This information is current as of May 1, 2017.

Michael L. Freeman, Claire E. Burkum, Meghan K. Jensen, David L. Woodland and Marcia A. Blackman

J Immunol published online 9 March 2012
http://www.jimmunol.org/content/early/2012/03/09/jimmunol.1102787
γ-Herpesvirus Reactivation Differentially Stimulates Epitope-Specific CD8 T Cell Responses

Michael L. Freeman, Claire E. Burkum, Meghan K. Jensen, David L. Woodland, and Marcia A. Blackman

The γ-herpesviruses are characterized by their ability to establish lifelong latency. Subsequent immune suppression leads to viral reactivation from latency and the onset of a variety of pathologies, including lymphoproliferative disease and cancers. CD8 T cells play a key role in preventing reactivation of latent virus. Therefore, to develop effective therapeutic immune strategies, it is essential to understand the maintenance of CD8 T cell responses during latency. Because the γ-herpesviruses are highly species-specific and mice cannot be infected with the human pathogens, EBV or Kaposi’s sarcoma-associated herpesvirus, we have used a natural rodent γ-herpesvirus experimental infection model, γ-herpesvirus-68. In this report, we show that during long-term latent infection, naive CD8 T cells are recruited into the ongoing immune response in an epitope-specific manner. When viral reactivation is induced in vivo, the recruitment of CD8 T cells for some, but not all, epitopes is enhanced. The variation in recruitment is not due to differences in epitope presentation. We also show that CD8 T cells that are newly stimulated during reactivation are functionally impaired compared with acutely stimulated cells in terms of cytokine production. Thus, our results demonstrate unexpected complexity in the response of CD8 T cells specific for different viral epitopes that were stimulated during acute infection, quiescent latency, and reactivation. The Journal of Immunology, 2012, 188: 000–000.

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fter a brief acute infection, the human γ-herpesviruses EBV and Kaposi’s sarcoma-associated herpesvirus (KSHV) are maintained in a latent state for the life of the host. EBV and KSHV are strongly species-specific and do not readily infect mice. The rodent pathogen murine γ-herpesvirus-68 (γHV68) is closely related to EBV and KSHV, and infection of mice with γHV68 provides an experimental animal model to study γ-herpesvirus pathogenesis and immunity. Because antiviral T cell activity is critical for the control of latent herpesvirus infections and the loss of T cell memory numbers or function can lead to viral reactivation and recurrent disease (1–3), it is of utmost importance to understand how antiviral CD8 T cell responses are maintained during γHV68 infection.

Memory T cells in persistent infections can be maintained by at least two mechanisms. First, a subset of memory T cells that retains the capacity for self-renewal can replenish the memory pool as nonreplicating memory T cells are eliminated. In this model, all of the memory T cells required for controlling the latent pathogen are derived from cells stimulated during the initial acute infection (4, 5). Alternatively, new naive cells with specificity for viral epitopes can be recruited into the ongoing T cell response, supplying a population of cells to replace pre-existing memory cells. In this model, over time the memory T cell population accumulates cells that were not stimulated during the acute infection, but rather were recruited into the response by presentation of Ags during viral latency or persistence (4, 6, 7). These two models are not mutually exclusive, and antiviral T cell populations may be maintained by both the replication of a subset of memory T cells and the recruitment of naive T cells (4, 6, 8). Which of these two mechanisms is used, and/or to what extent, might depend on the specific Ag involved, because viral Ags can be differentially expressed at various stages of viral persistence.

We and others have recently identified a panel of γHV68-specific CD8 epitopes that detect CD8 T cell responses with differential kinetics throughout the infection (9, 10). In this study, we used tetramers to track the response of T cells specific for individual epitopes and used adoptive transfers and generation of partial hematopoietic chimeras in infected mice (4, 6, 7) to track the response of naive and memory CD8 T cells during quiescent latency and during viral reactivation. We found that only naive CD8 T cells specific for ORF61524Kb were recruited during the initial acute infection, but they were not recruited into the ongoing immune response by presentation of Ags during reactivation. There was also a functional difference of Ag stimulation after primary infection or viral reactivation in that the ability to generate dual IFN-γ/TNF-α cytokines was impaired after stimulation of naive CD8 T cells by reactivating virus. Taken together, our data show that γHV68-specific CD8 T cell memory is differentially maintained during latent infection in an epitope-specific manner, there is little stimulation of new T cells during quiescent latency, and viral reactivation from latency stimulates naive CD8 T cells specific for some, but not all, epitopes. Furthermore, this epitope specificity

Trudeau Institute, Saranac Lake, NY 12983
Received for publication September 27, 2011. Accepted for publication February 8, 2012.

This work was supported by the National Institutes of Health (Grants AI042927, AI082919, and CA148250 to M.A.B.; Grant T32 AI049823 to D.L.W.; Grant F32 AI084327 to M.L.F.) and the Trudeau Institute.

Address correspondence and reprint requests to Dr. Marcia A. Blackman, Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983. E-mail address: mblackman@trudeauinstitute.org

Abbreviations used in this article: AC-RTA, β-actin chicken replication and transcription activator; B6, C57BL/6; gM, glycoprotein M; γHV68, γ-herpesvirus-68; KSHV, Kaposi’s sarcoma-associated herpesvirus; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102787

The Journal of Immunology
Material and Methods

Mice and viruses

Male or female 8- to 12 wk-old C57BL/6 (B6; CD45.2+Thy1.2+), B6.SIL-Ptp/rPep/cBoy (CD45.1+Thy1.2+), and B6.PL-Thy1+Cy (CD45.2+Thy1.1+) mice were obtained from the Trudeau Institute animal facility and maintained under specific pathogen-free conditions. Mice were anesthetized with 2,2,2-trichloroethanol and infected intranasally with 400 PFU of wild-type (WT) γHV68 (strain WUMS) or latency-deficient β-actin chicken replication and transcription activator (AC-RTA) (11). All experiments were approved by the Trudeau Institute Institutional Animal Care and Use Committee.

Tetramers and flow cytometry

Allophyocyanin-conjugated MHC class I-restricted tetramers specific for γHV68 epitopes ORF6487-495Db (AGPHNDME), ORF8504-612Ksy (KNY1- FEELK), ORF39167-174K (LVLYFYRPI), ORF4548-495K (TNYKFSLY), ORF54253-260K (AVQVFIRV), ORF61524-531K (TSINFVKV), ORF75276-284K (SAIENYETF), and ORF75404-410K (KSLTYYKL) were obtained from the Trudeau Institute Molecular Biology Core Facility. Cells were treated with Fc block (BD Biosciences) and then stained with tetramers for 1 h at room temperature. For intracellular cytokine staining, cells were incubated with congenic splenocytes, 10 μg/ml of the relevant peptide, and brefeldin A (Epicentre Biotechnologies) for 5 h at 37˚C, then washed, labeled, and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer’s instructions. Fluorochrome-conjugated Abs against CD8, CD19, CD44, CD45.1, CD45.2, CD62L, IFN-γ, Thy1.1, Thy1.2, and TNF-α were purchased from BioLegend, BD Biosciences, or eBioscience as needed. Samples were collected on a BD FACSCanto II cytometer and analyzed by FlowJo software (TreeStar).

Generation of partial hematopoietic chimeras

WT γHV68- or AC-RTA–infected CD45.1+Thy1.2+ mice 2 mo p.i. received 0.6 mg busulfan i.p. (Busulfex; Otsuka America Pharmaceuticals). One day later, 2×107 cells isolated from the bone marrow of naive CD45.2+Thy1.1+ mice were injected i.v. recipient B cells was previously shown to be 63.3 ± 6.585, in CD4 T cells, 43.38 ± 5.654, and in CD8 T cells, 33.06 ± 5.331 (3). Engraftment was in this range for the current studies.

Anti-Thy1.2 mAb treatment

Mice were treated with 0.25 mg mAb to Thy1.2 (clone 30H12; BioXcell), administered i.p. every 2–3 d for 12 d. T cell depletion efficiency was assessed by flow cytometry.

Quantitative real-time PCR

Host (CD45.1+) and donor (CD45.2+) CD19+ B cells were sorted via flow cytometry from spleens of WT γHV68-infected partial mixed bone marrow chimeras by CD45 marker expression. DNA was isolated from the purified B cell populations and quantitative real-time PCR for γHV68 ORF50 gene copy number was performed as described previously (12).

Adoptive transfers

To measure memory recall responses to reactivation, CD44hi CD8 T cells were positively sorted by flow cytometry from spleens of WT γHV68-infected CD45.2+Thy1.1+ mice 2 mo p.i., and 1×107 cells were transferred i.v. into WT γHV68- or AC-RTA–infected B6 mice.Recipient mice were then injected i.p. with PBS or anti-Thy1.2 mAb. Spleens were harvested 12 d after transfer and analyzed for donor tetramer-positive cells by flow cytometry. To measure naive T cell responses to reactivation, we sorted CD44hi CD8 T cells by flow cytometry from spleens of naive CD45.2+Thy1.1+ mice 2 mo p.i., and 2×106 cells were transferred i.v. into WT γHV68- or AC-RTA–infected B6 mice. Recipient mice were then injected i.p. with anti-Thy1.2 mAb. Spleens were harvested 12 d after transfer and analyzed for donor tetramer-positive cells by flow cytometry.

Statistical analysis

Data were analyzed for normality using the D’Agostino and Pearson omnibus normality test and compared using Student’s t test or one-way ANOVA with Bonferroni’s post test where appropriate. All analyses were performed using Prism 5 software (GraphPad). The p values ≤0.05 were considered statistically significant.

Results

Epitope-specific stimulation of naive T cells during γHV68 latency

To determine whether naive CD8 T cells could be recruited into an ongoing antiviral immune response during latent γHV68 infection, we treated mice with busulfan and then transferred congenic bone marrow to establish partial hematopoietic chimeras in latently infected mice (Fig. 1A). This technique has been used to demonstrate that new naive T cells can respond to persistent viral infections (4, 7). We have recently shown that busulfan treatment of γHV68-infected mice does not affect the latent viral load or impair the pre-existing humoral and cellular immunity (3). We allowed latently infected mice to reconstitute for up to 28 wk, then measured the phenotype of the host and donor CD8 T cells (Fig. 1B). The donor CD8 T cell population was less activated than the host T cells, consistent with the donor cells being mostly naive, despite existing in the presence of a latent viral infection. Using MHC class I-restricted tetramers for two well-characterized epitopes ORF6487-495Db and ORF61524-531K, we demonstrated that donor-derived CD8 T cells specific for ORF61524K, but not ORF6487Db, could be detected in the chimeric mice 28 wk after reconstitution (Fig. 1C). These data demonstrate that new naive T cells can contribute to an ongoing immune response during γHV68 latency, and interestingly, the level of contribution varies depending on the epitope. Using the detection of tetramer-positive cells shown in Fig. 1D, we measured the specific response of host (Fig. 1E, left) and donor (Fig. 1E, right) CD8 T cell populations specific for eight epitopes. Only the immunodominant ORF61524K-specific naive T cells expanded in the latently infected mice.

Epitope-specific stimulation of naive T cells during viral reactivation

In our recent report, we demonstrated that depletion of the host T cell response by anti-Thy1.2 mAb injections led to substantial reactivation of latent virus, leading to enhanced infection of the donor B cells (3). It is unknown, however, whether viral reactivation also stimulates the recruitment of naive T cells into the ongoing response. To test this, we treated latently infected mice that had been treated with busulfan and reconstituted ~6 wk previously with anti-Thy1.2 mAb injections over a period of 12 d (Fig. 2A). We chose to treat mice at 6 wk after reconstitution because there is not yet recruitment of naive donor-derived ORF61524K-specific CD8 T cells at that time. Anti-Thy1.2 mAb treatment profoundly depleted the host T cells but not the Thy1.1+ donor T cells (Fig. 2B). In addition, it led to reactivation, evidenced by the acquisition of viral genomes in the donor B cells (Fig. 2C). Anti-Thy1.2 mAb treatment led to the stimulation and expansion of several epitope-specific donor CD8 T cell populations, although some specificities were not expanded significantly (Fig. 2D, 2E).

Depleting the majority of host T cells from the chimeric animals can lead to lymphopenia and drive Ag-independent proliferation of the donor T cells (13, 14). To assess whether this occurred, we compared CD44 expression on donor CD8 T cells after anti-Thy1.2 mAb treatment in chimeric mice that were latently infected with WT γHV68 or had been infected with AC-RTA, a recombinant γHV68 that causes acute infection but is unable to establish latency (10, 11). A similar proportion of donor CD8 T cells increased expression of CD44 regardless of the presence of viral reactivation (Fig. 2F), consistent with conversion of cells to
a transient memory-like phenotype during lymphopenia (13–17). Notably, however, lymphopenia alone did not elicit the expansion of tetramer-positive donor T cells, as assessed after depletion of host CD8 T cells in mice infected with AC-RTA virus compared with WT virus (Fig. 2D, 2E). Thus, viral reactivation from latency differentially stimulates donor CD8 T cell responses in an Ag-driven, epitope-specific manner, which can be specifically detected above a background of lymphopenia-induced stimulation.

Expression of CD8 T cell epitopes during viral reactivation

Interestingly, ORF39167Kb-specific T cells did not seem to respond at all to anti-Thy1.2 treatment. This was surprising, because the ORF39167 epitope is one of the strongest stimulators of IFN-γ production, as measured by ELISpot assay 12 d after γHV68 infection (10). The ORF39 gene encodes the viral glycoprotein M (gM), which forms a complex with viral gN protein and is essential for viral lytic replication (18, 19). Whether gM is required for virus reactivation from latency is unknown, and as such, gM might not be expressed (or expressed at a very low level) during reactivation. Because memory CD8 T cells are more sensitive to cognate Ag expression than naive cells because of their reduced requirement for costimulation (20–22), we used memory T cells to determine whether the ORF39167Kb epitope was being expressed during reactivation by transferring congenic (Thy1.1+) memory CD8 T cells to latently infected mice coupled with depletion of the host T cells by anti-Thy1.2 mAb treatment (Fig. 3A). Before transfer, ORF39167Kb-specific cells represented a small but detectable proportion of the donor T cell pool (Fig. 3B). After transfer, ORF39167Kb-specific cells expanded in WT γHV68-infected anti-Thy1.2 mAb-treated mice 19.3 ± 6.36-fold over latency-deficient, AC-RTA–infected anti-Thy1.2 mAb-treated controls, indicating the ORF39167Kb Ag is expressed during virus reactivation. Indeed, all eight tetramer-specific populations we measured significantly expanded after transfer of memory CD8 T cells and anti-Thy1.2 mAb-induced viral reactivation (Fig. 3C), demonstrating that all the epitopes were expressed during reactivation.

Viral reactivation can stimulate naive epitope-specific CD8 T cell responses

Given that all the epitopes, including ORF39167Kb, were expressed during reactivation, it is interesting that reactivation did not elicit
an ORF39 167Kb-specific response in the anti-Thy1.2 mAb-treated partial bone marrow chimeras. This result could be because of several reasons, including a fundamental difference in the ability of naive cells that mature during latency to mount an antiviral T cell response or Ag presentation during reactivation being insufficient to stimulate naive CD8 T cells. As mentioned earlier, memory CD8 T cells have a lower threshold for stimulation by cognate Ag expression than naive cells (20–22). Therefore, even though virus reactivation induces expression of each epitope enough to stimulate memory T cells after transfer (Fig. 3C), naive cells might not expand in response to reactivation. To determine whether viral reactivation could elicit the priming and expansion of naive ORF39 167Kb-specific cells, we transferred 2 × 10^6 Thy1.2 mAb (0.25 mg) treated mice into Thy1.2 + mice that were infected with either WT γHV68 or AC-RTA 2 mo previously, then depleted the recipient mice of T cells by anti-Thy1.2 mAb treatment (Fig. 4A). We chose to transfer this number of cells because it resulted in robust epitope-specific T cell responses in an in vivo limiting dilution analysis assay, and it is the roughly the number of donor-derived CD8 T cells we recover from the spleens of partial mixed bone marrow chimeras (data not shown). Notably, even at this number of transferred cells, it appears some epitope-specific responses were not stimulated by viral reactivation, which may reflect precursor frequencies, 0.5 × 10^-6 or a lack of Ag presentation; however, naive cells specific for ORF39 167Kb were significantly stimulated by anti-Thy1.2 mAb treatment in WT γHV68- or AC-RTA–infected chimeras and analyzed by flow cytometry. (D) Representative dot plots show tetramer staining. (E) Combined data from two to six experiments showing the percentage of Thy1.1+ donor CD8 T cells that bind indicated tetramers under given infection and treatment conditions. (F) Representative histograms show expression of CD44 on donor CD8 T cells. n = 4–19/group; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA with Bonferroni’s post test.
We next sought to determine whether CD8 T cell responses induced by viral reactivation were functionally similar to responses primed during acute lytic infection. Considering there is no ORF39 167Kb-specific response to measure after anti-Thy1.2 mAb-induced reactivation, we focused our efforts on the two responses that are codominant early in infection, ORF6 487Db and ORF61 524Kb (23). The quality of CD8 T cell priming is highly dependent on the amount of Ag, the duration of Ag presentation, the expression of costimulatory molecules on the presenting APC, and the inflammatory milieu (24). Thus, it is likely that priming of new naive T cells during γHV68 reactivation would differ substantially from priming during acute infection, with considerably less costimulation and inflammation during reactivation. Such differences could result in functional differences of the reactivation-primed cells compared with their lytic infection-primed counterparts (4, 6, 7, 25–28).

To address this, we assayed the abilities of lytic infection-primed T cells and donor-derived T cells primed during anti-Thy1.2 mAb-induced reactivation (Fig. 5A) to produce the antiviral cytokines IFN-γ and TNF-α in response to cognate Ag presentation. Although the different priming conditions resulted in similar frequencies of ORF6 487Db- and ORF61 524Kb-specific cells (Fig. 5B), the frequency of cells that coexpressed IFN-γ and TNF-α...
α was significantly reduced for the reactivation-primed responses compared with responses 12 d after primary infection (Fig. 5B, 5C). These data suggest that the CD8 T cell response generated by reactivating virus is functionally impaired compared with the response generated during acute infection.

**Discussion**

The regulation of T cell responses during persistent infections may vary depending on several factors, including the quality of T cell priming, the anatomical sites of viral persistence, the inflammatory microenvironment, and the timing and duration of cognate Ag presentation (29–34). It is unclear how the antiviral T cell response is maintained long term during persistent γ-herpesvirus infections. Using the mouse γHV68 infection model, it has been shown that virus-specific CD8 T cells proliferate rapidly during latency, yet the majority of antiviral CD8 T cells express markers of terminal differentiation and replicative senescence (5, 10, 35–37). It is important to determine how antiviral memory T cell is maintained long term to design rational therapeutic vaccine strategies that target epitope-specific CD8 T cell responses. The goals of these studies were to identify whether naive virus-specific CD8 T cells could contribute to an ongoing immune response in latently infected animals and whether reactivation of virus from latency could stimulate naive CD8 T cells to enter the response.

In mice that are persistently infected with polyoma virus or lymphochoriomeningitis virus, naive T cells were shown to be recruited into the ongoing antiviral immune response (6, 7). However, the extent of recruitment of naive T cells to the maintenance of these T cell responses is unclear (7, 8, 38). During latent murine CMV infection, optimal maintenance of memory T cell responses was shown to require both the recruitment of naive T cells and the proliferation and differentiation of a population of cells primed early in infection (4). We have recently demonstrated that virus-specific naive CD4 T cells can enter the immune response during γHV68 latency (39). However, another recent report has suggested that the CD8 T cell response to γHV68 is maintained mainly by the continuous turnover of activated T cells that were primed during acute infection (5). Importantly, that report did not rule out the possibility that naive T cells could contribute to the ongoing antiviral T cell response and did not
examine epitope-specific T cell responses. In addition, it has been shown that naive CD8 T cells can respond to DCs from latently infected mice in vitro (40), suggesting that they might contribute to the ongoing immune response. In the studies presented in this article, we assessed whether naive CD8 T cells could enter the immune response during latent γHV68 infection in vivo. We observed a profound epitope-specific limitation in their recruitment; only cells specific for the immunodominant epitope ORF61L32K exhibited any appreciable recruitment into the antiviral T cell response during quiescent latency. This result supports the contention that Ag presentation drives recruitment of naive T cells, because it is likely that the ORF61L32K epitope is expressed substantially during latent infection given its immunodominance in the presence of latency and its loss of dominance in the absence of latency (10, 36).

Anti-γHV68 immunity is long-lasting and highly functional, yet latent infection is maintained for life. This is due, in part, to the ability of the virus to continually spread to naive B cells throughout infection, even in the presence of immunity (3). Should the immune system become compromised, however, γHV68 can reactivate from latency leading to recrudescence disease, morbidity, and even mortality (1, 3, 41). Whether the immune system can alleviate the detrimental effects of viral reactivation by generating a new T cell response to the reactivating virus is unknown. In this study, we induced reactivation by depleting host T cells in latently infected partial hematopoietic chimeric mice (3) and assessed whether reactivating virus could stimulate donor-derived naive T cells to expand. We observed significant expansion of several epitope-specific responses after induction of viral reactivation following anti-Thy-1.2 mAb treatment. The hierarchy of expanded responses 12 d after T cell depletion did not directly compare with the response hierarchy 12 d after primary infection (9, 10), and anti-Thy-1.2 mAb treatment did not seem to induce a response to ORF39 L32K. Several factors that could influence the generation of T cell responses might be different between primary infection and anti-Thy-1.2 mAb-induced reactivation. These include differences in APC type, costimulatory molecule expression, inflammatory milieu, and the timing of Ag expression. We sought to address the question of Ag presentation by transferring memory and naive Thy1.1+ CD8 T cells into latently infected Thy1.2+ animals and inducing reactivation by anti-Thy-1.2 mAb treatment. Intriguingly, ORF39 L32K-specific memory CD8 T cells responded vigorously, consistent with expression of that epitope during reactivation. Somewhat surprisingly, naive ORF39 L32K-specific cells also responded to reactivation. Our data therefore suggest that ORF39 L32K Ag expression during virus reactivation is sufficient to stimulate both memory and naive CD8 T cell responses, yet is insufficient to stimulate responses when the cells have matured in a latently infected mouse. Why this might be the case is of considerable interest because it may inform future therapeutic vaccine strategies designed to elicit or improve CD8 T cell responses in latently infected animals. One possibility that is difficult to directly address experimentally is epitope-specific thymic tolerance, because the γ-herpesviruses are thought to induce the thymus. The finding, if true, that some epitopes but not others drive thymic tolerance could provide key insight into the pattern of epitope expression during latency.

Another important implication of our findings is that reactivation-induced T cell responses appear to be functionally impaired in their ability to produce cytokines. Specifically, there was a deficiency in the generation of cells secreting dual cytokines, IFN-γ and TNF-α. These data raise the intriguing possibility, currently under investigation, that successive reactivation may drive the generation of dysfunctional T cells, leading to progressive dampening of the overall antiviral immune response.

In conclusion, our findings identify a complex system in which virus-specific naive T cells are highly restricted in their ability to enter the response during latency. Under conditions of immunosuppression, viral reactivation drives the expansion of dysfunctional T cells. This expansion is limited to only CD8 T cells of certain epitope specificities, even though the other epitopes that do not drive the expansion of naive T cells are capable of being expressed and presented to both memory and naive cells during reactivation. Thus, CD8 T cells that are generated and mature during latent viral infection are deficient in their ability to recognize cognate Ag and develop into fully functional effector T cells.

Acknowledgments

We thank Dr. Jacob E. Kohlmeyer for critically reading the manuscript and Otsuka America Pharmaceuticals for the generous gift of Busulfex.

Disclosures

The authors have no financial conflicts of interest.

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