CCL19 and CXCL12 Trigger in Vitro Chemotaxis of Human Mantle Cell Lymphoma B Cells

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ABSTRACT

Purpose: Few data are available in the literature on chemokine receptor expression and migratory capability of mantle cell lymphoma (MCL) B cells. Information on these issues may allow us to identify novel mechanisms of chemokine-driven tumor cell migration.

Experimental Design: The research was designed to investigate: (a) expression of CCR1 to CCR7 and CXCR1 to CXCR5 chemokine receptors; and (b) chemotaxis to the respective ligands in MCL B cells and in their normal counterparts, i.e., CD5⁺ B cells.

Results: Malignant B cells from MCL patients and normal counterparts displayed similar chemokine receptor profiles. MCL B cells were induced to migrate by CXCL12 and CCL19, whereas normal CD5⁺ B cells migrated to the former, but not the latter chemokine. Overnight culture of MCL B cells and their normal counterparts with CXCL12 cross-sensitized other chemokine receptors to their ligands in some tumor samples but not in CD5⁺ B cells.

Conclusions: CCR7 and CXCR4 ligands may play a key role in tumor cell migration and spreading in vitro. CXCL12 may additionally contribute by sensitizing MCL B cells to respond to the ligands of other chemokine receptors.

INTRODUCTION

Mantle cell lymphoma (MCL) is a B cell tumor that, in the majority of cases, originates from the clonal expansion of a naive CD5⁺ B cell localized in the mantle zone of secondary lymphoid follicles (1, 2). MCL B cells express monoclonal surface immunoglobulin usually belonging to IgM and IgD isotypes (3, 4), as well as CD5, CD20, CD79a, and CD79b (1–4), but not CD10 or CD23 (5). MCL is associated with the t(11;14)(q13;q32) which results in the positioning of the bcl-1 gene (11q13) near the immunoglobulin heavy-chain gene locus (14q32). This translocation causes bcl-1 up-regulation and cyclin D1 overexpression (6, 7). On clinical grounds, generalized lymphadenopathy and bone marrow infiltration are the most common manifestations (1, 2, 8–10). Splenomegaly is detected in 30–60% of the patients and peripheral blood lymphocytosis in 10–69% of cases (1, 2, 8–10); gastrointestinal (1, 2, 8, 9, 11) and central nervous system (1, 7–10, 12) involvement have been also reported.

Chemokines are low molecular weight cytokines, specialized in the mobilization of leukocytes and other cell types (13–16). Recent studies have shown that neoplastic cells of hematopoietic and nonhematopoietic origin express various chemokine receptors, and overexpression of some of these has been related to tumor progression and metastasis (17–19). As for B-cell derived lymphoproliferative disorders, CXC chemokine receptor (CXCR) 4 and CXCR5 have been detected in neoplastic B cells from B-cell acute lymphoblastic leukemia (B-ALL; Ref. 20–23), B-cell chronic lymphocytic leukemia (B-CLL; Ref. 20, 24, 25), follicular lymphoma (FL; Ref. 26), and hairy cell leukemia (HCL; Ref. 21). CXCR3 has been found to be expressed in B cells from B-CLL (20, 27), marginal zone lymphoma (20), and a fraction of HCL and B-cell acute lymphoblastic leukemia cases (20). CC chemokine receptor (CCR) 7 has been detected in B-CLL (28) and in tumor cells from classical Hodgkin’s disease with lymphocyte predominance (29). Finally, CCR5 has been detected in hairy cell leukemia cells and on the surface of B-CLL cells (21). In terms of functional in vitro activity, only a few studies have addressed chemokine-driven locomotion of neoplastic B cells. CXCL12, by interacting with its specific receptor (i.e., CXCR4), has been shown to enhance chemotaxis of B-ALL (22, 23), B-CLL (24, 25), and FL (26). The CCR7 ligands, CCL19 and CCL21, enhanced the chemotaxis of B-CLL and Hodgkin’s disease cells to CCL19 and CCL21 (28), whereas CXCR3 mediated the chemotactic response of B-CLL cells to CXCL10 (27).

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in the chemotaxis of MCL B cells, we have investigated here the expression of a panel of CXC (CXCR1 to CXCR5) and CC (CCR1 to CCR7) chemokine receptors in MCL B cells and in CD5+ B lymphocytes, and tested their in vitro migration to the respective ligands.

**PATIENTS AND METHODS**

**MCL Samples.** The study was approved by an Institutional Review Board. Diagnosis of MCL was established according to the criteria of the Revised European-American Classification of Lymphoid Neoplasms classification (30). Mononuclear cells (MNCs) were isolated on Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradients from peripheral blood (7 cases), lymph node fragments (2 cases), and one pleural effusion (1 case). Lymph node MNC were cryopreserved in a freezing solution composed of 50% RPMI 1640 (Sigma), 40% fetal bovine serum, and 10% DMSO (Sigma). Cells were kept in liquid nitrogen until tested.

Table 1 summarizes the main clinical and laboratory features of mantle cell lymphoma patients.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Sample</th>
<th>WBC/mm^3</th>
<th>Histological variant</th>
<th>Diagnostic assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>F</td>
<td>Lymph node</td>
<td>4.5 × 10^3</td>
<td>Typical</td>
<td>PCR^a</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>F</td>
<td>Lymph node</td>
<td>4.0 × 10^3</td>
<td>Typical</td>
<td>PCR</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>Blood</td>
<td>88 × 10^3</td>
<td>Blastoid</td>
<td>Immunohistochemistry^b</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>M</td>
<td>Blood</td>
<td>270 × 10^3</td>
<td>Typical</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>M</td>
<td>Blood</td>
<td>96 × 10^3</td>
<td>Typical</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>F</td>
<td>Blood</td>
<td>28 × 10^3</td>
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<td>Immunohistochemistry</td>
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<td>7</td>
<td>49</td>
<td>F</td>
<td>Blood</td>
<td>10.2 × 10^3</td>
<td>Typical</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>M</td>
<td>Blood</td>
<td>15.7 × 10^3</td>
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<td>FISH</td>
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<td>F</td>
<td>Blood</td>
<td>45.5 × 10^3</td>
<td>Typical</td>
<td>FISH</td>
</tr>
<tr>
<td>10</td>
<td>66</td>
<td>M</td>
<td>Pleural effusion</td>
<td>3.6 × 10^3</td>
<td>Typical</td>
<td>FISH</td>
</tr>
</tbody>
</table>

^a For bcl-1 rearrangement in lymph node cells.
^b For cyclin D1 overexpression in bone marrow cells.
^c FISH, fluorescence in situ hybridization, for t(11;14) detection in blood and pleural effusion cells.

7 patients with peripheral monoclonal lymphocytosis showed lymphadenopathy.

**Control B Cells.** Seven reactive lymph nodes with follicular hyperplasia from age-matched individuals were tested as sources of normal CD5+ B cells. In parallel experiments, circulating CD5+ B cells from the peripheral blood of four normal donors were tested.

MNC were isolated on Ficoll-Hypaque density gradients, washed in PBS containing 1% fetal bovine serum, and cryopreserved until tested. Immunophenotypic analyses showed that CD5+ B cells in reactive lymph node samples ranged from 5 to 10% MNC.

**Chemokines and Antibodies.** All of the chemokines (CCL2, CCL3, CCL19, CCL20, CCL22, CXCL8, CXCL10, CXCL12, and CXCL13) were purchased from Pepro Tech (Rochester, NY) and tested at the final concentration of 100 ng/ml. This concentration was chosen on the grounds of preliminary dose-response experiments carried out with normal tonsil B cells (33). In selected experiments, chemokine concentrations up to 1000 ng/ml were tested.

For B-cell immunophenotyping, the following mAbs were used: CD19-phycocerythrin (PE)-cy技能 (Cy5), CD5-FITC (Caltag Laboratories, Burlingame, CA); CD20-FITC, CD23-FITC, CD3-FITC, CD68-PE, and CD56-PE (Becton Dickinson Systems, San Jose, CA); and anti-immunoglobulin λ light chain-PE and anti-immunoglobulin λ light chain-FITC (PharMingen, San Diego, CA). Anti-CXCR1 to CCR7-FITC mAbs were purchased from R&D Systems Inc. (Minneapolis, MN). Controls were isotype-matched mAbs of irrelevant specificity conjugated with the same fluorochromes as test mAbs (PharMingen). Unconjugated anti-CXCR1 to CCR5 mAbs were purchased from Serotec Inc. (Raleigh, NC). Controls were isotype-matched unconjugated mAbs of irrelevant specificity (Serotec). Cells treated with anti-CXCR or control unconjugated mAbs were washed and incubated with PE-conjugated antimouse IgG subclass goat antisera (Serotec).

For fluorescence-activated cell sorter analysis, 10,000 events were acquired after double or triple gating for MCL or control B cells, respectively. The former cells were double stained with mAbs against the monoclonal immunoglobulin light chains and with each of the mAbs to chemokine receptors.

**Table 1** Main clinical and laboratory features of mantle cell lymphoma patients
Reactive lymph node mononuclear cells were subjected to tri-color staining with CD19 and CD5 mAbs, in combination with the different mAbs to chemokine receptors. Cells were scored using a FACSscan analyzer (Becton-Dickinson), and data were processed using CellQuest software (Becton-Dickinson). The threshold line was based on the maximum staining obtained with irrelevant isotype-matched mAb, used at the same concentration as test mAb. Negative cells were defined such that <1% of cells stained positive with control mAbs. Cells labeled with test antibody that were brighter than those stained with isotypic control antibody were defined as positive. Mean fluorescence intensity values of the isotype control and of test mAbs were used to evaluate whether the differences between the peaks of cells were statistically significant with respect to control. The Kolmogorov-Smirnov test for the analysis of histograms was used, according to the CellQuest software user’s guide.

**Migration Assay.** Chemotaxis of malignant and normal B-cell populations was tested using 24 transwell plates (5 μm pore-size, polycarbonate membrane; Costar, Cambridge, MA; Ref. 34). Five \( \times 10^5 \) mononuclear cells were dispensed in the upper chamber, and different chemokines (100 ng/ml concentration) or medium alone were added to the lower chamber. Unfractionated mononuclear cells, rather than purified cell fractions, from neoplastic samples and reactive lymph nodes were tested, to minimize the risk that chemotactic activity could be affected by the separation procedures. Plates were incubated for 2 h at 37°C. After removal of the transwell inserts, cells from the lower compartments were collected. Furthermore, 0.5 ml of 5 mM EDTA was added to the lower chamber for 15 min at 37°C to detach adherent cells from the bottom of the wells. Detached cells were pooled with the previously collected cell suspensions and counted by trypan blue staining. Transmigrated normal B cells were identified by double staining with CD19 and CD5 mAbs, whereas B lymphocytes from MCL samples were detected by single staining for the monoclonal immunoglobulin light chain expressed by the individual clones. Each experiment was performed in duplicate, and results were means from duplicate wells.

**Statistical Analysis.** Data were expressed as means ± 1 SD. Analyses were performed with GraphPad Instat (GraphPad Software, San Diego, CA). Differences were determined by one-way ANOVA with Bonferroni multiple comparison tests. Differences were accepted as significant when \( P < 0.05 \).

**RESULTS**

**Expression of Chemokine Receptors on Malignant B Cells from MCL Patients and Their Postulated Normal Counterparts.** MCL B cells from 2 lymph nodes (cases #1 and 2), 7 peripheral blood samples (cases #3–9), and 1 pleural effusion (case #10), as well as normal CD5+ B cells from 7 reactive lymph nodes (right), were investigated by flow cytometry. MCL B cells were double-stained with a monoclonal antibody (mAb) to the monoclonal immunoglobulin light chain expressed by the individual clones and with different mAbs to chemokine receptors. Normal CD5+ B cells were triple stained with CD19, CD5, and anti-CCR mAbs. Results are percentage of positive cells.

![Fig. 1 Expression of CC chemokine receptors on mantle cell lymphoma (MCL) B cells and CD5+ B cells from reactive lymph nodes.](image)

Fig. 1 shows the percentage of CCR expression in MCL samples (left columns) and in reactive lymph nodes (right columns). On the basis of mean fluorescence intensity analysis using the Kolmogorov-Smirnov test, MCL B cells were found to express CCR1 in 8 of 10 cases (#1–5 and 7–9), CCR2 in 7 of 10 (cases #1, 3, 4, and 6–8), CCR3 in 3 of 10 (cases #4, 6, and 7), CCR4 in 9 of 10 (cases #1–9), CCR5 in 2 of 10 (cases #6 and 8), CCR6 in 7 of 10 (cases #1–4, 6, 7, and 9), and CCR7 in 10 of 10. CCR1 was expressed in 2 of 7 CD5+ B cell samples from reactive lymph nodes, CCR2 in 1 of 7 (sample #6), CCR3 in 6 of 7 (samples #1–6), CCR4 in 4 of 7 (samples #1, and 4–6), CCR5 in 3 of 7 (samples #1, 3, and 4), and CCR6 and CCR7 in 7 of 7. Similar results were obtained with CD5+ B cells from normal peripheral blood; CCR3, CCR6, and CCR7 were detected in 4 of 4 samples tested, CCR4 and CCR5 in 2 of 4, CCR1 in 1 of 4, and CCR2 in 0 of 4 (data not shown).
Chemotaxis of MCL B Cells and CD5⁺ B Cells in Response to CCR and CXCR Ligands. MCL B cells were tested for chemotaxis to the ligands of the receptors investigated, with the exception of the CCR3 and CCR5 binding chemokines CCL11 and CCL4, respectively, because neither receptor had been detected on tumor cells. MNC isolated from 2 lymph nodes (cases #1 and 2), 5 peripheral blood samples (cases #4, 5, 6, 8, and 9), and 1 pleural effusion (case #10) were tested in a transwell assay. Neoplastic B cells that migrated in response to chemokines or medium (control) were identified by staining for the monoclonal immunoglobulin light chain expressed by each clonal population.

The chemotactic responses of the eight MCL cell suspensions are shown in Fig. 3. By assuming that a chemokine was active in the individual cases when it at least doubled the number of migrated cells in comparison with control cells, it was found that CXCL12 (the ligand of CXCR4) stimulated the chemotaxis of all of the MCL B cell suspensions. CCL19 (a ligand of CCR7) enhanced the locomotion of B cells in 6 of 8 cases (#1, 4, 5, 8, 9, and 10; Fig. 3). Other chemokines, *i.e.*, CCL3 (a ligand of CCR1), CCL2 (that binds to CCR2), CCL22 (a ligand of CCR4), CCL20 (that binds to CCR6), CXCL8 (a ligand of CXCR1 and CXCR2), CXCL10 (a ligand of CXCR3), and CXCL13 (that binds to CXCR5) displayed heterogeneous effects. In particular, CCL3 was found to enhance tumor cell locomotion in 2 of 8 cases (#4 and 8), CCL2 in 1 of 8 cases (#4), CCL22 in 2 of 8 cases (#4 and 5), CCL20 in 1 of 8 cases (#4), CXCL8 in 1 of 8 cases (#4), CCL10 in 3 of 8 cases (#1, 4, and 8), and CXCL13 in 1 of 8 cases (#4; Fig. 3). Notably, case #4 displayed special behavior, because malignant B cells responded to every chemokine tested (Fig. 3). When the results obtained in the individual cases were pooled, only CXCL12 and CCL19 were found to enhance significantly the chemotaxis of MCL B cells (*P* < 0.001 and < 0.05, respectively).

Because B cells from cases #9 and 10 were unresponsive to all of the chemokines other than CXCL12 and CCL19, tumor cells from these cases were tested for chemotaxis to CCL3, 2, 22, 20, CXCL8, CXCL10, and CXCL13 in concentrations ranging from 10 to 1000 ng/ml. No migration was ever detected even under these conditions (data not shown).

In subsequent experiments, chemotaxis of CD5⁺ B cells from 7 reactive lymph nodes with follicular hyperplasia to the following chemokines, CXCL12, CCL2, CCL19, CCL11, CCL22, CCL4, CCL20, CXCL8, CXCL10, and CXCL13 was investigated. These chemokines were selected on the ground of the consistent expression of the respective receptors on CD5⁺ B cells; for the same reason, CCL2 and CCL3 were not tested, because cognate receptors CCR1 and CCR2 had not been detected. In these experiments, MNC were tested, and CD5⁺ B cells migrating in the assay were identified by double staining for CD19 and CD5. As shown in Fig. 3, (bottom), only CXCL12 stimulated significantly the chemotaxis of normal CD5⁺ B cells (*P* < 0.001).

Taken together, these results demonstrate that CCR7 was functional in tumor cells but not in their normal counterparts, whereas CXCR4 mediated chemotaxis of both cell types.

Effects of CXCL12 Pretreatment of MCL and CD5⁺ B Cells on Their Subsequent Responsiveness to Chemokines. Cross-sensitization or desensitization of chemokine receptors may occur in differentiating normal B cells (35) and in follicular...
lymphoma B cells (36). To investigate this issue, MCL B cells and their normal counterparts were preincubated overnight with CXCL12 before being tested for chemotaxis to the ligands indicated above. CXCL12 was selected for these experiments because it was the only chemokine that attracted both MCL B cells and CD5\(^+\) normal B cells

As shown in Fig. 4, a significant response of malignant B cells to CXCL12 and CCL19 as compared with control cells (nil; \(P < 0.01\) and \(<0.05\), respectively) was consistently observed with five MCL cell suspensions. Notably, tumor cells from case #1 acquired migratory competence to CXCL13 under these conditions (Fig. 4 and, for comparison, Fig. 3). Malignant B cells from case #2, which were unresponsive to CCL19 in the experiments shown in Fig. 3, were found to migrate to the same chemokine after preincubation with CXCL12 (Fig. 4). Finally, tumor cells from case #5 did not migrate to CCL19 (Fig. 4), whereas freshly isolated cells from the same patient did (see Fig. 3). All of the remaining chemokines did not stimulate MCL B cell chemotaxis, as it was observed using freshly isolated cells (Fig. 4 and, for comparison, Fig. 3).

CD5\(^+\) normal B cells from the seven reactive lymph nodes did not migrate to any chemokine with the only exception of CXCL12, after overnight incubation with CXCL12 itself (data not shown).

Although the number of MCL samples tested in these experiments was limited, the above results indicate that tumor cells underwent cross-modulation of different chemokine receptors after exposure to CXCL12, whereas this was never observed with their normal counterparts.

**DISCUSSION**

In this study we have demonstrated that malignant B cells from MCL patients express different receptors for chemokines of the CC and CXC families, with a pattern common to all of the samples irrespective of their source (i.e., peripheral blood,
lymph node, or pleura). The chemotactic *in vitro* activity of tumor cells was enhanced significantly only by CXCL12 and CCL19. Nonetheless, scattered *in vitro* migration to other chemokines was observed in the individual MCL cases, demonstrating the heterogeneous behavior of each B cell clone.

In secondary lymphoid tissues, CXCL12 is expressed by stromal cells (37), whereas CCL19 and CCL21, the ligands of CCR7, are expressed on the surface of high endothelial venules and in the perivascular stroma (38). Similar patterns have been detected in infiltrated lymph nodes from B-CLL patients (28). Therefore, it is conceivable that interaction of CXCR4 with CXCL12 and of CCR7 with CCL19 and CCL21 in the lymph node microenvironment promotes migration of MCL tumor cells, thus contributing to their local invasive potential.

CD5+ B cells represent a lymphocyte subset specialized in the production of low affinity, polyreactive IgM autoantibodies (39, 40). They expand in fetal life, but are strongly down-regulated in postnatal life (40). In mice, CD5+ B cells represent a lineage separate from conventional, i.e., CD5−, B cells (41), whereas this has not yet been clearly established for their human counterparts (40). CD5+ B cell expansion takes place in autoimmune diseases, such as rheumatoid arthritis and some hemolytic anemias (42).

This study shows for the first time that human CD5+ B cells express various chemokine receptors, with an overall profile similar to that of MCL B cells, but migrate only to CXCL12. This latter observation is consistent with a previous report showing that mouse peritoneal CD5+ B cells are attracted by CXCL12 (43).

Failure of chemokine receptors to trigger chemotaxis upon interaction with their specific ligands may be related to low or absent expression or, alternatively, to functional impairment. In this study, many chemokine receptors, although expressed in malignant B cells or in their normal counterparts, were nonfunctional.

Potential mechanisms underlying this latter phenomenon may be receptor desensitization by homologous or heterologous ligands (35, 44), uncoupling of signal transduction pathways, or defective internalization of the receptor-ligand complex. For example, members of the RGS protein family, when expressed in normal or malignant B cells, turn off signal transduction initiated by the binding of chemokines to their receptors (45, 46). Defective internalization of the CXCL12-CXCR4 complex has been described in freshly isolated germinal center B cells, and related to their inability to migrate to CXCL12, despite high CXCR4 expression (37). A final explanation for the failure of some chemokine receptors to trigger chemotaxis upon interaction with their ligands may be that the model used in this study is not sensitive enough or just not representative, for example, because of the lack of important coactivators.

In this study, normal CD5+ B cells did not migrate in response to CCL19, indicating that functional CCR7 incompetence is characteristic of this cell type and that malignant transformation is associated with gain of CCR7 function.

In an attempt to overcome the refractoriness of various chemokine receptors in MCL B cells and their normal counterparts, cells were incubated overnight with CXCL12 and then subjected to chemotactic assays in the presence of a chemokine.

CXCL12 itself enhanced the *in vitro* migration of MCL B cells and their normal counterparts, indicating that CXCR4 was rapidly recycled to the cell surface after internalization of the CXCR4-CXCL12 complex. Tumor cells preincubated with CXCL12 showed a significant chemotaxis to CCL19, whereas normal CD5+ B cells did not. Finally, pretreatment of MCL B cells with CXCL12 endowed them with *de novo* responsiveness to CXCL13 in one case and to CCL19 in another, and, conversely, abrogated CCR7 function in a third case. In contrast, CD5+ B cells cultured with CXCL12 did not migrate to other chemokines. Thus, CXCL12, besides attracting directly MCL B cells, may also influence their *in vivo* migration by modulating other chemokine receptors.

A recent study has suggested the existence of two MCL subsets, one showing a gene expression profile comparable with that of normal pregerminal center and memory B cells, and
another resembling activated germinal center B cells (47). Furthermore, a small minority of MCL cases lacking cyclin D1, but overexpressing cyclin D2 or D3, has been reported (48). These findings, however, await confirmation from the analysis of a larger number of MCL cases. Differential expression of some chemokine receptor transcripts between MCL B cells and their normal counterparts has been reported (47). Thus, CCR6 mRNA was down-regulated and CXCR5 mRNA was virtually undetectable in MCL B cells as compared with their normal counterparts (47). In the present study, expression of the CCR6 protein on the cell surface was found to be significantly lower in MCL B cells than in their normal counterparts, whereas the CXCR5 protein was highly expressed on both normal and malignant B lymphocytes. Furthermore, a significant down-regulation of CCR3 and CXCR1 in MCL B cells versus their normal counterparts was detected here that has not been reported at the mRNA level (47). Taken together, these findings suggest that expression of discrete chemokine receptors in MCL B cells may be differentially regulated at the transcriptional and/or post-transcriptional levels.

In summary, key findings of this study are: (a) the identification of CXCL12 and CCL19 as chemotaxtactants for MCL B cells; (b) the gain of function of CCR7 in tumor cells versus their normal counterparts; and (c) the cross-sensitization of other chemokine receptors to the respective ligands by CXCL12 in tumor cells but not in their normal counterparts. These results provide novel and relevant information on the mechanisms controlling the in vitro migration of MCL B cells.

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Chemotaxis of Human

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