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

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. The recirculating capacity of CLL cells does, therefore, contribute 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 have been reported on a prognostic value of the chemokine receptors CXCR3 and CXCR4 in CLL.[1-4] Moreover, the complexity of the chemokine receptor network, with significant redundancy and cross-talk of receptors, e.g. via heterodimerization, represents a considerable hurdle in the development of chemokine-related drugs. A better understanding of interactive chemokine receptor signals will help in more reliable prediction of responses to therapy.

Here, we investigated both the independent and the combined prognostic significance of CXCR3 and CXCR4 expression in a cohort of 149 CLL patients. Patients' characteristics are shown in *Online Supplementary Table S1*.

Peripheral blood samples were collected after informed consent 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).

Time to first treatment was used in the receiver operating characteristic (ROC) analysis to define the optimal cut-offs of CXCR3 (3.7) and CXCR4 (31.5), measured as mean fluorescence intensity ratios. Using these cut-offs, 82 (55%) and 84 (56%) samples were characterized as CXCR3dim and CXCR4dim, and 67 (45%) and 66 (44%) samples as CXCR3bright and CXCR4bright, respectively. CXCR3 expression in CLL cells was a strong determinant of a worse clinical outcome predicting shorter time to first treatment [Figure 1Ai; hazard ratio (HR) = 2.6] and overall survival (HR = 2.1); (*Online Supplementary Figure S1A*), in agreement with previous observations.[2] Exhibiting similar power of significance compared to CXCR3, CXCR4 expression also served as a predictor for clinical outcome with CXCR4bright patients suffering from more rapid disease progression compared to CXCR4dim patients (HR = 2.5) (Figure 1Aii). These results suggest that the combination of CXCR3 and CXCR4 measurements could be clinically useful. Indeed, combining these two markers yielded an extremely robust and more effective prognostic tool than either receptor alone, with CXCR3dim/CXCR4bright patients having a significantly increased hazard of needing treatment compared to CXCR3bright/CXCR4dim patients (HR = 10.6) (Figure 1B).
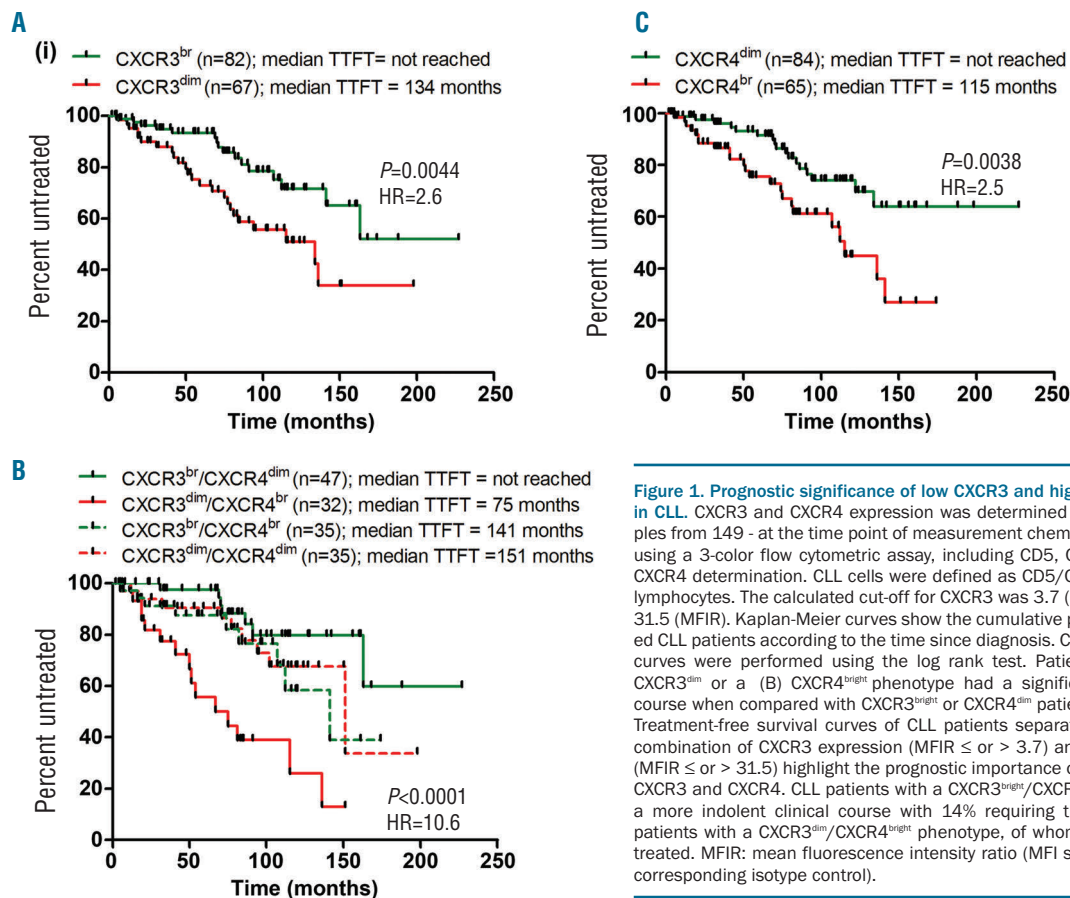

Figure 1. Prognostic significance of low CXCR3 and high CXCR4 expression in CLL. CXCR3 and CXCR4 expression was determined in whole blood samples from 149 - at the time point of measurement chemonaive - CLL patients using a 3-color flow cytometric assay, including CD5, CD19, and CXCR3 or CXCR4 determination. CLL cells were defined as CD5/CD19 double positive lymphocytes. The calculated cut-off for CXCR3 was 3.7 (MFIR) and for CXCR4 31.5 (MFIR). Kaplan-Meier curves show the cumulative proportion of untreated CLL patients according to the time since diagnosis. Comparisons between curves were performed using the log rank test. Patients displaying a (A) CXCR3dim or a (B) CXCR4bright phenotype had a significantly worse clinical course when compared with CXCR3bright or CXCR4dim patients, respectively. (C) Treatment-free survival curves of CLL patients separated according to the combination of CXCR3 expression (MFIR ≤ or > 3.7) and CXCR4 expression (MFIR ≤ or > 31.5) highlight the prognostic importance of the combination of CXCR3 and CXCR4. CLL patients with a CXCR3bright/CXCR4dim phenotype show a more indolent clinical course with 14% requiring therapy compared to patients with a CXCR3dim/CXCR4bright phenotype, of whom 50% needed to be treated. MFIR: mean fluorescence intensity ratio (MFI specific antibody/MFI corresponding isotype control).
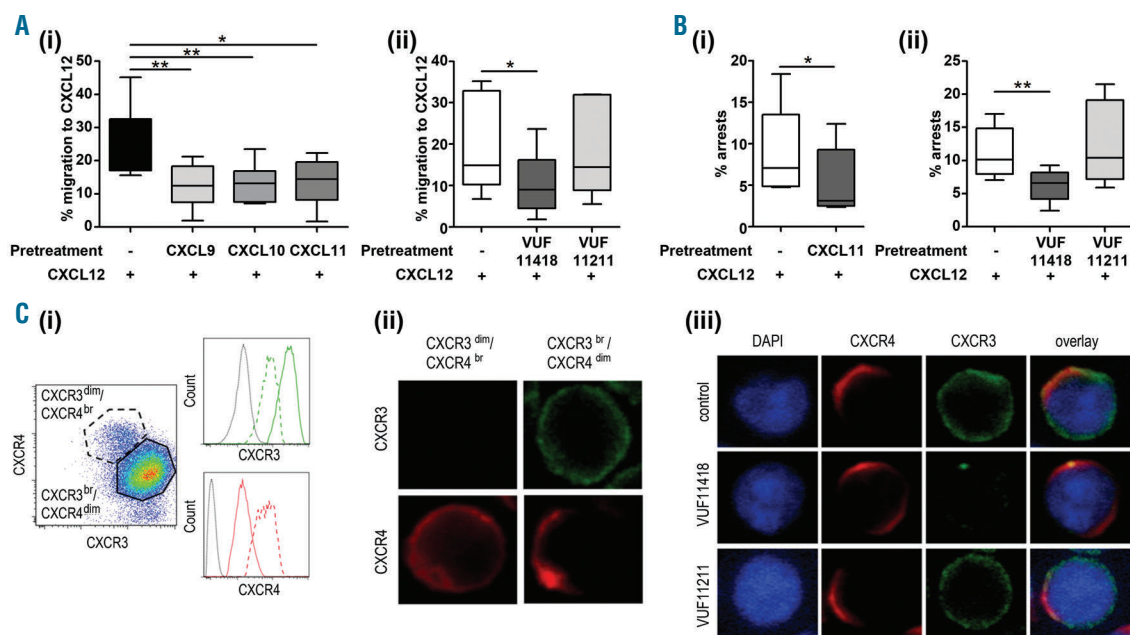
Figure 2. Stimulation of CXCR3 reduces CXCL12-induced chemotaxis and arrests of CLL cells and influences CXCR4 distribution on the cell membrane. Total CLL peripheral blood mononuclear cells (migration assay) or isolated CLL cells (adhesion assay, immunofluorescence) were incubated in RPMI-1640 containing 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 reduced chemotactic activity, whereas incubation with the CXCR3 antagonist VUF11211 did not affect chemotaxis. Each test was performed in duplicate. Data represent the results from six (i) and five (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 pretreated 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 five (i, ii) different samples tested that were analyzed using the paired Student *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^{dim}/CXCR4^{br} (dashed line) and CXCR3^{br}/CXCR4^{dim} (solid line) expressing subpopulations with differential CXCR4 distribution on the surface. Co-localization of CXCR3 and CXCR4 is shown in yellow.

The mutational status of immunoglobulin heavy-chain variable-region (IGVH) genes is considered the most powerful marker of clinical outcome in CLL.[5,6] However, patients with mutated IGVH genes (MCLL) are found to have a more heterogeneous clinical course than those harboring unmutated IGVH (UMCLL).[3] To test the prognostic power of the combined assessment of CXCR3 and CXCR4 in both MCLL and UMCLL, we categorized the cohort according to IGVH mutational status. The hazards in the MCLL subset were similar to those in the uncategorized CLL cohort with regard to CXCR3^{dim}/CXCR4^{bright} compared to CXCR3^{bright}/CXCR4^{dim} patients (*Online Supplementary Figure S1Bi*; HR = 9.5). Notably, the CXCR3^{bright}/CXCR4^{dim} subgroup was characterized by a remarkably indolent course with very little treatment requirement. Also in the UMCLL subset, patients with the CXCR3^{dim}/CXCR4^{bright} phenotype suffered from much more aggressive disease when compared with CXCR3^{bright}/CXCR4^{dim} patients (HR = 16.1) (*Online Supplementary Figure S1Bii*), suggesting that the combination of CXCR3 and CXCR4 is 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 CLL cells between blood and supportive lymphoid tissues.[1,3] To date, there is

no information on the mechanism by which CXCR3 might influence the pathogenesis of CLL. In principle, CXCR3 and CXCR4 can form heteromeric complexes with a negative ligand binding co-operativity.[7] The consequence of this receptor cross-inhibition is the constriction of CXCR4 to a conformation with lower affinity for CXCL12.[7] We investigated whether CXCR3 and CXCR4 functionally influence each other in CLL. Performing chemotaxis assays as previously described,[8] we found that CLL cells pre-stimulated with either CXCR3 ligand CXCL9, CXCL10, or CXCL11 exhibited significantly reduced directed cell migration towards CXCL12 (Figure 2Ai). Pre-stimulation with a small-molecule high-affinity CXCR3 agonist (VUF11418)[9] confirmed this observation and an antagonist (VUF11211)[9] did not interfere with chemotactic ability (Figure 2Aii).

A similar heterologous desensitization was recently observed in Th1 cells.[10] 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 ligand to the lymph node chemokine receptor CCR7 (*Online Supplementary Figure S2A*), nor general CXCR4 signaling to proximal targets such as Akt
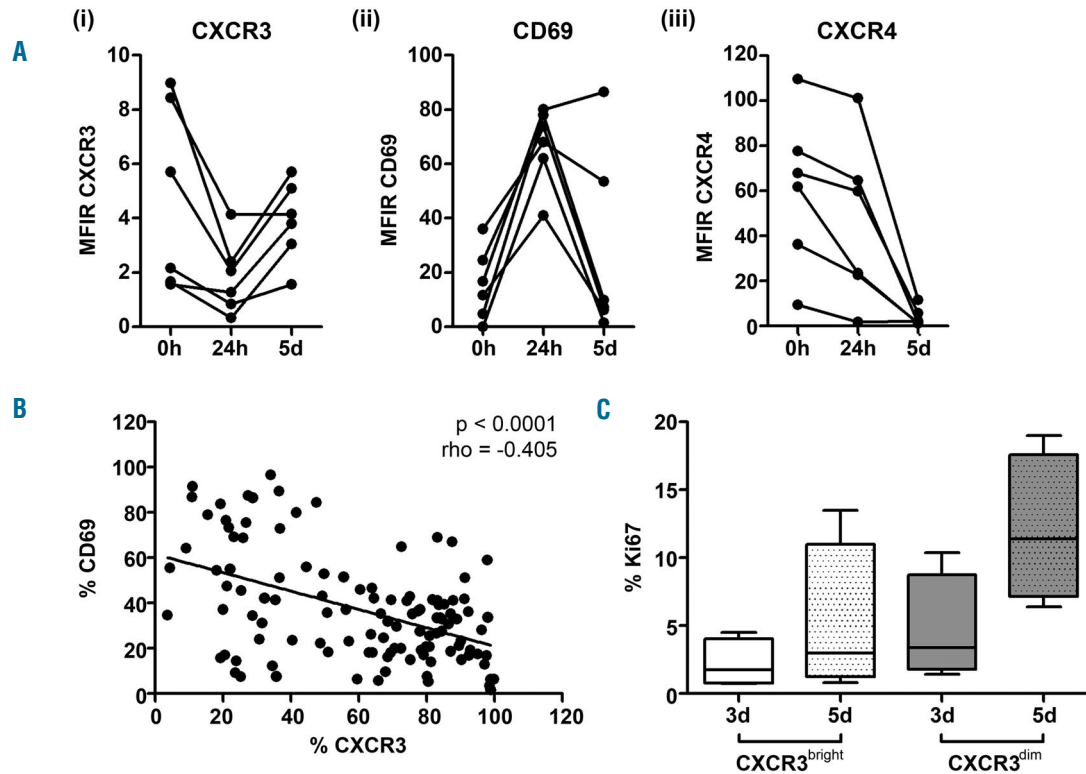
Figure 3. CXCR3 surface expression reflects the current activation status of the CLL cell. (A) Peripheral blood mononuclear cells (PBMC) from CLL patients were incubated with anti-CD3/CD28 beads in the presence of NIH 3T3 fibroblasts, and (i) CXCR3, (ii) CD69 and (iii) CXCR4 (MFIR) expression was cytometrically evaluated after 0, 24 h (h) and five days (d). In contrast to CXCR4, CXCR3 showed an inverse expression pattern compared to CD69. The data are representative of six 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 test. (C) PBMC from CLL patients exhibiting a CXCR3^{bright} or CXCR3^{dim} phenotype were cultured in the presence of NIH 3T3 fibroblasts over-expressing 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^{dim} CLL proliferated faster and more efficiently than CXCR3^{bright} CLL cells. Data show the results of four CLL samples tested in each subgroup.

and ERK (*Online Supplementary Figure S2B*) 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 bone marrow.[11] 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 elsewhere.[8] Pre-treatment of CLL cells with each CXCR3 ligand or the agonist VUF11418 resulted in significantly decreased CXCL12-induced cellular arrests whereas pre-treatment with the CXCR3 antagonist did not significantly affect tethering (*Online Supplementary Figure 2B and Figure S3*). 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 then used immunofluorescence to investigate whether CXCR3 and CXCR4 are co-localized, with parallel flow-cytometric quantification, and found two CLL cell subpopulations with inverse CXCR3 and CXCR4 expression within the samples (Figure 2Ci). Unstimulated CXCR3^{dim} CLL cells expressed CXCR4 with a uniform distribution (ring-shaped) while CXCR3^{bright} CLL cells displayed a more polarized CXCR4 expression (Figure 2Cii). In these polarized areas, co-localization of the two receptors could be seen (Figure 2Ciii). 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 2Ciii).

To confirm the *in vivo* presence of CXCR3 ligands in the blood of CLL patients, we determined the serum concentrations 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 more aggressive disease.[12] In line, we found all three CXCR3 ligands in CLL sera, with significantly higher levels in UMCLL compared to MCLL patients and CXCR3^{dim} compared to CXCR3^{bright} cases (*Online Supplementary Figure S4*). These higher levels may cause a chemokine-induced CXCR3 downregulation, 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,[13] with high CD69 expression being a prognostic marker predicting disease aggressiveness.[14] We measured CXCR3 and CXCR4 in parallel to CD69 expression during early CLL cell activation and proliferation. We used our recently described co-culture systems based on activated CD40L+ T cells, mimicking CLL-T-cell interactions in lymph nodes.[15] In a time course analysis, we observed a strong reduction in CXCR3 expression during early activation (24 h), which was paralleled by upregulation of CD69 (Figure 3Ai and ii). Using these assays, we found that CD69 was transiently up-regulated during the early activation period and diminished again during CLL cell proliferation and division occurring, on average, after five 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. We did indeed detect a significant inverse correlation of CXCR3 and CD69 (Figure 3B). Notably, this correlation was based on reduced CXCR3 expression in the CD69 positive fraction and *vice versa* (*Online Supplementary Figure S5*), suggesting that CLL cells expressing low CXCR3 are more activated. In order to determine whether this translates into differences of CXCR3bright and CXCR3dim samples in *in vitro* proliferation, we co-cultured CLL cells with CD40L-over-expressing fibroblasts and found that CXCR3dim CLL cells proliferated more efficiently than CXCR3bright 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 CLL patients with a good prognosis, and that combined CXCR3 and CXCR4 measurements are a powerful tool to identify both MCLL and UMCLL patients with a significantly lower risk of disease progression, which may also have implications on therapy decisions. Elucidating the functional role of CXCR3 in the pathogenesis of CLL, we observed that CXCR3 engagement by its ligands substantially modulated CXCR4-mediated effects during cell migration and VLA-4 mediated adhesion. We propose that CXCR3 has a negative impact on CXCR4 functionality by modifying its distribution on the cell membrane, with CXCR4 remaining the master player and major therapeutic target. Furthermore, we suggest that CXCR3 expression inversely mirrors the current activation status of the CLL cells and hence their proliferative propensity. In conclusion, 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.

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