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Lifelong Persistent Viral Infection Alters the Naive T Cell Pool, Impairing CD8 T Cell Immunity in Late Life

Megan J. Smithey,*† Gang Li,*† Vanessa Venturi,‡§ Miles P. Davenport,‡§ and Janko Nikolich-Zugich*†

Persistent CMV infection has been associated with immune senescence. To address the causal impact of lifelong persistent viral infection on immune homeostasis and defense, we infected young mice systemically with HSV-1, murine CMV, or both viruses and studied their T cell homeostasis and function. Herpesvirus* mice exhibited increased all-cause mortality compared with controls. Upon Listeria-OVA infection, 23-mo-old animals that had experienced lifelong herpesvirus infections showed impaired bacterial control and CD8 T cell function, along with distinct alterations in the T cell repertoire both before and after Listeria challenge, compared with age-matched, herpesvirus-free controls. Herpesvirus infection was associated with reduced naive CD8 T cell precursors above the loss attributable to aging. Moreover, the OVA-specific CD8 T cell repertoire recruited after Listeria challenge was entirely nonoverlapping between control and herpesvirus* mice. To our knowledge, this study for the first time causally links lifelong herpesvirus infection to all-cause mortality in mice and to disturbances in the T cell repertoire, which themselves correspond to impaired immunity to a new infection in aging. The Journal of Immunology, 2012, 189: 000–000.

Aging is associated with a pronounced impairment in immune defense to new pathogens. Factors thought to contribute to this progressive weakening of the immune system include cell-intrinsic defects (1), but also may include lifelong dietary, metabolic, and microbial influences and other environmental stressors (2–5). Understanding the relative weight of each of these potential contributors is a complex task, requiring careful examination of each individual factor in longitudinal studies in both humans and experimental model systems.

Nearly every human carries multiple latent persistent viral infections (6), including HSV, varicella-zoster virus, EBV, and, above all, CMV. Over a lifetime, repeated interactions between CMV and Ag-specific T cells lead to “memory inflation” (7, 8) of the antiviral T cell populations in both mice and humans, which can occupy up to 50% of the human T cell pool in late life (9–11). Whereas other herpesviruses can sometimes produce similar effects, they are much less pronounced than those of CMV, likely due to a combination of CMV’s exquisite immune evasion and reactivation properties.

At the time memory inflation was discovered, several studies had clinically associated CMV positivity with the manifestations of immune aging (reviewed in Ref. 12). It has therefore been proposed that antiviral memory T cell inflation comes at a cost to the immune system as a whole and that CMV may produce many of the signs and symptoms of immune aging (12). The competing hypothesis, that CMV-positive individuals may contain a deficiency that simultaneously predisposes them to CMV infection and to pronounced immune aging, could not be tested in humans and was not addressed so far in experimental animals.

Moreover, if the relationship between CMV and any of the components of the immune aging were to be causal, one would need to posit and prove the hypothesis about the precise mechanisms of how that would occur. A homeostatic hypothesis could posit that there is competition between memory and naive T cells for homeostatic survival signals with aging, which could impair the maintenance of a diverse naive T cell pool. There is evidence that age-related T cell clonal expansions in unimmunized mice result in holes in the naive T cell repertoire, particularly for new pathogens whose response would be dominated by T cells of the same TCR Vβ family to which the T cell clonal expansions belong (13). The implication was that the presence of large populations of (memory) T cells within a particular TCR Vβ family somehow impairs new immune responses to pathogens also dominated by that TCR Vβ family. This may be due to 1) loss of naive clonotype diversity within that TCR Vβ family, or 2) impaired recruitment of naive clonotypes into the new response, which could occur through several mechanisms (14), and where the immune response could be affected by the general or specific accumulation of T cell clonal expansions. All of this would occur alongside the general loss of naive T cell precursors that occurs as a consequence of aging, resulting in an even less complete T cell repertoire and even more impaired responsiveness to infectious challenge (15, 16).

We report in this study that lifelong persistent herpesvirus infection in mice erodes CD8 T cell responses to a new pathogen encountered in late life, over and above the effects of aging itself.
Beyond the previously reported age-related impact on the CD8 T cell compartment, infection with both HSV and MCMV (MCMV)-like infection) and/or MCMV in early life exacerbated functional defects in the CD8 response to challenge with recombinant *Listeria monocytogenes* expressing the OVA surrogate antigen *Lm-OVA* in late life. Aged animals demonstrated a reduced number of naive CD8 T cell precursors specific for either the H-2Kb-presented immunodominant H-2Kb-restricted vaccinia epitope TSYKFESV (B8R)20–27 or OVA257–264 epitopes relative to young mice, and lifelong MCMV infection further eroded the number of naive B8R20–27-specific precursors. Furthermore, the TCR Vβ repertoire of CD8 T cells recruited into the Lm-OVA response was completely different in lifelong MCMV-infected mice relative to aged controls. To our knowledge, this is the first evidence that lifelong, persistent infection with herpesviruses results in changes to the naive CD8 T cell repertoire, with functional consequences to the immune response to new pathogens in late life.

**Materials and Methods**

**Mice and herpesvirus infection**

Eight-week-old C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were tested for 1 wk prior to herpesvirus infection. At 9 wk age, mice were infected with either 106 PFU HSV-1 (strain 17, as described in Ref. 17) or 105 PFU MCMV (strain Smith) i.p. For mice infected with both HSV-1 and MCMV, mice were first infected with HSV-1 as above, rested for 7 wk, and then infected with MCMV. Twenty-three–month-old C57BL/6 mice were purchased from the National Institute on Aging Aged Mouse Colony (Charles River Laboratories), rested in-house for 1 wk, and then infected with HSV-1 or MCMV as above. All mice were maintained under specific pathogen-free conditions in the animal facility at the University of Arizona, and experiments were conducted under guidelines set by the University of Arizona Institutional Animal Care and Use Committee.

**Virus-specific memory T cell infection**

To confirm lifelong infection with herpesviruses and antiviral memory CD8 T cell infection, a subset of animals was monitored at 3- to 6-month intervals throughout life. PBLs were stained with fluorochrome-conjugated Abs specific for CD8 (GK1.5), CD8 (53-6.7), CD4 (IM7), CD26 (MEL-14), and KLrg1 (2F1), and then stained with B8R20–27 Kb (HSV gB inflating CD8 epitope) or m139.19-296 Kb (MCMV inflating CD8 epitope) tetrarmers (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA), and then evaluated by flow cytometry.

**Survival analysis**

Mice were infected with HSV-1, MCMV, or both viruses at 9 wk age, then monitored daily for all-cause mortality from 11 mo age through 23 mo age. Survival analysis was performed using the log-rank test for each group. Survival up to 23 mo age (prior to their challenge with HSV-1 or MCMV) was compared with age-matched uninfected control animals housed in the same room throughout the experiment by Mantel-Cox log-rank test using GraphPad Prism software (GraphPad Software, San Diego, CA).

**L. monocytogenes infections**

At 21 mo following herpesvirus infection (23 mo old), mice were systemically infected by i.v. injection in the lateral tail vein with 1 × 107 CFU *Lm-OVA* (18) in 100 μl sterile PBS. Ten-week-old, naive young C57BL6 mice (The Jackson Laboratory) as well as 23-month-old C57BL6 mice (National Institute on Aging) were included as additional control groups for analyses of the response to *Lm-OVA* challenge. The dose of inoculated bacteria was determined retrospectively by plating serial dilutions of the injected bacterial suspension onto brain heart infusion agar and counting colonies the next day.

**Intracellular cytokine staining and flow cytometric analysis**

Seven days following Lm-OVA challenge, spleenocytes were collected and passed through a 40-μm mesh screen to prepare single-cell suspensions. Cells were incubated for 6 h at 37°C in a total volume of 100 μl RPMI 1640 plus 5% FCS containing 0.1 μg/ml brefeldin A (eBioscience) plus either 10−6 M OVA257–264 peptide (SIINFEKL), 10−3 M Listeriolysin O (LLO)189–200 peptide (WNEKYAAYPVN), or no peptide. Alternatively, cells were stimulated for 6 h in the presence of PMA/ionomycin (BD Pharmingen leukocyte activation mixture). Cells were washed, stained overnight at 4°C with fluorochrome-conjugated Abs specific for the surface markers CD4 (GK1.5) and CD8a (53-6.7), washed, fixed and permeabilized, and then stained for intracellular IFN-γ (XM1.2), TNF-α (MP6-XT22), granzyme B (GB11), IL-2 (JES6-5H4), and IL-17A (eBio1B7) using the eBioscience Foxp3 Fix/Perm buffer kit according to the manufacturer’s directions. Data acquisition was performed on a custom-made, four-laser BD LSRFortessa flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). A minimum of 10,000 CD8+ events within the lymphocyte gate was collected for all files.

**Tetramer pull-down assay**

The tetramer-enrichment protocol was slightly modified (19). The spleen, inguinal, cervical, and axillary lymph nodes were pooled from individual mice in an attempt to collect most of the naive T cell population. Cells were resuspended in 1 ml isolation buffer (PBS with 0.2% NaN3, 0.5% BSA, and 2 mM EDTA) and stained with anti-CD8a (53-6.7), PE- and allophycocyanin-labeled OVA257–264-Kb or B8R20–27-Kb tetrarmers (National Institutes of Health Tetramer Core Facility), and Fc block for 1 h at room temperature. Cells were washed, resuspended in 500 μl isolation buffer plus 50 μl anti-allophycocyanin and anti-PE microbeads (Miltenyi Biotec), and slowly rocked for 30 min at 4°C. Cells were washed, resuspended in 500 μl isolation buffer, and passed over an LS SepElute column (Miltenyi Biotec) according to the manufacturer’s instructions. The columns were removed from the magnetic field, and bound cells were eluted by pushing 5 ml isolation buffer through the column a plunger. The resulting tetramer-enriched fractions were stained with a mixture of fluorochrome-labeled Abs for 30 min at 4°C that served as a “dump” gate: anti-CD4 (G4-100), anti-CD8 (53–6.7), anti-CD44 (IM7), and anti-MHC class II (M5/114.15.2), and anti-F4/80 (BM8). Cells were washed and the entire sample analyzed by flow cytometry. Precursors were also isolated from young adult animals as a control for every experiment, and they served as quality control, demonstrating that we can isolate the expected precursor numbers in young adult animals, and that therefore the lower precursor numbers in old animals were not caused by technical difficulties.

**Single-cell sorting for OVA-specific CD8 TCR sequencing**

Splenocytes collected 7 d following Lm-OVA challenge were processed into single-cell suspension as above, then negatively enriched for CD8+ cells using immunomagnetic beads (Miltenyi Biotec). Enriched CD8+ cells were stained with fluorochrome-conjugated anti-CD8a (53-6.7), anti-CD4 (GK1.5), anti-CD44 (IM7), anti-iV-β5.1/5.2 (MR-9-4), and OVA257–264-Kb tetramers (National Institutes of Health Tetramer Core Facility) intravenously on ice, then washed twice. CD8+ CD844-OVA254-Kb iV-β5 lymphocytes were sorted as single cells into 96-well plates with a FACSAria cell sorter (BD Biosciences). Control wells without sorted cells were included on every plate to control for contamination.

**cDNA synthesis and RT-PCR**

Our RT-PCR protocol was adapted from previous studies (20, 21). Single CD4+ CD8-OVA257–264-Kb iV-β5 CD4+ cells were sorted directly into 96-well PCR plates containing 5 μl cDNA reaction mix: 0.25 μl Sensiscript reverse transcriptase (Qiagen), 1× cDNA buffer (Qiagen), 0.5 mM 2′-deoxynucleoside 5′-triphosphate (Qiagen), 100 μg/ml tRNA (Invitrogen), 50 ng oligo(dT)12–18 (Invitrogen), 20 U RNaseOUT (Invitrogen), and 0.1% Triton X-100 (Sigma-Aldrich). cDNA synthesis was performed immediately after sorting by incubating plates at 37°C for 90 min, followed by 5 min at 95°C. Plates were immediately stored at −80°C.

VB5 transcripts were amplified by nested PCR with the entire 5′ cDNA reaction used for the first PCR reaction in a final 25 μl volume containing 1.25 μl DreamTaq polymerase (Fisher Scientific) in the manufacturer’s 1× buffer with 200 μM each 2′-deoxynucleoside 5′-triphosphate (Fisher Scientific) and 100 μM external degenerate sense VB5 primer (5′-GGGGTTGTCCAGTCTCC-3′) and antisense VB5 primer (5′-CCAGAGAACAGACTGGCC-3′). The PCR cycling program used was 5 min at 95°C; 40 cycles of 20 s at 95°C, 20 s at 56°C, and 45 s at 72°C; and ended with 5 min at 72°C. A 4-μl aliquot of each PCR product was used for the second PCR reaction with the internal degenerate VB5 sense primer (5′-CAGCAGATTTCTGACTGTC-3′) and the internal antisense VB5 primer (5′-GGTGGTCTGCTGCTGG-3′). The second PCR program was the same as the first, with 35 rounds of amplification. PCR products were purified with MinElute 96 UF PCR purification kits (Qiagen) and sequenced with 12 pmol internal degenerate VB5 sense primer using an Applied Biosystems 3730XL DNA analyzer at the University of Arizona Genomics Core (Tucson, AZ).
**TCRβ clonotype analysis**

OVA\textsubscript{257-264}-specific CD8\textsuperscript{+} TCRβ clonotypes were characterized by sequentially aligning each TCRβ sequence with the V\textsubscript{\beta}5.1 or 5.2 (TRBV12 in international ImMunoGeneTics information system nomenclature) gene and then the best match J\textsubscript{\beta} gene using the international ImMunoGeneTics information system reference alleles for the Mus musculus TRB genes (22). The CDR3\textsubscript{\beta} sequence was then identified between, and inclusive of, the conserved cysteine in the V\textsubscript{\beta} region and the conserved phenylalanine in the J\textsubscript{\beta} region.

**Statistical analysis**

All analyses were performed using GraphPad Prism software (GraphPad Software). Survival comparisons were performed by a Mantel–Cox log-rank test. Other analyses were performed by one-way ANOVA with a Dunnett posttest using herpesvirus-free aged mice as the comparison control. Probability values of \(p < 0.05\) were considered to be significant.

**Results**

**Study goals and design**

The goal of this longitudinal study was to evaluate how lifelong persistent systemic infections with the herpesvirus family members HSV-1 and MCMV might impact the ability of the adaptive immune system to mount a productive response to a new pathogen late in life. Specifically, we were interested in whether a lifetime of interactions between the immune system and a persistent virus might alter the function and/or repertoire diversity of the naive CD8 T cells remaining in old animals. To that end, four cohorts of 9-wk-old C57Bl/6 mice were infected systemically with either HSV (produces MCMV-like memory inflation) (10), MCMV, both viruses, or neither virus. Mice were then monitored for survival and allowed to age with their persistent virus(es) until they reached 23 mo age. The establishment of productive herpesvirus infection was monitored by memory CD8 T cell infection to either the gB\textsubscript{498-505} (HSV infection) or m139\textsubscript{419-426} (MCMV infection) determinants during the first 15 mo following infection (Fig. 1) (10, 11).

Our general strategy was to monitor animals for mortality up to the time they were challenged at 21 mo after herpesvirus infection (23 mo old) with recombinant *L. monocytogenes* expressing the OVA protein. Seven days following Lm-OVA challenge we evaluated 1) bacterial burden in the liver, 2) effector properties of the OVA-specific CD8 T cell response in the spleen by polyfunctional intracellular cytokine staining, and 3) CD8 effector repertoire diversity by TCR V\textbeta-chain sequencing of individually sorted OVA\textsubscript{257-264}-K\textsuperscript{\alpha} tetramer\textsuperscript{+} cells.

**Lifelong persistent herpesvirus infection impacts lifespan**

It has been observed in human subjects that the accumulation of memory CD8 T cells specific for CMV correlates with increased mortality in late life (3, 9, 23, 24). To determine whether such an effect is causal to CMV infection or whether, perhaps, genetic or lifestyle factors that predispose to early mortality may also predispose to CMV infection, we used a mouse model in which other potential contributors such as genetic background, lifetime pathogen exposure, diet, and environment were controlled for. We monitored mortality in our lifelong herpesvirus-infected cohorts to 23 mo age, just prior to challenge with *Lm*-OVA. Survival of each group (prior to *Lm*-OVA infection) was compared with age-matched herpesvirus-free control mice that were housed in the same animal room throughout the experiment. Comparisons of survival curves between these groups (in the absence of any deliberate pathogen challenge, and prior to all reaching natural deaths) found that animals that were dually infected with both HSV-1 and MCMV had just approached the threshold of significance for mortality relative to age-matched controls (Fig. 2, \(p = 0.05\)). Single infection with HSV-1 or MCMV did not significantly shorten lifespan by this measure, although importantly note that these animals were not allowed to live until natural death occurred, but rather they were monitored until their challenge with *Lm*-OVA at 23 mo age. Whether increased mortality would be observed in single herpesvirus-infected mice (HSV-1 or MCMV) beyond 23 mo age is unknown, although there was a trend for higher mortality with infection with HSV-1 alone (\(p = 0.0586\)).

**Lifelong viral infections further impair age-related CD8 T cell function**

We have previously characterized pronounced functional defects in the CD8 T cell responses of aged mice following acute infection with West Nile virus or *L. monocytogenes*. In these studies, aged mice showed decreased proliferation and upregulation of activation markers within the pathogen-specific CD8 T cell population, resulting in a numerically reduced effector CD8 T cell pool at the peak of the response, lower capacity to produce multiple effector...
molecules on a per-cell basis (including cytokines and lytic proteins, termed “polyfunctionality”), reduced quantities of the individual effector molecule(s)/cell, and a diminished ability to lyse targets bearing cognate Ag (25, 26). Similar age-associated defects in CD8 expansion and/or effector function have been observed in response to influenza, LCMV, and *Encephalitozoon cuniculi* infections as well (27–32). Collectively, these reports indicate that aging alone has severe consequences on the ability of the immune system to mount a robust CD8 T cell response to pathogens of varying classes, pathogenesis, and host-cell tropism.

We evaluated whether further impairment above and beyond age-associated CD8 T cell defects would be evident in animals that had experienced lifelong infections with herpesviruses. To that end, 23-mo-old mice that had been infected with HSV, MCMV, or both viruses in early life were challenged with *Lm-OVA*, and the splenic OVA257–264–specific CD8 T cell population was evaluated 7 d later. As expected, there was a marked decrease in the magnitude of the OVA-specific CD8 T cell response in aged mice, as measured by IFN-γ production following brief in vitro stimulation (Fig. 3) (26). This reduction was not significantly worsened in aged mice that had been infected with HSV-1, MCMV, or both viruses since 9 wk age, compared with their age-related uninfected littermates, suggesting that lifelong viral infection does not significantly impact the magnitude or expansion of a primary CD8 T cell response to an intracellular bacterium in late life.

Polyfunctional cytokine production, that is, the ability of individual cells to produce multiple different effector molecules in response to Ag recognition, is thought to be a hallmark of highly functional CD8 T cell populations that successfully control pathogens. As a measurement of the “robustness” of the effector CD8 T cells generated in our lifelong infected cohorts, we determined whether the OVA257–264–specific CD8 T cells elicited following *Lm-OVA* challenge were functionally altered. Following in vitro stimulation with OVA257–264 peptide, the ability of IFN-γ–producing cells to additionally make TNF-α, granzyme B, IL-17A, and/or IL-2 was measured. Very little production of IL-2 or IL-17A was seen in the OVA-specific CD8 T cell population, so these data have not been stratified out into separate populations for simplicity. Furthermore, IFN-γ–producing cells were the largest population seen in all groups, indicating this cytokine maintains its position at the top of the effector molecule hierarchy for all cohorts evaluated.

As shown in Fig. 4A, relative to the distribution of polyfunctional CD8 T cells seen in young adults, aged mice showed (as expected) a significant loss of most functional effector populations: those able to simultaneously produce IFN-γ, TNF-α, and Granzyme B (filled bars, I+T+G+) through those producing only IFN-γ (open bars, I+T+G2), with conservation of the small population seen in all groups, indicating this cytokine production following brief in vitro stimulation (Fig. 3) (26). This reduction was not significantly worsened in aged mice that had been infected with HSV-1, MCMV, or both viruses since 9 wk age, compared with their age-related uninfected littermates, suggesting that lifelong viral infection does not significantly impact the magnitude or expansion of a primary CD8 T cell response to an intracellular bacterium in late life.

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In animals that had experienced lifelong persistent infection(s) with HSV-1, MCMV, or both viruses, there was significant further erosion in two subpopulations of functional T cells beyond that attributable to aging alone: both the IFN-γ+/TNF-α– (hatched bars, I+T+G2) and IFN-γ-only (open bars, I+T+G2) subsets were significantly reduced in lifelong herpesvirus-infected cohorts relative to the response seen in virus-free aged mice (Fig. 4A). Thus, in addition to the effector molecule defects seen in primary CD8 T cell responses that result as a consequence of aging alone, to our knowledge these data show for the first time that lifelong infection...
with herpesviruses further erodes the functional capacity of effectors stimulated in response to a new infection in late life.

Lifelong viral infections do not affect CD4 T cell function

When we performed the same polyfunctional evaluation of the L. monocytogenes LLO189–200-specific CD4 T cell response, no such impact was seen (Fig. 4B). The distribution of polyfunctional L. monocytogenes–specific CD4 T cells into four functional categories defined by IFN-γ (with/without TNF-α and with/without granzyme B production) was comparable in aged mice with and without lifelong persistent herpesvirus infection. Although significant loss of all polyfunctional populations was observed as a consequence of aging (adult versus old), further attrition was not seen in any functional CD4 subsets in the lifelong-infected cohorts (old versus HSV, MCMV, or both). This suggests that the detrimental impact of lifelong persistent herpesvirus infection on T cell functionality is restricted to the CD8 T cell pool.

We next asked whether this impact on CD8 polyfunctionality might reflect a larger, global impairment of CD8 T cell function in general. To address this possibility we measured the polyfunctional responses of both the CD8 and CD4 subsets in the same animals following nonspecific stimulation with PMA and ionomycin, bypassing the TCR signal transduction machinery. Although age-related decreases in some functional subsets were observed (comparing adult versus old animals), for the most part these were not further depressed in the lifelong-infected cohorts (comparing old

### FIGURE 4.

Lifelong herpesvirus infections impair the polyfunctional cytokine responses of CD8 T cells responding in late life to Listeria challenge. Mice were infected with HSV, MCMV, or both viruses at 9 wk age. At 23 mo age, these mice plus 10-wk-old adult controls were challenged with 10^7 CFU Lm-OVA. Seven days later the frequency of splenic cytokine-producing (A) OVA257–264-specific CD8 T cells or (B) LLO189–200-specific CD4 T cells was determined by intracellular cytokine staining. Within each IFN-γ–producing population, the ability to also produce TNF-α and/or granzyme B was assessed. The composite response of all cytokine-producing, Ag-specific T cells in each category is shown on the left, with each polyfunctional subpopulation shown to the right with statistics. Floating bars represent the minimum and maximum response in each animal group, with a line at the mean. Significance was determined by one-way ANOVA with a Dunnett posttest relative to old mice (no herpesvirus infection). Data represent one of two replicate experiments with n = 8 mice/group. **p < 0.01, ***p < 0.001.
versus HSV, MCMV, or both; Supplemental Fig. 1), although MCMV infection (both with and without coinfection with HSV) significantly decreased the CD8 effector population capable of producing both IFN-γ and TNF-α (Supplemental Fig. 1A, hatched bars, I+T+G−). Thus, persistent infection with herpesviruses appears to primarily impact the functional properties of CD8 T cells recruited to a new infection, and it does not reflect an overall dampening of T cell responsiveness.

Persistent viral infection might influence antilisterial immunity as a side effect of proinflammatory cytokine production due to the ongoing anti-herpesvirus response (33, 34). Control experiments were performed to assess this issue. First, we determined whether short-term herpesvirus infection of aged mice would have a similar dampening effect on the CD8 T cell response to Lm-OVA challenge as did lifelong infection as shown in Fig. 4. To this end, 23-mo-old (naive) mice were infected with HSV or MCMV and then rested for 1 mo. Animals were subsequently challenged with Lm-OVA, and the polyfunctional analysis of OVA257–264-specific CD8 T cells in the spleen was determined 7 d later. Under this short-term viral infection condition, we found no unfavorable influence of either HSV or MCMV infection on the polyfunctional CD8 T cell response to Lm-OVA (Fig. 5). In fact, in HSV-infected animals, the IFN-γ+ and TNF-α+ subpopulations (gray bars, I+T+G+) of OVA257–264-specific effectors was significantly improved. Collectively, these data suggest that long-term, persistent infection with HSV or MCMV specifically impairs the ability to mount highly functional CD8 T cell responses to a new pathogen in late life, and that lifelong interactions between the immune system and the persistent virus are required for this erosion of CD8 T cell function.

Lifelong MCMV infection impairs bacterial clearance in late life

Because of its unique intracellular life cycle and ability to spread from cell to cell, pathogen clearance and protective immunity to Listeria are primarily mediated by CD8 T cells (reviewed in Ref. 35). As such, we measured whether the functional decay in the OVA257–264-specific CD8 T cell population in lifelong herpesvirus-infected animals impaired bacterial clearance after Listeria challenge. On day 7 after Lm-OVA infection, livers were homogenized and the bacterial burden was determined. Although there was a high degree of variability in the bacterial loads, animals harboring lifelong persistent MCMV infection had a significantly higher number of bacteria than did the herpesvirus-free, age-matched controls (Fig. 6, old versus MCMV). This suggests that there are biologic consequences to the impaired polyfunctional CD8 T cell responses in mice with lifelong herpesvirus infections (Fig. 4).

Lifelong MCMV and the maintenance of total and Ag-specific naive CD8 T cell pools

Aging, in the absence of persistent infections, results in decreased naive CD8 T cell precursor numbers and diversity (36, 37), leaving holes in the repertoire that increase susceptibility to new pathogens (15).

To evaluate how lifelong persistent herpesvirus infection influences the naive CD8 T cell pool over time, the number of naive CD8 T cells per spleen in HSV, MCMV, dually infected (HSV plus MCMV) mice, and uninfected age-matched controls was determined at various times after infection. We found no evidence that persistent herpesvirus infection further significantly eroded the global naive CD8 T cell pool in the spleen above and beyond the effect of aging alone (Fig. 7A). We also found that the number of naive CD8 T cell splenocytes held steady throughout most of the lifespan, with a precipitous drop in late life (Fig. 7A).

We next evaluated the influence of aging alone on the number of naive CD8 T cell precursors for two different Kb-restricted Ags: OVA257–267 used for our polyfunctional analysis above, and the B8R20–27 determinant shared by numerous poxvirus family members (including vaccinia, variola, ectromelia, and cowpox) (38). Pooled spleens and lymph nodes from individual adult and 18-mo-old naive C57BL/6 mice were negatively enriched for CD8 T cells, and then naive OVA- or B8R-specific CD8 T cell precursors were magnetically isolated by the tetramer pulldown technique (representative FACS plots are shown in Supplemental Fig. 2) (19, 39). We found that both Ag-specific precursor populations were significantly reduced in aged naive (herpesvirus-free) mice (Fig. 7B, 7C). In adult mice, the average number of OVA-and B8R-specific naive CD8 precursors was

![FIGURE 5. Short-term herpesvirus infection in aged mice does not influence CD8 functionality after Listeria challenge. Twenty-three–month-old mice were infected with HSV, MCMV, or neither and then rested for 1 mo before challenge with 10^5 CFU Lm-OVA. Seven days later the frequency of splenic cytokine-producing OVA257–264-specific CD8 T cells was determined by intracellular cytokine staining. Within each IFN-γ-producing population, the ability to also produce TNF-α and/or granzyme B was assessed. The composite response of all cytokine-producing, Ag-specific T cells in each category is shown on the left, with each polyfunctional subpopulation shown to the right with statistics. Floating bars represent the minimum and maximum response in each animal group, with a line at the mean. Significance was determined by one-way ANOVA with a Dunnett posttest relative to old mice (no herpesvirus infection) (n = 4–8 mice/group). **p < 0.01.](http://www.jimmunol.org/Downloadedfrom/0022-1767-190-5-6886-f0005.jpg)
viruses at 9 wk age and then challenged with 10^5 CFU Lm-OVA at 23 mo age. Seven days later, bacterial burdens in the liver were determined by plating serial dilutions of tissue homogenates onto brain heart infusion agar. Significance was determined by one-way ANOVA with a Dunnett posttest relative to age-matched old mice lacking persistent herpesvirus infection. Data were pooled from two replicate experiments (n = 13–17 mice/group). *p < 0.05 relative to old (no herpesvirus infection).

Because the OVA-specific naive precursor pool was already reduced in aged mice to near the limits of detection, we focused on the B8R-specific naive CD8 precursors to evaluate whether lifelong persistent herpesvirus infection would further diminish the naive CD8 precursor pool. Our rationale was that additional naive precursor loss as a consequence of lifelong viral infection would be difficult to conclusively measure in the OVA precursor population owing to the small pool present in aged mice (in the absence of lifelong infection). In contrast, the B8R-specific precursor population was still relatively large in aged mice (mean of 140 cells per aged mouse). Of interest, as shown in Fig. 7D, mice that had experienced lifelong infection with HSV had reduction in naive B8R-specific CD8 precursors (mean, 83 cells) that was not significant, whereas both MCMV and dually infected (HSV plus MCMV) mice showed a significant reduction in their B8R precursor pool as compared with age-matched controls (74 and 79 cells, respectively). These data indicate that lifelong persistent herpesvirus infection can further erode the naive CD8 precursor pool, exacerbating the pronounced loss of precursors that occurs due to aging alone.

**Lifelong MCMV infection changes the repertoire of cells recruited into a new response in late life**

Our experiments to determine the number of naive CD8 T cell precursors specific for the B8R peptide suggested that lifelong MCMV infection markedly changed the naive pool that remains in late life (Fig. 7D). Furthermore, our polyfunctional cytokine response suggested that the OVA257–264-specific CD8 T cells recruited into the response to Lm-OVA were functionally compromised in animals with lifelong persistent herpesvirus infection. To directly assess the impact of persistent viral infection on the T cell repertoire, TCR VB CDR3 sequencing was performed. Lifelong MCMV-infected mice (with and without HSV coinfection) and uninfected controls were challenged with Lm-OVA at 23 mo age. On day 7 after Lm-OVA challenge, VB55OVA257–264 Kb tetramer CD8 T cells were individually sorted for single-cell TCR VB sequencing analysis. The results from this analysis were striking. Across the herpesvirus-free, old mice, there were four TCR VB clonotypes that were each shared in two of three mice.

One of these four clonotypes was dominant within at least one of the mice, and all four were found to constitute ~65–70% of the OVA257–264-specific population in all uninfected animals (Fig 8, top row; clonotypes in red-orange). However, in old mice with lifelong MCMV infection, none of the clonotypes observed in the herpesvirus-free, old mice, including these four main shared clonotypes, was found. Rather, five different shared clonotypes were found in at least two of the four animals with lifelong MCMV infection (some also with HSV infection), and these clonotypes were not recovered from the herpesvirus-free, old mice (middle and bottom rows, dominant clones in blue-green). The dominance of the shared sequences was much lower in the MCMV-infected mice; in one animal these four sequences made up 94% of the entire

**FIGURE 6.** Lifelong MCMV infection impairs clearance of *Listeria* challenge in late life. Mice were infected with HSV, MCMV, or both viruses at 9 wk age and then challenged with 10^5 CFU Lm-OVA at 23 mo age. Seven days later, bacterial burdens in the liver were determined by plating serial dilutions of tissue homogenates onto brain heart infusion agar. Significance was determined by one-way ANOVA with a Dunnett posttest relative to age-matched old mice lacking persistent herpesvirus infection. Data were pooled from two replicate experiments (n = 8 mice/group). *p < 0.05 relative to old (no herpesvirus infection).

**FIGURE 7.** Lifelong MCMV infection further erodes the aged naive memory CD8 T cell pool. (A) Mice were infected with HSV, MCMV, neither virus, or both viruses at 9 wk age, and the number of CD44+ CD62L+ phenotypically naive CD8 T cells in the spleen was monitored over time. (B–D) The number of naive epitope-specific CD8 T cells within pooled splenocytes and lymph nodes from individual animals was determined by tetramer pull-down assay. Adult (10-wk-old) and Old (23-mo-old) uninfected C57BL/6 mice were evaluated for the number of (B) OVA257–264-Kb- and (C) B8R20–27-Kb-specific naive CD8 T cell precursors. (D) At 23 mo age, lifelong MCMV-infected, HSV-infected, MCMV plus HSV–infected, and uninfected control mice were assessed for the number of B8R20–27-Kb–specific naive CD8 T cell precursors. Significance was determined by either (A) Mann–Whitney U test or (B) one-way ANOVA with a Dunnett posttest relative to old mice lacking persistent herpesvirus infection (n = 8 mice/group). *p < 0.05.
connect lifelong persistent herpesvirus infection to manifestations associated with impaired naive T cell maintenance and function in aging.

CMV was associated with reduced residual life span in octo- and nonagenarians in a Swedish study (23) and with increased cardiovascular mortality in Hispanic-Americans (40). Although our study was not designed to assess full-course mortality at the end of life, it was nonetheless interesting to find that all-cause mortality was increased to just the point of significance at 23 mo age in the CMV plus HSV–infected group compared with controls. Of note, our systemic HSV infection model is, from the standpoint of infection and establishment of latency, much more similar to CMV in humans than HSV in humans (10). Proper longevity and mortality studies are currently in progress; however, given that in the Hadrup et al. (23) study T cell repertoire loss correlated to increased mortality, this raises a highly intriguing parallel between that study and our data in this study, and it mandates tracking the two in individual mice.

The second fundamental discovery was that herpesvirus+ mice exhibited perturbations in naive repertoire more profound than those seen in aging alone. A highly diverse T cell population is critical for protection against pathogens. The diversity of the T cell response to infection (i.e., the number of different clonotypes participating) is a better correlate of protection than the magnitude of the response (13, 41–43). As little as a 2- to 3-fold reduction in TCR repertoire diversity dramatically impairs Ag-specific responses (44, 45), and it is the T cell defects in the primary immune response that were identified as a major contributor to immune senescence. Thymic involution and the reduced generation of new naïve T cells, followed by an initially successful, but eventually failing, peripheral maintenance of a naïve T cell pool led to the loss of numbers and diversity within the naïve T cell pool over a lifetime, contributing to impaired immunity in the elderly. In terms of absolute cell numbers, we found a dramatic loss of splenic naïve CD8+ T cells between ages 18 and 22 mo in mice (16–20 mo post-infection; Fig. 7A) that was not further exacerbated by lifelong persistent herpesvirus infection. To our knowledge, this is the first formal demonstration of maintenance and loss of naïve T cell numbers in a longitudinal mouse study.

Although CMV in our experiments did not lead to global numerical loss of phenotypically defined naïve T cells, it led to reduction of precursors specific for the poxvirus B8r epitope, whose numbers were large enough to allow analysis using mPmhc tetramer pull-down. Of note, tetramer pull-down does not discriminate between different categories of cells that are Ag inexperienced, for example the truly phenotypically naïve CD44hiCD62Lhi cells (46). It is possible to track lifelong herpesvirus infection or not (represented in white). The list of recovered TCR Vβ sequences and their distributions in animals is provided in Supplemental Table I. Although performed on a limited sample, these data nonetheless suggest that substantial repertoire changes occur in mice that have experienced lifelong persistent infection. In herpesvirus-free old mice, the diversity of the naive repertoire pool appears to become narrowed to a few shared dominant clonotypes. In contrast, these clonotypes are absent in animals with persistent, lifelong MCMV infection, and others emerge in their place.

Discussion

In this study, we have deliberately infected mice with different herpesviruses and longitudinally explored the effects of such infection upon T cell homeostasis and function. We report three main and novel observations: 1) an increase of all-cause mortality in herpesvirus-infected animals that just nearly reached significance (p = 0.05) at the time of late-life challenge with a novel bacterial pathogen; 2) reduced bacterial clearance and depressed and altered CD8+ T cell functional reactivity; and 3) repertoire disturbances that were evident both in the unimmunized naïve and microbial pathogen-mobilized CD8+ T cell repertoires. To our knowledge, these findings, for the first time, causally response, whereas two other mice predominantly used “unshared sequences” that did not appear in any other mouse, MCMV infected or not (represented in white). The list of recovered TCR Vβ sequences and their distributions in animals is provided in Supplemental Table I. Although performed on a limited sample, these data nonetheless suggest that substantial repertoire changes occur in mice that have experienced lifelong persistent infection. In herpesvirus-free old mice, the diversity of the naive repertoire pool appears to become narrowed to a few shared dominant clonotypes. In contrast, these clonotypes are absent in animals with persistent, lifelong MCMV infection, and others emerge in their place.
would seem that, even in genetically identical mice, the possibility that a given clonotype (defined by its unique TCR sequence) will be found in separate animals is highly unlikely. However, numerous studies of Ag-specific responses to infection have found identical TCR β-chain sequences in the responding CD8 T cell populations in different animals, suggesting the presence of shared naive clonotypes in the T cell pool. Such “public” clonotypes stand in contrast to “private” clonotypes that are unique to individual animals (51, 52). In our evaluation of the CD8 TCR Vβ repertoire elicited in old mice challenged with Lm-OVA, we found a broad degree of sharing between aged mice, as well as the expected clonally limited response. However, the finding that no single sequences found in MCMV-infected mice were recovered in herpesvirus-free old controls (and vice versa) was unexpected. This suggests that the lifelong interactions between persistent viral infection shapes the naive CD8 T cell repertoire to such an aggressive degree that the entire naive pool shared no overlap with the repertoire that exists in the absence of persistent herpesvirus infection. Clearly, additional studies, including deep overlap with the repertoire that exists in the absence of persistent viral infection shapes the naive CD8 T cell repertoire throughout our longitudinal analysis, we cannot comment on whether the entire population of known inflating MCMV-specific CD8 epitopes was similarly dampened in these animals. Memory inflation is a complex and incompletely understood process that is likely driven by multiple factors, including microbial flora variations within animal facilities, and, based on the microbial barrier control in the facilities we have experienced at the Oregon Health and Science University (2001–2008) and the University of Arizona (2008–present), likely explains differences in memory inflation seen here and in prior work (9, 11). However, what is clear from our results is that even relatively modest levels of antiviral memory CD8 inflation can significantly alter immune function in aging.

Importantly, the observed repertoire alterations were accompanied in herpesvirus+ animals by altered functional responses of the remaining CD8, but not CD4, T cells, and with reduced ability to clear the bacterial infection, which was over and above the one seen in the absence of herpesvirus infection. While CMV and/or HSV-1 did not lead to significant numerical or percent reductions to the OVA-specific CD8 T cell population, the responses were already highly reduced by aging, and therefore the overall low numbers of the responding cells may have precluded detection of this effect.

Subtle differences were seen when comparing lifelong persistent infection with HSV versus MCMV (summarized in Table I). In general, defects in T cell function, pathogen clearance, and T cell repertoire were predominant in mice infected with MCMV (with or without coinfection with HSV). In contrast, increased all-cause mortality prior to late-life challenge with Lm-OVA, as well as the lack of effect on Lm-OVA clearance, was only observed in mice with lifelong HSV infection. Of consideration is that the levels of viral reactivation with persistent HSV versus MCMV infection may be quite different and thus have different impact(s) on the inflammatory environment generated in response to reactivation (both quantitatively in the frequency of viral reactivation, and qualitatively in the anatomical locations of reactivation and the immune response triggered at those sites). To the best of our knowledge, these interesting questions remain unanswered. One possibility is that the background inflammatory environment in old mice harboring persistent HSV may be somewhat more resistant to Listeria infection, as previously demonstrated for both γHV68 and MCMV infection, although it was not seen for HSV-1 infection in young mice (33). Furthermore, although this effect (with other herpesvirus family members) was shown to be transient in young animals (34), it is unclear how lifelong infection may alter the inflammatory environment over a lifetime and into old age.

It is of further note that such dramatic influences on the immune system were observed in our persistently infected cohorts that showed relatively lower levels of antiviral CD8 memory inflation compared with those we and others have previously reported (9, 11). Because we only monitored one inflating MCMV epitope throughout our longitudinal analysis, we cannot comment on whether the entire population of known inflating MCMV-specific CD8 epitopes was similarly dampened in these animals. Memory inflation is a complex and incompletely understood process that is likely driven by multiple factors, including microbial flora variations within animal facilities, and, based on the microbial barrier control in the facilities we have experienced at the Oregon Health and Science University (2001–2008) and the University of Arizona (2008–present), likely explains differences in memory inflation seen here and in prior work (9, 11). However, what is clear from our results is that even relatively modest levels of antiviral memory CD8 inflation can significantly alter immune function in aging.

Overall, together with the two other recent studies by our (53) and the Karrer group (54) investigating the impact of CMV upon antiviral immunity, we think that our data present a comprehensive picture of accumulating hits to the CD8 T cell compartment in mice experiencing a lifelong herpesvirus infection. Over time, age-associated loss of naive CD8 T cell precursors is further exacerbated in these animals. In addition to the loss of precursors, those that remain are of entirely different clonotypic composition compared with those in herpesvirus-free controls. The repertoire that remains in herpesvirus+ mice is functionally compromised, resulting in the priming of an effector CD8 T cell population with decreased polyfunctional capacity in response to Lm-OVA infection, as well as a reduced ability to control the infection. Moreover, as strongly suggested by the other studies (53, 54), the impact of infected herpesvirus-specific cells upon developing new immune responses could be deleterious and must be directly investigated. It has been proposed that peripheral turnover is most likely the primary mechanism for maintenance of a diverse memory T cell pool throughout life in humans, whereas thymic output is likely the primary mechanism in mice (55). If this holds true, then the impact of lifelong persistent herpesvirus infection on the naive CD8 T cell repertoire would be expected to be even more pronounced in humans, an issue that requires immediate attention.

Table I. Lifelong persistent viral infection alters the naive CD8 T cell pool, impairing immunity in late life

<table>
<thead>
<tr>
<th>Lifelong Viral Infection</th>
<th>HSV</th>
<th>MCMV</th>
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<tr>
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A summary of the impact of lifelong persistent herpesvirus infection compared with the expected age-associated immune defects observed in old mice is shown.

Single arrow indicates p values of 0.05–0.01; double arrow indicates p values of 0.01–0.0001 relative to herpesvirus-free old mice.

N/A, Not available.
Acknowledgments
We thank members of the Nikolich-Zugich Laboratory for help and stimulating discussions. Expert cell sorting assistance was provided by Paula Campbell at the University of Arizona Cancer Center/Arizona Research Laboratories-Division of Biotechnology Cytometry Core Facility. TCR Vβ sequencing was performed by the University of Arizona Genomics Core. Tetramers were provided by the National Institutes of Health Tetrramer Core Facility (Emory University).

Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTAL MATERIALS

Life-long persistent viral infection alters the naïve T-cell pool, impairing CD8 T-cell immunity in late life

Megan J. Smithey*, Gang Li*, Vanessa Venturi†, Miles P. Davenport† and Janko Nikolich-Zugich†

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Life-long persistent herpesvirus infection minimally impacts global T-cell responsiveness. Mice were infected with HSV, MCMV, or both viruses at 9 weeks of age, then challenged with 10^5 CFU Lm-OVA at 23 months of age. Seven days later, splenocytes were stimulated with PMA/ionomycin and the production of IFNγ, TNFα and Granzyme B within (A) CD8 and (B) CD4 T-cell populations was determined by intracellular cytokine staining. Within each IFNγ-producing population, the ability to also produce TNFα and/or Granzyme B was assessed. The composite response of all cytokine-producing, antigen-specific T-cells in each category is shown on the left, with each polyfunctional subpopulation shown to the right with statistics. Floating bars represent the minimal and maximal response in each animal group, with a line at the mean. Significance was determined by 1-way ANOVA with Dunnett’s post-test relative to Old mice (no herpesvirus infection). Data represents 1 of 2 replicate experiments with n=8 mice/group. * p <0.05, ** p <0.01, *** p <0.001.

Supplemental Figure 2. Representative flow cytometry plots for the tetramer pull-down assay. The spleen, inguinal, cervical and axillary lymph nodes were pooled from
individual mice in attempt to collect the majority of the naïve T cell population. Cells were stained with anti-CD8α and PE- and APC-labeled B8R20-27-Kb tetramers, followed by incubation with anti-APC and anti-PE microbeads, then magnetically enriched over columns. The resulting tetramer-enriched fractions were stained with a cocktail of fluorochrome-labeled antibodies that served as a “dump” gate: anti-CD19, -CD4, -MHC class II, and F4/80. Cells were washed and the entire sample analyzed by flow cytometry.

**Supplemental Table 1. Distribution and copy number of all CDR3 TCR Vβ sequences recovered from mice.** At 23 months of age, life-long MCMV-infected and uninfected controls were challenged with $10^5$ CFU *Lm*-OVA. Seven days later, individual CD8+ Vβ5+ OVA257-264-Kb tetramer+ cells were sorted and sequenced by single-cell PCR. Shown are the amino acid sequences spanning the V-D-J region of the CDR3 of the TCR Vβ chain, and the number of copies found in each animal.
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<th>Old #3</th>
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**Shared Sequences**

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| TRBV12-1 | CASSPRASDYTF | TRBJ1-2 | 16 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| TRBV12-1 | CASSPRASDYTF | TRBJ1-2 | 14 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
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**Unique Sequences**

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| TRBV12-1 | CASSPRASDYTF | TRBJ1-2 | 13 | 0 | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 1 |
| TRBV12-1 | CASSPRASDYTF | TRBJ1-2 | 13 | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 1 |
| TRBV12-1 | CASSPRASDYTF | TRBJ1-2 | 11 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 1 |
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