Acute Lymphoblastic Leukemia

**ActivinA: a new leukemia-promoting factor conferring migratory advantage to B-cell precursor-acute lymphoblastic leukemic cells**

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**ABSTRACT**

B-cell precursor-acute lymphoblastic leukemia modulates the bone marrow (BM) niche to become leukemia-supporting and chemoprotective by reprogramming the stromal microenvironment. New therapies targeting the interplay between leukemia and stroma can help improve disease outcome. We identified ActivinA, a TGF-β family member with a well-described role in promoting several solid malignancies, as a factor favoring leukemia that could represent a new potential target for therapy. ActivinA resulted over-expressed in the leukemic BM and its production was strongly induced in mesenchymal stromal cells after culture with leukemic cells. Moreover, MSCs isolated from BM of leukemic patients showed an intrinsic ability to secrete higher amounts of ActivinA compared to their normal counterparts. The pro-inflammatory leukemic BM microenvironment synergized with leukemic cells to induce stromal-derived ActivinA. Gene expression analysis of ActivinA-treated leukemic cells showed that this protein was able to significantly influence motility-associated pathways. Interestingly, ActivinA promoted random motility and CXCL12-driven migration of leukemic cells, even at suboptimal chemokine concentrations, characterizing the leukemic niche. Conversely, ActivinA severely impaired CXCL12-induced migration of healthy CD34+ cells. This opposite effect can be explained by the ability of ActivinA to increase intracellular calcium only in leukemic cells, boosting cytoskeleton dynamics through a higher rate of actin polymerization. Moreover, by stimulating the invasiveness of the leukemic cells, ActivinA was found to be a leukemia-promoting factor. Importantly, the ability of ActivinA to enhance BM engraftment and the metastatic potential of leukemic cells was confirmed in a xenograft mouse model of the disease. Overall, ActivinA was seen to be a key factor in conferring a migratory advantage to leukemic cells over healthy hematopoiesis within the leukemic niche.

**Introduction**

Acute lymphoblastic leukemia (ALL) is the most frequent childhood malignancy worldwide. B-cell precursor (BCP)-ALL represents about 80% of ALL cases and mainly affects children, with an incidence of 3-4 cases per 100,000 each year.1 Even
though the cure rate exceeds 80% in children, BCP-ALL is the leading cause of cancer-related death in children and young adults.\(^3\) In spite of the notable improvements in disease management, the emergence of chemoresistance decreases the probability that therapy will be successful, and leads to relapse in more than 20% of treated patients.\(^3\) BCP-ALL cells critically depend on interactions with the bone marrow (BM) microenvironment, which provides essential regulatory cues for proliferation, survival and drug resistance, and such interactions contribute to treatment failure and disease relapse.\(^4\) In particular, mesenchymal stromal cells (MSCs) have been recognized as an essential supportive element of the leukemic hematopoietic microenvironment because of their ability to define exclusive BM niches that sustain leukemic cells to the detriment of normal hematopoiesis and resist chemotherapy.\(^5\) In this complex network, it has been shown that chemokines could contribute to BCP-ALL development by driving the migration of leukemic cells toward protective BM niches, as well as by providing anti-apoptotic signals.\(^6\)

ActivinA is a pleiotropic cytokine that belongs to the TGF-\(\beta\) superfamily. It has a broad tissue distribution, being involved in multiple physiological and pathological processes, including inflammation, metabolism, immune response, and endocrine function. Recent studies have demonstrated that ActivinA is an important regulator of carcinogenesis. Indeed, it can directly modulate cancer cell proliferation and migration. It can also enhance tumor progression by regulating the tumor microenvironment.\(^7\) ActivinA sends signals through its transmembrane serine/threonine kinase receptors. It binds to type II Activin receptors (ACVR2A or ACVR2B), causing recruitment, phosphorylation and activation of type I Activin receptors (ALK2 or ALK4). ActivinA signaling is inhibited by Inhibins, through competitive binding for Activin receptors, and by Follistatin (FST) and Follistatin-like-3 (FSTL3), which act as trap molecules.\(^8\) The Activin receptor II ligand trap ACE-011 is currently under investigation in a Phase II clinical trial on multiple myeloma.\(^9\)

The aim of the current study was to explore the role of ActivinA in the leukemic BM niche, with a particular focus on its supportive role for BCP-ALL cells to the detriment of healthy hematopoiesis.

**Methods**

**Patients’ and healthy donors’ samples**

Bone marrow plasma samples were collected from 125 BCP-ALL patients at diagnosis and from 56 healthy donors (HDs). Primary BCP-ALL cells were isolated at diagnosis from 22 BM aspirates and used for *in vitro* assays. Details of the study cohort are shown in the Online Supplementary Appendix. The study was approved by the Institutional Review Board (AIEOP-BFM ALL 2009 protocol; EudraCT-2007-004270-43).

**Culture of B-cell precursor-acute lymphoblastic leukemia cell lines**

The leukemic cell lines 697, NALM-6, R54.11, SUP-B15 and REH were cultured as described in the Online Supplementary Appendix.

**Isolation of bone marrow-mesenchymal stromal cells**

Bone marrow-mesenchymal stromal cells (BM-MSCs) from 15 BCP-ALL patients (ALL-MSCs) and 15 age-matched HDs (HD-MSCs) (Online Supplementary Table S1) were cultured as described in the Online Supplementary Appendix.

**Cord blood-CDC4 and bone marrow-CDC4 cell isolation**

CD34+ cells were isolated from cord blood (CB) units and HD BM aspirates, as described in the Online Supplementary Appendix.

**Co-culture of primary leukemic cells with bone marrow-mesenchymal stromal cells**

Bone marrow-mesenchymal stromal cells were co-cultured with primary leukemic cells at an MSC:leukemia ratio of 1:30, either in the presence or in the absence of 0.4 \(\mu\)m Transwell inserts (Costar Transwell\textsuperscript{®} Permeable Supports, Corning Inc., MA, USA) in DMEM 2% FBS. After 72 hours (h), supernatants were collected and cryopreserved at -80°C for further analyses.

**ELISA assay for quantification of Activin, CXCL12 and pro-inflammatory cytokines**

The levels of ActivinA, pro-inflammatory cytokines (IL-\(\beta\), IL-6 and TNF-\(\alpha\)) and CXCL12 were assessed in BM plasma samples and culture supernatants using commercially available ELISA kits (R&D Systems, USA), according to the manufacturer’s instructions.

**Quantitative RT-PCR**

qRT-PCR was performed using LightCycler\textsuperscript{®} 480 (Roche, Basel, Switzerland), as reported in the Online Supplementary Appendix.

**Gene expression profile analysis**

Gene expression profile analysis of 697 cells treated or not with ActivinA (50 ng/mL) for 6 h and 24 h was evaluated by GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix Inc., Santa Clara, CA, USA). Details of the procedure are described in the Online Supplementary Appendix.

**Time-lapse microscopy**

Leukemic cells were seeded in Gelatin B-coated wells of an 8-well chamber slide (Ibidi, Martinsried, Germany). Cell tracks were recorded as described in the Online Supplementary Appendix.

**Chemotaxis assays**

Both leukemic and healthy CD34 \(^+\) cells, pretreated or not with ActivinA (24 h stimulation), were tested for chemotaxis in Transwell-based assays (Costar Transwell\textsuperscript{®} Permeable Supports, Corning Inc., MA, USA). Selected experiments were performed using the ALK4 blocker SB431542 (SigmaAldrich, St Louis, MO, USA). Details are described in the Online Supplementary Appendix.

**Invasion assays**

The invasive capacity of leukemic cells was evaluated using Matrigel-coated Transwells, as described in the Online Supplementary Appendix.

**Activin receptor analyses**

697 leukemic cell line and primary blasts were analyzed for ALK4, ACVR2A and ACVR2B expression by flow cytometry and qRT-PCR and for ALK2 expression by western blot, as described in the Online Supplementary Appendix.

**CXCR4 and CXCR7 staining**

Expression of CXCR4 and CXCR7 was analyzed by flow cytometry in 697 cells, pretreated or not with ActivinA (Online Supplementary Appendix).
Filamentous (F)-actin polymerization assay
F-actin polymerization was analyzed in 697 cells pretreated or not with ActivinA using AlexaFluor 647-labeled phalloidin (Invitrogen, Carlsbad, CA, USA) before and after CXCL12 stimulation, as reported in the Online Supplementary Appendix.

Calcium mobilization
Intracellular calcium mobilization was measured by flow cytometry using the Fluo-4NW Assay (Invitrogen), as reported in the Online Supplementary Appendix.

B-cell acute lymphoblastic leukemia xenograft model
Female 7-9-week-old NOD-SCID-γ-chain−/− (NSG) mice (Charles River, Calco, Italy) were intravenously (i.v.) transplanted with 0.5x10^6 697 cells or 10^6 NALM-6 cells, either pretreated or not with ActivinA for 24 h. Details are described in the Online Supplementary Appendix. The study was approved by the Italian Ministry of Health (approval n. 64/2014).

Statistical analyses
Differences between subgroups were compared with the Mann-Whitney test or Wilcoxon matched-pairs signed rank test in the case of matched values.

Results

Stroma-derived ActivinA increased in response to leukemia
It has been demonstrated that ActivinA could exert a pro-tumoral role in several types of cancer both through direct effects on tumoral cells and indirect effects on the tumor microenvironment. Therefore, we first measured ActivinA levels in BM plasma samples from 44 HDs and 108 BCP-ALL patients at disease onset. ELISA assay revealed that ActivinA was significantly increased in the BM plasma of BCP-ALL patients compared to that of HDs (Figure 1A). The median concentration (mc) of ActivinA was 400 pg/mL (range: 62.5-724 pg/mL) in BCP-ALL patients and 273.4 pg/mL (range: 62.5-2338 pg/mL) in HDs (P<0.05).

To determine whether ActivinA plasma levels at BCP-ALL diagnosis were related to disease outcome/severity, we analyzed 98 patients with available follow up out of the 108 tested, considering several clinical and biological parameters. In detail, 3- and 4-year event-free survival (EFS) and sensitive quantitative PCR-based minimal residual disease (MRD) at days +33 and +78 and final risk were taken into account. ActivinA levels did not impact on EFS, on PCR-MRD risk, or on patients’ final risk stratification (data not shown).

It had previously been shown that BM-MSCs exhibit a basal level of ActivinA secretion. In view of the pivotal role of BM-MSCs in sustaining the leukemic niche, we explored the regulation of MSC-derived ActivinA in the context of BCP-ALL. We first confirmed that MSCs isolated from the BM of HDs (HD-MSCs) were able to constitutively produce the molecule (mc: 103.2, range: 62.5-526.7 pg/mL). Then, to test whether BCP-ALL cells could modulate MSC-derived ActivinA, we set up co-culture experiments of HD-MSCs with primary leukemic blasts and quantified ActivinA in supernatants after 72 h of co-culture. Interestingly, we found that primary leukemic cells significantly induced ActivinA in MSCs both through...
soluble factors (mc: 268.1, range: 62.5-842.8 pg/mL) and a cell-to-cell contact-mediated mechanism (mc: 777.9, range: 96.2-1456 pg/mL), with a 2.6 and a 7-fold increase, respectively, compared to the basal condition (P<0.001 and P<0.0001). Notably, primary BCP-ALL cells secreted either very low or even undetectable levels of ActivinA (Figure 1B).

Leukemic cells expressed ActivinA receptors

To determine whether BCP-ALL cells could be targets of ActivinA, the expression of Activin receptors was assessed on five leukemic cell lines (697, NALM-6, RS4;11, SUP-B15, REH) (Figure 2A) and eighteen primary BCP-ALL blasts by flow cytometry and western blot analyses (Figure 2B). Western blot images are shown in Online Supplementary Figure S1.

Both type I and type II Activin receptors were found to be expressed by all primary blasts and cell lines tested, with a markedly wide range of expression. The expression level of ActivinA receptors in primary BCP-ALL cells was highly patient-specific and was shown to be independent of the commonly investigated leukemia-related genetic alterations.

Taken together, these data suggest that BCP-ALL cells could possibly respond to ActivinA. Moreover, we showed that ActivinA was able to significantly increase the expression of its type I receptors, thus suggesting a positive loop underlying the responsiveness of leukemic cells to ActivinA (Online Supplementary Figure S2).

Subsequent analyses were performed on the 697 and NALM-6 cell lines as their different Activin receptor expression makes them representative of the high inter-patient variability observed.

**Gene expression analysis revealed ActivinA involvement in regulating cell motility**

For a more in depth analysis of the molecular changes induced by ActivinA in BCP-ALL cells, we performed gene expression profile (GEP) analysis of 697 cells upon stimulation with ActivinA for 6 h and 24 h. We found that 122 genes were differentially expressed in ActivinA-treated cells versus untreated cells after 6 h of stimulation (FDR<0.05) and that 151 genes were differentially expressed after 24 h of stimulation (FDR<0.05). Gene Ontology (GO) analysis of differentially expressed genes identified enriched GO categories (Online Supplementary Figure S3A) critically linked to cancerogenesis such as “regulation of cell activation”, “positive regulation of antigen receptor-mediated signaling pathway”, “pathways in cancer”, etc.

Interestingly, we also observed that ActivinA was able to influence migration-associated pathways, such as “calcium ion homeostasis and transport into cytosol”, “PI3K/AKT activation”, “Ras signaling pathway”, “focal adhesion”, suggesting its possible effect on leukemic cell motility. These data are in agreement with the recently recognized role of ActivinA in the regulation of cell migration and invasion in the context of several solid malignancies. On the basis of this evidence, we first used qRT-PCR assays to validate the ActivinA-mediated changes in
the expression of several genes linked to Ca2+ homeostasis (ATP2B2, ATP2B4), Ras pathway activation (VAV3), and cell motility and movement regulation (ARHGAP25, CORO1A, DOCK4, LCK, PTPRC). Data obtained in qRT-PCR on 697 cells were highly concordant with microarray data (Online Supplementary Figure S3B). Raw data are shown in Online Supplementary Figure S5A. In addition, qRT-PCR analyses of the above-mentioned genes were performed on 7 primary BCP-ALL patients’ samples stimulated or not with ActivinA (Online Supplementary Figure S5). Among them, 3 presented the t(1;19) (gray dots) typical of the 697 cell line. Interestingly, four of the tested genes (ARHGAP25, CORO1A, DOCK4 and PTPRC) were significantly modulated in at least one stimulation time point (Online Supplementary Figure S5B) by ActivinA in more than 70% of patients, similar to our observations in 697 cells. It is worth noting that the t(1;19) patients showed a modulation of the ATP2B4, VAV3 and LCK genes (Online Supplementary Figure S5B) more similar to 697 cells than the translocation negative ones.

**ActivinA increased random motility, chemotaxis and invasion of B-cell precursor-acute lymphoblastic leukemia cells**

To test whether ActivinA was able to modulate BCP-ALL movement, we performed time-lapse microscopy (TLM) analyses and migration assays. TLM studies showed that ActivinA was able to increase random motility both in the 697 cell line (P<0.0001) (Figure 3A, left) and primary leukemic cells (P<0.0001) (Figure 3A, right).

It has been demonstrated that chemokines, and in particular the CXCR4/CXCL12 axis, play a key role in the homing and retention of ALL cells within the BM niche. Therefore, we tested whether ActivinA was able to modulate CXCL12-induced migration of leukemic cells using a Transwell-based migration assay. Notably, we found that ActivinA-pretreated 697 cells showed a significant increase in CXCL12-driven migration (P<0.05) (Figure 3B). Importantly, this finding was confirmed in primary leukemic cells obtained from the BM of 13 BCP-ALL patients collected at diagnosis (Figure 3C). The average percentage of primary unstimulated BCP-ALL blasts migrated in response to CXCL12 (mean: 15.6%, range: 1.9-43.5%) was significantly increased following ActivinA stimulation (mean: 22.9%, range: 4.1-61.1%) (P<0.001).

To ensure the specificity of the observed effect on CXCL12 mediated migration, we blocked ActivinA/Activin receptor axis by using SB431542, a well-characterized specific inhibitor of transforming growth factor-β superfamily type I Activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7.16,17 SB431542 inhibited the migration of ActivinA-pretreated 697,
NALM-6 cells (Online Supplementary Figure S6) and primary leukemic blasts (Figure 3D) in response to CXCL12 in a dose-dependent manner. In 3 different patients, we demonstrated that 10 μM SB431542 inhibited ActivinA stimulatory effect on CXCL12-driven migration of 78.8% (range: 74.5-84.0%; \( P < 0.05 \)).

Interestingly, it has been reported that ActivinA expression is associated with an invasive phenotype in several types of cancer, including ovarian cancer, esophageal adenocarcinoma, breast cancer, and oral squamous cell carcinomas.\(^{12-15}\) Therefore, we tested whether ActivinA was able to modulate leukemic cell invasive capacity using Matrigel-coated Transwells. We found that ActivinA increased the ability of primary BCP-ALL cells to migrate through a complex matrix in the presence of CXCL12 (Figure 3E), with a 2-fold increase compared to the untreated condition (\( P < 0.05 \)).

**ActivinA enhanced leukemic cells responsiveness to low levels of CXCL12**

CXCL12 reduction is one of the microenvironmental alterations occurring in the leukemic BM, as observed in both mice models and leukemic patients,\(^{18,19}\) which is associated with impairment of normal hematopoiesis.\(^{5}\) Here, in a large cohort of 70 patients, we confirmed a significant reduction of approximately 6 times the CXCL12 of BM plasma level in BCP-ALL patients (mc: 77.7, range: 15.7-488.9 pg/mL) compared to HDs (mc: 476.8, range: 99.1-1763 pg/mL, n=46) \( P < 0.0001 \) (Figure 4A). To test the potential ability of ActivinA to increase the responsiveness of leukemic cells to suboptimal concentrations of CXCL12, we performed dose-response chemotaxis assays. We demonstrated that ActivinA enhanced 697 cell line migration toward CXCL12 used at a concentration 10- or 100-fold lower than that classically used in in vitro migration assays (100 ng/mL). Indeed, ActivinA pretreatment induced a 10-fold increase in the CXCL12-driven chemotaxis toward 10 ng/mL CXCL12 \( P < 0.05 \) and a 7-fold increase toward 1 ng/mL CXCL12 \( P < 0.05 \), compared to untreated cells, that showed a responsiveness to these low chemokine concentrations comparable to that of empty medium (Figure 4B).

**Intracellular calcium levels and actin polymerization were increased by ActivinA in leukemic cells**

To further investigate the enhanced leukemic cell responsiveness to CXCL12, we first evaluated whether ActivinA treatment could affect chemokine receptor expression. Flow cytometry analysis of CXCL12 chemokine receptors on 697 cells showed that the levels of CXCR4 and CXCR7, evaluated both as extracellular receptors and intracellular pool, were not affected by ActivinA (Figure 5A).

We, therefore, performed flow cytometry analysis to determine the effect of ActivinA on the intracellular calcium level of BCP-ALL cells. Our data indicated that both in 697 cells (Figure 5B) and in primary BCP-ALL cells (Figure 5C) the basal intracellular calcium content was increased in ActivinA-pretreated cells as compared to untreated cells (mean range 1; \( P < 0.05 \)). Interestingly, on addition of CXCL12, ActivinA-treated cells showed a further significant increase in the concentration of free cytosolic Ca\(^{2+}\) compared to the untreated cells (Figure 5B and 5C, peak and mean range 2; \( P < 0.05 \)).

Moreover, we evaluated the effect of ActivinA on actin cytoskeleton dynamics. Since the conversion of globular
into filamentous actin (F-actin) is a prerequisite for site-directed migration, we analyzed whether the increased chemotactic response upon ActivinA treatment was associated with enhanced chemokine-induced actin polymerization. Pretreatment of 697 cells with ActivinA for 24 h resulted in a more prominent conversion of globular into F-actin starting from 15 seconds (s) after addition of CXCL12 (P<0.05) (Figure 5C). Notably, ActivinA-pretreated cells maintained a higher amount of F-actin compared to untreated cells, even 180 s after CXCL12 stimulation (P<0.001). These data strongly support our GEP results highlighting the role of ActivinA as a modulator of several genes involved in cytoskeleton remodeling and regulation of calcium dynamics.

Results on migration, invasion, chemokine receptors, and calcium flux were confirmed in the NALM-6 BCP-ALL cell line (Online Supplementary Figure S7).

ActivinA impaired CXCL12-driven migration of healthy CD34+ cells

We further evaluated whether ActivinA promoted a selective advantage to BCP-ALL cells compared to healthy CD34+ cells. CB- and BM-derived CD34+ cells expressed both type I and type II Activin receptors, thus suggesting that they could both respond to this molecule (Figure 6A).

The effect of ActivinA on CXCL12-driven chemotaxis of CD34+ cells was evaluated by Transwell-based migration assays. Surprisingly, we observed the opposite effect to that observed with leukemic cells. ActivinA pretreatment resulted in an average reduction of approximately 55% of CXCL12-driven chemotaxis, compared to untreated CB-CD34+ cells (Figure 6B). Of note, the regulation of cell viability did not account for the reduced chemotaxis (data not shown). These data were confirmed in BM-CD34+ cells derived from three HDs, with an average reduction of...
approximately 25% in CXCL12-driven migration (Figure 6B). This effect on CD34+ cell migration was not due to an ActivinA-mediated regulation of the CXCL12 chemokine receptors, CXCR4 and CXCR7, as demonstrated by flow cytometry analysis of both membrane-bound receptors and intracellular pool (Figure 6C). In addition, ActivinA pretreatment significantly decreased free cytosolic Ca2+ of CD34+ cells after the addition of CXCL12 in 2 out of 3 independent experiments (Figure 6D). Overall, these data suggest that leukemic cells could displace healthy hematopoietic stem cells from their niches through an ActivinA-mediated mechanism.

Acute lymphoblastic leukemia-mesenchymal stromal cells secrete high amounts of ActivinA

Finally, we focused our attention on the capacity of the...
BCP-ALL BM microenvironment to influence MSC-derived ActivinA. For this purpose, we isolated BM-MSCs from 15 HDs and 15 BCP-ALL patients at the onset of the disease. ALL-MSCs resulted comparable in terms of immunophenotype and adipogenic/osteogenic differentiation capacity to HD-MSCs (Online Supplementary Figure S8).

After 24 h of culture, ELISA assay showed a significantly higher production of ActivinA (P<0.05) by ALL-MSCs (mc: 222.2, range: 62.5-4855 pg/mL) compared to their normal counterparts (mc: 220.7, range: 62.5-518 pg/mL) (Figure 7A). Therefore, we hypothesized that BM-MSCs primed by the leukemic microenvironment could account for the high amount of ActivinA in the BM of BCP-ALL patients.

The role of inflammation in the editing of the microenvironment has been defined in several types of cancer, including hematologic malignancies. Recent evidence highlighted that the BM of ALL patients is a highly pro-inflammatory environment. These data were confirmed in our cohort of patients. Indeed, higher levels of the pro-inflammatory cytokines IL-1β (P<0.0001), IL-6 (P<0.01), and TNF-α (P<0.01) were detected in the BM plasma of BCP-ALL patients compared to HDs (Online Supplementary Figure S9).

We then investigated whether the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α could regulate ActivinA levels in the BM of BCP-ALL patients by stimulating both HD-MSCs and ALL-MSCs with a cocktail of the above-mentioned pro-inflammatory cytokines for 24 h. ELISA

![Figure 7. Inflammation contributed to ActivinA production by bone marrow-mesenchymal stem cells (BM-MSCs). (A) ActivinA secretion by BM-MSCs from B-cell precursor-acute lymphoblastic leukemia (BCP-ALL) patients (ALL-MSCs; n=15) and from healthy donors (HDs) (HD-MSCs; n=15) was assessed by ELISA after 24 hours (h) of culture ± IL-1β (50 ng/mL), IL-6 (40 ng/mL) and TNF-α (100 ng/mL). Each box plot shows the median and the mean (+), and extends from the lowest to the highest value. *P<0.05; **P<0.01: Wilcoxon matched-pairs signed rank test. (B) Primary BCP-ALL cells were co-cultured with HD-MSCs directly (bottom) or separated by a Transwell insert (top) in presence of IL-1β, IL-6 and TNF-α for 72 h. ActivinA expression was assessed by ELISA on culture supernatants (n=17 independent co-cultures). The expected additive effect was calculated as the sum of the single effects produced by the two stimulating factors, leukemic cells (second column) and inflammation (third column). Synergism was defined as a “greater-than-the-expected-additive effect”. Each box plot shows the median and the mean (+), and extends from the lowest to the highest value. §§§P<0.001; §§§§P<0.0001: stimulated versus unstimulated MSC; ***P<0.001; ****P<0.0001: measured effect versus expected additive effect, indirect contact and direct contact, respectively; Wilcoxon matched-pairs signed rank test.](image-url)
assays revealed a significant induction of ActivinA release in BM-MSCs compared to their respective basal condition. Indeed, upon stimulation, ActivinA production by HD-MSCs reached a 28-fold increase compared to the basal condition (mc: 5713, range: 1446-14221 pg/mL vs. basal condition) (P < 0.0001) (Figure 7A). Interestingly, the molecule was released to a higher extent by ALL-MSCs in a pro-inflammatory condition compared to their normal counterparts (mc: 10085, range: 2904-19776 pg/mL vs. inflamed HD-MSCs; P < 0.01).

Notably, by mimicking an inflamed BM niche through the simultaneous stimulation of HD-MSCs with leukemic blasts and pro-inflammatory cytokines (Figure 7B), we showed a strong increase in the secretion of ActivinA both in the direct (Figure 7B, bottom, mc: 27860, range: 13150-92391 pg/mL) and the indirect (Figure 7B, top, mc: 25409, range: 9050-65714 pg/mL) co-culture condition. Of note, the combination of both leukemic blasts and pro-inflammatory cytokines (Figure 7B, fourth column) produced a synergistic induction of ActivinA, since the extent of the release was higher compared to the sum of separately used stimuli (Figure 7B, expected additive effect: fifth column=second+third columns; top: P<0.0001; bottom: P<0.0001).

ActivinA increased the in vivo engraftment of B-cell precursor acute lymphoblastic leukemia cells to bone marrow and extramedullary sites in a xenograft mouse model.

With the aim of testing the efficacy of ActivinA to induce leukemia dissemination in vivo, we performed a set of experiments in which 697 or NALM-6 cells in vitro pretreated with ActivinA for 24 hours were injected (i.v.) into NSG mice.

Interestingly, on day +7, NSG mice injected with 697 cells (Online Supplementary Figure S10) pretreated with ActivinA showed a higher leukemic engraftment in the liver (median percentage of leukemic cells: 48.4%, range: 19.2-54.1%, n=9) compared to untreated cells (median percentage of leukemic cells: 27.0%, range: 10.9-43.2%, n=9), suggesting a migratory advantage in ActivinA-treated cells. As expected from the literature, our data showed a high tropism of 697 cells for the liver.22,23 On the contrary, the percentages of leukemia engraftment in BM and in other leukemic target organs were modest and were comparable between the two experimental groups.

To test a more physiological environment for leukemia and overcome the low engraftment of 697 in BM, we transplanted mice with NALM-6 cells that are known to
have high engraftment levels into the BM.24 NALM-6 cells, either pretreated or not with ActivinA, were injected (i.v.) at day 0 in NSG mice (1×10⁶/mouse) to evaluate their engraftment in different leukemia-targeted organs (Figure 8A). Mice were subsequently monitored for weight loss over two weeks after transplantation, and leukemia burden was evaluated 11 and 14 days after injection by flow cytometry, determining the percentage of human CD19 and CD10 positive cells in several organs.

First, we observed a significant difference in terms of change in body weight between the two experimental groups starting from day 4 after injection (P<0.05) with an even greater difference on days 11 and 14 after injection (P<0.01), suggesting a different disease progression (Figure 8B).

Moreover, we found that, on injection, both untreated and pretreated cells disseminated through the peripheral blood to different organs, such as BM, spleen, liver, meninges, and brain. Notably, we found that ActivinA-pretreated cells were able to engraft more rapidly in the BM of recipient mice (median leukemic percentage at day +11: 17.7%, range: 6.1-42.5%) compared to their untreated counterparts (median leukemic percentage at day +11: 10.3%, range: 0-21.4%, n=12). We also observed an increased leukemic percentage in the central nervous system (meninges and brain) of NSG mice following in vitro exposure to ActivinA (P<0.05) (Figure 8C), indicating that ActivinA stimulation was able to enhance the metastatic potential of leukemic cells in vivo.

Therefore, in these two mouse models, we were able to demonstrate that ActivinA stimulation makes leukemic cells more aggressive.

**Discussion**

There is ample evidence correlating aberrant TGF-β family growth factor activity to carcinogenesis. Despite its prominent role in solid cancer progression,27 the involvement of ActivinA, a member of the TGF-β family, in the pathogenesis of hematologic malignancies has never been explored. To the best of our knowledge, here we show for the first time that ActivinA is highly expressed in the BM of BCP-ALL patients at diagnosis compared to HDs. Interestingly, we demonstrated that BM-MSCs are an important source of ActivinA, the production of which is strongly up-regulated following direct contact with leukemic cells or through leukemia-released soluble factors. This finding is in accordance with the recently revised “seed and soil” theory, showing that leukemic cells are able to alter the BM stroma, creating a fertile ground which fuels tumor cell survival and progression.28 Of note, we observed that MSCs isolated from the BM of BCP-ALL patients were able to produce higher levels of ActivinA compared to HD-MSCs, even after several in vitro passages. This means that they ‘remember’ the profound alterations that had occurred within the leukemic BM niche.

Nowadays, there is general agreement that inflammation could play a pivotal role in the transformation, survival and proliferation of leukemic cells. In particular, several studies have demonstrated that BM cells in ALL are able to create a pro-inflammatory microenvironment that impairs frequency and function of normal HSCs within the BM.29 In line with this evidence, we demonstrate that the BM of BCP-ALL patients is characterized by increased levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, which can synergize with BCP-ALL cells in stimulating ActivinA production and release by BM-MSCs. Accordingly, it has been demonstrated that ActivinA expression is increased in several inflammatory diseases, such as septicemia, inflammatory bowel disease, and rheumatoid arthritis.27 The abundance of ActivinA within the leukemic BM niche, and its identification as a new MSC-secreted leukemia-driven molecule, prompted us to investigate its possible effects on BCP-ALL cells.

In accordance with recent literature reporting that ActivinA increases the migration and invasive properties of several solid tumors,24,25,26 our GEP analysis of ActivinA-treated leukemic cells showed a crucial effect on different biological processes linked to cell motility. In detail, we used time-lapse microscopy to demonstrate that this molecule was able to increase the spontaneous cell motility of both immortalized and primary BCP-ALL cells.

In addition to increased random motility, ActivinA-treated leukemic cells were more responsive to the CXCL12 chemokine, which plays an essential role in maintaining the quiescent BM HSC pool, thus regulating physiological hematopoiesis.34 The increase in BCP-ALL migration towards CXCL12 was selectively inhibited by ActivinA/Activin receptor blocking through SB431542,16 ensuring the specific contribution of ActivinA in mediating this advantage. Importantly, the effect of ActivinA on cell chemotaxis was highly cell-specific. While increasing leukemic cell migration in response to even suboptimal concentrations of CXCL12, ActivinA markedly impaired the ability of healthy CD34+ cells to migrate toward a CXCL12 gradient. This opposite effect could be particularly relevant in the context of the altered BCP-ALL BM niche, where we observed a significantly decreased CXCL12 concentration, in agreement with recent literature.19 Concerning the molecular mechanisms underlying ActivinA action, a protein-mediated regulation of the CXCL12 receptors CXCR4 and CXCR7 was ruled out. Moreover, in contrast to what Sozzani et al. described in dendritic cells,14 ActivinA did not stimulate either leukemic cell chemotaxis itself (Online Supplementary Figure S10) or their secretion of CXCL12 (data not shown). On the contrary, our GEP analysis demonstrated that this molecule mainly induces an overall positive regulation of pathways associated with cell motility, such as RAS, PI3K/AKT, and calcium homeostasis.

It has been demonstrated that Ca²⁺ co-ordinates structural components of the cell migration machinery and signaling molecules crucial for proper cell motility. Through the activation of actin-interacting molecules such as protein kinase C60 and calmodulin-dependent kinases,29 and the regulation of Rho GTPases, Ca²⁺ signaling finely tunes actin cytoskeleton dynamics.35 Interestingly, we demonstrated that ActivinA is able to increase the mobility of BCP-ALL cells through an increase in the pool of free cytosolic calcium, resulting in an increased rate of F-actin polymerization. Strikingly, the increase in intracellular Ca²⁺ was not observed in ActivinA-stimulated CD34+ cells, thus explaining the possible molecular mechanism mediating the differential activity of this molecule on the migration of leukemic versus healthy hematopoietic cells. Recent literature39 describing the ability of ActivinA to...
stimulate the migration of ovarian cancer cells suggests that Ca²⁺ increase could be achieved also in leukemic cells through the activation of non-canonical phospo-AKT, phospho-ERK and Rac1 signaling. In line with this hypothesis, our GEP data demonstrate an ActivinA-dependent increase in DOCK4 and CORO1A in the 697 cell line and primary BCP-ALL cells. This increase positively regulates the CDC42/RAC1 pathway, leading to the generation of phosphatidylinositol-3-phosphate, responsible for calcium release from intracellular stores. In agreement with this, ActivinA-mediated downmodulation of the Rac-GTPase activating protein ARHGAP25, that physiologically counterbalances the Rac-activating effect of nucleotide exchange factors, could play a prominent role in shaping calcium levels and modulating actin cytoskeleton also in leukemic cells. Moreover, ActivinA could also regulate the extent and duration of calcium responses through PTPRC downregulation. Indeed, in immature B cells, it has been demonstrated that the lack of the PTPRC product, CD45, induces enhanced levels of intracellular calcium that can last longer than in CD45 expressing cells, upon BCR engagement. Further studies will be crucial to better understand the possible molecular mechanisms mediating ActivinA differential activity in order to identify potential selective targets to counteract its action.

The ActivinA-mediated migratory advantage observed on BCP-ALL cells was further confirmed in a xenograft mouse model in which we demonstrated that leukemic cells prestimulated with ActivinA were able to engraft in the BM of NSG mice more rapidly than in their untreated counterparts. Overall, our in vivo data corroborate in vitro findings suggesting the effect of the molecule in favoring leukemia. Future studies testing the efficacy of ActivinA ligand traps on BCP-ALL patient-derived xenografts will be crucial to establish the impact of ActivinA on leukemia propagation.

Recent studies importantly linked ActivinA with the enhancement of cell invasion in several solid cancers (colorectal cancer, prostate cancer, breast cancer, glioblastoma, non-small cell lung cancer). In the context of BCP-ALL, relapse represents the most common cause of treatment failure, mainly occurring in the BM either in an isolated form or in combination with other extramedullary sites. Besides its key role in regulating homing processes in the BM niche, CXCL12 is thought to be involved in the widespread infiltration of other organs because of its constitutive expression in extramedullary tissues such as liver, spleen, thymus, lung, kidney, and brain. Interestingly, our in vitro observation demonstrated that ActivinA significantly increased the ability of leukemic cells to pass through an extracellular matrix in response to CXCL12. In addition, in vivo injected ActivinA-stimulated leukemic cells were more able than untreated cells to reach extramedullary disease target organs such as the meninges and the brain, suggesting a possible role for ActivinA in the promotion of leukemic cell invasiveness. Notably, ActivinA was able to up-regulate the expression of its type I receptors in leukemic cells, thus creating a self-reinforcing signaling cascade. Overall, our data suggest the establishment of a positive feedback loop between BCP-ALL cells and MSCs, which, through the key action of MSC-secreted ActivinA, generates a microenvironment favoring leukemia at the expense of normal hematopoiesis. Indeed, it is conceivable that the abundance of ActivinA, along with the decrease in CXCL12 within the BM niche, could lead to a reduction in the healthy HSC pool in favor of leukemic cells. On the other hand, the leukemic cells could access the BM sanctuaries where they can achieve signals necessary for cell survival and therapy resistance. Indeed, our in vitro findings were confirmed by in vivo studies and provide the biological rationale for designing therapeutic approaches targeting ActivinA in patients with BCP-ALL. Therefore, the direct targeting of ActivinA or its key downstream mediators could represent a valuable therapeutic option to be combined with conventional chemotherapeutic agents for decreasing the frequency of relapse in BCP-ALL.

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