TARGETING MIGRATORY AND PROSURVIVAL CASCADES INDUCED BY THE HOMEOSTATIC CHEMOKINES CCL19 AND CCL21 FOR THERAPY OF B-CHRONIC LYMPHOCYTIC LEUKEMIA

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Running Title: PI3K and Rho mediate the CCR7-dependent CLL migration

This work was supported by the grant 064700 from Immunological and Medicinal Products, Spain, and grant PETRI 2005_0908 from Ministerio de Educación y Ciencia to C.M. S.L. was supported by the Fundación de Investigación Biomédica del Hospital Universitario de La Princesa, Madrid, Spain. M.A. was supported by the Fundación LAIR, Spain.

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Keywords: CLL, Chemotaxis, Apoptosis, Chemokines, CCR7, Treatment, Signaling

ABSTRACT

The CCR7 chemokine receptor has been reported to promote the localization of chronic lymphocytic leukemia (CLL) cells into lymph nodes (LNs) where they may gain survival signals, but the mechanisms mediating these effects are largely unknown. We investigated the role of different signaling pathways in the migratory and prosurvival effects exerted by the chemokines CCL19 and CCL21, the CCR7 ligands, in CLL cells. Their chemotactic activity was markedly reduced in the presence of inhibitors of PI3K and the Rho effector molecule ROCK. Moreover, expression of dominant negative forms of PI3K and RhoA blocked the chemotactic effect induced through CCR7, whereas their constitutively activated mutants exerted an opposite effect. Finally, MAPKs were not clearly involved in CLL migration to CCL19/CCL21. Conversely, ERK and JNK, along with PI3K, were responsible for the prosurvival effects mediated by CCR7 in CLL cells. Biochemical experiments confirmed that CCL19/21 promote the PI3K-dependent phosphorylation of the Akt/PKB kinase, the activation of the Rho/ROCK/MLC pathway and the phosphorylation of MAPKs. The important role of PI3K, Rho GTPases and MAPKs in the migration and survival of CLL cells in response to CCL19/21 provides a rationale to explore these signal pathways as promising targets for the therapy of this condition.

INTRODUCTION

Chemokines play a key role in different physiologic and pathologic conditions, including lymphocyte homing and metastasis (1, 2). In particular, the chemokine receptor CCR7 ligands, CCL19 and CCL21, organize the physiological migration of lymphocytes and dendritic cells (DCs) to and in lymph nodes (LNs) (3). In addition, this chemokine receptor (CKR) is involved in the survival and migration of malignant cells (2).

When chemokines bind to their CKR, heterotrimeric GTP-binding proteins are activated and regulate the activation of different effectors, including phospholipase C and phosphatidylinositol-3-OH kinase (PI3K) (4) which, in turn, activate downstream effectors such as the Akt/PKB kinase and the MAPK signaling pathway. CKR also give rise signals that regulate the activity of small GTPases, their activators and effectors.

The activity of PI3Ks exert a relevant role in cell growth, proliferation, survival and migration (5). Class I PI3Ks are heterodimeric molecules composed by a regulatory and a catalytic subunit that generate phosphatidylinositol-3,4,5-triphosphate (PIP3), which have a key role in cell migration (6). Class I PI3Ks are further subdivided into class IA and class IB isoforms, which are involved in the chemotaxis and homing of B and T lymphocytes, respectively (7, 8).

The small GTPases Rho, Rac, and Cdc42 link CKR activation and the remodeling of the cytoskeleton (9). In this regard, Rho has an important role in integrin activation as well as in the adhesion and migration of lymphocytes in response to different chemokines (10-12). Important Rho effectors are the so-called Rho-associated coiled-coil forming protein kinases (ROCK) I and II, which enhance myosin light chain (MLC) phosphorylation, regulating actin myosin contraction (13). On the other hand, the three major sets of mammalian MAPKs (ERK, JNK and p38-SAPK) are involved in cell proliferation, survival, oncogenesis, differentiation, inflammation and stress responses, and members of this family appear to be essential for the migration of some cells (14) such as mature DCs in response to the CCR7 ligands (15).

We have previously described a high expression of CCR7 in those B-cell neoplasms that are characterized by the infiltration of secondary lymphoid tissue, like chronic lymphocytic leukemia (CLL) (16). CLL B cells show a marked tendency to invade the LNs (17), and we have demonstrated that the ability of these cells to migrate in response to CCR7 ligands correlates with lymphadenopathy, suggesting a functional role of CCR7 in guiding CLL cells to LNs (16). In addition, it has been reported a strong correlation between the expression of CCR7 by solid tumor cells and the presence of LN metastases (2). These findings prompted us to investigate a possible role of CCR7 as therapeutic target for CLL and demonstrated that anti-CCR7 monoclonal antibodies (mAbs) efficiently eliminated CLL cells *in vitro* (18).

In this work we have studied the CCR7-signaling pathways activated in CLL cells in order to gain insights into the pathogenesis of this disease as well as to explore new venues to modulate the activities of chemokines that either complement or improve the current treatments. Our data indicate that PI3K and Rho GTPases have an important role in the migration of CLL cells induced by CCR7 ligands whereas PI3K and MAPK are at least in part responsible for the prosurvival effects of CCL19 and CCL21 in the same cells. Some chemical inhibitors of these signal transduction pathways are currently in clinical testing (19-21), and as such, its potential to be implemented in CLL patients is high.

MATERIALS AND METHODS

Cells. After CLL patients (Table S1) and healthy donors had given their informed consent, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO). Only those samples from CLL patients with more than 85% of CD19⁺ CD20^{dim} CD5⁺ CD23⁺ malignant cells (Table S2) were studied. When indicated, cells were pretreated with the proper chemical inhibitors for 1 h at 37°C (Table S3).

Chemotaxis assay. Chemotaxis assays with PBMC in response to 1 µg/mL CCL19 or CCL21 (Table S3) were performed in Transwell chambers (6.5 mm diameter, 10 µm thickness, 5 µm diameter pore size; Costar, Cambridge, MA) for 4 hours. Migrated cells were stained with an anti-CD19 mAb (Table S3), counted by flow cytometry, and the percent of cell migration was calculated with the following formula: 100×(number of cells migrated / number of cells in the initial suspension).

Nucleofection and chemotaxis assay. CLL cells were nucleofected with plasmids encoding the dominant negative (DN) forms of PI3K-Δp85α-GFP and RhoA-pGFP-RhoA-N19, or their constitutive active mutants (AM) forms pCDNA3-p110CAAX and pEGFP-C1-V14, respectively (all provided by Dr. F. Sánchez-Madrid, Hospital Universitario de La Princesa, Madrid, Spain). The green fluorescent protein (GFP) expression vector (pEGFP-C1) was obtained from Clontech (Palo Alto, CA) and used as control. Transfections were performed using the Human B-cell Nucleofector Kit (Amaza, Köln, Germany). Sixteen hours post-nucleofection, chemotaxis assays in response to CCL19 were performed. Within the cells transfected with the same construct, fold induction is defined as the ratio between the percentage of input of chemokine-stimulated cells and chemokine-untreated cells. In all the cases only the viable B CD19⁺ GFP⁺ 7-AAD⁻ cells were counted.

Western blot assay. To determine total or phosphorylated forms of the studied proteins (Table S2), cells were starved in serum-free medium, exposed to the inhibitors selected or left untreated, and stimulated with CCL19 or

CCL21 for the time periods indicated. The cells were lysed in ice-cold RIPA buffer and proteins were resolved by 10% SDS-PAGE, probed with appropriate primary Abs and visualized using a horseradish peroxidase-conjugated secondary Ab and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). A densitometric analysis of each membrane was done and data were normalized with respect to the loading control. Arbitrary units obtained for each point of CCL19 stimulation were referred to the value of the untreated cells point (t-0) to obtain fold induction with respect to the untreated control.

RhoA pull-down assay. RhoA-GTP levels were assessed with recombinant purified GST-TRBD (Glutathion-S-Transferase Rhotekin Rho-binding domain) bound to glutathione beads (Amersham Biosciences Co., Piscataway, NJ). GST and GST-TRBD (a gift from Dr. M.A. del Pozo, CNIC, Madrid, Spain) were expressed in *Escherichia coli* and purified as previously described (22). Cells were stimulated with CCL19, lysed with ice-cold RIPA buffer, and cell lysates were incubated for 1 hour at 4°C with GST or GST-TRBD coupled to glutathione-Sepharose beads. Beads were then washed and boiled in SDS sample buffer. Precipitates were resolved by 15% SDS-PAGE, transferred to nitrocellulose membranes and blotted with an anti-RhoA antibody (Table S2).

Apoptosis assay. PBMC isolated from 13 patients were plated in 96-well, flat-bottomed culture plates at a density of 10^6 cells/mL and cultured in triplicates for 72 h in RPMI + 10% FCS and, where indicated, in the presence of chemokines (1 μ g/mL) and PI3K, ROCK and MAPK inhibitors at concentrations indicated (Table S3). When used along with CCR7 ligands, inhibitors were first added 3 h before chemokines. The evaluation of living cells was done by means of cell staining with FITC-labelled annexin-V and 7-AAD (Table S3), and flow cytometry analysis of necrotic (7-AAD⁺ annexin-V-FITC⁺ cells), apoptotic (7-AAD⁺ annexin-V-FITC⁺ cells) and viable cells (7-AAD⁻ annexin-V-FITC⁻ cells) (23).

Statistical analysis. On migration assays one-way analysis of variance (ANOVA) followed by a Dunnett or Bonferroni *post hoc* tests was used to detect significant differences among the studied groups. On survival assays significant differences between groups were determined using the non-parametric Wilcoxon-signed rank test.

RESULTS

PI3K and ROCK, but not MAPK, are involved in the migration of CLL cells induced by CCL19 and CCL21.

CLL cells marginate from the bloodstream to the secondary lymphoid organs (SLO) probably on the basis of their high CCR7 expression (Figure S1). In order to assess the possible role of PI3K, ROCK and MAPK in the

chemotactic response of CLL cells induced by CCR7 ligands, transwell assays were done and showed that baseline motility of CLL cells was very low (Figure 1a). In contrast, CCL19 and CCL21 induced a remarkable chemotactic effect of these cells, $33.25\% \pm 4.04$ and $36.62\% \pm 5.51$ of cell migration, respectively (Figure 1a). This chemotactic response was significantly inhibited by the PI3K inhibitors wortmannin and LY-294002. Similarly, the ROCK inhibitors Y-27632 and H4413 (hydroxyfasudil), markedly impaired the chemotaxis of CCL cells to the CCR7 ligands tested (Figure 1a). As expected, pretreatment of CLL cells with either pertussis toxin or a blocking anti-human CCR7 mAb almost blocked the chemotactic effect of CCL19 and CCL21 (Figure 1a). In contrast, chemotaxis assays performed in the presence of different chemical inhibitors, indicated that the three sets of MAPK are not apparently involved in the stimulation of motility of CLL cells through CCR7 (Figure 1a).

Normal B cells migrate to CCR7 ligands via PI3K, Rho and p38-SAPK pathways.

Next, we studied whether the pathways that regulate the chemotaxis of CLL cells also mediated the chemotaxis of normal B cells. We found that normal B cells efficiently migrated to CCL19 and CCL21 (Figure 1b). As in the case of CLL cells, PI3K and ROCK inhibitors caused a significant diminution of the chemotaxis of normal B cells induced by CCL19. Conversely the effect of CCL21 was not significantly diminished by the PI3K inhibitors although the migration of B lymphocytes to CCL21 was by trend reduced. The inhibition of p38-SAPK by SB203580 significantly impaired the chemotaxis of normal B lymphocytes towards CCL21, whereas the migration induced by CCL19 was not affected. Finally, the inhibition of ERK1/2 or JNK did not significantly inhibit the cell migration in any case (Figure 1b).

Mutants of PI3K and Rho reveal their important role in migration of CLL to CCL19.

To further assess the involvement of PI3K and Rho/ROCK in the migration of CLL cells to CCR7 ligands, transient transfection experiments were performed with different mutant forms of PI3K and RhoA. As expected, the chemotactic response of untransfected (UT) and GFP-transfected CLL cells to CCL19 was similar (Figure 2a and b). The expression of a DN form of the regulatory subunit p85 α of PI3K significantly reduced the fold induction of CLL chemotaxis to CCL19 from approximately 25 times in GFP-transfected CLL cells to 4 times in the DN-transfected CLL cells. In contrast, expression of a constitutively AM form of the PI3K catalytic subunit p110 (pCDNA3-p110CAAX), strongly augmented CCR7-induced migration (Figure 2a). Likewise, transfection of the constitutively AM form of RhoA induced a remarkable increment in CLL chemotaxis towards CCL19, whereas its DN form inhibited this process (Figure 2b). Control experiments showed that the constitutively activated forms of PI3K or RhoA mutants were unable to induce chemokinesis of CLL cells by themselves, and that their DN forms did not significantly modify their basal motility (Figure 2c).

CCR7 ligands induce Akt and MLC phosphorylation in CLL cells.

Additional experiments further supported the role of PI3K in the migration of CLL cells in response to CCR7 ligands. CCL19 induced the phosphorylation of Akt/PKB, a downstream effector of PI3K, in CLL cells, a phenomenon that was inhibited by wortmannin or LY-294002 (Figure 3a).

To confirm the involvement of the small GTPase RhoA in the migration of CLL cells induced through CCR7, its activation was assessed by a pull-down assay. We found very low levels of active bound RhoA in non-stimulated cells, which notably increased upon stimulation with CCL19 (Figure 3b). Next, we explored the possible phosphorylation of MLC, which is downstream of Rho/ROCK, and has an essential role in actin-myosin contraction and cell migration. Western blot analysis showed that CCL19 induced a significant increase in the baseline levels of phosphorylated MLC in CLL cells, which peaked at 5-10 minutes (Figure 3c), with a similar kinetics than that of RhoA activation. As expected, MLC phosphorylation induced by CCL19 was abrogated in the presence of the ROCK inhibitors Y-27632 and H4413/hydroxyfasudil (Figure 3c). Similar results were obtained with CCL21 (data not shown).

CCL19 and CCL21 induce a strong activation of ERK1/2 in CLL cells.

Whereas we did not observe significant constitutive phosphorylation of ERK1/2 in resting CLL cells, both CCL19 and CCL21 induced a strong activation of these kinases, which peaked at 2 minutes (Figure 3d and data not shown). As expected, treatment of cells with the MAPKK MEK inhibitors PD98059 and U0126 or with pertussis toxin, significantly diminished the chemokine-mediated phosphorylation of ERK1/2 (Figure 3d). On the other hand, JNK and p38-SAPK were constitutively phosphorylated in non-stimulated cells and CCL19 and CCL21 induced a variable increase in their activation that decreased in the presence of specific inhibitors of these MAPK (Figure 3e and f, and data not shown).

CCL19 and CCL21 increase B-CLL cells survival.

The apparent discrepancy between the strong phosphorylation of ERK induced by CCR7 ligands and its reduced role in CLL migration prompted us to analyze the effect of the kinases on functions other than the migration like CLL survival. Interestingly, exposure of CLL cells to either CCL19 or CCL21 induced a significant increment of the cell viability at 72 hours (Figure 4a). Inhibitors of PI3K, ERK and JNK markedly reduced the survival of untreated CLL cells (Figure 4a) and abrogated the augment in the cell viability mediated by the CCR7 ligands (Figure 4a, b and c). Conversely, inhibitors of ROCK did not modified the survival of CLL cells in any case (Figure 4a, b and c).

DISCUSSION

CLL is characterized by a widespread infiltration of tumoral CD5⁺ CD23⁺ B cells into the bone marrow, the blood and the SLOs, including the LNs and the spleen, which may provide survival and proliferative signals. The chemokine receptor CCR7 and its ligands, the homeostatic chemokines CCL19 and CCL21, can play a central role in these trafficking and survival processes (16, 24), but little is known about the molecular signals mediating these effects.

Our results indicate that Rho/ROCK and PI3K signaling pathways play a critical role in the CCR7-dependent migration of CLL cells. Among the different GEFs and GTPases that could participate in the migratory response of CLL cells to CCL19/21, we focused on the role of RhoA/ROCK axis because the ROCK inhibitor fasudil is a molecule with therapeutic interest as it has been already administered to patients for the treatment of cerebral vasospasm with no apparent serious side effects (25). The function of RhoA, and its downstream effector ROCK, is to regulate the retraction of uropod in migrating leukocytes and it has been suggested that Rho is also required for directional migration and not only for cell contraction (26, 27). In line with this, we confirmed that the inhibition of ROCK strongly impaired CLL cells migration towards CCR7 ligands, as it has been described in T lymphocytes (10), and that this effect is mediated, at least in part, by the inhibition of MLC phosphorylation, which participates in the cell contractility required to migrate.

PI3K and its effector Akt play relevant functions in promoting cell survival and chemotaxis of different cell types (6). CLL cells are not an exception and our data show that PI3K is partially responsible for the chemotactic effects of the CCR7 ligands. The resistance of CLL cells to apoptosis is one of the hallmarks of this leukemia and several reports have described the role of PI3K in CLL survival (28, 29). However, the prosurvival stimuli that activate PI3K are not well characterized. Interestingly, we have found that both CCR7 ligands significantly augment the *in vitro* viability of the leukemic cells, as it has been suggested for other cells, including DCs (30), CD8⁺ lymphocytes (31) and malignant cells from metastatic head and neck cancers (32). Thus, it is tempting to speculate that CCL19 and CCL21 not only attract CLL cells to LNs, but also promote prosurvival niches inside these SLOs where high concentrations of CCR7 ligands are found. We and others (33) have found that such prosurvival effect is partially dependent on PI3K and MAPKs pathways. Indeed, the strong activation of ERK by both the CCR7 ligands in the experiments of western blot is probably taking account for the anti-apoptotic effects of CCL19/21 opposite to their migratory activities that are not dependent on ERK. Different effects for ERK activation on CLL have been described, including the synthesis of the metalloprotease MMP-9, which is involved in cell invasion (34). Furthermore, CCR7-stimulation and ERK1/2 activation may participate in the

induction of proliferation of CLL cells. Finally, opposite to their chemotactic effects, the role of GTPases in the survival of CLL cells is limited, if any.

Our data on CCR7-dependent signaling on CLL are in agreement with the involvement of PI3K and GTPases in the migration of normal T and B lymphocytes in response to homeostatic chemokines, including CCL21 and CXCL13 (6-8). The relative contribution of different GTPases, GTPases exchange factors (GEF's) and PI3K isoforms to cell migration seems to be dependent on the chemokine and the cell type (4). In normal lymphocytes, optimal polarization and migration in response to CCL19, CCL21 or CXCL12 is dependent on the expression of DOCK2, a Rac GTPase exchange factor, and PI3K. Whereas in T cells PI3K-dependent migration is mainly mediated by PI3K γ , in B cells class IA PI3K is required for their efficient chemotaxis and homing to LNs (7, 8). In this work, we have described the important role of class IA PI3K in the migration of CLL cells to CCL21 and CCL19. Interestingly, our data show that CLL migration is more strongly inhibited by the DN form of p85 α than by the PI3K chemical inhibitors wortmannin or LY-294002. It is conceivable that this phenomenon is due to the important role of the class IA PI3K regulatory subunit, which seems to recruit/activate other molecules such as the small GTPases that mediate the polarization of the leukocytes, necessary for locomotion (35). The intracellular signaling pathways mediating the migratory response to CCR7 ligands are somewhat different between CLL and normal B cells. Thus, the chemotactic effects of CCL21 on normal B lymphocytes were not significantly blocked by PI3K inhibitors whereas the pharmacological inhibition of p38-SAPK decreased the CCL21-dependent migration of normal B cells, opposite to CLL cells. In this regard, our results are in accordance with previous records on different requirements of CLL and normal B cells to migrate through the endothelium (36). This is not surprising and probably reflects the phenotypic and functional diversity of these two B cell subpopulations.

In summary, the intracellular signals induced by CCL19/21 through CCR7 in CLL cells include the PI3K/Akt, Rho/ROCK/MLC, and MAPK pathways, and they seem to have an essential role in different relevant phenomena on CLL, including chemotaxis, invasion and survival (Figure 5). Therefore, the inhibition of some intracellular signals induced through CCR7 may be a commendable therapeutic approach in this condition. In this regard, a new generation of PI3K inhibitors with enhanced isoform selectivity are currently in clinical trials (www.ClinicalTrials.gov) (19, 20). In addition, the ROCK inhibitor H4413 or fasudil has been employed for the therapy of cerebral vasospasm without serious side effects, and its beneficial effect in an experimental model of cerebral thrombosis, is partially mediated by the prevention of neutrophil migration into the area of ischemic injury (21). As such, the potential of these drugs to be implemented in CLL patients is very high.

A better understanding of the molecular mechanisms mediating the migration and survival of tumor cells will allow the development of improved therapies for malignant conditions.

ACKNOWLEDGEMENTS

We thank Antonia Rodríguez, Ana Ramírez and Dr. Mercedes Rey for excellent technical assistance, Dr. Roberto González-Amaro, Dr. José Luis Rodríguez Fernández, Dr. Manuel Gómez and Dr. Sonia Chamorro for critical reading of the manuscript.

Supplementary information is available at The Leukemia's website.

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TITLES AND LEGENDS TO FIGURES

Figure 1. (a) Chemotaxis of CLL cells in response to CCR7 ligands is mediated by PI3K and Rho/ROCK pathways. Chemotaxis of CLL cells induced by CCL19 (solid bars, n=8) and CCL21 (open bars, n=12) was assayed in Transwell chambers, in the presence or not of the indicated chemical inhibitors or pertussis toxin or a blocking anti-CCR7 mAb, as stated in Materials and Methods. **(b) ROCK, PI3K and p38-SAPK regulate the chemotaxis of normal B-cells in response to CCL19 and CCL21.** Chemotaxis assays against CCL19 (solid bars, n=11) and CCL21 (open bars, n=9) of PBMC from healthy donors in the presence or not of the indicated chemical inhibitors or pertussis toxin or a blocking anti-CCR7 mAb. Cells were treated with the inhibitors indicated. In both (a) and (b) data correspond to the arithmetic mean $\pm$ SEM of the percent of cells that migrated in response to each chemokine. The final concentrations of the inhibitors are shown in Table S3. Abbreviations: CK, chemokine; WMN, Wortmannin; PTX, Pertussis toxin; ns, no-significant; *, $p < 0.05$; **, $p < 0.001$.

Figure 2. CCR7-mediated chemotaxis in transient transfected CLL cells with different PI3K and RhoA constructs. Chemotaxis assays towards CCL19 were performed with untransfected (UT) CLL cells or CLL cells transfected with either control (GFP), dominant negative (DN) or constitutively activated (AM) forms of **(a)** PI3K (open bars, n=5) or **(b)** RhoA (solid bars, n=4). Fold induction as defined in Material and Methods is shown. **(c)** Percentage of the transfected CLL cells that migrated without chemotactic signal. Data show the arithmetic mean $\pm$ SEM error bars. Abbreviations: ns, not significant; *, $p < 0.05$; **, $p < 0.001$.

Figure 3. (a) CCR7 stimulation induces the phosphorylation of Akt in CLL. CLL cells were stimulated for 2 minutes with CCL19. In some cases, cells were treated with 250 nM wortmannin (WMN) or 10 μ M LY-294002 prior to stimulation with the chemokine. Cells were lysed and phosphorylated Akt/PKB was assessed with an anti-phospho-Akt Ab. The membrane was stripped and reprobed with total Akt. **(b) Activation of endogenous RhoA in CLL cells in response to CCL19.** CLL cells were treated for 5 minutes with CCL19 and lysed. Active RhoA was evaluated as described in Materials and Methods. The upper panel shows the active fraction of RhoA and the lower panel shows the total amount of the GTPase. **(c) Phosphorylation of MLC is induced by CCR7 stimulation in CLL.** CLL cells were stimulated for 5 minutes with CCL19. When indicated, cells were treated with two ROCK inhibitors (10 μ M Y-27632 and 10 μ M H4413). Upper lane shows phosphorylation of MLC determined with specific anti-phospho-MLC Abs and the lower lane total MLC expression. **(d), (e) and (f) CCR7 ligands induce phosphorylation of ERK1/2, JNK and p38-SAPK in CLL cells.** When indicated, CLL cells were treated with 100 ng/mL PTX, 5 μ M U0126, 20 μ M PD98059, 20 μ M SP600125 or 20 μ M SB203580 prior to stimulation with CCL19 for 2 minutes. Phosphorylated and total ERK1/2, JNK and p38-SAPK were

analyzed by western blot. Fold induction as described in Materials and Methods is shown.

Figure 4. CCR7 ligands increase survival of B-CLL cells. (a) PBMC from 13 CLL patients were incubated at $10^6/\text{mL}$ in medium alone or with CCL19/21 (1 $\mu\text{g}/\text{mL}$), 5 μM U0126, 20 μM SP600125, 20 μM SB203580, 250 nM wortmannin, 10 μM LY-294002, 10 μM Y-27632 and 10 μM H-4413, respectively, for 72 h. (b) and (c) The effect of LY-294002, wortmannin, SP600125 and U0126 treatment on the prosurvival effects of CCL19/21. CLL cells from the same patients were coincubated with the chemokines and the inhibitors indicated for 72h. In all cases, cell survival was measured by annexin V-FITC/7-AAD double staining. The percentage of viable cells, defined as 7-AAD⁻/ annexin V-FITC⁻ cells, is shown, and results are expressed as Median, maximum and minimum, and 75%- and 25%- percentiles plotted by means a box -and- whiskers graph. The p-values (using the non-parametric Wilcoxon-signed rank test) are shown at the top of each box.

Figure 5. Proposed signal pathways and functions elicited by CCR7 and its ligands, CCL19 and CCL21, in CLL. The binding of CCL19/21 to its CKR in CLL cells produces the activation of PI3K and ROCK which play a central role in regulating the chemotaxis of CLL cells in response to CCR7 ligands. A low level of phosphorylated Akt is found in unstimulated cells, which increases after stimulation with CCL19/21 and participates in both the migration and survival of the CLL. The binding of chemokines CCL19/CCL21 to CCR7 increases RhoA-GTP levels which in turn activate its downstream effector ROCK resulting in the activation of MLC, a necessary requisite for an efficient migration; but in contrast to PI3K, ROCK is not implicated in CLL cells survival. The chemokines produce strong a activation of ERK1/2 in CLL cells, but this kinase does not play a main role in the chemotaxis of CLL cells. The CCL19/21-dependent activation of ERK1/2 is related to CLL cells survival and might be related to the proliferation in LNs. p38-SAPK and JNK are constitutively activated in CLL cells and while CCL19/21 moderately increase their phosphorylation, this effect presents donor-to-donor variability. Nevertheless, neither of these two kinases seem to play a crucial role in cell migration. Like ERK1/2, JNK regulates the apoptosis and along with p38-SAPK may be important in proliferation of CLL cells.

FIGURE1

a

b

FIGURE2

FIGURE 3

FIGURE 4

FIGURE 5