The lipid chemoattractant sphingosine 1-phosphate (SIP) guides cells out of tissues, where the concentration of SIP is relatively low, into circulatory fluids, where the concentration of SIP is high. For example, SIP directs the exit of T cells from lymph nodes, where T cells are initially activated, into lymph, from which T cells reach the blood and ultimately inflamed tissues. T cells follow SIP gradients primarily using SIP receptor 1 (SIPR1) to supply this SIP, in part because the expression of CD69 was associated with reduced levels of S1pr5 (which encodes SIP receptor 5). CD69 acted as a ‘stand-your-ground’ signal, keeping immune cells at a site of inflammation by regulating both the receptors and the gradients of SIP. Finally, increased levels of SIP prolonged the residence time of T cells in the lymph nodes and exacerbated the severity of experimental autoimmune encephalomyelitis in mice. This finding suggests that residence time in the lymph nodes might regulate the differentiation of T cells, and points to new uses of drugs that target SIP signalling.

We first asked whether extracellular levels of SIP in the lymph nodes change during an immune response. Exploration of this question is challenging because SIP is a lipid, so levels cannot be approximated by mRNA. Moreover, SIP has intracellular and extracellular roles, making mass spectrometry of whole tissues uninformative. Even when extracellular fluid can be obtained, interpretation is complicated because SIP is carried by proteins that may sequester or present the lipid. The most reliable measurements of ‘signalling-available’ SIP have been based on the observation that SIP receptor 1 (SIPRI) is internalized upon binding to SIP. All else being equal, a cell with a high level of SIPR1 on the surface is not sensing SIP, while a cell with a low level of SIPR1 on the surface is sensing SIP. This inference has been extensively validated for T cells in homeostasis, but is problematic in inflammation, when additional factors regulate surface SIPRI (ref. 2) (Extended Data Fig. 1a). Upon exposure to inflammatory cytokines or activation of the T cell receptor (TCR), T cells upregulate CD69; CD69 binds to SIPRI and the CD69–SIPRI complex is internalized. Moreover, activation of the TCR downmodulates SIPRI transcription. However, these effects are transient, leaving a large time window in which SIP might alter the trafficking of T cells.

To address whether the level of SIP in the lymph nodes changes during an immune response, we transferred Cd69−/− polyclonal T cells to a wild-type (WT) host, induced inflammation by injection of the viral double-stranded RNA mimic polynosinic:polycytidylic acid (pIC), and measured levels of surface SIPRI on the transferred cells in the draining lymph nodes (dLNs) (Fig. 1a). We observed reduced levels of surface SIPRI, consistent with increased levels of SIP in the lymph nodes (Fig. 1b, c, Extended Data Figs. 1, 2). To assess whether loss of SIPRI was ligand-independent (beyond the effects of CD69 and TCR signalling, avoided by assaying Cd69−/− polyclonal T cells), we used mice expressing an SIP sensor. The core of the sensor is SIPRI fused to GFP, which is internalized and partly degraded upon binding to SIP. The sensor also encodes a mutant SIPRI (SIPRIΔN), with an arginine-to-alanine substitution that prevents the binding of SIP, fused to RFP. SIPRIΔN–RFP stays on the cell surface regardless of extracellular SIP. The two receptors are linked by a 2A sequence, and hence translated and translated at a 1:1 ratio. The ratio of SIPRI–GFP to SIPRIΔN–RFP on the cell surface is a measure of the exposure to SIP of a cell (Fig. 1d). We transferred sensor-expressing Cd69−/− polyclonal T cells to WT hosts, induced inflammation with pIC and visualized the transferred cells. Surface SIPRI–GFP was reduced relative to SIPRIΔN–RFP (Fig. 1e, f, Extended Data Fig. 1k). This suggested that the internalization of SIPRI did not reflect transcriptional, translational or most post-translational modifications, and probably indicated increased SIP levels in the lymph nodes (Extended Data Fig. 2j).
If the loss of surface S1PR1 was due to increased levels of S1P, surface S1PR1 levels should be restored by inhibiting the synthesis of S1P. Mice that cannot produce S1P die at mid-gestation, so we made bone marrow chimaeras in which WT hosts were reconstituted with bone marrow from sphingosine kinase-deficient donors (SPHK-KO; Spkh2<sup>−/−</sup> Spkhk<sup>−/−</sup> Mx1-Cre<sup>−/−</sup>) or littermate controls. Loss of S1P production by haematopoietic cells blocked the internalization of S1PR1 after pIC injection, indicating that a haematopoietic source supplied S1P in inflammation (Fig. 1g, h, Extended Data Fig. 2k–p).

Last, if S1P levels in the lymph nodes increased, T cells should stay longer in the lymph nodes, because the increased levels of S1P in the lymph nodes would counter lymph S1P directing exit. To test this, we transferred polyclonal Cd69<sup>−</sup> T cells into SPHK-KO and control bone marrow chimaeras, treated the mice with pIC and waited 14 h. We then divided the mice into two groups. We euthanized one group at 't = 0' and counted T cells in the dLN. We treated the second group with LFA1-blocking and VLA4-blocking antibodies, preventing further T cell entry into the lymph nodes<sup>4</sup>. We waited 4 h, euthanized the second group of mice and counted the cells that remained in the dLN. While approximately 20% of cells exited the control lymph nodes in 4 h, approximately 50% exited the lymph nodes of SPHK-KO chimaeras (Fig. 1i–j, Extended Data Fig. 2q–r).

### iMo supply lymph node S1P

Our next question was which cells supply lymph node S1P in inflammation. CD11b<sup>+</sup>CCR2<sup>+</sup>Ly6ChighLy6G<sup>−/low</sup> inflammatory monocytes (iMo) accumulated in dLNs after treatment with pIC<sup>10</sup> (Fig. 2a, b, Extended Data Fig. 3a, b). Upon depletion of iMo and neutrophils with an antibody to Ly6C<sup>+</sup>/C<sup>+</sup>, we no longer observed the internalization of S1PR1 on T cells (Fig. 2c, d, Extended Data Fig. 3c–h). This suggested that iMo might supply lymph node S1P during an immune response.

To target the production of S1P by iMo more specifically, we used mixed bone marrow chimaeras. We reconstituted WT mice with a 1:1 mix of SPHK-KO bone marrow (which is unable to produce S1P) and bone marrow that was SPHK-WT but expressed the diphertheria toxin receptor (DTR) driven by the Ccr2 promoter (CCR2–DTR). CCR2 is highly expressed by iMo<sup>11</sup>. After treatment with diphertheria toxin, iMo from the CCR2–DTR donor were eliminated and the only remaining iMo were SPHK-WKO, while 50% of the CCR2-negative haematopoietic cells remained SPHK-WT. We also made a series of control chimaeras. We challenged diphertheria toxin-treated chimaeras with pIC. When CCR2-positive cells could no longer make S1P, S1PR1 was no longer downmodulated on T cells in the dLNs (Fig. 2e, Extended Data Fig. 3i–m). These results were consistent with iMo as a source of lymph node S1P.

If iMo supplied lymph node S1P during an immune response, iMo transferred to an otherwise healthy mouse should raise the levels of lymph node S1P. We injected control or SPHK-KO iMo into the lymph nodes of WT recipients at numbers comparable to those recruited following treatment with pIC. Control, but not SPHK-KO, iMo induced downmodulation of S1PR1 on T cells in the lymph nodes (Fig. 2f, Extended Data Fig. 4a–f). We also assayed iMo S1P release ex vivo. iMo secreted too little S1P for mass spectrometry measurement – T cells respond to as little as...
100 nM S1P, while mass spectrometry detects approximately 100 nM. As one alternative, we cultured iMo across a transwell from CD69<sup>+</sup> polyclonal T cells that expressed the S1P sensor. Control iMo secreted S1P, measured by the ratio of surface S1PR1–GFP to S1PR5<sup>-/-</sup>–RFP on the sensor-expressing T cells, while SPHK-KO iMo did not (Fig. 2g). We also tested whether supernatant from iMo could induce S1P-dependent chemotaxis. We transduced a B cell line with vector, S1PR1 or SIRP5. We plated the cells across a transwell from iMo. Vector-transduced cells did not migrate to iMo. S1PR1-transduced and SIRP5-transduced cells migrated to WT but not to SPHK-KO iMo, and their migration was blocked by a blocking antibody to S1P (Fig. 2h).
iMo require CD69 to supply S1P

We next asked what factors regulate the secretion of S1P by iMo. CD69 is expressed rapidly and robustly after activation by virtually all leukocytes, but the function of CD69 has remained unclear. Many phenotypes of CD69−/− mice may not be explained by the well-established interaction of CD69 with S1PR1 (refs. 11,12). To avoid potentially confounding factors in the experiments above, we adoptively transferred CD69−/− T cells to WT hosts. When we instead induced inflammation with pIC in CD69−/− mice, we did not observe downmodulation of S1PR1 on T cells (Fig. 2i, j, Extended Data Fig. 5a–h). This suggested that CD69 might be required for iMo to supply S1P.

To test whether iMo needed CD69 to supply S1P to T cells, we used mixed bone marrow chimaeras, similar to those described above. We lethally irradiated WT mice and reconstituted them with a 1:1 mix of CD69−/− bone marrow and CCR2−/−DTR bone marrow. When CCR2-positive cells no longer expressed CD69, S1PRI was no longer downmodulated on T cells in the dLN (Fig. 2k, Extended Data Fig. 5i–n). We also injected control and CD69−/− iMo into the lymph nodes of WT recipients, and found that control but not CD69−/− iMo induced S1PRI downmodulation on lymph node T cells (Fig. 2f, Extended Data Fig. 4a–f). Finally, we could not detect the secretion of S1P by CD69−/− iMo ex vivo (Fig. 2g, h, Extended Data Fig. 4g–k). These results were consistent with a requirement for CD69 expression by iMo to supply lymph node S1P.

We next addressed how CD69 enabled iMo to supply S1P to T cells. We performed RNA sequencing on CD69−/− and WT iMo sorted from the lymph nodes of mixed bone marrow chimaeras, so that they were taken from the same environment. Many genes were differentially expressed, and one change was in S1pr5, which was suppressed when iMo expressed CD69 (Extended Data Fig. 6a, k). We confirmed this change by quantitative PCR with reverse transcription (RT–qPCR) (Fig. 3a). We bred CD69−/−S1pr5−/− mice and found that loss of S1pr5 partially rescued T cell exposure to S1P (Fig. 3b, c). To explore the mechanism, we asked whether CD69 promoted the accumulation of iMo in the lymph nodes, potentially in part by blocking S1PR5-guided egress. However, we found no difference in the number of CD69−/− iMo compared to control iMo (Fig. 3d, Extended Data Fig. 6b, c). A second possibility was that CD69 might promote the positioning of iMo in the T cell zone, potentially in part by repressing S1PR5, which is known to hold natural killer cells in the lymph node periphery. We found that CD69−/− iMo poorly infiltrated the T cell zone, and their positioning was restored by loss of S1pr5 (Fig. 3e, Extended Data Fig. 6d, e). A third, not mutually exclusive, possibility was that CD69 promoted iMo S1P release. Part of the mechanism might be that S1PR5 on iMo ‘caught’ S1P secreted by iMo and prevented S1P from reaching nearby T cells. Indeed, the secretion of S1P by CD69−/− iMo was partially restored upon loss of S1pr5 (Fig. 3f, Extended Data Fig. 6f).

A cell line transduced with S1pr5 cleared S1P from culture medium more efficiently than controls, and CD69−/− iMo cleared S1P from culture medium more efficiently than CD69−/− S1pr5−/− iMo (Extended Data Fig. 6g–j).

iMo S1P regulates the course of EAE

Drugs that target S1P signalling are widely used to treat multiple sclerosis. We therefore assessed S1P gradients in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. We found reduced levels of surface S1PRI on T cells in the draining cervical lymph nodes at the onset of EAE symptoms, consistent with increased levels of S1P (Fig. 4a,b, Extended Data Figs. 7, 8a). iMo purified from the dLNs of mice with EAE secreted S1P ex vivo, and in mixed bone marrow chimaeras, downmodulation of S1PRI was lost when CCR2-positive cells could not make S1P or did not express CD69 (Fig. 4c, k, Extended Data Figs. 8b, c, 9a–f). At the same time point, we observed a substantial reduction in the number of total and MOG/Aβ-specific T helper 17 (TₐH17) and T follicular helper (TₐFH) cells in the dLNs, and a reduction in the number of TₐH17 cells in the central nervous system (few TₐH17 cells were in the central nervous system) (Fig. 4f, j, n, o, Extended Data Figs. 9g, h, 10a–g). The course of disease was delayed, despite being unusually aggressive as the mice were bone marrow chimaeras that were repeatedly treated with diphtheria toxin (Fig. 4d, e, l, m, Extended Data Fig. 10a).

Overall, our data indicate that the levels of S1P in the lymph nodes rise during an immune response. We used CD69−/− T cells as probes to avoid confounding effects of cell-intrinsic CD69-mediated S1PRI internalization, an interaction confirmed in this work (Extended Data Figs. 3m, 5l, 9b, f). However, most T cells express high levels of CD69 transiently, leaving a wide time window during which altered lymph node S1P could affect disease. This may occur early in an immune response; even after pIC injection, approximately 40% of CD4 T cells in the dLNs remained CD69+ (Extended Data Fig. 1j). This may also occur later in the immune response. At the onset of EAE symptoms, few lymphocytes expressed surface CD69, including MOG/Aβ-specific T cells (Extended Data Fig. 7a, b). The role of iMo in supplying lymph node S1P was unexpected, and the retention of T cells may synergize...
with the roles of iMo in antigen presentation and cytokine production. Lipopolysaccharide induces the activation of S1P1 in the liver, which requires haematopoietic S1P; we hypothesize that this is due to the infiltration of iMo. Our data also indicate that the early activation marker CD69 retains cells in an inflamed environment by regulating both the gradients and the receptors of SIP. Finally, this study raises the question of how S1P derived from iMo regulates the number of Tfh and Th17 cells. There are many possibilities, but several studies favor the possibility that residence time in lymph nodes regulates differentiation, and future work will address this 

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2. Olivera, A., Allende, M. L. & Proia, R. L. Shaping the landscape: metabolic regulation of S1P gradients. Biochim. Biophys. Acta 1831, 193–202 (2013).
3. Yanagida, K. & Hla, T. Vascular and immunobiology of the circulatory sphingosine 1-phosphate gradient. Annu. Rev. Physiol. 79, 67–91 (2017).
4. Liu, C. H. et al. Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. Mol. Biol. Cell 10, 1179–1190 (1999).
5. Bankovich, A. J., Shiow, L. R. & Cyster, J. G. CD69 suppresses sphingosine 1-phosphate receptor-1 (S1P1) function through interaction with membrane helix 4. J. Biol. Chem. 285, 22328–22337 (2010).
6. Shiow, L. R. et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature 440, 540–544 (2006).
7. Fang, V. et al. Gradients of the signaling lipid S1P in lymph nodes position natural killer cells and regulate their interferon-γ response. Nat. Immunol. 10, 1179–1190 (2009).
8. Ramos-Perez, W. D., Fang, V., Escalante-Alcalde, D., Cammer, M. & Schwab, S. R. A map of the distribution of sphingosine 1-phosphate in the spleen. Nat. Immunol. 16, 1245–1252 (2015).
9. Lo, C. G., Xu, Y., Proia, R. L. & Cyster, J. G. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J. Exp. Med. 201, 291–301 (2005).
10. Jakubzick, C. V., Randolph, G. J. & Henson, P. M. Monocyte differentiation and antigen-presenting functions. Nat. Rev. Immunol. 17, 349–362 (2017).
11. Hohl, T. M. et al. Inflammatory monocytes facilitate adaptive CD4+ T cell responses during respiratory fungal infection. Cell Host Microbe 6, 470–481 (2009).
12. Geissmann, F. et al. Development of monocytes, macrophages, and dendritic cells. Science 327, 656–661 (2010).
13. Ramos-Perez, W. D., Fang, V., Escalante-Alcalde, D., Cammer, M. & Schwab, S. R. A map of the distribution of sphingosine 1-phosphate in the spleen. Nat. Immunol. 16, 1245–1252 (2015).
14. Lo, C. G., Xu, Y., Proia, R. L. & Cyster, J. G. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J. Exp. Med. 201, 291–301 (2005).
15. Jakubzick, C. V., Randolph, G. J. & Henson, P. M. Monocyte differentiation and antigen-presenting functions. Nat. Rev. Immunol. 17, 349–362 (2017).
16. Hohl, T. M. et al. Inflammatory monocytes facilitate adaptive CD4+ T cell responses during respiratory fungal infection. Cell Host Microbe 6, 470–481 (2009).
17. Cibrián, D. & Sánchez-Madrid, F. CD69 from activation marker to metabolic gatekeeper. Eur. J. Immunol. 47, 946–953 (2017).
18. Kimura, M. Y. et al. Crucial role for CD69 in allergic inflammatory responses: CD69–Myδ system in the pathogenesis of airway inflammation. Immunol. Rev. 278, 87–100 (2017).
19. Xiong, H. & Pamer, E. G. Monocytes and infection. modulator, messenger and effector. Immunobiology 220, 210–214 (2015).
20. Geissmann, F. et al. Development of monocytes, macrophages, and dendritic cells. Science 327, 656–661 (2010).
21. Kono, M. et al. Sphingosine-1-phosphate receptor 1 reporter mice reveal receptor activation sites in vivo. J. Clin. Invest. 124, 2076–2086 (2014).
22. Lee, J. Y. et al. The transcription factor KLF2 restrains CD4+ T follicular helper cell differentiation. Immunity 42, 252–264 (2015).
23. Garris, C. S., Blaho, V. A., Hla, T. & Han, M. H. Sphingosine-1-phosphate receptor 1 signalling in T cells: trafficking and beyond. Immunology 142, 347–353 (2014).
24. Hatcher, S. E., Waubant, E., Nourbaksh, B., Crabtree-Hartman, E. & Graves, J. S. Rebound syndrome in patients with multiple sclerosis after cessation of fingolimod treatment. JAMA Neurol. 73, 790–794 (2016).

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HEK-293T cells were not authenticated or tested for mycoplasma. S1PR1-IRES–GFP group and 13% of total CD45+ in the IRES–GFP group. To generate bone marrow chimaeras, recipients were lethally irradiated by two doses of 6.6 Gray. Experiments previously described9,25 (retroviral backbone: MSCV2.2; packaging cell lines approved by the New York University Institutional Animal Care and Use Committee.

For FTY720 treatment, mice were injected intraperitoneally (i.p.) with 2 mg/kg FTY720 (Cayman Chemical) 12 h before analysis.

For 4-deoxypyridoxine treatment, mice received 30 mg/l 4-deoxypyridoxine (Sigma) and 1 g/l sucrose or 1 g/l sucrose alone in the drinking water from day 4 after EAE induction until analysis. To deplete CCR2+ cells, mice were treated with diphtheria toxin (Sigma), reconstituted at 1 mg/ml in PBS and frozen at −80 °C. Mice received 10 ng/g diphtheria toxin i.p. in 0.2–0.3 ml PBS. For pIC injection experiments, mice were treated 2 days before and on the day of piC treatment. For EAE experiments, mice were treated on the day of MOG immunization and every 3 days after.

To induce EAE, mice were immunized with 450 μg MOG 35–55 peptide in PBS emulsified 1:1 in incomplete Freund’s adjuvant (BD Biosciences) supplemented with a final concentration of 5 mg/ml of Mycobacterium tuberculosis (H37Rv; BD Biosciences). In each of three locations, 100 μl volume was injected s.c. Pertussis toxin (200 ng in 100 μl PBS; List Biological Laboratories) was injected i.p. on the day of immunization (day −1) and 2 days later (day 1). Animals were scored for clinical symptoms as follows: 0, no signs of disease; 1, decreased tail tone or flaccid tail; 2, weakness in the limbs and loss of righting reflex; 3, inability to move one or both hindlimbs, and urinary incontinence; 4, weakness of both forelimbs and hindlimbs, complete hindlimb paralysis, and atomic bladder; 5, moribund.

Methods

Confocal microscopy of tissue sections

Mice were lethally anaesthetized and perfused with 1% PFA in PBS. Organs were fixed in 4% PFA in PBS for 1 h at room temperature (22–25 °C) with gentle shaking; dehydrated overnight in 30% sucrose in PBS at 16 °C with gentle shaking; embedded in OCT (Sakura); and snap-frozen in dry-ice-cold 2-methylbutane. Sections (8–14 μm in thickness) were cut, fixed with ice-cold acetone for 10 min and air-dried. All staining was performed at room temperature (22–25 °C) in a humidified chamber.

For FTY720 treatment, mice were injected subcutaneously (s.c.) in the footpad or flank with 10 μg polyIC (GE Healthcare).

For FTY720 treatment, mice were injected intraperitoneally (i.p.) with 2 mg/kg FTY720 (Cayman Chemical) 12 h before analysis.

For 4-deoxypyridoxine treatment, mice received 30 mg/l 4-deoxypyridoxine (Sigma) and 1 g/l sucrose or 1 g/l sucrose alone in the drinking water from day 4 after EAE induction until analysis. To deplete Ly6C/G+ cells, mice were injected i.p. with 500 μg anti-Ly6C/G (clone RB6-8C5; BioXCell) 2 days before and on the day of piC treatment. To deplete Ly6C/G+ cells, mice were injected i.p. with 100 μg monoclonal antibody to integrin α4 (clone PS/2; BioXCell) and 100 μg monoclonal antibody to integrin α5 (clone M17/4; BioXCell).

For integrin blockade, mice were injected i.v. with 100 μg monoclonal antibody to integrin α4 (clone PS/2; BioXCell) and 100 μg monoclonal antibody to integrin α5 (clone M17/4; BioXCell). For integrin blockade, mice were injected i.v. with 100 μg monoclonal antibody to integrin α4 (clone PS/2; BioXCell) and 100 μg monoclonal antibody to integrin α5 (clone M17/4; BioXCell). For integrin blockade, mice were injected i.v. with 100 μg monoclonal antibody to integrin α4 (clone PS/2; BioXCell) and 100 μg monoclonal antibody to integrin α5 (clone M17/4; BioXCell). For integrin blockade, mice were injected i.v. with 100 μg monoclonal antibody to integrin α4 (clone PS/2; BioXCell) and 100 μg monoclonal antibody to integrin α5 (clone M17/4; BioXCell).
final concentration of 1.25 μg/ml; these sections were blocked with the avidin/biotin blocking kit from Vector Laboratories, and stained with streptavidin-BV421 and anti-CD4-Alexa Fluor 488 (RM 4-5, BioLegend) with a final concentration of 1.25 μg/ml.

Slides were mounted with G-Fluoromount (Southern Biotech). Slides were visualized using a Zeiss 710 inverted confocal microscope with a x25, or x63 oil-immersion objective and ZEN 2010 software. Images were processed with ImageJ v1.49. For all direct comparisons, samples were stained and imaged the same day with the same settings. The ratio of surface GFP to RFP for SIP sensor mice was analysed as previously described. The ImageJ macro to localize iMo is included as Supplementary Information.

Confocal microscopy of SIP sensor T cells

Transwell-cultivated T cells were cytospun at 800g for 6 min though a cytology funnel onto a slide (Thermo Scientific Shandon Coated Cytoslide). Then, cells were fixed in 4% PFA for 15 min at room temperature, fixed with ice-cold acetone for 2 min, air-dried, permeabilized for 5 min with 0.5% Triton X-100 in PBS and stained and analysed as above (without Lyve1).

Cell preparation for adoptive transfer, in vitro culture and flow cytometry

CD69-KO lymphocyte preparation for adoptive transfer. Lymphocytes were isolated from lymph nodes (axillary, brachial, inguinal, cervical and para-aortic) and in some cases, the spleen, by mechanical disruption and filtration through a 70-μm cell strainer. Red blood cells were lysed with ACK buffer. Cells were enumerated with a cell counter (Beckman Coulter Multisizer 3) set to detect nuclei between 3.5 and 7 μm. When necessary, the cells were stained with CFPSE before injection. For CFSE staining, lymphocytes were resuspended in PBS at 20 × 10^6 cells/ml and CFSE was added to a final concentration of 2 μM. Cells were incubated for 10 min at room temperature, and the reaction was stopped by adding FBS to a final concentration of 20% and incubating for 2 min. Labelled cells were washed three times in PBS before counting. Cells (12–16 × 10^6) were injected i.v.

CD69-KO lymphocyte preparation for cell culture. Cells were prepared as for adoptive transfer, but CD69-KO T lymphocytes were further purified by negative selection for CD4 and CD8 using anti-CD11b, anti-CD11c, anti-NKp46 and anti-CD19 biotinylated antibodies (biotin-negative selection kit, Stem Cell Technologies or eBioscience; used according to the manufacturer’s instructions).

iMo preparation for adoptive transfer or culture. Mice were injected i.v. with 200 μg pIC or PBS. Fourteen hours later, mice were euthanized. Organs were minced, and then digested with collagenase IV (1 mg/ml; Sigma) and DNase I (0.2 mg/ml; Roche) in HBSS. Collagenase IV was inactivated by washing with 5 mM EDTA and 3% FBS in PBS. The cell suspension was filtered through a 70-μm cell strainer. Further purification of lymphocytes was performed by density gradient centrifugation using 40% Percoll (GE Healthcare).

Cell preparation for flow cytometry: lymphoid organs. Lymphocytes were isolated from lymph nodes (axillary, brachial, inguinal, cervical and para-aortic) and in some cases, the spleen, by mechanical disruption and filtration through a 70-μm cell strainer. Cells were enumerated with a cell counter (Beckman Coulter Multisizer 3) set to detect nuclei between 3.5 and 7 μm.

Cell preparation for flow cytometry: CNS. After euthanasia, mice were perfused with PBS. The spinal cord and brain were minced, and then digested for 30 min at 37 °C with gentle rocking with collagenase IV (1 mg/ml; Sigma) and DNase I (0.2 mg/ml; Roche) in HBSS. Collagenase IV was inactivated by washing with 5 mM EDTA and 3% FBS in PBS, and the cells were filtered through a 70-μm cell strainer. Further purification of lymphocytes was performed by density gradient centrifugation using 40% Percoll (GE Healthcare).

In vitro cell culture

Cell line generation and culture. WEHI-231 cells (a gift from J. Cyster; ATCC CRL-1702) were transduced with retrovirus encoding murine S1PR1-IRES–GFP, S1PR5-IRES–GFP or IRES–GFP alone (retroviral backbone: MSCV2.2; packaging cell line: HEK-293T (ATCC CRL-11268)). The retroviral constructs have been previously described. GFP+ cells were sorted and maintained in complete RPMI 1640 (with 10% FBS, penicillin–streptomycin, 10 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids). Cells were maintained between 0.1 × 10^6 and 1 × 10^6 cells/ml. The cell lines were not authenticated. WEHI-231 cells were contaminated with mycoplasma; HEK-293T cells were not tested for mycoplasma.

Transwell migration. WEHI-231 cells (0.5 × 10^5) expressing the indicated constructs were tested for transmigration across 96-well uncoated 5-μm transwell filters (Corning Costar) to 0.2 × 10^6 sorted iMo resuspended in 148 μl with or without 10 μg/ml anti-SIP antibody clone LT1002 (Echelon Bioscience). After 3 h, migrated cells were enumerated by flow cytometry. The assay was performed in RPMI 1640 supplemented with 0.5% fatty acid-free BSA (Calbiochem), penicillin–streptomycin, 10 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

SIPRI downregulation. Purified CD69-KO SIP sensor T cells (or CD69-KO T cells, 0.1 × 10^6; Extended Data Fig. 4j, k) were cultured across a 96-well 0.4-μm transwell filter (Corning Costar) from 0.2 × 10^6 sorted iMo in 150 μl. Cells were incubated for 8 h (or 12 h for Extended Data Fig. 4j, k) in RPMI 1640 supplemented with 0.5% fatty acid BSA, penicillin–streptomycin, 10 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

SIP clearance. Sorted iMo (0.2 × 10^6) or WEHI-231 cells (0.5 × 10^5) were incubated with 5 h with 50 nM SIP (Sigma) in 150 μl RPMI 1640 supplemented with 0.5% fatty acid-free BSA (Calbiochem), penicillin–streptomycin, 10 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Then, 0.1 × 10^6 purified CD69-KO SIP Sensor T cells were cultured for 2 h across a 96-well 0.4-μm transwell filter (Corning Costar) from these cells.

Flow cytometry

For intracellular staining, cells were fixed and permeabilized with the FOXP3 kit from eBioscience, according to the manufacturer’s instructions. Staining for SIPRI was done on ice in PBS supplemented with 0.05% sodium azide, 1 mM EDTA and 0.5% FBS. Cells were stained for 90 min with anti-mouse SIPRI (7.2 μg/ml; MAB7089, R&D Systems), washed twice in buffer, stained for 45 min with anti-rat IgG-biotin F(ab′)2 (9.5 μg/ml; 2340649, Jackson Immunoresearch), washed twice in buffer and stained with streptavidin coupled with APC or PECy7 and the other surface membrane antibodies. To stain with the
MOGA<sup>A</sup> (GWYRSPFSRVVH) and hCLIP<sup>A</sup> (PVSKMRRMATPLLMQA) tetramers, cells were incubated for 60 min at room temperature with 7.5 μg/ml tetramer (NIH Tetramer Core Facility). Additional antibodies are described in Supplementary Table 1. Cells were analysed on a BD Biosciences LSRII flow cytometer running FACSDiva v8.02, and FlowJo software v9 or v10 was used for data analysis (including t-SNE plots).

RNA sequencing
Total RNA was extracted from sorted cell populations using TRIzol (Invitrogen) according to the manufacturer’s instructions. Phenol was removed using a Qiagen MiniElute Cleanup Kit, according to the manufacturer’s instructions. The quantity and quality of total RNA was assessed on a 2100 BioAnalyzer instrument (Agilent Technologies, Inc.). Of the total RNA, 1 μg was used to prepare libraries using the Trio RNA-Seq library prep kit (mammalian rRNA Deplete; part number 0506-96, Tecan Genomics, Inc.) following the manufacturer’s instructions (https://www.nugen.com/sites/default/files/M01440v2_User_Guide%3A_Trio_RNA-Seq_4270.pdf). Briefly, the library preparation consisted of the following steps: DNase treatment to remove genomic DNA, first strand and second stand cDNA synthesis from the input RNA, single primer isothermal amplification of the resultant cDNAs, enzymatic fragmentation and construction of unique barcoded libraries, PCR library amplification (for these samples, four cycles were used) and a final step to remove rRNA transcripts. The Agencourt AMPure XP bead (Beckman Coulter) purified libraries were quantified using qPCR and the size distribution was checked using Agilent TapeStation 2200. The libraries were subjected to paired-end 50-bp sequencing on HiSeq 2500 sequencing system (llumina, v4 chemistry). For the RNA sequencing analysis, sequencing reads were mapped to the mouse reference genome (GRCm38.85/mm10) using the STAR aligner (v2.5.0c)alignment. Alignments were guided by a Gene Transfer Format file. The mean read insert sizes and their standard deviations were calculated using Picard tools (v1.126) (http://broadinstitute.github.io/picard). The read count tables were generated using HTSeq (v0.6.0)alignment, normalized based on their library size factors using DEseq2<sup>34</sup>, and differential expression analysis was performed. The read per million normalized BigWig files were generated using BEDTools (v2.17.0)alignment and bedGraphToBigWig tool (v4). All downstream statistical analyses and generating plots were performed in R environment (v3.1.1) (http://www.r-project.org/).

RT-qPCR
Total RNA was extracted from sorted cell populations using TRIzol (Invitrogen) according to the manufacturer’s instructions. Before reverse transcription, RNA was treated with DNase I (Invitrogen). The RNA was converted to cDNA with the Superscript III First Strand Synthesis System from Invitrogen according to the manufacturer’s instructions, using a mix of oligo dT and random hexamers as primers. qPCR was performed on a Roche Light-Cycler 480 using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions, using a mix of oligo dT and random hexamers as primers. qPCR was performed in parallel for each sample–primer pair. To control for nonspecific amplification, the size of the reaction products was analysed by agarose gel electrophoresis. Primer pairs were tested for linear amplification over two orders of magnitude.

Wright–Giemsa stain
Freshly sorted CD11b<sup>+</sup>Ly6C<sup>+</sup> cells were cytospun onto a slide for 6 min at 800g (Thermo Scientific Shandon Coated Cytoslide). Slides were fixed for 1 min with ice-cold methanol and dried completely. Wright–Giemsa stain (Sigma) was performed for 30 s, washed in PBS for 5 min, washed in water for 1 min twice and dried overnight. A coverslip was affixed using Permount (SP15-100, Fisher). Purple shows nuclei, and blue-to-light pink shows the cytoplasm.

Statistical analysis
Graphpad Prism v.8.0.1 and v.9.0.0 were used for Mann–Whitney two-tailed t-test and two-way ANOVA with Geisser–Greenhouse correction.

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

Data availability
RNA sequencing data are available from the NIH/NCBI as Gene Expression Omnibus data set GSE139006. All other data will be available from the authors on reasonable request. Source data are provided with this paper.

Code availability
The ImageJ macro used for the localization of iMo is provided in the Supplementary Information.

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Author contributions A.B. designed, performed and conducted all of the experiments, analysed and interpreted the data, and wrote the manuscript. S.B. and V.S.C. performed the experiments. A.K.-J. analysed the RNA sequencing data. M.C. analysed the imaging data. S.R.S. designed the experiments, interpreted the data and wrote the manuscript.

Competing interests The authors declare no competing interests.

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Extended Data Fig. 1 | S1P increases in the dLN after pIC injection. a, CD69-KO lymphocytes (from LN) were incubated ex vivo with the indicated concentrations of S1P for 16h, and surface SIPRI on CD4+ T cells was measured by flow cytometry. Average of triplicates +/- SEM in 1 experiment. b–i, Experiment design and animals as in Fig. 1a–c. Compilation of 4 experiments for all panels except (h), which compiles 3 experiments. b, Absolute MFI of SIPRI for the cells shown in Fig. 1c. PBS (n = 8); pIC (n = 9). c, Percent CD69+ among endogenous (WT) CD4+ T cells in dLN. PBS (n = 8); pIC (n = 11). d, Relative number of the indicated CD4+ T cells in dLN. The number of endogenous CD4+ cells in the dLN of each mouse was divided by the mean number of endogenous CD4+ cells in the PBS-treated group; the number of transferred CD69-KO CD4+ cells in each mouse was similarly divided by the mean number of transferred CD69-KO CD4+ cells in the PBS-treated group. PBS (n = 9); pIC (n = 11). e, (left) Representative dot plots of CD69 vs SIPRI on endogenous CD4 T cells in PBS- or pIC-treated mice. (right) Representative of the data compiled in (f). f, The SIPRI MFI of endogenous CD69high or endogenous CD69low CD4+ cells in each mouse divided by the mean SIPRI MFI of endogenous CD69low CD4+ cells in the PBS-treated group. PBS (n = 8); pIC (n = 9). g, Representative histograms of surface SIPRI on endogenous (WT) CD69low or transferred CD69-KO CD8+ T cells in the dLN. FTY720-treated mouse served as a negative control. h, The SIPRI MFI of the transferred CD69low CD8+ cells in each mouse was divided by the mean SIPRI MFI of the endogenous CD69low CD8+ cells in the PBS-treated group; the SIPRI mean fluorescence intensity (MFI) of the transferred CD69-KO CD8+ cells in each mouse was similarly divided by the mean MFI of the transferred CD69-KO CD8+ cells in the PBS-treated group. PBS (n = 7); pIC (n = 6). i, Relative number of the indicated CD8+ T cells in the dLN. PBS (n = 9), pIC (n = 9). j, Experiment design as in Fig. 1a. Percent CD69+ among total CD4+ (n = 11), Treg (CD4+ Foxp3+) (n = 9), CD8+ (n = 12), NK (NK1.1+CD3-) (n = 5), and NKT (NK1.1+CD3+) (n = 5) cells in the dLN of pIC-treated mice. Compilation of 4 experiments. k, CD69-KO Sensor+ T cells were transferred i.v. into WT recipients. 24h later, mice were treated s.c. with PBS or pIC. 14h later, dLN were analysed by confocal microscopy. Representative section, showing T zone, B follicles, and subcapsular sinus (SCS). Arrows indicate transferred cells. Scale bar, 100 μm. Representative of 5 experiments, PBS n = 7, pIC n = 8. Data are presented as mean values +/- SEM. Mann–Whitney two-tailed t-test.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | S1P increases in the dLN of BM chimaeras after pIC injection. (a–j) C57BL/6 mice were lethally irradiated, reconstituted with a 1:1 mix of CD45.2 WT and CD45.1 CD69-KO BM, and allowed to recover for 12–16 weeks. The chimaeras were injected s.c. with PBS or pIC, and dLN analysed 14 h later. Compilation of 3 (for CD8 analysis)–4 (for CD4 analysis) experiments. **a**, Experiment design. **b**, S1PR1 on WT CD69low (left) and CD69-KO (right) CD4+ T cells. **c**, Compilation. The S1PR1 MFI of the WT CD69low CD4+ T cells in each mouse was divided by the mean S1PR1 MFI of the WT CD69low CD4+ T cells in the PBS-treated group; the S1PR1 MFI of the CD69-KO CD4+ T cells in each mouse was similarly divided by the mean MFI of the CD69-KO CD4+ T cells in the PBS-treated group. PBS (n = 11), pIC (n = 13). **d**, Percent CD69+ among WT CD4+ T cells. PBS (n = 11), pIC (n = 13). **e**, Relative number of the indicated CD4+ T cells in the dLN. The number of CD4+ WT cells in the dLN of each mouse was divided by the mean number of CD4+ WT cells in the PBS-treated group; the number of CD69-KO CD4+ cells in each mouse was similarly divided by the mean number of CD69-KO CD4+ cells in the PBS-treated group. PBS (n = 9), pIC (n = 11). **f**, S1PR1 expression on CD69low WT or CD69-KO CD8+ T cells. **g**, Compilation, as in (c). PBS (n = 9), pIC (n = 10). **h**, Percent CD69+ among WT CD8+ T cells. PBS (n = 9), pIC (n = 10). **i**, Relative number of the indicated CD8+ T cells, as in (e). PBS (n = 9), pIC (n = 10). **j**, RT-qPCR for S1pr1, normalized to Hprt. Compilation of 3 experiments. PBS (n = 9), pIC (n = 9). **k**, RT-qPCR for Sphk1 in bone marrow of pIC-treated Sphk1f/f Sphk2−/− (n = 5 animals, bone marrow cells split into 2 samples before RNA purification), pIC-treated Sphk1f/f Sphk2−/−Mx1-Cre− (n = 7 animals, bone marrow cells split into 2 samples before RNA purification), and Sphk1−/− (n = 2 animals). Compilation of 2 experiments. **l**, RT-qPCR for Sphk2 in blood of pIC-treated Sphk1f/f Sphk2−/−Mx1-Cre− (n = 4) and pIC-treated Sphk1f/f Sphk2−/−Mx1-Cre− (n = 4) animals. One experiment. **m**–r, UBC-GFP+ mice were lethally irradiated, reconstituted with pIC-treated Sphk1f/f Sphk2−/− Mx1-Cre− CD45.2 (SphK-KO) or littermate control (LitCtl) BM, and left to recover for 12–14 weeks. Chimaeras received CD45.1 CD69-KO T cells i.v. 24 h later, chimaeras were injected s.c. with pIC or PBS. 14 h later, dLN were analysed. **m**, S1PR1 on CD69-KO CD8+ T cells in LitCtl chimaeras (left) or SphK-KO chimaeras (right). FT720-treated mouse served as a negative control. **n**, Compilation of 3 experiments. The S1PR1 MFI on the CD69-KO CD8+ T cells in each mouse was divided by the mean S1PR1 MFI on the CD69-KO CD8+ T cells in the PBS-treated group. LitCtl (PBS n = 7, pIC n = 9); SphK-KO (PBS n = 7, pIC n = 7). **o**, Percent CD69+ among CD4+ T cells. Compilation of 3 experiments. LitCtl (PBS n = 7, pIC n = 9); SphK-KO (PBS n = 7, pIC n = 8). **p**, Percent CD69+ among CD8+ T cells. Compilation of 3 experiments. LitCtl (PBS n = 7, pIC n = 9); SphK-KO (PBS n = 7, pIC n = 7). **q**, r, CD69-KO CD45.1 lymphocytes were transferred into LitCtl or SphK-KO BM chimaeras. 24 h later, the chimaeras were injected s.c. with pIC. 14 h later, half of the mice in each group were euthanized and the cells in the dLN were counted. The remaining mice were injected i.v. with anti-αL and anti-α4 neutralizing antibodies. These antibodies blocked any further lymphocyte entry into the LN. 4 h later, these mice were euthanized and the cells in the dLN were counted. The decline in cell numbers in the LN over 4 h, with no further cell entry, indicated the exit rate. Compilation of 3 experiments. SphK-KO (t = 0 h n = 8; t = 4 h n = 8), LitCtl (t = 0 h n = 6, t = 4 h n = 8). **q**, Percent exit of CD69-KO CD8+ T cells. **r**, Percent exit of endogenous CD69low CD8+ T cells. Each point represents one mouse at t = 4 h relative to the average at t = 0. Data presented as mean values +/− SEM. Mann–Whitney two-tailed t-test.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | CD11b+Ly6ChiCCR2+ cells contribute to increased LN S1 P. a, Phenotype of cells infiltrating the dLN in WT mice 14h after pIC injection. Representative of 5 experiments. b, Phenotype of sorted iMo. Left panel, sort gate (Ly6ChiCD11bhi). Right top panel, staining of sorted cells for Ly6G and CCR2. Representative of 3 experiments. Right bottom panel, Wright-Giemsa stain of sorted cells. Representative of 2 experiments. c–h, C57BL/6 mice were injected i.p. with a depleting anti-Ly6C/G antibody on d0 and d2, or left untreated. On d1, the mice received CD69-KO CD45.1+ lymphocytes i.v. On d2, the mice were treated s.c. with PBS or pIC, and dLN were analysed 14h later. Compilation of 3 experiments. c, Representative flow cytometry plots. d, Number of iMo in the dLN (PBS n = 4, pIC n = 6, pIC anti-Ly6C/G n = 7). e, S1PR1 on CD69-KO CD8+ T cells. f, Compilation (PBS n = 4, pIC n = 5, pIC anti-Ly6C/G n = 7). g, Percent CD4+ T cells that were CD69+ (PBS n = 6, pIC n = 5, pIC anti-Ly6C/G n = 8). h, Percent CD8+ T cells that were CD69+ (PBS n = 4, pIC n = 5, pIC anti-Ly6C/G n = 7). i–m, Lethally irradiated C57BL/6 mice were reconstituted with a 1:1 mix of the indicated BM, and analysed 12-16 weeks later. On d0 and d2, the chimaeras were treated with DT. On d1, the chimaeras received CD69-KO CD45.1+ lymphocytes i.v. On d2, the chimaeras were injected s.c. with PBS or pIC, and 14h later the dLN were analysed. i, Percent of total CD45+ cells contributed by each genotype in dLN of the indicated chimaeras. Compilation of 5 experiments. LitCtl:LitCtl (PBS n = 6; pIC n = 6); CCR2 DTR:LitCtl (PBS n = 6, pIC n = 6). j, Number of iMo in the dLN of the indicated chimaeras. Compilation of 3 experiments. LitCtl:LitCtl (PBS n = 6, pIC n = 6); CCR2 DTR:LitCtl (PBS n = 6, pIC n = 4); LitCtl:SPHK-KO (PBS n = 4, pIC n = 4); CCR2 DTR:SPHK-KO (PBS n = 6, pIC n = 5). k, Representative histograms of S1PR1 expression on CD69-KO CD4+ T cells in the indicated chimaeras. l, Representative histograms of S1PR1 in the indicated chimaeras (top) and compilation (bottom) of S1PR1 expression on CD69-KO CD8+ T cells in the indicated chimaeras. Compilation of 3 experiments. LitCtl:LitCtl (PBS n = 5, pIC n = 5); CCR2 DTR:LitCtl (PBS n = 4, pIC n = 3); LitCtl:SPHK-KO (PBS n = 4, pIC n = 3); CCR2 DTR:SPHK-KO (PBS n = 5, pIC n = 6). m, Representative histograms (top) and compilation (bottom) of S1PR1 expression on endogenous CD4 T cells in the indicated chimaeras. For the compilation, the S1PR1 MFI of the CD69hiCD4+ T cells in each mouse in the PBS-treated group was divided by the mean S1PR1 MFI of the CD69hiCD4+ T cells in each mouse in the pIC-treated group; the S1PR1 MFI of the CD69loCD4+ T cells in each mouse of the pIC-treated group was similarly divided by the mean MFI of the CD69hiCD4+ T cells in the PBS-treated group. LitCtl:LitCtl (PBS n = 7, pIC n = 6); CCR2 DTR:LitCtl (PBS n = 6, pIC n = 6); LitCtl:SPHK-KO (PBS n = 4, pIC n = 4); CCR2 DTR:SPHK-KO (PBS n = 8, pIC n = 12). Compilation of 5 experiments. Bars represent mean +/- s.e.m. Mann-Whitney two-tailed t-test.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | iMo are sufficient to supply LN S1P. a–f, C57BL/6 mice received CD69-KO CD45.1+ lymphocytes i.v. 1d later, the mice received an intra-LN injection of Ly6G<sup>hi</sup>CD11b<sup>+</sup> iMo sorted from LN of mixed BM chimaeras (WT+CD69-KO or WT+SPHK KO). Alternately, mice were injected intra-LN with PBS (sham), injected s.c. with pIC, or left untreated. 14h later, the injected LN were analysed. Compilation of 3 experiments, untreated (n = 6); sham (n = 6); pIC (n = 8); iMo WT (n = 6); iMo SPHK-KO (n = 9); iMo CD69-KO (n = 7).
a, Representative dot plots (gated on live single cells) showing CD11b<sup>+</sup>Ly6G<sup>hi</sup> iMo in the injected LN. b, Number of iMo in the injected LN.
c, Percent CD69<sup>+</sup> among CD4<sup>+</sup> T cells in the indicated mice. d, S1PR1 on CD69<sup>−</sup>KO CD4<sup>+</sup> T cells in the indicated mice.
e, S1PR1 on CD69<sup>−</sup>KO CD4<sup>+</sup> T cells in the indicated mice. f, Compilation of S1PR1 on CD69<sup>−</sup>KO CD4<sup>+</sup> T cells in the indicated mice.
g, CD69-KO S1P sensor<sup>+</sup> T cells were cultured for 8h across a transwell from media or the indicated Ly6G<sup>hi</sup>CD11b<sup>+</sup> iMo sorted from LN of pIC-treated mixed BM chimaeras (either WT:CD69-KO or WT:SPHK KO). Sensor<sup>+</sup> cells were analysed by confocal microscopy. Images representative of cells quantified in Fig. 2g, scale bar 5μm.
h, i, CD69-KO Sensor<sup>+</sup> T cells were cultured for 8h across a transwell from media alone or Ly6G<sup>hi</sup>CD11b<sup>+</sup> iMo sorted from bone marrow of PBS-treated mice, spleen of PBS-treated mice, or LN of pIC-treated mice. Sensor cells were analysed by confocal microscopy. h, Representative images of the cells quantified in (i). Scale bar, 5μm. i, Quantification of S1P reporting, as in Fig. If. Compilation of 3 experiments. Each point is the ratio of surface GFP:RFP on one cell (media n = 26, iMo BM n = 60, iMo spleen n = 99, iMo LN n = 75).
j, k, CD69-KO T cells were cultured across a transwell from media or sorted iMo from LN of pIC-treated WT, SPHK-KO, or CD69-KO mice. After 12h, surface S1PR1 on the CD69-KO CD4<sup>+</sup> T cells was analysed by flow cytometry. j, Experiment diagram and representative histograms. k, Compilation of 5 experiments. Each symbol represents one well relative to the average of the media control wells. Media n = 7, iMo WT n = 11, iMo SPHK-KO n = 4, iMo CD69-KO n = 7. Data presented as mean values +/- SEM. Mann–Whitney two-tailed t-test.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | iMo require CD69 to supply S1P to T cells in the dLN.

a, CFSE-labelled CD69-KO or littermate control CD69<sup>low</sup> CD4 T cells were transferred i.v. into WT mice. The mice were injected s.c. with pIC or PBS, and the dLN were analysed 14h later. a, S1PR1 on the transferred cells, representative of the compilation in (b). b, Compilation of 3 experiments. LitCtl (PBS n = 6, pIC n = 6); CD69-KO (PBS n = 6, pIC n = 6). c, CFSE-labelled labelled CD69-KO or littermate control CD69<sup>low</sup> CD4 T cells were transferred i.v. into CD69-KO mice. The mice were injected s.c. with pIC or PBS, and the dLN were analysed 14h later. e, S1PR1 on the transferred cells. d, Compilation of 3 experiments. LitCtl (PBS n = 6, pIC n = 6); CD69-KO (PBS n = 6, pIC n = 6).

e, CD69-KO or littermate control mice were injected s.c. with pIC or PBS, and the dLN were analysed 14h later. e, S1PR1 on CD8<sup>+</sup> T cells. Controls are gated on CD69<sup>low</sup> cells.

f, Compilation of 3 experiments. LitCtl:LitCtl (PBS n = 4, pIC n = 4); CCR2DTR:LitCtl (PBS n = 10, pIC n = 12); LitCtl:CD69-KO (PBS n = 7, pIC n = 9); CCR2DTR:CD69-KO (PBS n = 8, pIC n = 9).

g, Dot plot of CD69 on CD11b<sup>+</sup>Ly6C<sup>+</sup> iMo in the dLN of a WT mouse 14h after pIC injection. CD69-KO iMo served as a negative staining control.

h, Compilation of 4 experiments (n = 10).

i–n, Lethally irradiated C57BL/6 mice were reconstituted with a 1:1 mix of the indicated BM, and analysed 12-16 weeks later. On d0 and d2, the chimaeras were treated with DT to deplete CCR2-DTR<sup>+</sup> cells. On d1, the chimaeras received CFSE-labelled CD69-KO lymphocytes i.v. On d2, the chimaeras were injected s.c. with PBS or pIC, and 14h later dLN were analysed. i, Percent of total CD4<sup>+</sup> cells contributed by each genotype in dLN of the indicated chimaeras. Compilation of 3 experiments. LitCtl:LitCtl (PBS n = 4, pIC n = 4); CCR2DTR:LitCtl (PBS n = 6, pIC n = 8); LitCtl:CD69-KO (PBS n = 5, pIC n = 5); CCR2DTR:CD69-KO (PBS n = 5, pIC n = 6). j, Number of iMo in the dLN of the indicated chimaeras. Compilation of 3 experiments. LitCtl:LitCtl (PBS n = 7, pIC n = 6); CCR2DTR:LitCtl (PBS n = 7, pIC n = 7); LitCtl:CD69-KO (PBS n = 5, pIC n = 5); CCR2DTR:CD69-KO (PBS n = 5, pIC n = 6).

k, Representative histograms of S1PR1 expression on CD69<sup>+</sup>KO CD4<sup>+</sup> T cells in the indicated chimaeras. i, Representative histograms (top) and compilation (bottom) of S1PR1 expression on endogenous CD4<sup>+</sup> T cells in the indicated chimaeras. For the compilation, the S1PR1 MFI of the CD69<sup>+</sup> CD4<sup>+</sup> T cells in each mouse in the PBS-treated group was divided by the mean S1PR1 MFI of the CD69<sup>+</sup> CD4<sup>+</sup> T cells in the PBS-treated group; the S1PR1 MFI of the CD69<sup>+</sup> CD4<sup>+</sup> T cells in each mouse of the pIC-treated group was similarly divided by the mean MFI of the CD69<sup>+</sup> CD4<sup>+</sup> T cells in the PBS-treated group.

l, Representative histograms (top) and compilation (bottom) of S1PR1 expression on endogenous CD4<sup>+</sup> T cells in the indicated chimaeras (top), and compilation of 5 experiments (bottom). LitCtl:CD69-KO (PBS n = 7, pIC n = 9); CCR2DTR:CD69-KO (PBS n = 8, pIC n = 9).

m, Representative histograms of S1PR1 expression on endogenous CD69-KO CD4<sup>+</sup> T cells in the indicated chimaeras (top), and compilation of 2 experiments (bottom). LitCtl:CD69-KO (PBS n = 2, pIC n = 3); CCR2DTR:CD69-KO (PBS n = 4; pIC n = 6).

n, Representative histograms of S1PR1 expression on endogenous CD69-KO CD8<sup>+</sup> T cells in the indicated chimaeras (top), and compilation of 2 experiments (bottom). LitCtl:CD69-KO (PBS n = 2, pIC n = 3); CCR2DTR:CD69-KO (PBS n = 4; pIC n = 6). Data presented as mean values +/- SEM. Mann–Whitney two-tailed t-test.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Characterization of CD69-KO iMo. a, Volcano plot showing transcripts from congenically marked WT and CD69-KO iMo, sorted from dLN of mixed BM chimaeras (1:1 WT CD45.2:CD69-KO CD45.1 BM) 14h after pIC injection. b–d, CD69-KO mice were injected s.c. with PBS or pIC, and dLN were analysed 14h later. b, Representative t-SNE plots (12-colour flow cytometry). NK cells (CD3\(^{−}\)NKp46\(^{+}\)), B cells (CD19\(^{+}\)), CD3\(^{+}\)CD4\(^{+}\) T cells, CD3\(^{+}\)CD8\(^{+}\) T cells, plasmacytoid dendritic cells (pDC) (CD11c\(^{+}\)B220\(^{−}\)SiglecH\(^{+}\)), classical dendritic cells (DC) (CD11c\(^{+}\)SiglecH\(^{−}\)B220\(^{−}\)), neutrophils (CD11b\(^{+}\)Ly6G\(^{+}\)Ly6C\(^{−}\)CCR2\(^{−}\)), iMo (CD11b\(^{+}\)Ly6C\(^{−}\)CCR2\(^{−}\)Ly6G\(^{+}\)), and other CD11b\(^{+}\) cells (CD11b\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{−}\)) are shown. Representative of 2 experiments. c, Number of the indicated cells. Compilation of 5 experiments (some experiments did not include all 12 antibodies). iMo (PBS \(n = 11\), pIC \(n = 11\)); NK cells (PBS \(n = 4\), pIC \(n = 4\)); neutrophils (PBS \(n = 5\), pIC \(n = 5\)); other CD11b\(^{+}\) cells (PBS \(n = 9\), pIC \(n = 9\)); pDC (PBS \(n = 7\), pIC \(n = 6\)); DC (PBS \(n = 7\), pIC \(n = 6\)). d, LN section from a pIC-treated CCR2-RFP mouse, stained with antibodies to Ly6C (green) and CD11b (blue). Left image shows Ly6C and CD11b, right image shows CCR2-RFP (red) and CD11b, arrows indicate double-positive cells (Ly6C\(^{+}\)CD11b\(^{+}\) or CCR2-RFP CD11b\(^{+}\)). Scale bar 50 μm. Image representative of 2 experiments. e, Littermate control (or WT), CD69-KO and CD69-KO S1PR5-KO mice were injected with PBS or PIC s.c. 14h later, dLN were analysed by confocal microscopy. Inflammatory monocytes (arrows) were identified as CD11b\(^{+}\)Ly6C\(^{−}\). T zone, B follicles, and medulla (M) were distinguished by CD4 and Lyve1 staining. Scale bar, 50 μm. LN section representative of data compiled in Fig. 3e. f, CD69-KO SIP-sensor\(^{+}\) T cells were cultured for 12h across a transwell from media alone or the indicated Ly6C\(^{−}\)CD11b\(^{+}\) inflammatory monocytes sorted from LN of pIC-treated mixed BM chimaeras (WT with CD69-KO, or WT with CD69-KO S1PR5-KO). Reporter cells were analysed by confocal microscopy. Images representative of cells quantified in Fig. 3f. Scale bar, 10 μm. g, h, The indicated sorted iMo were cultured for 5h with 50 nM S1P. Then CD69-KO SIP-sensor\(^{+}\) T cells were added across a transwell from the iMo for 2h. As controls, the T cells were cultured for 2h across a transwell from media alone or media + 50 nM S1P. g, Experiment design and representative images of the cells quantified in (h). Scale bar, 5 μm. h, Quantification of S1P reporting, as in Fig. 2g. Compilation of 4 experiments. Media (\(n = 65\)), S1P (\(n = 89\)), iMo CD69-KO + S1P (\(n = 21\)), iMo CD69-KO SIPR5-KO + S1P (\(n = 114\)). i, j, WEHI-231 cells were transduced with S1pr5 or vector control. The S1pr5\(^{+}\) or control lines were cultured for 5h with 50 nM S1P. Then CD69-KO SIP-sensor\(^{+}\) T cells were added across a transwell from the WEHI-231 cultures for 2h. As controls, the T cells were cultured for 2h across a transwell from media with 50 nM S1P or media alone. i, j, Experiment design and images representative of cells quantified in (j). Scale bar, 5 μm. J, Quantification of S1P reporting, as in Fig. 2g. Compilation of 3 experiments. Empty vector (media \(n = 39\), S1P \(n = 61\)); S1PR5 (media \(n = 36\), S1P \(n = 49\)). k, RT-qPCR analysis of sorted iMo from LN of pIC-treated WT, CD69-KO, and Sphk-KO mice. Sorted CD4\(^{+}\) T cells from WT mice served as a negative control for Spns2. Compilation of 3 experiments for Sphk1 (iMo WT \(n = 4\), iMo CD69-KO \(n = 7\), iMo Sphk-KO \(n = 3\) mice). Compilation of 4 experiments for Sphk2 (iMo WT \(n = 6\), iMo CD69-KO \(n = 8\), iMo Sphk-KO \(n = 3\) mice). Compilation of 5 experiments for Spns2 (iMo WT \(n = 7\), iMo CD69-KO \(n = 11\), WT T cells \(n = 3\) mice). For some mice, technical duplicates are included in the compilation (sorted cells were divided before RNA purification). Data presented as mean values +/- SEM. Mann–Whitney two-tailed t-test.
Extended Data Fig. 7} See next page for caption.
Extended Data Fig. 7 | S1P increases in the dLN in EAE.

a, (left) Percent CD69\(^{+}\) among total CD4 \(^{+}\) T cells in the cervical LN of healthy WT mice or WT mice with EAE (d9). Compilation of 4 experiments (no EAE \(n = 6\), EAE \(n = 11\)). (right) MOG/Ab\(^{+}\)specific 2D2 TCR transgenic T cells were transferred i.v. to WT recipients. After EAE induction, CD69 expression on the transferred 2D2 T cells, endogenous MOG/Ab\(^{+}\)tetrimer \(^{+}\) T cells, and endogenous MOG/Ab\(^{+}\) tetrimer \(^{+}\) T cells in the cervical LN was followed over time. Compilation of 2 experiments (3-5 mice per time point, 33 mice total) for endogenous cells, and 1 experiment (2-3 mice per time point, 18 mice total) for 2D2 T cells. b, Percent CD69\(^{+}\) among total CD4\(^{+}\) \((n = 11)\), Tet\(^{−}\) (CD4\(^{+}\) MOG/Ab\(^{−}\)-tetrramer\(^{−}\)) \((n = 10)\), Th17 (CD4\(^{+}\) Foxp3\(^{−}\) Rorγ\(^{+}\)) \((n = 10)\), Tfh (CD4\(^{+}\)CXCR5\(^{−}\)PD-1\(^{−}\)) \((n = 9)\), Th1 (CD4\(^{+}\)Foxp3 Tbet\(^{−}\)) \((n = 9)\), Treg (CD4\(^{+}\)Foxp3\(^{−}\)) \((n = 10)\), CD4\(^{+}\) Ki67\(^{−}\) (n = 9), CD4\(^{+}\)CD44\(^{−}\) (n = 9), CD8\(^{−}\) (n = 12), γδ T (n = 9), NK (NK1.1\(^{−}\)CD3\(^{−}\)) \((n = 7)\), and NKT (NK1.1\(^{−}\)CD3\(^{−}\)) \((n = 9)\) cells in the cervical LN of WT mice with EAE (d9). Compilation of 4 experiments. c, EAE was induced in WT mice. Left axis: Kinetics of surface S1PR1 expression on CD4\(^{+}\)CD69\(^{+}\) T cells in the cervical LN, relative to CD4\(^{+}\)CD69\(^{−}\) T cells in the cervical LN of healthy controls. 1-4 mice per time point, no EAE \(n = 11\) mice total, EAE \(n = 14\) mice total. Right axis: iMo recruitment to the cervical LN, as a percent of total CD45\(^{+}\) cells. 1-4 mice per time point, no EAE \(n = 8\) mice total, EAE \(n = 14\) mice total. Compilation of 2 experiments. d, e, Representative histograms (d) and compilation (e) of S1PR1 on transferred CD69\(^{−}\)KO, endogenous CD69\(^{−}\) and endogenous CD69\(^{−}\)CD4 T cells in the cervical LN of WT mice with EAE (d9-d12) or healthy controls. For the compilation, the S1PR1 MFI of endogenous CD69\(^{−}\) or endogenous CD69\(^{−}\)CD4\(^{+}\) cells in each mouse was divided by the mean S1PR1 MFI of endogenous CD69\(^{−}\)CD4\(^{+}\) cells in healthy controls. Compilation of 6 experiments (no EAE \(n = 8\), EAE CD69\(^{−}\) \(n = 15\), EAE CD69\(^{−}\)CD4\(^{+}\) \(n = 15\)). f, g, CD69\(^{−}\)KO CD45.1 lymphocytes were transferred i.v. into C57BL/6 mice. The following day, EAE was induced (controls were treated with PBS). 12d-14d after EAE induction, the dLN (cervical) was analysed. f, Representative S1PR1 on endogenous (WT) CD69\(^{−}\)CD8\(^{+}\) T cells (left) and CD69\(^{−}\)KO CD8\(^{+}\) T cells (right). g, Compilation of 2 experiments. (WT) CD69\(^{−}\)CD8\(^{+}\) T cells (no EAE \(n = 3\), EAE \(n = 4\)); CD69\(^{−}\)KO CD8\(^{+}\) T cells (no EAE \(n = 3\), EAE \(n = 4\)). h–m, Lethally irradiated C57BL/6 mice were reconstituted with a 1:1 mix of WT and CD45.1\(^{−}\)CD69\(^{−}\)KO BM, and allowed to reconstitute for 12-14 weeks. EAE was induced, or chimaeras were treated with PBS. 10d-12d later, the cervical LN was analysed. h, Experiment diagram. i, Number CD11b\(^{+}\)Ly6Chi cells in the dLN. Compilation of 5 experiments. No EAE \(n = 6\), EAE \(n = 6\). j, S1PR1 on WT CD69\(^{−}\) (left) and CD69\(^{−}\)KO (right) CD4\(^{+}\) T cells. k, Compilation of 3 experiments. No EAE \(n = 7\), EAE \(n = 7\). l, S1PR1 on WT CD69\(^{−}\) (left) and CD69\(^{−}\)KO (right) CD8\(^{+}\) T cells. m, Compilation of 2 experiments. No EAE \(n = 5\), EAE \(n = 5\). Data presented as mean values \(\pm\) SEM. Mann–Whitney two-tailed \(t\)-test.
Extended Data Fig. 8 | IMo supply S1P in the dLN during EAE. a, Left: Experiment design to test the effect of haematopoietic S1P on T cell exit from the cervical LN in EAE. Right: Percent cells exiting the LN in 6h. Each point represents one mouse at t = 6h relative to the average at t = 0. Compilation of 3 experiments. LitCtl (t = 0h n = 5; t = 6h n = 7), SPHK-KO (t = 0 n = 6; t = 6h n = 7). Note that the control and SPHK-KO chimaeras are at different stages of disease, so the change in T cell residence time may in part reflect differences in LN architecture. b, c. Total CD45+ cells and CD11b+Ly6Chi IMo were sorted from the cervical LN of littermate control or SPHK-KO BM chimaeras with EAE (d9). CD69-KO S1P-sensor+ T cells were cultured for 8h across a transwell from media alone or the indicated cells, and analysed by confocal microscopy. b. Representative images of the cells quantified in (c). Scale bar, 5μm. c. Quantification of S1P reporting, as in Fig. 1f. Each symbol represents the ratio of surface GFP:RFP on one cell. Compilation of 2 experiments. Media (n = 63), CD45+ (n = 101), IMo LitCtl (n = 147), IMo SPHK-KO (n = 83). Data presented as mean values ± SEM. Mann–Whitney two-tailed t test.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | IMo supply S1P in the dLN during EAE. a, b. The indicated chimaeras (as in Fig. 4c) received CD69-KO CD45.1 lymphocytes i.v. on d0. On d0 and every 3d after, they were injected i.p. with DT. On d1 EAE was induced (or mice were treated with PBS). 10d-12d later, S1PR1 levels on CD69-KO CD8+ (a) and endogenous (WT) CD4+ (b) T cells in dLN were analysed. Top, representative histograms. Bottom, compilation of 4 experiments. LitCtl:LitCtl (PBS n = 9, EAE n = 8); CCR2DTR:LitCtl (PBS n = 9, EAE n = 8); LitCtl:SPHK-KO (PBS n = 7, EAE n = 9); CCR2DTR:SPHK KO (PBS n = 9, EAE n = 9). For the compilation of endogenous CD4 T cells, the S1PR1 MFI of the CD69<hi> CD4+ T cells in each mouse in the PBS-treated group was divided by the mean S1PR1 MFI of the CD69<hi> CD4+ T cells in the PBS-treated group: the S1PR1 MFI of the CD69<hi> CD4+ T cells in each mouse of the pIC-treated group was similarly divided by the mean MFI of the CD69<hi> CD4+ T cells in the PBS-treated group. c–f. The indicated chimaeras (as in Fig. 4k) received CFSE-labelled CD69-KO lymphocytes i.v. on d0. On d0 and every 3d after, they were injected i.p. with DT. On d1 EAE was induced (or mice were treated with PBS). 10d-12d later, S1PR1 levels in the cervical LN were analysed. c. Representative histograms (top) and compilation of 4 experiments (bottom) for endogenous CD69-KO CD4+ T cells. LitCtl:CD69-KO (PBS n = 7, EAE n = 5); CCR2DTR:CD69-KO (PBS n = 7, EAE n = 5). d. Representative histograms (top) and compilation of 3 experiments (bottom) for transferred CD69-KO CD8+ T cells. LitCtl:LitCtl (PBS n = 4, EAE n = 5); CCR2DTR:LitCtl (PBS n = 5, EAE n = 4); LitCtl:CD69-KO (PBS n = 5, EAE n = 5); CCR2DTR:CD69-KO (PBS n = 6, EAE n = 6). e. Representative histograms (top) and compilation of 3 experiments (bottom) for endogenous CD69-KO CD8+ T cells. LitCtl:CD69-KO (PBS n = 5, EAE n = 5); CCR2DTR:CD69-KO (PBS n = 6, EAE n = 6). f. Representative histograms (top) and compilation of 4 experiments (bottom) for endogenous WT CD4+ T cells. For the compilation, the S1PR1 MFI of the CD69<hi> CD4+ T cells in each mouse in the PBS-treated group was divided by the mean S1PR1 MFI of the CD69<hi> CD4+ T cells in the PBS-treated group: the S1PR1 MFI of the CD69<hi> CD4+ T cells in each mouse of the pIC-treated group was similarly divided by the mean MFI of the CD69<hi> CD4+ T cells in the PBS-treated group. LitCtl:CD69-KO (PBS n = 5, EAE n = 5); CCR2DTR:CD69-KO (PBS n = 6, EAE n = 6). g. Representative staining of CD4+PD1<hi>CXCR5<hi> Tfh cells in the dLN. h. Representative staining of CD4+Rorγt+Foxp3+Th17 cells in the dLN (top) and in the CNS (bottom). For each graph bars represent mean ± s.e.m. Mann-Whitney two-tailed r-test.
Extended Data Fig. 10 | See next page for caption.
We sought to address how haematopoietic S1P regulates Tfh and Th17 accumulation in EAE. Possibilities include that this S1P may promote priming by delaying naïve T cell exit from LN; promote proliferation or differentiation by delaying activated T cell exit from LN; promote survival, proliferation, or differentiation due to residence-time independent effects of S1P signalling in T cells in LN34–36; or act indirectly through iMo S1P secretion in the CNS. a–g, The results of experiments similar to those in Fig. 4 but using BM chimaeras in which WT mice were reconstituted with SPHK-KO or littermate control BM, to avoid repeated DT injections. h–k, The results of experiments similar to those in Fig. 4 but using mice treated with an S1P lyase inhibitor, to test the effect of increased LN S1P. l–q, The results of experiments designed to distinguish effects of S1P signalling on T cell residence time from other effects. We generated T cells that overexpressed S1PR1. We expected these cells to exit tissues more quickly than control T cells34, similar to T cells that could not ‘see’ iMo-derived S1P, while experiencing enhanced S1PR1 signalling compared to control T cells due to their higher S1PR1 levels, unlike T cells that could not ‘see’ iMo-derived S1P. We observed that overexpression of S1PR1 reduced the frequency of Th17 and Tfh cells, consistent with a cell-intrinsic effect of trafficking on T cell numbers and with previous findings34. We also found that the influx of iMo into the cervical LN and the increased S1P peaked just before the onset of EAE symptoms, which is more consistent with an effect on differentiation than priming (Extended Data Fig. 7c). Although not definitive, these experiments provide impetus for future research on the effect of LN residence time on T cell differentiation. a–g, Lethally irradiated WT mice were reconstituted with BM from pIC-treated Sphk1f/f Sphk2f/f Mx1-Cre− (Sphk-KO) or littermate control (LitCtl) animals. 14–25 weeks after reconstitution, EAE was induced in the chimaeras. T cells in the cervical LN were analysed 9d after EAE induction. a, Symptoms over time. LitCtl (n = 9), SPHK-KO (n = 9), 1 experiment. b, Representative tetramer staining of CD4+ T cells. Number indicates mean percent tetramer+ +/- SEM. Compilation of 5 experiments, LitCtl (n = 11), SPHK-KO (n = 11), c, Number tetramer+ CD4 T cells. Compilation of 5 experiments. LitCtl (n = 11), SPHK-KO (n = 11). d, Representative contour plots identifying Th17 among CD4+ cells, top gated on MOG/Ab tetramer+ cells and bottom gated on MOG/Ab tetramer− cells. Number indicates mean percent Tfh+ +/- SEM. Compilation of 4 experiments, LitCtl (n = 11), SPHK-KO (n = 9), e, Compilation of the experiments in (d), showing ratio of the number of MOG/Ab tetramer+ Tfh cells (left) and MOG/Ab tetramer− Thfh cells (right) in SPHK-KO versus littermate chimaeras (each point represents the ratio of one SPHK-KO animal to the average of the LitCtl animals in the experiment). f, Representative contour plots identifying Th17 among CD4+ FoxP3+ cells, top gated on MOG/Ab tetramer+ cells and bottom gated on MOG/Ab tetramer− cells. Number indicates mean percent Th17+ +/- SEM from 5 experiments. LitCtl (n = 13), SPHK-KO (n = 13). g, Compilation of the experiments in (f), showing ratio of the number of MOG/Ab tetramer+ Th17 cells (left) and MOG/Ab tetramer− Th17 cells (right) in SPHK-KO versus littermate chimaeras (each point represents the ratio of one SPHK-KO animal to the average of the LitCtl animals in the experiment). h–k, WT mice were treated to induce EAE. 4d after EAE induction, the S1P lyase inhibitor 4-deoxypyridoxine (DOP) was added to the drinking water. After 5d of DOP treatment, T cells in the cervical LN were analysed. h, Representative gating for Tfh among CD4+ T cells. Number indicates mean percent Tfh +/- SEM from 2 experiments, Ctl (n = 6), DOP (n = 7). i, Compilation of 2 experiments. Ctl (n = 5), DOP (n = 7). j, Representative gating for Th17 among CD4+ FoxP3+ T cells. Number indicates mean percent Th17+ +/- SEM. Compilation of 2 experiments, Ctl (n = 6), DOP (n = 7). k, Compilation of 2 experiments. Ctl (n = 6); DOP (n = 7). l–q, WT BM progenitors were transduced with a vector encoding S1PR1, GFP or a control vector encoding IRES_GFP. Lethally irradiated WT hosts were reconstituted with S1PR1-overexpressing BM or control BM. Because transduction was inefficient, each mouse harboured a mix of GFP+ transduced and GFP untransduced BM. After reconstitution, EAE was induced in the chimaeras. T cells in the cervical LN were analysed 9d after EAE induction. l, Experiment diagram. m, Representative histograms of S1PR1 expression by GFP+ and GFP− CD4+ in a mouse that received S1PR1_IRES_GFP+ BM (left) and a mouse that received IRES_GFP− BM (right). n, Representative contour plots showing gating for Tfh cells among GFP+ CD4+ T cells in a mouse that received vector-transduced BM (left) or S1PR1-transduced BM (right). Number indicates mean percent Tfh +/- SEM, compiling 4 experiments, empty vector (n = 7), S1PR1 (n = 8). o, Each point represents, for a single mouse, the ratio of the % Tfh among GFP+ CD4+ T cells to the % Tfh among GFP− CD4+ T cells. Compilation of 4 experiments, empty vector (n = 7), S1PR1 (n = 8). p, Representative contour plots showing gating for Th17 cells among GFP+ FoxP3+ CD4+ T cells in a mouse that received vector-transduced BM (left) or S1PR1-transduced BM (right). Number indicates mean percent Th17 +/- SEM. Compilation of 4 experiments, empty vector (n = 8), S1PR1 (n = 7). q, Each point represents, for a single mouse, the ratio of the %Th17 among GFP+ CD4+ T cells to the %Th17 among GFP− CD4+ T cells. Compilation of 4 experiments, empty vector (n = 8), S1PR1 (n = 7). Mann–Whitney two-tailed t-test. For EAE curve, two-way ANOVA with Geisser–Greenhouse correction.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | FACSDiva v.8.02 |
|-----------------|-----------------|
|                 | ZEN 2010        |

| Data analysis   | FlowJo v.9.9 and FlowJo v.10; ImageJ v.1.49 (unpublished code is in Supplementary Information); STAR aligner v.2.5.0c; Picard tools v.1.126; HTSeq v.0.6.0; DEseq2; BEDTools v.2.17.0; bedGraphToBigWig tool v.4; R environment v.3.1.1; Graphpad Prism v.8.0.1 and v.9.0.0 |

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
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- A description of any restrictions on data availability

RNA-Seq data are available from NIH/NCBI as a Gene Expression Omnibus (GEO) dataset (GSE139006). All other data will be available from the authors upon reasonable request.
Field-specific reporting

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- Life sciences
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- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

Sample sizes were based on previous experiments, balancing statistical robustness and animal welfare. It is certainly possible that we missed subtle effects, and we do not over-interpret negative results. Because the effects we were studying were smaller than those induced by loss of S1P lyase (Schwab et al. Science 2005) or loss of S1PR1 (Matloobian et al. Nature 2004), we used more animals than in that work, with numbers comparable to those used in Blaho et al. Nature 2015.

**Data exclusions**

No animals were excluded from analysis unless they were clearly sick (hunched, low body weight). Experiments in which the positive or negative control failed were also excluded. These criteria were pre-established, and are standard in the laboratory.

**Replication**

For the experiments included in the manuscript, all attempts at replication were successful. Furthermore, each hypothesis was tested with multiple types of experiment. The number of independent repeats of each experiment is stated in the figure legend.

**Randomization**

No specific method of randomization was used to allocate mice into groups. Sex-matched littermates were used whenever possible, and experiments were intended to test a single variable. The order of sample collection and data acquisition was designed to avoid experimental bias: collection and processing of samples from control and knockout, as well as treated and untreated animals, were alternated.

**Blinding**

EAE scoring was blinded. Quantitative image analysis was automated (ImageJ macro), with the only user input being the definition of regions. Other experiments were not strictly blinded because the measurements were quantitative, without the subjectivity of disease scoring or qualitative image analysis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| Involved in the study | n/a |
|-----------------------|-----|
| Antibodies            | X   |
| Eukaryotic cell lines | X   |
| Palaeontology         |     |
| Animals and other organisms |   |
| Human research participants | |
| Clinical data         |     |

### Antibodies

All antibodies used are detailed in the methods section and SI. The flow cytometry antibodies are listed in a table (Antibodies for Flow Cytometry), and the immunofluorescence and blocking antibodies are listed in the appropriate subsections. The antibodies not in the table are noted again here: polyclonal chicken anti-GFP (Abcam, ab13970) final concentration 28 µg/ml; polyclonal rabbit anti-Tag-RFP (Evrogen, AB234) final concentration 40 µg/ml; polyclonal Alexa Fluor 488–conjugated goat anti-chicken (Jackson Immunoresearch, 103-045-155) final concentration 0.75 µg/ml; polyclonal Alexa Fluor 647–conjugated donkey anti-rabbit (Jackson Immunoresearch, 711-605-152) final concentration 6 µg/ml; anti-Lyve-1-AlexaFluor488 (eBiocience, ALY7) final concentration 1.25 µg/ml; anti-CD4-PE (Biolegend, RM-4) final concentration 0.25 µg/ml; anti-CD4-PE (Biolegend, RM-4) final concentration 0.25 µg/ml; anti-Lyve-1-biotin (eBiocience, ALY7) final concentration 1.25 µg/ml; anti-CD16/32 (BioLegend, clone 93) final concentration 0.25 µg/ml; anti-CD11b/APC (Biolegend, M1/70) final concentration 1 µg/ml; anti-CD4 AlexaFluor488 (Biolegend, RM 4-5) final concentration 1.25 µg/ml; anti-S1P antibody clone LT1002 (Echelon Bioscience) final concentration 10 µg/ml.

### Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**

The WEHI-231 cell line was provided by Jason Cyster, and is available from ATCC as CRL-1702. The HEK-293T cell line is available from ATCC as CRL-11268.

**Authentication**

We did not authenticate the cell lines, because their identity wasn’t important to our interpretation of the results. We transduced the same cells with vector, S1PR1, or S1PR5.

**Mycoplasma contamination**

WEHI-231 cells were contaminated with mycoplasma; HEK-293T cells were not tested for mycoplasma.

**Commonly misidentified lines**

(See ICLAC register)

WEHI-231 and HEK-293T cells are not on the ICLAC register of misidentified cell lines, v. 10.
Animals and other organisms

Policy information about **studies involving animals**: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | C57BL/6J (WT, CD45.2), B6 SJL-PtprcaPepcbr/BomJ (CD45.1), CD69-KO, UBC-GFP, Sphk1flf, Sphk2flf, Mx1-Cre+, S1pr5flf, CCR2-DTR, Sphk1flf, CCR2-RFP, and S1P reporter mice have been previously described (and are referenced in the methods section). All mice were on a C57BL/6 background. Mice were 5–42 weeks old at the time of analysis. Male and female mice were used depending on availability, as sex did not seem to affect the results. The only exception was the experiments tracking EAE clinical score over time, which were performed with females. (Analysis of S1PR1 and T cell subtypes in EAE was done with both sexes, and sex did not seem to affect the results.) Mice were compared to littermate controls or to WT C57BL/6 mice as indicated. Mice were housed in specific pathogen-free conditions in New York University School of Medicine animal facilities. All cages were on a 12:12-h light:dark cycle (lights on, 0700) in a temperature- and humidity-controlled room. Room temperature was maintained at 72 ± 2 °F (22.2 ± 1.1 °C), and room humidity was maintained at 30% to 70%.

| Wild animals | No wild animals were used in this study.

| Field-collected samples | No field-collected samples were used in this study.

| Ethics oversight | All animal experiments were performed in accordance with protocols approved by the New York University Institutional Animal Care and Use Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cell preparation for flow cytometry: lymphoid organs
Lymphocytes were isolated from LN (axillary, brachial, inguinal, cervical, paraaortic) and in some cases spleen by mechanical disruption and filtration through a 70-µm cell strainer. Cells were enumerated with a cell counter (Beckman Coulter Multisizer 3) set to detect nuclei between 3.5 and 7 µm.

Cell preparation for flow cytometry: CNS
After euthanasia, mice were perfused with PBS. Spinal cord and brain were minced, and then digested for 30 min at 37 °C with gentle rocking with collagenase IV (1 mg/ml, Sigma) and DNase I (0.2 mg/ml, Roche) in HBSS. Collagenase IV was inactivated by washing with 5 mM EDTA and 3% FBS in PBS, and the cells were filtered through 70µm cell strainer. Further purification of lymphocytes was performed by density gradient centrifugation using 40% Percoll (GE Healthcare).

Instrument

BD LSRII

Software

Collection: FACSDiva v.8.02
Analysis: FlowJo v.9.9 and FlowJo v.10

Cell population abundance

When a purity check was performed after sorting, samples were 95-98% pure.

Gating strategy

Gating strategies are shown in main or supplementary figures.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.