SUPPLEMENTARY APPENDIX

Reversible switching of leukemic cells to a drug-resistant, stem-like subset via IL-4-mediated cross-talk with mesenchymal stroma

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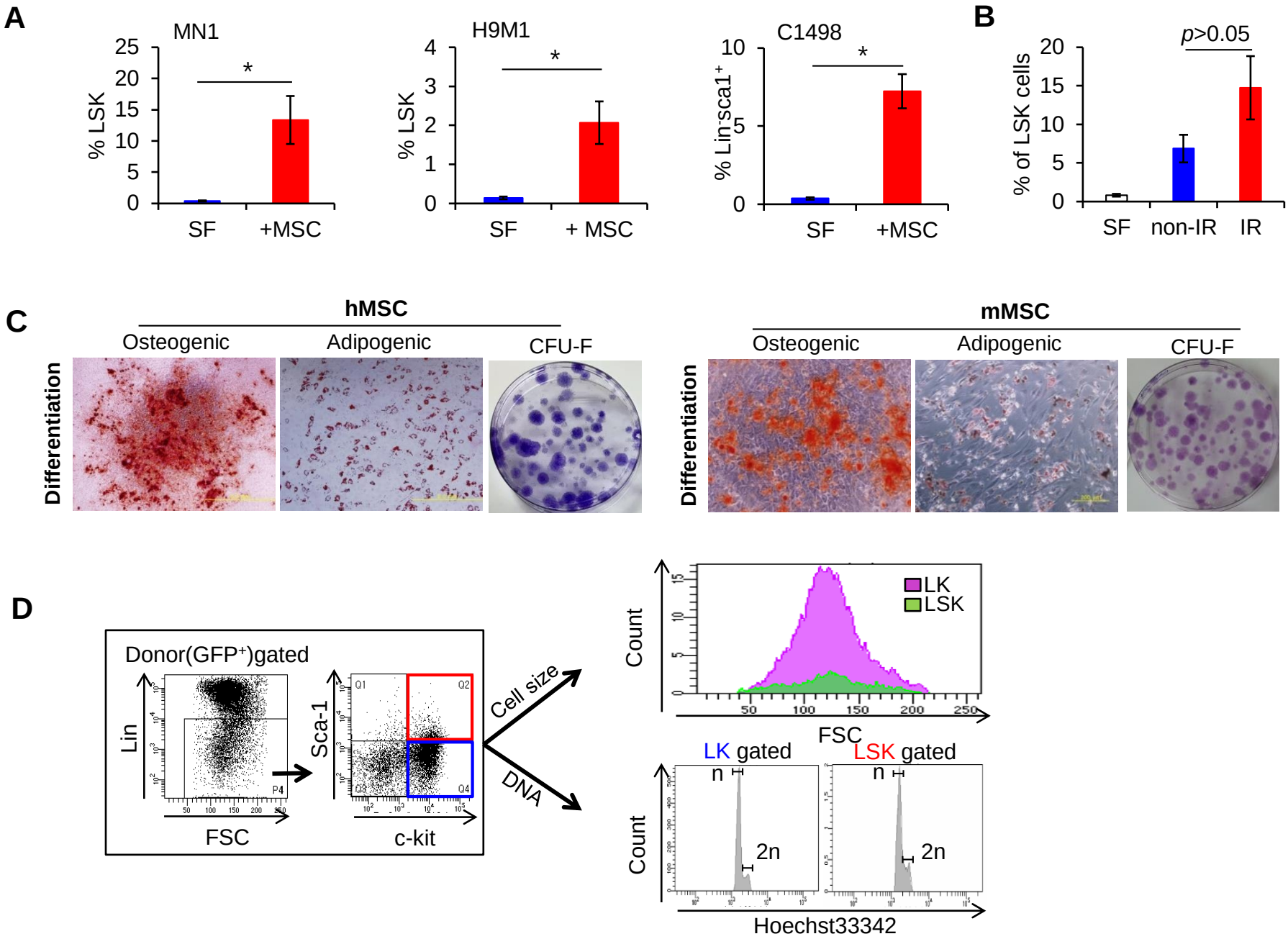
Supplementary Figure S1



Supplementary Figure S1. Generation of Sca-1(+) leukemic cells during co-culture with stromal cells.

(A) Generation of Sca-1(+) (Lin$^-$c-kit$^+$sca-1$^+$: LSK) cells during stromal co-culture in various murine leukemic cells. The indicated murine leukemic cells were cultured in the presence (+MSC) or absence (stroma-free: SF) of murine MSCs. The phenotype of leukemic cells after 3 days of co-culture was analyzed by flow cytometry by gating the leukemic cell population (CD45$^+$GFP$^+$) from MSCs (CD45$^-$GFP$^-$). Shown are quantitative analysis for generation of Sca-1(+) subsets (mean ± SEM , n=7 for MN1, 6 for H9M1, 7 for C1498).

(B) Influence of irradiation on MSCs for generation of Sca-1(+) subsets. Leukemic cells were co-cultured with irradiated or non-irradiated murine MSCs. The phenotype of leukemic cells after 3 days of co-culture was analyzed by flow cytometry by gating the leukemic cell population (CD45$^+$GFP$^+$) from MSCs (CD45$^-$GFP$^-$).

(C) Characterization of culture-established MSCs. MSCs established by in-vitro culture adherence were subcultured for 5-8 passages. MSCs were examined for colony formation (CFU-F) and differentiation into osteogenic or adipogenic lineages were examined by staining with Alizarin red S or Oil Red O, respectively. Shown are the representative images for differentiation of human (left) and murine (right) MSCs.

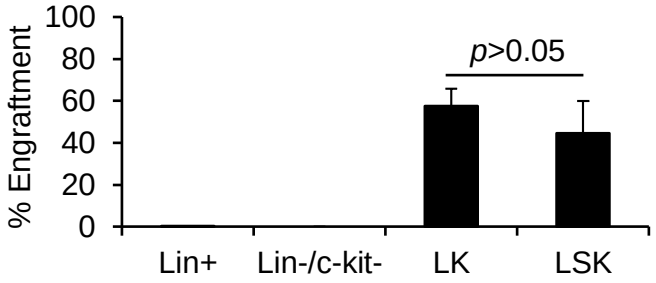
(D) Lack of evidence for cell fusion between leukemic cells and MSCs for in-vivo generation of LSK subsets. MN1 leukemic cells were transplanted into recipient mice, and LSK and LK subsets generated in the BM of recipient mice were compared for cell size (FSC) and DNA content (Hoechst33342).

Supplementary Figure S2

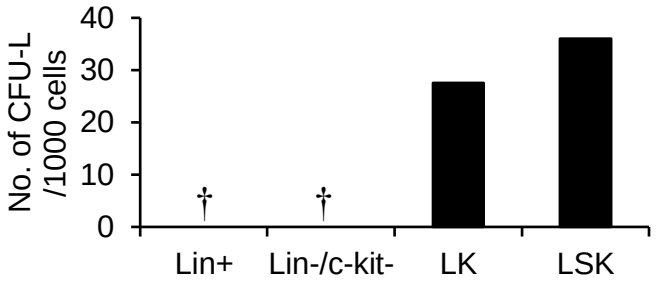
A

	input donor cell dose	frequency of leukemia (+) mice	frequency of leukemia initiating cells
Lin^+	200	0/5	1/28558 *(8707.6-93664)*
	2000	1/4	
	20000	2/5	
$Lin^-c\text{-}kit^-$	200	0/4	1/14778 *(2104.5-103789)*
	600	0/5	
	2000	1/6	
LK	60	1/3	1/179 *(80.2-400)*
	200	5/5	
	600	4/5	
	2000	4/4	
LSK	60	2/7	1/302 *(144.2-634)*
	200	4/5	
	600	4/6	
	2000	6/6	

B



C



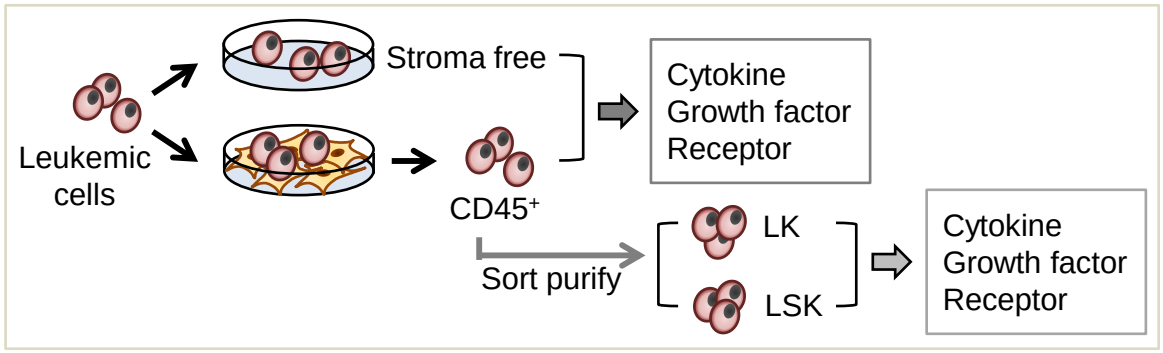
Supplementary Figure S2. Comparison of leukemogenic activity between leukemic subsets.

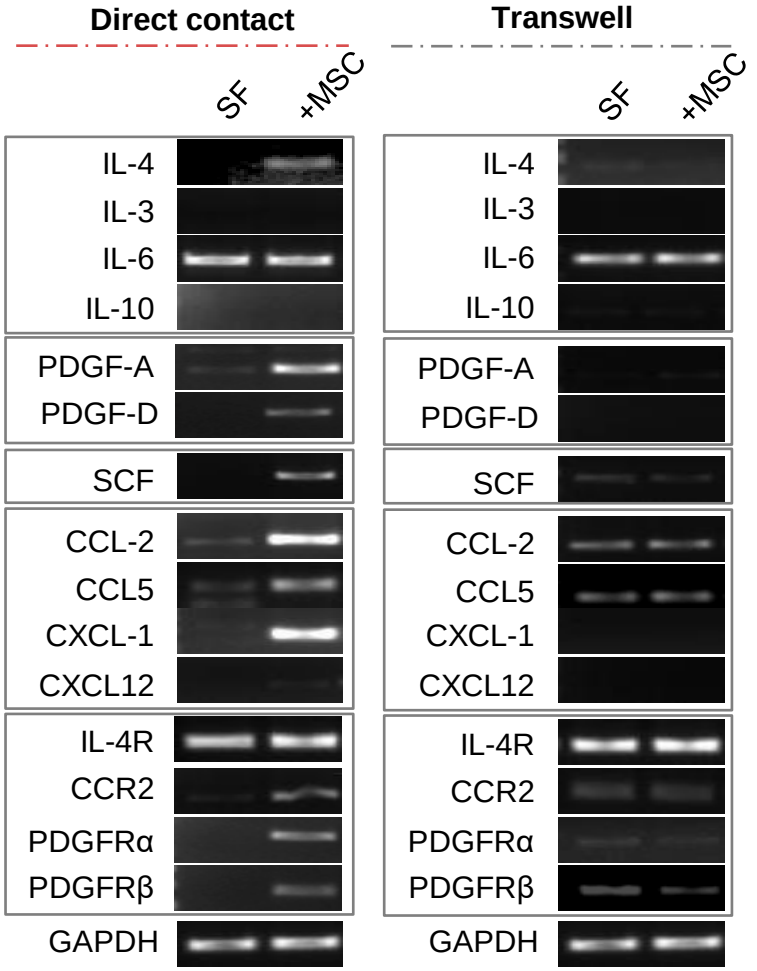
(A) Comparisons of leukemia-initiating cell (LIC) frequencies for each leukemic subset. MN1 leukemic cells were transplanted into mice and each subset of leukemic cells in BM were sort-purified for transplantation into secondary recipients in a limiting dilution dose. Shown are each leukemic subset analyzed by Poisson statistics, and the resulting LIC frequencies are shown with 95% confidence interval in parenthesis. The plot of limiting dilution analysis for frequencies of LIC are shown in Fig. 3D.

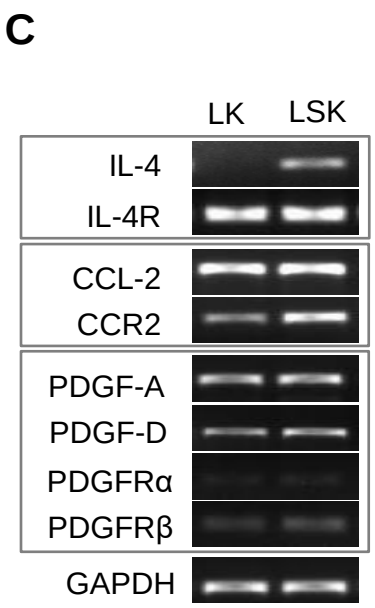
(B) MN1 leukemic cells were first engrafted into recipients, and various leukemic subsets generated in the recipient BMs were sort-purified for secondary transplantation. Shown are the mean ± SEM for % engraftment in secondary recipient mice transplanted with each subset (n= 5 for Lin^+, LK, and LSK, n=4 for Lin^-c-kit^-).

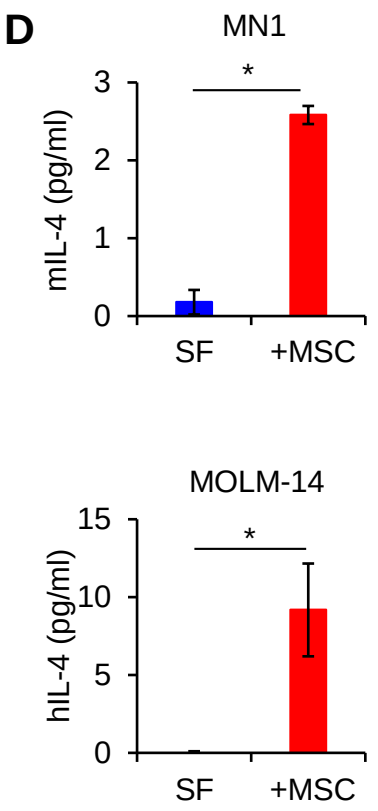
(C) Comparisons of leukemic proliferation between each subset. Leukemic subsets generated in recipient BMs were sort-purified and plated to analyze in-vitro leukemia cell proliferation. Shown are the mean numbers of leukemia-proliferating cells from 1000 leukemic cells plated on soft agar. (†; no colony found).

Supplementary Figure S3









Supplementary Figure S3. Induction of IL-4 in leukemic subsets by stromal contact

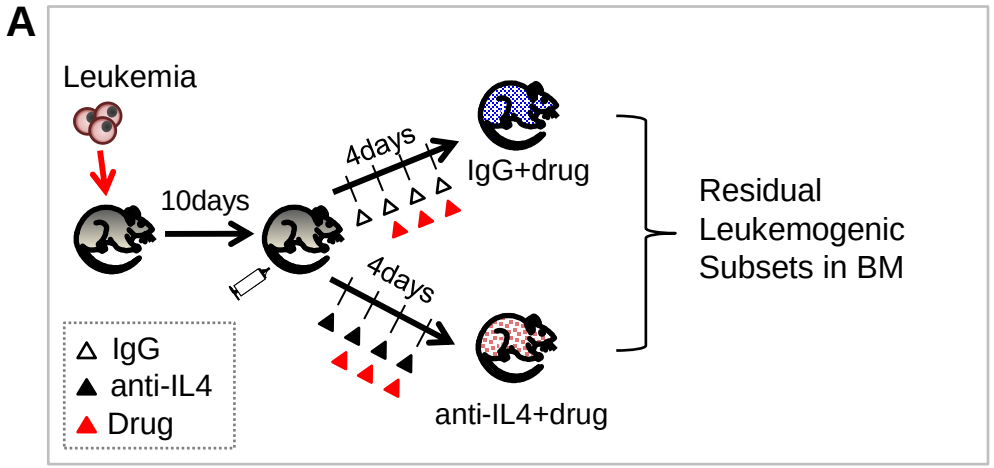
(A) Experimental design. Leukemic cells were cultured in the presence or absence of stroma or transwell filters between the cells, and expression of cytokines or growth factors were examined.

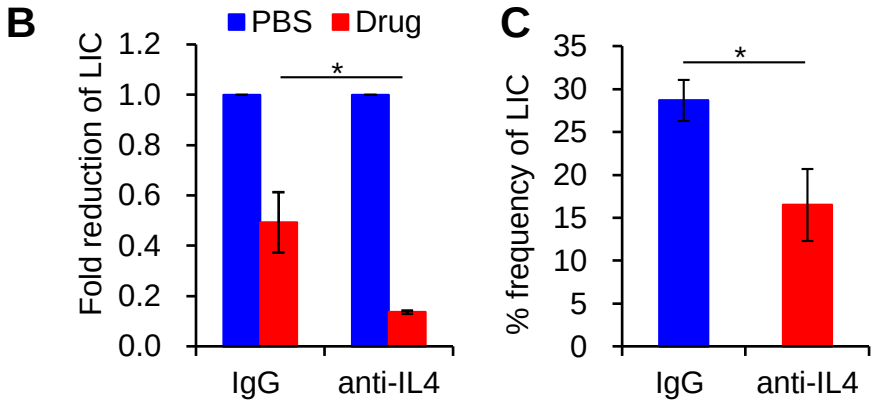
(B) Expression changes of cytokine and growth factor by co-culture with mesenchymal stroma. Shown are the representative profiles of RT-PCR for each indicated cytokines in the presence or absence of mesenchymal stromal feeder

(C) Comparisons for the expression of cytokines in purified leukemic subsets. Leukemic cells co-cultured with MSCs were sort purified for $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{-}$ (LK) or $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LSK), and the populations compared for the expression of indicated cytokine/growth factor genes.

(D) Detection of IL-4 protein in the supernatant. Leukemic cells were co-cultured with MSCs and enriched for adherent leukemic cells by removing non-adherent leukemic cells with fresh medium change. Co-cultures were continued for 72hrs and supernatants were measured for IL-4 protein by ELISA. IL-4 levels in MSCs or stroma-free leukemic culture were determined in the cultures that had been plated with equivalent numbers of leukemic cells or MSCs for same culture period (n=8, 2 expts, *;$p<0.05$)

Supplementary Figure S4



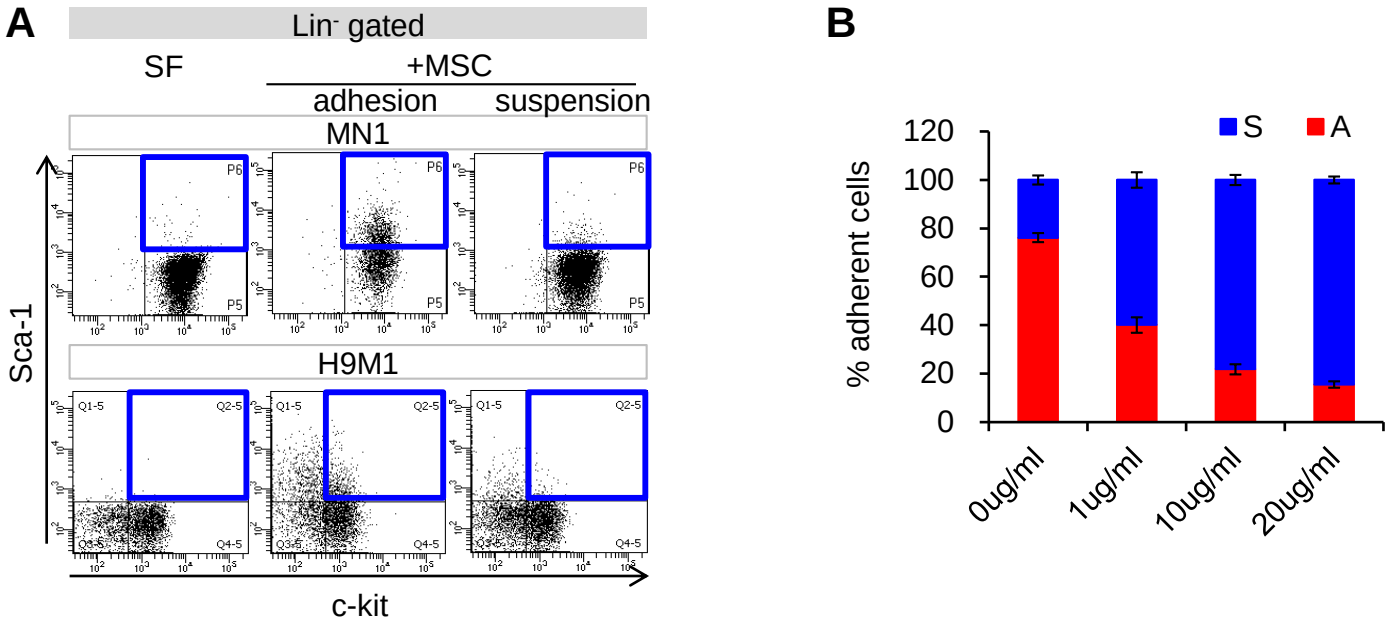


Supplementary Fig S4. Effect of IL-4 blocking on the chemotherapy of leukemia

(A) Experimental scheme. Mice engrafted with MN1 leukemic cells (10 days) were injected with either IgG or IL-4 antibody and chemotherapeutic drug (AraC+doxorubicin) for 4 days, and residual leukemia initiating cells (LICs) in BMs were measured.

(B, C) Effects IL-4 Ab on the reduction of LICs in BMs of chemotherapy treated mice. Mice treated with chemotherapy in combination of IL-4 Ab or IgG were analyzed for LICs in BM. LICs were counted by sum of LSK and LK subsets of leukemic cells. Shown are the fold redutions of LICs in BMs of mice treated with chemotherapy drugs in combination of IgG or IL-4 Ab (B) and % of LICs in total leukemic cells (GFP+) in BM of mice (C) (mean±SEM, n= 5, *; $p<0.05$).

Supplementary Figure S5

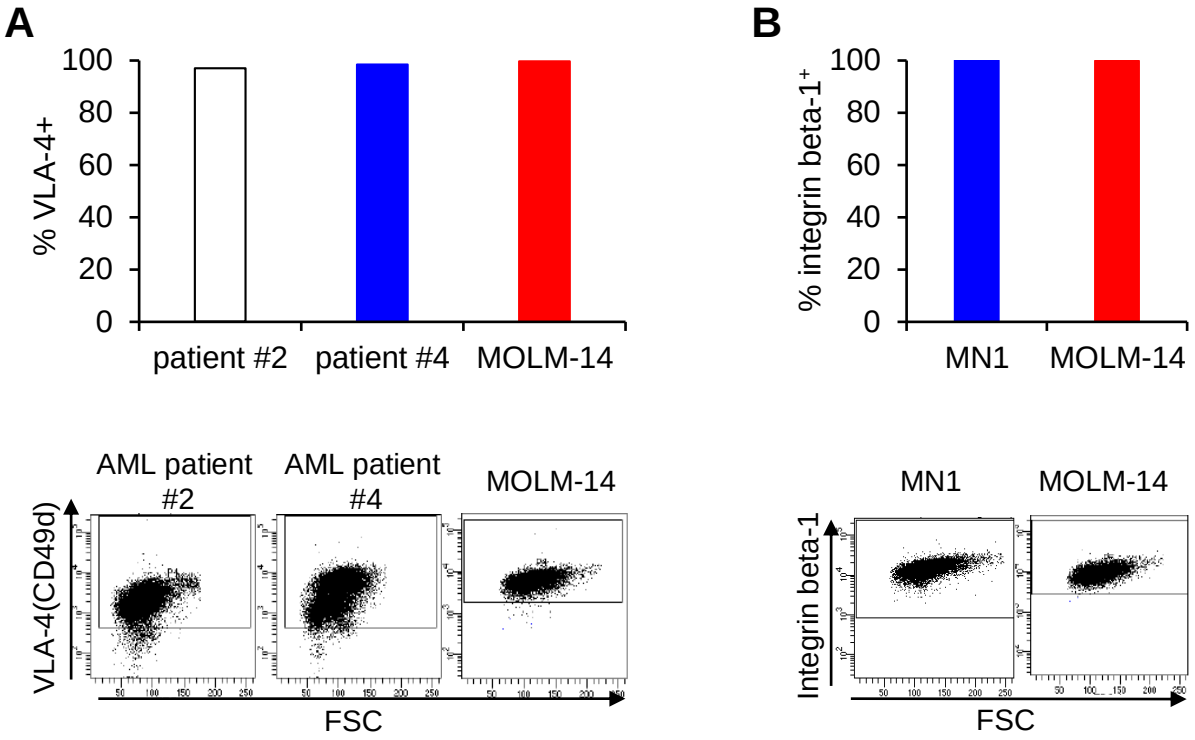


Supplementary Figure S5. Generation of LSK subsets is dependent on tight adherence to stromal cells.

(A) H9M1 or MN1 leukemic cells were co-cultured with stroma. After co-culture, leukemic cells in loose adherence/suspension were analyzed by taking the culture soup with PBS wash (2X), whereas leukemic cells tightly adherent to mesenchymal stroma were analyzed by trypsin/EDTA treatment of adherent cells after the PBS wash. Generation of LSK subsets among leukemic cells tightly adherent to stroma (adhesion) or those loosely adherent (suspension) cells were compared. Blue box shows the area for LSK subsets in each panel.

(B) Effects of blocking antibody against VCAM-1 on stromal adherence of leukemic cells. During co-culture of leukemic cells with stroma, indicated amount of antibody against VCAM-1 was added and changes in the leukemic cell adherence during the co-culture were analyzed (mean±SEM n= 6, 2 expts). (S; suspension/loose adherent cells, A; adherent leukemic cells).

Supplementary Figure S6

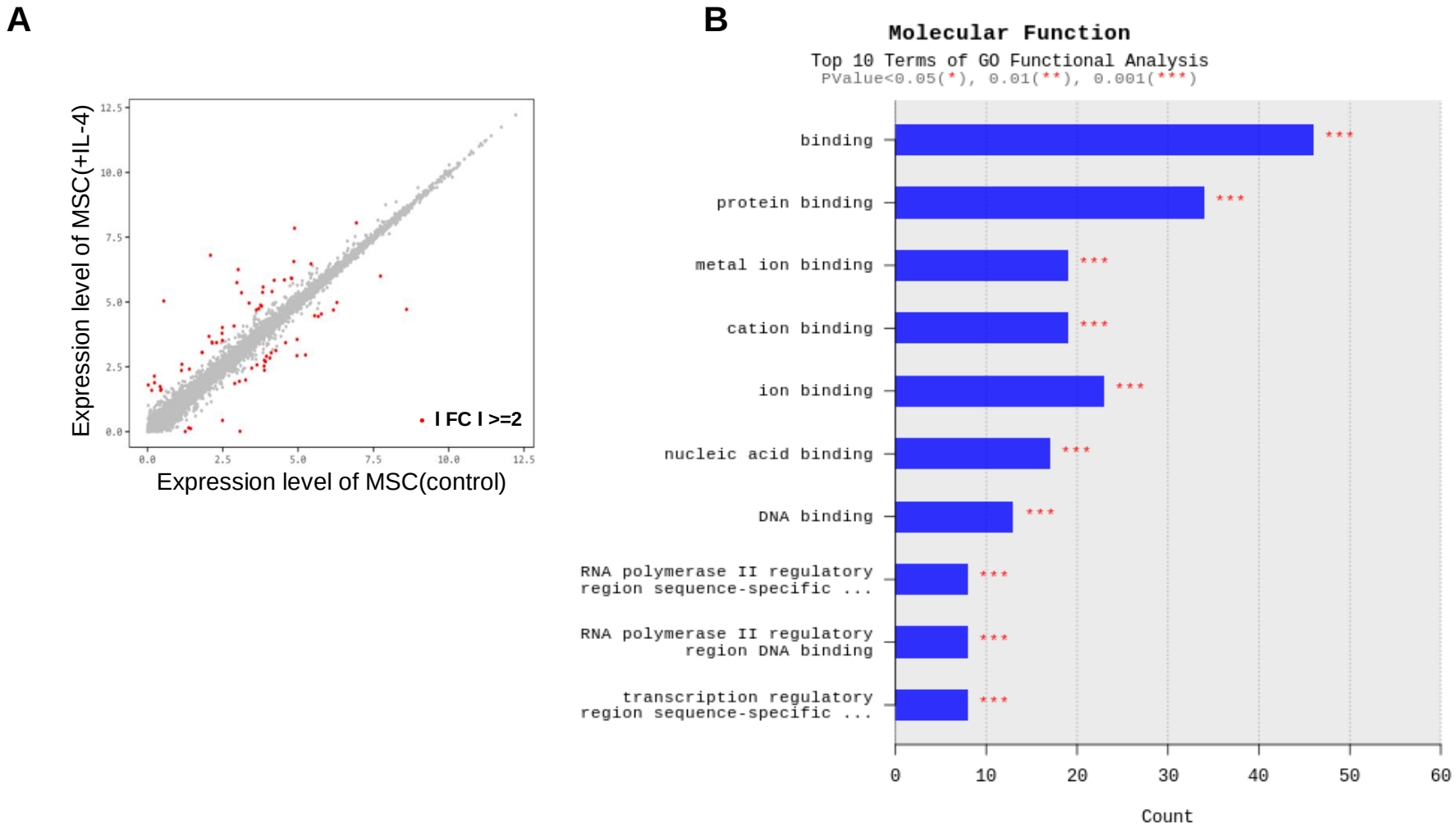


Supplementary Figure S6. Expression of VCAM-1 ligands in leukemic cells.

(A) Surface expression levels of VCAM-1 ligand VLA-4 on human primary AML patients' BM and MOLM-14 leukemic cells.

(B) Surface expression levels of the VCAM-1 ligand integrin beta-1 on MN1 and MOLM-14 leukemic cells.

Supplementary Figure S7

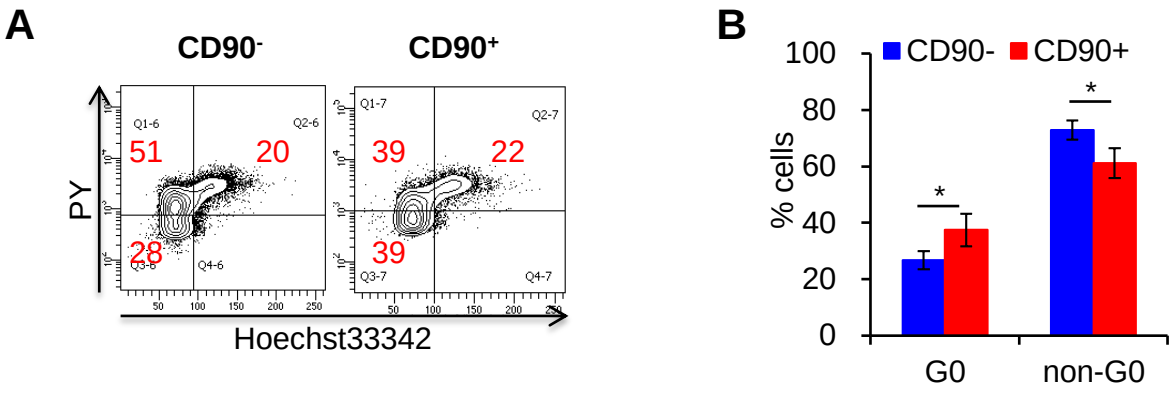


Supplementary Figure S7. Differentially expressed genes (DEGs) induced by IL-4 in MSCs.

(A) Plots of differential gene expression patterns between control MSCs and IL-4 stimulated MSCs.

(B) Gene ontology analysis of DEGs ($p<0.05$) between the MSCs induced by IL-4 stimulation.

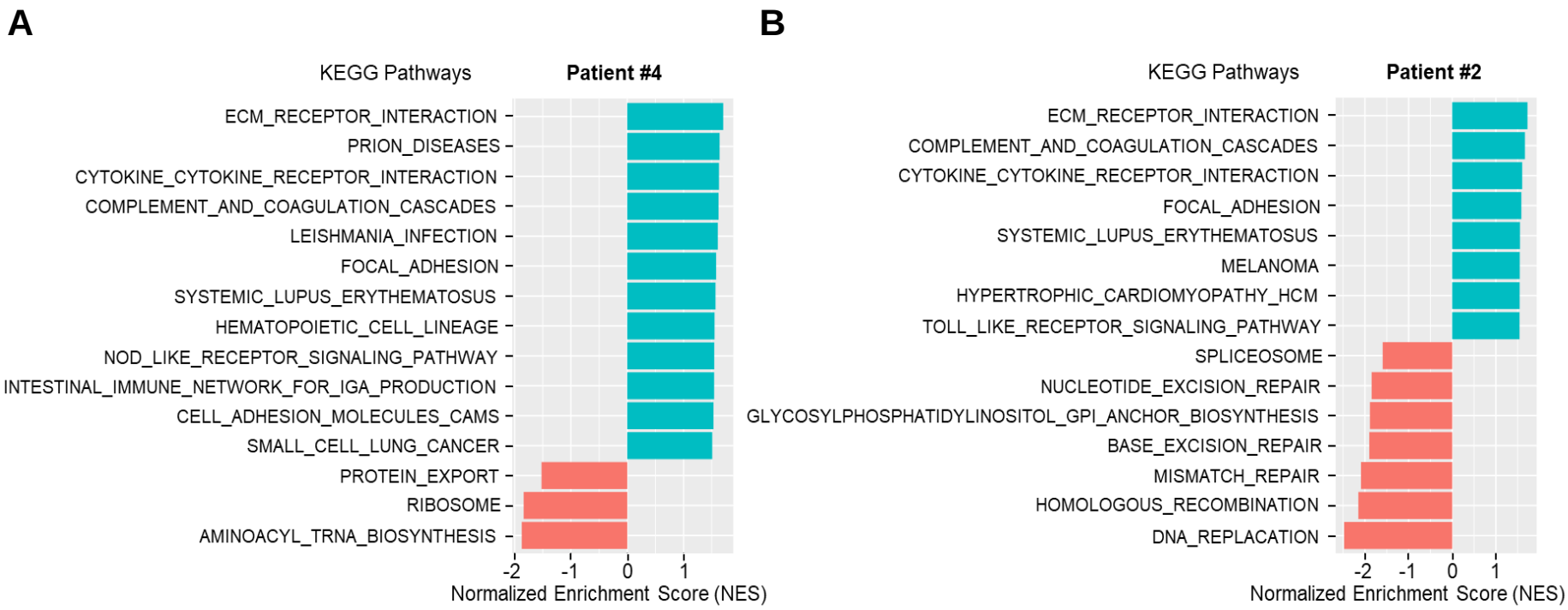
Supplementary Figure S8



Supplementary Figure S8. Cell cycling of CD90(+) leukemic subsets in human leukemic cells.

Human leukemic cells (MOLM-14) were co-cultured with human MSCs for 3 days and the cell cycling of leukemic cells were analyzed by flow cytometry by gating the leukemic ($CD45^+$) cell population. Shown are the representative flow cytometry plots for Hoechst33342/pyronin staining **(A)** and quantification of the % of cells in each stage: G0 and Non-G0 (G1/S/G2M) **(B)** (n=9, 3 expt *; $p<0.05$).

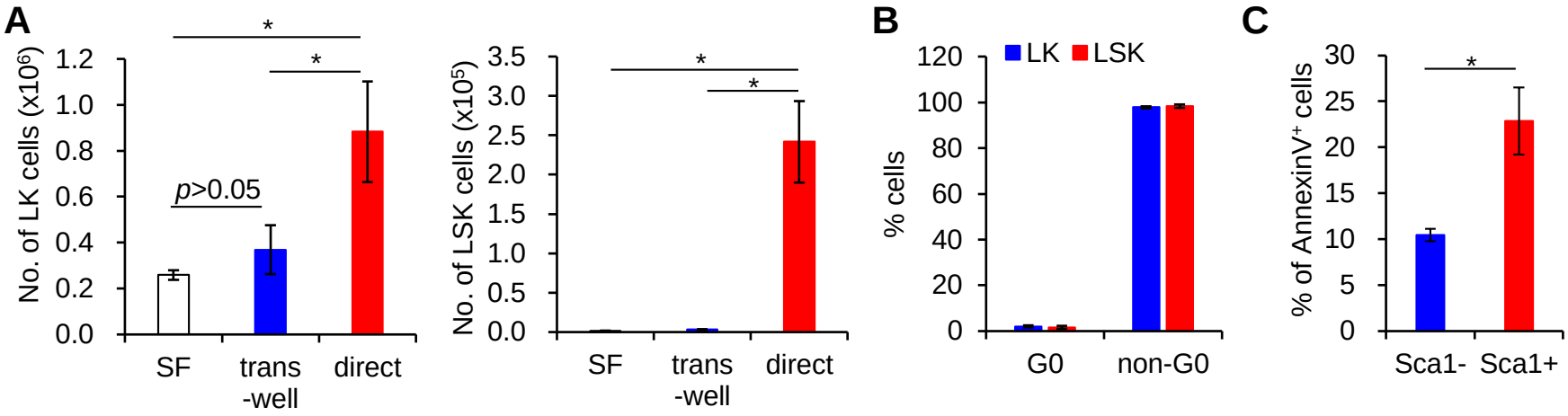
Supplementary Figure S9



Supplementary Figure S9. Enrichment of stem cell signatures in CD90(+) human leukemic cells.

Primary human leukemic cells from 2 AML patients (#2, #4) were co-cultured with human MSCs for 3 days, and the generated CD90(+) and CD90(-) cells were subjected to RNA seq analysis. Differentially expressed genes were analyzed by gene set enrichment analysis (GSEA-P). The Gene ontology enriched in CD90(+) cells compared to CD90(-) cells were analyzed by KEGG pathway analysis. Shown are the results for AML patients **(A for #4 patients, B for #2 patients).**

Supplementary Figure S10



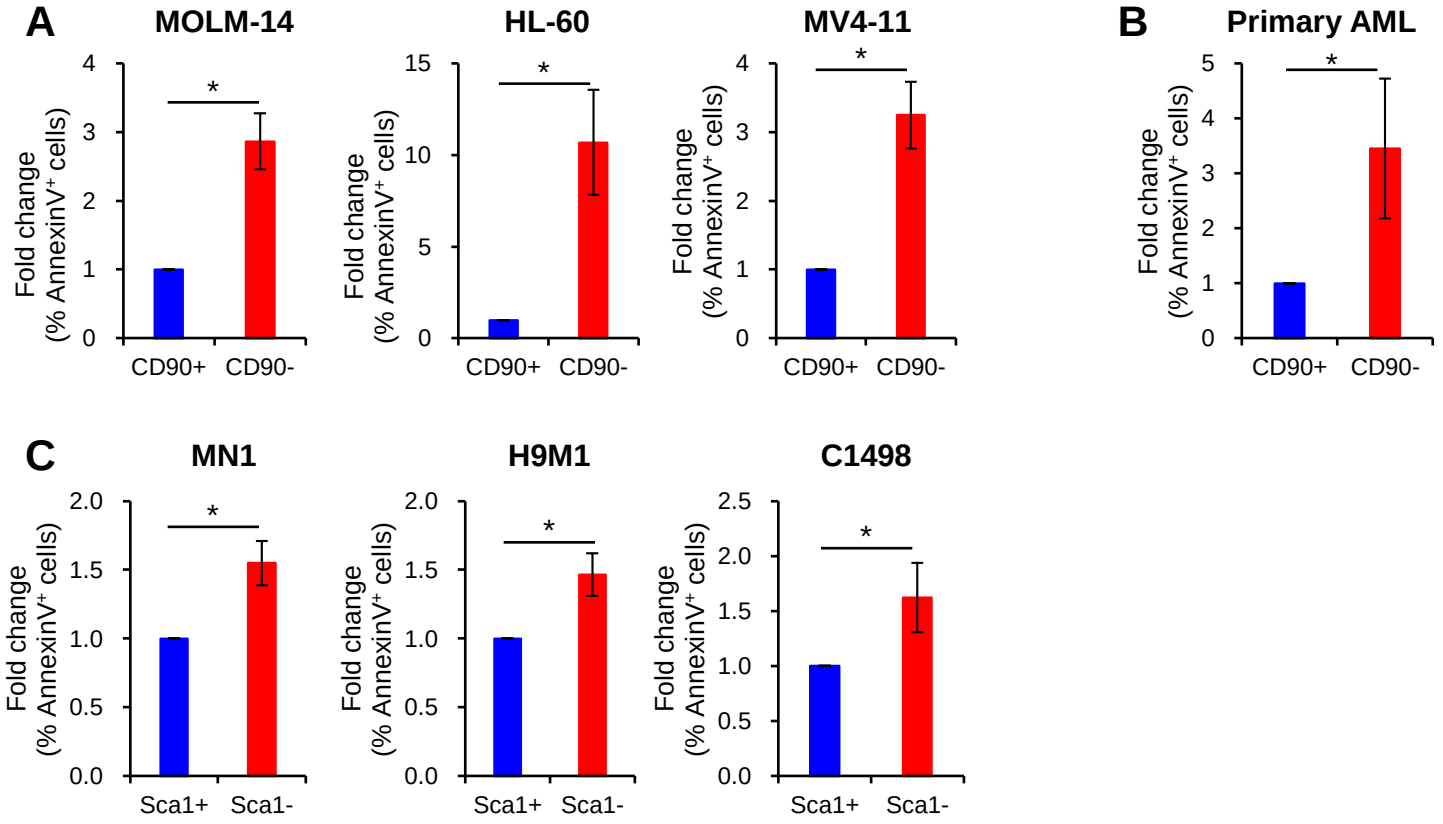
Supplemental Figure S10. Generation of Sca-1(+) subsets in leukemic cells independent of changes in cell cycle or apoptosis

(A) Generation of Sca-1(+) (Lin$^-$c-kit$^+$sca-1$^+$: LSK) leukemic cells during co-culture with murine mesenchymal stromal cells (mMSCs). Co-cultures with mMSCs for 3 days were performed in the presence (transwell) or absence (direct contact) of a transwell membrane between the cells in comparison to stroma-free (SF) culture. Phenotypes of leukemic cells (CD45$^+$GFP$^+$) from co-cultured MSCs (CD45$^-$GFP$^-$) were analyzed by flow cytometry. Shown are the numbers of LK (left) and LSK leukemic cells (right) before (SF) and after co-culture (mean ± SEM , n=7, *;p<0.05).

(B) Cell cycling of LSK leukemic subsets generated by in-vitro co-culture with MSCs. MN1 leukemic cells were co-cultured with murine MSCs for 3 days and the cell cycling of leukemic cells were analyzed by Hoechst33342/pyronin staining and flowcytometry after gating the leukemic (CD45$^+$) cell population. Shown are the quantification of the % of cells in each stage: G0 and Non-G0 (G1/S/G2M) (n=3, 1 expt).

(C) Apoptotic rate of leukemic subsets. Sca-1(+) and Sca-1(-) subsets generated from MN1 cells were compared for apoptosis by measuring % Annexin$^+$/PI$^-$ cells. Shown are the mean % apoptotic cells of each subsets (n=3, *; p<0.05). Note apoptosis of LSK is not lower than LK subsets.

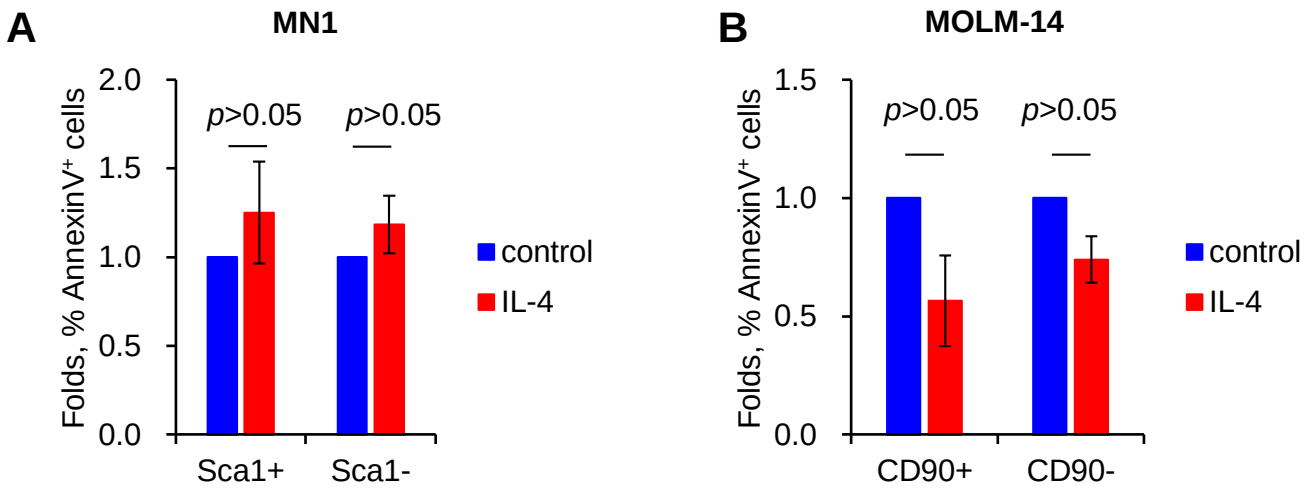
Supplementary Figure S11



Supplementary Figure S11. Comparisons of apoptotic response of leukemic subsets in response to Ara-C.

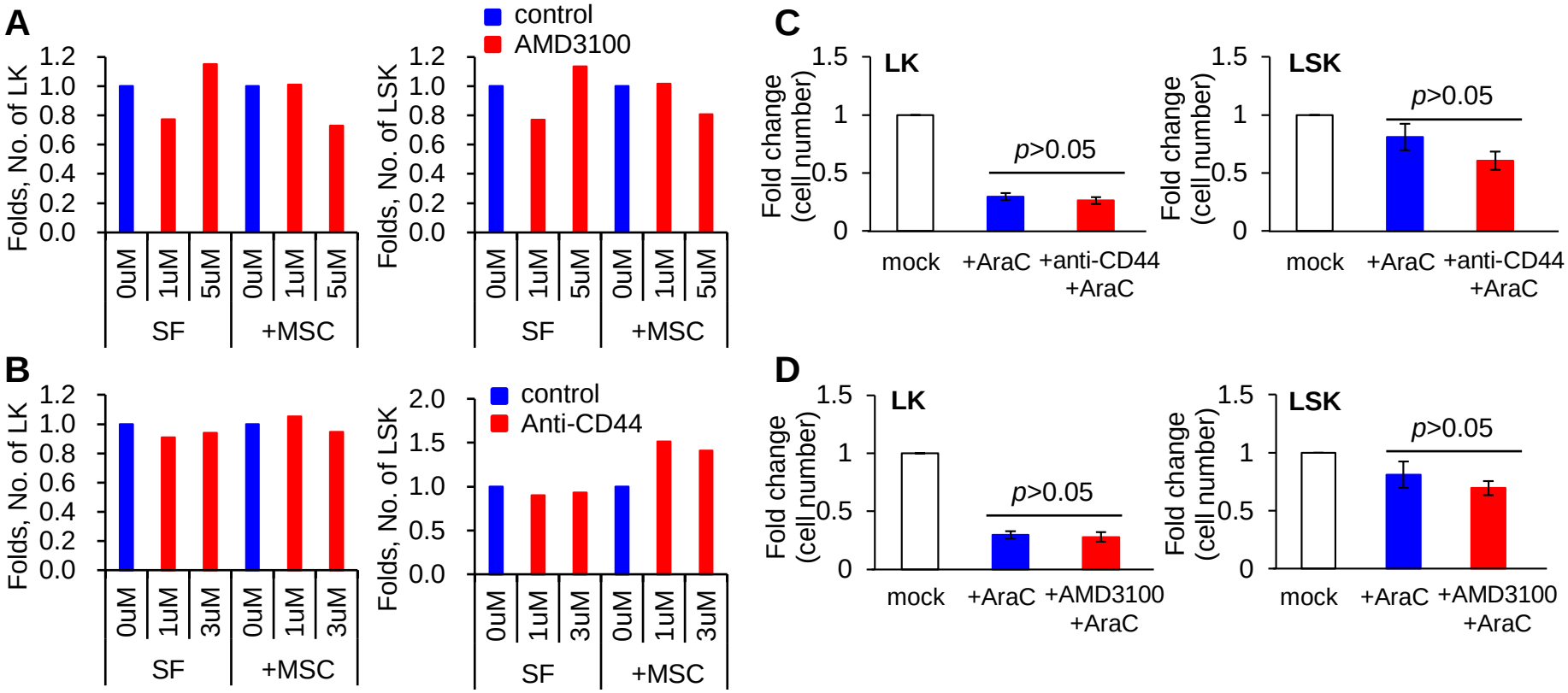
(A) Human leukemic cell lines were co-cultured with human MSCs in the presence or absence of Ara-C (200 nM) and % of apoptotic cells (AnnexinV$^+$ propidium iodide$^-$) in CD90(+) and CD90(-) cells were analyzed. Shown are the fold increase in the % of apoptosis in CD90(+) cells relative to the % in CD90(-) cells (mean ± SEM, n= 6, *; $p<0.05$). **(B)** Human primary leukemic cells from AML patients were analyzed for apoptosis in CD90 (+) and CD90 (-) subsets in response to Ara-C (mean ± SEM, n= 6, *; $p<0.05$). **(C)** Murine leukemic cells were analyzed for % apoptosis in Sca-1(+) or Sca-1(-) subsets in response to Ara-C (100 nM for MN1 leukemia, 30 nM for H9M1, 500 nM for C1498 leukemic cells) (mean ± SEM, n= 6, *; $p<0.05$).

Supplementary Figure S12



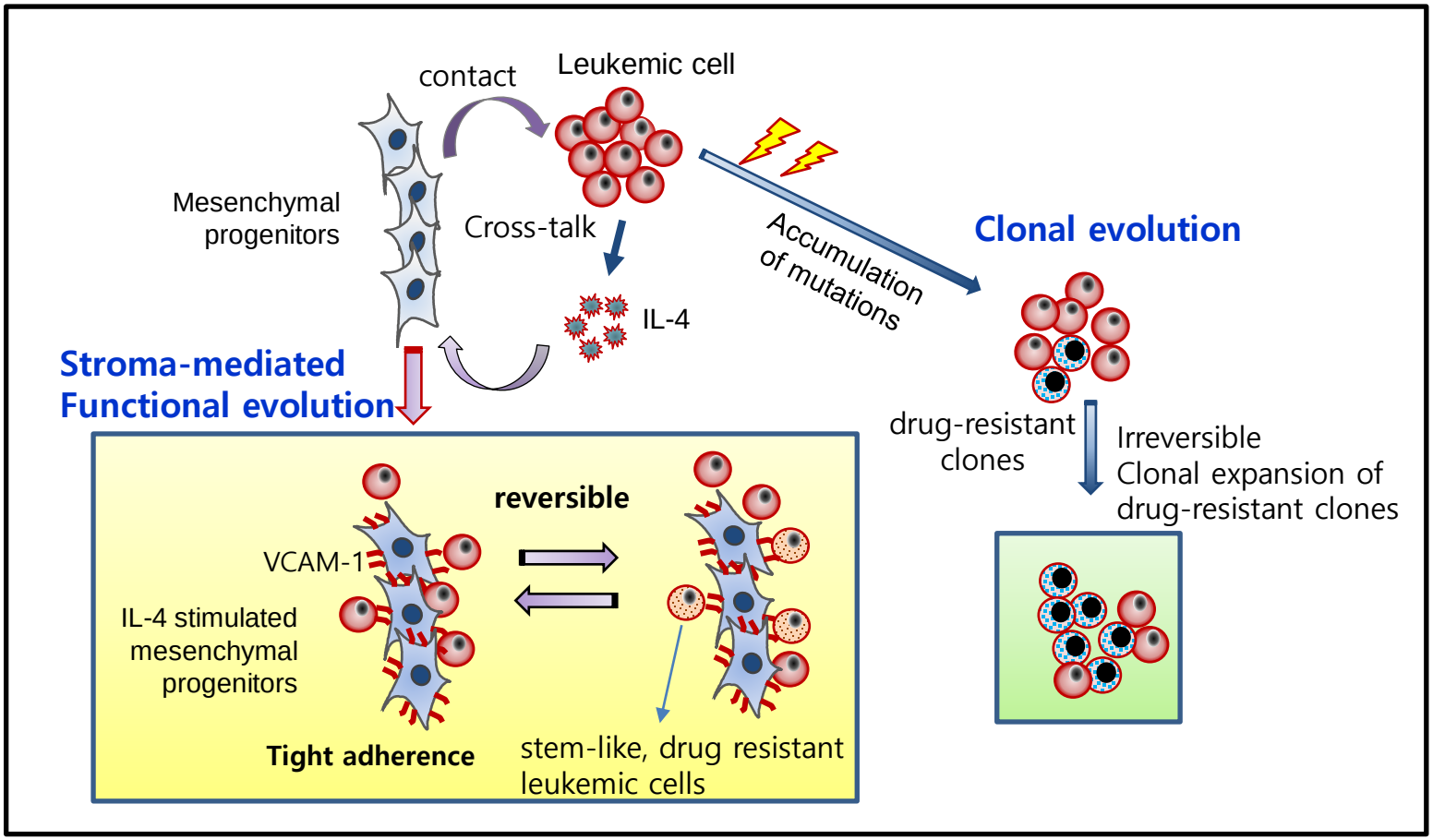
Supplementary Figure S12. Effects of IL-4 on the apoptosis of leukemic subsets. Murine or human leukemic cells were co-cultured with MSCs in presence or absence of recombinant IL-4. Apoptosis of each indicated leukemic subsets were measured by % Annexin V+ cells. Shown are the relative increase of % apoptosis in each subsets by IL-4 treatment compared to control (un-treated) group for murine **(A)** and human **(B)** leukemic cell model (mean ± SEM, n=3). Note no significant increase of apoptosis by IL-4 treatment in each subset.

Supplementary Figure S13



Supplementary Figure S13. Effects of blocking adhesion molecules on the stroma-dependent generation of stem-like leukemic subsets and chemoresistance. **(A, B)** MN1 leukemic cells were cultured under stroma-free (SF) conditions or co-cultured with MSCs in the presence of indicated doses of molecules blocking cellular adhesion (AMD3100 for CXCR4, anti-CD44 antibody for blocking CD44). Shown are the fold increases of LSK or LK cell numbers in the presence AMD3100 (A) or anti-CD44 antibody (B) (n=2). Note that LSK generation by stroma were not influenced by blocking CXCR4 or CD44. **(C, D)** Effects of blocking cellular interaction of leukemic cells with stromal cells on chemoresistance. MN1 leukemic cells were co-cultured with MSCs and treated with Ara-C in the presence or absence of blocking CD44 ligand (anti-CD44 antibody) or CXCR4 (AMD3100, the chemical inhibitor of CXCR4 signals). Shown are the fold changes of viable cell numbers in comparison to drug-untreated (mock) control (n= 6, 4 expts). Note no difference in cell protection by blocking CD44 or CXCR4-mediated cellular interaction (p>0.05)

Supplementary Figure S14



Supplementary Figure S14. Hypothetical model for additional type of drug-resistance in leukemic cells induced by microenvironmental cross-talk. Cellular interaction with mesenchymal stromal cells induce IL-4 in leukemic cells, which, in-turn, targets mesenchymal stromal cells. The IL-4 stimulated mesenchymal cells up-regulates VCAM-1(+) cells in mesenchymal progenitor cells for tight adherence of leukemic cells facilitating development of stem-like, drug-resistant subsets of leukemic cells. The acquisition of drug-resistant leukemic clones by microenvironmental cross-talk is reversible, stochastic process, representing a functional evolution of leukemic cells without clonal evolution driven by accumulated mutation.

Supplementary Methods

Cell lines and leukemia cell culture

C1498 cells (mouse myeloid leukemia cell line) were cultured in high glucose Dulbecco's Modified Eagle Medium (Hyclone, Waltham, MA, USA) supplemented with 10% FBS (Hyclone) and 1% (v/v) anti-biotics and anti-mycotics (Gibco, Grand Island, NY, USA), 2mM L-glutamin (Gibco). HL-60 cells (human promyelocytic leukemia cell line) and MOLM-14 (human acute monocytic leukemia cell line, AML-M5a) were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 1% (v/v) anti-biotics and anti-mycotics, 2mM L-glutamin. MV4-11 (human biphenotypic B myelomonocytic leukemia cell line) were cultured in IMDM (Gibco) supplemented with 10% FBS and 1% (v/v) anti-biotics and anti-mycotics, 2mM L-glutamin. C1498, HL-60, MV4-11 cell lines were obtained from ATCC (ATCC, Rockville, MD, USA) and MOLM-14 cell line was obtained from DSMZ (DSMZ, Braunschweig, Germany). Murine leukemic cells (MN1 or HoxA9/Meis1) were cultured in DMEM supplemented with 15% FBS, 20ng/ml of mouse stem cell factor (mSCF, Peprotech, Rehovot, Israel), 10ng/ml of human interleukin-6 (hIL-6, Peprotech, Rehovot, Israel), 6ng/ml of mouse interleukin-3 (mIL-3, R&D Systems Inc., Minneapolis, Minnesota, USA). Human primary AML cells were cultured in long-term culture media (H5100, STEMCELL Technologies) in the presence of a cytokine mixture (100 ng/mL human SCF; 100 ng/mL humanFlt3L; and 20 ng/mL human IL3, IL6, and G-CSF; ProSpec-TanyTechnoGene Ltd.).

Human and murine MSCs

Fresh, uncultured murine MSCs ($CD45^-31^-Ter119^-$) were analyzed by flowcytometry. Subsets of murine MSCs capable of colony formation (CFU-F) were analyzed by subsets of fresh MSCs expressing corresponding surface markers ($PDGFR^+CD51^+$, $PDGFR^+Sca\text{-}1^+$, or $CD44^-$). Fresh uncultured human MSCs ($CD45^-31^-235a^-$) were similarly analyzed by flowcytometry from BM samples. Clonogenic MSCs that are enriched with CFU-F were analyzed by additional marker ($CD146^+166^-$).

For in-vitro cultured MSCs, BM mononuclear cells from normal donors (allogenic) were separated by Ficoll-Paque^{TM} PLUS (GE Healthcare, Uppsala, Sweden), plated in the Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Non-adherent cells were discarded after one week and adherent populations were sub-cultured.

RT-PCR and ELISA

For detection at the transcriptional level of cytokines and receptors in leukemia cells, total RNA was extracted from leukemic cells using Trizol (Invitrogen). cDNA was synthesized from 1μg of total RNA with reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN). For detection at the transcriptional level of cytokines or receptors, RT-PCR was performed using Taq polymerase (TaKaRa, Otsu, Japan).

To detect protein levels of IL-4 in supernatant, non-adherent leukemic cells were removed after overnight culture with fresh medium changes and co-cultured for 3 days. Supernatant were measured for IL-4 using the murine or human cytokine-specific ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc) according to the instruction.

Mitochondrial transfer assay

To measure mitochondria transfer during co-culture, mouse MSCs were stained with 300nM of MitoTracker Red CMXRox (Invitrogen, USA) for 30 minutes, washed twice with PBS, and leukemic cells were seeded onto the MSCs for 3 days and analyzed for MitoTracker fluorescence at 635 nm wavelength by flow cytometry.

***In-vivo* repopulation and limiting dilution assay**

To quantitatively measure the number of leukemia initiating cells (LICs), in-vivo limiting dilution assay was performed by transplanting serially diluted cells into recipient mice as described(60). Briefly, LIC frequency was calculated by determining the cell dose that can result in negative engraftment (GFP <0.1%) in 37% of the mice tested. LIC frequencies and 95% confidence intervals (CI) representing ±2 SEM were calculated by using ELDA software (http://bioinf.wehi.edu.au/software/elda/). For leukemic cell engraftment in *bis* disrupted mice, cells were transplanted into irradiated recipient mice at postnatal day 2 and repopulation was analyzed 2 weeks after transplantation by flowcytometry.

Cell cycle

Cell cycles of leukemic cells were analyzed by staining the leukemic cells with 10 μmol/L Hoechst33342 at 37°C for 45 minutes, and Pyronin Y was then added to give a final concentration of 2.5 μg/mL, followed by an additional 15 minutes incubation at room temperature. Cell cycling of specific subsets of leukemic cells were determined by gating each specific phenotypes of leukemic cells in the flowcytometry (LSRII).