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Chemokine Unresponsiveness of Chronic Lymphocytic Leukemia Cells Results from Impaired Endosomal Recycling of Rap1 and Is Associated with a Distinctive Type of Immunological Anergy

Derek S. Pye,* Ignacio Rubio,†‡ Rico Pusch,† Ke Lin,*§ Andrew R. Pettitt,* and Kathleen J. Till*‡

Trafficking of malignant lymphocytes is fundamental to the biology of chronic lymphocytic leukemia (CLL). Transendothelial migration (TEM) of normal lymphocytes into lymph nodes requires the chemokine-induced activation of Rap1 and α4β7 integrin. However, in most cases of CLL, Rap1 is refractory to chemokine stimulation, resulting in failed α4β7 activation and TEM unless α4β1 is coexpressed. In this study, we show that the inability of CXCL12 to induce Rap1 GTP loading in CLL cells results from failure of Rap1-containing endosomes to translocate to the plasma membrane. Furthermore, failure of chemokine-induced Rap1 translocation/GTP loading was associated with a specific pattern of cellular IgD distribution resembling that observed in normal B cells anergized by DNA-based Ags. Anergic features and chemokine unresponsiveness could be simultaneously reversed by culturing CLL cells ex vivo, suggesting that these two features are coupled and driven by stimuli present in the in vivo microenvironment. Finally, we show that failure of Rap1 translocation/GTP loading is linked to defective activation of phospholipase D1 and its upstream activator Arf1. Taken together, our findings indicate that chemokine unresponsiveness in CLL lymphocytes results from failure of Arf1/phospholipase D1–mediated translocation of Rap1 to the plasma membrane for GTP loading and may be a specific feature of anergy induced by DNA Ags. The Journal of Immunology, 2013, 191: 1496–1504.

C hronic lymphocytic leukemia (CLL) is notable for its clinical variability. One clinical feature that varies widely between individual patients is the extent to which the malignant cells infiltrate lymphoreticular tissues. The extent of tissue invasion is important in CLL because it forms the basis of the Binet and Rai staging systems, which are powerful determinants of patient survival (1, 2). The shorter survival of patients with extensive tissue involvement likely reflects exposure of the malignant cells to survival/proliferation signals in the tissue microenvironment (3, 4). Understanding the mechanisms that regulate the entry of CLL cells into tissues is therefore a matter of considerable potential as a basis for developing novel therapy.

Tissue infiltration by CLL cells requires cell motility, transendothelial migration (TEM), and subsequent movement within lymphoid tissues, all of which depend on the activation of integrins by chemokines (5–7). We have previously reported that the CXCL12 (SDF-1)– and CCL21 (SLC)–induced TEM of CLL cells is reduced as a result of defective activation of the integrin α4β7 (LFA-1) (8, 9); CCL21 and CXCL12 are required for the migration of normal lymphocytes into lymph nodes and bone marrow, respectively (10–12). This defect of integrin activation was found on most CLL samples. Impaired α4β7 activation in CLL cells was associated with failure of the chemokines CCL21 and CXCL12 to increase the activation of Rap1, a small G protein known to be required for the activation of multiple integrins, including α4β7 (13), and which plays a crucial role in the trafficking of normal B cells into (14) and out of (15) lymph nodes and bone marrow. Furthermore, the absence of increased Rap1 activation accounted for the failure of chemokines to induce the conformational activation of α4β7 required for TEM (9). In summary, failure of chemokine-induced Rap1 activation appears to be a distinctive feature of CLL and one that is likely to be important in disease pathogenesis. Consequently, elucidation of the mechanisms underlying this phenomenon could provide a basis for developing novel therapeutic interventions.

The activation status of Rap1 is controlled by guanine nucleotide exchange factors (GEFs), which activate Rap1 by loading it with GTP, and GTPase-activating proteins (GAPs), which reverse Rap1 activation by enhancing the rate of Rap1-bound GTP hydrolysis.
We have previously shown that CLL cells express GTP-loaded Rap1, which is not further loaded in response to Ag stimulation (9). This situation is similar to that observed in anergic T cells (17). We also showed that chemokine, BCR cross-linking, and an EPAC agonist that activate the three different Rap-GEFs (CalDAG-GEFI, C3G, and EPAC, respectively) either all stimulated Rap1 GTP loading or all completely failed to stimulate Rap1 GTP loading (9). This suggests that the failure of CXCL12 to increase Rap1 activation in CLL cells reflects the inability of GEFs to activate Rap1 rather than the inability of CXCL12 to activate its GEF. One possible explanation for the refractoriness of Rap1 to activation by GEFs is that CLL cells exhibit extremely high GAP activity that keeps Rap1 in its GDP-loaded form independently of the extent of GEF activation. Alternatively, it is also conceivable that Rap1 could be already maximally GTP loaded as a result of reduced or absent GAP expression/activity. Along these lines, it is of interest that a proportion of mice with constitutive Rap1 activation due to absence of the Rap-GAP-SPA-1 develop a CLL-like disorder (18). As a third explanation, refractoriness of Rap1 to activation by GEFs could potentially arise through defective endosomal recycling. Thus, the chemokine-induced activation of Rap1 in T cells has been shown to require the phospholipase D (PLD)1– dependent recycling of endosomes containing Rap1 to the plasma membrane where Rap1 GEFs are located (19, 20). According to this model, Rap1 would essentially be constitutively uncoupled from its activator GEFs. In keeping with this idea, endosome recycling is altered in normal anergic B cells (21), which resemble CLL cells in a number of respects (22–24).

Following on from these considerations, we set out to examine whether the failure of chemokine-induced Rap1 activation in CLL cells was associated with defective GAP function or impaired recycling of endosomes containing Rap1 to the plasma membrane as a manifestation of anergy. Our findings suggest that failure of Rap1 activation is indeed associated with impaired endosomal recycling, and that this is linked with a form of anergy associated with chronic exposure to DNA-based Ags.

Materials and Methods

Patients and donors

The study involved 43 patients with typical CLL based on morphology and surface marker expression (CD5+ and CD23+ with dim L chain–restricted Ig). All patients had a total WBC count of $10^9/l, and run on the same blot.

Pull-down assays. CLL cells were incubated in the presence or absence of CXCL12 (R&D Systems, Oxon, U.K.) for 1 min and lysed as described above. Rap1-GTP was then pulled down from these lysates using Ral-GDS-RBD beads (Upstate Biotechnology, Hampshire, U.K.). Levels of the GTPase in the lysate and pull-down were then measured by Western blotting.

Arf6-GTP loading was measured as described previously (25). Briefly, Arf6 was pulled down from the lysates using GST-bound MT-2 (donated by Nicholas Vitale, University of Strasburg) that was conjugated to Sepharose beads. Arf1-GTP was pulled down using GGA3 protein binding domain–coated Sepharose beads (Pierce Biotechnology, Rockford, IL). Levels of Arf1 and Arf6 were measured as for Rap1.

Subcellular fractionation

Fractionation was performed according to the method of Bivona et al. (19). Fractions (150 μl) were collected, concentrated by TCA precipitation, and then run on a Western blot; the same membrane was probed for markers of the plasma membrane (Na/K ATPase; Cell Signaling Technology, Danvers, MA), the trans-Golgi network (58-kDa Golgi; Abcam), and endosomes (EEA-1; Abcam) and Rap1.

Quantitation of GTP– and GDP-bound Rap1

Levels of Rap1-GDP and Rap1-GTP were calculated according to the method of Boss and colleagues (26) (U.S. Patent no. 5,741,635). Briefly, Rap1 was immunoprecipitated from CLL cell lysates. The absolute amounts of GTP and GDP were then measured in these immunoprecipitates by coupled enzyme reactions. The levels of GTP and GDP were quantitated by reference to standard curves using known amounts of each nucleotide submitted to the above reactions (Sigma–Aldrich).

Immunofluorescent staining

Rap1. CLL cells were incubated with or without CXCL12 (R&D Systems) for 1, 2, 5, and 10 min and cytoplasm onto slides before being fixed and permeabilized. Slides were blocked in 10% BSA then stained with Rap1 (4 μg/ml) or control rabbit Ig (Santa Cruz Biotechnology), followed by goat anti-rabbit Ig-Alexa Fluor 488 (Invitrogen). The nucleus was then stained with TO-PRO-3 (Invitrogen). Staining was analyzed using confocal microscopy (LSM 710; Zeiss, Jena, Germany).

Endosome colocalization. Briefly, CLL cells were incubated with anti-IgM (Sigma–Aldrich) for 30 min for IgD plus LAMP-1; for Rap1 plus LAMP-1 or EEA-1, cells were treated with chemokine as above. Cells were then fixed, and for internal staining cells were permeabilized with 0.2% Triton X-100 (Sigma–Aldrich). After blocking, cells were stained with IgD (4 μg/ml; Santa Cruz Biotechnology)/Rap1 (as above) with or without LAMP-1/EEA-1 (1 μg/ml; Abcam) followed by goat anti-mouse Alexa Fluor 488 plus goat anti-rabbit Alexa Fluor 633. Cells were transferred onto poly-l-lysine–coated (Sigma–Aldrich) slides and examined by confocal microscopy, as above. The amount of colocalized staining was determined, in a single Z-section through the center of the cell, using the manufacturer’s software.

Cloning of Rap-GTP reporter plasmid

The Ras/Rap-GTP association domain of the Ras/Rap effector RalGDS (RGR) was used as an affinity probe to image Rap-GTP in live cells. Three RGR modules were cloned in series and in fusion with enhanced GFP (EGFP) to yield EGFP-RGRx3 fluorescent reporter constructs. The RGR sequence (amino acids 1–97 of human RalGDS) was amplified by PCR and cloned into pRSET B (Invitrogen) using forward and reverse primers encoding XhoI and KpnI sites, respectively, to yield pRSET B/RGR-1. RGR2 and RGRx3 construction involved two sequential rounds of PCR amplification of RGR using primer pairs encoding KpnI/EcoRI sites and EcoRI/HindIII restriction sites, respectively. PCR products were sequentially cloned on to the 3′ end of RGR in pRSET B/RGR to produce pRSET B/RGRx2 and pRSET B/RGRx3. Primers for RGRx2 and RGRx3 were designed such that concatenated RGR domains were separated by 5-aa linkers. K48D and other single-point mutant RGRx1, x2 and x3 constructs were generated in the same way, starting off with the corresponding single mutant RGRs. RGR sequences were transferred from pRSSET B to pEGFP-C2 (Clontech) by XhoI/HindIII restriction cloning to yield RGRx1, x2, x3 fused carboxyl terminally to EGFP. The integrity of all constructs was verified by sequencing.

Transfection

CLL clones were transfected using the Amaxa electroporator and cell line transfection kit (Lonza, Slough, U.K.). Briefly, 1 × 10^6 CLL cells were resuspended in the nucleofector solution together with the plasmid coding...
for the Rap1-GTP reporter probe EGFP-RGR(K48D)x3 (see Supplemental Fig. 1 for plasmid preparation and characterization). Cells were transfected using the U13 program and cultured for 24 h prior to use.

For analysis of the localization of active Rap1, transfected CLL cells were treated with CXCL12 for 1, 5, and 10 min (as above). The cells were fixed to stop further stimulation and the nucleus was stained with TO-PRO-3 before transferring the cells onto slides. The distribution of the Rap1-GTP probe was examined by confocal microscopy.

**Measurement of internal Ca^{2+}**

Intracellular calcium mobilization was measured by staining cells with the fluorescent dye Fluo-4/AM and analyzing the calcium-dependent fluorescent signal in real time by flow cytometry (23).

**Measurement of PLD**

PLD was measured according to the manufacturer’s instructions using the commercial Amplex Red PLD kit (Invitrogen). Protein levels were determined by reference to a standard curve generated using purified PLD (Sigma-Aldrich). In preliminary experiments enzyme activity was assayed at 10 and 60 s after exposure to CXCL12.

**Inhibition of PLD1.** CLL clones were incubated with an inhibitor of PLD1 (200 nM; VU0155069, Tocris Bioscience, Bristol, U.K.) for 2 h prior to treatment with CXCL12 and assaying for PLD activity as above.

**Results**

*Rap2 is expressed at low levels and is not GTP loaded in CLL cells*

In our previous study we reported that most CLL samples were unable to increase GTP loading of Rap1 in response to a range of stimuli (9). We also showed that in the absence of Rap1, activation CLL samples were unable to activate αβ2, a prerequisite for TEM, unless αβ1 was also expressed. However, we did not investigate the role of the Rap2 GTPase as a potential mediator of αβ2 activation and TEM in Rap1-unresponsive αβ1+ CLL cells. To do so seemed important because Rap2 is involved in the migration of normal lymphocytes (27), is activated by chemokine stimulation, and has functions that overlap with those of Rap1 (28).

**αβ2 activation and TEM in Rap1-unresponsive αβ1+ CLL cells.**

To investigate this possibility, confocal microscopy was employed to examine changes in the subcellular localization of Rap1 in response to chemokine stimulation. In the absence of stimulation, Rap1 was located in the intracellular compartment in both chemokine-responsive and -unresponsive CLL samples (Supplemental Fig. 1A–C). In CLL samples that were able to GTP load Rap1, CXCL12 rapidly (within 1 min, maximal effect at 5 min) induced the translocation of Rap1 to the plasma membrane (86.4 ± 3.4% of cells; p = 0.003). However, in chemokine-unresponsive cells, CXCL12 stimulation had no effect on the location of Rap1, which remained in the intracellular compartment (Supplemental Fig. 1A–C). These results indicate that Chemokine unresponsiveness is indeed associated with the failure of Rap1 translocation to the plasma membrane.

**CXCL12 induces Rap1 translocation to the cell membrane in chemokine-responsive but not chemokine-unresponsive CLL samples**

We next turned our attention to the possibility that chemokine unresponsiveness might be caused by a defect in endosomal recycling whereby Rap1 is denied access to GEFs located in the plasma membrane. In other cell types, Rap1-GDP has been shown to reside in the endosomal compartment, with GTP loading being dependent on translocation and fusion of endosomal-associated Rap1 with the plasma membrane (19, 31). We therefore speculated that the failure of CLL cells to GTP load Rap1 in response to CXCL12 might reflect a failure of such translocation to occur.

To investigate this possibility, confocal microscopy was employed to examine changes in the subcellular localization of Rap1 in response to chemokine stimulation. In the absence of stimulation, Rap1 was located in the intracellular compartment in both chemokine-responsive and -unresponsive CLL samples (Fig. 1A–C). In CLL samples that were able to GTP load Rap1, CXCL12 rapidly (within 1 min, maximal effect at 5 min) induced the translocation of Rap1 to the plasma membrane (86.4 ± 3.4% of cells; p = 0.003). However, in chemokine-unresponsive cells, CXCL12 stimulation had no effect on the location of Rap1, which remained in the intracellular compartment (Fig. 1A–C). These results indicate that Chemokine unresponsiveness is indeed associated with the failure of Rap1 translocation to the plasma membrane.

**Failure of CXCL12 to increase Rap1 GTP loading cannot be explained by altered GAP expression or activity**

We next addressed the question of why chemokine stimulation fails to increase Rap1-GTP loading in most CLL samples (hereafter referred to as chemokine unresponsive). In our previous report, we showed that the inability of CXCL12 to activate Rap1 reflected the refractoriness of Rap1 to activation by GEFs (9). We first explored the possibility that this refractoriness might result from Rap1 being aberrantly GTP loaded due to reduced or increased GAP activity. To address this question, the expression of Rap1 GAPS was investigated in chemokine-responsive and -unresponsive CLL samples. We focused on Rap1-GAP and SPA-1 owing to their established importance as Rap1-GAPS in hematopoietic cells (30). However, no correlation was observed between chemokine-induced Rap1 GTP loading and levels of SPA-1 or Rap1-GAP (Supplemental Fig. 2C, Supplemental Table 1).

To further investigate the possibility that chemokine unresponsiveness was associated with functional GAP defects, levels of Rap1-GTP and Rap1-GDP were measured in chemokine-responsive and -unresponsive CLL samples (Supplemental Fig. 2D). In keeping with our previous observations (9), Rap1-GTP was detectable in all unstimulated CLL samples tested. Rap1-GDP was also readily detectable in all unstimulated CLL samples. The ratio of Rap1-GTP/Rap1-GDP in unstimulated CLL samples varied between individual cases but was similar in chemokine-responsive and nonresponsive groups (Supplemental Fig. 2E). Taken together, these observations indicate that failure of chemokine-induced Rap1-GTP loading does not result from altered levels or functional aberrancies of GAPs.

**CXCL12 removes Rap1 from endosomes in chemokine-responsive but not chemokine-unresponsive CLL samples**

In T cells the translocation and activation of Rap1 involves trafficking via the endosomal compartment (19). To test whether this...
FIGURE 1. Subcellular localization of Rap1. (A) Representative images showing the localization of Rap1 (green) as determined by immunofluorescent staining. The nucleus is stained in blue. In the untreated cells, Rap1 was clearly seen throughout the cytoplasm. Following chemokine stimulation, most of the Rap1 moved from the cytoplasm to the plasma membrane in responsive cases, whereas in nonresponsive cases the distribution of Rap1 was similar before and after CXCL12 stimulation. (B) Line profile of representative cells from (A) showing distribution of Rap1. It can be clearly seen that CXCL12 causes Rap1 to be redistributed to a narrow peak at the periphery of the cell in the chemokine-responsive (R) but not in the nonresponsive (NR) cells. (C) Quantification of chemokine-induced Rap1 redistribution to the plasma membrane. For each treatment ≥50 cells were counted independently by two investigators who were blinded to other information about the cases. Mean values ± SEM are shown. Note that no ringed cells were seen in patient 4. Significantly more ringed cells were seen following chemokine treatment in the responsive CLL samples (p = 0.003). (D) CLL cells were transfected with an EGFP-tagged (green) reporter probe that binds to activated Rap1. The nucleus is stained in red. In the untreated cells, Rap1 was clearly seen throughout the cytoplasm. Following chemokine stimulation, the probe redistributed to the plasma membrane in responsive cases. In contrast, Rap1 remained in the cytoplasm in nonresponsive cases. (E) Line profiles of cells from (D). (F) Quantification of redistribution of Rap1-EGFP reporter probe to the plasma membrane. Note that the probe was redistributed to the plasma membrane of cells in only the responsive cases. Scale bars in (A) and (D), 10 μm (original magnification ×40).

was also the case in CLL cells, we first used cell fractionation and Western blotting to investigate the subcellular localization of Rap1. These experiments showed that Rap1 of unstimulated CLL cells (n = 3) was located in the plasma membrane, Golgi, and endosomal fractions (Fig. 2A). To confirm that Rap1 was present in endosomes, confocal microscopy experiments were performed to examine the colocalization of Rap1 and markers of early (EEA-1) and late (LAMP-1) endosomes. The results are shown in Fig. 2B and 2C. In unstimulated CLL cells, Rap1 was colocalized with both LAMP-1 and EEA-1 (not shown). Following treatment with CXCL12, Rap1 was no longer colocalized with the endosomal markers in chemokine-responsive CLL cells. However, in chemokine-unresponsive CLL cells, Rap1 remained in the LAMP-1/EEA-1 compartment. Applying these experiments to normal B cells produced results that were very similar to those obtained in chemokine-responsive CLL cells (average Rap1/LAMP-1 colocalization was 28 ± 4% in untreated cells and 12.0 ± 2% following treatment with CXCL12; p = 0.028). Taken together with the data presented in Fig. 1, these results strongly suggest that failure of CXCL12 stimulation to induce Rap1 activation in chemokine-unresponsive CLL cells results from failure of Rap1 translocation from endosomes to the plasma membrane where chemokine-induced GDP loading takes place.

Chemokine-unresponsive CLL cells show a distinct pattern of IgD distribution associated with anergy induced by DNA Ags

We next sought to investigate the mechanisms underlying the failure of endocytic recycling of Rap1 in chemokine-unresponsive CLL cells. A number of considerations led us to postulate that it might be related to cellular anergy. First, antigenic stimulation of anergic T cells fails to increase Rap1 GTP loading (17, 34). Second, anergy has been associated with sequestration of recycling endocytic vesicles in the perinuclear region (35) and suppression of phospholipid activation (36). Third, in mouse B cells, anergy has been linked to a reduction in the cellular response to CXCL12 (37). Fourth, CLL lymphocytes have been reported to share some of the features described in mouse models of B cell anergy (22, 38). We therefore speculated that the altered endocytic recycling responsible for the failure of Rap1-GTP loading might be linked to the anergic state of CLL cells. To investigate this hypothesis, chemokine-responsive and -unresponsive CLL cells were examined for features of anergy using normal (nonanergic) B cells for comparison.

Anergy is a complex process that is not fully understood. Two alternative types of anergy have been described in mice following chronic antigenic stimulation. Mouse B cells anergized to the protein Ag hen egg lysozyme display enhanced Ag-induced endocytosis of the BCR. The BCR accumulates in a large intracellular pool from which it rapidly recycles to the plasma membrane (39). In contrast, mouse B cells anergized by DNA-based Ags are characterized by failure of the BCR to enter LAMP-1 late endosomes after Ag engagement (35). Both of these models of anergy are characterized by the presence of intracellular IgD.

We therefore began our investigations by examining CLL cells for colocalization of IgD with LAMP-1+ endosomes (Fig. 3A–C). As expected, in the normal B cells, most of the IgD was located on...
The cell surface, with very little being associated with the LAMP-1+ endosomal compartment. However, upon IgM cross-linking, the IgD was internalized into the LAMP-1+ compartment. In contrast, in both chemokine-responsive and chemokine-unresponsive CLL cells, IgD was predominantly located internally rather than on the cell surface and was unaffected by cross-linking of surface IgM. Thus, chemokine-responsive and -unresponsive CLL cells both displayed a pattern of IgD distribution indicative of anergy. Interestingly, the internal IgD colocalized with LAMP-1 in the chemokine-responsive CLL cells but not in the chemokine-unresponsive CLL cells. It can therefore be deduced that chemokine-unresponsive CLL cells display a pattern of IgD distribution resembling that associated with chronic stimulation by DNA-based Ags, whereas chemokine-responsive CLL cells display a pattern of IgD distribution not associated with DNA-based Ags.

To confirm that the CLL samples used for these experiments were anergic, all 12 cases were analyzed for their ability to mobilize intracellular Ca\(^{2+}\) following cross-linking of surface IgM (Fig. 3D). No increase in intracellular Ca\(^{2+}\) was observed in 5 of 12 samples (2 chemokine-responsive and 3 chemokine-unresponsive cases). Furthermore, levels increased by only 3–20% in the other 7 cases compared with 47% in normal B cells with no difference between the two groups. This absence or marked attenuation of intracellular Ca\(^{2+}\) mobilization induced by BCR cross-linking supports the idea that all 12 cases of CLL were anergic (40).

**Ex vivo culture of chemokine-unresponsive CLL cells restores chemokine responsiveness and reverses anergy**

We next sought to establish to what extent chemokine unresponsiveness and anergy of the type associated with DNA Ags are functionally linked. We have previously shown that chemokine responsiveness can be restored to chemokine-unresponsive CLL cells by culturing them ex vivo for 48 h. In particular, CLL cells regained their ability to undergo TEM in response to chemokine stimulation, although chemokine receptor expression was unchanged (9). We therefore sought to establish whether restoration of chemokine responsiveness is accompanied by reversal of anergic features. As expected, incubation of chemokine-unresponsive CLL samples (n = 3) for 48 h restored CXCL12-induced Rap1 GTP loading (Fig. 4A), translocation of Rap1 from the intracellular compartment to the cell membrane (Fig. 4B), and \(\alpha_c\)-dependent TEM (Fig. 4C). We next examined the same CLL samples for reversal of anergic features by staining for surface and total IgD. After 48 h in culture, CLL cells resembled normal B cells in that most of the IgD was located on the cell surface. Furthermore, BCR cross-linking with anti-IgM resulted in internalization of most of the IgD into the LAMP-1+ endocytic compartment (Fig. 4D), indicating reversal of the anergic phenotype. Furthermore, ex vivo incubation of CLL cells for 48 h restored their ability to mobilize intracellular Ca\(^{2+}\) following BCR cross-linking (Fig. 4E). Taken together, these findings indicate that chemokine unresponsiveness and anergy of the type associated with DNA Ags are indeed linked and dependent on in vivo stimulation.

**Activation of PLD and Arf1 is defective in chemokine-unresponsive CLL samples**

We next sought to establish the mechanisms underlying the defective vesicular translocation of Rap1 in chemokine-unresponsive CLL samples. We initially focused our attention on PLD. There are two isoforms of PLD (1 and 2); both are found in the plasma membrane and in endosomes (41, 42). Because translocation of endocytic vesicles to the plasma membrane depends on PLD1 (20, 43), and because failure to activate PLD1 is a feature of anergy (36), we compared CXCL12-induced PLD activation in chemokine-responsive and chemokine-unresponsive CLL samples (Fig. 5A). Levels of active PLD were elevated in most CLL samples as compared with normal lymphocytes (CLL cells, 80 ± 34 U/ml; normal lymphocytes, 44 ± 14 U/ml). In chemokine-responsive
CLL samples, PLD activity was variably, but consistently, increased after exposure to CXCL12. In contrast, in chemokine-unresponsive CLL samples, no increase in PLD activity was observed after exposure to CXCL12; in fact, levels of active lipase were decreased following chemokine stimulation in most of these samples.

To determine the isoform of PLD that was involved we used a specific inhibitor of PLD1. The activity of PLD in untreated CLL cells was reduced by $\sim 50\%$ following incubation with the PLD1 inhibitor, suggesting constitutive PLD1 activity; the residual PLD activity in the presence of the PLD1 inhibitor can be reasonably attributed to PLD2. The PLD1 inhibitor prevented the CXCL12-induced increase in PLD activity seen in three of the four chemokine-responsive CLL samples tested (Fig. 5B). These findings indicate that chemokine-induced translocation of Rap1 to the plasma membrane of CLL cells is, at least in most cases, mediated by PLD1 and that chemokine unresponsiveness can therefore be attributed to failure of PLD1 activation.

PLD can be activated by a number of stimuli, including Arf and Rho family GTPases, protein kinase C, or phosphatidylinositol 4,5-bisphosphate (43–46), and thus failures in any of these signaling pathways may be responsible for the defective PLD1 activation in CLL. We focused on the Arf1 and Arf6 GTPases, as they are known to be key regulators of membrane trafficking (43, 44), have been implicated in anergy (47), and are both involved in the activation of PLD (43, 44). CLL samples differed in their ability to GTP load Arf6 in response to CXCL12 stimulation. However, this was not linked to chemokine responsiveness (Fig. 5C). In contrast, CXCL12-induced GTP loading of Arf1 was confined to chemokine-responsive CLL cells (Fig. 5D). These findings suggest that Arf1 plays a pivotal role in the chemokine-induced activation of PLD1 in CLL cells and that failure of PLD1 and Rap1 translocation to the plasma membrane in chemokine-unresponsive CLL samples can be attributed to defective Arf1 activation.

Because the inability of chemokine stimulation to activate Rap1 was reversed by ex vivo incubation (Fig. 4A, 4B), we sought to establish whether the same was true of chemokine-induced Arf1/PLD activation. To address this question, CLL cells were cultured for 48 h and examined for activation of Arf1 and PLD following treatment with CXCL12. Responsiveness of Arf1 and PLD to chemokine stimulation was indeed restored by ex vivo incubation for 48 h (Fig. 5E). These observations support the idea that the Arf1/PLD1/Rap1 pathway in chemokine-unresponsive CLL samples can be attributed to defective Arf1 activation.

Discussion

The aim of the present study was to investigate the mechanisms underlying defective Rap1 GTP loading in CLL cells in the ex-
pectation that this might reveal new insights into disease patho-
genesis and elucidate new molecular targets for drug therapy. However, we first sought to clarify the role of Rap2 in CLL cell motility given that it shares many functions with Rap1. Irrespective of Rap1-GTP loading, Rap2 was expressed at low levels in CLL cells and was not GTP loaded. This finding needs to be reconciled with the fact that the motility of normal lymphocytes requires GTP loading of either Rap1 or Rap2 to activate αL integrin (29, 48). It is evident that Rap1/Rap2-dependent motility cannot take place in CLL cells that are unable to GTP load either Rap1 or Rap2 and that the motility of such cells must therefore involve Rap1/Rap2-independent mechanisms. Our previous demonstration that CLL cells can activate αL directly via engagement of αL integrin provides a plausible explanation for such Rap1Rap2-independent motility (9).

With regard to Rap1, we showed that failure of CXCL12 to increase Rap1-GTP loading was not associated with aberrant GAP expression or function, but rather with failure of Rap1 translocation from endosomes to the plasma membrane where it is GTP loaded. We further showed that failure of chemokine-induced Rap1 translocation/GTP loading is linked to a distinct pattern of cellular IgD distribution resembling that observed in mouse B cells anergized by DNA-based Ags (IgD mostly internal, not altered by surface IgM cross-linking and not colocalized with LAMP-1+ endosomes). In contrast, chemokine-responsive CLL cells (39) displayed a different form of anergy (internal IgD colocalized with LAMP-1+ endosomes). Further evidence linking chemokine unresponsiveness and anergy was provided by our demonstration that ex vivo incubation of chemokine-unresponsive CLL cells for 48 h not only restored CXCL12-induced Rap1-GTP loading and function but also redistributed IgD to the cell surface and restored its ability to internalize following IgM cross-linking. Finally, we shed light on the mechanisms underlying the failure of chemokine-induced Rap1 translocation/GTP loading by showing that it is linked to defective activation of PLD1 and its upstream activator Arf1.

Our study makes three important observations regarding anergy in CLL. First, it confirms the results of previous studies showing that anergy is a frequently observed feature of CLL lymphocytes (22–24, 38, 49). Second, it provides evidence that individual cases of CLL display distinct patterns of anergy linked to specific types of antigenic stimulation. Third, it links these distinct patterns of anergy (and, by implication, distinct types of antigenic stimulation) to the ability of CLL cells to respond to chemokine stimulation. Taken together, these observations add significantly to our understanding of CLL biology and provide new insight into the mechanisms that govern how the leukemic burden is distributed between different body compartments and why this varies between patients.

Our observation that different cases of CLL display different types of anergy is in keeping with the known diversity of the BCR in CLL and with the idea that different CLL clones react to different Ags (49, 50). The literature on the relationship between anergy in CLL and IGHV mutation is contradictory; some suggest there is a link (23), whereas others suggest that there is not (22, 24). Our study failed to identify any association between chemokine responsiveness and IGHV gene usage, IGHV mutational status, or H chain isotype.

CDR3 stereotypy provides a more sophisticated approach to understanding BCR structure in relationship to the stimulating Ag. CDR3 sequences in our cohort were therefore compared with the most recently published CDR3 stereotype dataset involving 1967 IGHV sequences and 110 identified CDR3 stereotypes (51). We did not, in fact, find any association between chemokine responsiveness and CDR3 length or CDR3 stereotypes (data not shown). Importantly, however, note that our knowledge of CDR3 stereotypy is very incomplete. For example, the most recent study in-

FIGURE 4. Effect of ex vivo incubation on Rap1, TEM, and anergy. (A) Chemokine-unresponsive CLL cells were incubated ex vivo for 48 h and analyzed by confocal microscopy. The chart shows the percentage of cells with a ringed pattern of staining indicative of surface expression. (B) Chemokine-unresponsive CLL cells were incubated ex vivo for 48 h and analyzed for their ability to undergo TEM in the presence or absence of a blocking Ab to αL. The data shown form part of a previous report (9). (C) Chemokine-unresponsive CLL cells were incubated ex vivo for 48 h and analyzed by flow cytometry for intracellular Ca2+ levels following BCR cross-linking.
The effect of CXCL12 on Arf1-GTP loading and PLD activation as in (for comparison; the figures refer to the percentage increase in PLD activity. (C) Effect of CXCL12 on Arf6 activity. CLL cells were incubated in the presence or absence of CXCL12 and assayed for Arf6 GTP loading using a pull-down assay. The changes in Arf6 GTP loading induced by CXCL12 were inconsistent and not associated with chemokine responsiveness. (D) Effect of CXCL12 on Arf1 activity. CLL cells were incubated in the presence or absence of CXCL12 and assayed for Arf1 GTP loading. CXCL12 treatment increased Arf1-GTP loading in chemokine-responsive CLL samples (n = 6) but not in nonresponsive samples. Rap1-GTP loading in the same clones is shown for comparison; the figures refer to the percentage increase in PLD activity. (E) Chemokine nonresponsive cells were cultured for 48 h and analyzed for the effect of CXCL12 on Arf1-GTP loading and PLD activation as in (D). The ability of chemokine to activate both Arf1 and PLD was restored after culture.

FIGURE 5. Effect of CXCL12 on activation of PLD, Arf6, and Arf1. (A) Percentage change in PLD activity induced by CXCL12 in chemokine-responsive (R) and -unresponsive (NR) samples (n = 10 of each). (B) Effect of a specific PLD1 inhibitor on total PLD activity in responsive cases. PLD1 inhibition resulted in a reduction in constitutive PLD activity and blocked the increase in PLD activity induced by CXCL12 in three of the four cases tested. (C) Effect of CXCL12 on Arf6 activity. CLL cells were incubated in the presence or absence of CXCL12 and assayed for Arf6 GTP loading using a pull-down assay. The changes in Arf6 GTP loading induced by CXCL12 were inconsistent and not associated with chemokine responsiveness. (D) Effect of CXCL12 on Arf1 activity. CLL cells were incubated in the presence or absence of CXCL12 and assayed for Arf1 GTP loading. CXCL12 treatment increased Arf1-GTP loading in chemokine-responsive CLL samples (n = 6) but not in nonresponsive samples. Rap1-GTP loading in the same clones is shown for comparison; the figures refer to the percentage increase in PLD activity. (E) Chemokine nonresponsive cells were cultured for 48 h and analyzed for the effect of CXCL12 on Arf1-GTP loading and PLD activation as in (D). The ability of chemokine to activate both Arf1 and PLD was restored after culture.

Involving 7596 IGHV sequences has identified 952 CDR3 stereotypes (52). Sequences corresponding to these new CDR stereotypes have not been published, and thus we were not able to relate them to those in our cohort. It therefore follows that our inability to detect an association between chemokine responsiveness and CDR3 stereotypy in the present study does not necessarily mean that such association does not exist.

Perhaps the most interesting observation in our study is the association detected between the type of anergy in individual CLL cases and their chemokine responsiveness. Our data suggest that CLL samples with anergic features distinct from those induced by DNA Ags in mouse B cells are able to activate aβ2 integrin and undergo TEM in response to stimulation by CXCL12. In contrast, cases of CLL with anergic features resembling those induced by DNA-based Ags are unable to respond to chemokine stimulation unless they coexpress aβ1 integrin, which can activate aβ2 integrin and induce TEM independently of Rap1 (9). It is therefore intriguing to speculate that the ability of CLL cells to activate aβ2 integrin and undergo TEM into lymph nodes is governed partly by aβ1 expression and partly by the nature of the Ag to which the malignant clone reacts specifically. CLL clones should be well equipped to enter lymph nodes if they react to non-DNA Ags, or if they react to DNA-based Ags but express aβ1 integrin. In contrast, CLL clones that react to DNA-based Ags and do not express aβ1 integrin should have difficulty entering lymph nodes.

It is also intriguing to apply similar principles to the egress of malignant lymphocytes from lymph nodes. It is generally accepted that egress depends on Rap1 activation but not integrin expression (15, 53). Therefore, those CLL clones that react to non-DNA Ags should be able to freely exit from lymph nodes whereas those CLL clones that react to DNA-based Ags should become trapped in lymph nodes irrespective of aβ1 expression owing to their inability to activate Rap1.

Combining these hypotheses gives the following predictions: 1) CLL clones anergized by non-DNA Ags should be able to enter and exit lymph nodes freely, resulting in the leukemic burden being distributed in a balanced way between the blood and lymph nodes; 2) CLL clones anergized to DNA-based Ags and that lack aβ1 expression should have difficulty in both entering and exiting lymph nodes, again resulting in a balanced anatomical distribution of disease; and 3) CLL clones anergized to DNA-based Ags and that express aβ1 should be capable of entering but not exiting lymph nodes, resulting in disproportionate lymphadenopathy relative to the level of blood involvement. These considerations, although speculative, provide a plausible hypothesis to explain the marked variation in lymphadenopathy observed in CLL.

In summary, our study has elucidated the mechanisms underlying chemokine unresponsiveness in CLL and, in doing so, has revealed an association between chemokine responsiveness and a distinct form of immunological anergy, suggesting that the trafficking properties of CLL cells may be influenced by the nature of the Ag responsible for clonal selection. Because trafficking of CLL cells underpins the anatomical distribution of the leukemic burden, and because the latter influences therapeutic response and survival, our findings have inescapable clinical implications and could provide the basis for new therapeutic interventions that prevent CLL cells from entering, or stimulate their egress from, the protective lymph node microenvironment.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References

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Supplementary Fig. 1. Characterization of trivalent fluorescent probes for the visualization of cellular Rap-GTP.
Supplementary Fig. 2. Levels of Rap and GAPs
**Supplementary Figure Legends**

**Fig. 1. Characterization of trivalent fluorescent probes for the visualization of cellular Rap-GTP.**

**A.** COS-7 cells were co-transfected with the Rap-GTP reporter EGFP-RGRx3 along with the indicated mCherry tagged Rap variants and imaged confocally. EGFP-RGRx3 reported both wild type and GTP-loaded G12V versions of Rap1 and Rap 2. Colocalisation was specific because the Rap-effector-site mutation D38A, which reduces affinity to RGR by about 100 fold (1), abrogated colocalisation. Note that the probe reported Rap-GTP loading of wild-type Rap. (Bars = 10μm).

**B.** EGFP-RGRx3 activates Rap. Hela cells cotransfected with the indicated plasmids were subjected to a Rap-GTP pulldown assay 48 h post transfection. Levels of active endogenous Rap-GTP and mCherry-Rap1-GTP and total Rap1 protein levels are depicted in the upper and middle panels, respectively. Lower panel shows EGFP-reporter expression. Note that the wild-type (wt) RGRx3 probe activates endogenous Rap1 and Cherry- Rap1, whereas RGR(K48D)x3 is much less potent in this respect. Asterisk marks non-specific bands derived from the GST-RGR protein used to pulldown Rap1-GTP or degradation products thereof. C. RGR(K48D)x3 is a specific reporter of Rap-GTP versus Ras-GTP and does not report wild-type Rap. COS-7 cells transfected with the indicated constructs were imaged confocally 48 h post-transfection. (Bars = 10μm). Note that, firstly, RGR(K48D)x3 decorates GTP-loaded Rap but not Ras-GTP and secondly co-localization is contingent on RGR trimerization because RGR(K48D)x1 failed to re-distribute. D. Porcine aortic endothelial (PAE) cell lines expressing wt Platelet derived growth factor receptor β (PDGFRβ) activate Rap1 via PLCγ in response to PDGF stimulation. In analogous cell lines expressing the Y1009/1021F double tyrosine point mutant receptor PDGF does not activate PLCγ (as monitored by phosphorylation of the activatory Tyrosine residue Y783) and in consequence fails to induce Rap-GTP loading (1). Thus, the pair of PAE/PDGFRβ cell lines is optimally suited to investigate agonist induced Rap activation, the Y1009/1021F line serving as a negative control. Rap-GTP levels were assayed as described in the experimental section. PAE cell lines were a kind gift of Carl-Henrik Heldin, Uppsala, Sweden. E. Rap-GTP biosensor RGR(K48D)x3 decorates and reports agonist-induced Rap-GTP formation at the plasma membrane. PAE/PDGFRβ and PAE/PDGFRβ-Y1009/1021F cells transiently expressing RGR(K48D)x3 were stimulated with PDGF and imaged confocally. RGR(K48D)x3 specifically decorated plasma membrane resident Rap-GTP. To ascertain that the PAE/PDGFRβ-Y1009/1021F had responded to PDGF stimulation, the silhouette of one cell before and 10 min post stimulation was drawn to illustrate the contraction of the cell body triggered by PDGF. Arrows point to sites of RGR(K48D)x3 accumulation/Rap-GTP formation at the plasma membrane. Scale bars: 10 μm. Original magnification X63.

**Supplementary Fig. 2. Levels of Rap and GAPs.**

A. Levels of Rap1 and Rap2 were quantified by reference to a standard curve. Graphical representation of the data obtained (n=16, 8 responders and 8 non-responders).  

B. Effect of CXCL12 on Rap2 loading in responder and non-responder CLL samples. Representative examples are shown from among the 16 CLL samples and 3 normal B-cell samples tested. Levels of total Rap1 and Rap2 protein in the samples used for the Rap-GTP assay are shown to indicate equal loading. C. Western blot showing SPA-1 protein levels in chemokine-responsive and non-responsive CLL samples and normal B cells. D. Levels of Rap1-GDP and Rap1-GTP in chemokine-responsive and non-responsive CLL samples. Although Rap-
GTP levels varied between clones, Rap1-GDP was detected in all samples and therefore available for unloading by GEFs. Repetition of the experiment gave similar (<10% difference) results (data not shown). E. Ratios of Rap1-GTP/Rap1-GDP in chemokine-responsive and unresponsive CLL samples. Note that the ratios are within the same range for both responsive and non-responsive CLL samples.