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Down-Modulation of CXCR3 Surface Expression and Function in CD8⁺ T Cells from Cutaneous T Cell Lymphoma Patients

Dorian Winter,† Julia Moser,† Ernst Kriehuber,‡ Christoph Wiesner,† Robert Knobler,‡ Franz Trautinger,‡ Paula Bombosi,¶ Georg Stingl,† Peter Petzelbauer,§ Antal Rot,¶ and Dieter Maurer²

Viruses can escape detection by the immune system by exploitation of the chemokine-chemokine receptor system. It is less established whether human cancers can adopt similar strategies to evade immunologic control. In this study, we show that advanced cutaneous T cell lymphoma (CTCL) is associated with selective and efficient inactivation of CXCR3-dependent T cell migration. Our studies demonstrate that this alteration is at least in part due to CXCR3 down-regulation in vivo by elevated serum levels of CXCR3 ligands. The T cell population most affected by this down-regulatory mechanism are CD8⁺ cytotoxic effector T cells. In CTCL patients, cytotoxic effector T cells have strongly reduced surface CXCR3 expression, accumulate in peripheral blood, but are virtually absent from CTCL tumor lesions, indicating an inability to extravasate into lymphoma tissue. CTCL-associated inactivation of effector cell recruitment may be a paradigmatic example of a new type of immune escape mechanisms shielding the neoplasm from a tumoricidal attack. The Journal of Immunology, 2007, 179: 4272–4282.

CD8⁺ T cell function is associated with disease progression. Indeed, CD8⁺ T lymphocytes with specific cytotoxicity against parental tumor cells have been isolated from blood of mycosis fungoides patients. Candidate epitopes recognized by these lymphoma-specific CTL include variable and hypervariable regions of the clonal TCR and cancer-related Ags of the tumor/testis family (6–9). Interestingly, lymphoma-specific cytotoxic T cell responses of comparable specificity and magnitude could be induced from the blood of healthy controls and of lymphoma patients with a far advanced disease (6). This indicates that deletion or permanent anergy of lymphoma-specific circulating CD8⁺ T cells is not a viable explanation for a loss of immunologic control resulting in disease progression.

A relationship exists between a high percentage of cytotoxic CD8⁺ T lymphocytes in lymphoma skin infiltrates and a longer survival of patients (10, 11). This confirms that functional CD8⁺ CTLs have to enter lymphoma skin lesions to limit disease progression. Hence, an impairment of effector T cell homing into lymphoma lesions may underlie progression of CTCL from limited to advanced disease. Such condition could also explain why during disease progression from the plaque to the tumor stage, lesional cytotoxic effector CD8⁺ T cells are greatly reduced in numbers (10).

In this study, we investigate the possibility that CTCL impairs differentiation, cytotoxic function, and homing properties of CD8⁺ effector T cells. The patients studied in this work suffered from mycosis fungoides (subsequently referred to as CTCL) with or without hematologic tumor cell dissemination. We analyzed the expression and function of chemokine receptors on T cells of patients with advanced CTCL and found a selective defect in functional expression of CXCR3. In vitro studies suggest that this alteration is due to down-regulation of CXCR3 surface expression in vivo by elevated serum levels of cognate CXCR3 ligands (CXCR3L). These findings highlight a new chemokine receptor-based mechanism of hematologic malignancies to manipulate effector T cell migration and, thus, evade destruction. Consequently, therapeutic prevention of cancer-associated inhibition of effector T

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C utaneous T cell lymphoma (CTCL) is the most prevalent malignant lymphoproliferative disorder of the skin. Mycosis fungoides represents the vast majority of all CTCLs. In this disease, the transformed cell is a clonal CD4⁺/CD7⁻/CD26⁺ memory T cell with a bizarrely shaped nucleus (1–4). In early disease stage, these CTCL cells show a high degree of tropism for skin epithelium, whereas in later disease stages dermal infiltration predominates. Accordingly, scaly patches and plaques define early limited disease, whereas the occurrence of tumors indicates disease progression. Further disease progression is most often associated with hematogenic spreading of the tumor cells giving rise to T cell leukemia. The prognosis of mycosis fungoides patients is clearly linked to the extent of skin and, more importantly, systemic involvement. Cytodestructive and immunomodulatory therapies may induce partial remissions, but do not improve substantially the prognosis of advanced mycosis fungoides (5).

Recent studies indicate that CD8⁺ CTL can exert antitumor responses in mycosis fungoides, and that a down-regulation of
cell homing may be instrumental for the design of clinically effective cancer vaccines.

Materials and Methods

Patients

Heparinized blood (n = 25), serum, and 4- to 6-mm punch biopsies from lesional skin (n = 3) were obtained from mycosis fungoides (subsequently referred to as CTCL) patients with or without hematologic involvement (56.3% of patients had CD4+ CD7− tumor cells in blood with a range from 17.8 to 82.9% (mean 51.3%) of all peripheral blood (PB) T cells). The number of patient samples analyzed in the various assays is given in the respective figure legends. The diagnosis of mycosis fungoides was based on clinical criteria, typical histology, and immunohistology in accordance with the classification of cutaneous lymphomas by the European Organization for Research and Treatment of Cancer (12). T cell clonality in skin and/or blood was determined by a standard multiplex PCR approach and/or by mAb-based anti-TCRβ immunophenotyping (6). Blood and lesional skin were obtained from one patient with varicella zoster virus (VZV) infection. Control blood samples were obtained from healthy donors, patients suffering from systemic scleroderma (undergoing cycles of extracorporeal photochemotherapy (ECP) every 4 wk), or patients after surgery for skin were obtained from one patient with varicella zoster virus (VZV) and cells were squeezed out by applying gentle manual pressure. Debris purity of by mAb-based anti-TCRVβ immunophenotyping (6). Blood and lesional skin were obtained from one patient with varicella zoster virus (VZV) infection. Control blood samples were obtained from healthy donors, patients suffering from systemic scleroderma (undergoing cycles of extracorporeal photochemotherapy (ECP) every 4 wk), or patients after surgery for skin were obtained from one patient with varicella zoster virus (VZV) and cells were squeezed out by applying gentle manual pressure. Debris purity of.

Cells

PBMCs were prepared from heparinized blood by sedimentation over Ficoll-Paque® (Pharmacia). Remaining erythrocytes were lysed osmotically in a buffer containing 0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM 

Flow cytometry analysis

T cell populations were characterized using anti-CD3 (SK7) PerCP, anti-CD4 (Leu-3) PE, and anti-CD7 (4H9) FITC or anti-CD8 (SK1) FITC (BD Pharmingen). Anti-CD122 (2A3) PE, anti-HLA-DR (L243) PE, and anti-CD56 mAbs (all from Beckman Coulter)-based immunomagnetic depletion (MACS; Miltenyi Biotec). By this procedure, CD8+ T cells were routinely enriched to a purity of >95%.

Cells from CTCL tumor lesions and VZV-induced skin lesions were isolated, as described previously (6). Briefly, punch biopsies were minced and processed by applying manual pressure. Debris and fibers were removed by filtration through a 40-µm cell strainer (BD Discovery Labware Europe). The human erythrolymphocytic cell line K-562 was provided by O. Majdic, Institute of Immunology (Medical University of Vienna, Vienna, Austria).

Confluent cultures of CTCL skin lesions and VZV-induced skin lesions were obtained from one patient with varicella zoster virus (VZV) infection. Control blood samples were obtained from healthy donors, patients suffering from systemic scleroderma (undergoing cycles of extracorporeal photochemotherapy (ECP) every 4 wk), or patients after surgery for skin were obtained from one patient with varicella zoster virus (VZV) and cells were squeezed out by applying gentle manual pressure. Debris purity of.

Confocal microscopy

Purified CD8+ T cells were resuspended at a density of 2 × 10^6 cells/ml in PBS. Aliquots containing 4 × 10^5 cells were bound to adhesion slides (Bio-Rad) for 30 min at 4°C. Adherent cells were fixed and permeabilized using FIX&PERM. During permeabilization, cells were incubated with anti-CXCR3 (R&D Systems; diluted 1/20) or appropriate isotype control mAbs and rabbit anti-human CD3 (OKT-3; diluted 1/10; DakoCytomation). After washings with cold PBS/1% BSA, slides were incubated with Fab anti-mouse Alexa 488 (1:200; Molecular Probes) and Fab anti-rabbit Cy5 (1:200; Jackson ImmunoResearch Laboratories). Slides were mounted in Fluoroprep (bioMerieux). To define the intracellular localization of CXCR3, purified CD8+ T cells were fixed in ice-cold acetone for 5 min, blocked in PBS/1% BSA/5% goat serum, and exposed simultaneously to FITC-conjugated anti-CD8 mAbs (100 µg/ml; BD Pharmingen), and rabbit anti-early endosomal Ag (EEA1)1 (1:500; Abcam) and rabbit anti-calreticulin (at 1:500; Bio Research), according to the instructions of the manufacturer. Anti-CXCR3 FITC or appropriate isotype-matched control mAbs were used for intracellular staining. Cells were analyzed by flow cytometry on a FACSAria flow cytometer (BD Biosciences). By this procedure, CD8+ T cells were routinely enriched to a purity of >95%.

CD8+ T cells were enriched by anti-CD4, anti-CD25, anti-HLA-DR, and anti-CCR5, anti-CCR7, or anti-CXCR3. Five-color analyses and cell sorting were performed on a FACSAria flow cytometer (BD Biosciences).

Confocal microscopy

Purified CD8+ T cells were resuspended at a density of 2 × 10^6 cells/ml in PBS. Aliquots containing 4 × 10^5 cells were bound to adhesion slides (Bio-Rad) for 30 min at 4°C. Adherent cells were fixed and permeabilized using FIX&PERM. During permeabilization, cells were incubated with anti-CXCR3 (R&D Systems; diluted 1/20) or appropriate isotype control mAbs and rabbit anti-human CD3 (OKT-3; diluted 1/10; DakoCytomation). After washings with cold PBS/1% BSA, slides were incubated with Fab anti-mouse Alexa 488 (1:200; Molecular Probes) and Fab anti-rabbit Cy5 (1:200; Jackson ImmunoResearch Laboratories). Slides were mounted in Fluoroprep (bioMerieux). To define the intracellular localization of CXCR3, purified CD8+ T cells were fixed in ice-cold acetone for 5 min, blocked in PBS/1% BSA/5% goat serum, and exposed simultaneously to FITC-conjugated anti-CD8 mAbs (100 µg/ml; BD Pharmingen), and rabbit anti-early endosomal Ag (EEA1)1 (1:500; Abcam) and rabbit anti-calreticulin (at 1:500; Bio Research), according to the instructions of the manufacturer. Anti-CXCR3 FITC or appropriate isotype-matched control mAbs were used for intracellular staining. Cells were analyzed by flow cytometry on a FACSAria flow cytometer (BD Biosciences).

CD3-dependent killing assay

FcγR-positive K-562 cells were labeled with 0.1 µM CFSE (Molecular Probes). CFSE-labeled K-562 cells were incubated in PBS for 1 h at 4°C in the presence or absence of 50 µg/ml anti-CD3 mAb (OKT-3; Dako-Cytomation). After two washes in ice-cold PBS, 2 × 10^5 cells/well were plated in 96-well U-bottom plates (Corning Glass), anti-CD8 mAbs (three times the final concentration of ECP- and/or IFN-α-treated CTCL and control patients were taken upon informed consent at the end of the interval between consecutive treatments.

Confocal microscopy

Purified CD8+ T cells were resuspended at a density of 2 × 10^6 cells/ml in PBS. Aliquots containing 4 × 10^5 cells were bound to adhesion slides (Bio-Rad) for 30 min at 4°C. Adherent cells were fixed and permeabilized using FIX&PERM. During permeabilization, cells were incubated with anti-CXCR3 (R&D Systems; diluted 1/20) or appropriate isotype control mAbs and rabbit anti-human CD3 (OKT-3; diluted 1/10; DakoCytomation). After washings with cold PBS/1% BSA, slides were incubated with Fab anti-mouse Alexa 488 (1:200; Molecular Probes) and Fab anti-rabbit Cy5 (1:200; Jackson ImmunoResearch Laboratories). Slides were mounted in Fluoroprep (bioMerieux). To define the intracellular localization of CXCR3, purified CD8+ T cells were fixed in ice-cold acetone for 5 min, blocked in PBS/1% BSA/5% goat serum, and exposed simultaneously to FITC-conjugated anti-CD8 mAbs (100 µg/ml; BD Pharmingen), and rabbit anti-early endosomal Ag (EEA1)1 (1:500; Abcam) and rabbit anti-calreticulin (at 1:500; Bio Research), according to the instructions of the manufacturer. Anti-CXCR3 FITC or appropriate isotype-matched control mAbs were used for intracellular staining. Cells were analyzed by flow cytometry on a FACSAria flow cytometer (BD Biosciences).

Real-time quantitative PCR

CD8+ T cells were isolated from CTCL patients and healthy donors and divided in aliquots of 1 × 10^6 cells before or after 5-day incubation with 100 U/ml IL-2. mRNA was extracted (mRNA isolation kit; Roche Diagnostics) and reverse transcribed (first strand cDNA synthesis kit for RT-PCR; Roche). TaqMan RT-PCR (Applied Biosystems) was performed with the following primers and probes: CXCRC3A, FAM probe 5'-TGAATGCGA CACCAATGCTTAAATGAGC-3', forward primer 5'-ACCACAGCAC AGAGGACCG-3', and reverse primer 5'-TCGCGGCTATTAGC-3'.
ACTTG-3′, CXXR3B, FAM probe 5′-CCGGTTCCGGCCTCTCACAGG-3′, forward primer 5′-TGGCAAGGCTTTACACAGC-3′, and reverse primer 5′-TGGGCGTATTTAGCCTTGG-3′ (15). β2-microglobulin (β2m) quantification for normalization was performed using commercial TaqMan assay reagents (Invitrogen Life Technologies).

Depletion of CXXR3L from serum

Aliquots (1 ml) of patient serum were incubated with 50 μl of protein G-Sepharose 4B (Sigma-Aldrich) for 1 h at room temperature under continuous agitation. For depletion of CXXR3L, 50 μl of Sepharose was washed in PBS and incubated with 5 μg of anti-CXCL9 (49106), anti-CXCL10 (33036), and anti-CXCL11 (87328) mAbs (all from R&D Systems) for 1 h at 4°C. Control mAb-loaded Sepharose was prepared in parallel. After several washes, mAb-bound Sepharose was added to pre-clear serum aliquots and incubated for 2 h under agitation. Sepharose was removed by centrifugation, and sera were stored at −80°C until use.

Transwell insert chemotaxis assay

Chemotaxis assay was performed, as described previously (16). Briefly, PBMC, either freshly isolated or cultured for 5 days, were washed and resuspended in migration buffer consisting of HBSS (Invitrogen Life Technologies), 1 mM CaCl2, 0.5 mM MgCl2, and 0.1% BSA (all from Sigma-Aldrich) at a density of 2 × 106 cells/ml. Chemokine solution (600 μl) was added to wells of a 24-well plate (Corning Glass). Buffer alone was used as a negative control. Cells were allowed to migrate for 4 h at 37°C. Migrated cells were collected by complete aspiration of the content of the wells, followed by two washes of the wells. This rinsing fluid and the content of each well were pooled, and one-eighth (i.e., 100 μl) of each sample was used for cell counting on a FACScan. Absolute counts of migrated cells were obtained by multiplying the amount of cells found in the appropriate forward/sideward scatter gate by eight. Migration indices are calculated as follows: (percentage of chemokine-induced migration)/ (percentage of spontaneous migration). In desensitization experiments, T cells were incubated with CXXR3- or mock-depleted serum for 1 h before the migration assay.

Quantification of serum chemokine levels

Serum was obtained from healthy donors and CTCL patients, and chemokines were measured using a cytometric bead assay (Human Chemokine Kit I; BD Biosciences), according to the instructions of the manufacturer. The assay was performed on a FACScan equipped with BD CBA software (BD Biosciences).

Quantification of phosphorylated ERK1/2

Freshly isolated CD8+ T cells of healthy donors and CTCL patients were exposed to CXCL11 (100 ng/ml; R&D Systems), anti-CD3 (OKT-3, 1 μg/ml), or medium only for 5 min. Cells were washed in chilled PBS and pelleted. Pellets were lysed in denaturation buffer (BD Biosciences) and boiled for 5 min. The total protein amount of each sample was determined using the bicinchoninic acid assay (Pierce Biotechnology). Equal amounts of total protein were subjected to quantification of ERK1/2 phosphorylation using a cytometric bead assay (PhosphoERK1/2 (T202/Y204) Flex Set; BD Biosciences), according to the instructions of the manufacturer. Data analysis was performed on a FACScanLibur equipped with the FACSCalibur software and analyzed with FCAP software (both from BD Biosciences).

Immunohistochemistry

Cryostat sections (5 μm) from CTCL tumors were fixed in acetone and stained with MECA-79 (IgM; BD Pharmingen) (17), anti-VCAM-1, anti-CD154, anti-E-selectin Abs (18), or isotype-matched control mAbs. Bound Abs were visualized by biotinylated second-step Abs, peroxidase-labeled avidin biotin complexes, and aminoethylcarbazol. Sections were counterstained with hematoxylin and coverslipped.

Statistical analysis

Data was analyzed by SPSS 12.0 (SPSS).

Results

PB T cells of CTCL patients down-regulate the surface expression of CXXR3, but not of other chemokine receptors

To identify possible migratory alterations of the major T cell subsets in PB of CTCL patients with hematologic involvement, we analyzed chemokine receptor expression in circulating normal CD7+ CD4+ and CD8+ T cells as well as CD7− CD4+ lymphoma cells. As shown in Fig. 1, among 14 different CC and CXC chemokine receptors analyzed, selectively one chemokine receptor, CXXR3, is down-regulated in CD4+ and CD8+ T cells of CTCL patients as compared with the respective T cell subsets of healthy controls. In healthy subjects, CXXR3 is the chemokine receptor most abundantly expressed in CD8+ T cells (>70% of cells; Fig. 1) and is functionally associated with the ability of CD8+ T cells to extravasate and to exert cytotoxic functions in tissues (19, 20).

In CTCL patients’ blood, only a minor subset of CD8+ T cells expresses CXXR3 (Fig. 1) and the level of CXXR3 expression falls below that of cells from control subjects (data not shown). In contrast, other chemokine receptors that are expressed by CD8+ T cells (e.g., CCR5, CCR7, CXCR1) (20–23) showed no significantly altered expression in the T cells of CTCL patients. Circulating CD7− CD4+ CTCL lymphoma cells displayed only low levels of CXXR3, but expressed abundantly the chemokine receptors for homing to lymph nodes (CCR7) and skin (CCR4; Fig. 1). We noted a trend for increased CCR4 expression in CD4+ and CD8+ T cells of patients as compared with controls, but this difference did not reach statistical significance (Fig. 1, p > 0.05).

Down-regulation of CXXR3 expression is associated with the degree of tumor burden, but is not directly related to therapy

We next asked whether the phenomenon of CXXR3 down-regulation in T cells of CTCL patients is associated with specific treatment regimens or, alternatively, is linked to the pathophysiology of the disease process in CTCL. Current immunomodulatory therapies for medium to far advanced CTCL include ECP, systemic administration of IFN-α, or a combination of both. CTCL patients were grouped according to the therapeutic regimen used and analyzed for CD8+ T cell CXXR3 expression. Treatment controls included patients who underwent ECP as an experimental therapy of systemic scleroderma and patients who received IFN-α in an adjuvant setting after surgery for primary cutaneous melanoma. Although we noted a trend for lower CXXR3 expression in CD8+ T cells of these treatment controls as compared with those of healthy nontreated subjects, this difference did not reach statistical significance (p > 0.05; Fig. 2A). In contrast, CXXR3 expression in CD8+ T cells was significantly reduced in CTCL patients as compared with nontreated controls (p < 0.05) and in most cases was below that of the control treatment groups (Fig. 2A). Thus, the extent and the invariable occurrence of CXXR3 down-regulation in CD8+ T cells of CTCL patients, but not in control treatment groups, strongly suggest a direct relation of CXXR3 down-regulation to the disease process itself, although a certain contribution of treatment cannot be ruled out.

To analyze this possibility further, we asked whether the level of CXXR3 down-regulation correlates with the extent of disease. As a measure of tumor burden, we determined the numbers of tumor cells in the PB of CTCL patients. This parameter is justified by the fact that progression from local to systemic CTCL is almost invariably associated with the appearance of circulating tumor cells and the extent of systemic disease is closely reflected by the tumor cell counts in blood (2). Accordingly, we included CTCL patients with low to high tumor cell counts in the blood (>10% CD7− CD4+ circulating T cells) as well as patients who suffered from skin-restricted disease (<10% CD7+ CD4+ T cells). This analysis revealed a statistically significant inverse correlation between the level of CXXR3 expression on CD8+ T cells and the percentage of CD7− CD4+ tumor cells in PB (R² > 0.5; Fig. 2B).

No statistically significant correlation between CXXR3 expression
and total or relative counts of CD7+CD4+ and CD8+ T cells was observed ($R^2 < 0.5$; data not shown). Other chemokine receptors of the CD8+ T cells (i.e., CCR5, CCR7, CXCR1) failed to show a similar disease extent-related regulation (data not shown). Thus, it appears that the degree of CXCR3 down-regulation in CD8+ T cells is linked to the magnitude of the lymphoma cell burden, but not to a lesser degree to CTCL-related alterations in the homeostasis of normal T cells. Accordingly, we focused our experimental attention to the resolution of the mechanism of CXCR3 down-regulation in CTCL patients with substantial hematologic involvement.

![FIGURE 1](image1.png)

**FIGURE 1.** Comparative analysis of chemokine receptor expression on normal CD4+ T cells and CD8+ T cells from CTCL patients ($n = 4$) and healthy donors ($n = 3$). Values represent percentages of CD4+ T cells (left panel) and CD8+ T cells (center panel) that are reactive with mAbs specific for the indicated chemokine receptors. Right panel, Shows the chemokine receptor profile of circulating lymphoma cells from the same CTCL patients. Data were obtained by gating for CD3+CD4+CD7+ (left panel), CD3+CD8+ (center panel), and CD3+CD4+CD7+ PBMC (right panel) using four-color flow cytometry. Asterisks indicate significant statistic differences in chemokine receptor expression between corresponding cell populations as calculated using Student’s t test (**, $p < 0.001$).

![FIGURE 2](image2.png)

**FIGURE 2.** Low surface expression of CXCR3 on CD8+ T cells in CTCL patients is correlated with disease extent and is not a direct consequence of therapy. A, Lack of correlation between the expression of CXCR3 on CD8+ T cells and therapy. The percentage of CXCR3-expressing CD8+ T cells (y-axis) from controls (healthy donors (no Tx, $n = 5$), patients suffering from systemic scleroderma undergoing ECP ($n = 5$), patients treated with IFN-α after surgery for primary melanoma (IFN-α) ($n = 4$), and from CTCL patients treated with ECP ($n = 7$), with IFN-α ($n = 1$), or with ECP and IFN-α ($n = 3$) was assessed by flow cytometry. Asterisk indicates a significant difference in CXCR3 expression between groups ($p < 0.05$). B, Inverse correlation between the percentage of CD7+CD4+ circulating CTCL cells (x-axis) and the percentage of CXCR3-expressing CD8+ T cells (y-axis, $p < 0.05$). The curve depicts the logarithmic regression between the two data sets from patients (●, $n = 15$) and healthy donors (○, $n = 14$). The dotted line demarcates the upper limit of the range in which CD7+CD4+ T cells are found in healthy donors. In the patients with CD7+CD4+ T cell counts above this level, T cell clonality was detected in the PB (data not shown).
Down-regulation of CXCR3 on CD8+ T cells in CTCL is not linked to inefficient generation and function of CTL

On the basis of their surface receptor expression, CD3+CD8+ T cells can be subdivided into naive T cells (CD27+CD45RA+), memory T cells (CD27+CD45RA−), and effector T cells (CD27−CD45RA−); a fourth subset of CD27−CD45RA− cells can be resolved. It corresponds to CD8+ T cells that are in transition between the memory and the effector cell pool (Fig. 3A) (21). Because CXCR3 is of particular relevance for the migration of cytotoxic effector CD8+ T cells, we asked whether the down-regulation of CXCR3 expression in CD8+ T cells of CTCL patients can be explained by a reduction of effector T cells in PB. Intriguingly, our comparative analysis of the CD8+ T cell subset composition in CTCL patients and healthy controls revealed that this is not the case. Conversely, effector CD8+ T cells were enriched among CD8+ T cells in CTCL patients (Fig. 3, A and B). In absolute numbers, effector CD8+ T cells of both the CD27+CD45RA+ and the CD27−CD45RA− phenotype occurred in similar prevalence in patients and controls (Fig. 3C). Thus, the previously observed striking reduction in PB-CD8+ T cell counts in advanced CTCL (10) is due to a depletion of naive as well as of memory CD8+ T cells (Fig. 3C).

In support of an unperturbed CD8+ effector T cell function in CTCL patients’ blood is the finding of enhanced rather than reduced activation marker expression in these cells (Fig. 4A). Furthermore, the cytotoxic potential of patient-derived CD8+ T cells was found to be at least comparable to that of healthy donors’ CD8+ T cells (Fig. 4B). When we sorted patient-derived CD8+ T cells according to their naive, memory, and effector phenotypes and used them in a sensitive Europium-based cytotoxicity assay, we observed that cytotoxic function was clearly enriched in the immunophenotypically defined effector cell populations (Fig. 4C).
which is comparable to the situation seen in nondiseased individuals (data not shown) (21). When we comparatively analyzed CXCR3 expression in naive, memory, and effector CD8\(^+\) T cells of CTCL patients and controls, we observed that CXCR3 down-regulation is most pronounced in the effector CD8\(^+\) T cell population of patients (Fig. 4D). Thus, we conclude that CXCR3 down-regulation in PB-CD8\(^+\) T cells of CTCL patients mainly involves cytotoxic effector T cells. These cells, however, neither are compromised in their cytotoxic potential nor are reduced in numbers.

![Figure 5](https://example.com/figure5.png)

**FIGURE 5.** Down-regulated T cell CXCR3 expression in CTCL results from relocation of CXCR3 from the surface to endosomal organelles. 

A. CXCR3A and CXCR3B mRNA expression in purified CD8\(^+\) T cells of CTCL patients \((n = 3)\) and healthy donors \((HD, n = 3)\). The relative amount of CXCR3 mRNA was measured by real-time PCR and normalized to \(\beta_2\)m expression. 

B. Reduced surface, but high total cellular CXCR3 protein expression in CD8\(^+\) T cells of CTCL patients. CXCR3 expression was measured by flow cytometry on PBMC labeled with anti-CD3 allophycocyanin and anti-CD8 PE before (left panels) or after fixation and permeabilization (right panels). mAb reactivity with CXCR3 is shown by filled histograms, and isotype control reactivity by open histograms.

C. Intracellular localization of CXCR3 in CD8\(^+\) T cells from CTCL patients. Purified CD8\(^+\) T cells were mounted on slides, fixed, permeabilized, and immunostained with anti-CXCR3 (Alexa488, green) and anti-CD3 (Cy5, red). Merge of the emissions (yellow) demonstrates colocalization of CXCR3 and CD3 on the surface of CD8\(^+\) T cells from healthy donors (bottom panel). In CD8\(^+\) T cells from CTCL patients, CD3 surface expression is maintained, but CXCR3 accumulates in vesicular structures beneath the surface membrane (arrows, top panel). Images shown are representative for results obtained with CD8\(^+\) T cells from three different CTCL patients. 

D. Intracellular CXCR3 is contained in EEA1\(^-\) and EEA1\(^+\) LAMP2\(^+\) vesicular compartments. Purified CD8\(^+\) T cells from CTCL patients were mounted on slides, fixed, permeabilized, and immunostained with anti-CXCR3 PE (red), anti-LAMP2 FITC (green), and anti-EEA1 (allophycocyanin, blue). EEA1\(^-\)LAMP2\(^+\) endosomes containing CXCR3 appear in magenta (white arrows), whereas EEA1\(^+\)LAMP2\(^+\) endolysosomal compartments are white (gray arrows) in the overlay exposure.

E. Serum levels of CXCR3L. Sera were obtained from CTCL patients \((n = 5)\) and healthy donors \((n = 5)\). The amounts of the indicated chemokines in the serum were determined using cytometric bead assays. Error bars represent SD. The asterisk indicates significant statistic difference as determined using Student’s \(t\) test \((*, p < 0.05)\).

F. Rescue of CXCR3 expression on CD8\(^+\) T cells from CTCL patients by in vitro culture. CXCR3 surface expression was measured on CD8\(^+\) T cells, freshly isolated (left panels), or cultured for 5 days in medium containing IL-2. Dot plots (CD3, \(y\)-axis; CXCR3, \(x\)-axis) show cells gated for reactivity with anti-CD8 mAbs. Quadrants are set according to isotype control mAb reactivities.
Down-regulated surface expression of CXCR3 on CD8+ T cells is associated with the accumulation of the receptor in endolysosomal compartments

To see whether the selective defect in CXCR3 expression in T cells in CTCL patients is due to transcriptional silencing, we performed quantitative PCR experiments on purified CD8+ T cells. As shown in Fig. 5A, normalization of the amount of CXCR3 transcripts for housekeeping gene (β2m) expression revealed that CXCR3 transcripts in freshly isolated CD8+ T cells of CTCL patients are not reduced, but rather augmented, as compared with healthy donors. Culture of the isolated CD8+ T cells in the presence of IL-2 slightly up-regulated CXCR3A in cells from controls, but not patients (Fig. 5A). The migration-competent A form of CXCR3 (CXCR3A) was by far the most prevalent CXCR3 transcript expressed in CD8+ T cells of CTCL patients (Fig. 5A), mRNAs for the splice variant CXCR3B, a receptor that binds platelet factor-4 as well as classical CXCR3L without migration-inducing activity (15), and a truncated version of CXCR3 comprising only four transmembrane domains (22) were expressed at comparatively low abundance (Fig. 5A, data not shown). To analyze whether CD8+ T cells of CTCL patients display impaired translation of CXCR3, we comparatively analyzed surface expression and total cellular expression of CXCR3. Although down-regulated at the cell surface, CXCR3 protein was present intracytoplasmically in CD8+ T cells of CTCL patients (Fig. 5B). Thus, relocation of CXCR3 to intracellular sites, but not the impairment in transcription and translation is responsible for down-regulated cell surface expression in CD8+ T cells of CTCL patients.

To better define the cytoplasmic localization of CXCR3 in CD8+ T cells in CTCL patients, we performed image analyses using confocal microscopy. In CD8+ T cells from healthy donors, CXCR3 was expressed almost exclusively at the cell surface, as evidenced by colocalization with cell-surface-expressed TCR/CD3 (Fig. 5C). In striking contrast, CXCR3 in CTCL patients localized in vesicular structures juxtaposed to but clearly demarcated from cell membrane (Fig. 5C). Triple stainings for the endosomal marker EEA1 and the lysosomal Ag LAMP2 revealed that CXCR3 protein is present in endosomes (EEA1+ LAMP2−), lysosomes (EEA1− LAMP2+), and EEA1− LAMP2+ compartments that may represent endolysosomes (Fig. 5D). Thus, the reduced CXCR3 surface expression in T cells of CTCL patients can be the result of endosomal retention or lysosomal degradation or both. To investigate whether soluble CXCR3L may mediate the reduced CXCR3 surface expression in CTCL patients, we measured serum levels of the CXCR3L CXCL10 and CXCL9 in patients and healthy controls. A statistically significant increase in CXCL10 serum levels was observed in CTCL patients’ sera, whereas the increase in CXCL9 levels did not reach statistical significance (Fig. 5E). To see whether CXCR3 down-modulation in vivo can be reverted in vitro, we cultured CD8+ T cells under serum-free medium conditions. Although control CD8+ T cells only moderately up-regulated CXCR3, CD8+ T cells from CTCL patients strongly increased CXCR3 surface expression and reached expression levels close to those seen in controls (Fig. 5F).

CXCR3L fail to induce chemotaxis, but activate ERK in CD8+ T cells from CTCL patients

CXCR3 can activate at least two distinct signaling pathways in T cells, as follows: G proteins necessary for migration and the ERK/MAPK pathway involved in the regulation of survival. To see whether down-regulated CXCR3 surface expression in CD8+ T cells from CTCL patients results in functionally impaired responses to CXCR3L, purified CD8+ T cells from CTCL patients and healthy controls were subjected to Transwell migration and ERK activation assays. As shown in Fig. 6A, freshly isolated control CD8+ T cells exhibited weak, but reproducible chemotactic responses to CXCR3L. CXCL11 was used in these experiments because it induces stronger T cell migration than the other CXCR3L in vitro (23). In contrast to CD8+ T cells from controls,
CD8⁺ T cells from CTCL patients were deficient in their chemotactic response to CXCR3L (Fig. 6A). In vitro culture of CD8⁺ T cells from CTCL patients resulted in recovery of chemotactic activity to CXCR3L (Fig. 6A), which is in accordance with the observed rescue of CXCR3 surface expression. The magnitude of response was, however, invariably lower with cultured CD8⁺ T cells from CTCL patients than from healthy controls. In contrast, CD8⁺ T cells from patients and from controls were equally responsive to CCR5 ligands (Fig. 6B) and nonresponsive to the CXCR3B ligand PF-4 (data not shown). Although incapable of migrating to CXCR3L, fresh CD8⁺ T cells from CTCL patients showed vigorous phosphorylation of ERK upon exposure to CXCR3L (Fig. 6C). The level of phosphorylation of ERK induction by CXCR3 triggering was even comparable to that seen after optimized TCR/CD3 triggering (Fig. 6C). Thus, the redistribution of CXCR3 to endolysosomal compartments of CD8⁺ T cells in CTCL patients abolishes complex ligand-induced cellular processes, such as migration, whereas ERK activation remains functional.

CXCR3 is down-regulated not only in CD8⁺, but also in CD4⁺ T cells of CTCL patients (Fig. 1). Thus, we analyzed whether CD4⁺ T cells in CTCL have an impaired ability to migrate to CXCR3L. As shown in Fig. 6A, freshly isolated CD4⁺ T cells of CTCL patients do not migrate to CXCR3L, arguing for a down-regulation of CXCR3 function in the major circulating T cell subsets. The further observation that serum levels of CXCR3L are significantly increased in CTCL patients prompted us to investigate whether ligand-induced receptor down-regulation in vivo may contribute to the observed migratory defect. To address this possibility directly, sera from CTCL patients were depleted or were not depleted of CXCR3L by immunofinity chromatography. Normal T cells were incubated in CXCR3L- or mock-depleted patient serum before allowing them to migrate to CXCL11. As shown in Fig. 6D, CXCR3L in CTCL patients’ sera are capable of reducing CXCR3 functionality because T cell chemotaxis to CXCL11 was significantly increased upon removal of CXCR3L. Thus, contact with CXCR3L in serum is at least partially responsible for down-regulation of CXCR3 in T cells from CTCL patients.

**Impairment of CXCR3 function prevents the recruitment of circulating effector CD8⁺ T cells into tumor lesions**

The consequence of a loss of CXCR3-dependent T cell migration in CTCL patients could be the inability of circulating CD8⁺ T cells to extravasate into neoplastic tissue and to exert cytotoxic function. To directly address this possibility, we comparatively analyzed the subset composition of CD8⁺ T cells in the blood and in tumor lesions of three CTCL patients. In three of three patients, we observed only few effector CD8⁺ T cells in the lesions, whereas the same patients had a substantial population of effector CD8⁺ T cells in the blood (Fig. 7). Poor detectability of effector T cells in tissues is unlikely the reason for these observations because effector T cells of the memory/effector CD8⁺ T cell phenotype were the most prevalently enriched T cell population detected in virus-induced skin lesions (Fig. 7B). In contrast to effector T cells, memory CD8⁺ T cells that express CCR5 as their cardinal chemokine receptor (24, 25) (data not shown) were efficiently recruited into CTCL lesions (Fig. 7B). Interestingly, the memory CD8⁺ T cell population in CTCL lesions compared with that of blood contained more CCR5⁺ but not CCR7⁻ or CXCR3-expressing cells (CCR5⁺, +32.7%; CCR7⁻, +1.4%; CXCR3⁻, −10.9%; means of two independent experiments), as revealed by five-color flow cytometry. This further suggests that CCR5 is a critical receptor for memory T cell recruitment into CTCL lesions. In contrast, the very few effector CD8⁺ T cells found in CTCL lesions showed higher CXCR3, but not CCR5 or CCR7 expression than effector CD8⁺ T cells in blood (CCR3⁺, +12%; CCR5⁺, +1.4%; CCR7⁺, −10.9%). Thus, it appears that the impairment of CXCR3-dependent functionality in CTCL results in dramatically lowered effector CD8⁺ T cell recruitment into tumors, and that the few effector CD8⁺ T cells in CTCL tumors are recruited on the basis of residual CXCR3 functionality.

**Defective effector CD8⁺ T cell homing to CTCL lesions is not due to the absence of CXCR3L within the lesions**

To see whether the specific ligands of CXCR3 are present within CTCL lesions, we performed immunofluorescence analyses on sections from excised skin tumor material. As shown in Fig. 8A, anti-CXCL9 and anti-CXCL11 immunoreactivity was associated with the majority of the blastoid cells forming CTCL skin tumors. To exclude that infiltrating cells other than T cells (i.e., dendritic cells, macrophages, and B cells) may significantly contribute to the observed CXCL9 and CXCL11 expression in CTCL lesions, we...
performed double stainings using anti-MHC class II mAbs (Fig. 8A). Results obtained strongly suggest that CXCR3L expression is largely restricted to MHC class II-negative CTCL cells. Interestingly, CXCL9 was barely detected in tumor cells, but was present in granular cells within the lesions, most likely representing mast cells (data not shown). Thus, the defective effector CD8$^+$ T cell recruitment to CTCL lesions cannot be attributed to a lack of CXCR3L expression in CTCL lesions. Conversely, CXCR3L production in CTCL tumor lesions, most likely by tumor cells themselves, may contribute to the observed increased serum levels of these cytokines in patients with advanced CTCL.

Recruitment of memory and naive CD8$^+$ T cells correlates with high L-selectin ligand and low E-selectin and VCAM expression on endothelial cells in CTCL tumors

To further investigate receptor-ligand pairs possibly involved in the observed memory and naive CD8$^+$ T cell recruitment into CTCL skin tumors, CD8$^+$ T cell subsets were analyzed for the expression of receptors for vascular addressins. Lesional memory and naive CD8$^+$ T cells, but not effector CD8$^+$ T cells, expressed substantial levels of L-selectin, whereas all cell populations expressed the E- and P-selectin ligand-1 (data not shown). The expression of L-selectin ligands and other vascular addressins in CTCL skin tumors was analyzed by immunohistochemistry. As shown in Fig. 8B, the tumor vasculature in CTCL expresses abundantly MECA-79-reactive L-selectin ligands. In contrast, the activation-regulated endothelial adhesion molecules E-selectin and VCAM-1 are expressed in trace amounts only. Control stainings on tonsillar lymphoid tissue revealed strong L-selectin ligand along with poor E-selectin and VCAM-1 expression on the vasculature of T cell zones (data not shown). Thus, the vascular addressin pattern in CTCL tumors is similar to that seen in normal lymphoid organs. High-level L-selectin ligand expression by endothelial cell in CTCL tumors hence appears functionally relevant and responsible for the observed recruitment of memory and naive CD8$^+$ T cells into these lesions.

Discussion

CXCR3 is a prime receptor involved in targeting CD8$^+$ cytotoxic effector T cells into the skin (19) and other organs (20). In this study, we show that CXCR3 is down-modulated from the surface of CD8$^+$ cytotoxic effector T cells of CTCL patients with advanced disease. However, CXCR3 transcripts and total amounts of CXCR3 protein were not reduced in these cells. In patients’ CD8$^+$ T cells, CXCR3 protein accumulates in endolysosomal organelles, a feature indicative of ligand-induced receptor internalization (26). Indeed, we found strong evidence for ligand-induced down-regulation of CXCR3 surface expression and function in advanced
CTCL. In CTCL patients with systemic involvement, we observed the following: 1) increased serum levels of CXCR3L; 2) decreased expression of CXCR3, but not of other chemokine receptors on CD8+ and CD4+ T cells; 3) abolished migratory T cell responses to CXCR3L, but not to other chemokines; and 4) partial rescue of CXCR3-mediated T cell migration after ex vivo culture of patients’ T cells. Moreover, CXCR3L in patients’ serum were capable of strongly reducing CXCR3-dependent chemotaxis of normal T cells in Transwell migration assays.

Besides the defect in CXCR3 expression and function, we found rather unperturbed differentiation effecter CD8+ T cells in CTCL patients. This is evidenced by the following findings: 1) purified CD8+ T cell from patients and control subjects had a similar cytotoxic potential; 2) CD8+ T cell with the immunophenotype of cytotoxic effecter cells occurred in comparable absolute numbers in PB of patients and controls; and 3) these immunophenotypically defined effecter T cells had a higher cytotoxic potential than memory and naive CD8+ T cells from the same patient. Moreover, unlike CXCR3, other chemokine receptors that are associated with cytotoxic effecter cell differentiation, e.g., CXCR1 (27), were expressed at equal levels in patients and controls. Thus, we conclude that down-regulation of CXCR3 in CTCL patients is not associated with a global defect in CD8+ effecter T cell generation and function. Our investigations on patients with hematologic involvement reveal that naive CD8+ T cells and memory CD8+ T cells are the subpopulations most explicitly depleted from blood of CTCL patients. Thus, drastically lowered CD8+ T cell counts in PB are not necessarily associated with the inability to generate and maintain a circulating functional effector CD8+ T cell pool in advanced CTCL. It is, however, important to mention that the observed relative increase in effecter CD8+ T cells in patient blood is not necessarily due to an augmented efficacy in effecter CD8+ T cell generation. Our data suggest that effecter CD8+ T cells, but not the other CD8+ T cell subsets, have an impaired ability to extravasate. Thus, effecter CD8+ T cells are trapped in the circulation, which may further explain the well known propensity of CTCL patients to acquire infectious diseases with a severe clinical course (28).

To address directly the biological impact of CXCR3 down-regulation on effecter CD8+ T cell homing to tumor sites, we comparatively analyzed blood and CTCL skin tumors. Tumoral skin lesions were largely devoid of effecter CD8+ T cells. Because migration to CXCR3L was the only chemokine-dependent migratory activity deregulated in patients’ effecter CD8+ T cells, one could speculate that effecter CD8+ T cell homing is a nonredundant CXCR3-dependent mechanism (19, 20, 29). Interestingly, naive CD8+ T cells along with memory CD8+ T cells migrated in CTCL tumors. The endothelium of CTCL lesions fails to express activation-associated E-selectin and VCAM-1, but expresses ligands for L-selectin (17), a feature shared with high endothelial venules of normal lymphoid tissue. Our observation of naive T cell recruitment to CTCL tumors strongly suggests that L-selectin-binding molecules on CTCL tumor vessels are functionally relevant in vivo. The lack of E-selectin on these vessels may further contribute to the observed defect in effecter CD8+ T cell homing. This situation is contrasted by the preferential homing of CXCR3+ effecter CD8+ T cells to virus-induced skin lesions with CXCR3L presentation on E-selectin+ blood vessels (Fig. 7B) (30). Thus, modulation of endothelial selectin expression and down-regulation of effecter CD8+ T cell migration are two functionally distinct, but synergistically acting mechanisms of CTCL escape from cytotoxic T cell attack.

As the result of our study, one can argue that the prevention or the reversal of the migratory defect of effecter CD8+ T cells is a valid therapeutic option in advanced CTCL. IFN-α, currently used in CTCL, is a major inducer of CXCR3-binding chemokines. Thus, IFN-α may induce two effects with opposing outcome. On the one hand, IFN-α, when expressed locally, can promote CXCR3-mediated effecter CD8+ T cell recruitment. In contrast, IFN-α, when inducing high serum levels of CXCR3L, may also contribute to down-regulation of effecter CD8+ T cell migration. However, IFN-α treatment of control patients never resulted in CXCR3 down-regulation to the extent seen in IFN-α-treated CTCL patients. Thus, IFN-α treatment alone cannot explain the CXCR3 defect observed in CTCL patients. Increased serum levels of CXCR3L in CTCL patients could be also due to secretion from lymphoma cells autonomously, which is supported by the immunohistochemical detection of CXCR3L in CTCL tumors. Despite the fact that it is a plausible explanation, we currently cannot unequivocally prove that the increased serum levels of CXCR3L derive from the tumor cells exclusively. It is conceivable that mechanisms other than chemokine production contribute to high systemic levels of CXCR3L in CTCL patients, an argument supported by the observations of increased CXCL10 serum levels, but no apparent overexpression of this chemokine in the tumors. Accordingly, it is possible that serum levels of CXCR3L are further regulated by CTCL-associated alterations in chemokine metabolism. The enzyme dipeptidyl peptidase IV (CD26), which can initiate the digestion of CXCR3L, is expressed by all normal T cells, but is notoriously absent from CTCL cells (31). Reduction of normal T cells along with an increase in CTCL cells in blood may thus result in decreased catabolism of CXCR3L and effective receptor down-regulation by excess soluble chemokine as the consequence. This may also explain the correlation between CTCL cell counts in blood and the level of CXCR3 down-modulation on normal T cells in our patients. Thus, selective enzymatic or affinity-based removal of desensitizing chemokines from blood could be an interesting strategy to promote effecter T cell migration in the setting of cancers that escape immune surveillance by exploitation of chemokine-dependent immune cell migration.

In summary, our findings highlight a new mechanism by which CTCL can manipulate the immune system to evade T cell-mediated destruction. Interestingly, in advanced metastatic melanoma, down-regulation of CXCR3 on CD8+ T cells was identified as a predictor of a poor clinical outcome (32). Thus, it appears that histogenetically distinct cancers manipulate CXCR3 expression and function in T cells to lock themselves from effecter T cell attack. The development of specific interference strategies against these cancer-induced alterations of effecter T cell homing can be an important requirement for the design of cancer vaccines that are clinically effective.

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References


