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

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Graphical Abstract

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

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

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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” (Holttappels 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; Shendan 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
MCMV-specific TRM cells over time. These data suggest that mucosal TRM populations driven by persistent infections can be dynamically maintained.

RESULTS

MCMV-Specific TRM 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. Naïve mice were seeded with small numbers of naïve 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 1A). Expression of CD103 and CD69, the markers of TRM 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 TRM 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 acini and ducts (Figures 1B and S2). Endogenous CD8 T cells specific for MCMV epitopes also upregulated TRM 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.
period. The ongoing antigen-driven memory inflation causes a loss of BrdU-positive inflammatory 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 TRM cells in multiple mucosal tissues and that TRM 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 TRM 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
of the same mice (Figure 2D). The ratio of CD103−/− to WT OT-Is was slightly underrepresented in the salivary gland and the si-IEL relative to the spleen (Figure 2C). In the salivary gland, the ratio of CD103−/− and WT OT-Is was more pronounced in the si-IEL over time 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 TRM 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 (Dekhtיאrenko et al., 2013), and are maintained homeostatically (Figures S5A–S5D; Smith et al., 2014; Snyder et al., 2008). Because TRM 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 TRM 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 TRM 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 TRM cells in the salivary gland. To test whether viral replication in the salivary gland affects the rate of TRM 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 TRM phenotype cells in multiple mucosal tissues after an i.p. infection (Figure S6A). Any TRM 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...
accounts for the preferential maintenance of TRM cells with inflammation-specificities. Thus, we hypothesized that a continuous antigen-driven influx of inflationary T cells during latency might explain these data.

Levels of TGF-\(\beta\) in the Salivary Gland Are Not Markedly Altered by MCMV Infection or Latency

To investigate TRM formation at late times postinfection, we first wanted to determine how viral replication and latency in the salivary gland influences the expression of TGF-\(\beta\), IL-33, and TNF-\(\alpha\), which are critical for TRM 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-\(\beta\) and upregulated CD69 in response to IL-33 and TNF-\(\alpha\) (Figures 5A and 5B), consistent with previous work (Casey et al., 2012; Skon et al., 2013). Transcripts encoding TGF-\(\beta\), IL-33, and TNF-\(\alpha\) 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-\(\beta\) did not correspond to higher levels of total or active TGF-\(\beta\) in the gland as a whole (Figures 5D and 5E). Infection with \(\Delta\)GL-MCMV did not similarly increase TGF-\(\beta\) 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 TRM 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 TRM cells.

Inflationary Cells from the Spleen Can Become TRM 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 TRM, 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...
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 under-represented 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 TRM 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 TRM 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. Regardless of the donor, KLRG1+ cells failed to traffic to the salivary gland entirely (Figures 7A and 7B). Interestingly, TRM 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 infection but that TRM 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...
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 TRM differentiation during latency. To directly test the hypothesis that antigen stimulation outside of the salivary gland is sufficient to drive inflationary cells to become TRM, 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 TRM 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 TRM phenotype was antigen dependent. Furthermore, antigen recognition outside of the salivary gland was sufficient to promote new TRM formation and supplement established TRM 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 TRM markers on transferred OT-Is (Figures 7D and 7E), we would not expect all of these cells to develop into new TRM cells. Nevertheless, this represents a substantial pool from which new TRM 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 TRM populations independently of viral replication in the mucosa and, surprisingly, that these TRM populations can be dynamically supported by continuous TRM formation from the circulating pool.
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 TRM 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 TRM 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 TRM cells to form in multiple mucosal sites (Figure S6). Most surprisingly, we found that MCMV-driven TRM 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 TRM 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 TRM 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 TRM cells in multiple mucosal tissues.

The role of sustained local antigen in the formation and maintenance of TRM cells is unclear. This is noteworthy because CMV persists in many sites throughout the body. Antigen is not needed for the formation of TRM in the skin or small intestine after T cell priming (Casey et al., 2012; Mackay et al., 2012) and
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 \( \Delta g_{L} \)-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 inflammatory 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 TRM (Figures 6 and 7; Hofmann and Pincher, 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 re-stimulation of blood-localized cells drives memory inflation and also promotes the continuous migration of T cells to the mucosa and formation of TRM.

Recent work has indicated that responsiveness to TGF-β is a general requirement for the development of TRM 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 TRM 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 TRM after MCMV infection, even in the absence of viral antigens (Figures 2, S3, and S4).

Collectively, our data show that MCMV infection robustly induces mucosal TRM 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 TRM 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-Ptprca Pepcb/BoyJ) were used as recipients for adoptive transfers. OT-1s on a B6 background (C57BL/6-Tg(TcraTcrob)107 splenocytes/ml with either 1 μg/ml SIINFEKL or without peptide before transfer. MCMV K181 (WT-MCMV), MCMV K181-trf-OVA (MCMV-OVA), and MCMV-ΔgI were produced as described (Snyder et al., 2008; Zurbach et al., 2014). In all cases, mice were infected i.p. with 2 × 10^6 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 (Lefranc and Lycke, 2001). Intravascular staining was performed as described recently (Smith et al., 2014). Antigen-specific non-transgenic CD8+ cells 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 DECA-M1), 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 CD8+ cells 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.

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