Acute lymphoblastic leukemia cells create a leukemic niche without affecting the CXCR4/CXCL12 axis

Bob de Rooij,[1] Roel Polak,[1] Lieke C.J. van den Berk,[1] Femke Stalpers,[1] Rob Pieters[2] and Monique L. den Boer[1]

[1]Department of Pediatric Oncology, Erasmus MC, Sophia Children's Hospital, Rotterdam and [2]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

BdR and RP contributed equally to this work.

Correspondence: m.l.denboer@erasmusmc.nl

doi:10.3324/haematol.2016.159517

Acute lymphoblastic leukemia cells create a leukemic niche without affecting the CXCR4/CXCL12 axis

Bob de Rooij,[1] Roel Polak,[1] Lieke C.J. van den Berk,[1] Femke Stalpers,[1] Rob Pieters[2] and Monique L. den Boer[1]

[1]Department of Pediatric Oncology, Erasmus MC, Sophia Children's Hospital, Rotterdam and [2]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

BdR and RP contributed equally to this work.

Correspondence: m.l.denboer@erasmusmc.nl

doi:10.3324/haematol.2016.159517

SUPPLEMENTARY DATA

Acute lymphoblastic leukemia cells create a leukemic niche without affecting the CXCR4/CXCL12 axis

Authors: Bob de Rooij*, Roel Polak*, Lieke C.J. van den Berk, Femke Stalpers, Rob Pieters & Monique L. den Boer. * Authors contributed equally.

MATERIALS AND METHODS

Cell lines and reagents

B-cell precursor ALL (BCP-ALL) cell lines, NALM6 (B-Other) and REH (TEL-AML1), were obtained from DSMZ (Braunschweig, Germany), used at low cell passages, and routinely verified by DNA fingerprinting. Cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS), 1% penicillin-streptomycin at 37 °C and 5% CO_2.

Isolation of primary BCP-ALL leukemic blasts from patients

Primary BCP-ALL leukemic blasts were isolated from patients as described earlier (Polak *et al.* Blood 2015). In short, mononuclear leukemic cells were collected from bone marrow aspirates obtained from children with newly diagnosed BCP-ALL prior to treatment. All samples used in this study contained > 95% leukemic blasts.

Isolation and characterization of primary MSCs

Primary MSCs were isolated and characterized using positive (CD44/ CD90/ CD105/ CD54/ CD73/ CD146/ CD166/ STRO-1) and negative surface markers (CD19/ CD45/ CD34)(8). In addition multilineage potential was confirmed as described earlier (Polak *et al.* Blood 2015).

Isolation of $CD34^+$ cells

$CD34^+$ cells were obtained from umbilical cord blood using the Direct CD34 Progenitor Cell Isolation kit, human (Miltenyi Biotec, Gladback, Germany). $CD34^+$ cells were positively selected by magnetic microbeads using the MACS LS column (Miltenyi Biotec) in combination

with a MACS separator (Miltenyi Biotec). The purity of the isolated cells was confirmed by flow cytometry using the CD34-PE fluorescent antibody (BD Pharmingen, San Diego, USA).

Collection of bone marrow aspirates

Serum samples of bone marrow aspirates were collected from children with ALL at initial diagnosis and after completion of two courses of chemotherapy according to the ALL-10 protocol of the Dutch Childhood Oncology Group (day 79 after start of treatment) as previously described (van den Berk *et al.* Br J Haemat 2014). This study was approved by the Institutional Review Board and informed consent was obtained from parents or guardians.

Cell viability assays

Cell viability assays of primary material was performed as earlier described (Polak *et al.* Blood 2015). In short, primary patient cells (1 x 10^6 cells) were co-cultured with or without primary stromal cells (5 x 10^4) for five days in a 24-well plate at 37 °C and 5% CO_2. Stromal cells were allowed to attach prior to the start of an experiment. Before the start of each experiment, leukemic cells were screened for CD19 positivity (Brilliant Violet 421 anti-human CD19 antibody), which was used to discriminate leukemic cells from $CD19^{neg}$ MSCs. The percentage of viable leukemic cells was determined after 5 days of culture by staining with Brilliant Violet 421 anti-human CD19 (Biolegend), FITC Annexin V (Biolegend), and Propidium Iodide (PI; Sigma), after which the percentage of $AnnexinV^{neg}/PI^{neg}/CD19^{pos}$ cells within the MSC negative fraction was determined by flow cytometry (BD Biosciences).

Multiplexed fluorescent bead-based immunoassay (Luminex)

Primary leukemic cells were co-cultured with different sources of primary MSCs for 5 days at 37°C and 5% CO_2. Supernatant was collected upon which the viability of leukemic cells was assessed as described above. The concentration of 64 known cytokines/chemokines in these supernatants was analyzed using fluorescent bead-based immunoassay (Luminex Human Cytokine/Chemokine Panel I and II; Merck Millipore) according to the manufacturer's protocol. Serum of bone marrow aspirates of leukemia patients or healthy controls were used to determine the concentration of 64 known cytokines/chemokines using the same fluorescent bead-based immunoassay (Luminex Human Cytokine/Chemokine Panel I and II; Merck Millipore) according to the manufacturer's protocol

BCP-ALL migration assay

BCP-ALL migration experiments were performed using RPMI 10% FCS at 37°C and 5% CO_2. 5 x 10^4 primary MSCs and 2 x 10^5 BCP-ALL cells were cultured either separate or in co-culture in the lower compartment of a transwell in a volume of 750 µL for 24 hours prior to the start of the experiment. At the start of experiment, 4 x 10^5 NALM6-GFP cells in a volume of 250 µL were added to the upper compartment of a 6.5 mm diameter transwell system (Corning, NY, USA) with a pore size of 3.0 µm. Cells were allowed to migrate for 48 hours. At the end of experiment, transwells were removed and cells in the bottom compartment were harvested and stained with DAPI (Life Technologies). $DAPI^-/GFP^+$ cells were used as a measure for cell migration and quantified by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec, Gladbach, Germany).

$CD34^+$ migration assay

Experiments were performed using either RPMI Dutch Modified 15%FCS or Iscove modified Dulbecco medium (Gibco) containing 10% FCS, 50 µM β-mercaptoethanol, 1% penicillin-streptomycin, 2 mM glutamine, stem cell factor (SCF; 50 ng/mL; Peprotech) and fms-like tyrosine kinase-3 ligand (Flt3L; 50 ng/mL; Peprotech) at 37°C and 5% CO_2. Primary MSCs (5 x 10^4) and NALM6-GFP cells (2 x 10^5) were cultured either separate or in co-culture in a volume of 750 µL for 24 hours prior to start of experiment (bottom compartment). At the start of experiment, 5 x 10^5 $CD34^+$ cells in a volume of 250 µL were added to the top chamber of a 3.0 µm transwell system (Corning, NY, USA) and allowed to migrate for 48 hours at 37°C and 5% CO_2. At the end of experiment, transwell inserts were removed upon which the cells in the bottom compartment were harvested and stained with Propidium Iodide (PI; Sigma) and Brilliant Violet 421 anti-human CD45 antibody (Biolegend). $PI^-/GFP^-/CD45^+$ cells were quantified by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec, Gladbach, Germany).

MSC migration assay

Experiments were performed using DMEM medium containing 5% FCS, 1% penicillin-streptomycin at 37°C and 5% CO_2. REH cells (0.1 – 1.6 x 10^6) and primary MSCs (5 x 10^4) were cultured either separate or in co-culture in a volume of 750 µL for 48 hours prior to start of experiment. At the start of experiment, 5 x 10^4 MSCs were added to the top chamber of a 8.0 µm transwell system (Corning) and allowed to migrate through the membrane overnight (12-16 hours). Non-migrated cells were removed from the upper side of the transwell insert using a cotton swab. Cells that migrated through the membrane insert were fixed using 10% Formalin (Sigma) for 10 minutes, washed with PBS and stained for 30 minutes with Crystal violet at room

temperature. Images were captured using a Leica DM IL microscope mounted with a Leica DFC430C camera (Leica, Germany). Next, Crystal violet contained by MSCs was dissolved in 150 μL methanol, quantified by spectrophotometer using 540-570 nm wavelength settings (Versamax, San Francisco) and used as a measure of migration.

Statistical analysis

We used the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) to visualize gene expression data in supplemental Figure 4 and performed one way analysis of variance (ANOVA). For other statistics, Student's t-test was used and a Student's paired t-test was used when applicable. Bar graphs represent the mean of biological replicates. Error bars show standard error of the mean (SEM).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. ALL-MSC co-cultures induce leukemic cell migration

(**A**) Schematic overview of the experimental conditions used for experiments shown in Figure 1D-E. The migration of GFP-positive NALM6 cells is measured toward: culture medium (Control), primary MSCs (Normal Niche) or GFP-negative ALL-MSC co-cultures (Leukemic Niche).

(**B**) Representative microscope images depicting migrated GFP-positive NALM6 cells after 48 hours in a 3.0 μm transwell system. GFP-positive NALM6 cells were allowed to migrate toward a bottom compartment containing either culture medium, GFP-negative NALM6 cells or primary MSCs (n = 4).

(**C**) Gating strategy for quantification of migrated GFP-positive NALM6 cells.

(**D**) Representative flow cytometry plots showing the migration of GFP-positive NALM6 cells toward GFP-negative NALM6 cells, MSCs and GFP-negative ALL-MSC co-cultures.

(**E**) Representative microscope images showing the migration of GFP-positive NALM6 cells toward GFP-negative ALL-MSC co-cultures (right panel) or migration to a bottom compartment containing only MSCs (left panel; n = 4). No LCs = no leukemic cells in the bottom compartment.

(**F**) Graph showing the effect of CXCR4 inhibition by AMD3100 (10 μM) on migration of NALM6 cells towards MSC mono-cultures (normal niche, grey bars) and ALL-MSC co-cultures (leukemic niche, blue bars). Migration towards culture medium is used to calculate the fold-change migration. CXCR4 inhibition decreases migration of NALM6 cells towards the healthy and leukemic niche in a similar manner. However, the leukemic niche still induces migration of NALM6 cells in the presence of AMD3100.

Data are means ± SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, N6 = NALM6.

See also Figure 1.

Supplementary Figure 2. BCP-ALL cells recruit MSCs and stop recruiting after the leukemic niche is established

(**A**) Images of a representative experiment showing MSCs that migrated to the other side of a 8.0 μm transwell insert for 16 hours. Cells were fixed and subsequently stained with crystal violet. Images show MSCs migrated toward a bottom compartment containing culture medium, MSCs or BCP-ALL cells (REH) (n = 4).

(**B**) Schematic overview of experimental conditions used in Figure 1I and supplementary Figure 2C.

(**C**) Images of a representative experiment showing that MSCs migrate to the other side of a 8.0 μm transwell insert for 16 hours toward an increasing amount of BCP-ALL cells (REH) in mono-culture (upper panels) or in co-culture with MSCs (MSC + REH; lower panels).

See also Figure 1.

Supplementary Figure 3. Cytokine secretion of primary MSCs and BCP-ALL cells in mono-culture

(**A**) Graph showing the cytokine production of MSCs from four donors after 5 days of mono-culture.

(**B**) Graph showing the cytokine production of BCP-ALL cells from 10 different donors after 5 days of mono-culture.

Data was obtained using a multiplexed fluorescent bead-based immunoassay. Secreted levels of 64 cytokines/chemokines were analyzed. Cytokines/chemokines that were not detected in mesenchymal stromal cells or ALL cells were not depicted in this figure. < LOD = below limit of detection.

See also Figure 2.

Supplementary Figure 4. Chemokines and receptors upregulated in the BCP-ALL niche

(**A**) Plots showing the secretion of CXCL10 (IP10) by primary MSCs and primary BCP-ALL cells in mono-culture (circles represent the sum of cytokine secretion in mono-culture of BCP-ALL and mono-culture of MSC) compared to ALL-MSC co-cultures (squares). Data was obtained using a multiplexed fluorescent bead-based immunoassay. Boxes represent p25-p75 intervals. Raw data was logarithmically transformed to obtain a normal distribution of the data and upregulation of cytokines was tested using a one-tailed paired t-test * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

(**B**) Plot showing the serum levels of CXCL10 (IP10) in bone marrow aspirates from healthy controls (n = 7, circles), from untreated BCP-ALL patients at diagnosis (n = 10, blue squares) and from BCP-ALL patients after induction treatment (n = 10, triangles; lines indicate paired samples). Data was obtained using a multiplexed fluorescent bead-based immunoassay in which we measured the levels of 64 cytokines. Healthy (circles) and leukemic samples (blue squares) were compared using a two-tailed unpaired t-test. Leukemic samples before and after induction therapy (blue squares versus triangles) were compared using a one-tailed paired t-test * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

(**C**) Gene expression levels of the CCR4, CXCR1, CXCR2 and CXCR3 receptors in healthy hematopoietic cells (Andersson et al. BMC Med Genomics. 2010) and pediatric ALL cells (Den Boer et al. Lancet Oncol. 2009). Data was visualized using R2: microarray analysis and visualization platform.

(**D-E**) Graphs showing that the increase in patient-specific cytokines was not the effect of induced cellular survival in ALL-MSC co-cultures.

(**D**) Graph showing the viability of leukemic cells from patient ALL#4 cultured in absence or in presence of primary MSCs.

(**E**) Plots showing the secretion of cytokines by BCP-ALL cells from patient ALL#4 in mono-culture (grey circles) and the secretion of cytokines by ALL#4 cells in ALL-MSC co-culture corrected for the secretion of MSCs (grey boxes). The dashed boxes indicate significantly upregulated cytokines shown in Figure 2.

See also Figure 2.

Cytokines tested	Receptor
VEGF	VEGFR
sCD40L	C40; αIIbß3; α5ß1
EGF	EGFR
Eotaxin	CCR2; CCR3; CCR5
FGF-2	FGFR1
Flt-3 ligand	Flt-3
Fractalkine	CX3CR1
G-CSF	GCSFR
GM-CSF	GMCSFR
GRO	CXCR2
IFNα2	IFNAR
IFN-γ	IFNGR1; IFNGR2
IL-1α	IL1R
IL-1ß	IL1R
IL-1ra	IL1R
IL-2	IL2R
IL-3	IL3R
IL-4	IL4R
IL-5	IL5R
IL-6	CD126 and CD130 complex
IL-7	IL7R
IL-8	CXCR1; CXCR2
IL-9	IL9R
IL-10	IL10R1-IL10R2 complex
IL-12 (p40)	IL12R
IL-12 (p70)	IL12R
IL-13	IL13R
IL-15	IL15RaR
IL17A	IL17R
IP-10	CXCR3
MCP-1	CCR2 and CCR4
MCP-3	CCR2

Cytokines tested	Receptor
MDC (CCL22)	CCR4
MIP-1α	CCR1
MIP-1ß	CCR1;CCR5
PDGF-AA	PDGFR
PDGF-AB/BB	PDGFR
RANTES	CCR5
TGF-α	EGFR
TNF-α	TNFR1
TNF-ß	LTB
TSLP	CRLF2 and IL7-R complex
6Ckine	CCR7
BCA-1	CXCR5
CTACK	CCR10
ENA-78	CXCR2
Eotaxin-2	CCR3
Eotaxin-3	CCR3
I-309	CCR8
IL-16	CD4
IL-20	IL20R
IL-21	IL21R
IL-23	IL23R
IL-28A	IL28R
IL-33	IL1RL1
LIF	LIFR
MCP-2	CCR1; CCR2a; CCR5
MCP-4	CCR2; CCR3; CCR5
MIP-1d	CCR1; CCR3
SCF	c-Kit
CXCL12	CXCR4; CXCR7
TARC	CCR4
TPO	CD110
TRAIL	TRAILR-complex

Supplementary Table 1. Cytokine and chemokine screening panel

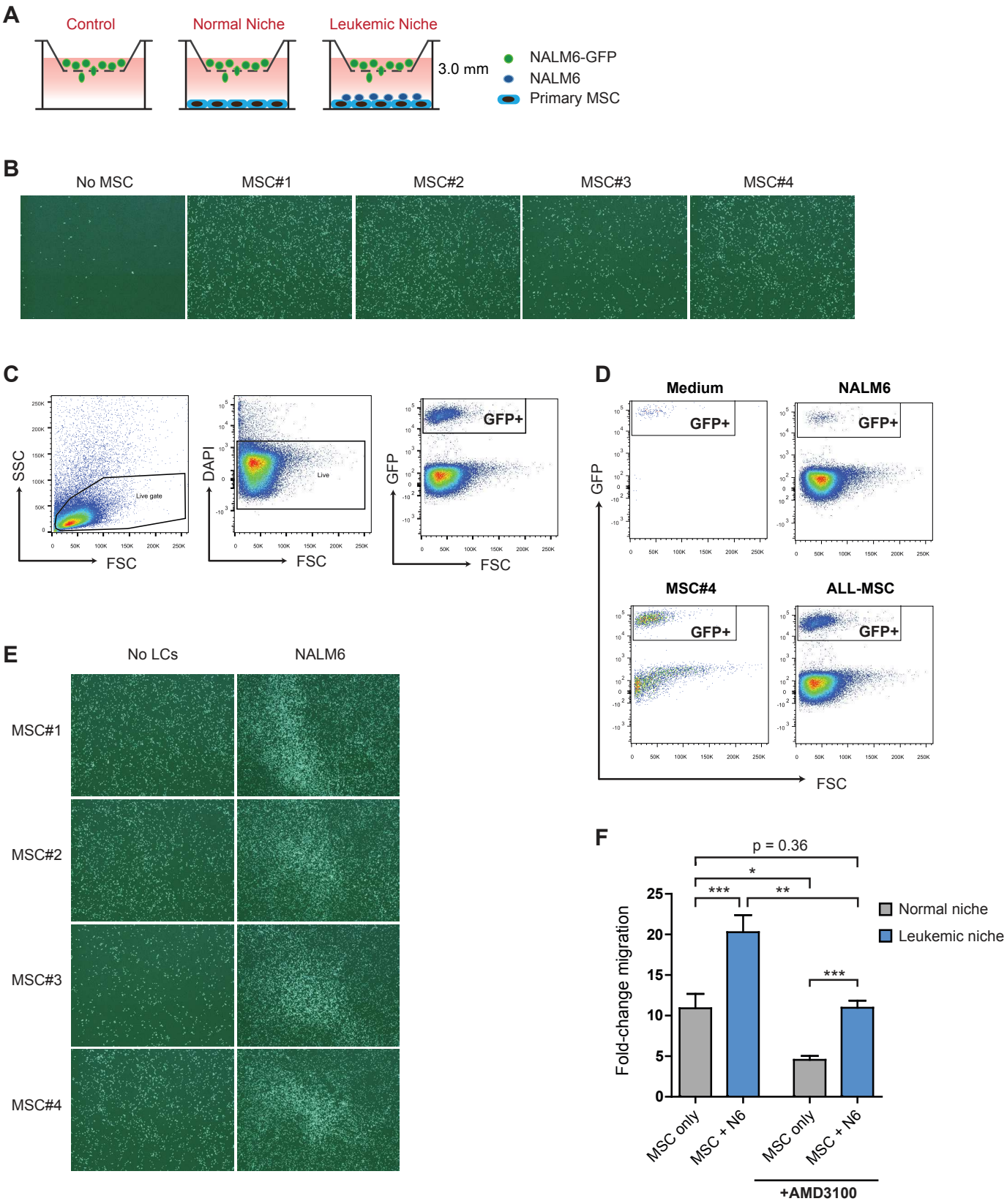
	ID	G-CSF CSF3	GM-CSF CSF2	GRO CXCL1	IP10 CXCL10	MCP-1 CCL2	MCP-3 CCL8	MDC CCL22	ENA-78 CXCL5	I-309 CCL1	IL-6	IL-8 CXCL8
BCR-ABL1	#1											
	#2											
	#3											
ETV6-RUNX1	#4											
	#5											
	#6											
MLL	#7											
	#8											
TCF3-PBX1	#9											
	#10											

Supplementary Table 2. Overview of cytokines upregulated in BCP-ALL/MSC co-cultures

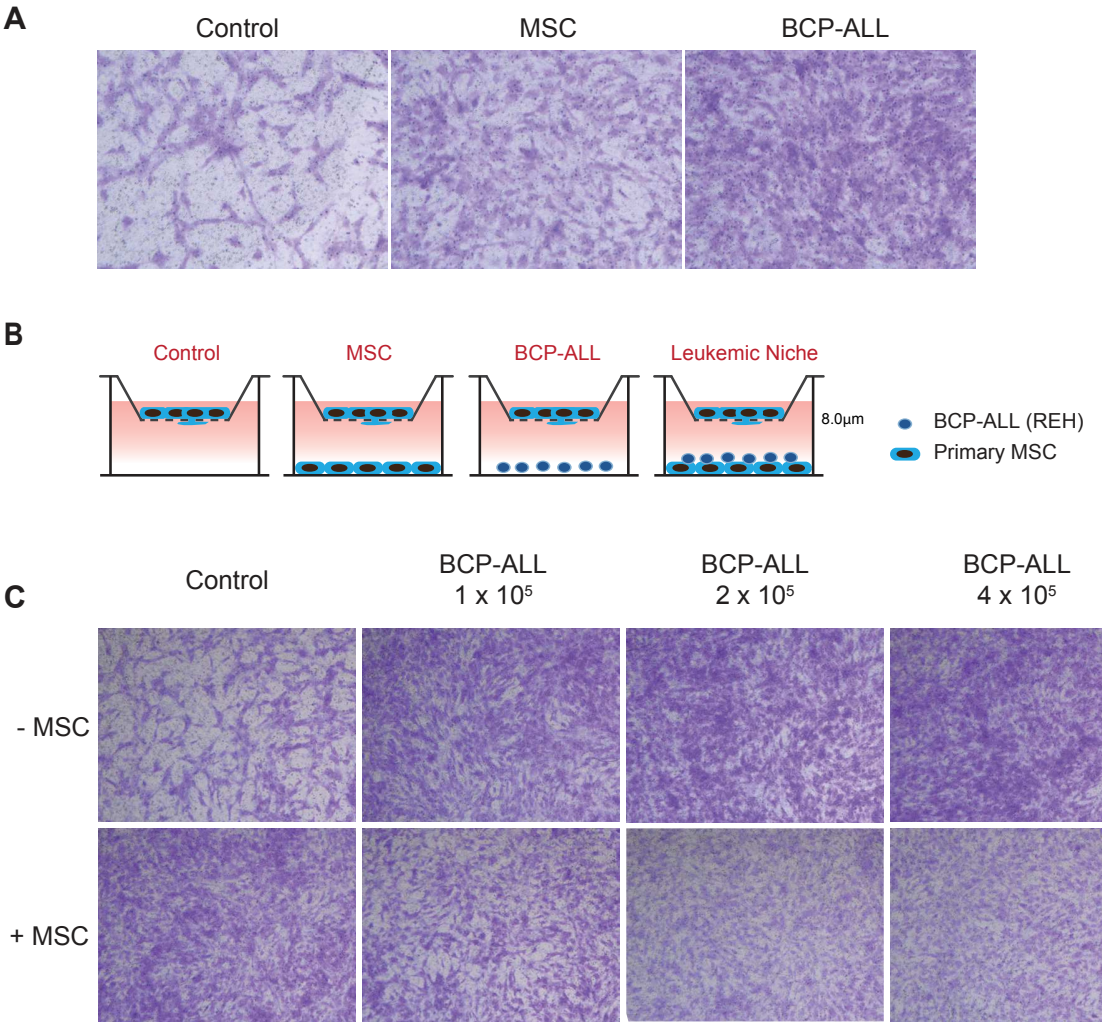
Name	Derived from	Subtype
MSC #1	Healthy BM	-
MSC #2	Healthy BM	-
MSC #3	Leukemic BM	B-Other
MSC #4	Leukemic BM	ETV6-RUNX1

Supplementary Table 3. Primary mesenchymal stromal cells used in this study

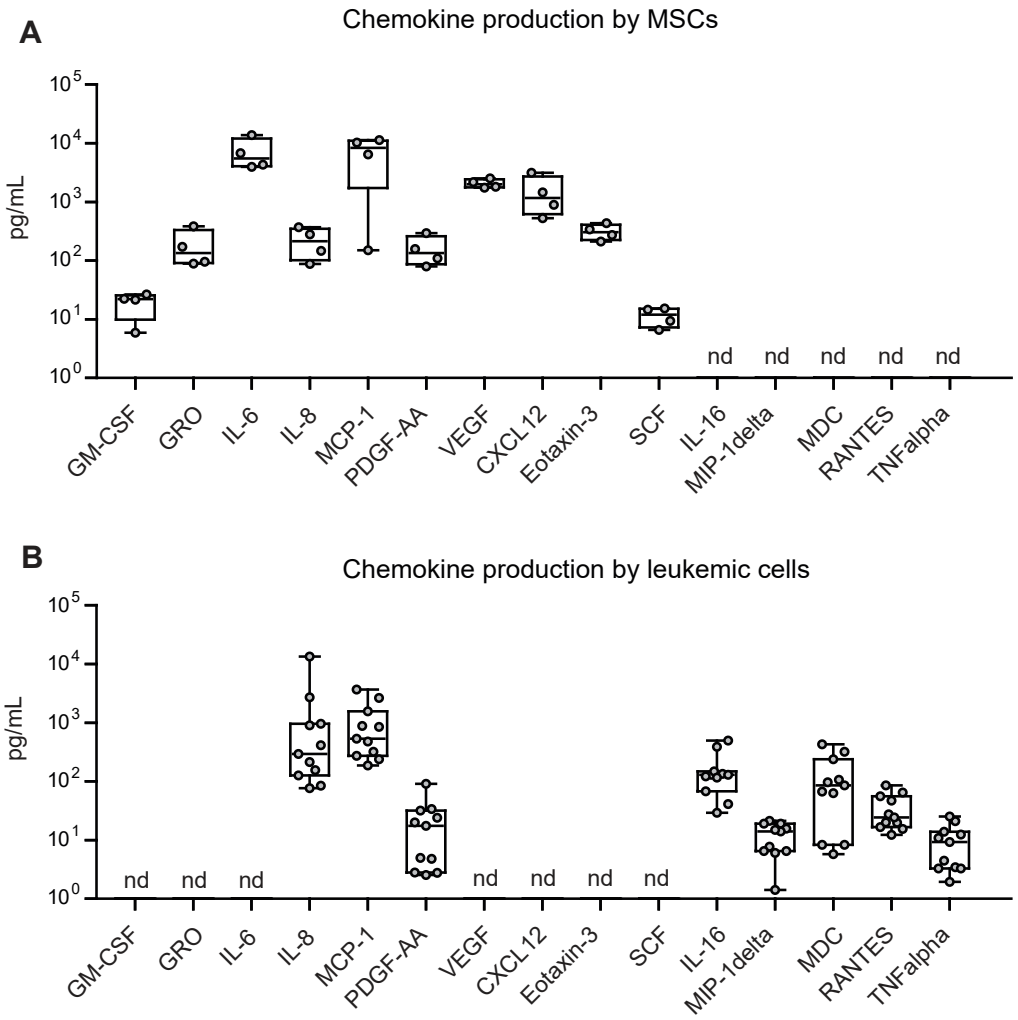
Supplementary Figure 1. BCP-ALL cells create a self-reinforcing niche with mesenchymal stromal cells



Supplementary Figure 2. BCP-ALL cells recruit MSCs and stop recruiting after the niche is established



Supplementary Figure 3. Cytokine secretion of MSC and ALL cells in mono-culture



Supplementary Figure 4. Chemokines and receptors upregulated in the BCP-ALL niche