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

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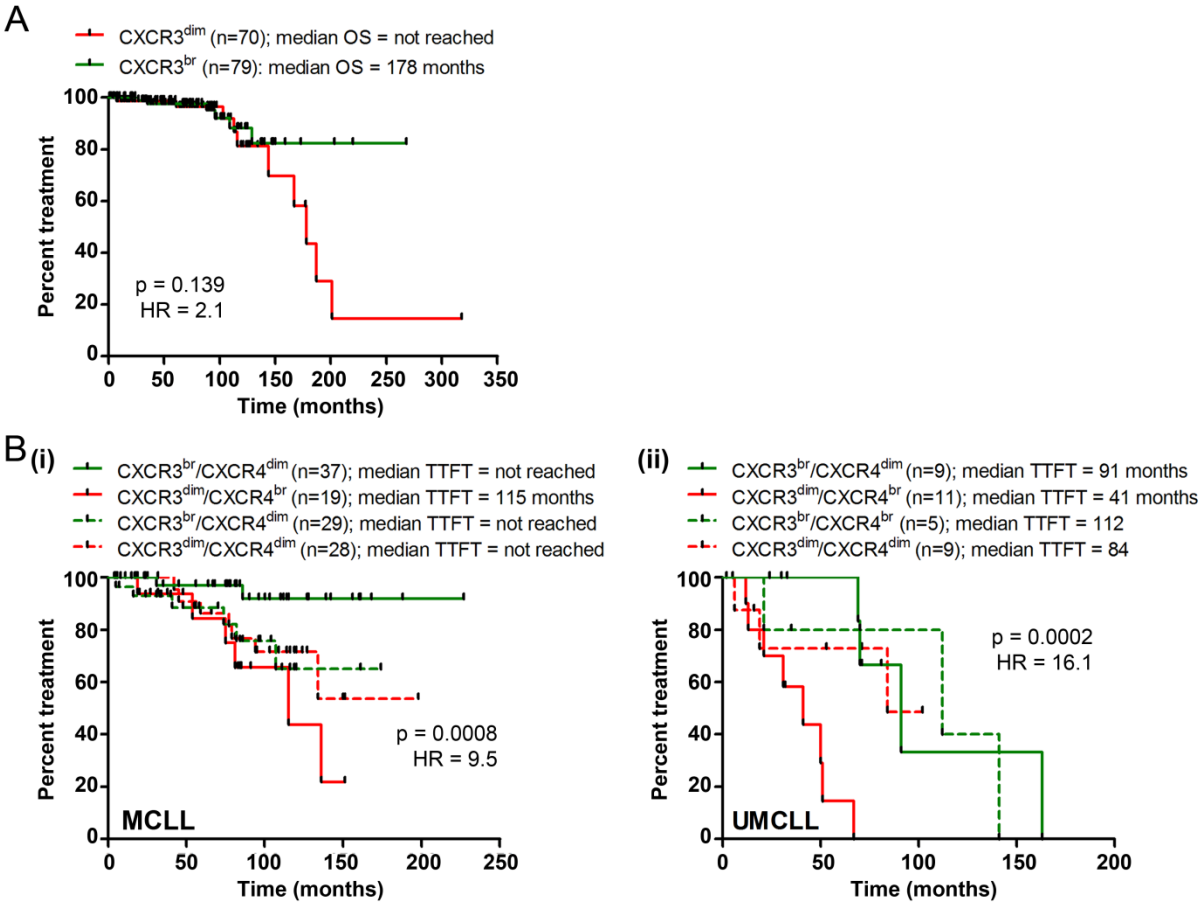
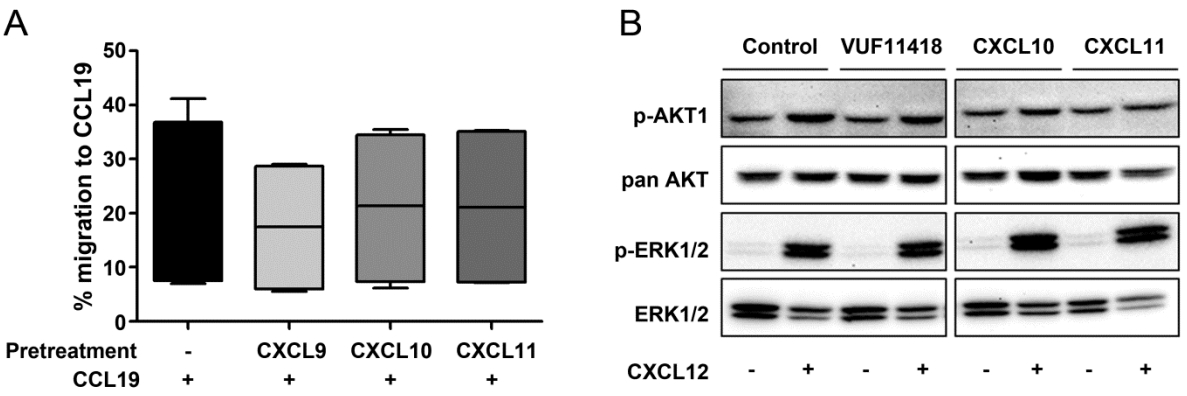
Table 1. Patient characteristics	
Sex	
Female	58
Male	91
Rai stage	
0	73
I-II	55
III-IV	2
ND	19
Treatment status	
Chemonaive	110
Treated	39
Risk parameters	
IgVH M	112
IgVH UM	34
ND	3
CD49d low	110
CD49d high	39
ZAP70 low	90
ZAP70 high	42
ND	15
CD38 low	112
CD38 high	37
del11q	4
del13q	69
del17p	0
del11q, del13q	7
del13q, del17p	6
tri12	13
tri12, del13q	3
no aberration	42
ND	5

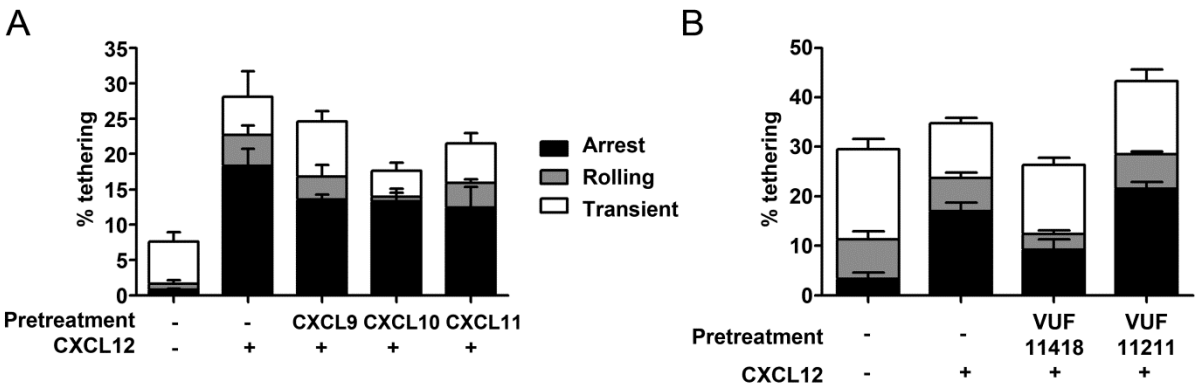
Ganghammer et al. Supp. Table 1.



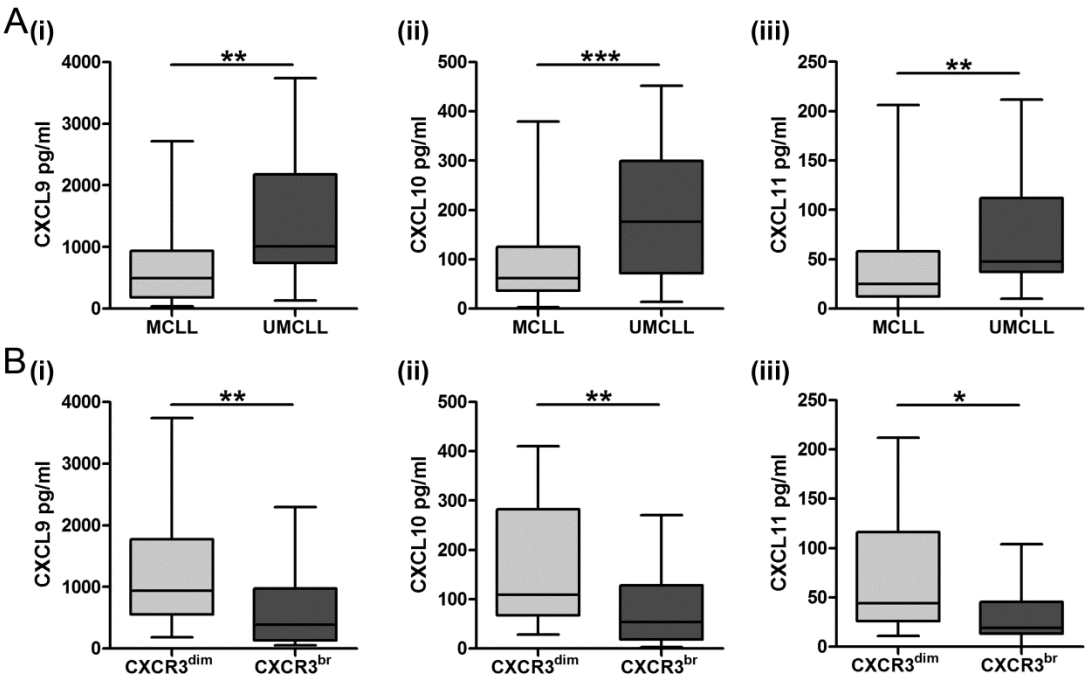
Ganghammer et al. Supplemental Figure 1.



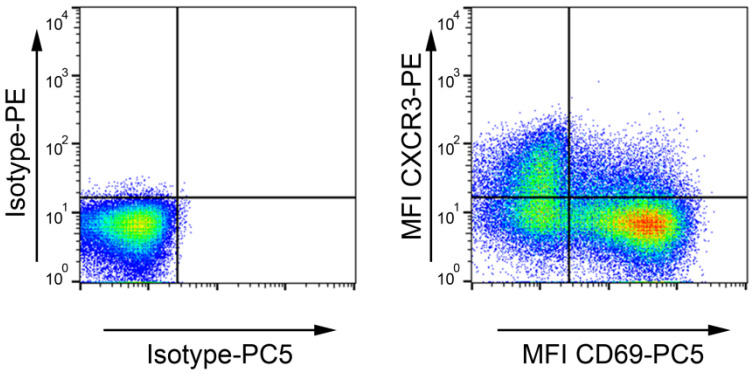
Ganghammer et al. Supplemental Figure 2.



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*.[1] CD49d expression (cutoff 30%) was analyzed as previously described[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, ≥98% homology). ZAP-70 expression was evaluated as recommended (low, NKT/B ratio >3.5; high, NKT/B ratio <3.1).[4] CD38 status was defined as described (low, <30%; high, ≥30%).[5] Chromosomal aberrations were determined according to Döhner *et al*.[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 $CXCR3^{dim}$ 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% CO_2. 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.[7] 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/cm^2 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.

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