

Down-regulation of CXCR4 and CD62L in Chronic Lymphocytic Leukemia Cells Is Triggered by B-Cell Receptor Ligation and Associated with Progressive Disease

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Abstract

Progressive cases of B-cell chronic lymphocytic leukemia (CLL) are frequently associated with lymphadenopathy, highlighting a critical role for signals emanating from the tumor environment in the accumulation of malignant B cells. We investigated on CLL cells from 30 untreated patients the consequence of B-cell receptor (BCR) triggering on the membrane expression of CXCR4 and CD62L, two surface molecules involved in trafficking and exit of B-lymphocytes from lymph nodes. BCR stimulation promoted a strictly simultaneous down-regulation of CXCR4 and CD62L membrane expression to a variable extent. The variable BCR-dependent decrease of the two proteins was strikingly representative of the heterogeneous capacity of the CLL cells to respond to BCR engagement in a given patient. Functionally, cells down-regulating CXCR4 and CD62L in response to BCR engagement displayed a reduction in both migration toward CXCL12 and adhesion to lymphatic endothelial cells. Remarkably, the ability of CLL cells to respond to BCR ligation was correlated with unfavorable prognostic markers and short progression-free survival. In conclusion, BCR signaling promotes decrease of CXCR4 and CD62L membrane expression in progressive cases only. These results are consistent with the hypothesis that BCR-mediated signaling pathways favor accumulation of a proliferative pool within the lymph nodes of progressive CLL cases. [Cancer Res 2009;69(16):6387–95]

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course. Aggressive CLLs are associated with enlarged lymph nodes (LN) and splenomegaly resulting from the accumulation of malignant B lymphocytes within secondary lymphoid organs (1, 2). This indicates that the recirculation process of CLL cells from blood to lymphoid tissues might be crucial for CLL cells accumulation and survival and highlights the predominant role of the tumor microenvironment in the pathogenesis of CLL (3, 4).

CLL microenvironment contains several cytokines acting through autocrine/paracrine mechanisms to affect CLL cell survival, migration, and resistance to drug-induced apoptosis (5, 6). Among them, the CXCL12 chemokine and its receptor CXCR4 seem to play an important role for homing of CLL cells into the bone marrow but also for CLL cell survival through cell-to-cell contacts with marrow stromal and/or nurse-like cells (7–9). Moreover, CXCR4-specific antagonists suppressed the protective effect of stromal cells on leukemic cell survival and drug-induced apoptosis (10, 11), thus highlighting the rationale for targeting the CXCR4/CXCL12 axis in CLL (12). Recent evidence indicates that CXCR4-CXCL12 interactions also play an important role in directing the movements of lymphoid cells within secondary lymphoid organs (13). CXCR4 expression on B-cells is essential for the organization of germinal center and for centroblast migration into the CXCL12-rich dark zone (14). On the other hand, the process of B-lymphocyte emigration from LNs and spleen is controlled in part via CXCR4-CXCL12 interactions. CXCR4 is critical for normal homing of activated B lymphocytes to the spleen red pulp and the LNs medullary cords, two CXCL12-enriched zones through which B lymphocytes egress from lymphoid organs and return to circulation (15, 16). During homing, B lymphocytes adhere then cross the sinusoidal endothelium before entering the efferent lymphatic vessel and exit from LNs. Recently, CD62L (L-selectin) was identified as crucial for B-lymphocyte binding to sinusoidal endothelial cells through interaction with mannose receptor (17). Thus, CD62L and its lymphatic receptor may participate in the mechanisms mediating lymphocytes exit from LNs.

B-cell receptor (BCR) activation is critical for B-lymphocyte development, proliferation, survival, and mobility within LNs (18). BCR activation could play a central role in CLL by participating in the selection and the expansion of the malignant clone (19, 20). Our group and others have recently reported that sustained activation of BCR with immobilized anti-IgM antibodies promoted *in vitro* CLL cell survival (21, 22) and that this survival advantage is restricted to a subset of patients with progressive disease and unfavorable prognostic factors (22). Considering the emerging role of BCR activation in CLL and the potential involvement of CXCR4 and CD62L in the physiologic B-lymphocyte recirculation process, we hypothesized that BCR stimulation may contribute to the accumulation of CLL cells in secondary lymphoid organs by regulating CXCR4 and CD62L membrane expression. We therefore explored *in vitro* the impact of BCR activation on cell surface expression of CXCR4 and CD62L. Our data show that sustained anti-IgM stimulation of CLL cells promoted a concomitant down-regulation of CXCR4 and CD62L only in a subset of patients with unfavorable prognostic markers and a risk of disease progression. Moreover, this

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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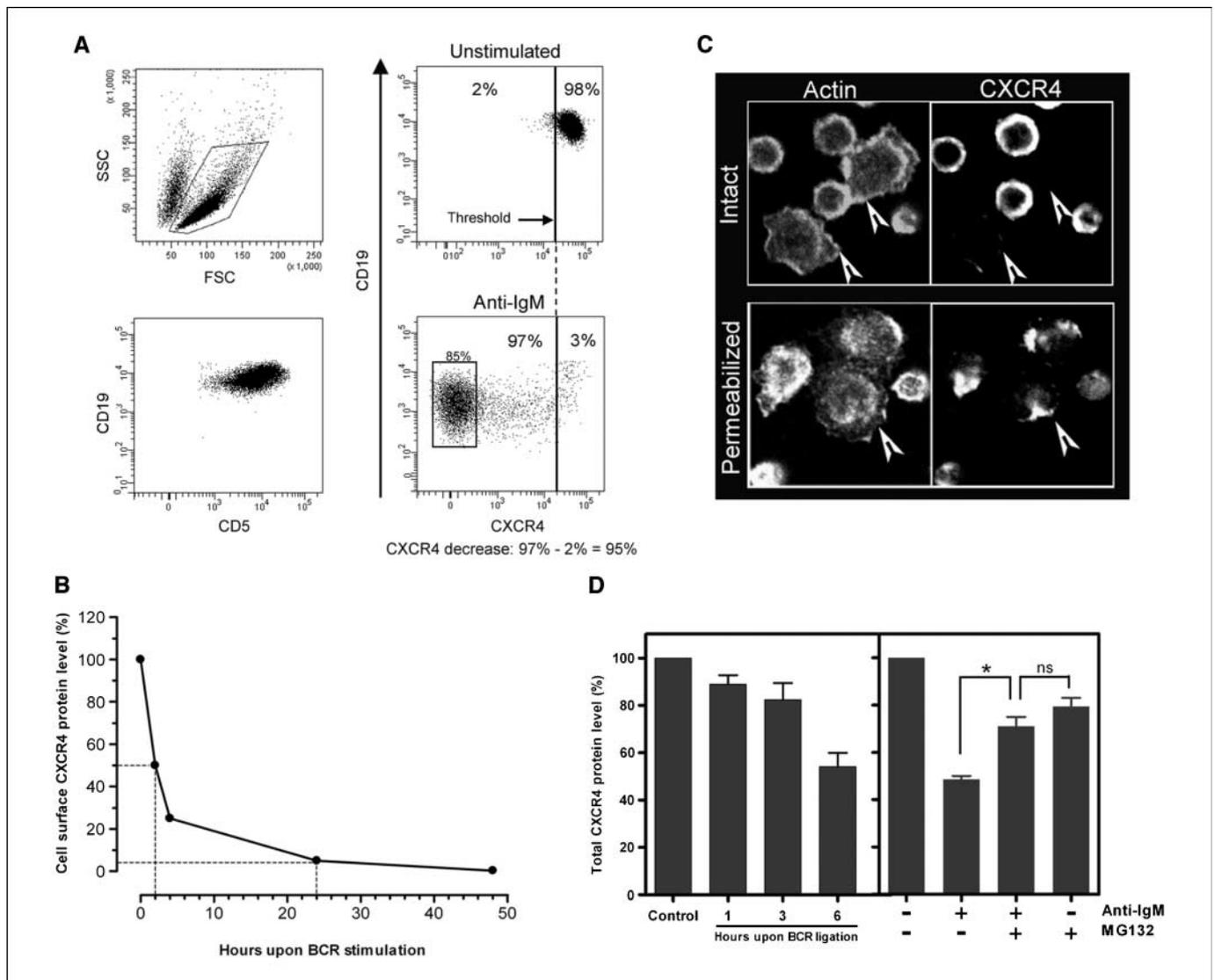


Figure 1. BCR stimulation of CLL cells leads to down-regulation of CXCR4 membrane expression. *A*, a representative flow cytometry analysis is shown (UPN 17). Freshly isolated lymphocytes were gated in a FCS/SSC dot plot and CD19/CD5 coexpression was analyzed indicating the purity of malignant cells (*left*). CLL cells were incubated for 48 h in the presence (*anti-IgM*) or absence (*unstimulated*) of immobilized anti-IgM antibodies and CXCR4/CD19 expressions were analyzed (*right*). The CXCR4 decrease represented the percentage of cells down-regulating CXCR4 upon BCR ligation. *B*, CLL cells were stimulated with anti-IgM and analyzed for their cell surface CXCR4 content by flow cytometry based on median fluorescence intensity. *C*, immunofluorescence analysis of CXCR4 down-modulation in response to BCR ligation. Cells were plated on anti-IgM-coated glass coverslips for 30 min and stained with phalloidin and anti-CXCR4 antibody either in intact or permeabilized cells. *Arrows*, BCR-activated cells (magnification, $\times 63$). *D*, CLL cells were subjected to anti-IgM stimulation for various period of time (*left*) or for 6 h in the presence of MG132 (5 $\mu\text{mol/L}$; *right*). Total CXCR4 content was analyzed in permeabilized cells by flow cytometry based on median fluorescence intensity ($n = 2$). *, $P < 0.05$; ns, not significant.

analysis reveals that progressive disease is closely linked to the proportion of CLL cells able to respond to BCR ligation in a given patient.

Materials and Methods

CLL B-cells samples. Peripheral blood mononuclear cells were freshly isolated from 30 previously untreated patients after informed consent and validation by the local research ethics committee from the Avicenne Hospital, in accordance with the Declaration of Helsinki. In this cohort, 27 patients were Binet stage A CLL, 3 were stage B or C (UPN 28,18, 25). CLL diagnosis was relied on typical morphology and RMH score of ≥ 4 immunophenotype.

Isolation of CLL cells and culture. Mononuclear cells were isolated from peripheral blood using Roset Sep kit and purity was verified by flow cytometry (T and natural killer cells, $< 2\%$). CLL cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 0.1 mg/mL

streptomycin, and 2 mmol/L L-glutamine. Upon BCR stimulation, CLL cells were cultured at 2×10^6 cells/mL in four-well plates coated with rabbit anti-IgM antibody (10 $\mu\text{g/mL}$; Jackson ImmunoResearch; ref. 22).

Flow cytometry analysis. Protein membrane expression was analyzed by flow cytometry (FACS-CANTO; Becton Dickinson) following labeling with the indicated antibody. Conjugated-antibodies included anti-CXCR4-APC (clone 12G5), anti-CD62L-FITC (clone SK11), anti-CD19-PerCPCy5.5 (clone SJ25C1), and anti-CD5-PECy7 (clone L17F12; BD Biosciences). Matching isotype monoclonal antibodies (IgG) conjugated to APC/FITC/PerCP/PE served as control (BD Biosciences). Lymphocytes were gated in a FCS/SSC dot plot. Almost 100% of gated lymphocytes were malignant CLL cells, as shown by the coexpression of CD19 and CD5 markers (see Fig. 1A). Data acquisition and analysis were performed using BD FACSDiva software. Overall CXCR4 protein content was measured by flow cytometry in CLL cells previously permeabilized using the Fix & Perm kit (Invitrogen).

The effect of BCR engagement on membrane CXCR4/CD62L protein expression was measured by determining the percentage of CLL cells present

below and above a threshold discriminating CXCR4/CD62L-negative and CXCR4/CD62L-positive cells, respectively. The threshold was arbitrarily set up case-by-case on unstimulated cells to include at least 95% positive cells at the left shoulder of the peak. The level of BCR-induced response was expressed as the percentage of cells decreasing CXCR4 or CD62L and will be called "CXCR4 decrease" or "CD62L decrease." This value was calculated as follows: (% of negative cells after anti-IgM stimulation) – (% of negative cells before anti-IgM stimulation). This type of quantitative analysis was chosen to take into account the cellular heterogeneity in the BCR response in a given patient.

Chemotaxis assay. Chemotaxis assays were performed as previously described (23). CLL cells (5×10^5) were either stimulated for 24 h with an anti-IgM or left unstimulated. Cells were resuspended in RPMI 1640 containing 0.5% bovine serum albumin and dispensed in the upper chamber of a Transwell culture insert (5 μ m pore size; Corning Costar). Inserts were then transferred into wells containing RPMI supplemented with CXCL12 (100 ng/mL). After incubation for 6 h, cells present in the upper and lower chambers were collected. Transmigrated CLL cells were then counted by fluorescence-activated cell sorting (FACS) for 1 min at 60 μ L/min to obtain a median of the relative number of total transmigrated cells. Cell surface CXCR4 expression of input cells, nonmigrated, and transmigrated cells was also analyzed by flow cytometry. All assays were performed in duplicate.

Detection of soluble CD62L by ELISA. Supernatants from CLL cells (2×10^6 /mL) either stimulated or not with an anti-IgM were collected and

concentrated (Amicon Ultra-4; Millipore). Soluble CD62L released into the supernatants were measured using a human sCD62L sandwich ELISA (Bender Medsystems).

In vitro adhesion assay of CLL cells to lymphatic endothelial cells. The *in vitro* adhesion assay was adapted from Akeson and colleagues (24). The murine endothelial cell line SVEC4-10 (American Type Culture Collection), which was recently characterized as lymphatic endothelial cells (25), was seeded at 1.5×10^5 cells per well onto Lab-Tek chamber slides (Nunc) in DMEM/10% FCS and allowed to adhere overnight. CLL cells either prestimulated with an anti-IgM (24 h) or unstimulated were labeled for 20 min at 37°C in RPMI with the fluorescent cell-permeant dye Cell Trace calcein green or Cell Trace calcein red-orange (Invitrogen), respectively. BCR-stimulated and unstimulated fluorescent cells (2.5×10^6 each) were mixed in RPMI 1640/10% FCS and added together onto the endothelial cell layer. After incubation for 2 h at 37°C, the nonadherent CLL cells were washed off. Remaining adherent cells were fixed, and 10 fields from duplicate chamber slides (average of 500 cells/field) were photographed under fluorescent microscope (magnification, $\times 10$). Red and green fluorescence were separately quantified using the Pixcavator IA 3.3 software (Intelligence Perception Co.). CLL cell-labeling efficiency using the two fluorescent dyes was measured both in BCR-stimulated and unstimulated cells. A high (90%) and identical labeling efficiency was observed in all tested combinations.

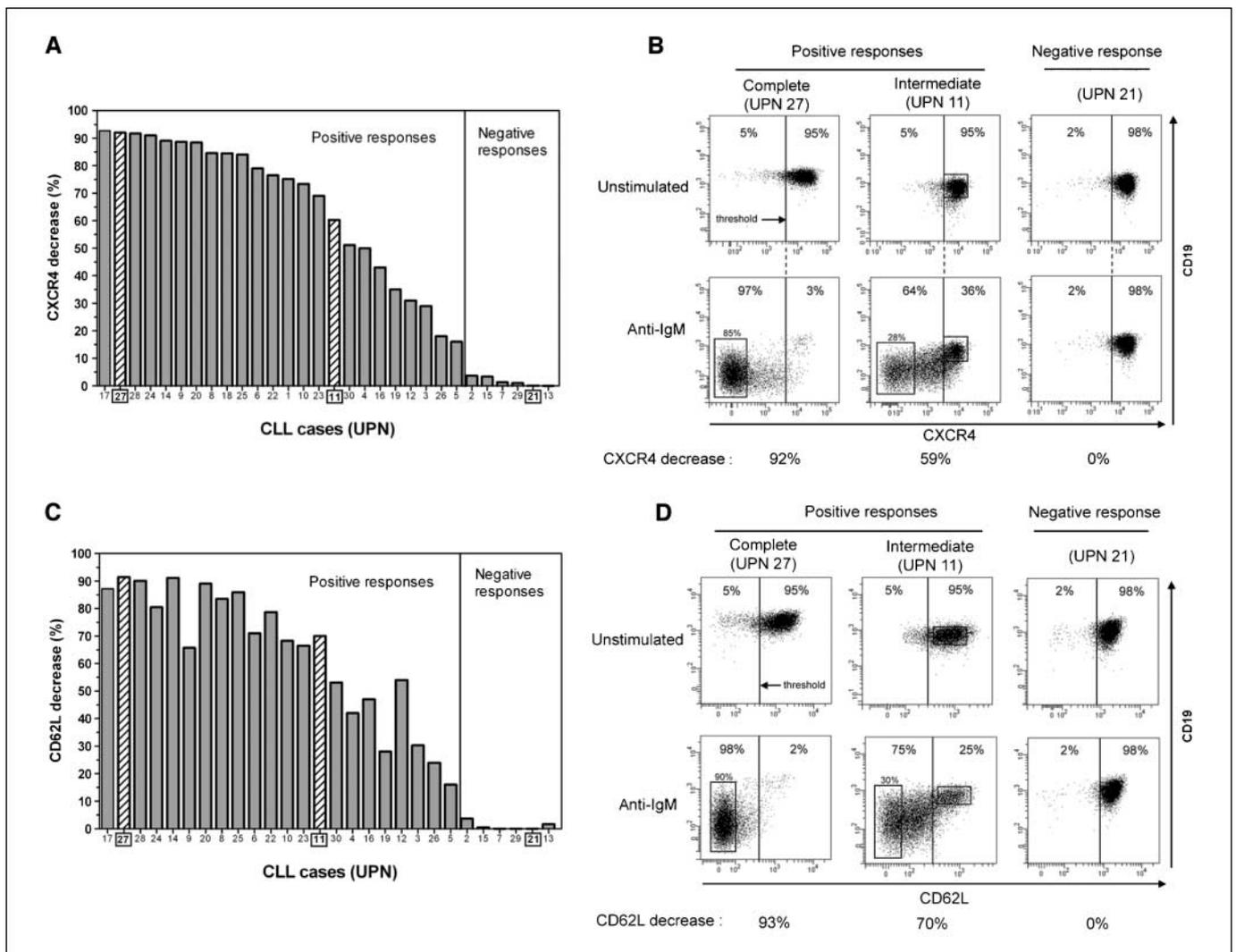


Figure 2. CXCR4 and CD62L down-regulation following BCR ligation is highly variable among CLL patients. CXCR4 decrease (A and B) and CD62L decrease (C and D) upon BCR engagement was measured on a series of CLL cases (48 h of anti-IgM stimulation). Representative flow cytometry analyses of different profiles of CXCR4 decrease (B) and CD62L decrease (D) are shown.

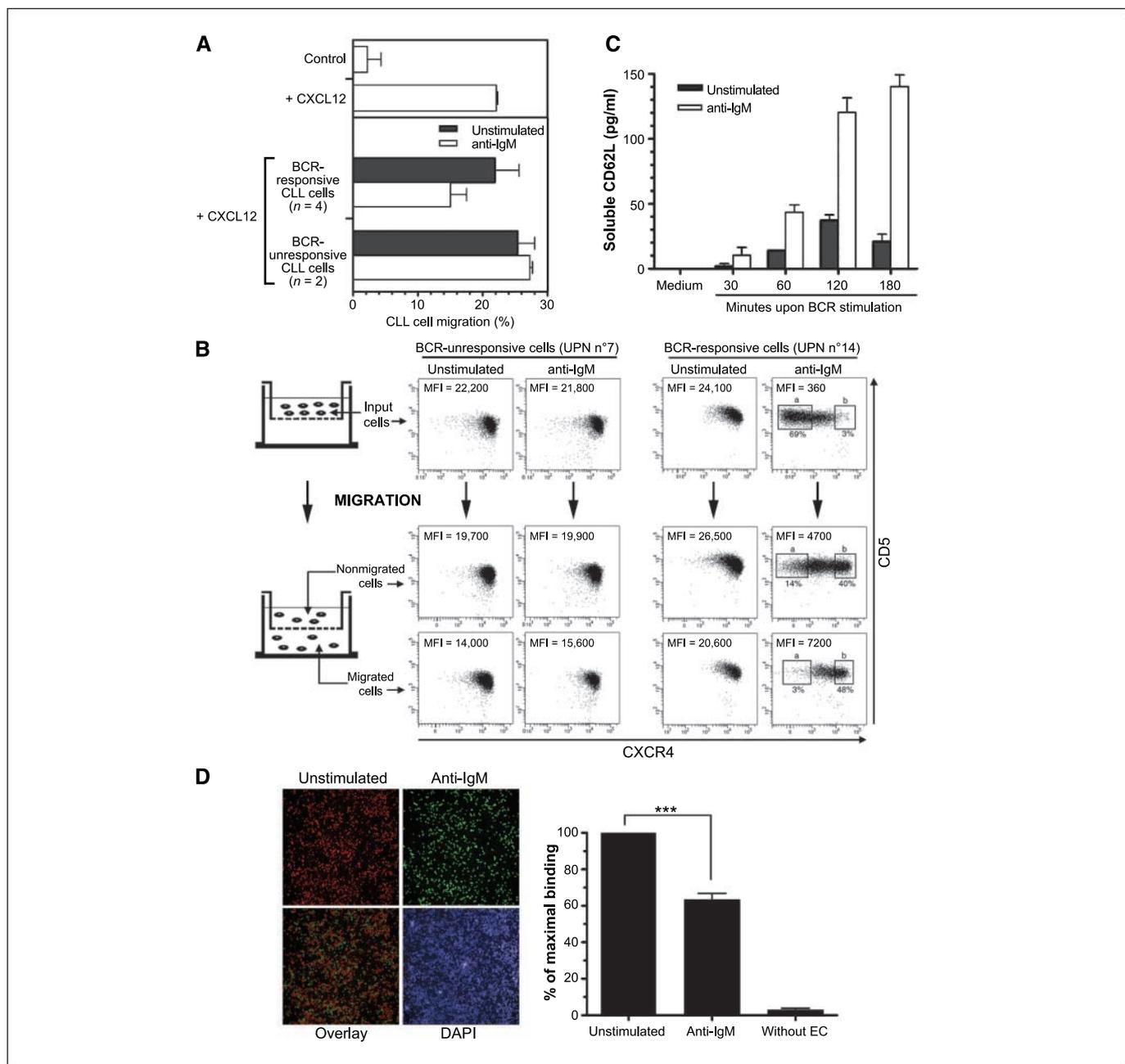


Figure 3. BCR engagement led to a reduction in both migration of CLL cells toward CXCL12 and adhesion to lymphatic endothelial cells. *A* and *B*, CLL cells either stimulated (24 h) or left unstimulated were subjected to migration assay for 6 h. *A*, transmigrated cells were counted to determine the percentage of the input cells that migrated into the lower chamber; *, $P = 0.02$. *B*, input, nonmigrated, and transmigrated cells were stained with anti-CXCR4 and anti-CD5 and analyzed by flow cytometry. FACS analyses of two representative BCR-induced responses are shown. *Inserts a* and *b*, delineated nonexpressing and reexpressing CXCR4 cells, respectively. *MFI*, median fluorescence intensity. *C*, CD62L is shed into supernatants from CLL cells during exposure to anti-IgM. *D*, an adhesion assay was performed to measure CLL cell binding to lymphatic endothelial cells. Unstimulated CLL cells (red fluorescence) and anti-IgM prestimulated (24 h) CLL cells (green fluorescence) were mixed, loaded in the same chamber slide onto the SVEC4-10 lymphatic endothelial cell layer, and allowed to adhere for 2 h. After washing, red and green fluorescent CLL cells were separately quantified as described in Materials and Methods. *Left*, a representative assay is shown (magnification, $\times 10$). *Right*, the results of three independent experiments are shown as mean percentage of maximal binding \pm SE. ***, $P < 0.0001$ using paired *t* test. Very few CLL cells adhere to the Lab-Tek plastic slide in absence of endothelial cell (without EC).

Immunofluorescence microscopy. Glass coverslips were coated overnight at 4°C with rabbit anti-human IgM antibody ($10\ \mu\text{g}/\text{mL}$). CLL cells were plated onto the coated surface and incubated at 37°C for 30 min. After fixation, cells were either left intact or permeabilized with 0.1% Triton and stained with biotin anti-human CXCR4 antibody (Clone 12G5; BioLegend) and streptavidin Alexa Fluor 647 conjugate (Invitrogen). Filamentous actin was stained with rhodamin-phalloidin (Sigma). Images

were acquired by confocal laser-scanning microscopy with high numerical aperture lens (63×2.8 ; Leica TCS SP2).

Statistics. When comparing data or CLL subgroups, *P* values were calculated using the unpaired *t* test (two-tailed, 95% confidence interval). For PFS study, statistical analyses were performed using the free software R (R Foundation for Statistical Computing, Vienna, Austria; ref. 26).

Results

BCR stimulation of CLL cells leads to down-regulation of CXCR4 membrane expression. The effect of BCR engagement on the cell surface expression of CXCR4 was investigated by flow cytometry on freshly isolated CLL cells after stimulation with immobilized anti-IgM for 48 h. Quantitative analysis of the BCR-induced response was performed as described in Materials and Methods. Anti-IgM stimulation induced a strong decrease of CXCR4 membrane expression (Fig. 1A). In the case highlighted, CXCR4 surface expression was decreased in 95% of the cells in response to BCR engagement (CXCR4 decrease, 95%). The strong down-regulation of CXCR4 was also associated with a concomitant but to a lesser extent, down-regulation of CD19 on the same subset of cells. As expected, the subset of cells down-regulating both CXCR4 and CD19 up-regulated both CD71 and CD23 expression (data not shown) due to the activation process resulting from BCR engagement (27, 28). The effect of immobilized anti-IgM on CXCR4 down-modulation was next compared with that of soluble anti-IgM. BCR stimulation with a soluble anti-IgM did not induce any change in cell surface CXCR4 level indicating that a continuous

antigenic stimulation, more representative of *in vivo* conditions, is likely to be necessary to induce this effect (Supplementary Fig. S1).

Down-regulation of CXCR4 in response to BCR ligation mainly occurs through a clathrin-mediated receptor internalization. We next investigated whether BCR-induced CXCR4 down-regulation could be attributed to an internalization process of the receptor. Kinetic studies indicated that ~50% of the cell surface CXCR4 down-regulation was achieved within the first 2 hours after BCR stimulation and maximal effect was reached after 24 hours of culture (Fig. 1B). BCR-induced cell surface decrease of CXCR4 was further analyzed by immunofluorescence on anti-IgM-coated coverslip. BCR-activated CLL cells were distinguished from inactivated cells using staining with phalloidin that highlighted actin-based cell spreading induced by clustering the BCR (29). After 30 minutes of stimulation, cell surface CXCR4 staining on intact cells was significantly decreased in spreading B cells compared with nonspreading/nonactivated cells (Fig. 1C, *top*). Concomitantly, in permeabilized cells, punctuated intracellular fluorescence increased markedly in spreading cells (Fig. 1C, *bottom*). Anti-IgM triggering effect

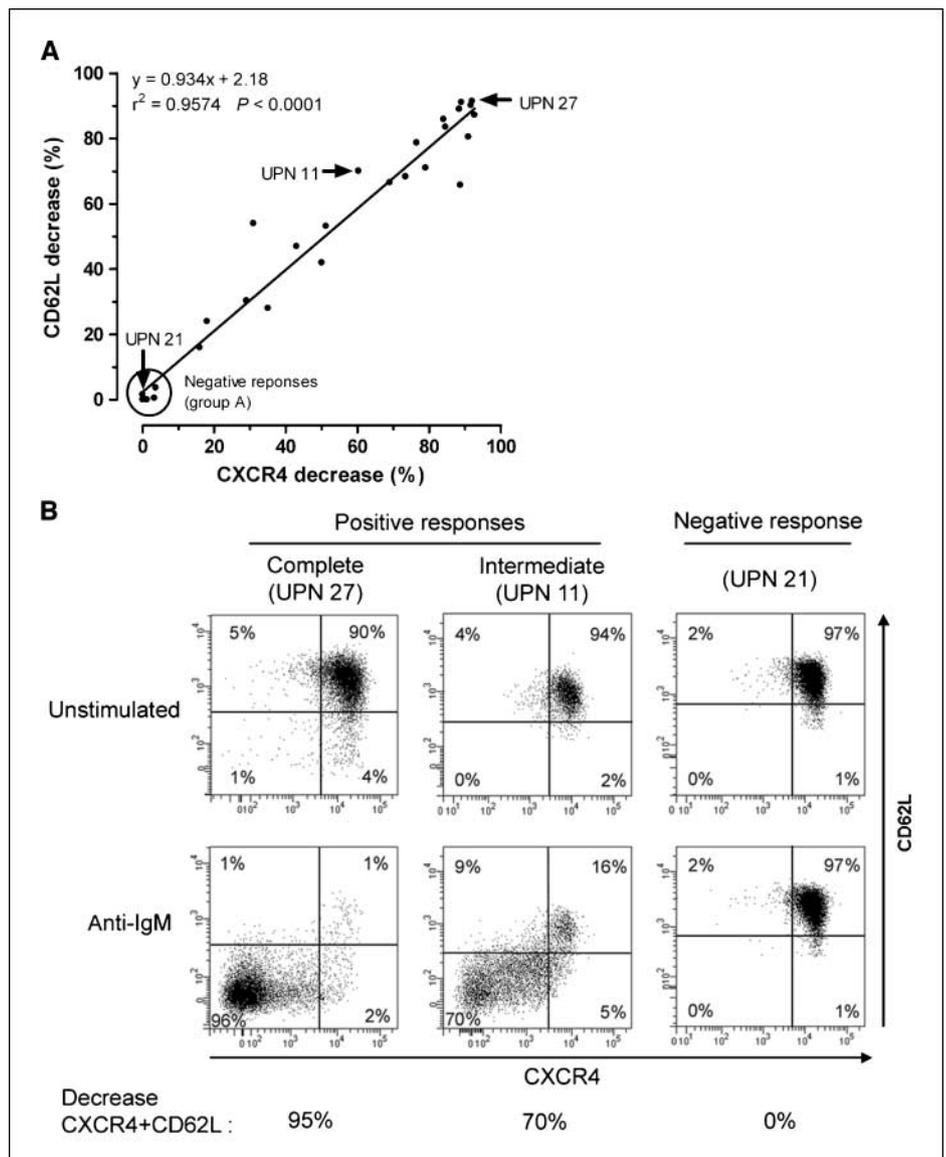


Figure 4. Down-regulation of CXCR4 and CD62L occurred simultaneously in response to BCR ligation. *A*, correlative diagram between BCR-induced CXCR4 and CD62L decrease. *Group A*, patients whose cells showed an absence of BCR-induced decrease of both CXCR4 and CD62L (negative response). *B*, FACS analysis of the CXCR4/CD62L coexpression in representative cases displaying positive (UPN 27, UPN 11) or negative (UPN 21) BCR-induced response.

Table 1. Biological variables of the 30 CLL cases (UPN)

UPN	CD62L*	CXCR4*	VH [†]	IgVH mutational status [‡]	ZAP-70 [§]	CD38	Cell survival [¶]
27	93	92	VH1-69	98	Pos	Neg	Pos
14	91,1	89	VH1-69	100	Pos	Pos	Pos
28	90,1	91,7	VH4-59	98	Pos	Neg	Pos
20	89	88,4	VH3-33	96	Neg	Neg	Pos
18	ND	84,5	VH4-39	99	Pos	Neg	Neg
17	87,2	95	VH3-30	89	Neg	Neg	Neg
25	85,9	84	VH3-21	100	Pos	Pos	Pos
8	83,5	84,6	VH2-5	100	Pos	Pos	Pos
24	80,5	91	VH1-69	95	Pos	Pos	Neg
22	78,7	76,5	VH3-23	97,7	Neg	Neg	Pos
6	71	79	VH4-34	95	Pos	Neg	Pos
1	ND	75,2	VH3-48	95	Neg	Neg	Neg
11	70	59	VH6-1	96	Neg	Neg	Pos
10	68,3	73,4	VH4-44	99	Pos	Pos	Pos
23	66,5	69	VH3-33	92	Neg	Neg	Neg
9	65,7	88,7	VH1-69	100	Pos	Pos	Pos
12	54	31	VH3-30	100	Pos	Pos	Pos
30	53,1	51,2	VH4-b	94	Neg	Neg	Pos
16	47	43	VH3-7	94	Neg	Neg	Pos
4	42	50	VH3-72	97	Neg	Neg	Neg
3	30,3	29	VH3-23	97	Neg	Neg	Neg
19	28	35	VH4-34	97	Neg	Pos	Neg
26	24	18	VH1-69	99	Neg	Neg	Pos
5	16	16	VH4-34	91	Neg	Neg	Neg
Group A							
2	3,7	3,7	VH3-30	97	Neg	Neg	Neg
13	1,6	0	VH4-34	95	Neg	Neg	Neg
15	0,5	3,3	VH1-3	85	Neg	Neg	Neg
29	0	1	VH3-48	91	Neg	Neg	Neg
7	0	1,3	VH3-23	94	Neg	Neg	Neg
21	0	0	VH3-7	93	Neg	Neg	ND

NOTE: Shaded boxes represent unfavorable factors.

Abbreviation: ND, not determined.

* BCR-induced CXCR4 or CD62L decrease (%).

† VH sequence.

‡ IgVH mutational status (%homology to the closet germ line VH gene).

§ Presence (*pos*) or absence (*neg*) of ZAP70 expression verified by reverse transcription-PCR (R. Letestu, unpublished data).

|| Cell surface CD38 expression (%) verified by flow cytometric analysis. (Pos, ≥10%; Neg, <10%).

¶ *In vitro* BCR-induced cell survival signal. Positive survival signal: inhibition of spontaneous apoptosis of >10% and increase of metabolic activity of >25%; Deglesne et al., 2006.

was next performed in hypertonic media (0.6 mol/L sucrose) inhibiting clathrin-coated pit pathway involved in the internalization of various receptors (30). As shown in Supplementary Fig. S2, the presence of sucrose inhibited the BCR-triggered decrease of CXCR4 cell surface expression. Furthermore, 15% to 20% of CXCR4 protein level was recovered at the cell membrane when CLL cells were stimulated for 24 hours and subsequently released from anti-IgM stimulation for a 6-hour period of time (Supplementary Fig. S3). BCR-induced transcriptional down-regulation was ruled out by CXCR4 mRNA quantitation as evidenced by a <2-fold decrease of CXCR4 mRNA synthesis after a 24-hour anti-IgM exposure (Supplementary Fig. S4). Analysis of the change in overall CXCR4 protein content after BCR engagement indicated that almost 50% of the CXCR4 protein content disappeared after a 6-hour BCR stimulation (Fig. 1D). This protein depletion seems to occur through a proteasome-mediated degradation mechanism since treatment with the

proteasome inhibitor MG132 significantly reversed this process. Taken together, these data show that BCR stimulation results in a strong decrease in CXCR4 membrane expression mainly through a clathrin-mediated receptor internalization.

CXCR4 and CD62L decrease in response to BCR ligation are highly variable among CLL patients. The CXCR4 decrease upon anti-IgM stimulation was further evaluated on a series of 30 previously untreated CLL cases and the extent of the decrease was quantified as described in paragraph 1. As shown in Fig. 2A, BCR stimulation induced a highly variable CXCR4 decrease within the CLL cases examined, ranging from >90% of CLL cells down-regulating CXCR4 expression to an absence of modulation of CXCR4 cell surface expression. These results allowed us to discriminate a group of six cases (UPN 2-7-13-15-21-29) exhibiting a BCR-induced CXCR4 decrease inferior to 5% called "negative response," whereas the other cases (24 of 30) displayed a marked decrease of CXCR4 (>5%) after BCR

engagement and was called “positive response.” Interestingly and as exemplified in Fig. 2B, two profiles of responses were observed among BCR induced positive responses and corresponded either to a complete down-regulation of CXCR4 (UPN 27) or to a partial down-regulation called intermediate response (UPN 11). The intermediate response was characterized by a mixture of cells with differential capability of internalizing CXCR4 upon anti-IgM stimulation. As seen in the Fig. 2B (middle), 36% of the overall cell population of UPN 11 maintained an identical level of CXCR4 membrane expression, whereas 28% had no remaining CXCR4 expression at the membrane. CXCR4 protein expression on unstimulated cells was not significantly different between groups displaying either responsiveness or unresponsiveness to BCR ligation (data not shown). Altogether, these results indicate that the ability of CLL cells to down-regulate CXCR4 in response to BCR engagement was highly variable among CLL patients and highlight the heterogeneous capacity of the cell population to respond to BCR in a given patient.

We next considered the effect of BCR engagement on the cell surface expression of CD62L (L-selectin) on the same series of CLL cases. BCR-induced CD62L down-regulation was also variable among cases (Fig. 2C), and similarly to CXCR4, two groups of response were identified, i.e., a group of six cases (UPN 2-7-13-15-21-29) showing no modulation of CD62L expression upon BCR stimulation (CD62L decrease, <5%; negative response), whereas the remaining cases (22 of 28) down-regulated CD62L upon BCR engagement (CD62L decrease, >5%; positive response) but to a variable extent. As observed for CXCR4, two profiles of BCR-induced response were indeed observed among the positive responses (Fig. 2D): a complete decrease of CD62L expression (UPN 27) or an intermediate decrease (UPN 11).

BCR-responsive CLL cells displayed a reduction in both migration toward CXCL12 and adhesion to lymphatic endothelial cells. Because BCR stimulation led to a CXCR4 down-regulation, we considered BCR impact on CXCL12-mediated chemotaxis of CLL cells. Figure 3A shows that the average proportion of CLL cells that migrated to lower chamber containing CXCL12 is 22% of input cells. The anti-IgM prestimulation of

CLL cells unresponsive to BCR ligation did not modify their migratory capacity (Fig. 3A), and a similar CXCR4 expression profile was observed for both nonmigrated and transmigrated cells (Fig. 3B, left). Conversely, CLL cells that strongly decreased CXCR4 upon BCR ligation exhibited a reduced ability to migrate (Fig. 3A) and remained mostly in the higher chamber (Fig. 3B, right, inset a). Almost half of migrated cells corresponded to CXCR4-reexpressing cells (Fig. 3B, right, inset b) due to surface readdressing of CXCR4 in absence of further anti-IgM stimulation as previously described.

We next showed that BCR-induced decrease of cell surface CD62L resulted from shedding of the protein as shown by a time-dependent increase of soluble CD62L in CLL cell supernatant during anti-IgM exposure (Fig. 3C). Because CD62L is involved in the binding of B-lymphocytes to lymphatic endothelium (14), we considered the *in vitro* BCR impact on CLL cell adhesion to the lymphatic endothelial cell line SVEC4-10 by using a static adhesion assay. As shown in Fig. 3D, lymphocyte binding to lymphatic endothelial cells was reduced by $37\% \pm 3\%$ when CLL cells were prestimulated with an anti-IgM ($P < 0.0001$). Taken together, these results indicate that CLL cells down-regulating CXCR4 and CD62L in response to BCR engagement exhibit a decreased ability to migrate toward CXCL12 and to adhere to lymphatic endothelial cells.

BCR-dependent decrease of CXCR4 and CD62L surface expression are strongly related. Because BCR ligation induced both CXCR4 and CD62L down-regulation with similar profiles, we assessed whether these responses might happen in the same subset of cells. As shown in Fig. 4A, CXCR4 and CD62L decrease in response to BCR ligation appeared strikingly correlated among cases (slope, 0.934; $r^2 = 0.9574$; $P < 0.0001$). As expected, this analysis underlined the previously identified subgroup of six patients (group A, UPN 2-7-13-15-21-29) with almost no BCR-dependent decrease of CXCR4 and CD62L (<5%), whereas the remaining cases showed variable but similar extent of down-regulation of both proteins. Figure 4B illustrates the different coregulation of the two proteins following BCR engagement: complete down-regulation (UPN 27), down-regulation of neither proteins (UPN21), and intermediate down-regulation (UPN 11). These intermediate cases were characterized by a mixture of cells decreasing both proteins (70% of the cells in this case) and cells decreasing neither of them (16% of the cells in this case). These results support a BCR-induced decrease of CXCR4 and CD62L occurring simultaneously in the same subset of cells leading to the interpretation that the extent of the down-regulation reflects the percentage of cells with a capacity to respond to BCR ligation.

BCR-induced decrease of CXCR4 and CD62L are indicators of the progressive disease. We previously reported that survival response to BCR ligation correlated with unfavorable markers and was restricted to progressive CLL cases (22). We thus investigated if the extent of CXCR4 or CD62L down-regulation upon BCR stimulation was of prognostic significance, and we found for both proteins a significant correlation with either immunoglobulin variable heavy-chain (IGVH) mutational status ($P = 0.001$) or ZAP70 status ($p \leq 0.03$) or BCR-induced *in vitro* cell survival response ($P \leq 0.01$; Supplementary Table S1). CLL cells isolated from group A cases displayed mutated IGVH sequence, ZAP70-negative status, CD38-negative status, and did not display an increased survival in response to BCR ligation (Table 1). Conversely, over 60% of cases whose cells exhibited a strong level ($\geq 80\%$) of BCR-induced CXCR4 decrease displayed unmutated IGVH sequence ($n = 6/9$), ZAP70 expression ($n = 7/9$), and a

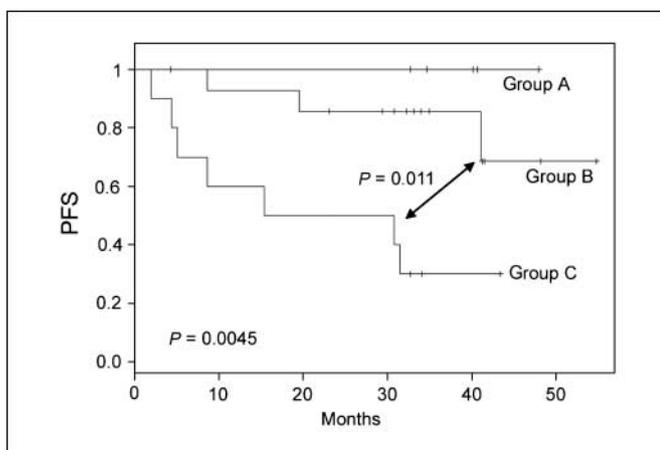


Figure 5. BCR-induced CXCR4 decrease is associated with a risk of disease progression. PFS curves (Kaplan-Meier) according to BCR-induced CXCR4 decrease. PFS was measured from diagnosis to first line treatment, death, or last follow-up. Group A ($n = 6$): CXCR4 decrease, <5%; group B ($n = 14$): $5\% \leq$ CXCR4 decrease < 80%; group C ($n = 10$): CXCR4 decrease, $\geq 80\%$. P values were calculated using log-rank test.

BCR-dependent survival response ($n = 6/9$). Furthermore, using a standard Cox analysis, we found a significant correlation between the extent of BCR-induced CXCR4 decrease and progression-free survival (PFS; $P = 0.039$; data not shown). We observed previously that there were essentially three groups of patients according to the ability of the cells to down-regulate CXCR4 after BCR ligation. We set up arbitrary cutoffs to better visualize the impact on PFS: in group A ($n = 6$), almost no cells were able to down-regulate CXCR4 in response to BCR ligation (CXCR4 decrease, $<5\%$); in group C ($n = 10$), the majority of the cells did (CXCR4 decrease, $\geq 80\%$); and in group B ($n = 14$), only a subset of the cells were able to do so. The PFS was significantly different among the three groups by log-rank analysis ($P = 0.0045$; Fig. 5). In group A, no patient experienced any disease progression. In contrast, PFS was shorter in the two other groups and significantly different in group C (median PFS, 23.1 months) versus group B (median PFS, not reached; $P = 0.011$). In conclusion, the ability of CLL cells to down-regulate CXCR4 and CD62L in response to BCR ligation correlates with unfavorable biological markers and the extent of this BCR-induced response is associated with a significantly shorter PFS.

Discussion

We previously showed that BCR engagement promoted *in vitro* CLL cell survival in patients with progressive disease and unfavorable prognostic markers only (22). Here, in primary CLL cells, we show that *in vitro* BCR engagement led to the decrease of CXCR4 and CD62L membrane expression, in particular in cells from patients with unfavorable prognostic factors and at risk of disease progression.

BCR-induced CXCR4 decrease was the consequence of the internalization of the chemokine receptor as evidenced by the appearance of intracellular CXCR4 following anti-IgM exposure, the inhibition of BCR-induced down-regulation in the presence of sucrose, and the rapid surface recovery 6 hours after cells were released from BCR stimulation. Moreover, we ruled out that BCR-induced decrease of membrane CXCR4 could be related to a transcriptional down-regulation, and this finding is in accordance with a recent work showing that BCR engagement using the same conditions of stimulation led to a moderate (1.5-fold) decrease of CXCR4 gene expression in CLL cells (31). In addition, we showed that BCR engagement promotes CD62L shedding from the cell surface of CLL cells. In other models, BCR engagement has been shown to induce CXCR4 internalization through a protein kinase C-dependent mechanism (32). Similarly, a protein kinase C-dependent shedding of CD62L was observed in B cells activated by formyl peptides and phorbol 12-myristate 13-acetate (33–35). Therefore, in CLL cells, antigen-driven down-regulation of CXCR4 and shedding of CD62L may occur through a common protein kinase C-dependent signaling pathway originating from the BCR. This interpretation is in line with the extremely tight correlation observed between the extent of CXCR4 and of CD62L down-regulation. Importantly, our results strongly suggest an underlying high heterogeneity of cellular capacity to respond to IgM ligation in a given patient, thus pointing out the existence of various CLL cell subclones with differential ability to respond to BCR engagement.

The capacity of CLL cells to down-regulate both CXCR4 and CD62L in response to BCR ligation was highly variable among CLL patients and allowed the discrimination of two groups of patients. One group of six cases had no CXCR4/CD62L down-regulation nor

increased cell survival after *in vitro* BCR ligation. These cases were all stable stage A CLL, harbored IGVH mutated genes, no ZAP-70 expression, and none of them experienced disease progression to date. In contrast, in a larger group exhibiting variable but significant responsiveness to BCR engagement, the intensity of BCR-induced CXCR4 down-regulation was correlated with a shorter PFS, indicating that disease progression was closely linked to the percentage of BCR-responsive cells. Interestingly, even if all ZAP70-positive cases were able to down-regulate CXCR4, a least two ZAP70-negative cases also did, confirming our previous finding that ZAP-70 is dispensable for BCR downstream signaling (22). Similarly, we observed that BCR-responsive cells only displayed an increased Syk activation following BCR engagement (Supplementary Fig. S5).

Importance of BCR signaling capacity after antigen stimulation in CLL disease progression is now well established (20, 22, 36–38). Functionally, cells down-regulating CXCR4 and CD62L in response to BCR engagement displayed a reduction in both migration toward CXCL12 and adhesion to lymphatic endothelial cells. Our data are in line with the CXCR4 internalization and subsequent impairment of CXCL12-mediated chemotaxis after BCR engagement in DT40 cell line (32). CD62L seems to be critical for B-lymphocyte exit from LNs through binding to lymphatic endothelium (17). Germinal center antigen-experienced B cells lack cell surface CD62L and fail to adhere to endothelium into LNs (39). In addition, CXCR4-deficient activated B-cells harbor an aberrant migratory behavior within spleen and LNs (15, 16) and BCR engagement through antigen-bearing dendritic cells leads to migration arrest and extrafollicular accumulation of B-cells into LNs (40). Altogether, these observations indicate that BCR-triggered down-modulation of CXCR4 and/or CD62L profoundly alters B-lymphocyte trafficking within secondary lymphoid organs. In CLL, our observations led to the hypothesis that BCR ligation trapped BCR-responsive CLL cells within the LNs. After entering the LNs, BCR engagement leads BCR-responsive CLL cells to behave like antigen-stimulated normal B cells by dramatically reducing their motility through CXCR4 down-regulation and shutting down their exit program through CD62L down-regulation. These cells remain in close contact with the microenvironment and antigenic stimulation and are prone to proliferate, resulting into enlarged LNs. Conversely, BCR-unresponsive CLL cells are able to exit rapidly from the lymphoid organs and recirculate, as most normal B-cells do because of their inability to detect a specific antigen (40). Thus, by coupling CXCR4/CD62L down-regulation and survival signal, BCR signaling capacity favors the maintenance of a proliferative pool within LNs and contributes to disease progression. Therefore targeting BCR signaling capacity seems of therapeutic interest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Down-regulation of CXCR4 and CD62L in Chronic Lymphocytic Leukemia Cells Is Triggered by B-Cell Receptor Ligation and Associated with Progressive Disease

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