Acute leukaemia in children : aspects of diagnosis and treatment

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Chemokine/Chemokine receptor interactions in extramedullary leukaemia of the skin in childhood AML: Differential roles for CCR2, CCR5, CXCR4 and CXCR7

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Abstract

Chemokine/chemokine receptor interactions orchestrate the migration of non-malignant as well as malignant cells to peripheral tissues. Understanding these homing patterns as well as the signals that are necessary for extramedullary leukaemic cell survival may yield new perspectives for the treatment of haemato-oncological diseases such as Acute Myeloid Leukaemia (AML). We analysed chemokine and chemokine receptor expression in skin biopsies (n=14), peripheral blood samples (n=7) and bone marrow aspirates (n=6) obtained from 15 paediatric AML patients who displayed skin involvement at the time of diagnosis; AML patients without skin involvement served as controls. High numbers of circulating CCR2pos AML cells were detected in patients with extramedullary disease but not in control patients. Skin residing AML cells, however, displayed a different set of receptors in situ, namely: CCR5, CXCR4, CXCR7 and CX3CR1. The CCR5 binding chemokine CCL3 and the CXCR4/CXCR7 binding chemokine CXCL12 could both be visualised in the same tissue sections. Additional in vitro experiments provided evidence that CXCR7/CXCL12 signalling supports the survival of bone marrow-derived AML cells. These preliminary results suggest the involvement of three different chemokine/chemokine receptor interactions in directing homing and retention of leukaemic blast cells in the skin.

Introduction

AML mostly originates in the bone marrow and is characterised by uncontrolled proliferation of myeloid progenitor cells, which are arrested in their maturation process (1). The prognosis of childhood AML has significantly improved over the past decades, given that approximately 60% of the paediatric AML patients now experiences long-term survival (2-4). Unfortunately, such a disease-free state can only be achieved after a very intensive course of chemotherapy and, if necessary, followed by haematopoietic stem cell transplantation. In 10-40% of the paediatric AML patients, extramedullary disease manifests at diagnosis, which is thought to correlate with poor prognosis (5-10). Extramedullary leukaemia (EML) is defined as an infiltrate of AML cells in soft tissues, skin, muscles, bone, gingival tissue or brain. This mostly occurs in patients suffering from M4 or M5 AML subtypes (5). Rarely, EML can also precede the diagnosis of bone marrow involvement. The latter scenario is predominantly seen in patients with aleukaemia cutis (11;12). In addition, AML may also be located at sanctuary sites such as testis or cerebrospinal fluid.

It has been increasingly recognised that chemokines play an important role in tumour cell migration and infiltration of distant organ sites. This multi-step process requires the sequential engagement of adhesion molecules and activation through chemokine receptors (13-17). To date, most studies addressing the involvement of chemokines and their receptors in the tropism of leukaemic cells have concentrated on the interaction of CXCL12 and its receptor CXCR4. In AML, the expression of CXCR4 is thought to be higher on blast cells residing in the bone marrow than on circulating blast cells. Due to co-localisation of its ligand, CXCL12, CXCR4 most likely facilitates retention of AML cells in the bone marrow (18;19). In fact, high CXCR4 expression by AML blast cells in the bone marrow is considered an independent risk factor for relapse and poor overall survival (20). CXCR4 has long been considered as the sole receptor for CXCL12. However, CXCL12 was also recently reported to be the ligand of a novel chemokine receptor named CXCR7 (21). CXCR7 is expressed by a range of primary tumours, many tumour cell lines and activated endothelial cells, but is rarely expressed by non-transformed cells (22). Unlike other chemokine receptors, CXCR7 lacks the ability to

mediate chemotaxis and calcium mobilisation after ligand binding. Yet, CXCR7 is thought to regulate several important biological processes including cell survival, cell clustering and tumour development as shown for prostate, lung and breast cancer cells (23;24).

Given the paucity of data regarding the role of specific chemokines and their receptors in the migration of myeloid blast cells to extramedullary sites, we investigated 1) the expression of chemokine receptors on leukaemic blasts derived from bone marrow and peripheral blood; 2) the in situ expression of corresponding ligands in affected skin biopsies and 3) the potential role of CXCR7 in the in vitro survival of primary AML blast cells.

Materials & Methods

Patients

Eighteen paediatric AML patients with proven skin involvement at diagnosis (of which 3 are aleukaemia cutis patients) and 10 additional patients without EML were enrolled in the study. Patient characteristics are described in Table 1. Routinely collected formalin fixed paraffin embedded skin biopsies were obtained from 14 AML patients with skin involvement. All biopsies were taken from affected sites containing tumour cells as confirmed by pathology reports. From 20 AML patients, frozen peripheral blood mononuclear cells (PBMC) and/or bone marrow mononuclear cells (BMMC) were available for flow cytometric analysis. Peripheral blood and/or bone marrow samples of 18 of these patients were obtained from the Dutch Childhood Oncology Group (DCOG, The Hague, the Netherlands; protocol: OC-2001-013). Skin biopsies and/or bone marrow samples of 5 patients were obtained from the Haematology/Oncology department of the Women's and Children's Hospital (Adelaide, Australia). Skin biopsies of 9 patients were obtained from 7 Dutch paediatric oncology centres, which all participate in the DCOG. All samples were obtained at diagnosis. In vitro survival assays were performed on primary bone marrow blasts derived from 4 AML patients. Informed consent was obtained from all patients and/or their parents.

Table 1. Patient characteristics at diagnosis

UPN	Sex	Age	Presentation	% Blasts in BM	% Blasts in PB	Extramedullary leukaemia site (if present) besides skin	Included in
Skin involvement at diagnosis							
1	*M*	*0*	*aleukaemia cutis*	*0*	*0*		*IHC*
2	*M*	*2*	*aleukaemia cutis*	*0*	*0*		*IHC*
3	*F*	*7*	*aleukaemia cutis*	*1*	*0*		*IHC*
4	*F*	*15*	*M2*	*77*	*99*		*FC, IHC, SA*
5	*F*	*15*	*M2*	*67*	*51*		*FC*
6	*M*	*0*	*M4*	*ND*	*82*		*FC*
7	*F*	*0*	*M4*	*55*	*16*		*IHC*
8	*F*	*1*	*M4*	*30*	*3*		*IHC*
9	*M*	*1*	*M5*	*ND*	*ND*		*IHC*
10	*F*	*0*	*M0*	*85*	*95*	*CNS*	*FC, IHC*
11	*M*	*12*	*M0*	*39*	*6*	*CNS*	*IHC*
12	*F*	*6*	*M2*	*65*	*65*	*CNS*	*FC, IHC, SA*
13	*M*	*14*	*M2*	*86*	*80*	*CNS*	*FC, IHC, SA*
14	*M*	*13*	*M5*	*87*	*48*	*CNS*	*FC, IHC*
15	*F*	*1*	*M4/M5a*	*35*	*0*	*Bone*	*IHC*
16	*F*	*3*	*M7*	*41*	*67*	*Eye*	*FC*
17	*M*	*16*	*M1*	*92*	*ND*	*CNS, lung, eye,*	*FC, IHC*
18	*M*	*1*	*M5*	*72*	*12*	*Eye, extradural chloroma, LN*	*FC*
No extramedullary manifestation of disease at diagnosis							
19	*M*	*12*	*M0*	*89*	*82*		*FC*
20	*F*	*5*	*M1*	*85*	*78*		*FC*
21	*M*	*14*	*M2*	*45*	*73*		*FC*
22	*F*	*2*	*M4*	*71*	*22*		*FC*
23	*F*	*16*	*M4*	*57*	*15*		*FC*
24	*F*	*10*	*M4*	*54*	*8*		*FC*
25	*M*	*13*	*M4*	*88*	*68*		*FC, SA*
26	*F*	*10*	*M5*	*97*	*ND*		*FC*
27	*M*	*9*	*M5*	*89*	*91*		*FC*
28	*M*	*2*	*M5A*	*95*	*95*		*FC*

UPN: Unique Patient Number; BM: Bone Marrow; PB: Peripheral Blood; CNS: Central Nervous System; LN: Lymph Node; Chloroma: granulocytic sarcoma mostly beneath the periosteum of the skull, spine or ribs; FC: Flow Cytometry; IHC: Immunohistochemistry and SA: Survival Assay.

Flow Cytometric analysis

Table 2 lists the primary antibodies that were used for flow cytometric analysis of chemokine receptor expression by blood or bone marrow-derived AML blast cells. For visualisation of unlabelled primary antibodies, PBMC or BMMC were stained with the relevant FITC-conjugated isotype-specific secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA) or Alexa Fluor 488-conjugated isotype-specific secondary antibody (Invitrogen, Breda, The Netherlands). Single stainings with these secondary antibodies were used as negative controls. Cells were fixed directly after staining using 4% paraformaldehyde. Intracellular expression of CXCR4 and CXCR7 was visualised after permeabilisation of the cells using the Fix and Perm permeabilisation kit according to the supplier's manual (Caltag, Burlingame, CA, USA) and subsequent staining with the appropriate primary and secondary antibodies. The percentage of positive cells was measured on a fluorescence-activated cell sorting (FACS) Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and data were analysed using Cellquest software. Selection of AML cells was based on their FSC/SCC pattern, combined with specific markers characterising the AML cells of each patient (CD7, CD13, CD14, CD33, CD34, CD56 and HLA-DR).

Immunohistochemical analysis

Immunohistochemical staining of a selected group of chemokine receptors and their ligands was performed on 11 formalin fixed paraffin embedded biopsies derived from AML patients with skin involvement and 3 aleukaemia cutis patients. Double immunofluorescent staining of tumour cell-specific markers and chemokine receptors was performed in cases where antibodies to the tumour specific markers were available. In all other cases single enzymatic staining of the chemokine receptor was performed. Single enzymatic staining was also performed for some of the corresponding ligands of chemokine receptors that were shown to be expressed by the leukaemic blasts in the skin. Table 2 lists the primary antibodies used for immunohistochemistry. Four μm paraffin sections were deparaffinised and subjected to heat-mediated antigen retrieval in a microwave using citrate buffer (10 mmol, pH 6.0) or Tris/EDTA buffer (10 mmol, pH 9.0).

Table 2. Overview of primary antibodies used for flow cytometry and immunohistochemistry

Antibody	Supplier
Flow cytometry	
CCR1	*R&D Systems (Minneapolis, MN, USA)*
CCR2	*R&D Systems*
CCR4	*BD Biosciences Pharmingen (San Diego, CA, USA)*
CCR5	*R&D Systems*
CCR10	*Capralogics Inc (Hardwick, MA, USA)*
CXCR4	*R&D Systems*
CXCR7	*Proteintech Group Inc (Chicago, IL, USA)*
CX3CR1	*Abcam (Cambridge, UK)*
CLA	*BD Biosciences Pharmingen*
CD7-PE	*Beckman Coulter (Fullerton, CA, USA)*
CD13-PE	*BD Biosciences (San Jose, CA, USA)*
CD14-APC	*BD Biosciences*
CD33-APC	*BD Biosciences*
CD34-PE	*BD Biosciences*
CD56-PE	*Beckman Coulter*
HLA-DR-PE	*BD Biosciences*
HLA-DR-APC	*BD Biosciences*
Immunohistochemistry	
CCR1	*Abcam*
CCR2	*R&D Systems*
CCR5	*Abcam*
CXCR4	*Abcam*
CXCR7	*Proteintech Group Inc*
CX3CR1	*Abcam*
CCL2	*R&D Systems*
CCL3	*R&D Systems*
CCL5	*R&D Systems*
CXCL11	*Peprotech EC Ltd (London, UK)*
CXCL12	*R&D Systems*
CX3CL1	*SantaCruz Biotechnology Inc (Santa Cruz, CA, USA)*
CD15	*Abcam*
CD34	*Neomarkers (Fremont, CA, USA)*
CD43	*Novocastra (Newcastle upon Tyne, UK)*
CD45	*Dako (Heverlee, Belgium)*
CD56	*Zymed (San Francisco, CA, USA)*
CD68	*AbD Serotec (Kidlington, UK)*

PE: phycoerythrin and APC: allophycocyanin.

Non-specific staining was blocked by incubating the slides with goat serum for 30 min. Subsequently, the slides were incubated overnight at room temperature with the primary unconjugated antibodies. For double stainings, primary antibodies were detected by fluorescence using the relevant isotype-specific, Alexa Fluor 488 or Alexa Fluor 594 labelled, secondary antibodies (Invitrogen). Replacement of the primary antibodies by PBS/bovine serum albumin (BSA) 1% was used as a negative control. Results were analysed by confocal microscopy using a LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

In the case of single enzymatic staining, bound primary antibodies were detected using Envision (Dako, Heverlee, Belgium) and 3,3'-Diamino-benzidine-tetrahydrochloride (DAB) detection or using the LSAB+ system (Dako) and DAB detection. Again the primary antibody was replaced by PBS/BSA 1% as a negative control. The staining intensity was scored as previously described (25;26). In short, the score for staining intensity was graded as follows: 0, absent; 1, weak; 2, moderate and 3, intense. Score for the percentage of positive tumour cells was graded as follows: 0 = absent; 1 = 1%-10%; 2 = 10%-25%; 3= 25%-50%; 4 = 50%-75% and 5 = >75% positive tumour cells. Sections were considered positive when a combined score for intensity and percentage positive cells was higher than three. Immunofluorescent slides were scored positive when more than half of the tumour cells expressed the relevant chemokine receptor.

Proliferation assay

The survival capacity of leukaemic blasts expressing CXCR7 was investigated using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay (Cell Titer96 Aqueous, Promega, Madison, WI, USA). For this pilot experiment, MTS assays were performed on primary bone marrow-derived AML blasts of 4 AML patients and 3 AML cell lines. The bone marrow samples of AML patients used in this assay were selected based on the expression of either intracellular or cell-bound CXCR7 (>5%). The samples used for these experiments contained >65% of AML blasts (Table 1). To study in vitro survival kinetics of AML blast cells, blocking experiments were performed by adding a CXCR4 antagonist, AMD3100 (1000 ng/ml) (Sigma-Aldrich, Zwijndrecht, The

Netherlands) or an anti-CXCR7 antibody (100 ng/ml) (Proteintech Group Inc) to the cells for 30 min and incubated at 37°C or at 4°C, respectively, before exposure to recombinant CXCL12 (100 ng/ml) (R&D Systems) for 24, 48 or 72 hrs. Control experiments with AML cell lines were used to determine the optimal experimental set up (data not shown). Subsequently, 20 µl of MTS was added to each well containing 100 µl of cell suspension. The MTS tetrazolium compound was bio-reduced by living cells into a coloured formazan product, which was measured using a VERSAmax Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 490-nm wavelength after 4 hours of incubation at 37°C. The measured absorbance (representing the viability of the cells) of the medium control, i.e. cells incubated without blocking agents and CXCL12, was set to 1 and all other conditions were hereto related, resulting in a ratio which expresses the difference in cell survival.

Statistical Analysis

Flow cytometric data on chemokine receptor expression are presented as median and interquartile range (Tukey) of the percentage of positive blast cells. Differences in expression between AML patients with skin involvement and without EML were assessed using a Mann–Whitney test. Proliferation assays were statistically analysed using a Friedman test (matched samples) and, when significant ($P<0.05$), followed by a Dunn's multiple comparison test.

Results and Discussion

We performed the first systematic analysis on chemokine receptor/ligand combinations that may be involved in the migration of AML cells to the skin. To this end, we analysed chemokine receptor expression not only on AML blast cells in peripheral blood or bone marrow, but also on blast cells present at this extramedullary site. The chemokine receptors analysed in this study were chosen based upon micro-array analysis of skin biopsies obtained from 3 different AML patients with aleukaemia cutis (skin infiltrates without detectable bone marrow involvement) performed in an unrelated study (personal communication, C.P. Tensen). This pilot analysis revealed high expression of mRNA encoding for CCR1,

CCR2, CCR5, CXCR4, CXCR7 and CX3CR1 as compared to the skin of CD4+CD56+ haematodermic neoplasm patients. In addition, we analysed three receptors known to direct skin-homing of non-malignant haematopoietic cells: CLA, CCR4 and CCR10 (27-29).

Chemokine receptor expression on AML blasts in the peripheral blood and bone marrow of AML patients

Using flow cytometry, the expression of CCR1, CCR2, CCR5, CXCR4, CXCR7, CX3CR1, on peripheral blood- and bone marrow-derived blast cells was investigated. This analysis was performed on samples derived from AML patients with skin involvement (n=6 to 7 for PBMC and n=6 for BMMC), and patients without EML (n=3 to 9 for PBMC and n=7 to 9 for BMMC).

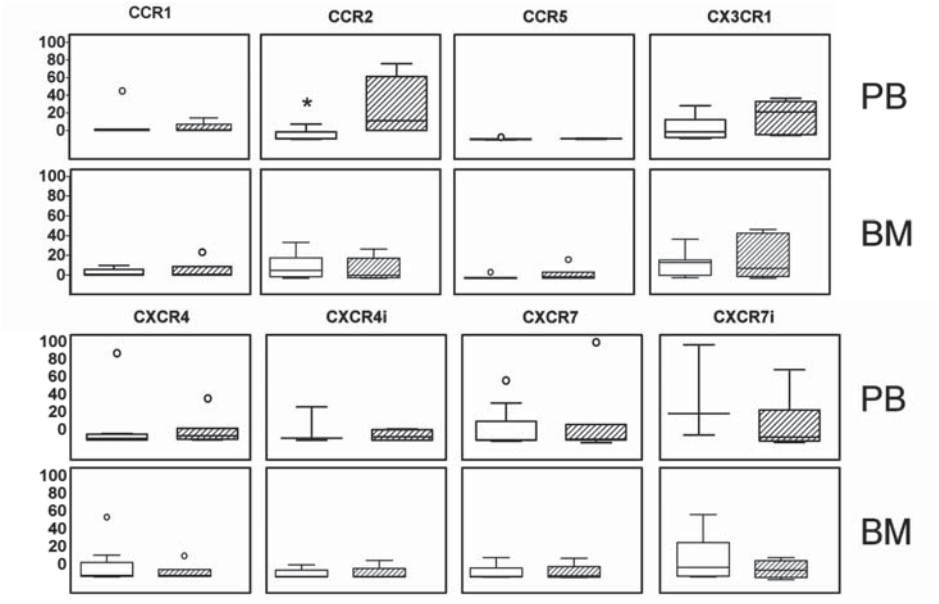


Figure 1: Flow cytometric analysis of chemokine receptors on AML blasts in peripheral blood and bone marrow. Flow cytometric results are given as median percentage of positive cells within the blast population with their interquartile range. Outliers are indicated with an open circle. The AML blast population was gated in the forward and side scatter plot and further gated using specific tumour markers for each individual patient. The upper row shows chemokine receptor expression on AML blasts from the peripheral blood (PB). The chemokine receptor expression by bone marrow-derived AML blasts is shown in the lower graphs (BM). Chemokine receptor expression by blasts of AML patients without extramedullary involvement is represented by the clear boxes (PB: n=3/9, BM: n=7/9). Hatched boxes represent chemokine receptor expression by blasts of AML patients with skin involvement (PB: n=6/7, BM: n=6). Significant differences between the groups ($P<0.05$) are indicated with an asterisk.

Because we observed mostly intracellular expression of CXCR4 in tissue sections prepared from skin biopsies (vide infra), we analysed intracellular expression of CXCR4 and CXCR7 (named CXCR4i and CXCR7i, respectively) as well as cell surface expression of these markers. Figure 1 shows the median and range of the percentage positive blast cells in PBMC and BMMC for each chemokine receptor. Traditional skin homing receptors and adhesion molecules like CCR4, CCR10 and CLA were expressed at very low levels on peripheral blood- and bone marrow-derived AML blast cells (data not shown). AML patients with skin involvement showed, however, significantly (P=0.009) increased percentages of CCR2pos AML blast cells in peripheral blood ranging from 8.8 to 75.0% (median 18.6%) compared to the patients without EML (range from 0.3 to 15.1%; median 0.9%). These findings are in line with a study published by Cignetti et al. reporting a correlation between co-expression of CCR2/CCL2 and extramedullary involvement in adult AML patients (30). No statistically significant differences between the two groups were observed for the other receptors, including CCR1, CCR5, CX3CR1, CXCR4 (i) and CXCR7 (i). However, expression of CX3CR1 tended to be higher on the circulating AML blast cells of patients with extramedullary involvement (P=0.18).

Analysis of bone marrow-derived AML blast cells demonstrated substantial inter-patient variation. Expression of CCR2 ranging from 0 to 33.3% (median 3.2%) in the patients with skin involvement and 0.2 to 41.3% (median 8.8%) in the patients without EML. CX3CR1 was expressed in a range from 0 to 56.1% (median 11.5%) and 0.4 to 44.6% (median 18.3%) in the patients with skin involvement and without EML, respectively. Intracellular expression of CXCR7 was observed, ranging from -3.1 to 22.4% (median 7.9%) and 0.1 to 39.8% (median 10.9%) in the two groups, respectively. However, no statistically significant differences in chemokine receptor expression were observed between the two groups for these three receptors. The other chemokine receptors (CCR1, CCR5, CXCR4, CXCR4i and CXCR7) were only expressed on bone marrow-derived AML blast cells in occasional patients, medians ranging from 0.4 to 1.6% in the patients with skin involvement and 0.5 to 1.6% in the patients without EML. We are aware of the heterogeneity in both study groups; however, age, gender and FAB classification are evenly distributed over the two main groups. Additionally, a major

difference in chemokine receptor expression in PBMC or BMMC between the different FAB classifications was not observed.

Expression of chemokine receptors and their ligands on skin infiltrating AML blasts

To gain more insight into the expression pattern of chemokine receptors on skin infiltrating AML blast cells, we performed immunohistochemical stainings on formalin fixed paraffin embedded skin biopsies collected from 11 paediatric AML patients with skin involvement at the time of diagnosis and 3 aleukaemia cutis patients. The results of these experiments are summarised in Table 3. Due to the scarcity of tissue, we could not test the complete set of available antibodies on each biopsy. Tissue sections were stained for CCR1, CCR2, CX3CR1, CCR5, CXCR4 and CXCR7. In line with the low levels of CCR1pos AML cells detected in peripheral blood and bone marrow samples, skin-infiltrating leukemic cells did not express CCR1. In contrast, CCR2pos cells were clearly detected in 8 out of 14 (57.1%) biopsies. These observations suggest the involvement of CCR2 in skin homing of leukaemic blast cells. However, in contrast to the Cignetti study (30), one of the ligands for CCR2, CCL2, was only observed in 2 out of 11 (18.2%) cases. This observation implies that CCR2 expressing AML cells probably exploit another ligand (i.e. CCL7, CCL8, CCL13 or CCL16 (31)) for homing to the skin. Like AML cells in peripheral blood and bone marrow, 7 out of 13 skin biopsies (53.8%) contained CX3CR1 expressing AML cells. However, CX3CL1, the ligand for CX3CR1, was only expressed in 3 out of 12 biopsies (25%). Thus, CX3CR1/CX3CL1 interactions probably do not play a major role in the migration of AML blasts to the skin.

A key observation in our study is the detection of CCR5 expressing AML cells in all available skin biopsies (n=14). The chemokines CCL3, CCL4, CCL5, CCL8 and CCL14 all bind to this receptor (31). While CCL5 could not be detected (0/10), CCL3 could be visualised in 7 out of 8 (87.5%) biopsies. Given that CCR5 was not expressed by leukaemic blast cells in either peripheral blood or bone marrow, we speculate that CCL3 might be involved in retention of AML blasts in the skin rather than in homing of these cells to the skin. To this end, the leukaemic cells first need to upregulate their CCR5 expression, as was already described for monocytes (32;33).

Table 3. In situ chemokine and chemokine receptor expression in skin biopsies derived from patients with aleukaemia cutis or AML. Figure 1. Flow cytometric analysis of chemokine receptor Figure 1. Flow

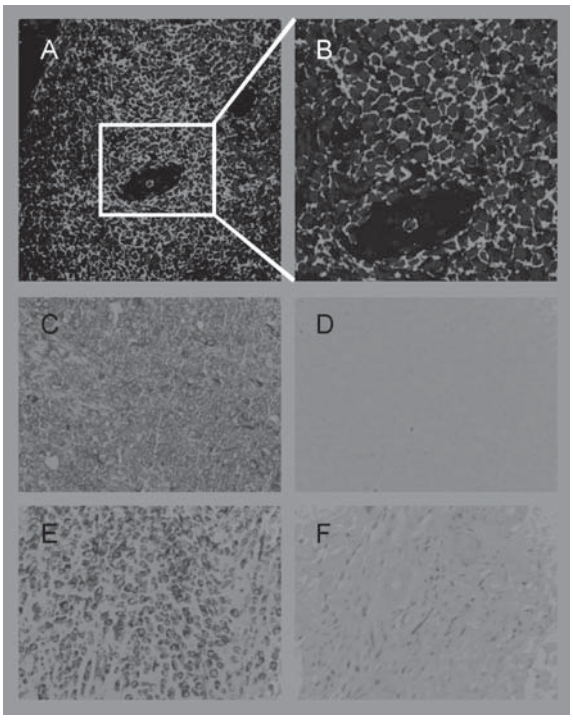
UPN	CCR1	CCR2	CCL2	CCR5	CCL3	CCL5	CXCR4	CXCL12	CXCR7	CXCL11	CX3CR1	CX3CL1
1	− (1)1	− (3)	− (0)	+ (8)	nd2	− (0)	+ (8)	+ (4)	+ (5)	− (3)	+ (5)	− (2)
2	Nd	− (0)	− (1)	+ (6)	+ (6)	− (1)	nd	− (3)	nd	nd	nd	nd
3	−	+ (8)	nd	+ (8)	nd	nd	+	+ (5)	+ (8)	− (0)	+ (8)	− (2)
4	− (2)	− (3)	− (0)	+ (8)	+ (8)	− (0)	+ (8)	− (3)	+ (8)	− (1)	+ (8)	− (3)
7	−	+ (6)	+ (7)	+ (8)	+ (8)	− (0)	+	+ (7)	+ (5)	− (2)	−	− (1)
8	−	+ (6)	− (3)	+ (8)	− (1)	− (0)	+	+ (8)	+	− (0)	−	− (3)
9	−	− (1)	− (3)	+ (8)	+ (8)	− (0)	+	+ (8)	+	− (0)	+	+ (7)
10	−	+ (7)	− (2)	+ (8)	nd	nd	−	+ (6)	−	− (0)	−	− (2)
11	−	− (1)	nd	+ (8)	nd	nd	+	+ (7)	+	− (0)	−	nd
12	−	+ (4)	− (3)	+ (8)	+ (5)	− (1)	+	+ (6)	+	− (0)	+	+ (6)
13	−	+ (6)	− (3)	+ (8)	nd	nd	+	+ (5)	+	− (0)	−	− (2)
14	−	+ (8)	+ (4)	+ (8)	+ (4)	− (1)	+	+ (6)	+	− (1)	−	− (1)
15	− (1)	+ (5)	− (0)	+ (8)	+ (8)	− (3)	+ (8)	− (3)	+ (7)	− (0)	+ (8)	+ (7)
17	Nd	− (2)	nd	+ (8)	nd	− (0)	+	− (2)	+	− (2)	+ (5)	− (1)
Total	0/123	8/14	2/11	14/14	7/8	0/10	12/13	10/14	12/13	0/13	7/13	3/12

1 Numbers in brackets represent the combined score for intensity and percentage positive tumour cells as described in materials and methods.

2 nd: not done.

3 Numbers in the lower row represent the number of positive cases out of the evaluable biopsies.

The expression of CXCR4 was mainly intracellular (Figure 2A-B), and present in almost all evaluable cases (12/13, 92.3%). Interestingly, also the recently described chemokine receptor CXCR7, associated with tumour development and survival, was expressed in the skin of the same patients (Figure 2C). CXCL12, the ligand for both CXCR4 and CXCR7, was expressed in 10 out of 14 skin biopsies (71.4%, Figure 2E). Skin expression of CXCL11, the alternative ligand for CXCR7, could not be demonstrated (0/13). CXCR4 has already been extensively described in relation to AML. Variable expression on blast cells in peripheral blood and bone marrow has been documented (34-39). Contradictive reports exist, however, on the level of CXCR4 expression and extramedullary localisation of blast cells. Given that bone marrow

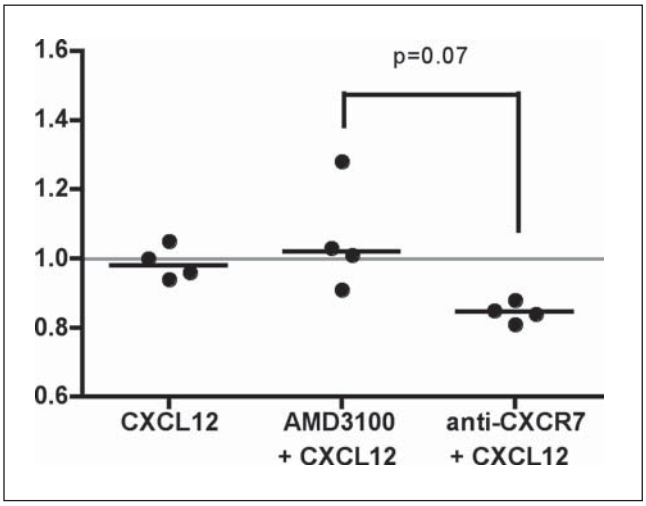


For full colour see figure section

Figure 2: Immunohistochemical staining of skin biopsies of AML patients with skin involvement.
(A) Double immunofluorescent staining on a skin biopsy of a representative patient (UPN 3) identified a large infiltrate of CD43+ tumour cells (green) with, in this case, intracellular localisation of CXCR4 (red).
(B) A cropped image of this picture. Single enzymatic staining (detected by the red/brown colour) showing CXCR7
(C, UPN 3) and CXCL12 (E, UPN 8). Omission of the primary antibodies was used as negative control
(D and F, UPN 1 and UPN 8, respectively). Magnification: 250x.

stromal cells are a major producer of CXCL12 (35;38), CXCR4/CXCL12 interactions likely facilitate the retention of AML blasts in bone marrow. On the other hand, high expression of CXCR4 by AML or ALL cells seems to be associated with a poor disease outcome due to migration of blast cells to extramedullary sites (34;39). Although we did not find a difference in the percentage of CXCR4 expressing cells in peripheral blood or bone marrow, CXCR4 expressing AML blasts were clearly detected in the skin. The relatively low expression of CXCR4 by either peripheral blood- or bone marrow-derived AML blast cells renders the role of this chemokine receptor in skin-specific homing of leukaemic blast cells, however, rather unlikely.

Whereas numerous studies have focused on the role of CXCR4/CXCL12 in AML, this is the first study also investigating the other receptor for CXCL12, i.e. CXCR7. CXCR7 is a recently discovered chemokine receptor and is thought to play a role in cell survival, cell adhesion and tumour development, but not in chemotaxis (21;23;24). High expression of CXCR7 and one of its ligands, CXCL12, was found in the skin of AML patients with skin involvement. However, variable expression was seen on AML blast cells in corresponding peripheral blood and bone marrow samples. Furthermore, no differences were observed between AML patients with and without skin involvement. These observations argue against a role for CXCR7 in migration of AML blast cells to the skin.



The measured absorbance (and thus the viability of the cells) of the medium control, i.e. cells incubated without blocking agents and CXCL12, was set to 1 (grey line). All other conditions were hereto related, resulting in an absorbance ratio which expresses the difference in cell survival. Absorbance ratios (after 72 hrs) are shown for bone marrow-derived AML blasts of 4 patients. These cells were either only stimulated with CXCL12 or blocked with a CXCR4 antagonist (AMD3100) or CXCR7 blocking antibody prior to CXCL12 stimulation.

Figure 3: Decreased survival capacity of AML blasts upon CXCR7 blockade.

The role of CXCR4 and CXCR7 in survival of bone marrow-derived AML blasts

Both CXCR4 and CXCR7 have been described to be involved in the survival of tumour cells. Tavor and colleagues (1;40) showed an increased in vitro viability of both AML cell lines and primary AML cells when cultured in the presence of CXCL12. This increase in viability was significantly reduced when the cells were pre-treated with the CXCR4 antagonist AMD3100. To find out whether the expression of CXCR4 or CXCR7 by AML blasts indeed imparts a survival advantage for these cells, we performed a pilot experiment using in vitro cell survival assays with three different AML cell-lines (data not shown) and primary bone marrow-derived AML blast cells obtained from 4 AML patients (Figure 3). The AML cell-lines were used to determine the most optimal experimental set up. The viability of AML cells was relatively unaffected when cells were exposed to CXCL12 or pre-treated with AMD3100 and followed by CXCL12 exposure as already shown by others (41;42). AMD3100 effectively blocks the CXCR4 receptor (42;43), leaving CXCR7 to be the only receptor on the cells that could interact with CXCL12. The small increase in cell survival when the cells were blocked with AMD3100 upon CXCL12 exposure might be due to the recently described agonistic property of AMD3100 on the binding of CXCL12 to CXCR7 (44). When the receptor for CXCR7 was blocked with anti-CXCR7 antibody prior to CXCL12 exposure, less viable cells were present at the end of the culture (P=0.07). A similar result was obtained for the AML cell-lines (data not shown). Note that the bone marrow samples analysed in our survival assay were selected based on either intra- or extracellular CXCR7 expression; intracellular CXCR7 was recently shown to be functional and susceptible to blocking with specific antibodies (45). The observations of this small pilot study suggest that only the CXCR7/CXCL12 pathway might play a role in the survival of AML blast cells in vitro. We speculate that the expression of CXCR7 and its ligand, CXCL12, by skin-residing AML blast cells is a poor prognostic value for the overall survival of such patients. This hypothesis needs to be investigated in a larger cohort of patients.

Based on our observations, we propose a three step model for the extravasation of AML cells into the skin. CCR2 expression by circulating AML blast cells facilitates homing to the skin in response to local production of an as yet unidentified chemokine. Subsequently,

CCR5/CCL3 and CXCR4/CXCL12 interactions facilitate the retention of AML cells in the skin. Finally, CXCR7/CXCL12 interaction may provide the necessary signals for leukaemic cell survival.

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