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Cancer-derived Exosomes Activate Immune Surveillance and Suppress Peritoneal Metastasis of Murine Colonic Cancer

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Abstract. *Background: Colonic cancer is associated with a low incidence of peritoneal metastasis compared with gastric cancer; however, the reason for this remains unclear. In this study, a model of peritoneal dissemination using the CT26 murine colon cancer cell line was used to analyze the physiological roles of cancer-derived exosomes. Materials and Methods: Exosomes were collected from the supernatant of CT26 cell culture by ultracentrifugation. The number of peritoneal disseminations in two mouse models of colonic cancer pre-administered exosomes or phosphate-buffered saline were compared. Results: Cancer-derived exosomes suppressed peritoneal dissemination compared to phosphate-buffered saline. After administration of exosomes, the number of intraperitoneal macrophages and the expression of inducible nitric oxide synthase increased. Furthermore, cancer-derived exosomes increased activated natural killer cells and interferon-γ expression. Conclusion: Tumor-derived exosomes from colonic cancer may suppress peritoneal metastasis via an immunological mechanism.*

Peritoneal metastasis is a type of distant metastasis in which the primary tumor invades the serosa. Cancer cells at the invasive front detach from the tumor, enter the intraperitoneal cavity, and ultimately attach and colonize the peritoneum. Distant metastases occur *via* a multi-step process, including matrix degradation, adhesion, and angiogenesis (1-3). Several other factors, such as the epithelial–mesenchymal transition and the immune system, are also involved in peritoneal metastasis (4, 5).

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Peritoneal carcinomatosis, specifically, is a typical pattern of metastasis in gastrointestinal cancer, which recurs after 29% of all surgical resections (6-8). Approximately 70% of cases of ovarian cancer also metastasize to the peritoneum (9). On the contrary, only 7% of patients with colorectal cancer are diagnosed with synchronous peritoneal carcinomatosis (10). The rate of peritoneal recurrence following surgical resection is only 2.3% (11); however, the underlying mechanisms responsible for the comparatively low incidence of peritoneal metastasis of colorectal cancer have not yet been elucidated.

Similar to angiogenesis and fibrosis, players of the immune system are an integral part of the tumor microenvironment. As part of the acquired immune response, dendritic cells present tumor antigens to T-cells and activated cytotoxic T-cells kill cancer cells (12, 13). Alternatively, M2 macrophages, which are myeloid-derived suppressor cells, produce anti-inflammatory cytokines, and promote tumor progression (14). Other cells that participate in innate immune responses, including M1 polarized macrophages and natural killer (NK) cells, also play important roles in eliminating cancer cells and producing cytokines to induce acquired immunity (15, 16), *e.g.* the interaction between CD11a/CD18 and intercellular adhesion molecule-2 activates NK cells and suppresses peritoneal dissemination (16, 17).

Exosomes are small membrane vesicles (50-150 nm in diameter) released by a myriad of cells, including immune cells, mesenchymal cells, and cancer cells (12). They contain proteins, DNA, mRNAs, and microRNAs of donor/host cell origin (12, 16-20). Host cell-derived exosomes can modulate the function of recipient cells (18). For instance, cancer-derived exosomes can modify the tumor microenvironment to accelerate cancer progression and metastasis (18). They can also promote angiogenesis in the tumor microenvironment and enhance tumor fibrosis (19). Cancer-derived exosomes express integrin, which alters the microenvironment pre-metastatically in organs, and fosters hematogenous metastasis (20). Furthermore, growing evidence suggests that these exosomes

may promote immune evasion by cancer cells (21). In contrast, exosomes derived from pre- or non-metastatic cancer stimulate nuclear receptor subfamily 4 group A member 1 (NR4A1)-positive monocytes and reduce lung metastasis (22). These data suggest that cancer-derived exosomes have multiple roles, and their involvement in tumor progression and metastasis remains controversial.

In this study, we analyzed the potential physiological roles of tumor-derived exosomes in the peritoneal microenvironment and in peritoneal metastasis using a murine colon cancer cell line and two mouse models of colon cancer.

Materials and Methods

Cell culture. The murine colon carcinoma cell line CT26 was purchased from the American Type Culture Collection (Manassas, VA, USA), and murine leukemia macrophage cell line RAW-264.7 were purchased from European Collection of Authenticated Cell Cultures (Public Health England, Portadown, UK). They were maintained in RPMI1640 (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (Sigma-Aldrich Japan. Inc., Tokyo, Japan) and incubated at 37˚C in 5% CO_2. Cells were tested for mycoplasma using e-Myco™ Mycoplasma PCR detection kit (iNtRON Biotechnology, Seongnam, Republic of Korea) according to the manufacture's protocol. Primary mouse intraperitoneal cells and splenocytes were isolated from BALB/c mice (7-9 weeks old) and cultured in RPMI with 15% fetal bovine serum.

Exosome isolation. CT26 cells in passage 4-8 were seeded in 225-cm^2 culture flasks at 5×10^6 cells/flask (25 ml/flask) and cultured to 80% confluency, and then they were cultured in 25 ml exosome-free medium for 48 h. The conditioned medium was the collected and centrifuged at 400 × *g* and 4˚C for 10 min to remove floating cells. The supernatant was filtered (0.22 μm, Hawach Scientific, Xi'an, PR China) to remove contaminating micro-vesicles and cell debris. The filtrate was centrifuged at 100,000 × *g* and 4˚C for 2 h. Exosome pellets were resuspended, washed with phosphate-buffered saline (PBS), centrifuged at 100,000 × *g*, 4˚C for 2 h, and resuspended in PBS (Nacalai Tesque). The density and total amount of proteins in exosome resuspended in PBS were measured with a Protein Assay Bicinchoninate kit (Nacalai Tesque) using Microplate reader (Infinite M200; Tecan Austria GmbH, Grödig, Austria). The numbers and size of exosomes were directly tracked using the Nanosight NS300 system (NanoSight Technology, Malvern, UK).

Western blotting. Exosomes and lysates from CT26 cells (4 μg of protein/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Merck Millipore, Burlington, Vermont, USA). Following a 1-h incubation with Blocking One (Nacalai Tesque), membranes were probed with primary antibodies against CD9 (rabbit monoclonal, dilution 1:2,000; cat. no. ab92726; Abcam, Cambridge, UK) and β-actin (rabbit monoclonal, dilution 1:1,000; cat. no.#4970s; Cell Signaling Technology, Danvers, MA, USA) at 4˚C, overnight. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, dilution 1:4,000; cat. no. ab6721; Abcam) for 1 h at 25˚C. Proteins were detected using enhanced chemiluminescence reagent (ECL; Amersham, Little Chalfon, UK) and ImageQuant LAS-4000 (GE Healthcare, Fairfield, CT, USA). Quantitative assay was performed using ImageQuant TL software (GE Healthcare, Chicago, IL, USA). The experiment was repeated twice.

Cell proliferation assay. CT26 or RAW-264.7 cells in passage 4-10 which reached 80% confluency were seeded in 96-well flat-bottom plates at a density of 2×10^3 cells/well (100 μl/well) and 10 μl PBS or exosomes (1 μg/10 μl) were added. The number of viable cells was measured every 12 h using CCK-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Following a 4-h incubation, the absorbance was read at 450 nm using an Infinite M200 microplate reader (TECAN; Männedorf, Zürich, Switzerland). The experiment was repeated three times independently.

Cell migration assay. The migratory capacity of CT26 cells was tested by a wound-healing assay. CT26 cells in passage 4-10 were incubated in 10-cm dishes to 80% confluency, and 100 μl PBS or exosomes (50 μg/100 μl) were added. Next, a thin scratch was generated on a confluent cell monolayer with a pipette tip. Images were captured every 12 h in the same three locations along the wound using a transmission light microscope equipped for live-cell imaging (IX71; Olympus Corporation, Tokyo, Japan) at ×40 magnification, and the gap was measured using ImageJ software ver. 1.50i (National Institutes of Health). The experiment was repeated three times.

Animals. Female mice (BALB/c, 7-9 weeks old) and female nude mice (BALB/c-nu, 6-8 weeks old) were purchased from SLC (Japan SLC, Inc., Shizuoka, Japan). The mice were reared in a pathogen-free environment under a 12/12 h light/dark cycle at room temperature and had free access to food and water. The health and behavior of the mice were monitored every day. All animal studies were approved and conducted according to the guidelines established by Shiga University of Medical Science (approval no. b2015-24). A total of 72 mice were used, and at the end of the study, all remaining mice were sacrificed by cervical subluxation after administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). After transplantation of cancer cells, difficulty eating and consuming water, abnormal posture, long-term appearance abnormalities with no signs of recovery (diarrhea, bleeding, dirt on the outside), weight loss of 20% or more in a few days, and tumors of 10% or more of body weight were considered to indicate ultimate cancer death, and the pain was eliminated by immediately performing sacrifice; 12 mice were deemed to have died of cancer under this definition.

Mouse models. Ten BALB/c mice and 28 BALB/c-nu mice were used for mouse models of peritoneal metastasis. BALB/c mice were injected intraperitoneally with CT26-derived exosomes (10 μg/300 μl) or 300 μl PBS every other day for 21 days. On day 22, 1×10^4 CT26 cancer cells were suspended in 0.5 ml PBS and injected intraperitoneally into the mice. Mice were euthanized by cervical subluxation under anesthesia on day 43 to evaluate tumors. BALB/c-nu mice were injected intraperitoneally with CT26-derived exosomes (20 μg/300 μl) or 300 μl PBS once per day for 3 days. On day 4, mice were injected intraperitoneally with 1×10^4 CT26 cancer cells suspended in 0.5 ml PBS. These mice were euthanized on day 18.

For examination of overall survival, mice were euthanized when severe disease symptoms developed.

After euthanasia, the small intestine was removed, and peritoneal metastatic nodules counted *via* visual inspection. The tumors, peritoneum, small intestines, livers and lungs were collected and prepared for pathological examination.

Forty-two BALB/c-nu mice were used for examination of the peritoneal microenvironment. BALB/c-nu mice were injected intraperitoneally with CT26-derived exosomes (20 μg/300 μl) or 300 μl PBS once per day for 3 days. On day 4, mice were euthanized and abdominal walls and intraperitoneal fluid containing cells were collected. The samples from each mouse were divided into abdominal wall for pathological examination, peritoneum for quantitative reverse transcription polymerase chain reaction (RT-qPCR), ascites for cytokine array, and intraperitoneal cells for flow cytometry.

Each experiment using the mouse model was performed once.

Hematoxylin and eosin and Immunohistochemistry. Paraffin-embedded specimens were sliced into 4 μm sections. For hematoxylin and eosin staining, after deparaffinization and rehydration, slides were stained with hematoxylin for 5 min at 25˚C (Muto Pure Chemicals, Tokyo, Japan). Slides were washed with distilled water, and stained with eosin (Muto Pure Chemicals) (diluted ×2 in 100% ethanol) for 2 min at 25˚C.

For immunohistochemistry, after deparaffinization and rehydration, slides were heated in ImmunoSaver (Nisshin EM, Tokyo, Japan) (diluted 200× in distilled water) for 45 min at 98˚C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Kanto Chemical, Tokyo, Japan) in methanol (Nacalai Tesque) for 10 min at room temperature. After blocking with Blocking One Hist (Nacalai Tesque), slides were incubated overnight with primary antibodies to mesothelin (rat monoclonal, dilution 1:100; cat. no. D053-3; MBL, Nagoya, Japan), α-smooth muscle actin (mouse monoclonal, dilution 1:100; cat. no. ab7817; Abcam), fibroblast activation protein (rabbit polyclonal, dilution 1:400; cat. no. ab28244; Abcam), and podoplanin (syrian hamster monoclonal, dilution 1:300; cat. no. ab11936; Abcam). Samples were incubated with a horseradish peroxidase-conjugated secondary antibody (Simple Stain Mouse MAX PO; Nichirei Bioscience, Tokyo, Japan) for 30 min at 25˚C. Visualization was performed using 3,3'-diaminobenzidine (Nichirei Bioscience). All samples were counterstained with hematoxylin. Images were obtained using a microscope (Eclipse 90i, Nikon, Tokyo, Japan) at ×400 magnification. The number of positive cells was qualitatively evaluated in five randomly selected fields of view.

RNA isolation and RT-qPCR. Peritoneum and intraperitoneal cells of BALB/c-nu mice which collected the day after intraperitoneal injection of cancer-derived exosomes or PBS once daily for 3 days, and primary mouse intraperitoneal cells and splenocytes from BALB/c mice were examined for mRNA expression by RT-qPCR. Total RNA from mouse peritoneum was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from RAW-264.7 cells and mouse intraperitoneal macrophages and splenocytes using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. Complementary DNA was generated from total RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. Detection and comparative expression analysis of mRNA levels were performed by real-time PCR using LightCycler 2.0 (Roche, Basel, Switzerland). A 20-μl reaction mixture contained 0.5-3 μg of target cDNA, 10 μM of each primer listed below and 4 μl Master SyBr Green I mix (LightCycler FastStart DNA MasterPLUS SYBR Green I; Roche). The PCR cycling conditions were 95˚C for 10 min followed by 50 cycles of 95˚C for 10 s, 68˚C for 10 s and 72˚C for 30 s. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23). glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as internal control, and all samples were assayed in triplicate and repeated three times. Forward (F) and reverse (R) PCR primers were as follows and synthesized by Eurofins Genomics (Tokyo, Japan): *Gapdh* F: 5'-AGGTCGGTGTGAACGG ATTTG-3', *Gapdh* R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; E-cadherin (*Cdh1*) F: 5'-TGTCTGGCGTATAATGTTGGC-3', E-cadherin (*Cdh1*) R: 5'-CTTGTGGGTTCGTAAGCTGCT-3'; integrin a2 (*Itga2*) F: 5'-TGTCTGGCGTATAATGTTGGC-3', integrin a2 (*Itga2*) R: 5'-CTTGTGGGTTCGTAAGCTGCT-3'; integrin b1 (*Itgb1*) F: 5'-ATGCCAAATCTTGCGGAGAAT-3', integrin b1 (*Itgb1*) R: 5'-TTTGCTGCGATTGGTGACATT-3'; matrix metallopeptidase 1 (*Mmp1*) F: 5'-CCAGGGTGTTGG ACTATGTTTG-3'), *Mmp1* R: 5'-CCCCGAGGAAGGTT CATCTTTA-3'; and inducible nitric oxide synthase (iNOS), *Nos2* F: 5'-GTTCTCAGCCCAACAATACAAGA-3', *Nos2* R: 5'-GTGGACGGGTCGATGTCAC-3'.

Isolation of mouse intraperitoneal cells. After the mice were sacrificed under anesthesia, 5 ml cold PBS was injected intraperitoneally. Peritoneal fluid containing cells was then collected and centrifuged at 250 × *g* and 4˚C for 5 min. The supernatant (ascites) was collected and used for cytokine array analysis. The cell pellets (intraperitoneal cells) were resuspended, quantified, and characterized by flow cytometric analysis. The number of intraperitoneal cells was determined using trypan blue (Thermo Fisher scientific, Tokyo, Japan).

Cytokine array. Ascites of BALB/c-nu mice were collected the day after intraperitoneal injection of cancer-derived exosomes or PBS once daily for 3 days. Cytokine levels in ascites were analyzed with a mouse cytokine array (Proteome profiler mouse cytokine array panel A; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Images were captured using ImageQuant LAS-4000, and the pixel density was determined by ImageQuant TL software.

Flow cytometry. The population of macrophages in intraperitoneal cells was detected by flow cytometry. The obtained intraperitoneal cells from the mice treated with exosome or PBS were incubated on ice in the dark for 20 min with antibodies of a surface marker, CD3-fluorescein isothiocyanate (rat monoclonal; cat. no. 11-0032-82; eBioscience, Santa Clara, CA, USA) and F4/80-phycoerythrin (PE)-cyanine7 (rat monoclonal; cat. no. 25-4801-82; eBioscience). After rinsing, cells were resuspended in 300 μl PBS. The expression of interferon-γ (IFNγ) by NK cell was determined by flow cytometry. Mouse spleen cells were seeded in 24-well plates at a density of 1×10^7 cells/well (500 μl/well) and incubated with 70 μl of PBS or exosomes (20 μg/70 μl) for 24 h. Six hours before cell collection, Brefeldin A (BioLegend, San Diego, CA, USA), a specific inhibitor of protein trafficking, was added to the medium to block the transport of secreted proteins from endoplasmic reticulum to Golgi apparatus. After culturing, the spleen cells were blocked on ice for 20 min with Fc receptor-blocking reagent, mouse (Miltenyi Biotec,

Bergisch Gladbach, Germany), and then incubated on ice in the dark for 20 min with antibodies to the surface markers CD3-fluorescein isothiocyanate, F4/80- Phycoerythrin (PE) -cyanine7, and CD49b-PE (rat monoclonal; cat. no. 12-5971-82; eBioscience). After washing with PBS, fixation and permeabilization reagents (FOXP3/Transcription Factor Staining Buffer Set; eBioscience) were used to detect intracellular cytokines. Cells were incubated on ice for 20 min with anti-mouse IFNγ-allophycocyanin (rat monoclonal; cat. no. 17-7311-82; eBioscience). After rinsing, cells were resuspended in 300 μl PBS. Flow cytometry was performed on a FACS Canto II (Becton Dickinson, Heidelberg, Germany) and analyzed with FlowJo v7 (Tree Star, Ashland, OR, USA).

Enzyme-linked immunosorbent assay (ELISA). Primary mouse splenocytes were stimulated for 24 h either with PBS or exosomes, and the culture supernatants were collected. Mouse IFNγ in culture supernatant was quantified using an ELISA kit (Mouse IFNγ Immunoassay; R&D Systems), according to the manufacturer's protocol. The absorbance was measured using an Infinite M200 microplate reader, and the reading at 540 nm was subtracted from the reading at 450 nm for wavelength correction. Samples were assayed in duplicated and repeated twice.

Statistical analysis. Statistical analyses were performed using International Business Machines Statistical Package for the Social Sciences Statistics v25 (IBM, Armonk, NY, USA). Data are expressed as the mean±standard error of the mean. Statistical analysis was performed using an unpaired Student's *t*-test to compare two groups. *p*-Values of less than 0.05 were considered statistically significant. The overall survival rate was calculated according to the Kaplan–Meier method and compared using log-rank test.

Results

CT26-derived exosomes did not affect CT26 cell growth or migration. To evaluate the potential autocrine effects of murine colon cancer (CT26)-derived exosomes on the CT26 parental cell line, we cultured CT26 cells for 48 h, collected the cell lysates and conditioned media, and isolated exosomes from the conditioned media by ultra-centrifugation. To verify that we had harvested exosomes, we evaluated the expression of β-actin (a protein expressed by all eukaryotic cell types) and CD9 (an exosome marker) in the cell lysates and particles extracted from the conditioned medium by western blot analysis. We found strong β-actin expression and CD9 expression in the cell lysates and conditioned medium isolate, respectively, indicating that we had indeed harvested CT26-derived exosomes (Figure 1A). To corroborate these findings, we measured the size of the particles isolated from the conditioned medium with a nanoparticle tracking system. The particles were approximately 200 nm in diameter, which is in the typical size range of exosomes (Figure 1B), verifying that we had isolated cancer-derived exosomes. To evaluate the potential effects of cancer-derived exosomes on the parental cell line, we incubated CT26 cells with PBS or CT26-derived exosomes and measured the absorbance at 450 nm every 12 h for 48 h to compare growth rates. We found that the exosome-treated cancer cells grew at a similar rate as those incubated with PBS (Figure 1C). We then evaluated migration with a wound-healing assay and found that cell migration was not altered by tumor-derived exosomes (Figure 1D and E). These results indicate that CT26 cells released exosomes which did not exert autocrine effects on the growth or migration of CT26 cells.

CT26-derived exosomes suppressed peritoneal dissemination of CT26 cells in immunocompetent mice. To evaluate the effects of colon cancer-derived exosomes *in vivo*, we used two mouse models of peritoneal metastasis. For the first model, we used BALB/c mice, which are immunocompetent mice with the same genetic background as the CT26 colonic cancer cell line. We injected the mice intraperitoneally with CT26-derived exosomes every other day for 21 days. On day 22, we injected the mice intraperitoneally with CT26 cancer cells. Mice were euthanized on day 43, small intestines were removed, and peritoneal metastatic nodules counted *via* visual inspection (Figure 2A). Peritoneal metastases were formed in the PBS-pretreated mice, whereas none were found in the exosome-pretreated mice (Figure 2B and C). We also found there were no lung or liver metastases. These results indicate that CT26-derived exosomes suppressed the formation of peritoneal metastases in immunocompetent mice.

CT26-derived exosomes suppressed peritoneal dissemination and prolonged survival in an immunodeficient mouse model of colonic cancer metastasis. Exosomes harbor host-derived DNA, miRNA, and proteins (12); therefore, exosomes may evoke acquired immunity in T- and B-cells. To exclude the effect of acquired immunity in the peritoneal metastasis of colonic cancer, we used an immunodeficient mouse model of peritoneal metastasis of colon cancer for the second model. We injected BALB/c-nu intraperitoneally with PBS or CT26-derived exosomes once per day for 3 days. On day 4, we injected the mice intraperitoneally with CT26 colonic cancer cells, euthanized them on day 18, and enumerated the peritoneal metastatic nodules in the small intestines (Figure 3A). The number of peritoneal metastases was significantly lower in the mice pretreated with cancer-derived exosomes compared with those pretreated with PBS ($p<0.05$) (Figure 3B and C). There were no lung or liver metastases. These data indicate that pretreatment with cancer-derived exosomes suppressed peritoneal metastasis in immunodeficient mice. These results suggest that CT26-derived exosomes suppressend the peritoneal metastasis of colonic cancer in a T-cell- and B cell-independent manner.

Next, to assess the effects of CT26-derived exosomes on survival, we injected BALB/c-nu mice intraperitoneally with PBS or CT26-derived exosomes once daily for 3 days. On

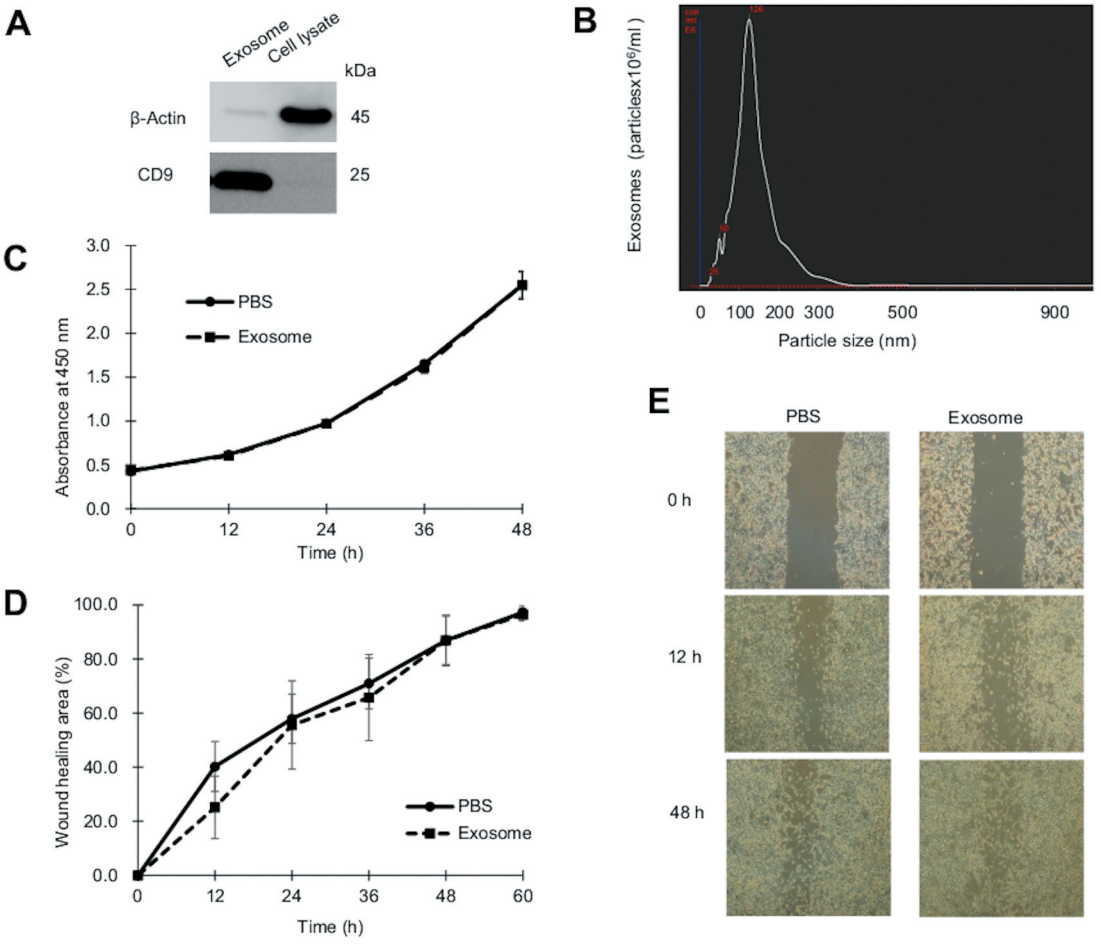

Figure 1. *CT26-derived exosomes did not affect the growth or migration of CT26 cells A: Western blot analysis of β-actin and CD9, an exosome marker, in the lysate of CT26 cells and the particles isolated from the media conditioned by CT26 cells (Exosome), respectively. B: Nanoparticle analysis of the size of particles isolated from the conditioned media, i.e., the exosomes. C: Analysis of the growth of CT26 cells incubated with PBS or with CT26-derived exosomes. Data are expressed as the mean±SEM (n=8). D: Quantitative cell migration analysis by wound-healing assay of CT26 cells incubated with phosphate-buffered saline (PBS) or with CT26-derived exosomes. Wound areas were measured every 12 h for 60 h in three different regions of the wounds. Data are expressed as the mean±SEM of three distinct locations along the wound. E: Representative images of the wound-healing assay acquired at 0, 12 and 48 h post-scratch.*

day 4, we injected the mice intraperitoneally with CT26 cells and recorded the survival duration of the mice until they all succumbed to cancer. We found that overall survival was significantly prolonged in mice pretreated with tumor-derived exosomes compared with the mice pretreated with PBS (Figure 3D). These data suggest that cancer-derived exosomes extended survival by suppressing peritoneal metastasis in this mouse model of colonic cancer.

Cancer-derived exosomes did not induce fibrosis or enhance expression of adhesion molecules in the peritoneum. The microenvironment of the metastatic site is crucial for cancer engraftment and growth (24). To evaluate the effects of cancer-derived exosomes on the peritoneal microenvironment, we injected BALB/c-nu mice intraperitoneally with PBS or CT26-derived exosomes once daily for 3 days. On day 4, we euthanized the mice and isolated the abdominal walls and adherent peritoneum, of which we created sections/slides and isolated total RNA. We assessed the expression of mesothelin, α-smooth muscle actin, fibroblast activation protein, podoplanin, and fibrosis- and lymphangiogenesis-related proteins by immunohistochemistry. There was no significant difference in the expression of these proteins in the peritonea of mice treated with exosomes compared with mice treated with PBS (Figure 4A). Since prior studies reported that proteases or adhesion molecules might be involved in peritoneal metastasis (3,25), we also evaluated the expression of *Cdh1, Itga2, Itgb1*, and matrix *Mmp1* in the mouse

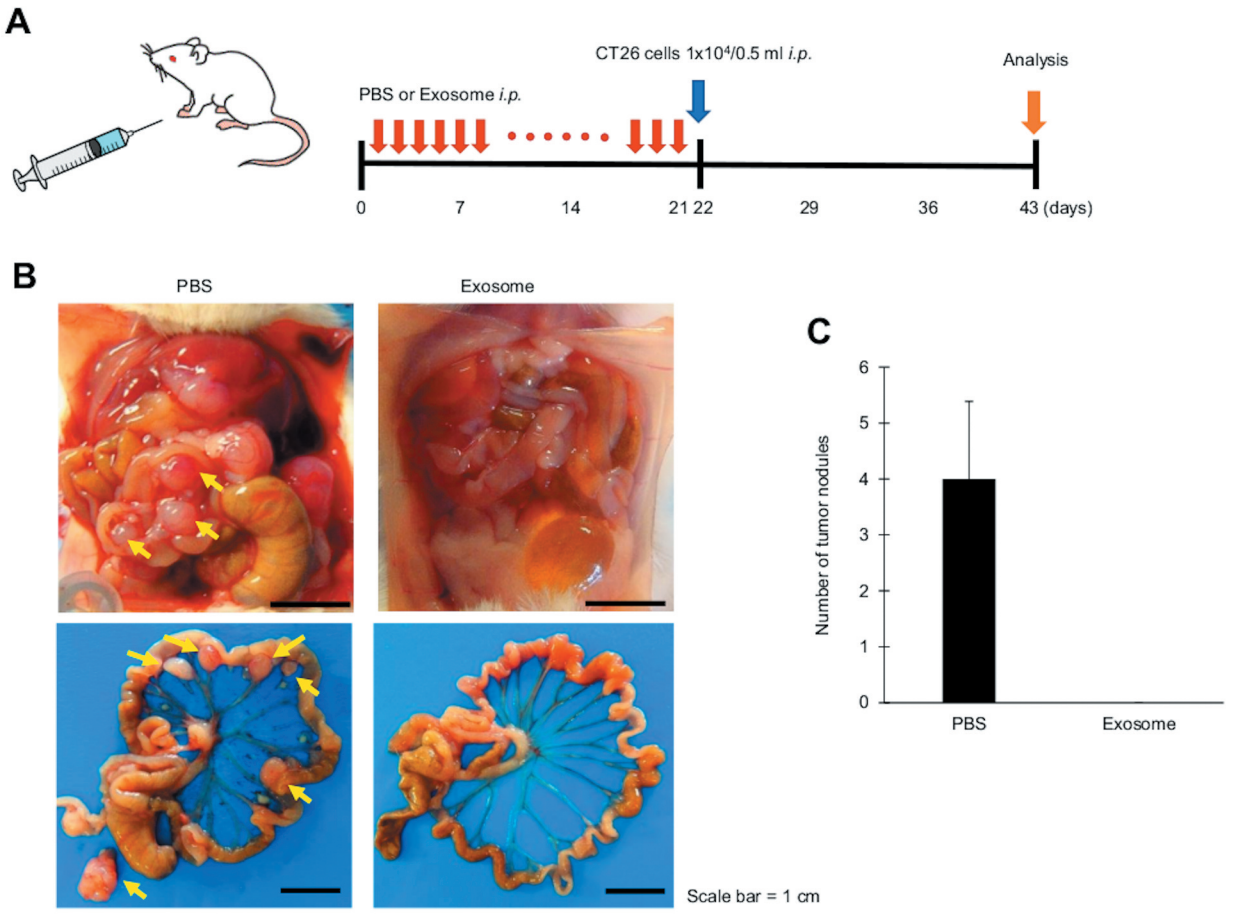

Figure 2. *CT26-derived exosomes suppress peritoneal dissemination in immunocompetent (BALB/c) mice. A: Schematic of an immunocompetent mouse model for peritoneal dissemination of colon cancer. B: Representative images of laparotomy (upper images) and small intestines (lower images) of mice pretreated with phosphate-buffered saline (PBS) or exosomes. Arrows indicate peritoneal metastases. C: Quantification of peritoneal metastatic nodules. Data are expressed as the mean±SEM (n=5).*

peritonea by qRT-PCR and found no significant difference in the expression of these mRNAs in the peritonea of the two treatment groups (Figure 4B). These data indicate that intraperitoneal injection of cancer-derived exosomes neither induced fibrotic changes nor upregulated the expression of adhesion molecules in the peritoneum.

Cancer-derived exosomes enhanced cytokine levels in ascites and induced migration of macrophages into the peritoneal cavity. To investigate the effect of cancer-derived exosomes on immune modulation in the peritoneal cavity, we injected BALB/c-nu mice intraperitoneally with PBS or CT26-derived exosomes once daily for 3 days. On day 4, we euthanized the mice and collected the intraperitoneal fluid, from which we isolated the ascites and cells. Since prior studies suggest that various cytokines may impact the cellular population of the peritoneum and alter its potential as a microenvironment for tumor metastasis (26-28), we assessed cytokine expression in the ascites with a cytokine array. We found that the expression of tumor necrosis factor a, chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11, CXCL13 and IFNγ, was higher in the ascites of mice injected with exosomes compared with those of mice injected with PBS (Figure 5A and B). These results suggest that cancer-derived exosomes enhance the levels of cytokines in ascites. Interestingly, when we compared the number of cells isolated from the intraperitoneal fluid in the two experiment groups, significantly more cells were isolated from the mice injected with cancer-derived exosomes compared with those injected with PBS (Figure 5C). To characterize the type of cells that migrated into the peritoneal cavity, we evaluated cell-specific markers by flow cytometry and found that the majority of intraperitoneal cells were $F4/80^{+}CD3^{-}$, indicating that they were macrophages (Figure 5D). These data suggest that

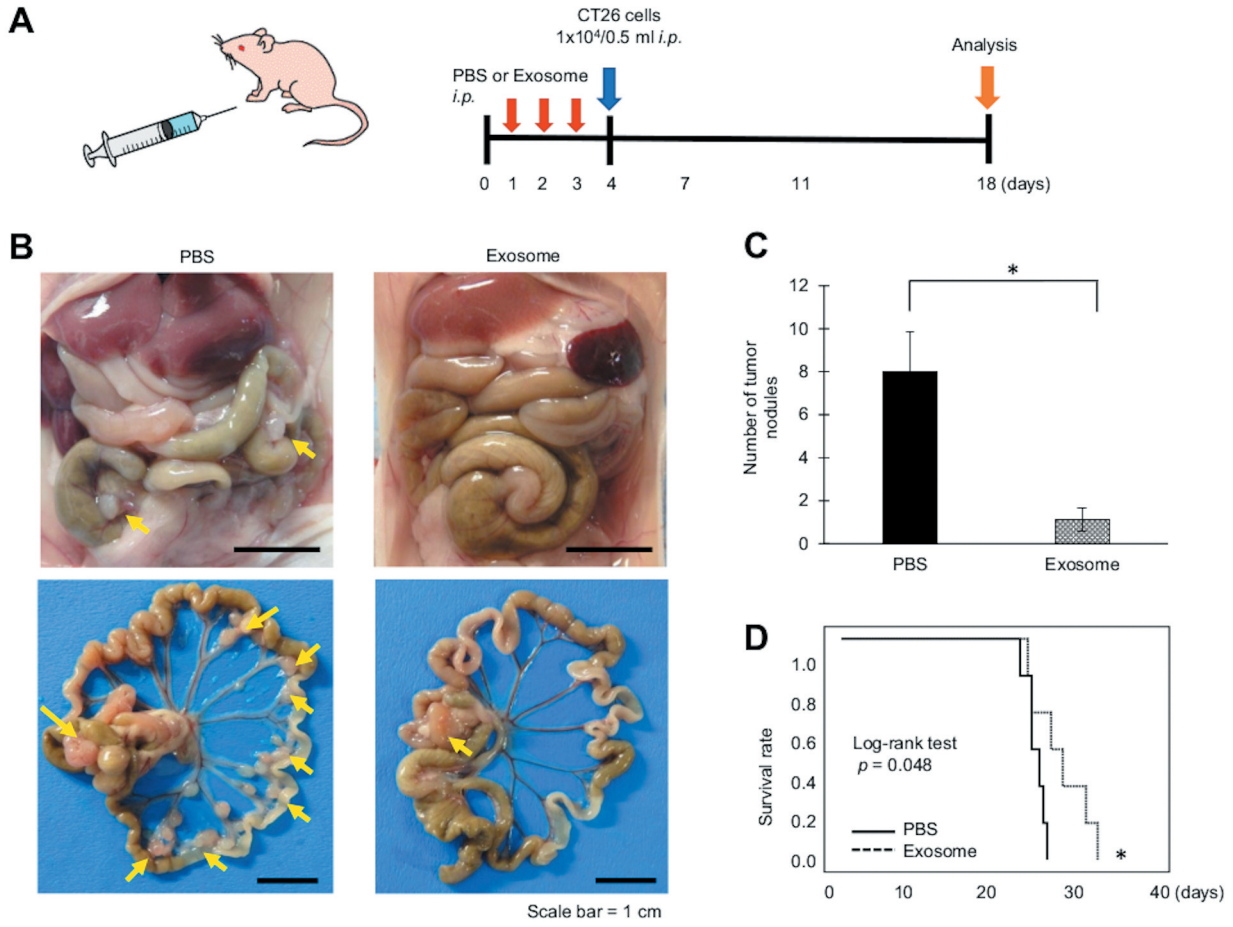

Figure 3. *CT26-derived exosomes suppress peritoneal dissemination and prolong survival in an immunodeficient mouse model of colonic cancer metastasis. A: Schematic protocol of an immunodeficient mouse model of peritoneal dissemination of colonic cancer. B: Representative images of laparotomy (upper images) and small intestines (lower images) of BALB/c-nu mice pretreated with phosphate-buffered saline (PBS) or exosomes prior to colonic cancer cell injection. Arrows indicate peritoneal metastases. C: Quantification of peritoneal metastatic nodules. Data are expressed as the mean±SEM (n=8). D: Kaplan–Meier analysis of the association of pretreatment and survival in immunodeficient mice pretreated with PBS or CT26-derived exosomes prior to colonic cancer cell injection. The log-rank test was used to compare the groups (n=6). *Significantly different at p<0.05.*

cancer-derived exosomes up-regulated peritoneal cytokine levels and induced the migration and accumulation of macrophages in the peritoneal cavity.

Cancer-derived exosomes induced NK cells to produce IFNγ and skewed differentiation toward M1 macrophages. Murine intraperitoneal cells were collected and incubated *in vitro* with tumor-derived exosomes or PBS for 24 h. mRNA levels of Nos2 significantly increased in cells incubated with cancer-derived exosomes compared with those incubated with PBS (Figure 6A). This result indicates that cancer-derived exosomes polarized macrophages to M1 status, which is an unfavorable condition for cancer metastasis. To elucidate the mechanism by which cancer-derived exosomes promoted macrophage accumulation and M1 polarization in the peritoneal cavity, we incubated the murine leukemia macrophage cell line RAW-264.7 with CT26-derived exosomes or PBS *in vitro*. Unexpectedly, incubation with cancer-derived exosomes did not affect the proliferation or iNOS expression of the macrophages (Figure 6B and C), indicating that cancer-derived exosomes did not affect macrophages directly, and the skewing toward M1 macrophage differentiation was induced by indirect effects elicited by cancer-derived exosomes.

Furthermore, cytokine array showed that IFNγ levels in the ascites of exosome-injected mice were elevated compared with that in mice injected with PBS, (Figure 5B). Since IFNγ induces M1 macrophage differentiation and exerts antitumor effects, we focused on NK cells, one of the major cell types that produce IFNγ. To this end, we harvested spleens from

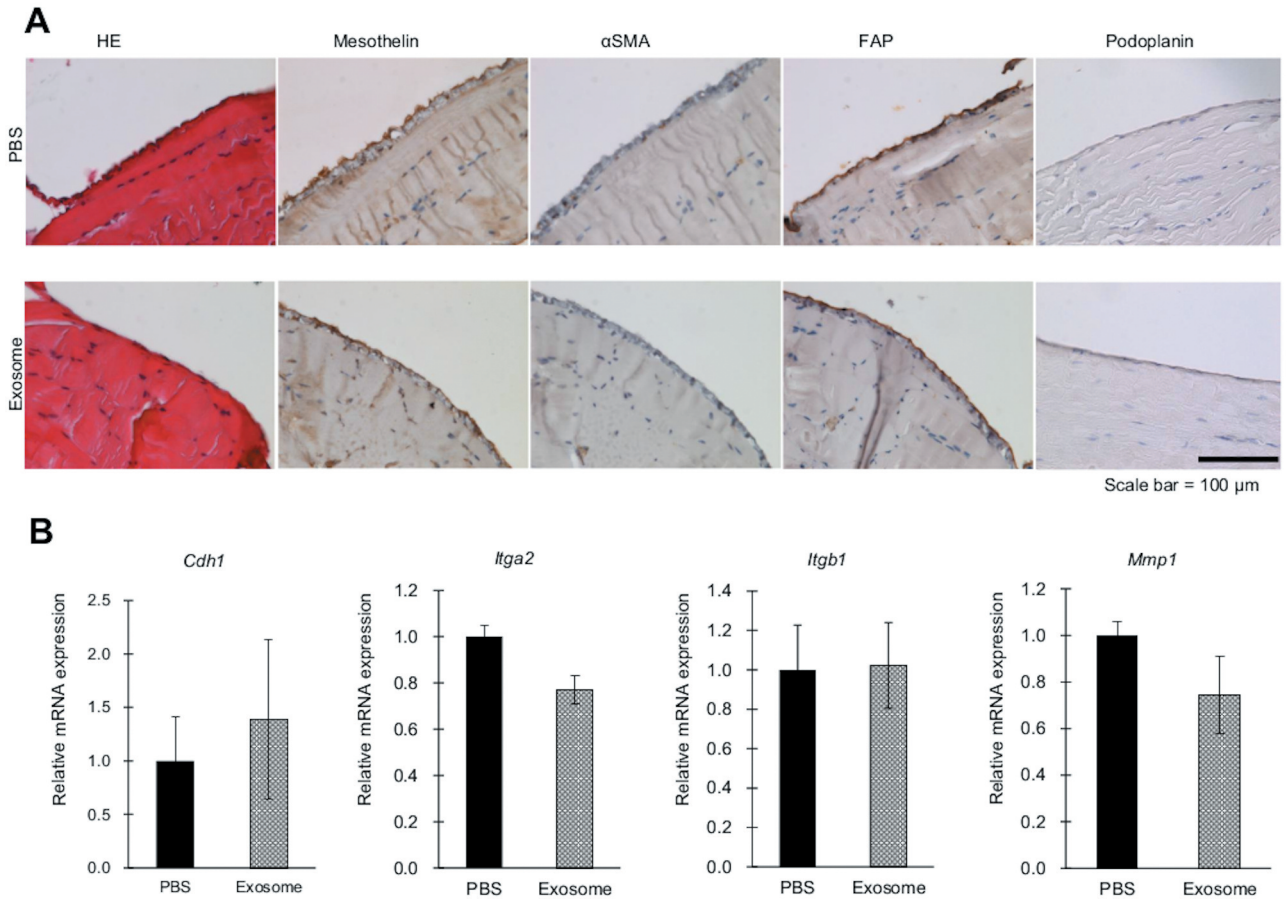

Figure 4. *CT26-derived exosomes did not induce fibrosis or enhance the expression of adhesion molecules in the peritoneum. Peritoneum/abdominal wall of BALB/c-nu mice were collected the day after intraperitoneal injection of cancer-derived exosomes or phosphate-buffered saline (PBS) once daily for 3 days. A: Representative images of hematoxylin and eosin (HE) staining and immunohistochemical analysis of the expression of the fibrosis- and lymphangiogenesis-related proteins, mesothelin, alpha-smooth muscle actin (αSMA), fibroblast activation protein (FAP) and podoplanin in peritoneum/abdominal wall sections of mice treated with PBS (upper images) or exosomes (lower images). B: Relative mRNA expression by quantitative reverse transcription polymerase chain reaction of E-cadherin (Cdh1), integrin a2 (Itga2), integrin a1 (Itga1), and matrix metallopeptidase 1 (Mmp1) in the peritoneum of mice injected intraperitoneally with PBS or cancer-derived exosomes. Data are expressed as the mean±SEM (n=3). Differences were not significant.*

BALB/c mice and created single-cell suspensions. We then cultured the splenocytes with tumor-derived exosomes or PBS for 24 had assessed the amount of IFNγ in the media by ELISA. IFNγ concentration was significantly higher in media conditioned by splenocytes incubated with exosomes compared with those incubated with PBS (Figure 6D). To confirm that NK cells were the source of the IFNγ, we incubated splenocytes with PBS or cancer-derived exosomes for 24 h prior to fluorescence-activated sorting analysis. Cells that were $CD49b^+$ and $CD3^-$ were considered NK cells. We found that incubation with cancer-derived exosomes induced NK cells to express significantly more IFNγ compared with PBS incubation (Figure 6E). These results suggest that cancer-derived exosomes promoted NK cells to secrete IFNγ and skewed differentiation toward M1 macrophages in the peritoneal cavity to prevent the peritoneal metastasis of colonic cancer cells.

Discussion

In this study, we investigated the effects of colonic cancer-derived exosomes on the peritoneal microenvironment and the incidence of peritoneal metastasis. Pretreatment with tumor-derived exosomes induced M1 peritoneal macrophages to express iNOS, and inhibited the formation of peritoneal metastasis in murine colonic cancer. Furthermore, tumor-derived exosomes induced NK cells to produce IFNγ. We, therefore, demonstrated that colonic cancer-derived exosomes

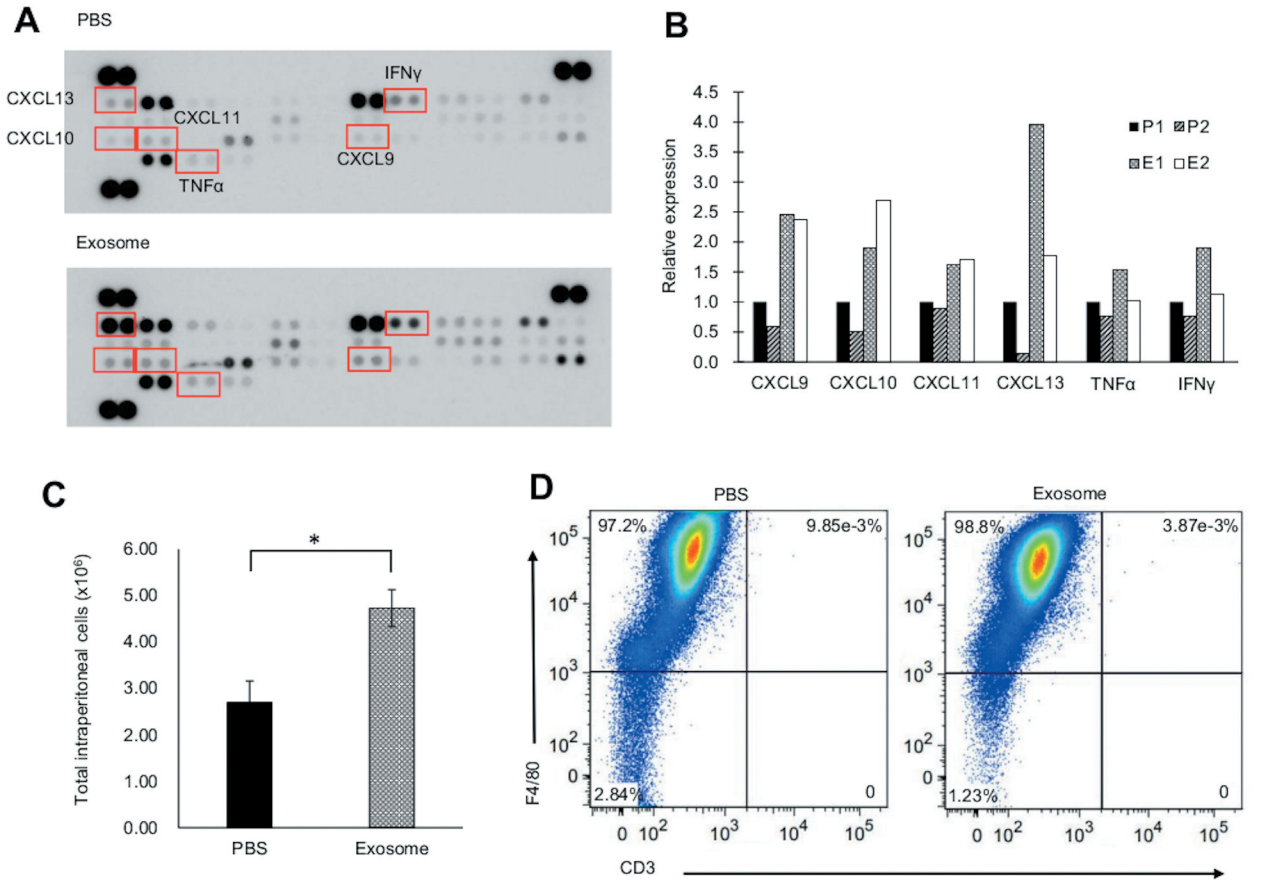

Figure 5. *Cancer (CT26)-derived exosomes enhanced the levels of cytokines in ascites and induced the migration of macrophages into the peritoneal cavity. The intraperitoneal fluid of BALB/c-nu mice were collected the day after intraperitoneal injection of cancer-derived exosomes or phosphate-buffered saline (PBS) once daily for 3 days. A: Cytokine array analysis of ascites isolated from the intraperitoneal fluid of mice injected with either PBS or exosomes. Tumor necrosis factor a (TNFα), chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11, CXCL13 and interferon-γ (IFNγ) are marked with red frames. B: Quantification of the relative levels of each cytokine of the cytokine array. P1; PBS treatment sample 1, P2; PBS treatment sample 2, E1; exosome treatment sample 1, E2; exosome treatment sample 2. C: Quantification by hemocytometry of the number of cells isolated from the intraperitoneal fluid of mice injected with either PBS or cancer-derived exosomes. Data are expressed as the mean±SEM (n=16). *Significantly different at p<0.05. D: Flow cytometric analysis of the F4/80⁺CD3⁻ population of intraperitoneal cells (macrophages) isolated as described in (C).*

may suppress colon cancer peritoneal metastasis by promoting the migration of M1 macrophages to the intraperitoneal cavity and by promoting the expression of IFNγ by NK cells.

Peritoneal metastasis requires multiple steps such as detachment of a cancer cell from the primary tumor and colonization in a distant organ (29, 30). In addition, tumor fibrosis can accelerate tumor progression and metastasis to local lymph nodes and beyond (24, 25, 29-33); however, we found that cancer-derived exosomes did not induce peritoneal fibrosis or alter the expression of cell adhesion molecules.

Some studies have evaluated the effects of tumor-derived exosomes on cancer cells, the tumor microenvironment, and metastasis (18-21). Tumor-derived exosomes can either induce or inhibit tumor-specific and non-specific immune responses (12, 21, 34). For example, tumor-derived exosomes were found to promote the expression of immune-activating cytokines and proteins, such as chemokine (C-C motif) ligand 5 (CCL5), major histocompatibility complex class II and heat-shock protein 70 (HSP70), to enhance antitumor effects (13, 35, 36); exosomes derived from pre-metastatic tumors suppressed lung metastasis by expanding the population of $Ly6C^{low}$ patrolling monocytes (22). In contrast, tumor-derived exosomes containing immune-suppressive cytokines or death receptor ligands such as transforming growth factor-b1, Fas ligand, or tumor necrosis factor-related apoptosis-inducing ligand suppress antitumor immunity (37, 38).

Peritoneal macrophages, one of the major components of the abdominal cavity microenvironment, are critical to

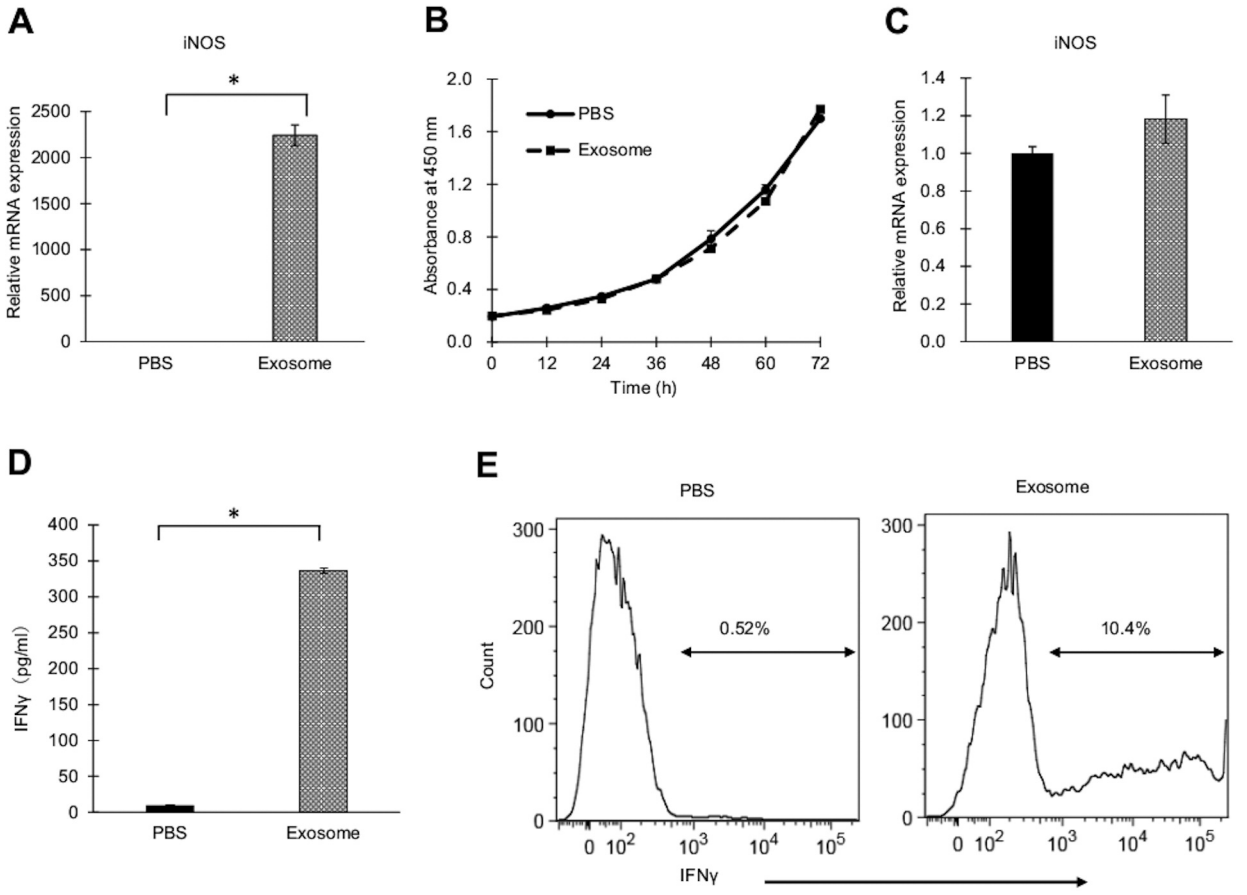

Figure 6. *CT26-derived exosomes activated natural killer cells and enhanced interferon-γ (IFNγ) secretion. A: Inducible nitric oxide synthase (iNOS) mRNA level by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in intraperitoneal cells ex vivo. B: Cell growth curve of the murine macrophage cell line, RAW264.7, incubated with phosphate-buffered saline (PBS) or with cancer-derived exosomes; (n=5). C: iNOS mRNA levels in RAW264.7 cells incubated with PBS or cancer-derived exosomes (n=2) by qRT-PCR. D: IFNγ levels in the media conditioned by splenocytes incubated with PBS or cancer-derived exosomes as determined by enzyme-linked immunosorbent assay. E: Flow cytometric analysis of the percentage of IFNγ-positive natural killer cells among splenocytes cultured for 24 h with PBS or cancer-derived exosomes. Data are expressed as the mean±SEM. *Significantly different at p<0.05 (n=2).*

preventing peritoneal implantation and metastasis of cancer cells. Tissue-specific macrophages are remarkably versatile, heterogeneous and plastic, and become activated *via* the effects of various cytokines, immune cells, and antigenic factors (14). Lipopolysaccharide and IFNγ induce M1 macrophages to produce pro-inflammatory cytokines and toxic agents such as nitric oxide and reactive oxygen species to protect against infection and tumor cells (15). Alternatively, interleukin-4 (IL4) and IL13 activate M2 macrophages to restrict inflammation and participate in tissue remodeling (14). Several reports have shown that induction of macrophage phenotype affects peritoneal metastasis (39, 40). In fact, Ryan *et al*. reported that media conditioned by nuclear factor-κB-deficient CT26 cancer cells induced M1 macrophages and reduced metastasis (41).

We found that compared to PBS, the intraperitoneal injection of cancer-derived exosomes enhanced expression of tumor necrosis factor α, CXCL9, CXCL10, CXCL11, IFNγ, and CXCL13 in ascites in the intraperitoneal fluid in BALB/c mice. CXCL11 is an IFNγ-inducible gene and an M1 macrophage marker (14, 42). Similarly to a previous study by Gastpar *et al*., which reported that HSP70 surface-positive, tumor-derived exosomes stimulated the cytotoxic activity of NK cells (36), we demonstrated here that tumor-derived exosomes markedly induced IFNγ production by NK cells. Notably, CT26 cancer cell lysates did not promote the expression of IFNγ by NK cells or reduce peritoneal metastasis in the mouse model. In addition, directly stimulating macrophages with tumor-derived exosomes did not induce M1 polarization. Although exosome contents can be modified by their parental cells (13), the

protein composition of exosomes and their parental cells differ, apart from sharing certain common membrane and cytosolic molecules (43). Rao *et al*. demonstrated that hepatocellular carcinoma cell-derived exosomes, displaying an array of HCC antigens, can elicit a stronger immune response than cell lysates *in vitro* and *in vivo* (44). A recent study reported that exosomal expression of HSP70 and toll-like receptor 2 can activate NK cells (45); however, additional studies are needed to characterize the molecular mechanism by which tumor-derived exosomes activate NK cells. Moreover, one of the limitations of our experiment is that it only used the murine colon cancer cell line, CT26. Exosomes are released by various cells but it is known that fewer exosomes are secreted from normal cells than from cancer cells. As the exosomes derived from fibroblasts accounted for 10 to 20% of CT26 cancer cells, we thought that exosomes derived from normal cells with such low secretion would not have sufficient physiological function, and we used PBS as a control. However, since exosomes contain proteins and nucleic acids peculiar to host cells, it is unclear whether tumors can be suppressed by exosomes from other cell lines of other organs.

In this study, we showed that treatment of CT26 colon cancer-derived exosomes from a mouse model of colon cancer, prior to intraperitoneal injection, promoted the production of IFNγ by NK cells, activated macrophages, and suppressed peritoneal metastasis, thereby prolonging overall survival. However, the specific mechanism by which tumor-derived exosomes activate NK cells remains unclear. Although the microenvironment of the intraperitoneal cavity serves as the site of multiple cell–factor interactions, characterizing the mechanism by which cancer-derived exosomes suppress peritoneal metastasis of colonic cancer, particularly through the use of such models, may inform the design of novel strategies to inhibit peritoneal metastasis of other cancer types.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

A.T. and T.M. conceived and designed the experiments. A.T., D.Y., and D.I. performed the experiments. A.T. performed the data analysis, wrote the main article and prepared the figures. T.M., K.M., S.M., T.S., and M.T. participated in the revision of the article critically for important intellectual content. All Authors read and approved the final article.

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