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Memory T cells specific for murine CMV reemerge after multiple challenges and recapitulate immunity in various adoptive transfer scenarios

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Abstract

Reconstitution of CMV-specific immunity following transplant remains a primary clinical objective to prevent CMV disease, and adoptive immunotherapy of CMV-specific T cells can be an effective therapeutic approach. Due to viral persistence, most CMV-specific $CD8^{pos}$ T cells become terminally differentiated effector cells (T_{EFF}). A minor subset retains a memory-like phenotype (T_M), but it is unknown whether these cells retain memory function or persist over time. Interestingly, recent studies suggest that CMV-specific $CD8^{pos}$ T cells with different phenotypes have different abilities to reconstitute sustained immunity following transfer. The immunology of human CMV (HCMV) infections is reflected in the murine model (MCMV). We found that HCMV- 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} cells. Strikingly, after transfer, T_M cells established sustained and diverse T cell populations even after multiple challenges. Although both T_{EFF} and T_M cells could protect Rag−/− mice, only T_M cells persisted after transfer into immune replete, latently-infected recipients and responded if recipient immunity was lost. Interestingly, transferred T_M cells did not expand until recipient immunity was lost, supporting that competition limits the antigen stimulation of T_M cells. Ultimately, these data show that CMV-specific T_M cells retain memory function during MCMV infection and can reestablish CMV immunity when necessary. Thus, T_M cells may be a critical component for consistent, long-term adoptive immunotherapy success.

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Conflict-of-Interest

The authors declare no conflict-of-interests.

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Introduction

Latent Cytomegalovirus (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 anti-viral 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 immune suppressed recipient. Due to the persistent nature of CMV infection, CMV-seropositive donors accumulate large numbers of CMV-specific $CD8^{pos}$ T cells (approximately 5-10% of the total $CD8^{pos}$ T cells), a process termed “memory inflation,” (19-28). Studies in humans and the well-characterized mouse model (MCMV) have shown that the majority of inflationary populations are composed of terminally differentiated effector phenotype (T_{EFF}) T cells that presumably develop as a result of repeated antigen 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 inflationary T cells retain a memory-like (T_M) phenotype, despite sharing epitope specificity and T cell receptor sequences with the 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 cells would be ideal to use in an adoptive immunotherapy setting. Recent evidence supports this hypothesis. In a non-human primate model, CMV-specific effector T cells that were expanded in vitro from sorted T_M cell 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 cells in a donor transfer and the long-term maintenance of donor derived cells (41).

The goal of our study was to utilize 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 cells share a common genetic program with their human CMV-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_M cells will improve consistency and clinical outcomes in patients at-risk for developing CMV disease.

Materials and Methods

Mice

Unless otherwise indicated, C57BL/6 mice, CD45.1 mice (B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ), Thy1.1 mice (B6.PL-$Thy1^a$/CyJ) and Rag−/− mice (B6.129S7-Rag1<tm1Mom>J) were purchased from Jackson Laboratory. OT-I transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) also purchased from Jackson, were bred with CD45.1 mice to produce double positive ($CD45.2^{pos}$/$CD45.1^{pos}$) 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 intraperitoneally (i.p.) with 2×10^5 pfu of MCMV strain MW97.01 (42). Mice were considered latently-infected at 8 weeks post-infection. Rag−/− mice were infected with 5×10^4 pfu of MCMV-TK virus (43). OT-I T cell transfer recipients were challenged with 2×10^5 pfu MCMV-SL8, which expresses the SIINFEKL peptide (44, 45).

Tetramer Staining, Antibodies and FACS Analysis

MHC-tetramers were provided by the NIH Tetramer 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 antibodies: [CD8(53-6.7); CD44(IM7); CD27(LG.3A10); CD127(A7R34); 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^{pos} CD8^{pos} T cells involved first gating lymphocytes and then singlets. CD8^{pos} cells were gated as frequency of singlets. Tetramer^{pos} cells were identified as a frequency of CD8^{pos} cells. A B8R tetramer (specific for the B8R peptide from Vaccinia), was used as a negative tetramer control. Tetramer^{pos} cells were phenotypically defined by they expression of KLRG1, CD27, CD127 or CD62L.

Adoptive Transfers

CD8^{pos} splenocytes from latently-infected donors were enriched using EasySep Biotin selection kit (StemCell Technologies) and biotinylated antibodies against RBCs(Ter-119), CD4(GK1.5) and CD19(6D5) according to the recommended protocol. Enriched cells were stained to determine the frequency of tetramer^{pos} cells within the enriched fraction and then sorted on either a MoFlo (Dako Cytomation) or an ARIA II (BD Biosciences) cell sorter. Sorted cells were counted, and 5×10^4 cells were transferred via the retro-orbital sinus. Sort purity was analyzed on an LSR II. The number of transferred tetramer-binding CD8^{pos} T cells was estimated using the tetramer frequency within the enriched CD8^{pos} population and the post-sort purity analysis. Fold change was calculated as the number of tetramer-binding T cells in the spleen 7 days post-challenge over the total number of tetramer^{pos} cells transferred (assuming 100% engraftment). The gating strategy for analyzing donor cells in the recipients was identical to that described above with antibodies 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, OT-I T_M CD8^{pos} T cells were FACS sorted and transferred as described above. Following challenge with MCMV-SL8, the frequencies of donor OT-Is were determined in the blood of recipients using the strategy described above except that singlets were not identified and OT-I donors were identified by expression CD45.1 and Vα2.

Intracellular Stimulation (ICS)

ICS and staining was performed as previously described (27, 45), with minor modifications. Specifically, cells were incubated with 1 μg/mL peptide (Genemed Synthesis), 1 μg/mL brefeldin A (GolgiPlug, BD Biosciences) and CD107a-specific antibody for 3 hours.

CD70 Blocking Antibody Treatment

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

Antibody Depletions

Antibody depletions were performed with Thy1.1(19E12), CD4(GK1.5) and NK1.1(PK136) antibodies. 300 μg of each antibody were administered i.p. in PBS. Three subsequent injections of 100 μg of each antibody were given at 7 day intervals.

Microarray

Splenocytes from latently-infected mice were co-stained with tetramers loaded with the antigenic peptides from M38, m139 and IE3 (25) and sorted on a MoFlo (Dako Cytomation) cell sorter. MCMV-specific T cells were identified as $CD8^{pos}$, $CD44^{hi}$ and tetramer binding and then further segregated into T_M and T_{EFF} cells subsets by expression of KLRG1 and CD127. Naïve $CD8^{pos}$ cells were $CD44^{lo}$. Total RNA was isolated using the Qiagen RNAeasy Plus Kit (Qiagen), quantified on a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and processed at the Microarray Core Facility at Thomas Jefferson University. Briefly, 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 Affymterix CEL files and perform background and RMA normalization (49). Annotation information was added using the mogene10sttranscriptcluster.db (50) package. Probes without valid annotations (7,196 of 35,556 probes) were removed before differential expression analysis using the limma package's (51) linear modeling and Bayes methods (52). Genes showing up- or down-regulation of at least twofold and p-value < 0.05 in each of three contrasts (T_{EFF} vs. Naïve, T_M vs. Naïve, and T_{EFF} vs. T_M) were considered for gene set enrichment analysis (GSEA). Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (53) (accession number: GSE61927 http://www.ncbi.nlm.nih.gov/genbank)

Gene Set Enrichment Analysis

Human data for series GSE24151 (54) was retrieved from NCBI's GEO database (53), extracted using Partek® Genomics Suite® software, version 6.6 (Partek Inc., St. Louis, MO) and curated for input into GSEA software (55) (http://www.broadinstitute.org/gsea/). Since 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 (up- or down-regulated in each of the three contrasts) were converted to human names

using data from NCBI'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 receptor 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 $CD8^{pos}$ T cells in humans

In the mouse model, MCMV infection of C57BL/6 (B6) mice results in inflation of select MCMV-specific $CD8^{pos}$ T cells specific for peptides from the viral proteins M38, m139 and IE3 (Fig. 1A and (25, 27, 28)). As in humans infected with human CMV (HCMV), the majority of MCMV-specific inflationary T cells express a T_{EFF} phenotype (often defined as T_{EMRA} in humans), while only a small fraction express a T_M-like phenotype, defined here as $KLRG1^{lo}/CD27^{hi}$ and further sub-divided into central memory (T_{CM} – $CD127^{hi}/CD62L^{hi}$) and effector memory (T_{EM} – $CD127^{hi}/CD62L^{lo}$) subsets (Fig. 1A and (22, 23, 27, 29-33, 56)). In contrast, “non-inflationary” MCMV-specific $CD8^{pos}$ 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 and (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 and (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 cells that were specific for inflationary antigens were stable over time and remarkably similar to the numbers of non-inflationary T_M cells, despite great differences between the numbers of inflationary and non-inflationary T_{EFF} cells (Fig. 1B, 1C). Thus, although continuous antigen stimulation maintains memory inflation, the inflationary T_M population remains stable.

The MCMV model is well characterized and the T cell responses clearly recapitulate those seen in HCMV-infected people. To determine whether MCMV-specific T_M and T_{EFF} cells share a common transcriptional program with their human counterparts, we sorted MCMV-specific T_M ($CD44^{hi}/CD127^{hi}/KLRG1^{lo}$) and T_{EFF} ($CD44^{hi}/CD127^{lo}/KLRG1^{hi}$) cells specific for the M38, m139 and IE3 antigens. Microarray analyses were performed on these cells. Genes that were significantly up- or down-regulated in T_M and T_{EFF} subsets relative to each other or to naïve ($CD44^{low}$) 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 as $CD27^{hi}/CD45RA^{lo}$ (T_M) or $CD27^{lo}/CD45RA^{hi}$ (T_{EFF}) (54). 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 $KLRG1^{lo}/CD27^{hi}$ cells (T_M) co-expressed CD127 (either T_{CM} or T_{EM}, Figure 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 T_M cells from each other were highly enriched within the corresponding human data set. That is:

genes up-regulated specifically in mouse T_M cells relative to mouse T_{EFF} cells were highly enriched within the genes that distinguish human T_M cells from human T_{EFF} and vice versa. Moreover, relative to naive T cells, mouse genes that were up and down-regulated by T_{EFF} or T_M cells 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 1. 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 T cell receptor signaling pathway or liver and the data exhibited expected patterns (Fig. 2B).

Overall, these data show that MCMV-specific and HCMV-specific T cells 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 mouse and human CMV-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} cells, both populations were sorted from spleens of latently-infected B6 mice (>3 months post-infection) using their differential expression of KLRG1 and CD27. Sorted cells were transferred into naive congenic recipients and re-challenged. The M45- and M38-specific T_M cells proliferated robustly within 7 days 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 less than 10-fold in the same time period. Importantly, while the T_{EFF} donor cells remained exclusively KLRG1^{hi}, 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 phenotype cells were present in the spleen 7 days after challenge at numbers that were approximately 50 to 100-fold higher than had been transferred (dotted line, Fig. 3D), indicating expansion of this subset without terminal differentiation. These data show that MCMV-specific T_M cells retain robust proliferative capacity and can produce phenotypically diverse progeny including new T_M-phenotype cells.

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 cells as above and blocked the CD27-CD70 interaction as described in the Methods. Blocking the CD27-CD70 interaction significantly decreased the expansion of the M38- and M45-specific T_M cells 7 days post-challenge by approximately 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 and 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 re-challenge. M38-specific T cells from T_M-sorted donors persisted at high frequencies in recipients, while 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 non-transgenic T cells (Fig. 4), secondary challenge of T_M OT-Is induced inflation and T_{EFF} formation as well as a persistent KLRG1^{lo} population (Supplemental Fig. 1B). These secondary T_M cells were again sorted (Supplemental Fig. 1C), transferred into a 3rd set of naïve recipients and re-challenged. Incredibly, the donor secondary T_M population inflated and produced both KLRG1^{hi} and KLRG1^{lo} progeny following this tertiary challenge (Fig. 5B-5E).

Repeated acute viral challenges of small numbers of T cells in naive mice drives 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 phenotype T cells specific for inflationary antigens can repeatedly recapitulate memory inflation upon viral challenge and produce functional T_{EFF} and T_M progeny.

Memory and Effector Subsets protect Rag−/− mice

To test the ability of transferred T_M cells to protect against a lethal MCMV challenge, T_M and T_{EFF} populations were sorted from latently-infected B6 mice as above and transferred 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 T_M or T_{EFF} cells expanded following 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 weeks and had to be sacrificed (Fig. 6B). Notably, the T_{EFF} population, which proliferated very poorly in immune replete mice (Fig. 3), expanded and persisted in immune deficient hosts for at least 11 weeks post-challenge (Fig. 6A, 6B). However, the T_{EFF} response in 5 out of 6 recipients M45-specific, non-inflationary T cells (Fig. 6A). These data show that MCMV-specific T_M cells are capable of protecting immune deficient mice and producing immune responses with broad specificities.

The T_M population can persist long-term and respond when necessary

Patients undergoing hematopoietic stem cell transplantation (HSCT) are most susceptible to late-onset (>100 days) reactivating CMV, as opposed to an acute CMV infection ((12, 64-66) and reviewed in (67)). Furthermore, transferred CMV-specific T cells will need to compete with host immunity. Therefore, we developed a model to test whether T_M and T_{EFF} subsets could respond to viral reactivation after a long delay. To this end, T_M and T_{EFF} cells were sorted from latently-infected mice (> 3 months post-infection) and transferred into immune replete, infection matched or naive, congenic recipients differing at the Thy1 locus (Fig. 7A). Following the transfer, the latently-infected recipients were rested as described in the figure legend. Donor T cells did not expand dramatically in any animal following 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 cocktail of depletion antibodies that targeted the host cells (Thy1.1^{pos}), but left the donor cells (Thy1.2^{pos}) intact (Fig. 7B and Supplemental Fig. 2B). This depletion protocol did not induce detectible viral transcription in any animal as assessed by nested RT-PCR (unpublished observations), likely due to the presence of anti-viral antibodies (68). Despite the 9-12 week rest period, MCMV-specific donor T_M cells responded robustly in all infected recipients after host depletion (Fig. 7C and 7D). Importantly, donor T_M cells did not expand to detectible levels in depleted naive recipients (Supplemental Fig. 2C). However, viral challenge of naïve mice that received T_M donor cells 12 weeks previously induced a robust donor response in 3 of the 4 animals, indicating that the T_M cells persisted in these mice, even without any antigen (Supplemental Fig. 2C). Thus, antigen rather than homeostatic mechanisms accounts for the donor T_M response in infected recipients.

In marked contrast, after depletion, donor T cells were only detectible in 2 animals that had received T_{EFF} cells and then only at very low frequencies (Fig. 7C, 7D). Control experiments (Supplemental Fig. 3A-3C) supported previous work (69) suggesting that the KLRG1-specific antibody did not induce depletion of the transferred T_{EFF} subset. Thus, the failure of T_{EFF} cells 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 antigen.

After expansion, all infected mice that received T_M cells had a donor population specific for multiple epitopes and the progeny had differentiated to form new T_{EFF} populations (Fig. 7E and unpublished observations). Furthermore, the four tetramers used only stained ~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 antigens. In contrast, in the two animals in which T_{EFF} donors expanded to detectible levels, each was skewed substantially towards a single inflationary epitope (Fig. 7E). Since these sorted T_{EFF} populations included large numbers of T cells specific for M38, m139 and IE3, this “hit-or-miss” expansion of donor T cells with select specificities implies that a very small number of non-T_{EFF} cells may have contaminated the transfer.

In the mice that received T_M donor cells, their diverse progeny persisted in recipients for more than 11 weeks after termination of the depletion regimen, even though host immunity had returned (Fig. 7F). These data suggest that T_M cells with inflationary specificities are

capable of surviving in an environment with very little or no antigen stimulation and then responding as needed during a period in which the host is immune compromised and viral antigen becomes available.

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

Discussion

Adoptive immunotherapy using CMV-specific $CD8^{pos}$ T cells can be a successful therapeutic strategy for combating CMV reactivations (5-18). However, the majority of CMV-specific $CD8^{pos}$ T cells isolated from healthy donors will express an effector-differentiated phenotype ($CD27^{lo}/CD127^{lo}/CD45RA^{hi}/KLRG\text{-}1^{hi}/CD57^{hi}$ - reviewed in (70)), and in vitro expansion of CMV-specific T cells drives their differentiation towards 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} cells protected Rag−/− mice (Fig. 6), humans are unlikely to remain completely immune depleted like Rag−/− mice, and bolus CMV infections are of lesser concern than reactivation following 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 correlated the transfer of $CD27^{hi}$ 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 that T_M and T_{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 man. These results highlight the usefulness of the MCMV model to: 1) perform CMV-specific $CD8^{pos}$ 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 antigen stimulation from CMV reactivations results in the majority of inflationary $CD8^{pos}$ T cells developing a T_{EFF} phenotype and function. However, our previous work showed that unsorted inflationary $CD8^{pos}$ T cells, containing primarily T_{EFF} cells, declined after transfer into congenic, latently-infected recipients (27). These data suggest that MCMV-specific T_{EFF} cells are unable to sustain themselves in an immune replete environment, even in the presence of

antigen. It has been proposed that the accumulation of T_{EFF} cells is the result of continual antigen stimulation of the T_M population. Our data show that a small, stable MCMV-specific T_M population has strong functional similarities to classical memory T cells that develop following acute infections and can recapitulate memory inflation. For example, the ability to proliferate in response to antigen without terminal differentiation is hallmark of functional memory T cells (71). In addition to producing differentiated progeny that accumulated after MCMV challenge (Figure 4D), donor T_M cells also produced T_M phenotype progeny that outnumbered the cells transferred (Fig. 3C and D) and persisted throughout our observation period (Fig. 4C and D). These data suggest that MCMV-specific T_M cells have the ability to replace themselves even while producing differentiated progeny in response to antigen. Importantly, this was true through at least three rounds of stimulation using sorted splenic T_M cells (Figure 5). Thus, MCMV-specific T_M cells have the capacity to respond repeatedly to viral antigen during this persistent infection.

It is interesting that transferred T_M cells failed to expand in immune-replete, latently-infected hosts. Indeed, detectible numbers of donor T cells were only evident in one out of six mice prior to 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 less than 1% of the total $CD8^{pos}$ 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_M cell recipients (Figure 7). The failure of transferred T_M cells to expand in the presence of host MCMV-specific immunity may reflect the relative lack of available antigen 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 antigen regulates the expansion of individual T cell clones (45). Thus, the combination of low antigen and large numbers of MCMV-specific T cells in the recipients may have “shielded” the majority of the donor T_M cells from the ongoing infection – an idea we have proposed previously (45, 74). Importantly, MCMV antigen is not required for MCMV-specific T_M cell survival. We have previously shown that MCMV-specific T_M cells divide at a consistent rate with or without antigen (28) and our new data (Supplemental Fig. 2C) show that inflationary T_M cells can survive in naïve mice without any antigen. Thus, homeostatic mechanisms can support the inflationary T_M population when it does not have access to antigen, which may partially explain the preservation of memory function within the T_M subset. Taken together, these data suggest that the highly functional T_M population, which can persist without access to antigen, proliferates robustly and produces new T_M cells as well as more differentiated progeny upon antigen stimulation.

Overall, our data further support the model that the burden of maintaining memory inflation falls on the functional T_M population, which can provide a stable and consistent source of new T_{EFF} progeny whenever needed, over for prolonged periods of time. However, T cell competition for limited antigen appears to prevent the continuous stimulation of most T_M cells. Nonetheless, the T_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 anti-viral therapy responses make it difficult to predict and standardize CMV prevention therapies. Our data suggest that the plasticity of

the T_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 antigen 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_M phenotype either *in vivo* or during *in vitro* expansion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CMV	Cytomegalovirus
HCMV	Human Cytomegalovirus
MCMV	Murine Cytomegalovirus
T_M	memory phenotype CD8+ T cells
T_{EFF}	terminal differentiated effector phenotype CD8+ T cells
T_{CM}	central memory CD8+ T cells
T_{EM}	effector memory CD8+ T cells
T_{EMRA}	terminal differentiated CD8+ T cell phenotype in humans
i.p.	intraperitoneal route
GSEA	gene set enrichment analysis
GEO	gene expression omnibus
SEM	standard error of the mean

References

1. Staras SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin. Infect. Dis. 2006; 43:1143–1151. [PubMed: 17029132]
2. Polić B, Hengel H, Krmpotić A, Trgovcich J, Pavić I, Lučin P, Jonjić S, Koszinowski UH. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. J. Exp. Med. 1998; 188:1047–1054. [PubMed: 9743523]
3. Simon CO, Holtappels R, Tervo H-M, Böhm V, Däubner T, Oehrlein-Karpi SA, Kühnapfel B, Renzaho A, Strand D, Podlech J. CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. J. Virol. 2006; 80:10436–10456. [PubMed: 16928768]
4. Holtappels R, Bohm V, Podlech J, Reddehase MJ. CD8 T-cell-based immunotherapy of cytomegalovirus infection: “proof of concept” provided by the murine model. Med. Microbiol. Immunol. 2008; 197:125–134. [PubMed: 18343947]

5. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N. Engl. J. Med. 1995; 333:1038–1044. [PubMed: 7675046]

6. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science. 1992; 257:238–241. [PubMed: 1352912]

7. Barron MA, Gao D, Springer KL, Patterson JA, Brunvand MW, McSweeney PA, Chang Z, Barón AE, Weinberg A. Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. 2009; 49:1777–1783. [PubMed: 19911937]

8. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, Mohty M, Or R, Maschan M, Schumm M, Hamprecht K, Handgretinger R, Lang P, Einsele H. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood. 2010; 116:4360–4367. [PubMed: 20625005]

9. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood. 1991; 78:1373–1380. [PubMed: 1652311]

10. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood. 1994; 83:1971–1979. [PubMed: 8142663]

11. Gratama JW, Boeckh M, Nakamura R, Cornelissen JJ, Brooimans RA, Zaia JA, Forman SJ, Gaal K, Bray KR, Gasior GH, Boyce CS, Sullivan LA, Southwick PC. Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. Blood. 2010; 116:1655–1662. [PubMed: 20508161]

12. Boeckh M, Leisenring W, Riddell SR, Bowden RA, Huang ML, Myerson D, Stevens-Ayers T, Flowers ME, Cunningham T, Corey L. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood. 2003; 101:407–414. [PubMed: 12393659]

13. Peggs KS, Verfuerth S, Pizzey A, Chow SL, Thomson K, Mackinnon S. Cytomegalovirus-specific T cell immunotherapy promotes restoration of durable functional antiviral immunity following allogeneic stem cell transplantation. Clin. Infect. Dis. 2009; 49:1851–1860. [PubMed: 19911966]

14. Peggs KS, Thomson K, Samuel E, Dyer G, Armoogum J, Chakraverty R, Pang K, Mackinnon S, Lowdell MW. Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. Clin. Infect. Dis. 2011; 52:49–57. [PubMed: 21148519]

15. Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M, Germeroth L, Ringhoffer M, Ringhoffer S, Wiesneth M, Greiner J, Michel D, Mertens T, Rojewski M, Marx M, von Harsdorf S, Dohner H, Seifried E, Bunjes D, Schmitt M. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. Transfusion. 2011; 51:591–599. [PubMed: 21133926]

16. Blyth E, Clancy L, Simms R, Ma CKK, Burgess J, Deo S, Byth K, Dubosq M-C, Shaw PJ, Micklethwaite KP. Donor-derived CMV-specific T cells reduce the requirement for CMV-directed pharmacotherapy after allogeneic stem cell transplantation. Blood. 2013; 121:3745–3758. [PubMed: 23435462]

17. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, Assenmacher M, Billingham L, Steward C, Crawley C, Olavarria E, Goldman J, Chakraverty R, Mahendra P, Craddock C, Moss PA. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J. Exp. Med. 2005; 202:379–386. [PubMed: 16061727]

18. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, Leung K, Carrum G, Gee AP, Vera JF, Krance RA, Brenner MK, Rooney CM, Heslop HE, Leen AM. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci. Transl. Med. 2014; 6:242ra283.
19. Holtappels R, Pahl-Seibert MF, Thomas D, Reddehase MJ. Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62L(lo) memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. J. Virol. 2000; 74:11495–11503. [PubMed: 11090146]
20. Holtappels R, Thomas D, Podlech J, Reddehase MJ. Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2d haplotype. J. Virol. 2002; 76:151–164. [PubMed: 11739681]
21. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, Phillips RE, Klenerman P. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J. Immunol. 2003; 170:2022–2029. [PubMed: 12574372]
22. Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, Muller CA, Pircher H, Pawelec G. Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp. Gerontol. 2003; 38:911–920. [PubMed: 12915213]
23. Sierro S, Rothkopf R, Klenerman P. Evolution of diverse antiviral CD8+ T cell populations after murine cytomegalovirus infection. Eur. J. Immunol. 2005; 35:1113–1123. [PubMed: 15756645]
24. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleath PR, Grabstein KH, Hosken NA, Kern F, Nelson JA, Picker LJ. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. 2005; 202:673–685. [PubMed: 16147978]
25. Munks MW, Cho KS, Pinto AK, Sierro S, Klenerman P, Hill AB. Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. J. Immunol. 2006; 177:450–458. [PubMed: 16785542]
26. Komatsu H, Inui A, Sogo T, Fujisawa T, Nagasaka H, Nonoyama S, Sierro S, Northfield J, Lucas M, Vargas A, Klenerman P. Large scale analysis of pediatric antiviral CD8+ T cell populations reveals sustained, functional and mature responses. Immun. Ageing. 2006; 3:11. [PubMed: 17156440]
27. Snyder CM, Cho KS, Bonnett EL, van Dommelen S, Shellam GR, Hill AB. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity. 2008; 29:650–659. [PubMed: 18957267]
28. Smith CJ, Turula H, Snyder CM. Systemic Hematogenous Maintenance of Memory Inflation by MCMV Infection. PLoS Pathog. 2014; 10:e1004233. [PubMed: 24992722]
29. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat. Med. 2002; 8:379–385. [PubMed: 11927944]
30. van Leeuwen EM, Gamadia LE, Baars PA, Remmerswaal EB, ten Berge IJ, van Lier RA. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8+ T cells. J. Immunol. 2002; 169:5838–5843. [PubMed: 12421965]
31. Gamadia LE, van Leeuwen EM, Remmerswaal EB, Yong SL, Surachno S, Wertheim-van Dillen PM, Ten Berge IJ, Van Lier RA. The size and phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and environmental cytokines. J. Immunol. 2004; 172:6107–6114. [PubMed: 15128796]
32. van Leeuwen EM, de Bree GJ, Remmerswaal EB, Yong SL, Tesselaar K, ten Berge IJ, van Lier RA. IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T cells. Blood. 2005; 106:2091–2098. [PubMed: 15947093]
33. Waller EC, McKinney N, Hicks R, Carmichael AJ, Sissons JG, Wills MR. Differential costimulation through CD137 (4-1BB) restores proliferation of human virus-specific “effector memory” (CD28 CD45RAHI) CD8+ T cells. Blood. 2007; 110:4360–4366. [PubMed: 17878400]

34. Wallace DL, Masters JE, De Lara CM, Henson SM, Worth A, Zhang Y, Kumar SR, Beverley PC, Akbar AN, Macallan DC. Human cytomegalovirus- specific CD8+ T- cell expansions contain long- lived cells that retain functional capacity in both young and elderly subjects. Immunology. 2011; 132:27–38. [PubMed: 20738423]
35. Rufer N, Zippelius A, Batard P, Pittet MJ, Kurth I, Corthesy P, Cerottini JC, Leyvraz S, Roosnek E, Nabholz M, Romero P. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. Blood. 2003; 102:1779–1787. [PubMed: 12750165]
36. Price DA, Brenchley JM, Ruff LE, Betts MR, Hill BJ, Roederer M, Koup RA, Migueles SA, Gostick E, Wooldridge L. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. J. Exp. Med. 2005; 202:1349–1361. [PubMed: 16287711]
37. Iancu EM, Corthesy P, Baumgaertner P, Devevre E, Voelter V, Romero P, Speiser DE, Rufer N. Clonotype selection and composition of human CD8 T cells specific for persistent herpes viruses varies with differentiation but is stable over time. J. Immunol. 2009; 183:319–331. [PubMed: 19542443]
38. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C. A human memory T cell subset with stem cell-like properties. Nat. Med. 2011; 17:1290–1297. [PubMed: 21926977]
39. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, Drexler I, Höfer T, Riddell SR, Busch DH. Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8+ Central Memory T Cells. Immunity. 2014; 41:116–126. [PubMed: 25035956]
40. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J. Clin. Invest. 2008; 118:294–305. [PubMed: 18060041]
41. Scheinberg P, Melenhorst JJ, Brenchley JM, Hill BJ, Hensel NF, Chattopadhyay PK, Roederer M, Picker LJ, Price DA, Barrett AJ. The transfer of adaptive immunity to CMV during hematopoietic stem cell transplantation is dependent on the specificity and phenotype of CMV-specific T cells in the donor. Blood. 2009; 114:5071–5080. [PubMed: 19776383]
42. Wagner M, Jonjic S, Koszinowski UH, Messerle M. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J. Virol. 1999; 73:7056–7060. [PubMed: 10400809]
43. Snyder CM, Cho KS, Bonnett EL, Allan JE, Hill AB. Sustained CD8+ T cell memory inflation after infection with a single-cycle cytomegalovirus. PLoS Pathog. 2011; 7:e1002295. [PubMed: 21998590]
44. Farrington LA, Smith TA, Grey F, Hill AB, Snyder CM. Competition for antigen at the level of the APC is a major determinant of immunodominance during memory inflation in murine cytomegalovirus infection. J. Immunol. 2013; 190:3410–3416. [PubMed: 23455500]
45. Turula H, Smith CJ, Grey F, Zurbach KA, Snyder CM. Competition between T cells maintains clonal dominance during memory inflation induced by MCMV. Eur. J. Immunol. 2013; 43:1252–1263. [PubMed: 23404526]
46. Welten SPM, Redeker A, Franken KL, Benedict CA, Yagita H, Wensveen FM, Borst J, Melief CJM, van Lier RAW, van Gisbergen KPJM. CD27-CD70 Costimulation Controls T Cell Immunity during Acute and Persistent Cytomegalovirus Infection. J. Virol. 2013; 87:6851–6865. [PubMed: 23576505]
47. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J. Bioconductor: open software development for computational biology and bioinformatics. Genome biology. 2004; 5:R80. [PubMed: 15461798]
48. Carvalho BS, Irizarry RA. A framework for oligonucleotide microarray preprocessing. Bioinformatics. 2010; 26:2363–2367. [PubMed: 20688976]
49. Irizarry RA, Hobbs B, Collin F, Beazer- Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4:249–264. [PubMed: 12925520]

50. MacDonald, JW. mogene10sttranscriptcluster.db: Affymetrix mogene10 annotation data (chip mogene10sttranscriptcluster).. R package version 8.1.0.
51. Smyth, GK. Bioinformatics and computational biology solutions using R and Bioconductor. Springer; 2005. Limma: linear models for microarray data.; p. 397-420.
52. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Molec. Biol. 2004; 3 Article 3.
53. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002; 30:207–210. [PubMed: 11752295]
54. Hertoghs KML, Moerland PD, van Stijn A, Remmerswaal EBM, Yong SL, van de Berg PJEJ, van Ham SM, Baas F, ten Berge IJM, van Lier RAW. Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. J. Clin. Invest. 2010; 120:4077–4090. [PubMed: 20921622]
55. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA. 2005; 102:15545–15550. [PubMed: 16199517]
56. Thimme R, Appay V, Koschella M, Panther E, Roth E, Hislop AD, Rickinson AB, Rowland-Jones SL, Blum HE, Pircher H. Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation. J. Virol. 2005; 79:12112–12116. [PubMed: 16140789]
57. Hutchinson S, Sims S, O'Hara G, Silk J, Gileadi U, Cerundolo V, Klenerman P. A dominant role for the immunoproteasome in CD8 T cell responses to murine cytomegalovirus. PLoS One. 2011; 6:e14646. [PubMed: 21304910]
58. Kern F, Khatamzas E, Surel I, Frommel C, Reinke P, Waldrop SL, Picker LJ, Volk HD. Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. Eur. J. Immunol. 1999; 29:2908–2915. [PubMed: 10508265]
59. Tomiyama H, Takata H, Matsuda T, Takiguchi M. Phenotypic classification of human CD8+ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function. Eur. J. Immunol. 2004; 34:999–1010. [PubMed: 15048710]
60. Jabbari A, Harty JT. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. J. Exp. Med. 2006; 203:919–932. [PubMed: 16567385]
61. Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J. Immunol. 2006; 177:831–839. [PubMed: 16818737]
62. Wirth TC, Xue HH, Rai D, Sabel JT, Bair T, Harty JT, Badovinac VP. Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. Immunity. 2010; 33:128–140. [PubMed: 20619696]
63. Fraser KA, Schenkel JM, Jameson SC, Vezys V, Masopust D. Preexisting High Frequencies of Memory CD8+ T Cells Favor Rapid Memory Differentiation and Preservation of Proliferative Potential upon Boosting. Immunity. 2013; 39:171–183. [PubMed: 23890070]
64. Kumar D, Chernenko S, Moussa G, Cobos I, Manuel O, Preiksaitis J, Venkataraman S, Humar A. Cell-mediated immunity to predict cytomegalovirus disease in high-risk solid organ transplant recipients. Am. J. Transplant. 2009; 9:1214–1222. [PubMed: 19422346]
65. Harvala H, Stewart C, Muller K, Burns S, Marson L, MacGilchrist A, Johannessen I. High risk of cytomegalovirus infection following solid organ transplantation despite prophylactic therapy. J. Med. Virol. 2013; 85:893–898. [PubMed: 23508914]
66. Manuel O, Husain S, Kumar D, Zayas C, Mawhorter S, Levi ME, Kalpoe J, Lisboa L, Ely L, Kaul DR. Assessment of cytomegalovirus-specific cell-mediated immunity for the prediction of cytomegalovirus disease in high-risk solid-organ transplant recipients: a multicenter cohort study. Clin. Infect. Dis. 2013; 56:817–824. [PubMed: 23196955]
67. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol. Oncol. Clin. North. Am. 2011; 25:151–169. [PubMed: 21236396]
68. Jonjic S, Pavic I, Polic B, Crnkovic I, Lucin P, Koszinowski UH. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J. Exp. Med. 1994; 179:1713–1717. [PubMed: 8163949]

69. Cush SS, Flaño E. KLRG1+ NKG2A+ CD8 T cells mediate protection and participate in memory responses during γ-herpesvirus infection. J. Immunol. 2011; 186:4051–4058. [PubMed: 21346231]

70. Appay V, Van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry Part A. 2008; 73:975–983.

71. Ciocca ML, Barnett BE, Burkhardt JK, Chang JT, Reiner SL. Cutting edge: asymmetric memory T cell division in response to rechallenge. J. Immunol. 2012; 188:4145–4148. [PubMed: 22467651]

72. Kurz SK, Rapp M, Steffens HP, Grzimek NK, Schmalz S, Reddehase MJ. Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. J. Virol. 1999; 73:482–494. [PubMed: 9847354]

73. Kurz SK, Reddehase MJ. Patchwork pattern of transcriptional reactivation in the lungs indicates sequential checkpoints in the transition from murine cytomegalovirus latency to recurrence. J. Virol. 1999; 73:8612–8622. [PubMed: 10482614]

74. Snyder CM. Buffered memory: a hypothesis for the maintenance of functional, virus-specific CD8(+) T cells during cytomegalovirus infection. Immunol. Res. 2011; 51:195–204. [PubMed: 22058020]

75. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009; 460:108–112. [PubMed: 19543266]

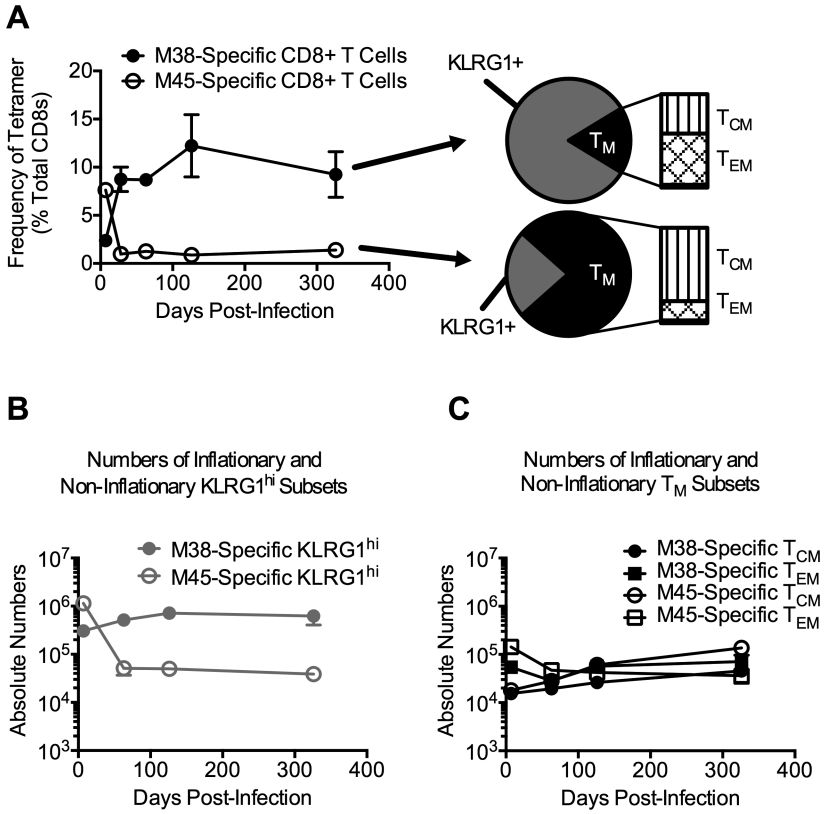

Figure 1. The non-inflationary and inflationary CD8^{pos} T cell populations retain similar numbers of T_M phenotype cells

Cohorts of age-matched B6 female 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^{pos} T cells in the blood at indicated time points. The phenotypic analysis shown was performed at d326 post-infection. T_M cells were identified as CD27^{hi}/KLRG1^{lo}. T_{CM} and T_{EM} were further identified as CD127^{hi} and either CD62L^{hi} or CD62L^{lo}, respectively. (B) Absolute numbers of KLRG1^{hi} tetramer-binding CD8^{pos} T cells in the spleen. (C) Absolute numbers of T_{CM} and T_{EM} tetramer-binding CD8^{pos} T cells. Data are displayed as mean ± SEM and represent two independent experiments.

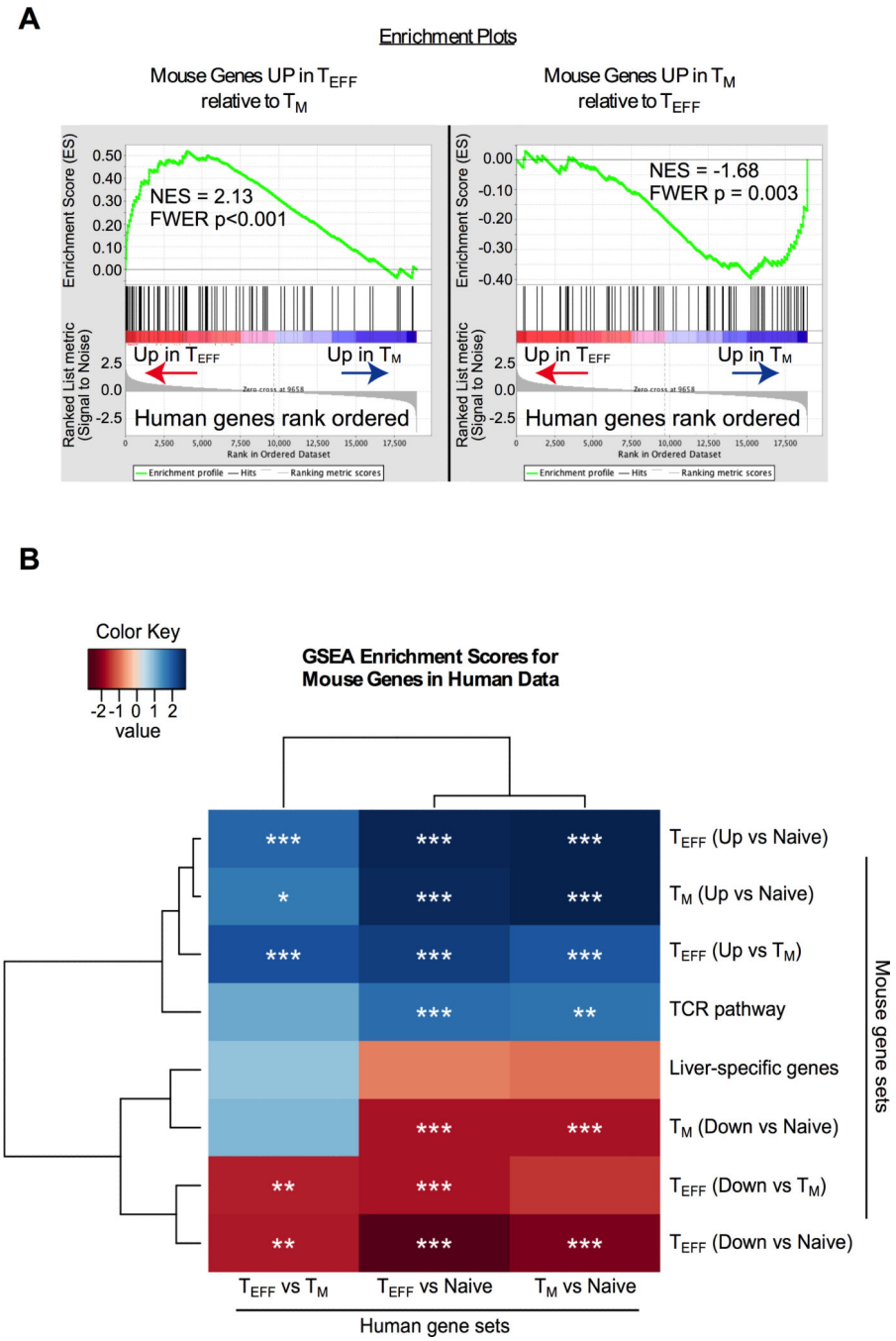

Figure 2. Gene Set Enrichment Analyses reveal significant overlap between the transcriptional profile of CMV-specific T cells in humans and mice

(A) Gene set enrichment was performed as described in the Methods. Shown are the enrichment plots for mouse genes that differed in a T_{EFF} vs. T_M comparison, plotted relative to human T_{EFF} and T_M cells. 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 $P<.05$) were generated for T_{EFF} and T_M cells relative to each other and relative to naive ($CD44^{low}$) T cells. GSEA analyses were performed with these mouse gene sets relative to each of the indicated human

data sets, rank ordered by expression (see methods). Stars indicate FWER corrected significance to control for multiple testing (* $P<.05$, ** $P<.01$, *** $P<.001$).

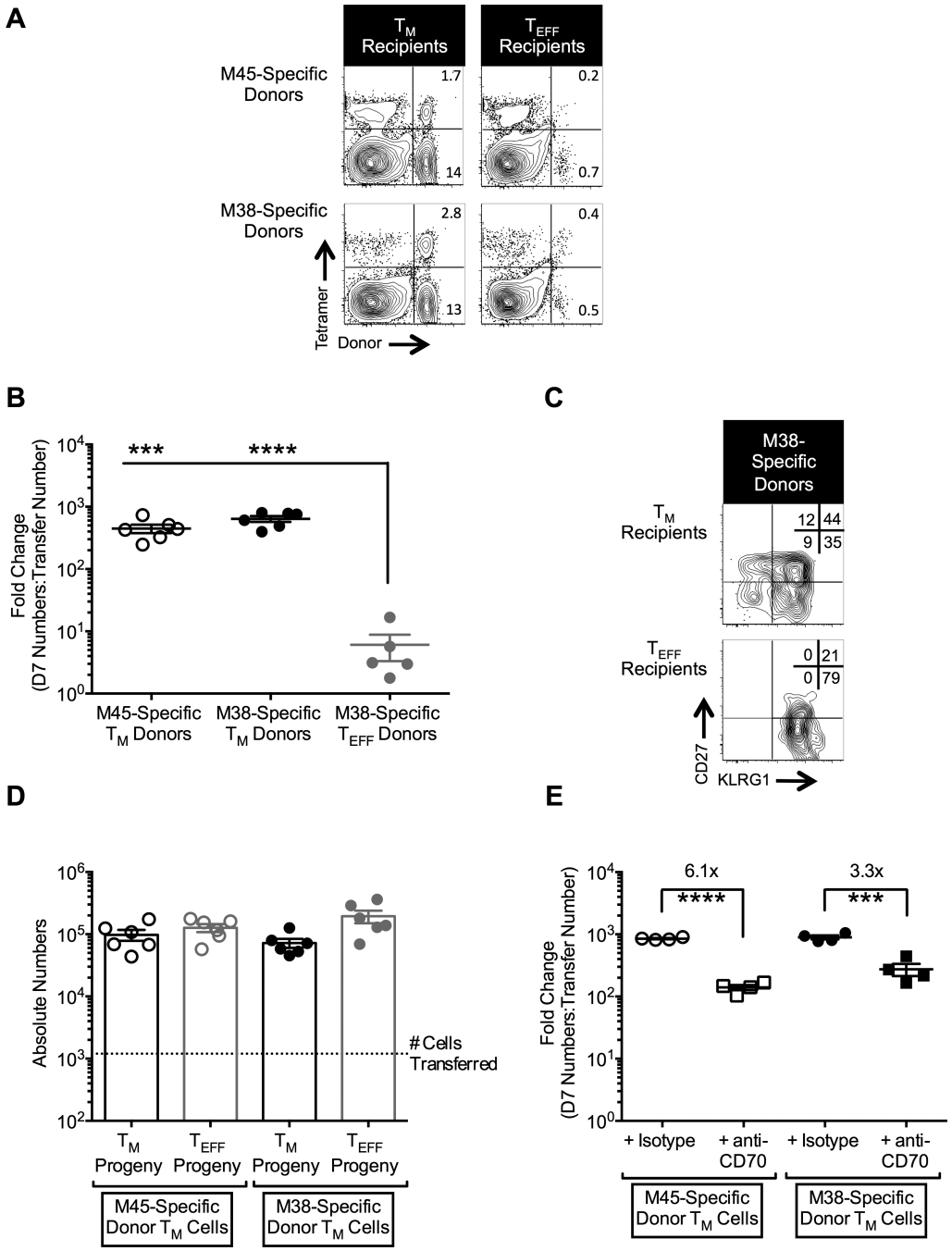

Figure 3. T_M cells dramatically expand 7 days post-challenge and produce both T_M and T_{EFF} progeny

Age matched B6 mice received either T_M or T_{EFF} cells and were challenged with MCMV as described in the Methods. Spleens were collected 7 days later for analysis. (A) Representative FACS plots of tetramer^{pos} donors in the spleen 7 days post-challenge. Frequencies in the corner are relative to total CD8^{pos} cells. (B) Fold change of donor cells in the spleen, calculated as described in the Methods, 7 days after challenge. As antigen-specific T cells were not sorted, approximately equal numbers of M38- and M45-specific T_M cells were transferred but ~10-fold more M38-specific T_{EFF} cells were transferred

compared with the T_M cells. Due to 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 were collected from two independent experiments (T_M: $n = 6$ total; T_{EFF}: $n = 5$ total) are shown. Statistical significance was determined by a Student's t-test (*** $P<.001$; **** $P<.0001$). (C) Representative FACS plots of M38-specific $CD8^{pos}$ T cell progeny from either T_M or T_{EFF} donors in the spleen at 7 days post-challenge. Frequencies in the corner are relative to M38-specific $CD8^{pos}$ cells. (D) Absolute number of T_M and T_{EFF} phenotypic progeny that were produced from T_M donors. Data are from the same experiments described in (B). (E) Fold change of donor cells in the spleen following treatment with either isotype control or anti-CD70 antibody. Data were collected 7 days post-challenge and represent two independent experiments ($n = 6$ total). Statistical significance was determined by a Student's t-test (*** $P<.001$; **** $P<.0001$). All graphical data are displayed as mean ± SEM.

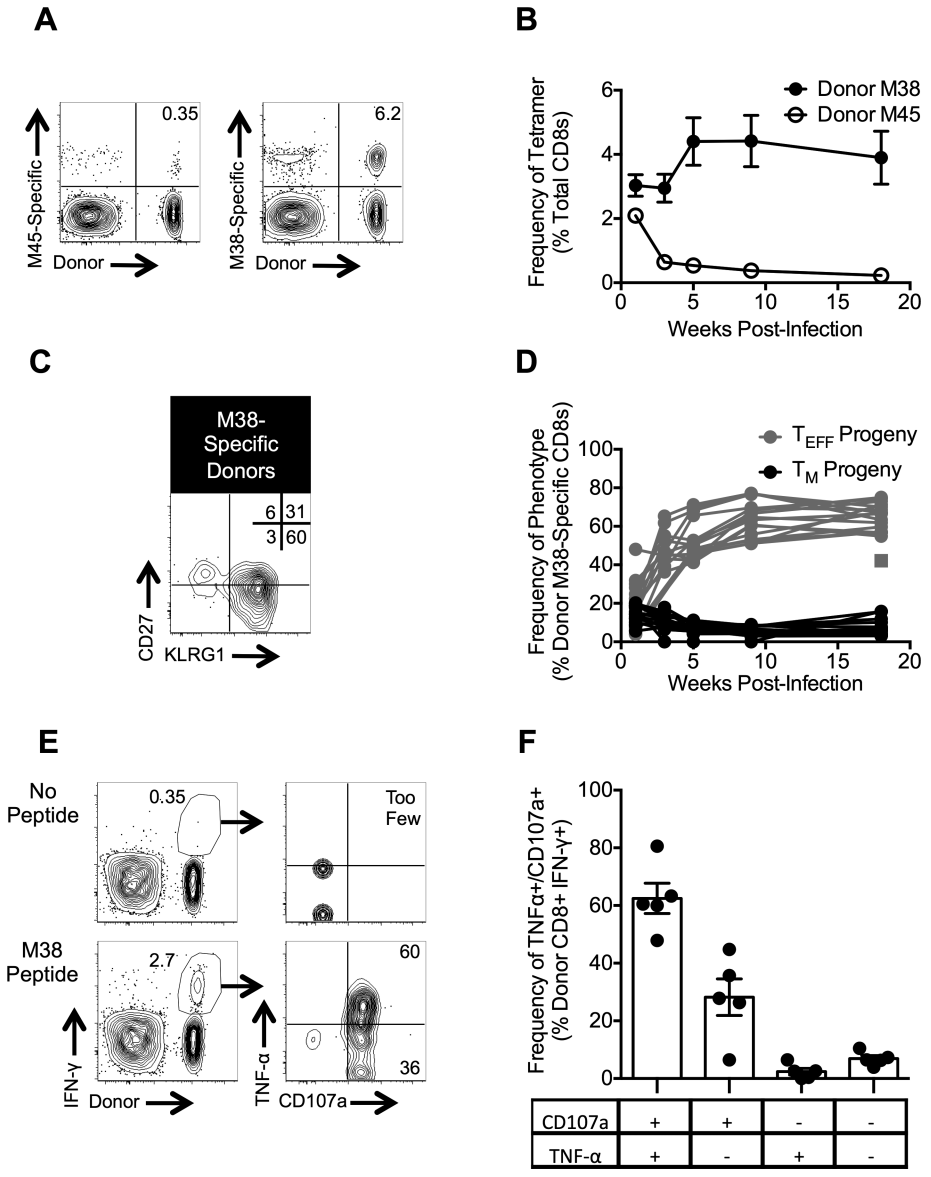

Figure 4. T_M cells reinflate following re-challenge and retain function

Age matched B6 mice received T_M cells and were challenged with MCMV as in the Methods. (A) Representative FACS plots of donor-derived T cells in the blood 126 days post-challenge. (B) Frequencies of tetramer-binding T cells in the blood over time. Data were collected from three independent experiments (*n* = 17 total). (C) Representative FACS plot of the phenotype of donor-derived M38-specific T cells in the blood 126 days post-challenge. (D) Frequencies of donor-derived, M38-specific T_M and T_{EFF} cells in the blood over time. Data are from the same experiments described in (B). Each line represents an individual mouse. The square datum point represents a mouse that appeared to lose the donor T cells after day 7 post-challenge, but effectors appeared ~20 weeks after challenge. (E-F) Intracellular cytokine staining was performed on splenocytes 221 days post-challenge. Shown are representative FACS plots of stimulated (with M38 peptide) and unstimulated cells (E) and the frequencies of IFN-γ positive cells that also express TNF-α and/or CD107a

(F). Data were collected from a single experiment (n = 5) described above. All graphical data are displayed as mean ± SEM.

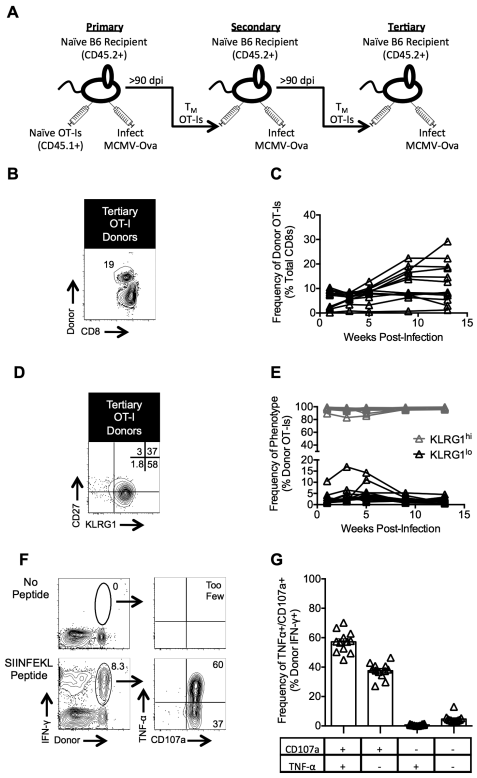

Figure 5. T_M cells can reinflate following multiple re-challenges

(A) Schematic of experimental design. To establish primary OT-I inflationary populations, 600 naïve OT-I T cells expressing CD45.1 were transferred into naïve B6 (CD45.2) recipients followed by infection with MCMV-SL8 (i.e. primary challenge). Thirteen weeks later, 6,000 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 3,500 T_M OT-Is into naive mice and challenging with MCMV-SL8 (i.e. tertiary challenge). (B-C) Representative FACS plot of the donor stain 91 days post 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-E) Phenotypic analyses of the mice described in (B-C). Representative FACS plot of the donor stain 30 weeks post-challenge Frequencies are relative to donor CD8s. (F-G) Intracellular cytokine staining was performed on splenocytes approximately 20 weeks after the tertiary challenge. Shown are representative FACS plots of stimulated (with SIINFEKL peptide) and unstimulated cells (F) and the frequencies of IFN-γ positive 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.

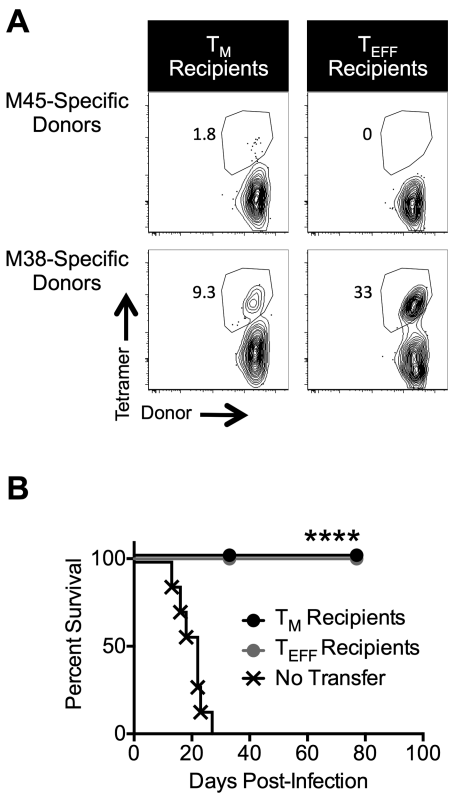

Figure 6. T_M and T_{EFF} cells protect Rag−/− mice following an acute MCMV challenge

Age matched Rag−/− mice received either T_M or T_{EFF} cells and were challenged with MCMV-TK as described in the Methods. Mice were monitored daily for signs of morbidity (lethargy, raised hair and shaking) and sacrificed if they displayed clear signs of morbidity. Data were collected from two independent experiments. One experiment was carried out until 77 days post-challenge. A second experiment was censored at 33 days post-challenge. (A) Representative tetramer staining of T cells in Rag−/− that received either T_M or T_{EFF} transfers. Data were collected 11 weeks post-challenge. Frequencies are relative to total CD8s. (B) Survival curve (n = 7 for control group; n = 7 for T_M group; n = 6 for T_{EFF} group). Statistical significance was determined by a log-rank (Mantel-Cox) test (**** P<. 0001).

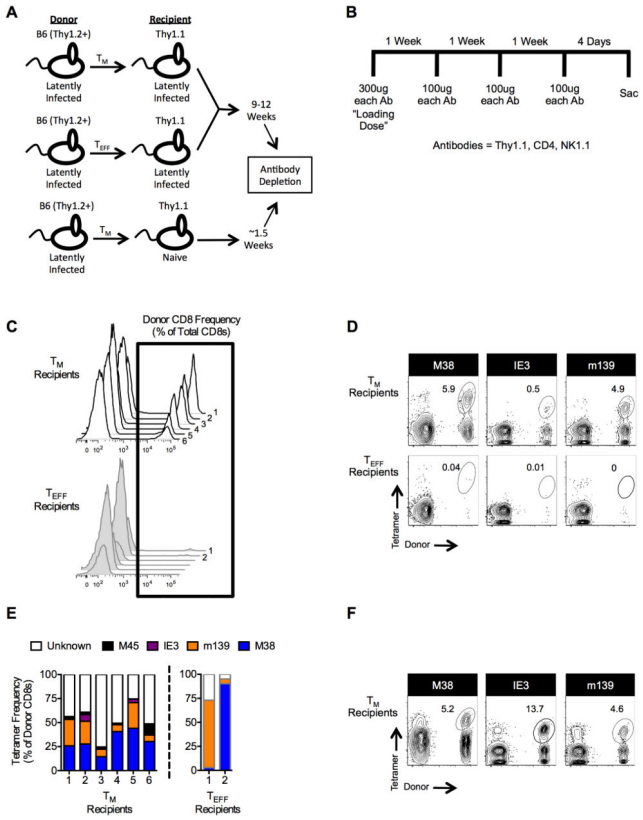

Figure 7. T_M cells persist in latently-infected, immune replete mice and expand when host immunity is lost

(A) Schematic of experimental design. Age matched B6 and Thy1.1 mice were infected with 1×10^6 pfu MCMV-Smith. Following the establishment of viral latency (>8 weeks post-infection), either T_M or T_{EFF} cells from the B6 donors were transferred, as described in the Methods, into the latently-infected Thy1.1 recipients or into naïve Thy1.1 mice. Latently-infected recipients were rested for 9-12 weeks, while the naïve recipients were rested for approximately 1.5 weeks. (B) Antibody depletion schedule. (C-E) The presence of tetramer^{pos} donors was analyzed by flow cytometry immediately following the depletion schedule. Data were collected from two independent experiments (n = 6 total). Three mice from each group were depleted 9 weeks after the transfer; three mice from each group were depleted 12 weeks after transfer. (C) Histograms of donor T cells within each individual recipient. (D) Representative FACS plots of tetramer^{pos} 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 weeks after depletion in one experiment described above (n = 3).