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J Immunol published online 15 August 2014
<http://www.jimmunol.org/content/early/2014/08/15/jimmunol.1400547>

Supplementary Material <http://www.jimmunol.org/content/suppl/2014/08/15/content.1400547.DCSupplemental.html>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The Expression of Sphingosine-1 Phosphate Receptor-1 in Chronic Lymphocytic Leukemia Cells Is Impaired by Tumor Microenvironmental Signals and Enhanced by Piceatannol and R406

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Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of clonal B lymphocytes. Proliferation occurs in lymphoid tissues upon interaction of leukemic cells with a supportive microenvironment. Therefore, the mobilization of tissue-resident CLL cells into the circulation is a useful therapeutic strategy to minimize the reservoir of tumor cells within survival niches. Because the exit of normal lymphocytes from lymphoid tissues depends on the presence of sphingosine-1 phosphate (S1P) and the regulated expression of S1P receptor-1 (S1PR1), we investigated whether the expression and function of S1PR1 can be modulated by key microenvironment signals. We found that activation of CLL cells with CXCL12, fibroblast CD40L⁺, BCR cross-linking, or autologous nurse-like cells reduces their S1PR1 expression and the migratory response toward S1P. Moreover, we found that S1PR1 expression was reduced in the proliferative/activated subset of leukemic cells compared with the quiescent subset from the same patient. Similarly, bone marrow-resident CLL cells expressing high levels of the activation marker CD38 showed a lower expression of S1PR1 compared with CD38^{low} counterparts. Finally, given that treatment with BCR-associated kinase inhibitors induces a transient redistribution of leukemic cells from lymphoid tissues to circulation, we studied the effect of the Syk inhibitors piceatannol and R406 on S1PR1 expression and function. We found that they enhance S1PR1 expression in CLL cells and their migratory response toward S1P. Based on our results, we suggest that the regulated expression of S1PR1 might modulate the egress of the leukemic clone from lymphoid tissues. *The Journal of Immunology*, 2014, 193: 000–000.

The homing of normal lymphocytes into tissues is a very well-studied process that involves a multistep cascade in which adhesion molecules, their ligands, chemokines, and

their receptors are the main characters (1). Normal lymphocyte exit from lymphoid tissues into the circulation depends on the presence of sphingosine-1 phosphate (S1P), a bioactive sphingolipid produced by phosphorylation of sphingosine, which participates in lymphocyte trafficking, vascular homeostasis, and cell communication in the CNS, among others functions (2). S1P mediates its extracellular functions by interacting with five G protein-coupled receptors, with S1P receptor-1 (S1PR1) being the main receptor involved in lymphocyte trafficking (3). Lymphocyte egress requires a differential in S1P concentration at the site of transmigration; accordingly, S1P concentration is high in blood and lymph and low in tissues, including the interstitial fluid of lymphoid tissues (4). The regulated expression of S1PR1 also drives lymphocyte exit into the circulation. Thus, S1PR1 can be downregulated rapidly in the S1P-rich circulatory fluids in a ligand-dependent internalization process and is then effectively re-expressed at the cell surface in S1P-poor lymphoid organs, favoring lymphocyte egress (5). In addition, during a normal immune response, AgR engagement at lymphoid tissues also downregulates S1PR1 transcript levels, ensuring that activated lymphocytes undergo sufficient clonal expansion and receive appropriate instruction before being released as effector cells (3).

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of clonal B lymphocytes in peripheral blood, bone marrow, lymph nodes, and other lymphoid organs. Although most circulating leukemic cells are arrested in G₀/G₁ of the cell cycle, proliferating CLL cells can be found in lymphoid tissues where

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Received for publication February 27, 2014. Accepted for publication July 21, 2014.

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica and Fundación Roemmers.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BSA-FAF, fatty acid-free BSA; Btk, Bruton's tyrosine kinase; CD40L, CD40 ligand; CLL, chronic lymphocytic leukemia; fCD40L, murine fibroblast expressing CD40L; FSC, forward scatter; NLC, nurse-like cell; PF, proliferative fraction; Pic, piceatannol; QF, quiescent fraction; Q-PCR, quantitative real-time PCR; S1P, sphingosine-1 phosphate; S1PR1, S1P receptor-1; SSC, side scatter; Syk, spleen tyrosine kinase.

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the supportive microenvironment drives tumor B cell accumulation by enhancing leukemic cell survival and proliferation (6). Stromal cells, monocyte-derived nurse-like cells (NLCs), and activated T cells are key players in the proliferation center through the production of different soluble factors or by direct cell–cell contact. Thus, CXCL12 and CXCL13 produced by the stroma and NLCs act through their main receptors, CXCR4 and CXCR5, respectively, favoring leukemic cells' homing to lymphoid tissues and enhancing survival of the malignant clone (7–9). In contrast, activated T cells may secrete antiapoptotic cytokines, such as IL-4 or IFN- γ , and express the molecule CD40 ligand (CD40L), which interacts with CLL cells through CD40, promoting their survival and expansion (10–13). In addition, BCR signaling, which involves critical downstream kinases, such as spleen tyrosine kinase (Syk), Bruton's tyrosine kinase (Btk), and PI3K δ , occurs primarily in lymph nodes and plays a central role in the maintenance and growth of CLL (14, 15). Upon contacting some of the array of signals provided by lymphoid tissues, CLL cells enhance the expression of a variety of molecules, including the activation markers CD38 (16), CD69 (17), and CD5 (18); the chemokines CCL3 and CCL4 (19); the antiapoptotic protein survivin (20); the proliferation marker Ki67 (21); and c-myc (22). Moreover, interaction with the microenvironment also confers drug resistance that may be responsible for residual disease after conventional therapy (10, 23–26). Therefore, mobilization of tissue-resident CLL cells into the circulation is a useful strategy to minimize the reservoir of tumor cells within survival niches. This is the case for BCR-associated kinase inhibitors, such as Syk, Btk, or PI3K δ inhibitors, which, in addition to their antiproliferative activity, induce an early transient redistribution of leukemic cells from lymph nodes into the circulation upon treatment (14, 27). The homing of CLL cells into lymphoid tissues has been studied in depth and involves the chemokines CXCL12, CXCL13, CCL19, and CCL21, as well as their specific receptors and diverse adhesion molecules and their ligands (28). In contrast, less is known about the mechanisms that regulate leukemic clone exit into the circulation. Because it was recently reported that CLL cells are able to migrate *in vitro* toward S1P and express variable levels of S1PR1 (29), in this study we investigated whether expression of the receptor and the migratory capacity toward S1P can be modulated by key microenvironment signals and BCR-associated kinase inhibitors, such as piceatannol (Pic) and R406.

Materials and Methods

Reagents and Abs

RPMI 1640, FCS, penicillin, and streptomycin were purchased from Life Technologies (Grand Island, NY). The FIX & PERM Kit for ZAP-70 intracellular staining was purchased from Caltag Laboratories (Burlingame, CA). S1P was purchased from Avanti Polar Lipids (Alabaster, AL). BSA used for Ab staining buffer was obtained from Weiner Laboratories (Santa Fé, Argentina), and fatty acid–free BSA (BSA-FAF) was purchased from Sigma-Aldrich (St. Louis, MO). CXCL12 was obtained from PeproTech (Mexico City, Mexico), Pic and DMSO were purchased from Sigma-Aldrich, and R406 was obtained from Santa Cruz Biotechnology (Dallas, TX).

FITC-, PE-, and PerCP-conjugated mAbs specific for CD19 (clone HIB19), CD3 (clone SK7), CD69 (FN50), CD38 (HB7), CD5 (L17F12), or CXCR4 (clone 12G5), as well as control Abs with irrelevant specificities (isotype control), were purchased from BD Biosciences, Pharmingen (San Jose, CA). FITC-ZAP-70 (clone SBZAP) and PC5-CD19 (J3-119) were obtained from Beckman Coulter (Brea, CA). PE-S1P1/EDG-1 (clone 218713) was purchased from R&D Systems, and anti-S1PR1 Ab (clone H60) was obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-rabbit FITC Ab was purchased from Jackson ImmunoResearch (West Grove, PA). For B cell purification, purified mAbs specific for CD14, CD56, CD3, or CD2 were purchased from BioLegend (San Diego, CA), and magnetic beads (Magna-Bind Beads) were purchased from Pierce (Rockford, IL).

CLL patients

Blood and bone marrow samples were collected from 36 and 4 CLL patients, respectively. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires, Argentina. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and either had received no treatment or had not received treatment for ≥ 3 mo before the investigation began. Characteristics of the patients enrolled in this study and the experiments performed with each sample are shown in Table I.

Cell separation procedures

PBMCs were isolated from fresh blood or bone marrow samples by centrifugation over a Ficoll-Hypaque layer (Lymphoprep; Nycomed Pharma, Oslo, Norway), washed twice with saline, and suspended in complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin). Cells were used immediately or were cryopreserved in FCS 20% DMSO for further experiments. B cells (CD19 $^{+}$) were purified by negative selection using mouse mAbs specific for CD14, CD56, CD3, or CD2 and magnetic beads, as described previously (30). The purity of B cells was checked using a FACS flow cytometer (BD Immunocytometry Systems) and anti-CD19 MoAb and was always $>98\%$. The percentage of CD5 $^{-}$ cells within CD19 $^{+}$ lymphocytes was always $<0.5\%$.

Analysis of CD38 and ZAP-70 expression by flow cytometry

CD38 expression on CLL cells was evaluated by flow cytometry using a FACScan flow cytometer (BD Immunocytometry Systems). To this end, PBMCs were stained with anti-CD38 PE mAb or PE-conjugated isotype control Abs and PerCP-conjugated mAbs specific for CD19. ZAP-70 expression in B cells from CLL patients was evaluated by flow cytometry, as previously described (31). Briefly, PBMCs were stained with anti-CD19-PerCP, anti-CD3 PE, or anti-CD56 PE. After washing twice with PBS supplemented with 0.5% BSA, cells were fixed and permeabilized with a FIX & PERM Kit (Caltag Laboratories), according to the manufacturer's instructions. Then, cells were stained with anti-ZAP-70 FITC and acquired using a flow cytometer.

In vitro CLL cell cultures

CLL cell cultures with CXCL12, anti-IgM, and murine fibroblasts expressing CD40L. Purified CLL cells were cultured in RPMI 1640 supplemented with 1% BSA-FAF (5×10^6 cells/ml) in medium alone (control) or in the presence of CXCL12 (500 ng/ml), immobilized anti-IgM Abs (20 μ g/ml; Jackson ImmunoResearch), or murine fibroblasts expressing CD40L (fCD40Ls). After 24 h of culture, CLL cells were collected, and activation was confirmed by flow cytometry by evaluating the surface expression of CD69. The migratory response toward S1P and S1PR1 expression was assessed by flow cytometry or by quantitative real-time PCR (Q-PCR), as described below.

NLC cocultures. *In vitro* NLC differentiation was performed as previously described (7, 32). Briefly, 5×10^6 freshly purified PBMCs from CLL patients were cultured in 1 ml complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in a 24-well cell culture plate for 14 d. Then, nonadherent cells were isolated, whereas attached NLCs were used for the following experiments. In the experiments in Fig. 2A and 2B, cryopreserved PBMCs from the same patients were thawed, and CD19 $^{+}$ cells were purified and cultured alone in RPMI 1640 with 1% BSA-FAF (control) or cocultured with autologous NLCs for 24 h. In contrast, in Fig. 2C and 2D, CLL cells were purified from nonadherent cells isolated after the *in vitro* NLC-differentiation cultures. Then, these purified CLL cells were washed with serum-free medium and cultured alone in RPMI 1640 with 1% BSA-FAF ("removed from NLC coculture") or plated back onto autologous NLCs ("NLC coculture") for 24 h.

Cultures with Pic and R406. CLL cells were cultured in RPMI 1640 1% BSA-FAF in the presence of 20 μ M Pic, 1 or 5 μ M R406, or vehicle (DMSO) for 24 h, and the expression of S1PR1 (flow cytometry and Q-PCR) and the migratory response toward S1P were evaluated as described below.

To evaluate the effect of Pic and R406 on cell survival, we first cultured CLL cells with different doses of Pic and R406 or vehicle alone (DMSO) for 24 h and then evaluated the viability by comparing forward scatter (FSC) and side scatter (SSC) parameters by flow cytometry analysis (33), which was corroborated by fluorescence microscopy using acridine orange and

ethidium bromide (34). We then confirmed that 20 μ M Pic and 5 μ M R406 were able to inhibit Syk-mediated functions, such as migration toward CXCL12 and activation by BCR cross-linking (35). To this end, we cultured CLL cells in the presence of the drugs or the vehicle (DMSO) for 1 h; subsequently, the chemotaxis assay toward 500 ng/ml CXCL12 was performed as described below. In other experiments, CLL cells treated for 1 h with Pic, R406, or vehicle were subsequently cultured with 20 μ g/ml immobilized anti-IgM Abs, and expression of the activation marker CD69 was evaluated by flow cytometry 24 h later.

Chemotaxis assay

Chemotaxis assay toward S1P was carried out using 96-well Transwell chambers with polycarbonate membranes (5- μ m pore size; Costar; Corning, NY), as previously described (29). Briefly, 0.5×10^6 purified CLL cells were placed in the upper chamber in 70 μ l serum-free RPMI 1640 with 0.5% BSA-FAF. The lower chamber of each well contained 200 μ l the same medium, with or without S1P. After 3 h of incubation at 37°C in humidified air with 5% CO₂, the cells in the lower chamber were counted by flow cytometry. The migration index was calculated by determining the ratio of migrated cells in response to S1P versus spontaneous migration, which was considered 100%.

We first performed a dose-response chemotaxis assay toward S1P to select the concentration of S1P to use in the other experiments. To this end, we cultured PBMCs from five CLL patients in serum-free RPMI 1640 with

1% BSA-FAF for 24 h to induce S1PR1 re-expression; we evaluated the migratory response to different doses of S1P and selected 10 and 100 μ M S1P for the other experiments (data not shown). The migration index was calculated by determining the ratio of migrated cells in response to S1P versus the spontaneous migration, which was considered 100%. Spontaneous migration in control wells was consistently <2% of CD19⁺ cells placed in the upper compartment. In accordance with a previous report (36), we found that the proportion of migrating cells in response to S1P ranges between 3 and 8% of CD19⁺ cells placed in the upper compartment (data not shown). The migratory response toward 500 ng/ml CXCL12 was performed as a positive control (data not shown), in which the proportion of migrating cells ranged between 20 and 40% of CD19⁺ cells placed in the upper compartment.

Q-PCR for S1PR1

Total RNA was obtained from 5×10^6 purified CLL cells lysed with TRIzol reagent (Invitrogen). Reverse transcription was carried out using M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Briefly, 2 μ g RNA was incubated for 50 min at 42°C in the presence of 50 pmol Oligo(dT) primer (Biodynamics SRL, Buenos Aires, Argentina) and 10 mM deoxynucleotide triphosphates mix. Q-PCR was performed using SYBR Green PCR Master Mix (Invitrogen) in 20- μ l reactions. S1PR1 cDNA was analyzed using Quantitect Primer Assay (QT00208733) from QIAGEN. Primers for GAPDH were 5'-GAGT-

Table I. Main characteristics of CLL patients enrolled in the study and experiments performed with each sample

CLL	Age	Gender	Binet	(Leukocytes $\times 10^3/\mu$ l)	CD19 ⁺ (%)	CD38 ⁺ (%)	ZAP-70 (%)	Experiment Performed
1	45	M	C	125	95	38	84	CLL activation culture; Pic culture; R406 culture
2	61	F	B	100	97	0.1	11	CLL activation culture, R406 culture
3	84	M	A	82	94	0.1	39	CLL activation culture; R406 culture
4	61	M	B	46	99	15	6	CLL activation culture
5	63	F	C	179	94	90	25	Pic culture
6	40	M	A	17	89	8	ND	NLC coculture
7	72	F	A	14.6	55	50	ND	NLC coculture
8	82	M	B	89	85	18	33	CLL activation culture
9	69	M	A	200	92	6	ND	CLL activation culture
10	70	F	A	68	86	1	35	CLL activation culture
11	57	M	C	330	98	50	90	CLL activation culture; Pic culture
12	80	F	C	171.9	94	1	4	CLL activation culture, R406 culture
13	77	M	A	29	72	29	10	Pic culture; NLC coculture
14	53	M	B	31	78	1	ND	NLC coculture
15	56	M	A	69.4	94	1	ND	CLL activation culture; Pic culture
16	78	F	A	24	76	1	ND	NLC coculture
17	62	M	C	110	91	4	93	Pic culture; R406 culture
18	79	M	A	19	87	1	83	Pic culture; NLC coculture
19	75	F	B	50	89	0.3	69	Pic culture
20	49	F	A	15	80	1	11	NLC coculture
21	77	F	NA	NA	96	0	2	CLL activation culture
22	74	F	A	55.7	84	1	10	CLL activation culture; NLC coculture
23	84	M	B	43.9	90	1	3	NLC coculture; R406 culture
24	67	M	A	67.8	96	1	23	CLL activation culture; Pic culture; R406 culture
25	74	M	A	35	94	88	6	CLL activation culture; Pic culture; R406 culture
26	68	M	B	92	49	57	63	NLC coculture; Pic culture
27	50	M	C	90	94	1	9	CLL activation culture; Pic culture; R406 culture
28	83	M	A	32	78	5	12	NLC coculture
29	74	M	B	180	98	0.5	7	CLL activation culture
30	69	M	A	16.5	77	97	41	Pic culture; NLC coculture
31	71	M	B	27.5	87	54	3	CLL activation culture, R406 culture
32	71	F	C	63	95	11	23	CLL activation culture
33	80	F	C	56	96	0.3	42	CLL activation culture
34	76	F	A	19	84	9	19	BM sample for CD38 sorter experiment
35	51	M	C	75	95	95	22	BM sample for CD38 sorter experiment
36	65	M	C	73.8	61	17	ND	BM sample for CD38 sorter experiment
37	56	M	B	69	88	1	ND	BM sample for CD38 sorter experiment
38	66	F	B	150	85	45	ND	QF/PF sorter experiment
39	51	M	C	65.2	90	7.5	ND	QF/PF sorter experiment
40	55	M	C	220	98	29	ND	QF/PF sorter experiment

CD19⁺ (%) refers to the percentage of CD19⁺ cells within the lymphocyte fraction. The percentages of CD38⁺ cells and ZAP-70⁺ cells were obtained as detailed in *Materials and Methods*. CLL activation culture refers to activations with CXCL12, anti-IgM, and fibroblast CD40L⁺, presented in Fig. 1.

BM, bone marrow; NA, not available; PF, proliferative fraction; Pic, piceatanol; QF, quiescent fraction.

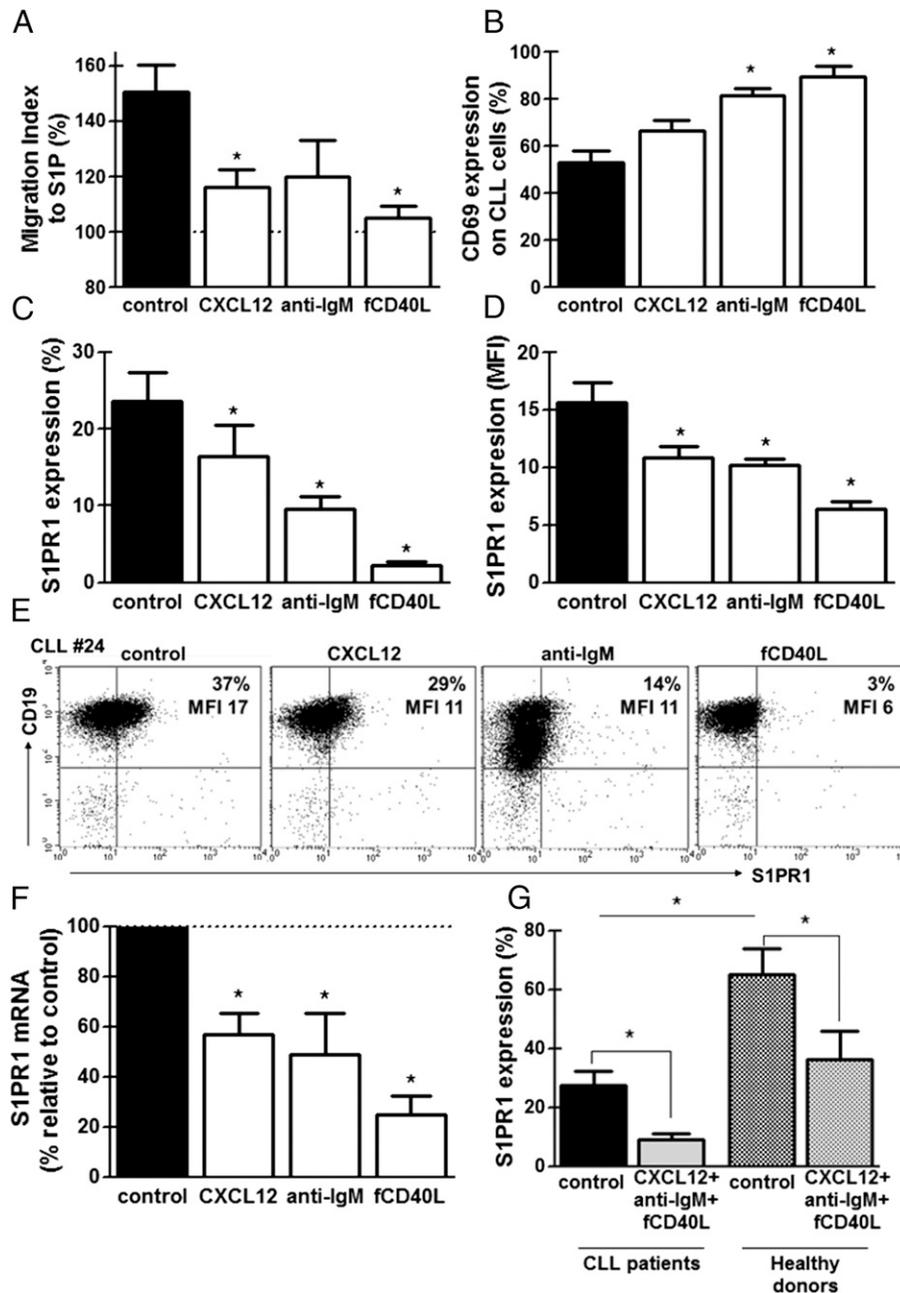


FIGURE 1. CXCL12, BCR triggering, and CD40L reduce S1PR1 expression and migratory response toward S1P of CLL cells. PBMCs from 19 CLL patients were cultured in RPMI 1640 medium supplemented with 1% BSA-FAF alone (control) or in the presence of 500 ng/ml of CXCL12, 20 μ g/ml of immobilized anti-IgM Abs, or fCD40Ls. After 24 h of culture, control and activated cells were collected, and CD19⁺ cells were purified using specific Abs and magnetic beads. The purity of the B cell population was checked by flow cytometry and was always >98%. Then, the following assays were performed. **(A)** The migratory response of CLL cells toward S1P (100 nM in RPMI 1640 0.5% BSA-FAF) was evaluated using the Transwell system, as previously described (29). All assays were performed in duplicate. After a 3-h incubation at 37°C, the migrating cells were stained with PE-Cy5-conjugated anti-CD19 Abs and counted with a FACSCalibur flow cytometer (number of cells acquired in 1 min under a defined flow rate). Data are mean \pm SEM of the migration indexes, which were calculated by determining the ratio of migrated cells in response to S1P versus the spontaneous migration, which was considered 100%. **(B)** The expression of CD69 on CLL cells was assessed by flow cytometry using mAbs anti-CD69 or the corresponding isotype control and anti-CD19. Data are mean \pm SEM of CD69⁺ CLL in the different cultures. **(C and D)** The expression of S1PR1 on CLL cells was assessed by flow cytometry using mAbs anti-S1PR1 or the corresponding isotype control and anti-CD19. The bars represent the mean \pm SEM of the percentage of S1PR1⁺ CLL cells (C) or the mean fluorescence intensity (MFI) of S1PR1 on CLL (D) in the different cultures. **(E)** Representative dot plots. **(F)** S1PR1-mRNA levels were analyzed by Q-PCR, as detailed in *Materials and Methods*. The bars represent the mean \pm SEM, considering the relative levels of S1PR1 mRNA from the control culture as 100%. **(G)** PBMCs from four CLL patients (1, 3, 12, and 27 from Table I) and four healthy donors were cultured alone or in the presence of CXCL12, anti-IgM, or fCD40Ls for 24 h, and the expression of S1PR1 was assessed by flow cytometry, as described above. The bars represent the percentage of S1PR1⁺CD19⁺ cells. * p < 0.05, treated versus control, Kruskal–Wallis test, followed by the Dunn multiple-comparison test (A, B, C, and D); * p < 0.05, treated versus control (100%), Wilcoxon signed-rank test (F); * p < 0.05, Kruskal–Wallis test, followed by the Dunn multiple-comparison test (G).

CAACGGATTTGGTCGT-3' (forward) and 5'-TTGATTTGGAGGGATCTCG-3' (reverse) and were used at a concentration of 250 nM. Reactions were carried out in an ABI 7500 cycler (Applied Biosystems). The cycling

program used was 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 25 s, and 72°C for 35 s. Data were analyzed by the $2^{-\Delta\Delta C_t}$ method using GAPDH as a reference gene.

Statistical analysis

Statistical significance was determined using nonparametric tests: Wilcoxon signed-rank test, Mann–Whitney test, Kruskal–Wallis test, Friedman test, and the Dunn post test. In all cases, $p < 0.05$ was considered statistically significant.

Results

The activation of CLL cells through CXCR4, CD40, and BCR reduces the expression of S1PR1 and the migration toward S1P

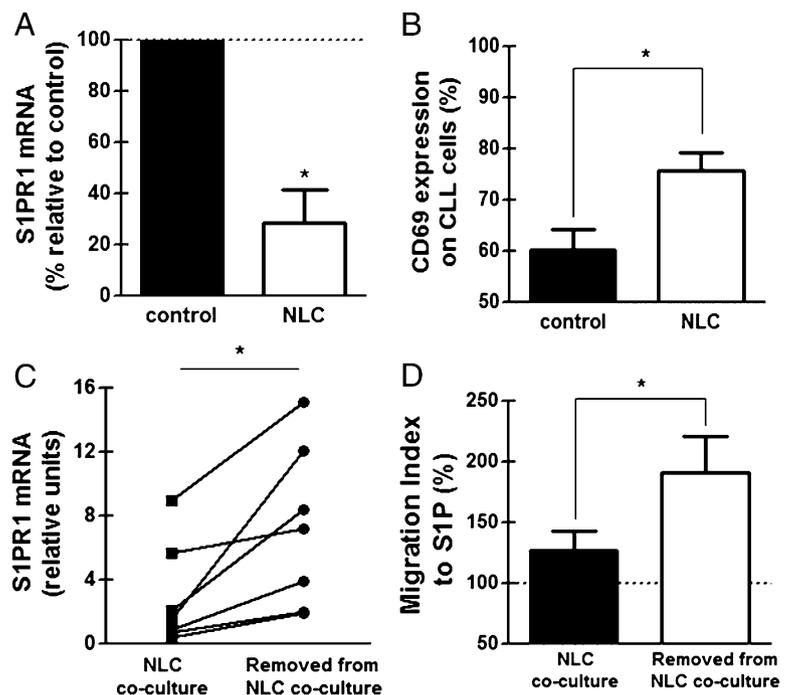
The migratory capacity of CLL cells toward different concentrations of S1P was confirmed using the Transwell system. To this end, CD19⁺ cells were purified from PBMCs from CLL patients by negative selection using magnetic beads and were incubated overnight in serum-free medium in the presence of BSA-FAF to allow recycling of receptors that had been internalized in the presence of the high S1P concentrations in blood. The chemotaxis assay was performed, as detailed in *Materials and Methods*; in accordance with a previous report (29), we found that CLL cells were able to migrate toward 10 and 100 nM of S1P (data not shown). Then, to evaluate whether key microenvironmental signals can modulate the migratory response toward S1P, PBMCs from 19 CLL patients (Table I) were cultured in RPMI 1640 supplemented with BSA-FAF alone (control) or in the presence of CXCL12, immobilized anti-IgM Abs, or fCD40Ls. After 24 h of culture, control and treated cells were collected, and CD19⁺ cells were purified as mentioned above. The purity of the B cell population was checked by flow cytometry and was always >98%. The viability of the cells was evaluated by fluorescent microscopy using acridine orange and ethidium bromide staining and was >90%. Then, the chemotaxis assay toward S1P (100 nM) was performed as mentioned above, and the migration index was calculated by determining the ratio of migrated CD19⁺ cells in response to S1P versus the spontaneous migration, which was considered 100%. As shown in Fig. 1A, we found that culture of CLL cells with the stimuli reduced their migratory response toward S1P, with statistically significant differences for CXCL12 and fCD40Ls. Similar results were obtained when 10 nM of S1P was used (data not shown). As expected, the stimuli increased the

activation of CLL cells, as measured by the surface expression of the activation marker CD69, with statistically significant differences for anti-IgM and fCD40Ls (Fig. 1B). Representative graphs of CD69 expression in control and treated CLL cells are shown in Supplemental Fig. 1A. In addition, the impairment in CLL migration toward S1P induced by the microenvironment was associated with a reduced expression of S1PR1, as evaluated by flow cytometry (Fig. 1C–E) and Q-PCR (Fig. 1F). Moreover, we also compared the expression of S1PR1 in B cells from healthy donors and CLL patients that were cultured or not with CXCL12 + anti-IgM + fCD40Ls. As reported recently (29, 37), we found that healthy B cells express higher levels of S1PR1 compared with B cells from CLL patients and reduce the surface S1PR1 expression (Fig. 1G, Supplemental Fig. 1B) and the migratory response toward S1P (Supplemental Fig. 1C) upon activation. Representative graphs showing the surface expression of S1PR1 in CD19⁺ cells from control and CXCL12 + anti-IgM + fCD40L cultures are shown in Supplemental Fig. 1D.

The presence of autologous NLCs impairs the expression of S1PR1 and the migratory response toward S1P

We next wanted to mimic the microenvironment of lymphoid tissues by obtaining NLCs from CLL patients. To this end, PBMCs from CLL patients were cultured for 14 d; nonadherent cells were separated while attached NLCs were used for coculture experiments. At that moment, cryopreserved PBMCs from the same patients were thawed, and CD19⁺ cells were purified as mentioned above and cultured alone (control) or with autologous NLCs. We found that, after 24 h of culture, mRNA expression of S1PR1 in leukemic cells was reduced by microenvironmental signals provided by autologous NLCs (Fig. 2A). As expected, the expression of CD69 in CLL cells was increased by coculture with NLCs (Fig. 2B). Interestingly, the reduced expression of S1PR1 induced by NLCs seemed to be partially reversible, because removal of CLL cells from this microenvironment restored, to some extent, the expression of the receptor (Fig. 2C) and the migratory response toward S1P (Fig. 2D). However, S1PR1 expression in leukemic cells detached from NLC cultures was still very low compared with that observed in control CLL cells (not shown).

FIGURE 2. NLCs impair the expression of S1PR1 and the migratory response toward S1P. In vitro NLC differentiation was obtained by culturing PBMCs from CLL patients in complete medium for 14 d, as detailed in *Materials and Methods*. Then, nonadherent cells were isolated, whereas attached NLCs were used for the following experiments. (**A** and **B**) Cryopreserved PBMCs from the same patients were thawed, and CD19⁺ cells were purified and cultured alone in RPMI 1640 with 1% BSA-FAF (control) or cocultured with autologous NLCs ($n = 4$; patients 6, 7, 14, and 23 from Table I). After 24 h, CLL cells were collected, and S1PR1 mRNA levels (**A**) and CD69 expression (**B**) on CLL cells were evaluated, as described above. (**C** and **D**) CLL cells were purified from nonadherent cells isolated from the in vitro NLC-differentiation cultures. Then, CLL cells were washed with serum-free medium and cultured alone in RPMI 1640 with 1% BSA-FAF (removed from NLC coculture) or plated back onto autologous NLCs (NLC coculture). After 24 h, the expression of S1PR1 mRNA on CLL cells (patients 6, 7, 14, 16, 20, 23, and 28 from Table I) (**C**) and their migration toward S1P (patients 13, 18, 22, 26, and 30 from Table I) (**D**) were assessed as described above. * $p < 0.05$, Wilcoxon signed-rank test.



In vivo-activated CLL cells express low levels of S1PR1

It was reported that the leukemic clone of CLL patients contains a variety of cells, from resting lymphocytes to leukemic cells that proliferate at different rates (38). Although the activation and proliferation of CLL cells take place in lymphoid tissue micro-environment, different groups identified a small proliferative subset of leukemic cells in peripheral blood (22, 38). The characterization of this small proliferative fraction (PF) and its quiescent counterpart (quiescent fraction [QF]) reveals that they differ in terms of their expression of several molecules, including chemokines, such as CCL22 (39), CCL3, or CCL4 (22); molecules, such as CD38 and Mcl-1 (38, 40); and apoptosis regulators, such as survivin (20) and BCL-2 (22). We aimed to evaluate the expression of S1PR1 in the QF and in the small PF of three CLL patients (Table I). As previously described by Palacios et al. (22), this PF is characterized by the presence of an active class-switch recombination process (Fig. 3A) and a high expression of proliferation-related genes, such as Ki-67, c-myc, CD49d, and p27-Kip1 (22). Interestingly, S1PR1 expression was reduced in the PF compared with the QF (Fig. 3B), showing that, *in vivo*, a small subset of circulating CLL cells expressed lower levels of S1PR1 compared with the rest of the leukemic clone. Additionally, when we evaluated S1PR1 expression in bone marrow-resident CLL cells segregated based on the surface expression of CD38, which defines a subpopulation of activated lymphocytes (38, 40), we found that, in all of the patients evaluated (Table I), bone marrow leukemic cells expressing high levels of CD38 showed a reduced expression of S1PR1 compared with CD38^{low/-} counterparts (Fig. 3C).

Pic and R406 enhance the expression of S1PR1 in CLL cells and their migratory response toward S1P

Because it was reported previously that treatment of CLL patients with Syk inhibitors induces a transient mobilization of leukemic cells from lymph nodes into the circulation (41), we wondered whether they enhanced the expression of S1PR1. To this end, purified CLL cells from 13 patients (Table I) were cultured for 24 h in the presence of the Syk inhibitor Pic or the corresponding vehicle (DMSO). The effective dose of Pic (20 μ M) was selected because it inhibits BCR-induced CLL activation (Fig. 4A) and CXCL12-induced migration (Fig. 4B) (35) without inducing significant apoptosis at 24 h (Fig. 4C). We found that *in vitro* treatment with Pic enhanced the migratory response of CLL cells toward S1P (Fig. 5A) and the expression of S1PR1 at both the mRNA level (Fig. 5B) and protein level (Fig. 5C, Supplemental Fig. 1E). A representative graph showing the expression of S1PR1 in CLL cells from control and Pic-treated cultures is shown in Fig. 5D.

The Syk inhibitor fostamatinib (R788), which is rapidly converted *in vivo* into its bioactive form R406, showed encouraging results in a phase I/II study in patients with CLL by inducing tumor cell death, clinical responses in a critical proportion of the patients, and transient lymphocytosis due to redistribution of CLL cells (41). Interestingly, we found that *in vitro* treatment with R406, in a clinically relevant dose (5 μ M) that was capable of inhibiting BCR-induced CLL activation (Fig. 4D) and CXCL12-induced migration (Fig. 4E) without inducing significant apoptosis at 24 h (Fig. 4F), also reduced the migratory response of CLL cells toward S1P (Fig. 5E) and the expression of S1PR1 evaluated by Q-PCR (Fig. 5F) and flow cytometry (Fig. 5G, Supplemental Fig. 1F). Representative dot plots showing the expression of S1PR1 in CLL cells from control and R406-treated cultures are shown in Fig. 5H.

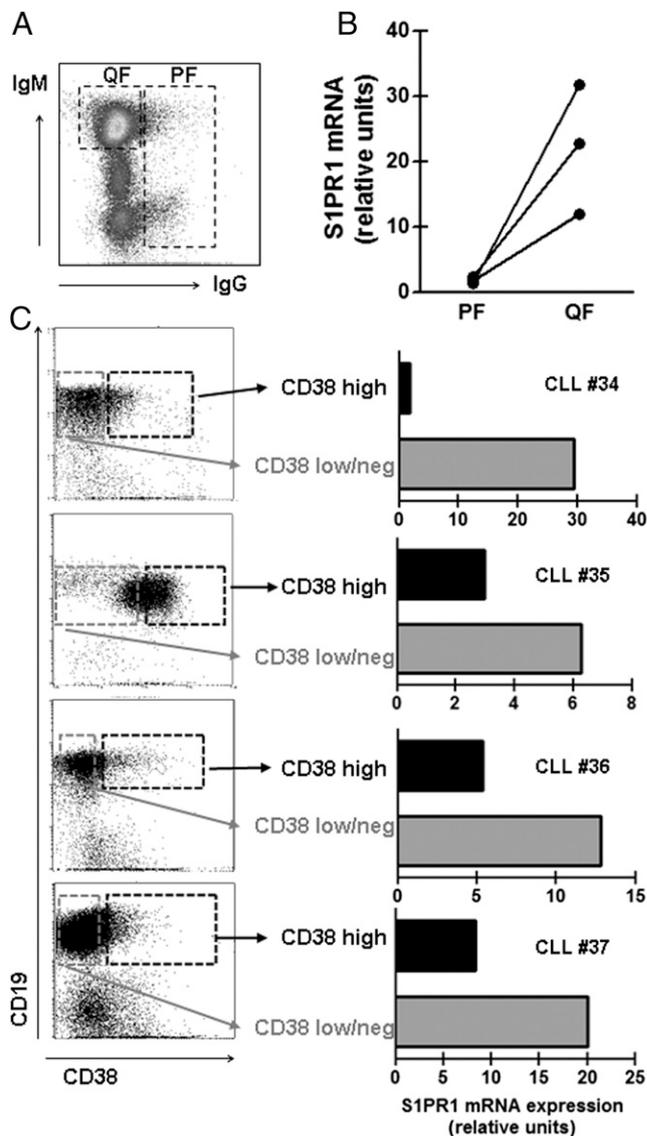


FIGURE 3. S1PR1 expression of *in vivo*-activated CLL cells. (**A** and **B**) PBMCs from three CLL patients (patients 38, 39, and 40 from Table I) were incubated with mAbs anti-IgG, anti-IgM, anti-CD5, and anti-CD19 for 20 min, washed twice, and sorted by FACS, as previously described (22). Cells were maintained ice-cold throughout the isolation procedure. Then, total RNA was purified from the IgG⁺ cells (PF) and IgM⁺IgG⁻ cells (QF), and the expression of S1PR1 mRNA was evaluated by Q-PCR, as described above. (**A**) Dot plot of a representative patient showing the expression of IgM and IgG. (**B**) The bars represent the mean \pm SEM, considering the relative levels of S1PR1 mRNA from the QF as 100%. (**C**) Bone marrow mononuclear cells from four CLL patients (patients 34–37 from Table I) were incubated with mAbs anti-CD19-PerCP and anti-CD38-PE for 20 min, washed twice, and sorted by FACS. Cells were maintained ice-cold throughout the isolation procedure. Then, total RNA was purified from the CD19⁺ CD38^{high} fraction and from the CD19⁺ CD38^{low/-} fraction, and S1PR1 mRNA levels were evaluated by Q-PCR. Dot plots of CD19 and CD38 expression of each patient (*left panels*) and S1PR1 mRNA relative units (*right panels*) are shown.

Discussion

The interest in understanding the mechanisms that regulate CLL cell recirculation between blood and lymphoid tissues increased rather *in vitro* and *in vivo* data undoubtedly identified the tumor microenvironment of lymphoid organs as the supportive milieu where the leukemic clone survives and proliferates (6). The homing

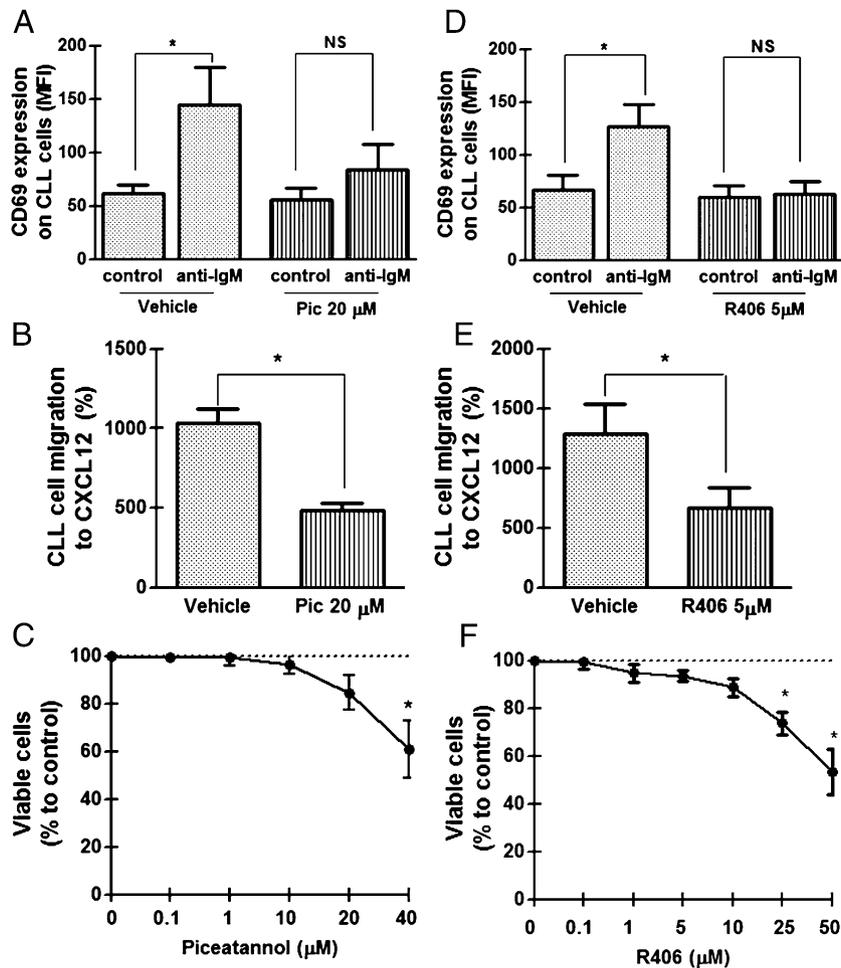


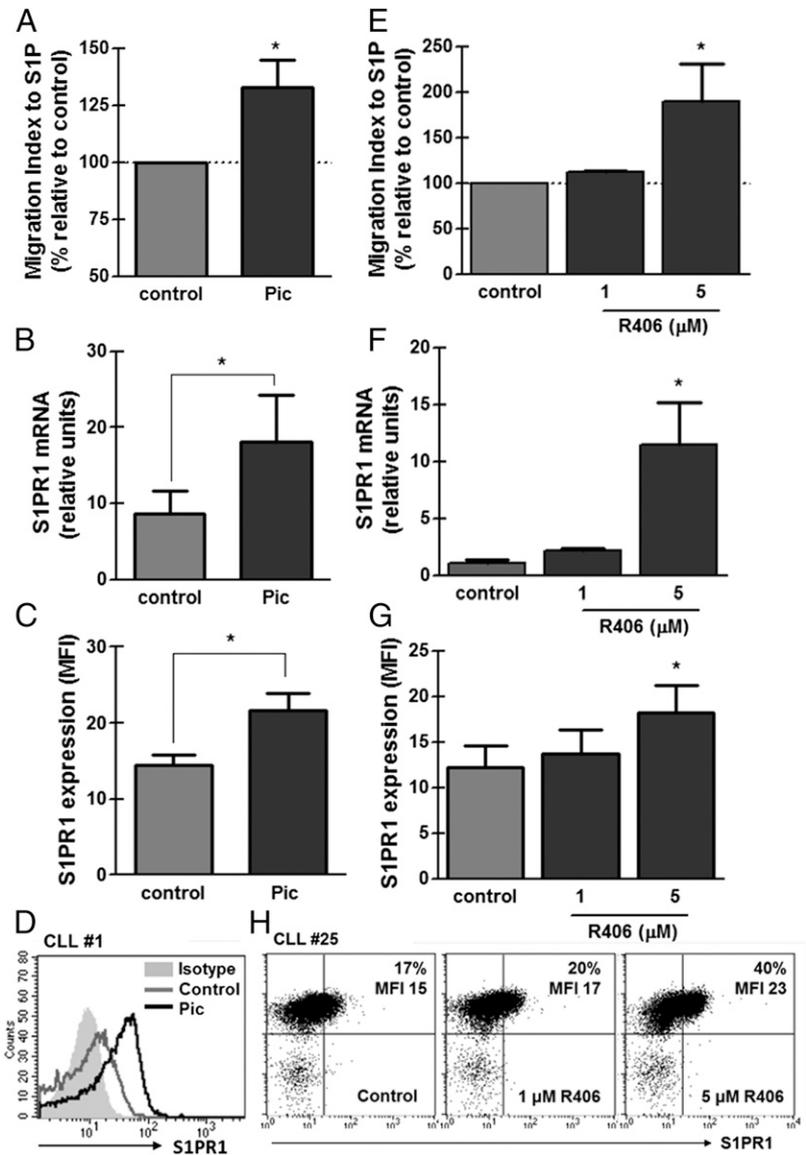
FIGURE 4. CLL cultures with Pic and R406. **(A)** PBMCs from six CLL patients were treated with 20 μM of Pic or vehicle (DMSO) for 1 h and then cultured with immobilized anti-IgM Abs for 24 h. Cells were collected, and CD69 expression was analyzed by flow cytometry. Data are mean \pm SEM of mean fluorescence intensity (MFI) of CD69 on CLL cells of each culture. $*p < 0.05$, Friedman test, followed by the Dunn post test. **(B)** PBMCs from four CLL patients were cultured in the presence of 20 μM of Pic or vehicle (DMSO) for 1 h, and the migratory response to CXCL12 was evaluated using the Transwell system, as described in *Materials and Methods*. Data are the mean migration indexes \pm SEM. $*p < 0.05$, Mann-Whitney *U* test. **(C)** CLL cells from six patients were cultured in the presence of different doses of Pic or vehicle alone (DMSO) at 37°C. After 24 h of culture, apoptosis was evaluated by comparing FSC and SSC parameters by flow cytometry analysis (33). The graph shows the percentage of viable cells in each condition relative to the control culture (with DMSO). $*p < 0.05$, treated versus control (100%), Wilcoxon signed-rank test. **(D)** PBMCs from six CLL patients were treated with 5 μM of R406 or vehicle (DMSO) for 1 h and then cultured with immobilized anti-IgM Abs for 24 h. Then, cells were collected, and CD69 expression was analyzed by flow cytometry. $*p < 0.05$, Friedman test, followed by the Dunn post test. **(E)** PBMCs from four CLL patients were cultured in the presence of 5 μM of R406 or vehicle (DMSO) for 1 h, and the migratory response to CXCL12 was evaluated using the Transwell system, as described in *Materials and Methods*. Data are mean of the migration indexes \pm SEM. $*p < 0.05$, Mann-Whitney *U* test. **(F)** CLL cells from six patients were cultured in the presence of different doses of R406 or vehicle alone (DMSO) at 37°C. After 24 h of culture, apoptosis was evaluated by comparing FSC and SSC parameters by flow cytometry (33). The graph shows the percentage of viable cells in each condition relative to the control culture (with DMSO). $*p < 0.05$, treated versus control (100%), Wilcoxon signed-rank test.

of CLL cells into lymphoid tissues is a very well-studied process that is mediated by chemokines and their receptors, as well as adhesion molecules and their ligands (28). In contrast, less is known about the mechanisms that regulate leukemic clone exit into the circulation. It was reported that S1PR1 is expressed at variable levels on leukemic cells from CLL patients, which are able to migrate toward SIP (29). The results presented in this article provide further insights into the dynamic regulation of S1PR1 expression in the leukemic clone. We found that expression of the receptor was reduced by different signals that leukemic cells may encounter within the supportive microenvironment of lymphoid tissues, including CXCL12, AgR cross-linking, and CD40-CD40L interactions. As expected, these stimuli induced the upregulation of the activation marker CD69 in our CLL samples, which was accompanied by an impaired migratory response toward SIP. Our results

suggest that leukemic cells that have contact with the supportive microenvironment may reduce the expression of S1PR1 and may delay their egress into the circulation, extending their stay at survival niches. In line with this, we speculate that CLL cells that enter lymphoid tissues but do not actively interact with the supportive microenvironment will not reduce their S1PR1 expression, favoring a more rapid return to the circulation. In accordance with a recent report (37), our results with healthy B cells suggest that the modulation of S1PR1 expression that is induced by B cell activation may also regulate their exit from lymphoid organs.

We also evaluated the effect of autologous NLCs on the expression of S1PR1, because NLCs can be found in the spleen and secondary lymphoid tissue of patients with CLL (9, 42); thus, they represent a useful model for the microenvironment in secondary lymphatic tissues (6). In addition, it was reported that gene ex-

FIGURE 5. Pic and R406 enhance S1PR1 expression and migration toward S1P. **(A–D)** Purified CLL cells from 13 patients (Table I) were cultured in the presence of 20 μM of Pic or vehicle (DMSO) for 24 h in RPMI 1640 with 1% BSA-FAF. After 24 h of culture, the migration toward 100 nM of S1P (A) and the expression of S1PR1 mRNA (B) were evaluated as described above. (C) Surface expression of S1PR1 also was evaluated by flow cytometry using anti-S1PR1 or the corresponding isotype control and anti-CD19-PeCy5. (D) Surface expression of S1PR1. **(E–H)** Purified CLL cells from 10 patients (Table I) were cultured in the presence of 1 or 5 μM of R406 or vehicle (DMSO) for 24 h in RPMI 1640 with 1% BSA-FAF. After 24 h of culture, the migration toward 100 nM of S1P (E) and the expression of S1PR1 mRNA (F) were evaluated, as described above. (G) Surface expression of S1PR1 also was evaluated by flow cytometry using anti-S1PR1 or the corresponding isotype control and anti-CD19-PeCy5. (H) Representative dot plots. * $p < 0.05$, Wilcoxon signed-rank test (A–D); * $p < 0.05$, treated versus control, Friedman test, followed by the Dunn multiple-comparison test (E–H).



pression profiles of CLL cells after NLC coculture (19) are strikingly similar to those of CLL cells isolated from secondary lymphatic tissues (21), which show clear signs of cellular activation. Our results showed that coculture with autologous NLCs reduced the expression of S1PR1 mRNA in CLL cells, whereas, as expected, it induced the upregulation of the activation marker CD69. NLCs express numerous factors that participate in the cross-talk with CLL cells (6), including CXCL12, which, by acting through its main receptor CXCR4, mediates leukemic cell homing to lymphoid tissues (8), displays a direct prosurvival effect in CLL (7), and also may be involved in the impaired S1PR1 expression induced by NLCs.

It was proposed by Calissano et al. (18) that, once inside lymphoid tissues, CLL cells that interact with the CXCL12-producing stroma or NLCs will be activated and divide, favoring the detachment of CLL cells from the microenvironment and their exit from the tissues (18). Our results showed that, when CLL cells were removed from the cultures with autologous NLCs, the expression of S1PR1 was partially restored. Therefore, it is tempting to speculate that the detachment proposed by Calissano et al. (18) may be accompanied by an increment in S1PR1 expression in these CLL cells.

Unlike the situation in T cells, in which the transcription factor Krüppel-like factor 2 is clearly responsible for S1PR1 expression

(43), B cell expression of S1PR1 does not show Krüppel-like factor 2-dependent regulation (44). However, it was reported recently that the molecule p66Shc, a Shc adaptor family member, controls S1PR1 transcription in leukemic cells from CLL patients through its pro-oxidant activity (29). Given that this study identified S1PR1 as a reactive oxygen species-responsive gene in CLL (29), the possibility exists that the supportive microenvironment signals reduce the expression of the receptor by modifying the redox status of the cells. In that sense, it was previously reported that tissue stromal cells modulate the endogenous effect of reactive oxygen species in CLL cells by providing cysteine, an essential nutrient that enables leukemia cells to produce glutathione and resist oxidative stress (45).

We then wanted to evaluate whether S1PR1 was reduced in CLL cells activated in vivo. We took advantage of the intracolon analyses of CLL cells that were reported, which suggest that the leukemic clone contains a spectrum of cells from the PF, enriched in activated, recently divided robust cells that are lymphoid tissue emigrants, to the QF enriched in older, less vital cells that need to immigrate to lymphoid tissue or die (18, 22). Considering that surface S1PR1 is extremely sensitive to desensitization and internalization in the continued presence of S1P, as well as the fact that, once lymphocytes reach circulation, the surface expression of

S1PR1 is rapidly downregulated (46) as a result of the high amounts of SIP in lymph and blood (47), we decided to evaluate S1PR1 by Q-PCR in the circulating PF and QF. As expected, S1PR1 expression was reduced in the PF compared with the QF of the same patient, suggesting a more recent contact between the first subpopulation and the activatory microenvironment of lymphoid tissues. Moreover, we also found that tissue-resident CLL cells from the bone marrow expressing high levels of the activation marker CD38 showed lower levels of S1PR1 compared with CD38^{low/-} counterparts. Altogether, these results suggest that the in vivo interaction with the microenvironment may reduce the expression of S1PR1.

Finally, we were interested in the kinase inhibitors that target BCR signaling. The most remarkable clinical observation common to inhibitors of Syk (41), Btk (48), and PI3K δ (49) is the rapid resolution of lymphadenopathies and splenomegaly within the first few weeks of therapy, which is accompanied by a transient increase in blood lymphocyte counts, suggesting CLL cell redistribution from the tissues into the peripheral blood. We evaluated the in vitro effects of two Syk inhibitors on the expression and function of S1PR1. Fostamatinib (R788), the prodrug of R406, is a clinically used oral formulation, which, although it is a more potent Syk inhibitor compared with Pic, might also have activity against other kinases (50). However, it is a clinically relevant drug in CLL because it showed exciting results in relapsed B cell non-Hodgkin's lymphoma and CLL; its clinical efficacy was observed in a variety of histologies, with the highest response rate in CLL patients (41). Moreover, it was reported recently that in vivo treatment using fostamatinib inhibits BCR signaling, cellular activation, and tumor proliferation in patients with relapsed and refractory CLL (51). Our in vitro results with Pic and R406 showed that these drugs enhanced the expression of S1PR1 in CLL cells both at the mRNA and protein levels and improved the migratory response toward SIP. Although we cannot rule out that kinases other than Syk are also inhibited, our observations suggest that S1PR1 upregulation may be involved in the in vivo leukemic cell mobilization that is observed in CLL patients after fostamatinib treatment.

In conclusion, because we found that S1PR1 expression was reduced by the supportive tumor microenvironment and was enhanced by Pic and R406, we suggest that the regulated expression of S1PR1 might modulate the egress of the leukemic clone from lymphoid tissues to peripheral blood. The mobilization of tissue-resident cells into the blood removes CLL cells from this nurturing milieu and sensitizes them to cytotoxic drugs. Therefore, a better understanding of the role of S1PR1 in CLL will contribute to our knowledge of leukemic cell biology and might assist in the discovery of potential targets for novel therapeutic treatments.

Acknowledgments

We thank Beatriz Loria, Gabriela Biscochea, and María Tejada for technical assistance. We also thank the CLL patients who participated in the study.

Disclosures

The authors have no financial conflicts of interest.

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