Homeostatic chemokines drive migration of malignant B cells in patients with non-Hodgkin lymphomas

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This study investigated the role of several chemokines and their receptors on malignant B lymphocytes recovered from 13 patients with chronic lymphocytic leukemia (CLL), 9 with hairy cell leukemia (HCL), 5 with mantle cell lymphoma (MCL), 5 with marginal zone B-cell lymphoma (MZL), 6 with small lymphocytic lymphoma (SLL), and 5 with follicular cell lymphoma (FCL). Flow cytometry analysis demonstrated that CXCR4 and CXCR5 were expressed on all malignant and normal B cells. Considering CC receptors, CCR1 was expressed in 70% of patients with CLL and 40% of those with HCL but was lacking in patients with MCL, MZL, SLL, and normal B cells. CCR2 showed a heterogeneous pattern of expression. CCR3 was found in almost all patients with CLL and in the majority of those with HCL, whereas it was usually lacking in patients with MZL and SLL and in healthy subjects. CCR5 was expressed in patients with HCL and MCL. Migration assays showed that different chemokines, mainly CXCL12 and CXCL13, are able to trigger migration of malignant B lymphocytes. Some of these chemokines induce calcium mobilization. These data indicate that different patterns of chemokine receptor expression identify different malignant B-cell subsets and that these receptors are functional and might play a role in malignant B-cell circulation. (Blood. 2004;104:502-508)

Introduction

Chemokines represent a group of molecules that regulate cell migration and can be distinguished in inflammatory and homeostatic chemokines. These latter are involved in the homeostasis of the immune system.1-8 Cells are exposed to a complex pattern of chemoattractant signals that, through a gradient of chemokine concentrations, drive the migration of lymphocytes to target tissues or lymphoid organs. Different chemokine receptors are able to bind the same chemokine and, in turn, different chemokines are able to bind the same receptor, indicating that redundancy is a definite feature of the chemokine network. Chemokine-receptor interactions are of crucial importance for homeostatic functions within the immune system, particularly for establishing the complex architecture of the secondary lymphoid organs.3,4,9

The role of chemokines for B-cell lymphopoiesis has been primarily demonstrated for CXCL12 (stromal-derived factor 1α [SDF-1α]) and its receptor CXCR4.10 CXCL12 can induce chemotaxis of B-cell progenitors, indicating that the CXCL12/CXCR4 system may be important in directing the migration of B-cell progenitors to the appropriate bone marrow microenvironment.11-13 In addition, it is highly expressed on B lymphocytes and it is likely to play a key role in the architecture of spleen and lymph nodes. To date, most information in terms of chemokines and their receptors has been provided on normal human B cells and in the mouse; little data are available on the role of these molecules on malignant B lymphocytes.14-17 In particular, it has been demonstrated that malignant B cells express CXCR3 in different neoplastic conditions and mediate migration following the binding of the chemokines CXCL10 (inflammatory protein 10 [IP-10]) and CXCL9 (Mig).14,16 Furthermore, CXCR4, a receptor constitutively expressed on normal B lymphocytes, has been detected on malignant B lymphocytes from patients with chronic lymphocytic leukemia (CLL).15

In this study, we investigated the expression and functional role of a large spectrum of chemokine receptors, including CCR1 to CCR6 and CXCR1 to CXCR5, in several B-cell non-Hodgkin lymphomas (NHLs). We demonstrated that these receptors recognize different B-cell malignancies and mediate chemotaxis following binding to their own ligands.

Patients, materials, and methods

Patient samples

Forty-three patients with different B-cell malignancies were studied at the time of diagnosis.18 This study was approved by the Università of Padova institutional review board. Informed consent was provided according to the Declaration of Helsinki. Thirteen patients (10 men and 3 women, ages 46-78 years) with the diagnosis of B-CLL were graded according to the Rai staging system19 as follows: stage 0 (1 case) stage I (5 cases), stage II (4 cases), stage III (4 cases), stage IV (4 cases). Supported by the Italian Association for Cancer Research (AIRC, Milan), by the Ministero dell’Università e della Ricerca Scientifica e Tecnologica (MURST), and by the Ministero della Salute (Rome).

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cases), stage III (2 cases), stage IV (1 case); the total lymphocyte count ranged from 21 to 98 × 10^9/L (21 000-98 000/mm³).

Nine patients with hairy cell leukemia (HCL; 6 men and 3 women, ages 46-75 years) were investigated. The diagnosis was established on the basis of clinical, morphologic, cytochemical, histologic, and immunologic features.18 Twenty-one patients with different histologic NHL entities (5 with mantle cell lymphoma [MCL], 5 with marginal zone lymphoma [MZL], 6 with small lymphocytic lymphoma [SLL], and 5 with follicular cell lymphoma [FLC]) in the leukemic phase were also included in the study.18

**Preparation of cell suspensions**

Peripheral blood mononuclear cells (PBMCs) from patients with NHL were obtained from freshly heparinized blood samples by centrifugation on Ficoll/Hypaque (F/H) gradient.20 Normal B lymphocytes were obtained from 5 tonsils after mechanic disruption.21 Mononuclear cells, recovered following centrifugation on F/H gradient, were washed 3 times with phosphate-buffered saline (PBS) and resuspended in endotoxin-free RPMI 1640 medium (Sigma Chemical, St Louis, MO) supplemented with 20 μM HEPEs (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 1-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS; ICN Flow, Costa Mesa, CA).

Cells with a percentage of monocytes, T cells, and natural killer (NK) cells more than 5% were further enriched in B lymphocytes by rosetting with neuroaminidase-treated (Sigma) sheep red blood cells (SRBCs) and by removing residual CD3+, CD16+, CD56+, and CD14+ cells using magnetic separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.22 Following this multistep negative selection procedure, more than 98% of the resulting cell population was CD19 negative.

**mAbs and cytokines**

CC and CXC receptor analysis was performed using anti-CCR and anti-CXCR (mouse or rat) monoclonal antibodies (mAbs) purchased from R&D Systems (Minneapolis, MN). The chemokines CXCL12 and CXCL13 anti-CXCR (mouse or rat) mAbs purchased from CC and CXC receptor analysis was performed using anti-CCR and mAbs and cytokines

**Migration assay**

Cell migration was measured in a 48-well modified Boyden chamber.14 Chemokines were diluted in RPMI 1640 medium at different concentrations and were used to evaluate the chemotactic properties of B lymphocytes from healthy donors and patients. Polyvinylpyrrolidone-free polycarbonate membranes with 5-μm pores were coated with fibronectin before use. Then 30 μL chemokine or control medium was added to the bottom wells, and 50 μL 5.0 × 10^6 cells/mL B cells, resuspended in RPMI 1640, were added to the top wells. The chemokines were used at the following concentrations: CXCL12 (both SDF-1α and SDF-1β) at 100 ng/mL, CXCL13 at 1 ng/mL, CCL3 at 5 ng/mL, CCL23 at 5 ng/mL, CCL20 at 5 ng/mL, CCL24 at 100 ng/mL, and CCL5 at 1 ng/mL. The concentrations indicated represent the doses that elicited the highest migration index in a dose-response curve with different chemokine concentrations (0.1, 1, 10, 100 ng/mL). At these concentrations the migration index was as follows: 0, 0, 1, 2, 3, 5, 10, 15 for CXCL12; 2, 3, 7, 10, 12, 10 to 11, 9 to 12 for CXCL13; 0, 4 to 6, 10, 10, 12, 10 to 11, 9 to 12, 10, 12 for CCL3; 2, 3, 7, 10, 11, 10, 11, 10, 12, 12 to 14 for CCL5; 0, 2, 5, 8, 15, 10, 12, 10, 12, 12 to 14 for CCL20; 0, 2, 4, 10, 15, 10, 12, 8, 10 for CCL23; and 0, 0, 2, 3, 10, 40 for CCL24.

The chamber was incubated at 37°C with 5% CO₂ for 2 hours. The membranes were then removed, washed with PBS on the upper side, fixed and stained with DiffQuik (Dade, Düdingen, Switzerland). Cells were counted at × 400 magnification in 3 fields/well. All assays were performed in triplicate.

**Cytosolic calcium measurement**

Changes in the intracellular calcium concentration [Ca²⁺], were measured in B lymphocytes from 10 patients with B-cell malignancies (6 CLL, 2 MZL, 1 HCL, 1 MCL) and in 2 healthy subjects, using the fluorescent indicator fura-2/am, as previously described.23 Briefly, 2 × 10^6 cells were incubated with 2 μM fura-2/am at 37°C for 40 minutes. After the loading procedure, aliquots of the cells (2 × 10⁶) were rapidly washed and resuspended in a magnetically stirred thermostatted cuvette. The incubation medium contained 1 mM CaCl₂. Excitation and emission wavelengths were 340 and 500 nm, respectively; the excitation slit width was 5 nm, and the emission slit was 10 nm. The inhibitor of organic anion transport sulfinpyrazone was added in experiments during [Ca²⁺] measurements at a final concentration of 250 μM to prevent fura-2 release into the medium.24 Control experiments without sulfinpyrazone gave essentially the same results except for a slowly increasing baseline due to fura-2 leakage.24 The chemokines were used at 100 ng/mL; anti-Ig was used at 500 ng/mL.

**Statistical analysis**

Data are expressed as mean ± SE of the mean and comparisons between values were made using the analysis of variance (ANOVA) test. A P value of less than .05 was considered as significant.

**Results**

**Expression of CC and CXC chemokine receptors on normal and malignant B cells**

The expression of chemokine receptors was analyzed by flow cytometry analysis on normal B lymphocytes obtained from healthy donors and on tumor B cells recovered from patients with different NHLs. Flow cytometry profiles for representative patients are shown in Figures 1 and 2 and the overall results are detailed in Tables 1 and 2. These latter report the number of positive cases for each receptor in different series of patients.

Considering the CC chemokine receptors, the data reported in Table 1 and Figure 1 demonstrate that CCR1 is usually expressed in...
B-CLL (9 of 13 patients tested) and in some HCL patients (4 of 9), whereas its expression is lacking on normal B lymphocytes and on malignant cells from patients with MCL, MZL, SLL, and FCL. CCR2 was heterogeneously found on normal B cells and in different groups of patients, being, however, consistently undetectable on tumor cells from patients with MZL. CCR3 was present in all patients with CLL and in the majority of those with HCL, whereas normal B cells and malignant B lymphocytes from patients having other NHLs (MCL, MZL, SLL, and FCL) were almost negative for CCR3. CCR5 is expressed in all subjects with MCL and in the majority of patients with HCL (78%), but barely detectable on B lymphocytes from control subjects and on malignant B cells from patients with CLL (7.7%). CCR6 is constitutively expressed on different B-cell subsets, both normal and malignant, with the exception of FCL.

The analysis of the CXC chemokine receptors (Table 1; Figure 1) showed that CXCR4 and CXCR5 receptors are constitutively present both on normal B cells from healthy subjects and on tumor B cells from patients with most NHLs, whereas a heterogeneous pattern of expression has been observed for CXCR1 and CXCR2 receptors. These 2 receptors are never found on malignant cells from MCL. CXCR3 expression was consistent with our data previously reported.14

We also evaluated the intensity of chemokine receptors by analyzing the MFI; data are reported in Table 2. Among different B-cell subsets that bear a particular receptor, the MFI changed from one B-cell population to another. In particular, CXCR4, a receptor expressed on all B lymphocytes under study, showed a lower MFI compared with patients with MZL and SLL compared with normal B lymphocytes and other NHLs (P < .05). Again, malignant cells from patients with CLL and HCL showed a higher expression of CXCR5, compared with patients with SLL (P < .05). Considering patients with B-CLL, there were not any statistically significant differences in the expression of CC and CXC receptors among subjects in different stages of the disease.

Migratory assay of CC chemokines

Due to the expression of CC chemokine receptors on malignant B cells, the migratory assay was performed in the presence of several CC chemokines.
CCL chemokines, including CCL24, CCL5, CCL3, CCL23, and CCL20. The assay was performed in 18 patients with NHL and in 3 healthy subjects, and the findings are reported in Figure 3. CCL24, a chemokine that binds CCR3, induced a high migration of malignant B cells recovered from all patients with HCL and in some with CLL. The effect on normal B lymphocytes was lower than that observed in patients with HCL. In addition, the migration induced in patients with MZL or SLL was very low or absent. This negative result is consistent with the lack of this receptor in these types of NHL (Tables 1 and 2).

CCR5 binds several receptors, including CCR1, CCR3, and CCR5. The migration induced by this chemokine was again more effective in patients with HCL or CLL as compared to normal B lymphocytes and patients with MZL or SLL.

The migration induced by CCL3, which binds CCR1 and CCR5 receptors, was principally induced on tumor cells obtained from patients with HCL and in some patients with CLL or MCL lymphoproliferative disorders (Figure 3).

CCL20, which selectively binds CCR6, induced migration of malignant B cells obtained from a small number of patients suffering from NHL. CCL23, which preferentially binds CCR1, seemed to display very low migration as compared to the other molecules tested.

Migratory assay of CXC chemokines

To further characterize the biologic properties of CXC receptors, normal and malignant B cells were assessed for their migratory properties in the presence of several chemokines. The data reported in Figure 4 were obtained at the concentration giving the highest migration in a previously determined dose-response curve (not shown). Figure 4 illustrates the migratory effect of CXC chemokines, that is, CXCL12 (SDF-1α and SDF-1β) and CXCL13, on normal B cells recovered from 3 healthy subjects, and on malignant B lymphocytes obtained from 18 patients with NHL. CXCL12 (SDF-1α and SDF-1β), 2 chemokines that specifically bind to CXCR4 receptor, displayed a low migratory capability on normal B lymphocytes, whereas the effect on B-cell migration was stronger in tumor cells from patients with CLL and HCL. The ability to induce migration in other NHLs was much lower in patients with MZL and SLL, whereas it was heterogeneous in patients with MCL. Even though cell chemotaxis was triggered by both molecules, the migration index was higher with SDF-1β rather than that obtained with SDF-1α.

Another chemokine receptor of particular interest for the recirculation of B lymphocytes is CXCR5. This receptor was widely expressed in both healthy subjects and in patients with lymphoproliferative disorders and mediates chemotaxis following binding of the relevant chemokine CXCL13 (Figure 4). Our data demonstrate that this chemokine displayed a discrete migratory capability in a few patients with B-CLL, HCL, and MCL malignancies, whereas it was unable to induce migration of normal B lymphocytes obtained from healthy subjects or of malignant cells recovered from patients with MZL and SLL.

Cytosolic calcium measurement

We examined the ability of CXC and CC chemokines to stimulate functional responses in malignant B cells from patients with NHL through the evaluation of cytosolic Ca\(^{2+}\) levels. The analysis was performed in B lymphocytes from 10 patients with NHL and 2 healthy subjects. The data are reported in Table 3 and 3 representative cases are shown in Figure 5. Our data demonstrate that a consistent increase in [Ca\(^{2+}\)], was observed on CXCL12 (100 ng/mL) addition. This effect was observed both on tumor B cells obtained from patients as well as on normal B lymphocytes obtained from healthy subjects. CXCL13 also displayed an increase in [Ca\(^{2+}\)], in patients with NHL and in healthy subjects; the effect, however, was very low when compared with the increase in [Ca\(^{2+}\)], observed with CXCL12 addition. Concerning the other chemokines we investigated, no [Ca\(^{2+}\)], increase was observed, with the only exception being CCL5 that displayed [Ca\(^{2+}\)], mobilization in a few patients with NHL (3 of 10 subjects tested, 2 CLL and 1 MZL). In all cases addition of anti-Ig resulted in an increase in [Ca\(^{2+}\)], indicating that the cells we were dealing with were normal.

### Table 1. Expression of CC and CXC receptors on normal B lymphocytes and on malignant B cells obtained from patients with lymphoproliferative disorders

<table>
<thead>
<tr>
<th>Patients</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR5</th>
<th>CCR6</th>
<th>CXCR1</th>
<th>CXCR2</th>
<th>CXCR3</th>
<th>CXCR4</th>
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<tbody>
<tr>
<td>B-CLL</td>
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<td>6/13</td>
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<td>13/13</td>
<td>3/13</td>
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<td>MCL</td>
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<td>2/5</td>
<td>5/5</td>
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</tr>
<tr>
<td>MZL</td>
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<td>0/5</td>
<td>0/5</td>
<td>3/5</td>
<td>5/5</td>
<td>2/5</td>
<td>2/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>SLL</td>
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<td>1/6</td>
<td>1/6</td>
<td>3/6</td>
<td>6/6</td>
<td>3/6</td>
<td>4/6</td>
<td>6/6</td>
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<td></td>
</tr>
<tr>
<td>FCL</td>
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<td>0/5</td>
<td>4/5</td>
<td>1/5</td>
<td>2/5</td>
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<tr>
<td>Normal B cells</td>
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<td>2/5</td>
<td>1/5</td>
<td>1/5</td>
<td>4/5</td>
<td>2/5</td>
<td>2/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Number of positive cases of the subjects tested.

### Table 2. Flow cytometry analysis of CC and CXC receptors in B-cell CLDs

<table>
<thead>
<tr>
<th>Cell types</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR5</th>
<th>CCR6</th>
<th>CXCR1</th>
<th>CXCR2</th>
<th>CXCR3</th>
<th>CXCR4</th>
<th>CXCR5</th>
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<tr>
<td>CLL</td>
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<td>2.1</td>
<td>2.1</td>
<td>2.7</td>
<td>0.9</td>
<td>0.3</td>
<td>2.1</td>
<td>5.3</td>
<td>2.6</td>
<td>0.7</td>
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<tr>
<td>HCL</td>
<td>51.8</td>
<td>33.4</td>
<td>30.0</td>
<td>94.0</td>
<td>60.0</td>
<td>1.5</td>
<td>118.2</td>
<td>30.1</td>
<td>4.2</td>
<td>0.7</td>
</tr>
<tr>
<td>MCL</td>
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<td>1.1</td>
<td>13.7</td>
<td>75.0</td>
<td>83.7</td>
<td>54.9</td>
<td>4.0</td>
<td>1.3</td>
<td>6.7</td>
<td>1.0</td>
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<tr>
<td>MZL</td>
<td>0.7</td>
<td>0.4</td>
<td>1.6</td>
<td>5.5</td>
<td>5.8</td>
<td>1.5</td>
<td>64.6</td>
<td>25.3</td>
<td>3.3</td>
<td>1.8</td>
</tr>
<tr>
<td>SLL</td>
<td>1.1</td>
<td>0.7</td>
<td>3.3</td>
<td>25.6</td>
<td>18.6</td>
<td>17.2</td>
<td>1.6</td>
<td>0.5</td>
<td>25.8</td>
<td>7.6</td>
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<tr>
<td>FCL</td>
<td>1.6</td>
<td>0.7</td>
<td>3.0</td>
<td>0.9</td>
<td>0.7</td>
<td>0.6</td>
<td>8.1</td>
<td>3.8</td>
<td>1.3</td>
<td>1.6</td>
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<tr>
<td>Normal B cells</td>
<td>5.1</td>
<td>2.2</td>
<td>6.4</td>
<td>4.7</td>
<td>28.1</td>
<td>6.3</td>
<td>2.2</td>
<td>1.2</td>
<td>30.7</td>
<td>9.5</td>
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</table>

Data are reported as mean ± SE of the MFI values for each group of subjects after subtracting the MFI of isotype control mAb from the MFI of positively stained samples.
Discussion

This study was designed to investigate the expression and functional role of CC and CXC chemokine receptors and their ligands in patients with NHL. Our data indicate that some receptors, that is, CCR1, CCR2, and CCR5, are expressed in patients affected by a limited number of histologic types of NHL and are usually absent on normal B lymphocytes. CXCR4, CXCR5, and CCR6 are constitutively expressed on normal and malignant B lymphocytes recovered from patients with different B-cell malignancies, whereas other receptors, that is, CXCR1 and CXCR2, are expressed in some malignancies but not in normal B lymphocytes. On functional grounds, migration in vitro assay demonstrated that these structures behave as fully functional receptors because they transduce chemotactic activity after binding relevant chemokines. Furthermore, some of these receptors are able to induce [Ca\(^{2+}\)] mobilization following binding of their specific ligands.

The trafficking and homing of normal B lymphocytes is a multistep process that requires the involvement of adhesion molecules and chemokine receptors. CXCL12 represents a CXC chemokine constitutively expressed by bone marrow cells and plays a key role in B-cell chemokine receptors. CXCL12 represents a CXC chemokine constitutively expressed by bone marrow cells and plays a key role in B-cell chemokine receptors. CXCL12 signaling in some B-cell malignancies, thus permitting an efficient migration of B lymphocytes via CXCR5. This receptor is commonly expressed on B cells obtained from NHL. CXCL12 expression on the cell surface of malignant B lymphocytes and migration assay. This finding might be related to the functional CXCL12 expression on different cell types and to the number of receptors on the cell surface.

B-cell attracting chemokine 1 (CXCL13) selectively drives the migration of B lymphocytes via CXCR5. This receptor is commonly expressed on resting B cells and is important for enabling the formation of B- and T-cell compartments of secondary lymphoid organs. In fact, mice lacking CXCR5 do not develop inguinal lymph nodes, have few Peyer patches, and have an altered spleen structure, as a consequence of the impairment of B-cell migration. In addition, mice with a deletion of tumor necrosis factor (TNF), TNFR1, or lymphotixin gene have decreased CXCL13 expression in follicular stromal cells of the spleen, a finding that is in agreement with the role of CXCL13 and CXCR5 in the homing of B lymphocytes. This observation suggests that the regulation of CXCL13 is under the control of some cytokines, such as TNF. This latter is constitutively expressed and produced by malignant lymphocytes from some chronic lymphoproliferative disorders (CLDs), that is, CLL and HCL. It might be suggested that the abnormal production of TNF by the transformed clone plays a role in the regulation of CXCL13 production and in the control of cell accumulation in lymphoid organs. The data herein provided showed that malignant B lymphocytes of different origin constitutively express CCL20 at 5 ng/mL, CCL24 at 100 ng/mL, and CCL5 at 1 ng/mL was tested on normal B cells obtained from 3 healthy subjects and on malignant B lymphocytes obtained from 18 patients with NHL. The concentrations were chosen in relation to a dose-response curve (see "Patients, materials, and methods"). The assay was performed in triplicate.
added and successively the Ca2+ concentration of 100 ng/mL. At the end of each experiment of 500 ng/mL), as a positive control. Where linking surface membrane immunoglobulins (at a concentration in malignant B cells obtained from patients with NHLs are equipped with different chemokine receptor profiles that might drive the recirculation of transformed cells from one site of the body to another. Future directions of research in this field rest on the study of the chemokines produced at several sites of involvement of NHL, that is, lymph nodes, spleen, and bone marrow. This might help us to understand the clinical presentation of different NHLs and might help us to design new therapeutic strategies using antagonistic molecules with the ultimate goal of limiting the spread of the disease.

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References

Homeostatic chemokines drive migration of malignant B cells in patients with non-Hodgkin lymphomas

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