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Supplemental information

TNF-α-induced alterations in stromal progenitors enhance leukemic stem cell growth via CXCR2 signaling

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Supplementary Figures and Tables

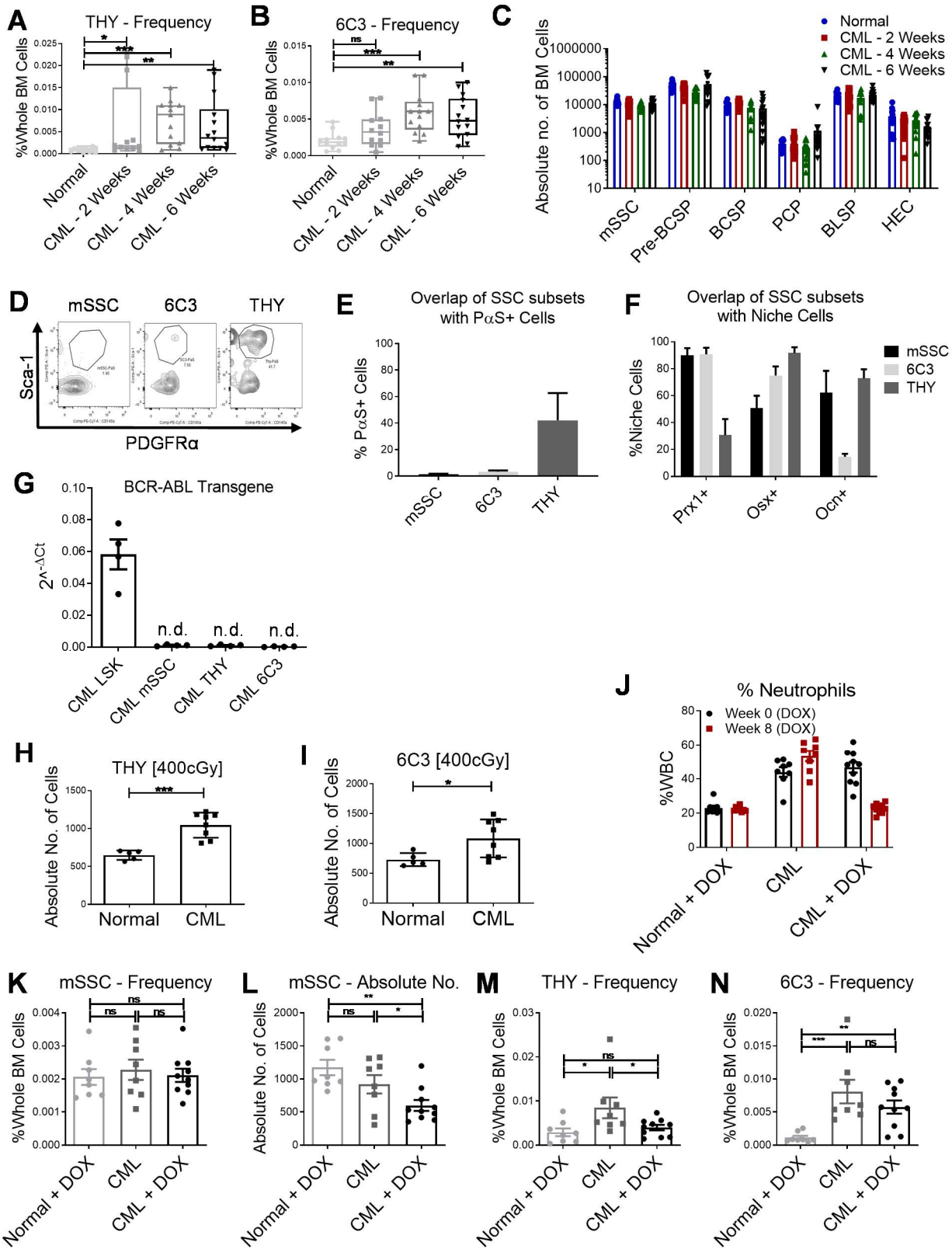

A
THY - Frequency
%Whole BM Cells
Normal
CML - 2 Weeks
CML - 4 Weeks
CML - 6 Weeks
B
6C3 - Frequency
%Whole BM Cells
C
Absolute no. of BM Cells
Normal
CML - 2 Weeks
CML - 4 Weeks
CML - 6 Weeks
mSSC
Pre-BCSP
BCSP
PCP
BLSP
HEC
D
mSSC
6C3
THY
Sca-1
PDGFRα
E
Overlap of SSC subsets with PαS+ Cells
% PαS+ Cells
F
Overlap of SSC subsets with Niche Cells
%Niche Cells
mSSC
6C3
THY
Prx1+
Osx+
Ocn+
G
BCR-ABL Transgene
2^-ΔCt
n.d.
n.d.
n.d.
CML LSK
CML mSSC
CML THY
CML 6C3
H
THY [400cGy]
Absolute No. of Cells
Normal
CML
I
6C3 [400cGy]
Absolute No. of Cells
Normal
CML
J
% Neutrophils
Week 0 (DOX)
Week 8 (DOX)
%WBC
Normal + DOX
CML
CML + DOX
K
mSSC - Frequency
%Whole BM Cells
Normal + DOX
CML
CML + DOX
L
mSSC - Absolute No.
Absolute No. of Cells
Normal + DOX
CML
CML + DOX
M
THY - Frequency
%Whole BM Cells
Normal + DOX
CML
CML + DOX
N
6C3 - Frequency
%Whole BM Cells
Normal + DOX
CML
CML + DOX

Figure S1. Increased stroma-forming and bone-forming progenitors in CML bone marrow (Related to Figure 1)

Frequency of THY+ (A) and 6C3+ cells (B), and the absolute number of subsets other than THY and 6C3 cells (C) within total bone marrow cells per four bones (2 femurs+2 tibias) are shown. Representative flow cytometry gating strategy (D) and the pooled data (n=4) for the overlap of indicated BM subsets with PDGFRα+Sca-1+ (PαS+) cells (E). Frequency of BM subsets expressing tdTomato in various Cre+ transgenic lines (n=3 mice) (F). Expression of the Bcr-Abl transgene in FACS sorted hematopoietic stem/progenitors (LSK, CD45+Lin-Sca-1+c-Kit+) and various BM mesenchymal subsets (n=4 mice/group); n.d. not determined (G). Absolute number of THY+ (H) and 6C3+ cells (I) from recipient mice irradiated at 400cGy from the experiment in Figure 1E. (J) Percentage of neutrophils in the PB of primary CML and aged-matched WT normal mice either re-exposed to doxycycline or not. Frequency (K) and absolute number of mSSC (L), THY+ (M), and 6C3+ cells (N) within the BM of indicated groups. Error bars represent mean ± sem. Significance values. *P< 0.05, **P< 0.01, ***P<0.001, ****P<0.0001.

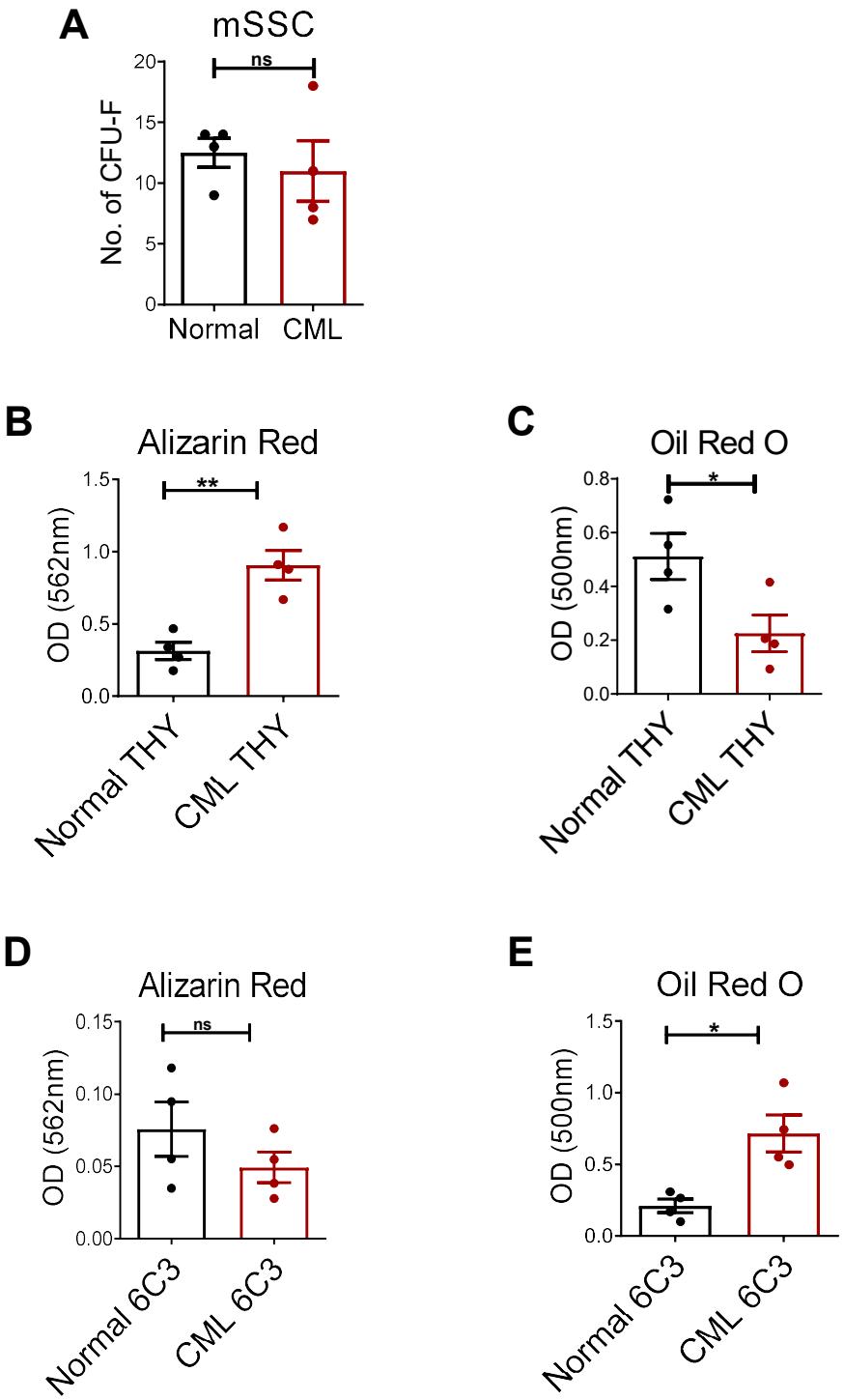

A
mSSC
ns
No. of CFU-F
20
15
10
5
0
Normal
CML
B
Alizarin Red
**
OD (562nm)
1.5
1.0
0.5
0.0
Normal THY
CML THY
C
Oil Red O
*
OD (500nm)
0.8
0.6
0.4
0.2
0.0
Normal THY
CML THY
D
Alizarin Red
ns
OD (562nm)
0.15
0.10
0.05
0.00
Normal 6C3
CML 6C3
E
Oil Red O
*
OD (500nm)
1.5
1.0
0.5
0.0
Normal 6C3
CML 6C3

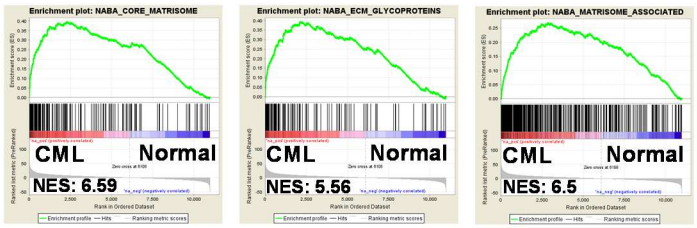
Figure S2. Altered cycling and differentiation of CML 6C3+ and THY+ progenitors (Related to Figure 2)

CFU-F colonies generated from normal and CML BM mSSC cells (A) (n=4 replicates/group). Quantification of osteoblast (B) and adipocyte (C) differentiation of normal and CML THY+ cells, and osteoblast (D) and adipocyte (E) differentiation of normal and CML 6C3+ cells (n=4 independent experiments). Error bars represent mean ± sem. Significance values. *P< 0.05, **P< 0.01, ***P<0.001, ****P<0.0001.

A

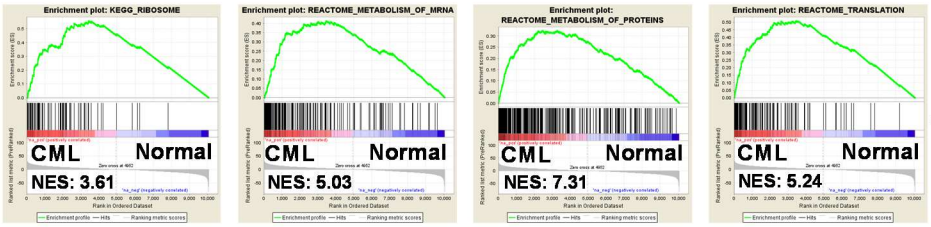
GENESETS POSITIVELY ENRICHED IN 6C3 CELLS				
NAME	SIZE	NES	NOM p-val	FDR q-val
ADIPOGENESIS RELATED				
BURTON_ADIPOGENESIS_8	76	3.87641	0	0
NAKAMURA_ADIPOGENESIS_EARLY_UP	47	2.09626	0	0.012447664
VERNOCHET_ADIPOGENESIS	18	1.84086	0.005859375	0.0401621
ECM RELATED				
NABA_CORE_MATRISOME	205	6.58709	0	0
NABA_MATRISOME_ASSOCIATED	440	6.5002	0	0
NABA_ECM_GLYCOPROTEINS	142	5.55783	0	0
NABA_ECM_REGULATORS	142	4.25922	0	0
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	70	4.08028	0	0
KEGG_ECM_RECEPTOR_INTERACTION	79	3.3602	0	0
GENESETS NEGATIVELY ENRICHED IN THY CELLS				
ADIPOGENESIS RELATED				
BURTON_ADIPOGENESIS_1	29	-2.2995	0	0.003625277
NAKAMURA_ADIPOGENESIS_LATE_UP	83	-2.152	0.004065041	0.008029568
STEGER_ADIPOGENESIS_UP	17	-2.1239	0.004282655	0.009231052
URS_ADIPOCYTE_DIFFERENTIATION_UP	59	-1.9031	0.006224067	0.025960509
ECM RELATED				
NABA_ECM_GLYCOPROTEINS	118	-4.2171	0	0
KEGG_ECM_RECEPTOR_INTERACTION	73	-3.2571	0	0
NABA_ECM_REGULATORS	117	-2.3451	0	0.002851269
BIOCARTA_ECM_PATHWAY	24	-1.8122	0.01980198	0.03808136

B



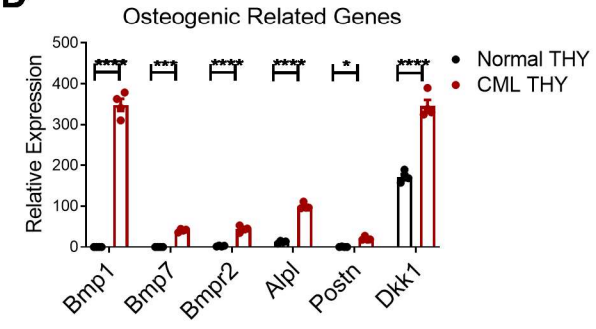
CML 6C3 (ECM Related)

C



CML THY (Cycling)

D



E

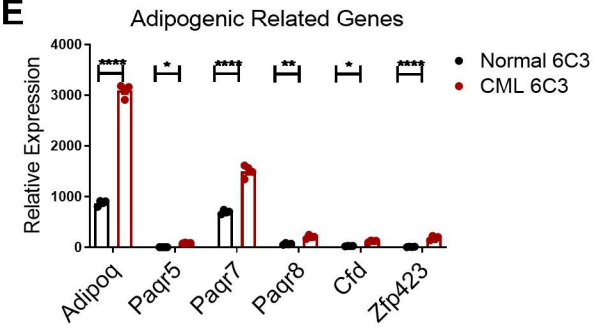


Figure S3. CML 6C3+ stromal progenitors demonstrate enhanced LSC and reduced normal HSC support (Related to Figure 4)

(A) Selected gene sets identified as significantly upregulated or downregulated in CML 6C3+ and THY+ cells compared to normal 6C3+ and THY+ cells (4 biological replicates) on GSEA analysis are shown with normalized enrichment score (NES), and statistical significance indicated by false discovery rate (FDR) and p-value. (B) Representative plots for significantly upregulated gene sets related to cell adhesion and extracellular matrix (ECM) in CML compared to normal 6C3+ cells. (C) Representative plots for significantly upregulated gene sets related to cell cycling in CML compared to normal THY+ cells. Relative mRNA levels from qPCR assay (n=4, each performed in duplicates) for osteogenesis-related genes in normal and CML THY+ cells (D), and for adipogenesis-related genes in normal and 6C3+ cells (E). Error bars represent mean ± sem. Significance values. ns (non-significant) P>0.05, *P< 0.05, **P< 0.01, ***P<0.001, ****P<0.0001.

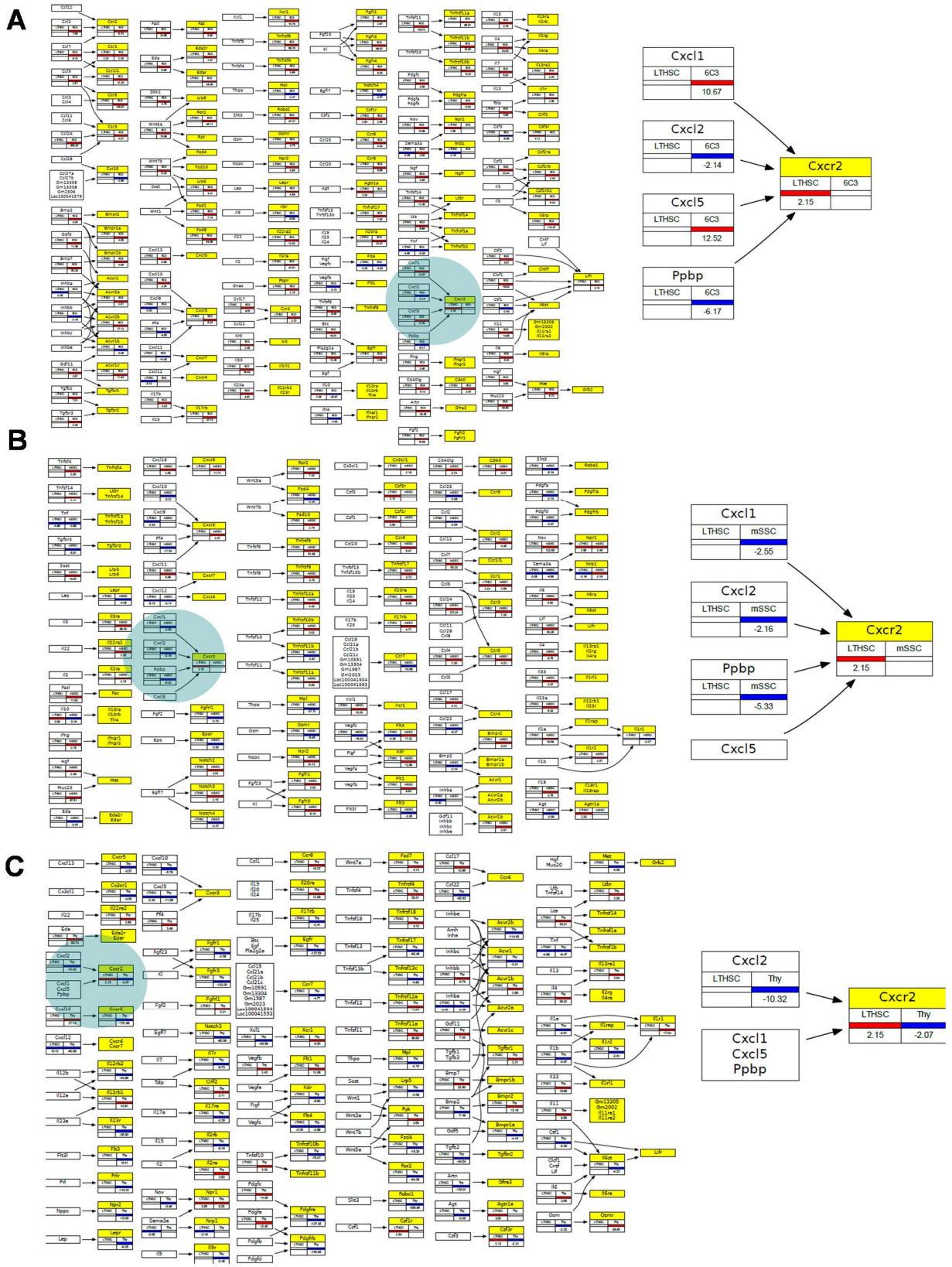

A
Cxcl1
LTHSC 6C3
10.67
Cxcl2
LTHSC 6C3
-2.14
Cxcl5
LTHSC 6C3
12.52
Ppbp
LTHSC 6C3
-6.17
Cxcr2
LTHSC 6C3
2.15
B
Cxcl1
LTHSC mSSC
-2.55
Cxcl2
LTHSC mSSC
-2.16
Ppbp
LTHSC mSSC
-5.33
Cxcl5
Cxcr2
LTHSC mSSC
2.15
C
Cxcl2
LTHSC Thy
-10.32
Cxcl1
Cxcl5
Ppbp
Cxcr2
LTHSC Thy
2.15 -2.07

Figure S4. Altered gene expression in CML compared to normal mesenchymal stem and progenitor cell subsets (Related to Figure 4)

Ligand-receptor interaction interface maps between CML 6C3+ and CML LSCs (A), CML mSSC and CML LSCs (B), and CML THY+ and CML LSCs (C) are shown. Ligand (white boxes)-receptor (yellow boxes) pairs are shown if at least one gene in a given ligand-receptor pair was differentially expressed (fold change > ~2x and FDR < 0.05). Numbers in each box represent fold change, with up- (red) or down-regulated (blue) fold changes. The paracrine CXCL1/CXCL5-CXCR2 interactions between CML subsets and CML LSCs are highlighted with blue circles and represented on the right side. See also Table S1.

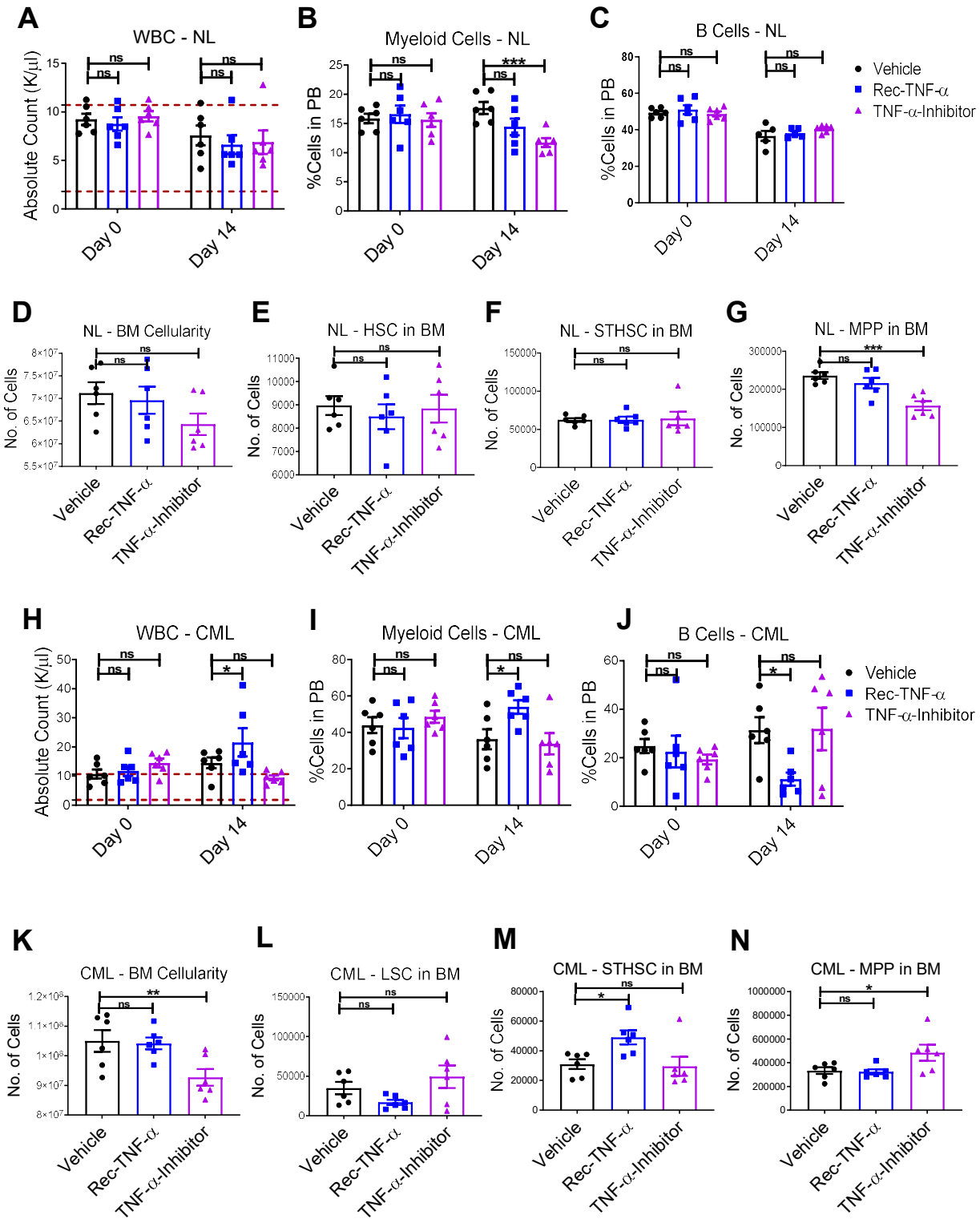

A
WBC - NL
Absolute Count (K/µl)
B
Myeloid Cells - NL
%Cells in PB
C
B Cells - NL
%Cells in PB
Vehicle
Rec-TNF-α
TNF-α-Inhibitor
Day 0
Day 14
D
NL - BM Cellularity
E
NL - HSC in BM
F
NL - STHSC in BM
G
NL - MPP in BM
No. of Cells
H
WBC - CML
I
Myeloid Cells - CML
J
B Cells - CML
K
CML - BM Cellularity
L
CML - LSC in BM
M
CML - STHSC in BM
N
CML - MPP in BM
ns

Figure S5. TNFα signaling contributes to expansion and altered gene expression in 6C3+ stromal progenitors in CML bone marrow (Related to Figure 5)

Total WBC (A), myeloid cell (CD45+Gr-1+CD11b+) frequency (B), and B cell (CD45+CD19+B220+) frequency (C) in PB of normal mice at start of treatment (Day 0) and end of recombinant TNF-α and anti-TNF-α treatment (Day 14). Total BM cellularity (D), LSCs (E), STHSC (F), and MPP (G), in the BM (2 femurs+2 tibiae) of normal mice. Total WBC numbers (H), and myeloid cell (CD45+Gr-1+CD11b+) (I) and B cell (CD45+CD19+B220+) frequency (J), at start of treatment (Day 0) and end of treatment (Day 14) in PB of CML mice. Total number of cells (K), LSCs (L), STHSC (M), and MPP (N) numbers in the BM (2 femurs+2 tibiae) of CML mice (n= 5-6 mice/group). Error bars represent mean ± sem. Significance values. ns (non-significant) $P>0.05$, *$P< 0.05$, **$P< 0.01$, ***$P<0.001$, ****$P<0.0001$.

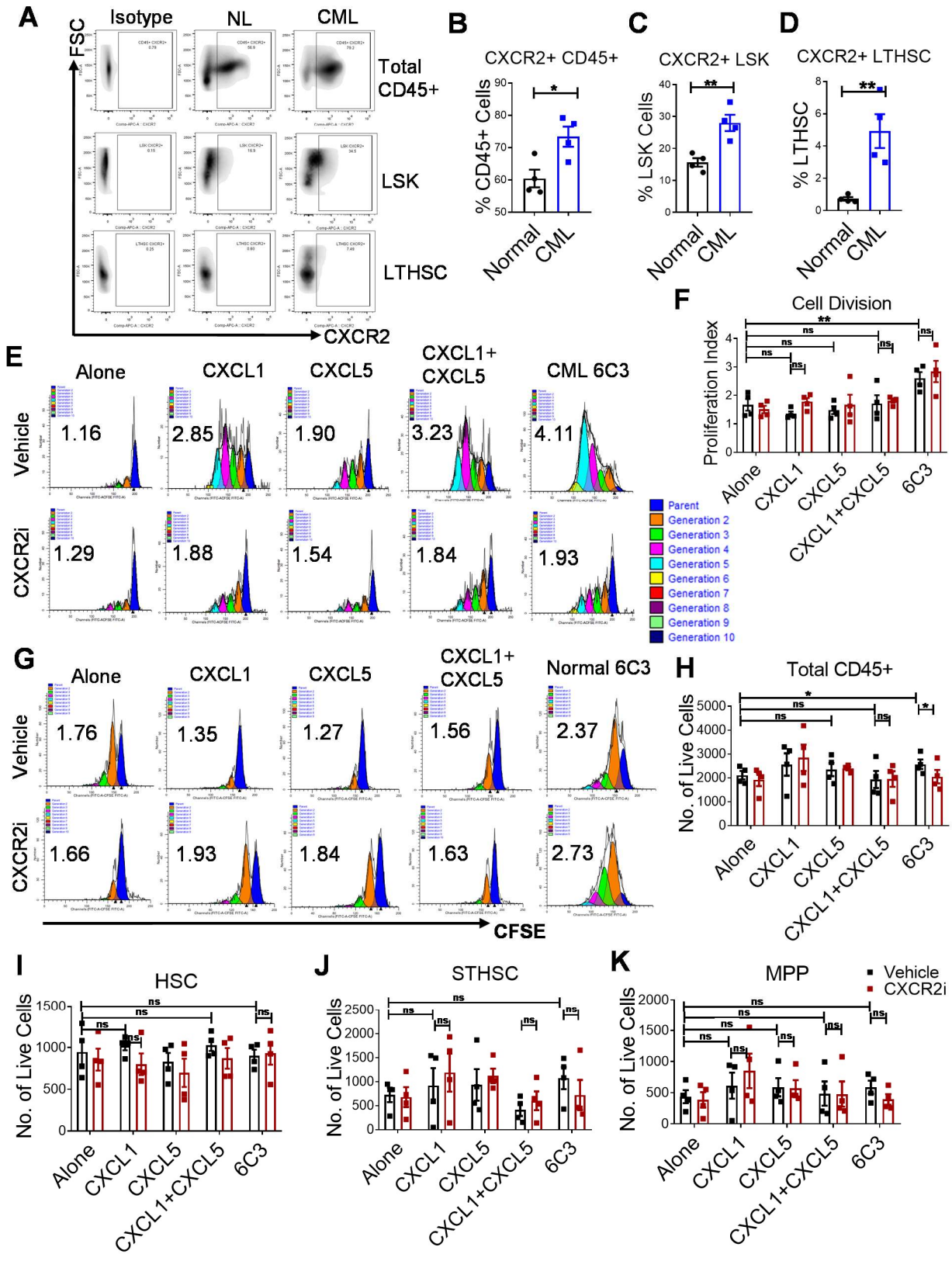

A
Isotype
NL
CML
FSC
Total CD45+
LSK
LTHSC
CXCR2
B
CXCR2+ CD45+
% CD45+ Cells
Normal
CML
C
CXCR2+ LSK
% LSK Cells
D
CXCR2+ LTHSC
% LTHSC
E
Alone
CXCL1
CXCL5
CXCL1+ CXCL5
CML 6C3
Vehicle
CXCR2i
1.16
2.85
1.90
3.23
4.11
1.29
1.88
1.54
1.84
1.93
Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10
F
Cell Division
Proliferation Index
Alone
CXCL1
CXCL5
CXCL1+CXCL5
6C3
G
Alone
CXCL1
CXCL5
CXCL1+ CXCL5
Normal 6C3
Vehicle
CXCR2i
1.76
1.35
1.27
1.56
2.37
1.66
1.93
1.84
1.63
2.73
CFSE
H
Total CD45+
No. of Live Cells
I
HSC
J
STHSC
K
MPP
Vehicle
CXCR2i
ns

Figure S6. CXCL1 and CXCL5 signaling through CXCR2 enhances growth of leukemic stem and progenitor cells (Related to Figure 6)

(A) Representative flow plots showing CXCR2 expression in freshly isolated BM populations from normal and CML mice using anti-CXCR2 antibody and isotype control antibody. Relative frequency of CXCR2+ cells as a percentage of parent population among total hematopoietic cells (CD45+) (B), hematopoietic stem/progenitor cells (LSK) (C) and primitive LSCs (D) (n=4 independent samples/group). (E) Representative CFSE plots of a CML sample. FACS purified $CFSE_{max}$ normal HSCs were cultured in the presence or absence of normal 6C3+ cells, CXCL1 (250ng/ml), CXCL5 (250ng/ml), CXCL1 and CXCL5, or vehicle, with or without CXCR2i (10μM) for 72hrs (2000 cells/condition), and proliferation and cell numbers analyzed. A proliferation index was calculated on the basis of reduction in CFSE levels. The calculated proliferation index for each plot is indicated. (F) Proliferation index of normal cells exposed to different treatments (n=4 samples/group). (G) Representative CFSE plots of normal sample. The absolute number of normal total CD45+ cells (H), HSCs (I), STHSC (J) and MPP (K). Error bars represent mean ± sem. ns (non-significant) $P>0.05$, *$P< 0.05$, **$P< 0.01$, ***$P<0.001$, ****$P<0.0001$.

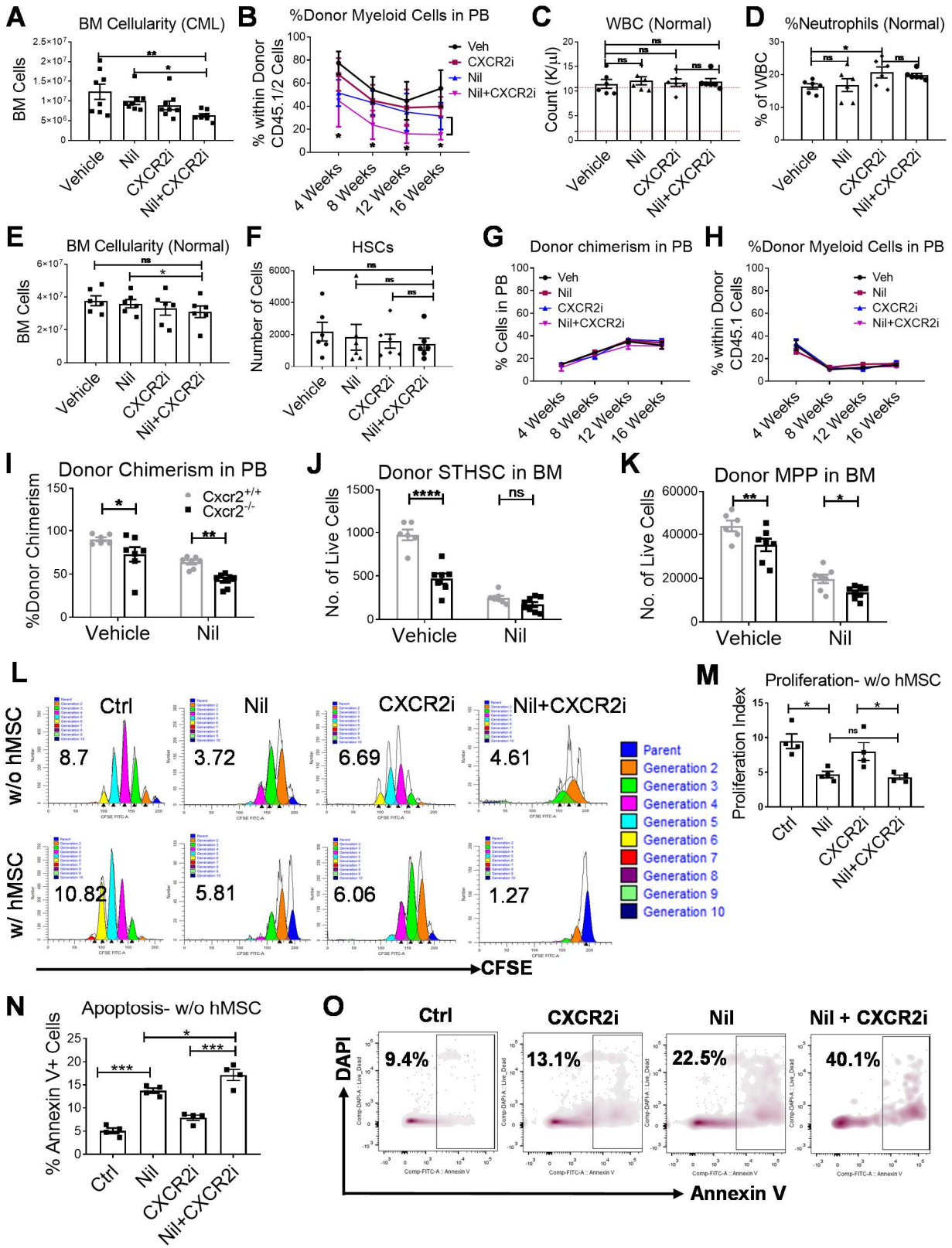

A
BM Cellularity (CML)
BM Cells
Vehicle
Nil
CXCR2i
Nil+CXCR2i
B
%Donor Myeloid Cells in PB
% within Donor CD45.1/2 Cells
Veh
CXCR2i
Nil
Nil+CXCR2i
4 Weeks
8 Weeks
12 Weeks
16 Weeks
C
WBC (Normal)
Count (K/µl)
D
%Neutrophils (Normal)
% of WBC
E
BM Cellularity (Normal)
F
HSCs
Number of Cells
G
Donor chimerism in PB
% Cells in PB
H
%Donor Myeloid Cells in PB
I
Donor Chimerism in PB
%Donor Chimerism
Cxcr2+/+
Cxcr2-/-
J
Donor STHSC in BM
No. of Live Cells
K
Donor MPP in BM
L
w/o hMSC
w/ hMSC
Ctrl
Nil
CXCR2i
Nil+CXCR2i
8.7
3.72
6.69
4.61
10.82
5.81
6.06
1.27
Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10
CFSE
M
Proliferation- w/o hMSC
Proliferation Index
N
Apoptosis- w/o hMSC
% Annexin V+ Cells
O
Ctrl
CXCR2i
Nil
Nil + CXCR2i
9.4%
13.1%
22.5%
40.1%
DAPI
Annexin V

Figure S7. CXCR2 targeting reduces leukemic stem cell growth and survival that is further enhanced in combination with TKI treatment (Related to Figure 7)

(A) Overall BM cellularity of CML mice from the experiment shown in Figure 7A (n= 6-8 mice/group). (B) Frequency of donor CML myeloid cells from serial blood draw performed every 4 weeks until 16 weeks from the experiment shown in Figure 7F. Total WBC (C) and frequency of neutrophils (D) in the PB, and total number of BM cells (E) and HSCs (F) in the BM of normal mice. After primary transplantation of normal WBM cells from the experiment shown in Figure 7A, LSCs were FACS purified and transplanted into secondary healthy WT mice irradiated at 800cGy. Serial blood draw was performed every 4 weeks until 16 weeks. Total donor chimerism (G), and donor myeloid cells (H) in the PB. Frequency of donor chimerism in the PB (I), and numbers of donor STHSC (J) and MPP (K) in the BM (2 femurs+2 tibiae) of CML mice. (L) Representative CFSE plots for a CML sample from the experiment shown in Figure 7L. A proliferation index was calculated on the basis of reduction in CFSE levels. The calculated proliferation index for each plot is indicated. Effects of treatment on the proliferation index in the absence of hMSC cells (M), and the percentage of apoptosis calculated based on Annexin V+ labeling of CML cells in the absence of hMSC (N) and representative FACS plots (O) (n=4 biological samples/group). Error bars represent mean ± sem. ns (non-significant) $P>0.05$, *$P< 0.05$, **$P< 0.01$, ***$P<0.001$, ****$P<0.0001$.

Table S2. A list of qPCR assays used (Related to Figures 5 and S3).

Target	Source	Cat No.
mActb	Thermo Fisher Scientific	4352933E
mBmp1	Thermo Fisher Scientific	Mm00802220_m1
mBmp7	Thermo Fisher Scientific	Mm00432102_m1
mBmpr2	Thermo Fisher Scientific	Mm00432134_m1
mAlpl	Thermo Fisher Scientific	Mm00475834_m1
mPostn	Thermo Fisher Scientific	Mm01284919_m1
mDkk1	Thermo Fisher Scientific	Mm00438422_m1
mAdipoq	Thermo Fisher Scientific	Mm00456425_m1
mPaqr5	Thermo Fisher Scientific	Mm01170057_m1
mPaqr7	Thermo Fisher Scientific	Mm00510958_m1
mPaqr8	Thermo Fisher Scientific	Mm00546979_m1
mCfd	Thermo Fisher Scientific	Mm01143935_g1
mZfp423	Thermo Fisher Scientific	Mm00677660_m1
mCxcl1	Thermo Fisher Scientific	Mm04207460_m1
mCxcl5	Thermo Fisher Scientific	Mm00436451_g1
mCxcr2	Thermo Fisher Scientific	Mm99999117_s1