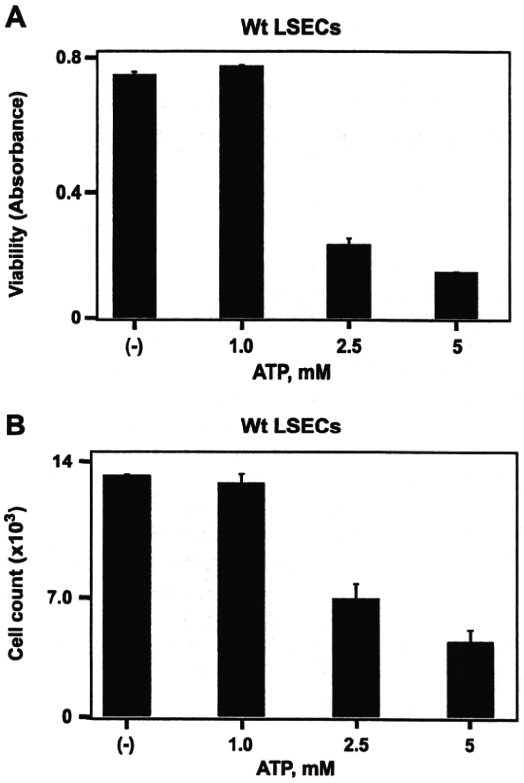

Figure W3. Effects of extracellular ATP on growth of wt LSECs. Extracellular ATP exhibited inhibitory effects on wt LSECs in a dose-dependent manner. Freshly purified wt LSECs were cultured for 24 hours before being exposed to ATP at the indicated concentrations for additional 16 hours. (A) Cell viability was evaluated using Cell Counting Kit-8. (B) Cells were counted using the Celigo Cell Counting application. Columns indicate mean of triplicate determinations; bars, SD.

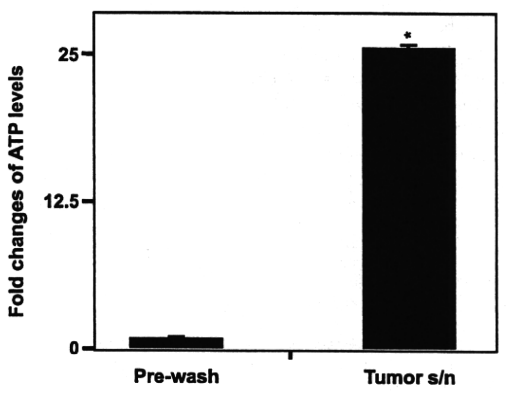

Figure W4. Release of ATP by damaged tumor cells. Extracellular ATP levels in prewash media (Pre-wash) and tumor supernatants (Tumor s/n) were measured using the ATP Colorimetric/Fluorometric Assay Kit (BioVision) and expressed as fold changes to the prewash medium. Columns indicate mean of triplicate determinations; bars, SD. $*P = 3.6e - 08$.

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Cancer Research

CCL11–CCR3 Interactions Promote Survival of Anaplastic Large Cell Lymphoma Cells via ERK1/2 Activation

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Abstract

CCR3 is a specific marker of anaplastic large cell lymphoma (ALCL) cells. ALCL cells also express CCL11, a ligand for CCR3, leading to the hypothesis that CCL11 may play an autocrine role in ALCL progression. In this study, we investigated a role of CCL11 in cell survival and growth of human Ki-JK cells, established from an ALCL patient, and murine EL-4 lymphoma cells. Both Ki-JK and EL-4 cells expressed cell surface CCR3. CCL11 increased cell survival rates of Ki-JK cells in a dose-dependent manner, whereas it promoted EL-4 cell proliferation. Furthermore, CCL11 induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in both Ki-JK cells and EL-4 cells. Cell survival and tumor proliferation promoted by CCL11 was completely blocked by inhibition of ERK phosphorylation. CCL11 induced expression of antiapoptotic proteins, Bcl-xL and survivin, in Ki-JK cells. CCL11 also enhanced tumor growth of EL-4 and Ki-JK cells *in vivo*. Consistent with these results, tumor cells of cutaneous ALCL expressed CCR3 and increased levels of phosphorylated ERK1/2, Bcl-xL, and survivin *in situ*. Thus, our findings prompt a novel therapeutic approach to treat relapses of an aggressive form of lymphoma based on the discovery that a cell surface marker of disease functions as a critical autocrine growth receptor. *Cancer Res; 71(6); 2056–65. ©2011 AACR.*

Introduction

Anaplastic large cell lymphoma (ALCL) is composed of anaplastic large cells expressing the CD30 antigen (1). Extranodal involvement is common and skin is the most frequently affected organ. In some cases with systemic ALCL, tumor cells express anaplastic lymphoma kinase (ALK), indicative of the 2;5 chromosomal translocation or its variants (1). Extracellular signal-regulated kinase (ERK) is one of the downstream mediators of nucleophosmin/ALK signaling in ALK^+ ALCL, which may be involved in the pathogenesis (2). It remains, however, unknown how tumor cells get activated and proliferate in cases with ALK^- ALCL including cutaneous ALCL.

Multiple factors contribute to the progression of neoplasms. Among them, chemokines can directly promote tumor cell growth (3–5). Chemokines are classified into 4 types: CC chemokines, CXC chemokines, C chemokine, and CX3C chemokine (6). There are emerging data suggesting that signaling through chemokine/chemokine receptor interactions is implicated with tumor growth and invasion of several cancer types, including lymphomas, in an autocrine/paracrine manner. For examples, interactions between CCL1/ CCR8 are involved in adult T-cell leukemia (7), CXCL13/CXCR5 in several mouse and human carcinoma cell lines (8), CCL20/CCR6 in colorectal cancer cells (9), CXCL1 and CXCL2/CXCR2 in esophageal cancers (10), and CXCL10/CXCR3 in nasal NK/T cell lymphoma (11). In most cases of ALCL, tumor cells express CCR3 (12, 13). ALCL cells also express CCL11, a ligand for CCR3 (12), leading to the hypothesis that CCL11 may play an autocrine role in ALCL pathogenesis (14).

CCL11 has potent chemotactic activity for CCR3-positive cells (15–17). In cutaneous tissue components, CCL11 is produced by fibroblasts, endothelial cells, and dendritic cells (18–20). Furthermore, CCL11 production is promoted in a variety of inflammatory diseases such as allergic asthma (21, 22), allergic rhinitis (23), and atopic dermatitis (24, 25).

In this current report, we demonstrate that CCL11–CCR3 interactions induce cell survival and proliferation of $CCR3^+$ lymphoma cells. Mitogen-activated protein kinase (MAPK)-ERK kinase (MEK) inhibitors completely blocked the cell survival and growth stimulatory effects of CCL11. CCL11 also promoted tumor growth of $CCR3^+$ lymphoma cells *in vivo*. Therefore, we propose that constitutive MAPK/ERK activation through the CCL11/CCR3 axis may be critical for the progression of ALCL.

Methods

Mice

C57BL/6 mice were purchased from SLC Japan (Hamamatsu) and C.B-17/Icr-scid/scidJcl (severe combined immunodeficient mice, SCID) mice were from CLEA Japan (Meguro). They were free of pathogenic bacteria and viruses.

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All experiments were performed using female mice between 8 and 14 weeks of age. All studies were approved by the animal ethics review board of the University of Tokyo and the Animal Committee of National Center for Global Health and Medicine.

Cell lines

Human ALCL cell line, Ki-JK, was purchased from the Health Science Research Resources Bank (Sennan). Ki-JK cells were established from a child with ALCL in the inguinal lesion, including skin, and they expressed CD25, CD30, CD45RO, HLA-DR, and epithelial membrane antigen (26). They were characterized by PCR assay using short tandem repeat. Mouse lymphoma cell line, EL-4, and mouse malignant melanoma cell line, B16, were kindly given by Dr Sam T. Hwang (Medical College of Wisconsin, Milwaukee, WI). Ki-JK cells were grown in RPMI 1640 (Millipore) with 10% FBS and supplements (penicillin G sodium, streptomycin sulphate, and amphotericin B). EL-4 and B16 cells were grown in Eagle's minimum essential medium (MEM; Sigma) with 10% FBS and supplements.

Real-time quantitative RT-PCR assay

Messenger RNA (mRNA) was obtained from lesional skin of patients with cutaneous ALCL ($n = 5$) and normal skin ($n = 5$) using illustra QuickPrep Micro mRNA Purification Kit (GE Healthcare). All samples were collected during daily clinical practice. The medical ethical committee of the University of Tokyo approved all described studies and the study was conducted according to the Declaration of Helsinki Principles. Informed consent was obtained to use skin samples. We also obtained mRNA from mouse skin before and 11 days after injection with EL-4 cells. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative reverse-transcriptase (RT)-PCR was performed as described previously (27). Primers for human genes were as follows: CCR3 forward, 5′-TCG TTC TCC CTC TGC TCG TT-3′ and reverse, 5′-GCC GGA TGG CCT TGT ACT TT-3′; CCL11 forward, 5′-GGG CCA GCT TCT GTC CCA AC-3′ and reverse, 5′-TTA TGG CTT TGG AGT TGG AGA TTT-3′; and GAPDH forward, 5′-ACC CAC TCC TCC ACC TTT GA-3′ and reverse, 5′-CAT ACC AGG AAA TGA GCT TGA CAA-3′. Primers for mouse genes were as follows: CCL11 forward, 5′-CAG ATG CAC CCT GAA AGC CAT A-3′ and reverse, 5′-TGC TTT GTG GCA TCC TGG AC-3′; and GAPDH forward, 5′-CGT GTT CCT ACC CCC AAT GT-3′ and reverse, 5′-TGT CAT CAT ACT TGG CAG GTT TCT-3′.

Flow cytometric analysis of CCR3 expression on cell lines

Cells (2×10^6) were stained with rat anti-human CCR3 or rat anti-mouse CCR3 monoclonal antibody (mAb; R&D systems) followed by fluorescein-conjugated secondary Ab (BD Biosciences Pharmingen). FACScan flow cytometer and CellQuest software (Becton Dickinson) were used. Mean fluorescence intensity (MFI) and percentages of cells in each quadrant were determined.

Apoptosis assays

Cells were plated onto 6-well plates at 1×10^5 per well. Human or mouse CCL11 (R&D systems) was added to final concentration of 0.01 to 1.0 μg/mL and cells were cultured as indicated (Ki-JK for 16 hours, EL-4 for 72 hours, and B16 for 48 hours) after preincubation with or without 10 μmol/L PD98059, U0126, or LY294002 (Cell Signaling Technology) for 1 hour. Apoptosis was analyzed using Annexin V FITC (fluorescein isothiocyanate) Apoptosis Detection Kit I (BD Biosciences Pharmingen) according to the manufacturer's instructions.

Proliferation assays by cell count

Cells were plated onto 6-well plates at 1×10^5 per well. Human or mouse CCL11 was added to final concentration of 0.1 to 1.0 μg/mL and cells were cultured as indicated (Ki-JK for 16 hours, EL-4 for 48 hours, and B16 for 24 hours) after preincubation with or without 10 μmol/L U0126 or LY294002 for 1 hour. Cells were counted using the Coulter Counter (Beckman Coulter).

BrdU cell proliferation assay

EL-4 cells (5×10^4) were cultured for 18 hours with mouse CCL11 at 0.1 to 1.0 μg/mL. Cells were stained with bromodeoxyuridine (BrdU) for 2 hours and reacted with anti-BrdU Ab peroxidase conjugate followed by peroxidase substrate using Cell Proliferation ELISA, BrdU (Roche Applied Science). Sulfuric acid was added to the solution to terminate enzyme activity. Optical densities were measured at 450 nm using a 550 microplate reader (Bio-Rad Laboratories).

Western blotting

Ki-JK and EL-4 cells were cultured in 6-well plates with human or mouse CCL11 at 1.0 μg/mL for 1 or 5 minutes or 16 hours. After collecting proteins, SDS-PAGE was performed as previously described (28). After transfer to nitrocellulose membrane (Invitrogen), the membrane was blotted with Abs against ERK1/2, phosphorylated ERK1/2 (phospho-ERK1/2), Bcl-xL, and survivin (Cell Signaling Technology) for overnight at 4°C and with the appropriate secondary Abs for 1 hour at room temperature. Visualization was performed by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Intracellular flow cytometric analysis of phosphorylated ERK1/2 and Akt

Ki-JK and EL-4 cells (1×10^5) were cultured with human or mouse CCL11 at 1.0 μg/mL as indicated (Ki-JK for 16 hours and EL-4 for 24 hours) after preincubation with or without 10 μmol/L U0126 after or LY294002 for 1 hour. After brief incubation with SYTOX Red Dead Cell Stain (Invitrogen), Ki-JK cells were permeabilized using BD Cytofix/Cytoperm Kit (BD Biosciences Pharmingen). Staining for phospho-ERK1/2 or phospho-Akt was performed with rabbit antiphosphorylated ERK1/2 monoclonal Ab or rabbit antiphosphorylated Akt monoclonal Ab, respectively (Cell Signaling Technology) followed by Alexa Fluor 488–conjugated secondary Ab (Invitrogen).

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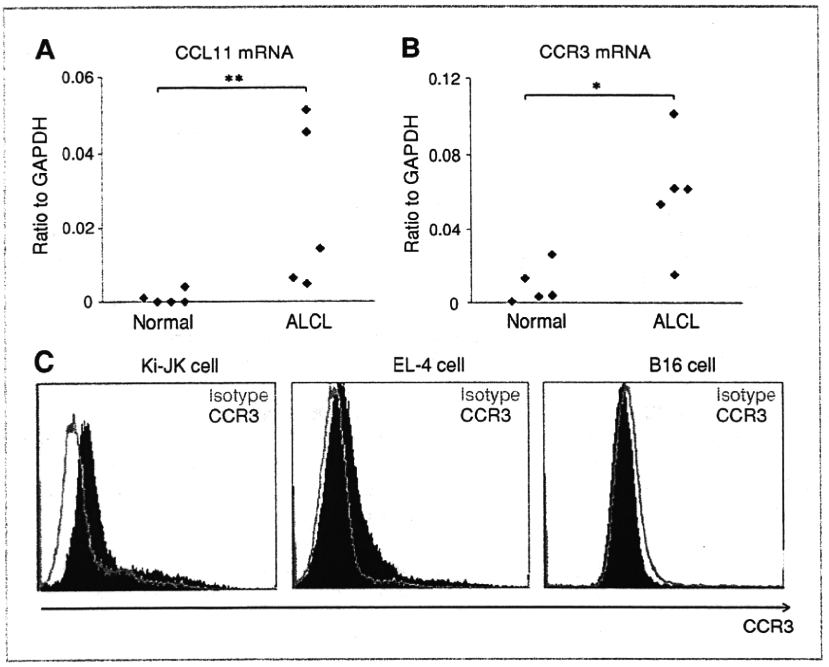

Figure 1. CCR3 expression on tumor cells of ALCL. A and B, quantitative RT-PCR was performed to measure expression levels of CCL11 and CCR3 using mRNA extracted from lesional skin of ALCL and normal skin. The measured values from individual patients were plotted by dots. *, $P < 0.05$; **, $P < 0.01$ by Mann–Whitney U test. C, CCR3 expression on Ki-JK cells (human ALCL cell line), EL-4 cells (mouse lymphoma cell line), and B16 cells (mouse malignant melanoma cell line) overlaid with isotype control (unshaded histogram).

In vivo animal experiments

EL-4 cells (5×10^6), B16 cells (2×10^6), or Ki-JK cells (5×10^6) in 100 μL of PBS with 1.0 μg/mL CCL11 (CCL11 group) or without CCL11 (PBS group) were injected subcutaneously into the shaved left flank of C57BL/6 mice or SCID mice. On days 4, 7, and 11, 1.0 μg/mL CCL11 in 100 μL of PBS was injected into the lesion in CCL11 group, whereas PBS was injected in PBS group. On days 4, 7, 11, and 14, we measured 2 perpendicular diameters and determined tumor size. In other experiments, EL-4 cells in 100 μL of PBS with 100 μmol/L U0126, 1.0 μg/mL mouse CCL11 + 100 μmol/L U0126 100 μg/ml, anti-mouse CCL11 Ab (R & D systems), and 1.0 μg/mL mouse CCL11 + 100 μg/mL anti-mouse CCL11 Ab were injected subcutaneously into the shaved left flank of C57BL/6 mice. Each reagent was injected under the same schedule as described above.

Immunohistochemistry

We performed immunohistochemical staining with lesional skin with cutaneous ALCL ($n = 8$) as previously described (29). Primary Abs against CD30, CCR3 (MBL International), phosphorylation of ERK1/2 (Santa Cruz), Bcl-xL, survivin (Cell Signaling Technology), and isotype-matched control were used.

Statistical analysis

All *in vitro* experiments were repeated at least 3 times and mean ± SD or SEM was determined. Statistical analysis between 2 groups was performed using the Welch's t test. Mann–Whitney's U test was used to determine statistical significance of quantitative real time RT-PCR differences and *in vivo* experimental differences. P values of < 0.05 were considered statistically significant.

Results

CCR3 expression on tumor cells of ALCL *in vivo* and *in vitro*

Tumor cells of cutaneous ALCL are reported to express CCL11 and CCR3 by immunohistochemistry (12). We first showed that expression levels of CCL11 and CCR3 mRNA in the lesional skin of ALCL were significantly higher than those in normal skin ($P < 0.01$ and $P < 0.05$, respectively; Fig. 1A and B). Expression levels of CCL11 and CCR3 mRNA significantly correlated with one another ($r = 1.0$, $P < 0.01$). We next investigated CCR3 expression on the cell surface of lymphoma cell lines by flow cytometry. Both Ki-JK cells and EL-4 cells expressed CCR3 on the surface, but B16 cells did not (Fig. 1C). For the following study, we decided to use Ki-JK and EL-4 cells to investigate function of CCR3 in human and mouse lymphoma cells.

CCL11 promotes cell survival of Ki-JK cells and proliferation of EL-4 cells

To assess effects of CCL11 on $CCR3^+$ cells, we stimulated Ki-JK cells, EL-4 cells, and B16 cells with CCL11 under serum deprivation conditions and examined cell survival by staining Annexin V. Annexin V-negative, propidium iodide (PI)-negative Ki-JK cells (live cells) were increased by stimulation with

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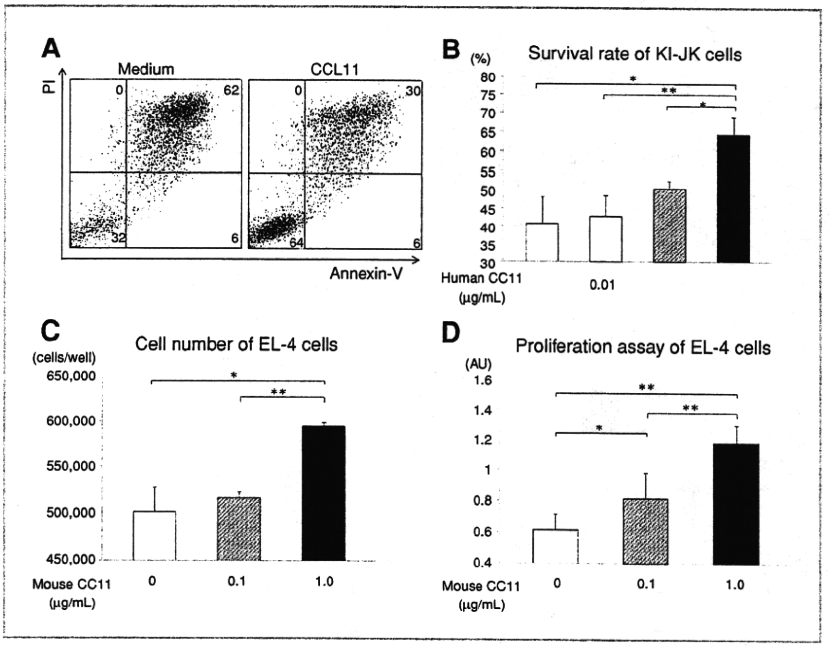

Figure 2. CCL11 promotes cell survival of Ki-JK cells and proliferation of EL-4 cells. A, Ki-JK cells were cultured with CCL11 (1.0 μg/mL) or medium only. Sixteen hours later, survival rate was analyzed by flow cytometry using Annexin V FITC Apoptosis Detection Kit. B, survival rate of Ki-JK cells cultured with CCL11 (0.01, 0.1, or 1.0 μg/mL) or medium. C, EL-4 cells were cultured with CCL11 (0.1 or 1.0 μg/mL) or medium. Forty-eight hours later, cell number in each well was counted. D, BrdU cell proliferation assay of EL-4 cells cultured with CCL11 (0.1 or 1.0 μg/mL) or medium. One representative result from 3 independent experiments with triplicates. Data are presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$ by Welch's t test (B–D).

CCL11 in a dose-dependent manner (Fig. 2A and B). CCL11, however, did not promote cell survival of EL-4 cells or B16 cells (data not shown). We further addressed if CCL11 may increase the absolute cell number. CCL11 dose dependently increased cell number of EL-4 cells (Fig. 2C). CCL11 slightly increased Ki-JK cell number (data not shown). Cell number of B16 cells was unchanged (data not shown). Moreover, CCL11 increased proliferation of EL-4 cells as shown by BrdU uptake (Fig. 2D). Thus, CCL11 promoted cell survival of $CCR3^+$ Ki-JK cells and augmented proliferation of $CCR3^+$ EL-4 cells.

CCL11 induces ERK1/2 phosphorylation in Ki-JK and EL-4 cells

CCL11 induces phosphorylation of ERK1/2 in several cell types expressing CCR3 (30–32). Thus, we then determined whether CCL11 induced phosphorylation of ERK1/2 in Ki-JK and EL-4 cells. CCL11 induced phosphorylation of ERK1/2 in Ki-JK cells (Fig. 3A). Phosphorylation of ERK1/2, which was detected in unstimulated EL-4 cells, was modestly increased by stimulation with CCL11 (Fig. 3B). Consistent with these results, we detected strong induction of ERK1/2 phosphorylation in Ki-JK cells and modest induction of ERK1/2 phosphorylation in EL-4 cells by CCL11 with flow cytometric analysis (Fig. 3C). To investigate the importance of ERK1/2 phosphorylation on Ki-JK cell survival, Ki-JK cells were stained with Dead Red in addition to intracellular phosphorylated ERK1/2. As shown in Figure 3D, most phosphorylated ERK1/2-positive Ki-JK cells cultured with CCL11 remained alive. The ratios (mean ± SD) of phosphorylated ERK1/2-positive live Ki-JK cells with or without CCL11 were 17.0% ± 4.4% and 3.3% ± 1.2%, respectively ($P < 0.05$). Thus, CCL11 induced ERK1/2 phosphorylation in Ki-JK and EL-4 cells.

MEK inhibitors suppress the cell survival and growth stimulatory effects caused by CCL11

To determine whether ERK1/2 phosphorylation is involved in CCL11-induced biological effects in $CCR3^+$ cells, Ki-JK and EL-4 cells were pretreated with a MEK inhibitor before CCL11 stimulation. We first stimulated Ki-JK cells with CCL11 after pretreatment with U0126 or DMSO (control) under serum deprivation conditions and examined survival rate and phosphorylation of ERK1/2 by intracellular flow cytometry. Pretreatment with a MEK inhibitor decreased live phosphorylated ERK1/2-positive cells (36.0% ± 5.0%–11.7% ± 4.0%), whereas it increased dead phosphorylated ERK1/2-negative cells in almost equal numbers (22.3% ± 5.1%–50.3% ± 5.9%; Fig. 4A). Using flow cytometric analysis for Annexin V staining, we confirmed that MEK inhibitors completely blocked the cell survival effects induced by CCL11 (Fig. 4B). A MEK inhibitor (U0126) was able to completely block CCL11-mediated cell growth in EL-4 cells (Fig. 4C). Thus, an MEK inhibitor completely abolished CCL11-promoted cell survival in Ki-JK cells and CCL11-augmented proliferation in EL-4 cells.

Akt pathway is not associated with the cell survival and growth effects of CCL11

Akt pathway is also important for cell survival and growth induced by chemokines. Thus, we determined to investigate Akt involvement in CCL11-mediated cell survival and growth.

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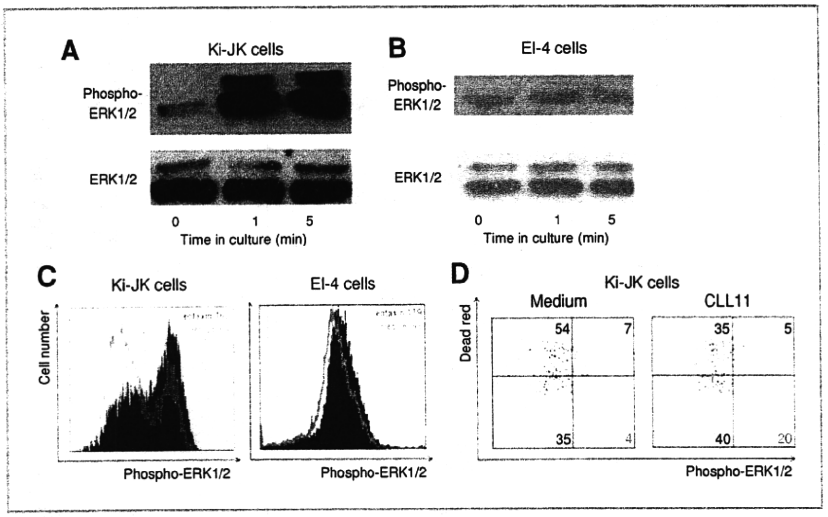

Figure 3. CCL11 induces ERK1/2 phosphorylation in Ki-JK and EL-4 cells. A, induction of ERK1/2 phosphorylation in Ki-JK cells stimulated for 1 or 5 minutes with 1.0 μg/mL CCL11 by Western blotting. B, ERK1/2 phosphorylation of EL-4 cells by Western blotting. C, induction of ERK1/2 phosphorylation in Ki-JK and EL-4 cells stimulated for 16 hours with 1.0 μg/mL CCL11 by intracellular flow cytometry, indicated with MFI number. D, Ki-JK cells were cultured with 1.0 μg/mL CCL11 or medium, and after 16 hours, ERK1/2 phosphorylation and survival rate were assessed by intracellular flow cytometry. One representative result from 3 independent experiments.

Phosphorylated Akt was detected in EL-4 cells, not in Ki-JK cells, without stimulation. We could not detect CCL11-induced phosphorylation of Akt by intracellular flow cytometry in Ki-JK cells or EL-4 cells (Fig. 5A and data not shown). We next pretreated those cells with a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) before CCL11 stimulation. In Ki-JK cells, we confirmed that PI3K inhibition did not affect CCL11-mediated cell survival (Fig. 5A and B). PI3K inhibition did not impair cell growth effects induced by CCL11 in EL-4 cells; although, total cell numbers decreased probably because

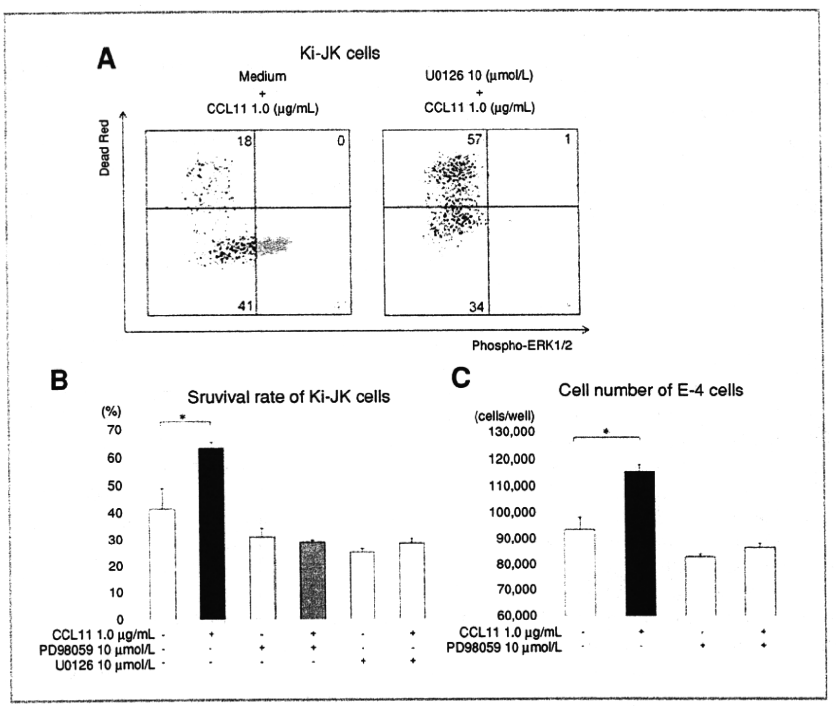

Figure 4. MEK inhibitors suppress the cell survival and growth stimulatory effects caused by CCL11. A, Ki-JK cells were precultured with 10 μmol/L U0126 or DMSO and were cultured with 1.0 μg/mL CCL11 for 16 hours. Phosphorylation of ERK1/2 and cell survival were assessed by intracellular flow cytometry. One representative result from 3 independent experiments. B, survival rate of Ki-JK cells cultured with 1.0 μg/mL CCL11 or medium for 16 hours with or without 10 μmol/L PD98059 or 10 μmol/L U0126. C, EL-4 cells were cultured with 1.0 μg/mL CCL11 or medium after preincubation with 10 μmol/L U0126 or DMSO for 48 hours. Cell number in each well was counted. One representative result from 3 independent experiments. Data are presented as mean ± SD. *, $P < 0.05$ by Welch's t test (B and C).

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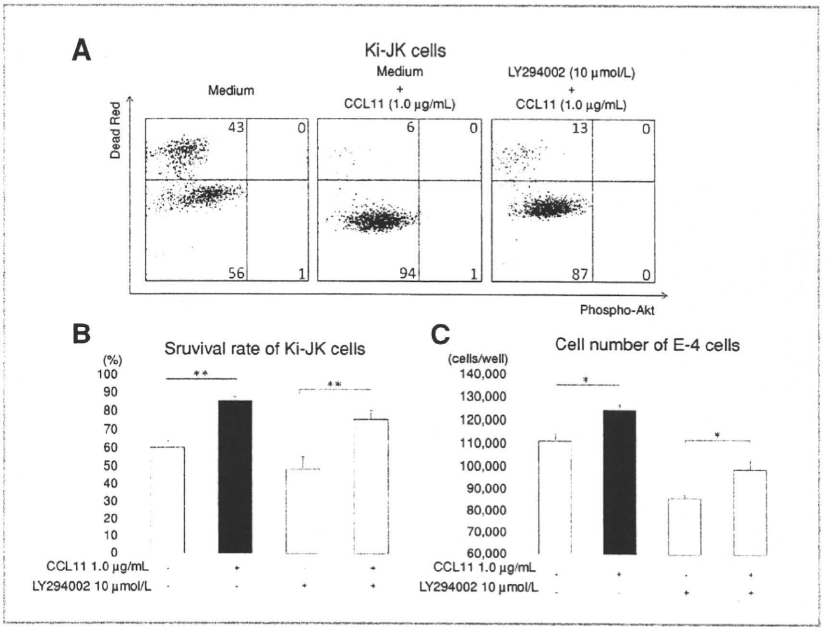

Figure 5. Akt pathway is not associated with the cell survival and growth effects of CCL11. A, Ki-JK cells were precultured with 10 μmol/L LY294002 or DMSO and were cultured with 1.0 μg/mL CCL11 or medium for 16 hours. Phosphorylation of Akt and cell survival were assessed by intracellular flow cytometry. One representative result from 3 independent experiments. B, survival rate of Ki-JK cells cultured with 1.0 μg/mL CCL11 or medium for 16 hours with or without 10 μmol/L LY294002. C, EL-4 cells were cultured with 1.0 μg/mL CCL11 or medium after preincubation with 10 μmol/L LY294002 or DMSO for 48 hours. One representative result from 2 independent experiments. Data are presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$ by Welch's t test (B and C).

of inhibition of CCL11-independent Akt phosphorylation (Fig. 5C). Thus, PI3K/Akt signaling pathway was not associated with the cell survival and growth effects induced by CCL11.

CCL11 enhances tumor growth of EL-4 cells *in vivo*, which is blocked by MEK inhibition or anti-CCL11 Ab

On the basis of the data above, we assessed effects of CCL11 on EL-4 cells *in vivo*. EL-4 cells were injected subcutaneously into the left flank of C57BL/6 mice, followed by repeated treatment with CCL11. Significantly larger tumors were formed in the mice treated with CCL11, compared with those treated with PBS (Fig. 6A). We also assessed effects of an MEK inhibitor on tumor growth of EL-4 cells *in vivo*. We observed significantly smaller tumors in the mice treated with U0126 or CCL11 + U0126 (Fig. 6A). To address the specificity, we next injected EL-4 cells with anti-CCL11 Ab or CCL11 + anti-CCL11 Ab. Anti-CCL11 Ab suppressed tumor formation to the same extent as U0126, and the effect of additional CCL11 injection was blocked by anti-CCL11 Ab (Fig. 6A). These results indicated the presence of CCL11 to active CCR3 in the injected skin. Indeed, CCL11 mRNA expression in the lesional skin was increased after tumor formation (Fig. 6B). As expected, CCL11 did not induce tumor growth of B16 cells (Fig. 6C). We also assessed effects of CCL11 on Ki-JK cells *in vivo*. Ki-JK cells were injected subcutaneously into SCID mice, followed by repeated treatment with CCL11 or PBS. Significantly larger tumors were formed in the mice treated with CCL11, compared with those treated with PBS (Fig. 6D). Thus, CCL11 promoted EL-4 and Ki-JK cell tumor growth *in vivo*, whereas MEK inhibition or neutralization of CCL11 suppressed it.

Bcl-xL and survivin expression in CCL11-stimulated Ki-JK cells *in vitro* and ALCL tumor cells *in situ*

We next investigated the expression of antiapoptotic proteins in Ki-JK cells. We stimulated Ki-JK cells with CCL11 under serum deprivation and measured protein levels of Bcl-xL and survivin, both of which are reported to be upregulated via phosphorylation of ERK1/2 (33–37). CCL11 increased Bcl-xL and survivin protein expression in Ki-JK cells (Fig. 7A).

We next immunolabeled samples of ALCL lesional skin for CD30, CCR3, phosphorylated ERK1/2, Bcl-xL, and survivin. Diffuse infiltration of $CD30^+$ atypical large cells was seen in all cases (Fig. 7B and data not shown). As previously reported (12, 13), tumor cells expressed CCR3 (Fig. 7B). Furthermore, in all cases of ALCL, phosphorylated ERK1/2, Bcl-xL, and survivin were constitutively expressed in tumor cells (Fig. 7B). On the other hand, inflammatory lymphocytes around the tumor were negative for phosphorylated ERK1/2, Bcl-xL, and survivin (Fig. 7B). Staining with isotype-matched control was also negative (data not shown). Thus, Bcl-xL and survivin were expressed in CCL11-stimulated Ki-JK cells *in vitro*, and ALCL tumor cells expressed these antiapoptotic proteins *in situ*.

Discussion

Tumor cells of cutaneous ALCL are reported to express CCR3 and its ligand, CCL11 (12, 13). In this study, we demonstrated cell survival effects of CCL11–CCR3 interactions in ALCL cells. CCL11 increased cell survival rates and promoted proliferation of $CCR3^+$ lymphoma cells via ERK pathway. We also showed Bcl-xL and survivin expression in CCL11-stimulated lymphoma cells *in vitro* and ALCL tumor cells *in situ*.

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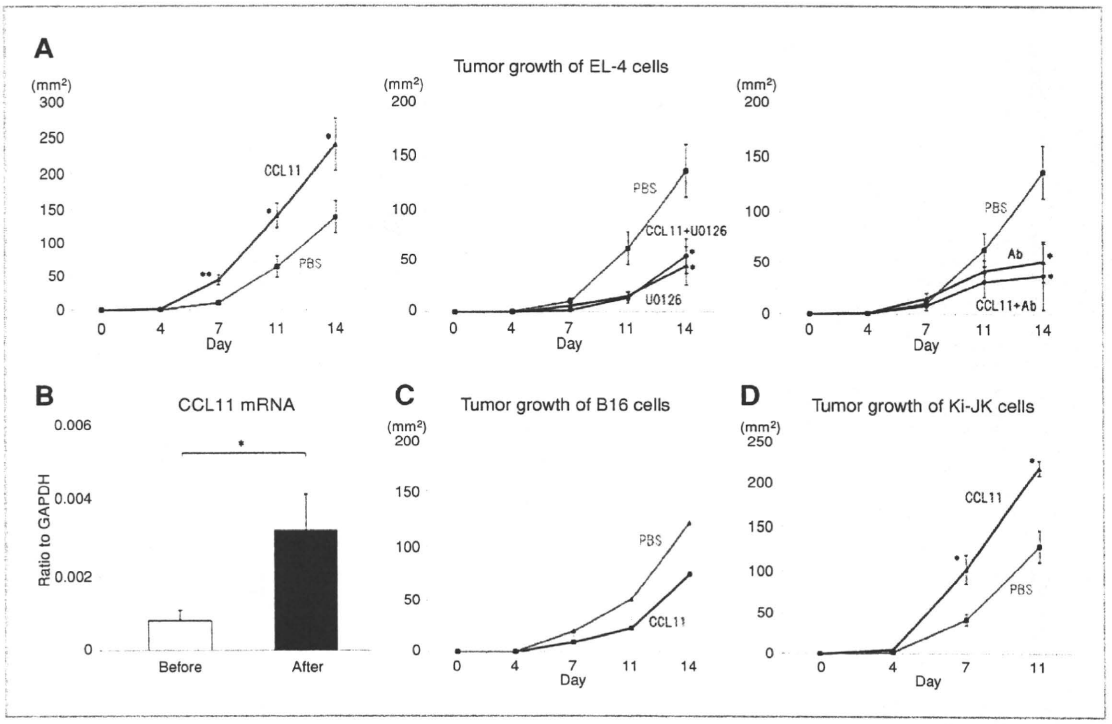

Figure 6. CCL11 enhances tumor growth of EL-4 cells *in vivo*, which is blocked by MEK inhibition or neutralization of CCL11. A, EL-4 cells were injected into shaved abdomen of C57BL/6 mice with PBS, CCL11 (1.0 μg/mL), U0126 (100 μmol/L), CCL11 + U0126, anti-CCL11 Ab (20 μg/mL), or CCL11 + anti-CCL11 Ab. B, CCL11 mRNA expression levels in mouse abdominal skin before and 11 days after EL-4 injection. Values are means and SEM ($n = 6$). *, $P < 0.05$ by Mann–Whitney *U* test. C, B16 cells were injected into C57BL/6 mice with CCL11 (1.0 μg/mL) or PBS. D, Ki-JK cells were injected into SCID mice with CCL11 (1.0 μg/mL) or PBS. Each reagent was injected on days 4, 7, and 11. Tumor size was calculated as (long axis × short axis) on days 4, 7, 11, and 14. Values are means and SEM ($n = 6$–10). *, $P < 0.05$; **, $P < 0.01$ by Mann–Whitney *U* test compared with PBS group (A, C, D).

Thus, these results suggest that signaling through CCR3 on ALCL cells is involved in tumor survival and growth via ERK1/2 activation.

CCR3 expression on ALCL tumor cells has been previously shown by flow cytometry and immunohistochemistry (12, 13). We first showed that CCL11 and CCR3 mRNA expression in lesional skin of ALCL was significantly higher than normal skin (Fig. 1A). Interestingly, CCL11 and CCR3 mRNA expression significantly correlated with one another ($r = 1.0$, $P < 0.01$). CCL11 is expressed by dermal fibroblasts, endothelial cells, and dendritic cells in the skin (18–20), whereas CCR3 is expressed on eosinophils, basophils, and subpopulations of T cells (15–17). Therefore, CCL11 may play a paracrine role as well as an autocrine role in the lesional skin of ALCL.

We next investigated effects of CCL11 on $CCR3^+$ cells *in vitro* using Ki-JK and EL-4 cells. We revealed that CCL11 increased cell survival in Ki-JK cells and induced cell proliferation in EL-4 cells (Fig. 2). We did not see increased cell survival of EL-4 cells stimulated with CCL11, probably because EL-4 cells showed quite high survival and proliferation rates under baseline culture conditions. On the other hand, Ki-JK cells did not proliferate well under serum deprivation conditions, which may be the reason why CCL11 did not induce cell proliferation of Ki-JK cells. In fact, in previous reports, CCL11 increased survival rate of eosinophils, which do not show high proliferation rate (38, 39). Moreover, CCL11 induced proliferation of a renal cell carcinoma cell line and an ovarian cell carcinoma cell line (40, 41). Taken together, it is likely that we can detect cell survival effects in cells showing little proliferation, whereas growth stimulatory effects can easily be detected in actively proliferating cells. CCL11 did not increase cell survival rate or proliferation of B16 cells (data not shown), suggesting that cell survival and growth stimulatory effects are specific to $CCR3^+$ cells.

CCL11–CCR3 interactions induce phosphorylation of ERK1/2 in eosinophils, bronchial epithelial cells, and mast cells (30–32). We also revealed that CCL11 induced phosphorylation of ERK1/2 in both Ki-JK and EL-4cells (Fig. 3). Moreover, most phosphorylated ERK1/2-positive Ki-JK cells were alive. Pretreatment with a MEK inhibitor decreased live phosphorylated ERK1/2-positive cells and increased dead phosphorylated ERK1/2-negative cells in almost equal

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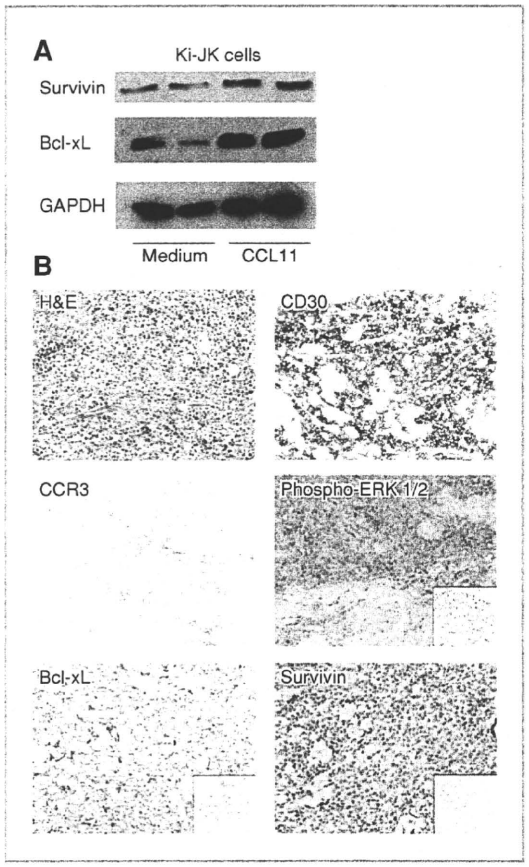

Figure 7. Bcl-xL and survivin expression in CCL11-stimulated Ki-JK cells *in vitro* and ALCL tumor cells *in situ*. A, Bcl-xL and survivin expression in Ki-JK cells stimulated for 16 hours with 1.0 μg/mL CCL11 by Western blotting. B, hematoxylin and eosin (H&E) staining and staining for CD30, CCR3, phosphorylated ERK1/2, Bcl-xL, and survivin with lesional skin of ALCL. Staining of inflammatory lymphocytes around the tumor was shown in lower right squares. Representative results of 8 cases.

numbers (Fig. 4A). These results suggest that phosphorylation of ERK1/2 is associated with the cell survival effects of CCL11 in Ki-JK cells. Recently, the same signal pathway was reported to be involved in the growth stimulatory effects of CCL11 on ovarian carcinoma cells (41). EL-4 cells showed phosphorylation of ERK1/2 even without stimulation (Fig. 3B and C), which may account for high survival and proliferation rates of these cells even under serum deprivation. The PI3K/Akt signaling pathway is also important for cell survival and growth induced by chemokines. However, in this study, CCL11-induced phosphorylation of Akt was not detected by intracellular flow cytometry (Fig. 5A and data not shown). In addition, PI3K inhibition had no effect on CCL11-mediated cell survival and growth effects (Fig. 5). Total cell numbers decreased when EL-4 cells were cultured with a PI3K inhibitor probably because of inhibition of CCL11-independent Akt phosphorylation. Therefore, the PI3K/Akt signaling pathway may be important for cell survival and growth, but is not a major pathway for CCL11–CCR3 interactions.

We also showed that CCL11 induced tumor growth of EL-4 cells *in vivo* (Fig. 6A). In addition, a MEK inhibitor or anti-CCL11 neutralizing Ab suppressed tumor growth of EL-4 cells to almost the same extent *in vivo* (Fig. 6A), suggesting that U0126 suppressed tumor growth not due to off target effects but by blocking CCL11-induced ERK pathway. We did not detect CCL11 expression by EL-4 cells as well as Ki-JK cells (data not shown). It is likely that MEK inhibition or CCL11 neutralization suppressed tumor growth *in vivo* because a variety of cells in murine skin can produce CCL11 (19, 42). CCL11 expression in dermal fibroblasts is enhanced by interleukin-4, which is abundantly expressed in lesional skin of ALCL (42, 43). Indeed, we detected enhanced CCL11 mRNA expression in the mouse skin where EL-4 cells had been injected, which was consistent with clinical data (Figs. 1A and 6B). We also showed that CCL11 induced tumor growth of Ki-JK cells *in vivo* (Fig. 6D). Collectively, in the lesional skin of ALCL, CCL11, which is produced by not only tumor cells but also surrounding cells, may play important roles for tumor growth via ERK1/2 phosphorylation. Indeed, tumor cells of cutaneous ALCL expressed phosphorylated ERK1/2 *in situ* (Fig. 7). Blocking ERK signaling pathway may offer a novel approach to treatment of ALCL.

We next investigated the expression of antiapoptotic proteins in ALCL. We focused on Bcl-xL and survivin, both of which are reported to be induced via phosphorylation of ERK pathway (33–37). We showed that CCL11 increased Bcl-xL and survivin expression in Ki-JK cells *in vitro* (Fig. 7A). Moreover, we detected expression of those proteins on tumor cells of cutaneous ALCL *in situ* (Fig. 7B). Bcl-xL expression in nodal ALCL has been previously reported, suggesting that antiapoptotic effects caused by this protein may explain development and growth of lymphoma cells (44, 45). Survivin, a member of the inhibitor of apoptosis family, is overexpressed in a variety of human cancers (46). Taken together, our results indicate that Bcl-xL and survivin may be involved in the cell survival and growth stimulatory effects induced by CCL11 in ALCL.

In summary, we showed that CCL11 increased survival rate and induces cell growth of ALCL through activation of ERK1/2. CCL11 also increased Bcl-xL and survivin expression. Thus, our findings prompt a novel therapeutic approach to treat relapses of an aggressive form of lymphoma based on the discovery that a cell surface marker of disease functions as a critical autocrine growth receptor.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

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References

1. Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, et al. WHO-EORTC classification for cutaneous lymphomas. Blood 2005;105:3768–85.
2. Turner SD, Yeung D, Hadfield K, Cook SJ, Alexander DR. The NPM-ALK tyrosine kinase mimics TCR signalling pathways, inducing NFAT and AP-1 by RAS-dependent mechanisms. Cell Signal 2007;19: 740–7.
3. Murooka TT, Ward SE, Fish EN. Chemokines and cancer. Cancer Treat Res 2005;126:15–44.
4. Rollins BJ. Inflammatory chemokines in cancer growth and progression. Eur J Cancer 2006;42:760–7.
5. Zlotnik A. Chemokines and cancer. Int J Cancer 2006;119:2026–9.
6. Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 2000;52:145–76.
7. Ruckes T, Saul D, Van Snick J, Hermine O, Grassmann R. Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309. Blood 2001;98:1150–9.
8. Meijer J, Zeelenberg IS, Sipos B, Roos E. The CXCR5 chemokine receptor is expressed by carcinoma cells and promotes growth of colon carcinoma in the liver. Cancer Res 2006;66:9576–82.
9. Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, et al. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. J Cell Biochem 2006;97:709–23.
10. Wang B, Hendricks DT, Wamunyokoli F, Parker MI. A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer. Cancer Res 2006;66: 3071–7.
11. Moriai S, Takahara M, Ogino T, Nagato T, Kishibe K, Ishii H, et al. Production of interferon-{gamma}-inducible protein-10 and its role as an autocrine invasion factor in nasal natural killer/T-cell lymphoma cells. Clin Cancer Res 2009;15:6771–9.
12. Kleinhans M, Tun-Kyi A, Gilliet M, Kadin ME, Dummer R, Burg G, et al. Functional expression of the eotaxin receptor CCR3 in CD30+ cutaneous T-cell lymphoma. Blood 2003;101:1487–93.
13. Yamaguchi T, Ohshima K, Karube K, Kawano R, Nakayama J, Suzumiya J, et al. Expression of chemokines and chemokine receptors in cutaneous CD30$^+$ lymphoproliferative disorders. Br J Dermatol 2006;154:904–9.
14. Sugaya M. Chemokines and cutaneous lymphomas. J Dermatol Sci 2010;59:81–5.
15. Forssmann U, Uguccioni M, Loetscher P, Dahinden CA, Langen H, Thelen M, et al. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. J Exp Med 1997;185:2171–6.
16. Uguccioni M, Mackay CR, Ochensberger B, Loetscher P, Rhis S, LaRosa GJ, et al. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. J Clin Invest 1997;100:1137–43.
17. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science 1997; 277:2005–7.
18. Bartels J, Schlüter C, Richter E, Noso N, Kulke R, Christophers E, et al. Human dermal fibroblasts express eotaxin: molecular cloning, mRNA expression, and identification of eotaxin sequence variants. Biochem Biophys Res Commun 1996;225:1045–51.
19. Rothenberg ME, Luster AD, Leder P. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. Proc Natl Acad Sci U S A 1995;92: 8960–4.
20. Beaulieu S, Robbiani DF, Du X, Rodrigues E, Ignatius R, Wei Y, et al. Expression of a functional eotaxin (CC chemokine ligand 11) receptor CCR3 by human dendritic cells. J Immunol 2002;169:2925–36.
21. Ganzalo JA, Jia GQ, Aguirre V, Friend D, Coyle AJ, Jenkins NA, et al. Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response. Immunity 1996;4:1–14.
22. Campbell EM, Kunkel SL, Strieter RM, Lukacs NW. Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. J Immunol 1998;161:7047–53.
23. Baraniuk JN. Pathogenesis of allergic rhinitis. J Allergy Clin Immunol 1997;99:S763–72.
24. Kaburagi Y, Shimada Y, Nagaoka T, Hasegawa M, Takehara K, Sato S. Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1alpha, MIP-1beta, and eotaxin) in patients with atopic dermatitis. Arch Dermatol Res 2001;293:350–5.
25. Yawalkar N, Uguccioni M, Schärer J, Braunwalder J, Karlen S, Dewald B, et al. Enhanced expression of eotaxin and CCR3 in atopic dermatitis. J Invest Dermatol 1999;113:43–8.
26. Shimakage M, Dezawa T, Tamura S, Tabata T, Aoyagi N, Koike M, et al. A Ki-1-positive cell line expressing Epstein-Barr virus antigens, established from a child with Ki-1-positive lymphoma. Intervirology 1993;36:215–24.
27. Sugaya M, Watanabe T, Yang A, Starost MF, Kobayashi H, Atkins AM, et al. Lymphatic dysfunction in transgenic mice expressing KSHV k-cyclin under the control of the VEGFR-3 promoter. Blood 2005; 105:2356–63.
28. Sugaya M, Fang L, Cardones AR, Kakinuma T, Jaber SH, Blauvelt A, et al. Oncostatin M enhances CCL21 expression by microvascular endothelial cells and increases the efficiency of dendritic cell trafficking to lymph nodes. J Immunol 2006;177:7665–72.
29. Miyagaki T, Sugaya M, Fujita H, Ohmatsu H, Kakinuma T, Kadono T, et al. Eotaxins and CCR3 interaction regulates the Th2 environment of cutaneous T-cell lymphoma. J Invest Dermatol 2010;130:2304–11.
30. Kampen GT, Stafford S, Adachi T, Jinquan T, Quan S, Grant JA, et al. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. Blood 2000;95:1911–7.
31. Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, et al. The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. Am J Respir Cell Mol Biol 2002;27:329–35.
32. Woo CH, Jeong DT, Yoon SB, Kim KS, Chung IY, Saeki T, et al. Eotaxin induces migration of RBL-2H3 mast cells via a Rac-ERK-dependent pathway. Biochem Biophys Res Commun 2002;298: 392–7.
33. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-xL and Bcl-2 repress a common pathway of cell death. J Exp Med 1995;182:821–8.
34. Jazirehi AR, Vega MI, Chatterjee D, Goodglick L, Bonavida B. Inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, Bcl-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B cells by Rituximab. Cancer Res 2004;64:7117–26.
35. Caja L, Sancho P, Bertran E, Iglesias-Serret D, Gil J, Fabregat I. Overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGF-{beta}-induced cell death through impairing up-regulation of the NADPH oxidase NOX4. Cancer Res 2009;69:7595–602.
36. Siddiqa A, Long LM, Li L, Marciniak RA, Kazhdan I. Expression of HER-2 in MCF-7 breast cancer cells modulates anti-apoptotic proteins survivin and Bcl-2 via the extracellular signal-related kinase

DOI:10.1158/0008-5472.CAN-10-3764

(ERK) and phosphoinositide-3 kinase (PI3K) signalling pathways. BMC Cancer 2008;8:129.

37. Li L, Gao Y, Zhang L, Zeng J, He D, Sun Y. Silibinin inhibits cell growth and induces apoptosis by caspase activation, down-regulating survivin and blocking EGFR-ERK activation in renal cell carcinoma. Cancer Lett 2008;272:61–9.
38. Shinagawa K, Trifilieff A, Anderson GP. Involvement of CCR3-reactive chemokines in eosinophil survival. Int Arch Allergy Immunol 2003;130:150–7.
39. Farahi N, Cowburn AS, Upton PD, Deighton J, Sobolewski A, Gherardi E, et al. Eotaxin/CC chemokine ligand 11: a novel eosinophil survival factor secreted by human pulmonary artery endothelial cells. J Immunol 2007;179:1264–73.
40. Jöhrer K, Zelle-Rieser C, Perathoner A, Moser P, Hager M, Ramoner R, et al. Up-regulation of functional chemokine receptor CCR3 in human renal cell carcinoma. Clin Cancer Res 2005;11:2459–65.
41. Levina V, Nolen BM, Marrangoni AM, Cheng P, Marks JR, Szczepanski MJ, et al. Role of eotaxin signaling in ovarian cancer. Clin Cancer Res 2009;15:2647–56.
42. Mochizuki M, Schröder J, Christophers E, Yamamoto S. IL-4 induces eotaxin in human dermal fibroblasts. Int Arch Allergy Immunol 1999;120 Suppl 1:19–23.
43. Yagi H, Tokura Y, Furukawa F, Takigawa M. Th2 cytokine mRNA expression in primary cutaneous CD30-positive lymphoproliferative disorders: successful treatment with recombinant interferon-gamma. J Invest Dermatol 1996;107:827–32.
44. Rassidakis GZ, Sarris AH, Herling M, Ford RJ, Cabanillas F, McDonnell TJ, et al. Differential expression of BCL-2 family proteins in ALK-positive and ALK-negative anaplastic large cell lymphoma of T/null-cell lineage. Am J Pathol 2001;159:527–35.
45. Coluccia AM, Perego S, Cleris L, Gunby RH, Passoni L, Marchesi E, et al. Bcl-XL down-regulation suppresses the tumorigenic potential of NPM/ALK *in vitro* and *in vivo*. Blood 2004;103:2787–94.
46. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.