

CXCL13 Is Highly Produced by Sézary Cells and Enhances Their Migratory Ability via a Synergistic Mechanism Involving CCL19 and CCL21 Chemokines

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Abstract

Chemokine and chemokine receptors expressed by normal and neoplastic lymphocytes play a key role in cell recruitment into skin and lymph nodes. The aim of this study was to get further insights into the role of chemokines in pathogenesis and progression of cutaneous T-cell lymphoma (CTCL) with particular regard to Sézary Syndrome (SS), a CTCL variant with blood involvement. Here, we show that functional CXCL13 homeostatic chemokine is strongly up-regulated in SS cells, well-detectable in skin lesions and lymph nodes, and measurable at high concentration in plasma of SS patients, at different levels during disease progression. Furthermore, we show that the addition of CXCL13 to CCL19 or to CCL21, the selective CCR7 agonists responsible for lymph node homing, strongly enhances the migration of CCR7+ SS cells. We also show that neutralization of the CCR7 receptor strongly impairs CCL19/21-induced chemotaxis of SS cells both in the absence or presence of CXCL13. Additional experiments performed to investigate the survival, adhesion, and metalloproteases secretion indicate that CXCL13 combined with CCL19 and CCL21 mainly affects the chemotaxis of SS cells. Our findings suggest that this newly described CXCL13 expression in SS represents a new pathogenetic mechanism of diagnostic significance. [Cancer Res 2008;68(17):7137–46]

Introduction

Cutaneous T-cell lymphoma (CTCL) represents a malignant expansion of CD4+CD45RO+ memory lymphocytes that show a strong propensity for the skin. The most common entities of CTCLs are Mycosis Fungoides (MF) and Sézary Syndrome (SS). Whereas MF is characterized by a slow clinical course that progresses over years through patches, plaques, and tumoral stage with an involvement of lymph nodes and visceral organs, SS represents an aggressive leukemic variant of CTCL with a short life expectancy, characterized by the presence of malignant lymphocytes in the skin, lymph nodes, and peripheral blood (1, 2).

In these last years, a number of studies have characterized the circulating neoplastic T lymphocytes of SS as CD45RO+, CCR4+CD26-CD27+CCR7+ cells (3–7). This immunophenotype resembles that observed for central memory T (T_{CM}) lymphocytes (8), and thus, as already proposed (6, 9), SS cells might represent a neoplastic expansion of this T lymphocytes subset. The mechanisms responsible for skin localization of these neoplastic cells are only partially disclosed: they express adhesion molecules such as cutaneous lymphocyte antigen that initiates skin homing by mediating E-selectin-dependent tethering, rolling, and transmigration within cutaneous venules (10).

Chemokines, by interacting with their specific receptors, are crucial regulators of cell chemotaxis/mobilization and homing to tissues. They play pivotal roles both in physiologic and pathologic conditions such as inflammation, tumor cell growth, angiogenesis, and metastasis (11), exerting multiple cell functions including cell locomotion, proliferation, and apoptosis (12). Furthermore, they represent key molecules involved in lymphocyte mobilization to and from the skin (10). Chemokines constitute a redundant and promiscuous system, where many receptors are able to bind to more than one ligand, and many chemokines are able to bind multiple receptors. An important control mechanism of this complex system is represented by posttranslational regulation involving the storage, release, and presentation of chemokines (13), their structure modification by proteases (14, 15) or by homodimeric (16) and heterodimeric complex formation (17, 18). Recent studies have already showed that chemokines are strongly involved in MF and SS pathogenesis and neoplastic cell homing to the skin (4–7, 19–22).

Here, we show, for the first time, that the homeostatic chemokine CXCL13 is up-regulated in SS, being highly expressed not only at skin and lymph node level but also in the plasma of SS patients. Furthermore, we observed a significant increase of the chemotaxis of CCR7+ SS cells when CXCL13 is added to CCL19 or CCL21, the lymphoid chemokines that exert their role controlling leukocytes trafficking during immune responses (23). Finally, we show that the CCR7 receptor neutralization strongly reduced the migration response of malignant T lymphocytes to CCL19 and CCL21 used alone or in the presence of CXCL13.

Materials and Methods

Patients and HCs. Peripheral blood samples from a total of 14 patients affected by SS (mean age, 57.5 ± 11.8 y; see Table 1 for clinical information), 10 by MF (64 ± 11), 3 by T-Prolymphocytic leukemia (T-PLL; 67 ± 13) and

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Table 1. SS patient characteristics on which RT-PCR and FACS analysis were evaluated

Patient	Sex/age	V β Family	V β clonal, %*	T/N/M	Disease duration (mo)	Current therapy
1	F/61	13.6	98	T4/N0/M0	46	30 ECP + IFN + Steroids syst
2	M/49	Failed	88	T4/N1/M0	8	5 ECP + IFN + Steroids syst
3	F/61	Failed	79	T4/N0/M0	38	30 ECP + Steroids syst
4	M/62	Failed	85	T4/N1/M1	6	1 ECP + IFN
5	M/86	13.2	86	T4/N1/M1	78	NONE
6	M/38	5.1	58	T4/N0/M0	22	10 ECP
7	M/45	17	91	T4/N2/M0	0	1 ECP + IFN
8	M/56	2	92	T4/N0/M0	10	1 ECP + Steroids syst
9	M/70	12	88	T4/N0/M0	0	1 ECP + IFN
10	F/58	7.2	80	T4/N0/M0	20	1 ECP
11	M/60	2	54	T4/N0/M0	0	IFN
12	M/46	5.1	77	T4/N0/M0	30	5 ECP + IFN + Steroids syst
13	M/52	12	99	T4/N0/M0	33	10 ECP + IFN
14	F/61	13.6	45	T4/N0/M0	36	24 ECP + IFN

NOTE: Numbers indicate the cycles.

Abbreviation: Nd, not done.

*% of dominant TCRV β +circulating neoplastic cells.

†% CCR7+, CXCR3+, and CXCR5+ cells within TCR-V β +CD3+CD4+ analyzed by FACS; FI, evaluated by qRT-PCR, of the chemokines and chemokine receptors expression of SS patients versus baseline represented by healthy donors.

compared with 10 age-matched healthy controls (HC; 59.6 ± 4.8) were analyzed in this study. Diagnosis of SS, MF, and T-PLL was based on clinical, histologic, and biological criteria. All patients analyzed in this study were enrolled in clinical protocols approved by the Ethical Committee of the Istituto Dermopatico dell'Immacolata, and informed consent was obtained in accordance with the Declaration of Helsinki.

Cell isolation. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Histopaque density gradient centrifugation (Sigma-Aldrich-Biochemicals) and CD4+ neoplastic and healthy lymphocytes (HL) were purified by positive selection using anti-human CD4-conjugated dynabeads according to manufacturer's instructions (Oxoid).

Cell lines. Raji cell line, from American Type Culture Collection (CCL-18), derived from peripheral blood of a patient affected by Burkitt lymphoma and HT-1080 (CCL-121) established from human fibrosarcoma cells were grown in complete RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Life Technologies).

Quantitative reverse transcription-PCR. Total RNA was extracted using Trizol (Life Technologies) and cDNA was generated by SuperScript II reverse transcriptase (Life Technologies) following manufacturer's instructions. Primers pairs are shown in Supplementary Table S1; quantitative reverse transcription-PCR (qRT-PCR) was performed with a SYBR Green I dye chemistry and AmpliTaq Gold DNA Polymerase on ABI PRISM 7000 machine (Applied-Biosystems). Quantitative gene expression were analyzed by the $\Delta\Delta C_T$ method as previously described (24).

Flow cytometry. SS samples showed a neoplastic clonal expansion from 45% to 99% as shown by the detection of a dominant TCR-V β rearrangement by fluorescence-activated cell sorting (FACS) analysis (Table 1). To analyze CCR7, CXCR3, and CXCR5 chemokine receptor expression, we followed the procedure already described (5). Antibodies used were purchased from (Becton Dickinson).

Intracellular CXCL13 expression was also evaluated by FACS analysis. Briefly, PBMCs from 4 SS, 2 MF patients, and 4 HC were fixed in 2% paraformaldehyde and permeabilized in 0.1% Triton-X in PBS. Cells stained with unconjugated mouse anti-human CXCL13 antibody (R&D) at 1 μ g/mL at room temperature for 30 min or with isotype-matched control Ab (Becton Dickinson) followed by FITC-conjugated goat anti-mouse IgG

(Becton Dickinson). To evaluate the coexpression of CXCL13 and CCR7 within CD4+ neoplastic and HLs, we performed a three-color flow cytometry with allophycocyanine-conjugated mouse anti-CD4 (Becton Dickinson) and phycoerythrin-conjugated mouse anti-CCR7 (R&D) monoclonal antibodies (mAb) and analyzed on FACS Aria Instrument (Becton Dickinson) using Diva software.

Immunohistochemistry. Paraffin-embedded skin tissues from SS ($n = 16$) and MF patients ($n = 14$) and metastatic lymph-nodes (SS, $n = 4$; MF, $n = 2$) were selected from the files of IDI Pathology. Pathologic specimens were classified according to the European Organization for Research and Treatment of Cancer classification (1, 2).

CXCL13 and CCL21 antigens were retrieved in citrate buffer at pH 6.0 (Dako) by incubating them in a bath at 96°C for 30 min and by microwave at 750 Watts for 10 min, respectively. A total of 30 skin and 6 lymph node biopsies from SS/MF were incubated overnight at +4°C with goat polyclonal anti-CXCL13 Ab (R&D) at 5 μ g/mL. A total of 18 skin and 6 lymph node biopsies from SS/MF were incubated overnight at +4°C with polyclonal goat anti-human CCL21 Ab (R&D) at 12.5 μ g/mL. Immunohistochemistry (IHC) was performed following the procedures already described in (5). Neoplastic T lymphocytes were recognized in the histopathologic sections by morphologic features.

ELISA. Plasmatic CXCL13 level was determined using specific Quantikine ELISA kits (R&D) according to the manufacturer's instructions. CXCL13 was measured in frozen plasma of the above described SS, MF, T-PLL patients, and HC. In 4 of the 14 SS patients, we had samples at different follow-up ($n = 12$). Totally, in SS, we measured CXCL13 concentration in 9 plasma at onset, 6 plasma after 1 y, and 11 plasma at 3 y postdiagnosis. Absorbances were measured at 450 nm by microplate reader (Bio-Rad). Protein level was calculated from a standard curve generated by a curve-fitting program. All samples were analyzed in duplicates.

Chemotaxis. Chemotaxis was performed in 24-well Transwell inserts with 5- μ m pore-size filters (Corning Costar) as previously described (5). For CXCL13 functional studies, Raji cells were chemoattracted by plasma of 4 HC and 4 SS patients diluted 1:25 in migration medium (MM). Response specificity was evaluated by preincubating Raji cells with an anti-CXCR5 neutralizing Ab (R&D) or an isotype-matched negative control (Becton

Table 1. SS patient characteristics on which RT-PCR and FACS analysis were evaluated (Cont'd)

FACS analysis			RT-PCR analysis				
CCR7 ⁺ SS cells% [†]	CXCR3 ⁺ SS cells% [†]	CXCR5 ⁺ SS cells% [†]	FI CXCL13	FI CXCR5	FI CXCR3	FI CCL19	FI CCL21
82	1	0.3	1,640 ± 0.006	2.40 ± 0.25	2.21 ± 0.12	1.1 ± 0.17	0.32 ± 0.67
38	1	0.5	724.08 ± 0.30	4.95 ± 0.20	1.65 ± 0.14	0.8 ± 0.23	0.41 ± 0.007
96	1.5	0.4	975.5 ± 0.03	2.50 ± 0.26	3.60 ± 0.03	3.7 ± 0.018	3.54 ± 0.38
82	1.5	<0.1	Nd	1.54 ± 0.17	3.75 ± 0.03	1.2 ± 0.17	1.03 ± 0.76
87	Nd	0.5	2,320 ± 0.28	1.68 ± 0.87	1.26 ± 0.04	4 ± 0.021	6.41 ± 0.04
63	Nd	<0.5	3.14 ± 0.26	1.74 ± 0.23	1.6 ± 0.56	0.9 ± 0.024	0.04 ± 0.28
51	50	0.1	87 ± 0.57	7.62 ± 0.34	11.23 ± 0.06	Nd	Nd
92	0	0.2	69 ± 0.17	1.61 ± 0.45	6.14 ± 0.48	1.4 ± 0.37	0.09 ± 0.4
82	1	0.4	685 ± 0.2	Nd	Nd	Nd	Nd
45	2	0.5	94 ± 0.3	Nd	Nd	Nd	Nd
90	2	0.3	121 ± 1.19	Nd	Nd	Nd	Nd
78	Nd	<0.1	1,234 ± 0.59	Nd	Nd	Nd	Nd
35	2.3	0.39	Nd	Nd	Nd	Nd	Nd
99	24	0.4	Nd	Nd	Nd	Nd	Nd

Dickinson) for 15 min before the assay. After 90 min, migrated cells were counted by a hemocytometer. Migration responses of Raji cells to increasing concentrations of human recombinant (rh) CXCL13 (from 0.01–500 ng/mL) were also evaluated. Results shown as MIs represent the ratio between the anti-CXCR5 or IgG1-treated cells, or untreated cells, able to migrate in response to plasma or rhCXCL13, and the cells migrated in response to MM.

For dose-response studies, PBMCs from 3 SS patients were allowed to migrate to rhCCL19 and rhCCL21 (R&D). Chemokines at 10, 100, 300, 500, 750, and 1,000 ng/mL were added to the lower chamber.

For synergistic studies, PBMCs from 7 SS patients and 6 HC were allowed to migrate in response to either rhCXCL13 (1.5 µg/mL), rhCCL19 (750 ng/mL), or rhCCL21 (750 ng/mL; R&D). Furthermore, the response to rhCCL19 or rhCCL21 was evaluated also in the presence of CXCL13 at 1.5 µg/mL. For inhibition studies, PBMCs isolated from 5 patients and from 5 HC were preincubated with 1 µg/mL of anti-CCR7 neutralizing Ab (R&D) or with an irrelevant murine isotype-matched IgG1. After 15 min at 37°C, cells were placed in the upper chamber and allowed to migrate for 2 h at 37°C in response to chemoattractants.

Migrated PBMCs were costained by phycoerythrin-conjugated mouse anti-CD4 and, for neoplastic samples, also by FITC-conjugated mouse-specific anti-Vβ mAbs. CD4+Vβ+ neoplastic and CD4+ HLs were counted by FACScan for 60 s at a flow rate of 60 mL/min, as described (25).

Adhesion assays. Four SS patients were analyzed by adhesion assay. Briefly, 10⁵ PBMCs from SS individuals untreated or stimulated as described in (Fig. 4C) were plated on 0.2% gelatin-coated (Sigma Aldrich Biochemicals) 96-well tissue culture plates and allowed to adhere for 30 min at 37°C. Cell adhesion was quantified by staining with 0.5% toluidine blue as previously described (26).

Gelatin zymography. PBMCs (2 × 10⁶/mL) from 4 SS patients were cultured in serum-free medium (X-vivo; Bio-Whittaker), stimulated for 6 h by chemokines, and conditioned medium were then collected by centrifugation. Matrix metalloproteinases (MMPs) activity was analyzed using SDS-PAGE under nonreducing conditions in 7.5% acrylamide gels containing 2 mg/mL gelatin. After separation of MMPs by electrophoresis, gels were treated to cleave the proforms to activated forms as previously described (27).

Statistical analysis. Results are expressed as mean ± SD or SE. Statistical analysis was performed using Student's *t* test or ANOVA.

Results

CXCL13 is highly expressed by circulating cells, skin, and lymph nodes of SS patients. Affymetrix expression analysis previously performed on CD4+ lymphocytes of SS patients versus

controls identified a number of differentially expressed genes including chemokine and chemokine receptors.⁴ Here, we performed qRT-PCR to validate a subset of these genes using mRNAs from sorted-circulating CD4+ cells of SS patients and controls.

As CXCL13 resulted to be up-regulated in our Affymetrix data, we validated the expression of CXCL13, at RNA level (qRT-PCR) and at protein level (FACS and IHC). As shown in Fig. 1A, qRT-PCR results, represented as mean expression values, show a statistical significant difference of CXCL13 expression (*P* = 0.001). In particular, the analysis of CXCL13 showed high level of expression of this chemokine in all SS individuals analyzed: when compared with controls, 7 of 11 (64%) showed a fold increase (FI) ranging from 121 to 2,320; 3 of 11 (27%) showed a FI close to 100; and only 1 out 11 (8%) showed a small FI of 3 (Table 1).

To assess whether the CXCL13 protein was detectable in circulating CD4+ cells, we also performed FACS analysis on PBMCs from 4 SS, 2 MF, and 4 HC used as controls. We observed an abundant CXCL13 expression in neoplastic CD4+ SS cells that also coexpressed CCR7 (Fig. 1B). On the contrary, we did not observe CXCL13 expression in CD4+CCR7+ HLs (Fig. 1C).

To verify whether CXCL13 was also expressed by skin-infiltrating neoplastic T lymphocytes, we performed an IHC analysis on 16 skin and 4 lymph node biopsies from SS patients and 14 skin and 2 lymph node biopsies from MF patients at different stages of disease (from Ia to III), as well as in normal and inflamed skin control samples. CXCL13 immunoreactivity was detected in 13 of 16 (81%) of SS and in 4 of 14 (28%) of MF cases studied. In substantial agreement with previously described data (28), we found that CXCL13 is moderately expressed by endothelial cells of healthy, inflamed, and neoplastic skin (data not shown). In particular, in the SS samples, we observed that 7 of 16 cases (44%) strongly expressed CXCL13 in almost the totality of neoplastic lymphocytes infiltrating the skin, often exhibiting a dot-like immunostaining (Fig. 1D and F). Interestingly, we frequently observed that the CXCL13 positivity is stronger in dermic neoplastic lymphocytes than in those present in the epidermis (Fig. 1E). In 6 of 16 cases (37%),

⁴ Unpublished data.

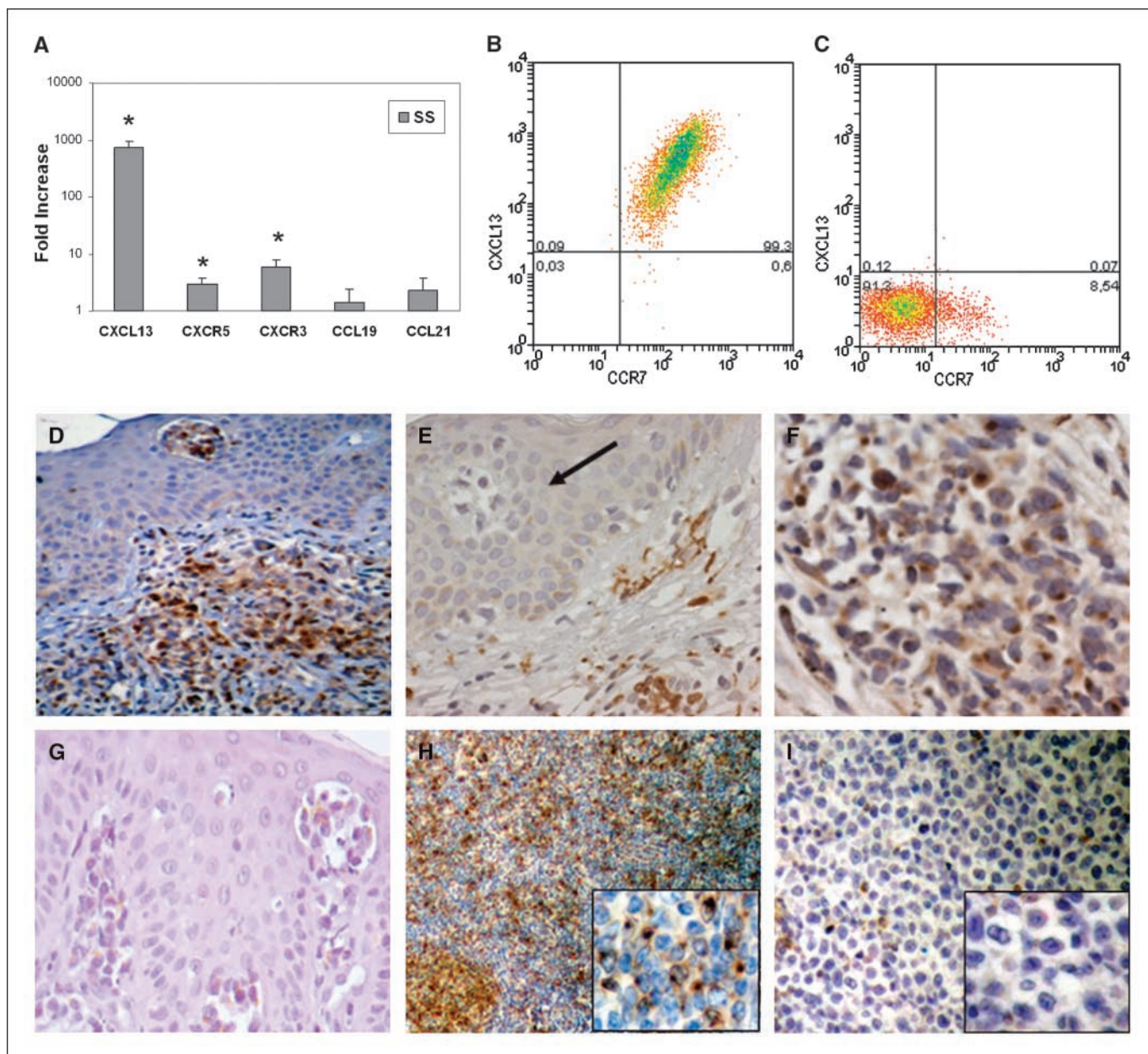


Figure 1. CXCL13 expression in SS and MF. **A**, qRT-PCR for chemokines. The graph represents the FI of chemokines expression calculated for SS patients versus baseline, represented by controls. *Columns*, mean of expression values of two experiments performed in triplicate; *bars*, SE. A significant statistical difference is observable for CXCL13 ($P = 0.001$), CXCR3, and CXCR5 expression ($P \leq 0.02$; *). **B** and **C**, FACS analysis for CXCL13 and CCR7 on PBMCs from SS patients and age-matched HC showing that a strong cytoplasmic CXCL13 expression is detectable within electronically gated CD4+CCR7+ neoplastic lymphocytes (**B**), whereas no CXCL13 expression is associated with CD4+CCR7+ HLs (**C**). The figure is a representative experiment relative to all SS and HC samples analyzed; **D–I**, IHC for CXCL13 in SS and MF skin lesions and lymph-node biopsies. **D**, SS skin lesion showing a strong CXCL13 immunoreactivity associated with neoplastic T lymphocytes of Pautrier's microabscess and neoplastic T lymphocytes that infiltrate dermis [$\times 20/0.40$ numerical aperture (NA)]. **E**, SS skin lesion exhibiting a CXCL13 positivity of dermic neoplastic T lymphocytes, whereas the T lymphocytes of Pautrier's microabscess (*arrow*) seem CXCL13 negative ($\times 20/0.40$ NA). **F**, SS skin lesion displaying neoplastic T lymphocytes with a CXCL13 dot-like immunoreactivity ($\times 60/0.80$ NA). **G**, MF skin lesion where a weak CXCL13 positivity of the neoplastic T lymphocytes of Pautrier's microabscess and infiltrating dermis is observable ($\times 60/0.80$ NA). **H**, lymph node of SS showing a strong CXCL13 immunoreactivity associated with follicular dendritic cells of a residual germinal center and with neoplastic T lymphocytes that infiltrate paracortex ($\times 10/0.25$ NA); *insert*, neoplastic T lymphocytes with the typical dot-like CXCL13 positivity ($\times 40/0.65$ NA). **I**, lymph node of MF with rare CXCL13-positive dendritic cells/histiocytes of the paracortex area, whereas the neoplastic T-lymphocytes seem CXCL13-negative ($\times 20/0.40$ NA); *insert*, neoplastic CXCL13-negative T lymphocytes (magnification, $\times 40/0.65$ NA).

we detected a CXCL13 positivity associated with 5% to 30% of malignant lymphocytes, whereas the remaining 3 of 16 cases (19%) were consistently negative.

On the contrary, in the MF samples analyzed, only 2 of 14 cases (14%) showed a strong CXCL13 immunostaining of the neoplastic lymphocytes. Furthermore, 2 of 14 cases (14%) displayed a CXCL13

immunoreactivity confined to scattered skin neoplastic lymphocytes with a positivity from moderate to strong (Fig. 1G). In the remaining 10 of 14 cases (71%), we did not detect any CXCL13 immunoreactivity associated with malignant cells.

The IHC analyses performed on healthy reactive, SS, and MF lymph node sections revealed, as previously shown (29), a strong

CXCL13 positivity of the endothelial and follicular dendritic cells (Fig. 1H). Furthermore, in 4 of 4 SS lymph nodes (100%), we observed a strong dot-like immunoreactivity for this chemokine associated with infiltrating SS neoplastic lymphocytes (Fig. 1H). On the contrary, in two of MF lymph nodes studied (0%), no infiltrating malignant cells exhibited a CXCL13 immunoreactivity, whereas a CXCL13 expression was observable in rare dendritic cells/histiocytes cells of paracortex (Fig. 1I).

Expression analysis of known CXCL13 receptors (CXCR5 and CXCR3) and of potential interacting chemokines (CCL19 and CCL21) in SS cells. It is widely known that CXCR5 is the main CXCL13 receptor (30) and CXCR3 is a receptor potentially able to bind CXCL13 (31). The expression of these two molecules was evaluated by qRT-PCR and FACS analysis in circulating SS cells (Fig. 1A; Table 1). As shown in Table 1, we observed at mRNA level, a slight increase of these two receptors ($P \leq 0.02$), whereas the expression at protein level was almost absent; in fact, CXCR5+ SS cells were almost undetectable ($n = 14$) as well as the majority of CXCR3+ SS cells ($n = 11$) with the exception of only 2 patients who showed 24% and 50%, respectively, of CXCR3+ cells within the circulating neoplastic population. These results are in agreement with those found in the literature reviewed in (9).

In the same way, we analyzed the expression of *CCL19* and *CCL21* by qRT-PCR because these chemokines are able to form complexes with CXCL13 that bind CCR7 (32), a receptor expressed on the majority of SS cells ($73\% \pm 23\%$). Also, these chemokines were not significantly augmented in circulating SS cells (Fig. 1A; Table 1). In conclusion, among all these chemokines and chemokine receptors, only *CXCL13* was really overexpressed in circulating SS cells.

CXCL13 is highly measurable in plasma of SS patients and is functionally active. We also measured by ELISA, the plasmatic concentration of this chemokine in patients affected by SS, MF, T-PLL, and HC as described in Materials and Methods section. Interestingly, CXCL13 concentration in SS patients was 800.24 ± 89.3 pg/mL ($n = 26$ including onset and disease progression), whereas the CXCL13 concentration in MF ($n = 10$), T-PLL ($n = 3$), and HC ($n = 5$) was 130 ± 128 , 200 ± 42 , and 12.8 ± 10 pg/mL, respectively. The comparison of SS samples with controls, MF, or T-PLL specimens showed in all cases statistical significance ($P \leq 0.002$). A statistical difference ($P \leq 0.05$) was also observed comparing MF or T-PLL with controls (Fig. 2A). Furthermore, to establish if the higher levels of CXCL13 observed in SS were correlated to tumor progression, we analyzed plasma of SS patients at the onset (0–3 months; $n = 9$) and at more advanced stages of disease (up to 36 months; $n = 11$). The comparison of these two groups revealed a significant increase of CXCL13 during the later phases of SS, increasing from 548 to 1,107 pg/mL ($P = 0.02$; Fig. 2B). Taken together, these results indicate that CXCL13 is augmented in all tumor samples analyzed, and its increment seems more closely related to SS and, particularly, to disease progression.

Before investigating the potential role played by CXCL13 as a soluble factor in SS pathogenesis, we tested its functionality in a chemotaxis assay using Raji cells, a CXCR5+CXCR3-B cell line. The plasma of four HC and four SS patients were used to assess the ability of CXCL13 to chemoattract these cells. To establish whether the observed chemotaxis was specifically induced by the CXCL13-CXCR5 axis, we performed the assay by preincubating Raji cells with an anti-CXCR5 neutralizing mAb or an irrelevant mouse IgG1. As shown in Fig. 2C, SS plasma was able to recruit untreated Raji cells with a migration index (MI) of 2.7 versus 1.2 of control plasma,

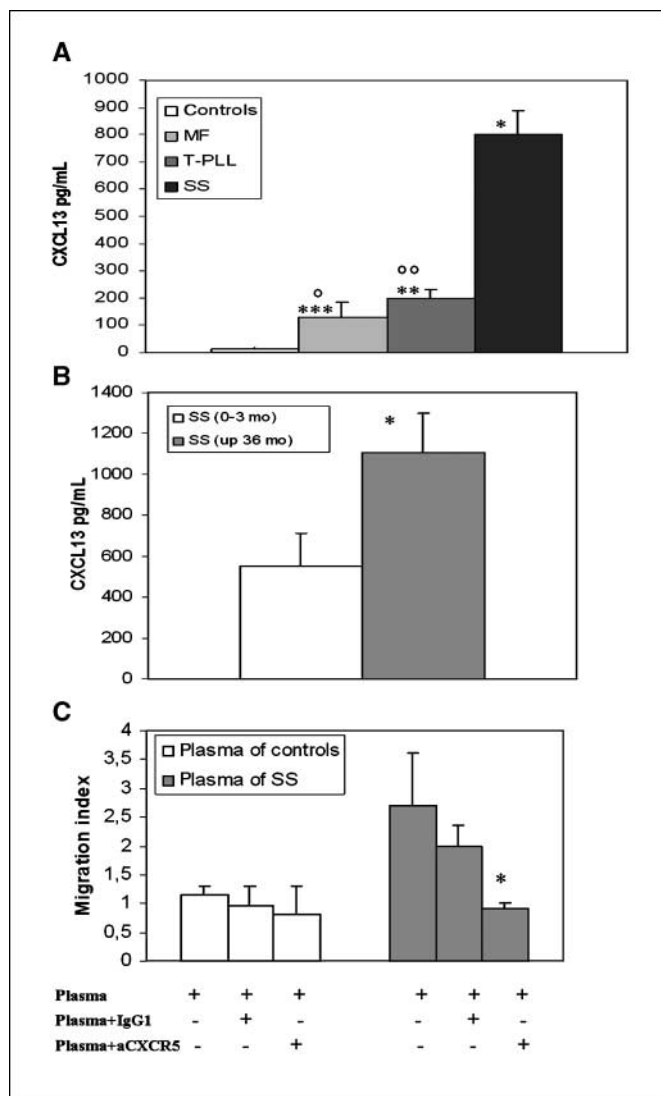


Figure 2. ELISA and chemotactic activity of plasmatic CXCL13. **A**, plasmatic CXCL13 levels from healthy individuals (white bar), MF (gray bar), T-PLL (dark gray bar), and SS patients (black bar) measured by ELISA. Statistical differences between SS and controls (*), SS and MF (***), and SS and T-PLL (**) with a P value of <0.002 and between MF (°) or T-PLL (°°) and controls ($P \leq 0.05$) were calculated by ANOVA. **B**, plasmatic CXCL13 levels of SS patients at the onset (0–3 mo) and at more advanced stages of disease (up to 36 mo) measured by ELISA. A statistical difference between these two groups was found (*, $P = 0.02$). **C**, chemotactic activity of plasmatic CXCL13 from SS patients and HC was evaluated by migration assay performed on CXCR5+ Raji cells. Results (mean $\pm$ SE) are shown as MI. A significant response decrease was observed between anti-CXCR5-treated cells and IgG1-treated cells migrated in response to SS plasma. *, $P = 0.01$.

IgG1-treated cells with a MI of 2 versus 0.95, and anti-CXCR5-treated cells with a MI of 0.9 versus 0.8. When Raji cells were preincubated with an anti-CXCR5 neutralizing Ab, we observed a significant decrease of chemotaxis induced by SS plasma ($P = 0.01$) compared with IgG1-treated cells. These results show that SS plasmatic CXCL13 is functionally active. To further validate this assay, we used also rhCXCL13 to chemoattract Raji cells applying either similar concentrations used in SS plasmatic CXCL13 recruitment (0.01–0.1 ng/mL) or relatively higher levels of recombinant chemokine (from 1–500 ng/mL; Supplementary Fig. S1). The results revealed that Raji cells showed a bell-shaped curve peaking at 100 ng/mL with a MI of 2.5, whereas at lower concentrations,

these cells showed a MI of 1.5 similarly to those observed with SS plasmatic CXCL13. On the contrary, we observed minimal chemotaxis of SS cells (MI, 1.2; $P = 0.07$) in response to rhCXCL13 used at concentration from 10 to 1,500 ng/mL (data not shown). These results indicate that although plasmatic CXCL13 produced from SS patients is functionally active, it is very unlikely that this chemokine acts, in autocrine way, on SS cell migration via CXCR3/5.

CXCL13 treatment enhances CCL19- or CCL21-induced chemotaxis of both CD4+ neoplastic and HLs. Next, we asked if other autocrine mechanisms could act on SS cells, through CCL19 and CCL21, as mentioned above. To address this point, we investigated whether CXCL13 might functionally enhance the CCL19- and/or CCL21-induced *in vitro* chemotaxis of CCR7+SS cells.

Dose-response migration assays performed on PBMCs of 3 SS patients toward CXCL13, CCL19, and CCL21 used alone (from 10–1,000 ng/mL) showed bell-shaped curves peaking at 750 ng/mL for both CCL19 and CCL21 (Fig. 3A). The addition of 1.5 µg/mL CXCL13 to 750 ng/mL CCL19 or CCL21 determined a synergistic effect on CCL19- or CCL21-induced chemotactic responses. For CCL19, an increase of 3.5-fold for SS cells ($P = 0.01$) and 2-fold for HL ($P = 0.02$) was observed (Fig. 4B). A similar effect was also noted when CXCL13 was added to CCL21: an increase of 2.2-fold for SS cells ($P = 0.03$) and 2.5-fold for HL ($P = 0.009$) was detected (Fig. 3B). Synergistic effects were also observed in SS using rhCXCL13, at 750 ng/mL, in combination with CCL19 and CCL21, with 2.6 and 1.5 FI, respectively (data not shown).

Finally, to determine whether the effect of CXCL13 was due to chemotaxis or chemokinesis, we performed a distinct set of experiments in which CXCL13 was added both in the lower and in the upper chamber during the chemotaxis assay in response to CCL19 and CCL21 used in combination with CXCL13. Such addition strongly impaired the CXCL13-induced synergistic effect on SS migration thus indicating that chemotaxis and not chemokinesis is at play (not shown).

The results obtained strongly suggest that CXCL13 might act, in a synergistic manner, with CCL19 and CCL21 in both pathologic- and physiologic-inducing chemotaxis.

CXCL13, CCL21, and CCL19, used alone or in combination, modulate adhesion and metalloprotease secretion of SS cells. To better understand which other mechanisms were affected by these interactions we assayed whether CXCL13, CCL21 and CCL19 could affect SS cell adhesion. Adhesion assay was performed using gelatin as matrix as adherence of PBMCs on fibronectin and collagen type I was very poor (data not shown).

PBMCs adherence to gelatin significantly decreased in the presence of CCL21 or CCL19 (Fig. 3C), whereas the addition of CXCL13 restored PBMCs adhesiveness. Furthermore, PBMCs from 4 SS patients were analyzed for MMP secretion by gelatin zymography in the presence of chemokines. Results obtained indicated that, as observed for HT-1080 cell line used as positive control, untreated and chemokine-treated PBMCs contained proform and active forms for MMP-9 and the proenzyme form of MMP-2 (Fig. 3D). Compared with untreated PBMCs, CXCL13, CCL19, and CXCL13+CCL19 treatments significantly decreased the level of total (pro-forms+active forms) and the active form of MMP-9 (Fig. 3D).

At last, experiments were performed to investigate whether chemokine treatment could also affect the survival of SS cells. Results obtained staining PBMCs with Annexin V and propidium iodide, by FACS analysis, revealed no significant difference, in term of apoptosis, between untreated and chemokine stimulated PBMCs (data not shown).

CCR7 blockade by neutralizing Ab strongly decreased the synergistic chemotactic response induced by CXCL13. To determine whether CCL19 and CCL21 used alone or in combination with CXCL13 act via CCR7, we tested the ability of an anti-CCR7 Ab to block the *in vitro* migration of SS cells and HL in response to these chemokines.

Treatment with anti-CCR7 mAb strongly reduced the SS cell migration induced by CCL19 alone by 67% ($P \leq 0.05$) and that obtained by CCL19 plus CXCL13 by 82% ($P = 0.05$). CCR7 blockade produced, in the same conditions, a chemotactic ability reduction of 30% and 74%, respectively ($P = 0.05$), of HL (Fig. 4). Similar results, with decreases of 61% and 64% ($P \leq 0.05$) of SS cells, 31% and 29% of HL chemotaxis were also observed using as chemo-attractants CCL21 and CCL21 plus CXCL13, respectively (Fig. 4).

These results indicate that the CCR7 neutralization was effective to reduce the migration of SS cells as well as HL toward CCL19 and CCL21 in absence or presence of CXCL13.

CCL21, a chemokine able to synergize with CXCL13, is expressed in skin and lymph nodes of SS and MF patients.

Finally, we sought to determine if CCL19 and/or CCL21 were produced *in vivo* in skin and lymph nodes of SS/MF patients. We analyzed their expression by IHC in 10 skin and 4 lymph node sections of SS individuals and 8 skin and 2 lymph node biopsies derived from MF patients. Although we were able to stain cells with anti-CCL21 Ab, the anti-CCL19 Ab did not yield satisfying results because of high background on skin sections. Coherently with CCL21 qRT-PCR results (Fig. 1A; Table 1), we did not observe significant CCL21 positivity associated with neoplastic skin infiltrating T lymphocytes of SS/MF biopsies, but we observed a strong CCL21 immunostaining associated with endothelial cells in 6 of 10 SS (60%) and 4 of 8 MF skin cases (50%; Fig. 5A and B). We found a strong expression of CCL21 associated to endothelial venules and dendritic cells of paracortical zone in 6 of 6 (100%) lymph nodes from SS/MF studied (Fig. 5C and D), in agreement to previous study (33) showing the expression of this chemokine by these cells in lymphoid districts in physiologic conditions.

Discussion

CXCL13 is a chemokine known to selectively attract B lymphocytes and to guide them via CXCR5 within follicles of lymphoid tissues (30). CXCL13 expression is associated with germinal center (GC) T-helper (Th) cells (34), and its overproduction has been described in Angioimmunoblastic T-Cell Lymphoma that has been proposed to be the malignant counterpart of GC-Th cells (35). More recently Mueller and colleagues (36) showed that a reduction of the CXCL13 and CCL19/21 homeostatic chemokines in responding inflammatory lymphoid tissues can turn off the immune response by the reduction of the recruitment of T-cell and antigen-presenting cells and, thus, promoting the generation of memory cells. Therefore, there are increasing evidences that this chemokine plays a fundamental role in both physiologic and pathologic conditions. However, especially in malignant T cells, this role has not been yet studied in detail.

In this study, we report that CXCL13 is strongly detectable in skin lesions and lymph nodes, measurable at high level in plasma of SS patients, and functionally active. Because SS cells do not express, at the protein level, the specific (CXCR5) and the potential (CXCR3) receptors for CXCL13 and these cells are not able to migrate in response to rhCXCL13, we wondered if CXCL13 could have a role in SS biology through different molecule(s). Interestingly,

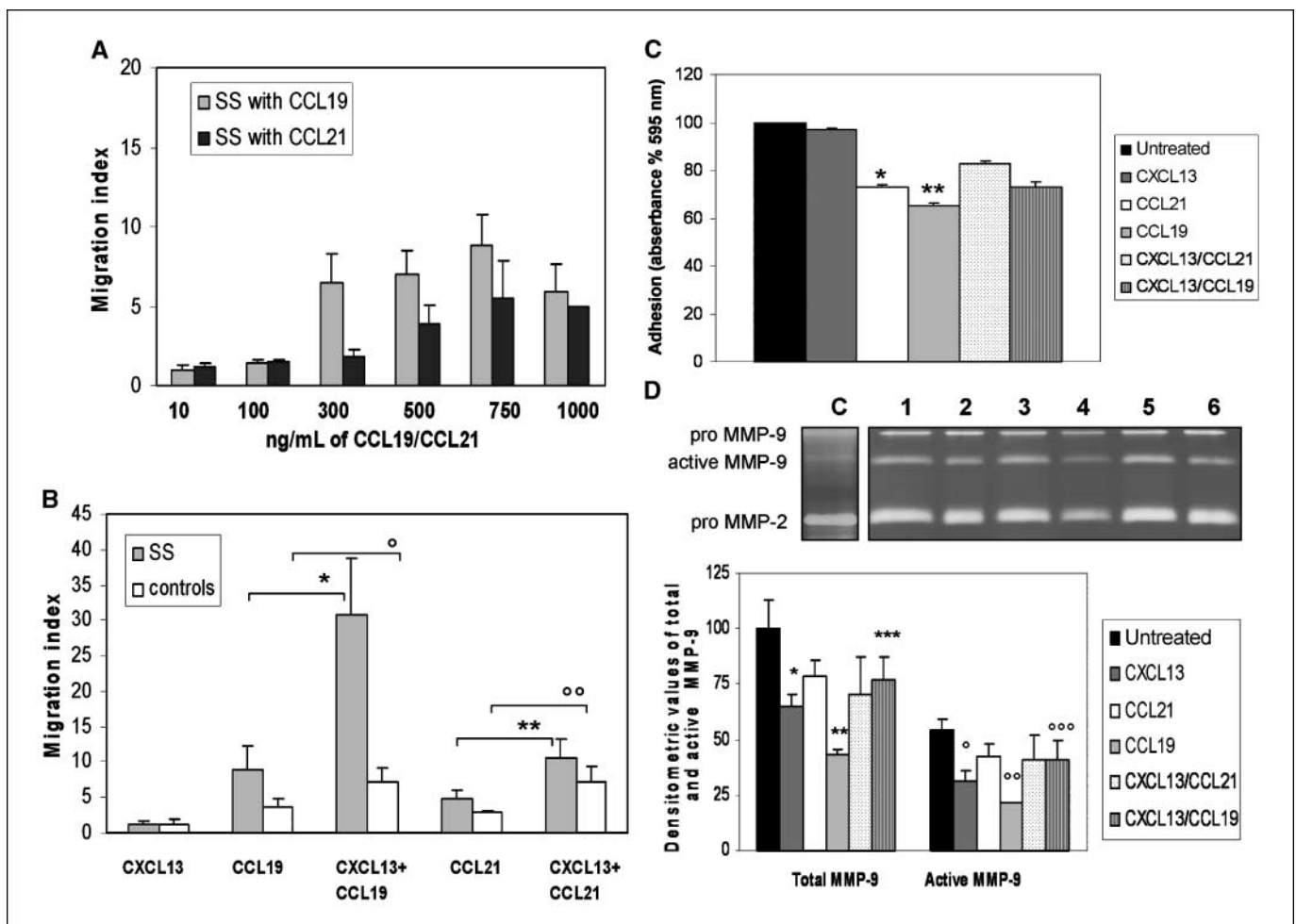


Figure 3. Effects of CXCL13, CCL19, and CCL21 chemokines on SS cell functions. **A**, dose response of PBMCs from SS patients to increasing concentrations of rhCCL19 and CCL21 (from 10–1,000 ng/mL) showing a typical bell-shaped dose response curve peaking at 750 ng/mL for both CCL19 and CCL21. **B**, chemotaxis of PBMCs from SS patients (gray bars) and controls (white bars) induced by rhCXCL13 alone, rhCCL19, and rhCCL21 in the absence or presence of rhCXCL13. Statistical differences were observed between SS cells (*) and HL (°) in response to CCL19+CXCL13 and CCL19 alone. Statistical differences were also noted for SS (**) and HL (°) that migrated in response to CCL21+CXCL13 and CCL21 alone, respectively. Columns, mean; bars, SE. *, $P = 0.01$; **, $P = 0.03$; °, $P = 0.02$; °°, $P = 0.009$. **C**, adhesion assay. Untreated PBMCs, CXCL13, CCL21, CCL19, CXCL13+CCL21, and CXCL13+CCL19-treated PBMCs were seeded on gelatin 0.2% in PBS for 30 min at 37°C. Cell adhesion was then quantified as absorbance at 595 nm and expressed as absorbance % versus control represented by untreated PBMCs. A statistical difference ($P \leq 0.03$) between untreated PBMC and CCL21 (*) or CCL19 (**) was observed. Columns, mean of four experiments carried out in triplicate; bars, SD. **D**, top, MMPs analysis. Conditioned medium from an equal cell number of HT-1080 cells used as positive control for MMPs secretion (lane C), untreated PBMCs (lane 1), CXCL13 (1.5 μ g/mL; lane 2), CCL21 (750 ng/mL; lane 3), CCL19 (750 ng/mL; lane 4), CXCL13 (1.5 μ g/mL) +CCL21 (750 ng/mL; lane 5), and CXCL13 (1.5 μ g/mL) +CCL19-treated (750 ng/mL; lane 6) PBMCs were analyzed for MMPs secretion using gelatin zymography. Bottom, densitometric analysis of gelatin zymograms. Values of total MMP-9 were expressed as percentage versus control represented by untreated PBMCs. Values of active MMP-9 were expressed as percentage of total MMP-9. Columns, mean of four independent experiments; bars, SD. Statistical differences observed (*, **, and ***, $P \leq 0.03$; ° and °°, $P \leq 0.007$; and °°, $P = 0.02$) were calculated by paired t test.

CXCL13 is able to synergize with CCL19 and CCL21, the selective CCR7 agonists that play crucial roles in lymph node homing (23, 32). As CCR7 is present at high levels in SS cells (3–6), we found *in vivo* that CCL21 is present in skin and lymph node of both SS and MF, and, *in vitro*, that the addition of CXCL13 to CCL19 and CCL21 enhances chemotaxis of SS cells, and finally, that this response is strongly reduced by CCR7 receptor neutralization. All together, these findings suggest that CXCL13 chemokine, coexpressed by CCR7 SS cells, might act as autocrine motility factor involved in lymph node and skin homing of these malignant cells.

Our findings might have direct clinical relevance: plasmatic concentration and expression of CXCL13 varies not only within CTCLs (MF and SS) but also during SS progression. This might indicate that CXCL13 detection could become an additional diagnostic tool for SS. For example, increasing amounts of this chemo-

kine in plasma could be indicative of the disease progression, whereas CXCL13 immunostaining in skin and lymph node could also represent an additional way to recognize neoplastic T lymphocytes in skin lesions.

MF and SS are clinical entities showing many similarities and differences and are referred to have an indolent and severe clinical outcome, respectively: the early stages of MF are characterized by cellular accumulation in the skin, whereas terminal (tumoral) stages of MF and SS show additional cellular colonization of lymph nodes and peripheral blood (1, 2). The selectivity of metastasis for specific districts may depend on a variety of factors (37, 38) including the ability of neoplastic cells to home to selected organs by cell migration by means of specific chemoattractants (39). There is a growing body of evidence that chemokines and their receptors might play a role in different and progressive stages of CTCLs.

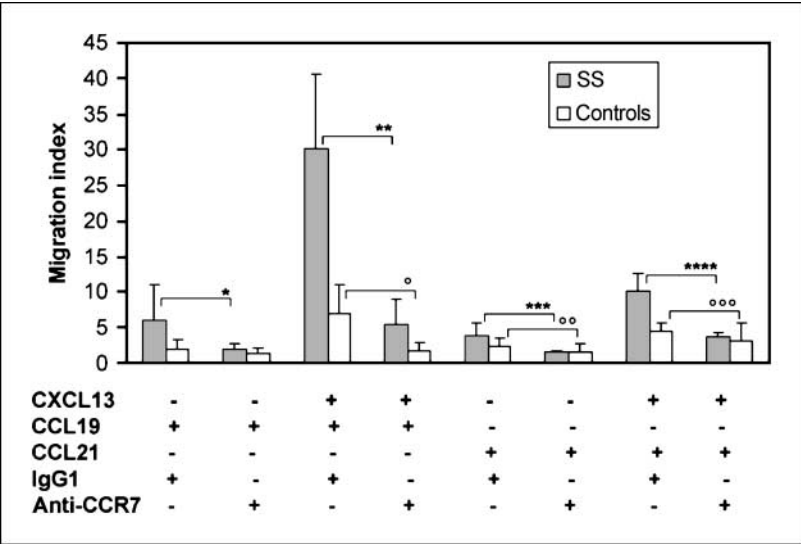


Figure 4. CCR7 receptor blockade strongly reduces the synergistic effect exerted by CXCL13 on CCL19- and CCL21-induced migration of CD4+ neoplastic and HLs. Chemotaxis assays were performed with PBMCs from SS patients (gray bars) and from HC (white bars) after preincubation with anti-CCR7 mAb or an irrelevant IgG1, both used at 1 µg/mL for 15 min at 37°C. Anti-CCR7-treated cell chemotaxis induced by CCL19 or CCL21 in the absence or presence of CXCL13 was compared with IgG1-treated cells. Columns, mean; bars, SE. *, **, ***, **** and °, °°, °°, °° indicate a statistical difference with $P \leq 0.05$ calculated by ANOVA. No statistical difference was observed between anti-CCR7 and IgG1-treated HL chemotaxis induced by CCL19.

Several of these factors are commonly found in progressive stages of MF and SS, such as CCR4/TARC (4, 21, 40), CXCR4/CXCL12 (5, 21), and CCR10/CCL27 (6, 19, 41). Conversely, the expression of other chemokines is more strictly correlated to clinical subtypes. One example is represented by CXCR3, a molecule mainly associated with epidermotropic neoplastic lymphocytes of early stage lesions of MF (22).

MF is characterized by an abundant tumor cells infiltration both in epidermis and dermis, whereas SS shows a tumoral infiltration mainly in dermis (2, 42). In this study, we report that the expression of CXCL13 and CCL21 is associated with malignant T lymphocytes and vessels, respectively, in the dermis of SS patients. Therefore,

these findings suggest that dermis might represent a favorable environment for the interaction *in vivo* between these chemokines. This interaction could be of particular relevance also for lymph node homing because CCL19/21 are constitutively expressed in these tissues and CXCL13 is carried by CCR7+ SS cells. Thus, similarly to central memory (CM) T-lymphocytes, known to be able to recirculate among peripheral tissues, blood, and via CCR7 to lymph nodes (8), SS cells, a category of malignant CD45RO+, CCR4+CD26-CD27+CCR7+CM T lymphocytes (3–7) that for chemokine receptors expression fits in pre-T_H2 effector CM subset (8) and here described also as CXCL13+, might be attracted to lymph nodes by CCL19/21 gradients mainly generated by high

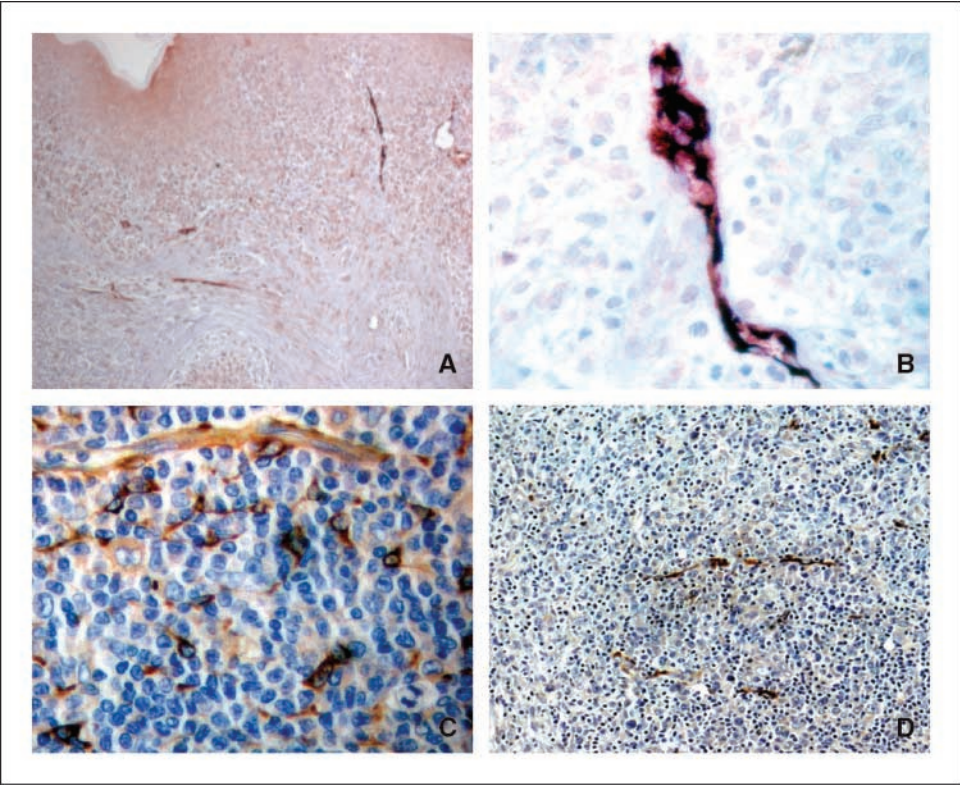


Figure 5. CCL21 immunoreactivity in SS and MF biopsies. A and B, SS skin lesion showing a CCL21 immunoreactivity associated with venule endothelial cells (×20/0.40 NA; 40×/0.65 NA). C, lymph node of SS exhibiting a CCL21 immunoreactivity of the dendritic and endothelial cells of paracortical zone (×40/0.65 NA). D, lymph node of MF displaying CCL21 positivity confined to dendritic and endothelial cells (×20/0.40 NA).

endothelial venules. Entrance into this favorable environment could lead to the exposure of SS cells to survival signals provided, for example, by lymphoid chemokines stimulations or mediated through direct contact between SS cells and cells, as the growth of circulating SS cells is stimulated by a direct contact with immature dendritic cells (43). Therefore, coherently with the data reported by Mueller and colleagues (36), the high level of CXCL13 observed in lymph node of SS patients might serve to increase cellularity and maintain the tumor process *in situ*. This hypothesis seems to be supported by additional molecular studies performed in this study that showed that CXCL13 (used alone or in combination with CCL19 and CCL21) did not affect both survival and adhesion of SS cells, whereas CXCL13 and CXCL13+CCL19 treatments significantly decreased the MMP-9 secretion. All these findings seem to indicate that CXCL13, in combination with CCL19 and CCL21, mainly influence the chemotaxis of SS cells. Our finding that CXCL13 is particularly elevated in the advanced stages of SS is in good agreement with this last observation. Although we did not observe any correlation between CXCL13 levels and the absolute number of circulating neoplastic cells (data not shown), we cannot exclude that the increase of this chemokine is associated to SS progression and/or therapeutic treatments here mainly represented by Extracorporeal-photochemotherapy (ECP) used in combination with IFN α . Furthermore, because MF revealed an increased level of CXCL13, although to a less extent than that observed in SS, either in blood or locally, it would be also of interest to investigate a possible correlation between the expression of CXCL13 and stages of MF.

Several Ab-based therapies have been already successfully used in the treatment of different tumors (44). Previous data from other laboratories (4, 6) and our own laboratory (5) showed that SS cells express high levels of CCR4, CXCR4, and CCR7. Recently, anti-CXCR4 Ab or antagonist peptides and anti-CCR4 Ab have been used with promising results in treatment of non-Hodgkin's lymphomas and T-cell leukemias (45, 46); the observation presented in this study also suggest that the interference of CXCL13 on CCL19/21-CCR7 axis might represent a novel therapeutic target and, similarly to the treatment recently proposed for B-CLL (47), the CCR7 neutralization might impair the migratory ability of SS cells. Experimental data *in vivo*, e.g., in immunodeficient mice transplanted with human CCR7+ SS cell line where CCR7 would be down-regulated with siRNA would be of great value in assessing the therapeutic potential of anti-CCR7 treatment in SS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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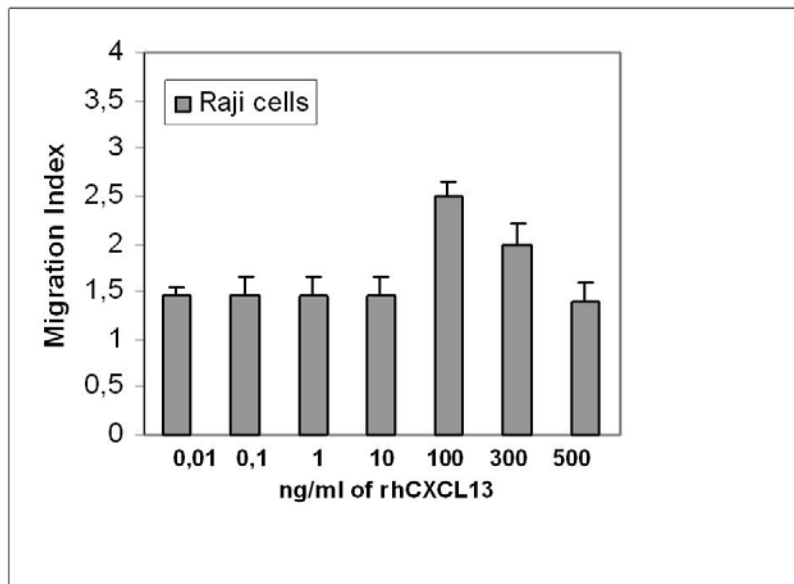
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SUPPLEMENTAL DATA

Table S1. Primer Sequences of selected genes for qRT-PCR

Gene Symbol/ Affymetrix ID	Forward Primer	Reverse Primer
CXCL13	5'-TTC AAC TCT ACC AGT TCC AGT GT-3'	5'-GAA TGC AGG TGT TCT TAG TGG A-3'
CXCR5	5'-CAG CCA TGA ACT ACC CGC TAA-3'	5'-GGC AGA GAT GAT TTT CCA CCA G-3'
CXCR3	5'-CAT GGT CCT TGA GGT GAG TGA-3'	5'-TAC AGC TAG GTG GAG CAG GAA G-3'
CCL19	5'-TCT CAT CAA GGA TGG CTG CAG-3'	5'-TGG ATG ATG CGT TCT ACC CAG-3'
CCL21	5'-AAC CAA GCT TAG GCT GCT CCA-3'	5'-TGT CTT GTC CAG ATG CTG CAT C-3'
GAPDH	5'-AGA TCA TCA GCA ATG CCT CCT G-3'	5'-ATG GCA TGG ACT GTG GTC ATG-3'

Figure S1



Dose-response of Raji cells induced by rhCXCL13. Chemotaxis of Raji cells to increasing concentration of rhCXCL13 showing typical bell-shaped response curve peaking at 100 ng/ml. Data represent mean $\pm$ SE of two separate experiments performed in duplicate