Memory T Cells Specific for Murine Cytomegalovirus Re-Emerge after Multiple Challenges and Recapitulate Immunity in Various Adoptive Transfer Scenarios

Michael Quinn, Holly Turula, Mayank Tandon, Berthony Deslouches, Toktam Moghbeli and Christopher M. Snyder

J Immunol 2015; 194:1726-1736; Prepublished online 16 January 2015;
doi: 10.4049/jimmunol.1402757
http://www.jimmunol.org/content/194/4/1726

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/01/16/jimmunol.1402757.DCSupplemental

References
This article cites 71 articles, 43 of which you can access for free at:
http://www.jimmunol.org/content/194/4/1726.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Memory T Cells Specific for Murine Cytomegalovirus Re-Emerge after Multiple Challenges and Recapitulate Immunity in Various Adoptive Transfer Scenarios

Michael Quinn, Holly Turula,1,2 Mayank Tandon,1,3 Berthony Deslouches,4 Toktam Moghbeli, and Christopher M. Snyder

Reconstitution of CMV-specific immunity after transplant remains a primary clinical objective to prevent CMV disease, and adoptive immunotherapy of CMV-specific T cells can be an effective therapeutic approach. Because of viral persistence, most CMV-specific CD8+ T cells become terminally differentiated effector phenotype CD8+ T cells (T_{EFF}). A minor subset retains a memory-like phenotype (memory phenotype CD8+ T cells [T_M]), but it is unknown whether these cells retain memory function or persist over time. Interestingly, recent studies suggest that CMV-specific CD8+ T cells with different phenotypes have different abilities to reconstitute sustained immunity after transfer. The immunology of human CMV infections is reflected in the murine CMV (MCMV) model. We found that human CMV– and MCMV-specific T cells displayed shared genetic programs, validating the MCMV model for studies of CMV-specific T cells in vivo. The MCMV-specific T_M population was stable over time and retained a proliferative capacity that was vastly superior to T_{EFF}. Strikingly, after transfer, T_M established sustained and diverse T cell populations even after multiple challenges. Although both T_{EFF} and T_M could protect Rag2−/− mice, only T_M persisted after transfer into immune replete, latently infected recipients and responded if recipient immunity was lost. Interestingly, transferred T_M did not expand until recipient immunity was lost, supporting that competition limits the Ag stimulation of T_M. Ultimately, these data show that CMV-specific T_M retain memory function during MCMV infection and can re-establish CMV immunity when necessary. Thus, T_M may be a critical component for consistent, long-term adoptive immunotherapy success. The Journal of Immunology, 2015, 194: 1726–1736.

L

atent CMV is present within a large percentage of the population but is effectively controlled by the immune system (1–6). However, in transplant patients, immune suppression can allow CMV reactivations to progress to disease and increase mortality. Despite the advancements of antiviral medications, long-term prevention of CMV disease is dependent on the reconstitution of CMV-specific immunity, which can be achieved through adoptive immunotherapy (5–18).

In adoptive immunotherapy, healthy CMV-seropositive donors provide CMV-specific T cells to an immunosuppressed recipient. Because of the persistent nature of CMV infection, CMV-seropositive donors accumulate large numbers of CMV-specific CD8+ T cells (~5–10% of the total CD8+ T cells), a process termed “memory inflation” (19–28). Studies in humans and the well-characterized murine CMV (MCMV) model have shown that the majority of inflammatory populations are composed of terminally differentiated effector phenotype CD8+ T cells (T_{EFF}) that presumably develop as a result of repeated Ag stimulation and may not possess the proliferative or survival capacity necessary for long-term maintenance of CMV immunity (22, 27, 29–34). Interestingly, however, a fraction of these inflammatory T cells retain a memory-like phenotype (memory phenotype CD8+ T cells [T_M]), despite sharing epitope specificity and TCR sequences with T_{EFF} subset (23, 25, 35–37). Studies with other infection models have shown that such a memory phenotype can identify cells that have stem cell–like characteristics (38, 39). If this model holds true for CMV immunity, the CMV-specific T_M would be ideal to use in an adoptive immunotherapy setting. Recent evidence supports this hypothesis. In a nonhuman primate model, CMV-specific effector T cells that were expanded in vitro from sorted T_M had a superior ability to survive after adoptive transfer (40). Moreover, a human study showed a positive correlation between the presence of CMV-specific T_M in a donor transfer and the long-term maintenance of donor-derived cells (41). The goal of our study was to use the mouse model (MCMV) to directly address the capacity of the CMV-specific T_M population to restore long-term CMV-specific immunity after transfer. Importantly, we found that the MCMV-specific T_M share a common
genetic program with their human CMV (HCMV)–specific counterparts and that these cells could repeatedly restore long-term CMV-specific immunity under a spectrum of transfer scenarios. Our data suggest that adoptive immunotherapy with CMV-specific T cell subsets retains similar numbers of T cell subsets and that these cells could repeatedly restore long-term CMV-specific immunity under a spectrum of transfer scenarios.

Materials and Methods

**Mice**

Unless otherwise indicated, C57BL/6 (B6), CD45.1 (B6.SJL-Ptprc<sup>−</sup> Pep8<sup>−</sup>BoyJ), Thy1.1 (B6.PL-Thy1<sup>−</sup>1CyJ), and Rag<sup>−</sup> mice (B6.129S7-Rag1<sup>tm1Mom/J</sup>) were purchased from The Jackson Laboratory. OT-I transgenic mice (C57BL/6-Tg(TcraTcrb<sup>−/−</sup>)100Mjb/J), also purchased from The Jackson Laboratory, were bred with CD45.1 mice to produce double-positive (CD45.2<sup>−</sup>/CD45.1<sup>+</sup>) OT-I mice. All protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

**Infections**

Unless otherwise indicated, mice were infected i.p. with 2 × 10<sup>6</sup> PFU MCMV strain MW97.01 (42). Mice were considered latently infected at wk postinfection. Rag<sup>−</sup> mice were infected with 5 × 10<sup>3</sup> PFU MCMV-TK virus (43). OT-I T cell transfer recipients were challenged with 2 × 10<sup>5</sup> PFU MCMV-SL8, which expresses the SINIFEKL peptide (44, 45).

**Tetramer staining, Abs, and FACS analysis**

MHC tetramers were provided by the National Institutes of Health Tetracer Core Facility (http://tetramer.yerkes.emory.edu) and have been described previously (27). Staining was performed as described previously (27) with tetramers and the following Abs: CD8<sup>+</sup>(53-6.7); CD44(IM7); CD27(LG.3A10); CD127(AR734); KLRG1(2F1); CD62L(MEL-14); CD45.1(A20); CD45.1(104); Thy1.1(OX-7); Thy1.2(30-H12); IFN-γ(XMG1.2); TNF-α(MP6-XT22); CD107a(1D4B). In all cases, samples were collected on an LSR II and analyzed with FlowJo software (Tree Star). The gating strategy for phenotypic characterization of tetramer<sup>+</sup> CD8<sup>+</sup> T cells involved first gating lymphocytes and then singlets. CD8<sup>+</sup> cells were gated as frequency of singlets. Tetramer<sup>+</sup> cells were identified as a frequency of CD8<sup>+</sup> cells. A Brkr tetramer (specific for the Brkr peptide from Vaccinia) was used as a negative tetramer control. Tetramer<sup>+</sup> cells were phenotypically defined by their expression of KLRG1, CD27, CD127, or CD62L.

**Adoptive transfers**

CD<sup>8</sup><sup>+</sup> splenocytes from latently infected donors were enriched using EasySep Biotin selection kit (Stemcell Technologies) and biotinylated Abs against RBCs (Ter119), CD4 (GK1.5), and CD19 (6D5) according to the recommended protocol. Enriched cells were stained to determine the frequency of transferred tetramer<sup>+</sup> CD8<sup>+</sup> T cells in the spleen. (OT-I T cells were transferred (assuming 100% engraftment). The gating strategy for analyzing donor cells in the recipients was identical to that described earlier with Abs specific for the relevant congenic marker (CD45.1 or Thy1.2). For OT-I adoptive transfers, splenocytes from naive mice containing 600 OT-I T cells were transferred. Recipients were challenged with MCMV-SL8. To establish secondary and tertiary populations, we FACS sorted and transferred OT-I T<sub>RM</sub> as described in the legend for Fig. 5 and in Supplemental Fig. 1. After challenge with MCMV-SL8, the frequencies of donor OT-Is were determined in the blood of recipients using the strategy described earlier except that singlets were not identified and OT-I donors were identified by expression of CD45.1 and V<sub>α</sub>2.

**Intracellular stimulation**

Intracellular stimulation and staining were performed as previously described (27, 45), with minor modifications. Specifically, cells were incubated with 1 μg/ml brefeldin A (GolgiPlug; BD Biosciences), and CD107a-specific Abs for 3 h.

**CD70 blocking Ab treatment**

CD70 Ab blockade was performed as previously described (46), with minor modifications. In brief, mice received either 150 μg anti-CD70 (FR70) or control rat IgG2b (both from BioXCell) via the i.p. route. Injections were administered at days −1, 0, and 3 postinfection.

**Ab depletions**

Ab depletions were performed with Thyl.1.1(9E12), CD4<sup>+</sup> (GK1.5), and NK1.1 (PK136) Abs. A total of 300 μg of each Ab was administered i.p. in PBS. Three subsequent injections of 100 μg of each Ab were given at 7-d intervals.

**Microarray**

Splenocytes from latently infected mice were cultured with tetramers loaded with the antigenic peptides from M38, m139, and I3 (25) and sorted on a MoFlo (Dako Cytomation) cell sorter. CMV-specific T cells were identified as CD8<sup>+</sup>, CD4<sup>+</sup> and tetramer binding, and then further segregated into T<sub>RM</sub> and T<sub>TEM</sub> subsets by expression of KLRG1 and CD127. Naïve CD8<sup>+</sup> cells were CD44<sup>+</sup>. Total RNA was isolated using the Qiagen RNeasy Plus Kit (Qiagen), quantified on a NanoDrop 2000c Spectrophotometer (Thermo Scientific), and processed at the Microarray Core Facility at Thomas Jefferson University. In brief, 2.5 μg fragmented and biotinylated cDNA was hybridized to Mouse gene 1.0 ST array (Affymetrix). Chips were scanned on an Affymetrix Gene Chip Scanner 3000, and data were analyzed using the R programming language and various packages from Bioconductor (47). The oligo package (48) was used to extract expression data from the Affymetrix CEL files and perform background and RMA normalization (49). Annotation information was added using the geneontologytranscriptcluster.db (50) package. Probes without valid annotations (7196 of 35,556 probes) were removed before differential expression analysis using the Limma package’s (51) linear modeling and Bayes methods (52). Genes showing upregulation or downregulation of at least 2-fold and p < 0.05 in each of three contrasts (T<sub>RM</sub> Versus naive, T<sub>RM</sub> versus naïve, and T<sub>TEM</sub> versus T<sub>RM</sub>) were considered for gene set enrichment analysis (GSEA). Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (53) under accession no. GSE61927.

**GSEA**

Human data for series GSE24151 (54) were retrieved from National Center for Biotechnology Information’s GEO database (53), extracted using SEM and t test analysis using the Limma package’s linear modeling and Bayes methods (52). Genes showing upregulation or downregulation of at least 2-fold and p < 0.05 in each of three contrasts (T<sub>RM</sub> Versus naive, T<sub>RM</sub> versus naïve, and T<sub>TEM</sub> versus T<sub>RM</sub>) were considered for gene set enrichment analysis (GSEA). Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (53) under accession no. GSE61927.

---

**FIGURE 1.** The noninflationary and inflationary CD8<sup>+</sup> T cell populations retain similar numbers of T<sub>RM</sub>. Cohorts of age-matched B6 mice were infected with MCMV and sacrificed at the indicated time points (n = 4 per time point). Tetramer staining and phenotypic analyses were performed on blood and splenocytes. (A) Frequency of tetramer-binding CD8<sup>+</sup> T cells in the blood at indicated time points. The phenotypic analysis shown was performed at 326 d postinfection. T<sub>RM</sub> were identified as CD27<sup>hi</sup>/KLRG1<sup>lo</sup>. T<sub>CM</sub> and T<sub>TEM</sub> were further identified as CD127<sup>hi</sup> and either CD42L<sup>hi</sup> or CD62L<sup>hi</sup>, respectively. (B) Absolute numbers of KLRG1<sup>hi</sup> tetramer-binding CD8<sup>+</sup> T cells in the spleen. (C) Absolute numbers of T<sub>CM</sub> and T<sub>TEM</sub> tetramer-binding CD8<sup>+</sup> T cells. Data are displayed as mean ± SEM and represent two independent experiments.
Partek Genomics Suite software, version 6.6 (Partek, St. Louis, MO) and curated for input into GSEA software (55) (http://www.broadinstitute.org/gsea). Because the data for GSE24151 have been deposited in GEO as log_{10} ratios of the reference pool to sample, each value was inverted by multiplying by –1. Gene names in the six mouse gene lists (upregulated or downregulated in each of the three contrasts described earlier) were converted to human names using data from National Center for Biotechnology Information’s HomoloGene database, Release 68 (http://www.ncbi.nlm.nih.gov/homologene). The converted gene lists along with genes specific to the liver and the TCR pathway from the Molecular Signature Database (MSigDB) (55) were analyzed for enrichment in the human data using recommended settings for the GSEA command-line interface.

Results
MCMV-specific inflationary T_{M} populations are stable and share a common transcriptional program with HCMV-specific CD8^{+} T cells in humans

In the mouse model, MCMV infection of B6 mice results in inflation of select MCMV-specific CD8^{+} T cells specific for peptides from the viral proteins M38, m139, and IE3 (Fig. 1A) (25, 27, 28). As in humans infected with HCMV, the majority of MCMV-specific inflationary T cells express a T_{EFF} phenotype (often defined as terminally differentiated CD8^{+} T cell phenotype in humans), whereas only a small fraction express a T_{M}-like phenotype, defined in this article as KLRG1^{lo}/CD27^{hi} and further subdivided into central memory CD8^{+} T cells (T_{CM}: CD127^{hi}/CD62L^{lo}) and effector memory CD8^{+} T cells (T_{EM}: CD127^{hi}/CD62L^{lo}) subsets (Fig. 1A) (22, 23, 27, 29–33, 56). In contrast, noninflationary MCMV-specific CD8^{+} T cell responses, represented by the response against the viral protein M45, contract after acute infection and are thought to be maintained by homeostatic mechanisms thereafter (Fig. 1A) (25, 27, 57). As expected, noninflators express a predominately memory (T_{M}) phenotype, which also includes both T_{CM} and T_{EM} subsets (Fig. 1A) (23, 27).

It remains unknown whether the constant immune stimulation needed to maintain memory inflation causes a decline of the T_{M} subset within inflationary populations over time. Using infection-matched cohorts, we found that the numbers of T_{M} that were specific for inflationary Ags were stable over time and remarkably similar to the numbers of noninflationary T_{M}, despite great differences between the numbers of inflationary and noninflationary T_{EFF} (Fig. 1B, 1C). Thus, although continuous Ag stimulation maintains memory inflation, the inflationary T_{M} population remains stable.

FIGURE 2. Gene set enrichment analyses reveal significant overlap between the transcriptional profile of CMV-specific T cells in humans and mice. (A) Gene set enrichment was performed as described in Materials and Methods. Shown are the enrichment plots for mouse genes that differed in a T_{EFF} versus T_{M} comparison, plotted relative to human T_{EFF} and T_{M}. Values represent the normalized enrichment score (NES) and family-wise error rate (FWER), which estimates the probability of a false-positive NES. (B) Lists of significantly altered mouse genes (2-fold up or down and p < 0.05) were generated for T_{EFF} and T_{M} relative to each other and to naive (CD44 low) T cells. GSEA analyses were performed with these mouse gene sets relative to the indicated human data sets, rank ordered by expression (see Materials and Methods). Asterisks indicate FWER-corrected significance to control for multiple testing (*p < 0.05, **p < 0.01, ***p < 0.001).
The MCMV model is well characterized and the T cell responses clearly recapitulate those seen in HCMV-infected people. To determine whether MCMV-specific TM and T EFF share a common transcriptional program with their human counterparts, we sorted MCMV-specific TM (CD44hi/CD127hi/KLRG1lo) and T EFF (CD44hi/CD127hi/KLRG1hi) specific for the M38, m139, and IE3 Ags. Microarray analyses were performed on these cells. Genes that were significantly upregulated or downregulated in TM and T EFF subsets relative to each other or to naive (CD44lo) T cells were mapped to the corresponding human genes and compared with the profiles of HCMV-specific T cells, previously defined by the van Lier group (54) as CD27hi/CD45RAlo (TM) or CD27lo/CD45RAhi (TEFF). The CD27 and CD127 (IL-7Rα) molecules both mark CMV-specific T cells with a memory phenotype in mice and humans (27, 29, 32, 58, 59), and nearly all MCMV-specific KLRG1lo/CD27hi cells (TM) coexpressed CD127 (either TM or TEM; Fig. 1A). Gene set enrichment analyses (GSEA) were used to measure the overall correlation between the mouse and human gene expression data. As shown in Fig. 2A, genes that distinguished mouse T EFF and TM from each other were highly enriched within the corresponding human data set; that is, genes upregulated specifically in mouse TM relative to mouse T EFF were...

FIGURE 3. TM dramatically expand 7 d postchallenge and produce both TM and T EFF progeny. Age-matched B6 mice received either TM or T EFF and were challenged with MCMV as described in Materials and Methods. Spleens were collected 7 d later for analysis. (A) Representative FACS plots of tetramer+ donors in the spleen 7 d postchallenge. Frequencies in the corner are relative to total CD8+ cells. (B) Fold change of donor cells in the spleen, calculated as described in Materials and Methods, 7 d after challenge. Because Ag-specific T cells were not sorted, approximately equal numbers of M38- and M45-specific TM were transferred, but ∼10-fold more M38-specific T EFF were transferred compared with the TM. Because of the extremely low number of M45-specific T EFF transferred and the minimal expansion at day 7, it was not possible to calculate a comparable fold change value for the M45-specific T EFF population. Data collected from two independent experiments (TM: n = 6 total; T EFF: n = 5 total) are shown. Statistical significance was determined by a Student t test (***, p < 0.001, ****p < 0.0001). (C) Representative FACS plots of M38-specific CD8+ T cell progeny from either TM or T EFF donors in the spleen at 7 d postchallenge. Frequencies in the corner are relative to M38-specific CD8+ cells. (D) Absolute number of TM and T EFF phenotypic progeny that were produced from TM donors. Data are from the same experiments described in (B). (E) Fold change of donor cells in the spleen after treatment with either isotype control or anti-CD70 Ab. Data were collected 7 d postchallenge and represent two independent experiments (n = 6 total). All graphical data are displayed as mean ± SEM. Statistical significance was determined by a Student t test (***, p < 0.001, ****p < 0.0001).
highly enriched within the genes that distinguish human T_M from human T_EFF and vice versa. Moreover, relative to naive T cells, mouse genes that were upregulated and downregulated by T_EFF or T_M were highly enriched within genes that distinguished their human counterparts from human naive T cells (Fig. 2B). The analyzed mouse genes and the core enrichment profiles of each comparison are listed in Supplemental Table I. Importantly, several of these genes corresponded to our sorting parameters and the known phenotypes of T_M and T_EFF populations. As controls, identical analyses were performed with genes associated with the TCR signaling pathway or liver, and the data exhibited expected patterns (Fig. 2B).

Overall, these data show that MCMV- and HCMV-specific T cell subsets share a common genetic program, validating the use of the MCMV model to investigate the function of HCMV-specific T cells. To our knowledge, this is the first direct comparison of MCMV-specific and HCMV-specific T cell gene expression profiles.

**The inflationary T_M population retains proliferative capacity**

To test the proliferative capacity of the T_M and T_EFF, both populations were sorted from spleens of latently infected B6 mice (>3 mo postinfection) using their differential expression of KLRG1 and CD27. Sorted cells were transferred into naive congenic recipients and rechallenged. The M45- and M38-specific T_M proliferated robustly within 7 d after challenge, each expanding almost 1000-fold in the spleen alone, assuming 100% engraftment of the donor cells (Fig. 3A, 3B). In contrast, the M38-specific T_EFF population expanded ∼10-fold in the same period. Importantly, whereas the T_EFF donor cells remained exclusively KLRG1high, the T_M donor cells produced large numbers of both T_EFF and T_M progeny (Fig. 3C). In fact, donor M45- and M38-specific T_M were present in the spleen 7 d after challenge at numbers that were ∼50- to 100-fold higher than had been transferred (Fig. 3D, dotted line), indicating expansion of this subset without terminal differentiation. These data show that MCMV-specific T_M retain robust proliferative capacity and can produce phenotypically diverse progeny including new T_M.

Recent work has shown that interaction between CD27 and its ligand CD70 plays a functional role in the proliferation of MCMV-specific inflationary T cells (46). To test the contribution of this interaction specifically within the T_M population, we sorted and transferred T_M as described earlier and blocked the CD27–CD70 interaction as described in Materials and Methods. Blocking the CD27–CD70 interaction significantly decreased the expansion of the M38- and M45-specific T_M 7 d postchallenge by ∼4- to 6-fold (Fig. 3E), which is in line with the impact of CD70 blockade on unsorted (i.e., combined T_M and T_EFF populations) inflationary T cells (46). These data further suggest that the majority of proliferative potential of inflationary T cells is contained within the minor T_M subset. It should be noted that even in the presence of CD70 blockade, the T_M population retained a proliferative capacity that was greater than the T_EFF population, suggesting that additional pathways contribute to the total proliferative potential of these cells (Fig. 3B, 3E) (M. Quinn and C.M. Snyder, unpublished observations).

**The inflationary T_M population persists and can repeatedly recapitulate memory inflation**

To determine the ability of the T_M donor cells to persist long term, we tracked the progeny from T_M donor cells in the blood after...
rechallenge. M38-specific T cells from T_M-sorted donors persisted at high frequencies in recipients, whereas the M45-specific donor cells contracted after their initial expansion in the same mice (Fig. 4A, 4B). Despite their initial T_M phenotype, the donor M38-specific T cells largely expressed a T_EFF phenotype after challenge (Fig. 4C, 4D), consistent with a typical inflationary population. The population as a whole retained its ability produce IFN-γ, TNF-α, and expose CD107a (Fig. 4E, 4F). Importantly, a small portion of donor T cells retained their T_M phenotype even after this secondary challenge (Fig. 4C, 4D).

To understand whether these persistent T_M phenotype donors continued to be functional, we turned to the OT-I transgenic system to facilitate sorting and avoid the possible selection of different T cell clones (Fig. 5A). As shown previously, transferred naive OT-Is undergo inflation and produce both T_M and T_EFF progeny after primary challenge with SIINFEKL-expressing MCMV-SL8 (45). We sorted the T_M phenotype OT-I cells that formed after primary challenge, transferred these cells, and challenged the recipients to establish secondary populations (Supplemental Fig. 1A). As with nontransgenic T cells (Fig. 4), secondary challenge of T_M OT-Is induced inflation and T_EFF formation, as well as a persistent KLRG1<sup>hi</sup> population (Supplemental Fig. 1B). These secondary T_M were again sorted (Supplemental Fig. 1C), transferred into a third set of naive recipients, and rechallenged. Incredibly, the donor secondary T_M population inflated and produced both KLRG1<sup>hi</sup> and KLRG1<sup>lo</sup> progeny after this tertiary challenge (Fig. 5B–E).

Repeated acute viral challenges of small numbers of T cells in naive mice drive T_EFF differentiation (60–63), and indeed, the overall frequency of tertiary inflationary cells that retained a T_M phenotype was reduced (Fig. 5E and Supplemental Fig. 1B). However, these tertiary stimulated OT-Is remained functional, producing both IFN-γ and TNF-α, as well as exposing CD107a (Fig. 5F, 5G). These data show that T_M specific for inflationary Ags can repeatedly recapitulate memory inflation upon viral challenge and produce functional T_EFF and T_M progeny.

**Memory and effector subsets protect Rag<sup>−/−</sup> mice**

To test the ability of transferred T_M to protect against a lethal MCMV challenge, we sorted T_M and T_EFF populations from la-

---

**FIGURE 5.** T_M can reinflate after multiple rechallenges. (A) Schematic of experimental design. To establish primary OT-I inflationary populations, we transferred 600 naive OT-I T cells expressing CD45.1 into naive B6 (CD45.2) recipients followed by infection with MCMV-SL8 (i.e., primary challenge). Thirteen weeks later, 6000 T_M phenotype primary OT-Is, isolated by FACS sorting, were transferred into new B6 recipients followed by MCMV-SL8 challenge (i.e., secondary challenge). This process was repeated a third time, transferring 3500 T_M OT-Is into naive mice and challenging with MCMV-SL8 (i.e., tertiary challenge). (B and C) Representative FACS plot of the donor stain 91 d after tertiary challenge (B) and frequencies of donor OT-Is (relative to total CD8s) in the blood at the indicated time points after tertiary challenge (C). Data were collected from two independent experiments (n = 12 total). Each line represents an individual mouse. (D and E) Phenotypic analyses of the mice described in (B) and (C). Representative FACS plot of the donor stain 30 wk postchallenge. Frequencies are relative to donor CD8. (F and G) Intracellular cytokine staining was performed on splenocytes ~20 wk after the tertiary challenge. Shown are representative FACS plots of stimulated (with SIINFEKL peptide) and unstimulated cells (F), and the frequencies of IFN-γ<sup>+</sup> cells that also express TNF-α and/or CD107a (G). Data were collected from two independent experiments (n = 12). Data are displayed as mean ± SEM.
ently infected B6 mice as described earlier and transferred them into Rag-/- recipients. One day later, the Rag-/- recipients were challenged with MCMV-TK, which lacks the m157 gene and is therefore resistant to NK-mediated control (43). Both transferred Tₘ and Tₑff expanded after the challenge and were sufficient to protect the recipients (Fig. 6A, 6B). In contrast, Rag-/- mice that received no T cell therapy became moribund in 2–4 wk and had to be sacrificed (Fig. 6B). Notably, the Tₑff population, which proliferated very poorly in immune-replete mice (Fig. 3), expanded and persisted in immune-deficient hosts for at least 11 wk postchallenge (Fig. 6A, 6B). However, the Tₑff responses lacked M45-specific, noninflationary T cells (Fig. 6A). These data show that MCMV-specific Tₘ are capable of protecting immune-deficient mice and producing immune responses with broad specificities.

The Tₘ population can persist long term and respond when necessary

Patients undergoing hematopoietic stem cell transplantation (HSCT) are most susceptible to late-onset (>100 d) reactivating CMV, as opposed to an acute CMV infection (12, 64–66 and reviewed in Ref. 67). Furthermore, transferred CMV-specific T cells will need to compete with host immunity. Therefore, we developed a model to test whether Tₘ and Tₑff subsets could respond to viral reactivation after a long delay. To this end, Tₘ and Tₑff were sorted from latently infected mice (>3 mo postinfection) and transferred into immune-replete, infection-matched, or naive, congenic recipients differing at the Thy1 locus (Fig. 7A). After the transfer, the latently infected recipients were rested as described in the legend for Fig. 7. Donor T cells did not expand dramatically in any animal after transfer (Supplemental Fig. 2A), supporting our previous conclusion that competition between T cells dictates MCMV-specific T cell expansion (45). Recipient T cells and NK cells were then eliminated in all mice using a mixture of depletion Abs that targeted the host cells (Thy1.1*) but left the donor cells (Thy1.2*) intact (Fig. 7B and Supplemental Fig. 2B). This depletion protocol did not induce detectable viral transcription in any animal as assessed by nested RT-PCR (M. Quinn, T. Moghbili, and C.M Snyder, unpublished observations), likely because of the presence of antiviral Abs (68). Despite the 9- to 12-wk rest period, MCMV-specific donor Tₘ responded robustly in all infected recipients after host depletion (Fig. 7C, 7D). Importantly, donor Tₘ did not expand to detectable levels in depleted naive recipients (Supplemental Fig. 2C). Thus, viral challenge of naive mice that received Tₘ donor cells 12 wk previously induced a robust donor response in three of the four animals, indicating that the Tₘ persisted in these mice, even without any Ag (Supplemental Fig. 2C). Thus, Ag rather than homeostatic mechanisms account for the donor Tₘ response in infected recipients.

In marked contrast, after depletion, donor T cells were only detectable in two animals that had received Tₑff and then only at very low frequencies (Fig. 7C, 7D). Control experiments (Supplemental Fig. 3A–C) supported previous work (69), suggesting that the KLRG1-specific Ab did not induce depletion of the transferred Tₑff subset. Thus, the failure of Tₑff to expand in this setting is not a sorting artifact, but rather the inability to persist and/or expand in response to low amounts of viral Ag.

After expansion, all infected mice that received Tₘ had a donor population specific for multiple epitopes, and the progeny had differentiated to form new Tₑff populations (Fig. 7E) (M. Quinn and C.M Snyder, unpublished observations). Furthermore, the four tetramers used stained only ~60% of the total donor population in each animal (Fig. 7E), suggesting that the remaining 40% of each donor population contained cells specific for additional MCMV Ags. In contrast, in the two animals in which Tₑff donors expanded to detectable levels, each was skewed substantially toward a single inflationary epitope (Fig. 7E). Because these sorted Tₑff populations included large numbers of T cells specific for M38, m139, and IE3, this hit-or-miss expansion of donor T cells with selective specificities implies that a very small number of non-Tₑff may have contaminated the transfer.

In the mice that received Tₘ donor cells, their diverse progeny persisted in recipients for >11 wk after termination of the depletion regimen, even though host immunity had returned (Fig. 7F). These data suggest that Tₘ with inflationary specificities are capable of surviving in an environment with very little or no Ag stimulation and then responding as needed during a period in which the host is immune compromised and viral Ag becomes available.

In total, these data show that protective MCMV-specific Tₘ persist throughout infection, retain superior proliferative function, and can respond to viral Ag as needed, in contrast with the numerically dominant Tₑff. Because MCMV-specific Tₘ share a transcriptional program with HCMV-specific Tₘ, our data suggest that Tₘ may be ideal candidates to restore functional immune surveillance in patients at risk for CMV reactivation.

Discussion

Adoptive immunotherapy using CMV-specific CD8⁺ T cells can be a successful therapeutic strategy for combating CMV reactivations (5–18). However, the majority of CMV-specific CD8⁺
T cells isolated from healthy donors will express an effector-differentiated phenotype (CD27 low/CD127 low/CD45RA high/KLRG-1 high/CD57 high) (reviewed in Ref. 70), and in vitro expansion of CMV-specific T cells drives their differentiation toward an effector phenotype (40). We used the MCMV model to show that the ability to restore MCMV immunity is contained almost entirely within the minor T M subset that retains CD27. Although both T M and T EFF protected Rag2−/− mice (Fig. 6), humans are unlikely to remain completely immune depleted like Rag2−/− mice, and bolus CMV infections are of lesser concern than reactivation after transplantation. The inability of the T EFF population to consistently expand after immune depletion in latently infected hosts suggests that these cells will only be protective under limited conditions. These data support a previous study in humans that

**FIGURE 7.** T M persist in latently infected, immune-replete mice and expand when host immunity is lost. (A) Schematic of experimental design. Age-matched B6 and Thy1.1 mice were infected with 1 × 10⁶ PFU MCMV-Smith. After the establishment of viral latency (>8 wk postinfection), either T M or T EFF from the B6 donors was transferred, as described in Materials and Methods, into the latently infected Thy1.1 recipients or into naive Thy1.1 mice. Latently infected recipients were rested for 9–12 wk, whereas the naive recipients were rested for ~1.5 wk. (B) Ab depletion schedule. (C–E) The presence of tetramer+ donors was analyzed by flow cytometry immediately after the depletion schedule. Data were collected from two independent experiments (n = 6 total). Three mice from each group were depleted 9 wk after the transfer; three mice from each group were depleted 12 wk after transfer. (C) Histograms of donor T cells within each individual recipient. (D) Representative FACS plots of tetramer+ donors immediately following the depletion regimen. (E) Frequency within each individual recipient of each analyzed tetramer as a percent of total donor CD8+ cells. T EFF recipients 3–6 are excluded because they did not have a donor population. (F) Tetramer staining was performed 11 wk after depletion in one experiment described earlier (n = 3).
correlated the transfer of CD27hi CMV-specific T cells with an increased likelihood of T cell persistence and expansion (41). To validate the use of the MCMV model, we compared human and mouse MCMV-specific T cells and show for the first time, to our knowledge, that T\textsubscript{M} and T\textsubscript{EFF} populations in mice and humans share a common transcriptional profile. The power of the GSEA analysis used for this comparison is that it identifies significant correlations across the entire transcriptional profile, rather than comparing individual genes. Nevertheless, we expect that future studies examining conserved and divergent genetic pathways will reveal significant and relevant information about CMV-specific immunity in mouse and human. These results highlight the usefulness of the MCMV model to: 1) perform CMV-specific CD8\textsuperscript{+} T cell functional studies that are difficult or impossible to perform in humans, and 2) provide translational insights into novel or improved therapeutic strategies.

Understanding how CMV-specific T cell immunity is maintained is critical for the improvement of CMV adoptive immunotherapy. Persistent Ag stimulation from CMV reactivations results in the majority of inflationary CD8\textsuperscript{+} T cells developing a T\textsubscript{EFF} phenotype and function. However, our previous work showed that unsorted inflationary CD8\textsuperscript{+} T cells, containing primarily T\textsubscript{EFF} declined after transfer into congenic, latently infected recipients (27). These data suggest that MCMV-specific T\textsubscript{EFF} are unable to sustain themselves in an immune-replete environment, even in the presence of Ag. Thus, we proposed that the accumulation of T\textsubscript{EFF} is the result of continual Ag stimulation of the T\textsubscript{M} population. Our data show that a small, stable MCMV-specific T\textsubscript{M} population has strong functional similarities to classical memory T cells that develop after acute infections. For example, the ability to proliferate in response to Ag without terminal differentiation is a hallmark of functional memory T cells (71). In addition to producing differentiated progeny that accumulated after MCMV challenge (Fig. 4D), donor T\textsubscript{M} also produced T\textsubscript{M} phenotype progeny that outnumbered the cells transferred (Fig. 3C, 3D) and persisted throughout our observation period (Fig. 4C, 4D). These data suggest that MCMV-specific T\textsubscript{M} have the ability to replace themselves even while producing differentiated progeny in response to Ag. Importantly, this was true through at least three rounds of stimulation using sorted splenic T\textsubscript{M} (Fig. 5). Thus, MCMV-specific T\textsubscript{M} have the capacity to respond repeatedly to viral Ag during this persistent infection and can recapitulate memory inflation.

It is interesting that transferred T\textsubscript{M} failed to expand in immune-replete, latently infected hosts. Detectable numbers of donor T cells were only evident in one of six mice before immune depletion (Supplemental Fig. 2A). In this case, the donors were not positive for any of the tetramers used in the analyses and made up <1% of the total CD8\textsuperscript{+} population. However, loss of the host T cell populations led to rapid and robust expansion of donor T cells with diverse specificities and phenotypes in all T\textsubscript{M} recipients (Fig. 7). The failure of transferred T\textsubscript{M} to expand in the presence of host MCMV-specific immunity may reflect the relative lack of available Ag during the latent phase of MCMV infection. Indeed, viral reactivations occur in only a fraction of latently infected cells at any given time, and only rarely produce infectious viral particles (72, 73). Moreover, we have found that competition between T cells for access to this limited Ag regulates the expansion of individual T cell clones (45). Thus, the combination of low Ag and large numbers of MCMV-specific T cells in the recipients may have “shielded” the majority of the donor T\textsubscript{M} from the ongoing infection, an idea we have proposed previously (45, 74). Importantly, MCMV Ag is not required for MCMV-specific T\textsubscript{M} survival. We have previously shown that MCMV-specific T\textsubscript{M} divide at a consistent rate with or without Ag (28), and our new data (Supplemental Fig. 2C) show that inflationary T\textsubscript{M} can survive in naive mice without any Ag. Thus, homeostatic mechanisms can support the inflationary T\textsubscript{M} population when it does not have access to Ag, which may partially explain the preservation of memory function within the T\textsubscript{M} subset. Taken together, these data suggest that the highly functional T\textsubscript{M} population, which can persist without access to Ag, proliferates robustly and produces new T\textsubscript{M}, as well as more differentiated progeny, upon Ag stimulation.

Overall, our data further support the model that the burden of maintaining memory inflation falls on the functional T\textsubscript{M} population, which can provide a stable and consistent source of new T\textsubscript{EFF} progeny whenever needed, over prolonged periods. However, T cell competition for limited Ag appears to prevent the continuous stimulation of most T\textsubscript{M}. Nonetheless, the T\textsubscript{M} population is capable of robustly responding if T cell competition is lost, a conclusion with important clinical implications for adoptive immunotherapy. Variations in transplant protocols, patients, and antiviral therapy responses make it difficult to predict and standardize CMV prevention therapies. Our data suggest that the plasticity of the T\textsubscript{M} population, transferred before any disease develops, may offer a personalized therapy, where the treatment adapts to the conditions of the patient and responds if and when Ag becomes available. Future studies will be needed to explore whether the addition of homeostatic cytokines (e.g., IL-15) or pharmacotherapeutics (e.g., rapamycin) (75) preserves the T\textsubscript{M} phenotype either in vivo or during in vitro expansion.

Acknowledgments
We thank the Kimmel Cancer Center Flow Cytometry Facility and Animal Facility at Thomas Jefferson University.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Representative plots for repeat transfers and data from secondary stimulated OT-Is

(A) Sorting strategy for isolating OT-I T\textsubscript{M} T cells after primary challenge. Briefly, 600 naïve OT-I T cells expressing CD45.1 were transferred into naïve B6 (CD45.2) recipients and challenged MCMV-SL8, (i.e. primary challenge). Thirteen weeks after the primary challenge, donor OT-I T cells were sorted using the CD45.1 marker and the T\textsubscript{M} subset was identified as CD27\textsuperscript{hi} and KLRG1\textsuperscript{lo}. (B) 6,000 sorted T\textsubscript{M} OT-I T cells were transferred into naïve B6 recipients and challenged with MCMV-SL8 (i.e. secondary challenge). Shown is the frequency and phenotype of blood-localized OT-Is over time, relative to total CD8\textsuperscript{pos} T cells. Data was collected from two independent experiments (n=7 total). (C) Sorting strategy for isolating OT-I T\textsubscript{M} T cells after secondary challenge. Briefly, thirteen weeks after the secondary challenge, donor OT-I T cells were sorted as described in (A).
Supplemental Figure 2: Antibody depletion does not induce T\textsubscript{M} proliferation in naïve recipients.

(A) Immediately prior to antibody depletion, the presence of donors in the blood was determined by flow cytometry. For T\textsubscript{M} and T\textsubscript{EFF} recipients, data was collected from two independent experiments (\(n=6\) total). For naïve recipients, data was collected from one experiment (\(n=4\) total) (B) Frequencies of total tetramer-binding CD8\textsuperscript{pos} cells were determined in the blood of the chronically-infected Thy1.1 recipient mice prior to and immediately following the depletion scheduled in Figure 7B. Data was collected from two independent experiments (\(n=6\) total) and is displayed as mean ± SEM. (C) Age matched naïve Thy1.1 recipients received T\textsubscript{M} cells from a MCMV chronically-infected donor. Recipients were bled “pre-depletion” at 1.5 weeks post-transfer. Mice were started on the antibody depletion regimen immediately following the “pre-depletion” bleed and were bled again “post-depletion” at approximately 5 weeks post-transfer. A B6 mouse (Thy1.2\textsuperscript{pos}) was used as the positive staining control. Naïve mice were challenged with MCMV approximately 12 weeks after the adoptive transfer. Data was collected from one experiment (\(n=4\) total).
**Supplemental Figure 3:** The KLRG1-specific antibody used for sorting does not deplete transferred cells.

(A) CD45.1 splenocytes were stained with the anti-KLRG1 antibody (clone 2F1), mixed with unstained CD45.2 splenocytes in a 1:1 ratio and transferred into B6.CB17-Prkdc<sup>scid</sup> (SCID) mice from Jackson. The representative FACS plots show the KLRG1 stain of CD45.1<sup>pos</sup>, but not CD45.2<sup>pos</sup> cells (left) and the proportion of transferred cells that were CD45.1<sup>pos</sup> (right). (B) Six days after transfer, SCID recipients were bled and analyzed by flow cytometry. CD45.1<sup>pos</sup> cells were still a similar proportion of the transferred T cells (compare to A), suggesting that the large CD45.1<sup>pos</sup> KLRG1-stained population was not depleted. (C) Anti-KLRG1 staining six days after the transfer showed that there were still KLRG1<sup>hi</sup> CD45.1<sup>pos</sup> cells. As KLRG1 expression has been shown to be dependent on antigen stimulation, it is unlikely that the KLRG1<sup>hi</sup> cells were newly formed. Importantly, the CD45.1<sup>pos</sup> cells detected 6 days after transfer were no longer positive for the KLRG1 antibody used prior to the transfer (not shown), suggesting that the cells have not been depleted and have cleared the antibody off their surface. Data was collected from a single experiment (n=3 total).