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CXC Chemokine Ligand 12 (Stromal Cell-Derived Factor 1α) and CXCR4-Dependent Migration of CTLs toward Melanoma Cells in Organotypic Culture

Tianqian Zhang,* Rajasekharan Somasundaram,* Klara Berencsi,* Laura Caputo,* Pyapalli Rani,* DuPont Guerry,† Emma Furth,‡ Barrett J. Rollins,§ Mary Putt,§ Phyllis Gimotty,‡ Rolf Swoboda,* Meenhard Herlyn,* and Dorothee Herlyn2*

Studies in experimental animal models have demonstrated that chemokines produced by tumor cells attract chemokine receptor-positive T lymphocytes into the tumor area, which may lead to tumor growth inhibition in vitro and in vivo. However, in cancer patients, the role of chemokines in T lymphocyte trafficking toward human tumor cells is relatively unexplored. In the present study, the role of chemokines and their receptors in the migration of a melanoma patient’s CTL toward autologous tumor cells has been studied in a novel organotypic melanoma culture, consisting of a bottom layer of collagen type I with embedded fibroblasts followed successively by a tumor cell layer, collagen/fibroblast separating layer, and, finally, a top layer of collagen with embedded fibroblasts and T cells. In this model, CTL migrated from the top layer through the separating layer toward tumor cells, resulting in tumor cell apoptosis. CTL migration was mediated by chemokine receptor CXCR4 expressed by the CTL and CXCL12 (stromal cell-derived factor 1α) secreted by tumor cells, as evidenced by blockage of CTL migration by Abs to CXCL12 or CXCR4, high concentrations of CXCL12 or small molecule CXCR4 antagonist. These studies, together with studies in mice indicating regression of CXCL12-transduced tumor cells, followed by regression of nontransduced challenge tumor cells, suggest that CXCL12 may be useful as an immunotherapeutic agent for cancer patients, when transduced into tumor cells, or fused to anti-tumor Ag Ab or tumor Ag.


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CXCL10, and CCL17 receptors), infiltrating T lymphocytes. These results indicate that CXCL9, CXCL10, and CCL17 chemokines influenced the response of Th2 cells in Hodgkin’s lymphoma. In human melanoma lesions, marked T cell infiltration was exclusively detected in those areas that exhibited strong CXCL9 expression (11). In human epithelial ovarian tumors, infiltrates of CD8<sup>-</sup> CD45RO<sup>+</sup> T cells were correlated with CCL2 and CCL5 expression by tumor cells (12). In glioma lesions, expression of CCL20 significantly correlated with the infiltration of CD8<sup>+</sup>, CD4<sup>+</sup>, and CD45RO<sup>+</sup> T cells (13).

Very few studies have identified chemokines and their receptors involved in human T cell migration toward tumor-derived or recombiant chemokines. CD4<sup>-</sup>, CD40L<sup>+</sup>, and CCR4<sup>+</sup> T cells were chemotactuated by CCL22 (macrophage-derived chemokine) present in supernatants of leukemia cells (14). Interaction of CD40L on T cells with CD40 on tumor cells led to tumor growth stimulation. In another study, human T cells, when retrovirally transduced with CXCR2, migrated toward recombinant or tumor-derived CXCL1 (melanoma growth-stimulating activity-α). The chemokine was able to induce IFN-γ in the transduced T cells (15).

Lastly, supernatants from CD40L-stimulated malignant human B cells containing CCL17, and CCL22, both ligands for CCR4, attracted activated T cells that expressed CCR4 (16).

In all of the T cell migration studies described above, migration of T cells was studied in chemotaxis assay using Transwell plates. In this chemotaxis system, T cells migrated from the upper culture chamber through a polycarbonate or nitrocellulose membrane to the lower chamber containing the chemoattractant. This culture system has several disadvantages: 1) tumor cells are missing from the culture system; 2) artificial high chemokine concentrations are often used, which do not reflect the concentrations produced by tumor cells; 3) the culture system is two-dimensional, whereas in vivo lymphocyte migration is three-dimensional; 4) the stromal components important for T cell migration and activation are missing; 5) T cells migrate only a short distance (~10 μm) through nonphysiological membranes (nitrocellulose or polycarbonate); and 6) study of the migration process is limited to a period of only a few hours.

We have developed a novel three-dimensional organotypic melanoma culture system (referred to hereafter as reconstruct) that allows the study of T cell migration toward tumor cells and the determination of chemokines and their receptors involved in T cell migration under in vivo-like conditions. This model consists of a bottom layer of collagen type I with fibroblasts, superimposed by a tumor cell layer, collagen/fibroblast separating layer, and finally a top layer of collagen, fibroblasts, and T cells, and has the following advantages over the Transwell plate cultures: 1) the cultures include growing tumor cells; 2) chemokines are produced by the growing tumor cells in concentrations similar to those found in tumor tissues in vivo; 3) the culture system is three-dimensional; 4) collagen and fibroblasts provide stromal components important for T cell migration and activation (17); 5) the model allows determination of T cell migration over 6–9 days; and 6) T cells migrate through a distance of at least 500 μm. Thus, the reconstruct has many advantages over the traditionally used Transwell culture system because it mimics the in vivo conditions that play a role in T cell migration.

We show here that CTL migrate toward autologous melanoma cells in the reconstruct, resulting in tumor cell apoptosis, and that migration is mediated by the chemokine CXCL12 (stromal cell-derived factor 1α) produced by tumor cells and the chemokine receptor CXCR4 expressed by T cells.

### Materials and Methods

#### Patient

Patient 793 (male Caucasian, 43 years of age) had excision of a “low risk” melanoma lesion of superficial spreading type with early vertical growth phase present; tumor thickness was 0.55 mm and the vertical growth phase had a brisk lymphatic infiltrate, with no evidence of metastases. The primary lesion was excised ~14 years earlier, and there has been no recurrence since. Patient 793 did not receive any chemotherapy after the removal of the primary lesion.

#### Cell lines

Melanoma cell line WM793 was established from the vertical growth phase of the primary lesion of melanoma patient 793 (18). Melanoma cells were maintained in MCD153-L15 medium (Sigma-Aldrich) supplemented with 2% FBS. HLA types of WM793 cells were A1, A29, B57[17], B35, DRB1 11, and DQB1 0301. Fibroblast cell line FF2441 was maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS. NK cell target K562 (human erythroleukemia cell line) and lymphokine-activated killer (LAK) cell target Daudi (human lymphoblastoid cell line) were obtained from American Type Culture Collection. All lymphoid cell lines were maintained in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS.

#### Reagents

The following mAbs were used: HLA-class I-specific mAb W6/32 and HLA-class II-specific mAb B331 and D1.B6 (obtained from Dr. B. Perussia (Thomas Jefferson University, Philadelphia, PA) and Dr. G. Trinchieri (The Wistar Institute)); mAb MA2.1 to HLA-A2 and B<sup>177/3</sup> (American Type Culture Collection); mAb Nok-1 to Fas ligand (BD Pharmingen); mAb CH-11 to CD95 (Immunotech); anti-CD11a mAb (Immunotech); fluoresceinated or PE-labeled anti-CD4, -8, -25, -40, -40L, -44, -49a, -49b, -61, -80, -86, and -54 mAbs (BD Pharmingen); anti-CXCL12 mAb; anti-human CCR1, -2, -3, -5, -6, -7, -9, and -49C1R; anti-human CXCR4, -8, and -10 mAbs (Imgenex); anti-CCR11 and -CX3CR1 polyclonal Abs (Abcam); fluoresceinated goat anti-mouse IgG (Molecular Probes). Recombinant human CXCL12 was purchased from R&D Systems. AMD3100, a CXCR4 antagonist (19), was purchased from Sigma-Aldrich.

#### Generation of anti-melanoma Ag CTL line

CTL were obtained from coculture of PBMC (10<sup>5</sup> cells/well of 96-well round-bottom microtiter plates) of melanoma patient 793 with irradiated (30,000 rad; Cs source) autologous melanoma cells WM793 (10<sup>5</sup> cells/well) in T cell medium containing RPMI 1640 medium, 10% human AB serum (Gemini), 10 mM HEPES (Sigma-Aldrich), t-arginine (116 mg/L); Invitrogen Life Technologies), t-asparagine (36 mg/L; Invitrogen Life Technologies), t-glutamine (216 mg/L; Invitrogen Life Technologies) and 2-ME (5 × 10<sup>−5</sup> M; Sigma-Aldrich). Cultures were stimulated weekly with irradiated autologous tumor cells in T cell medium containing partially purified IL-2 (20 U/ml; AB1). The phenotype of the CTL line changed from 9% CD4<sup>+</sup> and 61% CD8<sup>+</sup> at wk 1, to 63% CD4<sup>+</sup> and 5% CD8<sup>+</sup> at wk 4–8, and 99% CD4<sup>+</sup> and 0.4% CD8<sup>+</sup> at wk 16–24.

#### T cell proliferation assay

Lymphocytes (10<sup>5</sup> cultured cells per well of 96-well round-bottom microtiter plates; Corning) were stimulated with irradiated WM793 cells (10<sup>5</sup> cells/well) and rIL-2 (20 U/ml) for 4 days. Proliferative responses of the lymphocytes were determined by [3H]thymidine incorporation assay. All determinations were performed in triplicate.

#### Chemokine determination by RT-PCR, ELISA, or FACS analysis

mRNA was extracted from WM793 melanoma cells (3 × 10<sup>6</sup>) using Dyna-beads mRNA DIRECT kit (Dynal). The primers used were as follows: 5′-TGT AGG GCG ACG GTT TTA-3′ and 5′-TCC ACC ACA ACA TGC AG-3′ for CCL25 (TECK); 5′-ATG AAC GCC AAG TGC GTG GTC-3′ and 5′-TGG CGG TTG TGC TGG TCA TCT GTT GTC-3′ for CXCL12; 5′-ATG GCC CGC CTA CTT GGC CCC TTC AC-3′ and 5′-TTA ACT GCT GCC GGC CGG CCT CAT CCT GCCG-3′ for CCL19. PCR was performed for 35 cycles (94°C, 1 min; 60°C for CCL19, 57°C for CXCL12, 55°C for CCL25, 1 min; 72°C, 1 min) using the SuperScript One Step RT-PCR kit.

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3 Abbreviations used in this paper: LAK, lymphokine-activated killer; DC, dendritic cell.
(Invitrogen Life Technologies). CCL21 primers were purchased from BioSource. PCR for CCL21 involved a 1-min 30-s denaturation step at 94°C, followed by 35 cycles of 30 s each at 94°C, 45 s at 60°C, and 45 s at 72°C, with a final 7-min extension at 72°C. All PCR products were analyzed on 10% novex-TBE gel (Invitrogen Life Technologies).

Supernatants obtained from autologous melanoma cells WM793 on day 4 of culture were tested for the presence of CXCL12 using DuoSet ELISA kit (R&D Systems). CXCCL1 chemokine expression on the surface of melanoma cells was determined by FACS analysis as described below (see Phenotyping of tumor cells and T cells).

**Cytokine measurements**

Cytokines produced by CTL793-B were determined as described previously (18, 20). Briefly, cultured CTL were washed twice with serum-containing T cell medium (see above), incubated in serum-containing medium (without stimulants and IL-2) for 8 h at 37°C in a 5% CO2 incubator and washed once. CTL (10^4 cells/well) were stimulated with irradiated autologous tumor cells (10^5 cells/well) in 96-well microtiter plates, Supernatants obtained from cultured CTL after 2 days were tested for the presence of GM-CSF, TNF-α, and IFN-γ, and supernatants obtained from cultured CTL after 4 days were tested for the presence of IL-4. All cytokine determinations were performed using ELISA kits (Endogen).

**Cytotoxicity assay**

Cytotoxicity assay was performed as described previously (18). Briefly, labeled targets (15 μCi of 3^H Cr, as Na_2CrO_4, per 4 × 10^5 cells) were mixed in 96-well round-bottom microtiter plates with effector cells at various E:T ratios and incubated at 37°C for 6 h. Supernatants were harvested and tested for 3^H Cr release (experimental release). For maximal release, target cells were treated with 10 N HCl. Spontaneous release of radioactive activity by target cells was determined in the absence of effector cells. The percentage of cytotoxicity was determined by the following formula: % cytotoxicity = [(experimental release – spontaneous release)/maximal release – spontaneous release] × 100.

To determine HLA restriction of the CTL, tumor targets were incubated with various amounts of anti-HLA class I mAb W6/32 (lgG2a; 60 μg/ml), anti-HLA class II mAb B33.1 or D1.B6 (lgG2a; 60 μg/ml), or anti-HLA-B17/A2 mAb MA2.1 (IgG1; 20 μg/ml). Isotype-matched control mAbs were used at similar concentrations. All incubations were performed for 1 h at room temperature. Excess blocking mAbs were removed, and cytotoxicity assay was performed as described above. The percentage of CTL Lysis inhibition was determined using the following formula: % lysis inhibition = 100 – (% lysis with anti-HLA Ab% lysis with control Ab) × 100.

**Phenotyping of tumor cells and T cells**

Cultured T cells were incubated with saturating concentrations (5 μg/ml) of fluoresceinated or PE-labeled mAbs detecting human lymphocyte markers (see Table II) in RPMI 1640 medium supplemented with 5% human AB serum for 1 h at 4°C. Binding of the mAbs was analyzed by FACS. All values given in Results are corrected for irrelevant, isotype-matched control Ab binding.

**T cell migration in organotypic melanoma culture (reconstruct)**

Cultures were initiated by mixing 4.5 × 10^4 human fetal fibroblasts FF2441 with collagen matrix (1.6 ml of 10× Eagle’s MEM, 0.16 ml of t-glutamine, 1.82 ml of heat-inactivated human AB serum, 0.52 ml of NaHCO_3, and 14.8 ml of bovine collagen type I), and plating 450 μl of the mixture in wells of a 24-well plate. After 1 h, WM793 melanoma cells (1 × 10^5) were seeded on top of the collagen matrix. After 2 days, melanoma cells were stained with CellTracker Blue CMAC (Molecular Probes), and a separating layer of fibroblasts in collagen gel (100 μl; 500 μm) was added on top of the melanoma cells. Fibroblast-collagen overlay containing prestained (CFDA-Green; Molecular Probes) CTL was prepared by mixing 3 × 10^5 fibroblasts FF2441 and 1 × 10^5 CTL with collagen matrix, and adding 250 μl per well. For control reconstruct, PHA blasts prepared from PBMC of the same patient were used. Reconstructs were incubated in medium (50% DMEM, 50% MCD1153-L15 medium supplemented with 2% FBS). Four days after the addition of T cells, recon structs were fixed in 10% buffered formalin for 4 h at room temperature, and processed for histological evaluation. The percentage of apoptotic tumor cells was determined by counting apoptotic nuclei and intact tumor cells in sections stained with H&E, using the following formula: % apoptotic tumor cells = (no. of apoptotic tumor cells/total no. of tumor cells) × 100.

**Blocking of T cell migration in reconstruct**

The bottom layer of reconstructs contained 4.5 × 10^5 fibroblasts in 450 μl of type I collagen gel. After 1 h, WM793 melanoma cells (1 × 10^5) were seeded on top of the collagen matrix. After 48 h, a separating layer of fibroblasts in collagen gel (100 μl; 500 μm) was added on top of melanoma cells. After gel formation, anti-chemokine or chemokine receptor Abs (anti-CXCL12, anti-CXCR4, anti-CCR7, anti-CCR9, and anti-CX3CR1 Ab (10 μg/ml each Ab, corresponding to 10^4 the concentration recommended by the manufacturer for neutralization of the receptor or chemokine)), isotype-matched control Ab or AMD3100 (a CXCR4 antagonist; 5 μg/ml) were added, followed by the top layer containing CTL (1 × 10^5) mixed with 3 × 10^5 fibroblasts and type I collagen gel. To evaluate whether CXCL12 can block migration of T cells, CXCL12 (20 ng/ml) was added into the medium on top of T cell layer. The percentage of apoptotic tumor cells in the presence and absence of inhibitor was determined.

**Statistical analyses**

Differences between experimental and control values were analyzed for a statistical significance by two-sample Student’s t test.

**Results**

**Functional characteristics of CTL793-B in mixed lymphocyte tumor cell culture**

CTL793-B lysed autologous WM793 melanoma cells in a 6-h 51Cr release assay, and lysis was dependent on the E:T cell ratio. NK target cells K562 and LAK target cells Daudi were not lysed in the same assay (Fig. 1A). Lysis of melanoma cells by the CTL may be mediated by both perforin and granzyme as indicated by FACS analysis of T cell staining for the enzymes (Fig. 1B), and was inhibited by mAb W6/32 to HLA class I, as well as a mAb that recognizes a determinant shared by HLA-B7/57/58 and A2 allospecificities, compared with lysis in the presence of control Ab (Table I). Because patient 793 is HLA-B17 positive, but HLA-A2 negative, CTL most likely are HLA-B17 restricted. Partial inhibition of tumor cell lysis by anti-HLA class I mAb may be due to high-affinity binding of the TCR to the tumor Ag. Proliferation of CTL793-B is dependent on the presence of tumor cells (Fig. 1C), and proliferating lymphocytes secrete both IFN-γ and GM-CSF (D, but not TNF-α and IL-4 (not shown). Thus, the CTL are of T cytotoxic type (21).

**Functional characteristics of CTL793-B in reconstruct**

A schematic presentation of the four reconstruct layers, each consisting of cells embedded in collagen is shown in Fig. 2A. CTL793-B labeled with CFDA-Green migrated from the top layer of collagen and fibroblasts through a separating layer of collagen and fibroblasts toward WM793 tumor cells labeled with CMAC-Blue (Fig. 2Ba), whereas no T cells were found in cultures with tumor cells only (Bb). The CTL induced massive apoptosis in the autologous melanoma cells (Fig. 2C, e and f), compared with recon structs with tumor cells alone (C, a and d) or tumor cells plus PHA blasts (C, b and e). Apoptosis in melanoma cells was significantly (p < 0.0001; two-sample t test) higher on day 9 than on day 6 of culture with 90% apoptotic tumor cells on day 9 (Fig. 2, C and D). CTL values were significantly (p < 0.001 to p < 0.0001; two-sample t test) higher than either of the two control values on both days (Fig. 2D).

**Phenotypic characteristics of CTL793-B and WM793 melanoma cells**

CTL793-B and WM793 melanoma cells were phenotyped with special emphasis on molecules that might be involved in the interactions of these cells with each other and components of the reconstruct (Table II). CTL line 793-B is predominantly of CD4 phenotype (98.5% of the cells positive). The cells also express CD95 (FAS), and adhesion and costimulatory molecules (Table...
Expression of α₂ (CD49b) and β₁ (CD29) integrins by T cells (Table II) is important for T cell interaction with collagen in the reconstruct, which results in T cell activation (22, 23). LFA-1α (CD11a), ICAM-1 (CD54), and CD44 expressed by the CTL (Table II) facilitate interaction of the lymphocytes with fibroblasts in the reconstruct (24, 25). This interaction results in the activation of both lymphocytes and fibroblasts through secretion of growth and survival factors, cytokines, and fibronectin (24–27).

WM793 melanoma cells express both HLA class I and II molecules, FAS, ICAM-1, and various integrins. The cells express very low amounts of FAS ligand, B7-1 and B7-2 (Table II). Expression of α₂ and β₁ integrins by the melanoma cells (Table II) facilitates migration of the cells through collagen (28, 29). ICAM-1 on the melanoma cells interacts with LFA-1α on the CTL, resulting in T cell stimulation (30).

Thus, several phenotypic markers on CTL793-B, WM793 melanoma cells, and fibroblasts facilitate interactions between these cells and between the cells and collagen in the reconstruct, leading to activation of T cells and fibroblasts as well as T cell migration toward tumor cells.

### Chemokine and chemokine receptor involved in CTL793-B migration toward WM793 cells

CTL793-B expressed the chemokine receptors CCR7, CCR9, CXCR4, and CX3CR1 (Table III). WM793 melanoma cells produced CXCL12 (Fig. 3A). Significant amounts of CXCL12 are also produced by FF2441 fibroblasts used in the reconstruct, but only low amounts of the chemokine are produced by CTL793-B (Fig. 3A). CXCL12 produced by WM793 melanoma cells most likely binds to CXCR4 on CTL793-B cells (31, 32). Other chemokines known to bind to the other chemokine receptors on the CTL were not produced by the melanoma cells (Table III). CXCR4, although highly expressed by CTL793-B (47.2% of cells positive), showed low expression by WM793 melanoma cells (5.9%), and no expression by FF2441 fibroblasts (0.4%) (Fig. 3B).

We evaluated a possible role of CXCR4 expressed by CTL793-B cells and CXCL12 produced by WM793 cells in the migration of T cells toward melanoma cells in the reconstruct. T cell migration was measured as a function of tumor cell apoptosis and not absolute numbers of T cells at the tumor cell layer, because T cells may themselves apoptose after inducing tumor cell apoptosis, and one T cell may induce apoptosis in several tumor cells. CXCL12, when added on top of the T cell layer, significantly (p < 0.001) inhibited melanoma cell apoptosis induction by CTL793-B (Table IV). Similarly, anti-chemokine Ab, anti-chemokine receptor Ab, and CXCR4 antagonist AMD3100, when added on top of the separating layer, significantly (p < 0.001) inhibited tumor cell apoptosis (Table IV). Because FF2441 fibroblasts used in the reconstruct produced CXCL12 (Fig. 3A), we investigated whether they contributed to CTL793-B migration. Fibroblasts are evenly distributed over all layers in the reconstruct, and thus they are not expected to provide a chemokine gradient and contribute to T cell migration. However, fibroblasts in tumor cell vicinity may be stimulated by cytokines (produced by tumor cells or CTL) to produce chemokines, inducing a chemokine gradient that may induce CTL migration toward fibroblasts located in tumor or T cell vicinity (33–35). To evaluate whether fibroblasts play a role in CTL793-B migration toward WM793 melanoma cells, migration of T cells and subsequent tumor cell apoptosis was compared in the presence and absence of fibroblasts. CTL793-B induced significant (p < 0.001) apoptosis in WM793 tumor cells, both in the presence and absence of fibroblasts (Table V). Thus, CTL migration toward tumor cells is induced by tumor-derived CXCL12 chemokine and not by fibroblast-derived chemokine.

### Discussion

We have shown previously that CTL793 clone A lysed WM793 melanoma cells in culture and that lysis was HLA-B17 restricted.
Green) CTL793-B mixed with 3 of type I collagen gel. After 1 h, 1 followed by addition of a top layer containing 1/11003 graphed in the Nikon fluorescence microscope using appropriate filters.

Table I. Blocking of cytotoxicity of CTL793-B against WM793 melanoma cells by mAbs

<table>
<thead>
<tr>
<th>Designation</th>
<th>Specificity</th>
<th>Isotype</th>
<th>% Lysis</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG</td>
<td>NA</td>
<td>Predominantly IgG1</td>
<td>83.0 ± 9.9</td>
<td>13.4 ± 4.2</td>
</tr>
<tr>
<td>W6/32</td>
<td>HLA class I</td>
<td>IgG2a</td>
<td>48.3 ± 2.6d</td>
<td>41.7</td>
</tr>
<tr>
<td>MA2.1</td>
<td>HLA-B17/A2</td>
<td>IgG1</td>
<td>52.5 ± 2.4d</td>
<td>36.7</td>
</tr>
<tr>
<td>B33.1</td>
<td>HLA class II</td>
<td>IgG2a</td>
<td>69.3 ± 14.5</td>
<td>16.3</td>
</tr>
</tbody>
</table>

a Tumor targets were incubated with Ab (20–60 μg/ml) for 1 h at room temperature, and excess Ab was removed before addition of effector cells in a 6-h 51Cr release assay.

b Means ± SD of triplicate determinations.

c NA, Not applicable.

d Values are significantly (p < 0.05 or less) different from the values obtained with cultures that received control mouse IgG.

We have shown here that CTL migrate through a 500-μm collagen/fibroblast separating layer toward tumor cells, resulting in tumor cell apoptosis. We have also shown that migration is dependent on CXCL12 produced by tumor cells and CXCR4 expressed by T cells. To our knowledge, this is the first demonstration of chemokine dependency of human CTL migration toward tumor cells.

The reconstruct is a novel three-dimensional culture system in which the migration of leukocytes toward tumor cells and the factors that influence leukocyte migration can be studied under in vivo-like conditions. In the reconstruct, human melanoma is recapitulated in vitro using a mixture of collagen and fibroblasts (lattices or matrices). Ag-elicited T cells are stimulated by collagen, most likely through the interaction of α1 (CD49b) and β1 (CD29) integrins on T cells with collagen (22, 23). Because activated fibroblasts play an important role in the activation of T lymphocytes, they were included in the reconstruct. T lymphocytes bind to fibroblasts via LFA-1α (CD11a), ICAM-1 (CD54), and CD44. The

Table II. Phenotypic and functional markers of CTL793-B and WM793 melanoma cells

<table>
<thead>
<tr>
<th>Parameter Investigated</th>
<th>WM793</th>
<th>CTL793-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cells positive MFI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA class I</td>
<td>97.2</td>
<td>126.2</td>
</tr>
<tr>
<td>HLA class II</td>
<td>73.5</td>
<td>97.1</td>
</tr>
<tr>
<td>CD4</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD25</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD40</td>
<td>3.3</td>
<td>6.1</td>
</tr>
<tr>
<td>CD40L</td>
<td>6.0</td>
<td>3.5</td>
</tr>
<tr>
<td>CD44</td>
<td>92.5</td>
<td>139.8</td>
</tr>
<tr>
<td>CD95 (FAS)</td>
<td>98.7</td>
<td>103.0</td>
</tr>
<tr>
<td>CD95L (FASL)</td>
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<td>3.1</td>
</tr>
<tr>
<td>CD54 (ICAM-1)</td>
<td>99.1</td>
<td>120.5</td>
</tr>
<tr>
<td>CD11a (LFA-1a)</td>
<td>0.0</td>
<td>1.2</td>
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<tr>
<td>CD80 (B7–1)</td>
<td>7.1</td>
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<tr>
<td>CD86 (B7–2)</td>
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<td>11.0</td>
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<tr>
<td>CD49a (α1, integrin)</td>
<td>91.8</td>
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<tr>
<td>CD49b (α2, integrin)</td>
<td>98.0</td>
<td>11.3</td>
</tr>
<tr>
<td>CD29 (β1, integrin)</td>
<td>100</td>
<td>122.8</td>
</tr>
<tr>
<td>CD61 (β1, integrin)</td>
<td>99.2</td>
<td>38.2</td>
</tr>
</tbody>
</table>

a All markers were determined by FACS analysis.

b MFI, Mean fluorescence intensity per cell.

c NA, Not applicable.

FIGURE 2. CTL793-B migration toward autologous WM793 melanoma cells in reconstruct. A, Reconstruct schema. B, CTL793-B migration. The bottom layer of reconstructs contained 4.5 × 10⁵ fibroblasts in 450 μl of type I collagen gel. After 1 h, 1 × 10⁵ WM793 melanoma cells were seeded on top of the collagen matrix. After 48 h, melanoma cells were stained with CellTracker Blue CMAC. A separating layer of fibroblasts in collagen gel (500 μm) was then placed on top of the melanoma cell layer, followed by addition of a top layer containing 1 × 10⁵ prestained (CFDA-Green) CTL793-B mixed with 3 × 10⁵ fibroblasts and type I collagen gel (a). In control wells with no lymphocytes the top layer contained fibroblasts and collagen only (b). Reconstructs were harvested on day 4, fixed in buffered formalin, and embedded in paraffin. Sections were photographed in the Nikon fluorescence microscope using appropriate filters. C, Induction of apoptosis in WM793 cells by CTL793-B: qualitative analysis. The percentage of apoptotic tumor cells was determined by counting apoptotic nuclei and intact tumor cells in 30 fields of sections stained with H&E. Magnification, ×400. D, Induction of apoptosis in WM793 cells by CTL793-B: quantitative analysis. The percentage of apoptotic melanoma cells was determined by counting apoptotic nuclei and intact tumor cells in 30 fields of sections stained with H&E. Data represent means plus SD (bars) of 30 fields (D). Percentage of apoptosis in cultures with WM793 and autologous CTL793-B cells on day 9 is significantly (p < 0.0001; two-sample t test) higher than the value on day 6 (D). CTL values are significantly (p < 0.001 to p < 0.0001; two-sample t test) higher than either of the two control values on both days.
Chemokine receptors expressed by CTL793-B and chemokines produced by WM793 melanoma cells

<table>
<thead>
<tr>
<th>Chemokine Receptors Expressed by CTL793-B</th>
<th>Known to bind to receptor</th>
<th>Chemokines Expressed by WM793</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7</td>
<td>CCL21</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CCL19</td>
<td>–</td>
</tr>
<tr>
<td>CCR9</td>
<td>CCL25</td>
<td>–</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12</td>
<td>+</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3CL1 (Fractalkine)</td>
<td>–</td>
</tr>
</tbody>
</table>

a Chemokine receptors were detected by FACS. The following receptors were not expressed by CTL793-B: CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR10, CCR11, CXCR1, CXCR2, CXCR3, CXCR5, and CXCR6.

Table III. Chemokine receptors expressed by CTL793-B and chemokines produced by WM793 melanoma cells

The main biological role of IL-1 is the stimulation of T cells to express IL-2R and secrete IL-2 (36). IL-6 and IL-7 are T cell survival factors (37), and fibroblast stimulating predominantly resting lymphocytes (27). However, in our studies, induction of tumor cell apoptosis by CTL793-B was similar in the absence and presence of fibroblasts, suggesting that fibroblasts do not play a significant role in CTL activation and migration. This finding was surprising because fibroblast factors were expected to substitute for the absence of exogenous cytokines in the cultures. Thus, CTL793-B was IL-2 dependent in its growth outside the recon- struct, whereas the CTL migrated toward tumor cells and lysed these cells in the absence of exogenous IL-2 in the reconststruct.

Other investigators have used collagen matrices to study inter- action of leukocytes with tumor cells, but they have not demonstrated CTL migration resulting in tumor cell apoptosis in a culture system similar to the reconststruct shown here. Thus, Wei et al. (38) have demonstrated inhibition of mouse tumor cell growth by peptide-specific CTL in a three-dimensional collagen matrix. However, in contrast to our studies, T cells were not shown to migrate in that culture system. In an organotypic culture of human papil- loma virus-transformed keratinocytes, allogeneic lymphocytes in- duced apoptosis in tumor cells (39). This effect most likely was induced by NK, but not T cells. In another study, a two-layer collagen matrix culture model that consisted of a collagen gel con- taining human dendritic cells (DCs) as the lower layer and a col- lagen gel containing necrotic human tumor cells and T cells as the upper layer was used to demonstrate DC migration toward tumor and T cells and tumor Ag presentation to T cells by the DCs (40).

In the present study, CXCL12 produced by melanoma cells at- tracted CTL through binding to CXCR4 on T cells. This was demon- strated by blocking CTL migration toward tumor cells with high concentrations of CXCL12, applied on top of the T cell layer, or anti-CXCR4 Ab, anti-CXCL12 Ab, or small molecule CXCR4 an- tagonist, each applied on top of the separating collagen/fibroblast layer. Each of the compounds inhibited CTL migration, most likely by blocking CXCR4 on the CTL.

In preliminary studies, we have delineated the role of chemokine receptor/chemokine in the migration of five additional T cell lines or clones toward tumor cells. Only one of the five T cell lines used CXCR4 to migrate toward CXCL12 produced by the autologous tumor cells (our unpublished data). For the other four T cell lines, different receptor/ligand pairs were involved in T cell migration and induction of tumor apoptosis (CCR2/CCL2; CXCR3/ CXCL10; CCR4/CCL2; CXCR3/CXCL11; our unpublished data). These studies suggest that in general in different patients different chemokine receptors/chemokines are involved in T cell migration toward tumor cells.

CXCL12 acts synergistically with vascular endothelial growth factor to amplify angiogenic processes (41, 42). Therefore, trans- fusion of tumor cells with this chemokine may lead to enhanced tumor growth through stimulation of angiogenesis. However, this effect may be counterbalanced by inhibition of tumor growth through chemotraction of leukocytes into the tumor area by the chemokine. In this respect, mouse leukemia and melanoma cells transfected with human CXCL12 were rejected following injection into syngeneic mice (43). Furthermore, therapeutic vaccination of

Table IV. Blocking of CTL793-B migration in reconststruct

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percentage of Apoptotic Tumor Cells (mean ± SD/field (30 fields))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM793</td>
<td>6.5 ± 3.4</td>
</tr>
<tr>
<td>WM793 + CTL793-B</td>
<td>19.5 ± 8.3abc</td>
</tr>
<tr>
<td>WM793 + CTL793-B + CXCL12</td>
<td>5.6 ± 4.1bc</td>
</tr>
<tr>
<td>WM793 + CTL793-B + AMD3100</td>
<td>5.6 ± 4.1c</td>
</tr>
<tr>
<td>WM793 + CTL793-B + control Ig</td>
<td>17.5 ± 5.2de</td>
</tr>
<tr>
<td>WM793 + CTL793-B + α-CXCR4 Ab</td>
<td>4.8 ± 4.3de</td>
</tr>
<tr>
<td>WM793 + CTL793-B + α-CXCL12 Ab</td>
<td>4.9 ± 4.6f</td>
</tr>
</tbody>
</table>

* Reconstructs consisted of a bottom layer of collagen and fibroblasts, followed by a tumor cell layer and a separating layer of collagen and fibroblasts. Anti-chemokine or anti-chemokine receptor or control Abs were added at 10 µg/ml and AMD3100 was added at 20 ng/ml. Separate cultures received CXCL12 (20 ng/ml) on top of the T cell layer. Percentage of apoptotic tumor cells in 4-day cultures was determined as described in Materials and Methods.

b,c,d,e Values with the same letter differ significantly from each other (p < 0.001, Student’s two-sample t test).
mice bearing established tumors resulted in effective tumor rejection. Rejection most likely was mediated by CTL (43). Similarly, in mice immunized with tumor cells that were transfected with CXCL12, tumors regressed, and this effect was enhanced by IL-2 or GM-CSF (44). These studies, combined with our demonstration of chemotactic effects of the chemokine on human CTL in the reconstitute, suggest the potential usefulness of this chemokine in immunotherapeutic treatment of cancer patients. Thus, patients may be vaccinated with CXCL12-transduced tumor cells or tumor-associated Ags fused to CXCL12; alternatively, patients may be treated with anti-tumor Ab/CXCL12 fusion protein that will attract lymphocytes to the tumor area (1, 8, 9). However, patients will have to be selected for expression of CXCL12 by tumor cells and CXCR4 by T cells. Thus, patients’ treatment may have to be individualized.

In addition to therapeutic implications, our study has prognostic potential. Thus, infiltration of melanoma lesions with T lymphocytes is an independent and favorable prognostic biomarker (45–48), and CXCL12 expression by tumor cells of primary lesions as assessed by immunohistochemistry should be explored for its association with prognosis.

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Disclosures
The authors have no financial conflict of interest.

References