CXCL12-induced chemotaxis is impaired in T cells from patients with ZAP-70-negative chronic lymphocytic leukemia

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Design and Methods Appendix

Reagents and antibodies

RPMI 1640 was purchased from Life Technologies (Grand Island, NY, USA), fetal calf serum (FCS), penicillin and streptomycin were obtained from GIBCO Laboratories (Grand Island, NY, USA). The chemokines CCL19/ELC, CCL21/SLC and CXCL12/SDF-1 were purchased from PeproTech (DF, Mexico). Fluorescein isothiocyanate (FITC)-labeled phalloidin and 1-α-lysophosphatidylcholine were both from Sigma Aldrich (Saint Louis, MO, USA). The Fix&Perm Kit for intracellular staining was purchased from Caltag Laboratories (Burlingame, CA, USA). Bovine serum albumin (BSA) was obtained from Weiner Laboratorios (Santa Fé, Argentina).

FITC-, phycoerythrin (PE)- and peridin chlorophyll (PerCP)-conjugated monoclonal antibodies specific for CD3 (clone SK7), CD4 (clone RPA-T4), CD8 (clone HIT8a), CD19 (clone HIB19), CD45 (clone UCHL1), CXCR4 (clone 12G5), CCR7 (clone 3D12), and ZAP-70 (clone 1E7.2) and control antibodies with irrelevant specificities (isotype control) were purchased from BD Biosciences, Pharmingen (CA, USA). PE-conjugated monoclonal antibody specific for CD56 (clone N901) was obtained from Immunotech (Marseille, France). For T- and B-cell purification, purified monoclonal antibodies specific for CD19, CD14, CD56, CD3 and CD2 were purchased from Immunotech and magnetic beads (MagnaBind Beads) obtained from Pierce (Rockford, IL, USA).

Samples from chronic lymphocytic leukemia patients and healthy donors

Peripheral blood samples were obtained from CLL patients (mean age, 67.04 years; range, 41-86 years) and healthy age-matched donors (mean age 67.7 years; range, 45-80 years). The samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of the analysis all patients were free from clinically relevant infectious complications and were either untreated or had not received treatment for a period of at least 6 months before the investigation.

Cell separation procedures and cultures

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples by centrifugation over a Ficoll-Hypaque layer (Lymphoprep, Nycomed Pharma, Oslo, Norway), washed twice with saline and resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin). Purified T cells (pT) from CLL patients were obtained by negative selection as previously described.[1] Briefly, PBMC were incubated with mouse monoclonal antibodies specific for CD14, CD56 and CD19 for 30 min at 4°C, washed twice and treated with magnetic beads coated with anti-mouse IgG antibodies according to the manufacturer's instructions. The purity of the T-cell population was checked by flow cytometry analysis using anti-CD3 monoclonal antibody and was found to be always more than 95%. Purified B cells from CLL patients (pCLL) were also obtained by negative selection using mouse monoclonal antibodies specific for CD14, CD56, CD3 and CD2 and magnetic beads. The purity of B cells was checked by flow cytometry analysis using anti-CD19 monoclonal antibody and was always more than 99%.

Analysis of protein expression by flow cytometry

CD38 expression on B and T cells from CLL patients was evaluated by flow cytometry using a FACScan flow cytometer (BD, Immunocytometry System, San Jose, CA, USA). To this aim, PBMC were stained with PE-conjugated anti-CD38 monoclonal antibody or PE-conjugated isotype control antibodies and PerCP-conjugated monoclonal antibodies specific for CD19 or CD3. ZAP-70 expression in B cells from CLL patients was evaluated by flow cytometry as described previously.[2] Briefly, PBMC were stained with anti-CD19 PerCP, anti-CD3 PE and anti-CD56 PE. After washing twice with phosphate-buffered saline (PBS) supplemented with 0.5% BSA, cells were fixed and permeabilized with Fix and Perm kit (Caltag Laboratories) according to the manufacturer's instructions. The cells were then stained with 2 μg of anti-ZAP-70 FITC and acquired in the flow cytometer. Samples were analyzed using CellQuest software (BD Biosciences). $CD3^+$ cells and $CD56^+$ cells (T and NK cells) were used as internal positive controls for ZAP-70 expression in CLL cells. Patients were considered $ZAP\text{-}70^+$ when 20% or more of the $CD19^+$ cells expressed ZAP-70.[2] To determine the expression of ZAP-70 in T cells, PBMC were stained with anti-CD3 PerCP and anti-CD56 PE prior to ZAP-70 staining.

CXCR4 and CCR7 surface expression on T cells from CLL patients or healthy donors was performed by direct staining of whole blood samples in order to avoid Ficoll purification, which can transiently affect chemokine receptor expression.[3-5] Briefly, 100 μL of freshly collected whole blood were incubated with saturating concentrations of PerCP-conjugated anti-CD3 antibodies, FITC-conjugated anti-CD4 antibodies and PE-conjugated anti-CXCR4 (clone 12G5) or anti-CCR7 (clone 3D12), or the appropriate isotype control antibody for 30 min at 4°C. After selective red blood cell lysis with a hypotonic buffer ($Tris\text{-}NH_4Cl$), the white cell pellet was washed twice with PBS supplemented with 0.5% BSA, resuspended in Isoflow Buffer (BD Biosciences) and acquired in the flow cytometer. As previously report-

ed by other authors[3-5] we found that the PBMC isolation procedure decreases CXCR4 surface expression. Given that the expression of CXCR4 in T cells from whole blood samples recovered when PBMC were cultured for 2 h in complete medium at 37°C (*Online Supplementary Figure S1*), PBMC or pT were cultured in these conditions before functional studies were performed.

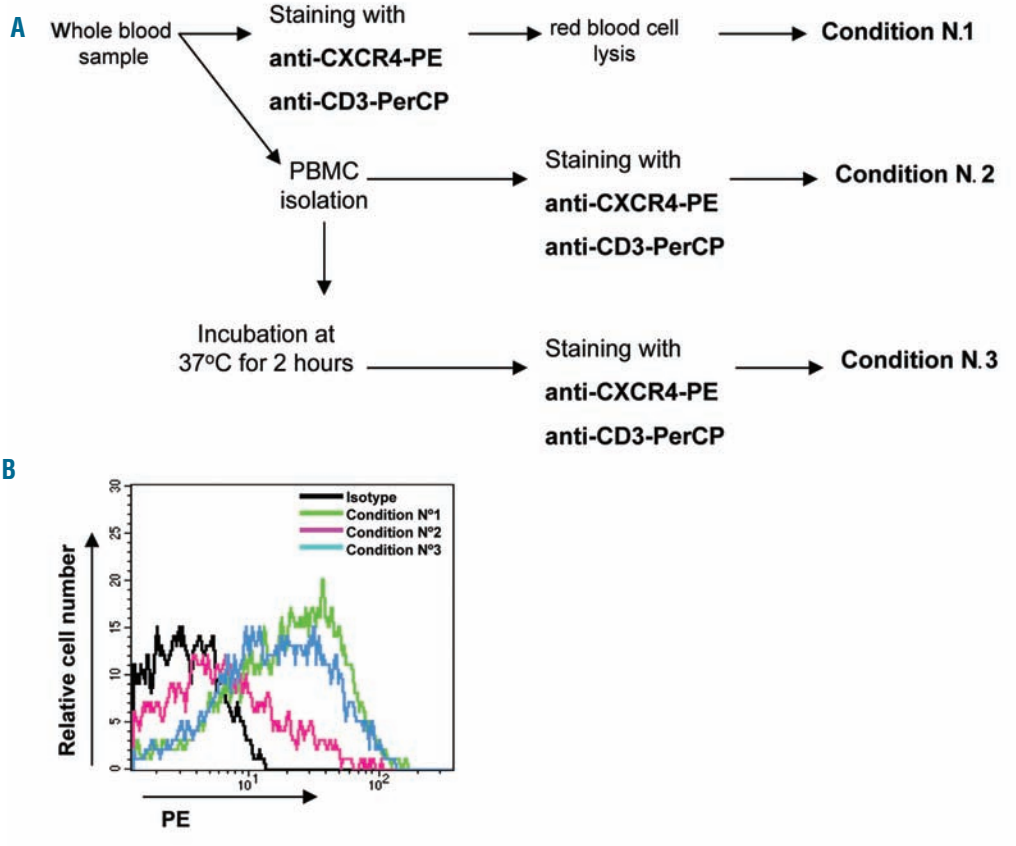
In order to evaluate surface CXCR7 expression, PBMC from CLL patients were stained with anti-CD3 PerCP and anti-CXCR7 PE clone 8F11-M16 (BioLegend, USA) or the appropriate isotype control antibody for 30 min at 4°C. The cells were then washed twice with PBS and 0.5% BSA and analyzed by flow cytometry. For intracellular CXCR7 detection, PBMC from CLL patients were stained with anti-CD3 PerCP and then fixed with paraformaldehyde 1.5% for 30 min at room temperature. Fixed cells were washed, permeabilized with Triton X100 0.2% in PBS and 1% BSA for 5 min and stained with anti-CXCR7 PE or the appropriate isotype control antibody for 20 min at room temperature. Cells were washed twice with PBS and 0.5% BSA before they were analyzed by flow cytometry.

Chemotaxis assay

The chemotaxis assay across bare polycarbonate membrane was performed as previously described.[6] Briefly, cells previously cultured for 2 h in complete medium to restore chemokine receptor expression (see above) were resuspended in RPMI-1640 supplemented with 1% FCS and a total of 100 μL, containing 1×10^6 cells, were placed in the upper chamber of a 6.5 mm diameter Transwell culture insert (Costar, Corning Incorporated, NY, USA) with a pore size of 5 μm. The lower chamber of each well contained 600 μL of medium alone (control wells) or medium with CXCL12, CCL21 or CCL19. All the assays were performed in duplicate. After incubation for 2 h at 37°C in humidified air with 5% CO_2, the contents of the lower chamber were recovered and the migrating cells were divided into aliquots for immunophenotyping with a saturating concentration of PE-conjugated anti-CD56 monoclonal antibody, FITC-conjugated anti-CD4 or -CD8 and PerCP-conjugated anti-CD3 monoclonal antibody or for counting with a FACSCalibur as the number of cells acquired in 1 min under a defined flow rate. The migration index was calculated by determining the ratio of migrated $CD3^+CD56^-$ ($CD4^+$ or $CD8^+$) cells in chemokine-treated wells *versus* control wells,[7] taking the spontaneous migration in control wells as 100%. Spontaneous migration in control wells was consistently found to be 1% of $CD3^+CD56^-$ cells placed in the upper compartment. We performed dose-response chemotaxis assays (*Online Supplementary Figure S2*) and selected 1000 ng/mL of CXCL12, 1000 ng/mL of CCL21 and 5000 ng/mL of CCL19 to evaluate T-cell migration of samples from CLL patients and healthy donors.

CXCR4 endocytosis assay

The time course and dose-dependency of CXCR4 endocytosis in T cells from CLL patients in response to CXCL12 were examined as previously described.[8] Briefly, PBMC of CLL patients were adjusted to a concentration of 5×10^6/mL in RPMI-1640 1% FCS. For the time course experiments, cells were treated with medium alone (control) or with CXCL12 (1 μg/mL) at 37°C in humidified air with 5% CO_2 and aliquots were removed at different time points (0, 2, 5, 15, and 60 min). The cells were then washed twice with ice-cold PBS supplemented with 1% BSA, stained at 4°C with a saturating concentration of PE-conjugated anti-CXCR4 monoclonal antibody and PerCP-conjugated anti-CD3 monoclonal antibody for 30 min and analyzed by FACS.



Online Supplementary Figure S1. CXCR4 expression in T cells from blood samples and peripheral blood mononuclear cells (PBMC). (A) The figure shows the three different conditions tested to determine CXCR4 expression levels on T cells by flow cytometry. PerCP-conjugated anti-CD3 antibodies and PE-conjugated anti-CXCR4 or the appropriate isotype control antibody were employed to stain freshly collected whole blood from CLL patients or healthy donors before selective red blood cell lysis (condition N. 1), freshly purified PBMC by Ficoll-hypaque purification (condition N. 2) or cultured PBMC (condition N. 3). (B) The figure shows a histogram for CXCR4 expression in T cells from a representative healthy donor under the three conditions detailed above. Comparable results were obtained with T cells from $ZAP\text{-}70^+$ or $ZAP\text{-}70^-$ CLL patients.

To determine dose-dependency of CXCR4 endocytosis, cells were cultured for 60 min with CXCL12 at various concentrations (0, 10, 100, and 1000 ng/mL) at 37°C in humidified air with 5% CO_2. The cells were then washed twice with ice-cold PBS supplemented with 1% BSA, stained at 4°C with a saturating concentration of PE-conjugated anti-CXCR4 monoclonal antibody and PerCP-conjugated anti-CD3 monoclonal antibody for 30 min and analyzed by FACS.

F-actin polymerization assay

Actin polymerization was evaluated as described elsewhere.[8] Briefly, pT from CLL patients or healthy donors were adjusted to a concentration of 5×10^6 cell/mL in RPMI 1640 1% FCS and then treated with medium alone (control) or medium with CXCL12 (1 μg/mL) at 37°C for different times (15, 30 and 300 sec). At the indicated time points, 400 μL of the cell suspension were added to 100 μL of a solution containing 4×10^{-7} M FITC-labeled phalloidin, 0.5 mg/mL of 1-α-lysophosphatidylcholine and 18% formaldehyde in PBS for 10 min at 37°C. The fixed cells were analyzed by flow cytometry and the percentage change in intracellular F-actin was calculated as the MFI of phalloidin in cells treated with CXCL12 relative to the control (without CXCL12).

Co-culture experiments

For co-culture experiments pT were cultured in complete medium alone (pT cultures) or at a 1:4 ratio with autologous pCLL (pT+pCLL cultures). Chemotaxis assays towards CXCL12 (1 μg/mL) were evaluated as previously described with freshly purified and 48 h cultured cells. In these experiments, the relative migration of $CD3^+$ cells was calculated by taking the migration index of T cells in pT+pCLL cultures as 100%.

CXCR4 expression on $CD3^+$ cells was checked by flow cytometry analysis after 48 h of culture. The viability of cultured cells included in this experiment was always more than 75%, as determined by fluorescence microscopy using acridine orange and ethidium bromide.[9]

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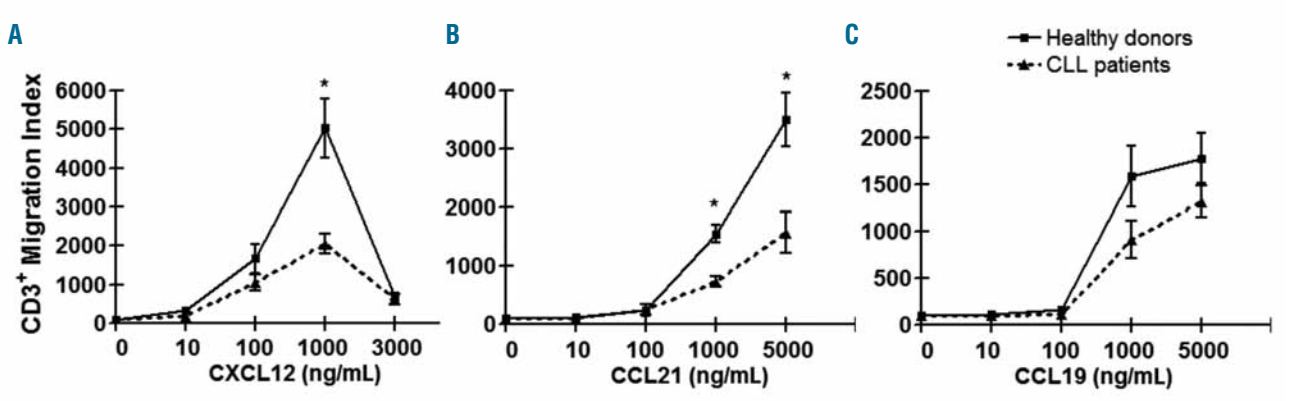
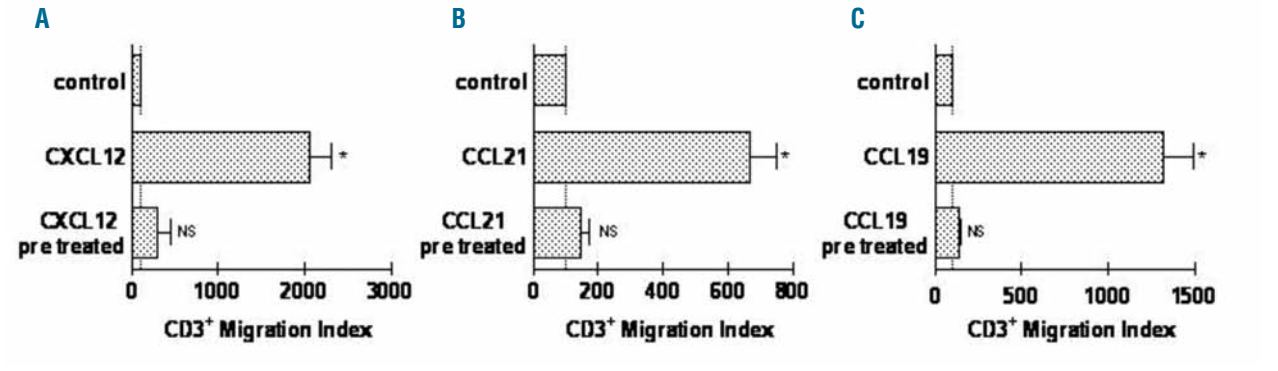
Online Supplementary Table S1. Expression of CXCR4 and CCR7 in T cells from CLL patients segregated by ZAP-70 or CD38 expression.

Risk parameter	CXCR4		P	CCR7		P
$CD38^+$ CLL cells	<7%	≥7%		<7%	≥7%	
Mean receptor expression in T cells (MFI)	328 (n=11)	300 (n=6)	0.366	33 (n=9)	40 (n=4)	0.315
$CD38^+$ CLL cells	<30%	≥30%		<30%	≥30%	
Mean receptor expression in T cells (MFI)	301 (n=14)	397 (n=3)	0.659	33 (n=10)	42 (n=3)	0.315
$ZAP\text{-}70^+$ CLL cells	<20%	≥20%		<20%	≥20%	
Mean receptor expression in T cells (MFI)	306 (n=11)	341 (n=6)	0.841	35 (n=8)	36 (n=5)	0.633

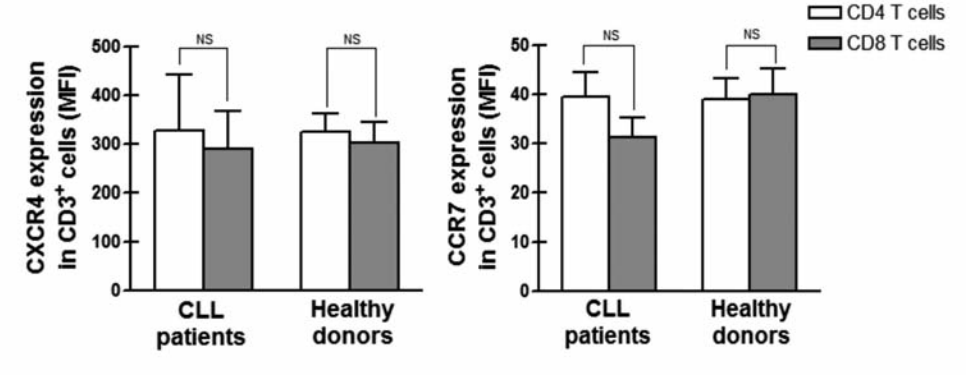
The expression of CXCR4 and CCR7 in T cells from CLL patients was evaluated by flow cytometry in 17 out of 28 patients for CXCR4 and 13 out of 28 patients for CXCR7. The expression of each chemokine receptor, in CLL samples divided by CD38 expression using the 7% and 30% cut-off values and in CLL patients divided by the 20% cut-off value for ZAP-70 expression, is shown as the mean value for CXCR4 or CCR7 mean fluorescence intensity (MFI) of each sample. The number of patients evaluated in each case is shown in brackets. The P value of the Mann-Whitney test shows that expression of the receptors was comparable between the groups.



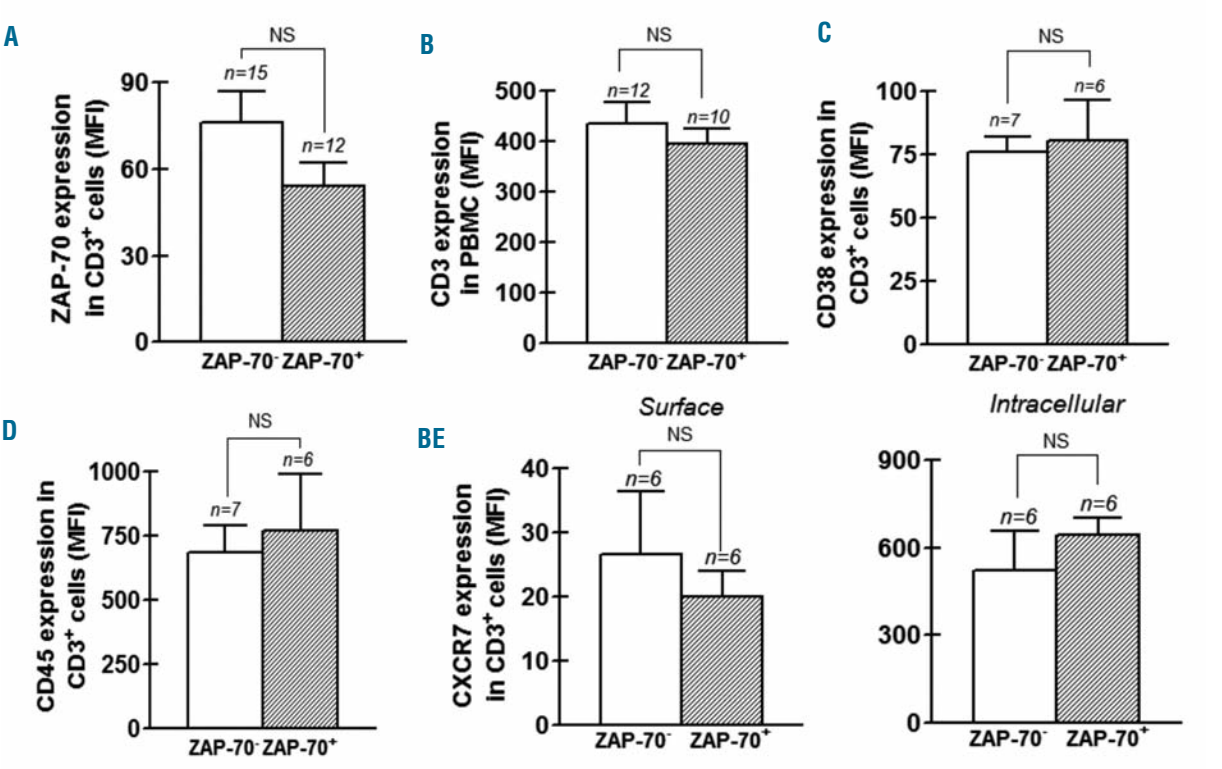
Online Supplementary Figure S2. T-cell chemotaxis towards different doses of CXCL12, CCL21 and CCL19. PBMC from healthy donors or CLL patients were placed in the upper chamber of a 24-transwell plate and medium alone (control wells) or with different doses of CXCL12 (10 to 3000 ng/mL) (A), CCL21 (10 to 5000 ng/mL) (B) or CCL19 (10 to 5000 ng/mL) (C) was placed in the lower chamber. After 2 h of culture the migrating cells were suspended and divided into aliquots for counting with a FACSCalibur or for immunophenotyping. The $CD3^+$ migration index for each sample was calculated by determining the ratio of migrated $CD3^+$ cells in chemokine-treated wells *versus* control wells. *P<0.01 Mann-Whitney test.



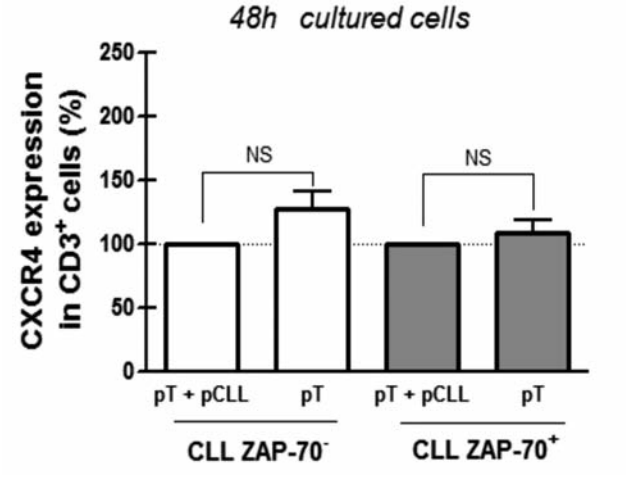
Online Supplementary Figure S3. T-cell chemotaxis towards CXCL12, CCL21 and CCL19. Untreated PBMC from CLL patients or PBMC from the same patient previously cultured for 1 h with the optimal concentration of the chemokines to induce chemokine receptor endocytosis were placed in the upper chamber of a 24-transwell plate and medium alone (control wells) or with the optimal dose of CXCL12 (1 μg/mL) (A), CCL21 (1 μg/mL) (B) or CCL19 (5 μg/mL) (C) as chemoattractant was placed in the lower chamber. After 2 h of culture the migrating cells were suspended and divided into aliquots for counting with a FACSCalibur or for immunophenotyping. The migration index for each sample was calculated by determining the ratio of migrated T cells in chemokine treated wells *versus* control wells. The bars represent the mean values ± SEM for the migration indices. NS: not significant, *P<0.0001 One sample t-test.



Online Supplementary Figure S4. The figure shows CXCR4 and CCR7 expression in T CD4 cells ($CD3^+CD4^+$) and T CD8 cells ($CD3^+CD4^-$) from CLL patients and healthy donors. Results are expressed as the mean values±SEM for CXCR4 and CCR7 mean fluorescence intensity (MFI) of each sample. There was no significant difference in the expression of the receptors between T CD4 and T CD8 cells. NS: not significant, Wilcoxon's signed rank test.



Online Supplementary Figure S5. (A) PBMC from ZAP-70$^-$ (n=15) and ZAP-70+ CLL patients (n=12) were stained with anti-CD3 PerCP and anti-CD56 PE prior to intracellular staining with anti-ZAP-70 FITC-conjugated monoclonal antibody. The bars represent the mean values ± SEM for the MFI of ZAP-70 in T cells (CD3$^+$CD56$^-$ cells). NS: not significant, Mann-Whitney test. (B) The bars represent the mean values ±SEM for the MFI of CD3 in PBMC from ZAP-70$^-$ (n=12) and ZAP-70$^+$ CLL patients (n=10). NS: not significant, Mann-Whitney test. PBMC from ZAP-70$^-$ (n=7) and ZAP-70$^+$ CLL patients (n=6) were stained with anti-CD3 PerCP and anti-CD38 PE (C) or anti-CD45 PE (D) and evaluated by flow cytometry. The bars represent the mean values ± SEM for the MFI of these molecules in CD3$^+$ cells. NS: not significant, Mann-Whitney test. (E) T cells from ZAP-70$^-$ (n=6) and ZAP-70$^+$ (n=6) CLL patients were stained for surface and intracellular CXCR7 expression and evaluated by flow cytometry. The bars represent the mean values ± SEM for the MFI. NS: not significant, Mann-Whitney test.



Online Supplementary Figure S6. Purified T cells from ZAP-70$^-$ and ZAP-70$^+$ CLL patients were cultured in complete medium alone (pTcultures) or at a 1:4 ratio with autologous purified CLL cells (pT+pCLL cultures). CXCR4 expression in T cells from 48 h cultured cells was analyzed by flow cytometry NS: not significant, Wilcoxon's signed rank test.