ZAP-70 enhances migration of malignant B lymphocytes towards CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation

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Abstract

ZAP-70 in chronic lymphocytic leukemia (CLL) has been associated with enhanced B-cell receptor (BCR) signaling, survival and migration. We investigated whether ZAP-70 can directly govern migration and the underlying mechanisms. In the ZAP-70 stably-transfected Ramos cell line, IgM stimulation but no IgD enhanced phosphorylation of ERK1/2, Akt and Syk, and delayed IgM and CD79b internalization. In contrast, in the Raji cell line, where ZAP-70 was constitutively phosphorylated, ERK1/2 but not Akt was phosphorylated, suggesting that MAPK pathway mediates ZAP-70 effects. BCR stimulation modulated the expression of CCR7, CXCR4, CXCR5, CD44, CD49d and CD62L, which were upregulated in ZAP-70-positive CLL primary subclones. The most dramatic change after BCR engagement in ZAP-70-transfected cells was CCR7 upregulation, this being impaired by ERK1/2 inhibition and translating into both increased signaling and migration towards CCL21. Primary CLL subclones with high ZAP-70 expression showed increased migration towards CCL21. In conclusion, ZAP-70 ectopic expression led to enhanced BCR signaling after IgM stimulation and increased the expression of CCR7 predominantly via ERK1/2, increasing the response and migration towards CCL21. In primary CLL samples, cellular subsets with high ZAP-70 expression had increased expression of adhesion molecules and chemokine receptors in addition to an enhanced ability to migrate towards CCL21.
Introduction

ZAP-70 protein is a 70 kDa member of the Syk family of protein tyrosine kinases that was firstly identified as a crucial element for proximal signaling from the T-cell receptor (TCR).\(^1\) Similar to Syk protein after the B-cell receptor (BCR) stimulation, ZAP-70 is recruited to the phosphorylated immunoreceptor tyrosine activation motifs (ITAMs) of the zeta and CD3 chains present in the TCR, where it subsequently becomes phosphorylated and initiates several signaling cascades.\(^2\) Expression of ZAP-70 protein was considered to be restricted to T lymphocytes and natural killer cells. However, it has also been found expressed in normal B-cell precursors and in some subsets of activated B-cells.\(^3\)-\(^6\) Among B-cell derived malignancies, ZAP-70 is mainly expressed in chronic lymphocytic leukemia (CLL) (37-57\% of cases),\(^7\)-\(^9\) B-acute lymphoblastic leukemia (B-ALL) (56-59\% of cases)\(^4\),\(^10\) and Burkitt lymphoma (8-31\% of cases).\(^11\),\(^12\)

An increased ZAP-70 expression in CLL has been associated with particular adverse biological features, such as the presence of unmutated IgHV genes\(^7\) or high CD38 expression,\(^13\) and correlates with a poor clinical outcome.\(^7\)-\(^9\) In the same vein, ZAP-70 expression in B-ALL correlates with a short survival as well.\(^10\) Despite the fact that ZAP-70 expression in B-cell malignancies has an adverse prognostic influence, its role in the biology of the tumoral B-cell is not fully defined. In this regard, the expression of ZAP-70 protein in CLL cells has been related to an enhanced BCR signaling.\(^13\)-\(^17\) In addition, increased ZAP-70 expression has been associated with increased migration capabilities of CLL cells towards different chemokines such as CXCL12, CCL19 and CCL21,\(^18\)-\(^20\) and with increased signaling and survival upon CXCL12 treatment.\(^18\),\(^21\)

However, whether these increased migrative capabilities are a direct effect of ZAP-70 expression or a mere reflection of the distinct biology features of ZAP-70 expressing cells needs to be further investigated.

In order to ascertain the direct implication of ZAP-70 in B-cell signaling and migration, we analyzed the phenotypic effects of ectopic ZAP-70 expression in a B-cell system and studied the expression of adhesion molecules and chemokine receptors in CLL
primary cells with high or low ZAP-70 expression within the same patient. Herein, we report that IgM but not IgD stimulation mobilizes and activates ZAP-70, which in turn enhances BCR-induced ERK1/2 and Akt phosphorylation and delays IgM and CD79b internalization. Moreover, we show that ZAP-70 induces the expression of the chemokine receptor CCR7 via ERK1/2 activation, thus directly enhancing the capacity of signaling and migration of the ZAP-70 expressing B-cells towards CCL21. Finally, we show that CLL cells with higher ZAP-70 expression within the same patient have a different expression profile of adhesion molecules and chemokine receptors and enhanced migration capacity towards CCL21.

Materials and methods

Cell lines and primary cells

The Burkitt lymphoma B-cell lines Raji and Ramos were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 37°C in a 5% CO₂ atmosphere.

Mononuclear cells from peripheral blood of patients with CLL were obtained by Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, United Kingdom) density gradient from 40 patients after informed consent. This procedure was done following the requirement of the local clinical investigation ethical committee and the principles of the Declaration of Helsinki.

Constructs, transfections and stable cell lines generation

The GFP-ZAP-70 expression vector (pEGFP-N2ZAP-70) was generated by subcloning the full-length human ZAP-70 coding sequence from the SRapuroZAP-70 plasmid (kindly provided by Dr. V. Di Bartolo) into the pGlow-TOPO vector (Invitrogen,
Carlsbad, CA). The ZAP-70 fragment was then subcloned into the EcoRI site at the polylinker of the mammalian expression vector pEGFP-N2 (Clontech, Mountain View, CA), which allowed the expression of a GFP-ZAP-70 fusion protein (97 kDa).

Raji and Ramos cells were stably transfected with plasmids expressing either GFP-ZAP-70 fusion protein or GFP only as a control. For this, cells were resuspended in 200 µl 1X Optimix electroporation buffer (Thermo Hybaid, Milford, MA), electroporated (150 µF/300 V), subsequently selected for the presence of the plasmids in standard growth medium containing 1.2 mg/ml of G418 (Invitrogen), and further sorted by GFP expression.

**Immunoblotting**

B-cell receptor (BCR) was stimulated with 5 µg/ml F(abʼ)2 anti-IgM (Invitrogen) or anti-IgD (Southern Biotech, Birmingham, AL). CCR7 was stimulated using synthetic human CCL21 (Peprotech, London, United Kingdom). ERK1/2 and Akt were inhibited with PD98059 (Cell Signaling Technology Inc., Beverly, MA) and LY294002 (Sigma, St.Louis, MO) respectively. Cell lysates from Ramos and Jurkat cells treated with phosphatase-inhibitor pervanadate (3 mM H2O2/1 mM NaVO4) for 5 minutes at 37°C were used as positive controls for phospho-proteins. Cells were lysed for 30 minutes at 4°C in 100 µl lysis buffer (20 mM Tris pH 7.4, 1 mM EDTA, 140 mM NaCl, 1% NP-40 supplemented with 2 mM sodium vanadate and 1X proteases inhibitor cocktail (Sigma). Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany). Equal amounts of denatured protein were resolved by 10% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hour at room temperature in 5% milk/TBST (Tris-Buffered Saline Tween-20). Membranes were incubated overnight with primary antibodies for phospho-ZAP-70<sup>Tyr319</sup>/Syk<sup>Tyr352</sup>, phospho-Akt<sup>Ser473</sup>, phospho-ERK1/2<sup>Thr202/Tyr204</sup>, Akt and ERK1/2 (Cell Signaling Technology Inc.), ZAP-70 (clone 2F3.2, Upstate Biotechnology, New York, NY), Syk
Flow cytometry

Cell surface antigens were detected using the following fluorochrome-labeled antibodies: IgM-Phycoerythrin (PE), IgD-PE, CD19-Phycoerythrin-Texas Red (ECD), CD5-Phycoerythrin-Cyanine 5.5 (PC5.5), CXCR3-Allophycocyanin (APC), CXCR4-APC, CD44-APC, CD49d-APC, CD62L-APC (BD Biosciences, San Jose, CA, USA), CCR7-APC, CXCR5-APC (R&D Systems, Minneapolis, MN), CD79b-PE and CD3-Phycoerythrin-Cyanine 7 (PE-Cy7) (Beckman Coulter, Brea, CA). For intracellular ZAP-70 detection in primary CLL cells, IntraSure™ kit and primary antibody anti-ZAP-70-PE (BD Biosciences) were used. Cells were acquired in a Navios™ cytometer (Beckman Coulter) and the results were analyzed using the FCS Express 4 software (De Novo Software, Los Angeles, CA).

Confocal microscopy

Cells were seeded at a density of 1.2×10^6 cells/ml in standard growth media on poly-l-lysine-coated glass coverslips for 30 minutes at 37°C and mounted in an Attoflour chamber (Molecular Probes, Invitrogen). The chamber was placed under a Leica TCS SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany) and all images were acquired with a 63X glycerol immersion objective lens. Cells were stimulated with 20 µg/ml F(ab')2 anti-IgM or IgD at 37°C and 5% CO2. Anti-human IgM-PE or IgD-PE were used to detect the receptor on surface. Image treatment was performed using the
Image Processing Leica Confocal and ImageJ Software (Wayne Rasband, Bethesda, MA).

**Calcium flux measurement**

For calcium mobilization measurement cells were washed three times with loading buffer (Hanks Balanced Salt Solution, 10 mM D-Glucose, 1.3 mM CaCl$_2$, 1.1 mM MgCl$_2$) and incubated with 2 µM Fluo-4 AM (Molecular Probes, Eugene, OR) in the presence of 0.02% Pluronic F-127 (Invitrogen, Carlsbad, CA). Afterwards, cells were washed twice with loading buffer, resuspended in RPMI with 25mM HEPES, and kept at room temperature for 15 minutes. 250 ng/ml of CCL21 were added to the samples and the mean fluorescence intensity (MFI) was recorded every 40 seconds for 11 minutes by flow cytometry in a FACS Calibur cytometer.

**Chemotaxis assay**

Chemotaxis assays were performed across bare polycarbonate membranes. Briefly, for cell lines, a total of 100 µl containing 5×10$^5$ cells were added to the top chamber of a 6.5 mm diameter transwell culture insert with a pore size of 5 µm (Corning, New York, NY). Filters were then transferred to wells containing 600 µl of standard growth medium with or without 1 µg/ml CCL21. Cells were allowed to migrate for 15 hours at 37°C in 5% CO$_2$. Transmigrated cells in the lower chamber were resuspended and collected for counting with a FACS Calibur cytometer under a defined flow rate for 5 minutes. The migration index was calculated as the number of cells transmigrating with chemokine divided by the number of transmigrating cells in the absence of chemokine.

Chemotaxis assays of primary CLL cells from 7 patients were performed adding 400 µl of RPMI-0.5%BSA containing 1.5×10$^7$ cells to the top chamber of a 24 mm diameter transwell culture insert. Cells were allowed to migrate towards media containing 1 µg/ml CCL21 for 6 hours and the percentage of CLL cells expressing ZAP-70 was then determined in both upper and lower chambers by flow cytometry.
**Statistical analysis**

Results are shown as mean ± standard error of the mean (SEM) of at least 3 replicates. For statistical comparisons between groups, the Mann-Whitney test was used and a p level inferior to 0.05 was considered significant. Paired-sample parametric test (t-test) was used to compare the differential expression of molecules between the ZAP-70 high and low CLL subpopulations within the same patient. Analyses were performed using the biostatistics software package SPSS v.17 (IBM, Somers, NY). Results were graphed with GraphPad Prism Ver.5.0. (La Jolla, CA).

**Results**

**Intensity and duration of Akt and ERK1/2 phosphorylation is enhanced in ZAP-70 expressing B-cells after IgM but not IgD stimulation**

In order to investigate the phenotypic effects of ZAP-70 expression in B-cells, we stably transfected the Burkitt cell lines Ramos and Raji with the pEGFP-N2ZAP-70 vector or the control vector pEGFP-N2. The expression of GFP protein could be detected by flow cytometry in all stable transformants whereas ZAP-70 expression was only detected in Ramos and Raji stably transfected with the GFP-ZAP-70 expression vector. Proper expression of the 97 kDa fusion protein was verified by western blot using anti-ZAP-70 antibody in both ZAP-70 transfected B-cell lines (Supplemental Figure S1).

Despite the fact that most CLL cells co-express surface IgM and IgD,22 there are several evidences that BCR stimulation can lead to different signaling depending on whether IgM or IgD are stimulated.23 Remarkably, almost all CLL cases respond to IgD ligation, whereas usually only CLL cells expressing unmutated IgHV genes respond to IgM.22;24-26 In this regard, the expression of ZAP-70 protein in CLL has been related to an enhanced signaling through the BCR upon IgM stimulation.13-17 However, the role of ZAP-70 in IgD-BCR signaling has not been investigated. Therefore, we analyzed the...
effects on BCR signaling of surface IgM and IgD stimulation according to the presence of ZAP-70. Firstly, Ramos cells were stimulated with 5 μg/ml F(ab’)2 anti-IgM for 5 minutes and 24 hours and afterwards the phosphorylation of key mediators of BCR signaling was examined by western blot. In ZAP-70 stable transfectants we found phosphorylation of ZAP-70 at activating tyrosine Tyr319 along with an increased intensity and duration of the BCR-induced phosphorylation of Syk, Akt and ERK1/2 kinases (Figure 1A). The selected Raji cell line lacked surface IgM and IgD and constitutively expressed phosphorylated ZAP-70 at Tyr319 after transfection. Remarkably, in this cell line we observed that ERK1/2 protein but no Akt protein was also constitutively phosphorylated (Figure 1B). Altogether, these results confirm the participation of ZAP-70 in the IgM-BCR signaling, they indicate that ZAP-70 is able to activate the MAPK pathway independently of BCR stimulation, and suggest that Akt activation may require BCR stimulation.

To elucidate whether ZAP-70 is also involved in IgD-BCR signaling, Ramos transfectants were stimulated with 5 μg/ml goat F(ab’)2 anti-IgD for 5 minutes. In contrast to what we observed after IgM stimulation, IgD did not induce phosphorylation of ZAP-70 or ERK1/2. However, IgD stimulation induced Akt phosphorylation which was not enhanced by the presence of ZAP-70 (Figure 1C). Further differences were observed by confocal microscopy after IgM or IgD stimulation of ZAP-70 expressing Ramos cells. After 5 minutes of F(ab’)2 anti-IgM stimulation we observed mobilization of ZAP-70 from the cytoplasm to the membrane, where it was distributed forming patches (Figure 1D, Supplemental Figure S2, Supplemental Video 1, 2 and 3). Remarkably, 40 minutes later, when the internalization of IgM was almost complete, ZAP-70 still remained associated to the membrane. In contrast, and in accordance with the previous results, BCR stimulation by IgD did not affect the intracellular localization of ZAP-70, thus confirming the lack of ZAP-70 activation upon IgD stimulation (Figure 1D). Taken together, these results indicate that the differences between IgD and IgM
signaling described in CLL cells could be explained in part by the fact that ZAP-70 has no influence in IgD signaling.

**ZAP-70 delays CD79b and IgM internalization after BCR stimulation**

The stimulation of the BCR is followed by the activation of several signaling cascades and by a fast internalization of the complex, so that the antigen can be processed and presented to T cells. Furthermore, under some conditions of antigen binding, the immunoglobulin is physically uncoupled from the signaling complex formed by CD79a/b molecules, being these signaling complexes preferentially retained in the membrane. The fact that ZAP-70 binds to CD79a/b phosphorylated ITAMS, along with our observation that ZAP-70 remained associated to the membrane long after IgM was internalized, prompted us to study the internalization kinetics of surface immunoglobulin and of CD79b upon BCR stimulation. For this, we analyzed by flow cytometry the expression of IgM and CD79b on surface at 10 and 60 minutes after BCR stimulation in Ramos stable transfectants. After 60 minutes of BCR stimulation, Ramos GFP internalized 53% ± 1.9 of surface IgM and 29.4% ± 2.9 of CD79b, whereas in Ramos GFP-ZAP-70 cells the internalization was significantly delayed, with a 25.7% ± 0.7 of IgM and 4.7% ± 1.5 of CD79b being removed from surface (Figure 2A). These phenomena were further confirmed by confocal microscopy, where after F(ab′)2 anti-IgM stimulation at 10, 30 and 60 minutes we observed a stronger surface IgM staining in the Ramos GFP-ZAP-70 cells, indicating that the internalization of the IgM was slower when ZAP-70 was present (Figure 2B). Overall, these results show that after IgM engagement the BCR complex is uncoupled and that the different components are internalized at different rates. In the presence of ZAP-70, the internalization of both IgM and CD79b is significantly delayed.

**ZAP-70 signaling induces the expression of CCR7 in B-cells through ERK1/2 phosphorylation**
Along with enhanced BCR signaling, the expression of ZAP-70 in primary CLL cells has been correlated with an increased response to survival and migratory signals in vitro.\textsuperscript{18-21} Moreover, it has been previously described that patients with CLL with high ZAP-70 expression (>20%) have an overall increase in expression of CCR7.\textsuperscript{18} Despite that, the mechanisms by which ZAP-70 is controlling the migration processes are not well defined. In order to analyze the influence of ZAP-70 in the expression of chemokine receptors and adhesion molecules, we studied CCR7, CXCR3, CXCR4, CXCR5, CD44, CD49d and CD62L surface levels by flow cytometry after ZAP-70 activation by BCR stimulation. The stimulation of the BCR in Ramos GFP cells significantly increased the levels of CCR7, CXCR4, CXCR5 and CD62L whereas we observed a modest downregulation of CD44 and CD49d and no change of CXCR3 after 16 hours (Figure 3A and Supplemental Figure S3). Moreover, BCR stimulation of Ramos GFP-ZAP-70 cells increased the expression of CCR7, CXCR5, CD44, CD49d and CD62L and decreased the expression of CXCR4, whereas CXCR3 expression remained unmodified (Figure 3A and Supplemental Figure S3). Interestingly, BCR stimulation of Ramos GFP cells increased CCR7 expression in 1.5 fold (MFIR from 2.13 ± 0.12 to 3.3 ± 0.04; \(p=0.02\)), whilst in Ramos GFP-ZAP-70 cells BCR stimulation enhanced the CCR7 expression in 8 fold (MFIR from 2.17 ± 0.005 to 17.5 ± 0.9; \(p=0.028\)) being this molecule the one with the greatest difference caused by ZAP-70 (Figure 3A, right panel). In the Raji cell line, the ectopic expression and activation of ZAP-70 also translated into an increase in CCR7 (MFIR from 1.8 ± 0.09 to 3.1 ± 0.19; \(p=0.0022\)), CD49d and CD62L expression (Figure 3B) and in a decrease in CXCR5 expression (MFIR from 76.3 ± 3.36 to 66.3 ± 1.79; \(p=0.028\)), while its effect on CXCR3, CXCR4, and CD44 expression was not significant. Of note, the stimulation of the BCR through IgD engagement in Ramos cells did not modulate the levels of CCR7 regardless of ZAP-70 expression, adding more evidences to the differences observed between IgM and IgD signaling (data not shown).
In order to determine whether Akt or ERK1/2 pathways were involved in the induction of CCR7 expression upon BCR stimulation and ZAP-70 activation, Ramos stable cell lines were stimulated with 5 μg/ml F(ab')2 anti-IgM after one hour of pre-incubation with either the ERK1/2 inhibitor PD98059 or the Akt inhibitor LY294002 (Figure 4A). As previously observed, BCR stimulation of Ramos GFP-ZAP-70 cells increased the levels of CCR7 expression to MFIR 6.37 ± 0.28 (Figure 4B). Interestingly, after ERK1/2 inhibition by PD98059 the CCR7 upregulation was significantly impaired, the levels raising only up to MFIR 2.5 ± 0.01 at 100 μM for Ramos GFP-ZAP-70 (p=0.002). The inhibition of Akt, although significant, had a biologically negligible effect on the expression of CCR7 (MFIR 5.5 ± 0.17 after LY294002 at 10 μM; p=0.026) (Figure 4B, right panel). The same pattern of inhibition was observed in Ramos GFP cells even though the increase of CCR7 after IgM stimulation was only to MFIR 1.47 ± 0.08 (Figure 4B, left panel). Taken together, these results show that the MAPK pathway has a prominent role in regulating the overexpression of CCR7 induced by IgM-activated ZAP-70.

**Differential expression of adhesion molecules and chemokine receptors by ZAP-70-high and ZAP-70-low cells within CLL primary clones**

As we observed that ZAP-70 can directly govern the expression of some chemokine receptors and adhesion molecules, we decided to depict the differential expression of these molecules in CLL cells expressing high and low ZAP-70 within the same patient. For this, we compared by flow cytometry the expression of CCR7, CXCR4, CXCR5, CD44, CD49d and CD62L in ZAP-70-high vs. ZAP-70-low CD19+/CD5+ gated cells from 40 CLL primary cases. The expression of ZAP-70 observed in the CD3+ T cells from each sample was used to define the threshold of high vs. low ZAP-70 and then the MFI of each corresponding cellular subset was obtained for each molecule (Figure 3C). Interestingly, we found that the expression of all the adhesion molecules and
chemokine receptors analyzed was significantly higher in the subset of CLL cells with higher expression of ZAP-70 (Figure 3D).

**CCR7 signaling is increased in ZAP-70 expressing B-cell lines**

CCR7 ligands (chemokines CCL21 and CCL19) are mainly expressed in high endothelial venules and the T zones from secondary lymphoid organs. The interaction of CCR7 with its ligands leads to the mobilization of intracellular calcium and activation of the Akt and MAPKs pathways. To elucidate whether the overexpressed CCR7 in ZAP-70 B-cells is functional, we stimulated cells with 1 μg/ml CCL21 for 30 minutes after 4 hours of stimulation with F(ab')2 anti-IgM (Figure 5A). In Ramos cells with BCR-activated ZAP-70, and therefore increased CCR7 expression, we observed an enhanced phosphorylation of Akt and ERK1/2 upon CCL21 stimulation compared to cells with BCR activation only. Moreover, the stimulation of CCR7 after BCR stimulation in Ramos GFP-ZAP-70 cells also induced higher phosphorylation of Akt and ERK1/2 compared to Ramos GFP cells. Besides, a slight increase in Akt phosphorylation was also observed after CCL21 stimulation in Ramos expressing ZAP-70 before BCR stimulation, this suggesting that ZAP-70 could be directly participating in the CCR7 signaling pathway. Moreover, the increase in intracellular calcium concentration was also higher in B-cells with activated ZAP-70 (Figure 5B). This enhanced signaling was also observed in the Raji GFP-ZAP-70 cell line, where phosphorylation of both Akt and ERK1/2 was greatly enhanced after CCR7 stimulation by CCL21 compared to Raji GFP cells (Figure 5A).

**Migration towards CCL21 chemokine is increased in B-cells expressing activated ZAP-70**

CCR7 ligation can mediate migration and survival of tumoral cells from CLL and B-ALL. Importantly, the upregulation of CCR7 alone may not be enough for CCR7-mediated migration; in this regard, it has been shown that in monocyte-derived
dendritic cells and B-ALL additional stimuli are needed after CCR7 upregulation for responsiveness to CLL19 or CCL21.\textsuperscript{37,38} Moreover, the levels of chemokine receptors in CLL do not always correlate with the migration towards their ligands.\textsuperscript{20,36,39} We therefore aimed to analyze if the increased CCR7 expression and signaling observed after ZAP-70 activation did translate into an increased migrative capacity of the B-lymphocytes. For this, we performed transmigration assays across bare polycarbonate transwell culture inserts placed on medium with or without CCL21. Before BCR stimulus we did not find significant differences caused by ZAP-70 expression in the average proportion of transmigrated Ramos cells towards CCL21 chemokine (migration index: 1.09 ± 0.15 vs. 1.09 ± 0.29; p=0.93). Moreover, BCR stimulation of Ramos GFP cells, although inducing a significant increase in CCR7 expression (Figure 3A), did not increase the migration towards CCL21 (1.09 ± 0.15 vs. 1.07 ± 0.12). In contrast, after BCR stimulation, the presence of ZAP-70 increased 2.9 times the migration index (1.07 ± 0.12 vs. 3.15 ± 0.82; p=0.016) (Figure 6A). In accordance, the migration index of Raji GFP-ZAP-70 cells was 3.2 times higher than the one observed for Raji GFP (1.9 ± 0.3 vs. 6.08 ± 2.13; p= 0.046) (Figure 6B). These results showed that, in this cell system, induction of CCR7 expression by ZAP-70 activation is a sufficient condition to increase the B-cell migrative capacity.

Primary CLL cells with high ZAP-70 expression have enhanced migrative capacity towards CCL21 compared to CLL cells with low ZAP-70 expression from the same patient.

Peripheral blood mononuclear cells from patients with CLL and high ZAP-70 expression (≥20%) have showed increased migration capacity towards CCL19 and CCL21\textsuperscript{18}. In this sense, we found that primary CLL cells with higher expression of ZAP-70 within the same patient had an increased expression of adhesion molecules and chemokine receptors. Therefore we investigated whether CLL subclones with higher ZAP-70 expression had also increased migratory potential. For this, we performed
chemotaxis assays towards CCL21 using peripheral blood mononuclear cells from 7 patients and measured the expression of ZAP-70 of the transmigrated cells. Of note, after 6 hours of migration we observed that, for all the cases analyzed, the percentage of ZAP-70-positive cells was significantly higher in the cells that had migrated compared to the cells present in the upper chamber indicating that ZAP-70-positive CLL cells have an enhanced ability to respond to and to migrate towards CCL21 (Figure 6C).

Discussion

The expression of ZAP-70 protein and its correlation with adverse prognosis in some subsets of B-lymphocyte derived malignancies prompted the analysis of its function in a B-lymphocyte setting. ZAP-70 and Syk proteins belong to the same protein family and have similar function in T and B-lymphocytes, respectively. Therefore, several parallelisms in their role in BCR signaling have been found, being ZAP-70 involved in enhancing the IgM-BCR signaling, thus mimicking the role of Syk. Moreover, in primary CLL cells other biological features have been correlated with ZAP-70 expression, such as increased response to migrative and survival stimuli. However, it has not been elucidated whether these features are a reflection of the distinct biological characteristics of cells expressing ZAP-70 or they are directly governed by ZAP-70. Therefore, we ascertained the effects on BCR signaling and migration induced by the ectopic expression of ZAP-70.

In our report, we showed that ZAP-70 enhances the signaling through IgM-BCR, increasing and prolonging Akt and ERK1/2 phosphorylation. In contrast, in a B-cell line lacking surface immunoglobulin where ectopic ZAP-70 became constitutively phosphorylated, we found that ERK1/2 but no Akt was also activated, suggesting that ZAP-70 would downstream signal through the MAPK pathway independently of BCR stimulation. Even though the majority of CLL cases co-express surface IgM and IgD, there is no information on the role of ZAP-70 in IgD BCR signaling. In CLL, BCR
signaling mediated by engagement of IgM has been found to be different from that observed after IgD stimulation: whereas only CLL cases with unmutated IgHV genes usually respond to IgM stimulation,\textsuperscript{22,24,25} virtually all cases respond to IgD;\textsuperscript{22,24,26} moreover, the response triggered by IgD or IgM ligation in the same sample can be qualitatively different.\textsuperscript{23} ZAP-70 enhances IgM signaling in B-cells, as previously described\textsuperscript{13-17} and further confirmed in this report. Intriguingly, however, in our cell system, ZAP-70 did not participate in IgD signaling. Upon IgD stimulation, ZAP-70 did not become phosphorylated, did not translocate from the cytoplasm to the cellular membrane, and did not enhance Akt phosphorylation. In addition, as opposed to signaling after IgM stimulation, IgD stimulation did not induce the phosphorylation of ERK1/2 protein. Altogether, these results show that ZAP-70 is not participating in IgD mediated signaling, this probably being responsible for some of the differences described between IgM and IgD signaling in CLL cells.

In B-lymphocytes, ZAP-70 protein binds to the signaling molecules of the BCR, namely CD79a and CD79b,\textsuperscript{13} and delays BCR internalization after stimulation.\textsuperscript{15} Herein, we observed that IgM and CD79b had different internalization kinetics, indicating that in our system BCR components are uncoupled and independently removed from the surface after BCR stimulation. Interestingly, both components of the BCR, particularly CD79b, were retained longer in the membrane of ZAP-70 expressing cells compared to BCR stimulated cells without ZAP-70 expression. These results show that CD79b-bound ZAP-70 is upholding longer the presence of both components of the BCR (CD79b and IgM) in the membrane, thus allowing for a sustained activation of the cells that express ZAP-70.

Several signals from the microenvironment of lymph node and bone marrow, mainly regulated by chemokines, can influence the accumulation and survival of B-lymphocytes, including B-ALL and CLL cells.\textsuperscript{33,34,40-43} Of note, in B lymphocytes the activation of the BCR can lead to a modulation in the expression of different chemokine receptors.\textsuperscript{20,25,44,45} In our B-cells without ZAP-70 we found that the stimulation of the
BCR caused variable modifications in the surface expression levels of several chemokine receptors and adhesions molecules, including the increase in CCR7 expression. The increase in the expression of CCR7 was regulated by the activation of the MAPK pathway, as it was shown by the impairment of CCR7 upregulation after ERK1/2 pharmacological inhibition. This inhibition did not completely abrogate CCR7 upregulation, indicating that other molecules are also involved in CCR7 regulation. In addition, Raji GFP-ZAP-70 cells, with constitutive ZAP-70 activation, also had a significantly higher level of CCR7 expression. Interestingly, these cells also had phosphorylated ERK1/2 but not phosphorylated Akt, thus supporting again that MAPK pathway is playing a role in the ZAP-70-dependent upregulation of CCR7 receptor. It has been described that CCR7 is variably expressed in B-ALL and overexpressed in CLL, where it has been associated with adverse prognostic characteristics such as clinical lymphadenopathy and increased ZAP-70 expression. However, it has not been depicted whether CLL cells expressing ZAP-70 are those with a high expression of CCR7. In our paper we found higher CCR7 expression in CLL cells with high ZAP-70 expression within the same patient sample, this indicating that ZAP-70 could be responsible for the increased expression of CCR7 observed in CLL patients with increased ZAP-70 expression as we observed in our cellular system. Moreover, CLL cells with high ZAP-70 had also significantly increased expression of additional adhesion molecules and chemokine receptors that had been found implicated in the interaction of CLL cells with their microenvironment, regarded as having a prominent role in CLL pathogenesis and progression.

CCR7 ligands, chemokines CCL19 and CCL21, are expressed in secondary lymphoid organs and can increase the migrative capacity and survival of CCR7 positive cells, including primary cells from patients with B-ALL and CLL. In this regard, we found that CCR7 signaling after CCL21 ligation was greatly increased in transfected cells with activated ZAP-70, probably due to the fact that these cells harbor higher levels of CCR7 on surface. However, we cannot exclude a direct participation of ZAP-70 protein.
in CCR7 signal transduction since we observed enhanced Akt phosphorylation upon CCL21 binding without BCR-mediated ZAP-70 activation and thus, with no upregulation of the CCR7 levels. In fact, ZAP-70 and Syk proteins have been implicated in BCR-independent signaling through CXCR4 and CXCR3,\textsuperscript{48-50} chemokine receptors with similar molecular structure to CCR7.

Despite the fact that ligation of chemokines to their receptors can induce survival and migration, additional stimuli may be needed to induce cells to migrate, as it has been reported for macrophage-derived dendritic cells and B-ALL cells.\textsuperscript{37,38} In addition, levels of chemokine receptor do not always correlate with migrative capacity towards ligands, as it has been shown for CXCR4 and CCR7 in CLL.\textsuperscript{20,36,39} Remarkably, in our experiments we did find a significantly higher migration towards CCL21 of B-cells expressing activated-ZAP-70. Therefore, upregulated CCR7 expression in ZAP-70 positive cells would induce them to migrate towards a more favorable microenvironment, like lymph nodes or secondary lymphoid organs, where they would receive additional survival and proliferative signals. In fact, we did observe a significant enrichment of ZAP-70 positive cells in primary CLL cells migrating towards CCL21 in an in vitro chemotaxis system.

In summary, this study described the participation of ZAP-70 in IgM-BCR signaling and the lack of involvement of ZAP-70 in IgD mediated signaling. Moreover, we showed that ZAP-70 was responsible for an ERK1/2-dependent upregulation of the chemokine receptor CCR7, this leading to an enhanced signaling and migration upon CCR7 ligation with CCL21. ZAP-70 was also involved in the regulation of CXCR4, CXCR5, CD44, CD49d and CD62L, molecules that had significantly higher levels in CLL primary subclones with high ZAP-70 expression; moreover, this primary CLL subclones with high ZAP-70 expression had enhanced migration capacity towards CCL21. These results are extending the knowledge of ZAP-70 function in B-cells and are contributing to explain the adverse clinical outcome of B lymphoproliferative disorders with increased ZAP-70 expression.
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Authorship

E.C. designed the research, performed experiments, analyzed data, designed figures and wrote the paper. C.C. designed the research and supervised the study. C. de C. performed experiments and analyzed data. M.J.B., P.A., and N.P. analyzed data. F.B. and M.C. designed the research, supervised the study, analyzed data and revised the manuscript.

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Figures

Figure 1. ZAP-70 enhances IgM-BCR signaling but does not participate in IgD-BCR signaling. (A) Ramos stable transfectants were stimulated with 5 µg/ml F(ab’)_2 anti-IgM for 5 minutes and 24 hours. Enhanced Syk, Akt and ERK1/2 phosphorylation was observed in IgM-BCR stimulated ZAP-70-expressing cells. (B) Immunoblotting analysis of Raji transfectants expressing constitutive phosphorylated ZAP-70 and ERK1/2. Ramos and Jurkat cells treated with pervanadate (PV) were used as positive controls. (C) Ramos stable transfectants were stimulated with 5 µg/ml F(ab’)_2 anti-IgD for 5 minutes. Upon IgD stimulation, activation of Akt but not of ERK1/2 or ZAP-70 was observed. Jurkat cells treated with pervanadate (PV) were used as positive control. (D) Confocal microscopy (magnification, 63X). Ramos GFP-ZAP-70 cells were activated with 20 µg/ml F(ab’)_2 anti-IgM or IgD for 40 minutes and then stained with anti-IgM-PE or anti-IgD-PE. ZAP-70 was translocated from the cytoplasm to the membrane and remained on surface after IgM activation while IgM internalization was almost complete (upper panel). In contrast, while IgD was also internalized, no mobilization of ZAP-70 was observed after IgD stimulation (lower panel). The scale bar in the image represents 5 µm.

Figure 2. ZAP-70 activation delays IgM and CD79b internalization. (A) Stimulation of Ramos transfectants was performed with 5 µg/ml F(ab’)_2 anti-IgM for 10 and 60 minutes at 37°C and stopped at 4°C. Density of IgM and CD79b on surface was analyzed by flow cytometry at the indicated time points with IgM-PE and CD79b-PE antibodies (upper panel). The internalization kinetics of IgM differed from that of CD79b. The expression of ZAP-70 delayed the internalization of both IgM and CD79b (lower panel). (B) Confocal microscopy (magnification, 63X). Ramos transfectants were stimulated with 5 µg/ml F(ab’)_2 anti-IgM and sequential images at 0, 10, 30 and 60 minutes after IgM-PE labeling were obtained. Density of surface IgM in Ramos GFP-
ZAP-70 cells was higher than in Ramos GFP cells. The scale bar in the image represents 5 µm.

**Figure 3. ZAP-70 enhances CCR7 upregulation after BCR activation**

(A) Expression of CCR7, CXCR3, CXCR4, CXCR5, CD44, CD49d and CD62L was assessed by flow cytometry in Ramos transfectants before and after stimulation for 16 hours with 5 µg/ml F(ab')2 anti-IgM. Left panel represents the MFIR (± SEM) of expression before and after IgM stimulation. The right panel is depicting the change of expression relative to unstimulated cells in Ramos GFP and Ramos GFP-ZAP-70. Asterisks indicate p<0.05. (B) Expression of CCR7, CXCR3, CXCR4, CXCR5, CD44, CD49d and CD62L was assessed by flow cytometry in Raji cells with or without ZAP-70 expression. (C) ZAP-70, CCR7, CXCR4, CXCR5, CD44, CD49d and CD62L expression were assessed by flow cytometry in peripheral blood mononuclear cells obtained from 40 patients with CLL. The expression of ZAP-70 in CD3+ gated T cells was used as internal control and allowed for the measurement of expression of chemokine receptor and adhesion molecules in CD19+/CD5+ gated CLL cells with high (black) or low (grey) ZAP-70 expression. (D) Expression of CCR7, CXCR4, CXCR5, CD44, CD49d and CD62L was significantly higher in CLL cells with high ZAP-70 expression within the same patient.

**Figure 4. CCR7 upregulation is dependent on ERK1/2 activation.**

(A) Ramos GFP (left panel) and Ramos GFP-ZAP-70 (right panel) cells were incubated for 1 hour in the presence of 50 µM or 100 µM PD98059 for ERK1/2 inhibition, and in the presence of 5 µM or 10 µM LY294002 for Akt inhibition prior to 5 minutes stimulation with 5 µg/ml F(ab')2 anti-IgM. Inhibition of phosphorylation of Akt and ERK1/2 was confirmed by immunoblotting. Jurkat cells treated with pervanadate (PV) were used as positive control. (B) IgM stimulation was performed for 4 hours after one hour of pre-incubation with Akt and ERK1/2 inhibitors and the levels of CCR7 were measured by flow
cytometry. Inhibition of ERK1/2 phosphorylation with PD98059 significantly reduced the IgM-mediated induction of CCR7 expression, while inhibition of Akt had only a minor effect on CCR7 expression. Asterisks indicate a p<0.05 respect to the IgM activated cells and MFIR was calculated relative to unstimulated samples.

**Figure 5.** CCR7 signaling is enhanced in B-cells expressing activated ZAP-70. (A) Ramos transfectants pre-stimulated for 4 hours with 5 µg/ml F(ab')\(_2\) anti-IgM were stimulated with 1 µg/ml CCL21 for 30 minutes (left panel). Only in IgM stimulated Ramos ZAP-70 transfectants, CCL21 estimation increased the phosphorylation of ERK1/2. CCL21 increased Akt phosphorylation with or without the presence of IgM stimulation. Akt phosphorylation was the highest when ZAP-70 was activated. A slight increase in Akt phosphorylation was observed in ZAP-70 expressing cells before IgM stimulation. In Raji transfectants treated with 1 µg/ml CCL21 for 5 and 30 minutes (right panel) the same enhanced signaling was observed. (B) Ramos transfectants pre-stimulated with 5 µg/ml F(ab')\(_2\) anti-IgM for 4 hours were stimulated with 250 ng/ml CCL21. MFIR was calculated relative to unstimulated samples. Significant increase in calcium mobilization was observed in BCR-stimulated ZAP-70 positive cells. Asterisks indicate p<0.05.

**Figure 6.** Migration towards CCL21 is enhanced after ZAP-70 activation. (A) Ramos transfectants either stimulated (6 hours, 5 µg/ml F(ab')\(_2\) anti-IgM) or unstimulated, and (B) Raji transfectants were subjected to migration assay towards CCL21 (1 µg/ml) for 15 hours at 37°C in 5% CO\(_2\) atmosphere. The absolute number of transmigrated cells was determined by flow cytometry, acquiring cells under a defined flow rate. Results are expressed as migration index, calculated as the number of cells transmigrating with chemokine divided by the number of transmigrating cells towards media only. ZAP-70-activated cells showed a significantly higher migrative capacity.
Asterisks indicate p<0.05. (C) Peripheral blood mononuclear cells from 7 patients with CLL were subjected to migration assay towards CCL21 (1 µg/ml) for 6 hours at 37°C in 5% CO₂ atmosphere. The percentage of CD19+/CD5+ CLL cells expressing ZAP-70 was determined in the cellular fraction remaining in the upper chamber and in the cellular fraction of transmigrated cells for each patient sample by flow cytometry. CLL cells transmigrating towards CCL21 after 6 hours had a significantly higher percentage of ZAP-70 positive cells (p=0.018).
Figure 1

A

5 min

24h

IgM

pZAP-70^{536}
pZAP-70^{519}

pSyk^{535}
pSyk^{519}

ZAP-70

ZAP-70

Syk*

pAkt^{5173}

Akt

pERK1/2

ERK1/2

GAPDH

* The control used for Syk protein was Ramos

B

Jurkat PV

Raji GFP

Raji GFP-ZAP-70

pZAP-70^{536}
pZAP-70^{519}

pSyk^{535}
pSyk^{519}

ZAP-70

ZAP-70

Syk

pAkt^{5173}

Akt

pERK1/2

ERK1/2

GAPDH

C

Jurkat PV

- + - +

ZAP-70

- - + +

IgD (5 min)

D

ZAP-70

IgM

Merge

Ramos GFP-ZAP-70 Control

Ramos GFP-ZAP-70 + IgM

Ramos GFP-ZAP-70 Control

Ramos GFP-ZAP-70 + IgD

Ramos GFP-ZAP-70 Control

Ramos GFP-ZAP-70 + IgD
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(A) Migration Index (+/− CCL21) for Ramos GFP and Ramos GFP-ZAP-70 with IgM.

(B) Migration Index (+/− CCL21) for Raji GFP and Raji GFP-ZAP-70.

(C) ZAP-70 expression (%) in Upper chamber and Lower chamber.

p = 0.018
ZAP-70 enhances migration of malignant B lymphocytes towards CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation

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