Chemokine C Receptor 7 Expression and Protection of Circulating CD8+ T Lymphocytes from Apoptosis

Jeong-Whun Kim,1,2,3 Robert L. Ferris,2,3 and Theresa L. Whiteside1,2,3

Abstract
Chemokine C receptor 7 (CCR7) expression is important for lymphocyte homing to tissues. We hypothesized that CCR7 also plays a role in CD8+ T-cell protection from apoptosis. Its expression was determined on circulating T cells in patients with cancer and related to that of molecules responsible for lymphocyte susceptibility/resistance to apoptosis. Peripheral blood mononuclear cells were obtained from 36 patients with squamous cell carcinoma of the head and neck and 16 normal controls. Multicolor flow cytometry was used to evaluate CCR7, Fas, Bax, and Bcl-2 expression in CD8+ T cells. Annexin V binding to CD8+CCR7+ and CD8+CCR7− T-cell subsets was compared. Fewer CD8+CCR7+ T cells bound Annexin V than CD8+CCR7− T cells in normal control and patients (P < 0.0001). CCR7 expression correlated with higher Bcl-2 but lower Bax and Fas expression levels in CD8+ T cells in both normal control and patients (P < 0.0001). In patients, the CD8+CCR7− subset was reduced relative to normal control (P = 0.008) and replaced with an excess of apoptosis-sensitive CD8+CCR7− T cells. To study CCR7 signaling, CD8+ T cells were stimulated with CCR7 ligands, chemokine C ligands 19 or 21. Ligand binding to CCR7 resulted in phosphorylation of Akt and increased Bcl-2 expression in CD8+CCR7− T cells, suggesting that CCR7 protects effector T cells from apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. The absence of CCR7 expression on the majority of CD8+ T cells in the peripheral circulation of patients with squamous cell carcinoma of the head and neck contributes to apoptosis and a rapid turnover of these effector cells.

Chemokines are a group of small (8-14 kDa) structurally related molecules. They regulate trafficking of various cells through binding to chemokine receptors, which are also structurally related and contain seven transmembrane domain coupled to a G protein (1). Chemokine C receptor 7 (CCR7) is involved in homing of mononuclear cells to secondary lymphoid tissues (2). Among hematopoietic cells, CCR7 is expressed on B and T lymphocytes and mature dendritic cells (3). There are two ligands for CCR7: chemokine C ligands (CCL) 19 and CCL21 (4). The two ligands are expressed on high endothelial venules and in the T-cell areas of lymph nodes (5, 6). Their physiologic role has not been established except that chemotaxis of immune cells as well as other cell types to secondary lymphoid tissues is dependent on CCL19 and CCL21 production by endothelial cells.

CCR7 has also been reported to play a key role in T-cell differentiation and its presence on the cell surface divides memory T cells into two subsets: effector memory (CCR7−) and central memory (CCR7+) T cells with different immune functions in secondary immune responses (7). In dendritic cells, CCR7 expression is up-regulated during maturation, and it has been recently shown that CCR7 may be involved in induction of endocytosis in mature dendritic cells (8). In addition to its functional importance in immune cells, CCR7 plays a role in metastasis of malignant cells (9, 10). Expression of CCR7 on cancer cells seems to be related to the ability of tumor cells to establish lymph node metastases in several malignant diseases, including breast cancer, malignant melanoma, and head and neck cancer (9, 10).

Recently, a novel role for CCR7 was described in mature dendritic cells, suggesting that CCR7 transduces signals that inhibit apoptosis of mature dendritic cells through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (11). In addition, CCR7 and its ligand, CCL21, seem to be potentially involved in mesangial proliferation, apoptosis, and tissue homeostasis in the kidney (12). In aggregate, these studies indicate that transmission of antiapoptotic signals could be an important mechanism through which CCR7 influences survival of various cells, including lymphocytes.

Apoptosis, a programmed cell death, is induced by the receptor-mediated and/or mitochondria-mediated pathways. We previously showed that both of these pathways are involved in inducing the onset of spontaneous apoptosis of circulating T cells observed in patients with malignant diseases (13–15). We showed that increased expression of proapoptotic molecules, Fas and Bax, and a decrease in expression of...
antiapoptotic molecules, e.g., Bcl-2, in circulating CD8+ T cells enhance their susceptibility to apoptosis. The proportions of CD8+ T cells undergoing spontaneous apoptosis in the peripheral circulation of patients with cancer, including those with squamous cell carcinoma of the head and neck (SCCHN), are significantly elevated relative to normal age-matched controls (13–15). Here, the potential involvement of CCR7 in inhibition of CD8+ T-cell apoptosis is investigated. We show that activation signals induced by CCL19 and CCL21, transmitted through CCR7 and processed via the PI3K/Akt pathway, protect CD8+CCR7+ T cells from apoptosis.

**Materials and Methods**

**Subjects and isolation of peripheral blood mononuclear cells.** Sixteen healthy normal controls and 36 patients with SCCHN, who were consecutively seen in the outpatient Otolaryngology Clinic at the University of Pittsburgh Medical Center from April through November 2003, are included in this study. Table 1 lists clinicopathologic characteristics of the patients. All patients and normal controls who donated specimens for this study signed the informed consent approved by the Institutional Review Board. The mean ages of normal controls (55 ± 8 years) and patients with SCCHN (58 ± 12 years) were comparable. The specimens from patients and normal control were processed and tested in the same assays. Thirty milliliters of venous blood was obtained from patients following surgical interventions, and immediately delivered to the laboratory. Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. Mononuclear cells were washed in AIM-V medium (Life Technologies, Grand Island, NY), counted, and resuspended in AIM-V medium (Life Technologies, Grand Island, NY), counted, and immediately used for experiments.

**Antibodies and reagents.** FITC-conjugated Annexin V was purchased from Immunotech (Marseilles, France). Phycoerythrin-conjugated anti-CCR7 (IgG2a) monoclonal antibody (mAb) was purchased from R&D Systems (Minneapolis, MN). The ECD-conjugated anti-CD3 and PE-Cy5-conjugated anti-CD8 mAbs and 7-aminomycin D were purchased from Immunotech. FITC-conjugated anti-Fas mAb was from Pharmingen (San Diego, CA). FITC-conjugated anti-Bcl-2 mAb (IgG1) was from Dako (Carpinteria, CA) and Bax (IgG2b) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Isotype control antibodies (IgG1, IgG2a, and IgG2b) were from Immunotech. Anti-Fas (CH-11) antibody was from CD8+ T-cell isolation kits and MACS columns were purchased from Miltenyi Biotec (Burlingame, CA). Etoposide (VP-16), a genotoxic agent used to induce apoptosis in T cells, was also obtained from Sigma.

**Table 1. Clinicopathologic characteristics of the patients with SCCHN included in the study.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. patients</th>
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<tbody>
<tr>
<td>Primary tumor site</td>
<td></td>
</tr>
<tr>
<td>Oral cavity</td>
<td>16</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>7</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>2</td>
</tr>
<tr>
<td>Larynx</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
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<td>Primary tumor status</td>
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<td>T2</td>
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<td>T3</td>
<td>4</td>
</tr>
<tr>
<td>T4</td>
<td>4</td>
</tr>
<tr>
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</tr>
<tr>
<td>Nodal status</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>21</td>
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<tr>
<td>N1</td>
<td>8</td>
</tr>
<tr>
<td>N2</td>
<td>6</td>
</tr>
<tr>
<td>N3</td>
<td>1</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>0</td>
</tr>
<tr>
<td>Disease status at blood draw</td>
<td></td>
</tr>
<tr>
<td>With active disease</td>
<td>21</td>
</tr>
<tr>
<td>Primary</td>
<td>20</td>
</tr>
<tr>
<td>Recurrence</td>
<td>1</td>
</tr>
<tr>
<td>With no evidence of disease</td>
<td>15</td>
</tr>
<tr>
<td>&lt;2 y</td>
<td>9</td>
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<td>&gt;2 y</td>
<td>6</td>
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permeabilized with 0.1% (w/v) saponin (Sigma) in PBS for 5 minutes at 4°C. Finally, the cells were stained for 30 minutes at 4°C with mAbs specific for Bcl-2 and Bax molecules. After washing in 0.1% saponin solution (×3), the cells were analyzed by multivariable flow cytometry on an EPICS XL instrument (Beckman Coulter, Hialeh, FL). The mean fluorescence intensity was determined for the Bcl-2-and Bax+ cells in the CCR7+ and CCR7− subpopulations, using the Expo32 v1.2 analysis software (Beckman Coulter, Inc, Miami, FL). Quantitation of fluorescence was done using quantitative FITC standard kit, Quantum 26 (Bangs Laboratories, Inc, Fishers, IN) as previously described (14).

In vitro T-cell stimulation and Western blotting. CD8+ T cells were isolated from PBMC of healthy donors by positive selection, using MACS immunobeads (Miltenyi Biotech, Auburn, CA) according to the recommendations of the manufacturer. When aliquots of the enriched fractions were examine by multivariable flow cytometry, >90% of the cells were positive for CD8, and >70% of CD8+ T cells were positive for CCR7. These CD8+ T cells were cultured in the presence of CCR7 ligands, CCL19 (200 ng/mL), or CCL21 (200 ng/mL) at 2 × 10^6 cells/mL in RPMI 1640 supplemented with 0.1% bovine serum albumin and 20 mmol/L HEPES buffer. Apoptosis of CD8+ T cells was evaluated by assays for Annexin V binding or for pan-caspase activity by flow cytometry. Activation of Akt and expression of Bcl-2 and Bax were evaluated by Western blots. For Western blots, aliquots containing 20 μg of the cell extract were resolved on SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking of the blotted membrane with skim milk, the blots were probed with specific antibodies and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The chemiluminescent signals were obtained by Supersignal West Femto detection kits. In experiments designed to evaluate the involvement of the PI3K pathway in the phosphorylation of Akt, cells were incubated with the inhibitor, wortmannin, at the final concentration of 100 nmol/L for 1 hour before stimulation with CCL19 or CCL21. The images of Western blots were captured and quantitative densitometry was done using Personal Densitometer PI (Molecular Dynamics, Inc., Sunnyvale, CA). The results shown are the mean of three independent experiments.

**Statistical analysis.** Statistical differences in Annexin V binding and expression of apoptosis-related molecules between CCR7+ and CCR7− subpopulations of CD8+ T cells were analyzed by Wilcoxon’s matched pairs sign-rank test and, when appropriate, by a Friedman χ^2 test. The difference in the percentage of CCR7+ cells between normal control and patients with SCCHN was determined by Mann-Whitney U test. P < 0.05 was considered to be statistically significant.

### Results

**Chemokine C receptor 7 expression and apoptosis of CD8+ T cells.** Annexin V binding is an early apoptotic event in circulating T lymphocytes (13–16). To determine whether Annexin V binding is dependent on CCR7 expression on CD8+ T cells, we compared the proportions of CD8+Annexin V+/CCR7+ T cells versus CD8−Annexin V+/CCR7− T cells in the circulation of patients with SCCHN and normal control. As shown in Table 2, both subsets of CD8+ T cells, CCR7+ and CCR7−, in the peripheral circulation of SCCHN patients were more sensitive to apoptosis (i.e., had significantly increased frequency of Annexin V binding events) than the same subsets in normal control of comparable age (P < 0.0001). This observation confirms previous reports from our laboratory that circulating CD8+ T cells in patients with cancer readily undergo spontaneous apoptosis (13–16). However, the frequency of CD8−CCR7+ T cells that bound Annexin V was significantly lower than that of CD8−CCR7− T cells in both normal control

#### Table 2. Annexin V binding to the CCR7+ and CCR7− subsets of circulating CD8+ T lymphocytes in SCCHN patients and normal controls

<table>
<thead>
<tr>
<th>Patients (n = 36)</th>
<th>NC (n = 16)</th>
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<tr>
<td><strong>CCR7+</strong></td>
<td><strong>CCR7−</strong></td>
</tr>
<tr>
<td>28 ± 18</td>
<td>46 ± 17</td>
</tr>
<tr>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
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NOTE: Data are mean ± SD percentages of Annexin V+CD8+ T cells that are CCR7+ or CCR7− as determined by multicolor flow cytometry. Abbreviation: NC, normal control.
Thus, CD8+CCR7+ T cells were less sensitive to apoptosis than CD8+CCR7−/C0 T cells.

To further investigate the possibility that CCR7 expression conferred resistance to apoptosis, circulating T lymphocytes obtained from three normal control and six patients with SCCHN were examined for Annexin V binding to CD8+CCR7+ and CD8+CCR7−/C0 subsets before and after 24 hours of starvation or VP-16 treatment to induce apoptosis (Fig. 1). Annexin V binding to freshly isolated (spontaneous apoptosis) or induced (VP-16 treated or starved) cells was significantly higher (P < 0.006) in SCCHN patients than normal control for all conditions. Representative flow cytometry data (Fig. 1A and B) also show that Annexin V binding to CD8+CCR7−/C0 cells was higher than that to CD8+CCR7+ cells. This difference was statistically significant at P < 0.03 for normal control and P < 0.02 for patients. Although CD8+CCR7+ cells were relatively resistant to induced apoptosis (spontaneous versus induced, no significant difference), Annexin V binding was significantly increased in VP-16-treated or VP-16-starved CD8+CCR7−/C0 T cells, especially in the patients’ T cells (spontaneous versus induced, P < 0.02). Similar results were obtained (data not shown) with agonistic anti-Fas (CH-11) antibody using not only Annexin V binding but also other flow-based assays measuring caspase activation, matrix metalloproteinase...
changes, or DiOC₆ staining. Our data are consistent with the hypothesis that CD8⁺CCR7⁻ T cells are more sensitive to spontaneous or induced apoptosis than their CD8⁺CCR7⁺ counterparts.

Chemokine C receptor 7 expression on CD8⁺ T cells correlates with expression of Fas, Bax, and Bcl-2. To determine whether expression of CCR7 correlates to that of proapoptotic (Fas and Bax) and antiapoptotic (Bcl-2) proteins, in CD8⁺ T cells, multicolor flow cytometry was done with lymphocytes obtained from patients and normal control. The CD8⁺CCR7⁻ T-cell subset contained significantly higher percentages of Fas⁺ cells than CD8⁺CCR7⁺ T-cell subset in normal control (mean values ± SD: 69 ± 11% versus 13 ± 9%, P < 0.0001) and SCCHN patients (78 ± 14% versus 41 ± 21%, P < 0.0001; Fig. 2A). Bax expression was also significantly up-regulated in the CD8⁺CCR7⁻ T-cell subset in normal control and patients with SCCHN (P = 0.01 for both; Fig. 2B). In contrast, antiapoptotic Bcl-2 expression was significantly down-regulated in CD8⁺CCR7⁻ T cells in normal control and patients with SCCHN (P < 0.0001 for both; Fig. 2C). When SCCHN patients were divided into the active disease and no evident disease groups (Table 1), no significant differences in expression of apoptosis markers in the CCR7⁺ and CCR7⁻ T-cell subsets were observed between these two cohorts. This suggests that the susceptibility/resistance to apoptosis is an innate characteristic of CD8⁺ T cells and is not influenced by disease activity. In aggregate, these results confirm that CD8⁺CCR7⁻ T cells are more susceptible to apoptosis than CD8⁺CCR7⁺ cells via the receptor- and mitochondria-mediated apoptotic pathways.

The CD8⁺CCR7⁺ T-cell population is reduced in the circulation of patients with squamous cell carcinoma of the head and neck. We compared the proportions of CD8⁺CCR7⁺ T cells in the peripheral circulation of normal control versus patients. As shown in Fig. 3, the percentage of CD8⁺CCR7⁺ T cells was significantly decreased in SCCHN patients (34 ± 21%) compared with normal control (49 ± 17%; P = 0.008). Expression levels of CCR7 were lower in the patients’ T cells than those of controls (data not shown). Representative flow cytometry data in Fig. 3 show that apoptosis-resistant CD8⁺CCR7⁺ T cells, which are the major CD8⁺ T cell subset in normal control, are replaced with apoptosis-sensitive CD8⁺CCR7⁻ T cells in the circulation of the patients with SCCHN. Thus, patients with SCCHN have an excess of CD8⁺CCR7⁻ T cells and few apoptosis-resistant CD8⁺CCR7⁺ T cells in the peripheral circulation.

Chemokine C receptor 7–mediated signals and protection of CD8⁺ T cells from apoptosis. The Annexin V binding data suggested that CCR7 expression is related to inhibition of apoptosis in CD8⁺ T cells. We hypothesized that the signals received through CCR7 are involved in promoting survival of CD8⁺ T cells. To test this hypothesis, CD8⁺ T cells were isolated from normal donors and cultured in the presence or absence of CCR7 ligands, CCL19 (200 ng/mL), or CCL21.
(200 ng/mL) in medium containing an apoptosis inducer, VP-16 (5 μmol/L), for 24 hours. As shown in Fig. 4, VP-16-induced apoptosis in both subsets was significantly elevated (0.001) relative to Annexin V binding in freshly harvested PBMC. However, VP-16-induced apoptosis was significantly reduced in CD8^+CCR7^+ cells incubated in the presence of either CCL19 or CCL21 relative to apoptosis of CD8^+CCR7^- cells (Fig. 4). The protective dose of chemokines (200 ng/mL) was selected after preliminary titrations; higher concentrations of CCL19 or CCL21 were not more effective. The data indicate that higher proportions of CD8^+CCR7^-/C0 T cells bind Annexin V and that protection from apoptosis is consistently greater in CD8^+ T cells that express CCR7 in normal control or patients with SCCHN. The results indicate that binding of the cognate chemokine ligands to CCR7 induce intracellular signals favoring survival of CD8^+ T cells.

Chemokine C receptor 7 signals via the phosphatidylinositol-3 kinase/Akt pathway. Receptors present on the surface of lymphocytes can transmit survival signals through diverse signal transduction cascades (16–20). A serine/threonine kinase, Akt, has been previously identified as an important component of the prosurvival signaling pathway (21–23) and, therefore, we studied Akt phosphorylation in CD8^+CCR7^+ T cells exposed to the CCR7 ligands. Figure 5A shows that CCL19 and CCL21 phosphorylated Akt within the first 2 minutes after ligand binding, and that Akt phosphorylation was optimal at 5 minutes and still demonstrable 10 minutes later. Akt phosphorylation was inhibited in the presence of a PI3K inhibitor wortmannin (Fig. 5B). The data support the hypothesis that CCR7 engagement, through the Akt pathway, is associated with the survival of CD8^+ T cells.
A wortmannin-treated CD8+CCR7+ T cells were no longer incubated in the presence of the CCR7 ligands. Further, CCL21. As shown in Fig. 7, phosphorylation of Akt was Akt plays a role in protecting CD8+ T cells from apoptosis.

Because Akt was phosphorylated in T cells incubated in the presence of CCL21, we next asked whether activation of apoptosis or death induced by a variety of extrinsic signals (VP-16, starvation, Fas cross-linking) is a generalized phenomenon. Further, we have established that apoptosis of CD8+ T cells takes place through both death receptor–mediated and mitochondria-mediated pathways (14). The proportions of CD8+ T cells binding Annexin V are markedly elevated in patients with cancer relative to normal controls (13, 14). However, not all CD8+ T cells are equally sensitive to apoptosis, as effector CD8+CD45RA CD27- or CD8+CD28+ cells are preferentially targeted for demise in patients with cancer (16, 17). This extensive spontaneous apoptosis may be one of the mechanisms responsible for immunosuppression found in cancer patients. It now seems that the CCR7+ subset of circulating CD8+ T cells in normal controls and patients with SCCHN is significantly less sensitive to apoptosis than are CD8+ T cells lacking CCR7. The more apoptotic subset is phenotypically characterized as CD8+CCR7- T cells. Also, expression levels of apoptosis-related proteins, such as the death receptor, Fas, proapoptotic protein, Bax, or antiapoptotic protein, Bcl-2, in CD8+CCR7- cells are consistent with their increased susceptibility to apoptosis.

In cancer patients, the apoptosis-resistant subset of CD8+CCR7+ T cells was found to be significantly diminished compared with normal control and, in a large part, replaced by the apoptosis-susceptible subset of CD8+CCR7- T cells. Thus, the greater susceptibility to apoptosis of CD8+ T cells seen in cancer patients relative to normal control could be attributed to this shift in CD8+ T cells expressing CCR7 receptor. The frequency of CD8+CCR7+ versus CD8+CCR7- subsets was not different in the SCCHN patients with active disease versus those with no evident disease, a finding that is consistent with our other data indicating that disease-associated changes in immune markers do not readily normalize in patients treated and presumably cured of their disease (14).

The chemokine receptor, CCR7, is well known for its role in migration of T cells and mature dendritic cells to secondary lymphoid tissues, which contain abundant CCR7 ligands, CCL19 and CCL21 (6, 25). It has been recently reported that CCR7 expression is important for survival of mature dendritic cells (11). Here, we report for the first time that CCR7 signals are also involved in survival of CD8+ T cells. CCR7, in addition to its chemotactic role, can protect CD8+ T cells from death by engaging the PI3K/Akt survival signaling pathway. Many cytokines can activate the PI3K, a family of evolutionarily

![Fig. 5. Akt phosphorylation following binding of the CCR7 ligands to CCR7.](Image)

A, an enriched population of CD8+ T cells was incubated in the presence of CCR7 ligands, CCL19 (200 ng/mL), or CCL21 (200 ng/mL) for various time periods, as indicated. Top, Western blots for phosphorylated Akt (p-Akt) and total Akt. Bottom, results of the densitometric analysis for phosphorylated Akt levels. Expression of phosphorylated Akt is shown as a relative band density; the band density of phosphorylated Akt was normalized to total Akt and data were further normalized to the 2-minute control protein level. Columns, mean of three separate experiments. B, CD8+ T cells were incubated in the absence (control) or presence of the PI3K inhibitor, wortmannin (100 nmol/L), for 1 hour in RPMI 1640 plus 0.1% bovine serum albumin before stimulation by CCR7 ligands, CCL19 (200 ng/mL) or CCL21 (200 ng/mL), for 2 minutes. Phosphorylation of Akt was blocked by wortmannin. Representative of three Western blots.

are consistent with the conclusion that Akt is phosphorylated via the PI3K signaling pathway in CD8+CCR7+ T cells incubated in the presence of the CCR7 ligands. Further, wortmannin-treated CD8+CCR7+ T cells were no longer protected by CCL21 from VP-16-induced apoptosis (data not shown).

Akt phosphorylation and protection of CD8+CCR7+ T cells from apoptosis. Because Akt was phosphorylated in T cells incubated in the presence of CCL21, we next asked whether activation of Akt plays a role in protecting CD8+ T cells from apoptosis.

When Akt was phosphorylated by binding of CCL21 to CCR7+ on CD8+ T cells, pan-caspase activation was simultaneously reduced (Fig. 6). The data suggest that activation of Akt signaling pathway induced by CCL21 is associated with survival of CD8+CCR7+ T cells.

Akt phosphorylation and Bcl-2 expression in CD8+CCR7+ T cells. In our experiments, the antiapoptotic protein Bcl-2 was found to be significantly up-regulated (see Fig. 2), whereas the proapoptotic protein Bax was down-regulated in CD8+CCR7+ T cells compared with CD8+CCR7- T cells. To determine whether Akt phosphorylation is associated with changes of Bcl-2 or Bax expression in CD8+CCR7+ T cells, we incubated these cells in the presence of the CCR7 ligand, CCL21. As shown in Fig. 7, phosphorylation of Akt was detectable within 5 minutes of CCL21 addition and thereafter progressively diminished. Bcl-2 expression was induced in the same cells at 30 minutes to 1 hour. In contrast, changes in Bax expression were not evident. In agreement with our protein expression data in CD8+CCR7+ T cells obtained from normal control or patients with cancer (see Fig. 2), these Western blot results suggest that CCR7 signaling is primarily associated with induction of Bcl-2 expression rather than with changes in Bax expression levels.

Discussion

We have previously reported that T cells, primarily CD8+ T lymphocytes, undergo apoptosis in the tumor environment and in the peripheral circulation of patients with malignant diseases, including SCCHN, melanoma, and ovarian cancers (14, 16, 24). Sensitivity of CD8+ T cells to spontaneous apoptosis or death induced by a variety of extrinsic signals (VP-16, starvation, Fas cross-linking) is a generalized phenomenon. Further, we have established that apoptosis of CD8+ T cells takes place through both death receptor–mediated and mitochondria-mediated pathways (14). The proportions of CD8+ T cells binding Annexin V are markedly elevated in patients with cancer relative to normal controls (13, 14). However, not all CD8+ T cells are equally sensitive to apoptosis, as effector CD8+CD45RA CD27- or CD8+CD28+ cells are preferentially targeted for demise in patients with cancer (16, 17). This extensive spontaneous apoptosis may be one of the mechanisms responsible for immunosuppression found in cancer patients. It now seems that the CCR7+ subset of circulating CD8+ T cells in normal controls and patients with SCCHN is significantly less sensitive to apoptosis than are CD8+ T cells lacking CCR7. The more apoptotic subset is phenotypically characterized as CD8+CCR7- T cells. Also, expression levels of apoptosis-related proteins, such as the death receptor, Fas, proapoptotic protein, Bax, or antiapoptotic protein, Bcl-2, in CD8+CCR7- cells are consistent with their increased susceptibility to apoptosis.

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conserved lipid kinases that play a central role in promoting survival of a wide range of cell types, including lymphocytes (26–28). Thus, CCR7 might be one signal among others that contributes to survival of T cells in vivo. Akt, a downstream target of PI3K, is a critical signaling component of cytokine-mediated survival pathways (21, 29, 30). It is possible that cytokines/chemokines other than CCL19 or CCL21 may protect CD8+ T cells from apoptosis using the same molecular pathway. In this study, we found that protection from apoptosis achieved in CD8+CCR7+ T cells by stimulation with CCR7 ligands, CCL19, and CCL21 induced a time-sensitive increase in levels of the antiapoptotic protein, Bcl-2. Cytoprotection by Bcl-2 is mediated in part by the inhibition of Bax homodimer formation in the mitochondrial outer membrane, which prevents the release of apoptosis-inducing proteins, such as cytochrome c (31, 32). It has been suggested that the PI3K/Akt signaling pathway promotes survival by inhibiting Bax translocation from cytoplasm to mitochondria (33) or by increasing Bcl-2 expression (34–36). In our ex vivo experiments, changes in Bax levels did not correlate with Akt phosphorylation or up-regulation of Bcl-2 expression. However, we measured total cellular Bax levels and thus cannot comment about Bax distribution among different cellular compartments.

A limited number of chemokine receptors have been reported to be involved in the promotion of cell survival. CXCR4, a member of CXCR family, is involved in survival of CD4+ T cells. CXCR4 activation in CD4+ T cells by its ligand, stromal cell–derived factor 1-α, leads to the activation of the prosurvival second messengers, Akt, and extracellular signal-regulated protein kinase (37). Chemokine involvement in cell survival through the PI3K/Akt signaling pathway has also been reported in nonimmune cells. For instance, the chemokine fractalkine activates the PI3K/Akt survival pathway in prostate cancer cells (38), and interactions between CXCR4 and its ligand, stromal cell–derived factor 1-α, are obligatory for maintaining survival of pancreatic ductal cells (39). These reports suggest that chemokine/chemokine receptor systems are required not only for chemotaxis but also for cell survival.

Because CCR7 expression on CD8+ T cells seems to play a role in cytoprotection from apoptosis, alterations in the normal balance between CCR7+ and CCR7− T cells are likely to have a profound effect on the host immune system. CCR7 is also a surface marker of central memory T cells (7). Differentiation of CD8+ T cells into effectors is associated with the loss of CCR7. As CD8+CCR7+ T cells leave the peripheral circulation, presumably to engage in interaction with dendritic

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**Fig. 6.** Akt phosphorylation and caspase activation in CD8+CCR7+ T cells. Enriched CD8+ T cells were incubated in the presence of CCR7 ligand, CCL21 (200 ng/mL), for 5 minutes. Alliquots of the cells were used for measurements of Akt phosphorylation. The remaining cells were incubated for 4 hours with the ligand and, thereafter, staurosporin (100 nmol/L) was added for 16 hours. The cells were harvested to measure caspase activity by intracellular staining using FITC-VAD-FMK. Representative experiment out of three. Akt phosphorylation
cells in lymph nodes, they are replaced in the circulation by more differentiated CD8+CCR7− effector T cells. The rate of this turnover is apparently very rapid in cancer patients, resulting in a conversion of the CCR7+/CCR7− ratio in the circulation, and a replacement of the apoptosis-resistant CD8+CCR7+ T-cell subset by apoptosis-sensitive CD8+CCR7− T cells. Interestingly, upon in vivo anti-OKT3 antibody activation, CD8+CCR7+ T cells proliferate more rapidly than the CD8+CCR7− subset (with the carboxy-fluorescein diacetate, succinimidyl ester proliferation index of 4.5 versus 1.5). This observation suggests that in vivo, rapidly driven expansion of CD8+CCR7+ T cells accompanied by activation-induced cell death could result in an accelerated turnover of this subset. Previous evidence from our laboratory, including T-cell receptor rearrangement excision circle and recent thymic emigrants analyses as well as decreased absolute T-cell subset counts in patients with SCCHN, suggest that a rapid turnover of CD8+ T cells is responsible for dysregulated lymphocyte homeostasis (16, 40, 41).

The apoptosis-sensitive subset of effector CD8+CCR7− T cells seems to be a major component of the lymphocyte population responsible for up-regulating T-cell immunity and specific memory in these patients. We have evidence that tumor peptide–specific (tetramer) CD8+ T cells are more sensitive to spontaneous apoptosis than tetramer-CD8+ T cells in patients. These effector cells rapidly become terminally differentiated and lose CCR7. As this shift becomes more pronounced in the circulation of the cancer patient, and as CD8+CCR7− T cells rapidly undergo apoptosis, more naive CD8+CCR7− T cells are recruited from the bone marrow stores and repopulate the naive and central cell compartments. The progressive replacement of CD8+CCR7− T cells with an enlarged pool of apoptosis-prone CCR7− cells in the patients with cancer may be one of the mechanisms of immune suppression orchestrated by the tumor. The CCR7/P13/Akt pathway transduces survival signals to CD8+CCR7− T cells. However, this pathway is not activated in CD8+ T cells devoid of CCR7. In the latter case, tumor escape from the immune system involves discrete cellular and molecular mechanisms designed to weaken the host antitumor defenses. Although it might seem that the enrichment in matured CD8+CCR7− effector cells would be beneficial for the host’s antitumor defense, the fact is that these effector cells are terminally differentiating at an accelerated rate and undergoing apoptosis, thus depleting the host of essential components of cellular antitumor immunity. Hence, future strategies in cancer therapy should consider protection of T cells from apoptosis possibly by up-regulation of CCR7-related molecular signaling.

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