11-10-2015

Murine CMV Infection Induces the Continuous Production of Mucosal Resident T Cells.

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Article

Cell Reports

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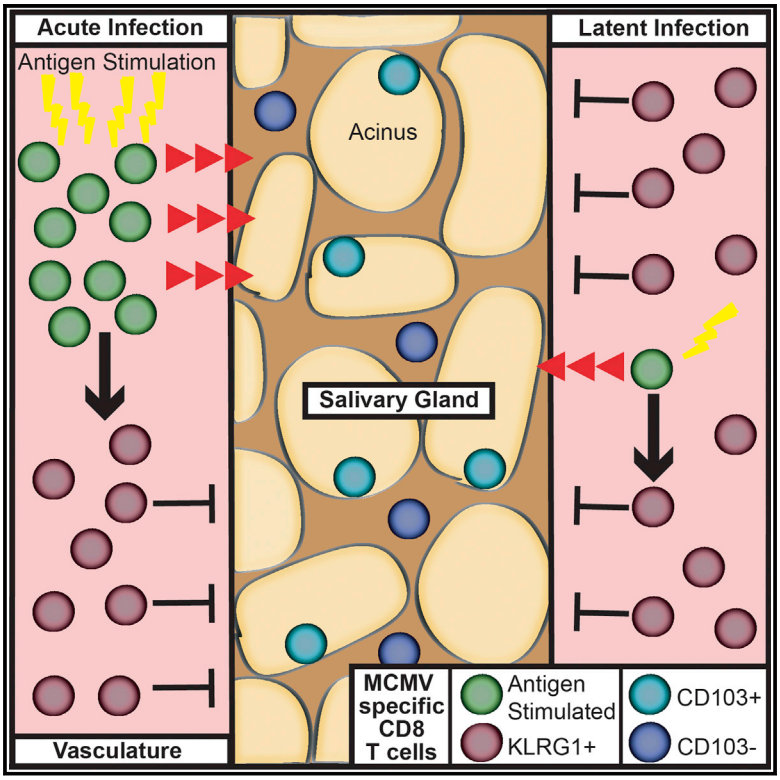
Graphical Abstract



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In Brief

Cytomegalovirus (CMV) is shed from the salivary gland and other mucosal tissues, yet the CD8 response at these sites is poorly understood. Smith et al. show that MCMV-specific tissue-resident CD8 populations are supported by continuous, low-level recruitment of circulating CD8s that become T_{RM} in an antigen-dependent manner.

Highlights

- MCMV induces resident memory CD8s in multiple mucosal tissues early after infection
- T_{RM} maintenance and IEL localization in the salivary gland does not depend on CD103
- MCMV-specific CD8s circulating during latency have reduced capacity to form new T_{RM}
- Antigen promotes the late recruitment and formation of T_{RM} in the salivary gland

Smith et al., 2015, Cell Reports *13*, 1137–1148
November 10, 2015
http://dx.doi.org/10.1016/j.celrep.2015.09.076

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Murine CMV Infection Induces the Continuous Production of Mucosal Resident T Cells

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http://dx.doi.org/10.1016/j.celrep.2015.09.076

SUMMARY

Cytomegalovirus (CMV) is a herpesvirus that persists for life and maintains extremely large numbers of T cells with select specificities in circulation. However, it is unknown how viral persistence impacts T cell populations in mucosal sites. We found that many murine (M)CMV-specific CD8s in mucosal tissues became resident memory T cells (T_{RM}). These cells adopted an intraepithelial localization in the salivary gland that correlated with, but did not depend on, expression of the integrin CD103. MCMV-specific T_{RM} cells formed early after infection, and spleen-localized cells had reduced capacities to become T_{RM} at late times. Surprisingly, however, small numbers of new T_{RM} cells were formed from the circulating pool throughout infection, favoring populations maintained at high levels in the blood and shifting the immunodominance within the T_{RM} populations over time. These data show that mucosal T_{RM} populations can be dynamically maintained by a persistent infection.

INTRODUCTION

Cytomegalovirus (CMV) is a β-herpesvirus that infects the majority of people in the world and establishes an asymptomatic latency, punctuated by periodic reactivation (Crough and Khanna, 2009). Controlling these reactivation events requires constant immune surveillance (Polić et al., 1998; Simon et al., 2006), which induces the accumulation of virus-specific T cells in a unique process called "memory inflation" (Holtappels et al., 2000; Karrer et al., 2003; Komatsu et al., 2003; Munks et al., 2006). This has led to great interest in using CMV as a vaccine vector, with pre-clinical success in a non-human primate model of HIV infection (Hansen et al., 2009, 2011, 2013). Like most herpesviruses, CMV displays strict species specificity. Thus, we use murine CMV (MCMV), a natural mouse pathogen and the homolog of human (H)CMV. The T cells induced by both viruses are broadly similar in phenotype, function, and genetic signature (Crough and Khanna, 2009; Krmpotic et al., 2003; Quinn et al., 2015; Snyder et al., 2011). Using the MCMV model, we found that most of the "inflationary" $CD8^+$ T cells (those that accumulate over time) are confined to the circulation after systemic MCMV infection (Smith et al., 2014). The major exception to this finding was the salivary gland, where MCMV and HCMV both persist and establish latency (Crough and Khanna, 2009; Krmpotic et al., 2003; Polić et al., 1998). It is unknown how CMV-specific T cells develop in this or other mucosal tissues.

It has become clear in recent years that many pathogen-specific T cells within the skin, brain, and mucosal tissues, including the salivary gland, are not in equilibrium with those circulating through the blood and lymphoid organs. These populations have been called tissue-resident memory T cells (T_{RM}), and they are thought to form early after infection, persisting in these tissues independently of circulation (reviewed in Schenkel and Masopust, 2014). In the small intestine, vagina, skin, and lung, pathogen-specific T_{RM} cells localize near or within the epithelial layer, which is thought to enable T_{RM} cells to be "first-responders": cells that do not require recruitment to rapidly respond to reinfection (Ariotti et al., 2014; Gebhardt et al., 2009; Mackay et al., 2012; Schenkel et al., 2013; Sheridan et al., 2014; Wu et al., 2014; Zhu et al., 2013). For these reasons, establishing T_{RM} in large numbers may be critically important in maintaining immune surveillance in these organs and is a major concern for vaccine design.

Several lines of evidence suggest that T_{RM} cells form independently of local antigen (Casey et al., 2012; Hofmann and Pircher, 2011; Mackay et al., 2012; Wakim et al., 2010). In fact, work with lymphocytic choriomeningitis (LCMV) clone 13, which induces a chronic infection that promotes T cell dysfunction, suggested that antigen may inhibit mucosal T_{RM} populations (Casey et al., 2012). Both MCMV and HCMV undergo prolonged replication in the salivary gland and persist for life in many sites in the body. However, unlike many persistent viruses, neither MCMV nor HCMV promotes T cell dysfunction. The persistence of low levels of antigen during CMV infection, along with the CMV-driven accumulation of functional $CD8^+$ T cells, raise the possibility that the dynamics of T cell maintenance in the mucosa do not reflect that of cleared infections or chronic infections that drive exhaustion.

We found that many MCMV-specific $CD8^+$ T cells in the salivary gland and other mucosal sites in the body developed a T_{RM} phenotype shortly after infection. Remarkably, our data suggest that persistent antigen stimulation during viral latency promotes the continuous, low-level recruitment of circulating inflationary MCMV-specific T cells to the T_{RM} population in the salivary gland, which resulted in a slow shift in the immunodominance of the

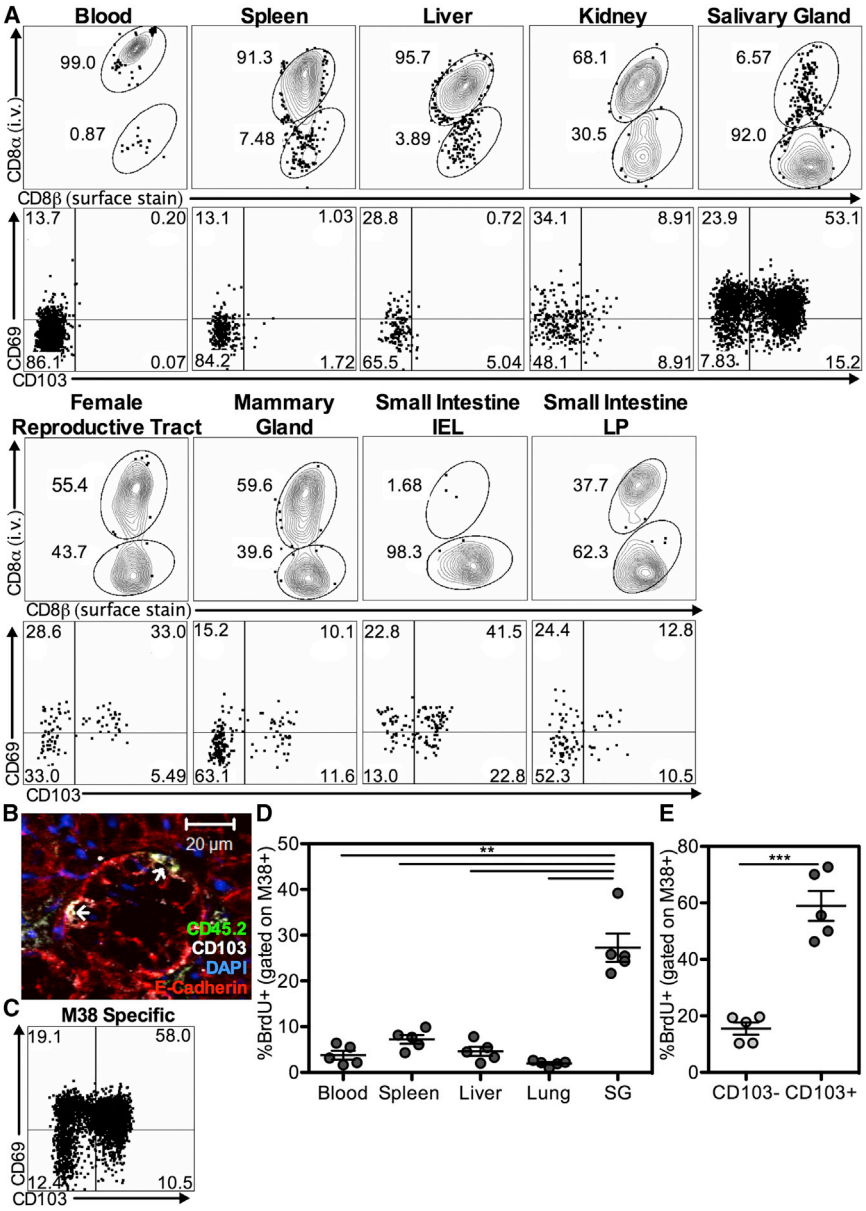

Figure 1. MCMV-Specific CD8s Become T_{RM} in the Salivary Gland and Other Mucosal Sites

(A) OT-Is in congenic B6 mice >12 weeks after infection with MCMV-OVA. (Top row) FACS plots of congenic OT-Is labeled with i.v. injected, fluorochrome-labeled anti-CD8α antibody to identify blood and tissue localized T cells and surface stained with anti-CD8β in the indicated organs are shown. (Bottom row) CD103 and CD69 expression on OT-Is in the i.v. unlabeled fraction of indicated organs (see also Figure S1) is shown. Data are representative of two independent experiments (n = 7).

(B) Immunofluorescent staining of OT-Is in the salivary gland >12 weeks postinfection. Shown is an OT-I (identified by CD45.2; green) expressing CD103 (white) co-localized with the epithelium (identified by E-cadherin; red). DAPI staining of the nucleus is shown in blue. Individual images and overlays are in Figure S2.

(C) FACS plot of CD103 and CD69 on M38-specific T cells in i.v. unlabeled fraction of salivary gland >12 weeks post-wild-type MCMV infection.

(D and E) Mice were treated with BrdU in drinking water for the first 9 weeks of MCMV infection and then sacrificed 15 weeks after the end of the pulse. The frequency of BrdU-labeled cells among M38-specific T cells in the indicated organ (D) and among $CD103^-$ or $CD103^+$ M38-specific cells in the salivary gland (E) after the 15-week chase period is shown. Data are representative of two independent experiments.

MCMV-specific T_{RM} cells over time. These data suggest that mucosal T_{RM} populations driven by persistent infections can be dynamically maintained.

RESULTS

MCMV-Specific T_{RM} CD8s Are Present in Large Numbers in the Salivary Gland

MCMV is a systemic, persistent pathogen that induces the accumulation of virus-specific CD8 T cells. We showed previously that, after an i.p. infection, the vast majority of MCMV-specific CD8s stimulated by viral antigen were in the blood or associated with the vasculature during the latent stage of infection (Smith et al., 2014). To investigate MCMV-specific CD8s in mucosal tissues, we used an MCMV virus expressing ovalbumin (MCMV-OVA) and OT-Is. Naive mice were seeded with small numbers of naive congenic OT-Is and infected with MCMV-OVA 1 day later. This protocol induces robust inflation of OT-Is (Turula et al., 2013), which were largely exposed to an i.v.-injected antibody, suggesting a vascular localization (Figure 1A). When blood-localized CD8s were excluded, the salivary gland harbored the most OT-Is of the organs studied (Figure S1A). Expression of CD103 and CD69, the markers of T_{RM} cells, was most pronounced on T cells within the salivary gland and the intraepithelial lymphocyte fraction of the small intestine (si-IEL) (Figures 1A and S1B). Moreover, cells in both of these sites tended to express low or intermediate amounts of the IL-7Rα (CD127) and lacked KLRG1 (Figures S1C and S1D), consistent with the T_{RM} phenotype (Mackay et al., 2013; Sheridan et al., 2014). Importantly, many of the MCMV-driven OT-Is in the salivary gland were IELs located within the acinii and ducts (Figures 1B and S2). Endogenous CD8 T cells specific for MCMV epitopes also upregulated T_{RM} markers in the salivary gland (M38-specific T cells shown in Figure 1C). To test whether these T cells were lodged in the salivary gland, we performed a BrdU pulse during the first 9 weeks of infection, followed by a prolonged chase

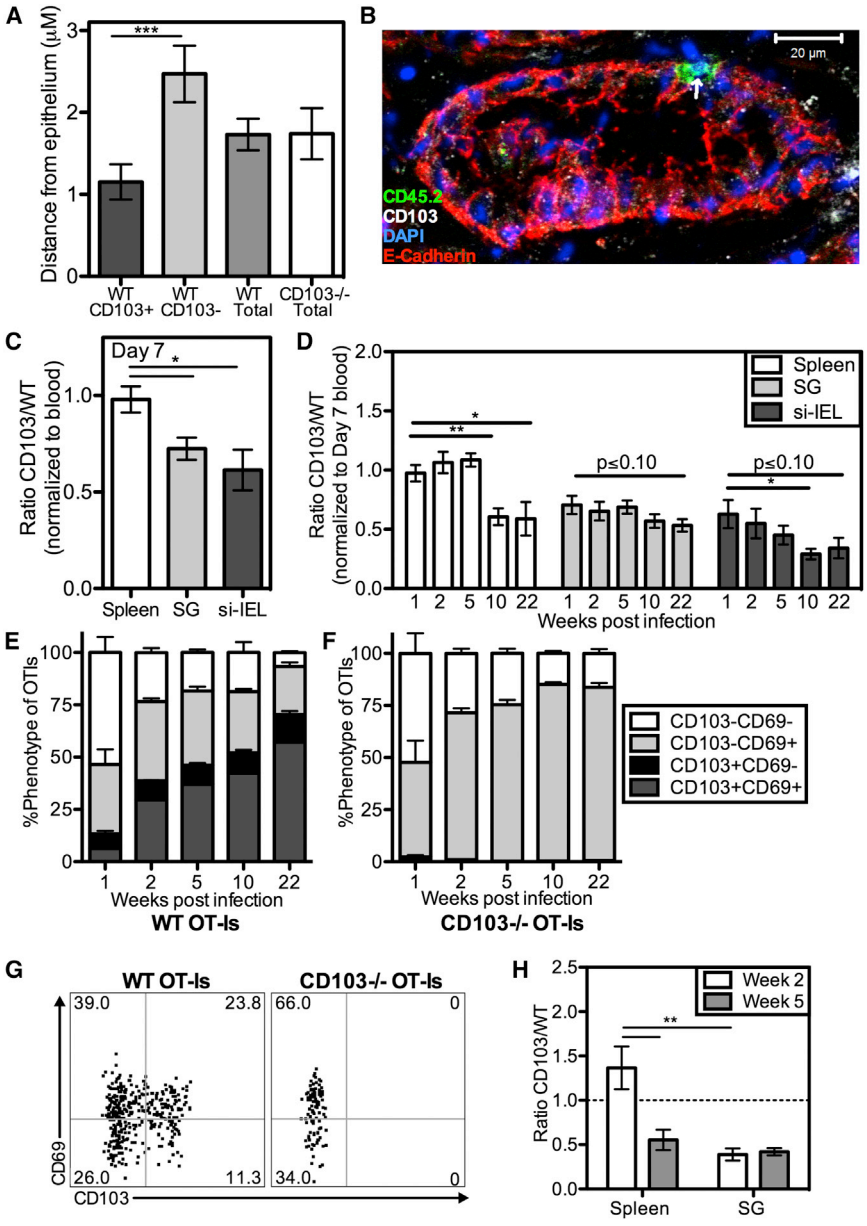

Figure 2. Localization and Maintenance of MCMV-Specific CD8s in the Salivary Gland Do Not Depend on CD103 Expression

B6 mice were seeded with wild-type or CD103$^{-/-}$ OT-Is and challenged with MCMV-OVA.

(A) Localization of OT-Is in the salivary gland was examined histologically by immunofluorescent staining >12 weeks after infection. (Left) Distance from the nearest epithelial surface of wild-type OT-Is in the salivary gland that did (n = 121 cells) or did not (n = 87 cells) express CD103 is shown. (Right) Distance of OT-Is from the nearest epithelial surface for wild-type OT-Is (whole population; n = 223 cells) compared to CD103$^{-/-}$ OT-Is (n = 83 cells) is shown.

(B) CD103$^{-/-}$ OT-Is (identified by CD45.2; green) embedded in the epithelium (identified by E-cadherin; red). CD103 is shown in white, and DAPI is shown in blue (see also Figure S3). Data are representative of two to four tissue sections per mouse from four mice containing wild-type OT-Is and three mice containing CD103$^{-/-}$ OT-Is.

(C–F) Wild-type and CD103$^{-/-}$ OT-Is were mixed and co-transferred before MCMV-OVA challenge. Cohorts were sacrificed at different times post-infection. (C) Ratio of CD103$^{-/-}$ to WT OT-Is in the spleen, salivary gland, and intestinal IEL normalized to ratio in the blood within the same mouse at 1 week postinfection is shown. (D) Ratio of CD103$^{-/-}$ to WT OT-Is in each organ over time normalized within an experiment to the average ratio in the blood at week 1 is shown. Frequency of indicated phenotype of WT OT-Is (E) and CD103$^{-/-}$ OT-Is (F) in the salivary gland over time is shown. See also Figure S4. Results are combined from two independent experiments (n = 7).

(G and H) Wild-type and CD103$^{-/-}$ OT-Is were mixed and co-transferred before MCMV-OVA infection. One week postinfection, WT and CD103$^{-/-}$ OT-Is were sorted from the spleen and equal numbers mixed and transferred into naive congenic recipients. (G) CD103 and CD69 on OT-Is in the salivary gland 2 weeks after transfer are shown. (H) Ratio of CD103/WT OT-Is in the indicated organ of naive recipients at 2 and 5 weeks posttransfer is shown. Results are combined from two independent experiments (n = 8).

Error bars represent the SEM. Statistical significance was measured by paired (A and C) or unpaired (D and G) Student's t tests (*p < 0.05; **p < 0.01; ***p < 0.001).

period. The ongoing antigen-driven memory inflation causes a loss of BrdU-positive inflationary T cells in circulation (Smith et al., 2014). However, inflationary T cells in the salivary gland retained BrdU at a much-greater frequency than cells extracted from the spleen, lungs, and liver (Figure 1D). The retention of the BrdU label was especially prominent in the CD103^{+} subset of MCMV-specific cells in the salivary gland (Figure 1E). Thus, salivary-gland-localized T cells are not in equilibrium with the circulating populations. Together, these data show that MCMV infection promotes T_{RM} cells in multiple mucosal tissues and that T_{RM} cells are lodged in the salivary gland, where they develop an IEL localization.

CD103 Expression Is Not Required for Maintenance or Localization of CD8s within the Salivary Gland

It has been suggested that expression of CD103 is important for enforcing the residency of lymphocytes in mucosal organs by tethering T cells to the epithelium (Casey et al., 2012; El-Asady et al., 2005). However, recent work has challenged this notion for T_{RM} cells within the si-IEL fraction (Sheridan et al., 2014). For MCMV-driven OT-Is in the salivary gland, CD103 expression was significantly correlated with epithelial localization as assessed by proportion or by distance from the epithelium (Figures 2A and S3B). Surprisingly, however, CD103$^{-/-}$ OT-I localization was comparable to wild-type

(WT) OT-Is within the salivary gland (Figures 2A, 2B, S3A, and S3B). These data suggest that CD103 expression marks IELs in the salivary gland but is not essential for the IEL localization. To determine whether CD103 is required for the maintenance of MCMV-specific T_{RM} in the salivary gland, we mixed congenically marked WT and CD103$^{-/-}$ OT-Is before transfer and MCMV-OVA infection. Although both populations had reached the salivary gland and small intestine in large numbers after 1 week, the CD103$^{-/-}$ OT-Is were slightly underrepresented in the salivary gland and the si-IEL relative to the spleen (Figure 2C). In the salivary gland, the ratio of CD103$^{-/-}$ to WT OT-Is was maintained stably over several weeks and only slightly favored WT OT-Is even after 22 weeks (Figure 2D). Importantly, the numbers of CD103$^{-/-}$ OT-Is did not decline over time (Figure S4A). Rather, this shift in ratio reflects a slight increase in the numbers of WT OT-Is in the salivary gland (Figure S4A). This subtle preferential maintenance of WT OT-Is was more pronounced in the si-IEL over time and unexpectedly, in the spleens of the same mice (Figure 2D), reflecting a greater loss of CD103$^{-/-}$ cells after day 7 (Figures S4B and S4C). The CD103$^{-/-}$ OT-Is in the salivary gland and si-IEL expressed high levels of CD69 from week 2 onward, mirroring the WT OT-Is in the same mice (Figures 2E, 2F, S4D, and S4E). These data suggest that CD103$^{-/-}$ OT-Is had a defect in their initial migration to the salivary gland but were maintained stably thereafter. To test whether persistent antigen was masking a defect in the maintenance of CD103$^{-/-}$ OT-Is, we isolated activated WT and CD103$^{-/-}$ OT-Is from the spleen of adoptive transfer recipients 1 week after infection and transferred equal numbers into naive mice. Both OT-I populations migrated to the salivary gland and upregulated markers of residency (Figure 2G). Again, CD103$^{-/-}$ OT-Is were underrepresented in the salivary gland 2 weeks after transfer, but the proportion of WT and CD103$^{-/-}$ OT-Is was unchanged over the next 3 weeks, indicating that both WT and CD103$^{-/-}$ OT-Is were maintained similarly in the salivary gland, even in the absence of antigen (Figure 2H). Thus, whereas CD103 marks T_{RM} cells with an IEL localization in the salivary gland, it is not required for their formation, localization, or maintenance.

MCMV-Specific CD8s that Undergo Memory Inflation Are Maintained at Higher Numbers than Non-inflationary CD8s in the Salivary Gland

Within the endogenous CD8$^+$ T cell populations, cells responding to different MCMV epitopes display different kinetics of accumulation or contraction. Inflationary populations (represented by M38- and IE3-specific CD8s in B6 mice) are maintained at high levels in the blood by ongoing antigen stimulation, whereas non-inflating populations (represented by M45- and M57-specific CD8s) undergo substantial contraction after the acute phase of infection, likely because their antigen becomes unavailable (Dekhtiarenko et al., 2013), and are maintained homeostatically (Figures S5A–S5D; Smith et al., 2014; Snyder et al., 2008). Because T_{RM} cells do not rely on antigen (Casey et al., 2012; Mackay et al., 2012), we did not expect these kinetics to be reflected in the T_{RM} populations. We sacrificed cohorts of B6 mice infected with WT-MCMV (strain K181) at different times postinfection. Inflationary M38- and IE3-specific CD8s were present in the salivary gland within 1–3 weeks of infection and were maintained stably once they'd reached their peak levels (Figures 3A and 3B), mirroring their kinetics in the spleen (Figures S5A and S5B). Surprisingly, non-inflationary populations in the salivary gland declined significantly in a prolonged manner over at least 10 weeks (Figures 3C and 3D). Whereas this contrasts to the dramatic contraction that occurred in the spleens of the same mice (Figures S5C and S5D), non-inflationary T cells had become subdominant to inflationary cells by late times after infection (Figures 3E and 3F). The change in immunodominance was not associated with any major differences in expression of CD69 or CD103 between populations (Figure S5E). Nevertheless, inflationary T cells were far more prevalent in the blood and thus it was possible that these cells were circulating passively through the salivary gland and driving the shift in immunodominance. To test this, we injected CFSE i.v. into latently infected mice, which labeled the vast majority of inflationary cells in the blood, with minimal labeling of T cells in the salivary gland (Figure 3G). Seventy-four days later, CFSE-labeled T cells had not equilibrated in the salivary gland (Figure 3H). Even though migrating cells could lose CFSE by dividing upon salivary gland entry, these data suggest that the shift in immunodominance is not caused by inflationary cells passively circulating through the salivary gland at steady state.

MCMV-Specific T_{RM} in the Salivary Gland Do Not Depend on Viral Replication

Circulating inflationary T cells quickly become immune dominant because they undergo prolonged antigen-driven division (Torti et al., 2011; Figures S6B–S6E). Unlike other organs, the salivary gland harbors replicating virus for prolonged periods of time. In our hands, MCMV transcripts encoding the late gene glycoprotein B (gB) were evident in the salivary gland for at least 10 weeks after infection (Figure 4A). The disappearance of gB transcripts correlated with the loss of non-inflationary T cells from the salivary gland (Figures 3C and 3D). Thus, it was possible that the presence of antigen promoted the proliferation of T_{RM} cells in the salivary gland. To test whether viral replication in the salivary gland affects the rate of T_{RM} division, we compared WT-MCMV to a spread defective version of MCMV that lacks the essential glycoprotein L (ΔgL-MCMV). This virus is a single-cycle virus in vivo that cannot spread but still induced memory inflation (Snyder et al., 2011) and T_{RM} phenotype cells in multiple mucosal tissues after an i.p. infection (Figure S6A). Any T_{RM} division driven by replicating virus in the salivary gland should be absent from mice infected with ΔgL-MCMV. However, there was no difference in salivary gland T cell division (assessed by KI-67 expression) between mice infected with WT- and ΔgL-MCMV beyond 1 week postinfection (Figures 4B–4E). We also adoptively transferred activated OT-Is into these mice on day 5 of infection. Because neither virus expresses OVA, these OT-Is serve as a reference population for antigen-independent division. Remarkably, OT-Is recovered from these salivary glands expressed KI-67 at an identical rate as endogenous T cells despite the complete absence of the OVA antigen (Figures 4B and 4C). Collectively, these data suggest that neither viral replication nor antigen in the salivary gland

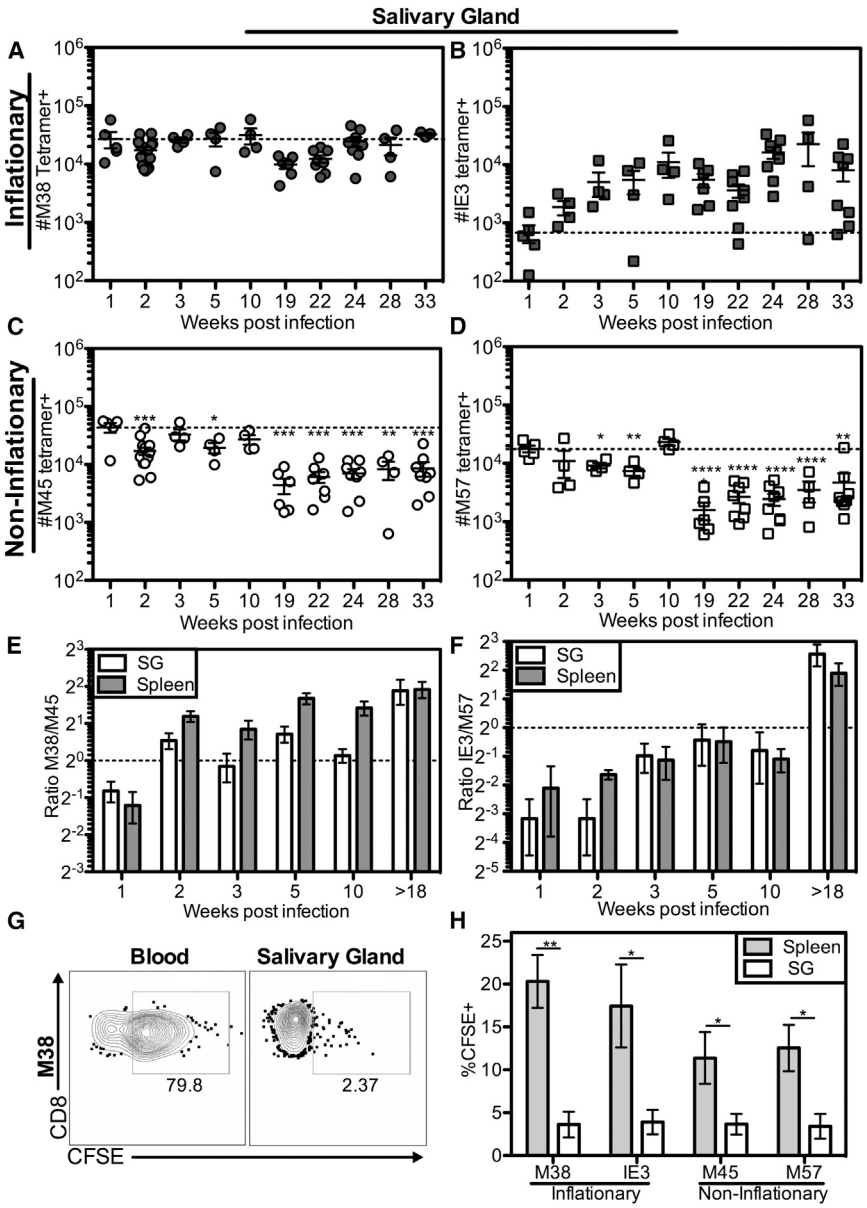

Figure 3. Inflationary CD8s Are Preferentially Maintained in the Salivary Gland during the Latent Stages of Infection

(A–D) B6 cohorts were infected with K181 MCMV and sacrificed at indicated times postinfection. Absolute numbers of CD8s in salivary gland specific for inflationary epitopes M38 (A) and IE3 (B) and non-inflationary epitopes M45 (C) and M57 (D) are shown. Dotted lines indicate average number of cells at week 1 for comparison. Asterisks indicate significant differences from week 1 (see also Figure S5).

(E and F) Ratio of M38-specific to M45-specific cells (E) or IE3-specific to M57-specific cells (F) in the salivary gland and spleen of each mouse calculated from the numbers shown in (A)–(D). Dotted lines indicate a ratio of 1. Data are combined from seven independent experiments (n = 4–16 mice per time point).

(G) FACS plots of blood and salivary gland from latently infected mice 45 min after i.v. injection with CFSE.

(H) Latently infected mice were given three i.v. injections of CFSE over 1 week and sacrificed 74 days later. Shown is the frequency of CFSE on inflationary and non-inflationary cells in spleen and salivary gland. Results are combined from two independent experiments (n = 6).

Error bars represent the SEM. Statistical significance was measured by unpaired (A–D) and paired (H) Student's t tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

accounts for the preferential maintenance of T_{RM} cells with inflationary specificities. Thus, we hypothesized that a continuous antigen-driven influx of inflationary T cells during latency might explain these data.

Levels of TGF-β in the Salivary Gland Are Not Markedly Altered by MCMV Infection or Latency

To investigate T_{RM} formation at late times postinfection, we first wanted to determine how viral replication and latency in the salivary gland influences the expression of TGF-β, IL-33, and TNF-α, which are critical for T_{RM} differentiation (Casey et al., 2012; El-Asady et al., 2005; Graham et al., 2014; Mackay et al., 2013; Sheridan et al., 2014). Indeed, MCMV-stimulated T cells upregulated CD103 in response to TGF-β and upregulated CD69 in response to IL-33 and TNF-α (Figures 5A and 5B), consistent with previous work (Casey et al., 2012; Skon et al., 2013). Transcripts encoding TGF-β, IL-33, and TNF-α were slightly increased at day 7 of infection with WT-MCMV (Figure 5C). However, levels of these cytokines were not markedly altered after day 7, even at late times, and the increased transcription of TGF-β did not correspond to higher levels of total or active TGF-β in the gland as a whole (Figures 5D and 5E). Infection with ΔgL-MCMV did not similarly increase TGF-β transcription (Figure 5F). These data suggest that MCMV replication and latency in the salivary gland have a minimal impact on the availability of cytokines responsible for inducing T_{RM} cells. Thus, if MCMV-specific cells were to arrive in the salivary gland at late times after infection, the available levels of cytokine and antigen are unlikely to be a limiting factor in their ability to form new T_{RM} cells.

Inflationary Cells from the Spleen Can Become T_{RM} with Reduced Efficiency

Memory inflation promotes the accumulation of T cells with an effector phenotype. To determine whether inflationary cells retain the capacity to become T_{RM}, we mixed naive OT-Is with inflationary OT-Is from the spleens of latently infected mice and co-transferred these cells into naive recipients. Infecting these mice with

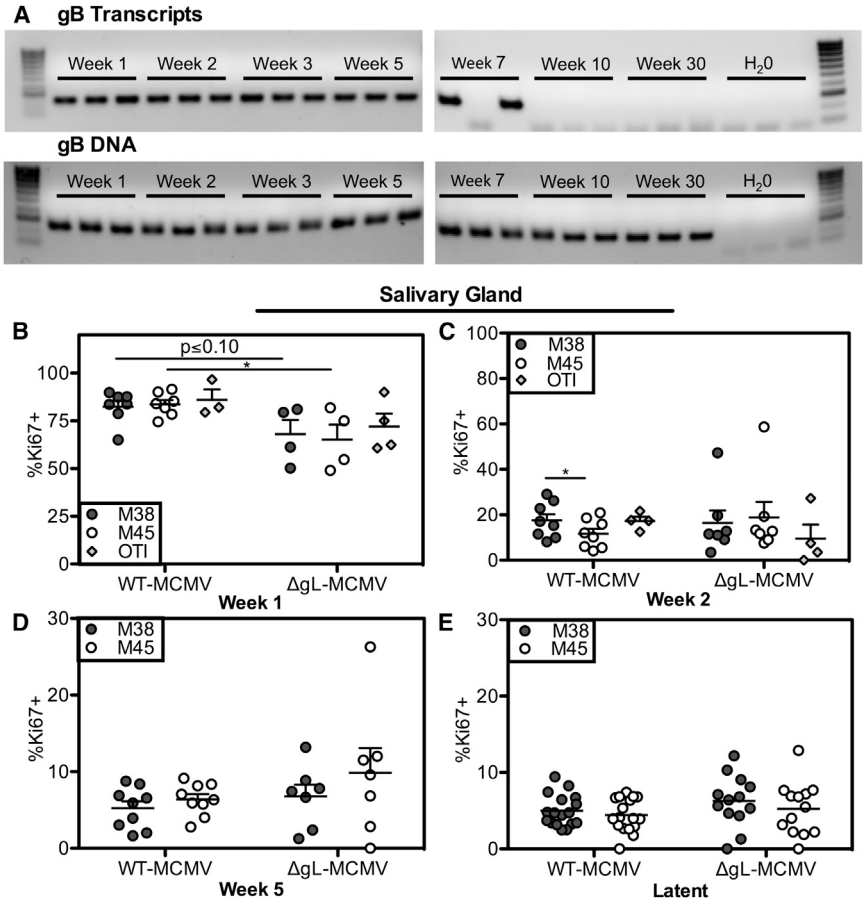

Figure 4. Viral Replication in the Salivary Gland Does Not Drive Replication of MCMV-Specific T_{RM}

(A) Nested RT-PCR for MCMV gB performed on cDNA (top) or total DNA (bottom) extracted from salivary glands of mice infected with wild-type MCMV. Each lane represents an individual mouse. Lanes labeled H_2O contained no template. All samples were positive in a PCR reaction for β-actin, and all reverse-transcriptase-negative cDNA samples were negative for gB (not shown). (B–E) Mice were infected with wild-type MCMV or ΔgL-MCMV. At 5 days postinfection, mice received transfer of OT-Is sorted from spleens of mice infected for 5 days with MCMV-OVA. Shown is the frequency of KI-67 among endogenous antigen-specific CD8s and donor OT-Is in the salivary gland at day 7 (B), day 14 (C), day 35 (D), or at latent times (E).

See also Figure S6. Error bars represent the SEM. Statistical significance was measured by unpaired Student's t tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

MCMV-OVA markedly expanded OT-Is from both donors and drove large numbers into the non-lymphoid tissues (Figure 6A). However, even though restimulated and primary OT-Is were approximately equal in the blood, restimulated OT-Is were underrepresented in the parenchyma of all organs tested and markedly so in both the salivary gland and si-IEL (Figure 6B). Importantly, similar results were obtained when naive and latent OT-Is were transferred into separate mice and challenged, ruling out the possibility that competition or an altered environment were affecting the results (not shown). In all cases, restimulated T cells were significantly less likely to express CD69 and CD103 compared to cells undergoing a primary infection (Figure 6C) consistent with previous work (Masopust et al., 2006). Indeed, when restimulated inflationary cells were treated with cytokines in vitro, they were less able to express CD103 in response to TGF-β compared to CD8s from an acute infection (Figure 6D compare to Figure 5A). Together, these data show that inflationary T cells from late times after infection can migrate to mucosal tissues and differentiate into T_{RM} upon restimulation but do so less efficiently than cells undergoing a primary response to infection.

Circulating Inflationary CD8s Traffic to and Become Resident in the Salivary Gland during Latent Infection

Inflationary cells were clearly able to respond to a new infection by becoming T_{RM} in the salivary gland. To assess the level at which this occurred in the absence of antigen, inflationary OT-Is were harvested from the spleen 1 week or >12 weeks after infection. These cells were transferred into naive mice to assess their migration to the salivary gland. Transferred OT-Is from latently infected mice infiltrated the salivary glands at a lower rate than OT-Is from 1 week postinfection (Figures 7A, 7B, and S7A). Regardless of the donor, KLRG1$^+$ cells failed to traffic to the salivary gland entirely (Figures 7A and 7B). Interestingly, T_{RM} phenotype cells were rare and almost completely absent when cells were derived from latently infected mice (Figure 7B). These data show that MCMV-specific T cells in the spleen during latency can migrate into the salivary gland at a low level irrespective of viral antigen or infection but that T_{RM} differentiation is rare without recent exposure to antigen.

To determine whether latent infection with MCMV increased the recruitment or T_{RM} differentiation of inflationary cells, we transferred CFSE-labeled OT-Is from the spleens of latently infected mice into mice latently infected with MCMV-OVA (with antigen) or WT-MCMV (lacking antigen). As above, KLRG1$^+$ OT-Is failed to access the salivary gland in all cases (Figure S7B). Comparing the number of donor OT-Is in the salivary gland to the spleens of the same mice normalized the results for variations in transfer efficiency or the impact of antigen-driven expansion. The presence of the OVA antigen slightly improved the rate of recruitment of OT-I T cells to the salivary gland over that of naive or WT infected mice (Figure 7C). However, OT-Is that made it to the salivary gland in MCMV-OVA-infected recipients were significantly more likely to have upregulated CD69 and CD103, although expression of both molecules was variable and reduced compared to OT-Is driven by a primary infection (Figures 7D and 7E). Moreover, OT-Is

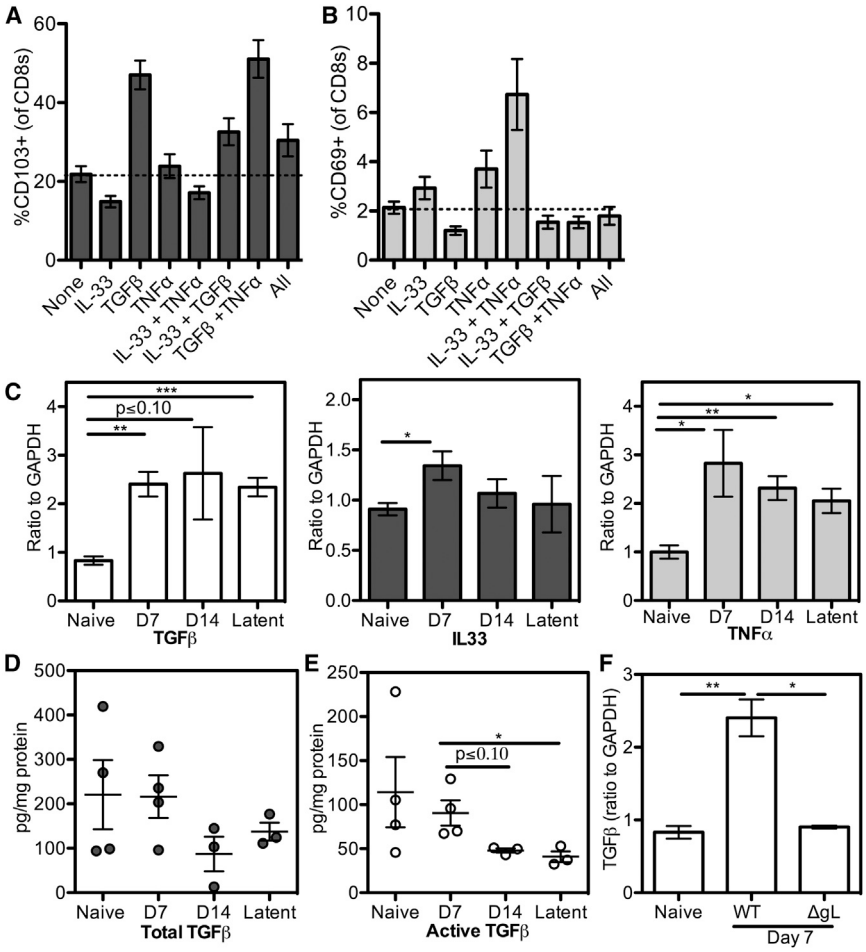

Figure 5. MCMV in the Salivary Gland Does Not Alter Cytokines Important for T_{RM} Differentiation

(A and B) Splenocytes isolated 4 days after infection with K181 MCMV were treated in vitro with TGF-β, TNF-α, and IL-33 alone or in combination for 40 hr (n = 9). Shown is the frequency of CD103 (A) and CD69 (B) on CD8s. Dotted lines indicate marker expression without cytokines.

(C) Transcription in salivary gland of the indicated cytokines normalized to GAPDH determined by qRT-PCR after infection with WT MCMV. Data shown are the average for one to four replicates per sample (n = 3 or 4 mice per group).

(D and E) Protein levels of total (D) and active (E) TGF-β determined by ELISA (n = 3 or 4 mice per group).

(F) Transcription of TGF-β in salivary gland 1 week after infection with WT MCMV or ΔgL-MCMV. Data shown are the average for one or two replicates per sample (n = 2–4 mice per group).

Error bars represent the SEM. Statistical significance was measured by unpaired Student's t tests (*p < 0.05; **p < 0.01; ***p < 0.001).

that reached the salivary gland in MCMV-OVA-infected recipients were enriched for cells that had fully diluted their CFSE (Figure S7C), and CD103-expressing OT-Is were exclusively CFSE low (Figure 7F, left), which is indicative of antigen-driven division. Interestingly, CD69 expression did not show a similar restriction and was expressed on both divided and undivided OT-Is in the salivary gland (Figure 7F, right). These data are consistent with the idea that antigen promotes T_{RM} differentiation during latency. To directly test the hypothesis that antigen stimulation outside of the salivary gland is sufficient to drive inflationary cells to become T_{RM}, we isolated spleens with inflationary OT-Is from latently infected mice, stimulated the T cells with SIINFEKL peptide in vitro, and adoptively transferred these cells into naive mice. OT-Is that had been stimulated with peptide reached the salivary gland in significantly greater numbers and upregulated T_{RM} markers more efficiently than unstimulated OT-Is, even in the absence of any inflammation or antigen in the recipient (Figures 7G, 7H, and S7D). Together, these data show that MCMV-specific T cells in the spleen during latency could migrate to the salivary gland with or without antigen at steady state but that the establishment of a T_{RM} phenotype was antigen dependent. Furthermore, antigen recognition outside of the salivary gland was sufficient to promote new T_{RM} formation and supplement established T_{RM} populations.

To estimate the recruitment of inflationary T cells at steady state, we used the numbers of salivary-gland-localized donor cells measured after transfer of inflationary OT-Is into recipients latently infected with MCMV-OVA (Figure 7C). This analysis suggests that, for every 1,000 OT-Is in the spleen, 8.6 (±2.3 SEM) OT-Is were recruited to the salivary gland over a 30-day period. Whereas this seems like a small number, it is important to note that, as a result of memory inflation, the average latently infected mouse contains 256,183 (±21,770) OT-Is in the spleen. Given these numbers, we would expect approximately 2,195 (±494.1) OT-Is to be recruited to the salivary gland over the course of 30 days, which would represent 5.5% (±1.47%) of the OT-Is in an average salivary gland. Given the variable expression of T_{RM} markers on transferred OT-Is (Figures 7D and 7E), we would not expect all of these cells to develop into new T_{RM} cells. Nevertheless, this represents a substantial pool from which new T_{RM} can be generated. Inflationary T cells are present at much higher numbers than non-inflationary populations during latent infection, precisely because they respond to viral antigen during this phase (Seckert et al., 2011; Torti et al., 2011). Thus, recruitment of inflationary populations would be heavily favored over non-inflating CD8s, which is consistent with the different maintenance of inflators and non-inflators in the salivary gland (Figure 3). Collectively, our data indicate that MCMV infections robustly induce intraepithelial T_{RM} populations independently of viral replication in the mucosa and, surprisingly, that these T_{RM} populations can be dynamically supported by continuous T_{RM} formation from the circulating pool.

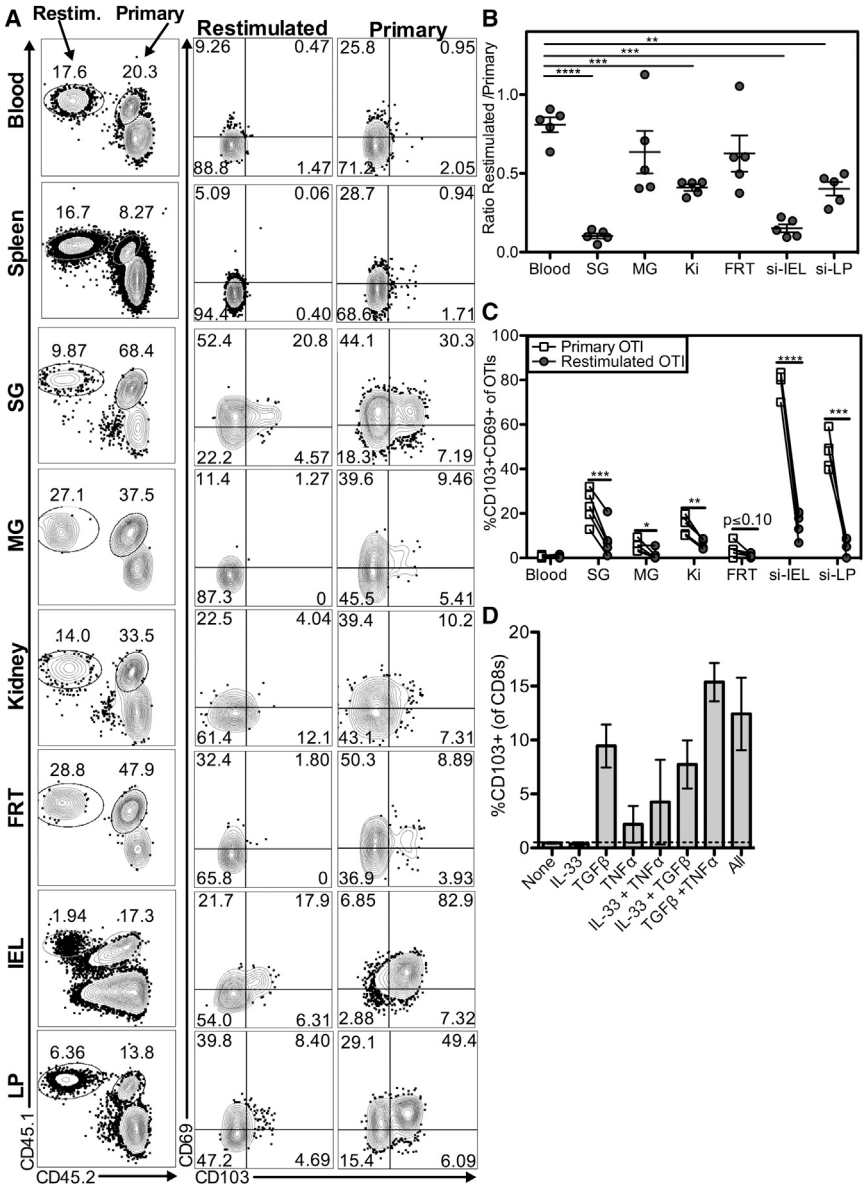

Figure 6. Inflationary CD8s Have a Reduced Capacity to Become T_{RM} after a New Infection

$CD45.1^+$ inflationary OT-Is from the spleen >12 weeks after infection and $CD45.1^+/CD45.2^+$ naive OT-Is were mixed and transferred into naive B6 recipients ($CD45.2^+$), which were then challenged with MCMV-OVA.

(A) FACS plots show frequency of OT-Is (left) and expression of CD69 and CD103 on restimulated OT-Is (middle) or OT-Is undergoing a primary response (right), in blood, spleen, or i.v. unlabeled fraction of the indicated organs 2 weeks after challenge.

(B) Ratio of restimulated to primary OT-Is in i.v. unlabeled fraction of the indicated organs.

(C) Frequency of CD103 and CD69 co-expression on restimulated and primary OT-Is in i.v. unlabeled fraction of the indicated organs. Each line connects populations in an individual mouse. Data are representative of two experiments (n = 5).

(D) Expression of CD103 on restimulated inflationary CD8s after treatment with cytokines as in Figure 5A (n = 3). Dotted line indicates CD103 expression without cytokines.

Error bars represent the SEM. Statistical significance was measured by paired Student's t tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

DISCUSSION

CMV has drawn much interest as a vaccine vector due to its unique ability to induce antigen-driven memory inflation. Most notably, work using Rhesus CMV (RhCMV) as a vaccine vector for simian immunodeficiency virus (SIV) has led to remarkable protection of the vaccinated animals (Hansen et al., 2009, 2011, 2013). These authors have speculated that CMV-based vaccines may be so effective because they can sustain large numbers of T cells at the mucosal sites of SIV (and HIV) entry (Masopust and Picker, 2012). Indeed, such T_{RM} cells are emerging as critical players in the surveillance of barrier tissues. Using the MCMV model, we have recently shown that most cells undergoing memory inflation are confined to the circulation and are not found within the parenchyma of mucosal or non-mucosal tissues (Smith et al., 2014). In this manuscript, we show that MCMV infection induces the early formation of T_{RM} cells that broadly distribute through the mucosal tissues of the body. Moreover, we found that a single-cycle spread-defective version of MCMV, which could be a useful platform on which to base a vaccine, also induced T_{RM} cells to form in multiple mucosal sites (Figure S6). Most surprisingly, we found that MCMV-driven T_{RM} populations can be dynamically supported by the recruitment of circulating T cells (Figure 7), which heavily favors the inflationary T cells. It is likely that most of these late-arriving T cells fail to differentiate into T_{RM} cells (Figures 7D and 7E). Indeed, previous work showed that T cells entering mucosal tissues from the circulation did not upregulate CD69 to the degree of cells already resident in the mucosa (Skon et al., 2013). Nevertheless, restimulation of splenic inflationary T cells could clearly drive the formation of new T_{RM} cells, albeit inefficiently, even in latently infected or naive mice (Figures 6 and 7). Thus, the large numbers of T cells stimulated by CMV-based vectors may be uniquely able to promote and sustain T_{RM} cells in multiple mucosal tissues.

The role of sustained local antigen in the formation and maintenance of T_{RM} cells is unclear. This is noteworthy because CMV persists in many sites throughout the body. Antigen is not needed for the formation of T_{RM} in the skin or small intestine after T cell priming (Casey et al., 2012; Mackay et al., 2012) and

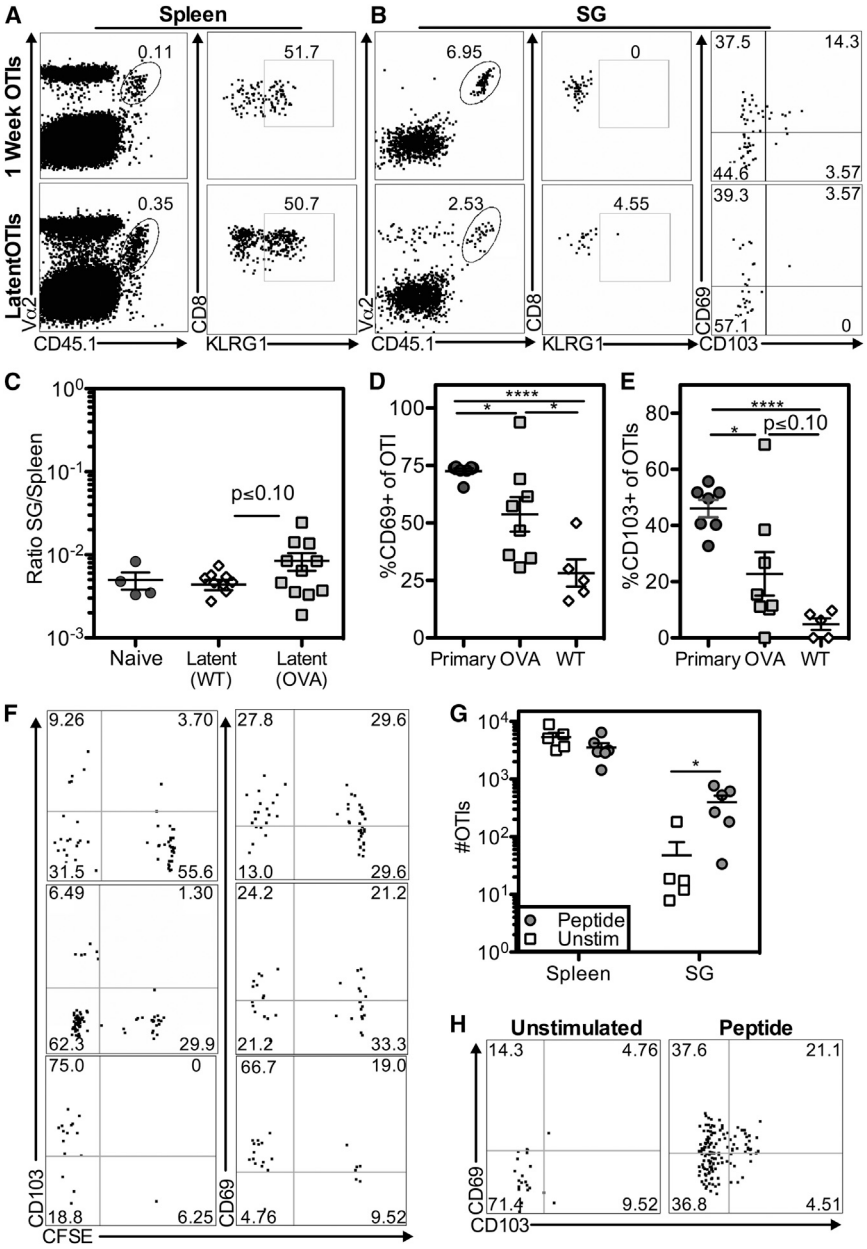

Figure 7. Inflationary CD8s Can Become T_{RM} in the Salivary Gland during Latent Infection

OT-Is were isolated from the spleens of mice at 1 week or >12 weeks after MCMV-OVA infection and transferred into congenic naive mice.

(A) FACS plots of donor OT-Is (left) and their expression of KLRG1 (right) in the spleen of recipients 2 weeks posttransfer.

(B) Donor OT-Is (left) and their expression of KLRG1 (middle) or CD69 and CD103 (right) in the salivary gland of recipients 2 weeks posttransfer.

(C–F) Mice latently infected with MCMV-OVA or MCMV-WT received CFSE-labeled OT-Is from spleens of mice >12 weeks postinfection with MCMV-OVA. (C) Ratio of the number of OT-Is in the salivary gland to the spleen of the same mouse 2–4 weeks after transfer into the indicated recipients is shown. Frequency of CD69 (D) and CD103 (E) on transferred OT-I in the parenchyma of the salivary gland compared to OT-Is in the salivary gland 4 weeks after primary infection (primary) is shown. (F) FACS plots of CFSE and CD103 or CD69 on OT-I donors in the salivary glands of latently infected MCMV-OVA recipients. Each plot shows an individual mouse.

(G and H) OT-Is from spleens of latently infected mice were harvested and stimulated with SIINFEKL in vitro for 3 hr before transfer into naive recipients. (G) Absolute numbers of OT-Is in spleen and salivary gland of naive recipients 2 weeks posttransfer are shown. (H) FACS plots of CD103 and CD69 on transferred OT-Is 2 weeks posttransfer are shown.

See also Figure S7. Error bars represent the SEM. Statistical significance was measured by unpaired Student's t tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

persistent antigen may antagonize T_{RM} in the small intestine (Casey et al., 2012), although chronic LCMV clone 13 infection may also promote sustained T cell migration to the small intestine (Zhang and Bevan, 2013). In contrast, sustained antigen has been proposed to bolster T_{RM} formation in the lung after influenza infection (Lee et al., 2011). Thus, the impact of persistent antigen on T_{RM} formation may vary by the infection or tissue. CMV infects and replicates for prolonged periods of time in the salivary gland and uses this mucosal barrier tissue as a primary means of transmission, along with breast milk, urine, and vaginal secretions (Crough and Khanna, 2009; Krmpotic et al., 2003; Kumar et al., 1984; Wu et al., 2011). We identified MCMV-driven T_{RM} in all of these sites to varying degrees. However, spread-defective ΔgL-MCMV clearly induced a similar pattern of T_{RM} formation, ruling out a direct role for viral replication in the formation of MCMV-specific mucosal T_{RM} cells. Moreover, we could find no evidence that viral antigen in the salivary gland resulted in any increase in the rate of T cell division within the salivary gland (Figure 4). Indeed, viral immune evasion genes are thought to markedly restrict $CD8^+$ T cell recognition of infected cells in the salivary gland (Walton et al., 2011). Moreover, a low dose of LCMV-WE, which does not lead to salivary gland infection, also promoted T_{RM} cells in this site (Thom et al., 2015 in this issue of *Cell Reports*). Nevertheless, the presence of antigen in latently infected mice may have caused a slight improvement in the rate of recruitment of inflationary cells to the salivary gland (Figure 7C) and clearly enhanced T_{RM} differentiation (Figures 7D and 7E). We have previously demonstrated that circulating MCMV-specific T cells undergo a constant low level of antigen stimulation primarily at sites that are accessible to the blood, such as the spleen or liver, where the virus is known to persist (Smith et al., 2014). It is clear that restimulated T cells

can traffic to the salivary gland and form new T_{RM} (Figures 6 and 7; Hofmann and Pircher, 2011), even when they were stimulated prior to infiltration of the salivary gland (Figures 7E–7G). Thus, we favor the model that, at late times after infection, the restimulation of blood-localized cells drives memory inflation and also promotes the continuous migration of T cells to the mucosa and formation of T_{RM}.

Recent work has indicated that responsiveness to TGF-β is a general requirement for the development of T_{RM} cells in multiple sites (Casey et al., 2012; El-Asady et al., 2005; Lee et al., 2011; Mackay et al., 2013; Sheridan et al., 2014; Skon et al., 2013; Zhang and Bevan, 2013), and it is thought that these TGF-β signals are received within the target tissue. TGF-β signals are particularly important for expression of CD103 on T cells, including MCMV-driven T cells (Figure 5; Thom et. al., 2015), which is thought to promote the retention of T_{RM} cells within the epithelium of the gut and lungs and the epidermis of the skin (Casey et al., 2012; El-Asady et al., 2005; Lee et al., 2011; Mackay et al., 2013; Zhang and Bevan, 2013). However, after an oral *Listeria monocytogenes* infection, CD103-deficient T cells were recruited poorly into the si-IEL but were maintained comparably to WT T cells (Sheridan et al., 2014). In agreement with this, our data suggest that CD103 expression correlates with the epithelial localization of MCMV-driven T cells in the salivary gland (Figure 2A) but that the absence of CD103 does not impair the localization or maintenance of T_{RM} after MCMV infection, even in the absence of viral antigen (Figures 2, S3, and S4).

Collectively, our data show that MCMV infection robustly induces mucosal T_{RM} cells. In the salivary gland, these cells adopt an intraepithelial localization and persist in large numbers, in part as a result of continual recruitment from the circulating T cell pool and antigen-driven differentiation. These data show that mucosal T_{RM} cells stimulated by a ubiquitous persistent infection can be maintained dynamically and support the use of CMV-based vaccine vectors to promote long-term mucosal immunity.

EXPERIMENTAL PROCEDURES

Mice and Infections

All mice were purchased from Jackson Laboratory and bred in house. C57BL/6 (B6) mice were used for all direct infections, and B6 or CD45.1 congenic mice (B6.SJL-Ptprc^a Pepc^b/BoyJ) were used as recipients for adoptive transfer experiments. OT-Is on a B6 background (C57BL/6-Tg(TcraTcrb) 1100Mjb/J) were bred to CD45.1 congenic mice (B6.SJL-Ptprc^a Pepc^b/BoyJ) or CD103$^{-/-}$ mice (B6.129S2(C)-*Itgae*^{tm1Cmp}/J) to generate congenic or CD103$^{-/-}$ OT-Is. Inflationary OT-Is were generated as described (Turula et al., 2013). For mixed WT and CD103$^{-/-}$ OT-I transfers, 1,000 splenocytes from each donor were transferred into naive mice, which were infected with MCMV-OVA 1 day later. Transfers from latently infected mice were performed as in Smith et al. (2014). For in vitro stimulation of OT-Is, splenocytes were harvested from latently infected mice harboring OT-Is and were incubated for 3 hr at 37°C at a concentration of 1×10^7 splenocytes/ml with either 1 μg/ml SIINFEKL or without peptide before transfer. MCMV K181 (WT-MCMV), MCMV K181-tfr-OVA (MCMV-OVA), and MCMV-ΔgL were produced as described (Snyder et al., 2008; Zurbach et al., 2014). In all cases, mice were infected i.p. with 2×10^5 pfu of virus. All protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Lymphocyte Isolation and FACS Staining

Lymphocytes from the blood, spleen, liver, salivary glands, mammary glands, kidneys, and female reproductive tracts were isolated as described (Smith et al., 2014). Lymphocytes from the small intestine IEL and lamina propria were isolated using the previously described protocol (Lefrançois and Lycke, 2001). Intravascular staining was performed as described recently (Smith et al., 2014). Antigen-specific non-transgenic CD8s were identified with tetramers produced at the NIH tetramer core facility (http://tetramer.yerkes.emory.edu/) as previously described (Snyder et al., 2008). Analyses of cellular phenotype and donor cells in adoptive transfers were performed as described previously (Smith et al., 2014) with the additions of antibodies specific for CD103 (clone 2E7) and CD69 (clone H1.2F3). All antibodies were purchased from Biolegend or BD Biosciences. Cells were analyzed on an LSR II flow cytometer (BD Biosciences) and using FlowJo software (TreeStar).

In Vivo Labeling

For the long-term BrdU pulse (Figure 1), mice were injected i.p. with 1 mg of BrdU (Sigma) on the day of infection and then subsequently provided with 0.8 mg/ml BrdU in their drinking water for the next 9 weeks. BrdU labeling was assayed using the BD Biosciences Flow kit. In vivo CFSE labeling was adapted from previous work (Becker et al., 2004). For the long-term pulse/chase, 45 μg of CFSE/mouse was injected retro-orbitally every other day for a total of three injections.

Immunofluorescent Microscopy

Sections of salivary glands were processed as described previously (Smith et al., 2014) and stained with antibodies against CD45.2 (clone 104), E-cadherin (clone DECMA-1), and CD103 (clone 2E7) and co-stained with DAPI (Prolong Gold antifade; Life Technologies). All antibodies were purchased from Biolegend. Images were generated with the LSM 510 Meta (Carl Zeiss) confocal laser scanning microscope and the LSM image browser software (Carl Zeiss) and analyzed with Fiji software (Schindelin et al., 2012).

PCR and ELISA

RNA was isolated from salivary glands with RNeasy Mini kit (QIAGEN), and cDNA was generated with the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). DNA was isolated using the Gentra Puregene Tissue kit (QIAGEN). Nested PCR for gB transcripts and latent gB DNA was performed as described (Cook et al., 2002). Transcript levels of TNF-α, TGF-β, and IL-33 were assessed by qPCR with a StepOnePlus system (Applied Biosystems) using SYBR green (Applied Biosystems) for detection. Primers are listed in Supplemental Information. Protein levels of active and total TGF-β were determined using Legend MAX ELISA kits (Biolegend).

In Vitro Cytokine Assay

Naive mice (Figures 5A and 5B) or naive mice that received an adoptive transfer of CD8s from latently infected mice (Figure 6D) were infected with MCMV-K181. Four days later, splenocytes were isolated and cultured for 40 hr in the presence of cytokines as described (Casey et al., 2012) and analyzed by FACS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.076.

AUTHOR CONTRIBUTIONS

Conceptualization, C.J.S. and C.M.S.; Investigation, C.J.S., S.C.-D., and H.T.; Writing, C.J.S. and C.M.S.; Funding Acquisition, C.M.S.

ACKNOWLEDGMENTS

This work was supported by grant AI106810 awarded to C.M.S.

Received: January 8, 2015
Revised: September 15, 2015
Accepted: September 24, 2015
Published: October 29, 2015

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