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

Mandy W. E. Smeets,[1,2*] Elisabeth M. P. Steeghs,[1*] Jan Orsel,[2] Femke Stalpers,[2] Myrthe M. P. Vermeeren,[2] Christina H. J. Veltman,[2] Lotte Slenders,[2] Stefan Nierkens,[2,3] Cesca van de Ven[2] and Monique L. den Boer[1,2]

[1]Department of Pediatrics, Erasmus MC-Sophia, Rotterdam; [2]Princess Máxima Center for Pediatric Oncology, Utrecht and [3]Center for Translational Immunology, University Medical Center, Utrecht, the Netherlands

*MWES and EMPS contributed equally as first authors.

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.

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common pediatric malignancy, and is characterized by malignant transformation and clonal expansion of B-precursor cells in the bone marrow.[1-3] The 5-year event-free survival rate of pediatric patients with BCP-ALL is currently ~90%.[4-8] However, despite a relatively high cure rate, BCP-ALL still represents a major cause of cancer-related mortality in children, which can be attributed to treatment-related death or relapse of the leukemia.[6,9]

Several studies have shown that the bone marrow microenvironment facilitates leukemogenesis and contributes to cellular drug resistance.[9-15] Bone marrow-derived mesenchymal stromal cells (MSC) are a major component of the bone marrow microenvironment.[16] *Ex vivo*, these cells provide a survival benefit to co-cultured leukemic cells and induce resistance to chemotherapeutic drugs. We previously showed that leukemic cells become re-sensitized to chemotherapeutics *ex vivo* when the interaction with MSC is disrupted, e.g., by interfering with tunneling nanotube formation.[10] Leukemic cells use tunneling nanotubes as an effective mechanism to communicate with MSC. Transfer of mitochondria, autophagosomes, and transmembrane proteins towards MSC has been established.[14] Disruption of tunneling nanotubes resulted in an altered profile of cytokines secreted by MSC and a reduced survival benefit for leukemic cells.[10,14] These findings indicate that MSC play an important role in the maintenance of BCP-ALL, although the mechanism is yet unknown. To elucidate potential mechanisms, we investigated the effect of leukemic cells on the gene expression profile of MSC. Subsequently, we studied whether differentially expressed genes contributed to the viability and drug responsiveness of BCP-ALL. Our study shows that the interferon (IFN) α/β pathway is selectively activated in MSC upon interaction with BCP-ALL cells but not with normal, healthy immune cells. However, interference with this pathway did not affect either the viability or the

level of drug resistance, suggesting that the IFNα/β response may serve a different role in the pathobiology of BCP-ALL.

Methods

More details on the methods involved in this research are provided in the *Online Supplementary Methods*.

Mesenchymal stromal cells and primary B-cell precursor acute lymphoblastic leukemia cells

MSC were obtained from bone marrow aspirates taken from pediatric BCP-ALL patients and healthy donors as specified in *Online Supplementary Table S1*, using previously described procedures.[17]

Primary BCP-ALL cells were collected from bone marrow aspirates taken from children with newly diagnosed BCP-ALL (<18 years) which were left over after diagnostic procedures, and for which written informed consent was given by patients, parents, or guardians in line with institutional review board guidelines. The patients' characteristics are presented in *Online Supplementary Table S2*. All samples were archived in liquid nitrogen and showed >90% leukemic blasts after having been thawed.

Healthy immune cells

Mononuclear cells were isolated from umbilical cord blood, with the approval of the ethics committee of Erasmus University Medical Center and the scientific research committee of Franciscus Vlietland Hospital. Antibodies against B-, T-, and natural killer (NK)-cell, monocyte, granulocyte, and dendritic cell markers were purchased for immune cell characterization using flow cytometry (Cytoflex LX; Beckman Coulter).

Co-culture of leukemic cells, healthy immune cells and mesenchymal stromal cells

MSC (0.225×10^6) were seeded in six-well plates with 3 mL of medium and cultured for 24 hours. This medium was replaced with 3 mL medium containing 5.0×10^6 freshly thawed primary BCP-ALL cells or healthy immune cells, or 0.9×10^6 cells in the case of BCP-ALL cell lines. Cells were cultured for 40 hours before supernatants were taken to measure cytokine levels by Luminex multiplex immunoassay.[18] In parallel, BCP-ALL and MSC were fractionated by flow sorting on a SH800S Cell Sorter (Sony, San Jose, CA, USA) and used as input for RNA sequencing. Both mono-cultured and co-cultured BCP-ALL cells and MSC were processed in the same way. Purity and viability checks were performed after sorting (*Online Supplementary Figure S1, Online Supplementary Table S3*).

Direct cell-cell contact-mediated interferon response

Dye transfer from Vybrant DiI-labeled (Invitrogen) ALL cells to MSC was determined in direct co-cultures and indirect co-cultures using a transwell setup (0.4 µm pore-size; Corning, New York, NY, USA). Accumulation of dye in MSC was measured after 40 hours of exposure to BCP-ALL cells by flow cytometry (Cytoflex S). Cells in the upper and lower compartments of the transwell were flow-sorted for RNA sequencing as mentioned above.

RNA sequencing

Total RNA (RNA integrity number >6.8) from sorted MSC and leukemic cells was used for ribonucleotide-depleted long-noncoding RNA-sequencing (NovaSeq 6000; Illumina, San Diego, CA, USA). EdgeR (version 3.28.1) was used to perform differential gene expression analysis. Pathway analysis was performed using Pathvisio software.[19]

Lentiviral silencing

Primary BCP-ALL cells (875,000; 1.0×10^6 cells/mL) were cultured for 72 hours with and without IFN signature gene-silenced MSC (50,000). The viability of BCP-ALL cells was determined by flow cytometry.

Inhibition of interferon signaling

Inhibitors of IFNα/β (1.8 ng/mL), and IFNγ (R&D Systems; 3.0 ng/mL) were used in unstimulated co-cultures of MSC and BCP-ALL cells for 40 and 120 hours. Cell viability and the IFN-related gene expression signature were assessed with flow cytometry and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR), respectively.

Drug exposure experiments

The sensitivity of leukemic cells to L-asparaginase, daunorubicin, and prednisolone in the presence of IFNα/β inhibitors was evaluated by flow cytometry after 120 hours' exposure. The drug concentration most discriminative for MSC-induced resistance was determined for each individual BCP-ALL sample prior to the inhibitor experiments (*Online Supplementary Figure S2*).

Quantitative reverse transcriptase polymerase chain reaction

RT-qPCR was performed using a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) (*Online Supplementary Methods*).

Statistical analysis

One-way analysis of variance tests and one-sided (un)paired *t* tests were performed using GraphPad Prism. Correction for multiple testing was included when appropriate. A (paired EdgeR) *P* value <0.05 was considered statistically significant.

Results

B-cell precursor acute lymphoblastic leukemia cells induce an interferon-related gene signature in mesenchymal stromal cells

We performed RNA sequencing on sorted fractions of MSC

after exposure to primary BCP-ALL patients' samples (n=15; Figure 1A) for 40 hours, a co-culture condition for which we previously established that a leukemic niche is created by the interaction of BCP-ALL cells with MSC.[14] Pathway analysis of RNA-sequencing data revealed enrichment for IFN-pathway-activated genes in the MSC after co-culture with BCP-ALL cells (*Online Supplementary Figure S3*). The

induction of IFN-related genes could not be attributed to other cell types, e.g., T-lymphocytes, present in the samples (*Online Supplementary Table S2*). All three tested origins of bone marrow-derived MSC (MSC#1-3) had similar levels of increased IFN-related gene expression in co-culture with BCP-ALL cells, suggesting that the leukemia-induced change in expression is independent from the origin of
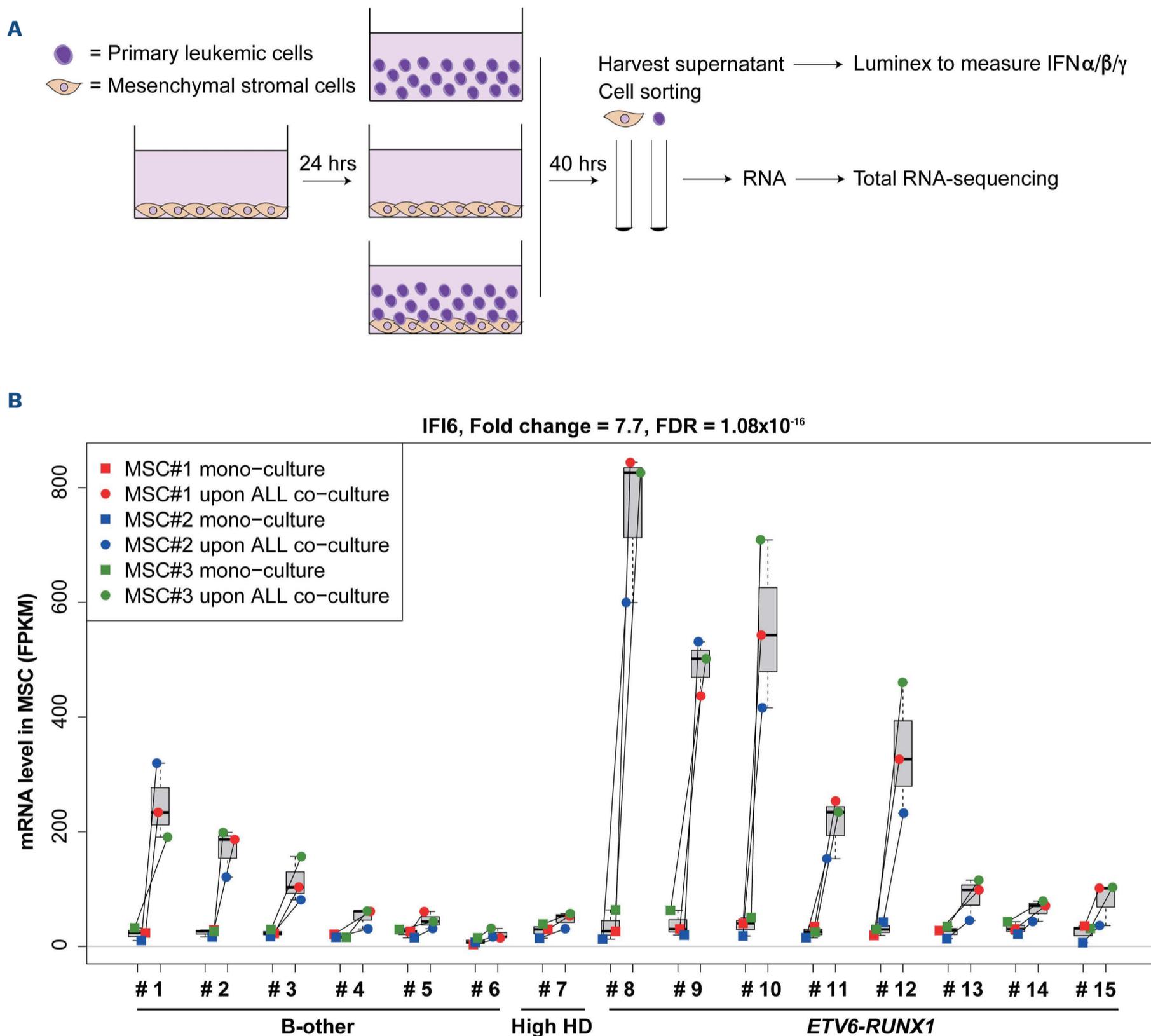
Figure 1. B-cell precursor acute lymphoblastic leukemia cells induce an interferon-related gene signature in mesenchymal stromal cells. (A) Diagram of the experimental setup. RNA sequencing was performed on sorted viable mesenchymal stromal cells (MSC) and acute lymphoblastic leukemia (ALL) fractions after mono-culture and after MSC/ALL co-culture; for the gating strategy see *Online Supplementary Figure S1*. Secreted interferon levels were measured in equal volumes of supernatant of mono-cultures and MSC/ALL co-cultures (Figure 6A). (B) *IFI6* mRNA levels expressed in paired samples of MSC sorted after mono-culture (squares; MSC#1-3 indicated in red, blue, and green, respectively.) and MSC sorted after co-culture with BCP-ALL cells from 15 individual patients (#1, *ETV6-RUNX1*-like; #2-6, B-other; #7, high hyperdiploid; #8-15, *ETV6-RUNX1*; circles). Boxplots represent the interquartile range, the median is depicted by a line. IFN: interferon; FDR: *P* value for the false discovery rate; FPKM: fragments per kilobase million; HD: high hyperdiploid.

MSC (Figure 1B and *Online Supplementary Figure S4*). This is strengthened by the fact that an *ETV6-RUNX1*-positive case induced a similar upregulation of IFN-related genes *IFI6* and *IFITM1* in *ETV6-RUNX1*-derived MSC as well as in B-other derived and healthy control MSC (*Online Supplementary Figure S5*). In addition to *IFI6* and *IFITM1*, other

IFN-related genes were also highly upregulated in MSC after co-culture with *ETV6-RUNX1*-positive cells (ALL#17; *Online Supplementary Figure S6*).

The IFN-related genes including *IFI6, MX1, IFI27*, and *OAS3*, were 4.3- to 7.7-fold upregulated in MSC upon co-culture with BCP-ALL compared to MSC kept in mono-cul-
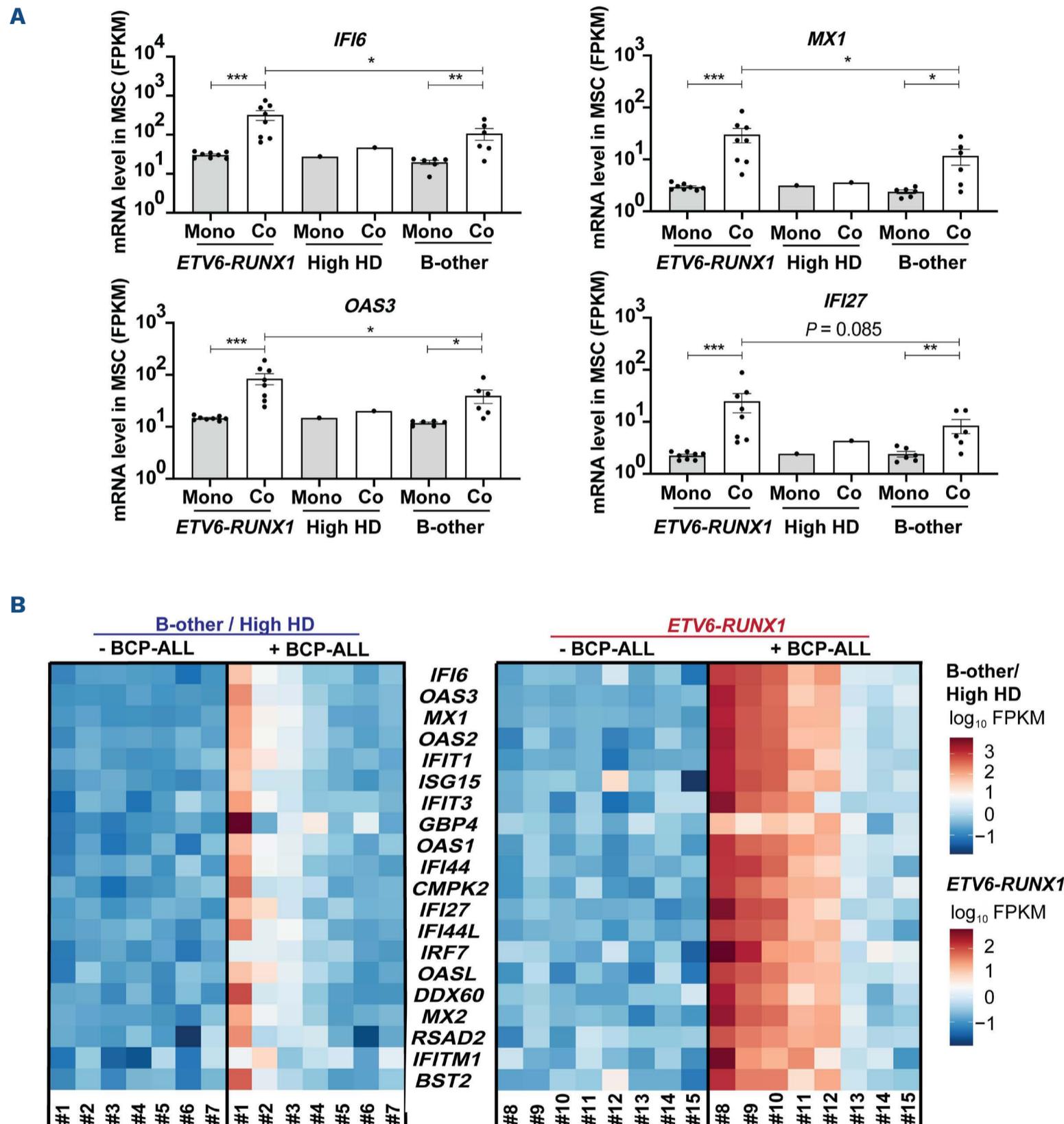
Figure 2. *ETV6-RUNX1* B-cell precursor acute lymphoblastic leukemia cells most potently induce an interferon-related gene signature in mesenchymal stromal cells. (A) mRNA expression levels in mesenchymal stromal cells (MSC) of *IFI6, MX1, OAS3* and *IFI27* upon mono-culture (gray bars) and co-culture (white bars) summarized according to B-cell precursor acute lymphoblastic leukemia (BCP-ALL) subtype (N=8 for *ETV6-RUNX1*, N=1 for high hyperdiploid, N=6 for B-other). Each dot represents the average expression of interferon (IFN)-related genes in three MSC per sort. The mean ± standard error of mean is indicated. (B) Heatmap of expression levels of IFN-related genes in MSC sorted after mono-culture (- BCP-ALL) and those after co-culture with 15 different ALL cases (+ BCP-ALL). The 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. *P<0.05, **P<0.01, ***P<0.001. MSC: mesenchymal stromal cells; FPKM: fragments per kilobase million; Mono: mono-culture; Co: co-culture; HD: high hyperdiploid; BCP-ALL: B-cell precursor acute lymphoblastic leukemia.

ture (false discovery rate [FDR]=1.08x10^{-16}, FDR=2.23x10^{-15}, FDR=3.81x10^{-11}, and FDR=1.44x10^{-15}, respectively.) (Figure 1B and *Online Supplementary Figure S7*). Other IFN-related genes such as *IFITM1, IFI44L, IFIT1* and *ISG15* were 3.1- to 4.3-fold upregulated (FDR=1.08x10^{-6}, FDR=5.21x10^{-11}, FDR=2.99x10^{-12}, and FDR=4.73x10^{-12}, respectively (*Online Supplementary Figure S7*). *STAT1, IRF7,* and *IRF9* were also upregulated in MSC (FDR=9.61x10^{-06}, FDR=1.47x10^{-10}, and FDR=0.00137, respectively), but to a lesser extent (1.5- to 1.9-fold) compared to the previously mentioned genes (*Online Supplementary Figure S7*). *Vice versa*, no MSC-induced change in expression of IFN-signature genes in BCP-ALL cells was found (*Online Supplementary Figures S8* and *S9*). We observed, however, that the intrinsic IFN-related gene profile was slightly higher in mono-cultured *ETV6-RUNX1* ALL cells compared to B-other or high hyperdiploid ALL samples (*Online Supplementary Figures S8* and *S9*). Expression of IFN-related genes was remarkably higher in MSC after co-culture with *ETV6-RUNX1*-positive BCP-ALL cells (n=8) compared to their expression in MSC sorted after co-culture with B-other BCP-ALL cells (n=6) as exemplified for *IFI6* (3-fold increase; *P*=0.031), *MX1* (2.6-fold increase; *P*=0.048) and *OAS3* (2.1-fold increase; *P*=0.04) in Figure 2A and for a set of 20 IFN signature genes in Figure 2B. Of particular interest is case ALL#1, which induced a similar IFN-related gene signature in MSC as *ETV6-RUNX1* cases. ALL#1 turned out to be an *ETV6-RUNX1*-like case as defined by gene expression.[20] In correspondence with

the observation for patients' ALL cells (Figure 2A, B), we noticed that MSC exposed to the *ETV6-RUNX1*-positive cell line REH consistently upregulated IFNα/β genes whereas this was more variable for the non-*ETV6-RUNX1* cell lines (*Online Supplementary Figure S10*). Exposure of MSC to healthy immune cells did not induce the typical IFN signature (n=5) (Figure 3A-C). However, we did observe induction of CXCL10 expression (*P*=0.018), which was most likely due to an interaction with monocytes since this induction was mainly observed in samples containing larger numbers of monocytes. No other IFN signature genes were induced. Thus, we found that the IFN 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.

In previous studies we showed that the viability of leukemic cells depends on a close and direct contact with MSC mediated via tunneling nanotubes,[10] as can be visualized by reduced DiI dye transfer from pre-labeled ALL cells in the upper compartment to MSC in the bottom compartment of a transwell setting (87.6% decrease, *P*<0.0001) (Figure 4A, B). Although preventing the formation of tunneling nanotubes significantly reduced the expression levels of *CXCL10* (3.3-fold, *P*=0.016), *IFI44L* (1.57-fold, *P*=0.009) and *IFITM1* (1.5-fold, *P*=0.005), not all tested genes were affected (i.e., *IFI6, IFITM1, MX1, IFI27*) (Figure 4C). This suggests that tunneling nanotube-dependent signaling

Figure 3. Normal cord blood, with the exception of monocyte-containing preparations, has a limited effect on expression levels of interferon-related genes in mesenchymal stromal cells. *IFI6, ISG15, IFIT1, MX1, CXCL10, IFI44L, IFI27, IFITM1,* and *OAS3* gene expression levels measured by reverse transcriptase quantitative polymerase chain reaction in mesenchymal stromal cells (MSC) (MSC#2) sorted after co-culture with mononuclear cells from five different cord blood samples: cord blood with relatively (A) low (N=2) or (B) high (N=2) percentages of monocytes (>20%), or (C) an unknown cellular composition (N=1). Bars are means of triplicate measurements ± standard error of mean for two independent co-culture experiments. The dashed line (---) indicates mRNA expression levels of MSC after mono-culture, set at 100%. *P*<0.05 MSC: mesenchymal stromal cells.

only partially contributes to the BCP-ALL-induced gene expression changes in MSC.

To examine whether the upregulation of IFN-related genes in MSC had a causal effect on the viability of primary BCP-ALL cells, *IFI6, MX1, IFI27, IFI44L* and *ISG15* were lentivirally silenced resulting in an efficient knockdown of 67-93% for individual genes (*Online Supplementary Figure*

S11A-E). The viability of MSC upon knockdown of several IFN-related genes was largely unaffected compared to controls, except for shIFI27-treated MSC (*P*<0.0001) (*Online Supplementary Figure S11F*). Efficient knockdown of *CXCL10* in MSC could not be achieved by four different short hairpin constructs tested (*data not shown*). Silencing of the strong IFN gene signature observed in MSC upon
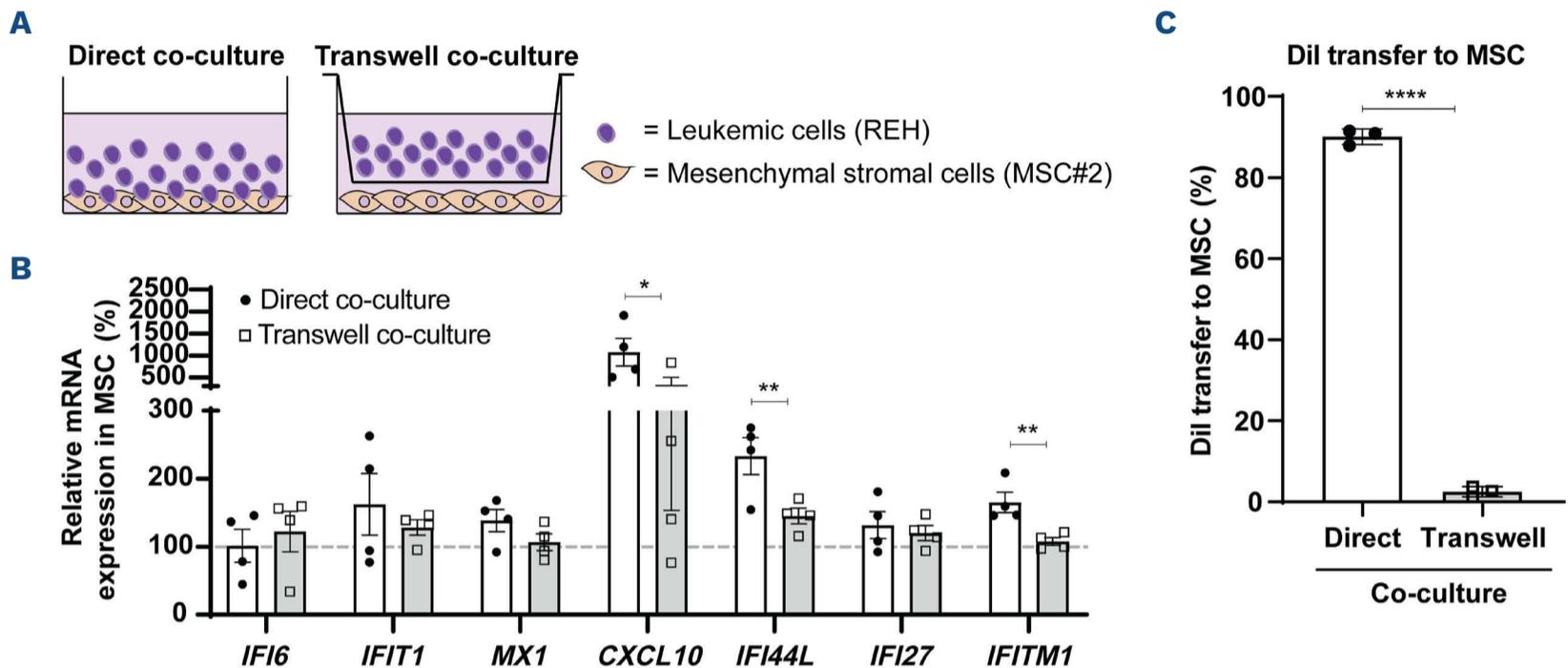
Figure 4. Direct cell-cell contact contributes to induction of Interferon-related gene expression in mesenchymal stromal cells. (A) Schematic overview of the experimental setup of direct co-culture and transwell co-culture of REH cells with mesenchymal stromal cells (MSC) (MSC#2) using 0.4 µm pore-size transwells. (B) Percentage of DiI-positive MSC after direct (circles, white bars) and transwell (squares, gray bars) co-culture with REH leukemic cells for 40 hours. Dots represent averaged duplicate measurements; bars represent means ± standard error of mean (SEM) for three independent experiments. (C) Interferon-related gene expression levels, measured by reverse transcriptase quantitative polymerase chain reaction, in MSC sorted after direct (circles, white bars) or transwell (squares, gray bars) co-culture with REH cells for 40 hours. Dots represent averaged triplicate measurements; bars represent means ± SEM for four independent experiments. The dashed line (---) indicates mRNA expression levels of MSC after mono-culture, set at 100%. *P<0.05, **P<0.01, ****P<0.0001. MSC: mesenchymal stromal cells.
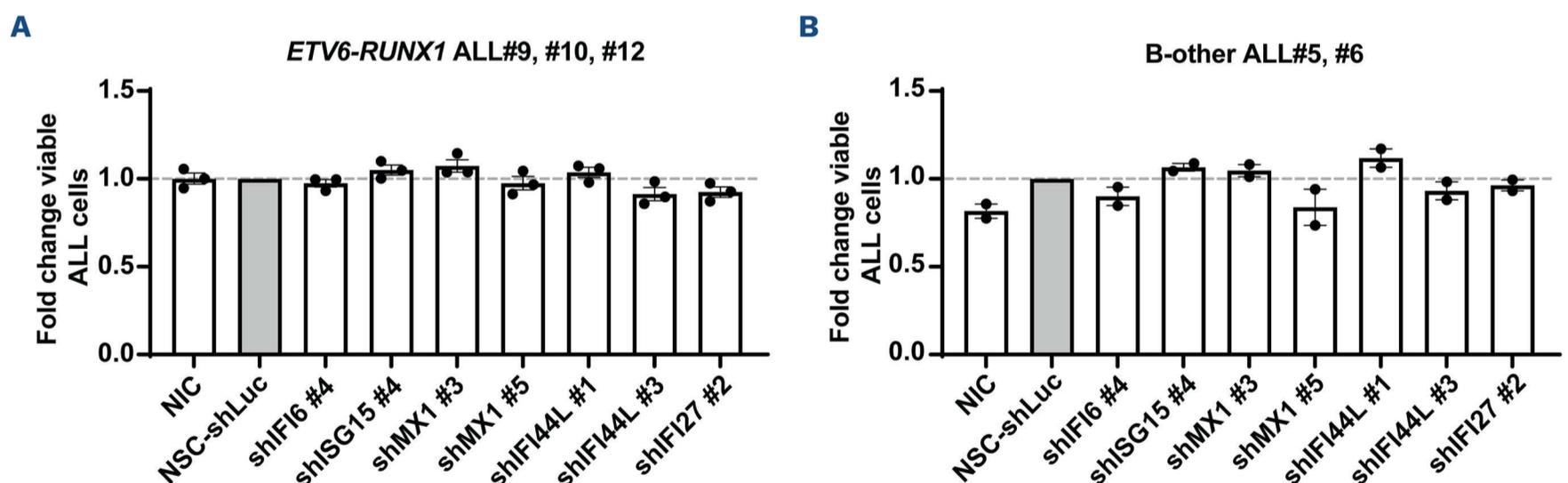
Figure 5. Silencing of interferon-related genes in mesenchymal stromal cells does not affect the viability of primary B-cell precursor acute lymphoblastic leukemia cells. Fold-change in percentage of viable leukemic cells after 72 hours of co-culture with transduced mesenchymal stromal cells (MSC) (MSC#2). A non-silencing control - short-hairpin luciferase (NSC-shLuc) is used as reference. (A) *ETV6-RUNX1* (N=3), and (B) B-other (N=2) B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. Five shRNA per gene were tested for knockdown efficiency; only shRNA resulting in >60% knockdown (shIFI6 #4, shISG15 #4, shMX1 #3/5, shIFI44L #1/3, and shIFI27 #2, see *Online Supplementary Methods*) were used. Bars represent means ± standard error of mean of duplicate measurements for three independent experiments. A dashed line (---) indicates that viability of ALL cells is equal to the viability of ALL cells co-cultured with NSC-shLuc-MSC (fold-change = 1; gray bars). ALL: acute lymphoblastic leukemia; NIC: non-infected control; NSC-shLuc: non-silencing control - short-hairpin-luciferase; sh: short hairpin.

exposure to *ETV6-RUNX1*-positive cells did not markedly decrease the viability of primary *ETV6-RUNX1* (nor B-other) BCP-ALL cells in 3-day culture assays (Figure 5). Moreover, silencing the key regulator of the IFN-signaling pathway, i.e., *STAT1*, in MSC also did not result in altered ALL via-

bility after 40 or 120 hours of co-culture, while there was a reduction of ≥60% in STAT1 protein expression (*Online Supplementary Figure S12*). Thus, the viability of leukemic cells was not affected by interference with IFN signaling via silencing of individual IFN-related genes.
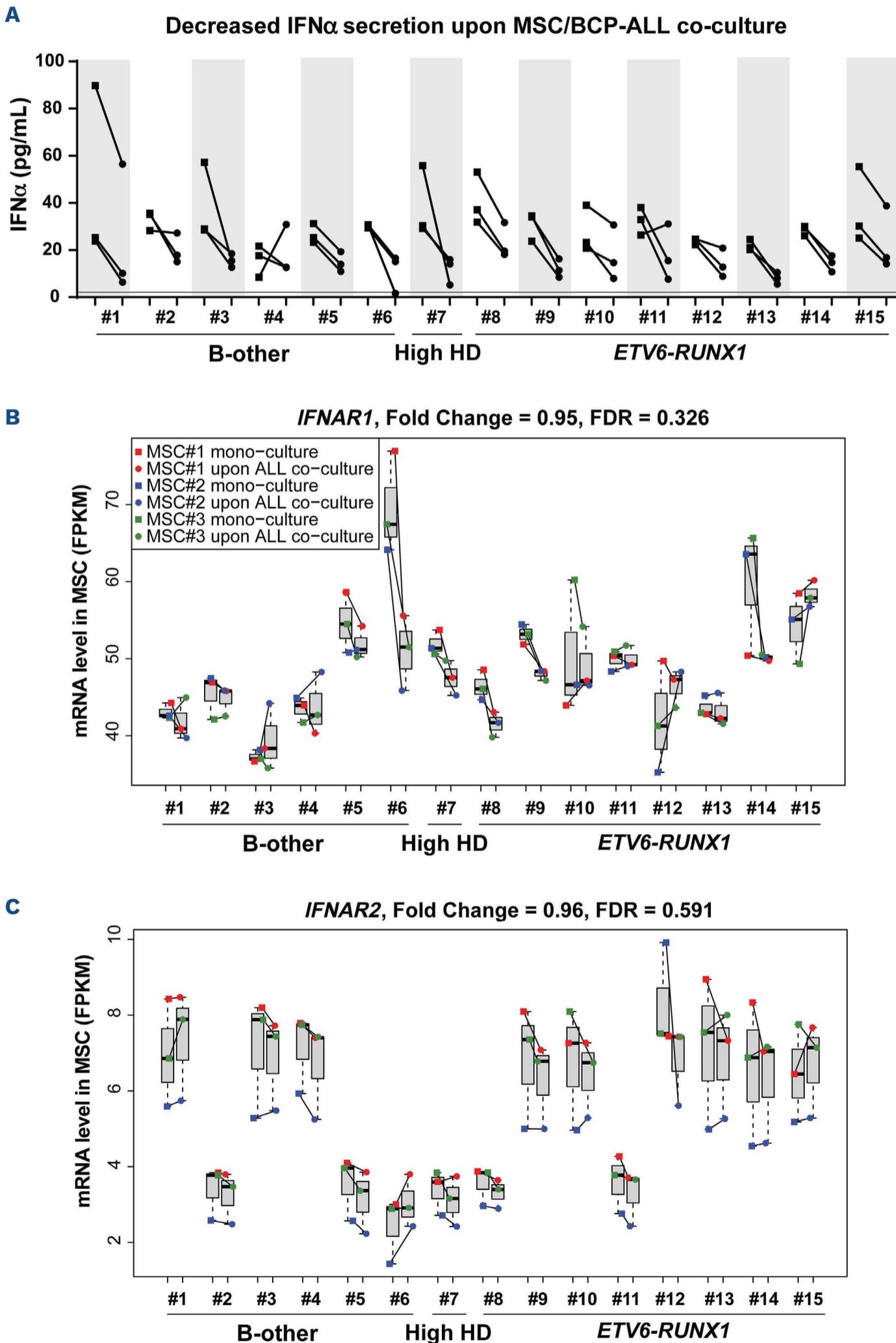
Figure 6. Interferon-α secretion and *IFNAR1* expression decreased in mesenchymal stromal cells after co-culture with primary B-cell precursor acute lymphoblastic leukemia cells. (A) Interferon-alpha (IFNα) concentration in supernatants collected after 40 hours from 15 mesenchymal stromal cells (MSC) (MSC#1-3) and B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (ALL#1-15) mono- and co-cultures. IFNα concentration is corrected for medium background level. Squares and circles indicate IFNα secretion level in the sum of MSC and BCP-ALL mono-cultures and their co-cultures, respectively. The limit of detection is indicated by a solid line. The range of detection for IFNα is 0.5-2,503 pg/mL. (B) *IFNAR1* and (C) *IFNAR2* mRNA levels expressed in paired samples of MSC sorted after mono-culture (squares; MSC#1-3 indicated in red, blue, and green, respectively.) and MSC 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. IFN: interferon; MSC: mesenchymal stromal cells; BCP-ALL: B-cell precursor acute lymphoblastic leukemia; HD: high hyperdiploid; FDR: *P*-value false discovery rate; FPKM: fragments per kilobase million.
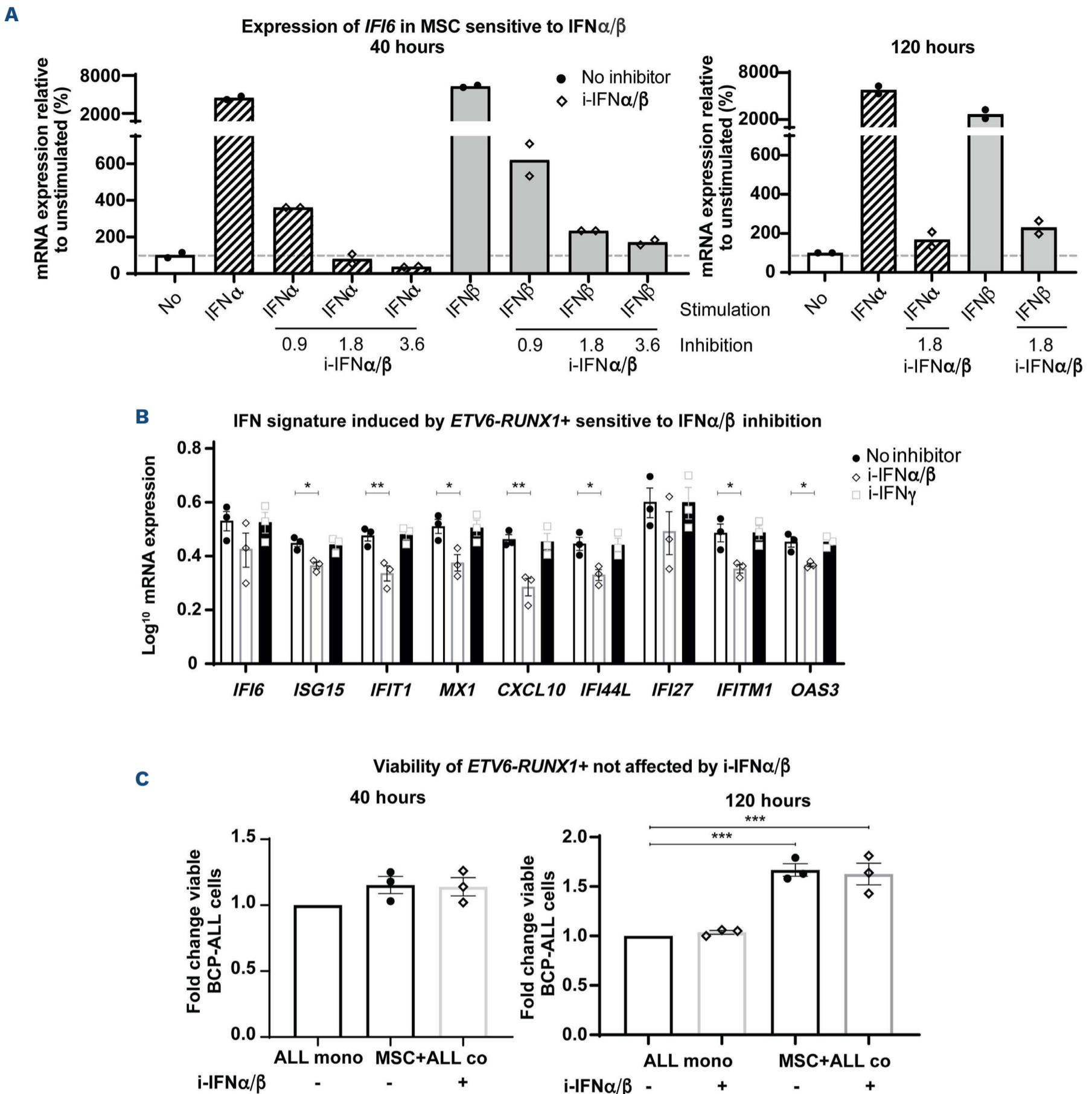
Figure 7. Interferon-mediated response in mesenchymal stromal cells does not affect primary *ETV6-RUNX1* B-cell precursor acute lymphoblastic leukemia viability. (A) mRNA expression levels of *IFI6* in mesenchymal stromal cells (MSC) (MSC#2) after 40 hours (left graph) and 120 hours (right graph) exposure to recombinant human interferon (IFN)α (3.0 U/mL; striped bars), IFNβ (60 pg/mL; gray bars) and inhibitors thereof (i-IFNα/β; 0.9, 1.8 and/or 3.6 ng/mL) (open diamonds). Unstimulated MSC served as the control (white bars; filled circles). The dashed line (---) indicates baseline mRNA expression levels in mono-cultures of MSC set at 100%. (B) Log$_{10}$-transformed IFN-related gene expression levels, measured by reverse transcriptase quantitative polymerase chain reaction, in MSC exposed to primary *ETV6-RUNX1*-positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells for 40 hours with and without inhibitors of IFNα/β (1.8 ng/mL; open diamonds) or IFNγ (3 ng/mL; open squares). Bars represent means ± standard error of mean (SEM) of triplicate measurements for three *ETV6-RUNX1*-positive samples (ALL#9-11). (C) Fold change in viability of *ETV6-RUNX1* BCP-ALL cells after 40 hours (left graph) and 120 hours (right graph) of mono-culture with i-IFNα/β (1.8 ng/mL), MSC co-culture without i-IFNα/β, or MSC co-culture with i-IFNα/β (1.8 ng/mL), normalized to mono-culture without i-IFNα/β. Bars represent means ± SEM of triplicate measurements for three independent experiments. *P<0.05, **P<0.01, ***P<0.001. MSC: mesenchymal stromal cells; i-IFNα/β: inhibitors of interferon α/β; i-IFNγ: inhibitor of interferon γ; BCP-ALL: B-cell precursor acute lymphoblastic leukemia; mono: mono-culture; co: co-culture.

B-cell precursor acute lymphoblastic leukemia cells trigger an IFNα/β but not IFNγ response in mesenchymal stromal cells

Remarkably, the amount of secreted IFNα (and IFNβ) was decreased in the co-culture setting compared with the levels detected in mono-culture of BCP-ALL cells and MSC, an effect unrelated to *ETV6-RUNX1* status (Figure 6A and *Online Supplementary Figure S13A, left*). This is remarkable since the *ETV6-RUNX1*-positive cells clearly induced the expression of IFN-related genes in MSC (Figures 1 and 2). The expression level of the IFNα/β-binding *IFNAR1*, but not *IFNAR2* receptor, was often decreased in MSC after co-culture with BCP-ALL, independently of *ETV6-RUNX1* status (Figure 6B). Secreted levels of IFNγ were below the level of quantifiable detection in virtually all samples and (mono/co)-culture conditions (*Online Supplementary Figure S13A, right*). Expression levels of the IFNγ receptor genes *IFNGR1* and *IFNGR2* were variable and not related to *ETV6-RUNX1* status (*Online Supplementary Figure S13C*).

The gene regulation in MSC is sensitive to IFNα/β since addition of recombinant IFNα and IFNβ to mono-cultures of MSC for 40 and 120 hours clearly induced the expression of the (most significantly upregulated) IFN-index gene *IFI6* in MSC. In correspondence, the *ETV6-RUNX1*-mediated induction of *IFI6* expression was prevented by simultaneous addition of inhibitors of IFNα/β signaling, resulting in 56-fold and 27-fold reductions, respectively, after 40 hours of incubation and 35-fold and 12-fold reductions, respectively, after 120 hours of incubation (Figure 7A). The *ETV6-RUNX1* BCP-ALL-induced expression of IFN-related genes in MSC was also reduced upon exposure to inhibitors of IFNα/β but not to inhibitors of IFNγ (Figure 7B). However, addition of these inhibitors did not affect the viability of BCP-ALL cells in co-culture with MSC, after either 40 hours or 120 hours of incubation, whereas MSC clearly provided a survival benefit to *ETV6-RUNX1* BCP-ALL cells at a prolonged incubation of 120 hours (Figure 7C). This MSC-induced survival benefit for *ETV6-RUNX1* BCP-ALL was not sensitive to IFNα/β inhibition (Figure 7C, right). Furthermore, inhibitors of IFNα/β did not modulate MSC-induced resistance of *ETV6-RUNX1* BCP-ALL cells to L-asparaginase, daunorubicin and prednisolone (Figure 8). In conclusion, the level of resistance of BCP-ALL cells to three chemotherapeutic agents was unaffected by blockade of IFN signaling in MSC.

Discussion

Leukemic cells communicate with components of the bone marrow microenvironment in such a way that protection against chemotherapy and survival of leukemic cells is stimulated.[9-13,21] In this study, we showed that *ETV6-RUNX1* BCP-

Drug resistance not affected by i-IFNα/β
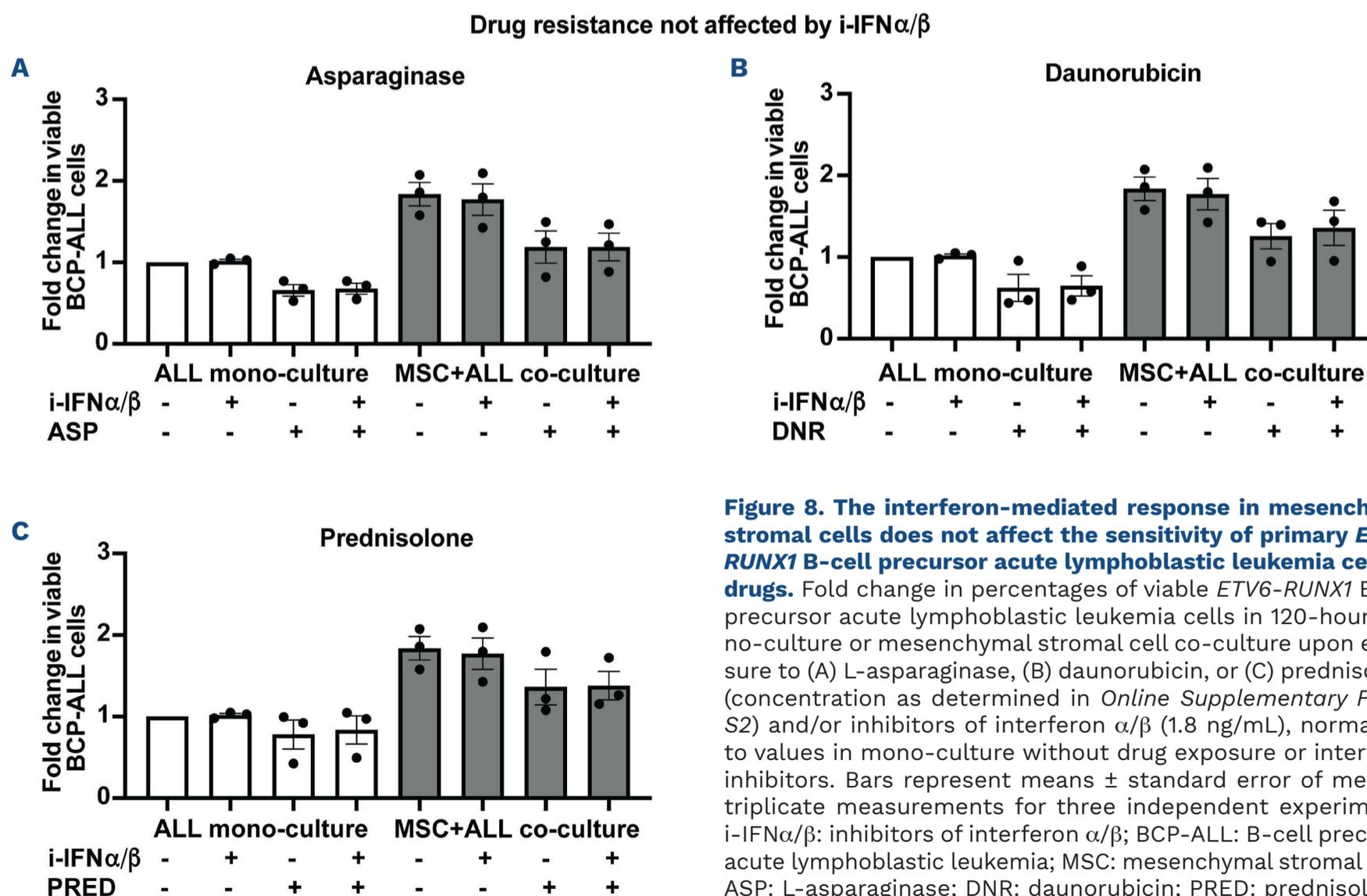
Figure 8. The interferon-mediated response in mesenchymal stromal cells does not affect the sensitivity of primary *ETV6-RUNX1* B-cell precursor acute lymphoblastic leukemia cells to drugs. Fold change in percentages of viable *ETV6-RUNX1* B-cell precursor acute lymphoblastic leukemia cells in 120-hour mono-culture or mesenchymal stromal cell co-culture upon exposure to (A) L-asparaginase, (B) daunorubicin, or (C) prednisolone (concentration as determined in *Online Supplementary Figure S2*) and/or inhibitors of interferon α/β (1.8 ng/mL), normalized to values in mono-culture without drug exposure or interferon inhibitors. Bars represent means ± standard error of mean of triplicate measurements for three independent experiments. i-IFNα/β: inhibitors of interferon α/β; BCP-ALL: B-cell precursor acute lymphoblastic leukemia; MSC: mesenchymal stromal cells; ASP: L-asparaginase; DNR: daunorubicin; PRED: prednisolone.

ALL cells induce an IFN-related gene signature in MSC, which was dependent on IFNα/β signaling, but independent of IFNγ. The IFN gene signature of MSC was leukemia-dependent since a mixed population of normal cord blood cells had a limited effect on expression levels of these genes in MSC, with an exception for monocyte-containing samples which induced, among others, CXCL10 expression. Monocytes, however, were absent in our BCP-ALL samples, strengthening the leukemia-driven origin of the IFN signature in MSC. BCP-ALL samples with an *ETV6-RUNX1* translocation were the most potent inducers of the IFN-related gene expression signature in MSC. This was true for both primary BCP-ALL samples as well as an *ETV6-RUNX1* cell line. In correspondence, we observed that addition of IFNα/β inhibitors (but not an IFNγ inhibitor) to co-cultures of *ETV6-RUNX1* BCP-ALL and MSC reduced the expression levels of IFN-related genes in the MSC compared to those in MSC that were co-cultured in the absence of these inhibitors.

ETV6 is known to be a transcriptional activator of a variety of IFN genes,[22] and in correspondence higher baseline expression levels of IFN-related genes have been found in *ETV6-RUNX1*-driven leukemia.[23] We also noticed some higher baseline levels in *ETV6-RUNX1*-positive ALL than in B-other ALL cases, but more remarkable was the upregulation of IFN-related genes in MSC upon exposure to *ETV6-RUNX1*-positive cells. This upregulation was partially dependent on close cell-cell contact, since we noticed that the expression was reduced when BCP-ALL cells and MSC were physically separated in transwell experiments. In line with this, Dander *et al.* showed that the *ETV6-RUNX1* fusion can trigger modifications in adhesion molecule expression and adherence capacity of B-precursor cells.[24]

Members of the IFN family are known to combat viral infections, modulate immune responses, and stimulate antitumor activities.[25-27] We noted that BCP-ALL cells, irrespective of *ETV6-RUNX1* status, downregulated expression of the MSC-IFNα/β receptor (*IFNAR1*) and secreted IFNα/β levels in co-culture with MSC. The most likely explanation is that this occurs due to receptor internalization upon ligand binding, which is part of the functional process of IFN-pathway activation.[28] Internalization of the IFNAR1/2-IFNα/β complex may be visualized by a fluorescence-based imaging method using a pH-sensitive dye that binds the receptor and will only fluoresce in acidic environments inside the cell.[29,30]

Recently, interferons were shown to elicit immune suppressive mechanisms in cancer, which may promote cancer progression and induce therapy resistance.[31,32] Several studies have investigated the expression profile of IFN-related genes in cells derived from distinct cancer types.[33-36] A subset of IFN-related genes, including the genes we found upregulated in MSC, i.e., *IFI27, OAS1, OAS3, MX1* and *ISG15*, was persistently overexpressed in tumor cells resistant to chemotherapy or radiotherapy.[33,34,36] We here observed that these same genes were induced in non-malignant stromal support cells, i.e., the MSC, upon interaction with *ETV6-RUNX1*-positive ALL cells. MSC provide a survival benefit to BCP-ALL cells and protect these cells against chemotherapeutic drugs.[9-12] However, we did not observe that the IFN-induced gene signature causally contributed to these processes since neither silencing of individual IFN-related genes or the key pathway regulator *STAT1* in MSC nor blockade of IFNα/β signaling counteracted this MSC-mediated benefit. Our data therefore imply that the *ETV6-RUNX1* BCP-ALL-induced IFNα/β signature in MSC serves a different role, e.g., attracting other cells, which are beneficial in maintaining leukemia, to the leukemic niche. IFNα/β are involved in recruitment, function, maturation and/or activation of immune cells, such as NK cells, monocytes and dendritic cells.[37,38] Cytokine and chemokine production can be induced by IFNα/β and may help to recruit inflammatory monocytes and NK cells to the site of inflammation.[31,38] In contrast, prolonged activation of IFNα/β responsive genes can also suppress normal immune cell functions. In solid tumors, migration of NK cells towards the tumor site was observed. Trapped in the tumor microenvironment these NK cells downregulated NKG2D, the receptor that is important in defense against cancer by the immune system.[39] This network of trapped, but not functional (innate) immune cells, resulted in a condition promoting tumor cell proliferation, survival, and metastasis.[39,40] However, increased IFN-related gene expression in MSC after contact with leukemic cells may also reflect an anti-leukemic response of MSC in the tumor microenvironment to attract other immune cells.[31,41,42] A recent study by Kumar *et al.* showed that reduced production of IFNα/β, leading to decreased NK-cell surveillance, promotes development of B-ALL *in vivo*.[42] Restoring this IFNα/β production allows for NK effector cells to reduce leukemia progression in mice that are prone to MYC-driven B-ALL. In high-risk acute myeloid leukemia, IFNα/β administration after hematopoietic stem cell transplantation may be effective in preventing relapse.[43] As interferons are often described as cytokines with a dual role,[26,31,38] it still needs to be determined whether the observed IFNα/β response in our study is advantageous or detrimental to leukemic cells. In conclusion, our study reveals that IFNα/β but not IFNγ contribute to an IFN signature elicited by BCP-ALL in MSC. The induction of IFN-related genes in MSC did not affect the viability and level of drug resistance of BCP-ALL cells observed upon exposure to MSC. Our data warrant further studies into the role of the BCP-ALL-induced IFNα/β response, especially in the context of *ETV6-RUNX1*-positive ALL. This may increase our understanding of how leukemic cells manipulate the immune microenvironment, as recently suggested for IFNα/β-dependent activation of NK cells and dendritic cell-presenting leukemia-specific antigens. This knowledge is important in the emerging field of cell-based immunotherapies that are being applied ever more frequently in BCP-ALL.[3,42]

Data-sharing statement
Prof. Monique L. den Boer (m.l.denboer@prinsesmaxima-centrum.nl) can be contacted for original data.

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