

DIFFERENTIAL CCR4 EXPRESSION AND FUNCTION IN CUTANEOUS T-CELL LYMPHOMA CELL LINES

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Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells primarily involving the skin. However, the mechanisms governing migration of CTCL cells have not been fully clarified. It has been shown that certain chemokine receptors are upregulated in CTCL cells, but it remains unanswered whether these chemokine receptors play a critical role in the migration dynamics of CTCL. Using cell lines originally derived from patients with different subtypes of CTCL, we have shown higher CCR4 expression in the line derived from the mycosis fungoides (MJ), compared with the line derived from Sézary syndrome (Hut78). In specific responses to CCL22 (a CCR4 ligand) treatments, MJ cells showed significant chemotactic migration, enhanced activation and adhesion of certain integrins (CD49d and CD29) *in vitro*, while the control cells (Hut78, CD4+CD45RO+ memory T cells, and Jurkat cells) did not. Furthermore, compared with Hut78 cells, MJ cells manifested significantly more transendothelial migration in responses to treatments with either CCL22 or conditioned medium from dendritic cells *in vitro*. These results provide further dynamic evidence, in line with the multistep cascade paradigm for leukocyte transendothelial migration, to support a critical role for CCR4 in CTCL migration.

Key Words: CCR4, chemokine, cutaneous T-cell lymphoma
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Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells (mTCs) primarily involving the skin. Mycosis fungoides (MF), the subtype of CTCL with the highest prevalence rate, typically presents as cutaneous patches or plaques, which may progress to tumor stages, while Sézary syndrome (SS), the erythrodermic variant of CTCL, may develop either *de novo* or following certain stages of classic MF [1]. Despite its diverse clinical features, one common feature of CTCL is its migration to skin, although to various extents in different subtypes. However, our current knowledge of the mechanisms regulating migration of CTCL is still limited.

It has been suggested that, for certain typical inflammatory skin diseases (e.g. atopic dermatitis), in order to migrate to skin, different lines of T cells adopt a complicated multiple-step adhesion cascade (tethering, rolling, and adhesion), in which chemokines and their receptors play a pivotal role, to leave the blood flow and transmigrate through endothelial cells into skin [2,3]. Importantly, certain chemokines and their receptors have been demonstrated to be highly expressed in inflammatory skin diseases, indicating possible important roles for them during the process. For example, the chemokine receptor CCR4 was found to be highly expressed by certain types of T cells in the peripheral blood of patients with atopic dermatitis, while CCL17 (also known as thymus and activation-regulated chemokine, or TARC), one of the ligands for CCR4, was found to be upregulated in lesional keratinocytes [4].

However, it remains elusive whether malignant clonal T cells of CTCL may also adopt multistep



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adhesion mechanisms to migrate. Among few reports that have addressed this issue, Heald et al have shown that T cells expressing cutaneous lymphocyte antigen (CLA), an adhesion molecule well known to mediate skin migration of T cells, correlate with disease extent in SS and decline with clinical remission [5]. Shiohara et al proposed that LFA-1 (CD11a/CD18) integrin expression by T cells may play a role in the epidermotropism of malignant T cells [6]. In contrast, Hwang and Fitzhugh reported a case of SS with downregulated expression of LFA-1 (CD11a/CD18), but upregulation of L-selectin (CD62L) [7]. More recently, several reports have focused on the expression of chemokines and chemokine receptors by CTCL. Kallinich et al demonstrated differential expression patterns of chemokine receptors in T cells at different stages (patch/plaque versus tumor stages) [8], finding that the chemokine receptors CCR4, CXCR3 and CXCR4 are highly expressed by MF T cells at early stages. Meanwhile, Kakinuma et al showed that the serum level of chemokine CCL17 (TARC), one of the ligands for CCR4, correlates with disease severity of MF, indicating that CCR4 and its chemokine ligands may play a role in the pathogenesis of MF [9].

We hence postulated that CCR4, beyond expression by CTCL cells, may play a critical role in the migration of CTCL cells through a multistep adhesion cascade causing transendothelial movement of leukocytes. By using different CTCL cell lines (MJ and Hut78; derived from MF and SS respectively), we found differential expression of CCR4 (much more in MF-derived MJ cells than in SS-derived Hut78 cells). In serial functional assays (in line with the current adhesion cascade paradigm) to test whether CCR4 may play an important role in CTCL migration, MJ cells showed greater responses to CCR4 ligands (mainly CCL22, also known as macrophage-derived chemokine, or MDC) than other cells (Hut78, CD4+CD45RO+ mTCs, and Jurkat cells as controls). Our results thus further support the postulation that CCR4 signaling may be functionally important in CTCL migration.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents

Originally from the American Type Culture Collection (Manassas, VA, USA), human HH, Hut78, MJ, and Jurkat (clone E6-1) cell lines, derived from

patients with non-MF CTCL tumors [10], SS [11], MF [12], and acute T-cell leukemia [13] respectively, were purchased from a local provider, National Health Research Institute Cell Bank (Hsin Chu, Taiwan). HH and Jurkat cells were cultured in RPMI 1640 (GIBCO, Invitrogen, Rockville, MD, USA) with 10% fetal bovine serum (GIBCO, Invitrogen). Hut78 and MJ cells were cultured in Iscove's modified Dulbecco's medium (GIBCO, Invitrogen) with 20% FBS. Human dendritic cells (DCs) were purchased from Lonza (Basel, Switzerland), and HaCaT cells (an immortalized keratinocyte cell line) were provided by Dr H.S. Yu of Kaohsiung Medical University. All cell lines were maintained at 37°C in a humid atmosphere of 5% CO₂. Recombinant human chemokine CCL22, recombinant human TNF- α , and recombinant human VCAM-1/Fc chimera were purchased from R&D systems (Minneapolis, MN, USA). The following monoclonal antibodies (mAbs) were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, phycoerythrin (PE)-conjugated anti-human CD45RO, PE-conjugated anti-human CD29, PE-conjugated anti-human CD49d, FITC-conjugated mouse IgG_{2b}, and PE-conjugated mouse anti-IgG₁ were from Biolegend (San Diego, CA, USA); FITC-conjugated anti-human CCR4 and anti-human CCL22 neutralizing antibody (Ab) were from R&D systems; FITC-conjugated AffiniPure F(ab')₂ fragment of goat anti-human IgG (Fc specific) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); and function-blocking anti-CD49d (α 4 integrin) mAb (clone: HP2/1) was from Serotec (Raleigh, NC, USA). The appropriate isotype controls used were unlabeled mouse IgG₁ and unlabeled mouse IgG_{2b}, purchased from R&D systems, and FITC-conjugated mouse IgG₁ and PE-conjugated mouse IgG_{2a} κ , purchased from BD Biosciences (San Jose, CA, USA). Other reagents used were calcein-acetoxymethyl ester (or known as calcein AM, Molecular Probe, Invitrogen), Ficoll-Paque (Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA), Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO, USA), HEPES (Sigma-Aldrich), manganese chloride (Sigma-Aldrich), and pertussis toxin (PTX; Calbiochem, San Diego, CA, USA).

Detection of CCR4 expression

CCR4 expression was detected by real-time quantitative PCR (qPCR) and flow cytometry. For qPCR, total RNA was isolation from cell pellets using RNeasy kits

(Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA was generated from total RNA using a commercial kit for reverse transcription (Superscript First-Strand Synthesis System; Invitrogen). Real-time qPCR was performed using an ABI PRISM® 7900 DNA Sequence Detection System and SYBR® Green (Applied Biosystems, Foster City, CA, USA) to quantify the PCR products as described [14]. Human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal control. Specific primer pairs for CCR4 (5'-CAATACTGTGGGCTCCTCCAA-3' and 5'-ATCCATGGTGGACTGCGTGTA-3') and G3PDH (5'-TGGGCTACACTGAGCACCAG-3' and 5'-CAGCGTCAAAGGTGGAGGAG-3') were designed using Primer Express software (Applied Biosystems). Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification. Fold differences in expression of gene x in cell populations y and z were derived by 2^k , where $k = (\text{Ct}_x - \text{Ct}_{\text{G3PDH}})_{\text{population } y} - (\text{Ct}_x - \text{Ct}_{\text{G3PDH}})_{\text{population } z}$ [14]. For flow cytometric analysis, cells were stained at 4°C with FITC-conjugated anti-human CCR4. After washing, cells were analyzed using a FACScan flow cytometer (BD Biosciences).

Chemotaxis assay

The chemotaxis response to chemokines was assessed using the ChemoTx® disposable chemotaxis assay system (Neuro Probe; Gaithersburg, MD, USA) as previously reported [15]. Cells were labeled with calcein AM and suspended in medium (2×10^6 cells/mL), and a 25- μ L cell suspension was added to the top side of the filter. Cells were then exposed to basal medium only or chemokine (CCL22 or CCL17) at 10 or 100 ng/mL, in the presence of other agents (neutralizing anti-CCL22 Ab at 16 μ g/mL, isotype for anti-CCL22 Ab at 16 μ g/mL, or PTX at 100 ng/mL) or not. Plates were then incubated for 3 hours at 37°C [16]. After incubation, cells migrating through the filter were counted in three randomly selected microscopic fields (40 $\times$) per well. The normalized migration index for each cell type was calculated using the control group (medium only) as the base level.

Integrin activation assay

To determine whether CCR4 signaling enhances integrin activation (conformation change to increase avidity for its ligand), cells were suspended in binding medium (HBSS containing BSA at 2 mg/mL, HEPES

at 10 mM, Ca^{2+} at 1 mM, and Mg^{2+} at 1 mM; pH at 7.4), with or without CCL22, in the presence of other agents to test CCL22 dependence (anti-CCL22 Ab, isotype for anti-CCL22, or PTX) or not, and incubated for 10 minutes at 37°C. Cells were then washed and suspended in binding medium in the presence of several mAbs against integrins, including PE-conjugated anti-human CD29 and anti-human CD49d (targeting $\alpha 4\beta 1$ integrin, from Biolegend), for 30 minutes at 4°C [17]. Cells were washed and then analyzed immediately with the flow cytometer.

Integrin adhesion assay

In order to further determine whether CCR4 signaling in MJ cells may actually enhance binding of integrin(s) to their ligands on endothelial cells, a soluble binding assay and static cell adhesion assay were performed. For the soluble binding assay, 2×10^5 cells were suspended in 50 μ L of binding medium and exposed to different conditions, including medium alone, CCL22 or CCL17 (100 ng/mL) only, or CCL22 with other agents, including anti-human CCL22 neutralizing Ab (16 μ g/mL for 10 minutes at 37°C), mouse IgG_{2B} isotype control for anti-CCL22 (16 μ g/mL for 10 minutes at 37°C), or PTX (100 ng/mL for 2 hours at 37°C) [18]. As described previously [19,20], soluble human VCAM-1/Fc chimera (ligand for $\alpha 4\beta 1$ integrin, at 20 μ g/mL) was added and incubated for 75 seconds at 37°C. Cells were then washed with ice-cold binding medium to stop binding. Binding of soluble VCAM-1/Fc was detected with FITC-conjugated AffiniPure F(ab')₂ fragments of goat anti-human IgG (specific for Fc) by flow cytometry. In some experiments (Figure 4C), pretreatment with function-blocking anti-CD49d (clone: HP2/1) mAb (10 μ g/mL for 10 minutes at 37°C) was also performed to determine whether binding of integrin ligand (VCAM-1) is also integrin dependent (except chemokine dependent). For the static cell adhesion assay, as described previously [20,21] with some modifications, 96-well plates were coated with recombinant human VCAM-1/Fc (1 μ g/mL in binding medium, 100 μ L) and incubated overnight at 4°C. Wells were then washed with bicarbonate buffer and incubated either with the presence of CCL22 (100 ng/mL), Mn^{2+} (1 mM MnCl_2 in binding medium), or binding medium only, for 30 minutes at room temperature. Cells (1×10^5 cells per 50 μ L of binding medium) were then added to the wells and incubated at 37°C for 30 minutes. Wells were washed four times

with binding medium to remove non-adherent cells. To quantify the adherent cells, cell proliferation assay kit from Biovision (Mountain View, CA, USA) was used. A total of 100 μ L of RPMI 1640 medium and 10 μ L of WST-1 solution was added to each well and incubated at 37°C for 2 hours. The plates were then analyzed using an ELISA plate reader to read absorbance at 450 nm.

Transendothelial migration assay with chemokine or conditioned medium treatment

Endothelial cells (ECs) derived from human skin (HMEC-1) [22] were grown to confluence on the inserts of Transwell plates (5- μ m pore size; Corning Costar, High Wycombe, United Kingdom). The confluent monolayers were incubated for 12 hours with TNF- α (10 ng/mL) to induce cell surface VCAM-1 expression [20]. Cells were labeled with calcein AM and suspended at 1×10^6 /mL in medium, and 100 μ L of this cell suspension was added to the inserts. Cells were then exposed to different conditions in the lower chambers of Transwell plates as indicated (Figure 5A), including medium alone, CCL22 (100 ng/mL) only, and CCL22 plus other agents including anti-human CCL22 neutralizing Ab (16 μ g/mL), mouse IgG_{2B} isotype control (16 μ g/mL), or PTX (100 ng/mL) [18]. Plates were incubated for 6 hours at 37°C [23]. After incubation, the inserts were removed, and the cells were counted in three randomly selected microscopic fields per chamber at a magnification of 40 $\times$. The transendothelial migration index was calculated by normalization against the medium alone group. In another series of experiments (Figure 5B), treatments with or without CCL22 were performed in the presence or absence of the function-blocking anti-CD49d (α 4 integrin) mAb (10 μ g/mL) or its isotype to determine whether transendothelial migration is also integrin dependent (except chemokine dependent). For the transendothelial migration assay involving treatment with conditioned medium (CM) from cells (DCs and HaCaT cells) (Figure 5D), MJ cells were exposed to basal medium or CM from DCs or HaCaT cells, plus other agents, including anti-CCL22 neutralizing mAb (or its isotypes), PTX, or anti-CD49d neutralized mAb (or its isotype).

Cell viability assay for MJ cells

Cells were seeded in 96-well plates (10^4 cells per well) in 90 μ L of culture medium containing FBS at only

0.5%. After incubation for 3 hours, cells were treated with 10 μ L of recombinant human CCL22, CCL17, or CXCL12 (also known as SDF-1) at 50 and 100 ng/mL concentrations in triplicate. As controls, cells were also treated with PBS only or culture medium containing 20% FBS. After incubation for 24 hours, a cell proliferation assay kit from Biovision (Mountain View, CA, USA) was used to quantify the cell viability.

Immunoblotting for CCR4 signaling in MJ cells

MJ cells (1×10^6 /mL) were cultured in 24-well plates for 4 hours at 37°C in serum-free medium and then treated with CCL22 (100 ng/mL). Cells were harvested at various time points (0, 5, 10, 15, and 20 minutes) for total protein extraction. The Coomassie Protein Assay Kit (PIERCE, Rockford, IL, USA) was used to standardize the protein concentrations of the supernatants. Cell lysates containing 30 μ g of protein were separated by 8% SDS-PAGE and transferred to PVDF membranes. Membranes were blotted with various mAbs (anti-human/mouse/rat pan-Akt, affinity-purified rabbit anti-phospho-Akt (S473), anti-human/mouse/rat pan-ERK1/ERK2, and anti-phospho-ERK1/ERK2(T202/Y204) (R&D systems) at 4°C overnight. Secondary Abs were added, and the bands were developed by enhanced chemiluminescence (ECL).

Statistical analysis

The data are presented as mean $\pm$ standard deviation. Data were analyzed using Student's *t* tests (two-sided, parametric). Statistical significance was determined at *p* < 0.05. Unless indicated otherwise, experiments were performed at least three times. Representative data are presented.

RESULTS

Expression of the chemokine receptor CCR4 by different CTCL cell lines

Using qPCR and flow cytometry, we measured and compared relative expression levels of the chemokine receptor CCR4 in two CTCL lines (MJ and Hut78), Jurkat cells (human leukemia T cell line), and CD4+CD45RO+ mTCs. By qPCR, higher expression of CCR4 was detected in MJ cells (~8.7-fold versus Hut78 cells by qPCR, Figure 1A), while Hut78 cells and other controls (Jurkat cells and CD4+CD45RO+ mTCs) showed

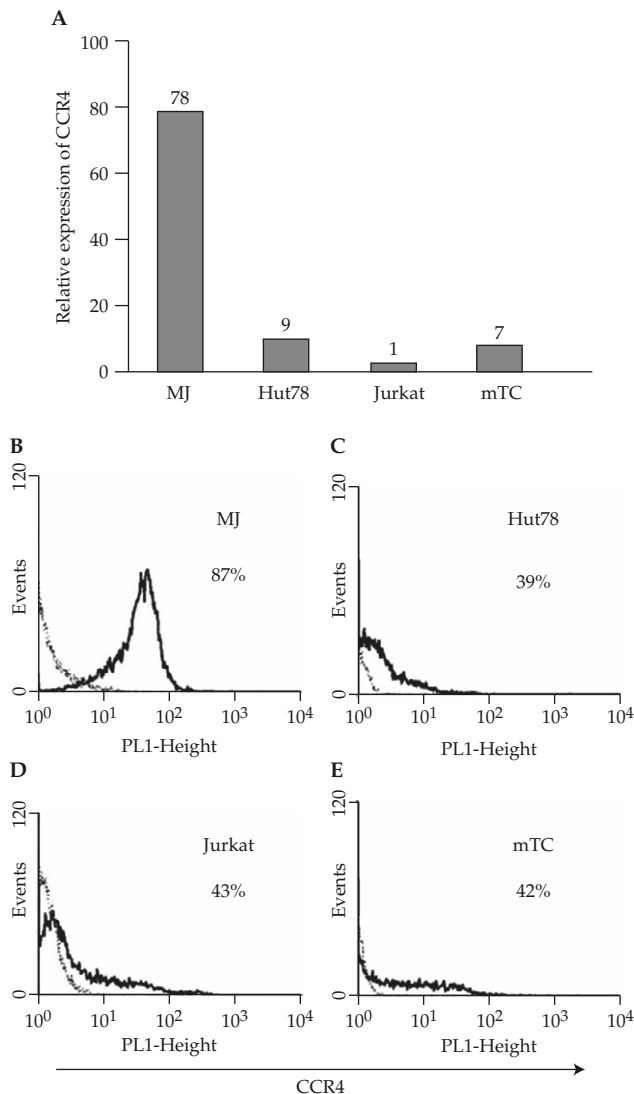


Figure 1. Relatively higher expression of CCR4 by CTCL MJ cells. (A) Relative expression levels of CCR4 (shown atop the black bar) in two CTCL lines (MJ and Hut78), Jurkat cells, and CD4+CD45RO+ mTCs were measured by qPCR. (B–E) Cells were stained with anti-CCR4 mAb (FITC-conjugated) and measured by flow cytometry.

relatively low expression of CCR4 (Figure 1A). A flow cytometry assay (Figure 1B–E) further demonstrated relatively high expression of CCR4 by MJ cells (~87%) compared with Hut78 cells (~39%) and controls (Jurkat cells, ~43%; mTCs, ~42%). Since the MJ cells originated from a patient with MF (the most typical CTCL subtype composed of predominantly skin-homing malignant T cells), whereas Hut78 cells were derived from a patient with SS (with many malignant T cells remaining in circulation instead of homing to skin), the differential expression of CCR4

by MJ and Hut78 cells appears to further substantiate CCR4's important role in CTCL skin homing.

MJ cells are more responsive to CCL22 (ligand for CCR4) in a chemotaxis assay

To further determine whether CCR4 signaling plays a critical role in the migration dynamics of MJ cells, we performed a chemokine functional assay to compare MJ cells with Hut78 cells and other control cells. As shown in Figure 2A, MJ cells demonstrated greater chemotaxis toward CCL22 (one of the two ligands for CCR4 tested) than Hut78 cells ($p < 0.01$) and control cells (Jurkat and CD4+CD45RO+ mTCs). The chemotaxis response of MJ cells to CCL22 is specific, since it could be negated by a neutralizing Ab (Anti-CCL22) and PTX (Figure 2A). Moreover, as shown in Figure 2B, CCL17 (another ligand for CCR4) may also induce chemotaxis of MJ cells in a dose-dependent manner, but to a lesser degree than CCL22, especially at a higher concentration (100 ng/mL; $p < 0.05$).

CCL22 treatment may enhance the activation of VLA-4 $\alpha 4\beta 1$ integrin (CD49d/CD29) on MJ cells

To address whether CCR4 signaling in MJ cells may induce a subsequent multiple-step adhesion cascade, which may lead to eventual transendothelial migration to skin, we next investigated if CCL22 treatments enhance integrin activation (that is, conformational change to increase avidity), integrin adhesion (to its ligand), and ultimate transendothelial migration. MJ cells were treated with CCL22 and then subjected to a flow cytometric assay to measure the expression of the active form (with increased avidity) of $\alpha 4\beta 1$ integrin using anti-CD49d and anti-CD29 mAbs able to detect the active form of integrin as previously described [17,24]. As shown in Figures 3A and 3B, MJ cells demonstrated enhanced expression (activation) of CD49d (Figure 3A) and CD29 (Figure 3B) after CCL22 treatment; this reaction is specific to the CCR4-CCL22 interaction, as anti-CCL22 and PTX, but not the isotype control, were able to attenuate the reaction. In contrast, enhanced integrin activation was not detected in similar assays using Hut78, Jurkat, and CD4+CD45RO+ mTC cells (data not shown). Furthermore, CCL17 was also able to enhance the expression of $\alpha 4\beta 1$ integrin by MJ cells (Figure 3C). In contrast, neither chemokine (CCL22 and CCL17) was able to induce enhanced expression of β_2 integrin

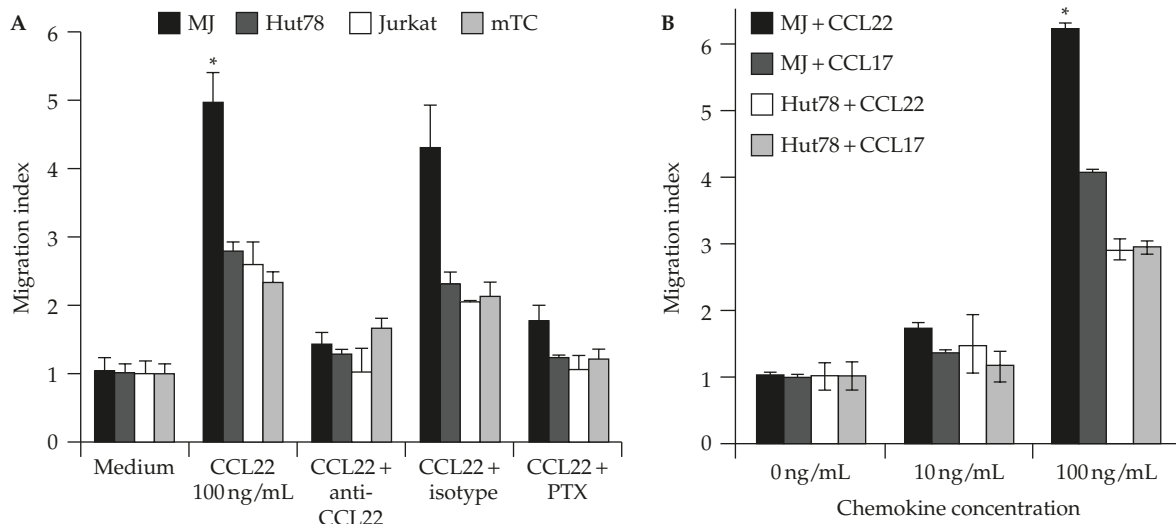


Figure 2. MJ cells show greater responses to CCL22 in the chemotaxis assay. (A) Migration of cells (MJ, Hut78, Jurkat, and mTC) was measured following exposure to medium only or CCL22 (100 ng/mL) in the presence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX) or not. * $p < 0.05$, MJ versus other types (Hut78, Jurkat, and mTC) of cells. (B) In another series of experiments, MJ and Hut78 cells were exposed to either CCL17 or CCL22 at 0, 10, or 100 ng/mL and chemotaxis was measured. * $p < 0.05$; MJ cells treated with CCL22 at 100 ng/mL versus other conditions (MJ with CCL17 at 100 ng/mL or Hut78 with either CCL22 or CCL17 at 100 ng/mL).

LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) by flow cytometry (data not shown).

CCR4 signaling may enhance $\alpha 4\beta 1$ integrin adhesion to its ligand (VCAM-1) on MJ cells

To further determine whether an interaction between CCR4 and CCL22 enhances *in vitro* adhesion of VCAM-1 (ligand for $\alpha 4\beta 1$ integrin; usually expressed on ECs by stimulation) to MJ cells via $\alpha 4\beta 1$ integrin (CD49d/CD29), MJ cells were subjected to a soluble binding assay by flow cytometry as described previously [20,25]. As shown in Figure 4A, following CCL22 treatment, CCR4 signaling by CCL22 treatment specifically enhanced adhesion of soluble VCAM-1, as this reaction could be blocked by either anti-CCL22 or PTX, but not the isotype control Ab. Moreover, the CCL22-enhanced VCAM-1 adhesion is indeed integrin dependent, as a neutralizing mAb for CD49d, but not its isotype control, was able to inhibit the VCAM-1 adhesion reaction (Figure 4B). In contrast, soluble VCAM-1 adhesion was not enhanced in Hut78, Jurkat, and CD4⁺CD45RO⁺ mTC cells (data not shown) after treatment with CCL22. Moreover, CCL17 treatment was also able to enhance VCAM-1 adhesion to MJ cells, but to a lesser degree (~15% less than the level of CCL22-enhanced binding, Figure 4C). To further substantiate the results of the soluble

VCAM-1 binding assay, a static cell adhesion assay using immobilized VCAM-1 was also performed. As shown in Figure 4D, CCL22 treatment may also enhance the binding of MJ cells to immobilized VCAM-1 (coated onto the plate), as shown in Figure 4D (compared with non-chemokine treatment, $p < 0.05$), which is comparable to a Mn^{2+} -induced reaction (a commonly used potent positive control for affinity regulation) [20].

CCR4-CCL22 interaction may enhance transendothelial migration of MJ cells

To determine whether CCR4-CCL22 interaction may ultimately enhance CTCL migration through ECs, an *in vitro* transendothelial migration assay to mimic the real skin milieu was set up by using skin-derived ECs (HMEC-1) and chemokine treatments (as described in the Materials and Methods section). As shown in Figure 5A, CCL22 treatment enhanced the migration of MJ cells through ECs relative to the migration of control cells (Hut78, Jurkat, and mTC) ($p < 0.05$, Figure 5A). This reaction was also CCR4-CCL22 interaction and integrin dependent, as it could be inhibited by neutralizing anti-CCL22 Ab, PTX (Figure 5A), and neutralizing Ab for CD49d (Figure 5B), but not by isotype controls. On the other hand, treatments with CCL17 moderately enhanced the transendothelial migration

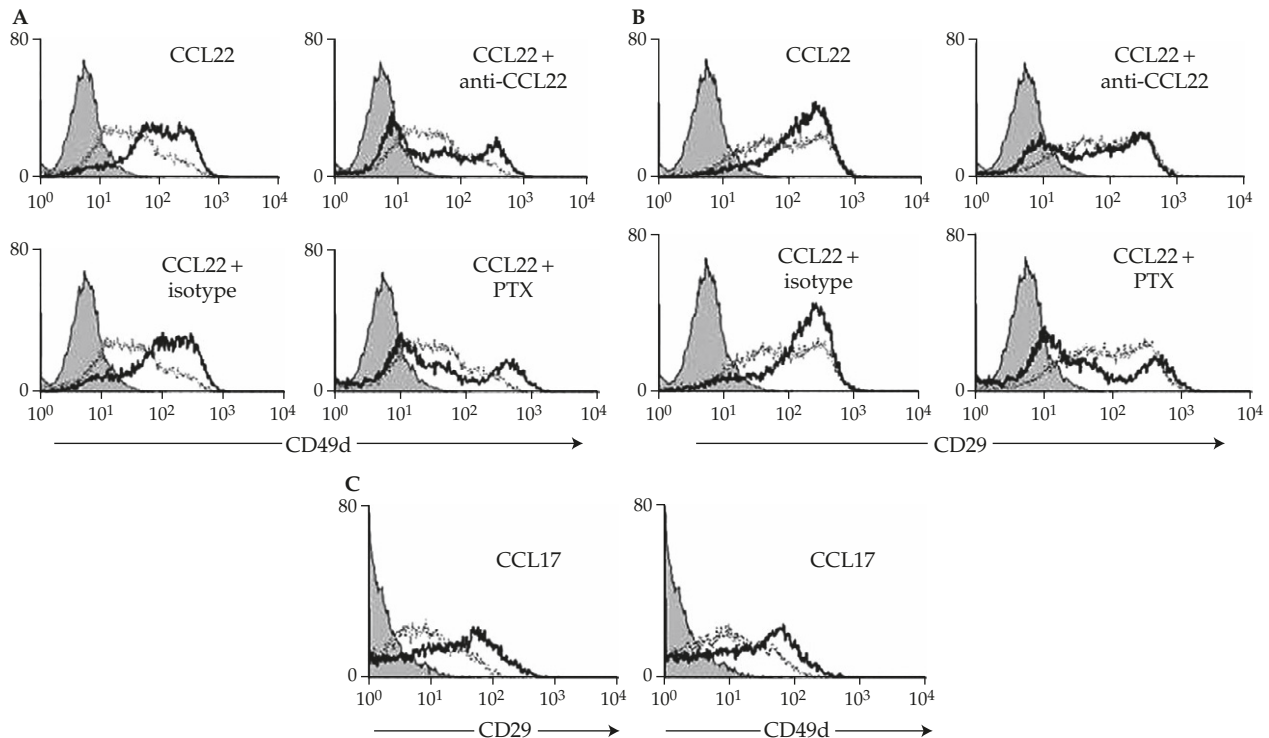


Figure 3. CCL22 treatment may enhance $\alpha 4\beta 1$ integrin activation on MJ cells. (A, B) MJ cells were exposed to CCL22 treatment or not, in the presence or absence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX), and then subjected to flow cytometric analysis of $\alpha 4$ (CD49d) and $\beta 1$ (CD29) integrin. Gray region: isotype control Ab for anti-CD49d or anti-CD29. Dotted line: anti-CD49d or anti-CD29 Ab, without CCL22. Solid line: anti-CD49d or anti-CD29 Ab, with the presence of CCL22, plus other agents or not. (C) MJ cells were exposed to CCL17 treatment or not, and then subjected to analysis of $\alpha 4$ (CD49d, right) and $\beta 1$ (CD29, left) integrin. Gray region: isotype control Ab for anti-CD49d or anti-CD29. Dotted line: anti-CD49d or anti-CD29 Ab, without CCL17 treatment. Solid line: anti-CD49d or anti-CD29 Ab, with CCL17 treatment. Results represent one of at least two experiments with similar results. X-axis: fluorescent intensity. Y-axis: cellular event number detected by flow cytometer.

of MJ cells, but to a lesser degree than treatments with CCL22 (~1.7-fold above basal level with CCL17 versus ~4.0-fold with CCL22, $p < 0.05$; Figure 5B).

DCs may also enhance the transendothelial migration of MJ cells by CCL22 secretion

It has been shown that DCs are the major source of CCL22 in the skin milieu [26,27], whereas CCL17 is mainly derived from keratinocytes (KCs) and ECs. To further substantiate our *in vitro* results (using exogenous chemokines CCL22 and CCL17 only), we next investigated whether human DCs and KCs enhance the transendothelial migration of CTCL via CCR4-CCL22 or CCR4-CCL17 interactions. High levels of expression of CCL22 and CCL17, by DCs and HaCaT cells (immortalized KCs), respectively, was confirmed by ELISA assays (using CM from cultured cells) (Figure 5C). Note that CCL22 is predominantly produced by DCs, but not by HaCaT or HMEC-1 cells, whereas

CCL17 is also found in DC- and HMEC-1-derived CM (Figure 5C). In the transendothelial migration assay, as shown in Figure 5D, DC-derived CM was able to significantly enhance the transendothelial migration of MJ cells in a similar fashion to CCL22, and this enhancement could also be blocked by neutralizing Abs (anti-CCL22 and anti-CD49d) and PTX, indicating that DC-enhanced transendothelial migration of MJ cells is also both CCR4-CCL22 interaction and integrin dependent. In contrast, HaCaT-derived CM could not significantly enhance the transendothelial migration of MJ cells beyond the basal level (~2.1-fold above basal level with HaCaT-derived CM, versus ~6.0-fold with DC-derived CM, $p < 0.05$; Figure 5D).

Cell survival of MJ cells in response to CCR4 signaling

As it has been well established that chemokine signaling may have multiple effects, besides cell migration,

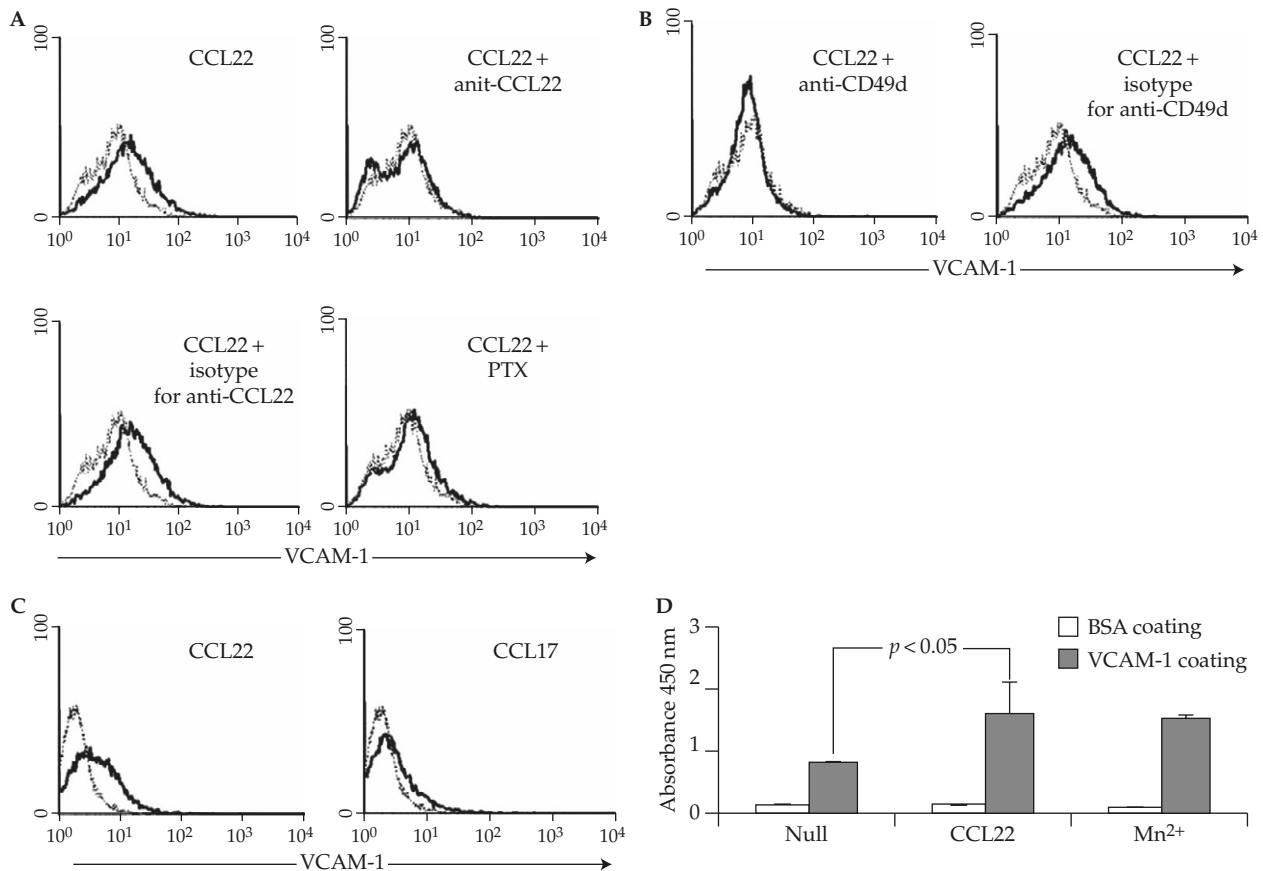


Figure 4. CCL22 treatment may enhance integrin-dependent adhesion to soluble or immobilized VCAM-1. (A) MJ cells were exposed to CCL22 treatment or not, in the presence or absence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX), to test the dependence on CCL22 treatment, and then incubated with soluble VCAM-1 before flow cytometric analysis of VCAM-1 adhesion. Dotted line: no CCL22 treatment. Solid line: with the presence of CCL22, plus other agents (anti-CCL22 Ab, isotype control for anti-CCL22 Ab, or PTX) or not. (B) In another series of experiments, MJ cells were likewise exposed to CCL22 treatment or not, followed by VCAM-1 incubation and flow cytometric analysis as in (A), but with the presence of function-blocking anti-CD49d mAb or isotype Ab to test the dependence on $\alpha 4$ integrin (CD49d). Dotted line: no CCL22 treatment. Solid line: with the presence of CCL22, plus neutralizing anti-CD49d Ab (or isotype for anti-CD49d Ab) or not. (C) In another series of experiments, MJ cells were exposed to either CCL22 or CCL17 treatment or not, and then subjected to VCAM-1 incubation and flow cytometric analysis. Dotted line: no chemokine treatment. Solid line: with CCL22 or CCL17 treatment. (A–C) X-axis: fluorescent intensity. Y-axis: cellular event number detected by flow cytometer. (D) MJ cells were treated with CCL22 or not (null), and put onto either VCAM-1- or BSA-coated dishes before washing. Adhesion to VCAM-1 (or BSA) is reflected by the number of wash-resistant (adherent) cells, measured by a commercial cell proliferation kit, shown as colorimetric reading at 450-nm absorbance. Mn²⁺ was used in some groups (without CCL22) as a potent positive control for affinity regulation as previously described [20]. Results represent one of at least two experiments with similar results (A–D), and are shown as mean $\pm$ SD in (D).

on cancer cells [28], we investigated whether CCR4 signaling also enhances survival of MJ cells. As shown in Figure 6, under nutrition deprivation (in 0.5% FBS), MJ cells showed enhanced survival ($p < 0.05$), compared with non-chemokine controls, in the presence of CCL22 or CCL17, which is comparable to the effect induced by CXCL12, another chemokine well established to enhance tumor cell survival [29].

Immunoblotting for tracing the CCR4 signal transduction route in response to CCL22

We further investigated the signaling events in MJ cells in response to CCL22. As shown in Figure 7, in response to CCL22 treatment, time-dependent accumulation of phosphorylated serine/threonine kinase, Akt (or PKB) was observed in MJ cells. In contrast, the phosphorylation of another signaling effector molecule,

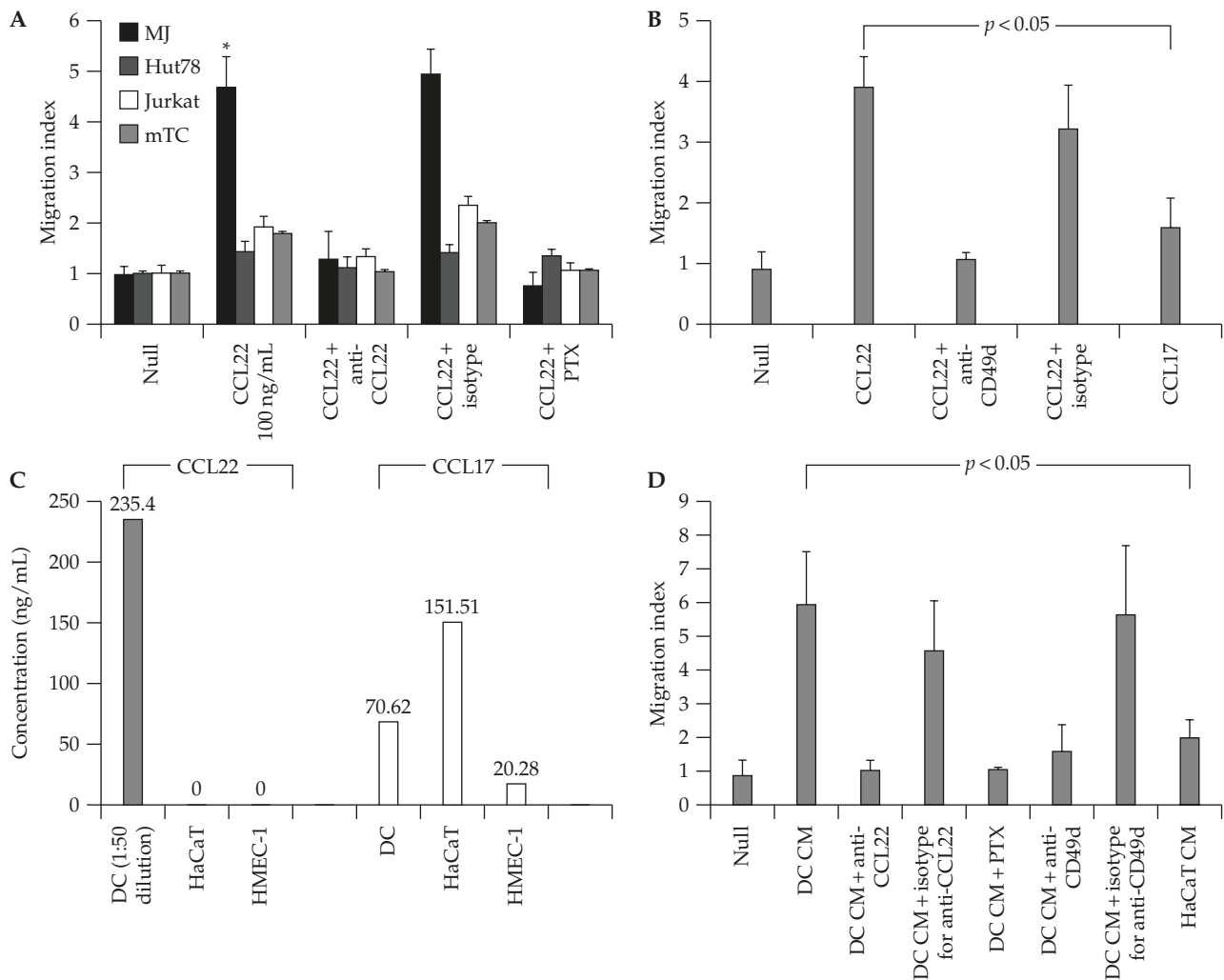


Figure 5. CCL22 and DC conditioned medium (CM) may enhance the transendothelial migration of MJ cells. (A) Transendothelial migration of cells (MJ, Hut78, Jurkat, and mTC) was measured following exposure to CCL22 (100 ng/mL), in the presence or absence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX). * $p < 0.05$, MJ versus other types (Hut78, Jurkat, and mTC) of cells. (B) In another series of experiments, MJ cells were likewise exposed to CCL22 treatment as in (A), but in the presence of neutralizing anti-CD49d Ab or isotype (for anti-CD49d Ab) to test the dependence on $\alpha 4$ integrin (CD49d). (C) Expression of CCL22 and CCL17 by DCs, HaCaT cells, and HMEC-1 cells was measured and compared by ELISA, using CM derived from the respective cell types. (D) In another series of experiments, MJ cells were exposed to DC- or HaCaT-derived CM, with or without the agents, to test the CCL22 dependence (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, or PTX), and the agents to test $\alpha 4$ integrin (CD49d) dependence (the neutralizing anti-CD49d Ab or isotype).

Erk, was not found to be increased above the basal level in the presence of CCL22.

DISCUSSION

CCR4 has long been considered an important mediator for CTCL skin homing; however, most evidence available to date to support this theory is based on expressional, rather than functional, studies. In light of the lack of sufficient functional evidence to support

a critical role of CCR4 in CTCL migration dynamics, we tried to address this issue and have demonstrated that CCR4 signaling, induced by CCL22 treatment, specifically enhances chemotaxis, integrin activation, integrin adhesion, and overall transendothelial migration of MF-derived MJ cells, while SS-derived Hut78 cells and other control cells (CD4⁺CD45RO⁺ mTCs and Jurkat cells) did not respond to CCL22 as well. The present study not only confirms the critical role played by CCR4 in CTCL trafficking, but also demonstrates that CCR4-mediated CTCL skin homing may occur in

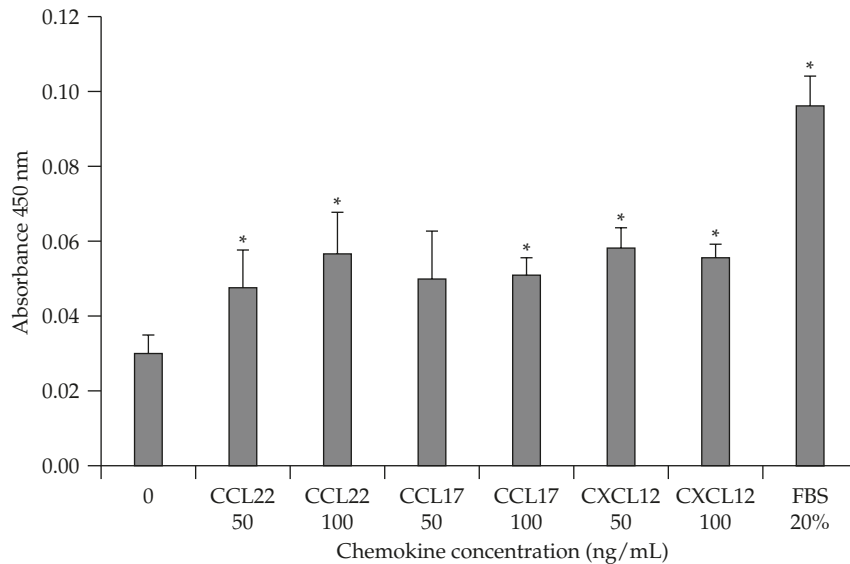


Figure 6. CCL22 and CCL17 mildly enhance cell survival of MJ cells under nutrition deprivation. Cells were seeded in 96-well plates with culture medium containing FBS at only 0.5%, and treated with CCL22, CCL17, or CXCL12 at 50 and 100 ng/mL concentrations in triplicate. Cells treated with PBS only or culture medium containing 20% FBS were used as controls. Cell viability was measured by colorimetric method with absorbance reading at 450 nm. * $p < 0.05$, versus non-chemokine control in low-serum conditions.

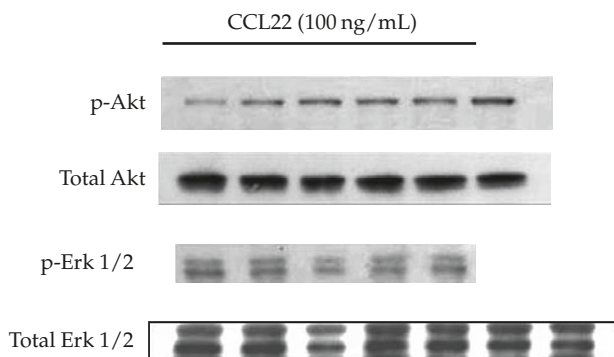


Figure 7. CCL22 treatment induces phosphorylation of Akt. MJ cells were treated with CCL22 (100 ng/mL) and cell lysates were subject to immunoblotting with various mAbs (anti-human/mouse/rat pan-Akt), affinity-purified rabbit anti-phospho-Akt (S473), anti-human/mouse/rat pan-ERK1/ERK2, and anti-phospho-ERK1/ERK2(T202/Y204) (R&D Systems) at 4°C overnight.

accordance with the current lymphocyte trafficking paradigm (adhesion and transendothelial migration cascade) for inflammatory skin diseases.

CCL22 and CCL17 are the only two ligands for CCR4 and both have been shown to be expressed by various types of cells in the skin milieu [26,30]. We were interested in whether there are any differences between CCL22 and CCL17, with regard to their roles and relative importance in mediating skin homing of CCR4-expressing CTCL. Using standard chemotaxis assays, we first tested the CCR4 function of CTCL

cells, in terms of their responses to treatments with CCL17 and CCL22. As shown in Figure 2B, CCL22 was able to induce more chemotaxis of MJ cells than CCL17 (at 100 ng/mL). In integrin activation and adhesion assays, we also found that CCL22 induces mildly greater responses in MJ cells than CCL17. Moreover, CCL22, but not CCL17, could enhance transendothelial migration of MJ cells beyond the basal level *in vitro*. These results are in line with the results of several previous studies showing that CCL22 is dominant over CCL17 in several CCR4-mediated reactions [31–33]. D'Ambrosio et al showed that CCL22 is much more powerful than CCL17 in the induction of integrin-dependent T cell adhesion to VCAM-1 [31], which is in accordance with our results (Figure 4). Mariani et al demonstrated that CCL22 is a more potent and rapid inducer of CCR4 internalization and recycling than CCL17 in Th2 cells [32]. They also noted that, while incubation of cells with CCL17 inhibited subsequent cell migration in response to CCL17, but not to CCL22, incubation of cells with CCL22 inhibited subsequent migration in response to both CCL22 and CCL17, thus suggesting a hierarchy (CCL22 over CCL17) of cross-desensitization action between these two CCR4 ligands. This report appears to provide a reasonable explanation for our results showing CCL22's superiority over CCL17 in terms of the enhancement of basal transendothelial migration of

MJ cells. Moreover, Cronshaw et al showed that CCL22 could induce a more lasting Akt phosphorylation in Th2 cells than CCL17 [34], which is also in line with our results regarding MJ cell CCR4 signaling (Figure 7).

In skin milieu, CCL22 has been found to be mainly expressed by DCs (both in the epidermis and dermis), while CCL17 appears to be mostly produced by KCs and ECs [26,35], but also by DCs [30]. Herein, we have confirmed this specific pattern of chemokine expression *in vitro* (Figure 5C). Note that while CCL17 is expressed by all three types of cells (KCs, ECs, and DCs), CCL22 seems to be predominantly expressed by DCs. More importantly, in the present study, we further demonstrated that CM from DCs has a superior effect to enhance transendothelial migration of MJ cells compared with KC-derived CM (Figure 5D), suggesting a pivotal role for DCs in the migration of CTCL cells toward the dermis through the vascular barrier. Given that CCL22 is a more potent (than CCL17) inducer of transendothelial migration of MJ cells and given the wide distribution (in both epidermis and dermis) and high motility of DCs, it is likely that DCs, rather than KCs or ECs, play a more decisive role in mediating transendothelial migration of MJ cells to skin dermis. Moreover, DCs may continue to impose major effects on CTCL cells after their migration into the dermis. It has also been found that CCL22 and CCR4 are involved in the formation of T lymphocyte-DC clusters in human inflamed skin [27]. Berger et al demonstrated that CTCL cells can be reproducibly grown in culture for 3 months when cocultured with DCs [36], and further found that CTCL cells adopt phenotypes of T-regulatory (Treg) cells (expression of CD25/CTLA-4 and FoxP3) after interaction with DCs [37]. The fact that CTCL cells are primed (by DCs) into Treg-like cells may explain the immunosuppressive nature of CTCL, in accordance with a recent report from Curiel et al [33] demonstrating that immune-suppressing Treg cells in ovarian cancer microenvironment have high CCR4 expression, and *in vivo* treatment with a mAb to CCL22 (but not a mAb to CCL17) decreased migration of Treg cells to tumors, thus further exemplifying the close relations among CTCL, Treg cells, cancer immunity, and the CCR4-CCL22 interaction. Collectively, these findings, including ours, imply that soluble mediators secreted by DCs, especially CCL22, play critical roles in many aspects of the skin homing process of CTCL cells, and also in subsequent survival and

remodeling of CTCL cells in skin. More importantly, in line with the report from Curiel et al [33], our findings may favor the selective targeting of CCL22, rather than CCL17, in the development of novel therapeutics against CCR4-mediated tumorigenesis, including CTCL.

Integrin-mediated firm arrest of CTCL cells on endothelial cells, activated by so-called "inside-out" signaling downstream of chemokine receptors [38], appears to also be important for CTCL skin homing. However, it has not been clearly characterized which integrins play a relatively more important role in the transendothelial migration of CTCL cells. Nevertheless, a previous report showed that the expression of VCAM-1, the main ligand for $\alpha 4$ integrin, was upregulated in the dermal endothelium of CTCL lesions [39]. Herein, we have demonstrated that $\alpha 4$ integrin, but not $\beta 2$ integrin, enhances the adhesion of MJ cells on VCAM-1 following CCR4 signaling *in vitro* (Figure 4). More importantly, we have shown that transendothelial migration of MJ cells, either enhanced by CCL22 or DC-derived CM, is dependent on $\alpha 4$ integrin (CD49d), indicating that in certain pathologic conditions like MF, certain sets of chemokine signaling (CCR4/CCL22) and integrin type ($\alpha 4$) may have a predominant role in controlling integrin-mediated adhesion, as suggested by a previous report [40].

We cannot exclude that chemokines other than CCL22 play some roles in the tumorigenesis of CTCL. Indeed, a previous report noted that skin homing of Sézary cells may involve CXCR4 signaling [41]. By qPCR, we also found that the expression level of CXCR4 in MJ cells is higher than that of CCR4; however, while CCR4 is differentially expressed by MJ and control cells (Hut78 and mTC), the CXCR4 expression level (by qPCR) is similar between MJ cells and other CTCL cell lines (HH and Hut78), but much lower than that in CD4+CD45RO+ T cells (~7-fold less), indicating a more specific role of CCR4 in CTCL pathogenesis. Given that CCL17 expression (production) is widely distributed in skin (from KCs, DCs, and ECs) it may still be able to contribute, to some extent, to the skin homing and tumorigenesis of CTCL (Figure 6). As suggested by Mariani et al [32], CCL17 may still play a role in promoting the arrest of rolling CTCL cells on ECs; meanwhile, the expression of CCR4 in CTCL cells remains sensitive to CCL22 stimulation and capable of mediating subsequent transendothelial migration of CTCL toward the dermis.

Using currently available CTCL cell lines, we have demonstrated the differential expression of CCR4 between one MF-derived cell line and one SS-derived cell line, and further shown the differential functional capacities of CCL22 and CCL17 in mediating CCR4-dependent adhesion and transendothelial migration cascades of MJ cells. Given the long-existing barriers in CTCL research caused by rare case number, difficulty in isolating CTCL cells, and lack of a proper animal model, these currently available CTCL cell lines may serve as legitimate tools for studies involving CCR4-mediated CTCL skin homing and tumorigenesis. More importantly, as CCR4 has become one of the main targets to develop novel therapies for cancer and viral infection [42], the MJ cell line, with its higher expression of CCR4 (versus Hut78 and mTC cells) and greater sensitivity to CCL22 (versus CCL17), may well serve as a decent model for studies involving CCR4- or CCL22-targeting therapies.

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CCR4 在不同類型皮膚 T 細胞淋巴瘤細胞株中的差異性表現與功能研究

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皮膚 T 細胞淋巴瘤屬於記憶型 T 淋巴球細胞所形成的腫瘤，主要影響的部位為人體的皮膚。雖然皮膚 T 細胞淋巴瘤在臨床上有許多不同的分型，然而大部份的皮膚 T 細胞淋巴瘤都有一共同之特色，就是移行至皮膚的能力。皮膚 T 細胞淋巴瘤移行的調控機轉大部分仍不明朗；過往的研究雖然顯示特定的趨化激素受體 (**chemokine receptor**) 於皮膚 T 細胞淋巴瘤中有提高之表現，然而這些趨化激素受體是否真正可以調控皮膚 T 細胞淋巴瘤移行仍屬未知。在本研究中，我們發現特定趨化激素受體 **CCR4** 在不同種類的皮膚 T 細胞淋巴瘤細胞株中有不同之表現：來自蕁狀肉芽腫 (**mycosis fungoides**，惡性 T 淋巴細胞絕大多數集中於皮膚) 的細胞株 (**MJ**) 相對於來自 **Sézary syndrome** (部分惡性 T 淋巴細胞存在於週邊血液) 的細胞株 (**Hut78**) 有較明顯的 **CCR4** 之表現。我們進一步進行一系列的 *in vitro* 功能測試 (趨化性，**integrin** 活化與黏著，血管內皮穿透)，我們發現具有較強 **CCR4** 表現之 **MJ** 細胞株對於趨化激素 **CCL22** (**CCR4** 之 **ligand**) 所引發之各種功能性表現都比對照組細胞株要明顯。這些結果顯示趨化激素受體 **CCR4** 可能在皮膚 T 細胞淋巴瘤移行的調控機轉中扮演重要角色。

關鍵詞：趨化激素受體 **CCR4**，趨化激素，皮膚 T 細胞淋巴瘤
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