[Frontiers in Bioscience 13, 1892-1898, January 1, 2008]

Immunostimulatory virotherapy using recombinant Sendai virus as a new cancer therapeutic regimen

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1. ABSTRACT

The utility of recombinant Sendai virus (rSeV) has been considerably examined over the last decade as a potent gene transfer candidate in a cytoplasmic gene expression system. Such risks as excessive immune responses associated with this virus administration *in vivo* however have limited its applicability in clinical settings as is the case with other viral vectors including adenoviruses. In consequence of extensive assessment on the mechanisms of immune responses against SeV, we found that *ex vivo* infection of immature dendritic cells (DCs) with SeV demonstrates their spontaneous maturation and activation. We applied this result to create a unique, representative, and powerful agent to activate DCs, namely rSeV-modified DCs (rSeV/DCs), for use in cancer immunotherapy. Use of this system *in vivo* resulted in the induction of efficient antitumor immunity against vascularized rodent tumors, including melanoma, hepatocellular carcinoma, neuroblastoma, squamous cell carcinoma, and prostatic cancer, and it even frequently associated with elimination of those tumors. These results indicate that rSeV could be a powerful immune booster for DC-based cancer immunotherapy that is worth investigating further. We propose a conceptual term "immunostimulatory virotherapy" to describe this new method of cancer therapy using the rSeV/DCs system.

2. RECOMBINANT SENDAI VIRUS (rSeV) VECTOR: A CUTTING EDGE TECHNOLOGY FOR HUMAN GENE THERAPY

2.1. Brief summary of structural and biological aspects of Sendai virus (SeV)

Sendai virus (SeV) is a member of the *Paramyxoviridae*: an enveloped virus with a linear, nonsegmented negative-strand RNA genome that encodes six unique genes for ribonucleotide–protein complex (RNP: NP, P/C/V, and L) and envelope-related proteins (F, HN, and M) (Figure 1). Wild-type SeV (SeV-WT) that is amplified in the chorioallantonic fluid of embryonated chicken eggs autonomously replicates in the presence of trypsin-like protease (identified as factor Xa)-mediated digestion of F-precursor (F0), forming active F-protein (1, 2). SeV-WT detects sialic acid residues on surface glycoproteins or asialoglycoproteins as receptors (3), and yields a broad spectrum of infection. SeV-WT however causes pneumonia in rodents after multiple infections, and of special note is that lungs are the only organs that are infected. This lung-specific tropism is determined by the presence of tryptase Clara: trypsin-like protease in the airway mucus which activates F-precursor (4).

One of the distinguished features of SeV is its rapid process of infection. When SeV is inoculated, the

viral particles adhere to cellular membrane and immediately start to fuse with it; this is also true for SeV-based recombinant vectors (5, 6). In addition, the life-cycle of SeV, including genome replication and transcription, occurs largely in the cellular cytoplasm: this virus does not show a DNA phase in contrast to retroviruses and lentiviruses. Furthermore, the level of gene expression is dramatically high. In the case of rSeV vector, transgene expression is frequently 100,000 times higher compared to the case when adenoviral vectors are used (5). These unique features of SeV prompted us to produce a new class of recombinant vectors based on SeV that would raise opportunities of what we term "cytoplasmic gene therapy" without genotoxicity.

2.2. Available rSeV

We have developed several types of rSeV by a stepwise strategy. rSeV vectors largely retain the features of SeV-WT as described above. The prototype vector has a simple structure with the addition of an exogenous gene (additive type; Figure 1), and most of our knowledge regarding its *in vivo* gene transfer efficiency has been obtained in studies using this additive type vector (5–10). Cells transfected by this replication-competent vector produce secondary vector particles containing a precursor form of F-protein; therefore, this type of vector evokes pneumonia to the extent comparable to the case when SeV-WT is used.

The first success in producing a non-transmissible rSeV vector was reported in 2000 (11). The genome of the first-generation rSeV vector lacks a gene encoding F-protein (vector rSeV/dF [fusion gene-deleted rSeV]), maintaining the basic characteristics of SeV-WT. Currently, rSeV/dF is being mass-produced according to the good manufacturing practice (GMP) protocol, and the first clinical study using rSeV/dF expressing human basic fibroblast growth factor (bFGF/FGF-2) is now under way on therapeutic angiogenesis to treat patients with critical limb ischemia (Gene Therapy Clinical Study Protocols Approved in Japan: http://www.nihs.go.jp/cgtp/cgtp/sec1/gt_prtcl/prtcl-e.html). Recent modifications of rSeV/dF to diminish its virus-induced cytopathic effects procreated a temperature-sensitive mutant rSeV/dF (ts-rSeV/dF), namely 1.5th generation vector (Figure 1) (12). No significant modification on the vector production system is required to reconstitute ts-rSeV/dF. This vector shows an apparent reduction of cytopathic effects on various types of cell *in vitro* as well as in mouse lungs *in vivo* (12).

Advanced designs of rSeV vectors with multiple depletion of envelope-related genes (rSeV/dFdM and rSeV/dFdMdHN, or second and third-generation vector, relatively) are being produced on a laboratory scale today. These vectors show almost complete elimination of budding of secondary virus-like particles (13). Multiple deletion of envelope-related genes has contributed to significant reductions in cytotoxic effects and host immune responses particularly in the case of innate immunity (including natural killer cell activity), and modest prolongation of transgene expression has been observed when these advanced vectors were used (13). Succeeding designs of SeV-based vector systems have derived a novel concept of "cytoplasmic gene therapy," assessing the feasibility to provide more efficient and safer therapy in clinical settings. However, cytoplasmic gene therapy itself also causes a variety of cellular responses related to host immune responses.

3. ESTABLISHMENT OF rSeV/DC-BASED CANCER IMMUNOTHERAPY

3.1. Responses of DCs to rSeV infection

As described above, infection with SeV-WT as well as rSeV evokes a considerable immune response in the host, even though envelope-related genes have been deleted from their genome. To assess this mechanism, we examined the responses of human, rat, and murine DCs to SeV-WT and rSeV. As shown in Figure 2, SeV-WT as well as additive rSeV expressing green fluorescent protein (GFP; SeV-GFP) amplified in chorioallantonic fluid of embryonated chicken eggs, stimulated the expression of CD80 on human peripheral blood-derived myeloid DCs (PB-DCs): similar consequences were observed upon expression of CD86, MHC class II, and CD40, and also with the use of rSeV/dF and ts-rSeV/dF (data not shown). Importantly, SeV-mediated upregulation of these surface proteins was completely diminished by the pretreatment of viruses with UV irradiation (Figure 2). These results attest that genome replication/transcription, but not envelope proteins or their structures, of SeV-WT as well as rSeV is critical for stimulating DCs. Furthermore, even in the presence of rSeV/dF or in the case where ts-rSeV/dF stimulated allogeneic mixed lymphocyte reaction (allo-MLR), rSeV/DCs produced various cytokines at high levels and showed a moderate loss of their phago-/endocytotic activity. These findings indicate that SeV-infected DCs have the identical properties with the mature phenotype as is the case with other animal studies. It is clear from these results that the potential of genome replication/transcription of rSeV in cytoplasm, not envelope proteins, was crucial for inducing host immune responses. For good measures, a possible use of rSeV/DCs for immunotherapy against malignancies is also implied.

3.2. Potency of rSeV/DC-based cancer immunotherapy

As an initial experiment to assess the potency of rSeV/DC-based immunotherapy against malignancies, we used additive rSeVs and typical tumor cell lines of murine malignant melanomas B16F1 and F10, which show modest susceptibility to immunotherapy (14). Lipopolysaccharide (LPS), a strong DC activator via Toll-like receptor 4 (TLR4), was used as a positive control. We also used rSeV expressing murine interferon-beta (mIFN-beta) since malignant melanoma is sensitive to IFN-beta (15).

Figure 3 shows the antitumor effects of DC therapies against B16F1 low-malignancy murine melanoma. rSeV/DC therapy that was started three days after tumor inoculation was attended with frequent elimination of tumors (Figure 3A) as well as superior survival (Figure 3B) compared with the use of LSP-DCs. We then tested the antitumor activity of rSeV/DCs against highly malignant murine melanoma B16F10. In this

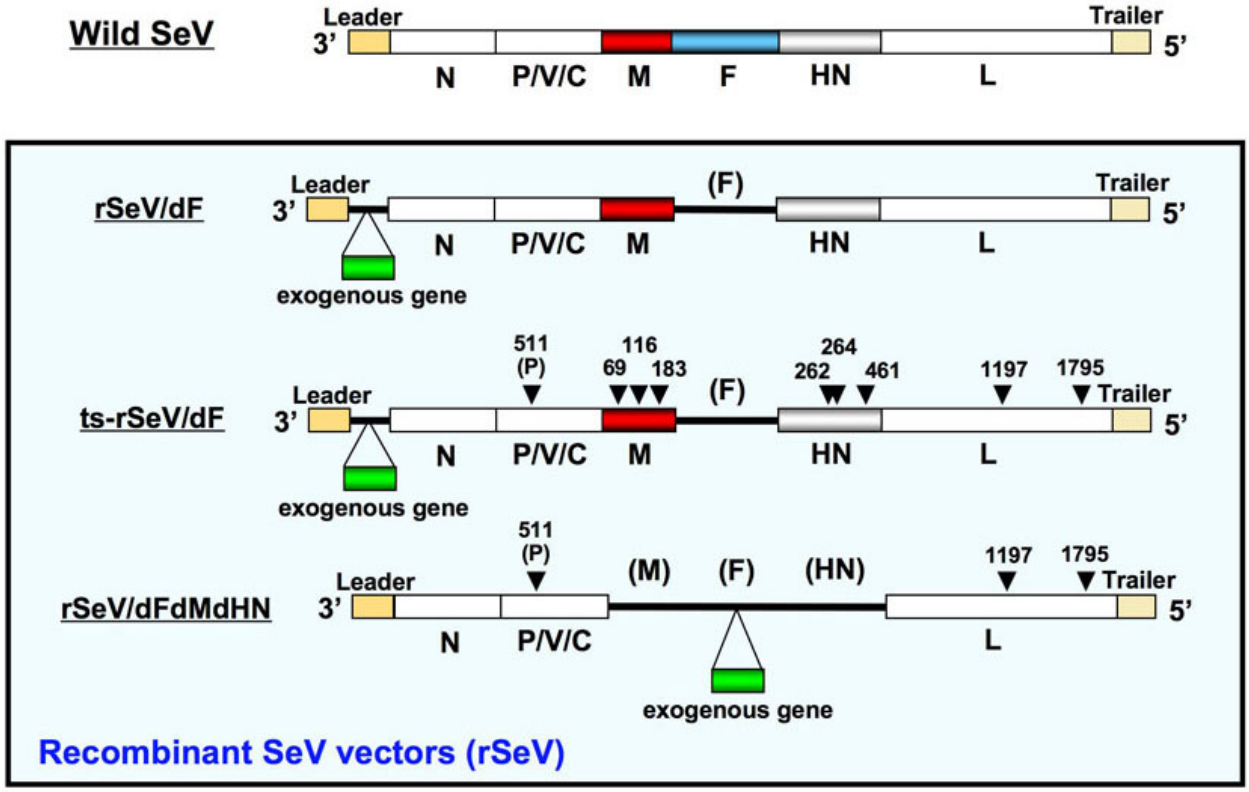

Figure 1. Schematic representation of the structures of rSeV used in this study. All recombinant viruses were based on the Z-strain of SeV, encoding six genes (M, F, and HN for membranous proteins, and NP, P/V/C, and L for negative-strand genomic RNP). Additive-type rSeV was simply inserted with the exogenous gene between the leader sequence and the open reading frame of the NP gene (5–11). Two schemes shown at the bottom demonstrate the structures of newly developed rSeVs (ts-rSeV/dF and rSeV/dFdMdHN). ts-rSeV/dF shows loss of expression of rest of membrane genes (M and HN), and some substitution of ribonucleotide sequences in the M, HN, and L genes, as indicated by arrowheads (12). rSeV/dFdMdHN has no membrane genes in the vector genome (13).

experiment, we started the treatment on day 10 after the tumor inoculation when the tumor had reached 7–9 mm in diameter (16). As shown in Figure 4, treatment with rSeV/DCs, not with either immature DCs or LPS-activated DCs, significantly improved the survival of tumor-bearing mice; notably, when rSeV/DCs expressing mIFN-beta were used, established B16F10 tumors became dormant. These effects were seen when rSeV/DCs were injected intratumorally and also when any types of rSeVs, including rSeV/dF and ts-rSeV/dF, were used. Furthermore, similar effects and antimetastatic activities have been observed in other tumor types with independent origins including murine hepatocellular carcinoma (MH134), murine neuroblastoma (c-1300), murine squamous cell carcinoma (SCCVII), murine osteoblastoma (LM8), and a rat prostate cancer (Dunning AT6.3). These results suggest the possible utility of rSeV as a new and promising tool for cancer immunotherapy in clinical settings.

3.3. Potential mechanism of the antitumor effect of rSeV/DCs

A precise mechanism by which rSeV/DCs therapy has its effects remains largely unknown. The fact that experimental studies indicating that UV-irradiated SeV did not produce any activation/maturation of DCs strongly suggest that cytoplasmic replication/transcription of the genome may be critical for its antitumor activity. Recent intensive studies by Dr. Akira and his colleagues have demonstrated that TLR-independent recognition of several RNA viruses by RNA helicases activates DCs and other cells; in particular a DExD/H-box RNA helicase, retinoic acid-inducible protein I (RIG-I), is critical for SeV (17–19). These findings prompted us to examine a role of the RIG-I-related signal transduction pathway (unpublished data). To perform this experiment, we newly constructed rSeV/dF expressing caspase-recruiting domain (CARD)-deleted dominant negative inhibitor RIG-IC (19). As expected, transfection of DCs with rSeV-dF stimulated the expression of IFN-beta as well as that of proinflammatory cytokines including interleukin (IL)-1beta, IL-6, tumor necrosis factor-alpha, and IL-12, which was completely disappeared by treatment with rSeV/dF expressing RIG-IC. On the other hand, expression of surface markers of DCs including CD80, CD86, and OX62 (an integrin) was not affected by expression of RIG-IC. Of interest, the antimetastatic effect of rSeV/dF-DCs was not impaired by RIG-IC expression: this implies that the antimetastatic effect of rSeV/DCs does not depend on the RIG-I-related pathway.

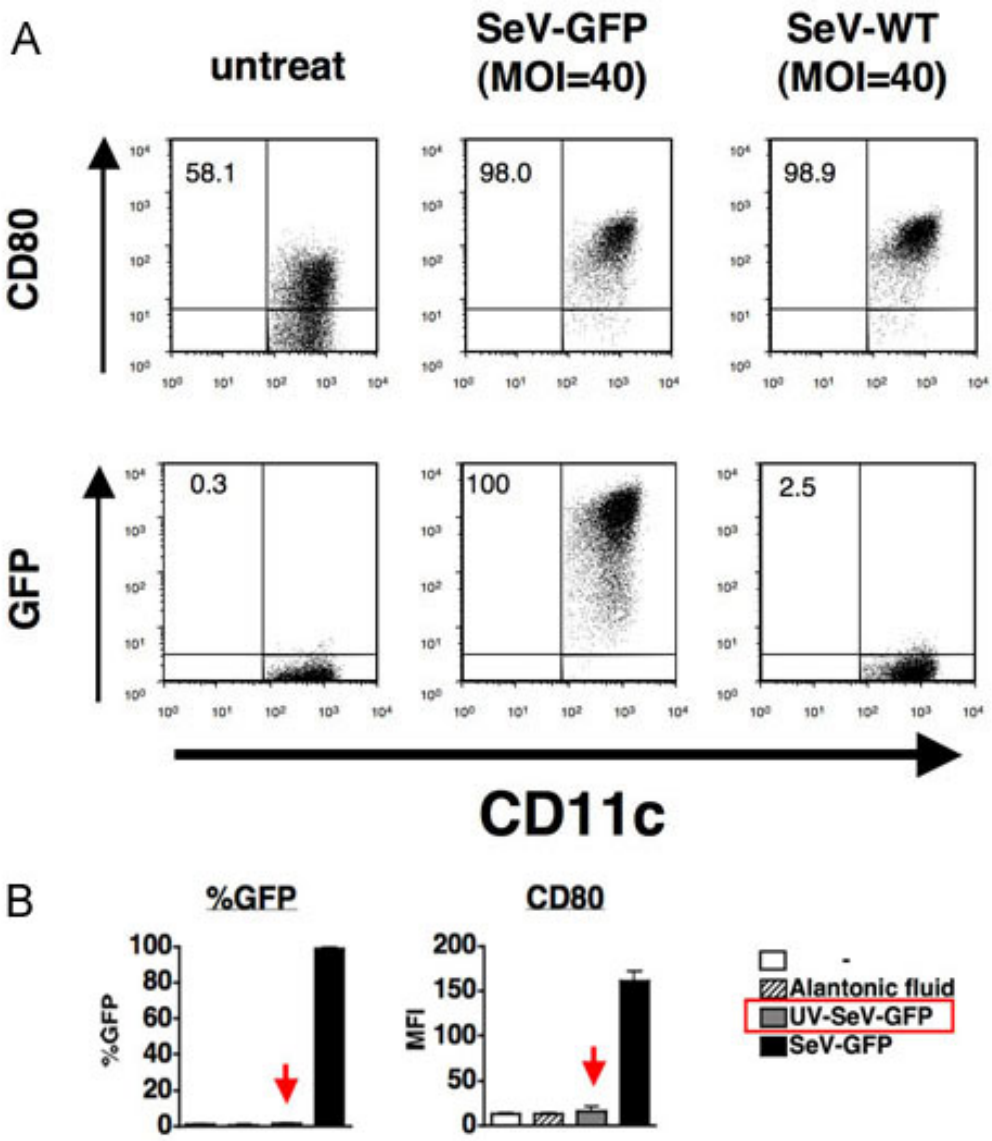

Figure 2. Optimization of tumor lysate-pulsed rSeV/dF-GFP-DC vaccine in a rat model of lung metastasis of AT6.3 prostate cancer. *P < 0.01. (A) Treatment regimen. (B) Optimization of dose–efficacy relationship. DCs were administered via a tail vein. The following numbers of animals were used: normal lung, n = 6; No Tx, n = 6; rSeV/dF, n = 17 (3×10^4 cells, n = 6; 3×10^5 cells, n = 5; 3×10^6 cells, n = 6). (C) Optimization of administration route. 3×10^6 DCs were used per vaccine. The following numbers of animals were used: normal lung, n = 6; No Tx, n = 9; rSeV/dF, n = 16 (s.c., n=7; i.v., n = 9).

4. CONCLUSIONS

Some concerns remain to be clarified. We however believe that lack of information regarding some of the mechanistic aspects of rSeV/DC-based immunostimulatory virotherapy does not diminish its value, because this system shows strong antitumor effects in some tumor models and species even when it is used in highly malignant tumor models (14). Since rSeV/dF is now available for clinical studies and the preparation of rSeV/dF-modified DCs does not require any special techniques or materials, we intend to continue investigating whether the use of this system has a significantly greater antitumor effect than current cancer vaccines in clinical settings.

5. ACKNOWLEDGMENTS

We thank Drs. Satoko Shibata, Sakura Tanaka, Kumi Yoshida, Kyosuke Tatsuta, and Shinji Okano of Kyushu University, and Tomonori Kato, Yasuo Yoneyama, Akinao Matsunaga, Atsushi Komaru, and Yui Harada of Chiba University for their contributions in *in vitro* and *in vivo* experiments. The authors also thank Drs. Makoto Inoue, Akihiro Tagawa, Takumi Kanaya, Hiroshi Ban, and Takashi Hironaka of DNAVEC Corporation for their

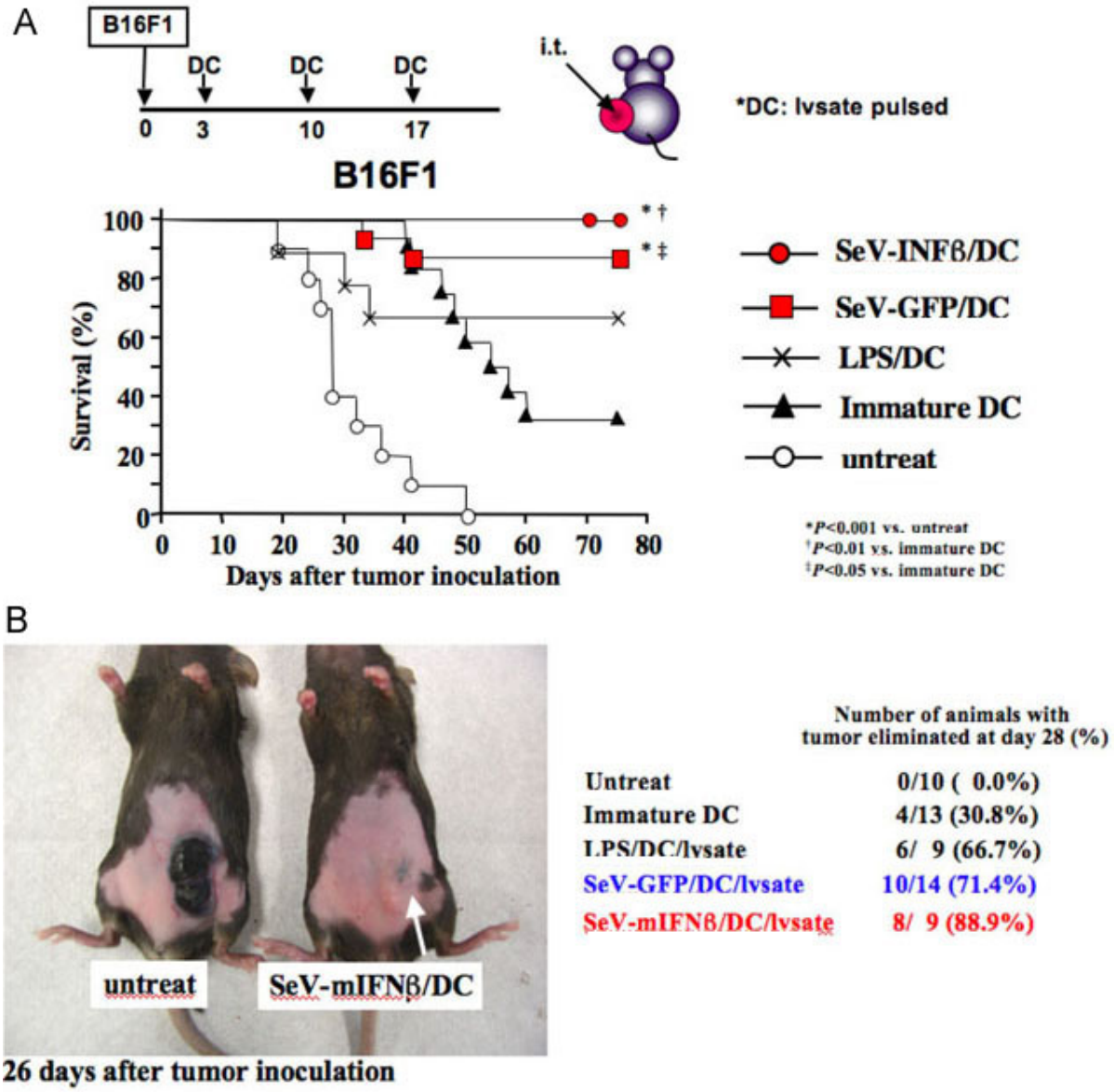

Figure 3. Assessment of antitumor activity of rSeV/DCs against low-malignant murine melanoma B16F1. Three days after tumor cell inoculation, DCs were injected intratumorally according to the indicated regimen (an early treatment regimen: a scheme shown at the bottom). Tumor lysate was pulsed to immature DCs, which were subsequently treated with LPS, SeV-GFP, or SeV-mINF-beta, and these DCs were used for DC immunotherapy eight hours later. DC treatment was done three times. The data are totals of three independent experiments. (A) Experimental design to assess the stimulator-dependent antitumor effect of DCs (upper panel). Survival curve of mice bearing B16F1 melanoma treated with various DCs. Significant prolongation of survival was seen in rSeV/DC groups (bottom). (B) Typical and representative gross observation of mice with B16F1 tumors treated with or without SeV-mIFN-beta/DC/lysate twenty-six days after tumor cell inoculation. Note the complete rejection of tumors after SeV-mIFN-beta/DC/lysate treatment (arrow), which was confirmed by histopathological examination (data not shown). Tumor rejection rate by mice at day 75 is also shown in the right column. Adapted from reference 14.

excellent technical assistance in the vector construction and large-scale production, and Ms. Chie Arimatsu (Department of Pathology, Kyushu University) and Ms. Kumi Ishida (Department of Gene Therapy, Chiba University) for their excellent help with animal experiments. We are also grateful to Ms. Kaori Tsukada for critically reading the manuscript. Dr. Yonemitsu is a member of Scientific Advisory Board of DNAVEC Corporation. This work was supported in part by Research Grants of 21st COE program, Chiba University Graduate School of Medicine, Grants-in-Aid (to TI and YY) from the Japanese Ministry of Education, Culture, Sports, Science,

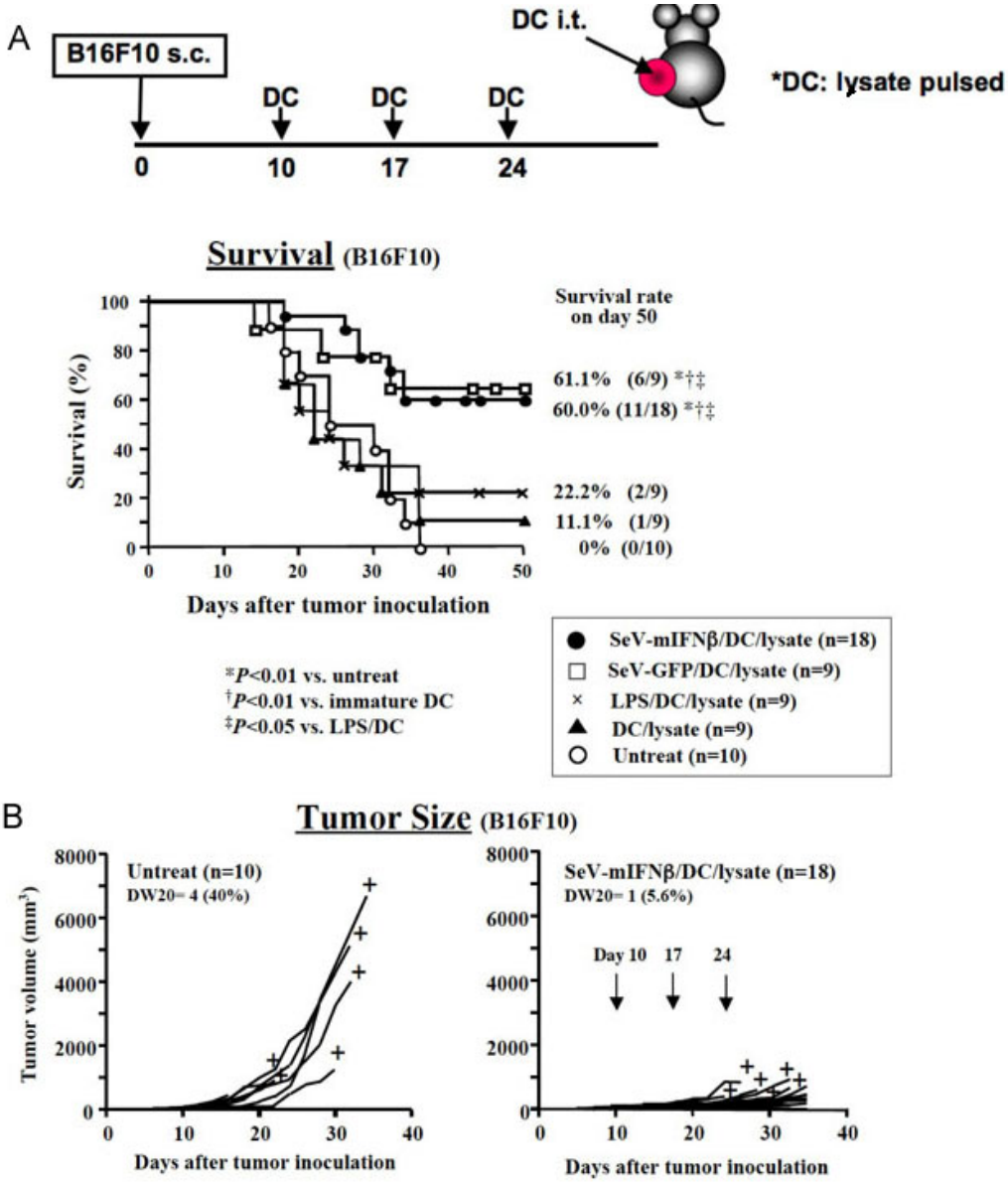

Figure 4. Tumor cell-dependent, divergent effects of rSeV/DCs immunotherapy expressing mIFN-beta (late treatment regimen). *$P < 0.01$. (A) Experimental design to assess the stimulator-dependent antitumor effect of DCs (upper panel). Survival curve of mice bearing B16F10 melanoma treated with various DCs. Significant prolongation of survival was seen in the rSeV/DC groups (bottom). (B) Time course of the volume of individual B16F10 tumors (IFN-beta-sensitive malignancy) treated with SeV-mIFNbeta/DC/lysate (right). Untreated animals were used as a control group (left). DW20 indicates the number of death animals within day 20. + indicates individual animals with death after day 21.

and Technology, and Research Grants from Sankyo Foundation of Life Science (to YY) and Mitsubishi Pharma Research Foundation (to YY).

6. REFERENCES

1. Yonemitsu Y, Kaneda Y: Hemagglutinating virus of Japan-liposome-mediated gene delivery to vascular cells and tissue. Ed.Baker AH, Molecular Biology of Vascular Disease. Method in Molecular Medicine: Humana press. *U.S.A. pp* 295-306 (1999)

2. Nagai Y: Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. *Rev Med Virol* 9, 83-99 (1999)

3. Markwell MA, Svennerholm L. Paulson JC: Specific gangliosides function as host cell receptors for Sendai virus. *Proc Natl Acad Sci USA*. 78, 5406-5410 (1981)

4. Kido H, Murakami M, Oba K, Chen Y, Towatari T: Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. *Mol Cell* 9, 235-244 (1999)

5. Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Dian J, Steel R, Phillipe S, Zhu J, Jeffery PK, Kato A, Hasan MK, Masaki I, Nagai Y, Fukumura M, Hasegawa M, Geddes DM, Alton EWFW: Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 18, 970-973 (2000)

6. Masaki I, Yonemitsu Y, Komori K, Ueno H, Nakashima Y, Nakagawa K, Fukumura M, Kato A, Hasan MK, Nagai Y, Sugimachi K, Hasegawa M, Sueishi K: Recombinant Sendai Virus-mediated Gene Transfer to Vasculature: a new class of efficient gene transfer vector to the vascular system. *FASEB J* 15, 1294-1296 (2001)

7. Yamashita A, Yonemitsu Y, Okano S, Nakagawa K, Nakashima Y, Iwamoto Y, Nagai Y, Hasegawa M, Sueishi K: Fibroblast growth factor-2 determines severity of joint disease in adjuvant-induced arthritis in rats. *J Immunol* 168, 450-457 (2002)

8. Jin CH, Kusuhara K, Yonemitsu Y, Nomura A, Okano S, Takeshita H, Hasegawa M, Sueishi K, Hara T: Recombinant Sendai virus provides a highly efficient gene transfer into human cord blood-derived hematopoietic stem cells. *Gene Ther* 10, 272-280 (2003)

9. Okano S, Yonemitsu Y, Nagata S, Sata S, Nakagawa K, Tomita Y, Nakashima Y, Hasegawa M, Sueishi K. Recombinant Sendai virus vectors for activated T-lymphocytes. *Gene Ther* 10:1381-1391, (2003)

10. Masaki I, Yonemitsu Y, Yamashita A, Sata S, Komori K, Fukumura M, Hou X, Hasegawa M, Sugimachi K, Sueishi K: Gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of VEGF165, but not of FGF-2. *Circ Res* 90, 966-973 (2002)

11. Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, Ueda Y, Lee YS, Fukumura M, Iida A, Kato A, Nagai Y, Hasegawa M: A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* 74, 6564-6569 (2000)

12. Inoue M, Tokusumi Y, Ban H, Kanaya T, Tokusumi T, Nagai Y, Iida A, Hasegawa M: Nontransmissible virus-like particle formation by F-deficient sendai virus is temperature sensitive and reduced by mutations in M and HN proteins. *J Virol* 77, 3238-3246 (2003)

13. Yoshizaki M, Hironaka T, Iwasaki H, Ban H, Tokusumi Y, Iida A, Nagai Y, Hasegawa M, Inoue M: Naked Sendai virus vector lacking all of the envelope-related genes: reduced cytopathogenicity and immunogenicity. *J Gene Med* 8, 1151-1159 (2006)

14. Shibata S, Okano S, Yonemitsu Y, Onimaru M, Sata S, Nagata-Takeshita H, Inoue M, Hasegawa M, Moroi Y, Furue M, Sueishi K: Induction of efficient antitumor immunity using dendritic cells activated by Sendai virus and its modulation of exogenous interferon-beta gene. *J Immunology* 177, 3564-3576 (2006)

15. Umeda T, Aoki K, Yokoyama A, Ohara H, Hayashi O, Tanaka K, Nishioka K: Changes in immunological parameters after combination adjuvant therapy with intravenous DTIC, ACNU, and VCR, and local injection of IFN-beta (DAV + IFN-beta therapy) into malignant melanoma. *J Dermatol* 25, 569-572 (1998)

16. Rosenberg SA, Yang JC, Restifo NP: Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 10, 909-915 (2004)

17. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S: IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6, 981-988 (2005)

18. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reise-Sousa C, Matsuura Y, Fujita T, Akira S: Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441, 101-105 (2006)

19. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, and Fujita T:The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol* 5, 730-737 (2004)

Abbreviations: DC: dendritic cells; rSeV: recombinant Sendai virus; rSeV/dF: fusion gene-deleted rSeV; LPS: lipopolysaccaride; PB-DC: blood-derived myeloid DC; GFP: green fluorescent protein; RIG-I: retinoic acid-inducible gene I; rSeV/dF-RIGIC: rSeV/dF expressing dominant negative mutant of RIG-I

Key Words: Recombinant Sendai virus, Dendritic cells, Cancer immunotherapy, Retinoic acid-inducible gene-I, Review

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