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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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1. ABSTRACT

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 (CLL) cells. We pursued a multiplexed comparative analysis of the role of the recently described chemokine-triggered rho-signaling module in human 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.

2. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world, with an age-adjusted incidence of 3.5 per 100,000 per year (USA, 1996-2000) and a prevalence of 46,000 individuals in the European Union (five years post diagnosis condition) [1,2]. It is more common in males than in females and it is largely a disease of the elderly, with a median age at diagnosis of 72 years [3].

CLL is characterized by the accumulation of mature-looking immunoincompetent lymphocytes that are typically positive for CD5, CD23, CD19 and CD20 [4]. Indeed, in 95% of the cases, CLL of the B-cell phenotype is diagnosed, explaining why the term CLL can readily be used instead of B-CLL (B-cell CLL). The accumulation of the clonal cell population occurs in the blood, the bone marrow, the lymph nodes and the spleen [5].

Chronic lymphocytic leukemia of the B-lymphocyte lineage is also characterized by enhanced trafficking and accumulation of CD5+ cells in the bone marrow and in secondary lymphoid organs [6]. As in normal lymphocytes, the combinatorial activity of chemokines and adhesion molecules controls tissue-selective dissemination of B-CLL cells [7]. For instance, neoplastic B-lymphocytes from B-CLL patients show increased levels of chemokine receptors such as CCR7, CXCR4 and CXCR5 [7,8], and previous papers show higher motility of neoplastic B-lymphocytes then normal B-lymphocytes *in vitro* [8,9], suggesting an intriguing correlation between changes in CXCR4-CXCL12 axis, integrin activation and migratory behavior of malignant B-lymphocytes induced by homeostatic chemokines [10,11]. CXCR4-CXCL12 axis, together with soluble factors of the microenvironment, is also critical to maintain malignant lymphocytes in the stroma and to protect them from apoptosis [12-15]. The β_1 integrin VLA-4 (very late antigen-4; also called $\alpha_4\beta_1$) and the β_2 integrin LFA-1 (lymphocyte function-associated antigen-1; also called $\alpha_L\beta_2$), which have a central role in mediating trafficking of circulating leukocyte [16,17] and specially lymphocytes

[18], are likely to play a similar regulatory role also in B-CLL lymphocyte accumulation in different tissues.

The functioning of the immune system depends on tissue-specific leukocyte recruitment and microenvironmental positioning. In turn, recruitment and positioning of leukocytes to sites of immune response relies on cell adhesion and motility (Fig. 1), which are both critically dependent on rapid integrin activation and engagement. Integrins are ubiquitously expressed, highly versatile type I transmembrane heterodimeric receptors [19]. The most relevant integrins to immune system regulation are the β_2 integrins Mac-1 and LFA-1, the β_1 integrin VLA-4 and the β_7 integrin $\alpha_4\beta_7$. These integrins regulate a variety of processes. For instance, they act as costimulatory molecules for T cell receptors in the immunological synapse [20], mediate interactions with bacteria [21] and phagocytosis [22], and support leukocytes adhesion to the endothelium and transendothelial migration during lymphocyte homing [23]. In this last case, integrins have a dual role: (a) they are the "ultimate brakes" for circulating leukocytes, stably arresting interacting cells on the endothelium; and (b) they contribute to the diversity in leukocyte recruitment [24]. Recently, in collaboration with prof. R. Alon's group, we demonstrated that LFA-1 also supports shear-resistant crawling of lymphocytes on cytokine-activated endothelial cells [25].

Then, the immune response critically depends on spatiotemporal control of leukocyte extravasation (Fig. 1) [18,26,27], and central paradigm in this phenomenon is represented by the adhesive function of integrins, regulated by inside-out signaling events triggered by chemokines and leading to rapid integrin activation [28-30].
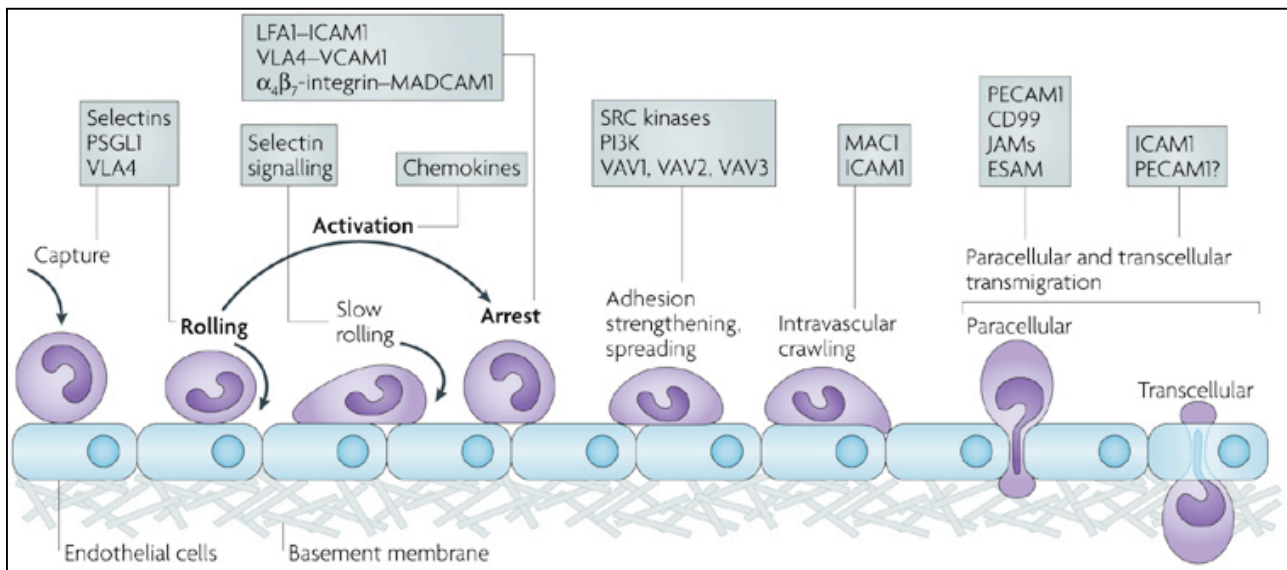
Figure 1. Leukocyte adhesion cascade [18].

Two main modalities of integrin activation are known, namely conformational changes, leading to increased affinity (Fig. 2*A, top*), and lateral mobility leading to increased valency (Fig. 2*A, bottom*), both enhancing cell avidity [31,32]. One of the most remarkable advances in the field is the interpretation of integrin activation as a dynamic equilibrium between different conformers, corresponding to conformational changes of the heterodimer with increasing affinity for the ligand (Fig. 2*B*) [33]. Although lateral mobility is also involved [31,34], published findings suggest a predominant function for affinity triggering in rapid leukocyte arrest under flow [35,36]. Particularly, in the context of adhesion mediated by LFA-1, the available data support a model by which chemokines induce a transition to a high-affinity state that is critical to support arrest of circulating leukocytes [18,31]. A more complex interpretation suggests that leukocyte arrest requires a chemokine-triggered transitional step of a non-extended LFA-1 low-affinity state to an extended form of low-to-intermediate affinity followed by transition to a high-affinity state induced by the interaction with the ligand, which is mandatory to full arrest [37]. Other data also suggest involvement of β_2 integrin–triggered outside-in signaling in leukocyte adhesion stabilization [38]. Notably, it is not clear whether leukocytes may respond to the quantitative variation of the various

pro-adhesive factors that could occur *in vivo* by triggering distinct modalities of integrin activation. Evidence obtained in *in vitro* experimental settings may suggest this possibility [39].
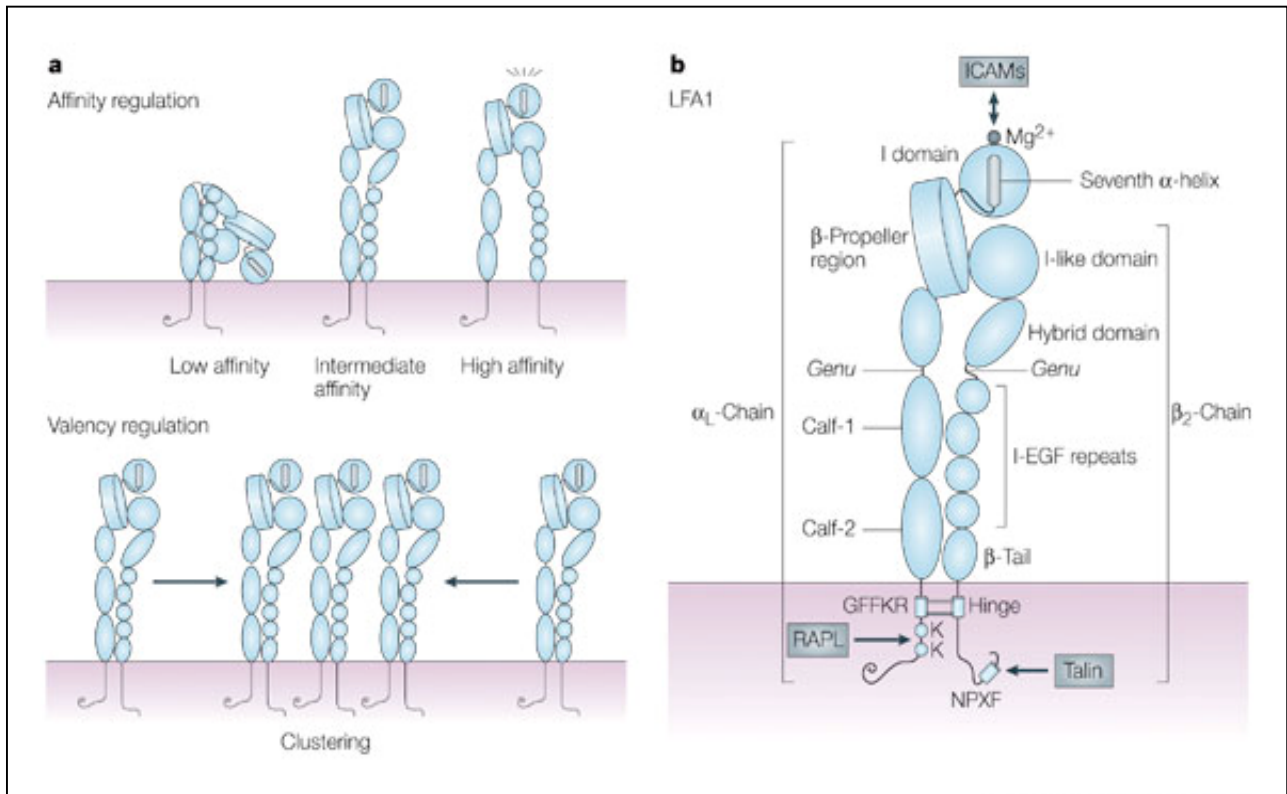

Figure 2. Integrin structure and regulation. *A,* model of affinity regulation (*top*) and of valency regulation (*bottom*); *B,* structural representation of LFA-1 at high-affinity state [28].

Recently, we have shown that, in human 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 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 [40,41].

3. AIM OF THE STUDY

Abnormalities in the expression and function of cell adhesion molecules may account for the patterns of intranodal growth and hematogenous spread of malignant cells [42-45] and thereby dramatically alter leukemic cell fate. Little is known about the capacity of circulating B-CLL cells to adhere and then 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 [46-48], the specific function of this integrin and of LFA-1, and the specific intracellular signaling governing their activation in CLL trafficking have been elusive.

The aim of this study was a comparative characterization of the regulatory role of the rho-module of LFA-1 affinity modulation and activation in human normal B-lymphocytes versus malignant B-lymphocytes isolated from B-CLL patients.

Our results highlight the universal relevance of the rho-module of LFA-1 activation in normal B-lymphocytes. In contrast, we show that in malignant B-CLL lymphocytes the regulatory relevance of the rho-module of LFA-1 affinity modulation is not conserved. 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 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.

4. MATERIALS AND METHODS

Reagents

FBS was from Irvine; human CXCL12, ICAM-1 and E-selectin were from R&D Systems; phycoerythrin-conjugated anti-mouse antibody was from Jackson; anti-LFA-1 monoclonal antibodies TS1-22 and KIM127 (reporter for extended conformation epitope possibly corresponding to intermediate affinity state [49]) were from American Type Culture Collection (ATCC); anti-LFA-1 monoclonal antibodies 327C (reporter for extended conformation epitope corresponding to high affinity state [50]) was from ICOS, Corp; rabbit monoclonal anti-RhoA and anti-CDC42 and anti-actin were from Cell Signaling; rabbit polyclonal anti-PIP5KC was from Abgent.

Isolation of B-lymphocytes from healthy subjects and B-CLL patients

Normal B-lymphocytes were isolated from healthy donors as in [49]. Purity of B-lymphocyte preparations was evaluated by flow cytometry with anti-CD19 mAb. CLL B-lymphocytes were isolated from PBMCs after blood separation on Lymphomed (MidiMed) and purification by negative selection. 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 [51] 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. Samples were obtained with informed consent and the approval of the Ethics Committee. Patients had 75 to 90% CLL cells. Normal and CLL B-lymphocytes were plated at 5×10^6/ml in RPMI + 2 mM Glutamine + 10% FBS for 3 hours

before treatment with Trojan peptides or silencing with siRNAs.

Trojan Peptide Technology

Penetratin-1 Technology. The 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 [36,40]; aa 34, mw 4430.29) and the PLD1-blocking (P1-PLD1) peptides were synthesized by GenScript. The P1-PLD1 peptide included the complete P1 sequence, an inserted glycine to allow flexibility of the fusion peptide, and the PLD1 sequence encompassing aa 944–962 corresponding to the complete RhoA binding and activating domain of human PLD1 [40,52,53]. Approximate prediction of secondary structure, charge distribution and solubility were computed by using the Biology Workbench service (http://workbench.sdsc.edu/; San Diego Supercomputing Center). The P1-PLD1 peptide displayed the following properties: aa 36, mw 4584.53, Pi 11.89; 4 mM stock solutions in DMSO were kept at -20°C and diluted in adhesion buffer immediately before the experiments. Standard treatment of cells with peptides was for 60–90 min at 37°C in 24- or 6-well plates.

TAT Technology. We constructed a series of specific TAT-fusion protein expression vectors based on pRSETa-b-c vectors (Invitrogen), designed for high-level protein expression and purification on nickel columns upon expression in *Escherichia coli*. The sequence coding for an epitope recognized by a specific antibody, between restriction sites *Nhe*I and *Bam*HI, was replaced by the sequence coding for TAT 16 aa peptide, derived from the 86-amino acid Tat transactivation protein involved in HIV replication. cDNAs for the small GTPases Rac1 and CDC42 isoforms (wild-type and point mutated) were provided by A. Hall and cloned into pRSETaTAT vector between *Bam*HI and *Eco*RI sites. Expression of recombinant TAT-fusion proteins was performed to allow purification under non-denaturing conditions. Briefly, overnight cultures of the *E. coli* strain BL21 (DE3) pLysS harboring recombinant plasmid expressing (His)$_6$-TAT-proteins were diluted in fresh LB medium containing ampicillin 50 μg/ml and chloramphenicol 35 μg/ml and grown at 37°C with vigorous

shaking to an OD$_{600}$ of 0.6. To induce the protein synthesis, isopropylthio-β-galactoside (IPTG) was added to the cultures to a final concentration of 0.1 mM and the bacteria were grown overnight at 25°C. Bacteria pellets from overnight induction were then resuspended in 20 mM phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.8 (binding buffer) containing 1 mM DTT and protease inhibitors without EDTA (Roche), and sonicated at 0°C 4 times for 15 s. After centrifugation, the supernatant was filtered in 0.45 μm filter unit and collected to the HisTrap™ HP 5 ml charged with nickel ions. The clarified lysate was applied to the column at 5 ml/min flow rate. After washing with binding buffer, proteins were eluted with binding buffer containing imidazole 500 mM. Protein purification was monitored by evaluation of apparent molecular weight on SDS-PAGE and by immunoblot using specific antibodies. The eluted proteins from IMAC were desalted using a HiTrap desalting column (Amersham Pharmacia Biotech) against 20 mM phosphate buffer pH 7.8 without NaCl and then applied to Äkta FPLC MonoSP or MonoQ column depending on the isoelectric point of each protein. Proteins were eluted with 20 mM phosphate buffer pH 7.8, containing 500 mM NaCl. Native proteins from anion-exchange chromatography were finally desalted using a HiTrap Desalting column against PBS buffer on Äkta FPLC system and concentrated on Amicon® Ultra-15 Centrifugal Filter Devices (Millipore) followed by 0.22 μm sterile filtration for direct use on living primary cells. Stock concentrated samples of purified native TAT-fusion proteins were stored at -80°C. Standard treatment of cells with TAT-fusion proteins was for 60 min at 37°C in 24- or 6-well plates.

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: (i)=GCGAAACCACCUACAAGAA;(ii)=CGAGAGCGACACAUAAUUU;(iii)=GCAACACGGU CUUUCGGAA; (iv)=GCACACAGUCGUCUGGACA). From these siRNAs, scrambled sequences were randomly generated by using Vector NTI software package and used as negative controls.

Silencing was performed in normal and CLL B-lymphocytes by nucleoporation, using the Amaxa Nucleofector (Amaxa Biosystems) [40]. The efficiency of siRNA nucleoporation was evaluated with fluorescein isothiocyanate-conjugated siRNA. Efficacy of gene silencing was evaluated by immunoblotting.

Immunoblot

After 24-48 hours, nucleoporated B-lymphocytes were lysed in 40 μl of ice-cold lysis buffer (50 mM Tris buffer, pH 7.5, 100 mM NaCl, 1% NP-40, 10% glycerol, 0.1% SDS, 1 mM PMSF, 1 mM DTT, containing Complete protease inhibitor "cocktail", from Roche). Cell lysates were separated by SDS-PAGE, transferred on nitrocellulose (Amersham Pharmacia Biotech) and analyzed by western blot using PIP5KC-specific antibody. Blots were also probed with actin-specific antibody, as an internal control.

Static Adhesion Assay

B-lymphocytes were resuspended at 5×10^6/ml in standard adhesion buffer (PBS, 10% FBS, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.2). Adhesion assays were performed on 18-well glass slides coated 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 [31].

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. The behavior of interacting

B-lymphocytes (shear stress was 2 dyne/cm^2) was recorded on digital videotape and analyzed frame by frame. Single areas of 0.2 mm^2 were recorded for at least 120 s. Interactions of > 20 ms were considered significant and were scored. Lymphocytes that remained 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 2x10^6/ml, were briefly pre-incubated with 10 μg/ml of KIM127 or 327C monoclonal antibody and then stimulated for 10 s with 0.5 μM CXCL12 under stirring at 37°C. After rapid washing, cells were stained with phycoerythrin-conjugated secondary polyclonal antibody and analyzed by cytofluorimetric quantification.

Biochemical Assays

RhoA activation. RhoA activity was evaluated by ELISA-based assay using the G-LISA™ RhoA Activation Assay kit (Cytoskeleton), following manufacturer's instructions.

Rac1 and CDC42 activation. Rac1 and CDC42 activation was evaluated by adaption of the Rac1 and CDC42 activation assays from Cytoskeleton. B-lymphocytes were stimulated in suspension at 37°C under stirring for 10 s with 0.5 μM CXCL12 and then lysed at 4°C in 0.5 ml of 100 mM HEPES buffer, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl$_2$, 2 mM EGTA, 2 mg/ml BSA, 20 mM benzamidine, containing Complete protease inhibitor 'cocktail' (Roche; reaction buffer). After centrifugation, equal amounts of protein lysates were added to 96-well plates coated with GST-Pak1 CRIB motif. After 60 min binding at 4°C, the wells were washed twice with reaction buffer followed by washing with PBS containing 10 mM MgCl$_2$, and 2 mg/ml BSA. The amount of bound Rac1 or CDC42 was quantified by enzyme-linked immunosorbent assay (ELISA) with absorbance reading at 492 nm by detecting the binding of

monoclonal anti-Rac1 or anti-CDC42 with a horseradish peroxidase-conjugated secondary antibody. The specificity of the assay was also evaluated by immunoblot analysis by desorbing bound Rac1 or CDC42 from the plates and evidencing the correct molecular weight by specific Rac1 or CDC42 monoclonal antibodies (data not shown).

PLD1 activation. Activation of phosphatidylcholine-specific PLD1 was evaluated by measuring choline release with the PLD activation Amplex Red Phospholipase D assay kit from Molecular Probes (A12219). Briefly, B-lymphocytes were stimulated in suspension at 37°C under stirring for 10 s with 0.5 μM CXCL12 and then lysed by sonication at 0°C immediately after the stimulation. Lysates were centrifuged, and the supernatants were subjected to colorimetric reaction following the manufacturer's indication in 96-well plates. Fluorescence was detected in fluorescence microplate reader with 530–560 nm excitation and 590 nm emission.

PIP5KC activation. PIP5KC activity was evaluated by measuring the accumulation of PtdIns(4,5)P$_2$. B-lymphocytes, suspended at 30×10^6 ml in Hanks' buffer pH 7.4, 1 mg/ml D-glucose, were labeled for 3 h at 37°C with 300 μCi of [^{32}P]PO$_4$. After stimulation in suspension at 37°C under stirring for 10 s with 0.5 μM CXCL12, 0.8 ml of cell suspension (24×10^6 cells) were directly lysed in 3 ml of methanol/chloroform 2:1. The chloroform phase was extracted, evaporated under N$_2$ stream, and the remaining lipid pellet was dissolved in 50 μl methanol/chloroform 2:1. Radioactive samples were spotted on Silica Gel 60 thin-layer chromatography plates, impregnated with 1.2% potassium oxalate and preactivated at 70°C, and then chromatographed in chloroform/methanol/2.5 N ammonium hydroxide (9:7:2, vol/vol). The ^{32}P-labeled products, identified by comparison with standards, were visualized by autoradiography. Radioactivity was quantified by a radioactivity plate reader (Instant Imager, Packard).

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.

5. RESULTS

The small GTPase RhoA regulates CXCL12-triggered LFA-1 activation in normal B-lymphocytes.

We first analyzed the consistency of the rho-module in human normal B-lymphocytes, by applying the Trojan peptide technology that has been already fully validated in the lymphocyte context [36,40].

We previously showed that the small GTPase RhoA, through the activity of distinct effector regions, differently controls LFA-1 high-affinity state and rapid lateral mobility induced by chemokines, in human T-lymphocytes [36]. So we verified whether RhoA is involved in chemokine-triggered LFA-1 activation in human B-lymphocytes.

RhoA was activated in B-lymphocytes by CXCL12 in a dose-dependent manner, with kinetics consistent with rapid adhesion triggering (Fig. 3*A*). In static adhesion assays, 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 [36], inhibited in a dose-dependent manner chemokine-stimulated rapid adhesion of B-lymphocytes to ICAM-1 (Fig. 3*B*). No effect was observed with the control Penetratin-1 peptide alone. Similar results were obtained in under-flow adhesion assays, either considering arrest for 1 or 10 seconds [40] (Fig. 3*C*). To support these observations, we investigated RhoA involvement in CXCL12-induced LFA-1 affinity up regulation by using the 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. 3*D*). Taken together, these data show that, in human normal B-lymphocytes, RhoA regulates LFA-1 affinity activation and dependent adhesion by CXCL12.
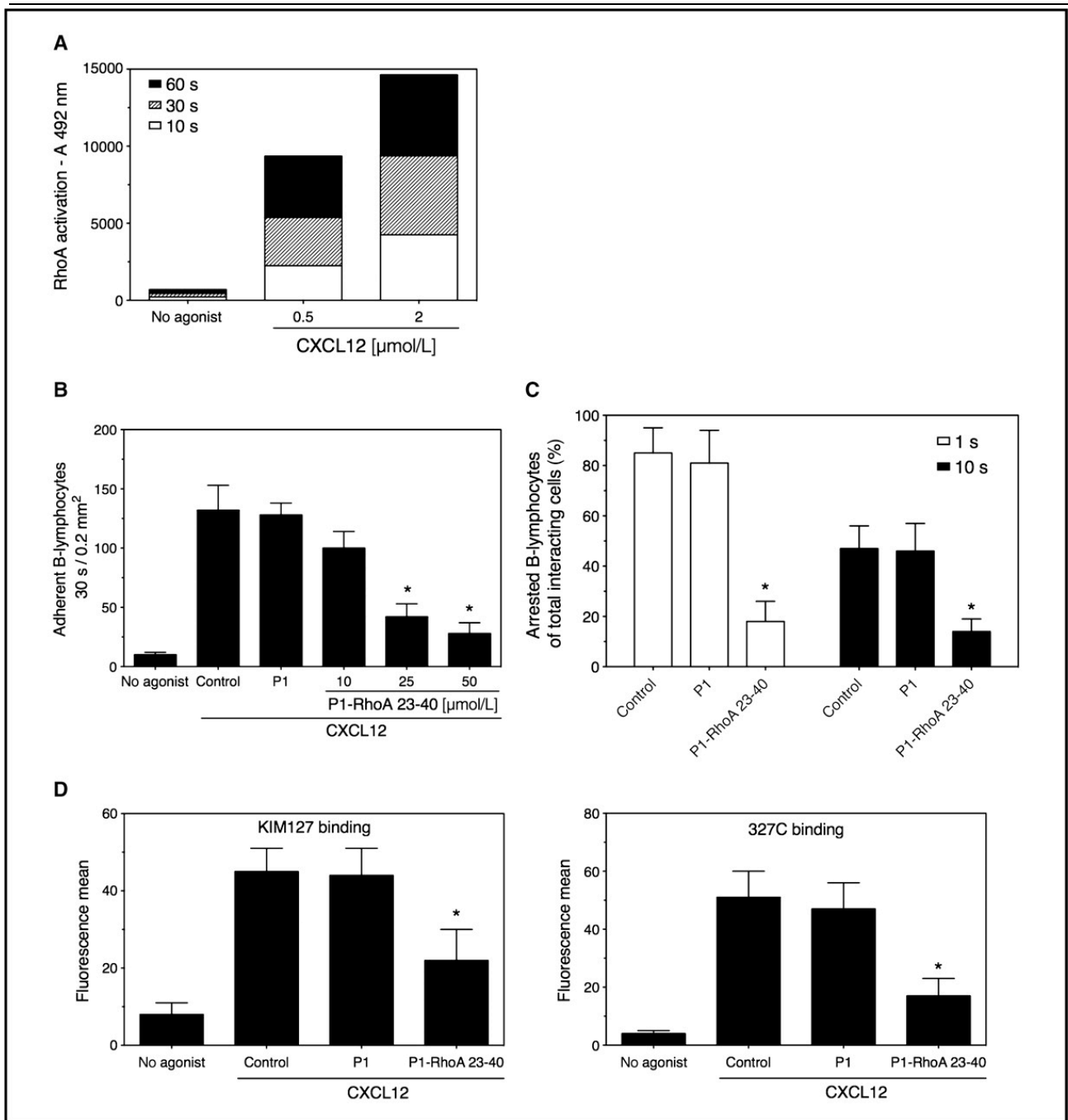
Figure 3. RhoA mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A*, measurement of RhoA activation by ELISA test; B-lymphocytes were treated with buffer (No agonist) or with the indicated concentrations of CXCL12 for the indicated times. *B*, static adhesion 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 as in (C) 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).

The small GTPase Rac1 regulates CXCL12-triggered LFA-1 activation in normal B-lymphocytes.

We next investigated the involvement of the small GTPase Rac1. Rac1 was activated in B-lymphocytes in a dose-dependent manner and with kinetics consistent with rapid adhesion triggering (Fig. 4*A*). To study Rac1 we applied a Tat-based Trojan peptide technology [40], that allows to put into the cell full-length functional proteins, mainly accumulated in the cytosol, as previously reported [54]. 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. 4*B*). Similar results were observed in under-flow adhesion assays (Fig. 4*C*). 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. 4*D*). The results are consistent with data obtained in static and under-flow adhesion assays and demonstrate that, along with RhoA, also Rac1 is critically involved in LFA-1 affinity modulation and dependent adhesion in human normal B-lymphocytes.
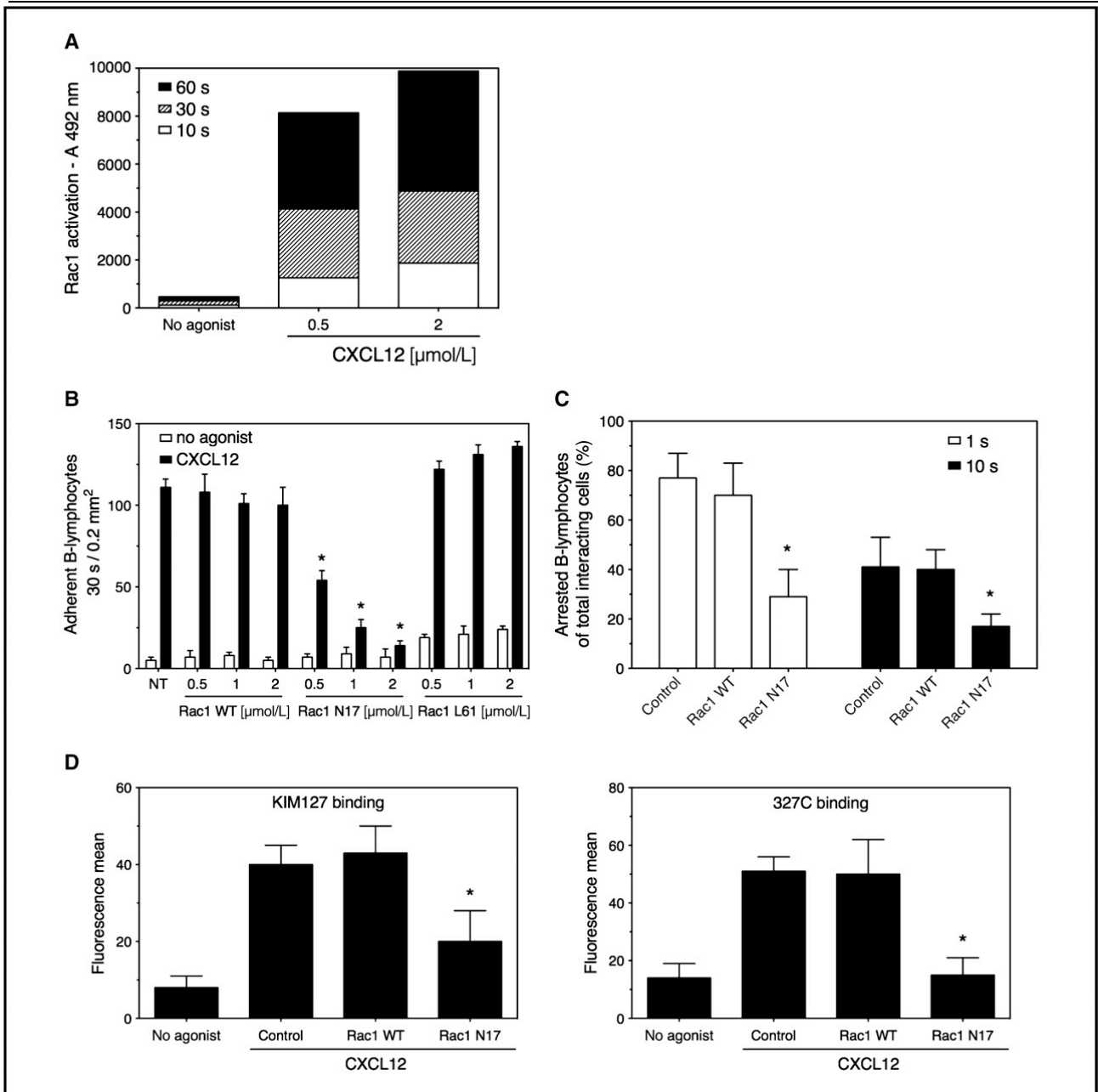
Figure 4. <u>Rac1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes.</u> *A*, measurement of Rac1 activation by ELISA test; B-lymphocytes were treated with buffer (No agonist) or with the indicated concentrations of CXCL12 for the indicated times. *B*, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (NT) 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. *D,* measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, *left*) or to high affinity state (327C, *right*); B-lymphocytes were treated as in (C) 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).

The small GTPase CDC42 is a negative regulator of CXCL12-induced LFA-1 activation in normal B-lymphocytes.

CDC42 is a RhoA- and Rac1-related small GTPase, showing an identity of 53% with RhoA and of 70% with Rac1, originally shown to regulate cytoskeleton dynamics and cell motility [55,56]. We recently discovered that CDC42 behaves as a general negative regulator of LFA-1 affinity modulation in human T-lymphocytes [40]. Thus, we verified whether CDC42 was negative regulator of LFA-1 activation also in human B-lymphocytes. CDC42 was activated in B-lymphocytes by CXCL12 although with slower kinetics with respect to RhoA and Rac1 activation (Fig. 5*A*). By using the Tat-based Trojan peptide technology we found that 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, Tat-CDC42-L61 or V12, two CDC42 constitutively active mutants, consistently blocked B-lymphocytes adhesion on ICAM-1, suggesting a negative function of CDC42 in CXCL12-triggered LFA-1 activation (Fig. 5*B*). Similar results were obtained in under-flow adhesion assays (Fig. 5*C*). 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. 5*D*). Indeed, the CDC42 constitutively active mutants (L61 and V12) reduced CXCL12-dependent LFA-1 affinity upregulation, 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 normal B-lymphocytes.
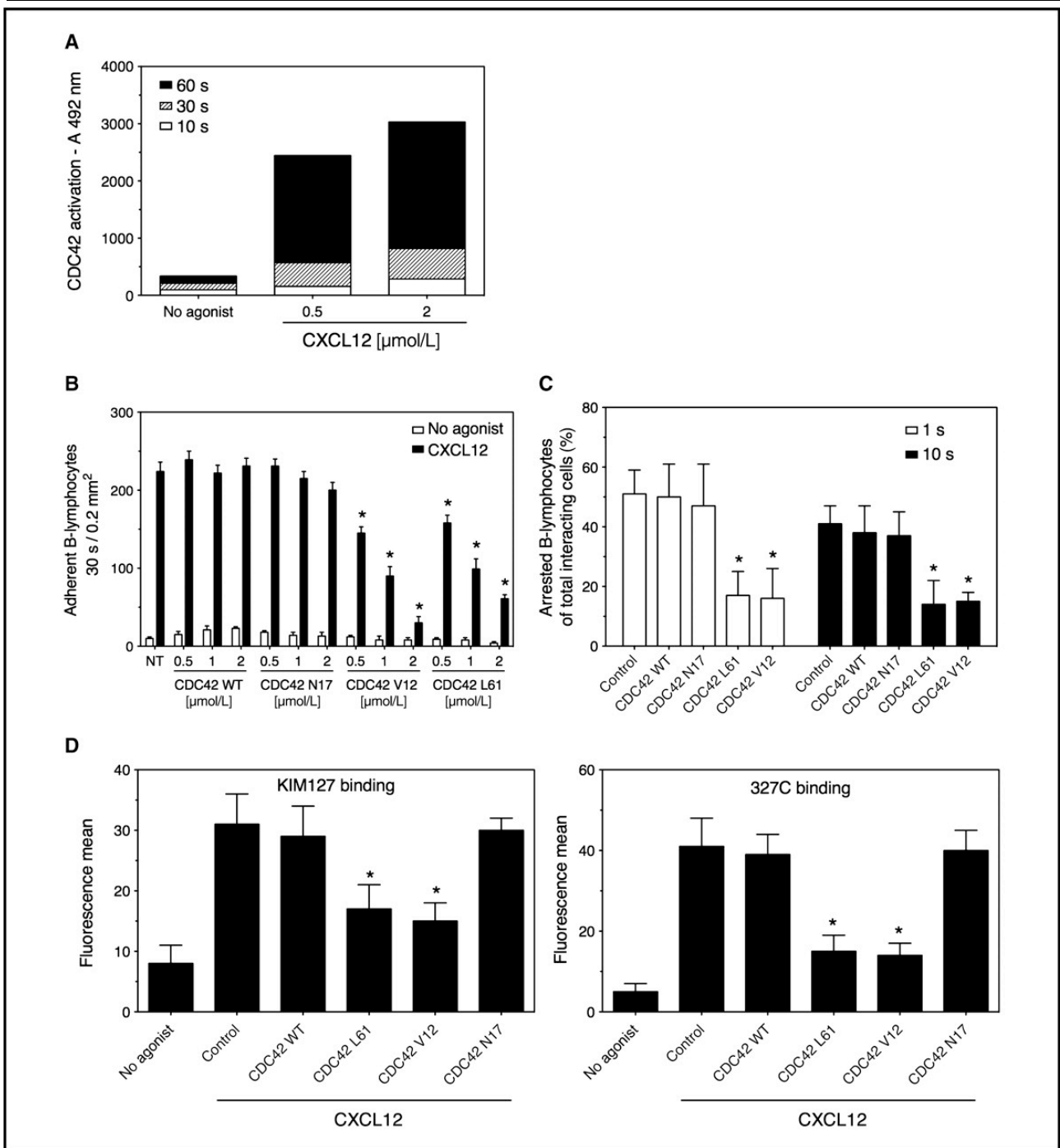
Figure 5. <u>CDC42 negatively modulates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes.</u> *A*, measurement of CDC42 activation by ELISA test; B-lymphocytes were treated with buffer (No agonist) or with the indicated concentrations of CXCL12 for the indicated times. *B*, static adhesion to ICAM-1; B-lymphocytes were treated with buffer (NT) 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. *D*, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, *left*) or to high affinity state (327C,

right); B-lymphocytes were treated as in (C) 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).

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

The positive regulatory function of RhoA and Rac1 on LFA-1 activation prompted us to characterize downstream effectors linking these two small GTPases to LFA-1 activation. We first focused on PLD1, which is a known common effector of RhoA and Rac1 [57,58] whose involvement in LFA-1 activation is suggested by the inhibitory effect of P1-RhoA 23-40 peptide on chemokine-induced LFA-1 affinity triggering [36]. Thus, we investigated PLD1 involvement in B-lymphocytes adhesion and LFA-1 activation by CXCL12. PLD1 was rapidly activated in a dose-dependent manner by CXCL12 in B-lymphocytes (Fig. 6*A*). In static adhesion assays, *n*-butanol, a commonly used PLD1 activity inhibitor able to prevent accumulation of phosphatidic acid (PA), strongly inhibited rapid CXCL12-induced adhesion to ICAM-1. In contrast, *ter*-butanol, an inactive isomer, was without effect (Fig. 6*B*). To further corroborate this data, we used a Penetratin-1-PLD1 fusion Trojan peptide, previously validated as an effective tool preventing PLD1 activation [40]. Inhibition of PLD1 by the peptide resulted in markedly reduced adhesion on ICAM-1; no effect was detected after treatment with the control Penetratin-1 peptide alone (Fig. 6*B*). Similar results were obtained in under-flow adhesion assays (Fig. 6*C*). To further support PLD1 role in LFA-1 activation in B-lymphocytes, we measured LFA-1 affinity triggering 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. 6*D*), clearly confirming PLD1 crucial role in regulating CXCL12-induced LFA-1 affinity regulation and mediated adhesion in B-lymphocytes.
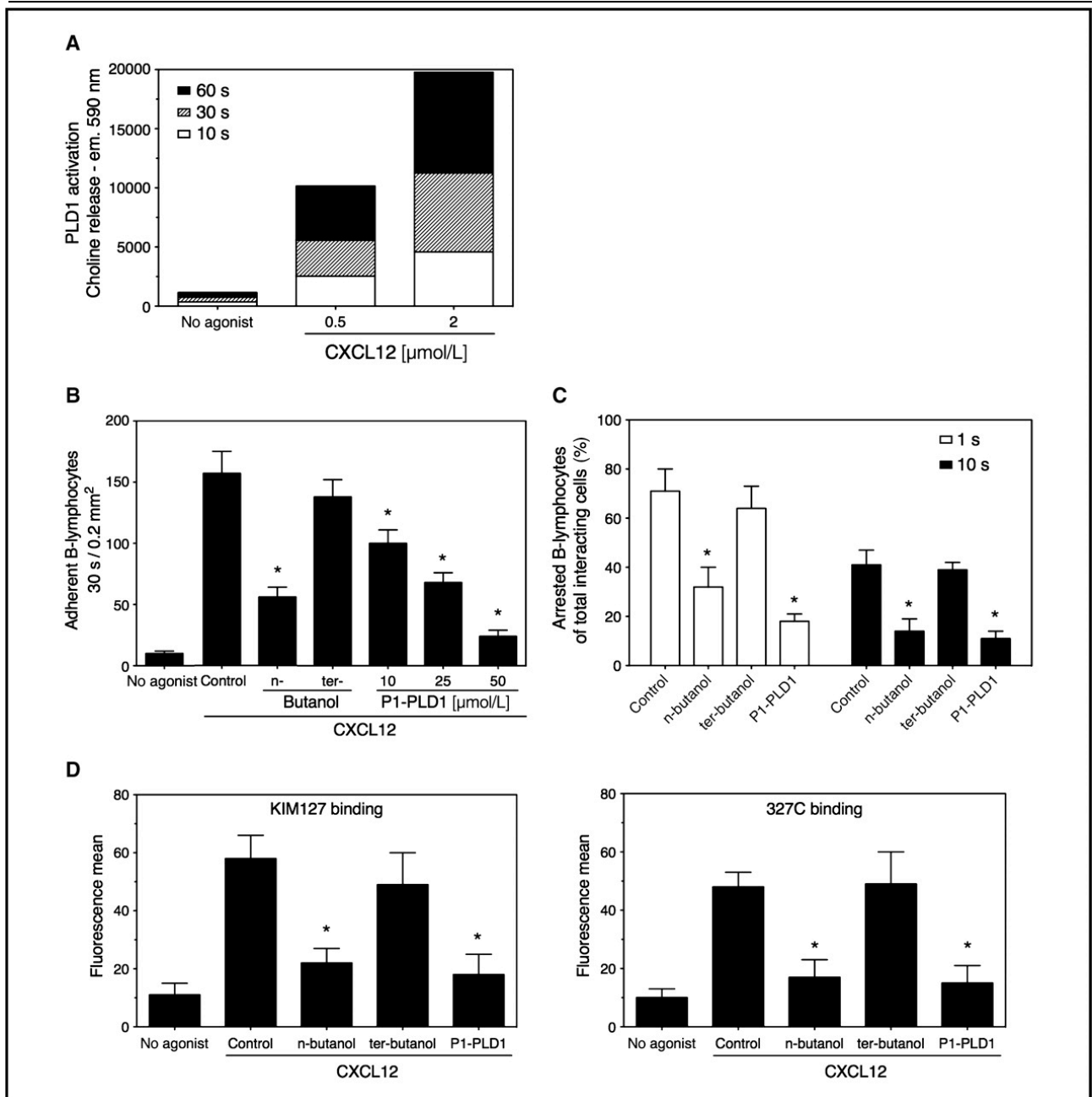
Figure 6. PLD1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A*, measurement of PLD1 activation by quantification of choline release; B-lymphocytes were treated with buffer (No agonist) or with the indicated concentrations of CXCL12 for the indicated 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. *D*, measurement of LFA-1 triggering to low-intermediate affinity state (KIM127, *left*) or to high affinity state (327C, *right*); B-lymphocytes were treated as in (C) 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).

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PIP5KC is a conformer-selective regulator of LFA-1 affinity triggering by CXCL12 in normal B-lymphocytes.

To completely characterize the role of the rho-signaling module controlling LFA-1 activation by CXCL12 in human normal B-lymphocytes, we tested the role of PIP5KC, a downstream effector of RhoA, Rac1 and directly activated by phosphatidic acid [59-61]. Notably, PIP5KC increases the local concentration of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) on the plsma membrane [62], which in turn activates talin1 (TLN1) [63], possibly leading to integrin affinity activation [64]. As the gamma isoform (PIP5KC) directly interacts with TLN1 [65-67], we next focused on PIP5KC as a potential component of the signaling cascade leading to integrin activation. PIP5KC activity was triggered with a time-course kinetic by CXCL12 (Fig. 5*A*). To explore the functional involvement of PIP5KC we exploited an established siRNA-based approach [40]. 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. 7*A*). 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. 7*B-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. 7*D*). However, and importantly, transition to high affinity state was consistently inhibited (Fig. 7*D*). Together, the data indicate that, as in T-lymphocytes, also in B-lymphocytes PIP5KC is a conformer-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.
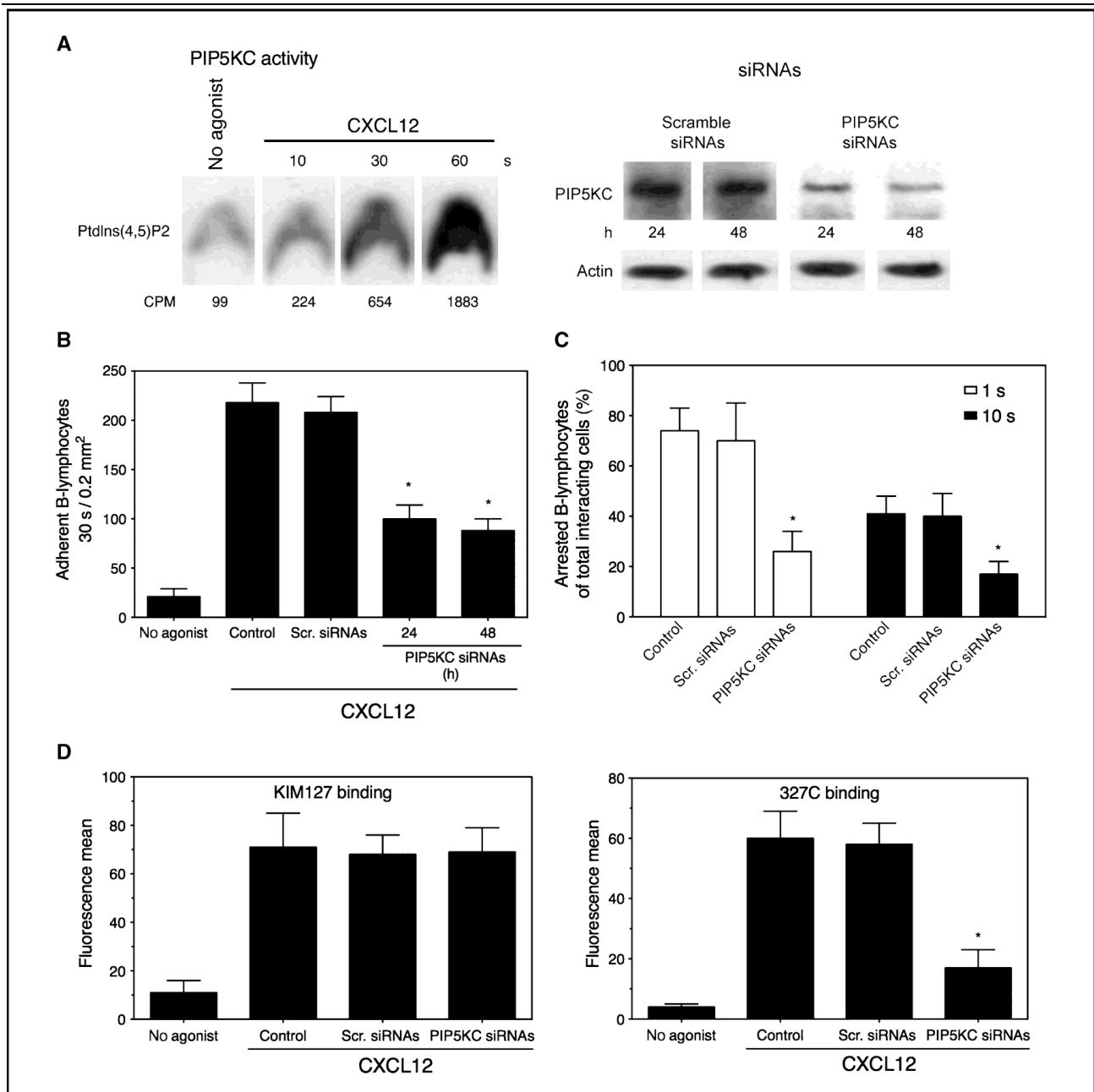
Figure 7. PIP5KC selectively controls CXCL12-induced triggering of LFA-1 to high affinity state and dependent adhesion in normal B-lymphocytes. *A, left*, evaluation of PIP5KC activity; B-lymphocytes were treated with buffer (No agonist) or with 0.5 μM CXCL12 for the indicated times. Shown is an autoradiogram of ^{32}P-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 the indicated times and treated with buffer (No agonist and Control) or 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. *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).

The regulatory role of the CXCL12-induced rho-module of LFA-1 affinity triggering is not conserved in CLL B-lymphocytes.

Having established the consistency of the pro-adhesive rho-module in normal B-lymphocytes, we investigated whether the rho-module of LFA-1 affinity regulation was conserved also in malignant B-lymphocytes directly isolated from CLL patients. 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.

Global analysis of the data immediately highlighted a consistent variability between patients regarding the relative regulatory role of the different signaling mechanisms (Supplementary Table S1). On this base, we could group the 31 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. 8*A-B*). Moreover, analysis of LFA-1 conformational changes showed that RhoA blockade resulted in a consistent inhibition of LFA-1 affinity triggering (Fig. 8*A-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 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 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 affinity by CXCL12. Treatment with Tat-Rac1 WT or L61 was without effect. In sharp contrast, in cluster (B), Rac1 inhibition did not affect adhesion to ICAM-1 nor LFA-1 affinity triggering. 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. 8*A*), and a second one with null sensitivity to Rac1 inhibition (Fig. 8*B*). Thus, 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. 8*A*) and a second one in which CDC42 did not have any role (Fig. 8*B*). 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. 8*A-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 affinity states induced by CXCL12 (Fig. 8*A*-*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 never bypassed by the neoplastic transformation.

Finally, we wished to test the role of PIP5KC by applying 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 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. 8*A*-*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. 8*A*-*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 B-lymphocytes, as it appears never involved in LFA-1 activation in B-CLL lymphocytes, thus establishing a sharp dichotomy with respect to normal B-lymphocytes.
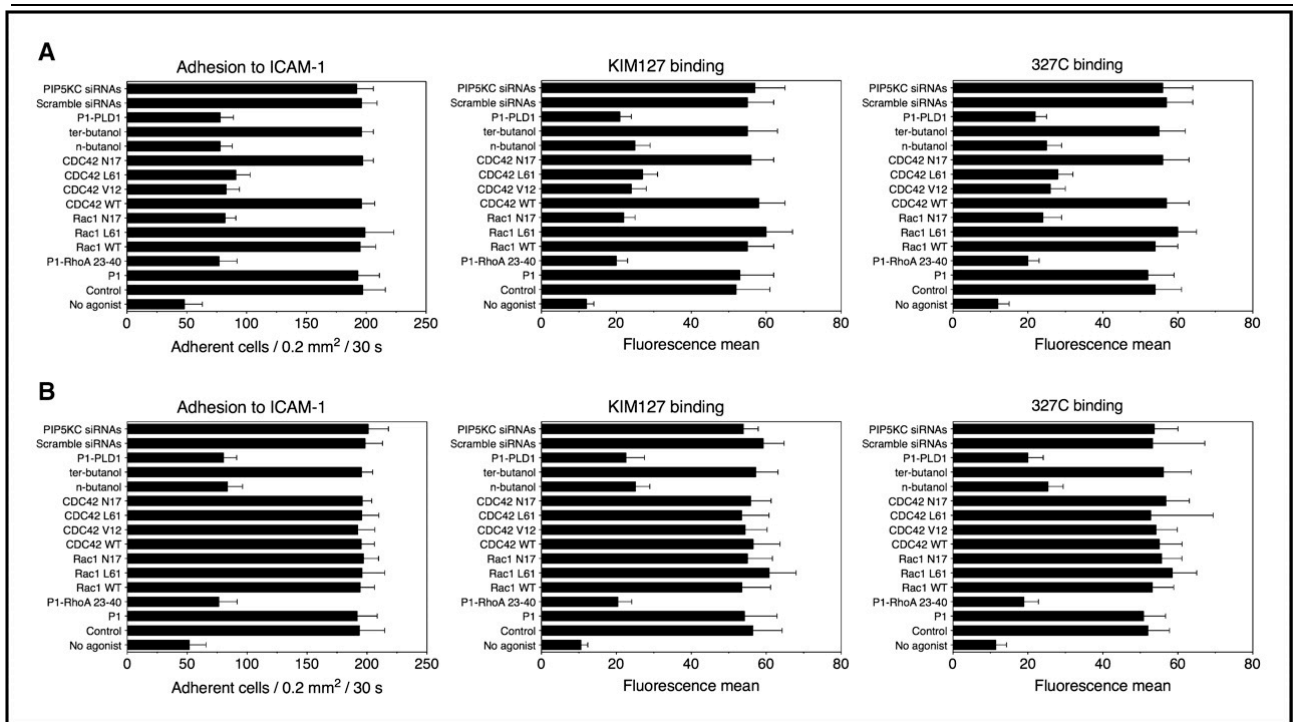
Figure 8. B-lymphocytes isolated from B-CLL patients show altered signaling mechanisms of LFA-1 affinity triggering by CXCL12. *A*, static adhesion to ICAM-1 (*left*), detection of LFA-1 conformers with low-intermediate affinity (KIM127, *middle*) or high affinity (327C, *right*); B-CLL lymphocytes from 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.

6. DISCUSSION

Cell migration is an universal process; most cell types in the body are capable of migrating at one or more distinct steps during their development and differentiation. This migration is essential for tissue morphogenesis and leukocyte trafficking, as well as for epithelial turnover and regeneration processes, such as wound healing. Furthermore, deregulated cell migration can take place in cancer, resulting in tumor invasion and metastasis.

B-cell chronic lymphocytic leukemia 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 [68,69]. A progressive dissemination and accumulation of malignant cells to secondary lymphoid organs and to bone marrow characterizes the progression and severity of the disease [68,70,71].

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. 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 normal T-lymphocytes [40]. 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 B-lymphocytes isolated from healthy donors; 2) in B-CLL patients, RhoA and PLD1 are conserved signaling events controlling LFA-1 activation by CXCL12; 3) in contrast, Rac1 and CDC42 display a consistent patient-by-patient variability, with a group of B-CLL patients showing LFA-1 affinity modulation completely independent of Rac1 and CDC42 signaling activity; 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 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 is also dispensable 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.

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 normal B-lymphocytes. This finding is of interest, since the critical regulatory role of this signaling module in normal T-lymphocytes [40] does not imply that identical signaling mechanisms regulate integrin activation also in normal B-lymphocytes, as also previously suggested [72]. Thus, our study suggests the universal relevance of the rho-module in regulating conformer-selective triggering of LFA-1 by chemokines.

A further important observation is that the signaling couple RhoA-PLD1 is critical in LFA-1 affinity regulation by CXCL12 in all 31 analyzed B-CLL patients. Notably, PLD1 activation by CXCL12 depends on RhoA and Rac1 activity [40]. Considering that in about half of analyzed patients (cluster (B)) Rac1 has no role, it is likely that in these patients only RhoA activates PLD1. Thus, in B-CLL, RhoA can fully bypass and compensate an eventually defective Rac1 signaling and ensure, alone, a competent PLD1 activation, thus establishing a sharp dichotomy with respect to normal lymphocytes.

A rather interesting finding of our study is that the regulatory role of Rac1 and CDC42 can be lost in a patient-selective manner. Thus, the neoplastic progression seems to affect, in certain CLL patients, the role of Rac1 and CDC42, with the 14 patients of group (B) showing LFA-1 affinity modulation by chemokines independent of Rac1 and CDC42 activity. Although variability in signaling mechanisms controlling integrin triggering could be expected, it was surprising to observe 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. It is difficult at this stage to

speculate about the mechanisms of such diversity. 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 mechanisms other then altered Rac1 and CDC42 expression or activation, possibly including a higher expression/activation of RhoA compensating defective Rac1 activity or altered intracellular localization and/or expression of Rac1 and CDC42 downstream effectors.

PIP5KC, which is the downstream component of the rho-module specifically controlling LFA-1 conformeric transition to high affinity state in normal lymphocytes [40], 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 shows that, in B-CLL lymphocytes, signaling mechanisms not involving PIP5KC activity 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 lymphocytes, in B-CLL lymphocytes the role of PIP5KC can be completely bypassed by the other two isoforms of PIP5K, A and B. Moreover, a possible regulatory role for the small GTPase Rap1 in B-CLL lymphocytes LFA-1 activation can be deduced by recent findings [73]. Notably, Rap1 can potentially mediate LFA-1 affinity triggering by means of RIAM-Talin1 interactions [74] or by modulating phosphorylation of LFA-1 α chain [75]. 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.
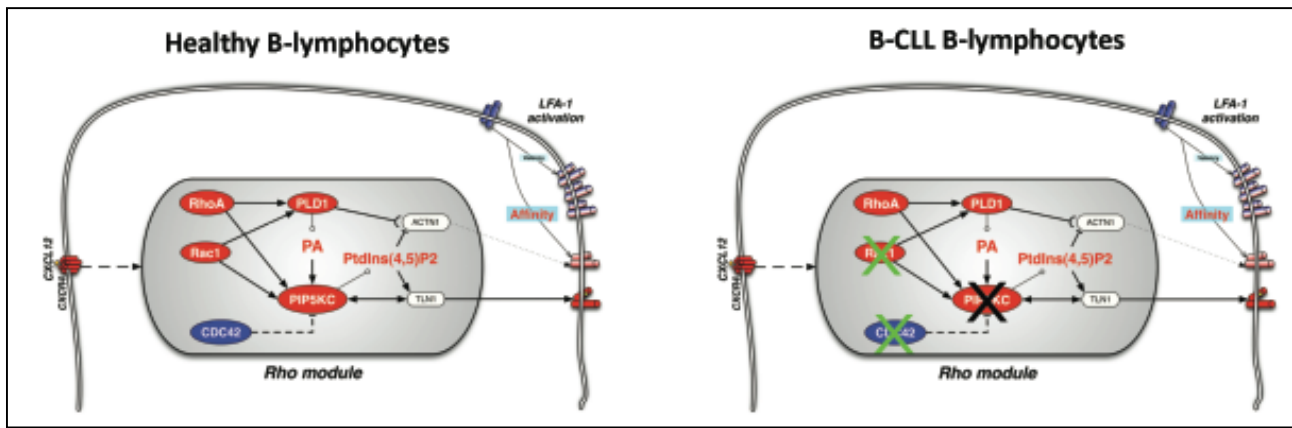
Figure 9. Network module regulating CXCL12-induced LFA-1 affinity activation in healthy (*left*) and B-CLL B-lymphocytes.

Overall, our study identifies a consistent diversity in signaling mechanisms controlling LFA-1 activation by chemokines in B-lymphocytes isolated from B-CLL patients. 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 contrasts with recent findings [73] 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 systematic analysis of the intracellular signaling events governing cell adhesion can be a helpful strategy to better categorize B-CLL patients, with possible impact on diagnosis, prognosis and therapy of leukemia diseases.

Finally, our findings show that RhoA, Rac1, CDC42, PLD1 and PIP5KC are functionally integrated to control different aspects of LFA-1 activation by chemokines in physiological conditions. According to the modern modular view of the functional architecture of biological networks [76,77], it is conceivable that chemoattractant-triggered intracellular signaling networks may be organized in discrete functional units, or modules, likely corresponding to co-compartmentalized proteins regulating specific cell activities [78,79], with some devoted to the control of dynamics of rapid integrin activation. In this context, our study 1) provides evidence supporting the existence

and critical regulatory function of a rho-based signaling module activated by chemokines and leading to conformer-selective modulation of LFA-1 affinity in normal B-lymphocytes and 2) identifies striking differences in the network architecture in chronic lymphocytic leukemia.

All the data showed and discussed in the present study, regarding the signaling pathway triggered by CXCL12 for LFA-1 activation in healthy versus B-CLL B-lymphocytes, were published last year in *Cancer Research* [80].

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