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IL-15-Independent Proliferative Renewal of Memory CD8$^+$ T Cells in Latent Gammaherpesvirus Infection

Joshua J. Obar,* Sarah G. Crist,* Erica K. Leung,† and Edward J. Usherwood$^2$

IL-15 is known to be critical in the homeostasis of Ag-specific memory CD8$^+$ T cells following acute viral infection. However, little is known about the homeostatic requirements of memory CD8$^+$ T cells during a latent viral infection. We have used the murine gammaherpesvirus-68 (MHV-68) model system to investigate whether IL-15 is necessary for the maintenance of memory CD8$^+$ T cells during a latent viral infection. IL-15 is not essential either for the initial control of MHV-68 infection or for the maintenance of MHV-68-specific memory CD8$^+$ T cells. Even at 140 days postinfection, the proportion of CD8$^+$ T cells recognizing the MHV-68 epitopes were the same as in control mice. The maintenance of these memory CD8$^+$ T cells was attributable to their ability to turn over in vivo, probably in response to the presence of low levels of Ag. IL-15$^{-/-}$ mice had a significantly higher turnover rate within the virus-specific memory CD8$^+$ T cell population, which was the result of increased levels of viral gene expression rather than an increase in viral load. These cells did not accumulate in the spleens of the IL-15$^{-/-}$ mice due to an increased sensitivity to apoptosis as a result of decreased Bcl-2 levels. Intriguingly, memory CD8$^+$ T cells from latently infected mice failed to undergo homeostatic proliferation in a naive secondary host. These data highlight fundamental differences between memory CD8$^+$ T cells engaged in active immune surveillance of latent viral infections vs memory CD8$^+$ T cells found after acute viral infections. The Journal of Immunology, 2004, 173: 2705–2714.

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3 Abbreviations used in this paper: γc, common γ-chain; MHV-68, murine gammaherpesvirus 6; QF, quantitative fluorescent; ORF, open reading frame.

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However, little is known about the requirements for the maintenance of CD8⁺ T cell memory in latent viral infections. To address this issue, we choose to study a gammaherpesvirus infection in mice using murine gammaherpesvirus-68 (MHV-68). This virus is closely related to the human gammaherpesviruses, EBV, and human herpesvirus-8 (23). After intranasal infection, MHV-68 establishes lifelong latency mainly within the host B cells (24) and lung epithelial cells (25), but also within macrophages (24, 26) and dendritic cells (24). During the latent infection, there is thought to be sporadic low-level reactivation allowing the release of infectious virus therefore re-exposing the immune system to viral Ags. This system allowed us to examine the effects of viral persistence on the maintenance of Ag-specific memory CD8⁺ T cells engaged in active immune surveillance.

In this study, we show that following MHV-68 infection the expansion of Ag-specific CD8⁺ T cells is unchanged in the absence of IL-15 and the acute lung infection was successfully cleared. Intriguingly, we show that in the absence of IL-15 the frequency of the Ag-specific memory CD8⁺ T cell population remains stable in the spleen and peripheral tissues, which is unlike what happens following acute viral infections (9, 10). In addition, the Ag-specific memory CD8⁺ T cells are still capable of proliferating in vivo in the absence of IL-15. Also, in contrast to what is seen with memory CD8⁺ T cells induced by acute viral infections (9, 27) or endogenously derived CD4⁺CD8⁻“memory” cells (11), CD8⁺ T cells were unable to undergo homeostatic proliferation when transferred to naive immunocompetent hosts. These data highlight important differences between memory CD8⁺ T cells actively engaged in immune surveillance of a latent viral infection vs memory CD8⁺ T cells found after acute viral infections.

Materials and Methods

Mice and virus

MHV-68 virus (clone G2.4) was originally obtained from Prof. A. A. Nash (University of Edinburgh, Edinburgh, U.K.). Virus was propagated and titered as previously described (28). C57BL/6 and B6.PL-Thy1.2/Cy mice were purchased from The Jackson Laboratory (Bar Harbor, ME), respectively. IL-15⁻/⁻ mice on the C57BL/6 background were obtained from Taconic Farms (Germantown, NY) and bred in the Dartmouth-Hitchcock Medical Center mouse facility. Mice were infected intranasally with 400 PFU of MHV-68 under anesthesia with 2,2,2-tribromoethanol. 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. Spleen cells were depleted of erythrocytes by treatment with buffered ammonium chloride, centrifuged, and counted. Lung and liver suspensions were resuspended in 80% isotonic percoll and subsequently overlaid with 40% isotonic percoll. Samples were then centrifuged at 400 g for 1 h before overlaying with carboxymethyl cellulose. After 5 days of incubation at 37°C, the assays were fixed with methanol and stained with Giemsa stain, and then the plates were enumerated microscopically.

Quantitative PCR for viral load and viral transcripts

Latent viral DNA was quantified by quantitative fluorescent (QF)-PCR as previously described (30). Briefly, DNA was extracted from splenocytes or homogenized lung samples using a Qiagen DNeasy kit (Qiagen, Valencia, CA) and then quantified using a UV spectrophotometer. DNA (300 ng) was subjected to QF-PCR using Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). 500 nM primers complementary to the ORF50 gene and 200 nM labeled probe complementary to the ORF50 gene. 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 (Bio-Rad, Hercules, CA). To construct a standard curve, a graded number of copies of the pTW-27 plasmid containing the genomic ORF50 gene, was mixed with 300 ng of normal splenocyte DNA and subjected to QF-PCR. No template controls containing 300 ng of normal splenocyte DNA were negative for all QF-PCR assays. The assay was able to detect fewer than 10 viral genomes per sample.

To analyze viral transcripts, total RNA was isolated from spleen or lung tissues using TRizol (Invitrogen) followed by a secondary purification using Perfect RNA columns (Brinkman Instruments, Westbury, NY). Following column purification, the RNA was treated with DNase I in the presence of 100 U of Protector RNase Inhibitor (Roche, Indianapolis, IN) for 75 min at 37°C to remove any contaminating DNA. After DNA digestion, 1–2 μg of RNA was reverse transcribed to cDNA with Omniscript RT (Qiagen) using 100 μg/ml oligo(dT) primer. QF-PCR was performed on 2.5 μl 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 β-actin, open reading frame (ORF) 61, or ORF6. Primers and probes were as follows: murine β-actin 3' primer, CAATATTGATACCT GCCGCT; murine β-actin, 5' primer, AGAGAGAAATCTGGTGAC; murine ORF6 5' probe, TGGTGGGATCAGATA; murine ORF6 3' probe, TCCAAAACTTTGGCACAGA; ORF61 5' primer, TGCAAACTTCTTG GCAGAGG; ORF61 probe, CTTGCTCACCCTGAATCCGTG; ORF63 5' primer, GTTGTGGGGTGTCACAGAG; ORF65 5' primer, GTGCCCC AACATCATTCAAAC; and ORF69 probe, CACACCTCCTTCCTCACACTTCAAGG. The samples were subjected to 40 cycles at 15 °C at 95°C and 1 min at 60°C. QF-PCR was performed using a Bio-Rad iCycler.

Flow cytometry analysis

Using standard protocols, 10⁶ cells were stained for FACS analysis in PBS containing 2% FCS and 0.09% sodium azide (staining wash buffer) using the following Abs: PE-Cy5-conjugated anti-CD45RA (53-6.7), FITC-conjugated anti-CD45RB (KT4), PE-conjugated anti-CD43 (1B11), FITC-conjugated anti-CD62L (ME14), FITC-conjugated anti-CD69 (H1.2F3) and FITC-conjugated anti-Ly-6C (AL-21) were all from BD Pharmingen (San Diego, CA); FITC-conjugated anti-CD127 (A7R34), PE-conjugated anti-CD11a (M17/4), PE-conjugated anti-CD25 (PC61), and PE-conjugated anti-CD122 (SH4) were from eBiosciences (San Diego, CA). In addition, cells were stained with MHC/peptide tetramer reagents labeled with allophycocyanin. The construction of folded MHC class I-peptide complexes and their tetramerization have been described previously (31). Two tetramers were used: Kb folded with the peptide ORF6 1524–1531 (AGPHNDMEI). Tetramers were stored as aliquots at −80°C. Upon thawing, tetramers were diluted in staining wash buffer plus 100 μM biotin and spun at 16,000 × g for 5 min to remove any aggregates. Staining with tetrameric reagents took place for 1 h at room temperature, followed by staining with anti-CD8α on ice for 20 min. For Vß4 staining, cells were stained with anti-CD4 and anti-Vß4 Abs on ice for 20 min. Stained samples were analyzed using a FACS Calibur flow cytometer and CellQuest software (BD Immunocytometry Systems, San Jose, CA). Control tetramers consisting of the same H chain folded with irrelevant peptides did not stain CD8⁺ T cells from MHV-68-infected mice.

Analysis of proliferation in vivo

C57BL/6 and IL-15⁻/⁻ mice latently infected with MHV-68 (>60 days) were given water containing 0.8 mg/ml BrdU (Fisher Scientific, Hampton, NH) for 10 days. Spleen cells were stained with both the ORF61524–1531/Kb or ORF6647–649/D9 tetramer with the anti-BrdU Ab according to the BrdU flow kit protocol (BD Pharmingen).

Adaptive transfer of CD8⁺ T cells

CD8⁺ T cells were enriched from pooled spleens of latently infected C57BL/6 mice (>60 days postinfection) using a StemCell CD8⁺ T cell purification kit (StemCell Technologies, Vancouver, Canada). Next, the cells were further purified using a BD FACStarPlus to obtain CD8⁺ T cells of >98% purity. The cells were then labeled with 0.5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min and extensively washed. At which point ~3 × 10⁶ cells were injected i.v. into B6.PL-Thy1.2/Cy mice and left for 28 days. The splenocytes were then stained with anti-Thy1.2, anti-CD69, and the appropriate tetramer as described above.
Intracellular Bcl-2 detection

For detection of intracellular Bcl-2 levels, 10^6 cells were stained with tetramer and anti-CD8α as described above. Following staining, cells were washed with PBS and fixed with 1% formaldehyde. After fixation, the cells were washed and permeabilized using 0.5% saponin for 10 min. The cells were then stained with an anti-Bcl-2 or isotype control Ab (BD Pharmingen) for 30 min on ice and washed twice with PBS.

Statistical analysis

All data were analyzed using the Student’s t test and a p < 0.05 was considered to be significant.

Results

IL-15 is not essential for controlling acute MHV-68 infection

Previous work has shown that IL-15 has a profound impact on the pathogenesis and replication of other herpesviruses, both in vivo (21) and in vitro (22). Infection of IL-15−/− mice with HSV-2 resulted in the mortality of all of the mice, as compared with zero mortality in C57BL/6 mice (21). Therefore, we first wished to examine whether IL-15 was needed for the control of MHV-68 infection in mice. To address this, IL-15−/− and C57BL/6 mice were infected with 400 PFU of MHV-68 intranasally. Control of the virus was monitored by quantifying infectious virus within the lungs for the first 2 wk and latent virus within the spleen at 14 days postinfection. In addition, we examined whether the expansion of the ORF6487−531/Kb-specific CD8+ T cell populations was compromised in the absence of IL-15 by staining spleen cells with anti-CD8α and the appropriate tetramer. As shown in Fig. 1A, IL-15 was not required for the clearance of MHV-68 from the lungs of infected animals, as both the IL-15−/− and C57BL/6 mice had similarly low viral titers at 14 days postinfection. However, 10 days after MHV-68 infection, IL-15−/− mice had significantly higher viral titers in their lungs when compared with those of C57BL/6 mice, even though both had similar viral titers at 7 days postinfection (Fig. 1A). The higher viral titers in the IL-15−/− mice did not cause any differences in the establishment of latency within the spleen (Fig. 1B). This indicated that IL-15 was not essential for clearance of the acute MHV-68 infection or control of early latency.

In addition to monitoring viral clearance from the lung, we also analyzed the frequency of the Ag-specific CD8+ T cells in the spleen by tetramer staining (Fig. 2A). This was done to determine whether the expansion of Ag-specific CD8+ T cells would be affected by the lack of IL-15. Fig. 2A shows an example of the tetramer staining for both the ORF6487−499/Dα and ORF61524−531/Kb-specific CD8+ T cell populations recognized in the absence of IL-15 by staining spleen cells with anti-CD8α and the appropriate tetramer. Our data demonstrate that peripheral CD8+ T cells (20, 32), we examined the total number of ORF61524−531/Kb-specific memory CD8+ T cells were maintained at equivalent levels in IL-15−/− and C57BL/6 mice for at least 139 days postinfection in the spleen. In addition, since IL-15−/− mice have a slight deficiency in naive CD8+ T cells (20, 32), we examined the total number of ORF61524−531/Kb-specific memory CD8+ T cells (data not shown) found in the spleen. We showed that the IL-15−/− mice had a marked decrease in the total number of virus-specific CD8+ T cells during the initiation and expansion of the response. This marked decrease carried into memory, but throughout memory the number of virus-specific CD8+ T cells in IL-15−/− mice remained stable when looked at as a percentage of cells found in C57BL/6 mice.

Memory CD8+ T cells do not decline during chronic MHV-68 infection in the absence of IL-15

It was recently shown that IL-15 is critical in the maintenance of Ag-specific memory CD8+ T cells induced by infection with lymphocytic choriomeningitis virus (9) and vesicular stomatitis virus (10). These studies demonstrated that in the absence of IL-15 Ag-specific memory CD8+ T cells were maintained at ~25–50% of the level found in the control mice at 100 days postinfection (9, 10). Since these systems used viruses that were cleared within ~2 wk, we wanted to evaluate whether IL-15 was important in the maintenance of Ag-specific CD8+ T cells during a latent viral infection. MHV-68 infected mice were left for >60 days, at which time the frequency of the ORF6487−499/Dα and ORF61524−531/Kb-specific memory CD8+ T cell cells was determined by anti-CD8α and tetramer staining. As shown in Fig. 3, A and B, both the ORF6487−499/Dα and ORF61524−531/Kb-specific memory CD8+ T cells were maintained at equivalent levels in IL-15−/− and C57BL/6 mice for at least 139 days postinfection in the spleen. In addition, since IL-15−/− mice have a slight deficiency in naive CD8+ T cells (20, 32), we examined the total number of ORF61524−531/Kb-specific memory CD8+ T cells (data not shown) found in the spleen. We showed that the IL-15−/− mice had a marked decrease in the total number of virus-specific CD8+ T cells during the initiation and expansion of the response. This marked decrease carried into memory, but throughout memory the number of virus-specific CD8+ T cells in IL-15−/− mice remained stable when looked at as a percentage of cells found in C57BL/6 mice.

Memory T cells can also be found within peripheral tissues, such as lung, liver, fat pads, kidneys, and intestine (33, 34). The maintenance of these peripheral memory CD8+ T cells has also been shown to be dependent on IL-15 (9, 10). To examine whether memory CD8+ T cells were maintained within the peripheral tissues, we stained lymphocytes isolated from either the lungs or livers of IL-15−/− or C57BL/6 mice that were infected with MHV-68 for >60 days with anti-CD8α and the appropriate tetramer. Our data demonstrate that peripheral CD8+ memory T cells

![Image](http://www.jimmunol.org/DownloadedFrom/doi.org/10.4049/jimmunol.2006187)
and 14 postinfection were stained with either the ORF6 487–495/D b (fi
s cells in those organs (data not shown). The ORF6 487–495/D b and ORF61 524–531/K b were also stained with anti-CD8a Ab. Tetramer staining
iv distribution of viral Ags is responsible for the continual proliferation of MHV-68-specific T cells. To monitor the distribution of these T cells, we used a combination of tetramers and antibodies specific for known viral epitopes (ORF6 487–495/D b and ORF61 524–531/K b specific) or the result of generalized virus-induced activation (V β4 + ) is independent of IL-15.

MHV-68-specific memory CD8 T cells turn over in the absence of IL-15

After acute viral infections, Ag-specific memory CD8 + T cells decline with time in the absence of IL-15 because those cells are unable to undergo proliferative renewal in the absence of this cytokine (9, 10). Since we observed no decrease in the size of the Ag-specific memory CD8 + T cell populations in IL-15−/− mice after MHV-68 infection, we asked whether those cells could turn over in vivo. To test this, IL-15−/− and C57BL/6 mice were infected with MHV-68 for >60 days, at which point they were treated with BrdU in their drinking water for 10 days. BrdU is a synthetic thymidine analog that is incorporated into the DNA of dividing cells and therefore labels all cells that proliferate during its administration. After 10 days of labeling, spleen cells were stained with anti-CD8a and the appropriate tetramer, at which point the cells were fixed, permeabilized, subjected to DNA digestion, and stained with an anti-BrdU Ab. As shown in Fig. 5, we observed that ORF6 487–495/D b and ORF61 524–531/K b-specific memory CD8 + T cells were able to turn over in the absence of IL-15.

Interestingly, in the absence of IL-15, the Ag-specific memory CD8 + T cells turned over at a significantly greater rate than in C57BL/6 mice during latent MHV-68 infection (Fig. 5). To ascertain why the Ag-specific CD8 + T cells turn over at a greater rate in IL-15−/− mice, we first asked whether they lose control of the latent infection. To address this question, we monitored the level of MHV-68 viral genomes by QF-PCR for the ORF50 gene, since viral reactivation is too low to measure at these time points (30). We found that the number of latent MHV-68 genomes in both IL-15−/− and C57BL/6 mice was the same in both the lungs (Fig. 6A) and spleen (Fig. 6B). Next, we asked whether the level of viral Ag was elevated in IL-15−/− mice. Since direct quantitation of viral Ag or reactivation at late times postinfection is not possible, we monitored the levels of viral transcripts for the ORF61 and ORF6 genes by QF-PCR using cDNA made from total RNA isolated from spleen or lung tissue taken from IL-15−/− or C57BL/6 mice infected with MHV-68 for >60 days. We show that the mRNAs for viral Ags were elevated ∼3-fold in both the spleens and lungs of latently infected IL-15−/− mice (Table I). This suggests that continual stimulation of the TCR by viral Ags is responsible for the maintenance of the Ag-specific CD8 + T cell population and the increased turnover observed in IL-15−/− mice.

In addition to the maintenance of Ag-specific CD8 + T cells, we looked at the maintenance of the V β4 + CD8 + T cell population. After the resolution of the acute MHV-68 infection, and concurrent with the establishment of latency, mice develop an infectious mononucleosis-like syndrome where the mice develop splenomegaly and large numbers of V β4 + CD8 + T cells (35). The reason for this huge increase in the size of the V β4 + CD8 + T cell population is currently unknown; however, this expansion is dependent on B cells, CD40L expression on CD4 + T cells, and is MHC haplotype independent (36, 37). The V β4 + CD8 + T cells are most prominent in the peripheral blood; to monitor this population, MHV-68 infected mice were bled at various time points and stained with anti-CD8a and anti-V β4 Abs. We found that maintenance of the V β4 + CD8 + T cell population in the blood was independent of IL-15 (Fig. 4), with similar results obtained from the spleen (data not shown). This shows that during latent MHV-68 infection maintenance of viral-specific memory CD8 + T cells, whether they are specific for known viral epitopes (ORF6 487–495/D b and ORF61 524–531/K b specific) or the result of generalized virus-induced activation (V β4 + ) is independent of IL-15.

MHV-68-specific memory CD8 T cells turn over in the absence of IL-15

After acute viral infections, Ag-specific memory CD8 + T cells decline with time in the absence of IL-15 because those cells are unable to undergo proliferative renewal in the absence of this cytokine (9, 10). Since we observed no decrease in the size of the Ag-specific memory CD8 + T cell populations in IL-15−/− mice after MHV-68 infection, we asked whether those cells could turn over in vivo. To test this, IL-15−/− and C57BL/6 mice were infected with MHV-68 for >60 days, at which point they were treated with BrdU in their drinking water for 10 days. BrdU is a synthetic thymidine analog that is incorporated into the DNA of dividing cells and therefore labels all cells that proliferate during its administration. After 10 days of labeling, spleen cells were stained with anti-CD8a and the appropriate tetramer, at which point the cells were fixed, permeabilized, subjected to DNA digestion, and stained with an anti-BrdU Ab. As shown in Fig. 5, we observed that ORF6 487–495/D b and ORF61 524–531/K b-specific memory CD8 + T cells were able to turn over in the absence of IL-15.

Interestingly, in the absence of IL-15, the Ag-specific memory CD8 + T cells turned over at a significantly greater rate than in C57BL/6 mice during latent MHV-68 infection (Fig. 5). To ascertain why the Ag-specific CD8 + T cells turn over at a greater rate in IL-15−/− mice, we first asked whether they lose control of the latent infection. To address this question, we monitored the level of MHV-68 viral genomes by QF-PCR for the ORF50 gene, since viral reactivation is too low to measure at these time points (30). We found that the number of latent MHV-68 genomes in both IL-15−/− and C57BL/6 mice was the same in both the lungs (Fig. 6A) and spleen (Fig. 6B). Next, we asked whether the level of viral Ag was elevated in IL-15−/− mice. Since direct quantitation of viral Ag or reactivation at late times postinfection is not possible, we monitored the levels of viral transcripts for the ORF61 and ORF6 genes by QF-PCR using cDNA made from total RNA isolated from spleen or lung tissue taken from IL-15−/− or C57BL/6 mice infected with MHV-68 for >60 days. We show that the mRNAs for viral Ags were elevated ∼3-fold in both the spleens and lungs of latently infected IL-15−/− mice (Table I). This suggests that continual stimulation of the TCR by viral Ags is responsible for the maintenance of the Ag-specific CD8 + T cell population and the increased turnover observed in IL-15−/− mice.

FIGURE 2. IL-15 is dispensable for the expansion of MHV-68-specific CD8 + T cells. Spleen cells from MHV-68-infected mice taken at days 10 and 14 postinfection were stained with either the ORF6 487–495/D b or ORF61 524–531/K b tetramer followed by anti-CD8a Ab. Tetramer staining of spleen cells at day 14 postinfection in both C57BL/6 and IL-15−/− mice was monitored by flow cytometry (A). The frequency of tetramer-positive cells within the CD8 + population was quantified for both the ORF6 487–495/D b (B) and ORF61 524–531/K b (C) tetramers in both C57BL/6 (○) and IL-15−/− (●) mice. Each data point represents a single mouse (p > 0.05).
MHV-68-specific CD8\(^+\) T cells do not accumulate in the spleen due to increased sensitivity to apoptosis

It is known that memory CD8\(^+\) T cells contain elevated levels of Bcl-2, which enables them to survive long-term in the host (38). In addition, it is known that IL-15 can induce elevated levels of Bcl-2, as memory CD8\(^+\) T cells failed to accumulate in IL-15\(^{-/-}\) mice even though they were proliferating at a greater rate than in C57BL/6 mice, we hypothesized that those cells are more sensitive to apoptosis. To address this, we evaluated intracellular Bcl-2 levels within the MHV-68-specific CD8\(^+\) T cells and also examined their binding of annexin V, an early marker of apoptotic cells. Spleen cells from IL-15\(^{-/-}\) and C57BL/6 mice infected with MHV-68 for >60 days were stained with anti-CD8\(\alpha\) and the appropriate tetramer, followed by annexin V or intracellular Bcl-2 staining. We have found that the Bcl-2 levels in the ORF6487-499/D\(^\beta\)-specific (data not shown) CD8\(^+\) T cells were decreased ~3-fold in IL-15\(^{-/-}\) mice compared with those in C57BL/6 mice. We also examined the binding of annexin V by the Ag-specific CD8\(^+\) T cells. We found that ~18% of the Ag-specific CD8\(^+\) T cells from latently infected IL-15\(^{-/-}\) mice stained with annexin V, whereas only 8% stained with annexin V in C57BL/6 mice. This indicates that the absence of IL-15 makes the Ag-specific CD8\(^+\) T cells more sensitive to apoptosis due to decreased Bcl-2 levels in those cells.

Memory CD8\(^+\) T cells from MHV-68 infection fail to proliferate in the absence of Ag

Since Ag appears to be critical in the maintenance of virus-specific CD8\(^+\) T cells during latent MHV-68 infection, we wanted to ask whether those cells could still proliferate in a naive host. To examine this, we isolated CD8\(^+\) T cells from C57BL/6 mice infected with MHV-68 for >60 days by FACS sorting, labeled those cells with CFSE, and adoptively transferred them into naive B6.PL-Thy1.2, anti-CD8\(\alpha\), and the ORF61524-531/K\(^\beta\)-tetramer to monitor the proliferation of the transferred cells. We found that the proliferation of the transferred cells was impaired because most of the cells had undergone no cell division and a small minority underwent one cell division (Fig. 8). This finding is strikingly different from what has previously been reported with memory CD8\(^+\) T cells induced by acute viral infections or endogenously derived memory cells that are able to undergo one to three cell divisions in 30 days (9, 11, 27).

Discussion

To date, the dependence of Ag-specific memory CD8\(^+\) T cells on IL-15 for survival and homeostatic proliferation has mainly been evaluated following acute viral infections (9, 10). In this report, we have examined the role that IL-15 plays in the survival of Ag-specific memory CD8\(^+\) T cells induced by infection with a latent virus. We have shown that IL-15 is not necessary for either the clearance of the acute lung infection or the maintenance of the Ag-specific memory CD8\(^+\) T cell populations. During latent viral infection, the Ag-specific memory CD8\(^+\) T cells were still able to proliferate in the absence of IL-15, unlike what was found for infection with viruses that are completely cleared (9, 10). This proliferation was likely attributable to the persistent low levels of viral Ags due to spontaneous reactivation. When CD8\(^+\) T cells were transferred to naive immunocompetent recipients, those cells

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**FIGURE 3.** Maintenance of ORF6487-499/D\(^\beta\)- and ORF61524-531/K\(^\beta\)-specific memory CD8\(^+\) T cells occurs in the absence of IL-15. C57BL/6 and IL-15\(^{-/-}\) mice were infected with 400 PFU of MHV-68 intranasally and left for >60 days. Lymphocytes from these latently infected mice were stained with either the ORF6487-499/D\(^\beta\) and ORF61524-531/K\(^\beta\) tetramers followed by anti-CD8\(\alpha\) Ab. The frequency of tetramer-positive cells within the CD8\(^+\) population was quantified for both the ORF6487-499/D\(^\beta\) (A) and ORF61524-531/K\(^\beta\) (B) tetramers in the spleen. Data for the ORF61524-531/K\(^\beta\)-specific CD8\(^+\) T cells were also expressed as total number of tetramer-positive cells per spleen, with the percentages above the bars representing the number of cell divisions. Data for C57BL/6 mice are represented by the open circles/bars whereas data for IL-15\(^{-/-}\) mice are represented by the filled circles/bars. Each data point represents a single mouse (p > 0.05).
ceased dividing and were unable to undergo homeostatic proliferation. These data highlight significant differences between memory CD8+ T cells induced by acute and latent infections.

Following intranasal administration of MHV-68, the acute infection was successfully cleared from the lungs. The virus replicated to higher titers in IL-15−/− mice at 10 days postinfection, but this had no impact on the establishment of splenic latency. The enhanced replication of MHV-68 in IL-15−/− mice was not due to a reduced frequency of the Ag-specific CD8+ T cells to the virus, as both the ORF6487−531/D)− and ORF61524−531/Kb-specific CD8+ T cell populations were induced to similar frequencies as found in C57BL/6 mice. One possible explanation for this difference is that although the frequency of the Ag-specific CD8+ T cells was the same, the total number of Ag-specific CD8+ T cells responding to the virus was less in the IL-15−/− mice because IL-15−/− and IL-15Rα−/− mice have ~50% of CD8+ T cells found in C57BL/6 mice (20, 32). Therefore, the absolute number of Ag-specific CD8+ T cells may not have been sufficient to limit viral replication at day 10 postinfection in the IL-15−/− mice. Another reason for the greater replication of the virus may be due to a lack of NK cells. It is known that the development and survival of NK cells is highly dependent on the presence of IL-15, and IL-15−/− mice are deficient in these cells (20, 32). NK cells have been shown to be important in the clearance of other herpesviruses in vivo (21, 40) and are known to inhibit the replication of EBV in vitro (22). However, by depletion studies NK cells do not appear to be important in the control of MHV-68 replication (E. J. Usherwood, S. Meadows, S. G. Crest, and C. L. Sentman, unpublished observations). In any case, by day 14 postinfection the adaptive immune response was effectively expanded and controlled the infection.

Since IL-15−/− mice were capable of resolving acute MHV-68 infection, we examined how the Ag-specific memory CD8+ T cells induced by MHV-68 behaved during the latent infection. Interestingly, we have found that during latent MHV-68 infection Ag-specific memory CD8+ T cells from the spleen of IL-15−/− mice remained at levels comparable to those found in C57BL/6 mice. MHV-68-specific CD8+ T cells were also maintained at similar frequencies within the peripheral tissues, such as the lungs and liver. In addition to the maintenance of the Ag-specific CD8+ T cell populations, the Vβ4+CD8+ T cell population was maintained independent of IL-15 during latent MHV-68 infection. Although the Ag-specific CD8+ T cells were maintained at similar frequencies in peripheral tissues, they trended toward being found at elevated frequencies. Recently, it has been shown that in vitro culturing of Ag-primed murine CD8+ T cells in the presence of IL-15 for 5 days skewed those cells toward a central-memory T cell phenotype, whereas culturing the same cells in the presence of IL-2 for 5 days skewed them toward an effector or effector-memory T cell phenotype (41). In our in vivo system, it is possible that continual antigenic stimulation of the CD8+ T cells in the absence of IL-15 will lead to the production of more effector or effector-memory T cells. These effector and effector-memory CD8+ T cells are known to preferentially home to peripheral organs where they are poised to clear or protect against pathogens entering the body from the environment (34). In addition to increased production of effector-memory cells, which can home to the periphery, it has been shown that effector-memory T cells are more resistant to apoptosis than central-memory T cells (42, 43). Taken together, the likely increased skewing of T cells to an effector-memory phenotype and those cells decreasing sensitivity to apoptosis will result in an increased frequency of Ag-specific CD8+ T cells within peripheral organs.

Since the Ag-specific CD8+ T cells did not decline in the absence of IL-15, we expected that those cells would be able to turn over in the absence of that cytokine. This was exactly what we found; in the IL-15−/− mice, the Ag-specific CD8+ T cells were still able to turn over in vivo, as measured by BrdU incorporation, and actually did so at a greater rate than in C57BL/6 mice. This
Since constant restimulation through the TCR appears to be important in the maintenance of the virus-specific CD8+ T cells in MHV-68 infection, we hypothesized that the increased proliferation rate in the virus-specific CD8+ T cells from IL-15−/− mice could be the result of elevated levels of viral Ags. One possible explanation for the elevated levels of viral Ags could be that the IL-15−/− mice were not able to control the long-term latent viral infection. This situation would be similar to what is seen in MHC II−/−, CD40−/−, and SAP−/− mice that are able to transiently control MHV-68 infection, but lose control of the latent infection around 30 days postinfection and new infectious virus is detectable (37, 49, 50). The loss of control over the latent infection in the MHC II−/− and CD40−/− mice is thought to be due to a defect in the CD8+ T cells (37, 49, 51). Since the IL-15−/− mice have a smaller CD8+ T cell compartment than C57BL/6 mice (20, 32), it was possible that the IL-15−/− mice may not control the long-term latent infection. To address this, we monitored the long-term latent infection by QF-PCR and found that the IL-15−/− mice had similar levels of MHV-68 genomes in both the spleen and lungs of latently infected mice. In addition, no infectious virus was detected in the spleens of IL-15−/− or C57BL/6 mice at these time points (data not shown).

Since the IL-15−/− mice did not appear to completely lose control of the latent MHV-68 infection, we asked whether the level of viral Ags was elevated in the absence of IL-15. It is possible that the latently infected cells are undergoing more spontaneous reactivation, but this process is stopped by the T cell response before the production of infectious virus. This would be analogous to what is believed to be occurring in the ganglion of mice latently infected with HSV-1, where gB-specific CD8+ T cells are constantly activated and prevent the production of new HSV-1 virions (52, 53). In our system, we used quantitative RT-PCR to examine the production of viral transcripts and found that IL-15−/− mice indeed had higher levels of the viral transcripts, suggesting more Ag was present. The increased levels of viral transcripts in IL-15−/− mice was not due to the CD8+ T cells being functionally compromised since those cells were able to produce IFN-γ during a 5-h restimulation with anti-CD3 or MHV-68 peptides (data not shown). In addition, Ag-specific CD8+ T cells from IL-15−/− mice also appear to be in a similar activation state as the same cells from C57BL/6 mice, they expressed similar levels of CD11a, CD25, CD43 (1B11), CD44, CD62L, CD122, and CD127, although cells from IL-15−/− mice did express slightly higher levels of CD69 and slightly lower levels of Ly6c (data not shown). Therefore, the reason for the increased viral transcripts in IL-15−/− mice is probably similar to why there was increased viral replication in the lungs at day 10 postinfection. This is most likely due to decreased CD8+ T cell numbers and the greater attrition of those cells. These two factors together would result in fewer Ag-specific CD8+ T cells actively patrolling for latently infected cells that are undergoing viral reactivation. When these reactivating cells are discovered by the CD8+ T cells, we propose that the reactivation will be inhibited or the cell will be killed by the activated CD8+ T cells before it is able to replicate its DNA and produce new infectious virions. This would be similar to what has been proposed for the control of latent HSV-1 infection in the sensory ganglia, although the effector mechanism may be different as sensory ganglia do not appear to be lost during long-term latent HSV infection (52, 53).

Even with the greater turnover rate of the Ag-specific CD8+ T cells in the IL-15−/− mice, the memory population within the spleen was the same size as in C57BL/6 mice, suggesting that in the IL-15−/− mice these cells might have a greater rate of cell death. Consistent with this idea, IL-15 has been shown to induce

**FIGURE 6.** Control of the latent virus is unaffected by the absence of IL-15. Latent viral load within the C57BL/6 and IL-15−/− mice was determined by QF-PCR for the ORF50 gene within the lungs (A) and spleen cells (B). Viral load is expressed as the number of viral genome copies per 300 ng of input DNA and was determined using a standard curve for a plasmid encoding the ORF50 (30). Open circles indicate samples from C57BL/6 mice, whereas filled circles indicate samples from IL-15−/− mice (p > 0.05). Finding was strikingly different from what others have found using memory CD8+ T cells induced by an acute viral infection where the number of Ag-specific CD8+ T cells decline over time and this decline is due to their inability to undergo homeostatic proliferation in response to IL-15 (8–11). Since the fundamental difference between an acute and latent infection is the persistence of viral Ag, we hypothesize that constant restimulation through the TCR would induce the proliferation of the CD8+ T cell.

During latent gammaherpesvirus infections, the majority of infected cells harbor latent virus and these cells express a limited number of viral Ags (44–46). However, a small population of latently infected cells will sporadically re activate to release infectious virus and spread to new hosts (47). This low-level reactivation results in the expression of many viral genes associated with viral replication, such as ORF6 and ORF61. The presentation of viral Ags to CD8+ T cells will then occur, providing a stimulus for these cells. This low-level restimulation of memory CD8+ T cells appears to induce their proliferation, as it has been reported that virus-specific memory CD8+ T cells turn over quicker during latent MHV-68 infection than following influenza infection (48).
Table 1. Expression of viral transcripts during latent MHV-68 infection (60 days postinfection)

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<th>Spleen</th>
<th>Lung</th>
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<tr>
<td></td>
<td>β-Actin ORF61</td>
<td>Relative Expression</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>14.5 31.7</td>
<td>1.00</td>
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<tr>
<td></td>
<td>14.15 32.7</td>
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<td></td>
<td>14.4 31.4</td>
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<td></td>
<td>14.3 32.0</td>
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<tr>
<td>IL-15−/−</td>
<td>14.5 29.65</td>
<td>3.05</td>
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<tr>
<td></td>
<td>14.0 28.65</td>
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a Viral gene expression during latent MHV-68 infection was monitored using QF-PCR to detect mRNA for the ORF61 gene. Data are representative of three experiments and similar data were obtained with the ORF6 gene.

b Relative expression was determined using the mean for each group and the formula: 2^{-ΔΔCt}. For each tissue, the expression level in C57BL/6 mice was set to a value of 1.

c ND. No message detected.

d Only the three detectable levels of ORF61 message were used to calculate the relative expression in this experiment.

Bcl-2 (39), a molecule that can prevent apoptosis in memory CD8+ T cells (38). We have shown that Ag-specific CD8+ T cells from the spleens of IL-15−/− mice latently infected with MHV-68 indeed had significantly lower levels of intracellular Bcl-2 than the same cells from C57BL/6 mice. This decrease in Bcl-2 levels made those cells more sensitive to apoptosis as measured by annexin V binding. This increase in early apoptotic cells was ~2-fold, with approximately the same increase seen in the proliferation of the Ag-specific CD8+ T cells, which probably accounts for why the levels of these cells remain the same in IL-15−/− mice as in C57BL/6 mice.

Since Ag appears to be crucial in the maintenance of the Ag-specific CD8+ T cells during latent MHV-68 infection, we evaluated the dependence of those cells on Ag for their proliferation. In this report, we show that virus-specific CD8+ T cells adoptively transferred from latently infected C57BL/6 mice into naive C57BL/6 underwent almost no proliferation after 30 days. This indicates a strict requirement for Ag in the proliferation and maintenance of the Ag-specific CD8+ T cells during latent viral infection. In addition, preliminary data indicate that the MHV-68-specific CD8+ T cells are unable to undergo homeostatic proliferation even in a lymphopenic environment (data not shown). These findings are strikingly

![FIGURE 7](Image) Bcl-2 levels are lower in the Ag-specific CD8+ T cells from IL-15−/− mice. C57BL/6 and IL-15−/− mice were infected with MHV-68 for 82 days, at which point spleen cells were then stained with the ORF61524-531/Kb tetramer and anti-CD8α Ab. After staining for cell surface markers, the cells were fixed, permeabilized, and stained with an anti-bcl-2 or isotype control Ab. Histograms are gated on the CD8+ tetramer-binding population. Values on the histograms indicate the average mean fluorescent intensity of Bcl-2 staining within the CD8+ tetramer-binding population ± 1 SD for that group of three to four mice. Staining with the isotype control Ab is shown by the filled histogram, Bcl-2 levels in cells from IL-15−/− mice are shown by the dark line, whereas C57BL/6 mice are represented by the light dotted line. These data are representative of four separate experiments, each containing three to four mice.

![FIGURE 8](Image) CD8+ T cells from latently infected C57BL/6 mice do not undergo homeostatic proliferation. CD8+ T cells were purified from C57BL/6 mice infected intranasally with 400 PFU of MHV-68 for >60 days by FACS sorting to >98% purity. Purified CD8+ T cells were labeled with CFSE. Five × 10^6 cells were injected i.v. into naive B6.PL-Thy1a mice and left for 28 days. At which point the cells were stained with anti-Thy1.2, anti-CD8α, and the ORF61524-531/Kb tetramer. The top histogram is the proliferation of all of the adoptively transferred CD8+ T cells, whereas the bottom histogram is the proliferation of the ORF61524-531/Kb-specific CD8+ T cells. These data are representative of two separate experiments, each of which contained four mice.
different from what is observed with memory CD8^+ T cells induced by acute viral infection or endogenously derived CD44^highCD8^+ memory T cells, since those cells will undergo one to three cell divisions over the same period time when adoptively transferred to naive recipients (9, 11, 27). This inability to undergo homeostatic proliferation could be attributable to the decreased cell surface levels of the IL-7 and/or IL-15 receptors, although their survival was improved (54). This suggests that MHV-68-specific CD8^+ T cells are unable to proliferate in response to IL-7 or IL-15 in vitro, although their survival was improved (54). This suggests that MHV-68-specific CD8^+ T cells are partially defective in their responses to IL-7 and IL-15, which will need to be explored further.

In conclusion, our work has shown that the requirements for the maintenance and survival of Ag-specific memory CD8^+ T cells are different following latent and acute viral infections. During latent MHV-68 infection, IL-15 was not essential for the maintenance of the Ag-specific CD8^+ memory T cell pool, whereas this cytokine is required for the survival of Ag-specific memory CD8^+ T cells following acute viral infections (9, 10). The survival of the Ag-specific memory CD8^+ T cells in IL-15^−/− mice during latent MHV-68 is likely due to the persistence of low levels of the virus. In addition, upon adoptive transfer to naive host the CD8^+ T cells fail to undergo homeostatic proliferation, indicating a strict requirement for the presence of Ag for the maintenance of the Ag-specific CD8^+ T cells in latent viral infection. These findings have important implications in understanding what is responsible for maintaining memory CD8^+ T cells during latent or chronic infections and also raises questions about what happens to memory CD8^+ T cells following clearance of chronic infections.

References

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