CC Chemokine Ligand 25 Enhances Resistance to Apoptosis in CD4+ T Cells from Patients with T-Cell Lineage Acute and Chronic Lymphocytic Leukemia by Means of Livin Activation


INTRODUCTION

CC chemokine ligand 25 (CCL25), also known as thymus-expressed chemokine (TECK), together with CC chemokine receptor 9 (CCR9), efficaciously induces chemotaxis of immature CD4+CD8+ double-positive, and mature CD4+ and CD8+ single-positive thymocytes, suggesting that CCL25/CCR9 interaction plays a pivotal role in T-cell migration in the thymus (1–4). CCL25/TECK delivers signals through CCR9 for developing of thymocytes (5), and developing T-cell migration in the thymus (1–4). CCL25/TECK delivers signals through CCR9 for developing of thymocytes (5), and developing and/or migrating of both αβ and γδ T cells (6). CCR9 activation leads to phosphorylation of glycogen synthase kinase-3β (GSK-3β) and forkhead transcriptional factor (FKHR) and provides a cell-survival signal (7). In the normal human, CCR9 is restrictedly expressed at high levels on CD4+ and CD8+ T cells in the small intestine (8), providing the evidence for distinctive mechanisms of lymphocyte recruitment. The importance of CCL25/TECK is to license effector/memory cells to access anatomic sites (9, 10). Thus, CCL25/TECK is important for the homing, development, and homeostasis of T cells, particularly, mucosal T cells. The functional importance of CCR9/CCL25 in the physiologic and pathophysiologic events of T-cell trafficking and selection is not fully understood despite a considerable body of investigations.

The inhibitor of apoptosis protein (IAP) family plays a key role in apoptosis regulation and has become increasingly prominent in the field of cancer (11). Thus far, eight human IAPs have been identified: c-IAP1, c-IAP2, NAIP, survivin, XIAP, Bruce, ILP-2, and Livin (11). IAPs can block apoptosis mainly through their ability to bind and inhibit specific caspases such as caspase-3 and -7 (12, 13). A novel IAP member, designated Livin/ML-IAP/KIAP (14, 15), is suggested to mediate suppression of cell death (14–16). A key function for Livin in the transformed phenotype suggests that this protein may be an important target for immune-mediated tumor destruction (17).

A common manifestation of T-cell lineage acute lymphocytic leukemia (T-ALL) and T-cell lineage chronic lymphocytic leukemia (T-CLL) is infiltration of various organs by leukemic cells (18). We have reported that CCR9 is selectively and functionally overexpressed on T-ALL and T-CLL CD4+ T cells (19). Despite these findings, little is known about the exact mechanisms and molecule regulation in the homing, migration, inappropriate proliferation, and resistance to apoptosis of T-ALL and T-CLL T cells.

MATERIALS AND METHODS

Patients and Cell Purification. All of the patients with T-ALL and T-CLL were diagnosed according to the French-American-British (FAB) Cooperative Group criteria (20) and to the guidelines of the National Cancer Institute Working Group on B-CLL (21, 22), and informed consent according to institutional guidelines. CD4+ T cells were purified by a positive selection procedure of Dynabeads (Dynal A/S, Dynal, Norway). The malignancy of purified T-ALL or T-CLL CD4+ T cells was checked by expression of CD25, CD45RO, and HLA-DR (19). Human thymus tissue was aseptically obtained after consent during surgical operative procedures involving the mediastinal region. Stromal cells were prepared by enzymatic digestion of thymus tissue, as described previously (23). CD4 and CD8 double-positive thymocytes were sorted with a FACStarPlus. The cell lines were obtained from the American Type Culture Collection, Manassas, VA (MOLT4 and human embryonic kidney cells 293T). The anti-CCR9 monoclonal antibody and chemokines (CCL25/TECK, CXC (CC) chemokine ligand 12 (CXC1L2)/SDF-1) were purchased from R&D Systems, Abingdon, United Kingdom.

Flow Cytometry. For detection of apoptosis, cells were stained in staining buffer with 1 μg/mL propidium iodide for 30 minutes at 4°C, then stained with FITC-conjugated annexin V with binding buffer (BD PharMingen, San Diego, CA) as described previously (7, 24). COULTER XL (Coulter, Miami, FL) was used for analyses. For detection of intracellular active caspases, cytofix/cytoperm buffer (BD PharMingen) was used to permeabilize cells, and cells...
were subsequently stained with anti-active-caspase-3 or anti-active-caspase-8 monoclonal antibody (BD PharMingen). Data were analyzed by means of the WinList program (The Streeter Research Associates, Inc., Bella, CA).

Real-time Quantitative Reverse Transcription-PCR Assay. All real-time quantitative reverse transcription-PCR (RT-PCR) reactions were done as described elsewhere (24–26). Briefly, total RNA from purified cells (1 × 10^5, purity >99%) was prepared by using Quick Prep total RNA extraction kit (Pharmacia Biotech, Hillerod, Denmark). RNA was reverse transcribed by using oligo(dT)12-18 and Superscript II reverse transcriptase (Life Technologies, Inc., Grand Island, NY). The real-time quantitative PCR was performed with an ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems, Foster City, CA). By using SYBR Green PCR Core Reagents kit, fluorescence signals were generated during each PCR cycle to provide real-time quantitative PCR information. The sequences of the specific primers were as follows: Livin sense, 5′-GTCCTGGCCTGCTGCT-3′, and Livin antisense, 5′-CAGGGAGGCCCATCCTGCA-3′.

All unknown cDNAs were diluted to contain equal amounts of β-actin cDNA. PCR reaction conditions were retained 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles with 15 seconds at 95°C, 60 seconds at 60°C for each amplification.

Northern Blot Assays. For mRNA detection, 2 μg of total RNA obtained from each sample were electrophoresed under denaturing conditions, followed by blotting onto Nytran membranes, and were cross-linked by UV irradiation as described previously (27). Livin cDNA probe, labeled by [α-32P]dCTP, was obtained by PCR amplification of total RNA from human melanoma cell lines (SK-Mel29). The membranes were hybridized overnight with 1 × 10^6 cpm/mL of [32P]labeled probe, followed by intensive washing before being autoradiographed.

Plasmids and Cell Transfection. Plasmid encoding CCR9, c-IAP1, c-IAP2, XIAP, survivin, Livin, and c-jun-NH2-kinase 1 (JNK1) had been described previously (16, 28, and 29). The cells were transiently transfected with vectors encoding target genes as described elsewhere (2, 7, 16, 28, and 29). Briefly, the cells were cultured with DMEM containing 10% FCS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were grown to 29% confluence in six-well plates. Cells were transiently transfected with indicated amounts of shRNA and plasmid DNA with standard calcium phosphate procedures at 50 to 70% confluence. The cells were washed three times for 20 minutes at 4°C, and 12 μL of M2 anti-FLAG monoclonal antibody (Sigma, St. Louis, MO) preincubated with anti-Myc antibody (5 μg/mL) was added and incubated for 15 minutes at 37°C. The cells were washed extensively and lysed in 200 μL of lysis buffer (30). Expression of kinase in indicated cells was analyzed by Western blotting onto Nytran membranes, and were cross-linked by UV irradiation. Expression of kinase was determined by blotting onto Nytran membranes, and were cross-linked by UV irradiation.

Immune Complex Kinase Assay and Immunoblotting. Cell lysates was performed for 30 minutes at 4°C with lysis buffer (30). Expression of kinase proteins was semi-quantified after Western blot analysis (30). Lysates were centrifuged at 10,000 rpm for 5 minutes at 4°C. Protein concentration was measured by Bio-Rad (Hercegova, CA) protein assay. Protein (10 μg) was loaded onto 16% SDS-PAGE, transferred onto polyvinilidene difluoride membranes after electrophoresis, and incubated with the appropriate antibodies at 0.5 μg/mL. All antibodies (Bel-2, Bel-X, c-FLIpL, c-IAP1, c-IAP2, XIAP, and survivin) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA, except anti-Livin was from Imgenex Corp. Sorrento Valley, San Diego, CA. The membrane was blocked in 5% bovine serum albumin-Tris-buffered saline, Myc-tagged proteins were immunoprecipitated with 20 μL of agarose-protein A (Pierce, Rockford, IL) preincubated with anti-Myc antibody (5 μg), and FLAG-tagged proteins with 20 μL of agarose conjugated with the M2 anti-FLAG monoclonal antibody (Sigma, St. Louis, MO). In vitro kinase assays were performed as described elsewhere (29). For coimmunoprecipitations, the cells were washed extensively and lysed in 200 μL of lysis buffer (29). After incubation for 30 minutes on ice, cell lysates were centrifuged (10,000 rpm for 10 minutes, 4°C) and the supernatants were recovered. Cell lysates were precleared three times for 20 minutes at 4°C with 20 μL of protein A-Sepharose beads and were mixed with specified antibodies for 3 hours at 4°C under constant agitation. Immune complexes were allowed to bind to 20 μL of protein A-Sepharose beads overnight, beads were washed three times with lysis buffer, then suspended in 20 μL of kinase buffer containing 10 μCi of [γ-32P]ATP and were incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 15 μL of 3× sample buffer. Immunoprecipitates were separated on SDS-12% polyacrylamide gels and were transferred to nitrocellulose membranes. Filters were blocked with 5% nonfat milk in blocking buffer and were incubated with specified antibody for 2 hours, followed by autoradiography.

Chromatasssism Assay. The chromatassism assay was performed in a 48-transwell microchamber (Neuro Probe, Bethesda, MD) technique (7, 19). Briefly, chemokinpe (CCL25/TECK) in RPMI 1640 with 0.5% bovine serum albumin was placed in the lower wells (25 μL). Cell suspension (25 μL, 2 × 10^6 cells/mL) was added to the upper well of the chamber, which was separated from the lower well by a 5 μm pore-size, polycarbonate, polivinylylidene-free membrane (Nucleopore, Pleasanton, CA). The cells were CD4^+ T cells that were positively selected by a procedure with anti-CD4 monoclonal antibody-coated Dynabeads. The chamber was incubated for 60 minutes at 37°C and 5% CO2. The membrane was then carefully removed and was stained for 5 minutes in 1% Coomassie Brilliant Blue.

Gene Silencing Assay. Short hairpin RNAs (shRNAs) were produced in vitro as described previously (31) with chemically synthesized DNA oligonucleotide templates (Sigma). Transcription templates were designed such that they contained U6 promoter sequences at the 5′ end. shRNA transcripts subjected to in vitro dicer processing were synthesized with a Riboprobe kit (Promega, Madison, WI). Double-stranded DNA oligonucleotides encoding shRNAs with 21 bases of homology to the targeted Livin gene were ligated into the EcoRV site to produce expression constructs. Sequences inserted immediately downstream of the U6 promoters were as follows: Livin sense, 5′-UAGAAACATGCTGCTGCTCCT-3′; Livin antisense, 5′-CAGGAGGCCCATCCTGCA-3′. The constructs were transfected with indicated amounts of shRNA and plasmid DNA with standard calcium phosphate procedures at 50 to 70% confluence in six-well plates. Cells were cultured in DMEM containing 10% of heat-inactivated fetal bovine serum, penicillin, and streptomycin. Cells were harvested 2 days after the transfection.

RESULTS

CCL25/TECK Selectively Rescues T-ALL and T-CLL CD4^+ T Cells from TNF-α-Mediated Apoptosis. The data in Table 1 showed that double-positive thymocytes, T-ALL and T-CLL CD4^+ T cells and MOLT4 T cells expressed high levels of CCR9, which were in agreement with previous reports (1, 2, 7, 18, and 32). By knowing that activation of CCR9 lead to phosphorylation of GSK-3β and FKHR and provided a cell survival signal (7), we examined the protective effects of CCL25/TECK and CXCL12/SDF-1 (7) on different types of cells from tumor necrosis factor α (TNF-α)-mediated apoptosis. The number of apoptotic and necrotic cells were significantly increased on culture of double-positive thymocytes in the presence of CCL25/TECK (Fig. 1A–e), in comparison with that in malignant cells. The apoptotic responses in T-ALL and T-CLL CD4^+ T cells as well as in MOLT4 T cells were significantly inhibited in the presence of CCL25/TECK (Fig. 1A-f, -g, and -h). CXCL12/SDF-1, to our surprise, could rescue malignant cells (Fig. 1A-j, -k, and -l), as well as double-positive thymocytes (Fig. 1A-i). Antibody against CCR9 could completely block the protective effect of CCL25/TECK (Fig. 1A-n, -o, and -p). As shown in Fig. 1B, the total dead cells (including apoptotic and necrotic) in different types of the cells tested were the same.

Table 1. CCR9 expression in different T cells

<table>
<thead>
<tr>
<th>Assay*</th>
<th>Thymocytes (DP)</th>
<th>T-ALL CD4^+</th>
<th>T-CLL CD4^+</th>
<th>MOLT4</th>
<th>Peripheral CD4^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL (%)</td>
<td>88 ± 6</td>
<td>81 ± 5</td>
<td>45 ± 7</td>
<td>72 ± 4</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Real-time RT-PCR (x 10^3)</td>
<td>7.3 ± 0.8</td>
<td>7.1 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>6.8 ± 0.7</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

Note: The cells were purified double-positive (DP) thymocytes, T-ALL CD4^+ T cells, T-CLL CD4^+ T cells, MOLT4 T-cell line, and purified peripheral CD4^+ T cells. Data are mean values ± SD of six experiments in each group.

* Applied assays were flow cytometry (FL) and real-time quantitative RT-PCR (x 10^3 mRNA copies/25 ng cDNA; ref. 19).

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patterns as the results in Fig. 1A. These results were suggesting that differential effects of CCR9 and TNF-α signaling existed between double-positive thymocytes and malignant T cells.

We examined the protective effects of CCL25/TECK on different types of malignant T cells from chemotherapy-induced apoptosis. CCL25/TECK itself induced no apoptosis in malignant T cells. Chemotherapeutic drugs induced significant apoptosis when the cells were pretreated without or with low concentration of CCL25, whereas high concentration of CCL25 significantly prevented the malignant T cells from chemotherapy-induced apoptosis (Table 2).

**Livin Expression in T-ALL CD4⁺ T Cells Is Selectively Increased.** To examine the mechanisms of cell-type selectivity of CCL25/TECK to rescue cells from TNF-α-mediated apoptosis, we first examined the expression levels of some important substrates of apoptosis pathways in the distinct types of cells. The protein levels of one antiapoptotic member Bcl-2 and c-FLIP_L in freshly isolated different types of T cells were identical (Fig. 2A). Interestingly, Bcl-2 expression level was significantly elevated in double-positive thymocytes after stimulation with CCL25/TECK and apoptotic induction with TNF-α, but not in the malignant cells (Fig. 2A). There were no significant differences of c-FLIP_L expression in various cell types before and after stimulation (Fig. 2A). We also observed a similar expression pattern of another antiapoptotic member, Bcl-X (data not shown). One antiapoptotic member protein in the IAP family, Livin, was selectively expressed at higher levels in the malignant cells, particularly in T-ALL CD4⁺ T cells, in comparison with the level in double-positive thymocytes (Fig. 2B). For a better comparison, samples from different un-stimulated cells were loaded in the same gel that was used for examining Livin expression (Fig. 2C). The expression levels of Livin in these malignant cells were significantly increased after stimulation with CCL25/TECK and TNF-α (Fig. 2B). Stimulation with TNF-α alone slightly changed (decreased) the expression of Livin in different types of cells. The antibody against CCR9 could significantly inhibit the elevation of expression level of Livin in these cells (data not shown). Another antiapoptotic member in the IAP family, survivin, expressed identical levels in the different conditions in various types of cells (Fig. 2B). The expression levels of other members in the IAP protein family (c-IAP1, c-IAP2, and XIAP) were not significantly changed in various cell types before and after stimulation with CCL25/TECK and TNF-α (data not shown).

To further confirm elevated expression of Livin in T-ALL CD4⁺ T cells, we examined the expression of Livin mRNA in the distinct types of cells. The Livin mRNA was expressed at a very low level in freshly isolated double-positive thymocytes (1.1 × 10³ copies) and was not substantially altered after stimulation with CCL25/TECK and TNF-α (Fig. 3A). In contrast, Livin mRNA expression was significantly higher in T-ALL CD4⁺ T cells (3.2 × 10³ copies), whereas there were significant increases in T-ALL CD4⁺ T cells (6.2 × 10³ and 5.8 × 10³ copies, respectively) after stimulation with CCL25/TECK alone or together with TNF-α (Fig. 3B). TNF-α stimulation alone slightly decreased the expression of Livin (2.5 × 10³ copies). The antibody against CCR9 could marginally inhibit the elevation in Livin mRNA expression (data not shown). The same patterns of Livin mRNA expression were seen in double-positive thymocytes (Fig. 3C) and T-ALL CD4⁺ T cells (Fig. 3D) by Northern blot. For a better comparison, samples from unstimulated thymocytes and T-ALL CD4⁺ T cells were loaded in the same gel that was used to examine Livin mRNA (Fig. 3E). Elevated Livin mRNA expressions were also observed in other malignant cells (T-CLL CD4⁺ T cells and MOLT4 T cells) by real-time quantitative RT-PCR assay and Northern blot (data not shown). The pattern of Livin mRNA expression in normal peripheral CD4⁺CCR9⁻ T cells was similar to that in double-positive thymocytes (Fig. 3F).

The caspase-3 and caspase-8 expression is essential for this type of cell death (33). Expression levels of activated caspase-3 and caspase-8 were measured by intracellular staining of double-positive thymocytes (Fig. 4A) and T-ALL CD4⁺ T cells (Fig. 4B) with the use of cleavage-specific caspase-3 and caspase-8 antibody. Double-positive thymocytes, normal peripheral CD4⁺CCR9⁻ T cells, and T-ALL CD4⁺ T cells
cells expressed substantially up-regulated cleaved caspase-3 and caspase-8 in TNF-/H9251 stimulation, but CCL25/TECK could only stabilize caspase-3 and caspase-8 in T-ALL CD4/H11001 T cells, but not in double-positive thymocytes nor in normal peripheral CD4/H11001 CCR9/H11001 T cells (Fig. 4). We observed similar results in other malignant cell types (T-CLL CD4/H11001 T cells and MOLT4 T cells; data not shown), which suggested that CCL25/TECK protection from apoptosis was limited in malignant cells.

Many signaling events of binding of certain chemokines to chemokine receptors were included, such as phosphoinositide-3 kinase (PI3K), mitogen-activated protein kinase (MAPK), or protein kinase C (PKC), which appeared to be involved in chemokine-mediated chemotaxis in certain cell types (7, 34–36). To determine whether these kinases were responsible for CCL25/TECK-mediated chemotaxis, T-ALL CD4/H11001 T cells were pretreated with various concentrations of pertussis toxin, an inhibitor of PKC; wortmannin, a potent PI3K inhibitor; PD98059, a MAPK inhibitor; or vehicle, DMSO. As seen in Fig. 5A, pertussis toxin and wortmannin significantly inhibited cell migration toward CCL25/TECK; PD98059 did not inhibit T-ALL CD4/H11001 T cell migration toward CCL25/TECK, which was in agreement with those who observed similar results in MOLT4 T cells (7). We observed similar results in other malignant cell types (T-CLL CD4/H11001 T cells and MOLT4 T cells; data not shown). To determine whether these kinases were responsible for CCL25/TECK-mediated protection from apoptotic response, we pretreated the cells with the inhibitors mentioned above. As seen in Fig. 5B, only pertussis toxin significantly inhibited the apoptotic protection effect in T-ALL CD4/H11001 T cells. Neither PD98059 nor wortmannin inhibited apoptotic protection effect in T-ALL CD4/H11001 T cells, suggesting that CCL25/TECK went through different signaling pathway to carry out chemotaxis and protection from apoptosis.

### Table 2. Apoptotic analysis in malignant T cells under different chemotherapeutic drug treatments in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>0.1</th>
<th>100</th>
<th>0</th>
<th>0.1</th>
<th>100</th>
<th>0</th>
<th>0.1</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>9 ± 2</td>
<td>8 ± 1</td>
<td>9 ± 6</td>
<td>6 ± 3</td>
<td>10 ± 2</td>
<td>7 ± 2</td>
<td>8 ± 3</td>
<td>9 ± 3</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Comp.</td>
<td>62 ± 12</td>
<td>55 ± 17</td>
<td>13 ± 6</td>
<td>57 ± 9</td>
<td>52 ± 12</td>
<td>9 ± 5</td>
<td>67 ± 3</td>
<td>59 ± 6</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>Cyclop.</td>
<td>65 ± 8</td>
<td>62 ± 9</td>
<td>12 ± 2</td>
<td>65 ± 7</td>
<td>61 ± 11</td>
<td>10 ± 5</td>
<td>59 ± 8</td>
<td>60 ± 10</td>
<td>14 ± 6</td>
</tr>
</tbody>
</table>

NOTE. Cells were pretreated for 2 hours in the presence (at concentration of 0.1 or 100 ng/mL) or absence (0) of CCL25, then were treated in the presence of camptothecin (Comp., 5 μmol/L) or cyclophosphamide (Cyclop., 7.5 mmol/L; ref. 48) for 12 hours, followed by apoptotic analysis by flow cytometry. The data for apoptotic cells are mean values ± SD of six experiments performed in each group.

Fig. 2. Occurrence of Bcl-2, c-FLIP<sub>L</sub>, survivin, and Livin expression in distinct cells. The Bcl-2 and c-FLIP<sub>L</sub> (A), and survivin and Livin (B) were examined with Western blot analyses. The cells were purified CD4 and CD8 double-positive thymocytes, T-ALL or T-CLL CD4<sup>+</sup> T cells, or MOLT4 cells that were pretreated as described in legend for Fig. 1. Different unstimulated cells were also loaded in the same gel for Livin test (C). Actins indicate the quantity of total cellular protein from the tested samples loaded in each lane. Arrows, markers used to verify equivalent molecular weights of appropriate proteins in each lane. The data were from a single experiment that was representative of six experiments.
apoptotic protection in T-ALL CD4⁺ T cells. The inhibitors themselves had no apoptotic effects on the T-ALL CD4⁺ T cells (Fig. 5B-g, h-i, and j). Z-VAD-FMK rescued T-ALL CD4⁺ T cells from TNF-α-mediated apoptosis. We observed similar results in other malignant cell types (T-CLL CD4⁺ T cells and MOLT4 T cells; data not shown).

**CCL25/TECK via CCR9 Activates Livin in JNK1 Kinase-Dependent Manner.** The inhibitor of apoptosis family of proteins was found to protect against the broadest spectrum of apoptotic signals (37, 38). We further investigated the ability of the IAP family members to induce down-stream kinase activation for protection from apoptosis by CCL25/TECK in the cells. CCL25/TECK selectively activated Livin (Fig. 6A). The phosphorylation of ATF-2 substrate was dose dependent on expression level of Livin in the system. In contrast, none of c-IAP1, c-IAP2, XIAP, or survivin showed activation. In the system with JNK1 transfection lacking, Livin could not be activated, phosphorylation of ATF-2 substrate was also dose-dependent on expression level of JNK1 (Fig. 6B), suggesting CCL25/TECK activating Livin was JNK1 kinase-dependent. To further elucidate whether the activation of JNK1 was also important for the protective activity of the IAPs against TNF-α-induced apoptosis, we applied short hairpin RNA of Livin (shRNAlivin) to knockdown Livin expression and activation in MOLT4 T cells before assay of CCL25/TECK-induced resistance to TNF-α-mediated apoptosis. The results (Fig. 8A) showed that culture with shRNAlivin at high concentration completely abolished expression of Livin in MOLT4 T cells at both mRNA and protein levels, whereas low-concentration shRNAlivin or DNAlivin or vector had no such effect. Only high-concentration shRNAlivin significantly blocked the effects of CCL25/TECK on induction of resistance to TNF-α-mediated apoptosis (Fig. 8B and C) and on stabilization of caspase-3 and caspase-8 in MOLT4 T cells (Fig. 8D and E). We observed similar results for shRNAlivin in T-ALL and T-CLL CD4⁺ T cells (data not shown). These results in MOLT4 and in T-ALL and T-CLL CD4⁺ T cells confirmed experimentally the observations that CCL25/TECK, via CCR9, activated Livin to induce resistance to TNF-α-mediated apoptosis.

Thus, CCL25/TECK rescued T-ALL and T-CLL CD4⁺ T cells as well as MOLT4 T cells from TNF-α-mediated apoptosis, but double-positive thymocytes did not. CCL25/TECK selectively activated Livin to induce resistance to TNF-α-mediated apoptosis in JNK1 kinase-dependent manner.
A majority of the G protein-coupled receptor supergene family has been shown to be capable of activating MAPK, which is an indication of providing proliferative or antiapoptotic signaling. Several chemokines are able to activate MAPK to function as proliferative or antiapoptotic signals (39). For example, CXCL12/SDF-1 is an important cytokine that, acting together with thrombopoietin, enhances the development of megakaryocytic progenitor cells and activates circulating CD34^+ cells and platelets (40, 41). CXCL1 and CXCL4 are able to support the survival of endothelial cells and monocytes,
respectively (42, 43). Chemokine receptor signaling may be able to provide antiapoptotic activity to hematopoietic cells in a natural context (44). A solid report shows that CCR9/CCL25 interaction provides a cell survival signal to the receptor-expressing cells (7). However, there are some controversial, even contradictory, reports. For example, CXCR4 has induced programmed cell death of human peripheral CD4+ T cells, malignant T cells, and CD4/CXCR4 transfectants (45). The interaction between HIV R5 Env and CCR5 activates the Fas pathway and caspase-8, as well as triggering FasL production, ultimately causing CD4+ T cell death (46). CCR3 expression, induced by interleukin (IL)-2 and IL-4, functions as a death receptor for B cells (24). We have investigated four different types of cells: double-positive thymocytes, T-ALL and T-CLL CD4+ T cells, and MOLT4 T cells. We have found that, even through all types of cells are CCR9 rich-expressing cells, the responsiveness to CCL25/TECK-mediated protection from apoptosis is quite differential; double-positive thymocytes use CCR9/CCL25 for migration, homing, development, maturation, selection, and cell homeostasis. Meanwhile, malignant cells, particularly T-ALL CD4+ T cells, take advantages of CCR9/CCL25 for infiltration, resistance to apoptosis, and inappropriate proliferation. To our knowledge, this study is the first report on differential functions of CCR9/CCL25 in distinct types of cells and is the direct evidence of the pathophysiologic activity of T-ALL and T-CLL CD4+ T cells induced by CCL25/TECK.

A genetic change that leads to blocking apoptosis may allow a cell to acquire additional mutations, survive inappropriately and eventually become malignant. Additionally, a defect in the inherent ability of a cell to undergo apoptosis may account for much of the resistance observed in leukemic cells. Therefore, much effort has gone into understanding how apoptosis is altered in leukemia cells and how it can be modulated to overcome resistance and improve clinical outcomes. Recent reports that CCR9 is selectively and functionally overexpressed on T-ALL and T-CLL CD4+ T cells (19) prompted us to investigate more closely the additional functions of CCR9 with regard to these malignant cells, which often manifest inappropriate survival and resistance to various clinical therapies. We have found that one antiapoptotic member protein in IAP family, Livin, is selectively expressed at higher levels in the malignant cells, particularly in T-ALL CD4+ T cells, in comparison with double-positive thymocytes. After stimulation with CCL25/TECK and apoptotic induction with TNF-α, the expression levels of Livin in these cells were cotransfected with vectors encoding CCR9 and JNK1 (400 ng each, A, or 200 ng, 400 ng, and 600 ng, respectively, B) in the absence or presence of increasing concentrations of c-IAP1, c-IAP2, XIAP, survivin, or Livin (200 or 600 ng). The amount of transfected cDNA was kept constant in each sample by adding control pcDNA3 vector. Cells were pretreated at absence or presence of CCL25/TECK (100 ng/mL) before cell lysis. An in vitro kinase assay was performed with ATF-2 as substrate. Kinase activity was semiquantitated and is expressed to the basal level of phosphorylation of each MAPK. (Ultra Violet stimulation) was used as positive controls. Western blotting (WB) show equal expression levels of JNK1 and CCR9 (A) or various expression levels of JNK1 and equal expression levels of Livin (B).
cells are significantly increased (Fig. 2B). With sufficient and direct evidence, we have shown that Livin, but not other IAP members (c-IAP1, c-IAP2, XIAP, and survivin), has been directly activated by CCL25/TECK in JNK1-dependent manner and that, subsequently, T-ALL and T-CLL CD4⁺ T cells, as well as MOLT4 T cells, inappropriately survive. Originally characterized in a number of melanoma cell lines (14, 15), Livin is a potent antiapoptotic protein and is not detectable in most normal adult tissues. Elevated expression of Livin renders melanoma cells resistant to apoptotic stimuli and potentially contributes to the pathogenesis of this malignancy (47). Our findings, together with those of others, indicate that Livin may be one of the critical cellular factors the increased expression of which confers resistance to apoptotic stimuli, thereby contributing to the pathogenesis and progression of malignant cells, such as melanoma cells and T-ALL and T-CLL CD4⁺ T cells. The observation interestingly indicates that Livin may be a suitable therapeutic target for gene therapy. Moreover, the results may strengthen the strategic ideal of identifying Livin as antigen linked with immune-mediated tumor destruction (immunotherapy; ref. 17).

PI3K, but not MAPK, is required for CCR9-mediated chemotaxis (7). Costimulation of MOLT4 T cells with CCL25/TECK and CXCL12/SDF-1 significantly blocks CHX-mediated apoptosis, whereas stimulation with only CCL25/TECK only partially blocks Fas-mediated apoptosis. Akt, GSK-3β, FKHR, and MAPK have been involved in cell survival signals in response to an array of death stimuli. Our results showing that CCL25/TECK rescues T-ALL and T-CLL CD4⁺ T cells, as well as MOLT4 T cells, from TNF-α-mediated apoptosis are largely in agreement with these observations. Of note, we have not been able to show that stimulation of CCR9 with CCL25/TECK can directly activate p38, ERK2, or PI3K in the presence of stimulation with TNF-α (Fig. 7) in our applied experimental system, except JNK1 is constantly activated (Fig. 7C). Taking into account that CCL25/TECK can directly activate JNK1, and subsequently Livin, to induce resistance to TNF-α-mediated apoptosis, we...
suggest that TNF-α can interfere with the activation of p38, ERK2, and PI3K, which is a necessary signal for cell survival. However, CCL25/TECK can bypass the obstacle, and directly activate JNK1. With facilitation of the environment of activated JNK1, Livin can, therefore, be activated to initiate a rescuing mechanism in the cells.

On the basis of our findings, we suggest that there are differential functions of CCR9/CCL25 in distinct types of cells: Double-positive thymocytes use CCR9/CCL25 for migration, homing, development, maturation, selection, and cell homeostasis. Meanwhile, the malignant cells use CCR9/CCL25 for infiltration, resistance to apoptosis, and inappropriate proliferation. The observation indicates that Livin may be a suitable therapeutic target for gene therapy.

REFERENCES


CC Chemokine Ligand 25 Enhances Resistance to Apoptosis in CD4+ T Cells from Patients with T-Cell Lineage Acute and Chronic Lymphocytic Leukemia by Means of Livin Activation

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