ORIGINAL ARTICLE

# Colon carcinoma cells induce CXCL11-dependent migration of CXCR3-expressing cytotoxic T lymphocytes in organotypic culture

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**Abstract** Adoptive immunotherapy of cancer patients with cytolytic T lymphocytes (CTL) has been hampered by the inability of the CTL to home into tumors in vivo. Chemokines can attract T lymphocytes to the tumor site, as demonstrated in animal models, but 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 colon carcinoma (CC) patient's CTL toward autologous tumor cells has been studied in a novel three-dimensional organotypic CC culture. CTL migration was mediated by chemokine receptor CXCR3 expressed by the CTL and CXCL11 chemokine secreted by the tumor cells. Excess CXCL11 or antibodies to CXCL11 or CXCR3 inhibited migration of CTL to tumor cells. T cell and tumor cell analyses for CXCR3 and CXCL11 expression, respectively, in ten additional CC samples, may suggest their involvement in other CC patients. Our studies, together with previous studies indicating angiostatic activity of CXCL11, suggest that CXCL11 may be useful as an immunotherapeutic agent for cancer patients when transduced into tumor cells or fused to tumor antigen-specific Ab.

**Keywords** CTL · Chemokines · Chemotaxis · Apoptosis · Tumor immunity

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## Abbreviations

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| CC | Colon carcinoma |
| CTL | Cytotoxic T lymphocyte |
| DC | Dendritic cell |
| EBV | Epstein–Barr virus |
| E:T | Effector-to-target ratio |
| FCFB/1 | Fetal colon fibroblast |
| GM-CSF | Granulocyte–macrophage colony-stimulating factor |
| IFN | Interferon |
| IL | Interleukin |
| LAK | Lymphokine-activated killer |
| mAb | Monoclonal antibody |
| MLTC | Mixed lymphocyte tumor cell culture |
| PBMC | Peripheral blood mononuclear cells |
| PCR | Polymerase chain reaction |
| TIL | Tumor infiltrating lymphocytes |
| TNF | Tumor necrosis factor |

## Introduction

Chemokines produced by tumor cells have been demonstrated to attract chemokine receptor-positive T lymphocytes into the tumor area, potentially leading to tumor growth inhibition in vitro and in vivo [13, 22, 48]. These studies have been largely confined to mouse tumor systems, whereas the role of human chemokines in human T-lymphocyte trafficking toward human tumor cells is relatively unexplored. In mouse systems, in vivo transduction of mouse tumor cells with chemokines led to T-cell infiltration into the tumor area and rejection of both transduced and non-transduced tumor cells [13, 22, 48]. $CD4^+$ T-cell subsets have been implicated in tumor rejection induced by vaccination of mice with CCL19-transduced tumor cells [6], and $CD8^+$ cytolytic T lymphocytes (CTL) were instrumental in tumor growth rejection in mice following intratumoral delivery of CCL20 via adenovirus vector [15] or injection of CCL16-expressing tumor cells [16]. Both $CD4^+$ and $CD8^+$ T cells were required for tumor growth inhibition to occur in mice injected intratumorally with CCL21 [41].

Adoptive immunotherapy of cancer patients with CTL has been hampered by the inability of the CTL to home to tumors in vivo [30]. Chemokines may be fused to tumor antigen (Ag)-specific Ab to attract adoptively transferred T cells to the tumor site.

To develop immunotherapeutic strategies for cancer patients based on chemokines and their receptors, similar to the approaches already successfully used in mice (see above), we must identify chemokines and their receptors involved in T-cell migration toward tumor cells. Such information is scarce in human tumor systems, including colon carcinoma (CC). In related studies Musha et al. demonstrated CCR5 and CXCR3 expressing infiltrating T cells in colorectal tumors, and the presence of CCL5 and CXCL10 in the same areas of tumor tissues [33]. In another study in colorectal cancer patients, the expression level of CX3CL1 in tumors correlated with the density of tumor infiltrating lymphocytes (TIL), and patients with strong CX3CL1 expression showed significantly better prognosis than those with weak expression [35].

Recently, we have shown that migration of CTL derived from melanoma patients toward autologous tumor cells is dependent on CXCR4 or CCR4 expressed by the CTL and CXCL12 or CCL2 produced by the melanoma cells, respectively [52, 53]. However, it was not known whether similar results would be obtained in patients with other malignancies.

In the present study, we have established a CTL line from the PBMC of a patient with CC and studied migration in a three-dimensional organotypic culture system (reconstruct) described previously [52, 53]. The CTL migrated toward and induced apoptosis of autologous CC cells. CTL migration was mediated by CXCR3 and CXCL11.

## Materials and methods

### Patients

Patient 020 (Caucasian female) underwent resection of a primary cecal adenocarcinoma, stage Dukes' B. There was transmural tumor penetration through the bowel wall, but no evidence of lymph node or distant metastatic disease. This patient's lymphocytes provided the basis for T-cell migration studies. Ten additional primary tumor tissues were obtained from CC patients of various disease stages (see Table 1). These patients' lymphocytes were used in chemokine receptor analyses. Blood and tissue were obtained under

**Table 1** CXCR3 expression by T-cell lines established from TIL of CC tissues in MLTC or reconstruct

| Patient | | T cell | | | Expression of CXCR3[a] |
|---|---|---|---|---|---|
| # | Disease stage (Dukes') | Culture | Phenotype | Function | (% positive cells) |
| 03123 | B | MLTC | $CD4^+$ | CTL | 76.2 |
| 05193 | B | MLTC | $CD4^+$ | CTL | 81.7 |
| 28712 | B | MLTC | $CD4^+$ | CTL | 87.7 |
| 274405 | B | Reconstruct | $CD8^+$ | NT | 42.4 |
| 280150 | C | MLTC | $CD4^+$ | Th | 88.7 |
| 280291 | A | Reconstruct | $CD4^+$ | Th | 43.7 |
| 280321 | A | MLTC | $CD4^+$ | NT | 71.7 |
| 281412 | C | Reconstruct | $CD4^+$ | NT | 85.0 |
| 281638 | B | Reconstruct | $CD4^+/CD8^+$ | NT | 30.4 |
| 1003485 | A | Reconstruct | $CD4^+/CD8^+$ | NT | 47.0 |

NT not tested

[a] CXCR3 expression was determined by FACS analysis

informed consent approved by the Institutional Review Board of the Fox Chase Cancer Center, University of Pennsylvania, and The Wistar Institute.

## Cell lines

Colon carcinoma cell lines were established from tumor tissues as previously described [23]. CC cells were maintained in 75% MCDB 201-L15 medium (Sigma-Aldrich, St Louis, MO, USA) and 25% DMEM (GIBCO-Invitrogen, Carlsbad, CA, USA) supplemented with 2% FBS. HLA types of WC020 CC cells were A30, B13, DR13, 15, and DQ06. Fibroblast cell line FCFB/1 was established from fetal colon and maintained in DMEM (GIBCO-Invitrogen) supplemented with 10% FBS. NK cell target K562 (human erythroleukemia cell line) and LAK cell target Daudi (human lymphoblastoid cell line) were obtained from ATCC (Rockville, MD, USA). Both lymphoid cell lines were maintained in RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS.

## Reagents

The following monoclonal antibodies (mAbs) were used: HLA-class I-specific mAb W6/32 and HLA-class II-specific mAb B33.1 and D1.B6 (obtained from Dr B. Perussia, Thomas Jefferson University, and Dr G. Trinchieri, The Wistar Institute); mAb Nok-1 to Fas ligand (PharMingen, Los Angeles, CA, USA); mAb CH-11 to CD95 and anti-CD11a mAb (Immunotech, Westbrook, ME, USA); fluoresceinated or phycoerythrin-labeled anti-CD:4, 8, 25, 29, 40, 40L, 44, 49a, 49b, 80, and 54 (PharMingen, Los Angeles, CA, USA); anti-CXCL-11 mAb; anti-human CCR:1, 2, 3, 5, 6, 7, 9, and CXCR:1, 2, 3, 4, 5, and 6 mAbs (R&D Systems, Minneapolis, MN, USA); anti-human CCR:4, 8, and 10 mAbs (Imgenex, San Diego, CA, USA); anti-CCR11 and -CX3CR1 polyclonal Abs (Abcam, Cambridge, MA, USA); fluoresceinated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Recombinant human CXCL11 was purchased from R&D Systems (Minneapolis, MN, USA).

## Generation of anti-CC CTL lines

CTL020 cells were obtained from co-culture of PBMC (obtained 7 months after surgery; $10^5$ cells/well of 96-well round-bottom microtiter plates) of CC patient 020 with irradiated (30,000 rads, Cs source) autologous CC cells WC020 ($10^5$ cells/well) in T-cell medium containing RPMI 1640 medium, 10% human AB serum (Gemini, Calabasas, CA, USA), 10 mM HEPES (Sigma-

Aldrich, St Louis, MO, USA), L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l; all from GIBCO-Invitrogen, Carlsbad, CA, USA) and 2-mercaptoethanol ($5 \times 10^{-5}$ M; Sigma, Aldrict, St Louis, MO, USA), in a humidified 5% $CO_2$ incubator. Cultures were stimulated with irradiated autologous tumor cells every 2 weeks in T-cell medium containing partially purified IL-2 (20 U/ml; ABI, Columbia, MA, USA), and characterized after 9–12 weeks in culture. Additional T-cell lines were established from TIL of fresh CC tissues. Tumor was minced and cultured in MLTC or in organotypic cultures (reconstructs). MLTC was initiated by culturing minced tumor in 24-well plates using 2 ml/well T-cell medium. Reconstructs were initiated by mixing minced tumor with collagen matrix [1.65 ml of $10 \times$ Eagle's MEM, 150 μl of 200 mM L-glutamine, 1.8 ml of heat inactivated human AB serum, 510 μl of 7.5% $NaHCO_3$ and 13.8 ml of bovine collagen type I (1.17 mg/ml; Organogenesis, Canton, MA, USA)], and plating 1 ml of the mixture and 1 ml T-cell medium into wells of a 24-well plate. Natural IL-2 was added to MLTC and reconstruct cultures weekly, starting on day 7. After 7–8 weeks, cultured cells were isolated from MLTC or reconstruct and stimulated every 2 weeks with cultured, irradiated (10,000 rads, Cs source) tumor cells or uncultured tumor cells derived from cryopreserved tissues. T-cell function and CXCR3 expression was tested after 8–12 weeks in culture.

## Chemokine determination by RT-PCR or ELISA

mRNA was extracted from CC cells ($5 \times 10^6$) using Fast Track 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA, USA). The primers used were 5′-TGT AGG GCG ACG GTT TTA-3′ and 5′-TCC ACC ACA ACA TGC AG-3′ for CCL25; 5′-ATG GCC CTG CTA CTG GCC CTC AGC CTG-3′ and 5′-TTA ACT GCT GCG GCG CTT CAT CTT GGC-3′ for CCL19; 5′-CAT GCT GGT GAG CCA AGC AGT TTG AA-3′ and 5′-CAC TTC TGT GGG GTG TTG GGG ACA AG-3′ for CXCL9; 5′-CGA TGC CTA AAT CCC AAA TCG AAG CA-3′ and 5′-AAT GCT GG ACT CCT TTG GGC AGT GG-3′ for CXCL11; 5′-ATG AAC GCC AAG GTC GTG GTC-3′ and 5′-TGG CTG TTG TGC TTA CTT GTT T-3′ for CXCL12; 5′-TCT CTC CAG GCC ACG GTA TTC-3′ and 5′ ACC ATT TGG CAC GAG GAT TCA C-3′ for CXCL13. PCR reactions were performed for 35 cycles (94°C, 45 s; 60°C for CCL19, CXCL9, and CXCL11; 56°C for CXCL12, 55°C for CCL25, 45 s; 72°C, 45 s) using the SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). CCL21 primers were purchased from Biosource (Camarillo, CA, USA). 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. CXCL10 primers were purchased from R&D Systems (Minneapolis, MN, USA), and PCR involved a 45-s denaturation step at 94°C, followed by 35 cycles of 45 s each at 94°C, 45 s at 55°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, Carlsbad, CA, USA). Supernatants obtained from CC cells on day 6 of culture were tested for the presence of CXCL9, CXCL11, CXCL12, CCL21, and CCL25 using DuoSet ELISA kits (R&D, Minneapolis, MN, USA).

Cytokine measurements

For cytokine production, cultured CTL were washed twice with serum-containing T-cell medium (see Sect. "Generation of anti-CC CTL lines"), incubated in serum-containing medium (without stimulants and IL-2) for 8 h at 37°C in a humidified 5% $CO_2$ incubator and washed once. CTL ($10^4$ cells/well of 96-well round-bottom microtiter plates) were stimulated with different numbers ($2.5 \times 10^3$, $5 \times 10^3$, or $10^4$ cells/well) of irradiated autologous tumor cells and 20 U/ml recombinant human IL-2 (gift from Biological Resources Branch, NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA) in 96-well microtiter plates. Supernatants obtained from cultured CTL after 2 days were tested for the presence of GM-CSF, TNF-$\alpha$, and IFN-$\gamma$, 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, Rockford, IL, USA).

Cytotoxicity assay

Labeled targets [5 $\mu$Ci of $^{51}$Cr, as $Na_2CrO_4$ (Perkin Elmer, Boston, MA, USA) per $5 \times 10^3$ cells] were mixed in 96-well round-bottom microtiter plates with effector cells at various E:T and incubated at 37°C for 10 h. Supernatants were harvested and tested for $^{51}$Cr release (experimental release). For maximal release, target cells were treated with 10 N HCl. Spontaneous release of radioactivity by target cells was determined in the absence of effector cells. The percentage of cytotoxicity was determined by the following formula:

% Cytotoxicity

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100 \tag{1}$$

To determine HLA restriction of the CTL, tumor targets were incubated with saturating concentrations of anti-HLA class I mAb W6/32 ($IgG_{2a}$), or anti-HLA class II mAb B33.1 ($IgG_{2a}$) at a final concentration of 0.45 mg/ml. Isotype-matched control mAb was used at similar concentrations. All incubations were performed for 1 h at room temperature. Excess blocking mAb was removed and cytotoxicity assay was performed as described above. The percentage of CTL lysis inhibition was determined using the following formula:

% Lysis inhibition

$$= 100 - \left[ \frac{\text{% lysis with anti} - \text{HLA mAB}}{\text{% lysis with control mAB}} \times 100 \right] \tag{2}$$

Phenotyping of tumor cells and T cells

Cultured T cells were incubated with saturating concentrations (5 $\mu$g/ml) of fluoresceinated or phycoerythrin-labeled mAbs detecting human lymphocyte and tumor markers (Tables 2, 3) in RPMI 1640 medium supplemented with 5% human AB serum for 1 h at 4°C. Binding of the mAbs was analyzed in the cytofluorograph. All values given in Sect. "Results" are corrected for irrelevant, isotype-matched control Ab binding.

T-cell migration in organotypic CC culture (reconstruct)

Cultures were initiated by mixing $4.5 \times 10^4$ FCFB/1 cells with collagen matrix (see at Sect. "Generation of anti-CC CTL lines"), and plating 450 $\mu$l of the mixture into wells of a 24-well plate. After 24 h, WC020 CC cells ($1 \times 10^5$) were seeded on top of the collagen matrix. After a further 24 h, a separating layer of fibroblasts in collagen gel (100 $\mu$l, 500 $\mu$m) was added on top of the CC cells. Fibroblast–collagen overlay containing CTL was prepared by mixing $2.5 \times 10^4$ fibroblasts FCFB/1 and $3 \times 10^5$ CTL with 250 $\mu$l collagen matrix per well. In some cultures CC cells were stained with CellTracker Blue CMAC (Invitrogen, Carlsbad, CA, USA) and CTL were pre-stained with CFDA-Green (Invitrogen, Carlsbad, CA, USA). For control reconstruct, PHA blasts prepared from the PBMC of the same patient by incubating them with 5 $\mu$g/ml of PHA were used. Reconstructs were incubated in medium (50% DMEM, 50% CC medium supplemented with 2% human AB serum). Three or 4 days after the addition of T cells,

reconstructs 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 (based on nuclear morphology) and intact tumor cells in sections stained with H&E.

This method has been superior to other methods used for staining of apoptotic cells, such as caspase-3, TUNEL, and cytokeratin staining [11].

### Blocking of T-cell migration in reconstruct

The bottom layer of reconstruct contained $4.5 \times 10^4$ fibroblasts in 450 µl type I collagen gel. After 24 h, WC020 CC cells ($1 \times 10^5$) 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 CC cells. After gel formation, anti-chemokine or chemokine receptor Abs, anti-CXCL11, anti-CXCR3, anti-CXCR4, anti-CCR7, and anti-CCR9 Ab (10 µg/ml each Ab), or isotype-matched control Ab were added, followed by the top layer containing CTL ($3 \times 10^5$) mixed with $2.5 \times 10^4$ fibroblasts and type I collagen gel. To evaluate whether excess CXCL11 can block migration of T cells, the chemokine (50 ng/ml) was added into the medium on top of the T-cell layer. The percentage of apoptotic tumor cells in the presence and absence of inhibitor was determined and the percentage of inhibition of apoptosis by Abs or chemokines was calculated.

### T helper assay

Helper activity of T cells was determined by measuring proliferative response of allogeneic PBMC in the presence of T cells. T cells ($1 \times 10^5$/ml) were added to the top chamber of Transwell plates (Costar No. 3413; 0.4 µm pore size), and allogeneic PBMC ($1 \times 10^5$/ml) to the bottom chamber. As negative control, medium or an EBV-transformed B cell line (EBVB013) was added to the top chamber. After 4 days of culture the top chamber was removed, and proliferative responses of the allogeneic PBMC in the bottom chamber were determined by $^3$H-thymidine (Amersham, Buckinghamshire, UK) incorporation assay. All determinations were performed in triplicate.

### Statistical analyses

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

## Results

### Functional characteristics of CTL020 cells in MLTC

The phenotype of the CTL020 cell line changed from 66.8% CD4$^+$ and 49% CD8$^+$ at week 7, to 88.7% CD4$^+$ and 5.8% CD8$^+$ at week 12, relative to the start of PBMC and CC tumor cell co-culture (data not shown). CTL020 cells lysed autologous WC020 CC cells in a 10-h $^{51}$Cr-release assay and lysis was dependent on the E:T cell ratio. NK target cells K562 and LAK target cells Daudi were not significantly lysed in the same assay (Fig. 1a). Lysis of CC cells by the CTL was significantly inhibited by mAbs to HLA class I as well as HLA class II, while isotype-matched control Ab had no effect on cytotoxicity (Table 2). The demonstration of dual HLA restriction of the CTL is not surprising since uncloned CTL020 cells presumably comprise cells with different specificities. Thus, the uncloned CTL020 cell population
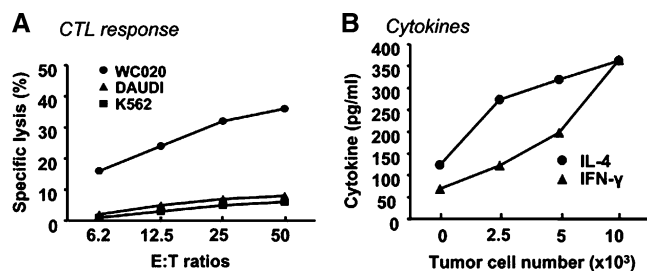


**Fig. 1** Functions of CTL020 cells in MLTC. CTL020 was generated in MLTC by stimulating PBMC of CC patient 020 with irradiated autologous WC020 CC cells. Cultures were restimulated every 2 weeks with irradiated autologous CC cells and 20 U/ml of natural IL-2. **a** CTL responses. CTL020 cells were stimulated for 4 days with irradiated autologous tumor cells and IL-2. CTL lysis of $^{51}$Cr-labeled autologous CC cells and control target cells [allogeneic Daudi (LAK target) and K562 (NK target)] was determined in 10 h $^{51}$Cr-release assay at various E:T ratios. **b** Cytokine production. CTL020 cells were incubated with various numbers of irradiated WC020 CC cells or without tumor cells in the presence of recombinant IL-2. IFN-$\gamma$ and IL-4 in supernatants obtained from cultured CTL after 2 and 4 days, respectively, were measured by ELISA. All values obtained from cultures stimulated with tumor cells are significantly ($P$ 0.01) different from controls (CTL020 cells incubated without tumor cells)

**Table 2** HLA restriction of CTL020

| Antibody treatment | % Specific lysis[a] | % Inhibition | Significance ($P$ value) |
|---|---|---|---|
| Isotype-matched control | 12.3 | – | |
| Anti-HLA class I | 3.7 | 69.7 | 0.003 |
| Anti-HLA class II | 6.9 | 44 | 0.028 |

[a]E:T = 12.5

most likely contains CD8$^+$ HLA class I-restricted, and CD4$^+$, HLA class II-restricted CTL. IFN-$\gamma$, and IL-4 production by CTL020 cells is dependent on the presence of tumor cells (Fig. 1b). CTL020 cells do not secrete measurable amounts of GM-CSF, or TNF-$\alpha$. The T cells did not display helper activity in an assay which measures proliferation of allogeneic PBMC in the presence of the T cells (not shown).

Functional characteristics of CTL020 cells in reconstruct

CTL020 cells labeled with CFDA-Green migrated from the top layer of collagen and fibroblasts through a separating layer of collagen and fibroblasts toward WC020 tumor cells labeled with CMAC-Blue (Fig. 2-A, Ba), whereas no T cells were found in cultures with tumor cells only (Fig. 2Bb). Tumor cell apoptosis was determined microscopically in H&E-stained cultures (Fig. 2C) and by enumerating apoptotic tumor cells (Fig. 2D). The CTL-induced significant apoptosis in the autologous CC cells, as compared to reconstructs with tumor cells alone or tumor cells plus PHA blasts (P 0.0001, Fig. 2D). Although a small proportion of PHA blasts migrated toward tumor cells (not shown), the PHA blasts did not induce apoptosis in the tumor cells (Fig. 2C, D). CTL020 cells did not mediate significant apoptosis of allogeneic HLA-mismatched 05193 or 281638 CC cell lines (Table 3).

Phenotypic characteristics of CTL020 and WC020 CC cells

CTL020 and WC020 CC 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 4). CTL020 cell line is predominantly of CD4 phenotype (88.7% of cells positive). The cells also express CD95 (FAS) as well as adhesion and co-stimulatory molecules (Table 4). Expression of $\alpha$2 (CD49b) and $\beta$1 (CD29) integrins by the T cells (Table 4) is important for T-cell interaction with collagen in the reconstruct, which results in T-cell activation [14, 37]. LFA-1a (CD11a), ICAM-1 (CD54), and CD44 expressed by the CTL (Table 4) facilitate interaction of the lymphocytes with fibroblasts in the reconstruct [1, 32]. This interaction results in the activation of both lymphocytes and fibroblasts through secretion of growth and survival factors, cytokines, and fibronectin [1, 10, 28, 32].

WC020 CC cells expressed both HLA class I and II molecules, FAS, ICAM-1, and various integrins. Expression of $\alpha$2 and $\beta$1 integrins by the CC cells
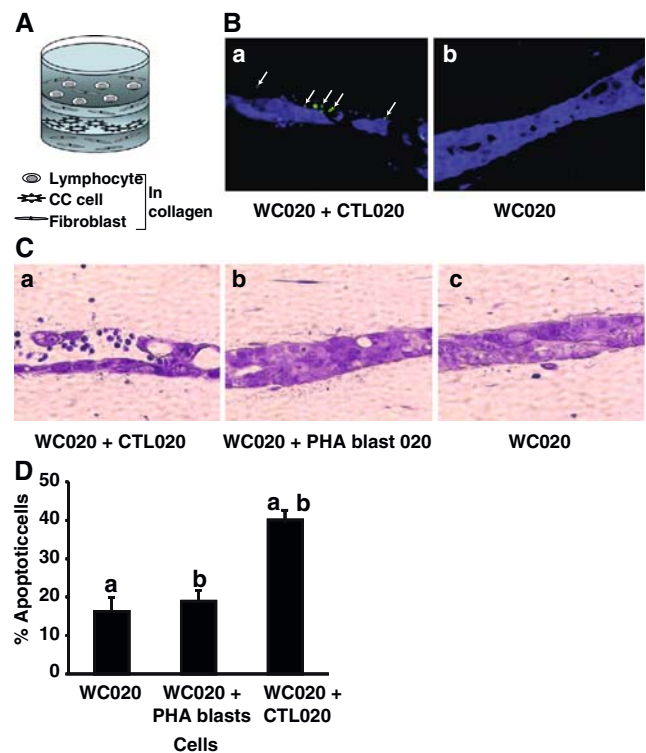


Fig. 2 CTL020 cell migration toward autologous WC020 CC cells in reconstruct. A Schema of reconstruct. B CTL020 cell migration. The bottom layer of reconstructs contained $4.5 \times 10^4$ fibroblasts in 450 µl type I collagen gel. After 24 h, CC cells ($1 \times 10^5$) were seeded on top of the bottom layer. After 24 h, CC cells were stained with CellTracker Blue CMAC. A separating layer of fibroblasts in collagen gel (500 µm) was then placed on top of the CC cell layer, followed by addition of a top layer containing $3 \times 10^5$ pre-stained (CFDA-Green, Invitrogen) CTL020, mixed with $2.5 \times 10^4$ fibroblasts and type I collagen gel (a); control cultures were prepared without lymphocytes (b). Reconstructs were harvested on day 6 (3 days after adding T cells), fixed in buffered formalin, and embedded in paraffin. Sections were photographed in the Nikon fluorescence microscope using appropriate filters. Magnification ×400 . Note the presence of CFDA-Green-labeled CTL in the tumor cell layer (a). C Induction of apoptosis in WC020 cells by CTL020—qualitative analysis. The reconstructs were harvested on day 7, fixed in buffered formalin, embedded in paraffin, and stained with H&E. Magnification ×400 . Note the presence of apoptotic tumor cells in cultures with WC020 plus CTL020 cells (a), but not in control cultures with WC020 plus PHA blasts (b), or WC020 only (c). D Induction of apoptosis in WC020 cells by CTL020—quantitative analysis. The percentage of apoptotic tumor cells was determined by counting apoptotic nuclei, based on nuclear morphology, and intact tumor cells in 20 fields of sections stained with H&E. Data represent means + SD of 20 fields. Values with the *same symbol* are significantly different from each other (P 0.0001)

indicates that these cells potentially can bind to collagen [27]. The cells did not express FAS ligand and B7-1 (Table 2). ICAM-1 on the CC cells potentially interacts with LFA-1a on the CTL, which may result in T-cell stimulation [3].

**Table 3** Apotosis induction by CTL020 cells in reconstructs with allogeneic CRC cells

| Tumor cells | | Apoptotic tumor cells (%) | |
|---|---|---|---|
| Code | Production of chemokines binding to CXCR3 | CTL020 | No CTL |
| 281638 | CXCL11 | 10.1 ± 2.7 | 10.0 ± 5 |
| 05193 | CXCL9 | 6.09 ± 3 | 5.6 ± 3 |
| WC020 | CXCL11 | 25.2 ± 5[a] | 10.5 ± 2.1[a] |

[a] Significant difference ($P$ 0.001)

**Table 4** Phenotypic and functional markers of anti-CC CTL020 cells and autologous WC020 tumor cell line

| Parameter investigated[a] | Cell lines (% cells positive) | |
|---|---|---|
| | CC WC020 | CTL020 |
| HLA class I | 99.6 | 99.5 |
| HLA class II | 51.7 | 92.5 |
| CD4 | NA | 88.7 |
| CD8 | NA | 5.8 |
| CD25 | 1.1 | 33.6 |
| CD40 | 0.3 | 0.3 |
| CD40L | 3.2 | 6.9 |
| CD44 | 94.9 | 66.7 |
| CD80 (B7-1) | 1.2 | 52.8 |
| CD49a?? ($\alpha$1 ?integrin) | 57.8 | 22.9 |
| CD49b?? ($\alpha$2 ?integrin) | 93.1 | 70.7 |
| CD29?? ($\beta$1 ?integrin) | 83.8 | 96.6 |
| CD95 (FAS) | 78.0 | 90.2 |
| CD95L (FASL) | 1.4 | 1.6 |
| CD54 (ICAM-1) | 20.0 | 71.5 |
| CD11a (LFA-1a) | 1.5 | 91.8 |

NA not applicable

[a] All markers were determined by FACS analysis

Thus, several phenotypic markers are expressed by CTL020 and WC020 CC cells, which may facilitate interactions between these cells and between the lymphocytes or tumor cells and collagen or fibroblasts in the reconstruct, leading to activation of T cells as well as T-cell migration toward tumor cells.

## Chemokine and chemokine receptor involved in CTL020 cell migration toward WC020 cells

CTL020 cells expressed the chemokine receptors CCR7, CCR9, CXCR3, and CXCR4 (Table 3). For each chemokine receptor, with the exception of CXCR4, the corresponding chemokine(s) was expressed by WC020 CC cells, as determined by RT-PCR (Table 5). However, chemokine protein expression by the tumor cells could only be shown for CXCL11 (Fig. 3a). No significant amounts of CXCL11 were produced by FCFB/1 fibroblasts used in the reconstruct

**Table 5** Chemokine receptors expressed by CTL020 cells, and chemokines produced by WC020 CC cells

| Chemokine receptors expressed by CTL020[a] | Chemokines | |
|---|---|---|
| | Known to bind to receptor | Expressed by WC020[b] |
| CCR7 | CCL21 | +/– |
| | CCL19 | – |
| CCR9 | CCL25 | +/– |
| CXCR3 | CXCL9 | +/– |
| | CXCL10 | – |
| | CXCL11 | + |
| | CXCL13 | – |
| CXCR4 | CXCL12 | – |

[a] The following receptors were not expressed by CTL020: CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR10, CCR11, CXCR1, CXCR2, CXCR5, CXCR6, CX3CR1

[b] Chemokine expression was detected by RT-PCR. CXCL11 protein was detected in WC020 supernatant by ELISA (see Fig. 3a). CCL21, CCL25, and CXCL9 mRNA were detected in WC020 cells by RT-PCR, but the protein was not expressed as determined by ELISA

or CTL020 cells (Fig. 3a). CXCL11 produced by WC020 CC cells most likely binds to CXCR3 on CTL020 cells [9]. CXCR3, while significantly expressed by CTL020 cells (48.7% of cells positive), showed no
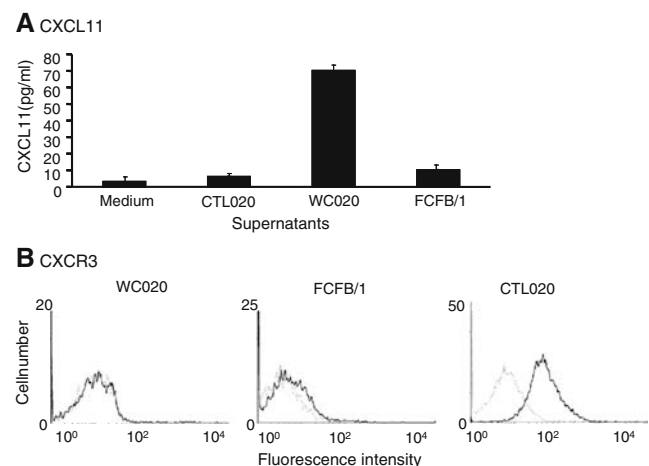


**Fig. 3** Autologous CC cells WC020 produce CXCL11, and CTL020 cells express the corresponding receptor CXCR3. **a** Production of CXCL11. CTL020 cells were cultured with irradiated WC020 tumor cells in T-cell medium for 4 days. WC020 CC cells were cultured in CC medium; after 2 days of culture the medium was replaced and supernatants were collected 4 days later. FCFB/1 fibroblast cells were cultured in DMEM for 4 days. CXCL11 production in supernatants obtained from various cells was measured in ELISA. **b** Expression of CXCR3. Cultured cells of CTL020, FCFB/1, and WC020 were incubated with saturating concentration (5 μg/ml) of anti-CXCR3 MAB (*solid line*) or mouse IgG control (*dotted line*) in RPMI-1640 with 5% FBS for 1 h at 4°C. After washing, fluoresceinated goat anti-mouse IgG Ab was added. The expression of CXCR3 was detected by flow cytometry

significant expression by WC020 CC cells or FCFB/1 fibroblasts (Fig. 3b).

We evaluated a possible role of CXCL11 produced by WC020 cells and CXCR3 present on CTL020 cells in the migration of the T cells toward CC 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, since T cells may themselves apoptose after inducing tumor cell apoptosis and one T cell may induce apoptosis in several tumor cells. Only apoptotic tumor cells, and not T cells, were counted. Apoptotic tumor cells could be easily distinguished from apoptotic T cells based on size difference. However, evaluation of apoptotic tumor cells does not allow us to distinguish between T cells with high migratory and low lytic activity and T cells with low migratory and high lytic activity. Blocking of CXCR3, but not CCR7, CCR9, or CXCR4 on CTL020 cells with antibodies significantly inhibited tumor cell apoptosis (Table 6). We therefore tested whether the ligand of CXCR3 expressed by WC020 tumor cells (CXCL11) was involved in T-cell migration and tumor cell apoptosis. Excess recombinant CXCL11 (50 ng/ml), when added on top of the T-cell layer, significantly ($P$ 0.01) inhibited CC cell apoptosis induction by CTL020 cells (Table 6). Anti-CXCL11 Ab when added on top of the separating layer, significantly ($P$ 0.01) inhibited tumor cell apoptosis (Table 6). In all migration blocking experiments shown in Table 6, blocking of tumor cell apoptosis was incomplete, suggesting that factors other than CXCL11 might attract CTL to the tumor site. Alternatively, Abs used for CTL migration blocking may have low affinity. CTL020 cells did not induce apoptosis of allogeneic HLA-mismatched 05193 or 281638 CC cells producing ligands of CXCR3 (CXCL9 or CXCL11, respectively) (Table 3).

FCFB/1 fibroblasts used in the reconstruct did not produce CXCL11 (Fig. 3) as detected by ELISA, and therefore they are not expected to contribute to T-cell migration. However, CXCL12 (the ligand for CXCR4 which is expressed by CTL020), was produced by the fibroblasts (1,991 pg/ml in culture supernatants). 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. Furthermore, anti-CXCR4 Ab did not inhibit tumor cell apoptosis (Table 6). These data suggest that CXCL12 produced by fibroblasts had no effect on T-cell migration.

## CXCR3 and CXCL11 expression by T cells and tumor cell lines, respectively, derived from additional CC patients

Since CXCR3 and CXCL11 mediated CTL020 cell migration toward tumor cells followed by tumor cell apoptosis (see above), we investigated the distribution of CXCR3 and CXCL11 in T cells and tumor cell lines, respectively, recently established from specimens of additional CC patients. Expression of the receptor and its ligand by the cells of additional CC patients would suggest that involvement of the receptor/ligand in T-cell migration toward tumor cells may not be a unique observation made in a single CC patient, but may be found in a larger population of these patients. Tumor-reactive T-cell lines derived from TIL of ten additional

**Table 6** Blocking of CTL020 cell migration in reconstruct

| Treatment[a] | Total number of tumor cells mean ± SD/field (10 fields) | Number of apoptotic tumor cells mean ± SD/field (10 fields) | Percentage of apoptotic tumor cells mean ± SD/field (10 fields) | Percentage of tumor cell apoptosis inhibition |
|---|---|---|---|---|
| WC020 | 69.9 ± 15.8 | 11.1 ± 3.4 | 15.8 ± 2.5 | NA |
| WC020 + CTL020 | 58.9 ± 7.5 | 23.9 ± 4.2 | 40.8 ± 6.9[b] | 0.0 |
| WC020 + CTL020 + control IgG | 49.3 ± 9.1 | 18.5 ± 4.0 | 38.9 ± 6.9[c,d] | 4.7 |
| WC020 + CTL020 + α-CXCR3 Ab | 72.7 ± 7.0 | 18.0 ± 4.3 | 24.9 ± 6.2[c] | 39.0 |
| WC020 + CTL020 + α-CCR7 Ab | 59.7 ± 8.2 | 24.0 ± 5.2 | 39.8 ± 4.6 | 2.5 |
| WC020 + CTL020 + α-CCR9 Ab | 59.9 ± 11.1 | 21.7 ± 4.8 | 36.4 ± 6 | 10.8 |
| WC020 + CTL020 + α-CXCR4 Ab | 60.4 ± 5.1 | 23.1 ± 5.2 | 38.7 ± 8.7 | 5.2 |
| WC020 + CTL020 + CXCL11 | 69.4 ± 6.8 | 20.1 ± 2.5 | 29.1 ± 4.8[b] | 28.7 |
| WC020 + CTL020 + α-CXCL11 Ab | 74.8 ± 6.0 | 17.4 ± 5.7 | 23.4 ± 8.3[d] | 42.7 |

*Ab* antibody, *NA* not applicable

[a] 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, followed by a top layer containing CTL mixed with fibroblasts and collagen (E:T = 3:1). Separate cultures received CXCL11 or CXCL9 (50 ng/ml) on top of the T-cell layer. Percentage of apoptotic tumor cells in 6-day cultures was determined

[b–d] Values with same letter differ significantly from each other ($P$ 0.01, Student's two-sample $t$-test)

CC patients all expressed CXCR3 (Table 1). Furthermore, two of the three established CC cell lines produced CXCL11 (not shown).

## Discussion

We have described here a CTL line (CTL020) that lysed autologous WC020 CC cells in culture. Only a few examples of CC-specific CTL have been described in cancer patients [17, 23, 42].

We have shown here that CTL020 cells 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 CXCL11 produced by the tumor cells and CXCR3 expressed by the T cells. To our knowledge, this is the first demonstration of chemokine dependency of human CTL migration toward CC cells. In addition to CXCL11 (this study), CC cells can produce CXCL8 [7], CXCL1, CXCL3, CCL2, CCL5 [51], CXCL9, and CXCL10 [20].

We have developed 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 physiological conditions [52]. In the reconstruct, human CC 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 α2 (CD49b) and β1 (CD29) integrins on T cells with collagen [14, 37]. Since 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-1a (CD11a), ICAM-1 (CD54), and CD44. The adhesive interaction stimulates fibroblasts to secrete inflammatory cytokines such as IL-1, IL-6 and IL-7 [1, 32], and fibronectin [28]. The main biological role of IL-1 is the stimulation of T cells to express IL-2 receptor and secrete IL-2 [44]. IL-6 and IL-7 are T-cell survival factors [38], and fibronectin stimulates predominantly resting lymphocytes [28].

Other investigators have used collagen matrices to study interaction of leukocytes with tumor cells, but they have not demonstrated CTL migration resulting in tumor cell apoptosis in a culture system similar to the reconstruct shown here. Thus, Wei et al. [49] 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 papilloma virus-transformed keratinocytes, allogeneic lymphocytes induced apoptosis in the tumor cells [24]. 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 containing human DCs as the lower layer and a collagen 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 [46].

In the present study, CXCL11 produced by CC cells attracted CTL through binding to CXCR3 on the T cells. This was demonstrated by blocking CTL migration toward tumor cells with high concentrations of CXCL11, applied on top of the T-cell layer, or antibodies to CXCR3 or CXCL11, each applied on top of the separating collagen/fibroblast layer. Each of the compounds inhibited CTL migration, most likely by blocking CXCR3 on the CTL.

CXCL11 binds to the receptor CXCR3 as does CXCL9, CXCL10, and CXCL13 [9, 25, 26]. Of the four CXCR3 ligands, three (CXCL9, CXCL10, and CXCL11) show close similarities in structure and function [34], are induced primarily by IFN-γ, and are produced by macrophages and some other cell types [9]. However, CXCL11 shows higher affinity for CXCR3 than either CXCL9 or CXCL10, and consequently is a more potent activator of the receptor [8].

CXCL11 mRNA is constitutively expressed by astrocytes, monocytes, SV-A3 microglial cell line, and in the pancreas, lung, and liver. Stimulation of these cells with IFN-γ results in increased CXCL11 production [9]. CXCL11 expression (along with CXCL9 and CXCL10) was demonstrated at inflammatory sites in human bronchoalveolar lavage fluid of patients with peribronchial inflammation after lung transplantation; keratinocytes in a variety of skin disorders, including allergic contact dermatitis and mycosis fungoides; and human atheroma-associated endothelial cells and macrophages [2, 12, 29, 47]. CXCL11 efficiently attracted activated T cells, NK cells, and a subset of B lymphocytes in in vitro chemotaxis assays [9, 19, 39]. CXCL11 is displayed on the surface of human endothelial cells, and was a potent inducer of transendothelial migration of activated T cells in an in vitro Transwell plate assay [40]. CXCL11 also induced migration of T lymphoblasts and spleen T cells in an in vivo rat model and had anti-tumor activity in a mouse model [21, 43]. In addition to its chemotactic effects, this chemokine stimulated proliferation of mouse CD4 T lymphocytes in a mixed leukocyte reaction assay [50].

In agreement with the above studies, high levels of the cognate CXCR3 receptor expression were found on

T cells activated by anti-CD3 Ab and IL-2; a proportion of circulating blood T cells, B cells, and NK cells from healthy individuals also expressed the receptor [26, 36]. Virtually all T cells within inflamed tissues expressed CXCR3, whereas far fewer T cells within normal lymph nodes were positive for the receptor [36]. CXCR3 expression by melanoma patients' lymphocytes was associated with enhanced patient survival, suggesting that CXCR3 is of central importance in maintaining effective anti-tumor immunity [31]. CXCR3 is also expressed by a small percentage of endothelial cells in human normal and pathological tissues, and CXCL11 blocks endothelial cell proliferation in vitro [45]. Thus, CXCL11 may inhibit tumor growth by attenuation of tumor angiogenesis.

In the present study, a new role for CXCL11 in human CTL migration toward tumor cells, which is followed by tumor cell apoptosis, has been established. The two functions of CXCL11, inhibition of angiogenesis [45] and attraction of tumor-specific CTL (this study) may act synergistically and lead to tumor regression.

Although a role of CXCL11 and CXCR3 in T-cell migration toward CC cells has been established in a single patient, this chemokine and its receptor may be involved in the migration of other patients' T cells. CC-reactive T cells from two additional CC patients expressed CXCR3 and the autologous tumor cells produced CXCL11. In addition, our studies suggest the potential usefulness of CXCL11 in immunotherapy of patients with CXCR3-expressing T lymphocytes, since T-cell lines of ten out of ten CC patients expressed CXCR3.

CXCL11 may be useful for immunotherapy of cancer patients. Thus, patients may be vaccinated with CXCL11-transduced tumor cells (analogous to CCL3-transduced tumor cells used as effective vaccines in mice [54]) or tumor-associated Ags fused to chemokines [5]; alternatively, patients may be treated with anti-tumor Ab/chemokine fusion protein, which will attract lymphocytes to the tumor area [4, 5, 22].

In addition to therapeutic implications, the results of our study have prognostic potential. Infiltration of CC with T lymphocytes is correlated with a favorable prognosis [18], and CXCR3 expression by T lymphocytes as well as CXCL11 expression by tumor cells should be explored for their possible association with prognosis. Thus, the expression of CXCR3 on CD8$^+$ T cells correlated with statistically significant survival advantage in melanoma patients with stage III disease [31], suggesting that expression of this receptor may serve as a biomarker of potential clinical responses to immunotherapy.

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