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Combined CXCR3/CXCR4 measurements are of high prognostic value in chronic lymphocytic leukemia due to negative cooperativity of the receptors

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Conflict of interest

The authors declare no conflict of interest.

Ethics

Peripheral blood samples were collected upon informed consent that was obtained in accordance with the Declaration of Helsinki and under the ethical approval of the Ethics Commission of the Province of Salzburg (415-E/1287/4-2011, 415-E/1287/8-2011).

Running title: CXCR3-CXCR4 interactions in CLL

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Letter to the Editor

Chronic lymphocytic leukemia (CLL) is composed of cell cycle-arrested leukemic cells circulating in the blood and activated cells that are located in supportive zones in lymphoid organs, driven into proliferation by signals from the microenvironment. Therefore, the recirculation capacity of CLL cells contributes to clinical aggressiveness and key molecules involved in extravasation, such as chemokine receptors and integrins, may represent important prognostic markers and therapeutic targets. Controversial data on a prognostic value of the chemokine receptors CXCR3 and CXCR4 have been reported in CLL.\(^1\)\(^-\)\(^4\) Moreover, the complexity of the chemokine receptor network with a significant redundancy and crosstalk of the receptors, e.g. via heterodimerization, represents a considerable hurdle in the development of chemokine-related drugs. A better comprehension of interactive chemokine receptor signals will help in more reliable prediction of therapy responses.

Here, we investigated CXCR3 and CXCR4 expression in regard to their independent and combined prognostic significance in a cohort of 149 CLL patients. The patient characteristics are described in Supplemental Table 1. Time to first treatment (TTFT) was used in the receiver operating characteristic (ROC) analysis to define the optimal cutoffs of CXCR3 (3.7) and CXCR4 (31.5), measured as mean fluorescence intensity ratios (MFIR). Using these cutoffs, 82 (55%) and 84 (56%) samples were characterized as CXCR3^dim and CXCR4^dim, and 67 (45%) and 66 (44%) samples as CXCR3^bright and CXCR4^bright, respectively. CXCR3 expression in CLL cells was a strong determinant of a worse clinical outcome predicting shorter TTFT (Figure 1A(i); hazard ratio (HR) = 2.6) and overall survival (OS) (HR = 2.1; Supplemental Figure 1A), in concordance with previous observations.\(^2\) Exhibiting similar power of significance compared to CXCR3, CXCR4 expression also served as a predictor for clinical outcome with CXCR4^bright patients suffering from a more rapid disease progression compared to CXCR4^dim patients (Figure 1A(ii); HR = 2.5). These results suggest that the combination of CXCR3 and CXCR4 measurements bears clinical utility. Indeed, combining these two markers yielded an extremely robust and more effective prognostic tool than each receptor alone, with CXCR3^dim/CXCR4^bright patients having a significantly increased hazard of needing treatment compared to CXCR3^bright/CXCR4^dim patients (Figure 1B; HR = 10.6).
The mutational status of immunoglobulin heavy-chain variable-region (IgVH) genes is considered the most powerful marker of clinical outcome in CLL. However, patients with mutated IgVH genes (MCLL) are found to exhibit a more heterogeneous clinical course than those harboring unmutated IgVH (UMCLL). To test the prognostic power of the combined assessment of CXCR3 and CXCR4 in both MCLL and UMCLL, we categorized the cohort according to the IgVH mutational status. In the MCLL subset, similar hazards were observed as in the uncategorized CLL in regard to CXCR3\textsuperscript{dim}/CXCR4\textsuperscript{bright} compared to CXCR3\textsuperscript{bright}/CXCR4\textsuperscript{dim} patients (Supplemental Figure 1B(i); HR = 9.5). Notably, the CXCR3\textsuperscript{bright}/CXCR4\textsuperscript{dim} subgroup here was characterized by a remarkable indolent course with very few treatment requirements. Also in the UMCLL subset, patients with the CXCR3\textsuperscript{dim}/CXCR4\textsuperscript{bright} phenotype suffered from a much more aggressive disease when compared with CXCR3\textsuperscript{bright}/CXCR4\textsuperscript{dim} patients (Supplemental Figure 1B(ii); HR = 16.1), suggesting the combination of CXCR3 and CXCR4 to be a powerful prognostic tool in both MCLL and UMCLL.

In CLL, high levels of CXCR4 are associated with advanced disease progression and are suggested to increase the migratory potential of the CLL cells between blood and supportive lymphoid tissues. To date, there is no information on the mechanism by which CXCR3 might impact on CLL pathogenesis. In principle, CXCR3 and CXCR4 can form heteromeric complexes with a negative ligand binding cooperativity. The consequence of this receptor cross-inhibition is the constriction of CXCR4 to a conformation with lower affinity for CXCL12. We investigated whether CXCR3 and CXCR4 functionally influence each other in CLL. Performing chemotaxis assays as previously described, we found that CLL cells pre-stimulated with either CXCR3 ligand CXCL9, CXCL10, or CXCL11 exhibited a significantly reduced directed cell migration towards CXCL12 (Figure 2A(i)). Pre-stimulation with a small-molecule high-affinity CXCR3 agonist (VUF11418) confirmed this observation and an antagonist (VUF11211) did not interfere with chemotactic ability (Figure 2A(ii)). A similar heterologous desensitization has been recently observed in Th1 cells. Notably, despite functional calcium responses and ERK activation, we did not observe a relevant chemotaxis of CLL cells towards CXCR3 ligands (data not shown). Moreover, the negative impact of CXCR3 stimulation was highly specific to CXCR4-induced migration as neither the migratory capacity towards CCL19, a
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ligand to the lymph node chemokine receptor CCR7 (Supplemental Figure 2A), nor general CXCR4 signaling to proximal targets such as Akt and ERK (Supplemental Figure 2B) was affected by CXCR3 ligands or the agonist.

Besides chemokine-mediated motility, CLL cell trafficking to lymphoid organs also requires chemokine receptor-integrin interactions that support the arrests of the cells on the endothelium under blood flow conditions. In this respect, the VLA-4 integrin is a key molecule for CLL homing to BM. Therefore, to corroborate our hypothesis of CXCR3–mediated CXCR4 desensitization in an in vivo-like situation, we performed adhesion assays under shear flow, using substrates of the VLA-4 ligand VCAM-1 co-immobilized with CXCL12, as described. Pretreatment of CLL cells with each CXCR3 ligand or the agonist VUF11418 resulted in significantly decreased CXCL12-induced cellular arrests whereas pretreatment with the CXCR3 antagonist did not significantly affect tethering (Supplemental Figure 3 and Figure 2B). Notably, this desensitization was not based on changes in chemokine receptor expression as pre-incubation with CXCR3 ligands did not alter CXCR4 surface levels (data not shown).

We next investigated by immunofluorescence whether CXCR3 and CXCR4 are co-localized, with parallel flow-cytometrical quantification, and found two CLL cell subpopulations with inverse CXCR3 and CXCR4 expression within the samples (Figure 2C(i)). Unstimulated CXCR3dim CLL cells expressed CXCR4 with a uniform distribution (ring-shaped) while CXCR3bright CLL cells displayed a more polarized CXCR4 expression (Figure 2C(ii)). In these polarized areas a co-localization of the two receptors was visible (Figure 2C(iii)). The surface stains show that CXCR3 agonism by VUF11418 resulted in diminished CXCR3 surface expression, most likely due to receptor internalization which was paralleled by the redistribution of CXCR4. CXCR3 antagonism did not alter CXCR4 surface expression or distribution (Figure 2C(iii)).

To confirm the in vivo presence of CXCR3 ligands in the blood of CLL patients, we determined the serum concentration of the three interferon-γ-inducible CXCR3 ligands CXCL9, CXCL10 and CXCL11. In high risk CLL, interferon γ is increased, contributing to an inflammatory environment and thereby to a more aggressive disease. In line, we found all three CXCR3 ligands in CLL sera,
with significant higher levels in UMCLL compared to MCLL patients and CXCR3\textsuperscript{dim} compared to CXCR3\textsuperscript{bright} cases (Supplemental Figure 4). These higher levels may cause a chemokine-induced CXCR3 downregulation, thereby resulting in less CXCR3-CXCR4 heterodimerization and consequently high CXCR4 functionality, allowing efficient CLL cell migration into protective environments.

Differences in disease progression may be based on a differential proliferative potential of the CLL cells. In this regard, CD69 has been identified as a readout of activation and propensity of CLL cells to proliferate\textsuperscript{13} with high CD69 expression being a prognostic marker predicting disease aggressiveness\textsuperscript{14}. We measured CXCR3 and CXCR4 in parallel to CD69 expression during early CLL cell activation and proliferation. Therefore, we used our recently described co-culture systems based on activated CD40L\textsuperscript{+} T cells, mimicking CLL-T cell interactions in lymph nodes\textsuperscript{15}. In a time course analysis, we observed a strong reduction in CXCR3 expression during early activation (24 hours), which was paralleled by the upregulation of CD69 (Figure 3A(i, ii)). Using these assays, we found CD69 to be transiently upregulated during the early activation period and diminished again during CLL cell proliferation and division occurring in average after 5 days of co-culture (data not shown). At this time point, CXCR3 but not CXCR4 expression, was recovered again, indicating an inversely regulated expression pattern of CXCR3 and CD69 (Figure 3A). To test whether CXCR3 expression inversely correlates with the percentage of CLL cells already pre-activated in the periphery, thus mirroring their current activation state, we performed parallel measurements of CXCR3 and CD69 in whole blood samples. Indeed, we detected a significant inverse correlation of CXCR3 and CD69 (Figure 3B). Notably, this correlation was based on a reduced CXCR3 expression in the CD69 positive fraction and vice versa (Supplemental Figure 5), suggesting that CLL cells expressing low CXCR3 are more activated. Determining whether this translates into differences of CXCR3\textsuperscript{bright} and CXCR3\textsuperscript{dim} samples in \textit{in vitro} proliferation, we co-cultured CLL cells with CD40L-overexpressing fibroblasts and found that CXCR3\textsuperscript{dim} CLL cells proliferated more efficiently than CXCR3\textsuperscript{bright} CLL cells (Figure 3C). The presence of CXCR3 ligands did not affect CLL cell proliferation (data not shown) suggesting an
indirect activation-associated effect rather than a direct inhibitory effect of CXCR3 responsible for CLL cell proliferation.

In summary, we demonstrated that high CXCR3 but low CXCR4 expression defines a subset of good prognosis in CLL, and that combined CXCR3 and CXCR4 measurements are a powerful tool to identify CLL patients with a significantly lower risk of disease progression in both MCLL and UMCLL, which may also have implications on therapy decisions. Elucidating the functional role of CXCR3 in CLL pathogenesis, we observed CXCR3 engagement by its ligands to substantially modulate CXCR4-mediated effects during cell migration and VLA-4 mediated adhesion. We propose a negative impact of CXCR3 on CXCR4 functionality by modifying its distribution on the cell membrane, with CXCR4 remaining the master player and major therapeutical target. Furthermore, we suggest that CXCR3 expression inversely mirrors the current activation status of the CLL cells and hence their proliferative propensity. Conclusively, we clarified the prognostic value of diminished CXCR3 expression in CLL and demonstrated here for the first time a functional role of CXCR3 in the process of leukemic infiltration and progression of the tumor.
References


Figure 1: Prognostic significance of low CXCR3 and high CXCR4 expression in CLL. CXCR3 and CXCR4 expression was determined in whole blood samples from 149 - at the time point of measurement chemonaive - CLL patients using a three-color flow cytometric assay, including CD5, CD19, and CXCR3 or CXCR4 determination, respectively. CLL cells were defined as CD5/CD19 double positive lymphocytes. Calculated cut-off for CXCR3 was 3.7 (MFIR) and for CXCR4 31.5 (MFIR). Kaplan-Meier curves depict the cumulative proportion of untreated CLL patients according to the time since diagnosis. Comparisons between curves were performed using the log-rank test. (A, B) Patients displaying a CXCR3$^{\text{dim}}$ (A) or a CXCR4$^{\text{bright}}$ (B) phenotype had a significantly worse clinical course when compared with CXCR3$^{\text{bright}}$ or CXCR4$^{\text{dim}}$ patients, respectively. (C) Treatment-free survival curves of CLL patients separated according to the combination of CXCR3 expression (MFIR $\leq$ or $> 3.7$) and CXCR4 expression (MFIR $\leq$ or $> 31.5$) highlight the prognostic importance of the combination of CXCR3 and CXCR4. CLL patients exhibiting a CXCR3$^{\text{bright}}$/CXCR4$^{\text{dim}}$ phenotype show a more indolent clinical course with 14% requiring therapy compared to patients with a CXCR3$^{\text{dim}}$/CXCR4$^{\text{bright}}$ phenotype of which 50% need to be treated. MFIR: mean fluorescence intensity ratio (MFI specific Antibody/MFI corresponding isotype control).

Figure 2: Stimulation of CXCR3 reduces CXCL12-induced chemotaxis and arrests of CLL cells and influences CXCR4 distribution on the cell membrane. Total CLL peripheral blood mononuclear cells (PBMCs, migration assay) or isolated CLL cells (adhesion assay, immunofluorescence) were incubated in RPMI-1640 containing 1% fetal calf serum (FCS) with or without CXCL9, CXCL10, CXCL11 (100 ng/ml), VUF11418 or VUF11211 (1 µM) where indicated for 30 min prior to the assay and washed with RPMI. (A) 5 x 10$^5$ cells were transferred to the upper chamber of a transwell culture insert with 5 µm pore size (Costar®, Fisher Scientific UK.). The transwells were placed into wells containing 600 µl RPMI/FCS supplemented with or without 100 ng/ml CXCL12 and incubated for 3 h at 37°C in 5% CO$_2$. Pre-incubation of the CLL cells with (i) the CXCR3 ligands and (ii) the CXCR3 agonist VUF11418 resulted in a reduced chemotactic activity, whereas incubation with CXCR3 antagonist VUF11211 did not affect chemotaxis. Each setting was
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performed in duplicates. Data represent the results from 6 (i) and 5 (ii) independent experiments and were analysed using the paired Student’s t-test. (B) Shear flow assays were performed as previously described.\(^8\) CLL cells were pre-treated with (i) CXCL11 or (ii) the CXCR3 agonist and antagonist where indicated and perfused for 1 min at 0.5 dyn/cm\(^2\) over VCAM-1 co-immobilized with CXCL12. Categories of interactions (tethers) are expressed as frequencies of cells in direct contact with the substrate. Pre-treatment of the isolated CLL cells with (i) CXCL11 or (ii) VUF11418 significantly reduced the total arrests whereas pre-incubation with (ii) VUF11211 did not influence the number of CXCL12 induced arrests. The data represent the results of 5 (i, ii) different samples tested that were analysed using the paired Student’s t-test. (C) 5 x 10\(^6\) isolated CLL cells were pre-incubated (i) or cultured on Poly-L-Lysine coated slides (ii, iii) for 1 h at 37°C in 5% CO\(_2\) with (iii) or without (i, ii) CXCR3 agonist and antagonist. CLL cells were stained with an anti-CXCR3 monoclonal antibody (green) and an anti-CXCR4 monoclonal antibody (red). Cells could be divided in CXCR3\(^{\text{dim}}\)/CXCR4\(^{\text{br}}\) (dashed line) and CXCR3\(^{\text{br}}\)/CXCR4\(^{\text{dim}}\) (solid line) expressing subpopulations with differential CXCR4 distribution on the surface. The yellow colour (iii) demonstrates co-localization of CXCR3 and CXCR4.

Figure 3: CXCR3 surface expression reflects the current activation status of the CLL cell. (A) PBMCs from CLL patients were incubated with anti-CD3/CD28 beads in the presence of NIH 3T3 fibroblasts, and (i) CXCR3, (ii) CD69 and (iii) CXCR4 (MFIR) expression was cytometrically evaluated after 0, 24 hours (h) and 5 days (d). In contrast to CXCR4, CXCR3 showed an inverse expression pattern compared to CD69. The data are representative for 6 independent experiments. (B) Flow-cytometric determination of CXCR3 and CD69 expression (% positive cells) in whole blood samples of 117 CLL patients. CXCR3 and CD69 expression were significantly inversely correlated. The data were analyzed using the Spearman’s test. (C) PBMCs from CLL patients exhibiting a CXCR3\(^{\text{bright}}\) or CXCR3\(^{\text{dim}}\) phenotype were cultured in the presence of NIH 3T3 fibroblasts overexpressing human CD40L for 5 d. After 3 d and 5 d, proliferation of the CLL cells (% Ki-67 expression) was determined by flow cytometry. CXCR3\(^{\text{dim}}\) CLL proliferated faster and more
efficiently than CXCR3$^{\text{bright}}$ CLL cells. The data show the results of 4 CLL samples tested in each subgroup.
A(i) CXCR3 br (n=82); median TTFT = not reached
CXCR3 dim (n=67); median TTFT = 134 months

p = 0.0044
HR = 2.6

CXCR4 dim (n=84); median TTFT = not reached
CXCR4 br (n=65); median TTFT = 115 months

p = 0.0038
HR = 2.5

B
CXCR3 br/CXCR4 dim (n=47); median TTFT = not reached
CXCR3 dim/CXCR4 br (n=32); median TTFT = 75 months
CXCR3 br/CXCR4 br (n=35); median TTFT = 141 months
CXCR3 dim/CXCR4 dim (n=35); median TTFT = 151 months

p < 0.0001
HR = 10.6

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Ganghammer et al. Supp. Table 1.
Ganghammer et al. Supplemental Figure 1.
Ganghammer et al. Supplemental Figure 2.
Ganghammer et al. Supplemental Figure 3.
Ganghammer et al. Supplemental Figure 4.
Ganghammer et al. Supplemental Figure 5.
Supplemental Table 1: Detailed patient characteristics. Rai stage was defined according to Rai et al.\textsuperscript{1} CD49d expression (cutoff 30%) was analyzed as previously described\textsuperscript{2,3}. The mutational status of the IgVH genes was defined by the percentage of sequence homology with the germline equivalent (M: mutated, <98% homology; UM: unmutated, \geq 98% homology). ZAP-70 expression was evaluated as recommended (low, NKT/B ratio >3.5; high, NKT/B ratio <3.1).\textsuperscript{4} CD38 status was defined as described (low, <30%; high, \geq 30%).\textsuperscript{5} Chromosomal aberrations were determined according to Döhner et al.\textsuperscript{6}

ND: not determined.

Supplemental Figure 1: Overall survival in CLL in respect to CXCR3 expression and time to first treatment in MCLL and UMCLL in respect to combined CXCR3/CXCR4 expression. CXCR3 and CXCR4 expression was determined in whole blood samples from 149 CLL chemonaive patients using a three-color flow cytometric assay, including CD5, CD19, and CXCR3 or CXCR4 determination, respectively. CLL cells were defined as CD5/CD19 double positive lymphocytes. Calculated cut-off for CXCR3 was 3.7 MFIR and for CXCR4 31.5 MFIR. Comparisons between curves were performed using the log-rank test. (A) Kaplan-Meier curves demonstrate reduced overall survival (OS) in CLL patients with a CXCR3\textsuperscript{dim} phenotype. (B) Kaplan Meier curves depict time to first treatment in the CLL cohort that was categorized into MCLL (i) and UMCLL (ii). In both cohorts, the combined assessment of CXCR3 and CXCR4 expression exhibited a significant power. MFIR: mean fluorescence intensity ratio (MFI specific Antibody/MFI corresponding isotype control).
**Supplemental Figure 2: Stimulation of CXCR3 does not influence CCL19-mediated chemotaxis of CLL cells or global CXCR4 signalling to proximal targets.** Total CLL peripheral blood mononuclear cells (PBMCs) were incubated in RPMI-1640 containing 10% fetal calf serum (FCS) with or without CXCL9, CXCL10 or CXCL11 (100 ng/ml) for 30 min prior to the assay and washed with RPMI. (A) 5x10^5 cells were transferred to the upper chamber of a transwell culture insert with 5 µm pore size (Costar®, Fisher Scientific UK.). The transwells were placed into wells containing 600 µl RPMI/FCS supplemented with or without 100 ng/ml CCL19 and incubated for 3 h at 37°C in 5% CO₂. Pre-incubation of the CLL cells with the CXCR3 ligands did not influence the chemotactic capacity towards CCL19. Each setting was performed in duplicates. Data show the results from 4 independent experiments. (B) 5x10^6 purified CLL cells were incubated with or without VUF11418 (1 µM), CXCL10 or CXCL11 (100 ng/ml) for 30 min prior to addition of CXCL12 (100 ng/ml) for 2 minutes. Cells were harvested, washed with pre-cooled PBS and disrupted in lysis buffer containing 1% Triton-X-100 supplemented with protease and phosphatase inhibitors. (Phospho-)Akt and (Phospho-)ERK protein expression were analyzed by Western blot. Data of a representative experiment out of two analyzed samples is shown.

**Supplemental Figure 3: CXCR3 stimulation reduces CXCL12-induced CLL cell tethering under shear flow.** Shear flow assays were performed as previously described. CLL cells were pre-treated with (A) the respective CXCR3 ligands (100 ng/ml) or (B) the CXCR3 agonist and antagonist (1 µM) where indicated and perfused for 1 min at 0.5 dyn/cm² over VCAM-1 co-immobilized with CXCL12. Pre-incubation of the CLL cells with the CXCR3 ligands and the agonist VUF11418 resulted in reduced tethering to the VCAM-1 substrate. Categories of interactions (tethers) are expressed as frequencies of cells in direct contact with the substrate. The depicted experiments are representative for 3 (A) and 5 (B) independent experiments and have been performed in triplicates.
**Supplemental Figure 4:** The chemokines CXCL9, CXCL10 and CXCL11 are differentially expressed in MCLL and UMCLL and in CXCR3^{dim} and CXCR3^{bright} subgroups. Levels of CXCL9, CXCL10 and CXCL11 were quantified in sera from CLL patients using a sandwich immunoassay-based protein system (Quantikine ELISA Kit, R&D). (A) CXCL9 (i), CXCL10 (ii) and CXCL11 (iii) levels were significantly higher in UMCLL compared to MCLL. (B) CXCL9 (i), CXCL10 (ii) and CXCL11 (iii) levels were significantly higher in the CXCR3^{dim} subgroup than in the CXCR3^{bright} cohort. All chemokine determinations have been performed in duplicates. Data represent the results from 70 (A) and 46 (B) different CLL samples. \( P \) values were calculated accordingly using the Mann Whitney test.

**Supplemental Figure 5:** CLL cells express reduced CXCR3 levels in the CD69 positive fraction. Cytometrical density plots demonstrating CXCR3 and CD69 expression of CLL cells, representative for whole blood samples of 117 patients. CLL cells were defined by gating on CD5^{+}/CD19^{+} cells.
References


