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Astrocyte reactivity impairs lymphopoiesis through cholesterol-dependent mTOR suppression in common lymphoid progenitors

Running Title: Cholesterol–mTOR control of lymphopoiesis

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Abstract

Immune homeostasis relies on coordinated communication between the central nervous system (CNS) and peripheral hematopoietic organs, yet whether astrocyte reactivity influences lymphoid progenitor function remains poorly understood. Here, using inducible Glast- and GFAP-lineage perturbation models, we uncover a cholesterol-dependent mechanism linking astrocyte reactivity-associated CNS perturbation to impaired bone marrow lymphopoiesis. These models exhibited reactive gliosis accompanied by reduced peripheral leukocyte counts, diminished B-cell output, and selective loss of common lymphoid progenitors (CLPs), whereas hematopoietic stem cells and myeloid progenitors were largely preserved. Transplantation assays indicated that this defect was predominantly cell-extrinsic to hematopoietic progenitors. Metabolomic profiling and filipin staining revealed increased cholesterol abundance in the brain and bone marrow, with preferential cholesterol accumulation in CLPs. Cholesterol exposure suppressed CLP proliferation, B-cell differentiation, and mTOR activity, whereas inhibition of cholesterol synthesis or restoration of mTOR signaling partially restored lymphoid development *in vivo*. Patient data from autoimmune encephalitis and neuromyelitis optica showed an inverse association between serum cholesterol levels and peripheral lymphocyte parameters. These findings suggest that astrocyte reactivity-associated cholesterol remodeling impairs CLP function by suppressing mTOR signaling.

37 **Introduction**

38 Immune homeostasis depends on the continuous production and balanced output of
39 hematopoietic and immune cells from peripheral hematopoietic organs(1, 2). Among the systemic
40 inputs that regulate this process, the CNS has emerged as an important regulator of bone marrow
41 function, the primary site of hematopoiesis(3). Within the bone marrow, the hematopoietic niche,
42 which sustains continuous production of blood and immune cell(4, 5), is regulated by both local
43 cues and systemic inputs, including sympathetic noradrenergic signaling(6), which modulates
44 niche components such as CXCL12 and controls hematopoietic stem and progenitor cell (HSPC)
45 retention and mobilization in a circadian manner(7, 8). These findings establish a neuronal route
46 by which the brain influences hematopoietic homeostasis. However, whether and how brain-
47 resident non-neuronal cells participate in the systemic regulation of hematopoietic progenitor
48 function remains largely unknown.

49 Astrocytes are abundant glial cells in the CNS and serve as central regulators of neural
50 homeostasis. They provide structural and metabolic support to neurons, regulate extracellular ion
51 balance and neurotransmitter levels, and contribute to the maintenance of the blood-brain barrier
52 (9, 10). In response to injury or disease, astrocytes undergo reactive changes that can include
53 proliferation, inflammatory signaling, and metabolic remodeling, thereby reshaping the local
54 neural environment(11-13). In the brain, astrocytes are major contributors to cholesterol
55 metabolism(14), which is critical for membrane organization, intercellular communication, and
56 tissue homeostasis. While astrocyte reactivity has been widely implicated in neuroinflammatory
57 and neurodegenerative disorders, whether astrocyte reactivity-associated metabolic remodeling
58 is linked to peripheral hematopoietic regulation remains unclear.

59 Within the hematopoietic system, lymphopoiesis is particularly sensitive to systemic and
60 metabolic perturbations(15-17). Common lymphoid progenitors (CLPs) give rise to B and T
61 lymphocytes, and defects at this stage contribute to impaired immune competence, chronic
62 inflammation, and dysregulated immune responses(18). Although alterations in the bone marrow
63 niche and systemic inflammatory cues have been linked to CLP fate determination(19, 20), the

upstream extramedullary signals that selectively affect lymphoid progenitor proliferation, survival, and lymphoid output remain incompletely defined. In particular, it remains unclear whether CNS-associated metabolic alterations can influence lymphoid progenitor homeostasis in the bone marrow.

Here, using complementary Glast- and GFAP-lineage perturbation models, we investigated whether astrocyte reactivity-associated CNS perturbation is linked to altered lymphoid progenitor function. We found that reactive gliosis was accompanied by impaired B-cell lymphopoiesis and selective dysfunction of CLPs, whereas HSCs and myeloid progenitors were largely preserved. Mechanistically, these changes were associated with cholesterol remodeling across the brain and bone marrow, preferential cholesterol accumulation in CLPs, and suppression of mTOR signaling. Pharmacological modulation of cholesterol synthesis or mTOR activity partially ameliorated lymphoid defects *in vivo*. Together, our findings suggest a cholesterol-dependent pathway linking astrocyte reactivity-associated CNS perturbation to mTOR-dependent suppression of CLP function, providing a potential metabolic route by which CNS pathology may influence systemic immune homeostasis.

Results

Astrocyte reactivity-associated CNS perturbation selectively impairs CLPs

To examine whether astrocyte reactivity-associated CNS perturbation is linked to hematopoietic regulation, we generated an inducible Slc1a3/Glast-lineage perturbation model by crossing Glast-CreERT mice with diphtheria toxin A (DTA) transgenic mice. In this system, tamoxifen administration activates CreERT in Slc1a3/Glast-expressing cells and induces DTA expression in the recombined lineage (**Fig. 1A**). Although DTA is commonly used to ablate Cre- target cells, tamoxifen-treated Glast-CreERT;DTA mice did not show widespread loss of GFAP⁺ astrocytes *in vivo*, consistent with prior reports(21, 22). Instead, this perturbation was accompanied by reactive gliosis, characterized by increased numbers of GFAP⁺ astrocytes in the hippocampus and elevated Ki-67 expression (**Fig. 1B-C**)(23). By contrast, astrocytes isolated from Glast-CreERT;DTA mice exhibited significantly increased apoptosis after tamoxifen exposure *in vitro*

(**Fig. 1D**), suggesting that the *in vivo* neural microenvironment may modify the cellular outcome of DTA expression and favor a reactive rather than overtly ablative response. We therefore used this model as an inducible Slc1a3/Glast-lineage CNS perturbation model characterized by astrocyte reactivity-associated gliosis, rather than as a model of complete astrocyte depletion.

We next assessed systemic hematopoiesis in these mice. Peripheral blood analysis revealed that white blood cell (WBC) counts were reduced by approximately 50% in Glast-CreERT;DTA mice compared with those in control animals (**Fig. 1E**). This reduction was predominantly attributable to a loss of B cells, with myeloid populations remaining largely unaffected (**Fig. 1F-G**). Consistently, spleen weight and total splenocyte counts were significantly lower in Glast-CreERT;DTA mice (**Fig. 1H-I**), accompanied by marked reductions in the numbers of splenic T and B cells and a moderate decline in the myeloid cell count (**Fig. 1J**). In bone marrow, the number of B cells was significantly reduced, whereas T cell numbers were increased, potentially reflecting altered peripheral T-cell distribution or homing (**Fig. 1K, Fig. S1A-C**). Conversely, analysis of brain leukocytes revealed that CD45⁺ T cells and myeloid cells, but not B cells, were expanded in Glast-CreERT;DTA mice (**Fig. S1D-E**), indicating that peripheral B-cell loss was not explained by B-cell accumulation in the brain.

We then analyzed bone marrow HSPC compartments. The numbers of hematopoietic stem cells (HSCs) (Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁻), LSK cells (Lin⁻Sca-1⁺c-Kit⁺), and LK cells (Lin⁻Sca-1⁻Kit⁺) (**Fig. S1F-G**) were unchanged in Glast-CreERT;DTA mice. By contrast, both the frequency and absolute number of CLPs (Lin⁻c-Kit^{low}Sca-1^{low}CD127⁺) were markedly reduced in Glast-CreERT;DTA mice relative to controls (**Fig. 1L**), while granulocyte-monocyte progenitors (GMPs; Lin⁻c-Kit⁺Sca-1⁻CD16/32^{high}CD34⁺), common myeloid progenitors (CMPs; Lin⁻c-Kit⁺Sca-1⁻CD16/32^{med}CD34⁺), and megakaryocyte-erythroid progenitors (MEPs; Lin⁻c-Kit⁺Sca-1⁻CD16/32^{med/low}CD34⁻) were unaffected (**Fig. S1H-I**). CLPs from Glast-CreERT;DTA mice also exhibited a shift in cell-cycle distribution patterns, reflected in an accumulation of cells in G₀ and fewer cells in the S/G₂/M phases, together with increased levels of apoptosis (**Fig. 1M-N**). These results indicate that Slc1a3/Glast-lineage CNS perturbation is associated with a selective

impairment of early lymphoid progenitors.

To validate these findings, we established an independent model by crossing GFAP-Cre mice with DTR mice, generating a GFAP-Cre;DTR line in which GFAP⁺ astrocytes can be selectively targeted by diphtheria toxin (DT) (**Fig. S2A**). DT administration induced a reactive gliosis-like phenotype in the hippocampus, characterized by increased numbers of GFAP⁺ and Ki-67⁺ cells (**Fig. S2B-C**). In line with observations from the Glax-CreERT;DTA model, cultured astrocytes from GFAP-Cre;DTR mice displayed increased apoptosis following exposure to DT treatment (**Fig. S2D**). Astrocyte activation was not accompanied by changes in the frequencies or composition of CD45⁺ brain-infiltrating leukocytes, with only a mild reduction in T cell numbers being observed (**Fig. S2E-F**), arguing against overt neuroinflammation as the primary driver of the hematopoietic phenotype.

Systemically, GFAP-Cre;DTR mice phenocopied Glax-CreERT;DTA mice, displaying reduced WBC counts, splenocyte numbers, and spleen size (**Fig. S2G-I**). Flow cytometry revealed a selective and consistent loss of B cells in peripheral blood, spleen, and bone marrow in GFAP-Cre;DTR mice, whereas T cells and myeloid cells were largely unaffected (**Fig. S2J-O**). In the bone marrow, both the frequency and number of CLPs were significantly reduced, while other progenitor populations remained stable (**Fig. S2P and Fig. S3A-E**). Consistent with findings in Glax-CreERT;DTA mice, CLPs from GFAP-Cre;DTR mice accumulated in the G₀ phase of the cell cycle, and exhibited reduced entry into S/G₂/M phases, along with increased apoptosis (**Fig. S2Q-R**), indicative of impaired proliferative capacity and survival. Collectively, these findings demonstrated that astrocyte reactivity-associated CNS perturbation is reproducibly linked to selective CLP dysfunction and impaired lymphoid differentiation.

Astrocyte reactivity-associated perturbation impairs CLP proliferation and B-cell differentiation

Having established that CLP numbers are selectively reduced in perturbation models of reactive astrocytes, we next investigated whether this reduction translates into impaired lymphoid differentiation. To this end, we first assessed lymphoid differentiation *in vitro* using OP9 stromal

co-culture assays with sorted Lin⁻ bone marrow progenitors. Compared with controls, Lin⁻ cells from Glast-CreERT;DTA mice generated significantly fewer B220⁺ cells, indicating that B-cell differentiation capacity was reduced (**Fig. 2A**). In line with this, pre-B cell colony formation by Glast-CreERT;DTA-derived B cell progenitors was markedly reduced (**Fig. 2B**). A similar phenotype was observed in GFAP-Cre;DTR mice, supporting a reproducible association between astrocyte reactivity-associated perturbation and impaired B-cell differentiation (**Fig. S2S-T**).

To examine this defect *in vivo*, we monitored CLP generation from HSCs using a lineage tracing strategy. Fgd5-CreER mice were crossed with Rosa26-tdTomato reporter mice, generating a Fgd5-CreER;tdTomato line. Bone marrow from these mice was transplanted into Glast-CreERT;DTA or control recipients, followed by tamoxifen administration to induce tdTomato labeling in HSCs and their progeny, including lymphoid cells (**Fig. 2C**). In Glast-CreERT;DTA recipients, the frequency of tdTomato⁺ CLPs in the bone marrow was significantly reduced, whereas upstream HSCs and multipotent progenitors (LSK, LK), as well as myeloid progenitors (GMPs, CMPs, MEPs), remained unchanged (**Fig. 2D-F**). These data revealed that the influence of astrocytes reactivity-associated perturbation on hematopoiesis is exerted predominantly at the CLP stage.

In Glast-CreERT;DTA mice, tdTomato-labeled CLPs additionally exhibited cell-cycle arrest, characterized by G₀ accumulation and reduced entry into the S/G₂/M phases (**Fig. 2G**), thereby recapitulating the proliferative and survival defects observed in bulk CLPs (**Fig. 1M-N**). Functionally, these deficiencies led to a reduction in tdTomato⁺ B220⁺ cell numbers in the bone marrow and peripheral blood (**Fig. 2H-I**). Together, these results indicate that astrocyte reactivity-associated CNS perturbation selectively impairs CLP hemostasis, thereby limiting B-cell differentiation and compromising systemic lymphopoiesis.

Lymphoid defects are mediated predominantly through a cell-extrinsic bone marrow microenvironmental mechanism

To determine whether lymphopoietic impairment in reactive astrocyte models stems from intrinsic defects in HSPCs or alterations in the bone marrow niche, we first performed competitive

reconstitution assays. Bone marrow cells (CD45.2⁺) were harvested from tamoxifen-treated Glast-CreERT;DTA or control mice, mixed 1:1 with competitor cells from congenic BoyJ mice (CD45.1⁺), and transplanted into lethally irradiated BoyJ recipients. After 4 months, bone marrow cells were harvested from these mice and transplanted into secondary recipients (**Fig. 3A**). In both primary and secondary recipients, donor-derived hematopoietic reconstitution was comparable between Glast-CreERT;DTA and control cells (**Fig. 3B-C**), indicating that HSPCs from mice with induced astrocyte reactivity-associated perturbation retained their reconstitution capacity.

We next assessed whether the bone marrow microenvironment contributed to the observed lymphoid defects. Wild-type (WT) bone marrow cells were transplanted into lethally irradiated Glast-CreERT;DTA or control recipients, followed by tamoxifen administration to induce Slc1a3/Glast-lineage CNS perturbation in the recipient environment (**Fig. 3D**). Glast-CreERT;DTA recipients displayed significantly reduced peripheral WBC counts at 4, 8, and 12 weeks post-transplantation (**Fig. 3E**), mainly attributable to impaired lymphoid, but not myeloid, reconstitution (**Fig. 3F-H**). At week 13, total cellularity in bone marrow and spleen was decreased in Glast-CreERT;DTA mice, primarily due to the loss of lymphoid cells (**Fig. 3I-L**). Conversely, transplantation of Glast-CreERT;DTA bone marrow cells into WT recipients resulted in hematopoietic reconstitution comparable to that of controls, thus excluding a direct effect of tamoxifen exposure or donor genotype on hematopoietic reconstitution (**Fig. 3D**).

To further test whether established hematopoiesis is sensitive to brain astrocyte reactivity-associated perturbation, we transplanted WT bone marrow into Glast-CreERT;DTA and control mice, allowed for full reconstitution, and subsequently induced astrocyte reactivity using tamoxifen (**Fig. 3M**). Consistent with earlier findings, Glast-CreERT;DTA mice showed reduced peripheral WBC counts, spleen weight, and splenocyte numbers, whereas total bone marrow cellularity remained unaffected (**Fig. 3N-Q**). Within the bone marrow, CLP numbers were significantly decreased, concomitant with a reduction in B-cell numbers in bone marrow, spleen, and blood (**Fig. 3R-U**). Other progenitor populations, including GMPs, CMPs, and MEPs,

remained unchanged, except for a modest decrease in LSK cell abundance (**Fig. 3V-W**). Collectively, these results indicate that astrocyte reactivity-associated CNS perturbation impairs lymphopoiesis predominantly through cell-extrinsic alterations in the bone marrow microenvironment rather than through an intrinsic loss of HSPC differentiation potential.

Cholesterol remodeling is associated with selective cholesterol accumulation in CLPs

Having established that lymphoid defects in reactive astrocyte models result from alterations in the bone marrow microenvironment rather than intrinsic HSPC dysfunction, we next sought to identify the mediators of this cross-organ regulatory effect. Astrocytes are metabolically active cells and major producers of bioactive metabolites, raising the possibility that astrocyte reactivity-associated perturbation influences hematopoiesis through metabolic cues. To test this, we performed untargeted metabolomic profiling of brain tissue and bone marrow fluid from Glast-CreERT;DTA and control mice (**Fig. 4A**). Forty-two and eight metabolites were significantly altered in the brain and bone marrow, respectively, with cholesterol being the only metabolite showing increased abundance in both compartments (**Fig. 4B-D**). Consistent with this, the fluorescence intensity of filipin-stained cholesterol was markedly enhanced in the brains of Glast-CreERT;DTA mice (**Fig. 4E-F**). As astrocytes are a principal source of cholesterol in the CNS, we measured the levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and observed that its expression was upregulated in astrocytes of Glast-CreERT;DTA mice relative to controls (**Fig. 4G**). These findings suggested that cholesterol synthesis is elevated in reactive astrocytes, which may explain the increase in cholesterol levels observed in the brain and bone marrow. We next examined whether cholesterol accumulation extends to hematopoietic progenitors. Flow cytometry showed that filipin fluorescence was significantly stronger in CLPs from Glast-CreERT;DTA mice relative to controls, but showed no change in HSCs, GMPs, CMPs, or MEPs (**Fig. 4H; Fig. S4A-D**). These data indicate that astrocyte reactivity-associated perturbation is accompanied by cholesterol remodeling across the brain and bone marrow, with preferential cholesterol accumulation in CLPs. To assess whether excess cholesterol is sufficient to impair lymphopoiesis, we supplemented

OP9 stromal cell/ Lin⁻ bone marrow progenitor co-cultures with cholesterol (**Fig. S5A**). Cholesterol exposure reduced total cell and B220⁺ B-cell numbers (**Fig. S5B-C**), while also decreasing CLP frequency and absolute number (**Fig. S5D-E**). Cell-cycle analysis showed that the G₀ fraction was increased among CLPs, while the S/G₂/M fractions were decreased (**Fig. S5F**), which was accompanied by a decline in the number of pre-B cell CFUs (**Fig. S5G**). These data demonstrate that excess cholesterol directly suppresses CLPs proliferation and B-cell differentiation *in vitro*.

We further examined the effects of systemic cholesterol administration on hematopoiesis *in vivo*. Mice given cholesterol by oral gavage displayed reduced WBC and splenocyte counts (**Fig. S5H-K**). CLP frequency and number were significantly decreased in bone marrow, while B-cell numbers were reduced in peripheral blood and spleen, but remained unchanged in bone marrow (**Fig. S5I-O**). CLPs from cholesterol-treated mice also exhibited elevated intracellular cholesterol levels (**Fig. S5P**). Together, these results indicate that elevated cholesterol is sufficient to recapitulate key aspects of CLP impairment and reduced lymphoid output, supporting cholesterol accumulation as a functional contributor to lymphopoietic defects.

Cholesterol suppresses mTOR signaling and impairs CLP function

Given that excess cholesterol impaired CLP differentiation (**Fig. 4**), we next sought to identify the signaling pathway mediating this effect. mTOR is a central regulator of nutrient sensing, progenitor proliferation, and lineage specification(24, 25). Although high-fat diet-mediated mTOR hyperactivation has been linked to myeloid-biased hematopoiesis(26), how excess cholesterol affects mTOR activity in lymphoid progenitors remains unclear. We found that cholesterol supplementation in *in vitro* CLP cultures reduced the levels of pS6 levels, which serves as a readout of mTOR activity (**Fig. 5A**). Similarly, pS6 expression was decreased in CLPs from both cholesterol-exposed mice and tamoxifen-treated Glax-CreERT;DTA mice (**Fig. S5Q; Fig. 5B-C**), indicating that elevated cholesterol levels suppress mTOR signaling in these cells.

To evaluate whether restoration of mTOR signaling could mitigate lymphoid defects, we treated Glax-CreERT;DTA mice with MHY1485, an mTOR agonist. MHY1485 administration partially

rescued spleen weight and total splenocyte counts (**Fig. 5D-G**), concurrently increasing both the proportion and absolute number of CLPs in bone marrow (**Fig. 5H**), and restoring pS6 levels in these cells (**Fig. 5I**). Moreover, MHY1485 treatment reduced the proportion of CLPs in the G₀ phase and increased that in the S/G₂/M phases, consistent with enhanced proliferative activity (**Fig. 5J**). Functionally, MHY1485 treatment also partially rescued B-cell numbers in bone marrow and spleen (**Fig. 5K-M**). Together, these findings suggest that cholesterol impairs CLP proliferation and lymphoid differentiation, at least in part, by suppressing mTOR signaling, and that pharmacological activation of mTOR can partially reverse these defects.

Targeting cholesterol synthesis or mTOR signaling partially ameliorates lymphoid defects

Given the association between cholesterol remodeling and impaired bone marrow lymphopoiesis (**Figs. 4,5**), we next asked whether reducing cerebral cholesterol levels alleviated these defects. For this, simvastatin (Sim), an HMGCR inhibitor that can be activated by NaOH to bypass first-pass metabolism, was delivered intracerebroventricularly (ICV) into Glast-CreERT;DTA mice to suppress cerebral cholesterol synthesis (**Fig. 6A**). Filipin staining confirmed that both brain and bone marrow cholesterol levels were reduced in Sim-treated Glast-CreERT;DTA mice (**Fig. 6B-C**), suggesting that brain-derived cholesterol can influence systemic cholesterol distribution.

Functionally, ICV Sim administration partially restored WBC counts, spleen cellularity, and spleen size (**Fig. 6D-F**). Notably, both the frequency and absolute number of CLPs in the bone marrow were significantly increased (**Fig. 6G**), whereas HSPC and myeloid progenitors were unaffected (**Fig. S6A-F**). CLPs from Sim-treated mice exhibited reduced intracellular cholesterol content (**Fig. 6H**), normalization of cell-cycle distribution with fewer cells in G₀ (**Fig. 6I**), and restoration of mTOR signaling activity, as indicated by increased pS6 expression (**Fig. 6J**). These changes were accompanied by a partial rescue of B-cell numbers in the bone marrow, spleen, and peripheral blood (**Fig. 6K-M**). Notably, oral Sim administration yielded comparable results (**Fig. S7A-O**). These results indicate that reducing cholesterol biosynthesis can ameliorate CLP dysfunction and impaired lymphoid output *in vivo*.

To examine the clinical relevance of cholesterol-associated lymphoid regulation in CNS

inflammatory disease, we assessed whether serum cholesterol levels correlated with peripheral lymphocyte parameters in patients with neuroimmune disorders. We analyzed peripheral blood samples from 25 patients diagnosed with autoimmune encephalitis (AE, $n = 8$) or neuromyelitis optica (NMO, $n = 17$), two conditions associated with astrocyte reactivity(27, 28). Spearman correlation analysis revealed that total serum cholesterol levels were inversely correlated with absolute lymphocyte counts in AE, NMO and the combined cohort (**Fig. S8A-C**). Similarly, negative correlations were observed between cholesterol levels and lymphocyte proportions among total WBCs across all groups (**Fig. S8D-F**). Although observational, these clinical data support the existence of a link between elevated systemic cholesterol levels and impaired lymphopoiesis in human disease, reinforcing the translational relevance of the astrocyte-cholesterol-lymphoid axis identified in murine models. Overall, these findings support a model in which astrocyte reactivity-associated cholesterol remodeling is coupled to cholesterol accumulation and mTOR suppression in CLPs, thereby limiting lymphoid progenitor function and B-cell lymphopoiesis.

Discussion

In this study, we identify a cholesterol-dependent pathway linking astrocyte reactivity-associated CNS perturbation to impaired bone marrow lymphopoiesis. Using complementary Glast- and GFAP-lineage perturbation models, we found that reactive gliosis was accompanied by selective loss and dysfunction of CLPs, reduced B-cell output, and largely preserved HSC and myeloid progenitor compartments. Mechanistically, these hematopoietic changes were associated with cholesterol remodeling across the brain and bone marrow, preferential cholesterol accumulation in CLPs, and suppression of mTOR signaling. These findings suggest a non-neuronal, metabolite-associated route through which CNS perturbation may influence lymphoid progenitor function and systemic immune homeostasis.

Previous studies have established sympathetic and hormonal pathways as key regulators of hematopoietic stem cell maintenance and mobilization (7, 29-35). Our findings extend this framework by linking astrocyte reactivity-associated CNS perturbation to the selective impairment

of lymphoid progenitor function. Unlike systemic metabolic stress or mTOR hyperactivation, which often promotes myeloid-biased hematopoiesis(25, 26, 36-40), the phenotype observed here was characterized by preferential CLP dysfunction and reduced B-cell output, while HSCs and myeloid progenitors were largely preserved. This lineage-restricted response suggests that CLPs may be particularly sensitive to cholesterol remodeling and mTOR suppression. Thus, beyond classical neuronal and endocrine routes, CNS perturbations may influence hematopoietic output through metabolite-associated mechanisms that converge on defined progenitor populations.

Our findings also place cholesterol–mTOR regulation of CLPs within a broader multi-organ network that governs hematopoiesis. A growing body of work demonstrates that extramedullary organs regulate hematopoietic output through endocrine, neural, and metabolic signals(41-51). By linking astrocyte reactivity-associated CNS perturbation to cholesterol remodeling and lymphoid progenitor dysfunction, our study expands the role of the brain beyond classical neuronal control and suggests that non-neuronal CNS responses may contribute to systemic hemato-immune regulation.

Functionally, pharmacological inhibition of cholesterol biosynthesis or restoration of mTOR signaling partially ameliorated CLP function and B-cell differentiation defects *in vivo*, supporting the functional relevance of cholesterol–mTOR signaling in this process. Consistent with this concept, we observed an inverse association between serum cholesterol levels and peripheral lymphocyte parameters in patients with autoimmune encephalitis and neuromyelitis optica, neurological conditions frequently accompanied by CNS inflammation and astrocyte reactivity. Although these clinical observations are correlative and based on a limited cohort, they are consistent with a potential link between cholesterol-related metabolic alterations and lymphoid immune dysregulation in CNS inflammatory diseases.

Several key questions remain. Although the convergent phenotypes observed in Glast- and GFAP-lineage perturbation models support a link between astrocyte reactivity-associated CNS perturbation and impaired lymphopoiesis, these models do not fully establish astrocyte-

autonomous causality. Glast and GFAP expression may not be restricted exclusively to astrocytes in all biological contexts, and contributions from other CNS or peripheral cell populations cannot be completely excluded. In addition, the precise cholesterol species, circulating carrier, and route by which CNS-associated cholesterol remodeling is coupled to cholesterol accumulation in bone marrow CLPs remain to be determined. Candidate mediators may include oxidized cholesterol species, apolipoproteins, or extracellular vesicles. Whether this pathway intersects with local bone marrow niche signals such as IL-7 or CXCL12 also warrants further investigation.

In summary, our study suggests a cholesterol-dependent pathway linking astrocyte reactivity-associated CNS perturbation to mTOR-dependent suppression of CLP function and impaired lymphopoiesis. By connecting CNS glial reactivity, cholesterol remodeling, and lymphoid progenitor dysfunction, this work expands the neuro-hematopoietic paradigm and identifies a potentially targetable metabolic route through which CNS pathology may influence systemic immune homeostasis.

Materials and methods

Mice and housing conditions

Glast-CreERT2, DTA, GFAP-Cre and DTR mice were obtained from the Shanghai Model Organisms Center. Fgd5-CreERT2 and B6.Cg-Gt (ROSA) 26S mice were provided by Professor Hui Peng (University of Science and Technology of China, USTC), and BoyJ CD45.1 mice were obtained from Professor Haiming Wei (USTC). Fgd5-creER/tdTomato mice were generated by crossing Fgd5-CreERT2 mice with B6.Cg-Gt (ROSA) 26S mice; the Glast-CreERT2;DTA line was generated by crossing Glast-CreERT2 mice with DTA mice; and GFAP-Cre;DTR mice were generated through crossing GFAP-Cre with DTR mice. All strains were maintained on a C57BL/6 background and housed under specific pathogen-free conditions with free access to food and water. All mouse experiments were performed in accordance with the National Guidelines for Animal Usage in Research (China) and approved by the Ethics Committee of USTC (Reference No. USTCACUC25120125012).

Cell lines

OP9 cells were a kind gift from Dr. Huan Li. The cells were maintained in MEM- α medium (Gibco, Cat# 12561-056) supplemented with 20% fetal bovine serum (FBS; Sigma, Cat# F7524) and 1% penicillin-streptomycin-amphotericin (Biosharp, Cat# BL142A), hereafter referred to as complete medium. The cell line was authenticated by short tandem repeat (STR) profiling and confirmed negative for mycoplasma contamination.

Human samples

Peripheral blood samples were retrospectively obtained from 25 adult patients (aged 21-75 years; median age, 48 years) diagnosed with AE ($n = 8$) or NMO ($n = 17$), at the Second Affiliated Hospital of Anhui Medical University. Their clinical characteristics are provided in **Table S1**. All participants provided written informed consent, and the study protocol was approved by the Ethics Committee of USTC (Reference No. 2022-KY-062). Inclusion criteria included a confirmed clinical diagnosis based on established criteria. Exclusion criteria comprised current statin therapy, active infection, hematologic malignancy, or immunosuppressive treatment within 4 weeks before sample collection.

Whole blood was collected via standard venipuncture and processed within 2 hours. Both absolute lymphocyte counts and serum total cholesterol levels were measured at the central clinical laboratory of the Second Affiliated Hospital of Anhui Medical University. Lymphocyte counts were obtained from routine complete blood count analysis, and cholesterol levels were determined using standard enzymatic colorimetric assays.

Culture of primary astrocyte from neonatal mice

Cerebral cortices were aseptically isolated from 2-3-day-old neonatal mice following isoflurane anesthesia. Meninges were removed and cortices were minced into $\sim 1 \text{ mm}^3$ fragments, which were digested in DMEM containing 0.25% trypsin for 30 min at 37 °C with gentle mixing every 10 min. Digested tissue was centrifuged ($300 \times g$, 5 min, 4 °C), resuspended in astrocyte culture medium (IMDM supplemented with 10% FBS and penicillin/streptomycin/amphotericin B), and

trituated to obtain a single-cell suspension. Cells were plated in poly-D-lysine-coated flasks and maintained at 37 °C with 5% CO₂. The medium was replaced after 48h, and subsequently refreshed every 3 days. At day 7-8, cultures were shaken first at 180 rpm for 30 min to detach microglia, and then at 240 rpm for 6 h to eliminate oligodendrocyte precursor cells. The adherent astrocyte layer was washed twice with phosphate-buffered saline (PBS), detached with trypsin, and subcultured. For functional assays, astrocytes were treated with tamoxifen or diphtheria toxin (24 h), and apoptosis was quantified by flow cytometry.

Hematopoietic progenitor analysis and functional assays

For hematopoietic progenitor cell analysis, bone marrow cells were flushed from femurs and tibias and washed. Then approximately 5×10^6 cells were stained for 30 min at 4 °C in PBS with the following antibodies: PE-CD11b (BioLegend, 101208), PE-B220 (BioLegend, 103208), PE-CD3 (BioLegend, 100308), PE-Ter119 (BioLegend, 116208), PE-Gr-1 (BioLegend, 108408), APC-c-Kit (BioLegend, 105812), APC-Cy7-Sca-1 (BioLegend, 108126), PE-Cy7-CD16/32 (BioLegend, 101318), AF700-CD34 (eBioscience, 56-0341-82), PerCP-Cy5.5-CD127 (BioLegend, 135022), BV510-CD48 (BioLegend, 103443), and BV425-CD150 (BioLegend, 115926). HSCs were defined as Lin⁻Sca-1^c-Kit⁺CD150⁺CD48⁻, CLPs as Lin⁻c-Kit^{low}Sca-1^{low}CD127⁺, CMPs as Lin⁻c-Kit⁺Sca-1⁻CD16/32^{med}CD34⁺, GMPs as Lin⁻c-Kit⁺Sca-1⁻CD16/32^{high}CD34⁺, and MEPs as Lin⁻c-Kit⁺Sca-1⁻CD16/32^{med/low}CD34⁻.

For CLP cell cycle analysis, 1×10^7 freshly isolated bone marrow cells were stained with lineage and progenitor markers (PE-CD11b, PE-B220, PE-CD3, PE-Ter119, PE-Gr-1, APC-c-Kit, APC-Cy7-Sca-1) for 30 min at 4°C, fixed, permeabilized using the Fixation/Permeabilization Concentrate and Diluent Kit (ThermoFisher, 00-5521-00), stained with BV650 conjugated Ki-67 antibody (Biolegend, 151215), and counterstained with DAPI (10 mg/mL).

For apoptosis assays, freshly isolated bone marrow cells were stained with CLP markers, incubated with APC-Annexin V (2.5 mg/mL) and DAPI (10 mg/mL), and immediately analyzed. Data were acquired on a Beckman CytoFlex flow cytometer and analyzed using FlowJo v10.

Isolation and immunophenotyping of brain-infiltrating immune cells

Brains from Glast-CreERT;DTA, GFAP-Cre;DTA, or control mice were harvested and minced into ~1 mm³ fragments in 2 mL of pre-chilled PBS. Tissue fragments were transferred to 15 mL tubes and digested in 5 mL of DMEM containing DNase I (100 U/mL; BioFroxx, 1211MG010) and collagenase IV (10 µg/mL; Biosharp, BS165-1g) at 37 °C (220 rpm) for 1 h, with inversion every 15 min. Following digestion, the cells were centrifuged (300 × g, 5 min, 4 °C), resuspended in 3 mL of 30% Percoll (Cytiva life sciences, 17089101) in a 15 mL tube. After centrifugation (300 × g, 30 min, 18 °C), immune cells were collected in the bottom of the tube, washed with 10 mL of PBS, centrifuged again (300 × g, 5 min, 4 °C), and treated with 1 mL RBC lysis buffer (BioLegend, 420301) for 5 min at 4 °C protected from light. The reaction was stopped with 10 mL of PBS, after which the cells were again centrifuged and resuspended in 1 mL of PBS. Cells were blocked in 100 µL of PBS containing 10% rat serum (Sigma-Aldrich, R9759), and stained at 4 °C for 30 min with the following antibodies: BV510-CD45 (BioLegend, 103138), APC-Cy7-CD3 (BioLegend, 100222), APC-B220 (BioLegend, 103212), PE-CD11b (BioLegend, 101208), and PerCP-Cy5.5-Gr-1 (BioLegend, 108428). After washing, the cells were analyzed on a Beckman CytoFlex flow cytometer and processed using FlowJo v10.

Colony-formation assay

Fresh bone marrow cells were seeded at a density of 1 × 10⁵ cells/mL in methylcellulose-based medium (MethoCult GF M3630, StemCell Technologies) in six-well plates, and cultured in a humidified incubator at 37 °C with 5% CO₂ for 7 days. Colony-forming units were counted manually under an optical microscope.

***In vitro* B cell differentiation assay**

Lin⁻ cells were isolated from the bone marrow of Glast-CreERT;DTA, GFAP-Cre;DTA, and littermate control mice using flow cytometry. A total of 50,000 Lin⁻ cells were resuspended in culture medium (90% DMEM, 10% FBS, 10 ng/mL IL-7 [Novoprotein, cc73], 20 ng/mL Flt3L [Novoprotein, cc19], and penicillin/streptomycin/amphotericin B), and seeded onto OP9 stromal cell-coated culture plates. The culture medium was replaced every 3 days. After 6 days, the cells

were harvested, filtered through a 70-µm sterile cell strainer (Biosharp, BS-70-XBS), and replated onto fresh OP9-coated plates. On day 12, the cells were collected and stained with BV510-CD45 (BioLegend, 103138), APC-Cy7-CD3 (BioLegend, 100222), and APC-B220 (BioLegend, 103212), and analyzed by flow cytometry.

To assess the impact of cholesterol on B-cell differentiation, 50,000 Lin⁻ cells from wild-type mice were cultured under the same conditions as above, but with 30 µg/mL cholesterol (MCE, HY-N0322) added to the medium. After 12 days, the cells were counted and assessed for CLP and B-cell frequencies by flow cytometry as described above.

Competitive repopulation assay

Fresh bone marrow cells (2×10^6) from Glast-CreERT;DTA (CD45.2⁺) mice were mixed 1:1 with bone marrow cells (2×10^6) from congenic BoyJ (CD45.1⁺) mice and transplanted into lethally irradiated (12 Gy) BoyJ recipient mice *via* tail vein injection. Peripheral blood was collected monthly post-transplantation for the flow cytometric assessment of donor-derived chimerism (CD45⁺) across hematopoietic lineages. Lineage composition was determined using antibodies against CD3, B220, Gr-1, and CD11b. At 4 months post-transplantation, recipient mice were euthanized, and the bone marrow and spleen were harvested to evaluate multilineage chimerism. For secondary transplantation, 1×10^6 bone marrow cells from primary recipients were transferred into lethally irradiated BoyJ mice. Donor-derived reconstitution (CD45⁺) in peripheral blood, bone marrow, and spleen was assessed by flow cytometry at 1, 2, 3, and 4 months post-secondary transplantation using the above-described staining and analysis protocols.

***In vivo* tracking of HSC differentiation using inducible lineage tracing**

Fresh bone marrow cells were isolated from Fgd5-CreERT2;tdTomato mice and intravenously transplanted into lethally irradiated (12 Gy) Glast-CreERT;DTA or control recipient mice at a dose of 1×10^6 cells per mouse. Six weeks post-transplantation, tamoxifen (120 mg/kg) was intraperitoneally administered for 5 consecutive days to induce tdTomato labeling in donor-derived HSCs. Two weeks after tamoxifen treatment, mice were sacrificed, and the differentiation of tdTomato⁺ cells into various hematopoietic lineages was assessed by flow cytometry. For

hematopoietic progenitor cell analysis, the following panel was used: FITC-Lineage cocktail (BioLegend, 133302), APC-c-Kit (BioLegend, 105812), APC-Cy7-Sca-1 (BioLegend, 108126), PE-Cy7-CD16/32 (BioLegend, 101318), AF700-CD34 (eBioscience, 56-0341-82), PerCP-Cy5.5-CD127 (BioLegend, 135022), BV510-CD48 (BioLegend, 103443), and BV425-CD150 (BioLegend, 115926). For mature immune lineage analysis, BV510-CD45 (BioLegend, 103138), APC-B220 (BioLegend, 103212), AC750-CD3 (BioLegend, 100330), FITC-CD11b (BioLegend, 101206), and PerCP-Cy5.5-Gr-1 (BioLegend, 108428). Data were acquired on a Beckman CytoExpert flow cytometer and analyzed using FlowJo v10.

Intracerebroventricular injection

ICV injections were performed as previously described (52), with minor modifications. Briefly, Glax-CreERT;DTA and littermate controls were anesthetized with 2.5% avertin (300 mg/kg, prepared with 0.25 g of 2,2,2-tribromoethanol [Sigma T48402] and 0.25 mL of 2-methyl-2-butanol [Sigma 240486]) via intraperitoneal injection. Erythromycin ophthalmic ointment was applied to the eyes to prevent corneal drying. The fur over the scalp was shaved, and the animal was placed in a stereotaxic frame. The surgical site was disinfected with iodophor, and a midline scalp incision (~1-1.5 cm) was made to expose the skull. The periosteum was gently removed with sterile cotton swabs to expose bregma and lambda. Bregma served as the reference point (0, 0, 0) to calibrate the stereotaxic coordinates. Using predetermined coordinates (AP - 0.5 mm, ML + 1.0 mm relative to bregma), a small opening was made in the skull using a dental drill. A microinjection needle connected to a Hamilton syringe was slowly lowered vertically to a depth of DV - 2.2 mm and was left in place for 1-2 min to allow for tissue equilibration and to reduce backflow. Sim (6 µg/µL in vehicle) or vehicle control was injected into the lateral ventricle at a rate of 1 µL/min, for a total volume of 2 µL. After injection, the needle was held in position for an additional 5 min and then slowly retracted. The incision was closed using sutures or surgical wound clips. Mice were placed in a clean recovery cage on a pre-warmed (37 °C) heating pad and monitored until fully recovered.

Immunoblotting

CLPs ($1-2 \times 10^5$) sorted from bone marrow were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5],

150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) at 4 °C for 1 h. The lysate was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatants were mixed with 5 × SDS-PAGE loading buffer (Biosharp, BL502A) and boiled for 10 min. Protein was separated by 10% SDS-PAGE (NCM biotech, P2200) and transferred onto PVDF membranes by wet transfer. The membranes were blocked for 1 h at room temperature with 5% fat-free milk dissolved in TBST, followed by incubation with primary antibodies overnight at 4 °C. After three 10-min washes in TBST, the membranes were incubated with HRP-conjugated secondary antibodies and washed again. Signals were detected using enhanced chemiluminescence and imaged a chemiluminescence imaging system (SHST, Compact 523).

Immunofluorescence staining

Mouse brains were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and sliced into 20-µm sections using a cryostat. The sections were mounted on glass slides, blocked in 1× PBS containing 1% BSA, 0.25% Triton X-100, and 10% goat serum at room temperature for 1 h, and incubated with primary antibodies overnight at 4 °C. After three 10-min washes in PBS on a shaker, the samples were incubated with secondary antibodies for 1 h at room temperature, counterstained with DAPI, mounted with antifade medium, and coverslipped.

For bone sections, fresh mouse leg bones were fixed, washed with ddH₂O, and decalcified in 14% EDTA (pH 7.4) at 4 °C for 3 days. Samples were then cryoprotected in 30% sucrose, cryosectioned at 20 µm, and stained using the same protocol as for brain sections. For cholesterol staining, sections were incubated with filipin III (50 µg/mL; Sigma, SAE0087-1ML) at 4 °C in the dark for 30 min before mounting. Fluorescence images were acquired using a Leica Thunder system.

Metabolomic analysis

Mouse brains and bone marrow fluids were rapidly collected and flash-frozen in liquid nitrogen. For extraction, tissues were placed in 2-mL centrifuge tubes containing two steel beads and 600 µL of a pre-cooled solution of 10% formic acid:methanol:water (1:1:1, v/v). Samples were vortexed for 30 s, homogenized using a tissue grinder at 55 Hz for 90 s, and centrifuged (12,000

rpm, 5 min, 4°C). For low-abundance metabolite detection, 100 µL of the supernatant was mixed with 100 µL of internal standard solution (10 ng/mL Trp-d3), vortexed, filtered through a 0.22-µm membrane, and transferred to LC-MS vials. For high-abundance metabolite detection, an appropriate volume of the supernatant was diluted 1:200 with the same extraction solution, and prepared as described above. Metabolomic profiling was performed using liquid chromatography (ExionLC, SCIEX) coupled with tandem mass spectrometry (Triple Quad™ 6500+, SCIEX). For data normalization, raw metabolite intensities were divided by the total ion intensity per sample. Differential metabolite abundance was assessed using two-tailed Student's *t*-tests implemented in the R stats package (v4.1.2).

Statistical analyses

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (s.d.). Normality was assessed using the Shapiro-Wilk test. For two-group comparisons, unpaired, two-tailed Student's *t*-tests were used when normality and equal variance (F-test) assumptions were met. For comparisons among three or more groups, one-way analysis of variance (ANOVA) with Tukey's post hoc test was used for normally distributed data with homogeneous variances, while the Kruskal-Wallis test with Dunn's correction was employed for non-parametric data. To assess clinical relevance, Spearman's rank correlation was used to evaluate potential associations between serum total cholesterol levels and absolute lymphocyte counts in patient samples. Normality was evaluated before correlation testing. Flow cytometric data and microscopic images are representative of at least three independent biological replicates. The number of biologically independent replicates for each experiment is indicated in the corresponding figure legends.

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Conflict of interests

The authors declare no competing interests.

Contributions

F.N. conceived and conducted the project; F.N. and R.Z. designed the experiments; R.Z., X.S., and Y.L. conducted the research and summarized the data; J.D. and X.S. collected clinical human samples, tested, and analyzed the data; R.Z. and T.L. analyzed the metabolomics data; R.Z. contributed to mouse model development and cell culture; Z.Y., L.Q., M.Z., J.Z., X.L., and X.H. provided critical advice on experimental design and data interpretation; H.Z. and C.K. provided comments and suggestions; R.Z., X.S., and F.N. wrote the manuscript; F.N. supervised the project.

Availability of Data and Materials

The data supporting the findings of this study are available within the paper. Untargeted metabolomics data generated in this study have been deposited in the China National Center for Bioinformation (CNCB) under accession number OMIX011805.

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Fig. 1

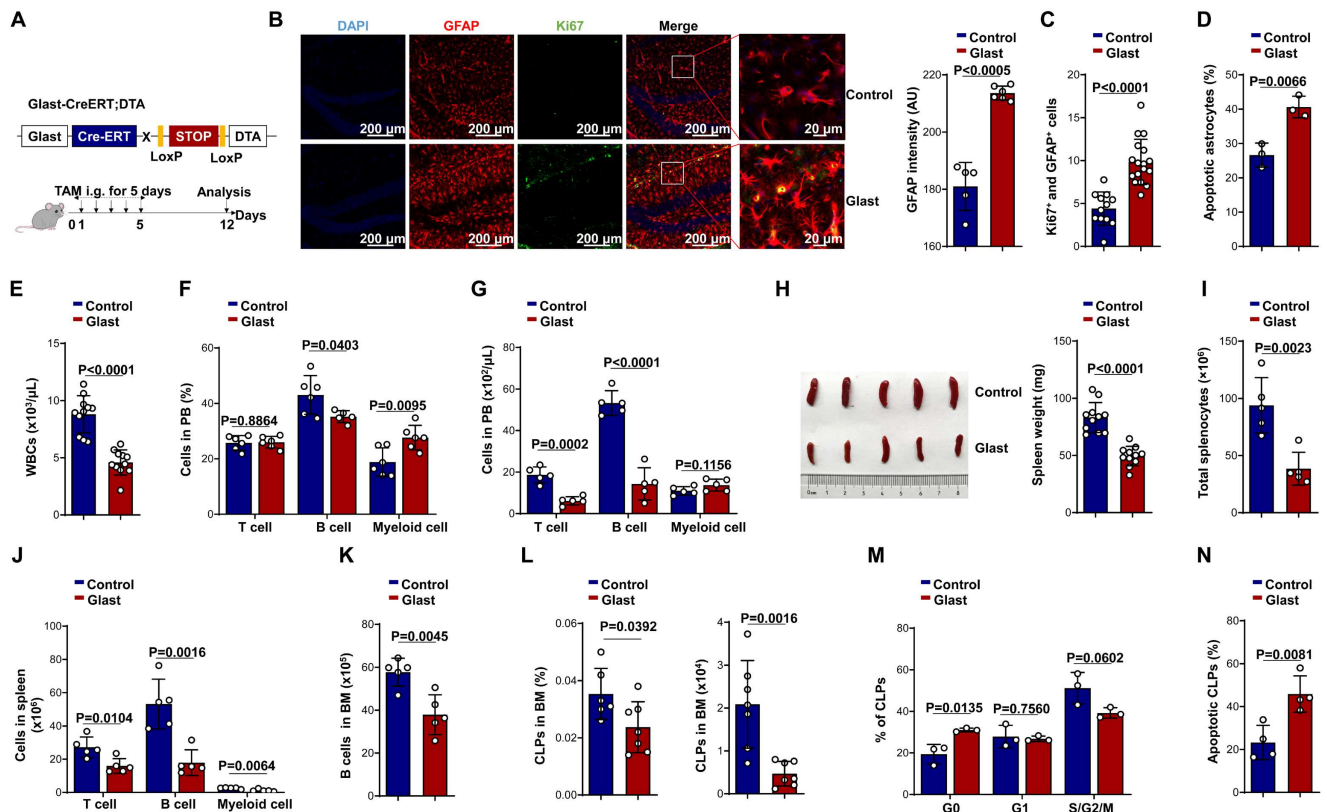


Fig. 1: Slc1a3/Glast-lineage CNS perturbation elicits reactive gliosis and selectively reduces common lymphoid progenitors.

A, Schematic of the Glast-CreERT;DTA mouse model. Tamoxifen (120 mg/kg) was administered by oral gavage for 5 consecutive days, and mice were sacrificed on day 12. **B**, Representative immunofluorescence staining for GFAP (red), Ki-67 (green), and nuclei (DAPI, blue) in the hippocampus of Glast-CreERT;DTA and control mice. Insets highlight proliferating GFAP⁺ astrocytes ($n = 5$ per group). **C**, Quantification of GFAP⁺ and Ki-67⁺ cells in the hippocampus ($n = 17$ for Glast-CreERT;DTA; $n = 12$ for controls). **D**, Flow cytometric analysis of apoptosis in cultured astrocytes from Glast-CreERT;DTA or control mice following tamoxifen treatment ($n = 3$ per group). **E**, Total white blood cell (WBC) counts in peripheral blood of Glast-CreERT;DTA and control mice ($n = 12$ per group). **F, G**, Frequencies (F) and absolute numbers (G) of T cells, B cells, and myeloid cells in peripheral blood of Glast-CreERT;DTA and control mice ($n = 5-6$ per group). **H**, Representative image of spleens (left) and quantification of spleen weight (right) from control and Glast-CreERT;DTA mice ($n = 11$ per group). **I**, Total splenocyte counts in Glast-CreERT;DTA and control mice ($n = 5$ per group). **J**, Absolute numbers of T cells, B cells, and

686 myeloid cells in the spleens of Glast-CreERT;DTA and control mice ($n = 5$ per group). **K**, Absolute
687 numbers of B cells in the bone marrow of Glast-CreERT;DTA and control mice ($n = 5$ per group).
688 **L**, Frequencies (left) and absolute numbers (right) of common lymphoid progenitors (CLPs) in the
689 bone marrow of Glast-CreERT;DTA and control mice ($n = 6$ per group). **M**, Cell cycle analysis of
690 CLPs in the bone marrow of Glast-CreERT;DTA and control mice ($n = 3$ per group). **N**, CLP
691 apoptosis (Annexin V⁺) in bone marrow of Glast-CreERT;DTA and control mice ($n = 4$ per group).
692 Data are presented as mean $\pm$ s.d. *P* values were calculated using two-tailed unpaired *t*-tests
693 between Glast-CreERT;DTA and control groups. All experiments were independently repeated
694 at least twice.

Fig. 2

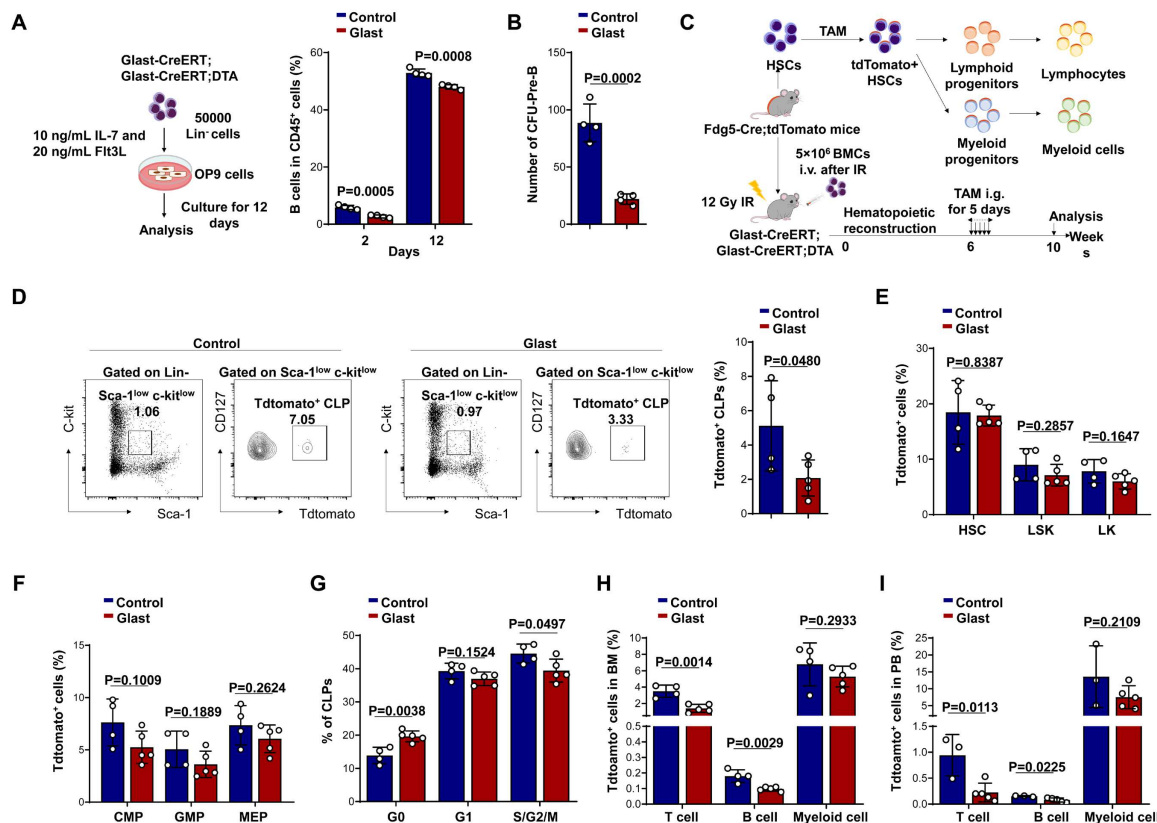


Fig. 2: Astrocyte reactivity-associated perturbation impairs CLP generation and B-cell differentiation.

A, *In vitro* B-cell differentiation assay. Lineage-negative (Lin⁻) cells (5×10^4) from Glast-CreERT;DTA or control mice were co-cultured with OP9 stromal cells in the presence of IL-7 and Flt3L for 12 days. The percentage of B220⁺ B cells was assessed by flow cytometry on day 2 and day 12 ($n = 4$ per group). **B**, Colony-formation assay for bone marrow (BM) cells from Glast-CreERT;DTA and control mice. Lymphoid progenitor-derived colonies were quantified after 12 days of culture ($n = 4$ per group). **C**, Schematic of hematopoietic stem cells (HSCs) lineage tracing. Bone marrow cells from Fgd5-CreER;tdTomato mice were transplanted into lethally irradiated Glast-CreERT;DTA or control recipients. At 6 weeks post-transplantation, tamoxifen was administered (120 mg/kg) for 5 consecutive days to induce tdTomato expression in donor-derived HSCs. Mice were analyzed 4 weeks later. **D-F**, Flow cytometric analysis of tdTomato⁺ cells in the BM of recipient mice. The percentages of tdTomato⁺ CLPs (**D**), HSCs, LSK cells, LK cells (**E**), and granulocyte-monocyte progenitors (GMPs), common myeloid progenitors (CMPs), and megakaryocyte-erythroid progenitors (MEPs) (**F**) are shown ($n = 5$ for Glast-CreERT;DTA; $n = 4$ for Control).

710 $n = 4$ for controls). **G**, Cell cycle analysis of CLPs showing the percentage of cells in the G_0 , G_1 ,
711 and $S/G_2/M$ phases in recipient mice ($n = 5$ for Glast-CreERT;DTA; $n = 4$ for controls). **H-I**,
712 Frequencies of tdTomato⁺ T (CD3⁺), B cells (B220⁺), and myeloid cells (CD11b⁺ and Gr-1⁺) in the
713 BM (**H**) and peripheral blood (**I**) ($n = 5$ for Glast-CreERT;DTA; $n = 3-4$ for controls). Data are
714 presented as mean $\pm$ s.d. P values were calculated using two-tailed unpaired t -tests. All
715 experiments were independently repeated at least twice.

A

Glast-Cre mice or Glast-CreERT;DTA mice (TAM, i.g., CD45.2*)

Mix 1:1

1st transplantation

12 Gy IR

2nd transplantation

12 Gy IR

Analysis

WT BoyJ mice (CD45.1*)

CD45.1 Recipient

CD45.1 Recipient

B

Control

Glast

CD45.2* cells in BM (%)

1 Primary transplantation

2 Secondary transplantation

C

Control

Glast

Primary transplantation

Secondary transplantation

CD45.2* cells in BM (%)

T cell

B cell

Myeloid cells

D

Transplantation

BM cells WT

BM cells WT

BM cells WT

Glast-CreERT (Control)

Glast-CreERT;DTA

WT

TAM i.g. for 5 days

PB analysis

Analysis

Weeks

E

WT → Ctrl

WT → Glast

Glast → WT

WBCs ($10^3/\mu\text{L}$)

Weeks

F

WT → Ctrl

WT → Glast

Glast → WT

T cells in PB ($10^2/\mu\text{L}$)

Weeks

G

WT → Ctrl

WT → Glast

Glast → WT

B cells in PB ($10^2/\mu\text{L}$)

Weeks

H

WT → Ctrl

WT → Glast

Glast → WT

Myeloid cells in PB ($10^3/\mu\text{L}$)

Weeks

I

WT → Ctrl

WT → Glast

Glast → WT

BM cells in two femurs and two tibias ($\times 10^3$)

J

WT → Ctrl

WT → Glast

Glast → WT

T cells in BM ($\times 10^3$)

B cells in BM ($\times 10^3$)

Myeloid cells in BM ($\times 10^3$)

K

WT → Ctrl

WT → Glast

Glast → WT

Cells in spleen ($\times 10^3$)

L

WT → Ctrl

WT → Glast

Glast → WT

T cells in SP ($\times 10^3$)

B cells in SP ($\times 10^3$)

Myeloid cells in SP ($\times 10^3$)

M

WT BM cells 5×10^6 i.v. after IR

12 Gy IR

Hematopoietic reconstruction

TAM i.g. for 5 days

Analysis

Weeks

Glast-CreERT; Glast-CreERT;DTA

N

Control

Glast

WBCs ($10^3/\mu\text{L}$)

O

Control

Glast

Spleen weight (mg)

P

Control

Glast

Total splenocytes ($\times 10^3$)

Q

Control

Glast

Cells in two femurs and two tibias ($\times 10^3$)

R

Control

Glast

CLPs in BM (%)

CLPs in BM ($\times 10^3$)

S

Control

Glast

Cells in PB ($\times 10^2/\mu\text{L}$)

T cell

B cell

Myeloid cell

T

Control

Glast

Cells in SP ($\times 10^3$)

T cell

B cell

Myeloid cell

U

Control

Glast

Cells in BM ($\times 10^3$)

T cell

B cell

Myeloid cell

V

Control

Glast

Cells in BM ($\times 10^3$)

HSC

LSK

LK

W

Control

Glast

Cells in BM ($\times 10^3$)

GMP

CMP

MEP

A, Schematic of competitive bone marrow (BM) transplantation. BM cells (CD45.2⁺) from Glast-CreERT;DTA or control mice were mixed 1:1 with CD45.1⁺ competitor BM cells and transplanted into lethally irradiated recipient mice. **B-C**, Donor chimerism analysis in recipient BM following primary (**B**) and secondary (**C**) transplantation. The percentages of CD45.2⁺ donor-derived cells were assessed by flow cytometry ($n = 3-6$ per group). **D**, Schematic of reciprocal transplantation.

723 Wild-type (WT) BM cells were transplanted into irradiated Glast-CreERT;DTA or control mice,
724 and Glast-CreERT;DTA BM cells were transplanted into WT recipients. Tamoxifen (TAM, 120
725 mg/kg, i.g.) was administered daily for 5 days beginning at week 6 post-transplantation. **E-H**,
726 Time-course analysis of white blood cells (**E**), CD3⁺ T cells (**F**), B220⁺ B cells (**G**), and CD11b⁺Gr-
727 1⁺ myeloid cells (**H**) in peripheral blood at 4, 8, and 12 weeks post-transplantation ($n = 3-5$ per
728 group). **I-L**, Quantification of absolute numbers of T, B, and myeloid cells in BM (**I, J**) and spleen
729 (**K, L**) at week 13 ($n = 4-5$ per group). **M**, Schematic of WT-to-Glast-CreERT;DTA transplantation.
730 WT BM cells were transplanted into irradiated Glast-CreERT;DTA or control mice. After 6 weeks
731 of reconstitution, TAM was administered for 5 consecutive days, and mice were analyzed at week
732 8. **N-W**, Hematopoietic profiling of recipient mice. Peripheral blood white blood cell (WBC) counts
733 (**N**), spleen weights (**O**), and total splenocyte numbers (**P**); BM cellularity (**Q**), frequencies and
734 absolute numbers of common lymphoid progenitors (CLPs) in BM (**R**); absolute numbers of T, B,
735 and myeloid cells in peripheral blood (PB) (**S**), spleen (SP) (**T**), and BM (**U**); absolute numbers of
736 hematopoietic stem cells (HSCs), LSK cells, LK cells (**V**); and common myeloid progenitors
737 (CMPs), granulocyte-monocyte progenitors (GMPs), and megakaryocyte-erythroid progenitors
738 (MEPs) (**W**) in BM ($n = 5$ for Glast-CreERT;DTA; $n = 4-5$ for controls). Data are presented as
739 mean $\pm$ s.d. P values were calculated using two-tailed unpaired t -tests (**B, C, N-W**) or two-way
740 ANOVA with Tukey's post hoc test (**E-H**). All experiments were independently repeated at least
741 twice.

Fig. 4

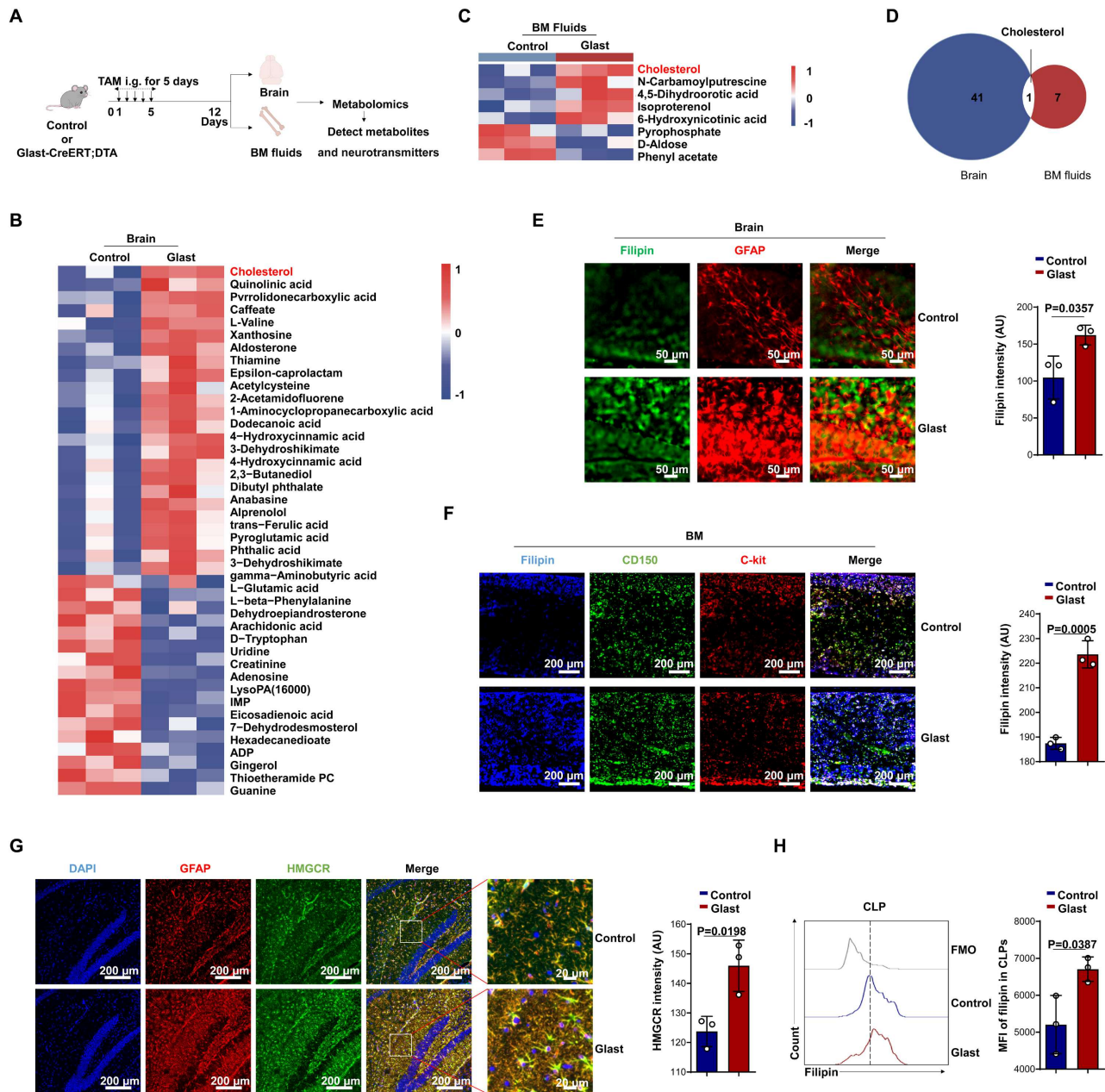


Fig. 4: Slc1a3/Glast-lineage perturbation is associated with brain–bone marrow cholesterol remodeling and selective cholesterol accumulation in CLPs.

A, Schematic of the untargeted metabolomics workflow. Metabolites were extracted from the brain and BM fluid of Glax-CreERT;DTA and control mice, then subjected to LC-MS/MS analysis and metabolite identification. **B–C**, Heatmaps showing differential metabolites ($P < 0.05$, two-tailed unpaired t -test) in the brain (**B**) and BM fluid (**C**) of Glax-CreERT;DTA versus control mice ($n = 3$ per group). **D**, Venn diagram showing overlapping differential metabolites between the

749 brain and BM fluid in Glast-CreERT;DTA mice. **E**, Representative immunofluorescence images
750 of filipin (green, cholesterol), GFAP (red, astrocytes), and DAPI (blue, nuclei) staining in
751 hippocampal sections from Glast-CreERT;DTA and control mice ($n = 3$ per group). **F**,
752 Representative immunofluorescence staining for filipin (blue, cholesterol), CD150 (green,
753 hematopoietic stem cell marker), and c-Kit (red, progenitor marker) in BM sections from Glast-
754 CreERT;DTA and control mice ($n = 3$ per group). **G**, Representative immunofluorescence staining
755 for GFAP (red), HMGCR (green, cholesterol synthesis enzyme), and nuclei (DAPI, blue) in
756 hippocampal sections. Insets show high-magnification views ($n = 3$ per group). **H**, Flow cytometric
757 analysis of filipin fluorescence intensity (mean fluorescence intensity, MFI) in CLPs from Glast-
758 CreERT;DTA and control mice ($n = 3$ per group). Data represent mean $\pm$ s.d. *P* values were
759 determined using two-tailed unpaired *t*-tests. All experiments were independently repeated at
760 least twice.

Fig. 5

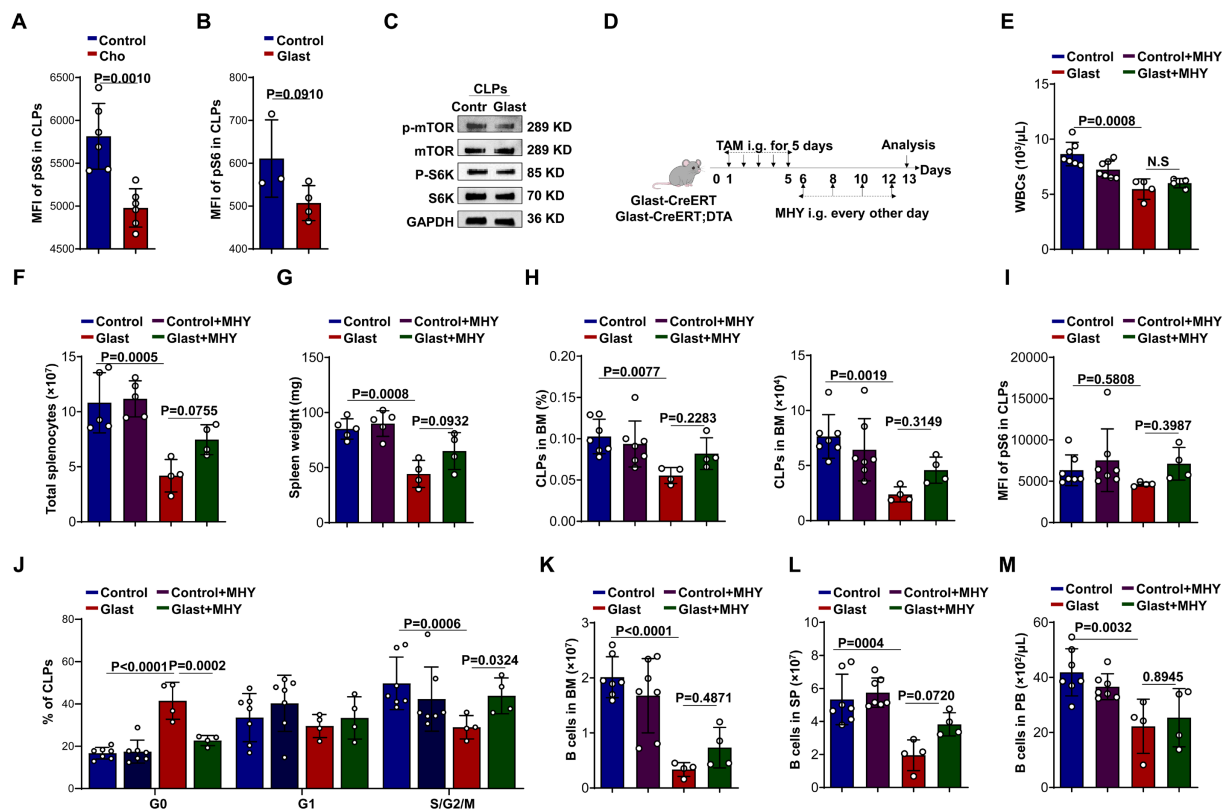


Fig. 5: Cholesterol suppresses mTOR signaling in CLPs and mTOR activation partially ameliorates lymphoid defects.

A, Flow cytometric analysis of phosphorylated S6 (pS6) fluorescence intensity (mean fluorescence intensity, MFI) in common lymphoid progenitors (CLPs) isolated from wild-type mice and cultured with cholesterol (30 $\mu\text{g/mL}$) or vehicle control for 20 min ($n = 6$ per group). **B**, Flow cytometric analysis of pS6 levels in CLPs from Glast-CreERT;DTA and control mice ($n = 4$ for Glast-CreERT;DTA and $n = 3$ for controls). **C**, Western blot analysis of phosphorylated and total mTOR and S6K levels in sorted CLPs from Glast-CreERT;DTA and control mice. GAPDH served as a loading control. Representative blots from three independent experiments are shown. **D**, Schematic of MHY1485 (MHY) treatment. Glast-CreERT;DTA and control mice were administered tamoxifen (120 mg/kg/day) by oral gavage for 5 days, followed by intraperitoneal injection of MHY (10 mg/kg) once every 2 days for 6 days. Mice were euthanized on day 13. **E**, Peripheral blood white blood cell (WBC) counts in Glast-CreERT;DTA, control, and MHY-treated groups ($n = 4-7$ per group). **F-G**, Total splenocyte numbers (**F**) and spleen weights (**G**) in the

775 experimental groups ($n = 5$ per group). **H**, CLP frequencies (left) and absolute numbers (right) in
776 bone marrow (BM) of the indicated groups ($n = 4-7$ mice per group). **I**, Flow cytometric analysis
777 of pS6 levels in CLPs from Glast-CreERT;DTA, control, and MHY-treated mice ($n = 4-7$ per group).
778 **J**, Cell cycle distribution of CLPs showing percentages in the G_0 , G_1 , and S/ G_2 /M phases ($n = 4-$
779 7 per group). **K-M**, Absolute numbers of B220⁺ B cells in peripheral blood (**K**), spleen (**L**), and
780 BM (**M**) from Glast-CreERT;DTA, control, and MHY-treated mice ($n = 4-7$ per group). Data are
781 presented as mean $\pm$ s.d. P values were calculated using two-tailed unpaired t -tests in **A** and **B**,
782 and two-way ANOVA followed by Tukey's post hoc test in **E-M**. All experiments were
783 independently repeated at least twice.

Fig. 6

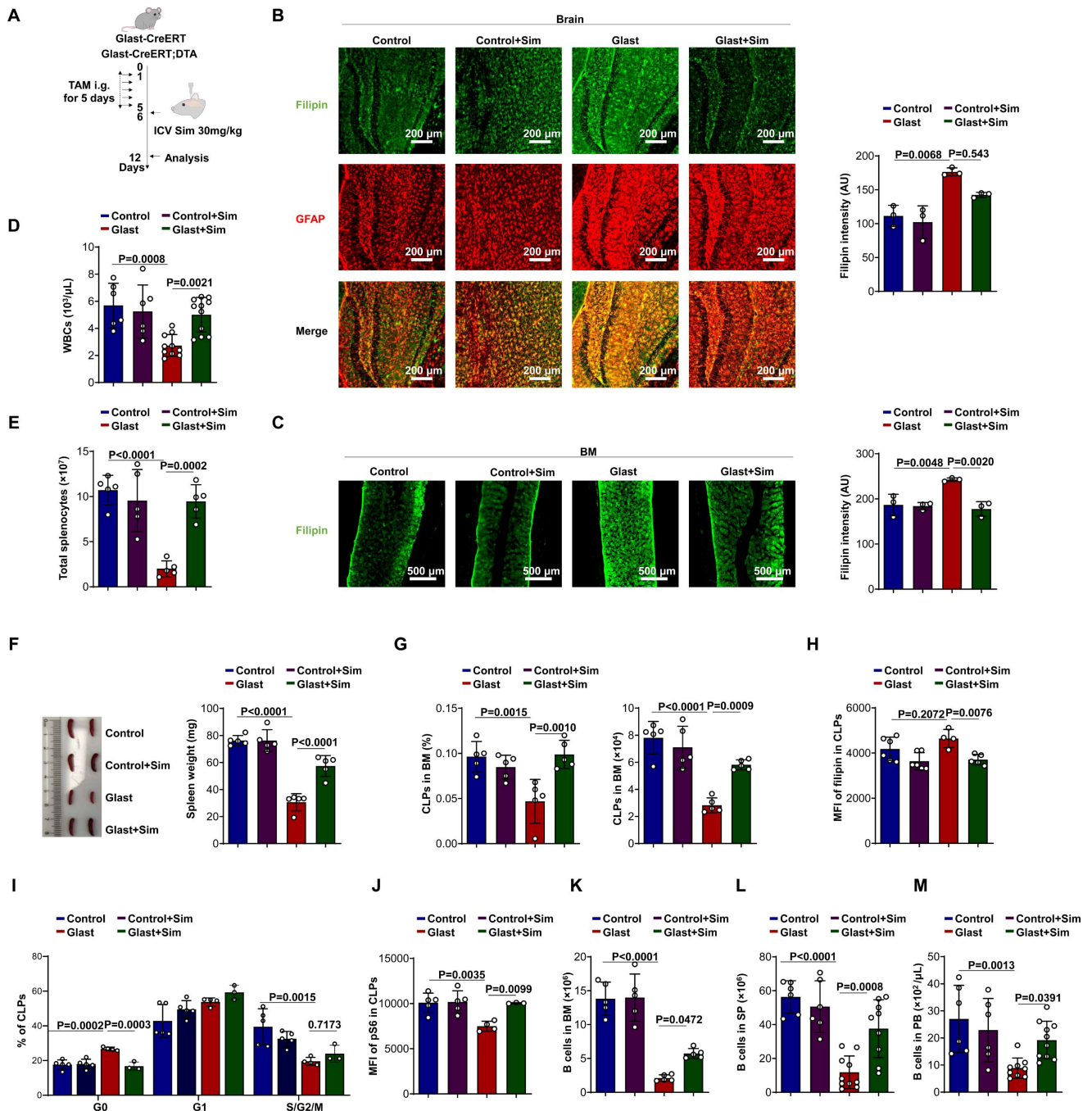


Fig. 6: Intracerebroventricular simvastatin reduces cholesterol accumulation and partially ameliorates lymphoid defects in Glax-CreERT;DTA mice.

A, Schematic of the experimental design. Glax-CreERT;DTA mice and littermate controls were administered tamoxifen (120 mg/kg) once daily for 5 consecutive days. On day 6, mice received an intracerebroventricular injection of simvastatin (30 mg/kg) or vehicle. Mice were sacrificed on day 12. **B**, Representative immunofluorescence images of hippocampal sections from the indicated groups stained for filipin (cholesterol, green), GFAP (astrocytes, red), and nuclei (DAPI,

791 blue). Scale bar, 200 μ m (n = 3 per group). **C**, Representative immunofluorescence images of
792 bone marrow (BM) cryosections stained with filipin (green). Scale bar, 500 μ m (n = 3 per group).
793 **D**, Peripheral blood white blood cell counts in Glast-CreERT;DTA, control, and simvastatin-
794 treated groups (n = 6-11 per group). **E-F**, Total splenocyte counts (**E**) and spleen weights (**F**)
795 across the indicated groups. (n = 5 per group). **G**, Flow cytometric quantification of common
796 lymphoid progenitors (CLPs) in BM of Glast-CreERT;DTA, control, and simvastatin-treated
797 groups. CLP frequencies (left) and absolute numbers (right) are shown (n =5 per group). **H**, Filipin
798 signal intensity in CLPs from the indicated groups (n = 4-6 per group). **I**, CLP cell cycle distribution
799 showing the percentages in the G₀, G₁, and S/G₂/M phases (n = 3-5 per group). **J**, pS6 levels in
800 CLPs as determined by flow cytometry (n = 3-5 per group). **K-M**, Absolute numbers of B220⁺ B
801 cells in BM (**K**), spleen (**L**), and peripheral blood (**M**) across groups (n = 5-10 per group). Data
802 are presented as mean $\pm$ s.d. P values were determined by two-way ANOVA followed by Tukey's
803 post hoc test. All experiments were independently repeated at least twice.

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