CD8 T Cells Recruited Early in Mouse Polyomavirus Infection Undergo Exhaustion

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CD8 T cells recruited early in mouse polyomavirus infection undergo exhaustion


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Abstract

Repetitive Ag encounter, coupled with dynamic changes in Ag density and inflammation, imparts phenotypic and functional heterogeneity to memory virus-specific CD8 T cells in persistently infected hosts. For herpesvirus infections, which cycle between latency and reactivation, recent studies demonstrate that virus-specific T cell memory is predominantly derived from naïve precursors recruited during acute infection. Whether functional memory T cells to viruses that persist in a non-latent, low-level infectious state (smoldering infection) originate from acute infection-recruited naïve T cells is not known. Using mouse polyomavirus (MPyV) infection, we previously showed that virus-specific CD8 T cells in persistently infected mice are stably maintained and functionally competent; however, a sizeable fraction of these memory T cells are short-lived. Further, we found that naïve anti-MPyV CD8 T cells are primed de novo during persistent infection and contribute to maintenance of the virus-specific CD8 T cell population and its phenotypic heterogeneity. Using a new MPyV-specific TCR transgenic system, we now demonstrate that virus-specific CD8 T cells recruited during persistent infection possess multi-cytokine effector function, have strong replication potential, express a phenotype profile indicative of authentic memory capability, and are stably maintained. In contrast, CD8 T cells recruited early in MPyV infection express phenotypic and functional attributes of clonal exhaustion, including attrition from the memory pool. These findings indicate that naïve virus-specific CD8 T cells recruited during persistent infection contribute to preservation of functional memory against a smoldering viral infection.

Introduction

The inflammatory microenvironment is a central determinant that directs pathogen-specific T cell differentiation. Strong early inflammatory responses divert pathogen-specific CD8 T cells toward effector and away from memory pathways of differentiation (1, 2). Alternatively, Ag presented in low-inflammatory settings favors CD8 T cell memory differentiation (3). These microenvironment-directed shifts in T cell differentiation are associated with changes in expression of select transcription factors, including T-bet.
Elucidation of the elements controlling CD8 T cell differentiation has been largely derived from experimental models where host immunity efficiently and completely eliminates cognate Ag.

For persistent infections, the pattern of CD8 T cell differentiation is perturbed by repetitive exposure to Ag and unresolved inflammation. In the setting of high-level persistent viremia, memory CD8 T cells express an effector phenotype (e.g., CD62Llo IL-7Rlo CCR7lo) and suffer progressive functional impairment that may culminate in deletion from the T cell pool. The severity of exhaustion experienced by these “chronic memory” T cells is dictated in large part by the level of persistent cognate viral Ag (6). However, chronic memory T cells also require cognate Ag for maintenance, but express a T-betlo Blimp-1hi transcription factor profile and upregulate cell surface receptors that inhibit their ability to clear viral infection (7). In contrast, virus-specific CD8 T cells maintained in the setting of low-level persistent infection typically preserve most of their effector functions, and the population remains stable or gradually increases in magnitude over time (8).

Polyomaviruses establish a lifelong, low-level infection in healthy hosts of a variety of avian and mammalian species, including humans (9). The human polyomaviruses BK and JC persist as clinically silent, nonviremic infections in most individuals. With immunocompromise resulting from HIV/AIDS or humoral immunotherapeutic agents affecting T cell trafficking (e.g., natalizumab, efalizumab, rituximab), JC virus may cause a life-threatening demyelinating brain disease; BK virus is a well-recognized cause of dysfunction and loss of kidney allografts (10, 11). Current evidence suggests that JC and BK virus-specific CD8 T cells control these smoldering persistent viral infections (12, 13).

During persistent infection by MPyV, virus-specific CD8 T cells express a predominantly effector phenotype, but retain cytokine and cytolytic effector functions and fail to express inhibitory markers upregulated by memory CD8 T cells that confront chronic viremia (e.g., PD-1, Lag-3, or Tim-3) (14, 15). In addition, the memory MPyV-specific CD8 T cell response is stable over the course of persistent infection (16). However, following transfer to persistently infected, congenic mice memory MPyV-specific CD8 T cells do not homeostatically proliferate and this population undergoes progressive attrition (17). Using a partial myeloablation approach to allow engraftment of congenic bone marrow in persistently infected mice, we recently demonstrated that naïve MPyV-specific CD8 T cells are primed de novo during persistent infection. Persistent infection-recruited memory antiviral T cells differ phenotypically from those recruited earlier in infection (17, 18). Whether these memory T cells differ functionally from those recruited during acute infection and to what degree they contribute to maintenance of the memory T cell compartment are not known.

Using novel mutant MPyVs and a TCR transgenic mouse model, we provide evidence that virus-specific CD8 T cells recruited during persistent MPyV infection not only favor expression of canonical markers of authentic memory T cells, but also possess superior effector functionality and secondary replicative potential over those memory cells derived from naïve precursors primed early in acute infection. Importantly, persistent infection-recruited memory CD8 T cells are maintained in all mice and do so at a higher magnitude than those recruited during acute infection, with acute infection-recruited memory cells falling below detection in a significant number of animals. Because virus-specific CD8 T cells recruited early in acute MPyV infection suffer clonal exhaustion, those recruited after acute infection likely play a central role in keeping this smoldering viral infection in check.
Materials and Methods

Mice

Female C57BL/6Ncr (B6) mice were purchased from the Frederick Cancer Research and Development Center (National Cancer Institute, Frederick, MD). B6.PL (Thy1.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.SJL-"Ptprca"/BoAiTac-H2-Ab1tm1GLM N13 mice (I-Aβ−/−CD45.1) were purchased from Taconic Farms (Germantown, NY) and both bred by the Emory University Division of Animal Resources. TCR-I transgenic mice bearing the TCR specific for LT206-215 from the Large T (LT) antigen of SV40 are previously described (19). TCR-I mice were crossed with B6.PL mice (Thy1.1) to create F1 mice expressing both the transgenic TCR and Thy1.1. Mice were bred and housed by the Division of Animal Resources in accordance with the guidelines of the Institutional Animal Care and Use Committee of Emory University. All mice were 6–8 wk of age at the time of infection.

Viruses and cell transfers

Mice were infected s.c in the hind footpads by 1 × 10^6 PFU of either MPyV strain A2 (MPyV.A2) or a mutant MPyV strain expressing the Dβ-restricted LT206-215 epitope from SV40 LT antigen (MPyV.LT206). MPyV.LT206 was generated using a QuikChange II XL sitedirected mutagenesis kit (Stratagene, Cedar Creek, TX) of MPyV.A2 genomic DNA to iteratively change the Dβ-restricted LT359-368 epitope from MPyV.A2 (SAVKNYSKLL) to the Dβ-restricted epitope LT206-215 from SV40 (SAINNYAQKL). The following primers were used: (ForLT359VI-AGGGTTTCAGCAATTAAGAATTATG, RevLT359VI-CATAATTCTTTATTGCCTGAACCCCT, ForLT359KN-GTTCAGCAATTAATTCGCTTAAG, RevLT359KN-CCTAGAGGCTATAATTTGCTGAACCC, ForLT359CA-GTTAAGAATTCGCTTAAGCTTTGC RevLT359CA-GCAAAGCTTCTATGCTGAACCC, ForLT359SQ-ATTATAATATATGCCAAGCTTCTATGCAAC, RevLT359SQ-GCTGCAAAAGCTTCTGCGATAATTATAAT). All primers were synthesized by Invitrogen (Grand Island, NY). Recombinant MPyV.LT206 DNA was ligated with T4 DNA ligase and baby mouse kidney cells were transfected using Lipofectamine 2000 (Invitrogen) as previously described (20). Because LT359-368 is not situated in a functional domain of LT, and does not overlap the coding sequence for the middle T oncoprotein, MPyV.LT206 is identical to MPyV.A2 in terms of replication and dissemination in vivo (Supplemental Fig. 1A), and tumor-induction when inoculated into newborn mice of an MPyV tumor-susceptible strain (unpublished data). A recombinant vaccinia virus encoding the SV40 LT206-215 epitope, designated rVV-ESI, is previously described (21); mice received 1 × 10^6 PFU of rVV-ESI i.v.

CD8 T cells from TCR-I transgenic mice or uninfected B6 mice were purified using a negative selection CD8 T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to manufacturer’s instructions. For all adoptive transfer experiments, the CD62L^hi status of the donor TCR-I cells was > 93% (range: 93.3% to 98.1%) and the CD44^hi status was < 8% (range: 2.4% to 8%). 100 or 1 × 10^6 TCR-I cells, or 10 × 10^6 or 20 × 10^6 polyclonal CD8 T cells were transferred. TCR-I cells were labeled with 5 µM CFSE (Invitrogen) for 10 minutes at 37°C prior to transfer of 1 × 10^6 cells (Supplemental Fig. 1). 100 purified TCR-I cells were transferred on d -1, d 60, or d 90 after MPyV.LT206 infection, and d -1 prior to rVV-ESI infection.

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Quantification of MPyV genomes

DNA isolation and Taqman-based PCR were performed as previously described (18). The detection limit of this assay is 10 copies of genomic viral DNA.

Synthetic peptides

LT206-215 (SAINNYAQKL) and LT359-368 (SAVKNYAbuSKL, where Abu is α-amino butyric acid, a cysteine structural analog) peptides were synthesized by the solid-phase method using F-moc chemistries on a Prelude peptide synthesizer (Protein Technologies, Inc.).

Cell isolation and flow cytometry

TCR-I cell numbers in the blood were determined by flow cytometric analysis using BD Trucount tubes (BD Biosciences) according to the manufacturer’s directions. Single-cell suspensions of RBC-lysed spleens were prepared. Antibodies to CD8α (53-6.7), Thy1.1 (OX-7), CD62L (MEL-14), CD44 (IM7), CD45 (30-F11), Vβ7 (TR310), CD3ε (2C11), Bcl-2, human Ki-67 (B56), IFN-γ (XMG1.2), and IL-2 were purchased from BD Biosciences (San Diego, CA). CD127 (A7R34), CXCR3 (XCXCR3-173), CD27 (LG.7F9), PD-1 (RMP1-30), TIM-3 (RMT3-23), and LAG-3 (eBioC9B7W) were purchased from eBioscience (San Diego, CA). Anti-KLRG-1 was purchased from SouthernBiotech (Birmingham, AL). Anti-CD43 (1B11) was purchased from BioLegend (San Diego, CA). Anti-Blimp-1 (C-21), anti-T-bet (4B10), and anti-Eomes (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V staining was performed as directed using a kit from BD Biosciences. DqLT206, DqLT359, KqMT246 and DqLT638 tetramers were constructed by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Tetramer staining was performed for 45 min at room temperature. Samples were acquired on a FACSCalibur or an LSR II (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Inc. Ashland, OR). For antigen recall experiments, TCR-I cells were stained with anti-Thy1.1 and anti-CD8α then sorted using a FACSARia (BD Biosciences) cell sorter.

Cells were stimulated with 1 µM to 1 pM LT206-215 peptide for 5 h in the presence of brefeldin A and intracellularly stained for IFN-γ and IL-2 as previously described (18, 22).

Gene expression analyses

Oligonucleotide primers for PCR amplification of T-bet, Blimp-1, and L9 cDNA were previously described (23). RNA was isolated from 1 × 10^5 – 1 × 10^6 FACS-sorted Thy1.1^ CD8^ cells using an RNasea mini kit (Qiagen, Valencia, CA), and cDNA prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Power SYBR green PCR master mix (Applied Biosystems) was used for quantitative PCR using an ABI PRISM 5700 sequence detection system (Applied Biosystems).

Statistical analyses

Statistical significance was determined by an unpaired, two-tailed Student’s t test, assuming unequal variance. Experiments with 3 groups were assayed for statistical significance using a Kruskal-Wallis one-way analysis of variance test. A value for p < 0.05 was considered statistically significant.
Results

MPyV-specific CD8 T cells recruited during acute vs. persistent infection differ in response profiles and maintenance

Dynamic recruitment of naïve virus-specific CD8 T cells in persistently infected hosts results in an amalgam of cells having different histories of encounter with Ag and pro-inflammatory mediators. The minimal myeloablation-congenic bone marrow transfer approach we used to observe de novo priming of antiviral CD8 T cells during persistent infection is unable to resolve the phenotypic and functional impact of recruitment at defined stages of infection, because antiviral T cells are recruited throughout the engraftment interval (17). To limit the timeframe over which naïve virus-specific T cells are recruited, and to eliminate variables of precursor frequency and TCR repertoire, we developed the following MPyV-specific CD8 T cell TCR transgenic system. By mutagenizing four codons in the genome of MPyV (designated MPyV.A2), we replaced the dominant Db-restricted LT359-368 (LT359) CD8 T cell epitope with the homologous Db-restricted LT206-215 (LT206) epitope in SV40 LT antigen (designated MPyV.LT206), which is recognized by CD8 T cells from the "TCR-I" TCR transgenic mouse (19). TCR-I cells exhibit no cross-reactivity to syngeneic APCs presenting the LT359 peptide or infected by MPyV.A2 in vitro (unpublished observations), and expand in Thy1 congenic recipients acutely infected by MPyV.LT206 but not MPyV.A2 (Supplemental Fig. 1B, upper 2 panels). In addition, an LT359-specific CD8 T cell response is not generated in B6 mice infected by MPyV.LT206. Finally, the endogenous LT206-specific CD8 T cell response to MPyV.LT206 infection is similar to that of the LT359-specific CD8 T cell response to infection by MPyV.A2, and there is no change in the frequency of the two subdominant anti-MPyV CD8 T cell responses (unpublished observations) (18).

TCR-I cells proliferate after transfer to congenic recipients infected with MPyV.LT206 for 60 d, showing that the DbLT206 epitope is expressed long-term and is available for recognition by virus-specific CD8 T cells (Supplemental Fig. 1B, bottom panel). However, only a portion of the $1 \times 10^6$ donor TCR-I cells expanded within 5 d post transfer. Antigenic competition by an excessive number of naïve cells faced with the limited Ag density of persistent MPyV infection likely impaired T cell recruitment. To minimize antigenic competition and approximate physiologic numbers of Ag-specific naïve cell precursors, we transferred only 100 TCR-I cells per recipient in all subsequent experiments (24). Transfer of 100 TCR-I cells one day prior to infection allowed the generation of an endogenous LT206-specific CD8 T cell response comparable to unmanipulated infected mice (unpublished observations); virus levels showed only a modest two-fold transient decrease at d 7 p.i. following transfer, with no decrease evident by day 15 p.i. or later post infection (Supplemental Fig. 1C). These data show that the MPyV.LT206/TCR-I approach can be used to faithfully monitor the fate and function of virus-specific CD8 T cells recruited during the acute and persistent phases of infection.

Donor TCR-I cells showed strikingly different response profiles when transferred to Thy1 congenic B6 mice acutely or persistently infected by MPyV.LT206. As depicted in Fig. 1A, TCR-I cells were transferred either 1 day before infection (acute infection recruitment) or at d60 or d90 p.i. (persistent infection recruitment); acute infection-recruited TCR-I cells were assessed at d 30 and d 90 p.i. to match the time after cell transfer and the time after infection, respectively (Fig. 1A). For acute infection recruitment, naïve TCR-I cells vigorously expanded, peaking in magnitude at 1 week p.i., then precipitously contracting to a small population (Fig. 1B). This response pattern mirrors the kinetics of Ag-specific CD8 T cell responses to acutely resolved infections. However, post-contraction, TCR-I cells fell below detection in the blood and spleen of several of these mice, with the proportion of TCR-I/low chimeric mice increasing over time (Fig. 1B & 5A). In contrast, when transferred

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to hosts infected 90 d earlier, TCR-I cells underwent a slow progressive expansion, without an appreciable contraction phase, over a 2-mo timeframe to reach a plateau maintenance level. Notably, by d90 post transfer, the pool of circulating TCR-I cells recruited during persistent infection was 4–5-fold larger than those recruited during acute infection, and 100% of these recipients had sizeable populations of memory TCR-I cells (Fig. 1B & 5A). This response profile difference was not simply a consequence of higher virus levels in acute than persistent infection. TCR-I cells transferred to B6 mice given $1 \times 10^6$ PFU MPyV.LT206 underwent a delayed but similar expansion-contraction response pattern as those given $1 \times 10^5$ PFU inocula (unpublished observations), as we previously described for the endogenous anti-MPyV CD8 T cell response (22). The increased frequency and numbers of TCR-I cells maintained in persistently infected recipients could not be accounted for by increased expression of the Bcl-2 anti-apoptotic molecule or decreased cell death as indicated by Annexin V staining (Fig. 1C and 1D). However, a significantly higher fraction of memory TCR-I recruited in persistently infected mice expressed the nuclear factor Ki-67, indicative of recent cell division, than TCR-I cells primed at the onset of infection (Fig. 1E). This finding suggests that persistent infection-recruited TCR-I cells retain higher replicative potential than those recruited in acutely infected recipients, which is further supported by differences in Ag recall responsiveness by these memory TCR-I populations (see Fig. 7). Together, these data indicate that the different response profiles and capacity for long-term maintenance by memory MPyV-specific CD8 T cells are instilled by recruitment during temporally distinct infection settings.

### Persistent infection-recruited TCR-I cells express a phenotype associated with improved memory potential

Compared to TCR-I cells recruited in acute MPyV infection, a higher proportion of persistent infection-recruited cells expressed the IL-7 receptor-α chain (CD127). This expression level mimicked that of true memory TCR-I cells (designated “Memory”) generated in response to acutely resolved infection by a recombinant vaccinia virus encoding the LT206 epitope (rVV-ES-I) (Fig. 2A). At d 30 after transfer, TCR-I cell expression of the lymphoid homing marker, CD62L, while trending higher for those recruited during persistent MPyV and resolved vaccinia virus infections, was statistically similar to acute MPyV infection-recruited TCR-I cells (Fig. 2B). Of note, a higher frequency of acute infection-recruited cells expressed CD127 at d 90 than at d 30 p.i. (Fig. 3B), which may be associated with the capacity of these cells to survive longterm. Interestingly, most TCR-I cells expressed KLRG1, a marker of T cell senescence (25), regardless of timing of recruitment (Fig. 2C and 3B), while only a fraction of cells recruited late in persistent infection expressed the CD43 marker of activation (Fig. 2D). These phenotypic differences among TCR-I cells as a function of recruitment were recapitulated using donor polyclonal CD8 T cells from uninfected mice (Supplemental Fig. 2). These data suggest that memory MPyV-specific CD8 T cells whose progenitors were recruited during persistent MPyV infection trended toward expression of canonical markers of bona fide memory.

Low expression of CD43 with coordinate upregulated expression of CD27 and CXCR3 have been associated with memory antiviral CD8 T cells having strong potential for antigenic recall (26). As shown in Fig. 3A, acute infection-recruited memory TCR-I cells skewed toward a CD43$^\text{hi}$ phenotype, with only a small fraction of the CD43$^\text{lo}$ cells co-expressing CD27 or CXCR3, regardless of the length of time after infection. However, persistent infection-recruited memory TCR-I cells expressed less CD43 (Fig. 2D and 3A) and higher CD27 and CXCR3 molecules (Fig. 3). These CXCR3$^\text{hi}$ cells were also more likely to express CD127 in persistent infection-recruited memory TCR-I cells than in acute infection-recruited cells. However, the majority of acute (both d 30 and d 90 p.i.) and persistent infection-recruited memory TCR-I cells were KLRG1$^\text{hi}$, with few TCR-I cells in either
group expressing the PD-1 or LAG-3 inhibitory receptors (Fig. 3B). Additionally, given the high KLRG1 expression by memory TCR-I cells generated under either acute or persistent infection settings, there were no phenotypically authentic CD127hi KLRG1lo memory cells (27). Taken in aggregate, these phenotyping analyses raise the possibility that memory TCR-I cells recruited during persistent infection have higher functional capabilities than those recruited during acute infection.

**Persistent infection-recruited MPyV-specific CD8 T cells possess superior functionality**

Although equal proportions of acute and persistent infection-recruited memory TCR-I cells produced IFN-γ upon ex vivo LT206 peptide stimulation, the latter cells were capable of multicytokine effector activity as shown by co-expression of IL-2 (Fig. 4A). Of note, the ability to coproduce IFN-γ and IL-2 improved with recruitment at later timepoints of persistent MPyV infection. In contrast, as shown in Fig. 4B, memory TCR-I cells recruited during acute infection failed to acquire the ability to co-produce IL-2 over time (compare d30 with d90 p.i.). LT206 peptide dose titration further showed that while the functional avidity of acute and persistent infection-recruited TCR-I cells was equivalent (i.e., similar slopes for IFN-γ production), those recruited during acute infection possessed significantly lower IFN-γ effector capability (Fig. 4C).

Because IFN-γ is an important anti-MPyV effector cytokine in vivo (14), an implication of this result is that acute infection-recruited MPyV-specific CD8 T cells are less effective in controlling this viral infection. In line with their diminished functional potential, acute infection-recruited memory TCR-I cells expressed more Blimp-1, both at the mRNA and protein level, than those recruited during persistent infection (Fig. 4D and 4E). As a transcriptional repressor of IL-2 gene expression, higher Blimp-1 levels may also underlie the lower IL-2 functional capability of acute infection-recruited memory TCR-I cells and their diminished durability in persistently infected hosts (28). By corollary, low Blimp-1 expression by memory TCR-I cells generated against acutely resolved rVV-ES-I infection is in line with their high IL-2-producing capability (Fig. 4A).

Although both acute and persistent infection-recruited memory TCR-I cells had similar T-bet transcript and protein expression levels, there was a notable increase in Eomes expression in cells recruited during the persistent phase of infection (Fig. 4F and 4G). Therefore, while T-bet levels were similar, consistent with each memory population retaining an effector differentiation state, increased Eomes expression may explain increased survival and functional competence of the persistent infected-recruited cells (29, 30). Alternatively, sustained high T-bet expression coupled with elevated Blimp-1 expression and low Eomes expression, may be linked to the attrition of acute infection-recruited memory TCR-I cells (29, 31).

Using D6LT206 tetramers, we further determined that the endogenous memory anti-MPyV CD8 T cell compartment closely resembled the stability and functionality of the memory TCR-I cells recruited during persistent infection, both in the spleen and lung (Fig. 5A & 5C). However, as shown in Fig. 5B, TCR-I cells recruited during acute infection progressively lost the ability to co-produce IFN-γ and IL-2, and fell below detection in a number of recipients. This functional deficit was also evident for acute infection-recruited memory TCR-I cells isolated from the lungs (Fig. 5D). Taken together, these findings imply that MPyV-specific CD8 T cells recruited during persistent infection make a larger contribution to the memory T cell compartment than those recruited during acute infection.
CD4 T cells are required for maintenance of memory MPyV-specific CD8 T cells during persistent infection

Using the myeloablation-congenic bone marrow engraftment approach, we previously reported that recruitment of MPyV-specific CD8 T cells depended on CD4 T cell help (16). These studies used MPyV-infected I-A\(^b\)-/- mice, which carry similar viral loads as MPyV-infected B6 mice during acute and persistent infection. Several host defense mechanisms control MPyV infection in MHC class II-deficient mice, including a strong protective T cell-independent MPyV-specific Ab response, exaggerated expansion of MPyV-specific CD8 T cells during acute infection, and the long-term maintenance of a small functional antiviral CD8 T cell population (16, 32). Thus, differences in efficiency of antiviral CD8 T cell recruitment between CD4 T cell-sufficient and -deficient mice cannot be ascribed to differences in viral loads. However, these experiments necessitated a long engraftment period to enable detection of de novo primed antiviral CD8 T cells in persistently infected mice, and therefore did not allow us to distinguish whether CD4 T cell deficiency impaired priming, expansion, or maintenance of anti-MPyV T cells. We revisited this question using the TCR-I/MPyV.LT206 model to determine how CD4 T cell insufficiency affected recruitment of virus-specific CD8 T cells during persistent infection. B6 and I-A\(^b\) deficient mice (I-A\(^b\)-/-) mice at d 60 p.i. received 100 TCR-I cells, and were serially bled to quantify the expansion and maintenance of the TCR-I cells. As shown in Fig 6A, TCR-I cells expanded in persistently infected, I-A\(^b\)-/- mice, but then underwent profound contraction similar to that seen by virus-specific CD8 T cells recruited in MPyV-infected, CD4 T cell-deficient animals (16). However, post-contraction, a small fraction of these persistent infection-recruited TCR-I cells, like the endogenous polyclonal LT206-specific CD8 cells, survived and retained cytokine effector function (compare Fig. 6B with Fig. 6C and D).

These data extend our previous findings by showing that the dependence on CD4 T cell help for recruiting MPyV-specific CD8 T cells during persistent infection involves mitigating the contraction and/or sustaining the memory phase.

Acute and persistent infection-recruited memory MPyV-specific CD8 T cells differ in recall potential

A cardinal property of T cell memory is rapid expansion upon re-encounter with cognate Ag. Because persistent infection-recruited TCR-I cells had an increased fraction of CD43lo CD27hi cells and higher IL-2 functionality, and possessed decreased levels of Blimp-1, we asked whether memory TCR-I cells recruited during persistent infection possessed higher replication potential than those recruited during acute infection. To test this possibility, we first compared the number of circulating TCR-I cells in acute and persistent infection, and then challenged these mice with rVV-ES-I. Five days following challenge, acute infection-recruited memory TCR-I cells showed no expansion in the blood (or spleen, unpublished observations); in marked contrast, memory TCR-I cells recruited during persistent infection expanded 50-fold (Fig. 7A).

To determine if this dramatic difference in recall potential was intrinsic to the TCR-I cells, TCR-I cells were transferred to naïve B6 recipients infected the following day or to B6 recipients infected 60 d earlier. Thirty days after transfer, Thy1.1+ CD8 T cells were FACSSorted and 1 x 10^4 memory TCR-I cells were re-transferred to naïve B6 mice. When challenged with rVV-ES-I, donor memory TCR-I cells that were recruited during persistent infection expanded 50-fold more than those recruited during acute infection. (Fig. 7B). These data are in line with the phenotypic and functional evidence that recruitment history impacts the differentiation of MPyV-specific CD8 T cells not only with respect to cytokine effector capability, but also in potential for proliferative expansion to Ag reencounter.
Discussion

In contrast to memory virus-specific CD8 T cells that encounter aggressive chronic infections, those co-existing with low-level persistent infections are often stably maintained and retain most of their effector capabilities. Previous studies from our group and others have shown that naïve virus-specific CD8 T cells are primed de novo during persistent infection. These persistent infection-recruited T cells upregulate expression of molecules associated with a central memory T cell phenotype (CD44hi CD62Lhi CD127hi); however, their functional integrity and contribution to the memory compartment have not been previously investigated. In this study, we reexamined the question of this apparent avoidance of dysfunction and loss (exhaustion) by antiviral CD8 T cells that are confronted by low-level persistent infections. By analyzing the function and fate of naïve TCR transgenic CD8 T cells primed at different stages of MPyV infection, we show that CD8 T cell exhaustion in the environment of a low-level persistent infection is revealed when kinetics of T cell recruitment are taken into account. In addition, we provide evidence supporting the concept that naïve virus-specific CD8 T cells recruited during persistent infection are important for the integrity of a stable and functional memory antiviral T cell population.

The timeframe over which naïve CD8 T cells are recruited to acutely resolved infections has been shown to impact the balance between effector and memory differentiation. Unlike naïve virus-specific CD8 T cells recruited at the inception of an acute viral infection, those recruited during later timepoints preferentially give rise to central memory-phenotype T cells capable of Ag-induced IL-2 production and antigenic recall (33, 34). These data are line with evidence that abbreviating the duration and reducing the peak magnitude of acute infection also favors generation of central over effector memory T cells (34, 35). Thus, even over the brief span of an acute infection, changes in Ag load, APCs, cytokines, costimulation, and availability of CD4 T cell help drive dynamic alterations in the differentiation program imprinted on viral Ag-activated naïve CD8 T cells (1). The data presented here show that even during smoldering infections such as MPyV, the direction of memory differentiation differs for virus-specific CD8 T cells recruited during acute vs. persistent infection.

In contrast to the latency-reactivation lifecycle of herpesviruses, polyomaviruses persist as smoldering infections, where viral replication may amplify in the setting of depressed immune surveillance. As a result, herpesvirus-specific CD8 T cells recognizing epitopes from lytic proteins may face a “prime-boost” scenario of antigenic encounter that guides a program of memory differentiation distinct from that imparted by non-latent persistent viral infections. This viral lifecycle difference may also underlie recent evidence that memory CD8 T cells to mouse cytomegalovirus and γ-herpesvirus infections are derived from naïve progenitors recruited during the acute phase of primary infection (8, 36). Data presented in this study indicate that naïve virus-specific CD8 T cells recruited after the acute phase of infection to MPyV are major precursors of durable functional memory. Further, we find that antiviral CD8 T cells recruited during acute infection possess diminished effector and proliferative capabilities, lack the capacity to recall upon Ag re-encounter, and are slowly lost from the memory pool – properties usually ascribed to exhausted CD8 T cells in chronic infection environments (37, 38). The precise stage of infection when differentiation is tipped toward effective memory (e.g., early after peak viremia or during persistent infection) remains to be determined. Preliminary evidence indicates that naïve TCR-I cells transferred two weeks after infection give rise to memory cells exhibiting phenotypic and functional characteristics intermediate between those recruited at the beginning and 30 days after MPyV infection. In this connection, we previously reported that decreasing MPyV-associated inflammation during acute infection results in qualitatively superior memory
antiviral CD8 T cells (22). Taken together, these data support the prediction that the proportion of naïve MPyV-specific CD8 T cells that progress toward effective memory increases over the course of infection.

During early acute viral infections, naïve T cells are exposed to high levels of Ag and inflammation, which preferentially drive differentiation toward effectors to optimize host antiviral defense. Using an acutely resolved LCMV infection model, Kaech and coworkers have identified Type I IFN and IL-12 as dominant determinants that induce expression of T-bet, Eomes, and Blimp-1, transcription factors critical for inducing CD8 T cell differentiation into cytotoxic- and cytokine-armed effectors (23). Coincident with viral clearance, T-bet and Blimp-1 transcription factors are downregulated, while Eomes expression gradually increases in concert with the emergence of self-renewing functional memory CD8 T cells (39). In contrast, during chronic LCMV infection, Blimp-1 levels remain high and closely correlate with upregulation of inhibitory receptors that mediate the dysfunction characteristic of clonal exhaustion; however, a basal Blimp-1 expression appears to be necessary for antiviral CD8 T cells to maintain effector function in the face of persistent infection. For memory TCR-I cells recruited during acute infection, we observed higher Blimp-1 expression, and lower expression of Eomes compared to persistent infection-recruited cells, suggesting a transcriptional profile indicative of senescent effector CD8 T cells (40, 41). Of note, although Blimp-1 expression was higher on acute infection-recruited cells, we were unable to identify an association between their functional deficits and upregulation of T cell exhaustion-associated inhibitory receptors. Whether inhibitory receptors other than PD-1, Lag-3, or Tim-3 mediate functional exhaustion by memory CD8 T cells recruited during acute MPyV infection remains to be determined. The lower, but still substantial, expression of Blimp-1 by persistent infection-recruited memory TCR-I cells may underlie their higher degree of fitness.

Eomes and T-bet also direct the differentiation of effector CD8 T cells, and are required for the ability of CD8 T cells to express effector molecules (29, 42). T-bet was initially described as a master regulator driving terminal effector T cell differentiation, and several studies suggested a redundant or complementary role for Eomes. However, T-bet has recently been shown to be necessary to sustain effector function by memory antiviral CD8 T cells during chronic infection by repressing PD-1 (43). Similarly, while Eomes may play a role in the induction of an effector differentiation state, recent studies suggest that Eomes promotes preservation of the memory CD8 T cell pool. (44, 45). Both acute and persistent infection-recruited MPyV-specific CD8 T cells express T-bet, while only persistent infection-primed cells show increased expression of Eomes. This sustained T-bet expression, coupled with increased Eomes expression, may be involved in improving the capacity of persistent infection-recruited cells survive and retain antiviral effector functionality.

Using the murine CMV (MCMV) infection model, Hill and coworkers recently showed that the memory antiviral CD8 T cell compartment is a composite of naïve T cells recruited during persistent infection and the progeny of cells primed early in infection (46). In this study, donor-derived, MCMV-specific CD8 T cells were detected months after transferring splenocytes from d7-infected mice into infection-matched, congenic recipients. Thus, the progenitors of these memory cells were primed at some point early in MCMV infection. Unlike the stable maintenance of the memory MPyV-specific CD8 T cell compartment, latent MCMV infection drives continuous expansion of CD8 T cells recognizing particular viral epitopes. Using adoptive transfer of MCMC-specific TCR transgenic CD8 T cells, Torti et al. similarly demonstrated that inflationary memory MCMV-specific CD8 T cells are recruited during acute infection, and further showed that CD8 T cell inflation results from viral Ag-driven restimulation of lymph node-resident central memory cells (47). These
findings reinforce the concept that mechanisms for maintaining memory virus-specific CD8 T cells vary depending on the nature of the infectious agent.

In summary, the findings presented here lead us to propose a revised model of memory CD8 T cell maintenance to low-level persistent viral infections. We currently envision a “conveyor belt” scenario where ongoing priming of naïve virus-specific CD8 T cells is required to resupply the pool of deteriorating antiviral T cells generated at early stages of infection. As viral load and/or inflammation diminish over time, bona fide memory T cells progressively emerge and assume an increasingly larger role in maintaining effective antiviral T cell memory. Understanding this dynamic balance between persistent infection and T cell recruitment will be essential for developing interventions to bolster immunity to smoldering viral infections.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**Abbreviations used**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>Eomes</td>
<td>eomesodermin</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<tr>
<td>LT</td>
<td>Large T antigen</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>MPyV</td>
<td>mouse polyomavirus</td>
</tr>
<tr>
<td>p.i.</td>
<td>postinfection</td>
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<tr>
<td>rVV</td>
<td>recombinant vaccinia virus</td>
</tr>
<tr>
<td>rVV-ES-I</td>
<td>rVV expressing SV40 LT aa 206–215</td>
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**References**


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Figure 1. Distinct response profiles for virus-specific CD8 T cells recruited during acute and persistent MPyV infection

A, Experimental design: 100 Thy1.1+ CD8+ TCR-I cells were transferred into Thy1.2+ B6 mice on d -1 (acute) or d 60 or 90 (persistent) of infection by MPyV.LT206. TCR-I cells were then evaluated either 30 d or 90 d post transfer. B, CD45+ CD8+ Thy1.1+ cells (TCR-I cells) in PBMCs were enumerated at the indicated timepoints after cell transfer. † and †† indicate 1 of 6 mice and 2 of 6 mice with no detectable TCR-I cells, respectively. Data is representative of 2 independent experiments of 3–6 mice. C & D, TCR-I cells were transferred either on d -1 or d 60 of infection with MPyV.LT206. At d 30 post transfer splenic TCR-I cells were analyzed for intracellular Bcl-2 and surface Annexin V binding. Left panel, representative histogram (open, acute infection-recruited; shaded, persistent infection-recruited); right panel, mean frequency ± SD. E, At d 30 and d 90 post transfer for acute infection recruitment, and d 30 post transfer for persistent infection recruitment (60 d p.i.), splenic TCR-I cells were analyzed for intranuclear Ki-67. Left panel, representative histograms stained for isotype control (gray) or Ki-67 (black); right panel, mean frequency ± SD. Data are representative of two independent experiments, with 3–6 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2. Expression of memory markers by CD8 T cells differs with timing of recruitment during MPyV infection

A–D, 30 d after TCR-I cell transfer, expression of cell surface CD127, CD62L, KLRG-1, and CD43 by splenic TCR-I cells was analyzed by flow cytometry. Memory TCR-I cells generated in response to a resolved infection were created by transferring 100 TCR-I cells to B6 mice 1 d before i.p. inoculation with rVV-ES-I (2 \times 10^6 PFU). Percentages are mean frequency ± SD. *p < 0.05, **p < 0.01.
Figure 3. Minimal changes in memory phenotype of acute infection-recruited anti-MPyV CD8 T cells over long-term maintenance

Representative flow cytometry plots and histograms of splenic TCR-I cells at 30 d or 90 d after d-1 p.i. transfer (acute) or 30 d after transfer to mice at d 60 p.i. (persistent). A, CD8+ CD44hi Thy1.1+ splenocytes were stained for CD27, CD43, CXCR3, and CD127. B, Representative histograms of CD8+ CD44hi Thy1.1+ splenocytes stained for CD127, PD-1, Lag-3, KLRG1, and CXCR3. Gates are based on isotype control staining of CD8+ CD44hi Thy1.1+ cells. Percentages are mean ± SD, of 2 independent experiments of 3–6 mice each.
Figure 4. Persistent infection-recruited memory CD8 T cells express higher multi-cytokine functionality and reduced Blimp-1

A & B, LT206 peptide-stimulated intracellular IFN-γ and IL-2 production by splenic TCR-I cells. A, TCR-I cells at d 30 post transfer in acutely (d -1 p.i.) or persistently (60 or 90 d p.i.) MPyV-infected mice, and at d 30 p.i. of rVV-ES-I infection (memory). B, Acute infection-recruited (d -1 p.i.) TCR-I cells at d 30 or d 90 p.i., or persistent infection-recruited TCR-I cells 30 d after transfer to mice infected 60 d earlier. Values represent the mean frequency ± SD of Thy1.1+ CD8+ TCR-I cells either singly producing IFN-γ or co-producing IFN-γ and IL-2. Data are representative of three experiments using 3–6 mice per group. C, Peptide dose-response curve for acute infection-recruited (30 d or 90 d post transfer) and persistent infection-recruited (d 60 p.i.) TCR-I cells expressing IFN-γ after stimulation with the indicated LT206 peptide concentration. Values represent the mean MFI ± SD of Thy1.1+ CD8+ TCR-I cells producing IFN-γ. Data are representative of 2–4 independent experiments of 3–6 mice per group. D & F, TCR-I cells were transferred either on d -1 (acute infection recruitment) or d 60 (persistent infection recruitment) of MPyV.LT206 inoculation, or d -1 of rVV-ES-I infection (memory) into Thy1.2’ B6 recipients. d 30 post transfer, splenic Thy1.1+ CD8+ TCR-I cells were FACS-sorted, lysed, and cDNA was prepared. Blimp-1 and T-bet transcript levels were determined by quantitative RT-PCR and normalized against L9 ribosomal protein mRNA levels. Values + SD were normalized to TCR-I cells purified from uninfected TCR-I mice. E, G, and H, Thy1.1+ CD8+ TCR-I cells from acute infection-
recruited cells on d 30 or d 90 p.i., or persistent infection-recruited cells on d 30 post transfer were stained for intranuclear Blimp-1, T-bet, or Eomes. Values represent MFI ± SD of Thy1.1+ CD8+ TCR-I cells for the indicated molecule. Goat IgG isotype controls (for Blimp-1 and Eomes) stained at an MFI of 3–5 and rat IgG isotype controls (for T-bet) stained at an MFI of 2–6. Data are representative of 2–4 independent experiments of 3–6 mice per group. *p < 0.05, **p < 0.01.
Figure 5. Acute MPyV infection-recruited CD8 T cells suffer exhaustion
Splenic (A) and pulmonary (C) Thy1.1− Db LT206 tetramer+ CD8 T cells (endogenous) were enumerated in mice at d 30 or d 90 p.i. that had received TCR-I cells on d -1 p.i. (acute), or 30 d after transfer of TCR-I cells at d 60 p.i. (persistent). Thy1.1+ CD8+ TCR-I cells from the spleen (B) and lungs (D) were enumerated for Db LT206 tetramer binding (top panels), LT206-stimulated intracellular IFN-γ production (middle panels) and intracellular IFN-γ and IL-2 coproduction (bottom panels). Data is compiled from 2–4 experiments of 3–6 mice each. Points on the x-axes represent individual mice with undetectable Thy1.1+ CD8+ cells. *p < 0.05, **p < 0.01.

Wilson et al. Page 20
J Immunol. Author manuscript; available in PMC 2013 May 01.
Figure 6. CD4 T cells are required for maintenance of memory MPyV-specific CD8 T cells during persistent infection
B6 or I-A<sup>b</sup>−/− mice received 100 TCR-I cells on d 60 p.i. of MPyV.LT206 infection A. Enumerations of Thy1.1<sup>+</sup> CD8<sup>+</sup> PBMCs (solid line, B6 mice; dotted line, I-A<sup>b</sup>−/− mice) over the course of infection. B, Total Thy1.1<sup>−</sup> CD8<sup>+</sup> CD44<sup>hi</sup> D<sup>b</sup> LT206 tetramer<sup>+</sup> splenocytes (endogenous) or TCR-I cells (Thy1.1<sup>+</sup>) were enumerated at d 30 after TCR-I cell transfer. Intracellular IFN-γ (C) and IFN-γ and IL-2 production (D) following LT206 peptide stimulation of the endogenous (Thy1.1<sup>−</sup>) CD8<sup>+</sup> T cells or TCR-I cells (Thy1.1<sup>+</sup>) were quantified. Values indicate mean ± SD. *p < 0.05, **p < 0.01.
Figure 7. MPyV-specific CD8 T cells recruited in persistent, but not acute, infection mount recall responses

A. B6 mice received 100 TCR-I CD8 T cells either 1 d before (acute infection recruitment) or at 60 d after infection (persistent infection recruitment) by MPyV.LT206. Thirty days post transfer, each mouse received 1 × 10^6 PFU rVV-ES-I. The number ± SD of Thy 1.1^+ CD8^+ cells in peripheral blood was enumerated at d 0 and d 5 p.i. as described in Fig. 1. B. 1 × 10^4 FACS-purified TCR-I T cells that were stimulated for 30 d during acute (d 0–30 p.i.) or persistent (d 60–90 p.i.) MPyV.LT206 infection (as described in Fig. 1A), were transferred i.v. into individual naïve B6 mice; 1 day post transfer, each mouse received an i.p. infection with 1 × 10^6 PFU rVV-ES-I. Thy 1.1^+ CD8^+ cells in the spleen were
enumerated at d 5 after rVV-ES-I inoculation. Values indicated mean ± SD. Data are representative of two independent experiments using 3 mice per group. *p < 0.05.