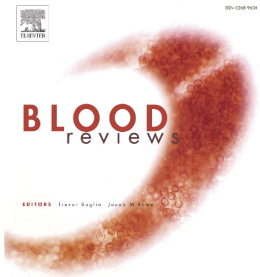

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The bone marrow microenvironment – home of the leukemic blasts

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Abstract

Acute Myeloid Leukaemia (AML) is a genetically, biologically and clinically heterogeneous set of diseases, which are characterised by an increased growth of abnormal myeloid progenitor cells within the bone marrow (BM). Ex-vivo AML exhibits a high level of spontaneous apoptosis. Furthermore, relapse for patients achieving remission occurs from minimal residual disease harboured within the BM microenvironment. Taken together, these observations illustrate the importance of the BM microenvironment in sustaining AML. While significant progress has been made elaborating the small-scale genetic mutations and larger-scale chromosomal translocations that contribute to the development of AML and its prognosis in response to treatment, less is understood about the complex microenvironment of the BM, which is known to be a key player in the pathogenesis of the disease. As we look towards future therapies, the consideration that the BM microenvironment is uniquely important as a niche for AML - coupled with the idea that leukaemic blasts are more likely to be genetically unstable and therefore evolve resistance to conventional chemotherapies - make the functions of the non-malignant cells of the BM attractive targets for therapy. In this review, we discuss the microanatomy of the BM and provide an overview of the evidence supporting the role of the BM microenvironment in creating conditions conducive to the survival and proliferation of AML blasts. Ultimately, we examine the therapeutic potential of uncoupling AML from the BM microenvironment.

Keywords: bone marrow, acute myeloid leukaemia, bone marrow microenvironment.

1. Introduction

The survival of patients with Acute Myeloid Leukaemia (AML) is presently poor. Two-thirds of younger adults and 90% of older adults die of their disease[1]. Even in patients who achieve remission with chemotherapy, relapse is common and occurs from minimal residual disease sequestered in protective niches in the bone marrow (BM) microenvironment.

The malignant blasts that form the AML tumour are presently understood to represent a variety of clinically, morphologically, genetically and epigenetically heterogeneous tumours collectively grouped under the classification of AML[2-5]. Even within individual patients, AML is now recognised as a number of distinct sub-clones of the disease, [3] and that these sub-clones evolve within patients as the disease is treated leading to potential relapse and progression.

Despite this inter- and intra-tumour heterogeneity, AML clones share many fundamental features. Clinically, these tumours are mitotically highly active, progressing rapidly within the patient. Historically, they have been clinically treated in a similar way (with the exception of acute pro-myelocytic leukaemia). Presently, treatment for those fit enough to withstand intensive therapy consists of multi-agent cytotoxic chemotherapy regimens with or without allogeneic stem cell transplant. Patients who are unfit for such intensive treatment may be managed with hypo-methylating agents and/or with supportive/palliative care. Biologically, all AMLs arise from myeloid haematopoietic progenitors and are characterised by the rapid accumulation of abnormal haematopoietic progenitor cells (HPC) within the BM. In addition to tumours, leukaemic stem cells (LSCs) or leukaemia-initiating cells (LICs),

a subpopulation of AML cells that have long-term repopulating potential, reside in the BM microenvironment and harbour one or more of the oncogenic mutations driving tumourigenesis. Relapse from AML is common and in such patients the cause of leukemic relapse is primarily due to remnant LSCs in the BM following chemotherapy. Leukaemia like most haematological malignancies, can mobilise from bone marrow to blood and the lymphatic system. It's also interesting to note that LICs can also be found in other sites including skin, the central nervous system (CNS) and other organs. [6, 7].

Accordingly, as all AML are dependent on the BM microenvironment, it has been hypothesised that better patient outcomes may come from novel treatment strategies derived from improved understanding of the biology of AML within the BM microenvironment[8]. Importantly, these treatments could be widely applicable to patients with AML across a spectrum of genetic subtypes.

Acute lymphoblastic leukemia (ALL), (another haematological malignancy of a heterogeneous nature, characterised by B-cell and T-cell progenitors) has also been studied in detail for its dependence on the BM microenvironement. While the survival outcomes of paediatric cases of this disease have greatly improved in the last six years, adult cases still carry a severe prognosis [9, 10]. Much like AML, relapses are attributed to the minimal residual disease of a pool of LIC in dormancy which are unaffected by chemotherapies, which target cycling cells. Various interactions between ALL cells and the microenvironment have been implicated in the progression of this disease; one of the most widely studied interactions is that of the BM fibroblasts and the ALL wherein the BM fibroblasts provide better support and survival for the B-cell ALL lineage[11].

In this review, we will highlight the fundamental importance of the BM in normal human haematopoiesis and will further investigate the key role(s) of the cell types therein in providing an environment that contributes to the survival, growth and migration of AML cells. The potential for inhibitory measures against the activity of these non-cancerous BM cells as a means for targeting AML survival will also be explored.

2. The bone marrow microenvironment and haematopoiesis

The BM is a soft viscous tissue that occupies cavities within the bone[12]. It is comprised of blood vessels and a heterogeneous population of cells that are either directly involved in the BM's primary function of haematopoiesis, or act in support of haematopoietic cell function via the cell types surrounding the haematopoietic cells. These supporting cells in the BM all contribute to the stimuli required for regulating normal haematopoiesis (Table 1). The bone marrow stromal cells, also called mesenchymal stem cells (MSC), are responsible for the establishment of the haematopoietic microenvironment as they reside in the bone marrow and give rise to cells such as marrow adipose tissue, bone cartilage and occasionally myofibres that are defined by their ability to differentiate into such cells[13]. A commonly shared view on MSCs is that they are ubiquitous in connective tissue and are phenotypically similar to skeletal progenitor cells and pericytes. In recent years, efforts have been made in this field to clearly define what MSCs are and how they can be better defined. A pioneer in the field of bone and marrow cell biology and development, Bianco et al., has recently identified a progenitor for these BM MSCs and has

redefined them more stringently, based on in vivo differentiation capability, as skeletal stem cells (SSC) [14, 15]. These cells are found on the surface of the blood vessels of the bone marrow (sinusoids) (Figure 1). Bianco et al. went further to prove that MSCs are not ubiquitous, have a different transcriptome for MSCs of different anatomical regions and are identified as CD34-/CD45-/CD146+ cells[17].

Due to the compartmental and heterogeneous nature of its stromal system, the BM is recognised as an organ with two separate yet co-operative systems exhibiting functional co-dependence: the haematopoietic tissue system and its associated supporting stromal tissue system. Originally, much interest was focused on the supporting nature of the stromal system and its contribution to haematopoiesis; however, recent studies have brought to light the unanticipated differentiation potential of stromal cells into special cell types that are phenotypically distinct from cells from the tissue of origin, an attribute termed 'transgermal plasticity' [17] The identification of this characteristic poses an exciting potential in terms of its manipulation for therapeutic applications. Plasticity of BM stromal cells could hold the key in identifying the switch from normal to malignancy-associated stromal cells and thereby identify a new field of therapeutic strategies for BM malignancies.

Haematopoietic stem cells (HSCs) reside in the BM and remain there until maturation. Here, they differentiate along one of two core lineages: the common lymphoid progenitor (CLP) line or the common myeloid progenitor (CMP) line. CLPs and CMPs subsequently differentiate into either leukocytes of the adaptive immune system (T cells and B cells), or cells of the megakaryocyte/erythrocyte lineage and granulocyte/macrophage lineage, respectively.

Table 1. Types of BM cells

Stromal cell type	Location	Function
Adipocytes	Central core that constitutes the yellow marrow	Indirectly but negatively regulate normal haematopoiesis [18]
Endothelial cells	Sinusoidal: Lining the sinusoids that infiltrate the red marrow Arteriolar: Lining of the arterioles originating from arterial vessels entering the marrow cavity through foramina nutricia [19]	Enable the exchange of molecules between the blood and surrounding bone marrow [20]
Fibroblasts	Red marrow	Synthesise structural components of marrow such as collagen [21]
Osteoblasts	Cortical regions of the red marrow	Synthesise bone tissue and regulation of BM angiogenesis [22]
Osteoclasts	Cortical regions of the red marrow	Resorb bone tissue [22, 23]
Chondrocytes	Cambium layer of the periosteum	Cartilaginous tissue synthesis [24]

Collectively, recent investigations have suggested that the BM can be divided into compartments termed ‘niches’ wherein the non-haematopoietic cells interact to influence several HSC functions, including proliferation, differentiation, adhesion and quiescence by producing a variety of cytokines, chemokines and other soluble factors [25], some of which are included in Table 2. The concept of a HPC and HSC niche, the constituents of which regulate cell fate, was first proposed by Schofield in 1978 with further studies highlighting the role of haematopoietic progenitor and stem cell (HPSC) niches in physically anchoring stem cells to the extracellular matrix (ECM) [26]. Recent studies have ascertained the specific regulators of HSCs and their progenitors in the BM, and have uncovered how a perturbation to one cell type can lead to an effect in another cell type without direct physical interaction between the two [27, 28]. The heterogeneous nature of HSCs has given rise to speculation that there could be specialised niches for particular types of HSCs and their progeny within each class of niche [29].

Table 2. AML microenvironment signalling axis

Receptor	**Ligand**	**Function**	**References**
CXCR2	IL-8	Chemotaxis	[30]
CXCR4	SDF-1	Chemotaxis	[31]
IL6R	IL-6	Immune response, haematopoiesis, acute phase response and inflammation	[32]
LFA	ICAM-1	Leukocyte adhesion	[33]

VLA-4	VCAM-1	Adhesion, signal transduction	[34]
RANK	RANKL	Bone remodelling	[35]
FAT/CD36	FFA	Transporter/regulator of fatty acid transport	[36]

3. Haematopoiesis in the BM niche

Many cells and their signals regulate haematopoiesis. Supporting cells within individual niches produce ligands and molecules that interact with their counterparts on the surface of HSCs, which contribute to several cellular functions. Recent studies have pointed towards migration as being of considerable importance across the different niches within the BM. Secretion of CXCL12 (a stromal-cell derived factor) along with other factors including IL-6, IL-8 and MCP-1 by MSCs has been shown to control HSPC retention in the BM[37]. SDF-1 is of particular importance to the retention of HSPCs in the BM through binding and activation of the CXCR4 receptor on HSPCs[38]. CXCR4 belongs to the C-X-C chemokine receptor family and is a G-protein-coupled receptor that is predominantly found on the surface of leukocytes. The primary function of CXCR4 is the regulation of leukocyte trafficking in haematopoiesis as well as during innate or acquired immune responses. By engaging CXCR4, CXCL12 is able to induce a rise in intracellular calcium ion levels, which consequently drives a chemotactic response[39]. The CXCL12/CXCR4 signalling axis has been widely studied in the context of leukaemia. One important study in this field showed that immunocompromised mice models had an increased blast circulation following CXCR4 antagonist introduction which consequently

enhanced the effect of chemotherapy-induced apoptosis. These effects are now being tested in clinical trials [40] . Other studies have implicated CXCL12 and CXCR4 binding in $CD34^+$ cells as a trigger for the production of VLA-4 and lymphocyte function-associated antigen-1 (LFA-1). These in turn induce $CD34^+$ cell adhesion to structures that carry vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Taken together, these studies illustrate the importance of SDF1/CXCR4 interactions in adhesion and retention of cells in the BM as a means of regulating normal haematopoiesis

4. The leukaemia–favourable microenvironment

The promotion of AML survival and proliferation by the BM microenvironment comes at the expense of the development and production of normal haematopoietic cells. Consequently in AML, there is a physiologic failure of the BM to produce adequate numbers of mature blood cells and platelets? and the impedance of maturation of the HSCs within the leukaemic BM[41]. BMSCs (including endothelial cells and fibroblasts) have all been shown to be manipulated by the AML blast[42-44]. This ultimately allows AML to reshape the microenvironment in a way that supports AML blast survival and proliferation. A schematic of the BM cell components and their interactions with the leukemic blast is illustrated in Figure 2.

4.1 Bone marrow stromal cells

Changes in expression of the adhesion molecules, cell cycle regulators and pro-angiogenic factors of BMSCs impart many features to malignant cells including pro-growth, anti-apoptotic and pro-invasive phenotypes[45]. The changes brought about in

the stroma by the presence of leukaemic cells have been shown to reflect alterations in their cytokine and chemokine profiles. BMSC secretion of IL-6, a pro-inflammatory cytokine, has been shown to protect AML via JAK/STAT pathway activation and other pro-survival pathways via integrin-linked kinases[46]. IL-8, a chemokine involved in chemotaxis, has recently been shown to be an important potential target for AML therapeutic strategies[47]. Schinke and colleagues have shown that IL-8 and its receptor CXCR2 were consistently highly expressed in $CD34^+$ cells from the pre-AML disorder myelodysplastic syndrome (MDS). IL-17, a cytokine responsible for inducing and mediating pro-inflammatory responses, has also been implicated in BMSC change with IL-17 signalling-related genes being over-expressed[30, 47]. Indeed, the therapeutic relevance of BMSCs has been demonstrated by several studies that show that co-culture of cancer-associated BMSCs with cancer cells induces proliferation and confers drug resistance [48-50]. This occurs either through intercellular contact or via the secretion of soluble factors[51, 52]. Evidence is accumulating that such alterations may also be relevant to AML, with one of the first studies to examine this demonstrating that direct cellular contact between AML blasts and adherent long-term stroma significantly decreased apoptosis of AML blasts[53]. This was followed by further studies showing that BMSCs provided protection against spontaneous and drug-induced apoptosis through direct contact with HS-5, a human stromal cell line[54]. Another recent study concluded that chemotherapeutic resistance could be conferred by activation of c-Myc in AML cells by the BMSCs[48]. Our own work has identified a novel pathway in which the chemokine macrophage inhibitory factor stimulates the BMSC to produce interleukin-8 which creates a pro-tumoral microenvironment.

The importance of BMSCs in initiating leukaemia was illustrated by experiments in which Phosphatase and tensin homolog (*PTEN)* deletion (tumour suppresser gene) in HSCs alone did not result in a proliferative phenotype; rather it depleted HSCs. By contrast, deletion of *PTEN* in both HSCs and cells of the BM resulted in malignant cell proliferation[55]. One study providing an insight into the interdependent relationship between the non-haematopoietic compartment and the myeloproliferative cells suggests that a pre-malignant state can be instigated by dysregulated non-haematopoietic cells. In this study, selective IκBα deletion in myeloid lineage of cells did not initiate a myeloproliferative disturbance, however a ubiquitous deletion of IκBα led to myeloproliferative disorder[56]. Together, these studies demonstrate the complex relationship between both haematopoietic and non-haematopoietic subpopulations of the BM. It is evident through several studies that the BMSCs play a critical role in the survival, proliferation and protection of leukaemic cells with the derivatives of BMSCs each playing an interdependent role in the sustainability and metastatic preference of these cells.

4.2 Endothelial cells and fibroblasts

4.2.1 Endothelial cells

Anatomically, endothelial cells are located in the sinusoid of the bone in close proximity to other cells types within the BM environment. Their location suggests a role as gatekeepers that regulate the movement of cells between the BM and the circulation[57]. Studies have hypothesised that the vascular network of the BM provided by the endothelial cells may serve as a protective environment that could

be advantageous to AML cells[58]. Matrix metalloproteinases (MMPs) are a group of enzymes structurally related to endopeptidases and are involved in the destruction of the extracellular matrix (ECM) by reabsorbing its macromolecules. They contribute towards connective tissue remodeling but also its pathological destruction. In addition to their structural role in the vasculature, endothelial cells also express E-selectin to which molecules on the surface of leukaemic cells are able to adhere. Another critical receptor of AML homing to and within the BM is very late antigen 4 (VLA-4), which is an α4β1 integrin that facilitates adhesion of AMLs to cellular vascular cell adhesion molecule-1 (VCAM-1). This pathway has been proven to be involved in leukaemic blast adhesion to vessel walls and is a key player in migration and survival of these cells[59]. As well as facilitating the spread of malignant cells, adhesion also encourages endothelial cells to proliferate via the VEGF-activated Notch/DII4 pathway, thereby rewiring the system to promote rampant angiogenesis[60]. In addition to blast survival, retention and proliferation, adhesion of these blasts to tumour-associated endothelial cells protects cells from chemotherapy-induced cytotoxicity[61].

4.2.2 Fibroblasts

The requirement of fibroblasts in AML progression was demonstrated upon co-culture of AML blasts with normal BMSCs and two fibroblast lines (HLF1 and Hs27), which the latter were partitioned by a semi-permeable membrane. In the absence of fibroblasts, the AML cells exhibited reduced proliferation, a reduced ability to evade apoptosis and lower levels of IL-8[43]. This suggests that contact between fibroblasts

and malignant blasts is important for the survival and migration of the cancer cells. EMMPRIN, also known as CD147, is a glycoprotein located on the surface of human tumour cells, and has been shown to stimulate tumour cells and stromal cells to produce higher levels of MMPs, resulting in ECM degradation and elevating tumour growth and metastasis[62]. Studies have shown that EMMPRIN can promote the release of MMP2 from fibroblasts in breast cancer [63] and several other tumour cell types[64]. However, little is known regarding MMP movement and how their activities are controlled once the target has been acquired[65]. Fu and co-workers demonstrated that the co-expression of EMMPRIN and VEGF in AML predicted poor clinical prognosis[66]. Moreover, it has recently been shown that EMMPRIN knockdown in the AML cell line U937, induced apoptosis; demonstrated anti-proliferative effects and also enhanced the activity of the cytotoxic drug Adriamycin[67].

The role of angiogenesis in many types of cancer - including AML - has been widely reported [42, 68, 69] and in theory represents a promising therapeutic target. Pro-angiogenic factors, which encompass VEGF, FGF, IL-8 and the MMPs, are released from BMSCs and osteoclasts, often stimulated by contact between AML cells and BMSCs, or changes at the genetic or transcriptional level. Gene expression signatures that identify a core set of angiogenic genes that may serve to identify angiogenic activity have been developed with broad applicability across many tumour types. These signatures, achieved by an integrative meta-analysis of several cancer types, delineate the underlying transcriptional pathways of angiogenesis. Interestingly, EGF, latrophilin and seven transmembrane domain- containing 1 (ELTD1), an unstudied G-protein coupled receptor and a highly ranked gene in the common angiogenesis signature, was shown to be significantly upregulated in

endothelial cells associated with solid tumours including renal, head and neck, colorectal, and ovarian cancer [70]. This evidence points to the importance of angiogenesis in terms of tumour regulation and the importance of ELTD1's role as a prognostic marker. Endothelial cells and fibroblasts control much of angiogenesis with fibroblasts synthesising the collagen and ECM that (i) provides the critical support required for vascularisation and (ii) releases the pro-angiogenic factors that recruit endothelial cells, which then line the blood vessels that penetrate the tumour mass.

4.3 Osteoclasts and osteoblasts

The homeostatic regulation of bone formation and reabsorption is often disrupted in malignancies of the BM. The result is progressive demineralisation of the bone driven by elevated osteoclast formation[71]. In multiple myeloma, the molecules implicated in this upregulation include RANKL, MIP1a, IL3 and IL6[72]. RANKL is upregulated as a consequence of BMSCs binding cancer cells, which in turn instigates the binding of RANKL to its receptor on the surface of osteoclasts, hence preventing them from undergoing apoptosis[73]. Evidence from allogeneic stem cell transplantation shows RANKL's counterpart, RANK, to be expressed on NK cells, which play a key role in immunosurveillance in AML. Driven by this evidence, recent studies have investigated the involvement of the RANKL/RANK signalling axis in NK and AML cell interaction. A study by Schmiedel and colleagues proposed that a RANKL-mediated "vicious cycle" is able to circumvent NK cell surveillance of AMLs. [35]. This hypothesis was drawn from observation of a feedback loop involving an upregulation of RANK on NK cells. RANKL-induced inhibitory effects allows RANK interaction with AML-expressed RANKL, which subsequently activates a bidirectional

signalling cascade that enables RANK-facilitated inhibitory signals' delivery to NK cells. This maintains a reversal of RANKL signalling in AML cells[74]. Therefore, targeting RANKL may enhance the anti-tumour action of NK cells in AML.

Osteoblasts have been the subject of a direct study in AML wherein immunocompetent mouse models were demonstrated to have reduced levels of osteocalcin, a secretary factor that promotes bone formation[75]. This phenomenon was observed in the absence of a substantial elevation in osteoclast numbers suggesting that unlike in myeloma, the osteoblastic arm in AML is of more importance than the osteoclastic aspect.

4.4 Adipocytes

Adipocytes are MSC derivatives and make up the majority of yellow marrow, which has been observed to expand with age. The abundance and proximity of fat cells to the leukaemic core coupled with work in other tumour types [76-78] has led to the hypothesis that such cells may well be involved in the deregulation of cellular energetics that is a hallmark of cancer. Physiologically, obesity is associated with poor clinical outcomes in leukemic patients [79] suggesting that in the context of cancer, adipose tissue may be a contributing factor to treatment resistance and relapse. In the context of haematology, Han et al. showed that adipose tissue can act as a reservoir for hematopoietic stem and progenitor cells [80] which supports the above notion that adipose tissue contributes to cancer protection and disease relapse. The proinflammatory element of leukaemia associated adipose tissue has been identified as a lipolysis-stimulating factor that contributes to adipose tissue atrophy in cancer[81, 82]. Lipolysis in adipocytes and fatty acid oxidation in AML cells

were the processes discovered to be underlying this phenomenon that has been described as a “metabolic symbiosis”[77]. One recent investigation into the role of adipocytes and free fatty acids in AML has uncovered that free fatty acids from BM adipocytes are able to activate a transcriptional programme that has been correlated with AML cell survival[83]. This investigation also reported fatty acid oxidation-dependent metabolism of the AML cell line U937, when co-cultured with MSC-derived adipocytes. An upregulation of pro-migratory and adhesion protein pathways along with a repression of oxidative phosphorylation was also reported in these co-cultured AML cell lines. Pharmacological inhibition of fatty acid oxidation (by inhibiting carnitine palmitoyltransferase 1a (CPT1a), which is a fatty acid chaperone, into the mitochondria) was reported to decrease the pro-survival effects of adipocytes on AML. Moreover, Lee and colleagues identified avocatin B (inhibitor of fatty oxidation (FAO)) to be a potent inhibitor of AML survival and proliferation[84]. In the context of fatty acid transfer, a recent candidate for targeted fatty acid transfer inhibition is CD36. Ye et. al. have shown that LSCs can be categorised into two distinct $CD36^+$ and $CD36^-$ subpopulations with the $CD36^+$ subpopulation displaying an increased FAO activity and drug resistance profile[81].

Several studies have shown that adipocytes promote resistance of cancer cells to conventional chemotherapies[85, 86]. One study in particular has shown that adipocytes confer breast cancer cell resistance to antibody-dependent cellular cytotoxicity by trastuzumab[87]. These crucial findings suggest that there is crosstalk between the cancer-associated adipocytes and the cancer cells. This is because the transcriptional activation of genes that regulate lipolysis in the adipocytes and fatty acid oxidation in the cancer cells act in concert with several other pathways including migration, adhesion and vascularisation. This hypothesis is further supported by a

study in which leukaemic cell subpopulation resident in the bone marrow adipose tissue are protected from chemotherapy[81].

More recently, our own work and that of another group has shown that AML relies on adipcytes for there survival and proliferation within the BM [83, 88]. Both groups show that the fatty acid chaperoning protein fatty acid binding protein 4 (FABP4) is increased in expression in the BM adipocyte when cultured with AML. Moreover, pharmacoligical targeting or knockdown of FABP4 reverses the protection of the conveyed by the adipocyte to the AML [83, 88]. We highlight through RNA-seq data of leukemic cells isolated from BM, peripheral blood and normal CD34+ cells that expression of FABP4 is also high in leukemic blast which is haboured in the BM. This suggests that directly targeting FABP4 or targting the pathway using inhbitors of B-oxidation is a potential therapy when combined with convential AML chemotherapy.

4.5 CXCR12–abundant reticular cells

CXCL12–abundant reticular cells (CAR) have been identified as high CXCL12 expressing reticular cells in the bone marrow forming a network like structure located in the perivascular region of the bone marrow. These cells surround the sinusoidal endothelial cells or are located near the endosteum and have the potential to differentiate into osteoblasts or adipocytes, forming a specialised niche for the HSCs[89, 90]. As previously mentioned, CXCL12/CXCR4 interaction is of particular interest in leukaemia in terms of its role in adhesion and migration. CXCR4 is expressed in both myeloid and lymphoid lineages of leukaemic cells, with its ligand SDF-1 secreted by the stromal cells in the bone marrow. Tavor et. al. have shown

these cells to be of significance for the collective retention of leukaemic blasts within the bone marrow [91] and targeting CXCR4 has been shown to upset migration and retention in the bone marrow, thus making it more susceptible to cytotoxic therapies [59, 92]. Furthermore, these cells also express adipocyte-associated PPARγ and osteoblast differentiation-associated transcription factor RUNX2 and Osterix, the disruption of which significantly impacts the number of HSPCs and the B cell and erythroid progenitors. The abundant CXCL12 release from these cells and the role of their differentiating capacity along with the release of various lineage dependant cytokines make these cells viable candidates for targeting factors crucial for leukemic blast retention and migration.

4.6 Sympathetic Neural cells

It has long been known that the arterioles that compose the BM microvasculature are innervated by the sympathetic nervous system (SNS)[93]. Accordingly, for many years the function of the SNS within the BM context was believed to be associated with the BM cell mobility[94]. Over 30 years ago, a study in which the BM was denervated produced an increase in the number leukocytes circulating in the peripheral blood [95]. Since then, there have been a number of studies conducted to investigate the role of adrenergic modulation of haematopoiesis. Despite these efferts, the role of sympathoadrenergic modulation of haematopoiesis is underexplored.

Recent evidence has shown that mobilisation of HSCs is enhanced by chemical stimulation of the β_2 adrenergic receptor[94]. The same study also showed that adrenergic neurotransmission controls granulocyte-colony stimulating factor (G-CSF)-induced mobilisation of HSCs, thus, further emphasising the role of the SNS in

migration of BM stem cells within their microenvironment. In the context of leukaemia, malignancy-containing BMs have been shown to have a reduced number of sympatho-adgrenergic fibres and supporting MSCs in mice that harbour human *JAK2* mutations. This reduction in MSCs results in the release of cytokines that favour the proliferation of abnormal HSCs within the BM, thereby accelerating the course of the disease. Upon treatment with β_3-adrenergic receptor stimulators, supporting MSCs are restored due to an apparent regulation by the restored sympathetic systems therein[96]. This report identified regulation of the neural capacity in the BM as a potential therapeutic target. Another recent study, that complements the above, investigated the role of the SNS in AML and described a novel mechanism by which leukaemic cells take advantage of the microenvironment and succumb to sympathetic neuropathy. The authors found that chemical removal of adrenergic nerves resulted in increased levels of leukaemic cell infiltration. This created a remodelled environment that favoured leukaemic cell expansion and malignancy-associated MSCs at the expense of healthy HSCs and their accessory cells[97]. Taken together, manipulation of the SNS can potentially preserve healthy HSCs and limit LSC development, and thus represents a promising therapeutic target.

5. Therapeutic opportunities in targeting AML and the BM microenvironment

Current and prospective systemic therapies for AML can be divided into at least three types: non-selective chemotherapies, immunotherapies and AML cell-targeted

therapies such as kinase inhibitors. Chemotherapies are typically administered with the aim of depleting the BM cell population. Stem cell or BM transplants may follow the depletion-based approach in selected patients. The primary drawback of chemotherapy is rooted in its non-selectivity, which results in a relatively high adverse effect profile that is often intolerable for older, frailer patients. Despite holding much promise, immunotherapies for AML are in the very early stages of development, are costly and may be very poorly tolerated in certain subpopulations of patients[98]. Notwithstanding some success, trials of AML cell-targeted therapies have been beset by the development of resistance in substantial numbers of AML patients. The genetic heterogeneity among the malignant cells of a substantial proportion of AML patients enables the rapid evolution of cellular mechanisms that confer resistance[99].

For this reason, two related albeit alternative approaches have been proposed: one that targets the LSCs that constitute the seed, and another that targets the BM microenvironment that is the soil to the LSC seed[100].

Table 3. Potential Inhibitors of signalling axis within the BM microenvironment

Targets	Potential inhibitors	Mechanism	References
Angiogenesis	Bevacizumab	Monoclonal antibody that binds to VEGF and blocks receptor binding.	101
	Combretastatin	Tubulin binding agents that induce vascular-	102, 103

		mediated necrosis	
	CD147 inhibitor	EMMPRIN silencing has shown to inhibit leukemia proliferation and increase chemosensitivity in vitro	[67]
	Sunitinib	Inhibits VEGFR1 and VEGFR2 signalling by inhibiting RTK	[104]
	Trenananib	Ang-1/2 neutralising peptide inhibiting its binding to Tie2 receptor	[105]
Migration	Ibrutinib	BTK inhibitor and inhibits AML migration and adhesion by targeting CXCR4/CXCL12 axis.	[92]
	AMD3100	CXCR4 antagonist and HSC mobilising agent.	[106, 107]
	SB-332235	Competitive inhibitor of CXCR2 over CXCR1. Block IL-8/CXCR2 binding and decreases	[30]

		AML viability.	
FAO Metabolism	Sulfosuccinimidyl oleate	Inhibits fatty acid uptake and sensitises leukemia to chemotherapy	[81]
	3-KAT inhibitors	Inhibits the catalysis of the last step of FAO	[108]

5.1 Targeting LSCs: the seed

There is now clear evidence that the leukaemic microenvironment is essential for the growth and proliferation of AML. However, there is the identification of features of LSCs that are potentially druggable and consistent in their absence on normal HPSCs is one selective way of targeting malignant cells in AML. One of the first characterisations of LSCs was conducted by Bonnet and colleagues in AML[109]. They described a subpopulation of $CD34^+$ $CD38^-$ human AML cells, which were able to constantly and progressively relocate themselves in a mouse xenograft model. Recently, Taussig and colleagues demonstrated that LSCs exhibit considerable phenotypic heterogeneity, hinting at a presence in different fractional populations with varying intensities of CD34 and CD38 expression, and not exclusively in a $CD34^+$ $CD38^-$ population[110]. This work highlighted the need to identify aberrant surface antigen expressions that specify LSC populations that can facilitate clinical monitoring strategies and the detection of minimal residual disease. These aberrant surface antigens can be used as markers for differentiating between healthy HPSC and LSCs. Therefore examining the LSC markers to identify novel targets are being

studies for potential immunotherapies. Recently, CD123 has shown promise in this regard as a marker specific to LSCs. CD123 is the IL-3RA receptor involved in proliferation, growth and differentiation of HPSCs. It was first reported as being expressed on the $CD34^+$ $CD38^-$ cells of AML patients but not on the $CD34^+$ $CD38^-$ cells of normal patients. Subsequent to this finding, Jordan and colleagues also showed that NF-κB activity was higher in $CD123^+$ $CD34^+$ AML cells than in $CD123^+$ $CD34^+$ normal HPSCs[111]. CD47 is another among the repertoire of promising LSC-specific markers demonstrated to have been linked with worse prognosis. Under normal homeostasis, this molecule is only expressed on HPSCs when these cells migrate out of the endosteal niche. The therapeutic potential of this target was revealed by monoclonal antibody blockade of CD47, which led to an elevation of phagocytosis and a reduction in LSC engraftment. The resistance-conferring capacity of LSCs has also come under scrutiny as a potential target for AML therapy. In order to confirm the localisation of the resistance-conferring capacity of AML, Ishikawa, et al. used immunodeficient/interleukin (NOD/SCID/IL)2r gamma(null) mice to show that LSCs are able to engraft AML and retain their self-renewing capability *in vivo* where they move to osteoblastic area of the BM, become quiescent and are protected from chemotherapy-induced apoptosis[112]. Saito and colleagues next demonstrated that the LSCs of these drug resistant osteoblastic regions enter the cell cycle upon in vivo treatment with G-CSF. This induction of AML LSCs into the cell cycle sensitises them to chemotherapy-induced apoptosis and lengthens their survival time in patient-derived xenograft models[113]. Taken together, these findings strongly suggest that quiescent AML LSCs underpin chemotherapeutic resistance and that targeting of the BM niches where these quiescent LSCs reside with agents such as G-CSF may have therapeutic value. In spite of this potential, many of the

markers discussed above are not present in all LSCs or in any LSCs of certain AML patients. This combined with the possibility that LSCs may harbour the genetic instability to rapidly adapt and overcome such targeted approaches, means that alternative, possibly complementary means of treating AML are much needed.

5.2 Targeting the BM microenvironment: the soil

As previously described, the BM microenvironment provides an environment that promotes the survival, differentiation, proliferation and migration of HPSCs and their progeny. However, not only is this microenvironment a harbour for normal haematopoiesis, it also provides a rich ecosystem for LSCs to proliferate as well as serving as a sanctuary for these malignant cells from chemotherapy[25, 114-116]. The relative genetic stability of normal BM cells [117, 118] coupled with the targeted approach needed to disrupt the interaction between AML cells and their environment may prevent drug resistance and side effect issues inherent to chemotherapeutic options. The routes to targeting the BM microenvironment's support of AML cell function can be divided into at least three forms that can be described in terms of Hanahan and Weinberg's updated hallmarks of cancer[119]. Deregulation of cellular energetics is a feature of tumours that is intimately linked with the tumour microenvironment[119]. Therapeutic opportunities for disrupting the supply of energy from free fatty acid metabolism abound with many drugs already approved for use in humans as treatment for cardiovascular-related diseases. Two enzymatic targets that may be of particular promise are CPT1, the rate-limiting enzyme of fatty acid oxidation, and 3-ketoacylthiolase (3-KAT), which catalyses the last step in this process. These two pharmacological targets have shown promising effects in mice models however, use of CPT-1 inhibitor, etomoxir which is widely used in vitro and in

mice models - has shown to result in hepatotoxicity in humans. Other CPT-1 inhibitors are still under pre-clinical assessment. 3-KAT inhibitors (Trimetazidine and Ranolazine), are currently approved for use in humans and have been used for the treatment of angina in some countries[108].

Another BM microenvironment-linked hallmark with potential as a target in AML is angiogenesis, which is promoted to facilitate the continuous delivery of oxygen, nutrients and growth factors to the ever-expanding population of malignant cells. Inhibiting angiogenesis has been conducted using a plethora of approaches, which include tyrosine kinase inhibitors, antibodies that neutralise VEGF receptors and other novel drugs such as statins which may indirectly affect the VEGF pathway[120-122]. Bevacizumab – which has been approved for the treatment of solid tumours – is a monoclonal antibody that binds to a VEGF isoform and blocks it from binding to its receptor[101, 123]. However, a recent randomised trial of Bevacizumab in AML patients alone and in combination with standard chemotherapy did not show any improvement in the therapeutic outcome[124]. Another promising angiogenesis inhibitor currently under trials is Combretastatin. It is a vascular disruption agent that induces mitotic arrest in proliferating endothelial cells. It is currently an experimental treatment under phase 1 clinical trials in AML patients showing promising results and is well tolerated[102]. Prominent angiogenesis factors within the leukaemia marrow are angiopoientins 1 and 2 (Ang-1/2) which are now subject of angiogenesis inhibition by neutralising Ang 1/2 antibody, Trenananib. This first-in-class neutralising antibody showed promising preliminary outcomes similar to that observed in solid tumors and is under further evaluation[105].Inhibition of angiogenesis via interruption of receptor tyrosine kinases is also currently being investigated. Sunitinib, which is an inhibitor of several receptor tyrosine kinases (and therefore inhibits angiogenesis by

antagonising signalling via VEGFR1 and VEGFR2) has been approved for several solid tumours; however, recent *in vitro* and *in vivo* studies in AML showed a marked decrease in VEGF, thus production warranting clinical trials in AML patients[104].

The capacity to invade and metastasise is a unique feature of malignant tumours. To achieve this, tumour cells enlist a variety of mechanisms including chemotaxis and adhesion. Chemokine molecules such as CXCR4, adhesion molecules such as CD44 and VLA4, and integrin are candidate targets of the BM microenvironment that have been shown to interact with the LSC niche and allow tumour migration[125, 126]. Using a CXCR4 antagonist, AMD3100, studies have shown an elevated white blood cell count as well as mobilisation of leukaemic blasts in the peripheral blood where they can be subjected to the cytotoxicity of chemotherapeutic drugs[106, 107]. Moreover, work in our laboratory shows in vitro that the BTK inhibitor, ibrutinib, inhibits AML adhesion and migration to BMSC, hence the initiation of clinical trials of ibrutinib in AML[92, 127]. Although these approaches have yet to be fully explored, the promise of an effective and durable response necessitates further study analysing the relationship between AML and its microenvironment. Table 3 provides a summary of potential inhibitors of these identified interactions.

6. Summary

Understanding the BM niches, their constituents and the mechanisms at play may hold the key to the development of methods/treatments that can directly affect the ability of LSCs to drive malignancy and avoid therapeutic-mediated destruction. Although systemic therapeutic approaches generally revolve around the direct elimination of malignant stem or progenitor cells, recent studies have shown that targeting abnormalities of the BM may have value[128]. Studies into how migration of quiescent HPSCs from the osteoblastic niche to the vascular niche wherein they acquire the ability to proliferate and differentiate may provide the key to the development of novel therapeutic approaches in the future.

Practice Points

- Clinical out-comes and prognosis in the aging population suffering from AML is poor as medical co-morbidities as well as a reduced haematopoietic reserve within the aging bone marrow present limiting factors.
- Patient relapse following remission is due to minimal residual disease harboured within the bone marrow which demonstrates the importance the leukaemic BM microenvironment in the retention and protection of this disease.
- Non- haematopoietic component of the bone marrow contributes to several functions such as migration, adhesion, metabolism and differentiation.

Research Agenda

- Dissection of the multi-faceted role played by the BMSC lineages and their non-malignant counterparts in the survival and regulation of AML.
- The genetic differences between the leukaemic and non-leukaemic BMSC may identify potential biomarkers that play a role in resistance and other cellular functions enhanced by the BM microenvironment.
- Targeting the BM microenvironment to limit blast malignancy and metastasis without non-selective destruction of haematopoietic tissue is a more attractive therapeutic strategy in both young and old AML populations.

Conflict of Interest

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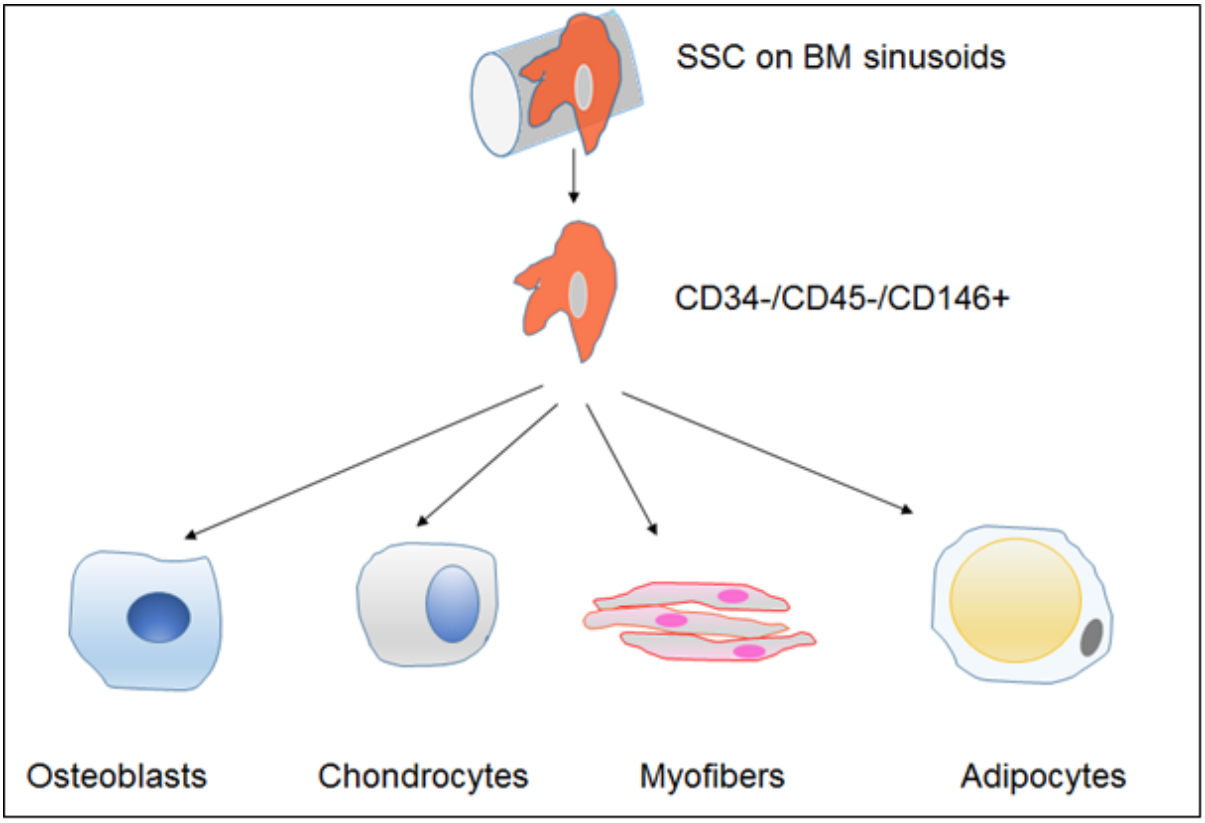

Figure 1. Skeletal stem cells CD146+ found in the BM are able to differentiate into osteoblasts, chondrocytes myofibers and adipocytes.

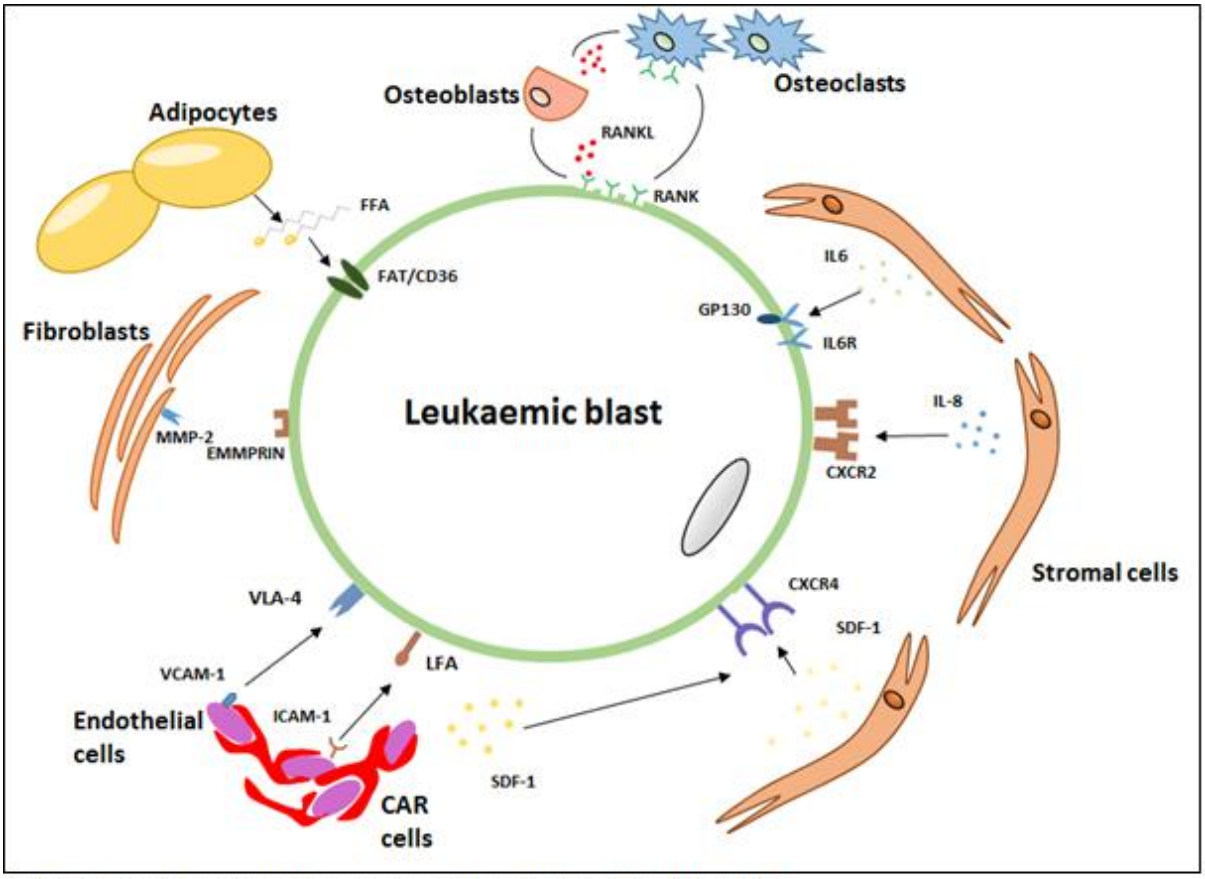

Figure 2. Leukaemic blast interaction with cells of the BM.