Do cytomegalovirus-specific memory T cells interfere with new immune responses in lymphoid tissues?

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Abstract In both mice and humans, the CD8 T cell compartment is expanded with age in the presence of a cytomegalovirus (CMV) infection due to an absolute increase in the CD8+ T cell effector memory (T_{EM}) cells. It has been hypothesized that in CMV+ subjects, such accumulated T_{EM} cells could interfere with responses to new infection by competing for space/resources or could inhibit new responses by other, undefined, means. Here we present evidence against this hypothesis. We show that MCMV-specific CD8 T cells accumulate in blood and bone marrow, but not lymph nodes (frequent sites of immune response initiation), in either persistent lifelong CMV infection or following reactivation. Moreover, adoptive transfer of effector memory T cells from MCMV positive mice into naïve animals did not interfere with either humoral or cellular response to West Nile virus or *Listeria monocytogenes* infection in recipient mice. We conclude that MCMV infection is unlikely to inhibit new immune responses in old animals through direct interference of MCMV-specific CD8 T cells with the priming.

Keywords Cytomegalovirus-specific memory T cells · Lymphoid tissues · West Nile virus

Abbreviations

CMV	Cytomegalovirus
LN	Lymph nodes
MCMV	Murine CMV
T _{EM}	T effector memory cells
WNV	West Nile virus

Introduction

Human (HCMV) and murine (MCMV) cytomegaloviruses are complex, large herpesviruses that establish latency in multiple host tissues (Leng et al. 2017) and reactivate periodically from tissue reservoirs. During both acute and latent infection, multiple lymphocyte subpopulations, such as NK cells, CD4 T cells, and CD8 T cells, cooperate to control viral replication and spread (Polic et al. 1998). CMV has been linked to diseases of aging, yet its causal role remains uncertain (Aiello et al. 2017).

Due to a lifelong “dance” between CMV and the immune system, it has been proposed that CMV may be one of the drivers of immune aging. Indeed, infected individuals exhibit an accumulation of highly differentiated (and, according to some authors, senescent) T effector memory (T_{EM}) cells and reduced ability to respond to new microbial challenges, over and above reduced responsiveness seen with aging alone (reviewed

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in Jackson et al. 2017; Nikolich-Zugich et al. 2017; Souquette et al. 2017).

The CD8 T cell compartment is strongly affected by CMV infection in adult, and even more in older, animals and humans. In both mice and humans, some MCMV-specific CD8⁺ T cell memory populations increase with time in absolute numbers and as a proportion of memory T cells (T_M)—"memory inflation" (Karrer et al. 2003; Sylwester et al. 2005). Memory inflation is driven by CMV antigens that are believed to be transcribed first during reactivation (Munks et al. 2006; Torti and Oxenius 2012), although it remains possible that some low-level production of "inflationary" epitopes may occur during latency. Other viral antigens induce a strong primary response, but are not inflationary, because they are produced late in the viral replicative cycle. Memory inflation leads to an absolute expansion of the CD8 T_{EM} cells in mice, and in humans also of T_{EM} cells that reexpress CD45RA (T_{EMRA}). Indeed, the unparalleled ability of CMV to attract very large CD8 T cell responses over time (up to 50% of all CD8 T_M cells) led some authors to hypothesize that this commitment of the immune system to control reactivation of latent CMV infection might negatively affect immune responses to other infections (Pawelec et al. 2004). While the mechanisms of this interference were never formally proven, they could theoretically occur by several mechanisms: (i) competition for nutrients, hormones, cytokines, chemokines, and other soluble factors; (ii) competition for antigen-presenting cells; and (iii) direct inhibition of other T or B cell activation via soluble (e.g., IL-10) or membrane contact mechanisms.

Along these lines, we and others have shown that immune responses to viral (Cicin-Sain et al. 2012; Mekker et al. 2012) and bacterial (Smithey et al. 2012) challenge were decreased in the secondary lymphoid organs of MCMV-infected old mice. This decrease inversely correlated to number of CD8 T_{EM} in the blood. However, the defects were rather discrete, were more pronounced in relative frequencies rather than in absolute numbers (likely due to an increase in CD8 T cells specific for CMV itself), and did not increase mortality against these infections (Marandu et al. 2015), and the specific mechanisms behind these defects were not elucidated. Here we investigated whether reduced heterologous immunity in CMV+ old mice may have been caused directly by inhibition of new immune responses by CMV-specific T_{EM} cells. We show that MCMV-specific cells did not accumulate in the secondary

lymphoid organs (sites of initiation of new immune responses) of latently infected mice at either steady state or following MCMV reactivation, and that adoptive transfer of T_{EM} cells from MCMV positive mice had no effect on the response to West Nile virus (WNV) or *Listeria monocytogenes* (Lm) infection. We conclude that T_{EM} cell interference with new immune responses is not a likely mechanism by which CMV infection affects immunity during aging.

Materials and methods

Ethics statement

Mouse studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. Institutional Animal Care and Use Committee at the University of Arizona (IACUC #08–102, PHS Assurance Number: A3248-01) approved all experiments. Euthanasia was performed by isoflurane overdose.

Mice and MCMV infection

Adult and old male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or were obtained from the NIA Aged Rodent Colony (via Charles River, Inc., Wilmington, MA). Mice were infected intraperitoneally (i.p.) with 10^5 pfu of MCMV Smith strain, obtained from Drs. M. Jarvis and J. Nelson (Vaccine and Gene Therapy Institute, OHSU, Beaverton, OR).

MCMV reactivation

MCMV reactivation was induced by treating mice (groups of four to five mice) with lipopolysaccharide (LPS) (5 μ g/kg i.p.) for 7 days, corticosterone (40 mg/kg, subcutaneous) mice for 21 days, or radiation (dose of 0.5 Gy followed by 0.7 Gy after 24 h).

Flow cytometric analysis and Ab neutralizing titers

Blood was collected into heparinized tubes and RBC lysed hypotonically. Single-cell blood, spleen, and lymph node suspensions were stained with surface Ab (15–30 min), dead cells excluded using vital stains (Life Technologies, Grand Island, NY), and MCMV epitope specificity diagnosed using m38_{316–323}:K^b, m45_{985–993}:D^b, and m139_{419–}

$426\cdot K^b$ tetramers (NIH Tetramer Core Facility, Emory University, Atlanta, GA). We used the following antibodies: CD3 (17A2), CD4 (RM4-5), CD8a (53-6.7), CD44 (IM7), and CD62L (MEL-14) (BioLegend, San Diego, CA). To assess cytotoxic function and expansion of CD8 T cells we stained intracellularly with antibodies for Granzyme B (QA16A02) and Ki67 (16A8). Intracellular staining was done following permeabilization of cells using the FoxP3 Fix/Perm Kit (Thermo Fisher, Waltham, MA), samples acquired using LSR Fortessa (BD Bioscience, San Jose, CA) and analyzed by FlowJo software (Tree Star, Ashland, OR). Positivity gates were set up using fluorescence minus one (FMO) control where needed. Absolute cell counts were calculated using Hemavet 950 (Drew Scientific, Miami Lakes, FL). The neutralization assay was performed exactly as described previously (Uhrlaub et al. 2011).

Adoptive transfer of effector memory T cells and infections

T_{EM} CD8⁺ T cells from MCMV-infected mice (> 10 months p.i.) were sorted on FACS Aria III sorter (BD Biosciences, San Jose, CA) based on the CD8⁺C62L[−]CD44^{hi} phenotype. Effector memory CD8 T cells isolated from the spleens were transferred into adult and old recipients (three donor mice per one recipient) and the recipients (groups of 5) infected with 2000- pfu WNV 24 h later by i.d. footpad infection. Tissue harvest and analysis were done on day 7 post-infection; no animals died as a result of the infection. Old B6 mice were intravenously infected with $\sim 2 \times 10^5$ colony-forming units (CFU) of *Lm*-OVA. Liver bacterial burden was measured as previously described (Smithey et al. 2011).

Statistical analysis

Differences were calculated by Mann-Whitney *U* test, one-way ANOVA, or Kruskal-Wallis test with post hoc correction using SPSS and GraphPad Prism. For all statistical differences, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

Results and discussion

We analyzed the frequency and distribution of inflationary and “non-inflationary” MCMV-specific CD8⁺ T cells across lymphoid organs of 24-month-old latently

MCMV-infected mice 21 months p.i. (see flow cytometric (FCM) gating schema in Supplemental Fig. 1). Percentage of CD8 T cells with T_{EM} phenotype (Fig. 1a) in both LN pools (cervical and superficial – brachial + inguinal + popliteal) was low (4–10-fold lower) compared to other niches. Ag-specific response against the inflationary epitope m38 was enriched in the blood compared to other niches (Fig. 1b), followed by spleen and bone marrow, but were barely detectable in the LN. Percentage of T_{EM} cells specific for the non-inflationary epitope m45 was dramatically lower in magnitude (< 1%) and slightly higher in the bone marrow compared to other tissues (Fig. 1c). Cells specific for the highest prevalent epitope m139 were particularly enriched in the blood compared to all the other tissues (Fig. 1d). Number of m139+ cells was increasing with age in the blood of mice infected with MCMV for 3, 12, or 21 months (Fig. 1e) as we have previously shown (Smithey et al. 2012). Therefore, we used mice infected for 21 months to investigate in more detail tissue distribution of MCMV-specific cells. Our results clearly demonstrate that even at its peak memory inflation primarily occurs in blood and bone marrow and not in secondary lymphoid tissue. Most of the MCMV-specific m38+ and m139+ cells were of the CD44^{hi}CD62L^{low} (T_{EM}) phenotype while m45+ cells exhibited a mixed T_{CM} (CD44^{hi}CD62L^{hi}) and T_{EM} phenotype (Supplemental Fig. 2A). Thus, MCMV-specific CD8 T cells were mostly T_{EM} , with a preference to remain in circulation or bone marrow and spleen while being excluded from the LN.

The above results were from mice carrying latent MCMV infection and kept under specific pathogen-free conditions, devoid of most stressors that cause MCMV reactivation, including immunosuppression, whole body irradiation, or infection/systemic inflammatory responses. We next examined distribution of MCMV-specific T_{EM} cells in mice exposed to stressors known to reactivate the virus (Cook et al. 2006; Goerig et al. 2016; Prösch et al. 2000): lipopolysaccharide (LPS) for 7 days, corticosterone (40 mg/kg) for 21 days, or a sublethal dose of radiation (0.7 Gy). Due to potential immunosuppressive effects of the three stressors we used low doses which did not lead to decrease in lymphocyte numbers (Supplemental Fig. 3), indicating no cell death or major disturbance of immune cells brought on by treatment. On day 1 following cessation of treatments, mice were sacrificed, and lymphoid organs tested for the presence and phenotype of CMV-specific cells.

Fig. 1 Effector memory CD8 T cells specific for inflationary CMV epitope are enriched in blood and rare in the LN 12-week-old C57/BL6 mice were injected with 10^5 pfu of MCMV; 21 months later, same mice blood, spleen, bone marrow, and LN were collected. **a** Percent of CD8 T cells with effector memory phenotype (CD44hiCD62L⁻) in circulation and different secondary lymphoid tissues (BM bone marrow, cLN cervical LN pool, sLN superficial LN pool). **b** EM CD8 T cells specific for

“inflationary” epitope m38 are enriched in the blood and rare in the LN. **c** EM CD8 T cells specific for “non-inflationary” epitope m45 are slightly increased in the LN compared to blood and spleen. **d** EM CD8 T cells specific for inflationary epitope m139 are enriched in the blood compared to all other tissues. **e** CD8 T cells specific for inflationary epitope m139 are increasing with time in MCMV-infected mice

All three treatment groups exhibited an increase in absolute numbers of m38-specific CD8 T cells in the blood (Fig. 2a) while the corticosterone group also showed an increase in the spleen (Fig. 2b). Yet, none of the groups exhibited an increase in m38-specific cells in the cervical (Fig. 2c) or superficial (not shown) LN. Similarly, the response against the non-inflationary epitope m45 was increased in the blood of all three groups (Fig. 2d) but not the spleen (Fig. 2e) or LN (Fig. 2f). These results indicate that MCMV reactivation does lead to an increase in absolute numbers of MCMV-specific T cells, but primarily in the blood and not in LN.

Overall, the low representation of MCMV-specific cells in the LN suggested that the inverse correlation between the number of EM cells in the LN and magnitude of naïve response to another infection (that is typically initiated in lymph nodes or spleen for non-mucosal infections) may not be causal. To test this, we

have sorted T_{EM} CD8 T cells from spleens of lifelong latently infected mice (> 10 months p.i.), transferred them into adult MCVN-negative CD45.1⁺ C57/BL6 mice at a ratio three donor mice per one recipient (schematic in Fig. 3a) and challenged them with WNV or *Listeria monocytogenes*. Both of these are intracellular pathogens that inflict significant morbidity and mortality specifically in older humans and mice (Bronze and Drevets 2008; Smithey et al. 2011; Yim et al. 2005). Moreover, in both infections, CD8 T cells are needed to clear the infection, a process known to be impaired with age (Jergović et al. 2018). Between 20 and 40% of the transferred cells were specific for the three MCMV epitopes—m38, m139, and m45 (Supplemental Fig. 2B). On day 7 p.i. we sacrificed recipients quantified engraftment and the CD8 T cell Ag-specific and humoral response. T_{EM} cells are known engraft poorly upon adoptive transfer (Busch et al. 2016). On day 7 we

Fig. 2 Viral reactivation leads to increase in CMV-specific memory CD8 T cells in blood and spleen but not LN. MCMV was reactivated in latently infected mice by administrating LPS, corticosterone, or low-dose radiation (0.7 Gy). **a** Absolute numbers of CD8 T cells specific for “inflationary” epitope m38 increased in the blood of all reactivated groups. **b** Absolute numbers of CD8 T cells specific for inflationary epitope m38 increased in the spleens

only in the group which received corticosterone treatment. **c** Absolute number of m38-specific cells in the cervical LN remained unchanged. **d** Absolute numbers of CD8 T cells specific for “non-inflationary” epitope m45 increased in the blood of all reactivated groups. **e**, **f** Absolute numbers of CD8 T cells specific for non-inflationary epitope m45 remained unchanged in the spleen and cervical LN of all groups

have detected around 45,000 (4.5%) / million transferred cells in the spleen and 15,000 (1.5%) in bone marrow from single femur and tibia. Previously it was shown that single femur and tibia contain ~10% of total bone marrow memory CD8 T cells (Geerman et al. 2016), implying that overall number of engrafted cells in the bone marrow was at least 5-fold higher than in the spleen. Number of transferred cells in the LN was very low (Fig. 3b), and this did not affect the absolute numbers of effector cells ($CD8^+Ki67^+GrzB^+$) recruited into the response against WNV in the draining LN was unaffected by T_{EM} transfer (Fig. 3c). WNV NS4b-specific CD8 response in the draining LN was also comparable between the control and recipient groups (Fig. 3d). Differentiation of antigen-specific CD8 T cells into short-lived effector cells (SLEC) was also unaffected by T_{EM} transfer (Fig. 3d). We further assessed

whether T_{EM} transfer inhibited B cell and humoral response. WNV-neutralizing antibody titer was not affected by effector memory T cell transfer (Fig. 4a) and neither was number of plasma cells ($CD19^+B220^{lo}CD183^+CXCR4^+$) (Fig. 4b) or activated B cells ($B220^+IgM^+IgD^+CD86^+CXCR4^-$) (Fig. 4c). Since percentage of CMV-specific cells, as well as engraftment of transferred EM cells was higher in the spleen than lymph nodes, we tested whether immune responses in the spleen may be more susceptible to inhibition by CMV-specific T cells. To that end, we performed the same transfer experiments (schematic in Fig. 5b) with aged recipients and infected them with Lm-OVA. *Listeria* is an intracellular pathogen and primary sites of infection in mice are spleen and liver (Conlan 1996) while CD8 T cells are necessary for pathogen clearance (Condotta et al. 2012). Similarly,

Fig. 3 Adoptive transfer of EM CD8 T cells from lifelong CMV-infected mice does not affect T cell response to WNV. **a** Transfer schematic. **b** Engraftment of transferred donor

cells. **c** Number of GrzB- and Ki67-positive effector cells on day 7 post-WNV infection was not affected by EM transfer. **d** Number of CD8 T cells specific for viral protein NS4B in the draining LN was equal in both groups. **e** Differentiation of antigen-specific CD8 T cells into short-lived effector cells was equal in both groups

Fig. 4 Adoptive transfer of EM CD8 T cells from lifelong CMV-infected mice does not affect humoral immunity after WNV infection. **a** Neutralizing antibody titer was not affected by transfer of effector memory T cells. **b** Number of plasma cells

(CD19⁺B220^{lo}CD183⁺CXCR4⁺) on day 7 post-WNV infection was not affected by EM transfer. **c** Number of activated B cells (B220⁺IgM⁺IgD⁺CD86⁺CXCR4⁺) was the same in both groups

Fig. 5 Adoptive transfer of EM CD8 T cells from lifelong CMV-infected mice does not affect T cell response to *Listeria monocytogenes* T_{EM} CD8 T cells from spleens of lifelong MCMV-infected mice were transferred into MCMV-negative aged (19 months) mice at a ratio three donor mice per one recipient. Mice were challenged with Lm-OVA 24 after transfer. **a** Transfer

schematic. **b** Number of GrzB- and Ki67-positive effector cells on day 7 post-Lm infection was not affected by EM transfer. **c** Number of CD8 T cells specific for OVA in the spleen was trending to be higher in the group which received T_{EM} transfer. **d** Liver bacterial burden on day 7 p.i. was equal in both groups

to the lymph nodes of WNV-infected mice, EM transfer did not affect the absolute numbers of Lm OVA-specific effector cells (CD8⁺Ki67⁺GrzB⁺) in the spleen of Lm-infected mice (Fig. 5b). The number of Ag-specific cells (OT-1) in the spleen was trending to be higher in the mice which received EM transfer (Fig. 5c) although the difference was not statistically significant. Finally, EM transfer had no effect on bacterial clearance in the liver (Fig. 5d) as number of CFU was same in both groups. We have previously shown cells that number of antigen cell-specific cells that express GrzB inversely correlate to ability of CD8 T cells to kill target cells (Brien et al. 2009). Similarly, we found that the number of Ki67⁺GrzB⁺ CD8 T cells inversely correlated to

bacterial CFU in the liver ($r = -0.616$, $p = 0.058$) but this was not affected by EM transfer as number of CFU was equal among two groups.

What mechanisms may be employed by CMV to interfere with ongoing immune responses? Possibilities proposed include reduction of diversity in responding naïve T cells with aging that would be pronounced by CMV inhibition or interference by accumulated T_{EM} cells, or potential interference by virus-produced or virus-induced soluble mediators (Nikolich-Zugich et al. 2017). Diversity of the naïve T cell pool has been assessed in CMV-negative humans, and the conclusions were that the reduction in the CD8 compartment exists (about 2–5-fold reduction), but may not be constraining

to new responses in light of the residual diversity, predicted to be of the order of 10^{8-9} (Qi et al. 2014). In the mouse, limited high-throughput sequencing did not reveal major differences between adult, old, or old mice carrying MCMV (Smithey et al. 2018). Therefore, at the present there is no experimental support, and there is some evidence against the virus-mediated naïve T cell repertoire constriction.

In this manuscript, we demonstrate that MCMV-specific CD8 T_{EM} cells are poorly represented in the lymph nodes compared to other niches (blood, bone marrow, spleen) and particularly enriched in blood. Even after exposure to stressors that induce viral reactivation, number of MCMV specific cells increased only in blood and not in secondary lymphoid tissues. Finally, adoptive transfer of T_{EM} cells from MCMV-positive mice had no effect on the response to WNV or *Listeria* in recipient mice. We therefore conclude that MCMV-specific T_{EM} cells are unlikely to directly interfere with an immune response against a new infection in the secondary lymphoid tissues. However, in light of the existing evidence, experiments should be conducted to test the role of soluble factors, produced by T or other host cells that may adversely impact responses to other infections in CMV-bearing hosts.

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