

Published in final edited form as:

*Blood Cancer Discov.* 2021 January 01; 2(1): 32–53. doi:10.1158/2643-3230.BCD-20-0155.

## Phingosine-1-phosphate receptor 3 potentiates inflammatory programs in normal and leukemia stem cells to promote differentiation

Stephanie Z. Xie<sup>1,\*</sup>, Kerstin B. Kaufmann<sup>#1</sup>, Weijia Wang<sup>#2</sup>, Michelle Chan-Seng-Yue<sup>#1,3</sup>, Olga I. Gan<sup>1</sup>, Elisa Laurenti<sup>1,4</sup>, Laura Garcia-Prat<sup>1</sup>, Shin-ichiro Takayanagi<sup>1,5</sup>, Stanley W.K. Ng<sup>6</sup>, ChangJiang Xu<sup>7,8</sup>, Andy G. X. Zeng<sup>1,14</sup>, Liqing Jin<sup>1</sup>, Jessica McLeod<sup>1</sup>, Elvin Wagenblast<sup>1</sup>, Amanda Mitchell<sup>1</sup>, James A. Kennedy<sup>1,9,10</sup>, Qiang Liu<sup>1</sup>, H el ena Boutzen<sup>1</sup>, Melissa Kleinau<sup>1</sup>, Joseph Jargstorf<sup>1</sup>, Gareth Holmes<sup>1</sup>, Yang Zhang<sup>2</sup>, Veronique Voisin<sup>7,8</sup>, Gary D. Bader<sup>7,8</sup>, Jean C.Y. Wang<sup>1,9,10</sup>, Yusuf A. Hannun<sup>11</sup>, Chiara Luberto<sup>12</sup>, Timm Schroeder<sup>2</sup>, Mark D. Minden<sup>1,9,10,13</sup>, John E. Dick<sup>1,14,\*</sup>

<sup>1</sup>Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada

<sup>2</sup>Department of Biosystems Science and Engineering, ETH Z urich, 4058 Basel, Switzerland

<sup>3</sup>PanCuRx Translational Research Initiative, Ontario Institute for Cancer Research, Toronto, Ontario, Canada <sup>4</sup>Wellcome Trust - Medical Research Council Cambridge Stem Cell Institute, Department of Haematology, University of Cambridge, Cambridge, United Kingdom <sup>5</sup>Cell Therapy Project, R&D Division, Kirin Holdings Company, Limited <sup>6</sup>Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada <sup>7</sup>The Donnelly Centre, University of Toronto, Toronto, Ontario, M5S3E1, Canada <sup>8</sup>Department of Molecular Genetics, University of Toronto, Toronto, Ontario, M5S1A8, Canada <sup>9</sup>Division of Medical Oncology and Hematology, Department of Medicine, University Health Network, Toronto, Ontario, Canada <sup>10</sup>Department of Medicine, University of Toronto, Toronto, Ontario, Canada <sup>11</sup>Stony Brook Cancer Center and Departments of Medicine, Biochemistry, and Pathology, Stony Brook University, Stony Brook, NY, USA <sup>12</sup>Department of Physiology and Biophysics, Stony Brook School of Medicine, Stony Brook, USA <sup>13</sup>Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada <sup>14</sup>Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

# These authors contributed equally to this work.

\* Corresponding Authors: Stephanie Z. Xie, Princess Margaret Cancer Centre, PMCRT, 8th Floor, Rm 8-362, 101 College Street, Toronto, Ontario, M5G 1L7, Tel:416-581-7470, Fax: 416-581-7476, sxie@uhnresearch.ca; John E. Dick, Princess Margaret Cancer Centre PMCRT, 8th Floor, Rm 8-358 101 College Street, Toronto, Ontario, M5G 1L7, Tel: 416-581-7472, Fax: 416-581-7476, jdick@uhnres.utoronto.ca.

### Author contributions

S.Z.X. conceived the study, performed research, and wrote the manuscript. K.B.K. and O.G. assisted with normal *S1PR3*OE experiments. W.W. and Y.Z. performed time-lapse imaging. E.L. assisted with the FTY720 drug study. L.J., J.M., A.M., Q.L., H.B., M.K., J.J., G.H. assisted with research. L.G.-P. and S.T. generated the normal CB hierarchy data, M.C.Y., C.X., S.W.K.N., A.G.X.Z., and V.V. performed bioinformatics analyses. M.D.M. provided AML and BM samples. C.L. assisted in lipid measurements. C.L. and Y.A.H. assisted in lipid data interpretations. G.D.B., T.S., and J.C.Y.W. supervised specific experiments. K.B.K., J.C.Y.W., T.S., Y.A.H., M.D.M., and J.E.D. edited the manuscript. J.E.D. supervised the study.

### Conflict of interests

J.E.D. Celgene: research funding; Trillium Therapeutics: Advisory Board. All other authors declare no conflicts of interests.

## Abstract

Acute myeloid leukemia (AML) is a caricature of normal hematopoiesis, driven from leukemia stem cells (LSC) that share some hematopoietic stem cell (HSC) programs including responsiveness to inflammatory signaling. Although inflammation dysregulates mature myeloid cells and influences stemness programs and lineage determination in HSC by activating stress myelopoiesis, such roles in LSC are poorly understood. Here, we show that S1PR3, a receptor for the bioactive lipid sphingosine-1-phosphate, is a central regulator which drives myeloid differentiation and activates inflammatory programs in both HSC and LSC. S1PR3-mediated inflammatory signatures varied in a continuum from primitive to mature myeloid states across AML patient cohorts, each with distinct phenotypic and clinical properties. S1PR3 was high in LSC and blasts of mature myeloid samples with linkages to chemosensitivity, while S1PR3 activation in primitive samples promoted LSC differentiation leading to eradication. Our studies open new avenues for therapeutic target identification specific for each AML subset.

## Keywords

Acute myeloid leukemia; hematopoietic stem cells; S1PR3; myeloid differentiation; TNF $\alpha$  via NF- $\kappa$ B

---

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by impaired myeloid differentiation that is hierarchically organized akin to the normal blood system(1–4). Leukemia stem cells (LSC) possess properties of self-renewal and lineage differentiation and are responsible for long-term clonal propagation in AML, similar to their normal long-term hematopoietic stem cells (LT-HSC) counterpart that sustains lifelong blood production(2). LSC are often resistant to standard chemotherapy and are responsible for clinical relapse(2,5–7). AML is a cellular hierarchy, driven by LSC that share many LT-HSC stemness properties, but remain impaired for normal myeloid lineage development(3,4,7). How myeloid fate networks at the stem cell level become perturbed during leukemogenesis remains poorly understood. Most studies of how HSC choose to differentiate into one lineage versus another have focused on the role of cytokines and the transcriptional factor networks that they engage(8,9). In parallel, investigations of AML primarily focus on how cytokines and their signaling networks are impaired by AML driver oncogenes(3,8,10,11). However, compelling evidence is emerging that lineage determination is not solely the consequence of lineage-specific cytokine exposure and signaling, but rather that metabolites generated from many pathways can play a role(12). Cellular metabolism is recognized as a hallmark of cancer and is known to be distinct between HSC and their downstream progenitors(12,13). We recently showed that sphingolipid composition is diverse across the human hematopoietic hierarchy and uncovered a novel role for sphingolipid metabolism in determining HSC fate(14). Sphingosine-1-phosphate (S1P) is a potent bioactive sphingolipid crucial for systemic health, particularly in disorders associated with inflammation(15–17). As an obligate ligand for a family of five G-protein coupled receptors (S1PR1–5), S1P plays pleiotropic roles in cellular proliferation, survival, and migration, processes that are dysregulated in inflammatory diseases(15,16,18,19). Notably, within the murine system S1P

signaling via S1PR1 regulates B-lymphopoiesis and neuroinflammation(20). In humans, S1P receptor modulators are used to target immune cells to treat multiple sclerosis, a chronic inflammatory disease(18,21–23). Aside from mature lineage cells, inflammation has pleiotropic effects within primitive hematopoietic cells, governing both lineage determination and HSC stemness functions<sup>10,11</sup>. There is also a role in hematopoietic malignancies, although studies exploring inflammation across the individual cells that make up the leukemia hierarchy are limited(11). Aging is also associated with increased inflammation, dysregulated lineage determination and myeloid skewing, and is a major risk factor for developing AML(24,25). Some reports suggest that both complex membrane sphingolipids such as sphingomyelin that are catabolized for S1P production, as well as levels of S1P itself are dysregulated during aging(26–28). Overall, the role of S1P signaling in the interplay between inflammation and normal hematopoiesis or in the development of stem cell malignancies like AML is poorly understood.

Dysregulated myeloid cells are the linchpin of inflammation within many chronic human conditions associated with aging including clonal hematopoiesis and atherosclerotic cardiovascular disease(29–31). Tumor necrosis factor (TNF $\alpha$ ) and interleukin-6 (IL-6) are two key inflammatory cytokines linked to chronic inflammatory diseases, myeloid dysregulation and stem cell function(11,23,32). Moreover, a large body of literature points to nuclear factor  $\kappa$  B (NF- $\kappa$ B), originally described as a gatekeeper for inflammatory control of immune cell responses, as a key transcriptional factor in the regulatory network activated by these inflammatory cytokines and other inflammatory inputs in normal and malignant cells of various tissues(11,33). Importantly, NF- $\kappa$ B is a crucial regulator of inflammatory activation in HSC and regulates quiescence, proliferation and survival particularly upon TNF $\alpha$  stimulation(23). NF- $\kappa$ B was shown to be constitutively active in LSC from AML patients(33). At the time, it was anticipated that modulating inflammation through inhibiting some of the key signaling molecules including TNF $\alpha$ , IL-6 and NF- $\kappa$ B could be exploited therapeutically for treating AML. However, some of these same pathways, for example TNF $\alpha$  via NF- $\kappa$ B, may actually promote differentiation and loss of LSC self-renewal(34). Uncovering novel lineage regulators that interact with such differentiation-inducing inflammatory programs would be pivotal for parsing the functional differences between the different cell types within each AML cell hierarchy and between different patients(3,35,36). Such studies could yield novel targets for developing therapeutic approaches focused on driving differentiation of LSC and blasts. Here, we show that the S1P signaling axis via S1PR3 promotes myeloid differentiation in human HSC, is dysregulated in AML, and intersects with the TNF $\alpha$  via NF- $\kappa$ B pathway. Furthermore, perturbation of this axis in AML leads to LSC eradication introducing a previously unreported therapeutic avenue for AML.

## Results

### **S1PR3 is an inflammation-activated S1P receptor regulating lineage differentiation in human HSC**

To gain deeper insight into the function of S1P in human hematopoiesis, we undertook gene expression analysis of ten S1P-related genes including its five receptors (S1PR1-5) and

enzymes involved in its synthesis/degradation across six human hematopoietic stem and progenitor cell (HSPC) populations and seven mature lineages from cord blood (CB) (Garcia-Prat, *et al.* (37)). Expression of *S1PR1*, *3* and *5* was most enriched in specific mature lineages (Fig. 1A). *S1PR1* was most highly expressed in T and B lymphoid, natural killer (NK), and dendritic cell (DC) populations, whereas *S1PR5* was restricted to NK and DC lineages, consistent with published reports primarily in the murine system(16,20). *S1PR3* was of particular interest as its expression was specific to monocytes and granulocytes and it had not been previously implicated in myeloid fate specification in either normal or malignant human hematopoiesis. Flow cytometric analysis confirmed that S1PR3 protein was highly expressed on the surface of CD33<sup>+</sup> myeloid cells, particularly within the CD14<sup>+</sup> subset of mononuclear cells where the majority of CD14<sup>+</sup> cells showed co-staining with S1PR3 (Fig. 1B-C; Supplementary Fig. 1A-H). Notably, S1PR3 was expressed at low levels in primitive CD34<sup>+</sup> cells and was not detectable in LT-HSC or other HSPC populations except for a very small proportion (~3%) of granulocyte-monocyte progenitors (GMP, Fig. 1B-D, Supplementary Fig 1H-J). These findings raised the question whether S1PR3 plays a role in myeloid differentiation.

As HSPC subpopulations are primarily quiescent until activated by mitogenic stimulation(14,38), we asked whether cellular activation alone or with an acute inflammatory stimulus would impact S1PR3 in primitive CB cells. Following 3 days of culture in a low cytokine media when the majority of LT- and ST-HSC have not yet undergone the first division from quiescence(38) (See Supplementary Fig. 2A,B for experimental scheme and HSPC gating scheme), we observed a 3-fold increase in the proportion of S1PR3<sup>+</sup> cells within the GMP subset over uncultured GMP; IL6 further enhanced the percentage of S1PR3<sup>+</sup> GMP cells 4.6-fold over uncultured GMP (Fig 1D-F). Whereas S1PR3 was not detected on the surface of uncultured LT-HSC (Fig 1D, Supplementary Fig. 1I-J), TNF $\alpha$  treatment in culture was sufficient to promote expression of S1PR3 on a small but significant population of immunophenotypic cultured LT-HSC (cLT-HSC) (0.5% S1PR3<sup>+</sup> cells in control media vs. 1.3% S1PR3<sup>+</sup> with TNF $\alpha$  treatment), despite no significant change in the percentage of cLT-HSC (Fig. 1D-F, Supplementary Fig. 2C). Moreover, TNF $\alpha$  treatment reduced surface S1PR3 expression on GMP at 3 days in culture to levels similar to those in uncultured GMP, and provoked loss of CD34<sup>+</sup> cells at 8 days culture with no significant effects on CD14 differentiation (Fig. 1D-G, Supplementary Fig. 2D, E), consistent with the distinct effects of TNF $\alpha$  on LT-HSC and GMP previously reported in the murine system(23). In contrast, IL6 treatment tended to increase expression of the CD14 differentiation marker following 8 days of culture (Supplementary Fig. 2E). These data suggest that surface expression of S1PR3 is regulated by pro-inflammatory cytokines with the potential for influencing stemness and lineage differentiation in response to acute demands.

To determine if S1PR3 activation is sufficient to regulate human HSC function, we utilized a lentiviral system(39) to enforce *S1PR3* overexpression (OE) coupled to blue fluorescent protein (BFP) for marking transduced cells. *S1PR3*OE cells exhibited increased levels of surface S1PR3 in the progeny of LT-HSC and short-term (ST)-HSC by flow cytometry relative to control cells (Supplementary Fig. 3A). *S1PR3*OE in LT-HSC and ST-HSC resulted in enhanced CD14<sup>+</sup> myeloid differentiation and decreased GlyA<sup>+</sup> erythroid

differentiation compared to controls following 9 to 10 days of culture (Fig. 1H-J). Next, we asked what effect perturbing *S1PR3* expression in human HSC subpopulations would have on lineage differentiation in culture. Two lentiviral knockdown (KD) constructs to *S1PR3* were generated that decreased *S1PR3* gene expression to less than 50% of control (shCtrl) in a cell line model (Supplementary Fig. 3B). There was a trend towards enhanced erythroid differentiation with both *S1PR3* KD constructs in ST-HSC while only shS1PR3-2 in LT-HSC increased GlyA<sup>+</sup> expression (Fig. 1K; Supplementary Fig. 3C). However, the level of *S1PR3* KD with these constructs did not appear to be sufficient to affect CD14<sup>+</sup> myeloid differentiation *in vitro* (Supplementary Fig. 3D). These data indicate that *S1PR3* is a myeloid-associated S1P receptor that regulates lineage differentiation in human HSC.

### ***S1PR3* overexpression is sufficient to promote myeloid differentiation in human hematopoietic stem and progenitor cells *in vitro* and *in vivo***

To determine if *S1PR3*OE was promoting myeloid differentiation in HSPC subpopulations with multilineage differentiation capacity at the single cell level, transduced (BFP<sup>+</sup>) LT-HSC, ST-HSC, common myeloid progenitor (CMP)-F1 and megakaryocyte erythroid progenitor (MEP)-F1 were isolated 3 days post-infection and plated in colony forming cell (CFC) or single cell MS5 stromal assays(14) (see Supplementary Fig. 3E,F for experimental design and HSPC sorting scheme). *S1PR3*OE did not significantly decrease clonogenic output by any HSPC population except MEP-F1 (Fig. 2A). However, the colony type distribution was significantly altered, with an increase in macrophage colonies generated from LT-HSC, ST-HSC and CMP-F1 populations and a marked decrease of erythroid burst forming unit (BFU) colonies for all subpopulations relative to controls (Fig. 2B). The alteration in myeloid-erythroid lineage distribution was confirmed by flow cytometry for one representative CFC assay (Fig. 2C). Following single cell plating on MS5 stroma(40,41), *S1PR3*OE reduced unilineage erythroid output (Fig. 2D) as well as total erythroid proliferative output except by MEP-F1 without decreasing cloning efficiency (Fig. 2E, Supplementary Fig. 3G). Total myeloid output was not disrupted except in LT-HSC (Fig 2F). The percentage of cells undergoing CD41<sup>+</sup> megakaryocytic differentiation was also increased with *S1PR3*OE(42) (Supplementary Fig. 3H). As *S1PR3* KD had a modest effect on erythroid differentiation in liquid culture, we evaluated effects at the single cell level in LT-HSC, ST-HSC and CMP-F1 in CFC assays. There was no alteration in clonogenic output, but *S1PR3* KD with shS1PR3-2 resulted in a modest increase in BFU colonies (Supplementary Fig. 3I-J). These data indicate that *S1PR3*OE is sufficient to promote myeloid differentiation on the single cell level at the expense of erythroid differentiation *in vitro*.

To investigate effects on lineage output *in vivo*, we xenotransplanted *S1PR3*OE CD34<sup>+</sup>CD38<sup>-</sup> CB cells, containing a mixture of transduced and non-transduced cells, into immunodeficient mice and evaluated engraftment and lineage differentiation after 4 weeks. Compared to controls, *S1PR3*OE significantly reduced overall human CD45<sup>+</sup> engraftment as well as the proportion of BFP<sup>+</sup> transduced cells (Fig. 2G,H; Supplementary Fig. 4A-C). Mice transplanted with *S1PR3*OE cells generated grafts with a higher proportion of CD33<sup>+</sup> myeloid cells at the expense of CD19<sup>+</sup> B lymphoid cells (Fig. 2I, J). Moreover, the proportion of GlyA<sup>+</sup> cells that were BFP<sup>+</sup> was lower in *S1PR3*OE engrafted mice, indicating



that *SIPR3*OE reduced erythropoiesis *in vivo* (Supplementary Fig. 4D,E). Overall, our findings demonstrate that *SIPR3*OE promotes myeloid and megakaryocytic differentiation at the expense of the erythroid and lymphoid lineages. These phenotypes are reminiscent of those observed in chronic inflammation-induced myelopoiesis models(22,42).

### ***SIPR3* induces a myeloid inflammatory program in HSC**

To ascertain the molecular and biological pathways altered by *SIPR3*OE in human CB HSCs, we performed RNA-sequencing (RNA-seq) of LT- and ST-HSC 3 days after transduction with control or *SIPR3*OE vectors (Supplementary Fig. 5A, B). Gene set enrichment analysis (GSEA) with the Hallmark gene sets (False Discovery Rate (FDR)  $q$ -value  $<0.01$ , Fig. 3A, Supplemental Table 1) showed that *SIPR3*OE upregulated many of the same pathways in both HSC subsets. Mitotic spindle, G2M checkpoint, and TNF $\alpha$  signaling via NF $\kappa$ B (NF- $\kappa$ B; Fig. 3B) were the top three enriched gene sets in LT-HSC relative to controls, suggesting that *SIPR3*OE induced an activated inflammatory program. Inflammation gene sets were enriched in *SIPR3*OE ST-HSC, with genes related to the interferon alpha response, interferon gamma response and NF- $\kappa$ B being the top 3 enriched gene sets (Fig. 3A, Supplemental Table 1; Fig. 3B). The differential expression patterns of selected inflammation and NF- $\kappa$ B related genes in *SIPR3*OE HSCs compared to controls resembled those of uncultured mature myeloid lineages relative to HSC subpopulations including TNF and IL6 receptor genes (IL6R, IL6T) (Fig 3C). Notable exceptions were a downregulation of IL1B(22) and upregulation of IL24(43) in LT-HSC, highlighting the complex interplay of pro- and anti-inflammatory processes governing human HSC fate. These data could also provide an explanation for why *SIPR3*OE was not sufficient to induce a *de novo* AML in xenografts in contrast to the malignancy described in a *SIPR3* transgenic mouse model(44). NF- $\kappa$ B and TNF $\alpha$  have been associated with S1P signaling in other contexts(15,45) and shown to regulate HSC survival and fate decisions(9,23). Hence, we performed a focused analysis comparing the significantly enriched leading edge genes from the NF- $\kappa$ B Hallmark gene set (Supplemental Table 2) and the genes that were differentially expressed between controls and *SIPR3*OE HSC subsets (Supplemental Table 3,  $q < 0.05$ , fold change=1). This identified a common set of eight myeloid lineage-associated genes including genes encoding the known myeloid differentiation factors Early Growth Response 1 and 2 (*EGR1/2*) and AML-associated Tribbles 1 (*TRIB1*)(46–49) (Fig. 3D). These results suggest that *SIPR3* regulates the well-known *ERG-NAB-GFI1* myeloid gene regulatory network(46) in human HSC. GSEA using a published human hematopoietic gene expression data set(50) showed that *SIPR3*OE LT- and ST-HSC had significant positive enrichment of granulocyte genes and negative enrichment for HSC genes, providing independent confirmation of myeloid lineage induction (Fig. 3E, Supplementary Fig. 5C,D). *SIPR3*OE cells resembled granulocytes with not only strong upregulation of inflammation gene sets but also downregulation of HSC-enriched metabolic gene sets (Supplementary Fig. 5E). If *SIPR3*OE promotes differentiation via an inflammation-induced emergency hematopoiesis-like mechanism, then *SIPR3*OE LT-HSC should exhibit cell cycle activation and a shorter division time as has been observed in murine HSC following treatment with inflammatory cytokines(22). Indeed, live cell imaging analysis(51) demonstrated that *SIPR3*OE resulted in faster cell division kinetics and better survival of LT-HSC compared to controls (Fig. 3E-H, Supplemental Movie). Overall, this strong induction of inflammatory gene sets upon

*S1PR3*OE together with the upregulation of S1PR3 membrane expression on HSC by TNF $\alpha$  treatment suggest that S1PR3 may function as a downstream potentiator of inflammatory signaling in human HSC to regulate survival and myeloid fate.

### **S1PR3 marks a subset of LSCs in human AML with a mature myeloid state and a distinct inflammatory signature**

Previous studies have suggested a possible link between inflammation and perturbed myeloid differentiation(30). We therefore investigated the role of S1PR3 in the pathogenesis of AML, a disease of impaired myelopoiesis(3). S1PR3 expression by flow cytometry was higher on CD14<sup>+</sup> myeloid and CD34<sup>+</sup>CD38<sup>-</sup> primitive cell subsets from AML patients (n=22) compared to CB (n=5) and normal adult bone marrow (n=3) (Fig. 4A, B, Supplemental Table 4), although there was considerable inter-patient heterogeneity. In keeping with the observation that AML cells at relapse are typically more primitive than at diagnosis, *S1PR3* gene expression was lower in relapsed patient samples compared to paired diagnosis samples(5) (Fig. 4C). Thus, we hypothesized higher S1PR3 expression may identify less primitive AML cases. We analyzed S1PR3 gene expression in a recently described single cell (sc) RNAseq data set(3) across 11641 malignant cells from 12 AML patients clustered by their similarity to six comparable populations along the normal bone marrow HSC to myeloid axis (HSC-like, progenitor-like, GMP-like, promonocyte-like, monocyte-like or cDC-like) (Fig. 4D). As seen in the normal hierarchy, S1PR3 in AML was enriched exclusively in samples classified as myeloid phenotype (mono-like and promono-like). As this scRNAseq platform was only able to capture the low abundance of S1PR3 transcript in <0.01% of cells, we analyzed transcriptomes from unfractionated AML patients generated by RNAseq or microarray analysis. Gene set variation analysis (GSVA) of the Beat-AML(35), TCGA-AML(52) and GSE6491(53) *de novo* AML datasets revealed that AML cases with the highest *S1PR3* expression were enriched for the myeloid signature and depleted of the HSC/Progenitor signature previously defined using the scRNAseq AML data set(3) (Fig. 4E, Supplemental Tables 5, 6). Hereafter, we will refer to these AML cases with high S1PR3 expression and enrichment of the myeloid signature as myeloid-like and the AML cases with low S1PR3 expression and enrichment of the HSC/progenitor signature as stem-like. Together, these findings point to a strong association between S1PR3 expression and myeloid differentiation in both normal hematopoiesis and AML.

To explore further the role of *S1PR3* in human AML, we investigated the *S1PR3*OE-induced signatures described in Fig 3 in cohorts of AML samples. AUCell (area under the curve Cell) analysis across 11641 single malignant cells revealed that the *S1PR3*OE LT-HSC signature was expressed in 86.5% of cells and most enriched in the mono-like subpopulation of AML patient samples(3) (Fig. 4F, 3A). Importantly, the *S1PR3*OE signature was significantly correlated to a number of inflammatory gene sets including the NF- $\kappa$ B Hallmark gene set, whereas MYC targets were negatively correlated, reminiscent of the phenotype observed in LT-HSC upon *S1PR3*OE (Fig. 3A, 4F, Supplementary Table 7). Moreover, GSVA analysis in an independent RNAseq AML cohort from Princess Margaret (PM) showed strong correlation of both single S1PR3 gene expression and the *S1PR3*OE signature to a number of inflammatory gene sets that were also induced by *S1PR3*OE in HSC (Fig. 4G). These data suggest that the malignancy state in *S1PR3*-expressing AML

cells is congruous with the transcriptional inflammatory state activated by *S1PR3*OE in LT-HSC.

We next investigated whether individual components of the *S1PR3*-mediated inflammatory pathways such as NF- $\kappa$ B were similarly regulated in individual myeloid-like or stem-like AML patients. To this end we derived a common 75 *S1PR3*-NF- $\kappa$ B-related gene signature from the Beat-AML(35), TCGA-AML(52) and GSE6491(53) cohorts ( $p < 0.05$ ) based on GSVA correlation between the NF- $\kappa$ B-Hallmark gene set and *S1PR3* expression, and validated it in the PM cohort (Fig. 4H, Supplementary Fig. 6A, Supplemental Table 8, clinical data in Supplemental Table 9). Importantly, the *S1PR3*-NF- $\kappa$ B-related gene signature also varied in a continuum from stem-like to mature-like myeloid states in functionally-validated LSC-containing (LSC+) cell fractions(7). Similar to observations in bulk patient samples (Fig. 4H, Supplementary Fig. 6A, Supplemental Tables 5, 6, 8), myeloid-like LSC+ and LSC- cell fractions were enriched for the common *S1PR3*-NF- $\kappa$ B signature (Fig. 4I, Supplementary Fig. 6B). The *S1PR3*-NF- $\kappa$ B signature included genes encoding the NF- $\kappa$ B subunits *REL*, *NFKB1*, and *NFKB2*, the anti-apoptotic *BCL2* family members *MCL1* and *BCL2A1*, and energy metabolism/oxidative stress related genes *NAMPT*, *SOD2*, *SLC2A3 (GLUT3)*, *SLC2A6*, and *SLC16A6*. *MYC* was a notable negatively correlated gene, in accordance with downregulation of *MYC* targets upon *S1PR3*OE in normal HSC. These results indicate that NF- $\kappa$ B regulation is heterogeneous amongst individual AML patients and raises the possibility that the NF- $\kappa$ B-*S1PR3* regulatory loop serves different functions in the LSCs isolated from stem-like patients or myeloid-like patients(11). Moreover, Beat-AML cases with high *S1PR3* expression were more resistant to *ex vivo* treatment with the *BCL2* inhibitor venetoclax, consistent with a recent report linking myeloid differentiation in AML and resistance to combination therapy with azacytidine and venetoclax(54,55) (Supplementary Fig. 6C). Overall, these results demonstrate that *S1PR3* expression can classify individual AML patients into two broad categories; more differentiated and more stem-like cases, each with distinct inflammatory, metabolic, stress response and cell survival programs.

### ***S1PR3* selects for less functional LSCs and regulates AML differentiation via TNF $\alpha$ via NF- $\kappa$ B signaling**

As high *S1PR3* expression is associated with a more differentiated phenotype in AML and marks a distinct activated inflammatory state, we evaluated whether *S1PR3* surface expression could resolve LSC+ from LSC- cells. We focused on AML samples classified as stem-like. *S1PR3* gene expression in LSC+ fractions (n=24) from 13 patients in the GSE76008 cohort(7) was highly correlated with *S1PR3* surface expression measured by flow cytometry ( $r=0.78$ , Fig. 5A, Supplemental Tables 10,11). CD34+ LSCs from three of these patients were fractionated based on surface expression of *S1PR3* (1-6% *S1PR3*<sup>+</sup>, see supplementary Fig. 7) and transplanted at limiting dilution into NSG mice (Fig. 5B). *S1PR3*<sup>+</sup> AML cells had reduced engraftment potential at 12 weeks and lower LSC frequency compared to *S1PR3*<sup>low/-</sup> cells (Fig. 5C, D). These data show that *S1PR3* expression is sufficient to identify a subset of cells with low LSC activity in AML patient samples.



To evaluate whether activation of S1PR3 could represent a therapeutic approach to disrupt LSC function in AML, we transduced AML cells with *S1PR3OE* and control vectors. *S1PR3OE* in LSC+ fractions from two AML patient samples virtually abolished leukemic engraftment in xenotransplantation assays (Fig. 5E, F). *S1PR3OE* in a hierarchical AML model and three primitive AML cell lines with low surface expression of S1PR3 resulted in acquisition of CD15 and loss of CD34 expression *in vitro*, suggesting that enforced expression of *S1PR3* is sufficient to promote myeloid differentiation in AML cells (Fig. 5G-I; Supplementary Fig. 8A-E). We found that TNF $\alpha$  enhanced S1PR3 surface expression in both the OCI-AML22 model we derived from a relapse patient (Boutzen, *et al.*(56)) and in Kasumi-1 cells, compared to controls (Fig. 5J; Supplementary Fig. 8F). Next, we interrogated the role of endogenous S1PR3 in AML. The S1PR3 antagonist TY51256 restricted myeloid differentiation in both AML cell line models during *in vitro* culture (Fig. 5K-M; Supplementary Fig. 8G-I). Importantly, in OCI-AML22, TY51256 counteracted the myeloid differentiation enacted by TNF $\alpha$  treatment (Fig. 5K-M). Moreover, shRNA-mediated *S1PR3* KD with shS1PR3-3 in Kasumi-1 CD34+ cells significantly decreased the CD34<sup>-</sup>CD15<sup>+</sup> and CD34<sup>+</sup>CD15<sup>+</sup> subpopulations at nine days post-transduction (Supplemental Fig. 8J). We also measured the intracellular levels of the p65 RelA subunit of NF- $\kappa$ B following *S1PR3OE* and KD in Kasumi-1 cells and found that *S1PR3OE* decreased p65 while shS1PR3-3 increased p65 relative to their respective controls (Fig. 5N). Overall, these findings suggest that S1PR3 is a biomarker that strongly anti-correlates with LSC activity in human AML, engagement of which disrupts LSC function in part by inducing differentiation via the TNF $\alpha$ -NF- $\kappa$ B axis.

### Sphingolipid genes including *S1PR3* predict outcomes in human AML

As no specific S1PR3 agonist has been shown to have *in vivo* efficacy to our knowledge, we asked if there were other possibilities for therapeutic targeting in the sphingolipid pathway. GSEA of functionally-defined LSC+ and LSC- subpopulations from AML patient samples in the GSE76008 data set demonstrated enrichment of *S1PR3* and other S1P pathway genes only in the LSC- fractions (Fig. 6A). *S1PR1*, *S1PR3* and *S1PR5* were in the lowest 10% of analyzed genes expressed in LSC+ fractions (Supplemental Table 12). These findings suggest that downregulation of S1P signaling may be required for LSC maintenance. We previously identified a lipid-stem signature that is distinct between HSC and committed progenitors, and showed that modulation of sphingolipid synthesis alters hematopoietic fate in HSC(14). This lipid-stem signature was enriched in LSC+ compared to LSC- fractions by GSEA (Fig. 6B). Among the significantly enriched genes in LSC+ samples were the *de novo* sphingolipid metabolism genes *CERS6*, *CERS5*, and *SPLTLC2*, suggesting that human AML retains features of sphingolipid regulation found in normal HSCs (Supplementary Table 12) and in particular upregulates the biosynthetic pathway generating S1P via ceramide as an intermediate (see Supplementary Figure 9 for biosynthetic pathway)(14,17).

To determine if sphingolipid levels are dysregulated in LSC+ populations, we profiled the sphingolipidome of LSC+ (n=7) and LSC- (n=7) fractions from ten AML patient samples(7) as well as CB CD34<sup>+</sup>CD38<sup>-</sup> (stem) and CD34<sup>+</sup>CD38<sup>+</sup> (progenitor) populations by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for S1P, sphingosine, ceramides, dihydroceramides (dhCer), hexosylceramides (HexCer, ceramides containing glucose or

galactose) and sphingomyelins (Supplementary Fig. 9A-H, Supplementary Table 9). Notably, dhCer, HexCer, and S1P levels were significantly different in LSC+ and LSC- samples (Supplementary Fig. 9C-E). Clustering samples by sphingolipid composition using uniform manifold approximation and projection (UMAP) showed that six of the seven LSC+ samples were most similar to each other and clustered away from both LSC- samples and CB stem and progenitor populations despite diverse immunophenotypes and molecular subtypes (Fig. 6C, Supplementary Fig. 9B). The seventh LSC+ sample (AML patient 9) that did not cluster with the others showed relatively higher S1PR3 surface and gene expression (See Supplemental Table 12). Thus, we speculated that a differential requirement for sphingolipid metabolism as well as S1PR3 signaling may exist in primitive AML cases (Fig. 4C). Regression analysis(7) of 54 sphingolipid genes including 10 S1P genes trained on survival outcomes in the GSE6891 cohort yielded an 8-gene sphingolipid signature (including *S1PR3* (-0.035), *S1PR5* (-0.183), and *SPHK1* (-0.111) that was strongly associated with overall survival (OS) in the independent GSE12417 and TCGA-AML cohorts (Fig. 6D, E). This signature was enriched in gene expression profiles from diagnostic relative to paired relapse samples by GSEA (Fig. 6F). These data suggest a link between sphingolipid metabolism and S1P signaling with stemness state and response to chemotherapy in subsets of AML patients.

### ***In vivo* S1P signaling modulation disrupts LSC function in human AML**

Since LSC exhibited depletion of S1P signaling genes, and higher *S1PR3* and *S1PR5* levels were associated with better prognosis in our sphingolipid signature, we evaluated the effects of modulating S1P signaling in patient AMLs using xenograft assays. FTY720, a prodrug whose phosphorylation generates the S1P mimetic FTY720-phosphate, possesses both agonist and antagonist activity for four S1P receptors including S1PR3. Clinically known as fingolimod, FTY720 is used for treatment of relapse-remitting multiple sclerosis patients, with the possibility of rapid repurposing for AML (18,21). Treatment of mice bearing primary AML xenografts with FTY720 resulted in decreased leukemia burden for three of twelve patient samples tested (Fig. 7A, B; Supplementary Fig. 10A, Supplemental Table 10). Notably, an interesting trend of modest increase in CD15 expression in the xenografts of the three FTY720 responders (AML1, AMI16, and AML19) was observed compared to controls (Fig. 7C) but was not seen in the non-responders except for AML21 (Supplementary Fig. 10B). Importantly, serial repopulation assays at limiting dilution demonstrated a reduction in LSC frequency following FTY720 treatment in five of the seven AML samples tested ( $p=0.018-0.158$ ), including relapsed and treatment-refractory cases (Fig. 7D, E, Supplemental Table 13), demonstrating that FTY720 treatment disrupted LSC activity even without overt immunophenotypic changes in myeloid differentiation markers. Engraftment of CB cells was not affected by FTY720 treatment, suggesting that a therapeutic window exists for targeting of LSCs by FTY720 (Fig. 7F). These data suggest that FTY720 treatment is acting in part as an S1PR3 agonist to promote myeloid differentiation and decrease LSC function in our xenograft studies. In summary, *in vivo* treatment with the S1P pro-drug FTY720 or *S1PR3*OE can target the disease-sustaining LSCs in AML.

## Discussion

Our study provides direct evidence that S1PR3 governs myeloid commitment in human HSC and LSC, and points to S1P signaling modulation as a potential therapeutic approach to target LSC in human AML. *S1PR3* OE alone was sufficient to induce myeloid differentiation in human HSC and LSC and S1PR3 antagonism or KD restricted myeloid differentiation in AML. Moreover, S1PR3 marks a subset of LSC from AML patients with a distinct inflammatory signature and decreased stemness properties. Thus, S1PR3 could have clinical utility as both a prognostic biomarker and a novel therapeutic target in AML. Although induction of differentiation (e.g. by retinoid agonists) is an effective approach in some AML cases(57) and metabolic processes(6,58,59) have been shown to control LSC function, this is the first demonstration to our knowledge of successful human LSC targeting by modulation of a bioactive lipid signaling pathway.

We hypothesize that the dichotomy of S1PR3 high and low AML patient samples mimics and can be traced to the diverse responses to inflammatory cytokines during stress hematopoiesis that are wired into the hematopoietic hierarchy(22,23). The S1PR3-mediated promotion of myeloid differentiation at the expense of other lineages, particularly erythropoiesis, and enhanced survival of LT-HSC are highly reminiscent of TNF $\alpha$ -induced emergency hematopoiesis(23). Thus, S1PR3 plays similar roles in human HSC to what has been reported for the TNF $\alpha$ -NF- $\kappa$ B axis in the murine system(23). Overall, our data point to S1PR3 as a potentiator of TNF $\alpha$  via NF- $\kappa$ B inflammatory signaling to promote pro-survival and myeloid differentiation in human HSC. Although TNF $\alpha$  levels are elevated during aging as well as in myeloid malignancies, it remains unclear how this inflammatory environment drives leukemia or which cellular subtypes are susceptible to transformation(11,25,30). TNF $\alpha$  has recently emerged as having complex roles in normal hematopoiesis with different effects on HSC versus myeloid progenitors(23). Notably, we found that acute TNF $\alpha$  treatment was sufficient to upregulate S1PR3 membrane levels in primitive AML cell lines *in vitro*. In contrast, S1PR3 antagonism was sufficient to counteract the myeloid differentiation enacted by TNF $\alpha$ . A previous study found that *S1PR3* OE driven by a lysosome M promoter, but not a F4/80 promoter, induced a leukemic transformation in the mouse system suggesting the cell of origin for transformation originated from a primitive subpopulation and not from a mature myeloid lineage; although a role for S1PR3 in inflammatory signaling was not identified (44). However, as we found that *S1PR3* OE in human HSC was not able to induce a *de novo* AML in xenografts, other factors such as the microenvironment and species differences need to be considered. We speculate that in a subset of patient AML cases, dependent on specific genetic lesions and metabolic states, the NF- $\kappa$ B/S1PR3 feed-forward loop is hijacked to promote pro-survival mechanisms. Although we were able to capture the same inflammatory signatures induced in HSC by *S1PR3* overexpression within AML patient samples, whether S1PR3 activity confers enhanced survival to AML cells *a priori* remains to be explored. Nonetheless, it is intriguing that AML cases with high *S1PR3* expression in the Beat-AML cohort were more resistant to venetoclax treatment, and *S1PR3* OE was sufficient to decrease p65 levels in AML cells *in vitro*. The nuances around how S1P signaling intersects with inflammation and NF- $\kappa$ B signaling to regulate LSC function is most likely different along the spectrum of stem-like to

myeloid-like AML samples and reflective of the differences in canonical and non-canonical NF- $\kappa$ B signaling within LSC(34). We wonder if the heterogeneity of response to venetoclax is related to such inflammatory wiring. The identification of a distinct NF- $\kappa$ B signature in a subset of *S1PR3* high AML cases raises the possibility that different strategies need to be employed for successful targeting of NF- $\kappa$ B in LSCs from stem-like and myeloid-like AML cases.

The FTY720 xenograft study along with our *S1PR3* OE, KD and *S1PR3* *in vitro* antagonist studies, suggest that FTY720 treatment is acting in part as an *S1PR3* agonist to downregulate NF- $\kappa$ B signaling and promote myeloid differentiation to decrease LSC function in stem-like AML. The transcriptome analysis of sphingolipid metabolism in AML and sphingolipidome profiling of LSC+ and LSC- subpopulations hints at connections between S1P signaling, sphingolipid metabolism and drug response in LSC beyond *S1PR3*. Stem-like AML cases may have a different requirement for sphingolipid metabolism and S1P signaling compared to more differentiated myeloid-like cases that appear to be connected to overall survival and chemotherapy response. Whether chemotherapy selects for pre-existing subclones with different sphingolipid metabolism/S1P downstream signaling or whether resistant clones survive by remodeling their underlying metabolism including the sphingolipid network remains to be determined. Importantly, our identification of an 8-gene sphingolipid signature associated with OS that includes *S1PR3*, *S1PR5*, and *SPHK1* (the kinase that produces S1P) raises the possibility that altering the balance in S1P signaling could be sufficient to disrupt LSC maintenance in a subset of AML patients. Our data suggest that next generation S1P modulators approved for multiple sclerosis(21) may have therapeutic efficacy in a subset of AMLs, although we cannot fully exclude the possibility that FTY720 targets LSC via factors beyond S1P signaling(60). Our data also provide strong evidence supporting further investigation of recently identified highly potent SPHK1 inhibitors for their effectiveness in targeting LSC(61–63). Another approach for therapeutic targeting of LSC could be direct modulation of sphingolipid metabolic/biosynthetic enzymes such as those in the glycosphingolipid pathway that have recently emerged as regulators of NF- $\kappa$ B(64) and were included in the 8-gene sphingolipid signature. Sphingolipid metabolism has been linked to ER stress, autophagy, and inflammatory stress programs in cancer, obesity and cardiovascular disease, but the mechanisms are ill-defined especially around drug resistance(14,15,65–67). Our study opens a rich avenue for future mechanistic studies to elucidate the interplay of sphingolipid metabolism and signaling with these potential drug resistance programs in stem cell diseases such as AML. In conclusion, our findings point to a functional role for *S1PR3* in myeloid differentiation of LSC via the TNF $\alpha$ -NF- $\kappa$ B axis and highlight modulation of sphingolipids and their associated signaling pathways as a means to identify and target inflammatory pathways for the treatment of human AML.

## Methods

### Human normal and malignant hematopoietic samples

All biological samples were collected with written informed consent according to the procedures approved by the University Health Network (UHN) Research Ethics Board (REB

01-0573-C). Human cord blood samples were obtained from Trillium and Credit Valley Hospital and William Osler Health Centre, processed as previously described, and stored viably as lineage depleted (lin<sup>-</sup>) cells at -80°C or -150°C(14). Normal human bone marrow samples or AML patient samples were obtained as viably frozen material from the Leukemia Tissue Bank at Princess Margaret Cancer Centre/ University Health Network. CB MNCs, lin<sup>-</sup> CB, bone marrow MNCs and AML patient samples were thawed by dropwise addition of X-VIVO + 50% fetal calf serum supplemented with DNase (100µg/mL final concentration, Roche) and resuspended in PBS+5% FBS for additional analyses below unless otherwise noted.

### **S1PR3 flow cytometry analysis and fluorescence-activated cell sorting Antibody reagents used throughout this study are listed in Supplemental Table 14**

S1PR3 surface protein content was analyzed with an antibody against human S1PR3 (S1P3/EDG-3-PE, 1:30 dilution) in 3 sets of flow cytometry studies at 1 million cells/ml. Each sample was also analyzed with a panel lacking S1PR3-PE (FMO). FlowJo 9.9 and 10 were used in this study for flow cytometry analysis and for calculation of S1PR3 mean fluorescence intensity (MFI).

1) S1PR3 expression for CD34<sup>+</sup> primitive cells and T (CD3<sup>+</sup>), B (CD19<sup>+</sup>), and myeloid (CD33<sup>+</sup>) cells in fresh human CB MNC samples (n=4; 2 samples were single CBs from male donors and 2 samples were pools of 6 CB) were analyzed with the following panel on a BD canto: FITC-anti-CD3, PECy5-anti-CD19, PE-anti-S1PR3, APC-anti-CD33, and APCCy7-anti-CD34. Live/dead discrimination was determined by sytox blue (ThermoFisher Scientific, S34857).

2) Analysis of S1PR3 on the surface of HSPC populations was performed on 3 pools of lin<sup>-</sup> CB with the following panel on a BD LSR II: FITC-anti-CD45RA, PE-anti-S1PR3, APC-anti-CD90, PECy5-anti-CD49f, V450-anti-CD7, BV421-anti-CD10, PECy7-anti-CD38, and APCCy7-anti-CD34. Propidium iodide (BD) was used for live/dead discrimination.

3) Analysis of myeloid subpopulations in CB MNCs (CD15<sup>+</sup> or CD14<sup>+</sup> cells) and comparison of previously frozen CB MNCs to bone marrow MNCs and AML patient samples were performed by flow cytometry with the following panel on a BD Canto: FITC-anti-CD15, PE-anti-S1PR3, PECy5-anti-CD14, PECy7-anti-CD38, and APCCy7-anti-CD34 or APC-anti-CD34. Live/dead discrimination was determined by sytox blue. Clinical characteristics are listed in Supplemental Table 9. S1PR3 relative fluorescence intensity (RFI) for each population is the ratio of S1PR3 MFI for the cells stained with S1PR3-PE divided by the FMO.

Lin<sup>-</sup> CB cells were stained with the following antibodies at a density of 5×10<sup>6</sup> cells/mL (1:50 dilution, unless stated otherwise) for isolation of HSPC subpopulations: FITC-anti-CD45RA, PE-anti-CD90, PECy5-anti-CD49f, V450-anti-CD7, PECy7-anti-CD38 (1:200), BV421-anti-CD10, APCCy7-anti-CD34 (1:200), APC-anti-CD71 and biotin-anti-Flt3. Cells were washed in PBS+5% FBS and then stained with streptavidin-Qdot605. Cells were washed, resuspended in PBS+2% FBS and propidium iodide and FACS-purified on FACS Aria III or Aria Fusion (BD Biosciences), consistently yielding >95% purity. Mature cell



populations including B cells, T cells, NK cells, DC, granulocytes, monocytes, and erythroid progenitors were isolated from CB MNCs for RNAseq (Garcia-Prat, *et al.* (37)).

Cell surface markers were used for each population are listed below.

LT-HSC: CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup>CD49f<sup>+</sup>

ST-HSC: CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>-</sup>CD49f<sup>-</sup>

Multipotent progenitors (MLP): CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>+</sup>CD90<sup>-</sup>

CMP: CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>-</sup>CD7<sup>-</sup>CD45RA<sup>-</sup>Flt3<sup>+</sup>

CMP-F1: CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>-</sup>CD7<sup>-</sup>CD45RA<sup>-</sup>Flt3<sup>+</sup>CD71<sup>-</sup>

GMP: CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>-</sup>CD7<sup>-</sup>CD45RA<sup>+</sup>

MEP: CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>-</sup>CD45RA<sup>-</sup>Flt3<sup>-</sup>

MEP-F1: CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>-</sup>CD7<sup>-</sup>CD45RA<sup>-</sup>Flt3<sup>-</sup>CD71<sup>-</sup>

T cell: CD3<sup>+</sup>CD19<sup>-</sup>

B cell: CD19<sup>+</sup>CD3<sup>-</sup>

NK cell: CD56<sup>+</sup>CD3<sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup>

Granulocyte: CD14<sup>+</sup>CD15<sup>+</sup>CD3<sup>-</sup>CD19<sup>-</sup>

Monocyte: CD14<sup>+</sup>CD15<sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>

DC: CD11c<sup>+</sup>CD45<sup>+</sup>

Erythroid cell: CD45<sup>-</sup>GlyA<sup>+</sup>CD71<sup>+</sup>)

For sphingolipidome profiling and xenotransplantation, nine samples were selected from the patient cohort previously analyzed for LSC activity(7). 30 million live cells from 9 patients (Supplemental Table 10) were stained with the following antibodies at a density of  $1 \times 10^7$  cells/mL: FITC–anti-CD45RA, PE–anti-S1PR3, PECy5–anti-CD33, V450–anti-CD15, PECy7–anti-CD38, V500–anti-CD45 (1:50), APC–anti-CD34, APCCy7–anti-CD3 and BV711–anti-CD19. Cells were washed, resuspended in PBS+2% FBS and propidium iodide and were sorted on CD34 and CD38 surface markers in the CD3<sup>-</sup>CD19<sup>-</sup> subpopulations into 4 fractions on a BD FACSAria Fusion, washed with PBS, and were frozen viably.

### Lentiviral overexpression and knockdown of S1PR3

Lentiviral cloning and VSV-G pseudotyped lentiviral vector particles were produced and titers calculated as previously described(39). A pLBC2 overexpression vector for human *S1PR3* was generated from a pENTR223\_S1PR3\_STOP plasmid (a stop codon insertion into HsCD00375638 obtained from PlasmID, DF/HCC DNA Resource Core at Harvard Medical School). The control vector for overexpression encodes *gp91<sup>phox</sup>P415H* (catalytic

inactive gp91<sup>phox</sup>/*CYBB*). shRNA sequences to *S1PR3* were predicted using the Sherwood algorithm and amplified as previously described (14). shCtrl is a sequence to Renilla was previously utilized(14,39). In order to assess shRNA knock-down efficiency, MOLM13 cells were infected at a multiplicity of infection of 0.3. Transduced cells were sorted for BFP<sup>+</sup> expression and total RNA was isolated and DNase treated using the RNeasy Micro Kit (Qiagen, 74004). RNA quality (RIN > 9) was verified using the Bioanalyzer RNA 6000 Pico Kit (Agilent) and cDNA was synthesized using SuperScript VILO (ThermoFisher, 11754050). qPCR was performed on the Roche Lightcycler 480 using Power SYBR Green (ThermoFisher, 4367659). All signals were quantified using the Ct method and were normalized to the levels of GAPDH.

#### shRNA sequences to *S1PR3*

shS1PR3-2:

TGCTGTTGACAGTGAGCGCGGTCCCCACTCTTCATCCTCATAGTGAAGCCACAGAT  
GTATGAGGATGAAGAGTGGGGACCATGCCTACTGCCTCGGA

shS1PR3-3:

TGCTGTTGACAGTGAGCGACCATCTGGAAAAACAATAAAATAGTGAAGCCACAGA  
TGTATTTTATTGTTTTTCCAGATGGCTGCCTACTGCCTCGGA

### **Xenotransplantation**

All animal experiments were done in accordance to institutional guidelines approved by the University Health Network Animal care committee. Aged match female or male NSG mice (NOD.Cg *Prkdcscid112rgtm1Wjl/SzJ*; Jackson Laboratory) 10-12 weeks of age were sublethally irradiated with 250 rads 1 day before intrafemoral injection. For CB xenotransplantation experiments, CD34<sup>+</sup>CD38<sup>-</sup> cells were transduced with *S1PR3OE* or control vectors at matching multiplicity of infection in a low cytokine gene transfer media(38) composed of X-VIVO 10 media, 1 % BSA supplemented with 1x Pen/Strep, L-Glutamine and the following cytokines: SCF (100 ng/ml), Flt3L (100 ng/ml), TPO (50 ng/ml) and IL7 (10 ng/ml) following 18 hours of pre-stimulation. Cells were collected and transplanted into NSG mice by intrafemoral injection 24 hours post-transduction. Cells were also left in culture to assay for transduction efficiency(39) marked by %BFP<sup>+</sup> at day 3 and 6 by flow cytometry on a BD Celesta and the mean of the timepoints was used to mark initial input. Following xenotransplantation of *S1PR3OE* into NSG mice for 4 weeks, mice were euthanized and the injected femur and other bones were flushed separately in Iscove's modified Dulbecco's medium (IMDM) with 5% FBS and human chimerism and transduced cells marked by BFP expression were assessed by flow cytometry on the BD LSR II with the following: PE-anti-CD19, PE-anti-GlyA or PECy7-anti-GlyA, APC-anti-CD33, PECy5-anti-CD45, APCCy7-anti-CD34, FITC-anti-CD71 and BFP.

For limiting dilution xenotransplantation experiments of patient AMLs fractionated by surface *S1PR3* protein expression, patient AMLs were thawed and stained with the same panel as for isolation of samples for lipid mass spectrometry. Within CD45<sup>+</sup>CD33<sup>+</sup> blasts, CD34<sup>+</sup> cells were gated and then isolated for *S1PR3*<sup>+</sup> and *S1PR3*<sup>-/lo</sup> cells based on unstained and *S1PR3*-PE FMO controls. Cells were counted and 3 different cell doses based

on previous xenotransplantation experiments were injected into NSG mice for 12 weeks and then analyzed for engraftment. The following flow cytometry analysis was performed: FITC-anti-CD15, PE-anti-S1PR3, PECy5-Anti-CD45, APC-anti-CD33, PECy7-anti-CD14, and APCCy7-anti-CD34. Mice were considered engrafted if CD33<sup>+</sup>CD45<sup>+</sup> cells >0.05% in the right femur. For *S1PR3OE* xenotransplantation experiments of human AML patient samples where CD34<sup>+</sup> cells were known to contain LSCs, control or *S1PR3OE* CD34<sup>+</sup> cells were FACS-purified, transduced with control or S1PR3OE vectors overnight with previously described AML cytokine culture conditions(39), and then transplanted into NSG mice by intrafemoral injection. Following 8 weeks, right femurs and other leg bones were isolated and analyzed for engraftment by flow cytometry on a BD Celesta with the following: FITC-anti-CD15, PE-anti-S1PR3, V500-anti-CD45, BV786-anti-CD33, PECy5-anti-CD15, APCCy7-anti-CD34 and BFP.

In the FTY720 drug study, 12 patient AMLs depleted of CD3<sup>+</sup> T cells using EasySep (Stem Cell Technologies) or 3 pools of lin- CB (66,000 cells/mouse) were transplanted into NSG mice for 4 weeks (clinical characteristics in Supplemental Table 10). Vehicle control (DMSO) or 1mg/kg FTY720 (Cayman Chemicals, 10006292, assuming average weight for NSG mouse to be 25 g) was diluted in PBS and administered to mice by intraperitoneal injections every other day (7x) over 2 weeks. On the day after the last injection, mice were sacrificed, bones isolated and engraftment analyzed by flow cytometry on a BD LSR or Celesta. CB engraftment was analyzed with APC-anti-CD33, PECy5-anti-CD45, FITC-anti-CD15, PE-anti-CD19, PE-anti-GlyA, PECy7-anti-CD14, and APCCy7-anti-CD34. AML engraftment (CD45<sup>+</sup>CD33<sup>+</sup> cells) was analyzed with APC-anti-CD45, PECy5-anti-CD33, FITC-anti-CD3, V450-anti-CD15, PECy7-anti-CD14, and APCCy7-anti-CD34. AML Samples were frozen viably and those samples which showed decreased engraftment with FTY720 or achieved engraftment over 20% were thawed, CD45<sup>+</sup>CD33<sup>+</sup> cells were isolated using APC-anti-CD45, PECy5-anti-CD33, FITC-anti-CD45 and propidium iodide for serial transplantation assays in NSG mice with the indicated cell doses to assess LSC function at 8 weeks post-transplantation (Supplemental Table 13; 9 of 12 samples tested for in the limiting dilution secondary transplantation assays but AML19 and AML21 did not engraft and hence are not listed). Secondary engraftment was assessed by flow cytometry as in the primary transplants and those mice that had greater than 0.5% CD45<sup>+</sup>CD33<sup>+</sup> engraftment were considered responders. LSC frequency was calculated with ELDA (<http://bioinf.wehi.edu.au/software/elda/>).

### TNF $\alpha$ or IL6 treatment

Three pools of CB lin- cells were cultured in gene transfer media alone or plus 10ng/ml human TNF $\alpha$  (Miltenyi Biotec, 130-094-018) or 50ng/ml IL6 and a portion of cells analyzed on a BD Celesta at 3 days post-treatment (FITC-anti-CD45RA, APC-anti-CD90, PECy5-anti-CD49f, AlexaFluor-anti-CD7, PE-Dazzle-anti-CD38 (1:100), BV605-anti-CD10, APCCy7-anti-CD34, S1PR3-PE and BV786-anti-CD33) and again at day 8 post-treatment (APC-anti-CD90, FITC-anti-CD14, V450-anti-CD15, AlexaFluor-anti-CD7, BV605-anti-CD10, APCCy7-anti-CD34, S1PR3-PE and BV786-anti-GlyA).

## In vitro CB HSPC assays

For *in vitro* assays, LT-HSC and ST-HSC isolated from 3 independent pools of CB transduced with indicated OE or knockdown lentiviral vectors as for xenotransplantation experiments, except cells were prestimulated for 4 hours prior to transduction. For liquid culture, cells were transferred from gene transfer media to a high cytokine StemPro media media starting at day 3 post-transduction (Stem Cell Technologies) supplemented with StemPro nutrients (Stem Cell Technologies), L-glutamine (GIBCO), Pen/Strep (GIBCO), human LDL (Stem Cell Technologies, 50 ng/mL) and the following cytokines (all from Miltenyi): SCF (100 ng/mL), Flt3L (20 ng/mL), TPO (100 ng/mL), IL-6 (50 ng/mL), IL-3 (10 ng/mL), GM-CSF (20 ng/mL), except EPO (3 units/mL, from Jansen). At day 9 or 10, cells were stained for flow cytometry analysis with PeCy5-anti-CD14 (1:200), APCCy7-anti-CD34 (1:200), and BFP. PE-anti-S1PR3 (1:33) was added into 1 experiment to confirm enhanced expression. For CFC assays, on day 3 post-lentiviral transduction of LT-HSC, ST-HSC, CMP F1, MEP F1 or GMP, transduced cells marked by BFP were sorted directly into methylcellulose (cat. No H4034, Stem Cell Technologies), supplemented with FLT3 Ligand (10 ng/ml) and IL6 (10 ng/ml). Samples were plated onto 35 mm dishes in duplicates and colonies were allowed to differentiate for 10 days and morphologically assessed for colonies in a blind fashion by a second investigator. At day 14, colonies from replicate plates were pooled and resuspended in PBS+5% FBS for flow cytometry analysis with PE-anti-Gly, PECy7-anti-CD33, APCCy7-anti-CD34 and BFP. For single cell erythroid/myeloid/megakaryocyte differentiation assays, on day 3 post-lentiviral transduction of LT-HSC, ST-HSC, CMP-F1, and MEP-F1, single BFP+ cells were sorted directly onto MS5<sup>44</sup> stroma in 96 well plates prepared as previously described<sup>28</sup> and cultured for 15 days. Flow cytometry analysis of wells containing single cells scored as successfully cloned by eye were detached from MS5 stroma and analyzed for FITC-anti-CD15, PE-anti-GlyA, APC-anti-CD71, PeCy5-anti-CD14, APCH7-anti-CD41, PECy7-anti-CD33 and BFP on an HTS unit of a BD Canto.

## Time-lapse imaging

Time-lapse experiments were conducted at 37°C, 5% O<sub>2</sub>, 5% CO<sub>2</sub> on  $\mu$ -slide VI<sup>0.4</sup> channel slides (IBIDI) coated with 20  $\mu$ g/ml anti-human CD43-biotin antibody(51). BFP+ cells 3 day post-lentiviral transduction were sorted and cultured overnight in phenol-red free X-VIVO 10 (Lonza) medium supplemented with BSA (1%), L-glutamine, Pen/Strep, human LDL and the cytokine cocktail described above before imaging. Brightfield images were acquired every 15 minutes for 4 days using a Nikon-Ti Eclipse equipped with a Hamamatsu Orca Flash 4.0 camera and a 10x CFI Plan Apochromat  $\lambda$  objective (NA 0.45). Single-cell tracking and fate assignment were performed using self-written software as previously described(51). Time to division was calculated using R 3.6.1.

## Sphingolipid quantitation by mass spectrometry

To profile the sphingolipid composition of LSC+ and LSC- fraction from AML patient samples, LSC+ and LSC- subpopulations (n=7) were selected from the previously FACS-isolated viably banked samples that contained a minimum of 4 million cells at the time of sort (samples ranged from 4 to 12 million cells). These were thawed concurrently, live cells

counted, washed twice with PBS and frozen as cell pellets with viable cells ranging from 1.5 to 5 million cells for LSC-samples and 3.4 to 5.3 million cells for LSC+ samples. The sphingolipid composition of HSPC CB cells (CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> from 3 pools of lin<sup>-</sup> CB) was previously reported(14). Subsequent lipid extraction and mass spectrometry of the 14 AML subpopulations and 6 HSPC samples were performed together to allow for direct quantitative comparison of SpL species levels between samples. LC/MS/MS analysis for sphingomyelin species, hexosylceramide species, ceramide species, dihydroceramide species and sphingoid species were performed by the Lipidomics Facility of Stony Brook University Medical Center on a SCIEX 4000 QTRAP mass spectrometer. Normalization to cellular inorganic phosphate (P<sub>i</sub>) was chosen to minimize the potential confounding effects of differences in cellular size and protein content between the profiled populations. Sphingolipid composition for each sample was calculated as proportion of all sphingolipids analyzed and subsequently scaled. Using scanpy(68), a neighbourhood graph was calculated with a local neighborhood size of 4 and a UMAP (*Uniform Manifold Approximation and Projection*) dimensionality reduction was performed.

### Cord Blood gene expression analysis

LT-HSC and ST-HSC from 3 pool of CB lin<sup>-</sup> were FACS-purified, transduced as described for *in vitro* culture assays in gene transfer media. At day 3, 2000-5300 BFP<sup>+</sup> cells were FACS-purified for RNA isolation with a PicoPure kit (ThermoFisher Scientific, KIT0214). Consistent with our observations that *S1PR3OE* increases the viability of LT-HSC over control vectors in live cell imaging studies (Fig. 2f-h), we were able to isolate only 1600-1800 BFP<sup>+</sup> cells from LT-HSC control samples as opposed to 4000-5400 BFP<sup>+</sup> cells from *S1PR3OE* samples. Thus, we pooled all control BFP<sup>+</sup> LT-HSC cells into one sample for RNA-seq analysis. BFP- LT-HSC from control vector transduction were purified from CB1 as an additional LT-HSC control. In total, Nextera libraries generated from 10 ng RNA from 5 LT-HSC samples (2 controls, 3 *S1PR3OE*) and 6 ST-HSC samples (3 controls, 3 *S1PR3OE*) were subjected to 125 bp, paired-end RNA-sequencing on the Illumina HiSeq 2500 with an average of 50 million reads/sample at the Centre for Applied Genomics, Sick Kids Hospital. *S1PR3OE*RNA-seq was aligned using STAR 2.5.2b(69) against GRCh38 and transcript sequences downloaded from Ensembl build 90. Default parameters were used except for the following: "--chimSegmentMin 12 --chimJunctionOverhangMin 12 --alignSJDBoverhangMin 10 --alignMatesGapMax 100000 --alignIntronMax 100000 --chimSegmentReadGapMax parameter 3 --alignSJstitchMismatchNmax 5 -1 5 5". Counts were obtained using HTSeq v0.7.2(70). Differential gene expression for the overexpression of *S1PR3* compared to control (mtGP91 and BFP-) for both LT-HSC and ST-HSC was performed with DESeq2 1.18.1 using recommended settings. Briefly, the HTSeq counts from the entire RNA-seq cohort was imported into R (3.4.1) and normalized using RLE from DESeq2. To identify differentially expressed genes, this was followed by a dispersion estimate and a quasi-likelihood negative binomial generalized log-linear model. For visualization, data underwent variance stabilization as recommended by DESeq2. Gene set enrichment was performed using GSEA PreRanked v3.0 (<http://www.broad.mit.edu/gsea/>) against the hallmarks gene set MSigDB v6.2 with recommended settings (Supplemental Table 1).



As independent verification for induction of a myeloid lineage gene expression program upon *SIPR3*OE, the HSC and granulocyte microarray data set from GSE24759(50) was retrieved for pathway and signature analysis using GSEA. Results were visualized as a network using Cytoscape 3.7.1 and EnrichmentMap 3.2.1. AutoAnnotate 1.3.2 automatically labelled pathway modules using most frequent words. HSC and GRAN gene lists were added as gene sets to the enrichment map and the significance of overlap with gene sets was calculated using the EnrichmentMap post analysis integrated hypergeometric test. The CB hierarchy RNAseq data set was normalized and pre-processed as noted above. For final visualization, the mean expression of the cord blood replicates was utilized.

### Human AML gene expression analysis

The single cell correlation analysis of *SIPR3* and the *SIPR3*OE LT-HSC DEGs was performed in a previously published scRNAseq data set consisting of 11641 malignant single cells from 12 AML patients at diagnosis(3). Counts were scrann normalized and signatures scored using AUCell with default settings. Signature enrichment was subsequently scaled to facilitate visualization. Four previously published bulk AML gene expression data sets and one data set described here were used to analyze *SIPR3* expression. 1) AML RNA-seq expression read counts and genomic analysis for mutations from the TCGA-AML study(52), consisting of 20442 genes and 179 patients, were downloaded from the companion website of the original publication ([https://tcga-data.nci.nih.gov/docs/publications/laml\\_2012](https://tcga-data.nci.nih.gov/docs/publications/laml_2012)). Gene expression is defined as  $\log_2(1 + \text{CPM})$ , where the CPM (counts per million) are computed using R package edgeR after filtering and normalizing the raw counts. 2) The Beat AML data was downloaded from Supplementary materials from Tyner, et al(35). The CPM table consisting of 22843 genes and 451 patients, listed in Supplementary Table 9, contained 440 AML samples (280 Dx, 23 Rx, 137 NA)). We utilized the 280 listed as Dx in our analysis here. 3) GSE6891(53) is a normalized RNA microarray AML data set (AFFY HG U133 plus 2) using basic RMA consisting of 495 patient samples previously analyzed in Ng, et al(7). The gene symbols were obtained from R database hgu133plus2.db. Gene expression was calculated using maximum values of multiple probes for each gene and those genes with  $\text{sd} < 0.01$  across samples were filtered resulting in 17325 genes. The correlation of genetic mutation data to *SIPR3* gene expression was analyzed for any genes reported with mutations in the Beat-AML, TCGA-AML and GSE6891. Briefly, the correlation coefficient and t-test p value were calculated for *SIPR3* gene expression to any mutation by comparing the two patient groups either containing the genetic mutation or wildtype using two-sample t-test. We computed single sample GSEA (ssGSEA) enrichment scores for the HSC/Progenitor and myeloid signatures (Supplemental Table 6) generated from the van Galen data set(3) and to the NFKB via TNFA hallmark gene set (gene lists in Supplemental Table 5) using the R GSVA\_1.30.0 package. The ssGSEA score and gene expression color key represents the normalized values that are equal to centered scores divided by maximum of the absolute centered scores. We analyzed correlations of *SIPR3* with the NFKB genes in these 3 AML cohorts using R. This resulted in 75 common NFKB genes correlated to *SIPR3* with  $\text{FDR} < 0.05$  in the three cohorts (correlation scores, correlation testing p-values, and Benjamini and Hochberg (BH) FDR are located in Supplemental Table 8. These analyses were also performed on 4) LSC+/LSC- microarray

data set and 5) a RNAseq data set generated from patients from the Princess Margaret Cancer Centre (PM) described below.

For LSC+/LSC- analysis, normalized RNA microarray (GPL10558 Illumina HumanHT-12 V4.0) data consisting of 138 LSC+ and 89 LSC- samples were obtained from the GEO database file GSE76008\_non-normalized.txt.gz(7). Official gene symbols from the HUGO Gene Nomenclature Committee were retrieved from R database illuminaHumanv4.db. Filtering of duplicated genes (the maximum value was used) resulted in 17685 genes. For GSVA and NFKB gene set analysis to *S1PR3*, we normalized the log2-transformed data using quantile normalization. Following differential gene expression using limma v3.38.3(71), GSEA was run using default settings against the custom gene sets for S1P associated or HSC-enriched lipid metabolism associated genes(14) (*SMPD4* was not found in the microarray data, hence we used 22/23 genes published previously as the lipid-stem signature) listed in supplemental table 12.

The PM-AML RNAseq dataset consists of 81 AML patient samples (clinical data in Supplemental Table 9), processed in two batches. This data set only contains patient samples that can engraft in the NSG mouse model. Five patients (90543, 598, 90240, 110484, 100500) were included in both batches. RNA was extracted from bulk peripheral blood mononuclear cells (PBMC) using the RNeasy Micro Kit (Qiagen Inc.). Libraries were constructed by SMART-Seq (Clontec inc). A paired-end 50 base-pair flow-cell lane Illumina High seq 2000 yielded an average of 240 million sequence reads aligning to genome per sample at the Genome Sciences Centre, BC Cancer Agency for cohort 1. Cohort 2 was subjected to 125 bp, paired-end RNA-sequencing on the Illumina HiSeq 2500 with an average of 50 million reads/sample at the Centre for Applied Genomics, Sick Kids Hospital. Subsequent alignment and normalization performed as for *S1PR3*OE RNAseq data set described above. Then R was utilized to perform pathway correlation of S1PR3 and S1PR3OE LT-HSC signature to the indicated Hallmark gene sets.

AML paired diagnosis-relapse gene expression analysis for S1PR3 was from RNAseq data of 12 patient pairs previously described(5).

RNA-seq and *ex vivo* drug screening data were acquired from Tyner et al(35). Gene expression data was normalized to TPM and AUC was used from the drug screening data to denote drug tolerance. Diagnostic AML samples were categorized as high *S1PR3* and low *S1PR3* by a median split on *S1PR3* gene expression, and Venetoclax tolerance was compared between the groups with a two-tailed t-test.

Sparse regression analysis within a set of 54 sphingolipid genes was utilized to derive a weighted sphingolipid gene score applied to a large training cohort of n=495 AML patients (GSE6891). The score was then tested on two independent data sets (GSE12417 and the TCGA AML cohorts) in univariate analysis.

### AML cell lines assays

AML cell lines (Kasumi-1, RRID:CVCL\_0589; KG-1, RRID:CVCL\_0374; ME-1 RRID:CVCL\_2110; PL-21 RRID:CVCL\_2161; U937, RRID:CVCL\_0007; and OCI-

AML3, RRID:CVCL\_1844) were obtained from ATCC and cultured in MEM alpha medium (12571-063, GIBCO) supplemented with 20% FBS (F1051, Sigma), 2mM PenStrep (15140-122, GIBCO) and 1% L-Glut (609-065-EL, Multicell), expanded for < 4 passages to generate a stock for subsequent experiments. OCI-AML22 cell line model (Boutzen, *et al.* (56)) was derived from the cells of a relapsed PMCC AML patient and were cultured in X-Vivo medium (04-380Q, Lonza) supplemented with 20% BIT (9500, Stem Cell Technologies), 1% GlutaMAX (35050061, ThermoFisher), 0.2% Primocin (ant-pm-1, Invitrogen), 100 ng/ml SCF (130-096-696, Miltenyi), 10 ng/ml IL-3 (130-095-068, Miltenyi), 10 ng/ml TPO (130-095-752, Miltenyi), 10 ng/ml FLT3 Ligand (130-096-474, Miltenyi) and 10 ng/ml G-CSF (130-093-866, Miltenyi). Experiments were conducted from cells within 2-3 months of thawing.

For Nanonstring analysis of the LSC104 score(7) from the indicated AML cell lines, RNA from FACS-sorted viable cells were isolated with TRIzol (Invitrogen) or for low cell numbers (<20,000 cells) with PicoPure RNA Isolation Kit (Thermo Fisher) according to manufacturer's protocol. The Nanostring nCounter system (NanoString Technologies) was used for multiplexed RNA quantification, and gene expression was analyzed as previously described(7). S1PR3 surface expression for the AML cell lines were analyzed by flow cytometry on a BD celesta with the following panel: FITC-anti-CD15, PE-anti-S1PR3, APC-anti-CD38, PeCy5-anti-CD14, APCCy7-anti-CD34 and sytox blue or an FMO panel lacking PE-anti-S1PR3. Statistical analysis of the LSC104 correlation score to S1PR3 MFI was calculated in R (3.6.1).

Cells were spinoculated with 5ug/mL polybrene (Sigma) at 1,400g for 90 minutes for lentiviral transduction. 72 hours after transduction with *S1PR3OE*, BFP+ cells from the KG-1, Kasumi-1, and ME-1 cell lines were FACS-purified on the Beckman Coulter MoFlo XDP or Sony SH800. For subsequent flow cytometry, BFP+ cells were stained with the following antibodies and analyzed on the BD Celesta flow cytometer: FITC-anti-CD15, PeCy5-anti-CD11b, APCCy7-anti-CD34 and AnnexinV-APC (BD). CD34+ from Kasumi-1 and OCI-AML22 were isolated on the FACS Aria III or Fusion (BD Biosciences), transduced with Control or *S1PR3OE* or shCtrl lentiviral vectors, BFP+ cells FACS-purified at 72 hours post-transduction and analyzed at the indicated timepoints with FITC-anti-CD15, PE-anti-S1PR3, and APCCy7-anti-CD34 on the BD Celesta. OCI-AML22 or Kasumi-1 CD34+ cells were cultured in 96-well bottom plates with media alone or with 10 ng/ml TNF $\alpha$  (Miltenyi Biotec, 130-094-018) with and without 5 $\mu$ M S1PR3 antagonist TY5126 (Tocris, 5238) or DMSO control (1:5000 dilution) and analyzed at day 5 and day 9 post-treatment by flow cytometry with FITC-anti-CD15, PE-anti-S1PR3, and APCCy7-anti-CD34 on the BD Celesta. For p65 NF $\kappa$ B flow cytometry analysis, Kasumi-1 CD34+ cells were transduced with indicated lentiviral vectors and BFP+ cells were isolated on the FACS Aria III or Fusion (BD Biosciences) at day 5 post-transduction and fixed for intracellular flow cytometry with BD cytoperm/cytofix buffers as described previously(38), stained with APC-anti-p65 overnight at 4°C and analyzed on a BD Celesta.

## Statistical Analyses

GraphPad Prism was used for all statistical analyses except for gene expression and other indicated studies where the analyses were performed with R. Unless otherwise indicated, mean  $\pm$  SD values are reported in the graphs.

## Data availability

The *S1PR3* RNAseq data for LT-HSC and ST-HSC (GSE149238) and the Princess Margaret bulk AML RNAseq data (GSE156914) were deposited in the Gene Expression Omnibus. Raw data is available under accession numbers EGAS00001004798 (*S1PR3*) and EGAS00001004792 (AML) in the European Genome-Phenome Archive. The human CB 13 population RNAseq data is available from the authors upon request (Garcia-Prat, *et al.* (37)) and includes 5 HSPC populations at GSE125345. All other data supporting the findings of this study are cited in the methods, available within the paper, or upon request from the authors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

J.E.D is supported by funds from the: Princess Margaret Cancer Centre Foundation, Ontario Institute for Cancer Research through funding provided by the Government of Ontario, Canadian Institutes for Health Research grants 130412, 89932, and 154293, International Development Research Centre Ottawa Canada grants 108401 and 109153, Canadian Cancer Society grant 703212, Terry Fox New Frontiers Program Project Grant 1047, University of Toronto's Medicine by Design initiative with funding from the Canada First Research Excellence Fund, and a Canada Research Chair. E.L. is supported by Wellcome grant 107630/Z/15/Z and a core support grant from the Wellcome and MRC to the Wellcome-Medical Research Council Cambridge Stem Cell Institute. C.L. is supported by NIH, NCI grant P01-CA097132. W.W. was supported by the Swiss Initiative in Systems Biology Transition Postdoc fellowship. We thank the obstetrics units of Trillium Health Partners, Credit Valley and William Osler, the PMH Leukemia Biobank, members of the UHN-SickKids Flow cytometry facility, I. Mileva at the Lipidomics Facility of Stony Brook University Cancer Center, K. Golan, Z. Tothova and T Hla for support. This work is dedicated to the memory of Lina Obeid. We thank all members of the Dick lab for critical feedback.

## Financial Support

J.E.D is supported by funds from the: Princess Margaret Cancer Centre Foundation, Ontario Institute for Cancer Research through funding provided by the Government of Ontario, Canadian Institutes for Health Research grants 130412, 89932, and 154293, International Development Research Centre Ottawa Canada grants 108401 and 109153, Canadian Cancer Society grant 703212, Terry Fox New Frontiers Program Project Grant 1047, University of Toronto's Medicine by Design initiative with funding from the Canada First Research Excellence Fund, and a Canada Research Chair. E.L. is supported by Wellcome grant 107630/Z/15/Z and a core support grant from the Wellcome and MRC to the Wellcome-Medical Research Council Cambridge Stem Cell Institute. C.L. is supported by NIH, NCI grant P01-CA097132. W.W. was supported by the Swiss Initiative in Systems Biology Transition Postdoc fellowship.

## References

1. Dohner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *N Engl J Med.* 2015; 373(12):1136–52. DOI: 10.1056/NEJMra1406184 [PubMed: 26376137]
2. Thomas D, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. *Blood.* 2017; 129(12):1577.doi: 10.1182/blood-2016-10-696054 [PubMed: 28159741]

3. van Galen P, Hovestadt V, Wadsworth Li MH, Hughes TK, Griffin GK, Battaglia S, et al. Single-Cell RNA-Seq Reveals AML Hierarchies Relevant to Disease Progression and Immunity. *Cell*. 2019; 176(6):1265–81 e24. DOI: 10.1016/j.cell.2019.01.031 [PubMed: 30827681]
4. Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet*. 2016; 48(10):1193–203. DOI: 10.1038/ng.3646 [PubMed: 27526324]
5. Shlush LI, Mitchell A, Heisler L, Abelson S, Ng SWK, Trotman-Grant A, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature*. 2017; 547(7661):104–8. DOI: 10.1038/nature22993 [PubMed: 28658204]
6. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, et al. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell*. 2016; 19(1):23–37. DOI: 10.1016/j.stem.2016.06.001 [PubMed: 27374788]
7. Ng SW, Mitchell A, Kennedy JA, Chen WC, McLeod J, Ibrahimova N, et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature*. 2016; 540(7633):433–7. DOI: 10.1038/nature20598 [PubMed: 27926740]
8. Wilkinson, AC, Göttgens, B. Transcriptional Regulation of Haematopoietic Stem Cells Transcriptional and Translational Regulation of Stem Cells. Hime, G, Abud, H, editors. Dordrecht: Springer Netherlands; 2013. 187–212.
9. Etzrodt M, Ahmed N, Hoppe PS, Loeffler D, Skylaki S, Hilsenbeck O, et al. Inflammatory signals directly instruct PU.1 in HSCs via TNF. *Blood*. 2019; 133(8):816–9. DOI: 10.1182/blood-2018-02-832998 [PubMed: 30301719]
10. Ye M, Zhang H, Yang H, Koche R, Staber PB, Cusan M, et al. Hematopoietic Differentiation Is Required for Initiation of Acute Myeloid Leukemia. *Cell Stem Cell*. 2015; 17(5):611–23. DOI: 10.1016/j.stem.2015.08.011 [PubMed: 26412561]
11. Hemmati S, Haque T, Gritsman K. Inflammatory Signaling Pathways in Preleukemic and Leukemic Stem Cells. *Frontiers in Oncology*. 2017; 7:265. [PubMed: 29181334]
12. Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nat Rev Mol Cell Biol*. 2014; 15(4):243–56. DOI: 10.1038/nrm3772 [PubMed: 24651542]
13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144(5):646–74. DOI: 10.1016/j.cell.2011.02.013 [PubMed: 21376230]
14. Xie SZ, Garcia-Prat L, Voisin V, Ferrari R, Gan OI, Wagenblast E, et al. Sphingolipid Modulation Activates Proteostasis Programs to Govern Human Hematopoietic Stem Cell Self-Renewal. *Cell Stem Cell*. 2019; doi: 10.1016/j.stem.2019.09.008
15. Maceyka M, Spiegel S. Sphingolipid metabolites in inflammatory disease. *Nature*. 2014; 510(7503):58–67. DOI: 10.1038/nature13475 [PubMed: 24899305]
16. Cartier A, Hla T. Sphingosine 1-phosphate: Lipid signaling in pathology and therapy. *Science*. 2019; 366(6463):eaar5551. doi: 10.1126/science.aar5551 [PubMed: 31624181]
17. Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol*. 2018; 19(3):175–91. DOI: 10.1038/nrm.2017.107 [PubMed: 29165427]
18. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, et al. The Immune Modulator FTY720 Targets Sphingosine 1-Phosphate Receptors. *Journal of Biological Chemistry*. 2002; 277(24):21453–7. DOI: 10.1074/jbc.C200176200
19. Golan K, Vagima Y, Ludin A, Itkin T, Cohen-Gur S, Kalinkovich A, et al. S1P promotes murine progenitor cell egress and mobilization via S1P1-mediated ROS signaling and SDF-1 release. *Blood*. 2012; 119(11):2478–88. DOI: 10.1182/blood-2011-06-358614 [PubMed: 22279055]
20. Blaho VA, Galvani S, Engelbrecht E, Liu C, Swendeman SL, Kono M, et al. HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation. *Nature*. 2015; 523(7560):342–6. DOI: 10.1038/nature14462 [PubMed: 26053123]
21. Cohan S, Lucassen E, Smoot K, Brink J, Chen C. Sphingosine-1-Phosphate: Its Pharmacological Regulation and the Treatment of Multiple Sclerosis: A Review Article. *Biomedicines*. 2020; 8(7)doi: 10.3390/biomedicines8070227
22. Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S, et al. Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid



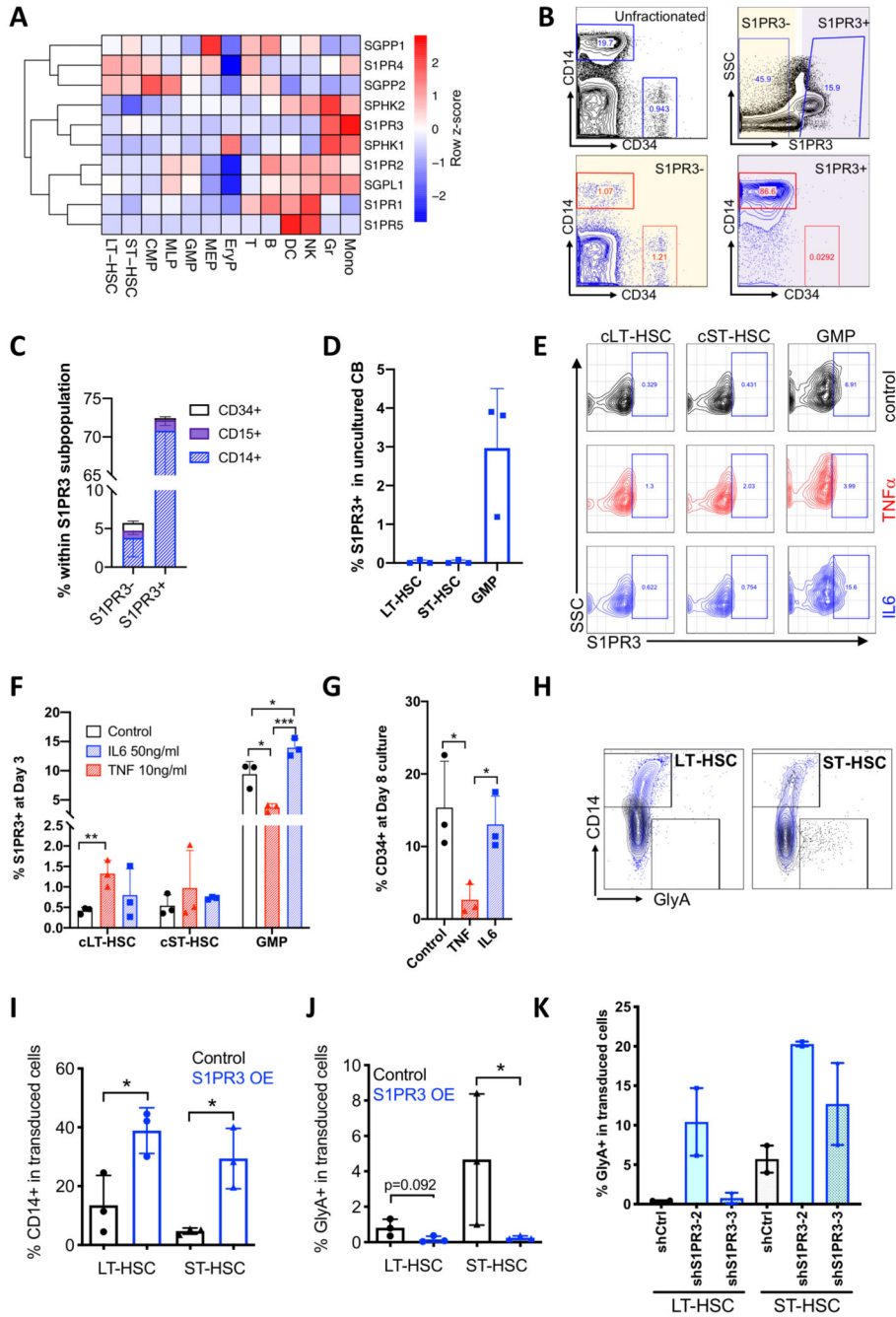
- differentiation at the expense of self-renewal. *Nat Cell Biol.* 2016; 18(6):607–18. DOI: 10.1038/ncb3346 [PubMed: 27111842]
23. Yamashita M, Passegue E. TNF-alpha Coordinates Hematopoietic Stem Cell Survival and Myeloid Regeneration. *Cell Stem Cell.* 2019; 25(3):357–72 e7. DOI: 10.1016/j.stem.2019.05.019 [PubMed: 31230859]
  24. Bruunsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. *Current Opinion in Hematology.* 2001; 8(3)
  25. Puchta A, Naidoo A, Verschoor CP, Loukov D, Thevaranjan N, Mandur TS, et al. TNF Drives Monocyte Dysfunction with Age and Results in Impaired Anti-pneumococcal Immunity. *PLOS Pathogens.* 2016; 12(1):e1005368.doi: 10.1371/journal.ppat.1005368 [PubMed: 26766566]
  26. Trabado S, Al-Salameh A, Croixmarie V, Masson P, Corruble E, Feve B, et al. The human plasma-metabolome: Reference values in 800 French healthy volunteers; impact of cholesterol, gender and age. *PLoS One.* 2017; 12(3):e0173615.doi: 10.1371/journal.pone.0173615 [PubMed: 28278231]
  27. Kowalski GM, Carey AL, Selathurai A, Kingwell BA, Bruce CR. Plasma sphingosine-1-phosphate is elevated in obesity. *PLoS One.* 2013; 8(9):e72449.doi: 10.1371/journal.pone.0072449 [PubMed: 24039766]
  28. Yu Z, Zhai G, Singmann P, He Y, Xu T, Prehn C, et al. Human serum metabolic profiles are age dependent. *Aging Cell.* 2012; 11(6):960–7. DOI: 10.1111/j.1474-9726.2012.00865.x [PubMed: 22834969]
  29. Fuster JJ, MacLauchlan S, Zuriaga MA, Polackal MN, Ostriker AC, Chakraborty R, et al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science.* 2017; 355(6327):842.doi: 10.1126/science.aag1381 [PubMed: 28104796]
  30. Abegunde SO, Buckstein R, Wells RA, Rauh MJ. An inflammatory environment containing TNF $\alpha$  favors Tet2-mutant clonal hematopoiesis. *Experimental Hematology.* 2018; 59:60–5. DOI: 10.1016/j.exphem.2017.11.002 [PubMed: 29195897]
  31. Jaiswal S, Natarajan P, Silver AJ, Gibson CJ, Bick AG, Shvartz E, et al. Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. *N Engl J Med.* 2017; 377(2):111–21. DOI: 10.1056/NEJMoa1701719 [PubMed: 28636844]
  32. Kagoya Y, Yoshimi A, Kataoka K, Nakagawa M, Kumano K, Arai S, et al. Positive feedback between NF-kappaB and TNF-alpha promotes leukemia-initiating cell capacity. *J Clin Invest.* 2014; 124(2):528–42. DOI: 10.1172/JCI68101 [PubMed: 24382349]
  33. Guzman ML, Neering SJ, Upchurch D, Grimes B, Howard DS, Rizzieri DA, et al. Nuclear factor- $\kappa$ B is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood.* 2001; 98(8):2301–7. DOI: 10.1182/blood.V98.8.2301 [PubMed: 11588023]
  34. Xiu Y, Dong Q, Li Q, Li F, Borcherding N, Zhang W, et al. Stabilization of NF-kappaB-Inducing Kinase Suppresses MLL-AF9-Induced Acute Myeloid Leukemia. *Cell Rep.* 2018; 22(2):350–8. DOI: 10.1016/j.celrep.2017.12.055 [PubMed: 29320732]
  35. Tyner JW, Tognon CE, Bottomly D, Wilmot B, Kurtz SE, Savage SL, et al. Functional genomic landscape of acute myeloid leukaemia. *Nature.* 2018; 562(7728):526–31. DOI: 10.1038/s41586-018-0623-z [PubMed: 30333627]
  36. Cancer Genome Atlas Research N, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med.* 2013; 368(22):2059–74. DOI: 10.1056/NEJMoa1301689 [PubMed: 23634996]
  37. García-Prat L, Kaufmann KB, Schneiter F, Voisin V, Murison A, Chan-Seng-Yue M, et al. Dichotomous regulation of lysosomes by MYC and TFEB controls hematopoietic stem cell fate.
  38. Laurenti E, Frelin C, Xie S, Ferrari R, Dunant CF, Zandi S, et al. CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell.* 2015; 16(3):302–13. DOI: 10.1016/j.stem.2015.01.017 [PubMed: 25704240]
  39. Kaufmann KB, Garcia-Prat L, Liu Q, Ng SWK, Takayanagi S-I, Mitchell A, et al. A stemness screen reveals C3orf54/INKA1as a promoter of human leukemia stem cell latency. *Blood.* 2019; 133(20):2198.doi: 10.1182/blood-2018-10-881441 [PubMed: 30796022]
  40. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science.* 2016; 351(6269)doi: 10.1126/science.aab2116

41. Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchino H, et al. Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. *Exp Hematol.* 1989; 17(2):145–53. [PubMed: 2783573]
42. Haas S, Hansson J, Klimmeck D, Loeffler D, Velten L, Uckelmann H, et al. Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. *Cell Stem Cell.* 2015; 17(4):422–34. DOI: 10.1016/j.stem.2015.07.007 [PubMed: 26299573]
43. Persaud L, De Jesus D, Brannigan O, Richiez-Paredes M, Huaman J, Alvarado G, et al. Mechanism of Action and Applications of Interleukin 24 in Immunotherapy. *Int J Mol Sci.* 2016; 17(6):869.doi: 10.3390/ijms17060869
44. Vorbach S, Grunder A, Zhou F, Koellerer C, Jutzi JS, Simoni M, et al. Enhanced expression of the sphingosine-1-phosphate-receptor-3 causes acute myelogenous leukemia in mice. *Leukemia.* 2019; 34:721–734. DOI: 10.1038/s41375-019-0577-7 [PubMed: 31636343]
45. Allende ML, Bektas M, Lee BG, Bonifacino E, Kang J, Tuymetova G, et al. Sphingosine-1-phosphate lyase deficiency produces a pro-inflammatory response while impairing neutrophil trafficking. *J Biol Chem.* 2011; 286(9):7348–58. DOI: 10.1074/jbc.M110.171819 [PubMed: 21173151]
46. Krumsiek J, Marr C, Schroeder T, Theis FJ. Hierarchical differentiation of myeloid progenitors is encoded in the transcription factor network. *PLoS One.* 2011; 6(8):e22649.doi: 10.1371/journal.pone.0022649 [PubMed: 21853041]
47. Jin G, Yamazaki Y, Takuwa M, Takahara T, Kaneko K, Kuwata T, et al. Trib1 and Evi1 cooperate with Hoxa and Meis1 in myeloid leukemogenesis. *Blood.* 2007; 109(9):3998–4005. DOI: 10.1182/blood-2006-08-041202 [PubMed: 17227832]
48. Murphy JM, Nakatani Y, Jamieson SA, Dai W, Lucet IS, Mace PD. Molecular Mechanism of CCAAT-Enhancer Binding Protein Recruitment by the TRIB1 Pseudokinase. *Structure.* 2015; 23(11):2111–21. DOI: 10.1016/j.str.2015.08.017 [PubMed: 26455797]
49. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, et al. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell.* 2006; 126(4):755–66. DOI: 10.1016/j.cell.2006.06.052 [PubMed: 16923394]
50. Novershtern N, Subramanian A, Lawton LN, Mak RH, Haining WN, McConkey ME, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell.* 2011; 144(2):296–309. DOI: 10.1016/j.cell.2011.01.004 [PubMed: 21241896]
51. Loeffler D, Wehling A, Schneider F, Zhang Y, Muller-Botticher N, Hoppe PS, et al. Asymmetric lysosome inheritance predicts activation of haematopoietic stem cells. *Nature.* 2019; 573(7774):426–9. DOI: 10.1038/s41586-019-1531-6 [PubMed: 31485073]
52. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. *New England Journal of Medicine.* 2013; 368(22):2059–74. DOI: 10.1056/NEJMoa1301689
53. Verhaak RGW, Wouters BJ, Erpelinck CAJ, Abbas S, Beverloo HB, Lugthart S, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica.* 2009; 94(1):131.doi: 10.3324/haematol.13299 [PubMed: 18838472]
54. Pei S, Pollyea DA, Gustafson A, Stevens BM, Minhajuddin M, Fu R, et al. Monocytic Subclones Confer Resistance to Venetoclax-Based Therapy in Patients with Acute Myeloid Leukemia. *Cancer Discov.* 2020; 10(4):536–51. DOI: 10.1158/2159-8290.CD-19-0710 [PubMed: 31974170]
55. Kuusanmäki H, Leppä A-M, Pölönen P, Kontro M, Dufva O, Deb D, et al. Phenotype-based drug screening reveals association between venetoclax response and differentiation stage in acute myeloid leukemia. *Haematologica.* 2019; doi: 10.3324/haematol.2018.214882
56. Boutzen H, Mandani A, Takayama N, Murison A, Mbong N, Mitchell A, et al. Uncovering non-coding epigenetic determinants that drive stemness programs in human leukaemia stem cells using a hierarchically-organized patient-derived model system.
57. Wang Z-Y, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood.* 2008; 111(5):2505–15. DOI: 10.1182/blood-2007-07-102798 [PubMed: 18299451]
58. Raffel S, Falcone M, Kneisel N, Hansson J, Wang W, Lutz C, et al. BCAT1 restricts alphaKG levels in AML stem cells leading to IDHmut-like DNA hypermethylation. *Nature.* 2017; 551(7680):384–8. DOI: 10.1038/nature24294 [PubMed: 29144447]

59. Jones CL, Stevens BM, D'Alessandro A, Reisz JA, Culp-Hill R, Nemkov T, et al. Inhibition of Amino Acid Metabolism Selectively Targets Human Leukemia Stem Cells. *Cancer Cell*. 2018; 34(5):724–40 e4. DOI: 10.1016/j.ccell.2018.10.005 [PubMed: 30423294]
60. Chen L, Luo LF, Lu J, Li L, Liu YF, Wang J, et al. FTY720 induces apoptosis of M2 subtype acute myeloid leukemia cells by targeting sphingolipid metabolism and increasing endogenous ceramide levels. *PLoS One*. 2014; 9(7):e103033.doi: 10.1371/journal.pone.0103033 [PubMed: 25050888]
61. Powell JA, Lewis AC, Zhu W, Toubia J, Pitman MR, Wallington-Beddoe CT, et al. Targeting sphingosine kinase 1 induces MCL1-dependent cell death in acute myeloid leukemia. *Blood*. 2017; 129(6):771–82. DOI: 10.1182/blood-2016-06-720433 [PubMed: 27956387]
62. Barth BM, Wang W, Toran PT, Fox TE, Annageldiyev C, Ondrasik RM, et al. Sphingolipid metabolism determines the therapeutic efficacy of nanoliposomal ceramide in acute myeloid leukemia. *Blood Adv*. 2019; 3(17):2598–603. DOI: 10.1182/bloodadvances.2018021295 [PubMed: 31488436]
63. Tan S-F, Liu X, Fox TE, Barth BM, Sharma A, Turner SD, et al. Acid ceramidase is upregulated in AML and represents a novel therapeutic target. *Oncotarget*. 2016; 7(50):83208–22. DOI: 10.18632/oncotarget.13079 [PubMed: 27825124]
64. Chao CC, Gutierrez-Vazquez C, Rothhammer V, Mayo L, Wheeler MA, Tjon EC, et al. Metabolic Control of Astrocyte Pathogenic Activity via cPLA2-MAVS. *Cell*. 2019; 179(7):1483–98 e22. DOI: 10.1016/j.cell.2019.11.016 [PubMed: 31813625]
65. Tam AB, Roberts LS, Chandra V, Rivera IG, Nomura DK, Forbes DJ, et al. The UPR Activator ATF6 Responds to Proteotoxic and Lipotoxic Stress by Distinct Mechanisms. *Dev Cell*. 2018; 46(3):327–43 e7. DOI: 10.1016/j.devcel.2018.04.023 [PubMed: 30086303]
66. Mann MJ, Hendershot LM. UPR activation alters chemosensitivity of tumor cells. *Cancer Biol Ther*. 2006; 5(7):736–40. DOI: 10.4161/cbt.5.7.2969 [PubMed: 16861918]
67. Halbleib K, Pesek K, Covino R, Hofbauer HF, Wunnicke D, Hanelt I, et al. Activation of the Unfolded Protein Response by Lipid Bilayer Stress. *Mol Cell*. 2017; 67(4):673–84 e8. DOI: 10.1016/j.molcel.2017.06.012 [PubMed: 28689662]
68. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biology*. 2018; 19(1):15.doi: 10.1186/s13059-017-1382-0 [PubMed: 29409532]
69. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2012; 29(1):15–21. DOI: 10.1093/bioinformatics/bts635 [PubMed: 23104886]
70. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)*. 2015; 31(2):166–9. DOI: 10.1093/bioinformatics/btu638
71. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015; 43(7):e47.doi: 10.1093/nar/gkv007 [PubMed: 25605792]

### Significance

S1PR3 is a novel regulator of myeloid fate in normal hematopoiesis that is heterogeneously expressed in AML. S1PR3 marks a subset of less primitive AML cases with a distinct inflammatory signature and therefore has clinical implications as both a therapeutic target and a biomarker to distinguish primitive from mature AML.

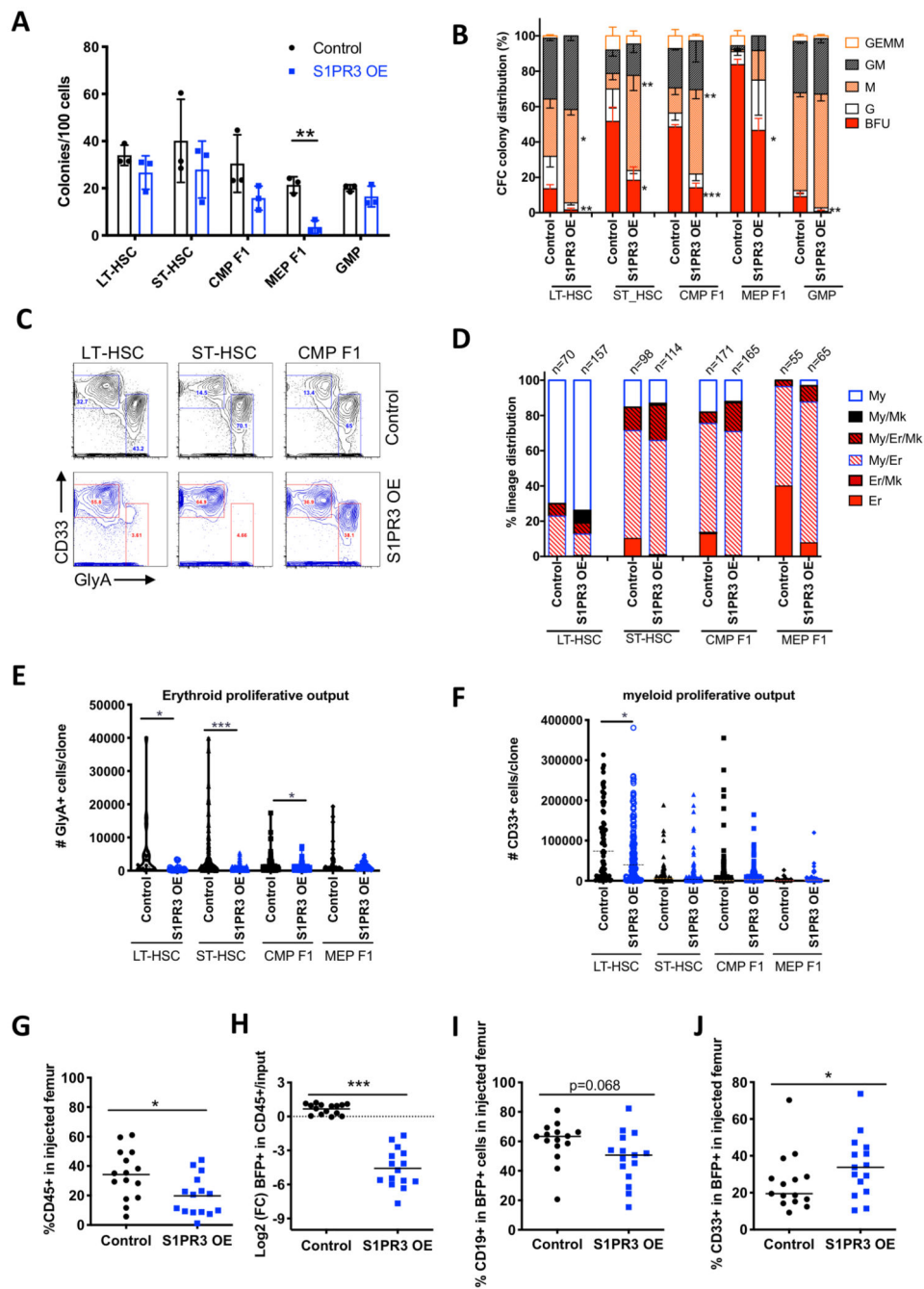


**Figure 1. S1PR3 is an inflammation-activated S1P receptor regulating lineage differentiation in human HSC.**

**A**, Heatmap of RNA-Seq gene expression (mean normalized counts) for 10 S1P genes in 13 human HSPC and mature populations isolated from CB shows myeloid lineage restriction for S1PR3. **B**, Representative flow cytometry plots showing gating scheme for CD14<sup>+</sup> myeloid and CD34<sup>+</sup> cells from 3 CB MNCs, in all live cells (black) and after gating for S1PR3<sup>-</sup> (gray) and S1PR3<sup>+</sup> (blue) cells. **C**, S1PR3 surface expression is absent in CD34<sup>+</sup> cells but present in CD14<sup>+</sup> monocytes and CD15<sup>+</sup> granulocytes shown by comparing the



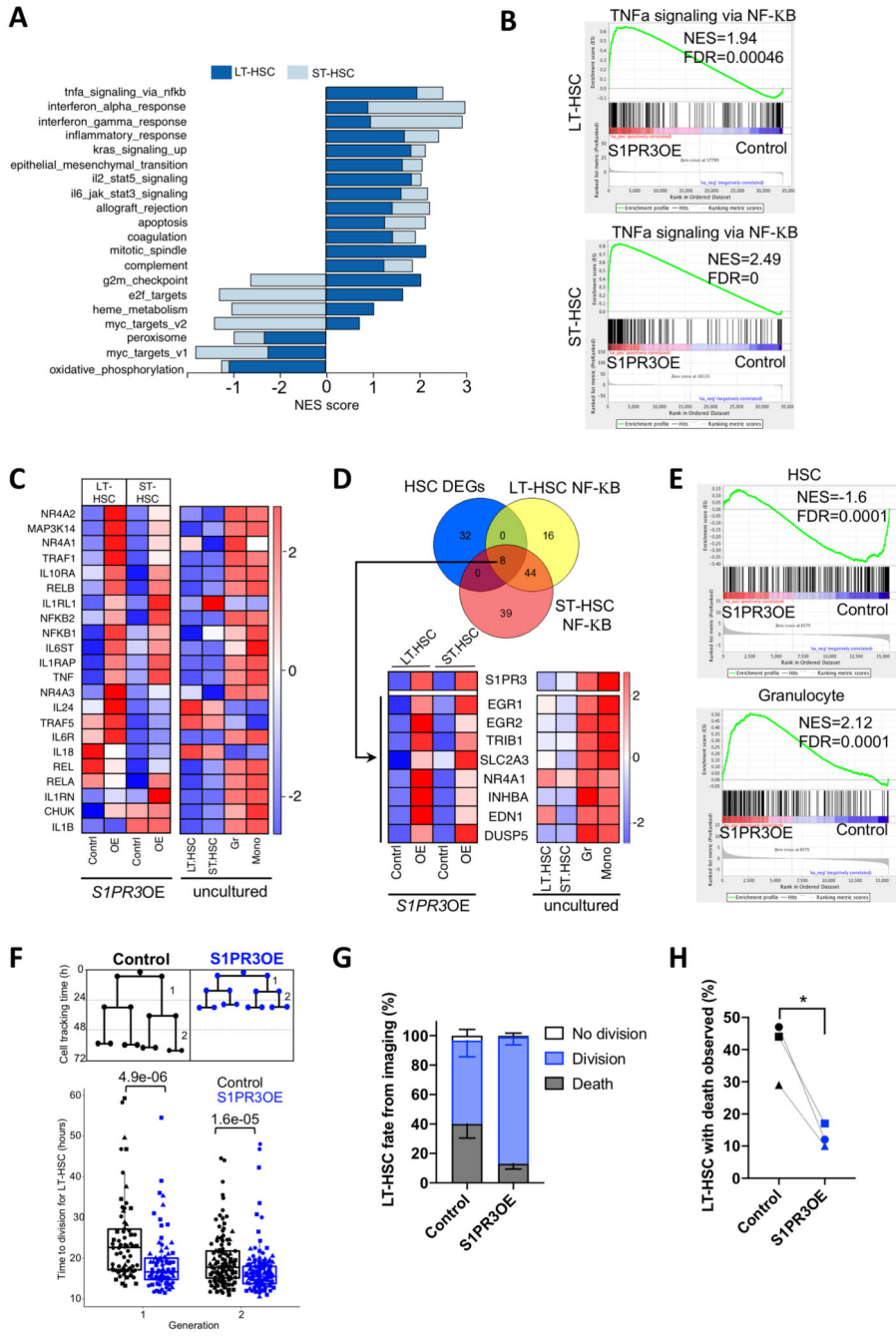
percentage of CD14<sup>+</sup>, CD15<sup>+</sup>, and CD34<sup>+</sup> cells within S1PR3<sup>-</sup> and S1PR3<sup>+</sup> gated populations. See Sup Fig. 1 for additional data including gating scheme and mean fluorescence intensity (MFI) quantification of S1PR3 related to 1B, C. **D**, Percentage of S1PR3<sup>+</sup> in indicated HSPC populations. **E**, Representative flow cytometry plots showing gating scheme for S1PR3<sup>+</sup> in indicated immunophenotypic HSPC populations (see Fig. S2A-B for culture conditions and gating scheme). **F**, Percentage of S1PR3<sup>+</sup> cells in HSPC populations on day 3 and **G**, Percentage of CD34<sup>+</sup> at day 8 culture in control media or with TNFa or IL6 treatment (n=3 CB pools). **H-I**, *S1PR3*OE alters lineage differentiation in the progeny of LT-HSC and ST-HSC in *ex vivo* culture at day 9 or 10 days. **H**, LT-HSC or ST-HSC progeny were analyzed for CD14 and GlyA and representative flow cytometry plots for control (black) or *S1PR3*OE (blue) are shown as overlays. Quantification of **I**, CD14<sup>+</sup> and **J**, GlyA<sup>+</sup> populations from **H**, (n=3 independent CB). **K**, GlyA expression following lentiviral knockdown of S1PR3 in LT-HSC and ST-HSC at 8 or 10 days post-transduction (n=2 CB). Statistical significance by unpaired Student t-test. \*\*\* P<0.001\*\* p<0.01, \*<0.05, unpaired Student t-test. Data are mean and s.d.



**Figure 2. S1PR3 overexpression is sufficient to promote myeloid differentiation in human HSPC *in vitro* and *in vivo*.**

**A**, The number of colonies for 100 transduced cells from the indicated populations at 10 days of a CFC assay for control and S1PR3OE lentiviral knockdown vectors ( $n=3$ ). **B**, Normalized colony distribution showing burst-forming units (BFU) are significantly decreased with *S1PR3*OE and macrophage (M) colonies are significantly increased from all scored cell populations ( $n=3$  CB). G (granulocyte); GEMM (granulocyte erythrocyte monocyte megakaryocyte). **C**, Myeloid (CD33<sup>+</sup>) vs erythroid (GlyA<sup>+</sup>) lineage distribution

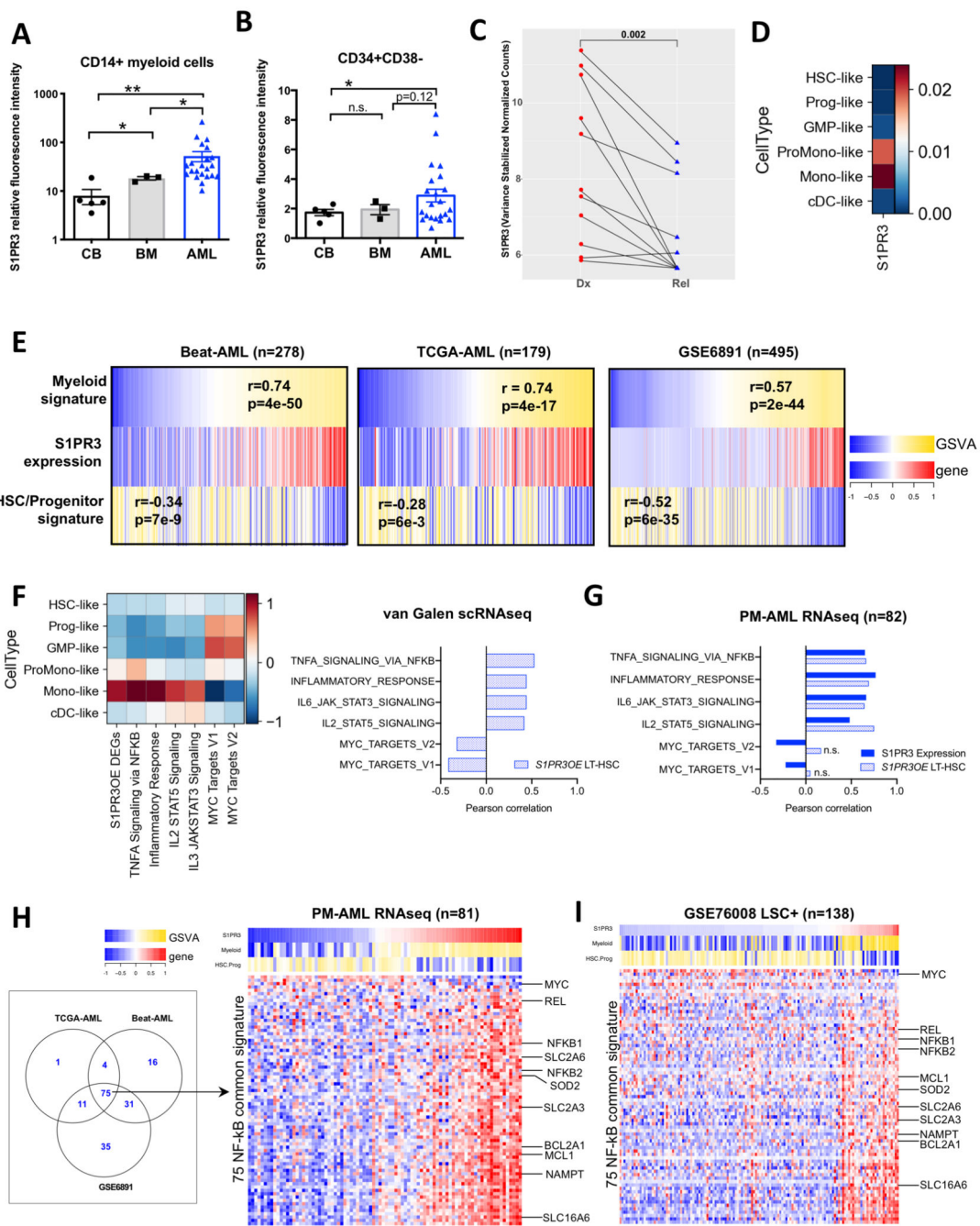
for all pooled cells from one representation assay at day 14 from **(A)**. **D**, Lineage distribution outcomes in single cell assays performed on MS5 stroma in erythroid-myeloid cytokine conditions. Data for indicated number of cells scored in each condition pooled from 3 CBs. **E-F**, Average number of GlyA<sup>+</sup> or CD33<sup>+</sup> cells from single cell differentiation assays **(D)**. **G**, Human CD45 chimerism, **H**, Log2 fold change (FC) in BFP which marks transduced cells relative to input, **I**, CD19<sup>+</sup> B lymphoid cells in the transduced fraction and **J**, CD33<sup>+</sup> myeloid cells in BFP<sup>+</sup> cells for control or *S1PR3*OE measured in the injected femur at 4 weeks post-transplant in xenotransplantation, 5 mice for each condition from 3 CB. \*\*\* P<0.001 \*\* p<0.01, \*<0.05, unpaired Student t-test. Data are mean and s.d. except for **E-F** where data are mean and s.e.m.



**Figure 3. *S1PR3* overexpression induces a myeloid inflammatory program in HSC.** **A**, Normalized enrichment scores (NES) for the pathways that are significantly different between control and *S1PR3OE* cells (FDR<0.01) in LT-HSC and ST-HSC (superimposed), following GSEA of the MSigDB v6.2 Hallmark gene sets in RNA-seq expression data, data from 3 CBs. *S1PR3OE* strongly upregulates inflammatory pathways in both HSC subsets. **B**, GSEA plots of TNFα via NF-κB Hallmarks gene set for LT-HSC and ST-HSC. **C**, Heatmap showing the median expression of selected NF-κB pathway genes, associated with both the canonical and non-canonical signaling pathway, and inflammatory cytokines and receptor

genes from the *S1PR3*OE RNA-seq and uncultured LT-HSC, ST-HSC, granulocytes (Gr) and monocytes (Mono). The majority of genes are highly expressed in myeloid lineages, and are upregulated with *S1PR3*OE in the HSC subsets. Of note, a number of inflammation genes exhibit distinct expression patterns in LT-HSC and ST-HSC with *S1PR3*OE including *TRAF5*, *IL1RL1*, *IL18*, *IL24* (an IL10 family member), and *IL1B*. **D**, Venn diagram representing the intersection of 40 significantly differentially expressed genes (DEGs, FDR<0.05) in *S1PR3*OE from both LT- and ST-HSC compared to control and leading edge genes of the NF- $\kappa$ B gene set in both LT-HSC and ST-HSC. The 8 genes found in the intersection pointed to upregulation of an *EGR1/2* associated myeloid transcriptional network. Heatmaps of the median expression of these 8 genes and *S1PR3* in control and *S1PR3*OE (OE) at day 3 compared to uncultured LT-HSC, ST-HSC, Gr and Mono are shown. **E**, GSEA plots for previously published HSC and granulocyte gene sets indicates *S1PR3*OE enriches for granulocyte and depletes HSC identity in genes common for LT-HSC and ST-HSC *S1PR3*OE RNA-seq (see Supplementary Fig. S4C-E for individual GSEA plots for LT-HSC and ST-HSC and enrichment map of pathways altered by *S1PR3*OE). **F**, Quantification of time to division for single cells tracked in the first two generations of control (n=34) or *S1PR3*OE (n=41) transduced LT-HSC beginning at day 3 post-transduction by time-lapse imaging (3 CB, marked by different symbols). Statistical significance by Mann-Whitney test. **G**, Distribution of division, death or no division fate for control (n=179) or *S1PR3*OE (n=198) LT-HSC cells tracked from 3 independent experiments. **H**, Percentage of LT-HSC (e) that died during time-lapse movies for control and *S1PR3*OE vectors. Paired T-test, \* p<0.05.

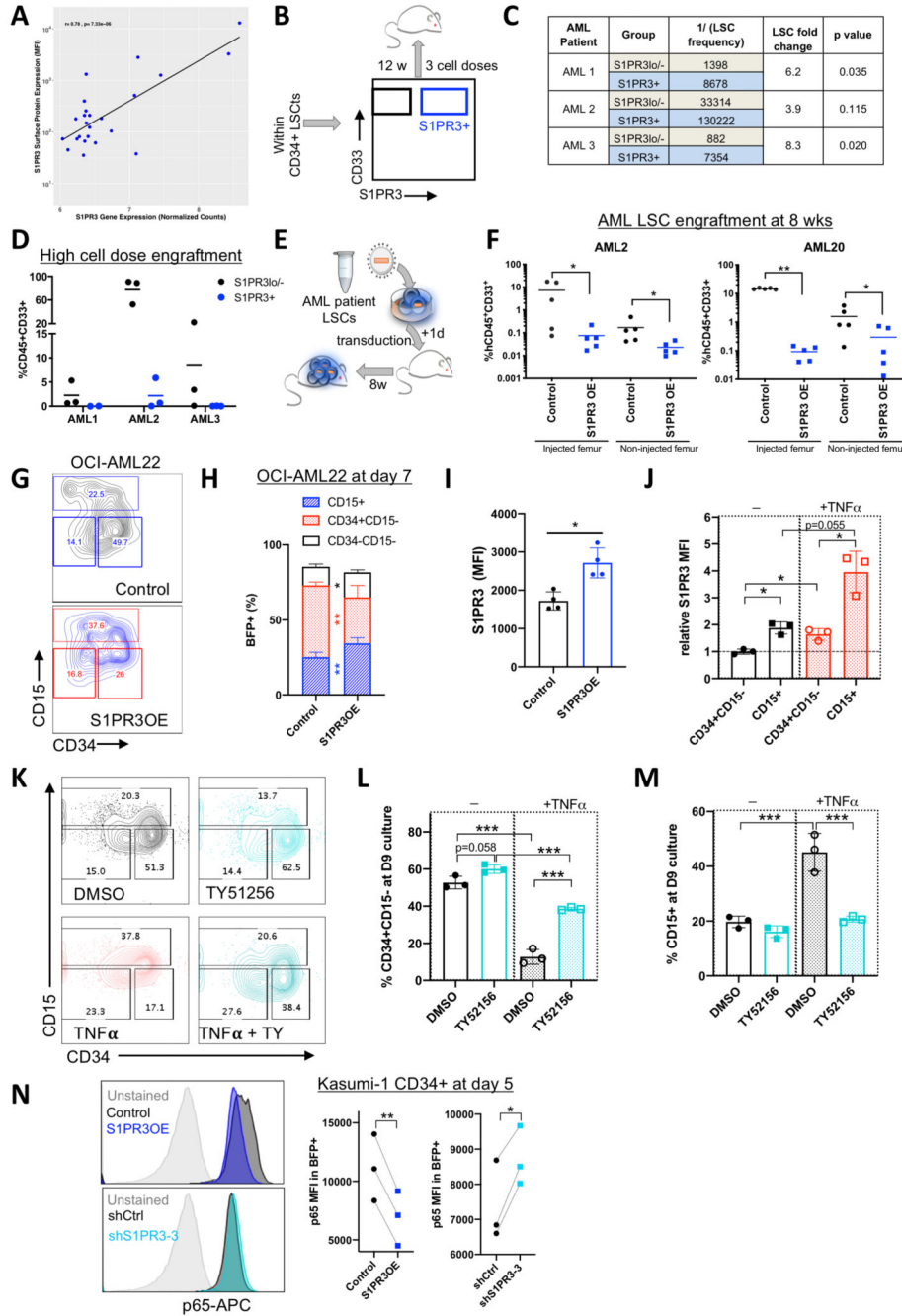




**Figure 4. S1PR3 marks a subset of LSCs in human AML with a mature myeloid state and a distinct inflammatory signature.**

Relative fluorescence intensity (RFI) of S1PR3 in **A**, CD14<sup>+</sup>myeloid cells and **B**, CD34<sup>+</sup>CD38<sup>-</sup> cells calculated as ratio of S1PR3 MFI to FMO controls from human CB MNCs (n=5), bone marrow (BM, n=3) and AML (n=22) samples. \*\* p<0.01, \*<0.05, unpaired Student t-test. Data are mean and s.d. **C**, *S1PR3* gene expression in paired diagnosis (Dx, red) and relapse (rel, blue) samples from 11 patients previously used to study the origin of AML relapse. Statistical significance by Wilcoxon signed-rank test. **D**, AUCell

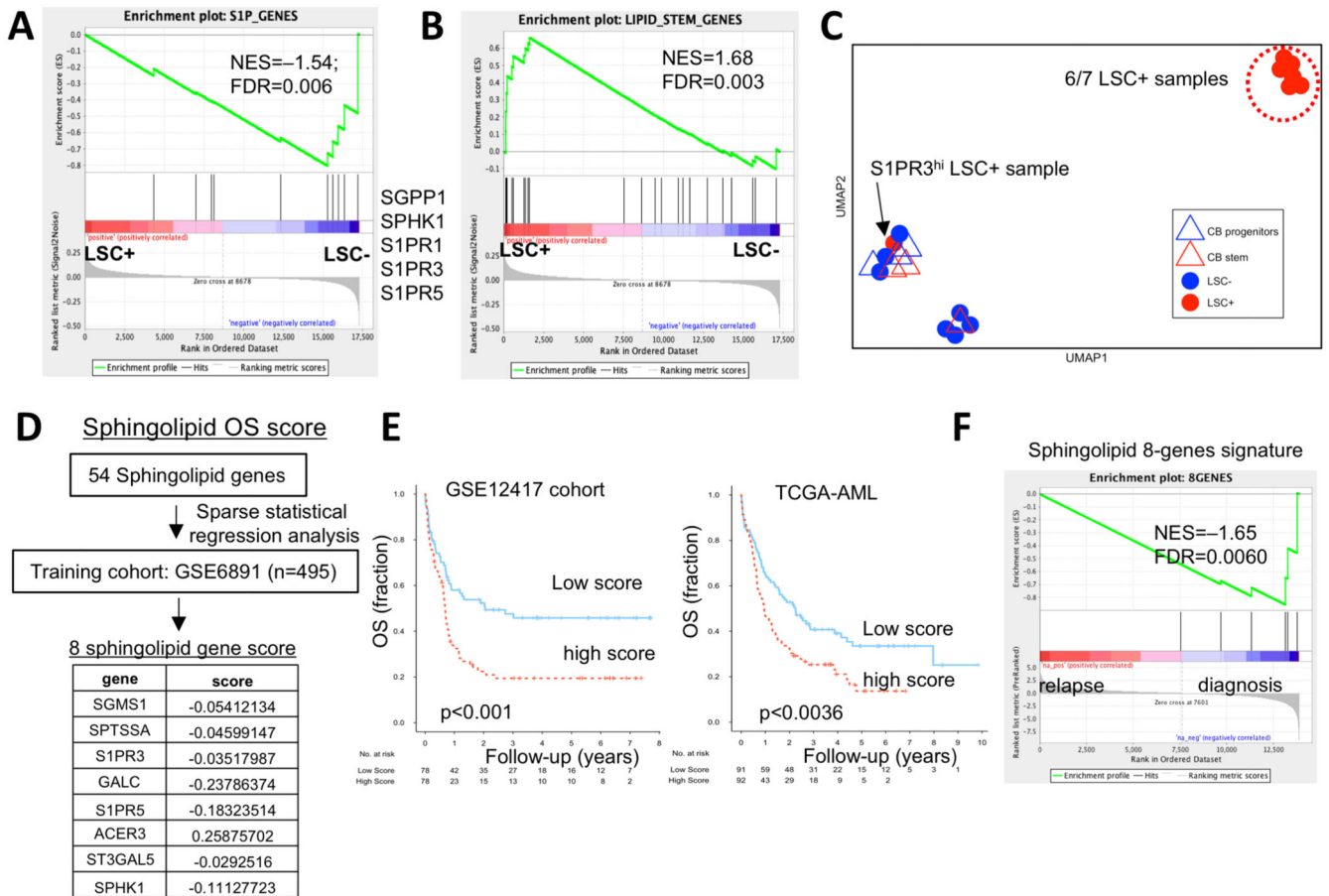
analysis across 11641 malignant cells from 12 AML patients for *S1PR3* gene expression as defined in indicated AML cell-types as described in Galen, et al.(3) **E**, Heatmaps depicting *S1PR3* gene expression with previously defined myeloid and HSC/progenitor (stem) signatures for *de novo* AML patients in the Beat-AML, TCGA-AML and GSE6891 cohorts. Rho correlation scores and p-values for *S1PR3* gene expression to the signatures indicated for each cohort showing correlation to the myeloid signature and anti-correlation to the stem signature. **F**, AUCCell analysis across van Galen scRNAseq data for the set of differentially expressed genes upon *S1PR3*OE in LT-HSC (*S1PR3*OE DEGs, Supplementary Table 3) and selected Hallmark gene sets upregulated upon *S1PR3*OE in LT-HSC with associated Pearson correlation. **G**, GSVA analysis of *S1PR3* gene expression and the set of *S1PR3*OE DEGs to selected Hallmark gene sets upregulated upon *S1PR3*OE in LT-HSC in a Princess Margaret Cancer Centre (PM) AML RNAseq patient cohort. **H**, Venn diagram of genes that show significant correlation from the NF- $\kappa$ B Hallmark gene set with *S1PR3* gene expression in diagnostic samples from Beat-AML, TCGA-AML and GSE6981 patient cohorts yielded 75 common genes. Heatmap of common genes (rows) for the PM-AML patient (columns) cohort shows myeloid-like AMLs have enrichment of NF- $\kappa$ B common genes. *S1PR3* gene expression and GSVA correlation values for the myeloid and HSC/Progenitor signatures are shown on top of the heatmap. **I**, Heatmap of 75 genes in the LSC+ subpopulations from GSE76008 indicates a similar relationship of *S1PR3* gene expression to enrichment for the set of 75 NF- $\kappa$ B genes as in unfractionated patient samples.



**Figure 5. S1PR3 selects for less functional LSCs and regulates AML differentiation via TNF $\alpha$  via NF $\kappa$ B signaling.**

**A**, Correlation of *S1PR3* gene expression from the GSE76008 cohort and S1PR3 MFI measured by flow cytometry for LSC<sup>+</sup> subpopulations (n=24) from 13 patients. **B**, Experimental schematic showing LSC<sup>+</sup> containing patient cell fractions (Supplementary Fig. 7) were sorted based on S1PR3 surface expression and transplanted in different cell doses into NSG mice for 12 weeks. **C**, Table with LSC frequency as calculated by ELDA. **D**, CD45<sup>+</sup>CD33<sup>+</sup> engraftment of mice injected with the highest cell dose of S1PR3<sup>+</sup> (blue) or

S1PR3<sup>-/lo</sup> (black) cells is shown. **E**, Experimental schematic for lentiviral transduction of control (black) or *S1PR3*OE (blue) vectors into patient AML LSCs and transplanted into NSG mice for (F). **F**, CD45<sup>+</sup>CD33<sup>+</sup> engraftment of human CD34<sup>+</sup>CD38<sup>-</sup> AML cells from 2 patients in NSG mice at 8 weeks post-transplantation in the injected and non-injected femurs. OCI-AML22 is an AML cell line model derived from an AML patient at relapse where the stem cell fraction is contained in the CD34<sup>+</sup> fraction. **G**, Representative flow cytometry plots for CD15 vs CD34 markers 7 days-post-transduction (n=2 experiments, in duplicate) **H**, Distribution of indicated immunophenotypic populations and **I**, S1PR3 surface expression (MFI) for control and *S1PR3*OE transduced OCI-AML22 cells at day 7 post-transduction. **J**, S1PR3 MFI in OCI-AML22 subpopulations normalized to an untreated CD34<sup>+</sup>CD15<sup>-</sup> sample 9 days post-treatment with 10ng/ml TNF $\alpha$  (n=3). K-M, Representative flow cytometry plots and quantitation for CD15 and CD34 markers 9 days post-treatment with DMSO or 5 $\mu$ M TY51256 alone or with 10ng/ml TNF $\alpha$  in OCI-AML22. N, Representative histogram plots of p65 RelA expression by intracellular flow cytometry and the quantitation of p65 mean fluorescence intensity (MFI) with *S1PR3*OE or shS1PR3-3 relative to controls in Kasumi-1 CD34<sup>+</sup> cells at 5 days post-transduction (n=3) \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Unpaired t-test (F,H,I); one-way ANOVA (J, L, M); paired t-test (N). Data are mean and s.d.

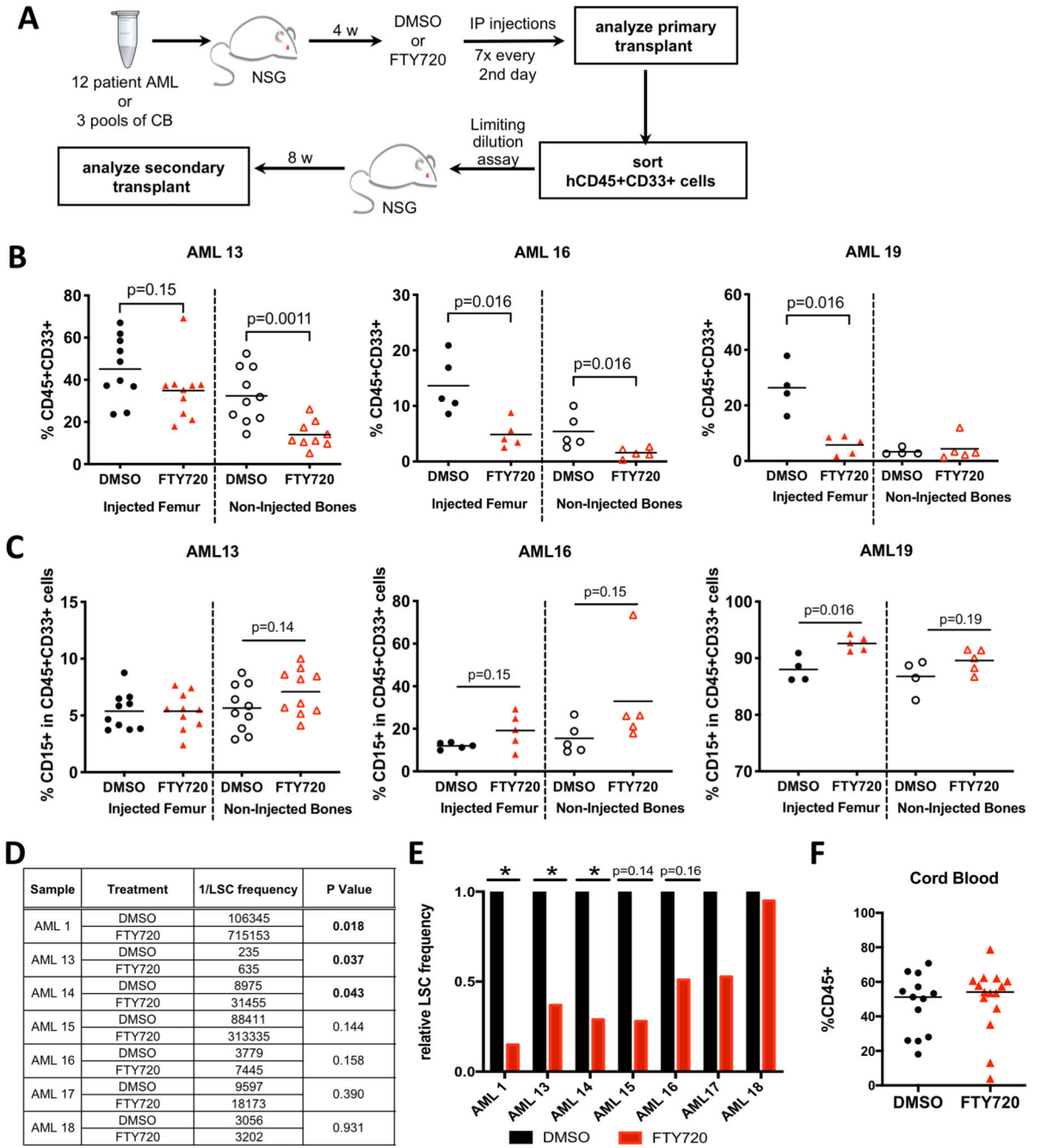


**Figure 6. Spingolipid genes including *S1PR3* predict prognosis in human AML.**

**A**, GSEA analysis of 10 S1P genes in AML LSC<sup>+</sup> and LSC<sup>-</sup> gene expression data shows enrichment of *S1PR1*, *S1PR3*, *S1PR5*, the S1P kinase (*SPHK1*) in LSC<sup>-</sup> samples. See Supplementary Table 12 for LSC ranking. **B**, GSEA analysis of 23 lipid-stem genes, previously shown to be enriched in HSC subsets relative to committed progenitor populations, in AML LSC<sup>+</sup> and LSC<sup>-</sup> gene expression data shows enrichment in LSC<sup>+</sup> samples. **C**, A uniform manifold approximation and projection (UMAP) plot for the spingolipid composition of samples measured by LC/MS spectrometry in functionally defined individual LSC<sup>+</sup> (n=7) and LSC<sup>-</sup> (n=7) fractions from 10 patient AMLs as determined by xenotransplantation and normal stem (CD34<sup>+</sup>CD38<sup>-</sup>) and progenitor (CD34<sup>+</sup>CD38<sup>+</sup>) fractions (CB, n=3). LSC<sup>+</sup> (red circles), LSC<sup>-</sup> (blue circles), CB stem (red triangles), CB progenitor (blue triangles). LSC<sup>+</sup> subpopulation from AML9, which is high for *S1PR3* gene expression, clusters away from other 6 LSC<sup>+</sup> samples. See Supplementary Figure 9C-G for spingolipid composition distribution of individual samples and quantification of specific spingolipid species and Supplementary Table 9 for patient AML data. **D**, Sparse regression analysis, as described in Ng, et al. (7), to derive a weighted spingolipid gene score predictive of overall survival (OS) applied to a large training cohort of n=495 AML patients yielded a set of 8 genes with their associated scores which were then validated on GSE12417 and TCGA-AML cohorts in univariate analysis. These included 3 genes associated with S1P production and signaling and 5 genes encoding spingolipid

enzymes. **E**, Kaplan-Meier estimates of OS based on above versus below median values of a weighted sphingolipid gene score. A low score is associated with greater OS. **F**, GSEA plot for sphingolipid 8-gene OS signature shows negative enrichment in patient samples at relapse.





**Figure 7. S1P signaling modulation *in vivo* targets LSC function in human AML.**

**A-F**, Pre-clinical targeting of the sphingolipid pathway with the S1P prodrug FTY720, clinically known as Fingolimod, in AML patient samples or CB (n=3) *in vivo*. **A**, Experimental schematic for xenotransplantation. AMLs with >20% engraftment in the injected femur and/or showed decrease in AML burden were taken through secondary transplant to enumerate LSC frequency in (D). **B**, AML engraftment for each mouse of the three FTY720 (red) responders relative to DMSO control (black), as defined by significant decrease of AML burden at 6 weeks post-transplant. AML13 is a treatment resistant sample.

AML16 is a relapse sample. AML 19 is a diagnosis sample. Significance calculated by Mann-Whitney test. Sup. Fig. 10 for additional engraftment data. **C**, Percentage of CD15<sup>+</sup> cells in the indicated xenografts. **D-E**, LSC frequency for indicated AMLs was determined in limiting dilution serial transplantation assays and calculated with ELDA. Mice were considered engrafters if CD45<sup>+</sup>CD33<sup>+</sup> cells >0.5%. The calculated LSC frequency and p value for FTY720 samples relative to DMSO control samples are shown in (D) and the relative secondary LSC frequency are shown in (E). **F**, Human CD45<sup>+</sup> engraftment of CB following treatment with DMSO or FTY720 at 6 weeks post-transplantation.