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Development of immunostimulatory virotherapy using non-transmissible Sendai virus-activated dendritic cells ☆

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Abstract

Dendritic cell (DC)-based immunotherapy has been clinically evaluated, however, still requires modification to improve its outcomes. We previously demonstrated that DCs activated by replication competent recombinant Sendai virus (SeV) showed dramatic efficacy over that seen in use of current DC vaccine for immunotherapy against malignancies; however, application of replication-deficient vector is more relevant in clinical setting. We here show that F-gene-deleted non-transmissible Sendai virus (SeV/dF)-activated DCs (DCs/SeV/dF) has strong antitumor effects against murine SCCVII tumor, that was well-known as a less immunogenic cell line. SeV/dF shows high transfection efficiency to DCs and leads them to upregulate costimulatory molecules. Intratumoral injection of DCs/SeV/dF resulted in a marked and representative inhibition of the tumor, even when the tumors were well-vascularized. This is the first demonstration that non-transmissible SeV vector, SeV/dF, could be a DC-activator; DC/SeV/dF-based cancer immunotherapy may, therefore, warrant further investigation.

Keywords: Squamous cell carcinoma; Non-transmissible Sendai virus; Dendritic cell

Despite the continuous efforts of physicians, the overall survival for patients with squamous cell carcinoma (SCC), including esophageal cancers, have not shown significant improvement over the last decade [1,2]. Among the clinically available therapeutics, surgical treatment has been the most effective when cancer foci could be resected without the involvement of or metastasis to other vital organs [3–5]. However, in the case of esophageal carcinomas, anatomical loss of serosa of the esophagus would be a cause of accelerated invasiveness of SCC to the aorta, trachea, etc., resulting in advanced diseases. Extensive surgical approaches, including extended esophagectomy with 3-field lymph node dissection could improve patient prognosis, but quality of life is occasionally reduced due to impairment of deglutition, phonation, and respiration [3,4]. Since such advanced esophageal SCC has also remained intractable to chemo-radiotherapy, the development of novel and less invasive therapeutic strategies has been much desired to treat patients with advanced SCC.

☆ *Competing interest statement:* The authors declare that they have no competing financial interests.

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Much attention has been paid to cancer immunotherapy using dendritic cells (DCs) that has shown good potential for antitumor immunity [6,7]; DC-based cancer immunotherapy is, therefore, currently being tested in clinical trials all over the world. Although reported clinical trials have shown the safety and feasibility of DC therapy in patients bearing advanced malignancies, objective clinical responses have remained limited [8,9].

The poor clinical response of DC therapy might be due to the insufficient activation, and, in turn, the state of activation of DCs for cancer vaccine is still under debate [10]. Immature DCs (iDCs) display a phenotype reflecting their specialized function as antigen-capturing cells; however, the activity of antigen presentation of this subset of DCs to T cells, however, is not efficient [10]. Thus, to achieve a dramatic improvement in the clinical efficacy of DC-based cancer immunotherapy, knowledge regarding the most appropriate condition of DCs for cancer immunization should be obtained.

Sendai virus (SeV) is a negative-strand RNA virus and a member of the *paramyxoviridae* family, and the genomic replication and transcription are carried out in the cytoplasm by its own RNA polymerase. Using a prototype vector based on SeV, replication competent SeV, we recently demonstrated that DCs activated by SeV showed dramatically enhanced antitumor effects on various murine tumors [11], suggesting the possible utility of SeV vector system for cancer immunotherapy. As a next step, we subsequently developed a new mode of non-transmissible viral vector based on SeV-lacking fusion (F) gene (SeV/dF) that is more relevant for clinical use [12]. Some previous observations have demonstrated that immature DCs are dramatically activated and leads to a maturation state by the infection of SeV, thus acquiring the capacity to promote cell-mediated immunity in a replication-dependent and toll-like receptor (TLR)-independent fashion [13,14].

We, therefore, investigated the therapeutic value and immune consequences of intratumoral administration of activated DCs by SeV/dF.

Methods

Mice and cell line. Female C3H/He mice (6–7 weeks old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan) and kept under specific pathogen-free conditions. All animal experiments were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animals, Recombinant DNA, and Experiments Using Infectious Pathogens at Chiba University, and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government. SCCVII (kindly provided by Dr. Shibamoto, Nagoya City University) is a spontaneously arising murine SCC that has been shown to be poorly immunogenic [15].

Rescue of SeV/dF. Preparation and recovery of F-defective and non-transmissible recombinant SeV used in this study (SeV/dF-GFP and SeV/dF-null) were performed as previously described [12,16]. Briefly, LLC-MK2 cells stably expressing F-gene were transfected with a plasmid mixture containing pSeV18+b(+)/F-EGFP, pGEM-NP, pGEM-P, and pGEM-L. The transfected cells were collected, resuspended, and lysed by three cycles of freezing and thawing. Subsequent genomic RNA–nuclear protein complex (RNP) transfection was performed by cationic lipid into F-expressing LLC-MK2/F7 cells. Virus yield is expressed in PFU and cell infectious units (CIU).

Murine bone marrow-derived DCs (mBM-DCs). DCs were obtained from mouse bone marrow precursors as described previously [11,17] with minor modification. Briefly, bone marrow cells were harvested from femurs and tibias. After washing, lineage antigen-positive (CD45R, CD5, CD11b, Gr-1, TER119, 7/4) cells were removed by using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell technologies, Canada). These lineage-negative cells were cultured under 20 ng/ml murine GM-CSF and 20 ng/ml murine IL-4 in RPMI 1640 medium. On day 4, the cultures were refreshed by adding a half volume of culture medium supplemented with GM-CSF and IL-4 at the same concentrations. On day 7, DCs were collected and seeded at 1×10^6/ml and then incubated with SeV vectors at each MOI or lipopolysaccaride (LPS) (1 μg/ml).

Flow cytometric analysis. DCs were replated in fresh medium and incubated with SeV-GFP at each MOI or 1 μg/ml LPS for 48 h. Biotin-conjugated anti-mouse H-2K, CD40, CD80, CD86, and FITC-conjugated anti-CD11c (Pharmingen, San Diego, CA) monoclonal antibodies (mAbs) were used for each primary antibody, and biotinylated Abs were detected by subsequent staining with streptavidin-PE (Pharmingen). Cells were analyzed using a FACScalibur (Becton–Dickinson Tokyo, Japan).

Cytotoxic assay. DCs were replated and incubated with SeV/dF-GFP at each MOI. At 48 h, cell viability was assessed with 7-amino-actinomycin D (7AAD) to count living cells for FACS analysis, as previously described [18].

Fluorescein isothiocyanate (FITC)–dextran uptake. FITC–dextran uptake was performed to assess the endo-/phagocytotic activity of DCs. Cells were then incubated with 1 mg/ml of FITC–dextran (MW = 40,000, Sigma–Aldrich, Tokyo, Japan) for 30 min at 4 or 37 °C, and washed with ice-cold phosphate-buffered saline (PBS) and labeled on ice with PE-conjugated mAb for CD11c. The uptake was measured by FACS at different times after stimulation, and was calculated as the change in MFI between cell samples incubated at 37 and 4 °C.

DC immunotherapy of the established tumor. SCCVII cells were harvested and processed by three rapid cycles of freezing and thawing. DCs were pulsed with tumor lysate (DC number: number of tumor cells for lysate = 1:3) for 18 h and were then incubated with SeV/dF or LPS for 8 h. Intradermal implantation was performed in the abdomen on day 0, and 1×10^6 DCs were injected intratumorally on days 10, 17, and 24. For all injections, materials were suspended in a 100-μl volume of PBS. The tumor size was assessed using microcalipers three times a week, and the volume was calculated by the following formula: (tumor volume; mm^3) = 0.5236 × (long axis) × (short axis) × (height).

Immunohistochemical staining. Standard immunohistochemical staining was performed using 5-μm-thick paraffin-embedded sections by standard avidin–biotin complex method. Blocking was done using 3% hydrogen peroxide and non-immune serum, and the primary antibodies (anti-vWF, Chemicon international, CA) was applied for 1 h at room temperature. Visualization was done using 3,3-diaminobenzidine substrate solution, and the sections were counter-stained with hematoxylin.

CTL activity. Seven days after the last immunization, spleen cells were pooled, and restimulated with irradiated (100 Gy) SCCVII cells for 6 days. These cells were harvested on day 6 and used as effector cells in a standard 4-h ^{51}Cr release assay. Target tumor cells (1×10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 90 min at 37 °C. The labeled target cells were incubated with the effector cells for 4 h at 37 °C in 96-well plates in 200 μl of T-cell medium at various E:T ratios. The radioactivity of the supernatants was counted using a γ-counter. Cytolytic activity was calculated using the following formula: percentage of specific Cr^{51} release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release). Assays were performed in triplicated wells.

Statistical analysis. The data were evaluated statistically by repeated measures one-way ANOVA. The statistical significance of difference groups was determined using the Scheff's test, and $P < 0.05$ was considered statistically significant.

Results

Characterization of DCs modified by SeV/dF

DCs infected by SeV/dF-GFP at each MOI were analyzed by flow cytometry for GFP expression. The ratio of GFP-expressing DCs increased in a MOI-dependent manner, with the transfection efficiency at MOI = 20 being estimated at approximately 80% based on similar findings in repeated experiments (Fig. 1A). Interestingly, LPS-activation of DCs reduced the transfection efficiency of SeV/dF-GFP (data not shown), indicating that the susceptibility of mBM-DCs to SeV/dF may depend on their maturation state. Higher viral doses of SeV/dF-GFP did not result in a significant cytopathic effect (Fig. 1A).

Phenotype of mBM-DCs treated by SeV/dF

We next evaluated the effects of SeV/dF transfection on the maturation and activation of DCs. Here, we used SeV/dF without a foreign gene (SeV/dF-null).

Two-day stimulation of iDCs with SeV/dF-null or LPS, a well-known strong DC-activator that is not clinically available, increased the expression of these costimulatory molecules. The expression levels of surface markers on the C3H strain-derived DCs after SeV/dF-null infection were milder than for those treated with LPS (Fig. 1B); a different finding indicating that C57BL6-derived DC/SeV/dF-null show comparable levels of expression to those seen with LPS [11].

We subsequently assessed the effects of SeV/dF on endo-/phagocytotic activity of mBM-DCs as compared with that of LPS (Fig. 1C). Endo-/phagocytosis of DC/LPS was mildly and transiently enhanced, peaked at 1 h after LPS stimulation, and then markedly downregulated in its time course. In contrast, endo-/phagocytosis of DC/SeV/dF-null was transiently decreased, and then caught up to levels comparable to those of immature DCs. Forty-eight hours after stimulation, the endo-/phagocytotic activity of DCs-SeV/dF-null was significantly higher than that of DC/LPS ($P < 0.05$, $n = 4$, each), which was equal to the levels of unstimulated DCs.

These results suggest that SeV/dF-null may activate mBM-DCs without any significant reduction in their endo-/phagocytotic activity.

Antitumor effects of SeV-activated DC in vivo

We next examined the antitumor effects of intratumoral administration of DCs activated with SeV/dF *in vivo*. We here used established (7–10 mm in diameter on day 10) and well-vascularized SCCVII tumors (Fig. 2A); because 'there are no cancer vaccine models that reproducibly demonstrate that vascularized tumors can be rejected' at present, as noted by Rosenburg et al. [9].

Tumor-bearing mice were given tumor lysate-pulsed mBM-DCs which were activated with LPS or SeV/dF-GFP three times (Fig. 2B and C). Administration of DCs without stimulation showed a modest antitumor response, probably due to their mild spontaneous activation during *ex vivo* cultivation. Antitumor activity was significantly enhanced with the use of DCs/LPS, and one animal showed a complete rejection of the tumor. Intratumoral injection of DCs/SeV/dF resulted in a marked inhibition of tumor growth in all animals, and with this regimen, 2 of 7 tumors were completely eliminated, a finding determined by histopathology.

Next, the cytolytic activity of spleen cells was investigated. Cytolytic activity against SCCVII cells was augmented in unstimulated DCs, DC/LPS, and the DCs/SeV/dF treatment group compared with that in the no-treatment groups (Fig. 3). The highest cytolytic activity was observed in the mice that received DCs/SeV/dF treatment.

These results confirmed that intratumor injection of DCs/SeV/dF enhanced the tumor-specific CTL response.

Antitumor effects of SeV-activated DC without pulsing tumor lysate

As shown in Fig. 1, endo-/phagocytosis of DCs was not impaired by SeV/dF treatment *in vitro*; we, therefore, evaluated the antitumor effects of SeV-activated DCs without pretreatment with tumor lysate. As shown in Fig. 4, the antitumor effects of DCs activated by SeV/dF were not impaired without pulsing tumor lysate, whereas no tumor showed complete elimination. These results suggest that intratumor injection of DC/SeV/dF may not always require the uptake of tumor antigen during preparation.

Discussion

The key observations of the current study were as follows: (1) SeV/dF efficiently transfected immature mBM-DCs without a significant reduction of cell viability; (2) SeV/dF leads mBM-DCs to a mature and activated state without a significant reduction in endo-/phagocytotic activity; (3) DC/SeV/dF-GFP demonstrates superior antitumor effects on less immunogenic murine SCCs, which were treated as well-established and well-vascularized, in comparison with the use of DC/LPS. This is the first report suggesting the potential utility of SeV/dF as an alternative activator for DCs in clinical cancer immunotherapy.

We demonstrate that SeV/dF efficiently transfects DCs at relatively lower MOI without significant cytopathic effects. Although other investigators have focused on adenovirus vectors for gene transfer to DCs, much higher titer (over MOI = 300) has been required [19,20] to achieve the comparable efficiency of SeV at lower MOI. It has been reported that adenoviral infection induces cytotoxicity and impairs antigen uptake [20], although there are some conflicting reports regarding this point. iDCs actively internalize antigen, and in contrast, mature DCs are poorly endocytotic and function to present antigens to T-cells [21,22]. Our findings, however, have demonstrated that DCs activated by SeV/dF can efficiently uptake antigens

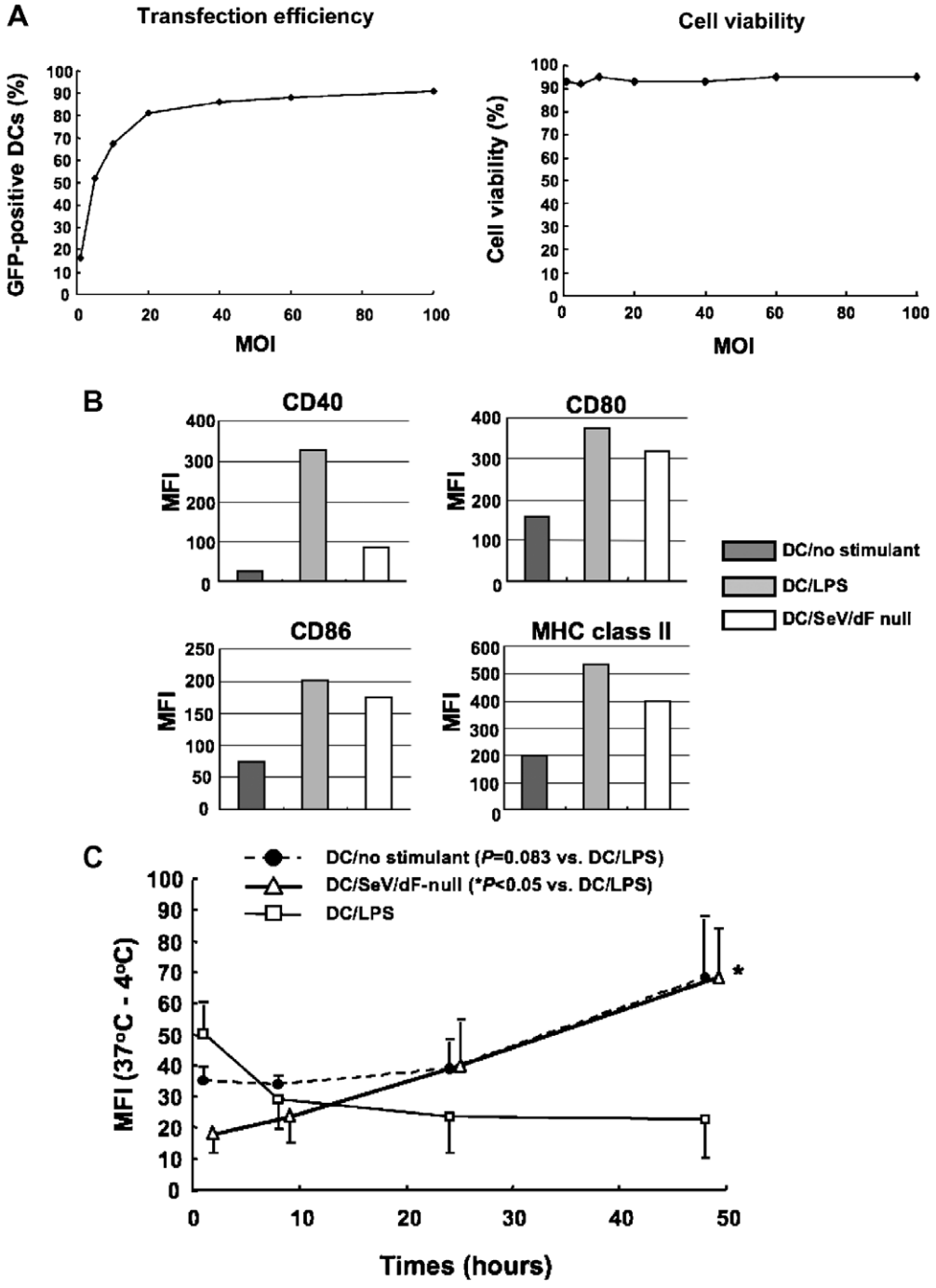

Fig. 1. Characterization of mBM-DCs modified by SeV/dF. (A) Gene transfer efficiency and cytopathic effects. Forty-eight hours after SeV/dF-GFP exposure, DCs were subjected to FACS analysis assessing the gene transfer efficiency (left), or to an assessment of cell viability by an early apoptotic cell-detecting technique using 7AAD (right). These experiments were performed at least in duplicate, and all produced similar results. (B) Effects of SeV/dF on surface markers of mBM-DCs. DCs were collected and transfected by SeV/dF-null at MOI = 40 or exposed to LPS (1 μg/ml), and 48 h later, DCs were subjected to FACS analysis assessing the expression of surface markers. Bar graph indicating the corresponding mean fluorescent intensity (MFI) is shown. The experiment was carried out in triplicate, produing similar results. (C) Effects of SeV/dF on endo-/phagocytic activities of mBM-DCs. DCs were transfected by SeV/dF-null at MOI = 40 or stimulated by LPS (1 μg/ml), and then exposed to 1 mg/ml of FITC–dextran for 30 min at 4 or 37 °C. The uptake was measured at each time and expressed MFI between cell samples incubated at 37 and 4 °C. The graph contains all data from four independent experiments using the same procedure.

and that the antitumor effects of DC/SeV/dF via intratumor injection were not impaired without pulsing tumor lysate, suggesting that the DC/SeV/dF may still have antigen uptake and processing activity *in vivo*.

Despite the higher expression of costimulatory molecules, CD40, CD80, and CD86 in DC/LPS than in DC/SeV/dF, the intratumor administration of DCs/SeV to established SCCVII tumors *in vivo* showed stronger antitu-

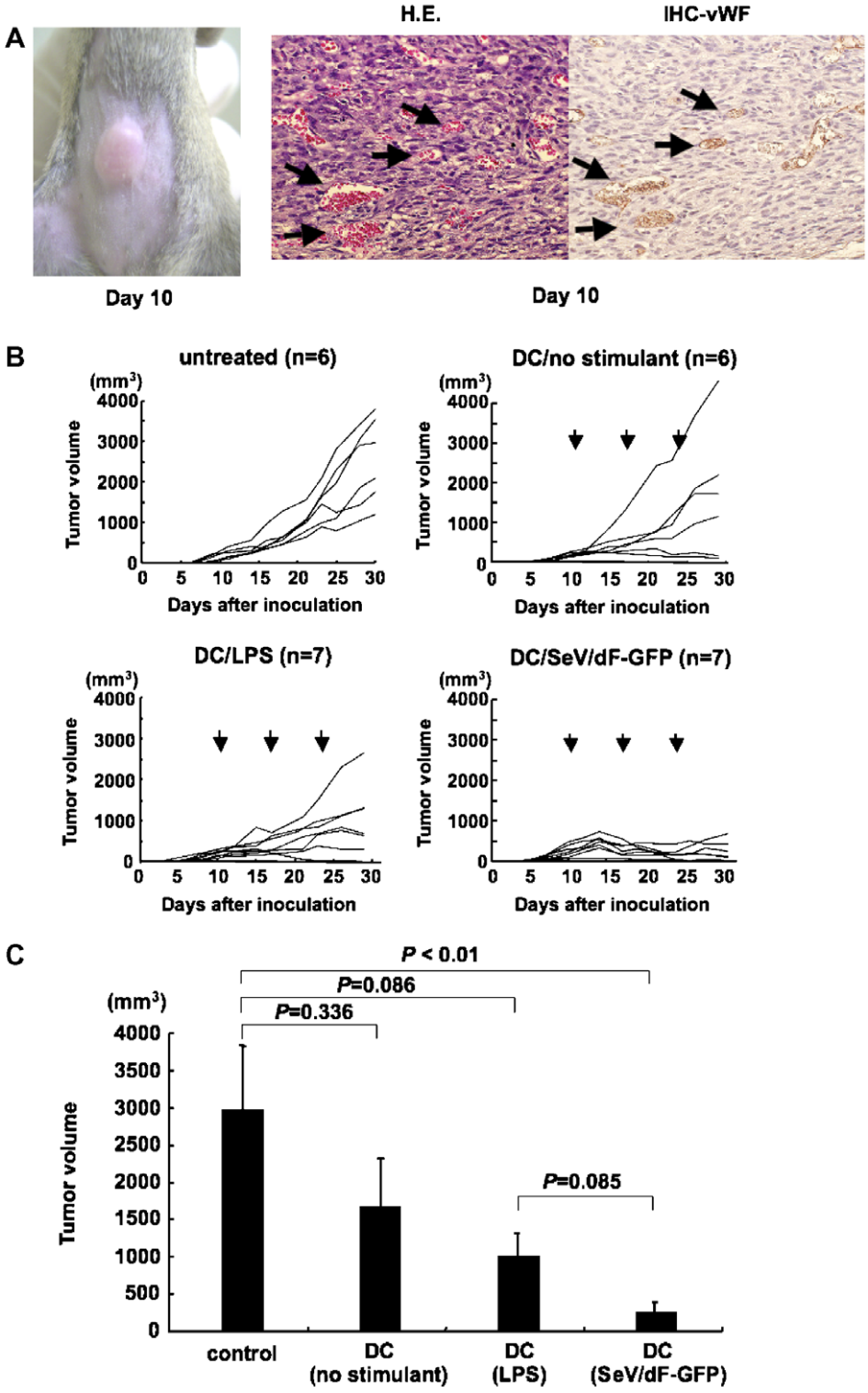

Fig. 2. Antitumor effects of intratumor administration of lysate-pulsed DC/SeV/dF-GFP against well-vascularized SCCVII tumors. (A) Characterization of the established SCCVII tumors at the time of the start of DC-therapy. Left: representative gross observation of intradermally inoculated SCCVII tumor of C3H mice. The size of the tumor is 7–10 mm in diameter. Middle and right (original magnification: 200×): histopathological findings in hematoxylin-eosin (H.E.: middle) and in immunohistochemistry for von Willebrand Factor (IHC-vWF) identifying intratumor neovascularization (arrows). (B) The time course of tumor volume of each animal. Ten days after tumor inoculation, DC-therapy (left upper: no treatment; right upper: DCs without stimulant; left bottom: DCs activated with LPS; and right bottom: DCs activated with SeV/dF-GFP) was performed three times at weekly intervals (indicated as arrows). One animal of the DC/LPS group and 2 of the DC/SeV/dF-GFP were completely eliminated. (C) Bar graph providing a direct comparison of tumor volume on day 30.

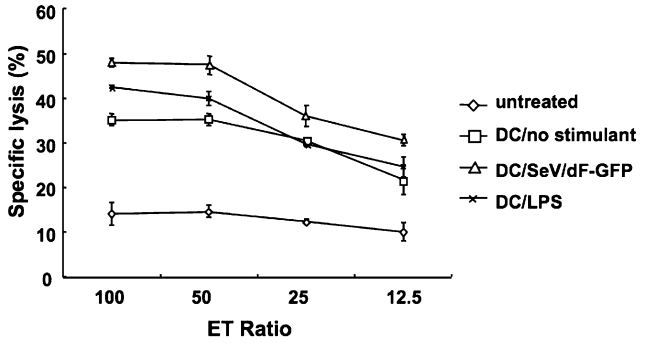

Fig. 3. Cr release assay to determine the CTL activity. Seven days after the third immunization, spleen cells isolated from immunized mice and ^{51}Cr release assay was performed.

mor effects and CTL activity than that of DC/LPS. The exact reason for these stronger effects remain unknown, but a possible mechanism related to the continuity of activity of DCs can be explained as follows. Recent studies related to the activity of DCs have shown that DCs exposed to stimuli for long periods undergo functional paralysis or exhaustion [23,24]. Considering the maintained endocytotic activity and modest upregulation of costimulatory molecules on DC/SeV, it may be possible that the paralysis or exhaustion of DC/LPS expressing higher surface markers resulted in more modest antitumor effects than those seen in the use of DC/SeV/dF.

Related to the above discussion, several important studies regarding the mechanisms of negative-strand RNA virus-induced activation of DCs have been reported in recent years. These are categorized as having two independent systems, namely toll-like receptors (TLRs) and TLR-independent systems; the former recognize dsRNA and ssRNA presuming pathogen-associated molecular patterns for viruses [25], and the latter is a TLR-independent pathway using RNA helicases, including RIG-I [26]. The latter pathway well explains that activated DCs by SeV are more powerful than DCs that are exposed to LPS *in vivo*; therefore, further characterization should provide a better understanding of the mechanism to improve the efficacy of cancer vaccines.

Immunotherapy for SCCs has not been well-studied, and there is at present sparse information regarding the response of SCCs to CTLs. Although previous experimental studies demonstrated that the pre-administration of lymphokine-activated killer cells prevented pulmonary metastases of SCCVII cells, which are a less immunogenic cell line used in this study, they have not demonstrated any significant reduction in established pulmonary metastases [15,27]. Since 'there are no cancer vaccine models that reproducibly demonstrate that vascularized tumors can be rejected' at present, as noted by Rosenburg et al. [9,28], the current results, indicating that the well-vascularized tumors were eliminated in more than 25 % of mice treated with DC/SeV/dF-GFP, suggest the potential utility of DC/SeV/dF.

In summary, we here demonstrated that mBM-DCs treated with non-transmissible SeV/dF without therapeutic

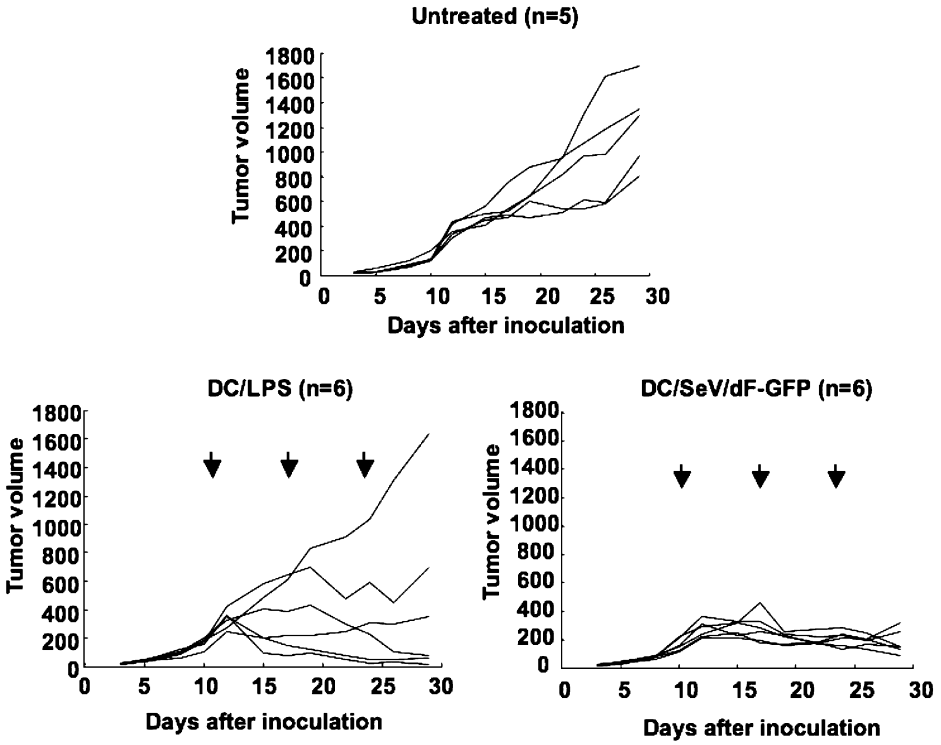

Fig. 4. The time course of tumor volume of each animal in response to DC-therapy without *ex vivo* tumor lysate pulsing. DC-therapy without pulse (upper: no treatment; left bottom: DC/LPS; and right bottom: DC/SeV/dF-GFP) was performed three times at weekly intervals from 10 days after tumor inoculation.

gene induced strong antitumor effects against less immunogenic murine SCC tumors. These results thus indicate for the first time that SeV/dF could be a promising candidate for DC-activator to treat intractable malignancies; therefore, DC/SeV/dF warrants further investigation for enhancing antitumor effects in a clinical setting.

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