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The bone marrow microenvironment as a sanctuary for minimal residual disease in CML

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Abstract

Bcr-abl kinase inhibitors have provided proof of principal that targeted therapy holds great promise for the treatment of cancer. However, despite the success of these agents in treating chronic myelogenous leukemia (CML), the majority of patients continue to present with minimal residual disease contained within the bone marrow microenvironment. These clinical observations suggest that the bone marrow microenvironment may provide survival signals that contribute to the failure to eliminate minimal residual disease. The bone marrow microenvironment is comprised of multiple sub-domains which vary in cellular composition and gradients of soluble factors and matrix composition. Experimental evidence indicates that exposure of tumor cells to either bone marrow derived soluble factors or matrixes can confer a multi-drug resistance phenotype. Together, these data indicate that targeting such pathways may be a viable approach for increasing the efficacy of chemotherapy. Moreover, we propose that personalized medicine must go beyond understanding predictive models inherent to tumors but rather build predictive models that consider diversity in response due to interactions with the tumor microenvironment. This review will focus on CML, however, understanding the contribution of the bone marrow microenvironment could contribute to rationale combination therapy in other types of leukemia, multiple myeloma and solid tumors which metastasize to the bone.

Keywords

Chronic myeloid leukemia; Drug resistance; Tumor microenvironment; CAM-DR; BCR-ABL

1. Introduction

Chronic Myeloid leukemia (CML) is a hematopoietic stem cell disease that is characterized by the existence of the BCR-ABL fusion oncogene resulting from the reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)) [1,2]. This single chromosomal abnormality called the Philadelphia chromosome (Ph) is the cytogenetic hallmark of more than 90% CML cases and about 25–30% acute lymphatic leukemia (ALL) cases. The expression of BCR-ABL oncogene, a chimeric bcr-abl protein with deregulated tyrosine kinase activity, is necessary and sufficient for the pathogenesis of CML [3]. The bcr-abl oncoprotein, unlike the primarily nuclear c-abl, is distributed throughout the cytoplasm and interacts with various proteins involved in signal transduction pathways leading to deregulated proliferation, differentiation and survival [4]. Progression of CML follows three distinct phases, namely the early indolent chronic phase, the intermediate accelerated phase and the terminal blast phase. The treatment goal for CML patients is to

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maintain remission and prevent relapse and disease progression to accelerated phase or blast phase.

STI571 or Imatinib mesylate (IM) was the first molecularly targeted therapy that was rationally designed to specifically inhibit bcr-abl tyrosine kinase activity [5]. IM interacts with the ATP-binding site of bcr-abl, thereby suppressing downstream signaling. The recent phase 3 International Randomized Study of Interferon and STI571 (IRIS) trial has confirmed the long-term efficacy and safety of IM, and it is currently the recommended first line therapy for CML in chronic phase [6-9]. However, despite the effectiveness and good tolerability of IM, drug resistance does emerge due to both intrinsic/innate and extrinsic/acquired factors. Intrinsic factors include point mutations in the abl-kinase domain, BCR-ABL gene amplification, BCR-ABL overexpression, clonal evolution and persistence of CML stem cells [10-13]. Extrinsic factors include decreased drug bioavailability, microenvironmental factors like cytokines, and growth factors leading to decreased apoptosis and increased survival, independent of bcr-abl mediated signaling and development of multidrug resistance in sanctuary sites like the bone marrow (BM) [12].

To overcome IM resistance in CML, more selective second generation bcr-abl kinase inhibitors like Nilotinib (NI) and a dual kinase inhibitor of bcr-abl and src kinases called Dasatinib (DA) were developed [14,15]. However, in clinical practice NI and DA failed to achieve sustained remission in IM-resistant CML blast crisis and ALL [16,17]. Hence, even though the targeted therapy against bcr-abl kinase induces hematologic and cytogenetic remission in chronic phase CML patients, the majority of patients still harbor minimal residual disease (MRD) that, if unchecked leads to relapse of CML.

For many hematopoietic tumors, including CML, following drug treatment, MRD is found in the BM compartment. Indeed, measuring Wilms' tumor 1 (WT1) gene transcript a marker for the diagnosis of MRD, has demonstrated that the WT1 expression level in peripheral blood was approximately 10 times lower than that in BM, regardless of the type of leukemia [18]. Consistent with this observation is the finding that bcr-abl transcripts in BM samples exceed the levels in the corresponding peripheral blood samples by at least 1 log unit in patients with bcr-abl positive ALL [19]. These clinical observations suggest that the BM microenvironment provides a very important protective sanctuary that contributes to drug resistance. In the following review, we describe the BM microenvironment including its various components and their functions, followed by a brief description of how the CML cells interact and manipulate this BM microenvironment to evade cell death and maintain MRD. Finally, we enumerate the various strategies utilized to overcome MRD, and thus potentially add to the arsenal of therapeutic tools that can be utilized along with standard therapy in order to seek a cure against CML.

2. Bone marrow architecture/microenvironment

Bone marrow (BM) is a hematopoietic organ that resides within the protected confines of the bones and is the major location for hematopoiesis. In adults, 4.6% of body weight is due to the BM that is distributed throughout the vertebrae, ribs, pelvis, skull and proximal ends of the long bones [20]. The fundamental objective of the BM is to maintain the numbers of differentiated hematopoietic cells in the peripheral blood at a constant level throughout the life time of a human being. The BM does this by producing an estimated 500 billion cells per day [21].

The structure of the BM consists of a rigid bone cortex enclosing a cavity containing the arterial vascular system, a complex sinusoidal system, hematopoietic cells and the stroma. Arterial vessels enter the BM and divide into arterioles and capillaries that span throughout the BM. These small vessels then supply sinusoids, which are radially distributed around a

draining central sinus which in turn follows into the emissary vein and finally joins the systemic venous circulation [21]. The BM sinusoids are unique, in that they are completely devoid of supporting connective tissue and, instead, the sinusoidal wall is made up of an overlapping single layer of endothelial cells [22]. Because of the lack of connective tissues, the major supporting structure for the sinusoids is the surrounding BM cells. Even with this blood supply, direct measurement of oxygen levels has revealed that BM, in general, is hypoxic (1–2% O_2) [23]. The cellular component of the BM can be divided into hematopoietic cells and mesenchymal-derived cells. The mesenchymal-derived cells, along with the macrophages and the extracellular matrix (ECM), form the BM stroma. The BM hematopoietic cells consist of hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and mature plasma cells. The HSCs and HPCs are held within the BM stroma not only through the adhesive interactions between VCAM-1, expressed by stromal cells, and integrin α4β1 expressed by the HSCs and HPCs [24,25], but also by the chemotactic interaction between the chemokine CXCL12 and its receptor CXCR4 expressed on the HSCs and HPCs [26,27]. Interestingly, BM is also innervated by myelinated and unmyelinated nerve fibers [28]. The following sections will discuss the components of BM and describe how it may contribute to tumor cell survival and drug resistance.

3. Hematopoietic stem cell niche

HSCs have the ability to differentiate into all myeloid and lymphoid cell lineages and reproduce the entire hematopoietic and immune systems. In steady-state conditions the majority of the HSCs reside in the BM with a few circulating in the peripheral blood [29,30]. Schofield identified the first HSCs in the BM, and he later proposed that HSCs needed to be localized in a particular niche within the BM to maintain their self-renewal capacity [31,32]. Indeed a large body of evidence suggests that the HSCs are localized in a nonrandom manner at the bone–BM interface (Osteoblastic niche) and around the blood vessels (vascular niche) [33-36]. In contrast, the more committed progenitors accumulate in the center of the BM [33,34].

3.1. Osteoblastic niche

The majority of HSCs in the endosteum are found to be in direct contact with the osteoblasts, and there is genetic evidence that increased bone formation leads to an increased number of HSCs in the BM [37,38]. For example, transgenic mice carrying an inducible deletion of the bone morphogenic protein receptor 1A, or mice expressing a constitutively active mutant of the parathyroid hormone receptor 1 in the osteoblasts, result in an increased number of osteoblasts and doubling of HSCs despite the fact that these mice have reduced BM cavity due to increased bone formation [35,37,38]. On the other hand, Visnjic et al. showed that in transgenic mice carrying the herpesvirus thymidine kinase suicide gene driven by an osteoblast-specific promoter, such that administration of ganciclovir specifically induces osteoblast death, a decrease in osteoblasts results in a reduction of HSCs in the BM [39]. These studies demonstrate the role of osteoblasts in the maintenance and survival of HSCs.

The HSCs home in on the endosteum with the help of a chemotactic Ca^{2+} sensing receptor that senses the Ca^{2+} gradient within the BM. The gradient is created by the release of Ca^{2+} in the BM due to the constitutive osteoclasts and osteoblasts-mediated bone remodeling [40]. On reaching the endosteum, the HSCs are held in the endosteum by the expression of adhesive molecules like osteopontin, N-cadherin, transmembrane c-KIT ligand stem cell factor and the polysaccharide hyaluronic acid [40-43] (Fig. 1). In addition to this, the interaction is strengthened by the cytokines and chemokines that are produced by the osteoblasts, like chemokine CXCL12, angiopoietin-1, the ligand for the tyrosine kinase receptor Tie-2 expressed by HSCs, and jagged-1, a ligand for Notch1 receptor also

expressed on HSCs [35,37,42]. Finally, these interactions regulate HSC self-renewal by controlling the survival, quiescence and proliferation of HSCs.

3.2. Vascular niche

In addition to the osteoblastic niche, a vascular niche was identified in the BM by using the signaling lymphocyte activation molecule (SLAM) family of receptors ($CD150^{+}CD48^{-}CD41^{-}$) [44]. The SLAM family are cell-surface proteins that act as adhesion and signaling receptors and are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness [45]. Interestingly, the HSCs in the vascular niche are devoid of N-cadherins and were specifically located adjacent to cells expressing high levels of CXCL12, which in turn surrounded the sinusoidal endothelial cells. The importance of CXCL12-CXCR4 interaction in the maintenance of the vascular niche was demonstrated by showing that a depletion of CXCR4 led to a reduction of the HSC pool [46]. One of the major physiological differences between the two niches is the high oxygen levels in the vascular niche compared to the hypoxic region of the osteoblastic niche [47] (Fig. 1). It has been postulated that the quiescent HSC located in the hypoxic region of the osteoblastic niche, on stimulation, could move to the vascular niche where the increased oxygen levels would induce the HSCs to undergo differentiation and provide the peripheral blood supply with the necessary mature hematopoietic cells [48]. When the differentiated hematopoietic cells are not required, HSCs move back to the endosteal niche and revert back to their original quiescent state, there by maintaining a constant number of differentiated hematopoietic cells in the peripheral blood [48].

4. Cancer stem cells and drug resistance

Cancer stem cells have been described in a number of cancers including acute myeloid leukemia (AML), breast cancer, brain cancer, colon cancer, pancreatic cancer and CML [49-54]. CML, is a stem cell derived hematopoietic cancer that can be effectively treated with the use of bcr-abl kinase inhibitors. However, failure to eliminate MRD found in the BM compartment due to the existence of the CML stem cells (that closely resemble normal HSCs), leads to the relapse of the disease [55]. *In vitro*, it was demonstrated that osteoblasts or osteoblast-like cells seeded on the bio-derived bone scaffold could maintain stem/progenitor cells from CML bone marrow primary cells in a long term culture [56]. *In vivo*, the CML stem cells utilize CD44 to home into and subsequently engraft in to the BM HSC niche [57]. Once within the BM, the CML stem cells utilize the survival pathways that are active in normal HSCs.

In CML stem cells, the resistance to apoptosis begins with quiescence, which favors survival against bcr-abl tyrosine kinase inhibitors that rely on cell cycle-dependent apoptotic machinery to induce cell death [58]. This was nicely demonstrated in an *in vitro* study where quiescent IM-resistant CML cells were rendered sensitive to IM by simply inducing cell cycle progression [59]. Tissue specific-stem cells, including HSCs, have an inbuilt characteristic of up regulating genes that translate to proteins involved in drug efflux and detoxification. Not surprisingly, when compared to mature CML cells, CML stem cells have decreased levels of the organic cation transporter-1 (OCT-1), a transporter involved in the active uptake of IM, and increased levels of drug-efflux-related surface molecules, including the multi-drug transporter, MDR1 [60,61]. Furthermore, it has been shown that pretreatment OCT-1 expression, but not expression of drug efflux transporter, was the most powerful predictor of complete cytogenic response achievement in CML [62].

Moreover, CML stem cell populations not only have higher BCR-ABL transcript levels than mature CML cells, but they are also composed of different subclones carrying different drug resistance bcr-abl kinase domain mutation [63-67]. Thus, the progeny of CML stem cells,

containing wild type bcr-abl, initially succumb to therapy with bcr-abl kinase inhibitors. However, with longer treatment there can be the emergence of bcr-abl kinase mutants through the process of subclone selection which in turn leads to drug resistance [61,64].

Another important feature of HSCs is their capacity for self-renewal through the Wnt/β-catenin and hedgehog activation pathways [68,69]. Sonic hedgehog was shown to induce expansion of CML stem cells and the Wnt/β-catenin pathway was found to be activated in granulocyte–macrophage progenitors, which resulted in the acquisition of 'stemness' in these CML cells [70,71]. This property of self-renewal ensures that there is always a reservoir of CML stem cells that can be activated to proceed and maintain a CML disease state. In summary, in CML a complete cure can only be established by abolishing MRD which in turn can only be achieved by the eradication of all the CML stem cells and their sub clone population. However, since the pathway for survival and self-renewal in HSCs and the CML stem cells are the same, elucidating the differentially regulated pathway will be critical for devising therapies that eradicate CML stem cells while sparing normal stem cells.

5. Overcoming cancer stem cell mediated drug resistance

Most of the conventional drugs currently used target actively dividing cells and not the quiescent cancer stem cells. Thus, even if the tumor burden is decreased after chemotherapy because of death in the actively dividing tumor cells, drug therapy leaves the quiescent tumorogenic cancer stem cells to survive leading to the reemergence of the disease state. In light of this, an attractive strategy would be to coax the cancer stem cells into cell division. As proof of principle, Ito et al. showed an improvement in the treatment outcome of CML by enhancing the cycling of quiescent leukemia stem cells with the induction of oxidative stress, using Arsenic trioxide (As_2O_3) [72]. In this study, not only did the number of quiescent leukemic stem cells decrease significantly but more of the stem cells entered the cell cycle when compared to normal stem cells following As_2O_3 treatment. The mechanism for this observation was attributed to the As_2O_3-induced degradation of promyelocytic leukemia protein (PML), which was found to be essential for HSC maintenance. Furthermore, *in vivo* addition of cytosine arabinoside (Ara-C) following As_2O_3 treatment induced significant cell death in the leukemic stem cell population resulting in complete remission of CML in recipient mice in a serial transplantation assay [72]. Surprisingly, Holtz et al. has shown that combining IM with As_2O_3 or Ara-C did not induce apoptosis in non-proliferating CML $CD34^+$ progenitors, indicating the use of caution when combining different drug regimens [73].

In addition to inducing cell cycling, oxidative stress also has an effect on the long term maintenance of HSCs. For example, repression of $p16^{lnk4a}$ and $p19^{Arf}$ senescence pathways promotes HSCs cell survival and self-renewal [74,75]. Oxidative stress can affect the long term HSC capacity for survival and self-renewal thorough the upregulation of tumor suppressors $p16^{lnk4a}$ and $p19^{Arf}$. Treatment with a p38 mitogen-activated protein kinase (MAPK) inhibitor or the antioxidant N-acetyl-L-cysteine blocked the oxidative stress-induced increase of $p16^{lnk4a}$ and $p19^{Arf}$ [76,77]. This suggests that oxidative stress induces the HSC-specific phosphorylation of p38 MAPK and this activation leads to defects in the maintenance of HSC capacity for self-renewal. However, it should be noted that oxidative stress does not differentiate between normal HSCs and CML stem cells, and a targeted therapy involving conjugation of oxidative stress inducing molecule with an antibody that specifically recognize CML stem cells might hold promise. An interesting candidate for targeted therapy may be interleukin (IL)-3 receptors, which is expressed in high abundance on the leukemic stem and very rarely on normal HSCs [78]. Another strategy to specifically target the CML stem cells was suggested in a study by Dierks et al., wherein they show that

Smo, a seven-transmembrane domain receptor protein involved in the hedgehog pathway, is specifically upregulated in CML cells and is essential for the expansion of the CML stem cell pool [79]. Interestingly, pharmacological inhibiton of Smo did not impact regular hematopoiesis, but it drastically reduced the CML stem cells *in vivo*. These results indicate that Smo, is a specific and 'druggable' target for CML stem cells that can help eradicate CML.

IM-mediated inhibition of the bcr-abl kinase activity in CML cells leads to suppression of all bcr-abl downstream signaling. This in turn induces a pro-apoptotic milieu that overcomes the pro-survival signaling of bcr-abl, ultimately resulting in cell death [80,81]. But, CML stem cells have high transcripts of BCR-ABL and resistance can arise due to inability of the drug to inhibit all the bcr-abl kinase activity. Dose escalation of bcr-abl kinase inhibitors might be a clinical strategy to circumvent this problem. Indeed, retrospective analysis of IM patient dose escalation in the IRIS trials supports this strategy [82]. Unfortunately, a more thorough investigation, namely the Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) study demonstrated that at the end of one year there was no significant difference in the cytogenic response between patients getting the standard dose of IM (400 mg/day) and the higher dose of IM (800 mg/ml) [83]. Switching to a more potent drug than IM, such as NI (30-fold more potent) or DA (325-fold more potent), is an option in IM-resistant CML patients [84,85].

The phenomenon of the 'emergence' of CML stem cells with point mutations in the ATP kinase domain is exhibited in patients receiving IM treatment [13,86]. Of the more than 40 different mutations that have been described, the most worrisome mutation is T315I, which renders the CML cell insensitive to any of the clinically available BCR-ABL kinase inhibitors [87-90]. Multi-kinase inhibitors like MK-0457 (VX-680), a potent aurora kinase and JAK2 inhibitor, PHA-739358 a pan-auroroa kinase inhibitor, and DSA compounds which are potent inhibitors of ABL, BCR-ABL T315I and src have shown very encouraging results in CML cells carrying the T315I mutations [91-93]. A novel compound referred to as DCC-2036 is a switch pocket inhibitor and binds to a regulatory site distant from the catalytic kinase domain of ABL and thus successfully circumvents the binding issues with mutations in the kinase domain [94]. Similarly, a selective allosteric bcr-abl inhibitor, GNF-2 and its N-hydroxyethyl carboxamide analogue, GNF-5 were shown to display enhanced inhibitory activity against T315I mutant in cellular assays and also in a murine BM transplantation model, when used in combination with IM [95]. Future and ongoing clinical trials will tell whether any of these agents will help eradicate CML.

The intracellular concentrations of bcr-abl kinase inhibitors in the CML stem cells can be increased by combining them with modulators of drug transporters. The efficiency of such combination in reversing drug resistance was demonstrated by Illmer et al., who showed using a novel high-performance liquid chromatography-based method, that intracellular levels of IM decreases in P-glycoprotein (Pgp)-positive leukemic cells. However, modulation of Pgp by cyclosporin A readily restored IM cytotoxicity in these cells [96]. Also, OCT-1 has been demonstrated to have a significant impact on the drug availability through inhibition of IM influx [97]. Since neither NI nor DA's cellular uptake is significantly affected by OCT-1 activity, examining the OCT-1 status of the patient prior to start of therapy may lead to a better clinical outcome of the patient [98,99].

Before the advent of bcr-abl tyrosine kinase inhibitors, inter-feron-α (IFNα) was used to treat CML with variable efficiency. Interestingly, IFNα is the only anti-leukemic drug that has more effect on CML stem cells than the conventional bcr-abl tyrosine kinase inhibitors. Whereas IFNα has little effect against the bulk of CML tumor burden, *in vitro* it can induce CML stem cells to differentiate [100]. To date, the only long term sustainable remission has

come from IFNα therapy. For example, 8% of patients treated with IFNα alone have maintained long-term disease-free status without any therapy [101]. Also, patients treated with IFNα who reached complete cytogenic response with follow up IM treatment remained disease free after more than 2 years without any type of therapy [102]. Taken together, these data indicate that a combination of IFNα with bcr-abl tyrosine kinase inhibitors would perhaps eliminate CML stem cells and thus eradicate CML.

Allogenic HSC transplant can also eliminate the primary refractory CML stem cells. HSC transplant in patients with drug resistant CML is the only established stem cell eradicating therapy in CML [103]. It has been hypothesized that in drug resistant patients one of the therapies detailed above can be utilized to induce remission which can then be followed by an allogenic transplant. After transplantation, these patients could be continued on a maintenance therapy utilizing novel bcr-abl tyrosine kinase inhibitors. However, this hypothesis and the success of such therapies remain to be investigated.

6. Bone marrow stroma

The BM stroma is a heterogeneous population of cells that as a whole contribute to hematopoiesis. The different components of the BM stroma include adipocytes, reticular cells, macrophages, vascular endothelial cells, smooth muscle cells and mesenchymal stromal cells (MSCs) [104]. This heterogeneous population is responsible for not only the production and deposition of a complex extracellular matrix (ECM), but also for the production and concentration of cytokines, chemokines and growth factors. The hematopoietic cells interact with the stroma through specific cell surface receptors, secreted growth factors and with adhesive ligands present on the ECM or stromal cells (Fig. 2). Thus, the BM stroma creates a permissive environment in which the process of myelopoiesis, lymphopoiesis and immunoregulation can occur in close proximity [105].

6.1. Mesenchymal stromal cells (MSCs)

Mesenchymal stromal cells (MSCs) are an important component of the BM stroma and are estimated to represent 0.01–0.0001% of the nucleated cells in adult human BM [106]. MSCs were first identified by Friedenstein et al. who described it as undifferentiated cells in the BM which were able to differentiate *in vitro* into bone- and cartilage-like colonies [107]. Later studies showed that MSCs were able to differentiate into various cell types, including osteoblasts, chondroblasts, adipocytes, myocytes, astrocytes, neurons, endothelial cells and lung epithelial cells [108-110]. Because of their plasticity they are often referred to as mesenchymal stem cells. However for this review we will refer to them as MSCs in accordance with the recommendation of the International Society for Cellular Therapy [111]. MSCs are characterized by the expression of a large number of adhesion molecules and stromal cell markers (CD73, CD105, CD44, CD29, CD90) in the absence of hematopoietic markers (CD34, CD45, CD14) and endothelial markers (CD34, CD31, von Willebrand factor) [112,113].

One of the main functions of MSCs is to provide, within the BM, a microenvironment that is conducive for hematopoiesis. It does so by not only differentiating into the various cellular components of the BM stroma, but also by providing a scaffold for cell to cell interactions and by secreting cytokines and growth factors. MSCs rely on the transforming growth factor-β (TGF-β) family of proteins in governing its development and differentiation. Even though TGF-β itself promotes the expansion of undifferentiated MSCs, other TGF-β family members in cooperation with specific transcriptional regulators promote the differentiation of MSCs. For example, transcription factor Runx2 drives MSCs to differentiate into osteoblasts and the transcription factor peroxisome proliferator-activated receptor (PPARγ) drives the differentiation of MSCs into adipocytes [114,115]. Interestingly, the

transcriptional coactivator PDZ-binding motif (TAZ) acts as a key modulator that ensures that this differentiation occurs independent of each other [116]. *In vitro*, TAZ was demonstrated to co-activate Runx2-dependent transcription while repressing PPARγ-dependent transcription thus ensuring only the differentiation of MSCs into osteoblasts.

Almost all the information of cytokine production from MSCs is derived from *in vitro* cell culture studies. MSCs have been shown to secrete cytokines and growth factors such as macrophage-CSF, Flt-3L, stem cell factor (SCF), interleukins like IL-6, IL-7, IL-11, IL-12, IL-14 and IL-15 [117,118]. MSCs are also considered the major source for the secretion of the homeostatic chemokine, CXCL12 which has a role in both homing of the circulating HSCs and in immune responses [119]. MSCs can also be stimulated to induce secretion of cytokines. For example, upon stimulation with IL-1α, MSCs have been demonstrated to secrete IL-1α, leukemia inhibitory factor, G-CSF and GM-CSF [120]. MSCs play a fundamental role in lymphopoiesis by aiding the development of the naïve B cells in the BM. The developmental process involves the interaction of MSCs with naïve B cells and providing the B cells with SCF and IL-7 [121,122]. MSCs are also implicated in the development of T cells via the process of extrathymic T cell lymphopoiesis [123]. MSCs, through the production of CXCL12, assists in the homing of $CD8^+$ T cells into the BM and thus mediate the induction of adaptive immunity in response to antigen [124].

In contrast, MSCs can also exert immunosuppressive activity through not only suppression of T cell proliferation but also by negatively modulating the functions of B cells, natural killer cells and dendritic cells [125-129]. Not surprisingly, the immunosuppressive properties of MSCs were utilized by Le Blanc et al. to treat a young patient with a severe grade IV acute graft-vs-host disease [130]. The exact mechanism for MSC-mediated immunosuppression remains to be clarified. However, *in vitro*, soluble factors like hepatocyte growth factor, prostaglandin E2, TGF-β1, indoleamine 2,3-dioxygenase and IL-10 have all been suggested to play a role [131,132].

6.2. Extracellular matrix (ECM)

The main constituents of the bone marrow ECM include fibronectin (FN), hualuronan, collagen types I and IV, laminin, glycosaminoglycans heparin sulfate and chondroitin sulfate. In addition to providing the structural scaffold for cellular elements of the BM, ECM also exerts its effect on cell growth, differentiation, motility and viability. The ECM molecules function as a reservoir of many secreted growth factors, cytokines, matrix metalloproteinases and other processing enzymes whose availability is regulated by arrangement of the matrix, thus creating stromal niches within the BM [133].

The BM cells sense their location within the BM through specific interactions within the ECM. Such interactions are very important for cell homeostasis, since inappropriate cell/ECM interaction leads to cell death through anoikis [134]. Most of the interactions between the ECM and the BM cells occur through specific cell-surface receptors named integrins. The integrins are composed of two distinct subunits, the α (18 α subunits) and the β (8 β subunits) subunits which form at least 24 different combinations of α/β sub units in humans [135]. The integrins function not only as mechano-transducers that transform mechanical forces created by the ECM into chemical signals, but they can also cross-talk with receptor tyrosine kinases in response to growth factors. Interestingly, integrins have the ability to communicate over the plasma membrane in both directions, distinguished as outside-in and inside-out signaling [136,137]. Thus, integrin signaling regulates many cellular processes such as proliferation, survival, migration and invasion.

7. Bone marrow stroma and drug resistance

Paget proposed the concept that the microenvironment of a developing tumor is a crucial regulator of tumor growth in his famous ‘seed and soil’ hypothesis [138]. This is clearly demonstrated in the study showing that BM stroma is abnormal in CML, as it has a reduced ability to support growth of normal progenitors cultured in contact with the stroma [139]. In contrast, the growth and proliferation of CML progenitors in contact with CML stroma was unimpaired, again showing that abnormal interactions between the CML cells and the stroma play an instrumental part in promoting survival and maintenance of MRD. This observation was further validated by the finding showing that leukemic cells create an abnormal stromal niche by secreting SCF. The high concentration of SCF directs the normal HSC to home into this niche which in turn makes the maintenance and self-renewal capacity of the normal HSC highly defective [140]. Another stromal niche is created due to the secretion of the chemokine CXCL12 within the BM by the stromal cells, predominantly MSCs [46]. Circulating CML cells, including the CML stem cells express CXCR4, which is the designated chemokine receptor for CXCL12 [141,142]. CML cells use this receptor for homing into the BM and thus evade cell death induced by bcr-abl tyrosine kinase inhibitors. Indeed, Vianello et al. have shown that MSCs in the BM protect and preserve the CML progenitor cells from IM-induced cell death via the CXCR4–CXCL12 axis [143]. Thus by manipulating the BM stroma, the CML cells control the population and fate of normal HSCs.

Cytokine signaling from the BM microenvironment can also lead to leukemic cell survival in the presence of bcr-abl kinase inhibitors. For example, Williams et al. have shown in their study that in spite of efficient inhibition of bcr-abl kinase activity in the tumor cells, mice with BCR-ABL-expressing Arf-null ALL are resistant to IM treatment [144]. The failure of the leukemic cells to respond was determined to be due to IL-7-mediated rescue within the host hematopoietic microenvironment by enabling cells to remain in cycle even when exposed to micromolar IM concentration. Interestingly, when the same resistant leukemic cells where isolated from the *in vivo* microenvironment and exposed to IM *in vitro* they became sensitive indicating that resistance was reversible [144]. Our lab addressed the contribution of soluble factors derived from the BM microenvironment in mediating resistance to bcr-abl kinase inhibitors in CML [145]. We showed that stable soluble factors secreted by HS-5 stromal cell line were sufficient to cause resistance to IM, NI and DA in CML cells. Specifically, soluble factors-mediated activation of STAT3 in the CML cells was sufficient to cause resistance to bcr-abl kinase inhibitors and may contribute to the failure of bcr-abl kinase inhibitors to eradicate MRD associated with CML [145]. Similarly, Wang et al. showed that conditioned media of IM-resistant LAMA84 cells protected IM-naïve LAMA cells and CML progenitors from IM- or NI-induced cell death [146]. The protection was due to the GM-CSF (from the conditioned media of IM-resistant LAMA84 cells) -mediated activation of janus kinases 2 (JAK-2)/signal transducer and activator of transcription 5 (STAT5) signaling pathway. Furthermore, in the same study they demonstrated that IM-resistant patient samples showed elevated mRNA and protein levels of GM-CSF, thus suggesting that GM-CSF also contributed to the IM and NI resistance *in vivo*.

The phenomenon of cell adhesion-mediated drug resistance (CAM-DR) has been observed in various tumor cells [147-149]. In CML cells, adhesion to FN via VLA-5 integrin induces CAM-DR [150]. In this study, K562 cells adhered to FN via the VLA-5 integrin, which in turn provided significant resistance to apoptosis induced by a number of DNA damaging agents including melphalan, mitoxantrone and γ-irradiation. In addition, cellular adhesion to FN also inhibited apoptosis in CML cells exposed to IM [150]. Decreased proliferation in CML cells adhered to FN via integrins might be one of the contributing factors for the manifestation of drug resistance [151]. Interestingly, in CML cells, β1 integrin mediated

adhesion reduced the levels of Bim, a proapoptotic protein that counteracts the effects of antiapoptototic proteins Bcl-2 and Bcl-x_L [152]. Furthermore, this decrease in Bim levels was attributed to increased degradation of Bim protein in CML cells adhered to FN. The importance of this finding was divulged in studies showing that incubation of CML cells with bcr-abl tyrosine kinase inhibitors results in cell death that is partially mediated by an increase in levels of Bim expression [153,154]. Thus, CML binding to the FN in the BM stroma might evade cell death induced by bcr-abl kinase inhibitor treatment through down regulation of Bim [152].

8. Overcoming bone marrow stroma-mediated drug resistance

Strategies to overcome BM stroma-mediated drug resistance should include not only disrupting the interaction between the CML cells and the BM stromal cells, but also, inhibit the signaling pathways mediated by cytokines and growth factors in the BM. Signals derived from the BM stroma can effectively reconstitute the downstream signaling pathway of bcr-abl protein, such that CML cells can achieve bcr-abl independent growth in the BM, making them resistant to bcr-abl tyrosine kinase inhibitors. Targeting the downstream pathway in such cases may offer an alternative pathway to induce cell death in resistant CML cells. For example, the PI3K-Akt-mTOR pathway is shown to be activated in CML cells after IM treatment and this activation was shown to be important in mediating cell survival, thereby resulting in the development of resistance [155]. This same pathway leads to the over expression of vascular endothelial growth factor in the CML BM, which in turn increases the microvascular density and the vascular niche within the BM and thus may be essential for the growth of CML progenitors [156]. Studies have shown that RAD001, a derivative of rapamycin, orthe PI3-kinase inhibitor, LY294002, significantly inhibits the PI3K-mTOR pathway resulting in, not only inhibition of the growth of CML cells, but also prolonging the survival of mice transplanted with BM retrovirally transduced with BCR-ABL [156,157]. Similarly, bcr-abl is known to couple with Ras pathway and play a critical role in the oncogenic transformation in CML. Ras activation requires the post-translational modification, involving the addition of 15-carbon farnesyl isoprenoid moiety to a conserved cysteine residue in a carboxy-terminal CAAX motif. This whole process called prenylation is catalyzed by farnesyltransferase (FT). FT inhibitors (FTI) like tipifarnib and lonafarnib were rationally designed drugs designed to target FT activity. In IM-resistant CML patients, tipifarnib showed modest activity when used either alone or in combination with IM [158,159]. Additionally, lonafarnib demonstrated selective activity against primary cells from patients with CML and also showed efficacy in a murine model of CML [160]. Finally, inhibitors of MAPK kinases (MEK1/2), PD18435273 and U0126, when combined with IM or DA, results in synergistic cell death in IM-resistant CML cell lines [161]. However, this effect was not reproduced in IM-resistant cell lines with T315I mutation in the ABL kinase domain, thereby limiting its utility [162].

Heat shock protein 90 (Hsp90) is a molecular chaperone that interacts and stabilizes signal transducing proteins like Raf, Akt and bcr-abl. 17-allylamino-17-demethoxygeldanamycin (17-AGG), a protein that binds Hsp90 and inhibits its ability to act as a chaperone results in down regulation of Raf, Akt and bcr-abl mediated downstream signaling leading to apoptosis in CML cell lines [163]. Also, 17-AGG targets the P-glycoprotein multi-drug resistance pump and has been successfully used in combination with IM to synergistically inhibit cell growth and induce cell death in IM-resistant CML cell lines [164]. Increased proteosomal activity in CML cells leads to resistance to apoptosis by decreasing the levels of pro-apoptotic protein Bim [152-154]. To overcome this, proteosome inhibitors like bortezomib have been used in IM-resistant CML cell lines to successfully inhibit proliferation, induce cell cycle arrest and promote apoptosis in these cells [165]. Growth factors and the cytokines within the BM stroma can synergize with bcr-abl activity to

regulate the cell cycling of the CML cells via the cyclin-dependent kinases. Two approaches have been utilized to inhibit this pathway, namely the use of either histone deacetylase inhibitors (HDI) or cyclin-dependent kinase inhibitors. Inhibtion of histone deacetylase by HDI causes the hyperacetylation of histones resulting in transcriptional upregulation of cyclin-dependent kinase inhibitor p21 leading to cell-arrest and apoptosis in CML cells [166]. HDI like suberoylanilide hydroxamic acid (SAHA), LBH589 and valproate have been used in combination with IM and NI and have shown to enhance BCR-ABL tyrosine kinase inhibitor-induced growth arrest and apoptosis in CML cells and in IM-resistant cell lines expressing T315I mutation [167-170]. Also, the multiple cyclin-dependent kinase inhibitor, falvopiridol has been used in combination with IM to cause selective apoptosis in CML and IM-resistant CML cells [168].

The CXCR4–CXCL12 axis functions as a major migration mechanism for CML cells to home into the BM. Use of CXCR4 antagonist offers an attractive approach to mobilize the CML cells from the BM and hence make them sensitive to bcr-abl tyrosine kinase inhibitor. One of such inhibitors that has been tested in CML is the bicyclam non-peptide antoagonist AMD3100. AMD3100 restored the sensitivity of CML progenitors cells to cell death by IM [143,171]. More importantly, CML progenitor burden within the BM was drastically reduced with the treatment with AMD3100 [143]. However, further studies have to be performed to warrant the use of inhibitors of the CXCR4–CXCL12 axis in combination with bcr-abl tyrosine kinase inhibitors.

Production of cytokines like IL-3, G-CSF and GM-CSF within the BM has been suggested to play a role in CML pathogenesis [146,172,173]. Studies have shown these cytokines have a role in development of resistance towards bcr-abl kinase inhibitors via the JAK/STAT pathway [145,146]. Indeed, use of JAK-2 inhibitor, AG490 reduced the GM-CSF-induced STAT5 activation and thus reversed the potential of GM-CSF to confer IM or NI resistance in CML [146]. Similarly, our lab has shown that reducing STAT3 levels with siRNA sensitized HS-5 stromal cell conditioned media-cultured BCR-ABL kinase inhibitor resistant K562 cells to IM, NI and DA [145]. Finally, it remains to be tested if the CAM-DR seen in CML can be reversed either by a VLA-4 inhibitor or by a novel integrin inhibitor like LFA703 [174]. In summary, we propose that effective testing of combination therapies should include strategies to test in multicellular models that include components of the bone marrow.

9. Conclusion

Survival among the CML patients is closely linked to the reduction of BCR-ABL positive cell burden in patients. Since the presence of bcr-abl protein can be cytogenetically identified by the presence of Philadelphia chromosome, the goal of treatment in CML is to effectively eradicate Philadelphia chromosome-positive cells. The current targeted molecular therapy utilizing bcr-abl kinase inhibitors is effective in inducing a complete hematological and cytogenic remission in a high percentage of patients, but the problem of resistance and intolerance to these agents still exists. We propose that personalized medicine must go beyond an understanding of tumor derived pathways and begin to delineate how variability within the context of tumor cell-microenvironment interactions may predict drug response and lead to the optimization of combination therapy. Taken together, this knowledge will provide the blueprint for a rational combination of drugs that is tailored to a specific individual patient, with the goal of helping overcome emergence of drug resistance and leading to a better patient outcome.

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Abbreviations

CML	chronic myeloid leukemia
ALL	acute lymphatic leukemia
IM	imatinib mesylate
BM	bone marrow
NI	nilotinib
DA	dasatinib
MRD	minimal residual disease
WT1	Wilms' tumor 1
HSC	hematopoietic stem cell
HPC	hematopoietic progenitor cell
ECM	extracellular matrix
MSC	mesenchymal stromal cell
AML	acute myeloid leukemia
SLAM	signaling lymphocyte activation molecule
SDF-1	stromal cell-derived factor-1
OCT-1	organic cation transporter-1
Pgp	P-glycoprotein
GMP	granulocyte–macrophage progenitors
CAM-DR	cell adhesion-mediated drug resistance
SCF	stem cell factor
IL	interleukin
INFα	interferon-α
TGF-β	transforming growth factor-β

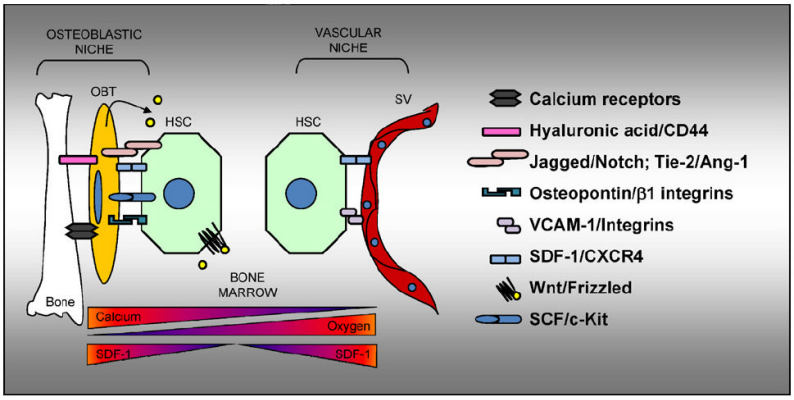

Fig. 1.

Schematic representation of the molecular interactions between hematopoietic stem cells and their niches in the bone marrow. In normal physiological conditions, the hematopoietic stem cells (HSCs) reside either at the endosteum, lodged with the osteoblasts (OBT) (osteoblastic niche) or at the sinusoidal vessels (SV) (vascular niche). The osteoblastic niche provides the microenvironment for HSC maintenance and quiescence and the vascular niche provides the microenvironment for HSC prolirferation and differentiation. The oxygen and the calcium gradient might play a crucial role in maintenance of the different niches within the bone marrow.

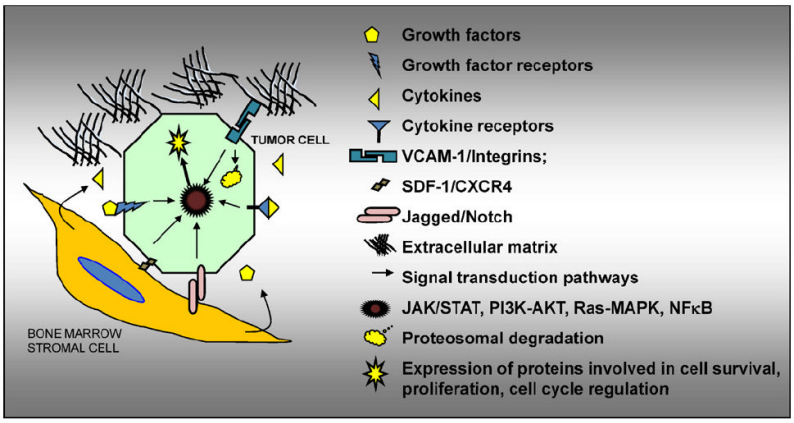

Fig. 2.

Schematic representation of the mechanism for the stroma-mediated resistance in the bone marrow. The tumor cells within the bone marrow (BM) is bathed in a mileu of cytokines (IL-6, IL-11, IL-1, TNF-α, IFNs) growth factors (VEGF, FGF, G-CSF, GM-CSF, TGFs) and chemokine factors (SCF, SDF-1) secreted by the BM stromal cells (paracrine) or the tumor cells (autocrine). In addition, the tumor cells interact with the stromal compartment with the help of integrins (VLA-4, ICAM, VCAM) and N-cadherins. All this interactions culminates in the activation of signaling pathways (MAPK, JAK/STAT, PI3K-Akt) within the tumor cells which ultimately leads to the proliferation and survival of the tumor cell in the BM stroma.