The Journal of Immunology

CD8 T Cells Recruited Early in Mouse Polyomavirus Infection Undergo Exhaustion

Jarad J. Wilson, Christopher D. Pack, Eugene Lin, Elizabeth L. Frost, Joshua A. Albrecht, Annette Hadley, Amelia R. Hofstetter, Satvir S. Tevethia, Todd D. Schell and Aron E. Lukacher

J Immunol 2012; 188:4340-4348; Prepublished online 23 March 2012;
doi: 10.4049/jimmunol.1103727
http://www.jimmunol.org/content/188/9/4340

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD8 T Cells Recruited Early in Mouse Polyomavirus Infection Undergo Exhaustion

Jarad J. Wilson,*[,1] Christopher D. Pack,*[,1] Eugene Lin,* Elizabeth L. Frost,* Joshua A. Albrecht,* Annette Hadley,* Amelia R. Hofstetter,* Satvir S. Tevethia,[†] Todd D. Schell,[†] and Aron E. Lukacher*

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 naive precursors recruited during acute infection. Whether functional memory T cells to viruses that persist in a nonlatent, low-level infectious state (smoldering infection) originate from acute infection-recruited naive 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 naive 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 multicytokine 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 naive virus-specific CD8 T cells recruited during persistent infection contribute to preservation of functional memory against a smoldering viral infection. ***The Journal of Immunology*****, 2012, 188: 4340–4348.**

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, eomesodermin (Eomes), and Blimp-1 (4, 5). Elucidation of the elements controlling CD8 T cell differentiation has been largely derived from experimental models in which 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., $CD62L^{lo}IL\text{-}7R^{lo}CCR7^{lo}$) 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 $T\text{-}bet^{lo}Blimp\text{-}1^{hi}$ 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 mouse polyomavirus (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., programmed cell death-1 [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

*Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322; and [†]Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, PA 17033

[1]J.J.W. and C.D.P. contributed equally to this work.

Received for publication December 21, 2011. Accepted for publication February 20, 2012.

This work was supported by National Institutes of Health Grants R01 CA71971 (to A.E.L.) and R01 CA025000 (to T.D.S.).

Address correspondence and reprint requests to Prof. Aron E. Lukacher, Department of Microbiology and Immunology, H107, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033. E-mail address: alukacher@hmc.psu.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6Ncr; Eomes, eomesodermin; $I\text{-}A^{b-/-}$, $I\text{-}A^{b}$–deficient mice; KLRG1, killer cell lectin-like receptor G1; LT, large T Ag; MCMV, murine CMV; MPyV, mouse polyomavirus; PD-1, programmed cell death-1; p.i., postinfection; rVV, recombinant vaccinia virus; rVV–ES-I, recombinant vaccinia virus expressing SV40 large T Ag aa 206–215.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103727

(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 naive 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 naive 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-*Ptprc*^{a}/BoAiTac-H2-*Ab1*^{tm1GLM} [^{13}N] mice (I-A$^{b-/-}$CD45.1) were purchased from Taconic Farms (Germantown, NY), and both were bred by the Emory University Division of Animal Resources. TCR-I–transgenic mice bearing the TCR specific for large T Ag (LT) 206–215 from the LT 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 MPyV strain A2 (MPyV.A2) or a mutant MPyV strain expressing the D^{b}-restricted LT206–215 epitope from SV40 LT Ag (MPyV.LT206). MPyV.LT206 was generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) of MPyV.A2 genomic DNA to iteratively change the D^{b}-restricted LT359–368 epitope from MPyV.A2 (SAVKNYCSKL) to the D^{b}-restricted epitope LT206–215 from SV40 (SAINNYAQKL). The following primers were used: forward LT359VI 5′-AGGGTTTCAGCAATTAAGAATTATG-3′, reverse LT359VI 5′-CATAATTCTTAATTGCTGAAACCCT-3′; forward LT359KN 5′-GTTTCAGCAATTAATAATTATGCCTCTAAG-3′, reverse LT359KN 5′-CTTAGAGGCATAATTATTAATTGCTGAAAC-3′; forward LT359CA 5′-GTTAAGAATTATGCCTCTAAGCTTTGC-3′, reverse LT359CA 5′-GCAAAGCTTAGAGGCATAATTCTTAAC-3′; and forward LT359SQ 5′-ATTAATAATTATGCCCAGAAGCTTTGCAGC-3′, reverse LT359SQ 5′-GCTGCAAAGCTTCTGGGCATAATTATTAAT-3′. 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 (J.J. Wilson and C.D. Pack, unpublished observations). A recombinant vaccinia virus (rVV) expressing the SV40 LT206–215 epitope (rVV–ES-I), is previously described (21); mice received 1×10^6 PFU of rVV–ES-I 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 the manufacturer's instructions. For all adoptive transfer experiments, the CD62L^{hi} status of the donor TCR-I cells was >93% (range: 93.3–98.1%), and the CD44^{hi} status was <8% (range: 2.4–8%). A total of 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 min at 37°C prior to transfer of 1×10^6 cells (Supplemental Fig. 1). A total of 100 purified TCR-I cells was transferred on days −1, 60, or 90 after MPyV.LT206 infection and day −1 prior to rVV–ES-I infection.

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, in which 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).

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. Abs 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 (CXCR3-173), CD27 (LG.7F9), PD-1 (RMP1-30), TIM-3 (RMT3-23), and LAG-3 (eBioC9B7W) were purchased from eBioscience (San Diego, CA). Anti-killer cell lectin-like receptor G1 (KLRG1) was purchased from Southern Biotechnology Associates (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. D^{b}LT206, D^{b}LT359, K^{b}MT246, and D^{b}LT638 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 an FACSCalibur or LSR II (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Ashland, OR). For Ag-recall experiments, TCR-I cells were stained with anti-Thy1.1 and anti-CD8α and then sorted using an 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 RNeasy mini kit (Qiagen, Valencia, CA), and cDNA prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, U.K.). 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 *t* test, assuming unequal variance. Experiments with three groups were assayed for statistical significance using a Kruskal–Wallis one-way ANOVA test. A p value <0.05 was considered statistically significant.

Results

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

Dynamic recruitment of naive virus-specific CD8 T cells in persistently infected hosts results in an amalgam of cells having different histories of encounter with Ag and proinflammatory 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 time frame over which naive 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 D^b-restricted LT359–368 (LT359) CD8 T cell epitope with the homologous D^b-restricted LT206-215 (LT206) epitope in SV40 LT Ag (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 (J.J. Wilson and C.D. Pack, unpublished observations) and expand in Thy1-congenic recipients acutely infected by MPyV.LT206 but not MPyV.A2 (Supplemental Fig. 1B, *top two 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 (J.J. Wilson and C.D. Pack, unpublished observations) (18).

TCR-I cells proliferate after transfer to congenic recipients infected with MPyV.LT206 for 60 d, showing that the D^bLT206 epitope is expressed long-term and available for recognition by virus-specific CD8 T cells (Supplemental Fig. 1B, *bottom panel*). However, only a portion of the 1×10^6 donor TCR-I cells expanded within 5 d posttransfer. Antigenic competition by an excessive number of naive 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 naive cell precursors, we transferred only 100 TCR-I cells per recipient in all subsequent experiments (24). Transfer of 100 TCR-I cells 1 d prior to infection allowed the generation of an endogenous LT206-specific CD8 T cell response comparable to unmanipulated infected mice (J.J. Wilson and C.D. Pack, unpublished observations); virus levels showed only a modest 2-fold transient decrease at day 7 postinfection (p.i.) following transfer, with no decrease evident by day 15 p.i. or later (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 d before infection (acute infection recruitment) or at days 60 or 90 p.i. (persistent infection recruitment); acute infection-recruited TCR-I cells were assessed at days 30 and 90 p.i. to match the time after cell transfer and the time p.i., respectively (Fig. 1A). For acute infection recruitment, naive TCR-I cells vigorously expanded, peaking in magnitude at 1 wk 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, postcontraction, 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). In contrast, when transferred to hosts infected 90 d earlier, TCR-I cells underwent a slow progressive expansion, without an appreciable contraction phase, over a 2-mo time frame to reach a plateau maintenance level. Notably, by day 90 posttransfer, the pool of circulating TCR-I cells recruited during persistent in-

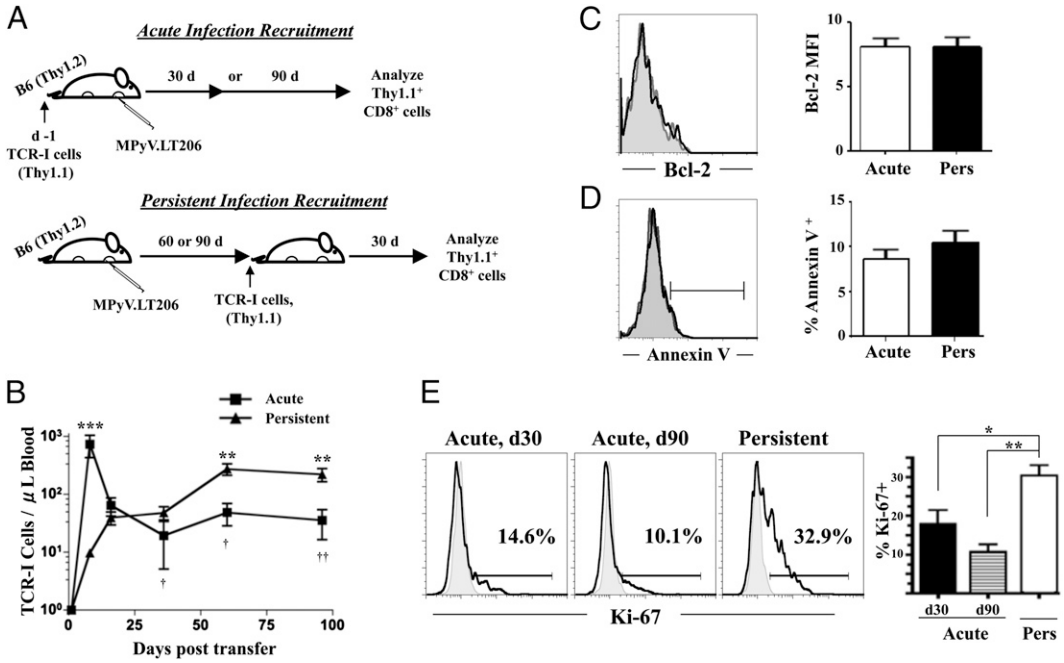

FIGURE 1. Distinct response profiles for virus-specific CD8 T cells recruited during acute and persistent MPyV infection. (**A**) Experimental design: 100 Thy1.1$^+$ CD8$^+$ TCR-I cells were transferred into Thy1.2$^+$ B6 mice on day −1 (acute) or days 60 or 90 (persistent) of infection by MPyV.LT206. TCR-I cells were then evaluated either 30 or 90 d posttransfer. (**B**) CD45$^+$CD8$^+$Thy1.1$^+$ cells (TCR-I cells) in PBMCs were enumerated at the indicated time points after cell transfer. Data are representative of two independent experiments of three to six mice. †, ††One of six mice and two of six mice with no detectable TCR-I cells, respectively. (**C** and **D**) TCR-I cells were transferred either on day −1 or 60 of infection with MPyV.LT206. At day 30 posttransfer, splenic TCR-I cells were analyzed for intracellular Bcl-2 and surface Annexin V binding. *Left panels*, Representative histogram (open, acute infection-recruited; shaded, persistent infection-recruited); *right panels*, mean frequency ± SD. (**E**) At 30 and 90 d posttransfer for acute infection recruitment and day 30 posttransfer 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 three to six mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

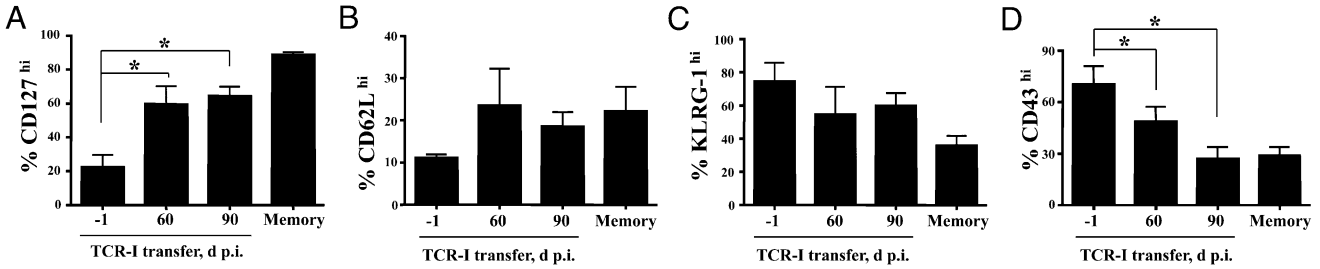

FIGURE 2. Expression of memory markers by CD8 T cells differs with timing of recruitment during MPyV infection. (**A**–**D**) Thirty days after TCR-I cell transfer, expression of cell-surface CD127, CD62L, KLRG1, and CD43 by splenic TCR-I cells was analyzed by flow cytometry. Memory TCR-I cells generated 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 (2×10^6 PFU). Percentages are mean frequency ± SD. *$p < 0.05$.

fection was 4- to 5-fold larger than those recruited during acute infection, and 100% of these recipients had sizeable populations of memory TCR-I cells (Fig. 1B). 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 1×10^3 PFU MPyV.LT206 underwent a delayed but similar expansion-contraction response pattern as those given 1×10^6 PFU inocula (C.D. Pack, 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 antiapoptotic molecule or decreased cell death as indicated by Annexin V staining (Fig. 1C, 1D). However, a significantly higher fraction of memory TCR-I recruited in persistently infected mice expressed the NF 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. 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-7R α-chain (CD127). This expression level mimicked that of true memory TCR-I cells (designated memory) generated in response to acutely resolved infection by rVV–ES-I (Fig. 2A). At day 30 after transfer, TCR-I cell expression of the lymphoid homing marker CD62L, although 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 day 90 than at day 30 p.i. (Fig. 3B), which may be associated with the capacity of these cells to survive long-term. Interestingly, most TCR-I cells expressed KLRG1, a marker of T cell senescence (25), regardless of timing of recruitment (Figs. 2C, 3B), whereas 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 for which 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 has 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 $CD43^{hi}$ phenotype, with only a small fraction of the $CD43^{lo}$ cells coexpressing CD27 or CXCR3, regardless of the length of time p.i. However, persistent infection-recruited memory TCR-I cells expressed less CD43 (Figs. 2D, 3A) and higher numbers of CD27 and CXCR3 molecules (Fig. 3). These $CXCR3^{hi}$ 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 day 30 and day 90 p.i.) and persistent infection-recruited memory TCR-I cells were $KLRG1^{hi}$, with few TCR-I cells in either group expressing the PD-1 or LAG-3 inhibitory receptors (Fig. 3B). Additionally, given

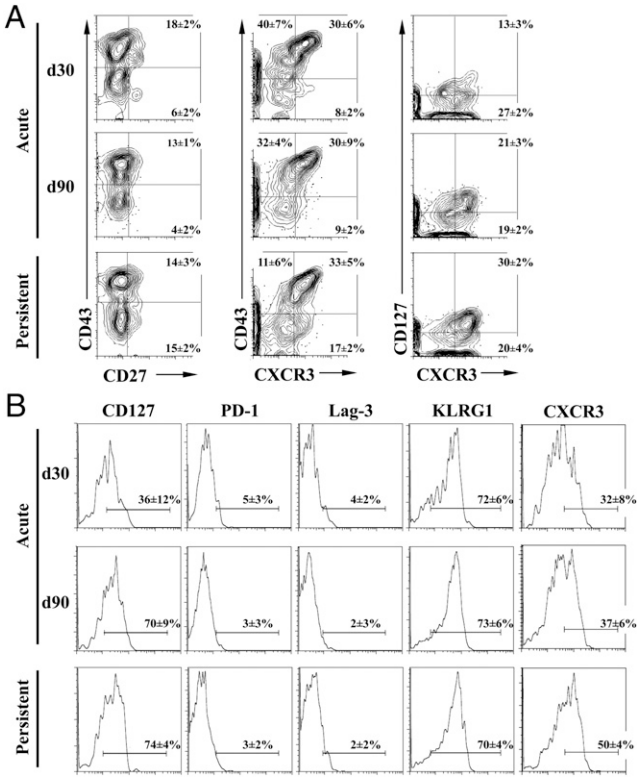

FIGURE 3. Minimal changes in memory phenotype of acute infection-recruited anti-MPyV CD8 T cells over long-term maintenance. Representative flow cytometry plots and histograms of splenic TCR-I cells at 30 or 90 d after day −1 p.i. transfer (acute) or 30 d after transfer to mice at day 60 p.i. (persistent). (**A**) $CD8^+CD44^{hi}Thy1.1^+$ splenocytes were stained for CD27, CD43, CXCR3, and CD127. (**B**) Representative histograms of $CD8^+CD44^{hi}Thy1.1^+$ splenocytes stained for CD127, PD-1, Lag-3, KLRG1, and CXCR3. Gates are based on isotype control staining of $CD8^+CD44^{hi}Thy1.1^+$ cells. Percentages are mean ± SD of two independent experiments of three to six mice each.

the high KLRG1 expression by memory TCR-I cells generated under either acute or persistent infection settings, there were no phenotypically authentic CD127^{hi}KLRG1^{lo} 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 coexpression of IL-2 (Fig. 4A). Of note, the ability to coproduce IFN-γ and IL-2 improved with recruitment at later time points 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 coproduce IL-2 over time (compare day 30 with day 90 p.i.). LT206 peptide dose titration further showed that whereas 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, 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

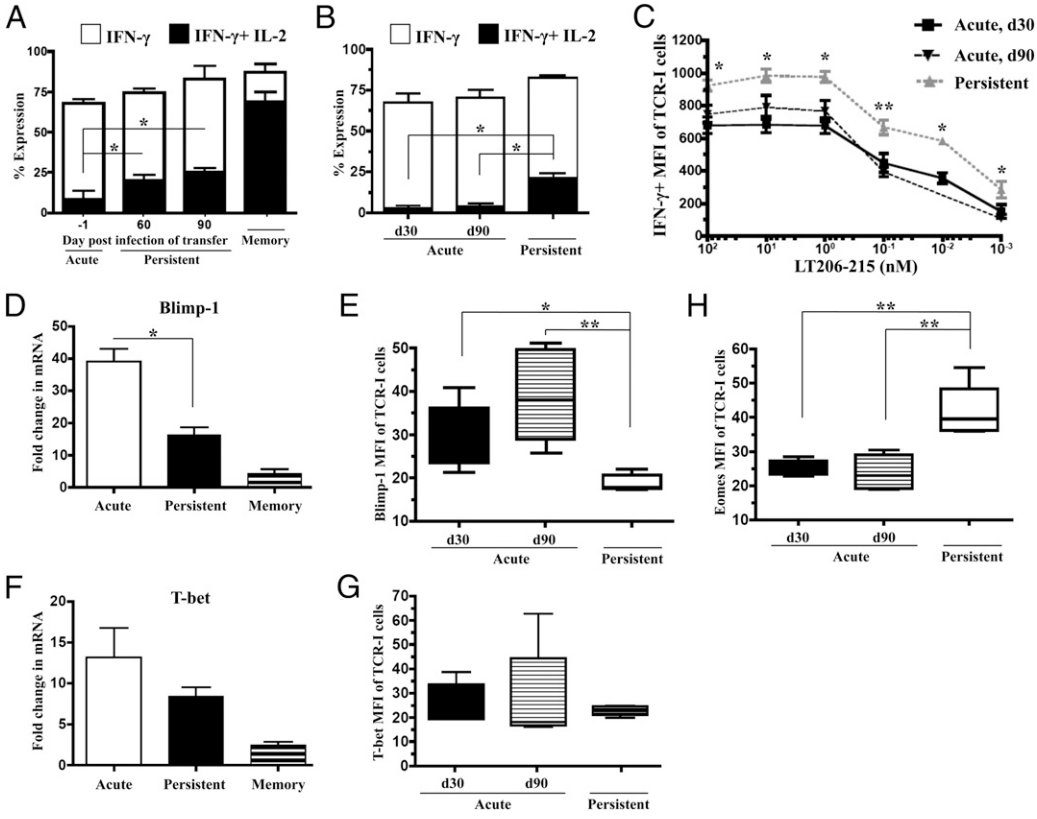

FIGURE 4. Persistent infection-recruited memory CD8 T cells express higher multicytokine functionality and reduced Blimp-1. (**A** and **B**) LT206 peptide-stimulated intracellular IFN-γ and IL-2 production by splenic TCR-I cells. (A) TCR-I cells at day 30 posttransfer in acutely (day −1 p.i.) or persistently (60 or 90 d p.i.) MPyV-infected mice, and at day 30 p.i. of rVV–ES-I infection (memory). (B) Acute infection-recruited (day −1 p.i.) TCR-I cells at day 30 or 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 coproducing IFN-γ and IL-2. Data are representative of three experiments using three to six mice per group. (**C**) Peptide dose-response curve for acute infection-recruited (30 or 90 d posttransfer) and persistent infection-recruited (day 60 p.i.) TCR-I cells expressing IFN-γ after stimulation with the indicated LT206 peptide concentration. Values represent the mean fluorescence intensity (MFI) ± SD of Thy1.1$^+$ CD8$^+$ TCR-I cells producing IFN-γ. Data are representative of two to four independent experiments of three to six mice per group. (**D** and **F**), TCR-I cells were transferred either on day −1 (acute infection recruitment) or day 60 (persistent infection recruitment) of MPyV.LT206 inoculation or day −1 of rVV–ES-I infection (memory) into Thy1.2$^+$ B6 recipients. At day 30 posttransfer, 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$^+$ D8$^+$ TCR-I cells from acute infection-recruited cells on days 30 or 90 p.i. or persistent infection-recruited cells on day 30 posttransfer 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 two to four independent experiments of three to six mice per group. *$p < 0.05$, **$p < 0.01$.

recruited during the persistent phase of infection (Fig. 4F, 4G). Therefore, although 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 D^bLT206 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 coproduce 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–deficient (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-$A^{b-/-}$ mice at day 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 6C, 6D). 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

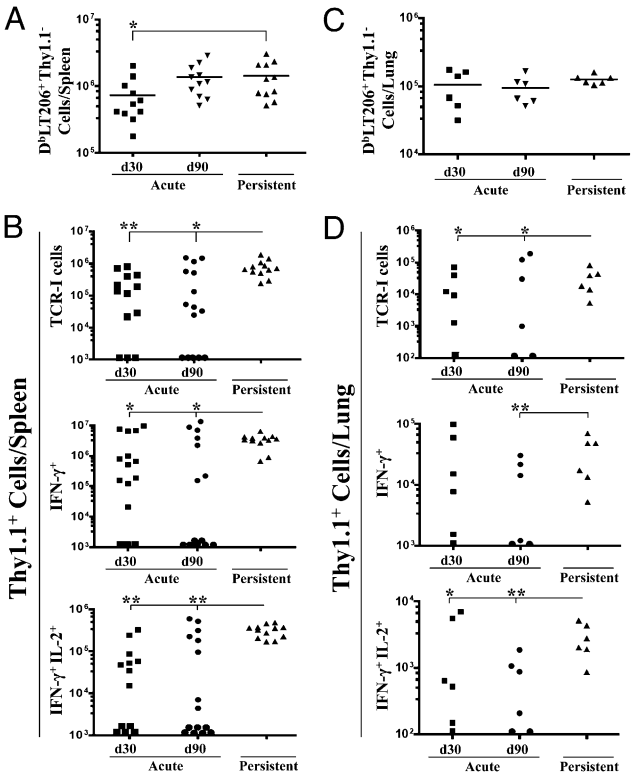

FIGURE 5. Acute MPyV infection-recruited CD8 T cells suffer exhaustion. Splenic (**A**) and pulmonary (**C**) Thy1.1$^-$$D^bLT206$ tetramer$^+$ CD8 T cells (endogenous) were enumerated in mice at day 30 or 90 p.i. that had received TCR-I cells on day −1 p.i. (acute) or 30 d after transfer of TCR-I cells at day 60 p.i. (persistent). Thy1.1$^+$ CD8$^+$ TCR-I cells from the spleen (**B**) and lungs (**D**) were enumerated for D^b LT206 tetramer binding (*top panels*), LT206-stimulated intracellular IFN-γ production (*middle panels*), and intracellular IFN-γ and IL-2 coproduction (*bottom panels*). Data are compiled from two to four experiments of three to six mice each. Points on the *x*-axes represent individual mice with undetectable Thy1.1$^+$CD8$^+$ cells. $^*p < 0.05$, $^{**}p < 0.01$.

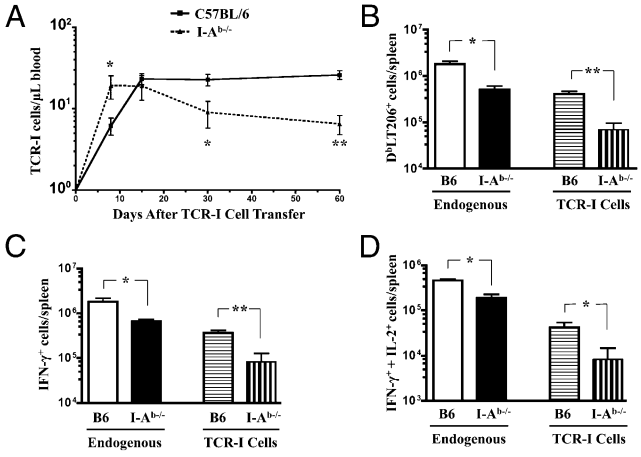

FIGURE 6. CD4 T cells are required for maintenance of memory MPyV-specific CD8 T cells during persistent infection. B6 or I-$A^{b-/-}$ mice received 100 TCR-I cells on day 60 p.i. of MPyV.LT206 infection. (**A**) Enumerations of Thy1.1$^+$ CD8$^+$ PBMCs (solid line, B6 mice; dotted line, I-$A^{b-/-}$ mice) over the course of infection. (**B**) Total Thy1.1$^-$CD8$^+$ CD44^{hi}D^bLT206 tetramer$^+$ splenocytes (endogenous) or TCR-I cells (Thy1.1$^+$) were enumerated at day 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$^-$) CD8$^+$ T cells or TCR-I cells (Thy1.1$^+$) were quantified. Values indicate mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

TCR-I cells had an increased fraction of $CD43^{lo}CD27^{hi}$ 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; C.D. Pack, 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 naive B6 recipients infected the following day or to B6 recipients infected 60 d earlier. Thirty days after transfer, $Thy1.1^+$ CD8 T cells were FACS-sorted, and 1×10^4 memory TCR-I cells were retransferred to naive 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 re-encounter.

Discussion

In contrast to memory virus-specific CD8 T cells that encounter aggressive chronic infections, those coexisting 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 naive 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 ($CD44^{hi}CD62L^{hi}CD127^{hi}$); however, their functional integrity and contribution to the memory compartment have not been previously investigated. In this study, we re-examined 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 naive 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 naive virus-specific CD8 T cells recruited during persistent infection are important for the integrity of a stable and functional memory antiviral T cell population.

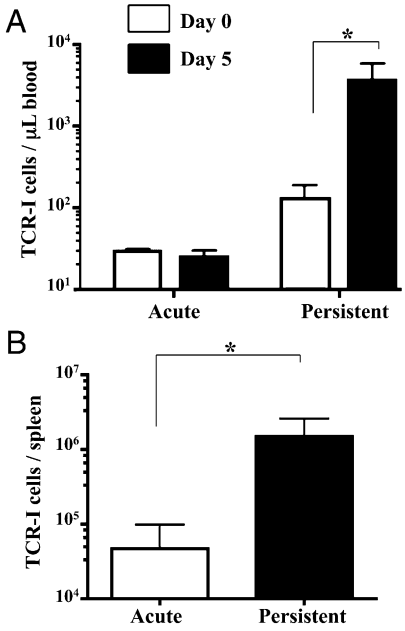

FIGURE 7. MPyV-specific CD8 T cells recruited in persistent, but not acute, infection mount recall responses. (**A**) B6 mice received 100 TCR-I CD8 T cells either 1 d before (acute infection recruitment) or at 60 d p.i. (persistent infection recruitment) by MPyV.LT206. Thirty days post-transfer, each mouse received 1×10^6 PFU rVV–ES-I. The number ± SD of Thy 1.1^+ $CD8^+$ cells in peripheral blood was enumerated at days 0 and 5 p.i. as described in Fig. 1. (**B**) Total of 1×10^4 FACS-purified TCR-I T cells that were stimulated for 30 d during acute (days 0–30 p.i.) or persistent (days 60–90 p.i.) MPyV.LT206 infection (as described in Fig. 1A) were transferred i.v. into individual naive B6 mice; 1 d posttransfer, 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 day 5 after rVV–ES-I inoculation. Values indicated mean ± SD. Data are representative of two independent experiments using three mice per group. $*p < 0.05$.

The time frame over which naive CD8 T cells are recruited to acutely resolved infections has been shown to impact the balance between effector and memory differentiation. Unlike naive virus-specific CD8 T cells recruited at the inception of an acute viral infection, those recruited during later time points 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, co-stimulation, and availability of CD4 T cell help drive dynamic alterations in the differentiation program imprinted on viral Ag-activated naive CD8 T cells (1). The data presented in this study show that even during smoldering infections such as MPyV, the direction of memory differentiation differs for virus-specific CD8 T cells recruited during acute versus persistent infection.

In contrast to the latency-reactivation lifecycle of herpesviruses, polyomaviruses persist as smoldering infections in which 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 nonlatent persistent viral infections. This viral lifecycle difference may also underlie recent evidence that memory CD8 T cells to mouse CMV and γ-herpesvirus infections are derived from naive progenitors recruited during the acute phase of primary infection (8, 36). Data presented in this study indicate that naive 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 naive TCR-I cells transferred 2 wk p.i. give rise to memory cells exhibiting phenotypic and functional characteristics intermediate between those recruited at the beginning and 30 d 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 naive MPyV-specific CD8 T cells that progress toward effective memory increases over the course of infection.

During early acute viral infections, naive 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 lymphocytic choriomeningitis virus infection model, Kaech and coworkers (23) 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. Coincident with viral clearance, T-bet and Blimp-1 transcription factors are downregulated, whereas Eomes expression gradually increases in concert with the emergence of self-renewing functional memory CD8 T cells (39). In contrast, during chronic lymphocytic choriomeningitis virus 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 with 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, although 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, whereas 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 (46) recently showed that the memory antiviral CD8 T cell compartment is a composite of naive T cells recruited during persistent infection and the progeny of cells primed early in infection. In this study, donor-derived, MCMV-specific CD8 T cells were detected months after transferring splenocytes from day 7-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 MCMV-specific TCR-transgenic CD8 T cells, Torti et al. (47) 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. 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 in this study 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 in which ongoing priming of naive 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.

Acknowledgments

We thank Dr. Susan Kaech for protocols for the RT-PCR analyses. We also thank Linda Stempora for expertise in cell sorting.

Disclosures

The authors have no financial conflicts of interest.

References

1. Harty, J. T., and V. P. Badovinac. 2008. Shaping and reshaping CD8$^+$ T-cell memory. *Nat. Rev. Immunol.* 8: 107–119.
2. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 25: 171–192.
3. Butler, N. S., and J. T. Harty. 2010. The role of inflammation in the generation and maintenance of memory T cells. *Adv. Exp. Med. Biol.* 684: 42–56.
4. Belz, G. T., and A. Kallies. 2010. Effector and memory CD8$^+$ T cell differentiation: toward a molecular understanding of fate determination. *Curr. Opin. Immunol.* 22: 279–285.
5. Rutishauser, R. L., and S. M. Kaech. 2010. Generating diversity: transcriptional regulation of effector and memory CD8 T-cell differentiation. *Immunol. Rev.* 235: 219–233.
6. Mueller, S. N., and R. Ahmed. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc. Natl. Acad. Sci. USA* 106: 8623–8628.
7. Angelosanto, J. M., and E. J. Wherry. 2010. Transcription factor regulation of CD8$^+$ T-cell memory and exhaustion. *Immunol. Rev.* 236: 167–175.
8. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity* 29: 650–659.
9. Abend, J. R., M. Jiang, and M. J. Imperiale. 2009. BK virus and human cancer: innocent until proven guilty. *Semin. Cancer Biol.* 19: 252–260.
10. Hirsch, H. H., P. Randhawa; and AST Infectious Diseases Community of Practice. 2009. BK virus in solid organ transplant recipients. *Am. J. Transplant.* 9 (Suppl 4): S136–S146.
11. Brew, B. J., N. W. Davies, P. Cinque, D. B. Clifford, and A. Nath. 2010. Progressive multifocal leukoencephalopathy and other forms of JC virus disease. *Nat. Rev. Neurol.* 6: 667–679.
12. Koralnik, I. J., R. A. Du Pasquier, M. J. Kuroda, J. E. Schmitz, X. Dang, Y. Zheng, M. Lifton, and N. L. Letvin. 2002. Association of prolonged survival in HLA-A2$^+$ progressive multifocal leukoencephalopathy patients with a CTL response specific for a commonly recognized JC virus epitope. *J. Immunol.* 168: 499–504.
13. Lacey, S. F. 2007. BK viral loads and immune monitoring in renal transplant recipients. *Am. J. Transplant.* 7: 1039–1040.
14. Wilson, J. J., E. Lin, C. D. Pack, E. L. Frost, A. Hadley, A. I. Swimm, J. Wang, Y. Dong, C. P. Breeden, D. Kalman, et al. 2011. Gamma interferon controls mouse polyomavirus infection in vivo. *J. Virol.* 85: 10126–10134.
15. Shin, H., and E. J. Wherry. 2007. CD8 T cell dysfunction during chronic viral infection. *Curr. Opin. Immunol.* 19: 408–415.
16. Kemball, C. C., C. D. Pack, H. M. Guay, Z. N. Li, D. A. Steinhauer, E. Szomolanyi-Tsuda, and A. E. Lukacher. 2007. The antiviral CD8$^+$ T cell response is differentially dependent on CD4$^+$ T cell help over the course of persistent infection. *J. Immunol.* 179: 1113–1121.
17. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J. Exp. Med.* 203: 2263–2269.
18. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8$^+$ T cell responses during a persistent virus infection. *J. Immunol.* 174: 7950–7960.

19. Staveley-O'Carroll, K., T. D. Schell, M. Jimenez, L. M. Mylin, M. J. Tevethia, S. P. Schoenberger, and S. S. Tevethia. 2003. In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes $CD8^+$ T cell effector function in SV40 T antigen transgenic mice. *J. Immunol.* 171: 697–707.
20. Andrews, N. P., C. D. Pack, and A. E. Lukacher. 2008. Generation of antiviral major histocompatibility complex class I-restricted T cells in the absence of CD8 coreceptors. *J. Virol.* 82: 4697–4705.
21. Fu, T. M., L. M. Mylin, T. D. Schell, I. Bacik, G. Russ, J. W. Yewdell, J. R. Bennink, and S. S. Tevethia. 1998. An endoplasmic reticulum-targeting signal sequence enhances the immunogenicity of an immunorecessive simian virus 40 large T antigen cytotoxic T-lymphocyte epitope. *J. Virol.* 72: 1469–1481.
22. Andrews, N. P., C. D. Pack, V. Vezys, G. N. Barber, and A. E. Lukacher. 2007. Early virus-associated bystander events affect the fitness of the CD8 T cell response to persistent virus infection. *J. Immunol.* 178: 7267–7275.
23. Rutishauser, R. L., G. A. Martins, S. Kalachikov, A. Chandele, I. A. Parish, E. Meffre, J. Jacob, K. Calame, and S. M. Kaech. 2009. Transcriptional repressor Blimp-1 promotes $CD8(^+)$ T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31: 296–308.
24. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the $CD8(^+)$ T cell response to infection. *Immunity* 26: 827–841.
25. Sarkar, S., V. Teichgräber, V. Kalia, A. Polley, D. Masopust, L. E. Harrington, R. Ahmed, and E. J. Wherry. 2007. Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation. *J. Immunol.* 179: 6704–6714.
26. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory $CD8^+$ T cells. *J. Exp. Med.* 204: 1625–1636.
27. Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector $CD8(^+)$ T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27: 281–295.
28. Shin, H., S. D. Blackburn, A. M. Intlekofer, C. Kao, J. M. Angelosanto, S. L. Reiner, and E. J. Wherry. 2009. A role for the transcriptional repressor Blimp-1 in $CD8(^+)$ T cell exhaustion during chronic viral infection. *Immunity* 31: 309–320.
29. Intlekofer, A. M., N. Takemoto, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. R. Palanivel, A. C. Mullen, C. R. Gasink, S. M. Kaech, J. D. Miller, et al. 2005. Effector and memory $CD8^+$ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6: 1236–1244.
30. Rao, R. R., Q. Li, K. Odunsi, and P. A. Shrikant. 2010. The mTOR kinase determines effector versus memory $CD8^+$ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity* 32: 67–78.
31. Kallies, A., A. Xin, G. T. Belz, and S. L. Nutt. 2009. Blimp-1 transcription factor is required for the differentiation of effector $CD8(^+)$ T cells and memory responses. *Immunity* 31: 283–295.
32. Szomolanyi-Tsuda, E., and R. M. Welsh. 1996. T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice. *J. Exp. Med.* 183: 403–411.
33. D'Souza, W. N., and S. M. Hedrick. 2006. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. *J. Immunol.* 177: 777–781.
34. Fousteri, G., A. Dave, A. Juedes, T. Juntti, B. Morin, L. Togher, D. L. Farber, and M. von Herrath. 2011. Increased memory conversion of naïve CD8 T cells activated during late phases of acute virus infection due to decreased cumulative antigen exposure. *PLoS ONE* 6: e14502.
35. Badovinac, V. P., and J. T. Harty. 2007. Manipulating the rate of memory $CD8^+$ T cell generation after acute infection. *J. Immunol.* 179: 53–63.
36. Bannard, O., M. Kraman, and D. T. Fearon. 2010. Cutting edge: Virus-specific $CD8^+$ T cell clones and the maintenance of replicative function during a persistent viral infection. *J. Immunol.* 185: 7141–7145.
37. Wherry, E. J. 2011. T cell exhaustion. *Nat. Immunol.* 12: 492–499.
38. Welsh, R. M. 2009. Blimp hovers over T cell immunity. *Immunity* 31: 178–180.
39. Takemoto, N., A. M. Intlekofer, J. T. Northrup, E. J. Wherry, and S. L. Reiner. 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced $CD8^+$ T cell differentiation. *J. Immunol.* 177: 7515–7519.
40. Parish, I. A., and S. M. Kaech. 2009. Diversity in $CD8(^+)$ T cell differentiation. *Curr. Opin. Immunol.* 21: 291–297.
41. Joshi, N. S., and S. M. Kaech. 2008. Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. *J. Immunol.* 180: 1309–1315.
42. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, et al. 2003. Control of effector $CD8^+$ T cell function by the transcription factor Eomesodermin. *Science* 302: 1041–1043.
43. Kao, C., K. J. Oestreich, M. A. Paley, A. Crawford, J. M. Angelosanto, M. A. Ali, A. M. Intlekofer, J. M. Boss, S. L. Reiner, A. S. Weinmann, and E. J. Wherry. 2011. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific $CD8^+$ T cell responses during chronic infection. *Nat. Immunol.* 12: 663–671.
44. Banerjee, A., S. M. Gordon, A. M. Intlekofer, M. A. Paley, E. C. Mooney, T. Lindsten, E. J. Wherry, and S. L. Reiner. 2010. Cutting edge: The transcription factor eomesodermin enables $CD8^+$ T cells to compete for the memory cell niche. *J. Immunol.* 185: 4988–4992.
45. Joshi, N. S., W. Cui, C. X. Dominguez, J. H. Chen, T. W. Hand, and S. M. Kaech. 2011. Increased numbers of preexisting memory CD8 T cells and decreased T-bet expression can restrain terminal differentiation of secondary effector and memory CD8 T cells. *J. Immunol.* 187: 4068–4076.
46. Snyder, C. M., A. Loewendorf, E. L. Bonnett, M. Croft, C. A. Benedict, and A. B. Hill. 2009. $CD4^+$ T cell help has an epitope-dependent impact on $CD8^+$ T cell memory inflation during murine cytomegalovirus infection. *J. Immunol.* 183: 3932–3941.
47. Torti, N., S. M. Walton, T. Brocker, T. Rülicke, and A. Oxenius. 2011. Non-hematopoietic cells in lymph nodes drive memory CD8 T cell inflation during murine cytomegalovirus infection. *PLoS Pathog.* 7: e1002313.