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Sustained and NK/CD4⁺ T Cell-Dependent Efficient Prevention of Lung Metastasis Induced by Dendritic Cells Harboring Recombinant Sendai Virus

Atsushi Komaru,† Yasuji Ueda,* Aki Furuya,* Sakura Tanaka,† Kumi Yoshida,§ Tomonori Kato,* Hiroaki Kinoh,* Yui Harada,* Hiroyoshi Suzuki,† Makoto Inoue,¶ Mamoru Hasegawa,¶ Tomohiko Ichikawa,† and Yoshikazu Yonemitsu2,3*§

We recently demonstrated efficient antitumor immunity against murine tumors using dendritic cells (DCs) activated by recombinant Sendai viruses (rSeVs), and proposed a new concept, “immunostimulatory virotherapy,” for cancer immunotherapy. However, there has been little information on the efficacy of this method in preventing metastatic diseases. In this study, we investigated the efficacy of vaccinating DCs activated by fusion gene-deleted nontransmissible rSeV (rSeV/dF) using a murine model of lung metastasis. Bolus and i.v. administration of DCs harboring rSeV/dF-expressing GFP without pulsation of tumor Ag (DC-rSeV/dF-GFP) 2 days before tumor inoculation showed efficient prevention against lung metastasis of c1300 neuroblastoma, but not of RM-9 prostatic cancer. We found that the timing of DC therapy was critical for the inhibition of pulmonary metastasis of RM-9, and that the optimal effect of DCs was seen 28 days before tumor inoculation. Interestingly, the antimetastatic effect was sustained for over 3 mo, even when administered DCs were already cleared from the lung and organs related to the immune system. Although NK cell activity had already declined to baseline at the time of tumor inoculation, Ab-mediated depletion studies revealed that CD4⁺ cells as well as the presence of, but not the activation of, NK cells were crucial to the prevention of lung metastasis. These results are the first demonstration of efficient inhibition of lung metastasis via bolus administration of virally activated DCs that was sustained and NK/CD4⁺ cell-dependent, and may suggest a potentially new mechanism of DC-based immunotherapy for advanced malignancies. The Journal of Immunology, 2009, 183: 4211–4219.

Dendritic cells (DCs) have received much attention as a new therapeutic tool for cancer immunotherapy against advanced malignancies, including advanced prostatic cancers (1–3). Although early clinical studies successfully demonstrated cancer cell-specific immune responses in tumor-bearing patients, the related clinical outcome of DC-based immunotherapy has been unsatisfactory (1, 2), similar to findings seen in other tumors (3). These results, together with those of similar clinical studies, indicate that a number of concerns need to be examined, including practical issues (optimal numbers of DCs and route, interval, and frequency of administration) as well as DC qualities (subtype, phenotype, and activation status) (3).

To provide a possible solution, we recently proposed a new concept, “immunostimulatory virotherapy,” that gives efficient, DC-based antitumor immunity against various tumor types; this immunity is induced by a new DC-activating modality, the replication-competent (4) as well as nontransmissible (5) recombinant Sendai viruses (rSeVs). SeV, a member of the family Paramyxoviridae, has a nonsegmented negative-strand RNA genome and shows a broad spectrum of gene transfer (6–8), including DCs (4, 5). rSeV can mediate gene transfer and expression to a cytoplasmic location using cellular tubulin (9), thereby avoiding possible malignant transformation due to the genetic alteration of host cells; this is a safety advantage of rSeV. Importantly, cytoplasmic replication and transcription of rSeV would also be recognized by a DExD/H-box RNA helicase, retinoic acid-inducible gene-I, leading to a variety of cellular responses, including induction of type I IFNs (10, 11).

Such unique features of rSeV modulation of DC functions may explain the efficient antitumor immunity induced by DC-rSeV. However, little information is now available regarding the efficacy of DC-rSeV for metastatic diseases, including pulmonary metastasis.

To answer this question, in this study, we investigated the antimetastatic activity of DCs modified by nontransmissible rSeV lacking the F-gene (rSeV/dF) (12) to treat a mouse model of lung metastasis of oncogene-induced RM-9 prostatic cancer (13). To examine the biological functions of DCs modified by rSeV/dF, we here used rSeV expressing a typical reporter gene, GFP, without any Ag loading. This study demonstrated that DC-rSeV/dF-GFP was effective for preventing pulmonary tumor metastasis in a...
murine model of prostate cancer. In addition, this antitumor effect depended on possible new mechanisms involving CD4+ cells and NK cells without the requirement of activation.

Materials and Methods

Animals

Male 7- to 8-wk-old C57BL/6 (for RM-9), A/J (for c1300), and BALB/c nu/nu (for RM-9) mice were obtained from Shizuoka Laboratory Animal Center. Mice were kept under specific pathogen-free and humane conditions in the animal care facility of Chiba University’s fushana campus. The majority of animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee and by the Biosafety Committee for Recombinant DNA experiments of Chiba University. The experiments using transgenic C57BL6 mice expressing enhanced GFP under the control of CAG promoter (chicken β-actin promoter plus cytomegalovirus enhancer plus bovine growth hormone poly(A); a gift from Dr. Okabe, Osaka University, Osaka, Japan) were approved by the Institutional Animal Care and Use Committee and by the Biosafety Committee for Recombinant DNA experiments of Kyushu University. These experiments were also done in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Cells and Abs

The RM-9 mouse prostate cancer cell line was provided by Dr. Thompson (School of Medicine, University of Texas Southwestern Medical Center, TX) (13, 14). The RM-9 cell line was derived from a rat plus mouse-induced primary tumor from the mouse prostate reconstitution model system using C57BL/6 mice as previously described (13, 14). Murine neuroblastoma c1300 was obtained from the RIKEN Cell Bank. The prostatic cancer cell line TRAMP-C2 and the NK-sensitive lymphoma cell line YAC-1 were purchased from the American Type Culture Collection. These cell lines were maintained in complete medium (DMEM for RM-9 and TRAMP-C2, and RPMI 1640 for others) supplemented with 10% FBS, penicillin, and streptomycin under a humidified atmosphere containing 5% CO2 at 37°C. Specifically, TRAMP-C2 cells were cultured in DMEM with supplementation of ν serum, FBS, insulin and dichotrostestosterone (15). The following mAbs were used in the study: FITC-conjugated anti-mouse CD11c, NK1.1, and H2-Kk (MHC class I for C57BL6); PE-conjugated CD4, I-Ak (MHC class II); and H2-Kk (MHC class I for A/J). All were purchased from BD Pharmingen.

Nontransmissible rSeVs (rSeV/dF)

F-defective nontransmissible recombinant rSeVs expressing jellyfish GFP (rSeV/dF-GFP) were prepared and recovered as previously described (12). In brief, vectors were prepared using recombinant LLC-MK2 cells carrying the F gene (LLC-MK2/F7). An adenovirus vector, AxCAN-Cre, expressing Cre recombinase was used to induce the F protein in LLC-MK2/F7 cells (referred to as LLC-MK2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave UV irradiation, and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units.

Generation of murine bone marrow-derived DCs

DCs were obtained as previously described with additional negative selection (4, 5). In brief, bone marrow cells were harvested from femurs and tibias. After collection, bone marrow cells were passed through a nylon mesh, and RBC and lineage-positive (B220, CD5, CD11b, Gr-1, TER119, 7/4) cells were depleted using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies). These lineage-negative cells were cultured in 20 ng/ml GM-CSF (PeproTech) and 20 ng/ml IL-4 (PeproTech) in endotoxin-free complete medium in 6-well plates. On day 4, the culture medium was refreshed using medium supplemented with GM-CSF and IL-4 at the same concentrations. On day 7, the obtained immature DCs (iDCs) were collected and used for subsequent experiments. For rSeV/dF-mediated transfection, DCs were incubated with rSeV vectors at multiplicity of infection of 100 as indicated.

Flow cytometric analysis

The cells reacted with appropriate Abs were analyzed using a FACSCalibur (BD Biosciences) with CellQuest software (BD Biosciences). Dead cells were excluded by staining with propidium iodide. Data analysis was performed using FlowJo 4.5 software (Tree Star) (4, 5).

DC preparation and administration, and lung metastasis model

DCs were plated in fresh medium (106 cells/ml) and incubated with SeV/dF-GFP at a multiplicity of infection of 50 for 48 h. Then DCs were collected and washed twice and resuspended in PBS. GFP expression was confirmed by FACS analyses and consistently showed >80%. Mice were subjected to one i.v. administration of 106 of iDC or DC-rSeV/dF-GFP in 200 μl of PBS via the tail vein. For the experimental lung metastasis model, 105 of tumor cells were inoculated i.v. Three weeks after tumor cell injection, mice were sacrificed by an overdose of anesthesia to determine the number of lung metastases. The metastases were counted by the number of metastatic nodules on the surface of the lung by two blinded independent examiners (A.F. and A.K.) under a dissecting microscope. For survival analysis, animals were evaluated at natural death.

51Cr release assay for NK cell and CTL activities

For the NK cell activity assay, splenocytes were obtained from animals that received DC-rSeV/dF-GFP on day −2 or −28, and erythrocytes were depleted. The splenocytes were used directly as effector cells (4, 5).

For CTL assay, splenocytes from animals that received DC-rSeV/dF on day −28 and RM-9 cells 7 days before sacrifice were obtained and erythrocytes were depleted. The effector cells (4 × 106 cells/ml) were cocultured with irradiated (100 Gy) RM-9 cells (stimulator; 3 × 105 cells/ml). Three days later, 30 IU/ml murine IL-2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells (4, 5).

Target cells (1 × 106) were labeled with 100 mCi of Na251CrO4 (Amersham Biosciences) in 200 μl medium for 90 min at 37°C. The labeled target cells (1 × 106 cells/well) were incubated with the effector cells for 4 h at 37°C in 96-well plates in 200 μl medium at various E:T ratios. The radioactivity of the supernatants was counted using a gamma counter. The maximum or spontaneous release was defined as counts from samples incubated with 2% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated using the following formula: percentage of specific 51Cr release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release). Assays were performed in duplicated wells.

In vivo depletion of immune cell subsets

Anti-asialo GM1 (Wako) was given i.p. (100 μg/dose) for NK cell depletion on days −8, −6, −4, 0, and 3 after tumor inoculation. Anti-CD4 and anti-CD8 mAbs (1 μg/dose) were derived from GK1.5 and TIB105 hybridomas, respectively (10, 11). CD4+ or CD8+ cells in the mice were eliminated by i.p. injection of mAbs on days −8, −6, −4, 0, and every 3 or 4 days until sacrifice after tumor inoculation. FACS analyses confirmed >98% depletion of the target cells in peripheral blood from the tail vein for at least 7 days after injection in animals.

Adoptive CD4+ cell transfer

C57BL mice were i.v. received DC-SeV/dF-GFP on day −30, and their spleens were collected on day −2. CD4+ cells were enriched from the splenocytes using the CD4 Negative Isolation Kit (Dynal Biotech), and then CD4+ cells were i.p. injected into naive mice (107 cells/head). The control CD4+ cells were obtained from mice that had i.v. received PBS instead of DC-SeV/dF-GFP. FACS analysis indicated that the positivity of CD4 was usually >85% after isolation. The mice with or without adoptive transfer of CD4+ cells were followed by i.p. inoculation of 105 cells of RM-9 on day 0. Metastatic nodules on the lung were counted on day 21.

Statistical analysis

All data were expressed as means ± SEM. The data were examined statistically using the Mann-Whitney U test. The survival curves were determined using the Kaplan-Meier method. The log-rank test was used to compare curves between study and control groups. A probability value of p < 0.05 was considered statistically significant. Statistical analyses were determined using StatView software (SAS Institute).

Results

Inhibition of lung metastasis via bolus injection of DC-rSeV/dF-GFP

We previously demonstrated that intratumor administration of DCs activated by rSeV without any exogenous gene or tumor Ag could induce efficient antitumor immunity against various tumor types
(i.e., B16 melanoma, MH134 hepatocellular carcinoma, SCCVII squamous cell carcinoma, c1300 neuroblastoma, etc.) (4, 5), resulting in the complete elimination of dermally established tumors (7–9 mm in diameter) at high ratios. However, there is only sparse information regarding the efficacy of this new modality for lung metastasis. Therefore, at the initial stage of this study, we examined the efficacy of a bolus injection of DC-rSeV/dF for preventing lung metastasis. In this study, we used rSeV/dF-expressing GFP as a marker (rSeV/dF-GFP) to confirm the representative transfection efficiency in each experiment (consistently > 80% of GFP-positive DCs without significant cytotoxicity; supplementary Fig. S1)⁵ (4, 5). In addition, the preliminary direct comparison assessing antitumor efficiencies between DC-rSeV/dF-GFP or DC-rSeV/dF-null (without an exogenous gene) for preventing lung metastasis did not reveal a significant difference (data not shown). Therefore, DC-rSeV/dF-GFP was used in the following experiments.

First, we administered DC-rSeV/dF-GFP i.v. 2 days before tumor inoculation (Fig. 1A). We expected that hemagglutinin/neuraminidase protein expressed on the surface of DC-rSeV/dF-GFP might show a somewhat beneficial effect in preventing tumor growth, because the other group previously suggested that the hemagglutinin/neuraminidase protein of SeV strongly stimulates NK cell activity to kill tumor cells (16–18). As expected, NK cell activity of splenocytes was dramatically increased 2 days after DC-rSeV/dF-GFP injection (data not shown; similar data would appear in Fig. 4A), resulting in the almost complete inhibition of lung metastasis and extensive dissemination of various sized tumor nodules of c1300 neuroblastoma (Fig. 1B). Injection of iDC showed a modest effect in this model. Similar findings were also observed using other murine models (B16F10 melanoma; C57BL/6, LM8 osteosarcoma; C3H, and CT26 colon cancer; BALB/C), as well as a rat model of lung metastasis of prostate cancer AT6.3 (data not shown). These results suggest the potential and consistent utility of bolus and i.v. administration of DC-rSeV/dF.

In contrast, it was unexpected that this was not the case with RM-9 prostate cancer. As shown in Fig. 1C, modification of the DC function by rSeV/dF-GFP did not contribute to the enhancement of the modest iDC-mediated antitumoral effect. In contrast, DC-rSeV/dF-GFP exhibited modest but significant prolongation of the survival of RM-9-inoculated mice with no detectable nodule at all (4/18 = 22.2% of survival over day 100, p < 0.01; Fig. 1D), suggesting the potential benefit in the use of rSeV/dF as a tool for DC activation. Therefore, we focused on the antitumoral activity of DC-rSeV/dF-GFP in subsequent experiments.

**Timing of injection is critical to optimal performance of DC-rSeV/dF-GFP preventing experimental lung metastasis of RM-9**

Next, we optimized the parameters (i.e., Ag loading, numbers of DCs, etc.) possibly affecting the efficacy of DC-rSeV/dF-GFP without Ag loading on the tumor formation of RM-9 in lung. We found that the timing of DC injection was the critical factor determining its preventive effect (experimental protocol is shown in Fig. 2A). As shown in Fig. 2B, mice receiving DCs 7, 14, or 28 days before tumor inoculation showed dramatically high efficacy compared with those treated with DCs on day −2 (p < 0.01); optimized efficacy (though statistically insignificant) was found for injection of DCs (0.25 + 0.16 nodules/head) on day −28. Interestingly, similar efficacy was maintained in the case of a much longer interval between DC injection and tumor inoculation (3 mo. Fig. 2C, left). However, the antitumoral effect of DC-rSeV/dF-GFP disappeared when tumor cells were inoculated 6 mo after DC injection (Fig. 2C, right).

Based on these findings, we next assessed the precise mechanism of the sustained antitumoral effect of bolus and i.v. administration of DC-rSeV/dF, and we established the regimen as treatment with DCs 28 days before tumor inoculation in subsequent experiments.

**Presence of DC-rSeV/dF-GFP in lung and systemic organs related to immune system is not required for its antitumoral activity**

To explain the antitumoral activity of DC-rSeV/dF-GFP, we hypothesized that i.v. injected DCs might be retained over 28 days in the lung or organs related to the immune system and systemic circulation, resulting in pronounced antitumor responses at local sites. To examine this possibility, we assessed the presence of GFP-labeled DCs during the 28 days after DC injection by two different methods (DCs from GFP-transgenic mice and DC-rSeV/ dF-GFP) in these organs.

Unexpectedly, the results were negative, as demonstrated by repeated experiments. As shown in Fig. 3A, no significant increase in C11c⁺/GFP⁺-positive cells was found in blood, bone marrow, thymus, spleen, or mesenteric lymph nodes in either method. In the lung, 4–5% of C11c⁺/GFP⁺ cells were detected in mice not only with DCs from GFP-TG mice and DC-rSeV/dF-GFP, but also naive ones (n = 5 in each group). As recently demonstrated by another group (19, 20), such FL1 of C11c⁺/GFP⁺ cells should be a population of alveolar macrophages that might show autofluorescence. To confirm this further, DCs generated from bone marrow cells of Ly5.1 mice (Fig. 3B), that were treated with rSeV/dF without GFP (rSeV/dF-null) were i.v. administered to Ly5.2 mice. The distribution of DCs in these mice was analyzed 2 days after injection, because rapid clearance of DCs, within 12 h, had already been demonstrated in the use of 111In-labeled DCs (21). As shown in Fig. 3C, FACS analyses demonstrated complete clearance of DCs from the systemic organs examined (n = 4).

These results indicated that i.v.-administered iDCs as well as DC-rSeV/dF-GFP were already cleared from systemic organs 28 days after administration.

**NK cell and CTL activities of splenocytes after bolus and i.v. administration of DC-rSeV/dF-GFP**

To examine whether or not bolus and i.v. administration of DC-rSeV/dF-GFP could evoke the systemic activation of NK and CTL, even when such DCs were cleared from systemic organs, we performed ⁵¹Cr release assays using splenocytes from mice treated with DC-rSeV/dF-GFP.

To assess NK cell activity, splenocytes form mice treated with DC-rSeV/dF-GFP 2 or 28 days before they were used for YAC-1 target cells (Fig. 4A). As expected, NK cell activity was strongly evoked by DCs 2 days after injection (p < 0.05 at E/T ratios = 100:1 and 50:1); however, that declined to baseline with the use of splenocytes 28 days after DC injection. Together with the findings obtained in Figs. 1 and 2, this result suggests that the optimal effect of DC-rSeV/dF-GFP for the prevention of lung metastasis of RM-9 cells might not depend on the “systemic activation of NK cells.” Next, we postulated that tumor cell inoculation itself might lead NK cells to rapid and spontaneous activation. To exclude this hypothesis, a similar assay was performed using splenocytes from mice receiving RM-9 cells 2 days before. As shown in Fig. 4B, no significant activation of NK cells was found.

To assess the possible activation of CTLs specific to RM-9 cells, splenocytes were subjected to a ⁵¹Cr release assay 7 days after RM-9 inoculation, which in turn was 28 days after bolus DC-rSeV/
As shown in Fig. 4C, only 2 of 10 animals exhibited specific CTL activity against RM-9 (Fig. 4C, left, with no significant difference), whereas no animal showed significant cytolytic activity for the third parties, TRAMP-C2 (Fig. 4C, right) as well as B16F10, LL/2 (data not shown).

These results may suggest that transient activation of NK cells does not make a significant contribution to preventing lung metastasis of RM-9 cells. In addition, it has been suggested that CTL might not be important for the antimetastatic effect of DC-rSeV/dF-GFP injection.

**Determination of effector cell subsets**

As final assessments, we assessed the effector cell subsets contributing to the antimetastatic effect of DC-rSeV/dF-GFP in vivo using athymic nude mice or C57BL6 mice that were administered Abs to deplete effector cells.
Surprisingly, a DC-rSeV/dF-GFP-mediated antimetastatic effect was still apparent in nude mice (Fig. 5A). Of interest, in this case, is that a 14-day interval between DC and tumor inoculation showed significantly better efficacy than that seen with a 28-day interval ($p < 0.05$). These results might suggest that the innate response would make a greater contribution than acquired immunity.

Next, we returned to immune-competent C57BL6 mice. NK cell depletion by intraperitoneal administration of anti-AGM1 around the time of RM-9 cell inoculation (days −8, −6, −4, 0, and 3) resulted in a dramatic increase of small and dispersed tumor nodules (Fig. 5B). Together with the data of Fig. 4C and Fig. 5A, these results highlighted the significance and necessity of the “presence” but not the “activation” of NK cells at the time of tumor inoculation for effective prevention of lung metastasis by DC-rSeV/dF-GFP therapy.

Regarding effector subsets concerning acquired immunity, the effector depletion study (days −8, −6, −4, 0, and every 3 or 4 days until sacrifice after the tumor inoculation), which was confirmed by FACS analyses of all animals (Fig. 5C), revealed that CD4$^+$ cells are the most potent contributor to DC-rSeV/dF-GFP-mediated inhibition of lung metastasis (Fig. 5C).

These results were also confirmed by a survival assay, indicating that the depletion-mediated loss of the antimetastatic effect was seen with the use of anti-AGM1 and anti-CD4, but not with the use of anti-CD8.

The impact of CD4$^+$ cells on the antitumor effect of DC-rSeV/dF-GFP was also confirmed by an adoptive transfer experiment. Thirty days before tumor inoculation, immunocompetent C57BL6 mice were i.v. administered with PBS or DC-rSeV-GFP, and on day 2, CD4$^+$ cells were isolated and i.p. injected to naive mice. Twenty-one days after tumor inoculation, the metastatic nodules were counted. As shown in Fig. 5E, adoptive transfer of CD4$^+$ cells from DC-rSeV/dF-GFP mice, but not from PBS mice, significantly inhibited lung metastasis ($p < 0.01$), indicating that CD4$^+$ cells have a critical role after immunization with DC-rSeV/dF-GFP.

**Discussion**

In this study, we investigated the mechanisms and therapeutic potential of our recently developed DC-based “immunostimulatory virotreatment” using non transmissible rSeV/dF in a mouse model of lung metastasis. The key observations obtained in this study were as follows: 1) the timing of bolus and i.v. administration of DC-rSeV/dF is critical to produce optimal antimetastatic activity, and the effect is sustained over at least 3 mo; 2) DC-rSeV/dF showed its optimal effect at 28 days after injection when DCs were absent from systemic organs; 3) the “presence,” but not the “activation,”...
of NK cells is crucial to DC-rSeV/dF-mediated inhibition of lung metastasis; 4) CD4+ cells are also important for DC-rSeV/dF-mediated prevention of lung metastasis; and 5) CD8+ cells are not a significant player, even though tumor-specific CTL activity is induced. This is the first demonstration of the high efficacy of DC-rSeV/dF in the prevention of lung metastasis and the first exploration of its possible mechanism of action. We believe that rSeV/dF-mediated immunostimulatory virotherapy warrants further

**FIGURE 3.** Clearance of DC-rSeV/dF at the time of tumor inoculation in vivo. A, A typical FACS profile for the expression of GFP and CD11c. On day −28, GFP-labeled DCs, DCs from GFP-transgenic mice, or DC-rSeV/dF-GFP was i.v. administered. On day 0, mice were sacrificed by over-anesthesia, and GFP/CD11c expression was assessed by FACS in use of peripheral blood (PB), bone marrow (BM), thymus, spleen, mesenteric lymph nodes (LN), and lung. Almost no GFP-positive DCs could be found in PB, BM, thymus, spleen, and LN in either treatment. In lung, a CD11chigh/GFPlow population was found in both treatments; however, a similar pattern was also found in the lungs of naive mice, a finding suggesting the previously known “autofluorescence” of a certain population of DCs in the lung (Refs. 18, 19). Each group was comprised of five animals showing similar results. B, A typical FACS profile for the expression of CD11c, Ly5.1, and Ly5.2 of DCs derived from Ly5.1 mice treated with rSeB/dF-null. This experiment was repeated four times and showed similar results. C, A typical FACS profile for the expression of Ly5.1 and Ly5.2 of DCs from lung and immune system-related organs of mice administered Ly5.1 DC-rSeV/dF-null. Two days after DC injection, the Ly5.1 DCs had already disappeared from the examined organs. Four animals were analyzed and showed similar results.
class I on tumor cells should closely correspond to NK cell-dependent cytotoxicity (22). The results of the present study, however, did not validate this “missing self” hypothesis, because, as shown in Fig. 1, DC injection-sensitive tumor cell c1300 expressed high levels of MHC class I Ags, while insensitive tumor cell RM-9 showed almost null expression of them without any stimuli (see supplementary Fig. S2). Therefore, other possible mechanisms besides direct cytotoxic activity via activated NK cells should be considered to explain this result. For example, it might be possible that activated NK cells play a role in immunoregulation in the systemic lymphatic organs (e.g., the spleen) stimulating CD4-dependent antitumor immunity, while effector functions of tumor-infiltrating NK cells would be inactivated and/or nonfunctional in the tumor microenvironment (23). This hypothesis seems to be attractive, and further assessment should be performed.

The second question is how DC-rSeV/dF administered on day −28 represents efficient and optimized antimetastatic activity to RM-9. At the time of tumor inoculation, almost all DC-rSeV/dF was cleared from systemic organs. Therefore, the participation of DCs having direct cell-killing activity should be excluded from consideration (24), and other indirect effectors/modulators should be sought out. Importantly, findings obtained by our current study strongly suggested that the presence, but not the activation, of NK cells should be involved in this process. This is because short-term depletion of the NK cell subset at the time of tumor inoculation resulted in the dramatic promotion of pulmonary metastasis.

Together, these results imply a novel antitumor mechanism of the presence, but not the activation, of NK cells, probably via their regulatory, but not directly cytotoxic, functions. Because it is premature to try to explain the precise mechanisms of such functions of NK cells, further studies are called for.

The next important question is the CD4+ cell-dependent antimetastatic activity of DC-rSeV/dF. Compared with CD8+ T-cell-mediated antitumor immunity, the CD4+ cell-mediated antitumor mechanism as an effector has attracted less attention among researchers. In this regard, sophisticated studies have demonstrated that the CD4+ cell-mediated antitumor effect on dermally implanted malignancies does not depend on CD8+ T cells (25); that it targets MHC class II-negative malignancies (25); that it mediates the antiangiogenic response via IFN-γ (26); and that it is potentially more powerful than that seen with CD8+ T cells (27). To our knowledge, our current study is the first demonstration of the prevention of lung metastasis efficiently mediated by CD4+ cells but not CD8+ cells. Very importantly, Perez-Diez and colleagues (27) demonstrated that NK1.1+ cells were involved in CD4+ -dependent tumor killing; however, such populations should include neither NKT cells nor NK-DCs, so the findings seem to agree with the present results.

The precise mechanism of CD4+ cell-mediated antitumor immunity is, at present, largely unknown. A pioneering study by Mumburg et al. (23) demonstrated the significance of an IFN-γ-dependent indirect mechanism to eliminate tumors by adoptive transfer of CD4+ T cells. More precisely, it has been shown that 1) antitumor immunity by CD4+ T cells can be induced even in MHC class II-negative tumors, and thereby, 2) tumor-specific Ag(s) should originate in host cells and not tumor cells. A large part of our study can be explained by a similar mechanism, because both tumors tested here, c1300 and RM-9, are negative for MHC class II even under the stimulation of IFN-γ (see supplementary Fig. S3). However, there is still an unsolved question in our current study; namely, why the timing was so critical for the prevention of lung metastasis of RM-9, as shown in Fig. 1. Further study is still called for to clarify this point.

**FIGURE 4.** Enhancement of NK and CTL activities by bolus and i.v. injection of DC-rSeV/dF without tumor Ag pulsation. **A.** Spontaneous activation of NK cell activity. Splenocytes from animals treated with DC-rSeV/dF at day −2 or −28 were subjected to cytolytic assay using YAC-1 as a target, as described in Materials and Methods. Splenocytes from naive or poly I:C-injected animals were used for negative and positive controls, respectively. Note that strong and significant enhancement of cytolytic activity was detected with the use of splenocytes with DC-rSeV/dF on day −2, a finding that was completely reversed at baseline when splenocytes from animals that received DC-rSeV/dF-GFP at day −28 were used. Each group was comprised of three animals. **B.** Spontaneous activation of NK cell activity by tumor cell inoculation. Splenocytes from animals treated with 10^6 cells of RM-9 on day −2 were subjected to cytolytic assay using YAC-1 as a target. Splenocytes from naive or poly I:C-injected animals were used for negative and positive controls, respectively. Each group was comprised of three animals. **C.** Loss of significant tumor-specific CTL activity due to bolus and i.v. administration of DC-rSeV/dF. Seven days after RM-9 tumor inoculation via the tail vein, splenocytes from animals treated with DC-rSeV/dF-GFP or not (naive control) at day −28 were subjected to cytolytic assay for syngeneic target tumor cells (C57BL6-derived RM-9 and TRAMP-C2). Splenocytes from naive animals were used as a negative control. Each graph contains the sum of three independent experiments.

investigation as a new concept and a promising tool for enhancing DC-mediated cancer immunotherapy.

Although the current study suggests a new antitumor mechanism preventing lung metastasis, namely NK/CD4-dependent antimetastatic activity of bolus and i.v. injection of virally activated DCs, this study also raises several new questions.

The first concern is why RM-9 was insensitive up to a few days before administration of DC-rSeV/dF-GFP, even though it was associated with increased activity of systemic NK cells. According to the classical ‘missing self’ hypothesis, the expression of MHC
Related to the consideration of DC-rSeV/dF-GFP-dependent and CD4<sup>+</sup>/NK cell-mediated antimetastatic activity, Ab-dependent cellular cytotoxicity, as well as complement-dependent cytotoxicity, should be encountered as possible mechanisms; because IgG generation (isotype switching) would require CD4<sup>+</sup>/NK T cells, and NK cells efficiently mediate Ab-dependent cellular cytotoxicity/complement-dependent cytotoxicity via specific Fc receptors (28, 29). Such a hypothesis is likely to explain well the delay of NK/CD4<sup>+</sup>-dependent efficacy of DC therapy against RM-9. However, the present study still contains paradoxical findings. This hypothesis cannot address the following: 1) how DC-rSeV/dF-GFP prevents the metastasis of c1300 neuroblastoma with higher expression of MHC class I (Fig. 1B and Supplementary Fig. S2) soon after DC injection; 2) which molecule(s) is (are) the possible...
target(s) of generated IgG; and 3) why the efficacy of DC-rSeV/dF-GFP lasted over 3 mo (Fig. 2C). Importantly, our preliminary Western blotting could not demonstrate the possible specific Ab in serum from mice treated with DC-rSeV/dF-GFP (data not shown). Therefore, further studies are called for to clarify this hopeful hypothesis.

In contrast, we here demonstrated the minor role of CD8+ cells in the prevention of lung metastasis and animal survival. Meanwhile, in a few of the animals treated with DC-rSeV/dF 28 days before tumor injection CTL activity specific for i.v. inoculated DCs had disappeared from systemic organs. These data, therefore, indicate that induced CTL activity may not be important in preventing lung metastasis.

In summary, we here demonstrated the efficient antitumorous activity of nontransmissible rSeV-modified DCs in a murine model of lung metastasis, and showed that the activity involved NK/CD4+. These results may point to a new mechanism of DC-based immunotherapy for advanced malignancies, which could be applicable to future clinical studies.

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Disclosures

The authors have no financial conflict of interest.

References


Supplementary Figure S1.

Legend: Transfection efficiency and cytopathic effect to murine mBM-DCs due to rSeV/dF-GFP

DCs were replated in fresh medium and incubated with rSeV/dF-GFP at each MOI for 48 hours. Percentage of cells expressing GFP (left graph), and of 7-amino-actinomycin D (7AAD)-positive living cells were examined by FACS Calibur.
Supplementary Figure S2.

Legend: Expression of MHC class I antigens and its responsiveness to IFN-γ
RM9, B16F10, LL/2, and TRAMP-C2 cells (C57 BL6 background) or c1300 cells (A/J background) were incubated with murine IFN-γ at various concentrations for 48h. The cells were incubated with appropriate antibodies (FITC-conjugated H2-Kb for C57BL6 background and PE-conjugated H2Kk, for A/J background) for 30 minutes at 4°C, and the cells were analyzed using a FACS Calibur. Dead cells were excluded by staining with propidium iodide.
Supplementary Figure S3.

Legend: Expression of MHC class II antigens and its responsiveness to IFN-γ
RM9, B16F10, LL/2, and TRAMP-C2 cells (C57BL6 background) or c1300 cells (A/J background) were incubated with murine IFN-γ at various concentrations for 48h. The cells were incubated with appropriate antibodies (PE-conjugated I-Aαb for C57BL6 background and PE-conjugated I-Aαk, for A/J background) for 30 minutes at 4°C, and the cells were analyzed using a FACS Calibur. Dead cells were excluded by staining with propidium iodide.
Methods for Supplementary Figures

Transfection efficiency and cytotoxic assay
DCs were replated in fresh medium and incubated with rSeV/dF-GFP at each MOI for 48 hours. Cells with GFP expression were analyzed using a FACScalibur (Becton Dickinson Tokyo, Japan). For cell viability assay, 7-amino-actinomycin D (7AAD) was used to count living cells for FACS analysis, as previously described (1-3).

Flow cytometric analysis
For murine MHC class I and class II induction, RM9, B16F10, LL/2, and TRAMP-C2 cells (C57 BL6 background) or c1300 cells (A/J background) were replated in fresh medium (10^6 cells/ml) were incubated with murine IFN-β or IFN-γ at various concentrations for 48h. The cells were incubated with appropriate antibodies (FITC-conjugated H2-K^b^, and PE-conjugated H2K^k^, -I-A^b^, and -I-A^c^, all from BD Pharmingen, San Diego, CA) for 30 minutes at 4°C, and the cells were analyzed using a FACS Calibur with the CellQuest software (BD Biosciences Japan, Tokyo, Japan). Dead cells were excluded by staining with propidium iodide. Data analysis was performed using FlowJo 4.5 software (TREE STAR, Inc., San Carlos, CA).