1 μg/ml), FITC-labeled Annexin-V (5 μl/TG; BD Pharmingen), and anti-CD16/CD32 (1:500), in 1X Annexin-V Binding Buffer (10X = 0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$; BD Pharmingen) overnight at 4ºC on a shaker. Binding of Annexin-V to phosphatidylserine molecules on the outer leaflet of the cell membrane is a marker for apoptosis as phosphatidylserines are only expressed on the outer leaflet of the cell membrane during apoptosis.

3.6.2 Fluorescently-labeled inhibitor of caspase activation (FLICA)

Single cell TG suspensions were incubated 1 h in serum-free media in the presence of FAM-VAD-FMK (Chemicon, Temecula, CA) at 37ºC. The cells were then labeled with appropriate antibodies and analyzed by flow cytometry.

3.6.2.1 FAM-VAD-FMK-based cytotoxicity assay

Single cell TG suspensions were incubated with B6WT3 fibroblasts labeled with the lipophilic tracer DiI (Molecular Probes) that had been pulsed with varying concentrations of $gB_{498-505}$ peptide or left nonpulsed for 6 h at 37ºC in the presence of FAM-VAD-FMK. After 6 h, the cells were stained with antibodies to CD8α and CD45, and then analyzed by flow cytometry.

3.7 DNA ISOLATION FROM TG

DNA was isolated from TG using the Qiagen DNAeasy™ Tissue Kit following the manufacturer's protocol. In short, collagenase-treated TG were resuspended in 200 µl PBS per sample, then treated with 20 µl proteinase K and 200 µl Buffer AL, mixed thoroughly, and incubated 10 min at 70ºC on a dry bath incubator (Fisher Scientific). Samples were then treated with 200 µl 100% EtOH and transferred to a mini-spin column and centrifuged. The columns were treated with Buffer AW1 and Buffer AW2, with spins between each treatment, and finally, samples were eluted in 100 µl Nuclease-Free H_2O (Ambion, Austin, TX). DNA was quantitated using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) using

SoftMax Pro 4.3 software (Molecular Devices) and 100 ng DNA per sample was resuspended to a 1 ng/ml concentration in nuclease-free H_2O.

3.7.1 Real-time PCR for glycoprotein H (gH) DNA

25 ng of DNA or water control was mixed in duplicate with 25 µl of TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) and an HSV-1 glycoprotein H (gH)-specific primer-probe set, custom designed and synthesized by ABI Assays-by-Design service (Applied Biosystems, Foster City, CA). Samples (50 µl/well) were assayed in 96-well plates with an ABI Prism 7700 sequence detector. ABI Primer Express v1.5a software default settings were used for instrument control and data analysis. The gH sequences were: forward primer (5'-GACCACCAGAAAACCCTCTTT-3'), reverse primer (5'ACGCTCTCGTCTAGATCAAAGC-3'), and probe [5'-(FAM)TCCGGACCATTTTC(NFQ)-3']. The HSV-1 genome contains a single copy of the gH gene; therefore, viral genome copy number can be determined quantitatively by comparing the experimental C_T value observed from the gH primer-probe assay with C_T values of known concentrations of gH-containing plasmid standards (226).

3.8 PREPARATION OF FROZEN TG SECTIONS

TG were excised from PBS-perfused mice, embedded in optimal cryogenic temperature (OCT) compound (Tissue-Tek, Naperville, IL), snap-frozen in an acetone dry ice bath, and 6 µm sections were cut on a Cryostat 3050 S (Leica, Wetzlar, Germany) at -20ºC. The sections were then quickly fixed in acetone and stored at -20ºC until staining.

3.8.1 Immunohistochemistry

Acetone-fixed 6 μm frozen TG sections were stained for HSV-1 Ags using a primary polyclonal rabbit anti-HSV-1 Ab (Accurate Chemical and Scientific) followed by goat anti-rabbit Ig secondary Ab, ABC reagent (Vectastain ABC, Vector Laboratories, Burlingame, CA), and detection with diaminobenzidine substrate (Vector Laboratories) as previously described (68). Sections were then dehydrated, mounted in Immu-mount (Thermo Electron, Waltham, MA), and covered with a coverslip. Stained sections were stored at -20ºC.

3.9 IN VIVO TREATMENTS

3.9.1 In vivo depletion of $CD8^+$ T cells

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

3.9.2 BrdU in vivo proliferation assay

Proliferation in vivo was assayed by injecting HSV-1 infected mice daily with 1 mg 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) in 1X PBS. BrdU is a synthetic nucleoside analogue of thymidine; it incorporates into newly synthesized DNA strands and can then be detected with specific antibodies by flow cytometry (229).

3.9.3 Restraint Stress

On 4 consecutive nights beginning at various times 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. Since 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. For experiments examining the effects of stress during latency, the stress paradigm was initiated at 9:00 PM, 30 d after infection (Figure 7); for experiments examining stress during the primary immune response, restraint was started 4 d after infection.

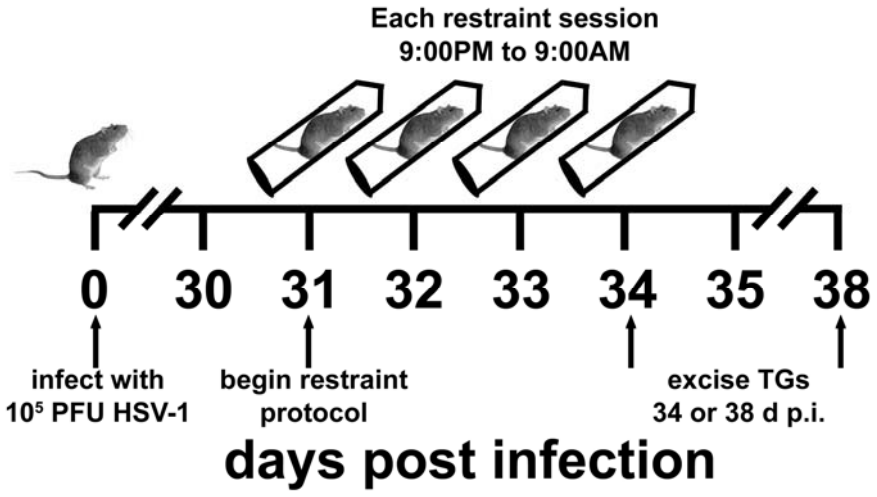

Figure 7. Model of restraint stress during latency.

Restraint stress was initiated on the evening prior to 31 d post infection and completed the morning of 34 d post infection. Figure adapted from (218), © The Journal of Immunology, used with permission.

3.9.4 Corticosterone treatment

For 4 consecutive days beginning at various times after infection, mice were supplied with 400 μg/ml corticosterone (MP Biomedicals, Irvine, CA) in the drinking water. Freshly made corticosterone was re-supplied every other day. Corticosterone was dissolved in 30% (2-hydroxypropyl)-β-cyclodextrin (HBC; Sigma-Aldrich), prepared by dissolving 9 g HBC in 30 ml H_2O. 100 mg corticosterone was then dissolved in 5 ml HBC and brought to 250 ml with H_2O per treated group. Thus, if two treated groups were required, 200 mg corticosterone was dissolved in 10 ml HBC and brought to 500 ml with H_2O.

3.9.5 Nadolol treatment

Mice were injected i.p. with 5 mg/kg nadolol (Sigma-Aldrich) in 500 μl PBS/0.2% EtOH per mouse beginning 1 d prior to stress, and continuing daily throughout the stress treatment, for a total of 5 injections per mouse. Control mice were treated with vehicle.

4.0 RESULTS

4.1 $CD8^+$ T CELL SURROUND SELECT NEURONS IN LATENTLY INFECTED TG

Following establishment of a latent HSV-1 infection, $CD8^+$ T cells remain in the TG for the life of the host (65,115). In humans, $CD8^+$ T cells selectively surround neurons that are latently infected with HSV-1, but not varicella zoster virus (VZV) (115). As depicted in Figure 8, in our murine corneal infection model, we observe $CD8^+$ T cells (red) surrounding some neurons (green), but not others. Some of these $CD8^+$ T cells polarize their TCR in an apparent immune synapse with the neuron (65).

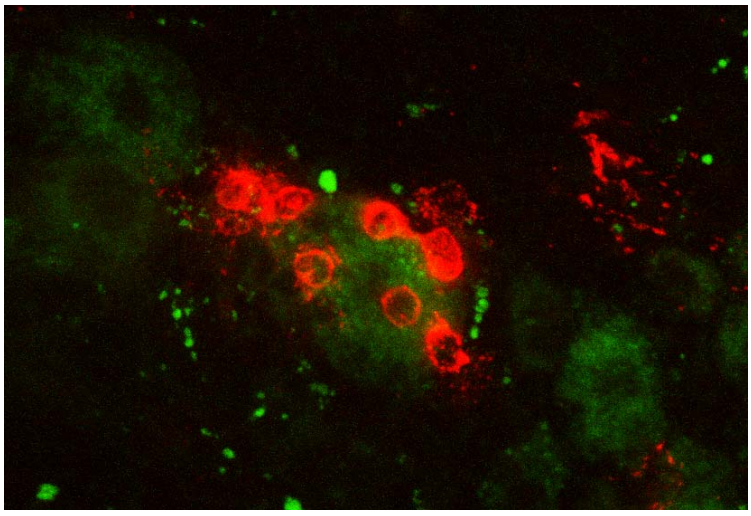

Figure 8. $CD8^+$ T cells surround some neurons in latently infected TG.

TG whole mounts were stained with anti-CD8α and examined by confocal microscopy. $CD8^+$ T cells (red) surround particular neurons (green) in latently infected TG 34 d p.i. Magnification 60X.

4.2 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 (64,65), a similar protective function has not been established *in vivo*. Here we show that within 3 d of a single injection of anti-CD8α mAb, CD8$^+$ T cells were effectively depleted from mouse TG that harbored latent HSV-1 (Figure 9A), and the HSV-1 genome copy number in the TG was significantly increased (Figure 9B). Thus CD8$^+$ T cells actively monitor latent HSV-1 in sensory neurons and inhibit replication of the viral genome.

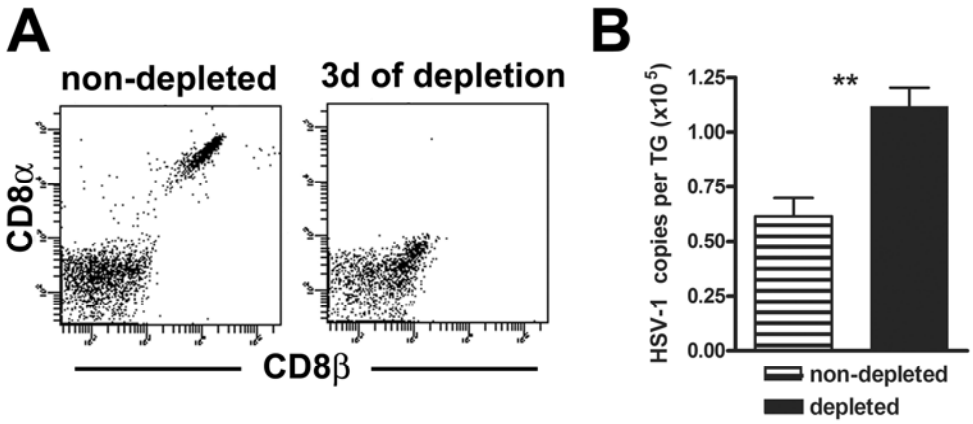

Figure 9. CD8$^+$ T cells are required to prevent viral DNA synthesis.

Thirty days after HSV-1 corneal infection mice harboring latent virus in their trigeminal ganglia (TG) received a single i.p. injection of anti-CD8α mAb. 3 d after treatment TG were excised from CD8-depleted and non-depleted mice and dispersed cells were analyzed by flow cytometry for their expression of CD8α and CD8β. **(A)** Dot plots demonstrate a lack of CD8$^+$ T cells in TG of anti-CD8α mAb treated mice, gated on CD45$^+$ cells. **(B)** DNA from ganglia of CD8-depleted mice (n = 5) contained a significantly ($p < 0.009$, Student's t test) higher viral genome copy number than that from non-depleted mice (n = 20) when analyzed by quantitative real-time PCR for the HSV-1 glycoprotein H gene. Figure adapted from (218), © The Journal of Immunology, used with permission.

4.3 STRESS OR GLUCOCORTICOID TREATMENT INCREASE SERUM LEVELS OF CORTICOSTERONE

The effectiveness of a stress response can be measured by elevated serum levels of HPA-derived glucocorticoids. Our stress protocol (as illustrated in Figure 7) uniformly elevated serum levels of corticosterone (Figure 10, left graph). Direct treatment with 400 μg/ml corticosterone in the drinking water increased serum levels of corticosterone to levels that were significantly greater than levels induced by stress (Figure 10, right graph).

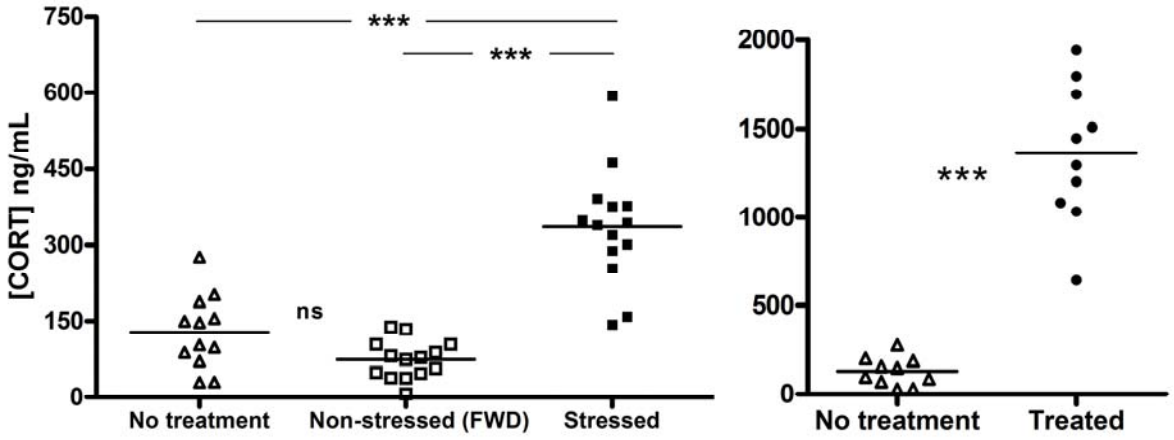

Figure 10. Elevated serum levels of corticosterone following stress or glucocorticoid treatment.

(A) Sera of nontreated (n = 12) mice or mice that were food and water deprived (FWD) but not stressed (nonstressed, n = 14) contained significantly ($p<0.0001$, one-way ANOVA and Tukey's post test) less corticosterone (CORT) than sera from stressed (n = 4) mice when analyzed by a standard radioimmunoassay. ns, not significant. **(B)** Sera of corticosterone treated (n = 10) mice contained significantly more corticosterone than nontreated (n = 10) mice. *** $p <0.0001$, Student's t test. Adapted from (218), © The Journal of Immunology, used with permission.

4.4 EFFECTS OF RESTRAINT STRESS ON HSV-1 REACTIVATION

Since 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 to four, 12-h restraint sessions as illustrated in Figure 7. That this stress protocol influenced the state of viral latency was established by an approximate 2-fold increase in the viral genome copy number in DNA from TG of stressed relative to non-stressed mice (Figure 11A), similar to the increased copy number observed following $CD8^+$ T cell depletion (Figure 9B).

Stress-induced reactivation was further established by direct ex vivo detection of infectious HSV-1 in 1 of 8 stressed TG, and detection of HSV-1 proteins in serial sections of 1 of 4 stressed TG (Figure 11B and data not shown). Neither infectious virus nor HSV-1 proteins were detected in any nonstressed TG tested. 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 (230).

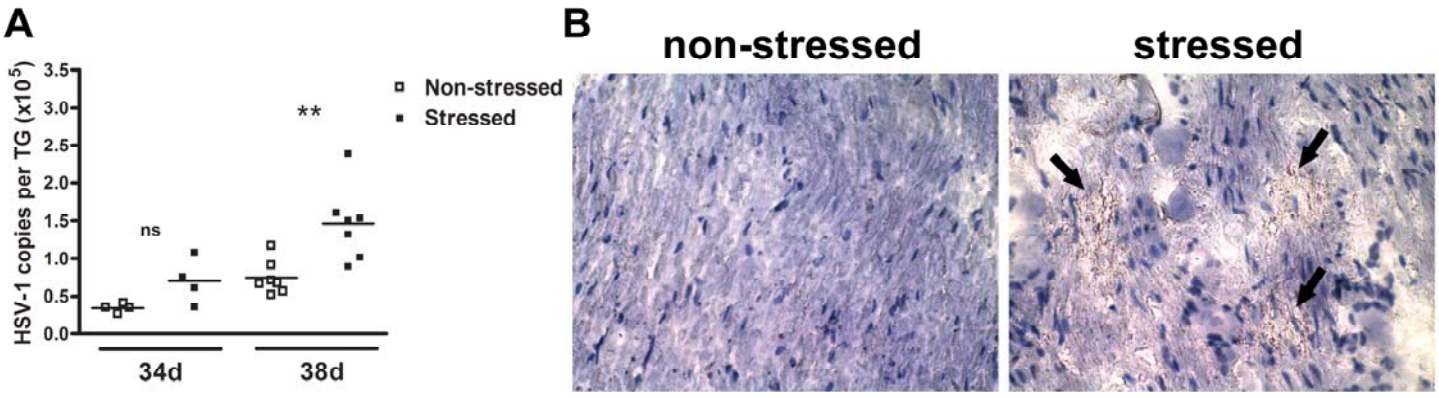

Figure 11. Restraint stress during latency increases HSV-1 genome copy number in the TG.

(A) TG were excised from stressed and non-stressed mice at the indicated time after infection and the HSV-1 genome copy number was determined by real-time PCR. ** p = 0.0039, Student's t test. **(B)** Frozen sections of TG obtained from stressed and nonstressed mice at 34 d after infection and stained for HSV-1 antigens. Arrows identify regions of positive staining for HSV-1 antigens. Figure adapted from (218), © The Journal of Immunology, used with permission.

Although observation of stress-induced reactivation in vivo by real-time PCR was particularly clear, stress-induced reactivation in ex vivo TG cultures was less obvious. When HSV-1 pgB-EGFP-infected TG were excised and plated in one-tenth TG/well cultures in a 96-well plate, we observed a slight increase in the percentage of cultures that showed reactivation, as measured by spread of fluorescence (Figure 12A). However, maximum reactivation was only about 10% of the cultures, making any differences between stressed and nonstressed cultures hard to discern. Using one-fifth TG/well cultures in a 48-well plate from mice infected with HSV-1 pgB-EGFP-pgC-mRed, we observed a greater increase in the percentage of cultures that were positive for reactivation, but again, differences between the groups were quite small (Figure 12B). Although there was a trend for higher reactivation rates in stressed cultures, in neither of these strategies to measure reactivation did we observe significant differences between nonstressed and stressed groups. Importantly, in neither of these conditions were the cultures

from stressed TG continuously exposed to stress hormones that would otherwise be present in vivo. Thus, these experimental designs may underestimate the stress-induced reactivation frequency. Additionally, although these cultures were pooled from several ganglia, the potential remains that repetition of these experiments could result in greater, biologically and statistically significant effects.

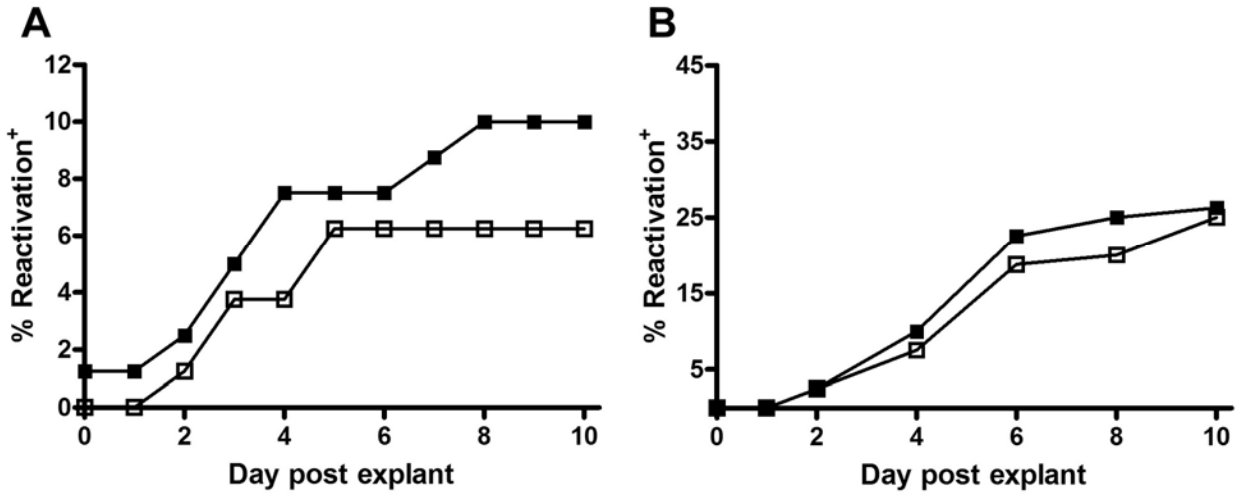

Figure 12. Reactivation in ex vivo TG cultures.

TG were excised from mice at 34 d p.i. that were latently infected with recombinant HSV-1 reporter virus and either stressed (solid squares, n = 40-90) or nonstressed (open squares, n = 40-84). **(A)** Rate of reactivation as measured by spread of fluorescence in 0.1 TG/well cultures. Mice were infected with HSV-1 pgB-EGFP. Data is from one experiment. **(B)** Rate of reactivation as measured by spread of fluorescence in 0.2 TG/well cultures. Mice were infected with HSV-1 pgB-EGFP/pgC-mRed. Data is pooled from two experiments, error bars are not shown.

$CD8^+$ T cells block HSV-1 reactivation through IFN-γ and lytic granule exocytosis in an MHC-restricted fashion (52,60,148,177). IFN-γ blocks reactivation in some, but not all, neurons that require T cell protection and this phenomenon correlates with the expression of IFN-γ receptor (IFN-γR) on a proportion of neurons (231). The nonclassical MHC-I molecule $Qa\text{-}1^b$ inhibits CD8+ T cell killing of infected neurons: blocking $Qa\text{-}1^b$ interactions with its receptor on

T cells, NKG2A/CD94, allowed for the T cell-mediated destruction of those neurons in an in vitro cytotoxicity assay (148). Therefore, we examined the effects of stress on neuronal expression of IFN-γR and Qa-1^b. Interestingly, we observed no overt qualitative differences in the expression of either of these two molecules on neurons in latently infected TG following stress (Figure 13). These data suggest that the stress-induced increase in viral genomes is not due to an impairment of the ability of latently infected neurons to respond to the protective capacities of T cells. However, this assay is qualitative, not quantitative, and at this time the conclusion is still tentative.

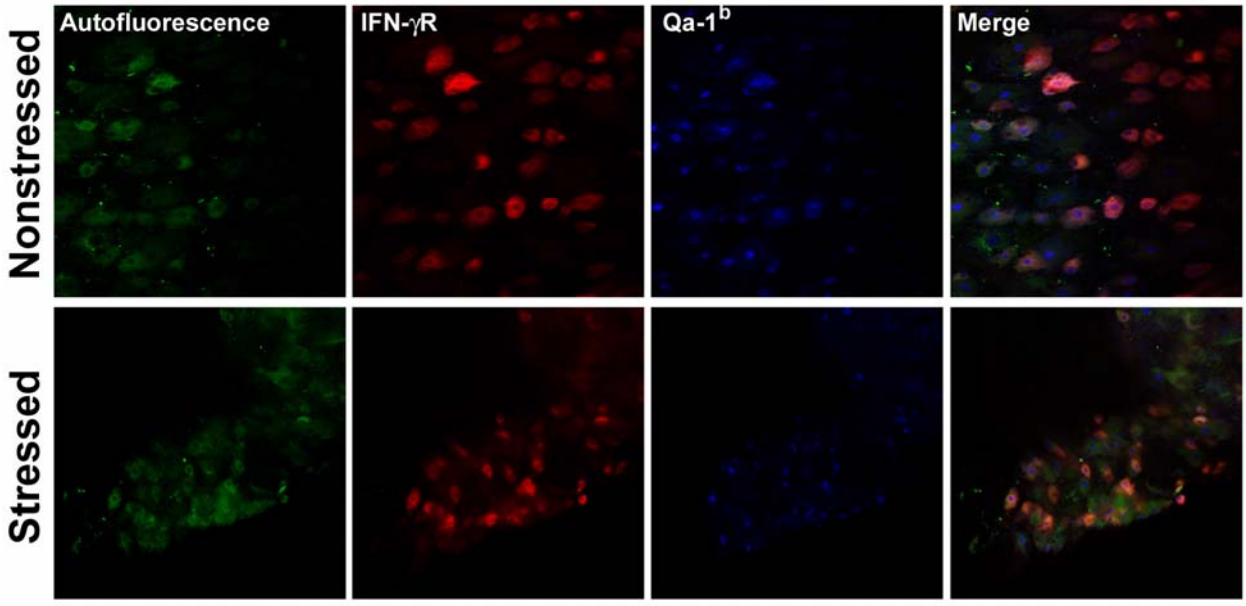

Figure 13. Effects of stress on neuronal expression of IFN-γR and Qa-1^b.

Whole TG from stressed or nonstressed mice were washed, fixed in 1% PFA, and labeled with antibodies to IFN-γ receptor (IFN-γR) or the nonclassical MHC-Ib molecule Qa-1^b and examined by confocal microscopy. Magnification 40X.

4.5 EFFECTS OF RESTRAINT STRESS ON T CELLS IN THE TG

4.5.1 Stress reduces $CD8^+$ and $CD4^+$ 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 (Figure 14A). This reduction was transient and by 2 d 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 $gB_{498\text{-}505}$ epitope and can be quantified by staining dispersed TG-derived immune cells with tetramers containing this H-$2K^b$ restricted epitope followed by flow cytometric analysis (65). Tetramer staining of $CD8^+$ T cells revealed a similar frequency of $gB_{498\text{-}505}$-specific $CD8^+$ T cells in the TG of stressed and nonstressed mice demonstrating that stress did not selectively deplete HSV-specific $CD8^+$ T cells (Figure 14B). Similar results were determined using $gB_{498\text{-}505}$ loaded dimers (data not shown). Given similar frequencies of virus-specific cells, but an overall reduction in total $CD8^+$ T cell number in the TG, the absolute number of TG-resident HSV-specific $CD8^+$ T cells was reduced by 30% following stress.

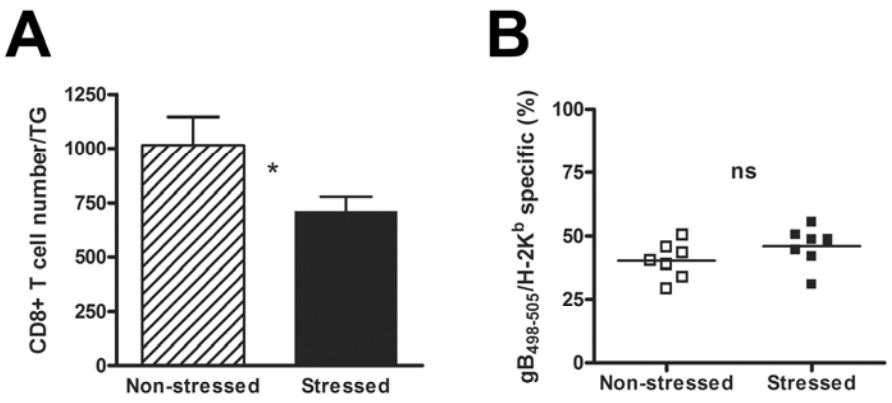

Figure 14. Restraint stress reduces CD8$^+$ T cell number in latently infected TG.

Latently infected mice were subjected to restraint stress as described in Figure 7. After the fourth restraint stress session (34 d after infection) TG were excised from stressed and nonstressed mice, dispersed into single cell suspensions, and simultaneously stained with anti-CD8α mAb and tetramers containing the immunodominant HSV-1 $gB_{498\text{-}505}$ epitope ($gB_{498\text{-}505}$ H-2K^b). **(A)** The mean absolute number of CD8$^+$ T cells/TG (± SEM) in stressed (n = 23) and non-stressed (n = 16) mice. * p = 0.03, Student's t test. **(B)** Percent of CD8$^+$ T cells that recognize the $gB_{498\text{-}505}$ epitope. Group differences were not significant (p = 0.1436, Student's *t* test). Figure adapted from (218), © The Journal of Immunology, used with permission.

TG-resident CD8$^+$ T cells express high levels of CD44, a marker for activated and memory cells, and CD69, an early activation marker (65). The frequency of CD44$^+$ memory CD8$^+$ T cells was slightly increased, but this result, while statistically significant, is of questionable physiological relevance (Figure 15A). Expression of CD69, although consistently elevated, was not significantly different (Figure 15B).

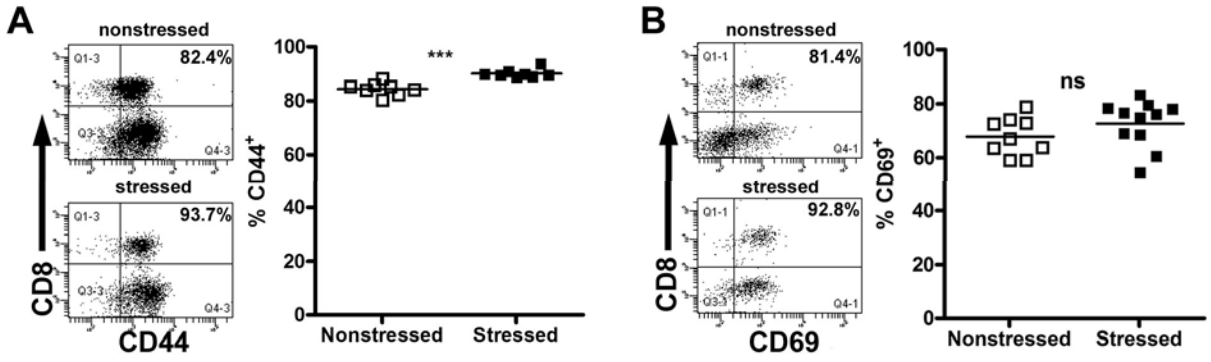

Figure 15. Activation phenotype of CD8$^+$ T cells in the TG after stress.

TG-resident CD8$^+$ T cells from stressed (n = 8-11) and nonstressed (n = 8-9) mice were assayed for cell surface expression of CD44 **(A)** and CD69 **(B)** by flow cytometry. Representative dotplots show the CD45$^+$ population. Numbers in the top right quadrant are percent of CD8$^+$ T cells that are positive for the given molecule. Graphs on the right are values pooled from 2 experiments. *** $p < 0.001$. ns = not significant, Student's t test.

The number of CD4$^+$ T cells is reduced approximately 30% following restraint stress, and like CD8$^+$ T cells, the reduction is transient (Figure 16, and data not shown). Although the antigen specificity of the CD4$^+$ T cell population is unknown, we examined the cells for expression of known activating and inhibitory receptors (Figure 17). There was a slight tendency toward a more activated phenotype (increased expression of activating receptors and decreased expression of inhibitory receptors), but there were no statistical differences from controls in the remaining cells. The presence of regulatory T cells (Tregs) was also unchanged, based upon intracellular expression of the transcription factor FoxP3 (Figure 17E).

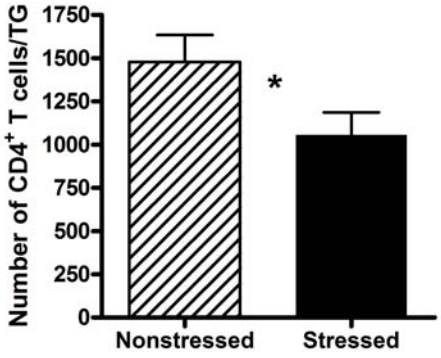

Figure 16. Restraint stress reduces $CD4^+$ T cell number in the latently infected TG.

Latently infected mice were subjected to restraint stress as described in Figure 7. After the fourth restraint stress session (34 d after infection) TG were excised from stressed and nonstressed mice, dispersed into single cell suspensions, and simultaneously stained with anti-CD4 mAb The mean absolute number of $CD4^+$ T cells/TG (± SEM) in stressed (n = 20) and non-stressed (n = 16) mice. * p = 0.04, Student's t test.

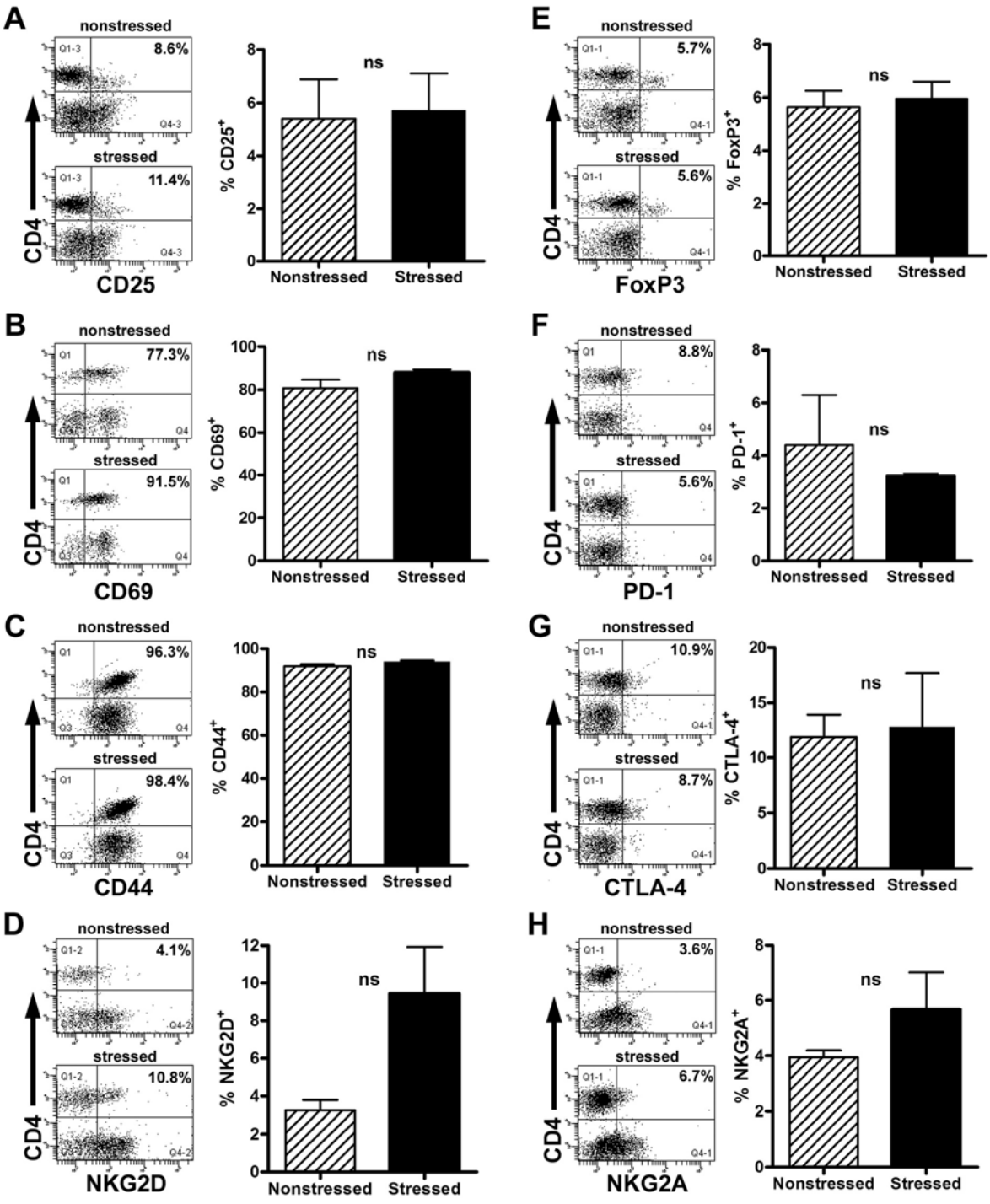

Figure 17. Phenotype of CD4$^+$ T cells after stress.

TG-resident CD4$^+$ T cells from stressed (n = 8) and nonstressed (n = 8) mice were assayed for cell surface or intracellular expression of CD25 **(A)**, CD69 **(B)**, CD44 **(C)**,

NKG2D **(D)**, FoxP3 **(E)**, PD-1 **(F)**, CTLA-4 **(G)**, or NKG2A **(H)** by flow cytometry. Representative dotplots show the $CD45^+$ population. Numbers in the top right quadrant are percent of $CD4^+$ T cells that are positive for the given molecule. Graphs on the right are values pooled from 2 experiments. ns = not significant, Student's t test.

4.5.2 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 (Figures 12 and 18), and reactivation can be inhibited by $gB_{498\text{-}505}$-specific $CD8^+$ T cells (65). Reactivating neurons present viral antigenic epitopes to T cells, but the amount of MHC-restricted viral epitopes presented is far less than the T cells are capable of recognizing. In vivo, the low level of antigen presentation during latency or at the beginning of reactivation is recognized and full reactivation to virion formation can be prevented. In cultures, however, a discrepancy develops between the magnitude of the T cell response to reactivating neurons and the T cell response to optimal stimulation by addition of exogenous peptide. Despite this, detailed observations can be made distuingishing stress-mediated effects at low densities of epitope versus a maximum response. 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 antigens) or when an optimal stimulatory dose of $gB_{498\text{-}505}$ peptide was added to the TG cultures. Two main functions of memory T cells in response to antigen are cytokine production and proliferation. Latently infected TG obtained from stressed and non-stressed mice were dispersed into single-cell suspensions, stained with CFSE, and cultured for 72 h with or without $gB_{498\text{-}505}$ 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.

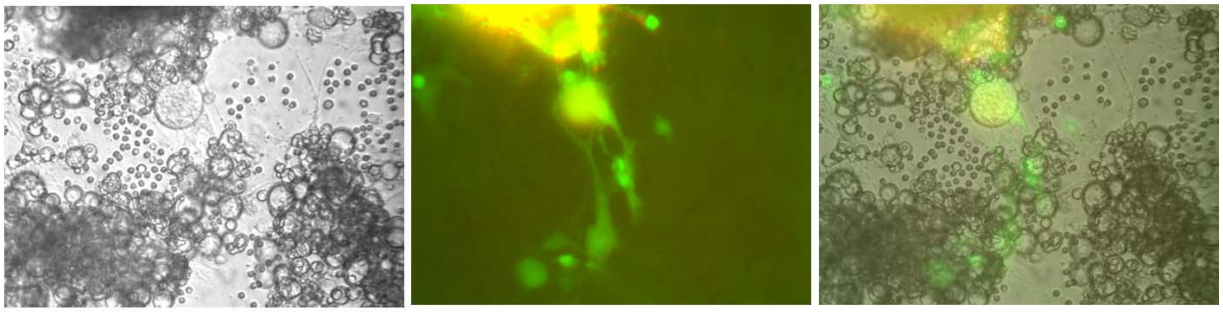

Figure 18. Viral late (γ1) gene expression in ex vivo TG cultures.

TG cultures from mice latently infected with HSV-1 pgB-EGFP/pgC-mRED were incubated for 48 h then imaged. Magnification 40X.

Figure 19A 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 for a rough comparison of the number of rounds of proliferation these cells underwent in the various groups. In cultures of non-stressed TG approximately 50% of $CD8^+$ T cells proliferated during the 72 h incubation in the presence or absence of $gB_{498\text{-}505}$ peptide. In contrast, significantly fewer $CD8^+$ T cells in stressed TG proliferated in response to reactivating virus (Figure 19B). The addition of $gB_{498\text{-}505}$ 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 to similarly stimulated $CD8^+$ T cells in non-stressed TG (Figure 19C). Moreover, those $CD8^+$ T cells in stressed TG that did proliferate in response to peptide underwent fewer rounds of proliferation than their counterparts in non-stressed TG as indicated by higher MFI (Figure 19C).

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 approximately 10% of $CD8^+$ T cells in non-stressed 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 (Figure 19D). The addition of $gB_{498-505}$ peptide to cultures greatly augmented IFN-γ production such that 40% of $CD8^+$ T cells in both stressed and non-stressed TG were IFN-γ positive (Figure 19E). These findings are consistent with the tetramer staining results showing a similar frequency of HSV-1 $gB_{498-505}$ specific $CD8^+$ T cells in the TG of stressed and non-stressed 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.

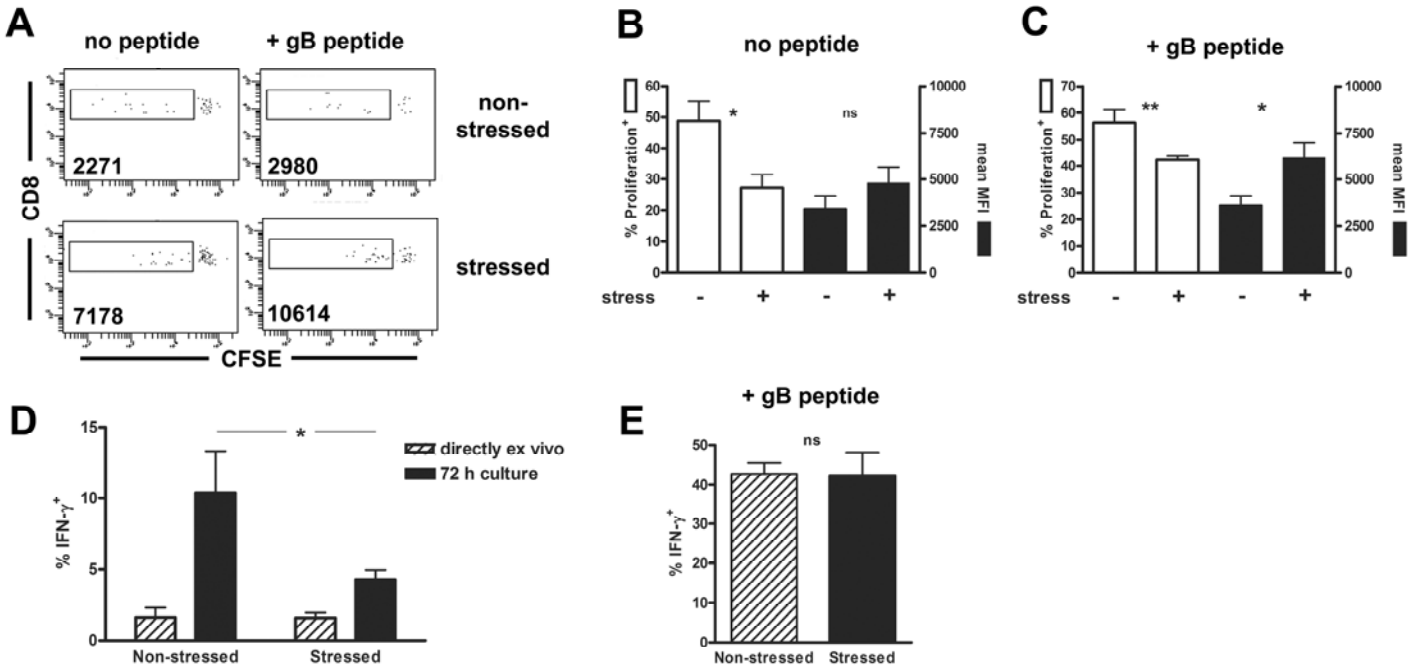

Figure 19. Stress compromises the function of HSV-specific $CD8^+$ T cells in latently infected TG.

TG were excised from stressed and non-stressed 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 $gB_{498-505}$ 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 the extent of CFSE dilution in stressed and non-stressed cultures, gated on $CD45^+CD8^+$ cells. The box designates cells that have undergone one or more rounds of cell proliferation, and the mean fluorescence intensity (MFI) of these cells is shown. **(B/C)** Data are presented as the mean (± SEM) percentage of $CD8^+$ T cells that underwent one or more rounds of proliferation, and MFI of proliferating cells in cultures without peptide **(B**, n = 8/group**)** or with added peptide **(C**, n = 13-14/group**)**. **(D)** Single cell suspensions of TG from stressed and non-stressed 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 = 13-17/group) for the last 6 h of incubation, stained for surface CD8 and intracellular IFN-γ, and analyzed by flow cytometry. Data are presented as the mean (± SEM) percentage of IFN-γ positive $CD8^+$ T cells. * $p<0.05$. **(E)** Cultures received HSV-1 $gB_{498-505}$ 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. ** $p<0.01$, all data were analyzed with Student's t test. Figure adapted from (218), © The Journal of Immunology, used with permission.

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 antigen production and presentation by neurons in stressed TG. To eliminate possible extrinsic effects, mixed cultures of latently infected TG from stressed (CD45.1) and non-stressed (CD45.2) congenic mice were employed (illustrated in Figure 20). 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 Figure 21A, 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 $gB_{498-505}$ 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 (Figure 21B). 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 $gB_{498-505}$ peptide to cultures.

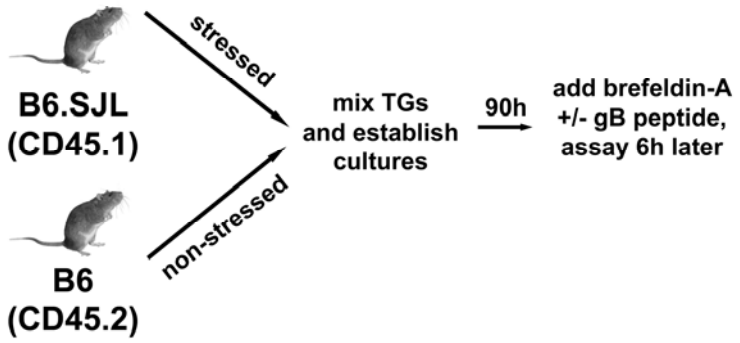

Figure 20. Experimental design for experiments shown in Figure 21

Illustration of the preparation of mixed TG cultures from stressed (CD45.1) and nonstressed (CD45.2) latently infected congenic mice. Figure adapted from (218), © The Journal of Immunology, used with permission.

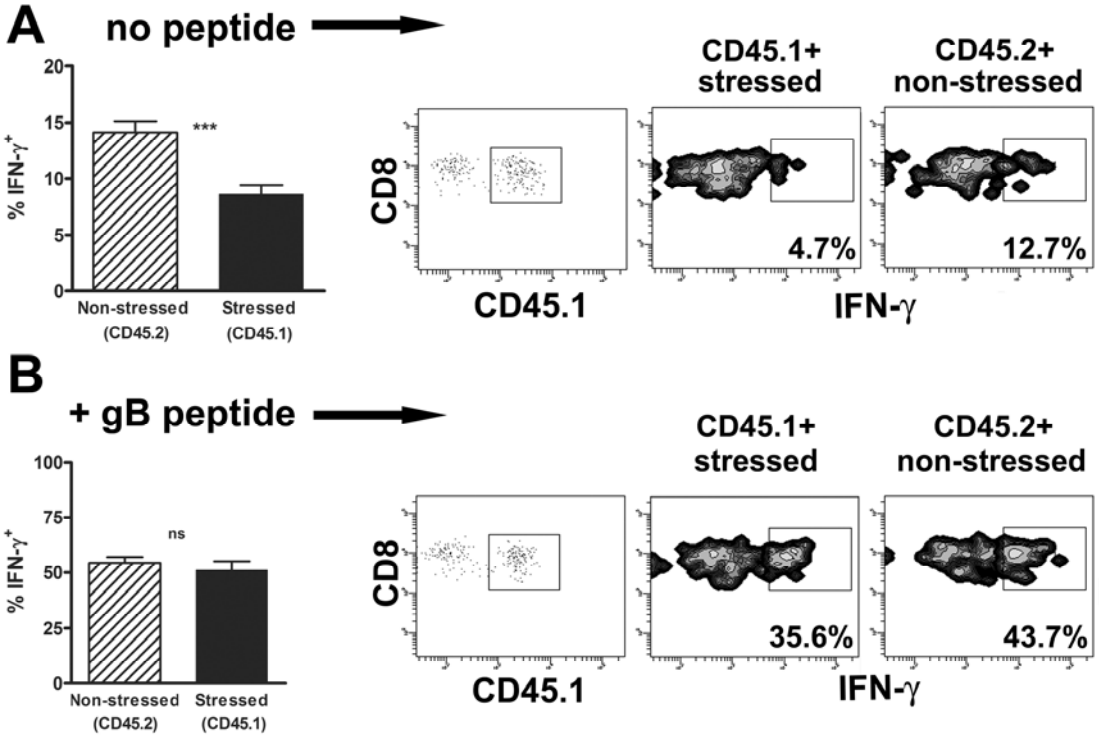

Figure 21. $CD8^+$ T cells in stressed mice exhibit an intrinsic compromise of IFN-γ production.

Congenic mice were infected with HSV-1 and 30 d later were stressed or not, TG were obtained and dispersed into single cell suspensions, and mixed cultures of TG from stressed and non-stressed mice were prepared as depicted. After 90 h, GolgiPlug™ was added to cultures without **(A)** or with **(B)** HSV-1 $gB_{498-505}$ peptide for the last 6 h of incubation. Cells were recovered from cultures, 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 non-stressed (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. Figure adapted from (218), © The Journal of Immunology, used with permission.

4.5.3 There is no extrinsic stress-induced deficiency.

Initial experiments suggested there might be an extrinsic effect of stress in addition to the intrinsic effect described above. Neurons from stressed TG (potentially undergoing active viral reactivation), or other stress-susceptible cells, could potentially secrete repressive molecules that might interfere with the function of the $CD8^+$ T cells. Subsequent experiments suggest otherwise, however, as IFN-γ production at 72 h in nonstressed cultures alone occurred in 10.4% of $CD8^+$ T cells (Figure 19D), and in mixed cultures at 96 h, 14.12% of the nonstressed cells synthesized IFN-γ (Figure 21A). The lack of a TG culture-mediated extrinsic effect was confirmed by directly comparing the IFN-γ response in TG cultures with or without CD8-depleted TG from stressed mice (Figure 22). Stressed TG were labeled with CFSE and then depleted of $CD8^+$ T cells by positive selection with magnetic beads. TG cultures from nonstressed mice were then mixed with CD8-depleted cultures, or cultured alone. Cultures were then assayed for IFN-γ synthesis during the final 6 h of a 72 h incubation as above. By depleting endogenous $CD8^+$ T cells before mixing the cultures, we were able to evaluate whether other components of the

stressed culture could influence the $CD8^+$ T cell response from nonstressed TG. We observed no difference in IFN-γ synthesis between nonstressed cultures alone or nonstressed cultures mixed with CD8-depleted stressed TG (Figure 22).

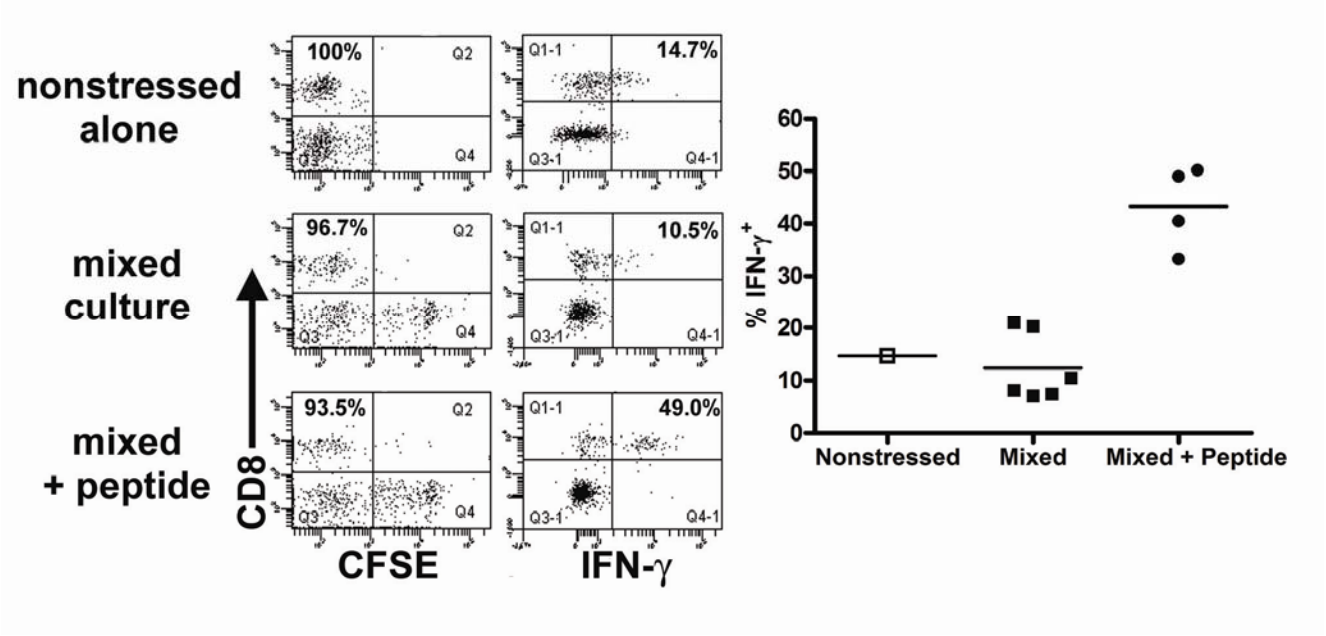

Figure 22. Stressed TG culture does not affect the nonstressed $CD8^+$ T cell response.

Stressed TG were labeled with 2.5 μM CFSE, depleted of $CD8^+$ T cells, then mixed with unlabeled nonstressed TG with or without 10^{-12}M $gB_{498\text{-}505}$ peptide. Cultures were incubated with GolgiPlug™ during the last 6 h of a 72 h incubation, then measured for IFN-γ by flow cytometry. Representative dotplots on the left show the extent of CD8 depletion. Numbers in top left quadrant indicate percentage of $CD8^+$ T cells in that quadrant. Representative dotplots on the right show IFN-γ production. Numbers in top right quadrant indicate percentage of $CD8^+$ T cells in that quadrant. Graph on the right shows the percentage of $CD8^+$ T cells that are IFN-γ^+. Nonstressed, nonstressed alone (n = 1), Mixed, CD8-depleted stressed TG plus nonstressed TG (n = 6), Mixed + peptide, similar to mixed, but with added $gB_{498\text{-}505}$ peptide.

The lack of extrinsic effect by the stressed TG culture is consistent with the observation that $FoxP3^+$ Tregs, important regulators of T cell function, were present at a similar frequency in ganglia of stressed and nonstressed mice (Figure 17E). Had Tregs been selectively retained in

stressed TG, there may have been a better case for an extrinsic effect. These data also imply that reactivating virus does not inhibit the response of surveillance memory $CD8^+$ T cells.

4.5.4 Cytokine response is dysregulated at low epitope densities.

Our previous studies revealed that $CD8^+$ T cells in stressed TG responded normally to optimal stimulation with $gB_{498\text{-}505}$ peptide, but exhibited an intrinsic defect in response to neurons harboring latent virus. Since latently infected neurons likely express low levels of viral proteinsand low MHC class I, we hypothesized that $CD8^+$ T cells in TG of stressed mice required a higher threshold of antigen stimulation than their counterparts in nonstressed TG. To test this, we recapitulated the effects of epitope density in vitro by stimulating stressed or nonstressed $CD8^+$ T cells with fibroblasts pulsed with varying concentrations of $gB_{498\text{-}505}$ epitope (Figure 23A). At 10^{-12} M gB-peptide concentration, stressed $CD8^+$ T cells responded 88.5% as well as nonstressed controls, dropping to 78.2% at 10^{-18} M, and 68.5% at 10^{-22} M.

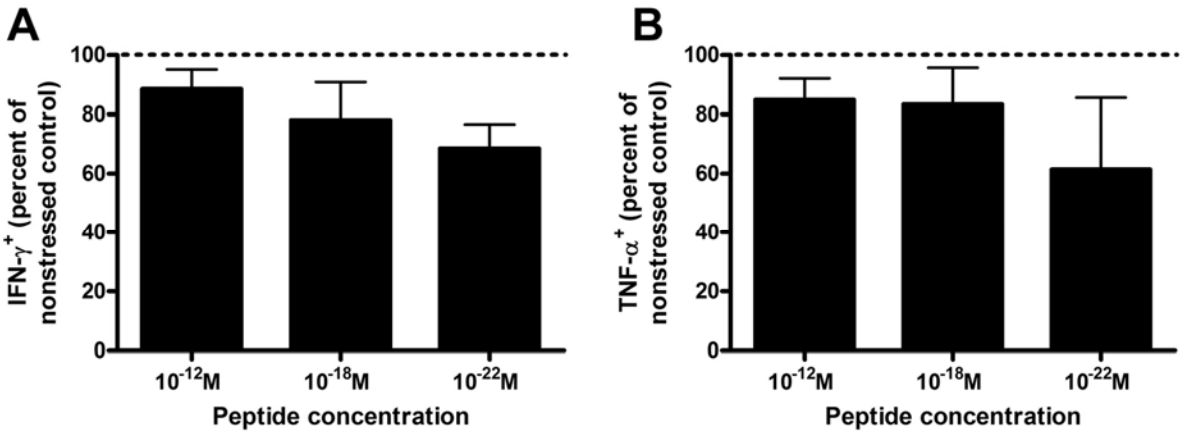

Figure 23. Cytokine response is compromised at low epitope densities.

TG from stressed or nonstressed mice were incubated for 6 h with B6WT3 fibroblasts pulsed with indicated concentrations of $gB_{498\text{-}505}$ peptide in the presence of GolgiPlug™. **(A)** The percentage of $CD8^+$ T cells from stressed TG that synthesized IFN-γ is shown as

a percentage (± SEM) of the nonstressed response at each peptide concentration. 10^{-12}M (n = 5), 10^{-18}M (n = 3), 10^{-22}M, (n = 2). **(B)** The percentage of $CD8^+$ T cells from stressed TG that synthesized TNF-α is shown as in part **(A)**. 10^{-12}M, (n = 3), 10^{-18}M, (n = 3), 10^{-22}M, (n = 2).

Synthesis of the cytokine TNF-α was also measured. As with IFN-γ, TNF-α production is optimal at 10^{-12} M gB-peptide concentration (67). Following stress, the $CD8^+$ T cell ability to produce TNF-α was impaired, dropping from 84.9% of nonstressed controls at 10^{-12} M to 61.5% at 10^{-22} M (Figure 23B).

We also measured the cytokine response in vivo during the last 12 h of stress. Using a modified protocol of the in vivo brefeldin-A treatment used by Liu and Whitton (232), we injected 250 mg brefeldin-A i.p. at 12- and 6 h prior to sacrifice assess IFN-γ and TNF-α synthesis in vivo (Figure 24). We observed a 6.67-fold reduction in IFN-γ production and a 7.24-fold reduction in TNF-α synthesis in the stressed group. It should be noted that the response was very low. However, at any given time, very few neurons are expressing viral antigens in normal ganglia (38), and thus, the amount of $CD8^+$ T cells that would be producing cytokines in response to viral antigens would necessarily be quite low.

Together, the preceding data show that stress compromises the memory $CD8^+$ T cell response at physiological concentrations of viral antigens.

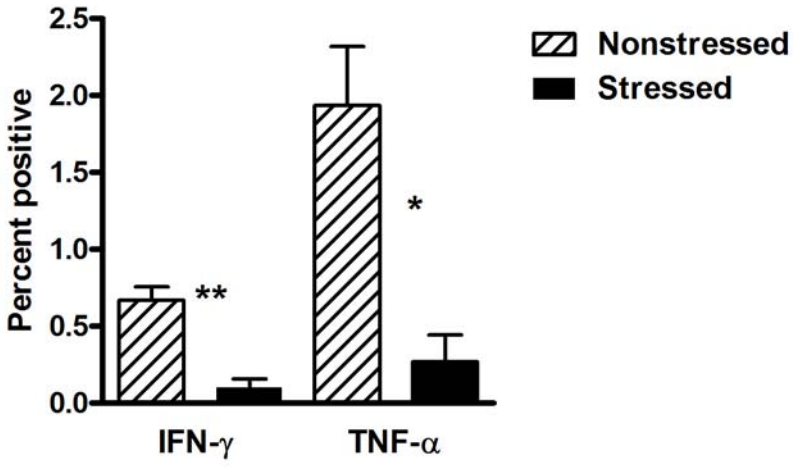

Figure 24. In vivo cytokine production during stress.

Mice were treated with 250 mg/mouse brefeldin-A i.p. at 12- and 6 h prior to sacrifice, during the final session of stress. The percentage (± SEM) of $CD8^+$ T cells in the TG that was positive for intracellular IFN-γ or TNF-α was measured by flow cytometry. All groups, n = 3. ** p = 0.0058. * p = 0.0170, Student's t test.

4.5.5 Stress impairs $CD8^+$ T cell ability to degranulate

Lysosomal associated membrane protein-1 (LAMP-1 or CD107a), is a protein embedded in the membranes of intracellular vesicles, including lytic granules. During exocytosis of these vesicles, CD107a is briefly expressed on the surface of the cell, where it can be labeled by antibody and assayed by flow cytometry. Labeling CD107a identifies cells that have recently degranulated and has been correlated lysis of target cells by both $CD8^+$ T cells and NK cells (228,233,234). In addition to synthesizing IFN-γ and TNF-α, TG-resident memory $CD8^+$ T cells have been shown to express CD107a on their cell surface during stimulation with peptide-pulsed target cells (67).

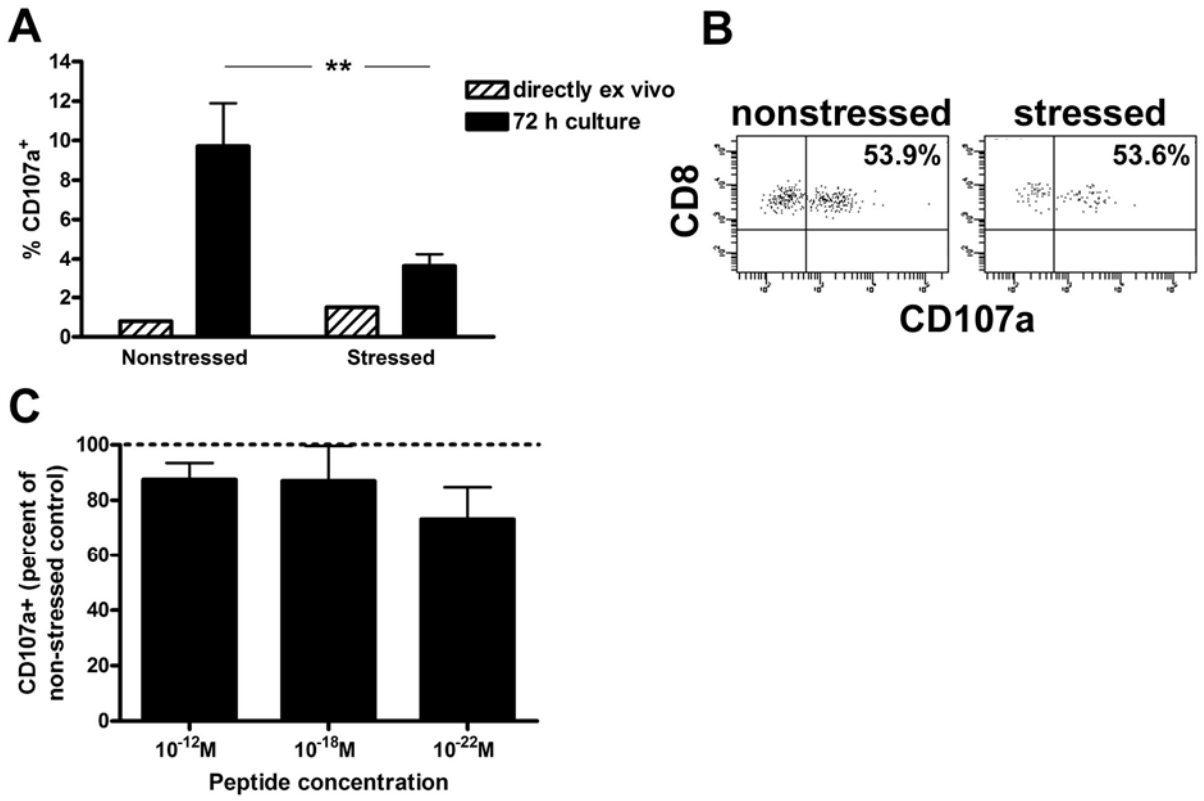

Figure 25. Stress impairs CD107a expression and granzyme B release.

Latently infected TG were excised from stressed or nonstressed mice and **(A)** assayed for surface expression of CD8, CD45, and CD107a by flow cytometry either directly ex vivo or for the last 6 h of a 72 h incubation. Directly ex vivo (n = 1 for both groups), 72 h culture (nonstressed, n = 14; stressed, n = 20). ** p = 0.0038, Student's t test. **(B)** TG were incubated for 6 h with 10^{-12}M peptide-pulsed B6WT3 fibroblasts and measured for CD8, CD45, and CD107a. Numbers in top right quadrant indicate percentage of $CD8^+$ T cells that expressed CD107a. **(C)** The percentage of $CD8^+$ T cells from stressed TG that upregulated surface CD107a is shown as a percentage (± SEM) of the nonstressed response at each peptide concentration. 10^{-12}M (n = 4), 10^{-18}M (n = 3), 10^{-22}M, (n = 2).

Expression of CD107a is negligible on the cell surface of TG-resident $CD8^+$ T cells directly ex vivo. Approximately 10% of the $CD8^+$ T cells upregulate CD107a from 66 to 72 h in culture in nonstressed controls, but this upregulation is not observed in cultures from stressed ganglia (Figure 25A). During 6 h of optimal stimulation, approximately 50% of $CD8^+$ T cells in the TG produce IFN-γ and express CD107a (67). As observed with IFN-γ synthesis (Figure 19E

and Figure 21B), stress had no effect of CD107a expression during optimal stimulation (Figure 25B). Reducing the antigen density revealed a functional compromise in degranulation; at an epitope density of 10^{-22}M, $CD8^+$ T cells from stressed TG degranulated only 73% as much as their nonstressed counterparts (Figure 25C).

A major component of preformed lytic granules is the serine protease granzyme B (235). Approximately 50% of virus-specific $CD8^+$ T cells in the TG contain intracellular granzyme B (148). Based on the deficit in lytic granule release by $CD8^+$ T cells in TG of stressed mice (Figure 25), we hypothesized that we would see a corresponding reduction in intracellular granzyme B. However, the amount of granzyme B strored in each cell increased significantly in the TG of stressed mice: the mean fluorescent intensity (MFI) of intracellular granzyme B was increased 80% following stress (Figure 26) in the virus-specific population, indicating that stress caused an accumulation of granzyme B within $CD8^+$ T cells. This accumulation could be due to proper granzyme B synthesis coupled with impaired granule release shown in Figure 25.

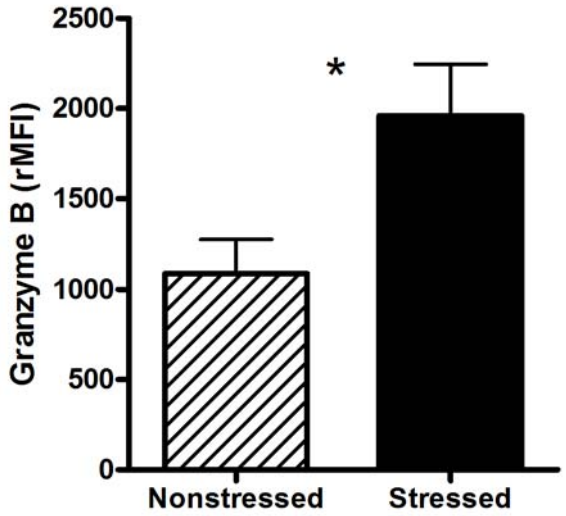

Figure 26. Increase in intracellular granzyme B MFI following stress.

TG were excised from latently infected stressed or nonstressed mice were labeled with anti-CD8α, anti-CD45, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers. Cells were then permeabilized and

measured for intracellular granzyme B by flow cytometry. Graph depicts the relative mean fluorescence intensity (MFI, ± SEM) of granzyme B antibody in the $CD8^+$tetramer$^+$ population. Nonstressed, n = 12, stressed, n = 12. * p = 0.0154, Student's t test.

Interestingly, granzyme B also accumulated within the gB-nonspecific $CD8^+$ T cell population (Figure 27). Much fewer gB-nonspecific $CD8^+$ T cells express granzyme B than gB-specific T cells, and the amount of granzyme B per cell is much less, demonstrating the efficacy of granzyme B as a marker for antigen-mediated activation versus other activation markers, such as CD69. As such, the granzyme B^+ gB-nonspecific cells could potentially represent a population of HSV-specific cells that recognize an as-of-yet undefined epitope. Regardless, as both gB-specific and gB-nonspecific cells accumulated granzyme B following stress, expression of granzyme B as a percentage of the population also increased slightly (Figure 28), albeit nonsignificantly.

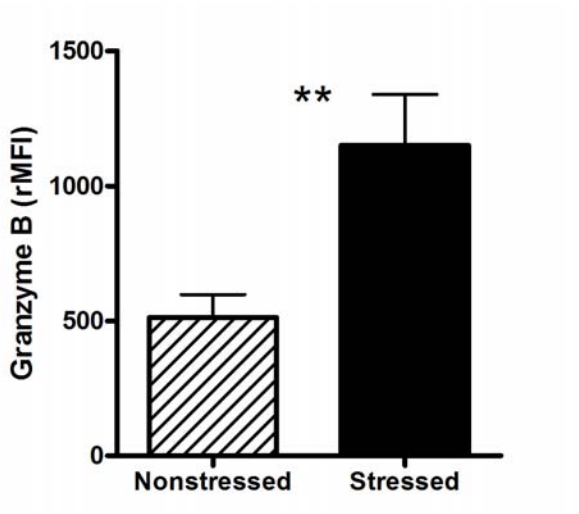

Figure 27. Stress increases granzyme B in the gB-nonspecific population.

TG were excised from latently infected stressed or nonstressed mice were labeled with anti-CD8α, anti-CD45, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers. Cells were then permeabilized and measured for intracellular granzyme B by flow cytometry. Graph depicts the relative MFI (± SEM) of granzyme B antibody in the $CD8^+$tetramer^{neg} population. Nonstressed, n = 12, stressed, n = 12. ** p = 0.0044, Student's t test.

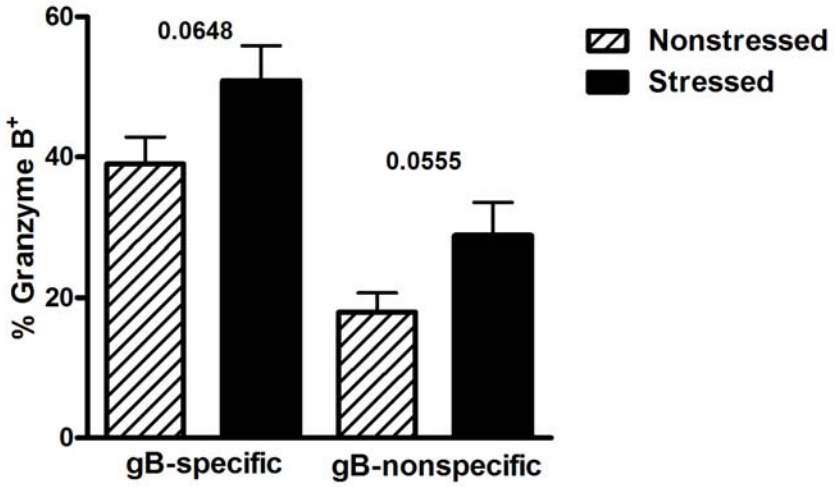

Figure 28. Stress increases the percentage of granzyme B^+ $CD8^+$ T cells.

TG were excised from latently infected stressed or nonstressed mice were labeled with anti-CD8α, anti-CD45, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers. Cells were then permeabilized and measured for intracellular granzyme B by flow cytometry. Graph depicts the mean (± SEM) percentage of the $CD8^+$ population that is positive for intracellular granzyme B. Nonstressed, n = 12, stressed, n = 12. p values determined by Student's t test.

CD107a expression is correlated with cytotoxicity (233). Restraint stress during primary infection has been shown to impair the cytotoxic function of T cells in the DLN, and this compromise is dependent on signaling through β-adrenergic receptors (203). During latency, restraint stress has been shown to compromise cytotoxicity by HSV-specific splenocytes (223). At low epitope densities, we observed a deficiency in the ability of TG-resident $CD8^+$ T cells to degranulate (Figure 25C). To directly relate CD107a expression with cytotoxic function within a small population of cells, we developed a novel flow-based cytotoxicity assay, fibroblasts labeled with the fluorescent membrane dye DiI are pulsed with varying concentrations of $gB_{498\text{-}505}$ peptide, then incubated for 6 h with TG cells in the presence of FAM-VAD-FMK, a fluorescently-labeled, cell permeable, non-cytotoxic molecule that binds activated caspases and inhibits their function (236). As the cells undergo caspase-mediated apoptosis they take up the

reagent, blocking their death and rendering them fluorescent (Figure 29). This assay will allow for the simultaneous measurement of apoptosis in target cells and CD107a expression by $CD8^+$ T cells.

This assay, currently still in development, should provide an excellent new tool with which to assess the cytotoxic potential of virus-specific $CD8^+$ T cells in the latently-infected TG.

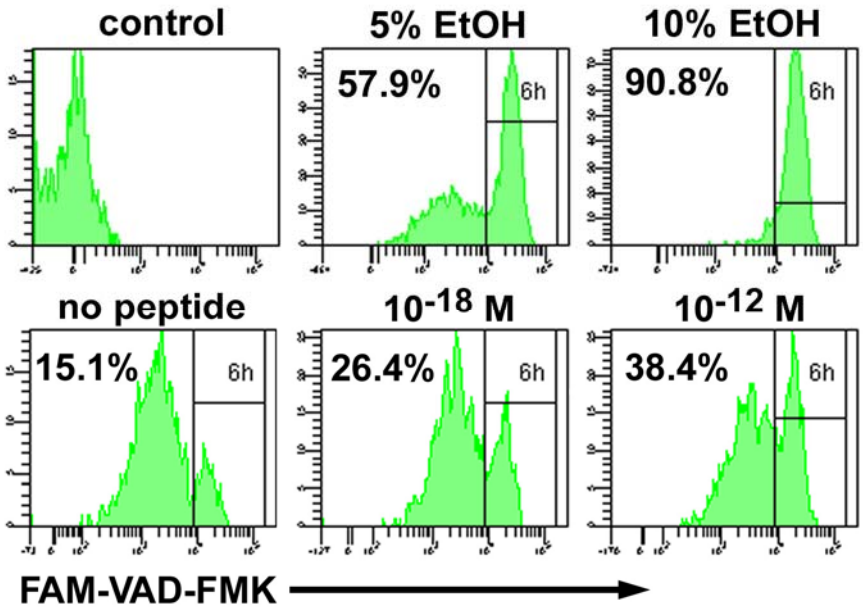

Figure 29. The FAM-VAD-FMK cytotoxicity assay.

$gB_{498-505}$-specific $CD8^+$ T cells were incubated with DiI-labeled B6WT3 fibroblasts pulsed with varying concentrations of $gB_{498-505}$ peptide for 6 h at a 1:1 effector to target ratio, with or without added ethanol, in the presence of FAM-VAD-FMK, a fluorescently-labeled inhibitor of caspase activation that binds activated caspases. Representative histograms show the percentage of fibroblasts that were postitive for incorportation of the FAM-VAD-FMK reagent. Control, 5% Ethanol (EtOH), 10% EtOH (n = 1 each), no peptide, 10^{-18}M, 10^{-12}M (n = 2 each).

4.5.6 T cell effector functions time course

At the optimal stimulatory dose of peptide, stressed T cells could respond as well as nonstressed controls by both IFN-γ production and degranulation (Figure 19E and Figure 21B). However, IFN-γ production and degranulation were measured at a single time (6 h of stimulation) leaving open the possibility that stress affects the rate of cytokine production or lytic granule exocytosis, potentially important for the ability of $CD8^+$ T cells to block reactivation prior to virion synthesis. To address this possibility, $CD8^+$ T cells from stressed or control mice were stimulated with fibroblasts pulsed with 10^{-12} M gB-peptide and examined at varying times within the 6 h assay. The percentage of cells that responded by expressing CD107a and synthesizing IFN-γ was similar between stressed and nonstressed TG at all time points examined (Figure 30A). Thus, at the optimal stimulatory concentration of peptide, stressed T cells were indeed as efficient at effector functions as controls. Interestingly, $CD8^+$ T cells that produced IFN-γ had all initiated production by 2 h of stimulation (Figure 30B), whereas degranulation continued steadily throughout the 6 h assay (Figure 30C).

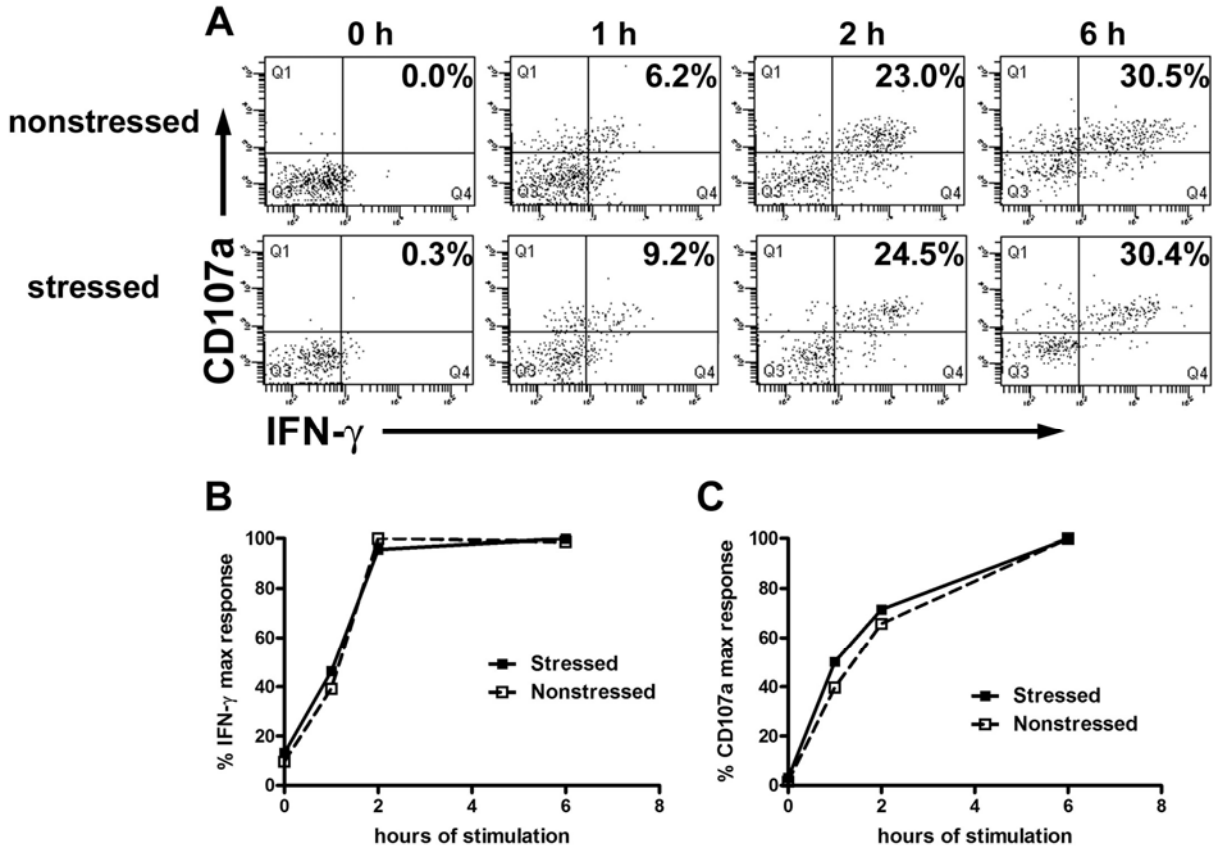

Figure 30. Time course of effector functions after stress.

TG were excised from stressed or nonstressed latently infected ganglia, incubated for varying times with 10^{-12}M peptide pulsed B6WT3 fibroblasts in the presence of GolgiPlug™ and anti-CD107a, labeled with anti-CD8α and anti-CD45, permeabilized, labeled with anti-IFN-γ and analyzed by flow cytometry. **(A)** Representative dot plots gated on $CD8^+$ T cells. Numbers in top right corner indicate percentage of $CD8^+$ T cells that are positive for both IFN-γ production and CD107a expression. **(B)** Graph depicts the percentage of $CD8^+$ T cells that produce IFN-γ as a percentage of the maximum response. **(C)** Graph depicts the percentage of $CD8^+$ T cells that express CD107a as a percentage of the maximum response.

4.5.7 Increase in apoptosis potential following restraint stress

The 30% reduction in $CD8^+$ T cell number after restraint stress could be due to several mechanisms, two of which are in situ apoptosis and migration out of the TG. Apoptosis of the

TG-resident $CD8^+$ T cells was measured by incubating single-cell TG suspensions with FAM-VAD-FMK for 1 h in serum-free media directly ex vivo. gB-specific $CD8^+$ T cells from stressed mice incorporated 1.34-fold more FAM-VAD-FMK than controls (Figure 31).

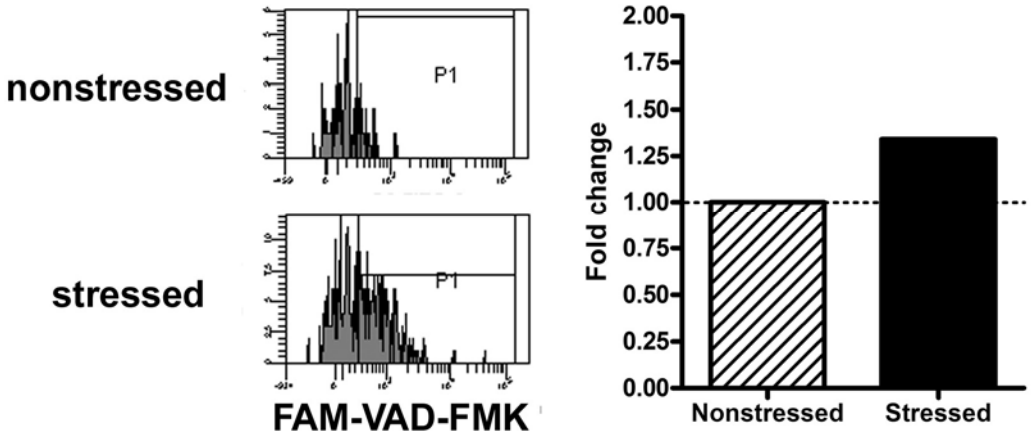

Figure 31. Apoptotic potential is increased following stress.

TG from stressed and nonstressed mice were dissociated into single cell suspensions and incubated with FAM-VAD-FMK for 1 h in serum-free media, then labeled with anti-CD8α and anti-CD45 and analyzed by flow cytometry. Representative histograms show extent of FAM-VAD-FMK incorporation in the $CD8^+$ T cell population. Graph shows the mean fold-change of the percent FAM-VAD-FMK^+ over nonstressed controls.

4.5.8 Proliferation in vivo is not disrupted following stress.

Proliferation of $CD8^+$ T cells from stressed TG was impaired in vitro following stress (see Figure 19A). We measured the rate of proliferation in the TG during the 4 d stress treatment by injecting stressed or nonstressed mice with 1 mg BrdU daily (Figure 32). BrdU is a thymidine analogue that is incorporated into new synthesized DNA strands, and BrdU incorporation is therefore a marker of cells undergoing proliferation (229). After 4 d, the rates of BrdU incorportation were remarkably low (<5%), and there were no significant differences between stressed groups and controls. Interestingly, there was a significant difference ($p = 0.0467$, Student's t test) between

the rates of proliferation of gB-specific and gB-nonspecific cells within the stressed mice. This data could indicate that the presence of reactivating virus and viral protein expression (Figure 11) was inducing a recall response in the virus-specific population. Whether the ratio of virus-specific to nonspecific cells would change over time given a longer exposure to stress is unclear, but after our 4 d stress protocol we did not observe a shift in the ratio (Figure 14B).

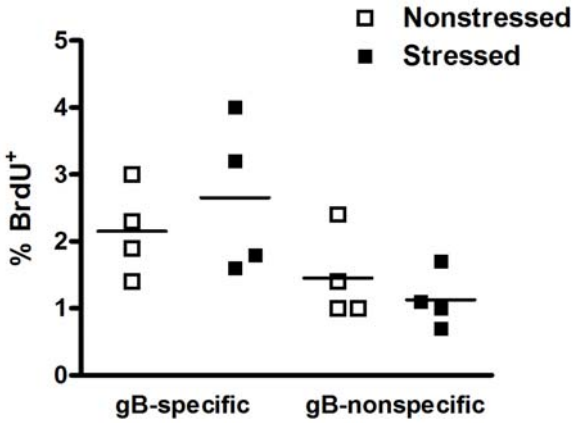

Figure 32. Incorporation of BrdU in the TG during stress.

Latently infected mice were treated with 1 mg BrdU i.p. daily for 4 d during the stress protocol. 34 d p.i. mice were sacrificed and BrdU incorporation was measured by flow cytometry in CD8α^+ cells gated on CD45$^+$ cells. Differences between stressed (n = 4) and nonstressed (n = 4) groups were nonsignificant. Among the stressed group, the difference in BrdU incorporation between the gB-specific and gB-nonspecific was significant (p = 0.0302, Student's paired *t* test).

4.5.9 Potential regulators of the stress effect

Combined, the above data describe a pervasive stress-induced compromise in CD8$^+$ T cell function. TG-resident CD8$^+$ T cell number in stressed mice is reduced, and the cells that remain have a greater apoptotic potential. The cells are also impaired in their abilities to proliferate in response to antigen and to synthesize cytokines and degranulate upon physiologic stimulation. Expression of several surface receptors has been correlated with T cell dysfunction. CD8$^+$ T cell

exhaustion during chronic viral infection is associated with increased PD-1 expression, and blocking PD-1/PD-L1 interactions restores function in exhausted T cells from chronically infected mice (165). Expression of the heteodimer CD94-NKG2A is associated with reduced cytotoxicity and blocking the interaction of CD94-NKG2A with its ligand can restore cytotoxic ability (148,237). We examined the surface expression of PD-1 and NKG2A on the $CD8^+$ T cells from stressed mice. Although we did note stark differential expression among gB-specific and gB-nonspecific cells (Figure 33), potentially signifying a differential mechanism for regulating the gB-nonspecific cell population in vivo, we did not observe significant differences in the expression of either of these receptors due to stress (Figure 34).

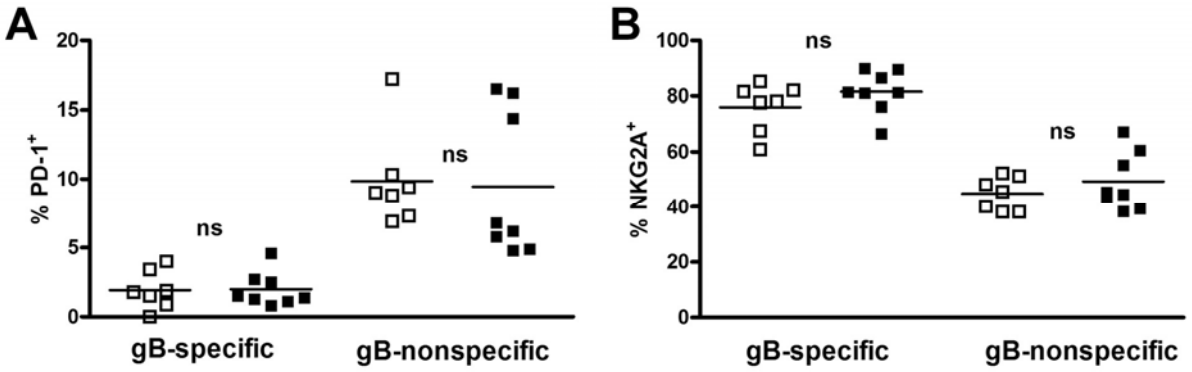

Figure 33. Differential expression of PD-1 and NKG2A.

TG were excised from stressed (closed squares, n = 8) or nonstressed (open squares, n = 7) mice and labeled with anti-CD45, anti-CD8α, $gB_{498\text{-}505}$/H-$2K^b$ tetramer, anti-PD-1, and anti-NKG2A, and analyzed by flow cytometry. **(A)** The percentage of $CD8^+$ T cells that expressed the PD-1 molecule. **(B)** The percentage of $CD8^+$ T cells that expressed the NKG2A molecule. ns, nonsignificant, Student's t test.

CTLA-4 expression, associated with impaired T cell responses, was also unchanged (Figure 34A). Diminished function could also be associated with reduced expression of activating receptors such as NKG2D. Roughly half of the $CD8^+$ T cell population expressed

NKG2D (Figure 34C), but we did not examine whether there was differential distribution of NKG2D or CTLA-4 between the gB-specific and gB-nonspecific populations.

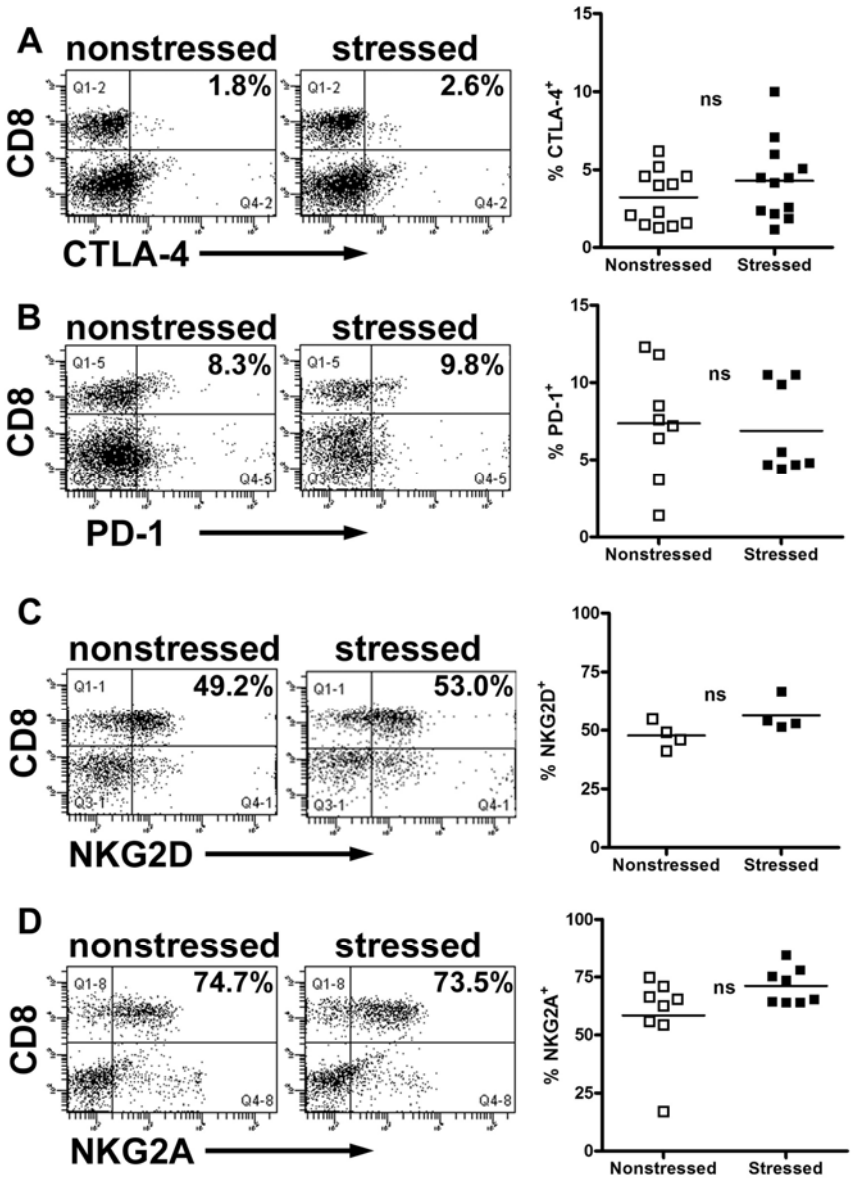

Figure 34. Expression of regulatory receptors on CD8$^+$ T cells in the TG after stress.

TG-resident CD8$^+$ T cells from stressed (n = 8) and nonstressed (n = 8) mice were assayed for cell surface expression of CTLA-4 **(A)**, PD-1 **(B)**, NKG2D **(C)**, or NKG2A **(D)** by flow cytometry. Representative dotplots show the CD45$^+$ population. Numbers in the top right quadrant are percent of CD8$^+$ T cells that are positive for the given molecule. Graphs on the right are values pooled from 2-4 experiments. ns, not significant, Student's t test.

We also examined the spleens of stressed mice, observing a significant decrease in $CD8^+$ and $CD4^+$ T cell numbers in stressed spleens (data not shown). To further investigate the splenocyte populations, we examined the susceptibility to IL-2 and IL-15 by measuring the surface expression of components of their receptors. IL-2 and IL-15 belong to the γc receptor family and share two out of 3 receptor components: the IL-2R/IL-15R β chain (CD122) and the common γ chain (CD132) (159). IL-2 binds the CD122/CD132 complex with low affinity, but when IL-2Rα (CD25) is present, IL-2 binds the tripartite receptor with high affinity. IL-15 binds the IL-15Rα subunit and can be presented to the CD122/CD132 receptor complex in *trans* (157,238), thus IL-15Rα is dispensible on the T cell surface (158). Therefore we investigated whether stress affected the surface expression of CD25, the IL-2Rα chain, and CD122, the IL-2R/IL-15R β chain (Figure 35) on $CD8^+$ and $CD4^+$ T cells in the spleen. We did not observe any overt differences between stressed and nonstressed spleens in the expression of CD25, CD122, or CD132 on $CD8^+$ or $CD4^+$ T cells. Thus, sensitivity of the remaining T cell populations to IL-2, and, perhaps, IL-15 as well, remains unaffected following stress.

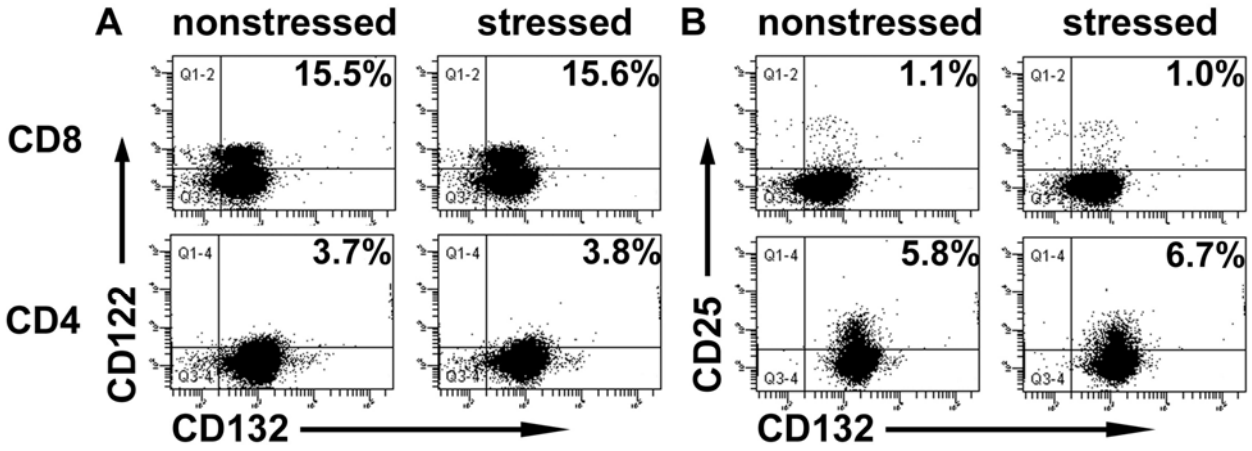

Figure 35. Surface receptor expression on splenocytes after stress.

Spleens were removed from stressed (n = 4) or nonstressed (n = 3) mice, labeled with antibodies to CD45, CD4, CD8, CD25, CD122, and CD132, then analyzed by flow

cytometry. Representative dotplots show the expression of CD122 **(A)** and CD25 **(B)** and CD132 on $CD8^+$ T cells (top row) and $CD4^+$ T cells (bottom row). Numbers in the top right quadrant indicate the percentage of the population that is double-positive for expression of CD132 and the receptor of interest.

4.6 ROLE OF CORTICOSTERONE IN STRESS RESPONSE

4.6.1 Direct corticosterone treatment dramatically reduces $CD8^+$ and $CD4^+$ T cells in the TG

The main effector molecules of the stress response are glucocorticoids. In mice, the major glucocorticoid is corticosterone. In order to recapitulate the effects of stress, mice were treated with 400 μg/ml corticosterone in their drinking water for 4 consecutive days during latency. This dose correlates with a greater-than-mild stress response, and is similar to doses used in corticosterone control-release implants (239,240). Figure 36 shows the results in $CD8^+$ T cells and $CD4^+$ T cells. For both cell types, corticosterone treatment dramatically reduced the number of cells, up to 90% for CD8s.

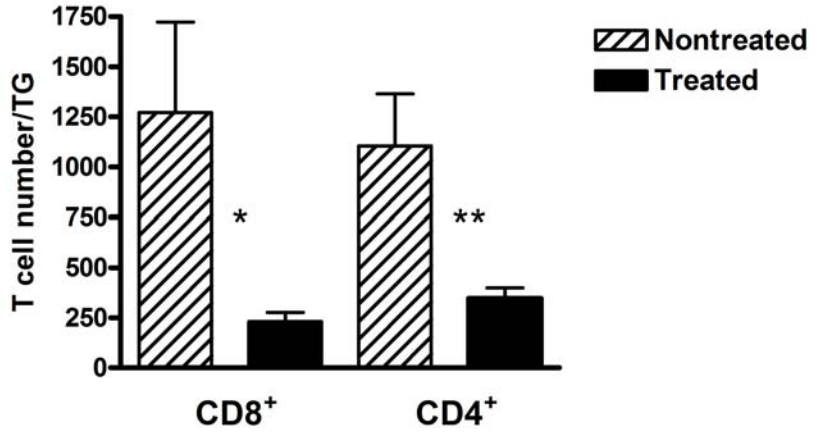

Figure 36. Reduction in T cell numbers following direct corticosterone treatment.

Mice were treated with 400 μg/ml corticosterone in their drinking water for 4 d during latency. TG from corticsosterone treated or nontreated mice were excised and labeled with anti-CD45, anti-CD4, and anti-CD8α and analyzed by flow cytometry. Nontreated (CD8, n = 4, CD4, n =10), Treated (CD8, n = 7, CD4, n = 13). * p = 0.0121, ** p = 0.0039, Student's t test.

4.6.2 Loss of $CD8^+$ T cells is due in part to apoptosis

Glucocorticoids are well-known mediators of apoptosis in T cells (181). The 90% reduction in $CD8^+$ T cell number following corticosterone treatment could be due to apoptosis of the TG-resident T cell population. TG cells were labeled with Annexin-V, a molecule that binds phosphatidylserine on the cell surface (241). Phosphatidylserine is a lipid molecule that is normally only found in the inner leaflet of the plasma membrane; expression of phosphatidylserine on the outside of the plasma membrane is an early marker of apoptosis. TG whole mounts from corticosterone treated or nontreated mice were stained with anti-CD8α and Annexin-V and analyzed by confocal microscopy (Figure 37). While nearly all $CD8^+$ T cells in the corticosterone-treated TG costained with Annexin-V, indicating they were apoptotic, no $CD8^+$ T cells in nontreated TG costained with Annexin-V.

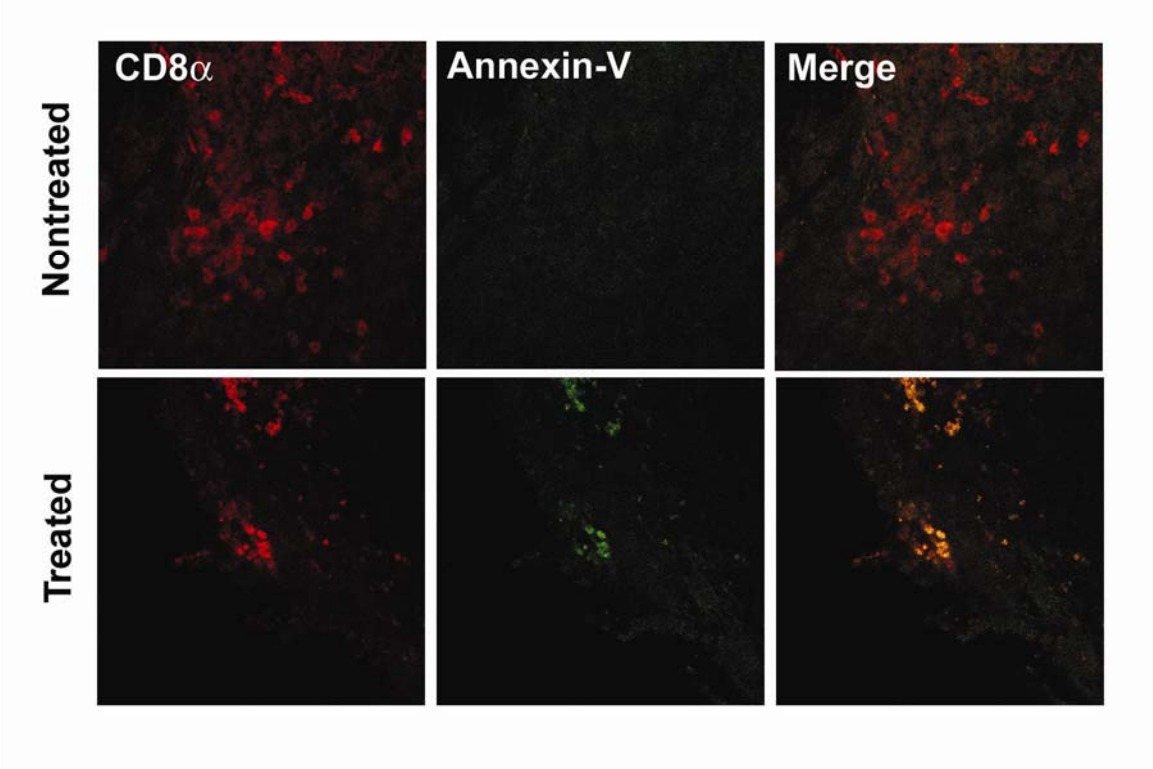

Figure 37. Corticosterone-mediated apoptosis of CD8$^+$ T cells in the TG.

Latently infected corticosterone treated or nontreated mice were perfused with 1% PFA. TG were excised and labeled with anti-CD8α (red) and Annexin-V (green), then analyzed by confocal microscopy.

4.6.3 Direct corticosterone treatment enriches for activated cells.

We examined the activation status of the remaining CD8$^+$ and CD4$^+$ T cells following corticosterone treatment. In both populations, the percentage of cells that were positive for the early activation marker CD69 was significantly increased (Figure 38). Whether corticosterone treatment leads to T cell activation, or whether CD69 expression designates cells that are more resistant to the apoptotic effects of stress is currently unknown. In latently infected TG, most of the cells are CD69$^+$, so the significance of our observed increase in CD69 expression is also unknown. A combination of corticosterone directly or indirectly activating cells, and activation

subsequently promoting the survival of these cells is most likely the explanation for these observations. It should be noted, though, that the absolute numbers of $CD69^+$ cells is still significantly lower in corticosterone treated mice versus controls, as there are much fewer cells total (see Figure 36).

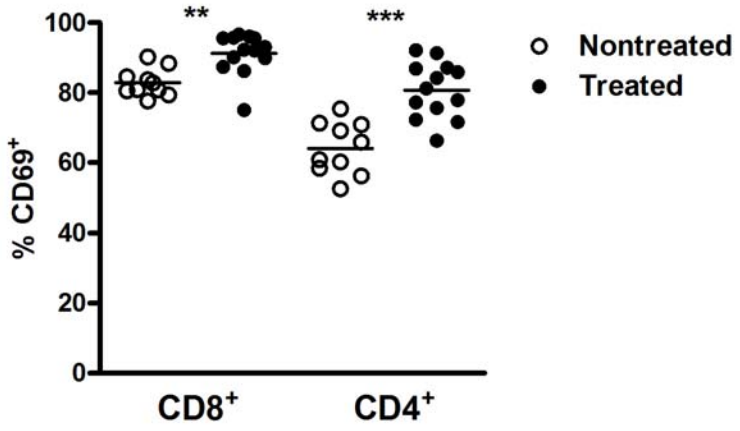

Figure 38. Corticosterone treatment enriches for activated T cells.

TG from corticosterone treated or nontreated mice were excised and labeled with anti-CD45, anti-CD8α, anti-CD4, and anti-CD69 and analyzed by flow cytometry. Graph shows the percentage of $CD8^+$ or $CD4^+$ T cells that express CD69. Nontreated, n = 9, Corticosterone treated, n = 13. ** p = .0010, *** p < 0.0001, Student's t test.

4.6.4 Frequency of virus-specific T cells is unchanged following corticosterone treatment.

The retention of activated cells did not translate to a selective retention of virus-specific cells (Figure 39A). Given that most of the T cells are already activated in the latently infected TG, this result is not altogether surprising. One interesting phenomenon occurred when mice were treated with corticosterone longer than 4 d. Following a 10 d corticosterone treatment, the numbers of $CD8^+$ T cell in the TG were similar to that seen after only 4 d of treatment (compare Figure 39B with Figure 36). Additionally, the percentage of $gB_{498\text{-}505}$-specific $CD8^+$ T cells in the TG after

10 d of treatment was similar to nontreated controls (Figure 39C). These data could be explained by at least two hypotheses: i) that approximately 10% of the $CD8^+$ T cells in the latently infected TG are resistant to corticosterone, or ii) the TG is replenished continuously by approximately 10% of the total TG cell pool. The former hypothesis implies that glucocorticoid resistance is evenly distributed between the virus-specific and nonspecific cells. The latter hypothesis implies that the infiltration of cells into the ganglia is preferential for virus-specific cells, since the even distribution of virus-specific and nonspecific cells that results derives from a much lower frequency of virus-specific cells in the circulation (data not shown).

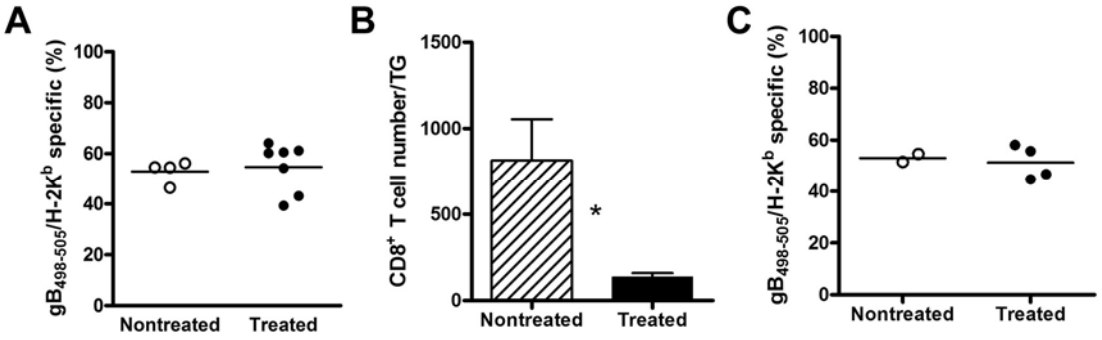

Figure 39. gB-specificity following corticosterone treatment.

TG from corticosterone treated or nontreated mice were excised and labeled with anti-CD45, anti-CD8α, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers and analyzed by flow cytometry. **(A)** The percentage of $CD8^+$ T cells that specific for $gB_{498\text{-}505}$ after 4 d of corticosterone treatment. Nontreated, n = 4, corticosterone treated, n = 7. **(B)** The absolute number of $CD45^+$ $CD8^+$ T cells per TG after 10 d of corticosterone treatment. Nontreated, n = 2, corticosterone treated, n = 4. * p = 0.0108. Student's t test. **(C)** The percentage of $CD8^+$ T cells that specific for $gB_{498\text{-}505}$ after 10 d of corticosterone treatment. Nontreated, n =2, corticosterone treated, n = 4.

4.6.5 Direct corticosterone treatment does not dysregulate granzyme B expression.

Surprisingly, the selective retention of activated cells after 4 d of treatment did not correlate with an increase in intracellular granzyme B, as observed following stress (see Figure 26). The virus-

specific $CD8^+$ T cell population did not increase the MFI for intracellular granzyme B (Figure 40). Although glucocorticoids have been shown to affect cytotoxicity in some studies (206,242), in others cytotoxicity is affected by catecholamines (203,243). Remarkably, in the gB-nonspecific population granzyme B expression was actually decreased 45% (Figure 41). The significance of this finding is currently unknown.

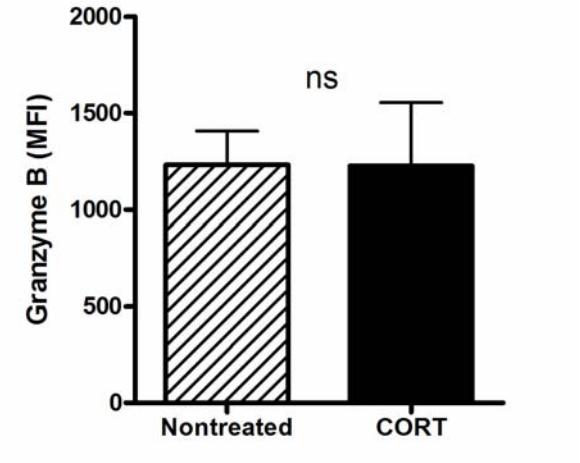

Figure 40. Direct corticosterone treatment does not affect intracellular granzyme B.

TG were excised from latently infected corticosterone treated (CORT, n = 12) or nontreated (n = 12) mice were labeled with anti-CD8α, anti-CD45, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers. Cells were then permeabilized and measured for intracellular granzyme B by flow cytometry. Graph depicts the relative MFI (± SEM) of granzyme B antibody in the $CD8^+$tetramer$^+$ population. ns, not significant, Student's t test.

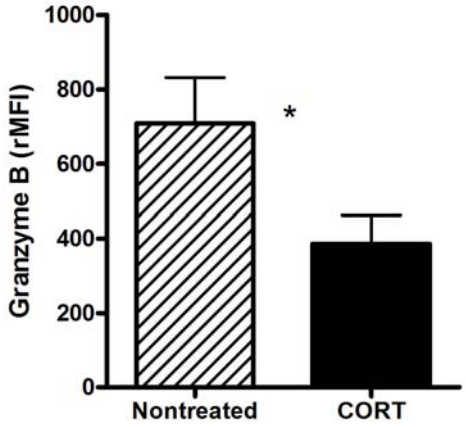

Figure 41. Corticosterone treatment decreases intracellular granzyme B in the gB-nonspecific population.

TG were excised from latently infected corticosterone treated (CORT, n = 12) or nontreated (n = 12) mice were labeled with anti-CD8α, anti-CD45, and $gB_{498\text{-}505}$/H-$2K^b$ tetramers. Cells were then permeabilized and measured for intracellular granzyme B by flow cytometry. Graph depicts the relative MFI (± SEM) of granzyme B antibody in the $CD8^+tetramer^{neg}$ population. * p = 0.0367, Student's t test.

4.6.6 IFN-γ synthesis is not affected following corticosterone treatment

As we observed following stress treatment, direct corticosterone treatment had no overt effect on the ability of $CD8^+$ T cells to produce IFN-γ in response to optimal stimulation. Figure 42 shows the percentage of $CD8^+$ T cells that respond to stimulation with IFN-γ is equivalent in corticosterone treated and nontreated mice. In addition, the amount of IFN-γ produced per cell is similar between the groups. These data suggest that although there is a nearly 90% reduction in the absolute number of $CD8^+$ T cells capable of producing IFN-γ, the actual IFN-γ synthesis machinery is not disrupted by glucocorticoid treatment.

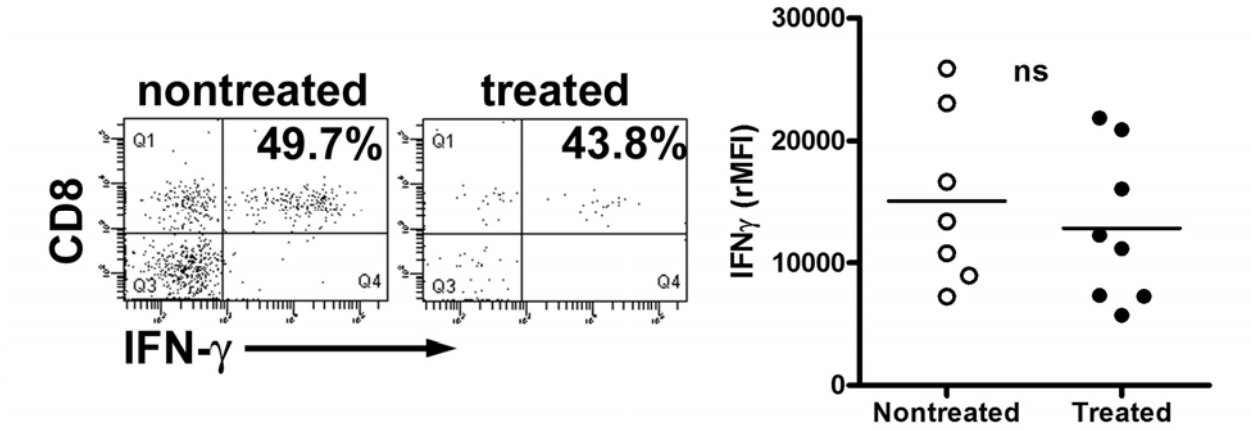

Figure 42. The IFN-γ response following direct corticosterone treatment.

TG from corticosterone treated or nontreated mice were excised, incubated for 6 h with 10^{-12}M $gB_{498-505}$ peptide pulsed B6WT3 fibroblasts in the presence of GolgiPlug™. Cell suspensions were then labeled with anti-CD8α and anti-CD45, permeabilized, stained for intracellular IFN-γ and analyzed by flow cytometry. Representative dotplots on the left gated on $CD45^+$ T cells. Numbers in top right quadrants indicate percentage of $CD8^+$ T cells positive for IFN-γ expression. Graph on the right is relative MFI of IFN-γ expression pooled from 2 experiments. Nontreated, n = 7, Treated, n = 8. ns, not significant, Student's t test.

The cytokine response of corticosterone treated $CD8^+$ T cells was not tested at suboptimal concentrations of peptide, so the possibility exists that glucocorticoids are responsible for the compromise in the cytokine response at low densities of antigen. Adrenalectomy, which eliminates glucocorticoids, has been shown to restore stress-induced compromises in the production of IL-6 and IFN-γ (220).

4.6.7 Degranulation is not affected following corticosterone treatment.

As depicted above, direct corticosterone treatment did not affect intracellular granzyme B retention in the gB-specific $CD8^+$ T cell pool (Figure 40). With stress treatment, the higher

granzyme B levels coincided with a reduced ability to degranulate at low epitope densities. Optimal stimulation restored the ability of the $CD8^+$ T cells to degranulate (Figure 25B), although proliferation was still reduced (Figure 19A and B). Given the gB-specific population was not compromised in their levels of intracellular granzyme B following corticosterone treatment, we would not hypothesize that degranulation capacity per cell would be impaired in corticosterone treated mice. Accordingly, under optimal stimulation conditions, the percentage of $CD8^+$ T cells that degranulated and the amount of surface CD107a expression per cell did not differ between treated or control groups in a 6 h assay (Figure 43). We did not measure the degranulation response at suboptimal epitope densities.

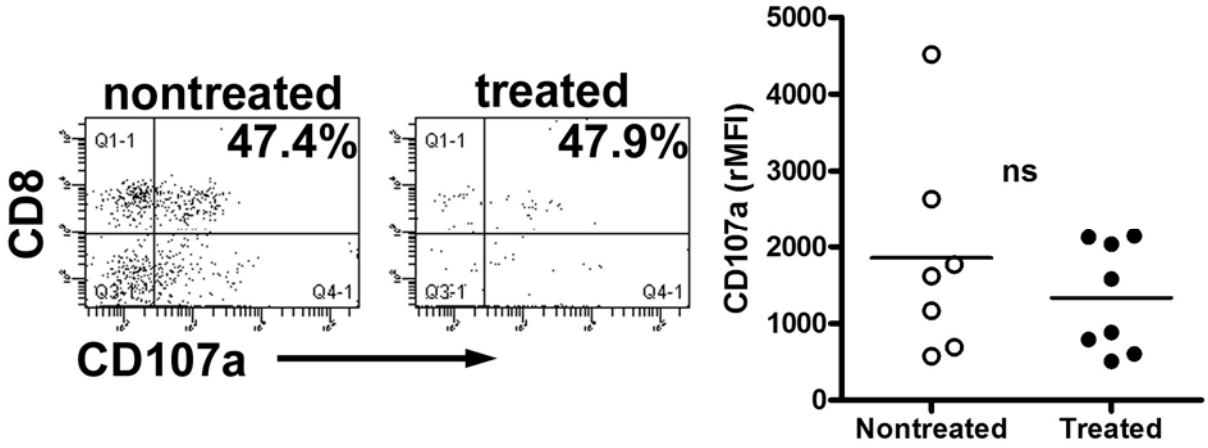

Figure 43. CD107a expression following direct corticosterone treatment.

TG from corticosterone treated or nontreated mice were excised, incubated for 6 h with 10^{-12}M $gB_{498\text{-}505}$ peptide pulsed B6WT3 fibroblasts in the presence of GolgiPlug™ and anti-CD107a. Cell suspensions were then labeled with anti-CD8α and anti-CD45, and analyzed by flow cytometry. Representative dotplots on the left gated on $CD45^+$ T cells. Numbers in top right quadrants indicate percentage of $CD8^+$ T cells positive for CD107a expression. Graph on the right is relative MFI of CD107a expression pooled from 2 experiments. Nontreated, n = 7, Treated, n = 8. ns, not significant, Student's t test.

4.6.8 Reactivation is not affected following corticosterone treatment.

Glucocorticoid treatment protocols have been shown to induce α-herpesvirus reactivation from latency in animal models and in culture (244-249). Restraint stress and in vivo depletion of $CD8^+$ T cells during latency each resulted in a significant increase in viral genome copies per TG. Given that corticosterone treatment drastically reduces the absolute number of IFN-γ producing cells, we anticipated that direct corticosterone treatment would also result in reactivation of latent HSV-1. To our surprise, glucocorticoid treatment did not result in an increase in viral DNA levels in the TG (Figure 44A). This data does fit with other published studies in which glucocorticoid treatment did not induce, or only mildly induced, viral reactivation (250,251). It would seem, therefore, that the uncompromised ability of the remaining $CD8^+$ T cells to degranulate is a major determinant in the prevention of HSV-1 reactivation from latency. Of course, we did not directly measure the ability of corticosterone treated $CD8^+$ T cells to produce cytokines in vivo or in response to neurons in culture, so, if the treated cells are not impaired in their cytokine production at low antigen concentrations, that, too, could potentially add to the success of the remaining $CD8^+$ T cells to protect from reactivation in vivo.

To asses the effect of corticosterone treatment on the abilities of $CD8^+$ T cells to prevent reactivation in culture, exogenous gB-specific T cells were added to T cell-depleted cultures from latently infected, nontreated mice and then treated with 10^{-6} M corticosterone in vitro (Figure 44B). Reactivation of latent virus was undetectable in treated cultures for 6 d, and only in 3% of wells at 7 d in culture. This suggests that the ability of the exogenous $CD8^+$ T cells to prevent reactivation was not compromised by direct corticosterone treatment in culture.

The lack of glucocorticoid-induced reactivation could also be attributable to a biphasic effect in which glucocorticoids disrupt T cell immunity but also act directly on the neuron to block viral DNA replication. To assess whether reactivation in neurons themselves could be inhibited by direct corticosterone treatment, TG were depleted of endogenous T cells and cultured with or without 10^{-6} M corticosterone (Figure 44C). Without allogeneic virus-specific $CD8^+$ T cells, ex vivo TG cultures reproducibly reactivate (64,65). Direct corticosterone treatment in culture did not inhibit the rate of reactivation in TG cultures through 9 d post explant.

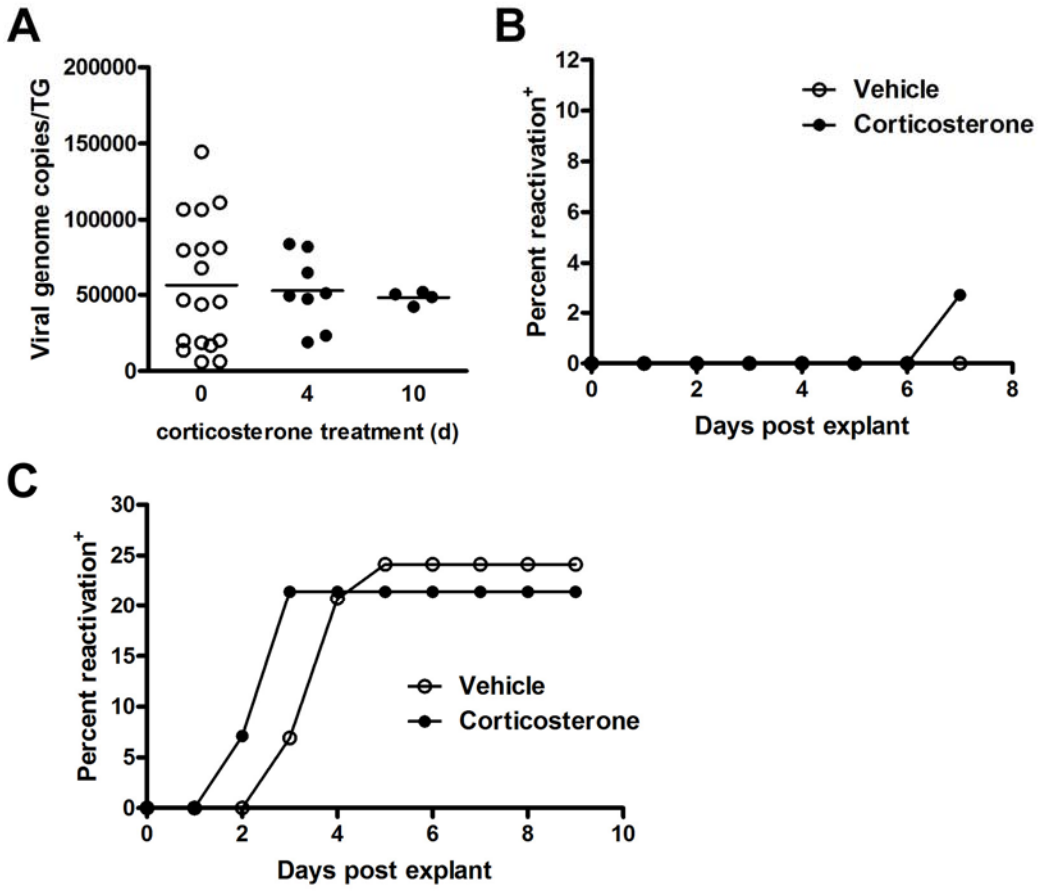

Figure 44. Reactivation following corticosterone treatment in vivo or in vitro.

(A) DNA was isolated from TG from mice that were either nontreated or treated with corticosterone for 4 or 10 d. **(B)** HSV-1 pgB-EGFP-infected TG were depleted of endogenous $CD8^+$ T cells by Dynabead™ negative selection. 10^6 exogenous gB-specific $CD8^+$ T cells were then added to the cultures that were either treated in vitro with 10^{-6}M corticosterone or nontreated, and reactivation was measured by examining cultures daily for spread of fluorescence. **(C)** HSV-1 pgB-EGFP-infected TG were depleted of endogenous $CD45^+$ T cells by Dynabead™ negative selection and left nontreated or treated with 10^{-6}M corticosterone in vitro. Reactivation was measured by examining cultures daily for spread of fluorescence.

4.7 BLOCKING β-ADRENERGIC RECEPTORS CAN INHIBIT THE STRESS-INDUCED GRANZYME B INCREASE

As described above, restraint stress impairs lytic granule release and cytotoxicity, evidenced by an increase in granzyme B. Surprisingly, direct corticosterone treatment did not result in a similar increase in intracellular granzyme B. These data suggest that the stress-induced compromise in lytic granule release may be a glucocorticoid-independent phenomenon. We sought to address the role of the SNS in our model by treating stressed mice with nadolol, a β-adrenergic receptor antagonist. In two out of 3 experiments, nadolol treatment reduced or blocked the stress-induced increase in the amount of intracellular granzyme B (Figure 45). These data are in accordance with previous studies in which nadolol treatment restored the stress-induced impairment in $CD8^+$ T cell cytotoxic ability (203). In addition to using nadolol to restore cytotoxicity, Dobbs, et al, treated stressed mice with the glucocorticoid receptor antagonist RU486, but did not observe a restoration of cytotoxic ability (203). Thus, glucocorticoid signaling was not required for the stress-induced defect in cytotoxicity. Indeed, in our hands, direct corticosterone treatment does not promote the accumulation of intracellular granzyme B, so most likely, cytotoxicity is governed by catecholamines and not glucocorticoids.

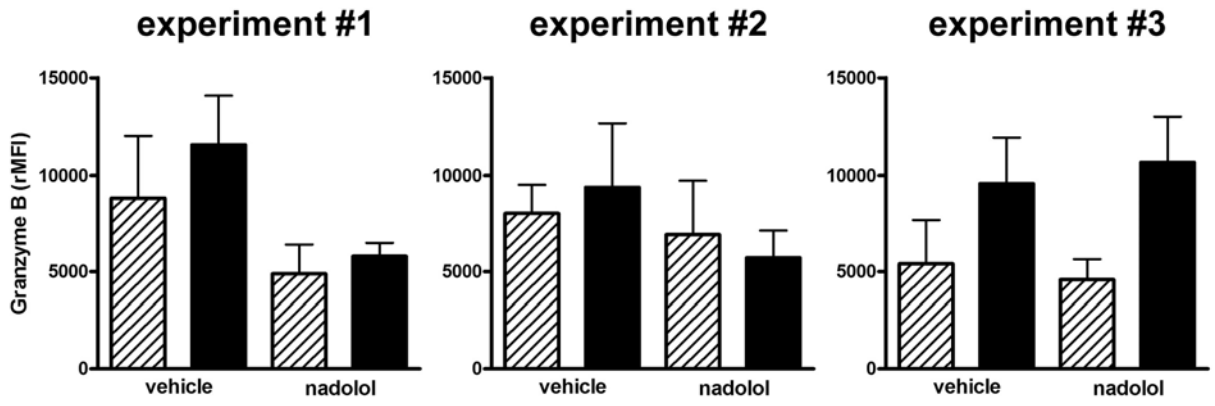

Figure 45. Nadolol treatment during stress can reverse the increase in granzyme B.

Mice were treated with nadolol (5 mg/kg in PBS/0.2% EtOH) i.p. beginning 1 d prior to stress and continuing daily. At 34 d p.i., TG were excised, permeabilized, and labeled with antibodies to CD45, CD8, and intracellular granzyme B, then analyzed by flow cytometry. Relative MFI (± SEM) is shown for three separate experiments. Nonstressed (hatched bars, n = 3-4/group/experiment), stressed (solid bars, n = 4/group/experiment).

Interestingly, our nadolol treatment protocol of daily i.p. injections appears itself to increase serum levels of corticosterone, in all 3 experiments (compare serum corticosterone levels for nonstressed mice in Figure 10 and Figure 46). Here, we see that the serum corticosterone levels of the nonstressed mice has either matched, or surpassed, the serum corticosterone levels in the stressed mice. These data add to the evidence that glucocorticoids are not responsible for the stress-induced increase in granzyme B, as even in the face of elevated serum glucocorticoids, nonstressed mice have consistently less intracellular granzyme B than stressed mice.

It has yet to be determined whether the stress-induced increase in intracellular granzyme B is correlated with reduced T cell cytotoxicity. The newly developed FAM-VAD-FMK cytotoxicity assay (Figure 29) will allow us to examine (i) whether stressed $CD8^+$ T cells are inhibited in their ability to induce apoptosis in target cells, (ii) if the 6 h stimulation causes a measurable decrease in intracellular granzyme B in nonstressed controls, indicative of

degranulation, (iii) if the stressed $CD8^+$ T cells are compromised in degranulation, and (iv) if in vivo nadolol treatment restores cytotoxicity and degranulation.

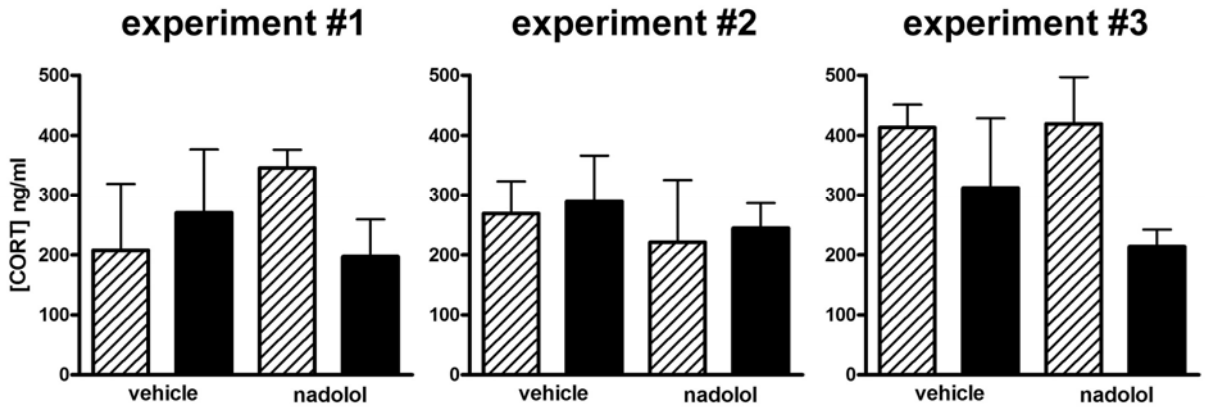

Figure 46. Serum corticosterone levels following stress and nadolol treatment.

Mice were treated with nadolol (5 mg/kg in PBS/0.2% EtOH) i.p. beginning 1 d prior to stress and continuing daily. At 34 d p.i., mice were sacrificed and serum corticosterone levels were determined by standard radioimmunoassay. Nonstressed (hatched bars, n = 3-4/group/experiment), stressed (solid bars, n = 3-4/group/experiment). CORT, corticosterone.

4.8 THE EFFECTS ON T CELL MEMORY BY STRESS OR CORTICOSTERONE TREATMENT DURING PRIMARY INFECTION

Given the changes in immunity seen following stress or glucocorticoid treatment, stress during an acute immune response could have severe complications on the development and quality of the immunologic memory response.

4.8.1 Stress reduces CD8$^+$ T cell number in the TG

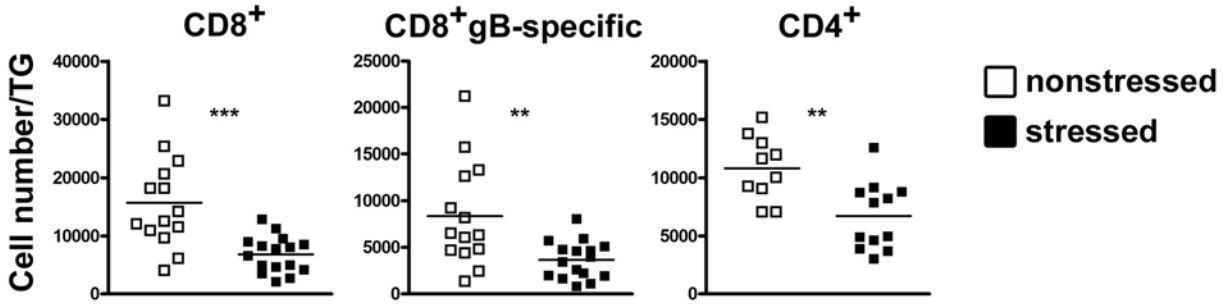

Figure 47. Effects of stress during primary infection.

HSV-1 infected mice were stressed 4-8 d p.i. At 8 d p.i., TG were excised, labeled with anti-CD45, anti-CD8α, anti-CD4, and $gB_{498\text{-}505}$/H-2K^b tetramers, and analyzed by flow cytometry. The numbers of nonstressed (n = 10-14) and stressed (n = 12-16) CD8$^+$, CD8$^+$$gB_{498\text{-}505}$-specific, and CD4$^+$ T cells is shown. ** $p < 0.004$, *** $p < 0.0001$, Student's t test.

Restraint stress beginning 4 d p.i. and continuing nightly for 4 consecutive nights results in an approximately 57% reduction in CD8$^+$ T cell number and virus-specific CD8$^+$ T cell number, and an approximately 40% reduction in CD4$^+$ T cell number in the TG (Figure 47). As both CD8$^+$ and gB-specific CD8$^+$ T cell numbers were reduced a similar percentage, the overall percentage of CD8$^+$ T cells that were specific for HSV-1 did not change following restraint stress. Corticosterone treatment during acute infection also resulted in a dramatic decrease in CD8$^+$ and CD4$^+$ T cell number in the TG. Somewhat surprising, however, was the observation that gB-specific CD8$^+$ T cells were selectively retained in the TG (Figure 48), suggesting early in infection gB-specific T cells are more resistant to glucocorticoids than the overall CD8$^+$ T cell population.

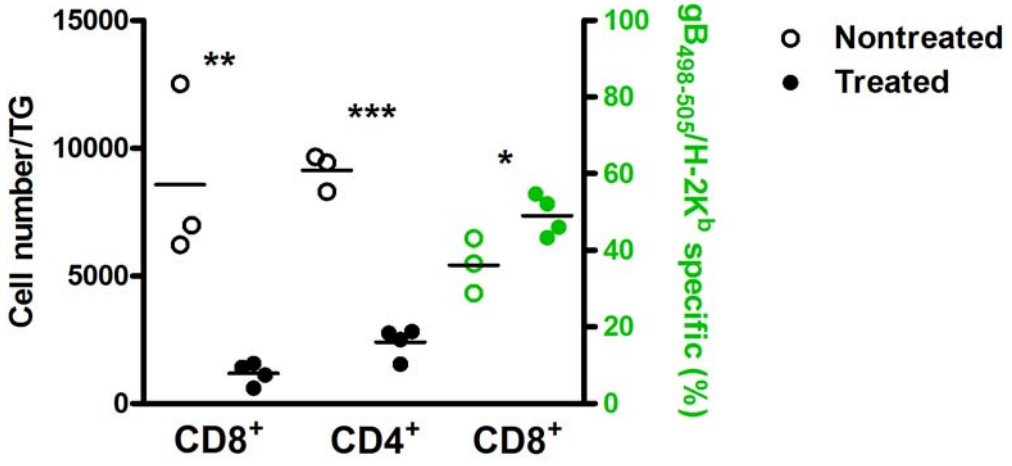

Figure 48. T cell number in the TG following early corticosterone treatment.

Mice were treated with 400 μg/ml corticosterone in their drinking water from 4-8 d p.i. At 8 d p.i., TG were excised, labeled with anti-CD45, anti-CD8α, anti-CD4, and $gB_{498-505}$/H-$2K^b$ tetramers, and analyzed by flow cytometry. The numbers of nonstressed (n = 3) and stressed (n = 4) $CD8^+$ and $CD4^+$ T cells are shown in black on the left axis. In green, the percentage of $CD8^+$ T cells that are specific for $gB_{498-505}$ is shown on the right axis. * $p = 0.0395$, ** $p = 0.0070$, *** $p < 0.0001$, Student's t test.

When mice were treated with corticosterone during latency, activated T cells were selectively retained in the TG (Figure 38). A similar phenomenon was observed when mice were corticosterone treated during acute infection (Figure 49). Together, these data provide evidence that CD69 expression correlates with glucocorticoid resistance. Interestingly, the percentage of $CD69^+$ T cells did not increase following restraint stress (data not shown). There are two potential hypotheses that may address this issue: (i) the glucocorticoid-mediated effects of restraint are not severe enough (compare serum corticosterone levels between stressed and corticosterone treated groups in Figure 10) to reduce T cell numbers enough to where activation-mediated cell survival is detectable, and (ii) stress-induced effects that are glucocorticoid-independent actually intervene in the pro-survival capabilities of activated T cells in the TG. These hypotheses are not mutually exclusive and are readily testable.

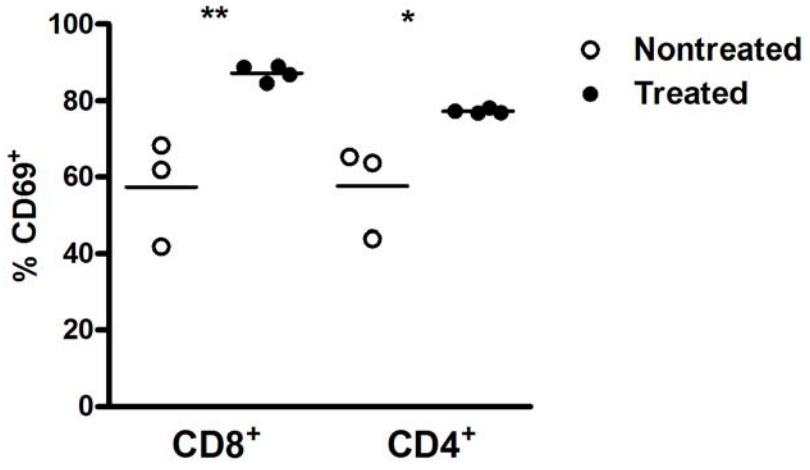

Figure 49. Expression of CD69 following early corticosterone treatment.

Mice were treated with 400 μg/ml corticosterone in their drinking water from 4-8 d p.i. At 8 d p.i., TG were excised, labeled with anti-CD45, anti-CD8α, anti-CD4, and anti-CD69, and analyzed by flow cytometry. The percentage of nonstressed (n = 3) and stressed (n = 4) $CD8^+$ and $CD4^+$ T that are $CD69^+$ is shown. * p = 0.091, ** p = 0.0068, Student's t test.

4.8.2 Control of viral infection in the face of stress during acute infection.

In our model, virus is reproducibly cleared from the eye by 8-10 d p.i. (252). Innate immune mechanisms are most important for the initial clearance of virus and for driving the virus to latency in the TG. The stress protocol was initiated 4 d p.i. and may have affected the ability of the innate immune response to adequately clear the virus. In addition, fewer T cells either enter, or are retained, in the TG at the peak of the response in the face of stress or corticosterone treatment (Figure 47 and Figure 48). We measured viral clearance in the eyes of mice during the stress treatment (Figure 50). By 8 d post infection, only 1 out of 8 nonstressed mice had detectable virus in the tear film but 4 out of 8 stressed mice had detectable virus (data not shown). The virus titer at 8 d was 34-fold higher in stressed mice (Figure 50A). In the TG, stress during acute infection had no effect on viral genome copy number (Figure 50B). Initial clearance

of virus is mediated mostly by the innate immune system, and therefore, beginning stress 4 d post infection may be too late in the infectious cycle in which to observe a change in the establish of viral latency. Interestingly, corticosterone treatment induced a nearly 4-fold increase in viral genome copies (Figure 50B). These data suggest that early in infection, the number of $CD8^+$ T cells correlates better with viral load than during latency. Conversely, direct corticosterone treatment could affect innate immune components, important in driving the virus to latency, more so than restraint, due to higher serum corticosterone levels.

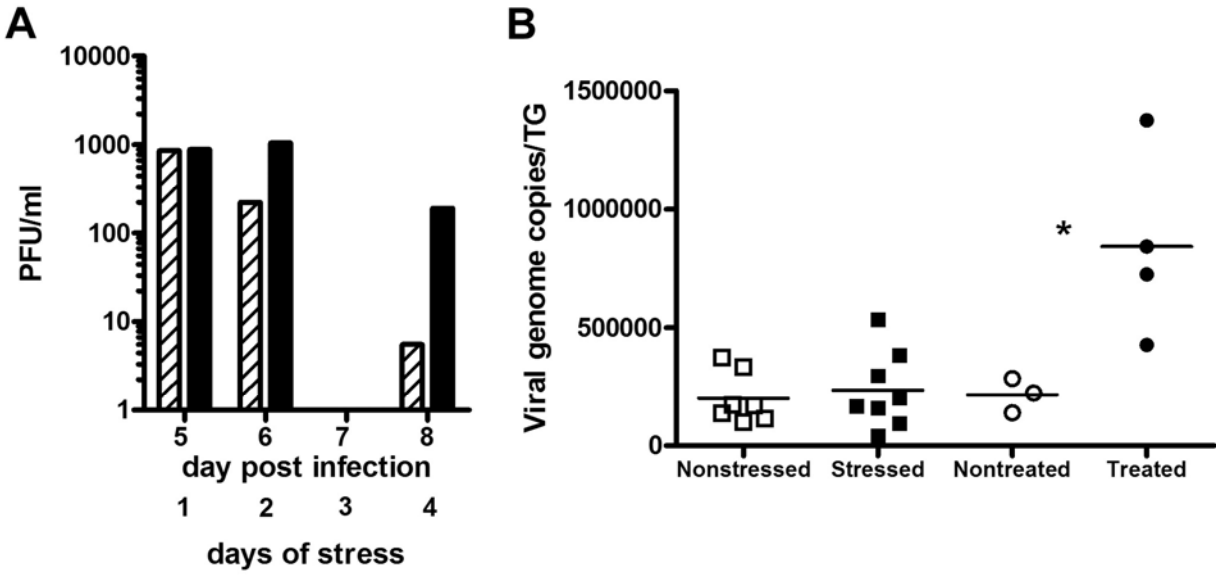

Figure 50. Viral clearance and control after early treatment.

Mice were stressed or corticosterone treated 4-8 d p.i. **(A)** Daily eye swabs were taken and plaque assays were performed on Vero cell monolayers from nonstressed (hatched bars, n = 2-8) or stressed (solid bars, n = 2-8) mice daily during the stress treatment. At 7 d p.i. no virus was detected. **(B)** At 8 d p.i., TG were excised from nonstressed (n = 7), stressed (n = 8), nontreated (n = 3), or corticosterone treated (n = 4) mice, DNA was isolated and analyzed by real-time PCR for the HSV-1 gH gene. * p = 0.0456, Student's t test.

4.8.3 Formation of CD8$^+$ T cell memory following stress

We examined the population of CD127$^+$ memory T cell precursors in the TG at 8 d p.i. Expression of CD127 (IL-7Rα) marks a population of T cells during the effector phase that are potentially destined to become memory T cells, although expression of CD127 alone is not sufficient to become a memory cell (125,126). Following stress, there was a significant 54% reduction in $gB_{498\text{-}505}$ specific CD8$^+$CD127$^+$ T cells, but surprisingly, there was no change in the gB-nonspecific CD127$^+$ population (Figure 51A). Because the total T cell pool in the TG was reduced (Figure 47), but the number of gB-nonspecific CD127$^+$ cells was unchanged, there was a 2.6-fold increase in the percentage of gB-nonspecific CD127$^+$ cells.

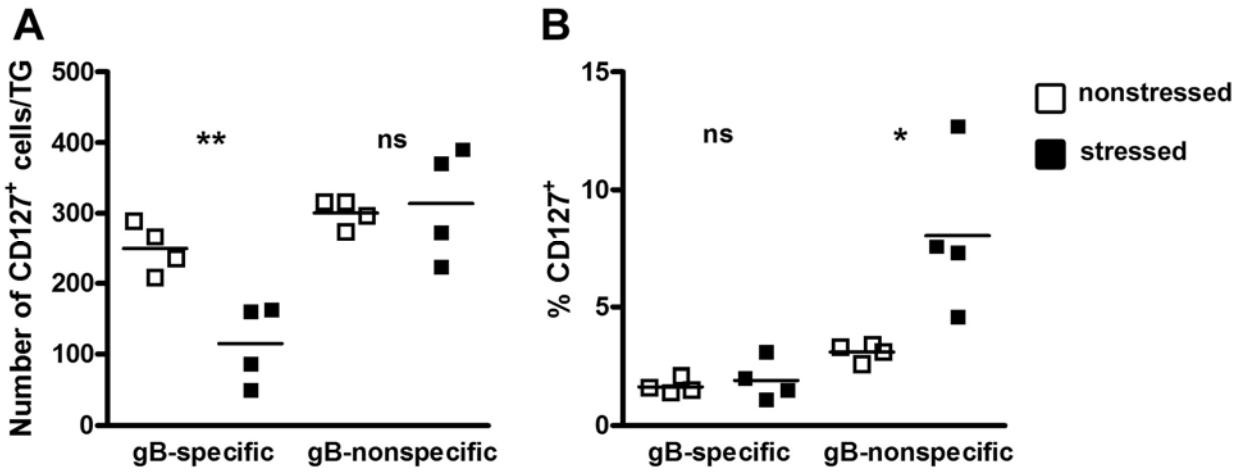

Figure 51. CD8$^+$ memory precursors following stress, 8 d p.i..

Mice were stressed 4-8 d p.i. (n = 4) or nonstressed (n = 4) and sacrificed 8 d p.i. TG were then excised and labeled with antibodies to CD45, CD8, CD127 (IL-7Rα), and $gB_{498\text{-}505}$/H-2K^b tetramers. **(A)** The number of CD127$^+$ cells in the gB-specific or gB-nonspecific populations. **(B)** The percentage of CD127$^+$ cells in the gB-specific or gB-nonspecific populations. * p = 0.0269, ** p = 0.0066, ns, not significant, Student's t test.

We next examined CD127 expression on CD8$^+$ T cells at a point early in latency (14 d p.i.) to measure if the stress-induced changes at 8 d p.i. influenced the memory T cell pool

(Figure 52). Surprisingly, the abundance of gB-nonspecific CD127$^+$ T cells at 8 d p.i. in stressed mice (2.7-fold more than gB-specific CD127$^+$ cells, see Figure 51A) was corrected by 14 d p.i. Comparing Figures 51A and 52C, the most interesting observation is that the absolute number of stressed, gB-nonspecific CD127$^+$ cells does not change from 8 to 14 d p.i., whereas the absolute number of stressed, gB-specific CD127$^+$ cells significantly increases ($p = 0.0045$). These data suggest that between 8 and 14 d p.i. gB-specific CD127$^+$ cells infiltrate into, or proliferate within, the TG at a much faster rate than gB-nonspecific memory precursors. Additionally, stress during the expansion phase does not appear to affect this increase.

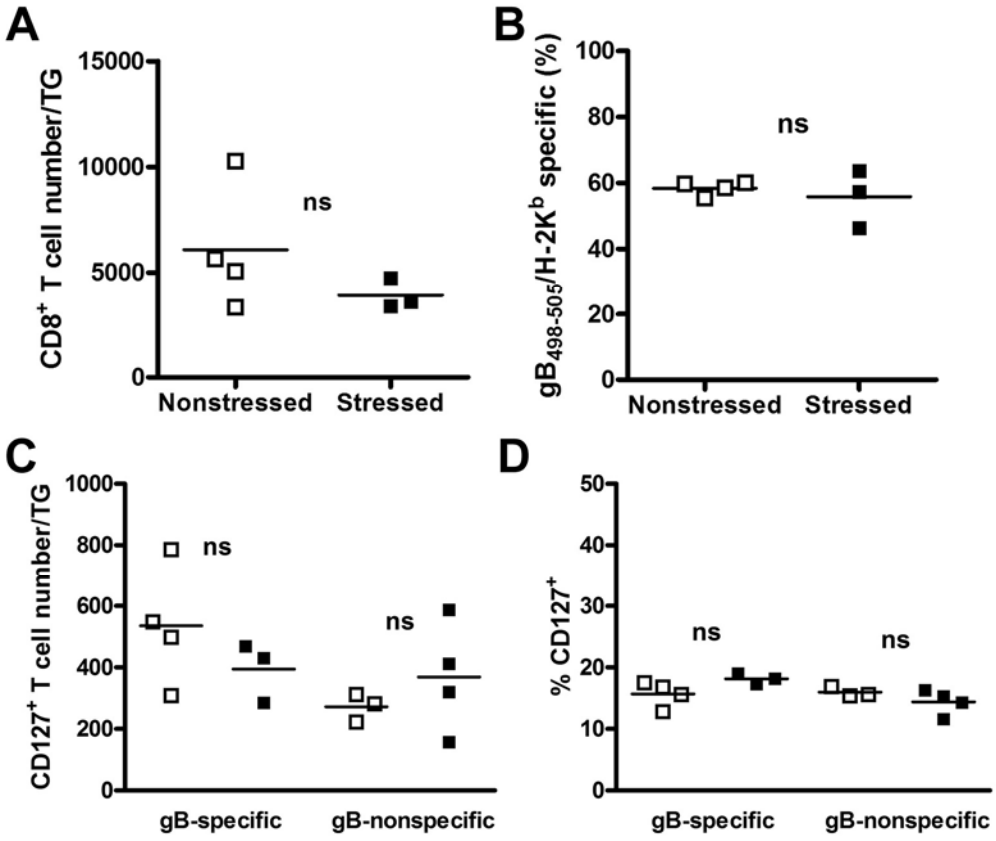

Figure 52. Phenotype of CD8$^+$ T cells 14 d p.i., after early stress treatment.

Mice were stressed 4-8 d p.i. (n = 3) or nonstressed (n = 4) and sacrificed 14 d p.i. TG were then excised and labeled with antibodies to CD45, CD8, CD127 (IL-7Rα), and $gB_{498\text{-}505}$/H-2K^b tetramers. **(A)** The number of CD8$^+$ cells in TG. **(B)** The percentage of CD8$^+$ T cells that are gB-specific. **(C)** The number of CD127$^+$ cells in the gB-specific or

gB-nonspecific populations. **(D)** The percentage of $CD127^+$ cells in the gB-specific or gB-nonspecific populations. ns, not significant, Student's t test.

The absolute number of stressed $CD8^+$ T cells is not significantly different from nonstressed controls at 14 d p.i. (Figure 52A). However, the number of cells at 14 d p.i. is similar to the number of stressed $CD8^+$ T cells 8 d p.i., the peak of T cell infiltrate. Whether this number of $CD8^+$ T cells remained constant between days 8 and 14, or if $CD8^+$ T cell number increases and then contracts post-stress, was not investigated in this study.

Memory cells undergo a basal level homeostatic proliferation that is independent of cognate antigen (123,156). Chronic infections, on the other hand, can lead to Ag-dependence or depletion of the memory cell pool (162,163). In latently infected TG, memory T cell numbers are maintained in the absence of IL-15 signals, but in the uninfected lung, the virus-specific memory cell pool is dependent on IL-15-mediated homeostatic signals (67). Whether the IL-15-independent memory cells require Ag to maintain their numbers is currently unknown. To investigate whether stress during expansion affects the ability of the memory response maintain stable numbers over time in the TG, we measured the amount of T cells in latently infected TG at 34 d p.i. It should be noted that although 14 d p.i. is an early time point in which the virus is already latent, it is still in the midst of the contraction phase, and thus T cell numbers are greater at 14 d than at 34 d p.i. We were unable to detect significant differences in the numbers of $CD8^+$ or $CD4^+$ T cells in the TG at 34 d p.i., regardless of whether the mice were stressed early (Figure 53A). Accordingly, the percentages of $CD8^+$ T cells that were gB-specific or activated ($CD69^+$) was unchanged (Figure 53B). These data suggest that the T cell memory pool is established properly, at least numerically, despite the deficiency in cell number at the peak of expansion due to the early stress treatment.

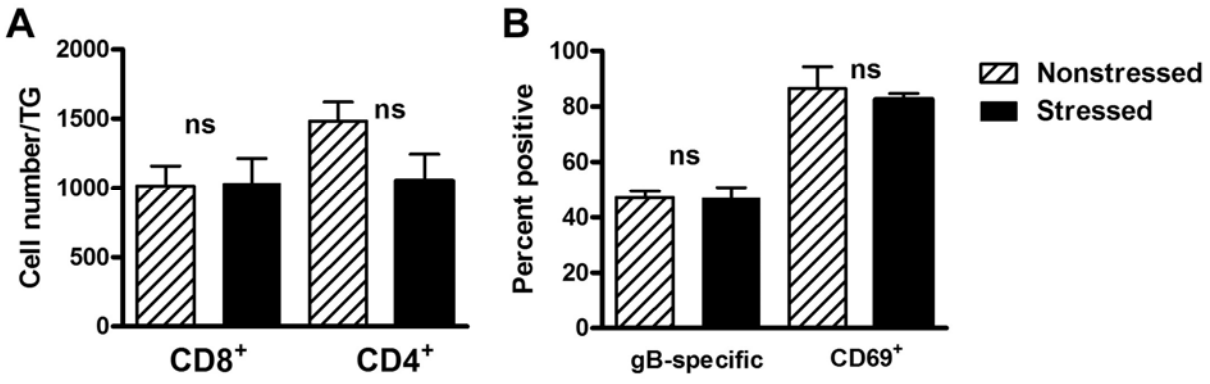

Figure 53. T cell phenotype at 34 d p.i., after early stress treatment.

TG were excised at 34 d p.i. from mice that were either nonstressed (n = 2-18) or stressed 4-8 d p.i. (n = 8). TG were stained with antibodies to CD45, CD8, CD4, CD69, and $gB_{498-505}$/H-$2K^b$ tetramers, and analyzed by flow cytometry. **(A)** The number (± SEM) of $CD8^+$ and $CD4^+$ T cells in the TG at 34 d p.i. **(B)** The percentage (± SEM) of $CD8^+$ T cells that were $gB_{498-505}$-specific or $CD69^+$ is shown. ns, not significant, Student's t test.

4.8.4 Memory function following stress.

The abilities of memory $CD8^+$ T cells in the TG to synthesize IFN-γ and degranulate were examined. Unlike previous studies (217,220,221,223), no functional deficiencies in the memory $CD8^+$ T cells within the TG were observed following stimulation with an optimum dose of $gB_{498-505}$ peptide (Figure 54) following stress during the primary infection. The discrepancy between our results and previous results could be due to several reasons, including differences between TG-resident cells and cells of peripheral immune organs, differences in the route of HSV-1 infection, and differences in the timing of the restraint sessions. The function of these memory $CD8^+$ T cells to respond at suboptimal doses of stimulation was not tested. Thus, our data presents a situation in which stress during the primary infection dysregulates the expansion of the $CD8^+$ T cell response, but does not seem to affect the establishment or functionality of the memory T cell pool.

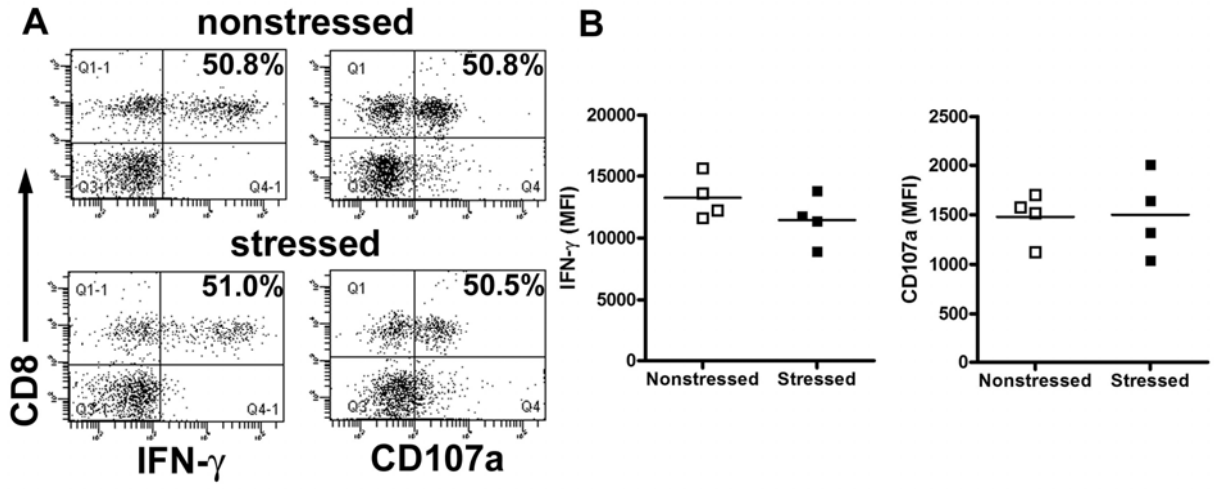

Figure 54. Memory T cell function following stress during the primary infection.

TG were excised at 34 d p.i. from mice that were either nonstressed (n = 4) or stressed 4-8 d p.i. (n = 4). TG were stimulated for 6 h with gB498-505 peptide-pulsed fibroblasts in the presence of GolgiPlug and anti-CD107a, then stained with antibodies to CD45, CD8, and intracellular IFN-γ, and analyzed by flow cytometry. **(A)** Representative dotplots show the production of IFN-γ (left column) or expression of CD107a (right column) on $CD45^+$ cells. Numbers in the top right quadrant indicate the percentage of $CD8^+$ T cells that express the given marker. **(B)** The MFI of IFN-γ (left graph) or CD107a (right graph) expression is shown.

One additional measurement of the memory T cell response in the TG following stress during the primary infection is whether the T cells are more or less susceptible to the effects of stress during latency. If stress early in infection sensitizes the memory pool to stressful stimuli during latency, then one explanation for why some individuals reactivate more often is that they could have been stressed when they were first infected. We measured the number of $CD8^+$ T cells in the TG at 34 d p.i., after stress 4-8 d p.i., 30-34 d p.i., or both (Figure 55A). Stress during both primary infection and latency did not result in an additional significant decrease in $CD8^+$ T cell numbers in the TG versus single stress treatment early in infection or single stress treatment during latency (as measured by one-way ANOVA). Student's t tests reveal significantly different cell numbers between mice stressed in both sessions and nonstressed mice ($p = 0.0495$), but no

significant differences in cell number compared to mice stressed early or late only. A repetition of this experiment to increase the n values of the stressed early and stressed both groups may provide enough power to observe significant differences. However, since T cell number does not seem to be the clearest indicator of $CD8^+$ T cell-mediated protection (compare the numbers of $CD8^+$ T cells following stress and the resulting viral genome copy number versus the numbers of cells and HSV-1 copy number following direct corticosterone treatment), we also examined the levels of HSV-1 genomes in the TG (Figure 55B). Although there was a general trend toward more genome copies after stress both early and late, there were no significant differences among the groups. More thorough investigation is certainly required to examine whether stress during the primary infection sensitizes the memory $CD8^+$ T cells to stress-mediated effects during latency.

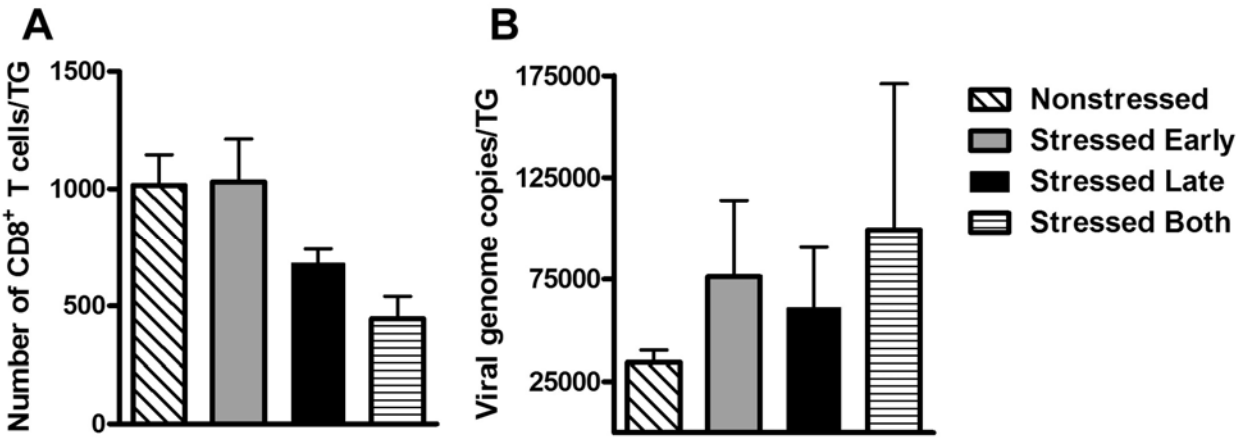

Figure 55. Effects of multiple stress treatments.

TG were excised at 34 d p.i. from mice that were either nonstressed (n = 16), stressed 4-8 d p.i. (stressed early, n = 8), stressed 30-34 d p.i. (stressed late, n = 27), or stressed 4-8 d p.i. and 30-34 d p.i. (stressed both, n = 4). **(A)** One TG equivalent per mouse was labeled with anti-CD45 and anti-CD8α, analyzed by flow cytometry, and $CD8^+$ T cells in the TG were enumerated. **(B)** DNA was isolated from one TG equivalent per mouse and viral genome copy number was measured by real-time PCR for the HSV-1 gH gene.

5.0 DISCUSSION

5.1 STRESS-INDUCED CD8$^+$ T CELL COMPROMISE PROMOTES REACTIVATION

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 (62,64,65,68,253). CD8$^+$ T cells surround HSV-1 infected neurons in latently infected TG of both mice and humans (65,114), maintain an activation phenotype (65), form an apparent immunologic synapse with neurons (65), and can block HSV-1 reactivation from latency in *ex vivo* cultures of latently infected TG (64,65). 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 (greater than 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 d 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 detected infectious virions in any latently infected nonstressed TG (38,47). A recent study showed only 30% of samples containing $1x10^5$ genome copies were culture positive (230). Thus, it is likely that only those stressed TG with the greatest increase in viral DNA achieved a necessary threshold for detection in culture. Additionally, the non-uniformity of stress induced reactivation could explain our inability to reproducibly detect increased reactivation in TG cultures from stressed mice compared to nonstressed controls, even when using sensitive recombinant promoter viruses.

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 which would be in agreement with the previous observation that restraint stress can impair mitogen-stimulated T cell proliferation (254). 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 antigen stimulation, whereas their counterparts in non-infected tissue are maintained by homeostatic proliferation in response to

cytokines, including IL-15 (67). Thus, a reduced capacity to proliferate in response to reactivating virus would likely result in a gradual reduction of 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 - less than 20% of $CD8^+$ T cells incorporate BrdU during a one-week treatment period (67). Moreover, we demonstrated a higher rate of apoptosis in $CD8^+$ T cells in stressed as compared to those in non-stressed TG. 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 in vivo or directly ex vivo less than 2% of $CD8^+$ T cells in stressed or nonstressed TG exhibited detectable IFN-γ production. However, when nonstressed TG were incubated for 72 h approximately 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. Since only neurons harbor latent virus in latently infected ganglia (64,255-257), and $CD8^+$ T cells prevent full HSV-1 reactivation with virion formation in TG cultures (64,65), 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 stressed 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 $gB_{498\text{-}505}$ peptide induced a similar frequency of IFN-γ producing $CD8^+$ T cells in stressed and nonstressed TG cultures, and the kinetics of IFN-γ production was similar.

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 γ1 gene that is expressed at low levels prior to 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 (175,258). Indeed, we found that stressed $CD8^+$ T cells were more impaired in cytokine production at low densities of epitope. Thus, it is likely that early in the reactivation process the density of $gB_{498\text{-}505}$ epitope on neurons fails to reach the elevated threshold of stimulation required for cytokine synthesis and degranulation.

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 antigen presentation or cytokine milieu accounted for the reduced IFN-γ production by $CD8^+$ T cells in stressed TG. We also tested the effects of CD8-depleted stressed TG culture on nonstressed cells and found no difference in the production of IFN-γ. 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 with or

without stressed $CD8^+$ T cells, 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 since the IFN-γ response was measured after the $CD8^+$ T cells were removed from the inhibitory microenvironment within the stressed animal. When we attempted to observe in vivo effects during the last stress session, we saw a significant decrease in the percentage of $CD8^+$ T cells that synthesized IFN-γ in stressed mice, suggesting that our ex vivo culture analyses were most likely demonstrating an actual stress-induced effect, even in the absence of the inhibitory microenvironment. However, as the numbers of cytokine-positive cells recovered in that assay were admittedly quite low, this conclusion is tentative. 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 (259,260). Glucocorticoids have also been shown to repress activation of the transcription factor NF-κB (261,262), as well as impair transcriptional activation by AP-1 (263). 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 lab.

5.2 DEFINING THE MECHANISMS OF RESTRAINT STRESS

5.2.1 Glucocorticoids and the HPA axis

The effects of stress on the TG-resident $CD8^+$ memory T cell pool are profound. Since our stress protocol increases serum corticosterone levels, we tested whether direct corticosterone treatment could recapitulate stress-induced effects. In general, the effects of direct corticosterone treatment were similar to stress, except to a greater degree, except in regard to HSV-1 reactivation from latency. Where we saw a 30% reduction in $CD8^+$ T cell number following stress, we observed an 80-90% drop in number following corticosterone treatment. The loss in T cell numbers was due mostly to apoptosis, as nearly all the TG-resident $CD8^+$ T cells costained with Annexin-V. Annexin-V is a molecule that binds phosphatidylserine when it is on the outer leaflet of the plasma membrane, an early event in apoptosis (241). In stressed mice, we observed a slight retention of activated cells, but following corticosterone treatment, the percentage of the population that expressed CD69 was significantly elevated. This result suggests that (i) activated cells are more resistant than non-activated cells to glucocorticoid-mediated apoptosis, (ii) glucocorticoids upregulate activation markers, or (iii) glucocorticoid-induced reactivation promotes viral gene expression resulting in increased epitope density on neurons, thereby activating the remaining cells. Although we cannot rule out the third hypothesis, we favor the first hypothesis in part because in vitro corticosterone treatment did not upregulate CD69 on splenocytes from naïve mice (data not shown) and in part because activated cells have been shown to be resistant to glucocorticoid-mediated cell death (264). In addition, CD69 is expressed equally by gB-specific and gB-nonspecific cells in the TG, and, like restraint stress, direct

corticosterone treatment did not change the ratio of gB-specific to gB-nonspecific cells in the TG, even after 10 d of treatment.

An interesting observation is that the number of $CD8^+$ T cells in corticosterone treated TG remains approximately the same, regardless of length of treatment. What governs this phenomenon is currently unknown, but these data suggest two intriguing hypotheses. First, that approximately 10-20% of $CD8^+$ T cells in the TG are resistant to the apoptotic effects of glucocorticoids. A second hypothesis is that the reduction is T cell number is a stochastic event, and the population of T cells is partially replenished throughout the 10 d of treatment, although the infiltration of cells to replace the dying cells would necessarily be enriched for gB-specific cells. We did not examine the expression of the intracellular glucocorticoid receptor (GR) in the context of these studies. Therefore, we do not know whether the cells that remain in the TG after 4 d of treatment are inherently resistant to the receptor-mediated effects of glucocorticoids. Interestingly, if the cells remaining after 4 d of corticosterone treatment were intrinsically incapable of responding to glucocorticoids, we would expect them to homeostatically proliferate in order to fill up the empty space. Lymphopenia-induced proliferation is a newly-characterized phenomenon in which naïve cells expand to fill up space created by the radiation-induced death of lymphocytes (265,266). In the process, the naïve cells take on characteristics of memory cells and are even able to protect from bacterial challenge as effectively as true memory cells (267,268). Importantly, proliferation appears to be partly governed by cytokines such as IL-15, so perhaps the lack of proliferation we see is due to a dearth of proliferation-inducing cytokines due to the glucocorticoid treatment (265,269-271). However, it should be noted that in the presence of antigen (as in the latently infected TG) the requirements for IL-2 and IL-15 seem to be reduced (67,164). It will be important in future studies to examine whether the GR is

expressed in the surviving cells after corticosterone treatment, and whether progeny from those cells maintain its expression, or lack thereof.

If the loss in T cell number is not due to differential GR expression, but is instead a stochastic process, then it would be expected that, with continuous glucocorticoid treatment, the numbers of cells in the TG would decrease over time, unless those cells are constantly replenished. Indeed, upon removing corticosterone, we observe a significant increase in proliferation throughout the mouse, including in the TG, spleen, bone marrow, and blood (data not shown). Thus, the proliferation potential is intact in the mouse, but the glucocorticoid treatment is preventing the increase in cell number, perhaps by killing cells as soon as they develop. Although we have not investigated the effects of glucocorticoid treatment in the thymus, it has been shown that thymocytes are highly susceptible to glucocorticoid-mediated effects (272,273). Moreover, glucocorticoids appear to be important for positive and negative selection (273-276). Thus, the direct corticosterone treatment may be inhibiting the replenishment of the T cell compartment by both killing the progeny of mature cells as well as by killing immature thymocytes before they can mature. It will be important in future studies to investigate whether the T cell repertoire following prolonged glucocorticoid treatment is impaired or limited in the cells that repopulate the mouse. The observation that T cell number in the TG remains constant between 4 and 10 d of treatment could indicate that there is a low level of cell trafficking into the TG at all times, but these newly emigrated cells are unable to fully recover T cell numbers until the glucocorticoids are removed from the system. This possibility is currently under investigation in our lab.

Surprisingly, we never observed an increase in viral genome copy number following corticosterone treatment, even with the prolonged 10 d treatment. These results suggest that T

cell function is more important than T cell number in the TG, although this hypothesis has yet to be rigorously tested. We investigated $CD8^+$ T cell ability to produce IFN-γ and degranulate. Under optimal stimulation conditions, we did not observe differences between the responses of cells from nontreated or corticosterone treated mice. Given that we did not observe functional compromise at optimal stimulatory doses in cells from stressed TG, it will be important to further examine the function capacity of corticosterone treated cells at suboptimal concentrations of HSV antigens. One major difference between the $CD8^+$ T cell populations of stressed and corticosterone treated mice was the level of intracellular granzyme B. Restraint stress induced a significant increase in granzyme B retention per cell, but we did not observe a similar effect after corticosterone treatment. Importantly, when serum corticosterone levels were increased endogenously by daily handling and i.p. injections, as opposed to direct exogenous treatment in the drinking water, the intracellular granzyme B levels were not increased. Therefore, either source of glucocorticoids (endogenous vs. exogenous) shows a similar phenotype.

5.2.2 Catecholamines and the SNS

The other main effector arm of the stress response is the sympathetic nervous system (SNS). There are two fairly common ways to disrupt the SNS, both of which have been used in context of HSV-1 infections: 6-hydroxydopamine (6-OHDA) induced chemical sympathectomy, and nadolol, a β-adrenergic receptor antagonist (203,206,242). In our hands, i.p. 6-OHDA treatment resulted in the death of all mice we treated, some as quickly as 2 d after initial treatment, in both HSV-1 infected and naïve mice (data not shown). However, treatment of mice with nadolol during stress was able to reduce the stress-induced granzyme B increase in two out of 3 experiments. Therefore, the potential exists that the increase in intracellular granzyme B, which

could be an indicator for reduced lytic granule release, is a catecholamine-mediated effect. Given that nadolol treatment has been shown previously to restore cytotoxic function to compromised HSV-specific effector cells (203,206), our results indicate a model of restraint stress-induced catecholamine release that can impair the $CD8^+$ T cell ability to degranulate, separate from the glucocorticoid-mediated loss of T cell number.

Perhaps the most intriguing observation is that direct glucocorticoid treatment does not induce reactivation of latent HSV-1, but depletion of T cells or restraint stress (which also compromises lytic granule release) does result in elevated genome copies. The obvious hypothesis is that degranulation is an important effector component of the $CD8^+$ memory T cells in controlling latent infections in vivo. Mice deficient in granzyme B survive initial HSV-1 infection (data not shown), but it is currently unknown whether these mice have deficiencies in maintaining latent virus or are more susceptible to reactivation than wild-type. Granzyme A, another lytic granule component with known non-cytolytic functions (277), has been shown to be important in restricting the spread of HSV-1 throughout the peripheral nervous system (278). We did not investigate granzyme A in our models of stress or direct corticosterone treatment. Moreover, mice deficient in perforin survive infection and control viral titers in the cornea as well as wild-type mice, suggesting that, in our model, the effects of granzyme B in control of latent virus may be mediated in vivo by a perforin-independent, noncytolytic mechanism (279).

The relative roles of lytic granules and cytokines in $CD8^+$ T cell-mediated control of latent HSV-1 infections are still under investigation. The studies presented here suggest that each mechanism may be important in vivo for proper maintenance of viral latency. We also present a model in which there is a minimum threshold required for T cell protection. In our hands, T cell function correlates with protection better than T cell number correlates with protection, at least

as far as degranulation is concerned. It should be noted, however, that we did not examine the potential caveat that in vivo corticosterone treatment may itself limit reactivation of latent virus, thereby bypassing the requirement for T cell-mediated protection. Indeed, a previous study has observed that treatment of mice with the synthetic glucocorticoid dexamethasone did not alone induce reactivation and dexamethasone treatment actually reduced the frequency of hyperthermic stress-induced reactivation (251). On the other hand, in vitro treatment of T cell depleted TG culture with 10^{-6} M corticosterone neither inhibited nor enhanced HSV-1 reactivation from latency, suggesting corticosterone most likely affects T cells more than neurons, although, we did not address the numbers or function of the surviving T cells in these cultures following corticosterone treatment. Moreover, when exogenous virus-specific $CD8^+$ T cells were added to the cultures, we observed nearly full protection from reactivation, suggesting corticosterone may affect T cell number but not function. Therefore, while our data does not indicate a reactivation-suppressing role of corticosterone, the possibility that direct corticosterone treatment inhibits HSV-1 reactivation has not been formally ruled out. Future studies are required to examine the ability of stress-induced catecholamines to compromise T cell cytotoxic and noncytotoxic effector functions mediated by release of lytic granules and the relative roles these mechanisms, along with T cell number, synthesis of IFN-γ, and production of other cytokines, to protect the host from HSV-1 reactivation from latency.

5.3 DISRUPTION OF IMMUNITY DURING PRIMARY INFECTION

Psychological stress during latent infection induces reactivation of latent virus in part by compromising the $CD8^+$ T cell efficacy. How would a similar compromise early in infection,

while T cell memory is still in development, affect the establishment, function, and susceptibility of the memory pool to stress?

We addressed this question by stressing mice from 4-8 d p.i., during the priming and initial expansion phase of the T cell effector response. We observed a 56% reduction of gB-specific $CD8^+$ T cells in the TG at 8 d, suggesting either the expansion of cells was reduced or infiltration into the TG was delayed or inhibited. Reductions in cell number were also seen for the overall $CD8^+$ T cell pool and $CD4^+$ T cells. These reductions corresponded with an inhibition of clearance of live virus from the cornea. Initial clearance of virus from the eye is dependent mostly on innate immune mechanisms, not the adaptive T cell response (68-71). We did not investigate whether our model of restraint stress affected the innate immune response, although there is plenty of evidence in other models for stress-mediated innate immune suppression (179,216,223,280). Restraint stress did not result in increased viral genome copies in the TG at 8 d p.i.; direct corticosterone treatment 4-8 d p.i., which resulted in a greater loss of T cell number in the TG than stress, led to a significant increase in viral genome copies in the TG. Perhaps the minimum number of innate and adaptive effector cells required to drive HSV-1 into latency was not met when mice were treated with corticosterone.

Interestingly, during the contraction phase (14 d p.i.), the numbers of T cells in the TG had equilibriated to wild-type levels in mice that had been stressed 4-8 d p.i. Given that there is a 54% reduction in gB-specific IL-7Rα^+ T cells in the TG at 8 d, the equilibriation to wild-type numbers at 14 d could indicate a restoration of infiltration into the TG. Future studies will address this possibility. Surprisingly, although we observed an overall reduction of $CD8^+$ T cells in the TG after stress early, without selectivity toward virus-specificity, the numbers of gB-nonspecific IL-7Rα^+ memory precursor cells was not reduced following stress. In fact, numbers

of gB-nonspecific IL-7Rα^+ cells did not even change between 8 and 14 d p.i. These data suggest that any infiltration of IL-7Rα^+ cells into the TG in the intervening period was nearly entirely a component of the gB-specific population. Alternatively, proliferation of IL-7Rα^+ cells between 8 and 14 d p.i. might only occur within the gB-specific population. The significance of these observations is still unknown.

Similar numbers of IL-7Rα^+ cells at 14 d led to similar numbers of CD8$^+$ T cells in the TG at 34 d p.i. Thus, stress early in infection does not affect the establishment of a CD8$^+$ memory T cell pool in the TG. Similar results have previously been observed in secondary lymphoid organs (221,223). However, in these previous studies and elsewhere, the functionality of the memory T cells was impaired as measured by cytokine production and cytotoxicity. Interestingly, we observed a significant decrease in the number of CD4$^+$ T cells following stress. CD4$^+$ T cells have been shown to be important during the generation of CD8$^+$ T cell effector responses to produce and maintain fully functional memory cells that can protect the host from pathogen rechallenge (281-284). Whether the stress-induced reduction of CD4$^+$ T cells (or a compromise in their functionality) during the initiation of the immune response has an effect on the generation, maintenance, or functionality of the CD8$^+$ memory T cell response is currently unknown but is an issue of considerable interest. Moreover, the ability of APC to present antigens and prime CD8$^+$ T cells may be impaired when mice are stressed 4-8 d p.i. Corticosterone has been shown to interfere with MHC antigen processing and presentation in dendritic cells (192). APC that are hindered in their presentation of antigen to T cells by stress could potentially induce compromised CD8$^+$ effector and memory responses. Admittedly, we have yet to fully characterize TG-resident CD8$^+$ memory T cell responsiveness to ex vivo stimulation with antigen, however, our initial results reveal the potential for subtle differences in

the efficacy of the memory population generated under stressful conditions. Indeed, optimal stimulation provides few differences between treatment groups whenever test, so to more efficiently measure the memory population after early stress, we will have to stimulate the cells under suboptimal antigen concentrations or examine their responsiveness to reactivating virus. These experiments will be very useful at determining the longterm effects of stress during the generation of a memory response. Stress may even prove to be a major component in explaining the variety in HSV-1 reactivation rates in people. As is shown here, psychological stress can lead to reactivation by disrupting T cell immunity at the site of latency. If the memory cells were already deficient due to stressful events early in the life of the host, then reactivation might be more facilitated and occur more often.

For the memory response that develops after stress early to be more susceptible to stress during latency, there would have to be some measurable difference between the cells that are generated after early stress and those that arise without stress. Some potential differences could be in expression of glucocorticoid or β-adrenergic receptors. Without knowing the exact differences between memory cells that develop after early stress versus wild-type, nonstressed memory cells, we chose to measure the susceptibility of each memory pool to stress during latency. Although not significant, there was a definite trend toward greater reduction in TG-resident $CD8^+$ T cell number 34 d p.i. after stress both early and late in infection (4-8 and 30-34 d p.i.) than either single stress treatment alone. Indeed, mice stressed during latency had fewer cells in the TG than nonstressed mice or mice stressed early, and mice stressed both early and late had an approximately 35% reduction from mice stressed late alone. Additonally, it appears that stress early may sensitize the memory pool to respond with a greater functional impairment to subsequent stressors, as we observe a slight increase in viral genome copies in mice stressed in

both sessions. It should be noted that we did not observe a significant difference in HSV-1 genome copy number when mice were stressed during latency until 38 d p.i., a few days after the cessation of stress. Therefore, it will be important to assess the viral genome copy number at 38 d, instead of 34 d presented here, to make the most accurate assessment of whether multiple stress treatments leads to greater reactivation than single stress treatments. Further investigation into the cumulative effects of multiple stress treatments may reveal fundamental properties of memory T cell maintenance in humans, as well as correlate the perception of stress in humans with susceptibility to HSV-1 reactivation from latency and pathogenesis of other diseases.

5.4 CONCLUDING REMARKS

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 (177,285). 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.

6.0 SUMMARY

The studies presented in this thesis address fundamental questions about the role of the adaptive immune response in the control of latent HSV-1 infections. Here we show for the first time that $CD8^+$ T cells are required during latency to inhibit the replication of viral genome, even in the absence of an overt reactivation stimulus. The association of stress with HSV-1 reactivation has long been proposed in humans, but no previous studies have adequately addressed potential mechanisms that involve the immune response at the site of latency, in animal models or in humans. Here we show, for the first time, that a psychological stressor can compromise the function and numbers of T cells in the ganglion during latency, and, that stress-induced disruptions of T cell efficacy are associated with reactivation of latent virus.

Additionally, we put forth a mechanism by which psychological stress can differentially regulate T cells by the two main arms of the stress response: the HPA axis and the sympathetic nervous system. Here, glucocorticoids mediate the survival of the T cells and catecholamines influence the effector functions of the T cells. Interestingly, in our model, it appears that the number of functional $CD8^+$ T cells is less important in inhibiting reactivation in vivo than the functional capacity of those cells. Whether the intensity of the stress changes the ratio of glucocorticoid to catecholamine release is unknown, but a model in which moderate stressors only mediate some effects while more intense chronic stressors can dyregulate the T cell response more thoroughly is intriguing.

The effects of stress during the primary infection are also important. Although it had been shown previously that stress prior to HSV-1 infection resulted in a general impairment of the memory T cell response, the direct effects on cells within the ganglion had never before been investigated. Whether the resulting TG-resident memory cells are impaired in their ability to control reactivation or respond to a induced reactivation event was not fully tested in these studies, however, the potential exists that the memory response could be compromised, and thus, individuals who underwent stressful experiences when they were first infected with HSV-1 might be more susceptible to the T cell compromising effects of stress many years later. An enhanced sensitivity to stress could explain why some people experience reactivation events much more often than others.

By understanding how stress influences the HSV-specific immune response we can ultimately design strategies for alleviating reactivation-inducing effects of stress, thereby reducing recurrent disease. Importantly, the data presented here suggest that memory cell effector functions are more important in preventing reactivation than the numbers of cells responding. Therefore, therapeutic vaccine strategies meant to only enhance memory cell number may be short-sighted in their goals; a better strategy may be to improve the effector functions of the cells that are already there.

APPENDIX

PUBLICATIONS

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