Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination

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Abstract: B cell neoplasms present heterogeneous patterns of lymphoid organ involvement, which may be a result of the differential expression of chemokine receptors. We found that chemokine receptor (CCR)7, CXC chemokine receptor (CXCR)4, or CXCR5, the main chemokine receptors that mediate B cell entry into secondary lymphoid tissues and their homing to T cell and B cell zones therein, were highly expressed in B malignancies with widespread involvement of lymph nodes. Conversely, those pathologies with little or no nodular dissemination showed no expression to very low levels of CCR7 and CXCR5 and low to moderate levels of CXCR4. These findings provide evidence for the role of CCR7, CXCR4, and CXCR5 in determining the pattern of lymphoid organ involvement of B tumors. Functional studies were performed on B malignancies expressing different levels of CCR7, CXCR5, and CXCR4. Multiple myeloma (MM) cells did not express CCR7 nor CXCR5 and did not migrate in response to their ligands; a moderate expression of CXCR4 on MM cells was accompanied by a migratory response to its ligand, CXCL12. By contrast, cells from B cell chronic lymphocytic leukemia (B-CLL) expressed the highest levels of these chemokine receptors and efficiently migrated in response to all ligands of CCR7, CXCR4, and CXCR5. In addition, the migration index of B-CLL cells in response to both of the CCR7 ligands correlated with the presence of clinical lymphadenopathy, thus indicating that the high expression of functional chemokine receptors justifies the widespread character of B-CLL, representing a clinical target for the control of tumor cell dissemination. *J. Leukoc. Biol.* 76: 462–471; 2004.

Key Words: *CCR7 · CXCR4 · CXCR5 · migration · lymphadenopathy*

INTRODUCTION

Chemokines are chemoattractant proteins that participate in homeostatic processes, including lymphocyte homing to secondary lymphoid organs, e.g., lymph nodes and Peyer's patches, as well as in pathologic conditions such as inflammation, tumor growth, and metastasis. A family of 7-transmembrane-spanning G-protein-coupled receptors mediates the attractant effects of chemokines [1–5].

Leukocyte passage across high endothelial venules (HEV) into peripheral lymphoid organs is a multistep process involving several families of adhesion molecules [6, 7]. Selectins and their counter-receptors on HEV mediate the first step, which consists of rolling and tethering leukocytes on the endothelium. In the second step, chemokines activate integrins, which in turn mediate firm arrest of white blood cells on the vessel walls [8, 9].

Chemokine receptor (CCR)7, CXC chemokine receptor (CXCR)4, and CXCR5 are the main chemokine receptors involved in B cell homing to secondary lymphoid tissues [10–15]. HEV express high levels of chemokine ligand (CCL)21, one of the ligands for CCR7, which plays a prominent role in triggering the adhesion of circulating B lymphocytes to this specialized endothelium [16, 17]. CCL19, the other ligand for CCR7, directs the movement of B cells toward the T cell areas, where this chemokine is produced [15, 18, 19]. CXCR4 and its ligand CXCL12 have been previously involved in diverse aspects of B cell homeostasis, including the retention of B cell precursors on bone marrow (BM) or the migration of plasmatic cells out of the lymph node [20–22]. Recent studies [23] have demonstrated that CCL19 and CXCL12 can be transcytosed and presented on the lumenal side of HEV, where they also contribute to lymphocyte recruitment into peripheral lymphoid tissues. CXCR5 and its ligand CXCL13 are needed for the

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positioning of B cells into follicles of Peyer's patches, lymph nodes, and spleen [11, 13, 24, 25].

The B cell neoplasms are characterized by a heterogeneous clinical behavior, which is determined, among others, by differences on the migratory and invasive patterns of the malignant cells [26, 27]. Whereas B cell chronic lymphocytic leukemia (B-CLL) has a wide dissemination to lymph nodes, BM, and peripheral blood (PB), multiple myeloma (MM) or hairy cell leukemia (HCL) is confined to a limited number of organs with minimal or no lymph node involvement. Little is known about the mechanisms that determine such migratory heterogeneity, although there is growing evidence about the role of homeostatic chemokines and their receptors in the dissemination pattern of the lymphoid neoplasias [28–37].

However, no studies have addressed the contribution of CCR7, which enables B lymphocytes to extravasate into lymphoid organs, to the differential pattern of lymphoid organ involvement that characterizes B cell malignancies. We therefore examined the expression of CCR7 on different B tumors ranging from B-acute lymphoblastic leukemia (B-ALL) to MM. The simultaneous analysis of CXCR4 and CXCR5 expression together with that of CCR7 was performed, as they also contribute to B cell entry into secondary lymphoid organs and their homing to T cell and B cell zones therein [11, 13, 14].

To further analyze a possible correlation between the expression of these chemokine receptors and clinical data related to malignant cell entry into lymphoid organs, we evaluated the function of CCR7, CXCR4, and CXCR5 on B-CLL and MM, which are B tumors with widespread and rare lymph node involvement, respectively. In addition, the high availability of B-CLL samples allowed us to perform a statistically reliable comparison between in vitro data and clinical data regarding stage and prognosis.

MATERIALS AND METHODS

Patients, cells, and cytokines

BM aspirates and/or PB and/or lymph node biopsy samples were obtained from patients with different B cell neoplasms whose diagnosis was based on the Revised European-American Lymphoma Classification [26]. The following diseases were included in the study: B-ALL (n=6), typical CD5+ B-CLL (n=79), atypical CD5– B-CLL (n=12), mantle cell lymphoma (MCL; n=15), follicular lymphoma (FL; n=16), splenic marginal zone lymphoma (SMZL; n=8), HCL (n=9), and MM (n=30).

Normal PB and BM B lymphocytes were obtained from healthy donors for allogenic BM transplantation with prior informed consent. Fresh tonsils were obtained from the department of Oto-Rhino-Laringology at Hospital Universitario de la Princesa (Madrid, Spain). Tonsillar and lymph node lymphocytes were isolated from surgical specimens as described [38]. Briefly, tonsils or lymph node biopsies were gently teased, and the cell suspension was subjected to a density gradient centrifugation. Cells were then cultured for 6–12 h at 37°C in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 50 μg streptomycin/penicillin, 2 mM glutamine, and 10% fetal calf serum (FCS) to avoid the eventual internalization of the chemokine receptors induced by the in vivo interaction with their corresponding ligands.

Recombinant human CXCL12 (stromal cell-derived factor-1), CCL19 (macrophage-inflammatory protein-3β), CCL21 (6Ckine), CXCL13 (B-cell attracting chemokine-1; B lymphocyte chemoattractant), and CCL5 (regulated on activation, normal T expressed and secreted) were purchased from R&D Systems (Minneapolis, MN).

Flow cytometry analysis

An initial immunophenotypic characterization of the B cell neoplasms was performed by using standard four-color flow cytometry with monoclonal antibodies (mAb) directed against the following human surface antigens: CD45, CD19, κ light-chain, λ light-chain, CD20, CD23, CD5, CD34, CD10, FMC7, CD22, CD11c, CD103, CD38, and CD56 (all from Becton Dickinson, Mountain View, CA). Analysis of chemokine receptor expression was subsequently performed on electronically gated tumor B cells.

Phycoerythrin (PE)-conjugated mouse anti-human CXCR4 was purchased from PharMingen (San Diego, CA). PE-conjugated mouse anti-human CCR7 and PE-conjugated mouse anti-human CXCR5 were purchased from R&D Systems. In all cases, appropriate isotype controls were included.

For staining, 100 μl whole blood from PB or BM samples was incubated for 30 min at 4°C with optimal concentrations (ranging from 1 to 3 μg/ml) of the appropriated antibodies. This incubation was followed by the lysis of red blood cells by using fluorescein-activated cell sorter lysing solution (Becton Dickinson) following the manufacturer's instructions. Finally, leukocytes were resuspended on 500 μl ice-cold phosphate-buffered saline (PBS). If lymphocytes from tonsils or lymph node were used, 10^6 cells suspended on 100 μl ice-cold PBS were stained as mentioned above without the lysis step.

Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using CellQuest software (Becton Dickinson). In all experiments, a minimum of 5000 neoplastic B cells was acquired.

Results are expressed as the mean fluorescence intensity (MFI) for a given chemokine receptor within the population of interest.

Chemotaxis assay

Chemotaxis was performed with isolated B cells from different PB and BM samples. PB mononuclear cells (PBMC) were isolated by centrifugation on standard Ficoll-Hypaque density gradient. Normal PB B cells were further isolated by immunomagnetic negative selection using a cocktail of anti-CD2, -CD4, -CD11b, -CD36, -CD16, and -immunoglobulin E (IgE) antibodies for the depletion of non-B cells and magnetic cell sorter LS columns (Miltenyi Biotech GmbH, Germany) following the manufacturer's instructions. In the case of B-CLL and MM, we chose those samples containing malignant cells in a proportion greater than 80% or 60%, respectively, of the isolated PBMC, and further isolation of tumor B cells was not performed. Cells were stored in liquid nitrogen before use. Frozen cells were reconstituted in RPMI 1640 containing 2% FCS, 2 mM L-glutamine, and 50 μg/ml penicillin/streptomycin and were allowed to recover for 3 h at 37°C in 5% CO_2 atmosphere before performing the assay.

Chemotaxis was determined by duplicate in Transwell cell culture chambers (6.5 mm diameter, 10 μm thickness, 5 μm diameter pore size, Costar, Cambridge, MA). A total of 5×10^5 cells suspended in 100 μl RPMI 1640 0.5% human serum albumin (HSA) was added to the upper chamber, and chemokines were added to the lower well in 600 μl of the same medium at the optimal concentration (100 ng/ml for CXCL12, 1 μg/ml for CCL19 and CCL21, 2 μg/ml for CXCL13, and 10 ng/ml for CCL5). Migration was allowed to proceed for 6 h at 37°C in 5% CO_2 atmosphere. Migrated cells were recovered from the lower chamber, stained with an anti-CD19 mAb or an anti-CD38 mAb (in the case of analyzing MM cells), and counted by flow cytometry for 60 s after calibrating the flow rate with Trucount tubes (BD Biosciences, San Jose, CA). Events were analyzed within the gated population of B or MM cells. Results are expressed as a migration index (number of CD19+ or CD38+ cells migrating in the presence of chemokine divided by the number of CD19+ or CD38+ cells migrating in the absence of chemokine).

Actin polymerization assay

PBMC from B-CLL patients were prelabeled with an anti-CD19 mAb to specifically determine actin polymerization in B-CLL cells. Cells (5×10^5) in 100 μl RPMI 1640 were incubated at 37°C with chemokines (100 ng/ml for CXCL12, 1 μg/ml for CCL19 and CCL21, and 10 ng/ml for CCL5) for different periods of time. Then, cells were fixed and permeabilized using the intracellular antigen detection kit Fix and Perm™ (Caltag Laboratories, San Francisco, CA) according to the recommendations of the manufacturers. Cells were stained with 5 μg/ml Alexa 488 phalloidin (Molecular Probes, Eugene, OR) and analyzed by flow cytometry. Results are expressed as the percentage of intracellular F-actin relative to the value found in untreated cells (t=0).

Receptor endocytosis assay

To study the down-modulation of chemokine receptor expression, 5×10^5 B-CLL cells in 100 μl RPMI 1640 supplemented with 0.1% HSA were incubated with several concentrations of CCL19, CCL21, and CXCL12 and for different periods of time (ranging from 1 min to 1 h) at 37°C in 5% CO_2 atmosphere. After washing with ice-cold PBS, CCR7 and CXCR4 expression was determined on B-CLL cells by flow cytometry analysis as indicated above.

IgV_H study

PBMC were isolated from 25 B-CLL cases, and genomic DNA was purified using a standard proteinase-K-based method.

Rearranged IgV_H genes were determined by a seminested polymerase chain reaction (PCR) method as described previously [39, 40] with some modifications. Briefly, in the first round of PCR, 500 ng genomic DNA was amplified using six framework 1 (FR1) V_H family-specific primers and a consensus primer for the J_H gene. These reactions were performed in a volume of 25 μl with 1× Master Mix (Promega, Madison, WI) and 1 μM each primer. The PCR conditions consisted of one cycle at 95°C for 1 min, 59°C for 2 min, and 72°C for 40 s, followed by 34 cycles at 95°C for 45 s, 59°C for 15 s, and 72°C for 40 s, and one final cycle of 72°C for 10 min. The seminested PCR was performed with the same six V_H FR1 primers and an internal J_H gene primer, 5 μl first-round PCR product, and the same concentrations of reagents. The second PCR conditions were 95°C for 45 s, annealing temperatures of 61°C for the V_H1, V_H2, and V_H6 primers and 65°C for the V_H3, V_H4, and V_H5 primers, and 72°C for 40 s. After 25 cycles, extension was continued for an additional 10 min. An aliquot of 10 μl PCR product was visualized in ethidium bromide-stained 2% agarose gel.

PCR products were sequenced directly with the same primers used in the amplification after purification with Spin-X kit (Costar) using an automated DNA sequencer ABI PRISM 3700 genetic analyzer (Applied Biosystem, Weiterstadt, Germany), following the manufacturer's procedure. Sequences were compared with the germline sequences in the IgBLAST and V BASE sequence directories (http://www.ncbi.nlm.nih.gov/igblast and http://www.mrc-cpe.cam.ac.uk/vbase). Rearranged IgV_H genes sequences were considered mutated when there was >2% deviation from a germline V_H sequence.

Statistical analysis

We used SPSS software (version 10) for data analysis. The normality of the distribution of the dependent variables was tested using the Kolmogorov-Smirnof test. A naperian log transformation of MFI values was performed to make data fit to the normal distribution. To identify any differences in terms of MFI among the groups, we used the ANOVA. Multiple comparisons were done with the Scheffé post-hoc test. All comparisons were made with two-tailed test. The data are expressed in medians and interquartile range of the original variable. The Mann-Whitney test was performed to compare the migration indexes and the MFI of chemokine receptors with the presence of lymphadenopathy, mutational status, or CD38 expression. The differences were considered statistically significant when $P < 0.05$.

RESULTS

CCR7, CXCR4, and CXCR5 are highly expressed on B-CLL and non-Hodgkin's lymphoma with nodular dissemination

Surface expression of chemokine receptors CCR7, CXCR4, and CXCR5 was analyzed on different B cell neoplasms ranging from B-ALL to MM (**Fig. 1** and **Table 1**). B cell neoplasms characterized by a widespread nodular dissemination, including B-CLL, MCL, and FL, showed consistently higher levels of CCR7, CXCR4, or CXCR5 than those pathologies with little or minimal lymphadenopathy such as SMZL, HCL, and MM, which showed no expression or very low levels of CCR7 and CXCR5 and low to moderate levels of CXCR4. Low to moderate levels of these molecules were also observed in precursor B-ALL, which presented no prominent lymphadenopathy in two of the six patients included in the study (data not shown). No significant differences were found on the levels of expression of the chemokine receptors in a given pathology when considering different cell sources (PB, BM, or lymph node biopsies).

To verify a possible relationship between B cell neoplasms and phenotypically related normal B cells, we analyzed chemokine receptor expression on different B cell subsets from PB, BM, and tonsils. We found that normal CD5+ B lymphocytes expressed consistently higher levels of CCR7 than the rest of B cells (**Fig. 2a** and Table 1). Conversely, the intensity of CCR7 expression in malignant CD5+ cells from B-CLL and MCL was even stronger than that of normal CD5+CD19+ B lymphocytes [301(176,577) and 100(53,143) vs. 68(51,72; Table 1)]. CXCR4 and CXCR5 expression was slightly stronger on normal CD5+ B cells than on the global B cell population (Fig. 2a and Table 1). As with CCR7, most B-CLL overexpressed CXCR4 and CXCR5 with a MFI that was twofold and fourfold higher, respectively, than that of normal CD5+ B lymphocytes [410(248,631) vs. 230(193,415) for CXCR4 and 474(279,838) vs. 123(101,143) for CXCR5].

Tonsillar CD10+CD38+ germinal center B cells, which are postulated to be the normal origin cell of CD10+ FL, expressed consistently lower CCR7 than the global B cell population (Fig. 2b and Table 1). Similar to CD5+ B neoplasms, CD10+ FL expressed significantly higher levels of CCR7 than normal CD10+CD38+ germinal center-derived B lymphocytes [47 (42,117) vs. 21(16,22)]. The MFI of CXCR4 and CXCR5 of germinal center-derived B cells was similar and higher, respectively, than that of the rest of B lymphocytes (Fig. 2b and Table 1). The intensity of expression of CXCR4 and CXCR5 of MCL and FL presented notable variability (Table 1) with a range of MFI which includes that of their related normal B lymphocytes.

Expression of CCR7, CXCR4, and CXCR5 was similar between normal BM-derived precursor B cells and B-ALL cells as well as between normal BM-derived plasmatic cells and MM cells (data not shown).

Chemotaxis pattern of different B cell populations is related to the levels of chemokine receptor expression

The expression data provide evidence for the role of CCR7, CXCR4, and CXCR5 in determining the pattern of lymphoid organ involvement of B lymphoproliferative disorders. Therefore, we next performed the functional characterization of these chemokine receptors.

First, we analyzed whether the in vitro chemotactic pattern of different B cell populations, including B-CLL, MM, and normal PB B cells, was related to its levels of chemokine receptor expression. The chemotactic effect of CCL19, CCL21, CXCL13, CXCL12, and CCL5 was analyzed in Transwell chamber assays.

MM cells, which present very low or negative expression of CCR7 and CXCR5, did not migrate in response to their ligands CCL19, CCL21, and CXCL13. By contrast, these cells migrate in response to CXCL12, whose receptor CXCR4 is expressed

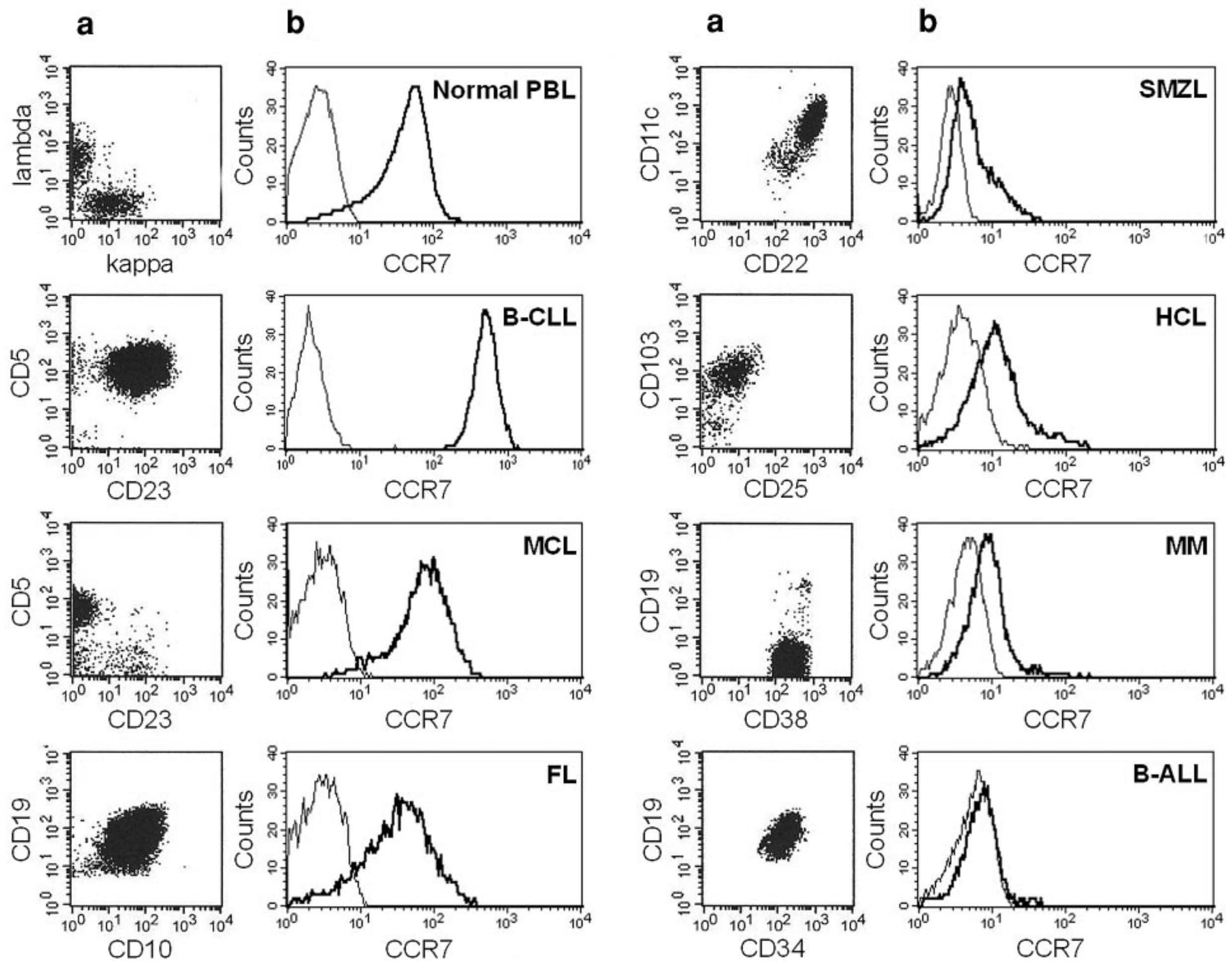

Fig. 1. Expression of CCR7 in different B cell malignancies. Surface expression of CCR7 was analyzed by flow cytometry in different B cell neoplasms. (a) The gated tumoral population that was subsequently analyzed for CCR7 expression in b. Thin lines correspond to the staining with an irrelevant isotype-matched antibody. Data correspond to representative cases of the different B cell malignancies studied. PBL, PB lymphocytes (n=10); B-CLL (n=91); MCL (n=15); FL (n=16); SMZL (n=8); HCL (n=9); MM (n=30); B-ALL (n=6).

TABLE 1. Expression of CCR7, CXCR4, or CXCR5 Is Significantly Higher in B Cell Neoplasms with Nodular Dissemination Than in Those with Minimal Lymphadenopathy

a. Malignant B cells	CCR7	CXCR4	CXCR5
B-CLL (*n*=91)	301 (176; 577)	410 (248; 631)	474 (279; 838)
MCL (*n*=15)	100 (53; 143)	173 (118; 289)	229 (77; 470)
FL (*n*=16)	47 (42; 117)	215 (82; 930)	98 (65; 381)
SMZL (*n*=8)	19 (7; 24)	17 (17; 50)	49 (7; 80)
HCL (*n*=9)	32 (21; 47)	36 (29; 62)	19 (14; 34)
MM (*n*=30)	11 (6; 22)	54 (26; 127)	17 (8; 24)
	P = 0.086*	P = 0.025	P = 0.076**
b. Normal B cells	**CCR7**	**CXCR4**	**CXCR5**
CD5+ B cells (*n*=5)	68 (51; 72)	230 (193; 415)	123 (101; 143)
CD10+ B cells (*n*=5)	21 (16; 22)	322 (204; 485)	210 (99; 312)
Total B cells (*n*=5)	29 (28; 30)	191 (92; 276)	98 (72; 125)

(a) The intensity of expression of chemokine receptors in B cell neoplasms with nodular dissemination (B-CLL, MCL, and FL) was compared with that of B cell neoplasms with little or no lymph node enlargement (SMZL, HCL, and MM) by using the Scheffé post-hoc test. (b) The intensity of expression of chemokine receptors was analyzed in tonsillar normal B cells. Medians and interquartile ranges of the CCR7, CXCR4, and CXCR5 MFI are shown for each pathology or normal B subpopulation. * P resulted higher than 0.05, as CCR7 MFI from FL cells was not statistically higher than CCR7 MFI from HCL cells. ** P resulted higher than 0.05, as CXCR5 MFI from FL cells was not statistically higher than CXCR5 MFI from SMZL cells.

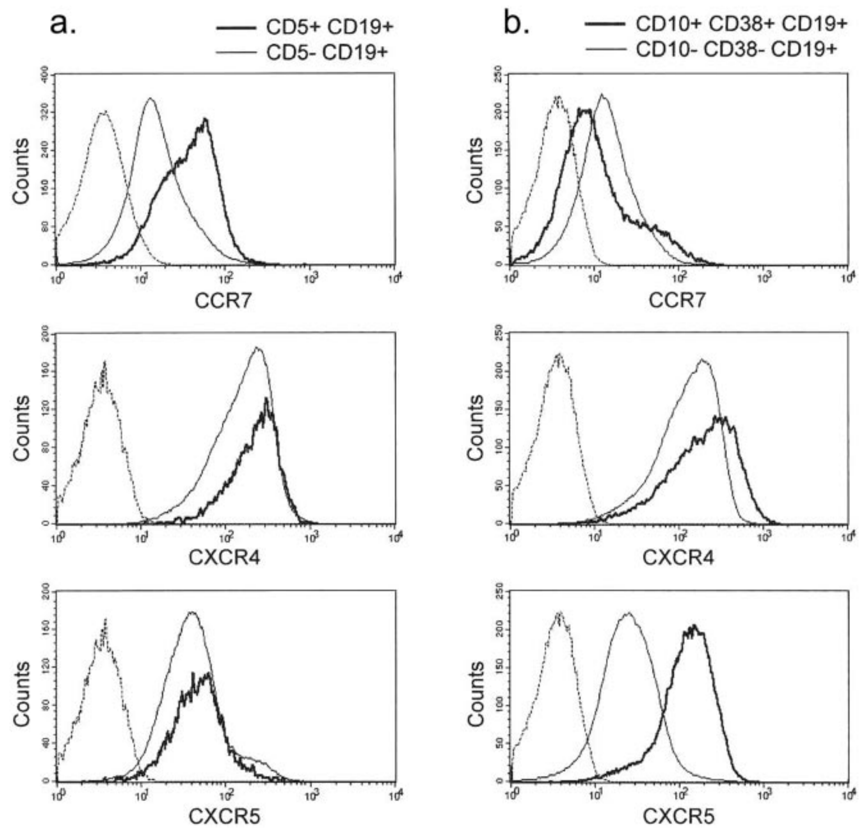

Fig. 2. Levels of CCR7, CXCR4, and CXCR5 in normal tonsillar B cell subpopulations. (a) Chemokine receptor expression in CD5+CD19+ lymphocytes (thick line) and CD5–CD19+ lymphocytes (thin line). (b) Chemokine receptor expression in germinal center-derived CD10+CD38+CD19+ lymphocytes (thick line) and CD10–CD38–CD19+ lymphocytes (thin line). Dotted lines correspond to the isotype-matched antibody. A representative experiment out of five is shown.

on their surface at low or moderate levels (**Fig. 3a**). Normal PB B lymphocytes showed a chemotactic response to the four chemokines, thus indicating that their respective receptors are functionally active (Fig. 3a).

Regarding B-CLL, the overexpression of CCR7, CXCR4, and CXCR5 may explain the widespread lymphoid organ dissemination that characterizes this malignancy. We found that B-CLL cells efficiently migrate in response to all four chemokines with donor-to-donor variability but with higher migration indexes than those of normal PB B lymphocytes (Fig. 3b). Dose-response experiments showed that optimal chemokine concentrations were the same for normal and B-CLL cells: 1 μg/ml in the case of CCL19 and CCL21, 2 μg/ml for CXCL13, and 100 ng/ml for CXCL12 (Fig. 3c and data not shown). The chemotactic efficacy of CCL19 and CCL21 was similar in the majority of B-CLL cases. The migration indexes induced by CXCL12 and CXCL13 were usually lower than those obtained with CCL19 or CCL21. As a negative control, CCL5, an inflammatory chemokine that binds to CCR1, CCR3, and CCR5, did not induce migration of B-CLL cells in any of the experiments performed (Fig. 3b).

A different approach to assess the responsiveness to chemokines is the study of the actin skeleton (F-actin) reorganization (Fig. 3d). We found that CCR7 ligands and CXCL12 induced a significant increase of intracellular F-actin, which was maximum at concentrations of 1 μg/ml for CCL19 and CCL21 and at 100 ng/ml for CXCL12. No effect on actin polymerization was observed with CCL5 (10 ng/ml).

Chemokines usually induce down-regulation of their receptor's expression to regulate their activity. Therefore, we assessed whether binding of CCL19 and CCL21 induced internalization of CCR7 on B-CLL cells (Fig. 3e). A down-modulation of CCR7 was observed 5 min after the stimulation with CCL19 (1 μg/ml), reaching a maximum of 55% ± 10 of internalization at 30 min. Higher concentrations of CCL19 did not induce a further down-modulation of CCR7. In contrast, CCL21 (1 μg/ml) had a very weak effect on CCR7 down-regulation with a maximum loss of surface expression of 20% in every case analyzed. No further uptake of CCR7 was observed by increasing the concentration of the chemokine or the incubation time (Fig. 3e and data not shown). It is very possible that this phenomenon is reflecting that cells, which have been extravasated by the action of CCL21, need to maintain their responsiveness to CCL19 to migrate toward the T cell zone of lymph nodes, as it has been proposed for T lymphocytes [41]. As a control, CXCL12, at doses ranging from 10 ng/ml to 2.5 μg/ml, had no effect on CCR7 expression (Fig. 3e). Conversely, CXCL12 at 100 ng/ml induced maximum internalization of CXCR4 (data not shown).

The ability of B-CLL cells to migrate in response to CCR7 ligands correlates with clinical lymphadenopathy but not with mutational status or CD38 expression

The functional data suggested a possible correlation between the migratory ability of B-CLL cells and the presence of clinical lymphadenopathy in these patients. To analyze this issue, we measured the migration index of B-CLL cells in response to CCL19, CCL21, and CXCL12 in a group of 25 patients: Ten cases presented no clinical lymphadenopathy (Rai stage 0), whereas 15 patients presented clinical nodal enlargement (this group included patients with Rai stages from

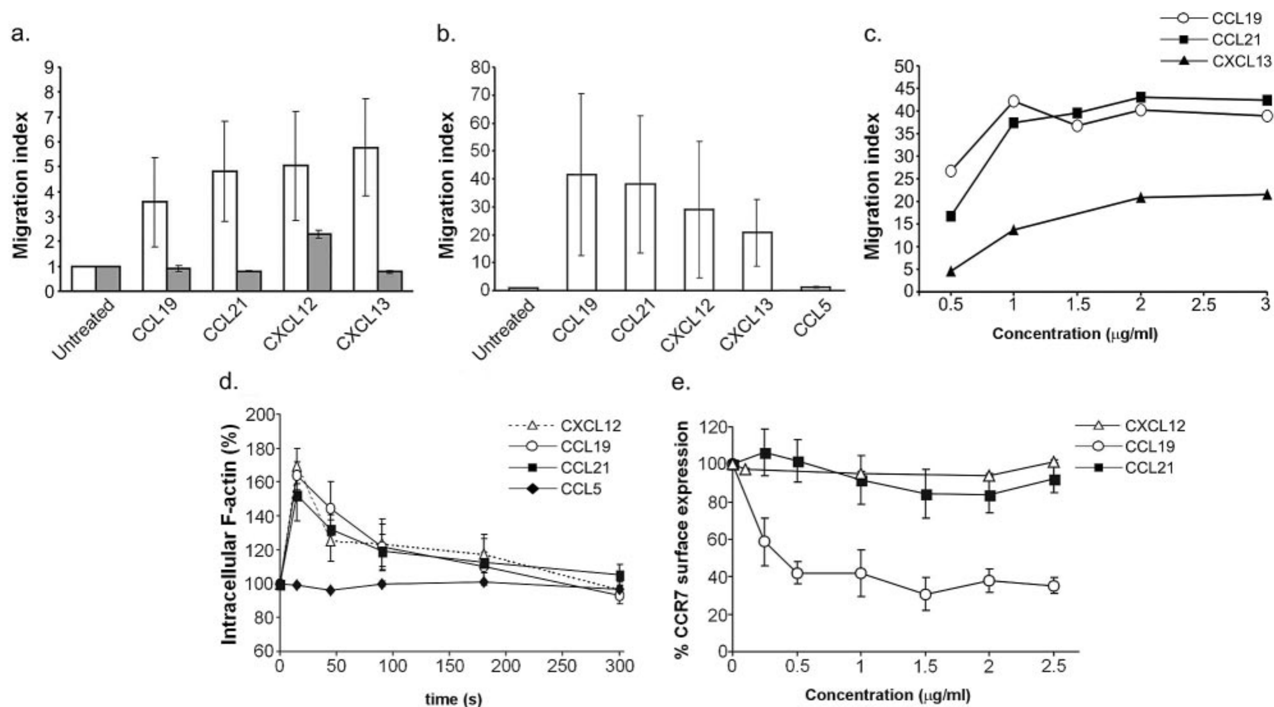

Fig. 3. Functional characterization of CCR7, CXCR4, and CXCR5. (a) Chemotactic responses to CCR7, CXCR4, and CXCR5 ligands of normal PB B lymphocytes and MM cells. Chemotaxis experiments were performed as described in Materials and Methods. The arithmetic mean and one standard deviation error bars of four independent experiments are shown. (b) Chemotactic responses to CCR7, CXCR4, and CXCR5 ligands of B-CLL cells. Chemotaxis experiments were performed as described in Materials and Methods. The arithmetic mean and one standard deviation error bars of 25 independent experiments are shown. (c) Chemotactic responses of B-CLL cells to different concentrations of CCL19, CCL21, and CXCL13. A representative experiment is shown. (d) CCL19, CCL21, and CXCL12 induce actin polymerization on B-CLL cells, which were incubated with chemokines at 37°C for different periods of time. Reorganization of the actin cytoskeleton in response to CCR7 and CXCR4 ligands was quantified by using Alexa 488 phalloidin, as described in Materials and Methods. (e) CCL19 but not CCL21 induces internalization of CCR7. B-CLL cells were incubated with increasing concentrations of CCL19, CCL21, and CXCL12 for 1 h at 37°C, and then, surface expression of CCR7 was determined by flow cytometry on CD19 + CD5 + B-CLL cells as described in Materials and Methods.

I to IV). Migration index of B-CLL cells in response to CCL19 and CCL21 was significantly higher in patients with clinical lymphadenopathy than in those without it ($P<0.05$; **Fig. 4a**). No correlation was found between migration index to CXCL12 and lymph node enlargement (data not shown). As the migratory effects of CCL21 and CCL19 are mediated by CCR7 and as a result of the variable expression of this chemokine receptor on B-CLL cells, we analyzed whether the level of CCR7 expression correlated with clinical lymphadenopathy in the same group of patients. We did not find correlation between the intensity of CCR7 expression and the presence of clinical lymph node enlargement ($P=0.5$; data not shown). Similar results were obtained when a greater number of patients comprising 33 and 37 subjects with and without lymphadenopathy, respectively, were analyzed ($P=0.22$; Fig. 4b). The same comparison was performed for CXCR4 and CXCR5 MFI obtaining similar results ($P=0.31$ and $P=0.9$; Fig. 4b).

The correlation between the migratory ability of B-CLL cells and prognostic factors of the disease, defined by the mutational status of the IgV_H or CD38 expression [42, 43], was examined on the former sample of 25 patients. With respect to the IgV_H mutational status, 13 cases (52%) displayed more than 98% sequence homology with the nearest germline IgV_H gene (unmutated), whereas the remaining 12 cases (48%) showed less than 98% homology with evidence of somatic hypermutation. There was no association between the mutational status of B-CLL cells and their migration index ($P=0.9$ for CCL19, and $P=0.6$ for CCL21; data not shown). Similar results were obtained when IgV_H mutational status was compared with the intensity of expression of CCR7 ($P=0.6$), CXCR4 ($P=0.9$), or CXCR5 ($P=0.1$; data not shown).

Regarding the expression of CD38, this molecule was considered positive when more than 20% of the B-CLL cells expressed it. Ten B-CLL samples were CD38+, and 15 samples were CD38–. Similar to the IgV_H mutational status, we did not find any correlation between CD38 expression and migration indexes to CCL21 ($P=0.9$) or CCL19 ($P=0.7$; data not shown). The correlation between CD38 expression and the intensity of expression of the chemokine receptors was investigated in a larger group of 70 cases. The intensity of expression of CCR7, CXCR4, or CXCR5 did not correlate with the expression of CD38 ($P=0.65$, $P=0.43$, and $P=0.17$, respectively; data not shown).

DISCUSSION

CCR7 is thought to be the main chemokine receptor involved in the entry of B cells into the lymph nodes. CXCR4 also contributes to this entry, playing a partially redundant role with

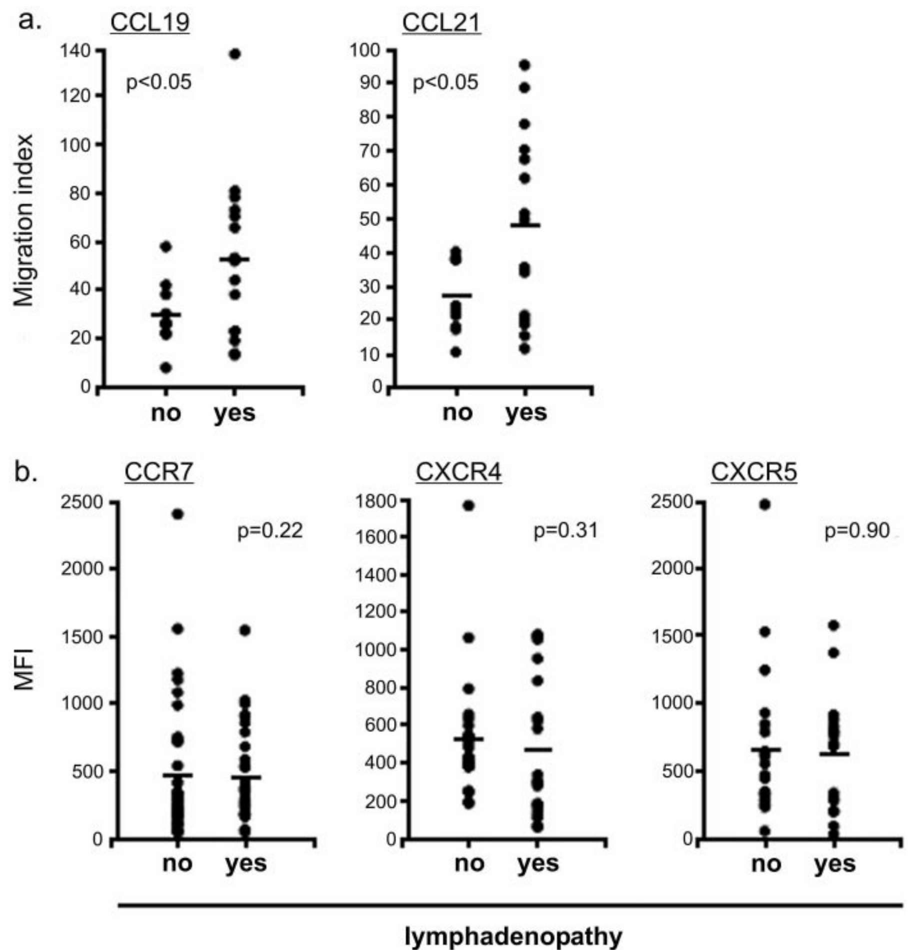

Fig. 4. Migration indexes to CCL21 and CCL19 but not the intensity of the expression of CCR7, CXCR4, or CXCR5 correlate with clinical lymphadenopathy. (a) Migration indexes to CCL19 and CCL21 were measured in a group of 25 patients comprising 15 cases with clinical lymphadenopathy and 10 cases without it. (b) CCR7, CXCR4, and CXCR5 MFI were measured in a group of 70 patients comprising 33 cases with clinical lymphadenopathy and 37 cases without it. The bar represents the average migration index (4a) or MFI (4b) for each patient group. no/yes, Patients without and with clinical lymphadenopathy, respectively.

that of CCR7. This also may be the case for CXCR5, whose main function is to guarantee the accumulation of B cells within the follicles of the lymph node [10, 11, 14, 44], hence the importance of analyzing CCR7 expression in B cell neoplasms with different patterns of lymph node involvement, separately or simultaneously with CXCR4 and CXCR5 expression. To date, no studies have addressed this issue.

The data reported here demonstrate that the intensity of expression of CCR7, CXCR4, or CXCR5 is significantly higher in those B cell neoplasms with a wide nodular dissemination pattern, including B-CLL, MCL, or FL, compared with B cell neoplasms, such as MM, SMZL, or HCL, which present with little or minimal lymph node involvement. Therefore, the expression of certain levels of functional CCR7, CXCR4, or CXCR5 in all probability guarantees the entry to the lymph nodes and the nodular dissemination character of some pathologies such as B-CLL, MCL, or FL. By contrast, B cell neoplasms with low or negative levels of these chemokine receptors would have little or no possibility to enter into the secondary lymphoid tissues.

In this regard, migration indexes of B-CLL cells in response to CCR7 ligands are related to clinical lymphadenopathy as shown in Figure 4a. However, there is a certain overlap between the positive and negative lymphadenopathy group. To better discuss this apparent discrepancy, it is important to highlight that nodal dissemination and clinical lymphadenopathy are related but not synonymous concepts. Thus, a nodal dissemination confirmed by histologic examination of the lymph node is possible in the absence of evident clinical lymphadenopathy, which is defined by an enlargement of the lymph node greater than 1 cm in diameter. Indeed, lymph nodes from B-CLL patients are almost always infiltrated, as referred to by the treatises on histopathology [45], but a substantial proportion of patients doesn't show clinical lymphadenopathy. Therefore, we propose that the capability of B-CLL cells to migrate in response to CCR7 ligands enables B-CLL cells to enter into the lymph nodes, but additional factors are probably involved in the development of clinical lymphadenopathy in certain patients including the differential expression of CCL19, CCL21, CXCL12, and CXCL13 in the lymph node; differences in the affinity of chemokine receptors and in the signaling evoked by the chemokines; differences in the expression of certain adhesion molecules in B-CLL cells; or differences in the proliferative ratio of the tumoral cells. Thus, the expression of certain chemokine receptors allows tumoral cells to enter into the lymph node, whereas additional factors influence the magnitude of the tumoral size.

The most obvious mechanism explaining the different migration indexes to CCR7 ligands is a differential expression of CCR7. In the majority of cases, patients with higher CCR7 expression presented a greater in vitro migratory response. However, we did not find a correlation between clinical lymphadenopathy and the intensity of expression of the chemokine receptors analyzed. These data contrast with those reported by

Till et al. [37], who showed that MFI of CCR7 correlated with the presence of clinical lymph node enlargement. It is possible that differences in the sample size or experimental procedures could explain this discrepancy. It may be relevant that we used a directly PE-conjugated anti-CCR7 antibody, whereas these authors used an indirect staining technique using a purified anti-CCR7 antibody that was revealed with a fluorescein isothiocyanate-conjugated goat antimouse Ig. Thus, the range of CCR7 MFI was much wider in our study. Furthermore, as mentioned above, the expression of certain chemokine receptors determines the disseminated character of B-CLL, but other factors, including differences in the affinity of chemokine receptors and in the signaling evoked by the chemokines, may be implicated in the development of clinical lymph node enlargement.

The tumoral dissemination is often related to the prognosis of the neoplasms. Therefore, we were also interested in a possible correlation between the migratory capability of B-CLL cells and certain prognostic factors of recent definition, such as the surface expression of CD38 molecule and the IgV_H mutational status of B-CLL cells. Patients whose B-CLL cells express CD38 or present unmutated IgV_H genes have a poorer prognosis than patients with CD38-negative B-CLL cells or patients with mutated IgV_H genes, respectively. We found that neither the intensity of expression of CCR7, CXCR4, or CXCR5 nor the migration indexes to CCR7 and CXCR4 ligands correlate with the IgV_H mutational status or with CD38 expression, in agreement with Till et al. [37]. Therefore, it seems unlikely that there is an association between the migratory capability of B-CLL cells and the biological variables that underlie the poorer prognosis of the B-CLL patients with unmutated IgV_H genes or CD38+ cells. These results indicate that the high expression of functional CCR7, CXCR4, and CXCR5 characterizes the cells of most B-CLL patients, defining a common B-CLL phenotype that allows the vast majority of B-CLL cases to disseminate into secondary lymphoid tissues with independence of certain prognostic indicators. Whether the different migratory capability of B-CLL cells would constitute an independent prognostic factor to the final outcome of the patient cannot be concluded from the current data, as our study was not designed with this objective, and a different kind of study would be required.

Of greater clinical interest is that the up-regulation of CCR7, CXCR4, and CXCR5 observed in almost all B-CLL samples makes of these molecules potential new therapeutic targets for all B-CLL patients regardless of the prognosis.

Lymphadenopathy may be present in up to 20–45% of the patients with B-ALL, which expresses low levels of CCR7 and CXCR5 and moderate expression of CXCR4 [46–48]. This would seem contradictory to the hypothesis proposed in our study. However, the lymphadenopathy of precursor B-ALL is seldom striking, and its incidence is much lower than in B-CLL, MCL, or FL, which often present with widespread central and peripheral lymphadenopathy. Again, differences in the affinity of chemokine receptors or a differential expression of adhesion molecules may explain the higher incidence of lymphadenopathy in B-ALL compared with other B cell lymphoproliferative disorders such as HCL or MM, which also present low CCR7, CXCR4, and CXCR5. The L-selectin molecule is a possible candidate to explain these differences, as its expression is particularly low in HCL, SMZL, and MM, whereas it is present in a moderate percentage of B-ALL blasts [49–53].

The high expression of CCR7, CXCR4, and CXCR5 observed in B cell neoplasms with widespread lymphadenopathy could be related to the oncogenic transformation process or to a recapitulation of the levels of chemokine receptors found on their postulated normal cell of origin, as it is known that chemokine responsiveness and chemokine receptor expression change during normal B cell differentiation and maturation [15, 21, 22, 54–56]. Thus, normal precursor B cells express very low or negative levels of CCR7 and CXCR5 and moderate levels of CXCR4. Peripheral and tonsillar B cell subpopulations express all the three chemokine receptors with the exception of germinal center cells, which are almost CCR7-negative. Plasmatic cells show little or no expression of CCR7 and CXCR5 and moderate levels of CXCR4. Our data suggest that the pattern of chemokine receptor expression on B cell neoplasms parallels that of their postulated normal counterparts, although we found that most of B-CLL, MCL, and FL expressed significantly higher levels of CCR7, CXCR4, or CXCR5 than their supposed cell of origin. This fact may be related to the oncogenic process or to other processes ongoing in the tumoral lymph node such as an inflammatory reaction or the antigenic stimulation of the malignant clone. In this regard, our protein expression data confirm a recent study by Klein et al. [57] showing that CCR7 mRNA levels in B-CLL are higher than in any normal B cell subpopulation, including CD5+ as well as naive and memory B lymphocytes.

Practical conclusions from our study include that the characterization of chemokine receptor expression can represent a useful diagnostic tool, particularly in the case of atypical CD5-negative B-CLL and SMZL, which have no distinctive phenotypical characteristics. Thus, the presence of high levels of CCR7, CXCR4, and CXCR5 molecules distinguishes atypical B-CLL from most other B lymphoproliferative syndromes. More important, these molecules can represent novel therapeutic targets in those B cell malignancies that express any of these chemokine receptors.

Further studies to assess how the oncogenic transformation modifies the migratory ability of lymphoid neoplasias will be of interest.

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