Inflation and Long-Term Maintenance of CD8 T Cells Responding to a Latent Herpesvirus Depend upon Establishment of Latency and Presence of Viral Antigens

Anna Lang, James D. Brien and Janko Nikolich-Zugich

* J Immunol 2009; 183:8077-8087; doi: 10.4049/jimmunol.0801117
http://www.jimmunol.org/content/183/12/8077

References

This article cites 50 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/183/12/8077.full#ref-list-1

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inflation and Long-Term Maintenance of CD8 T Cells Responding to a Latent Herpesvirus Depend upon Establishment of Latency and Presence of Viral Antigens

Anna Lang,*† James D. Brien,‡ and Janko Nikolich-Žugich2*†

Following the priming and contraction phases of the T cell response, latent persistent herpesviruses lead to an accumulation of large pools of virus-specific CD8 T cells, also known as memory inflation (MI). The mechanism of this inflation is incompletely understood, largely because the molecular reactivation of these viruses in vivo and its impact upon T cell biology have not been resolved in mice, and because the relevant observations in humans remain, by necessity, correlative. Understanding these processes is essential from the standpoint of the proposed critical role for latent herpesviruses in aging of the immune system. We studied the causes of memory CD8 T cell accumulation following systemic HSV-1 administration as a model of widespread latent viral infection in humans. A direct role of viral latency and Ag-specific restimulation in driving the accumulation and maintenance of inflated CD8 T cells and a strongly suggested role of viral reactivation in that process were shown by the following: 1) lack of MI in the absence of established latency; 2) prevention or delay of MI with drugs that curtail viral replication; and 3) abrogation of MI by the transfer of inflated T cells into a virus-free environment. These results strongly suggest that periodic, subclinical reactivations of a latent persistent virus cause dysregulation of memory CD8 T cell homeostasis, similar to the one in humans. Moreover, results with antiviral drugs suggest that this approach could be considered as a treatment modality for maintaining T cell diversity and/or function in old age. The Journal of Immunology, 2009, 183: 8077–8087.

Herpesviruses are among the most prevalent latent human pathogens, infecting the vast majority (>90%) of the human population in the Western hemisphere. These viruses persist for the life of the host and rarely cause manifest disease in the absence of profound immunosuppression. Because of that, there is considerable interest in understanding the impact of lifelong herpesvirus infections upon aging, long-term health, survival, and immunity (1). How exactly a balance is maintained between the virus and the immune system over long periods of time and into old age remains incompletely understood. The latent virus should be kept at bay to prevent systemic and potentially devastating reactivation; however, the long-term immune response against the virus must also remain under control. This can be challenging, as illustrated in the case of the CMV, a latent β-herpesvirus that elicits T cell responses unusual in strength, breadth, and complexity (2, 3). CMV seropositivity in humans has been correlated with an age-related increase in the fraction of CMV-specific memory T cells (reviewed in Ref. 1) and a shorter life span in octogenarians and nonagenarians (4). This spurred the hypothesis that immune aging is precipitated by persistent infections, yet direct causality and the mechanistic connection between the two remain to be established. This is due to both the ethical constraints in the human model and because it is difficult to demonstrate subclinical reactivation of infectious CMV in the blood of asymptomatic humans (5, 6) and mice (7, 8) late after the primary infection.

HSV-1 is a ubiquitous α-herpesvirus that establishes lifelong latent infection in the sensory ganglia and the CNS. In humans, this lifelong infection is associated with periodic viral reactivation and migration of the reactivated virus from the site of latency into the periphery. HSV readily infects mice; however, unlike in humans, spontaneous clinical HSV-1 reactivation does not occur in mice (9, 10). Much work has been done to analyze the relationship of the latent HSV virus with the sentinel CD8 T cells present in infected sensory ganglia (11–13), particularly as it pertains to CD8 T cells specific for gB-8p, the immunodominant, glycoprotein B-derived HSV-1 epitope recognized by CD8 T cells in C57BL/6 (B6) mice (14–16). By contrast, far less is known about the effect of the lifelong HSV-1 infection on the systemic memory CD8 T cell pool. In that regard, our recent analysis (17) of circulating memory T cells in ocularly infected B6 mice demonstrated that they were maintained at a stable frequency until late in life and exhibited a resting central memory phenotype. In addition, the size and phenotype of the memory CD8 T cell pool were the same in control mice and in mice treated with the antiviral drug famciclovir, suggesting that in the ocular infection model HSV-1 does not massively reactivate from the trigeminal ganglion (TG)3 to the extent so as to palpably influence the systemic CD8 T cell pool (17). Dysregulation of the

---

1 This work was supported by U.S. Public Health Service awards AG20719 (to J.N.-Ž.) and RR0163 (to the Oregon National Primate Research Center) from the National Institute on Aging and National Institute of Research Resources, National Institutes of Health, and by the E. Bowman Professorship in Medical Sciences (to J.N.-Ž.).

2 Address correspondence and reprint requests to Dr. Janko Nikolich-Žugich, Department of Immunobiology, College of Medicine, University of Arizona, 1501 N. Campbell Avenue, Tucson, AZ 85724. E-mail address: nikolich@ohsu.edu

References

Received for publication April 4, 2008. Accepted for publication October 10, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations used in this paper: TG, trigeminal ganglion; FCM, flow cytometry; i.c., intracorneal; MCMV, murine CMV; MI, memory inflation; p.i., postinfection; rVV, recombinant vaccinia virus; TCE, T cell clonal expansion.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00
systemic CD8 compartment in these mice occurred only late in life, appeared to be virus independent, and was indistinguishable from the spontaneously arising Ag-independent T cell clonal expansions (TCE) (17) (reviewed in Ref. 18). This stands in contrast to systemic murine CMV (MCMV) and human CMV infection, where there is an accumulation of virus-specific T cells several months to years after the priming phase (19–21), and these large cell populations are maintained at high numbers throughout the life span. This leaves several unanswered questions. First, is memory accumulation driven by viral reactivation or by the persistence of viral Ag regardless of viral reactivation? Second, are the expanded CD8 T cells programmed to expand, or are they able to contract upon antigenic withdrawal? Finally, which characteristics of latent herpesvirus infection (viral load, spread, number of latent sites, etc.) determine whether MI will occur?

To answer some of these questions and thereby elucidate the potential impact of latent herpesviruses on the adult and aging immune system, we established and characterized a model of systemic HSV-1 infection in which memory inflation (MI) readily occurs. This model permits precise dissection of the relative contributions of various virus-driven and virus-independent factors to the long-term (dys)regulation of the diversity and composition of the CD8 T cell compartment. In the present work, we report that the size and phenotype of HSV-1-specific CD8 T cell systemic memory is absolutely dependent on the establishment of HSV-1 latency and the presence of viral Ag and can be modulated by drugs that block viral replication and reduce viral Ag expression. We discuss these results from the standpoint of the maintenance of optimal T cell diversity over a lifetime.

Materials and Methods

Mice

Male C57BL/6-NCr (B6) mice and B6-Ly5.2/Cr (Ly-5.2) were purchased from the National Cancer Institute colony (Frederick, MD). B6.gBT-I (gBT-I in the text) TCR-transgenic mice (22) carrying rearranged tcr genes encoding TCR that recognize the immunodominant HSV-1 epitope gB-8p were generously provided by Dr. F. R. Carbone (University of Pennsylvania School of Medicine, Hershey, PA) and was grown on thymidine kinase-deficient L cells and titered on Vero cells. Mice were infected i.p. with 4 \(10^6\) PFU of HSV-1 (strain 17) per mouse were performed as described (23). Recombinant vaccinia virus (rVV) expressing the SSIEFARL minigene (rVV-gB-8p) was a generous gift from Dr. S. Tevethia (Pennsylvania State University, Hershey, PA) and was grown on thymidine kinase-deficient L cells and titered on Vero cells. Mice were infected i.p. with 4 \(10^6\) PFU of rVV-gB-8p.

Determination of titers of replicating virus

The amount of replicating virus in the indicated organs (see Table I) was determined by a plaque assay of organ homogenates as previously described (23).

Famciclovir treatment

Where indicated, famciclovir (Famvir; Novartis) was administered to mice in their drinking water at a concentration of 2 mg/ml. The Famvir water was changed twice a week.

BrdU labeling

Mice were given BrdU in drinking water at 0.8 mg/ml as described (24, 25) for 3 wk. BrdU incorporation was measured by flow cytometry (FCM) using a kit from BD Pharmingen as per the manufacturer’s recommendation.

Adoptive transfers and CFSE labeling

Splenocytes from naive gBT-I-transgenic mice or from HSV-immune mice were enriched for CD8 T cells and labeled with CFSE as previously described (23). Splenocytes containing 2 \(\times 10^6\) tetramer+ naive CD8 T cells (naive gBT-I cell transfers) were adoptively transferred into each congeneric B6-Ly5.2/Cr female recipient by i.v. injection 24 h before infection, as described previously (23). Alternatively, splenocytes containing 3 \(\times 10^6\) tetramer+ memory CD8 T cells (memory cell transfers) were transferred into congenic naive or HSV-1-infected B6-Ly5.2/Cr mice. The transferred and recipient T cells were distinguished by FCM detection of the CD45.2 (Ly-5.1) molecule (mAb clone 104; BD Pharmingen).

Reagents, Abs and FCM analysis

The gB-8p peptide (SSIEFARL) was purchased from SynPep, and the gB-8p-Kb tetramer was obtained from the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). mAbs were purchased from commercial sources.

FCM analysis was performed as previously described (23). Anti-Ki-67 mAb was purchased from BD Pharmingen and the intranuclear staining was performed per the manufacturer’s instructions. FCM data were acquired on the FACS LSR II instrument using FACSDiva software (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

Statistics

Student’s t tests were performed with Excel (Microsoft) using two-tailed analysis with equal variance.

Results

Systemic HSV-1 infection causes robust MI

In the present study, we analyzed the impact of HSV-1 infection on memory CD8 T cell maintenance in systemically infected B6 mice. Previously, we found that after i.c. (ocular) infection there was little or no evidence of continuous interaction between the latent virus (residing in the TG and the brain) and the systemic CD8 T cell pool (17). We speculated that the localized nature of ocular infection, where the virus spread is limited to the corneal epithelium, the TG and the brain (23), may contribute to this. A different situation could occur in the course of i.p. infection, where there is potential for broad initial viral dissemination and spread, conducive to wider establishment of latency, and consequently a wider base for viral reactivation. This could result in increased access to viral Ag, which could influence CD8 T cell priming and/or maintenance.

To test this prediction, we first compared the total viral titer in the nervous system of mice infected via the localized (i.c.) and systemic (i.p.) routes by comparing the total titers obtained from the brain, spinal cord, and sensory ganglia. We reasoned that the total amount of virus present in the nervous system would correlate with the viral load during latency, as has been suggested in the literature (26–28). Unexpectedly, the total viral titers in the nervous system (sum of viral titer in the brain, TG, spinal cord, and associated ganglia) at the peak of viral replication were identical on day 3 and somewhat higher, but not significantly so in systemic infection on day 5 (day 3 postinfection (p.i.), 1.3 \(\times 10^3\) \(\pm\) 3 \(\times 10^3\) PFU for both routes; day 5 p.i., 8 \(\times 10^2\) \(\pm\) 4 \(\times 10^2\) PFU for i.c. route and 4 \(\times 10^3\) \(\pm\) 1.1 \(\times 10^3\) PFU for i.p. route). However, we found that in many mice systemic infection resulted in variable but greater systemic viral spread than localized infection (Table I), with the virus reaching the fat pads, spinal cord, brain, spleen, lung, liver, kidney, and lymph nodes.

We next compared the dynamics of the CD8 T cell activation during localized and systemic infection. Cohorts of B6 mice were infected i.c. or i.p. and the expansion, contraction, and maintenance of Ag-specific cells were followed longitudinally in blood (Fig. 1A). Although i.c. infection produced a slightly lower percentage of tetramer+ cells, there was no statistically significant
difference in the extent of expansion during the effector phase. At the later time points, however, the HSV-specific CD8 T cells exhibited significant differences, with the systemically infected mice having significantly higher percentages of tetramer\(^+\) cells at all points starting from day 45 (Fig. 1A). Although Fig. 1A shows a particularly dramatic increase in HSV-specific memory T cells, such MI was consistently observed in all experiments and typically ranged between 12 and 20% of the total CD8 repertoire. This inflation was not only due to differences in the set point of memory (day 45); whereas after ocular infection the frequency of tetramer\(^+\) cells remained stable throughout the experiment, in systemic infection the frequency of memory tetramer\(^+\) cells continued to rise from the levels observed at the set point and was significantly higher than in localized infection at all time points tested (\(p < 0.00001\); Fig. 1A). Of note, inflation did not continue indefinitely in concert with the fact that the expanded cells were not malignantly transformed, thus suggesting strongly that some level of homeostatic control remained to keep these cells from continuous accumulation. Examination of cell numbers at 12 mo p.i. showed that this expansion was absolute and not only relative (Fig. 1B). Therefore, HSV-1, like CMV, was capable of producing early MI if administered systemically. The onset of MI was not likely a result of the particular conditions granted by the systemic infection route, because systemic infection with an acute virus, rVV, expressing the gB-8p epitope (rVV-gB), was not associated with MI early after infection (Fig. 1, C and D). This is consistent with our previous results (17) as well as those of others (29) showing that late-life T cell clonal accumulations are only rarely specific for viral Ags present during acute viral infections; if such T cell clonal accumulations occur, they then share the characteristics of spontaneous TCE (30, 31) in that they appear Ag independent and instead are maintained by cytokines.

**FIGURE 1.** Early onset of memory inflation following systemic HSV-1 infection. A and B. Two cohorts of young mice were infected with \(10^6\) PFU of HSV-1 via a localized infection route (triangles; \(n = 10\)) or a systemic (circles; \(n = 8\)) infection route. Blood samples were taken at the indicated time points (d, day; m, month) and stained with anti-CD8 and gB-8p:Kb tetramer. The values (A) represent the average percentage of tetramer\(^+\) cells within the CD8 T cell population over time (\(\pm\) SD), which was significantly different between groups from day 45 p.i. onward (at least at \(p < 0.05\)). The difference in numbers (\(B\)) of tetramer\(^+\) cells was determined at 12 mo p.i. and was also statistically significant. C and D. Infection with an acute virus, rVV-gB, does not result in memory inflation. Two cohorts of young mice were infected with \(10^6\) PFU of HSV-1 (C; \(n = 11\)) or rVV-gB (D; \(n = 11\)) via a systemic infection route. Blood samples were taken at the indicated time points and analyzed as in A. Each data point represents the percentage of tetramer\(^+\) cells within the CD8 T cell population of individual mice. Memory inflation was observed only in the HSV-1 infected group.

**Kinetics of CD8 T cell activation following systemic and local infection and in the course of MI**

Although the above data did not provide formal proof, they could be interpreted to suggest that the difference in memory CD8 T cell maintenance is linked to differences in viral spread. However, it was also possible that infection via different routes resulted in the generation of different CTLs with different responsiveness to homeostatic stimuli or different abilities to survive beyond the effector phase. The onset of MI could be either a result of early CTL programming unique to the systemic infection route or a response to different cell extrinsic stimuli, such as an ongoing exposure to viral Ag experienced by the memory cells during the latent phase of infection. To discriminate between these possibilities, we compared the detailed kinetics of CD8 T cell activation as determined by the expression/down modulation of markers associated with activation and also by expansion and contraction in the course of i.p. and i.c. infection (Fig. 2). Analysis of the proliferation kinetics of transferred indicator CD8 cells (gBT-1, specific for the immunodominant HSV gB-8p epitope; Ref. 22) (Fig. 2A) and the frequencies of endogenous gB-8p tetramer\(^+\) CD8 T cells (Fig. 2, B and C),

---

### Table I. Wide viral spread after systemic HSV-1 infection\(^a\)

<table>
<thead>
<tr>
<th>Infection Route</th>
<th>Day Postinfection</th>
<th>Spinal Cord</th>
<th>Brain</th>
<th>Trigeminal Ganglia</th>
<th>Spleen</th>
<th>Lungs</th>
<th>Liver</th>
<th>Eye</th>
<th>Draining Lymph Nodes</th>
<th>Kidneys</th>
<th>Peritoneal Wash</th>
<th>Fat Pads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized (corneal)</td>
<td>Day 1</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>0/13</td>
<td>4/13</td>
<td>9/9</td>
<td>0/13</td>
<td>0/5</td>
<td>0/5</td>
<td>13/13</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>0/9</td>
<td>7/9</td>
<td>9/9</td>
<td>0/9</td>
<td>0/5</td>
<td>0/5</td>
<td>9/9</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Systemic</td>
<td>Day 1</td>
<td>5/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td>2/5</td>
<td>4/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>13/22</td>
<td>1/14</td>
<td>0/14</td>
<td>13/25</td>
<td>1/9</td>
<td>2/9</td>
<td>0/9</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>3/9</td>
<td>4/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>1/9</td>
<td>0/9</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

\(^a\) Mice were infected as described in Fig. 1 and the presence of an actively replicating virus in the listed tissues at the time of harvest was determined by a plaque assay. The data show the number of mice with actively replicating viruses in the given tissue over the total number of mice tested. Boldfaced numbers denote organs where virus was detected.
as well as their activation phenotypes as measured by the down-regulation of CD62L and CD127 (Fig. 2, D and E), all revealed faster activation and proliferation in systemic infection, but these differences were greatly reduced or had disappeared by day 10 (Fig. 2, B–E), when the replicating virus became undetectable (Ref. 23) and data not shown). By that time CD8 T cells exhibited superimposable frequencies and absolute numbers (Fig. 2, B and C), as well as the activation phenotype (Fig. 2, D and E). Of importance, the activation phenotypes again became significantly different between the groups during the MI phase (Fig. 3), and these cells did not appear to exhibit any notable functional defects (Fig. 3, C and D). This is similar to the MI described in the MCMV model, where the memory CD8 T cells specific for the inflammatory MCMV epitopes retain their function (32). In contrast, the accumulation of functionally defective memory CD8 T cells has been documented in CMV-infected people, particularly the elderly, by the Pawelec/Wikby group (33–35). It is at present unclear what accounts for these differences, although it is tempting to speculate that the impact of MI on the accumulation of dysfunctional memory cells varies with the longevity of the host. We are currently investigating the combined effects of MI and aging on the functionality of memory CD8 T cells in the HSV infection model (A. Lang, J. D. Brien, and J. Nikolich-Zugich, manuscript in preparation).

MI in systemic infection was accompanied by an overwhelmingly effector/effector memory phenotype, with most tetramer+ CD8 T cells being CD62LlowCD127lowCD27+ cells (Fig. 2, D and E). Because the virus-specific CD8 T cells regain expression of CD127 early during the effector-to-memory transition phase (day 14 p.i.; Fig. 2E), the lack of CD127 expression by memory CD8 T cells at 12 mo p.i. (Fig. 3)
suggested that this molecule may have been down-regulated again by an activation event beyond the acute phase of infection. Moreover, down-regulation of CD27 has been linked with the presence of repeated Ag stimulation (36, 37). The above-described effector memory phenotype suggests that CD8 T cells from systemically infected mice have received additional stimulation in vivo following the resolution of acute infection. We sought to test the possibility that this stimulation resulted from the presentation of the gB-8p to the memory T cells as a result of viral reactivation.

**Ag dependence of development and maintenance of MI**

To decisively test whether the onset of MI is dependent on Ag, we performed adoptive transfer studies in which memory CD8 cells isolated from systemically infected mice were transferred into congenic naive or infected mice (Fig. 4). We first addressed the issue of MI development by using splenocytes from mice infected with HSV-1 via the localized or systemic route. We then performed adoptive transfer studies in which memory CD8 cells isolated from systemically infected mice were transferred into congenic naive or infected mice (Fig. 4). We first addressed the issue of MI development by using splenocytes from mice infected with HSV-1 via the localized or systemic route.

**FIGURE 3.** Cells undergoing MI are characterized by an effector/effector memory phenotype and retain their effector function. A, Examples of phenotypes displayed by inflating and noninflating memory CD8 T cells. At 12 mo p.i., blood samples from mice infected with HSV-1 via the localized or the systemic route were stained with CD8, tetramer, CD62L, CD127, and CD27. The graphs are gated on tetramer + cells (infected mice) or on total CD8 T cells from naive age-matched mouse. The numbers represent the percentage of gated cells expressing the given marker and are representative of values obtained from the entire experimental cohort. B, Summary of the expression of CD62L, CD127, and CD27 on mice infected with HSV-1 via the localized (n = 10) or the systemic (n = 8) route. The values show the average (± SD) percentage of CD62L high/CD127 high/CD27 high cells within the population of tetramer + CD8 T cells at 12 mo p.i. The data are representative of three independent experiments. C and D, Splenocytes from mice at 12 mo p.i. (n = 10) were stimulated for 6 h with 10−5 M gB-8p peptide in the presence of brefeldin A and then stained using gB-8p:Kb tetramer and CD8 (C, top plots) or CD8 and intracellular IFN-γ (C, bottom plots). Representative data from three individual mice are shown in C. The data from all tested mice are summarized in D. The x-axis values show the percentages of tetramer + cells (Tet +) and the y-axis values show the percentages of IFN-γ + cells within the total CD8− T cell pool of individual mice. The values for IFN-γ show the net IFN-γ (background IFN-γ staining from unstimulated wells was subtracted; background was <0.2%). Excellent correlation of the tetramer and IFN-γ staining was observed (R2 = 0.948), indicating that the expanded tetramer + cells are functional.
CD8 T CELL MEMORY INFLATION DEPENDS ON VIRAL REACTIVATION

Recipients and again saw MI only in systemically infected hosts. We next performed an adoptive transfer of CD8 T cells that had undergone MI for 4 mo (systemic infection) to test whether the maintenance of already expanded memory CD8 T cell populations requires the presence of Ag (Fig. 4B). The frequency of tetramer+ cells within the donor CD8 T cell population remained high early after transfer (day 2) but declined over time in naive recipients (Fig. 4B). By contrast, the same cell population continued to be maintained in infected mice (Fig. 4B). Although our results suggest that these cells may be even increasing in percentages and numbers over time, future experiments will be needed to decisively answer whether there is a net expansion of the transferred cells over long periods of time, particularly in aging mice. Therefore, the maintenance of an elevated frequency of memory CD8 T cells was absolutely dependent on the presence of the virus and, by inference, the presence of viral Ag.

**MI depends on establishment of latency and likely involves viral reactivation**

Next we sought to specifically address the role of latency and viral reactivation in MI. Famiclovir, also known as Famvir (Novartis), is an inhibitor of HSV-1 replication (38). Famvir is a nucleoside analog with strong affinity for the viral, but not the cellular, DNA polymerase, and the drug is ingested in its inactive form and becomes activated by viral thymidine kinase, selectively targeting cells with an actively replicating virus (38–40). We and others have previously demonstrated that Famvir treatment during acute ocular HSV-1 infection successfully blocks viral replication in vivo (17, 40) and significantly reduces, although it does not completely block, the gB transcription during viral reactivation (17). We therefore used this drug to test whether the establishment of latency and viral reactivation were necessary for MI.

We first administered Famvir to mice in a manner so as to prevent latency (17). Upon such treatment, the virus exhibited greatly curtailed replication at the site of infection (Fig. 5A) and showed no spread to the spleen (Fig. 5B), in contrast to the control mice. That, however, did not prevent CD8 priming in Famvir-treated animals (Fig. 5, C and D), which in absolute numbers was not significantly different from that in untreated animals on day 7 p.i. (Fig. 5D) and was most likely due to substantial amounts of preformed gB protein (41). Consequently, the control mice developed robust MI in relative (Fig. 5C) and absolute (Fig. 5D) terms, whereas Famvir-treated animals showed no MI. Analysis of the phenotypes of these cells further corroborated the Ag- and latency-dependent nature of MI, because while the control cells exhibited an effector memory phenotype, Famvir-treated cells exhibited a clear central memory phenotype (Fig. 5E).

Next, we sought to address the issue of reactivation from latency. The time of the onset of Famvir treatment directly correlated with MI, so that pretreatment with Famvir completely inhibited MI whereas a later onset of Famvir administration correlated with the increased magnitude and accelerated kinetics of MI (Fig. 6A). Even when Famvir treatment has been initiated as late as day 14, by which time acute viral replication was controlled by the immune system, there was an initial prevention and subsequently a delay in MI (data not shown and Figs. 6 and 7A). This delayed drug administration, however, was not enough to prevent MI over long time periods and was therefore very different compared with the sharp and nearly absolute differences between animals pretreated with Famvir (Fig. 5) and controls or between systemic and ocular infection (Figs. 1–3).

We used this delayed drug administration protocol to examine the proliferation and accumulation of CD8 T cells in the course of MI. Young animals were infected and the experimental group was maintained on Famvir as of day 14 p.i. Seven months later, there was significant MI in the untreated group but significantly lower MI in the Famvir-treated group (significant difference in the average percentage of tetramer+ CD8 cells, p = 0.005; Fig. 7A), at which time the mice were treated with BrdU in drinking water over 3 wk. This long pulse allowed the labeling of up to one-third of total CD8 T cells that had proliferated at least once over this time period. We next determined the kinetics of label loss as a measure of the long-term turnover among CD8 T cells. In the Famvir-treated group, HSV-specific cells did not show faster turnover than CD8 T cells of irrelevant specificity (Fig. 7B). By contrast, tetramer+ cells from control mice showed significantly greater loss of label (and therefore higher turnover) compared with tetramer+ cells (Fig. 7C). Finally, a direct comparison of tetramer+ cells from control and Famvir-treated mice revealed a significant increase in CD8 T cell turnover in the absence of Famvir (Fig. 7D). This was not a consequence of the direct action of the drug upon CD8 T cells, because we saw no difference between CD8 T cell turnover between naive, uninfected mice in the presence and the absence of a long-term administration of Famvir (day not shown).

Although the above differences in labeling rates were not dramatic, they could be expected to amplify over time and produce
substantial differences in the accumulation and maintenance of elevated numbers of memory CD8 T cells in older animals, as seen in Fig. 1. Alternatively, gain due to proliferation in these cells could be offset by simultaneous loss due to the death of some of these cells redistribution to other sites, and the recruitment of new naive cells into the response could also play a role in the overall population dynamics of this memory pool (32). Regardless of the precise source, an analysis of gB-specific CD8 T cell representation over the same period revealed a substantial increase in percentages and absolute numbers of gB-specific cells in the blood that was not seen in Famvir-treated mice, consistent with the above hypothesis. However, one should bear in mind that viral reactivation would be expected to occur asynchronously, and this linear sharp increase in this period is unlikely to represent the whole memory inflation period, as judged from the plateau effect we have seen in the absolute accumulation of inflated cells.

To better understand the mechanism of MI we next analyzed the maintenance of memory CD8 T cells by correlating their numbers and turnover over a longer period of time in blood (Fig. 8, A, C, E, and G) and spleen (Fig. 8, B, D, F, and H). Mice were infected via

FIGURE 5. Decreasing the initial viral load and interfering with viral reactivation can prevent memory inflation after systemic HSV-1 infection. A and B, Two groups of mice (n = 4 per group) were infected with HSV-1 via the systemic route and their CD8 T cell responses were followed longitudinally by staining blood samples with CD8 and tetramer. One cohort (HSV + Famvir (d−7)) was given the antiviral drug famciclovir (FVR) starting on day −7 (d−7) and continuing thereafter. The second cohort (HSV) was left untreated. On day 3 p.i., mice from the FVR-treated and control groups (n = 4/group) were sacrificed and the amount of actively replicating virus was determined in fat pads (A) and the spleen (B). C and D, In the FVR-treated group only two of four mice had any detectable virus in their fat pads, and no virus was detected in their spleens (n.d.). The average percentage (C) and number (D) of tetramer+ cells within the CD8 T cell population was determined. The asterisks in C and D indicate a statistically significant (p < 0.05; Student’s t test) difference between the FVR-treated and control groups. E, Significant differences in the expression of CD62L, CD127, and CD27 were observed between the FVR-treated and control groups at 180 days p.i. The values show the average percentage (± SD) of cells with a given phenotype within the tetramer+ CD8 T cell population.

FIGURE 6. Timing of famciclovir (FVR) treatment initiation determines the kinetics and extent of MI. Cohorts of mice (8–10/group) were infected systemically with HSV-1 and their gB-8p-specific CD8 T cell responses were monitored in blood over time. Four groups were analyzed: control (no famciclovir (no FVR)) and three groups of mice in which FVR treatment was started on day 5 p.i., day 1 p.i. or 7 days before infection (d−7)). Once started, FVR was administered continuously throughout the duration of the experiment. The number of tetramer+ cells/ml blood is shown for individual mice per group at 1 (A), 3 (B), and 6 mo p.i. (C). The data are representative of two independent experiments.
the i.p. route and either left untreated (HSV-only group) or treated with Famvir from day 5 p.i. onward (HSV plus FVR group). The timing of the initiation of Famvir treatment was based on the results presented in Fig. 6, which showed day 5 p.i. to be optimal for interfering with MI but not preventing it altogether. After resolution of the acute infection and establishment of gB-specific memory cell pools, mice were given BrdU for 3 wk (day 77–98 p.i.) and then the turnover of tetramer\textsuperscript{+} CD8 T cells was followed for 16 wk by measuring the loss of BrdU (day 98–210 p.i.). Similarly as in the experiment from Fig. 7, up to 30% of the tetramer\textsuperscript{+} cells found in blood got labeled with BrdU during the 3 wk-long pulse, both in control and Famvir-treated mice (Fig. 8E). However, over the course of 16 wk we found that the tetramer\textsuperscript{+} cells from control mice (HSV-only group) had a significantly greater turnover than tetramer\textsuperscript{+} cells from Famvir-treated mice, as shown by the greater loss of BrdU (Fig. 8E). This increased turnover in control mice correlated with the development of MI and the maintenance of elevated numbers of tetramer\textsuperscript{+} cells in blood (Fig. 8, A and C). Again, given that no major increase in percentages was seen over long periods of time, we conclude that there must be additional factors that result in the loss of tetramer\textsuperscript{+} cells to balance the increased proliferation so that the entire population is either maintained or increases only slightly. These factors remain to be elucidated. In addition, a significantly greater proportion of tetramer\textsuperscript{+} cells from control than from Famvir-treated mice had a phenotype indicative of recent stimulation (fewer CD62L\textsuperscript{high}CD127\textsuperscript{−}CD27\textsuperscript{+} cells; Fig. 8G), suggesting that there is ongoing (likely periodic) Ag presentation in HSV-infected mice and that its extent can be decreased by the treatment of latently infected animals with antiviral drugs.

Interestingly, we found that both the development of MI and the changes in the turnover of tetramer\textsuperscript{+} cells in control and Famvir-treated mice were less dramatic in the spleen than in blood (compare Fig. 8, A, C, and E with Fig. 8, B, D, and F). During the latent phase (sampled on days 98 and 210 p.i.), the HSV-infected mice had both higher percentages (Fig. 8B) and higher absolute numbers (Fig. 8D) of tetramer\textsuperscript{+} memory CD8 T cells in their spleens than did Famvir-treated mice. This correlated with the increased percentage of memory cells in the spleens of control mice expressing effector memory phenotype (fewer CD62L\textsuperscript{high}CD127\textsuperscript{CD27\textsuperscript{+}} cells; Fig. 8H). However, these differences were much smaller in the spleen than in blood. Similarly, the tetramer\textsuperscript{+} cells isolated from spleens of control mice showed a significantly greater loss of BrdU over the course of the experiment than did Famvir-treated mice; however, that difference was not dramatic (Fig. 8F) and was smaller than that seen among circulating lymphocytes (Fig. 8E). We interpret these results to suggest that T cells in the spleen, being at the site that does not contain latent virus (Table I and data not shown), are less exposed to the viral Ag upon reactivation compared with the cells that recirculate in the blood and presumably visit the sites of neuronal activation.
Overall, together with the prior results on Famvir efficacy and action (17, 40), these results strongly suggest that the increased proliferation rate of tetramer$^+$ cells is driven by viral reactivation and are consistent with the idea that this process is the key mechanism leading to the development and maintenance of MI in mice where viral reactivation is not controlled.

**Discussion**

In this study, we show in a murine model of latent herpesvirus infection that the dysregulation of CD8 T cells responding to the virus depends upon the establishment of latency and the presence of viral Ag, and we present results strongly suggesting that viral reactivation drives the extent of MI. These results have direct bearing upon the understanding of human T cell responses to latent herpesviruses, including the dysregulation of these responses with age. Specifically, the human memory T cell pool gets progressively obsessed with latent herpesviruses (35, 42), chiefly with CMV, so that up to one-half of the entire pool is specific for this virus (2). It remains unclear whether these responses are the “last man standing” as the rest of the immune system crumbles around them, or whether they may actively contribute to the phenomena associated with immune senescence. Our experiments show that systemic infection with a herpesvirus that is normally localized to the epithelial and neuronal tissues can produce CMV-like MI in mice, akin to the one described for CMV in mice (20, 21, 43) and humans (1, 42). We show that, similarly as in previously published results (21, 44), only latent but not acute viruses can produce intense MI. More importantly, we show that the establishment of latency must occur for the onset of MI and that viral Ags are necessary for the maintenance of inflated cells, and our data with

---

**FIGURE 8.** Comparison of in vivo proliferation and maintenance of memory CD8 T cells in blood and spleen. Cohorts of B6 mice were infected with HSV-1 as in Fig. 7. One group (HSV only; n = 8) was left untreated and the second was continuously treated with famciclovir (FVR) starting on day 5 p.i. (HSV + FVR; n = 8). Both groups were given BrdU in their drinking water for 3 wk (days 77–98 p.i.). The percentage (A and B) and number (C and D) of tetramer$^+$ cells, as well the loss of the BrdU label by tetramer$^+$ CD8 T cells (E and F) was analyzed in blood (A, C, and E) and spleen (B, D, and F) for 16 wk following the end of BrdU labeling (days 98–210 p.i.). G and H, The expression of CD62L, CD127, and CD27 was evaluated on tetramer$^+$ CD8 T cells in blood (G) and spleen (H) on day 210 p.i. The results are presented as the percentages of CD62L$^{hi}$ (CD6L$^{hi}$), CD127$, and CD27$^+$ cells within the tetramer$^+$ CD8$^+$ T cell population (± SD).
inhibitors of viral replication strongly suggest that viral reactivation is necessary for this process. All of this suggests that in mice and likely in humans too the constant subclinical reactivation of herpesviruses contributes heavily toward MI. It should be noted that the experiments discussed in this article focus solely on viral reactivation-induced MI, which is distinct from the reactivation-independent but age-dependent phenomenon of age-related TCE previously documented to take place in old mice and to sometimes be specific for acute viral Ags, but not be dependent on them for maintenance (17, 45).

The results presented in this article are consistent with recent work in the herpesvirus field suggesting that molecular reactivation of HSV-1 may occur in mice in the absence of clinical reactivation. Several studies have demonstrated the ongoing presence of inflammatory cell infiltrates at the site of latency that is associated with an elevated level of proinflammatory cytokines (9, 10, 46–48), both of which could be curtailed by the administration of acyclovir (49). With regard to HSV-1-specific CD8 T cells, effector memory phenotype cells were shown to be continually present in the TG of ocularly infected mice (11, 13) and to proliferate at a greater rate than the memory CD8 T cells present in an uninjured lung (12). In addition, only virus-specific cells were retained in the latently infected ganglia, and the maintenance of their activated phenotype depended on the ability of the host to present HSV Ag (50). This suggested the ongoing presentation of viral Ag(s) within the tissue harboring the latent virus. However, in these situations there is no infection of the virus-specific CD8 T-cells, and it was not clear how this latent virus would interact with the immune system if it was present systemically. We here show that systemic infection with HSV produces a strong MI of gB-specific CD8 T cells that is similar to the one described for MCMV (19, 21, 43) and akin to the situation encountered in humans (51).

Overall, the above results shed light on the molecular basis of this systemic memory accumulation and support the scenario whereby reactivation of a latent virus over a life time, followed by Ag presentation to virus-specific CD8 T cells, leads to MI. We show, to the best of our knowledge for the first time, that transferring infected cells into the virus-free environment leads to the contraction and/or loss of most of the activated cells. We therefore conclude that accumulating cells are not dysregulated in an absolute sense. This is consistent with the observation that although MI cells accumulate to impressive levels, they rarely occupy more than half of the total CD8 compartment and there is no absolute increase of the CD8 compartment in animals undergoing MI (21) (A. Lang and J. Nikolich-Zugich, manuscript in preparation). In addition, we found no evidence that the inflated memory cell population lost its ability to function in response to antigenic stimulation. We found that a minor population of memory CD8 T cells was still detectable 120 days p.i. upon these transfers. It will be of interest to determine, using sorted CD8 T cell subsets, whether these cells originated from effector memory cells converting into central memory or whether they were perhaps the central memory cells present in the original inoculum. Moreover, the limits of the expansion of cells already undergoing memory infection need to be established experimentally. Finally, it will be important to further understand the precise role of the various virus and host mechanisms involved in this phenomenon, as well as the impact of this form of clonally expanded T cells upon residual T cell diversity and the immune defense of the host. This is of particular importance in aging organisms, where the sizes of the expanded T cell populations can reach levels that have the potential to severely constrain the remaining T cells in the body. Such constriction could potentially impinge upon productive immune defense, as was shown in the case of spontaneously arising TCE in immune-fitted mice (52). If so, our results would open the possibility of intervention with antiviral treatments to attenuate viral reactivation and to consequent dampen the expansion of inflated virus-specific T cells. Experiments are in progress to address these possibilities.

Acknowledgments
We thank Dr. Ilhem Messaoudi for help with experimental troubleshooting and helpful discussions and the members of the Nikolich laboratory for input and help.

Disclosures
The authors have no financial conflict of interest.

References

Downloaded from http://www.jimmunol.org by guest on April 2, 2022


