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Activin A as a new key factor in the leukemic bone marrow niche

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“Chi dice che è impossibile,
non dovrebbe disturbare
chi ce la sta facendo”
Albert Einstein

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1. Introduction

1.1. B-cell precursor acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) comprises heterogeneous malignant hematological disorders arising from either B-cell (B-ALL) or T-cell (T-ALL) progenitors. B-ALL accounts for about 80% of ALL cases and mainly affects children with a peak incidence around 2-5 years of age. In children the incidence is 3-4 cases per 100’000 each year, whereas in adults the annual frequency is about 1 case per 100’000[1]. Despite cure rate has exceeded 90% in children, BCP-ALL (B-Cell Precursor Acute Lymphoblastic Leukemia) still represents the leading cause of cancer-related death in children and young adults[2].

1.1.1. Clinical features

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) arises from the malignant transformation and oligoclonal expansion of B cell precursors. Most of the clinical manifestations of the disease depend on the accumulation of leukemic blasts within bone marrow (BM), peripheral blood (PB) and extramedullary sites, such as liver, spleen, lymph nodes, mediastinum and central nervous system (CNS)[3]. Typical presenting symptoms include anemia, thrombocytopenia and leucopenia as effect of bone marrow failure. Other symptoms include the so-called “B symptoms” (fever, weight loss, night sweats), bleeding, bruising, fatigue, dyspnea and infections. Involvement of extramedullary sites causes lymphadenopathy, splenomegaly or hepatomegaly. CNS involvement presents

most commonly as cranial nerve deficits or meningismus[4]. Diagnosis is established by the presence of 20% or more lymphoblasts in the BM or PB. Evaluation of morphology, immunophenotype and cytogenetic are valuable both for confirming the diagnosis and for patients risk stratification.

1.1.2. Prognostic factors and genetic features

Studies in the pediatric population have identified some genetic syndromes that predispose to a minority of ALL cases, such as Down syndrome, Fanconi anemia, Bloom syndrome, ataxia telangiectasia and Nijmegen breakdown syndrome. Other predisposing factors include exposure to ionizing radiation, pesticides, viruses such as Epstein-Barr Virus (EBV) and Human Immunodeficiency Virus (HIV)[4].

The hallmark of ALL are somatic structural DNA rearrangements and mutations that altere lymphoid development, cytokine receptors, kinase signaling, tumor suppression and chromatin modification. Typical ALL translocations include t(12;21) [*ETV6-RUNX1*], t(1;19) [*TCF3-PBX1*], t(9;22) [*BCR-ABL1*] and rearrangement of *MLL*. More recently, a new group of patients characterized by a gene expression profile similar to (Philadelphia) Ph-positive ALL without presenting the classical BCR-ABL1 rearrangement has been identified. They are characterized by deletions in key transcription factors involved in B-cell development, including IKZF1, E2A, EBF1 and PAX5, and kinase-activating mutations, as rearrangements involving ABL1, JAK2, PDGFRB, CRLF2

and EPOR, activating mutations of IL7R and FLT3 and deletion of SH2B3, which encodes for the JAK2-negative regulator LNK[5-7]. Often, a single genetic lesion is not sufficient for BCP-ALL development: indeed, precursor B cells carrying such mutations are frequently found in newborns but the prevalence of leukemia is approximately a hundredfold lower[8]. Therefore, a two-step model was proposed according to which the leukemia-initiating genetic lesion occurs in utero, followed by an event that promotes expansion of the pre-leukemic clone and eventually leads to leukemia emergence. Epidemiological studies suggest that infections or immune responses to infections may promote B precursor oligoclonal expansion and BCP-ALL development, even though a specific pathogen responsible for this process has not been identified. It was hypothesized that a delayed exposure to common pathogens may lead to abnormal or dysregulated immune responses that promote growth of the leukemic clone[9]. Most recently, this model received mechanistic support: experimental mice predisposed for BCP-ALL development due to a PAX5 mutation only developed BCP-ALL upon transfer from specific pathogen-free (SPF) environment to an environment containing common pathogens[10].

1.1.3. Treatment of newly diagnosed Acute Lymphoblastic Leukemia

Treatment of newly diagnosed ALL contemplates different blocks of chemotherapy with increasingly intensive regimens corresponding to more aggressive disease categories. Remission induction is the first block of chemotherapy that aims to induce a complete remission, with approximately 95% of all patients achieving this benchmark. The agents used during induction include vincristine, corticosteroids, and asparaginase, with most regimens adding an anthracycline. This block is followed by consolidation, which aims to eradicate the residual disease remaining after complete remission obtainment. This phase of chemotherapy involves combinations of different chemotherapeutic agents, often including mercaptopurine, thioguanine, methotrexate, cyclophosphamide, etoposide and cytarabine. Maintenance chemotherapy is the final stage of treatment in childhood ALL: the cornerstone of this block is antimetabolite therapy with methotrexate and mercaptopurine. Finally, the fourth component of ALL treatment is therapy directed to CNS, because of the high rate of CNS relapse in absence of specific therapy directed toward this sanctuary site[11].

1.1.4. Targeted therapy and precision medicine

The past decade has been marked by ameliorations in disease treatment thanks to multiagent chemotherapy regimens and stratification of treatment intensity according to patient clinical features, reaching a survival rate of about 90%. However, recent advances regarding the genetic basis of ALL and the development of targeted therapies against specific molecular lesions have paved the way for the expanding use of precision-medicine approaches to cancer[3]. In particular, next generation sequencing technology has opened up new possibilities to identify cancer mutations involved in increased risk of leukemogenesis and relapse, but also “druggable” lesions, that could be used for developing individualized treatment for patients unresponsive to conventional therapies[12]. One notable example is the use of tyrosine kinase inhibitors that dramatically improves chronic myeloid leukemia management and that, in combination with cytotoxic chemotherapy, results to be highly effective in children with Ph-positive ALL[3]. Moreover, Ph-like ALL are characterized by genetic alterations that activate kinase signaling, therefore suggesting that this subgroup can be sensitive to tyrosine kinase inhibitors, such as JAK and PI3K/mTOR inhibitors[3].

A promising strategy to target ALL is immunotherapy, defined as harnessing the immune system for leukemia therapy. Cytotoxic T cells engineered with chimeric antigen receptors (CARs) are one example of modified adoptive cell transfer that aims at targeting leukemic antigens, such as for example CD19.

Another example of immunotherapic approach for B-ALL treatment is the CD3-CD19 bispecific T-cell engager Blinatumomab, able to enhance the cytotoxic killing of leukemic cells by binding at the same time the CD19 antigen expressed by the leukemic blasts and the CD3 antigen expressed on autologous T cells. Other immunotherapy targets for the treatment of B-ALL include CD22, CD20 and CD25 antigens[4]. Another promising anti-leukemic strategy is based on the correction of epigenetic changes observed during leukemogenesis, using histone deacetylase inhibitors (HDACi) or DNA methyltransferase inhibitors[13]. Finally, the inhibition of the proteasome machinery by Bortezomib in combination with conventional chemotherapeutic agents resulted able to produce a response rate of 80% in children with relapsed/refractory pre-B-ALL[14].

1.2. The hematopoietic stem cell niche

Hematopoietic stem cells (HSCs) are a population of multipotent cells resident in the bone marrow that are characterized by abilities to self-renew and to differentiate into all blood cell lineages. The process of blood-cell production, defined as hematopoiesis, continues throughout life and depends on interactions with the surrounding microenvironment, also referred as “niches”. BM niches are specialized areas involved in the control of number, quiescence, self-renewal, proliferation, differentiation and localization of HSCs and progenitor cells (HSPCs). During homeostasis, the niche is able to protect HSCs from exhaustion and genotoxic insults by limiting their entry into cell cycle and maintaining them in a quiescent state[15]. However, HSCs can quickly respond to niche or systemic signals by entering the cell cycle and proliferating to meet the needs of the organism[16]. Several cell types cooperate to regulate hematopoiesis and, among them, mesenchymal stromal cells (MSCs) exert a key supporting-role. Moreover, both adaptive and innate immune cells contribute to the complex network involved in the response of hematopoietic process to external stimuli[17]. Generally, regulatory signals derived from surrounding cells, in the form of membrane bound or soluble molecules, and from physical cues are responsible for the definition of HSC self-renewal and multilineage differentiation[18-20].

According to their localization and function, two distinct BM niches are defined: “osteoblastic (endosteal)” niche, lining the bone surface, and “vascular” niche, located in close proximity to blood vessels[18, 19]. The endosteal niche provides a hypoxic environment for the maintenance of HSC quiescent state, while the vascular niche, characterized by a higher oxygen availabilty, allows HSCs to proliferate and differentiate[21].

The BM is a highly vascularized environment as a result of a thick network of arterioles and capillaries that drain into densely organized sinusoids[22]. Arterioles preferentially locate nearby the endosteal region in close association with sympathetic nerve fibers and smooth muscle cells, while sinusoids pervade the central and endosteal marrow, thus defining a permeable barrier for the passage of mature blood cells into the circulation[23]. In the BM, blood vessels are closely associated with perivascular mesenchymal stromal cells (MSCs) defined as $CD146^+$ stromal cells and $Nestin^+$ mesenchymal stem cells. These cells, along with C-X-C motif chemokine (CXCL) 12-abundant reticular cells (CAR) cells surrounding sinusoidal endothelial cells, express high levels of key niche factors, including CXCL12 and SCF (Stem Cell Factor)[24]. In addition, Leptin receptor-expressing ($LepR^+$) cells and CAR cells participate, with overlapping functions, to HSC maintenance and retention[25].

The perivascular area contains several cell types, including endothelial cells, arteriolar pericytes, sympathetic nerves, nonmyelinating Schwann cells and megakaryocytes.

Endothelial cells (ECs) in the BM through the production of soluble factors, such as SCF, are able to promote HSC self-renewal and regeneration *in vivo*[26-28]. BM endothelial cells express E-selectin, that promotes HSC proliferation at the expense of population maintenance, and Jagged-1, implied in HSC maintenance[29, 30].

Glial non-myelinating Schwann cells are non-neuronal cells that envelop sympathetic nerves in the BM and are located in direct contact with a high number of HSCs. They maintain HSC dormant state through the secretion of TGF-β activator molecules, which exert an activatory effect on TGF-β/SMAD signalling pathway associated with HSC dormancy[31].

Recent findings identified a direct HSC regulation by megakaryocytes[32]: indeed, in mice approximately 20% of HSCs in the BM are physically associated with megakaryocytes, thus highlighting their role as HSC-derived niche cells that directly regulate HSC function[33]. In particular, they are able to mantain HSC quiescient state through the production of thrombopoietin[34], TGFβ[35] and TPO[36]. Moreover, megakaryocytes normally regulate HSC proliferation and reduce HSC numbers through the secretion of CXCL4[33].

The so-called “osteoblastic niche” is localized at the endosteum and includes osteoblasts, osteoclasts, adipocytes, glial non-myelinating Schwann cells and several subsets of immune cells[37]. Osteoblasts (OBCs) are specialized mesenchymal cells responsible for the deposition of bone matrix. They act in

concert with osteoclasts, the bone-reabsorbing cells, to maintain bone homeostasis[1]. Osteoblasts promote HSC niche maintenance through the production of several growth factors and cytokines, such as granulocyte colony-stimulating factor (G-CSF)[38]. Moreover, they are able to increase HSC pool through the activation of N-cadherin/β-catenin pathway and Notch/Jagged-1 axis, upon stimulation with bone morphogenetic protein (BMP) and parathyroid hormone (PTH)[39, 40]. Osteoblasts are responsible for the modulation of HSC quiescence via osteopontin and Angiopoietin-1/Tie2 signalling pathway[41, 42]. Finally, cell-to-cell contact with HSCs determines the creation of a signaling endosome that causes the downregulation of SMAD signaling and the increase of CXCL12 production in osteolineage cells, necessary for HSC survival[43].

Osteoclasts are specialized bone-reabsorbing cells derived from the myeloid/monocyte lineage, involved in the remodeling of the endosteal niche, therefore regulating both hematopoietic sites and the establishment of sinusoidal microcirculation[44]. RANKL-stimulated osteoclasts are responsible for the reduction of the stem cell niche factors, such as CXCL12, SCF and osteopontin, which causes progenitor mobilization[45]. Moreover, osteoclast activity is involved in HSC localization into the osteoblastic niche: indeed, Ca^{2+} released from the bone subsenquently to osteoclastic activity is bound by a seven-transmembrane-spanning Ca^{2+}-sensing receptor (CaR) implied in the retaining of HSCs in close proximity of the endosteal

surface[46]. Interestingly, bone resorption produces active TGFβ, which can act on HSCs[47].

Adipocytes could exert a regulatory function within the BM niche: indeed, they secrete cytokines, fatty acids and hormones, that influence the function of other neighbouring cells in the BM microenvironment[48] as leptin, a molecule that directly supports myelopoiesis[49]. Adipocytes are negative regulators of the hematopoietic niche since they can inhibit HSC growth and differentiation as well as hematopoietic reconstitution[50].

Lymphocytes could contribute to the regulation of hematopoiesis, through direct cellular interactions with HSCs: in particular, natural killer cells play a negative role in HSC differentiation[51], activated T cells modulate normal hematopoiesis[52] and regulatory T cells suppress colony formation and myeloid differentiation of HSCs[53]. Furthermore, regulatory T cells provide an immune-privileged site in the BM, protecting HSCs from immune attacks[54].

Macrophages in the BM are involved in bone remodeling and in HSC retention[55, 56]. Through the production of oncostatin M, they affect the expression of CXCL12 of nestin^{+} MSCs[57]. Moreover, a rare subpopulation of macrophages, expressing high levels of α-smooth muscle actin and cyclooxygenase 2, synthesizes prostaglandin E2 that positively regulates CXCL12 expression in nestin^{+} MSCs[58] and CXCR4 expression on HSCs[59], thus improving the survival and maintenance of HSCs in the BM.

Neutrophils are able to regulate HSC retention *in vitro* through the secretion of serine proteases necessary for the cleavage of several molecules such as CXCL12, CXCR4, VCAM-1, c-Kit, and SCF[60-64], thus contributing to HSC release from the BM. Moreover, the daily clearance of aged neutrophils operated by macrophages is associated to an increased number of CXCL12-expressing stromal cells and CXCL12 protein levels, which resulted in enhanced retention of HSCs in the BM[65].

1.2.1. Bone marrow niche signals

Emerging data suggest that stem cell niches in the BM are not static but instead responsive to external cues: indeed, soluble mediators, intrinsic signaling pathways and microenvironmental signals are responsible for HSC manteinance.

The different cell types that compose both endostal and vascular niche may account for the tight regulation of the hematopoietic process. The main molecular pathways involved in this mechanism comprise Notch, Wnt, TGF-β and CXCR4/CXCL12 signaling pathways.

Notch signaling pathway is involved in the regulation of developmental processes, such as adult stem cell fate decision, and in the maintenance of tissue homeostasis[66]. Notch receptors are expressed by HSCs early in hematopoiesis and are involved in cell differentiation decisions: in particular, Notch1 promotes T cell commitment and specifies megakaryocyte fate, whereas Notch2 marks primarily erythroid progenitor cells[18]. Interaction of Notch1 and Notch2 receptors

with Jagged-1 promotes the expansion of long-term repopulating HSCs to preserve self-renewal ability, while the interaction of these receptors with Jagged-2 causes short-term repopulating HSC expansion[18].

Wnt signalling pathway regulates the development of several tissues, including hematopoietic tissues[67]. In particular, activation of β-catenin in HSCs leads to their expansion *in vitro* contemporary maintaining their immature state[68]. Moreover, the lack of the canonical Wnt ligand, Wnt3a, leads to lower HSC and progenitor cell numbers in the fetal liver, decreases self-renewal and long-term repopulation ability[69]. On the other side, the non-canonical Wnt ligand Wnt5a inhibits canonical Wnt signaling, cell proliferation *in vitro* and increases the repopulating ability of HSCs through receptor-like tyrosine kinase (Ryk) receptor[70].

The activation of the TGF-β/SMAD signaling causes SMAD2 and SMAD3 phosphorylation that promotes HSC dormancy. Several sources of TGF-β are linked to HSC maintenance in the BM niche, i.e. Schwann cells and megakaryocytes[31, 32]. Moreover, TGF-β stimulates myeloid-biased HSCs to proliferate while inhibiting lymphoid-biased HSCs[71]. Finally, Cripto, an inhibitory molecule of TGF-β signaling, binds to the GRP78 receptor on hypoxic HSCs and activates the PI3K/Akt pathway, determining the maintenance of HSCs in the endosteal niche[72].

One of the most important key retention signal for hematopoietic stem and progenitor cells (HSPCs) in the BM is represented by CXCR4/CXCL12 axis. CXCL12 regulates HSC

quiescence and their multilineage reconstitution ability[73]. Of note, CXCL12 secreted by osteoblasts and perivascular stromal cells induces the production of common lymphoid progenitors, whereas its expression in osteoprogenitors is associated with higher numbers of committed B-lymphoid progenitors[74, 75].

Moreover, microenvironmental signals, such as hypoxia, infections and nervous stimuli, could affect the BM microenvironment thus contributing to the regulation of the hematopietic process.

Hematopoietic stem cells mainly reside in hypoxic areas that confer them a higher hematopoietic repopulating ability compared to those in more perfused areas[76]. These cells also express the stable form of hypoxia-inducible factor-1α (HIF-1α) and the angiogenic growth factor VEGF[77]: in particular, HIF-1α increases the production of CXCL12 in endothelial cells[78] and the expression of its receptor, CXCR4[79], therefore allowing HSCs to be retained in the BM. Moreover, in hypoxic conditions HSCs generate energy mainly via anaerobic metabolism maintaining a high rate of glycolysis, therefore limiting the production of reactive oxygen species[80].

Also nervous system can regulate HSC niche retention: indeed, the circadian noradrenaline secretion by the sympathetic nervous system causes a rapid downregulation of CXCL12, Angiopoietin-1 (Angpt1), Kit ligand (Kitl) and vascular cell adhesion molecule 1 (VCAM-1), through the β_3-adrenergic signaling, thus promoting HSC egress from the bone marrow

and their circulation between niches and bloodstream under steady-state conditions[81, 82]. Moreover, nervous system signaling through the release of neurotransmitters, such as norepinephrine, acts as direct chemoattractants for HSCs[83].

Finally, infectious status may account for the regulation of hematopoietic process. In particular, toll-like receptors (TLRs), a family of pattern recognition receptors expressed on HSPCs, endothelial cells and mesenchymal stromal cells[84], upon the binding to TLR ligands (lipopolysaccharide, single-stranded RNA, peptidoglycans, etc.) initiate the response of hematopoiesis to infections. For example, activation of TLR4 in endothelial cells, upon exposure to LPS, induces enhanced production of G-CSF resulting in increased granulopoiesis[85]. Moreover, upon infection with *Escherichia coli*, stromal cells downregulate CXCL12 expression, thus causing HSC mobilization[86].

1.2.2. Mesenchymal Stromal Cells as key regulators of the hematopoietic niche

Mesenchymal stromal cells (MSCs) were first described as spindle shaped cells derived from bone marrow which are able to adhere to plastic and to form colony-forming unit fibroblasts (CFU-F)[87]. Nowadays, they can be expanded from several other tissues including adipose tissue, umbilical cord blood, skin, tendon, muscle and dental pulp[88]. The International Society for Cellular Therapy formulated minimal criteria for defining a uniform way to characterize MSCs:

- adherence to plastic
- specific surface antigen (Ag) expression
- multipotent differentiation potential

Surface marker phenotype of MSCs is CD105^{+}, CD73^{+}, CD90^{+}, CD45^{-}, CD34^{-}, CD14^{-}, CD19^{-}, CD3^{-}, HLA-DR^{-}. MSCs must be able to differentiate into specialized cells of mesenchymal origin as osteoblasts, adipocytes and chondroblasts *in vitro*[89].

Apart from their stem/stromal features, MSCs are characterized by both anti-inflammatory and pro-inflammatory properties. Due to their immunomodulatory properties, MSCs are implied to facilitate HSC engraftment after T-cell depletion, HLA-haploidentical HSC transplantation (HSCT) to rescue patients with steroid-resistant acute graft-versus-host disease (GvHD) and to manage patients with chronic inflammatory diseases, such as Crohn's disease[90]. MSCs derived from bone marrow (BM-MSCs) are able to suppress the proliferation of activated T cells[91] and to promote T lymphocyte anergy through the induction of the so-called “tolerance arrest”[92]. Finally, BM-MSCs are able to direct naïve T cell differentiation toward regulatory T cell phenotype (CD4^{+} CD25^{+} FoxP3^{+}), which serves as a mechanism to suppress T cell response and dampen inflammation[93]. Human BM-MSCs are able to inhibit NK cell proliferation and cell-mediated cytolysis, through the downregulation of the activating NK receptors (NKp30, NKp44, and NKG2D) and the inhibition of NK production of IFN-γ[94]. BM-MSCs can suppress B lymphocyte proliferation by a cell-to-cell contact mechanism via the PD-1 (programmed death 1)

inhibitory molecule and its ligands PD-L1 and PD-L2. They also suppress the immune response of B cells along with their differentiation and chemotactic functions[95]. Increasing evidence have demonstrated that MSCs-mediated regulation of macrophages is critical for inflammation response and tissue injury repair. In particular, because of their immune-modulating features, MSCs induce preferentially a shift of the macrophage phenotype from M1 to M2, characterized by an anti-inflammatory profile[96].

Finally, BM-MSCs give a substantial contribution to the creation of the HSC niche, by both cytokine secretion and cell-to-cell interactions, thus playing a crucial role in the development and differentiation of the hematopoietic system[39]. Mesenchymal stromal cells have been identified as functional components of the bone marrow HSC niche. In particular, Nestin^{+} MSCs are characterized by higher levels of HSC maintenance factor transcripts, including CXCL12, SCF, Ang-1, IL-7, VCAM1 and osteopontin, compared with any other stromal cell type[24]. Moreover, CXCL12-abundant reticular (CAR) cells, similar to nestin^{+} MSCs, express HSC maintenance proteins such as CXCL12 and SCF, thus promoting HSC cycling and self-renewal[18]. Their supportive role has been demonstrated also in the context of hematological malignancies: indeed, it has been shown their promoting role in leukemia development through survival promotion and local immune suppression[97 98].

1.3. The Malignant Hematopoietic Stem Cell Niche

Leukemic stem cells (LSCs) are the main cause of treatment failure and disease relapse because of their resistance to irradiation, chemotherapy and to targeted therapies[99]. These cells are well defined in acute myeloid leukemia (AML), while they are poorly described in acute lymphoblastic leukemia (ALL). LSCs can be defined as the malignant counter part of HSCs since they share functional and molecular features modulated by signals derived from BM microenvironment. Moreover, LSCs are able to initiate and maintain the disease due to their self-renewal ability and their capacity to produce a differentiated leukemic progeny, defined as leukemic blasts[100]. LSCs, differently from HSCs, have a dysregulated activation of key pathways which regulate proliferation, survival and abilities to invade and spread[101].

1.3.1. BM niche and leukemic cells interplay as a mechanism for disease initiation and progression

Several studies highlight that stromal cells within the niche could be drivers of neoplasia by carring germline or somatic mutations. Leukemogenic transformation triggered by genetic alterations of the BM microenvironment has been observed in patients with BM failure syndromes such as aplastic anaemia, myeloproliferative neoplasm (MPN), chronic myelogenous leukemia (CML), acute leukemia, etc.[37]. Several genetically modified models have demonstrated this role of BM niche: indeed, deletion of *Dicer1*, *Rarg*, *Rb1*, *Mib1* or the DNA-binding

domain of Rbpj in BM stromal cells results in a myeloproliferative-like disorder in mice[102-106]. Also germline mutations in stromal cells can contribute to hematopoietic malignancy as in Shwachman Diamond syndrome, a congenital BM failure disease with known leukemic predisposition caused by the loss-of-function mutation of *SBDS* gene[107]: in mice deletion of *Sbds* gene in BM stromal cells induces an Myelodysplastic syndrome (MDS)-like disease[102].

Data about the presence of leukemia-associated genetic aberrations and immunoglobulin (IG) gene rearrangements in the contest of B-ALL are contrasting. Even if Shalapour et al. reported leukemia-associated genetic alterations in a variable proportion of MSCs from childhood B-cell precursor ALL patients[108], other groups failed to detect such abnormalities in MSCs isolated from leukemia patients[109]. So far, the idea that leukemic cells could alter the functional phenotype of normal MSCs is currently under investigation.

It has been recently proposed that leukemic cells *per se* are able to redirect BM stroma, thus sustaining pathogenesis, progression and drug resistance. Therefore, in the context of "malignant microenvironment", malignancy co-opts the normal microenvironment to increase its own support, while suppressing normal hematopoiesis.

A well characterized example of bidirectional crosstalk between malignant cells and niche cells is CML[110]: indeed, CML cells induce an abnormal differentiation of MSCs causing an overproduction of functionally altered OBCs, which accumulate

in the BM cavity as inflammatory myelofibrotic cells. These cells secrete low levels of HSC retention factors, including CXCL12, and high levels of pro-inflammatory cytokines, therefore defining a self-reinforcing malignant niche that favours LSCs at the expense of healthy HSCs[111].

In the aggressive MLL-AF9 model of AML, nestin^{+} niche cells show decreased levels of Angiopoietin, CXCL12, SCF, VCAM1 and increased levels of OPN, that induces HSC quiescence, causing a redistribution of HSCs out of the inhospitable BM niche towards the peripheral blood and spleen[112].

In the context of MPNs, HSCs carrying the *JAK2 V617F* mutation produce IL-1β that causes neural damage and Schwann cell apoptosis, thus negatively affecting the number of BM stromal cells[113]. Moreover, severe marrow fibrosis in MPNs is caused by the excessive production by megakaryocytes of TGF-β that acts on MSCs[33].

As regard BCP-ALL niche, few studies support the idea that leukemic cells could corrupt the normal BM niche, through different mechanisms. It has been shown that human ALL cell lines are able to induce a transient niche composed of mesenchymal stromal cells that surround clusters of leukemic cells, through the MSC-chemoattractant CCL3. Notably, these transient niches could confer resistance to chemotherapy[114]. Moreover, it has been demonstrated that in co-culture system BM-MSCs are able to protect B-ALL cells from both Ara-C and corticosteroids-induced apoptosis through the activation of Wnt and Notch signaling pathway, respectively[115, 116].

Furthermore, ALL-MSCs show higher levels of asparagine synthetase, compared to their healthy counterpart, that correlate with high levels of asparagine secretion. In this way, MSCs exert a protective effect and determine asparaginase resistance of ALL cells[117]. Chemoresistance of B-ALL cells could be also mediated by reciprocal leukemic cell-MSC activation of NF-*k*B signaling, dependent on the interaction between VLA-4 (expressed on leukemic cells) and VCAM-1 (produced by MSCs)[118]. Notably, in B-ALL relapsed patient samples, high expression of VLA-4 is associated with poor outcome and lower probability of event-free and overall survival[119].

Furthermore, changes in cancer metabolism represent a key chemoprotecive mechanism since, under hypoxic conditions, co-culture of B-ALL cells and MSCs shows an increase in HIF-1α expression which is associated to the upregulation of glucose transport and to the switch towards glycolytic metabolism in primary B-ALL samples[120].

Recently, it has been demonstrated the direct effect of BCP-ALL cells on BM-MSCs. De Vasconcellos et al. demonstrated that leukemic cells could induce the upregulation of inflammatory chemokines, such as CCL2 and IL-8, which directly affect BM-MSC survival, proliferation and adhesion to ALL cells[121]. Finally, it has been shown that, in co-culture system, BCP-ALL cells increase the levels of CCL2, CCL22, CXCL8 and CXCL1, thus triggering a possible mechanism for reducing healthy HSC homing to BM niche[122].

1.3.2. Chemokines: drivers of the malignant BM niche

Chemokines are small proteins implied in the regulation of cell migration, development, immune surveillance, inflammation and also in many pathological conditions[123]. Approximately 20 chemokine receptors and 50 chemokines have been identified in humans. Chemokines and their receptors belong to four families based on the pattern of cysteine residues: CXC, CC, CX3C and C, where C represents the cysteine and X represents non-cysteine amino acids. Moreover, chemokines can be classified according to their function into inflammatory chemokines and homeostatic chemokines, where inflammatory chemokines are specifically induced by inflammation while homeostatic chemokines are constitutively expressed and are involved in homeostatic immune regulation[124].

Chemokines are bound by seven transmembrane spanning proteins coupled to G-protein-coupled-receptors (GPCRs). The interaction between chemokines and their cognate receptors causes the activation of GPCRs as guanine nucleotide exchange factors (GEFs): indeed, GPCRs induce the activation of the Gα subunit through the loading of GTP and the dissociation from Gβγ and from the receptor[125]. The Gβγ dimer can act as a Gα inhibitor when bound to a Gα subunit, because it favours the interaction between Gα and GDP. However, when the Gβγ complex is dissociated from Gα, it can also participate in the signaling cascade regulating ion channels and causing

the phosphorylation of the extracellular signal-regulated kinases (ERK 1/2) via the protein kinase C/protein kinase A pathway. The system is switched off by regulators of G protein signaling that promote the GTPase activity of Gα subunit that in its GDP-bound form is able to re-associate with Gβγ. The newly reassembled inactive heterotrimer (Gαβγ) can couple again with available GPCRs[126].

Chemokines and their receptors are key players in several diseases. It has been demonstrated that an aberrant chemokine receptor signaling, due to inactivation of tumor suppressor genes or constitutive activation of oncogenes, promotes tumorigenesis[127]. In addition, the expression of many chemokines has been found to be altered in several malignancies overexpressing the nuclear factor-kappa B (NF-kappa B), which regulates chemokine production[128]. It has been proposed that tumor cells are able to exploit the so-called "cellular highways", that guide cells towards specific sites, and they express specific chemokine receptors, that allow them to acquire selective advantage and resistance to therapy[127].

As regard leukemia, the interaction of leukemic cells with BM niches has been identified as the main cause of leukemia relapse. ALL relapse has been associated to the survival of blasts in organs such as CNS or testes, where the levels of anti-leukemic drugs are diminished[129]. The main hypothesis about the enhanced ability of leukemic cells to infilitrate these sanctuary organs is that ALL blasts are characterized by a

deregulated expression of specific chemokine receptors, that are implied by immune cells in homeostatic conditions and that allow them to acquire a competitive advantage against chemotherapy[127].

The chemokine CXCL12 is one of the key factors that mediates this crosstalk and regulates the homing of leukemic cells into the BM niche: indeed, similarly to healthy HSCs, leukemic cells preferentially migrate to CXCL12-expressing vascular niches[130]. The activation of the CXCR4 signalling pathway leads to the upregulation of key molecules such as JAK/STAT, p38MAPK, MEK/ERK, PI3K/Akt and PKC[1] that mediate migration, adhesion, survival, proliferation and drug-resistance of ALL cells. Of note, a high expression of CXCR4 and its phosphorylated (active) form on B-ALL blasts has been related to a poor outcome[131, 132].

Consistently with the pro-migratory effects of CXCL12/CXCR4 axis, the high expression of CXCR4 on leukemic cells was strongly predictive for extramedullary organ involvement in B-ALL: indeed, a strong CXCR4 expression was detected in mature B-ALL, a disease characterized by massive leukemic infiltration of extramedullary sites, including liver, spleen, lymph nodes and central nervous system[133]. It has to be considered the impact of this axis on leukemia homing into the liver, since it has been documented that bile duct epithelial cells, which express high levels of CXCL12, define a hepatic niche that supports infiltration and proliferation of B-ALL cells in the liver of

NSG immunocompromised mice xenografted with primary human B-ALL cells[134].

Studies of MSCs derived from the BM of children with ALL revealed that the expression of several key lymphoid trophic factors, as CXCL12, is reduced, independently from the percentage of leukemic blasts[135]. In particular, pre-B leukemic cells are able to suppress stromal CXCL12 expression via SCF production, thus inhibiting support of stromal cells to normal HSCs[97]. In line with these data, it has been demonstrated that the expression of CXCR7, the other chemokine receptor for CXCL12, is often deregulated in leukemias and lymphomas and that its silencing in T-ALL cell lines negatively affects cell migration towards CXCL12. Therefore, it has been proposed that CXCR7 may potentiate CXCR4 response of ALL cells and contribute to leukemia maintenance by initiating cell recruitment to BM niches originally occupied by HSCs[136]

Under physiological condition, CCR7 drives the recruitment of naïve T cells and activated dendritic cells to lymph nodes, where they initiate adaptive immune responses thanks to CCR7 binding to CCL21 and CCL19[137]. Both CCR7/CCL21 and CCR7/CCL19 axes have been crucially linked to tumorigenesis and metastasis formation. In detail, it has been demonstrated that CCR7 mediates metastasis of T-cell leukemia to the central nervous system: indeed, the oncogenic expression of Notch1 signaling, typical of T-ALL patients, upregulates CCR7

expression in T-ALL cells that are able to infiltrate leptomeningeal spaces of the brain[138].
Additional studies show that CCR7/CCL21 interaction induces directional invasion of breast tumor cells, pseudopodia formation and actin polymerization, which increases the invasiveness of tumor cells. Since both CCL21 and CXCL12 are highly expressed in the lymph nodes and receptor-ligand interactions of both chemokines promote invasiveness, it is likely that the two ligands work together to promote metastasis to the lymph nodes[139]. Moreover, CCR7 also mediates the metastatic process in squamous cell carcinoma of the head and neck (SCCHN) through the activation of integrin αvβ3, implied in adhesion and migration of cancer cells to the extracellular matrix[140].
In addition to the well-studied CXCR4 and CCR7-dependent axes, recent works suggest that CXCR3 interaction with the chemokine CXCL10 could drive ALL relapse in the CNS, where neural cells, in response to inflammation, locally produce CXCL10[141].

In the last years, several studies have been aimed at improving leukemia cure rates by targeting deregulated chemokine axes, especially by using CXCR4 antagonists. CXCL12/CXCR4 inhibitor, Plerixafor, completely blocks CXCL12-induced chemotaxis, attenuates the migration of pre-B-ALL cells into BM stromal cell layers and enhances the cytotoxic and anti-proliferative effects of vincristine and dexamethasone[142].

Prolonged administration of CXCR4 antagonists to mice with either murine or human B-ALL results in a marked reduction in the number of leukemic cells and their dissemination into extramedullary sites, including liver and kidney. However, it was found that AMD3100 increases *in vivo* surface expression of both CD49a (the integrin α subunit of VLA-4) and CXCR7 in B-ALL cell lines, which could be considered as a mechanism of resistance to CXCR4 inhibition[142]. All these data suggest that targeting CXCR4/CXCL12 axis could be partially effective in BCP-ALL treatment and that other molecules could contribute to the definition of a protective microenvironment.

1.4. Inflammation and cancer

Inflammation is an immune mechanism that exerts a key role in the response against pathogens and tumor cells, through several cell types and mediators. Over the past decades, epidemiological, genetic and experimental evidence demonstrate that chronic nonresolving inflammation can increase cancer risk and promote cancer progression[143]. Therefore, inflammation is now considered a hallmark of cancer[144]. It has been demonstrated that the risk of carcinogenesis increases under conditions of persistent nonresolving inflammation and, in particular, chronic infections are at the basis of 15-20% of all cancers developed. Examples include viral infections as hepatitis B and C for liver cancer and papilloma virus for cervix carcinoma, bacterial infections, as Helicobacter pylori for gastric cancer or lymphoma, parasites, as Schistosoma for bladder cancer[145]. It has been shown that also non infectious agents as tobacco smoke, asbestos, silica, gastric reflux, chronic inflammatory disorders of the gastrointestinal tract and autoimmune diseases can trigger chronic inflammation, thus promoting cancer development[146]. Not all the tumors are triggered by infections or chronic inflammation, however for almost all tumors a reactive inflammatory microenvironment has been described: activation of oncogenes and/or inactivation of tumor-suppressor genes induces inflammatory genes, including cytokines and chemokines that recruit circulating leukocytes to the tumor tissues. Moreover, it has been shown that inflammatory cells

per se mediate tumor initiation and promote genetic instability[143, 144, 146]. The connection between inflammation and cancer can be defined both as inflammation-induced carcinogenesis (extrinsic pathway) and cancer-associated inflammation (intrinsic pathway). These two pathways both concur to build up an inflammatory microenvironment that accelerates cancer development and progression[143, 147].

Concerning the intrinsic pathway, different types of alteration, such as activation of oncogenes, mutations, chromosomal rearrangements or amplification, or inactivation of tumour suppressors, contribute to tumour progression. Cells that carry these alterations produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumours. Among molecules involved in cancer-related inflammation, there are transcription factors, such as NF-κB and STAT3[143].
NF-κB coordinates innate immunity and inflammation in homeostatic conditions, however it has emerged as an endogenous tumour promoter because of its deregulated expression both in tumour and inflammatory cells. This transcription factor is activated by both Toll-like receptor (TLR)-MyD88 signalling pathway and the inflammatory cytokines TNF-α and IL-1β. Moreover, it can be activated consequent to genetic alterations in tumour cells[148]. NF-κB activation causes the enhanced expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes of the prostaglandin-synthesis pathway, inducible nitric oxide synthase (iNOS) and

angiogenic factors. NF-κB pathway is tightly controlled: indeed, the balance of inhibitors and activators tunes the extent to which NF-κB operates as an endogenous tumour promoter[148].

Similar to NF-κB, STAT3 is a point of convergence for several oncogenic signalling pathways. It is constitutively activated both in tumour cells and in immune cells and is involved in oncogenesis and inhibition of apoptosis. The activation of STAT3 in tumour cells has been shown to increase the ability of tumours to evade the immune system, by suppressing the immune response[149, 150].

The extrinsic pathway is the mechanism by which inflammatory or infectious conditions increase the risk of developing cancer. In cancer, similarly to what happens in response to pathogens, the activation of pattern-recognition receptors (PRRs) responsible for sterile inflammation has been observed. In particular, among PRRs, toll-like receptors (TLRs) and NOD-like receptor (NLRs) are involved in the recognition of cancer-related Damage-Associated Molecular Patterns (DAMPs) released in response to apoptosis/necrosis, cellular stress and matrix remodelling[151]. While DAMPs-TLR interaction can produce IL-6 and TNF-α, the NLRs-containing protein complex, called inflammasome, controls the secretion of bioactive IL-1β. TLRs and inflammasome cooperatively increase inflammation in the tumor microenvironment through the production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α that, either directly or indirectly, stimulate tumour survival, proliferation and angiogenesis.

IL-1β is an important link between innate and adaptive immune responses since it promotes the polarization of IFN-γ-secreting $CD8^+$ T cells and induces generation of IL-17-producing γδT cells[152]. However, IL-1β is also a pro-cancer factor due to its immunosuppressive and chemoresistant properties. It has been shown that the injection of melanoma cells into *Il1b* knockout mice does not produce the formation of tumor, highlighting the role of IL-1β in carcinogenesis and cancer invasion[153]. The activation of IL-1/IL-1R signaling pathway engages multiple mediators, such as IL-6 and TNF-α and their soluble receptors, to join the inflammation[154].

IL-6 is a downstream target gene of NF-κB and also a strong inducer of STAT3 pathway. The IL-6/STAT3 signaling axis maintains the intra-tumoral inflammation and promotes tumor growth: IL-6 release by senescent cells can be sustained and enhanced in autocrine manner, while IL-6 promotes the proliferation of surrounding tumor cells in paracrine manner[154].

TNF-α produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor cell survival[145]. Binding of TNF-α to the TNF receptor 1 (TNFR1) causes the activation of NF-κB and c-Jun N-terminal kinase (JNK) signaling cascades that leads to the upregulation of pro-inflammatory, pro-angiogenic and invasiveness-promoting factors, and to the induction of anti-apoptotic molecules[155]. Furthermore, TNF-α impairs immune surveillance by affecting T cell responses and the activity of macrophages[156].

1.4.1. Inflammatory cytokines as critical regulators of the BM niche

The association between inflammation and cancer progression has been well recognized in solid cancers, however it has been recently described the role of pro-inflammatory milieu in the sustainment of leukemic cells at the detriment of healthy heamatopoiesis within the leukemic BM niche.

The acute exposure of HSCs to pro-inflammatory cytokines promotes effective host defense through the regulation of size and shape of the hematopoietic system[157], however a prolonged exposure to these molecule can be detrimental to HSC functions: in particular, overproduction of pro-inflammatory cytokines is often a feature of chronic inflammatory diseases and bone marrow failure syndromes[158]. Moreover, the crosstalk between pro-inflammatory cytokines and the BM niche may also play a critical role in the pathogenesis and/or progression of hematological diseases, such as CML and primary myelofibrosis (PMF), that are characterized by a disregulated production of inflammatory cytokines responsible for MSC reprogramming toward inflammatory OBCs, resulting in BM fibrosis. Strikingly, LSC maintenance is unaffected, suggesting that inflammation alters the BM niche into a self-reinforcing leukemic niche favouring the maintenance of transformed LSCs over normal HSCs[110].

1.4.2. Inflammatory pathways in invasion and metastasis

It has been recently demonstrated the existence of a link between inflammation and metastasis: indeed, the invasive capacity of malignant cells can be sustained by the exposure to inflammatory cytokines, such as TNF-α, IL-1β and IL-6[159].

TNF-α induces epithelial-to-mesenchymal transition (EMT) through transcriptional and post-transcriptional upregulation of Snail, a core regulatory factor for EMT[160]. Moreover, IL-6 is also responsible for induction of EMT phenotype in colorectal cancer via STAT3-dependent suppression of miR-34a[161]. Finally, IL-1β role in invasive process has been demonstrated in a *Il1b* knockout model of melanoma, since the lack of this molecule inhibit tumor formation and lung micrometastases of melanoma cells[153]. Therefore, along with the so-called "seed and soil" hypothesis, tumour cells ("seeds") preferentially metastasize to specific sites ("soil"), such as lung, liver and bone, where "pre-metastatic niches" are the results of inflammatory mediators derived from primary tumour sites[162].

The exposure of malignant cells to pro-inflammatory cytokines also induces upregulation of chemokine-receptors, that allows tumor cell migration to and survival at sites that are distant from the original tumour[143]. For example, autocrine TNF-α-mediated signaling upregulates the expression of functional CXCR4 by ovarian cancer cells[163], causing colonization of the peritoneal cavity, angiogenesis and spread to sites which are distant from the peritoneal cavity[164].

Moreover, it has been shown that TAMs are “obligate partners for tumour-cell migration, invasion and metastasis”: indeed, they promote invasive phenotype in tumor cells through a paracrine loop of tumour-cell M-CSF and macrophage epidermal growth factor, with intravasation assisted by direct interactions between tumour cells and TAMs[165, 166]. In addition, inflammatory macrophages increase peritoneal dissemination of tumour cells and metastatic spread in an ovarian cancer model in NF-κB-dependent and TNF-α-dependent manner[167].

1.5. Activins: emerging roles in physiology and cancer

Activin A is a member of the human transforming growth factor-β (TGF-β) superfamily, which is composed by 33 structurally similar proteins secreted as homodimers or heterodimers. Family members include TGF-βs, activins, growth and differentiation factors (GDFs) and bone morphogenetic proteins (BMPs). These molecules have a crucial role in development, during which they can act as morphogens since they elicit signaling responses to surrounding cells in a concentration dependent manner. Moreover, they contribute to tissue homeostasis in multicellular organisms and it has been demonstrated that perturbations in their mechanism of action can lead to cancer, fibrosis and vascular- and immune-related diseases[168].

TGF-β superfamily members communicate via structurally related type I and type II receptors, which are endowed with dual-specificity kinase activity. So far, seven type I, also defined as activin receptor-like kinases (ALKs), and five type II receptors have been described. TGF-β signals via TGF-β type II receptor (TβRII) and TβRI (also known as ALK5), activins signal via activin receptor type II (ACTRIIs) and ACTRIs (ALK2 and ALK4), and BMPs signal via BMP type II receptor (BMPRII) and ACTRII, or via BMPRI, ALK1, ALK2, ALK3 and/or ALK6. Activins and BMPs share some signalling receptors therefore competing for the binding to these receptors[169].

Activin A biological effects depend on its binding with type II receptor that causes the subsequent recruitment and phosporylation of type I receptor. This interaction can determine the activation of both SMAD (Small Mother Against Decapentaplegic)-dependent and independent signalling pathways, in a cell-type- and context-dependent manner[170]: indeed, accumulation of ligand and activation of receptors could activate the non-SMAD pathways either by phosphorylation or by direct activation, that could be mediated by ligand occupied receptors, for example. The non-canonical pathway can be activated in parallel to SMADs either downstream or upstream of SMAD signaling. Of note, SMAD-independent pathways could regulate SMAD signaling[170].

Concerning the SMAD pathway, once activated type I receptor directly phosphorylates and activates receptor-regulated SMADs (R-SMADs), SMAD2 and SMAD3, that, interacting with the common mediator SMAD4, generate a SMAD complex. This heterotetromeric complex exposes a nuclear localization signal, that causes its nuclear accumulation and transcription regulation[169]: indeed, SMADs can interact with transcriptional co-activators and co-repressors to regulate gene expression. Inhibitory SMADs have also been identified: in particular, SMAD7 inhibits Activin signalling by competing with R-SMADs for the interaction with activated type I receptors and by recruiting E3 ubiquitin ligases to activated type I receptors, thereby directing them to internalization and degradation[168].

In several cell types, such as epithelial cells, hepatocytes[171], osteoblasts[172], neural crest cells[173] and osteoclasts[174], TGF-β superfamily members can also signal independently of SMADs, through other signal transducers as p38 MAPK, c-Jun amino terminal kinase (JNK) and extracellular signal regulated kinases (ERKs) MAP kinase signalling pathways, phosphatidylinositol 3-kinase (PI3K)/AKT signalling and small GTPases, which are collectively known as non-SMAD signaling pathways[175, 176].

JNK and p38 MAPK signaling pathways are tipically activated by MAP kinase kinases (MKKs) in response to cytokines and environmental stress, as TGF-β[177]. In particular, ligand-bound TGF-β receptors interact with tumor necrosis factor receptor-associated factor 6 (TRAF6) and TGF-β-activated kinase 1 (TAK), thus inducing the activation of downstream kinases, such as JNK, p38 MAPK and IKK[178]. SMAD6 inhibits the TGF-β-induced JNK and p38 MAPK activation, whereas SMAD7 promotes TGF-β-induced JNK and p38 MAPK activation[176].
Activated JNK and p38 MAPK then phosphorylate their targeted transcription factors, whereas IKK phosphorylates nuclear factor-kB (NF-kB). These transcription factors cooperate with activated SMADs to regulate apoptosis[179] and epithelial-to-mesenchymal transition (EMT)[180]. TRAF6 promotes EMT and cancer cell invasion through a mechanism mediated by the proteolytic cleavage of TbRI: indeed, TRAF6 determines the release of an intracellular domain (ICD) of TbRI able to traslocate in the nucleus and to promote cell invasion in certain

types of cancer[181]. Moreover, TRAF4 is required for migration, EMT and metastatic dissemination in response to TGF-β[182]. It has been hypothesized that non-SMAD signaling through TRAF4 or TRAF6 and TAK1 is an obligatory step in TGF-β-induced EMT and cancer cell invasion.

TGF-β and BMP can also activate Rho-like GTPases, independently of SMAD signaling pathway[183]. The Rho-like GTPases, RhoA, RhoB, Rac and Cdc42, are involved in the control of the dynamics of cytoskeletal organization, cell motility and gene expression[184].

Rac1 is activated by tyrosine kinase receptors and its signaling mediators Ras-Raf-MEK-ERK: once activated, Rac1 can exert its function via multiple effectors, such as p21-activated kinase 1 (PAK1) and Rac1-dependent NADPH oxidases, which generate reactive oxygen species (ROS). Rac1 is a subunit of the NADPH oxidase (NOX) that intracellularly generates ROS in response to growth factors, cytokines or tumor promoters: indeed, ROS are able to oxidize critical target molecules, such as protein tyrosine phosphates (PTPs), thus triggering cell adhesion, EMT and migration[185]. Three of the downstream molecules regulated by ROS are p38, ERK MAPKs and PAK: in particular, MAPK cascade is considered the major signaling pathway involved in driving tumor cell metastasis downstream TGF-β/SMAD and integrin-mediated signaling, while PAK is an effector of Rac-mediated cytoskeletal remodeling responsible for cell migration[185]. Moreover, Rac1 enhances migration and

invasion through filopodia formation and PI3K dependent activation of Cdc42, or mediates EMT[186]. Finally, Rac1 activated by the atypical Rac1 activator DOCK4 mediates extravasation and metastasis in response to TGF-β/SMAD signaling[187].

The serine/threonine kinase protein kinase B, more commonly known as Akt, regulates cell survival, proliferation, increase in cell size and metabolism[188]. Growth factors, hormones and cytokines activate Akt through PI3K and, among these molecules, TGF-β and BMP[189]. TGF-β-induced PI3K and Akt signaling can be activated through receptor-mediated ubiquitylation of TRAF6, which induces membrane recruitment and activation of Akt[190].

TGF-β-induced EMT could be due to the activation of the PI3K/Akt pathway. In particular, mTOR, a target of the Akt kinase, plays a key role in the contribution of the PI3K/Akt pathway to TGF-β-induced EMT: indeed, TGF-β can induce a rapid activation of mTOR complex 1 (mTORC1) and S6 kinase (S6K), leading to increased protein synthesis, cell size, motility and invasion[191]. Moreover, TGF-β induces the activation of mTOR complex 2 (mTORC2), which promotes cytoskeletal reorganization, RhoA activation and cell migration[192].

1.5.1. TGF-β family: a crucial link with cancer

Several studies described the involvement of TGF-β family members in cancer pathogenesis and development. The best-characterized member of the family is TGF-β.

In physiological conditions, TGF-β, produced by both immune and stromal cell types, exerts a key role in initiation, maintenance and resolution of immune responses[193]. The dysregulation of TGF-β signalling components results in altered immune responses and systemic uncontrolled inflammation, that may account for the pathogenesis of several autoimmune and inflammatory diseases[168]. Moreover, TGF-β1 is a potent negative regulator of hematopoietic progenitor cells: indeed, *ex vivo* HSC pretreatement with TGF-β1 before transplantation into recipient mice causes a reduced *in vivo* long-term reconstitution ability of HSCs[194].

In normal and premalignant cells, TGF-β could suppress tumor progression directly through cell-autonomous tumor-suppressive effects, such as cytostasis, differentiation and apoptosis, or indirectly through effects on the stroma through the suppression of inflammation and stroma-derived mitogens. However, in advanced cancers, tumor cells lose tumor-suppressive responses and exploit TGF-β to initiate immune evasion, growth factor production, differentiation into an invasive phenotype, and metastatic dissemination[195-198].

Genetic and/or epigenetic changes that alter TGF-β signaling can lead to dysregulation and imbalance in signaling pathways required for normal hematopoiesis, thus resulting in hematological diseases and/or malignancies[199].

In CML, TGF-β signaling preserves the self-renewal capacity of the leukemic stem cells driving the disease[200]. Moreover, it has been demonstrated that a subset of natural killer (NK) cells

expressing KIR2DS1 secretes TGF-β, which drives the resistance of CML stem cells to tyrosine kinase inhibition[201]. Furthermore, phosporylation of SMAD3, along with p38 MAPK activation, contributes to CML stem cell mantainance *in vivo*[202].
Genetic alterations that attenuate TGF-β signaling promote ALL. The t(12;21) translocation generating the TEL-AML1 fusion oncoprotein, typical of childhood B cell precursor ALL, is able to bind SMAD3 and to inhibit its ability to activate transcription of target genes. Murine early B cell progenitor cells expressing TEL-AML1 transgene proliferate more slowly than parental cells but are more resistant to TGF-β-induced growth suppression. The inhibition of SMAD3 function exerted by TEL-AML1 sustains the proliferation of pre-leukemic cells in childhood B cell precursor ALL patients over healthy HSCs that are sensible for TGF-β anti-proliferative effect[203].

BMP signaling disregulation has been associated to the development of several pathological processeses and, among them, cancer[204]. BMPs are key molecules in embryogenesis, since are involved in development of many organ systems, by regulating cell proliferation, differentiation and apoptosis[205].
The best characterized molecule is BMP4 that has been implicated in cancer development, through the induction of epithelial-mesenchymal transition[206-208], migration[209-211], proliferation[212] and metastatic process[213, 214].
It has been demonstrated the pro-tumoral role of BMP4 in several type of hematological malignancies, such as AML, CML

and ALL. In particular, in CML BMP4 sustaines survival of LSCs and expansion of myeloid progenitors[215] as well as CML primitive cell resistance to Tyrosine Kinase Inhibitors[216]. In AML context, it has been demonstrated the role of BMP4 in the chemoresistance of leukemic cells through the activation of autophagy and inhibition of apoptosis[217].

Finally, a recent work highlighted a higher production of BMP4 in MSCs derived from ALL patients compared to healthy donors derived MSCs. Moreover, co-culture of ALL-MSC with leukemia cell line powerfully enhanced BMP4 production, suggesting the participation of this molecule in BM niche, even though the mechanism remains to be elucidated[218].

1.5.2. Activins

Three active forms of Activin have been identified, namely Activin A (βA/βA), Activin B (βB/βB) and Activin AB (βA/βB). Activin A is the best-characterized ligand of this subfamily.

It shares the SMAD2-SMAD3 canonical pathway with TGFβ, but uses distinct type II (ACTRIIs) and type I (ACTRIB) receptors. Follistatin, a glycoprotein that binds Activin A with high affinity and functions as a ligand trap, blocks the biological activity of Activin A. Activin A has emerged as a pivotal regulator of the immune system in mammals, with both anti- and pro-inflammatory characteristics depending on the cellular and temporal context[219].

Activin A can be produced by monocytes, macrophages, dendritic cells (DCs) and T helper 2(T_H2) polarized $CD4^+$ T cells in response to both pro-inflammatory and non-inflammatory stimuli[219]. Under physiological conditions, it has been shown to dampen inflammation through several mechanisms. In detail, it has been demonstrated that T_H2-secreted Activin A can act as a T_H2-like cytokine by promoting the alternative activation of macrophages into anti-inflammatory M2-like macrophages[220]. Moreover, it supports the development of $FOXP3^+$ T_{reg} cell populations synergizing with TGF-β1 to induce *Foxp3* gene transcription[221]. Furthermore, in the NK-DC crosstalk, Activin A results able to inhibit the expression of inflammatory cytokines and the NK cell-mediated DC maturation[222]. Finally, Activin A produced by DCs upon CD40L stimulation regulates DC migration by influencing chemokine receptor expression and also acting as indirect chemotactic factor through the polarized release of CXC-chemokine ligand 12 (CXCL12) and CXCL14[223, 224].

Besides its anti-inflammatory role, Activin A is a key player of hematopoiesis, with a crucial role in B cell development within the bone marrow, because of its capacity to control cytostasis and apoptosis. However, some evidence point out an opposite relationship between the stromal expression of Activin A and the onset of B cell development: indeed, inhibition of Activin A by follistatin in long-term bone marrow lymphopoietic cultures causes the early onset of B lymphopoiesis, suggesting a

negative effect of Activin A on normal B-lineage cells[225]. Along with these data, it has been shown an inverse relationship between functional Activin A titer and the abundance of B cells in long-term bone marrow lymphopoietic cultures. Finally, isolated B-cell precursors seeded *in vitro* in the presence of Activin A resulted halted at an earlier differentiation stage compared with cells seeded in the absence of this cytokine [226].

Activins play a fundamental role also in the regulation of erythropoiesis: mouse models showed that *in vivo* administration of Activin A is able to increase erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) in BM cultures established from anemic and healthy mice[227]. Moreover, *in vitro* treatment of primary human total BM cells or human CD34$^+$ progenitor cells with Activin A induces the expression of the erythropoietin receptor and hemoglobin, and promotes the proliferation of erythroid progenitors[228].

Intriguingly, Activin A has been reported to be either a pro- or anti-tumorigenic factor in different types of cancer. Activin A exerts a cytostatic effect on prostate, liver, breast and pancreatic carcinoma cells, whereas it promotes the growth of testis and ovarian cancer cells[229-231]. Moreover, it regulates self-renewal in colorectal cancer stem cells[232]. In addition, Activins have many immunoregulatory functions that may have a major impact on cancer development and progression[233]. Transgenic mice overexpressing Activin A in keratinocytes show increased

susceptibility to skin carcinogenesis since the molecule inhibits the proliferation of epidermal γδ T cells, leading to increased survival of transformed keratinocytes and higher tumour loads, and increases T_{reg} cell frequency, thus limiting anti-tumour immune responses[234].

Several works have defined a close connection between Activin A and several feautures of tumor aggressiveness, especially in solid tumor models. In particular, Activin A enhances migration, invasion, EMT and metastatic process in ovarian cancer, mesothelioma, esophageal adenocarcinoma, osteosarcoma, oral squamous cell carcinoma, prostate cancer, esophageal squamous cell carcinoma, breast cancer, head and neck squamous cell carcinoma[235-245]. Finally, in multiple myeloma it has been recongnized its role in tumor-associated osteolysis through a mechanism mediated both by osteoblast inhibition[246] and osteoclastogenesis induction[247]. Nowadays, the potential role of Activin A in leukemia is still unkown.

1.6. Scope of the thesis

Malignant cells are able to alter BM microenvironment to create a leukemic niche that protects tumor cells from the effects of chemotherapy and immune cells. This malignant process could be counteracted by targeting molecular axes crucial for the homing and support of leukemic cells within the “corrupted” leukemic BM niche.

The aim of the thesis was the identification and characterization of new molecules involved in the crosstalk between BCP-ALL cells and the BM niche, using BM-MSCs as BM stroma paradigm. In particular, we focused our attention on the stroma-derived factor Activin A, belonging to the TGF-β superfamily, whose pro-tumoral effects have been widely analyzed in several solid cancers.

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2. Activin A contributes to the generation of the leukemic bone marrow niche by modulating the migratory properties of BCP-ALL cells

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2.1. Abstract

B-Cell Precursor (BCP)-Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. Despite cure rates exceeding 90% in children, relapse still occurs in about 20% of patients. New therapeutical targets could emerge from studies on the "leukemic niche" where stromal cells confer support and chemoprotection to leukemic cells. Here we identified Activin A, a TGF-β family member, as a new crucial factor exploited by leukemic cells to create a self-reinforcing microenvironment. Interestingly, Activin A resulted overexpressed in the plasma and in the stroma surrounding leukemic infiltrate. Activin A resulted a leukemia-driven factor as BCP-ALL cells could specifically induce, *in vitro*, Activin A production by mesenchymal stromal cells (MSCs). Moreover, MSCs isolated from the BM of BCP-ALL patients showed an intrinsic ability to secrete a higher amount of the protein compared to their normal counterpart. The pro-inflammatory leukemic BM microenvironment synergized with BCP-ALL cells to induce stroma-mediated Activin A production. Gene expression analysis and *in vitro* biological studies on Activin A-treated cells showed that this protein was able to significantly promote both random motility and CXCL12-driven migration and invasion of BCP-ALL cells. This data was further confirmed *in vivo* in a mouse model of human leukemia: indeed, exposure to Activin A significantly enhanced leukemic cell ability to infilitrate extramedullary sites and, in particular, the liver which has been previously described as privileged organ of therapy-resistant

leukemic cells. This effect was due to Activin A regulation of several genes associated with cell motility, calcium homeostasis and actin reorganization. Of note, Activin A exerted an opposite effect on healthy $CD34^+$ cells, whose CXCL12-induced migration resulted severely impaired.

Our data highlighted that Activin A could confer an advantage to leukemic cells, making them able to generate a malignant BM niche at the detriment of the healthy hematopoiesis.

2.2. Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent childhood malignancy worldwide. B-ALL represents about 80% of ALL cases and mainly affects children with an incidence of 3-4 cases per 100'000 each year[1]. Despite cure rate has exceed 90% in children, BCP-ALL (B-Cell Precursor Acute Lymphoblastic Leukemia) represents the leading cause of cancer-related death in children and young adults[2]. Inspite of the notable ameliorations in disease management, the emergence of chemoresistance decreases the probabilities of therapy success causing relapse in more than 20% of the treated patients[3]. BCP-ALL cells critically depend on interactions with the BM microenvironment that provides regulatory cues essential for proliferation, survival and drug resistance leading 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 able to sustain leukemic cells to the detriment of normal hematopoiesis and protect them from chemotherapy[5]. In this complex network, it has been shown that chemokines could contribute to BCP-ALL pathogenesis by driving the migration of leukemic cells towards protective BM niches, as well as by providing anti-apoptotic signals[6].

Activin A is a pleiotropic cytokine that belongs to the TGF-β superfamily. It has a broad tissue distribution and it is involved in multiple physiological and pathological processes, including

inflammation, metabolism, immune responses and endocrine function. Recent studies have demonstrated that Activin A is an important regulator of carcinogenesis: indeed, it can directly modulate cancer cell proliferation and migration and it can also enhance tumor progression by regulating tumor microenvironment[7]. Activin A signals through transmembrane serine/threonine kinase receptors: it binds to a type II activin receptor (ACVR2A or ACVR2B), thus causing recruitment, phosphorylation and activation of type I activin receptor (ALK2 or ALK4). Classically, Activin A receptors phosphorylate SMAD2/3, however they can activate other signaling pathways, such as PI3K/AKT and ERK/MEK, in a ligand and cell dependent manner. Activin A signalling is inhibited by inhibins, through a competitive bounding for Activin receptors, and by follistatin (FST) and follistatin like-3 (FSTL3), that act as trap molecules[8].

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

2.3. Experimental procedures

2.3.1. Patient and healthy control samples

We collected BM plasma samples from 108 BCP-ALL patients (mean age: 7 years old) at the diagnosis of the disease. BM samples for healthy controls were obtained from 40 BM stem cell donors (mean age: 21 years old). 6 BM biopsy samples were collected from BCP-ALL patients to perform immunohistochemical analysis (Table 1).

Primary BM cells were obtained from BCP-ALL patients enrolled in the protocol of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) and the Berlin-Frankfurt-Munster (BFM) groups (AIEOP-BFM ALL 2009 protocol) at the Italian center of San Gerardo Hospital, Monza. BM-derived mononuclear cells (BM-MNCs) were isolated from BM samples by Ficoll (GE Healthcare, Uppsala, Sweden) gradient separation and cryopreserved in liquid phase nitrogen. All patients gave written informed consent.

AGE AT DIAGNOSIS	SEX	DIAGNOSIS	% OF BM INFILTRATE
60	F	B-II	90
27	F	B-II	100
32	M	B-II	70
61	M	B-II	100
30	F	B-II	100
53	F	B-II	90

Table 1. Clinical features of BCP-ALL patients implied for ICH analysis.

2.3.2. Culture of BCP-ALL cell lines

Leukemic cell lines, 697 and NALM-6, were maintained in Advanced RPMI (Roswell Park Memorial Institute) 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM) (Euroclone, Milan, Italy). The biologic identity of these cell lines was analysed by FACS analysis of cell surface phenotype (FACS Canto II, BD Bioscience, San Jose, CA, USA) (697: $CD3^-$ $CD34^-$ $CD10^+$ $CD38^+$ $CD19^+$ $MHC\text{-}II^+$; NALM-6: $CD3^-CD10^+$ $CD38^+$ $CD19^+$ $MHC\text{-}II^+$) (anti-human CD38: eBioscience, San Diego, CA, USA; anti-human MHC-II: BD Pharmingen, San Diego, CA, USA; anti-human CD3, CD10, CD19, CD34: BD Biosciences) and detection of cell-specific translocations [t(1;19) and t(5;12), respectively]. REH, RS4;11 and SUP-B15 cell lines were also implied for evaluation of Activin receptor expression by qRT-PCR.

2.3.3. BM-MSC isolation, culture and expansion

BM-MSCs were isolated from BM aspirates of 15 BCP-ALL patients (ALL-MSCs) and 15 age-matched healthy donors (HD-MSCs), from both fresh and cryopreserved samples (Table 2).
BM-MNCs were seeded in low glucose Dulbecco's Modified Eagle Medium (Lonza, Milan, IT), 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM). After 48 h, non-adherent cells were removed from the culture by washing with phosphate-buffered solution

(Euroclone). When 70-80% confluence was achieved, adherent spindle-shaped cells were harvested using 0.05% trypsin-EDTA (Euroclone) and used for further expansion. After four culture passages, BM-MSCs were checked for positive expression of CD105, CD73, CD90, CD54, MHC-I and the lack of expression of CD45, CD34, CD14 and MHC-II by flow cytometry (anti-human CD14, CD90 and CD105: eBioscience; anti-human CD45, CD54, CD73, MHC-I and MHC-II: BD Pharmingen; anti-human CD34: BD Biosciences). To evaluate their osteogenic and adipogenic differentiation ability, BM-MSCs were stimulated for 14-21 days with specific differentiation inductive media. Adipogenic inductive medium consisted of DMEM-High glucose (Euroclone) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM), dexamethasone (1μM), indomethacin (1μM), 3-isobutyl-1-methylxantine (IBMX) (500 μM) and human recombinant insulin (10 μg/ml) (all from SigmaAldrich, St Louis, MO, USA). Lipid droplets were stained with Oil Red O (SigmaAldrich). Osteogenic inductive medium, consisted of DMEM-Low glucose, 10% FBS, 2-phosphate-ascorbic acid (50 μM), β-glycerol phosphate (10 mM) and dexamethasone (100 nM) (all from SigmaAldrich). The presence of calcium deposits was detected by Alizarin Red staining (SigmaAldrich).

AGE AT DIAGNOSIS	SEX	DIAGNOSIS	% OF BM INFILTRATE	TRANSLOCATIONS	MRD RISK
3	M	B-II	90	NEG*	HR (SER)
3	M	B-II	98	t(12;21)	IR
4	M	B-II	90	NEG*	SR
4	F	B-II	77	NEG*	HR (SER)
4	M	B-II	86	t(12;21)	IR
6	M	B-II	80	NEG*	HR (SER)
6	F	B-II	95	NEG*	IR
7	M	B-II	59	t(12;21)	IR
7	F	B-II	98	t(12;21)	IR
8	F	B-II	88	t(12;21)	SR
9	F	B-II	94	NEG*	HR
9	M	B-II	78	NEG*	HR
10	M	B-II	N.A.	t(9:22) p210	HR
15	M	B-II	89	NEG*	IR
18	M	B-II	86	NEG*	IR

Table 2. Clinical and molecular features of BCP-ALL patients implied for BM-MSCs isolation. MRD risk was defined on the basis of patient state at day +15, +33, +78 after treatment beginning (HR: HIGH RISK, IR: INTERMEDIATE RISK, SR: STANDARD RISK, SER: SLOW EARLY RESPONDER). *NEG: negative for t(4:11), t(9;22) p190, t(12;21), t(1;19).

2.3.4. Immunohistochemistry

For Activin A detection, paraffin embedded sections from BM biopsies of BCP-ALL patients were used. The expression of inhibin beta A-subunit was assessed in accordance with standard streptavidin-biotin-peroxidase complex procedures using rabbit polyclonal to Inhibin beta A antibody (Abcam, Cambridge, MA).

2.3.5. Co-cultures of primary leukemic cells with BM-MSCs

2×10^5 BM-MSCs were plated in 6-well flat bottom plates and co-cultured with 6×10^6 primary leukemic blasts in the presence or not of a 0.4 µm transwell insert (Costar Transwell® Permable Supports, Corning Inc., MA, USA) in DMEM 2% FBS for 72h. Supernatants were collected and cryopreserved at -80°C for further analysis.

2.3.6. ELISA assay for quantification of Activin A, CXCL12 and pro-inflammatory cytokines

Levels of Activin A, pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and CXCL12 were assessed in BM plasma samples and culture supernatants using commercially aviable ELISA kits (R&D Systems, USA), according to the manufacturer's instructions.

2.3.7. Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA was used to perform first strand cDNA synthesis using Superscript (Invitrogen), according to the manufacturer's instructions. qRT-PCR was performed through a LightCycler® 480 platform (Roche, Basel, Switzerland) using primer and probes synthesized with Universal Probe Library (UPL, Roche) software. Primer sequences are listed in Table 3. Gene expression levels were normalized on GAPDH transcript levels.

TARGET	PRIMER	SEQUENCE
ALK2	Forward	acactccccacgggaaac
	Reverse	aaagaagagaagcacaggcaat
ALK4	Forward	gaagtgcagcccctctca
	Reverse	cgtctccactggcagtctc
ACVR2A	Forward	aaagcccagttgcttaacga
	Reverse	tgccatgactgtttgtcctg
ACVR2B	Forward	gcataagctgggttttctcct
	Reverse	cctgagcaactcatgcaaag
ATP2B2	Forward	tgcaggattggtggtgatt
	Reverse	tccctggaactggcatctac
ATP2B4	Forward	ccttgtctttgcgggtga
	Reverse	ggctgggtggtgaatgtaga
CCR7	Forward	ggggaaaccaatgaaaagc
	Reverse	acctcatcttgacacaggcata
CORO1A	Forward	agtttgtggccctgatctgt
	Reverse	cattcttgtccacacgtcca
DOCK4	Forward	agcccgatgagaccatctt
	Reverse	gctctctggaatgggagtca
GAPDH	Forward	agccacatcgctcagacac
	Reverse	gcccaatacgaccaaatcc
LCK	Forward	agtcagatgtgtggtcttttgg
	Reverse	cctccgggttggtcatc
PTPRC	Forward	ttcatgcagctagcaagtgg
	Reverse	gccgtgtccctaagaaacag
VAV3	Forward	ccttagatacaactctgcagtttcc
	Reverse	gcccagcacttttggactta

Table 3. Sequence of primers used for quantitative real-time PCR analysis.

2.3.8. Affymetrix Genechip-based gene expression

For microarray experiments, GeneChip Human Genome U133 Plus 2.0 (Affymetrix Inc., Santa Clara, CA, USA) was used. RNA was obtained from 697 cells treated or not with Activin A (50 ng/mL) (R&D Systems) for 6h and 24h from four independent experiments. RNA was extracted using TRIzol reagent (Invitrogen) and quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). RNA concentration was determined using NanoDrop ND-2000 Spectrophotometer (Thermo Scientific, Waltham, USA). *In vitro* transcription, hybridization and biotin labeling were performed according to Affymetrix One Cycle Target Labelling protocol (Affymetrix). Supervised analyses were performed using shrinkage test and multiplicity corrections were used to control FDR (false discovery rate); probes with local FDR lower than 0.05 were considered significant. Gene ontology (GO) analysis was performed on selected Affymetrix IDs by GO enrichment analysis tool.

2.3.9. Time-lapse microscopy

Target cells were prepared for live cell imaging by seeding 3 × 10^3 leukemic cells into each well of an 8-well chamber slide (Ibidi, Martinsried, Germany) coated with Gelatin B (SigmaAldrich). Cells were incubated overnight in Advanced RPMI 1640 1% FBS to promote adherence to the slide. Primary cells were stained with Propidium Iodide (PI) to discrimate live cells. Prior to recording, Activin A was added to the plates.

Chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C and constant CO_2 concentrations (5%). Images were acquired over 24h. Frame-by-frame displacements and velocities of randomly selected leukemic blasts were calculated by tracking individual cells using ImageJ software (NIH, USA) on manual tracking mode.

2.3.10. Chemotaxis assays

For end-point chemotaxis assays, leukemic and healthy $CD34^+$ cells were pretreated with Activin A in Advanced RPMI 1640 1% FBS. After 24h, they were loaded into Transwell inserts (Costar Transwell® Permable Supports, Corning Inc., pore size, 5 µm) and subsequntly incubated for 4h (BCP-ALL cell lines) or 1h (primary leukemic blasts and healthy $CD34^+$ cells). Advanced RPMI 1640 1% FBS containing CXCL12 (100 ng/mL, if not otherwise specified) (Peptrotech, Rocky Hill, NJ) was added to the lower chambers. Cells migrated in the lower chamber were counted by FACS after addition of a known number of fluorescent reference beads (BD Trucount tubes, BD Bioscience). Techical duplicates were set up for each condition. Cells harvested from each well were counted twice for 60 seconds. The percentage of migrated cells was determined by dividing the number of cells in the lower chamber by the total input of cells added to the upper chamber.

2.3.11. Invasion assays

The invasion capacity of leukemic cells was evaluated using a Matrigel-coated Transwell chamber system. Membrane filters (Costar Transwell® Permable Supports, Corning Inc., pore size 8 μm) were coated with 50 μL of Matrigel (1mg/mL) (Corning) that was allowed to form a gel layer for 1h at 37°C. Leukemic cell lines treated or not with Activin A (50 ng/mL) were loaded on the upper chamber (5×10^5 cells/well in 100 μL Advanced RPMI 1640 1% FBS). The medium containing or not CXCL12 (100 ng/mL) was added to the lower chamber. Transwell plates were incubated for 24h. The percentage of cells migrated through the matrigel barrier into the lower chamber were quantified as described for chemotaxis assay.

2.3.12. CXCR4 and CXCR7 staining

For CXCR4 and CXCR7 expression, 697 cells, pretreated or not with Activin A, were incubated with PE-conjugated human CXCR4 mAb (BioLegend, San Diego, CA) or PE-conjugated human CXCR7 mAb (BioLegend) for 30 minutes at 4°C. For intracellular detection, the cells were firstly permeabilized with Cytofix/Cytoperm (BD Biosciences) and then incubated with the above-mentioned antibodies for 30 minutes at 4°C. Cells were analyzed with a FACS Canto II cytometer and data analyzed by FlowJo software (Tree Star, Inc. Ashland, OR, USA).

2.3.13. F-actin polymerization assay

697 cells were cultured for 24h in Advanced RPMI 1640 1% FBS and they were subsequently stimulated or not with Activin A (50 ng/mL) for 24h. Cells were then harvested and resuspended in culture medium at 37°C with or without CXCL12 (100 ng/mL) for the indicated time points. Cells were permeabilized using Cytofix/Cytoperm solution at 4°C for 20 minutes and then incubated at room temperature for 20 minutes with AlexaFluor 647-labeled phalloidin (Invitrogen) to assess intracellular F-actin polymerization. Cells were analyzed with a FACS Canto II cytometer and the mean fluorescence intensity (MFI) was determined for each sample. The percentage change in the MFI was calculated for each sample at each time point over the basal value (unstimulated cells).

2.3.14. Calcium mobilization

Intracellular calcium mobilization was measured using the Fluo-4NW Assay (Invitrogen), according to the manufacturer's istructions. 697 and Nalm-6 cells were plated in 96-well plates in Advanced RPMI 1% FBS for 24h with or without Activin A (50 ng/mL). Cells were loaded with cell-permeant Fluo-4NW dye. Analysis was performed by FACS. After a 30-second of baseline recording, sample aspiration was briefly paused and CXCL12 (100ng/mL) was quickly added. The Ca^{2+} response was measured as fluorescence intensity of the cells as a function of time. Data analysis was performed using FlowJo “kinetics” tool.

2.3.15. CB- and BM-CD34$^+$ cell isolation

Cord blood (CB) units from healthy neonates were obtained from San Gerardo Hospital. Human BM samples were obtained from healthy paediatric BM donors at the San Gerardo Hospital. Mononuclear cells were isolated by Ficoll density gradient centrifugation and CD34$^+$ cells were purified using immunomagnetic CD34 microbeads (CD34 MicroBead Kit, Miltenyi Biotec, Cambridge, MA). The purity was assessed by flow cytometry and was consistently >95%.

2.3.16. Transplantation of leukemic cells into immune deficient mice

Non-obese diabetic/severe combined immunodeficiency IL-2Rγnull (NSG) mice were purchased from Charles River Laboratories. 5-7 weeks old female mice were i.v. injected with 5×10^5 697 cells pretreated or not with Activin A (50 ng/mL) for 24h. Before injection cells were washed twice in PBS and resuspended in 100 µL PBS/mouse. One week after transplantation, mice were sacrificed and cells from femur, spleen, liver, meninges and peripheral blood were collected. Cells were stained with anti-hCD45 (BD Bioscience), anti-mCD45 (eBioscience) and LIVE/DEAD fixable dead cell stain (Invitrogen). Leukemic engraftment was evaluated as the percentage of hCD45$^+$ on viable cells. To determine the absolute number of leukemic cells, the percentage of hCD45$^+$ was normalized on the total number of harvested cells, counted by a hemocytometer (Beckman Coulter, Fullerton, CA, USA).

2.4. Results

2.4.1. Stroma-derived Activin A increased in response to leukemia

It has been demonstrated that Activin A 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[7]. Therefore, we firstly evaluated Activin A levels in BM plasma samples from healthy donors (HDs) and BCP-ALL patients at the onset of the disease. ELISA assay revealed that Activin A was significantly increased in the BM plasma of BCP-ALL patients compared to healthy donors, as shown in Figure 1A. The mean concentration of Activin A (± SEM) was 864.6 ± 116.3 pg/mL in BCP-ALL patients and 487 ± 104.9 pg/mL in HDs ($P < 0.05$).

Given its role as tissue morphogen, we next evaluated Activin A expression in the BM biopsies of six BCP-ALL patients, by immunohistochemical analysis. As shown in Figure 1B, a very intense staining for Activin A co-localized with highly leukemic infiltrated area, while a lower signal was detected concurrently with healthy haematopoiesis. Furthermore, Activin A expression was specifically detected in the tumor surrounding stroma, suggesting a key role of the protein in the stroma-leukemia crosstalk. In view of the pivotal role played by BM-MSCs within the BM hematopoietic niche, we explored whether BCP-ALL cells could modulate MSC-derived Activin A. To test this hypothesis, we set up co-culture experiments of healthy donor-derived BM-MSCs (HD-MSCs) with primary leukemic blasts and

then we quantified Activin A in supernatants after 72h of culture. The analysis highlighted that primary leukemic cells significantly induced the molecule through a cell-to-cell contact-mediated mechanism (730 ± 111.4 pg/mL), with a 3-fold increase compared to the basal condition (223.9 ± 48.26 pg/mL) ($P < 0.001$). Of note, BCP-ALL primary cells secreted very low or undetectable levels of Activin A (Figure 1C).

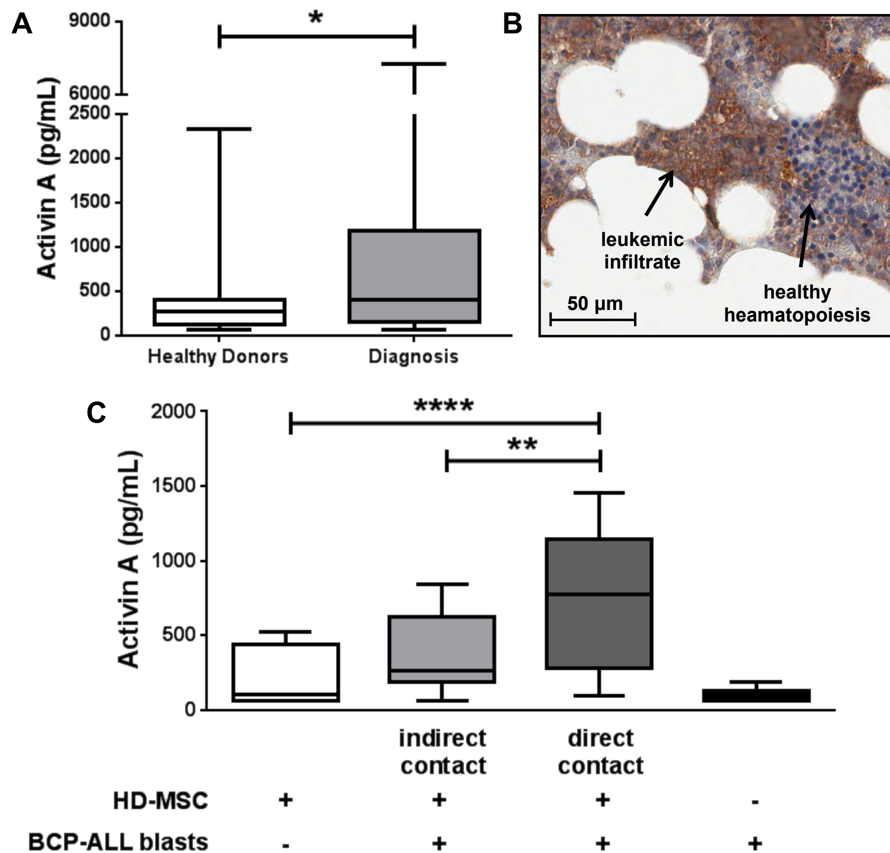


Figure 1. BCP-ALL cells reprogrammed the BM stroma to produce high levels of Activin A.

(A) BM plasma levels of Activin A were assessed by ELISA in HDs (n=44) and BCP-ALL patients at the onset of the disease (n=108).

(B) BM biopsies from BCP-ALL patients (n=6) were analyzed for Activin A expression by IHC. Positive staining was evidentiated by a brown staining on haematoxylin-eosin counterstained sections. Scale bar is 50 μm.

(C) Primary leukemic blasts were directly co-cultured or separated by a 0.4μm transwell insert with HD-MSCs. After 72h

of culture, supernatants were collected and Activin A concentration was analyzed by ELISA (n=17 independent co-cultures).

*P < 0.05: unpaired Student *t* test

**P < 0.01, ****P < 0.0001: one-way ANOVA and Bonferroni multiple comparison test

2.4.2. Leukemic cells expressed Activin A receptors

To determine whether BCP-ALL cells could be targets of Activin A, the mRNA expression of Activin receptors was assessed on five different leukemic cell lines (697, NALM-6, RS4;11, SUP-B15, REH) (Figure 2A) and nine primary BCP-ALL blasts by qRT-PCR (Figure 2B).

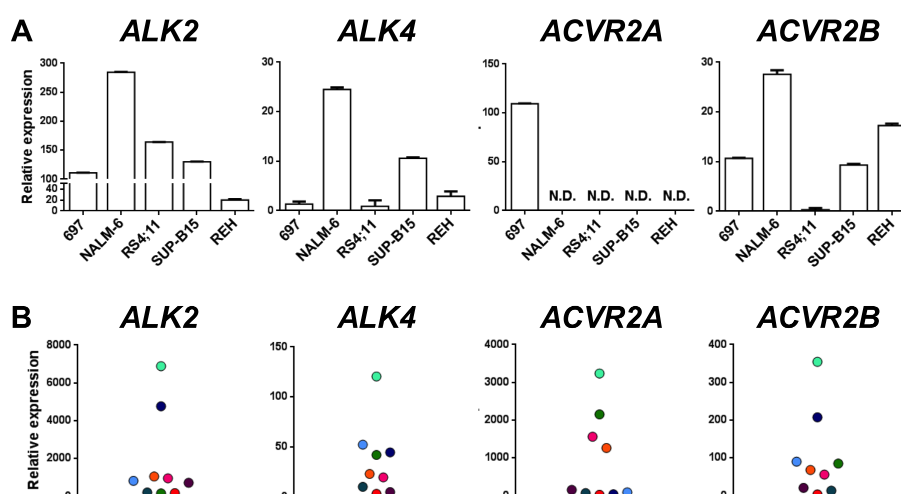
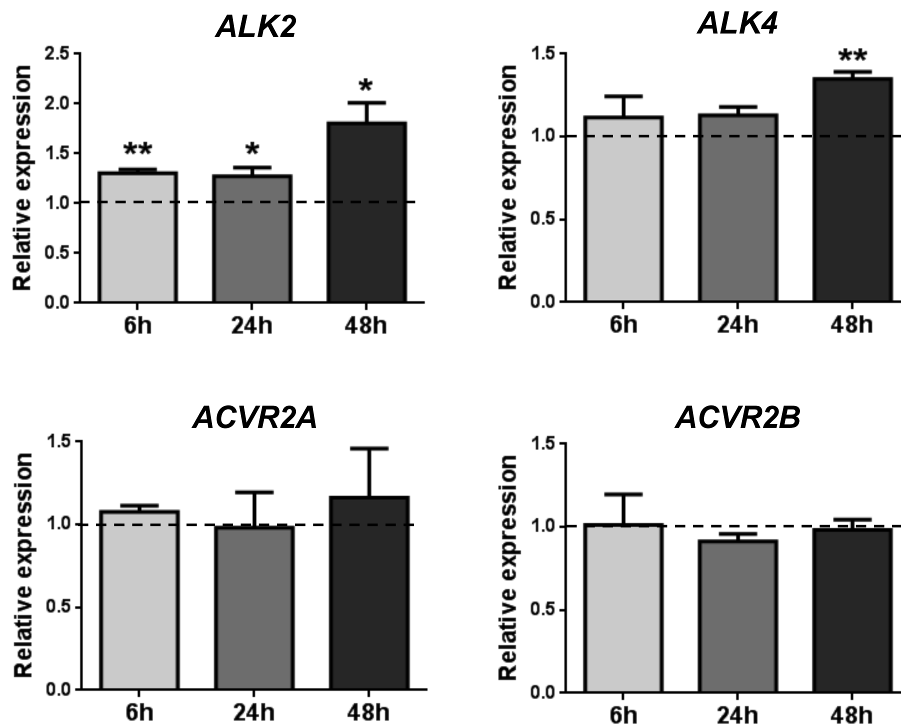


Figure 2. BCP-ALL cells expressed type I and type II Activin A receptors.

(A) The expression of the type I Activin A receptors *ALK2 and ALK4* and the type II Activin A receptors *ACVR2A* and *ACVR2B* was quantified in five leukemic cell lines (697, NALM-6, RS4;11, SU-B15, REH) and **(B)** in BM primary blasts from nine BCP-ALL patients by qRT-PCR. In the latter case a patient-specific colour was adopted. The data are presented as the fold change of mRNA levels of Activin A receptors normalized to *GAPDH* mRNA (endogenous control). DAUDI cell line was employed as

calibrator, because of its low expression of Activin A receptors (www.proteinatlas.org).

The analysis revealed that both type I and type II Activin receptors were expressed by all primary blasts and cell lines tested over a wide range of expression. Of note, *ACVR2A* was expressed only in 697 cell line and resulted undetectable in other cell lines tested. Concerning primary BCP-ALL cells, the level of Activin A receptors expression was highly patient-specific and resulted independent from commonly investigated leukemia-related genetic alterations. On the overall, these data suggest that BCP-ALL cells could possibly respond to Activin A. Moreover, we highlighted that Activin A was able to modulate the expression of its type I receptors, thus suggesting a positive loop underlying the responsiveness of leukemic cells to Activin A (Supplementary Figure 1).



Supplementary Figure 1. Modulation of Activin A receptor expression by Activin A

(A) Expression of *ALK2, ALK4, ACVR2A* and *ACVR2B* was assessed in 697 cells treated or not with Activin A (50 ng/mL) for 6h, 24h and 48h. The data are presented as the fold change of mRNA levels normalized to *GAPDH* mRNA (endogenous control). The graphs represented the results of three independent experiments.

*$P < 0.05$, **$P < 0.01$: unpaired Student *t* test

Subsequent analyses were performed on the 697 cell line, which resulted characterized, similarly to primary leukemic blasts, by an intermediate expression of Activin A receptors.

2.4.3. Gene expression analysis revealed Activin A involvement in regulating cell movement

To deeper analyze the molecular changes induced by Activin A in BCP-ALL cells, we performed gene expression profile of 697 cell line upon stimulation with Activin A for 6h and 24h.

The analysis revealed that 122 genes were differentially expressed in Activin A-treated cells vs. untreated cells after 6h of stimulation ($FDR < 0.05$) and that 151 genes were differentially expressed after 24h of stimulation ($FDR < 0.05$). Gene Ontology (GO) Analysis of differentially expressed genes identified 20 enriched GO categories (Figure 3A) 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 Activin A was able to influence migration-associated pathways such as "calcium ion homeostasis and transport into cytosol", "PI3K/AKT activation", "Ras signalling pathway", "focal adhesion", suggesting its possible effect on BCP-ALL motility. These data are in agreement with the recently recognized role of Activin A in the regulation of cell migration and invasion in the context of several solid malignancies[9-12].

On the basis of this evidence we firstly validated, by qRT-PCR assays, the Activin A-mediated changes in the expression of several genes linked to Ca^{2+} homeostasis (*ATP2B2*, *ATP2B4*), Ras pathway activation (*VAV3*), cell motility and movement regulation (*CCR7, CORO1A, DOCK4, LCK, PTPRC)*.

Data obtained by qRT-PCR were highly concordant with microarray data, as shown in Figure 3B. Raw data are shown in Supplementary Figure 2.

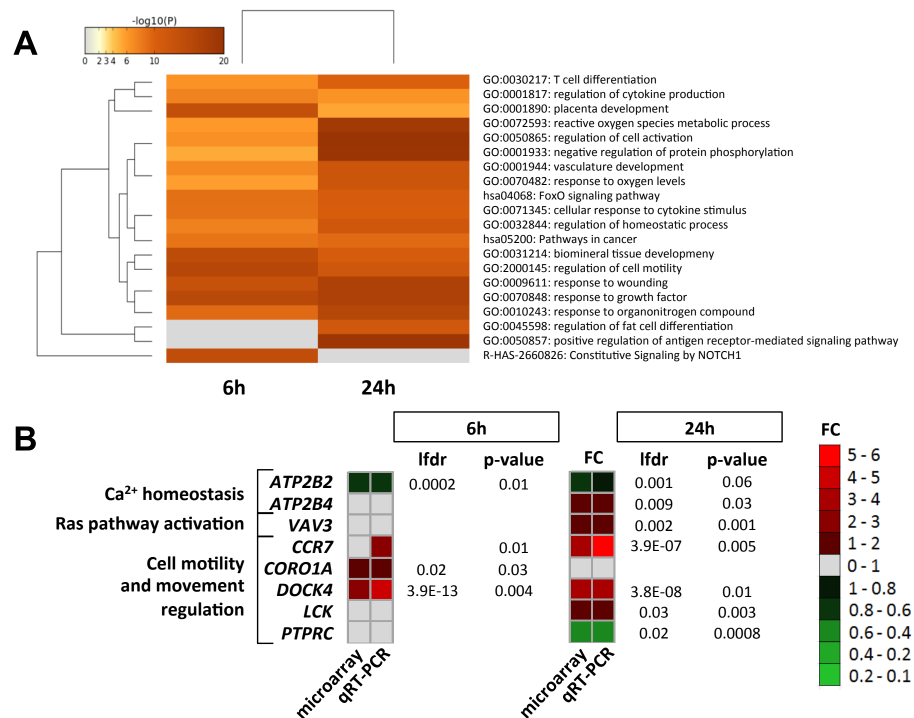
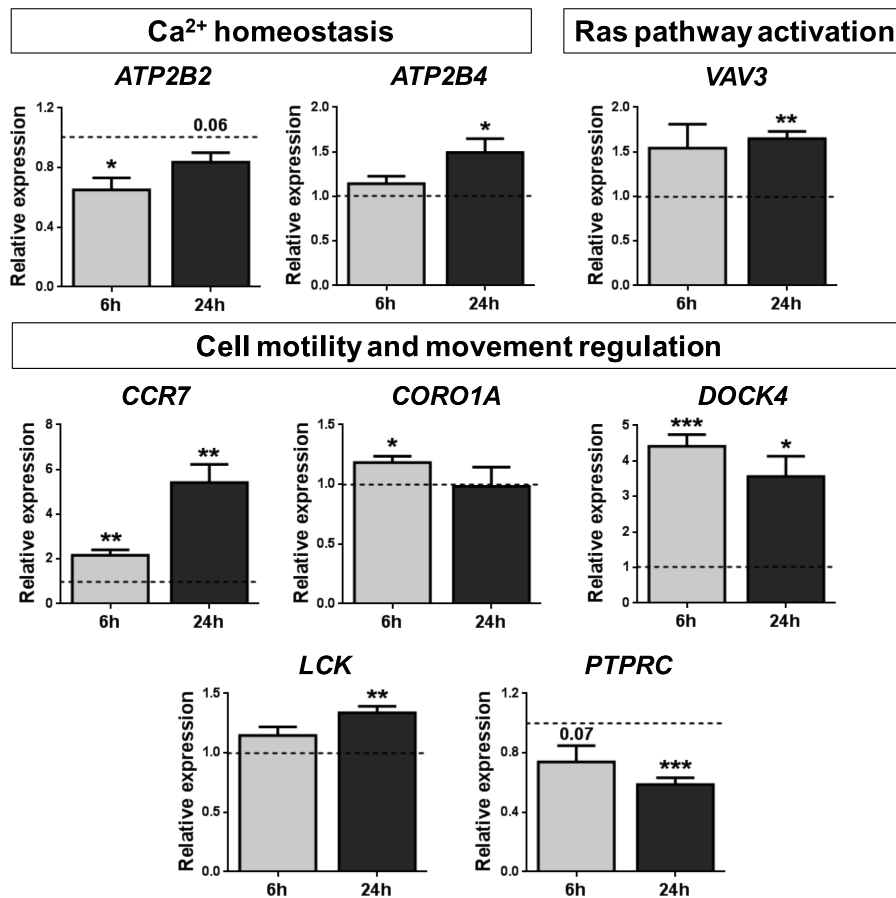


Figure 3. GEP analysis showed that Activin A positively modulates cell motility in leukemic cells

(A) Gene Ontology (GO) enrichment analysis of cell biological processes, based on differentially expressed genes in 697 cell line treated or not with Activin A (50 ng/mL) for 6h and 24h (n=4 independent experiments).

(B) Selected genes significantly modulated in Activin A-treated cells compared to the untreated control were validated by qRT-PCR. Expression levels were normalized to GAPDH mRNA levels. Lfdr (Shrinkage *t* test) for microarray data and p-value

(unpaired Student *t* test) for qRT-PCR data were respectively shown.



Supplementary Figure 2. Validation of gene expression analysis data

(A) Expression of *ATP2B2, ATP2B4, VAV3, CCR7, CORO1A, DOCK4, LCK, PTPRC* was assessed in 697 cells treated or not with Activin A (50 ng/mL). The data are presented as the fold change of mRNA levels normalized to *GAPDH* mRNA

(endogenous control). The results of three independent experiments are shown.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: unpaired Student t test

2.4.4. Activin A increased motility, chemotaxis and invasion of BCP-ALL cells

To test whether Activin A was able to modulate BCP-ALL movement, we performed time-lapse microscopy (TLM) analysis and migration assays.

TLM studies showed that Activin A was able to increase random motility of both 697 cell line ($P < 0.05$) and primary cells ($P < 0.001$) (Figure 4A, left and right panel, respectively). Indeed, Activin A addition to the chamber was able to trigger cells' motility machinery.

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 BM niche. Therefore, we tested whether Activin A was able to modulate CXCL12-induced migration of leukemic cells using a transwell-based migration assay. Firstly, end-point chemotaxis assays confirmed TLM analyses, since we found that Activin A pretreatment was able to increase the spontaneuos migration of 697 BCP-ALL cells towards empty medium compared to the untreated condition ($P < 0.001$) (Figure 4B). Moreover, Activin A-pretreated 697 cells showed a significant increase in CXCL12-driven migration ($P < 0.001$) (Figure 4B). Importantly, this data was confirmed in primary cells obtained from the BM of BCP-ALL patients at

disease diagnosis. In detail, Activin A pretreatment significantly increased the migration of primary BCP-ALL cells in response to CXCL12 in six out of eight tested patients (Figure 4C).

It has been demonstrated that Activin A expression is associated with an invasive phenotype in several type of cancers, such as ovarian cancer, esophageal adenocarcinoma, breast cancer, oral squamous cell carcinomas[9-13]. Therefore, we tested whether Activin A was able to modulate leukemic cell invasive capacity using matrigel-coated transwells. Here we found that Activin A increased the ability of 697 cells to spontaneously pass through a complex matrix ($P < 0.05$) (Figure 4D). 697 invasive ability was further enhanced in presence of the chemotactic factor CXCL12, since the migration of Activin A-pretreated cells through the matrigel matrix resulted in a 4-fold increased compared to untreated condition ($P < 0.05$) (Figure 4D).

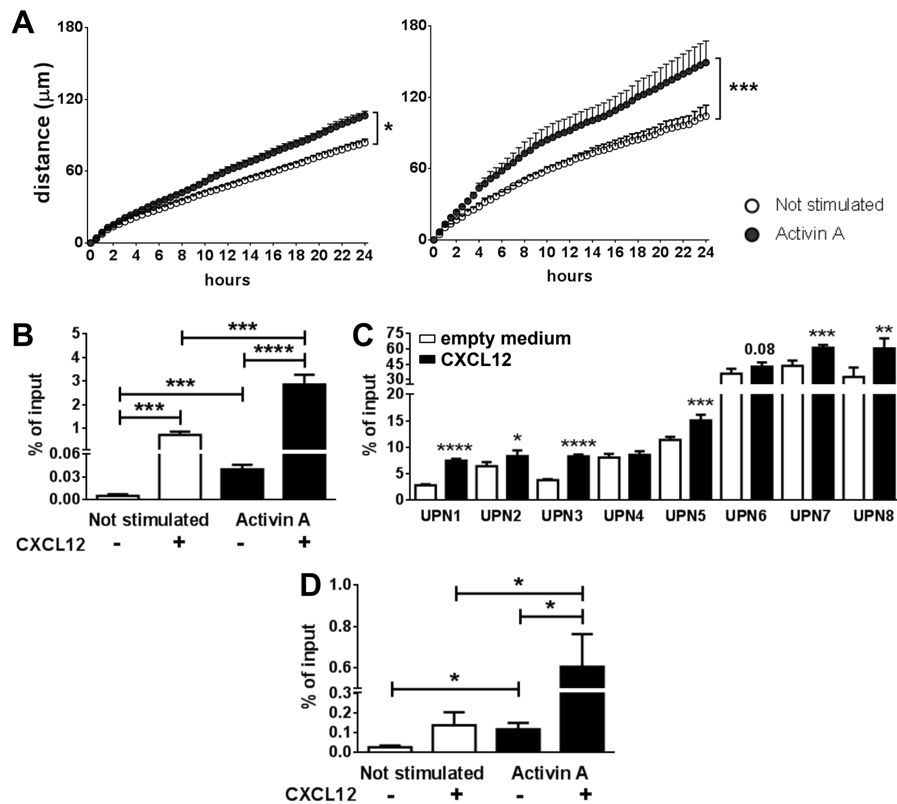


Figure 4. Activin A enhanced cell motility, migration and invasion of leukemic cells

(A) Leukemic cells were treated or not with Activin A and then tracked for 24h by timelapse microscopy. Dead cells were excluded using PI staining. Data represent the mean ± SEM of the migrated distance over time of three independent experiments. Left panel: 697 cells treated with 50 ng/mL Activin A. Right panel: BCP-ALL primary blasts treated with 100 ng/mL Activin A.

(B) Chemotaxis assay was performed using 697 cells stimulated with Activin A for 24h (50 ng/mL) and allowed to migrate towards empty medium or CXCL12-containing medium

(100 ng/mL) for 4h (5μM pores transwell). The percentage of migrated cells was determined as described in the *Experimental procedures*.

(C) Eight primary BCP-ALL cells were exposed to Activin A (100 ng/mL) for 24h and employed for chemotaxis assay towards CXCL12-containing medium (100 ng/mL). The average percentage ± SEM of cells migrated after 1h of culture is represented.

(D) 697 cells pretreated or not with Activin A for 24h (50 ng/mL) were allowed to pass through transwell inserts (8μM pores) coated with a matrigel-barrier (1 mg/mL) for 24h in presence or not of CXCL12 (100 ng/mL) in the lower chamber. The graph represents the average percentage ± SEM of five independent experiments.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: unpaired Student *t* test (Panels A and C); one-way ANOVA and Bonferroni multiple comparison test (Panels B and D).

2.4.5. Activin A enhanced leukemic cells responsiveness to low levels of CXCL12

Studies performed both in mouse models and leukemia patients highlighted that, among the microenvironmental alterations that occur in the leukemic BM, there is a reduction of the CXCL12 amount[14,15] that is associated to the impairment of normal heamatopoiesis[5]. Here we confirmed, in a larger cohort of patients, a significant 4-fold reduction of CXCL12 BM plasma levels in BCP-ALL patients (108.6 ± 15.87 pg/mL) compared to

HDs (489.6 ± 49.12 pg/mL) (*P* < 0.001) (Figure 5A). In this context, we tested the ability of Activin A to increase the responsiveness of leukemic cells to suboptimal concentrations of CXCL12. Activin A enhanced CXCL12-driven chemotaxis towards a 10-fold and 100-fold lower concentration of CXCL12 compared to those classicaly used in *in vitro* migration assays (100 ng/mL). In particular, Activin A pretreatment induced a 11-fold increase in the CXCL12-driven chemotaxis towards 10ng/mL CXCL12 (*P* = 0.06) and a 7-fold increase towards 1ng/mL CXCL12 (*P* < 0.05), compared to untreated cells characterized by a barely detectable migration (Figure 5B).

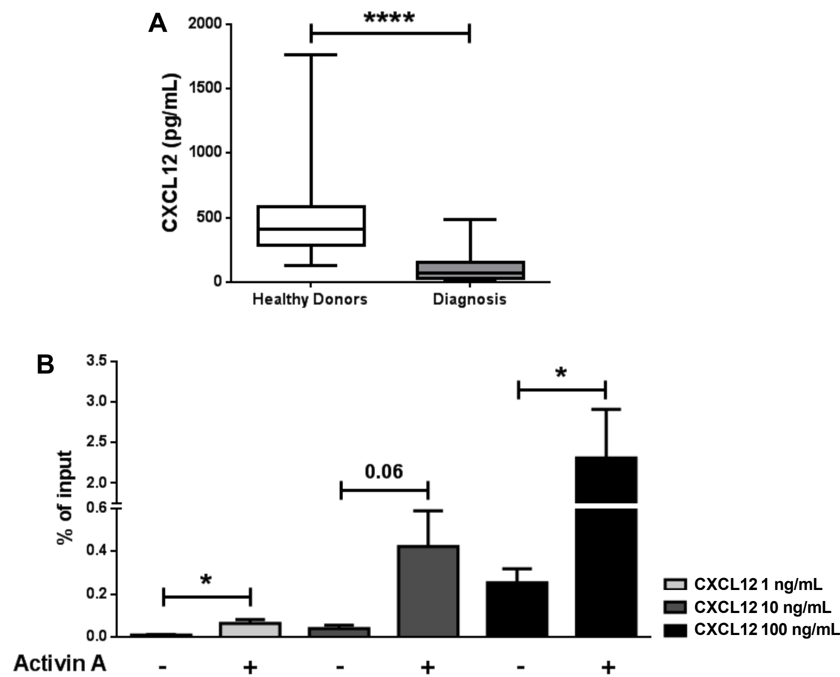


Figure 5. Activin A enhanced leukemic cell responsiveness to CXCL12

(A) CXCL12 BM plasma levels were assessed by ELISA in 44 HDs and 80 BCP-ALL patients at the onset of the disease.

(B) 697 cells were pretreated with Activin A (50 ng/mL) for 24h and then incubated for 4h in transwell chambers towards decreasing concentration of CXCL12 (100-10-1 ng/mL) (n=3). The graph represents the average percentage ± SEM.

*$P < 0.01$, ****$P < 0.0001$: unpaired Student *t* test

2.4.6. Intracellular calcium levels and actin polymerization were increased by Activin A in leukemic cells

To deeper investigate the enhaced leukemic cell responsiveness to chemotactic stimuli, we firstly evaluated whether Activin A treatment could affect chemokine receptor expression. Flow cytometic analysis of CXCL12 chemokine receptors showed that the levels of CXCR4 and CXCR7, evaluated both as extracellular receptors and intracellular pool, were not affected by Activin A (Figure 6A).

Starting from gene expression data, we evaluated the effect of Activin A on actin cytoskeleton dynamics: since the conversion of globular into filamentous actin is a prerequisite of site-directed migration, we analysed whether the increased chemotactic response upon Activin A treatment was determined by enhanced chemokine-induced actin polymerization. As shown in Figure 6B, pretreatment of leukemic cells with Activin A for 24h resulted in a more prominent conversion of globular into filamentous actin starting from 30 seconds upon addition of CXCL12 ($P < 0.01$). Notably, Activin A-pretreated cells maintained a higher amount of F-actin, compared to the untreated condition, even 180s after CXCL12 stimulation ($P < 0.05$).

Moreover, it has been demonstrated in several cell types[16-20] that Activin A is able to increase intracellular Ca^{2+} concentration, which is involved in cytoskeleton redistribution, traction force generation and relocation of focal adhesions[21].

Therefore, we performed flow cytometric analysis to determine the effect of Activin A on the intracellular calcium level of BCP-ALL cells. Our data revealed that the intracellular calcium content of 697 cells was basally increased in Activin A pretreated cells compared to untreated control, as shown in Figure 6C. Interestingly, upon the addition of CXCL12, Activin A-treated cells showed a significantly higher increase in the concentration of cytosolic free Ca^{2+} compared to the untreated control. These data strongly support our GEP results highlighting Activin A role as modulator of several genes involved in calcium ion transport and homeostasis.
Data on migration, invasion, chemokine receptors and calcium flux were confirmed in another BCP-ALL cell line, NALM-6, characterized by a high expression of Activin receptors (Supplementary Figure 3).

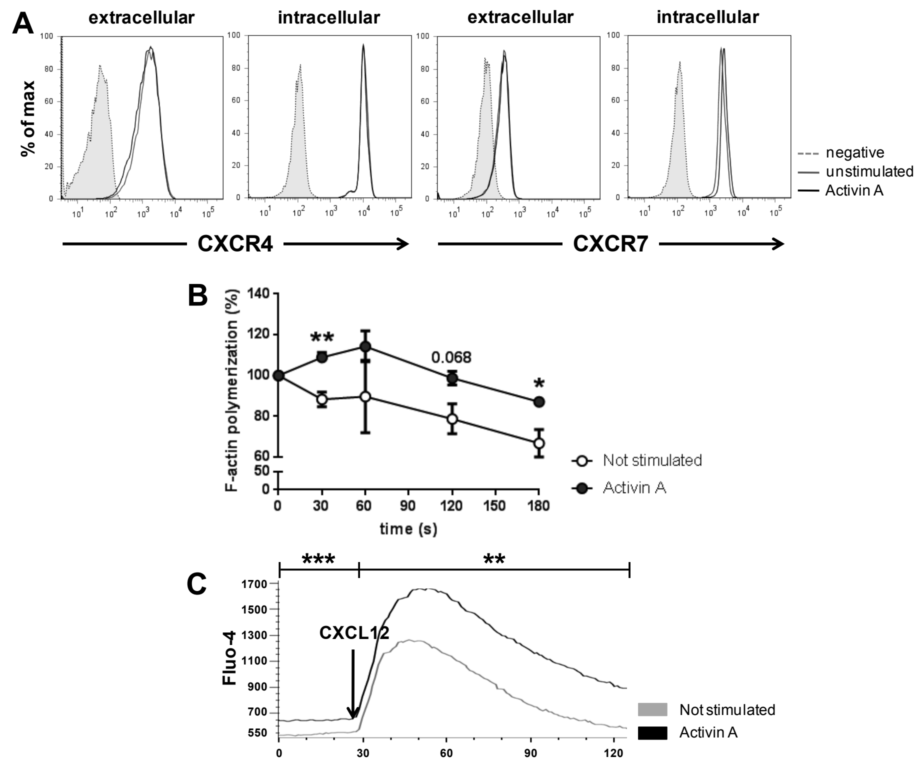


Figure 6. Activin A increased CXCL12-induced calcium mobilization and actin polymerization

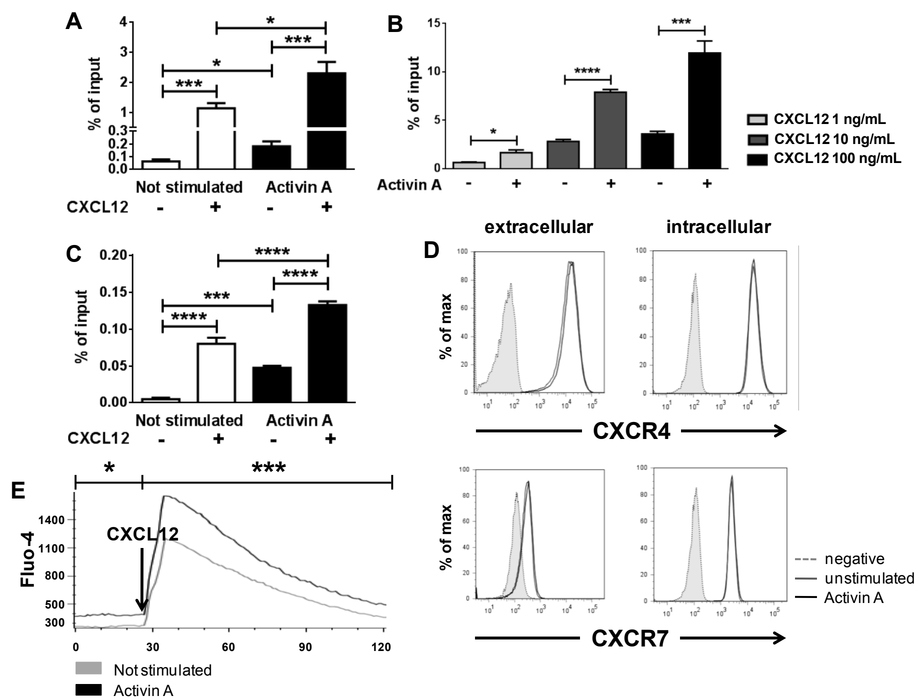
(A) The extracellular and intracellular levels of CXCR4 and CXCR7 were analysed by flow cytometry in cells treated (black line) or not (grey line) with Activin A for 24h. Representative data are shown from three independent experiments.

(B) 697 cells were starved in low serum medium for 24h and then stimulated or not with Activin A (50 ng/mL) for additional 24h. Cells were stained with AF647-phalloidin and mean fluorescence intensity (MFI) quantified by flow cytometry. Percentage of MFI change was defined as follows: (MFI after CXCL12 addition/ MFI before CXCL12 addition) x 100.

Mean values (±SEM) of three independent experiments are represented in the graph.

(C) 697 cells were cultured for 24h in presence or absence of Activin A (50 ng/mL). Cells were loaded with Fluo-4 NW and cytosolic free Ca^{2+} changes were measured by FACS. Background was recorded for 30s and signal upon CXCL12 addition was registered for additionl 90s. The black line represents the result obtained with Activin A-treated cells, while the grey line corresponds to untreated cells. The results are representative of three independent experiments.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: unpaired Student *t* test



Supplementary Figure 3. Activin A increased CXCL12-driven chemotaxis, invasion and calcium content in NALM-6 cell line

(A and B) Chemotaxis assay was performed using NALM-6 cells pretreated or not with Activin A for 24h (50 ng/mL) and allowed to migrate towards medium containing or not CXCL12 for 4h. The graphs represent the average percentage ± SEM of five independent experiments.

(C) Invasion assay was set up using NALM-6 cells stimulated with Activin A for 24h (50 ng/mL) and allowed to pass through matrigel-coated (1 mg/mL) transwell inserts for 24h in presence or not of CXCL12 (100 ng/mL). The graph represents the average percentage ± SEM of five independent experiments.

(D) The extracellular and intracellular CXCR4 and CXCR7 expression in cells treated (black line) or not (grey line) with Activin A for 24h was evaluated by flow cytometry. Representative data from one out of three independent experiments are shown.

(E) NALM-6 cells were cultured for 24h in the presence or not of Activin A (50 ng/mL). Cells were incubated with Fluo-4 NW dye and cytosolic free Ca^{2+} changes were measured by flow cytometry. The black line represents Activin A-treated cells, while the grey line corresponds to untreated cells.

Representative data from one out of three independent experiments are shown.

**P* < 0.05, ****P* < 0.001, *****P* < 0.0001: unpaired Student *t* test (Panels B and E); one-way ANOVA and Bonferroni multiple comparison test (Panels A and C)

2.4.7. Activin A impaired CXCL12-driven migration of healthy $CD34^+$ cells

To better characterize the role of Activin A in the BM niche, we evaluated its effect on the migratory properties of healthy $CD34^+$ cells. CB- and BM-derived $CD34^+$ cells expressed both type I and type II Activin A receptors, thus suggesting that they could both respond to this molecule (Figure 7A).

We next evaluated the effect of Activin A on the CXC12-driven chemotaxis of $CD34^+$ cells by using transwell-based migration assays. Surprisingly, we observed an opposite effect compared to leukemic cells: indeed, Activin A pretreatment resulted in an

average reduction of about 55% of CXCL12-driven chemotaxis, compared to untreated CB-CD34$^+$ cells (Figure 7B). 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 healthy donors, with an average reduction of about 25% in CXCL12-driven migration (Figure 7B). This effect on CD34$^+$ migration was not due to an Activin A-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 7C). On the overall, these data suggest that leukemic cells could displace healthy stem cells from their niches through an Activin A-mediated mechanism.

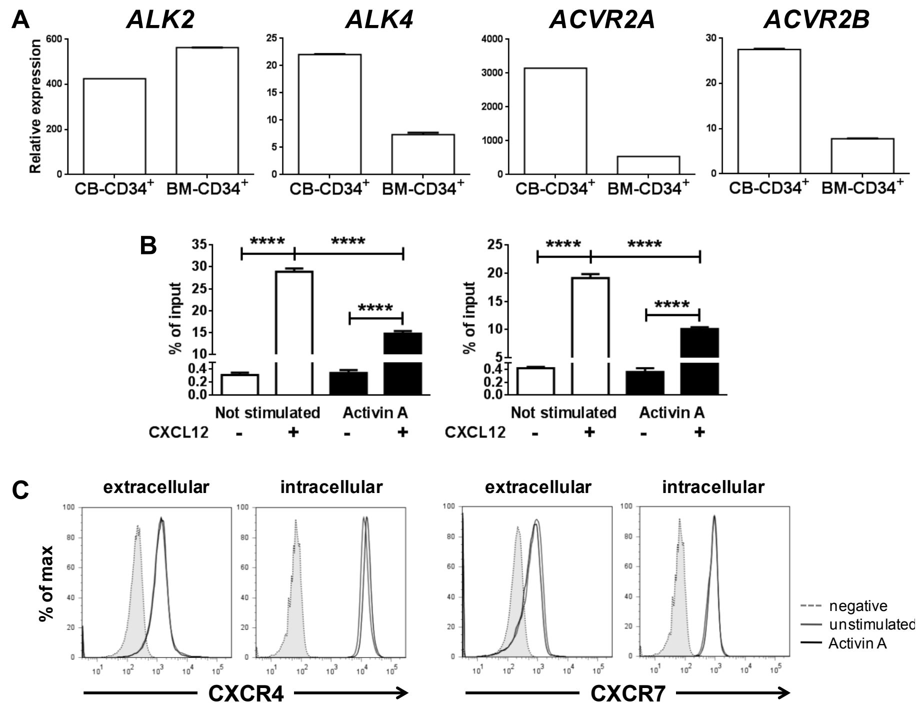


Figure 7. Activin A impaired CXCL12-driven migration of healthy CD34$^+$ cells

(A) Expression of Activin A receptors *ALK2, ALK4, ACVR2A* and *ACVR2B* was quantified in both CB- and BM-CD34$^+$ cells by qRT-PCR. Data are presented as Activin A mRNA fold change normalized to *GAPDH* mRNA (endogenous control), using DAUDI cell line as calibrator.

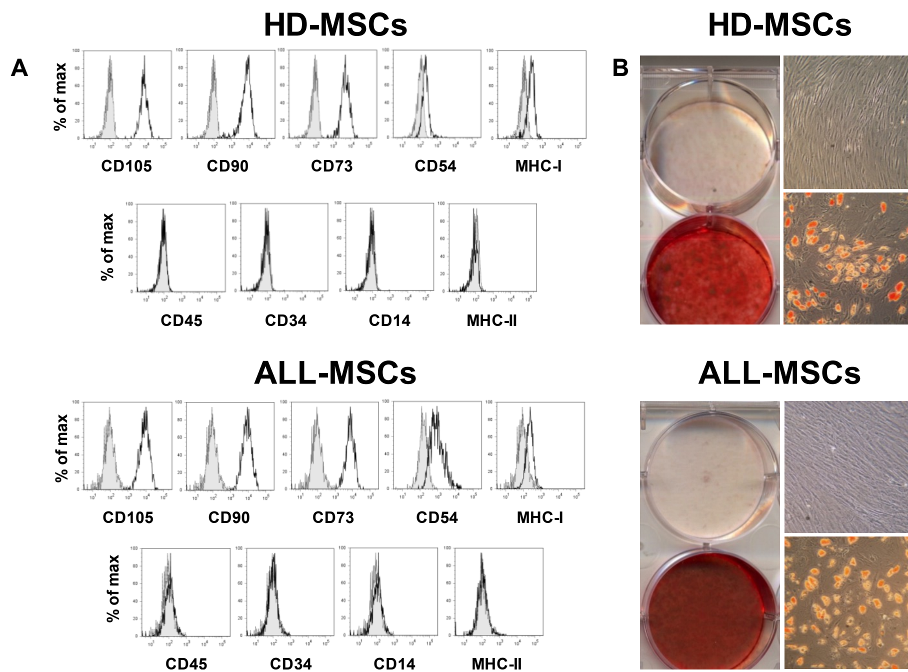
(B) CB-CD34$^+$ cells (left) and BM-CD34$^+$ (right) were pretreated with Activin A for 24h and allowed to migrate through 5μM pores in transwell chambers towards CXCL12 (100 ng/mL) for 1h. The graphs represent the average percentage ± SEM of one representative experiment (CB-CD34$^+$ n=5, BM-CD34$^+$

n=3). ****$P < 0.0001$: one-way ANOVA and Bonferroni multiple comparison test

(C) The extracellular and intracellular levels of CXCR4 and CXCR7 were analysed in cells treated (black line) or not (grey line) with Activin A for 24h by flow cytometry. Data from one representative experiment out of three are shown.

2.4.8. ALL-MSCs showed an intrinsic ability to secrete high amounts of Activin A possibly due to the synergistic effect of leukemic cells and inflammation

Finally, we focused our attention on the capacity of BCP-ALL BM microenvironment to influence MSC-derived Activin A. 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 to HD-MSCs (Supplementary Figure 5).

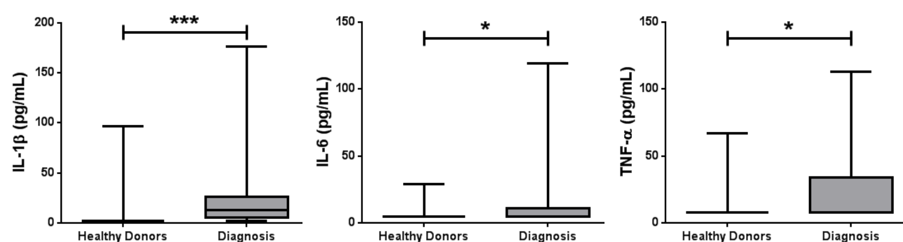


Supplementary Figure 5. ALL-MSCs were similar in terms of phenotype and differentiation ability to HD-MSCs.

(A) Immunophenotype of both HD-MSCs and ALL-MSCs was analyzed by flow cytometry. Mesenchymal stromal cells were positive for typical mesenchymal markers including CD105, CD90, CD73, CD54 and MHC-I, while lacked the expression of the hematopoietic markers CD14, CD34, CD45 and MHC-II.

(B) HD-MSCs and ALL-MSCs were induced to differentiate toward osteogenic (left panel) and adipogenic lineage (right panel), as showed by Alizarin Red staining of calcium deposits and Oil Red O liphophilic dye, respectively.

After 24h of culture we highlighted, by ELISA assay, a significantly higher production of Activin A ($P < 0.05$) by ALL-MSCs (983.6 ± 362.9 pg/mL) compared to their normal counterpart (218.5 ± 31.99 pg/mL), as shown in Figure 8A. Therefore, we hypothesized that BM-MSCs, primed by the leukemic microenvironment, could be responsible for the high amount of Activin A in the BM of BCP-ALL patients. The role of inflammation in the editing of the microenvironment has been defined in several type of cancer, including haematological malignancies. Recent evidence highlighted that the BM of ALL patients is a highly pro-inflammatory environment[22]. These data were confirmed in our cohort of patients: indeed, higher levels of pro-inflammatory cytokines IL-1β ($P < 0.001$), IL-6 ($P < 0.05$) and TNF-α ($P < 0.05$) were detected in the BM plasma of BCP-ALL patients compared to HDs (Supplementary Figure 4).



Supplementary Figure 4. Pro-inflammatory cytokines were highly represented in leukemic BM microenvironment

IL-1β, IL-6 and TNF-α BM plasma levels were assessed by ELISA in HDs and BCP-ALL patients at the onset of the disease.

*$P < 0.05$, ***$P < 0.001$: unpaired Student *t* test

Since it has been demonstrated that Activin A expression was increased in several inflammatory diseases such as septicemia, inflammatory bowel disease and rheumatoid arthritis[23], we investigated whether the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α could regulate Activin A levels in the BM of BCP-ALL patients. To explore this hypothesis, we stimulated both HD-MSCs and ALL-MSCs with a cocktail of the above-mentioned pro-inflammatory cytokines for 24h. ELISA assay revealed a significant induction of Activin A release in BM-MSCs compared to their respective basal condition: indeed, upon stimulation Activin A production by HD-MSCs reached a 28-fold increase compared to the basal condition (6030 ± 990 pg/mL). Interestingly, the molecule was released to a higher extent by ALL-MSCs in pro-inflammatory condition compared to their normal counterpart (10613 ± 1254 pg/mL) ($P < 0.01$), as shown in Figure 8A.

Notably, if we mimicked an inflamed BM niche by simultaneously stimulating HD-MSCs with leukemic blasts and pro-inflammatory cytokines, we evidenced a strong increase in the secretion of Activin A both in the direct (33822 ± 4670 pg/mL) and the indirect co-culture condition (28744 ± 3334 pg/mL). Of note, the combination of both leukemic blasts and pro-inflammatory cytokines produced a synergic induction of Activin A, since the extent of the release was higher compared to the levels obtained with singly used stimuli ($P < 0.001$) (Figure 8B).

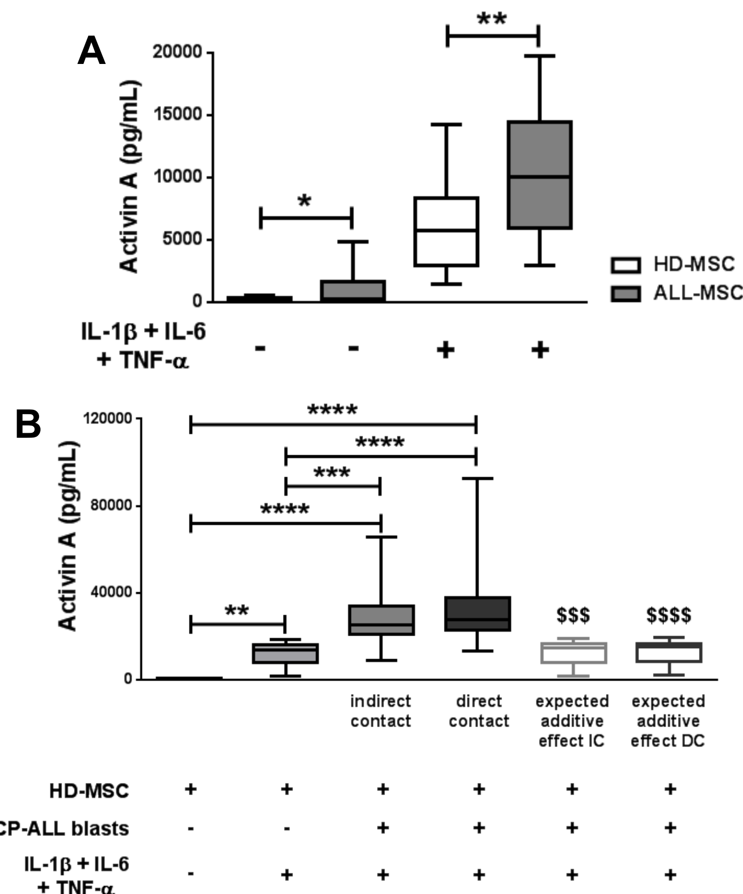


Figure 8. Inflammation synergized with leukemic cells to induce Activin A production by BM-MSCs

(A) Activin A secretion by BM-MSCs from HDs (HD-MSCs; n=15) and BCP-ALL patients (ALL-MSCs; n=15) was assessed by ELISA after 24h of culture ± IL-1β (50 ng/mL), IL-6 (40 ng/mL) and TNF-α (100 ng/mL).

(B) Primary BCP-ALL cells were co-cultured with HD-MSCs directly (DC) or separated by a transwell insert (IC) in presence of IL-1β, IL-6 and TNF-α for 72h. Activin A expression was assessed by ELISA on culture supernatants (n=17 independent

cocultures). The expected additive effect was calculated as the sum of the single effects produced by the two stimulating factors, inflammation and leukemic cells. Synergism is defined as a "greater-than-the-expected-additive effect".

$^{*}P < 0.05$, $^{**}P < 0.01$: unpaired Student *t* test (Panel A)

$^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: one-way ANOVA and Bonferroni multiple comparison test ($^{\$\$\$}P < 0.001$, $^{\$\$\$\$}P < 0.0001$: comparison between the expected additive effect and the measured effect, indirect contact and direct contact, respectively) (Panel B)

2.4.9. Activin A enhanced the homing ability of BCP-ALL cells in a mouse model of human leukemia

To validate our *in vitro* findings in an *in vivo* setting, we set up a xenograft murine model of human BCP-ALL. Indeed, 697 cells pretreated or not with Activin A (50 ng/mL) for 24h were intravenously injected into NSG mice. Animals were not subjected to any preconditioning to avoid any alteration or damage to the recipient niche, such as CXCL12 increase[24, 25].

As shown in Figure 9A, mice were weighed 4 and 7 days after transplantation. 7 days post-injection, animals were sacrificed and cells from BM, spleen, meninges, liver and PB were harvested to evaluate the percentage of engraftment of leukemic cells ($hCD45^{+}$) by flow cytometric analysis. We firstly evidenced a significant difference in the percentage of weight change between the two experimental groups both 4 days ($P < 0.05$) and 7 days ($P < 0.05$) after transplantation (Figure 9B).

The analysis of $hCD45^+$ cells revealed that the liver was the first engrafted organ, already four days after injection (data not shown). Of note, at day +7 Activin A pretreatment was able to increase of about 2-fold the liver engraftment compared to control group, evaluated both as percentage of $hCD45^+$ cells (Figure 9C, left panel) (Not stimulated: 18.65% ± 4.96, Activin A: 33.20% ± 6.38) and total number of $hCD45^+$ cells (Not stimulated: 29.76×10^6 ± 8.52, Activin A: 62.95×10^6 ± 13.66) (Figure 9C, right panel) ($P < 0.05$). Figure 9C shows one representative experiment out of three. The higher leukemic infiltrate may account for liver enlargement.

The leukemic infiltrate in the other organs harvested was lower than 0.01% in both groups (data not shown).

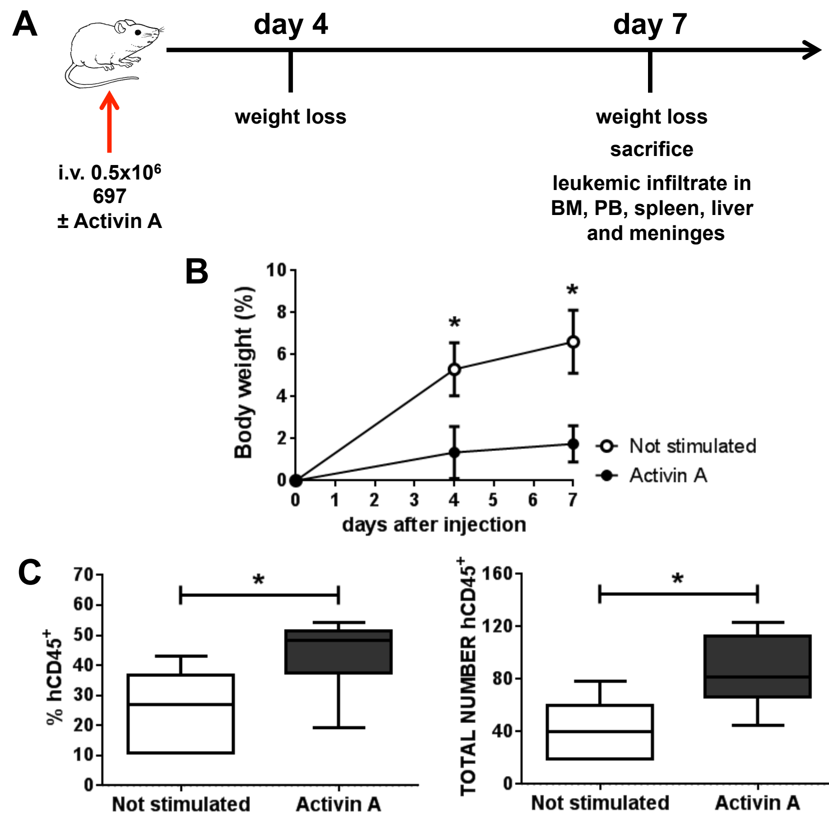


Figure 9. Activin A enhanced the homing ability of leukemic cells into the liver

(A) Experimental scheme of the xenograft murine model of human BCP-ALL**.**

(B) The percentage of body weight change was evaluated in each group 4 days (left panel) and 7 days (right panel) after transplanation (n=3 independent experiments).

(C) The engraftement of leukemic cells 7 days after transplantation was defined both as percentage of $hCD45^+$ cells (left) and as total number of $hCD45^+$ cells (right) in the liver.

*$P < 0.05$: unpaired Student *t* test

2.5. Discussion

There is ample evidence linking aberrant TGF-β family growth factor activity to carcinogenesis. Despite its prominent role in solid cancer progression[7], the involvement of Activin A, a member of the TGF-β family, in the pathogenesis of hematological malignancies has never been explored. We showed, for the first time, that Activin A is highly espressed in the BM of BCP-ALL patients at the diagnosis of the disease, compared to healthy donors. Interestingly, we demonstrated that BM-MSCs are an important source of Activin A, whose production is strongly upregulated following direct contact with leukemic cells. This data 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 disease survival and progression[1, 26, 27]. Of note, we observed that MSCs isolated from the BM of BCP-ALL patients were able to produce higher levels of Activin A, compared to HD-MSCs, even after several *in vitro* passages, meaning that they keep memory of the profound alterations occurred within the leukemic BM niche.

The concept that inflammation could play a pivotal role in the transformation, survival and proliferation of leukemias has been nowdays accepted. In particular, several works highlight that bone marrow cells in acute lymphoblastic leukemia are able to create a pro-inflammatory microenvironment, that impairs frequency and function of HSCs within the BM[14, 22].

In line with this evidence, we demonstrated that the BM of BCP-ALL patients presents increased levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α which can synergise with BCP-ALL cells in stimulating Activin A production and release by BM-MSCs. The aboundance of Activin A 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 work which describes Activin A-induced increase in the migratory and invasive properties of several solid tumours[9, 11, 28-36], our GEP analysis of Activin A-treated leukemic cells showed a crucial effect on different biological pathways linked to cell motility. In detail, we demonstrated, by using different *in vitro* assays (timelapse microscopy and transwell-based migration assays), that this molecule was able to increase the spontaneous cell motility of both immortalized and primary BCP-ALL cells.
We demonstrated that this Activin A-induced effect could be achieved through an increase of both cytosolic calcium and F-actin polymerization. It has been demonstrated that Ca^{2+} is a crucial regulator of multiple actin regulators, thus controlling actin cytoskeleton dynamics. Indeed, it activates actin-interacting molecules such as protein kinase C[37] and calmodulin-dependent kinases[38]. Moreover, Ca^{2+} signaling regulates the Rho GTPases, which are necessary for the formation of the major components of cell migration machinery, such as actin bundles for lamellipodia, focal

adhesion complexes and filopodia[39]. Data obtained with time lapse microscopy suggested that leukemic cells can respond to Activin A within few minutes by increasing cell motility. Moreover, besides this early response, Activin A could produce a long-lasting effect by regulating expression of molecules involved both in calcium homeostasis and cell motility, such as plasma membrane Ca^{2+} ATPase (ATP2B2 and ATP2B4[40]), regulators of Ras pathway (VAV3[41], ARHGAP25[42], RND3[43], RasGRP2[44] and RasGRP3[45]), regulators of cell motility and movement (LCK[46], DOCK4[47], DGKA[48], INPP4B[49], CORO1A[50]).

Along with the upregulation of random motility, we also observed a significant increase of CXCL12-mediated chemotaxis of leukemic cells upon exposure to Activin A. Importantly, this effect could be clearly observed even at suboptimal chemokine doses. Notably, Activin A was able to upregulate the expression of its type I receptors in leukemic cells, thus further enhancing Activin A signaling.

On the other side, it is worth to underline the differential effect exerted on healthy hematopoietic stem cells since Activin A is able to negatively modulate CXCL12-driven migration.

Our data and previously published data[15] highlighted that BCP-ALL BM is characterized by decreased levels of CXCL12, which not only drives migration, but is also responsible for the delivery of survival signals, thus providing an advantage for leukemic cells.

Overall, our findings support a novel pro-tumoral role of Activin A in the leukemic BM niche, where it contributes to the establishment of a positive feedback loop between BCP-ALL cells and MSCs that could result in the displacement of healthy hematopoiesis in favour of leukemic cells.

Besides its key role in the regulation of homing process in the BM niche, CXCL12 could be involved in the widespread extramedullary organ infiltration often seen in childhood ALL, because of its constitutively expression in extramedullary tissues such as liver, spleen, thymus, lung, kidney and brain[51]. Therefore, the Activin A-dependent increase of the leukemic cell ability to pass through a complex matrix in response to CXCL12, observed *in vitro*, could be considered a mechanism for organ invasion and metastatization, resulting in leukemia progression. In accordance with this hypothesis, data obtained in a xenograft mouse model of human leukemia suggested that leukemic cells previously exposed to Activin A could engraft at a higher rate into extramedullary sites.

In detail, the liver, which has been previously associated with the persistence of chemoresistant cells in BCP-ALL[52], resulted the most infiltrated organ at early timepoints, in line with the organ tropism of 697 cells. Of note, the upregulation of VAV3, observed after Activin A treatment of leukemic cells, could suggest a possible mechanism that may account for increased cell invasion. Indeed, it has been demonstrated that this molecules is associated to the enhancement of cell invasion in several type of solid cancers (colorectal cancer,

prostate cancer, breast cancer, glioblastoma, non small cell lung cancer)[53-57].

On the overall, our data suggest that at the onset of the disease leukemic cells could reprogram the BM stroma creating an altered malignant environment. Within the complex network of mediators regulating the leukemic niche, Activin A could play a crucial role by selectively enhancing the migratory and invasive properties of BCP-ALL cells. On the contrary, Activin A resulted able to modulate healthy HSCs CXCL12-driven migration in an opposite way, thus favouring leukemic cells in the competition for the niche. Therefore, Activin A could represent a key molecule in the creation of a self-reinforcing niche where BCP-ALL cells can achieve signals for survival and chemoresistence. This work opens new perspectives for the design of therapeutical approaches for BCP-ALL treatment, targeting the interplay between leukemic cells and the BM stroma.

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3. Summary, conclusions and future perspectives

BCP-ALL is the most common childhood malignancy. The persistance of minimal residual disease that survives chemotherapy can cause relapse with poor outcome[1]. To improve the prognosis of BCP-ALL patients, therapeutic strategies that aim at targeting BM niche-derived supporting signals are required to eliminate tumor cells.

In this work we focused our attention on the identification and characterization of a new regulatory molecule of the leukemic BM niche, Activin A. The present study describes for the first time the leukemia-supporting role of stroma-derived Activin A in the context of BCP-ALL.

In homeostatic condition, the main source of Activin A is represented by bone marrow neutrophil precursors, even though it is also expressed by megakaryocytes, osteoclasts, chondrocytes, endothelial cells, stromal barrier cells and bone-lining cells. On the contrary, the majority of mononuclear cells are negative for Activin A expression[2]. Here we found that in the leukemic BM niche this molecule is focally expressed within the areas of major leukemic infiltrate. Our i*n vitro* studies highlighted that BM-MSCs, key components of the BM stroma, are specifically induced to increase its production by leukemic blasts. Interestingly, this effect was mainly achieved through a direct contact-mediated mechanism.

Previous studies have described this mechanism defining the role of both JNK pathway and VLA4/VCAM-1 axis in the positive regulation of Activin A production by BM-MSCs, through NF-κB signaling activation[3,4]. Moreover, we also demonstrated that ALL-MSCs are endowed with a higher ability to secrete Activin A. This could be partially explained by the demonstration that ALL-MSCs are characterized by a higher nuclear translocation of NF-κB, compared to their normal counterpart[5].

Interestingly, in line with recent literature, we found that the leukemic BM is characterized by a high pro-inflammatory milieu: indeed, compared to healthy BM, we found high levels of IL-1β, IL-6 and TNF-α, suggesting that also in BCP-ALL an inflammatory microenvironment could promote tumor development, similarly to what happens in solid malignancies[6]. In particular, it is interesting to notice that leukemic blasts and the pro-inflammatory molecules cooperated to synergistically induce *in vitro* Activin A production by BM-MSCs. The main hypothesis is that stimulation of BM-MSCs with pro-inflammatory cytokines along with leukemic blasts could activate JNK, p38 and NF-κB pathways, that may converge on a common trasciptional target, Activin A[7-9].

To verify our hypothesis, we will inhibit the VLA4/VCAM-1 axis in BM-MSCs co-cultures with primary BCP-ALL cells by using a commercially available anti-VLA4b blocking antibody, able to prevent the interaction between VLA-4, expressed by leukemic cells, and VCAM-1, present on MSC membrane.

Activin A will be then quantified in culture supernatants by ELISA. In addition, we will evaluate the downstream involvement of the NF-kB pathway by Western Blot (WB) analysis of NF-κB p65 phosphorylation in BM-MSCs co-cultured or not with leukemic cells.

As revealed by gene expression analysis, Activin A mainly induces an overall positive regulation of pathways associated with cell motility, such as RAS and PI3K/AKT, and calcium homeostasis.

The Ras proteins are GTPases involved in the regulation of cell proliferation, survival, migration and cytoskeletal dynamism, through the effectors RAF, PI3K, RALGDS and PLCε, which in turn activate several downstream pathways, such as MAPK/ERK, AKT/PKB and PKC. Moreover, Ras pathway could determine increased diacylglycerol (DAG) and inositol trisphosphate ($InsP_3$) production, which is associated with calcium mobilization from intracellular stores. RAS signalling pathway has been well characterized for its involvement in actin cytoskeleton reorganization, which promotes cell motility and contributes to tumor cell invasion and metastasis[10, 11].

We pointed out that Activin A was able to positively regulate the expression of *RASGRP3* and *RASGRP2* (activators of Ras isoforms), *VAV3* and *DOCK4* (guanine nucleotide exchange factors for Rho family GTPases), *DGKA* (modulator of DAG intracellular signaling pathway). On the other hand, Activin A negatively regulates *RND3* (inhibitory molecule for Rho

GTPase), *INPP4B* (inhibitory molecule of PI3K pathway) and *ARHGAP25* (negative regulator of Rho GTPases). Notably, ARHGAP25 has been described for its role in the regulation of leukocyte recruitment[12] and stem cell progenitors mobilization[13]. In particular, *Arhgap25*-deficient mice showed enhanced neutrophils transendothelial migration towards inflammatory sites[12]. Furthermore, *Arhgap25*$^{-/-}$ mice showed higher frequency of HSPCs in the BM and lower frequency of peripheral blood HSPCs compared to controls[13].

Moreover, Activin A was able to contribute to calcium homeostasis through the regulation of *CORO1A*, *ATP2B2*, *ATP2B4*. In particular, CORO1A promotes the generation of the second messenger $InsP_3$, responsible for the release of Ca^{2+} from intracellular stores. ATP2B2 and ATP2B4 are plasma membrane Ca^{2+} ATPases (PMCA) involved in the extrusion of Ca^{2+} from the cytosol into the extracellular space. Since they resulted downregulated and upregulated, respectively, by Activin A, we could hypothesize that, according to their specific localization in the plasma membrane, they could contribute to the definition of the well-recognized front-low, back-high Ca^{2+} gradient during cell migration. The known association between increasing Ca^{2+} levels and induction of F-actin polymerization allow us to hypothesize the existence of a calcium-mediated mechanism involved in the Activin A-induced pro-migratory phenotype, that was further functionally confirmed by increased intracellular Ca^{2+} levels and F-actin polymerization in Activin A treated cells compared to control.

Therefore, as time lapse microscopy analysis and chemotactic assays revealed, Activin A produced an enhancement of random cell motility and shaped the migration machinery of leukemic cells, making them more responsive to chemotactic stimuli, such as CXCL12, known for its key role in the organization of both healthy and leukemic BM niche.

Notably, the above-mentioned effects were specifically observed only on leukemic cells, since Activin A exerted an opposite effect on the migration of healthy hematopoietic cells, which resulted impaired. We thus hypothesized that the healthy hematopoiesis could be displaced by its niche through two coordinated mechanisms in the face of a reduction of CXCL12 in the leukemic BM. Indeed, leukemic cells could increase Activin A that, on one side, highly increased their responsiveness even to very low levels of CXCL12 and, on the other side, strongly impaired the migration ability of $CD34^+$ cells towards CXCL12. Future studies will aim at deeper characterizing the role of Activin A, with a particular attention to the molecular pathways that could contribute to leukemic cell advantage over healthy hematopoiesis, for example by elucidating the molecules involved in the differential effect exerted on healthy $CD34^+$.

It is worth mentioning that Activin A was able to enhance the expression of LCK, a member of Src family of protein tyrosine kinases, involved in the chemotactic response of both T cells and NK cells to CXCL12. In particular, it is known that dexamethasone, which activates LCK and several downstream

kinases, could significantly enhance CXCR4-mediated signaling and function, thus suggesting its possible involvement in the increased Activin A-mediated CXCL12-driven migration. Therefore, to test this hypothesis, we will evaluate LCK activation by WB analysis on Activin A-treated leukemic cells.

In view of the peculiar pattern of genes modulated by Activin A on BCP-ALL treated cells, we would like to investigate the effect of this molecule on cell vesiculation. Extracellular vesicles (EVs) (including exosomes and microvescicles) represent an heterogeneous category of membrane vesicles of different sizes with a crucial role in intercellular comunication. There is increasing evidence for the role of EVs in the crosstalk between leukemic cells and stromal components. In the context of CLL, it has been shown that leukemia-derived exosomes can directly alter the BM niche by inducing an inflammatory cancer-promoting phenotype in stromal cells, by transferring proteins and microRNAs[14]. Interestingly, it has been demonstrated that microvesicles are formed by outward budding and fission of the plasma membrane in a process dependent on calcium and cytoskeleton[15, 16]. In view of the ability of Activin A to upregulate intracellular calcium content and actin polymerization, we are planning to investigate the effect of this molecule on the vesiculation of both BCP-ALL cells and BM-MSCs.
For this purpose, EVs will be isolated by an already set up method based on ultracentrifugation. EV size and count will be performed by Nanoparticle Tracking Analysis and EV identity

will be evaluated by immunoblotting using anti-human CD63, CD81 and HSP70 antibodies.

Furthermore, in view of our finding that both BCP-ALL co-culture and stimulation with inflammatory cytokines strongly increase Activin A secretion by BM-MSCs as soluble molecule, we would like to understand if this molecule could be further delivered by microvescicles for endocrine comunication. Therefore, we will evaluate Activin A content (WB and ELISA assays) in microvescicles obtained from ALL-MSCs, which, in our previous experiments, resulted intrinsically able to release a higher amount of the molecule, compared to their healthy counterpart.

The ability of Activin A to increase leukemic cell migratory properties was confirmed in a xenograft mouse model of BCP-ALL. Interestingly, our data showed that Activin A was able to enhance the aggressiveness of leukemic cells: indeed, 697 cells pretreated with Activin A resulted, upon i.v. infusion into NSG mice, able to invade at a higher extent extramedullary organs, such as the liver, which has been already described as an organ preferentially targeted by this BCP-ALL cell line[17, 18]. Previous work has established the role of CXCL12/CXCR4 axis in the re-expansion of BCP-ALL cells in the hepatic niche after chemotherapy: indeed, CXCR4 positive leukemic cells are able to infiltrate the portal area, surrounding CXCL12 positive bile duct epithelial cells.

Here we found that Activin A-treated leukemic cells invade the liver with a 2-fold increase compared to the control.

Besides CXCR4/CXCL12, it is necessary to consider that CCR7, one of the most significantly upregulated gene by Activin A, could be partially responsible for this *in vivo* effect. Indeed, this chemokine receptor is able to bind CCL19 and CCL21, which are expressed by lymph nodes in homeostatic conditions[19] and that can be quickly induced during inflammation, such as in chronic inflammatory liver disease[20, 21]. It will be interesting to evaluate whether CCL19 and CCL12 could be specifically enhanced by leukemic infilitrate in the liver. Moreover, it will be worth investigating other molecular pathways that emerged as modulated by Activin A, such as "vasculature development" and "response to oxygen levels" that may further contribute to Activin A leukemia-supporting role both *in vitro* and *in vivo.*

As regard *in vivo* studies, to possibly overcome the limited BM thropism of 697 cell line and the possible concerns raised by the fact that BCP-ALL lines are often less sensitive to extrinsic factor than primary cells, we will also set up a xenograft leukemia mouse model by using primary leukemic cells. For this purpose, primary BM BCP-ALL cells, pretreated or not with Activin A, will be i.v. injected into NSG mice. Mice will be daily monitored in terms of weight loss and weekly in terms of leukemic engraftment by FACS analysis of human $CD45^+$ cells on BM aspirates. When mice will present >80% of leukemic blasts infiltrating the BM (at least one out of the two groups) all

the animals will be sacrificed and human $CD45^+$ cells will be quantified by FACS in BM, peripheral blood, liver, spleen and meninges.

This mouse model will also represent an ideal platform to test new therapeutic drugs that specifically target Activin A. In this regard, data obtained with the specific inhibitor of TGF-β receptor kinase, SB-431542, in an AML mouse model indicate that the abrogation of this signaling axis may be, at least, partially responsible for the impaired engraftment of AML cells to their niche[22]. To test whether Activin A blocking could be an effective strategy for improving BCP-ALL management, we will use in our *in vivo* model RAP-011, a murine ortholog of Sotatercept (ACE-011: Phase II drug for several hematological diseases[23]). RAP-011 is an Activin receptor II ligand trap constructed to reduce the potential immunogenicity of Sotatercept in animal models undergoing chronic exposure to the drug. To pursue our aim, RAP-011 (30 mg/kg) will be repetitively injected (i.p.) to leukemia-bearing mice. Drug-treated mice and control mice (vehicle-treated) will be compared in terms of survival, weight loss and leukemia signs. The percentage of leukemic infiltrate (human $CD45^+$) will be weekly evaluated by FACS analysis of PB and BM aspirates. If the drug treatment will result effective, we will also plan additional experiments aimed at testing different drug doses alone or in combination with standard chemoterapic drugs used for B-ALL treatment.

Overall, our data support a novel pro-tumoral role of Activin A in the leukemic BM niche, where a positive feedback loop between MSCs and BCP-ALL cells able to displace healthy hematopoiesis is established. This work will potentially pave the way for the identification of new therapeutical molecules for BCP-ALL treatment aimed to target the interplay between leukemic cells and BM niche.

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4. Publications

Mikulak J, Oriolo F, **Portale F**, Tentorio P, Lan X, Saleem MA, Skorecki K, Singhal PC, Mavilio D
"Impact of APOL1 polymorphism and IL-1β priming in the entry and persistence of HIV-1 in human podocytes"
Retrovirology. 2016 Sep 6;13(1):63 doi: 10.1186/s12977-016-0296-3

Dander E, Palmi C, **Portale F**, Beneforti L, Biondi A, Cazzaniga G and D'Amico G
"Isolation and Characterization of Mesenchymal Stromal Cells Derived from Paediatric Patients with B Acute Lymphoblastic Leukemia" Blood, 126(23), 4771. Published online December 3, 2015 (http://www.bloodjournal.org/content/126/23/4771)

Alfano M, Cinque P, Giusti G, Proietti S, Nebuloni M, Danese S, D'Alessio S, Genua M, **Portale F**, Lo Porto M, Singhal PC, Rastaldi MP, Saleem MA, Mavilio D, Mikulak J
"Full-length soluble urokinase plasminogen activator receptor down-modulates nephrin expression in podocytes"
Scientific Report. 2015 Sep 18;5:13647 doi: 10.1038/srep13647
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