

Antitumor immune activity by chemokine CX3CL1 in an orthotopic implantation of lung cancer model *in vivo*

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Abstract. Due to their chemoattractant properties stimulating the accumulation of infiltrating immune cells in tumors, chemokines are known to have antitumor effects. Fractalkine, a unique CX3C chemokine, is expressed in activated endothelial cells, while its receptor, CX3CR1, is expressed in cytolytic immune cells, such as natural killer cells, monocytes and some CD8⁺ T cells. The biological properties of cancer cells are affected by the implantation organ and differences in immune systems, requiring cancer implantation in orthotopic organs in an *in vivo* experiment. To develop new therapy strategies for lung cancer, an animal model reflecting the clinical features of lung cancer was previously established. This study aimed to determine whether CX3CL1-induced biological functions should be used for immune cell-based gene therapy of lung cancer in the orthotopic implantation model. An orthotopic intrapulmonary implantation of CX3CL1-stable expression in mouse lung cancer (LLC-CX3CL1) was performed to analyze growth. Results showed a significant decrease in tumor growth in the lung compared to the control cells (LLC-mock). Furthermore, the antitumor effects of CX3CL1 were derived from natural killer cell activities in the depletion experiment *in vivo*. Therefore, CX3CL1 has the potential of a useful therapeutic target in lung cancer.

Introduction

Treatment outcomes of lung cancer are relatively poor and have not improved significantly in the past 20 years. In addition to common unresectable cases, resectable cases require systemic chemotherapy at a certain stage. Although improvement in the outcomes of chemotherapy in lung cancer has been

gradually observed, they remain unsatisfactory in circumstances whereby medication efficacy is only 2-3%. Therefore, gene therapy is clinically expected as the treatment strategy is different from that of the existing chemotherapy or radiation therapy. Subcutaneous implantation is widely practiced since cancer growth is easily observed. However, studies have shown that the biological behavior of cancer cells is influenced by the implantation site (1), and that the orthotopic implantation of human tumor cells into relevant organs of nude mice provides an *in vivo* model to study the biology and therapy of these cells (1,2).

On the other hand, chemokines are a family of small cytokines that primarily induce the directed migration of hematopoietic cells when bound to their seven-transmembrane, G protein-coupled receptors (3,4). Chemokines are attractive candidates for immune cell-based approaches to cancer gene therapy, as they function as chemoattractants for several immune effector cell types. CX3CL1 (also known as fractalkine) is a unique chemokine that functions as an adhesion and chemotactic molecule towards its receptor CX3CR1-expressing cells. Although chemokines activate adhesion molecules, such as integrins, to bind to target cells or the cellular matrix, the interaction of transmembrane CX3CL1-CX3CR1 strongly induces cell-to-cell contact in an adhesion molecule-independent manner (5,6). This study was conducted to examine the efficacy of mouse CX3CL1 for gene therapies by employing an orthotopic transplantation model of mouse lung cancer cells, a model reflecting cancer growth in the lung (7).

Materials and methods

Cell culture and transfection. Mouse lung cancer cells, Lewis lung carcinoma (LLC), were maintained in EMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂/95% air. LLC cells were transfected by Nucleofector™ (Amaxa Biosystems, Gaithersburg, MD, USA). Expression vectors (pIRES2-EGFP vector; Takara Bio, Inc., Shiga, Japan) for mouse membrane-bound CX3CL1, pIRES2-EGFP-CX3CL1 were used. DNA was adjusted to 1 µg with empty vectors.

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Subsequent to transfection, EGFP-positive LLC cells were sorted using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and cultured at 37°C with the antibiotic G148 (Invitrogen, Carlsbad, CA, USA). CX3CL1-stable expression cells (LLC-CX3CL1) and control cells (LLC-mock) were also cultured. CX3CR1-stable expression cells (L-CX3CR1) were maintained in RPMI-1640 supplemented with 10% FBS and antibiotics.

Animals. Specific pathogen-free C57BL/6 mice (7-week-old females) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were maintained in the Laboratory for Animal Experiments of the Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan, under laminar air flow conditions. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of the Toyama University.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed as previously described (8). Briefly, total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand cDNA was prepared from an RNA template (2 µg) using an oligo(dT)18 primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed at 42°C for 50 min and then at 70°C for 15 min. PCR amplification was performed using the Takara Ex Taq HS PCR kit (Takara Bio, Inc.) under the following conditions: 26 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 60 sec, and extension at 72°C for 60 sec. The primers were verified to yield the expected products under the indicated conditions. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The primer sequences were: GAPDH, sense: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and anti-sense: 5'-CATGTGGGCCATGAGGTCCACCAC-3'; mouse CX3CL1, sense: 5'-GAGCATTGGAAGTTTTGAGG-3' and antisense: 5'-GGTTGAAGGTGAAGTAGTGA-3'.

Fluorescence-activated cell sorting (FACS) analysis. LLC-CX3CL1 or LLC-mock cells were fixed with 4% labeled with paraformaldehyde, and incubated with anti-mouse CX3CL1 (provided by the Kan Research Institute, Kobe, Japan). Subsequent to incubation, the cells were washed three times with PBS, then stained with PE anti-Armenian and Syrian hamster immunoglobulin G (IgG) as the second antibody. FITC-labeled cells were then analyzed by flow cytometric analysis using FACSCalibur (BD Biosciences).

Migration assay. The migration assay was performed in Transwell cell culture chambers (Corning Incorporated Life Sciences, Tewksbury, MA, USA), as reported previously, with some modifications (9). Polyvinylpyrrolidone-free polycarbonate (PVFP) filters (8.0 µm pore size; Nuclepore, Pleasanton, CA, USA) were pre-coated with 1 µg fibronectin (Asahi Technoglass Co., Tokyo, Japan) on the lower compartment. LLC-CX3CL1 or LLC-mock cell suspension [1x10⁵ cells/100 µl in EMEM with 0.1% bovine serum albumin (BSA)] was added to the upper compartment and

incubated for 4 h at 37°C. Cells that invaded the lower surface were counted under a microscope in five pre-determined fields at x400 magnification.

Adhesion assay. The adhesion assay was performed as described previously, with some modifications (10). Triplicate wells of 96-well plates were coated with 1 µg fibronectin. LLC-CX3CL1 or LLC-mock cells (1x10⁴ cells/100 µl in EMEM with 0.1% BSA) were incubated for 25 min at 37°C. Cells that adhered to the well were counted under the microscope in five pre-determined fields at x400 magnification.

ELISA. Conditioned media of LLC-CX3CL1 or LLC-mock cells were collected in 1.5 ml tubes, centrifuged at 2,000 rpm for 5 min to remove cell debris, and kept at -80°C until assay. Assays were performed in a mouse CX3CL1 ELISA system (R&D Systems Inc., Minneapolis, MN, USA).

Chemotaxis assay. Chemotaxis assay was performed as described previously, with some modifications (11). LLC-CX3CL1 or LLC-mock cells were cultured in EMEM with 0.1% BSA for 24 h and conditioned media were collected. L-CX3CR1 cells labeled with DiA (Invitrogen) were applied to the upper wells of Transwell cell culture chambers of 8 µm pore size (Corning Incorporated Life Sciences) in 100 µl assay buffer (RPMI-1640 with 10 mM HEPES, pH 7.4, 1% BSA). Aliquots of conditioned media were pre-treated with or without anti-mouse CX3CL1 or control IgG for 30 min at 37°C and then applied to the lower wells in a volume of 350 µl. After 4 h at 37°C, cells that migrated into lower wells were lysed with 0.2% Triton X-100 in 20% ethanol and quantitated by fluorescence intensity measurement (excitation, 595 nm; emission, 485 nm).

Orthotopic implantation. The orthotopic intrapulmonary implantation of LLC cells was performed as described previously, with some modifications (7). Briefly, log-phase cell cultures of LLC-CX3CL1 or LLC-mock cells were suspended at a cell density of 2.5x10⁵ cells/ml in PBS containing 500 µg/ml Matrigel. Animals (n=6) were anesthetized and a small skin incision was made to the left chest wall (~5 mm in length). A 29-gauge needle attached to a 0.5-ml insulin syringe was inserted directly through the intercostal space into the lung to a depth of 3 mm. Cells (5x10⁵) were re-suspended in 20 µl of PBS containing 10 µg Matrigel, and injected into the lung parenchyma. To deplete the NK cells *in vivo*, mice (n=6) were intraperitoneally injected with 200 µl anti-asialo GM1 antibody twice a week.

Statistical analysis. The significance of differences between groups was determined by applying the two-tailed Student's t-test. P<0.05 were considered to indicate a statistically significant difference. The means and SDs were calculated for all variables

Results

Stable expression of mouse CX3CL1 in mouse lung cancer cells. We initially intended to obtain CX3CL1 stable-expression polyclonal LLC cells lines (LLC-CX3CL1) by combining

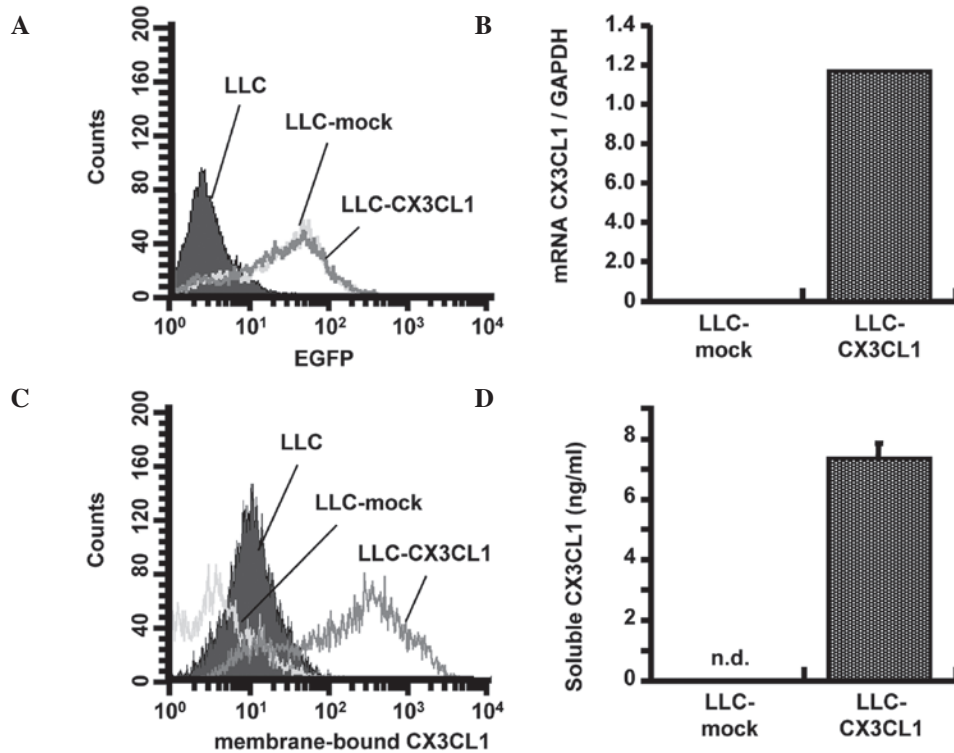


Figure 1. CX3CL1 expression of CX3CL1 gene-transfected cancer cells is shown. (A) After sorting EGFP-positive LLC cells using the FACSCalibur, (B) CX3CL1 transcript in LLC cells transfected with pIRES2-EGFP-CX3CL1 or pIRES2-EGFP-mock vector was detected by RT-PCR. Expression of membrane-bound and soluble CX3CL1 of sorted LLC cells (LLC-CX3CL1 and LLC-mock) were detected by (C) FACS and (D) ELISA, respectively.

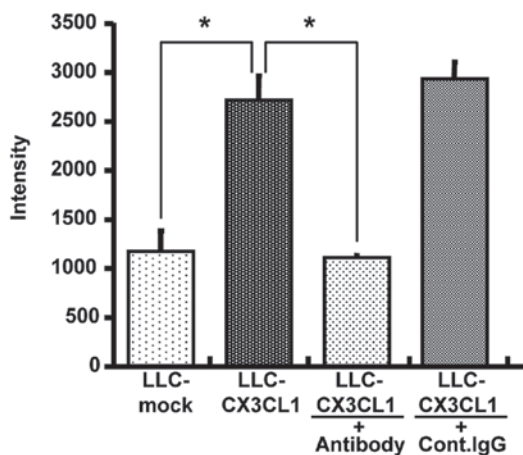


Figure 2. Chemotactic activity of CX3CL1 secreted by LLC-CX3CL1 is shown. LLC-CX3CL1 supernatants were added to the lower wells with or without neutralizing anti-CX3CL1 Ab. DiA-labeled L-CX3CR1 cells were placed in the upper compartments of Transwell chambers. After 4 h incubation, cells migrated into lower wells were lysed and quantitated by fluorescence intensity measurement.

antibiotic selection and flow cytometric sorting to obtain an index of the EGFP expression (Fig. 1A). LLC-CX3CL1 was found to express mRNA of mouse CX3CL1 (Fig. 1B). Moreover, as CX3CL1 has two phenotypes, flow cytometry and western blotting confirmed a higher expression of membrane-bound and soluble CX3CL1 in LLC-CX3CL1 compared to LLC-mock cells (Fig. 1C and D). Gene introduction into cells often generates various changes in the characteristics compared

to the control cells. No changes were observed regarding the cell proliferation, adhesion and migration towards the fibronectin of LLC-CX3CL1, when compared with CX3CL1-mock cells (data not shown).

Chemotactic activity of LLC-CX3CL1-generated CX3CL1. Given that LLC-CX3CL1 cells were stably expressed in mouse membrane-bound and soluble CX3CL1, and their properties were not different from those of CX3CL1-mock cells, polyclonal LLC cell line-generated CX3CL1 was verified to be biologically active. As a result of migration assay-induced chemotactic activity using L-CX3CR1 cells in culture supernatants, the supernatant of LLC-CX3CL1 significantly induced L-CX3CR1 as well as anti-mouse CX3CL1 cell migration, although the control IgG antibody did not completely neutralize chemotactic activity (Fig. 2).

Antitumor effect of CX3CL1 in the orthotopic transplantation of lung cancer models. The effect of CX3CL1-induced tumor growth inhibition was examined using orthotopic transplantation models. Seventeen days after LLC/CX3CL1 and LLC-mock were transplanted into the left lung of a mouse, the weight of the solid tumor was examined. The result showed a 65% inhibition of tumor growth compared to LLC-mock (Fig. 3).

Elucidation of CX3CL1-induced antitumor effects by immune cells. Anti-asialo GM1 antibody was used for NK cell depletion *in vivo*. Initially, FACS analysis was used to determine whether NK1.1-positive NK cells are eliminated by administering 200 μ g anti-asialo GM1 antibody via the tail vein to

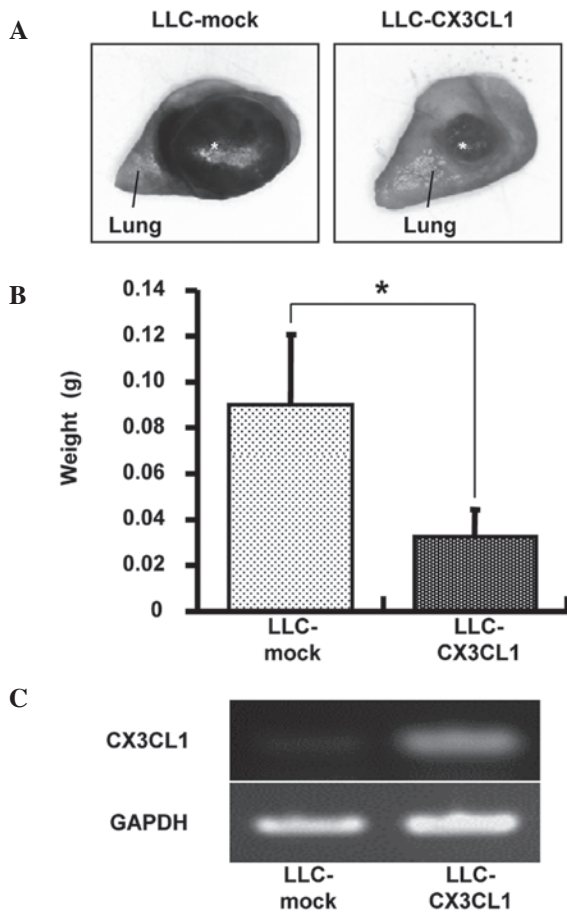


Figure 3. Effect of CX3CL1 on orthotopic intrapulmonary implantation of cancer growth is shown. LLC-CX3CL1 and LLC-mock cells (5×10^5) suspended in $20 \mu\text{l}$ PBS containing $10 \mu\text{g}$ Matrigel were injected into lung parenchyma. (A) Macroscopic observation of the resected lung on Day 17 after the orthotopic implantation of these cells is shown. (B) The weight of cancer at the implanted site was measured on Day 17 after implantation, $^*P < 0.05$. (C) CX3CL1 mRNA expression of implanted cells was detected on day 17 after implantation.

a C57BL/6 mouse twice with in a distance of 2 days (data not shown). In the non-treated group, LLC/CX3CL1 growth was significantly inhibited compared to the LLC-mock in the orthotopic intrapulmonary implantation of LLC cells (Fig. 4). In the depletion groups, the inhibition of LLC/CX3CL1 growth was suppressed. By contrast, the growth suppressive effects of CX3CL1 against LLC were affected by neither CD4 nor CD8 depletion (data not shown). These results suggest that NK cells contributed to the marked antitumor effect of CX3CL1.

Discussion

Chemokines are molecules attractive for cancer gene therapy (12), a method categorized as a cell-based cancer immunotherapy due to the involvement of chemokine-mediated immune cell chemotaxis, as well as the angiostatic activity of some chemokines (13). CX3CL1 is a type I transmembrane ligand expressed in activated vascular endothelial and dendritic cells (14-16). The secreted CX3CL1 is chemotactic for chemokine receptor, CX3CR1-expressing cells. The

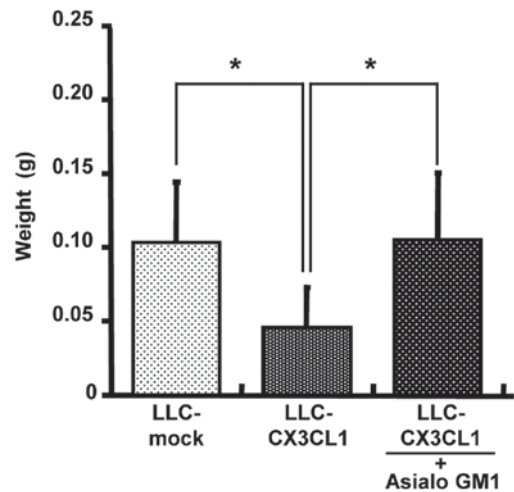


Figure 4. Growth of LLC-CX3CL1 with or without the depletion of NK cells is shown. After the orthotopic implantation of LLC-CX3CL1 and LLC-mock cells to deplete NK cells *in vivo*, mice ($n=6$) intraperitoneally injected with anti-asialo GM1 antibody were used for NK cell depletion *in vivo*, twice a week. The weight of cancer at the implanted site was measured on Day 17 after implantation, $^*P < 0.05$.

membrane form adheres to chemo-attracted cells, resulting in the interaction of dendritic cells, monocytes, T cells and natural killer cells (NK cells) locally (5,6). Initially, the chemotactic activity of CX3CL1 secreted by LLC-CX3CL1 stably expressing CX3CL1 was confirmed (Fig. 2). The secreted form of CX3CL1 is produced by a disintegrin-like metalloproteinase (ADAM)-mediated shedding of the extracellular chemokine domain, acting as a chemoattractant of mainly NK cells through CX3CR1 (17,18).

In this experiment, pIRES2-EGFP-encoded mouse CX3CL1 was used as the full-length transmembrane type, resulting in an efficient production of soluble CX3CL1 through cleavage at the membrane site by the ADAM family. Previous studies have showed that the biological behavior of cancer cells is influenced by the implantation organ (1), while properties of the immune system are also known to be different in each organ. A useful model for a solitary pulmonary tumor has previously been established by the intrapulmonary implantation of LLC cells in syngeneic immunocompetent mice (7,19-21). This model has the advantage of being simple and easy regarding the implantation procedure with a small skin incision at a pre-determined site followed by direct puncturing through the intercostal space to the lung parenchyma, without thoracotomy or intubation. Marked lung cancer regression was observed in LLC-CX3CL1 when compared to tumors comprising control cells (LLC-mock) (Fig. 3). Given the lack of cell functions, as well as the cell proliferation, adhesion and migration of LLC/CX3CL1 and LLC-mock (data not shown), these results suggest that the growth inhibition effect *in vivo* represents the antitumor effect depending on CX3CL1. CX3CL1 secreted from LLC/CX3CL1 *in vitro* generates the migration of CX3CR1-positive lymphocytes (Fig. 2). Therefore, to investigate the immune cell functions involved in the antitumor effects of CX3CL1, an *in vivo* depletion analysis was carried out using specific antibodies against CX3CR1-positive immune cells (NK, CD8⁺ T and CD4⁺ T cells), in the

orthotopic intrapulmonary implantation of LLC cells. The CX3CL1-dependent antitumor effect was derived from NK cell activities (Fig. 4).

In clinical studies, leukocyte accumulation in cancers directed by cancer cell-derived chemokines are crucial in cancer progression and metastasis. Chemokine expression was detected in several cancers, while cancer cell-derived chemokines were responsible for the infiltration of various types of leukocytes, mainly macrophages, into these cancers (22,23). CCL5 [also termed regulated on activation, normal T-cell expressed and secreted, (RANTES)] and CCL2 [also known as monocyte chemoattractant protein-1, (MCP-1)] are chemokines frequently observed in cancer. In breast cancer, a lower CCL2 expression was correlated with longer relapse-free survival and decreased tumor-associated macrophage (TAM) (24), while a higher level of CCL5 expression was associated with an increase of TAM and lymph node metastasis (25). In contrast to CCL2 and CCL5, a high-level expression of chemokine CXCL16 by tumor cells has recently been reported to correlate with a good prognosis and increased CD8⁺ T as well as CD4⁺ T cells in CRC (26). In addition, CX3CL1 is correlated with a better prognosis and an increased number of CX3CR1-positive CD8⁺ T and NK cells migrated into primary cancer in several cancers, such as CRC (27) or gastric adenocarcinoma (28). Although these clinical studies do not comprise cancer gene therapy, they indicate that the accumulation of CX3CR1-positive immune cells in primary cancer results in antitumor activity. Therefore, the finding that a cancer gene therapy strategy supports the accumulation of NK cells by CX3CL1, might be an effective therapeutic approach towards lung cancer, although the correlation of lung cancer-derived CX3CL1, migrated NK cells and good prognosis need to be further investigated.

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