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Buffered memory: a hypothesis for the maintenance of functional, virus-specific CD8(+) T cells during cytomegalovirus infection.

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**Buffered Memory: A hypothesis for the
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1 **Abstract:**

2 Chronic infections have been a major topic of investigation in recent years, but
3 the mechanisms that dictate whether or not a pathogen is successfully controlled
4 are incompletely understood. Cytomegalovirus (CMV) is a herpesvirus that
5 establishes a persistent infection in the majority of people in the world. Like other
6 herpesviruses, CMV is well controlled by an effective immune response and
7 induces little, if any, pathology in healthy individuals. However, controlling CMV
8 requires continuous immune surveillance and thus, CMV is a significant cause of
9 morbidity and death in immune compromised individuals. T cells in particular play
10 an important role in controlling CMV and both CD4+ and CD8+ CMV-specific T
11 cells are essential. These virus-specific T cells persist in exceptionally large
12 numbers during the infection, traffic into peripheral tissues and remain functional,
13 facts that make CMV an attractive vaccine vector for driving “CMV-like” T cell
14 responses against recombinant antigens of choice. However, the mechanisms by
15 which these T cells persist and differentiate while remaining functional are still
16 poorly understood and we have no means to promote their development in
17 immune compromised patients at risk for CMV disease. In this review, I will
18 briefly summarize our current knowledge of CMV-specific CD8+ T cells and
19 propose a mechanism that may explain their maintenance and preservation of
20 function during chronic infection.

21

22 **Introduction: CMV latency and persistence**

1 Herpesviruses are large DNA viruses that evolved an estimated 180 to 220
2 million years ago and have been co-speciating with their hosts ever since [1],
3 resulting in a very finely tuned host-pathogen balance. Herpesviruses establish
4 persistent or latent infections and induce little, if any, pathology in healthy hosts,
5 thanks largely to sustained, functional immune responses. Cytomegalovirus
6 (CMV) is a ubiquitous member of the β -subfamily of herpesviruses. Primary CMV
7 infection typically occurs in childhood and is usually asymptomatic. After an
8 active phase during which infectious virions are shed, CMV is controlled to levels
9 that are at, or just below what is readily detectable. Despite substantial
10 investigation, CMV remains an enigmatic virus and much of the viral behavior
11 during the persistent/latent phase of infection remains a mystery. However,
12 persistent CMV infection is characterized by periods of viral reactivation and
13 shedding, and constant immune surveillance is vital to keep CMV under control.
14
15 Our understanding of the ongoing host-virus interaction that keeps CMV in check
16 is somewhat limited, in part because the cells harboring latent virus or presenting
17 viral antigen during persistent infection are still incompletely defined. Several
18 groups have identified latent human CMV (HCMV) in hematopoietic progenitor
19 cells or monocyte/macrophages (reviewed in [2]). However, work in mice
20 suggests that hematopoietic cells are only part of the picture. Murine CMV
21 (MCMV) is a natural mouse pathogen and the homologue of human CMV. Both
22 viruses induce similar infections and are controlled by similar immune responses.
23 Like HCMV, MCMV is also thought to establish latency in macrophages [3, 4].

1 However, after primary infection, MCMV viral DNA is cleared from the blood and
2 bone marrow but not the salivary glands, lungs or spleen [5] suggesting that
3 relatively little of the MCMV genome is maintained by blood-derived cells.
4 Moreover, in the spleens of chronically infected mice, viral reactivation in explant
5 cultures was most easily detected in the stromal fraction and was unaffected by
6 depletion of lymphocytes or MHC class II bearing cells [6], again suggesting a
7 major non-hematopoietic site of MCMV latency. Endothelial cells have been
8 identified as one such non-hematopoietic site of MCMV latency [3, 7] and it is
9 likely that non-hematopoietic sites of latency, particularly endothelial cells [8], are
10 important for HCMV as well. Additional sites of latency are also likely and the
11 degree to which each contributes to the maintenance of the latent viral pool will
12 be of significant interest.

13

14 Initially, there was substantial debate about whether CMV remained persistent
15 (i.e. constantly replicating at a low rate) or achieved true molecular latency at late
16 times post infection. While it is now generally accepted that CMV achieves true
17 molecular latency, it is also evident that the virus undergoes frequent
18 reactivation. Landmark experiments by Reddehase and colleagues showed that
19 despite ubiquitous viral DNA in the lungs of mice infected for 12 months, viral
20 RNA in the lungs was expressed in a patchwork pattern [9]. These data
21 suggested that viral reactivation occurred frequently, but only in a fraction of
22 infected cells at any given time. This group further showed that the patchwork
23 viral reactivation rarely led to the production of infectious virions [10]. In order to

1 replicate, CMV transcribes its genes in an ordered cascade that begins with
2 immediate early (IE) genes followed by early (E) genes, DNA replication and
3 ultimately late (L) genes that encode structural virion components. While the
4 patchwork expression of viral RNA in the lungs frequently included IE genes, as
5 shown previously, E and L genes were undetectable [10]. Similarly, infectious
6 virus was undetectable even with techniques that allowed detection of as few as
7 5 viral genomes [11]. Importantly, this group has also shown that MCMV-specific
8 CD8+ T cells block the progression of viral gene expression during reactivation
9 [12], indicating that immune surveillance contributes to the suppression of virion
10 production. These data show that CMV is present in a latent state and that
11 reactivation occurs frequently, but also that reactivation is usually aborted or
12 blocked prior to complete gene expression and production of virions.

13

14 Overall, the data suggest that CMV is constantly pressuring the immune system.
15 Compromising the immune system of CMV-infected individuals frequently results
16 in disseminated viral replication and substantial morbidity and death. Many arms
17 of the immune system contribute to viral control. Interferons, natural killer (NK)
18 cells, CD4+ T cells, CD8+ T cells and antibody all play a recognized role in
19 controlling viral replication and spread [13]. Once latency is established, viral
20 reactivation seems to be largely controlled by the combined efforts of NK cells,
21 CD4+ and CD8+ T cells [14], though the specific contributions of these cells
22 remain more obscure. CD4+ T cells play a direct role in controlling viral
23 replication, likely by cytokine secretion or cytotoxicity [15-18]. In addition, CMV-

1 specific CD4+ T cells may help CMV-specific CD8+ T cell responses [19-21].
2 CD8+ T cells for their part, have been shown to directly limit viral reactivation in
3 healthy mice [12] and can control viral replication in otherwise
4 immunocompromised humans and mice [22-24].

5

6 **Memory Inflation: The CD8+ T cell response to CMV.**

7 CMV-specific CD4+ and CD8+ T cells are present in staggering numbers during
8 the persistent infection in healthy people, comprising approximately 5% of all
9 circulating T cells and 10% of all memory T cells in the average adult host [25].
10 Strikingly, the frequencies of virus-specific CD8+ T cells increase over the course
11 of infection. This slow increase in the numbers or frequency of virus-specific
12 CD8+ T cells has been referred to as “memory inflation” and occurs in both
13 MCMV infected mice and HCMV infected people [26-33]. For the purposes of this
14 review, “memory inflation” is used in its most basic sense: to describe the
15 increasing numbers and frequency of CMV-specific CD8+ T cells over time. The
16 general assumption has been that these “inflationary” CD8+ T cell populations
17 are the direct result of ongoing viral activity. How these inflationary cells develop,
18 how frequently they are stimulated and how they respond to each stimulation
19 event remain open questions.

20

21 Memory inflation of virus-specific CD8+ T cells is, in many ways, unique to CMV
22 infection. It is generally agreed that the majority of inflationary CMV-specific T
23 cells resemble extensively differentiated, but functional effector cells. This has

1 been investigated in both HCMV-infected people and MCMV-infected mice by
2 many laboratories [26, 28, 30, 32-45] and an interesting picture has emerged.
3 Most CMV-specific T cells express perforin and granzyme, they can kill antigen-
4 bearing target cells, and they can secrete IFN- γ and TNF- α , but only very little IL-
5 2. However, many of these cells bear phenotypic hallmarks of repeated antigen
6 stimulation. For example, most CMV-specific T cells lack expression of the co-
7 stimulatory molecules CD27 and CD28, as well as L-selectin (CD62L) and the
8 chemokine receptor CCR7 that allow for access to lymph nodes. Likewise, many
9 CMV-specific effectors have reduced expression of the receptors for IL-7 and IL-
10 15, cytokines important for homeostatic maintenance of memory T cells. In
11 humans, many of these T cells express the memory-marker CD45RO, but a
12 subset, considered to be the most terminally differentiated, express CD45RA, a
13 marker typically expressed by naïve T cells. CMV-specific CD8⁺ T cells with this
14 phenotype have short telomeres, indicating an extensive proliferative history [46].
15 Importantly, the frequency of these cells directly correlates with the amount of
16 virus at the peak of infection [37].

17

18 In addition to the phenotype described above, CMV-specific inflationary T cells
19 also upregulate a variety of NK-associated receptors including the activating
20 receptor NKG2D and the inhibitory receptors NKG2A and KLRG-1 along with
21 CD57 and CD85j in humans [31, 33, 47-50]. Expression of KLRG-1 in particular
22 is associated with repeated antigen stimulation [51], although it is also
23 upregulated by CD8 T cells during acute viral infections [52]. It is unclear how

1 CMV-specific T cells with this phenotype will be affected by this combination of
2 reduced co-stimulatory molecule expression and increased inhibitory molecule
3 expression. One possibility is that they have a restricted or decreased
4 proliferative capacity after stimulation. Indeed, expression of the inhibitory
5 molecules CD57 and KLRG-1, has been associated with reduced or absent
6 proliferative potential (senescence) [47, 48, 53-56]. However, CMV-specific
7 inflationary T cells do not appear to be senescent in healthy adult hosts. Although
8 CMV-specific T cells undergo relatively little proliferation at steady state during
9 chronic CMV infection [20, 28, 32, 38, 57, 58], T cells with the phenotype
10 described can be induced to divide by the correct combination of stimuli. In
11 particular, stimulating inflationary T cells through both the T cell receptor (TCR)
12 and common γ -chain cytokine receptors results in cell division as does the
13 simultaneous stimulation of both the TCR and 4-1BB (CD137, a member of the
14 TNF-receptor family) [42, 43, 59]. Likewise, MCMV-specific inflationary cells
15 divide after MCMV challenge in an adoptive transfer model [33]. It seems likely
16 that additional co-stimulatory pathways will also push these inflationary T cells
17 into cell cycle. Thus, CMV-specific inflationary T cells seem to be repeatedly
18 stimulated, but functional effectors during chronic infection, at least in healthy
19 adults, but require specific combinations of stimuli to enter cell cycle. For the
20 remainder of this article, I will refer to CMV-specific inflationary cells with this
21 phenotype as effector T cells (T_{EFF}) to distinguish them from small populations
22 that retain a more memory-like phenotype (described below).

23

1 **CMV-specific “memory-like” T cells**

2 The accumulation of the CMV-specific T cells with the phenotype described
3 above is contrasted by a persistence of other T cells with a more memory-like
4 phenotype. Many of these memory-phenotype cells do not recognize the same
5 antigens as the T_{EFF} cells that undergo memory inflation. CMV infection in both
6 mice and humans elicits T cells specific for a broad array of antigens [25, 60] and
7 many of these T cells contract after acute infection and persist at low levels
8 thereafter [30, 32, 33]. These “non-inflationary” T cells tend to develop a more
9 classical memory phenotype (i.e. retention of co-stimulatory molecules and
10 cytokine receptors and an absence of inhibitory receptors), suggesting that they
11 may not be repeatedly stimulated despite the persistent infection. Recent data
12 have suggested that these non-inflationary T cells may recognize peptides that
13 depend on the immunoproteasome for production [61]. Since non-professional
14 antigen presenting cells do not constitutively express the immunoproteasome,
15 these peptides may be rarely produced during chronic infection. However, there
16 are also small subsets of cells that retain a memory-like phenotype despite
17 sharing peptide specificity with large numbers of T_{EFF} cells, which can not be
18 explained by limited peptide presentation.

19

20 Using the mouse model of MCMV infection in C57BL/6 mice, we and others have
21 begun dissecting the array of CD8+ T cell responses elicited by MCMV over time.
22 We typically analyze T cells specific for 5 different viral antigens: M45, M57,
23 m139, M38 and IE3. Schematics of the kinetics of these T cell responses are

1 illustrated in Figure 1a. T cells specific for M45 and M57 contract after acute
2 infection and develop a memory-like phenotype at late times post infection,
3 illustrated here by high levels of CD127 (IL-7R α) expression and little KLRG-1
4 expression (Figure 1b and [30, 33]). These are prototypical examples of non-
5 inflationary T cells. In contrast, inflationary T cells specific for the viral antigens
6 m139, M38 and IE3 accumulate after MCMV infection, each with distinct kinetics
7 (Figure 1a). These cells mostly develop a T_{EFF} phenotype, illustrated here by a
8 downregulation of CD127 and expression of KLRG-1 (Figure 1b). However, there
9 is always a small subset of inflationary T cells (m139-, M38- or IE3-specific) that
10 retain CD127 expression and do not upregulate KLRG-1 (upper left quadrants in
11 Figure 1b). These memory-phenotype inflationary cells generally comprise less
12 than 10-15% of the total antigen-specific pool (Figure 1c) and there is only a little
13 data hinting at their functional capacity: HCMV-specific T cells that are memory-
14 phenotype tended to expand better than T_{EFF} HCMV-specific T cells after
15 allogeneic stem cell transplantation [62] and our data [33] implied that MCMV-
16 specific CD8⁺ T cells with a memory-phenotype expanded more robustly than
17 T_{EFF} cells with the same specificities after viral challenge. If memory is defined by
18 the speed and magnitude of recall responses, these data suggest that the
19 memory-phenotype cells are more “memory-like” than their T_{EFF} counterparts,
20 although more work is needed to define these differences.

21

22 The mechanisms dictating whether individual T cells with a given specificity will
23 remain memory-like or differentiate into effectors during chronic infection are

1 currently unclear. Analyses of HCMV-specific CD8+ T cells indicate that
2 individual T cell clones can be found within both T_{EFF} and memory-like
3 populations [63], indicating that segregation or selection into either subset is not
4 dependent on a particular T cell receptor. Are T_{EFF} cells, as their phenotype may
5 suggest, the only cells that respond to viral antigens during chronic infection? If
6 memory-like cells are stimulated, what is their fate? Likewise, what is the fate of
7 T_{EFF} cells stimulated during chronic infection? More precise measurements of the
8 development, maintenance, behavior and functional potential of these two
9 inflationary T cell subpopulations are needed to begin addressing these
10 questions. However, for the remainder of this article, I would like to consider the
11 idea that both of these populations work in tandem to enable the maintenance of
12 life-long, functional CMV-specific immunity.

13

14 **Short or long-lived CMV-specific CD8+ T cells.**

15 Given the absence of substantial proliferation within CMV-specific inflationary
16 populations [20, 28, 32, 38, 57, 58], there are two basic hypotheses describing
17 the homeostasis and persistence of inflationary T_{EFF} cells. The first suggests that
18 CMV-specific T cells are long-lived, resting effectors (discussed in [64]). This
19 model states that CMV-specific T cells are functional effectors that will circulate
20 for long periods of time and suppress reactivating or replicating virus when it is
21 encountered. These T_{EFF} cells may not undergo much or any cell division after
22 each stimulation event unless the conditions are ideal (e.g. appropriate co-
23 stimulation and/or cytokine signals). Supporting this model is the evidence that

1 individual CMV-specific clones of cells can persist for several years [63, 65]. In
2 addition, in humans, CD45RA⁺ CMV-specific T cells only emerge after acute
3 infection has been resolved [45]. As restimulation of these cells *in vitro* promotes
4 re-expression of CD45RO [44], these data may imply that the CD45RA⁺ subset
5 of CMV-specific T cells are resting and have not been recently stimulated. Thus,
6 in this model, a long half-life and only occasional cell division, driven by a specific
7 combination of stimuli, maintain the population.

8

9 The alternate hypothesis states that CMV-specific T cell populations are
10 extremely dynamic, comprising mostly short-lived effector cells that are
11 continually replaced as they die, thus maintaining the population as a whole
12 (Figure 2). We proposed this model because inflationary MCMV-specific T cells
13 from the spleens of chronically infected mice failed to sustain themselves after
14 adoptive transfer into either naïve or chronically infected animals [33]. In the
15 presence of virus, CMV-specific T cells underwent some cell division, but not
16 enough to maintain their numbers, and the transferred populations slowly
17 decayed with a half-life of approximately 1 to 2 months. Thus, we proposed that
18 the majority of inflationary T cells were actually short-lived effectors, similar in
19 phenotype (CD127^{low}, KLRG-1^{pos}, Figure 1b) and function to short-lived effector
20 cells elicited by acutely cleared infections [66]. Consistent with this model, it was
21 shown that CD127^{low} (T_{EFF}) inflationary T cells express less of the anti-apoptotic
22 molecule Bcl-2 than cells with the same specificity that retained CD127
23 expression (memory-like cells) [32]. This could imply that T_{EFF} inflationary cells

1 are more prone to apoptosis than memory-like inflationary T cells. Moreover,
2 recent evidence has shown that HCMV elicited CD45RA+, CD4+ T cells (the
3 most differentiated) are susceptible to apoptosis after antigen-stimulation *in vitro*
4 [67]. Although still unclear, it is interesting to speculate that both CD4+ and CD8+
5 CMV-specific T cell populations may be dominated by short-lived populations.

6

7 **Short-lived T_{EFF} cells might be repeatedly produced by more memory-like T**
8 **cells.**

9 In our model, there must be a continuous source of new short-lived effector cells
10 to replace those that die in order for the inflationary populations to be maintained
11 (Figure 2). Our data showed that naïve T cells can be recruited during chronic
12 infection to provide some new effector cells [33] and a similar model was
13 proposed to account for maintenance of murine polyomavirus-specific T cells [68,
14 69]. However, we further showed that insufficient numbers of T cells were
15 recruited from the naïve pool to account for maintenance of the MCMV-specific
16 inflationary populations [33]. Instead our data suggested that a population of T
17 cells produced early in the infection could maintain the inflationary T cell pool.
18 Thus, T cells transferred only 7 days after infection into infection matched
19 recipients could provide long-term maintenance of the donor populations. We
20 interpreted these data to suggest that some sort of memory or memory-like
21 population was generated during primary infection and then repeatedly
22 stimulated to produce new short-lived T_{EFF} cells *via* clonal expansion (Figure 2).
23 As long as the production rate of new T_{EFF} cells exceeded their rate of loss, the

1 result would be memory inflation. Once the production rate of T_{EFF} cells equaled
2 the rate of loss, the population would persist at an elevated, but stable level.
3
4 Since clones of memory-like cells would be responsible for repeatedly producing
5 T_{EFF} cells, this model is consistent with the fact that individual HCMV-specific
6 $CD8+$ T cell clones are found in both T_{EFF} and memory-like subsets [63]. In
7 addition, this model could explain the maintenance of certain T cell clones over
8 many years: a small pool of memory-like cells would produce T_{EFF} cells with a
9 limited clonal composition and individual clones would develop into effectors
10 repeatedly over time. If some clones were present at higher frequencies in the
11 memory-like pool, or possessed T cell receptors within an ideal avidity range,
12 these would undoubtedly be selected into the T_{EFF} pool more frequently. Thus,
13 competition between the memory clones could explain the selection of some but
14 not all clones over time. Interestingly, a study by Day et. al. [65] showed that
15 within a few weeks of primary HCMV infection, a relatively small number of T cell
16 clones were selected into the $CD8+$ T cell population specific for a given peptide
17 and that the relative frequency of individual clones fluctuated with time. Although
18 fluctuations in clonal frequency from time point to time point might represent
19 more or less comprehensive blood sampling, such fluctuations would also be
20 predicted by a model in which different clonal expansions contribute to the
21 maintenance of the whole population.

22

1 If memory-like T cells are responsible for clonal expansions that support a
2 circulating T_{EFF} pool, where might such memory clones reside? In our
3 experiments [33], CMV-specific populations from the spleen were unable to
4 sustain themselves. Thus, any memory population in the spleen was either
5 incapable of supporting memory inflation after adoptive transfer, or was
6 transferred in insufficient numbers to support the donor antigen-specific pool.
7 Alternative locations such as the bone marrow or lymph nodes may harbor
8 increased numbers of the relevant cell population. Interestingly, while the
9 frequency of memory-phenotype inflationary cells was greater in the spleen and
10 bone marrow relative to the blood, there was little or no difference between the
11 bone marrow and the spleen (Figure 1b and c). It remains formally possible that
12 bone marrow resident memory cells are functionally distinct from spleen resident
13 cells however, requiring further experiments to test the functional properties of
14 these subsets.

15

16 It must be noted however, that because we used adoptive transfer experiments
17 to test the persistence of MCMV-specific T cells, there is an alternative
18 explanation for our results: CMV-specific T cells (either T_{EFF} cells, memory-like
19 cells or both) may require a specific niche in which to survive. Thus, when we
20 transferred MCMV-specific T cells into chronically infected mice, the niches in the
21 host may have been filled with the host's own MCMV-specific T cells, thereby
22 excluding the donor cells. It is possible that this niche is not filled early
23 (explaining our ability to transfer self-sustaining populations at day 7 post

1 infection) but could fill over time with memory inflation. Studies specifically
2 designed to investigate the survival and engraftment of transfused MCMV-
3 specific T cells are required.

4

5 **CMV-specific T cells for adoptive therapy:**

6 The difference between these two models (short- vs. long-lived inflationary T
7 cells) may have a significant impact on the development of adoptive
8 immunotherapy for immune compromised patients. There are no therapies for
9 boosting CMV-specific T cell responses in immune compromised patients.

10 Therefore, several groups have been working to develop methods for the
11 isolation and passive transfusion of CMV-specific T cells from healthy donors into
12 immune compromised recipients at risk for CMV disease (recently reviewed in
13 [70]). Typically, CMV-specific T cells have been isolated based on their ability to
14 secrete IFN- γ after antigen-stimulation although in some cases, T cell clones or
15 directly sorted, tetramer-binding T cells have been used. These procedures have
16 yielded some promising results, but are still experimental and costly and
17 questions still remain about how to ensure persistence of effective donor T cells
18 after transfusion. Indeed, transfused T cells can only provide protection for as
19 long as they survive. If the majority of isolated T cells are short-lived and
20 continuously replaced, the challenge may be to transfer a self-sustaining
21 population that can control CMV over long periods of time. Interestingly, the
22 Riddell group, pioneers of this therapeutic approach to mitigate CMV infection
23 [19, 24], have shown using a non-human primate model of CMV infection, that T

1 cell clones derived from “central memory” (CD28+, CD95+) but not more
2 differentiated “effector memory” (CD28-, CD95+) subsets persist after adoptive
3 immunotherapy [71]. These data support the idea that CMV-specific T_{EFF} cells
4 may be less effective than more memory-like populations over long periods of
5 time. If we can develop an understanding of precisely how CMV-specific
6 populations are generated and sustained, it may become possible to transfuse
7 more protective or longer lasting CMV-specific populations or even to promote
8 these cells directly in immune compromised patients.

9

10 **T cell exhaustion: A paradigm for several chronic infections.**

11 Aside from the homeostasis of CMV-specific T_{EFF} and memory-like T cells, their
12 maintenance of effector function is also remarkable. The development of
13 dysfunctional CD8+ T cells, generally called T cell “exhaustion”, occurs during
14 several other chronic infections including Hepatitis C, Hepatitis B, HIV, and in the
15 mouse models of chronic lymphocytic choriomeningitis virus (LCMV) infection
16 (recently reviewed in [72]). Exhaustion during these infections is typically
17 characterized by a progressive loss in the ability of CD8+ T cells to produce
18 cytokines (first IL-2, then TNF- α and eventually IFN- γ), as well as to survive,
19 proliferate and kill targets. However, there is little evidence of such exhaustion
20 occurring during CMV infection except in immune compromised patients with
21 high titers of replicating CMV [73, 74]. Indeed, exhaustion appears to be a
22 defined molecular program induced in T cells by some chronic infections [75] and
23 gene expression profiles of HCMV-specific T cells do not conform to this

1 molecular signature [38]. Notably, during chronic CMV infection in healthy
2 humans and mice, CMV-specific T cells do not upregulate PD-1 [20, 38], one
3 inhibitory molecule that is strongly associated with antigen-driven T cell
4 exhaustion. Indeed, even in the absence of CD4⁺ T cell help, an environment
5 that accelerates and exacerbates CD8⁺ T cell exhaustion after chronic LCMV
6 infection [76, 77], only relatively mild CD8⁺ T cell dysfunction develops after
7 MCMV infection, despite the additional fact that MCMV is never fully controlled in
8 the absence of CD4⁺ T cells [20, 21].

9

10 One explanation for the lack of exhaustion during CMV infection is that CMV is a
11 “smoldering” infection [72, 76], with ongoing viral replication never reaching
12 levels that can drive T cell dysfunction in healthy hosts. In line with this, T cell
13 exhaustion in mice infected with LCMV-clone 13, a virus that replicates to high
14 titers, is associated with continuous antigen-driven T cell proliferation [78] while
15 CMV-specific T cells undergo relatively little proliferation during chronic infection
16 in both humans and mice [20, 28, 32, 38, 57, 58]. As mentioned above, this is not
17 because CMV-specific T cells are unable to proliferate. The correct combination
18 of stimuli will drive even T_{EFF} phenotype cells into cell cycle [42, 43, 59]. Thus,
19 either CMV-specific T cells rarely encounter antigen, or they are frequently
20 stimulated by antigen, but rarely driven into cell cycle. Perhaps the expression of
21 inhibitory molecules by T_{EFF} cells and their dependence on cytokines and/or
22 certain co-stimulatory signals limits cell division and thus, exhaustion. This may
23 be especially true since many T_{EFF} cells can be found within non-lymphoid

1 organs [26, 28, 32], where they may be exposed to much of the antigen
2 produced by reactivating virus [5, 79], but not the cytokines or co-stimulatory
3 molecules necessary for cell division [42, 43, 59]. Consistent with this idea,
4 continuous MCMV replication in CD4⁺ T cell deficient mice was necessary to
5 support the size of inflationary CD8⁺ T cell populations [21], but did not result in
6 markedly increased proliferation by inflationary CD8⁺ T cells [20]. Thus, even in
7 this scenario, where it was clear that MCMV-specific CD8⁺ T cells responded to
8 antigen from a continuously replicating virus, they still underwent relatively limited
9 cell division. Therefore, proliferation may be a poor indicator of how frequently
10 MCMV-specific T cells encounter antigen and it is possible that the T_{EFF} state,
11 with its specific requirements for entering the cell cycle, may itself prevent the
12 development of exhaustion, regardless of how frequently these cells are
13 stimulated.

14

15 **Buffered Memory: A hypothesis to explain the preservation of functional**
16 **memory CD8⁺ T cells during persistent CMV infection**

17 Regardless of how the CMV-specific T_{EFF} cells remain functional, an additional
18 mystery is the preservation of functional memory-like cells during chronic MCMV
19 infection. Perhaps the T_{EFF} cells, by recognizing the majority of viral antigen and
20 controlling the majority of viral gene expression, simply prevent the memory-like
21 T cells from being overstimulated during persistent CMV infection. If T_{EFF} cells
22 suppress viral reactivation in the tissue effectively, their presence should limit the
23 antigen burden placed on the more memory-like cells, thus preventing memory-

1 like cells from responding to all but a fraction of viral antigen (Figure 3). In this
2 way, perhaps the T_{EFF} cells act as a buffer for the memory-like cells and only
3 occasional “leaks” of viral antigen or perhaps antigen expression in a particular
4 environment (e.g. a specific cell type or organ) stimulate the memory-like T cells.
5 Perhaps, without T_{EFF} cells CMV-specific memory cells might otherwise be driven
6 to undergo terminal differentiation or become exhausted while occasional
7 stimulation might act more like a vaccine boost, inducing the memory-like cells to
8 undergo clonal expansion and produce more T_{EFF} cells, thereby adding to and
9 supporting the whole CMV-specific inflationary population. Thus, the immune
10 system may not be capable of accumulating or sustaining T_{EFF} cells without a
11 memory population to produce more T_{EFF} cells as needed (especially if T_{EFF} cells
12 are short-lived), while the memory population might not persist in a functional
13 state without being shielded from the majority of antigen.

14

15 **Concluding Remarks:**

16 The model presented here is obviously only relevant if the CMV-specific memory-
17 like cells play some role in the development or maintenance of memory inflation.

18 To investigate the models proposed here, it will be important to determine the
19 effect of stimulating both T_{EFF} and memory-like CMV-specific T cells during
20 chronic infection. Are the memory-like cells depleted over time or can they
21 sustain themselves through repeated rounds of stimulation? Are memory-like
22 cells stimulated at a different rate than T_{EFF} cells? Are T_{EFF} cells short- or long-
23 lived and can they persist in a functional state in the absence of memory-like

1 cells with the same specificity (e.g. when transferred into immune compromised
2 hosts infected with MCMV)? The answers to these questions will hinge on how
3 much antigen stimulation occurs during chronic infection, how it impacts the
4 virus-specific T cells and where that stimulation takes place for both T_{EFF} and
5 memory-like cells. Recent experiments have begun to shed light on some of the
6 details, suggesting that memory inflation depends on antigen-presentation by
7 non-hematopoietic cells [80] and that direct presentation of antigen by infected
8 cells is sufficient for memory-inflation [81]. Together, these observations fit nicely
9 with the evidence that inflationary T cells recognize peptides that can be
10 produced by non-professional antigen presenting cells lacking
11 immunoproteasomes [61]. In addition, our recent data showed that a limited
12 subset of cells infected by MCMV in the first round of infection were sufficient to
13 stimulate memory inflation (Snyder et. al, *PLoS Pathogens*, in press). It will be
14 interesting to determine which cells are responsible for presenting viral antigens
15 and whether they stimulate T cell division, recruitment or differentiation, or rather
16 prolong the survival of T_{EFF} cells so that the rate of T_{EFF} cell production exceeds
17 the rate of loss, resulting in accumulation.

18

19 Aside from the basic goals of understanding the complex relationship between
20 CMV and its host, the answers to the questions outlined in this review may be
21 relevant for promoting sustainable CMV-specific immunity in patients at risk for
22 CMV disease. Whether or not the models described here are ultimately
23 supported by the data, it will be important to determine how the homeostasis of

1 CMV-specific T cells is influenced by factors such as pro- and anti-inflammatory
2 cytokines, co-stimulatory and inhibitory receptors, and different types of antigen-
3 presenting cells. Finally, it will be interesting, as we learn more, to determine
4 whether the mechanisms that support life-long immunity to CMV are broadly
5 applicable to other chronic infections within the herpesvirus family or by unrelated
6 pathogens.

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10

11 **References**

12

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1 **Figure Legends:**

2

3 **Fig. 1** Antigen-specific T cells with an effector- or memory-phenotype develop
4 during MCMV infection in C57BL/6 mice. a) Schematic of the kinetics of CD8+ T
5 cells specific for 5 different MCMV antigens. b) Representative FACS plots of
6 inflationary and non-inflationary T cells in the blood, spleen and bone marrow of
7 mice infected for 19 weeks with MCMV. The data show the expression of CD127
8 (IL-7R α) and KLRG-1 for all CD8+ T cells (left most panels) or CD8+ T cells that
9 also bound to MHC-tetramers loaded with the indicated peptides. All FACS plots
10 were taken from a single animal for comparison. c) Average (n = 5 mice)
11 frequency of CD127^{hi}, KLRG-1^{neg}, memory-phenotype T cells specific for each of
12 the indicated antigens. Data were derived from the upper left quadrants of the
13 FACS plots gated as shown in b. p-values were derived from a student's t-test for
14 paired data.

15

16 **Fig. 2** Two proposed models to explain the persistence of MCMV-specific CD8+
17 T cells during memory inflation. The schematic on the left describes cells that are
18 long-lived or self-sustaining. The schematic on the right describes a situation in
19 which inflationary T cells are repeatedly replenished by clonal expansions,
20 perhaps from a small memory-like pool of cells specific for the same peptides. In
21 this model, a dominant T cell clone (represented by the light colored expansions)
22 is selected to undergo clonal expansion more frequently. Such clonal dominance
23 could be explained by an increased number of cells within this clone, or perhaps
24 a "better fit" T cell receptor. When sub-dominant clones are stimulated, the

1 composition of the inflationary pool is predicted to change (indicated by the
2 dotted lines).

3

4 **Fig. 3** Buffered memory: A model to explain the persistence of functional
5 memory during chronic CMV infection. In this model, a large number of effector-
6 phenotype T cells traffic to and accumulate in non-lymphoid organs, where the
7 majority of latent virus resides. These cells control most viral reactivation,
8 typically prior to virion production, and prevent memory-like T cells from
9 experiencing antigen stimulation. Occasional antigen “leaks” or expression of
10 viral antigens in a specific cell type or organ, might lead to stimulation of
11 memory-like cells (indicated by the down-facing arrow) and new clonal
12 expansions (upward, curved arrow) that produce large numbers of effector-
13 phenotype T cells to join the pool of effector cells in the periphery.

14