Functional expression of the eotaxin receptor CCR3 in CD30 positive cutaneous T cell lymphoma

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Abbreviations

CLA: Cutaneous lymphocyte associated antigen
CTCL: Cutaneous T cell lymphoma
FACS Fluorescence activated cell sorter
IHC: Immunohistochemistry
MCP: Monocyte chemoattractant protein
MoAb: Monoclonal antibody
NK: Natural killer
NHL: non-Hodgkin lymphoma
Th2: T helper 2
RANTES: Regulated upon activation normal T cell expressed and secreted
Abstract

Little is known about mechanisms involved in skin specific homing of cutaneous T cell lymphoma (CTCL). Chemokine/chemokine receptor interactions have been implicated in homing of lymphoma cells to various tissue sites. We investigated tissue samples and tumor cell suspensions of patients with CD30 positive CTCL (n=8) and CD30 negative CTCL (Mycosis fungoides, n=6; Sézary Syndrome, n=6) for expression of the chemokine receptor CCR3, CCR4, CCR8 and the CCR3 ligands eotaxin/CCL11, MCP-3/CCL7 and RANTES/CCL5. Of 8 CD30 positive CTCL, 7 expressed CCR3, 4 CCR4 and none CCR8. CCR3 expression was not found in skin tissue samples from 12 CD30 negative CTCL. Co-expression of CCR3 and CD30 was demonstrated by flow cytometry in tumor cell suspensions. Internalization experiments demonstrated functionality of CCR3 expressed by freshly isolated tumor cells. Actin polymerization as well as migration in response to eotaxin was demonstrated in a CD30+ cutaneous lymphoma cell line. CCR3 ligand eotaxin/CCL11 was detected in lesional skin of CD30 positive CTCL by immunohistochemistry preferentially in tumor cells. Eotaxin/CCL11 expression in tumor cells was confirmed by intracellular immunofluorescence. Analysis of cytokine expression pattern of CCR3 bearing infiltrating cells showed a predominance of IL-4 but not IFN-γ protein expression consistent with a Th2 profile. These results suggest that expression of CCR3 and its ligand eotaxin/CCL11 play a role in the recruitment and retention of CD30 positive malignant T cells to the skin.
Introduction

Cutaneous T cell lymphomas (CTCL) 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 positive large cell (anaplastic) CTCL lack the t(2;5) translocation (NPM/ALK negative) and have a significantly better prognosis than systemic CD30 positive 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 due 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 of the cutaneous lymphocyte associated antigen (CLA) has been implicated in skin specific homing patterns. Extravasation and active locomotion of malignant lymphocytes suggests the involvement of additional secreted factors such as chemokines. Chemokines are small molecules of about 8 kDa which are involved in leukocyte migration to specific tissue sites and are essential for the host immune response. The known chemokine system comprises approximately 50 ligands and 20 G protein coupled receptors. A new nomenclature was recently introduced. Investigation of the expression of chemokine receptors has been performed in extracutaneous non-Hodgkin lymphomas (NHL). CXCR3 was shown to be a marker of B-cell chronic lymphocytic leukemia and was expressed in a subset of B-cell lymphomas, while CXCR5 was expressed on all types of B-cell lymphomas. Subsets of T-cell NHL expressed various patterns of T-cell associated chemokine receptors. Expression of the TH-2 and skin associated chemokine receptor CCR4 was seen in 5 out of 5 ALK positive large cell lymphomas while the TH-1 associated receptor CXCR3 was only expressed in 1 out 15 of ALK positive large cell lymphomas.

Presence of eosinophils is prominent in certain types of CTCL. Eotaxin/CCL11 is one of the most potent eosinophil chemoattractants. The question of eotaxin/CCL11 receptor CCR-3 expression in CTCL has thusfar not been addressed.
Here we show that CD30 positive large cell CTCL express a functional CCR3 receptor and produce TH-2 like cytokines. These findings might have implications for homing of transformed T cells to skin.
Material and Methods

Tissue samples and Immunohistochemistry

Immunohistochemical analysis for chemokine receptors and chemokines was performed on frozen tissue samples. Biopsies were snap-frozen in liquid nitrogen and stored at −80°C. Representative serial 5-7 µm cryostat sections were mounted on poly-Lysine coated slides and fixed in 100% acetone for 10 minutes. After phosphate buffered saline w/o Ca, Mg (PBS) (Biochrom KG, Berlin, Germany) washing, nonspecific antibody binding sites were blocked using normal rabbit serum (DAKO A/S, Copenhagen, Denmark), for 15 min at room temperature. For chemokine receptor staining, 5 µg/ml of mouse IgG2a antihuman CCR3 (moAb 7B11, kindly provided by LeukoSite, Cambridge, MA, USA as well as monoclonal rat IgG2A, R&D Systems, Abingdon, UK), CCR4 (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, California, USA), CCR8 (Goat polyclonal IgG, Alexis Corporation, Lausen, Switzerland) antibodies were used. For chemokine staining the following antibodies were used: 1:100 anti-human eotaxin/CCL11 moAb (R&D Systems, Abingdon, UK), 1:30 anti-human MCP-3/CCL7 (Pharmingen/BD Biosciences) and 1:30 anti-human RANTES/CCL5 (Pharmingen/BD Biosciences). For chemokine receptor staining, negative controls with Antibody Diluent (DAKO A/S) instead of primary antibodies were included. For chemokine staining the following isotype controls were used: 10 µg/ml of mouse IgG1 (Ancell, Bayport, USA), mouse IgG2a (Ancell) and 1:300 of normal goat serum (DAKO A/S). Incubation time was 1h 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 A/S) as substrate, containing levamisole for blocking of endogenous alkaline phosphatase, was used as described. 18 All incubations were done at room temperature. Antibody dilutions were done using Antibody Diluent (DAKO A/S). After immunostaining slides were counterstained with hematoxylin. For evaluation of slides, 100 mononuclear cells were counted per x200 high power field and the percentage of positive cells was determined as
follows: - : negative, -/+ : 0-5%, + : 6-25%, ++ : 26-50%, +++ : 51-75%, ++++ : 76-100% 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 Nr. 1, 7, 8 by incubation with an enzyme-cocktail containing 200 U/ml collagenase (Sigma, Buchs, Switzerland), 200 U/ml hyaluronidase (Sigma), 0,01% DNAse (Böhringer Mannheim, Mannheim, Germany) in RPMI 1640 (Life Technologies Inc., Basel, Switzerland). Incubation was done at 37 °C for 1 h. Enzyme incubation was stopped adding RPMI 1640 (Life Technologies) supplemented with 10% FCS. Suspensions were washed twice with PBS (Biochrom KG) using 70 µm filters to remove tissue fragments. Cells were prepared for in-vitro investigation by re-suspending cells in PBS (Biochrom KG) containing 1% bovine serum albumin (Sigma, Buchs, Switzerland).

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, Buchs, Switzerland). 1x10^6 cells per reaction were stained with the following moAbs: 1:20 of FITC conjugated anti-human CD30 (Ki-1) moAb (DAKO A/S), 10 µg/ml of 7B11 anti-human CCR3 moAb. To control for non-specific staining or Fc-receptor-mediated binding of antibody, the following moAbs as negative controls were included: 1:10 of FITC conjugated mouse IgG1 (Becton Dickinson AG, Basel, Switzerland) and 10 µg/ml of mouse IgG2a (Ancell, Bayport, USA). Staining with anti CCR3 or IgG2a moAb was followed by 1:20 of PE conjugated goat anti-mouse F(ab’)2 moAb (DAKO A/S). All incubations were done at 4°C for 30 min. Samples were analyzed by flow cytometry with a FACScalibur System (BD Biosciences) equipped with a Cell Quest software (BD Biosciences).

For intracellular cytokine/chemokine staining cells were stimulated with 50 ng/ml TPA and 1µM ionomycin in RPMI 1640 (Gibco BRL) supplemented with 10% FCS (Serotech) in 5% CO2, 37°C. After 4 h transport inhibitor was added for additional 2 h: 0,75 µl/ml GolgiStop (PharMingen) and 1 µl/ml GolgiPlug (PharMingen). After cell surface antigen staining with 10 µg/ml of mouse IgG1 anti human CCR3 moAb or 1:10 of purified mouse
IgG1 (BD Biosciences) followed by PE-conjugated goat anti-mouse antibody (DAKO A/S). Intracellular cytokine staining was performed with 1:100 PE-conjugated anti-IL-4 mAb and 1:100 FITC-conjugated anti IFN-gamma mAb (PharMingen) after cell permeabilization with a saponin containing solution (Cytofix/Cytoperm, PharMingen). For intracellular chemokine staining, the following mAb was used: 1:25 of mouse IgG1 anti-eotaxin/CCL11 Ab, (Pharmingen). All antibody incubations for staining of cell surface antigens were done at 4°C for 30 min, staining of intracellular cytokines and chemokines at 4°C for 1 h. Flow cytometry acquisition was performed using a FACS Calibur® system (BD Biosciences) equipped with CellQuest® software (BD Biosciences), which was also used for flow cytometry analysis.

**Internalization experiments**

Recombinant chemokines eotaxin/CCL11, RANTES/CCL5, and MCP-3/CCL7 were obtained from R&D Systems, UK. Cell suspensions from lesional skin were obtained as described above and were cultured for 2 days in RPMI 1640 supplemented with 10% FCS. Short time cultures were incubated with 200 nM of the CCR3 eotaxin/CCL11, RANTES/CCL5, MCP-3/CCL7 or medium (untreated) respectively for 40 min at 37°C in a 5% CO₂ atmosphere. After chemokine incubation cells were washed extensively in PBS, last washing step was performed in acidic glycine buffer pH 3.0 to remove bound chemokine located on not internalized receptor for 1 min at 37°C. To determine the amount of surface-expressed CCR3, cells were stained with anti human CCR3 (10 µg/ml) followed by 1:20 of PE conjugated goat anti mouse F(ab´)2 antibody (DAKO A/S), 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 positive cutaneous lymphoma line (Mac-1) (1.25 × 10⁶/ml) were resuspended in RPMI-1640 medium containing 0.5% BSA at 37°C and incubated with 100 ng/ml eotaxin (R&D Systems, Abingdon, UK) for varying amounts of time. At the indicated time points, 400 µl of the cell suspension were added to 100 µl of a solution containing 4 × 10⁻⁷ mol/l FITC-labelled phalloidin, 0.5 mg/ml 1-α-lysophosphatidylcholine and 18% formaldehyde (all from Sigma, Buchs, Switzerland) in phosphate-buffered saline (PBS). After incubation
at 37°C for 10 min in the dark, fixed cells were centrifuged at 400 x g for 5 min at room temperature and subsequently resuspended in PBS containing 0.5% BSA. Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Allschwil, Switzerland) 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, Abingdon, UK) across 5 µm pore size polycarbonate filters (6.5-mm diameter) was assessed in 24-well Transwell chambers (Costar Corning Inc., Corning, NY, USA). 600 µl of warm assay (37°C) medium (RPMI containing 0.5% BSA) containing various concentrations of eotaxin were added to the lower wells. Lymphoma cells were suspended at 2 x 10⁶ cells/ml in warm assay medium (37°C) and 100 µl of the cell suspension was added to the upper chamber of each Transwell which were then transferred into the plate chambers. The plates were incubated for 3 hrs 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 positive large cell CTCL, but not in CD30 negative 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, CD45 RO, CD20, CD79a, and CD30 as published. 4 Twenty CTCL, including 8 CD30 positive large cell lymphoma, 6 mycosis fungoides and 6 Sézarys’ syndrome patient samples were analyzed by immunohistochemistry. CD 30 was expressed in all large cell CTCL (Table 1). Variation in the admixture of inflammatory cells accounted for the difference in numbers of CD30 positive cells in the infiltrate. Staining for the chemokine receptor CCR3 demonstrated expression of CCR3 in 7 out of 8 CD30 positive CTCL investigated (Table 1, Fig. 1 A and 1 B). No epidermotropism of CCR-3 positive cells was observed. 21 Mycosis fungoides (n=6) and Sézarys’ syndrome (n=6) sections were CD30 negative except one case (Nr. 17) where few CD30 positive cells were detected. In CD30 negative CTCL there were always less than 5% of cells positive for CCR-3. In most cases the extent of CD30 expression corresponded to the expression of CCR3 (Table 1). CCR4 expression was found in 4 out of 8 CD30 positive CTCL as well as 1 out of 6 MF cases and 1 out 6 Sezary’s syndrome cases. No CCR8 staining was observed in any of the samples.

CCR3 and CD30 co-expression in freshly isolated tumor cell suspensions from CD30 positive CTCL

To confirm our immunohistochemistry data on a single cell level and to assess expression levels of CCR3 on CD30 positive lymphoma cells, we performed flow cytometry analysis of tumor cell suspensions. Tumor cells suspensions were derived from fresh biopsy material, which was available in 3 patients with CD30 positive large cell CTCL (Nr. 1, 7, 8). Cells were double stained for CCR3 and CD30 and analyzed by flow cytometry. Compared with isotype control a strong co-expression of the chemokine receptor CCR3 and CD30 was detected (Figure 2, patient Nr. 7). Strong staining in immunohistochemistry
(Fig1A and B) translated into high level of CCR3 expression on CD30 positive tumor cells in flow cytometry analysis (Figure 2, patient Nr.7). The majority of CD30 positive cells were CCR3 positive. Expression levels were comparable to those seen in eosinophils (data not shown).

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

Detection of CCR3 protein on CD30 positive 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 downregulation which can be assessed by flow cytometry. After incubation with eotaxin/CCL11, RANTES/CCL5, MCP-3/CCL7 or medium alone, surface expression of CCR3 on short time cultured tumor cells was analyzed by flow cytometry using anti-CCR3 moAb. Receptor bound chemokine might interfere with antibody binding, therefore cells were washed in acidic glycine buffer as described to dissociate the chemokine ligand from its receptor. 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 due to a downregulation 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 positive cutaneous lymphoma cell line**

Investigation of receptor functionality in fresh tumor cell suspensions is limited both by the low number and 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 co-expressed CD30 and CCR3 (data not shown). As a first step, actin polymerization was investigated. Actin polymerization, which controls a number of processes regulating cell migration and reorganization of the actin cytoskeleton, has been shown to be an early event in the migratory response to chemokines. In order to examine whether eotaxin induces reorganization of the cytoskeleton in CD30+ lymphoma cells, filamentous actin was measured in the CD30+, CCR3+ lymphoma cell line using
fluorescein phalloidin. Stimulation with 100 ng/ml eotaxin induced a transient 38% increase in intracellular F-actin in CD30⁺ lymphoma cells within 15 seconds (Figure 3B) indicating the transduction of a migration signal to the cytoskeleton after binding of eotaxin to CCR3 on the CD30⁺ lymphoma cells.

Next we assessed whether engagement of CCR3 by its ligand eotaxin was leading to cell migration. Migration assays through 5 µm pore size polycarbonate membranes during 3 hours were performed using recombinant human eotaxin as chemoattractant (Figure 3C). Data show the typical curve of chemokine induced migration with a peak cell migration induced at 100 ng/ml.

**CCR3 ligand eotaxin/CCL11 is expressed by CD30 positive CTCL**

We investigated the expression pattern of CCR3 ligands in CD30 positive and negative 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 out of 18 CTCL (Table 1). In CD30 positive CTCL, eotaxin/CCL11 protein was detected in tumor cell aggregates demonstrated to be CD30 positive in serial sections (Figure 4B). Eotaxin/CCL11 protein was also detected in connective tissue cells surrounding tumor cells. Each of 8 CD30 positive CTCL 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 and Table 1). In contrast, positive staining for eotaxin/CCL11 in CD30 negative CTCL was confined to connective tissue cells only, while infiltrating lymphoma cells were negative (Figure 4A and Table 1). In 1 case of CD30 negative CTCL, eotaxin/CCL11 was also present in infiltrating lymphoma cells. Eosinophil infiltration was found in each of 5 CD30 positive large cell CTCL and in 3 out of 12 CD30 negative CTCL (Table 1). Staining for RANTES/CCL5 was positive in 4 CD30 positive CTCL, while MCP-3/CCL7 was expressed in only one 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 positive 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 positive tumor cells express IL-4 protein**

It has been previously shown that CD30 positive CTCL lesions contain mRNA related to TH-2 cytokine differentiation such as IL-4. To analyze the functional differentiation state of CCR3 bearing tumor cells, we performed three color staining of tumor cell suspensions obtained from lesional CD30 positive CTCL skin. Staining of tumor cell suspensions with IFN-γ, IL-4 and CCR3 showed a clear predominance of IL-4 positive cells within the CCR3 positive cell population (Figure 6, patient Nr. 8, representative of n=3 experiments). Ratio of IL-4/IFN-g expressing cells was 24 (12.3% vs. 0.5%). This indicates a skewing of CCR3 positive cells towards TH-2 cytokine production. Analysis of CCR3 negative lesional cells did not demonstrate a predominance of IFN-gamma or IL-4 protein (data not shown).
Discussion

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

CTCL 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’s 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 which 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 respectively. It has been shown that CCR3 is preferentially expressed in vitro by Th-2 cells. In this context presence of eosinophils as well as expression of Th-2 cytokine mRNA in lesions of CD30 positive cutaneous lymphomas are of interest. We reasoned that recruitment of CD30 positive Th-2 like lymphoma cells might be mediated by 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 eight CD30 positive CTCL demonstrated expression of CCR3 on lesional tumor cells (Table 1, Figure 1). This finding was confirmed by flow cytometry 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 using immunohistochemistry (Figure 1). CD30 negative 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 negative CTCL. The question of chemokine redundancy must be addressed early in any discussion of their potential role 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 receptor CCR4 might play a role in homing of lymphoma cells to skin. In our study 4 out of 8 CD30 positive CTCL, 1 out of 6 Mycosis fungoides and 1 out of 6 Sezary’s syndrome samples expressed CCR4. Significant CCR8 expression was absent in our samples. In most cases, temporal and spatial patterns of chemokine receptor expression are connected with ligand-receptor specificity to result in a disease specific pathology. An important question is the demonstration of a functional chemokine receptor expressed on skin infiltrating lymphoma cells. Most studies have demonstrated presence of chemokines or their receptors in-situ but have failed to provide data on fresh tumor cell suspensions. One recent investigation demonstrated differentially expressed chemokine receptors CCR3 and CCR5 on infiltrating leukocytes but not neoplastic cells in Hodgkin disease lymph nodes. We were able to demonstrate receptor functionality on CD30 positive tumor cells with an assay used in studies of CXCR4/SDF1-α interactions. In the presence of the CCR3 ligand eotaxin/CCL11 specific partial downregulation of CCR3 in tumor cell suspensions was observed (Figure 3A).

Investigation of receptor functionality in fresh tumor cell suspensions is limited both by the low number and heterogeneity of cells. To further address functionality of the CCR3 receptor we used the CD30 expressing cutaneous lymphoma cell line Mac-1. Engagement of the CCR3 receptor by its ligand eotaxin led to a signal towards the cytoskeleton involved in cell migration, i.e. actin polymerization (Figure 3B). Furthermore, directed migration of CD30 positive lymphoma cells towards an eotaxin gradient was observed (Figure 3C).

To establish a relationship between CCR3 expression and TH-2 cytokine production, we assessed the cytokine expression profile of CCR3 positive tumor cells. Earlier reports have demonstrated TH-2 cytokine mRNA in lesions of CD30 positive CTCL. On the protein level, we observed strong expression of IL-4 by the majority of CCR3 positive cells, while
only few cells expressed IFN-γ. This observation is compatible with a TH-2 like differentiation of CCR3 positive skin homing lymphoma cells.

The CCR3 ligand eotaxin/CCL11 is known to be produced by human dermal fibroblasts. Eotaxin/CCL11 does not only have agonistic functions but is also a natural antagonist for CCR2. Our data demonstrate the presence of eotaxin/CCL11 protein in skin of CTCL lesions (Table 1, Figure 4). In CD30 positive, as well as in CD30 negative CTCL we found an expression of eotaxin/CCL11, which was associated with connective tissue cells, most likely fibroblasts, located around the tumor. Only in CD30 positive CTCL, eotaxin/CCL11 expression was additionally 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, and an amplification of tumor cell homing to the skin. Downregulation 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 negative CTCL were shown to be associated with epidermal expression of interferon-γ inducible protein-10 (IP-10) and monokine induced by interferon-γ (Mig). 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 positive large cell CTCL as well as expression of its ligand eotaxin in skin. Expression of CCR3 on CD30 positive 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, pharmacological modulation of chemokine receptors may open the way to new treatment modalities.
Acknowledgements

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References


Legends

Table 1: Expression of CCR3 and its ligands in CTCL.

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 x200 high power field on 100 mononuclear cells and the percentage of positive staining cells was determined as follows: +/− : 0-5%, + : 5-25%, ++ : 25-50%, +++ : 50-75%, ++++ : 75-100% positive cells. Please note that CCR3 expression corresponds to CD30 expression. Staining for CCR3 ligands shows positivity for eotaxin in CD30 positive and CD30 negative CTCL (pos.: positive staining; neg.: negative staining), location of immunopositivity for chemokines is abbreviated as follows: tumor cells: TC; connective tissue cells: CT; eosinophils: Eos. Please note that tumor cells and connective tissue cells in CD30 positive CTCL are positive for eotaxin, while in CD30 negative CTCL tumor cells clusters are negative for eotaxin. n.d.: not done, MF: Mycosis fungoides.

Figure 1: Immunoreactivity of CCR3 in CTCL.

A: CCR3 immunoreactivity in cryosections of a CD30 positive large cell CTCL (Nr.7) using the alkaline phosphatase /anti-alkaline phosphatase method. Original magnification, x100.

B: CCR 3 immunoreactivity in cryosection of a CD 30 positive large cell CTCL (Nr.7) using the alkaline phosphatase/anti-alkaline phosphatase method. Original magnification, x400

Figure 2: Co-expression of CD30 and CCR3.

Flow cytometry analysis of tumor cell population. Staining of freshly isolated tumor cells shows co-expression of CD30 and CCR3. Please note high expression of CCR3 on tumor cells (patient Nr. 7). Experiment representative of n=3.
Figure 3: Functional expression of CCR3 on CD30 positive cutaneous lymphoma cells

A: CCR3 surface expression measured by flow cytometry on freshly isolated tumor cells (patient Nr. 8) after incubation with corresponding ligand eotaxin or medium control. Cells were incubated in pH 3.0 acid buffer to remove receptor bound chemokine on not internalized receptor. Internalization was not seen after incubation with low affinity ligands RANTES/CCL5 or MCP-3/CCL7 (data not shown). Experiment representative of n=3.

B: The F-actin content was measured in the CD30+ cutaneous lymphoma cell line Mac-1 upon stimulation with 100 ng/ml eotaxin for the indicated times. Fluorescein phalloidin was used to stain the cells and the filamentous actin content was then measured using flow cytometry. Data show mean of 3 different experiments.

C: To assess the chemotactic responses of the CD30+ cutaneous lymphoma cell line Mac-1 to different concentrations of eotaxin, migration across a 5 µm pore size polycarbonate membrane during 3 hours was measured by counting migrated cells using a flow cytometer. Migrated cells at each time point were measured in triplicates. Data are expressed as the mean number of migrated cells/well.

Figure 4: Immunoreactivity of eotaxin in CTCL.

A: Eotaxin/CCL11 immunoreactivity in cryosection of CD30 negative CTCL using the alkaline phosphatase/anti-alkaline phosphatase method. Eotaxin/CCL11 is only present in connective tissue cells, tumor cell staining is negative. Original magnification, x400.

B: Eotaxin/CCL11 immunoreactivity in cryostat section of CD30 positive large cell CTCL using the alkaline phosphatase/anti-alkaline phosphatase method. Eotaxin/CCL11 is present in connective tissue cells and tumor cells which were CD30 positive in serial sections (data not shown). Original magnification, x400.
Figure 5: Intracellular eotaxin expression in tumor cell suspensions

Intracellular flow cytometry staining of tumor cell suspensions for CCR3 ligand eotaxin/CCL11, after stimulation for 4h with PMA and ionomycin demonstrates expression of eotaxin in tumor cells. Analysis gated on high forward-sidescatter cell population which corresponds to CD30 positive tumor cells (patient Nr. 8). Experiment representative of n=3.

Figure 6: Production of IL-4 by CCR3 positive tumor cells

Intracellular flow cytometry analysis of cytokine expression shows that the majority of CCR3 bearing cells express IL-4 protein but not IFN-g (Ratio IL-4/IFN-g: 24). Analysis gated on CCR3 positive population (patient Nr. 8). Experiment representative of n=3.
Table 1

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Figure 1
Figure 2
Figure 3A

![Graph showing CCR3 expression with and without eotaxin incubation.](image)
Figure 3B
Figure 3C
Figure 4
Figure 5
Figure 6
Functional expression of the eotaxin receptor CCR3 in CD30 positive cutaneous T-cell lymphoma

Martin Kleinhans, Adrian Tun-Kyi, Michel Gilliet, Marshall E Kadin, Reinhard Dummer, Guenter Burg and Frank O Nestle