Comparative Analysis of Normal versus CLL B-Lymphocytes Reveals Patient-Specific Variability in Signaling Mechanisms Controlling LFA-1 Activation by Chemokines

Alessio Montresor,[1,3] Matteo Bolomini-Vittori,[1] Scott I. Simon,[4] Antonella Rigo,[2] Fabrizio Vinante,[2] and Carlo Laudanna[1,3]

[1]Department of Pathology, [2]Department of Clinical and Experimental Medicine, Section of Hematology, and [3]The Center for Biomedical Computing, University of Verona, Verona, Italy; and [4]Department of Biomedical Engineering, Genome and Biomedical Sciences Facility, University of California, Davis, California

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 patients with B-CLL, the role of the rho-module was not maintained, showing remarkable differences and variability. Specifically, RhoA and phospholipase D1 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 patients with CLL can be more accurately evaluated on the basis of the analysis of signaling mechanisms controlling integrin activation. Our findings could potentially affect the diagnosis, prognosis, and therapy of CLL disorders. [Cancer Res 2009;69(24):9281–90]

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). 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 patients with B-CLL show increased levels of chemokine receptors such as CCR7, CXCR4, and CXCR5 (2, 3). The CXCR4/CXCL12 axis is also critical to maintaining 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 plasma membrane leading to increased valency (7). LFA-1 is the most studied integrin in immune cells and its conformational changes leading to affinity increase are well characterized (8–10). 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 (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 normal B-lymphocytes versus malignant B-lymphocytes isolated from patients with B-CLL. Our results highlight the universal relevance of the rho-module of LFA-1 activation in normal 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 the 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, does not seem to have 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, neoplastic progression can make the regulatory role of signaling events—which is otherwise critical in normal lymphocytes—irrelevant. Based on this, we could group the patients into at least two categories, characterized by divergent signaling mechanisms regulating LFA-1 affinity triggering

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

Requests for reprints: Carlo Laudanna, University of Verona, Strada Le Grazie 8, Verona, VR 37134, Italy. Phone: 39-045-8027689; Fax: 39-045-8027127; E-mail: carlo.laudanna@univr.it.

©2009 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-09-2009

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

by CXCL12. These findings could potentially affect the diagnosis and treatment of CLL disease.

Materials and Methods

Reagents. Fetal bovine serum 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; ref. 12) were from American Type Culture Collection; anti–LFA-1 monoclonal antibodies 327C (reporter for extended conformation epitope corresponding to a high-affinity state; ref. 13) was from ICOS, Corp.

Isolation of B-lymphocytes from healthy subjects and B-CLL patients. Normal B-lymphocytes were isolated from healthy donors as in ref. (11). The purity of B-lymphocyte preparations was evaluated by flow cytometry with anti-CD19 monoclonal antibody. CLL B-lymphocytes were isolated from peripheral blood mononuclear cells 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 on clinical and laboratory parameters, including 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 (Verona, Italy). 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 mmol/L glutamine + 10% fetal bovine serum for 3 h before treatment with Trojan peptides or silencing with short interfering RNAs (siRNA).

Trojan peptide technology. Tat-fusion proteins were produced as described (11). P1-based peptides, including control penetratin-1 (P1), the RhoA blocking (P1-RhoA 23–40, which included P1 and the downstream switch I effector region of human RhoA encompassing amino acids 23–40; refs. 11, 15, 16) and the PLD1-blocking (P1-PLD1, which included P1 and the region of human PLD1 that specifically mediated the interaction of PLD1 with RhoA; refs. 11, 17), were synthesized by GenScript. Cell treatment with Tat proteins and P1 peptides was done for 60 to 90 min at 37°C.

Gene silencing of PIP5KC by siRNA. siRNAs targeting PIP5KC were designed according to GenBank (accession no. NM_012398), chemically synthesized by Dharmacon, and provided as a premixed pool (SmartPool). Silencing was performed in normal and CLL B-lymphocytes by nucleoporation, using the Amaxa Nucleofector (Amaxa Biosystems; ref. 11). The efficacy of gene silencing was evaluated by immunoblotting (11).

Static adhesion assay. B-lymphocytes were resuspended at 5×10^6/mL in standard adhesion buffer [PBS + 10% fetal bovine serum + Ca^{2+} 1 mmol/L + Mg^{2+} 1 mmol/L (pH 7.2)]. Adhesion assays were performed on 18-well glass slides coated with human ICAM-1, 1 μg/mL in PBS. A cell suspension (20 μL) was added to the well and stimulated at 37°C with 5 μL of CXCL12, at a final concentration of 0.5 μmol/L for 30 s. After washing, adherent cells were fixed in glutaraldehyde 1.5% in ice-cold PBS and counted by computer-assisted enumeration (7).

Underflow adhesion assay. Microcap glass capillary tubes (100 μL; 1 mm internal diameter, from Drummond) were first coated for 10 h at 4°C with 1 μg/mL of human E-selectin in PBS; tubes were then washed and coated overnight at 4°C with 1 μg/mL of human ICAM-1 in PBS. Before use, tubes were treated with FCS for 10 min, washed, and then coated with 2 μmol/L of CXCL12 in PBS for 30 min. The behavior of interacting B-lymphocytes (shear stress was 2 dyn/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 2×10^6/mL, were briefly preincubated with 10 μg/mL of KIM127 or 327C monoclonal antibodies and then stimulated for 10 s with 0.5 μmol/L of CXCL12 (final concentration) 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, Rac1, CDC42, and PLD1 activations were measured by using a commercial kits, as described in ref. (11). PIP5K activation was evaluated by TLC of ^{32}P-labeled lipid products, as described in ref. (11).

Statistical analysis. Statistical analysis was carried out by calculating mean and 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 normal B-lymphocytes. We first analyzed the consistency of the rho-module in human normal B-lymphocytes by applying the Trojan peptide technology, which has been already fully validated in the context of lymphocytes (11, 15, 16).

RhoA was activated in B-lymphocytes by CXCL12 in a dose-dependent manner, with kinetics consistent with rapid adhesion triggering (Fig. 1*A*). In static adhesion assays, the P1-RhoA 23–40 peptide, a synthetic peptide blocking the downstream switch I effector region of human RhoA (amino acids 23–40) and dependent downstream signaling pathway (15), inhibited the chemokine-stimulated rapid adhesion of B-lymphocytes to ICAM-1 in a dose-dependent manner (Fig. 1*B*). No effect was observed with the control penetratin-1 peptide alone. Similar results were obtained in underflow adhesion assays, either considering arrest for 1 or 10 seconds (ref. 11; Fig. 1*C*). To support these observations, we investigated RhoA involvement in CXCL12-induced LFA-1 affinity upregulation by using the monoclonal antibodies, KIM127 and 327C, which specifically recognize LFA-1–extended conformers expressing epitopes corresponding to low/intermediate and high-affinity states, respectively. Inhibition of RhoA signaling resulted in the blockade of LFA-1 conformeric transition to low/intermediate as well as to high-affinity states (Fig. 1*D*). Taken together, these data show that, in human normal B-lymphocytes, RhoA regulates LFA-1 affinity activation and dependent adhesion by CXCL12.

The small GTPase Rac1 regulates CXCL12-triggered LFA-1 activation in normal B-lymphocytes. We next investigated the involvement of the small GTPases Rac1. Rac1 was activated in B-lymphocytes in a dose-dependent manner and with kinetics consistent with rapid adhesion triggering (Fig. 2*A*). To study Rac1, we applied a Tat-based Trojan peptide technology (11). In static adhesion assays, inhibition of Rac1 signaling by Tat-Rac1-N17, a dominant-negative mutant, blocked CXCL12-triggered adhesion to ICAM-1 in a dose-dependent manner. In contrast, Tat-Rac1 wild-type (WT), and Tat-Rac1-L61 (constitutively active mutant form), had no effect on LFA-1–mediated adhesion on ICAM-1 (Fig. 2*B*). Similar results were observed in underflow adhesion assays (Fig. 2*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. 2*D*). The results are consistent with data obtained in static and underflow adhesion assays and show that, along with RhoA, Rac1 is also critically

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

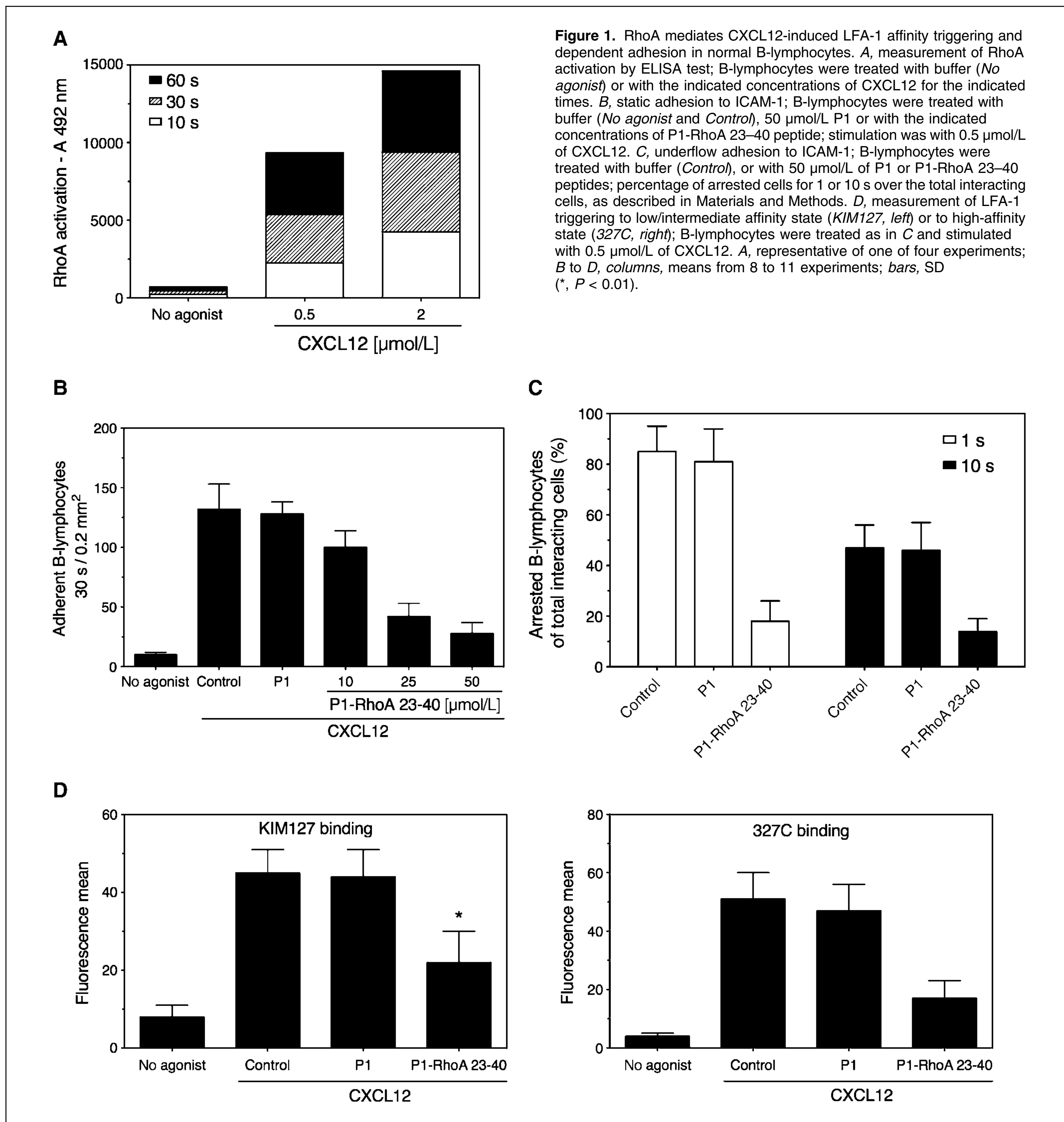

Figure 1. RhoA mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A,* measurement of RhoA activation by ELISA test; B-lymphocytes were treated with buffer (*No agonist*) or with the indicated concentrations of CXCL12 for the indicated times. *B,* static adhesion to ICAM-1; B-lymphocytes were treated with buffer (*No agonist* and *Control*), 50 μmol/L P1 or with the indicated concentrations of P1-RhoA 23–40 peptide; stimulation was with 0.5 μmol/L of CXCL12. *C,* underflow adhesion to ICAM-1; B-lymphocytes were treated with buffer (*Control*), or with 50 μmol/L of P1 or P1-RhoA 23–40 peptides; percentage of arrested cells for 1 or 10 s over the total interacting cells, as described in Materials and Methods. *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 μmol/L of CXCL12. *A,* representative of one of four experiments; *B* to *D, columns,* means from 8 to 11 experiments; *bars,* SD (*, $P < 0.01$).

involved in LFA-1 affinity modulation and dependent adhesion in human normal B-lymphocytes.

The small GTPase CDC42 is a negative regulator of CXCL12-induced LFA-1 activation in normal B-lymphocytes. We recently discovered that CDC42 behaves as a general negative regulator of LFA-1 affinity modulation in human T-lymphocytes (11). Thus, we verified whether CDC42 was also a negative regulator of LFA-1 activation in human B-lymphocytes. CDC42 was activated in B-lymphocytes by CXCL12 although with slower kinetics with respect to RhoA and Rac1 activation (Fig. 3*A*). By using 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 treatment. Conversely, Tat-CDC42-L61 or V12, two CDC42 constitutively active mutants, consistently blocked B-lymphocyte adhesion on ICAM-1, suggesting a negative

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

function of CDC42 in CXCL12-triggered LFA-1 activation (Fig. 3*B*). Similar results were obtained in underflow adhesion assays (Fig. 3*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. 3*D*). Indeed, the CDC42 constitutively active mutants (L61 and V12) reduced CXCL12-dependent LFA-1 affinity upregulation, whereas CDC42 WT and N17 constructs, in contrast, 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.

PLD1 mediates CXCL12-triggered LFA-1 activation in normal B-lymphocytes. PLD1 is a common downstream effector of RhoA and Rac1 (18). Its role in rapid LFA-1 triggering in human normal B-lymphocytes was never explored. Thus, we investigated PLD1 involvement in B-lymphocyte adhesion and LFA-1 activation by CXCL12. PLD1 was rapidly activated in a dose-dependent manner by CXCL12 in B-lymphocytes (Fig. 4*A*). In static adhesion assays, *n*-butanol, a commonly used PLD1 activity inhibitor able to prevent the accumulation of phosphatidic acid, strongly inhibited rapid CXCL12-induced adhesion to ICAM-1. In contrast, *ter*-butanol, an inactive isomer, had no effect (Fig. 4*B*). To further corroborate this data, we used a penetratin-1-PLD1

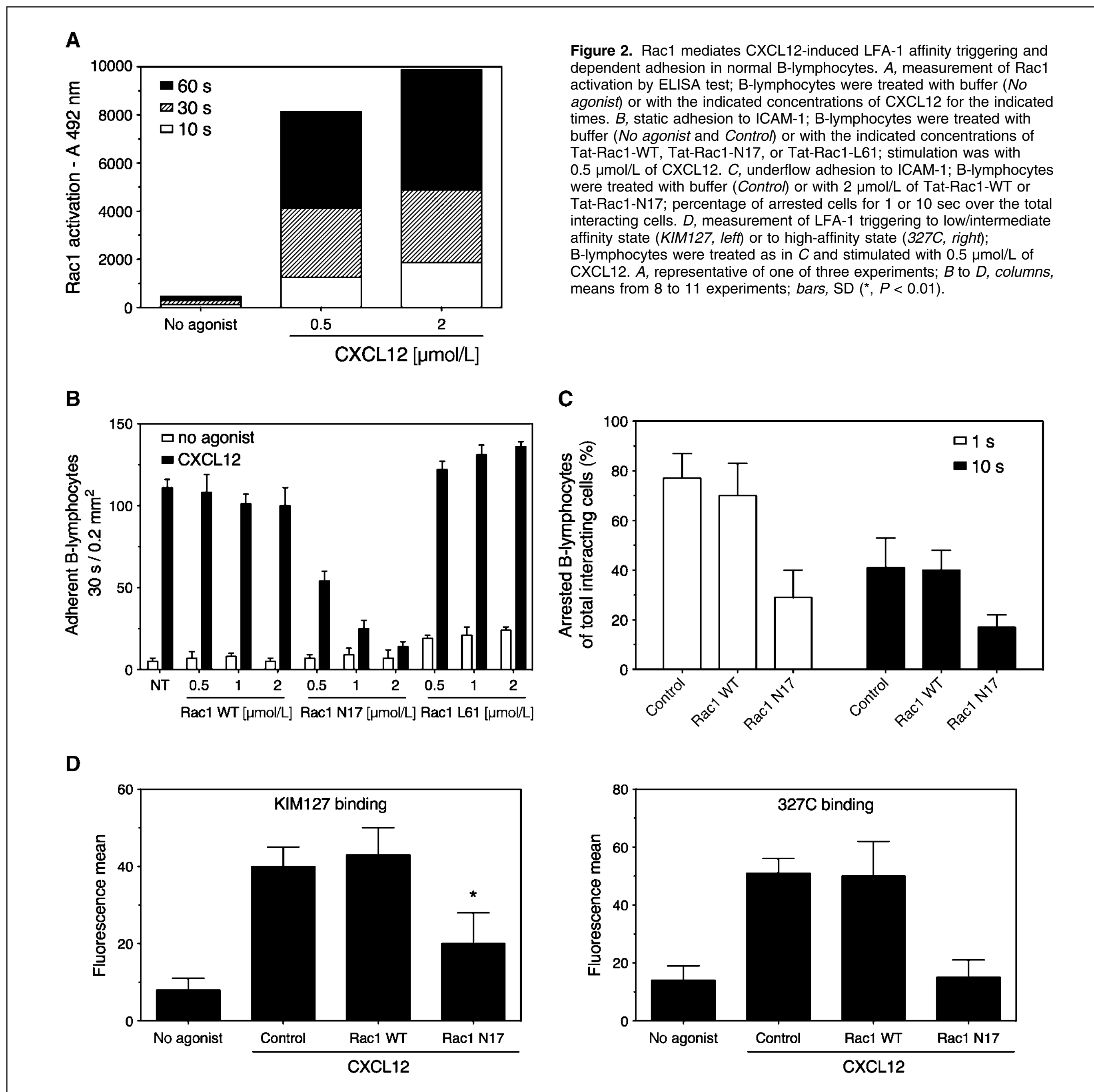

Figure 2. Rac1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A,* measurement of Rac1 activation by ELISA test; B-lymphocytes were treated with buffer (*No agonist*) or with the indicated concentrations of CXCL12 for the indicated times. *B,* static 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 μmol/L of CXCL12. *C,* underflow adhesion to ICAM-1; B-lymphocytes were treated with buffer (*Control*) or with 2 μmol/L of Tat-Rac1-WT or Tat-Rac1-N17; percentage of arrested cells for 1 or 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 μmol/L of CXCL12. *A,* representative of one of three experiments; *B* to *D, columns,* means from 8 to 11 experiments; *bars,* SD (*, $P < 0.01$).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

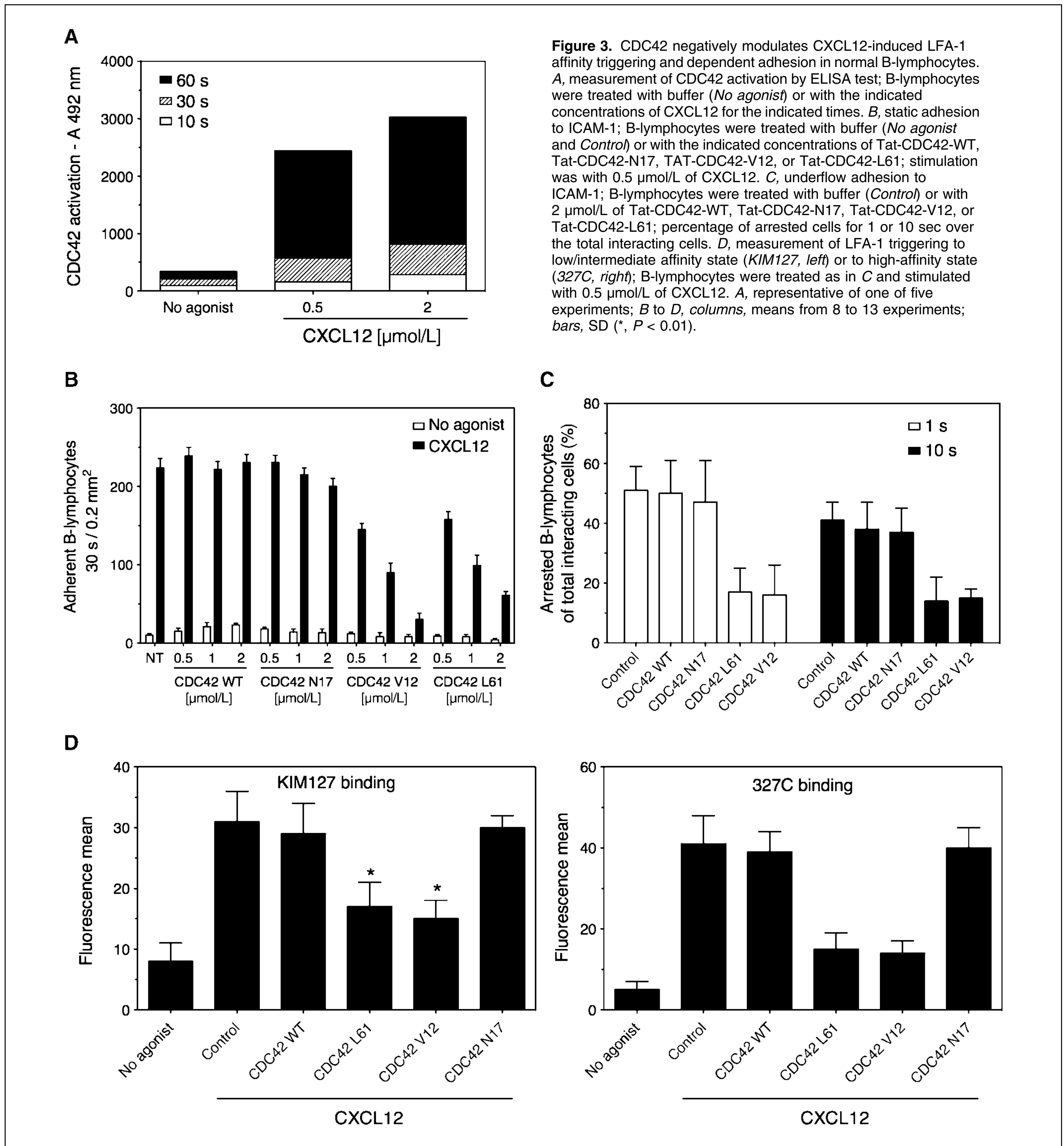

Figure 3. CDC42 negatively modulates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A,* measurement of CDC42 activation by ELISA test; B-lymphocytes were treated with buffer (*No agonist*) or with the indicated concentrations of CXCL12 for the indicated times. *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 µmol/L of CXCL12. *C,* underflow adhesion to ICAM-1; B-lymphocytes were treated with buffer (*Control*) or with 2 µmol/L of Tat-CDC42-WT, Tat-CDC42-N17, Tat-CDC42-V12, or Tat-CDC42-L61; percentage of arrested cells for 1 or 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 µmol/L of CXCL12. *A,* representative of one of five experiments; *B* to *D, columns,* means from 8 to 13 experiments; *bars,* SD (*, $P < 0.01$).

fusion Trojan peptide, previously validated as an effective tool preventing PLD1 activation (11). 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. 4*B*). Similar results were obtained in underflow adhesion assays (Fig. 4*C*). To further support the role of PLD1 in LFA-1 activation in B-lymphocytes, we measured LFA-1 affinity triggering after treatment with *n*-butanol and the PLD1 blocking peptide. Similar to adhesion data, both *n*-butanol and P1-PLD1 blocking peptides strongly inhibited rapid CXCL12-induced transition of LFA-1 to low/intermediate as well as to high-affinity states (Fig. 4*D*), clearly confirming the crucial role of PLD1 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 normal B-lymphocytes. To completely

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

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 PLD1 (19, 20). 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 (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 had no effect (Fig. 5*A*). Importantly, in B-lymphocytes with reduced expression levels of PIP5KC, rapid static as well as underflow adhesion to ICAM-1 triggered by CXCL12 were consistently blocked, suggesting the critical involvement of PIP5KC in LFA-1 activation (Fig. 5*B* and *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 a low/intermediate affinity state (Fig. 5*D*). However, and importantly, transition to a high-affinity state was consistently inhibited (Fig. 5*D*). Together, the data indicate that, as in T-lymphocytes, PIP5KC is also a conformer-selective regulator of LFA-1 affinity in B-lymphocytes, controlling triggering of LFA-1 to a high-affinity state, but not to a 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. Having established the consistency of the proadhesive rho-module in normal B-lymphocytes, we investigated whether the rho-module of LFA-1 affinity regulation was also conserved in malignant B-lymphocytes directly isolated from patients with CLL. To this end, we performed a multiplexed analysis of malignant B-lymphocytes directly isolated from a total of 31 patients with B-CLL 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). Based on this, we could group the 31 patients into two clusters (cluster A, 17 patients; cluster B, 14 patients) characterized by different sensitivities 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. 6*A* and *B*). Moreover, analysis of LFA-1 conformational changes showed that RhoA blockade resulted in a consistent inhibition of LFA-1 affinity triggering (Fig. 6*A* and *B*), comparable to the data obtained in healthy B-lymphocytes. These data were consistent in all 31 patients studied and clearly suggested that RhoA is a very conserved signaling mechanism, controlling LFA-1 activation by chemokines 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 with reduced adhesion to ICAM-1, accompanied by impaired triggering of LFA-1 affinity by CXCL12. Treatment with Tat-Rac1-WT or L61 had no 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 others totally insensitive to Rac1 inhibition. These observations allowed the grouping of the patients into two distinct groups (A and B), one characterized by high sensitivity to Rac1 inhibition (Fig. 6*A*), and a second one with null sensitivity to Rac1 inhibition (Fig. 6*B*). Thus, as a consequence of neoplastic transformation, the regulatory role of Rac1 on LFA-1 affinity modulation by chemokines could 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, CDC42 also manifests a patient-specific involvement in LFA-1 affinity modulation by CXCL12. As for Rac1, the CDC42 data also allowed the grouping of B-CLL patients into at least two different groups: one in which CDC42 had a negative regulatory role (Fig. 6*A*), and a second one in which CDC42 did not have any role (Fig. 6*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-lymphocytes, the neoplastic transformation could, in certain conditions, abolish the opposite regulatory roles of Rac1 and CDC42 at the same time.

The patient-specific regulatory role of Rac1 could also imply variability 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. 6*A* and *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 upregulate LFA-1 affinity states induced by CXCL12 (Fig. 6*A* and *B*). Again, this was evidenced in all 31 patients analyzed. Thus, as for RhoA, the regulatory role of PLD1 is highly conserved in normal as well as neoplastic B-lymphocytes and its regulatory role was apparently 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 had no effect (data not shown). In B-CLL lymphocytes showing reduced expression of PIP5KC, CXCL12-triggered adhesion to ICAM-1 was completely normal (Fig. 6*A* and *B*). Moreover, upregulation of both LFA-1 epitopes corresponding to low/intermediate and extended high-affinity state conformers were totally unaffected in all patients studied (Fig. 6*A* and *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 never seems to be involved in LFA-1 activation in B-CLL lymphocytes, thus establishing a sharp dichotomy with respect to normal 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

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

and to bone marrow characterizes the progression and severity of the disease (21, 22).

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 patients with CLL. 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 (11). The study involved 31 patients with B-CLL, and for each patient, we 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 conclusions could be drawn: (*a*) the rho-module of LFA-1 affinity triggering by chemokines is fully functional in B-lymphocytes isolated from healthy donors; (*b*) in patients with B-CLL, RhoA and PLD1 are conserved signaling events controlling LFA-1 activation by CXCL12; (*c*) 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; (*d*) in all studied B-CLL patients, PIP5KC has no role in LFA-1 affinity triggering by CXCL12. Overall, the data imply that in B-CLL lymphocytes (with respect to

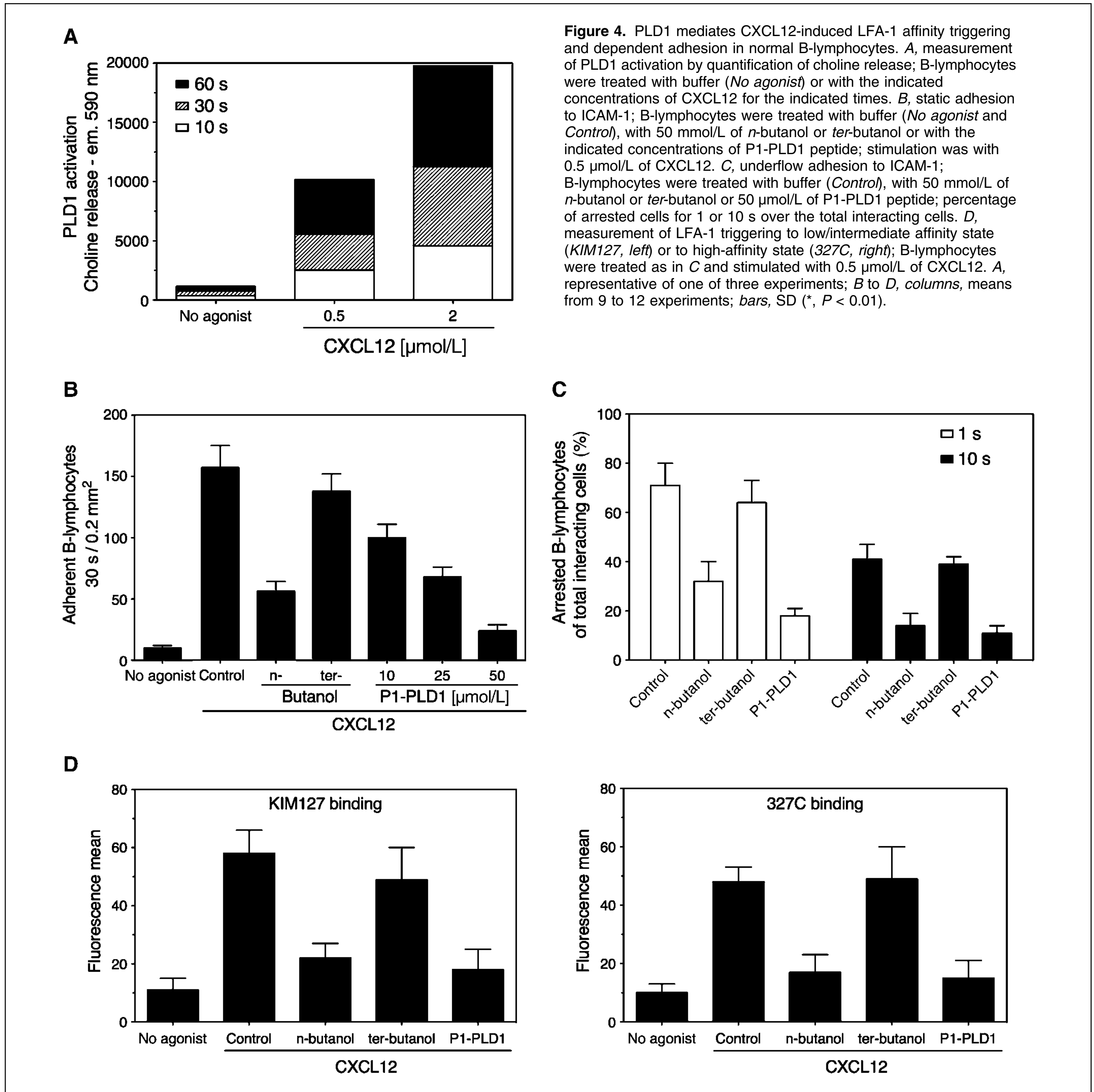

Figure 4. PLD1 mediates CXCL12-induced LFA-1 affinity triggering and dependent adhesion in normal B-lymphocytes. *A,* measurement of PLD1 activation by quantification of choline release; B-lymphocytes were treated with buffer (*No agonist*) or with the indicated concentrations of CXCL12 for the indicated times. *B,* static adhesion to ICAM-1; B-lymphocytes were treated with buffer (*No agonist* and *Control*), with 50 mmol/L of *n*-butanol or *ter*-butanol or with the indicated concentrations of P1-PLD1 peptide; stimulation was with 0.5 μmol/L of CXCL12. *C,* underflow adhesion to ICAM-1; B-lymphocytes were treated with buffer (*Control*), with 50 mmol/L of *n*-butanol or *ter*-butanol or 50 μmol/L of P1-PLD1 peptide; percentage of arrested cells for 1 or 10 s 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 μmol/L of CXCL12. *A,* representative of one of three experiments; *B* to *D, columns,* means from 9 to 12 experiments; *bars,* SD (*, $P < 0.01$).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

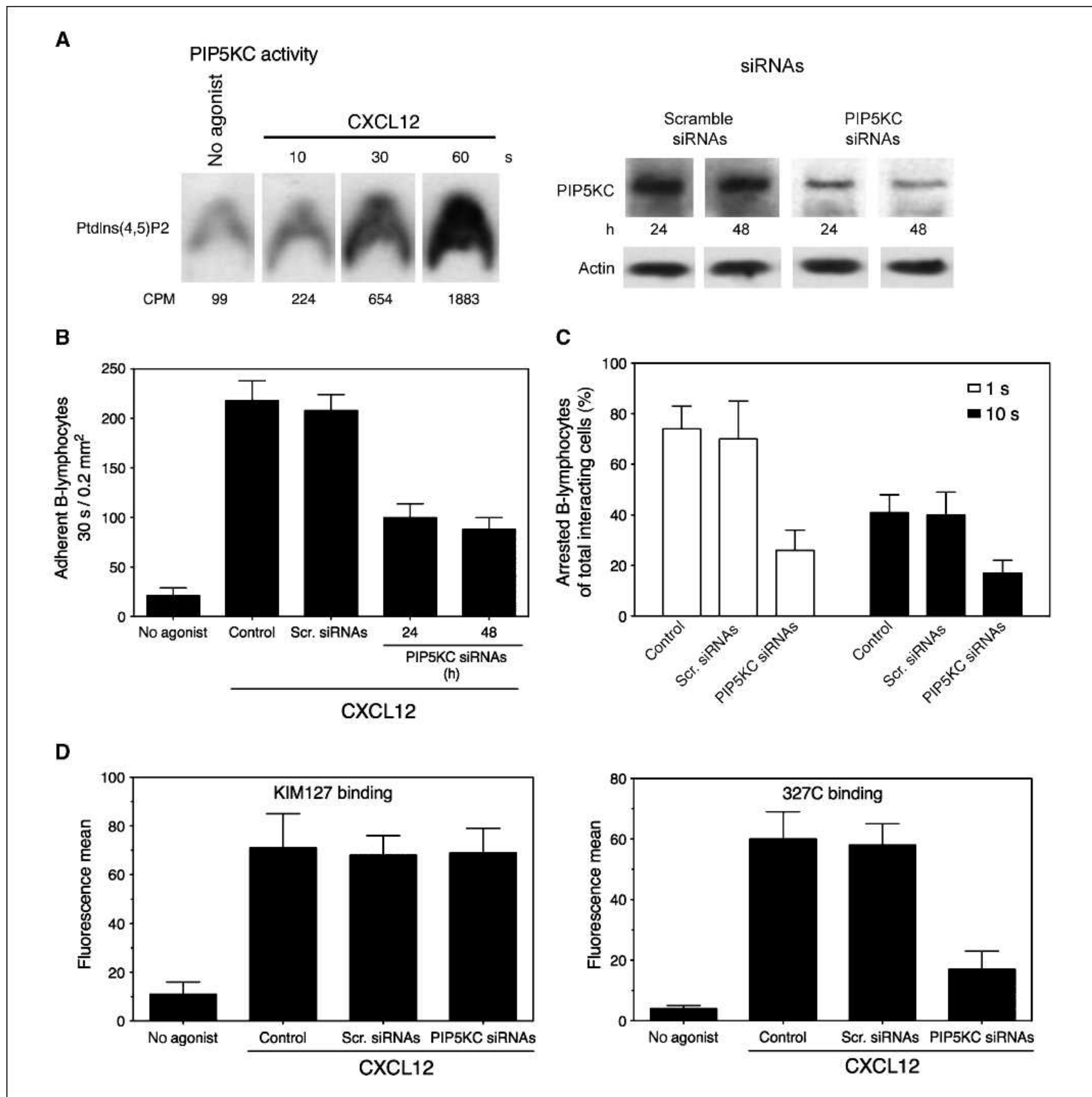

Figure 5. PIP5KC selectively controls CXCL12-induced triggering of LFA-1 to high-affinity state and dependent adhesion in normal B-lymphocytes. *A, left,* evaluation of PIP5KC activity; B-lymphocytes were treated with buffer (*No agonist*) or with 0.5 μmol/L of CXCL12 for the indicated times. Autoradiogram of ^{32}P-labeled PtdIns(4,5)P2. *Bottom,* 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; 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 h (*Scr*) or for the indicated times and treated with buffer (*No agonist*) with 0.5 μmol/L of CXCL12. *C,* underflow 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 h; percentage of arrested cells for 1 or 10 s 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 μmol/L of CXCL12. *A,* representative of 1 of 4 (*left*) and 1 of 10 (*right*) experiments; *B* to *D, columns,* means from 8 to 11 experiments; *bars,* SD (*, $P < 0.01$).

normal B-lymphocytes), neoplastic progression completely bypasses the regulatory role (which is otherwise critical in normal lymphocytes) of PIP5KC in the transition of LFA-1 to a 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 to be the most conserved signaling event controlling LFA-1 activation by chemokines in B-CLL lymphocytes.

The first outcome of our study is that the rho-module of conformer-selective LFA-1 affinity triggering by chemokines is also fully operative in human normal B-lymphocytes. This finding is of

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

interest because the critical regulatory role of this signaling module in normal T-lymphocytes (11) does not imply that identical signaling mechanisms also regulate integrin activation in normal B-lymphocytes, as previously suggested (23). Thus, our study suggests the universal relevance of the rho-module in regulating conformer-selective triggering of LFA-1 by chemokines.

Another important observation is that the signaling couple RhoA-PLD1 is critical in LFA-1 affinity regulation by CXCL12 in all 31 B-CLL patients analyzed. Notably, PLD1 activation by CXCL12 depends on RhoA and Rac1 activity (11). Considering that in about half of the 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 alone, ensure 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 could be lost in a patient-selective manner. Thus, the neoplastic progression seems to affect, in certain patients with CLL, the role of Rac1 and CDC42, with the 14 patients in 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, simultaneously lost 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 from 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 from 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 a high-affinity state in normal lymphocytes (11), was found without any regulatory role in B-CLL lymphocytes. This was verified in all 31 patients analyzed. 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, the lack of a regulatory role by PIP5KC shows that in B-CLL lymphocytes, signaling mechanisms not involving PIP5KC activity control the triggering of LFA-1 to a 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 from recent findings (24). Notably, Rap1 can potentially mediate LFA-1 affinity triggering by means of RIAM-Talin1 interactions (25) or

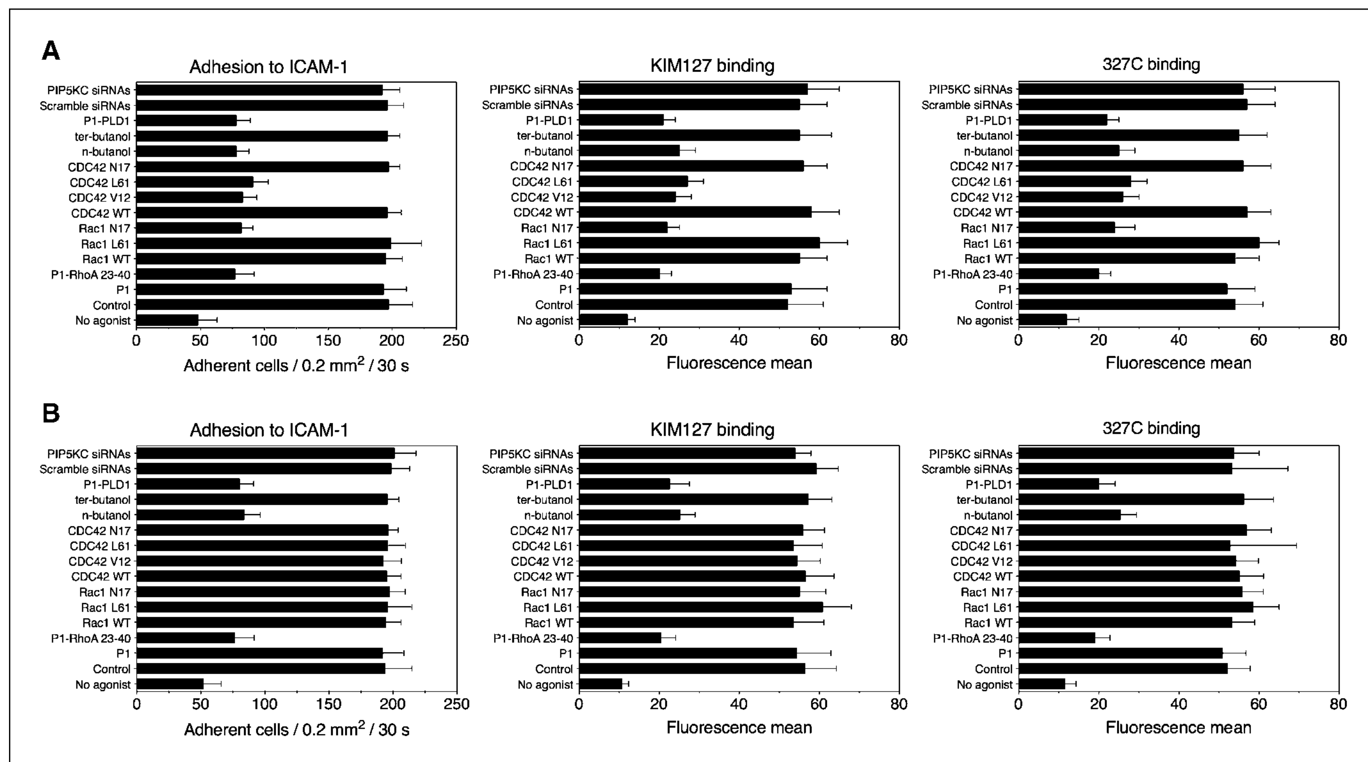

Figure 6. B-lymphocytes isolated from B-CLL patients show altered signaling mechanisms of LFA-1 affinity triggering by CXCL12. *A,* static adhesion to ICAM-1 (*left*), detection of LFA-1 conformers with low/intermediate affinity (*KIM127, middle*) or high-affinity (*327C, right*); B-CLL lymphocytes from the same patient (for a total of 31) were treated with buffer (*No agonist* and *Control*), with 50 µmol/L of P1, P1-RhoA 23–40, or P1-PLD1 Trojan peptides, with 50 µmol/L of Tat-fusion Rac1 and CDC42-WT or mutated proteins, with 50 mmol/L of *n*-butanol or *ter*-butanol, or were nucleoporated with a pool of four scrambled (*Scr*) or PIP5KC-specific siRNAs and kept in culture for 48 h; treated cells were stimulated with 0.5 µmol/L of CXCL12 as described in previous figures. *A, columns,* means from 17 experiments, corresponding to 17 B-CLL patients; *bars,* SD. *B, columns,* means from 14 experiments, corresponding to 14 B-CLL patients; *bars,* SD.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024

by modulating the phosphorylation of the LFA-1α chain (26). Thus, it is possible to hypothesize that in B-CLL lymphocytes, LFA-1 triggering to a 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 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 have systematically been able to detect LFA-1 affinity triggering by CXCL12. This contrasts with recent findings (24) in which 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 a uniform population and that a systematic analysis of the intracellular signaling events governing cell adhesion can be a helpful strategy to better categorize CLL patients, with a possible impact on diagnosis, prognosis, and therapy of leukemia diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 6/2/09; revised 9/7/09; accepted 9/30/09; published OnlineFirst 11/24/09.

Grant support: Italian Association for Cancer Research (2006-2008), MIUR and Fondazione Cariverona, Verona, Italy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Pangalis GA, Vassilakopoulos TP, Dimopoulou MN, Siakantaris MP, Kontopidou FN, Angelopoulou MK. B-chronic lymphocytic leukemia: practical aspects. Hematol Oncol 2002;20:103–46.
2. Lopez-Giral S, Quintana NE, Cabrerizo M, et al. Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination. J Leukoc Biol 2004;76:462–71.
3. Mohle R, Failenschmid C, Bautz F, Kanz L. Overexpression of the chemokine receptor CXCR4 in B cell chronic lymphocytic leukemia is associated with increased functional response to stromal cell-derived factor-1 (SDF-1). Leukemia 1999;13:1954–9.
4. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 2000;96:2655–63.
5. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678–89.
6. Laudanna C, Alon R. Right on the spot. Chemokine triggering of integrin-mediated arrest of rolling leukocytes. Thromb Haemost 2006;95:5–11.
7. Constantin G, Majeed M, Giagulli C, et al. Chemokines trigger immediate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity 2000;13:759–69.
8. Shi M, Foo SY, Tan SM, Mitchell EP, Law SK, Lescar J. A structural hypothesis for the transition between bent and extended conformations of the leukocyte β2 integrins. J Biol Chem 2007;282:30198–206.
9. Hogg N, Henderson R, Leitinger B, McDowall A, Porter J, Stanley P. Mechanisms contributing to the activity of integrins on leukocytes. Immunol Rev 2002;186:164–71.
10. Takagi J, Springer TA. Integrin activation and structural rearrangement. Immunol Rev 2002;186:141–63.
11. Bolomini-Vittori M, Montresor A, Giagulli C, Staunton D, Rossi B, Martinello M, Constantin G, Laudanna C. Regulation of conformer-specific activation of the integrin LFA-1 by a chemokine-triggered Rho signaling module. Nat Immunol 2009;10:185–94.
12. Robinson MK, Andrew D, Rosen H, et al. Antibody against the Leu-CAM β-chain (CD18) promotes both LFA-1- and CR3-dependent adhesion events. J Immunol 1992;148:1080–5.
13. Lum AF, Green CE, Lee GR, Staunton DE, Simon SI. Dynamic regulation of LFA-1 activation and neutrophil arrest on intercellular adhesion molecule 1 (ICAM-1) in shear flow. J Biol Chem 2002;277:20660–70.
14. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446–56.
15. Giagulli C, Scarpini E, Ottoboni L, et al. RhoA, ζ PKC. control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte *in vivo* homing. Immunity 2004;20:25–35.
16. Pasvolsky R, Grabovsky V, Giagulli C, et al. RhoA is involved in LFA-1 extension triggered by CXCL12 but not in a novel outside-in LFA-1 activation facilitated by CXCL9. J Immunol 2008;180:2815–23.
17. Cai S, Exton JH. Determination of interaction sites of phospholipase D1 for RhoA. Biochem J 2001;355:779–85.
18. Henage LG, Exton JH, Brown HA. Kinetic analysis of a mammalian phospholipase D: allosteric modulation by monomeric GTPases, protein kinase C, polyphosphoinositides. J Biol Chem 2006;281:3408–17.
19. Weernink PA, Meletiadis K, Hommeltenberg S, et al. Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. J Biol Chem 2004;279:7840–9.
20. Cockcroft S. Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases. Biochim Biophys Acta 2009;1791:905–12.
21. Chiorazzi N, Ferrarini M. Evolving view of the *in-vivo* kinetics of chronic lymphocytic leukemia B cells. Hematology Am Soc Hematol Educ Program 2006;512:273–8.
22. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804–15.
23. Nombela-Arrieta C, Lacalle RA, Montoya MC, et al. Differential requirements for DOCK2 and phosphoinositide-3-kinase γ during T and B lymphocyte homing. Immunity 2004;21:429–41.
24. Till KJ, Harris RJ, Linford A, Spiller DG, Zuzel M, Cawley JC. Cell motility in chronic lymphocytic leukemia: defective Rap1 and αLβ2 activation by chemokine. Cancer Res 2008;68:8429–36.
25. Lee HS, Lim CJ, Puzon-McLaughlin W, Shattil SJ, Ginsberg MH. RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. J Biol Chem 2009;284:5119–27.
26. Fagerholm SC, Hilden TJ, Nurmi SM, Gahmberg CG. Specific integrin α and β chain phosphorylations regulate LFA-1 activation through affinity-dependent and -independent mechanisms. J Cell Biol 2005;171:705–15.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/24/9281/2620402/9281.pdf by guest on 14 July 2024