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Trafficking of Acute Leukemia Cells – Chemokine Receptor Pathways that Modulate Leukemia Cell Dissemination

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1. Introduction

Recent advances in therapeutic regimens targeting aberrant proliferation of leukemia cells have led to a decline in the mortality of patients with acute leukemia. Nevertheless, a number of patients still suffer from refractory disease or relapse, indicating that more innovative and effective therapeutic strategies are required to achieve superior outcomes. One of the major indicators of poor prognosis in leukemia patients is extramedullary infiltration or dissemination of leukemia cells. In particular, leukemia cell infiltration into the central nervous system is one of the major complications negatively influencing prognosis. However, little is known about the mechanisms responsible for extramedullary dissemination of leukemia cells compared to those responsible for solid tumor metastasis.

The chemokine SDF1 (stromal derived factor-1) and its receptor CXCR4 regulate trafficking of normal hematopoietic stem cells (HSC) as well as metastasis of solid tumor cells. Similarly, the majority of acute myeloid leukemia (AML) cells express CXCR4 and migrate in response to SDF1, suggesting that the SDF1/CXCR4 axis may be involved in the dissemination of AML cells to various organs. A recent study also suggests that chemokine/chemokine receptor interactions orchestrate extramedullary dissemination in childhood AML. Moreover, signaling through the chemokine receptor CCR7 is crucial for infiltration of T-ALL cells in the central nervous system. Our recent studies indicate that internal tandem duplication mutations of the Flt3 gene (ITD-Flt3), found in patients with AML, significantly augments migration of hematopoietic cells by deregulating CXCR4 signaling that are qualitatively distinct from cells lacking ITD-Flt3 and facilitate their infiltration to visceral organs while decreasing their homing to the bone marrow. ITD-Flt3 regulates overlapping as well as functionally distinct signaling pathways down-stream of SDF1/CXCR4 compared to cells that do not harbor ITD-Flt3 mutations. The data suggest that ITD-Flt3 may facilitate dissemination of leukemia cells by modulating SDF1/CXCR4 signaling and that blocking this functional cross-talk between ITD-Flt3 and CXCR4 pathways may have therapeutic benefit. Therefore, genes differentially regulated by SDF1 specifically in ITD-Flt3 cells may represent key targets regulating aberrant migration by

ITD-Flt3 in response to SDF1 to prevent unnecessary dissemination and invasiveness of ITD-Flt3⁺ acute leukemia cells without affecting normal hematopoiesis. This chapter will describe gene and receptor signaling pathways responsible for aberrant trafficking of acute leukemia cells and discuss therapeutic implications of antagonizing chemokine receptor signaling to selectively block extramedullary dissemination of leukemia cells.

2. Dissemination of acute leukemia and the role of chemokines and chemokine receptors

Despite significant advances in treatments for patients with acute leukemia during the past decade, the prognosis of patients with minimal residual disease is generally poor and recurrence of the disease is common. One of the major complications that leads to poor outcomes of both adults and pediatric patients with acute leukemia is central nervous system (CNS) infiltration of leukemia cells. Intensified intrathecal chemotherapy and cranial irradiation for prophylaxis of CNS relapse can lead to serious adverse side effects, particularly secondary tumors, bone marrow suppression, growth impairment and endocrine complications (Pui CH & Howard SC, 2008). In order to minimize the treatment associated mortality, blocking leukemia cell invasion and migration may represent a rational alternative strategy. However, despite the clinical importance of CNS infiltration of leukemia cells, little is known about the underlying mechanism. In addition to CNS, leukemia cells are often found in other extramedullary sites, including skin and visceral organs, which is also an adverse prognostic factor (Byrd JC *et al.*, 1995, Kaneider NC *et al.*, 2002). It is conceivable that leukemia stem cells are derived from a single transformed clone in the bone marrow that subsequently peripheralize into the peripheral blood circulation and home to other organs or different sites within the marrow where they occupy normal hematopoietic niches and impair production of functionally normal blood cells. Similar processes can take place in simultaneously or successive manner, which in turn causes serious hematological complications. Dissemination of AML cells to other marrow sites increases the frequency for AML cells to interact with normal marrow niches and extracellular matrix (ECM), such as stromal fibronectin, via surface integrins, which is one of the mechanisms responsible for minimal residual disease in AML (Matsunaga T *et al.*, 2003). Therefore, dissemination of acute leukemia cells must be considered a crucial step in leukemic progression (Figure 1). The current chemotherapies and molecular targeting drugs were designed to kill leukemia cells but none of them are able to antagonize their movement and trafficking. Understanding molecular mechanisms regulating aberrant leukemia cell trafficking will aid in developing innovative therapeutic modalities to block leukemic infiltration to secondary organs, and will lead to superior outcomes of leukemia treatment.

One of the major causative molecules associated with AML and poor prognosis is internal tandem duplication mutation of the Flt3 kinase gene (ITD-Flt3). Several Flt3 kinase inhibitors have been developed and evaluated in early clinical trials with varied degrees of success, achieving >50% blast reduction in 12.5 to 81.3% of patients, with the duration of response ranging from two weeks to five months (Fiedler W *et al.*, 2003, Smith BD *et al.*, 2004, Stirewalt DL & Radich JP, 2003), indicating that targeting cell autonomous mechanism alone by these therapies was not sufficient to produce a complete cure. In this regard, the discovery of the leukemia cell niche identifies a new concept and suggests potential therapeutic approaches to antagonize interaction between the leukemia cell and their niche in addition to those targeting cell autonomous mechanisms. The leukemia cells destroy the

normal hematopoietic niche and create their own niche by down-regulating SDF1 in the lesion where leukemia cells invade in the bone marrow (Colmone *et al.*, 2008). Extramedullary infiltration of AML to distant organs likely accompanies creation of new niches in secondary organs by the leukemia cells, which in turn protects them from various chemotherapeutic stimuli.

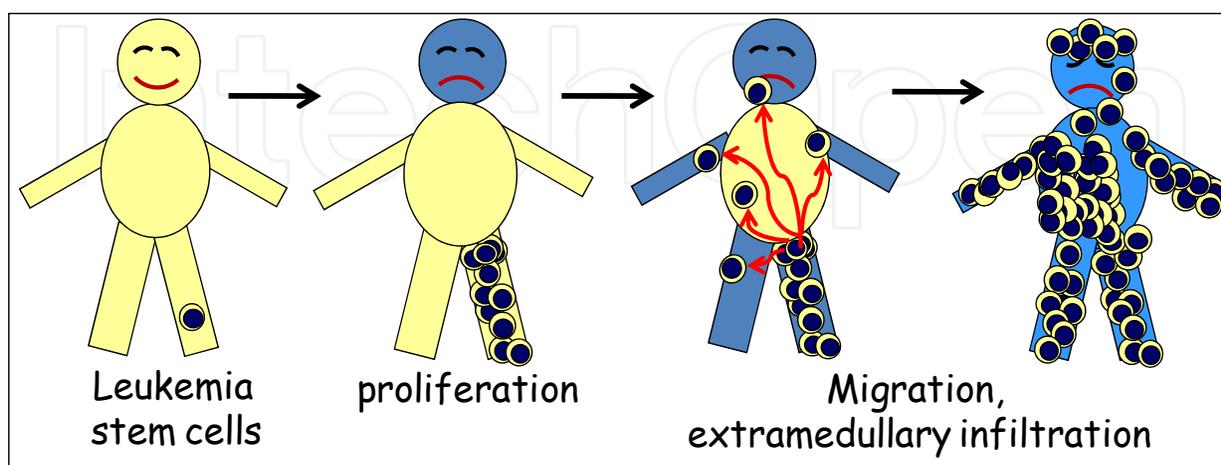


Fig. 1. Dissemination of leukemia cells is a crucial step for disease progression

Chemokines and their receptors play an important role in trafficking of hematopoietic stem cells (HSC) (Lapidot T & Petit I, 2000) and metastasis of tumor cells to distant organs (Muller A *et al.*, 2001). The major chemokine regulating homing and mobilization of HSC is stromal cell-derived factor-1 alpha (SDF1), a ligand for the cell surface CXCR4 receptor (Lapidot T & Petit I, 2000). SDF1 is expressed by osteoblasts, stromal cells and vascular endothelial cells in the bone marrow and attracts and retains HSC in the marrow niche (Lane SW, Scadden DT, & Gilliland DG, 2009). Within the niche, leukemia cells receive survival cues from osteoblasts and endothelial cells in the form of various cytokines and adhesion molecules provided by niche cells, which in turn increase their resistance to cytoreductive therapies (Lane SW, Scadden DT, & Gilliland DG, 2009). However, in skin, brain and visceral organs, extramedullary involvement is occasionally observed in patients with AML and ALL (Byrd JC, Edenfield WJ, Shields DJ, & Dawson NA, 1995, Pui CH & Howard SC, 2008). Leukemia cells need to leave their original bone marrow niche and find their home in the distant secondary niche for initiation of extramedullary dissemination. The mechanism for extramedullary infiltration of leukemia cells remains unexplored. Leukemia cells in the marrow express higher CXCR4 compared to their circulating counterparts, suggesting that interaction between SDF1 and CXCR4 facilitates retention of leukemia cells in the marrow niche (Spoo AC *et al.*, 2006). The majority of leukemia cells express CXCR4 and migrate in response to SDF1. Antagonizing CXCR4 inhibits engraftment and development of AML in a human xenograft human AML model, suggesting that CXCR4 is required for human AML to home to marrow niches (Tavor S *et al.*, 2004). Higher CXCR4 expression predicts extramedullary infiltration in pediatric patients with ALL (Crazzolara R *et al.*, 2001) and functional CXCR4 microparticles and SDF1 correlate with circulating AML (Kalinkovich *et al.*, 2006). These data suggest that chemokine signaling pathways are likely to play crucial roles in the dissemination of leukemia cells to secondary organs. Recent studies suggest that releasing leukemia cells from the marrow niche by blocking SDF1/CXCR4 interaction is effective in increasing their sensitivity to cytoreductive treatment (Nervi B *et al.*, 2009).

Instead of this strategy that principally targets aberrant leukemia cell proliferation, modulating functions of CXCR4 or other chemokine signaling in leukemia cells themselves may represent an alternative strategy to reduce their dissemination to secondary organs.

2.1 Physiological role of SDF1 and CXCR4

SDF1 is expressed in spleen, liver, lung, kidney, thymus, brain (Nervi B *et al.*, 2009), stromal cells (Nagasawa T *et al.*, 1994) and osteoblasts (Jung Y *et al.*, 2006, Semerad CL *et al.*, 2005) in bone marrow and regulates development of hematopoietic cells, immune cells, blood vessels, heart and brain (Tachibana K *et al.*, 1998, Zou YR *et al.*, 1998). Targeted disruption of CXCR4 gene results in impaired bone marrow myelopoiesis and B-lymphopoiesis (Zou YR *et al.*, 1998), which is a similar phenotype with SDF1^{-/-} mice (Nagasawa T *et al.*, 1996). One of the major physiological roles of the SDF1/CXCR4 axis is to regulate homing, retention, and survival of primitive hematopoietic stem and progenitor cells (HSPC) (Christopherson KW 2nd *et al.*, 2003, Kim CH & Broxmeyer HE, 1998, Levesque JP *et al.*, 2003, Liles WC *et al.*, 2003, Peled A *et al.*, 1999). Interaction between SDF1 and its receptor CXCR4 is believed to play an important role in these processes. SDF1 can attract HSPC that express CXCR4 to the marrow microenvironment (Kim CH & Broxmeyer HE, 1998, Peled A *et al.*, 1999), while disruption of SDF1/CXCR4 interaction within marrow can under appropriate circumstances facilitate their mobilization to the peripheral circulation (Liles WC *et al.*, 2003)(Figure 2).

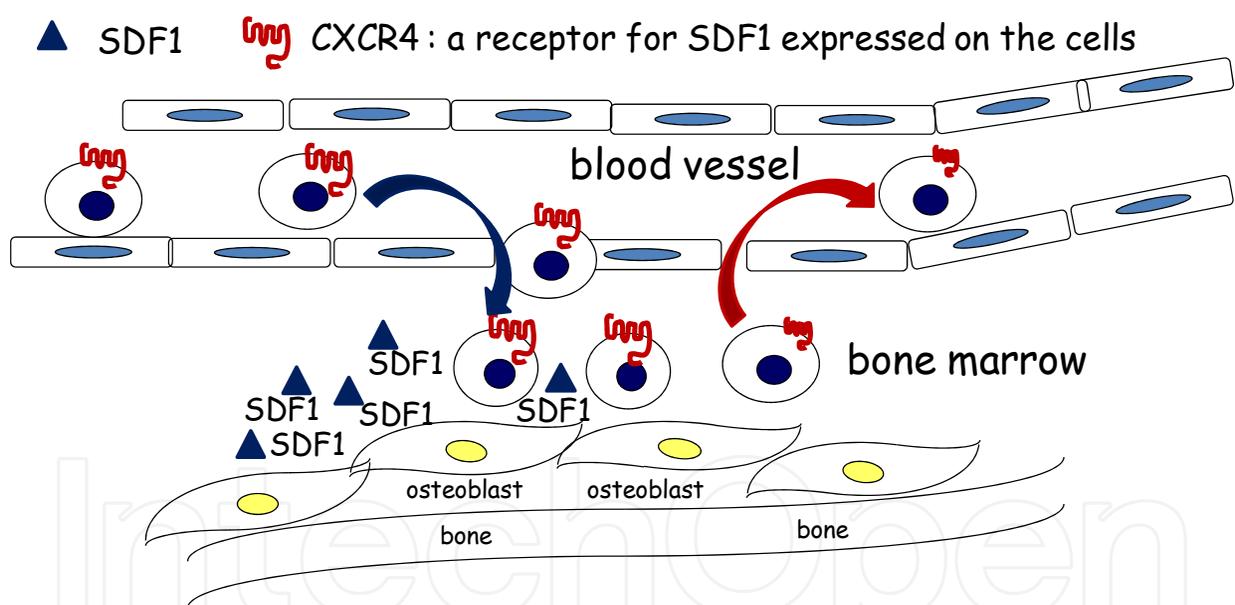


Fig. 2. SDF1 and CXCR4 regulate homing and mobilization of hematopoietic stem cells

HSCs can be found in contact with the cells expressing high amounts of SDF1 (Sugiyama T *et al.*, 2006). SDF1/CXCR4 signaling plays an essential role in maintaining the quiescent HSC pool (Sugiyama T *et al.*, 2006). SDF1 can activate adhesion molecules, particularly very late antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1) on HSPC, which also regulate the homing process (Peled A *et al.*, 2000). SDF1 enhances survival or proliferation of normal hematopoietic progenitor cells (Broxmeyer HE *et al.*, 2003) and regulates development of B-cells (Ma Q *et al.*, 1999). Expression of CXCR4 is up-regulated by various cytokines, including stem cell factor (Peled A *et al.*, 1999), VEGF, bFGF, EGF, IL2, IL4, IL6, IL7, IL10 and IL15 (Busillo JM & Benovic JL, 2007). In contrast, Flt3 ligand (Fukuda

S *et al.*, 2005), TNF α and INF γ down-regulate CXCR4 expression (Busillo JM & Benovic JL, 2007). Adrenergic inputs down-regulate SDF1 in the marrow environment during daytime (Mendez-Ferrer *et al.*, 2008), but up-regulate CXCR4 on HSC at night (Lucas D *et al.*, 2008). Although it was believed that CXCR4 was the only receptor for SDF1, a recent study identified CXCR7 as a secondary receptor for SDF1 (Balabanian K *et al.*, 2005). However, unlike CXCR4, CXCR7 mediates only cell survival, clustering and proliferation and lacks the ability to mediate chemotaxis (Burns JM *et al.*, 2006).

2.2 SDF1/CXCR4 in hematological malignancies

CXCR4 is expressed in most hematological malignancies including chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML) (Burger JA *et al.*, 1999), acute lymphoblastic leukemia (ALL) (Bradstock KF *et al.*, 2000, Mohle R *et al.*, 2000), acute myeloid leukemia (AML) (Kalinkovich A *et al.*, 2006, Rombouts EJ *et al.*, 2004) (Voermans C *et al.*, 2002), B-cell lymphoma (Burger JA *et al.*, 1999, Trentin L *et al.*, 2004), T-cell non Hodgkin lymphoma (NHL) (Weng AP *et al.*, 2003) and multiple myeloma (Weng AP *et al.*, 2003). High expression of CXCR4 predicts extramedullary organ infiltration in childhood ALL (Crazzolaro R *et al.*, 2001) and elevated CXCR4 expression is associated with poor prognosis of patients with AML (Rombouts EJ *et al.*, 2004, Spoo AC *et al.*, 2006). Extramedullary infiltration of M5 AML is associated with high CXCR4 expression (Mohle R *et al.*, 2000), suggesting that the SDF1/CXCR4 axis may be involved in the extramedullary disease of AML. In contrast, a recent report suggests that involvement of CXCR4 in skin infiltration of AML is unlikely (Faaij CMJM *et al.*, 2010). The extramedullary dissemination of AML may be associated with differentiation of AML that appears to affect CXCR4 expression, namely lower CXCR4 in undifferentiated M0, M1 and M2 compared to more differentiated M3, M4 and M5. Another study described that extramedullary dissemination of AML depends on single nucleotide polymorphism (SNP) of SDF1 (Dommange F *et al.*, 2006). Furthermore, SDF1 can recruit endothelial progenitor cells to the tumor microenvironment to facilitate neo-vascularization (Orimo A *et al.*, 2005), which may be required for continued growth of leukemia cells in the extramedullary niche. It has been suggested that targeting CXCR4 signaling pathways may be an important therapeutic strategy for ALL (Juarez J *et al.*, 2003), CLL (Burger M *et al.*, 2005a) and NHL (Bertolini F *et al.*, 2002). Recent data indicates that SDF1 plays an important role in protecting AML cells in the marrow niche, especially during intensive chemotherapy (Zeng ZH *et al.*, 2009). Blocking SDF1/CXCR4 signaling releases leukemia cells from marrow niches into blood circulation, dissociating them from the protective signal in the niche, and increases their sensitivity to chemotherapy (Nervi B *et al.*, 2009). Physiological circadian oscillation of SDF1 in the marrow niche and CXCR4 expression on HSC allows HSC to peripheralize into the blood circulation (Lucas D *et al.*, 2008, Mendez-Ferrer S *et al.*, 2008). It is likely that similar fluctuation in SDF1 and CXCR4 on leukemia stem cells that can affect their mobilization and homing may exist.

Similarly, recent evidence suggests that SDF1/CXCR4 signaling plays a central role in metastasis of solid tumors to the bone marrow or other tissues where SDF1 is expressed (Geminder H *et al.*, 2001, Muller A *et al.*, 2001) and serves as a survival factor for various solid tumor or leukemia cells (Burger JA *et al.*, 2000, Orimo A *et al.*, 2005, Zhou Y *et al.*, 2002). A growing body of evidence indicates that SDF1/CXCR4 signaling and oncogenic proteins, such as BCR/ABL in CML (Geay JF *et al.*, 2005, Mishra S *et al.*, 2006, Ptasznik A *et al.*, 2002, Salgia R *et al.*, 1999), HER2 in breast cancer (Cabioglu N *et al.*, 2006, Li YM *et al.*, 2004) and Internal Tandem Duplication (ITD)-Flt3 in AML (Fukuda S *et al.*, 2005, Fukuda S & Pelus

LM, 2006), functionally interact to modulate migration of malignant cells. Their concomitant expression with CXCR4 is often associated with poor patient prognosis (Li YM *et al.*, 2004, Mishra S *et al.*, 2006).

2.3 Hematopoietic growth factors that affect SDF1/CXCR4 signaling and migration of leukemia cells

While hematopoietic growth factors normally stimulate proliferation and survival of HSPC, some of them can stimulate migration. Stem cell factor (SCF) and Flt3 ligand (FL) are the respective ligands for the receptor tyrosine kinases c-kit and Flt3 that are expressed on HSPC. Although they have similar but distinct roles in HSPC proliferation and survival (Lyman SD & Jacobsen SEW, 1998), they both stimulate migration of human cord blood (UCB) CD34⁺ cells (Fukuda S *et al.*, 2005, Kim CH & Broxmeyer HE, 1998). In contrast to the chemotactic activity of SDF1 that stimulates directional cell migration towards the chemokine (chemotaxis), SCF and FL stimulate random cell migration (chemokinesis). SCF increases SDF1-induced chemotaxis of the AML line MO7e cells when combined with SDF1 (Kim CH & Broxmeyer HE, 1998). Similarly, FL enhances migration of the RS4;11 bi-phenotypic acute leukemia cell lines to SDF1 (Fukuda S *et al.*, 2005). These data suggest that hematopoietic growth factors regulate migration of acute leukemia cells by modulating the SDF1/CXCR4 axis.

Although both SCF as well as FL enhance migration of human UCB CD34⁺ cells induced by SDF1 in a similar manner, prolonged exposure the cells to SDF1 result in differential migratory responses. Prolonged incubation of UCB CD34⁺ cells with SCF up-regulates CXCR4 expression and enhances subsequent migration to SDF1 (Peled A *et al.*, 1999). In contrast, incubation of UCB CD34⁺ cells with FL over 24 hrs down-regulates CXCR4 expression coincident with a decrease in subsequent migration to SDF1 (Fukuda S *et al.*, 2005). Down-regulation of CXCR4 by incubation with FL is consistent with reduction of CXCR4 in CD34⁺ cells transfected with ITD-Flt3 that activate Flt3 without ligand binding (Jacobi A *et al.*, 2010). Similar to human CD34⁺ cells, the differential migration to SDF1 modulated by FL was also observed in Ba/F3 cells expressing human wild-type Flt3 receptor. The synergistic cell migration in response to the combination of SDF1 and FL was associated with synergistic phosphorylation of ERK, Akt and CREB. These results suggest that ERK, Akt and CREB are involved in the synergistic increase in migration by the combination of SDF1 plus FL. On the other hand, pre-incubation of Ba/F3 cells expressing Flt3 with FL for 24 hours down-regulates CXCR4 and significantly diminishes their subsequent migration to SDF1 compared to control cells pre-incubated without FL. In contrast to synergistic response, phosphorylation of ERK, Akt and CREB in response to SDF1 was reduced in the cells pretreated with FL compared to untreated control cells, further validating the differential modulation of SDF1/CXCR4 signaling by FL/Flt3 (Fukuda S *et al.*, 2005). Down-regulation of CXCR4 by FL may explain one of the mechanisms of HPC mobilization by FL administration (Brasel K *et al.*, 1997). A similar effect by SCF is not known. These data indicate overlapping but distinct roles of c-kit and Flt3 signaling on CD34⁺ cell migration induced by SDF1.

2.4 Bcr/abl alters signaling and chemotactic response mediated by SDF1/CXCR4

Chronic myeloid leukemia (CML) is caused by the Bcr/Abl oncogene with constitutive kinase activity, a result of a reciprocal translocation between chromosomes 9 and 22 (Kurzrock R *et al.*, 1988, Sawyers CL, 1999). Bcr/abl is occasionally observed in acute

lymphoblastic leukemia cells (ALL) as well (Mishra S *et al.*, 2006). One of the characteristics of CML is an early release of myeloid cells from the marrow and their accumulation in the blood and spleen, suggesting that cell retention in the marrow or migration may be impaired. CML cells show reduced adhesion to stromal cells and extracellular matrix (Gordon MY *et al.*, 1987, Verfaillie CM *et al.*, 1992) that may concomitantly contribute to impaired retention. Ectopic Bcr/Abl increases spontaneous motility of hematopoietic cell lines; however, it significantly reduces chemotactic response to SDF1 (Salgia R *et al.*, 1999). Homing of mouse hematopoietic Ba/F3 cells expressing Bcr/Abl to spleens in the mice that have been ectopically injected with SDF1 was significantly reduced compared to control cells, although their homing to the bone marrow, lung or blood was not affected. The migratory response of primary CML cells to SDF1 at blast crisis was profoundly reduced, which was associated with reduced CXCR4 expression, while CD34⁺ cells at chronic phase migrate normally in response to SDF1 (Geay JF *et al.*, 2005). In addition, higher Bcr/abl expression induces a marked down regulation of CXCR4, while treatment with STI-571 (Imatinib), the Bcr/abl antagonist, that blocks Bcr/abl activity restores CXCR4 expression in CD34⁺ cells from patients in blast crisis (Geay JF *et al.*, 2005). The reduction in CXCR4 expression and loss of response to SDF1 mediated by Bcr/abl may allow CML cells to exit bone marrow, resulting in infiltration to secondary organs, including blood and spleen. These data suggest that Bcr/abl regulates trafficking of hematopoietic cells by modulating SDF1/CXCR4 function, which is associated with disease progression and extramedullary dissemination. Conversely, SDF1 can increase resistance of acute lymphoblastic leukemia cells expressing Bcr/abl to STI-571, suggesting that SDF1/CXCR4 signaling may augment Bcr/abl signaling that enhances resistance to the therapy (Mishra S *et al.*, 2006).

2.5 ITD-Flt3 alters chemotaxis induced by SDF1/CXCR4 signaling

Genetic mutations (Internal tandem duplication: ITD) of the Flt3 gene that pathologically auto-activate Flt3 tyrosine kinase activity have been found in ~25-30% of patients with AML and elevated CXCR4 receptor expression is associated with poor outcome in patients with acute myeloid leukemia (AML) (Levis M & Small D, 2003, Spoo AC *et al.*, 2006), suggesting a role for ITD-Flt3 and CXCR4 in disease progression. Fifteen percent of patients with extramedullary AML were positive for ITD-Flt3 (Ansari-Lari *et al.*, 2004), suggesting that ITD-Flt3 may affect migration and trafficking of leukemia cells. ITD-Flt3 mutations cause extramedullary infiltration of hematopoietic cells with splenomegaly in a myeloproliferative disease model in mice (Kelly LM *et al.*, 2002). Consistent with this finding, ITD-Flt3 significantly increases accumulation of Ba/F3 cells in the spleen shortly after transplantation compared to wild-type Flt3 (Fukuda S & Pelus LM, 2006). Stable expression of ITD-Flt3 in mouse Ba/F3 and 32D cells significantly increases migration to SDF1 in addition to enhancing spontaneous motility (Figure 3) (Fukuda S *et al.*, 2005). This is in contrast to Bcr/abl that increases spontaneous motility while reducing migration in response to SDF1 (Geay JF *et al.*, 2005, Salgia R *et al.*, 1999). Importantly, CXCR4 expression on Ba/F3 cells harboring ITD-Flt3 mutations was significantly reduced, indicating that enhanced migration is not explained by CXCR4 expression (Fukuda S *et al.*, 2005) (Figure 3). This is consistent with down-regulation of CXCR4 in Ba/F3 cells expressing wild-type Flt3 or UCB CD34⁺ cells incubated with FL for over 24 hours. However, longer exposure to FL reduces migration to SDF1 (Fukuda S *et al.*, 2005). In contrast to synergistic phosphorylation of ERK, Akt or CREB coincident with enhanced migration to SDF1 and FL in Ba/F3 cells harboring wild-type Flt3, accentuated migration to SDF1 by ITD-Flt3 was not associated with an

increase in phosphorylation of these molecules (Table 1). These findings suggest qualitative differences between ITD-Flt3 signaling and FL / wild type Flt3 signaling, even if both stimuli result in similar enhancement in migration to SDF1.

	FL	prolonged stimulation with FL	ITD-Flt3
CXCR4	no change →	decrease ↓	decrease ↓
Migration to SDF1	increase ↑	decrease ↓	increase ↑
Phospho ERK, Akt, CREB	increase ↑	decrease ↓	no change →

Table 1. Differential regulation on CXCR4 expression and migration to SDF1 by FL/wild-type Flt3 signaling or ITD-Flt3 in Ba/F3 cells

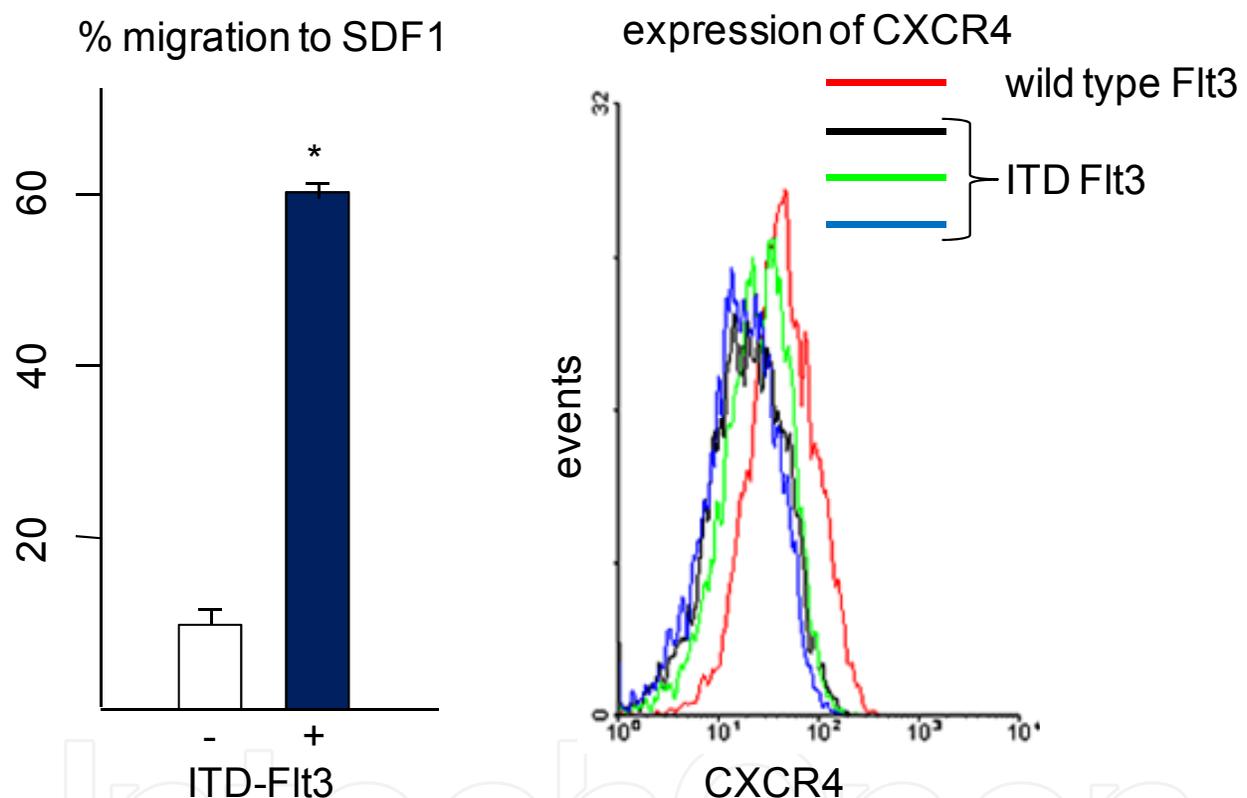


Fig. 3. Migration and CXCR4 expression of ITD-Flt3 + or ITD-Flt3 - Ba/F3 cells. Migration of ITD-Flt3+ cells towards SDF1 was significantly elevated compared to ITD-Flt3 - cells (* $P < 0.05$; left panel). However, CXCR4 expression was significantly reduced in ITD-Flt3 + cells compared to ITD-Flt3 - cells (right panel), suggesting that the enhanced migration is not a quantitative increase of CXCR4 signaling but more likely to be a qualitative alteration of CXCR4 signaling by ITD-Flt3.

Enhanced chemotactic response to SDF1 is partially mediated through Ras signaling, since dominant negative H-Ras dramatically inhibits spontaneous and SDF-mediated migration of Ba/F3 cells, while constitutively active H-Ras expression in Ba/F3 cells harboring wild-type Flt3 increases chemotactic response to SDF1 to a similar extent as ITD-Flt3 (Fukuda S & Pelus LM, 2006). Accentuated migration of Ba/F3 cells to SDF1 induced by ITD-Flt3 was barely inhibited by the Flt3 inhibitor AG1296 or the CXCR4 antagonist AMD3100 alone,

whereas it was partially inhibited by the combination of both compounds (Figure 4). Surprisingly, ITD-Flt3 mutations also increase cell migration away from an SDF1 gradient compared to control cells (Fukuda S *et al.*, 2005). This result implies that ITD-Flt3 may facilitate peripheralization of leukemia cells out of the bone marrow niche where SDF1 is present, in addition to the effects of enhancing cell homing towards SDF1 in the niche.

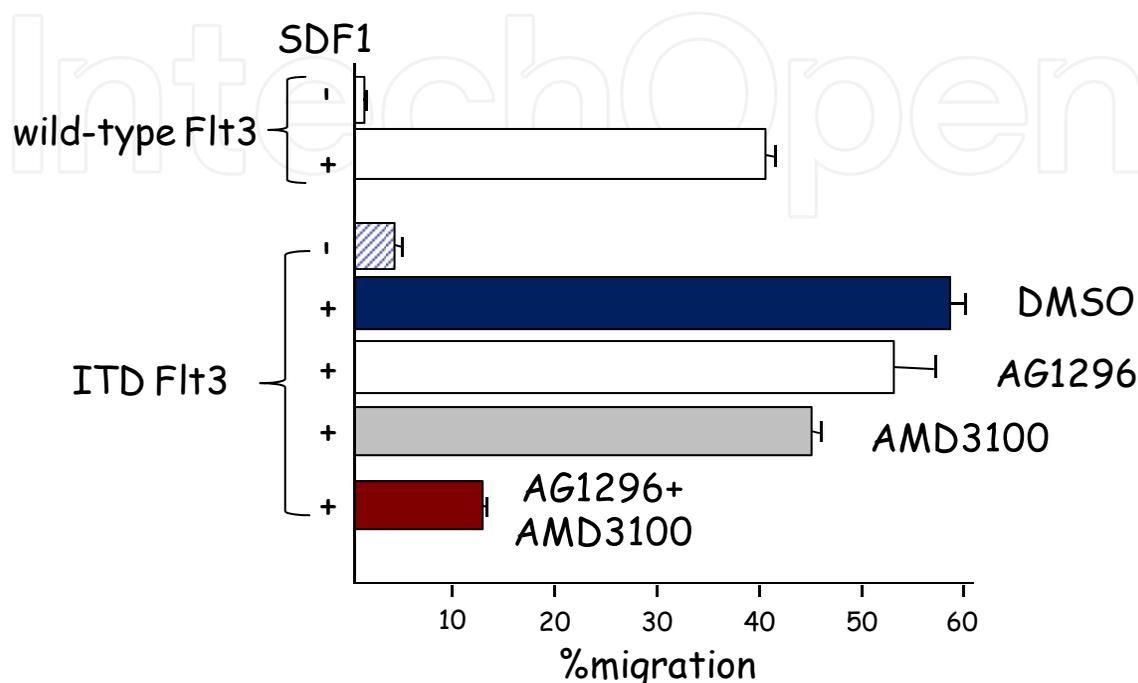


Fig. 4. Migration of Ba/F3 cells expressing ITD-Flt3 pretreated with AMD3100 and/or AG1296

In contrast to the mouse cell system where human ITD-Flt3 mutations were retrovirally transduced into Ba/F3 cells and 32D cells (Fukuda S *et al.*, 2005), a recent report has shown that transient expression of ITD-Flt3 in human CD34⁺ cells inhibited migration to SDF1 concomitant with reduction in cell surface CXCR4 expression (Jacobi A *et al.*, 2010). While reduction in CXCR4 in CD34⁺ cells by ITD-Flt3 is consistent with Ba/F3 cells transduced with ITD-Flt3, the differential effects on migration to SDF1 by these cells may reflect the timeframe of exposure to SDF1 following introduction of ITD-Flt3 into the cells. Shortly after introduction of ITD-Flt3, expression of CXCR4 is down-regulated, thereby inhibited migration to SDF1. In contrast, prolonged expression of ITD-Flt3 leads to functional activation of CXCR4 signaling pathways and increases cell migration, while maintaining lower CXCR4 expression, suggesting that ITD-Flt3 may differentially regulate chemotactic response to SDF1. These findings suggest that migration of leukemia cells to SDF1 may be diminished shortly after emergence of ITD-Flt3 in the patients due to reduction of CXCR4 level and concomitant quantitative decline in CXCR4 signaling. This does not accompany qualitative changes in CXCR4 signaling. In contrast, their migration to SDF1 is enhanced at a later stage of the disease long after ITD-Flt3 appearance, most likely due to subsequent functional alteration of CXCR4 signaling by ITD-Flt3 that is not coupled with CXCR4 expression level. Reduced migration to SDF1 by ITD-Flt3 can facilitate their peripheralization into blood at an early stage of the disease whereas enhanced migration

to SDF1 may aid to increase homing of the leukemia cells to the organs where SDF1 is expressed at a later stage of the disease.

2.6 Identification of CXCR4 pathways that are selectively regulated by ITD-Flt3

CXCR4 is expressed in the majority of hematopoietic cells, including HSC, T-cells, B-cells and myeloid cells. While antagonizing CXCR4 has been shown to be a safe way to collect HSC from the healthy donors (Liles WC *et al.*, 2003), it is known that SDF1/CXCR4 signaling provides survival effects to the primitive hematopoietic cell compartment (Broxmeyer HE *et al.*, 2003), therefore the long term effect of CXCR4 antagonist on normal hematopoietic cell function should be monitored with caution. In this regard, concomitant use of a CXCR4 antagonist with chemotherapeutic drugs may enhance toxicity, not just to leukemia cells, but also to normal HSC. In order to minimize toxicity on HSC by CXCR4 antagonist, it would be necessary to identify selective signaling molecules downstream of CXCR4 that are specifically regulated in leukemia stem cells but not in normal HSC. Similarly, identification of selective CXCR4 related molecular pathways regulating homing of leukemia cells distinct from normal hematopoietic cells will aid to antagonize aberrant trafficking of leukemia cells without affecting normal hematopoiesis.

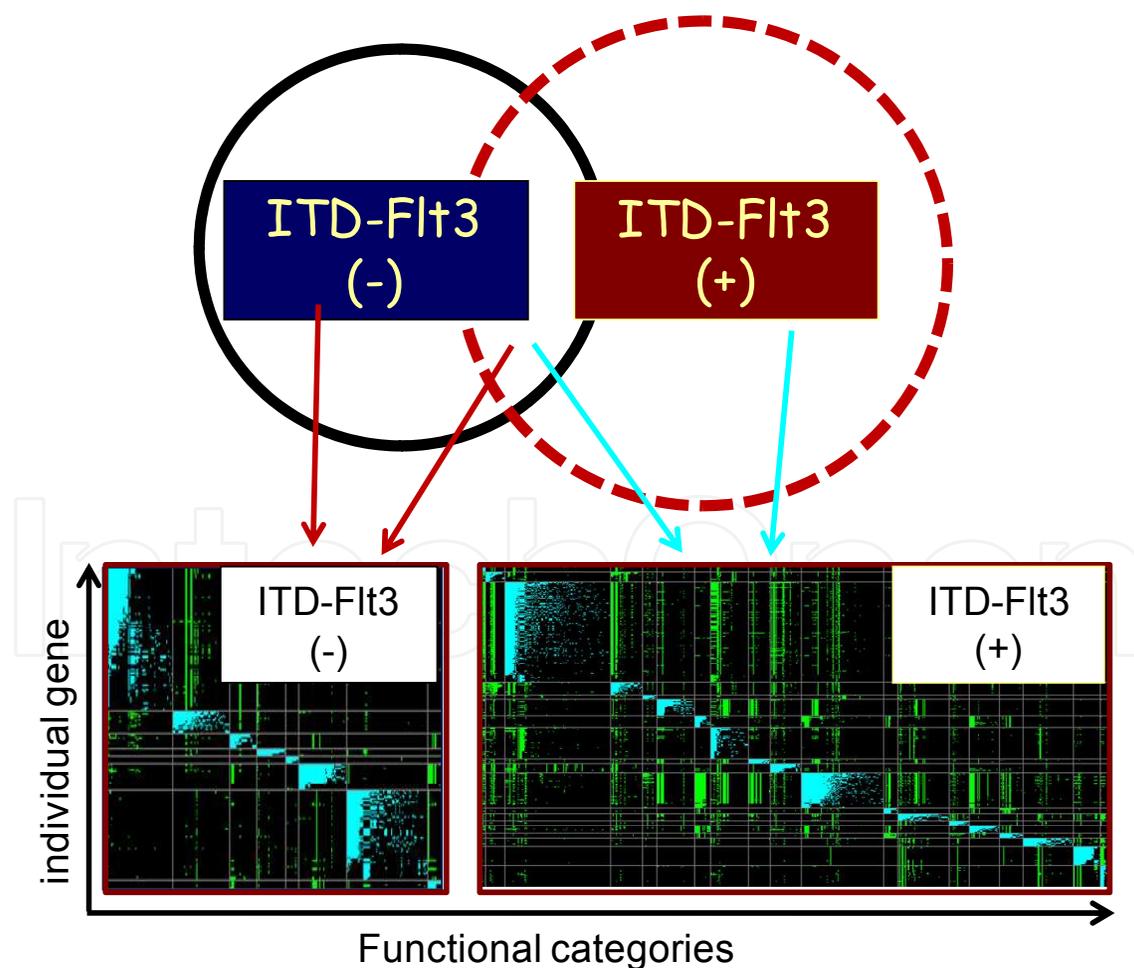


Fig. 5. Functional classification of genes downstream of SDF1/CXCR4 in ITD-Flt3 + versus ITD-Flt3 - mouse Ba/F3 cells.

As we previously described, ITD-Flt3 mutations enhance migration of hematopoietic cells to the chemokine SDF1 (Figure 3) (Fukuda S *et al.*, 2005), suggesting that ITD-Flt3 may facilitate dissemination of leukemia cells by modulating the SDF1/CXCR4 signaling pathway. The enhanced migration to SDF1 by ITD-Flt3 positive cells was associated with down regulation of CXCR4 compared to control cells lacking ITD-Flt3. This suggests that the enhanced migration to SDF1 by ITD-Flt3 is not a consequence of a quantitative increase in SDF1/CXCR4 signaling, which led us to investigate qualitative alteration of CXCR4 signaling by ITD-Flt3. Analysis of gene expression in ITD-Flt3⁻ and ITD-Flt3⁺ Ba/F3 cells migrating to SDF1 indicated that SDF1 modulates 4.0% of 40,000 genes analyzed in ITD-Flt3⁺ cells, of which 2.5% were regulated by SDF1 exclusively in ITD-Flt3⁺ cells. Figure 4 indicates comparison of changes in gene expression before and after migration to SDF1 (SDF1 responsive genes) between ITD-Flt3⁻ and ITD-Flt3⁺ Ba/F3 cells. Genes modulated in ITD-Flt3⁻ cells or ITD-Flt3⁺ cells following migration to SDF1 compared to these cells analyzed before migration were functionally classified based on Gene Ontology Term using DAVID software (Huang DW *et al.*, 2009)(Figure 5). X-axis represents functional categories of the individual genes analyzed in the Y-axis. Functional classification indicated that several functional signaling pathways were significantly enriched exclusively in ITD-Flt3 but not in control cells. These data indicate that enhanced cell migration to SDF1 induced by ITD-Flt3 is likely mediated through activation of selective CXCR4 signaling pathways that are functionally distinct from ITD-Flt3⁻ cells and that are not coupled with CXCR4 expression. Importantly, several of these CXCR4 downstream molecules selectively regulated by ITD-Flt3 are products of genes known to be deregulated in AML stem cells (Majeti R *et al.*, 2009). Leukemia stem cells (LSC) are likely responsible for dissemination to secondary organs in addition to disease initiation and recurrence. Genes deregulated by LSC and regulated by SDF1 specifically in ITD-Flt3⁺ cells may represent key targets to prevent unnecessary dissemination and invasion of ITD-Flt3⁺ acute leukemia cells without affecting normal hematopoiesis. In addition to the genes selectively regulated by SDF1 in ITD-Flt3⁺ cells, approximately 30 mRNAs that are known to be functionally associated with cell motility or migration were deregulated in ITD-Flt3⁺ cells compared to ITD-Flt3⁻ cells. This data suggests that ITD-Flt3 itself may affect cell migration. This is consistent with enhanced spontaneous migration by ITD-Flt3 in Ba/F3 cells (Fukuda S *et al.*, 2005).

Our working hypothesis on ITD-Flt3⁺ AML cell trafficking regulated by interaction between ITD-Flt3 and SDF1/CXCR4 pathway is shown in Figure 6. The presence of ITD-Flt3 mutations decrease CXCR4 expression, thereby reducing interaction between SDF1 and AML cells and allowing their release from bone marrow. In addition, ITD-Flt3⁺ cells can migrate away from SDF1 more efficiently compared to ITD-Flt3⁻ cells, which can also contribute to their peripheralization/egress. Alternatively, migration to SDF1 may not be enhanced shortly after ITD-Flt3 emergence or at an early stage of the disease owing to the reduction of CXCR4 that without activation of CXCR4 signaling. Prolonged exposure to aberrant signaling generated by ITD-Flt3 functionally activates SDF1/CXCR4 signaling in the ITD-Flt3⁺ cells that is different from ITD-Flt3⁻ cells (Figure 5) while maintaining lower levels of CXCR4 expression (Figure 3). This suggests that enhanced migration to SDF1 is a consequence of functional alteration of CXCR4 signaling rather than CXCR4 up-regulation. This will help AML cells to home to the distant organs or secondary bone marrow where SDF1 is present. Circadian oscillation of SDF1 in the marrow niche and CXCR4 expression on HSC (Lucas D *et al.*, et al 2008, Mendez-Ferrer S *et al.*, 2008) may also exist in the

leukemia niche and leukemia stem cells expressing ITD-Flt3. This fluctuation of SDF1 and CXCR4 can also affect their mobilization and homing. De-sensitization of CXCR4 signaling following exposure to SDF1 may also be distinct in leukemia cells versus normal HSC, which can affect their response to SDF1.

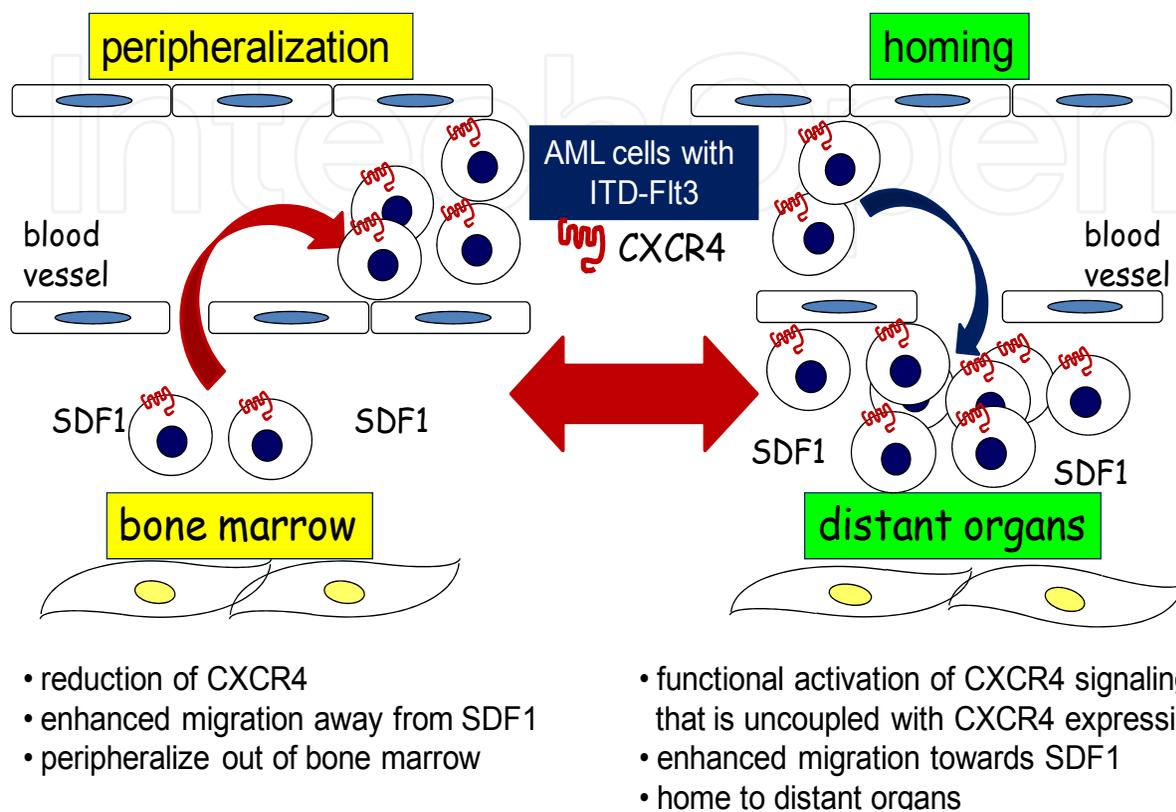


Fig. 6. Suggested model for ITD-Flt3⁺ AML cell trafficking regulated by interaction between ITD-Flt3 and SDF1/CXCR4 pathway

2.7 Role of other chemokines and their receptors for leukemia cell dissemination

Childhood T-ALL often relapses in the CNS (Pui CH & Howard SC, 2008). Oncogenic Notch1 signaling that is frequently activated in T-ALL regulates CCR7 expression. CCL19 expressed in central nervous system serves as a chemo-attractant for T-ALL cells with elevated expression of CCR7. Antagonizing CCR7 and its chemokine ligand CCL19 inhibit CNS infiltration of T-ALL in an animal model (Buonamici S *et al.*, 2009). On the other hand, overexpression of CCR7 was sufficient to recruit T-ALL cells into the CNS. The data indicates that CCL19/CCR7 signaling activated by oncogenic Notch1 regulates CNS infiltration of T-ALL and that targeting the CCR7 pathway may represent a novel therapeutic strategy for treatment of CNS prophylaxis of T-ALL (Buonamici S *et al.*, 2009) (Figure 7).

While antagonizing CCR7 signaling is a promising strategy to block invasion of T-ALL cells, it may impair immune surveillance by normal T-cells, since CCR7 is one of the chemokine receptors required for T-cell trafficking (Burger M *et al.*, 2005b). Unnecessary immune impairment that can cause serious life threatening infection by pathogens needs to be avoided during the intensified treatment for leukemia. In this regard, identification of selective signaling molecules in the CCR7 pathway in T-ALL that are functionally distinct

from normal T-cells is important in order to develop selective strategy with minimal toxicity on T-cells.

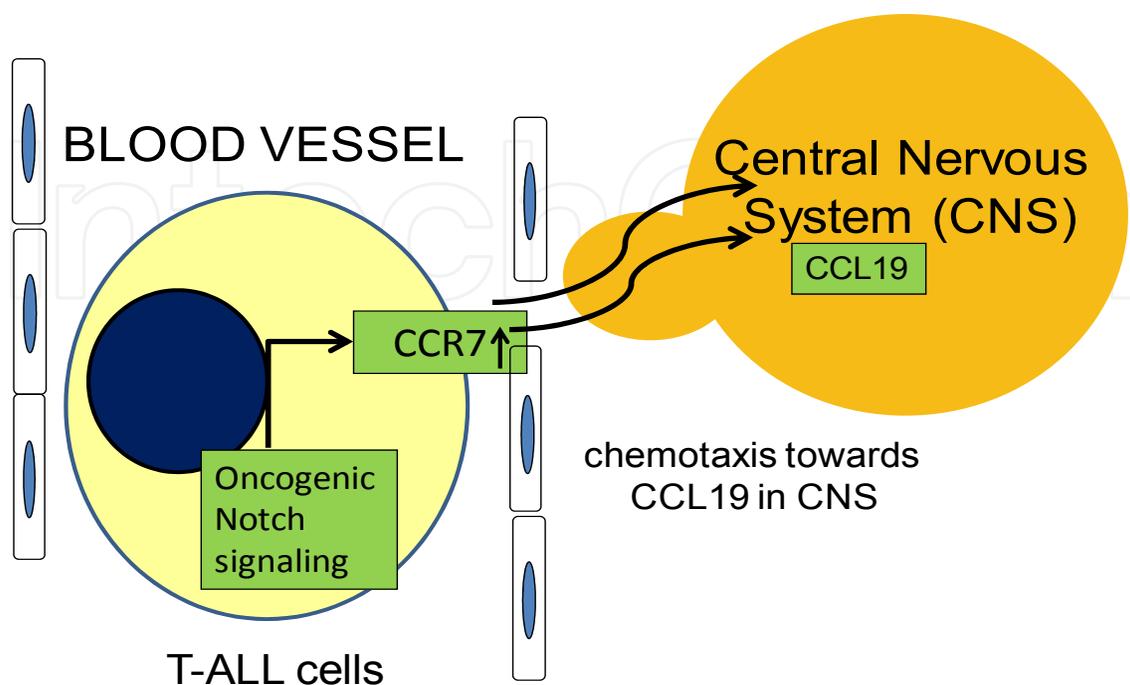


Fig. 7. Migration and invasion of T-ALL cells into central nervous system mediated through CCL19/CCR7 pathway

Mixed lineage leukemia (MLL) frequently found in infant ALL is caused by a chromosomal translocation that involves 11q23 (Pieters R, 2009). This is associated with poor outcome with relatively high frequency of central nervous involvement compared to older children, suggesting that MLL gene rearrangement modulates leukemia cell dissemination. A recent report indicates that MEF2C regulates homing and invasiveness of MLL/ENL leukemia cells without affecting establishment and maintenance of leukemia stem cells (Schwieger M *et al.*, 2009). MEF2 regulates expression of the chemokine receptors CXCR4, CCR2 and CCR5 and chemokines, such as CCL2, CCL3, CCL4 and CCL6 (Schwieger M *et al.*, 2009). This suggests that MLL gene rearrangement regulates homing and invasion of leukemia cells through MEF2C by affecting expression of chemokines and their receptors.

Chemokine receptor expression is differentially regulated in AML patients with skin involvement. Skin residing AML cells displayed a different set of chemokine receptors in situ, for instance: CCR5, CXCR4, CXCR7 and CX3CR1. However, a recent report indicates a high percentage of circulating CCR2^{pos} AML cells were only detected in patients with extramedullary disease (Faaij CMJM *et al.*, 2010). High expression of CCR2 was not observed in bone marrow blasts. This study showed that there was no difference in the expression of CXCR4, suggesting that CCR2 may be a dominant regulator for skin dissemination of AML.

3. Conclusion

Given that various oncogenic molecules responsible for hematological malignancies such as ITD-Flt3, Bcr-abl and Ras, modulate response to SDF1, these oncogenes likely modulate trafficking of leukemia cells. Although there is no definitive evidence that CXCR4 is indeed

involved in the dissemination of acute leukemia, multiple data support that this is most likely the case. Blocking CXCR4 appears to be a promising strategy to sensitize leukemia cells to chemotherapy by releasing/mobilizing them into the peripheral blood circulation. Targeting CXCR4 signaling pathways may also be useful to minimize leukemia cell dissemination in addition to sensitizing them to chemotherapy by releasing them from marrow niches. This idea is consistent with targeting the CCR7 receptor in T-ALL that blocks their CNS dissemination (Buonamici S et al., 2009). Although promising, precautions need to be taken since antagonizing CXCR4 initiates mobilization of leukemia cells into circulation, which may facilitate secondary organ infiltration.

4. Acknowledgment

The authors have no conflict of interest associated with this work. This work was supported by Naito Memorial Foundation, the Mother and Child Health Foundation and the Grant-in-Aid for Scientific Research (B) (20390298) from Japan Society for the Promotion of Science (to SF).

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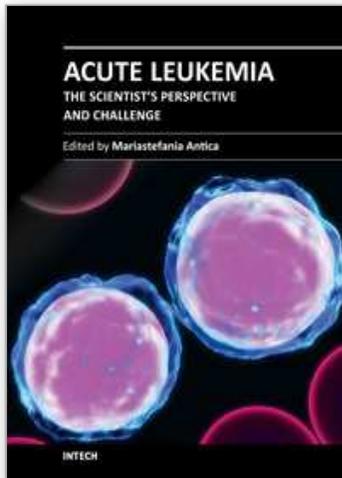
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Acute Leukemia - The Scientist's Perspective and Challenge

Edited by Prof. Mariastefania Antica

ISBN 978-953-307-553-2

Hard cover, 428 pages

Publisher InTech

Published online 22, December, 2011

Published in print edition December, 2011

This book provides a comprehensive overview of the basic mechanisms underlying areas of acute leukemia, current advances, and future directions in management of this disease. The first section discusses the classification of acute leukemia, taking into account diagnoses dependent on techniques that are essential, and thankfully readily available, in the laboratory. The second section concerns recent advances in molecular biology, markers, receptors, and signaling molecules responsible for disease progression, diagnostics based on biochips and other molecular genetic analysis. These advances provide clinicians with important understanding and improved decision making towards the most suitable therapy for acute leukemia. Biochemical, structural, and genetic studies may bring a new era of epigenetic based drugs along with additional molecular targets that will form the basis for novel treatment strategies. Later in the book, pediatric acute leukemia is covered, emphasizing that children are not small adults when it comes to drug development. The last section is a collection of chapters about treatment, as chemotherapy-induced toxicity is still a significant clinical concern. The present challenge lies in reducing the frequency and seriousness of adverse effects while maintaining efficacy and avoiding over-treatment of patients.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Seiji Fukuda, Chie Onishi and Louis M. Pelus (2011). Trafficking of Acute Leukemia Cells – Chemokine Receptor Pathways that Modulate Leukemia Cell Dissemination, *Acute Leukemia - The Scientist's Perspective and Challenge*, Prof. Mariastefania Antica (Ed.), ISBN: 978-953-307-553-2, InTech, Available from: <http://www.intechopen.com/books/acute-leukemia-the-scientist-s-perspective-and-challenge/trafficking-of-acute-leukemia-cells-chemokine-receptor-pathways-that-modulate-leukemia-cell-dissemin>

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