# B-cell precursor acute lymphoblastic leukemia elicits an interferon-α/β response in bone marrow-derived mesenchymal stroma

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## Abstract

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) can hijack the normal bone marrow microenvironment to create a leukemic niche which facilitates blast cell survival and promotes drug resistance. Bone marrow-derived mesenchymal stromal cells (MSC) mimic this protective environment in *ex vivo* co-cultures with leukemic cells obtained from children with newly diagnosed BCP-ALL. We examined the potential mechanisms of this protection by RNA sequencing of flow-sorted MSC after co-culture with BCP-ALL cells. Leukemic cells induced an interferon (IFN)-related gene signature in MSC, which was partially dependent on direct cell-cell signaling. The signature was selectively induced by BCP-ALL cells, most profoundly by *ETV6-RUNX1*-positive ALL cells, as co-culture of MSC with healthy immune cells did not provoke a similar IFN signature. Leukemic cells and MSC both secreted IFNα and IFNβ, but not IFNγ. In line, the IFN gene signature was sensitive to blockade of IFNα/β signaling, but less to that of IFNγ. The viability of leukemic cells and level of resistance to three chemotherapeutic agents was not affected by interference with IFN signaling using selective IFNα/β inhibitors or silencing of IFN-related genes. Taken together, our data suggest that the leukemia-induced expression of IFNα/β-related genes by MSC does not support survival of BCP-ALL cells but may serve a different role in the pathobiology of BCP-ALL.

# Supplementary tables

**Supplementary Table S1. Characteristics of mesenchymal stromal cells derived from pediatric BCP-ALL patients and healthy donors.**

| Mesenchymal stromal cells | Subtype ALL | Remark |
|---|---|---|
| **MSC#1** | *ETV6-RUNX1* | Relapse |
| **MSC#2** | Hyperdiploid | Relapse |
| **MSC#3** | B-other | Initial (diagnosis) |
| **MSC#4** | Healthy bone marrow | Normal |
| **MSC#5** | Healthy bone marrow | Normal |
| **MSC#6** | *ETV6-RUNX1* | Initial (diagnosis) |
| **MSC#7** | *ETV6-RUNX1* | Initial (diagnosis) |
| **MSC#8** | B-other | Initial (diagnosis) |
| **MSC#9** | B-other | Initial (diagnosis) |

## Supplementary Table S2. Characteristics of primary BCP-ALL samples

| Patient characteristics | | | | | | Specifications of used ALL samples | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **ALL #** | **Subtype** | **Remark** | **Gender** | **Age (years) at diagnosis** | **Risk Group** | **Blast %*** | **Other cell types *** | **Viability after thawing (%) **** | **Viability after mono-culture (%) ***** | **Viability after co-culture (%) ****** |
| **1** | B-other | *ETV6-RUNX1*-like | F | 3 | High-risk | 96 | 3% lymphocytes/1% metamyelocytes | 81 | 53.2 | 66.2 ± 7.3 |
| **2** | B-other | No fusion detected by RNAseq | M | 2 | High-risk | 99 | 1% lymphocytes | 88.1 | 50.3 | 23.6 ± 13.6 |
| **3** | B-other | *P2RY8-CRLF2* fusion | M | 4 | High-risk | 97 | 2% lymphocytes/1% metamyelocytes | 88.5 | 22.0 | 37.2 ± 10.1 |
| **4** | B-other | *IGH-DUX4*-rearranged | M | 9 | High-risk | 96 | 4% lymphocytes | 80.9 | 63.6 | 61.0 ±36.0 |
| **5** | B-other | *IGH-EPOR*-rearranged | F | 14 | High-risk | 99 | 1% granulocytes | 94.5 | 36.3 | 68.9 ± 8.6 |
| **6** | B-other | *MEF2D-BCL9* fusion | M | 15 | High-risk | 98 | 1% lymphocytes/1% metamyelocytes | 94.8 | 66.0 | 44.6 ± 20.7 |
| **7** | High hyperdiploid | | M | 4 | Medium-risk | 98 | 1% lymphocytes/1% metamyelocytes | 92.3 | 19.2 | 25.8 ± 4.6 |
| **8** | *ETV6-RUNX1* | | M | 4 | ND | 99 | 1% lymphocytes | 93.3 | 27.5 | 34.6 ± 11.0 |
| **9** | *ETV6-RUNX1* | | F | 9 | Standard-risk | 99 | 1% lymphocytes | 91.7 | 52.7 | 53.5 ± 13.0 |
| **10** | *ETV6-RUNX1* | | F | 4 | Medium-risk | 93 | 7% lymphocytes | 96.1 | 64.3 | 70.1 ± 7.0 |
| **11** | *ETV6-RUNX1* | | M | 3 | Medium-risk | 98 | 2% lymphocytes | 95.5 | 70.2 | 64.8 ± 5.3 |
| **12** | *ETV6-RUNX1* | | F | 3 | Standard-risk | 98 | 1% lymphocytes/1% metamyelocytes | 92.1 | 41.7 | 48.0 ± 5.4 |
| **13** | *ETV6-RUNX1* | | F | 4 | Standard-risk | 95 | 4% lymphocytes/1% metamyelocytes | 92.8 | 64.4 | 55.0 ± 16.1 |
| **14** | *ETV6-RUNX1* | | M | 10 | Non-high risk | 94 | 6% lymphocytes | 94.3 | 58.2 | 47.3 ± 4.2 |
| **15** | *ETV6-RUNX1* | | F | 4 | Standard-risk | 90 | 10% lymphocytes | 86.5 | 66.8 | 59.6 ± 6.4 |
| **16** | *ETV6-RUNX1* | | M | 3 | Standard-risk | 95 | 5% lymphocytes | 96.4 | 63.2 | 71.9 ± 1.6 |
| **17** | *ETV6-RUNX1* | | M | 7 | Medium-risk | 98 | 2% lymphocytes | 95 | 58.4 | 74.4 ± 4.7 |

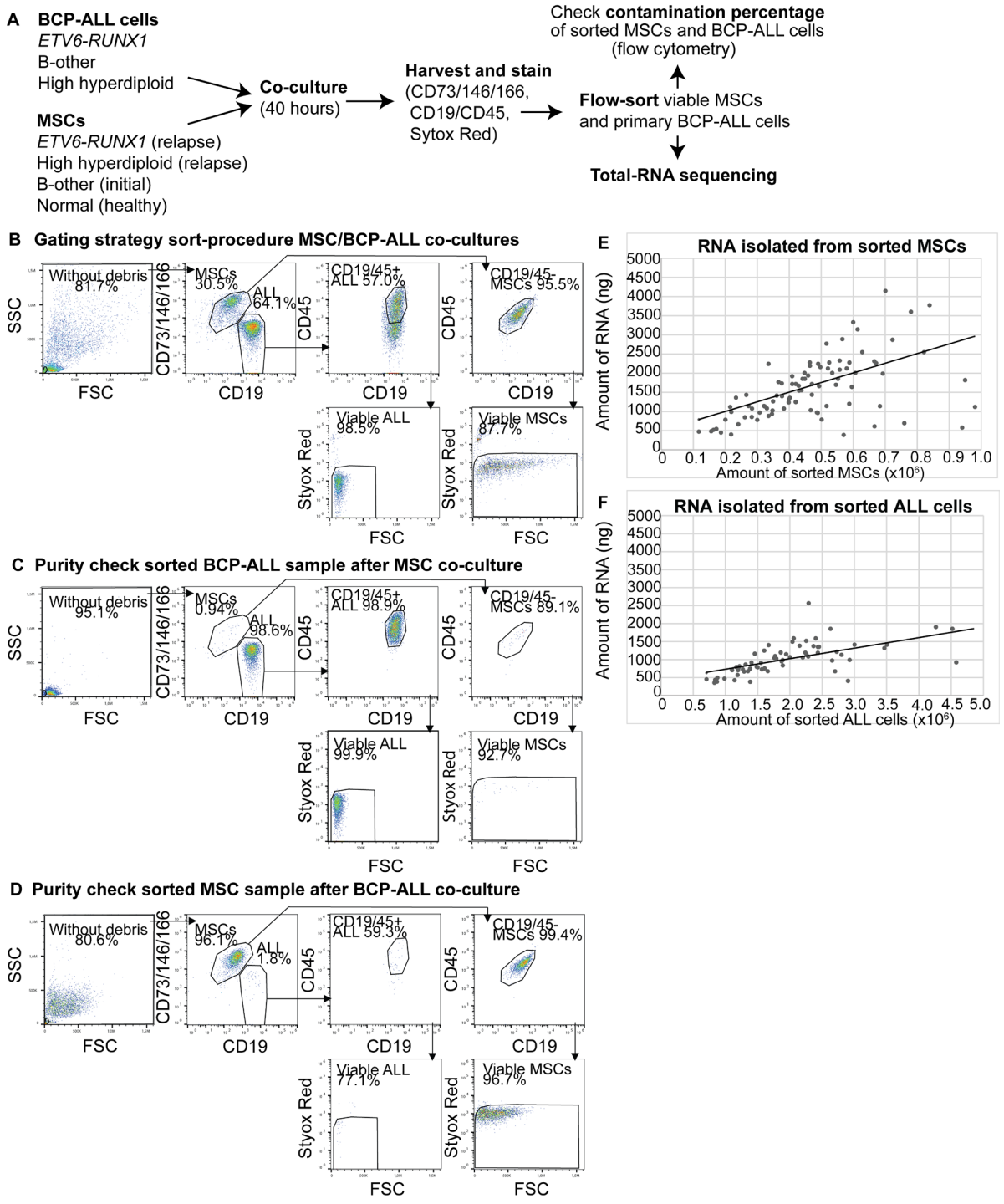
* Percentage of blasts and other cell types in the processed samples used for the described experiments. ** Percentage of viability after thawing of cryopreserved samples. *** Percentage of viable leukemic cells after 40-hour mono-culture. **** Average percentage (±SD) of viable leukemic cells after 40-hour co-culture with MSCs of different origins.

**Supplementary Table S3. Purity of sorted mesenchymal stromal cells and BCP-ALL fractions.** The table summarizes the percentage of ALL cells in sorted MSC fractions and the percentage MSCs in sorted ALL fractions.

| Sample | % ALL | Sample | % MSC |
|---|---|---|---|
| **MSC#1 after ALL#1** | 0.80 | **ALL#1 after MSC#1** | 0.26 |
| **MSC#2 after ALL#1** | 0.82 | **ALL#1 after MSC#2** | 0.26 |
| **MSC#3 after ALL#1** | 1.56 | **ALL#1 after MSC#3** | 0.37 |
| **MSC#1 after ALL#2** | 0.46 | **ALL#2 after MSC#1** | 0.26 |
| **MSC#2 after ALL#2** | 0.60 | **ALL#2 after MSC#2** | 0.50 |
| **MSC#3 after ALL#2** | 0.69 | **ALL#2 after MSC#3** | ND |
| **MSC#1 after ALL#3** | 0.88 | **ALL#3 after MSC#1** | 0.58 |
| **MSC#2 after ALL#3** | 3.65 | **ALL#3 after MSC#2** | 0.47 |
| **MSC#3 after ALL#3** | 0.71 | **ALL#3 after MSC#3** | 0.50 |
| **MSC#1 after ALL#4** | 1.10 | **ALL#4 after MSC#1** | 0.27 |
| **MSC#2 after ALL#4** | 2.80 | **ALL#4 after MSC#2** | 0.11 |
| **MSC#3 after ALL#4** | 1.10 | **ALL#4 after MSC#3** | 0.12 |
| **MSC#1 after ALL#5** | 1.70 | **ALL#5 after MSC#1** | 0.19 |
| **MSC#2 after ALL#5** | 0.50 | **ALL#5 after MSC#2** | 0.13 |
| **MSC#3 after ALL#5** | 2.12 | **ALL#5 after MSC#3** | 0.24 |
| **MSC#1 after ALL#6** | 7.13 | **ALL#6 after MSC#1** | 0.17 |
| **MSC#2 after ALL#6** | 8.60 | **ALL#6 after MSC#2** | 0.17 |
| **MSC#3 after ALL#6** | 17.60 | **ALL#6 after MSC#3** | 0.12 |
| **MSC#1 after ALL#7** | 1.76 | **ALL#7 after MSC#1** | 0.45 |
| **MSC#2 after ALL#7** | 2.00 | **ALL#7 after MSC#2** | 0.20 |
| **MSC#3 after ALL#7** | 1.50 | **ALL#7 after MSC#3** | 0.24 |
| **MSC#1 after ALL#8** | 1.50 | **ALL#8 after MSC#1** | 0.04 |
| **MSC#2 after ALL#8** | 2.77 | **ALL#8 after MSC#2** | 0.19 |
| **MSC#3 after ALL#8** | 3.35 | **ALL#8 after MSC#3** | 0.22 |
| **MSC#1 after ALL#9** | 1.44 | **ALL#9 after MSC#1** | 0.27 |
| **MSC#2 after ALL#9** | 3.40 | **ALL#9 after MSC#2** | 0.10 |
| **MSC#3 after ALL#9** | 1.20 | **ALL#9 after MSC#3** | 0.25 |
| **MSC#1 after ALL#10** | 3.20 | **ALL#10 after MSC#1** | 0.77 |
| **MSC#2 after ALL#10** | 1.50 | **ALL#10 after MSC#2** | 0.21 |
| **MSC#3 after ALL#10** | 2.20 | **ALL#10 after MSC#3** | 0.18 |
| **MSC#1 after ALL#11** | 9.30 | **ALL#11 after MSC#1** | 0.82 |
| **MSC#2 after ALL#11** | 4.60 | **ALL#11 after MSC#2** | 0.39 |
| **MSC#3 after ALL#11** | 0.83 | **ALL#11 after MSC#3** | 0.14 |
| **MSC#1 after ALL#12** | 0.25 | **ALL#12 after MSC#1** | 0.10 |
| **MSC#2 after ALL#12** | 0.47 | **ALL#12 after MSC#2** | 0.77 |
| **MSC#3 after ALL#12** | 0.39 | **ALL#12 after MSC#3** | 0.63 |
| **MSC#1 after ALL#13** | 4.50 | **ALL#13 after MSC#1** | 0.48 |
| **MSC#2 after ALL#13** | 1.99 | **ALL#13 after MSC#2** | 0.45 |
| **MSC#3 after ALL#13** | 0.34 | **ALL#13 after MSC#3** | 0.31 |
| **MSC#1 after ALL#14** | 1.18 | **ALL#14 after MSC#1** | 0.24 |
| **MSC#2 after ALL#14** | 6.92 | **ALL#14 after MSC#2** | 0.14 |
| **MSC#3 after ALL#14** | 2.19 | **ALL#14 after MSC#3** | 0.15 |

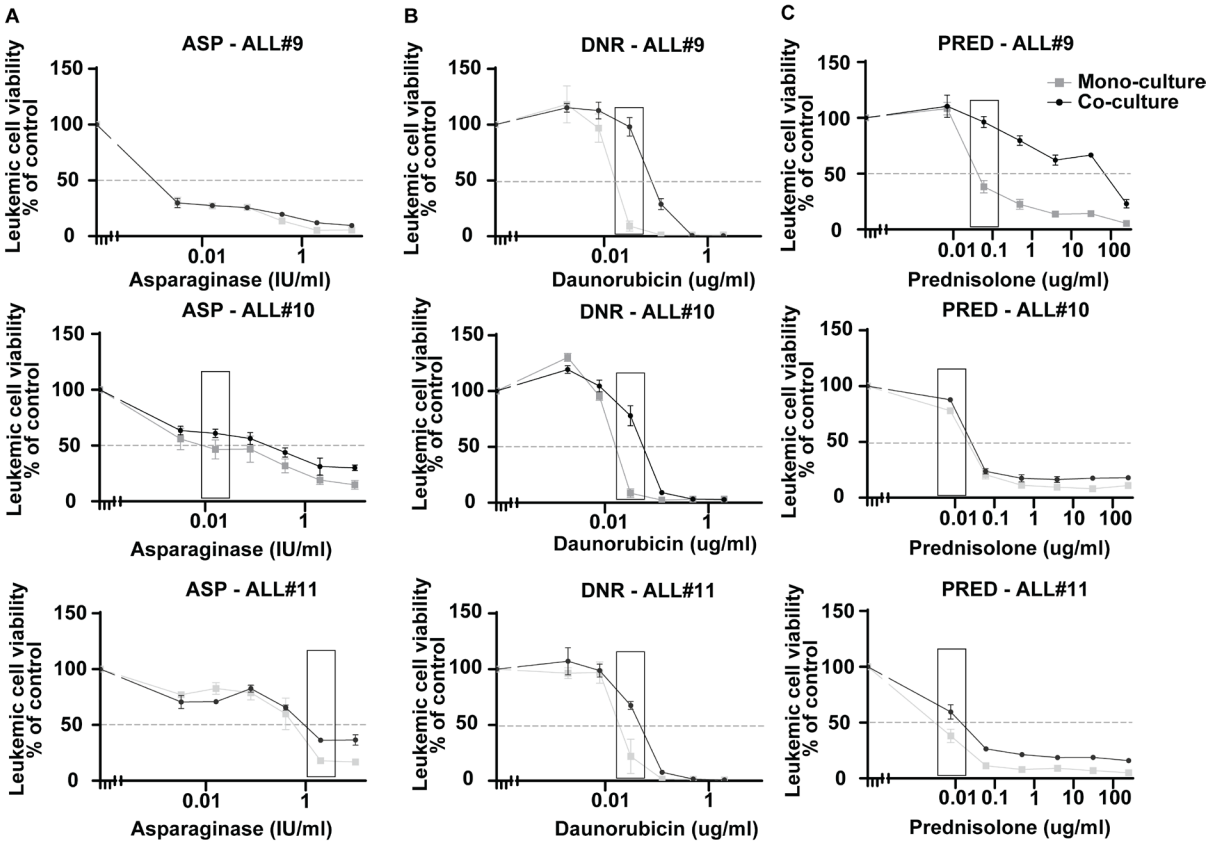
| | | | |
|---|---|---|---|
| **MSC#1 after ALL#15** | 4.10 | **ALL#15 after MSC#1** | 0.71 |
| **MSC#2 after ALL#15** | 3.60 | **ALL#15 after MSC#2** | 0.49 |
| **MSC#3 after ALL#15** | 1.50 | **ALL#15 after MSC#3** | 0.70 |

# Supplementary Figures

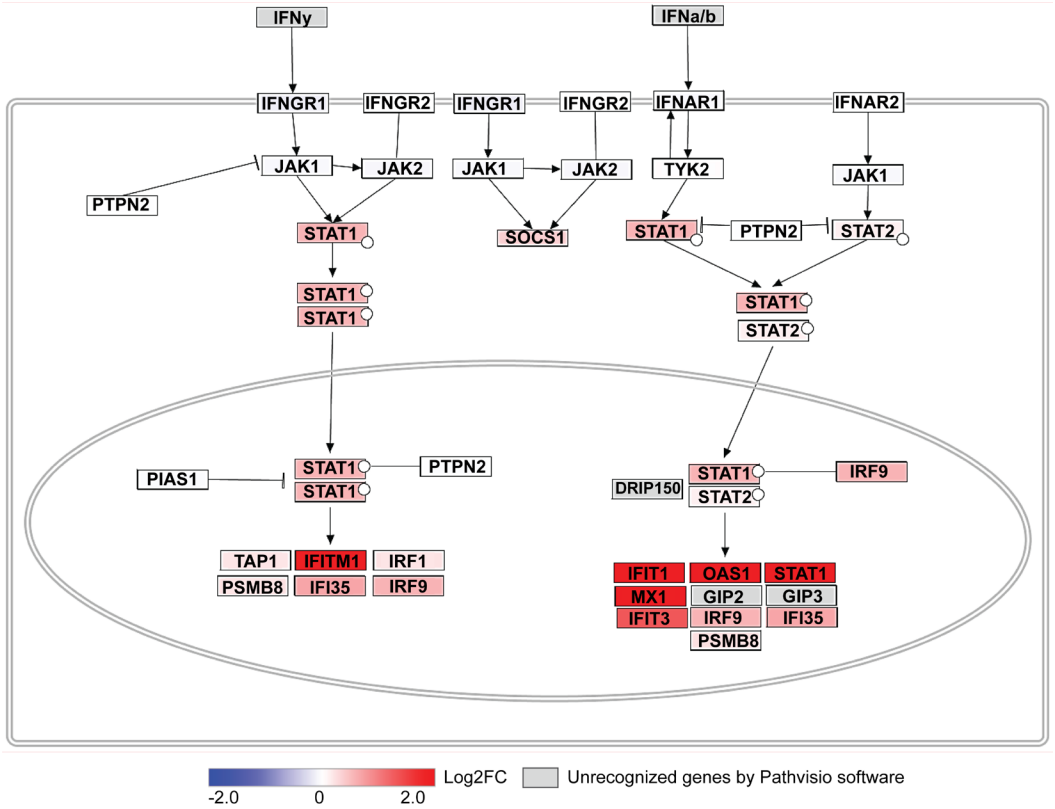



**Supplementary Figure S1. Analysis of flow cytometry data and purity check of mesenchymal stromal cells and BCP-ALL mono- and co-cultures. (A)** BCP-ALL cells from 15 pediatric patients (B-other, high hyperdiploid, *ETV6-RUNX1*: ALL#1-15)) were co-cultured for 40 hours with MSCs derived from 3 different origins (MSC#1: *ETV6-RUNX1*, relapse; MSC#2: high hyperdiploid, relapse; MSC#3: B-other, initial). BCP-ALL cells from 4 pediatric patients (B-other: ALL#3/#4, and *ETV6-RUNX1:* ALL#16/#17) were co-cultured for 40 hours with MSCs derived from 3 different origins (MSC#4/#5: healthy bone marrow; MSC#6/#7: *ETV6-RUNX1*, initial; MSC#8/#9: B-other, initial). MSCs and BCP-ALL cells were separated using flow sorting. Purity (contamination percentage) of sorted samples was determined by flow cytometry. RNA from sorted MSCs and BCP-ALL cells was used for performing total-RNA sequencing. **(B)** Gating strategy used for sorting MSCs and ALL#13 cells during FACS. Cell population was selected based on FSC and SSC. MSCs were selected for CD73+CD146+CD166+CD19-CD45-. BCP-ALL cells were selected for CD19+CD45+CD73-CD146-CD166-.

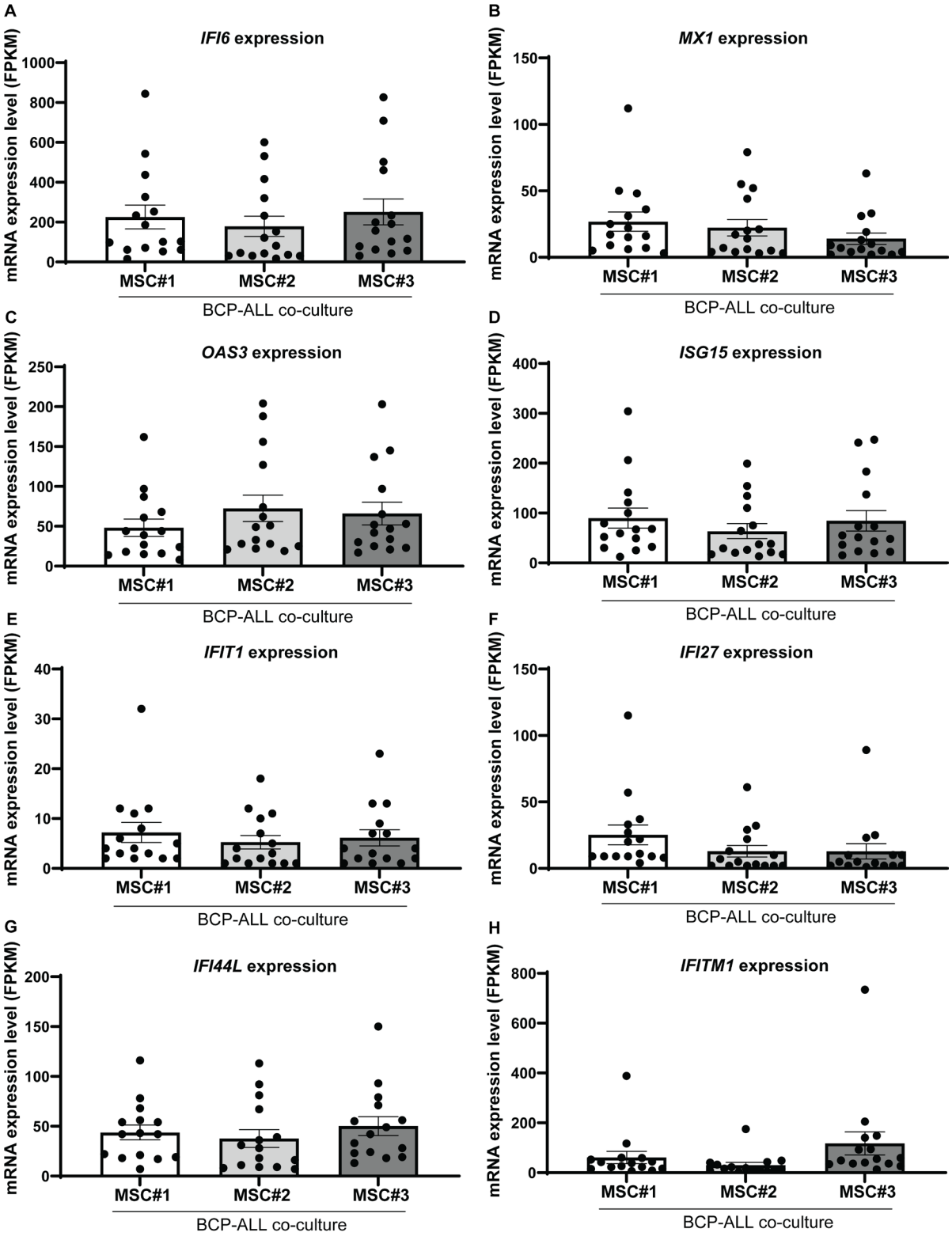
The Sytox Red negative population represents viable cells. FSC = forward scatter, SSC = side scatter. Purity of the sorted **(C)** viable BCP-ALL cells and **(D)** MSCs after co-culture was determined by flow cytometry. **(E)** Graph representing the amount of RNA isolated (y-axis) from increasing amounts (100,000-1,000,000) of sorted MSCs (x-axis). Each dot represents one sorted sample. **(F)** Same as **(E)** but for ALL cells (600,000-4,500,000). BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells.



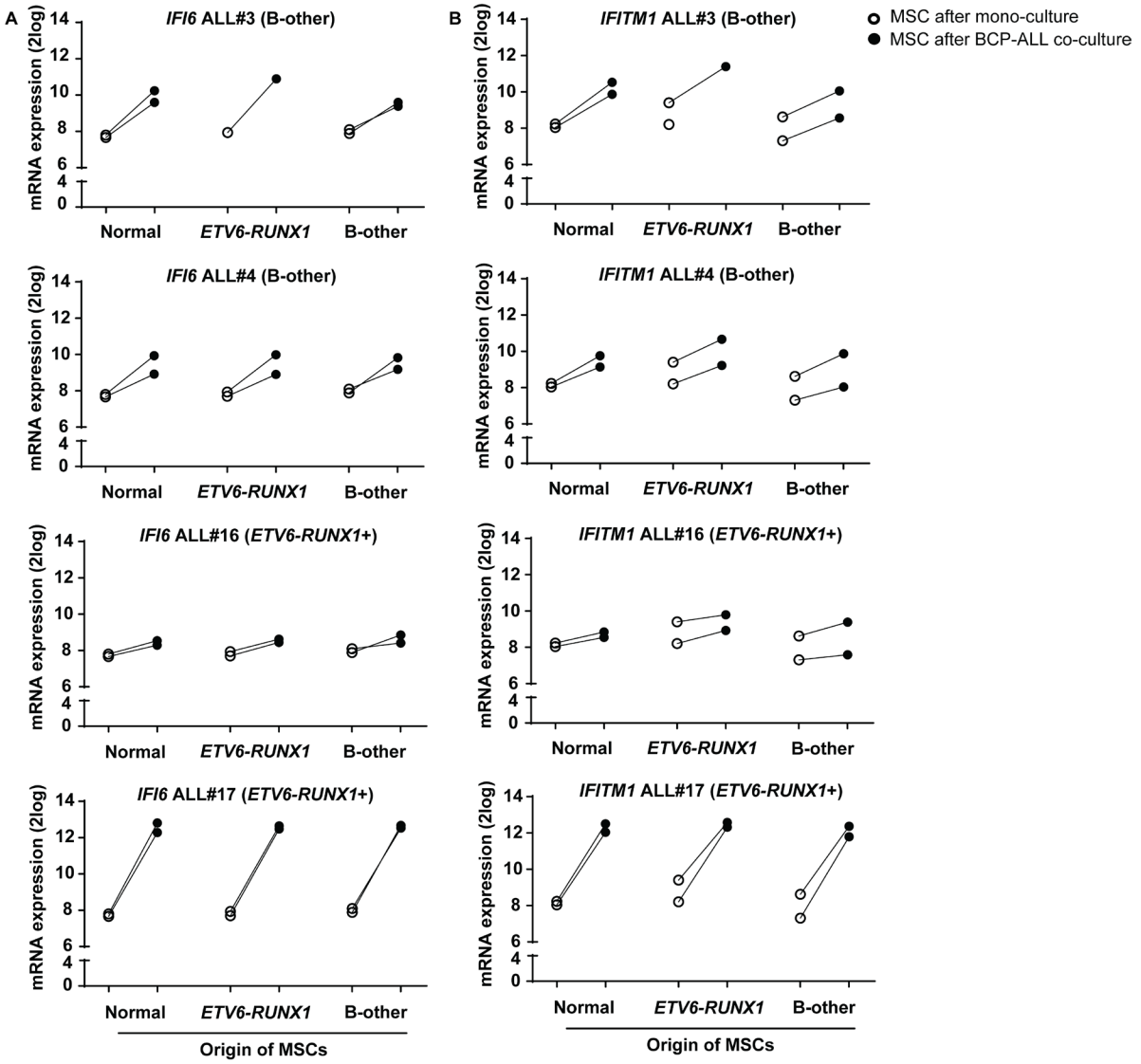

**Supplementary Figure S2. Mesenchymal stromal cells protect primary BCP-ALL cells against drugs.** Graphs represent the percentage of viable primary leukemic cells (ALL#9, #10 and #11) when treated with **(A)** L-asparaginase (ASP; 0.003, 0.016, 0.08, 0.4, 2.0 and 10 IU/ml), **(B)** daunorubicin (DNR; 0.002, 0.008, 0.031, 0.125, 0.5 and 2 µg/ml) or **(C)** prednisolone (PRED; 0.008, 0.06, 0.49, 3.9, 31.25 and 250 µg/ml) normalized to untreated control. Data points represent means of triplicate measurements ± SD. Grey squares and black circles indicate leukemic cell viability upon mono-culture and MSC co-culture, resp. Boxes indicate the drug concentration most discriminative for resistance induced by co-culturing BCP-ALL cells with MSCs. This drug concentration was used to investigate the effect of i-IFNs as shown in Figure 7/8. In case of ALL#9, 0.001 IU/ml asparaginase was selected as no clear effect was observed within the chosen range. Dashed line (---) indicates LC50. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, i-IFN = interferon inhibitors.



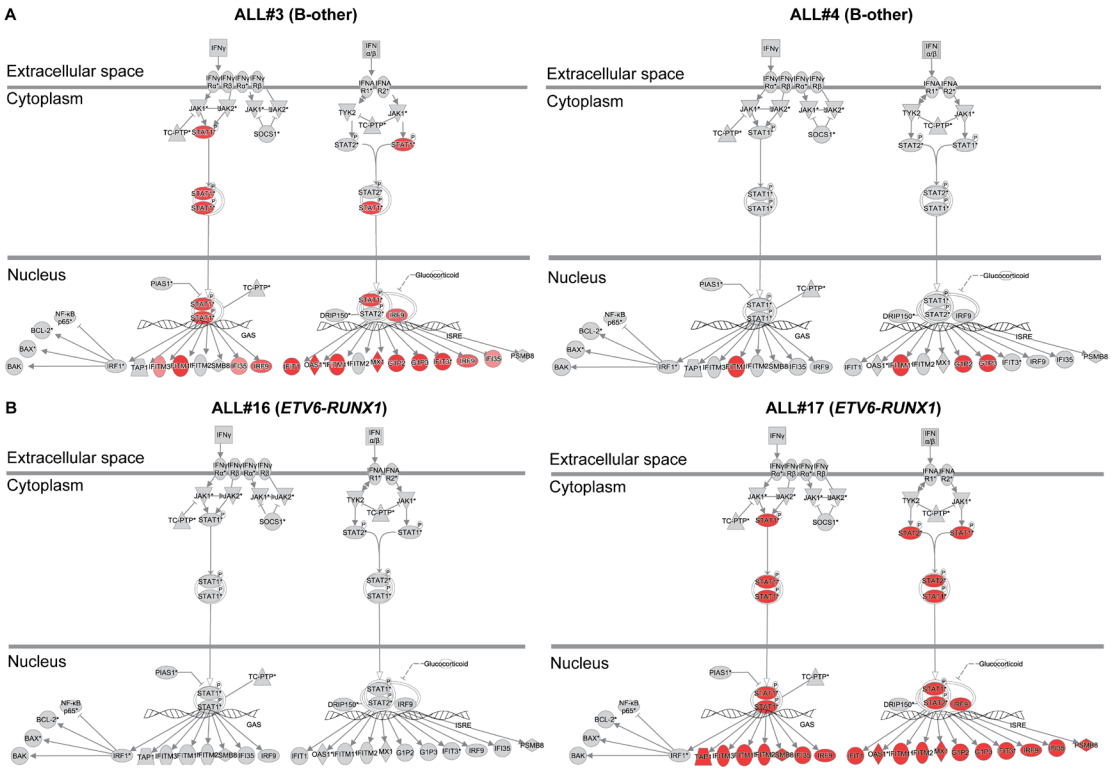

**Supplementary Figure S3. Pathway analysis reveals changes in the mesenchymal stromal cells' IFN gene profile induced by BCP-ALL cells**. Differentially expressed genes (FDR < 0.05) in MSCs (MSC#1, #2, and #3) after co-culture with primary pediatric BCP-ALL samples (n=15) analyzed by pathway analysis (Pathvisio). Blue, low expression. Red, high expression. Grey boxes indicate unrecognized genes by Pathvisio software. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.



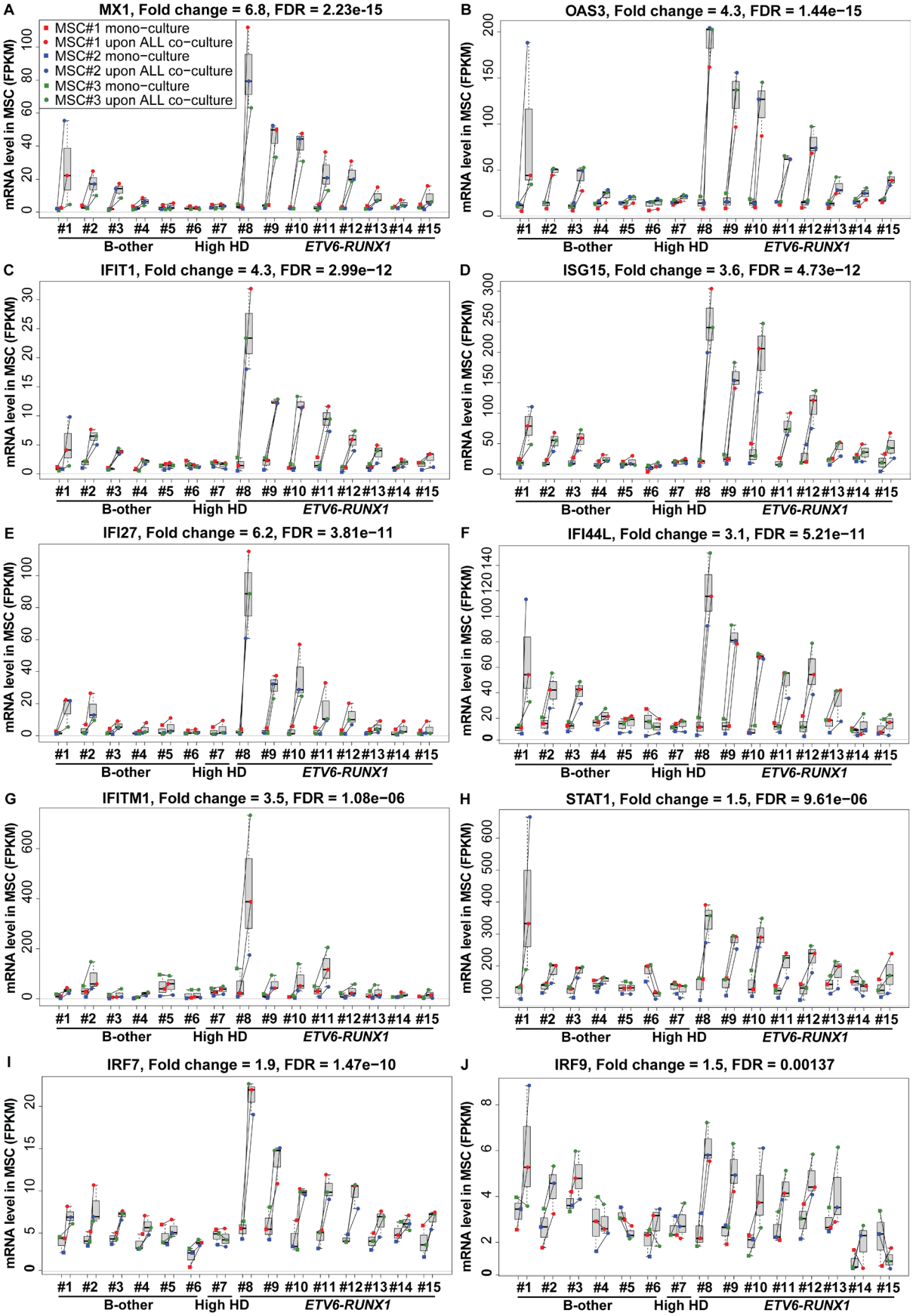

**Supplementary Figure S4. Induction of IFN-related genes by BCP-ALL cells is comparable between mesenchymal stromal cells from different origins. (A)** *IFI6*, **(B)** *MX1*, **(C)** *OAS3*, **(D)** *ISG15*, **(E)** *IFIT1*, **(F)** *IFI27*, **(G)** *IFI44L*, and **(H)** *IFITM1* mRNA levels (FPKM) expressed in MSCs, from three different origins (MSC#1, #2, and #3), sorted after co-culture with BCP-ALL cells from 15 individual patients (#1-15). Bars represent means ± SEM for 15 independent experiments. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.




**Supplementary Figure S5. ALL-induced expression of an IFN-signature gene in MSCs does not depend on the origin of the mesenchymal stromal cells**. **(A)** *IFI6* and **(B)** *IFITM1* mRNA expression in normal MSCs (MSC#4 and #5), MSCs derived from *ETV6-RUNX1* ALL patients (MSC#6 and #7), or B-other ALL patients (MSC#8 and #9) after 40-hour mono-culture (open circles) or co-culture with primary pediatric BCP-ALL samples (closed circles, B-other n=2, ALL#3 and #4; *ETV6-RUNX1* n=2, ALL#16 and #17). BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.

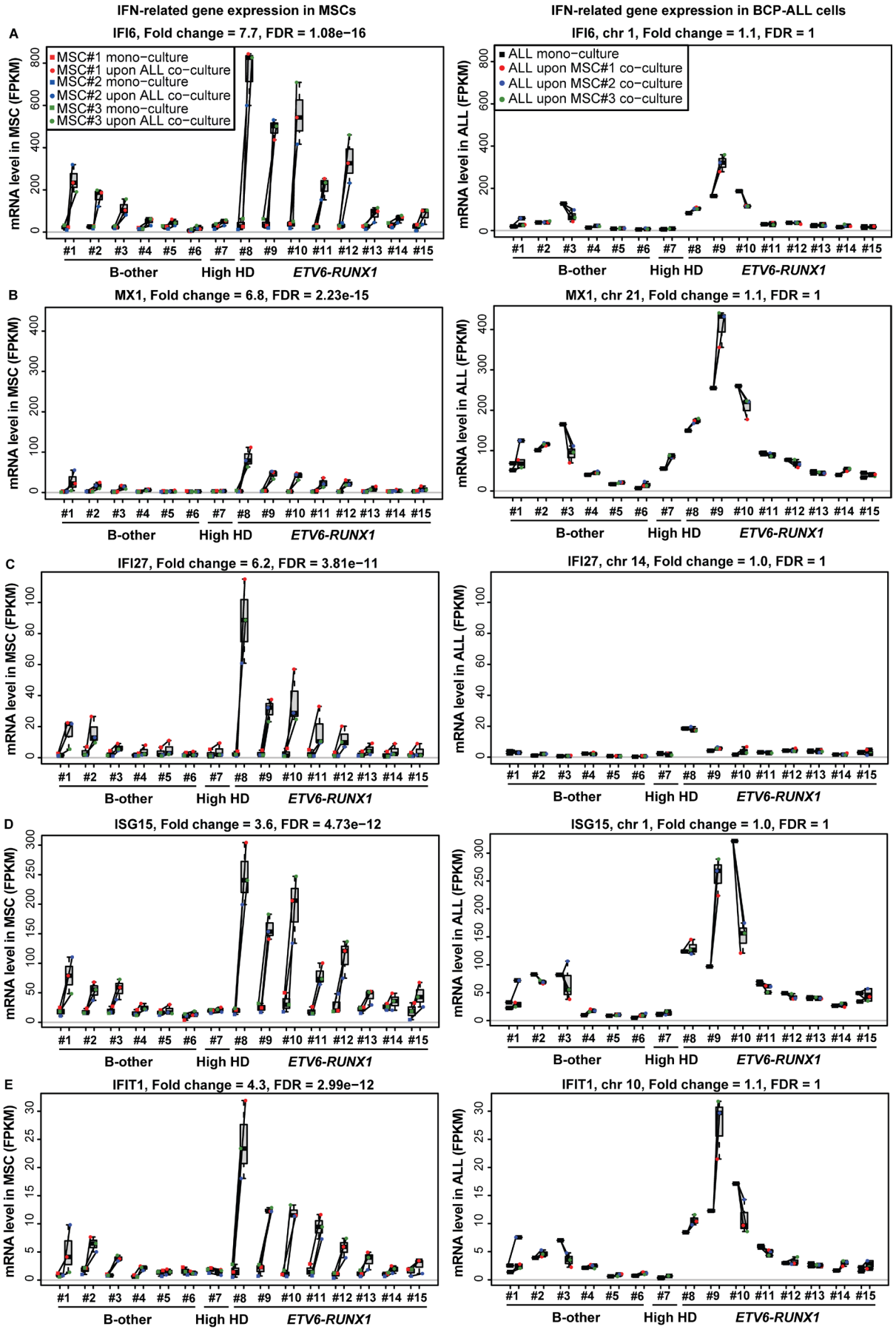



**Supplementary Figure S6. Pathway analysis in pilot study reveals changes in the mesenchymal stromal cells' IFN gene profile induced by BCP-ALL cells**. Differentially expressed probesets (Limma; adj. $p < 0.05$) in six MSCs (MSC#4, #5, #6, #7, #8 and #9) after co-culture with primary pediatric BCP-ALL samples: **(A)** B-other (n=2, ALL#3 and #4) and **(B)** *ETV6-RUNX1* (n=2, ALL#16 and #17) analyzed by ingenuity pathway analysis (IPA). Red, high expression. Grey boxes indicate unrecognized genes by IPA software. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon, G1P3 = IFI6.



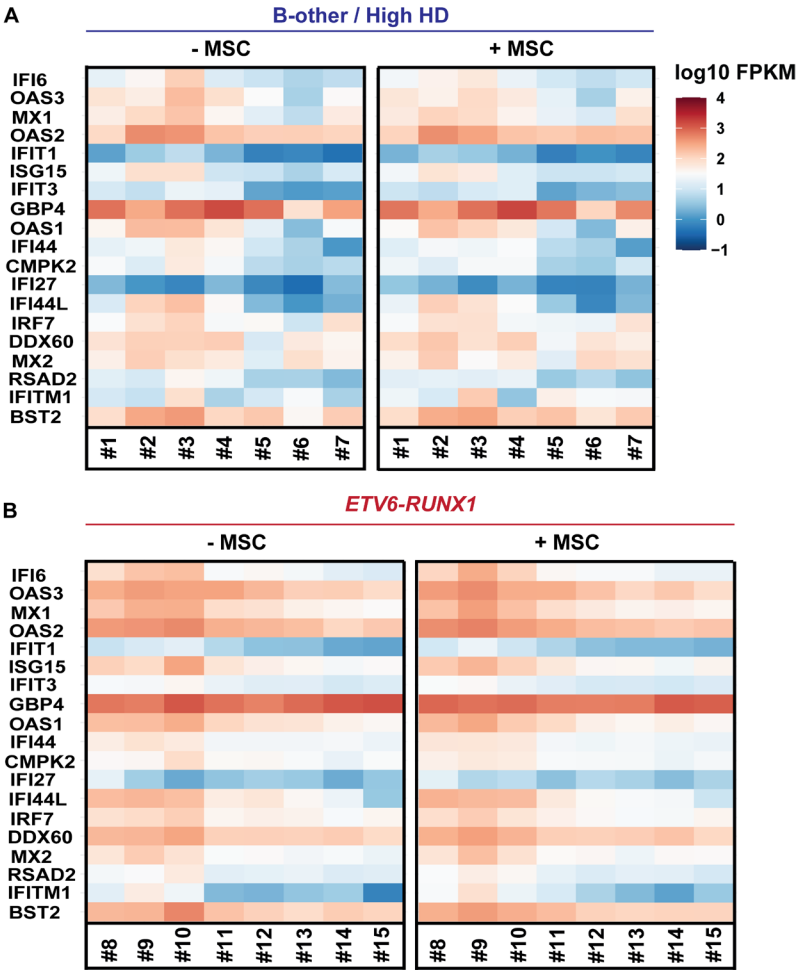

**Supplementary Figure S7. IFN-related gene signature in mesenchymal stromal cells upon BCP-ALL co-culture. (A)** *MX1*, **(B)** *OAS3*, **(C)** *IFIT1*, **(D)** *ISG15*, **(E)** *IFI27*, **(F)** *IFI44L*, **(G)** *IFITM1*, **(H)** *STAT1*, **(I)** *IRF7*, and **(J)** *IRF9* expression levels (FPKM) for paired MSC mono-culture (squares; MSC#1-3 indicated in red, blue,

and green, resp.) and MSC after co-culture with BCP-ALL cells from 15 individual patients (#1-15; circles). Boxplots represent the interquartile range, the median is depicted by a line. FDR, p-value false discovery rate. Solid grey line indicates no expression. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.

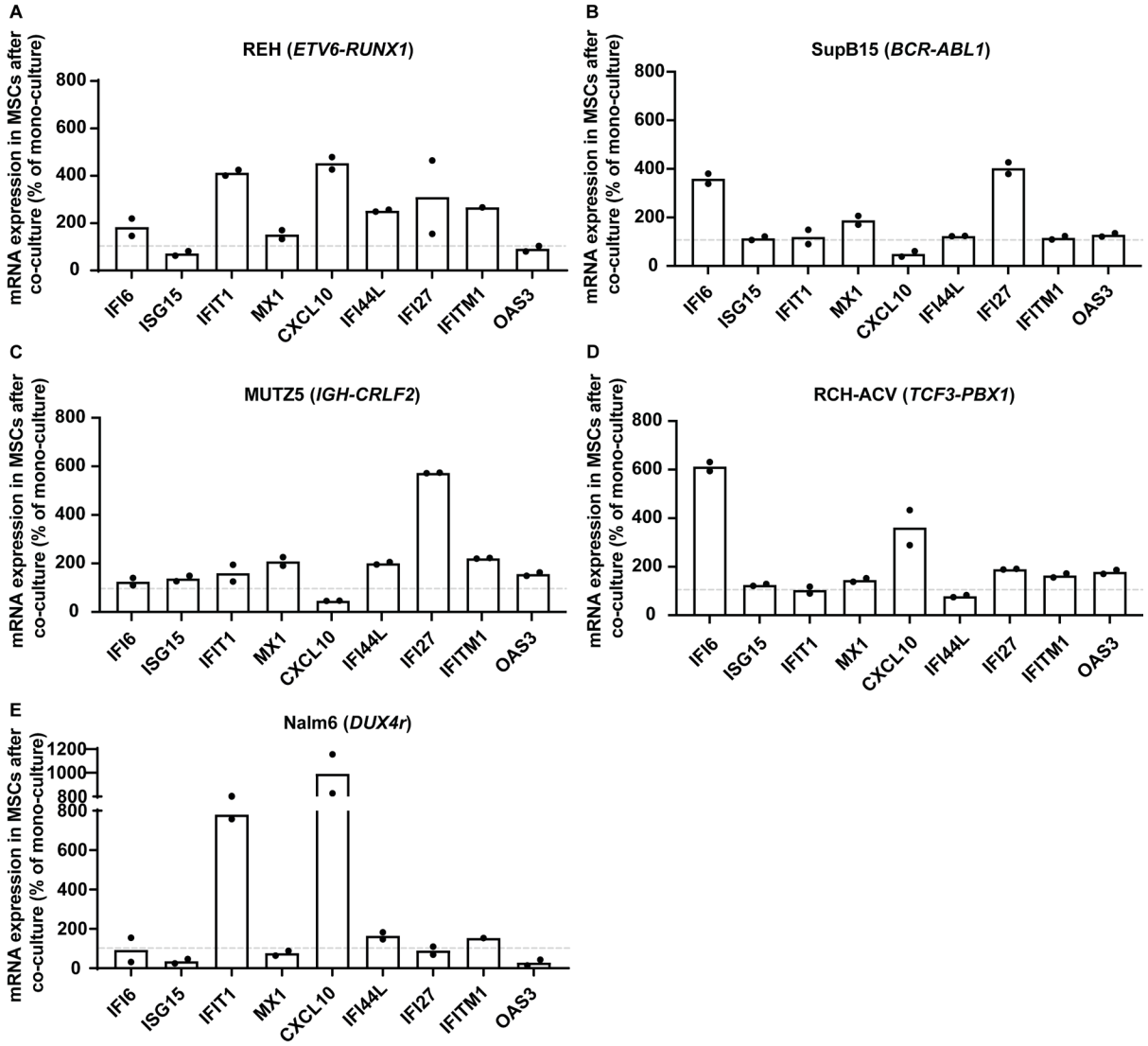



**Supplementary Figure S8. IFN-related expression in BCP-ALL cells after mesenchymal stromal cells co-culture. (A)** *IFI6*, **(B)** *MX1*, **(C)** *IFI27*, **(D)** *ISG15*, and **(E)** *IFIT1* expression levels (FPKM) for the left plots:

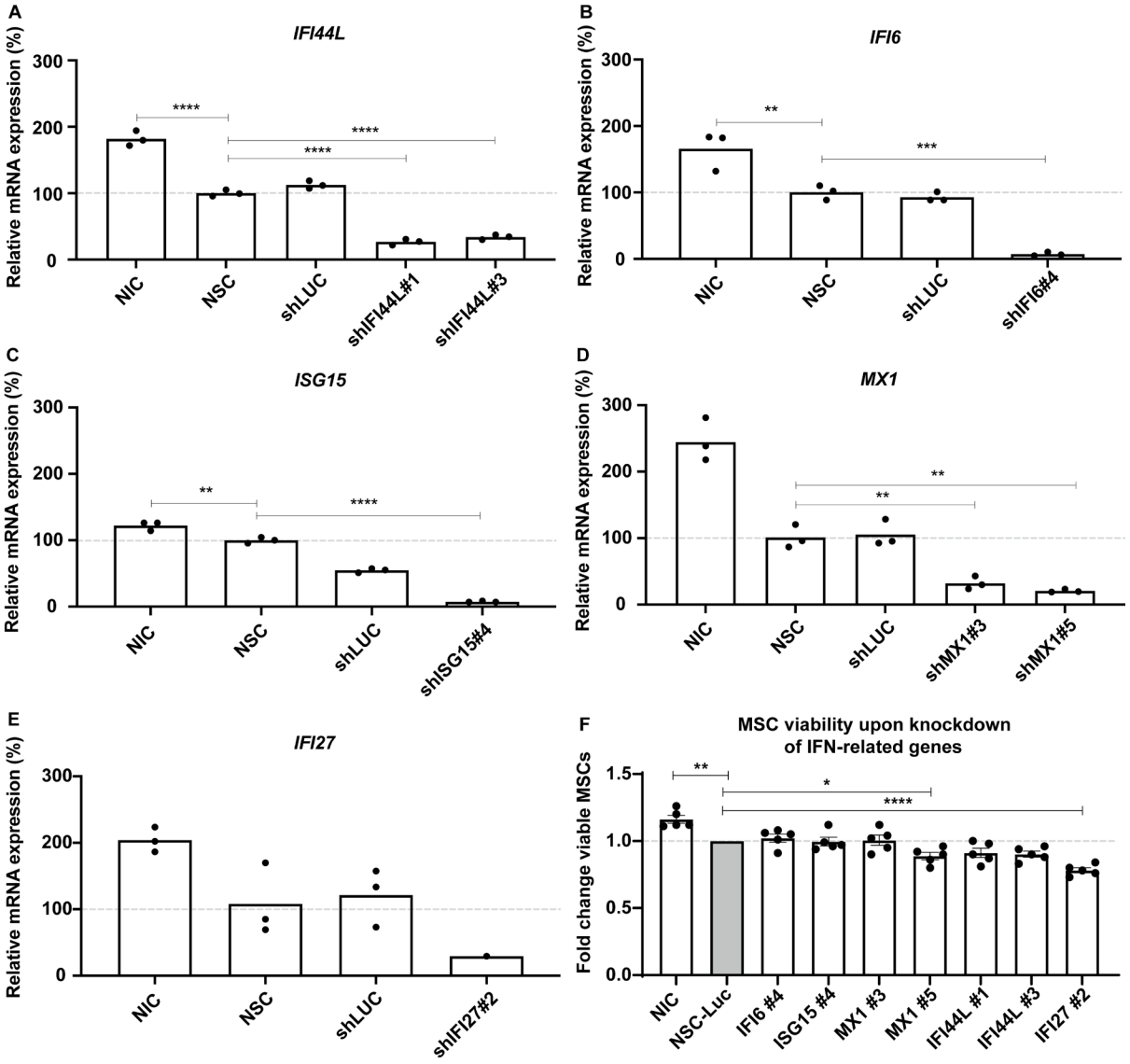
paired MSC mono-culture (squares; MSC#1-3 indicated in red, blue, and green, resp.) and MSC after co-culture with BCP-ALL cells from 15 individual patients (#1-15; circles), and the right plots: paired BCP-ALL mono-culture (black squares) and BCP-ALL cells from 15 individual patients (#1-15) after co-culture with MSC#1-3 (indicated in red, blue, and green circles, resp.). Boxplots represent the interquartile range, the median is depicted by a line. FDR, p-value false discovery rate. Solid grey line indicates no expression. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.



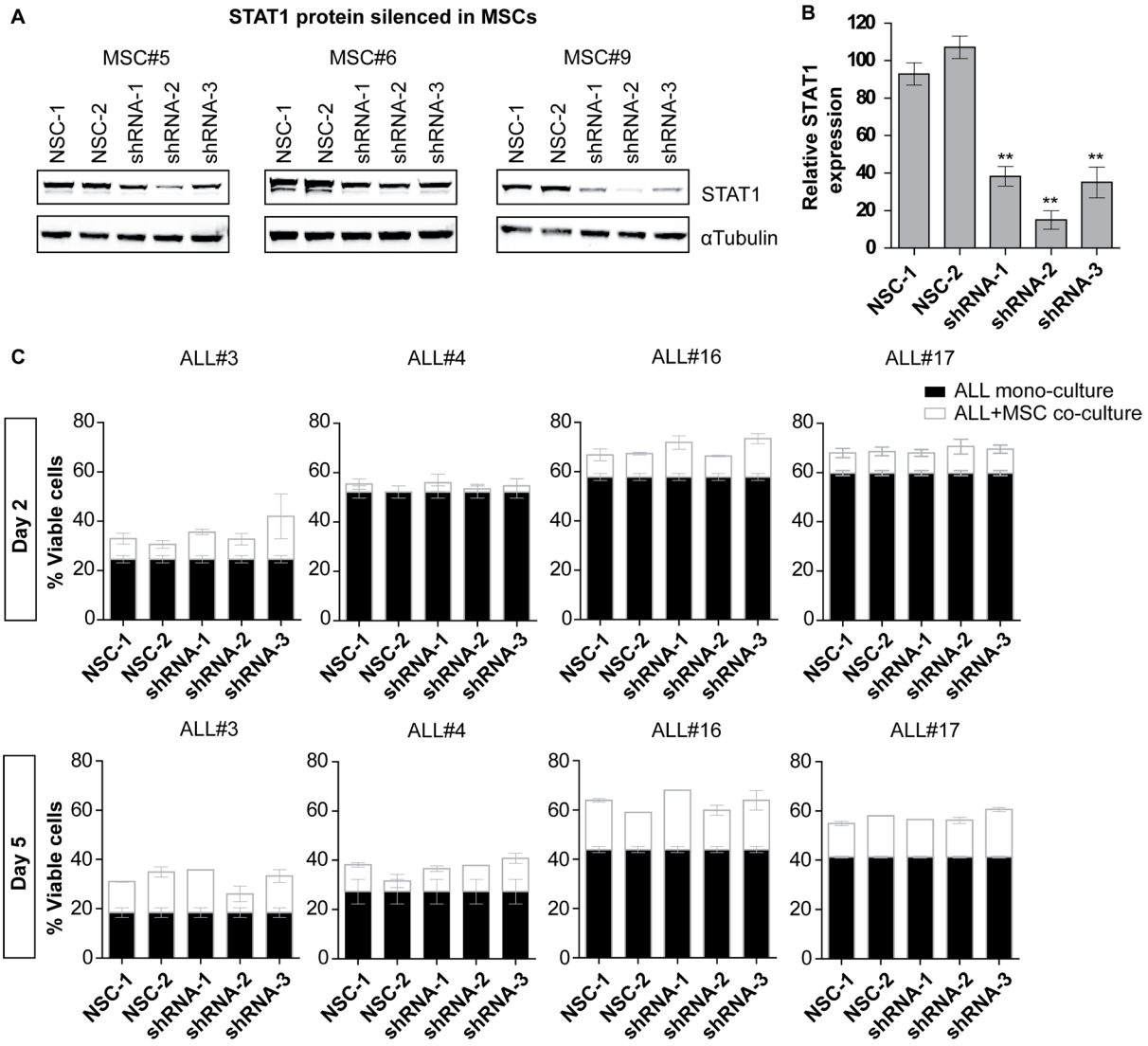

**Supplementary Figure S9. *ETV6-RUNX1* BCP-ALL cells show a slightly higher intrinsic IFN gene signature compared to B-other BCP-ALL samples, which is not affected by mesenchymal stromal cell co-culture.** Heatmap of RNA expression levels of IFN-related genes in ALL cells sorted after mono-culture (- MSC) and those after co-culture with MSCs of the corresponding subtype (+ MSC). Heatmap is grouped by BCP-ALL subtype: high hyperdiploid (HD) and B-other ALL cases (#1-7) or *ETV6-RUNX1* ALL cases (#8-15). Blue, low expression. Red, high expression. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.



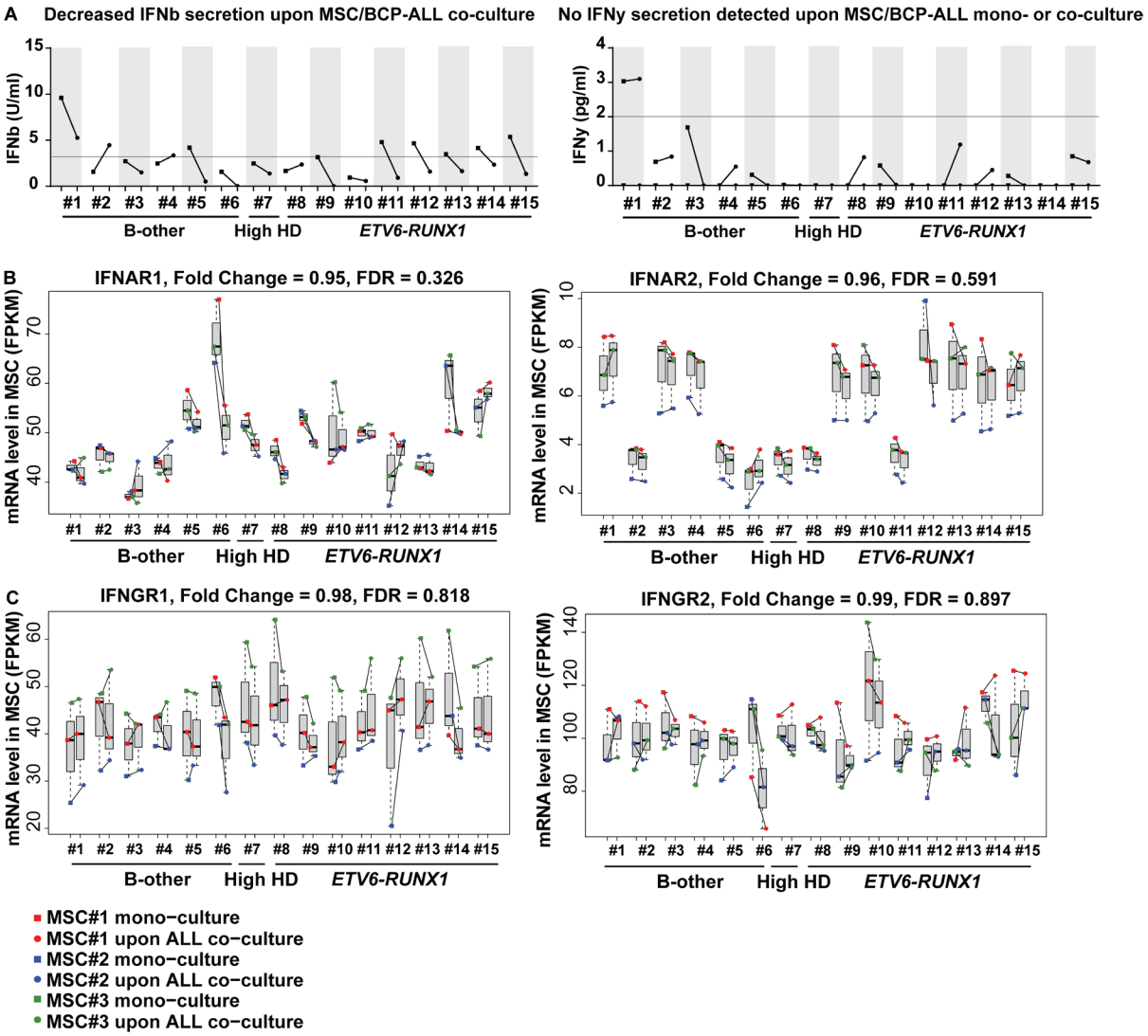

**Supplementary Figure S10. IFN-gene expression levels in mesenchymal stromal cells upon co-culture with leukemic cell lines.** mRNA expression levels of IFN-related genes in MSCs upon 40 hours co-culture with **(A)** REH (*ETV6-RUNX1*), **(B)** SupB15 (*BCR-ABL1*), **(C)** MUTZ5 (*CRLF2*-rearranged), **(D)** RCH-ACV (*TCF3-PBX1*), and **(E)** Nalm6 (*DUX4*-rearranged B-other) relative to MSC mono-culture. Bars are means of duplicate measurements for 1 experiment. Dashed line (---) indicates mRNA expression levels of MSC after mono-culture, set to 100%. MSCs = mesenchymal stromal cells, IFN = interferon.




**Supplementary Figure S11. Knockdown efficiency of IFN-related genes in mesenchymal stromal cells and effect on mesenchymal stromal cell viability.** A representative experiment showing relative mRNA expression levels of **(A)** *IFI44L*, **(B)** *IFI6*, **(C)** *ISG15*, ***(D)*** *MX1*, and ***(E)*** *IFI27* in MSCs (MSC#2) after lentiviral silencing. Bars represent means of technical triplicates for one independent experiment. mRNA expression is normalized to the non-silencing control (NSC). Dashed line (---) indicates mRNA expression levels of MSCs treated with shNSC, set to 100%. **(F)** Fold change in percentage of viable MSCs (MSC#2) transfected with IFN-related short-hairpin (sh)RNAs after 72 hours of co-culture. Average viability of Non-Silencing Control and short-hairpin-Luciferase (NSC-shLuc) is used as reference. Five shRNAs per gene were tested for knockdown efficiency; only shRNAs resulting in >60% knockdown (shIFI6 #4, shISG15 #4, shMX1 #3/5, shIFI44L #1/3, shIFI27 #2, see supplemental methods) were used. Bars represent means ± SEM of duplicate measurements for 5 independent experiments. Dashed line (---) indicates that viability of MSCs is equal to viability of NSC-shLuc-treated MSCs (fold-change = 1; grey bars). NIC = Non-Infected Control, shLUC = short-hairpin-Luciferase, MSCs = mesenchymal stromal cells, IFN = interferon.




**Supplementary Figure S12. Silencing of STAT1 expression in mesenchymal stromal cells does not affect the viability of BCP-ALL cells. (A)** Western blot analysis of STAT1 expression levels in MSCs (n=3) after lentiviral silencing using non-silencing control (NSC) short-hairpin (sh)RNA or three different *STAT1* shRNAs. αTubulin was used as a loading control. **(B)** Quantified STAT1 protein expression levels relative to αTubulin after lentiviral silencing in 3 MSC samples (MSC#5, #6 and #9). Bars represent means ± SEM. **(C)** Percentage of viable primary BCP-ALL cells (n=4) after two and five days in mono-culture (white bars) and co-culture with MSCs (n=3; black bars) as determined by flow cytometry. Bars represent means ± SEM. shRNA, short hairpin RNA. Three different samples of MSCs were used throughout this experiment, for which the results were comparable. MSCs used are from healthy donor (MSC#5), *ETV6-RUNX1* positive (MSC#6) and B-other (MSC#9) patients. ALL = acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells.




**Supplementary Figure S13. IFNβ and IFNγ secretion levels in mesenchymal stromal cell/BCP-ALL co-cultures and mRNA expression of IFNAR1/2 and IFNGR1/2 in mesenchymal stromal cells after BCP-ALL co-culture**. **(A)** Concentration of IFNβ (left; MSC#2) and IFNγ (right; MSC#1, #2 and #3) in supernatant from 15 MSC and BCP-ALL (ALL#1-15) mono- and co-cultures collected after 40 hours. IFNβ and IFNγ concentration is corrected for medium background level. Squares and circles indicate IFNβ and IFNγ secretion level in the sum of MSC and BCP-ALL mono-cultures and their co-cultures, resp. Limit of detection is indicated by solid line. Range of detection: IFNβ: 2.8 – 14.926 U/ml, IFNγ: 2.0 – 12.34 pg/ml. IFNγ levels were below the level of linear detection for all but two samples. **(B)** *IFNAR1* and *IFNAR2*, or **(C)** *IFNGR1* and *IFNGR2* mRNA levels (FPKM) expressed in paired samples of MSCs sorted after mono-culture (squares; MSC#1-3 indicated in red, blue, and green, resp.) and MSCs sorted after co-culture with BCP-ALL cells from 15 individual patients (#1-15; circles). Boxplots represent the interquartile range, the median is depicted by a line. FDR, p-value false discovery rate. BCP-ALL = B-cell precursor acute lymphoblastic leukemia, MSCs = mesenchymal stromal cells, IFN = interferon.

# Supplemental Methods

### Mesenchymal stromal cells

Primary MSCs were obtained from whole bone marrow aspirates taken from pediatric BCP-ALL patients (at diagnosis or at relapse of leukemia) and healthy donors as described previously (van den Berk et al., 2014). MSCs were selected based on adherence capacity, followed by confirmation of negativity for surface markers CD19/CD34/CD45, and positivity for CD44/CD54/STRO-1/CD73/CD90/CD105/CD146/CD166. MSCs were passaged twice a week in DMEM low glucose/pyruvate/HEPES medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% fetal calf serum (FCS) (Bodinco BV, Alkmaar, The Netherlands), amphotericin B (1:166; 250µg/ml, Gibco), gentamicin (1:1000; 50ng/ml, Gibco), fresh 1ng/ml recombinant human fibroblast growth factor (FGF) basic (Bio-Rad, Veenendaal, The Netherlands) and 0.1mM L-ascorbic acid (Sigma Aldrich, Zwijndrecht, The Netherlands). MSCs were cultured at 37°C/5%$CO_2$ and used for experiments until passage 10. For this study, MSCs from nine different donors were selected (***Supplementary Table S1***).

### Primary BCP-ALL cells

Primary BCP-ALL cells were collected from bone marrow samples originating from children with newly diagnosed BCP-ALL (<18 years). This study was performed in agreement with the Institutional Review Board, and written informed consent was given by patients, parents, or guardians. As described by Den Boer *et al*. (Den Boer et al., 2003), isolation and processing of the leukemic blasts was performed by density gradient centrifugation using Lymphoprep (1.077 g/ml, Nycomed Pharma, Oslo, Norway) for 15 minutes at 1500rpm. When necessary, normal hematopoietic cells were depleted using magnetic beads coupled to monoclonal antibodies to enrich for leukemic cells, resulting in ≥ 90% blasts for all samples prior to experiments. Primary BCP-ALL cells were kept in (short-term) culture at 37°C/5%$CO_2$ using RPMI 1640 Dutch Modified medium (Gibco) containing 20% FCS (Bodinco), 2% PSF (penicillin 5.000U/ml, streptomycin 5.000U/ml, fungizone 250µg/ml, Gibco, Thermo Fisher Scientific), gentamicin (0.2mg/ml, Gibco), 1% insulin-transferrin-selenium (ITS) (Sigma Aldrich), and 2mM L-glutamine (Life Technologies, Thermo Fisher Scientific). A total of 17 primary BCP-ALL samples was selected for this study, including 10 *ETV6-RUNX1*, 1 high hyperdiploid, and 6 B-other cases (***Supplementary Table S2***).

### Isolation and characterization of healthy immune cells from umbilical cord blood

Cord blood was collected by and received from the Franciscus Vlietland Hospital in Schiedam. Mononuclear cells were isolated from umbilical cord blood using Lymphoprep (1.077 g/mL, Nycomed Pharma) as described above. The collection and use of umbilical cord blood from donors for our studies was approved by the ethics committee of the Erasmus University Medical Center and the scientific

research committee of the Franciscus Vlietland Hospital. Antibodies against B-cell, T-cell, NK cell, monocyte, granulocyte, and (plasmacytoid) dendritic cell markers were purchased for characterization of immune cells using flow cytometry: PerCP/Cy5.5 anti-human CD3 and CD11c (Biolegend, San Diego, CA, USA), PE anti-human CD4 and CD56 (Biolegend), Brilliant violet (BV)421 anti-human CD8 (Biolegend), Vioblue anti-human CD14 (Miltenyi Biotec), BV650 anti-human CD16 (Biolegend), BUV395 anti-human CD19 and CD123 (BD Biosciences, San Jose, CA, USA), BV605 anti-human CD45 (Biolegend), APC anti-human CD66b, and HLA-DR (Biolegend). Antibody panel 1 includes CD3/CD4/CD8/CD16/CD19/CD45/CD66b antibodies; antibody panel 2 includes CD11c/CD14/CD16/CD45/CD56/CD123/HLA-DR antibodies. ViaKrome808 Fixable Viability Dye (Beckman Coulter, Brea, CA, USA) was used to select for the viable population. Immune cells were characterized using the Cytoflex LX (Beckman Coulter). Flow cytometry data were analyzed using FlowJo (version 10.7.1).

**BCP-ALL cell lines**

The cell lines REH (*ETV6-RUNX1*), Nalm6 (*DUX4*-rearranged B-other), RCH-ACV (*TCF3-PBX1*), SupB15 (*BCR-ABL1*), and MUTZ5 (*CRLF2*-rearranged) were cultured at 37ºC/5%$CO_2$ in RPMI 1640 GlutaMAX medium (Gibco) containing either 10 or 20% FCS (Bodinco) and 2% PSF (Gibco) and were passaged twice a week. Authentication of cell lines was confirmed regularly by short tandem repeat (STR) profiling by using the GenePrint 10 kit (Promega, Madison, WI, USA) on an ABI 3730 DNA analyzer (Applied Biosystems, Waltham, MA, USA).

**Co-culture of BCP-ALL cells, healthy immune cells and MSCs**

MSCs ($0.225x10^6$) were seeded in a 6-well plate in 3 ml DMEM medium (15% FCS, gentamicin, FGF, and L-ascorbic acid), and cultured for 24 hours at 37ºC/5%$CO_2$. The MSC monolayer (~95% confluent) was washed with PBS, followed by adding 2 ml RPMI-1640 Dutch Modified medium. A volume of 1 ml RPMI 1640 Dutch Modified medium containing freshly thawed $5.0x10^6$ primary BCP-ALL cells or healthy immune cells was added to the MSCs. In case of BCP-ALL cell lines, 1 ml RPMI 1640 medium containing $0.9x10^6$ cells was added. Mono-cultures of BCP-ALL cells and MSCs, with cells kept in same concentration and total volume of 3 ml, were used as controls. The MSCs and BCP-ALL cells were cultured for 40 hours at 37ºC/5%$CO_2$.

**Fluorescent activated cell sorting**

Cells were harvested and stained with antibodies against B-cell, MSC and hematopoietic cell markers. Antibodies used to distinguish the different cell populations included Brilliant violet 421 anti-human CD19 (Biolegend, San Diego, CA, USA), PE anti-human CD45 (Biolegend), Alexa Fluor 750-conjugated human 5'-Nucleotidase/CD73, MCAM/CD146, and ALCAM/CD166 (R&D Systems, Minneapolis, MN, USA). Sytox Red Dead Cell Stain (Thermo Fisher Scientific) was used to select for the viable population. Viable cells were sorted by the SH800S Cell Sorter (Sony, San Jose, CA, USA)

with a 100µm nozzle into an MSC- and leukemic cell fraction. A purity check of both fractions was performed by measuring 10,000 events after sorting (***Supplementary Table S3***). The maximum percent of contaminating cells allowed in sorted fractions was determined by the effect these contaminating cells had on the expression levels of genes. This was previously determined in our lab by performing a quantitative RT-PCR experiment in which increasing amounts of MSCs, or ALL cells were added to ALL cells or MSCs, resp. In this study, we aimed for a maximum of 5% ALL cells in sorted MSC fractions, and only 0.3% MSCs in sorted ALL fractions as MSCs contain more RNA than ALL (***Supplementary Figure S1***). 17/45 (37.8%) sorted ALL samples contained >0.3% MSCs, while only 5/45 (11.1%) sorted MSC samples contained >5.0% ALL cells (***Supplementary Table S3)***. Although some samples showed high contamination, all samples were included for further analysis. The sorted MSCs and leukemic cells were pelleted, and dissolved in 1ml Trizol (Invitrogen, Bleiswijk, The Netherlands) for RNA isolation. Samples were stored at -80ºC until further use.

**Multiplexed fluorescent bead-based immunoassay (Luminex)**

Supernatants of mono- and co-culture experiments were centrifuged for 10 minutes at 1200rpm to remove cellular debris. Supernatants were immediately stored at -80°C. An in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP, Luminex, Austin, TX, USA) was used to measure cytokines of interest at the Center for Translational Immunology (University Medical Center Utrecht, the Netherlands) (Smids et al., 2017). Archived supernatants were incubated with antibody-conjugated MagPlex microspheres for 1 hour at room temperature with constant shaking. Samples were incubated 1 hour with biotinylated detecting antibodies, followed by 10 minutes of incubation with phycoerythrin (PE)-conjugated streptavidin. PE-conjugated streptavidin was diluted in high performance ELISA buffer (HPE) (Sanquin, Amsterdam, The Netherlands). The Bio-Rad FlexMAP3D (Bio-Rad laboratories, Hercules, CA, USA) and xPONENT software version 4.2 (Luminex) were used to determine the concentration of the secreted cytokines in supernatants. Bio-Plex Manager software, version 6.1.1 (Bio-Rad) was used to analyze and to quantify the data using standard dilution series.

**RNA sequencing**

RNA extraction was performed with Trizol (Invitrogen) as described by manufacturer. The quantity, and A260/A230 and A260/280 ratios of RNA were determined by the Qubit 4 fluorometer (Thermo Fisher Scientific), and the DeNovix Spectrophotometer DS 11 (DeNovix Inc., Wilmington, DE, USA), respectively. RNA quality was determined using the Bioanalyzer Agilent 2100 (Agilent, Santa Clara, CA, USA) combined with the Agilent RNA 6000 Nano Kit according to manufacturer's protocol. 600ng RNA (25 ng/µl; RIN>6.8) was used for ribonucleotide-depleted long-noncoding RNA sequencing on a NovaSeq 6000 (Illumina, San Diego, CA, USA). Fastq reads were aligned to the human reference genome GRCh38 using STAR (version 2.6.0c) and data were analyzed using R (version 3.6.3). Genes

with zero counts across all samples, or <1.0 counts per million mapped reads in all but one sample were removed. Normalization factors were calculated using the trimmed mean of M values (TMM) method. Dispersion in the data was estimated taking batch effect and subtype of BCP-ALL cells into consideration. EdgeR (version 3.28.1) was used to perform differential gene expression analysis. A paired EdgeR p-value corrected for multiple testing (FDR) below 0.05 was considered significant. Fragments per kilobase per million reads mapped (FPKM) values were generated using the transcript length of the longest known transcript (Gencode v29). FPKM values were log10-transformed to create a heatmap. Pathway analysis was performed using Pathvisio software (van Iersel et al., 2008).

**Microarrays**

In our pilot study, microarrays were performed to determine differentially expressed genes in MSCs after BCP-ALL co-culture. RNA (RIN > 6.9) was labeled, fragmented, and linearly amplified with the Affymetrix GeneChip 3'IVT Express Kit (Santa Clara, CA, USA) according to the manufacturer's protocol. Fragmented, amplified RNA was hybridized on Affymetrix U133 Plus 2.0 GeneChip arrays for 16 hours. Arrays were washed and stained using the GeneChip Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000. Affymetrix Microarray Suite version 5.0 was used to assess gene-expression values. Expression signals were scaled to the target intensity of 500 and log transformed. Arrays were variance stabilization and normalization 2 (VSN2) normalized. To identify differentially expressed probesets, Limma R Package (version 3.26.9) was used in R 3.0.1. Pathway analysis was performed using Ingenuity Pathway Analysis software (IPA; Ingenuity Systems Inc., Redwood City, CA).

**Tunneling nanotube inhibition**

Vybrant DiI Cell-Labeling Solution (Invitrogen) was used to pre-stain BCP-ALL cell line REH for 24 hours. Transwells (0.4μm pore-size; Corning, New York, USA) were pre-incubated with RPMI medium for 30 minutes at 37°C/5%$CO_2$ before DiI-stained ALL cells were added. Dye transfer from DiI-stained ALL cells cultured in transwells towards MSCs cultured in 6-well plates (Sigma Aldrich) was determined after 40 hours by flow cytometry, visualized by excitation with 561nm laser and emission detection with 585/42 filter using the Cytoflex S (Beckman Coulter). MSCs cultured with DiI-labeled ALL cells without transwell were used as control. Flow cytometry data were analyzed by using FlowJo (version 10.7.1) and graphs were created by GraphPad Prism (version 9.1.2).

**Lentiviral silencing**

pLKO.1-puro Mission vectors (Sigma Aldrich), containing short-hairpin RNA (shRNA), were purchased to target genes involved in the Interferon (IFN) pathway. The pLKO.1-puro Mission vectors non-mammalian shRNA control plasmid and luciferase shRNA control plasmid (SHC002 and SHC007, resp.) were used as control vectors. Human embryonic kidney 293T (HEK293T) cells were cultured in

DMEM, high glucose, GlutaMAX medium (Gibco) supplemented with 2% PSF (Gibco) and 10% FCS (Bodinco). HEK293T cells were transfected at 80% confluence according to manufacturer's protocol. In short, transfection was performed with 1.6µg pVSV-G (Addgene plasmid 12259; Addgene, Cambridge, Massachusetts, USA), 3.7µg pPAX2 (Addgene plasmid 12260), and 10.7µg plasmid DNA (Sigma Aldrich; *MX1* (#3: TRCN0000056880; #5: TRCN0000056882), *IFI6* (#4: TRCN0000057501), *IFI27* (#2: TRCN0000115858), *IFI44L* (#1: TRCN0000167757; #3: TRCN0000242541) *ISG15* (#4: TRCN0000007423) or *STAT1* (#1: TRCN0000004267, #2: TRCN0000004265, #3: TRCN0000004264) in the presence of X-tremeGENE HP DNA Transfection Reagent (Sigma Aldrich). Five shRNAs per gene were tested for knockdown efficiency, but merely shRNAs resulting in efficient knockdown (>60%) were used for consecutive experiments. Fresh medium was added 24 hours after transfection. Virus supernatant was harvested two and three days after transfection of HEK293T cells and filtered with a 0.45µm filter to eliminate cell debris. Virus particles were collected by centrifugation at 32,000rpm for 2 hours at 4°C using the Optima XE-90 ultracentrifuge (Beckman Coulter). Virus was aliquoted and stored at -80°C until further use. Virus titration was performed on primary MSCs to determine the optimal virus concentration. Genes of interest were silenced by transfecting MSCs using spin-infection (1800rpm, 45 minutes, 21°C), followed by selection for puromycin resistance for 1 week. RNA was extracted by means of the RNeasy Mini Kit (Qiagen, Hilden, Germany), and was used to determine the knockdown efficiency by quantitative RT-PCR.

Next, lentiviral transduced MSCs were seeded in 24-well plates (50,000 cells/well) and incubated for 5 hours at 37°C/5%$CO_2$ in 700µl RPMI-1640 Dutch Modified medium. A volume of 175µl RPMI 1640 medium with 875,000 primary BCP-ALL cells was added to the MSCs ($1.0x10^6$ BCP-ALL cells/ml). Mono- and co-cultures were performed for 72 hours, after which viability of MSCs and BCP-ALL cells was determined by flow cytometry (Cytoflex S) with the antibodies described previously.

**Western blotting**

MSCs were pelleted and lysed in RIPA basic lysis buffer (Thermo Fisher Scientific), freshly supplemented with phosphatase/protease inhibitors (Thermo Fisher Scientific). A BCA assay (Thermo Fisher Scientific) was performed prior to Western blotting to determine the protein quantity. Per sample, 15µg protein was loaded on a 10% mini protean precast gel (Bio-Rad) to separate proteins by electrophoresis. A 1x Trans-blot Turbo Mini 0.2µm Nitrocellulose transfer pack (Bio-Rad) was used to transfer the proteins. The membrane was washed with 5% milk blocking buffer for 1 hour at room temperature, followed by incubation with primary antibody according to specifications of the manufacturer. Antibodies against αTubulin (loading control; #2144, Cell Signaling Technology, Danvers, MA, USA) and STAT1 (#9176, Cell Signaling Technology) were used at 4°C overnight. Secondary antibodies were ordered from LI-COR Biosciences (Lincoln, NE, USA): IRDye 680RD-labeled anti-mouse and IRDye 800CW-labeled anti-rabbit. After 1 hour of incubation at room

temperature, the blot was analyzed using the Odyssey CLx infrared imager (LI-COR), and Image Studio Lite software (version 4.0). Signal intensities were normalized to αTubulin.

**Activation and inhibition of interferon signaling**

MSCs were stimulated with recombinant human IFNα (0.03 U/ml) or IFNβ (60 pg/ml) protein (R&D Systems), in combination with different concentrations of inhibitor of IFNα/β (i-IFNα/β) (Recombinant Viral B18R, R&D Systems; 0.9, 1.8 or 3.6 ng/ml) for 40 and 120 hours. Inhibitors of IFNα/β (1.8 ng/ml), and IFNγ (Recombinant Viral B8R, R&D Systems; 3.0 ng/ml), by functioning as decoy receptors, were used in unstimulated co-cultures of MSCs and BCP-ALL cells for 40 and/or 120 hours. Cell viability and the IFN-related gene expression signature were assessed with flow cytometry and quantitative RT-PCR, resp.

**Drug exposure experiments**

BCP-ALL cells in mono- or co-culture with MSCs were exposed to a range of six concentrations of L-asparaginase (ASP, 0.003 – 10 IU/ml), daunorubicin (DNR, 0.002 – 2 μg/ml) or prednisolone (PRED, 0.008 – 250 μg/ml) to create a dose-response curve. MSCs (5,500) were seeded in 96-well flat-bottom plates and incubated for 24 hours. Primary BCP-ALL cells (137,500; $1.0x10^6$/ml) were added with or without ASP, DNR or PRED, and were incubated for 4 days at 37ºC/5%$CO_2$. Cells were harvested, stained with the antibodies described previously, and the percentage of viable Sytox Red negative cells was measured by means of flow cytometry (Cytoflex S). Sensitivity of leukemic cells to ASP, DNR and PRED in mono- and MSC co-culture in the presence of i-IFNα/β was tested. The concentration of drug most discriminative for resistance induced by MSCs was determined per BCP-ALL sample (***Supplementary Figure S2***). These drug concentrations were tested with and without i-IFNα/β (1.8 ng/ml). In detail, 16,500 MSCs were seeded in 48-well plates, incubated for 24 hours, followed by addition of 412,500 primary leukemic cells ($1.0x10^6$/ml) with or without i-IFNα/β. Cells were cultured for 24 hours to establish a leukemic niche. The previously determined optimal drug concentration was then added to the cells and incubated for 4 days at 37ºC/5%$CO_2$. Cells were harvested and measured using flow cytometry.

**Quantitative RT-PCR (RT-qPCR)**

Primer-probe pairs were purchased at Thermo Fisher Scientific targeting IFN-related genes. These genes included *MX1* (Hs00895608_m1), *IFI6* (Hs00242571_m1), *IFI27* (Hs01086370_m1), *ISG15* (Hs00192713_m1), *IFI44L* (Hs00915292_m1), *CXCL10* (Hs00171042_m1), *IFIT1* (Hs01675197_m1), *OAS3* (Hs00196324_m1), *IFITM1* (Hs00705137_s1), and housekeeping gene *GAPDH* (Hs99999905_m1). RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. cDNA was synthesized by using the SensiFAST cDNA Synthesis kit (Bioline,

London, England). Gene expression levels were measured using a MicroAmp Fast Optical 96-well Reaction Plate with Barcode, 0.1 ml (Thermo Fisher Scientific), and a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). mRNA expression values were corrected for GAPDH ($\Delta Ct = Ct_{gene} - Ct_{GAPDH}$). Fold change in relative gene expression was calculated using the $2^{\wedge- \Delta\Delta Ct}$ method. Graphs were made using GraphPad Prism (Version 9.1.2). The IFN-related gene signature in MSCs upon (in)direct co-culture was measured by means of quantitative RT-qPCR on flow-sorted cells. mRNA expression levels in MSCs upon co-culture were normalized to MSC mono-culture (100%).

**Statistical analysis**

One-way ANOVA tests and one-sided (un)paired t-tests were performed using GraphPad Prism, and correction for multiple testing was included when appropriate. A (paired EdgeR) p-value below 0.05 was considered statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.