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Chemokine CXCL16 suppresses liver metastasis of colorectal cancer via augmentation of tumor-infiltrating natural killer T cells in a murine model

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Abstract. Colorectal cancer (CRC) is a typical lifestyle-related disease, and it metastasizes mostly to the liver. It is important to understand the molecular mechanisms of CRC metastasis in order to design new and effective treatments for CRC patients. Chemokines are known to have antitumor effects as their chemoattractant properties stimulate the accumulation of infiltrating immune cells (TILs) in tumors. Chemokine (C-X-C motif) ligand 16 (CXCL16), also known as SR-PSOX, is a unique membrane-bound chemokine that induces the expression of its specific receptor CXCR6. We previously reported that the expression of CXCL16 by cancer cells enhances the recruitment of TILs, thereby improving the prognosis of CRC. It has since been reported that CXCL16/CXCR6 expression is involved in the metastasis of various types of cancer. However, there is no report of the association between CXCL16 expression and liver metastasis in CRC. In this study, we investigated the role of cancer-derived CXCL16 and the possibility of gene therapy using CXCL16. Therefore, we examined the metastasis of colon 38 SL4 cells to the liver in an experimental model. Following injection of cancer cells into the intraportal vein, CXCL16-expressing CRC cells drastically inhibited liver metastasis. We also found that CD8 T cells and natural killer T (NKT) cells, known as CXCR6-expressing cells, increased in CXCL16-expressing metastatic tissue. Collectively, the inhibitory effect on metastasis to the liver by CXCL16 was observed in NKT cell-depleted mice but not in CD8 T cell-depleted mice. These results demonstrate the inhibitory effect of CXCL16 on liver metastasis via NKT cells in CRC.

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Introduction

Colorectal cancer (CRC) is the second and third most commonly diagnosed cancer in females and males, respectively (1). Approximately 25% of patients with CRC have liver metastasis at initial diagnosis and an additional 50% of patients develop metachronous liver metastasis. Surgery is considered the only curative treatment option for patients with resectable liver metastasis, and the five year survival rate is approximately 35% (2).

It has been shown that chemokines play a role in cancer progression and/or organ-specific metastasis (3-5). Chemokine (C-X-C motif) ligand 16 (CXCL16) is a membrane-bound chemokine that exists in a transmembrane form (TM-CXCL16) and a soluble form (sCXCL16) cleaved by proteolytic enzymes, ADAM10 and ADAM17 (6-11). CXCL16 is expressed in macrophages, dendritic cells, monocytes, and B cells (6-8) as well as in various cancer cell lines and tumor tissues (12-20). CXCL16 has multiple biological functions. TM-CXCL16 can act as a cell adhesion molecule for CXCR6-expressing cells and also as a scavenger receptor for phosphatidylserine and oxidized lipoprotein (7), whereas sCXCL16 is a chemotactic factor for cells expressing its receptor CXCR6 such as activated CD8 T cells, CD4 T cells, and natural killer T (NKT) cells (21,22).

NKT cells are important regulatory immune cells which express NK1.1 and TCR on their surface. Among mouse NKT cells, type I NKT (iNKT) cells such as a Vα14^{+} NKT express an invariant TCR containing the gene segment Vα14-Jα281 (23).

Activated iNKT cells with exogenous glycolipids presented via CD1d molecules are directly cytotoxic to a number of tumor cell lines and play important roles in antitumor and anti-metastatic responses (24-30). Of note, localized iNKT cell infiltration into primary colorectal cancer is associated with a good prognosis (31).

Mouse iNKT cells express high levels of CXCR6 and CXCL16/CXCR6 regulate iNKT cell functions (6,22,31-35). Recent studies have demonstrated that CXCL16 and its receptor CXCR6 are important in the homeostatic distribution of iNKT cells to the liver (7,34,35). In addition to regulating iNKT cell homing, CXCR6 and CXCL16 have been shown to play a critical role in iNKT cell activation in response to glycolipid antigens (7,35). As CXCL16 is expressed as a transmembrane protein on the surface of APCs (7), it is likely that CXCR6 and CXCL16 mediate costimulatory interactions between iNKT cells and glycolipid-presenting cells that modulate activation and effector functions (35).

Previously, we showed that primary tissues highly expressing CXCL16 generated tumor-infiltrating lymphocytes (TIL) and improved the prognosis of CRC patients (15). It has since been reported that CXCL16/CXCR6 expression is involved in the metastasis of various types of cancer (36,37). By contrast, the role of CXCL16 in controlling the metastasis of CRC is unknown. In this study, we investigated the involvement of CXCL16 and metastasis to the liver by CRC cells in an experimental model.

Materials and methods

Cell culture. Colon 38 SL4 cells, a highly metastatic variant of the mouse colon 38 colon cancer cell line, were used. This cell line was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Transfection of plasmid DNAs. Colon 38 SL4 cells were transfected by Nucleofector™ (Amaxa, Inc., Gaithersburg, MD, USA). Expression vectors (pcDNA 3.1(+) vector; Life Technologies Ltd., Japan) for mouse membrane-bound CXCL16, pcDNA 3.1(+)-CXCL16 were used. DNA was adjusted to 1 μg with empty vectors. After transfection, CXCL16-positive colon 38 SL4 cells were selected by using the antibiotic G148 (Invitrogen). CXCL16-stable expression cells (SL4-CXCL16) and control cells (SL4-Mock) were maintained in DMEM/F12 supplemented with 10% FBS and antibiotics.

Assays of cell growth. Cell growth was quantified using the cell proliferation reagent WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo, Kumamoto, Japan). SL4 cells were seeded in 96-well microplates at $2x10^3$ cells/well and incubated 24 h. The medium was then changed (90 μl), 10 μl of WST-8 solution was added, and absorbance was measured at 450 nm.

Migration and adhesion assay. The migration assay was performed in Transwell cell culture chambers (Corning Incorporated Life Sciences, Tewksbury, MA, USA). Polyvinylpyrrolidone-free polycarbonate (PVFP) filters (8.0 μm pore size; Nuclepore, Pleasanton, CA, USA) were pre-coated with Matrigel (BD Biosciences) on the lower part. SL4-Mock or SL4-CXCL16 cell suspension [$1x10^5$ cells/100 μl in DMEM/F12 with 0.1% bovine serum albumin (BSA)] was added to the upper compartment and incubated for 4, 8 and 12 h at 37˚C. Cells that invaded the lower surface were stained with hematoxylin and eosin and counted under a microscope at x400 magnification. The adhesion assay was performed in 96-well plates. Triplicate wells were precoated with Matrigel and after removing the Matrigel, SL4-Mock or SL4-CXCL16 cells ($1x10^4$ cells/100 μl in DMEM/F12) were incubated for 30 min at 37˚C. Cells were aspirated and fixed with 4% formaldehyde approximately 30 min. Adhered cells were stained with hematoxylin for 20 min and counted under the microscope in five predetermined fields at x400 magnification.

Mice. C57BL/6 female and KSN/slc male 5-week-old nude mice were purchased from Sankyo Lab Service (Hamamatsu, Japan). The study was conducted in accordance with the standards established in the guidelines for the Care and Use of Laboratory Animals of Toyama University.

Experimental liver metastasis of colon 38 SL4 cells. Colon 38 SL4 cells were harvested and resuspended in 1 mM EDTA in phosphate-buffered saline (PBS). For experimental liver metastasis, colon 38 SL4 cells were injected into the intraportal vein. The mice were sacrificed 17 days later and the livers were removed. The increase in tumor weight and the number of tumor colonies in the liver were measured to evaluate tumor metastasis.

CD8 T cell depletion model. Hybridomas producing a 53-6.72 CD8 T cell a specific monoclonal antibody (ATCC, Manassas, VA, USA) were injected intraperitoneally into KSN/slc mice. C57BL/6 female mice were intraperitoneally injected with the ascites fluid of KSN/slc mice 3 days prior to and 3 and 6 days following the inoculation with cancer cells. CD8 T cell depletion was verified by flow cytometry.

NKT cell depletion model. di-palmitoyl-phosphatidylethanolamine polyethyleneglycol (DPPE-PEG) which is used as a reagent of NKT cell depletion (38), purchased from NOF Corporation, was dissolved with saline and injected intraperitoneally (250 μg/50 μl/mouse/day) until the mice were sacrificed.

Real-time PCR. Total-RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. First-strand cDNA was synthesized from an RNA template (2 μg) using SuperScript II reverse transcriptase (Invitrogen). The primer sequences are as follows: CD4, 5'-ACACACCTGTGCAAGAAGCA-3' (forward) and 5'-GCTCTTGTTGGTTGGGAATC-3' (reverse); CD8, 5'-CTCACCTGTGCACCCTACC-3' (forward) and 5'-ATCCGGTCCCCTTCACTG-3' (reverse); Vα14-Jα281, 5'-TGGGAGATACTCAGCAACTCTGG-3' (forward) and 5'-CAGGTATGACAATCAGCTGAGTCC-3' (reverse); CD3ε, 5'-AACACGTACTTGTACCTGAAAGCTC-3' (forward) and 5'-GATGATTATGGC

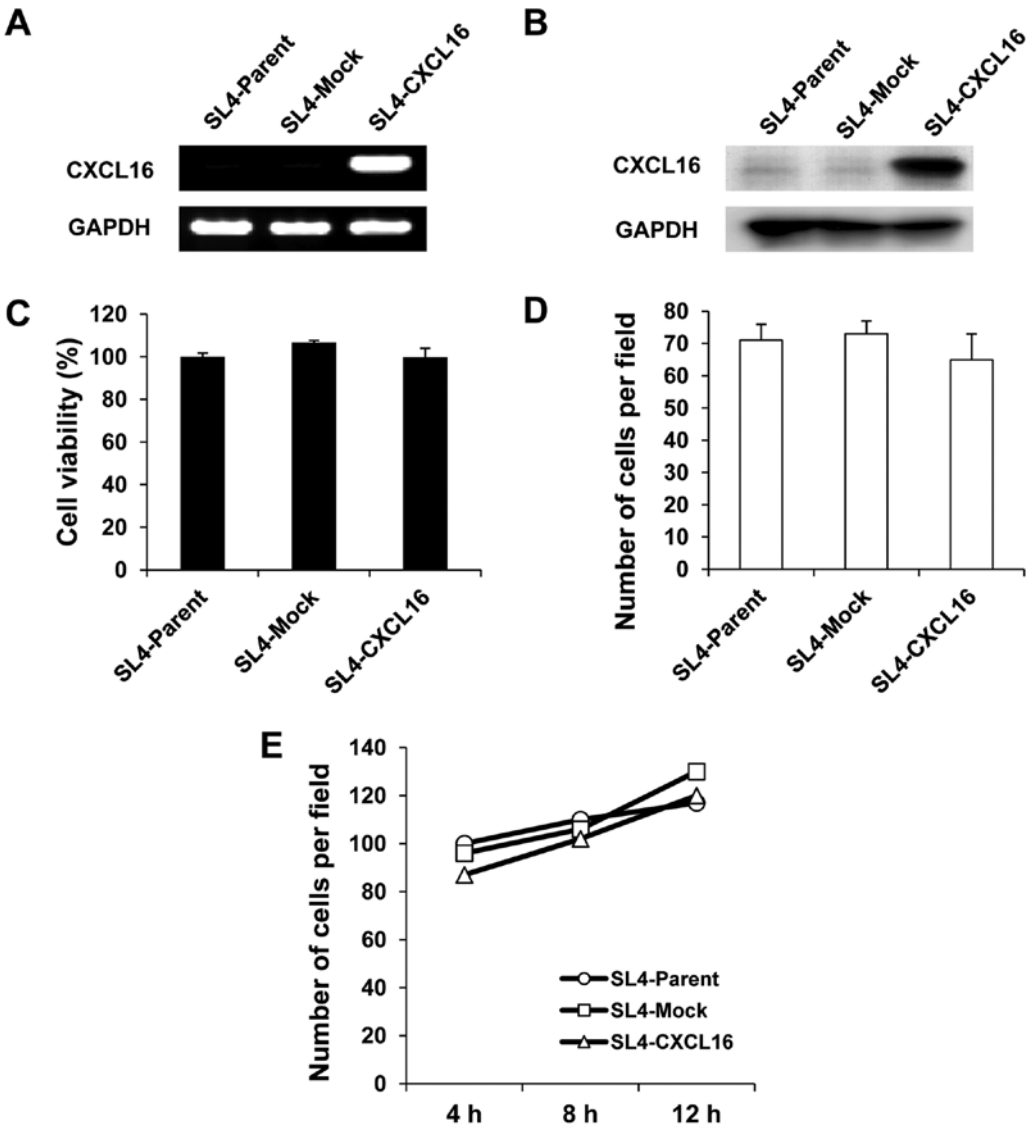

Figure 1. Established CXCL16-expressing metastatic CRC cells. (A and B) We established CXCL16-overexpressing colon 38 SL4 cells. The cells stably expressed (A) CXCL16 mRNA and (B) protein. (C) Cell viability, (D) migration and (E) adhesion ability were not changed by CXCL16 overexpression.

TACTGCTGTCA-3' (reverse); β-actin, 5'-CTAAGGCCAACC GTGAAAAG-3' (forward) and 5'-ACCAGAGGCATACAGG GACA-3' (reverse). Real-time quantitative RT-PCR was performed using a SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) and Lightcycler nano system (Roche, Pleasanton, CA, USA). All data were normalized to β-actin mRNA.

Lymphocyte isolation and flow cytometry. To isolate lymphocytes from the liver, normal and tumor tissues from the liver were dissected in 20% RPMI-1640 medium. Samples were then further homogenized through wire mesh and mononuclear cells were isolated using a 30% Percoll gradient (GE Healthcare, Little Chalfont, UK) 5 ml, 10X PBS 365 μl, 7% $NaHCO_3$ (Meylon) 65 μl, Heparin 50 μl, and RPMI-1640 10 ml/mouse. Red blood cells were lysed by lysis buffer (NH_4Cl solution: 155 mM NH_4Cl, 100 mM $KHCO_3$ and 1 mM EDTA-2Na to 1 L with DW, Tris-HCl solution: Trizma 20.6 g was dissolved in 900 ml DW, adjusted pH 7.6 with concentrated HCl, diluted to 1 L with DW mix 900 ml NH_4Cl solution and 100 ml Tris-HCl solution, autoclaved, stored at 4˚C). To isolate lymphocytes from the spleen, the Percoll gradient was used. For flow cytometry, lymphocytes were first pre-incubated with CD16/32 (2.4G2) mAb and then incubated with a saturating amount of mAb. Antibodies to CD3ε (145-2C11), NK1.1 (PK136), and CD1d (RA3-6B2) were purchased from eBioscience (San Diego, CA, USA). The samples were analyzed with the FACSCanto II system (BD Biosciences).

Statistical analysis. Data were analyzed for statistical significance using the Student's t-test. P-values <0.05 were considered to indicate statistically significant differences. The mean and SD were calculated for all variables.

Results

Stable expression of mouse CXCL16 in mouse colon cancer cells. We initially intended to obtain CXCL16 stable-expression colon 38 SL4 cells (SL4-CXCL16) by the antibiotic selection. Higher CXCL16 expression in SL4-CXCL16 than SL4-Mock cells was confirmed by RT-PCR and western

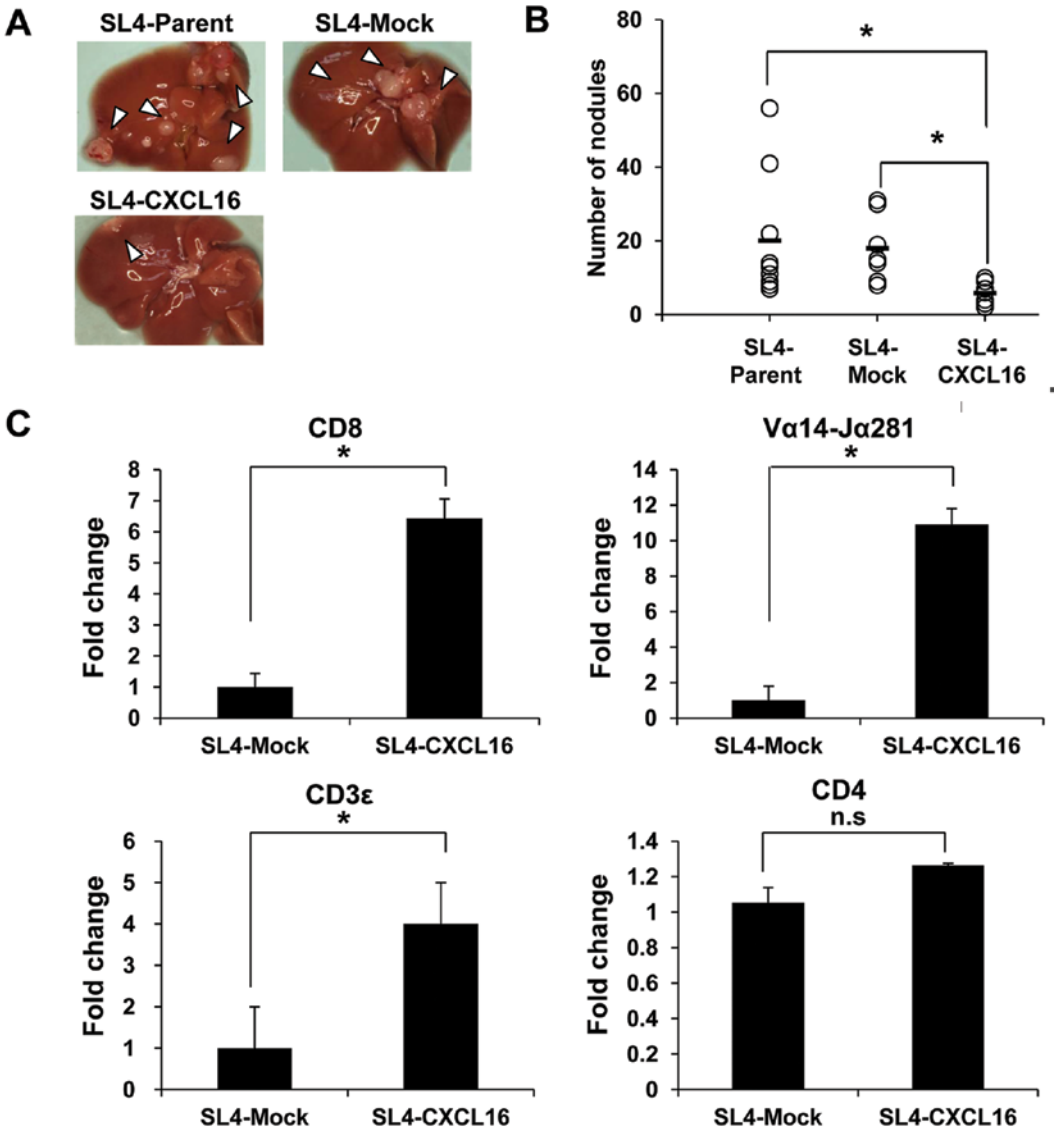

Figure 2. CXCL16 expression inhibited colorectal liver metastasis. (A) Results of injecting SL4 cells ($7.5x10^4$ cells/200 μl/mouse) i.p.v into C57BL/6 mice. SL4-CXCL16 group compared with SL4-Parent and SL4-Mock group. White arrows indicate tumor nodules. (B) The number of tumor nodules. The mean number for each group is expressed with a horizontal bar; 8 mice were used for each group. Similar results were obtained in 3 independent experiments. *P-values <0.05 denote statistically significant differences. (C) mRNA expression of markers of CXCR6-expressing cells. CD8 T cell (CD8), NKT cell (Vα14-Jα281) and T cell (CD3ε) markers were increased 6, 10 and 4-fold in SL4-CXCL16 compared with SL4-Mock.

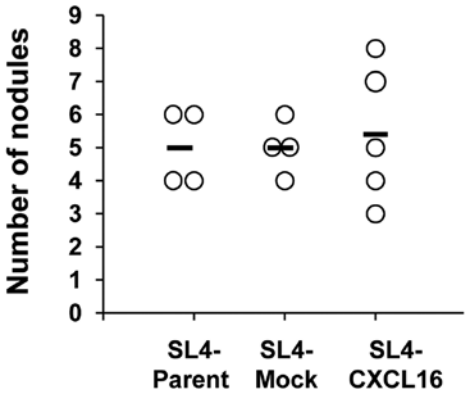

Figure 3. CXCL16 expression did not inhibit liver metastasis in KSN/slc mice. Results of injecting SL4 cells ($7.5x10^4$ cells/200 μl/mouse) i.p.v into KSN/slc mice. SL4-Parent, SL4-Mock and SL4-CXCL16. The mean number for each group is expressed with a horizontal bar. Similar results were obtained from repeated experiments using 5 mice/group and a representative result for each group is shown.

blotting (Fig. 1A and B). Gene introduction into cells often causes various property changes, compared with control cells. Compared with SL4-Mock cells, there was no significant difference in cell growth (Fig. 1C), migration (Fig. 1D) and adhesion (Fig. 1E) in SL4-CXCL16 cells.

Liver metastasis is inhibited by CXCL16-expressing CRC cells. Colon 38 SL4 cells (Parent, Mock and CXCL16) were injected into the intraportal vein to confirm the effect of CXCL16 expression on liver metastasis. Mice were sacrificed on Day 17 and liver metastasis was evaluated by counting nodules. Metastasis into the liver was significantly decreased by SL4-CXCL16 cells (Fig. 2A). Also, the number of nodules was significantly decreased in CXCL16-expressing tumor tissue (Fig. 2B). Since CXCL16 acts as a chemoattractant as well as a cell adhesion molecule for CXCR6-expressing cells (21,22), we conducted a real-time PCR analysis with tumor

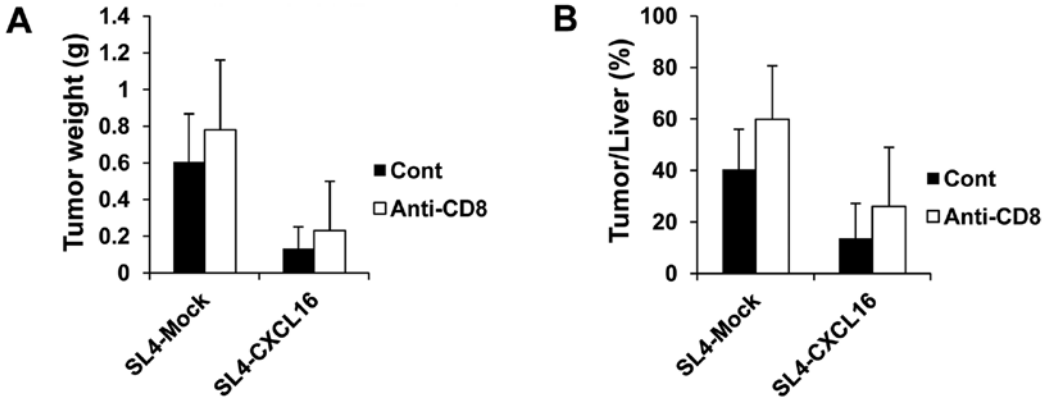

Figure 4. Inhibition of liver metastasis by CXCL16 expression was not changed in CD8 T cell depleted mice. (A and B) Results of injecting SL4 cells ($1.5x10^5$ cells/200 μl/mouse) i.p.v. into CD8 T cell-depleted mice. (A) The weight of tumor and (B) tumor weight/liver weight as a percentage. Similar results were obtained from repeated experiments using 8 mice/group and a representative result for each group is shown.

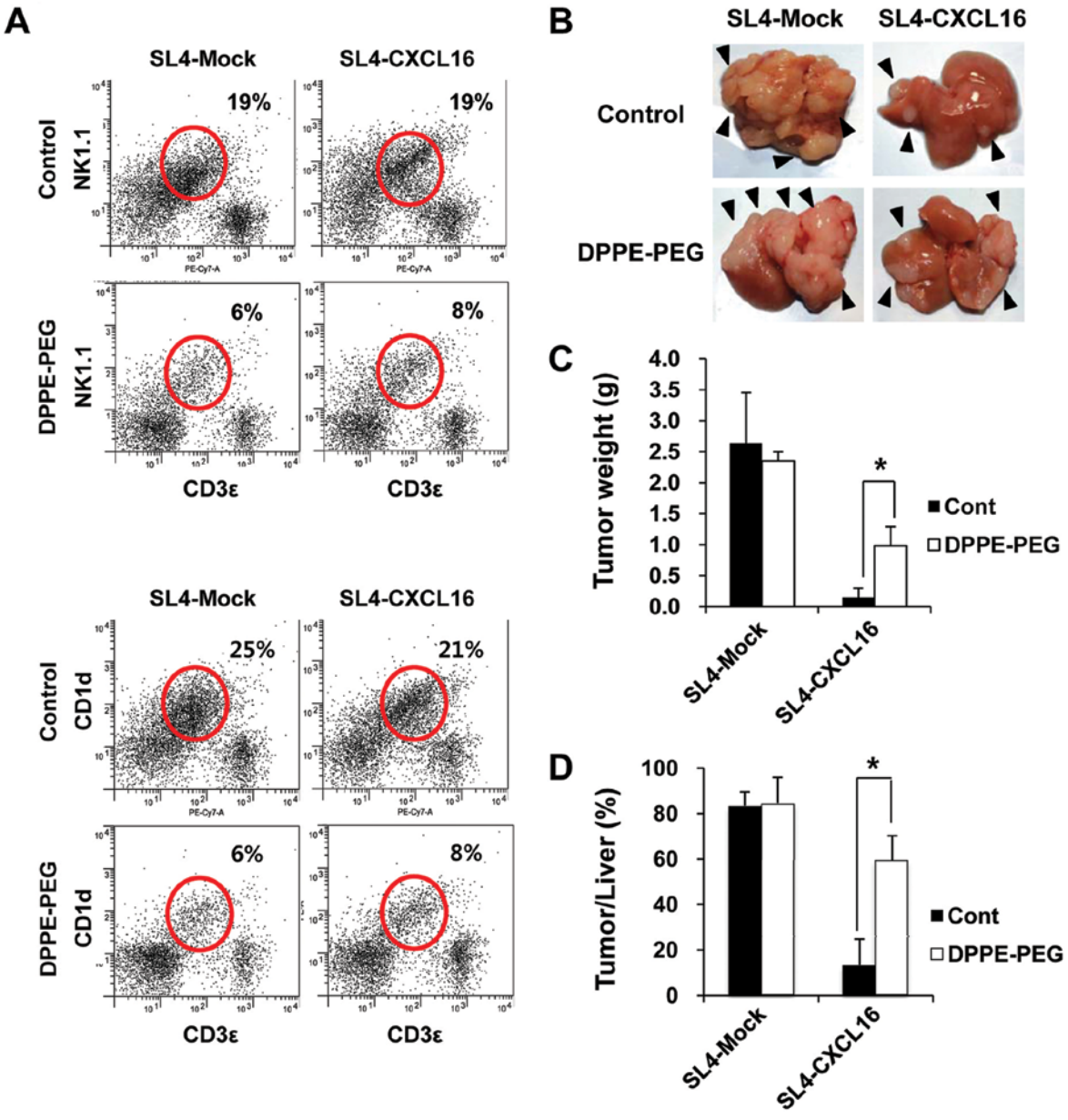

Figure 5. Inhibition of liver metastasis by CXCL16 expression was recovered in NKT cell depleted mice. (A) NKT cells were depleted by DPPE-PEG treatment. (B) Results of injecting SL4 cells ($1.5x10^5$ cells/200 μl/mouse) on liver metastasis in control and DPPE-PEG-treated mice. Black arrows indicate nodules of tumors. (C) The weight of tumors and (D) tumor weight/liver weight as a percentage. Similar results were obtained from repeated experiments using 8 mice/group and a representative result for each group is shown. *P-values <0.05 denote statistically significant differences.

tissues to confirm which immune cells were recruited to the liver by CXCL16. Among the CXCR6-expressing cells, mRNA levels of CD8 T cell (CD8), NKT cell (Vα14-Jα281) and T cell (CD3ε) markers, but not CD4 T cell marker (CD4), were increased 6-, 10- and 4-fold compared with SL4-Mock (Fig. 2C). These results suggested that CXCL16 expression in metastatic tissues recruited CXCR6-expressing cells and then inhibited metastasis to the liver.

Inhibition of liver metastasis by CXCL16 expression is recovered in nude mice. To confirm that CXCR6-expressing T cells are involved in liver metastasis by CXCL16- expressing CRC cells, we performed intraportal vein (i.p.v.) injections of SL4 cells ($7.5x10^4$ cells/200 μl PBS/mouse) into nude mice (T cell-deficient mice). Metastasis to the liver did not differ significantly in the SL4-CXCL16 group (Fig. 3). Inhibition of metastasis to the liver by CXCL16 expression was not observed in T cell-deficient mice. These results indicate that T cells mediated the inhibition of liver metastasis by CXCL16.

CD8 T cells are not related to inhibition of liver metastasis by CXCL16. Activated CD8 lymphocytes were recruited to inflamed liver in a mouse model and human patients (39,40). Thus, we examined the role of CD8 T cells in the inhibition of metastasis by CXCL16 in CD8 T cell-depleted mice. To analyze lymphocytes in tumor tissues, we injected SL4-Mock and SL4-CXCL16 cells ($1.5x10^5$ cells/200 μl PBS/mouse) and sacrificed the animals at 17 days. In the CD8 T cell-depleted group, liver metastasis was decreased by CXCL16 expression the same as in the control group. Tumor weight and tumor/liver weight percentage were not changed in the CD8 T cell-depleted mice (Fig. 4). These results suggested that CD8 T cells were not related to the inhibition of liver metastasis by CXCL16.

Inhibition of liver metastasis by CXCL16 is recovered in NKT cell-depleted mice. NKT cells present in the liver and the activation of NKT cells regulate tumor surveillance in the liver microenvironment and suppresses liver metastasis (24-30). Thus, we examined whether NKT cells are involved in the inhibition of metastasis by CXCL16. We injected SL4-Mock and SL4-CXCL16 cells ($1.5x10^5$ cells/200 μl PBS/mouse) into C57BL/6 mice and DPPE-PEG-treated mice. DPPE-PEG has been reported to be used to inhibit the action of NKT cells (38). The expression of NK1.1 and CD1d in metastatic tumor tissues was measured by FACS analysis. Thus, we used DPPE-PEG as a NKT cell-specific antagonist. As expected, levels of NK1.1 and CD1d were decreased by the DPPE-PEG treatment (Fig. 5A). In addition, the inhibition of liver metastasis by CXCL16 recovered in NKT cell-depleted mice (Fig. 5B). Tumor weight and tumor/liver weight percentage were significantly recovered in NKT cell-depleted mice (Fig. 5C and D). These results indicate that NKT cells are necessary for the inhibition of liver metastasis by CXCL16-expressing cancer cells.

Discussion

Previously, we reported a relationship between the expression of CXCL16 and prognosis of CRC patients, therefore CXCL16 is expected to be a new biomarker for CRC (15). From our report, a recent study found CXCL16 and its receptor CXCR6 to be related to various types of cancer, such as prostate cancer and epithelial ovarian carcinoma, respectively (36,37). However, there is no appropriate treatment for liver metastasis. Therefore, we determined the relationship between CXCL16 and the liver metastasis of CRC cells.

In this study, we investigated the role of cancer-derived CXCL16 and the possibility of gene therapy using CXCL16. We established cells stably overexpressing CXCL16 using the mouse colon 38 SL4 cell line and inoculated SL4-Mock and SL4-CXCL16 cells into the intraportal vein to confirm the effect of CXCL16 expression on liver metastasis. Liver metastasis of CRC cells was decreased by CXCL16 in tumor tissues (Fig. 2A and B). We carried out a microarray assay to confirm the difference between WT and CXCL16-expressing cells. The microarray data showed that there was no significant difference in metastasis-related factors (data not shown). Therefore, we focused on organ-specific immune cells which related to CXCL16. CXCR6, a specific receptor of CXCL16, is expressed in CD4 T cells, CD8 T cells and NKT cells. Real-time PCR data suggested that CD8 T cells and NKT cells were generated in CXCL16-expressing tumor tissues (Fig. 2C).

Since activated CD8 lymphocytes were recruited to inflamed liver in a mouse model and human patients (39,40), we focused on the involvement of CD8 T cells. Thus, we examined the effect of CXCL16 on liver metastasis in CD8 T cell-depleted mice, however, there was no difference from the CD8 T cell-depleted group (Fig. 4). This is probably because among the CD8 T cells, $CD8^+$ Treg cells may be produced and suppress the proliferation and function of effector T cells (41).

Nevertheless, NKT cells make up approximately 1-2% of the lymphocytes in the spleen and account for approximately 30% of lymphocytes in the liver and these NKT cells express high levels of CXCR6. The CXCL16 and CXCR6 axis has been reported to act as a regulator of NKT cell functions such as inhibition of cancer and metastasis (21,34,35). It was reported that neutralization of CXCL16 enhanced metastasis to the liver by B16 melanoma cells, but not lung metastasis (28). These reports suggested that CXCL16 and CXCR6-expressing NKT cells are involved in metastasis to the liver. Therefore, we examined the effect of CXCL16 on the liver metastasis of CRC cells in NKT cell-depleted mice.

Liver metastasis in the control SL4-CXCL16 group was increased by NKT cell depletion (Fig. 5). However, this is an unsettled issue that remains to be clarified in future studies. Markedly, liver metastasis was not increased in the SL4-Mock group, whereas NKT cells were depleted by DPPE-PEG. α-GalCer (NKT cell activator) treatment did not reduce the progression of tumors, as Th1 response by NKT cells was reduced in $CXCL16^{-/-}$ mice (28). It is possible that NKT cells, recruited by CXCL16, are CXCR6 positive and invariant NKT cells which has cytotoxicity to the tumor cells, conversely immature NKT cells did not effect to the metastasis. Further studies are needed to investigate the population and functions of NKT cells augmented by CXCL16-positive CRC compared with CXCL16-negative CRC in the experimental model and clinical samples.

Overexpression of chemokines in cancer can be used to recruit immune cells for antitumor responses and cytotoxicity to cancer cells. It has been reported that CCL21 and CX3CL1

overexpression reduced colon adenocarcinoma growth and inhibited primary cancer and metastasis of neuroblastoma, respectively. However, these results are conducted in an experimental model (42). Reasonable evidence exists that CXCL16 shows the possibility of application in antitumor therapy, as we reported that spontaneous CXCL16 expression is related to the pathology of CRC in clinical cases (15), not only in an experimental model.

Herein we reported that cancer-derived CXCL16 suppresses the liver metastasis of CRC cells by augmenting NKT cells to the metastatic organ. Therefore, our findings indicate that a cancer gene therapy strategy supporting the accumulation of NKT cells by CXCL16, might be an effective therapeutic approach against liver metastasis in CRC.

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