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IL-15 Complexes Induce Migration of Resting Memory CD8 T Cells into Mucosal Tissues

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IL-15 is an essential cytokine known to promote T cell survival and activate the effector function of memory phenotype CD8 T cells. Blocking IL-15 signals also significantly impacts tissue-specific effector and memory CD8 T cell formation. In this study, we demonstrate that IL-15 influences the generation of memory CD8 T cells by first promoting their accumulation into mucosal tissues and second by sustaining expression of Bcl-6 and T-bet. We show that the mechanism for this recruitment is largely dependent on mammalian target of rapamycin and its subsequent inactivation of FoxO1. Last, we show that IL-15 complexes delivered locally to mucosal tissues without reinfection is an effective strategy to enhance establishment of tissue resident memory CD8 T cells within mucosal tissues. This study provides mechanistic insight into how IL-15 controls the generation of memory CD8 T cells and influences their trafficking and ability to take up residence within peripheral tissues. ***The Journal of Immunology*****, 2017, 199: 2536–2546.**

CD8 T cells are a critical component of the immune system's ability to mount protective responses against infection and tumors. A key element of CD8 T cell–mediated protection is that memory CD8 T cells are positioned at sites of frequent pathogen exposure. In response to infection, CD8 T cells expand profoundly, migrate into tissues, and eliminate infected cells. After inflammatory signals subside, most effector CD8 T cells are eliminated from the body. Effectors capable of surviving the contraction either take up residence in tissues or circulate throughout the blood and secondary lymphoid organs (SLOs) as long-lived memory CD8 T cells. Tissue resident memory (T_{RM}) CD8 T cells are characterized by their persistence within tissues and lack of recirculation (1–3). T_{RM} cells have been identified in many nonlymphoid tissues (NLTs) including the skin, brain, lungs, liver, gastrointestinal tract, and reproductive tract (3–11). In addition to CD69, T_{RM} cells within the mucosal tissues express CD103, and both of these molecules are involved in their retention within the epithelium (9). They play an important part in pathogen surveillance at barrier sites, and when T_{RM} cells are reactivated they can stimulate local innate immune responses and recruit circulating T cells into the tissues (3). T_{RM} cells originate from a common $KLRG1^-$ memory precursor cell that also gives rise to circulating central and effector memory CD8 T cell populations (12). Thus, the formation of T_{RM} cells is largely dependent on local environmental cues, such as TGF-β and IL-15, that they receive when they arrive in inflamed tissues (13, 14).

IL-15 is an important cytokine for maintaining survival and homeostasis of memory CD8 T cells, and it plays an essential role in promoting survival of effector CD8 T cells and generating memory CD8 T cells (15, 16). IL-15 can be supplied to CD8 T cells bound to IL-15Rα chain (IL-15Rα)−IL-15R on neighboring cells in a contact-dependent mechanism called *trans*-presentation. Many cellular sources of IL-15/IL-15Rα have been identified and their roles in T cell homeostasis have been characterized (17–19). Soluble IL-15/IL-15Rα (IL-15 complexes) are also generated during inflammation and virus infection and may act on local or distal CD8 T cells to influence effector responses (20). The role of IL-15 complexes in the generation of T_{RM} cells is unclear, but given that IL-15 is required for optimal generation of CD8 T cell responses and that IL-15 complexes are detected early postinfection suggests that IL-15 complexes may have an important role in regulating either effector CD8 T cell generation or the effector–T_{RM} transition. Another possibility is that IL-15 may serve as a chemotactic factor that directs T cells into peripheral tissues.

We recently reported that accumulation of effector CD8 T cells within mucosal tissues depends on a signal that is mediated through the mammalian target of rapamycin (mTOR) pathway (21). In

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R.T.S. and A.L.M. conceived the project and designed experiments; R.T.S., J.W.G., and A.L.M. analyzed data and wrote the manuscript; R.T.S., M.R., and Z.Q. performed experiments; Y.C. performed quantitative RT-PCR experiments; Y.C. and A.F. analyzed the quantitative PCR data; and E.F.C. designed experiments relating to mitochondria analysis.

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Abbreviations used in this article: dpi, d postimmunization; FRT, female reproductive tract; IL-15Rα, IL-15Rα chain; iVAG, intravaginal; mTOR, mammalian target of rapamycin; NLT, nonlymphoid tissue; PLN, peripheral lymph node; RV, retrovirus; shRNA, small hairpin RNA; SI, small intestine; SI IEL, SI intraepithelial lymphocyte; SI LP, SI lamina propria; SLO, secondary lymphoid organ; SOCS, suppressor of cytokine signaling; S1pr1, sphingosine-1-phosphate receptor 1; T_{RM}, tissue resident memory; VM, vaginal mucosa; VSV, vesicular stomatitis Indiana virus; VSV-ova, VSV encoding OVA; WT, wild-type.

addition, IL-15 has been reported to activate the mTOR pathway in NK cells and induce their activation (22). In this study, we demonstrate that IL-15 can promote the accumulation of CD8 T cells within mucosal tissues by activating mTOR and sustaining T-bet expression. Our data suggest that IL-15/mTOR signaling early during effector differentiation is an important event that enables CD8 T cells to circulate away from initial priming sites and populate mucosal tissues. Moreover, we propose that locally administered IL-15 complexes therapeutically enhance the quantity of T_{RM} cells within sites of frequent pathogen exposure.

Materials and Methods

Mice and infections

C57BL/6 mice were purchased from the National Cancer Institute. IL-15$^{-/-}$ (C57BL/6NTac-*IL15*^{tm1Imx} N5) mice were purchased from Taconic. Tsc2 dominant-negative OT-I (Tsc2-DN OT-I) mice were generated in our facility by crossing Tsc2-DN (C57BL/6-Tg[CMV-Tsc2*]1Arbi/KlanJ, purchased from Jackson Laboratories) with CD45.1$^+$ OT-I transgenic mice. Mice were immunized with 10^6 PFU of vesicular stomatitis Indiana virus (VSV) or VSV encoding OVA (VSV-ova) by i.v. tail-vein injection. VSV-specific CD8 T cell responses were measured against VSV-N (RGYVYQGL) with tetramers provided by the National Institutes of Health Tetramer Facility. To generate memory cells for analysis, 5×10^3 naive CD45.1$^+$ OT-I cells were adoptively transferred by i.v. injection into naive CD45.2$^+$ C57BL/6 recipients, immunized 24 h later with VSV-ova, then rested for >60 d. To generate in vivo–activated OT-I cells, Rag$^{-/-}$ OT-I TCR transgenic mice were i.v. immunized with 10^7 PFU. VSV-ova and OT-I cells were harvested from the spleen 24 h later. All mice were maintained in the specific pathogen free facility at Rush University in compliance with the Rush University Institutional Animal Care and Use Committee.

In vivo rapamycin treatment and small hairpin RNA knockdown

Mice were treated by i.p. injection of a low dose of rapamycin (75 μg/kg) daily starting 24 h prior to immunization and persisting to the peak of the effector response (7 d postimmunization [dpi]). small hairpin RNA (shRNA) knockdown of mTOR and Raptor were done as previously described (21, 23, 24).

IL-15 complex treatment

Recombinant IL-15 was purchased from eBioscience and IL-15Rα–Fc chimeric molecule was purchased from R&D Systems. IL-15/IL-15Rα–Fc complexes were generated by mixing and incubating IL-15 and IL-15Rα–Fc together in PBS for 30 min at 37˚C. Dosing/concentrations were as follows: for in vitro administration, 0.5 μg/ml IL-15 and 1 μg/ml IL-15Rα–Fc; intravaginal (iVAG) administration, 0.25 μg IL-15 and 1.45 μg IL-15Rα–Fc in 15 μl PBS per mouse; and i.p. injection, 2 μg IL-15 and 12 μg IL-15Rα–Fc in 200 μl PBS per mouse. For iVAG administration of IL-15 complexes, mice were anesthetized with Avertin and the vaginal cavity was swabbed with calcium alginate. IL-15 complexes were administered into the vagina using a 20-μl, gel-loading pipette tip.

FACS analysis of intracellular proteins

For analysis of transcription factors, cells were stained with surface Ags and then fixed and permeabilized using the eBioscience FoxP3 Fix/Perm Kit according to manufacturer's recommendations. Abs to detect transcript factors were T-bet (clone eBio4B10; eBioscience), Bcl-6 (clone BCL-DWN; eBioscience), and Blimp-1 (clone 5E7; BD Biosciences). For analysis of phosphorylated proteins, cells were promptly fixed in Phosflow Lyse/Fix Buffer (BD Biosciences) and then permeabilized with Phosflow Perm Buffer III (BD Biosciences) prior to staining with phospho-ribosomal protein S6 (clone D57.2.2E; Cell Signaling), phospho-Akt Ser^{473} (clone D9E; Cell Signaling), and phospho-FoxO1 Ser^{253} (Cell Signaling).

Quantitative RT-PCR analysis

RNA was isolated from CD8 T cells using TRIzol-chloroform extraction. Relative mRNA levels were determined by obtaining quantification cycle values normalized to two reference genes, 18s and *Hprt*. mRNA levels were determined relative to untreated cells.

Statistical analysis

Results indicate means and error bars are shown for SEM. Results were compared using either Student *t* test or ANOVA and *p* values were indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Accumulation of effector CD8 T cells within the vasculature is promoted by mTOR signals

To examine the role of mTOR in trafficking of effector CD8 T cells into mucosal tissues, we generated mTOR-deficient, Ag-specific CD8 T cells by transducing in vivo–activated CD45.1$^+$ OT-I CD8 T cells with GFP-expressing retroviruses (RVs) containing shRNA targeting mTOR or empty vector as a control. We then adoptively transferred RV-transduced OT-I cells into infection-matched recipients and measured their accumulation in the spleen 8 dpi. To discriminate between OT-I cells localized within the tissue vasculature and the parenchyma, we i.v. injected 3 μg anti–CD8α-PE Abs prior to harvesting tissues (25). We detected fewer mTOR-deficient effector OT-I cells in the i.v. anti-CD8α$^+$ population compared with controls, suggesting that mTOR regulated the accumulation of these effectors within the vasculature (Fig. 1A). It is reported that KLRG1$^+$ cells are more reliant on IL-15 for their survival, and their frequency is selectively reduced when mTOR signals are blocked (24, 26). At the peak of the CD8 T cell response, we detected KLRG1$^+$ cells primarily in the i.v. CD8α$^+$ population, and this population was especially reduced in absence of mTOR (Fig. 1A). mTOR signals through two complexes and both have effects on memory CD8 T cell formation. Previously, we reported that rapamycin, a selective inhibitor of mTORC1, can disrupt the generation of T_{RM} CD8 T cells (21). Specifically, we showed that blocking mTOR during differentiation of virus-specific CD8 T cells results in effectors that are defective in their ability to traffic to the small intestine (SI) (21). Therefore, we determined the effect of a specific loss in mTORC1 signals on the accumulation of effector CD8 T cells in peripheral tissues by transducing OT-I cells with RVs targeting Raptor (key component of the mTORC1 complex). We observed fewer mTOR- and mTORC1-deficient OT-I effector cells 4 dpi in the spleen, SI lamina propria (SI LP), and SI intraepithelial lymphocytes (SI IEL), but not in the peripheral lymph nodes (PLN); suggesting that mTORC1 signals are responsible for the accumulation of effector CD8 T cells in peripheral tissues (Fig. 1B). One signature of T_{RM} cells within the SI epithelium is the expression of CD103 and CD69. Consistent with our previously reported results (21), low-dose rapamycin (attenuates mTORC1 signaling but does not completely block mTOR) significantly decreased the percentage of CD103$^+$CD69$^+$ effector CD8 T cells that accumulated in the SI (Fig. 1C). shRNA-targeted knockdown of mTOR in OT-I cells also resulted in reduced numbers of effector CD8 T cells in the SI IEL, however most of the mTOR-deficient OT-I cells found in the SI IEL were CD103$^+$CD69$^+$ (Fig. 1D). These data suggest that mTOR does not directly control expression of CD103 and CD69 in the mucosal tissues but the trafficking of effector cells into the tissues. shRNA knockdown of mTORC1 and Raptor are validated in Supplemental Fig. 1A.

Early effector CD8 T cell infiltration into mucosal tissues requires IL-15 signals

IL-15 is a critical regulator of the generation and homeostatic maintenance of memory CD8 T cells (27). Moreover, the generation of T_{RM} cells in the skin is perturbed in absence of IL-15 produced by a radiation-resistant cell population, possibly keratinocytes (12). In the small intestine, IL-15 produced by intestinal epithelial cells promotes the development of CD8αα IEL (18). We then asked whether IL-15 was required for the early infiltration of effector CD8 T cells into mucosal tissues which would eventually seed the T_{RM} cell population. To do this, we adoptively transferred wild-type (WT) naive OT-I cells into WT or IL-15–deficient (IL-15$^{-/-}$) recipients,

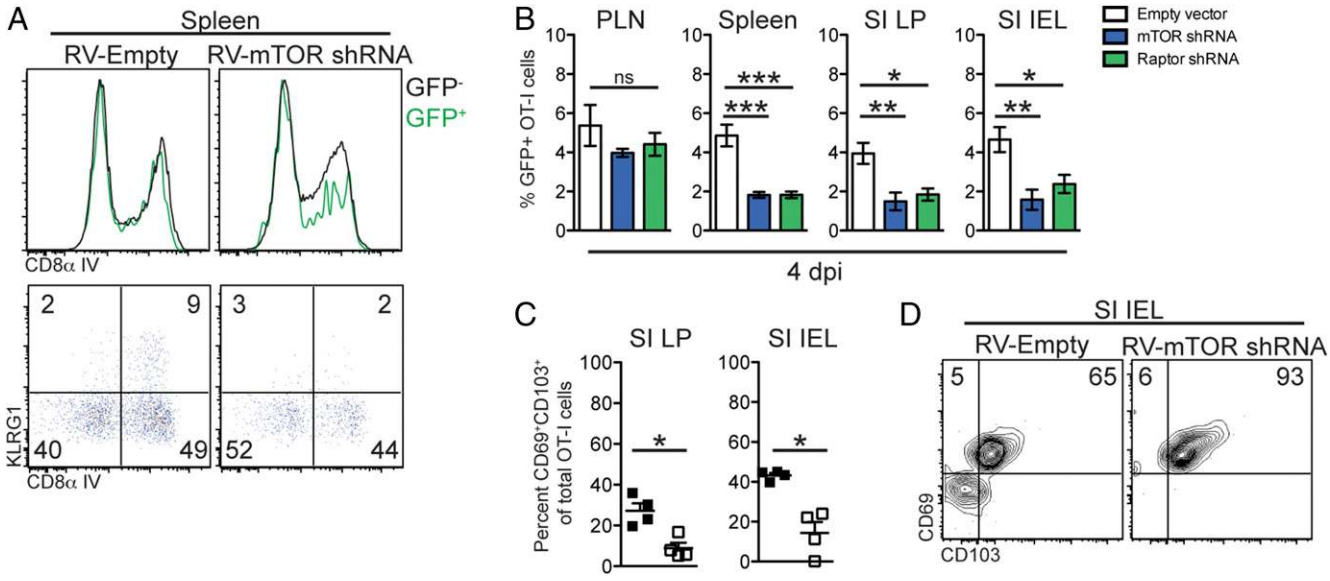

FIGURE 1. Accumulation of effector CD8 T cells within vasculature is promoted by mTOR. (**A**) CD45.1$^+$ OT-I cells were transduced with RV encoding empty-GFP or mTOR shRNA-GFP and immunized with VSV-ova. Cells were intravascularly labeled with anti–CD8α-PE 8 dpi. Histograms show the proportion of vascular localized (CD8α IV$^+$) versus nonvascular (CD8α IV$^-$) OT-I cells RV-transduced (GFP$^+$) or nontransduced (GFP$^-$) (top). Dot plots show the frequency of vascular and nonvascular KLRG1$^+$ cells of total CD45.1$^+$ OT-I cells (bottom). (**B**) Percent OT-I cells RV-transduced with empty-GFP, mTOR-shRNA–GFP, and Raptor-shRNA–GFP RVs within PLN, spleen, SI LP, and SI IEL 4 dpi with VSV-ova. (**C**) Percent CD69$^+$CD103$^+$ effector OT-I cells within the SI LP and SI IEL 6 dpi ± low-dose rapamycin (75 μg/kg days −1 to 6). Open boxes represent mice treated with rapamycin, filled boxes with media. (**D**) FACS plots show expression of CD69 versus CD103 on retroviral empty-GFP and mTOR-shRNA–GFP transduced cells within the SI IEL. Data in histograms and dots plots are from one mouse representative of an experiment with five mice per group (A and D). Data are means (± SEM) from a representative experiment with n = 4–5 mice per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (B and C).

and i.v. immunized with VSV-ova. These mice were treated with rapamycin from the time of adoptive transfer until 7 dpi. Loss of IL-15 significantly inhibited the accumulation of effector CD8 T cells in both the SI LP and IEL, and to a lesser degree in the female reproductive tract (FRT) vaginal mucosa (VM) (Fig. 2A). The reduced numbers of mucosal effector OT-I cells in the absence of IL-15 phenocopies the effect of rapamycin on the accumulation of the mucosal OT-I effector. To determine if the defective accumulation of effector CD8 T cells was a result of decreased integrin expression, which is required for entry into mucosal tissues, we examined the cell-surface levels of α4β7 in effector CD8 T cells from the spleen and SI LP. In the spleen, OT-I effectors in the presence of rapamycin or absence of IL-15 had higher levels of α4β7 (Fig. 2B). In contrast to the spleen, the expression level of α4β7 was decreased on OT-I cells in the SI LP in the presence of rapamycin or absence of IL-15, suggesting that IL-15 and mTOR affect the distribution of effector CD8 T cells and not their capacity to express α4β7. A similar decrease in the number of CD103$^+$ effector OT-I cells in the SI LP and SI IEL in the presence of rapamycin and absence of IL-15 signals was observed (Fig. 2C). Interestingly, memory CD8 T cells generated in the absence of IL-15 were enriched within the population that is resistant to in vivo intravascular staining (Fig. 2D). Because blockade of mTOR signaling can lead to the generation of IL-15–independent, homeostatic proliferation–induced memory CD8 T cells (28), we next determined whether blocking mTOR with rapamycin would generate memory CD8 T cells that would develop and/or be maintained in absence of IL-15. We adoptively transferred WT OT-I cells into WT or IL-15$^{-/-}$ recipients, immunized with VSV-ova, and treated with low-dose rapamycin (−1 to 8 dpi). We were unable to detect memory CD8 T cells in the absence of IL-15 in spleens of mice 300 dpi (Fig. 2E), suggesting that IL-15 did not have the same effect on the generation of virally induced memory CD8 T cells compared with those memory CD8 T cell generated from homeostatic proliferation (28). Taken together, these data suggest the possibility that IL-15 and mTOR may signal through a shared conduit to direct the generation of T_{RM} cells in mucosal tissues.

Local IL-15 signals can promote infiltration of effector CD8 T cells into the mucosa

In vivo, IL-15 is delivered to CD8 T cells bound to IL-15Rα either through *trans*-presentation by neighboring IL-15Rα–expressing cells or through soluble IL-15/IL-15Rα complexes. Thus, we determined tissue-specific expression of IL-15Rα 2 d after VSV infection and found that IL-15Rα is increased during immunization in the PLN and SI LP (Fig. 3A). We next asked whether exogenous IL-15 complexes applied locally to mucosal tissues could increase accumulation of effector CD8 T cells. To do this, we immunized mice with VSV i.v. and 2 dpi we iVAG administered IL-15 complexes or sham treatment. The use of IL-15 complexes in vivo has been shown to significantly increase the half life and bioavailability of IL-15 (29). Four days later (6 dpi) we quantified the number of VSV-specific CD8 T cells localized within the VM. As a control, we also determined the numbers of VSV-specific cells in the spleen. We found the numbers of effector CD8 T cells were significantly increased in the VM with IL-15 complex treatment but not in the distal tissues (Fig. 3B). A previous report demonstrated that administration of IL-15 complexes could increase CD8 accumulation in the lungs of IL-15$^{-/-}$ mice, partially rescuing the defective accumulation of Ag-specific CD8 T cells seen in IL-15$^{-/-}$ mice (30). Therefore, we next wanted to determine if, by providing IL-15 complexes iVAG in mice that cannot respond to IL-15, the effector CD8 T cells could be induced to accumulate in the VM. To do this we adoptively transferred WT-naive OT-I cells into IL-15–deficient recipients, i.v. immunized with VSV-ova, and administered 4 dpi IL-15 complexes or sham treatment iVAG. We show that the recruitment of Ag-specific CD8 T cells upon VSV-ova immunization was not

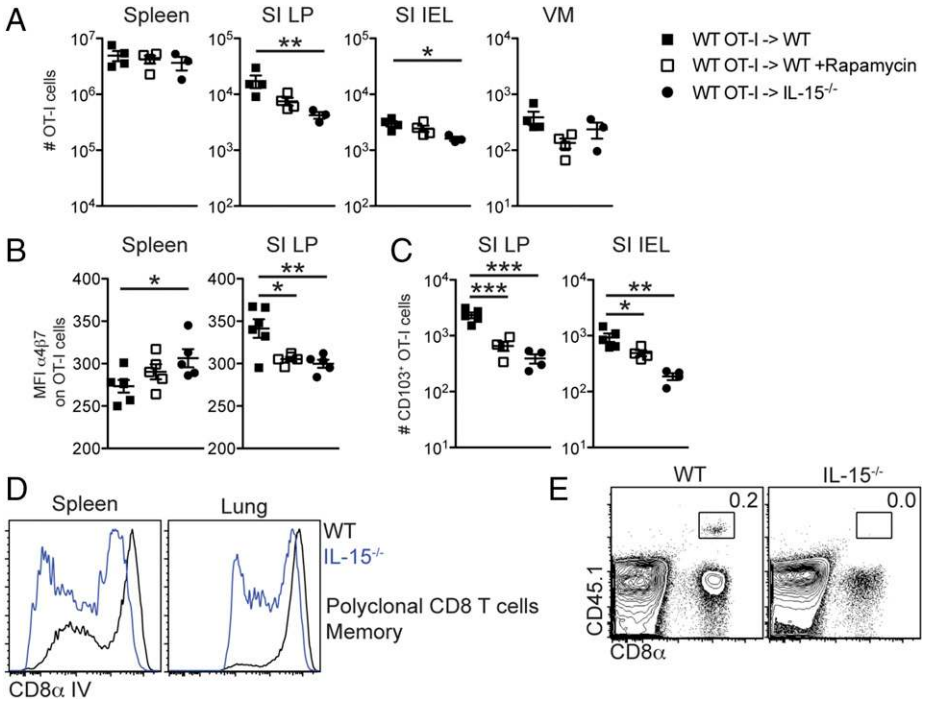

FIGURE 2. Effector CD8 T cell infiltration into mucosal tissues requires IL-15 signals. OT-I cells were adoptively transferred into WT, low-dose rapamycin treated WT, or IL-15$^{-/-}$ recipients and immunized with VSV-ova. (**A**) Comparison of the total number of OT-I cells in spleen, SI LP, SI IEL, and VM 6 dpi. (**B**) Graphs show mean fluorescence intensity (MFI) of α4β7 on OT-I cells in the spleen and SI LP 6 dpi. (**C**) Graph represents the number of CD103$^+$ OT-I cells in the SI LP and SI IEL 6 dpi. (**D**) CD8a intravascular staining of polyclonal CD44^{hi}CD8$^+$ T cells from the spleen and lung of WT and IL-15$^{-/-}$ mice >30 dpi. (**E**) Dot plot shows frequency of 300-dpi memory CD45.1$^+$ OT-I cells (gated on total lymphocytes) in spleens of low-dose rapamycin–treated (75 μg/kg, i.p. daily, day −1 to 7) WT or IL-15$^{-/-}$ mice. Plot is representative of $n = 3$ mice per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

rescued in IL-15$^{-/-}$ mice that received iVAG administration of IL-15 complexes and sham treated (Fig. 3B). Therefore, the observations made by Verbist and colleagues may not be universal to all mucosal tissues, and mechanisms for recruitment of Ag-specific CD8 T cells into the FRT may require IL-15 signals at different stages, whether at priming or exiting into the circulation as suggested by our data in Fig. 2D.

CXCR3 is an important chemokine receptor that enables CD8 T cells to migrate into inflamed tissues. Therefore, we next tested whether IL-15 induced changes in chemokine-receptor expression in recently activated effector CD8 T cells. To do this we activated OT-I cells in vivo by immunizing CD45.1$^+$ Rag$^{-/-}$ OT-I mice with VSV-ova (i.v.) and harvested splenocytes 24 h later. In vivo–activated OT-I cells were cultured ex vivo with IL-15 complexes for 24–72 h, and expression of CXCR3 and CXCR4 was determined. We found that both control and IL-15 complex–treated OT-I cells upregulated CXCR3 after 24 h of culture, but the effect was short-lived in IL-15–treated cells (Supplemental Fig. 2A). After 72 h, control OT-I cells continued to increase their level of CXCR3 expression but IL-15–treated OT-I cells did not. Similar results were observed with CXCR4 expression (Supplemental Fig. 2B). We also examined the expression of CXCR3 on effector CD8 T cells in WT and IL-15$^{-/-}$ mice, and saw similar upregulation of CXCR3 expression on Ag-specific CD8 T cells from the spleen (Fig. 3C). However, when compared with effector CD8 T cells in the spleen, CXCR3 expression is significantly lower on effector cells accumulating in the FRT. Additionally, the IL-15 complex increases the percentage of CXCR3$^+$ effector T cells in the FRT of WT mice, but not IL-15$^{-/-}$ mice (Fig. 3C). These data suggest that IL-15 complexes may not directly signal to increase the expression of CXCR3, but alternatively recruit a larger number of effector cells, some of which are CXCR3$^+$, into the FRT from the blood.

IL-15 complexes increase activity of the mTOR pathway in effector CD8 T cells

To test whether IL-15 activates the mTOR pathway in effector CD8 T cells, we treated in vivo–activated OT-I cells with IL-15 complexes in the presence or absence of rapamycin. In addition, we treated CD8 T cells with a combination of IL-15 complexes and TGF-β. We found that IL-15 robustly increased phosphorylation of ribosomal protein S6 (Ser$^{240/244}$) and Akt (Ser^{473}) after

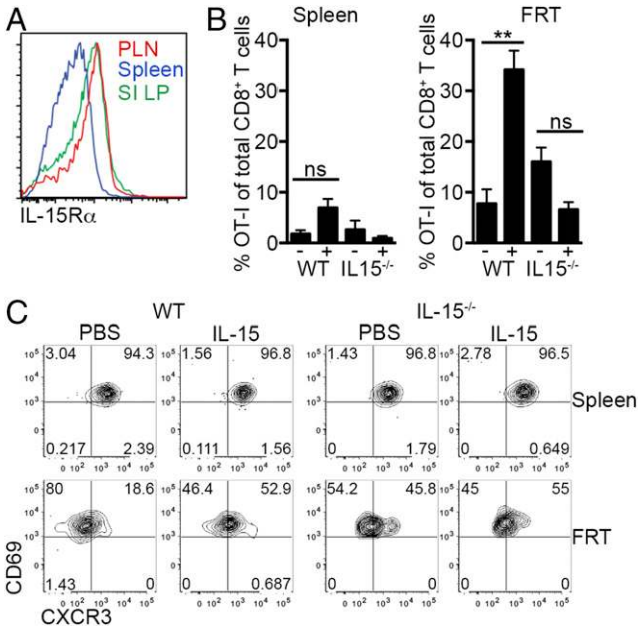

FIGURE 3. Local IL-15 signals promote infiltration of effector CD8 T cells into mucosal tissues. (**A**) Histograms compare expression of IL-15Rα on CD11c$^+$MHCII$^+$ cells isolated from the PLN, spleen, and SI LP from mice 2 dpi with VSV. (**B**) Bar graphs show mean number of OT-I TCR transgenic T cells isolated from the spleen and FRT 7 d after immunization with VSV-ova. Five thousand naive OT-I cells were adoptively transferred into congenic B6 recipients 24 h prior to VSV-ova immunization. IL-15 complexes were then iVAG delivered to mice 2 dpi and the frequency of OT-I cells were assessed 5 d later (7 dpi). (**C**) Expression of CXCR3 and CD69 on antigenic CD8$^+$ T cells (OT-I) within the spleen and FRT 7 dpi with VSV, after receiving iVAG IL-15 complexes or PBS 2 d after immunization. Contour plots are representative of one mouse per group ($n = 3$–5). **$p < 0.01$. ns, $p > 0.05$.

stimulating for 24 h (Fig. 4A). This increase in phosphorylation could be blocked when cells where stimulated with IL-15 complexes in the presence of rapamycin. The inclusion of TGF-β partially decreased S6 phosphorylation but had no detectable effect on IL-15–mediated Akt phosphorylation. Ribosomal protein S6 is phosphorylated by S6 kinase, which is a direct downstream target of mTORC1. Whereas Akt can activate mTORC1, phosphorylation at Ser^{473} is mediated by mTORC2. These data suggest that IL-15 complexes induce activation of both mTORC1 and mTORC2, which can be inhibited by rapamycin. Rapamycin is known to selectively inhibit mTORC1 activity (31, 32), indicating mTORC2 activation by IL-15 is downstream of mTORC1 signals. We found that another common γ-chain cytokine family member, IL-7, which shares the γ-chain subunit of the IL-15R, also activated the mTOR pathway, albeit to a lesser extent and with slower kinetics. IL-7 complexes sustained mTORC1 activity in a rapamycin-insensitive manner (Fig. 4B). IL-15, but not IL-7, increased the proliferation and recovery of live cells after 48 h ex vivo culture (Fig. 4C).

FoxO1 is a transcriptional regulator of many genes and has been identified as an important mediator of CD8 T cell trafficking and memory formation (33–38). Phosphorylation of Akt on Ser^{473} leads to Akt translocation to the nucleus, where it phosphorylates and inactivates FoxO1 by inducing its export to the cytosol. To determine whether IL-15–mediated activation of mTOR can lead to inactivation of FoxO1, we assessed the phosphorylation state of FoxO1 (Ser^{253}) in activated CD8 T cell effectors 48 h after treatment with IL-15 complexes. We found FoxO1 phosphorylation to be increased upon treatment with IL-15 complexes, and this was partially sensitive to rapamycin (Fig. 4D). IL-7 complexes also increased phosphorylation of FoxO1 but through a rapamycin-insensitive mechanism. The addition of TGF-β together with IL-15 complexes, while increasing FoxO1 phosphorylation, was similar to IL-15 alone.

Cytokine-directed control of transcriptional regulators Bcl-6, T-bet, and Blimp-1 through mTOR

We next asked whether cytokine-induced activation of mTOR which could lead to functional changes in CD8 T cell effectors could potentiate their ability to traffic to tissues and form T_{RM} cells. To do this we treated effectors for 48 h with IL-7, IL-15, TGF-β, or TGF-β/IL-15. IL-15 induced a broad effector program that led to the increase in expression of CD8α, CD69, Bcl-6, T-bet, Blimp-1, and CD122 (Fig. 5A, 5B). Furthermore, the increased expression of T-bet after cytokine treatment was limited to the Bcl-6–positive OT-I cells. The IL-15–mediated effector program was diminished in the presence of rapamycin, indicating that this effector program was mTOR dependent. IL-15 downregulated β7 expression, a subunit that associates with CD103 and α4 to form αEβ7 and α4β7 integrins, respectively (Fig. 5C). More importantly we show that, even in the presence of rapamycin, IL-15 was still capable of decreasing β7 expression, suggesting that at least this feature of IL-15 signaling occurred independently of mTOR. TGF-β increased β7 expression and in conjunction with IL-15 was also capable of increasing its expression (Fig. 5C).

We also noted that the IL-15 induction of Blimp-1, T-bet, and to some degree Bcl-6 was not sustained after prolonged treatment with IL-15 (data not shown). One possible explanation for these data are that increased mTORC1 signaling in effector cells leads to a negative feedback loop inhibiting mTOR activity after prolonged IL-15 stimulation. A major regulator of mTOR signaling is the upstream Tsc1–Tsc2 complex, which when activated represses mTORC1 activity. When Akt is activated it represses Tsc2 activity, leading to activation of mTORC1. In the presence of excess growth factors, mTORC1 can provide negative feedback to inhibit Akt activity and thus limit energy consumption (31, 39, 40). Therefore, we tested the ability of IL-15 to induce Bcl-6, T-bet,

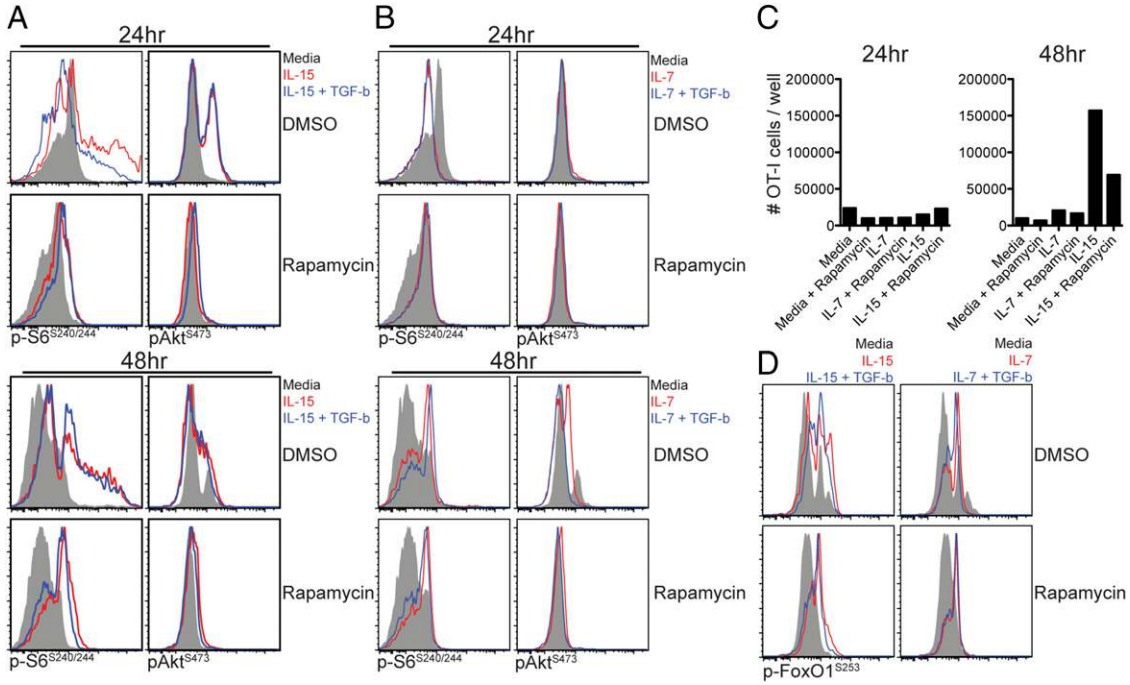

FIGURE 4. IL-15 activates mTOR and FoxO1 in a rapamycin-sensitive mechanism. OT-I cells were activated in vivo by immunizing a $Rag^{-/-}$ OT-I mouse with VSV i.v. and 24 h later harvested from the spleen. Histograms show levels of phospho-$S6^{S240/244}$ and phospho-Akt^{S473} of in vivo–activated OT-I cells cultured with indicated cytokines for 24 and 48 h and treated with DMSO or rapamycin beginning 60 min prior to culture with (**A**) IL-15 complexes or (**B**) IL-7 complexes. (**C**) Graphs indicate the number of live cells recovered after 24 and 48 h in indicated culture conditions. (**D**) Histograms show the relative level of phospho-$Foxo1^{S253}$ after treating in vivo–activated OT-I cells with either IL-15 complexes, IL-15 with TGF-β, IL-7 complexes, or IL-7 with TGF-β in the presence or absence of rapamycin.

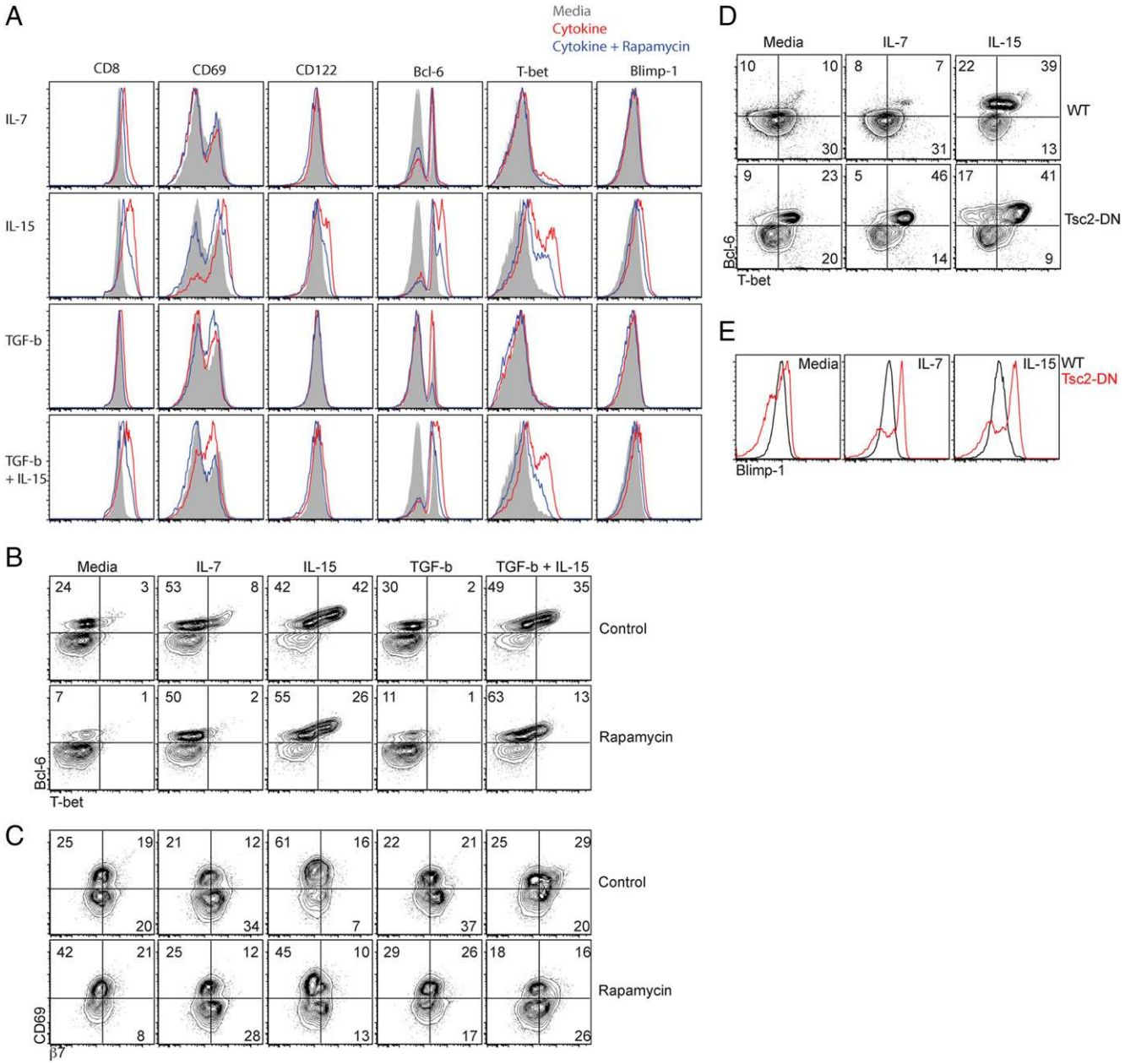

FIGURE 5. IL-15 sustains expression of Bcl-6, T-bet, and Blimp-1 in effector CD8 T cells. In vivo–activated OT-I cells were cultured with IL-7, IL-15, TGF-β, and IL-15 with TGF-β and treated with rapamycin or DMSO as control. (**A**) Histograms show expression of CD8α, CD69, CD122, Bcl-6, T-bet, and Blimp1 48 h after treatment with indicated cytokines (red line), cytokines with 100 μM rapamycin (blue line), or media alone (black line). (**B**) Representative FACS plots of Bcl-6 and T-bet expression or (**C**) CD69 and β7 after treatment with indicated cytokines for 48 h ± 100 μM rapamycin. In vivo–activated WT or Tsc2-DN OT-I cells were cultured in the presence of IL-7, IL-15, or media alone for 48 h and the expression of Bcl-6 and T-bet were assessed. (**D**) Representative FACS plots of Bcl-6 versus T-bet on WT or Tsc2-DN OT-I treated with indicated cytokines. (**E**) Histogram showing comparison of Blimp-1 expression between WT and Tsc2-DN in vivo–activated OT-I cells treated with indicated cytokines for 48 h. Histograms and FACS plots are representative of two independent experiments.

and Blimp-1 expression in effector CD8 T cells that are unable to turn off mTORC1. To do this, we generated OT-I cells that expressed a form of Tsc2 with mutations in the rabaptin-5 and rap1GAP motifs (Tsc2-DN OT-I), resulting in a dominant-negative function of Tsc2. Cells that express Tsc2-DN have increased phosphorylation of S6 and S6K, indicating dysregulated mTORC1 activity (41). Tsc2-DN OT-I effectors had higher basal levels of Bcl-6 and T-bet after ex vivo culture for 48 h. Tsc2-DN OT-I cells were hyperresponsive to IL-7 and IL-15 complexes compared with WT OT-I cells and expressed higher levels of T-bet and Bcl-6 (Fig. 5D). We also observed increased expression of Blimp-1 in Tsc2-DN OT-I cells compared with WT cells after IL-7 and IL-15 complex treatment (Fig. 5E). These data suggest that feedback inhibition of mTOR in a Tsc2-dependent mechanism controls expression of Blimp-1, T-bet, and Bcl-6.

mTOR influences cytokine signaling in effector cells by modulating suppressor of cytokine signaling 1 and 3 expression

To gain a better mechanistic insight into how mTOR may be controlling cellular responses to IL-15 complexes, we established whether IL-15 complexes could modulate mRNA levels of several members of the STAT pathway (Fig. 6A). STAT3 signals can sustain Bcl-6 during the effector-to-memory transition, and CD8 T cells lacking STAT3 have reduced expression of Bcl-6 and

Blimp-1 (42). IL-15 can activate STAT3 and STAT5, and mTOR inhibition did not have a detectable effect on STAT3 mRNA levels (Fig. 6A). mTOR can regulate STAT signaling through increasing levels of suppressor of cytokine signaling (SOCS) proteins (43). SOCS3 is a well-described target of STAT3, and SOCS3 is reduced in STAT3$^{-/-}$ effector and memory CD8 T cells (42, 44, 45). Moreover, SOCS3 expression was higher in memory precursors than short-lived effector CD8 T cells (42). Therefore, we asked whether IL-15 could regulate expression of *Socs3* and also *Socs1*, a gene target of STAT5. After IL-15 treatment, we found relative levels of *Socs1* mRNA to be increased in effectors cells, whereas levels of *Socs3* mRNA were decreased (Fig. 6A, 6B). IL-7 was capable of inducing similar changes in *Socs1/3* expression albeit to a lesser extent (Fig. 6A). The changes in *Socs1* and *Socs3* expression were dependent on mTOR signaling as cells cultured with rapamycin prior to and during IL-7 and IL-15 treatment abolished their ability to modulate SOCS expression (Fig. 6B).

IL-15 complex treatment activates mTOR to reduce Klf2 expression

Klf2 expression is controlled by active (nuclear, nonphosphorylated) FoxO1 and is central for trafficking of naive CD8 T cells (35). *Klf2* enhances while mTOR suppresses the expression of L-selectin and the chemokine receptor CCR7, regulating CD8 T cell entry into lymph nodes (46). *Klf2* also controls the expression of sphingosine-1-phosphate receptor 1 (*S1pr1*), a G protein–coupled receptor, and downregulation of S1pr1 is a critical event during the establishment of T_{RM} cells (47). Therefore, we tested if IL-15 complexes could reduce *Klf2* expression, which would provide a mechanism by which IL-15 is able to increase trafficking of CD8 T cells into peripheral tissues. We found that after 24 h of IL-15 treatment, effector CD8 T cells displayed decreased *Klf2* expression compared with media alone (Fig. 6A, 6B). Conversely, inhibition of mTOR increased the relative levels of *Klf2* mRNA, even in the presence of IL-15. However, after 96 h *Klf2* levels were dramatically increased in the presence of IL-15 and IL-15 with rapamycin (Fig. 6C). *Klf2* mRNA levels are lower in effectors compared with naive and memory cells. Thus, our data suggest that IL-15 may serve to sustain low levels of *Klf2* expression acutely, but after prolonged stimulation feedback inhibition of the mTOR pathway increases *Klf2*. Increased *Klf2* through inhibition of mTOR may then limit the ability of effector CD8 T cells to egress from lymph nodes and take up residence within mucosal tissues.

IL-15 complex treatment in vivo is sufficient to induce memory CD8 T cells into mucosal tissues in the absence of infection

To understand if IL-15 signals alone were capable of influencing migration of CD8 T cells, we examined the effect of IL-15 on resting memory CD8 T cells. We generated VSV-specific memory CD8 T cells by immunizing mice i.v. with VSV and resting them for >60 d. We harvested memory CD8 T cells from spleens by CD8 enrichment, and then CFSE labeled the memory CD8 T cells and adoptively transferred them into naive congenic recipients. Twenty-four hours after transfer, mice were injected i.p. with IL-15 complexes or PBS (control) and then 7 d later the accumulation of VSV-specific CD8 T cells into peripheral tissues was determined. Transferred CD8 T cells were detected in the SI LP and VM of the FRT of IL-15–treated mice (80 ± 17 cells and 30 ± 2 cells, respectively) but not in PBS-treated mice (8 ± 2 cells versus 4 ± 2 cells, respectively) (Fig. 7A). In contrast, within the spleens we detected transferred cells in both IL-15– and PBS-treated animals. IL-15 complex treatment also induced proliferation of memory cells which accumulated within the tissues. We then asked whether memory CD8 T cells recruited into the tissues in response to IL-15 were capable of persisting long term after IL-15 withdrawal. Forty seven days after IL-15 treatment we were still able to detect increased numbers of transferred memory CD8 T cells within the SLO and mucosal tissues (Fig. 7B). These data suggest that a single treatment of IL-15 complexes could have lasting effects on memory CD8 T cells recruited into peripheral sites, in that they are able to become T_{RM} cells and persist long term. Next we asked whether the effect of IL-15 treatment was specific to Ag-experienced cells. We assessed the effect of IL-15 on the accumulation of naive cells within the SLO (PLN and spleens) and mucosal tissues (SI LP and FRT VM) and found that IL-15 also increased the numbers of naive cells recovered within both SLO and mucosal tissues (Fig. 7C). Additionally, IL-15 treatment converted naive T cells into effectors (Fig. 7D).

Because IL-7 was also capable of inducing moderate changes in effector cells and memory CD8 T cells express high levels of IL-7, we next assessed the ability of IL-7 complexes to recruit resting memory CD8 T cells into peripheral tissues. Similar to IL-15, we detected increased numbers of adoptively transferred memory CD8 T cells in the PLN and SI LP after administering systemic IL-7 complexes (Fig. 7E). However, unlike IL-15, IL-7 complexes did not induce proliferation of memory CD8 T cells, suggesting that accumulation of memory CD8 T cells within mucosal tissues precludes proliferation (Fig. 7F).

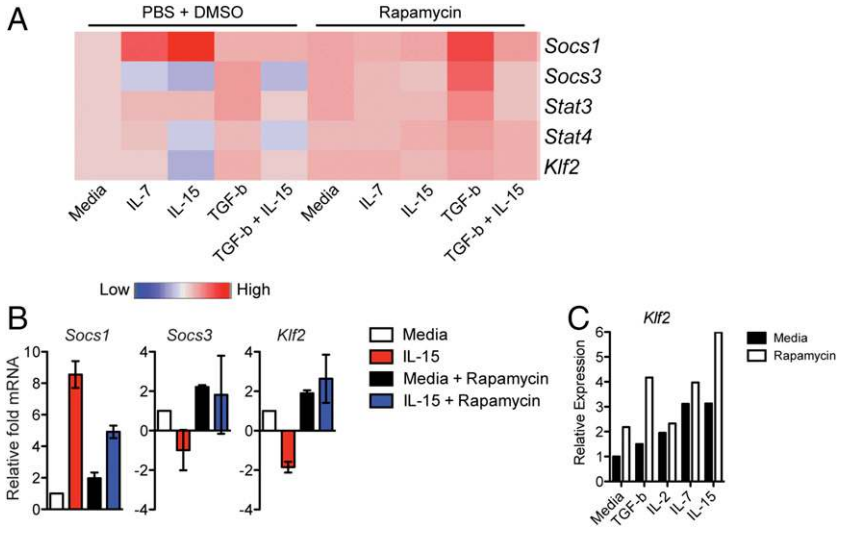

FIGURE 6. mTOR controls IL-15 regulation of SOCS1, SOCS3, and Klf2 expression. In vivo–activated OT-I cells were cultured with IL-7, IL-15, TGF-β, TGF-β with IL-15, or media alone with/without 100 μM rapamycin for 24 h. (**A**) Heat map shows mean relative mRNA levels for indicated genes normalized to 18s mRNA and relative to culture in media alone. (**B**) Bar graphs show relative levels of *Socs1*, *Socs3*, and *Klf2* mRNA after culture of activated OT-I cells with or without IL-15 complexes ± 100 μM rapamycin for 24 h. (**C**) Bar graph shows relative levels of *Klf2* mRNA in response to culture with indicated cytokines for 96 h ± 100 μM rapamycin. Data is representative from two independent experiments (A–C). Data are means of two biological replicates (± SEM) (B).

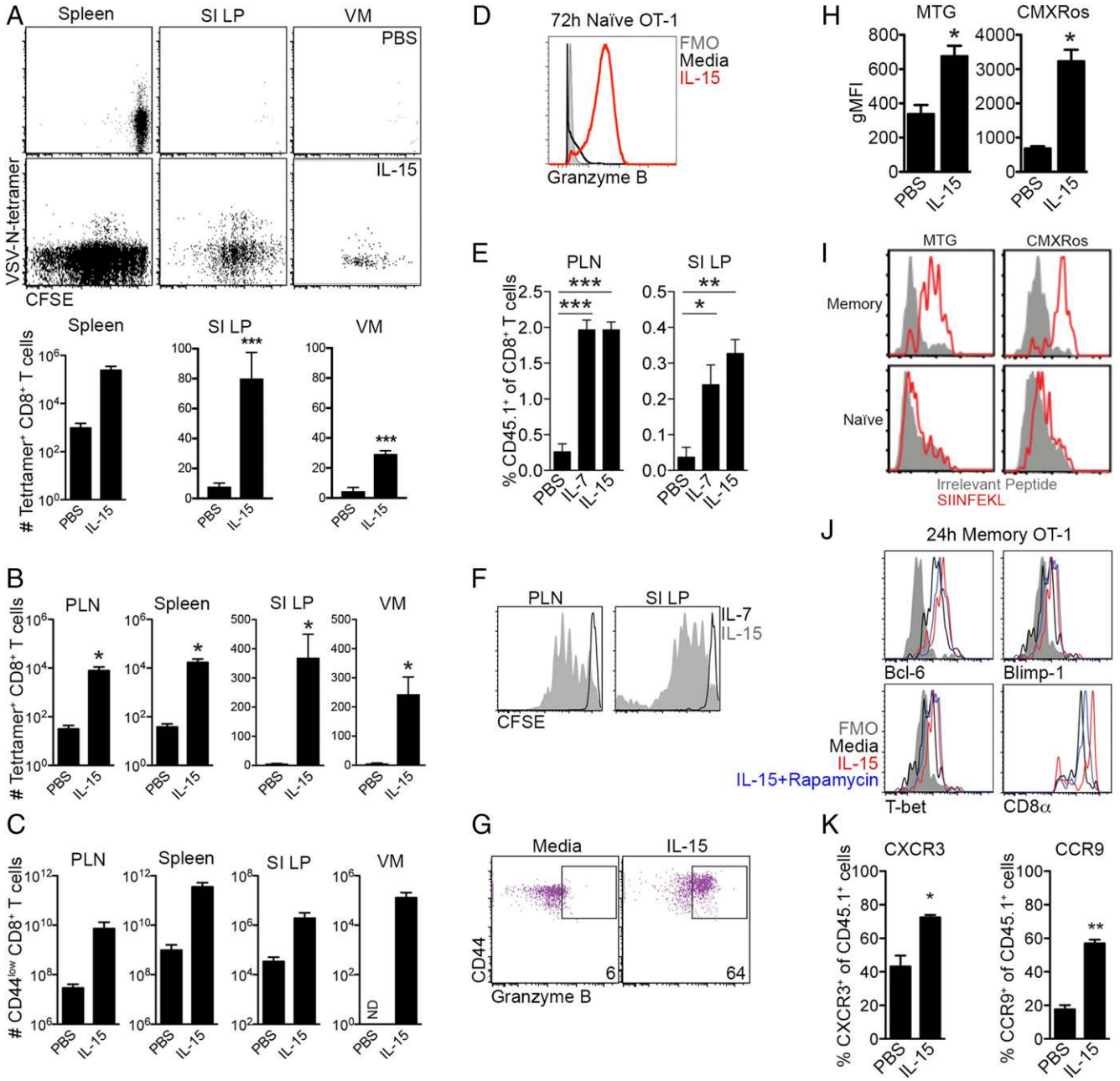

FIGURE 7. IL-15 complexes are sufficient to induce migration and a broad effector program in memory CD8 T cells. (**A**) Representative FACS plots and bar graphs of mean number of VSV-specific memory CD8 T cells 6 d after adoptive transfer and systemic treatment with IL-15 complexes or PBS as a control. (**B**) Forty-seven days after IL-15 complex administration, the number of VSV-specific memory CD8 T cells in the SLO and mucosal tissues were enumerated. (**C**) Numbers of naive ($CD44^{low}$) CD8 T cells were enumerated in the SLO and mucosal tissues 6 d after i.p. administration of IL-15 complex. (**D**) Analysis of Granzyme B expression gated on naive splenic CD8 T cells cultured with IL-15 complexes for 72 h. (**E**) Memory cells were generated by immunizing $CD45.1^+$ C57BL/6 mice with VSV and resting for >60 d. $CD8^+$ T cells were enriched by magnetic bead separation and adoptively transferred into naive $CD45.2^+$ mice. Polyclonal ($CD44^{hi}$) memory CD8 T cells were enumerated in the PLN and SI LP 6 d after i.p. administration of either IL-7 or IL-15 complexes. (**F**) Polyclonal memory CD8 T cells were CFSE labeled and adoptively transferred into naive recipients and i.p. injected with IL-7 or IL-15 complexes. Histograms show CFSE dilution as a measure of memory CD8 T cell proliferation 6 d after treatment. (**G**) Analysis of Granzyme B expression gated on splenic VSV-specific memory CD8 T cells cultured with IL-15 complexes for 72 h. Dot plots were previously gated on $CD8^+$N-$tetramer^+$ cells. (**H**) Relative levels of total mitochondrial mass (MitoTracker green [MTG]) and total polarized mitochondria (MitoTracker CMXRos [CMXRos]) staining in VSV-specific memory CD8 T cells isolated from the spleen and treated with IL-15 complexes ex vivo for 24 h. (**I**) Antigenic stimulation increases mitochondrial biogenesis in memory CD8 T cells. Histograms demonstrate relative total mitochondrial mass (MTG) and relative polarized mitochondria (CMXRos). Memory OT-I cells were stimulated with 1 μg/ml SIINFEKL (red) or irrelevant peptide (gray) ex vivo overnight. (**J**) Memory OT-I cells were cultured ex vivo for 24 h with IL-15 complexes (red), IL-15 complexes and rapamycin (blue), or media alone (black) with fluorescence minus one (FMO) control (gray), and then transcription factors were intracellular stained and analyzed by flow cytometry. (**K**) Graphs show percent $CXCR3^+$ and $CCR9^+$ of total adoptively transferred memory CD8 T cells in the SI LP after IL-15 complex treatment. Data are means (± SEM) representative of three independent experiments (n = 3–5 mice per group). Histograms and dot plots are from one animal representative of the group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

IL-15 complexes further promote the effector program in memory CD8 T cells

Migration into mucosal tissues is limited to effector CD8 T cells, whereas memory CD8 T cells do not redistribute to the SI and FRT under steady-state conditions. Moreover, overexpression of *Klf2* leads to dramatic changes in expression of a broad range of genes related to trafficking, effector function, cytokine receptors, and transcriptional regulation (48). Therefore, we next asked whether IL-15 complexes could alter effector functions of memory CD8 T cells. Ex vivo culture of memory CD8 T cells in the presence of IL-15 increased Granzyme B expression (Fig. 7G). Effector CD8 T cells differentiated in the presence of IL-15 showed increased mitochondrial mass and, as a result, increased mitochondrial spare respiratory capacity (49). IL-15 significantly increased mitochondrial staining in memory CD8 T cells cultured for 24 h, indicating both increased total mitochondrial mass and polarized mitochondria, suggesting IL-15 induces rapid mitochondrial biogenesis (Fig. 7H). We also observed increased mitochondrial staining after ex vivo peptide stimulation of memory CD8 T cells (Fig. 7I), in line with a previous report that TCR/CD28 stimulation of T cells increases mitochondrial biogenesis (50). In addition, IL-15 induced a change in cell-surface expression patterns similar to that of effector CD8 T cells (i.e., increase cell size, increased CD8α expression, decreased CD127, and increased CD27) on memory and naive CD8 T cells (Supplemental Fig. 3). IL-15 increased protein levels of T-bet, Bcl-6, and Blimp-1 in memory CD8 T cells and upregulation of T-bet was mediated by mTOR as IL-15 stimulation in the presence of rapamycin attenuated the increase in T-bet (Fig. 7J). Furthermore, more resting memory CD8 T cells infiltrating the mucosal tissues after i.p. administration of IL-15 treatment were $CXCR3^+$ and $CCR9^+$ (Fig. 7K).

Discussion

Mucosal tissues represent a vast barrier from the external environment that is often the site of initial pathogen exposure. Vaccine strategies that promote the establishment of T_{RM} cells within the mucosal tissues hold promise for limiting early dissemination of virus infections such as HIV-1 and influenza (7, 51). We demonstrated that CD8 T cells depend on mTORC1 signaling to specifically accumulate with mucosal tissues. Moreover, the common γ-chain cytokine IL-15 activates mTOR and recruits CD8 T cells into the mucosa. We found that IL-15 further promotes the effector program mediated through mTORC1 and T-bet signaling. This study reveals a currently unappreciated function of IL-15 in promoting the trafficking of CD8 T cells away from SLOs and into mucosal tissues. (See Supplemental Fig. 4 for a schematic overview of how IL-15 controls the generation of memory CD8 T cells and influences their trafficking and ability to accumulate within peripheral tissues.) We demonstrate that administration of IL-15 leads to local recruitment of $CXCR3^+$ cells, and thus CXCR3 might potentiate the ability of effector cells to enter the circulation and traffic to mucosal sites after IL-15 treatment. Blimp-1 and T-bet promote the trafficking of effector CD8 T cells from the T cell zone of the spleen into the red pulp (52). In this study, overexpression of T-bet increased the accumulation of effector cells in the splenic red pulp. Our data show that without either mTOR or IL-15 signals, effector CD8 T cells were enriched within the splenic nonvascular staining populations, suggesting that IL-15 and mTOR functioned to increase exit from the splenic T cell zone and into the red pulp. Blockade of mTOR signaling decreased T-bet levels and increased expression of CD62L (*Sell*) and CD127 (*Il7r*), signals which favor survival of memory CD8 T cells and their localization to SLOs (53).

The transcription factor FoxO1 controls expression of *Sell*, *Il7r*, as well as *Ccr7* and *S1pr1*. FoxO1 is a well-documented transcriptional regulator of T cell trafficking, memory formation, and homeostasis (33–38). FoxO1 activity is regulated by the phosphorylation of Akt (Ser^{473}) by mTORC2, which leads to the inactivation of FoxO1 by exclusion from the nucleus. Recently, the contributions of mTORC1 and mTORC2 in memory differentiation were characterized, and they appear to control effector and memory cell formation, respectively (54). In this study, the absence of mTORC2 partially diminished FoxO1 phosphorylation (Ser^{253}) within in vitro–expanded CD8 T cells. We demonstrate that IL-15 inactivates FoxO1 (indicated by Ser^{253} phosphorylation), which was partially dependent on mTOR signaling. IL-15 complexes could activate not only mTORC1 but also mTORC2. We also show that IL-7 complexes were capable of recruiting T cells into mucosal tissues. Whereas IL-7 and IL-15 signal through the common γ-chain (CD132), IL-7 does not signal through CD122 like IL-15. Both IL-15 and IL-7 can induce phosphorylation of Akt Ser^{473} and inactivate FoxO1, suggesting this signal is transduced, at least in part, through CD132. One possibility is that CD122 signals activate mTORC1 and CD132 signals activate mTORC2. This is supported by our observation that IL-7 does not induce activation of mTORC1 as rapidly as IL-15. Moreover, IL-15 activation of mTORC1 can be inhibited by anti-CD122 blockade in NK cells (22). FoxO1 also controls *Klf2* transcription to control T cell trafficking, and we show that *Klf2* mRNA levels are decreased after IL-15 treatment. We also demonstrate that mTOR blockade with rapamycin increased *Klf2*, even in the presence of IL-15. Taken together, our findings indicate that IL-15 and mTOR control the trafficking of effector CD8 T cells into the circulation by modulating the levels of T-bet and FoxO1.

Other factors besides IL-15 that can increase levels of T-bet, including TCR signaling, are IL-2 and IL-12. IL-2 also increases the proliferation of regulatory T cells and IL-12 promotes the terminal differentiation of CD8 T cells. However, IL-12 has also been shown to sustain T-bet expression in effector CD8 T cells through an mTORC1-dependent mechanism, and rapamycin treatment can limit terminal differentiation (53). Early effector CD8 T cells express T-bet and Bcl-6 and removal of inflammatory stimuli results in the rapid loss of both Bcl-6 and T-bet. IL-15, like IL-12, has been reported to increase T-bet expression (55). We show that IL-15 also sustains the expression of Bcl-6 and Blimp-1 in effector CD8 T cells. Bcl-6, a transcription factor and gene target of STAT3, promotes the accumulation memory precursor ($CD127^{hi}KLRG1^-$) cells. STAT3 activation is impaired in mTORC1-deficient T cells (43), and this is consistent with our observations that IL-15–sustained Bcl-6 was ablated upon mTOR inhibition. We found that, at the peak of the effector response, terminally differentiated effector CD8 T cells were restricted to the vascular-staining populations in the spleen. Although an increase in the level of T-bet is important for promoting differentiation of effector CD8 T cells, repression of T-bet by FoxO1 and/or receding inflammation promotes the transition of effectors to memory cells (38). Moreover, the formation of short-lived effector cells and the effectors that develop into memory precursor cells appear to be controlled by a gradient of T-bet expression (56). Overexpression of T-bet in effector CD8 T cells limits formation of T_{RM} cells in the lung (57). Mechanistically, T-bet may directly repress TGF-β–mediated upregulation of CD103 by competing with Smad3 binding on the *Itgae* (CD103) promoter. Although we found that knockdown of mTOR in effector CD8 T cells resulted in a reduced quantity of effectors in the gut epithelium, the majority of cells were $CD103^+CD69^+$. Unlike shRNA-knockdown experiments, low-dose rapamycin reduced the frequency of $CD103^+CD69^+$ cells in the gut epithelium. We reconciled this

difference with the fact that mTOR knockdown impairs both mTORC1 and mTORC2 signaling, whereas low-dose rapamycin only partially impairs mTOR, presumably by selective attenuation mTORC1 signaling.

In $CD4^+$ T cells, a T-bet/Bcl-6 complex binds to the promoters of *Socs1* and *Socs3* and represses their expression (58). In absence of T-bet, Th1 cells have increased SOCS1 and SOCS3 levels. We found that IL-15 complexes altered expression of *Socs1* and *Socs3* in effector CD8 T cells. Unlike CD4 T cells, IL-15 (and IL-7 to a lesser extent) increased levels of *Socs1* mRNA despite increased T-bet and Bcl-6. IL-15's ability to increase *Socs1* was partially dependent on mTOR. SOCS1 is an important negative regulator of STAT5 signaling and, as such, *Socs1*-deficient CD8 T cells are hyperresponsive to IL-15 (59). The upregulation of SOCS1 may serve as a negative feedback loop for effectors in response to increased IL-15 and IL-7 signaling. SOCS3 negatively regulates IL-12–mediated STAT4 activation and blockade of mTORC1 signaling in CD4 T cells increases SOCS3 levels (43, 44, 60). STAT3 activation sustains SOCS3 and Bcl-6, and in STAT3-deficient CD8 T cells effectors fail to form memory CD8 T cells as they are hyperresponsive to IL-12 (42). In accordance with this, we observed an mTOR-dependent decrease in *Socs3* expression after IL-15–induced upregulation of Bcl-6 and T-bet.

It is possible that activation of mTOR is required for early egress out of SLOs and into the circulation; then once arriving within NLTs, mTOR is inhibited to promote retention within the tissues and establishment of long-lived memory cells. This possibility is supported by: 1) our observations that mTOR deficiency leads to sequestration of effectors in SLOs, 2) previous reports that T-bet and Blimp-1 can promote trafficking of effectors in the spleen and their exit into the circulation, and 3) reports that subsequent repression of T-bet is required for the formation of T_{RM} cells within the lung (57).

Therefore, we propose a model where activated CD8 T cells are recruited into the circulation after priming according to their level of T-bet expression. IL-15 signals produced by locally inflamed tissues promote the recruitment of CD8 T cells into mucosal tissues. Once in the tissue, mTOR signaling is repressed, decreasing the level of T-bet and enabling effectors to take up residence and persist long after the infection is resolved. IL-15's ability to activate STAT3 and STAT5 may serve as an early survival signal for memory precursors that migrate to NLTs. In contrast, terminally differentiated effectors are promptly cleared by apoptosis from within the epithelium of NLTs due to their dependence on mTOR-mediated survival signals.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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