Lymphatic endothelial cells (LECs) chemoattract naïve T cells and promote their survival in the lymph nodes, and can cross-present antigens to naïve CD8+ T cells to drive their proliferation despite lacking key costimulatory molecules. However, the functional consequence of LEC priming of CD8+ T cells is unknown. Here, we show that while many proliferating LEC-educated T cells enter early apoptosis, the remainders comprise a long-lived memory subset, with transcriptional, metabolic, and phenotypic features of central memory and stem cell-like memory T cells. In vivo, these memory cells preferentially home to lymph nodes and display rapid proliferation and effector differentiation following memory recall, and can protect mice against a subsequent bacterial infection. These findings introduce a new immunomodulatory role for LECs in directly generating a memory-like subset of quiescent yet antigen-experienced CD8+ T cells that are long-lived and can rapidly differentiate into effector cells upon inflammatory antigenic challenge.
The lymphatic endothelium is actively involved in shaping T cell responses, both directly and indirectly\(^1\). In the lymph nodes (LNs), lymphatic endothelial cells (LECs) have been shown to present peripheral tissue-restricted antigens on MHC class I molecules (MHC-I) to induce tolerance in autoreactive CD8\(^+\) T cells\(^2\). In addition to endogenous antigen presentation, we and others have demonstrated that LECs abundantly scavenged and can cross-present exogenous antigens to naive CD8\(^+\) T cells and drive proliferation, both in the context of a tumor\(^6\) as well as under homeostatic conditions\(^10\). These LEC-activated T cells lack effector cytokine secretion and some undergo early-generation apoptosis, suggesting a dysfunctional or anergic phenotype. LECs have therefore been established as antigen-presenting cells (APCs) that can sample the peripheral exogenous antigen repertoire and contribute to CD8\(^+\) T cell regulation and tolerance.

The induction of peripheral tolerance has been primarily assigned to professional APCs\(^11\). Under homeostatic conditions, LN-resident immature dendritic cells (DCs) acquire self-antigens from peripheral tissues and cross-present them without co-stimulation, inducing T cell tolerance or deletion\(^12\). In this case, peripheral tolerance of CD8\(^+\) T cells remain functionally impaired, even in response to pathogenic challenge\(^13\). In contrast, apparently tolerized CD8\(^+\) T cells that were primed by non-hematopoietic cells—such as intestinal epithelial cells and liver sinusoidal endothelial cells (LSECs)—can exit the non-responsive state and be functionally reactivated under pro-inflammatory conditions\(^14,15\). We therefore asked whether quiescent LEC-educated CD8\(^+\) T cells can be functionally activated upon inflammatory challenge, thereby representing a memory-like state rather than an irreversibly anergic or tolerized state.

Canonically, memory T cells are considered to arise during the contraction phase of an immune response (e.g., after primary infection or vaccination), differentiated from a subset of effector T cells. However, other models propose that memory T cells can arise directly from naïve cells, either as precursors to effector memory and effector T cells (i.e., the loss of memory correlates with terminal differentiation), or as asymmetric divisions reminiscent of stem cell behavior\(^16,17\). The memory T cell pool is also highly heterogeneous, as their phenotypic state is tightly governed by the integration of numerous signals including antigen load and persistence, TCR affinity, co-stimulation, cytokine environment, and CD4\(^+\) T cell help\(^16-18\). An emerging consensus suggests that higher levels of TCR-pMHC affinity, antigen load, co-stimulation, and pro-inflammatory cytokines promote differentiation into terminal effectors, while weaker activation signals promote a shift towards memory T cells\(^19\). This new understanding raises important considerations with regard to CD8\(^+\) T cell priming by LECs and other non-hematopoietic cells, which occurs in the context of lower levels of co-stimulation and pro-inflammatory cytokines and higher levels of co-inhibitory signals relative to priming by activated DCs\(^10\).

Here, we describe a new role for LECs in directly cross-priming naïve CD8\(^+\) T cells into long-lived memory T cells capable of potent effector differentiation following inflammatory challenges. This has implications for understanding T cell memory and also helps to reconcile the seemingly contradictory roles of lymphangiogenesis in tumors versus in chronic inflammatory diseases or transplantation. We suggest that LEC-educated T cells may function as a reserve of antigen-experienced T cells in a memory-like state, enhancing immune responses upon immunogenic challenge or promoting tolerance upon challenge under immunosuppressive conditions, as in the tumor microenvironment.

**Results**

**Priming by stromal cells promotes CD8\(^+\) T cell memory.** First, we sought to determine the relevance of antigen uptake and cross-presentation by LECs on CD8\(^+\) T cell responses in vivo. Creating mice in which MHC-I presentation is restricted to LECs was not feasible, since there is no known truly lymphatic-specific gene (e.g., Lvyel is expressed by macrophage subsets, Proxl1 by hepatocytes, and podoplanin (Pdpn) by fibroblast subsets). Instead, we generated bone marrow chimeric mice in which donor CD45\(^+\) cells lacked MHC-I (\(\beta_2\)-m\(^-/-\)WT, denoting the status of the hematopoietic population:non-hematopoietic remainder of the host), and therefore presentation of the H-2K\(^\text{b}\) restricted SIINFEKL epitope after intradermal (i.d.) injection of OVA would be restricted to radiosensitive lymph node stromal cells (LNSCs). LNSCs include fibroblastic reticular cells (FRCs), follicular DCs, and blood endothelial cells (BECs) as well as LECs. However, among these LNSC subtypes, LECs take up the majority of antigen in the draining LNs (dLNs) following i.d. administration under steady-state conditions\(^10\). Furthermore, when loaded with antigen ex vivo, FRCs induced negligible T cell proliferation in vitro compared to LECs (Supplementary Fig. 1). We therefore reasoned that LECs would be the primary Ag-presenting cells for OVA following i.d. administration in \(\beta_2\)-m\(^-/-\)WT chimeric mice, with little contribution from FRCs\(^20\). As controls, we also generated inverse chimeras (WT:\(\beta_2\)-m\(^-/-\)) as well as positive (WT:WT) and negative (\(\beta_2\)-m\(^-/-\)-\(\beta_2\)-m\(^-/-\)) controls.

We transferred naïve CFSE-labeled OT-I CD8\(^+\) T cells (CD45.1/2\(^+\)) intravenously (i.v.) into the mice and the following day administered i.d. OVA (50 µg) or PBS (Fig. 1a). Five days later, we analyzed the OT-I cells in skin-dLNs and observed substantial levels of antigen-specific proliferation in \(\beta_2\)-m\(^-/-\)-WT mice compared to those injected with PBS, suggesting that indeed, MHC-I-antigen presentation by LNSCs can induce T cell proliferation in vivo (Fig. 1b). As expected, transferred OT-I T cells proliferated the most in WT:WT mice and the least in \(\beta_2\)-m\(^-/-\)-\(\beta_2\)-m\(^-/-\) mice, while interestingly, proliferation was comparable in WT:\(\beta_2\)-m\(^-/-\) and \(\beta_2\)-m\(^-/-\)-WT mice (both ~70% of levels in WT:WT mice), indicating that LNSC participation in T cell priming under steady-state conditions is important and physiologically relevant compared to that induced by DCs and other hematopoietic cells.

Next, we evaluated the phenotype of the LNSC-educated OT-I cells. In all mice except \(\beta_2\)-m\(^-/-\)-\(\beta_2\)-m\(^-/-\) chimeras, the majority of transferred cells expressed CD44\(^+\) with both central memory (T\(_{\text{CM}}\): CD44\(^+\)CD462L\(^+\)) and effector/effector memory (T\(_{\text{DE/EM}}\): CD44\(^+\)CD462L\(^-\)) subsets in all groups (Fig. 1c). Interestingly, the T\(_{\text{CM}}\) fraction was highest in the \(\beta_2\)-m\(^-/-\)-WT mice (Fig. 1d), where the ratio of T\(_{\text{CM}}\) to T\(_{\text{DE/EM}}\) was close to unity (Fig. 1e), while in mice lacking MHC-I on LNSC (WT: \(\beta_2\)-m\(^-/-\)), ~30% of total OT-I cells were T\(_{\text{CM}}\). Upon ex vivo SIINFEKL re-stimulation, the OT-I cell fractions that were IFN\(\gamma\) or IL-2\(^+\) were similar among the groups (Fig. 1f, g), but the fraction of double-positive cells was lowest in WT: \(\beta_2\)-m\(^-/-\) mice (Fig. 1h) and the fraction of IFN\(\gamma\) cells expressing IL-2 was highest in \(\beta_2\)-m\(^-/-\)-WT mice (Fig. 1i). These results established that (i) LNSCs are capable of antigen presentation under non-inflammatory conditions to induce the proliferation and activation of CD8\(^+\) T cells, and (ii) T cell education by LNSCs promotes a memory phenotype.

**LECs uniquely generate CD8\(^+\) memory.** Although LECs represented the dominant LNSC subset that both take up i.d.-delivered antigen\(^10\) and can cross-present antigen to naïve CD8\(^+\) T cells (Supplementary Fig. 1), we could not rule out the unlikely possibility that migratory DCs such as Langerhans cells, which are less radiation-sensitive than circulating DCs\(^21\), could contribute to the memory skewing that we saw (although previous in vitro studies using co-cultures of such cells with CD8\(^+\) T cells did not recapitulate the phenotype we observed\(^21-23\)). To address this, we first exposed LN-resident cells to antigen in vivo and then tested
their ability to activate cognate T cells ex vivo. Specifically, we injected WT mice i.d. with OVA or PBS, and after 2 h, isolated the dLNs and sorted for four subsets: DCs (CD45^+ CD11c^+), macrophages (Mψ; CD45^+ F4/80^+), LECs (CD45^- gp38^+ CD31^-), and FRCs (CD45^- gp38^- CD31^-). These subsets (or antigen-pulsed, LPS-activated bone marrow-derived DCs (BMDCs) as a positive control) were immediately placed into culture with naïve OT-I CD8^+ T cells for 3 days (Fig. 2a).

We reasoned that after 2 h, injected antigens would have already drained to and perfused the sinuses of the dLNs, whereas migratory DCs that picked up antigen from the skin would not yet have arrived24. Consistent with this, 71 ± 4% of LECs were positive for antigen at this time, versus 14 ± 4% of LN-resident cells (Fig. 2b, c). Nevertheless, the antigen-positive DCs had taken up more antigen per cell than the antigen-positive LECs, showcasing their propensity to sample and internalize antigen (Fig. 2d).

Without antigen, overall OT-1 cell survival was highest when co-cultured with LECs and lowest with FRCs, consistent with previous reports that LECs are the major source of the pro-survival cytokine IL-7 in the LN27,28. With antigen, LECs were the only subset that increased T cell numbers (Fig. 2e) and activated T cells (Fig. 2f) relative to PBS. There was no appreciable increase in T cell numbers when cultured with DCs, presumably due to inefficient in vivo targeting of DCs compared to LECs after 2 h (Fig. 2c). As expected, the overall differences seen in OVA-treated vs. PBS-treated mice for all groups were small compared to when antigen pulsing is done in vitro, where all of the cells would be exposed to antigen (Supplementary Fig. 1).

In addition, antigen education by LECs increased intracellular IFNγ^+ in OT-1 CD8^+ T cells (Fig. 2g). Interestingly, this was not reflected in the levels of IFNγ in the supernatant, which were low in all cultures compared to BMDC-educated T cells (Fig. 2h). On the other hand, IL-2 levels were significantly elevated in LEC and DC co-cultures from OVA-treated vs. PBS-treated mice. These results suggest that LEC education activates the CD8^+ T cells to a state where they are poised to generate IFNγ without secreting it, while increasing secretion of IL-2 to support homeostatic proliferation and survival18.

We next explored the phenotype of these activated OT-1 cells. Interestingly, the IFNγ^+ cells activated by LECs isolated from OVA-treated vs. PBS-treated mice occurred mainly in CD62L^- cells (Fig. 2i); indeed, the TCM compartment accounted for ~55% of IFNγ^+ T cells (Fig. 2i). These IFNγ^+ OT-1 cells also stained positively for other markers of memory cells, including the receptors to IL-2, IL-7, and IL-2/15 (CD25, CD127, and CD122, respectively) as well as the co-stimulatory receptor CD27 (Fig. 2k, l). Collectively, these data suggest that in the absence of inflammatory signals, antigen cross-presentation by LECs under steady-state conditions may directly promote CD8^+ T cell memory.

To further test whether LECs can indeed promote the acquisition of a memory phenotype by CD8^+ T cells in vivo, we designed a method to introduce βm^-competent LECs into βm^-/- mice, such that the transplanted LECs would be the sole
source of peptide-MHC systemically. For such a system, we used LECs isolated from Actb-mOVA mice, where expression of membrane-bound OVA is driven by the β-actin promoter in all cells. We confirmed that in vitro, these LECs could stimulate the acquisition of CD44^CD62L^ phenotype by co-cultured naïve OT-I cells (Fig. 3a; 28 ± 13%, relative to 7.6 ± 0.4% with wild-type, unpulsed LECs), albeit to a lesser degree than OT-I cells co-cultured with OVA-pulsed control LECs (84 ± 2%).

In our hands, injections (i.d., intraperitoneal, intravascular) of LEC cell suspensions produced poor survival and recovery even within 1 day post-administration, so we opted to produce 3D spheroid cultures for implantation, based on the observation that many endothelial cells maintain better structure and function in the presence of self-derived extracellular matrix proteins. Spheroids (18 ± 3 μm diameters) were implanted into i.d. pockets formed between the ear cartilage and the ventral skin (Fig. 3b), where they survived more than 8 days in fully immunocompetent mice (Fig. 3c), β₂m^−/− mice implanted with WT or OVA-expressing LEC spheroids later received a co-transfer of OT-I and bystander CD8^+ T cells at a 1:5 ratio (Fig. 3d). While there were...
no differences in the overall numbers of OT-1 or bystander CD8+ T cells recovered in the blood at 1 week post-transfer (Fig. 3e), there were fewer naïve (CD44hiCD62L−) cells among OT-1 cells in mice that had received OVA-expressing LECs (Fig. 3f). Moreover, among CD8+ T cells isolated from the implant site, mice that received OVA-expressing LECs specifically expanded OT-1 cells, and a higher frequency of those OT-1 cells expressed CD69 (Fig. 3e, g, h). This increased activation was only seen at the implant site, rather than the superficial cervical LNs draining the implant site (Fig. 3g, h). Together with the data shown in Figs. 1 and 2, these data indicate that antigen presentation by LECs in vivo results in the acquisition of memory phenotype by cognate CD8+ T cells.

**LECs prime naïve CD8+ T cells into TCM/TSCM-like states.** To further examine APC-intrinsic differences in the education and activation of cognate CD8+ T cells, we turned to in vitro activation studies using CD8+ T cells from OT-1 mice in co-culture with primary LN LECs or LPS-matured BMDCs (herein referred to as mDC). For antigen, we used a nanoparticle delivery platform for SIINFEKL with a reduction-sensitive linkage, NP-ss-COVA250−264 (Fig. 4a–h, Supplementary. Fig. 2a–e), which our group has extensively characterized as a tool to detect efficient cross-presentation. Within 3 days, LEC-educated CD8+ T cells proliferated robustly and increased in size compared to naïve cells, indicative of their activated state, although they did not acquire the large blastoid morphology of mDC-educated cells (Fig. 4a, b). Interestingly, we observed that LEC-educated cells primarily expressed a CD44hiCD62L− phenotype, which was clearly distinct from the effector-like phenotype of mDC-educated cells. This was also distinct from the phenotype of OT-1 cells co-cultured with LN FRCs in the presence of OVA257−264, which proliferated significantly less than LEC-educated cells and also induced some effector cells (Supplementary Fig. 1). Therefore, OT-1 CD8+ T cells co-cultured in vitro with LECs, but not with FRCs, acquired a similar phenotype as those educated in vivo in the βm-/-;WT chimeras, further confirming that among the major non-hematopoietic LNSC populations, LECs rather than FRCs promote the acquisition of memory phenotype in CD8+ T cells.

We next analyzed the kinetics of T cell phenotype of LEC-educated versus mDC-educated OT-1 cells, and we observed that CD62L was initially downregulated on both LEC-educated and mDC-educated cells (Fig. 4b, day 1), consistent with reports that CD62L is cleaved from the cell surface following TCR engagement. However, as LEC-educated cells proliferated, they predominantly expressed CD62L, whereas the mDC-educated...
**Fig. 4 Surviving LEC-primed CD8+ T cells express T_{CM}/T_{SCM}-like phenotype.** Flow cytometry analysis of phenotype of naïve CFSE-labeled OT-I cells following 3 days co-culture (unless otherwise noted) with Ag-pulsed LECs or mDCs. As a control, resting naïve OT-I cells have been shown (Educ: Naive). a Representative dot plots showing the size and granularity (top) and CD62L and CD44 expression (bottom) of OT-I cells following various co-culture conditions. b Representative dot plots of CD62L expression versus CFSE dilution. c-h Absolute recovery of live LEC or mDC-educated cells versus time in co-culture. d Percentage of OT-I cells within each generation that stain positive for the apoptotic marker Annexin V (AnV) following 2 days co-culture. e Frequency of CD62L+ cells per generation gated on AnV+ or AnV− OT-I cells, or conversely, f AnV+ cells gated on the T_{eff/EM}-like (CD44+CD62L−) or T_{CM-like} (CD44+CD62L+) cells following 2 days co-culture. g Normalized mean fluorescence intensity (MFI; fold-change relative to isotype control) for indicated phenotypic markers in CD44−CD62L− naïve, versus CD44+CD62L+ LEC-educated, versus CD44+CD62L+ mDC-educated cells. h Composition of total recovered OT-I cells classified as naïve (CD44−CD62L−); central memory (CM)-like or stem cell memory (SCM)-like (CD44+CD62L−CD127+CD122−Sca-1−); effector memory (EM)-like (CD44+CD62L−CD127+); effector (Ef)-like (CD44+CD62L−CD127−). i, j LECs or mDCs were co-cultured with Pmel CD8+ T cells in the presence of gp10025−33 peptide for 3 days prior to analysis of Pmel phenotype by flow cytometry. Gated on live Pmel CD8+ T cells, i dot plots indicate expression of CD44 and CD62L with inset numbers indicating percentages of Pmel cells that occurred within the gate. j Histograms depict expression of the indicated phenotypic markers depending on co-culture condition. k, l Naïve OT-I cells were sorted by FACS based on CD44 expression as naïve or ‘virtual memory’ (VM) cells prior to 3 days co-cultures. k Frequency of T_{CM-like} (CD44+CD62L+) OT-I cells, l ratio of T_{CM-like} to T_{eff/EM-like} (CD44+CD62L−) OT-I cells, both gated on live OT-I cells. Gating strategy in Supplementary Fig. 3b. Data points/columns and error bars indicate mean ± SD from a representative of two a-f, i to three g-j independent experiments (n = 3 technical replicates per experiment); stars indicate significant difference between groups in the same generation d-f or compared to naïve group g; *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA c, g, k, l or two-way ANOVA d-f with Bonferroni post-test.
T cells followed the opposite trend. Moreover, mDC-educated cells expanded continuously, while LEC-educated cell numbers fluctuated over time (Fig. 4c). In agreement with our earlier findings for skin-derived, immortalized LECs, a higher fraction of LN LEC-educated T cells were positive for the apoptosis marker AnnexinV (AnV) compared to those educated by mDCs on day 2, when both central and effector memory subsets are detectable (Fig. 4d). However, when we compared the expression of CD62L per generation among AnV− versus AnV+ cells, apoptotic LEC-educated OT-1 cells were enriched for the CD62L− phenotype, whereas both apoptotic and non-apoptotic mDC-educated cells shared similar frequencies of CD62L− cells (Fig. 4e). Conversely, the T eff/EM-like cells were significantly more apoptotic than the T CM-like cells (Fig. 4f). This observation implies that LEC education induces two divergent populations among CD8+ T cells: apoptotic effector–like cells (which are presumably deleted) and surviving predominantly T CM-like cells.

To further investigate the phenotype of LEC-educated T CM-like cells in terms of canonical markers of memory versus effector cell differentiation, we measured a variety of memory-related surface markers using flow cytometry (Fig. 4g, Supplementary Fig. 2a, b). LEC-educated cells expressed CD127 (IL-7R), CD122 (IL-2Rβ), and CCR7 at levels similar to or higher than in mDC-educated cells. These markers are notable as CD127 and CD122 functionally mediate IL-7 and IL-15-mediated signaling for the homeostatic proliferation of memory cells, while CCR7 allows homing of T CM cells to secondary lymphoid organs. Furthermore, the relative expression in LEC-educated T cells of the transcription factors (Tfs) Eomes and Tbet (Tbx21), which are mutual antagonists that counter-regulate memory vs. effector differentiation, was consistent with a T CM-like phenotype (increasing Eomes and decreasing Tbet) (Fig. 4g, Supplementary Fig. 2b). Interestingly, LEC-educated cells expressed additional markers that may delineate a subset of stem cell-like memory T cells (TSCM)33–35, including stronger expression of Sca1, B cell lymphoma 2 (Bcl2), CD27, CD95 (Fas), CXCR3, and LFA1 compared to mDC-educated cells.

Further illustrating phenotypic differences between LEC-educated and mDC-educated CD8+ T cells (Fig. 4h), most LEC-educated cells were CD44+CD62L+ (T CM-like), with the largest subset expressing high levels of CD127, CD122, and Sca1, whereas mDC-educated cells were mostly CD44−CD62L−CD127− (T eff/EM-like). We further observed a distinct cytokine milieu in LEC- versus mDC-educated T cell co-cultures (Supplementary Fig. 2c), with increased G-CSF, eotaxin (CCL11), CXCL10, CCL7, IL-31, IL-7, IL-2, IL-22, TNFa, GM-CSF, and RANKL. In both LEC and mDC co-cultures with OT-1 cells, IL-7 and IL-15, which are known to contribute to the induction of TSCM cells, were similarly present, but at the mRNA level, LEC expression of these cytokines were increased upon cognate T cell activation (Supplementary Fig. 2d, e).

LEC-educated CD8+ T cells similarly acquired memory characteristics regardless of whether we used free or nanoparticle-bound SIINFEKL peptide (NP-ss-COVAS5O-26A) or full-length ovalbumin (Fig. 3a, Supplementary Figs. 1, 2f). We also examined if TCR affinity may affect memory phenotype acquisition by adapting the co-culture experiments to naïve OT-3 CD8+ T cells, which possess TCRs with a lower affinity for the SIINFEKL peptide than OT-1 CD8+ T cells. TCR affinity accounted for a reduction in the overall frequency of activated OT-3 cells (~50% when educated by LECs and ~80% by mDCs compared to ~98% of OT-1 cells for both education conditions). Nonetheless, OT-3 cells preferentially acquired a memory phenotype when educated by LECs (Supplementary Fig. 2g). Importantly, these same observations also held true in other antigen-transgenic TCR systems, such as with Pmel CD8+ T cells co-cultured with gp100 peptide-pulsed LECs (Fig. 4i, j).

Because naïve OT-1 mice often harbor a significant population of CD44+CD8+ T cells, or so-called ‘virtual memory’ (VM) cells, we wanted to rule out the possibility that the activated phenotype we saw in LEC-educated OT-1 cells in these studies was not due to those cells being re-activated, rather than from priming of naïve cells. To this end, we sorted OT-1 splenocytes based on naïve (CD44−CD62L+) or VM phenotype (CD44+) prior to co-culture with LECs or mDCs pre-pulsed with full-length OVA protein (Fig. 4k, l, Supplementary Fig. 3). We found that >80% of LEC-educated naïve OT-1 cells acquired a CD44+CD62L+ phenotype, compared to ~57% for LEC-reactivated ‘VM’ OT-1 cells (Fig. 4k, l, Supplementary Fig. 3b). This correlated with increased IL-2 production (Supplementary Fig. 3c), further confirming the T CM-like phenotype of these cells.

Collectively, these data indicate that LEC-educated CD8+ T cells phenotypically show characteristics of both T CM and TSCM subsets, independently of the transgenic TCR tested (i.e., CD8+ T cells from OT-1, OT-3, and Prf1 transgenic mice) or the antigen formulation (peptide, full-length protein, or nanoparticle-bound), and are distinct from the T eff/EM-like phenotype acquired by DC-educated cells under inflammatory conditions.

LEC-primed CD8+ T cells express a memory-like transcriptome. To determine whether the phenotypic differences we saw between LEC-educated and mDC-educated T cells are reflected at the transcriptomic level, we performed RNA sequencing to characterize and quantify the transcriptional profiles of naïve, LEC-educated and mDC-educated CD8+ T cells after up to 3 days of co-culture. Unsupervised principal component analysis (PCA) (Fig. 5a) and hierarchical clustering (Supplementary Fig. 4a) were performed to visualize the kinetics of T CM-like and T eff/EM-like phenotype acquisition by LEC-educated and mDC-educated CD8+ T cells, respectively, and to potentially identify sets of genes that were modulated by differences in the APCs across time. The first and second principal components (PC1 and PC2) explained ~52% and 18%, respectively, of the total variance in gene expression profiles. Interestingly, while LEC and mDC-educated cells clustered closely to one another on day 1, both shifted along the PC2 axis over the 3 days co-culture period, with LEC-educated cells diverging on the last day from mDC-educated cells along the PC1 axis toward naïve cells (Fig. 5a).

Therefore, we reasoned that PC1 is likely to primarily contain genes that differentiate antigen-experienced from naïve T cells, while PC2 likely accounted for genes associated with memory/effector differentiation.

Focusing solely on the expression of memory/effector signature genes, we saw that LEC-educated cells (Fig. 5b, Supplementary Fig. 4b) displayed higher expression of genes related to the T CM/TSCM subsets (Sell, Cd27, Cd28, Ly6e, Ccr7) by d3, whereas mDC-educated cells expressed higher levels of genes consistent with an effector signature (Gzm’s, Prf1, Ifng, il2ra, Cxcr5, Sema7a), with the exception of Pdcd1 (encoding PD-1), which was highly upregulated in LEC-educated cells as previously reported. Notably, Sell (CD26L) expression was initially downregulated in LEC-educated cells on day 1, but then upregulated to levels similar to those in naïve cells, consistent with our observations at the protein level (Fig. 4b). This trend was also observed in LEC-educated cells for other memory-associated genes (Cd27, Cd28, Ly6e, Sph, Il7r, and Ccr7), while mDC-educated cells either failed to upregulate or upregulated them to a lesser extent (Fig. 5b, c, Supplementary Fig. 4b). Finally, consistent with our observations that some LEC-educated cells
Fig. 5 Transcriptome and metabolic signaling in LEC-educated CD8⁺ T cells is consistent with TCM/TSCM-like phenotype. Naïve OT-I cells were educated in the presence of antigen by LECs or mDCs for up to 3 days and subsequently analyzed by RNA sequencing. Resting naïve OT-I cells have been shown for comparison. a (top) Principal component analysis (PCA) of most-modulated genes across time and APC type suggest PC1 (52% of variance) includes genes related to antigen experience and APC type, whereas PC2 (18% of variance) contains genes related to the effects of time. (bottom) Heatmap depicting the most significant differences in gene profiles following 3 days of education by LECs or mDC, filtering for changes in expression (log2FC > 2.89) between mDC/LEC-educated CD8⁺ T cells and p < 0.01. b Heat maps depicting normalized expression levels of various memory-associated and effector-associated genes on OT-I cells according to co-culture condition and time. Each column represents data originating from a single biological replicate. c Normalized expression (rpkm; reads per kilobase per million mapped reads) of genes encoding the central mediators of the mTOR-signaling pathway, including components of the mTORC1 (the most significant changes), and the most significant changes in expression (|log2FC| > 2.89) between mDC/LEC-educated CD8⁺ T cells and p < 0.01. d Unsupervised hierarchical clustering analysis to identify dynamics of gene expression encoding TFs regulating effector/memory differentiation. e (left) Normalized expression of genes encoding mTOR-related proteins. f Volcano plot highlighting most-modulated genes following LEC education (magenta) or mDC education (blue), and kinetics of normalized expression of selected genes. g, h pair-wise comparison of transcriptomes as volcano plots highlighting sets of genes linked to specific biological processes, as annotated on the Gene Ontology Atlas. i Heatmap plots for genes associated with anabolic (glycolysis) and catabolic processes (oxidative phosphorylation, aerobic respiration, and fatty acid oxidation). j Normalized gene expression (rpkm) of genes encoding the central mediators of the mTOR-signaling pathway, including components of the mTORC1 (Rictor) or mTORC2 (Rictor) complexes. k I SIINFEKL-pulsed LECs or mDCs were co-educated with naïve OT-I cells for 2 h in vitro, and phosphorylated mTOR (S2448) and Akt (S473) was quantified on OT-I by intracellular flow cytometry. Data points and error bars indicate mean ± SD of n = 3 biological replicates. a–j or n = 2–8 technical replicates.
Since metabolism and T cell differentiation state are interrelated processes, we examined the expression of genes related to respiratory chain and fatty-acid metabolic programs, typically associated with resting naive and memory T cells, and glycolysis, which is dominant in proliferating effector cells (Fig. 5i). Both LEC/mDC-educated cells upregulated genes related to these metabolic programs compared to naive cells, while glycolysis-associated genes were more upregulated in mDC-educated cells, confirming that LEC-educated cells are less prone to effector differentiation.

Crucially implicated in the regulation of these metabolic processes is the mammalian target of rapamycin (mTOR) pathway, which integrates intracellular and extracellular signals, leading to the formation of either of two multiprotein complexes, mTORC1 or mTORC2, which propagate different downstream signals. Blockade of mTORC1 was shown to favor memory T cell generation and induce TSCM differentiation, while suppression of mTORC2 activity enhanced memory CD8+ T cell formation. We detected consistently lower levels of mTOR in LEC-educated compared to mDC-educated cells at all time points examined (Fig. 5j, Supplementary Fig. 4g). Furthermore, while they both expressed similar levels of Rictor, LEC-educated cells displayed significantly lower Raptor expression on d3, suggesting a lower induction of the mTORC1 complex. To further confirm these observations at the protein level, we assessed the phosphorylation of mTOR (pmTORS2448) and Akt (pAktS473, indicative of mTORC2 activity) by flow cytometry (Fig. 5k, l). Within 2 h of co-culture, mDC education was superior to LEC education in inducing pmTORS2448+ and pAktS473+ in OT-I cells, suggesting that both mTORC1 and mTORC2 were indeed less active and that PI3K–Akt–mTOR activity is less sustained in LEC-educated cells.

Altogether, these data validate the TCM/TSCM-like phenotypic properties of LEC-educated CD8+ T cells, which exhibit transcriptional and metabolic programs consistent with a memory-like differentiation state, distinct from mDC-educated cells.

In vitro LEC-primed CD8+ T cells have memory-like LN-homing. The elevated expression levels of CD62L in LEC-educated cells prompted us to investigate whether they exhibit preferential migration to secondary lymphoid organs, because CD62L enables naive and TCM cells to localize to lymphoid tissue. To this end, we transferred naive or LEC/mDC-educated OT-I cells into mice and analyzed their homing into various organs 1 week later (Fig. 6a). LEC-educated cells homed primarily to secondary lymphoid organs (LN and spleen, 53%) whereas mDC-educated cells migrated mainly to the periphery (lungs and liver, 67%) (Fig. 6b). A smaller fraction of mDC-educated cells was found in lymphoid tissues (33%), to which naive cells almost exclusively homed (91%), as expected.

LEC-educated cells that homed to the LNs and spleen primarily expressed a TCM-like (CD44+CD62L+) phenotype (Fig. 6c, d), as well as in the rest of the organs examined (Supplementary Fig. 5a). In contrast, mDC-educated cells were predominantly TEM-like (CD44+CD62L−) in all organs except in the LN, where they were mostly TCM. LEC-educated cells possessed a higher ratio of TCM/TEM than mDC-educated cells in all organs examined, with the highest ratio in the LN, while for mDC-educated cells, the ratio TCM/TEM was <1 in all organs except in the LN, underlining the phenotypic divergence due to their education (Fig. 6e, Supplementary Fig. 5b, c). To determine whether LEC-educated cells preferentially localized to specific sub-anatomic sites in the LN, we co-transferred LEC-educated and naive CD8+ T cells, each labeled...
with different fluorophores, and after a week, imaged thick sections of the brachial LN using confocal microscopy. We observed the naïve cells primarily in the paracortical T cell zone (Fig. 6f), while the LEC-educated cells localized mostly near the cortical and medullary sinuses, around B cell follicles and particularly in the interfollicular areas. These homing patterns are consistent with recent descriptions for naïve and memory CD8\(^+\) T cells\(^{52,53}\).

Collectively, these data suggest that CD62L expression in LEC-educated cells is functional and corresponds to preferential migration to secondary lymphoid organs.

**Re-stimulation of LEC-educated CD8\(^+\) T CM generates cytotoxic lymphocytes.** The most important function of memory T cells is their reactivation potential, so we next assessed their potential of T CM cells. More importantly, reactivated LEC-educated cells exited their non-responsive state and produced increased levels of TNF\(\alpha\) and IL-2 compared to mDC-educated cells (Fig. 7c, Supplementary Fig. 6b), and acquired more of an effector-like phenotype with downregulation of CD62L and even higher levels of TNF\(\alpha\) and IL-2 compared to mDC-educated cells (Fig. 7c, Supplementary Fig. 6b), and acquired more of an effector-like phenotype with downregulation of CD62L and increased expression of granzyme-B (GzB) (Fig. 7d–f), although not to the same extent as mDC-educated cells.

Because LEC-educated cells responded to an in vitro antigen recall with a strong, multifunctional response, we asked whether this translates to rapid recall responses following secondary challenge under inflammatory conditions in vivo. For this purpose, we transferred CFSE-labeled LEC-educated or mDC-educated OT-I cells into mice, and after 5 days, we administered OVA with LPS (+RECALL) or saline (−RECALL) s.c. (Fig. 7g). Both LEC-educated and mDC-educated CD8\(^+\) T cells (which
may have been induced to divide at different rates before the time of CFSE labeling) expanded significantly within the LNs and spleens of recalled recipients, even to the point of completely diluting CFSE before the endpoint (Fig. 7h, i). Based on cell recovery, compared to non-recalled recipients, recalled LEC-educated T cell numbers increased four-fold in the LN and the spleen, while recalled mDC-educated cells increased 0.5-fold.

This surprising difference may be attributable to the tendency of LEC-educated T cells to be enriched in memory-like cells, which in theory, possess superior expansion potential than the effector-like cells generated by mDC education. Additionally, LEC-educated cells were particularly enriched in the LN compared to the spleen, a result attributable to the early time point following challenge and their homing properties (Fig. 6). Along with the vigorous expansion, and in agreement with our in vitro observations, LEC-educated cells also exhibited the functional potential expected of memory cells by upregulating effector cytokines (Fig. 7, k). Following ex vivo re-stimulation, we noted increased production of IFN-γ and TNF-α in antigen-recalled mice, and furthermore, the transferred cells acquired effector function, expressing GzB and demonstrating cytolytic capacity (Fig. 7l), although mDC-educated CD8+ T cells were superior by all measures.

Because true memory cells persist even in the absence of antigen, we repeated the above experiment but with re-challenge after >5 weeks of LEC-educated or mDC-educated OT-I cell transfer (Fig. 7g, long-term recall). Both LEC-educated and mDC-educated T cells expanded following recall (Fig. 7m), with LEC-educated cells giving rise to effector-like cells in both the LN and spleen (Fig. 7o, o), though to a less extent than mDC-educated T cells. Similar frequencies of LEC-educated and mDC-educated cells acquired the short-lived effector phenotype (KLRG1+CD127−), which was predominantly expressed by OT-I cells in the spleen, especially by mDC-educated cells (Fig. 7o). Overall, the phenotypes of LEC-educated T cells resembled those of mDC-educated cells, although LEC-educated cells achieved significantly lower frequencies of TCM/TEM (CD44+CD62L−) cells (Fig. 7o, Supplementary Fig. 6c). The differences seen in the magnitude of responses in LEC-educated vs. DC-educated T cells upon recall are likely due to differences in their phenotypes and degree of activation at the time of adoptive transfer.

Taken together, these data suggest that LEC-educated CD8+ memory-like T cells can acquire cytotoxic function and effector phenotype upon antigen re-encounter. The ability of antigen-experienced T cells to proliferate and differentiate into cytotoxic lymphocytes upon secondary challenge constitutes a hallmark of immunological memory, adapted to long-term immune protection in the host.

LEC-educated CD8+ T cell memory protects against infection. These findings raised the question of whether LEC-educated CD8+ T cells can mediate protective immunity against a pathogen in a manner comparable to mDC-educated cells. To first measure the cellular response to memory recall, we co-transferred LEC-educated (CD45.1+2+) and mDC-educated (CD45.1+) OT-I cells (1:1) in mice and after 5 weeks, challenged the mice with OVA-expressing Listeria monocytogenes (L.m.-OVA) (Fig. 8a). Within a week post-infection, LEC-educated cells expanded from making up 0.5 ± 0.4% of all circulating CD8+ T cells to 11.2 ± 5.0%, and mDC-educated cells further expanded by at least two-fold (Fig. 8b, c). These trends were not observed in the dLNs (Supplementary Fig. 7a), due to the systemic route of L.m.-OVA infection.

Importantly, LEC-educated cells acquired effector function to a similar extent as mDC-educated cells upon ex vivo re-stimulation, exhibiting comparable cytotoxic potential as demonstrated through the frequency of cells producing effector cytokines (IFN-γ, TNF-α, IL-2) and expressing CD107a, which signifies cytolytic granule release (Fig. 8d, e, Supplementary Fig. 7b). Interestingly, LEC-educated cells trended towards higher poly-functional than DC-educated cells, defined as double or triple positive for IFN-γ, TNF-α and IL-2 (p = 0.07; Fig. 8f, Supplementary Fig. 7c).

Consistent with our previous observations, LEC-educated cells acquired a predominantly effector phenotype (CD44+CD62L−) by day post-infection (Fig. 8g, h), and most were found within the KLRG1+CD127− subset. Remarkably, we also observed a significant TCM-like (CD44+CD62L+) subset among LEC-educated cells, which led to a significantly larger TCM/TEM ratio relative to mDC-educated cells (Fig. 8h). We additionally noted a significantly higher ratio of memory precursors (KLRG1−CD127+) to short-lived effectors within the LEC-educated population in both the spleen and LN (Supplementary Fig. 7d).

To directly compare the protective functions of LEC-educated versus mDC-educated cells, we injected mice with either LEC-educated or mDC-educated cells (Fig. 8a, bottom branch). In comparison to mice that received resting CD8+ T cells (Educ. Naïve), LEC-educated CD8+ T cells and mDC-educated T cells were similarly competent at controlling the bacterial load following infection with L.m.-OVA (Fig. 8i).

We also noted that LEC-educated T cells uniquely retained a population of TCM-like phenotype following antigen re-encounter (Fig. 7d, n) or pathogenic infection (Fig. 8g, h), so we asked whether LEC-educated cells were preferentially capable of generating a secondary TCM-like subset. We co-transferred LEC-/mDC-educated OT-I cells (Fig. 8a, top branch) and tracked the transferred cells for 2 weeks post-infection. While the cytotoxic effector fraction (CD44+GzB+) decreased over time in both groups, only the LEC-educated cells showed a concomitant and significant increase in the TCM-like phenotype within 9 days post-infection (Fig. 8j, Supplementary Fig. 7e). Furthermore, 3 weeks post-infection, LEC-educated cells possessed a higher ratio of CD127+CD62L+ to CD127+CD62L− cells in the spleen and LN (Fig. 8k). Additionally, we observed similar trends for the TCM/TEM in the memory precursor-to-short-lived effector ratios in both organs (Supplementary Fig. 7f). Finally, the frequency of CD44+CD62L+CD122+ cells was significantly higher among LEC-educated cells in both the LN and spleen.

We followed up with an evaluation of LEC-educated TCM responses to homeostatic signals in vitro (Supplementary Fig. 8a). Similar to conventional TCM/TEM cells, LEC-educated cells underwent homeostatic turnover and displayed increased survival in response to IL-15 compared to mDC-educated cells (Supplementary Fig. 8b). Notably, LEC-educated cells retained their initial CD44+CD62L+ phenotype following IL-15-dependent proliferation (Supplementary Fig. 8c), suggesting self-renewal capacity.

Overall, our findings indicate that LEC-educated CD8+ TCM/TEM cells can be reactivated to participate in protective immunity while also possessing the capacity for self-renewal, giving rise to a persistent secondary-memory population.

Requirements for LEC-educated CD8+ TCM polyfunctionality. Finally, we sought to identify the signals that may lead to functional re-activation of LEC-educated CD8+ T cells. To evaluate the roles of TCR stimulation and co-stimulation, LEC-educated OT-I cells were treated with αCD3 with or without αCD28 (Fig. 9a). TCR ligation by αCD3 was sufficient to induce IFNγ production; but co-stimulation through αCD28 was required to induce polyfunctional cells (Fig. 9b).
To ask if undesirable reactivation of LEC-educated cells could be triggered under homeostatic conditions, we examined their functional profile following reactivation with immature DCs (iDCs) in the presence of immunogenic or immunosuppressive signals. LEC-educated or mDC-educated OT-I cells were cultured together with iDCs (iDC-recall), with or without SIINFEKL, either in the presence of LPS + IFNα + IL-12 or IL-10 + TGFβ (Fig. 9c). LEC-educated cells only became functional effectors when reactivation took place under Th1-polarizing conditions (LPS + IFNα + IL-12; Fig. 9d), but not in a suppressive setting (IL-10 + TGFβ), as demonstrated by their low production of IFNγ and GzB. Importantly, re-stimulation with iDCs in the...
Fig. 8 LEC-educated CD8+ T cells protect against infection and give rise to a persistent secondary memory population. a Experimental setup: LEC-educated (CD45.1/2+) and mDC-educated CD45.1+ OT-I cells were co-transferred (1:1, 10^5 total cells/mouse) into healthy wild-type mice a, h, j, k, and challenged 5 weeks later with L.m.-OVA (10^4 cfu/mouse) and sacrificed 8 days later j, k, b, c. Frequency of OT-I cells among all CD8+ T cells in the b blood and c spleen. d–h Spleenic OT-I cells were further characterized for: d Intracellular staining for IFNγ, TNFα, and IL-2 after ex vivo re-stimulation; e frequency of IFNγ+ and CD107α+ cells; f frequency of multifunctional (IFNγ, TNFα, and IL-2)+ cells; g expression of CD44, CD62L, CD127, and KLRG1; and h (top) ratio of TCM (CD44+CD62L+) to Tmem (CD44+CD62L-) cells and (bottom) ratio of memory precursor effector cells (CD44+CD127+KLRG1+) to short-lived effector cells (CD44+CD127- KLRG1+). j Colony-forming assay performed 3 days following challenge with L.m.-OVA (10^4 cfu/mouse), which occurred 5–9 weeks after adoptive transfer of LEC-educated or mDC-educated OT-I cells, or resting OT-I cells (educ: naïve) as a control. (left) Representative photographs of spleen suspensions from mice receiving indicated OT-I cell populations, cultured on BHI agar plates overnight. (Right) Quantification of bacterial burden, normalized to the c.f.u. detected in the negative control (no transfer) group (n = 5–6 per experimental session: 1.5 × 10^6 c.f.u., 5.6 × 10^6 c.f.u., and 3.9 × 10^5 c.f.u. for the three experiments). k Kinetics in the frequency of CD44+CD62L+ and CD44+GzB+ phenotype on circulating OT-I at the indicated timepoints post-challenge.

Discussion

This study illuminates a new role for LECs in immunomodulation: the generation of antigen-experienced CD8+ T cells, which emerge from antigen encounter as memory-like cells. The ability of non-hematopoietic APCs to drive memory formation has been previously reported[44]. In one example, SLEC-educated T cells exhibit an antigen-experienced phenotype with TCM-like characteristics[45]. Here, we explored whether by analogy it is feasible that LEC education of naïve CD8+ T cells can generate CD8+ T cells with multiple differentiation states, including those with memory-like functional phenotypes. Because LECs are known to be capable of scavenging and cross-presenting exogenous antigen[10], we hypothesized that they may contribute to the generation of memory T cells.

Education of CD8+ T cells by LECs versus by professional APCs leads to their acquisition of vastly different phenotypes, which may be dependent on a variety of signals, including TCR affinity, duration of antigen exposure, cytokines, and the balance between co-stimulatory/inhibitory signals, which are all known to regulate the outcomes of T cell differentiation[25,56]. The conditions of LEC education are characterized by low co-stimulation coupled with inhibitory PD-L1/PD-1 signaling[7,10], which are typically observed in transient inflammation and would favor memory rather than terminal effector differentiation. The cytokine milieu in LEC–OT-I co-cultures was consistent with these findings (Supplementary Fig. 2c): we failed to detect IL-12, a key regulator of effector T cell generation that antagonizes memory generation[57]. Notably, we detected IL-7 and IL-15 expressions in LECs (Supplementary Fig. 2d,e), two cytokines known to promote
challenge (Fig. 7i, m). Most importantly, under the correct conditions, LEC-educated T_{CM} cells gave rise to potent cytotoxic lymphocytes that protected the host from pathogen challenge (Fig. 8b–i). Interestingly, LEC-educated cells also appeared to sustain a persistent secondary T_{CM}-like population (Fig. 8c, j, k, Supplementary Fig. 7e, f), showcasing their potential for self-renewal (Supplementary Fig. 8). While we did not confirm if LEC-educated cells exhibit multipotency and self-renewal at the level of individual cells in similar fashion to canonical T_{SCM} and T_{CM} cells, LEC-educated cells appeared to be more differentiated than T_{SCM} cells based on CD44 expression, while simultaneously being distinct from effector-like mDC-educated cells (Fig. 4a). Therefore, LEC-educated cells may possess an intermediate differentiation state between T_{SCM} and T_{CM} cells, distinct from terminal effector cells.

We speculate that the functional significance of LEC-educated memory CD8^{+} T cells is their contribution to diversity of memory T cell states. As much as they have in common to conventional T_{CM} cells, they differ in important functional properties. Canonical T_{CM} cells are known to produce IFNγ as a bystander effect during inflammation despite the absence of cognate antigen. In contrast, naïve cells, LEC-educated cells required all three signals—cognate antigen, co-stimulation, and T_{H}1-polarizing cytokines, to become reactivated (Fig. 9). A similar phenomenon was observed for LSEC cross-primed CD8^{+} T cells and in other non-hematopoietic APCs, where functional reactivation of antigen-experienced CD8^{+} T cells required multiple synergistic signals. Such prerequisites may safeguard against undesired reactions and hinder the onset of auto-immunity, given that LECs would normally collect endogenous antigens under homeostatic conditions. These key differences may also highlight the need for CD4^{+} T cell help, in the form of co-stimulation or accessory cytokines, to generate canonical T_{CM} cells, although LECs express low levels of co-stimulatory molecules, such as CD80 and CD86 and thereby are unlikely to stimulate optimal CD4^{+} T cell responses.

These more stringent reactivation requirements may be mistaken for tolerance induction by LECs, as reported in tumors and tumor-draining lymph nodes. Based on our findings, efficient reactivation of LEC-educated cells and subsequent cytotoxic lymphocyte generation would be quite unlikely in such suppressive microenvironments due to the strong immunoregulatory factors present. After all, the tumor-draining microenvironment rather resembles reactivation in the presence of IL-10 + TGFβ, which completely failed to induce functional GzB-expressing effector T cells among LEC-educated cells (Fig. 9d). However, in the setting of chronic inflammation rich in inflammatory cytokines and danger signals, re-stimulation of LEC-educated cells would likely favor generation of cytotoxic effector T cells. Indeed, while controversial, lymphangiogenesis has been frequently associated with aggravation of inflammatory diseases and transplant rejection.

We envision that LEC-educated T_{CM}-like CD8^{+} T cells are physiologically significant in at least two scenarios. First, they may be very important in first-line containment against systemic antigen spread. Both viral and bacterial pathogens employ different strategies to circumvent innate immunity, which results in serious pathology. Therefore, LEC priming may spare pathogen-specific T cells from elimination due to first non-immunogenic encounter, generating antigen-experienced cells that may be activated into functional effectors under the correct conditions. A similar role was recently suggested for LSEC-primed CD8^{+} T cells. Second, LEC priming may initiate the generation of antigen-experienced CD8^{+} T_{CM}-like cells following infection, because freely draining antigens arrive in the LN much earlier than antigen-loaded migratory DCs from peripheral tissues. Consistent with this, in the first moments of a viral
infection, virions were reported to drain to the LN and infect resident cells, as well as the sub-anatomic locations where LECS are reportedly capable of antigen archiving. Given their constant contact with the antigen-rich pool of the lymph, LECS can generate a population of antigen-experienced T cells, which may be readily expanded by activated professional APCs. In line with our hypothesis, stromal cells were able to induce naïve T cell recruitment and activation early during infection, even though their sustained expansion at later time points was markedly limited. Of course, different pathogens engage different immunostimulatory pathways and produce different response kinetics, which directly affect the balance of antigen presentation by professional APCs versus by LECS, possibly explaining seemingly contradictory data with regard to LEC-induced T cell proliferation.

In conclusion, our findings demonstrate a new immunomodulatory role for LECS. Exogenous antigen cross-presentation by LECS induces an expandable pool of memory-like T cells, leading to effector T cell generation under inflammatory conditions to fight ongoing infection, while preserving a subset of long-lived cells to combat future pathogen encounter. Our work reveals the multifaceted effects of antigen education by LECS, highlighting their dynamic roles in response to the local microenvironment for the regulation of immunity. These findings also help explain and reconcile the seemingly contradictory effects of lymphangiogenesis in tumors versus chronic inflammatory diseases or transplants. As we begin to better understand the impact of antigen presentation by LECS in immunomodulation and unravel the various signals implicated, a new challenge emerges: how can we harness this function to improve protection against pathogens and tumors, or to prevent autoimmunity and transplant rejection?

Methods

Reagents

Chemicals were purchased from Sigma-Aldrich (Buchs, CH) unless otherwise noted. The mature MHC I/II peptide, OVA25–33 (SIINFEKL), peptide, was from GenScript (Piscataway, NJ, USA) and the gp10025–33 peptide was from AnaSpec (Fremont, CA, USA). The Endotoxin-free OVA was from Hyglos GmbH, (Bennemed am Starnberger See, DE). Ultra-pure lipopolysaccharide (LPS; 0.11 μg) was from Invivogen (Irvine, CA, USA). CpG-1826 oligonucleotide (CpG) was obtained from Microsynth (Balgach, CH). Recombinant murine GM-CSF and IL-15 were purchased from Peprotech (Oak Park, CA, USA).

Generation of ex vivo LN LECS and mDC-educated CD8+ T cells

CD8+ T cells from human-individualized LL-OT-3 mice and nude mice were negatively selected from total splenocytes using the EasySep Mouse CD8+ T Cell Isolation Kit from Stemcell Technologies (Vancouver, BC, Canada). In some experiments, the resulting CD8+ T cells were further purified by FACS based on naïve (CD44+CD62L−) or VM (CD44+CD62L+CD45R+CD25−) phenotypes. Subsequently, CD8+ T cells were directly co-cultured with LN LECS or mDCs in the presence of either 1 nM NPs (OVA25–33) or 1 μM full-length OVA protein, 1 nM SIINFEKL peptide, or 1 μM of gp100 peptide as indicated (human gp100:25–55 KVPVRQNDWLL, which differs from the murine sequence by only two amino acids and is functional in reports using murine cells). In some experiments, LN LECS or mDCs were pulsed with antigen, then washed with PBS prior to co-culture.

Synthesis of peptide-conjugated nanoparticles (NPs)

To explore the mechanisms of cross-presentation poly(propylene sulfide)-NPs with ~30 nm diameter were synthesized and characterized as described. The long peptide containing the mature MHC I/II epitope SIINFEKL-G-G-G-G-G-G (OVA25–33) was synthesized in-house and activated with a 2-pyridylthioxyl as previously described. Cells were then processed and stained for immunological markers to be analyzed by flow cytometry (Supplementary Fig. 9c). Cellular proliferation was monitored by CFSE dilution and apoptosis was determined by Annexin V staining (BioVision, Milpitas, CA, USA).

Preparation and implantation of LECS spheroids

From Hugh Robson MacDonald. Constitutively OVA-expressing mice (C57BL/6-2m−/−−/−) were a gift from the manufacturer’s protocol using a standard culture conditions. In the E-Toxate® endotoxin standard (Sigma-Aldrich). Endotoxin levels of antigens were bluntly assessed by a colorimetric assay based on the HEK-Blue® TLR4 cell line (InvivoGen, San Diego, CA, USA) according to the manufacturer.

Primary cell isolation

To obtain primary LN LECS and LN FRCSs, LN cells (axillary, cervical, brachial, inguinal, and popliteal) were digested with 1.3 Wünsch Units/mL Liberase DH and 200 Kunitz/mL DNase I (Roche, Basel, CH) to obtain a single cell suspension and cultured as described. Cells were then processed and stained for immunological markers to be analyzed by flow cytometry (Supplementary Fig. 9c).

In vitro reactivation

LECM-d Educated Mouse CD8+ T cells were harvested on day 3 of co-culture, washed at least twice with basal medium and counted. CD8+ LECD- or mDC-educated CD8+ T cells were subsequently co-cultured with fresh 106 mDCs loaded with or without the SIINFEKL peptide (1 μM) for another 24 h in 200 μL of co-culture media (mDC-recall). Supernatants were harvested and frozen for cytokine analysis. T cells were then processed and stained for immunological markers to be analyzed by flow cytometry (Supplementary Fig. 9c). Cellular proliferation was monitored by CFSE dilution and apoptosis was determined by Annexin V staining (BioVision, Milpitas, CA, USA). T cell proliferation was determined by assessing CFSE intensity using the proliferation algorithm in FlowJo 9.4.1.

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for cytokine analysis. Cells were then processed and stained for immunological markers to be analyzed by flow cytometry. The levels of intracellular cytokines were determined after activation and 2 h of RNA extraction with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories AG, Cressier, CH) according to the manufacturer’s instructions. Quantitative RT-PCR was performed using SYBR Green mix and a LightCycler® 96 instrument (both Roche). Primer sequences were as follows: IL-7, 5'-TCTCCATCAGTACCTTGTGTC-3' and 5'-GCCATGCTTGCGAAGACAT-3'; IFN-γ, 5'-CTTGTCACCTGCTTGTC-3' and 5'-TTCTGTTGGACCAAGTTTGTG-3'.

Antigenic challenge. To determine whether exogenous antigen uptake by LNSCs can drive CD8⁺ T cell proliferation in vivo, we i.d. administered 50 μg of endotoxin-free OVA in all four limbs at a volume of 10 μL/limb, or saline as a control. For one study (Fig. 2), a total dose of 100 μg of endotoxin-free OVA was administered i.d. in six locations, including all four footpads (10 μL per limb) and two locations along the spinal axis of the mouse in the dorsal skin (30 μL per spot). To test the capacity of LN LEC/mDC-educated T cells to respond to secondary antigen encounter in vivo, we subcutaneously administered 50 μg of endotoxin-free OVA plus 100 μg of ultra-pure LPS.

Infection with OVA-expressing Lm. To assess the functional potential of LN LEC/mDC-educated CD8⁺ T cells upon pathogenic challenge, mice were infected i.v. with 10⁷ OVA-Lm. The challenge dose was selected to induce splenic, mice were infected with 10⁷ cfu Lm-OVA at least 9 weeks following adoptive transfer of LN LEC/mDC-educated CD8⁺ T cells and sacrificed 3 days after. Splenocytes were collected, homogenized, and resuspended in 10 mL sterile PBS. Cell suspensions were then plated on BHI agar plates and incubated overnight at 37 °C. The following day, colony-forming units (cfu) were counted and the amounts of Lm-OVA were calculated with respect to the relative dilutions.

Ex vivo killing assay. To assess the specific killing capacity of reactivated LEC- and mDC-educated CD8⁺ T cells, the cells were cultured, following in vivo antigen re-exposure, with a 1:1 mixture of Fls3+/- labeled (5 μM) SIINFEKL-loaded and Fls3 low-labeled (0.1 μM) mismatched-peptide-loaded DCs (mixed target cells) at different effector to target cell ratios. We kept the number of mixed target cells constant at 5 × 10⁵ cells. Mixed target cells alone served as a control for non-specific death. After 12–16 h, the ratio of the surviving Fls3 high and Fls3 low cell populations was assessed by flow cytometry to calculate the percentage of specific killing as follows: % specific killing = 100–[(Fls3 high/Fls3 low) sample/(Fls3 high/Fls3 low) control] × 100.

Flow cytometry. Cells were washed and stained with a cocktail of surface antibodies in staining buffer, containing HBSS (Life Technologies) supplemented with 0.5% bovine serum albumin. Cell viability was determined by propidium iodide incorporation in staining buffer after surface antibody staining or with live/dead fixable cell viability reagent (Life Technologies) in PBS before surface antibody staining. For blocking non-specific binding of immunoglobulin to Fc receptors, cells were also pre-incubated with purified anti-CD16/CD32 antibody (clone J555) for 10 min on ice prior to immunostaining. For intracellular/intranuclear staining, cells were fixed and permeabilized with the Foxp3/Transcription Factor Fixation/Permeabilization kit (ebioscience) according to the manufacturer’s instructions. Cells were stained in permeabilization buffer with a cocktail of monoclonal antibodies. For phospho-specific staining, cells were fixed with IC fixation buffer (ebioscience) and subsequently incubated with ice-cold 100 μM methanol for membrane permeabilization. Cells were stained with a cocktail of surface and phospho-flow antibodies in staining buffer. The following anti-mouse antibodies were used: CD62L (MEL-14), CD44 (IM7), KLRG1 (2F1/KLRG1), CD107 (1D4B), Granzyme-B (NGZB), ki-67 (SolA15), pS6S235/236 (D57.2.2E), phospho-Permeabilization kit (eBioscience) according to the manufacturer's instructions. Quantitative RT-PCR was determined after 3 h reactivation and 2 h of Brefeldin A (BfA) treatment. When studying the effect of BfA on the transcriptional response, reactivation of LEC-educated or mDC-educated CD8⁺ T cells was also conducted with plate-bound anti-CD3 antibody (145.2C11) at 5 μg/mL and/or soluble anti-CD28 (37.51) at 2 μg/mL (eCD3/CD28, ebioscience). The same procedures, described above, were performed to analyze the phenotype and function of the reactivated cells.

RNA sequencing. RNA sequencing was performed with naive or LEC/mDC-educated CD8⁺ T cells, coming from 3 OT-I mice, at three different time points during control (day 1, 2, 3), making for a total of 93 samples across to different conditions. Total RNA was extracted with the RNeasy mini Plus Kit (Qiagen, Hombrechtikon, CH). The Agilent RNA ScreenTape assay was performed in combination with the 4200 TapeStation system (Agilent Technologies) for the determination of quality and quantity of the RNA samples. Additionally, concentration of the RNA samples was determined by Qubit DNA HS and proﬁle analysis was performed by Fragment Analyzer (Advanced Analytical). Seven samples were then pooled to create one library for each group. For each sample, NextSeq 500 million reads (nominally ~55 million reads/sample) of 75 bps in single-end mode.

To visualize global changes in gene expression as a function of T cell education and antigen presentation, we applied PCA on all 45,707 detected genes, and hierarchical clustering on a pre-filtered subset of these genes with a median rpkms > 5, and with adjusted p < 0.01 versus expression on naive OT-I cells. This subset of 16,752 genes essentially represented substantially expressed genes that were differentially regulated in naive or in antigen-experienced CD8⁺ T cells. The rpkms of these 16,752 genes were used as a control to the GENE-E platform (http://geneontology.org/GENE_E/index.html) for hierarchical clustering, using Pearson correlation coefficients among the genes to potentially identify modules of genes that appear co-regulated or induced uniquely due to LEC- or mDC education.

To compare data expression in LEC-educated cells with memory/effector cell function, we calculated the 16,752 genes with the greatest difference in expression (all of those with an adjusted p value of <0.05) and a change in expression of >1.5-fold between day 3 LEC/mDC-educated cells) were selected. To detect genes most differentially regulated in LEC-educated cells on day 3, we selected genes that fulfilled the following conditions: (1) an adjusted p < 0.01 between any pair of comparisons andante-B1 cells and LAbCDs, mDCs, or mDC-educated OT-I cells) 3, and (2) [log(FC)] ≥ 2.89-fold difference in expression between LEC-educated versus mDC-educated OT-I cells. These criteria led to a final panel of ~200 genes, which represent the genes that are differentially regulated in antigen-experienced T cells, and furthermore, most differentially modulated by the nature of the APC used (Supplementary Table 1). These ~200 genes were mapped onto biological functions and pathways using Gene Ontology Enrichment Analysis (Gene Ontology Consortium—http://geneontology.org/). Furthermore, we used the Gene Ontology Atlas to build lists of genes related to specific biological processes, and used R and Excel to map these genes on the volcano plots to better visualize gene expression patterns between naive OT-I cells, or LEC-educated or mDC-educated OT-I cells.

Transcriptional profile analysis. Raw-sequencing files were first checked for sequence quality and potential contamination using the FastQC program (Babraham Bioinformatics, Cambridge, United Kingdom). From here, read mapping and read counting was performed on the HTSplatform [68], which is an integrated modular platform using the Bowtie2 algorithm to map reads to a reference mouse genome [69] and the (Bioconductors) GAGE pipeline. After read alignment and expression statistical analyses [70] and generation of adjusted p-values values via one ANOVA, adjusting for the false-discovery rate using the Benjamini–Hochberg method.

To visualize global changes in gene expression as a function of T cell education and antigen presentation, we applied PCA on all 45,707 detected genes, and hierarchical clustering on a pre-filtered subset of these genes with a median rpkms > 5, and with adjusted p < 0.01 versus expression on naive OT-I cells. This subset of 16,752 genes essentially represented substantially expressed genes that were differentially regulated in naive or in antigen-experienced CD8⁺ T cells. The rpkms of these 16,752 genes were used as a control to the GENE-E platform (http://geneontology.org/GENE_E/index.html) for hierarchical clustering, using Pearson correlation coefficients among the genes to potentially identify modules of genes that appear co-regulated or induced uniquely due to LEC- or mDC education.

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Statistical analysis. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) or unpaired, two-tailed Student’s t-test followed by Bonferroni post-test with Prism 5 software (Graphpad, San Diego, CA, USA) unless otherwise stated. If the groups to be compared had significantly different variances (p < 0.05 by F test) then Welch’s correction was performed. In all the animal studies, mice were randomly assigned to the different groups and treated in a random order. The investigators were not blinded to group allocation during the experiment or when assessing the outcome. The number of animals chosen per experiment was based on previous publications using similar models and addressing similar questions, as well as on previous studies performed in our lab when statistical significance was actually achieved. To account for data reproducibility, the experiments were performed at least twice. For in vitro experiments, samples were randomized from cells in culture in multi-well plates and the processing order was random. The investigators were not blinded to sample identity. Sample sizes were chosen as the minimum number of samples.
mice to sort enough cells to perform three to four technical replicates per group per assay. Results are shown as mean ± standard deviation, unless noted otherwise, with significance indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. When Box-and-Whiskers plots are used, the line in the middle of the box indicates the median and the whiskers go down to the smallest and up to the largest value.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The RNA sequencing data have been deposited to the NIH Gene Expression Omnibus under accession number GSE88330. Source data are provided as a Source Data file. All other data are included in the supplemental information or available from the authors upon reasonable request.

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