Cell Motility in Chronic Lymphocytic Leukemia: Defective Rap1 and αLβ2 Activation by Chemokine

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Abstract

Chemokine-induced activation of α4β1 and αLβ2 integrins (by conformational change and clustering) is required for lymphocyte transendothelial migration (TEM) and entry into lymph nodes. We have previously reported that chemokine-induced TEM is defective in chronic lymphocytic leukemia (CLL) and that this defect is a result of failure of the chemokine to induce polar clustering of αLβ2; engagement of α4β1 and autocrine vascular endothelial growth factor (VEGF) restore clustering and TEM. The aim of the present study was to characterize the nature of this defect in αLβ2 activation and determine how it is corrected. We show here that the αLβ2 of CLL cells is already in variably activated conformations, which are not further altered by chemokine treatment. Importantly, such treatment usually does not cause an increase in the GTP-loading of Rap1, a GTPase central to chemokine-induced activation of integrins. Furthermore, we show that this defect in Rap1 GTP-loading is at the level of the chemokine-induced activation of both α4β1 and αLβ2 and that combined stimulation by α4β1 engagement and autocrine vascular endothelial growth factor (VEGF) overcomes this defect, allowing αLβ2-dependent motility and TEM. We also showed that CLL cells, which express little or no α4β1 at their surface, display a markedly reduced ability to undergo chemokine-stimulated TEM (3). These observations have substantial clinical relevance because we have shown a strong correlation between α4β1 expression and clinical lymphadenopathy (3). The importance of α4β1 for tissue invasion by malignant cells is further underscored by the recent demonstration that expression of this integrin is a major prognostic indicator in CLL (5).

Introduction

It has been known for many years that extensive accumulation of malignant lymphocytes in the lymph nodes, spleen, and bone marrow of chronic lymphocytic leukemia (CLL) patients is associated with progressive disease and an adverse prognosis. This tissue infiltration provides a microenvironment favoring the survival and proliferation of the malignant cells and also leads to suppression of hematopoiesis and immune function. Indeed, tissue invasion by malignant cells is such an important pathogenetic process in CLL that it forms the basis of current clinical staging systems (1, 2). For all these reasons, understanding the mechanisms governing tissue invasion in CLL is a subject of major clinical importance.

Tissue invasion in CLL requires the motility and transendothelial migration (TEM) of the malignant cells. These processes involve chemokines, activation of both α4β1 (VLA-4; CD49d/29) and αLβ2 (LFA-1; CD11a/18) integrins, and ligand binding to VCAM-1 and intercellular adhesion molecule-1 (ICAM-1), respectively. We have therefore focused on the role of chemokines, and these two integrin heterodimers, in CLL cell motility on and through endothelium (3, 4). Our most recent work (4) showed that the chemokine-induced clustering of αLβ2 is defective in CLL cells and that combined stimulation by α4β1 engagement and autocrine vascular endothelial growth factor (VEGF) overcomes this defect, allowing αLβ2-dependent motility and TEM. We also showed that CLL cells, which express little or no α4β1 at their surface, display a markedly reduced ability to undergo chemokine-stimulated TEM (3). These observations have substantial clinical relevance because we have shown a strong correlation between α4β1 expression and clinical lymphadenopathy (3). The importance of α4β1 for tissue invasion by malignant cells is further underscored by the recent demonstration that expression of this integrin is a major prognostic indicator in CLL (5).

The aims of the present study, therefore, were to define the nature of the defect in activation of αLβ2 in the pathophysiology of CLL. [Cancer Res 2008;68(20):8429–36]

Materials and Methods

Patients and Donors

The study involved 19 patients with CLL, all of whom had typical CLL with respect to morphology and surface marker expression (CD5-positive and CD23-positive with dim light chain-restricted immunoglobulin). All patients had a total WBC count of >50 × 10^9/L, and mononuclear cell preparations therefore always had ≥90% CLL cells. A given CLL clone was regarded as α4+ when surface fluorescence-activated cell sorting (FACS) analysis showed a clear peak shift with an MFI of >10. For α4− clones, the
stained peak virtually overlapped that of the class-specific immunoglobulin control. Further patient details are given in Supplementary Table S1.

Human umbilical vein endothelial cells (HUVEC) were prepared as previously described (3). Normal peripheral blood mononuclear cells (PBMC) were either from the blood of normal volunteers or from buffy coats prepared by National Blood Transfusion Service.

All samples were obtained with informed consent and with the approval of the Liverpool Research and Ethics Committee, Royal Liverpool and Broadgreen University Hospitals Trust and the Research and Development Committee, Liverpool Women’s Hospital.

Cell Preparation and Culture

CLL cells and PBMC were isolated from peripheral blood and buffy coats by Ficoll-Hypaque density gradient centrifugation. Normal B cells were purified from PBMC, either by positive selection using CD19-conjugated magnetic beads or by negative selection using a B-cell isolation kit (Miltenyi Biotec; >98% and >95% CD20+ respectively). Lymphocytes were cultured (5% CO2 in air) at 37°C in RPMI containing 1% bovine serum albumin (BSA; Sigma), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

In certain experiments, cells were cultured on polyHEMA-coated plates (Sigma) in serum-free medium (Invitrogen) containing 0.1% BSA. Cells cultured under these conditions are deprived of the exogenous stimuli received in vivo from serum and adhesion; however, endogenous stimuli persist and the cells remain viable (15).

Live Cell Imaging and TEM

CLL cells (5 × 105) were added to 3.5 mm2 Petri dishes (IWAKI) coated with HUVEC or with VCAM-1 or ICAM-1 (both at 10 μg/mL; R&D Systems), and motility was measured as previously described (4). TEM of CLL cells was measured after 6 h (3).

Inhibition Studies

Cells were incubated at 4°C for 30 min with blocking monoclonal antibodies (mAb) to either α4 (5 μg/mL) or αL (10 μg/mL) or with isotypic control mAbs at the relevant concentrations (all mAbs from R&D). In addition, cells were incubated with an inhibitor of VEGF receptor kinase activity, SU5416 (10 μmol/L Merck Biosciences), or with 0.25% DMSO (the diluent) alone for 2 h at 37°C.

Staining

Activation antigens. Lymphocytes ± chemokine (CXCL12 (100 ng/mL), CXCL13 (10 ng/mL), CCL21 (1 μg/mL) or ± Mg2+ + EGTA were stained with conformation-dependent anti-αL/α2 mAbs (all at 10 μg/mL) according to the method of Canadas and Hogg (16); staining was analyzed by FACS or confocal microscopy. The chemokines (all from R & D) were used at concentrations that induced maximal CLL cell TEM (ref. 3; data not shown). The conformation-dependent mAbs used were mAb24 (16), NKI-L16 (17), and 327A and 327C (ref. 9; kindly donated by Nancy Hogg, LRF London; Carl Fidgter, University Medical Center Nijmegen; and ICOS Corporation, respectively). As a measure of total αL, cells were also stained with a conformation-independent anti-αL mAb (10 μg/mL; Santa Cruz Biotechnology).

Rap1. CLL cells were either untreated, incubated with CXCL12 (100 ng/mL) for 5 min or with an exchange protein directly activated by cAMP (EPAC) agonist (8-Br-2′-0-Me-cAMP, 10 μg/mL; Biolog) for 1 min and then cytospun before fixation in methanol. Slides were blocked in 10% BSA before being stained with an antibody to Rap1 (4 μg/mL) or with control rabbit immunoglobulin (both from Santa Cruz), followed by goat anti-rabbit immunoglobulin-AlexaFluor488.

Adhesion Assay

A bead assay was used to measure adhesion (18). Briefly, slides were coated with BSA (10%), ICAM-1 (10 μg/mL), or VCAM-1 (10 μg/mL) ± CXCL12 (100 ng/mL). Latex beads (Sigma) were also coated with ICAM-1, VCAM-1, or BSA (all 10 μg/mL). CLL cells were then added to the coated slides and incubated with the labeled beads for 4 h, fixed in 1% formaldehyde, and stained with hematoxylin. The number of beads that had bound to 100 cells was counted.

Rap1 Assay

1 × 107 cells were lysed, and an aliquot was removed to examine total protein levels. Rap1-GTP was then pulled down from the remaining lysate using Rap1-GDS-RBD beads (Upstate Biotechnology; ref. 19). Levels of the GTPase were then measured in the lysate and pull-down by Western blotting using a Rap1 antibody (Santa Cruz).

The effects of various treatments on Rap1-GTP levels were examined. Cells were treated with chemokines or the EPAC agonist (both for 1 min) or with VCAM-1 ± CXCL12 for 5 min. The B-cell receptor (BCR) was stimulated with either F(ab)2 goat anti-human IgM (10 μg/mL) cross-linked with F(AB)2 rabbit anti-goat immunoglobulin (both from Jackson Immunoresearch Laboratories) or with antihuman IgM coated beads (Sigma) for 5 min.

Results

Our previous studies of CLL cell motility, which showed that chemokines fail to induce polar clustering of αLβ2, involved only clones expressing α4β1 (4). These investigations were restricted in this way because clones expressing α4β1 had a markedly greater ability to undergo TEM in response to chemokine, and because such TEM correlated with clinical lymphadenopathy (3, 4). Therefore, it seemed important to investigate αLβ2 function in clones lacking α4β1 to establish whether or not the defect in αLβ2 function is a general feature of CLL cells.

The αLβ2 of α4β1−/− CLL cells does not cluster or mediate motility in response to chemokine. Using live cell imaging, we first examined the distribution of αLβ2 on α4β1−/− CLL cells in contact with HUVEC (which express ICAM-1 and produce chemokines) or with purified ICAM-1 + chemokines (CXCL12 or CCL21). The αLβ2 of the CLL cells, in contrast to that of normal B cells, did not become clustered in a polar fashion on either HUVEC (n = 4; 3 ± 3% clustered versus 47 ± 5% for normal B cells) or ICAM-1 + chemokine (n = 4; 4 ± 6% clustered versus 31 ± 1% for normal B cells). Furthermore, the cells did not undergo αLβ2-dependent motility.

We concluded at this stage that CLL clones, regardless of their expression of α4, have a defect in the polar clustering of αLβ2 induced by chemokines and that this is associated with defective movement dependent on this integrin heterodimer.

Clustering of integrins during cell activation is often accompanied by changes in their conformation (6, 7, 20). Therefore, we next examined the conformation of αLβ2 on untreated and chemokine-stimulated CLL cells and compared it with that of normal B cells.

The αLβ2 of CLL cells is variably activated and not altered by chemokine stimulation. In unstimulated cells, αLβ2 has a bent conformation, which has low affinity for ligand (8). Upon cell stimulation, the molecule extends to an intermediate affinity form on which an epitope detected by the antibody NK1-L16 is exposed (8, 21). Further conformational changes in the ligand-binding I domain are responsible for high-affinity ligand binding (21–23); these changes are detected by mAb24 and/or mAbs 327A and 327C.

The immobilization of chemokine and engagement of αLβ2 affect the expression of the activation epitopes in a different way than do soluble chemokines (21). Therefore, using the above mAbs, we first examined unstimulated cells and then determined the effects of both soluble and immobilized chemokine(s) + ligand. In these experiments, Mg2+ treatment was used as a positive control because this divalent cation forces the exposure of the epitopes detected by mAb24 and 327A and 327C while decreasing that of
NKI-L16 (21). Normal B cells were examined for comparison, and because their expression of αLβ2 activation epitopes and the effect of chemokines thereon had not been clearly defined in the literature.

α4β1+ CLL cells resembled normal B cells in their expression of the αLβ2 activation epitopes, except that the conformation detected by NKI-L16 was more strongly expressed \((P = 0.032; \text{Fig. 1})\). For both α4β1+ CLL and normal B cells, Mg2+ markedly increased the expression of the mAb24 epitope \((P < 0.03)\) and had a less marked, but significant, effect \((P < 0.03)\) on the expression of the 327A and 327C epitopes. In contrast, unstimulated α4β1+ CLL cells, in addition to expressing the NKI-L16 epitope, consistently displayed higher levels of the activation epitopes detected by mAbs 327A, 327C, and mAb24 \((P < 0.05)\). Moreover the expression of these epitopes was not enhanced by Mg2+, suggesting that the αLβ2 of these cells is already fully activated (Fig. 1).

Although the baseline levels of αLβ2 expression were different in α4β1+ and α4β1+ CLL clones, soluble chemokines (CXCL12, CXCL13, and CCL21; all relevant to the pathogenesis of CLL; refs. 3, 24, 25) had no effect on the expression of the activation epitopes. As expected (9, 16, 21), soluble chemokines increased the expression of the 327A and 327C epitopes on normal B cells, but had no effect on mAb24 epitope expression. However, in contrast to T cells (21), the unstimulated normal B cells expressed little or no NKI-L16 epitope and levels were increased after exposure to soluble chemokines (Fig. 1 and Supplementary Fig. S1). The observed changes were not attributable to altered expression of total αLβ2 because, as shown using a conformation-independent mAb, levels of the integrin were not affected by chemokine or Mg2+ (data not shown).

To test the effect of immobilized chemokine and ligand binding (9, 16, 26), we examined cells on either HUVEC or ICAM-1 + chemokine. On both surfaces, activation epitopes were not increased on either α4β1+ and α4β1+ CLL cells (Fig. 2A and Supplementary Fig. S2). In contrast, on normal B cells, which become motile on both surfaces, the expression of mAb24 (Fig. 2A)

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of soluble chemokine stimulation on the activation of αLβ2 as measured by FACS analysis of cells stained with mAbs detecting activation epitopes. The values shown are the MFISEM of the test mAb, whereas the MFI values of the IgG1 class-specific control ± SEM indicate background staining. The percentage of positive cells is not shown, because for all the mAbs, >95% of cells were stained. For normal B cells, lymphocytes from three donors were examined, whereas for CLL, five α4+ and five α4+ clones were examined. Where the error bars for the α4+ clones were relatively large, this was the result of variations in expression of epitopes from clone to clone, but for each individual clone, stimulation by chemokine did not significantly increase reactivity (in a paired t test, \(P > 0.05\); see also Supplementary Fig. S1); in particular, the apparent increase in NKI-L16 (asterisk) after chemokine treatment was not significant.
were stimulated with two other chemokines (CCL21 and CXCL13), protein expressed (12 clones, levels of GTP-Rap1 correlated with the amount of Rap1 cases studied, but levels varied from clone to clone (Fig. 3

CLL clones. GTP-loaded Rap1 was readily detected in the cells of all pull-down assay to determine the GTP loading of Rap1 in the same

A

ICAM-1

F

coated beads. In contrast, CLL clones lacking α4β1 bound the ICAM-1–coated beads when plated on either BSA or VCAM-1 (Fig. 2B).

These results indicate that the fully extended αLβ2 on α4β1– cells is able to bind ICAM-1 without stimulation, whereas the form of αLβ2 on α4β1+ cells requires stimulation via α4β1–VCAM-1 interaction for adhesion to become demonstrable.

We next examined the effect of chemokine. Treatment of α4+ or α4− clones with CXCL12 had no effect on the binding of ICAM-1–coated beads to cells incubated on BSA and did not enhance such binding when the cells were plated on VCAM-1 (data not shown). These findings are in accord with our observations that chemokine does not affect either the conformation (data above) or clustering (4) of the αLβ2 of CLL cells.

We then turned to the mechanism(s) involved in the defect in chemokine-induced activation of CLL cell αLβ2. Because signaling via Rap1 is central to chemokine-induced activation of αLβ2 (27–29), we next examined the activation of this GTPase in CLL cells.

The chemokine-stimulated GTP loading of Rap1 is defective in most CLL clones. We first used Western blotting to examine Rap1 protein in CLL cells and found that the protein is variably expressed (Fig. 3A, n = 12, α4+ n = 6, α4− n = 6). We then used a pull-down assay to determine the GTP loading of Rap1 in the same CLL clones. GTP-loaded Rap1 was readily detected in the cells of all cases studied, but levels varied from clone to clone (Fig. 3A). In all 12 clones, levels of GTP-Rap1 correlated with the amount of Rap1 protein expressed ($R^2 = 0.91$; Supplementary Fig. S3A). However, there was no significant difference between α4+ and α4− clones with regard to total Rap1 protein, Rap1-GTP, or Rap1-GTP/Rap1 ratios ($P > 0.3$; Supplementary Fig. S3B).

We next examined the effect of CXCL12 on GTP loading of Rap1 in the same and additional cases. In 11 of the 16 clones studied (9 α4+ and 7 α4−), chemokine did not enhance the GTP loading of Rap1 (Fig. 3B). As expected, CXCL12 enhanced Rap1-GTP loading in highly purified normal B cells ($n = 3$; Fig. 3B). When CLL clones were stimulated with two other chemokines (CCL21 and CXCL13), identical results were obtained (Supplementary Fig. S3C). Taken together, these results indicate that chemokine-induced activation of Rap1 is defective in ~70% of CLL clones.

We next sought to confirm this conclusion using a different method. On activation, Rap1 is translocated to the cell membrane where it regulates integrin activation (30, 31). We therefore used confocal microscopy to examine the location of Rap1 in CLL cells before and after chemokine stimulation. In CLL clones, which failed to GTP load their Rap1 in response to chemokine, the distribution of the GTPase was unchanged (Fig. 3C). In contrast, in the minority of CLL clones in which chemokine did induce Rap1 activation, the GTPase was translocated to the membrane in a clustered fashion (Fig. 3C).

Having established that the chemokine-induced activation of Rap1 is frequently defective in CLL, we next examined possible mechanisms involved.

The defect in chemokine-induced Rap1-GTP loading in CLL cells is at the level of the GTPase. The GTP loading of Rap1 in response to chemokine is mediated by CalDAG-GEFI (32). However, Rap1 can be activated by a number of other guanine nucleotide exchange factors (GEF), including EPAC, which is highly expressed in CLL cells (33). Therefore, we next measured Rap1-GTP in CLL

Figure 2. A, effect of immobilized chemokine and ligand engagement on the expression of the mAb24 activation epitope of αLβ2 on normal B and CLL cells. B, adhesion of CLL cells to ICAM-1–coated beads. A, representative confocal images of CLL clones (n = 8 each for α4+ and α4−) and highly purified normal B cells (n = 3) stained for mAb24; reactivity with the other MAb is shown in Supplementary Fig. S2. The images are composites of more than one field; at least five cells are present in each panel. However, in certain panels, the cells are only faintly seen because of their low or absent expression of the epitope detected by mAb24. For reasons that are unclear, incubation of α+4 cells on ICAM-1 + CXCL12 resulted in reduced expression of the epitope detected by all the conformation-dependent antibodies. B, CLL cells were incubated on either BSA-coated or VCAM-1–coated plates. The number of ICAM-1–coated beads that adhered to these cells was then counted. The numbers assigned to a particular clone in this and other figures (3 and 5) correspond to the patient no. in Supplementary Table S1.
cells treated with an agonist of EPAC, arguing that if the defect is upstream of Rap1, the EPAC agonist would stimulate Rap1-GTP loading. In fact, in cases in which CXCL12 did not increase GTP loading, the EPAC agonist also did not increase Rap1-GTP levels ($n = 3$; Fig. 3B). In the minority of clones where chemokine did enhance the GTP loading of Rap1 ($n = 5$) and in normal B cells ($n = 3$), the EPAC agonist increased GTP-Rap1 to the same extent as chemokine (Fig. 3B).

We next used confocal microscopy to confirm these results. As expected, where EPAC did not induce GTP loading of Rap1, the GT-Pase was not translocated to the cell membrane and vice versa (Fig. 3C).

We also stimulated the Rap1 of CLL cells by BCR cross-linking, which activates the GTPase via a third GEF (34). BCR stimulation using both immobilized and soluble anti-IgM failed to induce further GTP loading of Rap1 in the clones that did not respond to chemokines or to the EPAC agonist and vice versa ($n = 4$ clones; all responded to BCR cross-linking by increasing [Ca$^{2+}$]; Supplementary Fig. S3C).

The fact that stimulation by pathways involving three different GEFs did not induce GTP loading of Rap1 indicates that the defect in chemokine-induced activation of Rap1 is not upstream of the GTPase.

The defect in chemokine-induced Rap1-GTP loading is the result of in vivo stimulation. There is now considerable evidence that CLL cells have been activated in vivo (35, 36), and the results presented, thus far, support this view. We consequently hypothesized that the absence of Rap1-GTP loading in response to chemokine is the result of such stimulation. We therefore cultured CLL cells in serum-free medium on polyHEMA-coated plates (to prevent exogenous stimulation; ref. 15) and examined Rap1-GTP levels ± chemokine stimulation. In clones that did not increase their Rap1-GTP loading in response to chemokine, Rap1-GTP levels dropped after 24 to 48 h of culture and loading in response to chemokine became demonstrable (Fig. 4A). Furthermore, after such culture, α$^4^+$ clones displayed a markedly increased ability to undergo αL$\beta^2$-dependent TEM in response to chemokine (Fig. 4B). This confirms the notion that in vivo stimulation leads to GTP loading of Rap1 and chemokine unresponsiveness.

We next turned to the minority of CLL clones, which were able to GTP load their Rap1 in response to chemokine. We hypothesized that such CLL clones would differ from the majority of cases (4) in being able to cluster their αL$\beta^2$ and undergo TEM in response to chemokine in a manner independent of autocrine VEGF and α4. In fact, the chemokine did indeed induce polar clustering of αL$\beta^2$ in these cells and they underwent αL$\beta^2$-dependent TEM even when α4 engagement and autocrine VEGF were blocked ($n = 3$, two α$^4^+$ and one α$^4^-$; data not shown).

Having shown that the defect in αL$\beta^2$ function observed in most CLL clones is the result of in vivo stimulation of Rap1, we next examined the mechanism(s) by which α4 engagement and autocrine VEGF overcome this defect.

Autocrine VEGF and chemokine activate α4β1 for ligand binding, and together, these stimuli induce αLβ2 clustering in a Rap1-independent manner. It is well established that cross-talk from α4β1 can induce activation of αLβ2 by an unknown mechanism (37, 38). We hypothesized that this cross-talk occurs independently of Rap1. We therefore incubated α$^4^+$ CLL cells (which did not GTP load their Rap1 in response to chemokine) on VCAM-1, in the presence or absence of CXCL12; no increase in Rap1-GTP was seen (data not shown; $n = 3$) despite the induction of αL$\beta^2$ clustering in the presence of chemokine. This indicates that α4β1 engagement in the presence of chemokine induces clustering independently of Rap1.

In our previous work, we showed that the αLβ2-dependent motility/TEM of CLL cells requires autocrine VEGF in addition to α4β1 engagement and chemokine (4). We therefore next examined the relative contributions of α4β1 engagement, autocrine VEGF, and chemokine to the polar clustering of αLβ2 required for motility and TEM. We first showed that activation of α4β1 for adhesion to ligand requires combined stimulation by both VEGF and chemokine. Thus, unstimulated CLL cells were unable to bind VCAM-1–coated beads. However, chemokine markedly enhanced the binding of such beads, but this was largely abrogated by a VEGF receptor kinase inhibitor, SU5416 (Fig. 5A). We next showed that α4β1 engagement alone does not generate the
stimulus for the polar clustering of α4β2. To do this, we incubated CLL cells on an immobilized anti-α4 mAb and showed that the α4β2 did not become clustered (Fig. 5B), and the cells did not become motile (data not shown). However, addition of chemokine-induced marked clustering and motility, which were inhibited by SU5416 (Fig. 5A), induced marked clustering and motility, which were inhibited by SU5416.

Therefore, autocrine VEGF and chemokine act in concert to stimulate α4 for adhesion to ligand, and this, together with autocrine VEGF and chemokine, is required for the polar clustering of α4β2, which is necessary for CLL cell TEM (these interactions are shown schematically in Fig. 6).

Discussion

The aim of the present study was to characterize the defect in chemokine-induced α4β2 activation that we have previously identified in α4β1+ CLL cells (3, 4).

Because many CLL clones express little or no α4β1 (3, 5), we started the present studies by examining chemokine-induced α4β2 clustering on these cells. The αL of α4− CLL cells also failed to undergo polar clustering or αLβ2-dependent motility when incubated on ligand in the presence of chemokine. Because the αLβ2 of normal B cells becomes clustered under these conditions (4), this indicates that defective αL clustering in response to chemokine is a feature of CLL cells, regardless of their expression of α4β1.

We next found that the αLβ2 of CLL cells tested directly ex vivo is in an activated conformation, but the degree of activation varied among cases, with α4− clones expressing the high-affinity, fully activated form of αLβ2; furthermore, these cells were able to bind ICAM-1. In contrast, α4β1+ cells expressed the intermediate-affinity form of αLβ2 and were unable to bind ligand. Because the intermediate affinity form of αLβ2 is important in mediating motility (39), it is likely that the in vivo activation of αLβ2 in α4− cells contributes to the defect in motility/TEM of such CLL clones.

In both α4+ and α4− CLL clones, chemokines and ligand binding did not induce further conformational activation of αLβ2. In contrast, the αLβ2 of normal B cells, like that of unstimulated T lymphocytes (9, 16, 21), was in a nonactive conformation and became activated after chemokine stimulation. Thus, the αLβ2 of CLL cells differs from that of normal B cells, not only with regard to its activated conformation and inability to undergo clustering, but also in its failure to undergo chemokine/ligand-induced conformational changes. Because the αLβ2 of α4− clones became clustered on HUVEC (4) but did not change its conformation (present study), our results indicate that α4 engagement overcomes the defect in αLβ2 by inducing clustering without changing the conformation of the integrin heterodimer. In contrast, in the absence of the possibility of α4 engagement, the αLβ2 of α4− CLL clones does not cluster and also cannot undergo conformational changes in response to chemokine, because the integrin heterodimer is already in its fully extended high-affinity state. Because polar clustering is necessary for αLβ2-mediated motility (7, 20, 29)
and this integrin is essential for lymphocyte TEM (40, 41), the present results explain why α4− CLL cells display markedly reduced TEM in response to chemokine.

We next examined Rap1 activation, because this GTPase is central to integrin activation by chemokines (27, 30). We found that Rap1-GTP is readily detected in the cells of all CLL clones. Rap1-GTP has been shown to induce expression of the intermediate affinity form αLβ2, whereas induction of the high-affinity form is Rap1-independent (42). This is fully in accord with our results, which show that both the GTPase and the NKI-L16 epitope are detected in all CLL clones and that the high-affinity conformation observed in the α4+ cells is not linked to levels of Rap1-GTP.

Importantly, the GTP loading of Rap1 was not increased by chemokine in the majority of CLL clones examined. The fact that stimuli activating Rap1 via different GEFs all failed to increase Rap1-GTP levels in these cells indicates that the defect is at the level of Rap1 loading. Taken together, these data suggest that the inability of chemokine to induce Rap1 activation is central to the defective function of αLβ2 observed in CLL.

We next returned to the majority of CLL clones in which chemokine failed to induce GTP loading of Rap1 and attempted to define the cause of this defect. The fact that stimuli activating Rap1 via different GEFs all failed to increase Rap1-GTP levels in these cells indicates that the defect is at the level of Rap1 loading. Furthermore, the defect is clearly the result of in vivo stimulation because cell culture in the absence of exogenous stimulation led to a reduction of Rap1-GTP levels and to a restoration of chemokine responsiveness as measured by Rap1 activation and TEM. We have not identified the nature of this in vivo stimulus, but it is tempting to speculate that the BCR is involved because stimulation of this receptor by (auto)antigen is central to the pathogenesis of CLL (43, 44). It is possible that such stimulation of CLL cells causes their Rap1 to be fully loaded and that these cells have been more stimulated in vivo than have the cells which responded to chemokine. Also, because deficiency of the Rap1–GTPase-activating protein, SPA-1, has been linked to a CLL-like disorder in knockout mice (45), it is possible that SPA-1 levels/function are reduced in human CLL, thereby reducing the ability of Rap1 to convert GTP to GDP. These possibilities will be the subject of a future study.

Finally, we examined the mechanism by which α4β1 engagement overcomes the defect in chemokine-induced activation of αLβ2. We found that such engagement does not activate Rap1 and then, using combinations of stimuli and a VEGF kinase inhibitor, we showed that autocrine VEGF and chemokine together activate α4β1–VCAM-1 adhesion, and the three stimuli together then induce Rap1-independent polar clustering of αLβ2 and TEM in response to chemokine.

**Figure 6.** Cartoon summarizing the nature of the defect in activation of CLL-cell αLβ2 and its correction by engagement of α4β1, chemokine, and autocrine VEGF. A, the sequence of events leading to chemokine-induced αLβ2 and α4β1 activation in normal B cells. B, in CLL cells, αLβ2 and Rap1 are already activated as a result of in vivo stimulation, and chemokine does not induce additional loading of Rap1. As a result, neither αLβ2 or α4β1 are further activated by chemokine alone. VEGF and chemokine activate α4β1–VCAM-1 adhesion, and the three stimuli together then induce Rap1-independent polar clustering of αLβ2 and TEM in response to chemokine.
changes lead to a defect in the function of both this integrin heterodimer and α4β1. We have already shown that most CLL clones differ from normal B cells in requiring α4β1 engagement and autocrine VEGF to undergo TEM and entry to the proliferation centers of lymph nodes (4). Therefore, the present study, by defining the individual roles of chemokine, autocrine VEGF, and α4β1-VCAM-1 binding in correcting the defect in chemokine-induced activation of αLβ2, emphasizes the therapeutic potential of inhibitors of α4β1 and/or VEGF.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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