Little is known about mechanisms involved in skin-specific homing of cutaneous T-cell lymphoma (CTCL). Chemokine/chemokine receptor interactions have been implicated in the homing of lymphoma cells to various tissue sites. We investigated tissue samples and tumor cell suspensions of patients with CD30⁺ CTCL (n = 8) and CD30⁻ CTCL (mycosis fungoides, n = 6; Sézary syndrome, n = 6) for expression of the chemokine receptors CCR3, CCR4, and CCR8 and the CCR3 ligands eotaxin/CCL11, monocyte chemoattractant protein 3 (MCP-3)/CCL7, and RANTES (regulated on activation, normal T expressed and secreted)/CCL5.

Introduction

Cutaneous T-cell lymphomas (CTCLs) are part of the spectrum of extranodal non-Hodgkin lymphomas and are characterized by proliferation of clonally expanded helper T-cells in skin, but without detectable systemic involvement at least 6 months following diagnosis. Primary cutaneous CD30⁺ large-cell (anaplastic) CTCLs lack the t(2;5) translocation (NPM/ALK) and have a significantly better prognosis than systemic CD30⁺ large-cell anaplastic lymphomas. Expression of CD30 is not specific for systemic large-cell anaplastic lymphoma, and identification of abnormally expressed anaplastic large-cell lymphoma kinase 1 (ALK-1) is favored. However, in skin lymphomas, owing to the usual absence of ALK expression, CD30 is still a valuable marker.

Little is known about mechanisms leading to accumulation of large numbers of transformed lymphocytes in skin expression. The presence of eosinophils is prominent in certain types of CTCLs. Eotaxin/CCR ligand 11 (CCL11) is one of the most potent eosinophil chemoattractants. The question of eotaxin/CCR3 receptor expression in CTCL has thus far not been addressed.

Here we show that CD30⁺ large-cell CTCLs express a functional CCR3 receptor and produce Th-2–like cytokines. These findings might have implications for the homing of transformed T cells to skin.

Materials and methods

Tissue samples and immunohistochemistry

Immunohistochemical analysis for chemokine receptors and chemokines was performed on frozen tissue samples. Biopsies were snap-frozen in

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liquid nitrogen and stored at −80°C. Representative serial 5- to 7-µm cryostat sections were mounted on polylysine-coated slides and fixed in 100% acetone for 10 minutes. After washing in phosphate-buffered saline (PBS) without Ca or Mg (Biochrom, Berlin, Germany), nonspecific antibody binding sites were blocked by means of normal rabbit serum (DAKO, Copenhagen, Denmark), for 15 minutes at room temperature. For chemokine receptor staining, the following antibodies were used: 5 µg/mL mouse immunoglobulin G2a (IgG2a) antihuman CCR3 (monoclonal antibody [moAb] 7B11 [kindly provided by LeukoSite, Cambridge, MA]) as well as monoclonal rat IgG2a [RA6-8D5, Abingdon, United Kingdom]; CCR4 (rabbit polyclonal IgG [Santa Cruz Biotechnology, CA]); and CCR8 (goat polyclonal IgG [Alexis, Lausen, Switzerland]). For chemokine staining, the following antibodies were used: 1:100 ratio of antihuman eotaxin to CCL11 moAb (R&D Systems); 1:30 ratio of antihuman monocyte chemoattractant protein 3 (MCP-3) to CCL7 (Pharmingen/BD Biosciences, Basel, Switzerland); and 1:30 ratio of antihuman RANTES (regulated on activation, normal T expressed and secreted) to CCL5 (Pharmingen/BD Biosciences). For chemokine receptor staining, negative controls with antibody diluent (DAKO) instead of primary antibodies were included. For chemokine staining, the following isotype controls were used: 10 µg/mL mouse IgG1 (Ancell, Bayport, MN); mouse IgG2a (Ansell); and 1:300 dilution of normal goat serum (DAKO). Incubation time was 1 hour at room temperature for chemokine receptor staining; chemokine Abs and corresponding isotype controls were incubated overnight at room temperature. As detection system, the alkaline phosphatase/anti–alkaline phosphatase system followed by New Fuchsin (DAKO) as substrate, containing levamisole for blocking of endogenous alkaline phosphatase, was used as described. All incubations were done at room temperature. Antibody dilutions were done with the use of antibody diluent (DAKO). After immunostaining, slides were counterstained with hematoxylin. For evaluation of slides, 100 mononuclear cells were counted per high-power field (original magnification, ×200), and the percentage of positive cells was determined as follows: − indicates 0; +/−, 0% to 5%; +, 6% to 25%; +++, 26% to 50%; +++, +, 51% to 75%; and ++++, ++, 76% to 100% positive cells. Only slides with at least 5% stained cells were regarded as positive.

Preparation of tumor cell suspensions

Tumor cell suspensions were obtained from biopsy material of patients 1, 7, and 8 by incubation with an enzyme-cocktail containing 200 U/mL collagenase (Sigma, Buchs, Switzerland), 200 U/mL hyaluronidase (Sigma), and 0.01% DNase (Böhringer Mannheim, Germany) in RPMI 1640 (Life Technologies, Basel, Switzerland). Incubation was done for 37°C for 1 hour. Enzyme incubation was stopped by adding RPMI 1640 supplemented with 10% FCS (Serotech, Basel, Switzerland). After washing twice with PBS (Biochrom), and 70-µm filters were used to remove tissue fragments. Cells were prepared for in vitro investigation by resuspending cells in PBS (Biochrom) containing 1% bovine serum albumin (Sigma).

Flow cytometry, intracellular cytokine/chemokine staining

All antibody dilutions and washing steps were done in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma). Then, 1 × 10⁶ cells per reaction were stained with the following moAbs: 1:20 dilution of fluorescein isothiocyanate (FITC)–conjugated antihuman CD30 (Ki-1) moAb (DAKO) of 10 µg/mL 7B11 antihuman CCR3 moAb. To control for nonspecific staining or Fc–receptor–mediated binding of antibody, the following moAbs were included as negative controls: 1:10 dilution of FITC–conjugated mouse IgG1 (Becton Dickinson, Basel, Switzerland) of 10 µg/mL mouse IgG2a (Ansell); Staining with anti-CCR3 or anti-IgG2a moAb was followed by phycoerythrin (PE)–conjugated goat antimouse F(ab′)₂ moAb (DAKO) at a 1:20 dilution. All incubations were done at 4°C for 30 minutes. Samples were analyzed by flow cytometry with a FACScalibur System (BD Biosciences) and equipped with CellQuest software (BD Biosciences).

For intracellular cytokine/chemokine staining, cells were stimulated with 50 ng/mL PMA and 1 µM ionomycin in RPMI 1640 (Gibco BRL, Basel, Switzerland) supplemented with 10% FCS (Serotech, Basel, Switzerland) in 5% CO₂, 37°C. After 4 hours, transport inhibitor was added for an additional 2 hours: 0.75 µL/mL GolgiStop (Pharmingen) and 1 µL/mL GolgiPlug (Pharmingen). After cell-surface antigen staining with 10 µg/mL mouse IgG1 antihuman CCR3 moAb or by purified mouse IgG1 (BD Biosciences) followed by PE–cytoxin 5 (PE-Cy5)–conjugated goat antimouse antibody (DAKO) at a 1:10 dilution, intracellular cytokine staining was performed with 1:100 PE-conjugated anti–IL-4 moAb and 1:100 FITC-conjugated anti–IFN-γ moAb (Pharmingen) after cell permeabilization with a saponin-containing solution (Cytofix/Cytoperm; Pharmingen). For intracellular chemokine staining, the following moAbs was used: 1:25 dilution of mouse IgG1 antieotaxin CCL11 Ab (Pharmingen). All antibody incubations for staining of cell surface antigens were done at 4°C for 30 minutes; staining of intracellular cytokines and chemokines was performed at 4°C for 1 hour. Flow cytometry acquisition was performed by means of a FACScalibur system (BD Biosciences) equipped with CellQuest software (BD Biosciences), which was also used for flow cytometric analysis.

Internalization experiments

Recombinant chemokines eotaxin/CCL11, RANTES/CCL5, and MCP-3/CCL7 were obtained from R&D Systems. Cell suspensions from lesional skin were obtained as described in “Preparation of tumor cell suspensions” and were cultured for 2 days in RPMI 1640 supplemented with 10% FCS. Short-term cultures were incubated with 200 nM CCR3 ligands eotaxin/CCL11, RANTES/CCL5, MCP-3/CCL7, or untreated medium, respectively, for 40 minutes at 37°C in a 5% CO₂ atmosphere. After chemokine incubation, cells were washed extensively in PBS; the final washing step was performed in acidic glycine buffer, pH 3.0, to remove bound chemokine located on noninternalized receptor for 1 minute at 37°C. To determine the amount of surface-expressed CCR3, cells were stained with antihuman CCR3 (10 µg/mL) and PE-conjugated goat antimouse F(ab′)₂ IgG (DAKO) at a dilution of 1:20; each Ab was incubated for 30 minutes at 4°C.

Actin polymerization assay

Actin polymerization was tested as previously described. Briefly, cells derived from a CD30+ cutaneous lymphoma line (Mac-1) (1.25 × 10⁶/mL) were resuspended in RPMI-1640 medium containing 0.5% bovine serum albumin (BSA) at 37°C and incubated with 100 ng/mL eotaxin (R&D Systems) for varying amounts of time. At the indicated time points, 400 µL cell suspension was added to 100 µL solution containing 4 × 10⁷ M FITC-labeled phalloidin, 0.5 mg/mL 1-α-lysophosphatidylcholine, and 18% formaldehyde (all from Sigma) in phosphate-buffered saline (PBS). After incubation at 37°C for 10 minutes in the dark, fixed cells were centrifuged at 400g for 5 minutes at room temperature and subsequently resuspended in PBS containing 0.5% BSA. Cells were analyzed by flow cytometry on a FACS Calibur (Becton Dickinson), and all time points are plotted relative to the mean relative fluorescence of the sample before addition of eotaxin.

Migration assay

The number of CD30+ lymphoma cells migrating in response to recombinant eotaxin (R&D Systems) across 5-µm pore size polycarbonate filters (6.5-mm diameter) was assessed in 24-well Transwell chambers (Costar Corning, NY). First, 600 µL warm (37°C) assay medium (RPMI containing 0.5% BSA) containing various concentrations of eotaxin were added to the lower wells. Lymphoma cells were suspended at 2 × 10⁶ cells per milliliter in warm assay medium (37°C), and 100 µL cell suspension was added to the upper chamber of each Transwell chamber. The plates were incubated for 3 hours at 37°C in 10% CO₂. The migrated cells in the lower chambers were collected, and the number of migrated cells was counted by acquisition for 60 seconds with a flow cytometer.

Results

Chemokine receptor CCR3 is expressed in CD30+ large-cell CTCL, but not in CD30- CTCL

The diagnosis of CTCL was established by assessment of the clinical appearance, analysis of T-cell receptor clonality, conventional histology, and immunohistochemical staining for various
T-cell, B-cell, and activation markers, such as CD3, CD4, CD5, CD8, CD45RO, CD20, CD79a, and CD30, as published.²

Twenty CTCLs, consisting of 8 CD30⁺ large-cell lymphoma, 6 mycosis fungoides, and 6 Sézary syndrome, patient samples, were analyzed by immunohistochemistry. CD30 was expressed in all large-cell CTCLs (Table 1). Variation in the admixture of inflammatory cells accounted for the difference in numbers of CD30⁺ cells in the infiltrate. Staining for the chemokine receptor CCR3 demonstrated expression of CCR3 in 7 of 8 CD30⁺ CTCLs investigated (Table 1; Figure 1). No epidermotropism of CCR3⁺ cells was observed. There was faint staining of keratinocytes, which is explained by the recently described CCR3 expression on these cells.²¹ Mycosis fungoides (n = 6) and Sézary syndrome (n = 6) sections were CD30⁻ except one case (no. 17) where few CD30⁺ cells were detected. In CD30⁻ CTCL, there were always fewer than 5% of cells positive for CCR3. In most cases, the extent of CD30 expression corresponded to the expression of CCR3 (Table 1). CCR4 expression was found in 4 of 8 CD30⁺ CTCLs as well as 1 of 6 MF cases and 1 of 6 Sézary syndrome cases. No CCR8 staining was observed in any of the samples.

**CCR3 and CD30 coexpression in freshly isolated tumor cell suspensions from CD30⁺ CTCL**

To confirm our immunohistochemistry data on a single-cell level and to assess expression levels of CCR3 on CD30⁺ lymphoma cells, we performed flow cytometric analysis of tumor cell suspensions. Tumor cell suspensions were derived from fresh biopsy material, which was available in 3 patients with CD30⁺ large-cell CTCL (nos. 1, 7, and 8). Cells were double-stained for CCR3 and CD30 and analyzed by flow cytometry. Compared with isotype control, a strong coexpression of the chemokine receptor CCR3 and CD30 was detected (Figure 2; patient no. 7). Strong staining in immunohistochemistry (Figure 1) translated into a high level of CCR3 expression on CD30⁺ tumor cells in flow cytometric analysis (Figure 2; patient no. 7). The majority of CD30⁺ cells were CCR3⁺. Expression levels were comparable to those seen in eosinophils (data not shown).

**Eotaxin/CCL11 induces CCR3 down-regulation on CD30⁺ cutaneous tumor cells**

Detection of CCR3 protein on CD30⁺ tumor cells does not necessarily imply the existence of a functional receptor. Freshly isolated cell suspensions did not provide sufficient material to perform migration assays. A different approach to demonstrate the existence of a functional chemokine receptor is receptor internalization. Binding of ligand to its cognate receptor leads to receptor down-regulation, which can be assessed by flow cytometry.²² After incubation with eotaxin/CCL11, RANTES/CCL5, MCP-3/CCL7, or medium alone, surface expression of CCR3 on short-term cultured tumor cells was analyzed by flow cytometry with the use of anti-CCR3 moAb. Receptor-bound chemokine might interfere with antibody binding; therefore, cells were washed in acidic glycome buffer, as described, to dissociate the chemokine ligand from its receptor.²² As shown in Figure 3A, the CCR3 ligand eotaxin/CCL11, but not medium alone, is able to induce a 1.9-fold decrease of CCR3 expression owing to down-regulation of CCR3. In contrast, CCR3 ligands RANTES/CCL5 and MCP-3/CCL7, which possess a lower affinity to CCR3 than eotaxin/CCL11, did not induce an internalization of CCR3 (data not shown).

**Functional expression of CCR3 on a CD30⁺ cutaneous lymphoma cell line**

Investigation of receptor functionality in fresh tumor cell suspensions is limited by both the low number and the heterogeneity of cells. To further explore functional CCR3 expression in CD30⁺ lymphoma cells, we investigated the expression of CCR3 in CD30⁺ lymphoma cell lines. The cell line Mac-1 coexpressed CD30 and

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Table 1. Expression of CCR3 and its ligands in CTCL

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Staining of different subtypes of cutaneous T-cell lymphoma for CD30, CCR3, CCR4, CCR8, and its ligands eotaxin, MCP-3/CCL7, and RANTES/CCL5. Positive mononuclear cells were counted per high-power field (magnification × 200) on 100 mononuclear cells, and the percentage of positive staining was determined as follows: +/− indicates 0% to 5%; +, 5% to 25%; ++, 25% to 50%; +++, 50% to 75%; and ++++, 75% to 100% positive cells. Please note that CCR3 expression corresponds to CD30 expression. Staining for CCR3 ligands shows positivity for eotaxin in CD30⁺ and CD30⁻ CTCL. Please note that tumor cells and connective tissue cells in CD30⁺ CTCL are positive for eotaxin, while in CD30⁻ CTCL tumor cell clusters are negative for eotaxin.

Eos indicates eosinophils; pos, positive staining; TC, tumor cells; neg, negative staining; CT, connective tissue cells; ND, not done; and MF, Mycosis fungoides.
F-actin in CD30

100 ng/mL eotaxin induced a transient 85% increase in intracellular cell line 20 with the use of reorganization of the cytoskeleton in CD30 magnification of the CD30 cell line 20 with the use of the alkaline phosphatase/anti-cryosections of a CD30 and CD30 after binding of eotaxin to CCR3 on the CD30 indicating the transduction of a migration signal to the cytoskeleton.

We investigated the expression pattern of CCR3 ligands in CD30 CCR3 ligand eotaxin/CCL11 is expressed by CD30/CTCL. We found selective expression of the chemokine receptor CCR3 in CD30+ large-cell CTCL. No CCR3 expression was seen in CD30- CTCL. Coexpression of CCR3 and CD30, as well as Th-2 cytokine production, was detected on freshly isolated tumor cells. Functionality of the CCR3 receptor was shown by receptor-internalization experiments and actin polymerization, as well as by migration of tumor cells toward an eotaxin gradient.

Figure 1. Immunoreactivity of CCR3 in CTCL. (A) CCR3 immunoreactivity in cryosections of a CD30+ large-cell CTCL (no. 7) with the use of the alkaline phosphatase/anti-alkaline phosphatase method. Original magnification, ×100. (B) CCR3 immunoreactivity in cryosection of a CD30+ large-cell CTCL (no. 7) with the use of the alkaline phosphatase/anti-alkaline phosphatase method. Original magnification, ×400.

CCR3 ligand eotaxin/CCL11 is expressed by CD30+ CTCL

We investigated the expression pattern of CCR3 ligands in CD30+ and CD30- CTCL. Immunohistochemical staining of lesional skin for eotaxin/CCL11, MCP-3/CCL7, and RANTES/CCL5 was performed. Positive dermal immunoreactivity for eotaxin/CCL11 occurred in 17 of 18 CTCLs (Table 1). In CD30+ CTCL, eotaxin/CCL11 protein was detected in tumor cell aggregates demonstrated as being CD30+ in serial sections (Figure 4B). Eotaxin/CCL11 protein was also detected in connective tissue cells surrounding tumor cells. Each of 8 CD30+ CTCLs showed an immunopositivity for eotaxin/CCL11 associated with tumor cell aggregate, and in 5 of 8 there was expression by connective tissue cells (Figure 4B; Table 1). In contrast, positive staining for eotaxin/CCL11 in CD30- CTCL was confined to connective tissue cells, while infiltrating lymphoma cells were negative (Figure 4A; Table 1). In one case of CD30- CTCL, eotaxin/CCL11 was also present in infiltrating lymphoma cells. Eosinophil infiltration was found in each of 5 CD30+ large-cell CTCLs and in 3 of 12 CD30- CTCLs (Table 1). Staining for RANTES/CCL5 was positive in 4 CD30+ CTCLs, while MCP-3/CCL7 was expressed in only 1 case (Table 1).

To verify eotaxin/CCL11 expression in infiltrating cells on a single-cell level, we performed intracellular eotaxin/CCL11 staining of cells isolated from freshly obtained biopsy specimens of patients with CD30+ CTCL. As shown in Figure 5, eotaxin/CCL11 protein was detected in cells with high forward- and side-scatter properties corresponding to infiltrating tumor cells.

CCR3+ tumor cells express IL-4 protein

It has been previously shown that CD30+ CTCL lesions contain mRNA related to Th-2 cytokine differentiation such as IL-4.24 To analyze the functional differentiation state of CCR3-bearing tumor cells, we performed 3-color staining of tumor cell suspensions obtained from lesional CD30+ CTCL skin. Staining of tumor cell suspensions with IFN-γ, IL-4, and CCR3 showed a clear predominance of IL-4+ cells within the CCR3+ cell population (Figure 6; patient no. 8; representative of 3 experiments). Ratio of IL-4- to IFN-γ-expressing cells was 24 (12.3% versus 0.5%). This indicates a skewing of CCR3+ cells toward Th-2 cytokine production. Analysis of CCR3+ lesional cells did not demonstrate a predominance of IFN-γ or IL-4 protein (data not shown).

Discussion

We found selective expression of the chemokine receptor CCR3 in CD30+ large-cell CTCL. No CCR3 expression was seen in CD30- CTCL. Coexpression of CCR3 and CD30, as well as Th-2 cytokine production, was detected on freshly isolated tumor cells. Functionality of the CCR3 receptor was shown by receptor-internalization experiments and actin polymerization, as well as by migration of tumor cells toward an eotaxin gradient.

Figure 2. Coexpression of CD30 and CCR3. Flow cytometric analysis of tumor cell population. Staining of freshly isolated tumor cells shows coexpression of CD30 and CCR3. Please note high expression of CCR3 on tumor cells (patient no. 7). Data shown are representative of 3 experiments.
CTCLs are a group of lymphoproliferative disorders with clonal expansion of transformed T cells in skin. Surface expression of CD30 distinguishes a subtype of large-cell CTCL with slow progression, indolent behavior, and favorable prognosis. CD30 is a member of the tumor necrosis factor receptor family, which was originally described as Ki-1 antigen on Hodgkin and Reed-Sternberg cells in Hodgkin disease. The favorable prognosis of CTCL may be related in part to the fact that dissemination to other body compartments occurs only late during disease development. This might be suggestive of chemotactic forces that keep lymphoma cells confined to the skin. The insight into the field of chemokine/chemokine receptor interactions has been developing rapidly. Recently, chemokine receptor expression has been correlated with differential recruitment of polarized Th-1 or Th-2 T cells as well as secretion of Th-1 or Th-2 cytokines. It has been shown that CCR3 is preferentially expressed in vitro by Th-2 cells. In this context, the presence of eosinophils as well as expression of Th-2 cytokine mRNA in lesions of CD30+ cutaneous lymphomas is of interest. We reasoned that recruitment of CD30+ Th-2–like lymphoma cells might be mediated by the presence of CCR3 ligands in skin in association with constitutive expression of CCR3 on lymphoma cells. The availability of a monoclonal antibody to CCR3 allowed us to perform the present study.

Seven of 8 CD30+ CTCLs demonstrated expression of CCR3 on lesional tumor cells (Table 1; Figure 1). This finding was confirmed by flow cytometric staining of tumor cell suspensions (Figure 2). Interestingly, there was high expression of CCR3 on lymphoma cells, comparable to the expression on eosinophils, which was also reflected by strong staining intensity for CCR3 with the use of immunohistochemistry (Figure 1). CD30+ CTCL samples did not express CCR3 in spite of a suggested Th-2 differentiation of tumor cells. However, there is also recent evidence that Th-2 cytokine mRNA might be absent in CD30+ CTCL. The question of chemokine redundancy must be addressed early in any discussion of the potential role of chemokines in disease. There might be an enormous overlap in ligand-receptor specificity. Even though eotaxin/CCL11 has a very specific binding to CCR3, other chemokine receptors, such as the skin-associated chemokine...
To establish a relationship between CCR3 expression and Th-2 cytokine production, we assessed the cytokine expression profile of CCR3+ tumor cells. Earlier reports have demonstrated Th-2 cytokine mRNA in lesions of CD30+ CTCL.24 On the protein level, we observed strong expression of IL-4 by the majority of CCR3+ cells, while few cells expressed IFN-γ. This observation is compatible with a Th-2–like differentiation of CCR3+ skin-homing lymphoma cells.

The CCR3 ligand eotaxin/CCL11 is known to be produced by human dermal fibroblasts.35,45 Eotaxin/CCL11 not only has agonistic functions but is also a natural antagonist for CCR2.46 Our data demonstrate the presence of eotaxin/CCL11 protein in skin of CTCL lesions (Table 1; Figure 4). In CD30+ as well as in CD30− CTCL, we found expression of eotaxin/CCL11, which was associated with connective tissue cells, most likely fibroblasts, located around the tumor. Only in CD30+ CTCL was eotaxin/CCL11 expression also observed in aggregates of tumor cells. Eotaxin/CCL11 expression at the single-cell level was shown by intracellular eotaxin/CCL11 staining of fresh tumor cell suspensions (Figure 5). Eotaxin/CCL11 expression by lymphoma cells may lead to homotypic aggregation, observed as cohesive clusters of tumor cells, a characteristic of CD30+ anaplastic lymphomas,28 and an amplification of tumor cell homing to the skin. Down-regulation of the receptor in the presence of high eotaxin/CCL11 concentrations may keep lymphoma cells in skin. In this regard, it is of interest that epidermotropic CD30− CTCLs were shown to be associated with epidermal expression of IFN-γ–inducible protein-10 (IP-10) and monokine induced by IFN-γ (Mig).47 Thus, different sets of chemokines produced by different cellular constituents might have an impact on the nature of the malignant infiltrate.

In summary, we have shown functional CCR3 expression in CD30+ large-cell CTCL as well as expression of its ligand eotaxin in skin. Expression of CCR3 on CD30+ T cells may provide a link between the recruitment of lymphoma cells and their functional state as Th-2 cells. As suggested for breast cancer,48 pharmacological modulation of chemokine receptors may open the way to new treatment modalities.

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