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Review

Advances in understanding the acute lymphoblastic leukemia bone marrow microenvironment: From biology to therapeutic targeting☆

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ABSTRACT

The bone marrow (BM) microenvironment regulates the properties of healthy hematopoietic stem cells (HSCs) localized in specific niches. Two distinct microenvironmental niches have been identified in the BM, the "osteoblastic (endosteal)" and "vascular" niches. Nevertheless, these niches provide sanctuaries where subsets of leukemic cells escape chemotherapy-induced death and acquire a drug-resistant phenotype. Moreover, it is emerging that leukemia cells are able to remodel the BM niches into malignant niches which better support neoplastic cell survival and proliferation. This review focuses on the cellular and molecular biology of microenvironment/leukemia interactions in acute lymphoblastic leukemia (ALL) of both B- and T-cell lineage. We shall also highlight the emerging role of exosomes/microvesicles as efficient messengers for cell-to-cell communication in leukemia settings. Studies on the interactions between the BM microenvironment and ALL cells have led to the discovery of potential therapeutic targets which include cytokines/chemokines and their receptors, adhesion molecules, signal transduction pathways, and hypoxia-related proteins. The complex interplays between leukemic cells and BM microenvironment components provide a rationale for innovative, molecularly targeted therapies, designed to improve ALL patient outcome. A better understanding of the contribution of the BM microenvironment to the process of leukemogenesis and leukemia persistence after initial remission, may provide new targets that will allow destruction of leukemia cells without adversely affecting healthy HSCs. This article is part of a Special Issue entitled: Tumor Microenvironment Regulation of Cancer Cell Survival, Metastasis,Inflammation, and Immune Surveillance edited by Peter Ruvolo and Gregg L. Semenza.

1. Introduction

Acute lymphoblastic leukemia (ALL) comprises highly 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. B-ALL can occur at any age, however it mainly affects children where it is by far the most common malignancy, 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 lower, about 1 case per 100,000. While the outcome for pediatric B-ALL patients has dramatically improved over the last two decades with survival rates of over 80% at 5 years, the prognosis of adult B-ALL patients is much more severe [1,2]. This is at least partly due to the fact that 25–30% of B-ALL adult patients display the BCR-ABL1 fusion protein, which portends a poorer prognosis, while only 2–10% of pediatric B-ALL patients display such protein [3]. However, less than 50% of BCR-ABL1$^{(-)}$ adult patients maintain their remission at 5 years [2].

T-ALL accounts for 10–15% and 25% of pediatric and adult ALL cases, respectively. Thanks to the current intensified multi-agent chemotherapy protocols, the 5-year event-free survival (EFS) of children with T-ALL is 70–75%, whereas the EFS is 30–40% for adults below 60 years of age, and only 10% above this age [4]. However, the prognosis of T-ALL patients with primary chemoresistant or relapsed leukemia is still very poor [1].

Chemoresistance and relapse of acute leukemias are believed to be driven by a pool of rare leukemia initiating cells (LICs) (Table 1). LICs share several properties with healthy hematopoietic stem cells (HSCs)

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Table 1
Definition and main characteristics of HSCs, progenitor cells, MSCs, and LICs.

Abbreviation	Cell lineage name	Definition and main characteristics
HSCs	Hematopoietic stem cells	HSCs are cells within the hematopoietic system that possess the potential for both multi-potency and self-renewal, and are characterized by infrequently cycling under homeostatic conditions. Because normal hematopoiesis is organized as a hierarchy, HSCs are at the apex and continuously provide differentiated blood cells. In addition, HSCs self-renewal activity maintains the HSC pool size throughout life [15].
LT-HSCs	Long-term HSC	These cells belong to the HSC compartment and are defined as $CD34^-FLT3^-Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells. LT-HSCs are rare, relatively dormant cells which possess self-renewal potential and are able to provide long-term hematopoietic reconstitution (>4 months) after transplantation. During hematopoietic differentiation, LT-HSCs give rise to short-term HSCs (ST-HSCs, $Lin^-CD34^+Sca\text{-}1^+c\text{-}Kit^+$) which loose self-renewal potential and are able to reconstitute early (2 weeks) after transplantation [16] [17].
Progenitor cells	-	In the hierarchical structure of hematopoiesis, progenitors are classified as cells in which multi-potency is progressively restricted. Progenitors are further classified into highly proliferative multipotent progenitors (MPPs, $Lin^-CD34^+CD38^-CD90^-CD45RA^-$) which no longer possess self-renewal ability yet keeping full-lineage differentiation potential; oligopotent progenitors [common lymphoid progenitor (CLP) and common myeloid progenitor (CMP)]; lineage restricted progenitors (e.g., granulocyte-macrophage and megakaryocyte-erythrocyte progenitors) [15].
MSCs	Mesenchymal stromal cells	These are multipotent cells characterized by the expression of CD105, CD73 and CD90, whereas they lack the expression of CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR. MSCs support hematopoiesis and can differentiate *in vitro* along mesenchymal (adipocytic, osteoblastic and chondrocytic) and non-mesenchymal (neuronal, hepatocyte-like, and cardiac-like) lineages. MSCs can be isolated from different tissues. Bone marrow or human bone marrow MSCs are indicated as BM-MSCs and hBM-MSCs, respectively [18].
LICs	Leukemia-initiating cells	Within the hematopoietic compartment, LICs are the cells susceptible to leukemic transformation which give rise to more differentiated blasts. LICs were first identified in acute myeloid leukemia (AML) within the $CD34^+CD38^-$ compartment, and were capable of initiating human AML in NOD/SCID mice [19].

which are the only cells capable of producing all blood cell lineages throughout life [5]. Indeed, LICs are mostly dormant and therefore they escape chemotherapeutic drugs that preferentially target cycling cells [6]. Moreover, LICs are able to self-renew and generate more LICs and have the capacity to differentiate into a progeny with a limited self-renewal potential [6,7].

In 1978, Schofield proposed that healthy HSCs reside in bone marrow (BM) "niches", where they are protected from environmental stresses and receive adequate support for maintaining self-renewal and multi-lineage differentiation capacity [8]. Niches are specialized BM areas consisting of different cell types that control the number, quiescence, self-renewal, proliferation, differentiation, and localization of HSCs. At least two types of HSC have been identified, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) [8]. LT-HSCs can provide long-term (>4 months) hematopoietic reconstitution in the recipient and display a higher self-replicating activity [9] (Table 1).

Two distinct BM niches were identified, referred to as the "osteoblastic (endosteal)" and the "vascular" niches [9,10]. The osteoblastic niche, which is localized at the endosteum, comprises osteoblasts, osteoclasts, glial non-myelinating Schwann cells, and regulatory T-cells (T-regs), while the vascular niche, localized at the sinusoidal walls, is composed of C-X-C motif chemokine (CXCL) 12-abundant reticular cells (CAR), endothelial cells, nestin-positive (NES^+) mesenchymal stromal cells (MSCs) (Table 1), and leptin receptor positive [$LepR^{(+)}$] perivascular stromal cells [11] (Fig. 1). These cells regulate HSC properties though a myriad of different signals, including cytokines, chemokines, and adhesion molecules.

Like healthy HSCs, LICs remain at least partly dependent on signals originating from the hematopoiesis-regulating BM microenvironment for their survival and proliferation [12]. Nevertheless, LICs can outcompete HSCs, thus hijacking the BM microenvironmental niches [13]. In other words, leukemic cells are thought to disrupt the healthy BM niches and to create "leukemic" niches. Indeed, leukemic cells, by releasing exosomes/microvesicles, induce changes in the BM microenvironment, that is reprogrammed to support leukemic persistence [14]. The BM leukemic niches act as "sanctuaries" where LICs evade chemotherapy-induced death and acquire drug-resistance, as well as metastatic potential [11].

Last but not the least, it is becoming apparent that also the microenvironment of other tissues/organs that are infiltrated by leukemic cells, plays a critical role in the evolution of the disease process [20–22].

The key roles played by the BM microenvironment in sustaining ALL is documented by the seminal observation that both in B- and T-ALL patients, a higher lymphoblast recovery after culturing leukemic cells with BM-derived stromal cells, was associated with a poorer outcome [23,24].

In this review, we shall describe the main signals, emanating from the BM microenvironment of both $BCR\text{-}ABL1^{(-)}$ B-ALL and T-ALL, that are involved in leukemia support. We will also highlight the potential role played by exosomes/microvesicles released by leukemic cells in influencing the biology of microenvironmental cells. A better understanding of the contribution of microenvironmental signals to the process of leukemogenesis and leukemia relapse/persistence after initial treatment, may provide new therapeutic targets that should allow eradication of LICs without adversely affecting HSCs.

1.1. Osteoblastic niche

Osteoblasts are bone-forming cells that line the endosteum and act in concert with osteoclasts, the bone-reabsorbing cells, to maintain bone homeostasis [25]. Osteoblasts are critical components of the osteoblastic niche, as demonstrated by several studies that highlighted their roles in HSC maintenance. Human osteoblasts were able to sustain and expand *in vitro* HSCs through osteoblast-derived growth factors, e.g. granulocyte colony-stimulating factor (G-CSF) [26,27], whereas the absence of osteoblasts resulted in loss of HSCs [28]. Subsequent *in vivo* studies in a murine model correlated the increase in osteoblast number with that of HSCs, demonstrating the osteoblast roles in regulating niche size and HSCs number. The importance of osteoblasts in controlling the HSC number was also substantiated by the finding that bone morphogenetic protein (BMP) signaling through the BMP receptor type IA (BMPRIA) increased the number of spindle-shaped $N\text{-}cadherin^+$ $CD45^-$ osteoblastic cells that correlated with an increase in the number of HSCs, presumably through the interactions between N-cadherin on osteoblasts and β-catenin on HSCs [29]. Similarly, the osteoblast number increase induced by the overexpression of parathyroid hormone (PTH), resulted in high levels of the Notch ligand, Jagged-1, and a parallel increase in HSC number with evidence of Notch1 activation [30]. Moreover, osteoblasts induced a quiescent status in HSCs that was mediated by the interactions between Angiopoietin-1 (Ang-1) produced by osteoblasts and its receptor, the Tie2 tyrosine kinase, expressed by HSCs [31]. Osteoblasts could also negatively regulate HSCs proliferation through the expression of osteopontin [32,33]. All of these findings underscored the essential roles played by osteoblasts in HSC physiology (Fig. 1). However, recent evidence failed to demonstrate that osteoblasts directly supported HSCs, suggesting that these bone-forming cells may promote HSC niche maintenance through other BM cells that co-localize with bony surfaces [10].

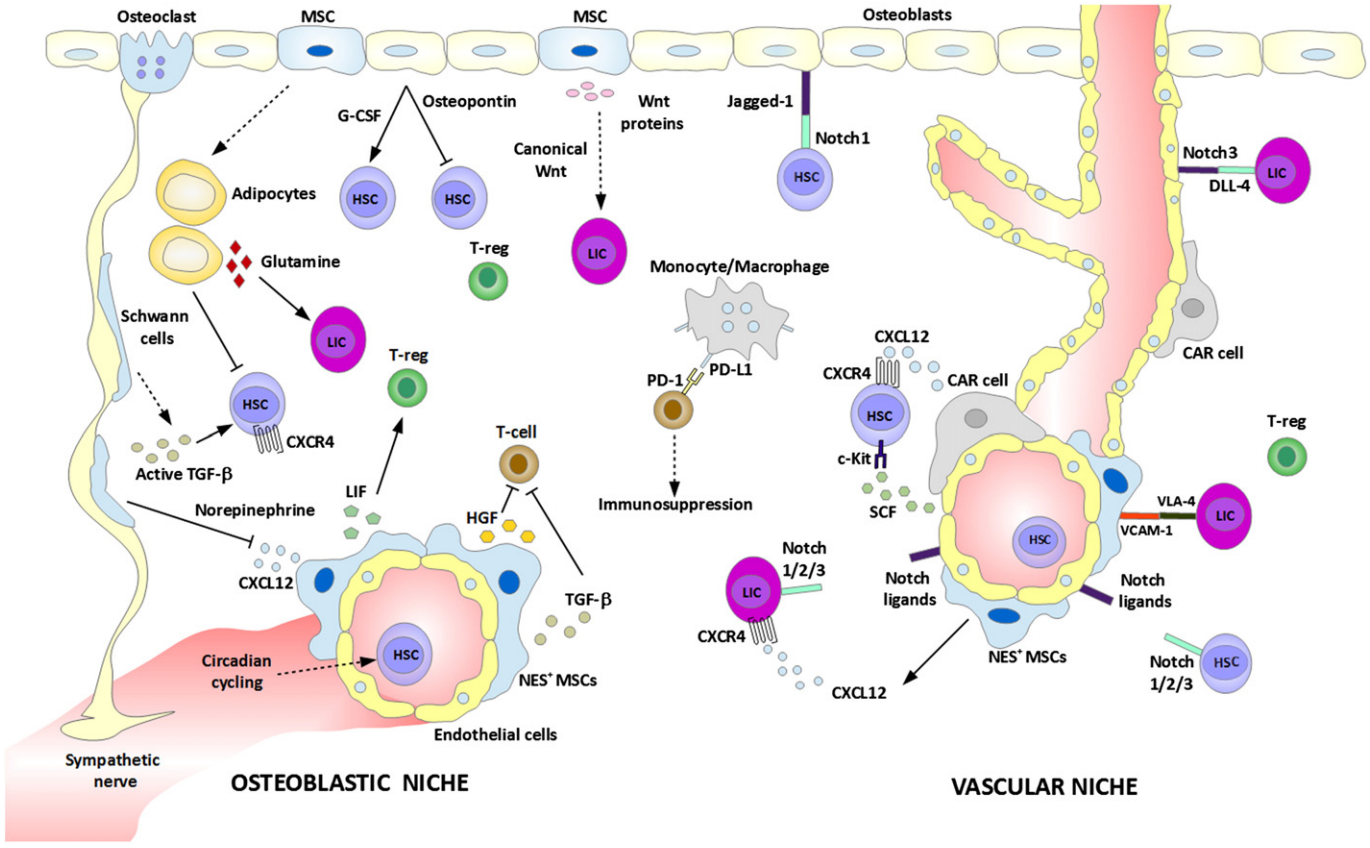

Fig. 1. Mechanisms involved in HSCs and LICs maintenance in osteoblastic and vascular niches. Osteoblastic niche is mainly involved in maintaining hematopoietic stem cell (HSC) quiescence, while vascular niche presumably promotes proliferation, differentiation, and mobilization of HSCs. Osteoblasts are able to both sustain and negatively regulate HSC proliferation, through granulocyte colony-stimulating factor (G-CSF), Jagged-1/Notch1, and osteopontin signaling. HSC dormant state is regulated through transforming growth factor-β (TGF-β) signaling, whose activation is regulated by non-myelinating Schwann cells. The sympathetic nervous system also regulates HSC bloodstream release by means of circadian norepinephrine secretion that down-regulates chemokine C-X-C motif ligand 12 (CXCL12) expression in nestin^{+} mesenchymal cells (NES^{+} MSCs). Adipocytes decrease the frequency of quiescent HSCs, whereas they may also sustain leukemic initiating cells (LICs), for instance through glutamine secretion. Due to the fact that niches are an immune suppressive environment, immune system T-regulatory (T-reg) cells participate in creating a localized zone protected from immune attacks. Leukemia inhibitory factor (LIF) could promote T-reg cell expansion, while both hepatocyte growth factor (HGF) and TGF-β display an inhibitory activity on T-cells. Also programmed death-1 (PD-1)/PD-L1 signaling has immunosuppressive effects on T-cells. Endothelial cells express Notch ligands and stem cell factor (SCF), the ligand of c-Kit, and sustain HSC self-renewal and proliferation. High levels of Notch ligand Delta-like 4 (DLL-4) are expressed in endothelial cells and specifically activate Notch3 signaling in LICs, thus promoting tumor escape from dormancy. CXCL12/C-X-C Receptor 4 (CXCR4) signaling is essential for both HSC homing and maintenance. CXCL12 is secreted by several stromal cell types, especially CAR cells (CXCL12-abundant reticular cells). The canonical WNT pathway is involved in the protective effects toward leukemic cells: Exogenous WNT proteins provided by MSCs are involved in WNT signaling activation in LICs, thus sustaining their proliferation and cell cycle progression. Adhesion molecules play also a prominent role in BM homeostasis, providing signals for quiescence and proliferation of HSCs/LICs. For example, very late antigen-4 (VLA-4) expressed by HSCs/LICs interacts with vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, retaining these cells into BM niches.

Osteoclasts are specialized bone-reabsorbing cells derived from the monocyte/macrophage lineage that participate to the normal bone remodeling [34]. There is evidence that osteoclast activity could also influence hematopoiesis. Osteoclasts may be involved in the formation/maintenance of the cavities that constitute the endosteal niche through the receptor activator of nuclear factor κ-B ligand (RANKL) [35], and this event is likely related to the sinusoidal microcirculation establishment. On the other hand, absence of osteoclast activity in an osteopetrosis mouse model, severely affected HSC niche formation [36]. Also treatment with bisphosphonates (a class of drugs that prevent loss of bone mass by negatively affecting both osteoclastogenesis and osteoclast survival and that are widely used for treating osteoporosis and bone metastasis [37]), decreased the HSC number [38]. Moreover, RANKL-stimulated osteoclasts induced HSCs mobilization by reducing the levels of CXCL12 (also referred to as stromal-derived factor 1 α or SDF1α), stem cell factor (SCF), and osteopontin, which all have crucial roles in HSC anchorage, survival, and quiescence [39]. Finally, osteoclast activity seems to be crucial for HSC localization to the osteoblastic niche. Indeed, it has been shown that osteoclasts released Ca^{2+} from the bone and the Ca^{2+} content of the osteoblastic niche dictated the preferential localization of HSCs. This mechanism relied on the seven-transmembrane-spanning Ca^{2+}-sensing receptor (CaR), as antenatal mice deficient in CaR displayed HSCs in the circulation and spleen, whereas few were found in BM [40]. However, recent findings suggested that osteoclasts could be dispensable for HSC maintenance and may function as negative regulators in the hematopoietic system, as osteoclast absence in mice models did not negatively affect HSC mobilization, but even increased it [41].

Transforming growth factor-β (TGF-β)/Smad signaling is involved in maintenance of the dormant state in HSCs [42]. TGF-β is produced as a latent form by a variety of cells, including HSCs, which, however, cannot activate it by themselves. It has been recently shown that non-myelinating Schwann cells expressed TGF-β activator molecules [43]. These glial cells, which are wrapped around sympathetic nerves in the BM, are in direct contact with a significant percentage of HSCs, and their contribution to the regulation of hematopoiesis has been demonstrated by the drastic reduction of both cells expressing active TGF-β and HSCs following ischiatic nerve denervation, as well as selective sympathetic denervation. These findings support the notion that non-myelinating Schwann cells are components of BM niches and maintain HSC quiescence [43] (Fig. 1). The sympathetic nervous system is also involved in regulating HSC circulation between the niches and the bloodstream under steady-state conditions [44] and this process follows circadian rhythms [45]. Indeed, sympathetic nerves in the BM, by means

of circadian norepinephrine secretion, induced a down-regulation of CXCL12 expression in stromal cells that could result in HSC release from the BM [45].

1.2. Vascular niche

It has been estimated that a large proportion of mice HSCs, identified as $CD150^{+}CD48^{-}CD41^{-}Lin^{-}$ cells, are associated with sinusoidal endothelium in the BM [46]. It is worth remembering here that CD150, CD48, and CD41 are members of the signaling lymphocyte activation molecule (SLAM) protein family [47]. SLAM proteins are immune cell-specific receptors that have the ability to regulate the function of several immune cell types. SLAM proteins are differentially expressed among hematopoietic progenitors in a way that correlates with primitiveness [46]. Sinusoids are wide, thin-walled blood vessels which ramify throughout the marrow cavity. Whereas the osteoblastic niche is mainly involved in maintaining HSCs quiescence, vascular niche presumably promotes proliferation, differentiation, and mobilization of HSCs by providing a more nutrient-rich microenvironment marked by higher concentrations of oxygen and growth factors [48,49]. In addition to endothelial cells, other cell types associate with vascular structures and contribute to the HSCs transiting into or out of the bloodstream.

Endothelial cells are involved in HSC maintenance both directly and indirectly. Endothelial cells expressed the Notch ligands Jagged-1, Jagged-2, and Delta-like (DLL) -1 and −4, and sustained *in vitro* self-renewal of LT-HSCs and *in vivo* reconstitution of the LT-HSC pool after myeloablation, through activation of the Notch pathway [50] (Fig. 1). Endothelial cells also modulated HSC homeostasis and differentiation by producing a specific set of angiocrine factors. In particular, endothelial cells in which the serine/threonine protein kinase Akt was activated, supported HSC self-renewal and expansion by up-regulating fibroblast factor 2 (FGF2), insulin-like growth factor binding protein 2 (IGFBP2), Ang-1, BMP4, and desert hedgehog (DHH). In contrast, endothelial cells where the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) signaling cascade was up-regulated, stimulated the expression of factors that promoted HSCs differentiation, including Ang-2 and interleukin (IL) -6 [51].

SCF, the ligand for c-Kit tyrosine kinase, is a key niche component that maintains HSCs and is expressed by different BM cell types, including endothelial and $LepR^{(+)}$ perivascular stromal cells (Fig. 1). It has been reported that deletion of *Scf* specifically in these cells resulted in reduced HSC number [52], thus providing a direct evidence that endothelial and $LepR^{(+)}$ perivascular stromal are functionally essential for HSC maintenance. Moreover, the adhesion molecule E-selectin, expressed exclusively by endothelial cells, promoted HSC proliferation, as demonstrated by the increased HSC quiescence and self-renewal potential in E-selectin knockout ($Sele^{-/-}$) mice [53].

Several stromal cell types surrounding the vessels express factors that regulate HSCs [54] and can be identified based on the expression of specific markers that, however, are partially overlapping among the different cellular populations. CXCL12 interacts with CXC-chemokine receptor 4 (CXCR4) expressed on HSCs. CXCL12/CXCR4 signaling is essential for both HSC homing into BM and for maintaining the HSC pool [55]. CXCL12 is mainly produced by CAR cells, which are located both around endothelial cells and at the endosteum [55]. Although the exact nature of CAR cells is still unclear, they comprise cells positive for intermediate filament protein nestin or NES^{+} MSCs, as well as $LepR^{(+)}$ perivascular stromal cells [56]. Deletion of CXCR4 in adult mice, resulted in a severe reduction of HSC number, because of an enhanced exit of HSCs from the quiescent status [55]. Moreover, ablation of CAR cells *in vivo* severely impaired the adipogenic and osteogenic differentiation potential of BM stromal cells, as well as production of SCF and CXCL12 [57], suggesting that CAR cells are adipo-osteogenic progenitors required for proliferation of HSCs as well as for the maintenance of HSCs in an undifferentiated state. NES^{+} MSCs are spatially associated with HSCs and adrenergic nerve fibers, and highly expressed HSC maintenance genes, including *Cxcl12*, *Ang-1*, *IL-7*, and *osteopontin* [56]. In *in vivo* models, depletion of NES^{+} MSCs strongly impacted on HSCs and reduced both HSC content and their homing in the BM [56].

In addition to the above mentioned cell types, the BM contains adipocytes, whose roles in HSC regulation has only recently begun to emerge. Indeed, it has been shown that adipocytes decreased the frequency of quiescent HSCs [58] (Fig. 1). Adipocytes could also sustain leukemia cells. Of note, obesity is a recognized risk factor in leukemia progression and relapse [59]. MSCs can differentiate into different cell lineages, including osteoblasts, chondrocytes, and adipocytes. The connective tissue growth factor (CTGF) is a negative regulator of adipocyte differentiation, as demonstrated in both *in vitro* and *in vivo* models [60]. Most importantly, in murine models where MSCs were CTGF-depleted, leukemic cells exhibited a preferential engraftment and BM homing. CTGF-depleted MSCs expressed higher leptin (an adipocyte-derived hormone) and CXCL12 levels, as compared with their normal counterpart [60], and similarly, *in vitro*, murine B-ALL cell lines migrated toward adipose tissue in a CXCL12-mediated manner [20], suggesting a role for adipocytes in promoting leukemia cell engraftment and growth. These observations were further supported by detection of high leptin expression in human BM specimens derived from ALL patients, when compared with normal BM [60]. The protective role of adipocytes has been observed in case of L-asparaginase (ANSase) treatment, a first line therapy for ALL, which depletes asparagine and glutamine from plasma and results in leukemic cell cytotoxicity [61]. Indeed, *in vitro*, adipocytes protected leukemic cells from ANSase, likely through glutamine secretion, and BM collected from patients after ASNase treatment displayed an increase in glutamine expression localized in areas occupied by adipocytes [61]. On the other hand, ALL cells induced asparaginase synthase (*ASNS*) gene expression, and consequent ASN secretion, in MSCs [62]. This effect was mediated by IGFBP7 and enhanced by insulin, which is elevated in obesity [62]. *In vitro*, *ASNS* expression correlated with the protective role of MSCs and increased ASNase resistance [63]. Furthermore, in primary B-ALL cells, sensitivity to ASNase was dependent on *ASNS* expression [63], and higher *IGFBP7* mRNA levels were associated with a worse prognosis in B-ALL patients [62]. A similar mechanism, involving MSCs, supported T-ALL cell lines survival after pegylated arginase I (BCT-100) treatment, that is another amino acid depleting agent [64]. Interestingly, this protective effect could be overcome by pretreatment with vincristine, a commonly used chemotherapeutic drug that suppresses the proliferation and function of MSCs [64]. These findings shed further light on the mechanisms that protect ALL cells in BM microenvironment, and suggest novel potential therapeutic approaches/strategies (e.g. targeting leptin-producing adipocytes, or blocking leukemia cell migration toward adipocytes with CXCR4 antagonists) for ameliorating current therapies.

1.3. Immune system cells and BM niches

Although the BM is an immune reactive site [65], niches seem to exert their protective roles toward HSCs also through the establishment of an immune suppressive environment or an immune privileged site where multiple mechanisms cooperate to prevent immune attacks, even enabling prolonged survival of foreign allografts without exogenous immunosuppression [66]. B- and T- lymphocytes, plasma cells, dendritic cells, neutrophils, and macrophages reside in BM stroma and parenchyma, while the BM regulates immune cells through the production of cytokines, chemokines, and growth factors [67].

In this context, it should be highlighted the role of programmed death-1 (PD-1), because its inhibition is emerging as an applicable strategy for cancer therapy [68].

PD-1 is a CD28 family member cell surface receptor that belongs to the immunoglobulin superfamily. PD-1 is expressed on activated T- and pro-B cells and binds two ligands, PD-L1 and PD-L2 [69]. While PD-L2 is expressed primarily on macrophages and dendritic cells, PD-L1 is expressed on almost all types of lymphohematopoietic cells,

as well as on non-lymphoid cells. Moreover, PD-L1 is abundant in tumor cells, including leukemia cells [69]. The interactions between PD-1 and its ligands, inhibit T-cell activation and cytokine production, and in normal tissues this inhibitory pathway cooperates with the T-cell receptor (TCR) signaling to maintain homeostasis of immune response in order to prevent autoimmunity. Indeed, ligand engagement of PD-1 results in recruitment of phosphatases, particularly Src homology region 2 domain-containing phosphatase-2 (SHP2), and consequent dephosphorylation of TCR down-stream effectors. This induces an attenuation of TCR signaling [70]. It is worth underscoring the existence of a feedback loop that decreases immune responses, as T-cell activation induces PD-1 expression, whereas different cytokines produced in response to T-cell activation, up-regulate PD1-ligands [69]. However, in the tumor microenvironment, PD-1 signaling provides an immune escape for tumor cells by turning off cytotoxic T-cells [71] (Fig. 1). Indeed, PD-1 is highly expressed in T-cells from tumor patients, and PD-L1 is enriched on the surface of tumor infiltrating macrophages, tumor cells, and antigen-presenting cells, as well as on the surface of the majority of malignant hematological cells [69]. Therefore, in recent years immunotherapy based on PD-1/PD-L1 inhibition was introduced in the clinic for cancer therapy for sensitizing tumor cells to cytotoxic T-cell attack [68].

Another important role for preventing immune attacks is played by T-regs, a small lymphocyte subpopulation, characterized by the $CD3^+CD4^+CD25^+CD127^-$ immunophenotype, which is involved in the regulation of adaptive immune responses [72]. In mouse models, it was observed the persistence of HSCs from allogeneic donor mice (allo-HSPCs) in non-irradiated recipient mice for 30 days without immunosuppression with the same survival frequency compared to syngeneic HSCs. However, HSCs were lost after the depletion of T-reg cells. Allo-HSCs co-localized with T-regs on the endosteal surface in the calvarial and trabecular BM, suggesting that T-regs participate in creating a localized zone protected from immune attacks [66].

For the scopes of this review, it is worth emphasizing that a significantly increased percentage of T-regs was observed in the BM of B- and T-ALL patients where it was implicated as a poor prognostic factor [73–75]. Thus, it is reasonable to assume that similarly to HSCs, LICs are at least partially protected in an immunosuppressive microenvironment because of the higher frequency of T-regs in the leukemic BM.

It should be considered, however, that also MSCs have been implicated in immunosuppression in the leukemic BM as these cells released cytokines, such TGF-β and hepatocyte growth factor (HGF) that mediated T-cell suppression [76]. On the other side, it has been reported that leukemia inhibitory factor (LIF) released by MSCs could expand the T-reg subpopulation [77]. Understanding the mechanisms of MSC-mediated immunosuppression will require further investigations in the context of hematopoietic malignancies. Indeed, a better understanding of the immune cells and their functions in the BM microenvironment may certainly help to develop specific therapies targeting leukemia at the level of LICs.

2. Signaling pathways sustaining LICs in the BM niches

For LIC dormancy, survival, proliferation, and differentiation, both the osteoblastic and vascular niches are essential [78–81]. LIC share certain biological features with HSCs, therefore several of the signals that mediate interactions between the LICs and the BM niches, are similar to those of HSCs. However, LICs differ from HSCs in their aberrant activation of key signaling pathways that control survival, proliferation, drug-resistance, and ability to invade and spread [82].

2.1. CXCL12–CXCR4 axis

CXCL12 is a member of the chemokine family that binds to CXCR4, one of the 18 currently known G-protein-coupled seven-span transmembrane receptors [83]. Intriguingly, CXCL12 was first cloned from a BM–derived stromal cell line and identified as a pre-B cell growth stimulating factor [84].

The CXCL12/CXCR4 axis is involved in development and maintenance of healthy tissues and organs [85,86], however it appears to play an important role in the dissemination of both solid tumors and hematopoietic disorders, including ALL [87–89]. Indeed, CXCL12/CXCR4 signaling is involved in the regulation of components of focal adhesion complexes, including the very late antigen 4 (VLA-4), vascular cell adhesion molecule 1 (VCAM-1), and fibronectin [88]. In addition, the CXCL12-CXCR4 axis mediates chemotaxis of a wide variety of cell types, including lymphocytes, HSCs, endothelial and epithelial cells, as well as cancer cells [90,91]. In the BM microenvironment, CXCL12 is synthesized and released by osteoblasts, CAR cells, and NES^+ MSCs (Fig. 1).

Activated CXCR4 up-regulates various signaling pathways which include Janus kinase/signal transducer and activator of transcription (JAK/STAT), p38 mitogen-activated protein kinase (p38MAPK), MEK/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and protein kinase C (PKC) [89]. All of these signaling cascades play key roles in mediating migration, adhesion, survival, proliferation, and drug-resistance of ALL cells [92–103]. For example, Jak/STAT, MEK/ERK, PI3K/Akt, and PKC signaling pathways regulate cell survival by controlling the expression/phosphorylation of Bcl-2 family proteins, including Bcl-2 itself [96,97], Bim [104], Mcl-1 [105], Bad and Bax [106]. PI3K/Akt signaling could control cell cycle progression by regulating the expression/phosphorylation of proteins such as cyclin B1 and stathmin [106]. The expression of membrane transporters involved in drug resistance (ABCB1 and ABCG2) has been documented to be under the control of p38MAPK and MEK/ERK [95]. Finally, both MEK/ERK and p38 MAPK kinase promoted T-ALL migration through interactions with α2β1 integrin [92].

2.1.1. CXCL12–CXCR4 axis in B-ALL

The importance of the CXCL12/CXCR4 axis for the BM homing of B-ALL cells was first highlighted in 2001 [107]. It was subsequently shown that VLA-4 was involved in the BM homing of human B-ALL cells xenotransplanted in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Among the signaling pathways activated downstream of CXCR4 in B-ALL cells, the PKC cascade was the most prominent for BM migration and homing [108]. Nevertheless, CXCL12 was able to cause a transient activation of p38 MAPK in a model of B-ALL. p38 MAPK inhibition in stromal cells resulted in reduced production and secretion of a number of cytokines and chemokines with potential roles in leukemic cell proliferation and survival. However, no effects on adhesion molecules (VCAM-1, fibronectin and other adhesive ligands) were observed. It was thus concluded that inhibition of p38 MAPK could be important for the supportive role of stromal cells in B-ALL progression [109].

Importantly, an elevated expression of CXCR4 on B-ALL blasts has been related to a worse patient outcome [110,111]. Also the expression of phosphorylated (active) CXCR4 has been associated with a poor survival in adult B-ALL patients [112].

Consistently with the pro-migration effects of CXCL12/CXCR4 signaling, up-regulated expression of CXCR4 on leukemic cells was strongly predictive for extra-medullary organ involvement in B-ALL, independently from the peripheral lymphoblast count. In particular, a very high CXCR4 expression was detected in mature B-ALL, a disease characterized by a high incidence of massive leukemic infiltration of extra-medullary anatomical sites, including liver, spleen, lymph nodes, and central nervous system [113].

The expression of CXCL12/CXCR4 was also evaluated in the BM-MSCs from a cohort of adolescents and young adults with ALL. It was found that CXCR4 levels decreased, while CXCL12 level increased in these cells when compared with similar cells from healthy donors. These changes were reversed by chemotherapy, however the relevance of these findings is unclear [114].

The interest in CXCR4/CXCL12 signaling stems also from the fact that several CXCR4 antagonists have been developed over the years, initially for the treatment of HIV, where CXCR4 functions as a co-receptor for virus entry into T-cells [115]. However, since CXCR4 plays a key role in the cross-talking between leukemic cells and their microenvironment, acute leukemia treatment may become the ultimate application of CXCR4 antagonists [116]. Several studies have addressed the issue of the efficacy of CXCR4 antagonists in pre-clinical models of B-ALL.

T140 and AMD3100 (Plerixafor) are two CXCR4 antagonists that were able to block CXCL12-driven chemotaxis both in the B-ALL cell line, NALM6, and in primary B-ALL cells [117]. Regarding the survival of B-ALL cells in co-culture with stromal cells, it was demonstrated that AMD3100 had little effect, but the inhibitor significantly impacted on the proliferation of B-ALL cells, due to the co-stimulatory role of CXCL12 in enhancing the effects of cytokines and growth factors released in the BM microenvironment [117].

Prolonged administration of CXCR4 antagonists to mice with either murine or human B-ALL, resulted in a reduction in the number of leukemic cells in the peripheral blood and spleens of treated animals when compared with control animals. There was also a marked reduction in the dissemination of leukemic cells to extra-medullary sites, including liver and kidney, in all cases where this occurred [117]. The effects on liver localization appear particularly interesting in light of subsequent findings that have documented how bile duct epithelial cells formed a hepatic niche that supported infiltration and proliferation of B-ALL cells in the liver of NOD/SCID/IL-2rγ- (NSG) immunocompromised mice xenografted with primary human B-ALL cells [118]. NSG mice, besides displaying no activity of T- and B-cells, also lack NK-cell function and show alterations in dendritic cell functions, due to the absence of IL-2rγ [119]. Intriguingly, in this study it was demonstrated that the functions of the hepatic niche were also maintained by the CXCL12/CXCR4 axis.

Furthermore, the CXCL12/CXCR4 signaling pathway is involved in drug-resistance of B-ALL cells, as documented by several lines of evidence. NOD/SCID/NSG mice transplanted with human B-ALL cells and treated with vincristine and AMD11070 (yet another CXCR4 antagonist) had few circulating leukemic cells, a normal spleen, and reduced human $CD19^+$ cells in the BM at the end of the treatment [120]. AMD3100 was also effective *in vivo* in a model of *MLL*-rearranged (*MLL*-R) ALL xenografted in NOD/SCID mice. It should be underlined that *MLL* rearrangements confer a poor outcome to infants with ALL and are responsible for increased expression of the tyrosine kinase, fms-related tyrosine kinase (FLT) 3 [121]. High levels of FLT-3 may be associated with worse prognosis in acute leukemias [122]. A combination treatment consisting of AMD3100/G-CSF and lestaurtinib (an FLT3 inhibitor), markedly decreased LIC engraftment at 10 weeks indicating that the AMD3100/G-CSF combination potently enhanced the efficacy of FLT3 inhibitors against *MLL-R* ALL LICs. These findings indicated that interruption of leukemia/BM-MSC signaling is critical for the treatment of *MLL*-R ALL [123].

It has also been reported that B-ALL cell lines up-regulated surface expression of CXCR4 in response to chemotherapeutic drugs (Ara-C, daunorubicin, vincristine). AMD3100 preferentially decreased BM-MSC protection to a greater extent in chemotherapy-pretreated cells, when compared with untreated cells [124]. However, it was found that AMD3100 increased *in vivo* surface expression of both CD49a (the integrin α subunit of VLA-4) and CXCR7 (the second CXCL12 receptor, but also the receptor for CXCL11) in B-ALL cell lines, which could be interpreted as mechanisms of resistance to CXCR4 inhibition.

2.1.2. CXCL12–CXCR4 axis in T-ALL

There is much less information on the CXCL12/CXCR4 axis of T-ALL cells. However, CXCR4 is highly expressed in human T-ALL cell lines [125] and inhibition of CXCR4 activity with the RCP168 peptide partially overcame resistance to chemotherapy (Ara-C treatment) induced in Jurkat T-ALL cells by co-culturing with BM-MSCs [126]. Treatment with RCP168 down-regulated pro-survival signaling pathways activated by CXCL12 in Jurkat cells, i.e. PI3K/Akt and MEK/ERK. Overall, these findings indicated CXCR4 inhibitors combined with chemotherapeutic agents as a potential strategy for targeting T-ALL cell/BM microenvironment interactions.

Two very recently published papers have highlighted the importance of CXCL12–CXCR4 stromal cell signaling for T-ALL pathophysiology.

It was found that direct T-ALL cell interaction with CXCL12-producing vascular endothelial cells was required for disease maintenance and progression *in vivo*, suggesting the importance of the vascular niche in T-ALL pathophysiology [127]. Characterization of murine and human T-ALL cells revealed an increased cell-surface expression of CXCR4 compared with healthy T-cells. Inducible CXCR4 deletion or treatment with the CXCR4 antagonist, AMD3465, significantly reduced murine and human T-ALL burden and LIC activity. Furthermore, it increased overall survival via altered T-ALL cell localization, enhanced apoptosis, and decreased c-Myc protein expression [127].

Consistently with these findings, Passaro and coworkers [128] have documented that CXCL12 promoted T-ALL cell motility through a mechanism dependent on calcineurin, a serine/threonine protein phosphatase previously implicated in T-ALL LIC activity [129]. Cell motility defects in calcineurin-deficient mice were linked to reduced CXCR4 surface expression due to down-regulation of the actin-binding protein, cortactin, and defective CXCR4 recycling. Silencing of CXCR4 in either murine or human T-ALL cells inhibited leukemia cell motility and proliferation and promoted T-ALL cell apoptosis *in vitro*. In addition, it impaired T-ALL cell homing *in vivo*, leading to decreased LIC activity and suppression of leukemia engraftment.

In addition, it has been reported that BM stromal cells up-regulated IL-8 mRNA in T-ALL cells through the activity of CXCR4. Accordingly, exogenous CXCL12 increased the synthesis of IL-8 mRNA and protein in primary T-ALL cells. It was shown that CXCL12 activated the nuclear factor-κB (NF-κB) and c-Jun. N-terminal kinase/activator protein-1 (JNK/AP-1) pathways and that these events were necessary for the increased expression of IL-8 [130].

Overall, the results obtained in both B- and T-ALL preclinical models, have highlighted the importance of cross-talks between CXCR4-expressing ALL cells and CXCL12-producing stromal cells in leukemia initiation, maintenance, and progression. They have also provided a rationale for the use of CXCR4 antagonists as a novel therapeutic strategy in ALL patients. Of note, there is an active phase I clinical trial (NCT01319864) in which AMD3100/Plerixafor is being combined with chemotherapeutic drugs (Ara-C and etoposide) for treating pediatric patients with hematological malignancies, including ALL.

As stated above, CXCR7 is the second receptor for CXCL12 [131]. A recent study has highlighted that CXCR7 mRNA was more expressed in ALL primary cells, than in acute myelogenous leukemia (AML) blasts or healthy BM mononuclear cells [132]. Among ALL-diagnosed patients, CXCR7 expression was more pronounced in the T-ALL subtype. Western blot analysis of ALL cell lines confirmed a much higher expression of CXCR7 in T-ALL cell lines (Jurkat and MOLT-4) when compared with B-ALL cell lines (Raji and Daudi), whereas the expression of CXCR4 was similar in both T- and B-ALL cell lines. CXCR7 silencing by shRNA negatively affected cell migration, but not proliferation or survival, of Jurkat and MOLT-4 T-ALL cells [132]. It was thus hypothesized that CXCR7 may potentiate CXCR4 response of ALL cells and contribute to leukemia maintenance by initiating cell recruitment to BM niches that were originally occupied by HSCs. There is fairly solid evidence for CXCR7 acting as a scavenger receptor for CXCL12, thereby preventing desensitization of CXCR4 also in cancer cells [133].

2.2. Notch signaling

Notch proteins are transmembrane receptors consisting of an extracellular amino-terminal and an intracellular carboxy-terminal subunits that are non-covalently linked by the heterodimerization domain (HD).

In humans, there are 4 Notch proteins (Notch 1–4) and 5 ligands (Jagged 1, Jagged 2, DLL-1, DLL-3, DLL-4). Ligand binding induces Notch proteolytic cleavage, mediated by a γ-secretase protein complex, and translocation of the Notch intracellular domain (NICD) to the nucleus where it regulates the expression of down-stream target genes [134]. Notch signaling has been found to be functional in the main cell components of BM niche, including HSCs, osteoblasts, osteoclasts, and MSCs, where it regulates HSC self-renewal, quiescence, and apoptosis [135]. Nevertheless, because of the utmost complexity of this signaling pathway, it is still unclear whether Notch signaling is dispensable for the healthy BM niche homeostasis. Moreover, despite the important functions played by Notch during T-cell development [136], only limited information is available regarding the roles of Notch signaling in the cross-talks between leukemic and BM niche cells. Alterations in Notch signaling have been extensively reported in T-ALL, and recently its role has begun to emerge also in B-ALL [134]. Endothelial cells of the BM microenvironment blood vessels expressed high level of DLL-4 and activated specifically Notch3 signaling in T-ALL cells, thus promoting tumor escape from dormancy [137] (Fig. 1). Importantly, it was documented that when DLL-4 was neutralized *in vivo* by the monoclonal antibody YW152F, tumor size and levels of active Notch3 were significantly reduced. In addition, *in vitro* Notch3 silencing affected T-ALL cell survival, thus providing a mechanism by which the vascular niche may sustain tumor development [137]. The notion that DLL-4 is a critical component of tumor microenvironment has been strengthened by the observation that *in vitro* recombinant murine or human DLL-4 stimulated both Notch1 and Notch3 signaling in human primary T-ALL samples, while *in vivo* antibody-mediated DLL-4 blockade inhibited Notch signaling and delayed leukemia growth [138]. Recently, an important role of the spleen in recruiting and sustaining proliferation of T-ALL cells has emerged in a Notch1-induced murine model of disease. Indeed, T-ALL cells showed a preferential localization to the spleen as compared with BM and other organs, including liver, kidney, lung, and thymus, especially at an early stage of disorder [22]. This is likely due to cytokine macrophage inflammatory protein (MIP)-3β, whose concentrations were higher in the spleen than in BM [22]. MIP-3β specifically binds to CXCR7, whose expression is promoted by Notch1 [22]. In addition, an important role for the spleen microenvironment in leukemia cell maintenance was further supported by the observation that splenectomy prolonged the survival of leukemic mice [22]. These findings suggested that other microenvironments in addition to the BM **one** should be considered as a therapeutic target for leukemia treatment. Very recently, the role of Notch1 signaling has been analyzed in relationship with the invasion potential of T-ALL cells. Indeed, it has been reported that human BM-MSCs (hBM-MSCs) allowed T-ALL cell lines to migrate in *in vitro* cell invasion assay [139]. This process associated with and was dependent on Notch1 signaling activation, that likely mediated up-regulation of pro-invasive matrix metalloproteinase (MMP)-2 and MMP-9 [139]. Importantly, since the authors employed hBM-MSCs obtained from both healthy donors and T-ALL patients, they observed a higher invasive potential of T-ALL cells in the presence of malignant hBM-MSCs than healthy hBM-MSCs, as well as a higher expression of Jagged-1 and DLL-4 [139]. These findings provided new insight for treatment of infiltrating T-ALL.

The role of Notch signaling and its relevance in the cross-talks with hBM-MSCs has been explored also in B-ALL. It was found that B-ALL cells expressed all Notch receptors and ligands, some of which were modulated in co-culture with hBM-MSCs, in particular Notch1, 3, and 4 were up-regulated in B-ALL cells, while Notch3 and 4 were increased in hBM-MSCs. In contrast, Notch2 was down-regulated in both cell types [140]. In co-culture, active Notch signaling sustained B-ALL cell proliferation, as treatment with the γ-secretase inhibitor, GSI XII, significantly reduced B-ALL cell viability [140]. Importantly, specific blocking experiments demonstrated that the hBM-MSCs supportive effects were mainly sustained by Notch3 and 4, as well as by Jagged-1/−2 and DLL-1 ligands, which interacted through both cell contact and in solution in the microenvironment [140]. Finally, in co-culture Notch3 and 4 signaling induced chemoresistance of primary B-ALL cells after treatment with corticosteroids [140], suggesting a novel mechanism for leukemic cells to escape this therapy.

2.3. WNT/β-catenin signaling

The WNT/β-catenin signaling pathway is involved in the development of different tissues, including the hematopoietic tissue [141]. There are three WNT/β-catenin pathways: The canonical WNT pathway, the non-canonical WNT–planar cell polarity (WNT-PCP) pathway, and the WNT-calcium (WNT-Ca^{2+}) pathway [142]. In the canonical pathway, WNT ligands bind to a receptor complex comprised of frizzled (FZD) and either low-density lipoprotein receptor-related protein (LRP) 5 or LRP6, and this interaction relieves β-catenin from its constitutive proteasomal degradation, thus allowing β-catenin translocation into the nucleus where it associates with transcription factors to control target gene transcription [141]. Activated WNT pathway is involved in the protective effects exerted by MSCs toward B-ALL cells. It was demonstrated that both B-ALL cells and BM-MSCs expressed WNT signaling components to a higher level as compared with their normal counterparts. Exogenous WNT proteins (e.g. WNT-3a) were required to induce β-catenin stabilization and nuclear accumulation, which in turn sustained proliferation and cell cycle progression, by modulating the expression of cell cycle regulators, including *E2F1*, *MYBL2* and *CDC25B* [143]. Because B-ALL cell autonomous production of WNT proteins was not sufficient to induce the same proliferative effects, it could be argued that a non-autocrine mechanism, likely provided by MSCs, was involved in WNT signaling activation [143]. Indeed, in co-culture, MSCs protected B-ALL cell lines and primary samples from Ara-C-induced apoptosis, and gene expression microarray profiling uncovered an increased expression of several WNT pathway members, including lymphoid enhancer-binding factor (Lef) 1, c-Myc, and cyclin D-type binding-protein 1 (CCNDBP1), as compared with cultures without MSCs [144]. Consistently, co-cultured B-ALL cells displayed increased phosphorylation and thus inhibition of glycogen synthase 3 (GSK3) β (which acts as a β-catenin inhibitor). Furthermore, treatment with the β-catenin inhibitor, XAV939, sensitized leukemia cells to Ara-C chemotherapy both *in vitro* and *in vivo* [144]. Another mediator of the WNT pathway which has been recently identified is the pleiotropic protein Galectin-3 (Gal-3), which is likely involved in GSK3β inhibition and consequent β-catenin up-regulation at a post-transcriptional level [145]. Gal-3 expression was induced in both T- and B-ALL cell lines co-cultured with hBM-MSCs and was associated with stroma-induced drug-resistance [145]. Intriguingly, Gal-3 expression was elevated in relapsed/refractory ALL patient samples [145]. Therefore, targeting WNT signaling through inhibition of either GSK3β or β-catenin, could represent a novel interesting therapeutic approach for ALL.

2.4. Hypoxia and hypoxia inducible factor 1 α signaling

Self-renewal, differentiation, and mobilization of healthy HSCs are the result of interactions between the osteoblastic and vascular niches of the BM. The osteoblastic niche is thought to home mostly quiescent LT-HSCs. While the importance of the different microenvironmental domains for HSCs maintenance is still under investigation, it is commonly thought that a gradient of decreasing oxygen levels exists from the vascular to the osteoblastic niche and the most immature HSCs are sequestered in a highly hypoxic microenvironment, where low oxygen levels play a fundamental role in the maintenance of healthy HSC functions [146]. Indeed, quiescent HSCs from BM regions with minimal blood perfusion comprise a high percentage of LT-HSCs [147].

However, recent evidence obtained by *in vivo* measurements of local oxygen tension (p_{O2}) in the BM of live mice seems to indicate that the lowest p_{O2} (1.3%) was found in deep peri-sinusoidal regions, while the endosteal region is less hypoxic (p_{O2}: 1.8%) [148].

Whatever the case, the hypoxic BM microenvironment could also support LICs. Protective signals arising from the BM microenvironment maintain residual leukemic cells after chemotherapy, potentially contributing to disease relapse. Data obtained in rat leukemia models demonstrated that leukemic cells infiltrating the BM preferentially resided in markedly hypoxic areas, when compared with cells in the BM of healthy rats [149]. Furthermore, it has been shown that progression of leukemia is associated with a vast expansion of BM hypoxic areas and that hypoxia contributed to chemoresistance of leukemic cells [150].

Due to the fact that hypoxic tumors are in general more resistant to both chemotherapy and radiation, a hypoxic microenvironment may promote resistance of LICs, suggesting that hypoxia could be a therapeutic target. Hypoxia inducible factor 1 α (HIF1α) is the best characterized marker of hypoxia, being a transcriptional regulator of cellular response to hypoxia. HIF1α subunits are normally degraded by the protein encoded by the von Hippel-Lindau tumor suppressor gene *VHL* (which encodes a E3-ubiquitin ligase) in the presence of oxygen, but are stabilized under conditions of hypoxia. HIF1α is stabilized post-transcriptionally by levels of oxygen tension less than 2% [151]. Activation of the HIF1α signaling pathway induces a vast array of gene products that control energy metabolism, glycolysis, angiogenesis, apoptosis, and cell cycle progression [150].

HIF1α has been shown to be overexpressed in clusters of leukemic cells in the BM of pediatric ALL patients, while it was absent in healthy BM biopsies. Likewise, BM from adult patients with ALL frequently displayed HIF1α expression, which was associated with a worse outcome [150]. Intriguingly, HIF1α was recently shown to be activated in LICs also under normoxic conditions [152]. Indeed, HIF1α activity can also be induced in tumor cells through a variety of oncogenic stimuli and growth factors, primarily following the activation of the PI3KAkt/mammalian target of rapamycin (mTOR) and MER/ERK signaling pathways [153]. In particular, mTOR activation is one of the central mechanisms leading to up-regulation of HIF1α. On the other hand, BM microenvironment and hypoxia play a crucial role in activating several signaling pathways, including PI3K/Akt/mTOR [126].

A recent study was performed to investigate the molecular mechanisms of survival of leukemic cells growing under hypoxic conditions in co-culture with BM-MSCs. Under hypoxic conditions, co-culture of B-ALL cells and MSCs led to increased level of HIF1α protein expression, suggesting that the activation of the mTOR pathway by means of MSCs, was able to enhance HIF1α stabilization. The sensitivity of B-ALL samples to chemotherapy under hypoxic conditions was assessed and it was observed that chemotherapeutic drugs were not effective. It was also documented that BM-MSCs enhanced HIF1α-dependent up-regulation of glucose transport and promoted a switch to glycolytic metabolism in primary B-ALL samples. Modulation of HIF1α expression or treatment with the allosteric mTOR inhibitor, everolimus (RAD001), were able to restore drug sensitivity. Everolimus also reverted several phenotypic attributes of hypoxia, including up-regulated HIF1α, facilitated glucose uptake, and accelerated glycolytic rate of leukemic cells [154].

A recent paper has highlighted the importance of HIF1α for the pathophysiology of T-ALL LICs, where HIF1α up-regulated WNT/β-catenin signaling under hypoxic conditions. Importantly, either genetic inactivation of β-catenin or HIF1α deletion severely impaired LIC activity, whereas the growth or viability of bulk leukemic cells were not affected by these manipulations, suggesting that WNT/β-catenin and HIF1α pathways specifically support T-ALL LICs [155].

Novel strategies to target HIF1α are being developed to counter the hypoxia pro-survival effects on cancer cells. These strategies include the inhibition of HIF1α or signaling pathways that have an impact on HIF1α expression. However, due to the fact that HIF1α is regulated in a complex manner by several factors other than oxygen, and not all hypoxic cells express HIF1α (conversely not all HIF1α expressing cells are hypoxic), it is emerging that other strategies are necessary to tackle this issue [156].

In this context, promising candidates are represented by hypoxia activated prodrugs (HAPs), which are inert in normoxia, and active only in hypoxic conditions. A common mechanism by which a nontoxic prodrug can be activated is enzymatic addition of one electron, which initiates the formation of DNA reactive species, a process that could be inhibited in presence of oxygen. The prodrug PR104 is a phosphate ester, rapidly hydrolyzed *in vivo* to the corresponding alcohol, PR104A, which acts as a HAP. Under extremely low oxygen concentrations, PR104 is also reduced to the amine and hydroxyl-amine nitrogen mustards, PR104H and PR104M, which induce DNA cross-linking in hypoxic cells [157]. Another mechanism of PR104 activation is through a hypoxia-independent two electron reduction, by the enzyme aldo-keto reductase 1C3 (AKR1C3). PR104 demonstrated remarkable activity in *in vivo* B-ALL murine models, suggesting that targeting hypoxia is feasible and could be a valuable tool in the treatment of human ALL [150]. A relevant aspect highlighted by this study was the observation of great expansion of the hypoxic areas in the BM of leukemic mice, accompanied by a loss of vessels integrity with increased leukemic cell burden. BM samples from B-ALL patients, immunostained for HIF1α, showed a strong positivity at diagnosis, which was impressively reduced when patients achieved a complete remission. HIF1α was expressed not only in leukemic cells, but also in the surrounding stromal cells. This observation indicated the expansion of hypoxic niches and that hypoxia is an intrinsic property of the leukemia microenvironment [150]. In preclinical studies, PR104 demonstrated impressive single agent activity also in xenograft models of human B-ALL, providing a rationale for exploiting HAPs [147]. The efficacy of PR104 has been shown also in preclinical models of pediatric T-ALL where expression of AKRC13 correlated with PR104/PR104A sensitivity *in vivo* and *in vitro* [158].

Phase I clinical trials employing PR104 in advanced solid cancers established a maximum tolerated dose and toxicity profiles of the drug [159]. However, it is important to highlight that the usefulness of PR104 in leukemia settings should be evaluated also in light of sensitivity of leukemia subgroups to cross-linking agents, given the ability of active metabolites of the drug to exploit defects in DNA repair, and of the possibility of activation through AKR1C3, as well as the action of hypoxic reductases. PR104 entered phase I/II clinical trials in relapsed and refractory ALL and results of the first clinical have been recently published [160]. This study confirmed that the BM of leukemic patients was markedly hypoxic and PR104 showed clinical activity. The drug was administered at 3 and 4 g/m^2, which was threefold higher than the dose used in solid tumors trials. This could explain the gastrointestinal toxicity observed in several patients (severe in 14% of cases treated with the highest doses), probably due to the biliary excretion of the O-glucuronide metabolite, PR104G. The most severe adverse effects were neutropenia and myelosuppression, pointing to a possible toxicity against healthy HSCs, residing in the hypoxic niches of the BM, as a serious issue for a successful long term therapy with PR104 [160]. Studies are underway to generate HAPs that are triggered by hypoxia to release molecularly targeted inhibitors that potentially could provide better tolerated therapies [147].

2.5. Adhesion molecules

Among the different mechanisms involved in BM homeostasis, a prominent role is played by the adhesion molecules, including osteopontin, VLA-4/VCAM-1, LFA-1/intercellular adhesion molecule-1 (ICAM-1), and N-cadherin, which regulate the binding of HSCs to the BM niche cell components required for HSC mobilization, homing, and engraftment. Furthermore, adhesion molecules provide signals for either quiescence or proliferation of HSCs [161]. The importance of LFA-1/ICAM-1-mediated adhesion between T-ALL cells and BM-MSCs was documented for the first time in a paper where it was reported as LFA-1/ICAM-1 adhesive interactions promoted survival of T-ALL cell lines and patient-derived T-ALL lymphoblasts on BM stroma [162]. LFA-1/ICAM-1 binding is presumably an early event necessary, but not

sufficient, to sustain T-ALL cell survival, as a complexity of additional interactions involving other adhesion molecules (e.g. VCAM-1 and/or E-selectin) are required [162]. VLA-4 is an integrin dimer expressed by HSCs whose interaction with VCAM-1 could retain these cells into the BM endothelial or osteoblastic niche [161]. In B-ALL relapsed patient samples, high expression of VLA-4 represents an independent prognostic factor which associated with a poor outcome and a significantly lower probability of event-free and overall survival [163]. Therefore, VLA-4 mediated cytoprotective effects of stromal cells could be a potential therapeutic target in relapsed B-ALL [163]. Moreover, VLA-4 expression correlated with a distinctive gene expression signature in risk-stratified patient subgroups, and interestingly most of the differentially expressed genes are involved in the PI3K/Akt/mTOR, WNT, and Rho-GTPase signaling pathways, suggesting that the cellular events related to VLA-4 expression are critical for leukemic cell survival and/or response to therapy [163].

Furthermore, it has been recently reported that chemoresistance of B-ALL cell lines could be mediated by reciprocal leukemic cell-MSC activation of NF-*k*B signaling which was dependent on interactions between VLA-4 (expressed on leukemia cells) and VCAM-1 (produced by MSCs) [164].

Also ion channels could participate in cell-cell adhesion. Thus, investigating their roles in the context of ALL is very attractive [165]. Recently, a novel protective mechanism exploited by MSCs and involving the human ether-à-go-go related gene 1 (*hERG1*) has been described. This gene codes for a protein, also referred to as Kv11.1, that is the α subunit of a voltage-dependent K^+ channel [166]. hERG1 is overexpressed in B-ALL primary cells and cell lines, where it interacts with β_1-containing integrins (VLA-4 and VLA-5) and CXCR4 to form a macromolecular complex. This complex activated the down-stream signaling molecule integrin linked kinase (ILK), as well as the PI3K/Akt and MEK/ERK pathways in a β_1-integrin-dependent manner. Indeed, integrin blockade inhibited signaling down-stream of hERG1 [167]. hERG1 activity was essential for the protective effects of MSCs on leukemic cells, as either specific hERG1 blockers (e.g. E4031) or non-specific inhibitors (sertindole, erythromycin, R-roscovitine), dampened activation of both signaling pathways and enhanced doxorubicin, prednisone, or methotrexate pro-apoptotic effects on ALL cells [167]. Treatment with the hERG1 inhibitor E4031 was also significantly effective *in vivo* both as a single agent or in combination with dexamethasone, underscoring the relevance of blocking hERG1 as a novel therapeutic strategy for ALL [167].

2.6. Cytokines

Cytokine signaling is essential for BM niche homeostasis, by finely tuning cross-talks between the different cellular components. Initial studies on relapsed B-ALL patients, aimed to better characterize the leukemic cells, led to the discovery that the expression levels of a broad spectrum of cytokines (which included IL-7, IL-10, IL-15, and IFN-γ) and their receptors were higher as compared with healthy BM samples. This seminal observation suggested the existence of a complex autocrine/paracrine regulation of leukemic cell functions [168]. Moreover, also stromal cells from leukemic BM expressed cytokines in a pattern different to their normal counterpart, and *in vitro* interactions between leukemic cells and BM stromal cells further modified cytokine expression which was also influenced by drug treatment [169]. The effects of IL-7 in sustaining ALL progression were initially documented in *in vitro* studies [170], and subsequently confirmed in *in vivo* murine models [171]. For instance, B-ALL primary cells co-cultured with stromal cells displayed a CXCL12-dependent proliferation which increased in the presence of IL-7 and, to a lower extent, of IL-3. This synergistic/additive effect was partially reflected by the increased phosphorylation of components of CXCL12-activated signaling pathways (e.g. PI3K/Akt, p38MAPK, and MEK/ERK) [172]. BM-MSCs could also increase survival and proliferation of T-ALL cells through IL-7 [173]. Similarly, thymic epithelial cells, which exert a dominant inductive role in the survival and maturation of healthy immature T-cells, increased survival of T-ALL primary cells through IL-7, suggesting a functional role for the thymic microenvironment in the acquisition of selective growth advantage of leukemic cells [174]. Importantly, in both of these experimental models (BM stromal or thymic epithelial cells co-cultured with T-ALL cells) enhanced survival specifically required IL-7/IL-7R interactions, as selective blocking of either IL-7 or IL-7R significantly reduced apoptosis inhibition mediated by the microenvironment [173,174]. *In vivo*, absence of IL-7 did not affect tumor engraftment or homing, nor it prevented leukemia progression-related death of mice, however it delayed disease progression and reduced tumor infiltration. These effects were related to IL-7 dependent up-regulation of Bcl-2 and down-regulation of $p27^{Kip1}$, two mediators of leukemic cell survival and proliferation [171]. The interactions between leukemic and BM stromal cells are mutually important to support proliferation and survival. Microarray analysis of hBM-MSCs co-cultured with B-ALL primary samples, documented a significant increased expression of several inflammatory response genes, including IL-8, whose levels were found higher also in patient plasma samples [175]. In leukemic cells, IL-8 partially increased cell adhesion capacity without affecting survival and migration. However, IL-8 promoted hBM-MSC survival and this could contribute to the establishment of a malignant BM microenvironment [175].

3. Exosomes and microvesicles

Ligand-receptor interactions and direct cell-to-cell contacts have long been considered as the predominant means of intercellular communication. However, the past few decades have seen the development of a new chapter in membrane dynamics: the release of specific extracellular vesicles (EV) from the cell [176]. These vesicles have been recognized as sources of communication between cells, either for short or long distance [177]. At present, EVs are envisioned as vehicles for the transfer of signaling molecules, as well as of microRNAs (miRs) and genes, in a functionally active form [178]. EVs can be classified into three main classes: Exosomes, ectosomes or microvesicles (MVs), and apoptotic bodies. Exosomes are of endocytic origin and are the smallest among the EVs (diameter: 40–150 nm). They are released by both healthy and cancer cells after the fusion of multivesicular bodies with the plasma membrane. Ectosomes are larger vesicles (diameter: 50–1000 nm), directly formed from the plasma membrane, while apoptotic bodies are produced via membrane blebbing by dying cells [176]. This nomenclature does not adequately discriminate exosomes from ectosomes, as an overlap in size and the ability of the same cell to produce both types of vesicles confuse the classification. Moreover, both vesicle types are commonly found in the same extracellular fluids and may exert similar biological effects. Nevertheless, exosomes have emerged as particularly interesting class of information-carrying vehicles because of their potential use as reservoirs of disease biomarkers and putative importance in therapeutics.

3.1. Exosomes

While exosome secretion occurs under physiologic conditions and all live cells produce and release exosomes, cells under stress are especially active exosome producers. For example, it has been reported that inducible formation and release of exosomes depends on the DNA damage–inducing p53-dependent secretory pathway [179]. Also hypoxic conditions may favor exosome release [180].

Once released from the cell, exosomes have to interact with recipient cells to be able to deliver their cargos [181]. The results of proteomic studies, available in the EXOCarta and EVPedia databases, indicate that exosomes have a defined protein signature which consists of conserved as well as cell-type specific subsets of proteins [182,183]. Exosomes carry a wide variety of proteins, including MHC molecules, chaperones

such as HSP90 and HSP70, receptors, receptor ligands, cytokines [184]. They are highly enriched in tetraspanins, a superfamily of transmembrane proteins thought to act as scaffolding proteins, anchoring multiple proteins to one area of the cell membrane [185]. Therefore, CD9, CD23, CD37, CD81, CD82, and CD151 have been proposed as exosome markers [184]. Moreover, various miRs and mRNA species carried by exosomes can be transferred and translated into functional proteins [186]. Importantly, the composition of an exosome is not a mere reflection of the donor cell. Indeed, it has been documented that the profiles of the exosomal cargo can be substantially different from the originating cell, which indicates the existence of a highly controlled sorting process.

Studies on exosome molecular profiles have indicated that there are significant differences in protein and nucleic acid contents in exosomes derived from healthy cells, as compared with exosomes released by neoplastic cells. For example, exosomes recovered from the plasma of AML patients displayed an enrichment in leukemic blast-associated molecules, including CD34, CD33, CD117, CD90, chemokine ligand 1 (CCL1), FasL, and others [178]. Therefore, exosomes derived from both solid tumors and acute leukemias may play a critical role in cancer development and progression, as well as in chemoresistance and metastasis. However, tumor cell exosome release may be inhibited by previously secreted exosomes that are still present in the microenvironment, creating a balancing negative feedback control loop. Also, cells of the tumor microenvironment release exosomes to affect both themselves and tumor cells. Meanwhile, tumor cells release exosomes to reprogram their microenvironment to be both tumor permissive and tumor promoting [187,188]. The ability of tumor-derived exosomes to modify the BM microenvironment was highlighted in a melanoma model [189]. In this study, melanoma-derived exosomes transferred into the BM microenvironment in mice caused an alteration in the BM progenitor cells (but not in HSCs), creating a pro-angiogenic ($c\text{-}Kit^{+}\,Tie2^{+}$) pre-metastatic niche. The mechanism responsible was the transfer of the MET oncogene from tumor cells to BM progenitor cells [189]. Jung et al. demonstrated how cancer-associated exosomes participated in the formation of the pre-metastatic niche in a rodent pancreatic cancer model [190]. Moreover, EVs isolated from serum-deprived MSCs, were protective, by transporting supportive miRs and promoting breast tumor growth *in vivo* [191]. Collectively these studies indicates that tumor-derived exosomes play a crucial role in manipulating the tumor microenvironment for the benefit of cancer cells. However, also the tumor microenvironment releases exosomes with a content that is beneficial to neoplastic cells.

3.2. Leukemic EVs and BM microenvironment

When compared to solid tumors, there still are few published papers that have investigated the roles played by EVs in the context of the interactions between hematologic tumor cells and BM microenvironment cells. Furthermore, most of these papers deal with either AML or multiple myeloma (MM), so that the role of EVs in ALL awaits to be investigated. Exosomes isolated from AML cells were enriched in transcripts relevant for leukemia prognosis and stem cell niche function: FLT3, nucleophosmin 1 (NPM1), CXCR4, MMP-9, and IGF-1R. IGF-1R played an important role in stimulating induction of down-stream gene expression and changes in cell proliferation [14]. Another study demonstrated that exosomal miR cluster 17–92 (and especially miR92a), released from K562 AML cells, could interact with HUVEC endothelial cells, thus stimulating their migration properties and vascular tube formation activity [192].

The role of exosomes released by MM BM-MSCs cells has been investigated in both murine models and human primary samples. It was documented that BM-MSCs released and transferred exosomes to MM cells and that the miR profile of healthy BM-MSC-derived exosomes was different to that of MM BM-MSC-derived exosomes. In particular, the expression of miR-15a (which has tumor suppressive properties) was lower in exosomes released by MM BM-MSCs, and could contribute to increased tumor burden [193]. It was also demonstrated that exosomes derived from BM-MSCs were able to promote migration of MM cells. This was at least partially due to the up-regulation of CXCL12/CXCR4/monocyte chemoattractant protein-1 (MCP-1) axis [194]. Moreover, BM MSC-derived exosomes were able to increase MM cell survival and proliferation by up-regulating anti-apoptotic Bcl-2, and activating signaling cascades that included p38 MAPK, p53/JNK, and PI3K/Akt [194]. However, it should not be forgotten that exosome transfer is bidirectional. Indeed, a recent paper investigated the role of the exosomes secreted by MM cells, chronically exposed to hypoxia. These exosomes were enriched in oncogenic miR-135b, which was capable of influencing the surrounding BM microenvironment and accelerated vascular tube formation by targeting HIF1α signaling and the hypoxic response [195]. These findings support the hypothesis that cancer cell–derived exosomes act as vesicles responsible for modeling the microenvironment surrounding MM cells, leading to cancer cell growth and dissemination, ultimately resulting in disease progression.

Regarding ALL, so far there are only two published papers dealing with EVs. An interesting study performed on a T-ALL cell line (CCRF-CEM) documented how the combined effects of EVs (exosomes and ectosomes) and cytokines differed from their single effects, and highlighted that different EVs could modify the effects of inflammatory cytokines, by comparing the effects of EVs in the presence/absence of tumor necrosis factor (TNF) on the global gene expression profile of recipient myelomonocytic U937 cells. In particular, it was demonstrated how the combination of EVs and TNF caused a synergistic up-regulation of IL-8 mRNA [196]. Therefore, it would be important to test the combined effects of soluble molecules and different EVs, as such an approach could model the *in vivo* effects of these mediators more accurately than testing separately. In keeping with this, the complexity of the system could give an explanation why targeting of certain soluble mediators does not always lead to a therapeutic effect.

In another very recent study, it was found that a marked induction of Gal-3, which is a multifunctional galactose-binding lectin implicated in numerous type of cancers, correlated with a poor outcome in B-ALL. The levels of Gal-3 mRNA and protein expression in B-ALL cells were in relation with the appearance of drug-resistance. When B-ALL cells were in contact with stromal cells and subjected to drug treatment, there was a marked synthesis of Gal-3 [197]. The induction of Gal-3 synthesis correlated with MEK/ERK activation and the induction of drug-resistance. Gal-3 synthesis required cell-to-cell contact between stromal and B-ALL cells and was stimulated by chemotherapeutic drugs, suggesting that Gal-3 may protect B-ALL cells from drug treatment. It was established that not only Gal-3 originated by stromal cells, but also that it was present in exosomes released by these cells, thus highlighting the fundamental role of these vesicles in carrying molecules with key roles in drug sensitivity/response [197]. These results are also intriguing in light of the existence of Gal-3 antagonists, such as GCS-100 [198].

At present there is an explosion of interest for the roles of EVs and their relationship with the microenvironment. There are however many issues which have to be addressed in order to fully exploit EV potential. Very little is known of EV physiologic role in tissue homeostasis, moreover *in vitro* studies could provide only limited information. Thus, a major challenge will be to answer numerous pending questions about EV relevance to cancer progression *in vivo*, as well as the elucidation of the molecular mechanisms responsible for their release, trafficking, and uptake by recipient cells.

4. Conclusions and future perspectives

The impact of the leukemic microenvironment on the therapeutic outcome or the potential targets of the leukemic microenvironment have not been well characterized yet. Nevertheless, the rapid pace of discovery in the field of microenvironment-ALL cell interactions, many

of which were first molecularly defined over the last 10 years, points toward a future with the potential for major clinical advances. Examples from murine models and primary human disease have documented that with the potential to both cause and treat leukemia, the BM microenvironment is an emerging focus of studies with significant potential to help identify druggable targets for pharmacologic therapeutic intervention. The identification of the exact mechanisms involved in leukemic cell–host interactions that contribute to leukemia drug-resistance and relapse, will provide important insights for development of novel therapies that, however, should likely target both ALL cells and the cells in their surrounding microenvironment. Our increasing knowledge of the specific leukemic BM microenvironment should ultimately result in therapeutic strategies in a framework for targeting niche cells for attenuating leukemic progression or targeting LICs without adversely affecting healthy HSCs. Many formidable hurdles need to be overcome. For example, one potential serious drawback of inhibiting CXCL12/CXCR4 interactions, is that leukemic cells will be massively released into the peripheral blood. While CXCR4 inhibition will drive leukemic cells out of their protective bone marrow niches and likely make them more sensitive to chemotherapy, some experts have expressed concern that mobilized leukemic cells could have the potential for infiltrating to a greater extent extra-medullary organs. Therefore, all of the currently open clinical trials examining CXCR4 inhibitors as chemosensitizing agents, are using the inhibitors in combination with more than one chemotherapeutic drug, thereby increasing the probability that mobilized leukemic cells will be killed [87].

We also believe that a better knowledge of EVs in ALL holds the promise not only for gaining information on its pathobiology and select specific biomarkers for the diagnosis and prognosis of the disorder, but also for engineering modified exosomes for the selective administration of therapeutic miRs, siRNAs, signal transduction modulators or traditional chemotherapeutics to leukemic cells. Extensive studies will definitely improve the understanding of exosome functions in ALL drug-resistance, progression, and treatment.

Conflict of interest

The authors have nothing to disclose.

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