

Circulating B-Cell Chronic Lymphocytic Leukemia Cells Display Impaired Migration to Lymph Nodes and Bone Marrow

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Abstract

Homing to secondary lymphoid organs and bone marrow (BM) is a central aspect of leukemic pathophysiology. We investigated the roles of the two major lymphocyte integrins LFA-1 and VLA-4 on B-cell chronic lymphocytic leukemia (CLL) cells in these processes. We found that the majority of CLL cells expressed significantly reduced LFA-1 due to low $\beta 2$ integrin transcripts. VLA-4 expression was heterogenous but underwent rapid activation by the BM chemokine CXCL12. CLL cells failed to transmigrate across VCAM-1-expressing, ICAM-1-expressing, and CXCL12-expressing endothelium, whereas when LFA-1 expression was regained in subsets of CLL cells, these lymphocytes rapidly transmigrated the endothelium. Furthermore, when injected into tail veins of immunodeficient mice, normal B cells rapidly homed to lymph nodes (LN) in a LFA-1-dependent manner, whereas CLL cells did not. Nevertheless, only residual CLL subsets could reenter BM, whereas both normal and CLL cells homed to the mice spleen in an LFA-1-independent and VLA-4-independent manner. Our results suggest that CLL cells have a reduced capacity to adhere and transmigrate through multiple vascular endothelial beds and poorly home to lymphoid organs other than spleen. Integrin blocking could thus be an efficient strategy to prevent circulating CLL cells from reaching prosurvival niches in LNs and BM but not in spleen. [Cancer Res 2009;69(7):3121–30]

Introduction

B-cell chronic lymphocytic leukemia (CLL) is marked by the accumulation of CD5⁺ B lymphocytes within the blood, bone marrow (BM), and secondary lymphoid tissues but the molecular signals that determine CLL trafficking to these organs are largely unknown. Abnormalities in the expression and function of cell adhesion molecules may account for the patterns of intranodal growth and hematogenous spread of the malignant cells (1, 2). Chemokine- and integrin-mediated adhesion and transendothelial migration (TEM) are central aspects in trafficking and retention of

hematopoietic cells in the BM and lymphoid organs. To enter these organs, circulating cells need to arrest on specific endothelial barriers, to locomote over the endothelial surface toward interendothelial junctions, and to cross these junctions while resisting disruptive shear forces (3, 4). Both firm adhesion and ability to locomote and transmigrate across endothelial barriers depend on the ability of circulating cells to establish dynamic adhesive interactions through their $\alpha 4$ integrins VLA-4 ($\alpha 4\beta 1$) and $\alpha 4\beta 7$ and the $\beta 2$ integrins LFA-1 ($\alpha L\beta 2$) and Mac-1 ($\alpha M\beta 2$; ref. 5). During these interactions, the integrins undergo reversible activation by endothelial-presented chemokines (6).

The major endothelial integrin ligands, chemokines, selectins, and selectin ligands expressed on the main lymphoid organs are conserved between human and mice. Indeed, human stem cells interact with the murine BM vasculature and consequently can home to and repopulate the BM of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (7–9). This high conservation between the human and murine vasculature enables the comparison between normal and leukemic cell trafficking to the murine BM, and thus, adoptive transfer experiments into NOD/SCID mice have been used as a functional, preclinical model for *in vivo* dissemination of human leukemic cells (10, 11). Human precursor-B acute lymphoblastic (ALL) cells adoptively transferred into these mice rapidly entered the murine BM in an integrin-dependent manner (12). These early findings prompted us to use the NOD/SCID model to address the possible involvement of LFA-1 and VLA-4 in CLL cell trafficking to peripheral lymph nodes (LN), the BM, and the spleen using short-term homing assays.

In the current study, we analyzed the migratory behavior of CLL cells at the beginning of the disease and intended to exclude chemotherapeutic influence. We therefore concentrated on CLL samples of currently untreated patients of early and intermediate stages. We report a severely impaired *in vivo* homing of these peripheral blood (PB) CLL cells to murine peripheral LNs compared with human B lymphocytes, which efficiently migrated into these organs. This attenuated CLL cell trafficking to LNs was the result of reduced LFA-1 levels on the majority of the CLL samples of our cohort. CLL cells also poorly interacted with various ICAM-1-expressing endothelial cells *in vitro* under shear flow conditions and displayed severely impaired *in vitro* TEM in response to stromal cell-derived factor-1 (CXCL12). CLL homing to BM was lower than that of normal B lymphocytes and depended on functional VLA-4 expression, whereas spleen homing was integrin independent. In summary, our data suggest that PB CLL cells fail to mount integrin-mediated adhesions to multiple vascular endothelial beds, poorly extravasate through LNs and BM, and preferentially home to the spleen.

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

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Materials and Methods

Patients and cell preparation. The study was approved by the local ethics committees and conducted according to the Declaration of Helsinki. After informed consent, PB samples were obtained from 105 patients fulfilling diagnostic and immunophenotypic criteria for common CLL at the Kaplan Hospital (Rehovot, Israel), Freiburg University Hospital (Freiburg, Germany), and Salzburg University Hospital (Salzburg, Austria; Supplementary Table S1). Additional BM aspirates were obtained from 12 CLL patients and 3 healthy donors. PB mononuclear cells (PBMC) of CLL or healthy donors were isolated via density gradient centrifugation and freshly used or viably frozen in FCS plus 10% DMSO for storage in liquid nitrogen. Thawed cells were cultured overnight in RPMI 1640 with 10% FCS and antibiotics. No difference in the integrin expression or behavior of thawed and cultured or fresh cells was observed ($P = 0.458$, $n = 34$). For functional *in vitro* assays, B lymphocytes were enriched untouched (EasySep, StemCell Technologies). For RNA isolation, CLL cells were purified by CD19⁺ selection (MACS, Miltenyi Biotec). Analysis of the immunoglobulin heavy chain variable (IgVH) mutational status, ZAP-70, and CD38 expression and fluorescence *in situ* hybridization analysis of chromosomal abnormalities were routinely performed as described

(13, 14). ZAP-70 risk was assessed as T-cell/CLL expression ratio <2.5 versus ≥ 2.5 as previously recommended as consensus of the optimization of ZAP-70 evaluation procedures by multicenter efforts (15).

Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were cultured as described (16). The endothelial-derived cell line ECV-304 (LS12), stably transfected with α -1,3-fucosyltransferase and *N*-acetylglucosamine 6-*O*-sulfotransferase and expressing functional sulfated L-selectin ligands and ICAM (herein ECV-304-LL), was kindly provided by Dr. R. Kannagi (Aichi Cancer Center, Nagoya, Japan). Cells were maintained in RPMI 1640, 10% FCS, glutamine, and antibiotics.

Reagents and monoclonal antibodies. Recombinant human CXCL12 was purchased from R&D Systems, and human serum albumin (HSA) was from Calbiochem. Soluble purified seven-domain human VCAM-1 and the anti- α 4 HPI/2 monoclonal antibody (mAb) were kindly provided by Dr. B. Pepinsky (Biogen). The β 2 blocking antibody TS1/18 was provided by D. Staunton (ICOS). Phycoerythrin-conjugated anti-CD49d, anti-CD11a, FITC-conjugated anti-CD19 antibodies, and isotype controls were purchased from BD Biosciences. PC7-conjugated anti-CD5 antibody was from Beckman Coulter.

Flow cytometric analysis. Cells were stained with a mixture of fluorescence-labeled anti-CD19, anti-CD5, and anti-VLA-4 or anti-LFA-1

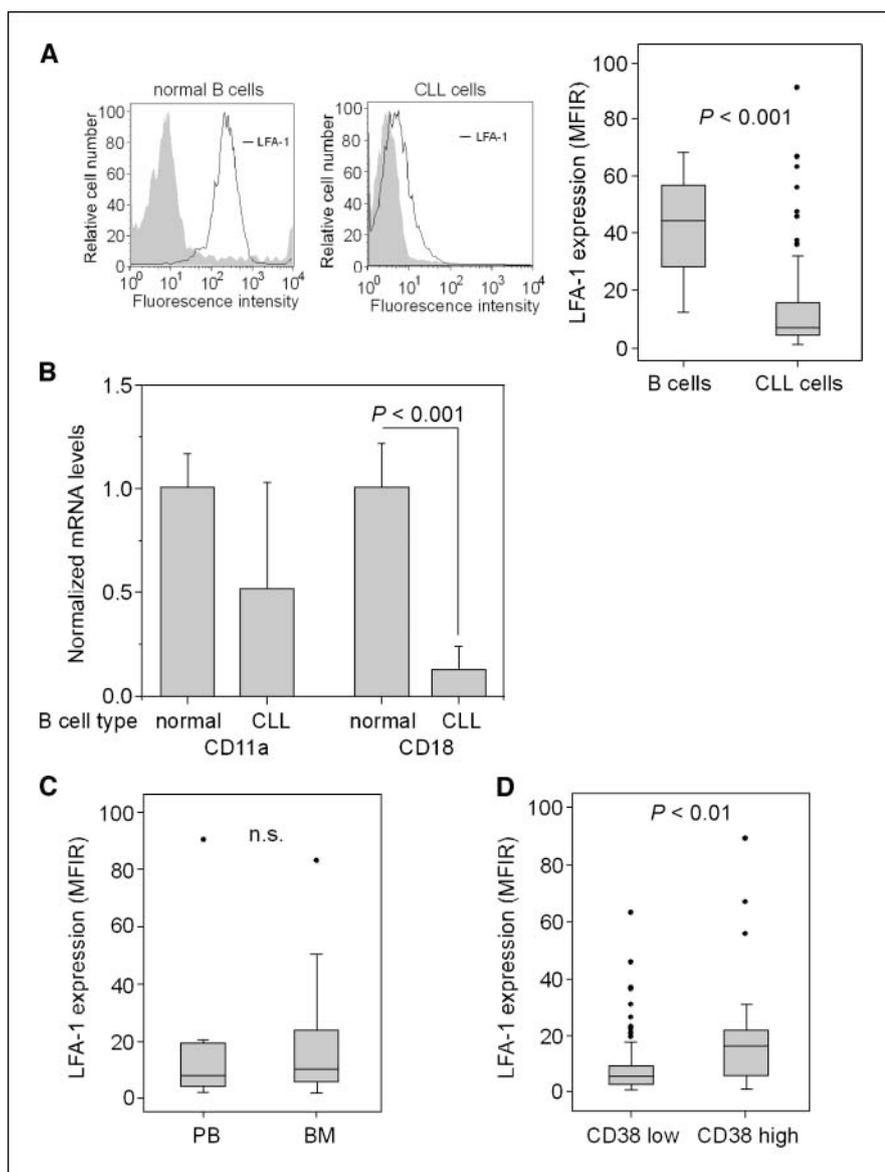
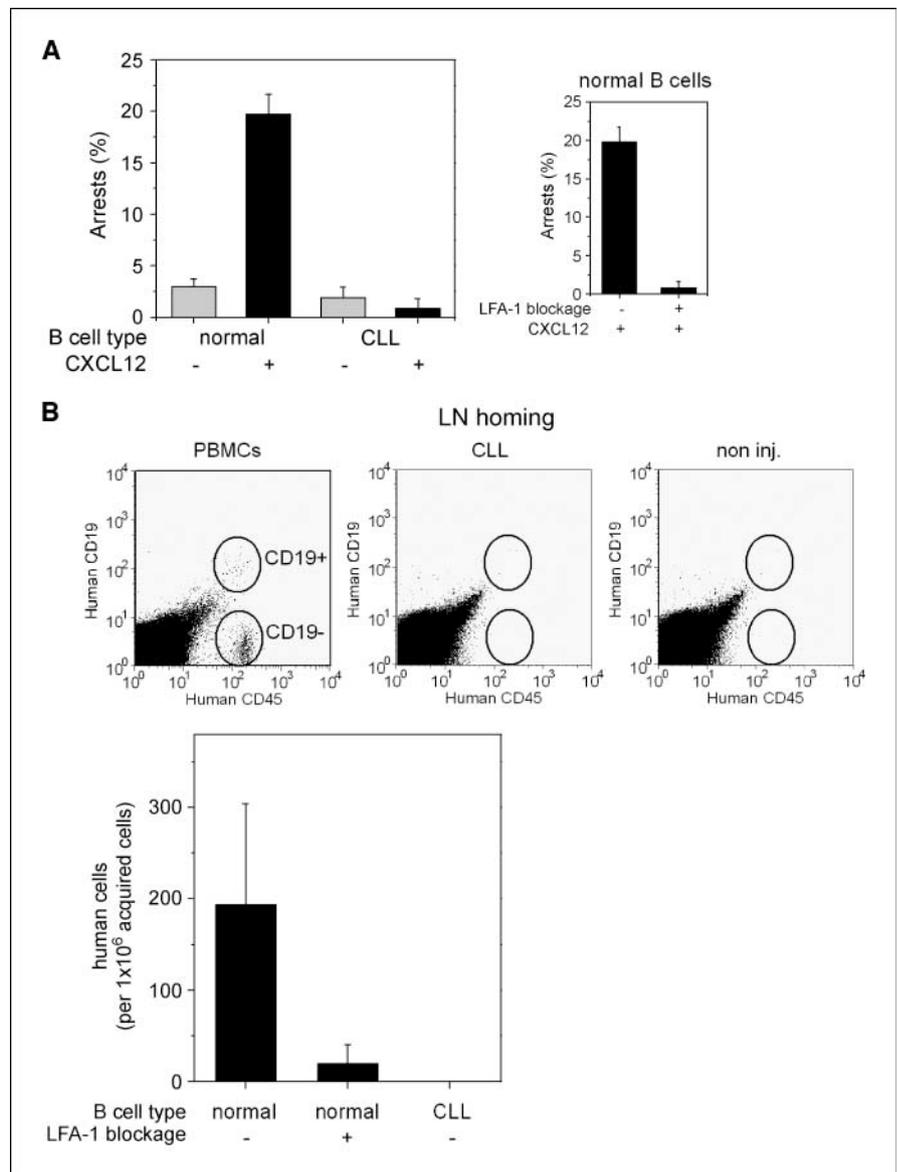


Figure 1. LFA-1 expression is decreased in CLL lymphocytes. *A, left*, representative fluorescence histograms of normal B cells and CLL cells stained with anti-LFA-1 (black lines) or the corresponding isotype control (tinted histograms). CLL cells were defined by gating on CD19⁺ CD5⁺ expression. *Right*, MFI ratios (MFI) of LFA-1 expression on B lymphocytes of 11 healthy donors and 105 CLL patients. Data are presented in box-and-whisker format: the 25th and 75th percentile form the box, with the median marked as a line; the smallest and the largest observation form whiskers; and outliers are presented as circles. Significance levels are indicated in the figures. *B*, relative mRNA levels of α L (CD11a) and β 2 (CD18) subunits were determined from purified B and CLL cells by real-time quantitative PCR as described in Materials and Methods and normalized to healthy B-cell levels. CD18 levels were dramatically reduced in CLL cells at $n = 13$. *C*, LFA-1 expression (MFI) of CLL cells on PB compared with BM samples of 12 CLL patients as determined by FACS. *D*, LFA-1 expression in clinical CD38 high-risk group compared with CD38 low-risk group, defined by the standard prognostic threshold defining CD38 expression on $<30\%$ of the cells as low risk versus on $\geq 30\%$ as high risk (49).

Figure 2. CLL cells fail to arrest on ICAM-expressing endothelium and cannot home to LNs due to reduced levels of LFA-1. *A, left*, purified CLL cells or normal B cells were perfused at 1.5 dyn/cm² for 30 s over ECV-304-LL cells, expressing ICAM, and overlaid with CXCL12 where indicated. Arrested cells are expressed in frequencies of flowing cells passing close to the substrate. Data are expressed as the mean \pm range of two fields in view and depict one experiment representative of three. *Right*, pretreatment with 10 μ g/mL LFA-1 blocking antibody for 5 min abolished CXCL12-induced arrests of normal B cells on ECV-304-LL cells. *B*, PBMCs ($15\text{--}25 \times 10^6$) of CLL patients or healthy donors were injected into NOD/SCID mice either untreated or after blocking with anti-LFA-1 ($\beta 2$)-neutralizing antibodies. Human B cells or CLL cells in the LNs were detected after 3 h by flow cytometry using specific anti-human CD19 and anti-CD45 antibodies as illustrated on the top panels. *Columns*, mean of detected human CD19⁺, CD45⁺ cells per 1×10^6 million injected cells, and 1×10^6 acquired LN cells from healthy donors ($n = 3$) or CLL patients ($n = 8$); bars, SD.



mAbs and at least 5,000 CLL cells were analyzed by gating CD19⁺ CD5⁺ CLL cells using a FC-500 flow cytometer and CXP2-2 software (Beckman Coulter) or using a FACSCalibur (BD Biosciences) compared with the corresponding isotype controls.

Real-time PCR. Quantitative real-time PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems) using Taqman Gene Expression Assay numbers Hs01047127_m1, Hs01051751_m1, Hs01035609_m1, and Hs00559595_m1. Results were quantified based on the relative expression of the target genes versus a reference gene (18s rRNA) and normalized to the expression levels of normal B lymphocytes.

In vitro shear flow assays. VCAM-1 was mixed in coating medium [PBS, 20 mmol/L sodium bicarbonate (pH 8.5), and 2 μ g/mL HSA] and adsorbed overnight at 4°C, alone or with chemokine. HUVECs were tumor necrosis factor- α (TNF- α) stimulated for 16 h (2 ng/mL, 5 units/mL; R&D Systems). B cells and CLL cells were suspended in HBSS, 2 mg/mL bovine serum albumin, and 10 mmol/L HEPES (pH 7.4) and, at 37°C, perfused over endothelium or VCAM-1/chemokine-coated substrates, assembled as the lower wall of a flow chamber (260- μ m gap) mounted on inverted phase-contrast microscope (Diaphot 300, Nikon). Cell perfusion was videotaped and all cellular interactions were tracked (17). Interactions were defined as transient if cells attached briefly (<2 s) to the substrate and as stable arrests

if remaining stationary for at least 3 s. Frequencies of adhesive categories were determined as percentages of cells flowing immediately over the substrates, as described (18). For transmigration assays, cells were perfused over HUVECs at 0.75 dyn/cm² for 1 min followed by 5 dyn/cm² for 15 min. Motion analysis was done manually in high-power field (magnification, $\times 20$). Distinct categories (as percentages of originally accumulated lymphocytes) were as follows: (a) lymphocytes that rolled away or detached from the substrate during the shear application phase were considered detaching; (b) lymphocytes that remained stationary throughout the shear application phase were considered arrested; (c) lymphocytes that spread and migrated over the endothelium without crossing were considered locomoting; and (d) lymphocytes that spread, migrated for variable distances, and transmigrated through the ECs were considered transmigrating.

Mice. NOD/SCID mice were maintained under defined flora conditions in individually ventilated, sterile microisolator cages at the Weizmann Institute. All experiments were approved by the animal care committee of the Weizmann Institute. Human PBMCs ($15\text{--}25 \times 10^6$) of either healthy donors or CLL patients were injected into the tail vein of nonirradiated mice. For *in vivo* blocking experiments, cells were first preincubated for 30 min with 6 μ g/mL blocking anti- $\alpha 4$ (HPI/2) or anti- $\beta 2$ (TS1/18) antibody

and washed before the injection. Cells were recovered from LNs, BM, and spleen of the mice 3 h after transplantation and presence and number of human B cells were detected by flow cytometry using human-specific anti-CD45 and anti-CD19 antibodies.

Statistical analysis. Statistical analysis was performed using Statistical Package for the Social Sciences 14.0. All data were tested for normal distribution and *t* test was performed in normally distributed data sets only. PB-BM (data normally distributed) comparison was done with paired *t* test. In nonnormally distributed data sets, as, for example, association of LFA-1/VLA-4 expression with CD38, ZAP-70, IgVH gene mutations, and chromosomal aberrations, rank sum test (Mann-Whitney) was used.

Results

CLL cells do not home to LNs due to reduced LFA-1 expression. LFA-1 has a predominant function in lymphocyte trafficking to LNs (19). LN infiltration with malignant B lymphocytes frequently occurs in progressive CLL but how efficient CLL cells reach LNs and other lymphoid organs remains elusive. We therefore first compared the expression and functionality of the major lymphocyte integrin, LFA-1 (α L β 2, CD11a/CD18), in CLL and normal B lymphocytes. When LFA-1 expression on PB CLL cells derived from 105 patients was compared with that on B lymphocytes of 11 healthy age-matched persons, highly

significantly reduced LFA-1 expression was observed on CLL cells (Fig. 1A). This reduction did not result in decreased fractions of LFA-1-positive cells but rather of a global reduction in the mean fluorescence intensity (MFI) of LFA-1 expression on all CLL cells. Quantitative real-time PCR indicated that this lowered surface LFA-1 expression was the result of reduced transcripts of the β 2 integrin subunit (CD18; Fig. 1B) rather than of the α L (CD11a) subunit of the LFA-1 heterodimer. Interestingly, reduced LFA-1 expression on CLL cells was not restricted to the PB CLL pool because CLL cells derived from BM aspirates expressed similarly reduced levels of LFA (Fig. 1C). Dissecting LFA-1 expression on healthy BM B-cell types, we found lowest LFA-1 levels on pro-B and pre-B cells, comparable with those found in CLL cells, whereas at later maturation stages of normal B lymphocytes LFA-1 expression was very high (Supplementary Fig. S1). Thus, CLL cells express lower LFA-1 than PB and BM mature normal B lymphocytes but similar levels to those on pro-B-lymphocyte and pre-B-lymphocyte maturation stages from normal donors. We next evaluated the relationship between LFA-1 expression and commonly used prognostic parameters. LFA-1 expression was significantly higher in CD38 high-risk than low-risk groups (Fig. 1D) by trend higher in patients with clinically unfavorable chromosomal aberrations but not associated with any other tested risk parameter (Supplementary Table S2).

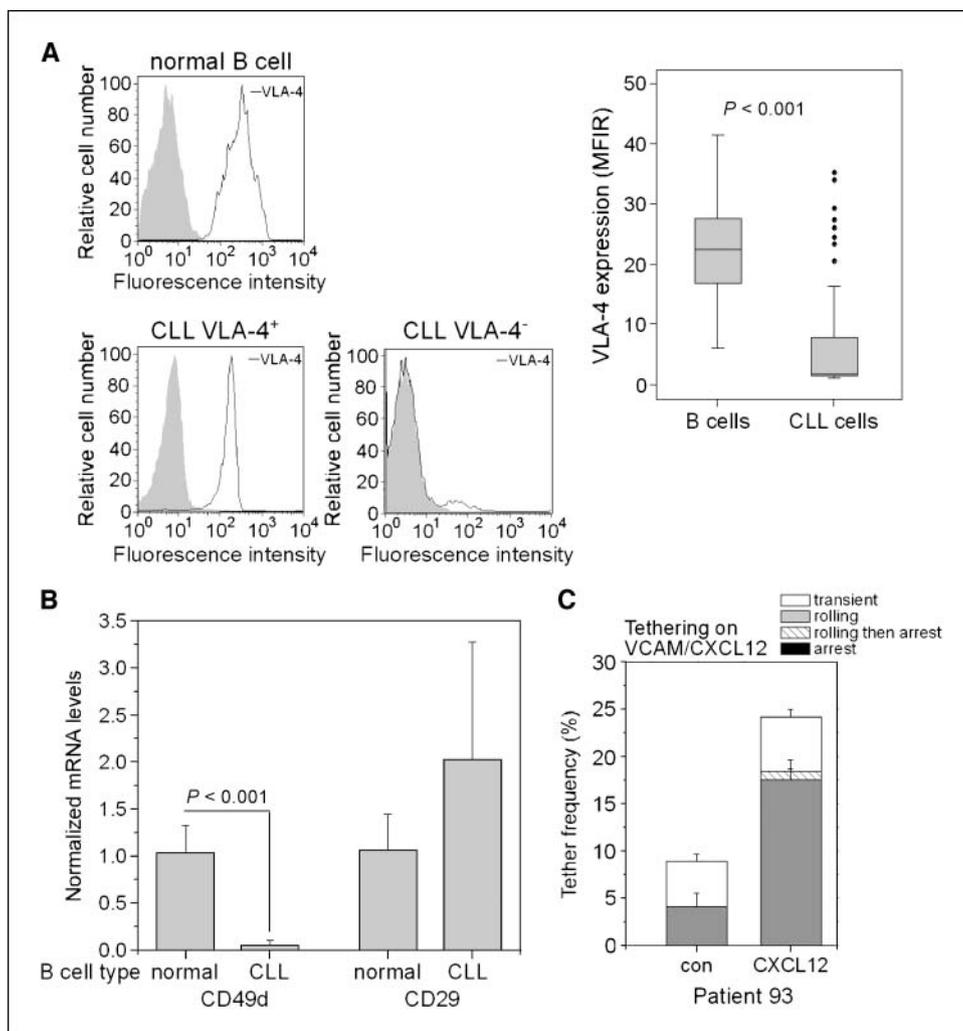


Figure 3. VLA-4 expression is decreased in subsets of CLL lymphocytes. **A**, MFIRs of VLA-4 expression on B lymphocytes of 11 healthy donors (defined by gating CD19⁺ expression) and 105 CLL patients (defined by gating CD19⁺ CD5⁺). Data are presented in box-and-whisker format. **B**, relative mRNA levels of α 4 (CD49d) and β 1 (CD29) subunits were determined from purified CLL or B cells by real-time quantitative PCR as described in Materials and Methods and are shown normalized to healthy B-cell levels. CD49d levels were significantly reduced in CLL cells ($n = 13$). **C**, CLL cells of VLA-4⁺ patients were perfused for 1 min at 0.75 dyn/cm² over VCAM-1 coimmobilized with heat-inactivated (–) or functional (+) CXCL12. Categories of interactions (tethers) are expressed as frequencies of cells in direct contact with the substrate. Data are expressed as the mean \pm range of two fields in view and depict one experiment representative of experiments with eight patients.

Table 1. Expression of VLA-4 in different prognostic risk groups

Risk parameters			P
Total	1.3 (n = 105)		
Median VLA-4 expression on CLL cells (MFIR)			
CD38 B-CLL cell risk	Low	High	0.0004
Median VLA-4	1.13	7.02	
	n = 62	n = 18	
ZAP-70 risk	Low	High	n.s.
Median VLA-4	3.12	2.44	
	n = 33	n = 48	
IgVH mutation status	Mutated	Unmutated	0.001
Median VLA-4	1.05	4.48	
	n = 46	n = 26	
Cytogenetic aberrations	Favorable	Unfavorable	0.01
Median VLA-4	1.05	5.61	
	n = 31	n = 16	

NOTE: Expression of VLA-4 in different prognostic risk groups. VLA-4 expression was compared in different favorable or unfavorable clinical risk groups as defined by the following standard prognostic thresholds: CD38 (expression on <30% versus \geq 30% of CLL cells), ZAP-70 (NKT/B ratio >2.5 versus \leq 2.5), IgVH mutational status (>2% as mutated versus \leq 2% regarded unmutated), and cytogenetics [favorable (normal, del13q) versus unfavorable (tri12, del11q, del17p)].

Abbreviation: n.s., not significant.

To determine if the residual LFA-1 on CLL cells can be activated by endothelial-displayed chemokines and support adhesiveness to the key LFA-1 ligand, ICAM-1, we used an *in vitro* flow chamber assay to measure CLL interactions with a monolayer of ECV-304-LL (17). This cell line expresses ICAM-1 and L-selectin ligands expressed by LN high endothelial venules (HEV), but not the VLA-4 ligand VCAM-1 or the endothelial E-selectin or P-selectin, and serves as a model substrate for the analysis of LFA-1–dependent but VLA-4–independent arrest of lymphocytes under shear flow (17, 20). When overlaid with CXCL12, ECV-304-LL cells supported robust LFA-1–mediated arrest of normal B lymphocytes (Fig. 2A, *left*) under physiologic shear flow. Importantly, all CXCL12-triggered B-lymphocyte arrest on the ECV-304-LL monolayer was inhibited by LFA-1 blockage (Fig. 2A, *right*). In contrast, CLL cells failed to arrest on ECV-304-LL even upon CXCL12 activation (Fig. 2A, *left*). This failure was not the result of impaired CLL responsiveness to CXCL12 because all CLL expressed high levels of CXCR4 and migrated toward CXCL12 in chemotaxis assays (data not shown). Furthermore, the residual LFA-1 on CLL cells could still undergo conformational activation by CXCL12, as evident from the induction of the β 2 activation epitopes KIM127 and 327C in CLL cells incubated with the chemokine (Supplementary Fig. S2; ref. 21). Thus, the inability of CLL cells to arrest on endothelial ICAM-1 in response to CXCL12 signals was the outcome of reduced LFA-1 expression rather than a defect of direct CXCL12 signaling to the LFA-1 heterodimer.

Lymphocyte entry into LNs is critically regulated by LFA-1 (22). We next compared the capability of normal human B lymphocytes and CLL cells to home to LNs of NOD/SCID mice in short-term adoptive assays. PBMCs of either healthy donors or CLL patients were injected into the tail veins of the mice and human B lymphocytes were identified 3 hours after injection in explanted peripheral and mesenteric nodes (Fig. 2B). Whereas normal B lymphocytes were easily detected in both types of LNs, CLL cells could be hardly detected. As expected, functional blocking LFA-1

on the normal B lymphocytes almost completely abrogated their LN homing (Fig. 2B). Collectively, these data suggest a major extravasation defect of CLL cells into LNs.

Subgroups of CLL cells express functional and chemokine-responsive VLA-4 but require LFA-1 to migrate through activated endothelium under shear flow conditions. We next analyzed VLA-4 expression on CLL cells by flow cytometry. VLA-4 (α 4 β 1, CD49d/CD29) was found to be the exclusive member of the α 4 integrin subfamily expressed by CLL cells. The second family member, the α 4 β 7 integrin, a counterreceptor to the gut-enriched endothelial ligand MadCAM-1 (23), was essentially undetectable, and therefore, all subsequent α 4 staining results were attributed to α 4 β 1 (data not shown). We found that VLA-4 expression was variable on CLL cells and significantly reduced compared with normal B cells (Fig. 3A). Reduced VLA-4 expression was due to a major reduction in α 4 subunit transcripts as determined by real-time PCR (Fig. 3B). VLA-4 (CD49d) was expressed heterogeneously in CLL subgroups and variable patterns of VLA-4 fluorescence intensity clearly corresponded to different percentages of VLA-4–positive (VLA-4⁺) CLL subpopulations as previously described (24). Comparing VLA-4 and LFA-1 expression, we also found lowest VLA-4 levels on CLL cells deficient in LFA-1 expression. Highly significant associations of VLA-4 and IgVH status, CD38 expression, and cytogenetic aberrations were detected, but no significant associations between VLA-4 and ZAP-70 could be established (Table 1).

Immobilized CXCL12 rapidly activates VLA-4 on lymphocytes tethered to the chemokine in the presence of purified VCAM-1 (18). We tested if VLA-4 expressed on CLL cells is able to undergo chemokine-induced activation and support CLL arrest on VCAM-1 under shear stress conditions. VLA-4 on CLL cells perfused over a VCAM-1–presenting substrate underwent significant activation by surface-bound CXCL12 (Fig. 3C). CXCL12-induced arrests on VCAM-1 were highly susceptible to CXCR4 blocking with AMD3100 (data not shown). VLA-4–negative (VLA-4[–]) CLL

samples were not able to interact with a VCAM-1-presenting surface and did not respond to any CXCL12 signals, suggesting that CXCL12-CXCR4 interactions on their own are not adhesive (data not shown).

Interaction of VLA-4 ($\alpha 4$, CD49d) with VCAM-1 was implicated in B-cell adhesion to activated endothelium under shear stress (25). We therefore compared VLA-4⁺ and VLA-4⁻ CLL cell accumulation

on TNF- α -activated HUVECs, a prototype for cytokine-activated vascular endothelium expressing high levels of E-selectin, VCAM-1, and ICAM-1 (26), using normal B cells as reference lymphocytes. TNF- α -activated HUVECs supported normal B-lymphocyte rolling, but only small numbers of lymphocytes could arrest on the endothelial monolayer unless encountering a potent integrin stimulatory chemokine, such as CXCL12 (Fig. 4A, left). Accordingly,

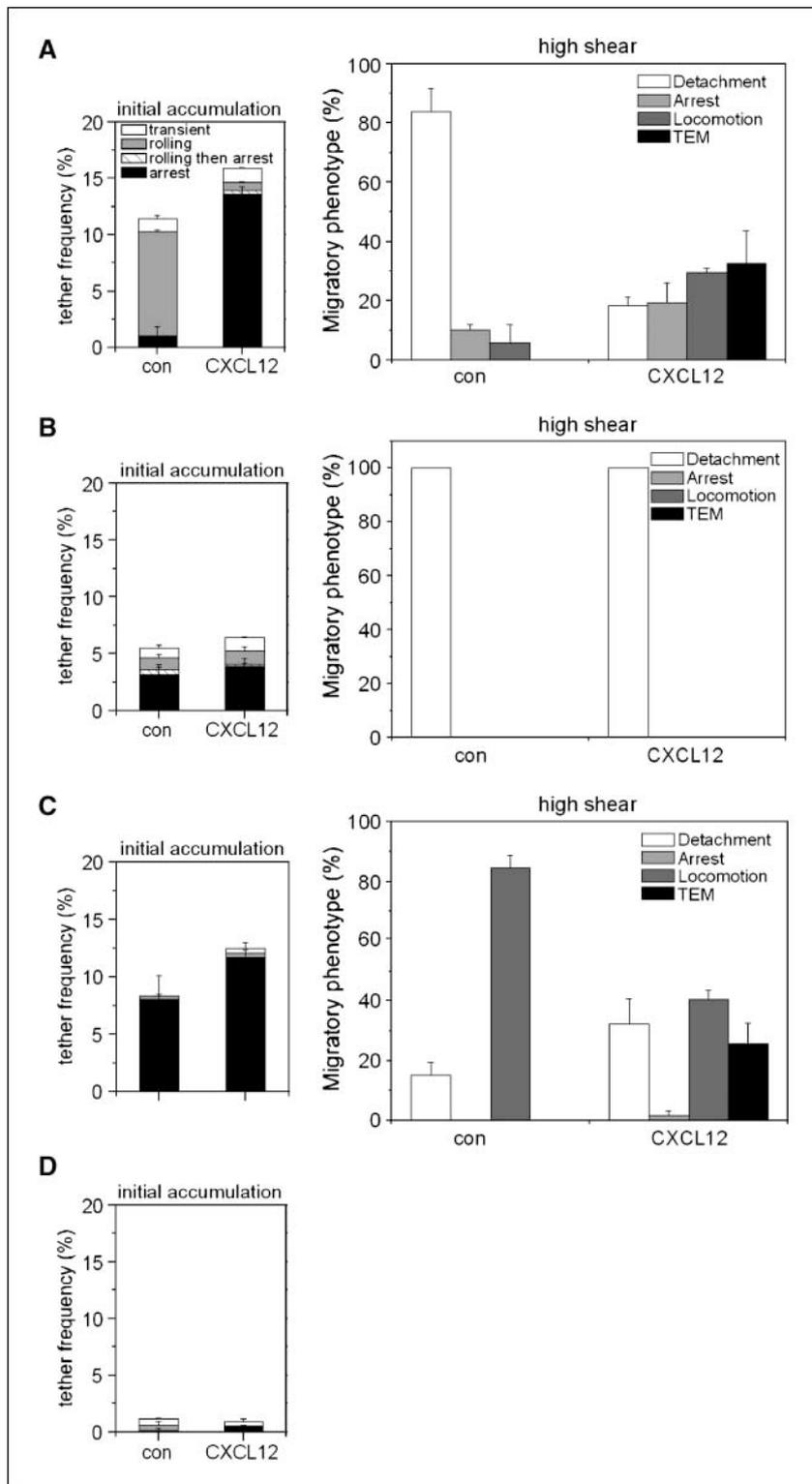


Figure 4. CLL cells require LFA-1 to transmigrate across activated endothelium under shear flow. *A, left*, normal B-cell accumulation on TNF- α -stimulated HUVEC cells. Purified B cells were perfused over the HUVEC monolayer at 0.75 dyn/cm² for 60 s. Where indicated, HUVECs were preoverlaid with CXCL12. Categories of interactions were determined in frequencies of interacting cells within the cell flux in direct contact with the monolayer and are expressed as the mean \pm range of two fields in view. *Right*, B cells accumulated at 0.75 dyn/cm² for 60 s on HUVECs alone or overlaid with CXCL12 were subjected to physiologic shear stress (5 dyn/cm²) for 15 min. Shown are relative numbers of each migratory phenotype. *B, left*, VLA-4⁺ CLL cell accumulation on TNF- α -stimulated HUVEC cells with and without CXCL12 overlay; *right*, effects of CXCL12 on the adhesive and migratory phenotype of the accumulated CLL cells when subjected to physiologic shear stress after the accumulation phase. Example patient 105, Supplementary Table S1, representative for five tested patients. *C, left*, effects of CXCL12 on the accumulation of LFA-1 and VLA-4⁺ CLL cells on TNF- α -stimulated HUVEC; *right*, effects of CXCL12 on the adhesive and migratory phenotypes of the initially accumulated CLL cells shown in the left panel. Example patient 74, Supplementary Table S1. *D*, accumulation of VLA-4⁻ LFA-1 low CLL cells on TNF- α -stimulated HUVEC cells. Example patient 95, Supplementary Table S1, representative for three tested patients.

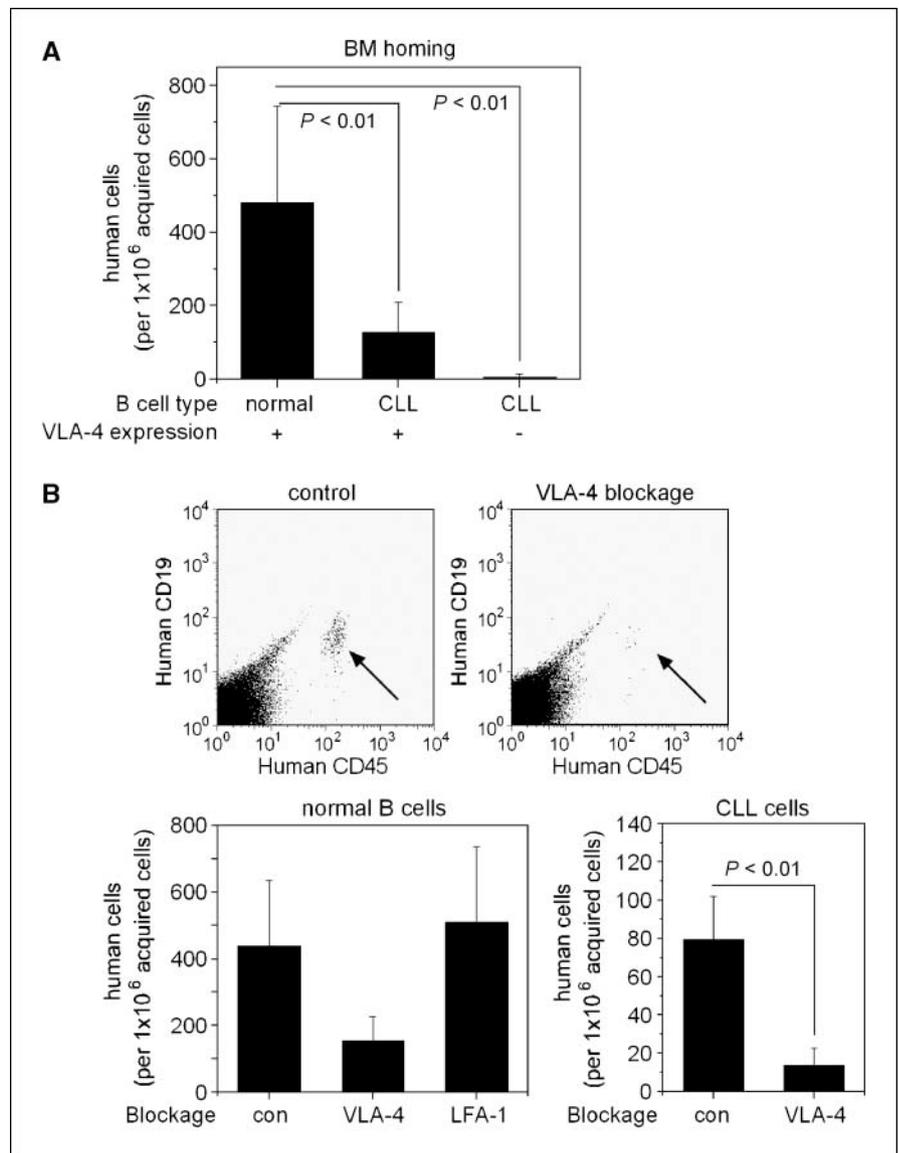


Figure 5. Functional VLA-4 expression is indispensable for the homing of normal and CLL cells to the BM. **A**, PBMCs ($15\text{--}25 \times 10^6$) of either healthy persons or CLL patients were injected into NOD/SCID mice, and after 3 h, human B cells or CLL cells were detected in the BM by flow cytometry using specific anti-human CD19 and anti-CD45 antibodies. *Columns*, mean of detected human CD19⁺, CD45⁺ cells per 10^6 acquired BM cells and 10^6 injected PBMCs either from healthy donors ($n = 10$) or from VLA-4⁺ ($n = 7$) or VLA-4⁻ ($n = 4$) CLL samples; *bars*, SD. **B**, PBMCs were treated with blocking LFA-1 or VLA-4 antibodies and injected into NOD/SCID mice. Human cells were determined as in **A**. *Columns*, mean of experiments with three healthy donors and four VLA-4⁺ CLL patients; *bars*, SD.

occasionally arrested B lymphocytes readily detached on application of physiologic shear flow and did not transmigrate the endothelium unless encountering CXCL12 activation (Fig. 4A, right). CXCL12 stimulated not only B-lymphocyte arrest but also adhesion strengthening, locomotion (crawling) on the EC monolayer, and TEM (Fig. 4A, right). In contrast to normal B cells, VLA-4⁺ LFA-1 low CLL cells accumulated on the HUVECs but exhibited very poor response to CXCL12 (Fig. 4B, left). Consequently, all of these arrested CLL cells detached from the endothelium and none could transmigrate under shear flow (Fig. 4B, right). Interestingly, a rare subset of VLA-4⁺ CLL cell samples, with exceptionally high LFA-1 expression (Supplementary Table S1, patient 74, see also outliers Fig. 1A), resisted detachment from the TNF- α -activated HUVECs after initial arrest, and in response to CXCL12, a significant fraction of these cells also successfully transmigrated through HUVECs (Fig. 4C). CLL cells, negative for VLA-4 and low in LFA-1, failed to transiently interact with the activated HUVECs even in the presence of CXCL12 (Fig. 4D). Taken together, these results suggest that VLA-4 on CLL cells, although responsive to chemokine signals, must cooperate with LFA-1 to establish optimal

adhesion strengthening and TEM capacity across inflamed endothelial barriers under shear flow.

Functional VLA-4 is indispensable for residual CLL homing to BM, whereas LFA-1 is not. Our *in vitro* experiments suggested that the loss of LFA-1 adhesiveness and the defective trans migratory capability of CLL cells across E-selectin-expressing, VCAM-1-expressing, and ICAM-1-expressing HUVECs may result in impaired *in vivo* CLL crossing of BM endothelial cells, known to also coexpress these adhesion molecules (27). We therefore next investigated the capability of CLL cells and normal B lymphocytes to home to BM of NOD/SCID mice in short-term adoptive assays. Consistent with their *in vitro* properties, VLA-4⁺ CLL samples homed in significantly lower numbers to the murine BM than normal B lymphocytes ($P = 0.005$; Fig. 5A) and VLA-4⁻ CLL completely failed to reach the BM ($P = 0.002$; Fig. 5A). Consistent with their *in vitro* properties, VLA-4⁺ CLL samples homed in significantly lower numbers to the BM than normal B lymphocytes ($P = 0.005$; Fig. 5A) and VLA-4⁻ CLL completely failed to reach the BM ($P = 0.002$; Fig. 5A). Residual VLA-4⁺ BM homing of CLL cells could be blocked by anti- $\alpha 4$ antibody ($P = 0.013$), showing that

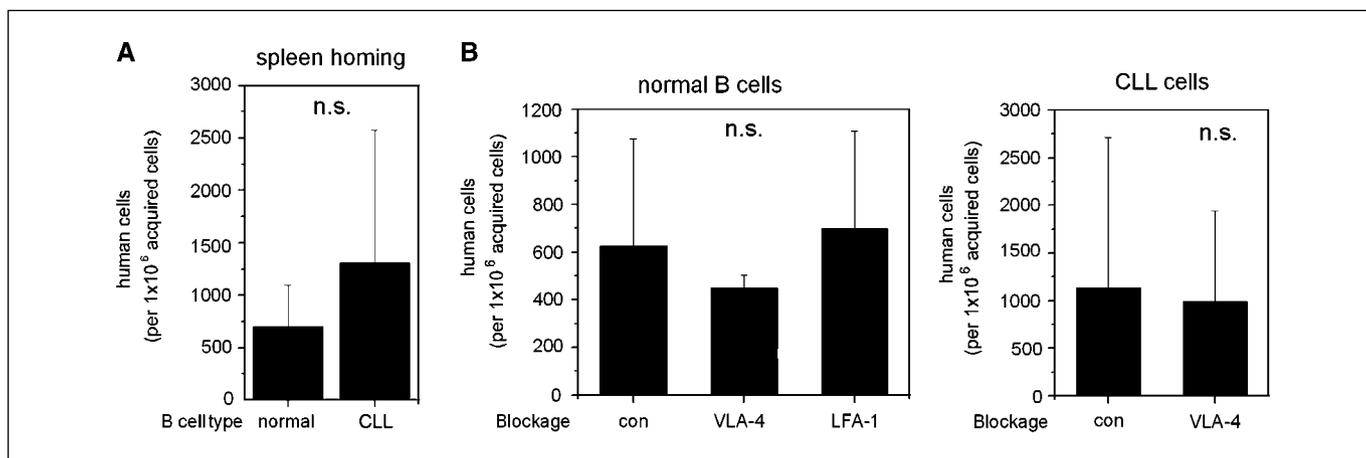


Figure 6. CLL cells preferentially home to spleen. *A*, PBMCs ($15\text{--}25 \times 10^6$) of either healthy persons or CLL patients were injected into NOD/SCID mice, and after 3 h, human B cells or CLL cells were detected in the spleen by flow cytometry using specific anti-human CD19 and anti-CD45 antibodies. *Columns*, mean of detected human CD19⁺, CD45⁺ cells normalized to 10^6 acquired spleen cells and 10^6 PBMC cells from either healthy donors ($n = 7$) or CLL patients ($n = 8$); *bars*, SD. *B*, PBMCs were treated with blocking LFA-1 or VLA-4 antibodies and injected into NOD/SCID mice. Human cells were determined as in *A*. *Columns*, mean of three to four experiments; *bars*, SD.

CLL migration across BM endothelial barriers is VLA-4 integrin dependent (Fig. 5*B*).

CLL cells home to the spleen in LFA-1-independent and VLA-4-independent manner. Blood lymphocytes recirculate through the spleen (28). In light of their deficient LFA-1 functions, we next wished to assess the homing of CLL cells to the spleen. Notably, in contrast to the poor homing rates of VLA-4⁺ CLL cells to BM, the lack of VLA-4⁻ CLL cell homing to BM, and the complete failure of both VLA-4⁺ and VLA-4⁻ CLL cells to home to LNs, CLL cells readily homed to the spleen. Relative spleen homing of CLL cells was by trend higher than that of normal B cells (Fig. 6*A*) without any difference related to LFA-1 or VLA-4 expression. In absolute numbers, we recovered in average ~8% (range, 2–25%) of the injected human B or CLL lymphocytes in LNs, BM, and spleen 3 hours after the injection. The highest absolute numbers of human cells were thereby consistently observed in the murine spleens. After injection of normal B lymphocytes, 85% of the recovered B cells were located in the spleen, and after injection of CLL lymphocytes, 98% of the recovered cells were located in the spleen, whereas only 2% and 0% of the recovered CLL cells entered the BM or LN, respectively, further showing the deficient CLL homing capability to these organs. Interestingly, either pretreatment of normal B lymphocytes with VLA-4 or LFA-1 blocking antibodies or pretreatment of VLA-4⁺ CLL cells with anti-VLA-4 blocking antibody did not alter their homing to the spleen (Fig. 6*B*). Thus, both normal and CLL lymphocyte migration to the spleen was VLA-4 or LFA-1 integrin independent. These results collectively suggest that the defective ability of CLL to home to the LNs and the BM is mirrored by enhanced retention in the circulation and a preferential tendency to enter the spleen.

Discussion

The TEM of lymphocytes and their localization in primary and secondary lymphoid organs depend on multiple intercellular adherence mechanisms, primarily governed by integrins (29). Changes in expression or function of integrins on malignant lymphocytes can lead to different extravasation, homing, and retention properties of these cells and thereby dramatically alter

leukemic cell fate (30). Little is known about the capacity of circulating CLL cells to extravasate at distinct lymphoid organs and the specific roles of the two major lymphocyte integrins LFA-1 and VLA-4 in this process. Furthermore, although VLA-4 was recently suggested as a novel prognostic marker in CLL (24, 31, 32), the specific functions of this integrin and of the second major lymphocyte integrin, LFA-1, in CLL trafficking have been elusive. Importantly, our present results suggest that CLL trafficking through the BM, the spleen, and the LNs shares similar integrin usage with the trafficking of normal B lymphocytes through these organs. We find that normal B cells use VLA-4 but not LFA-1 to enter the BM, require LFA-1 to enter LNs, and enter the spleen independently of either VLA-4 or LFA-1. Whereas CLL trafficking seems to be governed by the same molecular pathways, the low LFA-1 and variable VLA-4 expression result in significant deficiencies in entering lymphoid organs other than the spleen. Consequently, the majority of CLL samples tested by us while failing to enter LNs and poorly migrating to the BM preferentially homed to the spleen despite inherently deficient LFA-1 or VLA-4 expression and function.

The severity of CLL is generally described using staging systems devised by Rai and colleagues (33) and Binet and colleagues (34). To exclude chemotherapeutic influences on integrin expression and signaling, and to study migratory behavior of CLL cells at the beginning of the disease, our study concentrated on CLL samples of currently untreated patients. Consequently, our cohort is mostly composed of early and intermediate stages (Rai 0, I, and II; Supplementary Table S1). The majority of these CLL samples expressed severely reduced LFA-1 levels and failed to home to LNs but exhibited spleen tropism (19). Lymphocyte spleen homing does not necessarily require integrin activation (35). Our CLL homing data are reminiscent of those of murine LFA-1 knockout mice, which exhibit impaired LN entry but high tropism toward spleen (19). In addition, VLA-4 blockage did not alter the spleen homing of either normal B cells or CLL cells in our experiments. Our results are furthermore highly consistent with a recently published CLL xenograft model in which highest absolute numbers of human cells were recovered in murine spleens (36). These recovered CLL cells displayed an activated and proliferative phenotype with

paraimmunoblast- or polymphocytic-like morphology, thus indicating the spleen microenvironment to be favoring for CLL cells (36). It is therefore possible that in early and intermediate stages of CLL, malignant B lymphocytes released from the BM fail to return to the BM or enter LNs and prefer integrin-independent pathways to enter and temporally accumulate in the spleen where they may gain survival and proliferation signals. Indeed, progressive spleen enlargement is a feature of Tc1-1 transgenic mice developing a CLL-like disorder resembling human B-CLL (37).

Our results are in accordance with previous reports on low integrin expression of CLL cells (38, 39). In addition, consistent with our findings, a very early human study using ^{51}Cr labeling indicated that CLL cells leave the circulation less rapidly than lymphocytes from healthy persons (40). Furthermore, [^3H]thymidine-labeled CLL cells were shown to survive in the circulation of a CLL patient for many weeks without any evidence of extravasation (41). As we observe that CLL cases with high LFA-1 expression and transmigratory capacity can enter LNs at low numbers (data not shown), we cannot exclude that small CLL subsets, underrepresented in the circulating pools characterized by us, successfully home to LNs. As LN HEVs express VCAM-1 (42), VLA-4 might also contribute to a very low entry of CLL into LNs. In spite of the overall reduced entry of CLL cells to LN, these cells may nevertheless accumulate in LNs due to decreased egress through efferent lymphatics in light of reduced expression or activity of egress receptors, a possibility that should be addressed in the future. Another nonmutually exclusive possibility is high proliferation of small CLL subsets within pseudofollicles (43). Interestingly, we find significantly higher LFA-1 expression in CD38 high-risk CLL samples. LFA-1 expression also defines the proliferative and/or progressive pool in multiple myeloma and acute myeloid leukemia and LFA-1 antagonists are under evaluation (see ref. 30 for review). In addition, small lymphocytic leukemia cells express high LFA-1 and are essentially compartmentalized within LNs with few circulating cells (44). In contrast, in CLL, the circulating pool may be disproportionately higher, probably due to their reduced LFA-1 and VLA-4 integrins obligatory for constitutive extravasation from blood vessels into LNs and BM. Interestingly, in a subgroup of CLL patients with clinical lymphadenopathy, autocrine vascular endothelial growth factor (VEGF) was argued to promote a cross-talk between VLA-4 and LFA-1 and thereby augment CLL TEM (45). Nevertheless, the role of VEGF in LFA-1 activation was shown in the absence of shear flow (45), and so, its ability to activate CLL LFA-1 within the vasculature should be further addressed.

Our results emphasize the importance of VLA-4 expression and function in CLL homing to BM. VLA-4 plays a key role not only in lymphocyte adhesion to and extravasation through endothelial barriers expressing its key ligand, VCAM-1, but also in hematopoietic cell retention to the BM stroma, which is a prerequisite for progenitor cell proliferation (7). Only VLA-4⁺ CLL cells were able to residually home to BM. Interestingly, pre-B-ALL cells also display the low LFA-1 expression (46) and use VLA-4 rather than LFA-1 to home to the BM (12). This central role of VLA-4 in CLL-BM interactions is clinically interesting because VLA-4-dependent CLL

adhesion to VCAM-1 or fibronectin at BM niches might contribute to cell adhesion-mediated drug resistance.

Our study was mostly conducted on stages Rai 0, II, and II, and within those groups, the majority of CLL cells expressed highly reduced levels of both investigated integrins, VLA-4 and LFA-1. Furthermore, our data support recent observations achieved on a xenoplant CLL model (36) in which human CLL cells were recovered at low numbers from BM but at high numbers from spleen. BM recovery, and possibly also LN recovery, may correlate with clinical disease activity. Very recently, three groups suggested VLA-4 (CD49d) as a novel independent prognostic marker for CLL predicting overall survival (24, 31) and/or progressive disease (31, 32). The studies differ due to cohort variations in their results about associations of VLA-4 expression to several prognostic parameters, such as ZAP-70 or IgVH mutational status, but unequivocally observe high association of VLA-4 expression with CD38 (31). In complete accordance to these recent reports, we observed highly significant higher VLA-4 expression in CD38 high-risk groups compared with CD38 low-risk groups with nearly total loss of VLA-4 in the low-risk group. We also found significant higher VLA-4 expression in CLL samples of patients with unmutated IgVH status and with unfavorable cytogenetic abnormalities (trisomy 12, deletion11q, deletion 17p).

The low expression of both VLA-4 and LFA-1 in low-risk CLL groups might explain the more favorable clinical course of these patients because it obviously restricts circulating CLL cells from reentering the BM and from homing to the LNs, organs at which these cells are likely to encounter antiapoptotic factors. Our results also highlight the possibility that VLA-4 and LFA-1 detection could be useful prognostic markers of CLL severity. More importantly, it can aid in evaluating the susceptibility of CLL cells to VLA-4 and LFA-1 blocking therapy. Anti-VLA-4 antibody treatment also causes CD34⁺ progenitor cell mobilization from the BM (47). Stem cell mobilization is generally associated with functional inactivation or down-regulation of VLA-4-VCAM-1 interactions within the BM niches (48). Therefore, anti-VLA-4 blocking therapy could be highly effective in releasing VLA-4⁺ CLL from the BM and preventing their reentry to the BM. Together with their reduced levels of LFA-1, these VLA-4-blocked CLL cells will be restricted from emigration into supportive LN and BM niches, home to the spleen, and may be rendered more susceptible to CLL chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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