Sphingosine 1-phosphate receptor 5 (S1PR5) regulates the peripheral retention of tissue-resident lymphocytes

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Tissue-resident memory T (T_{RM}) cells provide long-lasting immune protection. One of the key events controlling T_{RM} cell development is the local retention of T_{RM} cell precursors coupled to downregulation of molecules necessary for tissue exit. Sphingosine-1-phosphate receptor 5 (S1PR5) is a migratory receptor with an uncharted function in T cells. Here, we show that S1PR5 plays a critical role in T cell infiltration and emigration from peripheral organs, as well as being specifically downregulated in T_{RM} cells. Consequentially, T_{RM} cell development was selectively impaired upon ectopic expression of S1pr5, whereas loss of S1pr5 enhanced skin T_{RM} cell formation by promoting peripheral T cell sequestration. Importantly, we found that T-bet and ZEB2 were required for S1pr5 induction and that local TGF-β signaling was necessary to promote coordinated Tbx21, Zeb2, and S1pr5 downregulation. Moreover, S1PR5-mediated control of tissue residency was conserved across innate and adaptive immune compartments. Together, these results identify the T-bet–ZEB2–S1PR5 axis as a previously unappreciated mechanism modulating the generation of tissue-resident lymphocytes.

Introduction

Effective immune protection relies on the generation of long-lived memory T cells. During a typical immune response, naive T cells surveying lymphoid organs are activated by cognate antigen and differentiate into short-lived effector cells (SLECs) or long-lived memory T cells (Kaech and Cui, 2012; Omilusik and Goldrath, 2019). Following their activation, T cells exit lymphoid organs and infiltrate infected tissues where they can mediate pathogen clearance. Memory T cells persist after infection resolution to provide protection against subsequent encounters with the same pathogen. These memory cells can be partitioned into distinct subsets based on their migratory and functional properties. Nonrecirculating tissue-resident memory T cells (T_{RM} cells) reside in a wide range of organs, such as the skin, gut, and lung. There, they form a defensive barrier, providing potent immune protection against a plethora of infections and cancer (Masopust and Soerens, 2019; Park et al., 2019; Szabo et al., 2019). After effector T cells enter inflamed tissues, multiple factors determine whether T_{RM} cell precursors “stay” to receive local signals that promote T_{RM} cell differentiation, or “go” and return to the circulation (Mackay et al., 2013). These decisions fundamentally shape the course of the immune response and the magnitude of protective immunity that ensues, yet the signals orchestrating these processes are not yet fully understood.

T_{RM} cell differentiation is not a default pathway for T cells entering peripheral tissues but is instead a multistep process that requires the infiltration, retention, and long-term survival of T cells in the tissue. At epithelial surfaces, local tissue recruitment is achieved via the guidance of effector T cells through chemokine receptors, such as CCR9, CXCR3, and CXCR6 (Mackay et al., 2013; Masopust et al., 2010; Takamura et al., 2019). In contrast, tissue egress of effector and memory T cells is facilitated via the lymphatic system that is rich in chemottractants, including CCL19/CCL21 (recognized by CCR7; Bromley et al., 2005; Debes et al., 2005) and sphingosine 1-phosphate...
(SIP; Skon et al., 2013). The sphingolipid SIP is highly abundant in blood and lymph and can bind to five different receptors (SIPRI-5; Baeyens and Schwab, 2020; Ishii et al., 2004; Kihara et al., 2014). Among these receptors, SIPRI controls key aspects of T cell trafficking from the migration of naive T cells out of LNs to the egress of effector T cells from peripheral organs (Matloubian et al., 2004; Skon et al., 2013). At the transcriptional level, SIPRI expression is induced by Kruppel-like factor 2 (KLF2; Carlson et al., 2006), and downregulation of KLF2 and SIPRI are essential for T RM cell differentiation in a wide variety of organs (Skon et al., 2013). In addition, the activation-induced surface molecule CD69, which is found on most T RM cells, can directly bind to SIPRI and promote its degradation, thereby antagonizing tissue egress and bolstering T RM cell differentiation in the skin (Mackay et al., 2015a; Shiow et al., 2006; Walsh et al., 2019).

While the role of SIPRI in regulating T cell migration is well established, the contribution of the other SIPR family members is less clear (Baeyens and Schwab, 2020). Among SIPRs, we have previously observed that SIPR5 is expressed by memory CD8+ T cells and selectively downregulated in T RM cells (Mackay et al., 2013, 2016). Although SIPR5 has no ascribed function in T cells, it has been reported that SIPR5 mediates natural killer (NK) cell migration, promoting NK cell egress from the bone marrow (BM) and LNs (Mayol et al., 2011; Walzer et al., 2007). Mice carrying a mutation in Tbx21, the gene encoding the T-box transcription factor expressed in T cells (T-bet), exhibit reduced SIPR5 expression in NK cells, suggesting that T-bet induces Sipr5 (Jenne et al., 2009). In addition, unlike SIPRI, SIPR5 does not interact with CD69 (Jenne et al., 2009), together highlighting salient differences in the molecular regulation of these two receptors. Nonetheless, whether the control of SIPR5 expression is similar in CD8+ T cells and to what extent this receptor may regulate their trafficking remain unclear.

Here, we characterized the expression, regulation, and function of SIPR5 in effector and memory T cell subsets. We found that akin to SIPRI, downregulation of SIPR5 was required for efficient T RM cell differentiation. However, in contrast to SIPRI, which is uniformly expressed in naive and circulating memory T cells under the control of KLF2, SIPR5 expression was only induced following antigen experience and was predominantly driven by the transcription regulator zinc finger E-box binding homeobox 2 (ZEB2), which acts downstream of T-bet. We found that tissue-derived TGF-β was necessary to promote the downregulation of Tbx21 and Zeb2, and ultimately Sipr5, thereby hindering tissue traversal and promoting T RM cell formation. Moreover, we identified a similar role for SIPR5 in potentiating the development of certain tissue-resident innate lymphoid cell (ILC) populations. Collectively, our study identifies SIPR5 as a novel regulator of T cell trafficking that governs formation of tissue-resident lymphocyte populations.

**Results**

**Differential expression of SIPRs by circulating and resident CD8+ T cells**

To dissect the roles of the different SIPRs in memory T cell generation, we first examined the pattern of SIPR expression in T RM cells and circulating memory T cell subsets. We began by comparing established transcriptomic profiles (Mackay et al., 2013) of antigen-specific CD6+CD103+CD8+ T RM cells isolated from the skin, lung, and small intestine after HSV-KOS, influenza virus (WSN.gB), or acute lymphocytic choriomeningitis virus (LCMV-Armstrong) infection, respectively, with their circulating effector memory T cell (T EM cell) and central memory T cell (T CM cell) counterparts. Whereas Sipr2, Sipr3, and Sipr4 were similarly expressed by resident and circulating T cell populations, Sipr1 and Sipr5 were selectively downregulated in T RM cells across all three tissues (Fig. 1 A). Consistent with the known role for KLF2 in driving Sipr1 expression (Carlson et al., 2006), Klf2 and Sipr1 transcripts were concordantly elevated in splenic naive T cells and T EM and T CM cells compared with T RM cells (Fig. 1 B). In contrast, Sipr5 was not expressed by naive CD8+ T cells (Fig. 1 B), suggesting that Sipr5 was likely regulated by factors other than KLF2. Analysis of analogous human CD8+ T cell populations revealed that Sipr5, KLF2, and SIPRI were likewise extinguished in CD103+ skin T RM cells compared with circulating memory T cells in peripheral blood (Fig. 1 C), implying similar regulation of SIPRs in mouse and human CD8+ T cells.

To determine the kinetics of Sipr5 expression during memory T cell differentiation, we transferred congenically marked CD45.1+ gB-T-I transgenic CD8+ T cells specific for the immunodominant HSV epitope (gB498-505) into C57BL/6 mice before HSV skin infection. While Sipr5 was not detected in naive gB-T-I T cells, its expression was induced in splenic effector T cells as early as 4 d postinfection (dpi), increased over time, and was then maintained in circulating memory T cells for at least 30 d thereafter. Conversely, Sipr5 expression diminished upon T cell entry into the skin, with expression continuing to decline between 14 and 30 dpi (Fig. 1 D). We found that Sipr5 expression was extinguished before the upregulation of the integrin CD103 (Fig. 1 E), which is expressed following epidermal entry and indicative of full acquisition of the T RM cell program (Mackay et al., 2015b). Taken together, our data indicate that akin to SIPRI, SIPR5 is an SIPR expressed by circulating effector and memory T cells that is downregulated during T RM cell differentiation.

**T-bet cooperates with ZEB2 to regulate SIPR5 expression in CD8+ T cells**

Discordant patterns of SIPR5 and SIPRI expression in naive T cells suggested a differential role of KLF2 in regulating SIPRI and Sipr5. To examine this, we first employed ribonucleoprotein (RNP)-based CRISPR/Cas9 technology (Nüssing et al., 2020; Seki and Rutz, 2018) to disrupt Klf2 in vitro-activated CD8+ T cells. While KLF2-ablated cells showed reduced Sipr1 expression in agreement with previous studies (Carlson et al., 2006; Takada et al., 2011), Sipr5 expression remained unchanged (Fig. S1 A). Interestingly, although KLF2 ablation did not affect Sipr5 transcript levels, overexpression of KLF2 in CD8+ T cells via retroviral transduction increased both Sipr1 and Sipr5 in vitro (Fig. S1 B). Previous studies have shown that forced KLF2 expression is sufficient to induce T-bet in CD4+ T cells (Lee et al., 2015) and that SIPR5 expression in NK cells is controlled by...
T-bet (Jenne et al., 2009), implying an indirect link between KLF2 and S1PR5. Indeed, ablation of T-bet in KLF2-overexpressing cells markedly reduced S1pr5 mRNA levels without changing S1pr1 expression, demonstrating that S1PR5 is upregulated via T-bet and that KLF2 does not directly drive S1pr1 expression, without changing Klf2 expression (Figs. 2A and S2A). Furthermore, forced expression of T-bet by retroviral transduction in HSV-primed gBT-I T cells led to S1pr5 upregulation following retroviral transduction and transferred these cells into HSV-infected mice. While T-bet overexpression in gBT-I T cells potentiated SLEC formation, the differentiation of SLEC was reduced when T-bet expression was forced in ZEB2-ablated gBT-I T cells (Fig. S2E), consistent with the known role of ZEB2 in driving SLEC differentiation (Dominguez et al., 2015; Omilusik et al., 2015). Conversely, forced expression of ZEB2 in effector CD8+ T cells using retroviral transduction was sufficient to drive S1pr5 upregulation in vitro (Figs. 2D and S2D). Hence, ZEB2 appeared to be the primary regulator of S1pr5 in CD8+ T cells, with T-bet acting upstream of ZEB2. To corroborate this, we forced T-bet expression in ZEB2-ablated or control gBT-I T cells via retroviral transduction and transferred these cells into HSV-infected mice. While T-bet overexpression in gBT-I T cells potentiated SLEC formation, the differentiation of SLEC was reduced when T-bet expression was forced in ZEB2-ablated gBT-I T cells (Fig. S2E), in agreement with the known role of ZEB2 in driving SLEC differentiation (Dominguez et al., 2015; Omilusik et al., 2015). Importantly, whereas forced T-bet expression increased S1pr5 expression in control gBT-I T cells, this effect was greatly diminished in the absence of ZEB2, although T-bet could still promote low levels of S1pr5 in ZEB2-ablated cells (Fig. 2E). In contrast, ZEB2 overexpression in Tbx21−/− cells was sufficient to maintain S1pr5 expression in vitro (Fig. S2F), reminiscent of observations in NK cells (van Helden et al., 2015). Overall, these findings support a model whereby T-bet indirectly regulates ZEB2. To this end, we used CRISPR/Cas9 to ablate Zeb2 in effector gBT-I T cells before transfer into HSV-infected mice. Critically, S1pr5 transcripts were substantially reduced in ZEB2-ablated cells, despite similar expression of Tbx21, Klf2, and S1pr1 compared with control T cells (Figs. 2C and S2C). This was in agreement with data indicating reduced T-bet binding to S1pr5 promoter in the absence of ZEB2 (Dominguez et al., 2015).

Heatmap representations of gene expression (z-score normalized by row) of mouse and human CD8+ T cell subsets. (B) Gene expression of indicated mouse CD8+ T cell subsets was analyzed from the Mackay et al. (2013) dataset (GEO accession no. GSE47045) and plotted after log2 transformation. (C) Gene expression of human CD8+ T cell subsets analyzed by qPCR and plotted as a heatmap (z-score normalized by row). Memory T cells were identified as CD8+CD45RO+ or CD8+CD69+CD103− in PBMCs or skin, respectively. (D and E) qPCR analysis of gBT-I T cells isolated from the spleen or skin at indicated time points following HSV infection. (D) Gene expression was normalized to naive gBT-I T cells. Graph shows mean ± SD. (E) Gene expression of mouse CD8+ T cell subsets analyzed by qPCR and plotted as a heatmap (z-score normalized by row). In D and E, data are from two independent experiments, with n = 5–10 mice per time point. rel., relative; Sk., skin; Spl., spleen; Tm., naive T cell.
S1PR5 by activating expression of ZEB2, which then acts as the major proponent of S1PR5 induction.

**Forced expression of S1PR5 perturbs T cell localization in secondary lymphoid organs**

S1PR5 upregulation is required for NK cell trafficking from the BM and LNs to the periphery (Jenne et al., 2009; Mayol et al., 2011; Walzer et al., 2007). However, a potential role for S1PR5 in regulating T cell migration has not been explored. To this end, we used RVs to drive S1PR5 expression in CD8+ T cells, which yielded a fourfold increase in S1pr5 gene expression compared with effector CD8+ T cells primed by LCMV infection (Fig. S3 A). To test the impact of S1PR5 expression on CD8+ T cell migration, we transduced congenically marked effector CD8+ T cells with S1PR5 (S1PR5-RV) or control (Ctrl-RV) RVs and cotransferred these cells into naïve mice. While forced expression of S1PR5 did not appear to influence T cell localization to the spleen, S1PR5-RV cells were severely underrepresented in LNs (Fig. 3 A).
hypothesized that the discrepancy between S1PR5 control of spleen and LN localization might be attributed to distinct tissue architecture in each lymphoid organ, with the spleen being highly vascularized compared with LNs. To address this, we performed intravascular labeling to distinguish cells located within blood vessels from those in the tissue parenchyma (Anderson et al., 2014). Relative to Ctrl-RV cells, S1PR5-RV cells exposed to the vasculature were increased in both number and frequency (Fig. 3 B and Fig. S3, B and C). Immunofluorescence staining confirmed that while similar numbers of Ctrl-RV and S1PR5-RV cells were observed in the spleen, the majority of Ctrl-RV cells localized to the T cell zone of the white pulp (WP), whereas S1PR5-expressing cells redistributed to the red pulp (RP) at higher frequencies (Fig. 3, C–E).

While S1PR5 has been described to drive cellular egress from lymphoid tissues (Jenne et al., 2009), it remained unclear whether S1PR5 expression could also limit T cell entry into tissues. To investigate this, we analyzed the ability of S1PR5-RV and Ctrl-RV cells to migrate to the spleen and LN 2 h after cell transfer and observed a reduction of S1PR5-RV in the splenic WP and LN parenchyma (Fig. 3 F). Overall, these findings indicate that S1PR5 expression can hinder the entry of CD8+ T cells in lymphoid tissues such as LNs and spleen WP and promote their relocalization into vascular beds.

S1PR5 expression controls bidirectional tissue trafficking and impairs TRM cell formation

TRM cells arise from precursor cells recruited to peripheral tissues during acute inflammation (Mackay et al., 2013; Sheridan et al., 2015). However, the role of S1PR5 in the fate of TRM cells is not clear. Here, we found that S1PR5 expression reduced the number of TRM cells in LNs and spleen (Fig. 3 G). This is likely due to the decreased entry of S1PR5-expressing cells to these tissues, as observed in our ex vivo experiments (Fig. 3 F).

Confocal images of the spleen showing the localization of GFP+ Ctrl-RV or S1PR5-RV cells (green) in relation to B cells (B220+, blue) and endothelial cells (CD31+, red). Selected areas are magnified (right panels) to highlight the boundary between WP and RP. Lack of CD31 staining was used to delineate WP/RP separation (dotted lines). Frequencies of Ctrl-RV and S1PR5-RV cells located in the WP and RP were calculated. n = 4 mice per group. (F) Effector OT-I T cells carrying distinct congenic markers were transduced with Ctrl-RV or S1PR5-RV, co-transferred into recipient mice, and isolated from the spleen and LNs 2 h later. Mice were i.v. labeled before harvest. Ratio of Ctrl-RV and S1PR5-RV cells isolated from the indicated organs were normalized to splenic i.v.+ cells. In A, B, and F, data are representative of two independent experiments, with n = 8–10 mice per experiment. Wilcoxon test was used in B, Mann–Whitney test in E, and one-way ANOVA in F. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Graph shows mean ± SD. TEFF, effector T cell.
et al., 2014). G-coupled protein receptors, including CCR7 and SIRP1, can promote egress of these cells to oppose TRM cell generation (Bromley et al., 2013; Mackay et al., 2013; Skon et al., 2013). To determine whether S1PR5 can also facilitate T cell migration from peripheral tissues and preclude TRM cell development, we injected effector CD8+ T cells transduced with S1PR5-RV or Ctrl-RV directly into the skin. This approach precipitates local TRM cell formation in the absence of local antigen recognition (Mackay et al., 2013). 8 d after intradermal injection, S1PR5-RV cells were numerically reduced in the skin but not the spleen compared with Ctrl-RV cells, suggesting that sustained S1PR5 expression promotes departure of effector T cells from the skin (Fig. 4, A and B). Corroborating this, culturing explanted skin containing control and S1PR5-RV cells cultured overnight revealed increased emigration of S1PR5-RV cells from the skin tissue (Fig. 4 C). Given our results showing that S1PR5 limited cellular homing to lymphoid organs (Fig. 3 F), we asked whether S1PR5 signals could also inhibit T cell entry into the skin. To address this, we forced S1PR5 expression in effector CD8+ T cells that were transferred i.v. into mice on the skin flank with the contact sensitizer 1-flour-2,4-dinitrobenzol (DNFB) to induce T cell migration to this tissue (Frizzell et al., 2020). While equivalent numbers of S1PR5-RV cells were recovered from the spleen, reduced numbers of S1PR5-expressing cells were isolated from the skin as early as 4 d after transfer, a defect that was augmented over time (Fig. 4 D). To extend these observations to other organs, we forced S1PR5 expression in effector CD8+ T cells that were transferred i.v. (Fig. 4, E and F) and also examined P14 transgenic CD8+ T cells specific for the LCMV glycoprotein (gp33^-/-) that were transferred into LCMV-Armstrong-infected mice (Fig. 4 G). In these settings, TRM cells naturally develop in a wide range of tissues (Casey et al., 2012), and to induce T cell lodgment in the skin, mice were treated with the contact sensitizer DNFB (Frizzell et al., 2020). 8 d after transfer, S1PR5-overexpressing CD69+ T cells were comparatively reduced in the liver, salivary glands, small intestine intra-epithelial lymphocytes (SI-IELs), and skin. Furthermore, the expression of TRM cell-associated molecules, including CD69, CXCR6, and CD103, were diminished in S1PR5-overexpressing T cells (Fig. 4 F). This loss of S1PR5-RV cells did not appear linked to increased cell death in the tissue (Fig. S3 D) but likely reflects their relocalization to the vasculature (Figs. 3, B–D; and S3 C). Collectively, these data suggest that irrespective of the mode of TRM cell generation, S1PR5 expression hinders CD8+ TRM cell differentiation by dampening T cell extravasation and promoting T cell egress from nonlymphoid tissues.

While these findings showed that S1PR5 expression was sufficient to induce T cell exit from peripheral tissues, it was unclear how S1PR5 might intersect with other molecules coordinating tissue retention. CCR7 promotes memory T cell egress from the skin via lymphatics (Bromley et al., 2013), and CCR7 deficiency enhances skin TRM cell differentiation (Mackay et al., 2013). We therefore asked whether S1PR5 could interface with CCR7 signaling to influence CD8+ T cell tissue egress decisions. Consistent with our prior work (Mackay et al., 2013), Ccr7^-/- effector T cells transduced with a Ctrl-RV displayed enhanced retention in the skin after intradermal injection compared with WT T cells (Fig. 4 H). Forced expression of S1PR5 in Ccr7^-/- T cells restored their capacity to egress from the skin (Fig. 4 H), indicating that S1PR5 signaling can override loss of CCR7 to facilitate tissue egress of CD8+ T cells.

**TGF-β enforces retention of TRM cell precursors in the skin via Zeb2 and S1pr5 downregulation**

Tissue-derived cytokines, including IL-15 and TGF-β, play a crucial role in shaping multiple aspects of TRM cell differentiation (Mackay et al., 2013, 2015b; Schenkel et al., 2016; Zhang and Bevan, 2013). Among these, TGF-β has previously been shown to extinguish expression of Klf2 and S1pr1 (Skon et al., 2013), T-bet (Mackay et al., 2015b), and Zeb2 (Guan et al., 2018). To explore the impact of TGF-β signaling on S1PR5 expression, we cultured effector CD8+ T cells with TGF-β in vitro and found that this cytokine drives S1pr5 downregulation alongside Klf2, S1pr1, and Zeb2 (Figs. 5 A and S4 A). To assess the importance of TGF-β signaling in vivo, we transferred effector OT-I T cells that were either sufficient or deficient in the TGF-β receptor II (TGF-βRII; OT-I WT or Tgfb2^-/-) into HSV-OVA-infected mice. While TGF-βRII–deficient OT-I T cells were compromised in their ability to form TRM cells and almost entirely lacking from the skin, those that were recoverable from the skin epidermis 2 wk after cell transfer exhibited elevated expression of Klf2 and S1pr1 compared with OT-I WT T cells. Importantly, TGF-βRII–deficient cells also displayed increased expression of Zeb2 and S1pr5, suggesting that TGF-β signaling might also repress the ZEB2–SIPR5 axis to promote skin TRM cell formation (Fig. 5 B).

Given that ZEB2 can induce S1pr5 expression (Fig. 2, C and D) and that S1PR5 signaling drives T cell egress from the skin (Fig. 4, A–D), we reasoned that Zeb2 downregulation via TGF-β signaling may be necessary to promote efficient TRM cell formation. To address whether loss of ZEB2 might enhance skin TRM cell development, we transferred CRISPR/Cas9 Zeb2-ablated naive gBT-I T cells to mice that were subsequently infected with HSV. Consistent with previous reports (Domínguez et al., 2015; Omlusik et al., 2015), ZEB2 ablation led to a defect in SLEC differentiation without altering the number of circulating memory precursor cells (Fig. S4 B). In addition, ZEB2 disruption led to increased formation of CD69+CD103+ TRM cells in the skin 2 wk after HSV infection (Figs. 5 C and S4 C). To assess the relative importance of S1PR5 in this process, we cotransferred congenically distinct OT-I WT T cells (OT-I WT) or S1pr5-deficient OT-I T cells (OT-I S1pr5^-/-) into CD45.1 recipient mice before HSV-OVA infection (Fig. 5 D). Akin to ZEB2 ablation, we found that S1PR5 deficiency led to enhanced retention of CD8+ T cells in the skin as early as 8 dpi, with an increased potential to form CD69+CD103+ TRM cells (Fig. 5, D and E; and Fig. S4 D). In contrast, equal proportions of WT and S1pr5-deficient CD8+ T cells localized to the spleen (Fig. 5, D and E; and Fig. S4 D). Together, our data highlight the importance of TGF-β signaling in downregulating Zeb2 and S1pr5 expression, which ultimately enforces the local retention of T cells in peripheral tissues and their differentiation into TRM cells.
Figure 4. **S1PR5 induces CD8+ T cell egress from nonlymphoid tissues.** (A–C) Effector gBT-I T cells carrying distinct congenic markers were transduced with Ctrl-RV or S1PR5-RV and cotransferred intradermally (i.d.) into the flank of naive mice. (A) Ratios of Ctrl-RV and S1PR5-RV cells isolated from the indicated organs were normalized to those in the spleen. (B) Skin was harvested 4 or 8 d after i.d. cell transfer and cultured overnight. Transduced cells were enumerated from the skin and culture supernatant (S/N) and shown as a ratio. (D–F) Effector gBT-I or OT-I T cells were transduced with control or S1PR5 RVs and cotransferred i.v. into naive mice whose flanks were treated with DNFB. (D) Mice were i.v. labeled before harvest. (D and E) Ratios of Ctrl-RV and S1PR5-RV cells were enumerated from the indicated organs and normalized to numbers obtained from the spleen at the indicated times after transfer. (F) CD69, CXCR6, and CD103 expression on Ctrl-RV (black) or S1PR5-RV (blue) cells isolated from the indicated organs 8 d after transfer.

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S1PR5 regulation of tissue-resident lymphocytes

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...tissue distribution of NK cells and ILC1

Tissue residency is not just a feature of CD8+ T cells but also a property shared by both innate and adaptive lymphocytes that use conserved mechanisms to differentiate and persist in peripheral tissues (Gasteiger et al., 2015; Mackay et al., 2016; Robinette et al., 2015). While S1PR5 controls the migration of NK cells from the BM and LNs to the peripheral circulation (Jenne et al., 2009; Walzer et al., 2007), the importance of this receptor in regulating the tissue retention of other ILCs is not known. To explore whether the ZEB2–S1PR5 axis may influence the distribution of additional populations of tissue-resident lymphocytes, we compared the expression of various molecules influencing tissue egress in NK cells and tissue-resident ILC1 in publicly available datasets (Fig. 6 A; Robinette et al., 2015). Analogous to CD8+ TRM cells, tissue-resident ILC1 displayed lower expression of Klf2, S1pr1, S1pr5, and Zeb2 compared with their circulating NK cell counterparts (Fig. 6 A). To understand the importance of S1PR5 during ILC1 differentiation in peripheral tissues, we generated mixed BM chimeras by transferring equal proportions of WT (CD45.1+CD45.2+) and S1pr5−/− (CD45.2+) cells to CD45.1+ hosts. In agreement with prior studies (Jenne et al., 2009; Mayol et al., 2011; Walzer et al., 2007), S1pr5−/− NK cells were found in lower frequencies within the blood and liver. In stark contrast, S1PR5 deficiency led to an increased accumulation of ILC1 in the...
intestine and salivary glands (Fig. 6, B and C). Taken together, these results argue that S1PR5 commonly regulates the retention of multiple tissue-resident lymphocyte populations across both innate and adaptive cell lineages.

**Discussion**

T<br><sub>RM</sub> cells are recognized as essential mediators of host protection against cancer and infections in the periphery (Park et al., 2019; Szabo et al., 2019), yet the processes regulating their generation are not fully understood. One of the key events controlling T<br><sub>RM</sub> cell commitment is the downregulation of molecules that promote tissue exit. In the current study, we identify the ZEB2–S1PR5 axis as a novel pathway balancing peripheral T cell trafficking and T<br><sub>RM</sub> cell formation, with S1PR5 downregulation being required for efficient T<br><sub>RM</sub> cell differentiation. We find that while S1PR1 and S1PR5 display distinct expression patterns and transcriptional regulation. Despite this, local cues, such as TGF-β, act in an overarching manner to suppress both S1PR1 and S1PR5 expression and in doing so, coordinate tissue retention. As a consequence, while multiple independent molecular mechanisms converge to control T cell tissue exit, there appears to exist a unifying process that harmonizes these pathways and enforces T<br><sub>RM</sub> cell commitment.

It is known that S1P signaling regulates T cell migration and positioning in nonlymphoid organs (Baeyens and Schwab, 2020). We have previously shown that the downregulation of S1PR1 and S1PR5, but not other S1PRs is a conserved feature of the T<br><sub>RM</sub> cell differentiation program across distinct tissues (Mackay et al., 2013). Seminal work by Skon et al. (2013) showed that the loss of S1pr1 is required for CD8<sup>+</sup> T<br><sub>RM</sub> cell development and further, that the forced expression of S1pr1 precludes T<br><sub>RM</sub> cell formation. Here, we extend this observation by showing that in addition to S1pr1, S1pr5 is selectively downregulated in T<br><sub>RM</sub> cells and similarly required for T<br><sub>RM</sub> cell formation. More importantly, we identified key differences between these receptors with regard to their transcriptional regulation, expression patterns, and impact on T cell trafficking over the course of CD8<sup>+</sup> T cell differentiation. While S1pr1 expression is primarily regulated by KLF2 (Carlson et al., 2006), the loss of KLF2 had a negligible effect on S1pr5 expression. Instead, we observed that the induction of ZEB2 downstream of T-bet is necessary to promote S1pr5 expression. Interestingly, the sole expression of T-bet does not seem sufficient to induce S1pr5 since T-bet-expressing lymphocytes, including CD4<sup>+</sup> and TCRγδ T cells, typically show negligible S1pr5 mRNA levels. This is likely because T-bet acts as a gradient (Dominguez et al., 2015; Joshi et al., 2007) whereby only high concentrations of T-bet can induce ZEB2 and, consequently, S1PR5. Together, this differential molecular regulation explains discordant S1PR1 and S1PR5 expression in naive CD8<sup>+</sup> T cells, which express KLF2 (Carlson et al., 2006) but not ZEB2 (Dominguez et al., 2015; Omilusik et al., 2015). Importantly, while S1PR1 primarily acts by promoting T cell egress from peripheral tissues (Skon et al., 2013), we find that S1PR5 additionally affects T cells by hindering...
tissue infiltration and promoting the exit of interstitial T cells. Given that S1PR5 expression is limited to specific T cell subsets, S1PR5-controlled migration is likely restricted to effector and memory CD8+ T cells and may explain why ZEB2-dependant terminal effector T cells, which show the highest S1PR5 expression, are mostly confined to the vasculature and poorly infiltrate peripheral organs (Gerlach et al., 2016). Consequently, targeting S1PR5 or molecular components that induce this molecule may permit more selective manipulation of antigen-experienced T cells without impacting naïve T cell trafficking.

After entry to peripheral tissues, effector T cells receive extrinsic cues that either promote their local retention or oblige their return to the circulation. While chemokine gradients can entice migration of TRM cell precursors toward epithelial surfaces (Mackay et al., 2013; Masopust et al., 2010; Takamura et al., 2019), other pathways, including CCR7, S1PR1, and S1PR5 signaling, actively drive their egress via lymphatics (Bromley et al., 2005; Debes et al., 2005; Ledgerwood et al., 2008; Skon et al., 2013). However, the integration of these signals and their impact on the decision to become tissue resident likely depend on the inflammatory context, tissue location, and cell type. For example, although S1PR1 degradation via CD69 complexing is required to enforce the local retention of CD8+ T cells in the skin, kidney, and lung (Mackay et al., 2015a; Takamura et al., 2016; Walsh et al., 2019), CD69 expression is dispensable for T cell retention in other tissues, such as the small intestine (Walsh et al., 2019). Differences observed in distinct tissue sites suggest a certain degree of redundancy with regard to egress mechanisms, which could be orchestrated by local cues from the microenvironment. Interestingly, the cytokines IL-15 and TGF-β, which drive keys aspects of TRM cell differentiation, survival, and function (Mackay et al., 2015b; Schenkel et al., 2016; Zhang and Bevan, 2013), are also essential to enforce the downregulation of tissue egress molecules. For instance, IL-15 induces the expression of Hobit, which in turn represses Ccr7 and Sipr1 (Mackay et al., 2016). Here, we found that TGF-β promotes the downregulation of both Klf2 and Zeb2, thereby suppressing Sipr1 and Sipr5, respectively, in the skin. While not all TRM cells rely on TGF-β for their development, it is unclear whether such TGF-β-independent TRM cells show a less stable form of tissue residency or might rely more stringently on other retention mechanisms, such as integrins (Christo et al., 2021; McNamara et al., 2017).

The shutdown of tissue egress signals is a key step toward the formation of TRM cells, yet it is not a feature unique to T cells. For instance, the downregulation of egress receptors, such as SIPR1, is widespread among innate lymphocyte populations isolated from peripheral tissues, including NK T cells, mucosal-associated invariant T cells, and ILCs (Huang et al., 2018; Mackay et al., 2016; Salou et al., 2019). Downregulation of tissue egress genes can be enforced via common mechanisms in innate and adaptive lymphocytes, with the transcription factors Blimp1 and Hobit directly repressing Ccr7 and Sipr1 in cells spanning both lineages (Mackay et al., 2016). Here, we found that extinguishment of Sipr5 is an additional conserved program acting to promote tissue residency in both ILCs and CD8+ T cells.

While tissue-resident cells provide critical protection against infections and malignancies, they can also be the driving cause of immune pathologies (Park and Kupper, 2015; Sasson et al., 2020). In such settings, therapeutic intervention aiming to selectively remove these deleterious T cells could be highly beneficial but may prove challenging given the durability of TRM cell populations in some tissues. For instance, skin TRM cells are numerically stable and not displaced over time (Park et al., 2018); furthermore, they are inaccessible to targeted depletion using monoclonal antibodies in some circumstances (Watanabe et al., 2015). Therefore, strategies aiming to remove TRM cells by promoting tissue exit via activation of the KLF2–SIPR1 or ZEB2–SIPR5 pathways may help to overcome these obstacles. On the other hand, while skin TRM cells are highly durable, those that reside in the lung decay over time (Slüter et al., 2017). Interestingly, lung CD8+ TRM cell attrition can be attributed in part to “retrograde migration” whereby long-term tissue-resident cells relocate from the periphery to the circulation at steady state, but the mechanisms facilitating this process are unknown (Stolley et al., 2020). It is possible that tissue egress gene pathways may be specifically reactivated in certain populations of TRM cells, such as those lacking expression of Id3 (Kurd et al., 2020; Milner et al., 2020). Interestingly, it has recently been shown that compared with Id3+ TRM cells, the Id3+ population can progressively reexpress ZEB2 and appears to contract within the SI-IEL over time (Milner et al., 2020), potentially via an ZEB2–SIPR5 emigration axis. Elucidating the role of SIPRs in coordinating this process may provide new avenues to promote long-term local immunity for vaccination against mucosal pathogens. Furthermore, SIPR5 may serve as an exit strategy for TRM cells shown to reenter the circulation upon antigen restimulation (Behr et al., 2020; Fonseca et al., 2020; Stolley et al., 2020). Whether antigen recognition may lead to the induction of Zeb2 and Sipr5 in TRM cells to facilitate retrograde migration remains an open question.

Overall, we reveal that S1PR5 acts as a novel checkpoint that controls bidirectional lymphocyte tissue trafficking and impacts the formation of tissue-resident cells across both innate and adaptive cell lineages. Importantly, we demonstrate that tissue T cell retention is a highly coordinated process dictated by the integration of cytokine cues from the local microenvironment, which unaniomously enforce the shutdown of multiple tissue egress genes, even when they are induced by distinct transcriptional regulators. Together, these signals enforce the establishment of tissue residency and promote TRM cell formation (Fig. S5). Further studies will be required to determine how S1PR5 interfaces or combines with other tissue egress factors to control peripheral lymphocyte migration and differentiation. Understanding these interactions will unveil how these pathways could be harnessed for therapeutic gain, such as to improve TRM cell formation during vaccination or elicit the departure of malevolent tissue-resident lymphocytes in the context of autoimmunity.

**Materials and methods**

**Mice**

C57BL/6, B6.SJL-PtpcrPep3b/BoyJ (CD45.1), B6.SJL-PtpcrPep3b/BoyJ × C57BL/6 (CD45.1 × CD45.2), gBT-1 CD45.1, gBT-1
GFP, P14 CD45.1, P14 Thy1.1, OT-I CD45.1, OT-I GFP, OT-I Tbx21^−/− CD45.1, OT-1 Tgfbra2^ΔΔ^ΔΔ.Dlc-Lck-cr CD45.1 (OT-1 Tgfbra2^−/−), OT-I Sippr5^−/−, and Sippr5^−/− mice were bred in the Department of Microbiology and Immunology at The University of Melbourne. Female mice were used for experiments at 6–12 wk of age. All animal experiments were approved by the University of Melbourne Animal Ethics Committee. Sippr5^−/− mice were kindly provided by J. Chun. P14 mice express a transgenic TCR recognizing the LCMV glycoprotein-derived epitope gp33-41; gBT-I mice express a transgenic TCR recognizing the HSV glycoprotein-B-derived epitope gBαω8-105. OT-I mice express a transgenic TCR recognizing the OVA epitope OVA257-264. BM chimera were generated by irradiation of recipient mice (550 rad 3 h apart × 2) followed by reconstitution with 2–5 × 10^4 donor BM cells. Residual lymphocytes were depleted the next day (100 μg anti-Thy1 [T24] i.p.).

**T cell transfer**

Adoptive transfers of naive gBT-I, P14, or OT-I T cells were performed i.v. with LN suspensions. Naive gBT-I, P14, or OT-I T cells were transferred at a total number of 5 × 10^4 or 2.5 × 10^4 cells/population in cotransfer experiments, where cell types were transferred at a ratio of 1:1.

**Infections and DNFB treatment**

HSV infection was performed by scarification using 1 × 10^6 PFU of the KOS strain of the virus (HSV-KOS) or the KOS strain modified to express OVA protein (HSV-OVA). LCMV infection was performed by i.p. injection of 2 × 10^5 PFU of the Armstrong strain of LCMV. For DNFB treatment, mice were shaved and depilated, and an area of 1 cm^2 was excised. Skin was incubated in Dispase II (2.5 mg/ml; Roche) for 12 h at 37°C. Digested skin was homogenized into a single-cell suspension and filtered using 30-μm nylon meshes.

**Mouse tissue processing**

Mice were i.v. injected with 4 μg biotin-conjugated anti-CD45 (30-F11) or CD8β (YTS156.7.7) 4 min before euthanasia, as indicated. Blood was collected via an incision in the submandibular region and then lysed using 1× RBC lysis buffer (eBioscience). Spleens and LNs were processed into a single-cell suspension using metal meshes. Femurs were flushed using a 23-gauge syringe filled with 1× PBS to obtain BM single-cell suspension.

**Flow cytometry and cell sorting**

Mouse cells were stained at 4°C for 60 min with the following antibodies (all purchased from BD Biosciences, BioLegend, or Thermo Fisher Scientific): anti-B220 (RA3-6B2), anti-CD8α (S3-6.7), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD49a (Ha31/8), anti-CD49b (DSX), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD103 (2E7), anti-CD127 (A7R34), anti-CXCR3 (CXCR3-173), anti-CXCR6 (SA051D1), anti-CX3CR1 (SA011F11), anti-KLRG1 (2F1), anti-Ly6C (HK1.4), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-TCRβ (H57-597), anti-TCRγδ (GL3), and anti-VO2 (B20.1). PE-conjugated annexin V was purchased from BioLegend, and staining was performed according to the manufacturer’s instructions. In some experiments, cells were fixed and permeabilized using a FoxP3 transduction factor staining buffer set and stained with the following antibodies (purchased from BD Biosciences or Cell Signaling Technology): anti-Bcl2 (3F11) and anti-Bim (C345C). Human cells were stained with the following antibodies (all purchased from BD Biosciences or BioLegend): anti-CD3 (UCHT1), anti-CD8α (SK1), anti-CD45RO (UCHL1), anti-CD69 (FN5D), and anti-CD103 (Ber-ACT8). Dead cells were excluded from analysis using DAPI (0.5 μM; BioLegend), Zombie Yellow, or Zombie NIR fixable live/dead (BioLegend). For flow cytometry experiments, samples were acquired on a five-laser BD LSRFortessa (BD Biosciences) or a five-laser Cytomax Aurora analyzer. For cell sorting experiments, murine T cells from the spleen (CD8α^+Vα2^CD45.1^+^) and skin (TCRβ^+Vα2^CD45.1^+^) or human T cells from PBMCs (CD3^+CD8α^CD45RO^) and skin (CD3^+CD8α^CD45RO^CD69^CD103^ and CD3^+CD8α^CD45RO^CD69^CD103^) were sorted using a five-laser BD FACSAria III (BD Biosciences; >95% purity). Data were analyzed using FlowJo version 10 (TreeStar).

**RV transduction of CD8+ T cells**

RVs were produced using Plat-E cells (Cell Biolabs), which were transfected with pCL-Eco and pMSCV-IRE-S-FIP II (pMIG II) or...
pMScV-IRES-Thy1.1-based vectors. Briefly, Plat-E cells were seeded in 96-mm dishes at a density of 7 x 10⁶ cells 12 h before transfection with 14 μg of pMIG II and 7 μg of pCL-Eco plasmid DNA using the CalPhos Mammalian Transfection Kit (Takara). Viral supernatants were harvested 48 h later and filtered (0.45 μm; Millipore). T-bet and KLF2 vectors have been previously described (Mackay et al., 2015b; Skon et al., 2013). Sipr5 and Zeb2 cDNA were cloned into pMIG II vector. Purified naive gBT-I, P14, or OT-1 CD8+ T cells were in vitro activated with anti-CD3 (145-2C11) and anti-CD28 (37.51, 5 μg/ml for each; both from Bio X Cell) for 24 h and were “spinfected” with 0.5 ml of retroviral supernatant in 24-well plates coated with Retronectin (32 μg/ml; Takara). CD8+ T cells were further expanded for 3 d in the presence of IL-2 (25 U/ml; Peprotech). Transduction efficiency was determined by GFP expression. Cells transduced with an empty vector (Ctrl-RV) or overexpression vectors (Tbct2-RV or Sipr5-RV) were mixed at a 1:1 ratio. 2 x 10⁵ transduced cells of the relevant specificity were administered i.v. in mice that were infected with HSV 2 d before or LCMV 1 d before. In the absence of infection, 2-5 x 10⁶ transduced cells were i.v. or intradermally injected into naive recipients. In some experiments, cells transduced with an empty vector (Ctrl-RV) or overexpression vector (Zeb2-RV) were maintained in culture for 3 d in the presence of IL-15 (10 ng/ml; Peprotech) and GFP+ cells were sorted by flow cytometry before quantification of gene expression using quantitative PCR (qPCR).

Skin explant migration assay

Flank skin was shaved and depilated, and an area of 1 cm² was excised. Skin samples were cultured overnight in complete RPMI medium. The next day, cells that migrated out of the explant were collected from the culture supernatant, whereas cells that did not migrate from the skin tissue were isolated as indicated above. After processing, cells were enumerated by flow cytometry.

T cell culture with cytokines

Mice received naive P14 T cells and were infected with LCMV as described above. 7 dpi, P14 T cells were sorted from the spleen (CD8α+Vα2+CD45.1+) and cultured in the presence of IL-15 (10 ng/ml) and TGF-β (10 ng/ml) for 48 h before quantification of gene expression by qPCR.

CRISPR/Cas9 gene editing of CD8+ T cells

Single guide RNA (sgRNA) targeting Cdx9 (5'-CCUGGCUGUGGA UUGCAAGU-3' and 5'-GAGAACGCUUGGUAUCG-3'), Klf2 (5'-CCUGCGCGAAUAAGACCG-3' and 5'-UCCAUGGAUUG GACGCUU-3'), Zeb2 (5'-GGCAUGACUCAUGAACC-3' and 5'-GGAUUCUGAGAGACUGC-3'), or scramble control (5'-ACUACUGGAA GACGCAACG-3' and 5'-CAGAUGCAUCAUGACACU-3') or scramble control (5'-GGCAUGACUCAUGAACC-3') were produced from Synthego (CRISPRRevolution sgRNA EZ Kit). sgRNA/Cas9 RNPs were formed by incubating 1 μl sgRNA (0.3 nmol/μl) with 0.6 μl Alt-R S.p. Cas9 Nuclease V3 (10 mg/ml; Integrated DNA Technologies) for 10 min at room temperature. Naive or in vitro–activated (anti-CD3 and anti-CD28, 5 μg/ml each for 24-48 h) gBT-I T cells were resuspended in 20 μl P3 buffer (P3 Primary Cell 4D-Nucleofector X Kit S; Lonza), mixed with sgRNA/Cas9 RNP, and electroporated using a Lonza 4D-Nucleofector system (pulse code: DN100 for naive T cells and CM137 for activated T cells). Naive gBT-I T cells were rested for 10 min at 37°C before i.v. transfer, whereas activated gBT-I T cells were further expanded for 3 d in the presence of IL-2 (25 U/ml; Peprotech). 2 x 10⁶ gBT-I T cells edited with control (sgCtrl) and Zeb2 (sgZeb2) guides were mixed at a 1:1 ratio and transfected i.v. Mice receiving edited naive T cells were infected with HSV 4 d after cell transfer, whereas mice that received edited in vitro–activated cells were infected with HSV 2 d before transfer. In some experiments, in vitro–activated P14 T cells were electroporated as mentioned above with control Cdx9 (sgCdx9) or Klf2 (sgKlf2) targeting guides in P4 buffer (P4 Primary Cell 4D-Nucleofector X Kit S). Cells were further maintained in culture for 3 d in the presence of IL-15 (10 ng/ml; Peprotech) and then performed quantification of gene expression using qPCR. In other experiments, in vitro activated P14 T cells were transduced as mentioned above with pMScV-IRES-Thy1.1 empty vector (Ctrl-RV) or KLF2 overexpression vectors (KLF2-RV) and were subsequently nucleofected 2 d later with Cdx9 (sgCdx9) or Tbx2i (sgTbx2i) targeting guides in P4 buffer (P4 Primary Cell 4D-Nucleofector X Kit S; Lonza). Cells were further maintained in culture for 3 d in the presence of rhIL-2 (100 U/ml, Peprotech), and Thy1.1+ cells were sorted by flow cytometry before quantification of gene expression using qPCR.

qPCR

RNA was extracted from sorted samples using RNeasy Plus Micro Kit (QIAGEN) according to the manufacturer's instructions. mRNA was converted into cDNA using High Capacity cDNA Reverse Transcription Kit or SuperScript IV VILO Master Mix (Thermo Fisher Scientific), and genes of interest were preamplified using TaqMan PreAmp Master Mix (Thermo Fisher Scientific). Gene expression was analyzed by real-time PCR using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific), TaqMan Fast Advanced Master Mix, and the following TaqMan probes (purchased from Thermo Fisher Scientific or Integrated DNA Technologies): mouse probes, Hprt Mm00446968_m1, Klf2 Mm01244979_g1, Sipr1 Mm00514644_m1, Tbp Mm00446973_m1, Tbx2i Mm00450960_m1, and Zeb2 Mm:PT.58.7239300. Sipr5 probe was custom made using the following primers: forward primer 5’-ACCAAGACTCTTCACA ACA-3’, TaqMan probe 5’-AACCTTTGATCGCTTACTGAGCC-3’, reverse primer 5’-GGAGACAACTGTCTTGATG-3’, and human probes, KLF2 Hs00360439_g1, SIPR1 Hs00173499_m1, SIPRS Hs00928195_s1, UBC Hs00824723_m1. In some experiments, gene expression was assessed with an ABI 7000 sequence-detection system, and amplification was detected with PowerUp SYBR Green Master Mix (Applied Biosystems). The sequences of the primer pairs used were as follows: Klf2, forward 5’-ACCAACTGCGGCAAGACCTA-3’ and reverse 5’-CATCTTCCAGTTGACATGA-3’; Sipr1, forward 5’-TCCTGGTCTGACTGTCCTACAGG-3’ and reverse 5’-CACAAAACCCCTTTGCCAAAGAG-3’ and reverse 5’-TCCCTACCCAGCAGTTG.
ACAGT-3'; Zeb2, forward 5'-CATGAACCCATTTAGGCA-3' and reverse 5'-AGCAAGTCTCCGTGAATCC-3' or forward 5'-CCA GAGAAAAGAGGTTCG-3' and reverse 5'-AGCCCTGACATG TAGGTCTTTG-3'; and Hprt, forward 5'-CATTTAGGCCAGGAT TTAGA-3' and reverse 5'-CACAGACGGCCACAAATG-3'. Cycle-threshold values were determined for genes individually, and gene expression was normalized according to the 2−ΔΔCt method to the housekeeping gene Hprt or Tbp for mouse samples or UBC for human samples.

RNA sequencing
cDNA library preparation, sequencing, normalization of sequenced reads, and analysis of differentially expressed genes have been described elsewhere (Christo et al., 2021). Heatmap representation of selected differentially expressed genes was generated using pheatmap. RNA sequencing data have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO) public database under accession no. GSE178768.

Confocal microscopy and image analysis
Spleens were prepared for immunofluorescence staining as described previously (Kato et al., 2015). Briefly, spleens were fixed for 8 h at 4°C in PLP buffer (0.2 M NaH2PO4, 0.2 M Na2HPO4, 0.2 M L-lysine, and 0.1 M sodium periodate with 2% paraformaldehyde), washed twice in PBS, embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek), snap frozen in liquid nitrogen, and stored at −80°C. Frozen tissues were sectioned to 12-μm thickness with a cryostat (CM3050S; Leica). Sections were fixed with acetone, blocked for 10 min with Serum-Free Protein Block (Dako), and stained with the following antibodies (all from BioLegend): anti-B220 (RA3-6B2) Pacific Blue, anti-GFP (FM264G) AF488, and anti-CD31 (390) AF647. Slides were mounted using ProLong Gold Antifade Reagent (Invitrogen). Mosaic imaging covering ∼3,700 μm were acquired with a 20× objective for each spleen on an LSM 700. Images were processed using Imaris (Bitplane). Delineation of spleen WP and RP and quantification of retrovirally transduced GFP+ cells in these areas were performed semiautomatically using the Imaris “surface” tool.

Statistical analysis
All statistics analyses were calculated by performing unpaired $t$ test, unpaired Mann–Whitney test, paired Wilcoxon test, one-way ANOVA test with Dunnet posttest, or two-way ANOVA test with Šidák posttest using Prism 8 (GraphPad Software). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

Online supplemental material
Fig. S1 shows the effect of KLF2 ablation or overexpression on the expression of Ifi2, Sipr1, Sipr5, Tbx2l, and Zeb2 in effector CD8+ T cells. Fig. S2 shows the effect of T-bet or ZEB2 ablation or overexpression on the expression of Klf2, Sipr1, Sipr5, Tbx2l, and Zeb2 and SLEC differentiation in effector CD8+ T cells in various settings. Fig. S3 depicts the degree of Sipr5 overexpression in SIP5-responsive cells and its effect on their vascular localization and regulation of apoptosis. Fig. S4 illustrates the impact of TGF-β signaling on the expression of various genes, including Sipr5, and shows the effect of Zeb2 and Sipr5 ablation of CD8+ T cell differentiation. Fig. S5 shows a model of how SIP5 expression is regulated in CD8+ T cells and how it affects their trafficking and differentiation into T RM cells.

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**Figure S1.** KLF2 does not directly regulate S1pr5 expression. (A and B) Effector P14 T cells were nucleofected with control Cd19-targeting (sgCd19), Klf2-targeting (sgKlf2), or Tbx21-targeting (sgTbx21) sgRNA/Cas9 RNPs and were maintained in culture for 3 d. (B) Effector P14 T cells were transduced with a control (Ctrl-RV) or a KLF2 (KLF2-RV) R (RVs) before nucleofection with sgRNA/Cas9 RNPs. Expression of the indicated genes was quantified by qPCR. In A and B, data are pooled from two independent experiments, with n = 2–6 samples per condition. Multiple Mann–Whitney test was used in A and multiple t test in B. n.s., nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 rel., relative; T_EFF, effector T cell.
Transcriptional control of SLEC CD8+ T cell differentiation and S1pr5 expression via T-bet and Zeb2. (A) Mice were adoptively transferred with naive GFP+ OT-I (OT-I WT) and CD45.1+ OT-I Tbx21−/− (OT-I Tbx21−/−) and infected with HSV-OVA. Expression of indicated genes quantified by qPCR on OT-I T cells from the spleen 8 dpi and shown normalized to a housekeeping gene. (B) Effector gBT-I T cells were transduced with control (Ctrl-RV) or T-bet (Tbet-RV) GFP-expressing RVs and cotransferred into mice infected with HSV. Expression of indicated genes quantified by qPCR on sort-purified gBT-I T cells from the spleen 8 dpi and shown normalized to a housekeeping gene. (C) Effector gBT-I T cells were nucleofected with control-nontargeting (CD45.1+CD45.2+ gBT-I sgCtrl) or Zeb2-targeting (CD45.1+ gBT-I sgZeb2) sgRNA/Cas9 RNPs and cotransferred into HSV-infected mice. Expression of indicated genes quantified by qPCR on sort-purified transgenic T cells from the spleen 8 dpi and shown normalized to a housekeeping gene. (D) Effector gBT-I T cells were transduced with control or ZEB2 (ZEB2-RV) RVs and maintained in culture with IL-15 for 3 d. Expression of indicated genes was quantified by qPCR in GFP+ gBT-I T cells and shown normalized to a housekeeping gene. (E) Ctrl-RV– or Tbet-RV–transduced gBT-I T cells were nucleofected with control nontargeting (sgCtrl) or Zeb2 targeting (sgZeb2) sgRNA/Cas9 RNPs and cotransferred into HSV-infected mice. gBT-I T cells were sort-purified from the spleen 8 d after transfer. Tbx21 expression was quantified by qPCR and shown normalized to a housekeeping gene (left), and frequencies of SLECs were analyzed by flow cytometry (right). (F) Effector OT-I WT of Tbx21−/− were transduced with Ctrl-RV or ZEB2-RV and cultured with IL-15 for 3 d. Expression of the indicated genes in transduced cells was quantified by qPCR. In A–E, data are representative of two independent experiments, with n = 4–5 mice per group per experiment. In F, data are pooled from three independent experiments. *, P < 0.05; **, P < 0.01 by Mann–Whitney test. Graph shows mean ± SD. A.U., arbitrary units; rel., relative.
Figure S3. S1PR5 promotes T cell relocation to vascular beds. (A) P14 T cells were transduced with control (Ctrl)-RV or S1PR5-RV and transferred into LCMV-infected mice. Transduced cells were sorted from the spleen 8 dpi. Expression of S1p5 was quantified by qPCR and normalized to a housekeeping gene.

(B–D) OT-I T cells were transduced with Ctrl-RV or S1PR5-RV and cotransferred i.v. into mice treated with DNFB. Mice received anti-CD45 antibody i.v. to label vasculature-associated cells before harvest. Shown are numbers (B) and frequencies (C) of Ctrl-RV and S1PR5-RV cells isolated from the indicated organs 8 d after transfer and the Bcl2/Bim ratio (left) and percentage annexin* live/dead* (L/D) apoptotic cells (right) of Ctrl-RV and S1PR5-RV cells isolated from the indicated organs 8 d after transfer (D). In A, data are pooled from two independent experiments, with n = 3 mice per group per experiment. In B–D, data are representative of two independent experiments, with n = 5–10 mice per experiment. Multiple paired t test was used in B and multiple unpaired t test in C and D. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Graph shows mean ± SD. A.U., arbitrary units; TEFF, effector T cell.
Figure S4. **ZEB2 and S1PR5-deficient CD8+ T cells show increased TRM cell formation in the skin.** (A) Naive P14 T cells were transferred into LCMV-infected mice. P14 T cells were isolated from the spleen 7 dpi and cultured with TGF-β in vitro for 2 d. Expression of indicated genes was quantified by qPCR and normalized to a housekeeping gene. (B and C) Effector or naive gBT-I T cells were nucleofected with control-nontargeting (GFP+ gBT-I sgCtrl) or Zeb2-targeting (CD45.1+ gBT-I sgZeb2) sgRNA/Cas9 RNPs and cotransferred into recipient mice infected with HSV. gBT-I T cells were quantified in the spleen (CD127+CXCR3+KLRG1−CX3CR1− and CD127−CXCR3−KLRG1+CX3CR1+) 8 dpi (B) or spleen and skin (total gBT-I or CD69+CD103+ gBT-I) 14 dpi (C). (D) Naive OT-I WT and OT-I S1pr5−/− T cells were cotransferred into recipient mice infected with HSV-OVA. Shown are numbers of WT and S1pr5−/− OT-I T cells isolated from the spleen and skin (total OT-I or CD69−CD103− OT-I) 14 and 30 dpi. In A, data are pooled from two independent experiments, with n = 6 mice per group. In B and C, data are representative of two independent experiments, with n = 4 mice (B) and n = 7–9 mice (C) per experiment. In D, data are representative of four independent experiments, with n = 9–10 mice per group per experiment. Mann–Whitney test was used in A, paired t test in B, and paired Wilcoxon test in C and D. *, P < 0.05; **, P < 0.01; ***, P < 0.001. A.U., arbitrary units; n.s., not significant; TEFF, effector T cell; TN, naive T cell.
Figure S5. Model of T cell trafficking in peripheral tissues impacting the generation of TRM cells. Multiple factors can influence the decision of effector T cells (TEFF) to enter inflamed tissues, return to the circulation, or remain locally and differentiate into TRM cells. Local T cell retention is classically achieved via the downregulation of tissue egress molecules, including CCR7 and S1PRs. In this study, we identified S1PR5 as an additional regulator of these decisions, with S1PR5 expression limiting T cell extravasation and promoting T cell egress, thereby impeding TRM cell development. While S1PR1 and S1PR5 share the same ligand, these two receptors are controlled by distinct transcriptional regulators, with KLF2 and ZEB2 being the main drivers of S1PR1 and S1PR5, respectively. Importantly, despite exhibiting distinct transcriptional regulation, the tissue-derived cytokine TGF-β promotes downregulation of both pathways and ultimately S1pr1 and S1pr5, thereby enforcing tissue retention and TRM cell differentiation. TCIRC, circulating T cell.