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Dissecting the Requirements for Maintenance of the CMV-Specific Memory T-Cell Pool

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Abstract

Cytomegalovirus (CMV) infection promotes a broad T-cell response, with the resulting memory cells displaying diverse phenotypes. CMV establishes lifelong persistence/latency, and it is thought that viral antigens expressed during this period may regulate the expansion and/or maintenance of "inflationary" CD8 T-memory populations that display an effector memory phenotype. We show here that mouse CMV (MCMV)-specific inflationary memory T cells do not decrease in number after thymectomy, indicating that recent thymic emigrants are not strictly required for their maintenance. Furthermore, persistent MCMV replication in the salivary gland does not significantly impact the T-cell memory compartment, as surgical removal did not alter its composition. These results shed light upon the mechanisms required for maintenance of the large, MCMV-specific T-cell memory pool.

Human cytomegalovirus (HCMV/HHV5, a b-herpesvirus) establishes a largely asymptomatic infection in $\geq$50% of the healthy population. In contrast, immune-compromised individuals can suffer serious consequences when facing HCMV (e.g., newborns, transplant recipients, and AIDS patients) (1,2). While it is established that HCMV causes disease in settings in which immunity is decreased, increasing evidence indicates that CMV "shaping" of the immune system over a lifetime of infection may also be a cofactor for disease. This hypothesis stems partly from the fact that CMV-specific T cells expand to large numbers and compose a huge percentage of the circulating lymphocyte pool in infected persons, a process termed "memory inflation" (3–7).

Data gleaned from several model systems indicate that memory T cells are a heterogeneous population of cells requiring diverse stimuli for their generation and maintenance (8–10). The CMV-specific T-cell response is directed against many viral orfs ($\geq$151), but ones that ultimately comprise the majority of the memory pool have a limited number of epitope specificities and display an effector memory phenotype (T_{EM}, for example H-2K^b M38$_{316–323}$, m139$_{419–426}$, and IE3$_{416–423}$) (3). In turn, the remainder of the CMV-specific memory T-cell pool is more diverse in specificity, has a central memory phenotype, and remains stable in number following its contraction after acute infection ($T_{CM,}$ for example H-2D^b M45$_{985–993}$) (6,11–14).

T_{EM} differ from T_{CM} in their maintenance requirements and functional properties, as they primarily reside in tissues and likely function as first lines of defense against mucosal infection (15–17). Heterologous epitopes derived from LCMV or influenza proteins expressed by a recombinant mouse CMV (MCMV) can give rise to inflationary T_{EM} cells that are protective in a vaccine setting (19). Furthermore, a rhesus CMV vaccine-vector engineered to express SIV proteins promoted far superior protection by inducing cells with a T_{EM} phenotype compared to T_{CM} of the same specificity generated by different means (18). Consequently, even though it is not fully understood why some CMV orf-derived peptides induce T_{EM} cells, there is a movement to utilize the fact that CMV induces large populations of these cells to clinical advantage. In this study, we address two fundamental questions regarding CMV-specific T_{EM} cells: (1) are naïve recent thymic emigrants required to maintain the numbers of these cells, and (2) does persistent salivary gland replication play a role in their generation and/or maintenance.

After thymectomy, the number of circulating naïve T cells gradually decreases due to the lack of recent thymic emigrants (RTE) (T½ naïve T cells $\sim$49 d) (20–24). To test how this might affect the CMV-specific T-cell pools, female young adult (6–8 wk) C57BL/6 (B6) mice were thymectomized or sham operated, and after a $\sim$10-d recovery period were infected intraperitoneally (IP) with 5×10^4 PFU of salivary

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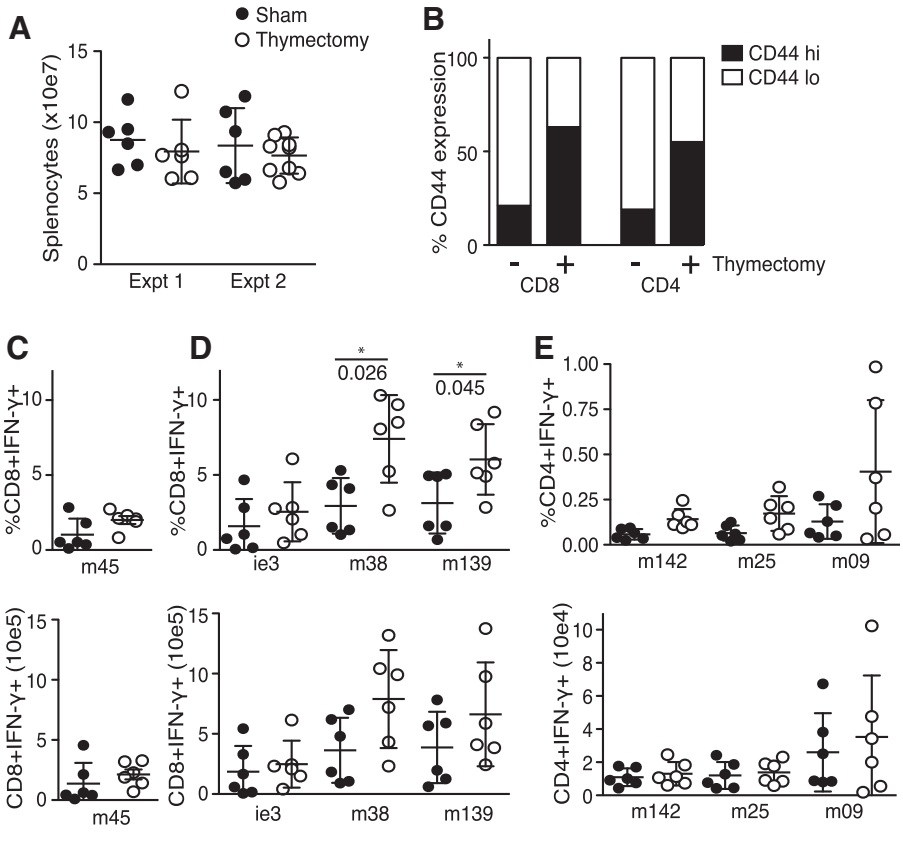
gland propagated MCMV-Smith (ATCC VR-194) for ~100 d. At that time, splenocytes were quantified and re-stimulated with 2 μg/mL of MCMV orf-derived peptides in the presence of 1 μg/mL brefeldin A for 5 h (CD8 T cells), or incubated first with peptide for 3 h and then for an additional 5 h in the presence of golgi-plug (BD, CD4 T cells). Virus-specific CD8 and CD4 T cells were identified by flow cytometry for intracellular IFN-γ expression. Re-stimulation with BFA alone resulted in <0.4% IFN-γ positive cells, and golgi-plug alone <0.03%. MCMV epitope-specific inflationary CD8 memory T cells (peptides derived from the MCMV proteins $M38_{316–323}$, $m139_{419–426}$, and $IE3_{416–423}$), and stable CD8 memory T cells ($M45_{985–993}$), as well as CD4 memory T cells ($M25_{409–423}$, $m142_{24–38}$, and $m09_{133–147}$), were measured in a blinded fashion, segregating the groups after examining sacrificed mice for the presence of a thymus (3). The percentages of M45-specific CD8 T cells trended slightly higher in the spleens of thymectomized animals (Fig. 1C), but this was less dramatic when comparing total cell numbers (Fig. 1C), as spleens of thymectomized animals showed modestly reduced cellularity (Fig. 1A). The inflationary CD8 T populations specific for M38, M139, and IE3 consistently trended higher in thymectomized animals (Fig. 1D and F), while only the M09-specific CD4 T-cell response was slightly increased, leaving M142- and M25-specific CD4 T cells largely unaffected by the thymectomy (Fig. 1E).



F

	Expt 1		Expt 2	
	sham	thy	sham	thy
m45	1.4 (1.7)	2.1 (1.0)	1.0 (0.3)	2.0 (1.0)
m38	3.6 (2.7)	7.9 (4.1)	3.1 (2.0)	4.8 (3.9)
m139	3.9 (3.0)	6.6 (4.3)	2.6 (0.7)	3.1 (3.0)
IE3	1.9 (2.1)	2.5 (2.0)	1.0 (0.4)	1.9 (1.6)

CD8+ T cells (x10e5)

FIG. 1. The effect of thymectomy on CMV-specific memory T cells. Thymectomized (open circles) or sham operated (solid circles) wild-type (wt) B6 mice were MCMV infected. (**A**) On day 100, mice were sacrificed and splenocyte numbers were determined. (**B**) Splenocytes were stained for CD4, CD8, and CD44 expression. (**C**, **D**, and **E**) Splenocytes were stimulated *in vitro* with the indicated MCMV orf-derived peptides, and virus-specific CD8 (**C** and **D**) or CD4 (**E**) T cells were identified by flow cytometry for expression of IFN-γ. Results are displayed as percentages (top row) or numbers (bottom row) of peptide-specific CD8 or CD4 T cells/spleen, and are shown as mean $\pm$ SD of 6 mice/group. (**F**) Mean numbers and SD of epitope-specific CD8 T-cells/spleen at day 100 in two different experiments. CD4 T-cell determinations were done once. Statistical analysis was performed using the two-way Mann-Whitney *U* test.

Although HCMV-specific CD4 T cells may undergo memory inflation in infected patients (25), almost nothing is known about the phenotype of these cells. In MCMV-infected B6 mice, M142-specific CD4 T-cell responses show a canonical kinetic, peaking at ~day 8 of primary infection, and contracting ~90% by days 15–20. In contrast, M09-specific CD4 T cells only become detectable by ~day 15 of infection, peak at ~day 35, and then contract very slowly over the following months (26). However, even given the vastly different time course for development of these CD4 T-cell responses to MCMV, neither was found to depend upon RTE for their expansion and/or maintenance.

During acute infections with vaccinia virus and LCMV Armstrong, the size of the CD8 T_{CM} pool is maintained primarily by low-level, cytokine-driven homeostatic proliferation after virus is cleared (i.e., IL-7 and IL-15), and does not require either "lingering" viral antigen or newly-primed, naïve T cells (27,28). Consequently, the fact that M45-specific CD8 memory T-cell numbers do not decrease in thymectomized animals was not entirely surprising (10,29). In a thorough series of T-cell transfer experiments, Snyder *et al.* estimated the half-life of circulating MCMV-specific CD8 T_{EM} at ~45–60 d in both naïve and infected animals, and also proposed that the overall maintenance of this population requires both sporadic expansion and the contribution of RTE (30). Our experimental time course was equivalent to ~2 half-lives of MCMV-specific CD8 T_{EM}, and therefore we anticipated a potential reduction in their numbers following thymectomy (20,22–24). Instead, however, we observed a slight expansion of MCMV-specific inflationary T-cell

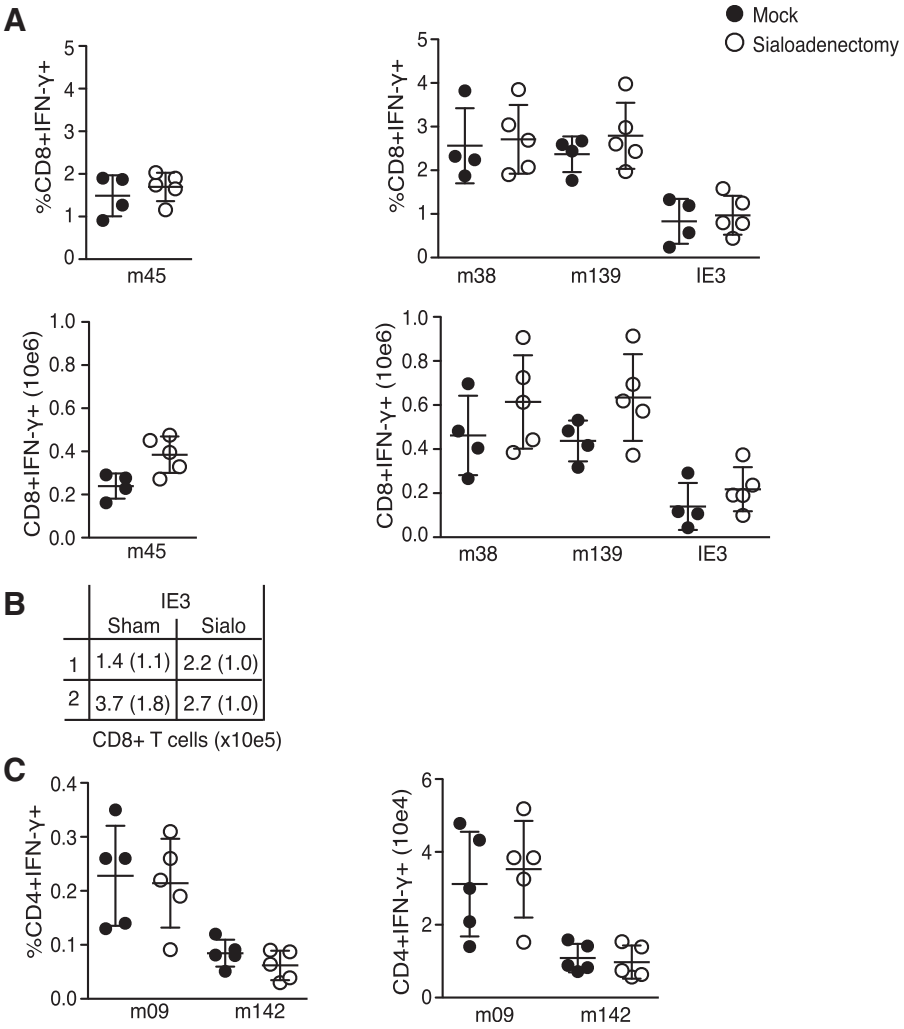


FIG. 2. Salivary gland replication is not required for the development of CMV-specific memory T cells. (**A** and **C**) WT B6 mice were sialoadenectomized (open circles) or sham operated (solid circles) and MCMV infected. (**A**) One hundred days later, splenocytes were stimulated with the indicated viral peptides and virus-specific CD8 T cells were identified by flow cytometry. Results are displayed as percentages or numbers of peptide-specific CD8 T cells/spleen, and are shown as mean ± SD of 6 mice/group. (**B**) Mean numbers and SD (in parentheses) of IE3-specific CD8 T cells/spleen at day 100 and are shown as mean ± SD of 5 (top row, Expt 1) or 6 (bottom row, Expt 2) mice/group. (**C**) Splenocytes were stimulated with the indicated viral peptides, and MCMV-specific CD4 T cells expressing IFN-γ were identified by flow cytometry. Results are displayed as percentages or numbers of CD4 T cells/spleen. Statistical analysis was performed with the two-way Mann-Whitney *U* test.

numbers. In turn, the total circulating T-cell pool was composed of a significantly higher percentage of cells that displayed a "memory" phenotype 100 d after operation (~20% or ~3×10e6 $CD44^{hi}CD8+$ T cells in sham operated versus ~60% or ~6×10e6 $CD44^{hi}CD8+$ T cells in thymectomized mice; Fig. 1B). The apparent insensitivity of inflationary CD8 T cells specific for MCMV antigens to thymectomy differs from mice infected with LCMV clone 13 or polyoma virus, in which CD8 T_{EM} and $CD62^{lo}$ memory populations are reduced, respectively (31,32). Consistently, memory T-cell populations specific for these pathogens show phenotypic differences from inflationary cells generated during MCMV infection. An example is that CD8 T_{EM} that arise during LCMV clone 13 infection express high levels of PD-1 and become functionally exhausted, whereas this does not generally occur for inflationary CD8 T_{EM} in either human or mouse CMV infection (33–35). One likely possibility is that the level, locale, and/or duration of antigen expression during the persistent phase of infection is a differentiating factor in regulating both the maintenance and phenotype of these cells during diverse infections. In the case of LCMV clone 13 and polyoma virus, as well as during bacterial infection with *Leishmania major* and Bacille Calmette-Guerin, the maintenance of these memory CD8 T cells is critically dependent upon continued exposure to antigen (31,36–38). However, during MCMV infection the precise role that prolonged antigen expression plays in maintaining the inflationary CD8 T_{EM} remains incompletely understood.

High levels of MCMV replication continue in the salivary gland (SG) for several weeks/months, suggesting this organ may contribute to shaping the virus-specific T-cell memory pool. To address this, a sialoadenectomy was performed to remove the SG, a minimally-invasive surgery from which the mice recover quickly with no alterations in weight or feeding behavior (39). Wild-type B6 mice were sialoadenectomized or sham operated on day 0 and subsequently infected IP with 5×10^4 PFU of MCMV. One hundred days later splenocytes were analyzed as already described.

M45-specific CD8 T_{CM} numbers were comparable in the operated and sham groups. Somewhat surprisingly, CD8 T cells specific for peptides derived from M38, M139, and IE3 were also equivalent, with an apparent trend towards being slightly higher in number, although not reaching statistical significance (Fig. 2A and B). This result indicates that replication in the SG does not impact the development of either stable or inflationary MCMV-specific CD8 T-cell memory populations. Next, CD4 T-cell responses specific for peptides derived from MCMV orfs M142 and M09 were analyzed (Fig. 2C) (26). CD4 T cells are essential for the eventual control of MCMV in the SG (40), and are localized to this organ during times of replication, suggesting some of these cells may require SG-expressed antigen for their priming, expansion, and/or maintenance. However, similarly to what was observed for MCMV-specific CD8 T cells, neither the numbers nor the percentages of M142- or M09-specific CD4 T cells were altered in sialoadenectomized mice.

The surface marker phenotype of T_{EM} cells suggests a recent encounter with peptide-MHC complex (41); however, the specific requirements for maintaining this phenotype are still incompletely understood, and almost certainly vary based on the specific pathogen or vaccination regimen, as already discussed. While the maintenance of the total CMV-specific CD8 T_{EM} pool over time is thought to involve expression of persistent viral antigen (30), spread-defective MCMV mutants can induce both antibody and T-cell responses to some degree (42,43). Snyder *et al.* have recently shown that the immunodominance hierarchy of CD8 T cells is similar between wild-type and a spread-defective mutant MCMV at early times of infection, although the overall magnitude was lower (42,43). In turn, Mohr *et al.* demonstrated that a spread-defective MCMV mutant provides protection against viral challenge up to 20 wk later, indicating that the initial populations of cells infected by MCMV can function as efficient "antigen sources" to provide lasting immunity (42,43). However, neither of these studies analyzed the effect that these mutant viruses had on the inflationary populations of CD8 T_{EM} specific for MCMV antigens.

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Author Disclosure Information

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