Original Article

L-Selectin Expression Is Influenced by Phosphatase Activity in Chronic Lymphocytic Leukemia

Ildikó Beke Debreceni,[1] Róbert Szász,[2] Zoltán Kónya,[3] Ferenc Erdődi,[3] Flóra Kiss,[1] and János Kappelmayer[1]*

[1]Faculty of Medicine, Department of Laboratory Medicine, University of Debrecen, Debrecen, Hungary

[2]Faculty of Medicine, Division of Hematology, Department of Internal Medicine, University of Debrecen, Debrecen, Hungary

[3]Faculty of Medicine, Department of Medical Chemistry, MTA-DE Cell Biology and Signaling Research Group, University of Debrecen, Debrecen, Hungary

Background: Adhesion receptors have important role in cellular invasiveness and L-selectin is a primary determinant in the binding of chronic lymphocytic leukemia (CLL) cells to several glycated proteins on endothelial cells. We investigated L-selectin expression on CLL cells and explored the mechanisms that lead to their shedding.

Methods: Surface and soluble L-selectin expression levels were studied by flow cytometry and immunoassay, respectively. Magnetically isolated B-cells from patients and controls were investigated for total and protein phosphatase-2A activities. Flow cytometry of permeabilized cells was utilized for the determination of phosphorylated mitogen-activated protein kinase (pp38MAPK) and surface tumor necrosis factor alpha-converting enzyme expression (TACE).

Results: In CLL patients elevated absolute lymphocyte cell counts, high soluble and low surface L-selectin expression were observed. Similarly, TACE surface expression was significantly lower on B-CLL cells compared to normal B-cells. Both total phosphatase and protein phosphatase-2A activities were also significantly lower in B-CLL cells compared to normal B-cells and we found a consequently higher level of pp38 MAPK in B-CLL cells. Based on in vitro experiments a MAPK inhibitor could attenuate the phosphatase inhibitor's effect on L-selectin shedding.

Conclusions: The lower phosphatase activity detectable in chronic lymphocytic leukemia, results in a downstream signaling cascade with subsequent reduction of surface L-selectin expression and this effect is mediated by enhanced phosphorylation of p38MAPK and an altered TACE expression.

Key terms: chronic lymphocytic leukemia; L-selectin expression; soluble L-selectin; protein phosphatases; tumor necrosis factor alpha converting enzyme

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Chronic lymphocytic leukemia (CLL) is a common and rather heterogeneous disorder, characterized by progressive accumulation of immunologically incompetent B-lymphocytes with autonomous B cell receptor (BCR) activation in the bone marrow (BM), blood and lymphoid organs. Most CLL patients do not entail treatment until evident progression of the disease. Mere staging by Binet or Rai criteria is not satisfactory to anticipate the clinical course (1).

*Correspondence to: János Kappelmayer, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Nagyerdei krt. 98, Debrecen 4032, Hungary. Email: kappelmayer@med.unideb.hu

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Numerous adhesion molecules apparently contribute to the cellular processes involved in CLL pathogenesis, via influencing homing of CLL cells to lymphoid organs, survival, and proliferation (2). Leukocytes use a multistep adhesion cascade consisting of rolling, sticking, and crawling to firmly adhere and transmigrate into tissues (3). The lymphocytes first undergo chemokine-induced activation of their integrins, which mediate lymphocyte arrest (4,5). Activation of leukocytes leads to shedding of L-selectin (CD62L) from the cell surface, and this may facilitate detachment of leukocytes from endothelium as they migrate into the tissues. Both the low levels of cell surface L-selectin and high level of soluble L-selectin can account for impaired rolling and migration ability of leukocytes in vivo (6–8). Cell surface L-selectin expression that participates in B-cell homing to lymph nodes and other secondary lymphoid organs is likely to be also important for CLL cell migration, chemokine and integrin-mediated adhesion, and transendothelial migration. These processes are central in trafficking and retention of hematopoietic cells in the BM and lymphoid organs. L-selectin is profoundly overexpressed on malignant B-cells located within the lymph nodes and the BM particularly in the proliferation centers.

In vitro BCR activation results in downregulation of cell surface L-selectin on CLL cells, in particular on cells from patients with unfavorable prognostic factors and at risk of disease progression. In progressive cases, BCR-responsive CLL cells within the lymph nodes are trapped by the BCR-mediated signaling pathway and are prone to proliferate, resulting in enlarged lymph nodes. Conversely, BCR-unresponsive CLL cells are able to exit rapidly from the lymphoid organs and recirculate, as most normal B-cells do. The dysregulation of L-selectin expression on CLL cells is likely to contribute to the pathological disturbance in total CLL cell number and the accumulation of pathological B-cells in the peripheral blood and lymph nodes (9,10). Gu et al., already in 2001 (11–13) showed that in advanced B-CLL, L-selectin expression on malignant cells is less than half than on normal peripheral blood lymphocytes and lower levels of L-selectin expression on CLL cells has been associated with impaired transendothelial migration. Compared to soluble P- and E-selectin levels, soluble L-selectin concentration is at least 20-fold higher (6) and this value can further be increased in pathological disorders. Here, we investigated untreated CLL cases and confirmed the downregulation of cell surface L-selectin on circulating CLL cells. We highlight a possible molecular mechanism leading to decreased surface L-selectin expression and to enhanced shedding that results in increased soluble L-selectin levels.

MATERIALS AND METHODS

Antibodies and Reagents

The following directly conjugated monoclonal antibodies, isotype control, Quantibrite™ PE fluorescence quantitation kit, and Phosflo™ reagents were purchased from Becton Dickinson (San Jose, CA): Mouse IgG2a-FITC, CD5-PerCPCy5.5, CD62L-PE, Mouse anti-p38MAPK (pT180/pY182)-PE. CD19-PC7 was from Beckman Coulter (Indianapolis, IN) and anti-hTACE/-FITC was purchased from R&D Systems (Minneapolis, MN). Paraforaldehyde (PFA), dimethylsulfoxide (DMSO), Histopaque 1077, and Protein G Sepharose were from Sigma-Aldrich (St. Louis, MO). Calyculin-A (CLA) and SB203580 were ordered from Calbiochem (La Jolla, CA) and R&D Systems (Minneapolis, MN), respectively. Human B cell separation kit was purchased from Life Technologies (Oslo, Norway, Dynabeads® Untouched Human B cell). Soluble L-selectin and soluble ADAM17/TACE were measured from plasma using commercially available enzyme-linked immunoassay (Bender Med System and Cloud-Clone Corp., Houston, TX). Antibodies for immunoprecipitation and Western blot were purchased R&D Systems (Minneapolis, MN). Avidin-biotin complex kit was from Vector Laboratories (Burligame, CA).

Patients and Controls

Blood was drawn into EDTA tubes (K_2EDTA, Becton Dickinson vacutainer tube, San Jose, CA) from a total number of 117 untreated CLL patients including 84 patients in the observational group and an additional 33 patients in the investigative group, who were recruited at the Division of Hematology, Department of Internal Medicine at the University of Debrecen. Informed consent was obtained from all participants in accordance with the regulations set by the Ethics Committee of the University of Debrecen (registration number: 4674–2016). All patients examined in this series had immunophenotypically defined CLL as outlined by the NCI criteria. Healthy volunteers as controls were recruited from the staff of the Department of Laboratory Medicine.

Purification of Normal B-Cells and CLL Cells

Normal and leukemic B-cells were purified from peripheral blood. Briefly, peripheral blood mononuclear cells (PBMC) were separated on Histopaque-1077 than normal B-cells and leukemic B-cells of CLL patients were isolated by a magnetic Untouched Human B Cell Separation kit. In case of normal B-cell separation, the kit was used according to the manufacturer's instructions. We obtained an >94% purity of CD19+ lymphocytes as determined by flow cytometry. Some modifications were applied for the separation of B-CLL cells and the purity of the cell suspension was >98%.

Hematology Analysis, Labeling, and Flow Cytometric Assays

Peripheral blood cell count was performed according to our routine clinical laboratory procedure on ADVIA 120 hematology analyzer (Siemens, Forcheim, Germany).

For the analysis of cell surface L-selectin and TACE expression of normal B-cells and CLL cells, we used a four-color staining. CD19-PC7 and CD5-PerCPCy5.5

antibodies were used for cell identification. We used FITC-labeled TACE and PE-conjugated CD62L monoclonal antibodies for detection of cell surface expression of TACE and L-selectin. Mouse IgG2a-FITC was used as an isotype control for the investigation of TACE expression. A quantitative flow cytometric determination of cell surface L-selectin was carried out by a direct immunofluorescence assay using Quantibrite™ PE fluorescence quantitation kit. Results were expressed as antibody binding capacity (ABC). Cells were labeled for 30 min at room temperature (RT) in dark. Then, they were washed twice with PBS and finally were fixed in 500 μL 1% PFA.

Pp38MAPK expression was detected by flow cytometry according to the following procedure. PBMCs were stained with cell surface markers and were immediately fixed with BD Phosflow™ Fix buffer I. in a 37°C water bath for 10 min. Thereafter, cells were washed with BD Phosflow™ Perm/Wash buffer I. and resuspended cells were stained with Mouse Anti-pp38 MAPK (pT180/pY182)-PE antibody for 30 min at RT in dark. After washing cells were resuspended in BD Pharmingen™ Stain buffer. In each experiment, 30,000 events were collected by an FC500 flow cytometer and results were analyzed with the Kaluza software (Beckman Coulter).

Plasma Preparation and Measurement of Soluble Proteins

Plasma from patients with CLL and healthy volunteers were separated from K_2EDTA- anticoagulated blood by centrifugation at 1,000*g* for 15 min at RT. Soluble L-selectin and soluble TACE were measured from plasma samples using commercially available enzyme-linked immunoassay according to the manufacturer's instruction.

Immunoprecipitation and Western Blot Analysis of Soluble L-Selectin

Briefly, plasma of CLL patients was purified from immunglobulins by Protein G Sepharose for 2 h at 4°C. After centrifugation anti-human L-selectin antibody and another aliquot of Protein G Sepharose were added to the supernatant of the immunglobulin free plasma. Immunoprecipitation was done overnight at 4°C. After washing, precipitates mixed with Loading Dye (61.90 mM TRIS, 2% SDS, 10% glycerol, 0.025% bromphenol blue) were boiled for 5 min. Samples were applied to SDS-PAGE using 10% polyacrylamide gel. Proteins were blotted to nitrocellulose membrane that was blocked in 3% gelatin. Subsequently the membrane was incubated with biotinylated anti-human L-selectin antibody for 90 min followed by avidin-biotin complex for 60 min. Bands were visualized by enhanced chemiluminescence (ECL).

Protein Phosphatase Assay

Magnetically separated normal B-cells and CLL-B cells (5×10^5) were suspended in lysis buffer containing 50 mM Tris–HCL (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 0.5% protease inhibitor cocktail. The lysate was sonicated, and then centrifuged at 15,000*g* at 4°C for 10 min. The phosphatase activity of the supernatants was determined at 30°C using 1 μM ^{32}P-labeled 20 kDa light chain (^{32}P-MLC20) of turkey gizzard myosin as substrate in the absence or presence of PP1 inhibitor-2 (I-2, 2 μM) as previously described (14). The reaction was initiated by addition of the substrate and after 10 min incubation was terminated by the addition of 10% TCA and 6 mg/ml BSA. Following centrifugation, the released $^{32}P_i$ was determined from the supernatant in a scintillation counter (Perkin Elmer). The total phosphatase activity in normal B-cells was regarded as 100%.

In Vitro Experiment with CLA and p38MAPK Inhibitor

These experiments were carried out in suspension of magnetically separated normal B-cells and B-CLL cells. Cells were incubated with DMSO or CLA in different concentrations at 37°C for 30 min. For those experiments, where we wanted to inhibit p38MAPK, cells were preincubated with 5 μM SB203580—as specific inhibitor of p38MAPK—before CLA treatment. After treatments pp38MAPK level and surface L-selectin were examined as describe above.

Statistical Analysis

GraphPad Prism 4.0 program was used for the statistical analysis. The statistical significance of the differences between groups was determined by unpaired Student's *t*-test in case of Gaussian distribution, and by Mann–Whitney test in case of non-Gaussian distribution, as appropriate. Differences were considered significant when *P* value was below 0.05.

RESULTS

Surface and Soluble L-Selectin Expression

Peripheral blood samples of CLL patients were extensively characterized by 8-color immunophenotyping. In case of CLL patients, surface L-selectin analysis was conducted on $CD5^+/CD19^+$ B-CLL cells while in control samples L-selectin expression was investigated on $CD5^-/CD19^+$ normal B-cells (Fig. 1). We have found that surface L-selectin was significantly reduced on B-CLL lymphocytes in RAI 0 stage of CLL, compared to normal B-cells. Simultaneously soluble L-selectin was significantly elevated in the plasma of B-CLL patients (Fig. 2A,B), but when the soluble L-selectin level of plasma of CLL patients was normalized for 10^6 leukocyte counts, soluble L-selectin values became lower in CLL patients (Fig. 2C). In cases of patients, where absolute lymphocyte count was above 32 G/L, B-CLL cells expressed low surface L-selectin levels and at the same time plasma samples derived from these patients displayed elevated soluble L-selectin values (Fig. 2D,E). L-selectin expression on T-cells in the CLL group was similarly lower but the differences was less pronounced (Fig. 3A). The soluble form of L-selectin can derive

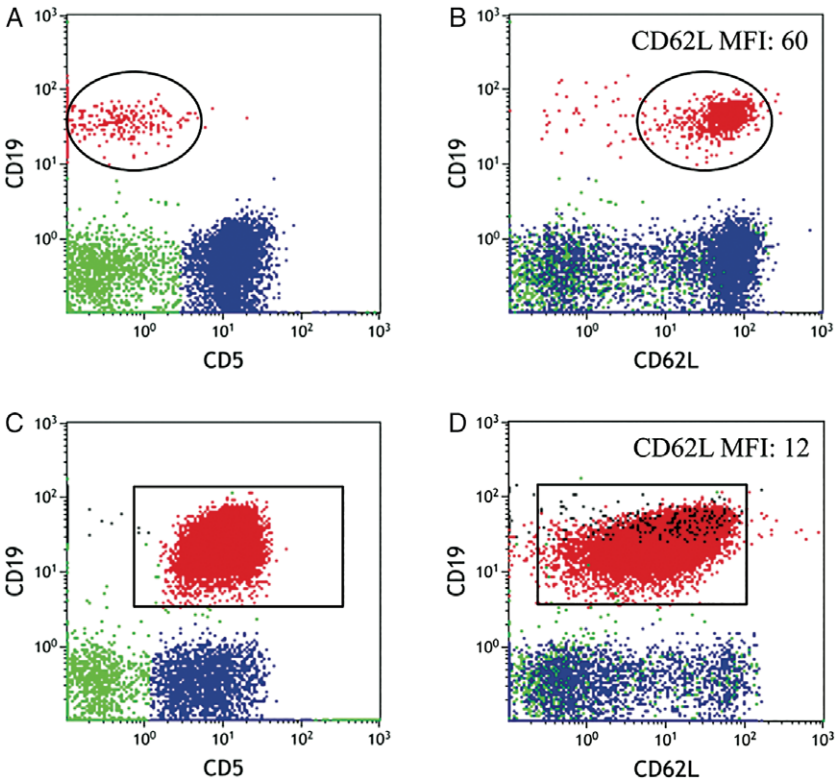

FIG. 1. Expression of L-selectin (CD62L) by normal B-cells and B-CLL cells. Flow cytometry dot-plots of peripheral blood (PB) lymphocytes from a healthy donor (panels **A** and **B**) and of a PB sample from a B-CLL case (panels **C** and **D**). CD19+ normal B-cells marked by circular gates on panels A and B show high and homogenous CD62L expression (MFI: 60) when compared to the heterogenous and much lower CD62L expression (MFI: 12) of the CD5+ B-CLL lymphocytes which are in rectangle gates on panels C and D. Black dots on panel D were residual normal B-cells of B-CLL sample. [Color figure can be viewed at wileyonlinelibrary.com]

from both lymphocytes and neutrophils as could be detected in samples containing considerable number of neutrophils (Fig. 3B). Nevertheless, since the mean neutrophil count in the investigated samples was 4.5 G/L, this represented a neglectable quantity compared to the mean absolute lymphocyte count (43.6 G/L). By flow cytometry 78% of these lymphocytes were B-CLL cells, thus soluble L-selectin represented primarily the B-CLL cell derived protein.

TACE Expression and pp38MAPK Level

TACE expression was significantly lower on the surface of CD5+/CD19+ malignant B-cells while pp38MAPK level was significantly higher in these cells compared to normal B-cells as determined in permeabilized cells using a phospho-specific antibody (Fig. 4A–D).

Protein Phosphatase Activity

Phosphatase activity of B-CLL and normal B-cell lysates was assayed in the absence and presence of PP1 inhibitor-2 (I2), accordingly total and PP2A phosphatase activities (the activity in the presence of I2) were determined. We have found that both total and PP2A phosphatase activities were significantly lower in B-CLL cells compared to normal B-cells (Fig. 5).

In Vitro Studies with a Phosphatase Inhibitor CLA

Leukemic B-cells from CLL patients showed higher basic pp38MAPK level compared to normal B-cells, after CLA treatment the pp38MAPK level was elevated in a dose-dependent manner in both groups but the pp38MAPK level was higher in B-CLL cells compared to normal B-cells at all investigated CLA concentrations (Fig. 6A). Similarly, a rapid downregulation of L-selectin was observed by CLA treatment with different kinetics in the two groups. In case of malignant B-cells, L-selectin almost entirely was shed from the cell surface at 10 nM CLA concentration (Fig. 6B).

L-Selectin Shedding Is Mediated via the p38MAPK Phosphorylation in Normal B-Cell

Normal B-cells were preincubated with a highly specific inhibitor of p38MAPK (SB203580) at 5 μM concentration before CLA treatment. The p38MAPK inhibited cells showed decreased level of pp38MAPK after CLA treatment and 5 μM SB203580 almost completely inhibited the CLA induced phosphorylation of p38MAPK at 5 nM CLA concentration (data not shown). Similarly to this change the CLA-induced shedding of L-selectin was also attenuated at 5 nM CLA by p38MAPK inhibitor, as depicted in Figure 6C.

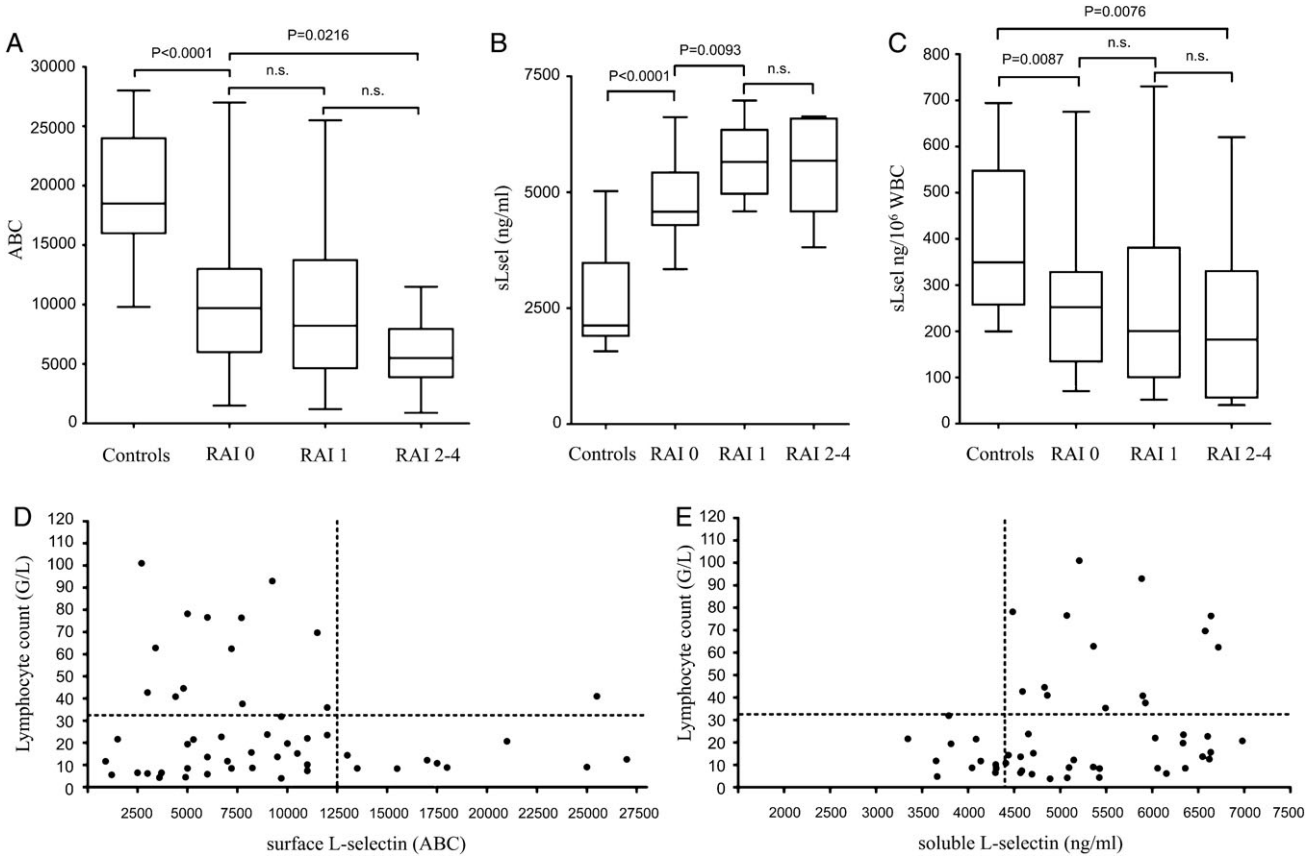

FIG. 2. Cell surface and soluble L-selectin (sLsel) analysis of B-CLL samples. L-selectin was significantly reduced ($P < 0.0001$) on B-CLL lymphocytes, compared to control B-cells and L-selectin ABC was the lowest in progressing cases (panel **A**). The numbers of patients in the Rai groups were the followings: Rai 0: 27, Rai 1: 12 and in the pooled Rai 2–4: 12. Rai 2–4 patients were pooled in one group due to the low number of cases. Contrary to surface L-selectin, soluble L-selectin was significantly elevated in the plasma of B-CLL patients (panel **B**), but when these data were normalized for leukocyte counts, soluble L-selectin values became lower in CLL patients (panel **C**). Patients where an absolute lymphocyte count was above 32 G/L expressed low surface and high soluble L-selectin levels (panels **D** and **E**, respectively).

DISCUSSION

Reversible protein phosphorylation is essential for a myriad of biochemical processes and in a healthy state it is tightly controlled by a network of protein kinases and protein phosphatases. In numerous hematological malignancies, unrestricted activation of oncogenic kinases occur that can either be achieved by the constitutive activation of the upstream kinases or via the inactivation of their antioncogenic phosphatases. Functional defects of PP2A in leukemias are due to the overexpression of

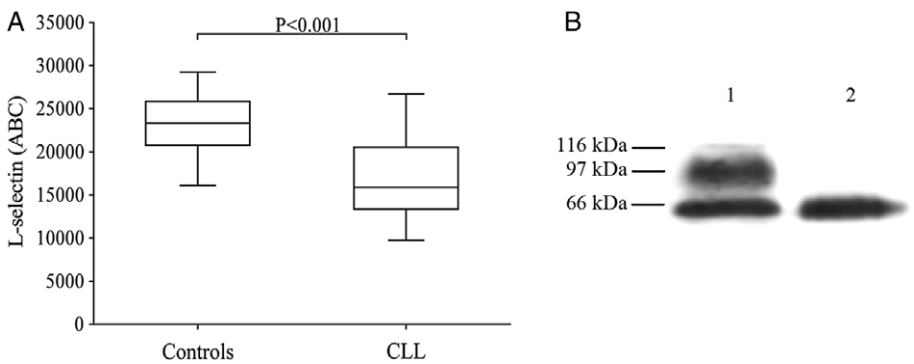

FIG. 3. Cell surface L-selectin on T-cells and soluble L-selectin in plasma of CLL patient. T-cells from CLL patients ($n = 28$) show significantly lower L-selectin expression compared to T-cells from controls ($n = 15$) on panel **A**. The soluble form of L-selectin can derive from lymphocytes and neutrophils too. Western blot image illustrates soluble L-selectin in case of two CLL samples (panel **B**). Lane 1 displays a CLL sample that contain 55% B-CLL cells and 42% neutrophils (upper band neutrophil derived, lower band lymphocyte derived L-selectin). Lane 2 shows soluble L-selectin from a patient with 89% B-CLL cells and 5% neutrophils.

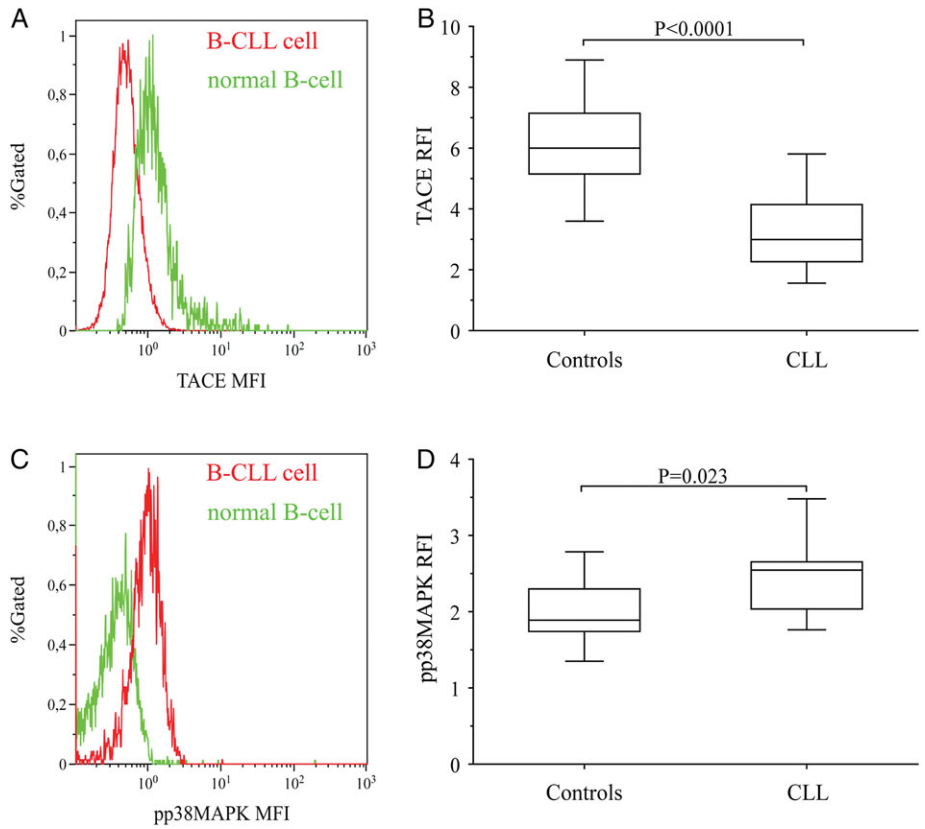

FIG. 4. Cell surface TACE and phosphorylated p38MAPK expression. Cell surface TACE expression was detected by flow cytometry, the representative histogram of panel **A** shows TACE expression of control B-cells and B-CLL cells. Results of TACE expression (panel **B**) were expressed as relative fluorescent intensities (RFI) in samples of controls ($n = 18$) and CLL patients ($n = 30$). In CLL samples, TACE RFI was significantly lower ($P < 0.0001$) compared to controls. The pp38MAPK was examined in permeabilized cells by flow cytometry, the histogram of panel **C** shows pp38MAPK expression of control B-cells and B-CLL cells. Results of controls ($n = 11$) and CLL patients ($n = 19$) were expressed as RFI (panel **D**). Malignant B cells showed significantly higher ($P = 0.023$) pp38MAPK levels compared to normal B-cells. [Color figure can be viewed at wileyonlinelibrary.com]

proto-oncogenic PP2A inhibitors termed CIP2A (Cell Proliferation Regulating Inhibitor of Protein Phosphatase 2A) and SET; a putative oncoprotein in several leukemias (15).

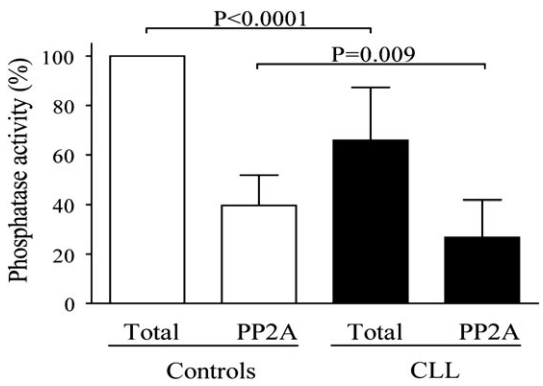

FIG. 5. Protein phosphatase activity of normal B-lymphocytes and CLL cells. Phosphatase activity (PA) of B-cell lysates was assayed with ^{32}P-MLC20 substrate and the released ^{32}Pi was measured. Total and PP2A phosphatase activity were determined and the results were normalized for cell counts. Total phosphatase activity in normal-B cells ($n = 9$) were regarded as 100%. Both total PA and PP2A were significantly lower in B-CLL ($n = 24$) compared to normal B-cells. The results are displayed as the mean and standard deviation (SD).

The SET oncoprotein has been long known to be associated with myeloid malignancies (16) and later it was also described that chronic lymphocytic leukemias and related non-Hodgkin lymphoma cell lines also significantly overexpress SET (17). There are several ways how SET may influence the development of malignancies, but it is primarily thought to be attributed to the inhibition of PP2A. More recently, another important mechanism, the enhanced oncogenic signaling by the tyrosine kinase Lyn has been described in CLL that strengthens the SET-mediated inhibition of PP2A (18). PP2A is involved in the regulation of several oncogenic signal transduction pathways including cell transformation (19). Disease progression is mediated by several mechanisms and one aspect in CLL is the expression of adhesive receptors on the surface of leukemic cells.

The primary aim of this work was to delineate mechanisms that determine surface expression of L-selectin in CLL and to elucidate the regulatory mechanisms of this adhesive protein on lymphocytes. CLL consists of a reservoir of proliferating cells within the bone marrow and the lymph nodes and a pool of quiescent cells circulating in the peripheral blood. L-selectin expression in these two populations is largely different being

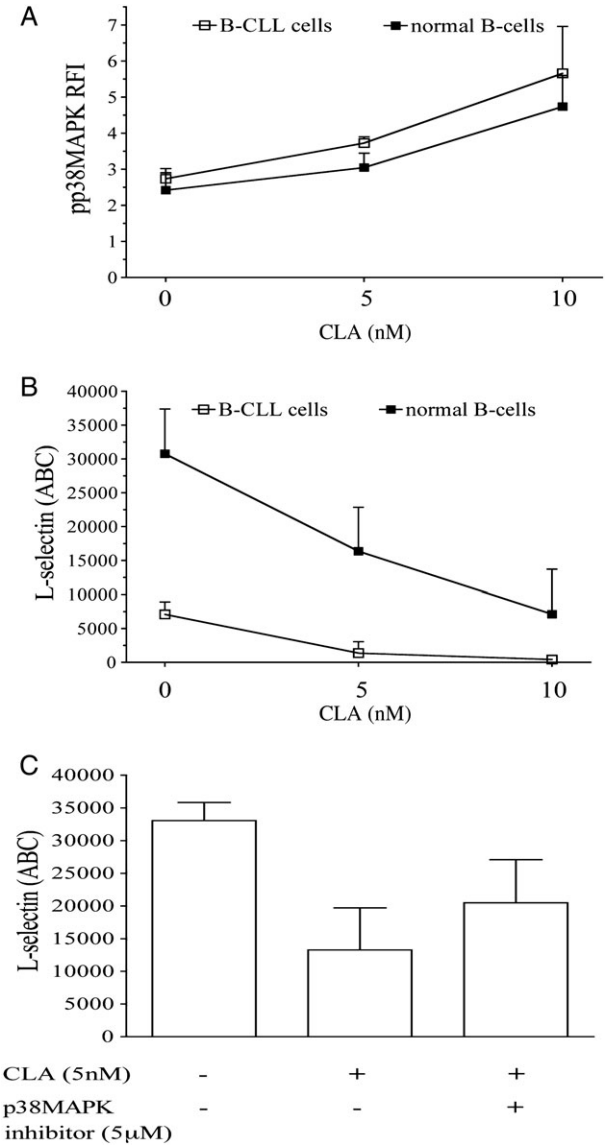

FIG. 6. Effect of phosphatase inhibitor (CLA) and MAPK inhibitor (SB203580) on the p38MAPK phosphorylation and L-selectin shedding. Inhibition of serine/threonine phosphatases with CLA caused phosphorylation of p38MAPK, the degree of phosphorylation was slightly larger in B-CLL cells with increasing CLA concentrations (panel A). These intracellular changes induced the downregulation of cell surface L-selectin (panel B). Panel C displays results of in vitro experiment in which control PBMCs were pretreated with MAPK inhibitor before CLA treatment. CLA-induced shedding of L-selectin was also attenuated at 5 nM CLA, in the presence of the MAPK inhibitor. The results are the mean and standard deviation (SD) of three different experiments.

elevated in the former and decreased in the latter (9). Previous works have demonstrated that the transendothelial migration of lymphocytes in CLL is accompanied by a downregulation of L-selectin on circulating B-CLL cells (7) and it was also demonstrated that low L-selectin expression on these cells is a negative prognostic factor (20). In a recent publication (21), it was also found, that L-selectin levels on T-lymphocytes in chronic myeloid leukemia patients are associated with the success of treatment, that is, low surface L-selectin on T-cells and elevated soluble L-selectin levels were found to be an unfavorable sign for tyrosine kinase inhibitor treatment.

In our studies, we found in an observation cohort of CLL patients that the low surface L-selectin expression of B-CLL cells is accompanied by an elevated soluble L-selectin level. The low surface expression was found to be associated with the clinical status of the CLL patients as determined by the Rai-stages. The soluble L-selectin values were also significantly elevated in more advanced stages but evidently—when normalized for the absolute lymphocyte count of the patients—these values became actually lower.

In a separate investigative cohort, we performed a stepwise evaluation for the investigation of decreased surface L-selectin expression on B-CLL cells. We hypothesized that the enhanced SET oncoprotein described for B-CLL cells would result in a downregulation of PP2A activity. Using a ^{32}P-based phosphatase activity assay with selective phosphatase inhibitor, we verified that lysates of B-CLL cells possess significantly lower total phosphatase activity as well as PP2A activity when compared to normal B-cells. Cytogenetic alterations are frequent in CLL and the 11q deletion is an unfavorable prognostic marker that may result in a diminished PP2A acivity. In our investigative cohort of B-CLL patients, we found that 11q deletion did not influence the PP2A activities measured in cell lysates. Other cell types in our cohort were also investigated and T-cells from B-CLL patients were also shown to display significantly lower values compared to T-cells of healthy individuals, although the differences were considerably more subtle compared to that observed between normal and leukemic B-cells. L-selectin expression of neutrophils of B-CLL patients showed no difference when compared to healthy individuals (data not shown) although neutrophil soluble L-selectin was detectable in samples with normal neutrophil percentage.

The observed lower phosphatase activity can result in an enhanced phosphorylation of downstream phosphoproteins. We found significantly higher basic pp38MAPK level in permeabilized B-CLL cells compared to normal B-cells by flow cytometric assay ($P = 0.023$). We further investigated this phenomenon using the phosphatase inhibitor CLA. As expected, after CLA treatment the pp38MAPK level was elevated in a dose-dependent manner in both groups but the pp38MAPK level was higher in B-CLL cells compared to normal B-cells at all investigated CLA concentrations. The functional consequence of p38MAPK phosphorylation is the subsequent further phosphorylation of numerous proteins one being the metalloprotease TACE. In a work of Peschon and coworkers on mouse embryos with non-functional TACE, it was found that in addition to their inability to release TNF-α, the L-selectin shedding of thymocytes from these mice was

also drastically reduced that proved that TACE is responsible for L-selectin shedding in this setting (22). Detailed biochemical studies have revealed the association of L-selectin shedding and TACE (23). The shedding process involves the simultaneous decrease of surface expression of both the shed protein (L-selectin) as well as the sheddase TACE as has been described previously (24). We also found a significantly lower expression of functional TACE on the surface of B-CLL cells when compared to normal B-cells. It may be anticipated that TACE downregulation would result in a succeeding significant elevation of soluble form of TACE in the plasma. However, this is not evidently the case as we only observed a mild elevation of soluble TACE (data not shown). This finding is in agreement with previous results (24) where the authors also did not detect soluble fragments of TACE in their THP1 and Jurkat cell lines, but they observed that instead of release to the medium the TACE was internalized upon activation.

Because of the already diminished phosphatase activity, 5 nM CLA basically abrogated L-selectin expression on B-CLL cells but only decreased L-selectin expression by 50% on normal B-cells. This observation is also consistent with the known high activity of kinases due to the autonomous BCR activation in CLL. Overactive kinases of CLL cells are losing their brake with depressed phosphatase inhibition and release their exaggerated effect on inhibition of L-selectin expression. This phosphatase inhibitor elicited L-selectin downregulation was investigated using a highly specific inhibitor of the p38MAPK, that prevented the CLA-elicited pp38MAPK formation even in the presence of relatively high CLA concentration and it also attenuated the CLA-elicited L-selectin downregulation. L-selectin shedding however is not restricted to the aforementioned processes, several pathophysiological stimuli including as simple as oxidation–reduction processes can modify cysteinyl sulfhydryl groups in the mature TACE and may provide further means for regulating L-selectin shedding (25).

In our both cohorts, we deliberately selected non-treated CLL patients as we wanted to explore the phenomena of adhesive protein expression and some underlying mechanism. Studying adhesion protein expression on CLL-cells may be extended to treatment follow-up since there are several drugs that either inhibit PP2A like FTY720 (26) or inhibit p38MAPK activity like Rituximab (27).

Our findings have limitations, since we did not study longitudinal changes of L-selectin expression and phosphorylation processes in CLL cases with or without treatment and it is quite likely that low L-selectin expression already occurs in monoclonal B-cell lymphocytosis (28). Furthermore, it also remains an open question whether novel therapeutic agents like ibrutinib and idelalisib therapy may affect L-selectin expression or function as has been shown for an adhesion receptor with prognostic significance (29,30). Nevertheless our data probably have pathophysiological significance as human soluble L-selectin at concentrations around 8,000 ng/mL completely inhibit L-selectin mediated lymphocyte binding to activated endothelial cells (31) and the migratory capabilities of lymphocytes expressing lower surface L-selectin like in B-CLL, can already be significantly modified by soluble L-selectin values of 5–6,000 ng/mL observed in our samples.

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CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

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