Developmental plasticity allows outside-in immune responses by resident memory T cells

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# Abstract

Central memory T (T

# Introduction

Antigen-specific CD8+ T cells protect mammalian hosts from intracellular infections. The extensive repertoire of T cells needed to protect the host from a variety of foreign antigens limits naive cell clonal abundance**{^PMC7096285\_R1$}**. Naive T cell recirculation is thus restricted to secondary lymphoid organs (SLOs), facilitating its encounter with cognate antigen presented by antigen presenting cells**{^PMC7096285\_R2$}**. After activation, CD8+ T cells proliferate to become numerically relevant and migrate outwards to nonlymphoid tissues to seek infected cells**{^PMC7096285\_R3$}**. After a return to homeostasis, clonally expanded memory T cells (relative to their naive predecessors) are left behind, and persist in lymphoid and nonlymphoid tissues, providing enhanced protection against subsequent infections**{^PMC7096285\_R4$}{^PMC7096285\_R5$}{^PMC7096285\_R6$}{^PMC7096285\_R7$}{^PMC7096285\_R8$}**.

Memory T cells are functionally specialized and often partitioned into putatively discrete subsets with uncertain developmental relationships**{^PMC7096285\_R9$}{^PMC7096285\_R10$}{^PMC7096285\_R11$}{^PMC7096285\_R12$}{^PMC7096285\_R13$}**. Like naive T cells, TCM recirculate amongst lymph nodes (LNs), and when reactivated, fulfill the canonical properties of self-driven expansion, differentiation into diverse T cell types, and acquisition of new homing properties**{^PMC7096285\_R10$}**,**{^PMC7096285\_R14$}**. Effector memory T cells (TEM) are a heterogeneous population that patrols blood**{^PMC7096285\_R12$}**,**{^PMC7096285\_R15$}**. Immune surveillance of nonlymphoid tissues is mostly assumed by TRM that park within tissues during the effector phase of the response**{^PMC7096285\_R16$}{^PMC7096285\_R17$}{^PMC7096285\_R18$}{^PMC7096285\_R19$}**. TRM act as first responders against local reinfection and accelerate pathogen control**{^PMC7096285\_R7$}**,**{^PMC7096285\_R20$}**,**{^PMC7096285\_R21$}**. Indeed, they share many properties with recently activated effector T cells, supporting that they may constitute a terminally differentiated population**{^PMC7096285\_R11$}**,**{^PMC7096285\_R22$}**,**{^PMC7096285\_R23$}**.

In summary, in the event of reinfection at barrier sites, immune organisms have an opportunity for local control by TRM cells. If that immunity fails, the recall response can be modeled as a faster recapitulation of a primary response, originating in LNs, but being driven by TCM instead of naive T cells. This can be visualized as an ‘inside-out’ model, where immune responses originate inside LNs and migrate out toward peripheral tissues. This model fails to capture the observation that TRM cells proliferate**{^PMC7096285\_R24$}**,**{^PMC7096285\_R25$}** and contribute to durable expansion of the local memory population in response to antigen restimulation**{^PMC7096285\_R26$}**. Here, we show that re-stimulated TRM cells undergo retrograde migration, exhibit developmental plasticity, join the circulation, give rise to TCM and TEM cells, yet retain biased homing and TRM differentiation potential. Collectively, this supports a new ‘outside-in’ model of protective immunity.

# Results

To assess whether local reactivation of TRM cells precipitates egress to circulation, we generated C57BL/6J mice that contained CD90.1+ OT-I TRM cells within skin through Vesicular stomatitis virus expressing ovalbumin (VSVova) viral infection (OT-I chimeras, see Methods). After viral clearance, skin was engrafted onto infection matched CD45.1+ OT-I immune chimeric C57BL/6J mice. 30 days later, we reactivated TRM cells within the skin graft by injecting SIINFEKL peptide, which is recognized by OT-I T cells (Fig. 1a). 2–3 weeks later, displaced residents were observed within the draining lymph node, and circulating TCM and TEM cells were observed in distant lymph nodes (Fig. 1b), suggesting that reactivated TRM may give rise to TRM, TEM, and TCM cells.

To further test TRM retrograde migration and plasticity, we depleted CD90.1+ circulating TEM and TCM from OT-I chimeras via titrated injection of depleting anti-CD90.1 antibody (which depletes circulating cells while sparing many TRM cells**{^PMC7096285\_R27$}**). As a control, these mice were seeded with an independent population of undepleted CD90.1–CD45.1+ circulating OT-I memory T cells. Mice were then challenged with SIINFEKL peptide in the skin (Fig. 1c). 10 days later, CD90.1+ OT-I appeared in the blood, and many of these cells transiently retained a phenotype that distinguish skin TRM from circulating TCM and TEM (CD103+ CD49ahiLy6Clo) and exhibited other properties shared by TRM and long lived TEM, including lack of KLRG1 and CD62L expression (Fig. 1d and e). We performed similar experiments, except TRM were reactivated in the female reproductive tract (FRT) (Fig. 1f). Ex-TRM appeared in blood within 10 days, and these cells bore marks reminiscent of mucosal TRM, including slight underexpression of CD44 and Ly6C relative to circulating memory T cells (Fig. 1g). These observations suggest that TRM cells exhibit migrational plasticity and undergo retrograde migration after re-stimulation.

It was previously reported that antigen rechallenge at barrier sites induced CD69+ TRM within draining lymph nodes and that these cells had emigrated from the upstream nonlymphoid tissue**{^PMC7096285\_R28$}**. Here, we transferred naive P14 CD8+ T cells to naive mice, and infected recipients with Lymphocytic choriomeningitis virus (LCMV, Armstrong strain) intraperitoneally (i.p.) the following day. This established memory P14 CD8+ T cells throughout organism, including within the FRT**{^PMC7096285\_R29$}**. 30 days later, we treated the mice with FTY720 for 8 days, which inhibits S1P-mediated cell egress**{^PMC7096285\_R30$}**. On the second day of treatment, cognate gp33 peptide was delivered transcervically to reactivate local P14 memory CD8+ T cells**{^PMC7096285\_R27$}**,**{^PMC7096285\_R31$}**. When draining iliac lymph nodes were assessed 30 days later, we observed a substantial reduction in CD69+ LN TRM cells in mice that were treated with FTY720 during T cell activation (Supplementary Fig. 1). These data suggest that S1P contributes to the egress of reactivated T cells from nonlymphoid tissues.

We wished to compare the potential developmental plasticity of CD8+ TRM cells with other CD8+ T cell lineages including naive, TCM and TEM cells. We focused on small intestine intraepithelial lymphocytes (SI IEL) TRM because they are uniformly (>99%) resident after LCMV infection based on parabiosis studies**{^PMC7096285\_R29$}**. Moreover, SI IEL TRM express a highly differentiated TRM phenotype (CD103+, CD69+, granzyme B+, CD62L-, Ly6Clo, IL-1MP5Rblo), whereas CD8+ T cells, including TRM cells in other tissues, are more heterogeneous**{^PMC7096285\_R22$}**,**{^PMC7096285\_R32$}**,**{^PMC7096285\_R33$}** (Fig. 2a). SI IEL TRM also express CCR9 (Fig. 2a). To generate naive, early and late effector, and memory CD8+ T cell subsets expressing identical TCRs, we transferred naive CD90.1+ P14 CD8+ T cells to naive C57BL/6J mice. The following day, mice were infected with 2×105 PFU LCMV Armstrong i.p., which causes an infection that is cleared within approximately one week**{^PMC7096285\_R34$}**. Four and eight days later, effector cells were sorted into memory precursor cells (MPs, CD127hiKLRG1lo) and terminal effector cells (TEs, CD127loKLRG1hi). Memory P14 were isolated from LN or spleen at least three months later and sorted into CD62L+ (TCM) and CD62L- (TEM) subsets or were isolated from small intestine epithelium and sorted to ensure a uniform CD103+CD69+CD62L-Ly6Clo (TRM) phenotype. Naive CD62L+ cells were sorted from LNs of naive CD90.1+ P14 Tg mice.

The transcriptome of LCMV-specific P14 TRM cells isolated from gut has previously been reported**{^PMC7096285\_R35$}**. Principal component analysis (PCA) revealed that TCM and TEM cells were nearly identical and more similar to naive T cells than TRM cells (published dataset reanalyzed in Fig. 2b), supporting the contention that TRM cells represent a distinct cell type or lineage.

Transcriptional profiling indicates what genes are currently being transcribed by a cell population. However, it does not inform which genes have the potential to be expressed under changing conditions or as a result of external stimuli. For instance, mRNA profiling fails to capture key biological differences between resting naive and memory T cells, such as the ability to synthesize IFNγ rapidly in the event of antigen recognition**{^PMC7096285\_R36$}**. Whole genome bisulfite sequencing (WGBS) indicates which genes have been silenced by DNA methylation. In other words, it provides a readout more closely aligned with gene expression potential, rather than an indication of which genes are actively undergoing transcription. We performed whole genome bisulfite sequencing (WGBS) on naive, TCM, TEM, and TRM cell subsets (Fig. 2c). Because TRM cells share phenotypic properties with effector T cells, as a basis of comparison we also analyzed recently primed MPs and TEs.

Principal component analyses (PCA) of genome wide CpG methylation status in naive, recently activated effector cells, TCM, TEM, and TRM cells revealed that there was little variance in methylation status in the memory T cell subsets. Specifically, the TCM, TEM, and TRM cell subsets clustered together in a separate cluster while naive cells and the recently activated effector subsets were in separate clusters (Fig. 2c). Supplementary Fig. 2a details the methylation status of several TRM specific genes among the naive, effector, and memory T cell subsets. It should also be noted that even when effector cells were removed from PCA, all memory subsets clustered together and away from naive T cells (Supplementary Fig. 2b). These data suggest that although TRM cells share some phenotypic signatures with recently activated effector T cells (Fig. 2a), they may be resting memory T cells at the epigenetic level and share commonalities with other resting memory T cell subsets, including TCM cells.

To explore developmental potential, we subjected our WGBS methylation datasets to machine learning algorithms designed to assign relative plasticity among cell types**{^PMC7096285\_R37$}**. Here naive T cells, which biologically exhibit the most multipotency, are assigned a score of 1. Exhausted CD8+ T cells exhibit a plasticity score of close to zero, in keeping with numerous observations supporting senescence and little developmental potential. Here we found that the TRM plasticity score was intermediate between that defined for TCM and TEM, raising the possibility that TRM cells may not be as terminally differentiated as previously proposed. Recently activated cells had plasticity scores that were almost as high as TRM at D4 but diminished by D8 (Fig. 2d). Interestingly, SI IEL have been shown to be seeded approximately 4 days after infection in this LCMV infection model, and by day 7, effector cell migration to the SI is significantly diminished**{^PMC7096285\_R32$}**. Moreover, KLRG1+ CD8+ T cells (a subset usually associated with terminal differentiation) are largely incapable of differentiating into CD103+ TRM cells**{^PMC7096285\_R38$}{^PMC7096285\_R39$}{^PMC7096285\_R40$}**. Although our PCA analysis, which was based on the DNA methylation status of the CD8+ T cells, broadly segregates the effector subsets from the memory subsets, our machine learning-derived plasticity index supported the hypothesis that the developmental potential of TRM cells (as well as TCM cells) is more comparable to CD8+ T cells that have not undergone terminal differentiation.

The results above (Fig. 1 and 2) raised the possibility that TRM cells may have developmental plasticity rather than represent a terminal effector stage of differentiation. This concept had been explored years prior, but without sorting cells on TRM markers, leaving interpretation subjective**{^PMC7096285\_R32$}**. To address this question, 90 days after transfer of naive P14 CD8+ T cells and LCMV infection, memory P14 cells were isolated from pooled lymph nodes and sorted into CD62L+ (TCM) or spleen into CD62L– (TEM) cells. In addition, P14 were isolated from SI IEL and sorted to ensure a uniform CD103+CD69+CD62L–Ly6Clo (TRM) phenotype (see Fig. 3a for pre- and post-sort analysis).

To directly test developmental plasticity, 20,000 sorted naive, TCM, TEM, or SI IEL TRM P14 cells were transferred i.v. into separate naive C57BL/6J recipients (Fig. 3a). Mice were then infected with LCMV, and the primary or recall response from transferred memory cells was monitored in blood (Fig. 3b). We observed that CD62L was gradually upregulated at the population level in blood by all subsets except TEM (Fig. 3c). Hierarchically, naive T cells most rapidly produced CD62L+ memory T cell progeny, followed by TCM, then TRM cells (Fig. 3c and d).

These data indicate that after isolation and re-stimulation, purified bona fide SI IEL TRM cells have the capacity to differentiate into TCM cells. Consistent with TRM developmental plasticity, we found that bloodborne secondary Ex-TRM cells had downregulated CD69 and CD103 (Fig. 3e). However, it should be noted that we found phenotypic traces of their non-lymphoid history imprinted on circulating secondary Ex-TRM cells: both Ly6C and CCR9 expression only slowly conformed to the canonical circulating phenotype, and even at the latest time point analyzed (100 days after re-stimulation), bloodborne secondary Ex-TRM cells still exhibited phenotypic traces of their former tissue of residence (Fig. 3f and g).

We next assessed the anatomic distribution of the progeny of transferred and re-stimulated TCM and TRM cells performed in Fig. 3. Again, primary memory T cells after transfer of naive P14 T cells was included as a basis of comparison. In spleen, TCM cells produced more secondary memory CD8+ T cells than did re-stimulated SI IEL TRM cells. However, the progeny of intravenously transferred SI IEL maintained a predilection for repopulating the small intestine and were observed in both the lamina propria (SI LP) and epithelium by immunohistochemistry (Fig. 4a, b and d). This propensity did not extend to other TRM compartments, such as salivary gland (SG) and FRT (Fig. 4a and b). Similar observations were made after transfer and recall of polyclonal endogenous N-specific CD8+ TRM cells isolated from SI IEL of VSV-infected mice (Supplementary Fig. 3). Accordingly, previous evidence suggested that T cells isolated from SG or lung retained a predilection for their tissue of origin**{^PMC7096285\_R41$}**,**{^PMC7096285\_R42$}**.

If re-stimulated SI IEL returned to the small intestine, they retained the naive T cell-like capacity to acquire canonical site-associated residence signatures, including high expression of CD103 and CD69, and low expression of Ly6C (Fig. 4c). In contrast, TCM progeny were moderately less likely to express CD103 and down regulate Ly6C. Secondary memory SI IEL that derived from transferred TRM cells also maintained higher expression of Granzyme B long after clearance of LCMV Armstrong infection (Fig. 4c). These data indicate that although SI IEL TRM exhibited developmental plasticity (Fig. 2), compared to TCM cells they retain a bias to home to their parental tissue and reacquire SI IEL TRM signatures.

To further test imprinting of memory T cell fates, we tested trans-generational developmental plasticity. First, P14 immune chimeras were generated as described in Fig. 3, which provided a source of flow sorted primary CD90.1+CD62L+ TCM and CD103+CD69+CD62L–Ly6Clo SI IEL TRM cells. 20,000 of each population was transferred into separate recipients, followed by LCMV infection to induce a recall response. 100 days later, the secondary memory progeny of each population (referred to as 2º Ex-TCM and 2º Ex-TRM, respectively) were isolated from spleen (phenotype shown in Fig. 3e and Supplementary Fig. 4). 1×105 P14 cells (or 2.5×104 in some experiments) of each population were transferred to new naive recipient mice, these mice were infected with LCMV to induce a tertiary immune response, and the fate of donor cells was evaluated 50 days later. Primary memory cells and secondary Ex-TCM cells provided a basis for comparison (Fig. 5a).

Tertiary Ex-TRM cells retained properties of primary TRM cells, even after proliferating and differentiating outside of the mucosa (as secondary Ex-TRM cells were isolated from spleen). For instance, the population retained its predilection to repopulate the intestinal mucosal epithelium and to reacquire the phenotype observed among primary SI IEL TRM cells (Fig. 5b-e). In contrast, tertiary Ex-TCM cells increasingly deviated from the acquisition of a canonical SI IEL TRM phenotype. These data imply that induction of a TRM differentiation program during the primary response can influence subsequent fate upon recall, even when the cells are removed from the tissue where the residence program was acquired, and this program could be maintained through two generations of proliferation and transfer. In other words, these data indicate that a history of residence is epigenetically maintained despite the developmental plasticity of TRM cells.

We further note that in the absence of reinfection upon transfer, neither secondary Ex-TRM nor Ex-TCM cells were recovered from the small intestine (Supplementary Fig. 5), consistent with a model whereby secondary Ex-TRM cells require restimulation to migrate and re-differentiate into mucosal residents.

TRM precursors are thought to differentiate in response to local cytokine cues encountered upon migration to nonlymphoid tissues**{^PMC7096285\_R22$}**,**{^PMC7096285\_R32$}**,**{^PMC7096285\_R38$}**,**{^PMC7096285\_R43$}**. Here, we compared the capacity of Ex-TRM, Ex-TCM, and Ex-TEM cells (generated as in figure 3, except cells were isolated 70 days after secondary infection) to acquire TRM signatures after culture with TGFβ, IL-2, and IL-15. Ex-TRM cells were most poised to adopt SI IEL TRM signatures in response to cytokines, including upregulation of CD69 and CCR9 and down regulation of Ly6C (Fig. 6a and b).

# Local reactivation of T

To assess whether local reactivation of TRM cells precipitates egress to circulation, we generated C57BL/6J mice that contained CD90.1+ OT-I TRM cells within skin through Vesicular stomatitis virus expressing ovalbumin (VSVova) viral infection (OT-I chimeras, see Methods). After viral clearance, skin was engrafted onto infection matched CD45.1+ OT-I immune chimeric C57BL/6J mice. 30 days later, we reactivated TRM cells within the skin graft by injecting SIINFEKL peptide, which is recognized by OT-I T cells (Fig. 1a). 2–3 weeks later, displaced residents were observed within the draining lymph node, and circulating TCM and TEM cells were observed in distant lymph nodes (Fig. 1b), suggesting that reactivated TRM may give rise to TRM, TEM, and TCM cells.

To further test TRM retrograde migration and plasticity, we depleted CD90.1+ circulating TEM and TCM from OT-I chimeras via titrated injection of depleting anti-CD90.1 antibody (which depletes circulating cells while sparing many TRM cells**{^PMC7096285\_R27$}**). As a control, these mice were seeded with an independent population of undepleted CD90.1–CD45.1+ circulating OT-I memory T cells. Mice were then challenged with SIINFEKL peptide in the skin (Fig. 1c). 10 days later, CD90.1+ OT-I appeared in the blood, and many of these cells transiently retained a phenotype that distinguish skin TRM from circulating TCM and TEM (CD103+ CD49ahiLy6Clo) and exhibited other properties shared by TRM and long lived TEM, including lack of KLRG1 and CD62L expression (Fig. 1d and e). We performed similar experiments, except TRM were reactivated in the female reproductive tract (FRT) (Fig. 1f). Ex-TRM appeared in blood within 10 days, and these cells bore marks reminiscent of mucosal TRM, including slight underexpression of CD44 and Ly6C relative to circulating memory T cells (Fig. 1g). These observations suggest that TRM cells exhibit migrational plasticity and undergo retrograde migration after re-stimulation.

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# Epigenetic profiling of T

We wished to compare the potential developmental plasticity of CD8+ TRM cells with other CD8+ T cell lineages including naive, TCM and TEM cells. We focused on small intestine intraepithelial lymphocytes (SI IEL) TRM because they are uniformly (>99%) resident after LCMV infection based on parabiosis studies**{^PMC7096285\_R29$}**. Moreover, SI IEL TRM express a highly differentiated TRM phenotype (CD103+, CD69+, granzyme B+, CD62L-, Ly6Clo, IL-1MP5Rblo), whereas CD8+ T cells, including TRM cells in other tissues, are more heterogeneous**{^PMC7096285\_R22$}**,**{^PMC7096285\_R32$}**,**{^PMC7096285\_R33$}** (Fig. 2a). SI IEL TRM also express CCR9 (Fig. 2a). To generate naive, early and late effector, and memory CD8+ T cell subsets expressing identical TCRs, we transferred naive CD90.1+ P14 CD8+ T cells to naive C57BL/6J mice. The following day, mice were infected with 2×105 PFU LCMV Armstrong i.p., which causes an infection that is cleared within approximately one week**{^PMC7096285\_R34$}**. Four and eight days later, effector cells were sorted into memory precursor cells (MPs, CD127hiKLRG1lo) and terminal effector cells (TEs, CD127loKLRG1hi). Memory P14 were isolated from LN or spleen at least three months later and sorted into CD62L+ (TCM) and CD62L- (TEM) subsets or were isolated from small intestine epithelium and sorted to ensure a uniform CD103+CD69+CD62L-Ly6Clo (TRM) phenotype. Naive CD62L+ cells were sorted from LNs of naive CD90.1+ P14 Tg mice.

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# Transdifferentiation of T

The results above (Fig. 1 and 2) raised the possibility that TRM cells may have developmental plasticity rather than represent a terminal effector stage of differentiation. This concept had been explored years prior, but without sorting cells on TRM markers, leaving interpretation subjective**{^PMC7096285\_R32$}**. To address this question, 90 days after transfer of naive P14 CD8+ T cells and LCMV infection, memory P14 cells were isolated from pooled lymph nodes and sorted into CD62L+ (TCM) or spleen into CD62L– (TEM) cells. In addition, P14 were isolated from SI IEL and sorted to ensure a uniform CD103+CD69+CD62L–Ly6Clo (TRM) phenotype (see Fig. 3a for pre- and post-sort analysis).

To directly test developmental plasticity, 20,000 sorted naive, TCM, TEM, or SI IEL TRM P14 cells were transferred i.v. into separate naive C57BL/6J recipients (Fig. 3a). Mice were then infected with LCMV, and the primary or recall response from transferred memory cells was monitored in blood (Fig. 3b). We observed that CD62L was gradually upregulated at the population level in blood by all subsets except TEM (Fig. 3c). Hierarchically, naive T cells most rapidly produced CD62L+ memory T cell progeny, followed by TCM, then TRM cells (Fig. 3c and d).

These data indicate that after isolation and re-stimulation, purified bona fide SI IEL TRM cells have the capacity to differentiate into TCM cells. Consistent with TRM developmental plasticity, we found that bloodborne secondary Ex-TRM cells had downregulated CD69 and CD103 (Fig. 3e). However, it should be noted that we found phenotypic traces of their non-lymphoid history imprinted on circulating secondary Ex-TRM cells: both Ly6C and CCR9 expression only slowly conformed to the canonical circulating phenotype, and even at the latest time point analyzed (100 days after re-stimulation), bloodborne secondary Ex-TRM cells still exhibited phenotypic traces of their former tissue of residence (Fig. 3f and g).

# Developmental plasticity and tissue redistribution of T

We next assessed the anatomic distribution of the progeny of transferred and re-stimulated TCM and TRM cells performed in Fig. 3. Again, primary memory T cells after transfer of naive P14 T cells was included as a basis of comparison. In spleen, TCM cells produced more secondary memory CD8+ T cells than did re-stimulated SI IEL TRM cells. However, the progeny of intravenously transferred SI IEL maintained a predilection for repopulating the small intestine and were observed in both the lamina propria (SI LP) and epithelium by immunohistochemistry (Fig. 4a, b and d). This propensity did not extend to other TRM compartments, such as salivary gland (SG) and FRT (Fig. 4a and b). Similar observations were made after transfer and recall of polyclonal endogenous N-specific CD8+ TRM cells isolated from SI IEL of VSV-infected mice (Supplementary Fig. 3). Accordingly, previous evidence suggested that T cells isolated from SG or lung retained a predilection for their tissue of origin**{^PMC7096285\_R41$}**,**{^PMC7096285\_R42$}**.

If re-stimulated SI IEL returned to the small intestine, they retained the naive T cell-like capacity to acquire canonical site-associated residence signatures, including high expression of CD103 and CD69, and low expression of Ly6C (Fig. 4c). In contrast, TCM progeny were moderately less likely to express CD103 and down regulate Ly6C. Secondary memory SI IEL that derived from transferred TRM cells also maintained higher expression of Granzyme B long after clearance of LCMV Armstrong infection (Fig. 4c). These data indicate that although SI IEL TRM exhibited developmental plasticity (Fig. 2), compared to TCM cells they retain a bias to home to their parental tissue and reacquire SI IEL TRM signatures.

# Ex-T

To further test imprinting of memory T cell fates, we tested trans-generational developmental plasticity. First, P14 immune chimeras were generated as described in Fig. 3, which provided a source of flow sorted primary CD90.1+CD62L+ TCM and CD103+CD69+CD62L–Ly6Clo SI IEL TRM cells. 20,000 of each population was transferred into separate recipients, followed by LCMV infection to induce a recall response. 100 days later, the secondary memory progeny of each population (referred to as 2º Ex-TCM and 2º Ex-TRM, respectively) were isolated from spleen (phenotype shown in Fig. 3e and Supplementary Fig. 4). 1×105 P14 cells (or 2.5×104 in some experiments) of each population were transferred to new naive recipient mice, these mice were infected with LCMV to induce a tertiary immune response, and the fate of donor cells was evaluated 50 days later. Primary memory cells and secondary Ex-TCM cells provided a basis for comparison (Fig. 5a).

Tertiary Ex-TRM cells retained properties of primary TRM cells, even after proliferating and differentiating outside of the mucosa (as secondary Ex-TRM cells were isolated from spleen). For instance, the population retained its predilection to repopulate the intestinal mucosal epithelium and to reacquire the phenotype observed among primary SI IEL TRM cells (Fig. 5b-e). In contrast, tertiary Ex-TCM cells increasingly deviated from the acquisition of a canonical SI IEL TRM phenotype. These data imply that induction of a TRM differentiation program during the primary response can influence subsequent fate upon recall, even when the cells are removed from the tissue where the residence program was acquired, and this program could be maintained through two generations of proliferation and transfer. In other words, these data indicate that a history of residence is epigenetically maintained despite the developmental plasticity of TRM cells.

We further note that in the absence of reinfection upon transfer, neither secondary Ex-TRM nor Ex-TCM cells were recovered from the small intestine (Supplementary Fig. 5), consistent with a model whereby secondary Ex-TRM cells require restimulation to migrate and re-differentiate into mucosal residents.

# Ex-T

TRM precursors are thought to differentiate in response to local cytokine cues encountered upon migration to nonlymphoid tissues**{^PMC7096285\_R22$}**,**{^PMC7096285\_R32$}**,**{^PMC7096285\_R38$}**,**{^PMC7096285\_R43$}**. Here, we compared the capacity of Ex-TRM, Ex-TCM, and Ex-TEM cells (generated as in figure 3, except cells were isolated 70 days after secondary infection) to acquire TRM signatures after culture with TGFβ, IL-2, and IL-15. Ex-TRM cells were most poised to adopt SI IEL TRM signatures in response to cytokines, including upregulation of CD69 and CCR9 and down regulation of Ly6C (Fig. 6a and b).

# Discussion

Our data indicate that TRM cells share more epigenetic signatures with circulating memory T cell subsets than recently activated effector T cells and furthermore, support a model by which TRM cells express the memory-like qualities of anamnestic expansion, migration, and differentiation after antigen recognition. This contrasts with models that view TRM as effector-like immediate responders that are terminally differentiated, with host recall responses relying on antigen dissemination to draining SLOs where anamnestic recall responses are induced solely by TCM cells.

Primary immune responses can be considered ‘inside-out’, meaning they are induced in deeper tissues (e.g. LNs that drain barrier sites of infection), and then proliferate and migrate out towards infected tissues. This topology is likely compelled by adaptive immune systems that rely on extreme clonal diversity. Our data support the existence of ‘outside-in’ recall immune responses, whereby anamnestic responses are initiated and expand at frontline sites of infection and tissue barriers by local non-lymphoid TRM cells**{^PMC7096285\_R26$}**,**{^PMC7096285\_R31$}**, and form progeny that redistribute and even contribute to the circulating memory T cell pool. We speculate that this might provide some advantages for maintaining host protective immunity. For instance, in the event of reactivation, Ex-TRM cells from SI IEL retained a bias to repopulate the intestinal mucosa and the capacity to reacquire TRM signatures after arrival. In contrast, iterative re-stimulation of TCM and Ex-TCM cells resulted in populations of cells that lose TRM differentiation capacity. Thus, in the event that TRM-mediated front-line immunity wanes, or if an iterative environmental re-exposure were to exceed the capacity of local TRM cells to contain the infection or antigen locally, previous exposures could have populated the circulating memory T cell compartment with cells predisposed to preferentially migrate back to the parent tissue and to re-establish local resident immunity. Such a process could better prepare the organism for defense against future reinfections. The fact that Ex-TRM cells share minor phenotypic commonalities with their TRM predecessors raises the possibility that analysis of blood could, in theory, give some indication of tissue-specific immunity.

This study demonstrates that naive T cells exhibit the greatest developmental plasticity, whereas both TCM and TRM cells bias against producing reciprocal subsets. However, this biasing is not absolute. TRM cells are not terminally differentiated. And because both TCM and TRM cells can interconvert, it indicates that each subset is not a fixed discrete cell type or lineage, which rejects many models of memory T cell subset ontogeny, particularly those that define linear unidirectional subset relationships. We did note that SI IEL did not proliferate as well as TCM cells upon i.v. transfer and reinfection. This might be due to extrinsic variables, for instance TRM cells may not survive well or may not migrate to locations optimized for reactivation after isolation and transfer. These issues might be unphysiological products of experimental design. Alternatively, there may be cell intrinsic differences in activation and proliferation potential. Perhaps both intrinsic and extrinsic variables explain differences in observed TRM and TCM expansion.

As KLRG1 expression is sometimes associated with terminal T cell differentiation, our data is consistent with observations that TRM derive from KLRG1lo precursors**{^PMC7096285\_R38$}**,**{^PMC7096285\_R39$}**. That said, some contexts may not allow productive TRM recall responses that expand the population, lead to a redistribution of TRM, or reveal developmental plasticity, as reported for HSV-specific recall responses in mouse skin**{^PMC7096285\_R25$}**. It is unclear whether these differences are related to HSV-specific memory or other aspects of site-specific immunity, but we previously found that VSV-specific memory T cells positioned in skin were capable of recall responses that expanded the local population**{^PMC7096285\_R26$}**.

It was previously observed that LN TRM cells accumulate in regional lymph nodes after local reinfection**{^PMC7096285\_R28$}**. We further demonstrated that LN TRM cells derived from cells previously present in the upstream nonlymphoid tissue, and we showed evidence of retrograde migration. Whether these cells derived from either bona fide nonlymphoid TRM cells or transient migrants was not concluded. It should also be noted that T cells can recirculate through nonlymphoid tissues**{^PMC7096285\_R15$}**,**{^PMC7096285\_R44$}{^PMC7096285\_R45$}{^PMC7096285\_R46$}{^PMC7096285\_R47$}**. Retrograde migration occurs from skin xenografts by CD4+ T cells that retain TRM markers**{^PMC7096285\_R33$}**,**{^PMC7096285\_R48$}**. As these cells were postulated to recirculate in the steady state (reenter skin from blood in the absence of intentional restimulation), it is unclear whether this phenomenon relates to the Ex-TRM biology we describe, or rather indicates that nonlymphoid recirculating cells may retain some markers in common with residents**{^PMC7096285\_R2$}**,**{^PMC7096285\_R44$}{^PMC7096285\_R45$}{^PMC7096285\_R46$}**. Indeed both resident and recirculating memory CD4+ T cells have been identified in skin**{^PMC7096285\_R33$}**,**{^PMC7096285\_R47$}**. CD69+ T cells are increased in the blood of psoriatic arthritis patients and CD103+ T cells appear in human celiac disease patients after in vivo challenge with gluten**{^PMC7096285\_R49$}**,**{^PMC7096285\_R50$}**. Our study raises the possibility that these phenomena may be accounted for by Ex-TRM cells.

In summary, this study demonstrates that TRM cells share key features of developmental and migration plasticity with circulating memory T cells, including TCM cells. Further evidence indicates that Ex-TRM cells may shape the circulating pool to be predisposed to mount site-specific recall responses that preferentially maintain TRM redifferentiation capacity.

# Materials and Methods

C57BL/6J female mice were purchased from The Jackson Laboratory and were maintained in specific-pathogen-free conditions at the University of Minnesota. CD90.1+ P14, CD45.1+ P14, CD90.1+ OT-I, and CD45.1+ OT-I mice were fully backcrossed to C57BL/6J mice and maintained in our animal colony. B6.SJL mice were purchased from JAX and bred in-house. All mice used were 6–10 weeks of age and used in accordance with the Institutional Animal Care and Use Committees guidelines at the University of Minnesota.

We generated P14 immune chimeras by transferring 5×104 naive CD90.1+ or CD45.1+ P14 T cells i.v. into naive C57BL/6J mice and infecting mice with 2×105 plaque-forming units (PFU) of LCMV (Armstrong strain) i.p. the following day. OT-I immune chimeras were generated by transferring 5X104 naïve CD90.1+ or CD45.1+ OT-I CD8+ T cells into naive C57BL/6J mice. Mice were infected with 1X106 PFU Vesicular Stomatitis Virus expressing chicken ovalbumin (VSVova) i.v. the following day. For cell sorting experiments, memory and naive P14 CD8+ T cells were sorted using fluorescently labeled CD45.1 (A20), CD8β (YTS156.7.7), CD62L (MEL-14), Ly6C (AL-21 and HK1.4), CD127 (SB/199), CD44 (IM7), CD69 (H1.2F3) and CD103 (M290) antibodies in a Becton Dickinson FACSAria II. Following sort purification, 2×104 memory and naive P14 cells were transferred i.v. to new C57BL/6J recipients, followed by infection with 2×105 PFU of LCMV Armstrong i.p. in the same day.

Circulating CD90.1+ P14 CD8+ memory T cells were depleted by injecting 0.75 to 1.5 μg of anti-CD90.1 antibody (HIS51, eBioscience) i.p. as previously described**{^PMC7096285\_R27$}**. 4 days after administration of antibody, 2×105 CD45.1+ P14 or CD45.1+ OT-I memory lymphocytes from the spleen and lymph node were transferred i.v. into depleted mice. Local TRM reactivation was performed by delivering 50 μg of gp33 peptide trans-cervically (t.c.) in a 30 μl volume by modified gel loading pipet**{^PMC7096285\_R27$}**. To reactivate OT-I CD8+ TRM cells positioned in skin, a 2cm2 area of the flank skin was shaved and 0.5 μg of SIINFEKL peptide was applied using a tattoo gun as previously described**{^PMC7096285\_R28$}**. PBS was used in control animals.

To discriminate intravascular from extravascular cells, we injected mice i.v. with biotin-conjugated anti-CD8α as described**{^PMC7096285\_R51$}**. Three minutes after the injection, we sacrificed the mice and harvested tissues as described**{^PMC7096285\_R52$}**. Isolated cells were stained with antibodies to CD45.1 (A20), CD8α (53–6.7), CD8β (YTS156.7.7), CD27 (LG.3A10), CD62L (MEL-14), Ly6C (AL-21 and HK1.4), CD127 (A7R34), CCR9 (CW-1.2), CD44 (IM7), CD69 (H1.2F3), CD103 (M290 or 2E7), CD90.1 (OX-7 or His51), CD122 (TM-β1), CD49a (Ha31/8), CX3CR1 (SA011F11), α4β7 (DATK32) and KLRG1 (2F1), all from BD Biosciences, Tonbo Biosciences, Biolegend or Affymetrix eBiosciences. LCMV-specific T cells were stained with fluorescently conjugated H-2Db/gp33 MHC I tetramers. Ova-specific T cells were stained with fluorescently conjugated H-2Kb/SIINFEKL MHC I tetramers. Endogenous VSV-specific cells were stained with fluorescently conjugated H-2Kb/N MHC I tetramers. Allophycocyanin (APC) or Phycoerythrin (PE)-conjugated anti-Granzyme B (GB12 or GB11, Invitrogen) antibody intracellular staining was performed using the Cytofix/Cytoperm kit (BD Pharmigen) following manufacturer’s instructions. Cell viability was determined with Ghost Dye 780 (Tonbo Biosciences). The stained samples were acquired on LSRII or LSR Fortessa flow cytometers (BD) and analyzed with FlowJo software (Treestar).

Murine tissue was harvested, embedded and sectioned as described**{^PMC7096285\_R29$}**. Briefly, 7 μm tissue sections were obtained from frozen tissue blocks in a Leica CM1860 UV cryostat. The sections were stained with CD8β (YTS156.7.7, Biolegend) and CD45.1 (A20, Biolegend) as above. The collagen-IV signal was amplified using AF488 Bovine anti-goat IgG (Jackson ImmunoResearch). Microscopy was performed using a Leica DM6000 B microscope and images were analyzed in Adobe Photoshop CS6.

Skin transplant was performed as described earlier**{^PMC7096285\_R53$}**. CD90.1+ OT-I immune chimeric mice skin was harvested. The recipient (infection matched CD45.1+ OT-I immune chimeric mice) graft bed was prepared by removing a ∼1 cm2 piece of skin from the upper left flank. The donor skin was attached on to the graft site using silk sutures (Sofsilk, Covidien) and a band aid was used to keep the graft in place. The band aid and sutures were removed 7 days post-surgery and the graft was allowed to heal for at least 30 days before peptide recall.

DNA was isolated from 50,000 FACS-purified P14 CD8+ T cells per sample using the Qiagen DNeasy blood and tissue kit. Genomic DNA was bisulfite-treated using the Zymo Research EZ DNA methylation kit. Bisulfite-induced deamination of cytosine was used to determine the allelic frequency of cytosine methylation of the target genomic region**{^PMC7096285\_R54$}**.The PCR amplicon was cloned into the pGEM-T TA cloning vector (Promega) then transformed into XL10-Gold ultracompetent bacteria (Stratagene). Individual bacterial colonies were grown and the cloning vector was isolated and sequenced. Library preparation and sequencing for the generation of whole genome DNA methylation profiles of naive and memory CD8+ T cell subsets (TCM, TRM, and TEM) were performed using previously established protocols**{^PMC7096285\_R13$}**,**{^PMC7096285\_R55$}**. The M values for the 3,000 most variable CpG sites were used for hierarchical clustering and PCA analyses in RStudio (v1.0.136). The built-in R prcomp and autoplot functions were used to perform PCA.

To identify the methylation state of the CpG sites associated with the T cell multipotent potential, a supervised analysis was performed between the methylomes from 3 wild type naive and 2 wild type after exhausted (35 days post chronic LCMV infection) gp33-specific CD8+ T cells (methylation difference >=0.6 and FDR<= 0.01). A minimum of 10,000 cells were used per sample to establish the whole-genome methylation profiles of naïve and exhausted T cells. These whole genome methylation profiles were then used for the machine learning approach to develop the multipotency index. This analysis resulted in identification of 598 CpGs sites that were hypomethylated in naive CD8+ T cells compared to exhausted CD8+ T cells. This set of CpGs was then used as an input to the one-class logistic regression to calculate the multipotency signature using naive samples**{^PMC7096285\_R37$}**,**{^PMC7096285\_R56$}**. Once the signature was obtained, it was then applied to naive, TCM, TEM, TRM, day 4 MP and TE, and day 8 MP and TE CD8+ T cell methylomes. Exhausted CD8+ T cell and effector CD8+ T cell profiles were obtained from previously published data sets**{^PMC7096285\_R13$}**,**{^PMC7096285\_R55$}**. The score was calculated as the dot product between the DNA methylation value and the signature. The score was subsequently converted to the [0, 1] range. Data sets with multipotency indices closer to 1 were more similar to naive cells.

Previously published mouse CD8+ T cell transcriptome data was downloaded from GEO(GSE70813)**{^PMC7096285\_R35$}**. Principal Component Analysis (PCA) was performed with the naive, TEM, TCM, and gut TRM data samples in this data set. The built-in R prcomp and autoplot functions were used to perform the PCA and a plot of the first two principal components, respectively in RStudio (v1.0.136). Differential gene expression between the naive and gut TRM samples was assessed with DESeq2(v1.12.4) using HOMER’s getDiffExpression.pl using a false discovery rate (FDR) of 5% and a log2 fold change of 2**{^PMC7096285\_R57$}**.

2×104 primary memory CD45.1+ P14 TCM from macroscopic lymph nodes, TEM from spleen, and TRM from SI IEL were transferred i.v. into individual naive C57BL/6J recipients and infected with 2×105 PFU LCMV-Armstrong the same day. 50–70 days post infection, single cell suspensions from the spleen and all macroscopic lymph nodes except mesenteric were isolated and CD8+ T cells were purified using a CD8+ T cell Negative Isolation Kit (StemCell Technologies). Cells were cultured as described previously**{^PMC7096285\_R58$}**, with modifications. Briefly, purified cells were incubated in individual wells with 20 IU/mL rhIL-2 (R&D Systems) and 50 ng/mL rhIL-15 (R&D Systems) for 2 days, followed by 2 day incubation with 20 IU/mL rhIL-2 and 50 ng/mL rhTGFβ−1 (R&D Systems) in complete RP-10 (RPMI-1640 containing 10% heat inactivated FBS, 100 U/ml penicillin/streptomycin, 1× nonessential amino acids, 1× essential amino acids, and β-mercaptoethanol). Cells were analyzed after 4 days.

Sample distribution was evaluated using the D’Agostino and Pearson omnibus normality test. Parametric tests (unpaired two-tailed Student’s t-test for two groups and one-way or two-way ANOVA with Tukey’s multiple comparison test for more than two groups as indicated) or nonparametric (two-tailed Mann-Whitney U test) were used when specified. All statistical analysis was done in GraphPad Prism (GraphPad Software Inc.).

All original data are available from the corresponding author upon request.

Further information on experimental design can be found in the Life Sciences Reporting Summary.

# Mice

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# Adoptive transfers and infections

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# Intravascular staining, lymphocyte isolation and phenotyping

To discriminate intravascular from extravascular cells, we injected mice i.v. with biotin-conjugated anti-CD8α as described**{^PMC7096285\_R51$}**. Three minutes after the injection, we sacrificed the mice and harvested tissues as described**{^PMC7096285\_R52$}**. Isolated cells were stained with antibodies to CD45.1 (A20), CD8α (53–6.7), CD8β (YTS156.7.7), CD27 (LG.3A10), CD62L (MEL-14), Ly6C (AL-21 and HK1.4), CD127 (A7R34), CCR9 (CW-1.2), CD44 (IM7), CD69 (H1.2F3), CD103 (M290 or 2E7), CD90.1 (OX-7 or His51), CD122 (TM-β1), CD49a (Ha31/8), CX3CR1 (SA011F11), α4β7 (DATK32) and KLRG1 (2F1), all from BD Biosciences, Tonbo Biosciences, Biolegend or Affymetrix eBiosciences. LCMV-specific T cells were stained with fluorescently conjugated H-2Db/gp33 MHC I tetramers. Ova-specific T cells were stained with fluorescently conjugated H-2Kb/SIINFEKL MHC I tetramers. Endogenous VSV-specific cells were stained with fluorescently conjugated H-2Kb/N MHC I tetramers. Allophycocyanin (APC) or Phycoerythrin (PE)-conjugated anti-Granzyme B (GB12 or GB11, Invitrogen) antibody intracellular staining was performed using the Cytofix/Cytoperm kit (BD Pharmigen) following manufacturer’s instructions. Cell viability was determined with Ghost Dye 780 (Tonbo Biosciences). The stained samples were acquired on LSRII or LSR Fortessa flow cytometers (BD) and analyzed with FlowJo software (Treestar).

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# Plasticity score calculation

To identify the methylation state of the CpG sites associated with the T cell multipotent potential, a supervised analysis was performed between the methylomes from 3 wild type naive and 2 wild type after exhausted (35 days post chronic LCMV infection) gp33-specific CD8+ T cells (methylation difference >=0.6 and FDR<= 0.01). A minimum of 10,000 cells were used per sample to establish the whole-genome methylation profiles of naïve and exhausted T cells. These whole genome methylation profiles were then used for the machine learning approach to develop the multipotency index. This analysis resulted in identification of 598 CpGs sites that were hypomethylated in naive CD8+ T cells compared to exhausted CD8+ T cells. This set of CpGs was then used as an input to the one-class logistic regression to calculate the multipotency signature using naive samples**{^PMC7096285\_R37$}**,**{^PMC7096285\_R56$}**. Once the signature was obtained, it was then applied to naive, TCM, TEM, TRM, day 4 MP and TE, and day 8 MP and TE CD8+ T cell methylomes. Exhausted CD8+ T cell and effector CD8+ T cell profiles were obtained from previously published data sets**{^PMC7096285\_R13$}**,**{^PMC7096285\_R55$}**. The score was calculated as the dot product between the DNA methylation value and the signature. The score was subsequently converted to the [0, 1] range. Data sets with multipotency indices closer to 1 were more similar to naive cells.

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# In vitro T

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# Statistics

Sample distribution was evaluated using the D’Agostino and Pearson omnibus normality test. Parametric tests (unpaired two-tailed Student’s t-test for two groups and one-way or two-way ANOVA with Tukey’s multiple comparison test for more than two groups as indicated) or nonparametric (two-tailed Mann-Whitney U test) were used when specified. All statistical analysis was done in GraphPad Prism (GraphPad Software Inc.).

# Data availability

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# Life Sciences Reporting Summary

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# Supplementary Material