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Maintenance of T Cell Function in the Face of Chronic Antigen Stimulation and Repeated Reactivation for a Latent Virus Infection

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Persisting infections are often associated with chronic T cell activation. For certain pathogens, this can lead to T cell exhaustion and survival of what is otherwise a cleared infection. In contrast, for herpesviruses, T cells never eliminate infection once it is established. Instead, effective immunity appears to maintain these pathogens in a state of latency. We used infection with HSV to examine whether effector-type T cells undergoing chronic stimulation retained functional and proliferative capacity during latency and subsequent reactivation. We found that latency-associated T cells exhibited a polyfunctional phenotype and could secrete a range of effector cytokines. These T cells were also capable of mounting a recall proliferative response on HSV reactivation and could do so repeatedly. Thus, for this latent infection, T cells subjected to chronic Ag stimulation and periodic reactivation retain the ability to respond to local virus challenge. The Journal of Immunology, 2012, 188: 2173–2178.

Certain viruses persist as a direct consequence of their ability to inactivate T cells that would otherwise result in their elimination. These include persisting infections by pathogens such as lymphocytic choriomeningitis virus (LCMV) (1, 2) and hepatitis C virus (3, 4), both of which can be cleared by an effective T cell response (5, 6). For this class of pathogen, it is thought that chronic or ongoing T cell stimulation is an essential contributor to the inactivation required for virus persistence and ongoing viremia (7, 8). This inactivation takes the form of T cell exhaustion and senescence, where the cells progressively lose various properties associated with full effector function (9). In contrast, other viruses, especially those belonging to the herpesvirus family, do not require compromised immunity for their prolonged survival. Once established, these viruses are never cleared from the infected individual even in the face of effective T cell immunity; instead, they survive by use of various immune evasion strategies (10). In addition, many of these pathogens persist in a state of latency, which is characterized by limited transcriptional activity and little if any virus replication (11). Importantly, effective immunity is actually crucial for maintenance of latency, as for a range of herpesviruses, reactivation from latency is often a major complication associated with generalized immunosuppression (12, 13).

In certain instances, active suppression of herpesvirus reactivation involves direct T cell recognition of persistently infected cells (14). The chronically stimulated T cells involved in virus control are unlikely to be compromised by such events, as their inactivation would result in disease recrudescence. However, although recent reports suggest that T cells associated with latent infection are indeed functional (15–17), there has been no formal demonstration that the cells actually involved in suppressing reactivation are not exhausted by the recognition event or inactivated by the reactivation process. In this study, we show that for persisting HSV infection, T cells involved in latency control remain fully functional and maintain a self-renewing ability despite undergoing chronic stimulation during the latent phase of infection.

Materials and Methods

Mice

C57BL/6 and gBT-I × B6.CD45.1 (gBT-LCD45.1) mice were bred at the Department of Microbiology and Immunology of The University of Melbourne. The gBT-LCD45.1 TCR and gzmBCreERT2/ROSA26EYFP transgenic mice have been described previously (18, 19). gzmBCreERT2/ROSA26EYFP mice received daily injections of tamoxifen (1 mg) i.p. injection as described (18).

Viral infections, ganglia transplantations, and adoptive transfer of transgenic CD8+ T cells

Viruses used were the KOS strain of HSV-1 (HSV) and K.L8A with a position 8 alanine mutation in the glycoprotein B (gB)p8a.505 immuno-dominant determinant derived by recombining HSV.gB-L8A (20) with the KOS strain of HSV and selecting for a recombinant that gave equivalent replication and lesions as found with the wild-type virus. Mice were infected with 1 × 10^6 PFU virus via flank scarification as previously described (21). Latently infected ganglia (T8–T12) were transplanted under the kidney capsule of syngeneic recipients as described (17). C57BL/6 mice received 5 × 10^6 naive gBT-LCD45.1 lymph node cells via i.v. injection. For LCMV infections, mice were infected with 2 × 10^6 PFU LCMV clone 13 by i.v. injection.
Flow cytometry, mAbs, and BrdU staining

T cells were recovered from skin, ganglia, and ganglia grafts as described (17, 22). The following fluorescently conjugated Abs were from BD Pharmingen: anti-CD45.1 (A20), Vα2 (B20.1), anti-CD8α (53-6.7), anti–IFN-γ, anti–TNF-α, anti–IL-2, and anti-CD107a/b. Anti–Tim-3, anti–Lag-3, and anti–CD160 were purchased from eBioscience. Anti–granzyme B was from Invitrogen, and anti–PD-1 was from BioLegend. H-2Kb/gB(498–505)–PE tetramer was generated at the Department of Microbiology and Immunology of The University of Melbourne. For analysis of T cell proliferation, 1.25 mg BrdU was injected i.p. as two injections 12 h apart, and mice were analyzed 12 h later. Uptake was detected with a BrdU Flow Kit according to the manufacturer’s instructions (BD Pharmingen).

Intracellular assays with peptide stimulation

Lytic granule release and cytokine production by gBT-I–CD45.1 T cells was measured by stimulating lymphocytes from pooled ganglia (T8–T12 from two mice) with 0.1 μM gB498–505 peptide in the presence of brefeldin A, monensin, and anti–CD107a/b. Cells were cultured for 5 h before staining for surface markers. Cells were then fixed using a Cytofix/Cytoperm kit (BD Pharmingen) and stained with intracellular Abs before analysis by flow cytometry.

Results

HSV-specific T cells in sensory ganglia harboring latent infection express granzyme B as a consequence of ongoing Ag recognition

HSV forms a prototypic latent infection confined to a localized compartment, the sensory ganglia that innervate the site of initial infection, usually skin or mucosal tissue (23). It is known that latent infection is maintained under tight immune control, partly as a result of the action of CD8+ T cells and their secretory progranzyme B and IFN-γ (14, 24, 25). As a consequence, inhibition of virus reactivation likely requires ongoing local T cell stimulation by the persistently infected neurons. To show this is the case, we transferred HSV-specific T cells from the TCR-transgenic mouse, gBT-I, into C57BL/6 mice prior to subjecting the animals to skin infection and examining the T cells in skin and ganglia at the end of the lytic (day 8) and during the latent (after day 20) phase of infection. Fig. 1A shows that gBT-I cells in skin, a tissue that clearing HSV at the cessation of lytic infection, expressed low levels of granzyme B during latency. In contrast, those T cells in ganglia harboring latent HSV infection had high levels of granzyme B during the latent phase of infection.

It has recently been shown that granzyme B can be upregulated in a nonspecific manner by virus infection (26). To demonstrate that the presence of local infection was not a major driver of nonspecific granzyme B expression within the ganglia, we made use of a recombinant virus (K.L8A) that carries a mutation within the immunodominant determinant from the gB. The mutant virus did not stimulate HSV-specific gBT-I T cells, which otherwise proliferated and expanded after adoptive transfer and flank skin infection with wild-type HSV (Fig. 1B, left panel). The failure of gBT-I T cells to recognize mutant virus was not limited to just the priming phase of the response, and memory gBT-I cells transferred into naive hosts were also unable to respond after challenge by the mutant virus (Fig. 1B, right panel). Note that both wild-type and K.L8A viruses replicate with equal efficiency within the sensory ganglia and exhibit similar levels of skin disease and peak virus load (Supplemental Fig. 1).

In mice simultaneously infected with wild-type and mutant virus on opposing flanks, responding gBT-I transgenic T cell populations lodged and then persisted in sensory ganglia infected with either virus (Fig. 1C). Ganglionic gBT-I cell numbers were largely equivalent in wild-type HSV- and K.L8A-infected ganglia in early latency and diverged as latency progressed, although even at day 35 postinfection, the latter still outnumbered those found in control noninfected ganglia from the same animals. Importantly, activation of gBT-I cells, as measured by granzyme B upregulation, was dependent on specific recognition, with high levels of this effector molecule only found in sensory ganglia harboring wild-type virus infection (Fig. 1D). Thus, because gB-specific T cells have been implicated in latency control (14), these results argue that granzyme B expression by T cells within sensory ganglia was a marker for recognition of cells persistently infected with HSV.
**HSV-specific CD8⁺ T cells undergoing chronic stimulation during latency show a polyfunctional phenotype and limited expression of inhibitory receptors**

Given that the T cell stimulation within the latently infected ganglia is ongoing, and chronic stimulation has been associated with T cell inactivation (1, 2, 27), we next sought to determine whether the T cells persisting in the sensory ganglia retained full functional capabilities. To this end, we examined a number of functional parameters; specifically, the secretion of cytokines TNF-α and IFN-γ and the upregulation of surface expression of CD107, a marker of granule exocytosis. Simultaneous expression of multiple effector molecules has been used as a measure of T cell fitness (28), and their expression is progressively lost as T cells become exhausted from chronic stimulation (9). The extent of this polyfunctionality was assessed during the lytic and latent phases of HSV infection. In addition, T cells were also examined after HSV reactivation, which was initiated by ganglionic excision and transplantation under the kidney capsule of naive recipients, a method previously shown to initiate this event (17). As can be seen in Fig. 2, a high proportion of effector T cells recovered during the lytic phase of infection had multifunctional capabilities. After a 5-h stimulation with gB peptide, ~70% of transgenic T cells recovered from acutely infected ganglia were able to produce IFN-γ. Furthermore, the majority of these IFN-γ-expressing T cells were also able to produce TNF-α and expressed CD107. Importantly, the frequency of polyfunctional T cells as measured by these parameters was similar for ganglia harboring lytic or latent infections, and this was also true when it came to IL-2 production (Supplemental Fig. 2). In addition, T cells that had been subjected to virus reactivation also retained these capacities. Thus, sustained and repeated exposure to virus Ag in the ganglia did not result in functional inactivation of virus-specific CD8⁺ T cell responses.

After prolonged antigenic exposure during chronic infections such as LCMV, virus-specific CD8⁺ T cells have been shown to upregulate multiple inhibitory receptors, including PD-1, which correlates with T cell exhaustion (29, 30). To determine whether intraganglionic T cells showed an exhausted phenotype, we assessed the expression level of markers associated with this condition, notably PD-1, Lag3, Tim-3, and CD160 (29–31), during the lytic, latent, and reactivation phases of infection. As shown in Fig. 3, T cells in sensory ganglia showed only modest increases in expression of these exhaustion markers compared with memory T cells in the spleen. There was no difference between the respective lytic and latent phases of infection, when T cells are either actively involved in virus clearance or undergoing chronic stimulation. Critically, expression levels were less than those on control exhausted T cell populations from mice chronically infected with LCMV. Overall, these data suggest that T cells found in latent ganglia do not exhibit an overtly exhausted phenotype.

**HSV-specific CD8⁺ T cells undergoing chronic stimulation during latency can mount a proliferative response on virus reactivation**

The results above demonstrated that T cells within HSV-infected ganglia maintained a polyfunctional phenotype and did not bear markers of exhaustion. In addition, we had previously shown that ganglionic T cells underwent proliferation and expansion during HSV reactivation (17). Thus, despite ongoing or chronic stimulation within the sensory ganglia, local T cells appeared fully functional and retained self-renewing capability. However, it remained possible that such capabilities were confined to a subpopulation of sequestered cells not involved in active virus re-

![FIGURE 2. Functionality of HSV-specific CD8⁺ T cells during lytic and latent phases of infection. Mice were seeded with naive gBT1.CD45.1 cells and flank infected with HSV. T cells were isolated from the ganglia at day 8 (lytic) or day 30 (latent) postinfection or from latent ganglia that had been transplanted beneath the kidney capsule of naive syngeneic mice (reactivated). T cells subject to transplant-induced reactivation were harvested from ganglia grafts 9 d posttransplantation. T cells were stimulated with gB498–505 peptide for 5 h and then analyzed for lytic granule release (CD107a/b) and IFN-γ and TNF-α production. Shown are representative flow cytometry profiles of gBT1.CD45.1 T cells (gated on CD8⁺ Va2⁺ CD45.1⁺ events) coexpressing IFN-γ and TNF-α (left panels) and IFN-γ and CD107 (right panels). Control panels are gBT1.CD45.1 cells (isolated from latently infected ganglia) incubated without gB498–505 peptide. Frequencies represent means of six mice ± SEM. Data are representative of three independent experiments.](http://www.jimmunol.org/)

To ensure that T cells were marked only during latency, we administered tamoxifen before days 30 and 35 postinfection, well after viral Ag presentation in the lymphoid compartment is extinguished (22) and lytic infection has subsided (21). HSV reactivation was initiated at day 40 by means of transplantation-induced reactivation (Fig. 4A), and the reactivating ganglia, isolated from tamoxifen-treated gzmBCreERT2/ROSA26EYFP transgenic mice, contained a population of YFP-expressing T cells (Fig. 4B). Because the drug was administered between days 30 and 35 after the initiation of infection, this meant that the T cells marked by Cre recombination had recognized infected neurons.
during latency. Most of the YFP⁺ cells, but only a minority of non-marked T cells, were virus specific, recognizing the immunodominant determinant from gB as determined by tetramer staining (Fig. 4C). Consequently, the bulk of T cells responding to virus infection during latency appeared specific for this single immunodominant determinant. Importantly, both YFP⁺ and YFP⁻ T cells specific for gB had undergone equivalent proliferative bursts after virus reactivation, as assessed by BrdU incorporation (Fig. 4D). Overall, HSV-specific T cells actively involved in recognition of virus-infected cells during latency appeared fully capable of proliferation and self-renewal during the process of virus reactivation.

**Ganglion-resident T cells respond to successive rounds of reactivation**

Certain HSV-infected individuals undergo periodic bouts of reactivation, sometimes in a subclinical, continuous cycling pattern

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**FIGURE 3.** Limited expression of inhibitory receptors by HSV-specific CD8⁺ T cells in the ganglia. Mice were seeded with naive gBT-I.CD45.1 cells and flank infected with HSV. T cells were isolated from the ganglia during lytic (day 8) or latent (day 30) phases of infection or from ganglia subjected to transplantation-induced reactivation (day 9 posttransplantation). Shown is expression of PD-1, Lag3, Tim-3, and CD160 on lytic (red line), latent (blue line), and reactivated (black line) ganglion gBT-I.CD45.1 T cells. Appropriate isotype staining is also shown (gray-shaded). Expression of these markers is shown on naive and memory (day 30 after HSV infection) gBT-I.CD45.1 T cells in the spleen (middle row). As a positive control, C57BL/6 mice were infected with LCMV clone 13, and splenic T cells were isolated at day 14 postinfection and stained for inhibitory receptor expression (gated on CD45.2⁻ CD8⁺ events, bottom row). Flow cytometry profiles are representative of five to eight mice per group.

**FIGURE 4.** Chronic Ag stimulation does not impair the ability of ganglion-resident T cells to proliferate upon virus reactivation. gzmBCreERT2/ROSA26EYFP transgenic mice were flank infected with HSV and treated with tamoxifen from day 30 to 35 postinfection. Grafts were recovered on day 8. (A) Schematic depicting the strategy for the analysis of proliferation between YFP⁺ and YFP⁻ labeled T cells. (B) Representative flow cytometry profiles depicting the frequency of YFP⁺ CD8⁺ cells from pooled grafts of BrdU-treated mice. (C) The proportion of gB-tetramer staining cells of YFP⁺ CD8⁺ (black line) and YFP⁻ CD8⁺ (gray-shaded) subsets. (D) BrdU incorporation by YFP⁺ gB-tetramer⁺ and YFP⁻ gB-tetramer⁻ cells. Flow cytometry profiles are representative of five to eight mice per group and three independent experiments.

**FIGURE 5.** Ganglion-resident T cells can undergo further expansion after multiple rounds of virus reactivation. Latent ganglia containing gBT-LCD45.1 cells were transplanted beneath the kidney capsule of naive syngeneic mice. On day 15 posttransplantation, the grafts were recovered and either directly grafted into secondary recipients (without culture) or cultivated in vitro for 2 d before being placed under the kidney capsule of secondary naive recipient mice. On day 15 posttransplantation, the grafts were recovered and either directly grafted into secondary recipients (without culture) or cultivated in vitro for 2 d before being placed under the kidney capsule of secondary naive recipient mice. (A) Levels of infectious virus isolated from the graft (with or without 2 d of culture) on day 3 after re-transplantation. Bars represent the mean + SEM with three mice per group. (B) Number of gBT-I.CD45.1 cells in the grafts (with or without cultivation) on day 0 or day 9 after re-transplantation. Bars represent the mean + SEM with 6–14 mice per group. The data are pooled from three independent experiments.
expansion as shown in this study. Such T cell expansion seen on reactivation would be impossible if the cells were inactivated by chronic herpes simplex virus infection. Thus, our results demonstrate that ganglion-resident T cells remain effective at virus control, either during prolonged periods of latency or when HSV repeatedly cycles from quiescent to active phases of infection.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References
established lytic infections from skin and nerves and can partially limit the early spread of virus after cutaneous inoculation. *J. Immunol.* 172: 392–397.


Supplemental Data

Supplementary Figure 1: Equivalent infection with HSV and gB-mutant K.L8A virus. Representative photographs of lesion development and viral titres in the skin and ganglia 6 days post virus challenge in HSV-infected or K.L8A-infected mice. Viral titres were measured by standard PFU assays.

Supplementary Figure 2: Functionality of HSV-specific CD8+ T cells during lytic and latent phases of infection. Mice were seeded with gBT-I.CD45.1 cells and flank infected with HSV. T cells were isolated from the ganglia at day 8 (lytic) or day 30 (latent) following infection, or from latent ganglia that had been transplanted beneath the kidney capsule of naïve recipient syngeneic mice (reactivated). T cells subject to transplant-induced reactivation were harvested from ganglia grafts 9 days post transplantation. T cells were stimulated with gB498-505 peptide for 5 hours and then analysed for IFNγ and IL-2 production. Control is gBT-I.CD45.1 cells incubated without gB498-505 peptide. Frequencies represent means of 5 mice +/- SEM.

Supplementary Figure 3: Ganglion-resident T cells undergo further expansion following virus reactivation in recipient mice and fail to escape into the circulation. Latent ganglia containing gBT-I.CD45.1 cells were transplanted beneath the kidney capsule of naïve recipient mice. On day 15 post transplantation the grafts were recovered and either directly grafted into secondary recipients (without culture) or cultivated in vitro for 2 days before being placed under the kidney capsule of secondary naïve recipient mice. (A) Section of graft following 2 days of culture staining for HSV Ag (red) and DAPI (blue) (B) Representative FACS profiles
depicting the expansion of gBT-1.CD45.1 cells in the graft with or without culture.

(C) Representative FACS profiles of spleen from mice on day 15 post transplantation (primary recipient) and on day 9 post secondary transplantation (secondary recipient).
Supplementary Figure 1

Skin

n.s. p=0.2965

Ganglia

n.s. p=0.0768

PFU / sample (log)

HSV K.L8A

HSV K.L8A
Supplementary Figure 3

A

B

day 9 post re-transplantation

d2 post culture  |  with in vitro culture  |  without in vitro culture

C

primary recipient  |  secondary recipient

CD45.1  |  CD45.1

CD8  |  CD8