Comparative analysis of normal versus CLL B-lymphocytes reveals patient-specific variability in signaling mechanisms controlling LFA-1 activation by chemokines

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Running Title
Diversity in pro-adhesive signaling mechanisms in B-CLL lymphocytes

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CLL leukemia, chemokines, signal transduction, adhesion, integrins
Abstract

Integrins critically regulate leukocytes trafficking and are also involved in widespread dissemination of leukemia cells to tissues. Activation of lymphocyte function-associated antigen-1 (LFA-1) by chemokines is fine-tuned by inside-out signaling mechanisms responsible for integrin-mediated adhesion modulation. In the present study we investigated the possibility of qualitative variability of signaling mechanisms controlling LFA-1 activation in chronic lymphocytic leukemia cells. We pursued a multiplexed comparative analysis of the role of the recently described chemokine-triggered rho-signaling module in primary normal versus CLL B-lymphocytes. We found that the rho module of LFA-1 affinity triggering is functionally conserved in normal B-lymphocytes. In contrast, in malignant B-lymphocytes isolated from B-CLL patients the role of the rho module was not maintained, showing remarkable differences and variability. Specifically, RhoA and phospholipase D1 (PLD1) were crucially involved in LFA-1 affinity triggering by CXCL12 in all analyzed patients. In contrast, Rac1 and CDC42 involvement displayed a consistent patient-by-patient variability, with a group of patients showing LFA-1 affinity modulation totally independent of Rac1 and CDC42 signaling activity. Finally, phosphatidylinositol-4-phosphate 5-kinase isoform 1γ (PIP5KC) was found without any regulatory role in all patients. The data imply that the neoplastic progression may completely bypass the regulatory role of Rac1, CDC42 and PIP5KC and show a profound divergence in the signaling mechanisms controlling integrin activation in normal versus neoplastic lymphocytes, suggesting that CLL patients can be more accurately evaluated on the basis of the analysis of signaling mechanisms controlling integrin activation. Our findings may potentially impact diagnosis, prognosis and therapy of CLL disorders.
Introduction

Chronic lymphocytic leukemia (CLL) of the B-lymphocyte lineage is characterized by enhanced trafficking and accumulation of CD5+ cells in the bone marrow and in secondary lymphoid organs (1). It is well known that widespread accumulation of malignant lymphocytes is associated with progressive disease and an adverse prognosis. Indeed, tissue invasion by malignant cells is an important pathogenetic process in CLL and represents the basis of current clinical staging procedures. Thus, understanding the molecular mechanisms governing tissue invasion in CLL is of evident clinical relevance.

As in normal lymphocytes, the combinatorial activity of chemokines and adhesion molecules controls tissue-selective dissemination of B-CLL cells (2). For instance, neoplastic B-lymphocytes from B-CLL patients show increased levels of chemokine receptors such as CCR7, CXCR4 and CXCR5, all involved in B-lymphocyte homing, and probably contributing to the accumulation of B-CLL cells in secondary lymphoid organs (2, 3). CXCR4-CXCL12 axis is also critical to maintain malignant lymphocytes in the stroma and to protect them from apoptosis (4). The β1 integrin VLA-4 (very late antigen-4) and the β2 integrin LFA-1 (lymphocyte function-associated antigen-1), which have a central role in mediating trafficking of normal circulating lymphocytes (5), are likely to play a similar regulatory role also in B-CLL lymphocyte accumulation in different tissues.

Integrin activation by chemokines is a key step in leukocyte recruitment and is commonly described as a complex process characterized by changes in heterodimer conformation, leading to increased affinity (6), and in redistribution on the plasmamembrane leading to integrin clustering, responsible for increase in valency (7). Overall, enhanced integrin affinity and valency lead to increased cell avidity (adhesiveness) for the substrate. The lymphocyte function-associated antigen-1 (LFA-1; αLβ2; CD11a/CD18) is the most studied integrin in immune cells and its conformational changes leading to affinity increase are well characterized (8-10). Upon activation, LFA-1 undergoes dramatic structural changes implying rapid and reversible transitions from a bent to a fully extended conformationic structure responsible for a progressive increase of binding energy (affinity) for the
ligand supporting rapid arrest under flow of circulating cells (6). These conformational transitions are under the control of complex intracellular signaling mechanisms triggered by chemokine receptors. Recently, we have shown that, in human primary normal T-lymphocytes, the concurrent activity of the three main rho small GTPases, RhoA, Rac1 and CDC42, as well as of two main rho effectors, phospholipase D1 (PLD1) and phosphatidylinositol-4-phosphate 5-kinase isoform 1γ (PIP5KC), generates a coherent signaling module controlling LFA-1 affinity modulation by CXCL12 in a conformer-selective manner, with PIP5KC specifically controlling LFA-1 transition to high, but not to intermediate, affinity state (11).

The aim of this study was a comparative characterization of the regulatory role of the rho-module of LFA-1 affinity modulation in human primary normal B-lymphocytes versus malignant B-lymphocytes isolated from B-CLL patients. Our results show that primary B-lymphocytes share the same mechanisms of LFA-1 affinity modulation by chemokines with primary T-lymphocytes, thus suggesting the universality of the rho-module of LFA-1 activation in normal lymphocytes. In contrast, we discovered that in malignant B-CLL lymphocytes the regulatory relevance of the rho-module of LFA-1 affinity modulation is not conserved. Indeed, by analyzing several B-CLL patients, we observed a consistent variability of the regulatory role of molecules forming the rho-module, with RhoA and PLD1 always critical to regulation of LFA-1 affinity induced by CXCL12, whereas, in contrast, Rac1 and CDC42 displayed a patient-restricted role. Interestingly, PIP5KC, which critically regulates LFA-1 in a conformer-selective manner in normal lymphocytes, appears without any regulatory role in neoplastic B-lymphocytes. These data shed light on the mechanisms of integrin activation in leukemic cells and suggest that in B-CLL lymphocytes the neoplastic progression can make irrelevant the regulatory role of signaling events otherwise critical in normal primary lymphocytes. On this basis, we could group the patients in at least two categories, characterized by divergent signaling mechanisms regulating LFA-1 affinity triggering by CXCL12. These findings may potentially impact diagnosis and treatment of CLL disease.
Materials and Methods

Reagents

FBS was from Irvine; human CXCL12, human ICAM-1 and human E-selectin were from R&D Systems; phycoerythrin-conjugated anti-mouse antibody was from Jackson ImmunoResearch; anti-LFA-1 monoclonal antibodies TS1-22 and KIM127 (reporter for extended conformation epitope possibly corresponding to intermediate affinity state (12)) were from ATCC; anti-LFA-1 monoclonal antibodies 327C and A (reporters for extended conformation epitope corresponding to high affinity state (13)) were by ICOS, corp. USA.

Isolation of B-lymphocyte population from healthy subjects and B-CLL patients

Peripheral blood mononuclear cells (PBMCs) from Ficoll-processed buffy coats derived from healthy donors were resuspended in PBS + 2% FBS at 5x10^7/ml; purification of B-lymphocyte population was performed by negative selection (EasySep® Human B Cell Enrichment Kit, #19054, StemCell Technologies Inc.), according to manufacturer's instructions. Purity of B-lymphocyte preparations was evaluated by flow cytometry after staining with a fluorochrome-conjugated antibody against CD19. CLL B-lymphocytes were isolated from PBMCs after blood sample separation on Lymphomed (Midimed) and purification by negative selection as described above. The study involved 31 patients with B-CLL. The diagnosis of B-CLL was made upon clinical and laboratory parameters, including the complete blood cell count, peripheral blood smear, immunophenotype of the circulating lymphoid cells, bone marrow aspirate and biopsy, and cytogenetics, according to the current guidelines (14) and fulfilling diagnostic and immunophenotypic criteria for common B-CLL at the hematology section of the Department of Clinical and Experimental Medicine, University of Verona. All samples were obtained with informed consent and with the approval of the local Ethics Committee. Patients had between 75 to 90% CLL cells. Normal and CLL B-lymphocytes were plated at 5x10^6/ml in RPMI + 2 mM Glutamine + 10% FBS for 3 hours before treatment with Trojan peptides or silencing with siRNAs.
**Trojan Peptide Technology**

Tat-fusion proteins were produced, FPLC-purified and tested for their specific activities as previously described (11). P1-based peptides, including control Penetratin-1 (P1), the RhoA blocking (P1-RhoA 23-40, that included P1 and the downstream switch I effector region of human RhoA encompassing amino acids 23-40 (11, 15, 16) and the PLD1-blocking (P1-PLD1, that included P1 and the region of human PLD1 that specifically mediated the interaction of PLD1 with RhoA (11, 17, 18)), were synthesized by GenScript. Cell treatment with Tat-proteins and P1-peptides was for 60-90 min at 37°C.

**Gene Silencing of PIP5KC by siRNA**

siRNAs targeting PIP5KC were designed according to GenBank accession number (NM_012398), chemically synthesized by Dharmacon and provided as premixed pool (SmartPool). Silencing was performed in normal and CLL B-lymphocytes by nucleoporation, using the Amaxa Nucleofector (Amaxa Biosystems) (11). Briefly, cells were resuspended in nucleofection buffer at 5x10⁷/ml and 3 μg of siRNAs were added according to the manufacturer's instructions. Nucleoporation was performed using U-15 program and then the cells were cultured as described above. Efficacy of gene silencing was evaluated by immunoblotting. Cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham). The primary antibody used was rabbit polyclonal anti-PIP5KC from Abgent (11). The corresponding peroxidase-labeled secondary antibody was detected with chemiluminescence HRP reagent (Millipore).

**Static Adhesion Assay**

B-lymphocytes were resuspended at 5x10⁶/ml in standard adhesion buffer (PBS + 10% FBS + Ca²⁺ 1 mM + Mg²⁺ 1 mM, pH 7.2). Adhesion assays were performed on 18-well glass slides coated overnight at 4°C with human ICAM-1, 1 μg/ml in PBS. 20 μl of cell suspension were added to the
well and stimulated at 37°C with 5 µl of CXCL12, 0.5 µM final concentration, for 30 sec. After washing, adherent cells were fixed in glutaraldehyde 1.5% in ice-cold PBS and counted by computer-assisted enumeration (7).

Under-flow Adhesion Assay
100 µl microcap glass capillary tubes (1 mm internal diameter, from Drummond) were first coated for 10 h at 4°C with 1 µg/ml human E-selectin in PBS; tubes were then washed and coated over night at 4°C with 1 µg/ml human ICAM-1 in PBS. Before use, tubes were treated with FCS for 10 min, washed and then coated with 2 µM CXCL12 in PBS for 30 min. After extensive washing with PBS, the behavior of interacting B-lymphocytes (shear stress was 2 dyne/cm²) was recorded from fast CCD videocamera on DV digital videotape recorder (25 frames/s, capable of 1/2 sub frame 20 msec recording) and analyzed sub frame by sub frame. Single areas of 0.2 mm² were recorded for at least 120 s. Interactions of > 20 ms were considered significant and were scored. Lymphocytes that remained firmly adherent for at least 1 s were considered fully arrested. Cells arrested for at least 1 s and then detached (a sign of rapid inside-out affinity triggering) or for 10 s and remained adherent (possibly implying post-binding adhesion stabilization) were scored separately and plotted as independent groups.

Measurement of LFA-1 Affinity States
B-lymphocytes, resuspended in standard adhesion buffer at 2x10⁶/ml, were briefly preincubated with 10 µg/ml of KIM127 or 327C mAbs and then stimulated for 10 s with 0.5 µM CXCL12 (final concentration) under stirring at 37°C. After rapid washing, the cells were fixed in paraformaldehyde 1%, stained with phycoerythrin-conjugated secondary polyclonal antibody and analyzed by cytofluorimetric quantification.

Biochemical Assays
RhoA, Rac1, CDC42 and PLD1 activations were measured by using commercial kits, as described in (11). PIP5K activity was evaluated by thin-layer chromatography of $^{32}$P-labeled lipid products, as described in (11).

**Statistical analysis**

Statistical analysis was carried out by calculating mean and standard deviation (SD) between different experiments. Significances were calculated by Student’s $t$-test or one-way ANOVA. P<0.05 was considered significant.
Results

The small GTPase RhoA regulates CXCL12-triggered LFA-1 activation in primary normal B-lymphocytes. We have recently described the regulatory role of a complex rho small GTPase-based signaling module activated by the chemokine CXCL12 and controlling conformer-selective LFA-1 activation and dependent rapid adhesion to ICAM-1 (inter-cellular adhesion molecule-1) in human primary T-lymphocytes (11). To perform a comparative study of the regulatory role of this signaling mechanism in human B-CLL leukemia cells isolated from patients, we first analyzed the consistency of the rho module in normal human primary B-lymphocytes. To this end, we took advantage of the Trojan peptide technology, which allows to load cells with small peptides or functional full length proteins, and that has been already fully validated in the lymphocyte context (11, 15, 16). Trojan peptides are a convenient approach in case of less abundant, patient-derived, cell samples.

We first characterized the role of RhoA in CXCL12-induced LFA-1 activation. RhoA was activated in B-lymphocytes by CXCL12 in a dose-dependent manner, with kinetics consistent with rapid adhesion triggering (Fig. 1A). In static adhesion assays, pre-treatment with the P1-RhoA 23-40 peptide, a synthetic peptide blocking the downstream switch I effector region of human RhoA (aa 23-40) and dependent downstream signaling pathway (15) by competitive inhibition, inhibited in a dose-dependent manner chemokine-stimulated rapid adhesion of B-lymphocytes to ICAM-1 (Fig. 1B). No effect was observed after incubation with the control Penetratin-1 peptide alone. Similar results were obtained in under-flow adhesion assays, either considering arresting times of 1 or 10 seconds, which possibly corresponds to distinct adhesive phenomena (11) (Fig. 1C). To support these observations, we investigated RhoA involvement in CXCL12-induced LFA-1 affinity up regulation by evaluating the induction of LFA-1 conformer transitions to low-intermediate and to high affinity states, detected by using monoclonal antibodies KIM127 and 327C, that specifically recognize LFA-1 extended conformers expressing epitopes corresponding to low-intermediate and high affinity states, respectively. Inhibition of RhoA signaling resulted in blockade of LFA-1
conformeric transition to low-intermediate as well as to high affinity states (Fig. 1D). Taken together, these data show that, in human primary B-lymphocytes, RhoA regulates LFA-1 affinity activation and depended adhesion by CXCL12.

The small GTPase Rac1 regulates CXCL12-triggered LFA-1 activation in primary normal B-lymphocytes. We next investigated the involvement of the small GTPases Rac1 in the regulation of CXCL12-induced LFA-1 affinity increase in human primary B-lymphocytes. To study Rac1 we applied a Tat-based Trojan peptide technology that was previously validated as an efficient and selective approach to interfere with Rac1 signaling (11). Rac1 was activated in B-lymphocytes, similarly to RhoA activation, in a dose-dependent manner and with kinetics consistent with rapid adhesion triggering (Fig. 2A). In static adhesion assays, inhibition of Rac1 signaling by Tat-Rac1-N17, a negative dominant mutant, blocked CXCL12-triggered adhesion to ICAM-1 in a dose-dependent manner. In contrast, Tat-Rac1-WT (wild type), and Tat-Rac1-L61 (constitutively active mutant form), had no effect on LFA-1 mediated adhesion on ICAM-1 (Fig. 2B). Similar results were observed in under-flow adhesion assays (Fig. 2C). To further characterize the role of Rac1 in LFA-1 function modulation in B-lymphocytes by chemokines, we analyzed LFA-1 affinity triggering. Inhibition of Rac1 function blocked rapid CXCL12-triggered LFA-1 transitions to low-intermediate and to high affinity states (Fig. 2D). The results are consistent with data obtained in static and under-flow adhesion assays on ICAM-1 and demonstrate that, along with RhoA, also Rac1 is critically involved in LFA-1 affinity modulation and dependent adhesion in human primary B-lymphocytes.

The small GTPase CDC42 is a negative regulator of CXCL12-induced LFA-1 activation in primary B-lymphocytes. CDC42 is a small GTPase highly related to RhoA and Rac1, shown to regulate cytoskeleton dynamics and cell motility (19, 20). We recently discovered that CDC42 behaves as a general negative regulator of LFA-1 affinity modulation in human T-lymphocytes (11). Thus, we wished to verify whether CDC42 could behave as negative regulator of LFA-1 affinity up regulation and mediated adhesion also in human B-lymphocytes. CDC42 was activated
in B-lymphocytes by CXCL12 stimulation but, differently of RhoA and Rac1 activation, dose-
dependency was less pronounced and the kinetic of activation of CDC42 was slower, with
significant activation detected only after 30 seconds of stimulation (Fig. 3A). Thus, RhoA, Rac1 and
CDC42 are activated by CXCL12 in B-lymphocytes but with different kinetics. A first set of
experiments performed by using the Tat-based Trojan peptide technology, demonstrated a negative
regulatory role of CDC42 in chemokine-induced static adhesion on ICAM-1 (Fig. 3B). Indeed,
inhibition of CDC42 by Tat-CDC42-N17, a CDC42 dominant negative mutant, had no effect on
rapid chemokine-induced adhesion and a similar effect was observed with Tat-CDC42-WT (wild
type) treatment. Conversely, treatment of B-lymphocytes with Tat-CDC42-L61 or V12, two
CDC42 constitutively active mutants, resulted in marked inhibition of adhesion on ICAM-1,
suggesting a negative function of CDC42 in CXCL12-triggered LFA-1 activation. Similar results
were obtained in under-flow adhesion assays (Fig. 3C). To further characterize the negative role of
CDC42, we investigated its function in LFA-1 affinity triggering. The data clearly confirmed the
inhibitory effect of CDC42 on LFA-1 transition to low-intermediate and to high affinity states (Fig.
3D). Indeed, the CDC42 constitutively active mutants (L61 and V12) significantly reduced
CXCL12-dependent LFA-1 affinity up regulation, whereas, in contrast, CDC42 WT and N17
constructs did not exert any effects. Altogether, these data clearly imply CDC42 as a negative
regulator of LFA-1 affinity modulation by chemokines in human primary B-lymphocytes.

**PLD1 mediates CXCL12-triggered LFA-1 activation in primary normal B-lymphocytes.**

PLD1 is a common downstream effector of RhoA and Rac1 (21, 22). Its role in rapid LFA-1
triggering in human primary B-lymphocytes was never explored. Thus, we wished to investigate
PLD1 involvement in B-lymphocytes adhesion and LFA-1 affinity up regulation by CXCL12.
PLD1 was rapidly activated in a dose-dependent manner by CXCL12 in B-lymphocytes with
kinetics consistent with rapid LFA-1 triggering by chemokines (Fig. 4A). In static adhesion assays,
treatment of cells with n-butanol, a commonly used PLD1 activity inhibitor able to prevent
accumulation of phosphatidic acid (PA), resulted in strong inhibition of rapid CXCL12-induced
adhesion to ICAM-1. In contrast, ter-butanol, an inactive isomer, was without effect, thus excluding possible unspecific effects of n-butanol (Fig. 4B). To further corroborate this data, we used a Penetratin-1-PLD1 fusion Trojan peptide that was previously validated as specific and effective tool to prevent PLD1 activation by chemokines (11). Inhibition of PLD1 by the blocking peptide resulted in markedly reduced adhesion on ICAM-1; no effect was detected after treatment with the control Penetratin-1 peptide alone (Fig. 4B). Similar results were obtained in under-flow adhesion assays (Fig. 4C). To further support PLD1 role LFA-1 activation in B-lymphocytes, we measured the conformeric transition of LFA-1 to low-intermediate and to high affinity states after treatment with n-butanol and the PLD1 blocking peptide. Similarly to adhesion data, both n-butanol and P1-PLD1 blocking peptide strongly inhibited rapid CXCL12-induced transition of LFA-1 to low-intermediate as well as to high affinity states (Fig. 4D), clearly confirming PLD1 crucial role in regulating CXCL12-induced LFA-1 affinity regulation and mediated adhesion in B-lymphocytes.

**PIP5KC is a conformer-selective regulator of LFA-1 affinity triggering by CXCL12 in primary normal B-lymphocytes.** To completely characterize the role of the rho-signaling module controlling LFA-1 activation by CXCL12 in human primary B-lymphocytes, we tested the role of PIP5KC, a downstream effector of RhoA, Rac1 and PLD1 (23-25). PIP5KC activity was triggered with a time course kinetic by CXCL12 (Fig. 5A). To explore the functional involvement of PIP5KC we exploited a previously established siRNA-based approach (11). The expression level of PIP5KC was very efficiently reduced by nucleoporating B-lymphocytes with a pool of four different PIP5KC-specific siRNAs. Scrambled siRNAs were without effect (Fig. 5A). Importantly, in B-lymphocytes with reduced expression levels of PIP5KC, rapid static as well as under-flow adhesion to ICAM-1 triggered by CXCL12 were consistently blocked, suggesting the critical involvement of PIP5KC in LFA-1 activation (Fig. 5B-C). We then investigated the role of PIP5KC in LFA-1 affinity triggering in B-lymphocytes. The data show that PIP5KC was not involved in structural changes leading to low-intermediate affinity state (Fig. 5D). However, and importantly, transition to high affinity state was consistently inhibited (Fig. 5D). Together, the data indicate that, as in T-
lymphocytes, also in human primary B-lymphocytes PIP5KC is a conformation-selective regulator of LFA-1 affinity, controlling triggering of LFA-1 to high affinity state, but not to low-intermediate affinity state by CXCL12 and this correlates with rapid adhesion triggering to ICAM-1.

**The regulatory role of the CXCL12-induced rho-module of LFA-1 affinity triggering is not conserved in CLL B-lymphocytes.** Altogether, the previous data show that the rho-module of conformation-selective regulation of LFA-1 affinity triggering by chemokines is fully functional in primary B-lymphocytes. This clearly suggests the universal relevance of the rho-module in controlling LFA-1 activation by chemokines in normal human primary lymphocytes.

Having established the full consistency of the pro-adhesive rho-module in normal B-lymphocytes, we proceeded to investigate whether the rho-module of LFA-1 affinity regulation was conserved also in malignant B-lymphocytes directly isolated from CLL patients or if, in presence of oncogenic transformation and progression, diversity in normal versus B-CLL subjects and between various B-CLL patients could appear. To this end, we performed a multiplexed analysis of malignant B-lymphocytes directly isolated from a total of 31 B-CLL patients by evaluating, for each patient, the regulatory role of RhoA, Rac1, CDC42, PLD1 and PIP5KC in LFA-1 activation by CXCL12.

In Supplementary Table S1 are reported the data for every single patient. Global analysis of the data immediately highlighted a consistent variability between patients regarding the relative regulatory role of the different signaling mechanisms. On this base, we could group the 31 analyzed patients in two clusters, (cluster (A) = 17 patients; cluster (B) = 14 patients) characterized by different sensitivity to signaling inhibition. In both B-CLL clusters (A) and (B), blockade of RhoA activity by the Trojan peptide P1-RhoA 23-40 resulted in significant inhibition of CXCL12-induced rapid adhesion to ICAM-1 (Fig. 6A-B). Moreover, analysis of LFA-1 conformational changes leading to affinity increase, showed that RhoA blockade resulted in a consistent inhibition of the complete LFA-1 transition to low-intermediate and to high affinity states (Fig. 6A-B), comparable to the data obtained in healthy B-lymphocytes. These data were consistent in all 31 studied patients and clearly
suggested that RhoA is a very conserved pro-adhesive signaling mechanism, controlling LFA-1 activation by chemokines also in malignant B-CLL lymphocytes.

In contrast, analysis of Rac1 displayed a marked difference with respect to RhoA, with a consistent heterogeneity in the regulatory role of Rac1. Indeed, in cluster (A), Rac1 signaling inhibition by treatment with Tat-Rac1-N17 fusion mutant was systematically associated to reduced adhesion to ICAM-1, accompanied by impaired triggering of LFA-1 to low-intermediate and to high affinity states by CXCL12. Treatment with Tat-Rac1 WT or L61 was without effect. In sharp contrast, in cluster (B), Rac1 signaling inhibition by treatment with Tat-Rac1-N17 fusion mutant did not affect adhesion to ICAM-1 nor LFA-1 affinity triggering to low-intermediate and to high affinity states by CXCL12. Thus, the analysis highlighted a marked heterogeneity between patients, with some patients rather sensitive and other totally insensitive to Rac1 inhibition. These observations allowed grouping the patients in the two distinct groups (A) and (B), one characterized by high sensitivity to Rac1 inhibition (Fig. 6A), and a second one with null sensitivity to Rac1 inhibition (Fig. 6B). This data show that the role of Rac1 is not universal and that, as a consequence of neoplastic transformation, the regulatory role of Rac1 on LFA-1 affinity modulation by chemokines can be bypassed and become irrelevant.

A similar pattern was found with CDC42. Indeed, activation of CDC42 signaling, by Tat-CDC42-L61 manifested a broad heterogeneity in the inhibitory capability of CDC42 on LFA-1-mediated adhesion of B-CLL lymphocytes. The data were also confirmed by cytofluorimetric analysis of the activation epitopes of LFA-1. Thus, as for Rac1, also CDC42 manifests a patient-specific involvement in LFA-1 affinity modulation by CXCL12. As for Rac1, also the CDC42 data allowed grouping B-CLL patients in at least two different groups: one, in which CDC42 had a negative regulatory role (Fig. 6A) and a second one in which CDC42 did not have any role (Fig. 6B). Notably, and interestingly, patient grouping based on Rac1 and CDC42 analysis generated coincident groups, with patients insensitive to Rac1 inhibition also displaying insensitivity to
CDC42 activation. Thus, in B-lymphocyte the neoplastic transformation can, in certain conditions, abolish at the same time the opposite regulatory roles of Rac1 and CDC42.

The patient-specific regulatory role of Rac1 could imply variability also in downstream signaling events regulated by Rac1 in B-CLL lymphocytes. Thus, we analyzed the role of PLD1, which is commonly regulated by RhoA and Rac1. PLD1 blockade by \( n \)-butanol and by P1-PLD1 blocking peptide consistently prevented adhesion triggering to ICAM-1 by CXCL12 in all 31 analyzed patients (Fig. 6A-B). These data were further supported by analysis of LFA-1 affinity modulation. Indeed, upon treatment with \( n \)-butanol or with P1-PLD1, B-CLL lymphocytes displayed a clear defect in the capability to up regulate LFA-1 epitopes corresponding to both low-intermediate and high affinity states induced by CXCL12 (Fig. 6A-B). Again, this was evidenced in all 31 analyzed patients. Thus, as for RhoA, the regulatory role of PLD1 is highly conserved in normal as well as neoplastic B-lymphocytes and its regulatory role seems not bypassed by the neoplastic transformation.

Finally, we wished to test the role of PIP5KC, possibly the most downstream component of the Rho-signaling module controlling LFA-1 affinity modulation by chemokines (11). To test PIP5KC role in B-CLL lymphocytes we applied the siRNA-based approach. In all the studied patients, the expression level of PIP5KC was efficiently reduced by nucleoporating B-CLL cells with the pool of four different PIP5KC-specific siRNAs. Scrambled siRNAs were without effect (data not shown). In B-CLL lymphocytes showing reduced expression of PIP5KC, CXCL12-triggered adhesion to ICAM-1 was completely normal (Fig. 6A-B). Moreover, up-regulation of both LFA-1 epitopes corresponding to low-intermediate and extended high affinity state conformers was totally unaffected in all studied patients (Fig. 6A-B). This shows that PIP5KC is, along with Rac1 and CDC42, a dispensable signaling mechanism in the transduction machinery modulating chemokine-triggered LFA-1 activation in B-CLL lymphocytes. However, and importantly, PIP5KC represents a major point of divergence with respect to normal primary B-lymphocytes, as it appears never
involved in LFA-1 activation in B-CLL lymphocytes, thus establishing a sharp difference with respect to normal primary B-lymphocytes.
Discussion

B-CLL is a clinically heterogeneous disease originating from either somatically non-rearranged or rearranged antigen-experienced B-lymphocytes that may differ in activation, maturation state, or cellular subgroup. A progressive dissemination and accumulation of malignant cells to secondary lymphoid organs and to bone marrow characterizes the progression and severity of the disease. Widespread dissemination of leukemic cells is likely facilitated by survival signals delivered, to migrating malignant cells, by the microenvironment through a variety of receptors including B-cell receptor, chemokine and cytokine receptors (26-28).

In this study we pursued a multiplexed comparative characterization of the intracellular signaling mechanisms differentially controlling the adhesion of normal versus neoplastic B-lymphocytes isolated from CLL patients. The logic of the investigation derives from the possibility that, as a consequence of the neoplastic transformation and/or progression, the intracellular signaling mechanisms controlling \textit{in vivo} trafficking of leukemia cells can be altered with respect to normal lymphocytes. We focused our analysis on the regulatory role of the rho-module of LFA-1 affinity triggering by the CXC chemokine CXCL12, which we have recently characterized in human primary T-lymphocytes (11). The study involved 31 B-CLL patients and for each patient we have analyzed the regulatory role of the signaling proteins RhoA, Rac1, CDC42, PLD1 and PIP5KC on LFA-1 affinity modulation by CXCL12. From this study the following conclusion can be drawn: 1) the rho-module of LFA-1 affinity triggering by chemokines is fully functional in human primary B-lymphocytes isolated from healthy donors; 2) in B-CLL patients, RhoA and PLD1 appear to be the most conserved signaling events controlling LFA-1 activation by CXCL12; 3) in contrast, Rac1 and CDC42 display a consistent patient-by-patient variability, with a restricted group of B-CLL patients showing LFA-1 affinity modulation completely independent of the signaling activity of Rac1 and CDC42; 4) in all studied B-CLL patients, PIP5KC has no role in LFA-1 affinity triggering by CXCL12. Overall, the data imply that, with respect to normal primary B-lymphocytes, in B-CLL lymphocytes the neoplastic progression completely bypasses the regulatory role, otherwise critical
in normal lymphocytes, of PIP5KC in LFA-1 transition to high affinity state. Furthermore, the regulatory role of Rac1 and CDC42 may also become totally irrelevant but in a patient-specific fashion. Thus, the signaling couple RhoA-PLD1 seems the most conserved signaling event controlling LFA-1 activation by chemokines in B-CLL lymphocytes. Our data clearly show a profound divergence in the signaling mechanisms activated by chemokines and controlling integrin activation in normal versus neoplastic B-lymphocytes.

A first outcome of our study is that the rho-module of conformer-selective LFA-1 affinity triggering by chemokines is fully operative also in human primary B-lymphocytes. This finding is of interest, since the critical regulatory role of this signaling module in primary T-lymphocytes (11) does not automatically imply that identical signaling mechanisms regulate integrin activation also in primary B-lymphocytes, as also previously suggested (29). Thus, our study suggests the universal relevance of the rho-module in regulating conformer-selective triggering of LFA-1 by chemokines. Further studies will be necessary to verify whether this complex signaling mechanism also regulates integrin activation in other primary leukocyte sub-types, such as monocytes and polymorphonuclear cells. Moreover, it will be of interest to test the regulatory role of the rho-module in other integrin contexts, such as VLA-4 and α4β7.

A further important observation is that the signaling couple RhoA-PLD1 is critically involved in LFA-1 affinity regulation by CXCL12 in all 31 analyzed B-CLL patients. We previously showed that, in the CXCL12-triggered signaling context, PLD1 activation depends on RhoA and Rac1 activity (11). We also verified this data in primary B-lymphocytes (data not shown). Notably, we found that about half of analyzed patients (14 on a total of 31, corresponding to cluster (B)) do not rely on Rac1 activity to control LFA-1 affinity triggering by CXCL12. Thus, in these patients, activation of PLD1 is likely to be dependent only on RhoA activity. This suggests that, in B-CLL, RhoA can fully bypass and compensate an eventually defective Rac1 signaling and ensure, alone, a competent PLD1 activation. This establishes a sharp dichotomy with respect to normal primary lymphocytes, where both RhoA and Rac1 are necessary to PLD1 activation leading to LFA-1
A rather interesting finding of our study is that the regulatory role of Rac1 and CDC42 can be lost in a patient-selective manner. This finding allowed us to cluster the patients in two groups, characterized by divergent regulatory roles of Rac1 and CDC42 on LFA-1 affinity modulation. Thus, the two groups of patients, although characterized by the same disease diagnosis, are substantially different at the level of chemokine-triggered intracellular signaling mechanisms controlling LFA-1 mediated adhesion. Particularly, the neoplastic progression seems to affect, in certain CLL patients, the centrality of the regulatory roles of Rac1 and CDC42, with the 14 patients of group (B) showing LFA-1 affinity modulation by chemokines totally independent of Rac1 and CDC42 activity. Although qualitative and/or quantitative variability in signaling mechanisms controlling integrin triggering could be expected, it was surprising to observe such a dramatic dichotomy, in which two signaling molecules, with opposite regulatory activities, lost at the same time their regulatory role in a coincident group of patients. This finding may suggest the occurrence of specific genetic and/or functional signatures characterizing the patients of group B and it will be of great interest to try to correlate, by analyzing a much larger number of patients, this specific pro-adhesive signaling signature to clinical data such as severity of the disease and response to therapies. Notably, with respect to normal B-lymphocytes, Rac1 and CDC42 were normally expressed and activated in B-lymphocytes derived from some patients of the group B (data not shown). Although this aspect needs to be systematically verified, this suggests that the defective role of the rho-module in patients of group B is possibly generated by more complex mechanisms other than altered protein expression or activation.

PIP5KC, which is the downstream component of the rho-module controlling LFA-1 conformeric transition specifically to high affinity state in normal primary lymphocytes (11), was found without any regulatory role in B-CLL lymphocytes. This was verified in all 31 analyzed patients. This finding establishes a remarkable difference with normal lymphocytes and further highlights the altering effect of neoplastic transformation and/or progression on signaling mechanisms controlling
integrin triggering in leukemia cells. Moreover, this data raises important mechanistic questions. Indeed, lacking of regulatory role of PIP5KC in B-CLL lymphocytes shows that signaling mechanisms not involving PIP5KC activity can still control triggering of LFA-1 to high affinity state. In these alternative mechanisms RhoA, Rac1 and PLD1 are still critical but do not converge on PIP5KC. One possibility is that, in contrast with normal primary lymphocytes, in B-CLL lymphocytes the role of PIP5KC can be completely bypassed by the other two isoforms of PIP5K, A and B. This specific hypothesis will need to be verified in a second group of patients. Moreover, a possible regulatory role for the small GTPase Rap1 in B-CLL lymphocytes LFA-1 activation can be deducted by recent findings (30). Notably, Rap1 can potentially mediate LFA-1 affinity triggering by means of RIAM-Talin1 interactions (31). Thus, it is possible to hypothesize that, in B-CLL lymphocytes, LFA-1 triggering to high affinity state by CXCL12 is controlled by alternative signaling modules involving RhoA, (Rac1), PLD1, PIP5K (A or B isoforms) and Rap1.

Overall, our study identifies a consistent diversity in signaling mechanisms operating in B-lymphocytes isolated from B-CLL patients and controlling LFA-1 activation and mediated adhesion by chemokines. It is of interest that although we identified defective signaling components, we systematically have been able to detect LFA-1 affinity triggering by CXCL12. This contrast with recent findings (30) where an impaired capability of chemokines to increase LFA-1 affinity in B-CLL lymphocytes was observed and related to Rap1 defective activation. This further suggests that, concerning the signaling mechanisms controlling integrin triggering by chemokines, the B-CLL phenotype should not be regarded as uniform population and that a deep, systematic, analysis of the intracellular signaling events governing cell adhesion can be a helpful strategy to better categorize CLL patients, with possible impact on diagnosis, prognosis and therapy of leukemia diseases.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Figure Legends

Figure 1. RhoA mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. A, measurement of RhoA activation by ELISA test; B-lymphocytes were treated with buffer (no agonist) or with the indicated concentrations of CXCL12 for the indicated times. B, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (no agonist and control), 50 μM P1 or with indicated concentrations of P1-RhoA 23-40 peptide; stimulation was with 0.5 μM CXCL12. C, under-flow adhesion to ICAM-1; B-lymphocytes were treated with buffer (control), or with 50 μM of P1 or P1-RhoA 23-40 peptides; shown is percentage of arrested cells for 1 sec or for 10 sec over the total interacting cells, as described in the Material and Methods section. D, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, left) or to high affinity state (327C, right); B-lymphocytes were treated with buffer (no agonist and control) or with 50 μM of P1 or P1-RhoA 23-40 peptides and stimulated with 0.5 μM CXCL12. Panel A is one representative experiment of 4; values in panels B to D are means with SD from 8 to 11 experiments. (* = P<0.01).

Figure 2. Rac1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. A, measurement of Rac1 activation by ELISA test; B-lymphocytes were treated with buffer (no agonist) or with the indicated concentrations of CXCL12 for the indicated times. B, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (no agonist and control) or with the indicated concentrations of Tat-Rac1-WT, Tat-Rac1-N17 or Tat-Rac1-L61; stimulation was with 0.5 μM CXCL12. C, under-flow adhesion to ICAM-1; B-lymphocytes were treated with buffer (control) or with 2 μM of Tat-Rac1-WT or Tat-Rac1-N17; shown is percentage of arrested cells for 1 sec or for 10 sec over the total interacting cells, as described in the Material and Methods section. D, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, left) or to high affinity state (327C, right); B-lymphocytes were treated with buffer (no
agonist and control) or with 2 μM of Tat-Rac1-WT or Tat-Rac1-N17 and stimulated with 0.5 μM CXCL12. Panel A is one representative experiment of 3; values in panels B to D are means with SD from 8 to 11 experiments. (** = P<0.01).

**Figure 3.** CDC42 negatively modulates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. **A**, measurement of CDC42 activation by ELISA test; B-lymphocytes were treated with buffer (no agonist) or with the indicated concentrations of CXCL12 for the indicated times. **B**, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (no agonist and control) or with the indicated concentrations of Tat-CDC42-WT, Tat-CDC42-N17, Tat-CDC42-V12 or Tat-CDC42-L61; stimulation was with 0.5 μM CXCL12. **C**, under-flow adhesion to ICAM-1; B-lymphocytes were treated with buffer (control) or with 2 μM of Tat-CDC42-WT, Tat-CDC42-N17, Tat-CDC42-V12 or Tat-CDC42-L61; shown is percentage of arrested cells for 1 sec or for 10 sec over the total interacting cells, as described in the Material and Methods section. **D**, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, left) or to high affinity state (327C, right); B-lymphocytes were treated with buffer (no agonist and control) or with 2 μM of Tat-CDC42-WT, Tat-CDC42-N17, Tat-CDC42-V12 or Tat-CDC42-L61 and stimulated with 0.5 μM CXCL12. Panel A is one representative experiment of 5; values in panels B to D are means with SD from 8 to 13 experiments. (** = P<0.01).

**Figure 4.** PLD1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. **A**, measurement of PLD1 activation by quantification of choline release; B-lymphocytes were treated with buffer (no agonist) or with the indicated concentrations of CXCL12 for the indicated times. **B**, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (no agonist and control), with 50 mM n- or ter-butanol or with the indicated concentrations of P1-PLD1 peptide; stimulation was with 0.5 μM CXCL12. **C**, under-flow adhesion to ICAM-1; B-
lymphocytes were treated with buffer (control), with 50 mM of n- or ter-butanol or 50 μM of P1-PLD1 peptide; shown is percentage of arrested cells for 1 sec or for 10 sec over the total interacting cells, as described in the Material and Methods section. D, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, left) or to high affinity state (327C, right); B-lymphocytes were treated with buffer (no agonist and control) or with 50 mM of n- or ter-butanol or 50 μM of P1-PLD1 peptide and stimulated with 0.5 μM CXCL12. Panel A is one representative experiment of 3; values in panels B to D are means with SD from 9 to 12 experiments. (* = P<0.01).

**Figure 5.** PIP5KC selectively controls CXCL12-induced triggering of LFA-1 to high affinity state and dependent adhesion in normal B-lymphocytes. A, left, evaluation of PIP5KC activity; B-lymphocytes were treated with buffer (no agonist) or with 0.5 μM CXCL12 for the indicated times. Shown is an autoradiogram of 32P-labeled PtdIns(4,5)P2. The bottom values are quantifications of incorporated radioactivity in PtdIns(4,5)P2; right, evaluation of PIP5KC content by immunoblot; B-lymphocytes were nucleoporated with a pool of four scrambled or PIP5KC-specific siRNAs and kept in culture for the indicated times; shown is the PIP5KC protein content compared with the total amount of actin. B, static adhesion to ICAM-1; B-lymphocytes were nucleoporated with a pool of four scrambled (scr) or PIP5KC-specific siRNAs, kept in culture for 48 hours (scr) or for the indicated times and treated with buffer (no agonist) with 0.5 μM CXCL12. C, under-flow adhesion to ICAM-1; B-lymphocytes were nucleoporated with a pool of four scrambled (scr) or PIP5KC-specific siRNAs and kept in culture for 48 hours; shown is percentage of arrested cells for 1 sec or for 10 sec over the total interacting cells, as described in the Material and Methods section. D, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, left) or to high affinity state (327C, right); B-lymphocytes were nucleoporated as in (C) and stimulated with 0.5 μM CXCL12. Panel A is one representative experiment of 4 (left) and of 10 (right); values in panels B to D are means with SD from 8 to 11 experiments. (* = P<0.01).
Figure 6. B-lymphocytes isolated from B-CLL patients show altered signaling mechanisms of LFA-1 affinity triggering by CXCL12. A, static adhesion to ICAM-1 (left), detection of LFA-1 conformers with low-intermediate affinity (KIM127, middle) or high affinity (327C, right); B-CLL lymphocytes from the same patient (for a total of 31) were treated with buffer (no agonist and control), with 50 µM of P1, P1-RhoA 23-40 or P1-PLD1 Trojan peptides, with 50 µM of Tat-fusion Rac1 and CDC42 WT or mutated proteins, with 50 mM n- or ter-butanol or were nucleoporated with a pool of four scrambled (scr) or PIP5KC-specific siRNAs and kept in culture for 48 hours; treated cells were stimulated with 0.5 µM CXCL12 as described in previous figures. Values in panels A are means with SD from 17 experiments, corresponding to 17 B-CLL patients; values in panels B are means from 14 experiments with SD, corresponding to 14 B-CLL patients.

Supplementary Table S1. The table summarizes the results of all experiments of static adhesion assays to ICAM-1 and of the detection of LFA-1 conformational activation epitopes corresponding to low-intermediate affinity state (KIM127) or high affinity state (327C) performed in B-lymphocytes from all 31 B-CLL patients. Data are clustered in two different groups, (A) and (B), corresponding to patients showing increasing diversity in the regulatory role of the rho-module of LFA-1 affinity triggering by CXCL12. The values at the bottom of each column are calculated means and SD, which have been plotted in Fig. 6.