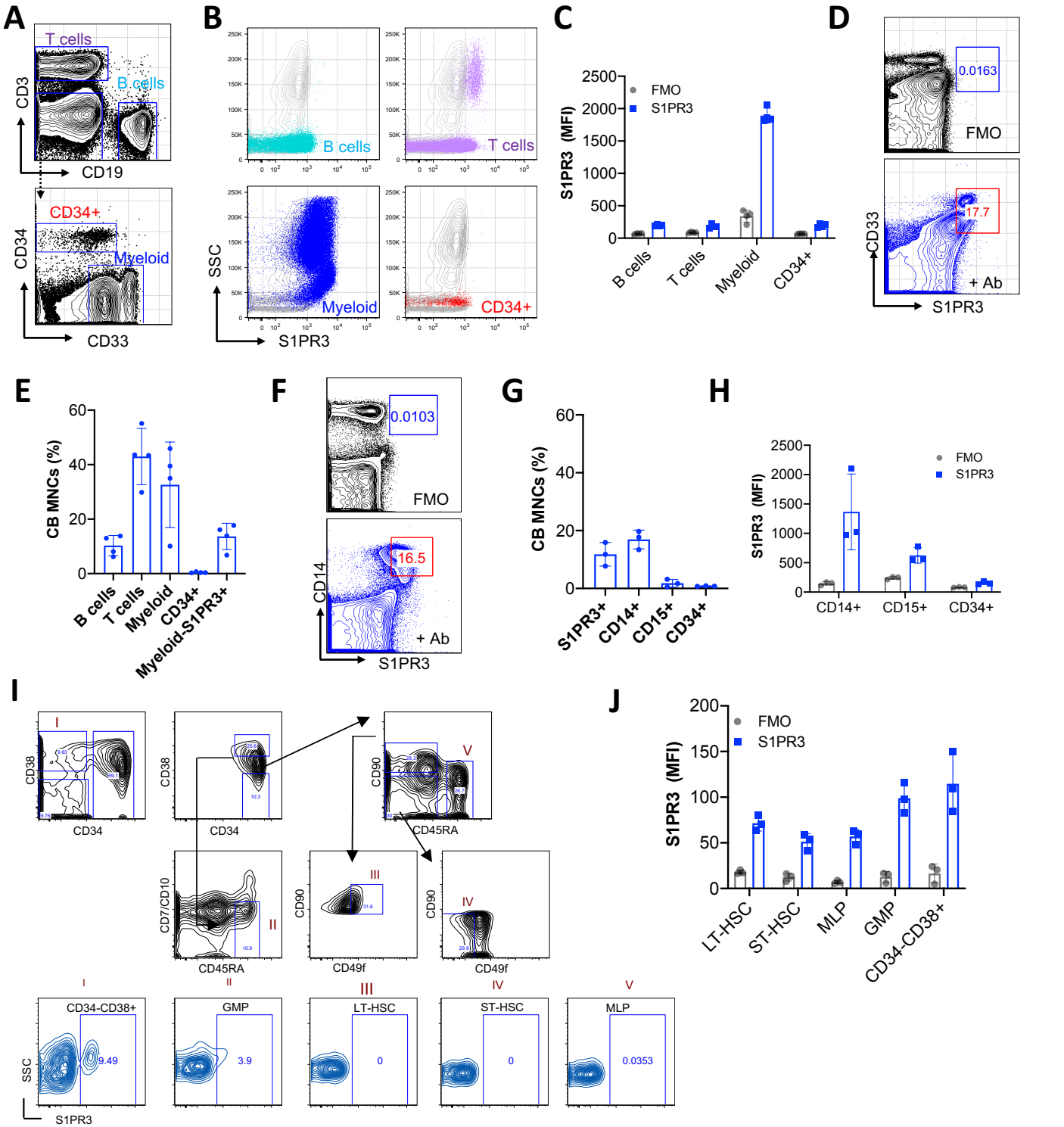
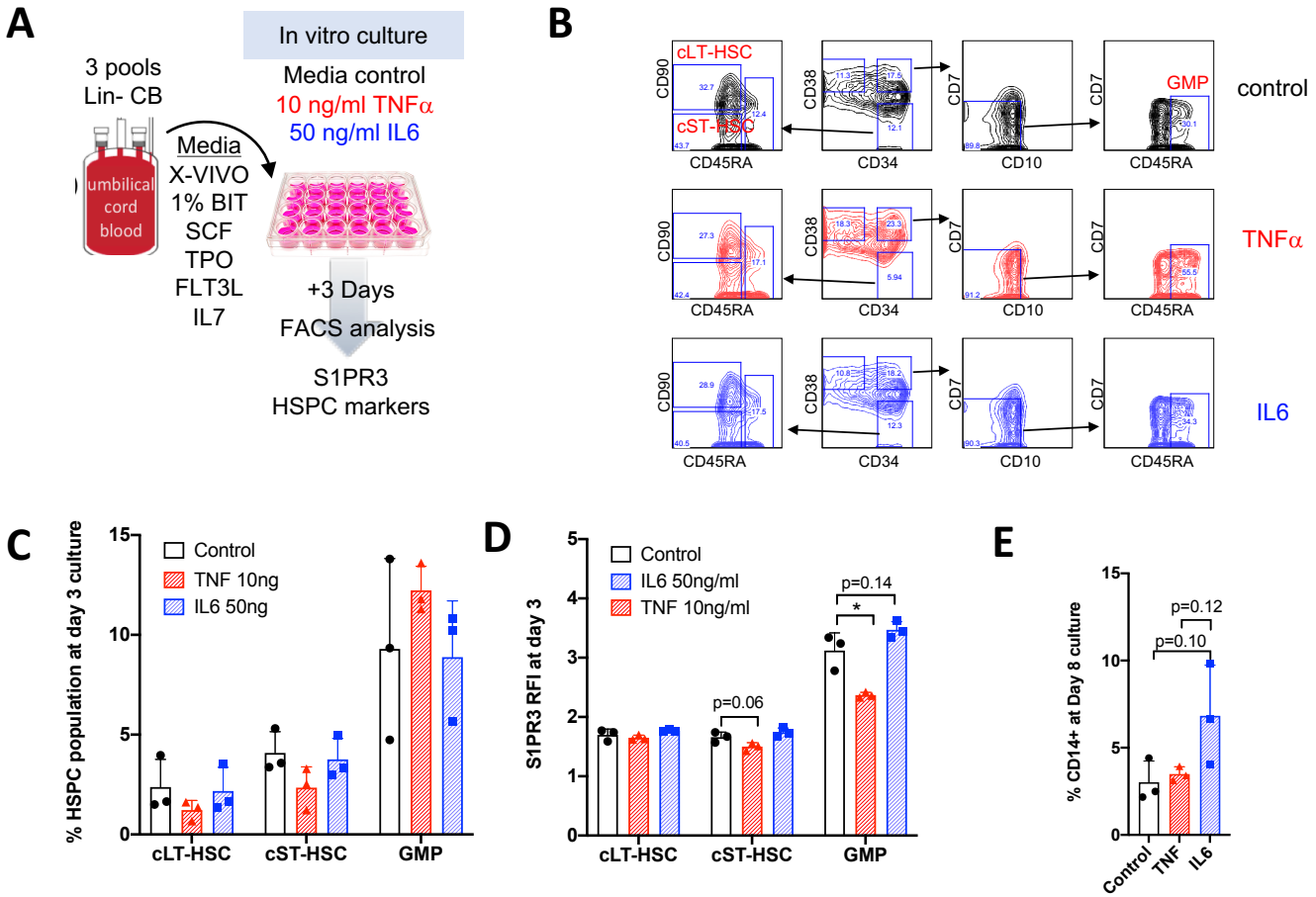
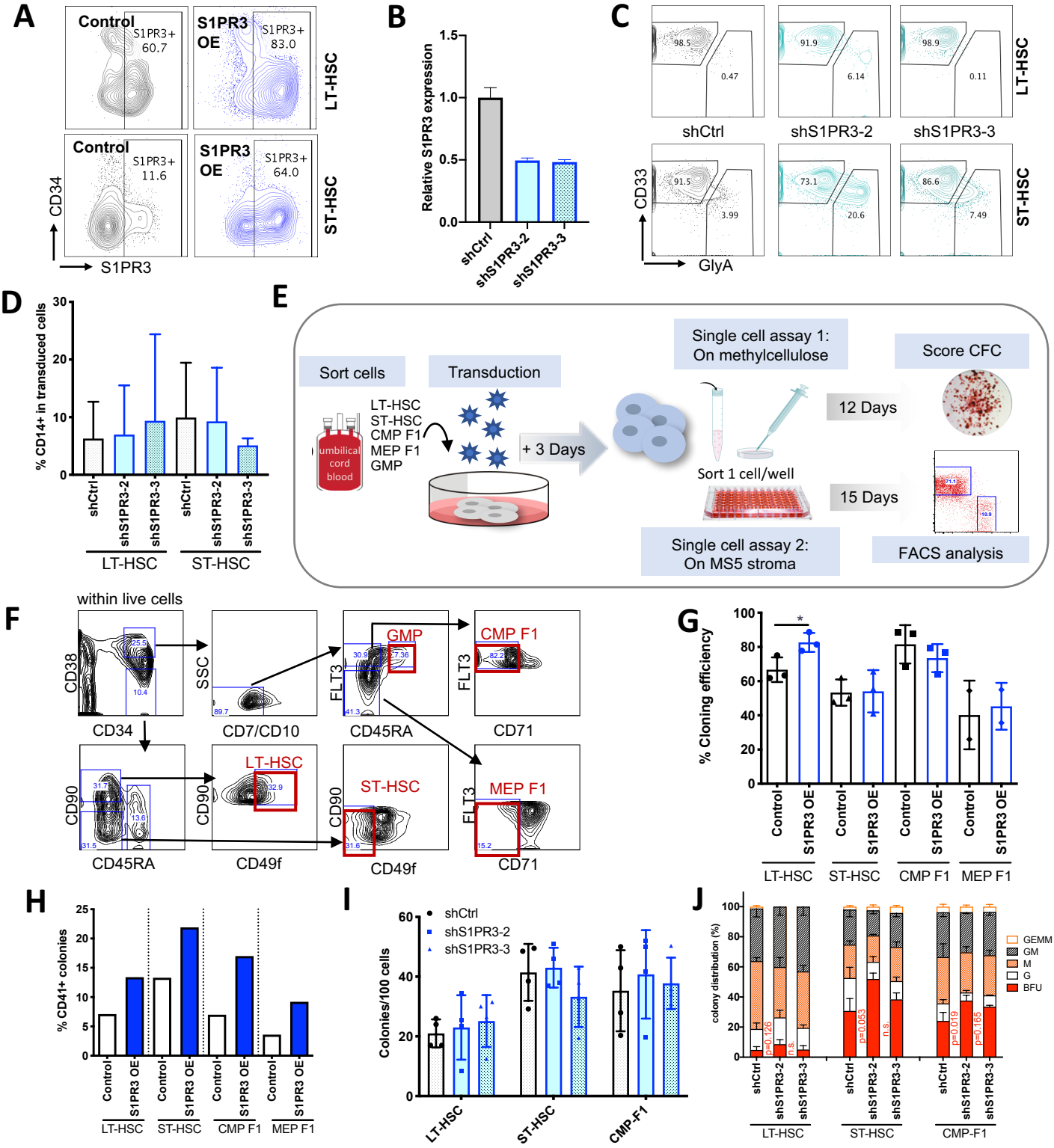


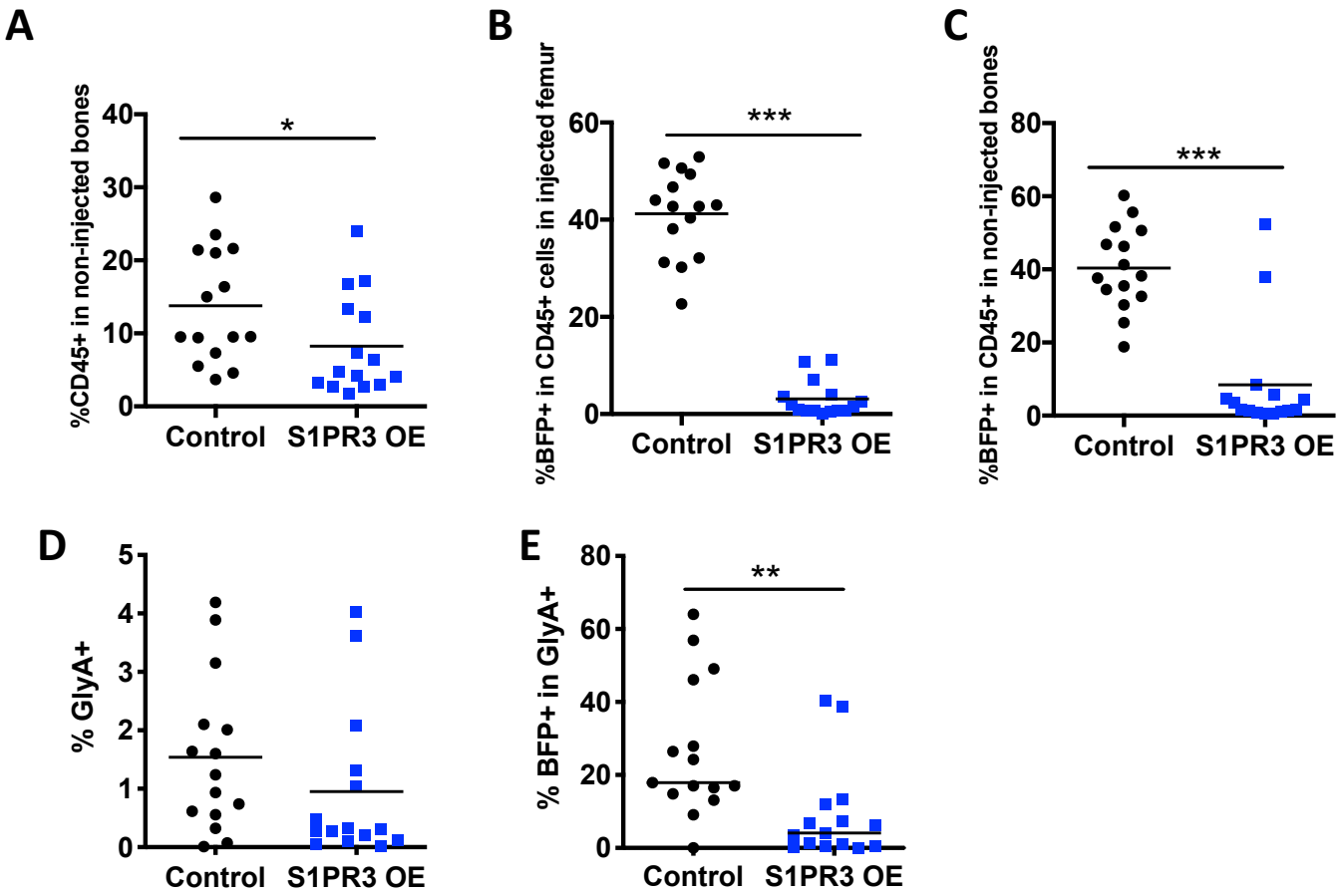
Supplementary Figure 1. S1PR3 is a myeloid-associated S1P receptor not expressed in human HSPC. A, Representative flow cytometry plots showing gating scheme for T cells, B cells, CD33$^+$ myeloid, and CD34+ cells from freshly isolated CB mononuclear cells (MNCs, n=4), 2 samples were single units and 2 samples were pools of 6 CB units. **B**, Representative flow cytometry plots showing S1PR3 staining for T cells, B cells, CD33+ myeloid, and CD34$^+$ overlaid on all cells from FMO control (gray). **C**, S1PR3 mean fluorescence quantification with S1PR3 antibody (blue) or FMO control (gray) (n=4). **D**, Representative flow cytometry plots showing overlapping expression of S1PR3 and the myeloid marker CD33 in CB MNCs for control (FMO, black) and S1PR3 antibody containing sample (+Ab, blue). **E**, The distribution of T cells, B cells, CD33$^+$ myeloid and CD34$^+$ are shown. A subset of CD33$^+$ cells co-stains for S1PR3 (myeloid-S1PR3$^+$). **F**, The percentage of S1PR3$^+$, CD14$^+$ monocytic, CD15$^+$ granulocytic, and CD34$^+$ primitive cells in previously frozen CB MNCs (n=3), related to Fig. 1b-c)**. G**, Representative flow cytometry plots showing co-expression of S1PR3 and the myeloid marker CD14 in CB MNCs (n=3) for a Fluorescence minus one (FMO, black) control and S1PR3 antibody stained sample (+Ab, blue). **H**, S1PR3 MFI in CD14$^+$, CD15$^+$, and CD34$^+$ cells**. I**, Representative flow cytometry plots showing gating scheme and S1PR3 staining for LT-HSC, ST-HSC, MLP, GMP, and CD34$^-$CD38$^+$ cells from 3 pools of lin$^-$ CB. **J**, Quantification of S1PR3 MFI in indicated populations. *** P<0.001** p<0.01, *<0.05, unpaired Student t-test. Data are mean and s.d.



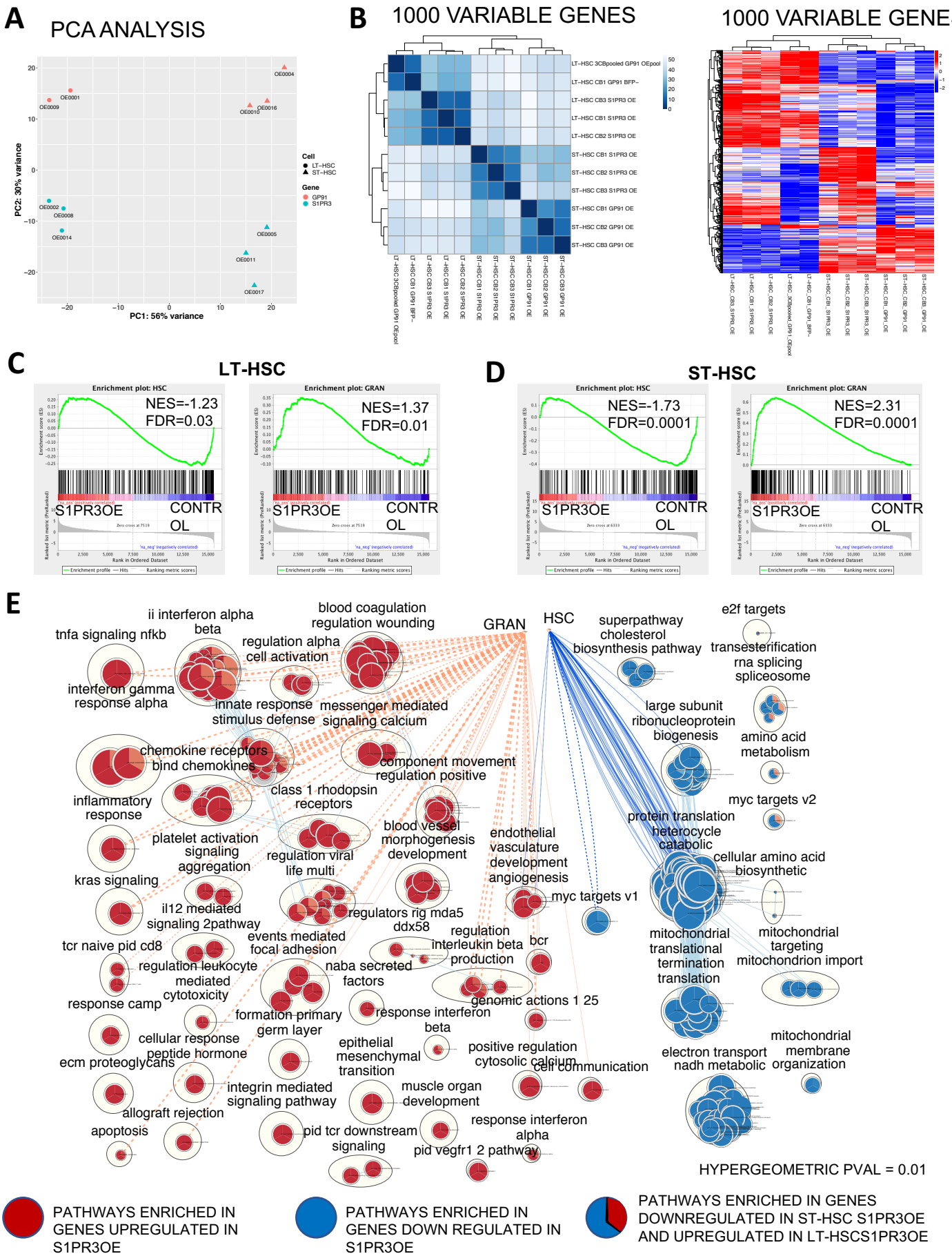
Supplementary Figure 2. Pro-inflammatory cytokines regulates S1PR3 surface expression in human HSPC. A, Experimental schematic for in vitro treatment of TNFα or IL6 of CB cells, lineage depleted to enrich for HSPC, in *ex vivo* culture and then analysis for S1PR3 surface expression in immunophenotypic HSPC populations by flow cytometry. **B**, Gating scheme for cultured LT-HSC (cLT-HSC, $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$), cultured ST-HSC (cST-HSC, $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$), and GMP ($CD34^{+}CD38^{+}CD7^{-}CD10^{-}CD45RA^{+}$) for control media or with TNFα or IL6 treatment. **C**, Percentage of cLT-HSC, cST-HSC and GMP cells at day 3 culture. **D**, Relative fluorescence intensity (RFI) of S1PR3 calculated as ratio of S1PR3 MFI to FMO controls in the indicated populations at day 3 culture. **E**, Percentage of CD14+ cells at day 8 culture. Statistical significance by unpaired Student t-test. Data are mean and s.d.



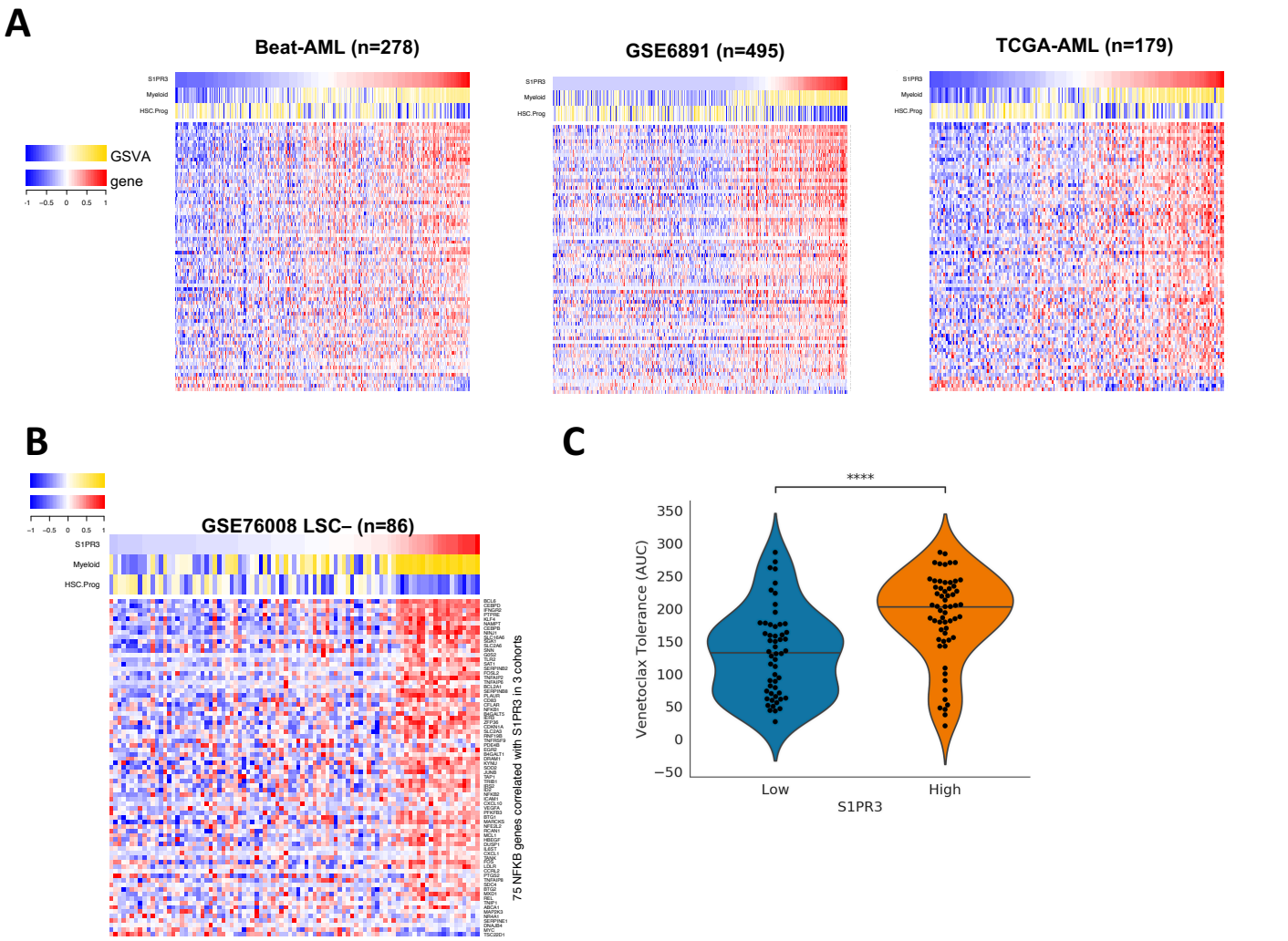
Supplementary Figure 3. *S1PR3* overexpression is sufficient to promote myeloid differentiation at the expense of erythroid differentiation in human HSPC. A, Representative flow cytometry plots for S1PR3 and CD34 expression in progeny of LT-HSC or ST-HSC at day 9 following lentiviral transduction with control or *S1PR3*OE. **B**, Relative S1PR3 expression determined by real-time PCR in MOLM13 cells transduced with the indicated lentiviral vectors. **C**, Representative flow cytometry plots for CD33 and GlyA following lentiviral knockdown with shCtrl or 2 vectors to S1PR3. **D**, CD14 expression following lentiviral knockdown of S1PR3 in LT-HSC and ST-HSC at 8 or 10 days post-transduction (n=2 CB). **E**, Experimental scheme for *in vitro* single cell colony forming cell (CFC) assays on methylcellulose or MS5 stromal differentiation assays. **F**, Representative sorting scheme for human HSPC populations with multilineage differentiation capacity analyzed in **(E).** F1 populations for CMP and MEP were previously described to contain myeloid, erythroid, and megakaryocytic differentiation capacity**. G**, Cloning efficiency of single cell stromal assays (**Fig. 2D**). **H**, Percentage of cells scored in stromal assays that yielded CD41^{+} megakaryocytic progeny. **I**, The number of colonies for 100 transduced cells from the indicated populations at 10 days of a CFC assay for shCtrl or S1PR3 lentiviral knockdown vectors (n=3). **J**, Normalized colony distribution for (**I**). BFU (burst-forming units); macrophage (M); G (granulocyte); GEMM (granulocyte erythrocyte monocyte megakaryocyte). , *<0.05, unpaired Student t-test. Data are mean and s.d.



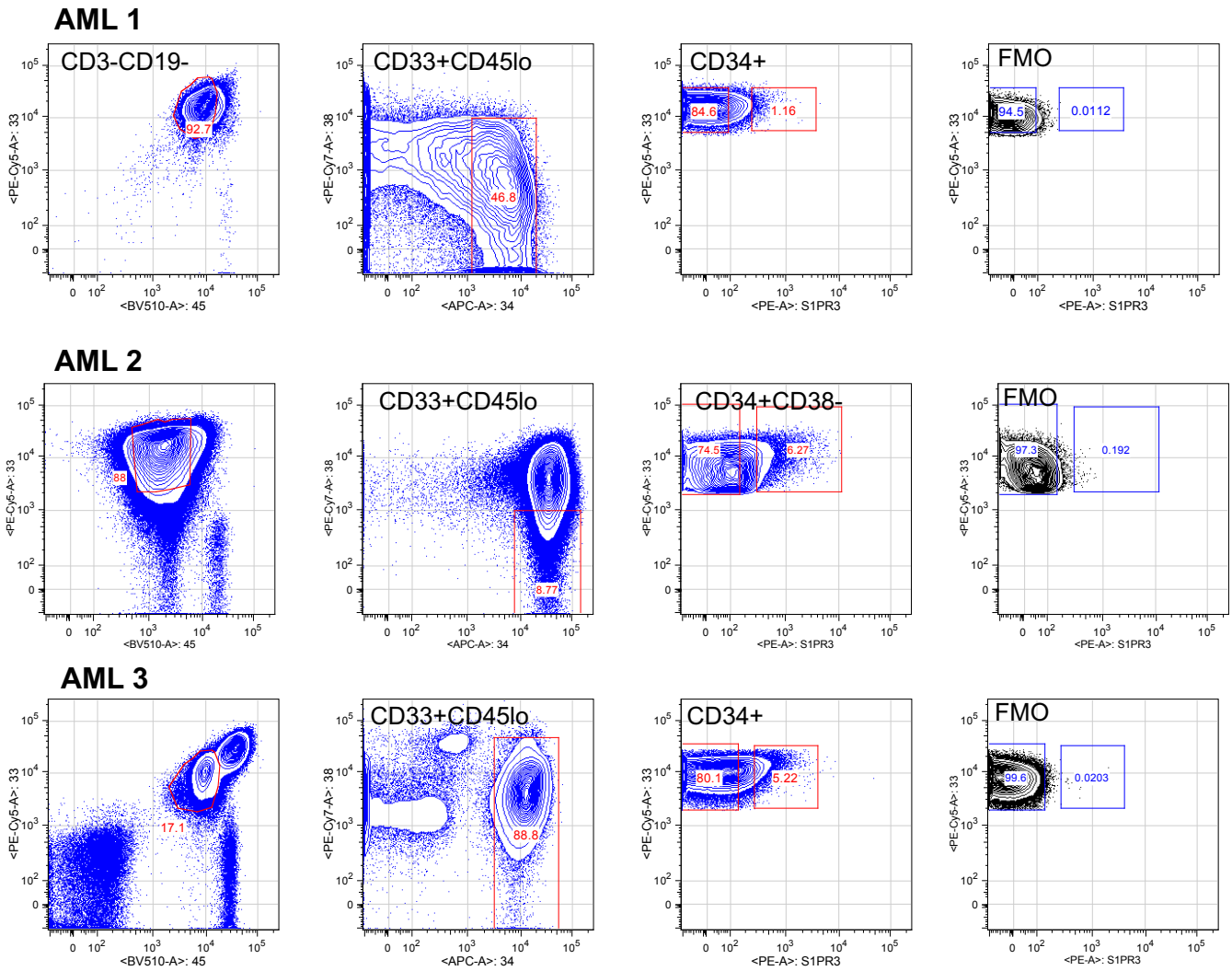
Supplementary Figure 4. *S1PR3* overexpression in CD34^{+}CD38^{-} HSPC disrupts engraftment in xenotransplantation assays. A, Human CD45 chimerism in non-injected bones at 4 weeks post xenotransplantation. The percentage of transduced cells marked by BFP^{+} in human CD45^{+} cells of the **B**, injected femur and **C**, non-injected bones. **D,** The percentage of human GlyA^{+} cells in the injected femur. **E,** Percentage of transduced cells marked by BFP+ in human GlyA^{+} cells from (**D**). *** P<0.001** p<0.01, *<0.05, unpaired Student t-test. Data are mean and s.d.



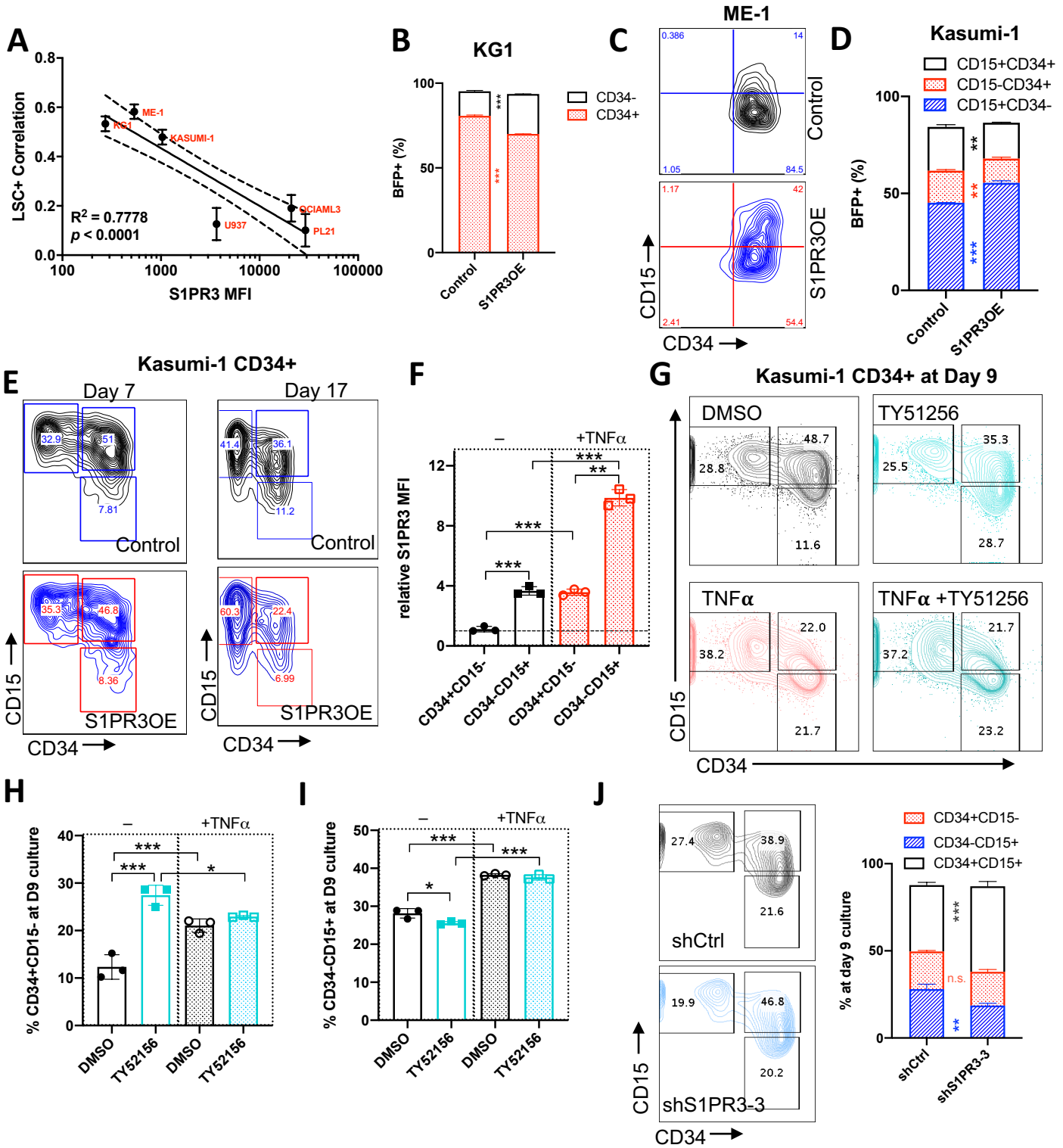
Supplementary Figure 5. RNAseq analysis of LT-HSC and ST-HSC following *S1PR3* overexpression A, Principal component analysis of RNAseq samples. **B**, Unsupervised clustering of samples based of gene expression in 1000 most variable genes with heatmap visualization. GSEA plots shows enrichment of S1PR3OE samples in granulocyte (GRAN) and depletion in HSC gene sets in **C,** LT-HSC and **D,** ST-HSC. **E,** Pathway enrichment map of the GSEA results using the BaderLab gene-sets at FDR value = 0.0001. 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 integrated hypergeometric test at p = 0.01.



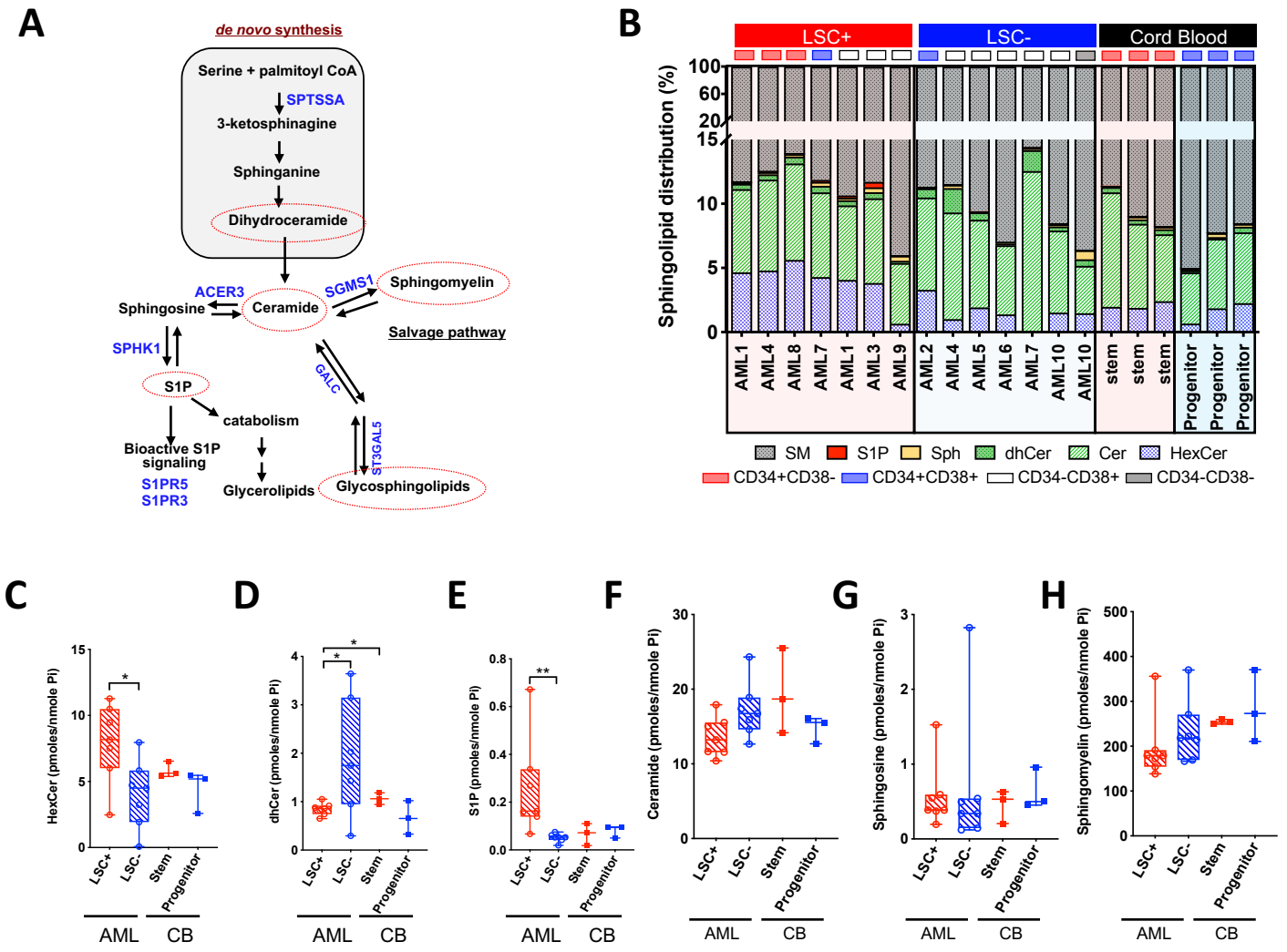
Supplementary Figure 6. AML patients enriched for a differentiated myeloid signature show high expression of *S1PR3* and of a subset of NF-κB pathway genes. A, Heatmaps of NF-κB common genes (rows) in the Beat-AML, TCGA-AML and GSE6891 cohorts. *S1PR3* gene expression and GSVA correlation values for the myeloid and HSC/Progenitor signatures are shown on top of the heatmap. **B**, Heatmap of 75 genes in the LSC^{-} subpopulations from GSE76008 indicates samples with high *S1PR3* gene expression are enriched for the myeloid signature and deficient for the HSC/Progenitor signature while also enriched for the set of 75 NF-κB genes as in unfractionated patient samples. However, those LSC^{-} samples with low *S1PR3* expression show heterogeneity for the myeloid and HSC/Progenitor signatures as well as expression of the 75 NF-κB gene set. **C**, Venetoclax tolerance expressed as AUC (area under the curve) values for 114 AML samples from Beat-AML based on 50/50 split at median *S1PR3* expression. *S1PR3*^{high} samples have greater tolerance for Venetoclax.



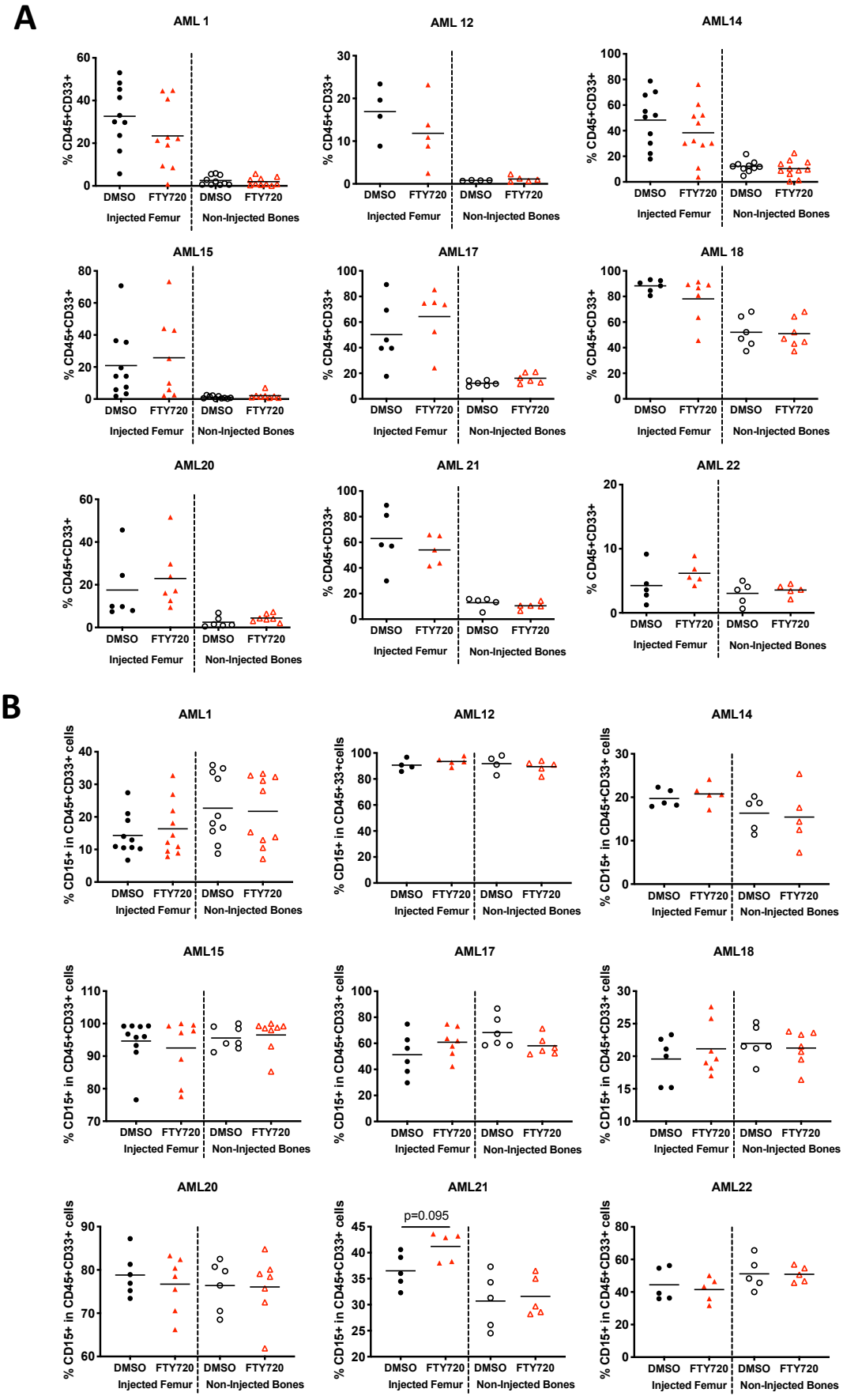
Supplementary Figure 7. Gating scheme for S1PR3 in patient AMLs for xenotransplantation. Related to **Fig. 6B-D**. Flow cytometry data for AML patients analyzed for LSC frequency in limiting xenotransplantation experiments are shown.



Supplementary Figure 8. S1PR3 in S1PR3low AML cell lines regulates myeloid differentiation. **A**, LSC104 correlation score by nanostring analysis relative to S1PR3 MFI in 6 AML cell lines shows AML lines with high LSC104 score indicating a more stem-like state has lower S1PR3 expression. **B**, *S1PR3*OE induces a decrease in phenotypic CD34+ and increase in CD34- cells in KG1 cells at day 7 post-transduction (n=3). **C**, ME-1 cells are predominantly $CD34^+$, but showed an immunophenotypic increase in $CD15^+$ cells with *S1PR3*OE (n=1). **D**, Kasumi-1 cells exhibit increased CD15+ and decreased CD34+ immunophenotypic changes with *S1PR3*OE relative to controls (n=3) 7 days following lentiviral transduction. **E**, $CD34^+$ Kasumi cells were isolated, transduced with control or *S1PR3*OE lentivectors, and flow cytometry analysis were performed at day 7 and day 17 for CD15 and CD34 expression showing a delay in immunophenotypic CD15 and CD34 changes. *S1PR3OE* induced loss of $CD34^+$ populations and increase in $CD15^+CD34^-$ cells from day 7 to day 17 (n=1, in duplicate). **F**, S1PR3 expression in Kasumi-1 subpopulations normalized to an untreated CD34+CD15- sample 9 days post-treatment with 10ng/ml TNF**α** (n=3). **G-I**, Representative flow cytometry plots and quantitation for CD15 and CD34 markers 9 days post-treatment with DMSO or 5μM TY51256 alone or with 10ng/ml TNF**α** in Kasumi-1 CD34+ cells at day 9 post-treatment (n=3). **J**, Representative flow cytometry plots and quantitation for CD15 and CD34 markers 9 days post-transduction in transduced cells marked by BFP (n=3). *<0.05, ** p<0.01, *** P<0.001. Unpaired Student t-test for B,D and one-way ANOVA for F-K. Data are mean and s.d.



Supplementary Figure 9. Lipidomics confirms gene expression analysis that AML patient LSC+ and LSC- subpopulations have distinct sphingolipidome profiles. A, Schematic showing the sphingolipid metabolic pathway and the species analyzed by LC/MS mass spectrometry. **B**, Sphingolipid distribution profiled by LC/MS mass spectrometry in functionally defined individual LSC+ (n=7) and LSC^{-}(n=7) fractions from 10 patient AMLs as determined by xenotransplantation and normal stem (CD34^{+}CD38^{-}) and progenitor (CD34^{+}CD38^{+}) fractions (CB, n=3). CD34 and CD38 marker status of each sample are indicated. **C-H,** Quantification of sphingolipid species measured as pmoles of indicated sphingolipid/nmoles of inorganic phosphate (Pi) including **C,** hexosylceramides (HexCer) which are sphingolipids containing glucose or galactose sugar moieties, **D,** dihydroceramide (dhCer), **E,** S1P, **F**, ceramide, **G**, sphingosine, and **H**, sphingomyelin.



Supplementary Figure 10. The S1P prodrug FTY720 decreases AML burden and decreases LSC frequency in AML including relapse and treatment refractory patients. Related to **Fig. 7A-F**. **A**, Human AML engraftment marked by $CD45^{+}CD33^{+}$ cells at 6 weeks post-transplantation for individual mice engrafted with the indicated AML patient samples in the injected femur or non-injected bones following treatment with DMSO vehicle or FTY720. These are the 9 samples that did not show significant change in engraftment between vehicle and FTY720. **B.** Percentage of CD15+ cells in A**.**