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"FUNCIONES DEL NICHO ESTROMAL MESENQUIMAL DE LA MEDULA ÓSEA EN LA BIOLOGÍA DE LAS CÉLULAS INICIADORAS DE LA LEUCEMIA LINFOBLÁSTICA AGUDA INFANTIL"

TESIS

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"BONE MARROW MESENCHYMAL STROMAL NICHE FUNCTIONS IN THE BIOLOGY OF LEUKEMIA INITIATING CELLS OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA"

By

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RESUMEN

Actualmente, la leucemia linfoblástica aguda (LLA) infantil es una de las principales preocupaciones de la salud en todo el mundo y una prioridad biomédica. La disminución de la mortalidad por leucemia en niños requiere una comprensión exhaustiva de su patobiología. Se ha demostrado que la intercomunicación maligna de célula y su nicho, y las señales microambientales que controlan las decisiones iniciales del destino celular son críticas para la progresión tumoral.

En este trabajo demostramos que las células estromales mesenquimales (CEM) derivadas de médula ósea (MO) de pacientes con LLA difieren de su contraparte normal en una serie de propiedades funcionales y tiene un papel clave durante el desarrollo leucémico. Su bajo potencial de proliferación, concomitante a la fuerte capacidad de producir citocinas pro-inflamatorias y una pérdida aberrante de CXCL12 y SCF, sugiere que los nichos linfoides leucémicos en la MO durante la LLA son únicos y excluyentes de la hematopoyesis normal.

Las estructuras leucémicas de tipo organoide $Nestin^{+}$ $LepR^{+}$ proporcionan santuarios para la población de células iniciadoras de leucemia (CIL) capaces de escapar de la muerte inducida por la quimioterapia con características biológicas similares a las células de la Enfermedad Mínima/Medible Residual (EMR) responsables de las recaídas. Nuestro modelo imita mejor el microambiente de BM durante la leucemia, produciendo señales microambientales mínimas pero necesarias para que las CIL crezcan *ex vivo*.

Confirmamos la existencia de un microambiente tumoral inducida por células leucémicas con propiedades de troncalidad e inmunofenotipo irrelevante, pero con capacidad de iniciar la leucemia en ratones inmunodeficientes y probablemente de ser la responsable de la EMR antes de la recaída. Las CIL secuestran los nichos de las células troncales hematopoyéticas (CTH) para establecerse, mientras que los factores pro-inflamatorios remodelan otros nichos de CEM a través de la disminución de CXCL12 y SCF concomitante con un agotamiento de las CTH a través del desplazamiento celular y su proliferación.

En conjunto, nuestros datos sugieren la existencia de dos nichos en LLA.

ABSTRACT

Pediatric oncology, notably childhood acute lymphoblastic leukemia (ALL), is currently one of the Health leading concerns worldwide and a biomedical priority. Decreasing overall leukemia mortality in children requires a comprehensive understanding of its pathobiology. It is becoming clear that malignant cell-to-niche intercommunication and microenvironmental signals that control early cell fate decisions are critical for tumor progression.

We show here that the mesenchymal stromal cell component of ALL bone marrow (BM) differs from its normal counterpart in a number of functional properties and may have a key role during leukemic development. A decreased proliferation potential, contrasting with the strong ability of producing pro-inflammatory cytokines and an aberrantly loss of CXCL12 and SCF, suggest that leukemic lymphoid niches in ALL bone marrow are unique and may exclude normal hematopoiesis.

In this work, leukemic MSC $Nestin^{+}LepR^{+}$ organoid-like structures provide sanctuaries for leukemia-initiating cell (LIC) population able to escape chemotherapy-induced death with biological characteristics resembling Minimal/Measurable Residual Disease (MRD) cells responsible for relapse. Our model better mimic BM microenvironment during leukemia, producing minimal but necessary microenvironmental signals for LIC to grow *ex vivo*.

We confirmed the existence of a tumor microenvironment/niche induced population of leukemic cells with stemness properties and irrelevant immunophenotype, able to initiate leukemia in immunodeficient mice and probably for be the responsible for MRD before relapse. LIC hijack normal hematopoietic stem cells (HSC) from CAR niches where LIC is stablished, while pro-inflammatory factors remodel other MSC niches downregulating CXCL12 and SCF concomitant with a HSC exhaustion by cell displacement and proliferation. Our data also suggest the existence of two niches in ALL.

INTRODUCTION

The increasing evidence of the tumor microenvironment role in carcinogenesis has substantially modified the perspective of cancer pathobiology, favoring a more integrative view that consider the convergence of cellular genetics and an abnormal microenvironment as crucial elements for the development and maintenance of the disease[1]. In concordance with most types of cancer, induced chronic inflammation and microenvironment editing by tumor cells have been reported in some hematological malignancies, including acute lymphoblastic leukemia (ALL)[2–4].

ALL, characterized by a multi-clonal uncontrolled production of lympho-hematopoietic precursors within the bone marrow (BM), represent the most frequent childhood malignancy worldwide, showing a higher incidence in the Hispanic population and specifically, an increased ratio of high risk patients in Mexico[5–7]. Despite the notable progress in the disease management[8,9], the emergence of mixed-lineage leukemias, chemoresistance and minimal residual disease, decrease the probabilities for therapy success and determine relapse in more than 20% of the treated patients. As observed for the normal hematopoietic progenitors, neither pre-malignant cells nor leukemic blasts work as independent and autonomous entities, but they are surrounded in all dimensions by bone marrow (BM) niche components, providing regulatory cues essential for their cell fate decisions such as proliferation and survival[10–13]. Even when in the initial stages of leukemogenesis, normal and malignant populations apparently share BM niches, the disease progression transits in parallel with microenvironmental alterations that drive the establishment of exclusive leukemic structures unable to support normal hematopoiesis[14–17]. Data from mice models and leukemia patients have revealed that abnormal production of CXCL12 and

SCF is one of the crucial BM microenvironmental alterations in leukemia[2,15,18]. On the other hand, previous work in our lab suggest an important damage in the frequency and function of hematopoietic stem and progenitor cells (HSPC), accompanied by an abnormal and pro-inflammatory secretion profile in BM of ALL patients (e.g. increased secretion of IL-1β and TNFa)[19–21]. Thus, our experimental and theoretical observations strongly indicate a fundamental role of mesenchymal stromal cells in leukemia progression[22].

BACKGROUND

1. Early human lymphopoiesis

In normal conditions, hematopoietic stem cells (HSC) are responsible of producing all blood lineages through a highly regulated process called hematopoiesis, which take place in BM after birth[23,24] (**Figure 1**). HSC maintain blood cell production throughout the life by long-term reconstitution and their biological stemness properties. Immunophenotypically, HSC is enriched in the $CD34^{+}CD38^{-}Lin^{-}$ BM cell compartment, although in a very low frequency (0.01% of BM mononuclear cells). Further lineage cell fate decisions result from the continuing intercommunication between HSC and their microenvironment that regulate diverse transcription factors which promote differentiation options.

B, T, NK, NKT, some categories of dendritic cells and other innate lymphoid cells comprise the lymphoid lineage. The earliest commitment event of lymphopoiesis is the transcription of recombinase RAG1 and concomitant downregulation of VCAM-1, as reported in mouse models[25]. In humans, the earliest lymphoid progenitor is the $CD34^{+}CD45RA^{+}CD10^{+}CD19^{-}$ Multi-lymphoid progenitor (MLP)[26] is the major source of B/NK progenitors

retaining some myeloid residual potential. Multipotent progenitor development is critically dependent of PU.1 and Ikaros, while B-lineage specification depends on E2A, EBF and Pax5 functional expression levels[4]. Downstream B/NK progenitors, ProB ($CD34^{+}CD10^{+}CD19^{+}$) precursor cells are generated and sequentially, PreB ($CD34^{-}CD10^{+}CD19^{+}$) and mature B cells. Rigorously, formation of the B-immunorepertoire requires critical regulation with several checkpoints and B-lymphopoiesis occurs within BM specialized niches (further detailed). In contrast, T-lymphopoiesis progress in the thymus upon import of T-cell early progenitors from the BM (**Figure 1**).

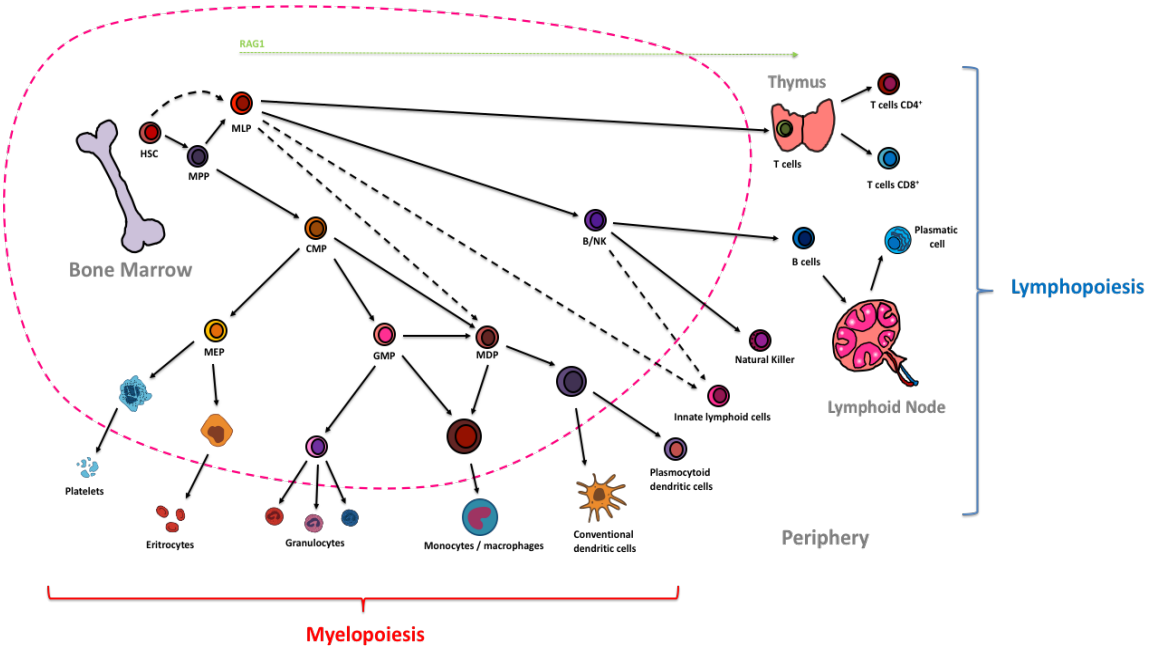


Figure 1. Human hematopoiesis differentiation map.
All blood cells derive from hematopoietic stem cells (HSC) and, except T cells, complete their differentiation programs within the bone marrow (BM) before concluding their maturation at the peripheral secondary lymphoid organs. T-lymphopoiesis occurs in the thymus after T-cell early progenitors are imported from BM. Multi-Lymphoid Progenitor (MLP), Multipotent Progenitor (MPP), B/N cell perogenitors (B/NK) Common Myeloid Progenitor (CMP), Megakaryoblastic-Erytroid Progenitor (MEP), Granulo-monocytic Progenitor (GMP), Monocytic-dendritic Progenitor (MDP), ILC (Innate Lymphoid Cells). (*Modified from Pelayo, Balandrán and Ruiz-Argüelles. Academia Nacional de Medicina 2018).*

2. Acute Lymphoblastic Leukemia

Among all hematopoietic malignancies, childhood leukemia is currently a global Health priority[5,27], characterized by uncontrolled proliferation of lymphoid or myeloid precursors in BM, which represents the most common pediatric oncological disease around the world with a variable incidence rate of 10-50 per million children per year with a cumulative risk up to the age of 15 years[28,29]. Multiparametric flow cytometry have been crucial to define the affected cellular linage which is important to classify into risk groups as well as to most properly follow-up the disease evolution after treatment (**Figure 2A**). Acute lymphoblastic leukemia (ALL) represents around 80-85% of the total leukemia cases and around 75-80% comprises B-cell lineage[6,21]. Only 10-15% of pediatric ALL show T cell immunophenotype while 15-20% are of myeloid lineage[21,30] (**Figure 2B**). In developed countries and high-income regions, 90% of patients can achieve total remission. A slight but gradual increase in the incidence of ALL appears to be highest in Latin America with abundant rates of high risk patients[27,31]. Currently, the most useful prognostic indicators are age, white blood cell count, immunophenotype, minimal residual disease (MRD) detection and poor response to induction therapy[32]. In contrast to previous notion of monoclonality in cancer, the combination of genomics and clonal studies with xenotransplantation approaches has revealed unsuspected genetic diversity in B-ALL, supporting its multiclonal co-evolution in the context of tumor microenvironment and suggesting that it is an oligoclonal disease[4,33].

Leukemic initiation has been proposed to occur through discrete steps during embryogenesis and at the V(D)J recombination stage to form a pre-leukemic clone. More recently, it has been suggested that the absence of common infections in newborns can trigger secondary mutations so that pediatric leukemias are potentially preventable[28]. In this sense, other

interesting studies have postulated that at least two-thirds of mutations in human cancer are caused by random errors occurring when cell DNA is copied, making the microenvironment manipulation a promising strategy to potentially prevent some cancer types[34].

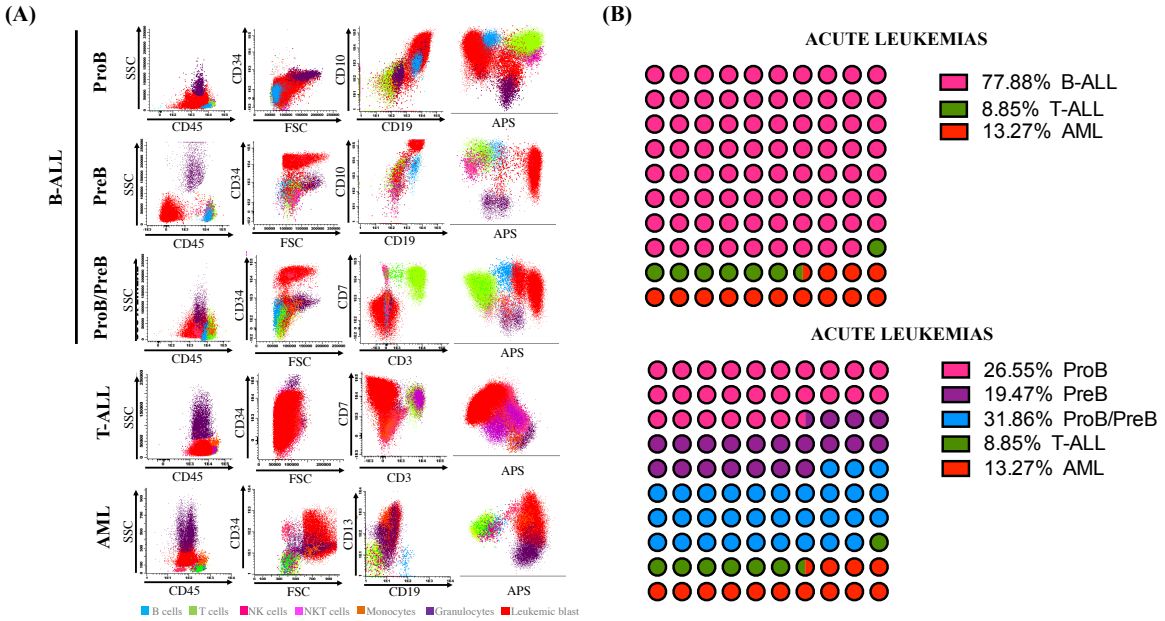


Figure 2. Classification and frequencies of childhood acute leukemias.
B precursor cell acute lymphoblastic leukemias (B-ALL) were appropriately subclassified into three types according to the differentiation stages of the BM ALL blasts by multiparametric flow cytometry: ProB-ALL, PreB-ALL and ProB/PreB-ALL. T-ALL and AML were also immunophenotyped (A). Frequencies of AL cases are shown (B). ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APS, Automatic Population Separator (*Modified from Balandrán et. al. Arch of Med Res 2016*).

3. Leukemia-initiating cells

The hematopoietic system is organized in a hierarchical structure where only a few HSC is able of producing all hematopoietic lineages by serial transplants. Similar to normal hematopoiesis, a seminal work by John Dick demonstrated that hierarchy is also applied for myeloid leukemias where only primitive $CD34^{+}CD38^{-}$ cancer stem cells are able to initiate leukemia in mice[35,36]. Leukemia-initiating cells (LIC) constitute a rare leukemic population sharing stem cell properties with their normal counterpart but variant immunophenotype among patients[37]. For B-ALL, several pieces of work have demonstrated lack of immunophenotype-based hierarchy but the function of a rare population with stem-like properties[38–41]. That is, from the experimental view, an stochastic model in B-ALL is more accepted than the cancer stem cell model[32]. To address this point, patient-derived xenografts (PDX) have been extensive used for propagation of primary cells and pre-clinical drug testing, showing that primary B-ALL usually engraft at low numbers in immunodeficient mice[39,40,42].. Existence of a putative-LIC in B-ALL have been reported as a rare, dormant and treatment-resistant leukemic population residing at the niche, that resemble MRD cells by RNAseq of MRD^{+} patients[43]. Of note, LIC may have important clinical implications as they have been proposed as responsible of treatment failure and relapse, which are the major challenge in leukemia. Because ALL is an oligoclonal disease, the probability to find different LIC subsets among patients is high so novel approaches to identify functional LIC should be investigated.

4. Bone marrow microenvironment

Normal and malignant lymphopoiesis are strongly dependent on BM microenvironment. In non-pathological conditions, HSPC reside in specialized niches and are constantly in a very close communication with BM stroma, soluble factors and extra-cellular matrix proteins[4,44]. BM niches are conformed by non-hematopoietic cells (endothelium, osteoblasts, mesenchymal stromal cells, adipocytes, chondrocytes and some nervous system cells) and hematopoietic cells (osteclasts, megakaryocytes, platelets, Tregs, etc.) within specific anatomical structures to control normal blood production[45–48]. At least three different types of niches have been described in humans and mice, although most knowledge have come from mouse models: i) osteoblastic niche, supported by osteoblast located at the bone surface where several mechanisms regulate stemness properties mediated by CXCL12, stem cell factor (SCF), Wnt, Notch, thrombopoietin, N-cadherin and angiopoietin-1; ii) the vascular niche, formed by endothelial cells located at the sinusoids and arterioles, which express some adhesion molecules such as E-selectin, P-selectin, VCAM-1, ICAM-1, PECAM-1 and VE-cadherin that interact with hematopoietic cells and, iii) the perivascular/reticular niche, formed by mesenchymal stromal cell (MSC) populations including Nestin$^+$ expressing cells, leptin receptor$^+$ (LepR$^+$) cells which also express Prx1 and overlap with the CXCL12-abundant reticular (CAR) cells. Some MSC populations endowed with stemness properties are the source of adipocytes, chondrocytes and osteoblasts. The identity of a unique mesenchymal stem cell is still under investigation[49–51]. Although it has been reported low adipogenic potential in Nestin$^+$ MSC, LepR$^+$ cells are the main source of this cellular lineage in BM[49]. CAR cells overlap with LepR+ and are also the major stem cell factor (SCF) producers, play important roles in

HSPC & lymphoid progenitor maintenance and immune cell production (**Figure 3**).

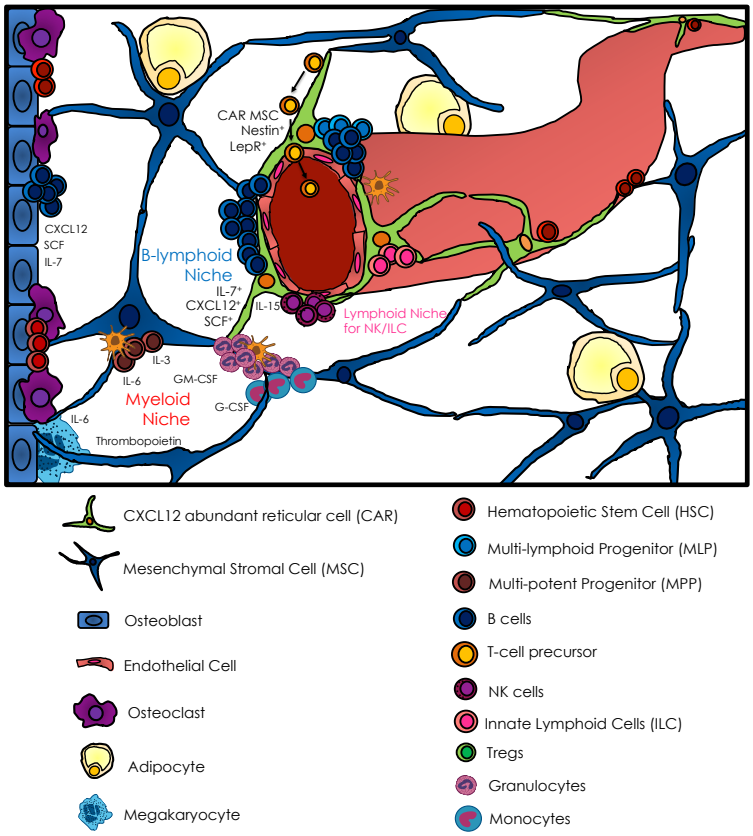


Figure 3. Cellular complexity of the bone marrow microenvironment.
Normal development of immune-hematopoietic system is contributed by non-hematopoietic cell populations residing within bone marrow and creating specialized niches in a highly organized architecture embedded in low oxygen tension conditions. Endosteal niche is located at the bone surface in coexistence with osteoclasts that achieve bone remodeling, while endothelial cells in the sinusoids and arterioles share medullar space to form perivascular/reticular niches supported by mesenchymal stromal cell (MSC) subsets. The microenvironment is a cellular continuum with "hematopoietic units" which provide hematopoietic cells in development, growth factors and cytokines required for differentiation, maintenance and proliferation of blood and immune cells (*Modified from Pelayo, Balandrán and Ruiz-Argüelles. Academia Nacional de Medicina 2018)*.

The hypoxic condition of BM has an important role in HSPC, particularly to maintain quiescence[52]. Low oxygen tension measurement in the BM have been achieved by several techniques[53,54] The most relevant transcriptional regulator of CXCL12 is the hypoxia-inducible factor 1α (HIF-1α) and its activation is related to poor leukemic prognosis[55]. Inhibitory signals from HIF-1α impair BM homing and retention in chronic lymphocytic leukemia and diminish the expression levels of CXCR4 and CXC12.

5. Bone marrow niche for early lymphopoiesis

Like HSPC, lymphoid progenitors are critically dependent on CAR cells due to their CXCL12 and SCF production. Of note, *in vitro* lymphopoiesis cannot be achieved without stromal feeders[56] and plastic-adherent BM stromal cells have shown to properly support B-cell development[57]. CXCL12 is a chemokine required for HSPC maintenance and retention in the BM. Observations from CXCL12 and CXCR4 knockout mice have indicated that CXCL12 is critical for early B-lymphopoiesis in the fetal liver and post-natal BM[58]. Conditional knockout of CXCL12 in BM osteoblasts deplete BM from lymphoid progenitors but not HSPC, suggesting that endosteal niche is predominately occupied by early lymphoid cells[59].

IL-7 signal is also critical for the earliest stages and commitment of the lymphoid program (though not rigorously in human) and IL-7 receptor (IL-7R) expression identifies lymphoid restricted progenitors in the BM. Classical models of lymphoid niche include two stage-specific cellular niches moving one to another as differentiation occurs on CXCL12 enriched (CAR) niche and other releasing IL-7[4]. More recently, it has been reported that IL-7 is also produced by a subset of CAR cells in the perivascular niche, then excluding osteoblasts as a significant IL-7 producer[60].

6. Leukemic microenvironment and bone marrow remodeling

Loss of normal hematopoietic function is a clinical feature of leukemias. Biopsies analysis of ALL BM samples have described alterations in BM architecture and composition[61]. During leukemogenesis different mechanisms can occur to explain disrupted BM homoeostasis: a competition of tumor cells for normal HSC niches, the manipulation of normal environments led by tumor cells, and disruption of HSC-niche communication[4]. The first report demonstrating how B-ALL cells hijack normal HSPC niche was made by Dr. Sipkins' group by using an elegant mice xenograft model. Importantly, B-ALL cells colonized specific $CXCL12^{+}$ perivascular niches triggering its downregulation and displacement of normal $CD34^{+}$ cells[15]. Increasing evidence indicates the prevalence of functional defects in both soluble and cellular components of the ALL microenvironment[20], although it is still unclear whether these are intrinsic abnormalities or if they appear as a consequence of the activity of the leukemic cells. B-ALL soluble factors are able to remodel MSC derived from healthy BM donors and altering its normal CXCL12 production[62]. Pro-inflammatory settings can be mediated by microRNAs released by extracellular vesicles (ECV) as a ligand for TLR7/8 signaling[63] (Ríos de los Ríos, in preparation). MSC remodeling during leukemia seems to be stable to retain an abnormal phenotype even after leukemic interactions suggesting epigenetic changes but completely reversible after chemotherapy total cure when normal hematopoiesis is restored[64]. Evasion of therapy by ALL cells is one of the main obstacles in leukemia treatment. Surviving cells are surrounded by stromal cells in BM creating a chemotherapy-derived protective niche composed by MSC and no necessary protected by inducing quiescent stages. Importantly, the efficacy of chemotherapy was enhanced by interfering with the protective niche formation or function[65].

Similar to normal lymphopoiesis, B-ALL precursors are highly dependent on their microenvironment and rapidly undergo apoptosis when ALL blast are cultured even with the most sophisticated conditions. Particularly, functional assays with primary B-ALL have been hampered by the inability to expand tumor *ex vivo*. B-ALL blast short-term co-cultures with MSC have been used to test therapeutic drugs before culture decline and chemotherapy protection mediated by MSC have been vastly documented[66–69]. However, MSC protective niches as well as the leukemogenic support cannot be completely mimicked by conventional monolayers [65], where lack of hypoxic signals might contribute to poor leukemia maintenance[70].

Interestingly, BM stromal cells have been shown to display chromosomal abnormalities in malignancies such a myelodysplastic syndrome, multiple myeloma and myeloid and lymphoid leukemias, but the role of this gene aberrations under hematopoietic malignances is still under investigation[4].

RESEARCH JUSTIFICATION

Acute lymphoblastic leukemia (ALL) is a serious public health problem in the pediatric population of our country and in the world, contributing to 85% of deaths from childhood neoplasms. Understanding of the establishment and maintenance of the disease is of crucial importance for its clinical management and the development of therapeutic strategies. Leukemia-initiating cells (LIC) are strongly dependent of their niche, where they can live in the context of biological regulation, expand and maintain protected during chemotherapy. Current knowledge of LIC niche properties has emerged from mice models or co-culture systems on stromal monolayers, which has substantially limited the progress and therefore, the understanding of micro-environmental elements regulating them.

HYPOTHESIS

Mesenchymal stromal $Nestin^{+}LepR^{+}$ bone marrow organoid-like structures can select and maintain leukemia-initiating cells from ALL B-cell precursors by mimicking their microenvironmental requirements and promoting stemness properties.

AIM

To investigate the mesenchymal stromal niche functional identity in a bone marrow organoid-like co-culture system in childhood B-precursor cell acute lymphoblastic leukemia.

PARTICULAR AIMS

- To characterize primary bone marrow (BM) mesenchymal stromal cells (MSC) derived from B-ALL pediatric patients (ALL-MSC).

- To identify ALL-MSC BM organoid-like structures functioning as stem-like niches for leukemia-initiating cells (LIC).

- To test LIC potential in cells collected from organoid-like structures through *in vivo* xenotransplantation assays.

- To study chemotherapy-induced protective niche functions in residual disease or relapse preclinical models.

METHODS

Patient Characteristics and Sample Collection.

58 children referred to Toluca Children's Hospital (Toluca, Mexico) and diagnosed with B-ALL were included in the study, 7 of them resulted in B-ALL relapse. BM specimens were collected by aspiration before any treatment and according to international and institutional guidelines. All procedures were approved by the Ethics, Research and Biosafety Committee of the Hospital and by the National Committee of Scientific Research at the Mexican Institute for Social Security (CIEICE-007-01-13, R-2012-3602-29 and R-2015-785-120). Control BM specimens were obtained from 12 healthy children undergoing minor orthopedic surgery. All samples were collected after informed consent from the parents (**Table 1**).

Inclusion criteria:

- Patients younger than 18 years
- Patients newly diagnosed with ALL without treatment
- Patients with ALL relapse

Exclusion criteria

- Patients whose parents did not agree to participate
- Samples stored at room temperature or at 4°C for more than 24 hours
- Samples from patients in remission or under treatment

Table 1. Patient characteristics

Patient	Age (y)	Sex	Immunophenotype	WBC x10³/mm3	BM Blast (%)	Chromosomal aberrations	Risk
1	3	F	PreB	293.4	94.6	-	High
2	2	F	PreB	7.1	67	-	High
3	9	M	PreB	-	-	-	High
4	4	M	PreB	281	89	t(9;22)(q34;q11) BCR ex13 - ABL1 ex2 (b2a2)	High
5	9	M	PreB	123	73.5	-	High
6	9	F	PreB	188	48.3	-	High
7	8	F	PreB	23	67	-	High
8	11	M	PreB	143	56	-	High
9	10	M	PreB	121	78	-	High
10	9	F	PreB	109	67	-	High
11	10	M	PreB	71.7	90.4	-	High
12	15	M	PreB	-	-	-	High
13	13	F	PreB	-	-	-	Standard
14	7	F	PreB	45.6	98.6	-	High
15	8	M	PreB	112	78.6	-	Standard
16	7	M	PreB	108.6	94.6	-	Standard
17	14	M	PreB	110.6	56.4	-	High
18	7	F	PreB (relpase)	110.8	84	-	High
19	8	F	PreB (relapse)	-	-	-	High
20	14	F	ProB	108.3	67	t(15;17)(q22;q21) PML ex3-RARA ex5	High
21	5	F	ProB	53.5	87.8	t(12;21)(p13;q22) ETV6 ex5 - RUNX1 ex4:	High
22	10	F	ProB	164.5	85	t(9;22)(q34;q11)	High
23	12	F	ProB	4.9	72	t(1;19)(q23;p13)TCF3-PBX1(PRL)	High
24	4	F	ProB	58.6	83.1	t(12;21)(p13;q22) ETV6 ex5 - RUNX1 ex3	High
25	6	F	ProB	34	12	-	Standard
26	14	F	ProB	45.6	65	-	High
27	9	F	ProB	121.4	78	-	High
28	9	F	ProB	112	56	-	High

29	5	F	ProB	60.4	49.5	-	Standard
30	16	M	ProB	45.7	28.5	-	High
31	5	F	ProB	34.5	86.4	-	Standard
32	15	F	ProB	-	-	-	High
33	8	M	ProB (reapse)	89.4	70	-	High
34	7	M	ProB (relapse)	-	-	-	High
35	8	F	ProB/PreB	48.6	80	-	High
36	7	F	ProB/PreB	117	71.23	t(15;17)(q24;q21)	High
37	9	M	ProB/PreB	100.7	81	t(1;19)(q23;p13)TCF3-PBX1(PRL)	High
38	3	M	ProB/PreB	119.2	83	-	High
39	2	M	ProB/PreB	160.5	86.35	t(1;19)(q23;p13)	High
40	4	F	ProB/PreB	37.5	87	t(12;21)(p13;q22) ETV6 ex5 - RUNX1 ex4:	High
41	12	F	ProB/PreB	25.6	62.4	t(1;19)(q23;p13)TCF3-PBX1(PRL)	High
42	9	M	ProB/PreB	55	86.6	t(12;21)(p13;q22) ETV6 ex5 - RUNX1 ex4:	High
43	16	M	ProB/PreB	9.5	74.9	t(16;21)(p11;q22) FUS ex7 ,Äì ERG ex13	High
44	4	M	ProB/PreB	64.6	89.7	t(12;21)(p13;q22) ETV6 ex5 - RUNX1 ex4:	High
45	3	F	ProB/PreB	123	66	-	High
46	7	M	ProB/PreB	89	65.4	-	Standard
47	5	M	ProB/PreB	45.3	65.4	-	Standard
48	6	F	ProB/PreB	12.4	37.8	-	Standard
49	6	M	ProB/PreB	27.6	68.4	-	Standard
50	15	F	ProB/PreB	26.9	78.6	-	High
51	5	M	ProB/PreB	29.4	76.1	-	Standard
52	4	M	ProB/PreB	145.2	67.8	-	High
53	6	F	ProB/PreB	43.2	25	-	Standard
54	2	F	ProB/PreB	134.5	89.4	-	High
55	14	M	ProB/PreB	23.5	76.4	-	High
56	7	M	ProB/PreB (relapse)	45.2	22.8	-	High
57	2	F	ProB/PreB (relapse)	59.9	91.47	t(12;21)(p13;q22)	High
58	1	M	ProB/PreB (relapse)	135.6	67.3	-	High

Cell lines.

B-ALL cell lines RS4;11, REH and Nalm6 were obtained by ATTC. Only low passages were used.

Isolation of hematopoietic primary CD34$^+$ cells.

Mononuclear cells (MNC) were prepared by Ficoll-Paque Plus (GE Healthcare Bioscience) gradient separation. CD34$^+$ fraction containing HSC and progenitor cells was enriched from MNC using the human CD34 progenitor cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Cell purity and yield were measured by flow cytometry using a BD FACSCanto II. In order to identify biological heterogeneity on the basis of the primitive cell behavior, and to limit heterogeneity due to patient diversity and/or disorder variations, no sample pooling was performed for any of the experimental strategies.

Flow Cytometry.

Immunophenotyping and identification of stem and progenitor populations were performed by multiparametric flow cytometry on a FACSCanto II (BD Biosciences) after staining with anti-lineage markers (CD3, CD8, CD56, CD11b, CD14, CD16, CD19, CD20, and CD235a), anti-CD34, fluorochrome-conjugated antibodies (BioLegend). Hematopoietic stem cells and progenitor cells (HSPC) were identified as Lin$^-$CD34$^+$. For cell co-cultures, the fraction containing HSPC or leukemic blasts (Pro-B blast population was identified as CD34$^+$CD19$^+$ while Pre-B cells as CD34$^-$CD19$^+$) was highly purified using a FACSAria II flow cytometer (BD Biosciences). Cell purity was confirmed by post-sorting analysis.

Isolation of Mesenchymal Stromal Cells (MSC).

MNC from ALL patients and controls were isolated by the classical adhesion method cultured in low glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/mL of penicillin/streptomycin (Gibco) at density of 1,000-2,000 MNC/μL in a 5% CO_2-in-air incubator at 37°C. After a 3-day culture, the nonadherent cells were removed and fresh medium was added to the cultures. Once the cultures reached 80-90% confluence, the cells were harvested with trypsin (0.05% trypsin-EDTA; Gibco). Cell viability was assessed by trypan blue staining, and the cells were expanded in secondary and tertiary passages. At the second passage, cells were harvested, analyzed, and cryopreserved for future use. Harvested cells were investigated by flow cytometry for the expression of typical MSC and lacking of hematopoietic cell markers (CD105, CD90, CD73, CD45, CD34 and HLA-DR); (All experiments were performed with cells harvested between the third and fourth passage).

Multi-linage differentiation capacity of MSC.

To evaluate the chondrogenic differentiation potential of normal and ALL-MSC, they were cultured with chondrogenic medium (DMEM-alpha glucose, supplemented with 0,1μM dexamethasone, 50μg/mL ascorbic acid, 110μg/mL sodium pyruvate, 40μg/mL prolin, 10ng/mL TGF-β1, 50mg/ml ITS+ premix (Becton Dickinson), 6.25μg/ml insulin, 6.25μg/ml transferrin, 6.25 ng/ml selenic acid, 1.25mg/ml bovine serum albumin (BSA) and 5.35 mg/ml linoleic acid), followed by alcian blue staining after 14 days. For adipogenic differentiation, MSC were growth in medium supplemented with 0.5mM isobutil-metilxantin, 1μM dexametasone, 10μM insulin and 200μM indomethacin, before red oil staining. Osteogenic differentiation was investigated using low glucose DMEM medium supplemented with 15% of

osteogenic supplement, 10^{-7}M dexamethasone and 0,2 mM ascorbic acid. Cells were growth during 3 weeks and then stained with von Kossa dye.

Three-dimensional (3D) cultures based on stromal spheroids.

10,000-50,000 stromal cells were plated per well of a 96 well round bottom plates previously coated with 1% agarose (Invitrogen) to induce the spheroid formation. After 24 hours spheroids were formed under this condition and used in further experiments.

Colony-forming units-fibroblast (CFU-F) assay.

MSC cell progenitor frequencies were recorded by fibroblast colony formation assay. Crescent numbers of MNC were plated in DMEM supplemented with 10% of FBS and 14 days later Wright-Giemsa staining was performed to determine the CFU-F content.

MSC proliferation assay.

A carboxifluorescein (CFSE) dilution assay was conducted using 10mM CFSE per $5x10^5$ MSC and dye dilution evaluated by flow cytometry at 24 and 48 hours.

RT-PCR analyses for MSC differentiation.

Transcription of differentiation related genes were evaluated by RT-PCR using the following primers: PPARγ2, Runx2, Sox9, CXCL12, HIF-1a and IL7, with the 18s ribosomal RNA as housekeeping control. cDNAs were subject of amplification by RT-PCR (95ºC initial denaturalization for 5 minutes, 35 amplification cycles of 5 seconds at 95ºC, and aligning temperature in function of every single gene during 30 seconds and 72ºC during 10 minutes) in a thermal cycler T100 ™BIORAD. AlphaView software (FluorChem System) was used for analysis.

Lymphoid differentiation co-culture system.

1000 lymphoid progenitors were seeded (input value) in alpha-MEM 10% FBS supplemented with 100 ng/ml rhSCF, 100 ng/ml rhFlt3-L, 20 ng/ml rhIL-7 and 10 ng/ml rhIL-15. Co-cultures were maintained during 3-6 weeks and B precursor cells production was evaluated by multiparametric flow cytometry. Results were recorded as yield per input (absolute number of B cell precursors that are produced per each initial progenitor).

Induction of hypoxic conditions.

MSC were treated with 100µM cobalt chloride II ($CoCl_2$) during 24 hours. Cytoplasmic stabilization of HIF-1a was measured by flow cytometry and its nuclear translocation by indirect immunofluorescence microscopy.

Pimonidazole incorporation.

Detection of cell and tissue hypoxia was performed using Hypoxyprobe-1 Plus Kit (pimonidazole hydrochloride, Chemicon International, Temecula, CA, USA) by flow cytometry and fluorescent microscopy following the manufacturer's recommended protocols.

Hematopoietic proliferation by BrdU incorporation assay.

MSC-hematopoietic cell co-cultures were supplemented with BrdU during 24 hours and leukemic cells harvested, fixed and permeabilized. DNAse treatment was performed for 1 hour at 37ºC before cell incubation with anti-BrdU. Proliferation rates were analyzed by multiparametric flow cytometry.

Co-culture of primitive hematopoietic cells with 3D spheroids.

10,000-20,000 hematopoietic cells were cultured with 3D spheroids. Cultures were maintained with DMEM 10%FBS and antibiotics supplemented with

2ng/mL SCF, 1ng/mL Flt3-L, 5ng/mL and 5ng/mL IL-7 and 10ng/mL IL-15 (Preprotech) in a 5% CO_2-in-air incubator at 37ºC. Spheroids were washed with PBS-EDTA (EDTA 0.5mM) and then enzymatically digested by using TrypLE Express (Gibco), released cells were called as "3D-in" while outer cells were named as "3D-out". Cellular count, viability and phenotype were evaluated by flow cytometry using 7-AAD or DAPI, and CD45, CD34 and CD19 staining.

Cell Tracking strategy.

MSC were stained with the fluorescent dyes Cell Trace Violet® while hematopoietic cells CellTrace CFSE® or CellTrace FarRed® (all from Life Technologies). Staining procedure was done according to manufacturer's instructions.

Fluorescence Microscopy.

MSC were fixed with paraformaldehyde 4% and permeabilized with PBS-Triton 0,01%. Fc receptors were blocked with 3% PBS-FBS and cells were incubated overnight at 4ºC with primary unlabeled antibodies (anti-human CXCL12, anti-human SCF, anti-human IL-7, anti-human Nestin, anti-human PDGFRα, anti-human VCAM-1, anti-human HIF-1α or anti-human Cx43, all from Abcam) diluted in PBS 3% SFB, washed three times with PBS and incubated for 1 hour at RT with conjugated secondary antibodies. Fixed spheroids were stained or embedded in Tissue-Tek O.C.T. and quickly frozen to get cryostat slices of 5-20 mm. Wholly spheroids were treated with 0.01% Triton X-100 (Bio-Rad) for 2 hours and non-specific sites were bloked with 3% BSA for 1 hour and then incubated over night with with primary unlabeled antibodies. Slices were treated with 0.01% Triton X- 100 and blocking with 3% BSA for 30 minutes at 37ºC and then incubated over night at 4ºC with primary unlabeled antibodies, slides were washed three times with PBS and it were

mounted with vectashield-DAPI or vectashield-IP. Images were acquired using the manufacturer-provided Nikon Elements software using a Nikon Ti Eclipse microscope, equipped with an A1 imaging system at the National Laboratory of Advanced Microscopy or with the EVOS FL Cell Imaging System.

Cytokine Detection.

Supernatants were collected and stored at −20∘C until analysis. Thee cytokine, chemokine, and growth factor content in supernatants were measured by Multiplex Milliplex Map Immunoassay (Merck Millipore), following the manufacturer's recommended protocols. The assay included: SDF-1, BCA-1, LIF, TRAIL, SCF, TSLP, FGF-2, Flt-3L, G-CSF, GM-CSF, IFNα, IFNγ, IL-1α, IL-1β, IL-2, IL- 3, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-15, IP-10, TNFα, and VEGF.

Proliferation Assay.

Primary B-ALL CD34^{+} cells were stained with CellTrace CFSE® according to manufacturer's instructions and co-cultured with one 3D spheroid, 48 hours later spheroids were vigorously washed with PBS to remove cells attached to their surface then spheroids were mechanically and enzymatically dissociated with 0,125% trypsin (0,78 mM EDTA). Cells recovered for the inside of the spheroid or in surrounded suspension were collected and evaluated by flow cytometry.

Side population assay.

Hoechst staining was conducted following Margaret Goodell's protocol. [71] Briefly, harvested cells were adjusted at 10^6 cells per ml in DMEM and were incubated with Hoechst 33342 to a final concentration of 5 μg/mL (Sigma-Aldrich) at 37ºC during 2 hours. Cells were washed with PBS 1x and were

acquire in a BD Influx at the Flow Cytometry Facility.

Xenotransplantation Assays in NSG mice.

In vivo experiments were carried out under an Institutional Animal Care and Use Committee (IACUC) approved protocols (Weill Cornell Medicine) and the *Comité Interno para el Cuidado y Uso de los Animales de* Laboratorio (CICUAL) (CINVESTAV) and institutional guidelines for the proper and humane use of animals in research were followed. NOD/SCID gamma (NSG) mice were obtained from Jackson Laboratories (JAX). Primary B-ALL cells were harvested from the different culture conditions after 72H (stromal-free system, 2D co-culture and 3D-in and 3D-out recovered from the organoids) then injected i.v. Animals were euthanized after 5-7 weeks of injections or until they began to exhibit clinical symptoms of the disease. Engraftment was ascertained by determining the percentage of human $CD45^+$ cells in peripheral blood by bleeding or in the mouse (BM) by femoral bone marrow aspiration.

Statistics.

Flow cytometry data were analyzed by using FlowJo 10.0.8 (LLC) or Infinicyt 1.8 (Cytognos) software. Prism V3.02 (GraphPad) software was used to perform statistical data analysis. Differences within groups were established by non-parametric tests, considering significant probability values <0.05. U Mann-Whitney test with a of 5% to define statistical significance was applied.

RESULTS

Functional alterations in mesenchymal stromal cells (MSC) set up a pro-inflammatory microenvironment in acute lymphoblastic leukemia (ALL).

BM MSC from childhood ALL patients were characterized according to their morphological, phenotypical and functional properties established as the minimal criteria in the International Society for Cellular Therapy ([72]). No differences in morphological aspects between cells from both sources were apparent. However, ALL-MSC showed intercellular disorganization when monolayer structures were observed (**Figure 4A**). Remarkably, although CFU-F capacity and cell proliferation were reduced (**Figure 4-C**). As known, specific MSC markers are still under investigation and currently, most accepted phenotype is $CD34^{-}CD45^{-}CD14/CD11^{-}CD90^{+}CD73^{+}CD105^{+}$ with a variable expression of MHC II, CD29 (integrin β1), CD49b (integrin a2), CD44 (HCAM), CD54 (ICAM-1), CD58 (LFA-3) and CD166 (ALCAM). Here, our data indicate a correspondence in the phenotype of the MSC from ALL and NBM with minor differences in expression of some MSC-markers suggesting potential abnormalities in the capacity of cell-cell intercommunication with leukemic precursors within the BM. Interestingly, as previously reported for angiogenic-stimulated abnormal MSC in hematological malignancies[73], CD90 was significantly reduced in ALL (**Figure 4D**). Whether this molecule may discriminate more primitive stages of mesenchymal linage as in the hematopoietic system, remains elusive. Importantly, their differentiation potential to chondrogenic, adipogenic and osteogenic linages were not truly compromised (**Figure 4E**) but differentiation gene transcripts, including Runx2 (osteogenesis), Sox9 (chondrogenesis) and PPARG2 (adipogenesis) revealed a substantial reduction in Sox9 expression by ALL cells, suggesting altered lymphopoiesis associated to defects in osteochondrogenesis (**Figure 4F**) as recently reported [74]. ALL-MSC promoted proliferation on primary B-ALL

cells as well as the hematopoietic support capability when compared to their normal MSC counterpart (**Figure 5A**), though it is uncertain the normal or leukemic origins of such supported cells.

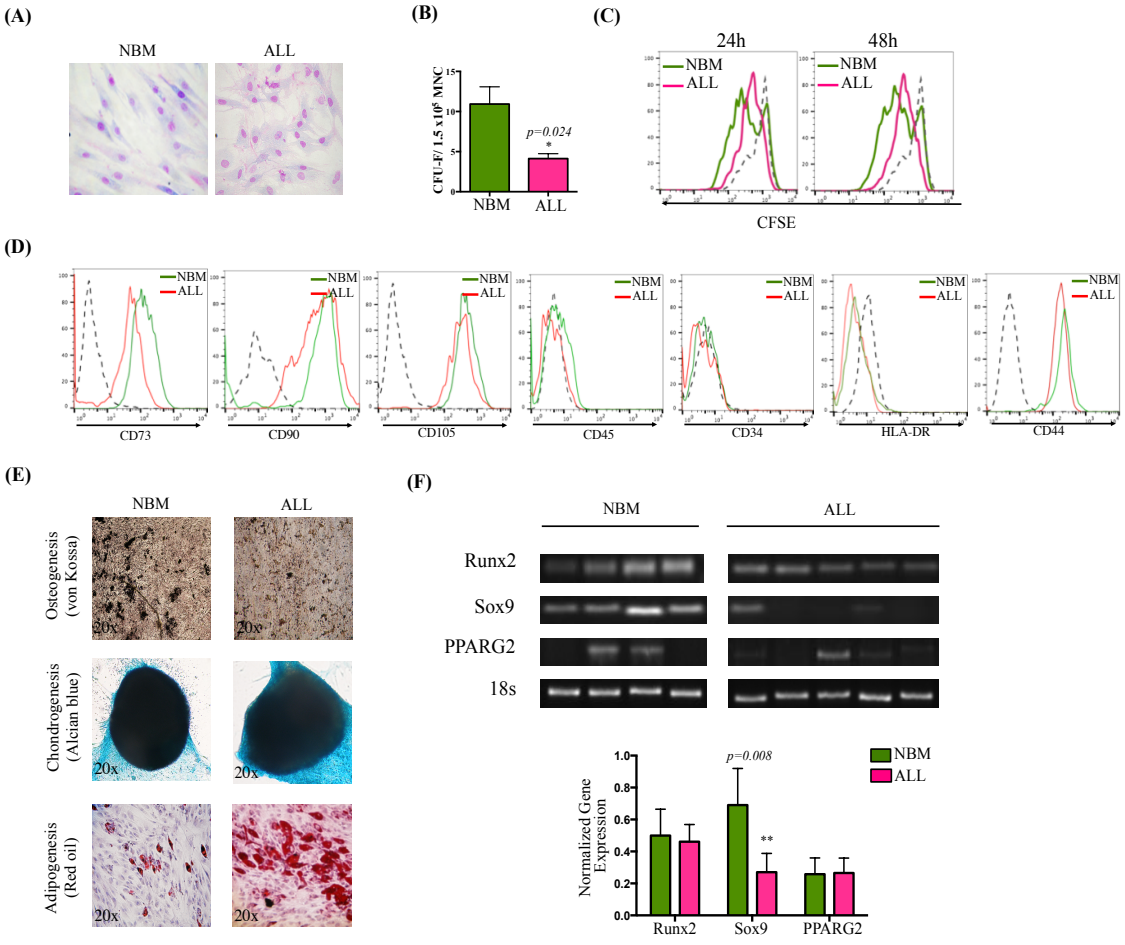


Figure 4 Biological characterization of mesenchymal stromal cells (MSC) derived from bone marrow of acute lymphoblastic leukemia (ALL) patients.
MSC were evaluated according to morphology (A), colony-forming unit-fibroblast capacity (B), proliferation through CFSE dilution assay (N_{NBM}=4, N_{ALL}=6) (C), minimal criteria immunophenotyping (N_{NBM}=5, N_{ALL}=7) (D), multiple differentiation potential (N_{NBM}=5, N_{ALL}=7)(E) and differentiation genes expression (N_{NBM}=4, N_{ALL}=5) (F). NBM, normal bone marrow; ALL, acute lymphoblastic leukemia.

At least 16 soluble factors relevant to lymphoid development were aberrantly secreted, as supernatant contents indicated high levels of pro-inflammatory cytokines IL-1a, IL-6, IL-12p70 and TNFa, as well as interferon type I and type II and early growth factors like Flt3, G-CSF and IL-7 (**Figure 5B**). Remarkably, CXCL12 (stromal derived factor-1, SDF-1) and SCF (stem cell factor), two components of the hematopoietic microenvironment and main regulators of stem cell maintenance that have shown to be key players in the early lymphopoiesis pathway, were critically reduced. Accordingly, ALL-MSC conditioned medium contained high amounts of G-CSF, which has shown the ability of mobilizing hematopoietic cells from BM through a CXCL12/CXCR4 axis breaking mechanism. The observed pro-inflammatory profile was supported by the nuclear NF-κB translocation in ALL-MSC (**Figure 5C**). Taking together, our findings suggest a pro-inflammatory microenvironment contributed by activated MSC that may impact normal and leukemic developmental dynamics.

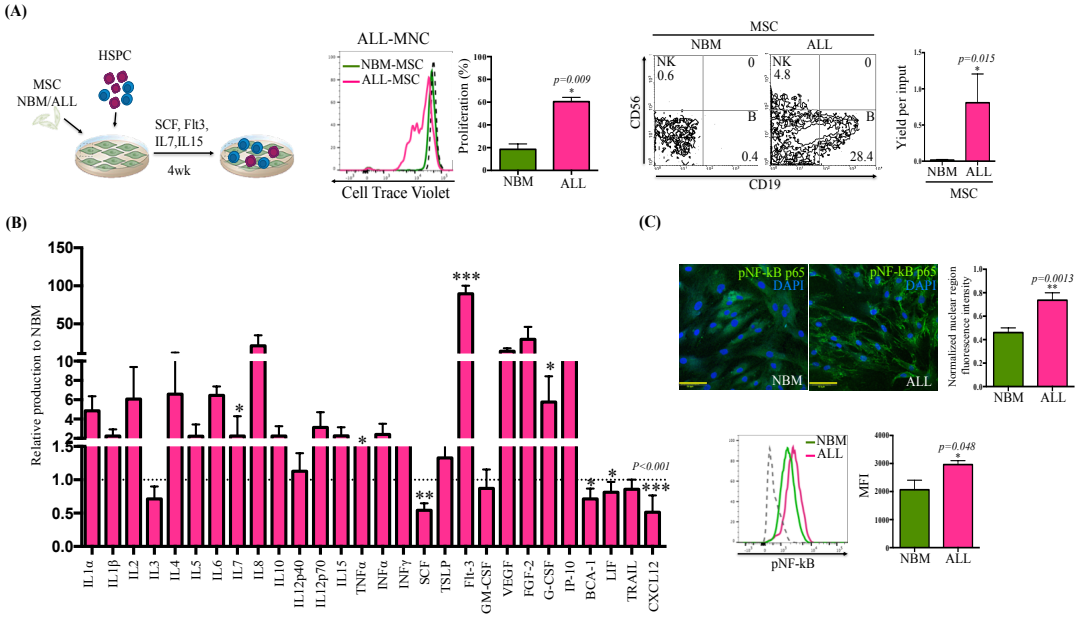


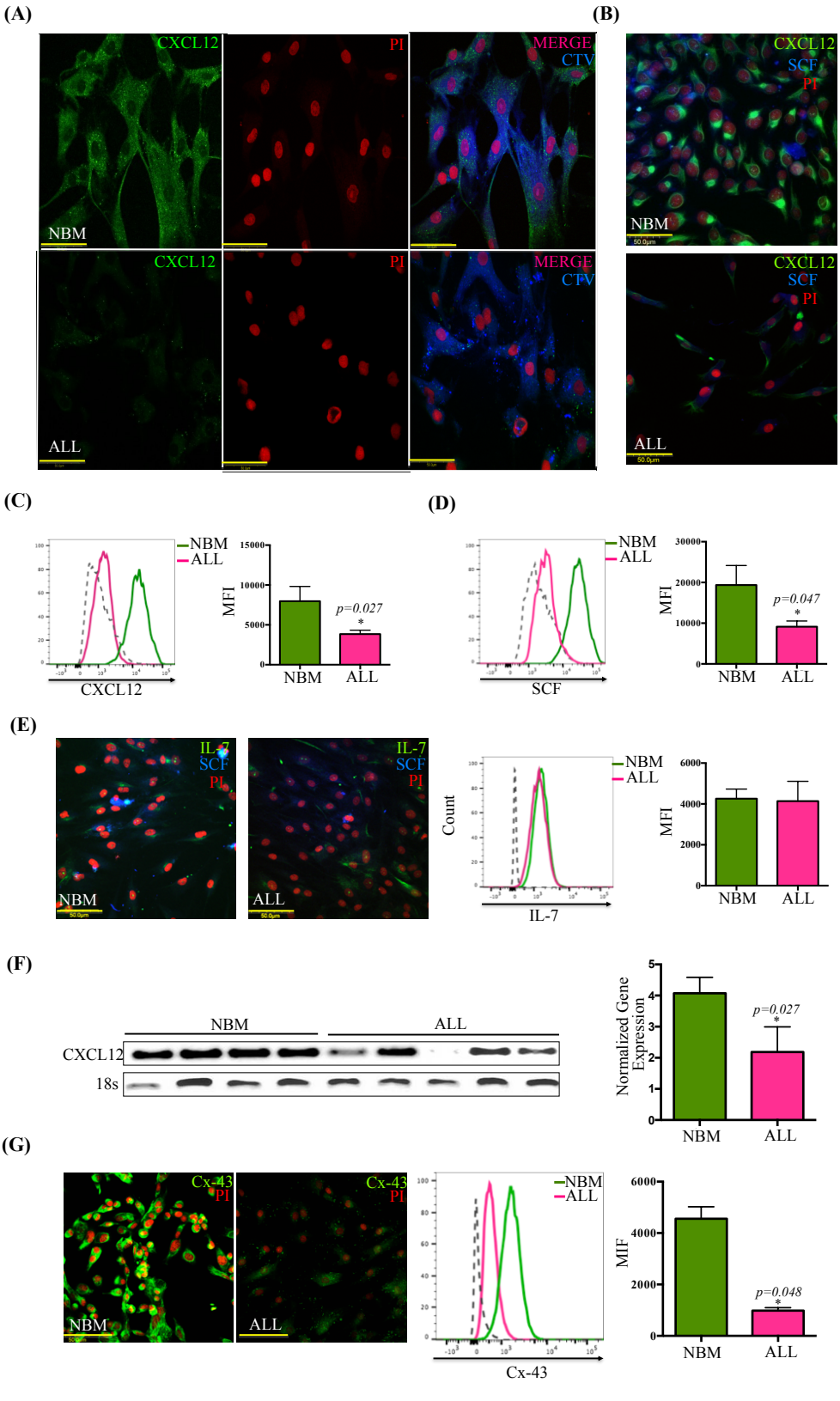
Figure 5. ALL-MSC contribute to leukemic cell maintenance by creating a pro-inflammatory microenvironment.

Hematopoietic stem and progenitor cells (HSPC) from ALL BM were co-cultured on NBM-MSC or ALL-MSC monolayers in lymphoid conditions during 4 weeks before flow cytometry analysis of the newly produced cells and the B cell yield per input were recorded (N_{NBM}=5, N_{ALL}=7) (A). Cytokines, chemokines and growth factors production by NBM and ALL MSC were evaluated after collection of 24 hour supernatants. Data are normalized to NBM MSC secretion (B) (N_{NBM}=5, N_{ALL}=13). Phosphorylated p65 (NF-κB) was evaluated by immunofluorescence microscopy (N_{NBM}=3, N_{ALL}=4) (C, upper panel) and flow cytometry (C, lower panel) of MSC (N_{NBM}=3, N_{ALL}=5). Normalized nuclear fluorescence intensity and mean fluorescence intensity (MFI) from flow cytometry are shown.

Loss of CXCL12 marks the leukemic niche within the bone marrow.

A crucial regulatory axis of the crosstalk between lymphoid progenitors and the hematopoietic microenvironment is CXCL12/CXCR4. CXCL12 and SCF highly producer MSC constitutes the primary niche for B lymphoid progenitor cells[75]. Concurring with our previous supernatants observation, confocal microscopy revealed the per-cell-basis reduced production of both factors (**Figure 6A-D**). In contrast, IL-7 lymphoid niche appears unaltered in ALL (**Figure 6E**). To explore the biological mechanism associated to CXCL12 loss, we investigated the gene transcription extent and found it heterogeneously impaired among patient samples (**Figure 6F**). In addition, the intercommunicating stromal cell Gap-junction molecule, connexin-43 (Cx-43), that drives secretion of CXCL12, appeared frankly down-regulated in ALL derived MSC (**Figure 6G**). As it is believed that in BM, MSC maintain hematopoietic cells attached to them in a semi-quiescence state through the dynamic production of CXCL12, lack of Cx-43 impacting the release of CXCL12[76], may have important implications in architecture and function of BM niches. Moreover, homing to the endosteal niche might relate to Cx-43, due to its participation in the bi-directional traffic from BM to the periphery and vice versa during ALL[77].

Figure 6. Abnormal CXCL12 and SCF production by mesenchymal stromal cell forming the B-lymphoid niche in acute lymphoblastic leukemia (ALL).
CXCL12 (A, B, C), SCF (B, D, E) an IL-7 (E) production was evaluated by immunofluorescence microscopy and flow cytometry in mesenchymal stromal cells (MSC) from normal (NBM) and ALL bone marrow (N_{NBM}=6, N_{ALL}=12). CXCL12 mRNA expression was determined by RT-PCR in normal and ALL MSC, and their normalized gene expression tabulated (N_{NBM}=4, N_{ALL}=6) (F). The gap-junction connexin-43 (Cx-43) displayed by ALL MSC and their normal counterpart was investigated by immunofluorescence microscopy and flow cytometry (G). CTV, Cell Trace Violet; NBM, normal bone marrow; ALL, acute lymphoblastic leukemia; MFI, mean fluorescence intensity.


(A)
CXCL12
PI
MERGE
CTV
NBM
ALL
(B)
CXCL12
SCF
PI
NBM
ALL
(C)
NBM
ALL
CXCL12
MFI
p=0.027
*
(D)
SCF
p=0.047
(E)
IL-7
SCF
PI
Count
(F)
CXCL12
18s
Normalized Gene Expression
p=0.027
(G)
Cx-43
MIF
p=0.048

HIF-1α is a major transcriptional regulator of CXCL12[78]. Here, a critical reduction of its expression with a cytoplasmic localization instead of the typical nuclear translocation seen in their normal counterpart was recorded in ALL-MSC (**Figure 7A**). Furthermore, experimental hypoxia was not able to increase HIF-1a expression to normal levels. Chemical imitation of hypoxia by Chloride Cobalt ($CoCl_2$) has been reported to create a hypoxic-like condition by disturbing the constant proteasomal degradation of HIF-1α and allowing its cytoplasmic accumulation and further nuclear translocation even during normoxic settings. Although expression of CXCL12 showed a slight boost, the end balance was never as high as from normal bone marrow (NBM) (**Figure 7B**).

Thus, in ALL, the CXCL12-dependent lymphoid niche might be intrinsically altered due at least to an impaired transcriptional regulation by HIF-1a and to an abnormal functional activity of Cx-43.

To investigate the biological relevance of loss of CXCL12 in leukemic progression, we conducted an *in vitro* cell proliferation assay by incorporation of BrdU under distinct CXCL12 scenarios. Leukemia cell line REH was treated either with $CoCl_2$ to increase CXCL12 expression, or with carbenoxolone (CBX), a Cx-43 inhibitor, to indirectly reduce CXCL12, and their proliferative status analyzed (**Figure 7C**).

Of interest, malignant proliferation extent was inversely proportional to CXCL12 expression in MSC. Since the role of CXCL12 in early lymphopoiesis has been vastly studied[58,59], our findings suggest that CXCL12 lymphoid niche may have an additional function in normality by limiting proliferation of malignant precursor cells.

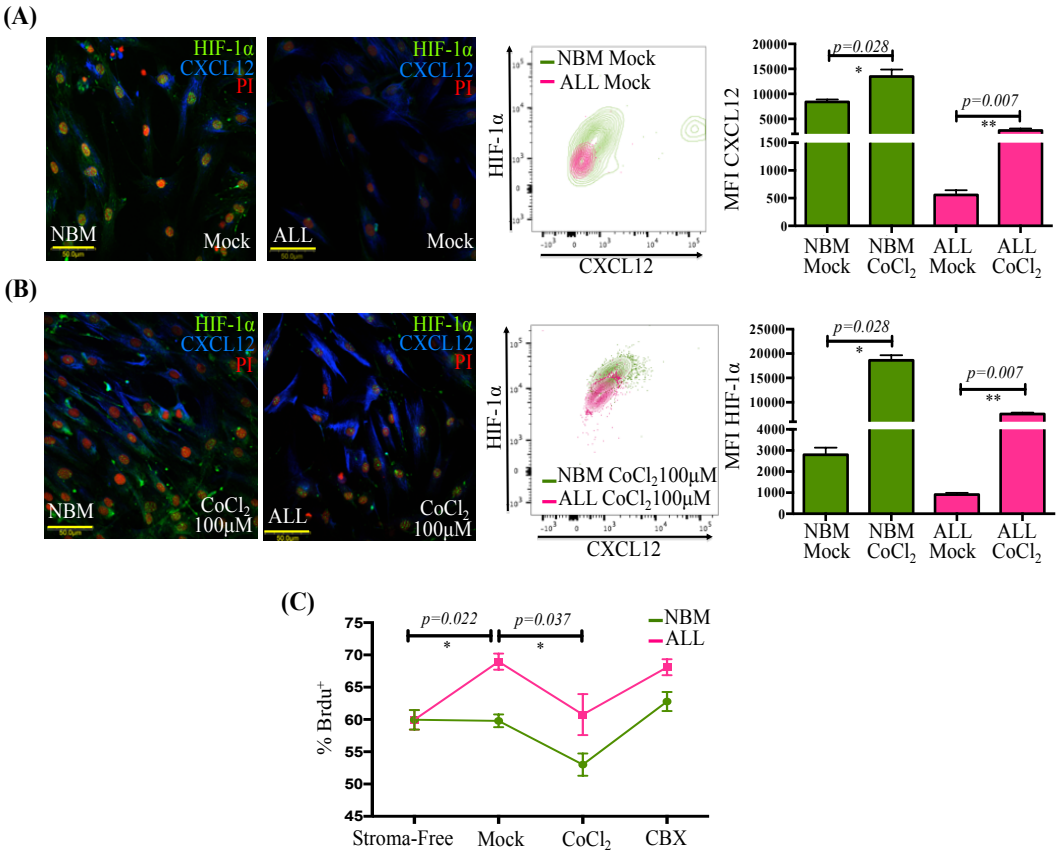


Figure 7. The CXCL12 mesenchymal niche disruption is associated to an abnormal HIF-1α regulation.

HIF-1α and CXCL12 displayed by MSC from normal or ALL BM were evaluated by immunofluorescence microscopy and flow cytometry upon standard cultures (A, left panel) or hypoxic-like conditions promoted by $CoCl_2$ treatment for 2 hours (B, left panel). HIF-1a increase was monitored by flow cytometry (A and B, middle panels). Mean fluorescence intensity values are shown in bar graphs (A, B, right panels). Primary ALL cells were co-cultured with NBM- or ALL-MSC previously treated with $CoCl_2$ or carbenoxolone (CBX) to regulate CXCL12 expression. The culture system was pulsed with bromodeoxyuridine (BrdU) and its incorporation by leukemic cells investigated by flow cytometry (C) (N_{NBM}=4, N_{ALL}=5).

Characterization of three-dimensional (3D) bone marrow mesenchymal stromal spheroids derived from ALL patients.

Because *in vitro* maintenance of primary ALL cells in conventional culture conditions and *in vivo* BM structure is critical to generate specialized hematopoietic niches, we explored the possibility of improving the microenvironmental conditions and increasing the yield per input values by mimicking the structural BM properties through 3D co-cultures in stromal spheroids. Bone marrow MSC from B-ALL patients can form functional spheroids at the first hours of culture in nonadherent conditions, spheroid diameter is proportional to the number of plated MSC (**Figure 8A-B**). MSC phenotype were stable under 3D conditions (**Figure 8C**) but proliferation activity was diminished compared to monolayers (2D) (**Figure 8D**). Interestingly, secretion of CXCL12, SCF and GM-CSF increased when normal and leukemic-derived MSC growth in spheroids (**Figure 8E**).

Of note, the highest levels of CXCL12 and SCF were observed in NBM-MSC. *In situ* analysis of some clue factors described for the lymphoid-mesenchymal niche by fluorescent microscopy and flow cytometry did not suggest strong differences between normal and leukemic spheroids, and gradients of CXCL12 and SCF levels were detected in both settings (**Figure 9E**). 3D BM stromal spheroids resembling normal cellular architecture increasing critical soluble factor for normal and malignant hematopoiesis with stable phenotype and lower proliferation activity.

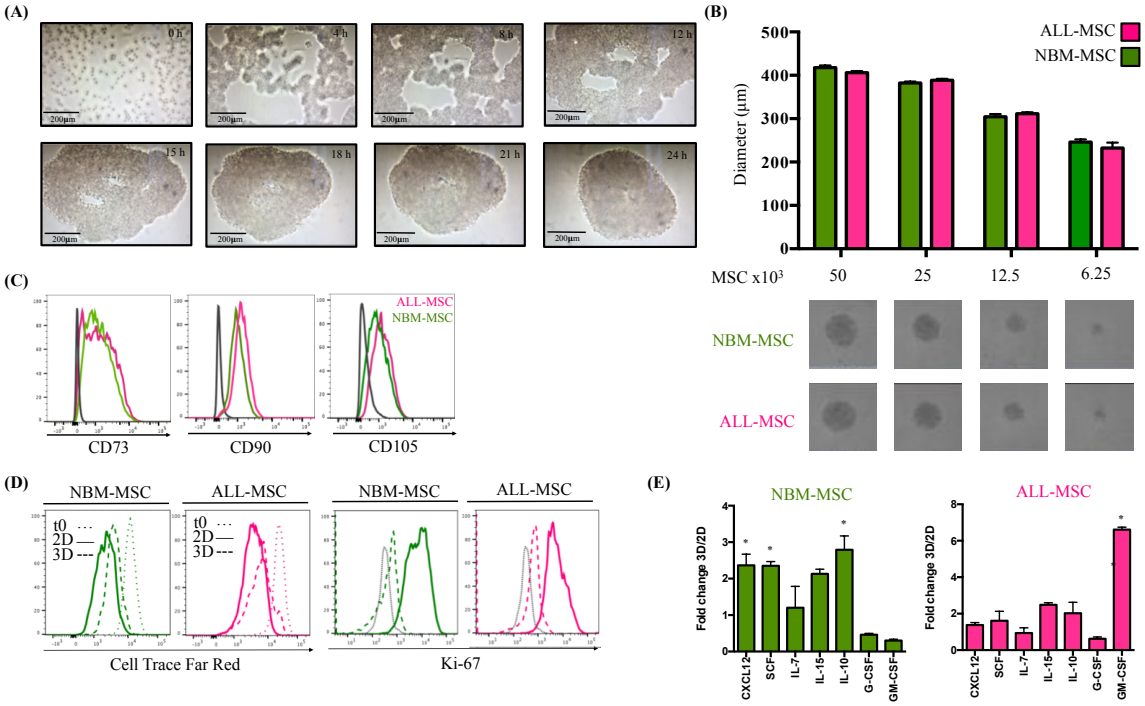


Figure 8. ALL-MSC form GM-CSF highly producing 3D low proliferative spheroids. MSC from NBM and ALL were cultured in non-adherent conditions and 24 hours time-lapse recorded (A). NBM and ALL spheroids were formed with different MSC numbers and their sizes were determined (B). 2D and 3D MSC immunophenotype (C) are shown. Proliferation analyses in 2D and 3D cultures of NBM or ALL MSC by CTFR or Ki-67 methods (D). Soluble factor production was investigated in supernatants from 2D and 3D MSC cultures and fold change 3D/2D is shown (E) (N_{NBM}=5, N_{ALL}=12) ($p < 0.05$). MSC, Mesenchymal Stromal Cell; NBM, Normal Bone Marrow; ALL, Acute Lymphoblastic Leukemia.

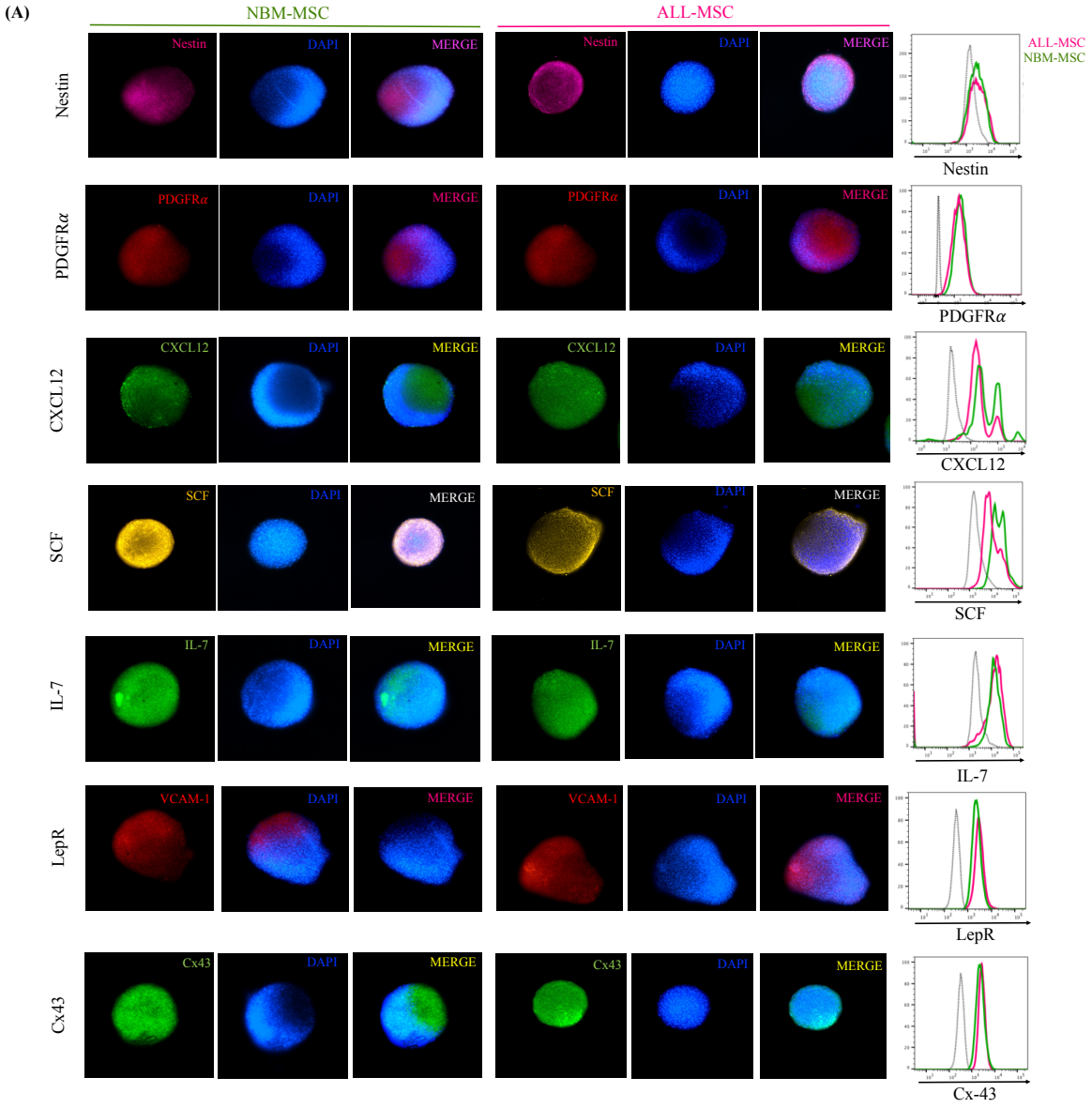


Figure 9. The 3D reticular- like niche in ALL differs from its normal counterpart in the low expression of CXCL12 and SCF.

Mesenchymal cell related expression molecules, including Nestin, PDGFRα and LepR), CAR-cell derived factors (CXCL12, SCF and IL7) and the thight junction protein Cx43 were evaluated by indirect fluorescence microscopy (left panel) and flow cytometry (right panel) to investigate abnormalities in the niche formation in ALL. CAR, CXCL12-abundant reticular.

Bone marrow organoid-like culture increase maintenance and function of primary B-ALL cells.

The lack of appropriate culture systems to allow leukemic cell growth/maintenance has delayed research in leukemia due to the strict microenvironment requirements from primary leukemic cells. Co-cultures with MSC monolayers can slightly increase primary B-ALL viability but cell to cell interactions cannot be totally mimicked[68]. To date, generation of patient-derived xenografts (PDX) is the gold standard to expand primary B-ALL cells but this model is extremely expensive and limited for mechanistic research. To investigate if normal or leukemic spheroids could increase viability and function of primary B-ALL cells, co-cultures with MSC in 2D and 3D controlled systems were conducted, creating a leukemic BM organoid-like model. Remarkably, viability and primitive fraction $CD34^{+}CD19^{+}$ was

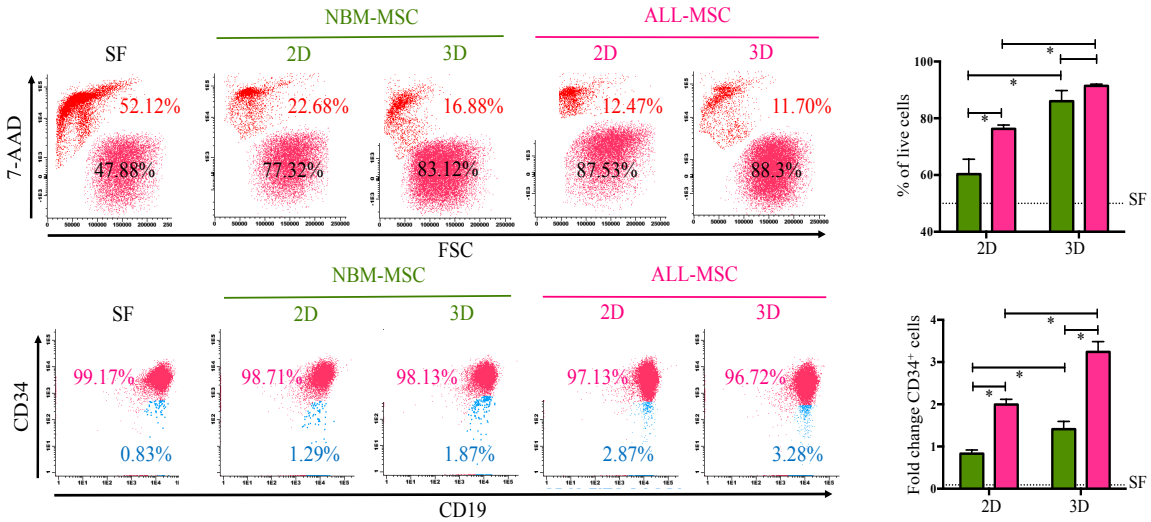


Figure 10. MSC-ALL 3D cultures favor viability and maintenance of primitive ALL $CD34^{+}$ B precursors.

Primitive $CD34^{+}$ B-ALL were co-cultured in stromal free (SF), monolayer (2D) and spheroid (3D) conditions with MSC from NBM and ALL for 7 days, followed by immunophenotyping (upper panel) and viability (lower panel) analyses by multiparametric flow cytometry. Fold changes are shown as bar graphs. (N_{NBM}=5, N_{ALL}=13) ($p < 0.05$).

relative superior compared with stromal-free cultures and better supported when leukemic-derived MSC was used (**Figure 10**).

In addition, yield per input was higher when normal lymphoid differentiation was tested (**Figure 11**). Fascinatingly, leukemogenesis function is also rescued when primary B-ALL was co-cultured with 3D BM structures, of note, leukemic engraftment resulted higher when 3D ALL-MSC was previously used (**Figure 12**).

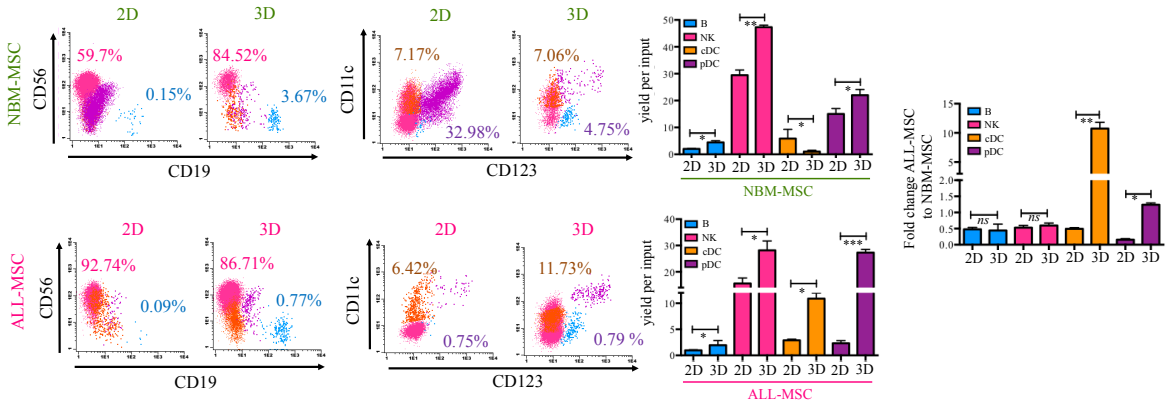


Figure 11. 3D niches in ALL capable of supporting normal lymphopoiesis highly promote cDC and pDC differentiation.

Normal CD34⁺ cells from mobilized peripheral blood were co-cultured in stromal-free (SF) systems, monolayers (2D) and spheroids (3D) in lymphoid conditions (SCF, Flt3-L, IL7 and IL15) with normal (NBM) and leukemic (ALL) MSC. Newly produced B, NK and dendritic cells were determined by flow cytometry. Yield per input are shown in bar graphs. (N_{NBM}=3, N_{ALL}=6) ($p < 0.05$).

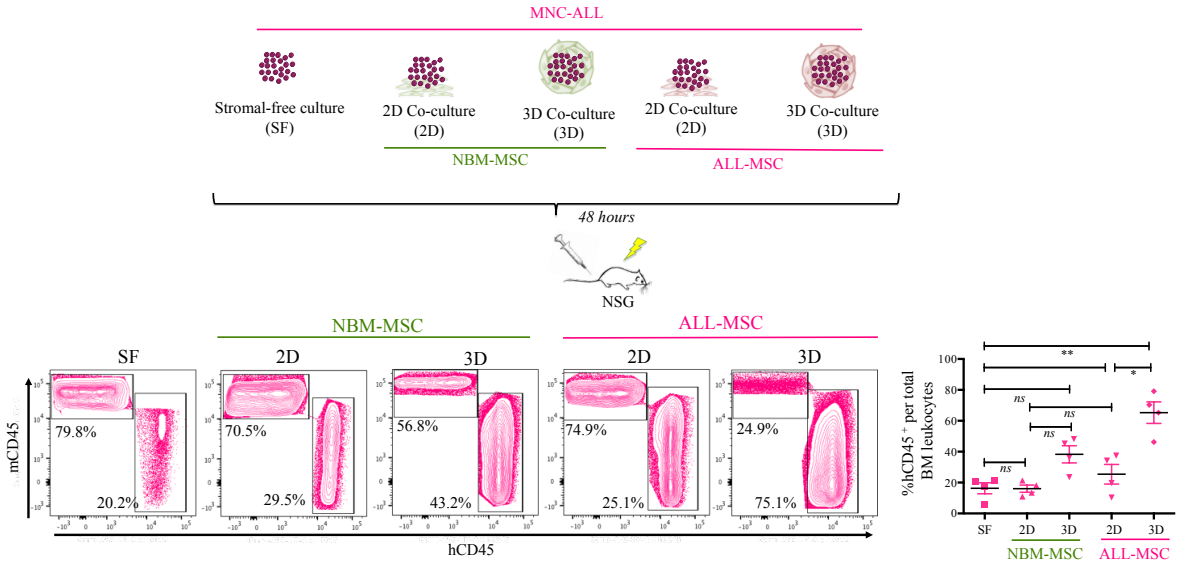


Figure 12. The leukemogenesis potential of B-ALL cells is substantially increased in 3D-tumor microenvironment.
Mononuclear cells from B-ALL patients were co-cultured with normal (NBM) or leukemic (ALL) MSC in stromal-free (SF), monolayer (2D) and spheroid (3D) systems. After 48 hours, cells were harvested and transplanted to NSG mice. Human engraftment was determined by flow cytometry of $hCD45^+$ 6 weeks after transplant in mouse bone marrow (N=4) ($p < 0.05$).

Thus, our data suggest that 3D restructuration allows a better communication between blast and ALL-MSC increasing viability of primary B-ALL cells and its function to initiate leukemia in mice.

Leukemic stemness properties are enriched by BM mesenchymal organoid-like system in B-ALL cells.

LIC cannot be distinguished by phenotypic markers but its existence has been tested by generation of serial PDX[39,42,79,80]. *In vivo*, transplanted LIC should arrive to BM niches by homing mediated mainly by CXCL12/CXCR4 axis. Establishment of LIC in particular BM niches has been reported in mice with high capacity to hijack normal mesenchymal niches and then edited in favor to support tumor growth[15].

3D co-cultures suggest that about 3.6 % of primary B-ALL cells were able of colonizing ALL-MSC spheroids, with no differences in B-ALL colonization after 24h of co-cultures with normal or leukemic BM spheroids (**Figure 13A**). However, when CXCR4 is inhibited by AMD3100 (Plerixafor) B-ALL colonization critically decreases (**Figure 13B**). Interestingly, $CD34^+$ were preferentially recruited by BM spheroids (3D-in) with less proliferative activity compared with the outer cells (3D-out) (**Figure 14A-B**). Cell cycle status analysis suggested a quiescent B-ALL subpopulation and an abundant side population inside BM organoids (**Figure 14C-E**). Recovered 3D-in B-ALL cells from primary organoids re-colonize secondary BM structures with higher efficiency compared with the outer cells (**Figure 14F**). Taking these observations together, a primitive and less proliferative B-ALL cell population colonizes preferentially BM spheroids. Capacity of colonize secondary BM spheroids suggest a LIC activity with stemness properties.

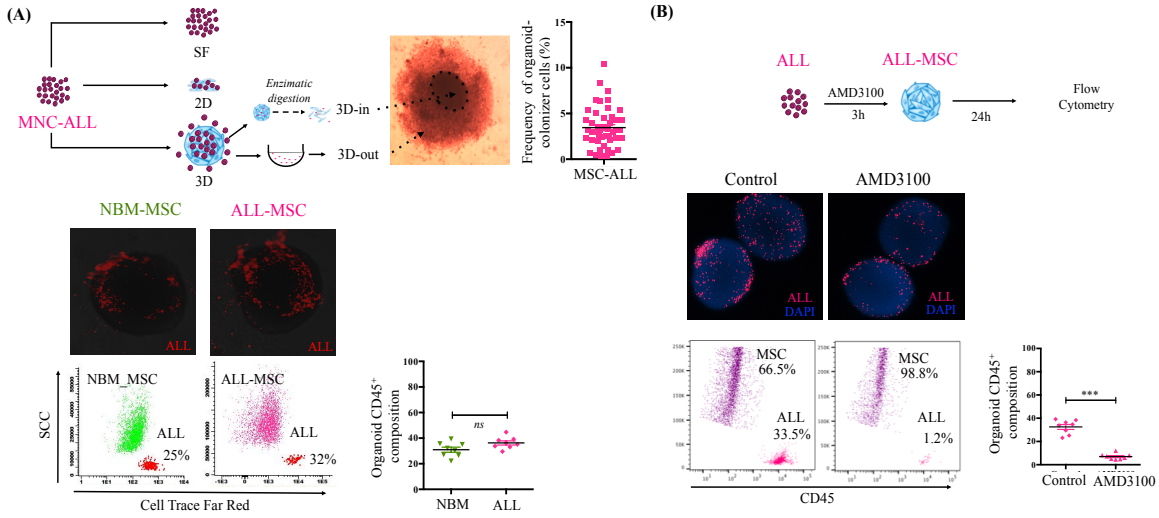


Figure 13. A conspicuous ALL B precursor cell population is able of migrating and colonizing BM organoid-like structures in a CXCR4/CXCL12 axis-dependent manner.

Bone marrow mononuclear cells from ALL patients (MNC-ALL) were labeled with Cell Trace Far Red and co-cultured with normal (NBM) and leukemic (ALL) MSC spheroids for 24h. Frequencies of colonizer cells were determined upon enzymatic digestion of organoid-like 3D structures (upper panel) and CD45$^+$ cells recovered per organoid-like structure were also quantified (lower panel). Primary B-ALL cells were treated with a CXCR4 inhibitor (AMD3100 5mM) during 3 h and then co-cultured with ALL-MSC BM-organoids for 24h and organoid CD45$^+$ composition was determined (B) ($p < 0.05$).

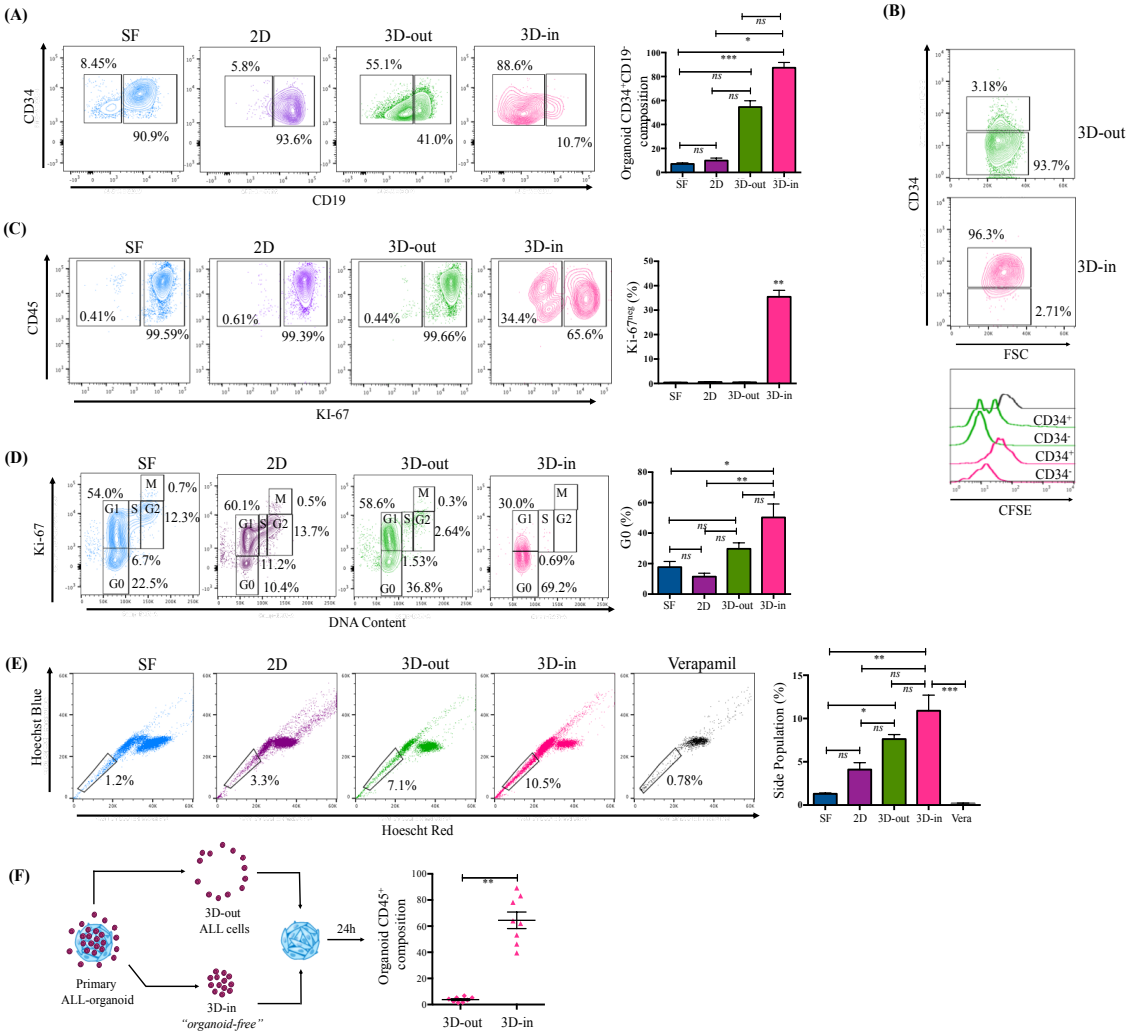


Figure 14. Mesenchymal stromal organoid structures promote stem cell-like phenotype, quiescence and long-term repopulating properties in a subset of ALL cells.

Primary B-precursor ALL cells were co-cultured for 48 hours in stromal-free (SF), monolayers (2D) and MSC spheroids (3D-in and 3D-out) and then harvested for immunophenotype analysis (A). B-ALL proliferative capacity by CFSE dilution (B) or Ki-67 was analyzed (C). Cell cycle status (D) and side population cell contents are shown (E). Secondary spheroids colonization assay was performed with 3D-in and 3D-out purified leukemic cells from primary organoid-like structures (F) ($p < 0.05$).

B-ALL mesenchymal niche is predominantly hypoxic.

Low oxygen tension is a hallmark of BM, normal hematopoietic stem and progenitor cells reside predominantly in hypoxic niches[52,54,81]. Hypoxia cultures of B-ALL cells increased viability concomitant with chemoresistance acquisition features by quiescence promotion[70,82]. Our 3D model mimic those hypoxic MSC niches increasing the hypoxia inducible factor 1-alpha (HIF-1α) and promoting incorporation of pimonidazole only in the inner cell population (**Figure 15A-B**).

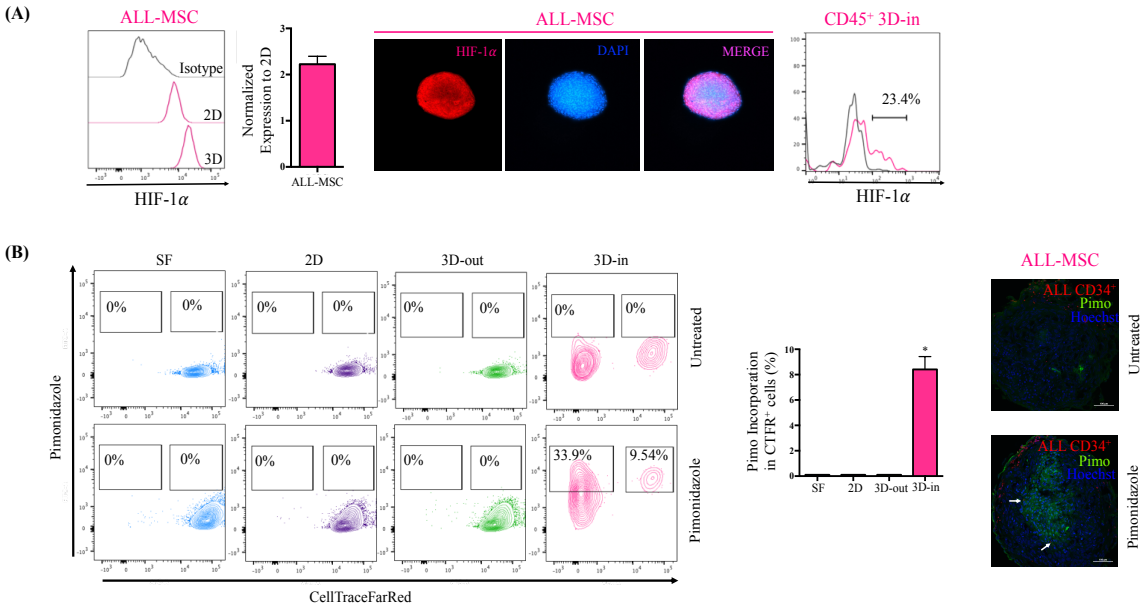


Figure 15. Organoid- like structures from ALL BM create inner hypoxic niches.
HIF-1α expression was evaluated in MSC 3D spheroids by flow cytometry (left panel) and fluorescent microscopy (A, middle panel), as well as in CD45+ intra-organoid cells by flow cytometry (A, right panel). Pimonidazole incorporation (Pimo) was evaluated by flow cytometry in different culture derived cells, including SF, 2D, 3D outer and 3D inner cells (B, left panel). Fluorescent microscopy of leukemic organoid-like structures is shown (B, right panel) ($p < 0.05$).

BM organoid-like model can segregate primary B-ALL LIC population.

To determine LIC activity, same numbers of RS4;11 (B-ALL cell line) after SF, 2D or 3D cultures (3D-in and 3D-out) were injected into NSG mice. Remarkably, leukemia engrafted better when 3D-in cells were transplanted, concomitant to much lower mice overall survival (**Figure 16A**). In parallel, a limiting dilution assay was done by using crescent number of B-ALL cells ($3x10^4$, $3x10^5$ and $3x10^6$). Data suggest that engraftment from 3D-in cells arise same level when 10 times less RS4;11 cells were transplanted (**Figure 16A**). Similar results were obtained when primary B-ALL cells were co-cultured and then transplanted into NSG mice (**Figure 16B**), strongly supporting a functional LIC hierarchy mediated by BM specialized niches.

The $CXCL12^+$ lymphoid niche is remodeled by leukemia precursor cells, favoring tumor progression at expense of normal lymphopoiesis.

Population dynamics was studied by a competence assay between purified $CD34^+$ cell populations from ALL BM or healthy donors mobilized peripheral blood (MPB) that were previously stained with blue and red commercial fluorescent dyes, respectively. Of special interest, ALL-MSC spheroid were less colonized by normal progenitors compared to the NBM-MSC counterparts at the first 24 hours (**Figure 17A**). However, 120 hours later, the normal mesenchymal niche was colonized by leukemic cells simultaneously to an apparent decrease of CXCL12 and displacement of normal progenitors (**Figure 17B-C**).

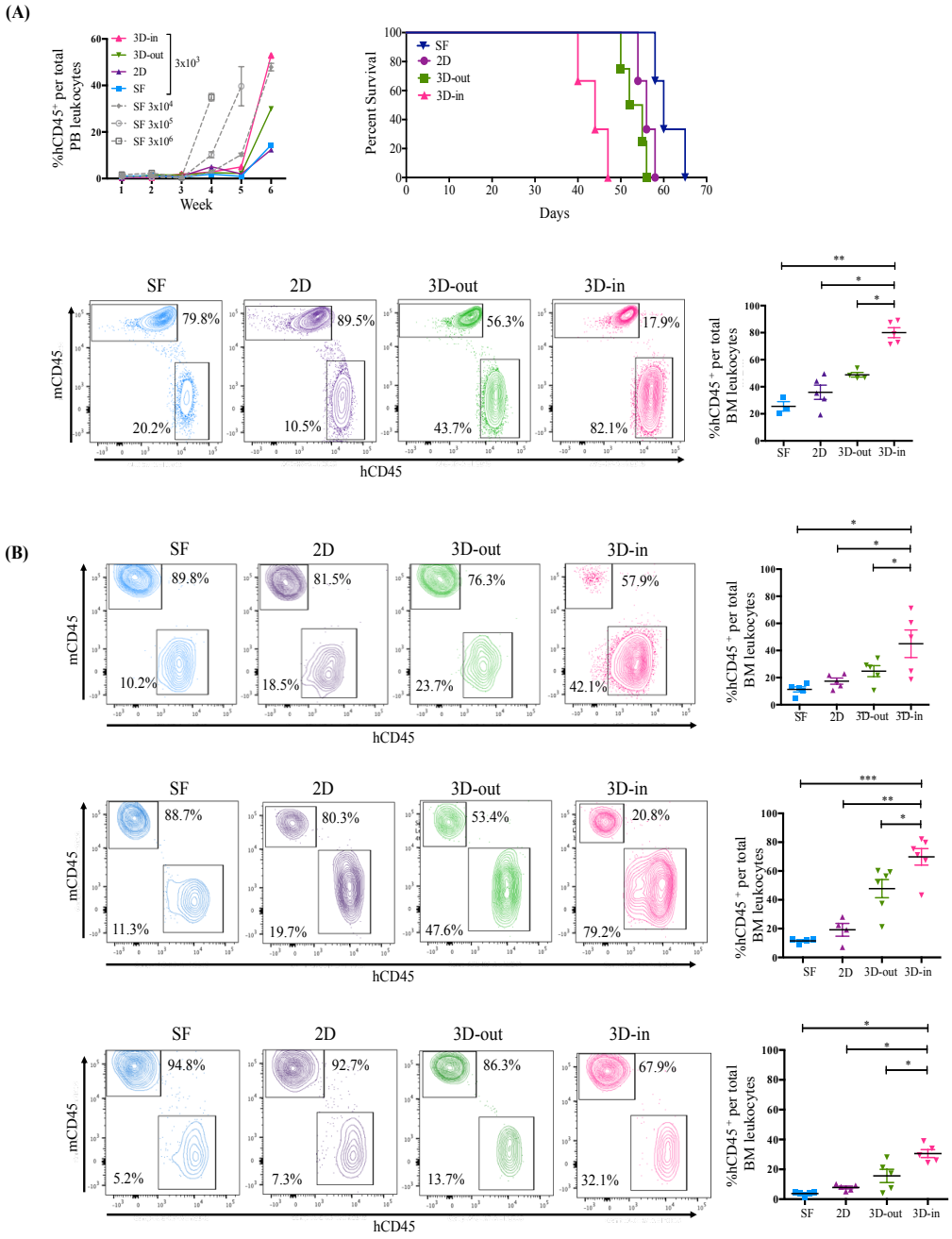


Figure 16. Putative leukemic initiating cells (LIC) are enriched in BM organoid-like inner niches.

RS4;11 leukemic cells (A) or primary B-ALL (B) were cultured during 48h co-cultures on stromal-free (SF) systems, on MSC monolayers (2D) and organoids (3D) and then transplanted into NSG mice in parallel with a limiting dilution assay of stromal-free (SF) cultured cells (grey bars), leukemia was monitored in peripheral blood by flow cytometry and overall survival was plotted (A, upper panel). BM engraftment was determined after BM aspiration after 6 weeks of transplantation (A, lower panel) (N=5) ($p<0.05$).

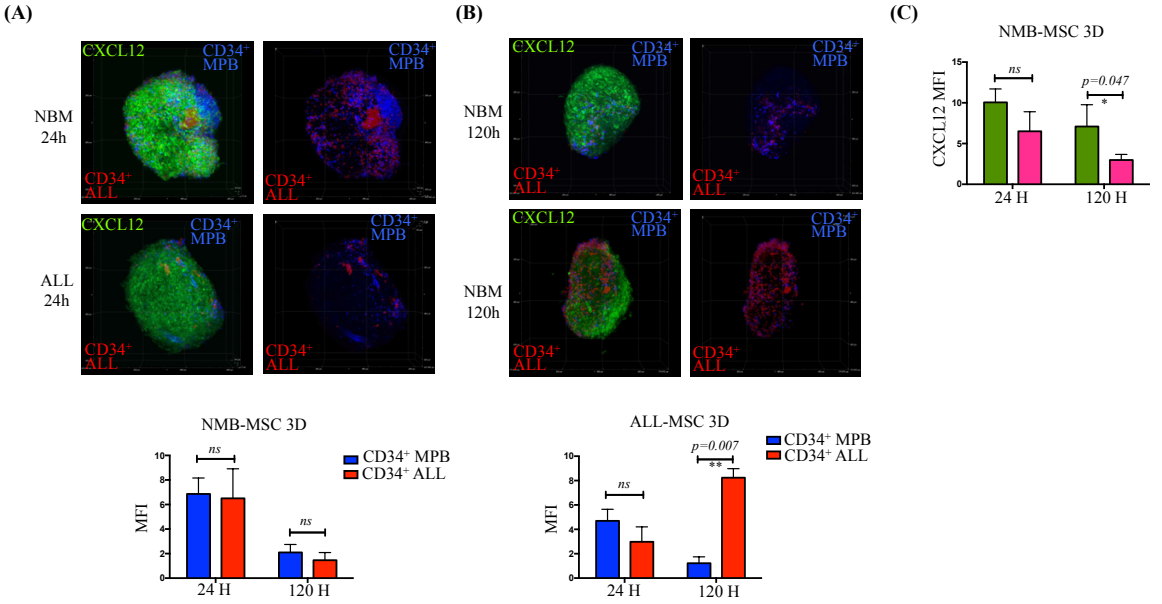


Figure 17. Remodeling of the normal lymphoid niche is associated with leukemic colonization and results in the displacement of normal progenitor cells. CD34+ cells from mobilized peripheral blood (MPB) and CD34+ ALL cells were co-cultured in a competence ratio 1:1 for 24 hours in NBM-MSC and ALL-MSC spheroids (A) or during 120 hours in NBM-MSC spheroids (B). Organoids were washed and CXCL12 expression (green) evaluated (C) ($N_{NBM\text{-}CD34+}$=2, $N_{ALL\text{-}CD34+}$=2, $N_{NBM\text{-}MSC}$=3, $N_{ALL\text{-}MSC}$=4).

LIC are protected by the chemotherapy in BM organoid-like system.

Relapse, treatment failure and organ infiltration have been associated to LIC with special biological attributes and resistance to drug therapy[66]. Microenvironment protection from chemotherapy has been vastly reported by several groups[67]. To investigate whether BM-organoids could protect LIC from standard therapy, we treated leukemic organoids with dexamethasone, prednisolone, vincristine, daunorubicin and its combination (D-P-V-D) in a 1:1:1:1 ratio. As daunorubicin is fluorescent itself,

we demonstrated that B-ALL inner cells uptake drug without compromise its viability (**Figure 18A**).

All drugs tested here resulted to be able to kill B-ALL cells in SF cultures, but can be protected by MSC-ALL. Interestingly, viability on 3D-in cells stayed superior even when high drug concentrations were tested even in combination (**Figure 18B**). Of note, resistant profiles were just observed in some B-ALL patients with poor prognosis or in relapse (**Figure 18C**). To explore if chemoresistance was facilitated by special niches, 3D-in cells were purified and re-treated with drugs. Interestingly, viability was lower but 40% of the cells stayed intact (**Figure 18D**). Novel mobilization strategies are being applied to detach LIC from their niches[37], we tested if a CXCR4 inhibitor (AMD3100, plerixafor) could take B-ALL cells out from the BM organoid-like. Not surprising, not all LIC can be eliminated by using this strategy, indicating that not only CXCR4/CLXC12 is implicated in LIC-MSC communication. Thus, Minimal/Measurable Residual Disease (MRD) were imitated when D-P-V-D treated BM-organoids were cultured again and leukemic cells reemerged (**Figure 18E-F**).

Here, we provided experimental evidence to support a LIC hierarchy not necessary identified by immunophenotype but strongly dependent by mesenchymal niche interaction. In non-pathological conditions, bone marrow microenvironment acts like a leukemic repressor but once tumor is stablished normal stem and progenitors are displaced presumably because B-ALL cells are remodeling MSC creating a pro-inflammatory microenvironment unsuitable for normal lymphopoiesis. LIC are a very small cell population in B-ALL with different metabolic and microenvironment requirements, which seems to be not responsible for BM remodeling but responsible to maintain tumor by promoting MRD after chemotherapy. Mesenchymal leukemic niche could be identified by MSC niche-markers like

Nestin and PDGFR-α, which in normal conditions are able to produce high levels of CXCL12, SCF and IL-7. During B-ALL progression, low production of these factors favor tumor expansion concomitant with a very weak lymphopoiesis support. Importantly, BM organoid-like model can be applied to identify other LIC characteristics or identify patients with a higher probability of recurrence risk (putative MRD^+) with poorest prognosis as well as to test novel therapeutic drugs to target leukemic niche interactions (**Figure 19**).

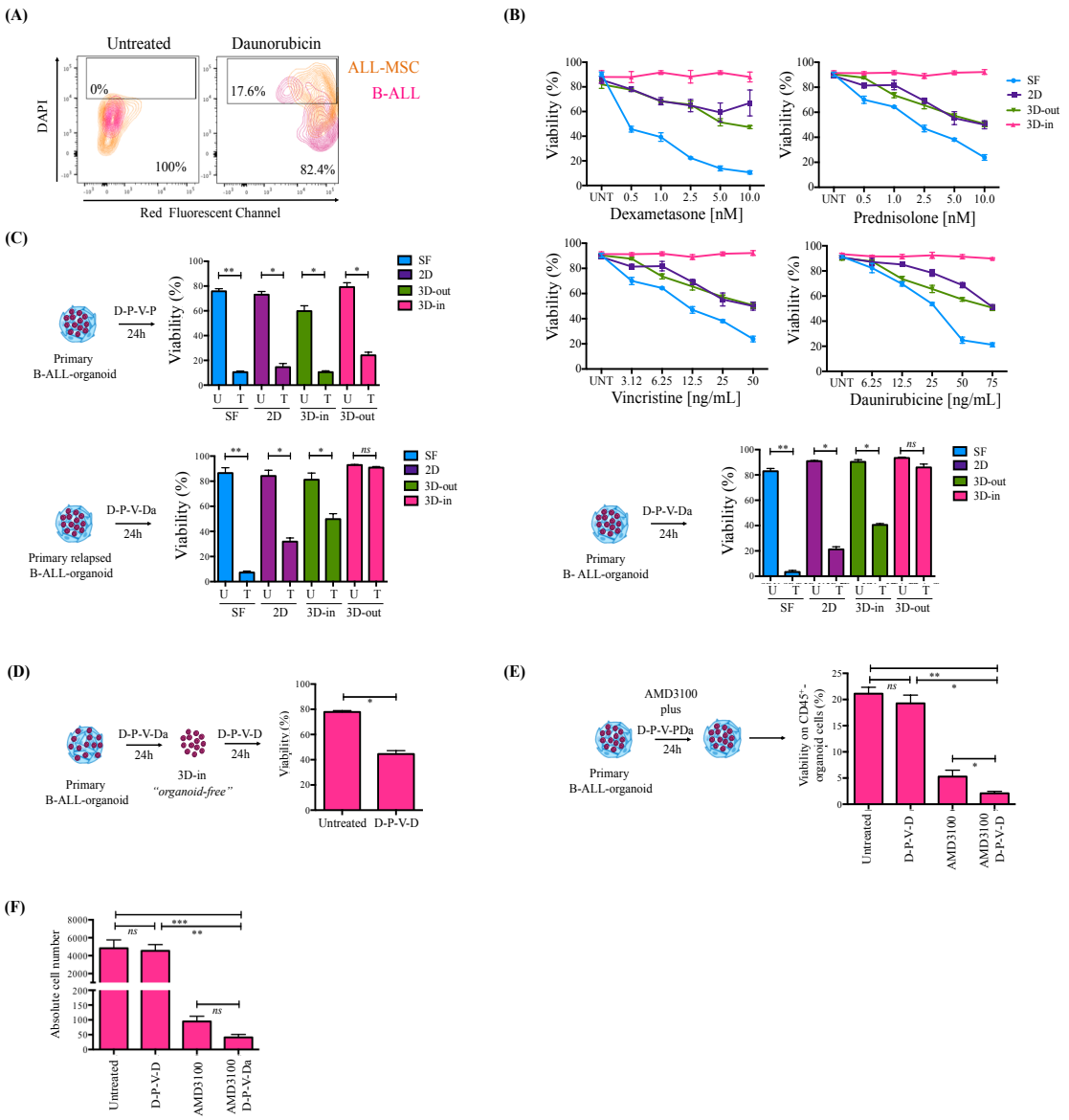


Figure 18. Leukemia-initiating cells from MRD-BM relapse are protected from chemotherapy treatment by the tumor microenviroment.

B-ALL organoid-like structures were incubated with daunorubicin (100 ng/mL) during 2 hours and fluorescence in PE-channel was determined in enzymatically disrupted organoids by flow cytometry (A); primary B-ALL were cultured in different scenarios (SF, 2D and 3D) and viability of $CD45^+$ cells were evaluated after 24 hour treatment with dexamethasone (D), prednisolone (P), vincristine (V) and daunorubicin (Da) or in a 1:1:1:1 drug mixture (lower panel) (B). Newly B-ALL diagnosed (upper panel) and B-ALL relapse (lower) were tested for drug sensibility (C) Surviving cells recovered from the 3D-in condition were re-exposed to drug mixture again and their viability was measured by flow cytometry (D). Primary B-ALL organoid-like structures were co-treated with AMD3100 (plerixafor) and drug mixture during 24 hours and viability was assessed (E) previous replating (F) ($p<0.05$).

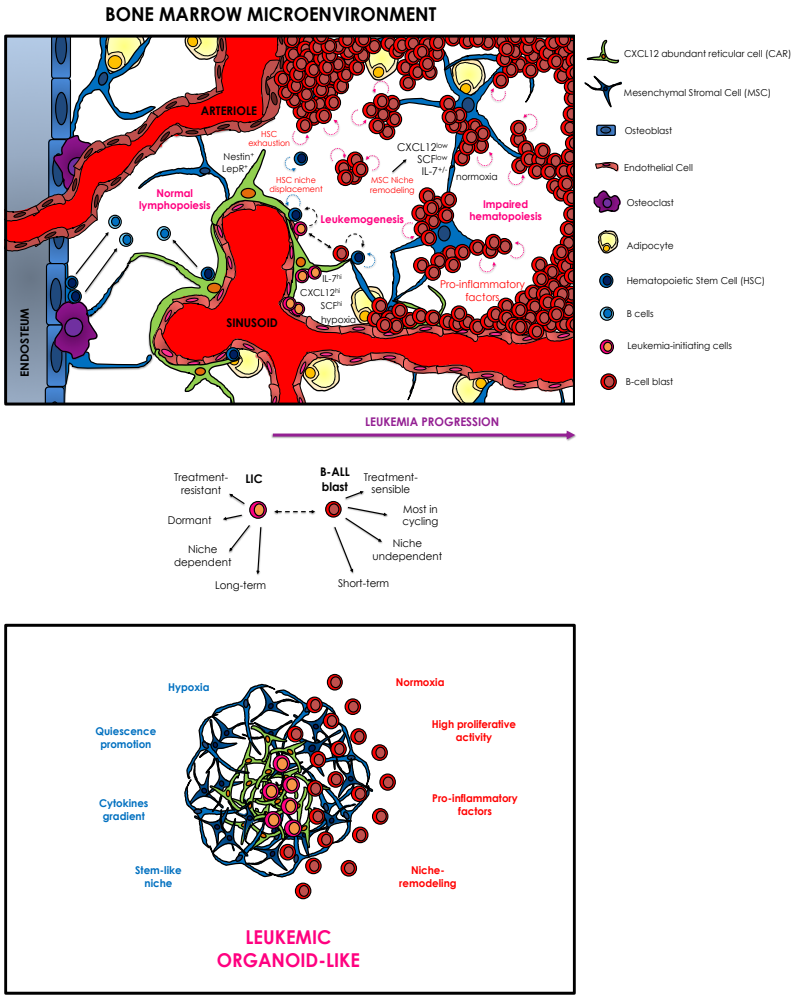


Figure 19. Two BM mesenchymal niches within leukemia units sustain B-cell acute lymphoblastic leukemia progression.

Suitable hematopoietic niches which support B-cell lymphopoiesis are formed by CXCL12-abundant reticular cells (CAR niche) able to produce high levels of SCF and IL-7 that may function by limiting the proliferation of lymphoid progenitors in the absence of pro-inflammatory factors and within a normal hypoxic microenvironment. In leukemogenesis settings, emerging leukemic clones hijack normal niches that are useful for leukemia-initiating cells or tumor cells to establish. As normal hematopoietic stem cell (HSC) get exhausted the niche is progressively induced to remodeling by compromising CXCL12 and SCF expression until a CXCL12/SCF scarce reticular niche is dominant (CSS leukemic niche). In this new sanctuary, unsuitable for normal hematopoiesis. B-precursor ALL blasts proliferate advantageously. In contrast, deeper zones with hypoxic properties resembling normal primitive CAR niches function by protecting leukemia initiating cells (LIC) so they may continuously feed the tumor pool or reemerge as residual disease in a relapse condition. Organoid-like systems mimic better bone marrow microenvironment allowing *ex vivo* leukemogenesis by favoring LIC microenvironmental requirements (lower panel).

DISCUSION

Tumor cells coexist with their normal counterparts in the same microenvironment. During cancer progression, they both contend for the niche and conditioned it for the sake of tumor benefit[83], as has been documented for myeloid leukemias[84] or in murine models[15]. CXCL12 is an essential chemokine showing constitutive expression by bone marrow (BM) cells that directly impacts retention, quiescence and proliferation of hematopoietic stem cells and B-cell progenitors[85].

Accordingly, CXCL12 knockout mouse prenatally die due the lacking of B lymphopoiesis and myelopoiesis in the BM while leukemia-initiating cells express CXCR4[86–88] and respond to CXCL12 by delaying apoptosis processes[89].

The cell-cell intercommunication as key factor for tumor progression has become a research priority. Evidence of leukemic blasts supported by $Nestin^{+}$ MSC indicates their ability of long-term maintenance at the disease onset[68,90]. MSC are part of the complex tissue network of the bone marrow microenvironment[85] and establish an essential niche for hematopoiesis[90]. Here, our data suggest that ALL-MSC are defective in their proliferation and CFU-F capabilities, in concordance to observations in myeloid leukemias[91]. This behavior may relate to the apparent decreased levels of Sox9 that regulates proliferation, survival and chondrogenesis[92]. Moreover, similar defects have been found in myelodysplastic syndrome patients[93,94], in association to chromosomal abnormalities[95]. Evaluating the existence of chromosomal alterations in ALL-MSC should be imperative, as it could compromise their biological functions.

We have previously reported a critical decrease in the content of hematopoietic stem and progenitor cells (HSPC) in BM from ALL patients and a concomitant impaired lymphoid differentiation potential[4,21]. When

hematopoietic support is tested for ALL-MSC, B-cell differentiation is favored, suggesting that this stroma is providing the necessary signals to developing B cells. Although their origins –normal or leukemic- remains elusive, our *in vivo* assays after short-term co-cultures suggest a permissive role for leukemia cells.

One of the biological properties of MSC is their immune-regulatory capability mediated by TLRs and cytokine receptors[96], suggesting a dual role: anti-inflammatory and pro-inflammatory. Typically, when MSC are exposed to pro-inflammatory cytokines, including IFNγ, TNFa, IL-1a and IL-1β, they adopt an immune-suppressive phenotype to limit inflammation previous activation. ALL-MSC expressed high levels of activated NF-κB that may result from a number of mechanisms operating together, e.g., inflammation or a VCAM-1/VLA-4 axis activation signaling[97]. Interestingly, NF-κB activation via TNFa on MSC stimulates its osteogenic differentiation[98], but we did not find an ostegenesis-bias behavior in this study. In contrast to normal MSC that inhibit NK cytotoxic activity, recent data has shown that ALL-MSC are capable of activating NK cells and that this profile can be adopted by NBM-MSC when they are exposed to leukemia cells[99], supporting the notion of local activating factors that promote a feedback loop between MSC and leukemic cells.

Secretion and transcription of CXCL12 by MSC is regulated through gap-junctions mainly formed by connexins where Cx-43 and connexin-45 (Cx-45) orchestrate cellular communication within BM[100]. CXCL12-abundant reticular (CAR) cells in the reticular niche, which express the highest levels of CXCL12, also express the highest levels of Cx-43[100]. Furthermore, adherence properties are dependent on cell interconnections where CXCL12 functions as an indispensable anchor molecule[100]. Regulation of its production in BM samples has been controversial[89,101].

We don't discard the participation of additional regulatory components other than hypoxia-related HIF-1α, based on the partial reestablishment of CXCL12 in hypoxia-like conditions. Taking together, our data suggest that the final defect in the CXCL12 production is modulated at distinct levels in ALL, that may include FGF-2-dependent miR-31 high activity[102].

Other compensatory mechanisms could be operating through untouched subsets of MSC or in the vascular or endosteal niche in order to maintain residual normal hematopoiesis. We confirmed that CXCL12 levels on MSC regulates proliferation of leukemic cells using a co-culture system with perturbed levels of CXCL12 by treatment with $CoCl_2$ or by inhibition of Cx-43[100]. Our results suggest that $CXCL12^{hi}$ niche is acting like a leukemic repressor, contrary to a leukemic niche that favor proliferation by inhibiting the leukemic cell attachment to its stroma and promoting the entrance to cell cycle[103].

So far, our findings concur with observations in animal models of changes in adhesion molecules and production of soluble factors controlled by malignant cells[15,104,105]. Remarkably, the cellular communication between leukemic cells and MSC through exosomes or across nanotubes[66,67], and disruption of the CXCR4/CXCL12 axis[101,106], suggesting that G-CSF has a key role in this phenomena are consistent with our previous reports of production of pro-inflammatory factors by tumor cells[20], related to G-CSF and Gfi-1[19]. Accordingly, G-CSF administration in a pre-clinical model of ALL showed an increased tumor burden[107] and its mechanism may be operating in the niche because B cell malignances rarely expressed G-CSF receptor and are unable to respond *in vitro* to this cytokine (63). In contrast, *in vitro* normal B-cell production in a stromal-free model is more efficient when G-CSF is supplied[108].

Loss of normal hematopoiesis is a classical event during human myeloproliferative neoplasias as well as leukemias where the niche down-regulate essential HSPC retention factors as CXCL12, SCF, LepR, Angpt1, Cdh2, Slit2 and TGF-β1 though pro-inflammatory factors secreted by leukemia cells [104].

Recent research indicates that the CXCR4/CXCL12 pathway is also involved in modulating mitochondrial activity, regulating the production of ATP and reactive oxygen species (ROS) though the precise mechanism has not been elucidated[109] (65). Incubation of leukemic cells with CXCL12 diminishes the mitochondrial activity, coherent with a repressor profile to maintain HSPC population within BM. Nevertheless, the sustained stimulus increases the mitochondrial activity and may be due to the internalization of CXCR4 or the participation of factors as TGF-β1[110,111]. Therefore, the study of primitive populations *in vitro*, and specifically its maintenance in regulatory niches mediated by CXCR4/CXCL12 axis requires a model that better mimics the structural complexity *in vivo*.

A mathematical model approaching has also predicted instability in this axis following TLR ligation and/or inflammation, where an aberrant expression of NF-κB contribute to create a tumor microenvironment that allows effective growing of leukemia cells[22].

We propose the existence of two distinct niches of MSC at ALL diagnosis: one supported by $CXCL12^{hi}SCF^{hi}$ MSC, which better support normal hematopoiesis and is endowed with repressor functions for leukemia, and a second niche composed by remodeled MSC which CXCL12 and SCF production is abated and favors growing of malignant cells. During ALL progression, leukemic cells hijack and remodel normal niches inducing alterations in hematopoiesis at central levels that displace and exhaust primitive cells via inflammation-derived proliferation.

Lack of suitable *in vitro* conditions to maintain primary ALL cells plus the absence of phenotypic markers to distinguish normal and leukemic cells have delayed our understanding of the disease. Currently, the number of reports about the benefits of using 3D cultures is growing and their use to increase the survival of leukemic cells may consummate important questions[112]. Here, this notion is supported by showing a better maintenance of primary ALL cells. Moreover, this novel model may allow the study of human-human and patient-patient microenvironment interactions and further contribute to Precision Medicine advance. The design of strategies that reconstitute the main axes of normal cell-to-cell communication will be of fundamental importance.

Generation of patient-derived xenografts (PDX) is the gold standard to maintain primary human leukemic cells suggesting a strict microenvironmental cues that conventional co-cultures with BM stromal cells did not achieve[39,41,42]. BM stromal spheroids have been used to study migration between niches and expansion for normal hematopoietic progenitors[50,112–114] but never explored in B-ALL. Even the biological changes on MSC-ALL described already, primary MSC derived from patients can form functional multicellular spheroids with stable immunophenotype. As proliferation is inhibited by cell-contact, spheroids reduce their proliferative activity. Increasing the cell-to-cell contact can boost the capability to release soluble factors as well as extra-cellular vesicles (ECV)[67,112,114,115].

We provide experimental evidence that critical soluble factors for early lymphopoiesis are enhanced by 3D organization, of note supernatants obtained from ALL-MSC release higher levels of GM-SCF creating a loop for CXCL12 downregulation in BM.

Re-organization of MSC on 3D structures change cell architecture reducing their cell size and complexity, the high cell connectivity concomitant with hypoxic signals are inducing upregulation of some

molecules, including CXCL12 and SCF[78,100]. Primary MSC derived by their adherent properties contain a very heterogeneous population, BM perivascular cells include some Nestin$^+$ cells which are the major osteoblast progenitors while CXCL12-abundant reticular (CAR) cells (which overlap with the leptin receptor$^+$ (LepR$^+$) population) have been shown to be an important adipocyte source.

Normal B-lymphopoiesis is inhibited by adipocytes[116]. During pediatric leukemogenesis MSC *in vitro* can differentiate to adipocyte easier so investigation of the adipocyte progenitor contents in ALL BM should be imperative to propose novel strategies to restore the adipogenic balance.

When leukemogenic support was tested on MSC spheroids, ProB-ALL blast viability were higher when MSC source were ALL. In contrast with the 2D co-cultures, normal CD34$^+$ progenitor cells can differentiate into NK, B and dendritic cells suggesting that normal niches are also functional during 3D reconstitution during ALL settings.

We have developed an innovative culture tool to study leukemia-initiating cells (LIC) by forming BM mesenchymal organoid-like structures which mimic better the *in vivo* biological properties. Hypoxia has been vastly documented to control biology of normal hematopoietic stem cells as well as in several tumors[52,70]. In fact, normal stem cell niche is hypoxic[54]. During B-ALL, low oxygen tension reduce proliferation on leukemic cells and increase drug resistance[70]. For this reason, novel therapy strategies with hypoxia-activated drug have started to be applied to target BM hypoxia[55].

We also recently suggested that LIC BM occupation may be operated by cortactin, an actin binding protein which play an important role in cell migration (Velázquez-Avila, in revision). Our data revealed high levels of cortactin in B-cell lines and primary lymphoid precursors from BM and cerebrospinal fluid (CSF) of B-ALL patients when compared with normal B cells. Interestingly, downregulation of cortactin diminish BM colonization and

extra-medullar infiltration. Thus, cortactin inhibition can impair BM relapse and may prevent secondary organs from leukemia infiltration.

Chemoresistance and Minimal/Measurable Residual Disease (MRD) are the worse early events after leukemia treatment. B-ALL organoid-like structures can be treated with drugs resembling bioavailability and diffusion trough tissue. LIC have been hypothesized to hide in protecting niche sanctuaries during chemotherapy. Interestingly, when relapse primary B-ALL cells were harvested from organoid-like structures resulted be resistant to the standard care of therapy and were able to reemerge when were plated again. Mobilization strategies have been also applied in leukemia to disrupt niche interactions and induce cell cycle on LIC and then to become more therapy-sensible. Our observations demonstrated that LIC can be mobilized out from organoid-like structures but did not result as successful as the described in patients[117,118].

Experimental evidence confirmed the existence of a population of leukemic cells with stemness properties with irrelevant immunophenotype, able to initiate leukemia in immunodeficient mice and probably for be the responsible for MRD before relapse. LIC hijack HSC from CAR niches where LIC is stablished, while pro-inflammatory factors remodel other MSC niches downregulating CXCL12 and SCF concomitant with a HSC exhaustion by cell displacement and proliferation. This novel microenvironment $CXCL12^{low}$ allowing blast proliferation while CAR niches promote quiescence in LIC.

Implementation of our model may predict LIC content in patients as an original prognosis tool reducing the use of laboratory animals, as well as have a personalized platform to test current and novel therapeutics drugs and prognosticate clinical responses including immunotherapy (Ramirez-Ramirez D, work in progress). More importantly, mRNA-seq of LIC should be imperative to find novel therapeutic targets to eliminated LIC in B-ALL.

CONCLUSION

Mesenchymal stromal $Nestin^{+}LepR^{+}$ bone marrow organoid-like structures can maintain leukemia-initiating cells (LIC) from B-cell acute lymphoblastic leukemia (ALL) by mimicking their microenvironmental requirements. Taken together our findings strongly suggest that this approach will be relevant to the understanding of the pathobiology of the disease and further characterization of LIC as well as for the study of interactions of the B-ALL cells and their tumor microenvironment without affect normal hematopoiesis. We show evidence of functional leukemia initiating induced or selected by the niche, and become endowed with stem cell properties.

FUTURE WORK AND PERSPECTIVES

Niche-selected leukemia-initiating cells by using our organoid-like model will be purified to obtain the transcriptome to propose novel therapeutic targets focused on particular adhesion molecular by comparing mesenchymal LIC-interactions with normal interactions with normal hematopoietic stem cells (HSC).

Currently, our 3D model is now in an experimental phase to try to predict clinical responses to the current standard care of therapy for childhood and adult acute leukemias in a personalized high throughput system (HTS).

Finally, we hypothesize that BM 3D structures will be predict homing of normal hematopoietic stem and progenitor cells for those to require bone marrow transplantation.

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ANEXOS

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