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Memory Inflation during Chronic Viral Infection Is Maintained by Continuous Production of Short-Lived, Functional T Cells

Christopher M. Snyder,[1,3,*] Kathy S. Cho,[1,3,4] Elizabeth L. Bonnett,[1] Serani van Dommelen,[2] Geoffrey R. Shellam,[2] and Ann B. Hill[1]
[1]Department of Molecular Microbiology and Immunology, Oregon Health and Sciences University, 1381 SW Sam Jackson Park Rd, Portland, OR 97239, USA
[2]Microbiology and Immunology, School of Biomedical, Biomolecular & Chemical Sciences, M502, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia
[3]These authors contributed equally to this work
[4]Present address: Astellas Research Institute of America, 8045 Lamon Ave, Skokie, IL 60077, USA
*Correspondence: snydechr@ohsu.edu
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SUMMARY

During persistent murine cytomegalovirus (MCMV) infection, the T cell response is maintained at extremely high intensity for the life of the host. These cells closely resemble human CMV-specific cells, which compose a major component of the peripheral T cell compartment in most people. Despite a phenotype that suggests extensive antigen-driven differentiation, MCMV-specific T cells remain functional and respond vigorously to viral challenge. We hypothesized that a low rate of antigen-driven proliferation would account for the maintenance of this population. Instead, we found that most of these cells divided only sporadically in chronically infected hosts and had a short half-life in circulation. The overall population was supported, at least in part, by memory T cells primed early in infection, as well as by recruitment of naive T cells at late times. Thus, these data show that memory inflation is maintained by a continuous replacement of short-lived, functional cells during chronic MCMV infection.

INTRODUCTION

The development and maintenance of memory T cell responses during persistent or chronic infection is poorly understood. Most of our knowledge is derived from the Lymphocytic choriomeningitis virus (LCMV) clone 13 model system, which involves very high titers of persistently replicating virus. In this environment, antigen-specific T cells continuously divide and ultimately become nonfunctional, a phenomenon known as clonal exhaustion (Shin et al., 2007; Wherry et al., 2004; Wherry et al., 2003; Zajac et al., 1998). In contrast, cytomegalovirus (CMV) is a β-herpesvirus that undergoes systemic infection and establishes true latency but probably also maintains a low degree of persistent infection. The T cell response to CMV in humans and mice is exceptionally large, comprising up to 10% of all $CD8^+$ T cells (Gillespie et al., 2000; Karrer et al., 2003; Lang et al., 2002; Munks et al., 2006a; Sylwester et al., 2005). Most of these T cells are functional, and the populations are maintained for the life of the host.

CMV-specific cells accumulate over time before stabilizing at a high frequency, a phenomenon known as memory inflation (Holtappels et al., 2000; Karrer et al., 2003; Karrer et al., 2004; Munks et al., 2006a; Sierro et al., 2005). Inflated CMV-specific T cells generally express low amounts of the costimulatory molecules CD27 and CD28 and are also deficient in expression of the IL-7Rα and IL-15Rβ chains (Appay et al., 2002; Karrer et al., 2003; Munks et al., 2006a; Sierro et al., 2005; van Leeuwen et al., 2005; van Leeuwen et al., 2002; Weekes et al., 1999b). In addition, most cells express large amounts of KLRG-1, an inhibitory molecule that has been linked to replicative senescence (i.e., failure to proliferate in response to antigen) (Ibegbu et al., 2005; Thimme et al., 2005). In total, this phenotype is indicative of extensive antigen-driven differentiation.

Despite their phenotype, CMV-specific T cells can clearly be driven to divide and seem to respond to viral reactivation by expanding in vivo (Gamadia et al., 2004; Karrer et al., 2004; van Leeuwen et al., 2005; van Leeuwen et al., 2002; Waller et al., 2007). These cells can also kill targets ex vivo and provide protection in vivo (Cobbold et al., 2005; Holtappels et al., 2001; Holtappels et al., 2002; Karrer et al., 2004; Pahl-Seibert et al., 2005; Reddehase et al., 1988; Steffens et al., 1998; van Leeuwen et al., 2002). Perhaps most importantly, it is thought that the establishment and maintenance of these inflated T cell populations is critical for control of the persistent viral infection (Avetisyan et al., 2006; Boeckh et al., 2003; Reusser et al., 1991; Simon et al., 2006; van Leeuwen et al., 2007). Thus, these cells are maintained at a high frequency and remain functional despite expressing markers indicative of antigen-driven differentiation and, possibly, senescence.

These data have led to the general assumption that most CMV-specific T cells are long-lived and maintained by cell division resulting from infrequent but repeated antigen stimulation (van Leeuwen et al., 2007). In contrast, we found that the inflated T cell populations consisted primarily of functional, short-lived T cells that divided only a little and decayed even in the presence of persistent infection. Naive T cells could be recruited during

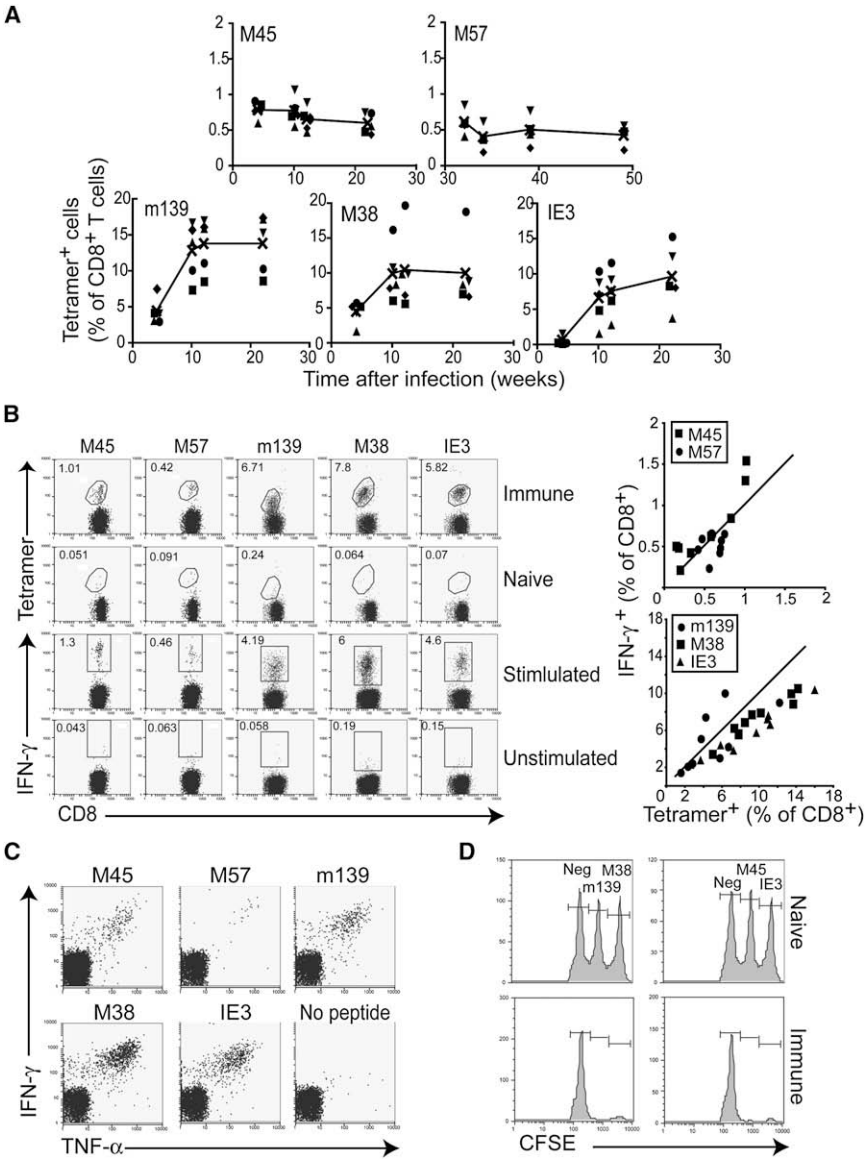

Figure 1. Inflationary T Cells Remain Functional throughout Chronic MCMV Infection

(A) The percentage of MCMV-specific T cells with the indicated specificity were measured over time by MHC-tetramer staining. Each symbol represents an individual mouse. The black line represents the average of all mice in this experiment. Note that the scale is different for M45- and M57-specific T cell populations. The data are representative of > 20 experiments.

(B) For assessment of the percentage of antigen-specific cells that can produce IFN-γ, peripheral blood from individual mice infected for > 3 months was divided and stained with the indicated tetramer or stimulated with the indicated peptide and assessed for IFN-γ production. Except for those of the naive, tetramer-staining control, the FACS plots shown in each column represent the same mouse. A summary of the data from multiple mice and two independent experiments is shown in the graphs to the right. The solid line in each graph represents an ideal 1:1 correlation between tetramer staining and IFN-γ production.

(C) Peripheral-blood cells from chronically infected mice were stimulated as described above and stained for IFN-γ and TNF-α production. The data are representative of three independent experiments.

(D) The survival of peptide-loaded targets was assessed 18 hr after injection into chronically infected mice (two mice per group, one experiment).

chronic infection to replace the decaying short-lived pool, but maintenance of the total population was also largely dependent on the progeny of cells primed early in infection. This suggests that inflated CMV-specific T cell populations are not static and long-lived but, rather, are extremely dynamic.

RESULTS

Inflationary CD8+ T Cells Remain Functional Over Time

Murine cytomegalovirus (MCMV) infection drives two major types of T cell populations, stable memory T cells and inflationary T cells, which can be distinguished on the basis of antigen specificity (Karrer et al., 2003; Munks et al., 2006a). T cells that will become stable memory T cells decline rapidly after acute infection and then persist at low frequencies. In C57BL/6 mice (B6), the stable memory T cell pool is typified by T cells specific for M45 and M57 (Figure 1A and Munks et al., 2006a). In contrast, inflationary T cells increase in number after acute infection before stabilizing at a high frequency, at which they are maintained for the life of the host (Munks et al., 2006a; Munks et al., 2007). In B6 mice, populations specific for proteins encoded by m139, M38, and IE3 inflated dramatically over the 8–12 weeks following infection (Figure 1A).

In contrast to the LCMV clone 13 model of chronic viral infection, MCMV-specific T cells remain functional during chronic infection. Most cells detected in the blood (Figure 1B) and spleen (not shown) by tetramer staining also secreted IFN-γ, although some cells in the largest responses failed to secrete IFN-γ. Though we have previously demonstrated that very few MCMV-specific T cells secrete IL-2 at these time points (Munks et al., 2006a), most cells that secreted IFN-γ also produced TNF-α (Figure 1C). In addition, peptide-pulsed targets were readily killed in vivo, indicating that the T cells also retain their cytotoxic capacity (Figure 1D). Thus, MCMV-specific T cells remain functional, even at late times after infection.

Phenotype of Stable and Inflationary T Cells

Sierro et al. described a phenotypic dichotomy between inflationary and stable memory T cells (Sierro et al., 2005). Consistent with that report, we found that the prototypical stable memory T cell populations (M45- and M57-specific T cells) generally expressed a central memory T cell-like phenotype (Figure 2). This is best illustrated by high expression of the cytokine receptors CD127 (IL-7Rα) and CD122 (IL-15Rβ), as well as high

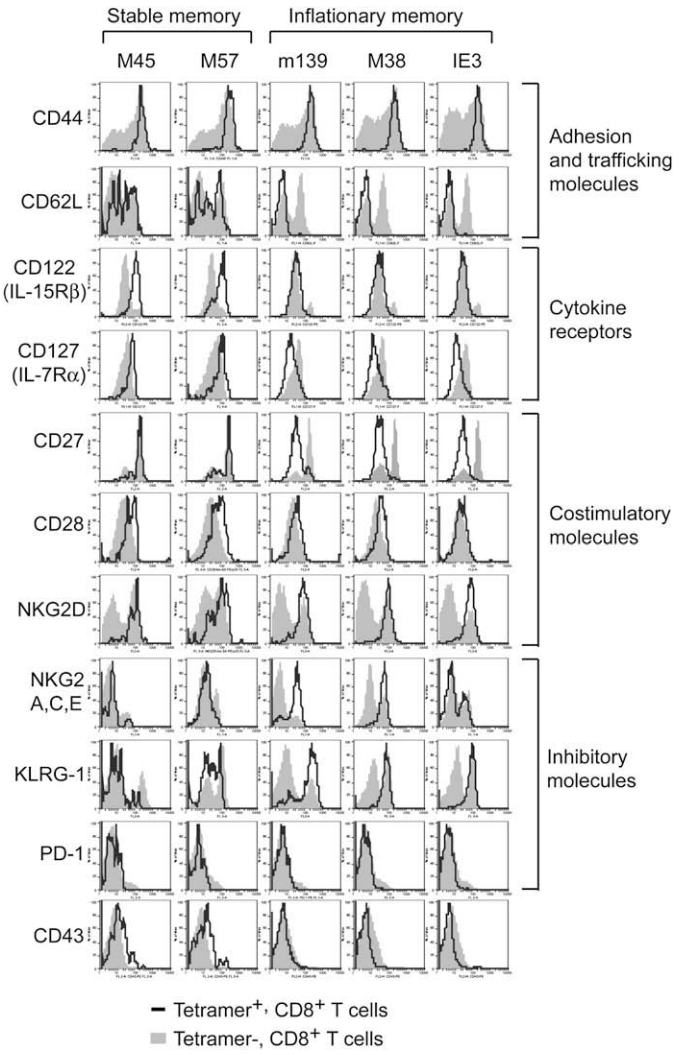

Figure 2. Phenotype of Stable and Inflationary CD8⁺ T Cells

Peripheral blood and/or splenocytes from mice infected for > 3 months was stained with the indicated tetramer, as well as with antibodies specific for CD8 and the indicated cell-surface molecule. The plots shown are gated on tetramer⁺, CD8⁺ T cells (black line) or tetramer⁻, CD8⁺ T cells from the same sample (shaded histogram). Data are compiled from different sets of infected mice measured at different time points during chronic infection (at least 3 months after infection) and are representative of at least four individual mice per stain. CD43 stains are from a single experiment; all others are representative of at least three independent experiments.

expression of the costimulatory molecule CD27. In addition, these cells did not express the NK cell-inhibitory molecules NKG2A or KLRG-1, and a substantial portion of the cells in the blood and spleen expressed CD62L (L-selectin).

In contrast, the prototypical inflating populations (m139- and M38-specific T cells) were almost uniformly differentiated. Almost all of these cells expressed low amounts of CD62L, CD27, CD127, and CD122 and had high expression of the NK cell-inhibitory molecules NKG2A and KLRG-1. IE3-specific cells, which are almost undetectable during acute infection but inflate dramatically thereafter, were almost identical to the m139- and M38-specific populations, with one exception: approximately 30%–60% of IE3-specific cells were NKG2A-negative. It is unclear what this might mean, because these NKG2A-negative cells were otherwise indistinguishable from the NKG2A-positive cells. Similar results were seen with the NKG2A-specific antibody (clone 16a11) that does not crossreact with the NKG2C and E molecules (not shown). Interestingly, most of the inflationary T cells retained expression of CD28, although we sometimes observed CD28 downregulation (Figure 2 and Figure S1A, available online). HCMV-specific T cells and inflationary T cells in BALB/c mice are typically CD28-negative (Appay et al., 2002; Gamadia et al., 2001; Khan et al., 2002a; Sierro et al., 2005; Weekes et al., 1999a).

Both stable and inflationary T cells had high expression of the costimulatory molecule NKG2D, and no cells expressed the inhibitory molecule PD-1 (Figure 2), in agreement with data from HCMV-specific T cells (Day et al., 2006; Trautmann et al., 2006). Finally, most cells were not stained with the CD43-specific antibody 1B11, although there was usually a fraction of M45- and M57-specific T cells that were stained. This antibody has recently been used to distinguish memory T cells with a reduced capacity to proliferate after challenge (Hikono et al., 2007).

In total, the phenotype of m139-, M38-, and IE3-specific T cells is consistent with that seen after repeated antigen stimulation (Jabbari and Harty, 2006; Masopust et al., 2006). It is worth noting, however, that a very small fraction of the m139-, M38-, and IE3-specific populations express a more central memory T cell-like phenotype, similar to most M45- and M57-specific T cells (Figure S1B).

T Cells within the Inflating Population Do Not Respond to Homeostatic Signals but Can Respond to a New Viral Infection

To address how inflationary T cell populations are maintained during chronic infection, we performed a series of adoptive transfer experiments. Responsiveness to homeostatic signals was investigated by transferring CFSE-labeled splenocytes from chronically infected mice (>3 months after infection) into SCID recipients and tracking tetramer-positive cells. As predicted from their low expression of CD122 and CD127 (Figure 2), m139-, M38- and IE3-specific T cells mostly failed to divide in the lymphopenic host, whereas tetramer-negative cells underwent extensive division in the same time period (Figure 3A).

To test whether these inflationary T cells had lost the capacity to divide, we transferred CFSE-labeled cells from chronically infected mice into naive congenic mice and infected recipients 1 day later. Strikingly, a large fraction of donor-derived T cells specific for all antigens had proliferated extensively during the first week of an acute infection (CFSE-negative; Figure 3A), although there were also cells that had failed to divide. To test whether all of the divided inflationary T cells were derived from the small proportion of the inflationary T cell population that is less differentiated, we sorted the total CD8⁺ pool into CD27^{hi} (less differentiated) and CD27^{lo} (more differentiated) populations, transferred them into naive congenic mice, and infected the recipients. Inflationary T cells from both donor populations divided in the recipient mice (CFSE-negative; Figure 3B, top

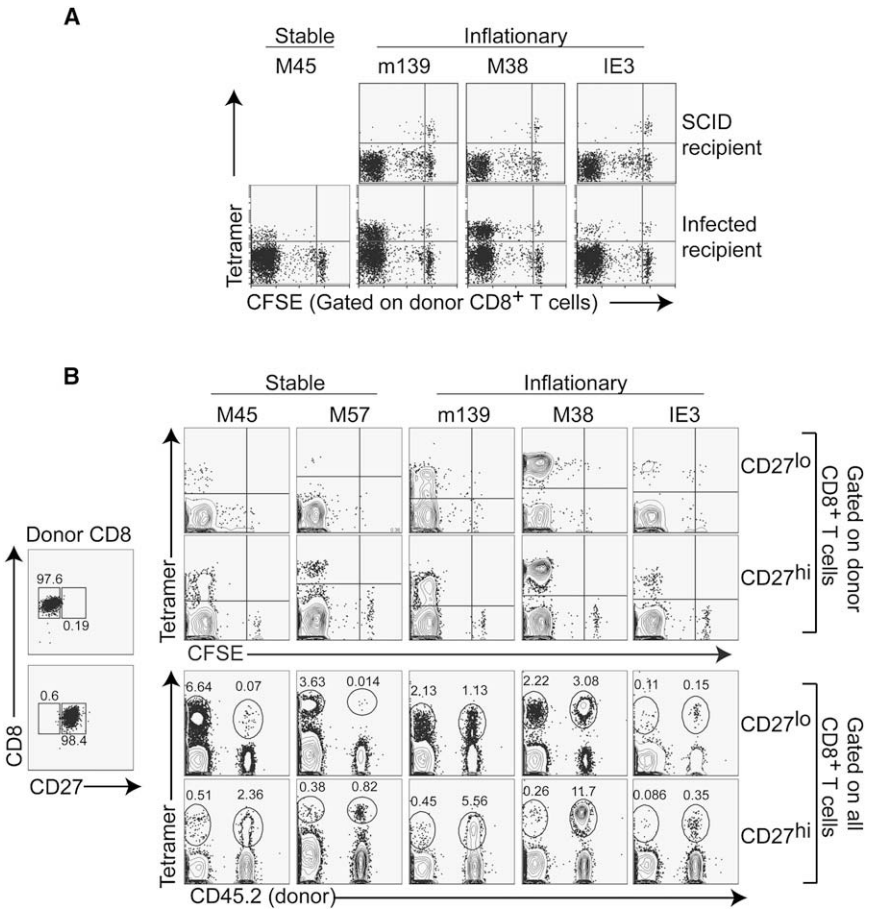

Figure 3. Inflationary T Cells Fail to Divide in SCID Recipients but Can Respond to a New Viral Infection

(A) Splenocytes from C57BL/6 mice infected for > 3 months were transferred into C57BL/6-SCID mice or naive CD45.1 congenic mice. Naive, CD45.1 congenic recipients were infected with MCMV 2 days later. Peripheral blood from SCID recipients (day 12 after transfer) or splenocytes from CD45.1 congenic recipients (day 7 after infection) were stained with the indicated tetramers. The FACS plots have been gated on $CD8^+$ donor-derived T cells in all cases. Data are representative of two independent experiments.

(B) For assessment of the ability of differentiated T cells to divide in response to antigen, $CD8^+$-enriched splenocytes were FACS sorted for $CD8^+$, $CD27^{hi}$, and $CD27^{lo}$ cells. Sorted cells were CFSE labeled and transferred into naive, congenic mice. Recipients were infected 1 hr later, and donor-derived cells were analyzed in the peripheral blood on day 7 after transfer. Numbers represent the percentage of all $CD8^+$ T cells that fall into the indicated gates in the shown FACS plots. Data are from a single experiment.

panels). However, the $CD27^{hi}$ cells appeared to have a greater proliferative capacity (Figure 3B, lower panels). Because so few inflationary T cells are $CD27^{hi}$ (Figure 2), many more cells specific for inflationary epitopes were transferred in the $CD27^{lo}$ donor-derived pool than in the $CD27^{hi}$ donor-derived pool. Nevertheless, donor-derived cells from the $CD27^{hi}$ pool contributed > 90% of the entire inflationary T cell response in the recipient mouse 7 days later, whereas donor-derived cells from the $CD27^{lo}$ pool composed only approximately half of the overall inflationary T cell response (Figure 3B, lower panels). Together, these data suggest that $CD27^{lo}$ inflationary T cells retain some ability to divide in response to viral antigen but are probably not as responsive as the few $CD27^{hi}$ inflationary T cells within each population.

Inflationary T Cells Undergo Sporadic Antigen-Dependent Division

Because inflationary T cells are capable of dividing in response to infection, we wanted to asses whether inflationary T cells undergo antigen-driven proliferation in chronically infected mice. CFSE-labeled splenocytes from chronically infected mice were transferred into infection-matched (infected on the same day) or naive CD45 congenic recipients (Figure 4). $Tetramer^+$ donor-derived cells were tracked in the peripheral blood of recipients (e.g., Figure S2). At these late time points after infection, there is no detectible replicating virus in the spleen of an infected mouse. Nevertheless, as a control for the transfer of virus to the naive recipients, splenocytes were simultaneously transferred into SCID mice, which were monitored for survival for 3 months. All mice survived, and no plaque-forming virus was detected in any organ by plaque assay at the end of the experiment (not shown).

Very little division was seen in any donor population at early times. At late times after transfer, most of the M45- and M57-specific T cells had undergone some division, but only a few cells had fully diluted their CFSE. The extent of division was the same in naive and infected recipients and is consistent with homeostatic turnover (Figures 4A and 4B).

The inflationary T cells (m139-, M38-, and IE3-specific) failed to divide significantly in naive recipients at any time point. In contrast, there was clear evidence of antigen-driven proliferation in immune recipients, as evidenced by extensive CFSE dilution in a fraction of the inflationary, donor-derived T cells (Figures 4A and 4B). Yet, division was infrequent and sporadic. Even 4 months after transfer, the majority of the transferred inflationary T cells had not divided (Figures 4A and 4B), and division within one epitope-specific inflationary T cell population did not correlate with division in the other inflationary T cell populations in the same mouse (Figure S3A). Thus, even though inflationary, MCMV-specific T cells can divide in response to a new viral infection, they do not frequently divide during chronic infection.

The Inflationary T Cell Populations Are Exchanged Regularly

Despite the sporadic antigen-driven proliferation in infected recipients, the donor-derived inflationary T cells (m139-, M38-, and IE3-specific) disappeared from the blood of both immune and naive recipients, with an approximate half-life of 45–60 days

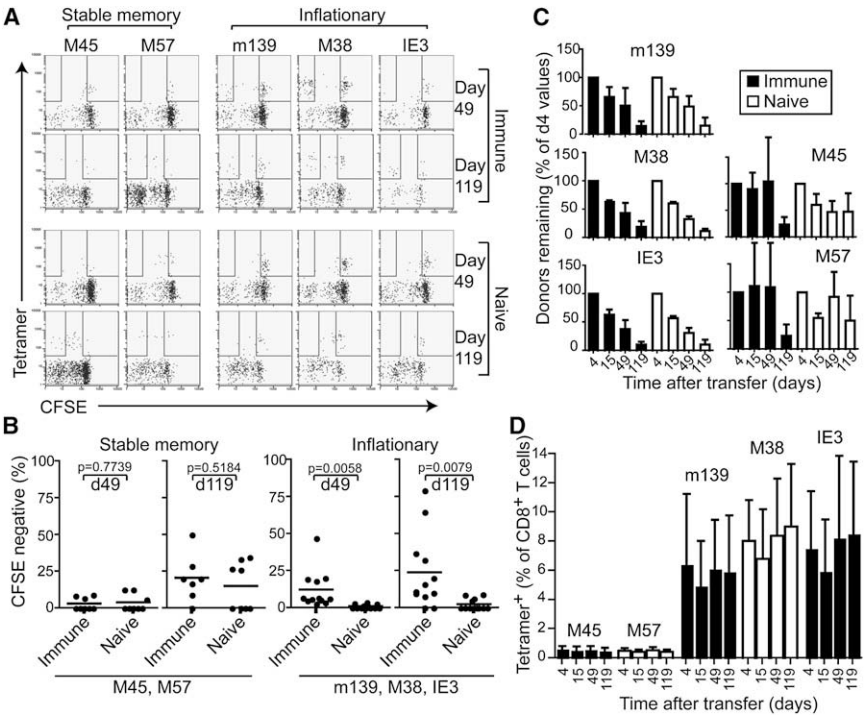

Figure 4. Inflationary T Cells Decline Over Time in both Immune and Naive Recipients

Splenocytes from C57BL/6 mice infected for > 3 months were harvested and transferred into infection-matched, CD45.1 congenic recipients. Donor-derived tetramer$^+$ T cells were tracked over time as a percentage of all CD8$^+$ T cells in the peripheral blood of recipients.

(A and B) Extensively proliferated populations of inflationary T cells (CFSE negative, on the basis of recipient cells) were measured over time. (A) FACS plots at two time points from the same individual mice gated on donor-derived CD8$^+$ T cells. (B) Percentage of donor-derived tetramer$^+$ stable (left panels) or inflationary (right panels) T cells that were CFSE$^-$ either 49 or 119 days after transfer in this experiment. The Student's t test was used for assessing significance.

(C) Donor-derived MCMV-specific T cells were measured over time. When measured as a percentage of all CD8$^+$ T cells, donor-derived CD8$^+$ T cells engrafted ~1.5 to 2 times better in naive recipients compared to immune recipients. As a correction for these differences in the "take" of the donor-derived population, the percentage of the donor-derived MCMV-specific T cells was normalized to 100% at the first time point. The error bars represent the standard deviation from the average. T cell half-life was estimated by the length of time it took for the population to be reduced by 50% in circulation, relative to all CD8$^+$ T cells.

(D) The total (donor + recipient) tetramer-staining population from the same mice as in (C) is shown. Error bars show the standard deviation. Data are representative of four independent experiments.

(Figure 4C). A similar half-life was estimated regardless of whether we counted cells on the basis of IFN-γ production or tetramer staining (not shown). The fact that inflationary T cells parked in both immune and naive recipients decayed at a similar rate suggests that this decay occurred in an antigen-independent manner and that the sporadic antigen-driven proliferation seen in Figures 4A and B had little impact on the size of the total inflationary T cell population. This is supported by the data in Figure 4A, which show that the divided T cells did not accumulate. The FACS plots for each epitope shown in Figure 4A represent the same recipient at different time points, and it can be seen that there was not a noticeable shift of cells from the undivided to the divided fraction over time. Interestingly, we occasionally saw some mouse-to-mouse variation in the rate of decline of an individual inflationary T cell population, particularly in immune recipients (Figure S3B). However, in only one experiment was an accumulation of any transferred inflationary T cells observed (Figure S3C). This example of inflation in the M38-specific population correlated with a large antigen-specific population that had undergone division (CFSE negative). But this was only seen once out of all three inflationary T cell responses followed in 15 infected recipients from four independent experiments. It is important to note that the overall inflationary T cell populations (donor + recipient) were maintained in all immune mice (Figure 4D), suggesting that the circulating cells are being replaced as they decay.

In contrast to the donor-derived inflationary T cells, donor-derived stable memory T cell populations (M45- and M57-specific), largely persisted in naive recipients, consistent with homeostatic cell division (Figure 4C, right side). Interestingly, at very late times, there appeared to be some loss of donor-derived M45- and M57-specific cells in immunological recipients.

Similar experiments were set up in parallel as a control for antigen-independent effects of viral infection (i.e., cytokine environment, etc.) through a recombinant MCMV that expresses Ovalbumin (MCMV-Ova). The SIINFEKL-specific T cells observed after MCMV-Ova infection act like inflationary T cells and are extensively differentiated (S4A and S4B). Splenocytes from mice that were chronically infected with MCMV-Ova were transferred into mice that were naive or were infected with wild-type MCMV or MCMV-Ova. As above, extensive division of donor-derived T cells was seen only in the presence of antigen (MCMV-Ova-infected recipients), but antigen-specific T cells decayed after transfer in all mice (Figures S4C–S4E).

Altogether, these data show that the inflationary T cell populations decay from circulation during chronic MCMV infection. Though we found evidence of antigen-dependent T cell division within the inflated populations, this appeared to be due to sporadic events that may have reduced the rate of decay but only involved a small number of cells and did not sustain the populations.

Recruitment of Naive T Cells at Late Time Contributes to the Inflated CD8$^+$ T Cell Populations

If most cells in the circulating inflationary T cell population decay, how is the overall population maintained? It has been recently

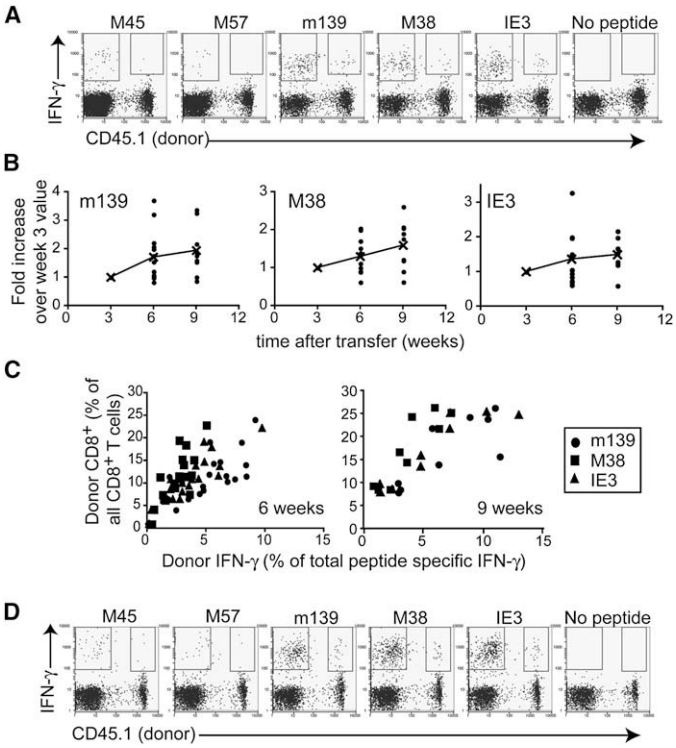

Figure 5. Recruitment of Naive T Cells Occurs during Chronic Infection

C57BL/6 mice that were infected for > 3 months were treated with busulfan and injected with bone marrow from naive CD45.1 congenic donors. Donor-derived MCMV-specific T cells in the peripheral blood were tracked by intracellular cytokine staining over time.

(A) Donor-derived MCMV-specific T cells were measured in the peripheral blood of recipients. Shown are representative FACS plots (from three independent experiments) gated on all $CD8^+$ T cells from mice that received naive bone marrow 9 weeks earlier.

(B) Data from all three independent experiments were combined. The graphs show peripheral-blood donor-derived cells that produced IFN-γ in response to the indicated peptides as a percentage of the total peptide-specific IFN-γ response. The data were normalized to the amount of donor-derived IFN-γ found at the first time point. The lines represent the average of the donor responses over time.

(C) The data from (B) was replotted to show the correlation between the percentage of $CD8^+$ T cells that were donor derived and the percentage of MCMV-specific T cells that were donor derived.

(D) Chronically infected mice received CD3-depleted donor bone marrow after busulfan treatment. Shown are representative FACS plots from four individual mice in one experiment.

shown that naive T cells can be recruited during chronic polyomavirus and LCMV clone 13 infections (Kemball et al., 2005; Vezys et al., 2006). As a test of whether recruitment of naive T cells occurs during chronic MCMV infection, chronically infected mice were treated with busulfan, a DNA-alkylating agent used to condition recipients for bone-marrow transplant. One day after busulfan treatment, mice were given bone marrow from naive, congenic donors (Figure 5). In this way, we could track donor-derived lymphocytes that developed in the presence of chronic infection. Importantly, busulfan is thought to have minimal impact on the established memory T cells, allowing us to analyze recruitment of new cells without dramatically disrupting the overall T cell compartment (Kemball et al., 2005; Vezys et al., 2006).

The data shown in Figure 5 demonstrate that naive T cells were readily recruited into the inflationary T cell pool. As early as 3 wk after bone-marrow transfer, a substantial population of donor-derived $CD8^+$ T cells could be detected, and these increased as a percentage of the total MCMV-specific pool over time (Figures 5A and 5B). Importantly, the overall amount of recruitment seemed to be dependent on the degree of chimerism achieved in a given mouse (Figure 5C), and the overall T cell responses were not affected by the busulfan treatment (Figure S5A). In contrast to the inflationary T cell populations, few if any M45- and M57-specific T cells were derived from the donor, which was expected because these populations don't inflate (Figure 5A). Recruitment of cells from the donor-derived bone marrow into the inflationary T cell pool was also seen when mature T cells were depleted from the donor-derived bone marrow prior to transplantation (Figure 5D and Figure S5B). Thus, T cells that develop in the presence of chronic MCMV infection can respond to the ongoing infection and join the inflationary T cell populations.

Cells Primed Early in Infection Also Contribute to the Maintenance of the Inflationary T Cell Populations

If recruitment of naive T cells were solely responsible for maintaining the inflationary T cell populations, then all inflationary T cells should eventually be replaced from the naive T cell pool. Thus, in mice that developed a high degree of chimerism (most new $CD8^+$ T cells were donor derived), most MCMV-specific T cells should have eventually become donor derived as well. However, we found that even though the majority of all thymocytes were donor derived in some mice, the percentage of donor-derived MCMV-specific T cells remained low 10–11 months after transplant (Figures 6A and 6B). Although animals with a higher percentage of donor-derived thymocytes had more donor-derived MCMV-specific T cells (not shown), the two populations did not equalize, even after more than five half-lives of the circulating, differentiated T cells had elapsed. This result suggests that naive T cells are not essential for maintaining the inflationary T cell populations. In addition, at 92 days after infection of thymectomized mice with salivary-gland-derived MCMV, memory inflation is not abrogated, adding support to this hypothesis (A. Loewendorf, R. Arens, and C. Benedict, personal communication). These results were initially puzzling, but an experiment originally performed for a different reason suggested an explanation. We transferred splenocytes from day 7 infected mice into infection-matched, congenic recipients. This time point represents the peak of T cell expansion after acute infection. Donor-derived cells were measured in the peripheral blood of recipients 2 weeks and 6–7 months after transfer (Figures 6C–6E). As expected, donor-dervied M45-specific T cells persisted in most recipients. Strikingly, however, many donor-derived inflationary T cells also persisted or inflated in these recipients, even after what should have been 3–4 half-lives on the basis of our previous findings (compare Figure 6E to Figure 4C). This was most evident for m139-specific T cells, which underwent dramatic inflation or were maintained in most mice (Figure 6E). Persistence of M38-specific donor-derived T cells was less obvious but was also seen in several animals.

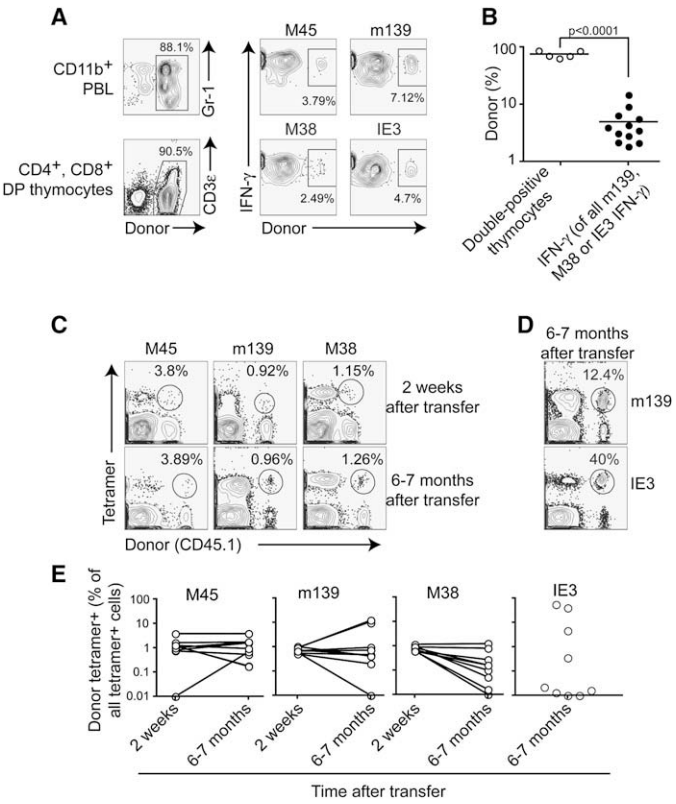

Figure 6. Naive T Cells Do Not Account for the Replacement of all Decaying, Circulating, Differentiated T Cells

(A) The FACS plots show an individual mouse (from the experiments described in Figure 5) in which most of the newly produced T cells were donor derived but most MCMV-specific T cells were recipient derived, 10 months after transplant.

(B) MCMV-specific chimerism (from the experiments in Figure 5) is shown for several mice with a high degree of chimerism within double-positive thymocytes.

(C) Donor-derived MCMV-specific T cells were transferred at the peak of T cell expansion (day 7 after infection) and measured at 2 weeks and at 6–7 months after transfer. Sample FACS plots are from the same mice at each time point. The numbers indicate the percentage of all tetramer$^+$ cells that are donor derived. Data are representative of two independent experiments.

(D) FACS plots show two examples of dramatic inflation of the donor-derived MCMV-specific T cell populations 7 months after transfer.

(E) Combined data (as in panels C and D) from multiple mice and two independent experiments. Shown is the donor-derived tetramer$^+$ population as a percentage of all tetramer$^+$ T cells to adjust for inflation of the m139- and M38-specific populations over time. Lines connect individual mice at either 2 wk after transfer (3 wk after infection) or 6–7 months after transfer. IE3-specific T cells were not measured at 2 wk after transfer.

Strikingly, donor-derived IE3-specific T cells, which are almost undetectable at day 7 of infection, when the transfer was performed, were found to have inflated in several mice. Thus, at day 7 of infection, we were able to transfer MCMV-specific cells whose progeny could replenish the inflationary T cell pool, whereas such cells were not transferred with splenocytes from chronically infected mice. Altogether, these data show that the circulating, short-lived, inflationary T cell populations can be replaced by the progeny of cells primed during acute infection, as well as by naive T cells primed at late time points.

DISCUSSION

Our work demonstrates that the inflationary, MCMV-specific T cell populations are highly dynamic. Most inflationary T cells in the spleen and blood appear to be functional but are short-lived, disappearing from circulation with a half-life of approximately 45–60 days. As they decay, these cells are constantly being replaced by an influx of differentiated cells. In our model (Figure S6), cells primed early in infection will consistently produce progeny that can replace the decaying, short-lived T cells in circulation. This may be the main way that inflationary T cell populations are maintained, resulting in the persistence of some clones over time even as individual cells within those clones decay. However, naive T cells can also be primed during chronic infection and may effectively alter the clonality of the population over time. Although naive T cells are not essential for maintaining inflationary T cell populations, the impact of naive T cell recruitment is unclear and may depend on the time that has elapsed since infection and the age of the animal. Regardless, it is clear that the populations are highly dynamic, and individual analyses of circulating cells reveal only snapshots of a complex process that also includes a small amount of antigen-driven cell division. It is possible that the disappearance of the short-lived populations from the blood indicates a migration into tissues over this time. Indeed, we have seen large numbers of inflationary T cells in the lungs (not shown). However if this was the cause of the loss of donor-derived T cells from the circulation, we would have to conclude that these cells rarely re-enter the blood after extravasation and probably die in the tissues. Importantly, most HCMV-specific T cells also bear a highly differentiated phenotype, similar to that described here for the majority of inflationary T cells (Gamadia et al., 2001; Kuijpers et al., 2003; van Leeuwen et al., 2005; van Leeuwen et al., 2004; Weekes et al., 1999a; Weekes et al., 1999b), and some, but not all, HCMV-specific T cell clones have been shown to persist over time (Day et al., 2007; Weekes et al., 1999a), agreeing with our results.

We can imagine three possible explanations for our failure to transfer memory T cells from chronically infected mice. First, it is possible that the memory T cells are not present in the spleen at late times. They may reside selectively in some other compartment, perhaps the bone marrow or lymph nodes, and continuously produce the differentiated cells that we see in circulation at late times. Second, it is possible that the memory T cells cannot find a niche to survive in after transfer. If all of the available niches are occupied in chronically, but not acutely, infected mice, we would expect donor-derived memory T cells to die shortly after transfer. Finally, it is possible that the memory T cells are not extracted from the spleen before transfer, perhaps remaining associated with the splenic stroma when the spleens were crushed through a filter. Experiments to discriminate between these possibilities are ongoing and should enhance our understanding of the nature of T cell memory.

The phenotype of the circulating, inflationary, MCMV-specific T cells resembles cells that have been repeatedly stimulated with antigen and not allowed to rest (Jabbari and Harty, 2006;

Masopust et al., 2006). However, we saw no reversion of these cells to a more "central memory T cell" phenotype after transfer into naive animals, suggesting that the inflationary T cells may be terminally differentiated (not shown). Yet, these inflationary T cells clearly retained the capacity to divide in response to viral antigen, even though they largely failed to do so during chronic infection. This lack of proliferation may reflect the fact that differentiated inflationary T cells express low amounts of costimulatory molecules and high amounts of inhibitory molecules, a combination that should increase the activation threshold for these cells. Recent data indicating that expression of 4-1BBL by APCs is essential for driving the proliferation of HCMV-specific T cells supports the idea that CMV-specific T cells have "special" stimuation requirements (Waller et al., 2007).

It is interesting to speculate that MCMV, which is a natural murine pathogen, may have evolved to allow cells to be replaced as they become extensively differentiated, rather than overstimulating an existing population. This could allow T cell immunity to be maintained without exhaustion so that the virus and its host can both persist. It is likely, however, that many factors influence this equilibrium. First, the inflammatory environment of the host clearly increases viral reactivation from latency (Cook et al., 2006; Haagmans et al., 1994a; Haagmans et al., 1994b; Prosch et al., 1995; Simon et al., 2005; Stein et al., 1993). Therefore, any ongoing infections are likely to drive more viral replication. In addition, there are likely to be age-associated effects on the host-virus relationship. As thymic output decreases, the host may be more dependent on the memory and short-lived T cell populations. Indeed, CMV infection is associated with dramatic T cell clonal expansions in aged people (Khan et al., 2002b; Ouyang et al., 2003).

In contrast to the inflationary T cell populations, T cells specific for M45 and M57 appear to be more similar to classical central memory T cells, and we were clearly able to transfer self-maintaining populations into naive mice. Although the cause for this dichotomy between stable and inflationary T cells is not clear, we speculate that it reflects different patterns of viral gene expression during acute infection and latency. Interestingly, upon viral reactivation, patterns of gene expression vary dramatically in different tissues (Streblow et al., 2007).

Overall, our data describe a dynamic host-virus balance that may shed light on the behavior of T cells in the face of chronic antigen. Our data suggest that antigen persistence alone is insufficient for inducing T cell dysfunction and that the context of antigen presentation (that which determines whether previously primed T cells are driven to proliferate) is likely to play a major role in determining T cell fate during chronic infections.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (B6.SJL-CD45.1 congenic) and B6.CB17-Prkdc^{scid}/SzJ (B6.SCID) were also initially purchased from The Jackson Laboratory and then bred in house. Mice were between the ages of 6 and 16 weeks, and all studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at Oregon Health and Sciences University.

Virus Strains and Infections

Unless otherwise indicated, mice were infected i.p. with 1×10^6 pfu of MCMV strain MW97.01, which is derived from a bacterial artificial chromosome of the Smith strain (Wagner et al., 1999). In some experiments, MCMV-Ova was used. This virus was produced as described elsewhere (Lloyd et al., 2003). In all cases, virus stocks were grown on mouse-embryo fibroblasts that were sonicated or dounced to release the viral particles.

Tetramer Staining, Antibodies, Intracellular-Cytokine-Stimulation Assay, and FACS Analysis

Tetramers were synthesized by the National Istitutes of Health tetramer core facility (http://www.niaid.nih.gov/reposit/tetramer/overview.html). Tetramer staining was performed on 50 μl of whole blood or 1×10^6 splenocytes. Red blood cells were lysed with BD lysis buffer after staining (Becton Dickenson). Various fluorescently conjugated antibodies were used (CD8α [53-6.7], CD27 [LG.7F9], CD28 [37.51], CD43 [1b11], CD44 [IM7], CD45.1 [A20], CD45.2 [104], CD62L [MEL-14], CD122 [TM-b1], CD127 [A7R34], KLRG-1 [2F1], NKG2A,C,E [20d5], NKG2D [C7], PD-1 [RMP1-30]). For measurement of intracellular IFN-γ, red blood cells were lysed with 3 ml of lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$), and the remaining cells were stimulated and stained as described previously (Munks et al., 2006a; Munks et al., 2006b). In all cases, samples were acquired on an LSR II or a FACSCalibur (both from BD) and analyzed with FlowJo software (Tree Star).

Adoptive Transfers and Bone-Marrow Chimeras

For in vivo CTL assays, CD45.1 congenic splenocytes were stained with 2 μM, 0.5 μM, or 0.1 μM CFSE, loaded with 2 μM of the indicated peptide, and i.v. transferred into C57BL/6 recipients that were infected for > 3 months or naive controls. FACS analysis of the surviving donor-derived cells (CD45.1^{+}) in the spleens of recipient mice was performed approximately 18 hr later.

For adoptive transfer of mature T cells, splenocytes from congenic mice were labeled with 1 μM CFSE, suspended in PBS, and injected i.v. into recipient mice. Typically, recipients were given 2–5 × 10^7 unfractionated splenocytes per injection. In one experiment (Figure 3C), CD8^{+} T cells were purified by negative selection (EasySep, Stem Cell Technologies) and then sorted for CD8^{+}, CD27^{hi}, and CD27^{lo} cells with a FACSVantage cell sorter (Becton Dickenson). Sorted cells were i.v. transferred into naive, congenic mice, and recipients were infected 1 hr later.

To measure recruitment of naive T cells, we adapted a protocol recently described (Kemball et al., 2005; Vezys et al., 2006). In brief, chronically infected mice were injected i.p. with 600 μg of busulfan dissolved in 50% DMSO. The following day, 2×10^7 whole or CD3-depleted bone-marrow cells from naive CD45 congenic mice were injected i.v. into treated mice. CD3^{+} T cell depletion was carried out with 1.5 μg of PE- conjugated CD3ε-specific antibody per 1×10^8 bone-marrow cells and the PE selection kit (EasySep, Stem Cell Technologies), according to the manufacturer's recommended protocol.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00418-4.

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