HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation

Victoria A. Blaho1,2, Sylvain Galvani1, Eric Engelbrecht1, Catherine Liu1, Steven L. Swendeman1, Mari Kono3, Richard L. Proia3, Lawrence Steinman4, May H. Han4 & Timothy Hla1,2

Lipid mediators influence immunity in myriad ways. For example, circulating sphingosine-1-phosphate (SIP) is a key regulator of lymphocyte egress1–3. Although the majority of plasma SIP is bound to apolipoprotein M (ApoM) in the high-density lipoprotein (HDL) particle, the immunological functions of the ApoM–SIP complex are unknown. Here we show that ApoM–SIP is dispensable for lymphocyte trafficking yet restrains lymphopoiesis by activating the SIP1 receptor on bone marrow lymphocyte progenitors. Mice that lacked ApoM (Apop−/−) had increased proliferation of Lin− Sca-1+ cKit+ haematopoietic progenitor cells (LSKs) and common lymphoid progenitors (CLPs) in bone marrow. Pharmacological activation or genetic overexpression of SIP1 suppressed LSK and CLP cell proliferation in vivo. ApoM was stably associated with bone marrow CLPs, which showed active SIP1 signalling in vivo. Moreover, ApoM-bound SIP, but not albumin-bound SIP, inhibited lymphopoiesis in vitro. Upon immune stimulation, Apom−/− mice developed more severe experimental autoimmune encephalomyelitis, characterized by increased lymphocytes in the central nervous system and breakdown of the blood–brain barrier. Thus, the ApoM–SIP–SIP1 signalling axis restrains the lymphocyte compartment and, consequently, adaptive immune responses. Unique biological functions imparted by specific SIP chaperones could be exploited for novel therapeutic opportunities.

We determined whether ApoM levels affected the SIP concentrations in blood and lymph, which are known to be important for lymphocyte egress. Although plasma SIP was decreased by ~60% (ref. 3), lymph SIP concentrations were not changed in Apom−/− mice (Extended Data Fig. 1a). ApoM in lymph was estimated to be approximately half of plasma levels (Extended Data Fig. 1b). Albumin

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1Center for Vascular Biology, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, New York 10065, USA. 2Brain and Mind Research Institute, Weill Medical College of Cornell University, New York, New York 10065, USA. 3Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland 20892, USA. 4Department of Neurology and Neurological Sciences, Stanford University, Stanford, California 94305, USA.

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Figure 1 | Increased lymphocytes and their progenitors in Apom−/− mice. a, b, CD4+ and CD8+ T cells, CD19+ B cells, monocytes (Mono.) and neutrophils (Neut.) in blood (a) or lymph (b) from wild-type (WT; white) and Apom−/− (blue) mice. c, LSK cells in blood of wild-type and Apom−/− mice. d, Stem and progenitor cell populations in bone marrow (BM) of wild-type and Apom−/− mice. e, Pro-, pre-, immature, and mature B-cell populations in bone marrow of wild-type and Apom−/− mice. f, ETPs in thymuses of wild-type and Apom−/− mice. g, Proliferation of progenitors in bone marrow of wild-type and Apom−/− mice determined by BrdU incorporation. KO, knockout. a–g, Circles indicate values from individual mice and bars represent means. Data are compiled from two (b, c, e, f) or four (a, d, g) independent experiments. h, Representative histograms of Hoechst-33342-stained LSK bone marrow cells from wild-type and Apom−/− mice demonstrating entry into cell cycle. Numbers above bars represent percentage of cells in S/G2/M phase. i, Quantification of cycling LSK, CLP and LKSCa− cell subsets in wild-type and Apom−/− mice. Bars represent means ± standard deviation (s.d.). n = 4. Graphs are representative of at least two experiments. *P < 0.05, **P < 0.005 as compared to wild type.
consistently with a requirement for S1P1 in lymphocyte egress from secondary lymphoid organs and the thymus (Extended Data Fig. 1d). In contrast, global knockout of S1pr1 resulted in severe lymphopenia, consistent with a requirement for S1P1 in lymphocyte egress from secondary lymphoid organs and the thymus (Extended Data Fig. 1d). While examination of lymph nodes (brachial and inguinal) revealed similar lymphocyte numbers in Apom–/– mice compared to wild-type (Extended Data Fig. 2a), thymuses of Apom–/– mice contained significantly more CD4+/CD8+ double-positive and CD4+ or CD8+ single-positive cells (Extended Data Fig. 2b). B-cell populations in spleens of Apom–/– mice were slightly increased but there were no differences in the T-cell populations or spleen weights (Extended Data Fig. 2c, d). Surface expression of lymphocyte activation markers CD69 and CD62L were unchanged in the lymph nodes, thymus and spleen (Extended Data Fig. 3a–c). Administration of anti-integrin antibodies, which block lymphocyte entry into lymph nodes, had similar effects on wild-type and Apom–/– lymph node cell numbers (Extended Data Fig. 3d), implying that ApoM+ HDL is not critical for lymphocyte egress.

FTY720, which induces internalization of S1P1, induces lymphopenia13,14. In both wild-type and Apom–/– mice, administration of FTY720 resulted in marked lymphopenia in blood and lymph 2 h after administration, with similar retention patterns of increased CD4+ and CD19+ cells in lymph nodes and spleen (Extended Data Fig. 4a–d). Double-negative thymocytes were decreased whereas double-positive and single-positive cells increased in thymuses of both wild-type and Apom–/– mice (Extended Data Fig. 4e). A similar degree of lymphopenia was seen using two S1P1-selective agonists, AUY954 and SEW2871 (Extended Data Fig. 4f, g)15,16. Collectively, these data suggest that lymphocyte trafficking out of the thymus and secondary lymphoid organs into blood and lymph is not dependent on ApoM+ HDL.

To determine the cause of lymphopenia seen in Apom–/– mice, we examined haematopoietic cell populations in blood and bone marrow (Extended Data Fig. 5a–c). LSK cells, a designation encompassing several distinct haematopoietic stem and progenitor populations, were more abundant in blood and bone marrow of Apom–/– mice than wild-type mice (Fig. 1c). The bone marrow of Apom–/– mice also contained increased numbers of CLPs (Lin– Flt3+ IL7Rα+ cKit+ Sca1+), whereas granulocyte macrophage progenitors (GMPs; LSKca1– CD34+ FcγRII/IIIhi), common myeloid progenitors (CMPs; LSKca1– CD34+ FcγRII/IIIhi) and megakaryocyte/erythroid progenitors (MEPs; LSKca1– CD34+ FcγRII/IIIhi) were unchanged (Fig. 1d)18–20. Pre-, immature and mature B cells in the bone marrow (Fig. 1e) and early thymic progenitors (ETPs)/double-negative 1 cells (DN1; CD4+ CD8– CD3– cKit+ CD44+ CD25–) in the thymus were increased in Apom–/– mice (Fig. 1f). Although S1P may have a role in LSK recirculation from tissues to the bone marrow18–20, B-cell bone marrow egress, which is insensitive to pertussis toxin, is probably not involved in the lymphopenia seen in Apom–/– mice21. Two and a half hours after bromodeoxyuridine (BrDU) injection, Apom–/– LSK cells and CLPs had significantly more BrDU incorporation compared to wild-type cells (Fig. 1g and Extended Data Fig. 5d). No measurable difference was detected in myeloid progenitors. Cell cycle analysis demonstrated increased fractions of LSK cells and CLPs, but not LSKca– cells in S/G2/M phase in Apom–/– bone marrow (Fig. 1h, i)15. These data suggested that ApoM restrained cell cycle entry specifically in bone marrow progenitors destined for lymphoid lineages. Genetic deletion of the cholesterol transporters ATP-binding cassette A1 (ABCA1) and ABCG1, which interact with HDL to export cellular cholesterol, results in significant increases in LSK and myeloid progenitor cells18,22. ABCG1 single knockout or deletion of scavenger

**Figure 2** | ApoM–S1P–S1P1 signalling suppresses LSK- and CLP-cell-subset expansion. a. Per cent BrdU incorporation (top) and total BrdU+ (bottom) LSK and CLP cells in wild-type (WT) and Apom–/– bone marrow 24 h after treatment with SEW2871 (SEW), n = 6. *P < 0.05 versus wild type; †P < 0.05 versus vehicle-treated control. b, c. LSK and CLP cells in bone marrow (b) and percentage and total number of LSK cells (c) in blood of S1pr1 OE mice and wild-type littermates. S1pr1 OE, n = 6; wild type, n = 7. d. BrdU incorporation by bone marrow LSK and CLP cells of wild-type and S1pr1 OE mice. e, f. ETPs (e) or blood CD4+ and CD8+ and CD19+ cells (f) from wild-type and S1pr1 OE mice. S1pr1 OE, n = 6; wild type, n = 7. g. Immunofluorescence of ApoM (red) bound to the surface of Lin– Sca1– (LSK and CLP) cells from wild-type or Apom–/– bone marrow. *I, 6-Diamidino-2-phenylindole (DAPI) nuclear counterstain, blue. h, Representative histogram and quantitative median fluorescence intensity (MFI) of GFP expression by CLPs from bone marrow of S1P1 OE, GFP signalling (S1P1GS, green) or control (black) mice. i. Immunofluorescence of IL-7R (red) expression and GFP (green) by CLPs. DAPI nuclear counterstain, blue. j, k. Representative flow cytometry histograms of phosphorylated (p)-Stat5 (j) and p-Erk1/2 (k) in wild-type (red) and Apom–/– (blue) CLPs. MFI of p-Stat5 and p-Erk1/2 in CLPs of 3–4 wild-type or Apom–/– mice are shown in inset. d, h, k. Bars represent means and circles represent values from individual animals. b, c, e, f. Bars represent means ± s.d. *P < 0.05; **P < 0.005 versus wild type or control.

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receptor (SR)-B1 (ref. 24), a high-affinity HDL receptor, resulted in increased mature T and B cells, respectively, while not affecting their progenitors. Our findings suggested a bone marrow compartment-specific function of ApoM<sup>+</sup> HDL in lymphocyte ontogeny. We next investigated whether interleukin (IL)-7, a key regulator of lymphopoiesis<sup>26</sup>, was influenced by ApoM–S1P signalling. IL-7 concentrations in the bone marrow (Extended Data Fig. 5e) and CLP IL-7R<sub>x</sub> surface expression (Extended Data Fig. 5f) were similar in wild-type and Apom<sup>−/−</sup> mice. Thus, modulation of the IL-7–IL-7R interaction is unlikely to be responsible for ApoM-mediated suppression of lymphopoiesis.

To examine the role of S1P in progenitor proliferation regulated by ApoM<sup>+</sup> HDL, wild-type and Apom<sup>−/−</sup> mice were treated with SEW2871 and bone marrow cell BrdU incorporation was examined (Fig. 2a). SEW2871 treatment suppressed BrdU incorporation in Apom<sup>−/−</sup> LSK and CLP cells but not in their wild-type counterparts. Treatment with AUY954 or FTY720 also had similar effects (Extended Data Fig. 6a, b), suggesting that pharmacological activation of S1P<sub>1</sub> on LSK and CLP cells suppresses their proliferation. We next determined LSK and CLP cell numbers in the bone marrow of mice with an inducible S1p1 transgene (S1p1 OE; Extended Data Fig. 7a–c).<sup>27</sup> Induction of S1P<sub>1</sub> expression led to decreased numbers of LSK and CLP cells (Fig. 2b). Blood LSK cell numbers were also reduced, ruling out increased bone marrow egress of haematopoietic progenitors (Fig. 2c). Both LSK and CLP populations showed decreased BrdU incorporation (Fig. 2d). S1P<sub>1</sub> overexpression also resulted in decreased ETPs in the thymus (Fig. 2e). Mature T- and B-cell numbers in the blood were unchanged and the secondary lymphoid organs had reduced cell numbers (Fig. 2f and Extended Data Fig. 7d–f), suggesting increased egress of mature lymphocytes from lymphoid organs upon S1P<sub>1</sub> overexpression. These pharmacological and genetic data further confirm that ApoM<sup>+</sup> HDL signalling via bone marrow progenitor–cell S1P<sub>1</sub> restrains lymphopoiesis.

Real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis found that S1p1 messenger RNA is expressed by CLPs and confirmed a previous report of its expression by LSK cells (Extended Data Fig. 8a).<sup>25</sup> Immunofluorescence analysis of Lin<sup>−</sup> Sca-1<sup>−</sup> bone marrow cells from wild-type mice showed stable association of ApoM with the cell surface (Fig. 2g). To determine if S1P<sub>1</sub> is active in bone marrow progenitor cells in vivo, we used S1P<sub>1</sub> green fluorescent protein (GFP) signalling mice, which show a nuclear GFP signal upon receptor activation (Extended Data Fig. 8b–i), and found that LSK and LSK cells show active S1P<sub>1</sub> signalling (Fig. 2h, i).

We next examined the signal transducer and activator of transcription 5 (Stat5) pathway, which is activated by IL-7 signalling and regulates lymphocyte development, proliferation and differentiation<sup>28–30</sup>. Phosphoflow analysis (Extended Data Fig. 8g, h) revealed significantly increased phospho-Stat5 in Apom<sup>−/−</sup> CLPs compared to wild-type cells (Fig. 2i). Conversely, extracellular–signal-regulated kinases 1 and 2 (Erk1/2) antagonize the proliferative signals induced by Stat5 (ref. 26). CLPs from mice lacking ApoM–S1P had significantly less phospho-Erk1/2 than wild-type CLPs (Fig. 2k). Collectively, these data suggest that ApoM–S1P directly interacts with bone marrow lymphocyte progenitors and activates the S1P<sub>1</sub>/Erk pathway to restrain their proliferation.

To test whether ApoM<sup>+</sup> HDL modulated lymphocyte development directly, we used in vitro lymphopoiesis assays. Using Lin<sup>−</sup> cells from wild-type bone marrow, we observed that the generation of B220<sup>−</sup> cells (pre-, pro- and immature B lymphocytes) was strongly suppressed by ApoM<sup>+</sup> HDL but not by albumin-bound S1P (Fig. 3a, b and Extended Data Fig. 9a). HDL from Apom<sup>−/−</sup> mice, which lack S1P (ApoM HDL) (Extended Data Fig. 9b), did not suppress lymphopoiesis (Fig. 3c). ApoM<sup>+</sup> HDL-mediated suppression of lymphopoiesis was blocked by pre-incubation of HDL with an anti-S1P immunoglobulin M (IgM) but not by an irrelevant IgM (Fig. 3d). These findings suggest that ApoM<sup>+</sup> HDL-bound S1P is critical to restrain lymphocyte progenitor differentiation and proliferation.

To determine the involvement of S1P<sub>1</sub>, cultures were incubated with AUY954 or phosphorylated FTY720 (FTY720p), resulting in significantly decreased numbers of B220<sup>−</sup> cells (Fig. 3e). However, incubation of Lin<sup>−</sup> bone marrow cells with W146, a selective S1P<sub>1</sub> antagonist, abrogated the suppressive effect of ApoM<sup>+</sup> HDL. Next, we used Lin<sup>−</sup> bone marrow from S1P<sub>1</sub>−/− mice, which express a mutated S1P<sub>1</sub> that is internalization defective, resulting in extended surface residency and sustained signalling<sup>15,17,27</sup>. S1P<sub>1</sub>−/− bone marrow cells were more
sensitive to ApoM–SIP than wild-type bone marrow, since lower SIP concentrations were sufficient to repress B220+ development (Fig. 3f). Despite greater SIP sensitivity, albumin–SIP was unable to suppress lymphopoiesis by SIP1,SSA cells, similar to wild-type cells. ApoM– HDL did not suppress B220+ generation from SIP1,SSA bone marrow cells (Fig. 3g). Collectively, these findings support the hypothesis that ApoM+ HDL activation of SIP1, on lymphocyte progenitors inhibits their proliferation.

We hypothesized that modulation of the lymphocyte compartment size by ApoM+ HDL could impact the magnitude of immune responses. The experimental autoimmune encephalomyelitis (EAE) model recapitulates many aspects of multiple sclerosis pathology, including increased bone marrow cell proliferative capacity, blood–brain barrier (BBB) disruption, and autoreactive immune cell CNS infiltration. Interestingly, increased plasma HDL correlates with decreased acute inflammation in multiple sclerosis patients, although the mechanism and the role of specific HDL populations are unknown.28–29. To ascertain whether the expanded lymphocyte compartment in Apom−/− mice could affect the extent and intensity of the adaptive immune response, we induced EAE in wild-type, Apom−/− mice, and mice overexpressing a human Apom transgene (APOM12) by immunization with myelin oligodendrocyte protein (MOG35–55) peptide. Mice lacking Apom developed symptoms earlier and exhibited more severe CNS pathology compared to wild-type mice (Fig. 4a). Remarkably, APOM12 mice developed milder symptoms, supporting a protective role for ApoM–SIP (Fig. 4a). More severe inflammation in the CNS was evident at day 16 after immunization in Apom−/− brains as compared to the wild-type counterparts (Fig. 4b), and increased lymphocytes were found in Apom−/− brains at the peak of disease (Fig. 4c). Lymphocytes from MOG-immunized Apom−/− mice were highly activated, as demonstrated by increased splenocyte proliferative responses to ex vivo MOG restimulation (Fig. 4d). Thus, the increase in bone marrow proliferation of lymphocyte progenitors and increased circulating lymphocytes in Apom−/− mice resulted in an exaggerated autoimmune neuroinflammatory response, whereas overexpression of Apom was protective.

Previously, our laboratory has reported that the loss of Apom results in enhanced pulmonary vascular leakage.1 To determine whether altered vascular barrier function was contributing to increased EAE severity, wild-type and Apom−/− mice were injected intravenously with tetramethylrhodamine (TMR)-labelled dextran (70 kilodalton) before or 8 days after MOG35–55 immunization to assess BBB functionality. Before immunization, no differences in dextran extravasation were evident in the brain or spinal cord (Fig. 4e, f and Extended Data Fig. 10a). After immunization, Apom−/− brains and spinal cords displayed significantly increased vascular leakage. This was dependent upon the immune response, since pertussis toxin alone was insufficient to induce vascular leakage (Extended Data Fig. 10b). Thus, the BBB was intact in mice lacking Apom before MOG35–55 immunization, and induction of an immune response was necessary for the generation of EAE and associated BBB breakdown.

SIP1, ECKO mice have increased pulmonary vascular leakage (Extended Data Fig. 10c); however, deletion of endothelial Sipr1 did not influence EAE disease severity (Fig. 4g), suggesting that alterations...
in vascular barrier function are not sufficient to modulate autoimmune responses in the CNS. Similarly, S1P₁ expression by astrocytes has been shown to affect EAE severity. Since neurovascular leakage would be a necessary antecedent for plasma ApoM-S1P to gain access to astrocytes, it seems unlikely that the increased disease severity seen in Apom¹⁻/⁻ mice is due to direct ApoM effects on neural cells. Collectively, these data indicate that ApoM-S1P signalling in lymphocytes is a key factor in the increased EAE pathology displayed by Apom¹⁻/⁻ mice.

In summary, these data reveal an unexpected mechanism by which ApoM³ HDL specifically restrains bone marrow lymphopoiesis by inducing S1P/S1P₁ receptor signalling in bone marrow progenitors, thereby limiting the lymphocyte compartment. These findings also suggest that unique functions of S1P are imparted by its chaperone, ApoM³ HDL, and that ApoM³ HDL-dependent S1P/S1P₁ signalling induces distinct physiological outcomes. Although dispensable for lymphocyte trafficking, ApoM-S1P attenuation of lymphopoiesis under homeostasis may be an important regulator of the lymphocyte life cycle, and therefore have a role in autoimmune responses. Our studies indicate that ApoM³ HDL-mediated regulation of lymphocyte ontogeny impacts the extent and intensity of autoimmune neuro-inflammation in the EAE model. Furthermore, chaperone-dependent S1P signalling may allow the development of novel strategies for the control of immune responses.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions V.A.B. designed and performed the experiments, analysed and interpreted data, and wrote the manuscript. S.G., E.E., C.L. and S.L.S. performed experiments and analysed data. L.S. and M.H.H. contributed to EAE studies. R.L.P. and M.K. contributed to the reporter mouse studies. T.H. supervised the overall project, designed experiments, interpreted data, and wrote the manuscript. All authors read and commented on the manuscript.

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METHODS

Animals. Apom–/– and Apom15 mice were crossed at least nine generations to the C57BL/6 genetic background31. C57BL/6 mice purchased from Jackson Laboratory were used as wild-type controls. To generate mice that overexpress S1P1, mice expressing the S1p1floxed transgene were crossed to mice expressing the tamoxifen-inducible Rosa26-Cre-ER2 to yield S1p1floxed Rosa26-Cre-ER2 animals. To generate mice with endothelial-cell-specific deletion of S1pr1, mice with floxed S1p1 genes (S1p1flox/flox) were crossed with mice expressing a tamoxifen-inducible Cre under the control of the VE-cadherin gene (Cd55-Cre-ER2)32. For both S1pr1-overexpressing mice (S1pr1 OE) and endothelial-cell-specific deletion (ECKO), Cre-negative littermates were used as controls. To induce deletion of the endogenous S1p1 or floxed ‘stop’ sequence, mice were treated with tamoxifen by oral gavage, 4 mg every third or fifth day for a total of three treatments. S1pr1 OE mice were analysed 3–4 days after the final tamoxifen administration; ECKO mice were analysed ≥10 days after the final tamoxifen administration. S1P1 GFP signalling mice were generated as previously described4. Briefly, mice expressing a histone–GFP reporter gene under the control of a tTA-responsive promoter. The transgene were crossed to mice expressing the S1p1 knock-in were considered controls. For all bone marrow studies, animals were killed and tissues collected between 12:00 and 15:00 to limit circadian effects. No randomization method was used and investigators were blinded to the genotype of animals during data analysis. The minimum number of animals per group was four and for all inducible Cre strains wild-type littermate controls were used. Male and female mice were used as age-matched controls for all studies except for EAE experiments, for which only female animals were used. No animals were excluded from the analysis. Animals were housed in a specific pathogen-free facility and provided food and water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee of Well Cornell Medical College.

Verification of S1pr1 mRNA overexpression and floxed ‘stop’ excision. After completion of the tamoxifen treatment regimen, RNA was extracted from whole bone marrow with Trizol according to the manufacturer’s instructions, and TaqMan real-time multiplex RT–PCR was performed as described later to assess overexpression of S1pr1 mRNA. For extraction of genomic DNA from the same samples, back extraction buffer (1 M Tris, pH 8.0, 50 mM guanidine thiocyanate, 50 mM sodium citrate) was added to the remaining organic phase and interphase, vortexed, and DNA was precipitated with 400 μl isopropanol and samples centrifuged again at 15 000 g for 30 min. Amplification of the intact floxed stop cassette yielded a 1.4 bp fragment and, when deleted, a 308 bp fragment.

Cell and plasma collection from blood and lymph. Mice were euthanized with CO2. Blood was recovered via terminal cardiac puncture and collected in tubes containing 35 μl 0.1 M EDTA. Five-hundred millilitres of whole blood was then removed to a new tube, centrifuged at 2000g for 15 min, and plasma removed and stored at −20 °C. Cells were then subjected to ammonium chloride (ACK) erythocyte lysis, washed with PBS, and stained with eFluor718 fixable viability dye (eBioscience) according to manufacturer’s instructions. Lymph was recovered from the cisterna chyli, as previously described33, and collected in 5 μl 5% citrate-dextrose. Total lymph volume was recorded before the entire sample was centrifuged at 2000g for 15 min to isolate plasma. Plasma was then removed and cells were washed with PBS before staining with the eFluor718 viability dye. After a PBS wash, blood and lymph cells were then briefly fixed for 30 min in 0.1% paraformaldehyde (PFA), washed, and stained with antibody cocktails for flow cytometry, as described later.

Tissue cell isolation. Single-cell suspensions were created from spleen, thymus and lymph nodes (two inguinal and brachial lymph nodes per mouse) by gently disaggregating tissue between frosted glass slides. Bone marrow cells were collected after removing the epiphyses and a brief centrifugation in a 1.5 ml tube. Cells were then washed with 1% PBS in PBS and subjected to ACK lysis buffer. After another PBS wash, cells were stained with eFluor718 viability dye. For some studies, such as cell sorting, cells were stained for and analysed by flow cytometry unfixed. Except for studies requiring live sorted cells, samples were gently fixed with 0.1% PFA for 10 min and then washed with 1% PBS in PBS, unless noted otherwise. Although absolute numbers were lower after fixation, this did not have a dramatic effect on staining patterns and gave results similar to those obtained using unfixed cells.

Flow cytometry. After fixation, cells were washed with 1% FBS in PBS (flow buffer (FB)) and Fc receptors were blocked with TrueStain FCx (Biolegend) in 3% FBS overnight at 4 °C. Cells were then aliquoted and stained in their respective antibody cocktails for 30 min on ice. After washing with FB, liquid counting beads (BD) were added to each sample and data were obtained on a BD LSRII (Becton Dickson) and analysed using FlowJo software (Tree Star, Inc.).

Antibodies were purchased from BioLegend (BL), eBioscience (eB), Southern Biotech (SB), or Becton Dickson (BD). For the determination of blood and lymph cell populations the following clones and antibody–fluorescence conjugates were used: CD4–PE (RM4-4, BL), CD8–PerCP/eFluor710 (H35-17.2; eB), CD19–eFluor450 (ID3; eB), CD11b–APC–eFluor780 (M1/70; eB), Ly6G–FITC (HK1.4; SB), Ly6–PE–Cy7 (1A8; BL) and CD115–APC (AF598; BL). For the determination of lymph node and splenocyte populations: CD4–APC–Cy7 (GK1.5; BL), CD8–eFluor450 (H53-17.2; eB), B220–AF700 (RA3-6b2; eB), CD62L–PerCP-Cy5.5 (ME-14; BL) and CD69–PE–Cy7 (H1.2F3; eB). For the determination of thymocyte populations: CD3–APC–Cy7 or –AF700 (17A2; eB), CD4–PE–RM4-4, BL, CD8–APC (53-6-7, eB), CD62L–PerCP-Cy5.5 (ME-14; BL), CD69–PE–Cy7 (H1.2F3; eB), CD44–AF700 or –PECy7 (IM7; eB), CD25–AF488 (3C7; eB), CD11b–APC–eFluor710 (CD11b; 2B8; eB). DN1 cells (ETPs) were defined as CD25+CD8–CD3–CD44+CD25– (Kit2); DN2 cells were defined as CD4–CD8–CD3–CD44– (Kit–); DN3 cells were defined as CD4+CD8–CD3+CD44+ (Kit+); DN4 cells were defined as CD4+CD8+CD3–CD44+ (Kit+). For the determination of bone marrow B-cell populations: B220–AF700, nkIT–APC–eFluor780, CD8–PE–Cy7, FLt3–PE, CD24–BV421, CD43–AF488, IgM–PerCP-Cy5.5, IgA–APC. Pro-B cells were defined as B220+ ‘kit’ CD19+ Flt3+ CD24+ ‘IgM-’; pre-B cells were defined as B220+ ‘kit’ CD19+ Flt3+ IgM+ ‘IgM-’; and mature B cells were defined as B220+ ‘kit’ CD19+ Flt3+ IgM+ “IgD-”. To quantify bone marrow cell populations with or without BrdU binding and stained to identify bone marrow populations.

Phosphoflow for phospho-Stat5 and phospho-Erk1/2. Whole bone marrow cells were resuspended in Lyse/Fix buffer (BD Biosciences) and processed according to manufacturer’s instructions. To permeabilize, 100% methanol pre-cooled at −80 °C was added and cells were vortexed for 15 s before 45 min incubation on ice. After washing with 3% FBS in PBS, cells were incubated in Fc block (anti-CD16/32) and Innex Biosciences peptide Fc block at room temperature for 20 min before incubation with antibody cocktails. For detection of phospho epitopes in CLPs, the following antibodies were used: Lineage cocktail–PE, nkIT–PerCP–eFluor710 (IA72, RA3-6b2, M1/70, Ter-119, RB6-8C5; eB), B220–eFluor450 (ID3; eB), CD11b–APC–eFluor710 or –APC–eFluor780 (CD117; 2B8; eB), Sca-1–PE–Cy7 (LY6A/Ed; D7; eB), IL-7R–APC–eFluor780, –FITC; or BR24–BD421 (CD127; A7R34; eB or SB/199; BD); CD4–APC–RAM34 (BD); FCrYII/III–AF700 (93; eB), Brdu–PerCP–eFluor710 (BU20A; eB); for low abundance markers (Sca1, IL-7Rz, CD34, FcRyI/II/III, CD25 or CD45), fluor-esscence minus one (FMO) controls were used to set gating stages. For S1P1 staining, tiabone bone marrow were spun into a tube containing 35 μl EDTA, then immediately fixed with 0.1% PFA on ice for 1 h. After washing with 100 nM HEPEs in PBS, nonspecific interactions were blocked with anti-CD16/32 at room temperature for 15 min. Cells were then aliquoted for anti-SIP1, or isotype staining (R&D) overnight at 4 °C. The next day, cells were washed twice with PBS-HEPEs and stained to identify bone marrow populations.

Drug administration. FTY720 (fingeromil, trade name Gilenya) was administered orally at a single dosage of 0.5 mg kg−1 in 200 μl of 2% hydroxypropyl-b-cyclodextrin (HPC; Sigma-Aldrich). AUT954 was dissolved in dimethylsulphoxide (DMSO) and administered orally at a single dosage of 3 mg kg−1 in 200 μl of 2% HPC. Both FTY720 and AUT954 were gifts from Novartis Pharma AG. E220781 (Cayman Chemical) was dissolved in 1:4 Tween-20 and ethanol, then administered twice at 0 h and 12 h at a dosage of 20 mg kg−1 orally in 200 μl of HPC before mice were killed at 24 h. For all studies, control animals were treated with the appropriate vehicle.

Albumin ELISA. Albumin concentrations in blood and lymph plasma were determined using the mouse albumin ELISA kit from Bethyl Labs, according to
manufacturer’s instructions. Blood plasma was diluted 1:1.6 × 10^6 and lymph plasma 1:8 × 10^5 before analyses.

ApoM and ApoA1 immunoblots. One microtitre of mouse blood plasma was first diluted in 10 μl PBS, then diluted serially 1:1 in PBS, and 1 μl of diluted lymph plasma was further diluted in 10 μl PBS. Samples were then denatured by boiling in SDS sample buffer and separated on a 12% denaturing gel. After transfer to PVDF membrane, blots were incubated overnight at 4 °C with 1:1,000 dilution of antibodies against ApoM (rabbit anti-human; Genetex) or ApoA1 (goat anti-mouse; AbCam). Primary antibodies were detected with 1:20 Tris-buffered saline (TBS-T). Secondary antibodies were incubated at 1:10,000 dilutions in 1% TBS-T for 1 h at room temperature. For approximation of ApoM concentrations, bands for the blood plasma lymph plasma were compared to the standard curve and an approximate concentration calculated. For approximation of ApoM concentrations, bands for the blood plasma were further diluted in 10% plasma was first diluted in 10 μl PBS and then resuspended in DNase buffer (10% plasma 1:8 plasma was further diluted in 10% BSA before incubation with a 1:5,000 dilution of donkey anti-rabbit AF546 (Life Technologies) for 30 min at room temperature. After washing again on the magnetic column, cells were incubated in PBS containing DAPI before imaging with an Olympus Fluoview FV10i confocal microscope using the ×60 water objective. Intensities for the blue and red channels were adjusted for the Apom−/− cells in accordance with their spectra and, once set, the same settings were used for the wild-type images. Thus, channel intensity correction was the same for each sample and no gamma correction was used.

Ex vivo IL-7Rα staining of bone marrow cells from S1P, GEP signalling mice. Bone marrow was spun into a tube containing 35 μl EDTA and the femurs flushed with, and resuspended in, 0.1% paraformaldehyde. Cells were fixed at room temperature for 12 min and washed with 1% FBS in PBS before being subjected to lineage depletion followed by Sca-1 positive selection. Cells were then stained with either Lin cocktail or biotinylated anti-il-7Rα primary antibody (clone SB/199; BD) followed by streptavidin-AF596 secondary antibody (Jackson Immuno-research). All cells were incubated in DAPI in PBS before imaging by confocal microscopy, as described earlier.

In vitro haematopoiesis and lymphopoiesis. Lineage-negative (Lin−) bone marrow cells from wild-type or SAA mice were isolated using a negative selection magnetic bead kit (StemCell Technologies). In some experiments, CLPs from wild-type mice were sorted by FACS. Five-thousand cells were then incubated in 350 μl methylcellulose medium HPP-S1P (MC; HemoGenix) containing IL-1, IL-6, stem cell factor (SCF), thrombopoietin (TPO), Flt3L, BSA and 5% FBS and supplemented with 10 ng ml−1 rmIL-7 (Peprotech). Control wells contained only this complete medium. Data from at least two duplicate wells per experimental condition were averaged. Cells from each mouse were assayed individually and not pooled. The stock solution of W146 was prepared in filtered Na2CO3 buffer and sonicated until dissolved, then added to MC at a final concentration of 1 μM. AU9Y54 was dissolved in DMSO and diluted in MC for a final concentration of 100 nM and 0.001% DMSO. FTY720p was dissolved in 1% PBS and added to MC. The vehicle control for the in vitro agonist/antagonist studies was 0.001% DMSO. Mouse and human HDL molecules were isolated by ultracentrifugation of plasma from healthy donors or mice as previously described and added to MC at the concentrations indicated44, 46. The human S1P in HDL preparations was determined by high-performance liquid chromatography (HPLC) and I/S/MS/MS as described earlier46. For S1P neutralization by anti-S1P antibody, 50 μg ml−1 HDL was incubated with 25 ng ml−1 of either anti-S1P antibody (Alfresa Pharma Corp) or mouse IgM (Bethyl Labs) at 37 °C for 1 h, then added to the MC. Albumin−S1P was prepared by reconstituting stock S1P (in ethanol) in 0.1% fatty-acid-free BSA (F AF-BSA; Fisher), then sonication for 20 s. Dilutions were then made in 0.1% FAF-BSA before adding to the MC. All MC incubations of Lin− cells were incubated for 10 days at 37 °C and 5% CO2.

Flow cytometric analysis of in vitro incubations. For cell collection, 500 μl of MC was added to each well, incubated for 10 min, then gently triturated to resuspend the cell culture/MC mixture. Resuspended cells were removed to a 1.5 ml microcentrifuge tube and cells remaining in the well were collected with an additional 500 μl of PBS. Cells were then centrifuged at 700g for 10 min at 4 °C, washed once with PBS, then processed for incubation with antibody cocktail and flow cytometric analysis as earlier.

Induction of EAE. EAE was induced by subcutaneous injection on both flanks and base of the tail of 200 μg myelin oligodendrocyteprotein (MOG35-55) peptide (Alpha Diagnostic Intl.) emulsified in 200 μl of complete Freund’s adjuvant (Difco). Two-week-old C57BL/6 mice were intraperitoneally injected with Bordetella pertussis toxin (List Biological Laboratories) was injected intraperitoneally in 200 μl on the day of immunization and 48 h later14. Clinical scores were determined based on the following scale: 0, normal; 1, tail weakness; 2, tail paralysis and hind limb weakness; 3, complete hind limb paralysis; 4, hind limb paralysis with forelimb weakness; and 5, moribund or death15. For histopathological analysis of brain tissue sections, brains were removed and fixed in 10% neutral-buffered formalin overnight at room temperature. After washing in PBS, brains were placed in 70% ethanol before paraffin embedding. Tissue sections were then stained with haematoxylin and eosin (Histoserve).

Isolation of CNS-infiltrating immune cells. Brains from wild-type and Apom−/− mice were minced, then digested in a mixing incubator for 1 h at 37 °C and 1,300 r.p.m. in a cocktail of Liberase (1 U per 1 ml Roche) and DNase (1 mg ml−1; Sigma) in Hank’s balanced salt solution (HBSS). Tissues were further disaggregated by repeated passage through an 18G needle, then brought to 1 ml with HBSS. Leukocytes were isolated on a discontinuous Percoll gradient according to Pino and Cardona48 and analysed by flow cytometry.
**In vitro lymphocyte restimulation.** Spleens from wild-type or Apom<sup>+/−</sup> mice were isolated 9 days after MOG<sub>35–55</sub> peptide immunization. After disaggregation and erythrocyte lysis with ammonium chloride, cells were cultured in 96-well plates at a density of 5 × 10<sup>5</sup> cells with increasing concentrations of MOG<sub>35–55</sub> peptide. After 96 h of incubation, numbers of CD<sup>4+</sup> cells were quantified by flow cytometry<sup>34</sup>.

**Evans blue dye extravasation measure of vascular permeability.** Pulmonary vascular leakage was measured by Evans blue dye (EBD) accumulation assay. EBD (0.5% in PBS) was injected via tail vein 90 min before tissue harvest. Pulmonary vasculature was perfused with 5 ml PBS through the right ventricle, while allowing the perfusate to drain from an incision in the left ventricle. Lungs were then weighed and dried at 56 °C overnight. Dry lung weight was measured again, and EBD was dissolved in formamide at 37 °C for 24 h and quantitated spectrophotometrically at 620 and 740 nm.

**TMR–dextran brain and spinal cord vascular permeability.** Eight days after MOG immunization, MOG-immunized mice, mice injected with pertussis toxin alone, or unimmunized mice were injected via tail vein with 100 μl of 25 mg ml<sup>−1</sup> TMR–dextran in 1 mM CaCl<sub>2</sub>. After 18 h, mice were killed by CO<sub>2</sub> and perfused with 1% Triton X-100 in PBS then spun at 4 °C for 25 min at 20,000 g. Supernatants were transferred to new tubes until fluorometric analysis at 540/590.

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism software v.6.0. Two-tailed Student’s <i>t</i>-test was used for direct comparison of two groups. Analysis of variance (ANOVA) followed by Dunnett’s for comparison to control group or Tukey’s to compare all groups was used to determine significance between three or more test groups. Differences in EAE clinical scores were determined using the Mann–Whitney <i>U</i>-test for analysis of the area under the curve and at individual time points. For all analyses, <i>α</i> was set to 0.05 and <i>P</i> values ≤ 0.05 were considered significantly different.

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Extended Data Figure 1 | Blood and lymph ApoM and albumin in wild-type and Apom−/− mice and blood cell numbers in S1pr1 global and ECKO mice.

a, Concentrations of blood and lymph S1P were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). Bars represent means ± s.d. n = 4 as described in Methods. WT, wild type.
b, Western blots of ApoA1 and ApoM in lymph of wild-type mice. One microlitre of wild-type blood plasma was serially diluted 1:1, and 1 μl of diluted lymph plasma from 5 animals was analysed for ApoA1 and ApoM protein levels.
c, Determination by enzyme-linked immunosorbent assay (ELISA) of albumin concentrations in the blood and lymph of wild-type or Apom−/− (KO) mice. Bars are mean ± s.d. Wild type, n = 5; Apom−/−, n = 7.
d, Quantification of CD4+, CD8+ and CD19+ cells in the blood of wild-type, S1pr1fl/fl Rosa26-Cre-ERT2 (global) or S1pr1fl/fl Cdh5-Cre-ERT2 (ECKO) mice. Bars are mean ± s.d. Wild type, n = 5; global or ECKO, n = 6. *P < 0.05, **P < 0.005, as compared to wild-type values.
Extended Data Figure 2 | Cell populations of the lymph node, thymus and spleen in Apom<sup>−/−</sup> and wild-type mice. **a**, Quantification of CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells in two brachial and two inguinal lymph nodes combined. Data are compiled from five experiments. **b**, Quantitation of CD4<sup>+</sup> single-positive (SP), CD8<sup>+</sup> single-positive, CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP), and total CD4<sup>−</sup>CD8<sup>−</sup> double-negative (DN) cells in thymuses of wild-type or Apom<sup>−/−</sup> mice. Data are compiled from three experiments. **c**, Quantitation of CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells in spleens of wild-type or Apom<sup>−/−</sup> mice. Data are compiled from three experiments. **a**–**c**, Bars represent means and circles represent values obtained from individual mice. *P < 0.05. **d**, Total spleen weights (grams of wet weight) from wild-type or Apom<sup>−/−</sup> mice. n = 6. Bars represent means ± s.d.
Extended Data Figure 3 | Surface expression of the maturation markers CD62L and CD69 and lymph node egress are unchanged in Apom⁻/⁻ mice. a–c, Surface expression of the lymphocyte maturation markers CD62L and CD69 was determined by flow cytometry and representative histograms are shown of staining by lymph node cells (a), thymocytes (b) or splenocytes (c) quantified in Extended Data Fig. 2 from wild-type (WT; red) and Apom⁻/⁻ (blue) mice. d, Percentage of CD4⁺, CD8⁺ and CD19⁺ cells remaining in brachial and inguinal lymph nodes 14 h after administration of alpha-4 and alpha-L integrin-blocking antibodies. Circles represent mean value acquired from three mice in independent experiments; bars represent means.
Extended Data Figure 4 | ApoM expression is not required for the lymphopenia response to treatment with FTY720 or the S1P1-specific agonists AUY954 and SEW2871. a–e, Wild-type (WT; red) and Apom<sup>−/−</sup> (blue) mice were treated with a single dose of FTY720 (0.5 mg kg<sup>−1</sup> orally), and samples were collected at 0, 2 or 24 h post-treatment. CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells from blood (a), lymph (b), lymph node (c) and spleen (d), and CD4<sup>+</sup> single-positive, CD8<sup>+</sup> single-positive, CD4<sup>+</sup> CD8<sup>+</sup> double-positive and CD4<sup>+</sup> CD8<sup>−</sup> double-negative cells from the thymus (e) were quantitated by flow cytometry. Symbols represent means ± s.d. and graphs are of data compiled from two experiments. 0 h, n = 5; 2 h, n = 6; 24 h, n = 4. f, g, Wild-type and Apom<sup>−/−</sup> (KO) mice were treated with AUY954 (AUY; 1 mg kg<sup>−1</sup>) (f), SEW2871 (SEW; 20 mg kg<sup>−1</sup>) (g), or respective vehicle controls. Twenty-four hours after treatment, CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells in the blood were quantitated by flow cytometry. Bars represent means ± s.d., n = 4 for all treatment groups and data are representative of two experiments.
Extended Data Figure 5 | Multiparameter flow cytometry gating scheme for determination of CLPs and other bone marrow populations. a. Cells are first gated as Lin^{-} and IL-7R\alpha^{-}. Cells are then gated by Flt3 (Flk2/CD135) versus IL-7R\alpha expression, then further gated by cKit versus Sca-1 expression to define CLPs. Representative staining examples of wild-type (WT; top) or Apom^{-/-} (bottom) bone marrow cells are shown. b, c. Representative flow cytometric plot (b) and quantitation (c) of B220^{+} CD11b^{-} and CD11b^{+} B220^{-} cells generated from CLPs in vitro. CLPs, gated according to the gating hierarchy shown in a, were sorted from the bone marrow of wild-type mice and incubated in methylcellulose medium containing growth factors to support both lymphoid and myeloid lineage development. Cells were analysed for B220 and CD11b expression after 12 days of culture. n = 6. d. Per cent BrdU incorporation by LSK cells (left) or CLPs (right) in bone marrow of wild-type (red) or Apom^{-/-} (blue) mice in two independent experiments (A and B). *P < 0.05, **P = 0.006, ***P = 0.0009 versus wild type. Equality of variance was determined by an F test. Bars represent means and circles represent values obtained from individual mice. e. IL-7 protein in bone marrow supernatants from wild-type and Apom^{-/-} mice was quantified by ELISA. n = 6 combined from two studies. Bars represent mean ± s.d. f. Representative histogram of IL-7R\alpha (CD127) expression on the surface of wild-type (red) or Apom^{-/-} (blue) CLPs. The fluorescence minus one (FMO) control is represented by the grey shaded histogram.
Extended Data Figure 6 | Treatment with the S1P₃ modulator FTY720 or the S1P₃-specific agonist AUY954 suppresses BrdU incorporation by LSK and CLP cells in Apom⁻/⁻ bone marrow. a, b, Per cent BrdU incorporation 24 h after treatment with 0.5 mg kg⁻¹ FTY720 (a) or 1.0 mg kg⁻¹ AUY954 (b). a, Wild type, n = 4; Apom⁻/⁻, n = 5 for vehicle-treated and wild type, n = 7; Apom⁻/⁻, n = 8 for FTY720-treated groups. b, Vehicle-treated, n = 3; AUY954-treated, n = 6. Bars represent means ± s.d. and data are compiled from two experiments. *P < 0.05 versus wild type; †P < 0.05 versus vehicle-treated control.
Extended Data Figure 7 | Overexpression of S1P1 results in marked decreases in lymphocyte populations in the thymus and secondary lymphoid organs. a, Representative flow cytometry plots and quantitative MFI of S1P1 expression by Lin^− cells from S1pr1 OE and wild-type (WT) littermates. n = 3. b, Relative expression levels of S1pr1 mRNA in bone marrow cells of S1pr1 OE mice relative to wild-type mice, as determined by multiplex qRT–PCR. c, Representative agarose gel of Cre activation and excision of the floxed stop cassette as assessed by PCR of genomic DNA from bone marrow cells of S1pr1 OE mice, lox/stop/lox littermate, or wild-type littermate. Arrows indicate amplified DNA fragments corresponding to undeleted or deleted segments. d–f, Four days after the final dose of tamoxifen, CD4^+^, CD8^+^, and CD19^+^ cells in brachial and inguinal lymph nodes (d) and spleen (e), and thymic CD4^+^, CD8^+^, double-positive (DP), CD4^+^ and CD8^+^ single-positive (CD4 or CD8 SP), total double-negative (DN), and double-negative subpopulations DN2, DN3, and DN4 (f) were quantified by flow cytometry in S1pr1 OE mice and wild-type littermates. Bars represent means ± s.d.; S1pr1 OE, n = 6; wild type, n = 7 and data are compiled from two experiments.
Extended Data Figure 8 | GFP expression by bone marrow cells and splenocytes of S1P1 GFP signalling mice and stimulation of Stat5 or Erk1/2 phosphorylation in splenocytes. a, Relative expression levels of S1pr1 mRNA in LSK and CLP cells from bone marrow, or splenic B or T cells of wild-type mice relative to mouse endothelial cells, as determined by multiplex qRT–PCR. Bars represent means and circles represent values obtained from individual mice. b, Representative histograms of GFP expression by LSK cells from bone marrow of S1P1 GFP signalling mice (S1P1GS; green) or control (black) mice. c, Representative histogram of GFP expression by splenic B cells (CD19+) from S1P1 GFP signalling mice (green) or control (black) mice, demonstrating high in vivo GFP expression. d, Representative immunofluorescence image of IL-7Rα cell with CLP morphology from bone marrow of littermate control of S1P1 GFP signalling mice. Cells were subjected to the same selection process before immunofluorescence staining. d–f, IL-7Rα, red; blue, DAPI. e, f, Staining of bone marrow cells from S1P1 GFP signalling mouse littermate controls demonstrates IL-7Rα staining specificity: cells with CLP morphology stained with secondary alone (e) or myeloid cell morphology from bone marrow stained with anti-IL-7Rα (f) exhibit no IL-7Rα positivity. g, p-Stat5 staining after in vitro stimulation of wild-type splenocytes with IL-7 (10 ng ml⁻¹) for 15 min. CD19⁻ cells serve as positive controls for p-Stat5 staining. CD11b⁻ cells serve as negative controls, since they do not have IL-7R and therefore do not respond to IL-7 stimulation with Stat5 phosphorylation. h, p-Erk1/2 staining after in vitro stimulation of wild-type splenocytes with PMA (5 ng ml⁻¹) for 15 min. CD19⁻ cells serve as positive controls for p-Erk1/2 staining.
Extended Data Figure 9 | In vitro lymphopoiesis in the presence of ApoM⁺ HDL generates B cells at different stages of development. a, Phenotyping of B220⁺ cell populations generated from wild-type (WT) Lin⁻ bone marrow after 8 days of culture in methylcellulose medium. Initially, cells were gated as B220⁻, B220⁺, or B220hi. From populations expressing B220, pro-B cell (Hardy fractions B/C) and pre-B cell (Hardy fraction D) equivalents were identified. Pro-B cells were defined as IL-7R⁺, CD19⁺, cKit⁺, CD43⁻ and IgM⁻. Pre-B cells were defined as IL-7R⁺, CD19⁺, cKit⁻, CD43⁻ and IgM⁻. 

b, Western blot analysis of two batches of mouse HDL (batches n1 and n2) isolated from wild-type or Apom⁻/⁻ mice showing the absence of contaminating albumin.

c, LC-MS/MS analysis of HDL isolated from pooled plasmas of wild-type or Apom⁻/⁻ mice. *P < 0.05, **P < 0.005. n = 3. dhSph, dihydrosphingosine; dhS1P, dihydro-sphingosine-1-phosphate; Sph, sphingosine.
Extended Data Figure 10 | Background fluorescence and vascular permeability analysis in brain, spinal cord, and lung. a, Representative photographs of auto-fluorescence in whole brains or spinal cord in wild-type mice at day (d)0 or day 8 after MOG35–55 immunization. 'No rhodamine' denotes tissues from animals that were not injected with TMR–dextran. b, Representative photograph of whole brain 16 h after TMR injection of a mouse injected with pertussis toxin (PTX) alone, without accompanying MOG35–55 immunization. c, Pulmonary vascular permeability as determined by Evans blue dye extravasation in wild-type (WT), Apom<sup>−/−</sup> and S1P, ECKO mice. Bars represent means and circles represent values from individual animals. **P < 0.005, ****P < 0.0001, as compared to wild-type controls.