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Early CD4+ T Cell Help Prevents Partial CD8+ T Cell Exhaustion and Promotes Maintenance of Herpes Simplex Virus 1 Latency

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HSV-specific CD8+ T cells provide constant immunosurveillance of HSV-1 latently infected neurons in sensory ganglia, and their functional properties are influenced by the presence of latent virus. In this study, we show that ganglionic HSV-specific CD8+ T cells exhibit a higher functional avidity (ability to respond to low epitope density) than their counterparts in noninfected lungs, satisfying a need for memory effector cells that can respond to low densities of viral epitopes on latently infected neurons. We further show that lack of CD4+ T cell help during priming leads to a transient inability to control latent virus, which was associated with a PD-1/PD-L1 mediated reduced functional avidity of ganglionic HSV-specific CD8+ T cells. CD4+ T cells are not needed to maintain CD8+ T cell memory through 34 d after infection, nor do they have a direct involvement in the maintenance of HSV-1 latency. The Journal of Immunology, 2010, 184: 000–000.

The typical CD8+ T cell response paradigm is illustrated during acute infections. Naïve T cells initially encounter Ag in draining lymph nodes (DLNs), where they expand and acquire the functional program of effector cells. Subsequently, a polyfunctional Tc1 program including expression of the cytokines IFN-γ, TNF, and IL-2, and the lytic granule (LG) components perforin and granzyme B is associated with efficient viral eradication. After elimination of the infection, the effector T cell population undergoes contraction, with retention of a long-lived memory population that is maintained through homeostatic proliferation (1, 2).

In most infectious models CD4+ T cell help during the initial differentiation of naïve CD8+ T cells is required for programming of CD8+ T cells for functional memory. Nonhelped CD8+ T cells develop a memory population that exhibits functional defects and provides an inadequate recall response on subsequent exposure to Ag (3, 4). Emerging from recent studies is a scenario in which CD4+ T cells augment the programming of CD8+ T cells directly through the production of IL-2 (5, 6), and indirectly by enhancing the provision of costimulatory signals by dendritic cells (7–9).

A departure from this T cell response paradigm is observed during persistent infections with viruses such as lymphocytic choriomeningitis virus (LCMV), hepatitis C virus, and HIV (10–12). The initial programming of CD8+ T cells appears to be dysregulated, resulting in functional exhaustion of the CD4+ and CD8+ memory T cell populations. In persistent infections induced by LCMV clone 13, the exhaustion of virus-specific memory CD8+ T cells is characterized by the serial loss of production of IL-2, TNF, IFN-γ, and granzyme B (10, 13). The CD8+ T cell exhaustion appears to result in part from exposure to IL-10, and in part from their acquisition of inhibitory receptors such as PD-1, LAG-3, and CD160 (14, 15). Interestingly, mice that are deficient in CD4+ T cells at the time of clone 13 LCMV infection exhibit a more profound CD8+ T cell functional exhaustion and never clear the virus (10, 16, 17). Thus, CD4+ T cell help during the initial programming of LCMV-specific CD8+ T cells attenuates the aberrant programming that leads to functional exhaustion.

A hallmark of the herpesvirus family is their capacity to induce latent infections in which the viral genome persists for prolonged periods without production of infectious virions. Recent studies demonstrate that latency with many herpesviruses is maintained through constant immunosurveillance by CD8+ T cells (18–21). Studies in murine models of HSV-1 latency demonstrate that HSV-specific CD8+ T cells are persistently exposed to viral Ags, forming immunologic synapses and releasing LGs into the junction with latently infected neurons (22–25). Despite persistent antigenic exposure, these CD8+ T cells do not become functionally exhausted. This might reflect the fact that replicating virus is rapidly eradicated during acute infection, preventing prolonged Ag exposure during the programming of CD8+ T cells. Once programming is complete, persistent exposure to low levels of Ag does not appear to have an adverse effect on the virus-specific memory T cell population. Interestingly, CD4+ T cell deficiency during the acute phase of murine gamma herpesvirus 68 infection renders virus-specific memory CD8+ T cells incapable of maintaining viral latency, suggesting that nonhelped CD8+ T cells might be susceptible to functional exhaustion in the context of a latent viral infection (26, 27).

In this study, we show that CD4+ T cell deficiency during the programming of HSV-specific CD8+ T cells gives rise to a memory population that exhibits transient partial exhaustion characterized...
by elevated PD-1 levels and a reduced functional avidity that is associated with reduced ability to control HSV-1 latency in sensory ganglia.

**Materials and Methods**

**Mice and virus**

HSV-1 strain RE was grown in Vero cells, and intact virions were isolated on Optiprep gradients according to manufacturer’s instructions (Accurate Chemical and Scientific, Wesbury, NY). Six- to 8-wk-old female wild-type C57BL/6 mice were anesthetized by i.p. injection of 2.0 mg ketamine hydrochloride and 0.04 mg xylazine (Phoenix Scientific, San Marcos, CA) in 0.2 ml HBSS (BioWhittaker, Walkersville, MD). The abraded central corneas of anesthetized mice were infected by topical application of 3 μl RPMI (BioWhittaker) containing 1 × 10^6 PFU of HSV-1. All animal experiments were conducted in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Tissue preparation**

At the indicated days postinfection (dpi), anesthetized mice were sedated and euthanized by perfusion with sterile PBS. Prior to perfusion DNLs were harvested. Trigeminal ganglia (TGs) and lungs were digested in 100 μl (TGs) or 1 ml (lungs) of DMEM (BioWhittaker) containing 10% FCS (Atlanta Biologicals, Norcross, GA) and 400 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C. Tissues were then dispersed into single-cell suspensions and treated with RBC lysis buffer before staining with the designated antibodies. Data were collected on a FACSArria cytometer and analyzed by FACS DIVA software (BD Biosciences, San Jose, CA).

**Reagents**

The gB498-505 (SSIEFARL) peptide was purchased from Research Genetics (Huntsville, AL). PE-conjugated H-2Kb tetramers complexed with the gB498-505 peptide were kindly provided by the NIAID Tetramer Core Facility (Emory University Vaccine Center, Druid Hills, GA). Rat anti-mouse allophycocyanin-alexa750-conjugated and Pacific Blue-conjugated anti-CD8α (clone 53-6-7); FITC-conjugated rat anti-mouse CD107α (1d45), PE-Cy7-conjugated rat anti-mouse CD4 (RM4-5) and PE-D1 (RMP1-30), and anti-mouse TNF (MP6-XT22); allophycocyanin-conjugated rat anti-mouse IFN-γ (XM12.1), and PerCP-conjugated rat anti-mouse CD45 (30-F11) were purchased from BD Pharmingen (San Diego, CA). The appropriate isotype control Abs were purchased from BD Pharmingen or eBiosciences (San Diego, CA).

**In vivo mAb treatment**

Mice were treated with an i.p injection of 0.15 mg rat anti-mouse CD4 Ab clone GK1.5 (BioXcell, West Lebanon, NH) for in vivo depletion of CD4 T cells. For depletions prior to infection, injections were given 2 d before infection, followed by 1, 3, 8, 15, 22, 29, 36, 43, and 50 dpi. For depletions during contraction, treatments were given at 8, 15, 22, and 29 dpi. For PD1 blockade, mice were treated with 0.2 mg rat anti-mouse PDL1 Ab clone 10F.9G2 (BioXcell) every 3 d for 2 wk before the desired observation point.

**Phenotypic analysis of T cells**

For all phenotypic analyses, cells were stained for CD45 to permit gating exclusively on bone marrow–derived cells. For analysis of CD8 and CD4 T cell populations and their phenotype, cells were stained with anti-CD8α and anti-CD4. For analysis of HSV-specific CD8+ T cells (gB-CD8α), cells were additionally stained with anti-CD8α and gB498-505 H-2Kb tetramers.

**Fluorescently assisted cell sorting**

At indicated time points, lungs and DNLs were dispersed into single-cell suspension and stained with anti-CD8 and gB498-505 H-2Kb tetramers. Cells were sorted at high purity (purity-32) for CD8+ gB tetramer+ cells on a FACSArria cytometer (BD Biosciences).

**Intracellular cytokine staining and LG exocytosis**

Dispersed TGs or polyomavirus B (gB) tetramer sorted cells were stimulated directly with either 10^{-6} M (optimal) or 10^{-11} M (suboptimal) gB498-505 peptide pulse or 10^{-6} M (optimal) or 10^{-11} M (suboptimal) gB498-505 peptide pulse. Six wells were run on each plate to avoid tetramer rebinding (28-8-6; BD Pharmingen) at 37°C for the designated times. Cells were then stained with anti-CD8α and anti-CD45 mAB, and analyzed via flow cytometry to observe loss of tetramer over time. Data are CD8+ tetramer+ cells as a percentage mean fluorescence intensity (MFI) ± SEM of the maximal binding observed at time zero (n = 10 mice per group).

**Detection of infectious virus on corneas**

The corneal surfaces of mice were swabbed with sterile Weck-Cel surgical sponges (Medtronic Solan, Jacksonville, FL) on days 2, 4, 6, and 8 after HSV-1 infection; sponges were placed in 0.5 ml RPMI and frozen at −80°C until assayed. Samples were added to confluent Vero cells, incubated for 1 h at 37°C, and overlaid with 0.5% methylcellulose. The cultures were incubated for 72 h, fixed with formalin, and stained with crystal violet. Viral cytopathic effect was detected with the aid of a dissecting microscope.

**Quantitative real-time PCR**

Total DNA was isolated from single-cell TG suspensions using DNeasy columns according to manufacturer’s instructions (Qiagen, Valencia, CA). DNA was quantified by spectrophotometry and diluted to 1 ng/μl in nuclease-free dH2O. Twenty-five nanograms DNA or water control was mixed in duplicate with a 25-μl mixture of TaqMan Universal PCR Master Mix (Roche, Basel, Switzerland) 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 per 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 (Applied Biosystems). The gH sequences were: forward primer (5'-CGACCAACAGAAAACCCCTTCT-3'), reverse primer (5'-ACGGCTCTCGTCTAGATCAGG-3'), and probe [5'-FAM]TCCGGACCATTTTC(NFQ)-3'].

**Statistics**

All statistical analyses were performed with GraphPad Prism 5 (San Diego, CA) software using a two-tailed unpaired t test with 95% confidence.

**Results**

**CD4+ T cell ablation influences the maintenance of HSV-1 latency**

Initial studies tested the effect of CD4+ T cell ablation starting before HSV-1 corneal infection on the clearance of virus from the cornea, the establishment of viral latency in the TG, and on the maintenance of latency. Our studies used an Ab treatment protocol that effectively ablated CD4+ T cells from mice beginning 2 d before HSV-1 corneal infection and continuing through 60 dpi (Supplemental Fig. 1). In this study, we show that depletion of CD4+ T cells through 8 dpi did not influence the clearance of HSV-1 from the cornea (Fig. 1A), the kinetics of latency establishment within the TG (not shown), or the load of latent viral genome at 8 dpi (Fig. 1B). However, the CD4+ T cell ablated mice failed to maintain the virus in a latent state between 8 and 35 dpi, as indicated by a significant increase in the viral genome copy number in the TG (Fig. 1C). The latent viral load returned to normal levels by 56 dpi in CD4+ T cell ablated mice. The failure of CD4+ T cell ablated mice to control the early stages of HSV-1 latency did not reflect a direct requirement for CD4+ T cells in control of viral latency because initiating CD4 depletion at 8 dpi did not result in a similar increase in the latent viral load (Fig. 1D).

**CD4+ T cell help does not influence the size of the HSV-specific CD8+ T cell effector or memory pool**

We previously demonstrated that diminished function of memory HSV-specific CD8+ T cells within latently infected TGs was
associated with failure to maintain latency, as indicated by an increase in viral genome copy number (32, 33). Therefore, we hypothesized that the absence of CD4+ T help during the first 8 d after HSV-1 infection might have resulted in an HSV-specific CD8+ effector or memory T cell population within the TG that was incapable of maintaining HSV-1 latency. We used an HSV-1 corneal infection model in C57BL/6 (B6) mice in which tetramers containing an immunodominant glycoprotein B (gB498–505) epitope were used to identify and quantify a majority of gB-CD8s. The number of CD4+ T cell–helped (helped) and CD4+ T cell–nonhelped (nonhelped) gB-CD8s peaked at similar levels in the DLNs (data not shown) and the infected TG (Fig. 2) at 8 dpi. In the infected TG, the nonhelped gB-CD8s underwent a more rapid contraction between 8 and 14 dpi (Fig. 2A), but ultimately established a stable and numerically normal memory population (Fig. 2A). CD4+ T cell ablation starting after the peak of the effector response (8 dpi) resulted in accelerated contraction similar to that seen when CD4+ T cells were ablated from the time of infection, suggesting a direct role for CD4+ T cells in gB-CD8 cell contraction (Fig. 2B).

**Nonhelped gB-CD8s exhibit functional alterations upon infiltrating the infected TG**

In this study we quantified four separate populations of gB-CD8s. As previously reported (28), one population of low functional avidity gB-CD8s produces effector cytokines only in response to an optimal epitope density (1 × 10^-6 M gB498–505 peptide loaded fibroblasts); whereas a second high functional avidity population responds to both optimal and suboptimal (1 × 10^-11 M gB498–505 peptide loaded fibroblasts) epitope densities. Both the high and low functional avidity gB-CD8 populations contain subpopulations that express different functional programs: all responding gB-CD8s produce IFN-γ and release LGs (IFN-γ/LG) when stimulated, but a subpopulation here referred to as polyfunctional also produces TNF-α (IFN-γ/TNF/LG). No gB-CD8s were single producers of TNF-α. These four subpopulations are illustrated in Fig. 3B.

We sorted for gB-CD8s in the DLN at 8 dpi to test their functionality (Fig. 3A). Within the DLN, ~75% of both helped and nonhelped gB-CD8s peaked at similar levels in the DLNs (data not shown) and the infected TG (Fig. 2) at 8 dpi. In the infected TG, the nonhelped gB-CD8s underwent a more rapid contraction between 8 and 14 dpi (Fig. 2A), but ultimately established a stable and numerically normal memory population (Fig. 2A). CD4+ T cell ablation starting after the peak of the effector response (8 dpi) resulted in accelerated contraction similar to that seen when CD4+ T cells were ablated from the time of infection, suggesting a direct role for CD4+ T cells in gB-CD8 cell contraction (Fig. 2B).

The most dramatic difference between the gB-CD8 effector cells in the DLN and TG at 8 dpi was in the low frequency of polyfunctional (IFN-γ/LG/TNF) cells in the TG (Fig. 3D). Although both the helped and nonhelped gB-CD8s in the TG showed a marked reduction in the frequency of polyfunctional cells in response to optimal gB peptide stimulation relative to their counterparts in the DLN, high functional avidity polyfunctional cells were nearly undetectable in the nonhelped gB-CD8s in the TG.
Early CD4+ T cell help is required to avert gB-CD8+ functional compromise

The frequency of helped gB-CD8s in the TG that responded to an optimal gB peptide stimulation remained constant during 8–56 dpi (Fig. 4A). Interestingly, the frequency of those cells that exhibited a high functional avidity by responding to a suboptimal gB peptide dose increased dramatically over the same period (Fig. 4A). In contrast, the nonhelped gB-CD8s showed a sharp decline in functionality at 8–14 dpi, when only 14% exhibited a high functional avidity. At 14–35 dpi, the frequency of nonhelped gB-CD8s that responded to an optimal dose of gB peptide declined further, but the frequency of high functional avidity cells increased. The frequency of both populations remained significantly reduced (p < 0.05), relative to the helped gB-CD8s at 35 dpi. By 56 dpi, the nonhelped and the helped gB-CD8s showed the same frequency of cells capable of responding to an optimal and suboptimal dose of gB peptide.

It is noteworthy that in addition to an increased frequency of high functional avidity cells within the overall gB-CD8 responding population in the TG at 8–56 dpi (IFN-γ/LG; Fig. 4A), the helped gB-CD8s also exhibited a gradual increase in the polyfunctional (IFN-γ/LG/TNF) subpopulation (Fig. 4B), and most of these cells exhibited high functional avidity by 56 dpi. Thus, in the helped gB-CD8 population in the TG, there was a marked enrichment of polyfunctional and high functional avidity cells over time within the latently infected TG. In contrast, at 8–14 dpi the nonhelped gB-CD8s exhibited a further decline in polyfunctional cells, which was particularly prominent among the high functional avidity cells that represented only 4% of the nonhelped gB-CD8s at 14 dpi (Fig. 4B). This reduced functionality combines with a reduced number of nonhelped gB-CD8s in the TG at 14 dpi (Fig. 2A) to define a highly compromised gB-CD8 population. The frequency of polyfunctional cells within the nonhelped gB-CD8 population increased at 14–35 dpi, but remained significantly reduced (p < 0.01) relative to their helped counterparts after optimal and suboptimal gB peptide stimulation. By 56 dpi, the nonhelped gB-CD8s regained full functionality. The augmentation of gB-CD8 functionality was imprinted by CD4+ T cells during the first 8 d after infection, because depletion of CD4+ T cells beginning at 8 dpi had no effect on gB-CD8 function at 35 dpi (Fig. 4C).

Latently infected TGs selectively promote the enrichment of high functional avidity gB-CD8 during establishment of memory

We have previously demonstrated that the microenvironment of the latently infected TG can influence functional properties of the resident gB-CD8 memory population, presumably owing to persistent low-level exposure to viral Ags (28). To determine whether the increased frequency of polyfunctional and high functional avidity gB-CD8s was unique to latently infected TGs, we analyzed the functionality of gB-CD8s in the noninfected lungs of the same mice at 35 and 56 dpi (Fig. 5A). As shown in Fig. 5B and 5C, the gB-CD8 population in the lungs already showed a high frequency of polyfunctional cells by 35 dpi, and these remained high at 56 dpi. Thus, the acquisition of polyfunctional cells over time appeared to be inherent to the transition of gB-CD8s into memory. In contrast, the frequency of high functional avidity cells within both the overall responding gB-CD8 population (IFN-γ/LG) and within the polyfunctional (IFN-γ/LG/TNF) population declined significantly (p < 0.01) between 35 and 56 dpi, when they constituted only 22% of the gB-CD8 population. The selective increase (total response p < 0.01; polyfunctional p < 0.001) in high functional avidity cells in the TG relative to the lungs is illustrated in Fig. 5D and 5E. Although acquisition of polyfunctional property appears to occur normally in gB-CD8s during the transition from an effector to memory population, latently infected tissue appears to provide a selective pressure for accumulation of high functional avidity gB-CD8.

TCR affinity does not correlate with functional avidity in nonhelped gB-CD8

The reduced functionality of nonhelped gB-CD8s in the TG at 35 dpi could be due to low TCR affinity or exposure to a variety of immunomodulatory signals in vivo (31, 34, 35). To differentiate these two types of influences, we used a tetramer release assay to compare the TCR affinity of helped and nonhelped gB-CD8s obtained from TGs at 35 dpi. As illustrated in Fig. 6, the nonhelped gB-CD8s exhibited an average TCR affinity that was essentially identical to that of the helped gB-CD8s, suggesting that the reduced functionality of the nonhelped gB-CD8s detected directly ex vivo resulted from in vivo exposure to immunoregulatory influences.

The reduced functionality of nonhelped gB-CD8 is associated with increased PD-1 expression

We next determined whether the functional compromise observed in nonhelped gB-CD8s in TGs at 8–35 dpi was associated with enhanced expression of the inhibitory receptor PD-1. At 8 dpi, PD-1+ expression was significantly elevated on nonhelped gB-CD8s in both the DLN (Fig. 7A) and TG (Fig. 7B, 7C). Increased PD-1 expression was manifest as both an increased frequency and increased level (MFI) of PD-1 expression. The frequency of PD-1+
nonhelped gB-CD8s gradually declined in the TG, but remained significantly elevated through 35 dpi. The increased PD-1 expression during this time was associated with reduced functionality (Fig. 5A, 5B). By 56 dpi, PD-1 expression on nonhelped gB-CD8s in the TG was further reduced and no longer significantly different from that on helped gB-CD8s; this was concurrent with complete functional recovery.

**Blockade of PD-L1 restores function to nonhelped gB-CD8 in the TG**

Because transient functional dysregulation of nonhelped gB-CD8s in the TG was associated with elevated PD-1 expression, we predicted that blocking PD-1/PD-L1 interaction would restore normal function to these cells. TGs were obtained from mice at 35 or 56 dpi following 2 wk of systemic treatment with anti-PD-L1 or control mAb. At 35 dpi the nonhelped gB-CD8s in the TGs of mice that were treated with control mAb exhibited the anticipated reduction in the frequency of total gB-CD8s (Fig. 8A) and polyfunctional (Fig. 8C) capable of responding to gB peptide-pulsed targets. However, functionality was completely restored to the nonhelped gB-CD8s following 2 wk of anti-PD-L1 mAb treatment (Fig. 8A, 8C). In contrast, anti-PD-L1 treatment did not influence the function of helped gB-CD8 in TG at 35 dpi or the function of helped or nonhelped gB-CD8s in the TGs at 56 dpi (Fig. 8B, 8D).

**Discussion**

The involvement of CD4+ T cell help in CD8+ T cell responses is complex and likely influenced by factors such as the nature of the immunogen, the activation status of APCs, and the microenvironment in which the effector and memory CD8+ T cells reside. In this study, we use a unique model system in which CD8+ T cell priming occurs in the context of an acute HSV-1 infection, but the
Functional properties of the resulting HSV-specific CD8+ T cell memory population can then be examined within disparate tissue microenvironments with or without persistent antigenic exposure (28). Moreover, in this model, persistent low-dose antigenic exposure in latently infected ganglia does not lead to CD8+ T cell functional exhaustion, as seen with chronic viral infections that are characterized by extended exposure to high levels of viral Ags after initial infection. In this model, long-term functionality of the ganglionic gB-CD8+ T cell population is required to maintain the virus in a latent state (24, 32, 33). We use this model system to explore the influence of CD4+ T cells on the gB-CD8 response. We demonstrate that CD4+ T cells influence the gB-CD8 response in two ways. First, the gB-CD8 effector population in the TG undergoes a more rapid contraction in the absence of CD4+ T cells. This effect is exerted at the time of contraction, versus during the initial programming of the naive gB-CD8, because contraction is accelerated when CD4+ T cell ablation is initiated before infection or after the peak accumulation of effector gB-CD8 in the TG at 8 dpi. The mechanism by which CD4+ T cells influence CD8+ T cell contraction is currently unclear.

Accelerated contraction alone did not influence the functional characteristics, immunosurveillance capability, or size of the gB-CD8 memory pool established in the TG. Mice that were depleted of CD4+ T cells beginning at 8 dpi showed an ~50% reduction in gB-CD8 at 14 dpi, but the capacity of the remaining CD8+ T cells to maintain HSV-1 latency was not compromised, as indicated by a similar latent HSV-1 genome copy number in TGs. This finding is probably a reflection of the fact that the remaining gB-CD8 were

**Figure 4.** Early CD4+ T cell help is required to avert gB-CD8 functional compromise. B6 mice that received HSV-1 corneal infections were not depleted or continuously depleted of CD4+ T cells beginning 2 d before infection (nonhelped, A and B), or beginning at 8 dpi (C). At the indicated dpi, dispersed TG cells were stimulated with an optimal or suboptimal dose of gB peptide and IFN-γ, and TNF-α production and LG release were quantified as in Fig. 2. Data are presented as (A) mean percent ± SEM of gB-tetramer+ CD8+ T cells (gB-CD8+) from CD4-depleted and nondepleted mice that responded (IFN-γ/LG) to optimal or suboptimal gB peptide stimulation, (B) mean percent ± SEM of polyfunctional (IFN-γ/LG/TNF) gB-CD8 in response to optimal or suboptimal gB peptide stimulation, and (C) mean percent ± SEM of total (IFN-γ/LG) or polyfunctional (IFN-γ/LG/TNF) gB-CD8 in response to optimal or suboptimal gB peptide stimulation at 35 dpi. *p < 0.05; **p < 0.01; ***p < 0.001; n = 8–10 mice per group.
functionally normal, and at any given time only a small number of latently infected neurons appear to require CD8+ T cell protection from reactivation (36). These findings establish that CD4+ T cells are not required for either the establishment or maintenance of HSV-1 latency in the TG following corneal infection.

Of greater consequence was the influence of CD4+ T cells on the initial programming of gB-CD8. Depriving mice of CD4+ T cell help during the initial programming and expansion phase of the CD8+ T cell response to HSV-1 had no effect on the size of the gB-CD8 effector population in the TG at 8 dpi. This contrasts with findings from a flank HSV-1 infection model, in which CD4 T cell deprivation resulted in reduced gB-CD8 effector expansion (37).

Important differences in the two studies include the strain of HSV-1 used and the route of infection, both of which might influence the amount of CD4+ T cell help required for the expansion of HSV-specific CD8 effector cells. We also saw no functional differences between the helped and nonhelped gB-CD8 effector cells in the DLNs at 8 dpi. However, the nonhelped gB-CD8 did exhibit a progressive functional compromise following infiltration of the TG at 8–35 dpi. The compromise was initially most pronounced among the high functional avidity polyfunctional (IFN-γ/LG/TNF) cells at 8 dpi, but progressive loss of the capacity to produce IFN-γ and release LGs was observed at 14 and 35 dpi. The concurrent nature of the functional compromise in the nonhelped gB-CD8 population in the TG and escape of HSV-1 from latency, as indicated by elevated HSV-1 genome copy number, underlines the importance of gB-CD8 functionality in maintaining HSV-1 in a latent state.

We previously demonstrated that gB-CD8s are maintained in the latently infected TG of IL-15 knockout mice, but lost in their noninfected lungs (28). Those findings were consistent with the

**FIGURE 5.** Latent virus influences some of the functional changes that occur in gB-CD8 during the establishment of memory. 

A. Lungs were harvested at 35 or 56 dpi, dispersed cells were stained with gB498-505 tetramers, and sorted gB-CD8 were ≥90% gB-tetramer+ as illustrated in a representative dot plot. Sorted gB-CD8s were stimulated with an optimal or suboptimal dose of gB peptide and IFN-γ and TNF-α production and LG release were quantified as in Fig. 2.

B. Representative flow plots depicting background activation from tetramer binding, as well IFN-γ, TNF-α, and LG responses to optimal and suboptimal gB peptide stimulation. C. Bars represent mean ± SEM of total (IFN-γ/LG) responders or polyfunctional (IFN-γ/LG/TNF) responders. The total bar represents the frequency of cells responding to optimal gB peptide stimulation, and the dark gray bar represents the frequency of high functional avidity cells responding to a suboptimal gB peptide dose. Bars represent the frequency of high functional avidity total (D) (IFN-γ/LG) and polyfunctional (E) (IFN-γ/LG/TNF) gB-CD8 responders in the TG and lungs. *p < 0.05; **p < 0.01; ***p < 0.001; n = 8–10 mice/group.
We cannot formally rule out the possibility that nonhelped gB-express higher levels of PD-1 than their CD4-helped counterparts. Expression on neurons in latently infected TGs (R. L. Hendricks, B. infected neurons (10, 23, 24). Finally, we have observed PD-L1 closely associate and form immunologic synapses with latently infected ganglia. Our findings are consistent with this prediction, because the source of PD-L1 that engages PD-1 on gB-CD8s in latently infected neurons would be best suited to provide immunosurveillance and survive within latently infected ganglia. Our findings are consistent with this prediction, because high functional avidity gB-CD8s are highly enriched in the latently infected TG, but not in the noninfected lungs, and transient compromise in the nonhelped high functional avidity gB-CD8s was associated with failure to maintain HSV-1 latency. These findings are also consistent with the recent demonstration that CD8 T cells in latently infected TGs represent a tissue-resident subpopulation of memory CD8 T cells that possess unique properties (38).

In a chronic LCMV infection model, intermediate levels of PD-1 expression on LCMV-specific CD8 T cells was associated with partial exhaustion that could be reversed by in vivo PD-L1 blockade (10). In our model, intermediate PD-1 expression (based on both frequency of positive cells and level of expression per cell) on nonhelped gB-CD8s at 8–35 dpi was associated with partial functional exhaustion. This exhaustion was similar to that seen in the LCMV infection model, with TNF-α being more severely affected before other effector mechanisms as exhaustion progressed (39). Moreover, in vivo blockade of PD-1/PD-L1 interaction effectively reversed the exhaustion in nonhelped gB-CD8s. This finding is in agreement with a recent study that observed cells that were PD-1int but not PD-1hi were capable of functional recovery (40).

The source of PD-L1 that engages PD-1 on gB-CD8s in latently infected ganglia is not clear, but several observations point to latently infected neurons as likely candidates. First, it appears that PD-1 inhibits TCR signaling when the TCR and PD-1 ligands are expressed on the same cell (41). Second, extended PD-1/PD-L1 exposure is required for CD8 T cell functional exhaustion and gB-CD8s closely associate and form immunologic synapses with latently infected neurons (10, 23, 24). Finally, we have observed PD-L1 expression on neurons in latently infected TGs (R. L. Hendricks, B. S. Sheridan, and T. L. Cherpes, unpublished observations).

This of course, begs the question of why nonhelped gB-CD8 express higher levels of PD-1 than their CD4-helped counterparts. We cannot formally rule out the possibility that nonhelped gB-CD8s upregulate PD-1 expression because of encountering higher levels of viral Ag in the DLNs or TG. However, we consider this unlikely because CD4-sufficient and CD4-deficient mice eliminate replicating virus from the cornea and TG with similar kinetics and establish latency with a similar load of viral DNA at 8 dpi. Instead, we favor the hypothesis that the threshold level of TCR signaling required for PD-1 expression is set at a lower level when gB-CD8s are programmed in the absence of CD4 T cell help. The threshold level of TCR signaling required to induce PD-1 expression might then be met by nonhelped, but not helped, gB-CD8s through an encounter with Ag. Of interest is how the nonhelped gB-CD8 pool still retained functionality despite elevated PD-1 expression within the DLN at 8 dpi. We believe concordant expression of CD8 TCR affinity does not correlate with functional compromise in nonhelped memory. TGs were harvested and dispersed at 35 dpi. TG suspensions were stained with gB tetramer and incubated with anti-H-2Db/Kb Ab at 37°C for the designated times to observe tetramer dissociation. Cells were then stained with anti-CD8a and anti-CD45 mAb, and analyzed via flow cytometry. Data represent the MFI of CD8+ tetramer+ cells as a percentage ± SEM of the maximum MFI observed at time zero. n = 10 mice per group.

FIGURE 6. TCR affinity does not correlate with functional compromise in nonhelped memory. TGs were harvested and dispersed at 35 dpi. TG suspensions were stained with gB tetramer and incubated with anti-H-2Db/Kb Ab at 37°C for the designated times to observe tetramer dissociation. Cells were then stained with anti-CD8a and anti-CD45 mAb, and analyzed via flow cytometry. Data represent the MFI of CD8+ tetramer+ cells as a percentage ± SEM of the maximum MFI observed at time zero. n = 10 mice per group.

FIGURE 7. The dysregulated function of nonhelped gB-CD8s is associated with increased PD-1 expression. B6 mice that received HSV-1 corneal infections were not depleted or continuously depleted of CD4 T cells beginning 2 d before infection. DLNs were excised at 8 dpi, TGs were excised at the designated dpi, and dispersed cells were stained simultaneously with anti-CD8a, anti-CD45, anti-PD1, and gB tetramer and analyzed via flow cytometry. Representative dot plots illustrate PD1 expression (based on isotype control) by (A) non–CD4-depleted (helped) gB-CD8 in DLN at 8 dpi and (B) helped and CD4-depleted (nonhelped) gB-CD8 in the TG at 8, 21, and 56 dpi. The MFI and frequency of the PD1+ cells is indicated in each plot (C). Symbols represent the mean ± SEM frequency of PD1+ helped and nonhelped gB-CD8 in the TG at the designated dpi. The wedge under the graph represents the corresponding change in functionality. *p < 0.05; **p < 0.01; ***p < 0.001; n = 8–10 mice per group.
costimulatory molecules with PD-L1 on APCs within the DLN may slow or prevent the onset of exhaustion. As these cells enter the TG and undergo prolonged interactions with neurons, they undergo progressive functional exhaustion. Elucidating the mechanisms leading to reduced PD-1 expression and functional recovery of nonhelped gB-CD8 in HSV-1 latently infected TGs at 35–56 dpi will require further study.

Several recent reports emphasize the importance of CD8+ T cells in maintaining HSV-1 in a latent state within the TG. Effective immunosurveillance of latently infected neurons requires CD8+ T cells with the capacity to respond to low levels of viral epitopes that likely exist on infected neurons. Our current findings demonstrate that CD4+ T cell help during initial programming is important in generating a memory CD8+ T cell population with sufficient sensitivity to provide this surveillance. Because HSV-1 reactivation from latency is the primary cause of recurrent herpetic disease, any vaccine designed to prevent reactivation will need to incorporate both CD4 and CD8 epitopes.

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Disclosures
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References


