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## Gammaherpesvirus Persistence Alters Key CD8 T-Cell Memory Characteristics and Enhances Antiviral Protection

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**In herpesvirus infections, the virus persists for life but is contained through T-cell-mediated immune surveillance. How this immune surveillance operates is poorly understood. Recent studies of other persistent infections have indicated that virus persistence is associated with functional deficits in the CD8<sup>+</sup> T-cell response. To test whether this is the case in a herpesvirus infection, we used a mutant murine gammaherpesvirus that is defective in its ability to persist in the host. By comparing the immune response to this virus with a revertant virus that can persist, we were able to dissect the changes in the antiviral CD8<sup>+</sup> T-cell response that are induced by virus persistence. Surprisingly, persistently infected mice controlled a secondary challenge infection more rapidly than nonpersistently infected mice, indicating enhanced rather than diminished effector functions. Consistent with this, virus-specific CD8 T cells from these mice exhibited faster upregulation of the cytotoxic mediator granzyme B. Another unexpected finding was that CD8<sup>+</sup> T cells from neither infection responded efficiently to homeostatic cytokines. The unresponsiveness of the memory cells from the nonpersistently infected mice appears to be linked to the prolonged replication of virus within the lungs. Other changes seen in different chronic infection models were also observed, such as changes in Bcl-2 levels, interleukin-2 production, and the immunodominance hierarchy. These data show persistence of gammaherpesvirus type 68 alters the properties of CD8<sup>+</sup> T cells and illustrates that immune surveillance does not require CD8 T cells with the same attributes as “classical” memory CD8<sup>+</sup> T cells.**

Herpesviruses have a unique lifestyle in that they can very effectively evade the immune response and persist in the infected host indefinitely. The virus exists in a latent state undergoing sporadic, often subclinical reactivation, which releases infectious virions, allowing the virus to spread to new hosts. Clinical reactivation during alphaherpesvirus infections, such as herpes simplex virus and varicella-zoster virus, occurs spontaneously, whereas clinical reactivation is only seen in the context of immune suppression during beta- and gammaherpesvirus infections, such as cytomegalovirus and Epstein-Barr virus. This illustrates that immune surveillance is very effective for the latter viruses. However, the exact nature of this immune surveillance is poorly understood.

Immunosurveillance during persistent viral infection is thought to be mediated largely by T cells. However, most of our knowledge about memory T cells is from acute virus infections. These studies have shown that memory CD8 T cells can be divided into two categories: effector memory cells (T<sub>EM</sub>) and central memory cells (T<sub>CM</sub>) (32, 41, 42, 56, 59). Effector memory cells are found predominantly in peripheral tissues and display immediate effector functions. In contrast, T<sub>CM</sub> cells are found predominantly in lymphoid tissue and lack immediate effector function but have a higher proliferative capacity and likely provide better longer-term immunity than T<sub>EM</sub> cells (59). By comparing the gene expression in naive, effector, and memory CD8 T cells, it appears that the full pattern of gene expression associated with memory cells is only

attained several weeks following the clearance of viral antigen (25). This includes up-regulation of antiapoptotic genes, such as Bcl-2, genes for cytokines such as interleukin-2 (IL-2), and up-regulation of the receptors for cytokines that enhance memory cell survival, such as IL-15 or IL-7. Responsiveness to these homeostatic cytokines is believed to be critical for memory cell survival, and expression of IL-7 receptor  $\alpha$  (IL-7R $\alpha$ ) specifically marks T cells that are destined to become memory cells (23, 26). Responsiveness to IL-15 has been shown to be critical for long-term memory cell survival following acute viral infection (6, 9, 18, 24, 44, 49).

The functional consequences of persistent exposure to viral antigens are poorly understood. From studies on human persistent infections, it is clear that the memory T-cell response does not fit easily into the central memory/effector memory T-cell model (1, 2). In the murine lymphocytic choriomeningitis virus (LCMV) model, a high dose of persistent virus results in exhaustion of the T-cell response, leading to various states of functional deficits or death of virus-specific CD8<sup>+</sup> T cells (17, 34, 35, 50, 58). This is particularly marked in the absence of CD4<sup>+</sup> T-cell help (62). Functional exhaustion does not appear to be a feature of the T-cell response to herpesviruses, as the antiviral response is vigorous and apparently fully functional. However, little is known about what, if any, impact herpesvirus persistence has upon the memory CD8<sup>+</sup> T-cell response. To date, the data from chronic virus infections in mice and humans has been difficult to interpret. This is because it is not clear whether the effects seen relate to the peculiarities of T-cell priming inherent to a particular virus infection or whether the effects are due to persistence of the virus. In this report, we examine this issue in detail using a recently de-

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scribed mutant gammaherpesvirus that induces a strong immune response but fails to persist in the host (16, 33). By comparing this virus with a revertant strain that is competent to persist, we show that viral persistence has a marked impact on the properties of the CD8<sup>+</sup> T-cell response. However, the ability of these cells to protect against virus infection is actually enhanced by viral persistence.

## MATERIALS AND METHODS

**Mice and virus.** C57BL/6 and BALB/c mice were purchased from the National Cancer Institute (Bethesda, MD). A recombinant gammaherpesvirus type 68 ( $\gamma$ HV-68) strain containing a frameshift mutation in ORF73 (FS73) and the revertant virus (FS73R) were obtained from Stacey Efsthathiou (University of Cambridge, United Kingdom) (16). A recombinant vaccinia virus expressing the ORF61<sub>524-531</sub>/K<sup>b</sup> epitope (rVV-p79) of  $\gamma$ HV-68 was obtained from Peter Doherty (St. Jude Children's Research Hospital, Tennessee) (8). For  $\gamma$ HV-68 infections, mice were infected intranasally (i.n.) with 400 PFU of FS73 or FS73R under anesthesia with 2,2,2-tribromoethanol. For vaccinia virus challenge, mice were given  $1.5 \times 10^6$  PFU of rVV-p79 i.n. The Animal Care and Use Program of Dartmouth College approved all animal experiments.

**Tissue preparation.** Single-cell suspensions of the spleen, lungs, and liver were prepared by passage through cell strainers. Lung and liver suspensions were resuspended in 80% isotonic Percoll and subsequently overlaid with 40% isotonic Percoll. Samples were then centrifuged at  $400 \times g$  for 25 min at 4°C. The cells at the 80%-40% interface were collected, washed, and counted. To obtain the bone marrow, thigh and shin bones were collected and the marrow was flushed from the bones. Peripheral blood was obtained through a tail bleed and collected into Hanks balanced salt solution containing 10 U/ml of heparin. Spleen cells, bone marrow, and peripheral blood were depleted of erythrocytes by treatment with buffered ammonium chloride, centrifuged, and counted.

**Viral plaque assay.** Infectious  $\gamma$ HV-68 virus titers in the lungs were determined by plaque assays as previously described (48). To measure vaccinia virus titers, ovaries were homogenized in 1 ml of medium and then subjected to three freeze-thaw cycles. Then the samples were serially diluted and assayed on monolayers of 143B cells in minimal volume. The virus was allowed to adsorb for 1 h before being overlaid with carboxymethyl cellulose. After 2 days of incubation at 37°C, the assay mixtures were fixed with a solution of 50% acetone–50% methanol for 15 min and stained with Giemsa stain for 4 h, and then the plaques were enumerated microscopically.

**Quantitative PCR for viral load and viral transcripts.** Latent viral DNA was quantified by quantitative fluorescent (QF)-PCR as previously described (54). To analyze M2 gene expression, total RNA was isolated from spleen or lung tissue using TRIzol (Invitrogen), followed by a secondary purification using the RNeasy mini kit (QIAGEN, Valencia, CA) and utilizing on-column DNA digestion to get rid of residual DNA. After column purification, 1 to 2  $\mu$ g of RNA was reverse transcribed to cDNA using Omniscript RT (QIAGEN) with 100  $\mu$ g/ml of oligo(dT) primer. QF-PCR was performed on 2.5  $\mu$ l of the cDNA mixture using Platinum Quantitative PCR SuperMix-UDG, 400 nM primers, and 100 nM FAM/Black Hole Quencher-1-labeled probes for either murine  $\beta$ -actin or M2. Primers and probes were as follows: murine  $\beta$ -actin 3' primer, CAATAGTGA TGACCTGGCCGT; murine  $\beta$ -actin 5' primer, AGAGGGAAATCGTGCGT GAC; murine  $\beta$ -actin probe, TGCAATCTGGCTCAACGCCCG; M2 3' primer, TGTGGTTCGGGAGTTTAGG; M2 5' primer, CGAGGTCTTAATGATG TTGG; M2 probe, TCTGGCTCGACTGACAGTCCAGAAA. The samples were subjected to 40 cycles of 15 s at 95°C and 1 min at 60°C. QF-PCR was performed using a Bio-Rad iCycler.

**Flow cytometry analysis.** All surface and intracellular staining was performed as previously described (37). For granzyme B staining, tetramer staining was performed, and cells were fixed with 1% formaldehyde and permeabilized with 0.5% saponin. Cells were then stained with anti-human granzyme B (GB11; Caltag, Burlingame, CA), washed, and then analyzed on a FACSCalibur. All tetramers were made by the NIH Tetramer Core Facility (Emory University, Atlanta, GA).

**Intracellular staining for IFN- $\gamma$  and IL-2.** Cells were incubated with 1  $\mu$ g/ml of the appropriate peptide plus 10 U/ml IL-2 and 10  $\mu$ g/ml brefeldin A (BFA) in complete medium at 37°C for 5 h. Cells were stained with anti-CD8 antibody and then fixed and rendered permeable before staining with antibodies to gamma interferon (IFN- $\gamma$ ) and/or IL-2 as described previously (53). Analysis was performed on a FACSCalibur flow cytometer using CellQuest software. For restimulation of vaccinia virus-specific CD8<sup>+</sup> T cells, we used the DC2.4 dendritic

cell line infected in vitro with vaccinia virus, as previously described (52). The DC2.4 dendritic cell line was obtained from the Dana Farber Cancer Institute.

**Measuring responsiveness to cytokines in vitro.** CD8<sup>+</sup> T cells were purified using the EasySep murine CD8 $\alpha$ -positive selection kit (StemCell Technologies, Vancouver, BC, Canada) to >95% CD8<sup>+</sup>. Cells were then labeled with 0.5  $\mu$ M carboxyfluorescein (diacetate) succinimidyl ester (CFSE) for 10 min at room temperature and then extensively washed. Cells were then cultured in 96-well plates at  $5 \times 10^6$  cells/ml in the presence or absence of 500 U/ml IL-2 (Tecin; National Cancer Institute), 125 ng/ml IL-7 (Peprotech, Rocky Hill, NJ), 125 ng/ml IL-15 (Peprotech), or 50 ng/ml IL-21 (R&D Systems, Minneapolis, MN) for 4 days at 37°C. Antigen-specific cells were detected by intracellular cytokine staining for IFN- $\gamma$ , and CFSE dilution was monitored using a FACSCalibur flow cytometer. The precursor frequency of the cells responding to the cytokines was determined using the ModFit software.

**Treatment of mice with antiserum and cidofovir.** Immune serum was collected from C57BL/6 mice which had been infected with  $\gamma$ HV-68 for >21 days postinfection. To shorten the infectious period of the FS73 infection, mice were treated with 150  $\mu$ l of  $\gamma$ HV-68 immune serum intraperitoneally (i.p.) on day 6 postinfection. This was followed by treatment with 25 mg of cidofovir/kg of body weight given subcutaneously as previously described (14). Cidofovir was administered every other day from day 7 until day 28 postinfection.

**Statistical analysis.** All data were analyzed using Student's *t* tests, and a *P* value of less than 0.05 was considered significant. Viral titer data were log transformed before performing Student's *t* test, as recommended by Richardson and Overbaugh (40).

## RESULTS

**In vivo analysis of the growth and establishment latency by the FS73 strain of  $\gamma$ HV-68.** Previous work has shown that recombinant  $\gamma$ HV-68 viruses engineered to be deficient in the ORF73 gene were unable to establish latency, although these viruses replicated normally during the acute phase of the virus infection (16, 33). By comparing this mutant with a revertant virus in which the ORF73 gene was repaired, and therefore could persist in the host, we could test whether herpesvirus persistence influences the antiviral immune response. We obtained a strain of  $\gamma$ HV-68 containing a frameshift mutation in the ORF73 gene (FS73) along with a genetic revertant containing the wild-type ORF73 gene (FS73R) (16). To ensure these viruses grew similarly in our hands, we infected C57BL/6 mice i.n. with 400 PFU of either FS73 or FS73R and monitored the viral titers within the lungs at days 7 and 14 postinfection. We found that both FS73 and FS73R grew to similar titers at day 7 postinfection, and both were markedly reduced by day 14 postinfection (Fig. 1A). Next, we sought to determine whether the FS73 virus was able to establish latency. To do this, C57BL/6 mice were infected with either of the two viruses, and the number of viral genomes was quantified within the spleen using QF-PCR for the ORF50 gene of  $\gamma$ HV-68. We found that, at day 14 postinfection, only a few animals had detectable levels of virus following FS73 infection and, at later time points, no virus was detectable following FS73 infection (Fig. 1B). This contrasts with the FS73R infection, where virus was reliably detected at all time points. Similarly, we could detect no persistence of FS73 in the lungs at later time points (data not shown). Thus, consistent with published reports, we confirmed that the FS73 mutant virus replicates as well as the control virus but fails to persist in mice.

As the FS73 mutant fails to persist, we wished to test if certain latency-associated events were altered in infected mice. During the establishment of latency, mice develop an infectious mononucleosis-like syndrome marked by a transient splenomegaly and a dramatic expansion of the V $\beta$ 4<sup>+</sup> CD8<sup>+</sup>

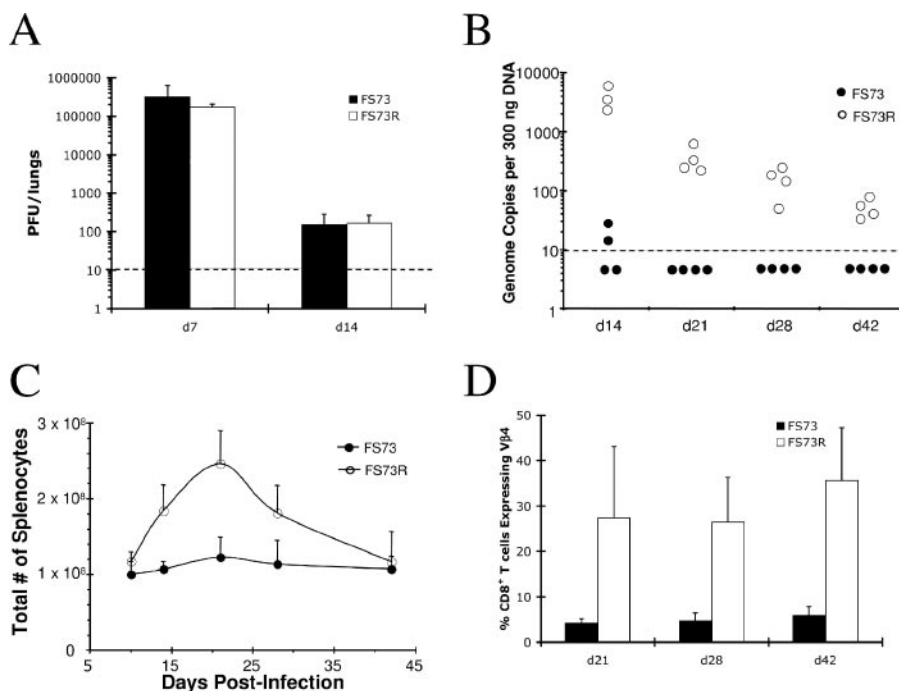


FIG. 1. FS73 and FS73R grow similarly during lytic replication but fail to establish long-term latency. C57BL/6 mice were infected i.n. with 400 PFU of either the FS73 or FS73R strain of  $\gamma$ HV-68. (A) Growth of the viruses during the lytic phase of replication was measured in the lungs at days 7 and 14 postinfection using a standard plaque assay. (B) The establishment and maintenance of latency within the spleens was measured by QF-PCR for the ORF50 gene at days 14, 21, 28, and 42 postinfection. In addition, spleen cellularity (C) and expansion of the CD8<sup>+</sup> Vβ4<sup>+</sup> T-cell population (D) were measured at the indicated times points after infection, as these events are associated with the establishment of latency. Panels A, C, and D show the average values for 4 mice per group, with the error bars each indicating one standard deviation. In panel B, each dot represents an individual mouse, with 3 to 4 mice per group. In all graphs, open symbols represent mice infected with FS73R, and filled symbols indicate mice infected with FS73. Data are representative of results from at least two experiments.

T-cell population (48, 51). To test if these events were attenuated in mice infected with the nonpersistent virus, C57BL/6 mice were infected with either the FS73 or the FS73R strain of  $\gamma$ HV-68. At the times indicated, the number of spleen cells was determined and the expansion of the Vβ4<sup>+</sup> CD8<sup>+</sup> T-cell population was monitored within the blood, where this expansion is most dramatic (51). Mice infected with the FS73R strain of  $\gamma$ HV-68 displayed a transient splenomegaly (Fig. 1C) and expansion of the Vβ4<sup>+</sup> CD8<sup>+</sup> T-cell population (Fig. 1D). In contrast, infection with the FS73 strain of  $\gamma$ HV-68 resulted in neither splenomegaly nor expansion of the Vβ4<sup>+</sup> CD8<sup>+</sup> T-cell population. Therefore, lymphocytosis normally associated with persistent  $\gamma$ HV-68 infection was absent in mice infected with the nonpersistent FS73 virus. Taken together with the virological data, these data indicate that the FS73 mutant of  $\gamma$ HV-68 is unable to establish latency effectively and persist long-term in the host, making this strain an effective tool to dissect the effects of viral persistence on CD8<sup>+</sup> T-cell function.

**CD8<sup>+</sup> T-cell expansion and memory during FS73 and FS73R infection.** Since little is known about the effects of herpesvirus persistence on the size of the CD8<sup>+</sup> T-cell response, we next monitored the CD8<sup>+</sup> T-cell response elicited by both the nonpersistent FS73 strain and the persistent FS73R strain of  $\gamma$ HV-68 over the first 90 days postinfection. In C57BL/6 mice, two major epitopes have been defined within lytic proteins, ORF6<sub>487-495</sub>/D<sup>b</sup> and ORF61<sub>524-531</sub>/K<sup>b</sup> (47). In these experiments, C57BL/6 mice were infected i.n., and the T-

cell response against the ORF6<sub>487-495</sub>/D<sup>b</sup> and ORF61<sub>524-531</sub>/K<sup>b</sup> epitopes was monitored using major histocompatibility complex peptide tetramers. Examining the ORF61<sub>524-531</sub>/K<sup>b</sup> response, we found that both the FS73 and FS73R viruses induced CD8<sup>+</sup> T-cell responses of similar frequency throughout the primary response and into the memory phase (Fig. 2A), although the total number of ORF61<sub>524-531</sub>/K<sup>b</sup>-specific cells was decreased at 21 and 28 days after infection due to the lack of splenomegaly in FS73-infected mice (Fig. 2C). The CD8<sup>+</sup> T-cell response against the ORF6<sub>487-495</sub>/D<sup>b</sup> epitope was similar for both viruses on days 10 and 14 postinfection. However, on day 21 postinfection, the response induced by the persistent and nonpersistent strains began to diverge. Whereas the CD8<sup>+</sup> T-cell response induced by the nonpersistent FS73 virus continued to expand until day 21, the response induced by the revertant virus began to contract (Fig. 2B and D). Therefore, infection with the nonpersistent FS73 virus resulted in ORF6<sub>487-495</sub>/D<sup>b</sup> and ORF61<sub>524-531</sub>/K<sup>b</sup> being codominant, whereas after infection with the revertant virus, the ORF61<sub>524-531</sub>/K<sup>b</sup> response was strongly dominant over that of ORF6<sub>487-495</sub>/D<sup>b</sup>.

Since memory CD8<sup>+</sup> T cells are known to be found in every organ of the body (21, 32), we also wanted to test if similar differences in immunodominance were seen in other organs. To do this, C57BL/6 mice were infected i.n. and left for >60 days. Then the peripheral blood, liver, lungs, and bone marrow were collected and analyzed by tetramer staining for both the



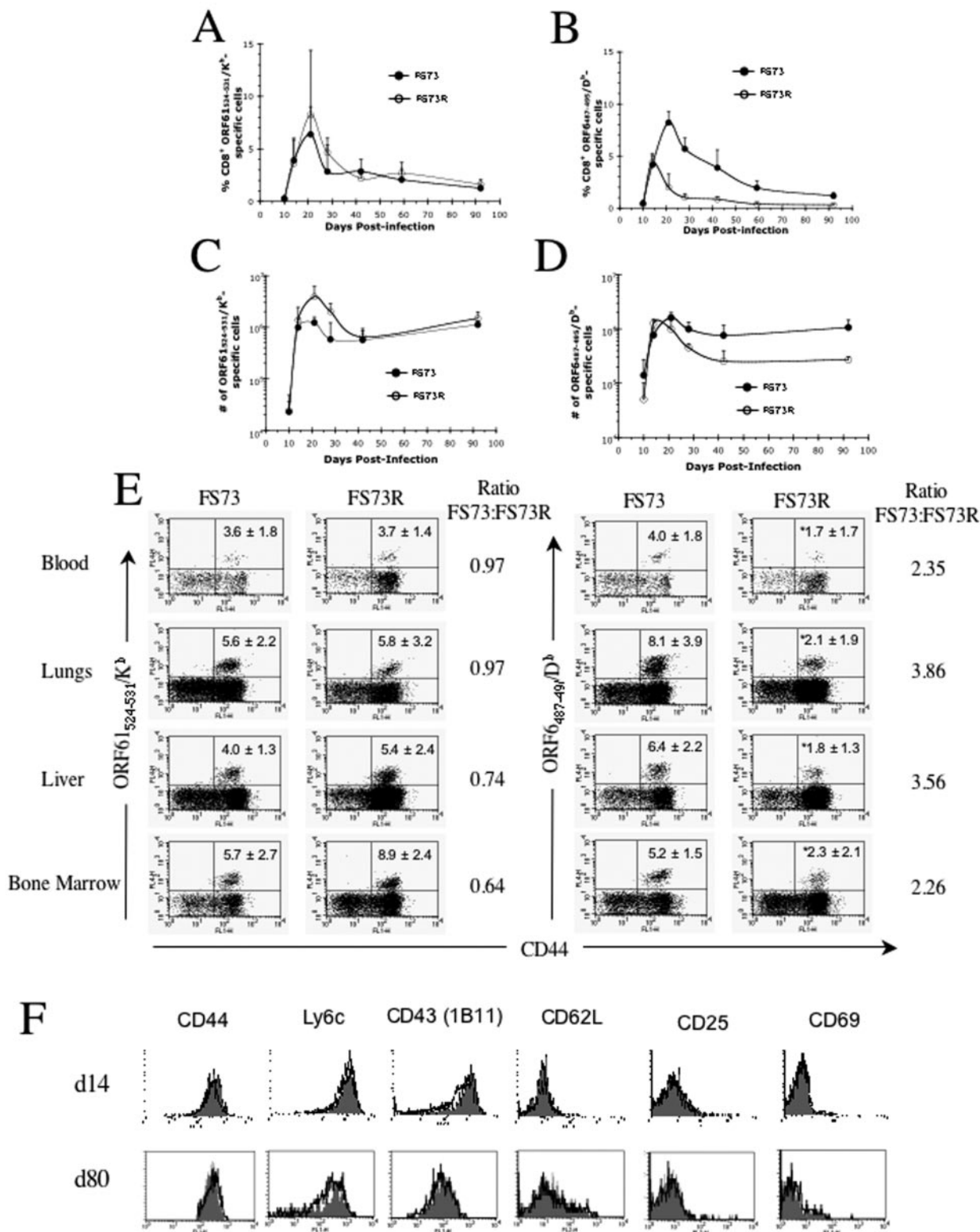


FIG. 2. Virus persistence alters the CD8<sup>+</sup> T-cell immunodominance hierarchy in C57BL/6 mice. C57BL/6 mice were infected i.n. with 400 PFU of either the nonpersistent FS73 or the persistent FS73R strain of  $\gamma$ HV-68 and then were analyzed by tetramer staining for either the ORF61<sub>524-531</sub>/K<sup>b</sup> or ORF6<sub>487-495</sub>/D<sup>b</sup> population plus anti-CD8a and antibodies to the appropriate activation markers. The kinetics of the immune response against the ORF61<sub>524-531</sub>/K<sup>d</sup> epitope (A, C) and the ORF6<sub>487-495</sub>/D<sup>d</sup> epitope (B, D) were monitored at the indicated times within the spleen. In panels A and B the data are presented as percentages of antigen-specific CD8<sup>+</sup> T cells, while panels C and D present the same data as the absolute numbers of antigen-specific CD8<sup>+</sup> T cells. Each symbol represents the average for 4 to 5 mice per group, with the error bars each

ORF6<sub>487-495</sub>/D<sup>b</sup> and ORF61<sub>524-531</sub>/K<sup>b</sup> epitopes. Similar to what was observed in the spleen, the CD8<sup>+</sup> T-cell immunodominance hierarchy was altered in all organs tested during persistent infection (Fig. 2E).

Since viral persistence could result in the continual activation of the CD8<sup>+</sup> T cells to effector cytotoxic T lymphocytes (CTLs), we examined the cell surface phenotype of CD8<sup>+</sup> T cells from the spleen to determine if the populations in the two infections were in different states of activation. We chose to stain with a panel of the most commonly used markers for identifying activated/memory CD8<sup>+</sup> T cells. At 14 days postinfection, antigen-specific CD8<sup>+</sup> T cells from both groups had the same phenotype, CD44<sup>hi</sup> CD62L<sup>lo</sup> CD43<sup>hi</sup> Ly6c<sup>hi</sup> (Fig. 2F), as was expected considering the initial replication of these viruses was the same during the acute infection. We did not observe upregulation of CD25 and CD69 at day 14, likely because these molecules are only transiently upregulated after T-cell activation. At later time points, the phenotype of the antigen-specific CD8<sup>+</sup> T cells induced by the nonpersistent and persistent  $\gamma$ HV-68 infections was identical. This was true at all time points tested during the persistent infection (31 to 120 days postinfection). The antigen-specific CD8<sup>+</sup> T cells were uniformly CD44<sup>+</sup> and Ly6c<sup>+</sup> but expressed lower levels of CD43 (1B11) compared with the day 14 response (Fig. 2F). Interestingly, the majority of the antigen-specific CD8<sup>+</sup> T cells remained CD62L<sup>lo</sup> following infection with the nonpersistent FS73, similar to what is seen with the persistent FS73R. Thus, infection of mice with the nonpersistent or persistent strain of  $\gamma$ HV-68 results in a robust CD8<sup>+</sup> T cell response that at later time points results in cells of similar phenotypes even though virus persists during FS73R infection.

**Viral persistence is not responsible for the phenotypic differences observed between lytic and latent antigen-specific CD8<sup>+</sup> T cells.** The effect of ORF73 deficiency on the expression of latency-associated genes and the corresponding immune response to the gene products is not known. The M2 gene has been shown to be associated with viral latency. It is expressed transiently around 14 days postinfection and contains an H-2K<sup>d</sup>-restricted CD8<sup>+</sup> T-cell epitope (22). Therefore, we sought to determine whether the M2 gene was still expressed following FS73 infection and whether the CD8<sup>+</sup> T-cell response to this protein was mounted effectively. These studies used the BALB/c (H-2<sup>d</sup>) mouse strain, as no CD8<sup>+</sup> T-cell responses to latent antigens have been identified in C57BL/6 mice. BALB/c mice were infected with either the nonpersistent FS73 or persistent FS73R virus, and viral gene expression was monitored at day 14 postinfection in the spleen, while the M2<sub>91-99</sub>/K<sup>d</sup>-specific CD8<sup>+</sup> T cell response was examined at days 18 and 188 postinfection in the spleen. We found that expression of the latency-associated M2 gene was decreased

approximately fivefold in the absence of ORF73 (Fig. 3A). An M2<sub>91-99</sub>/K<sup>d</sup>-specific CD8<sup>+</sup> T-cell response was present by day 18 postinfection in both groups, although it was greater in size in the FS73R group (Fig. 3B). By day 188 postinfection, the M2<sub>91-99</sub>/K<sup>d</sup>-specific CD8<sup>+</sup> T-cell population was of equivalent size in both groups.

Previously, we had reported that CD8<sup>+</sup> T cells specific for lytic antigens had a markedly different cell surface phenotype than those recognizing latent antigens. CD8<sup>+</sup> T cells recognizing the latency-associated M2 protein were predominantly CD62L<sup>hi</sup> or CD43<sup>hi</sup> (1B11), whereas those recognizing the lytic cycle antigen ORF65 expressed significantly lower levels of these markers (36). In that report, we hypothesized that the different cell surface phenotype was due to the persistence of the virus and differing levels of viral antigen seen by the responding cells during latency. As the FS73 strain of  $\gamma$ HV-68 is unable to establish latency, we were able to test this directly. Spleen cells from BALB/c mice infected with either the nonpersistent FS73 or persistent FS73R virus for >60 days were stained with either the M2<sub>91-99</sub>/K<sup>d</sup> or ORF65<sub>131-140</sub>/D<sup>d</sup> tetramer plus antibodies to CD8 and activation/memory markers. We found that viral persistence had no impact on the phenotype of the lytic or latent antigen-specific CD8<sup>+</sup> T cells at late time points (Fig. 3C), demonstrating that viral persistence was not responsible for these phenotypic differences.

**Memory CD8<sup>+</sup> T cells turn over faster during persistent viral infection.** To measure the proliferation of the antigen-specific CD8<sup>+</sup> T cells, C57BL/6 mice were infected with either the persistent FS73R or nonpersistent FS73 virus for >60 days, at which time they were treated with bromodeoxyuridine (BrdU) in their drinking water for 10 days. BrdU incorporation was then measured in virus-specific CD8 T cells, which were identified using tetramer staining. As shown in Fig. 4, approximately twice the proportion of antigen-specific CD8<sup>+</sup> T cells were BrdU<sup>+</sup> in the FS73R group compared with the FS73 group. This difference was observed with both the ORF6<sub>487-495</sub>/D<sup>b</sup> and ORF61<sub>524-531</sub>/K<sup>b</sup> epitopes. Therefore, the turnover of the antigen-specific CD8<sup>+</sup> T cells was significantly faster in the presence of persistent infection.

**Bcl-2 is expressed at lower levels during persistent  $\gamma$ HV-68 infection.** Studies examining the maturation process of memory CD8<sup>+</sup> T cells indicate that complete maturation requires the absence of antigen to gain a full memory phenotype (25, 59). One important characteristic of memory CD8<sup>+</sup> T cells is their high level of antiapoptotic molecules, especially Bcl-2 (19). To address whether viral persistence affects the Bcl-2 levels found in antigen-specific CD8<sup>+</sup> T cells during viral latency, spleen cells from C57BL/6 mice infected with either the FS73 or FS73R virus for >60 days were stained with anti-CD8 and the appropriate tetramer, followed by intracellular stain-

indicating one standard deviation. (E) ORF61<sub>524-531</sub>/K<sup>b</sup>- and ORF6<sub>487-495</sub>/D<sup>b</sup>-specific CD8<sup>+</sup> T cells were measured in a range of tissues at >60 days postinfection. Plots are gated on CD8<sup>+</sup> T cells, with the values indicating the average percentages of antigen-specific CD8<sup>+</sup> T cells for that group (4 mice per group)  $\pm$  1 standard deviation, while an asterisk (\*) indicates a statistically significant difference between the two groups ( $P < 0.05$ ). (F) The activation status of the antigen-specific CD8<sup>+</sup> T cells at late time points (>60 days) was determined following either persistent FS73R or nonpersistent FS73 infection. The filled gray histograms represent ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells from mice infected with the nonpersistent FS73 virus, whereas the solid black lines represent this population from mice infected with the persistent FS73R virus. Data are representative of results from at least two experiments.

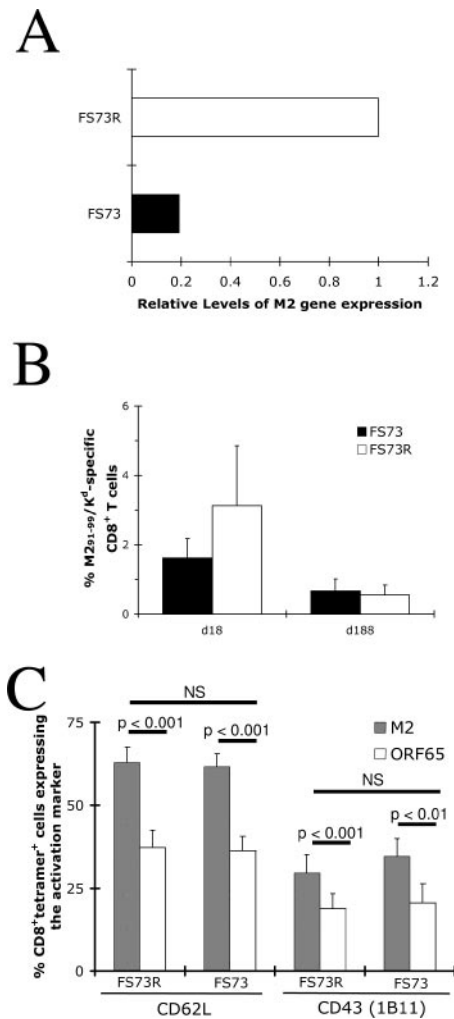


FIG. 3. Expression of a latency-associated gene is decreased, but the CD8<sup>+</sup> T cell response is induced and has a similar phenotype during long-term latency. BALB/c mice were infected i.n. with 400 PFU of either the nonpersistent FS73 or persistent FS73R strain of  $\gamma$ HV-68. (A) At 14 days postinfection, spleen samples were taken and total RNA was isolated as described in Materials and Methods. The level of M2 gene expression was determined by QF-PCR and was normalized to  $\beta$ -actin expression. Data are expressed as the mean relative expression of the group, with the expression level in the FS73R group being defined as 1.0 and the data ranges (lowest and highest values) were as follows: FS73, 0.11 to 0.34; FS73R, 0.14 to 6.82. (B) The M2<sub>91-99</sub>/K<sup>d</sup>-specific CD8<sup>+</sup> T-cell population was monitored during both expansion and long-term latency by tetramer staining. Data represent the average percentages of M2<sub>91-99</sub>/K<sup>d</sup>-specific CD8<sup>+</sup> T cells in the spleen, with the error bars each indicating one standard deviation. (C) The phenotype of lytic versus latent antigen-specific memory CD8<sup>+</sup> T cells was compared in BALB/c mice infected with either the FS73 or FS73R virus for >60 days. The graph shows the average percentages of M2- or ORF65-specific CD8<sup>+</sup> T cells that express either CD62L or CD43 (1B11), and the error bar indicates one standard deviation. Data are representative of results from at least two experiments.

ing to detect Bcl-2. We found that Bcl-2 levels in the ORF61<sub>524-531</sub>/K<sup>b</sup>-specific memory CD8<sup>+</sup> T cells during persistent FS73R infection were significantly lower than the levels found following nonpersistent FS73 infection (Fig. 5). Similar

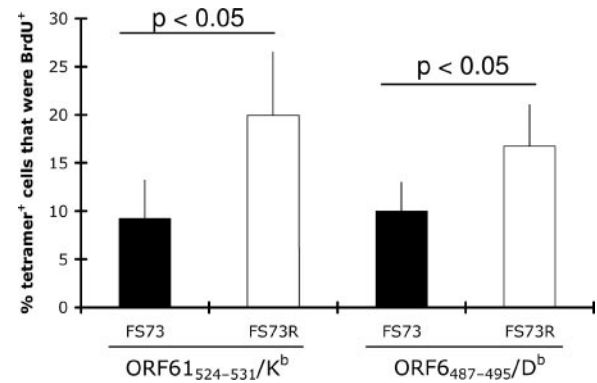


FIG. 4. In vivo turnover of virus-specific CD8<sup>+</sup> T cells is increased during FS73R infection. C57BL/6 mice that had been infected with either the persistent FS73R or nonpersistent FS73 virus for >60 days were treated with 0.8 mg/ml BrdU in their drinking water for 10 days. Spleen cells were then stained with either the ORF61<sub>524-531</sub>/K<sup>b</sup> or ORF6<sub>487-495</sub>/D<sup>b</sup> tetramer followed by anti-CD8a antibody. Cells were then fixed, rendered permeable, DNA digested, and stained with an anti-BrdU antibody. The graph represents the average percentage of antigen-specific CD8<sup>+</sup> T cells that have incorporated BrdU into their DNA. Each group contained 4 mice, and the error bars each indicate one standard deviation. Black bars represent mice infected with the nonpersistent FS73 virus, whereas white bars represent mice infected with the persistent FS73R virus. Data are representative of the results from two experiments.

results were also obtained when the ORF6<sub>487-495</sub>/D<sup>b</sup> epitope was examined (data not shown).

**Fewer antigen-specific CD8<sup>+</sup> T cells can produce IL-2 during persistent infection.** The ability to produce IL-2 is an important function of memory CD8 T cells, as this cytokine is required for rapid expansion following secondary exposure to

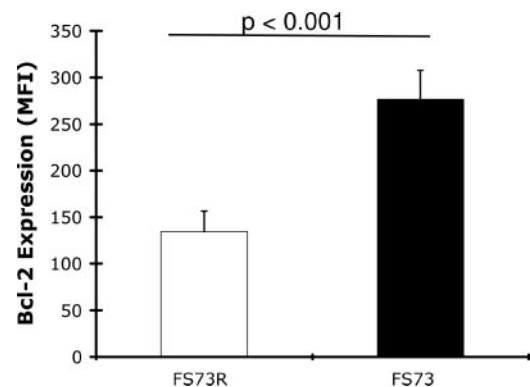


FIG. 5. Virus persistence results in antigen-specific CD8<sup>+</sup> T cells which fail to completely up-regulate Bcl-2. C57BL/6 mice were infected i.n. with 400 PFU of either the persistent FS73R or nonpersistent FS73 virus for >60 days. Spleen cells were stained with the ORF61<sub>524-531</sub>/K<sup>b</sup> tetramer plus anti-CD8a antibody. Cells were then fixed, rendered permeable, and stained with an anti-Bcl-2 antibody. Data are shown as the mean fluorescent intensities (MFI) for Bcl-2 within the ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells. The graph depicts the average for each group of 4 mice, with the error bars each indicating one standard deviation. The black bar represents mice infected with the nonpersistent FS73 virus, whereas the white bar represents mice infected with the persistent FS73R virus. Data are representative of results from two experiments, with similar data obtained for the ORF6<sub>487-495</sub>/D<sup>b</sup>-specific population.

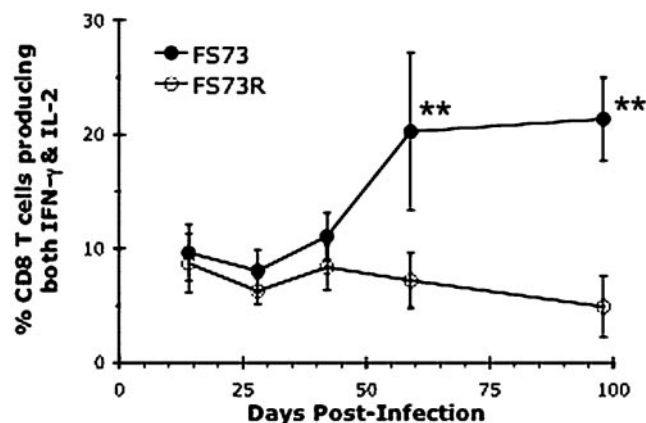


FIG. 6. IL-2 production by virus-specific CD8<sup>+</sup> T cells in the presence or absence of persistent virus. C57BL/6 mice were infected i.n. with 400 PFU of either persistent FS73R or nonpersistent FS73 virus for the indicated period of time. Spleen cells were then restimulated with ORF61<sub>524-531</sub> peptide for 5 h. The cells were then stained with an anti-CD8 $\gamma$  antibody, fixed, rendered permeable, and stained with anti-IFN- $\gamma$  and anti-IL-2 antibodies. Each symbol is the average for the group (4 mice per group) and represents the percentage of cells that produce IL-2 in addition to IFN- $\gamma$ . The open circles represent the persistent FS73R infection, whereas the filled circles represent the nonpersistent FS73 infection. Asterisks (\*\*) indicate a statistically significant difference between the groups ( $P < 0.05$ ). Data are representative of results from at least three experiments, with similar data obtained using the ORF6<sub>487-495</sub> peptide.

antigen. In other systems, approximately 20% of memory CD8<sup>+</sup> T cells produce IL-2 in response to antigen restimulation (27). It has previously been shown in  $\gamma$ HV-68 infection that the number of antigen-specific CD8<sup>+</sup> T cells observed by tetramer analysis and the number of IFN- $\gamma$ -producing cells detected using intracellular cytokine staining are equivalent (7, 46). Therefore, we identified antigen-specific CD8 T cells using intracellular IFN- $\gamma$  staining and also measured how many of these cells produced IL-2. During the first 45 days postinfection, only a small proportion of cells in either group made IL-2 (Fig. 6); however, at 60 days postinfection and later, the proportion of cells making IL-2 rose significantly in the FS73 group. In contrast, the proportion in the persistent FS73R group remained low. These changes were observed for both the ORF6<sub>487-495</sub>/D<sup>b</sup>, and ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8 T-cell populations. Therefore, virus persistence decreases the proportion of virus-specific CD8 T cells that can produce IL-2.

**Proliferation of antigen-specific CD8<sup>+</sup> T cells in response to homeostatic cytokines in vitro.** Following acute viral infections, memory CD8<sup>+</sup> T cells are maintained in the absence of antigen due to their ability to proliferate in response to IL-15 (6, 9, 18, 24, 44, 49), although IL-7 can substitute for the absence of IL-15 in lymphopenic mice (18, 39, 49). However, we have shown that, during wild-type  $\gamma$ HV-68 infection, the antigen-specific CD8<sup>+</sup> T cells appear hyporesponsive to these cytokines and that these cells are actually maintained in the absence of IL-15 (37). To examine whether this unresponsiveness to IL-15 and other members of the common  $\gamma$ -chain cytokines was a result of viral persistence, CD8<sup>+</sup> T cells were purified from C57BL/6 mice, which had been infected with

either the persistent FS73R or nonpersistent FS73 virus for >60 days. Cells were CFSE labeled and then cultured in vitro for 4 days without antigen in the presence of IL-2, IL-7, or IL-15. Antigen-specific CD8<sup>+</sup> T cells were identified by intracellular cytokine staining for IFN- $\gamma$ , and CFSE dilution was monitored to assess proliferation in response to cytokines. As a positive control, vaccinia virus-specific memory CD8<sup>+</sup> T cells were shown to proliferate vigorously in response to IL-2 and IL-15 at the concentrations we used (Fig. 7A). Surprisingly, we found that antigen-specific CD8<sup>+</sup> T cells induced by both the nonpersistent FS73 and persistent FS73R viruses responded poorly to all cytokines tested (Fig. 7A). Proliferation in response to IL-15 and IL-2 was reproducibly better in the antigen-specific CD8<sup>+</sup> T cells induced by the nonpersistent FS73 virus; however, the difference was small. In addition, proliferation was not enhanced by the addition of IL-21, which has been shown to synergize with IL-7 and IL-15 (63). Therefore, CD8<sup>+</sup> T cells responding to both the persistent and nonpersistent gammaherpesviruses had a poor response to homeostatic cytokines. One possible reason for this lack of proliferation could be reduced expression of the receptors for these cytokines. To examine this issue, we took mice infected for >60 days with either the persistent or nonpersistent viruses and analyzed the expression of IL-7R $\alpha$  (CD127), IL-2/IL-15R $\beta$  (CD122), and  $\gamma_c$  receptor (CD132) on the antigen-specific CD8<sup>+</sup> T cells from the spleen. As expected, we found that vaccinia virus-specific CD8<sup>+</sup> T cells expressed all three receptors, whereas both nonpersistent and persistent  $\gamma$ HV-68 infection resulted in antigen-specific CD8<sup>+</sup> T cells which had a heterogeneous expression of IL-7R $\alpha$  and expressed lower levels of IL-2/IL-15R $\beta$  but had normal levels of  $\gamma_c$  receptor (Fig. 7B). This is consistent with the responsiveness of the antigen-specific CD8<sup>+</sup> T cells to these cytokines. Levels of CD127 or CD122 on the FS73 group did not change at later times postinfection, as at day 121 postinfection, both groups had the same expression pattern of these markers (Fig. 7C). CD127 expression was slightly elevated in both groups relative to that seen during the acute infection (day 14); however, levels of CD122 were similar at both time points.

Since the antigen-specific CD8<sup>+</sup> T cells induced by the nonpersistent strain of  $\gamma$ HV-68 were unable to undergo homeostatic proliferation, we wanted to understand the mechanism behind this impairment. Comparing the viral replication kinetics of a typical acute infection (i.e., LCMV, vesicular stomatitis virus [VSV], or vaccinia virus) with the nonpersistent strain of  $\gamma$ HV-68, we noticed that infection with the nonpersistent  $\gamma$ HV-68 resulted in prolonged viral replication (approximately 7 days versus 14 days, respectively). To test whether this prolonged acute replication of the nonpersistent FS73 virus was responsible for the lack of homeostatic proliferation, we shortened the time period where infectious virus could be detected using an antiviral treatment. C57BL/6 mice were infected with the FS73 virus, and then half of the mice were treated with antiserum from  $\gamma$ HV-68-infected animals on day 6 postinfection, followed by treatment with the antiviral drug cidofovir for 2 weeks. We found that this treatment was able to rescue the ability of the antigen-specific CD8<sup>+</sup> T cells to respond to homeostatic cytokines (Fig. 7D).



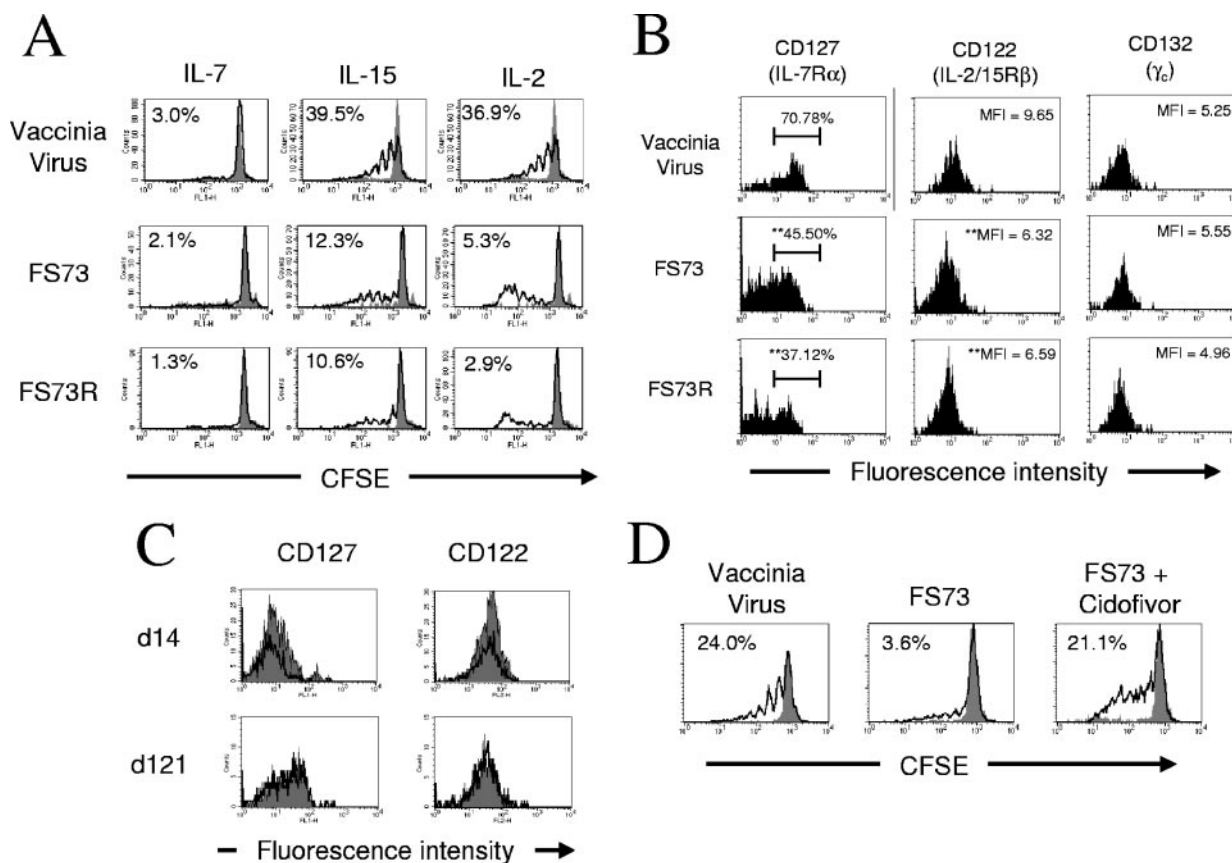


FIG. 7. In vitro proliferation of antigen-specific CD8<sup>+</sup> T cells in response to homeostatic cytokines is impaired following both FS73 and FS73R infection. (A) C57BL/6 mice were infected with vaccinia virus, the persistent FS73R virus, or nonpersistent FS73 virus for >60 days. CD8<sup>+</sup> T cells from the spleens were purified by magnetic bead selection (purity, >98% CD8<sup>+</sup>), then labeled with CFSE, and cultured with 125 ng/ml IL-15, 125 ng/ml IL-7, or 500 U/ml IL-2 for 4 days. Antigen-specific CD8<sup>+</sup> T cells were then identified by intracellular IFN- $\gamma$  staining, and CFSE dilution was monitored. For each plot, the filled histogram is representative of no cytokine and the open histogram represents the addition of cytokine. The numbers at the top of each plot represent the percentage of the starting population that has undergone at least one division. (B) Expression levels of the IL-2/IL-15R $\beta$ , IL-7R $\alpha$ , and  $\gamma_c$  receptors on the antigen-specific CD8<sup>+</sup> T cells from mice infected for >60 days were determined by tetramer staining. For IL-7R $\alpha$ , values represent the percentages of antigen-specific CD8<sup>+</sup> T cells expressing the receptor, whereas for IL-2/IL-15R $\beta$  and the  $\gamma_c$  receptor, the values are the mean fluorescence intensity (MFI). Asterisks (\*\*) indicate a significant difference compared to the expression on the vaccinia virus-specific CD8<sup>+</sup> T cells ( $P < 0.01$ ). (C) Expression of CD127 and CD122 during acute infection and at late times postinfection. Filled histograms represent FS73R-infected mice; solid black lines represent FS73-infected mice. (D) CD8<sup>+</sup> T cells from the spleens of C57BL/6 mice infected with either vaccinia virus, the nonpersistent FS73, or the nonpersistent FS73 plus cidofovir treatment as described in Materials and Methods were purified by magnetic beads (purity, >98% CD8<sup>+</sup>). Homeostatic proliferation in response to IL-15 was measured as described above. For each plot, the filled histogram is representative of no cytokine and the open histogram represents the addition of cytokine. The numbers represent percentages of the starting population that had undergone at least one division. These data are representative of results from at least two experiments.

**Antigen-specific CD8<sup>+</sup> T cells induced by persistent infection afford better protection from virus rechallenge than those induced by acute infection.** Since the purpose of memory T-cell populations is to protect the host from future infection, we wanted to test whether the antigen-specific CD8<sup>+</sup> T cells following nonpersistent FS73 or persistent FS73R infection were able to protect the mice against a secondary viral challenge.  $\gamma$ HV-68-infected mice are very resistant to rechallenge with the same virus, presumably due to a potent neutralizing antibody response. Therefore, we challenged mice that had been infected with either the nonpersistent FS73 or persistent FS73R virus for >60 days with a recombinant vaccinia virus expressing the ORF61<sub>524-531</sub>/K<sup>b</sup> epitope (rVV-p79). Two days after i.p. challenge with rVV-79, we found that FS73 memory mice had slightly reduced titers, while FS73R memory mice

had largely controlled the infection at this time (Fig. 8A). By day 4 postchallenge, both the FS73 and FS73R memory populations were able to control the vaccinia virus to the same extent (Fig. 8A). This indicates that CD8<sup>+</sup> T cells from both persistently and nonpersistently infected mice have potent antiviral activity, but the cells from the persistently infected mice were more effective at protecting against secondary challenge. Equivalent viral titers were seen in all groups when mice were infected with wild-type vaccinia virus (data not shown), ruling out nonspecific effects associated with persistent  $\gamma$ HV-68 infection. Two factors that contribute greatly to the efficiency of controlling a secondary infection are the speed of expansion of the responding T cells and the kinetics with which the antiviral functions of the CD8<sup>+</sup> T cells are induced. We first examined the expansion of the antigen-specific CD8<sup>+</sup> T cells at day 2 and

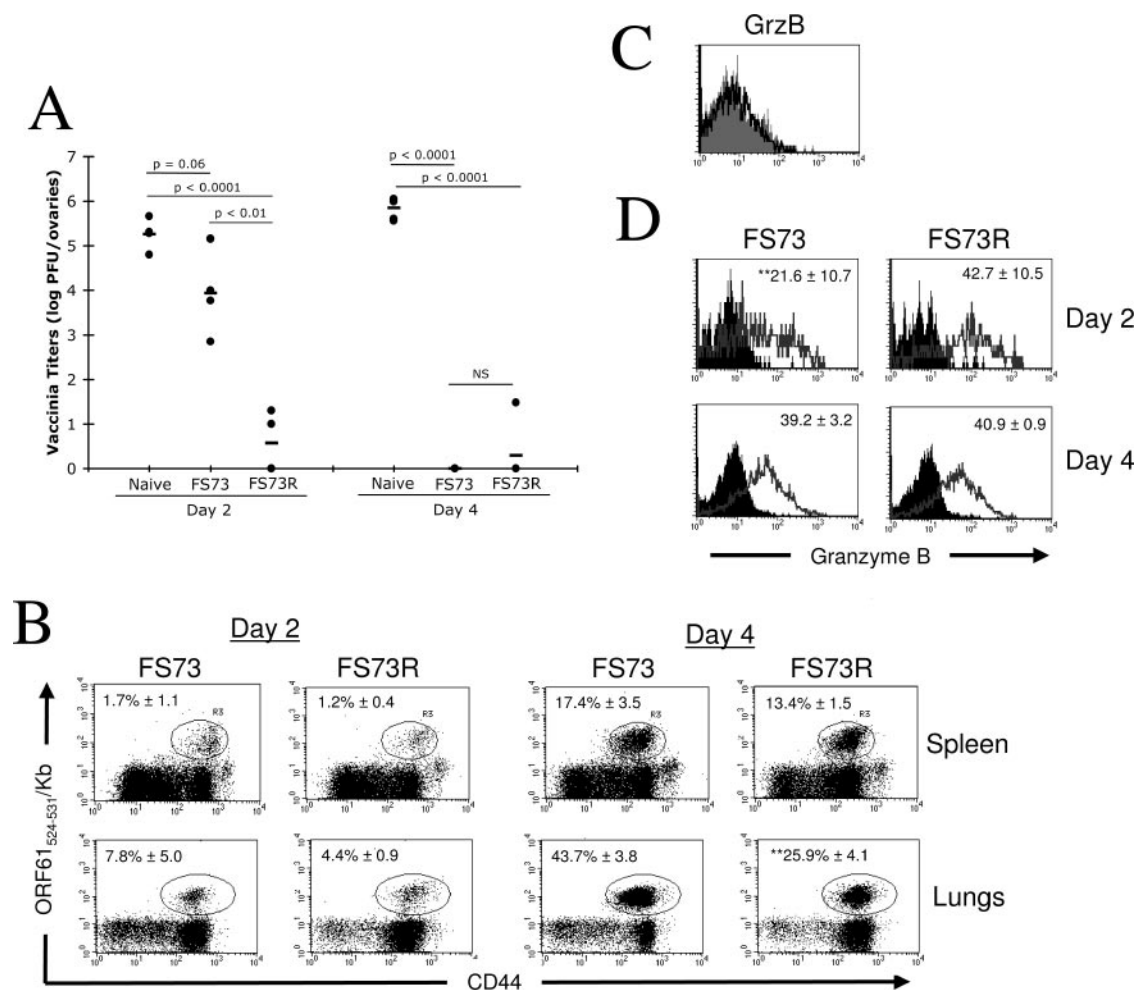


FIG. 8. Antigen-specific CD8<sup>+</sup> T cells induced by both FS73 and FS73R are protective against secondary viral challenge. C57BL/6 mice infected with either the persistent FS73R or nonpersistent FS73 virus for >60 days were rechallenged with  $1.5 \times 10^6$  PFU of rVV-p79 i.p. (A) Vaccinia virus titers were measured in the ovaries using a standard plaque assay at day 2 or day 4 postchallenge. Each data point represents a single mouse, and the bars indicate the means for each group. (B) The expansion of the ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells in the spleen was analyzed by tetramer staining at both day 2 and day 4 postchallenge. Data are gated on CD8<sup>+</sup> T cells, and the values represent the average percentages of ORF61<sub>524-531</sub>/K<sup>b</sup>-specific T cells  $\pm$  one standard deviation. (C) Granzyme B levels in mice infected with FS73 or FS73R for >60 days postinfection. (D) Reactivation of effector functions in ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells was monitored by staining for intracellular granzyme B at both day 2 and day 4 postchallenge. The data are gated on the ORF61<sub>524-531</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells, and the values represent the average mean fluorescence intensities (MFI)  $\pm$  one standard deviation. The filled histograms represent the isotype control, while the open histograms represent the average mean granzyme B staining. Asterisks (\*\*) indicate a significant difference between the groups at the same time point ( $P < 0.01$ ). For all experiments, the data are representative of the results from at least three experiments containing 4 to 6 mice per group.

day 4 postchallenge by tetramer analysis. No difference in the expansion of the antigen-specific CD8<sup>+</sup> T cells was observed at either time point (Fig. 8B). Next we examined the levels of granzyme B, a key mediator of target cell cytotoxicity. Virus-specific CD8<sup>+</sup> T cells from both the nonpersistent and persistent infections expressed minimal granzyme B levels in the absence of rVV-p79 challenge (Fig. 8C). At day 2 postchallenge, virus-specific CD8<sup>+</sup> T cells from the persistently infected mice had increased levels of granzyme B compared with those from the nonpersistently infected mice (Fig. 8D). By day 4 postchallenge, both groups expressed high levels of granzyme B. Thus, it appears that persistently infected mice are able to more quickly reactivate CTL effector functions and control a secondary infection more rapidly.

## DISCUSSION

In this report we have shown that the CD8<sup>+</sup> T-cell response is affected in several specific ways by persistent gammaherpesvirus infection. Using a mutant virus that does not persist but nevertheless induces a potent CD8<sup>+</sup> T-cell response, we were able to compare directly between responses, which matured in the presence or absence of virus persistence. Our data demonstrate that virus persistence is associated with lower levels of Bcl-2, a smaller proportion of cells capable of producing IL-2, and a faster turnover of antigen-specific CD8<sup>+</sup> T cells. In addition, the antigen-specific CD8<sup>+</sup> T cells found during the persistent infection exhibited more potent antiviral effects than those from the nonpersistent infection. Surprisingly, CD8<sup>+</sup> T

cells from neither the nonpersistently nor persistently infected mice were able to undergo homeostatic proliferation, which appears to be related to prolonged acute lung infection during  $\gamma$ HV-68 infection.

Infection of mice with  $\gamma$ HV-68 results in a persistent latent infection. During the latent infection, the antigen-specific CD8<sup>+</sup> T cells are restimulated with antigen, as evidenced by their increased turnover rate. One possible outcome of viral antigen persistence is the continual activation of CD8<sup>+</sup> T cells to become effector CTLs. However, we observed no evidence of the continual presence of activated effector CTLs in the persistently infected mice, as they did not express any early activation molecules and had minimal amounts of stored lytic granules, as determined by granzyme B expression. The expression of these molecules was similar to what was observed for memory CD8<sup>+</sup> T cells reactive against vaccinia virus (data not shown) and is similar to what was observed during acute and chronic LCMV infection (26, 57).

Studies examining the differentiation of CD8<sup>+</sup> T cells into memory cells have demonstrated that following the clearance of antigen, effector CTLs progressively differentiate into effector memory cells and eventually into central memory cells (25, 59). Our data indicate that this may not always be the case, as antigen-specific CD8<sup>+</sup> T cells induced by the nonpersistent FS73 infection resulted in cells with the same phenotype as those elicited during persistent  $\gamma$ HV-68 infection. Specifically, the majority of those cells remained CD62L<sup>lo</sup>, which is reexpressed on central memory cells (59), and had a heterogeneous expression of IL-7R $\alpha$ , which is expressed at high levels on memory cells following acute infections (23, 26). Impairment in the phenotypical maturation to classical memory cells has been observed in other systems, such as recombinant adenovirus infection, where the virus is present for approximately 3 weeks but then cleared (60). More recently, the model of T<sub>EM</sub> cells converting to T<sub>CM</sub> cells has been challenged by several reports which have shown that the T<sub>EM</sub> and T<sub>CM</sub> populations may be largely distinct subsets of memory cells (5, 11, 31).

Epstein-Barr virus (EBV)-specific CD8<sup>+</sup> T cells in humans are largely CD27<sup>+</sup> CD28<sup>+</sup> and considered to be at an early stage of differentiation, similar to what was observed for influenza-specific CD8<sup>+</sup> T cells (1, 2). The fact that both EBV and influenza virus-specific memory CD8<sup>+</sup> T cells have the same phenotype implies that virus persistence alone does not determine the cell surface phenotype. Although CD27 and CD28 are less useful markers of memory cell differentiation in mice, our results indicate that the phenotype of the antigen-specific CD8<sup>+</sup> T cells following nonpersistent or persistent  $\gamma$ HV-68 infection are identical, thus corroborating these human studies and extending their findings to include the phenotype of latent antigen-specific CD8<sup>+</sup> T cells. This was surprising, as we and others had postulated that viral persistence in  $\gamma$ HV-68 and EBV was responsible for the differences in the phenotype observed between lytic and latent antigen-specific CD8<sup>+</sup> T cells (13, 20, 36). We are currently trying to understand what causes these phenotypic differences observed in both EBV and  $\gamma$ HV-68 infection.

Another aspect of the CD8<sup>+</sup> T-cell response that viral persistence could influence is the size of the resulting antigen-specific T-cell response. In our system, the immune response against the immunodominant epitope, ORF61<sub>524-531</sub>/K<sup>b</sup>, was

induced and maintained at similar levels regardless of whether the virus persisted in the host. This indicates that the persistence of viral antigens, or other factors associated with persistent infection, are not required to maintain the relatively high level of virus-specific CD8<sup>+</sup> T cells observed in this model. Interestingly, there was a change in the immunodominance hierarchy when the virus failed to persist. After infection with the nonpersistent FS73 mutant virus, the responses to both the ORF61<sub>524-531</sub>/K<sup>b</sup> and ORF6<sub>487-495</sub>/D<sup>b</sup> epitopes were very similar in magnitude, both during acute infection and at extended times postinfection. However, the ORF6<sub>487-495</sub>/D<sup>b</sup>-specific response declined much sooner than the ORF61<sub>524-531</sub>/K<sup>b</sup>-specific response (starting at day 14) during persistent infection. This led to a much lower frequency of ORF6<sub>487-495</sub>/D<sup>b</sup>-specific cells during the long-term persistent infection. Similarly, an altered immunodominance has been observed in other persistent infections (50, 58). Regulation of the immunodominance hierarchy is subject to a very complex interplay of many different factors (61), and how persistence of antigen impacts this process is still very poorly understood.

Many factors can influence the immunodominance hierarchy during virus infection (61). We hypothesize that this altered immunodominance hierarchy is due to a combination of two factors, the kinetics of antigen presentation and inflammation which occurs as a result of splenomegaly. During the  $\gamma$ HV-68 infection, both the ORF61<sub>524-531</sub>/K<sup>b</sup> and ORF6<sub>487-495</sub>/D<sup>b</sup> epitopes are presented early after infection, but only the ORF61<sub>524-531</sub>/K<sup>b</sup> epitope is presented in the spleen at later time points, during the establishment of latency (29). Therefore, while the ORF6<sub>487-495</sub>/D<sup>b</sup> response expands but subsequently contracts, the ORF61<sub>524-531</sub>/K<sup>b</sup> response contracts less, likely due to this second wave of antigen presentation. During persistent infection, this contraction is probably due in part to the pronounced splenomegaly, which likely involves the production of inflammatory mediators such as IFN- $\gamma$ , that have been shown to induce contraction of the T-cell response (4). However, in nonpersistent infection there is no splenomegaly and also no second wave of presentation of the ORF61<sub>524-531</sub>/K<sup>b</sup> epitope, as the virus does not establish latency. Therefore, the contraction of the two epitopes will be similar and survival of the ORF61<sub>524-531</sub>/K<sup>b</sup> population is not enhanced relative to the ORF6<sub>487-495</sub>/D<sup>b</sup> population, as there is no second wave of antigen presentation.

Importantly, we have uncovered that viral persistence can have both positive and negative effects on the maturation and function of the responding CD8<sup>+</sup> T cells. Viral persistence impaired the ability of antigen-specific CD8<sup>+</sup> T cells to up-regulate Bcl-2 and resulted in a smaller proportion of those cells being capable of producing IL-2. A reduction in the levels of Bcl-2 and/or the ability of CD8<sup>+</sup> T cells to produce IL-2 has also been reported following infection with chronic LCMV or murine cytomegalovirus in mice (27, 45, 57) and human immunodeficiency virus, EBV, and cytomegalovirus infection in humans (10, 30, 38, 43). Therefore, these may be general events that occur during persistent virus infections. Thus, the difference observed in our systems and others is likely a result of chronic exposure to antigen. However, there could also be other explanations for the changes we observed. Persistent infection may be accompanied by a low-level inflammatory response, which may play a role in shaping the maturation of

the CD8<sup>+</sup> T-cell response. However, coinfection of mice with both  $\gamma$ HV-68 and vaccinia virus had no impact on the development of the CD8<sup>+</sup> T cells responding against vaccinia virus (data not shown), which strongly argues against the idea that the cytokine milieu during priming or persistence influences memory cell maturation. In addition, the idea that antigen persistence results in these defects is strongly supported by a recent study where chronic antigen exposure from repeated administration of incomplete Freund's adjuvant and peptide resulted in reduced levels of Bcl-2 and continued down-regulation of IL-7R $\alpha$  (28).

Our finding that the functional capabilities of the virus-specific CD8<sup>+</sup> T cells are maintained during  $\gamma$ HV-68 persistence is in sharp contrast to what is seen in other persistent infections, where the continued presence of virus causes severe impairments in antiviral effector functions (17, 34, 35, 50, 58). The key difference which may account for these results is likely the quantity of virus that the immune system is exposed to during the persistent infection. In systems like chronic LCMV, there is a high viral load for an extended period (58), whereas during gammaherpesvirus infections, the viral load is extremely low and gene expression is tightly regulated. Nevertheless, viral reactivation does occur and is sensed by the T cells, as demonstrated by the fact that the turnover rate of virus-specific CD8<sup>+</sup> T cells doubles in the presence of persistent virus. This low-level stimulation of the T cells is probably not sufficient for the development of severe deficits in effector functions, but nevertheless prevents the expression of some memory characteristics, such as the ability to synthesize IL-2 or up-regulate Bcl-2 as discussed. However, the question still remains as to why  $\gamma$ HV-68 persistence would result in antigen-specific CD8<sup>+</sup> T cells that can afford better protection against subsequent viral challenge? One possibility is that during the persistent infection there is a low level of inflammation, producing type I and type II interferons which can inhibit vaccinia virus growth (39a). This is likely not the case, as infection of persistently infected mice with the Western Reserve strain of vaccinia virus resulted in the rapid growth of the virus, similar to what was seen in the nonpersistently infected mice or naive mice (data not shown). Recently, it has been shown that the number of antigen-specific CD8<sup>+</sup> T cells present in the peripheral tissue before challenge correlates with the clearance of vaccinia virus from the host (3). Following either nonpersistent or persistent  $\gamma$ HV-68 infection, we observed no statistically significant differences in the number of antigen-specific CD8<sup>+</sup> T cells in any of the tissue tested. Therefore, it is unlikely that the enhanced clearance is due to an elevated precursor frequency at the time of challenge. Finally, it is possible that viral reactivation only occurs sporadically; thus, responding T cells are not continuously exposed to antigen. Periodic exposure to antigen followed by periods of rest would constantly restimulate the T-cell response, perhaps resulting in T cells that are poised and ready to initiate very rapid antiviral functions upon reexposure to virus, such as their expression of granzyme B.

We had previously found that antigen-specific CD8 T cells induced by  $\gamma$ HV-68 failed to undergo homeostatic proliferation in response to IL-7 or IL-15, but those cells had an increased survival when cultured with those cytokines (37). Surprisingly, in this study, we found that antigen-specific CD8<sup>+</sup> T cells elicited even by a nonpersistent  $\gamma$ HV-68 infection re-

sponded poorly to homeostatic cytokines. Typically, following acute infections, memory CD8<sup>+</sup> T cells gain the ability to be maintained in the absence of antigen through their proliferation to IL-15 (6, 9, 18, 24, 44, 49), although IL-7 can compensate for the lack of IL-15 in some circumstances (18, 39, 49). However, following chronic LCMV infection, the resulting memory CD8<sup>+</sup> T cells population is left unresponsive to these homeostatic cytokines (57). This is at least partly due to their lower expression of the receptors for both IL-7 and IL-15. In contrast, memory CD8<sup>+</sup> T cells induced by acute LCMV infection expressed high levels of both the IL-7R and IL-15R complexes and are able to proliferate in response to these cytokines without antigen (57). Similarly, a recent study examining antigen-specific CD8<sup>+</sup> T-cell responses in humans demonstrated that CD8<sup>+</sup> T cells specific for cleared infections (influenza and RSV) homogeneously expressed high levels of IL-7R $\alpha$ , whereas those specific for cytomegalovirus and EBV had a mixed expression for IL-7R $\alpha$  (12, 55). Our results demonstrate that, following either nonpersistent or persistent  $\gamma$ HV-68 infection, the antigen-specific CD8<sup>+</sup> T cells express lower levels IL-2/IL-15R $\beta$  and were heterogeneous in their expression of IL-7R $\alpha$ . These lower levels of IL-2/IL-15R $\beta$  and IL-7R $\alpha$  in our system were consistent with the finding that neither group of virus-specific CD8 T cells responded efficiently to homeostatic cytokines. However, other factors could be playing a role in the unresponsiveness of these cells to homeostatic cytokines, such as increased expression of SOCS proteins, which have recently been shown to regulate homeostatic proliferation (15) and need to be further explored in our system. Therefore, our data argue that responsiveness to homeostatic cytokines does not only correlate with the persistence of virus but also varies according to the identity of the virus. There are many differences between the  $\gamma$ HV-68 and LCMV. These discrepancies between the two systems include the viral replication kinetics, cellular tropism, and/or antigen-presenting cells initiating the immune response, which singly or in combination may result in the differences observed between the systems. It appears that the length of the initial viral replication is important in establishing the responsiveness to homeostatic cytokines, as shortening the length of viral replication following nonpersistent infection with FS73 allowed those antigen-specific CD8<sup>+</sup> T cells to proliferate in response to IL-15. Importantly, our data with the nonpersisting mutant virus also show that, in this system, memory CD8<sup>+</sup> T cells do not need to express high levels of IL-7 $\alpha$  or IL-2/IL-15R $\beta$  to persist long-term in the absence of antigen.

In conclusion, we present evidence that the persistence of a gammaherpesvirus shapes the antiviral cellular immune response. A mutant virus that fails to persist elicits a CD8<sup>+</sup> T-cell response that has a marked shift in the immunodominance hierarchy, expresses higher levels of an antiapoptotic protein, and has an enhanced ability to produce IL-2. Surprisingly, these CD8<sup>+</sup> T cells responded poorly to homeostatic cytokines regardless of whether or not the virus persisted in the host, indicating that, in the  $\gamma$ HV-68 infectious model, high levels of IL-7R $\alpha$  and IL-2/IL-15R $\beta$  are not always necessary for the survival of memory CD8<sup>+</sup> T cells either in the presence or absence of viral antigen. Finally, we show that viral persistence actually enhances the ability of the virus-specific CD8<sup>+</sup> T cells to mount a secondary response *in vivo* and resist challenge with



a recombinant vaccinia virus. This work raises several interesting questions, such as what factors are responsible for the generation of these different types of memory cells? Does continued antigen presentation by specific cell types influence the character of the memory CD8<sup>+</sup> T-cell response? Also, it will be interesting to determine if similar changes are observed in the antiviral CD4<sup>+</sup> T-cell response in this system. These questions and others will form the basis of our future investigations.

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