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Understanding the bone marrow microenvironment in hematologic malignancies: a focus on chemokine, integrin, and extracellular vesicle signaling

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Abstract

Signaling between leukemia cells and non-hematopoietic cells in the bone marrow microenvironment contributes to leukemia cell growth and survival. This complicated extrinsic mechanism of chemotherapy resistance relies on a number of pathways and factors, some of which have yet to be determined. Research presented at the 2016 annual Therapeutic Advances in Childhood Leukemia (TACL) investigator meeting explored the importance of cell-cell crosstalk the bone marrow microenvironment in acute leukemia. This review summarizes the mini-symposium proceedings and focuses on chemokine signaling via the cell surface receptor CXCR4, adhesion molecule signaling via integrin $\alpha 4$, and crosstalk between leukemia cells and the bone marrow microenvironment that is mediated through extracellular vesicles.

INTRODUCTION

Crosstalk between normal, non-cancerous cells and tumor cells in the bone marrow microenvironment has been shown to mediate leukemogenesis, extrinsic drug resistance and relapse and to help populations of leukemia cells evade chemotherapy-induced death.¹ For example, interaction between the chemokine CXCL12, which is produced by stromal cells in the bone marrow microenvironment, and the cell surface receptor CXCR4, which is present on the surface of leukemic blasts, facilitates microenvironment-mediated protection of leukemic blasts.^{1,2} Another important receptor on leukemia cells is integrin $\alpha 4$, which binds to the ligands fibronectin and VCAM-1 in the bone marrow microenvironment.¹ Like CXCR4, integrin $\alpha 4$ is expressed on the surface of hematopoietic stem cells (HSCs) and

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CONFLICT OF INTEREST STATEMENT

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acute leukemia cells.³ Integrin $\alpha 4$ is an important mediator of leukemia-stromal cell adhesion.⁴ Finally, extracellular vesicles (EV), which contain protein and/or RNA and are constitutively secreted by cells, may play an important role in leukemogenesis, leukemic cell survival, and hijacking of bone marrow niches.⁵

The importance of CXCR4 in acute leukemia

CXCR4 expression as a prognostic marker in acute leukemia—CXCR4 is a seven transmembrane domain, GTP-binding protein coupled-receptor that is present on the surface of hematopoietic stem cells (HSCs), white blood cells, acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML) cells.^{1,2} Similar to its role in the regulation of hematopoietic stem cells (HSCs), activation of CXCR4 by CXCL12 is critical for the homing and retention of leukemia cells within the protective bone marrow niche.¹

Low surface CXCR4 expression in leukemic blasts (as measured by flow cytometry) has been associated with longer overall and relapse-free survival in adults with AML or myelodysplastic syndrome (MDS).⁶ Low CXCR4 expression in AML blasts (as measured by immunohistochemistry) has also been associated with longer overall and relapse-free survival in adults with newly-diagnosed, cytogenetically normal, non-*FLT3*-mutated AML.⁷ In pediatric ALL, patients with leukemia-induced hepatomegaly and/or splenomegaly had significantly higher surface expression of CXCR4 in their blasts.⁸ Similarly, higher surface CXCR4 expression in ALL blasts was associated with a shorter disease-free survival.⁹ Recently, CXCR4 deletion and/or pharmacologic antagonism of CXCR4 was shown to significantly decrease leukemic burden in a mouse model of T-ALL.¹⁰ Collectively, these findings suggest that CXCR4 may be a therapeutic target in ALL and AML.

Pre-clinical and clinical studies of CXCR4 antagonism in acute leukemia—

Antagonism of CXCR4 may enhance sensitivity to anti-leukemic therapies by disrupting interactions between leukemic blasts and supportive cells in the bone marrow microenvironment. Several CXCR4 antagonists have been evaluated in the pre-clinical and clinical settings (Table 1). Plerixafor (Mozobil, AMD3100) is a bicyclam, small molecule CXCR4 antagonist that has been shown to decrease proliferation of AML cell lines,¹¹ decrease engraftment of pediatric ALL in a xenograft model,¹² and enhance sensitivity to anti-leukemic therapies in mouse models of acute promyelocytic leukemia (APL)¹³ and infant *MLL*-rearranged ALL.^{14,15} A single-institution phase 1/2 trial combined plerixafor with mitoxantrone, etoposide, and cytarabine in adults with relapsed or refractory AML (n=52).¹⁶ No dose-limiting toxicities (DLT) were reported and plerixafor induced an average 2-fold mobilization of leukemic blasts into the peripheral blood. In the phase 2 portion of the trial, the complete remission (CR) rate was 39% and the combined rate of CR and CR with incomplete blood count recovery (CRi) was 46%. A subsequent multi-center phase 1 clinical trial combined plerixafor with the 7+3 regimen (cytarabine and daunorubicin) in adults with newly-diagnosed AML.¹⁷ At the time of abstract presentation at the 2011 American Society of Hematology (ASH) Annual Meeting, 21 subjects had available response data. There were no DLTs identified and plerixafor caused an average 3-fold increase in peripheral leukemic blasts. The CR rate was 67% and the CR+CRi rate was 76%; to date, the completed trial results have not been presented or published. A phase 1 clinical trial in adults with relapsed

or refractory *FLT3*-ITD AML combined plerixafor and granulocyte-colony stimulating factor (G-CSF) with the tyrosine kinase inhibitor sorafenib.¹⁸ Interim analysis of 13 subjects with response data were presented in abstract form at the 2012 ASH Annual Meeting. Plerixafor and G-CSF induced a 41-fold increase in circulating AML blasts; 1 subject achieved CR and 2 achieved CRi. At this time, completed trial results have yet to be presented or published. A multi-center phase 1 trial combined plerixafor with high-dose cytarabine and etoposide in pediatric subjects (n=19) with relapsed or refractory acute leukemias or myelodysplastic syndrome (MDS).¹⁹ Plerixafor induced a median 3.4-fold increase of leukemic blasts into the peripheral blood and no DLTs were observed. Three subjects with AML achieved CR or CRi; no subjects with ALL or MDS had responses. Plerixafor is approved by the Food and Drug Administration (FDA) for autologous hematopoietic stem cell (HSC) mobilization in combination with G-CSF in adults with multiple myeloma²⁰ or non-Hodgkin lymphoma.²¹ However, there are no active clinical trials evaluating plerixafor as a chemosensitizing agent. Current clinical trials of plerixafor are mostly focused on HSC mobilization, perhaps due, in part, to suboptimal CR rates from the early phase chemosensitization trials.

BL-8040 (BKT-140, T140) is a cyclic, synthetic peptide antagonist of CXCR4 that inhibits CXCR4 antibody binding and CXCL12-induced chemotaxis in ALL.²² A phase 2 dose-escalation study in adults with relapsed or refractory AML (n=22 in dose-escalation phase) combined BL-8040 with cytarabine.²³ At the time of abstract presentation, BL-8040 induced an average 40-fold mobilization of AML blasts into the peripheral blood, and subjects who were treated at the 3 highest dose levels had a CR+CRi rate of 38%. No DLTs were observed and 1 patient experienced Sweet syndrome that may have been related to study drug. Ongoing phase 2 trials include BL-8040 and nelarabine in adults with relapsed/refractory T-ALL/lymphoblastic lymphoma and BL-8040 and cytarabine in adults with relapsed/refractory AML.²⁴

Ulocuplumab (BMS-936564, MDX-1338) is a human, monoclonal anti-CXCR4 antibody that is effective as a single agent against AML, NHL, and multiple myeloma in mouse xenograft models.²⁵ A phase 1 dose-escalation study combined ulocuplumab with mitoxantrone, etoposide, and cytarabine in adults with relapsed or refractory AML (n=73).²⁶ Ulocuplumab induced a median 5-fold mobilization of AML blasts into the peripheral blood. At the time of abstract presentation, no DLTs were observed and 51% of subjects treated at the maximum dose of ulocuplumab had achieved CR or CRi. A phase 1 trial of ulocuplumab in combination with low-dose cytarabine for adults with AML who are unfit for intensive induction therapy is currently ongoing.²⁴

LY2510924 is a peptide antagonist of CXCR4 decreases leukemic burden and increases survival in mouse models of AML, either as monotherapy or in combination with chemotherapy.²⁷ A phase 1 of LY2510924 in combination with idarubicin and cytarabine in adults with relapsed/refractory AML is currently ongoing.²⁴

POL6326 is a cyclic, peptide-like antagonist of CXCR4. Its tool compound, POL5551, blocks CXCR4 antibody binding in pediatric B- and T-ALL primary samples and enhances

sensitivity to cytarabine in a mouse model of infant *MLL*-R ALL.²⁸ POL6326 is not currently being studied in clinical trials for patients with leukemia.

In conclusion, targeting leukemia-microenvironment interactions through CXCR4 antagonism may be an effective strategy in the treatment of pediatric leukemia.

Integrin $\alpha 4$ as a therapeutic target in ALL

Integrin expression in leukemia—Integrins are a family of glycoprotein cell surface receptors that are composed of 2 subunits, α and β .²⁹ There are 18 different α chains and 8 β subunits in humans, which form at least 24 distinct α/β integrin heterodimers.^{30–32} Integrins are responsible for cell adhesion to the extracellular matrix (ECM) and help mediate the protection of leukemic cells from apoptosis by bone marrow stromal cells. In addition, integrins elicit intracellular (outside-in) signals through a variety of cell-type dependent pathways, including survival/apoptosis, cell cycle, and metabolism, among others.^{29,33–40} Integrins thus mediate physical adhesion to suitable matrices/ligands while at the same time providing outside-in signals that regulate cell fate. Integrin $\alpha 4$, which combines with integrin $\beta 1$ to form VLA-4, is expressed by HSCs, a variety of white blood cells, and B-precursor ALL cells.^{3,41} Importantly, an analysis of the ALL-REZ BFM 2002 trial found that high mRNA expression of integrin $\alpha 4$ (*ITGA4*) was an adverse risk factor in childhood ALL at first relapse.⁴² Therefore, targeting integrin $\alpha 4$ may be beneficial in the treatment of ALL.

Integrin $\alpha 4$ antagonists—An elegant study that demonstrated the importance of integrin $\alpha 4$ in HSCs suggests that antagonism of integrin $\alpha 4$ may be extrapolated to leukemia. Deletion of the integrin $\alpha 4$ gene in a mouse model resulted in an efflux of HSCs into the circulation, suggesting that HSCs were no longer being retained within the bone marrow microenvironment.⁴³ When these integrin $\alpha 4$ -deleted HSCs were then transplanted into recipient mice, homing to the bone marrow was partially inhibited, early engraftment of HSCs into the bone marrow was impaired, and HSCs were rapidly taken up by the spleen rather than the bone marrow.⁴³ Taken together, these data support the role of integrin $\alpha 4$ in adhesion, migration, and survival of HSCs.⁴³

Because leukemia cell survival is dependent upon adhesion, inducing de-adhesion through integrin $\alpha 4$ antagonism may decrease survival and sensitize leukemia cells to chemotherapy. Pre-clinical and clinical testing of new therapies against various integrins involved in different diseases is a rapidly emerging field (Table 2).^{44–49} Approximately 240 different integrin $\alpha 4$ -directed therapies are under evaluation for various conditions. The only FDA-approved anti-integrin $\alpha 4$ therapy is natalizumab (Tysabri), a humanized anti-integrin $\alpha 4$ monoclonal antibody that blocks the binding of integrin $\alpha 4$ to VCAM-1.⁴⁷ While natalizumab is approved for the treatment of relapsing forms of multiple sclerosis and some patients with moderately to severely active Crohn's disease, it has been shown to have HSC mobilizing activity.⁵⁰ Additional work has demonstrated that natalizumab can de-adhere ALL cells from VCAM-1 and can sensitize ALL cells to chemotherapy.⁵¹ However, because natalizumab increases the risk of progressive multifocal leukoencephalopathy, which is an

opportunistic viral infection of the brain that is caused by JC virus,⁵² patients with hematologic malignancies are not candidates for treatment with natalizumab.

Several small molecules have been studied in an attempt to target integrin $\alpha 4$ and VLA-4-dependent adhesion.⁵³ Screening of a chemical library containing small molecules, many of which are FDA- or European Medicines Agency (EMA)-approved agents, identified several structurally related compounds that were able to reduce the binding affinity of VLA-4-specific ligand and block VLA-4/VCAM-1-dependent cell adhesion.⁴⁸ These compounds also mobilized mouse HSCs from the bone marrow to peripheral blood in a manner that was comparable to AMD3100/plerixafor.⁴⁸ BIO5192 is a selective small molecule antagonist that is specific for integrin $\alpha 4\beta 1$, or VLA-4.^{54,55} BIO5192 has been shown to mobilize hematopoietic stem and progenitor cells in a mouse model.⁵⁴ TBC3486 is a non-peptidic small molecule inhibitor of integrin $\alpha 4$.⁵⁶ *In vitro* treatment of ALL cells with TBC3486 led to decreased adhesion to VCAM-1 and stromal cells.⁵⁶ TBC3486 also sensitized ALL cells co-cultured with stromal cells to chemotherapy. Notably, TBC3486 prolonged survival of murine recipients of primary ALL, both as monotherapy or in combination with chemotherapy.⁵⁷ Most recently, a small molecule antagonist that targets both integrins $\alpha 9\beta 1$ and $\alpha 4\beta 1$ was shown to mobilize HSCs in a mouse model.⁵⁸ Combination of the integrin $\alpha 9\beta 1/\alpha 4\beta 1$ antagonist with AMD3100 resulted in a synergistic increase in HSC mobilization, suggesting that anti-CXCR4 and anti-integrin $\alpha 4$ agents may be used in combination.⁵⁸ Additional molecules targeting integrin $\alpha 4$ are under evaluation for a variety of diseases.⁵³

In summary, a role of microenvironment in protecting ALL cells from chemotherapy has been implicated.⁵⁹ Disruption of the adhesion between ALL cells and the bone marrow stroma by antagonism of integrin $\alpha 4$ promotes a release of ALL cells from bone marrow to peripheral blood where the chemotherapy might more effectively attack leukemia cells.^{53,60} Pre-clinical evidence suggests that interference with integrin $\alpha 4$ -mediated adhesion can sensitize ALL cells to chemotherapy and thus facilitate their eradication.^{51,57,61} Therefore, blockade of adhesion molecules, such as integrin $\alpha 4$ or VLA-4, using either antibodies or small molecules may be a novel adjuvant therapeutic strategy in ALL.

An emerging role for extracellular vesicles in the leukemia microenvironment

Vesicle biogenesis—As a biological process vesicle biogenesis is broadly conserved across evolution and well-defined roles for vesicles exist in transferrin receptor extrusion, crosstalk at the immune cell synapse, cancer metastasis promotion, and peripheral insulin resistance tuning.^{62–65} Extracellular vesicle (EV) biogenesis is a constitutive cellular function and EVs are distinct from apoptotic bodies arising during the course of cellular disintegration.⁶⁶ EV of various shapes and sizes have been demonstrated in every biofluid tested to date (Figure 1).⁶⁷ EV size continues to serve as a common surrogate for their biogenesis mechanism, as exosomes emerge from endocytic compartments, whereas larger microvesicles bud from the limiting cell membrane.⁶⁸ Typically purified using a series of extended high speed centrifugation steps, vesicle preparations from most cells reveal diverse types of EV.⁶⁸ With sizes ranging from 30–800 nM, many vesicles fall in size below the visible spectrum, complicating isolation and characterization immensely. Accordingly,

structure, content, and function for each EV vary substantially. Moreover, vesicles can be transported inside cytoplasmic extensions, traffic at short distance, or via the bloodstream in a paracrine, or endocrine manner, respectively.^{63,69}

The lipid bilayer vesicle membrane is embedded with proteins that can serve direct signaling function, or confer activity via attachment, uptake (endocytosis, pinocytosis or fusion at the membrane), and cellular delivery of cargo protein,⁷⁰ lipids,⁷¹ RNA,⁷² and DNA.⁷³ Several studies suggest that biogenesis is a highly orchestrated process and certain rules for the cargo composition and occupancy are emerging.^{74–78}

Vesicle release is subject to a range of cellular stimuli, including cytokine activation, ionizing radiation, and low oxygen conditions.^{5,79,80} Remarkably, vesicle entry is not a requirement for downstream target cell activation, and several examples appear to indicate that membrane-embedded proteins can serve to trigger cellular events, in part, by carrying cytokines.^{67,81,82} EV surface composition may also be responsible for directed trafficking and preferential binding capacity, including through the modulation of integrin expression.⁸³ Entry mechanisms are diverse and likely both EV- and cell-specific, ranging from endocytosis to membrane fusion and pinocytosis.^{84–87}

Involvement of EV in leukemogenesis—EV trafficking in the bone marrow has been widely studied in chronic myeloid leukemia (CML),⁸⁸ multiple myeloma (MM),⁸⁹ AML,⁹⁰ ALL,⁹¹ and to a lesser extent in the non-malignant bone marrow microenvironment.⁸² In AML, work by several groups has revealed that the bone marrow niche undergoes a functional conversion, whereby the presence of AML cells sets off a series of functional changes that successively erode physiologic organ function, leading to enhanced leukemic survival and spread.⁹² There are several potential means by which EVs can promote leukemia.^{93,94}

- **Angiogenesis / Vasculogenesis:** One of the most widely reproduced phenotypes is the enhancement of angiogenesis by EV transfer.⁹⁵ Specifically, microvesicle transfer between tumor and endothelial cells has been shown to result in enhanced EGFR signaling and VEGF expression.⁷⁰ Increased angiogenesis, structural reorganization of the tumor microenvironment, and increased microvascular density are all characteristic features of acute and chronic leukemia. Not surprisingly, low tissue oxygen enhances this EV-mediated activity in MM as well as in a model of CML.^{79,96}
- **Direct disease promotion:** Tumor promotion through extrinsic EV activity has been shown to occur in a range of cancers.⁹⁷ Several groups have shown that EV transfer between cell lines can confer a more aggressive disease phenotype.⁹⁸ Experiments in MM show that EV from stromal cells enhance proliferative activity in MM cells.⁸⁹ In a cell culture model of CML, EVs induce IL-8 secretion and disease promotion, a response that was abrogated *in vivo* by inhibition of IL-8 reduced in CML xenografts.⁹⁹
- **Drug resistance:** The mechanisms by which EVs can enhance drug resistance appear to differ widely. For example, EVs can sequester drug (imatinib) into

lysosome compartments, leading to reduced cellular accumulation in leukemic cells.¹⁰⁰ In B-cell lymphoma, an ATP binding cassette transporter appears to regulate EV release, including the release of EVs that can serve as CD20-binding decoys.¹⁰¹ Drug resistance in ALL also appears to be mediated by galectin 3 that is transferred to leukemic cells from stromal cells via EVs.⁹¹ Galectin 3 was also shown to be simultaneously exported by ALL cells via EV in a paracrine manner.⁹¹ Finally, in AML, exposure to patient stromal cell-derived EVs led to increased resistance to tyrosine kinase inhibitors.¹⁰²

- Hematopoiesis regulation: Several groups have reported effects of mesenchymal stem cell (MSC)-derived vesicles on stem and progenitor cell functions, and EVs are released from MSC under homeostatic conditions.^{82,103,104} Also, the miRNA-mediated suppressive impact that EVs have on hematopoietic progenitors in AML was recently described.¹⁰⁵

A perspective on therapeutic targets derived from EV trafficking—Where cytokines, chemokines and integrins typically confer a predictable paracrine activity that is restricted to cells that express the cognate receptor with engagement of a molecularly defined signaling pathway, such rules have yet to be defined for EV. It stands to reason that a greater understanding of cell type specific biogenesis, cargo composition and cellular uptake restrictions will reveal the cells and molecular pathways in the multicellular crosstalk in the BM. In turn, the knowledge of how tissues use EV to adapt during leukemic evolution should provide insight for the design of more selective therapies that minimize collateral damage during treatment and reverse the very changes by which the BM reinforces disease and contributes to relapse.

CONCLUSIONS AND FUTURE DIRECTIONS

Interactions between leukemic blasts and stromal cells in the bone marrow microenvironment are likely to be important and targetable mechanisms of therapy resistance. Because CXCR4 and integrin $\alpha 4$ are important in retaining leukemia cells within the protective bone marrow microenvironment, selective inhibition of these receptors could allow leukemia cells to be released from niches that contribute to chemotherapy resistance.¹ Interference with integrin $\alpha 4$ not only de-adheres leukemia cells from the chemoprotective bone marrow niche, but inhibition may also interrupt intracellular signaling changes that are critical for the survival and therapeutic resistance of leukemia cells.^{61,106} Similarly, disabling leukemia EV-mediated signalling, or rather interfering with the cellular targets of EV cargo, may further help to achieve and sustain remissions. Ultimately, an improved understanding of interactions between the bone marrow microenvironment and leukemic blasts promises to uncover new, selective and highly effective therapies for children with leukemia.

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Figure 1. EM image of K562 erythroleukemia cells with EVs.

Electron micrograph of extracellular vesicles and the K562 erythroleukemia cells of origin. Note the range in EV sizes.

Table 1.

CXCR4 antagonists in clinical development.

Agent	Molecule type	Phase	Disease	Regimen
Plerixafor	Bicyclam small molecule	FDA-approved	Autologous HSC mobilization in multiple myeloma and non-Hodgkin lymphoma	+ granulocyte colony-stimulating factor
POL6326	Peptide	1/2 1	Allogeneic HSC mobilization Metastatic breast cancer	Monotherapy + eribulin
Ulocuplumab	Human IgG(4) monoclonal antibody	1 1 1 1	R/R AML, DLBCL, CLL, FL R/R multiple myeloma AML unfit for induction therapy Pancreatic cancer and SCLC	Monotherapy + len/dex or bort/dex + low-dose cytarabine + nivolumab
BL-8040	Peptide	1/2 2 2 2 2 2	Autologous HSC mobilization Allogeneic HSC mobilization R/R T-ALL/LL R/R AML Metastatic pancreatic cancer Aplastic anemia, hypoplastic MDS	Monotherapy Monotherapy + nelarabine + cytarabine + pembrolizumab + hATG, CSA, methylprednisolone
LY2510924	Peptide	1 1 2 2	R/R AML Refractory solid tumors Metastatic clear cell RCC Extensive-stage SCLC	+ idarubicin and cytarabine + durvalumab + sunitinib + carboplatin and etoposide

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CSA, cyclosporine; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; hATG, horse anti-thymocyte globulin; HSC, hematopoietic stem cell; LL, lymphoblastic lymphoma; MDS, myelodysplastic syndrome; RCC, renal cell carcinoma; R/R, relapsed/refractory; SCLC, small cell lung cancer.

Trial listings available at reference 24.

Table 2.Integrin $\alpha 4$ antagonists under investigation.

Agent	Molecule type	Notes
Natalizumab	Humanized IgG4 κ monoclonal antibody	FDA-approved to treat 1) relapsing forms of multiple sclerosis and 2) patients with moderately to severely active Crohn's disease with evidence of inflammation who have had an inadequate response to or are intolerant of conventional therapies or TNF- α inhibitors Sensitizes ALL cells to chemotherapy <i>in vitro</i> and in mouse ALL xenografts
BIO5192	Small molecule	Mobilizes HSCs and HPCs in a mouse model
TBC3486	Small molecule	Sensitizes ALL cells to chemotherapy in an <i>in vitro</i> stroma co-culture model Prolongs survival of mouse ALL xenografts as monotherapy or in combination with chemotherapy

ALL, acute lymphoblastic lymphoma; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; TNF, tumor necrosis factor.