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BRIEF COMMUNICATION

Activin A contributes to the definition of a pro-oncogenic bone marrow microenvironment in t(12;21) preleukemia

Federica Portale[a], Linda Beneforti[a], Alessandra Fallati[a], Andrea Biondi[a,b], Chiara Palmi[a], Giovanni Cazzaniga[a], Erica Dander[a], and Giovanna D'Amico[a]

[a]*Centro Ricerca Tettamanti, Pediatric Department, University of Milano-Bicocca, Fondazione MBBM, Monza, Italy;* [b]*Clinica Pediatrica Ospedale S. Gerardo, Fondazione MBBM, University of Milano-Bicocca, Monza, Italy*

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The *TEL-AML1* fusion gene, generated by the t(12;21) chromosome translocation, arises in a progenitor/stem cell and could induce clonal expansion of a persistent preleukemic B-cell clone which, on acquisition of secondary alterations, may turn into full-blown leukemia. During infections, deregulated cytokine signaling, including transforming growth factor β (TGF-β), can further accelerate this process by creating a protumoral bone marrow (BM) microenvironment. Here, we show that activin A, a member of the TGF-β family induced under inflammatory conditions, inhibits the proliferation of normal progenitor B cells but not that of preleukemic TEL-AML1–positive clones, thereby providing a selective advantage to the latter. Finally, we find that activin A inhibits BM-derived mesenchymal stromal cell-mediated secretion of CXCL12, a major chemoattractant in the BM compartment, thereby contributing to shape a leukemia-promoting environment. Overall, our findings indicate that activin A, in concert with TGF-β, could play an important role in the creation of a pro-oncogenic BM microenvironment and provide novel mechanistic insights into TEL-AML1-associated leukemogenesis. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

The t(12;21) is the most frequent chromosomal lesion in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [1]. The translocation gives rise to the *TEL-AML1* fusion gene, which results in the generation of a persistent preleukemic clone [2]. Because this alteration is insufficient for leukemogenesis, additional secondary postnatal genetic events are necessary for the transition of silent preleukemic cells to overt ALL [3]. Previous epidemiological and experimental studies have demonstrated the impact of infections and inflammation in the definition of an oncogenic environment able to favor TEL-AML1–expressing clones [4]. Notably, we have previously reported that transforming growth factor β (TGF-β) confers a selective advantage to translocation-bearing clones over healthy cells [5]. In particular, we found that TEL-AML1–expressing clones are insensitive to the growth-inhibitory effect of TGF-β caused by genetic blockade of SMAD signaling. By this mechanism, TEL-AML1–expressing cells, despite displaying reduced proliferation levels, may acquire a selective advantage over their normal counterparts [5].

Activin A is a TGF-β family member highly produced by mesenchymal stromal cells (MSCs) [6,7] and specifically induced by pro-inflammatory stimuli [7,8]. It signals through transmembrane serine/threonine kinase type II receptors (ACVR2A or ACVR2B), which transphosphorylate type I receptors (ALK2, ALK4 or ALK7), initiating both SMAD-dependent and -independent signaling pathways [9]. Recent studies on solid cancers have indicated that activin A acts as a key regulator of carcinogenesis by directly modulating cancer cell behavior and creating a

ED and GD equally contributed equally to this work.

Offprint requests to: Giovanna D'Amico, Centro Ricerca Tettamanti, Pediatric Department, University of Milan-Bicocca, Fondazione MBBM, Centro Maria Letizia Verga, Via Cadore, 20900 Monza (MB), Italy.; E-mail: giovanna.damico@asst-monza.it

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tumor-supportive microenvironment [10]. In addition, our group has recently demonstrated that activin A exerts a leukemia-promoting role by enhancing the migratory and invasive properties of BCP-ALL cells to the detriment of healthy hematopoiesis within the leukemic niche [7].

To assess a potential role of activin A in preleukemia-to-leukemia transition, we first aimed to determine whether this molecule could favor the persistence of preleukemic clones using an already established inducible TEL-AML1 system derived from murine pro-B Ba/F3 cells [5]. Furthermore, we sought to determine whether activin A could generate a leukemia-favoring microenvironment by modulating stroma-derived CXCL12, a crucial regulator of normal hematopoietic progenitors.

Methods

Ba/F3 culture

Ba/F3 cells (kindly provided by Dr. Anthony Ford) were cultured as previously described [5]. Cells induced to express TEL-AML1 (T/A^+) and control cells (T/A^-) were obtained and cultured as described in the Supplemental Material (online only, available at www.exphem.org).

Co-culture experiments of T/A^+ and T/A^- cells

After induction of *TEL-AML1*, T/A^+ cells were co-cultured with T/A^-, at a ratio of about 90% to 10%, respectively, in the presence or not of murine MSC (mMSC) monolayers, as described in the Supplemental Material.

Activin receptor analyses on Ba/F3 and hMSCs

Activin receptors were evaluated as described in the Supplemental Material.

Human BM-MSC stimulation and CXCL12 evaluation

MSCs were isolated from the bone marrow (BM) of 10 pediatric healthy donors (HDs) as previously described [7]. Human BM specimens were obtained from healthy BM donors at the Pediatric Department of Fondazione MBBM/San Gerardo Hospital (Monza, Italy; AIEOP-BFM ALL 2009 Protocol). MSCs were cultured and stimulated as described in the Supplemental Material.

CXCR4, CXCR7, and CXCL12 staining

CXCR4, CXCR7, and CXCL12 expression levels in hBM-MSCs treated or not with activin A were determined by flow cytometry (Supplemental Material).

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. An analysis of variance (ANOVA) test was used in cases of multiple comparisons.

Results

To investigate the impact of activin A on the proliferation of TEL-AML1–expressing preleukemic clones, we first evaluated the expression of activin receptors on murine Ba/F3 B-cell precursors, induced to express the fusion gene. Although Ba/F3 cells expressed *Alk4* and *Acvr2b* genes, they were negative for *Alk2, Alk7,* and *Acvr2a* mRNA expression (Supplemental Figure E1, online only, available at www.exphem.org). Interestingly, induction of the *TEL-AML1* (*T/A*) fusion gene significantly upregulated *Alk4* expression at both the mRNA (Supplemental Figure E1) and protein levels (median MFI T/A^- cells: 23.0, range: 6.1–83.2, vs. median MFI T/A^+ cells: 124.4, range: 64.6–295.4, $P < 0.05$) (Figure 1A). In addition, Acvr2b was overexpressed on T/A^+ cells (median MFI: 1633, range: 803.2–2288) compared with T/A^- control cells (median MFI: 656.3, range: 164–1010, $P < 0.05$) only at the protein level (Figure 1A).

Because we have previously reported that TGF-β selectively inhibits proliferation of T/A^- but not T/A^+ clones, thereby favoring clonal expansion of the latter [5], we asked whether activin A would similarly exert a permissive role in T/A^+ preleukemic clone persistence. Indeed, although activin A treatment inhibited T/A^- Ba/F3 cell proliferation by 43.6% at day +3 of culture, it did not affect T/A^+ Ba/F3 cell growth (Supplemental Figure E2, online only, available at www.exphem.org).

Next, we sought to determine whether activin A would also favor preleukemic clone persistence under co-culture conditions. To this end, we co-cultured T/A^+ and T/A^- Ba/F3 cells at a ratio of about 90% to 10%, respectively and measured the percentage of T/A^+ cells over a 3-day period. In line with our previous findings [5], the percentage of T/A^+ cells decreased from 90% at day 0 to a median of 36.8% (range: 13.9%–39.6%) at day +3 of co-culture, whereas the median percentage of TGF-β-treated T/A^+ clones, similarly co-cultured, decreased to a much lesser extent, with a median value of 51.8% (range: 24.1%–69.1%, $P < 0.0001$ vs. unstimulated condition) (Supplemental Figure E3, online only, available at www.exphem.org). As expected from our monoculture experiments, activin A also conferred a selective advantage to T/A^+ cells, with the median reaching a value of 49.2% after 3 days of co-culture (range: 28.2%–54.4%, $P < 0.0001$, vs. unstimulated condition) (Supplemental Figure E3). Lastly, combined treatment with activin A and TGF-β did not have an additive effect (Figure 1B and Supplemental Figure E3).

Next, to mimic the BM niche, we performed co-culture experiments in which the above-mentioned mixed Ba/F3 population (T/A^+ to T/A^- cells: 90%–10%) was cultured on a confluent monolayer of murine BM-MSCs in the presence or not of activin A and/or TGF-β (Figure 1B, 7 selected experiments out of 13 shown in Supplemental Figure E3). Also, in this more physiological context, both molecules were able to provide a growth advantage to T/A^+ cells (activin A: $P < 0.05$, TGF-β: $P < 0.001$, vs. unstimulated condition). On the other hand, the single addition of the MSC monolayer to the mixed culture did not result in improved survival

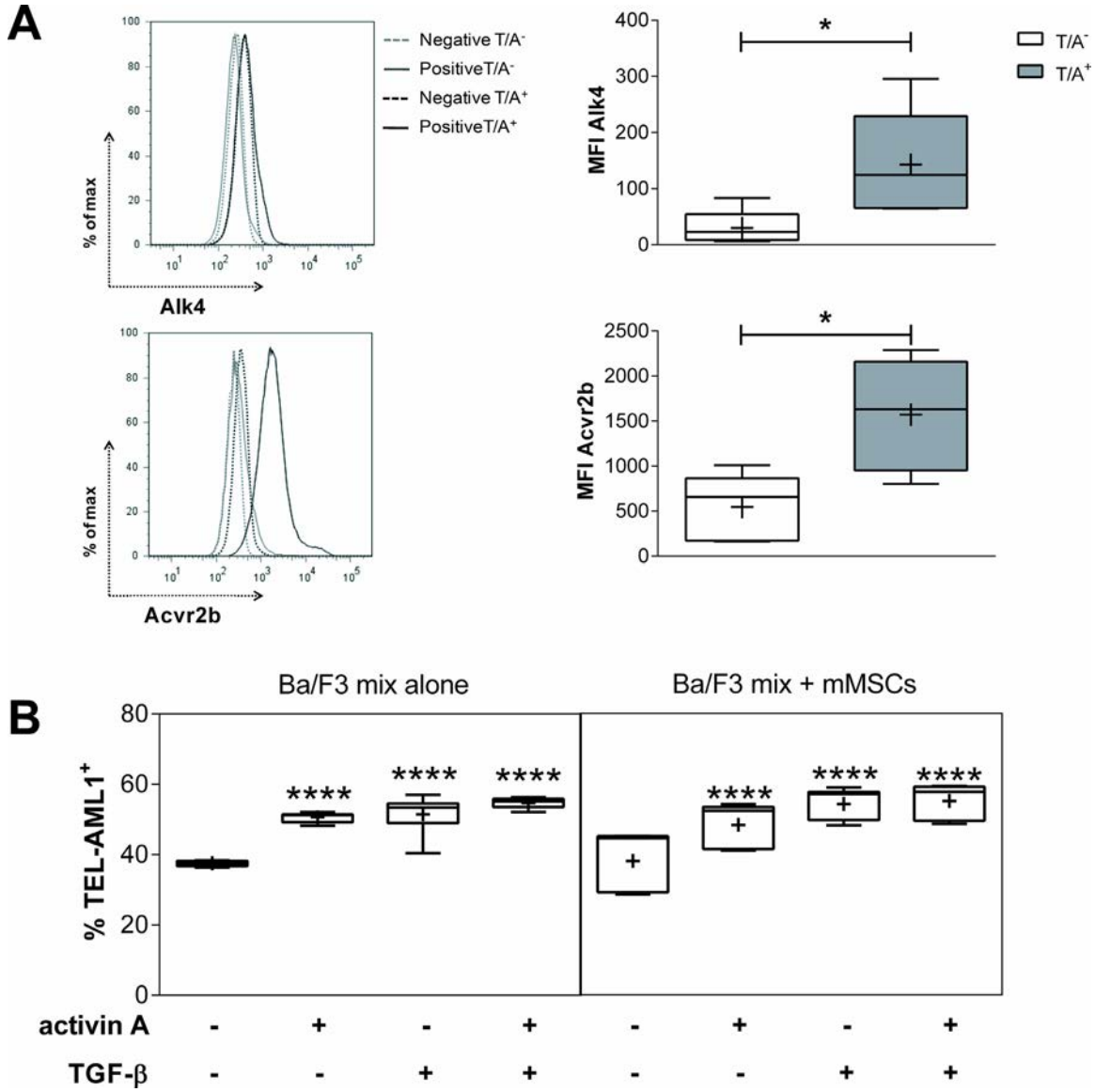

Figure 1. Activin A sustains TEL-AML1–expressing Ba/F3 cell expansion. **(A)** Membrane expression of the murine activin receptors Alk4 and Acvr2b was assessed in both TEL-AML1–positive (T/A^+) and –negative (T/A^-) Ba/F3 cells by flow cytometry. Representative histograms are shown on the left. Data pooled from five independent experiments are presented on the right as the mean fluorescence intensity (MFI) of activin receptors in T/A^+ and T/A^- Ba/F3 cells. Positive: cells stained with fluorescent dye-conjugated antibody. Negative: cells stained with isotype-matched control in the case of Alk4 or stained with only secondary antibody in the case of Acvr2b. Each boxplot illustrates the median and the mean (+) and extends from the lowest to the highest value. $*P < 0.05$: Mann–Whitney test. **(B)** T/A^+ and T/A^- Ba/F3 cells were co-cultured at a ratio of about 90% to 10%, alone (left panel) or in the presence of a murine MSC monolayer (right panel) and stimulated with activin A (50 ng/mL) ± TGF-β (10 ng/mL) for 3 days. The percentage of T/A^+ cells was evaluated by flow cytometry. $*P < 0.05$, $***P < 0.001$, **** $P < 0.0001$: analysis of variance test ($n = 7$ independent experiments).

of T/A^+ cells (Figure 1B), suggesting that activin A and TGF-β are required for this process.

To study whether activin A could promote a pro-leukemic BM microenvironment, we evaluated activin receptors on healthy BM-derived MSCs. As illustrated in Figure 2A and B, we could readily detect ALK2, ALK7, and ACVR2A protein expression in MSCs, indicating that these cells are indeed potential targets of activin A.

As leukemic BM has recently been reported to exhibit reduced expression of CXCL12, a well-known regulator of normal hematopoiesis [7,11], we next asked whether activin A, alone or in combination with TGF-β, would impair MSC-mediated secretion of CXCL12. Remarkably, MSC treatment with either cytokine led to a similar and significant drop in CXCL12 production (activin A: 29.6% median reduction, range: −2.6% to 47.3%, $P < 0.01$, vs. unstimulated condition; TGF-β: 29.8% median reduction,

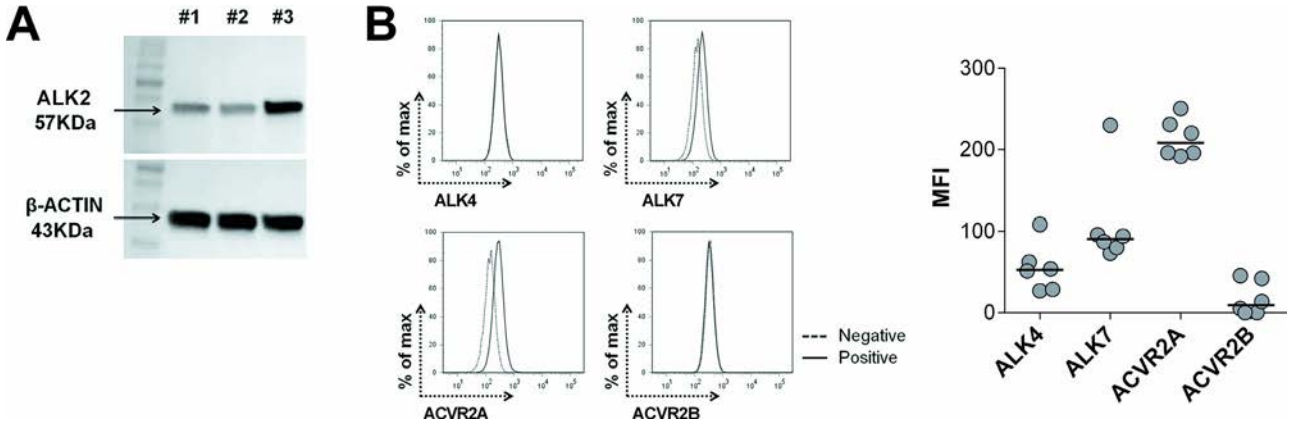

Figure 2. Activin receptor expression on human BM-MSCs. **(A)** Western blot analysis of hBM-MSC protein extracts from three different healthy donors (HDs) using an antibody anti-human ALK2 protein (57 kDa) or anti-human β-actin (43 kDa) as loading control. **(B)** On the left are flow cytometry histograms for the identification of cell surface ALK4, ALK7, ACVR2A, and ACVR2B expression of one of six MSC specimens tested (positive: cells stained with fluorescent dye-conjugated antibody; negative: cells stained with isotype-matched control in the case of ALK4, ALK7, and ACVR2A or stained with only secondary antibody in the case of ACVR2B). On the right are the mean fluorescence intensity (MFI) values for ALK4, ALK7, ACVR2A, and ACVR2B expression in six different HD-MSCs (*horizontal lines* represent median values).

range: 6.0%–69.9%, $P < 0.01$, vs. unstimulated condition) (Figure 3A). The decrease in MSC-derived CXCL12 after 24 and 48 h of stimulation with activin A was further demonstrated at the mRNA level (Supplemental Figure E4, online only, available at www.exphem.org). Furthermore, MSC stimulation with a cocktail of the pro-inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor α significantly decreased CXCL12 production by MSCs (Supplemental Figure E5, online only, available at www.exphem.org).

Trying to dissect the contribution of membrane-bound CXCL12 to the observed effect, we demonstrated that activin A stimulation did not alter the expression of CXCR4 and CXCR7 on MSC membrane (Figure 3B). We next evaluated the fraction of CXCL12 that can be sequestered and presented on the cell membrane by glycosaminoglycans (GAGs) [12], independently of its receptors CXCR4 and CXCR7. By using an anti-CXCL12 antibody, we found that activin A stimulation for 24 or 48 h did not alter the amount of membrane-bound CXCL12. In contrast, after 72 h of activin A stimulation, we observed a slight upregulation of membrane-bound CXCL12, albeit not statistically significant when compared with unstimulated control cells (Figure 3C).

Discussion

The onset of ALL has been linked to the cooperation of environmental exposures with genetic lesions. It has been shown that the t(12;21)-derived TEL-AML1 fusion protein is a frequent initiating event in childhood ALL able to induce a preleukemic phenotype. Inflammatory conditions, such as infections, may provide a promoting background for overt leukemia [4]. TGF-β has been identified as a leukemia-favoring factor, mediating a marked advantage to TEL-AML1–expressing clones [5].

Activin A is a member of the TGF-β family, whose expression is increased in infections and inflammatory diseases [13]. Evidence of its pro-tumoral role within the BCP-ALL BM niche [7] prompted us to investigate whether activin A favors the preleukemia-to-leukemia transition. Here, we demonstrated that activin A, in combination with TGF-β, can exert dual leukemia-promoting activity by directly acting on both preleukemic cells and the surrounding BM stroma.

In co-culture experiments we found that activin A is able to sustain TEL-AML1–expressing clones to the detriment of their normal counterparts. Indeed, while T/A$^+$ cell count was stable on activin A, TGF-β [5], or activin A + TGF-β stimulation, the growth of T/A$^-$ Ba/F3 cells was significantly impaired. It is conceivable that activin A, similarly to TGF-β, could exert a SMAD-mediated anti-proliferative effect [14] on normal B progenitors, as already observed in other cell types [15,16]. On the other hand, as previously demonstrated for TGF-β [5], TEL-AML1–expressing clones are insensitive to activin A, likely because of constitutive inhibition of SMAD signaling, which confers on them a growth advantage over their non-expressing counterparts. The blockade of SMAD signaling in TEL-AML1–expressing cells does not seem to preclude these cells from being responsive to activin A stimulation through other non-SMAD-mediated pathways, as suggested by enhanced expression levels of the Alk4 and Acvr2b activin receptors observed on these cells. In this regard, ALK4 overexpression has been previously associated with cancer stem cell persistence in different tumors [17,18].

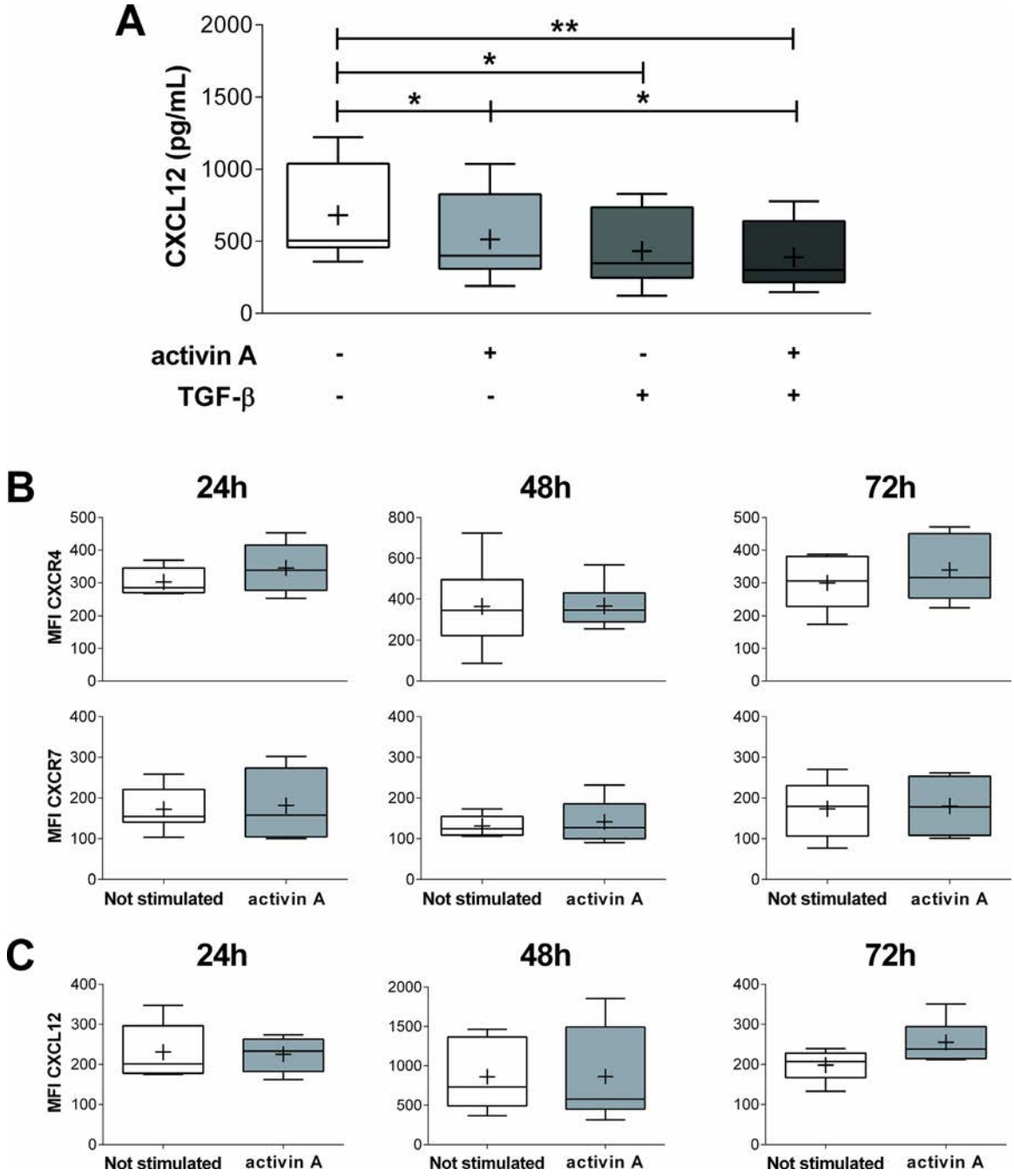

Figure 3. Activin A impairs CXCL12 release by human BM-MSCs. **(A)** CXCL12 secretion by HD-derived BM-MSCs ($n = 8$ for each condition) was assessed by enzyme-linked immunosorbent assay (ELISA) after 72 h of stimulation with activin A (100 ng/mL) ± TGF-β (10 ng/mL). Each boxplot illustrates the median and the mean (+) and extends from the lowest to the highest value. *$P < 0.05$, **$P < 0.01$: analysis of variance test. **(B)** CXCR4 and CXCR7 levels were evaluated by flow cytometry on the membranes of six different BM-MSC lines stimulated or not with activin A (100 ng/mL). The graphs illustrate the mean fluorescence intensity (MFI) values for CXCR4 and CXCR7 measured at three different time points (24, 48, or 72 h after stimulation). Each boxplot depicts the median and the mean (+) and extends from the lowest to the highest value. **(C)** The amount of membrane-bound CXCL12 was evaluated by flow cytometry on six different BM-MSC lines stimulated or not with activin A (100 ng/mL). Each boxplot depicts the median and the mean (+) and extends from the lowest to the highest value.

We recently reported that BM-MSCs can produce high activin A levels under inflammatory conditions [7]. In line with its ability to promote tumors by editing the surrounding stroma in solid cancers [10], here we report that activin A can skew MSCs to a leukemia-favoring phenotype in an autocrine fashion. Specifically, we show that MSCs express type I and II receptors, required for activin A signaling, and that activin A/TGF-β can significantly impair the release of CXCL12 by MSCs, possibly through a SMAD-mediated pathway, as previously demonstrated

[19]. In accordance, our data indicate that the reduction of CXCL12 secretion by activing A-stimulated MSCs can be, at least in part, explained by a decrease in its mRNA levels, thereby ruling out a contribution of CXCL12 sequestration on MSC membrane by its cognate receptors or GAGs. In addition, the stimulation of MSCs with pro-inflammatory cytokines, typically overexpressed in the BCP-ALL BM niche [7], results in a significant decrease in CXCL12 expression. Intriguingly, reduced expression of CXCL12 appears to be a common feature of the BCP-ALL BM niche, supporting the hypothesis that this decrease might be responsible for leukemogenesis at the expense of normal hematopoiesis [7,11,20]. In addition, De Rooij et al. [21] reported that B-ALL cells are able to alter the BM microenvironment, creating a self-reinforcing niche independent of the CXCR4/CXCL12 axis. Accordingly, the reduction of CXCL12 within the preleukemic BM niche could also be a relevant step in the transition to overt leukemia. In addition to its well-known role as a supporting factor for hematopoietic stem cell quiescence and retention into the BM, recent works have indicated that CXCL12 can mediate protection from oxidative stress and myelotoxic injury [22,23]. This scenario could increase the susceptibility of preleukemic progenitors to the acquisition of new genetic aberrations leading to disease onset.

Overall, we propose that activin A, in concert with TGF-β, could play an important role in the creation of a pro-oncogenic BM microenvironment by directly favoring preleukemic clone expansion and genetic instability through regulation of the CXCL12/CXCR4 axis. Even though molecular targeting of TGF-β and activin A is still far from clinical application, our study paves the way for the identification of new therapies to avoid the transition of preleukemic clones to primary leukemia or its relapse.

Acknowledgments

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Conflict of interest disclosure

The authors declare no conflicts of interest.

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Supplemental Materials

Ba/F3 culture and V5 staining

The IL-3-dependent murine pro-B cell line Ba/F3 cell line was cultured in Advanced RPMI medium (Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biosera, UK), 2% L-glutamine (Euroclone, Italy), 10 ng/mL rmIL-3 (ImmunoTools GmbH, Germany), 10 μM 2-mercaptoethanol and 0.2 mg/mL hygromycin B (Invitrogen, USA). *TEL-AML1* expression was induced by adding mifepristone (Invitrogen).

Control cells (T/A$^-$) are Ba/F3 cells transfected with the pSwitch plasmid (Invitrogen, Carlsbad, CA, USA) expressing a GAL4 regulatory fusion protein. TEL-AML1-expressing cells (T/A$^+$) were obtained by transfecting Ba/F3 cells with pGene plasmid (Life Technologies) containing the *ETV6-RUNX1* cDNA (coding for the TEL-AML1 fusion protein) fused to a V5 epitope tag controlled by a promoter regulated by the GAL4 regulatory fusion protein (inducible TEL-AML1 cells), as previously described [1]. Addition of mifepristone (Invitrogen) to the culture medium for 3 days induced the expression of TEL-AML1 only in T/A$^+$ cells.

Induction efficiency was verified by intracellular detection of V5 tag in TEL-AML1-expressing cells. Ba/F3 cells were first permeabilized with Cytofix/Cytoperm (BD Bioscience, USA) and then incubated with FITC-conjugated anti-V5 tag antibody (Abcam, UK). Data were analyzed by FACS Canto II flow cytometry using BD FACSDiva software (BD Biosciences, USA).

TEL-AML1–expressing and control cells were stimulated with 50 ng/mL activin A or 10 ng/mL TGF-β (R&D Systems, USA), for 3 days, and the number of cells was evaluated by trypan blue dye exclusion (Thermo Fisher Scientific, USA).

Co-culture experiments of T/A$^+$ and T/A$^-$ cells

After induction of *TEL-AML1*, T/A$^+$ cells were co-cultured with T/A$^-$ (at a ratio of about 90% to 10%), and stimulated with 50 ng/mL activin A $\pm$ 10 ng/mL TGF-β for 3 days. To mimic a physiological BM niche, mixed Ba/F3 co-cultures were repeated in presence of a monolayer of murine (m) BM-MSCs (Ba/F3 mix + mMSCs). mMSCs were isolated and characterized in terms of phenotype and differentiation potential, as previously described [2]. Cultured cells were harvested by using trypsin-EDTA (Euroclone) and stained by Fixable Viability Stain 450 (BD). Cells were then stained with a PE-conjugated anti-mouse CD45 antibody (eBioscience, San Diego, CA). Finally, the percentage of T/A$^+$ cells was measured by flow cytometry after staining for intracellular V5 tag.

Activin receptor analyses on Ba/F3 and hMSCs

To evaluate mRNA levels of activin receptors on murine Ba/F3 cells expressing or not *TEL-AML1*, total RNA was isolated using TRIzol reagent (Invitrogen) and used for cDNA preparation (Superscript reverse transcriptase, Invitrogen). QRT-PCR was performed using LightCycler® 480 (Roche, Switzerland). Primers and probes, synthesized with Universal Probe Library (UPL, Roche) software, are listed in Supplementary Table 1. Gene expression levels of target genes were normalized on *Hprt* levels.

The protein levels of the activin receptors Alk4/ALK4, Alk7/ALK7, Acvr2a/ACVR2A and Acvr2b/ACVR2B were evaluated on Ba/F3 cells and hMSCs by flow cytometry, using the following anti-human/mouse antibodies: anti-human/mouse activin RIB/ALK4 Alexa Fluor® 488-conjugated mAb (R&D Systems); anti-human activin RIC/ALK7 APC-conjugated mAb (R&D Systems); anti-human activin RIIA/ACVR2A APC-conjugated mAb (R&D Systems); primary anti-human/mouse activin receptor type IIB/ACVR2B mAb used in combination with secondary goat anti-mouse IgG H&L DyLight® 488-conjugated mAb (Abcam, Cambridge, UK). To ensure the specificity of the staining, Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience, CA, USA) was used. Mouse IgG APC-conjugated antibody (R&D Systems), mouse IgG FITC-conjugated antibody (BD Biosciences) were used as isotype controls for directly stained antibodies. Concerning ACVR2B indirect staining, the secondary antibody mouse IgG H&L DyLight® 488-conjugated mAb (Abcam) was used alone as negative control. Cells were analyzed by FACS Canto II cytometer (BD Biosciences), and data analyzed by FlowJo software (Tree Star, Inc. Ashland, OR, USA).

ALK2 expression was evaluated by Western Blot analyses on human BM-MSC cell extracts. For this purpose, cells were washed with ice cold PBS and lysed for 30 min on ice. Cell extracts were prepared in lysis buffer containing 1% NP-40 (SigmaAldrich), 0.5% Na-deoxyxholate (SigmaAldrich), 350 mM NaCl (SigmaAldrich), 0.1% SDS (SigmaAldrich), 1% protease inhibitor (SigmaAldrich) and 0.25 mM PMSF (SigmaAldrich) in PBS. Cell debris was removed by centrifugation at 21000 $\times$ g at 4°C for 5 min and protein concentration measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were then separated on Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, CA, USA). Gels were blotted onto Immun-Blot® PVDF Membranes (Bio-Rad), blocked with 10% dried milk in tris-buffered saline with 0.1% Tween 20 (SigmaAldrich) (TBS-T) and incubated with primary antibodies. Primary antibodies used were rabbit monoclonal anti-human ALK2 antibody (Abcam) and mouse monoclonal anti-human β-Actin antibody (SigmaAldrich). Blots were washed in TBS-T before incubation with horseradish peroxidase conjugated secondary antibodies, goat anti-rabbit IgG (Invitrogen) and rabbit anti-mouse IgG (SigmaAldrich). The blots were washed thoroughly with TBS-T before bands detection using LiteAblot Extend (Euroclone) as

luminescence substrate. Image detection was performed with Alliance LD2-77WL system (Uvitec, Cambridge).

Human BM-MSC stimulation and CXCL12 evaluation

HMSCs were cultured in DMEM low glucose 2% FBS and stimulated with 100 ng/mL activin A ± 10 ng/mL TGF-β. In selected experiments hMSCs were stimulated with a cocktail of pro-inflammatory cytokine consisting of IL-1β (50 ng/mL), IL-6 (40 ng/mL) and TNF-α (100 ng/mL). Culture supernatants were harvested and tested for CXCL12 levels by ELISA assay (R&D Systems). In addition, in selected experiments, hMSCs were collected and total RNA was isolated using TRIzol reagent (Invitrogen) and used for cDNA preparation (Superscript reverse transcriptase, Invitrogen). QRT-PCR was performed using LightCycler® 480 (Roche, Switzerland). Gene expression levels of target genes were normalized to *GAPDH* levels. Primers and probes, synthesized with Universal Probe Library (UPL, Roche) software, are listed in Supplementary Table 2.

CXCR4, CXCR7 and CXCL12 staining

BM-MSCs were grown in a confluent monolayer and stimulated or not with 100ng/mL activin A for 24, 48 and 72 h. Cells were then collected by using trypsin-EDTA, maintained and stained at +4°C with PE-conjugated anti-human CXCR4 mAb (BioLegend, San Diego, CA), PE-conjugated anti-human CXCR7 mAb (BioLegend) or anti-human CXCL12 mAb (R&D). Cells were analyzed by FACS Canto II cytometer (BD Biosciences), and data analyzed by FlowJo software (Tree Star, Inc. Ashland, OR, USA).

Supplemental Figures

Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4
Supplemental Figure 5

References

[1] Ford AM, Palmi C, Bueno C, et al. The TEL-AML1 leukemia fusion gene dysregulates the TGF-beta pathway in early B lineage progenitor cells. The Journal of clinical investigation. 2009;119:826-836.

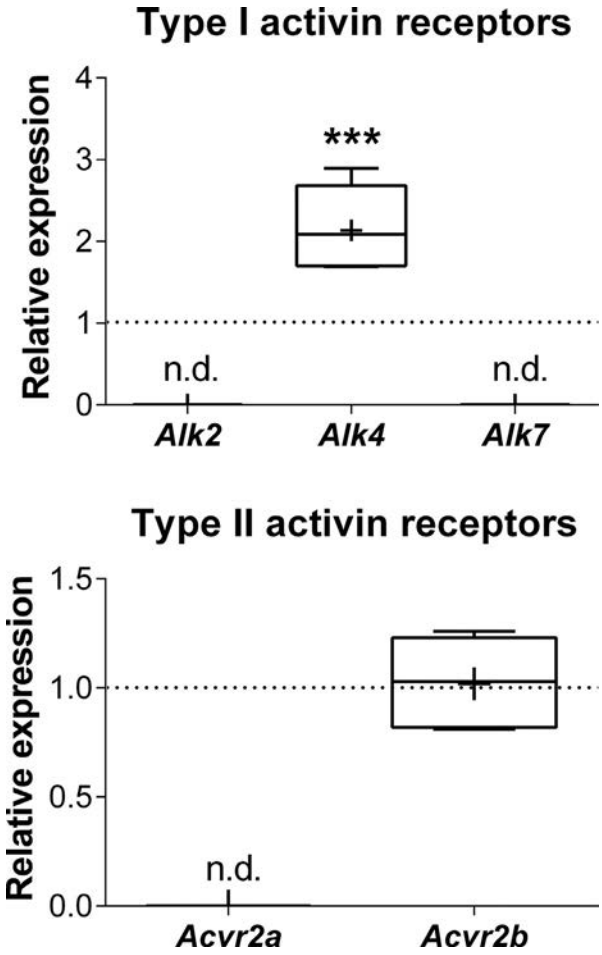
[2] Cappuzzello C, Doni A, Dander E, et al. Mesenchymal Stromal Cell-Derived PTX3 Promotes Wound Healing via Fibrin Remodeling. The Journal of investigative dermatology. 2016;136:293-300.

Supplementary Table 1. Primer sequences for quantitative real-time PCR in Ba/F3 cells.

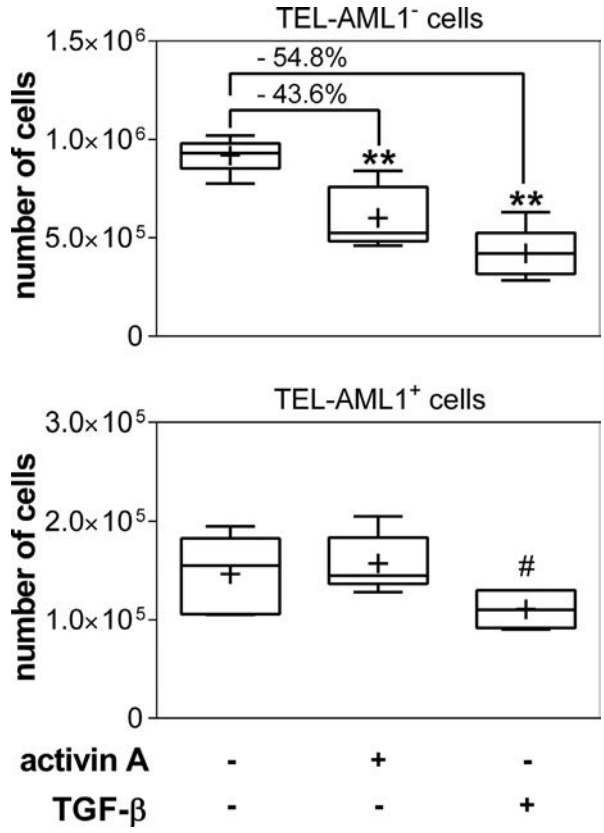
TARGET	PRIMER	SEQUENCE
mALK2	Forward	aggggaagatgacgtgtaagac
	Reverse	gtgttccggggaaggact
mALK4	Forward	agagggtggggaccaaac
	Reverse	tgcttcatgttgattgtctcg
mALK7	Forward	tgggaaatagctcgaaggtg
	Reverse	gtaaggcaactggtactcctcaa
mACVR2A	Forward	ccctcctgtacttgttcctactca
	Reverse	gcaatggcttcaaccctagt
mACVR2B	Forward	gctgcgtttggaaagctc
	Reverse	gccacgactgcttgtcct
mHPRT	Forward	ggagcggtagcacctcct
	Reverse	ctggttcatcatcgctaatcac

Supplementary Table 2. Primer sequences for quantitative real-time PCR in hBM-MSCs.

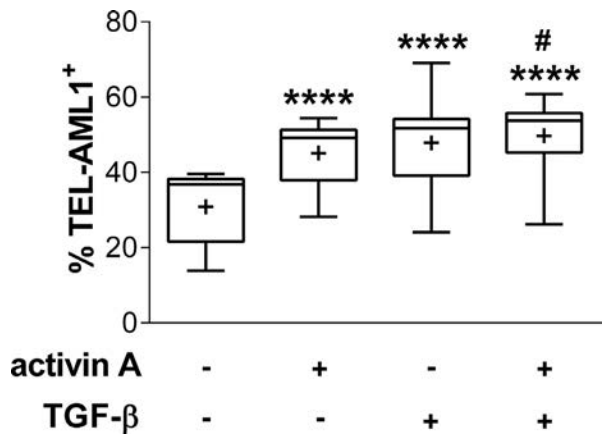
TARGET	PRIMER	SEQUENCE
hCXCL12	Forward	gctggtcctcgtgctgac
	Reverse	gcatgggcatctgtagctc
hGAPDH	Forward	agagggtggggaccaaac
	Reverse	tgcttcatgttgattgtctcg



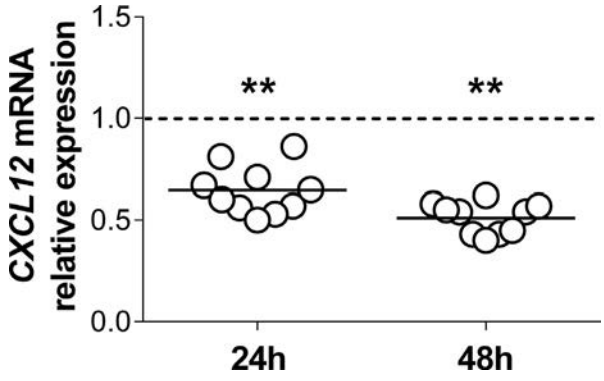
Supplemental Figure 1. Evaluation of activin receptor gene expression in T/A$^+$ and T/A$^-$ Ba/F3 cells. mRNA expression levels of type I (*Alk2, Alk4, Alk7)* and type II activin receptors (*Acvr2a* and *Acvr2b*) were determined by qRT-PCR in both TEL-AML1 positive (T/A$^+$) and negative (T/A$^-$) Ba/F3 cells. Data are presented as mRNA fold change of activin receptors in T/A$^+$ over T/A$^-$ cells (dotted line), normalized to *Hprt* mRNA (endogenous control). Each box plot shows the median and the mean (+) and extends from the lowest to the highest value. ***$P < 0.001$: Mann-Whitney test ($n = 7$ independent experiments).



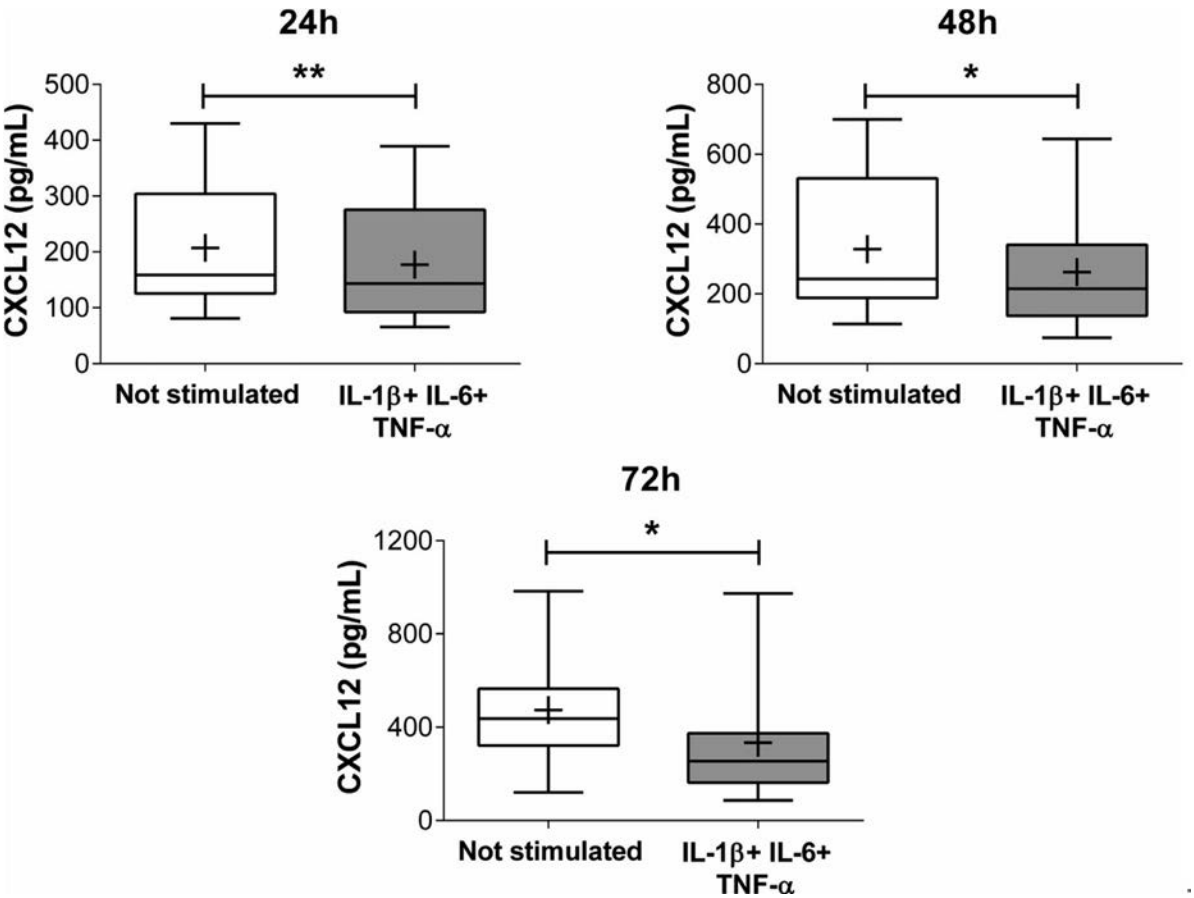
Supplemental Figure 2. T/A$^+$ Ba/F3 cells are resistant to the anti-proliferative effect of activin A and TGF-β. T/A$^+$ and T/A$^-$ Ba/F3 cells were cultured for 3 days in the presence or absence of activin A (50 ng/mL) or TGF-β (10 ng/mL). The number of viable cells was evaluated by trypan blue exclusion. Each box plot shows the median and the mean (+) and extends from the lowest to the highest value. **$P < 0.01$ vs. unstimulated cells; # $P < 0.05$ vs. activin a stimulation, analysis of variance test ($n = 5$ independent experiments).



Supplemental Figure 3. Activin A and TGF-β effect on a mixed co-culture of T/A$^+$ and T/A$^-$ Ba/F3 cells. T/A$^+$ and T/A$^-$ Ba/F3 cells were co-cultured at a ratio of about 90% to 10% and stimulated with activin A (50 ng/mL) ± TGF-β (10 ng/mL) for 3 days. The percentage of T/A$^+$ cells was evaluated by flow cytometry. Each box plot shows the median and the mean (+) and extends from the lowest to the highest value. ****$P < 0.0001$ vs. unstimulated cells; # $P < 0.05$ vs. activin A stimulation, analysis of variance test ($n = 13$ independent experiments).



Supplemental Figure 4. Downregulation of *CXCL12* mRNA levels in activin A-stimulated hMSCs. Expression of *CXCL12* was assessed in 10 BM-MSC lines treated or not with activin A (100 ng/mL) for 24 or 48 h. The mean *CXCL12* mRNA fold change in activin A-treated cells over unstimulated control was calculated (dotted line) after normalization to *GAPDH* mRNA (endogenous control). Solid lines represent the median values. $^{**}P < 0.01$, Mann-Whitney test.



Supplemental Figure 5. Downregulation of CXCL12 secretion by inflamed hMSCs. CXCL12 protein expression levels were evaluated in 8 BM-MSC lines treated or not with IL-1β (50 ng/mL), IL-6 (40 ng/mL) and TNF-α (100 ng/mL) for 24, 48 or 72 h by ELISA. 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.