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 HSCs by activating stress myelopoiesis, such roles in LSCs are poorly understood. Here, we show that S1PR3, a receptor for the bioactive lipid sphingosine-1-phosphate, is a central regulator that drives myeloid differentiation and activates inflammatory programs in both HSCs and LSCs. S1PR3-mediated inflammatory signatures varied in a continuum from primitive to mature myeloid states across cohorts of patients with AML, each with distinct phenotypic and clinical properties. S1PR3 was high in LSCs and blasts of mature myeloid samples with linkages to chemosensitivity, whereas 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.

**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.

See related commentary by Yang et al., p. 3.
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 cell (LT-HSC) counterpart that sustains lifelong blood production (2). LSCs are often resistant to standard chemotherapy and are responsible for clinical relapse (2, 5–7). AML is a cellular hierarchy, driven by LSCs 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 hematopoietic stem cells (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 HSCs 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 (10, 11). 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 and 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.

Disregulated myeloid cells are the linchpin of inflammation within many chronic human conditions associated with aging, including clonal hematopoiesis and atherosclerotic cardiovascular disease (29–31). TNFα and IL6 are two key inflammatory cytokines linked to chronic inflammatory diseases, myeloid dysregulation, and stem cell function (11, 23, 24). Moreover, a large body of literature points to NF-κB as a crucial regulator of inflammatory activation in HSCs and regulates quiescence, proliferation, and survival, particularly upon TNFα stimulation (23). NF-κB was shown to be constitutively active in LSCs from patients with AML (33). At the time, it was anticipated that modulating inflammation through inhibition of some of the key signaling molecules, including TNFα, IL6, and NF-κB, could be exploited therapeutically for treating AML. However, some of these same pathways, for example TNFα via NF-κB, 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 LSCs and blasts. Here, we show that the S1P signaling axis via S1PR3 promotes myeloid differentiation in human HSCs, is dysregulated in AML, and intersects with the TNFα via NF-κB 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 HSCs

To gain deeper insight into the function of S1P in human hematopoiesis, we undertook gene expression analysis of 10 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; 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 S1PR3 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 CD34+ myeloid cells, particularly within the CD14+ subset of mononuclear cells (MNC) where the majority of CD14+ cells showed co-staining with S1PR3 (Fig. 1B and C; Supplementary Fig. S1A–S1H). Notably, S1PR3 was expressed at low levels in primitive CD34+ 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. S1H–S1J). These findings raised the question of 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 affect S1PR3 in primitive CB cells. Following 3 days of culture in a low-cytokine media when the majority of LT- and short-term (ST)-HSCs have not yet undergone the first division from quiescence (ref. 38; see Supplementary Fig. S2A and S2B for experimental scheme and HSPC gating scheme), we observed a 3-fold increase in the proportion of S1PR3+ cells within the GMP subset over uncultured GMPs; IL6 further enhanced the percentage of S1PR3+ GMP cells 4.6-fold over uncultured GMPs (Fig. 1D–F). Whereas S1PR3 was not detected on the surface of uncultured LT-HSCs (Fig. 1D; Supplementary Fig. S1I and S1J), TNFα treatment in culture was sufficient to promote expression of S1PR3 on a small but significant population of immunophenotypic cultured LT-HSCs (cLT-HSC; 0.5% S1PR3+ cells in control media vs. 1.3% S1PR3+ with TNFα treatment) despite no significant change in the percentage of cLT-HSCs (Fig. 1D–F; Supplementary Fig. S2C). Moreover, TNFα treatment reduced surface S1PR3 expression on GMPs at 3 days in culture to levels similar to those in uncultured GMPs, and provoked
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**A**

Heatmap showing gene expression of SGPP1, S1PR4, SGPP2, SPHK2, S1PR3, SGPL1, S1PR2, S1PR1, and S1PR5 in various cell populations.

**B**

Flow cytometry images showing CD4 and SSC markers for S1PR3 subpopulations.

**C**

Bar graph showing percentage of CD34+ within S1PR3 subpopulation.

**D**

Graph showing percentage of S1PR3+ in uncultured CB.

**E**

Flow cytometry images showing GlyA+ in transduced cells.

**F**

Bar graph showing percentage of S1PR3+ at day 3 for cLT-HSC, cLT-HSC, and GMP.

**G**

Graph showing CD34+ at day 8 culture for Control, TNF, and IL6.

**H**

Graph showing GlyA+ at day 8 culture for LT-HSC and ST-HSC.

**I**

Bar graph showing percentage of CD4+ in transduced cells for LT-HSC and ST-HSC.

**J**

Bar graph showing percentage of GlyA+ in transduced cells for LT-HSC and ST-HSC.

**K**

Bar graph showing percentage of GlyA+ in transduced cells for shCtrl, shS1PR3-2, and shS1PR3-3 for LT-HSC and ST-HSC.

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**Supplementary Information**

**S1PR3 Promotes Myeloid Differentiation**

**Figure Legends**

**A**
- Heatmap showing gene expression of various S1PR family members

**B**
- Flow cytometry images assessing S1PR3 subpopulations

**C**
- Bar graph illustrating CD34+ within S1PR3 subpopulation

**D**
- Graph depicting S1PR3+ in uncultured CB

**E**
- Flow cytometry images evaluating GlyA+ in transduced cells

**F**
- Bar graph showing S1PR3+ at day 3 for different cell types

**G**
- Graph demonstrating CD34+ at day 8 culture under control, TNF, and IL6 conditions

**H**
- Graph illustrating GlyA+ at day 8 culture for LT-HSC and ST-HSC

**I**
- Bar graph comparing CD4+ in transduced cells for LT-HSC and ST-HSC

**J**
- Bar graph assessing GlyA+ in transduced cells for LT-HSC and ST-HSC

**K**
- Bar graph showing GlyA+ in transduced cells for various shRNA conditions
loss of CD34+ cells at 8 days in culture with no significant effects on CD14 differentiation (Fig. 1D–G; Supplementary Fig. S2D and S2E), consistent with the distinct effects of TNFα on LT-HSCs and GMPs 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. S2E). These data suggest that surface expression of S1PR3 is regulated by proinflammatory 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. S1PR3OE cells exhibited increased levels of surface S1PR3 in the progeny of LT-HSCs and ST-HSCs by flow cytometry relative to control cells (Supplementary Fig. S3A). S1PR3OE in LT-HSCs and ST-HSCs resulted in enhanced CD14+ myeloid differentiation and decreased GlyA+ erythroid differentiation compared with 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. S3B). There was a trend toward enhanced erythroid differentiation with both S1PR3 KD constructs in ST-HSCs, whereas only shS1PR3-2 in LT-HSCs increased GlyA+ expression (Fig. 1K; Supplementary Fig. S3C). However, the level of S1PR3 KD with these constructs did not appear to be sufficient to affect CD14+ myeloid differentiation in vitro (Supplementary Fig. S3D). These data indicate that S1PR3 is a myeloid-associated S1P receptor that regulates lineage differentiation in human HSCs.

**S1PR3OE Is Sufficient to Promote Myeloid Differentiation in Human HSPCs In Vitro and In Vivo**

To determine if S1PR3OE was promoting myeloid differentiation in HSPC subpopulations with multilineage differentiation capacity at the single-cell level, transduced (BFP+) LT-HSCs, ST-HSCs, common myeloid progenitor (CMP)-F1, and megakaryocyte erythroid progenitor (MEP)-F1 were isolated 3 days after infection and plated in colony-forming cell (CFC) or single-cell MS5 stromal assays (ref. 14; see Supplementary Fig. S3E and S3F for experimental design). S1PR3OE reduced erythropoiesis S1PR3+ mice, indicating that S1PR3OE reduced erythropoiesis in vivo (Supplementary Fig. S4D and S4E). Overall, our findings demonstrate that S1PR3OE 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 myelopoeisis models (22, 42).

**S1PR3 Induces a Myeloid Inflammatory Program in HSCs**

To ascertain the molecular and biological pathways altered by S1PR3OE in human CB HSCs, we performed RNA...
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A

B

C

D

E

F

G

H

I

J
sequencing (RNA-seq) of LT- and ST-HSCs 3 days after transduction with control or S1PR3OE vectors (Supplementary Fig. S5A and SSB). Gene set enrichment analysis (GSEA) with the hallmark gene sets (FDR q value < 0.01; Fig. 3A; Supplementary Table S1) showed that S1PR3OE upregulated many of the same pathways in both HSC subsets. Mitotic spindle, G2–M checkpoint, and TNFα signaling via NF-κB (Fig. 3B) were the top three enriched gene sets in LT-HSCs relative to controls, suggesting that S1PR3OE induced an activated inflammatory program. Inflammation gene sets were enriched in S1PR3OE ST-HSCs, with genes related to S1PR3 activated inflammatory program. Inflammation gene sets S1PR3 (Fig. 3B) were the top three enriched gene sets in LT-HSCs q induction with control or S1PR3 dataset (50) showed that OE LT- and ST-HSCs had S1PR3 using a published human hematopoietic gene expression Tribbles 1 (EGR1/2 Growth Response 1 and 2 (23) of eight myeloid lineage–associated genes, including genes q OE HSC subsets (Supplementary Table S3; controls and S1PR3 using a published human hematopoietic gene expression dataset (9, 23). Hence, we performed a focused analysis comparing the significantly enriched leading-edge genes from the NF-κB hallmark gene set (Supplementary Table S2) and the genes that were differentially expressed between controls and S1PR3OE HSC subsets (Supplementary Table S3; 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; refs. 46–49; Fig. 3D). These results suggest that S1PR3 regulates the well-known ERG–NAB–GFI1 myeloid gene regulatory network (46) in human HSCs. GSEA using a published human hematopoietic gene expression dataset (50) showed that S1PR3OE LT- and ST-HSCs had significant positive enrichment of granulocyte genes and negative enrichment for HSC genes, providing independent confirmation of myeloid lineage induction (Fig. 3E; Supplementary Fig. S5C and S5D). S1PR3OE cells resembled granulocytes with not only strong upregulation of inflammation gene sets but also downregulation of HSC-enriched metabolic gene sets (Supplementary Fig. S5E). If S1PR3OE promotes differentiation via an inflammation-induced emergency hematopoiesis-like mechanism, then S1PR3OE LT-HSCs should exhibit cell-cycle activation and a shorter division time, as has been observed in murine HSCs following treatment with inflammatory cytokines (22). Indeed, live-cell imaging analysis (51) demonstrated that S1PR3OE resulted in faster cell-division kinetics and better survival of LT-HSCs compared with controls (Fig. 3E–H; Supplementary Movie). Overall, this strong induction of inflammatory gene sets upon S1PR3OE together with the upregulation of S1PR3 membrane expression on HSC by TNFα treatment suggests that S1PR3 may function as a downstream potentiator of inflammatory signaling in human HSCs 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+ myeloid and CD34+CD38- primitive cell subsets from patients with AML (n = 22) compared with CB (n = 5) and normal adult bone marrow (n = 3; Fig. 4A and B; Supplementary Table S4), although there was considerable interpatient 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 with paired diagnosis samples (ref. 5; Fig. 4C). Thus, we hypothesized that higher S1PR3 expression may identify less primitive AML cases. We analyzed S1PR3 gene expression in a recently described single-cell RNA-seq (scRNA-seq) dataset (3) across 11,641 malignant cells from 12 patients with AML clustered by their similarity to six comparable populations.
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A

- tnta_signaling_via_rtkb
- interferon_alpha_response
- interferon_gamma_response
- inflammatory_response
- kras_signaling_up
- epithelial_mesenchymal_transition
- il1_jak_stat3_signaling
- allograft_rejection
- apoptosis
- coagulation
- mitotic_spindle
- complement
- g2m_checkpoint
- e2f_targets
- heme_metabolism
- myc_targets_v2
- peroxisome
- oxidative phosphorylation

NES score

B

- TNFa signaling via NF-κB

NES = 1.94
FDR = 0.00046

- TNFa signaling via NF-κB

NES = 2.49
FDR = 0

C

D

- HSC DEGs
- LT-HSC NF-κB

E

- HSC

NES = -1.6
FDR = 0.0001

- Granulocyte

NES = 2.12
FDR = 0.0001

F

- Control
- S1PR3OE

Cell tracking time (h)

Time to division for LT-HSC (hours)

0.4e-06

Control
S1PR3OE

1.6e-05

Time to division for LT-HSC (hours)

G

- LT-HSC fate from imaging (%)

No division
Division
Death

Control
S1PR3OE

H

- LT-HSC with death observed (%)

Control
S1PR3OE
S1PR3 from GSE76008 indicates a similar relationship of human CB MNC (n = 40) and S1PR3 in CD14+CD38− cells as in unfractionated patient samples. Relative fluorescence intensity of S1PR3 in CD14+ myeloid cells (A) and CD34+CD38− cells (B) calculated as ratio of S1PR3 mean fluorescence intensity to fluorescence minus one controls from human CB MNC (n = 5), bone marrow (BM; n = 3), and AML (n = 22) samples. n.s., not significant; **, P < 0.05; and ***, P < 0.01, unpaired Student t test. Data are mean and SD. 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 11,641 malignant cells from 12 patients with AML for S1PR3 expression as defined in indicated AML cell types as described in study by van Galen and colleagues (3). Mono-like, monocyte-like; Prog-like, progenitor-like; ProMono-like, promonocyte like. Heatmaps depicting S1PR3 gene expression with previously defined myeloid and HSC/progenitor (stem) signatures for patients with de novo AML 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 anticorrelation to the stem signature. E, AUCell analysis across van Galen scRNA-seq data for the set of differentially expressed genes upon S1PR3OE in LT-HSC (S1PR3OE DEGs; Supplementary Table S3) and selected hallmark gene sets upregulated upon S1PR3OE in LT-HSCs with associated Pearson correlation. G, GSVA of S1PR3 gene expression and the set of S1PR3OE DEGs to selected hallmark gene sets upregulated upon S1PR3OE in LT-HSCs in a PM-AML RNA-seq patient cohort. H, Venn diagram of genes that show significant correlation from the NF-κB 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-κB 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-κB genes as in unfractionated patient samples.

Figure 4. S1PR3 marks a subset of LSCs in human AML with a mature myeloid state and a distinct inflammatory signature. Relative fluorescence intensity of S1PR3 in CD14+ myeloid cells (A) and CD34+CD38− cells (B) calculated as ratio of S1PR3 mean fluorescence intensity to fluorescence minus one controls from human CB MNC (n = 5), bone marrow (BM; n = 3), and AML (n = 22) samples. n.s., not significant; **, P < 0.05; and ***, P < 0.01, unpaired Student t test. Data are mean and SD. 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 11,641 malignant cells from 12 patients with AML for S1PR3 gene expression as defined in indicated AML cell types as described in study by van Galen and colleagues (3). Mono-like, monocyte-like; Prog-like, progenitor-like; ProMono-like, promonocyte like. Heatmaps depicting S1PR3 gene expression with previously defined myeloid and HSC/progenitor (stem) signatures for patients with de novo AML 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 anticorrelation to the stem signature. E, AUCell analysis across van Galen scRNA-seq data for the set of differentially expressed genes upon S1PR3OE in LT-HSC (S1PR3OE DEGs; Supplementary Table S3) and selected hallmark gene sets upregulated upon S1PR3OE in LT-HSCs with associated Pearson correlation. G, GSVA of S1PR3 gene expression and the set of S1PR3OE DEGs to selected hallmark gene sets upregulated upon S1PR3OE in LT-HSCs in a PM-AML RNA-seq patient cohort. H, Venn diagram of genes that show significant correlation from the NF-κB 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-κB 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-κB genes as in unfractionated patient samples.
along the normal bone marrow HSC to myeloid axis [HSC-like, progenitor-like, GMP-like, promonocyte-like, monocyte-like, or conventional DC (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 scRNA-seq platform was only able to capture the low abundance of S1PR3 transcript in <0.01% of cells, we analyzed transcriptomes from unfractonated patients with AML generated by RNA-seq or microarray analysis. Gene set variation analysis (GSVA) of the Beat-AML (35), The Cancer Genome Atlas (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 scRNA-seq AML dataset (ref. 3; Fig. 4E; Supplementary Tables S5 and S6). 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 S1PR3OE-induced signatures described in Fig. 3 in cohorts of AML samples. AUCell (area under the curve Cell) analysis across 11,641 single malignant cells revealed that the S1PR3OE LT-HSC signature was expressed in 86.5% of cells and most enriched in the mono-like subpopulation of patients with AML (ref. 3; Figs. 3A and 4F). Importantly, the S1PR3OE signature was significantly correlated to a number of inflammatory gene sets, including the NF-κB hallmark gene set, whereas MYC targets were negatively correlated, reminiscent of the phenotype observed in LT-HSCs upon S1PR3OE (Figs. 3A and 4F; Supplementary Table S7). Moreover, GSVA in an independent RNA-seq AML cohort from Princess Margaret (PM) showed strong correlation of both single S1PR3 gene expression and the S1PR3OE signature to a number of inflammatory gene sets that were also induced by S1PR3OE in HSCs (Fig. 4G). These data suggest that the malignancy state in S1PR3-expressing AML cells is congruous with the transcriptional inflammatory state activated by S1PR3OE in LT-HSCs.

We next investigated whether individual components of the S1PR3-mediated inflammatory pathways such as NF-κB were similarly regulated in individual myeloid-like or stem-like AML patients. To this end, we derived a common 75 S1PR3–NF-κB–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-κB hallmark gene set and S1PR3 expression, and validated it in the PM cohort (Fig. 4H; Supplementary Fig. S6A; Supplementary Table S8; clinical data in Supplementary Table S9). Importantly, the S1PR3–NF-κB–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. S6A; Supplementary Tables S5, S6, and S8), myeloid-like LSC+ and LSC− cell fractions were enriched for the common S1PR3–NF-κB signature (Fig. 4I; Supplementary Fig. S6B). The S1PR3–NF-κB signature included genes encoding the NF-κB subunits REL, NFKB1, and NFKB2; the antiapoptotic BCL2 family members MCL1 and BCL2A1; and the 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 S1PR3OE in normal HSCs. These results indicate that NF-κB regulation is heterogeneous among individual patient with AML and raise the possibility that the NF-κB–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 azacitidine and venetoclax (refs. 54, 55; Supplementary Fig. S6C). Overall, these results demonstrate that S1PR3 expression can classify individual patient with AML 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 TNFα via NF-κB 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; Supplementary Tables S10 and S11). CD34+ LSCs from three of these patients were fractionated based on surface expression of S1PR3 (1%–6% S1PR3+; see Supplementary Fig. S7) and transplanted at limiting dilution into NSG mice (Fig. 5B). S1PR3+ AML cells had reduced engraftment potential at 12 weeks and lower LSC frequency compared with S1PR3low−/− cells (Fig. 5C and D). These data show that S1PR3 expression is sufficient to identify a subset of cells with low LSC activity in samples of patients with AML.

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 and 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. S8A–S8E). We found that TNFα enhanced S1PR3 surface expression in both the OCI-AML22 model we derived from a relapse patient (S6) and in Kasumi-1 cells, compared with controls (Fig. 5J; Supplementary Fig. S8F). Next, we interrogated the role of endogenous S1PR3 in AML. The
**A** S1PR3 gene expression (normalized counts) and S1PR3 surface protein expression (MFI).

**B** Expression of S1PR3 in AML LSCs.

**C** Summary of AML patient LSC fold change.

**D** High dose engraftment.

**E** Transduction of OCI-AML2 cells with S1PR3OE or shS1PR3-3.

**F** AML LSC engraftment at 8 wks.

**G** OCI-AML2 cells at day 7.

**H** P65 MFI in BFP.

**I** Relative S1PR3 MFI.

**J** Number of CD15+CD34+LSCs at day 5.

**K** Effects of DMSO and TY51256 on CD15+CD34+ LSCs.

**L** Kasumi-1 CD34+ at day 5.

**M** Kasumi-1 CD34+ at day 5.
S1PR3 Promotes Myeloid Differentiation

S1PR3 antagonist TY51256 restricted myeloid differentiation in both AML cell line models during in vitro culture (Fig. 5K–M; Supplementary Fig. S8G–S8I). Importantly, in OCI-AML22, TY51256 counteracted the myeloid differentiation enacted by TNFα treatment (Fig. 5K–M). Moreover, shRNA-mediated S1PR3 KD with shS1PR3-3 in Kasumi-1 CD34+ cells significantly decreased the CD34+ CD15+ and CD34+CD15− subpopulations at 9 days after transduction (Supplementary Fig. S8J). We also measured the intracellular levels of the p65 RelA subunit of NF-κB 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 anticorrelates with LSC activity in human AML, engagement of which disrupts LSC function in part by inducing differentiation via the TNFα–NF-κB 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 samples of patients with AML in the GSE76008 dataset 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 (Supplementary Table S12). 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 HSCs (14). This lipid-stem signature was enriched in LSC+ compared with 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 S12) and in particular upregulates the biosynthetic pathway generating S1P via ceramide as an intermediate (see Supplementary Fig. S9 for biosynthetic pathway; refs. 14, 17).

To determine if sphingolipid levels are dysregulated in LSC+ populations, we profiled the sphingolipidome of LSC+ and LSC− samples were the sphingolipidome of LSC+ and 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 S12) and in particular upregulates the biosynthetic pathway generating S1P via ceramide as an intermediate (see Supplementary Fig. S9 for biosynthetic pathway; refs. 14, 17).

In Vivo S1P Signaling Modulation Disrupts LSC Function in Human AML

Because LSCs 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 patients with AML using xenograft assays. FTY720, a produg whose phosphorylation generates the S1P mimetic FTY720-phosphate, possesses both agonist and antagonist activities for four S1P receptors including S1PR3. Clinically known as fingolimod, FTY720 is used for treatment of patients with relapsing remitting multiple sclerosis, with the possibility of rapid...
even without overt immunophenotypic changes in myeloid refractory cases (Fig. 7D and E; Supplementary Table S13), following FTY720 treatment in five of the seven AML samples (AML patient 1, AML patient 16, and AML patient 19) was nonresponders except for AML patient 21 (Supplementary Fig. S10B). Importantly, serial repopulation assays at different time points in the nonresponders except for AML patient 21 (Supplementary Table S10). Notably, an interesting trend of modest increase in CD15 and B; Supplementary Fig. S10A; Supplementary Table S10).

Figure 6. Sphingolipid gene expression levels show enrichment of SPHK1 lipid-stem genes, previously shown to be enriched in HSC subsets relative to committed progenitor populations, in AML LSC samples. GSEA of 23 lipid-stem genes, previously shown to be enriched in HSC subsets relative to committed progenitor populations, in AML LSC and LSC− gene expression data shows enrichment in LSC− samples. A, A UMAP plot for the sphingolipid composition of samples measured by LC/MS spectrometry in functionally defined individual LSC− (n = 7) and LSC+ (n = 7) fractions from 10 patient AML samples as determined by xenotransplantation and normal stem (CD34+CD38−) and progenitor (CD34+CD38+) fractions (CB, n = 3). LSC− (red circles), LSC+ (blue circles), CB stem (red triangles), and CB progenitor (blue triangles). CA 2018-2019 figures and tables are available in Supplementary Data. CB progenitor, CB progenitor, CB progenitor, CB progenitor, CB progenitor.

Differencing 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 prodrug FTY720 or S1P3OE can target the disease-sustaining LSCs in AML.

**DISCUSSION**

Our study provides direct evidence that S1PR3 governs myeloid commitment in human HSCs and LSCs, and points to S1P signaling modulation as a potential therapeutic approach to target LSC in human AML. S1PR3OE alone was sufficient to induce myeloid differentiation in human HSC...
**Figure 7.** S1P signaling modulation in vivo targets LSC function in human AML. 

**A-F,** Preclinical targeting of the sphingolipid pathway with the S1P prodrug FTY720, clinically known as fingolimod, in samples of patients with AML or CB (*n* = 3) in vivo. 

**A,** Experimental schematic for xenotransplantation. AMLs with >20% engraftment in the injected femur and/or that 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 after transplant. AML 13 is a treatment-resistant sample, AML 16 is a relapse sample, and AML 19 is a diagnosis sample. Significance calculated by Mann–Whitney test. 

**C,** Percentage of CD15+ cells in the indicated xenografts.

**D,** LSC frequency for indicated AMLs was determined in limiting dilution serial transplantation assays and calculated with ELDA. Mice were considered engrafters if CD45+CD33+ 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 is shown in E. 

**E,** Human CD45+ engraftment of CB following treatment with DMSO or FTY720 at 6 weeks after transplantation.
and LSC and S1PR3 antagonism or KD-restricted myeloid differentiation in AML. Moreover, S1PR3 marks a subset of LSCs from patients with AML 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-HSCs are highly reminiscent of TNFα-induced emergency hematopoiesis (23). Thus, S1PR3 plays similar roles in human HSCs to what has been reported for the TNFα–NF-κB axis in the murine system (23). Overall, our data point to S1PR3 as a potential tor of TNFα via NF-kB inflammatory signaling to promote prosurvival and myeloid differentiation in human HSCs. Although TNFα 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). TNFα has recently emerged as having complex roles in normal hematopoiesis, with different effects on HSC versus myeloid progenitors (23). Notably, we found that acute TNFα 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 TNFα. A previous study found that S1PR3OE driven by a lysosome M promoter, but not an F4/80 promoter, induced a leukemic transformation in the mouse system, suggesting that 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 S1PR3OE in human HSCs 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 cases of patients with AML, dependent on specific genetic lesions and metabolic states, the NF-kB–S1PR3 feed-forward loop is hijacked to promote prosurvival mechanisms. Although we were able to capture the same inflammatory signatures induced in HSCs by S1PR3OE within samples of patients with AML, 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 S1PR3OE was sufficient to decrease p65 levels in AML cells in vitro. The nuances around how S1P signaling intersects with inflammation and NF-kB signaling to regulate LSC function are most likely different along the spectrum of stem-like to myeloid-like AML samples and reflective of the differences in canonical and noncanonical NF-kB signaling within LSCs (34). We wonder if the heterogeneity of response to venetoclax is related to such inflammatory wiring. The identification of a distinct NF-κB signature in a subset of S1PR3-high AML cases raises the possibility that different strategies need to be employed for successful targeting of NF-kB in LSCs from stem-like and myeloid-like AML cases.

The FTY720 xenograft study along with our S1PR3OE, KD, and S1PR3 in vitro antagonist studies suggests that FTY720 treatment is acting in part as an S1PR3 agonist to downregulate NF-kB 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 LSCs beyond S1PR3. Stem-like AML cases may have a different requirement for sphingolipid metabolism and S1P signaling compared with more differentiated myeloid-like cases that appear to be connected to OS and chemotherapy response. Whether chemotherapy selects for preexisting 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 eight-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 patients with AML. Our data suggest that next-generation S1P modulators approved for multiple sclerosis (21) may have therapeutic efficacy in a subset of patients with AML, although we cannot fully exclude the possibility that FTY720 targets LSCs 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 LSCs (61–63).

Another approach for therapeutic targeting of LSCs could be direct modulation of sphingolipid metabolic/biosynthetic enzymes such as those in the glycosphingolipid pathway that have recently emerged as regulators of NF-kB (64) and were included in the eight-gene sphingolipid signature. Sphingolipid metabolism has been linked to endoplasmic reticulum 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 LSCs via the TNFα–NF-kB 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...
S1PR3 Promotes Myeloid Differentiation

Health Network (UHN) Research Ethics Board (REB 01-0573-C). Human CB samples were obtained from Trillium and Credit Valley Hospital and William Osler Health Centre, processed as previously described, and stored via lyophilization (lin−) cells at −80°C or −150°C (14). Normal human bone marrow samples or samples of patients with AML were obtained as viable frozen material from the Leukemia Tissue Bank at PM/UHN. CB samples or samples of patients with AML were obtained as viable cells at −80°C or −150°C (14). Normal human bone marrow samples were obtained from Trillium and Credit Valley Hospital.

Multipotent progenitor (MLP): CD34+ 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, PE–anti-CD19, PE-anti-S1PR3, APC–anti-CD33, and APC–anti-CD34. Live/dead discrimination was determined by sytox blue (Thermo Fisher Scientific, S34857).

1) S1PR3 expressions for CD34+ primitive cells and T (CD3+), B (CD19+), and myeloid (CD33+) 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, PE–anti-CD19, PE-anti-S1PR3, APC–anti-CD33, and APC–anti-CD34. Live/dead discrimination was determined by sytox blue. (Thermo Fisher Scientific, S34857).

2) Analysis of S1PR3 on the surface of HSPC populations was performed on three pools of lin− cells with the following panel on a BD LSR II: FITC–anti-CD45RA, PE–anti-S1PR3, APC–anti-CD90, PE–anti-CD19, V450–anti-CD7, BV421–anti-CD10, PE–anti-CD38, and APC–anti-CD34. Propidium iodide (BD) was used for live/dead discrimination.

3) Analysis of myeloid subpopulations in CB MNCs (CD15+ or CD14+ cells) and comparison of previously frozen CB MNCs with 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, PE–anti-CD14, PE–anti-CD90, PE–anti-CD38, and APC–anti-CD34. Live/dead discrimination was determined by sytox blue. Clinical characteristics are listed in Supplementary Table S9. S1PR3 relative fluorescence intensity for each population is the ratio of S1PR3 MFI for the cells stained with S1PR3-PE divided by the FMO.

Lin− CB cells were stained with the following antibodies at a density of 5 × 10^6 cells/mL (1:50 dilution, unless stated otherwise) for isolation of HSPC subpopulations: FITC–anti-CD45RA, PE–anti-CD90, PE–anti-CD19, V450–anti-CD7, BV421–anti-CD10, PE–anti-CD38, and APC–anti-CD34. Live/dead discrimination was determined by sytox blue. Clinical characteristics are listed in Supplementary Table S9. S1PR3 relative fluorescence intensity for each population is the ratio of S1PR3 MFI for the cells stained with S1PR3-PE divided by the FMO.

For splingolipidome profiling and xenotransplantation, nine samples were selected from the patient cohort previously analyzed for LSC activity (7). Thirty million live cells from 9 patients (Supplementary Table S10) were stained with the following antibodies at a density of 1 × 10^7 cells/mL: FITC–anti-CD45RA, PE–anti-S1PR3, PE–anti-CD33, V450–anti-CD15, PE–anti-CD38, V500–anti-CD45 (1:50), APC–anti-CD34, APC–anti-CD38, and BV711–anti-CD19. Cells were washed, resuspended in PBS + 2% FBS and propidium iodide, sorted on CD34 and CD38 surface markers in the CD3+ CD19− subpopulations into four fractions on a BD FACSAria Fusion, washed with PBS, and frozen viably.

Lentiviral OE and Knockdown of S1PR3

Lentiviral cloning and VSV-G pseudotyped lentiviral vector particles were produced, and titers calculated as previously described (39). A PLB2 OE vector for human S1PR3 was generated from a pENTR223_S1PR3_STOP plasmid (a stop codon insertion into S1PR3). shRNA sequences to S1PR3 were predicted using the Sherwood algorithm and amplified as previously described (14). shCtrl is a sequence to Renilla that was previously utilized (14, 39). In order to assess shRNA KD efficiency, MOLM13 cells were infected at a multiplicity of infection of 0.3. Transduced cells were sorted for BFP− expression, and total RNA was isolated and DNase-treated using the RNeasy Micro Kit (Qiagen, 74004). RNA quality (RNA integrity number > 9) was verified using the Bioanalyzer RNA 6000 Pico Kit (Agilent), and cDNA was synthesized using SuperScript VILO (Thermo Fisher Scientific, 11754050), qPCR was performed on the Roche Lightcycler 480 using Power SYBR Green (Thermo Fisher Scientific, 4367659). All signals were quantified using the ΔCt method and were normalized to the levels of GAPDH.

shRNA sequences to S1PR3 are listed below:

| shS1PR3-2 | TGCTTGTGACATGGGCGGTTCCCACTCTTCTACTCATGATGGAGCTGTAAGTGTGGGGACATCTGCTACTGGG | shS1PR3-3 | TGCTTGTGACATGGGCGGTTCCCACTCTTCTACTCATGATGGAGCTGTAAGTGTGGGGACATCTGCTACTGGG |
| shS1PR3-4 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG | shS1PR3-5 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG |
| shS1PR3-6 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG | shS1PR3-7 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG |
| shS1PR3-8 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG | shS1PR3-9 | TACATGGAAGCCACAGATGTAGGCTGATTAGAGTGTGGGGACATCTGCTACTGGG |

Xenotransplantation

All animal experiments were done in accordance with institutional guidelines approved by the UHN animal care committee. Aged-match female or male NSG mice (NOD.Cg PrkdcscidIl2rgtm1Wjl/SzJ; The Jackson Laboratory) 10 to 12 weeks of age were sublethally irradiated with 250 rads 1 day before intrafemoral injection. For CB xenotransplantation experiments, CD34+CD38− 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 1× Pen/Strep, 1-glutamine, and the cytokines SCF (100 ng/mL), Flt3L (100 ng/mL), and GM-CSF (100 ng/mL).
TNFa or IL6 Treatment

Three pools of CB lin- cells were cultured in gene transfer media alone or plus 10 ng/mL human TNFa (Millenyi Biotec, 150-094-018) or 50 ng/mL IL6 and a portion of cells analyzed on a BD Celesta at 3 days after treatment [FITC–anti-CD54RA, APC-anti-CD90, PE-Cy5–anti-CD49F, Alexa Fluor-anti-CD7, PE-Dazzle-anti-CD38 (1:100), BV605–anti-CD10, APC-Cy7–anti-CD34, S1PR3-PE, and BV786–anti-CD133] and again at day 8 after treatment [APC–anti-CD90, FITC–anti-CD14, V500-anti-CD15, Alexa Fluor-CD7, BV605–anti-CD10, APC-Cy7–anti-CD34, S1PR3-PE, and BV786–anti-GlyA].

In Vitro CB HSPC Assays

For in vitro assays, LT-HSCs and ST-HSCs were isolated from three independent pools of CB transduced with indicated OE or KD 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 starting at day 3 after 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), IL6 (50 ng/mL), IL3 (10 ng/mL), and GM-CSF (20 ng/mL), except EPO (3 units/mL, from Janssen). At day 9 or 10, cells were stained for flow cytometry analysis with PE-Cy5–anti-CD14 (1:200), APC-Cy7–anti-CD34 (1:200), and BFP. PE-anti-S1PR3 (1:33) was added into one experiment to confirm enhanced expression. For CFU assays, on day 3 after lentiviral transduction of LT-HSCs, ST-HSCs, CMP-F1, MEP-F1, or GMPs, transduced cells marked by BFP were sorted directly into methylcellulose (cat. no. H4034, Stem Cell Technologies), supplemented with FLi3 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, PE-Cy7–anti-CD33, APC-Cy7–anti-CD34, and BFP. For single-cell erythroid/myeloid/megakaryocyte differentiation assays, on day 3 postlentiviral transduction of LT-HSCs, ST-HSCs, CMP-F1, and MEP-F1, single BFP+ cells were sorted directly onto MS5 (44) stroma in 96-well plates prepared as previously described (28) and cultured for 15 days. Wells containing single cells scored as successfully cloned by eye were detached from MS5 stroma and analyzed by flow cytometry analysis for FITC-anti-CD15, PE-anti-GlyA, APC-anti-CD71, PeCy5–anti-CD14, APC-Cy7–anti-CD34, and BFP on a high-throughput sampler unit of a BD Canto.

Time-lapse Imaging

Time-lapse experiments were conducted at 37°C, with 5% O2 and 5% CO2 on μ-slide VI68 channel slides (IBIDI) coated with 20 μg/mL anti-human CD43-biotin antibody (51). BFP+ cells 3 days after 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 λ 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 samples of patients withAML, LSC+ and LSC− subpopulations were selected...
(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 4–12 million cells). These were thawed concurrently, and live cell counts, 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+CD38- and CD34+CD38+ from three pools of lin-) 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 was performed by the Lipidomics Facility of Stony Brook University Medical Center on a SCIEX 4000 QTRAP mass spectrometer. Normalization to cellular inorganic phosphate (P) 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 neighborhood graph was calculated with a local neighborhood size of 4, and a UMAP dimensionality reduction was performed.

**CB Gene Expression Analysis**

LT-HSCs and ST-HSCs from three pools of CB lin- were FACSpurified and transduced as described for *in vitro* culture assays in gene transfer media. At day 3, 2,000 to 5,300 BFP+ cells were FACSpurified for RNA isolation with a PicoPure kit (Thermo Fisher Scientific, KIT0214). Consistent with our observations that S1PR3OE increases the viability of LT-HSCs over control vectors in *in cell-* imaging studies (Fig. 2F–H), we were able to isolate only 1,600 to 1,800 BFP+ cells from LT-HSC control samples as opposed to 4,000 to 5,400 BFP+ cells from S1PR3OE samples. Thus, we pooled all control BFP+ LT-HSC cells into one sample for RNA-seq analysis. BFP+ LT-HSC cells from control vector transduction were purified from CB1 as an additional LT-HSC control. In total, Nextera libraries generated from 10 ng RNA from five LT-HSC samples (two controls and three S1PR3OE) and six ST-HSC samples (three controls and three S1PR3OE) were subjected to 125 base-pair (bp), paired-end RNA-seq on the Illumina HiSeq 2500, with an average of 50 million reads/sample at the Centre for Applied Genomics, SickKids 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 –alignSJoverhangMin 10 –alignMatesGapMax 100000 –alignIntronMax 100000 –chimSegmentReadGapMax 5 -1 5 5.” Counts were obtained using HTSeq v0.7.2 (70). Differential gene expression to any mutation by comparing the two patient groups was analyzed with DESeq2 (58). For each sample, we compared the S1PR3 gene expression to any mutation by comparing the two patient groups. In the Beat-AML, TCGA-AML, and GSE6891 cohorts. Briefly, the correlation coefficient and t test P value were calculated for S1PR3 gene expression to any mutation by comparing the two patient groups either containing the genetic mutation or wild-type using two-sample t test. We computed single-sample GSEA (ssGSEA) enrichment scores for the HSC/progenitor and myeloid signatures (Supplementary Table S6) generated from the van Galen dataset (3) and the NF-kB via TNFα hallmark gene set (gene lists in Supplementary Table S5) using the R package edgeR after filtering and normalizing the raw counts.

**Human AML Gene Expression Analysis**

The single-cell correlation analysis of S1PR3 and the S1PR3OE LT-HSC DEGs was performed in a previously published scRNA-seq dataset consisting of 11,641 malignant single cells from 12 patients with AML at diagnosis (3). Counts were scran normalized, and signatures were scored using AUCell with default settings. Signature enrichment was subsequently scaled to facilitate visualization. Four previously published bulk AML gene expression datasets and one dataset described here were used to analyze S1PR3 expression.

- (i) AML RNA-seq expression read counts and genomic analysis for mutations from the TCGA-AML study (52), consisting of 20,442 genes and 179 patients, were downloaded from the companion website of the original publication. Gene expression is defined as log2 ([1 + counts per million (CPM)], where the CPM are computed using R package edgeR after filtering and normalizing the raw counts.
- (ii) The Beat-AML data were downloaded from Supplementary Materials from Tyner and colleagues (35). The CPM table consisting of 22,843 genes and 451 patients, listed in Supplementary Table S9, contained 440 AML samples (280 diagnosis, 23 relapse, 137 not available). We utilized the 280 listed as diagnosis in our analysis here. (iii) GSE6891 (53) is a normalized RNA microarray AML dataset (AFFY HG U133 plus 2) using basic robust multivar-ray average consisting of 495 patient samples previously analyzed in Ng and colleagues (7). The gene symbols were obtained from R database hg133plus2.db. Gene expression was calculated using maximum values of multiple probes for each gene, and those genes with SD < 0.01 across samples were filtered, resulting in 17,325 genes. The correlation of genetic mutation data to S1PR3 gene expression was analyzed for any genes reported with mutations in the Beat-AML, TCGA-AML, and GSE6891 cohorts. Briefly, the correlation coefficient and t test P value were calculated for S1PR3 gene expression to any mutation by comparing the two patient groups either containing the genetic mutation or wild-type using two-sample t test. We computed single-sample GSEA (ssGSEA) enrichment scores for the HSC/progenitor and myeloid signatures (Supplementary Table S6) generated from the van Galen dataset (3) and the NF-kB via TNFα hallmark gene set (gene lists in Supplementary Table S5) using the R package edgeR after filtering and normalizing the raw counts. The ssGSEA score and gene expression color key represent the normalized values that are equal to centered scores divided by maximum of the absolute centered scores. We analyzed correlations of S1PR3 with the NF-kB genes in these three AML cohorts using R. This resulted in 75 common NF-kB genes correlated to S1PR3 with FDR < 0.05 in the three cohorts (correlation scores, correlation testing P values, and Benjamini and Hochberg FDR are located in Supplementary Table S8). These analyses were also performed on (iv) LSC+/LSC- microarray dataset and (v) an RNA-seq dataset generated from patients from 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 Gene Expression Omnibus (GEO) database file GSE76008 Non-normalized.cts.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 17,685 genes.
For GSEA and NF-kB gene set analysis to S1PR3, we normalized the log-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 (ref. 14; SMDP4 was not found in the microarray data, hence we used 22 of 23 genes published previously as the lipid-sterm signature) listed in Supplementary Table S12.

The PM-AML RNA-seq dataset consists of 81 AML patient samples (clinical data in Supplementary Table S9), processed in two batches. This dataset only contains patient samples that can engraft in the NSG mouse model. Five patients (90543, 598, 90240, 110484, and 100500) were included in both batches. RNA was extracted from bulk peripheral blood MNCs using the RNeasy Micro Kit (Qiagen Inc.). Libraries were constructed by SMART-Seq (Clontech Inc.). A paired-end, 50 bp 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-seq on the Illumina HiSeq 2500 with an average of 50 million reads/sample at the Centre for Applied Genomics, SickKids Hospital. Subsequent alignment and normalization were performed as for the S1PR3OE RNA-seq dataset 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 RNA-seq data of 12 patient pairs previously described (5).

RNA-seq and ex vivo drug screening data were acquired from Tyner and colleagues (35). Gene expression data were normalized to transcripts per million, 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 using 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 the TCGA-AML cohorts (38), stained with APC–anti-p65 overnight at 4°C, and analyzed on a BD Celesta. For p65 NF-κB gene set analysis in univariate analysis.

AML Cell Line Assays

AML cell lines (Kasumi-1, RRID:CVC1_0589; KG-1, RRID:CVC1_0374; ME-1 RRID:CVC1_2110; PL-21 RRID:CVC1_2161; U937, RRID:CVC1_0007; and OCI-AML3, RRID:CVC1_1844) were obtained from the ATCC and cultured in MEM alpha medium (12571-063, Gibco) supplemented with 10% FBS (F1051, Sigma), 2 mmol/L Pen/Strep (15140-122, GIBCO), and 1% β-glutamine (609-065-EL, Multicell), expanded for ≤4 passages to generate a stock for subsequent experiments. OCI-AML22 cell line model (S6) was derived from the cells of a relapsed PM-AML patient and was cultured in X-Vivo medium (04-380Q, Lonza) supplemented with 20% BIT (9500, Stem Cell Technologies), 1% Glutamax (35050061, Thermo Fisher Scientific), 0.2% Primocin (ant-pm-1, Invitrogen), 100 ng/ml SCF (130-095-696, Miltenyi), 10 ng/ml IL3 (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 to 3 months of thawing.

For Nanonstring analysis of the LSC104 score (7) from the indicated AML cell lines, RNA from FACS-sorted viable cells was isolated with TRIzol (Invitrogen) or for low cell numbers (~<20,000 cells) with PicoPure RNA Isolation Kit (Thermo Fisher Scientific) according to the 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 expressions 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, APC-Cy7-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 5 µg/ml polybrene (Sigma) at 1,400 x g for 90 minutes for lentiviral transduction. Seventy-two 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, APC-Cy7-anti-CD34, and Annexin V-APC (BD). CD34+ cells from Kasumi-1 and OCI-AML22 were isolated on the FACS Aria III or Fusion (BD Biosciences) and transduced with control or S1PR3OE shCtrl lentiviral vectors, and BFP+ cells were FACS-purified at 72 hours after transduction and analyzed at the indicated time points with FITC-anti-CD15, PE-anti-S1PR3, and APC-Cy7-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 TNFα (Miltenyi) Biotec, 130-094-018) with and without 5 µmol/L S1PR3 antagonist TY5126 (Tocris, 5238) or DMSO control (1:5,000 dilution) and analyzed at days 5 and 9 after treatment by flow cytometry with FITC-anti-CD15, PE-anti-S1PR3, and APC-Cy7-anti-CD34 on the BD Celesta. For p65 NF-kB flow cytometry analysis, Kasumi-1 CD34+ cells were transduced with indicated lentiviral vectors. Then, BFP+ cells were isolated on the FACS Aria III or Fusion (BD Biosciences) at day 5 after transduction and fixed for intracellular flow cytometry with BD cytperm/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 ± SD values are reported in the graphs.

Data Availability

The S1PR3OE RNA-seq data for LT-HSC and ST-HSC (GSE149238) and the PM bulk AML RNA-seq data (GSE156914) were deposited in the GEO. Raw data are available under accession numbers EGAS00001004798 (S1PR3OE) and EGAS00001004792 (AML) in the European Genome-phenome Archive. The human CB 13 population RNA-seq data are available from the authors upon request (37) and includes five HSPC populations at GSE125345. All other data supporting the findings of this study are cited in the Methods, available within the article, or upon request from the authors.

Authors’ Disclosures

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Authors’ Contributions


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REFERENCES


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