

A septin requirement differentiates autonomous and contact-facilitated T cell proliferation

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T cell proliferation is initiated by T cell antigen receptor (TCR) triggering, soluble growth factors or both. In characterizing T cells lacking the septin cytoskeleton, we found that successful cell division has discrete septin-dependent and septin-independent pathways. Septin-deficient T cells failed to complete cytokinesis when prompted by pharmacological activation or cytokines. In contrast, cell division was not dependent on septins when cell-cell contacts, such as those with antigen-presenting cells, provided a niche. This septin-independent pathway was mediated by phosphatidylinositol-3-OH kinase activation through a combination of integrins and costimulatory signals. We were able to differentiate between cytokine- and antigen-driven expansion *in vivo* and thus show that targeting septins has strong potential to moderate detrimental bystander or homeostatic cytokine-driven proliferation without influencing expansion driven by conventional antigen-presentation.

T cell proliferation rapidly expands the number of antigen-specific cells, a process that is necessary to control infection. Typically, this type of cell division is initiated by a T cell interaction with its cognate antigen on an antigen-presenting cell (APC), and its magnitude is determined by the strength of the TCR recognition event in that cell-cell contact^{1–3}. Antigen-specific T cell clonal expansion has been reported to occur in the lymph node, where swarming T cells engage in cell-cell contacts with proximal APCs and other activated T cells^{4,5}, and this may represent a 'niche' for cell division. Yet cell division can also be driven by high local cytokine concentrations in the environment, in the possible absence of such cell-cell interaction. This scenario is considered a possible hazard for autoimmunity, as when non-virus-specific 'bystander' cells encounter high concentrations of cytokines produced by viral-specific T cells during an immune response in a lymph node^{2,6}. Cytokine-driven cell division is also important for homeostatic maintenance, wherein cytokines such as interleukin-7 (IL-7) or IL-15, in conjunction with transient low-affinity peptide-MHC (p-MHC)-TCR interactions, support turnover of clones⁷. Asymmetric cell division has been proposed to be a pathway that can influence the individuality of daughter cells⁸, but completion of cytokinesis has been considered invariant. To our knowledge, it has not been possible to clearly distinguish cytokine-driven cell division from TCR-driven cell division.

The physical event of cell division requires many processes, including the functions of specific kinases⁹, specific cytoskeleton proteins such as myosins and, notably, septins^{10–13}. Septins are GTP-binding proteins that self-assemble into tetrameric, hexameric or octameric quaternary structures and then into large filaments, rings and gauzes, *in vitro* and *in vivo*, and are assembled on the cell cortex^{14–16}. The mammalian septins, of which there are 13, can be divided into four groups; one from each class is required to form a canonical complex. In mammals, septin 7, the only one in its class, appears to

be indispensable for the generation of filaments, and its depletion leads to loss of other septin proteins^{14,17–19}, presumably as a result of quality-control processes.

Septins were originally identified as 'cell-division cycle' mutations²⁰ and are evolutionarily conserved in their crucial role in cytokinesis. Similarly to yeast, septins in mammals have been found to be essential for completion of cytokinesis^{12,21}. Septins are usually, but not exclusively, found assembled as a ring at the cleavage furrow^{12,22}. They have been suggested to be essential in coordinating myosin motor proteins during cell abscission^{10,23}, remodeling the membrane as cytokinesis progresses²⁴ and anchoring the midbody ring structure to the membrane²⁵ as mother and daughter cell separation is resolved.

One exception to the requirement of septins for mammalian cytokinesis is in T cells; septin 7 depletion in D10 cell lines leads to loss of the other septins but results in near-normal cell division in response to cues driven by APCs¹⁷. T cell cytokinesis in the absence of septins has also been confirmed in *Sept7* knockout mice¹⁹. To investigate how T cells evade this highly conserved requirement, we generated mice with T cell-specific depletion of septin 7 and examined CD8⁺ T cell activation and function under a variety of conditions. We found that the requirements for septins in T cell division differ depending on whether T cells engage in cell contacts during cytokinesis. This finding led us to examine how proliferation occurs in septin-deficient (septin-null) CD8⁺ T cells in order to isolate the compensatory pathways. Our results provide insight into the possibility of attenuating cytokine-driven expansion specifically while leaving antigen-driven expansion unaffected.

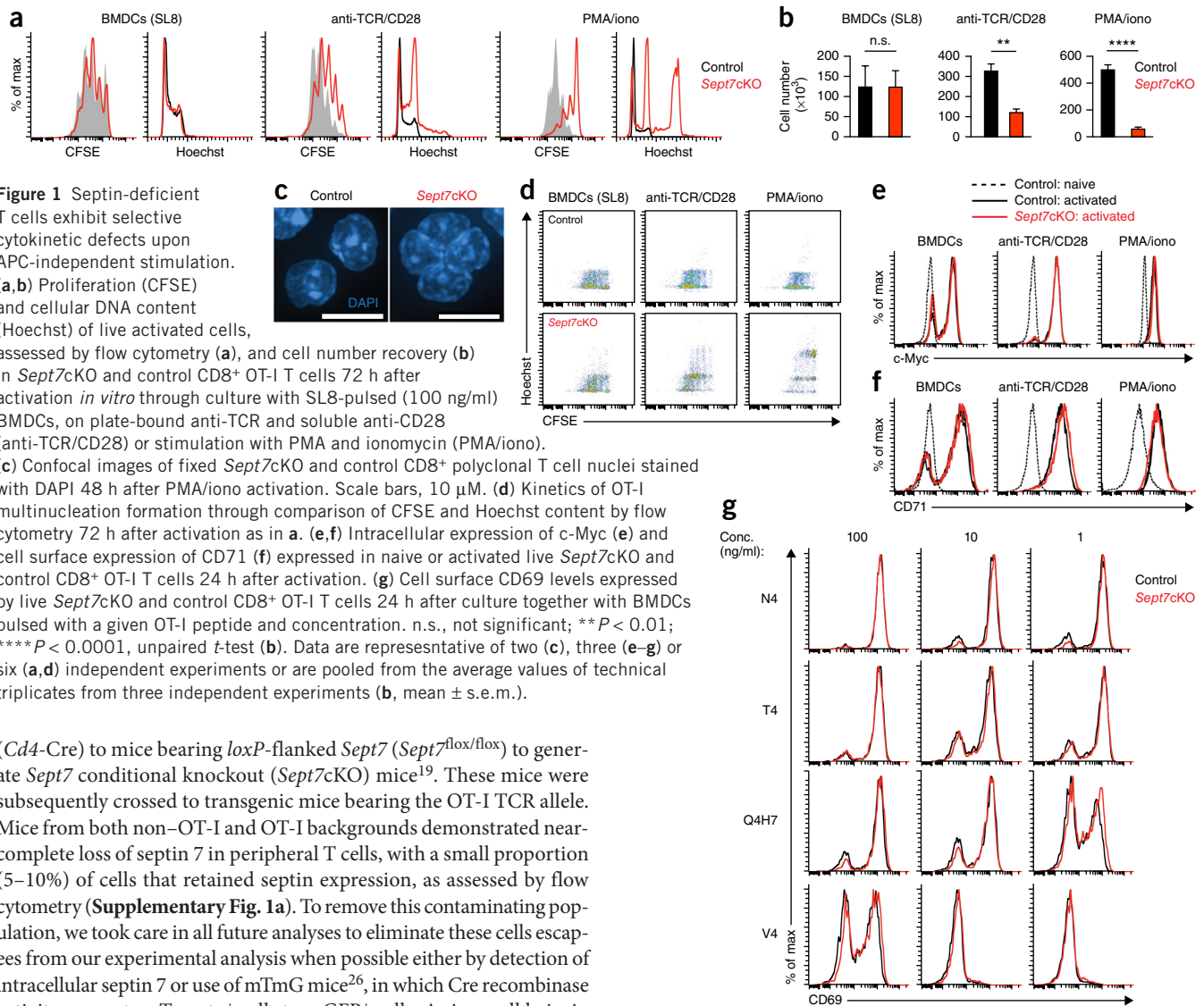
RESULTS

Development of septin-deficient T cells is unimpaired

We engineered T cells to lack all septins by crossing mice bearing a Cre recombinase expressed from the promoter and enhancer of *Cd4*

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(*Cd4*-Cre) to mice bearing *loxP*-flanked *Sept7* (*Sept7*^{fllox/fllox}) to generate *Sept7* conditional knockout (*Sept7*ckO) mice¹⁹. These mice were subsequently crossed to transgenic mice bearing the OT-I TCR allele. Mice from both non-OT-I and OT-I backgrounds demonstrated near-complete loss of septin 7 in peripheral T cells, with a small proportion (5–10%) of cells that retained septin expression, as assessed by flow cytometry (Supplementary Fig. 1a). To remove this contaminating population, we took care in all future analyses to eliminate these cells escapes from our experimental analysis when possible either by detection of intracellular septin 7 or use of mTmG mice²⁶, in which Cre recombinase activity converts mTomato⁺ cells to mGFP⁺ cells. As in small hairpin RNA (shRNA) studies in cell lines¹⁷, genetic deletion of septin 7 led to coordinate loss of other key T cell-expressed septin family members in peripheral T cells as assessed by immunoblotting (Supplementary Fig. 1b). The *Cd4*-Cre allele is expressed in a pre-double-positive (pre-DP, for CD4 and CD8 expression) phase of thymic development²⁷, and we observed initial onset of septin loss in double-negative (DN, CD4[−]CD8[−]) thymocytes with maximal loss by the single-positive (SP) stage in *Sept7*ckO mice (Supplementary Fig. 1c). We observed similar proportions of DN, DP and SP thymocyte populations (Supplementary Fig. 1d), equivalent cellularity in secondary lymph node organs (Supplementary Fig. 1e) and similar frequencies of CD4⁺ and CD8⁺ T cells within those organs (Supplementary Fig. 1f). Additionally, naive resting septin-deficient CD8⁺ T cells maintained normal amounts of filamentous actin (Supplementary Fig. 1g), and septin-deficient OT-I T cells showed morphological defects that phenocopied published findings with septin knockdown in T cell clones^{17,22} (Supplementary Fig. 1h,i). These results demonstrate that development of septin-deficient T cells in this mouse model is largely intact.

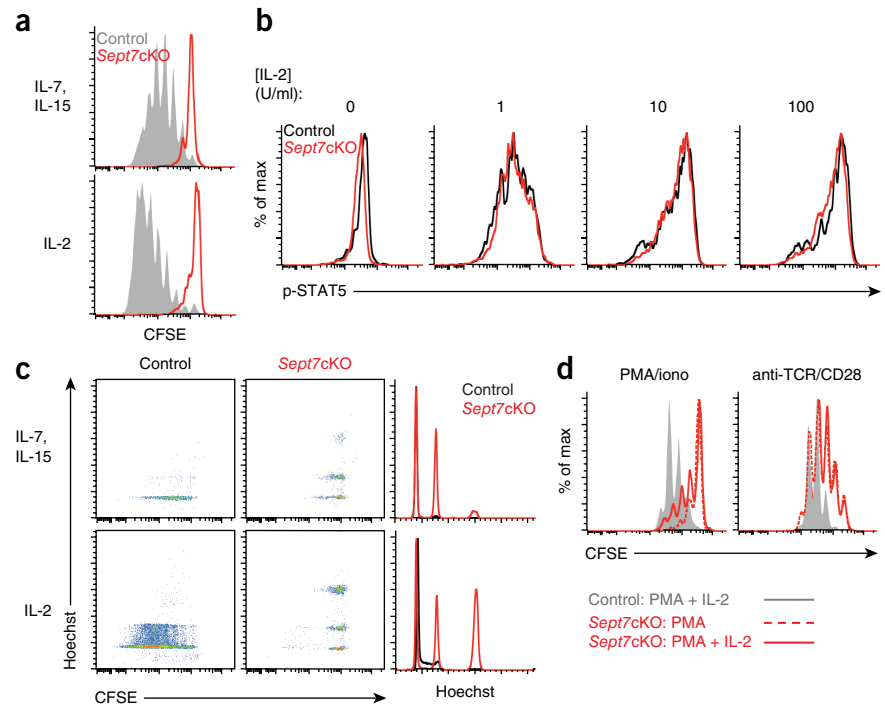
Selective proliferation defects in the absence of septins

We sought to examine T cell proliferation in the context of control (*Cd4*-Cre⁺ *Sept7*^{fllox/+} or *Cd4*-Cre[−] *Sept7*^{fllox/fllox}) or *Sept7*ckO

CD8⁺ T cells isolated from these respective mice. Although septins are required for cell division in various types of eukaryotic cells, T cells can proliferate proficiently in the absence of septin 7 and associated septin family members^{17,22,19}. Consistent with this, we found that, when cultured *in vitro* with bone marrow-derived dendritic cells (BMDCs) pulsed with the OT-I peptide antigen SL8, CD8⁺ OT-I T cells divided (Fig. 1a and Supplementary Fig. 2a), progressed in the cell cycle and expanded in number at a similar rate to wild-type cells (Fig. 1b). Unexpectedly, however, when activated with plate-coated anti-TCR antibody or soluble phorbol myristate acetate (PMA) and ionomycin, septin-deficient OT-I T cells underwent fewer cell divisions, as assessed by dilution of the cytosolic dye CFSE (Fig. 1a and Supplementary Fig. 2a) and cell recovery (Fig. 1b) after 72 h. Polyclonal CD8⁺ *Sept7*ckO T cells exhibited these cell-division defects as well (data not shown). Additionally, whereas stimulation with BMDCs generated largely conventional G1–S–G2/M cell-cycle profiles, stimulation in the absence of APCs resulted in bi- and multinucleated cells, as detected by flow cytometry (Fig. 1a and Supplementary Fig. 2b) and confocal microscopy (Fig. 1c). This differential block in cell division was also accompanied by an accumulation of septin-deficient cells of increased size (FSC-A^{hi}) as

Figure 2 Septin-deficient T cells undergo cytokinetic failure after cytokine exposure.

(a) Proliferation, as indicated by CFSE dilution, of live naive *Sept7*KO and control CD8⁺ OT-I T cells after *in vitro* culture with IL-7 (5 ng/ml) and IL-15 (100 ng/ml) (top), or IL-2 (5,000 U/ml) (bottom) for 5 d. (b) Intracellular levels of phosphorylated STAT5 (p-STAT5) in live control and *Sept7*KO CD8⁺ OT-I T cells after IL-2 exposure 24 h after cells were stimulated with PMA and ionomycin. (c) CFSE dilution and Hoechst levels in live *Sept7*KO and control CD8⁺ OT-I T cells cultured *in vitro* with IL-7 and IL-15 (top) or IL-2 (bottom). (d) Proliferation of live *Sept7*KO and control CD8⁺ OT-I T cells 72 h after *in vitro* activation, with or without IL-2 (10–20 U/ml) at initial plating. Data are representative of at least three independent experiments. *iono*, ionomycin; *anti-TCR/CD28*, anti-TCR and anti-CD28.



compared to septin-competent cells from *Sept7*KO OT-I mice in T cell cultures stimulated with anti-TCR or PMA and ionomycin (Supplementary Fig. 2c). Together, these data demonstrate that T cells are not intrinsically unique in not requiring septins for cytokinesis, as has been proposed¹⁹; rather, only certain cytokinetic pathways are septin independent. Moreover, in assessing how the generation of multinucleated cells relates to cell division, we observed that septin-deficient cells were susceptible to cytokinesis failure with every division, not just the first one (Fig. 1d). This observation suggests that failure to divide is a stochastic event, with the limited expansion of *Sept7*KO T cells to APC-independent stimuli resulting from a breakthrough event with each division.

The division defect was not an obvious result of differential loss of filamentous actin with some stimuli and not others, as phalloidin staining at 24 h was identical between *Sept7*KO and control OT-I cells (Supplementary Fig. 2d). Additionally, proximal signaling in response to all cues was unaffected by septin 7 depletion. This was apparent in equivalent upregulation of c-Myc and CD71 after stimulation (Fig. 1e,f). Further, *Sept7*KO OT-I cells stimulated by APC-dependent or APC-independent stimuli upregulated CD69 and CD25 similarly (Supplementary Fig. 2e,f). Finally, *Sept7*KO OT-I T cell calcium flux in response to anti-CD3 crosslinking or thapsigargin blockade of sarcoendoplasmic reticulum calcium transport ATPase (SERCA) uptake was also equivalent to that of wild-type cells (Supplementary Fig. 2g). To determine whether the distinctions among these stimuli related to strength of signal, we cultured T cells from control and *Sept7*KO OT-I mice with BMDCs that had been pulsed with peptides differing in OT-I TCR-pMHC affinity across a range of concentrations and measured CD69 upregulation after 24 h (Fig. 1g). Weak agonist peptides and lower doses induced less activation by this measure, but *Sept7*KO cells behaved identically to controls, demonstrating that *Sept7*KO T cell 'sensed' the density and identity of TCR signals similarly to wild-type cells. Thus, the differences we observed in *Sept7*KO T cell proliferative capacity stimuli did not stem from defective TCR signaling or cell-cycle entry but suggest, rather, that APCs contribute key cellular factors that facilitate division of *Sept7*KO T cells.

***Sept7*KO division defects with APC-independent stimuli**

Soluble cytokines also substantially drive T cell expansion, so we tested whether this stimulus would lead to cell-division defects in

septin-null T cells. We found that naive septin-deficient CD8⁺ OT-I T cells did not divide *in vitro* after exposure to homeostatic cytokines IL-7 and IL-15 or to high concentrations of IL-2 (ref. 28) (Fig. 2a and Supplementary Fig. 3a). Defects in *in vitro* proliferation did not appear to result from dysfunctional signaling, as the extent of phosphorylation of STAT5, a target of these cytokine receptors, was similar between *Sept7*KO CD8⁺ OT-I cells and control T cells (Fig. 2b). As with TCR stimulation in the absence of APCs, soluble cytokines induced multinucleated septin-deficient CD8⁺ T cells (Fig. 2c). The combination of APC-independent activation (by PMA or anti-TCR) with addition of IL-2 also failed to rescue *in vitro* proliferation, which suggests that the defect we observed did not result from inadequate cytokine production (Fig. 2d and Supplementary Fig. 3b). We concluded instead that, in contrast to stimuli from BMDCs, cytokines alone do not support cytokinesis of septin-null T cells.

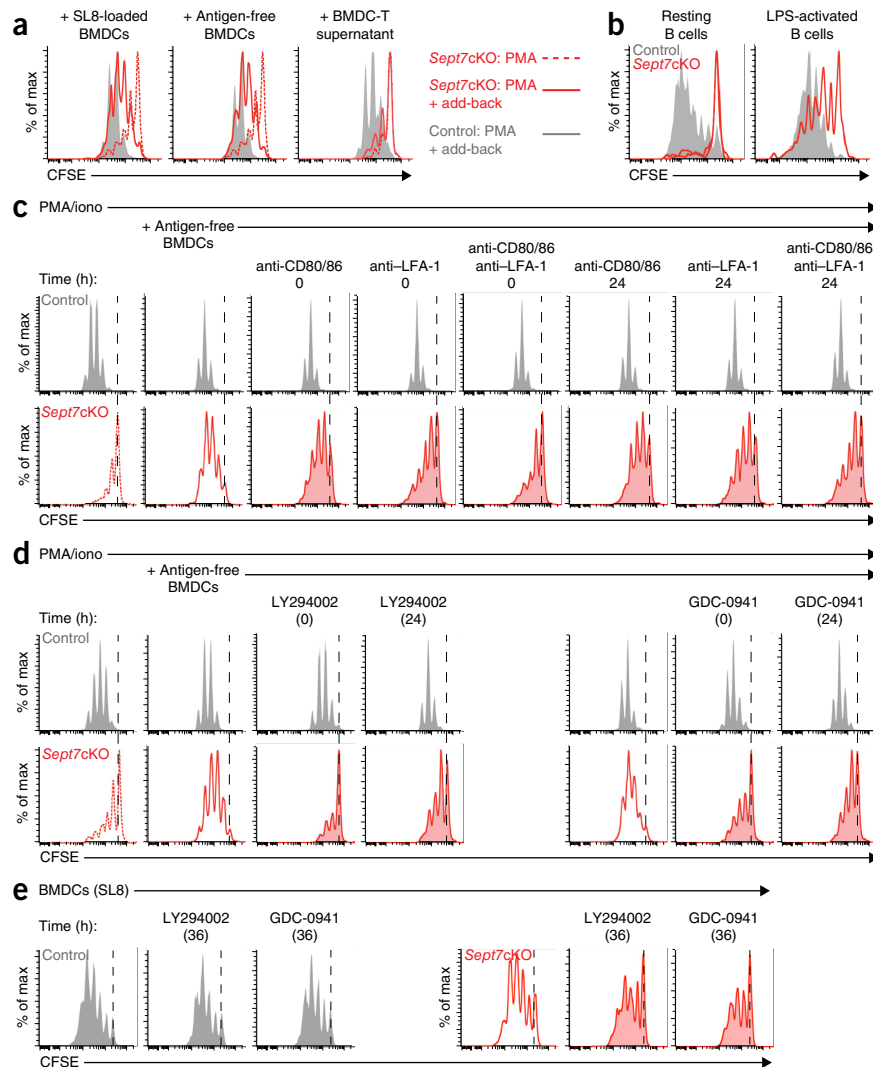
Rescue of defective proliferation through cell contacts

We next sought to determine whether BMDCs were providing additional signals that overcame a block in proliferation in *Sept7*KO cells. To do so, we again activated T cells *in vitro* with PMA and ionomycin as a base stimulus. Addition of peptide-pulsed BMDCs to these PMA-activated OT-I T cell cultures as an 'add-back' largely restored cell division, as assessed by CFSE dilution (Fig. 3a and Supplementary Fig. 4a). This partial rescue was equivalent when BMDCs lacking antigenic peptide were added, demonstrating that BMDCs mediate rescue independently of their ability to generate strong TCR signals. However, supernatant from competent BMDC-T cell cultures, added at 20% of the total culture volume, was unable to restore division, which suggests that cell-cell contact was primarily responsible for this rescue effect. In addition, we found that resting B cells were unable to support cell division in *Sept7*KO CD8⁺ OT-I T cells, whereas lipopolysaccharide (LPS)-activated B cells facilitated enhanced proliferation (Fig. 3b and Supplementary Fig. 5b), though not to the same extent as BMDCs (Fig. 1b). That BMDCs and LPS-treated B cells supported *Sept7*KO OT-I T cell division suggested that this rescue was mediated by cellular properties unique to highly activated APCs.

Figure 3 APCs mediate rescue of septin-null CD8⁺ T cell cytokinetic defect through costimulatory PI(3)K signaling. **(a)** CFSE dilution of live *Sept7*^{CKO} or control CD8⁺ OT-I T cells stimulated with PMA and ionomycin and cultured at the time of plating with an add-back of SL8-loaded BMDCs (left, 100 ng/ml SL8), antigen-free BMDCs (middle) or 20% supernatant generated from wild-type BMDC-T cell cultures (right). **(b)** CFSE dilution of live *Sept7*^{CKO} or control CD8⁺ OT-I T cells after 72 h of culture with resting or LPS-treated, SL8-pulsed splenic B cells. **(c,d)** CFSE dilution of live *Sept7*^{CKO} or control CD8⁺ OT-I T cells stimulated with PMA and ionomycin (PMA/iono), cultured with antigen-free BMDCs and treated with anti-CD80 and anti-CD86 (anti-CD80/86) and/or anti-LFA-1 **(c)** or PI(3)K inhibitors LY294002 (10 μ M) or GDC-0941 (10 μ M) **(d)** at various times after plating. **(e)** CFSE dilution of *Sept7*^{CKO} or control CD8⁺ OT-I live activated T cells cultured with SL8-pulsed (1–100 ng/ml) BMDCs and treated with LY294002 (10 μ M) or GDC-0941 (10 μ M) 36 h after plating. Data are representative of three **(a,e)**, four **(b)** or five **(c,d)** independent experiments.

In addition to TCR signals, APCs provide numerous accessory cues for T cells, and we investigated several of these. We found that signaling from costimulatory molecules to CD28 and from ICAM adhesion molecules to integrin LFA-1 represented a prominent portion of the rescue; antigen-free BMDCs restored cell division to *Sept7*^{CKO} OT-I cells, and this effect was partially inhibited with blocking antibodies to CD80 and CD86 or antibodies to LFA-1 (Fig. 3c and Supplementary Fig. 4b). This blocking was even more profound when anti-CD80 and anti-CD86 were combined with anti-LFA-1. Surprisingly, blockade was nearly as effective when these antibodies were added 24 h after the initial stimulation with PMA and ionomycin plus BMDCs. This finding suggests that rescue of cell division was mediated by BMDCs in cell-cell contacts that take place well after the initiation of TCR signals.

To test whether these interactions were purely adhesive or resulted from signaling, we repeated the BMDC add-back experiments with several inhibitors that target phosphatidylinositol-3-OH kinase (PI(3)K), a key downstream signal transduction molecule in CD28 and LFA-1 pathways. We found that pan-PI(3)K inhibitor compounds LY294002 and GDC-0941 blocked the BMDC-mediated rescue of *Sept7*^{CKO} OT-I cell division, with a modest reduction in control T cell proliferation (Fig. 3d and Supplementary Fig. 4c). Wild-type cell viability, however, was not grossly affected at the dose used (data not shown). Notably, blockade of PI(3)K signaling reduced septin-null OT-I T cell proliferation, whether inhibitors were added 24 h after BMDC addition or at the onset of culture. To extend these findings to a more physiological setting, we inhibited PI(3)K signaling 36 h after culturing *Sept7*^{CKO} and control OT-I T cells with SL8-pulsed BMDCs and found that treatment reduced *Sept7*^{CKO} T cell proliferation (Fig. 3e and Supplementary Fig. 4d). Notably, control T cell proliferation was modestly inhibited, with a more substantial loss in proliferation when PI(3)K was inhibited 24 h after initial



culture with BMDCs (Supplementary Fig. 4e). Although the magnitude of this effect was larger for septin-null T cells, these results imply that ongoing PI(3)K activity is required for maximal proliferation even in wild-type T cells.

Consistently with these results, the differing capacities of resting and LPS-treated B cells in facilitating *Sept7*^{CKO} OT-I T cell division were not due to differences in proximal T cell activation, as assessed by CD69 upregulation (Supplementary Fig. 5a). T cell division driven by LPS-treated B cells was mediated through prolonged signaling via CD28 and LFA-1 and dependent on PI(3)K (Supplementary Fig. 5b,c). Together, these findings support a model in which APCs establish a niche of cell-cell contact interactions that is characterized by PI(3)K signaling and complements or compensates for septin function in CD8⁺ T cell division.

Polarized contacts support *Sept7*^{CKO} T cell division

BMDCs bearing CD80, CD86 and ICAM-1 are likely to represent a polarized surface during cell division, and we sought to address whether that feature is sufficient to complement septin deficiency. To address this, we cultured PMA-activated OT-I T cells in wells coated with adhesive molecules including ICAM-1, fibronectin and antibodies against CD44 and CD28. Of these, ICAM-1 and anti-CD28 were uniquely capable of enhancing division of septin-null CD8⁺ T cells (Fig. 4a and Supplementary Fig. 3c). These findings suggest that

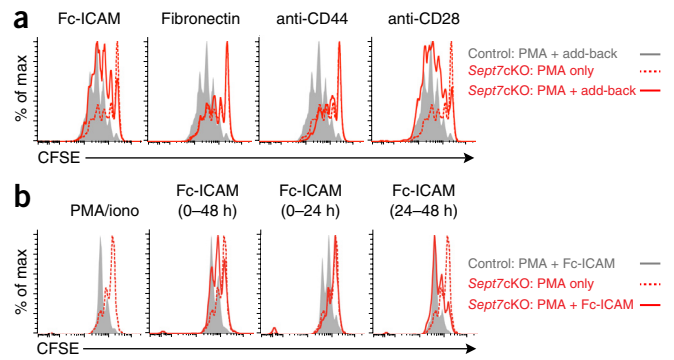
Figure 4 Costimulatory signaling is sufficient to enhance septin-null CD8⁺ T cell division. **(a)** CFSE dilution of live *Sept7*^{CKO} or control CD8⁺ OT-I T cells stimulated with PMA and ionomycin and cultured on uncoated surfaces or plate-bound Fc-ICAM, fibronectin, anti-CD44 or anti-CD28 for 72 h. **(b)** CFSE dilution of live *Sept7*^{CKO} or control CD8⁺ OT-I T cells stimulated with PMA and ionomycin (PMA/iono) for 48 h and cultured on plate-bound Fc-ICAM during various intervals after initial plating. Data are representative of four independent experiments.

BMDCs facilitate septin-null division through specific signaling, not merely through general adhesion and cellular contact. Furthermore, by selectively plating OT-I T cells with ICAM-1 at various time points during stimulation, we determined that rescue was taking place at least 24 h after initial activation but not at the time of initial stimulation (**Fig. 4b** and **Supplementary Fig. 3d**). T cells begin dividing at least 24 h after activation⁴, so this temporal window coincided with the kinetics of T cell entry into the cell cycle and ongoing division.

Septin dependence separates proliferation drivers *in vivo*

Our *in vitro* findings suggest that the requirement for septins in T cell division distinguishes whether cell division is driven by the presence or absence of specific contacts or niches. We therefore compared different activating stimuli *in vivo*. Using the Dec-OVA model of antigen delivery, in which an antibody to the dendritic cell (DC) marker DEC-205 is conjugated to ovalbumin (OVA), to load antigens onto lymph node-resident DCs²⁹, we found that adoptively transferred septin-null CD8⁺ OT-I T cells expanded similarly to their cotransferred wild-type counterparts (**Fig. 5a**). To test whether *Sept7*^{CKO} T cells continue to require the presence of antigen-loaded APCs after initial activation, we cultured CFSE-labeled *Sept7*^{CKO} and control CD8⁺ OT-I T cells with SL8-pulsed BMDCs for 36 h, at which point we isolated the T cells. We then replated *Sept7*^{CKO} and control T cells or cotransferred them to antigen-free mice. When the T cells were cultured *in vitro*, *Sept7*^{CKO} T cells showed notable cell division defects (**Fig. 5b**). *Sept7*^{CKO} T cells, however, did not divide as proficiently as control T cells when transferred to antigen-free host mice (**Fig. 5b**). These findings suggest that although *Sept7*^{CKO} T cells do not require antigen-bearing APCs during cell division, endogenous *in vivo* interactions that are absent from the T cell-only cultures are sufficient to support successful cell division.

Figure 5 Septin deficiency differentiates APC- and cytokine-driven division *in vivo*. **(a)** Frequency of cotransferred control or *Sept7*^{CKO} CD8⁺ OT-I T cells expanded in draining inguinal lymph nodes 6 d after subcutaneous immunization of host mice with Dec-OVA and anti-CD40. Each symbol represents an inguinal lymph node (left or right flank) from a host mouse. **(b)** Top, ratio of control to *Sept7*^{CKO} live T cells 48 h and 72 h after *in vitro* culture (left) and representative CFSE dilution profile 48 h after T cell isolation (right) of naive *Sept7*^{CKO} and control CD8⁺ OT-I T cells cultured with SL8-pulsed (100 ng/ml) BMDCs *in vitro* for 36 h, isolated and cultured *in vitro* with low-dose IL-2 (10 U/ml). Bottom, ratio of control to *Sept7*^{CKO} T cells recovered from host-mouse spleen 48 h after cotransfer (left) and CFSE dilution profile (right) of naive *Sept7*^{CKO} and control CD8⁺ OT-I T cells cultured with SL8-pulsed (100 ng/ml) BMDCs *in vitro* for 36 h, isolated and transferred to antigen-free host mice. $P = 0.0351$, one-sample *t*-test comparing distribution to theoretical mean of 1. Each symbol represents an individual mouse (bottom) or *in vitro* culture sample (top). **(c)** Frequency of control or *Sept7*^{CKO} CD45.2⁺CD8⁺ OT-I T cells that expanded in the spleen and inguinal lymph nodes (LN) of CD45.1⁺ host mice after 7 d of daily intraperitoneal delivery of IL-2 complex. **(d)** Frequency of cotransferred *Sept7*^{CKO} and control polyclonal CD8⁺ T cells in the spleen and skin-draining LN of sublethally irradiated mice. Each symbol represents an individual mouse (**c,d**). n.s., not significant; **** $P < 0.0001$, with paired (**a,d**) or unpaired (**c**) *t*-test. Data are representative of three independent experiments with $n = 3$ –5 mice (**a,d**) or pooled from two (**b**, top) or three experiments with $n = 7$ (**b**, bottom) or 8 mice (**c**); error bars, mean \pm s.e.m. (**a–d**).



In contrast, when anti-IL-2 complexes were delivered to generate cytokine-mediated proliferation in the absence of overt APC involvement, expansion of adoptively transferred septin-null OT-I T cells was significantly reduced compared to that of control cells (**Fig. 5c**). As an additional cytokine-mediated *in vivo* challenge, we transferred polyclonal wild-type and *Sept7*^{CKO} cells into sublethally irradiated mice. In these mice, T cells typically undergo a slow form of lymphopenia-induced proliferation, which is thought to mimic an acute form of homeostatic expansion³⁰. Again, the overall expansion of septin-null CD8⁺ T cells was impaired (**Fig. 5d**). These findings support a context-dependent requirement for septins in T cell division and extend our model to critical *in vivo* processes.

Septin requirement for CD8⁺ T cell homeostasis

We found evidence for defects in steady-state homeostatic maintenance of naive and memory CD8⁺ *Sept7*^{CKO} T cells, which is in agreement with findings that septin 7 is required for cytokine-driven proliferation. Although peripheral CD8 compartments were comparable between *Sept7*^{CKO} and control OT-I mice at 6–8 weeks of age, the frequency of CD8⁺ T cells declined after 6 months (**Fig. 6a**). In addition, we found that decline was accompanied by a significant loss of phenotypically naive CD44^{lo-int} CD8⁺ T cells in aged *Sept7*^{CKO} OT-I mice. Of the CD8⁺ T cells that remained in aged *Sept7*^{CKO} mice, most were CD44^{hi}, in contrast to control cells that showed bimodal expression (**Fig. 6a**), which suggests a lymphopenic environment in which surviving *Sept7*^{CKO} CD8⁺ T cells may be responding to antigen^{31,32}. Given that memory CD8⁺ T cell homeostasis also relies on

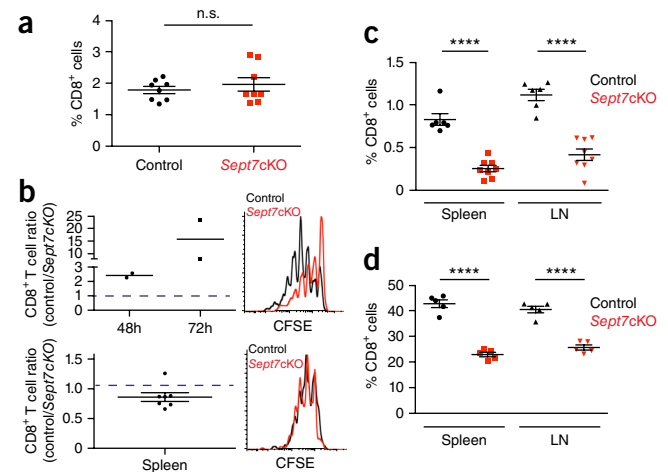
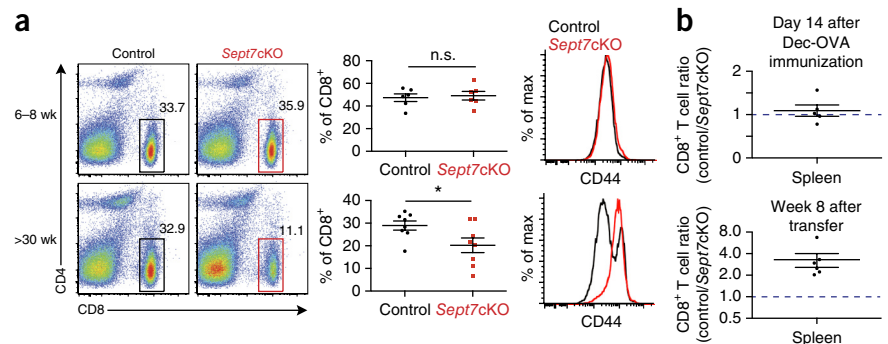


Figure 6 Septins are required for homeostatic maintenance of naive and memory CD8⁺ T cells *in vivo*. **(a)** Frequency of CD8⁺ T cells (left) and CD8⁺ T cell CD44 surface expression (middle and right) in pooled lymph nodes from 6- to 8-week-old (top) or 6-month-old (bottom) OT-I mice. **(b)** Ratio of control to *Sept7*CKO cells sorted from spleens of mice 14 d after intravenous immunization with Dec-OVA (top) or detected in spleens of antigen-free host mice 8 weeks after transfer of sorted OT-I T cells from immunized mice (bottom). n.s., not significant; * $P < 0.05$, unpaired *t*-test (**a**); $P > 0.05$ (**b**, top) and $P = 0.0243$ (**b**, bottom), one-sample *t*-test comparing distribution to theoretical mean of 1. Each symbol represents an individual mouse (**a** and **b**, bottom) or an independent experiment (**b**, top). Data are pooled from 4 (**b**, bottom), 5 (**a**, bottom; **b**, top) or 6 (**a**, top) independent experiments with total $n = 6$ (**a**, top), 8 (**a**, bottom), 5 (**b**, top), or 6 (**b**, bottom) mice (mean \pm s.e.m.).



cytokines, we examined maintenance of memory CD8⁺ *Sept7*CKO T cells. Naive *Sept7*CKO and wild-type CD8⁺ OT-I T cells were cotransferred to host mice that were immunized intravenously (i.v.) with Dec-OVA. *Sept7*CKO T cells were detected at similar frequencies to that of wild-type T cells in the spleen 14 d later (**Fig. 6b**) but were slightly lower in number in lymph nodes (**Supplementary Fig. 6a**) and showed high expression of memory precursor cell markers CD44 and CD62L (**Supplementary Fig. 6b**). *Sept7*CKO and wild-type OT-I T cells were then sorted from the spleen and lymph nodes and cotransferred to antigen-free mice. The frequency of *Sept7*CKO and control T cells in these mice was determined 8 weeks later, as studies have demonstrated that many rounds of homeostatic turnover occurs over this period of time³³. Indeed, the frequency of *Sept7*CKO T cells was significantly lower than that of control T cells in antigen-free host spleens after 8 weeks (**Fig. 6b**), which indicates that septins have a role in facilitating memory T cell homeostasis. In contrast, although we noted a trend toward preferential wild-type T cell maintenance in the lymph node, it was not as severe as in the spleen (**Supplementary Fig. 6c**). This observation may indicate different milieus between the two organs that support homeostatic division but remains an area of future investigation. Together, these data demonstrate that a lack of septins impedes T cell division in a stimulus-dependent manner *in vivo*.

DISCUSSION

We have identified a situation and mechanism that differentiates APC- and niche-driven proliferation from niche-independent division. This suggests that it might be possible to target specific types of T cell division with, for example, cytoskeletal inhibitors. This is of particular importance, as homeostatic expansion arising from lymphopenic conditions can result in rejection of organ transplants and, possibly, in autoimmunity and is not responsive to costimulatory blockade^{34–38}. A preponderance of clinical immunosuppressive agents are directed against antigen-specific T cell expansion, typically targeting pathways downstream of T cell activation, such as those involving mTOR or calcineurin³⁵. Drugs that are available to restrict homeostatic division, such as mycophenolic acid, target nonspecific processes, such as DNA synthesis³⁹, that are characteristic of all forms of T cell division and thus leave patients ill-equipped to face pathogen challenge. Our work suggests that targeting septins and the pathways they use might yield better therapeutic strategies in such cases.

Published studies have found that T cells are among a select few eukaryotic cells that do not appear to require septins to complete cell division^{17,19}. We now understand that the T cell exception reflects a role for septins in cell division that is not cell-type dependent but rather context dependent. In the case of T cells, the context that

rescues cell division is the presence of a highly adhesive and activated surface or cell type. *In vivo* lymph node imaging studies of proliferating T cells have shown that antigen-specific T cells divide independently of contact with labeled APCs⁵, though division after DC contact has also been noted⁴⁰. Our findings that APCs or surfaces high in specific costimulatory molecules and integrins rescue septin-null T cell cytokinetic defects *in vitro* and *in vivo* provide support that a niche in which T cells engage in contact interactions during the temporal window of division serves a functional role. We observed a spectrum of septin-null T cell proliferative competence across conditions of activation (PMA and ionomycin; anti-TCR and anti-CD28; and antigen-pulsed APCs), an effect that corresponds to the degree of PI(3)K signaling generated by these stimuli. It is thus possible that a threshold of total PI(3)K signaling must be reached for T cells to divide in the absence of septins; however, cytokines such as IL-7 and IL-2 generate PI(3)K activity through their receptors, and we did not find partial or full rescue of the cytokinesis defect in the presence of these cytokines. Distinctions in how and when PI(3)K is active probably underlie this observation, and it is also possible that the activating PI(3)K cue is counterbalanced by additional cell-cell cues. On the basis of the data, we favor a model in which APCs mediate septin-null cell division by providing a highly polarized source of PI(3)K signaling and/or stabilized cell cortex at or near the time of cell division.

As septins have been described to establish polarity in yeast and reinforce local membrane compartmentalization by serving as a diffusion barrier^{41–43}, it is tempting to speculate that septins and PI(3)K ‘cooperate’ or act redundantly to maintain polarity while T cells undergo cell division. Notably, compartmentalization of phospholipids has been described as important during cytokinesis, as the cytokinetic furrow is enriched for phosphatidylinositol-(4,5)-bisphosphate^{44,45}. Although studies have found PI(3)K signaling within the first 8 h of T cell activation to have the most crucial impact on eventual proliferation⁴⁶, our data using PI(3)K inhibitors suggest that PI(3)K signaling even 24 h after T cell activation continues to regulate wild-type T cell proliferative potential and kinetics. Indeed, dual PI(3)K–septin pharmacological inhibition might prove selective in blocking antigen-independent proliferation of leukemic cells while sparing the proliferation of antigen-dependent host T cells.

One notable result of our work is that T cell signaling, even at a synapse, is independent of septins. Our original impetus for studying these proteins was the observation that they assembled densely as a ring around the immune synapse (J.K.G. and M.F.K., unpublished observations). To this extent, our collective data are at odds with studies that use shRNA approaches to link septins to efficient colocalization of the calcium sensor STIM1 and the calcium channel

ORAI and subsequent calcium flux in HeLa, Jurkat and human embryonic kidney (HEK) cells^{47,48}. We, in contrast, did not find that differences in septin-deficient T cell division capacity stemmed from defects in TCR signaling processes. One possible reason for this difference would be some form of compensation in our cells for this specific septin function. The presence of cell division defects argues against ubiquitous compensation, and the idea of signaling-specific compensation is not supported by data in which normal calcium flux was observed despite Cre transfection of *Sept7^{flx/flx}* T cell blasts (data not shown). An alternative explanation for the apparent septin defect reported in cell lines may be that these cells are experiencing cytokinetic failure and, thus, that defective signaling is relatively distal to septin deficiency. At present, although we cannot confirm that there is a requirement of septins for T cell calcium signaling, further work may be needed to determine how and when our findings align with published signaling studies.

Why were we able to isolate T cells from the periphery of cKO mice if cell division is possibly compromised? One answer is that although the *Cd4*-Cre allele depletes septins in the pre-DP window, T cells in the post-DP stage typically do not divide again unless called upon to do so for homeostatic expansion. Additionally, with respect to homeostatic processes, although septin-null cells were clearly defective in expanding *in vivo* when transferred to sublethally irradiated mice, the effect was not as profound as the division defect to pure cytokines *in vitro*. It may be that some maintenance, if not expansion, of T cells in developing mice can involve synaptic cell-cell encounters that are septin independent. For example, IL-15 is trans-presented to CD8⁺ T cells by another cell bearing the α -chain⁴⁹. Additionally, T cells at steady state engage in cellular contact with APCs presenting self-peptide-MHC and/or fibroblastic reticular cells that produce homeostatic cytokines such as IL-7 (ref. 7).

What is the breadth of this septin requirement in homeostatic processes? Septin-null T cells appeared to selectively lose CD44⁺CD8⁺ populations in lymph nodes^{31,32}, and we also observed defective homeostasis of memory CD8⁺ OT-I *Sept7*cKO T cells in the spleen. In light of the trend in septin requirement, these findings suggest that endogenous cell-cell contacts in these settings do not mediate *Sept7*cKO T cell division as do endogenous cell-cell contacts for activated T cells. Although we found no specific defects in septin-deficient T cells for effector generation, as assessed by surface markers or interferon- γ at day 6 after immunization (data not shown), late-stage cellular contacts such as those during asymmetric cell division or in late-stage T-T interactions^{8,50} may supply an additional, higher degree of complexity in T cell effector and memory development as well as clonal burst size and sustained survival. In sum, the observed distinction between septin-dependent and septin-independent immune cell cytokinesis indicates possibilities for enhanced selective targeting of proliferative processes.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M.M. and M.F.K. designed the experiments for all primary text figures; A.M.M. did experiments; J.K.G. performed and helped with experiments related to initial characterization of *Sept7*cKO mice; A.G. contributed to experimental design and analysis; M.K. provided the *Sept7^{flx/flx}* mice and consulted on experiments; A.M.M. and M.F.K. wrote and revised the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Sept7^{fllox/flox}* mice¹⁹ were crossed to *Cd4-Cre* transgenic mice²⁷ to deplete septin 7 in T cells. These mice were bred to ovalbumin (OVA)-specific TCR transgenic OT-I mice⁵¹ and/or mTmG mice²⁶ (Jackson Laboratory). Cells designated as experimental controls were generated from *Sept7^{fllox/flox};Cd4-Cre^{-/-}* or *Sept7^{fllox/flox};Cd4-Cre⁺* mice. These mice, along with C57BL/6 (The Jackson Laboratory and Simonsen) and CD45.1⁺ mice, were housed and bred under specific pathogen-free conditions at the University of California Animal Barrier Facility. All experiments with mice were approved by the Institutional Animal Care and Use Committee of the University of California.

Cell isolation. CD8⁺ polyclonal or OT-I T cells were isolated from lymph nodes of 6- to 8-week-old mice using EasySep CD8 negative-selection kits (STEMCELL Technologies). B cells were isolated from spleens of 6- to 8-week-old mice using EasySep B cell negative selection kits (STEMCELL Technologies). In specified experiments, B cells were treated with 1 µg/ml LPS (Sigma-Aldrich) for 5 h before use. BMDCs were generated from treating cultured bone marrow cells with granulocyte macrophage-colony-stimulating factor (GM-CSF) for 7–11 d. IL-4 was added for the last 2 d, with 1 µg/ml LPS stimulation 6–24 h before use. BMDCs and B cells were incubated with 1–100 ng/ml SL8 OVA peptide (SIINFEKL) (Anaspec) or variant peptides N4, T4, Q4H7, V4 (gift from E. Palmer and D. Zehn) for at least 30 min at 37 °C and washed three times.

Immunoblotting. Naive CD8⁺ T cells were isolated from 6-week-old *Sept7^{cKO}* and littermate control OT-I mice. 10⁶ cells per sample were lysed in PBS containing 1% Triton X-100 in the presence of a cocktail of protease and phosphatase inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 mM PMSF, 10 mM sodium fluoride, 10 mM iodoacetamide and 1 mM sodium orthovanadate). After 15 min lysis on ice, lysates were centrifuged at 21,000 × g for 10 min to remove insoluble material, and protein concentration in the supernatant was quantified by the Bio-Rad detergent-compatible protein assay to ensure equal loading. Samples were resolved by SDS-PAGE, and immunoblot analysis was performed using rabbit polyclonal primary antibodies to Sept1, Sept6C, Sept9 (ref. 52), Sept7 (IBL-America, Inc.), and HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) Relative protein abundance was quantified using ImageJ software (NIH).

In vitro T cell activation assays. Isolated T cells were labeled with 0.5–5 µM CFSE (Invitrogen) or CellTrace Proliferation Dye (BD Biosciences) for 15 min at 37 °C, cultured in 96-well plates at a density of 0.1 × 10⁶ per ml in complete RPMI and harvested for analysis 72 h later, unless noted otherwise. CD8⁺ T cells were cultured on plate-bound anti-CD3 (2C11; UCSF Hybridoma Core) or anti-TCRβ (H57-597; produced in M.F.K.'s lab) with addition of soluble anti-CD28 at 2 µg/ml (PV-1; UCSF Hybridoma Core). Alternatively, T cells were stimulated with 100–1,000 ng/ml PMA and 125 ng/ml ionomycin. In specified experiments 10–20 U/ml of recombinant human IL-2 (NIH AIDS Reagent Program) or 20% supernatant harvested from previous wild-type BMDC-T cell cultures was added at time of plating. For other indicated experiments, 96-well plates were coated with the following antibodies or proteins: 10 µg/ml anti-CD44 (IM7; eBiosciences), 10 µg/ml anti-CD28 (PV-1; UCSF Hybridoma Core), 5 µg/ml recombinant mouse ICAM-1 Fc chimera protein (R&D Systems), or 10 µg/ml fibronectin, bovine plasma (EMD Millipore). In some experiments, cells were moved to or removed from plates containing ICAM-1 Fc Chimera protein, prepared as detailed above.

In coculture experiments, OT-I T cells were plated with activated BMDCs at a 10:1 ratio in flat-bottom wells or with isolated B cells at a 1:1 ratio in round-bottom wells. In specified experiments, 10 µM of PI(3)K inhibitors LY-294002 (Sigma-Aldrich) or GDC-0941 (gift from J. Roose) was added to culture 24 h or 36 h after plating. For BMDC rescue assays, T cells were stimulated with PMA and ionomycin, and mature activated BMDCs were added at time of plating. To block costimulatory signaling, 10 µg/ml anti-CD80 (16-10A1; UCSF Hybridoma Core) and 10 µg/ml anti-CD86 (GL-1; UCSF Hybridoma Core) and/or anti-CD11a (M17/4; UCSF Hybridoma Core) was added to culture 0 or 24 h after plating. Alternatively, 10 µM of LY-294002 or 10 µM GDC-0941 was added 0 or 24 h after plating. In other experiments, T cells were plated

with activated BMDCs at a 5:1 ratio, isolated with a CD8 negative selection kit, and re-plated with 10 U/ml of human recombinant IL-2.

Cytokine exposure. Isolated CD8⁺ OT-I T cells were cultured in 96-well plates at a density of 0.1 × 10⁶ cells per ml. 5,000 U/ml human recombinant IL-2 or murine recombinant 5 ng/ml IL-7 (Peprotech) and 100 ng/ml IL-15 (Peprotech) were added to medium at time of plating, and cells were harvested for analysis 5 d later.

Surface and intracellular flow cytometry staining. Cells were harvested from lymph nodes, spleens or *in vitro* culture, washed with PBS and nonspecific binding blocked with flow cytometry buffer (PBS, 2% FCS) and anti-CD16/32 (2.4G2; UCSF Hybridoma Core). Surface proteins on cells were stained with the following antibodies for 25 min at 4 °C: anti-CD69 (H1.2F3; eBiosciences), anti-CD25 (PC61.5; eBiosciences), anti-CD71 (C2, BD Pharmingen), anti-CD8α (53-6.7; eBiosciences), anti-CD4 (RM4-5; BioLegend), anti-CD44 (IM7; eBiosciences), anti-CD45.1(A20; eBiosciences), or anti-CD45.2 (104; BioLegend). Cells were again washed and resuspended with flow cytometry buffer before data collection, with addition of black latex beads to samples to quantify cellular number.

For intracellular stains, cells were washed with PBS and incubated with Zombie NIR fixable viability dye (BioLegend) at 4 °C for 30 min to dye dead cells. Cells were next washed and fixed with 4% PFA for 15 min at 20 °C. Last, cells were washed and stained in flow cytometry buffer with 0.2% saponin along with anti-septin 7 (ref. 52) or phalloidin probe (Invitrogen) for 30 min before a final wash and resuspension in flow cytometry buffer. Alternatively, fixed cells were treated with cold (–20 °C) methanol for 30 min at 4 °C. Cells were then stained for 1 h at 20 °C in staining buffer (PBS, 1% BSA) containing anti-c-Myc (D84C12; Cell Signaling Technology), washed, and stained with fluorescence-labeled donkey F(ab')₂ anti-rabbit (Abcam) for 30 min at 20 °C.

For assessing cellular DNA content with Hoechst dye, harvested cells were washed with PBS, fixed in 70% ethanol for 30 min on ice and washed twice with PBS. Cells were then incubated with PBS containing 0.1% Triton X-100, 0.1 mM EDTA, 100 µg/ml RNase A (Thermo Scientific), and 5 µg/ml Hoechst dye (Thermo Scientific). Cells were again washed and resuspended in flow cytometry buffer.

STAT5 phosphorylation assay. Isolated CD8⁺ OT-I T cells were cultured in 96-well plates and activated through PMA and ionomycin stimulation or culture together with SL8-pulsed activated BMDCs. Anti-mouse IL-2 (15 µg/ml, JES6-1A12) was added to cultures at time of plating to restrict IL-2 delivery. After 24 h, recombinant human IL-2 (0.1–100 U/ml) was added to wells for 20 min at 37 °C. Cells were washed, fixed with 4% PFA for 15 min at 20 °C and washed. Cells were resuspended with cold (–20 °C) methanol and washed with flow cytometry buffer four times. To stain, cells were incubated in PBS containing 2% BSA and anti-phospho-STAT5 (C71E5; Cell Signaling Technology) before a final wash and resuspension in flow cytometry buffer.

Calcium flux signaling. Isolated CD8⁺ OT-I cells were labeled with 1 µM ratiometric calcium-binding dye Indo-1, AM (Life Technologies) in PBS for 15 min at 37 °C. Cells were washed twice with complete RPMI and incubated at 37 °C for 15 min to allow for complete de-esterification. Cells were coated with 5 µg/ml anti-CD3 (2C11; UCSF Hybridoma Core) on ice, washed and transferred to 37 °C 10 min before data collection. To induce CD3 crosslinking, 10 µg/ml of anti-Armenian hamster (BioLegend) was added to CD3-coated cells after 1 min of sample collection. Alternatively, 1 µM of thapsigargin (Sigma-Aldrich) was added to uncoated cells. Cell samples were kept in a heating chamber (37 °C) during data collection by flow cytometry.

In vivo cell transfer and Dec-OVA immunization. Isolated CD8⁺ T cells were resuspended in PBS and adoptively transferred by retro-orbital or tail-vein injection to recipient mice. 0.5 × 10⁶ CFSE-labeled wild-type and *Sept7^{cKO}* polyclonal T cells were cotransferred to host mice that had been sublethally irradiated. Inguinal lymph nodes and spleen were harvested and analyzed 14 d after transfer. 0.5–1 × 10⁶ CFSE-labeled wild-type and *Sept7^{cKO}* CD8⁺ OT-I T cells that had been cultured together *in vitro* with SL8-pulsed BMDCs were



isolated and cotransferred to antigen-free host mice. Spleens were harvested and analyzed 48 h later.

Alternatively, 2.5×10^3 wild-type and *Sept7cKO* OT-I T cells were cotransferred to congenic wild-type mice, respectively. Dec-OVA complexes were generated in house from conjugation of anti-DEC-205 (NLDC-145) to OVA. 1 μ g Dec-OVA, along with 10 μ g anti-CD40 (1C10; eBiosciences), was injected subcutaneously in the left and right flanks of host mice. Inguinal lymph nodes were harvested and analyzed 6 or 7 d later.

For memory homeostasis experiments, 2.5×10^6 naive CD8⁺ OT-I wild-type and *Sept7cKO* T cells were cotransferred to host mice that were immunized i.v. with 10 μ g Dec-OVA and 50 μ g anti-CD40. Transferred T cells were sorted from pooled spleens and lymph nodes of these mice 14 d later, counted, and $0.5\text{--}1 \times 10^6$ wild-type and *Sept7cKO* cells were cotransferred to antigen-free mice. Spleens and lymph nodes were harvested and analyzed 8 weeks later.

In vivo cell transfer and IL-2 complex delivery. 1×10^6 isolated *Sept7cKO* or control OT-I CD8⁺ T cells were adoptively transferred by retro-orbital injection to congenic recipient mice. IL-2 complexes were formed by incubating 1 μ g recombinant murine IL-2 peptide (Peprotech) with 5 μ g murine anti-IL-2 (S4B6-1; BioXCell) for 1 h at 20 °C. PBS was added and the solution administered by intraperitoneal injections. IL-2 complexes were freshly made and injected daily for 7 d before harvesting and analysis of inguinal lymph nodes and spleen.

T cell morphology analysis. T cells were activated and imaged on ICAM-coated coverslips with 0.25% low-melt agarose for morphological analysis. Cell length was measured using Metamorph software's Integrated Morphometry Analysis (IMA) measurement tool. Images were acquired on a modified ZeissAxiovert 200M microscope with a Plan Neofluar 63 \times objective (Carl Zeiss) using Metamorph imaging software (MDS Analytical Tech).

Confocal microscopy. Isolated CD8⁺ T cells were stimulated with PMA and ionomycin and cultured for 48 h. Cells were then transferred to poly-L-lysine-coated chamber slides. Once adherent, cells were washed twice with PBS, fixed with 4% PFA (in PBS) for 15 min at 20 °C and washed. Cells were permeabilized with 0.1% Triton X-100 (in PBS) for 5 min, and washed twice with 1% BSA (in PBS). Cells were then stained with DAPI (Invitrogen) before a final wash and application of a cover slip. Images of cells were generated using an inverted Yokogawa CSU-10 spinning-disk confocal microscope (Zeiss) with Micro-Manager imaging software (<http://micro-manager.org>).

CFSE quantification. A mean CFSE peak number was quantified for each *in vitro* proliferation experiment with CFSE-labeled T cells. For a given sample's CFSE profile of live cells, each CFSE peak was gated and the percentage of cells populating the peak was generated. A weighted mean of the number of CFSE peaks diluted was then calculated.

Statistics and general methods. Comparisons between groups were analyzed by Student's *t*-test with GraphPrism software. Data were considered significant if *P* values were ≤ 0.05 . No statistical method was used to predetermine sample size. Experimental group allocation was determined by genotype, and investigators were not blinded to allocation during experiments and outcome assessment.

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