Systemic Administration of a Novel Immune-Stimulatory Pseudovirion Suppresses Lung Metastatic Melanoma by Regionally Enhancing IFN-γ Production

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Abstract

**Purpose:** Cancer immunotherapy has encountered many difficulties in the face of the expectation to eradicate cancer, and new breakthroughs are required. We have previously shown that UV-inactivated Sendai virus particles (hemagglutinating virus of Japan envelope; HVJ-E) induce immunity against multiple tumor types. In this study, a novel pseudovirion that stimulates more robust antitumor immunity was designed for cancer treatment.

**Experimental Design:** First, we found that culturing murine splenocytes with HVJ-E in combination with interleukin (IL)-12 resulted in a remarkable increase in IFN-γ production compared with that observed in splenocytes cultured with IL-12 alone. The synergistic effects of HVJ-E and IL-12 on IFN-γ production were caused by viral F proteins independently of HVJ-E fusion activity and not by hemagglutination from hemagglutinin-neuraminidase (HN) proteins. We next constructed HN-depleted HVJ-E expressing the Fc region of immunoglobulin G (IgG) on the envelope and single-chain IL-12 containing the ZZ domain of protein A to produce an IL-12–conjugated HVJ-E particle without hemagglutinating activity.

**Results:** IL-12–conjugated HVJ-E dramatically enhanced the amount of IFN-γ produced by immune cells. Intratumoral injection of IL-12–conjugated HVJ-E eradicated murine melanomas more effectively than injection of wild-type HVJ-E through increased production of melanoma-specific CTLs. IL-12–conjugated HVJ-E preferentially accumulated in the lungs after systemic administration. When small metastatic melanoma foci were formed in the lungs, systemic administration of IL-12–conjugated HVJ-E significantly reduced the number of metastatic foci by inducing local production of IFN-γ in the lungs and generating large numbers of melanoma-specific CTLs.

**Conclusion:** IL-12–conjugated HVJ-E is a promising tool for the treatment of cancers, including lung metastasis. *Clin Cancer Res;* 19(3); 668–79. ©2012 AACR.

Introduction

Cancer tissues use several systems to induce immunotolerance, including the activation of FoxP3⁺CD4⁺CD25⁺ regulatory T cells (Treg; ref. 1). Therefore, although many types of anticancer drugs have been developed, the cure for cancer remains elusive. In recent years, much attention has been paid to cancer immunotherapy because it may suppress tumor metastasis and recurrence by activating immune cells to target cancer cells. Recently, several cancer immunotherapy systems (Provenge, ipilimumab, and anti-PD1) were developed. Provenge induces the activation of effector lymphocytes specific for cancer cells, and ipilimumab (anti-CTLA4 antibody) and the anti-PD1 antibody inhibit the downregulation of effector lymphocyte activity; these systems have shown beneficial effects for the treatment of cancer (2–6). Therefore, more effective immunotherapy should result from the activation of cancer-targeting effector lymphocytes and the suppression of immunosuppressive factors (7–9).

The Sendai virus (hemagglutinating virus of Japan; HVJ) belongs to the paramyxovirus family and has a negative-sense, single-strand RNA genome (10, 11) and 2 membrane glycoproteins. One of the glycoproteins is hemagglutinin-neuraminidase (HN), which binds to cell surface receptors, and the other is fusion protein (F), which allows for membrane fusion after binding to the receptors (10, 11). We recently reported that UV-inactivated HVJ (HVJ-envelope; HVJ-E) suppresses murine colon carcinoma (CT26) tumors by activating CTLs and eradicates murine renal cancer by activating natural killer (NK) cells (12, 13). Several cytokines and chemokines, such as IFN-β, CXCL10, and interleukin (IL)-6, are produced by dendritic cells in tumor...
The importance of IFN-γ in tumor cells (19, 20). Previous reports have shown the activation of CTLs and NK cells (16), the induction of MHC class I expression in cancer immunotherapy.

First, the inability of HVJ-E to directly induce IFN-γ or IFN-α, HVJ-E needs to be improved in the following ways. The HVJ-E induces hemagglutination in the blood. In this study, we attempted to overcome these limitations by developing a high-performance HVJ-E for more robust antitumor activity. Therefore, to develop a second generation HVJ-E with enhanced antitumor activity, we constructed interleukin (IL)-12-conjugated, hemagglutinin-neuraminidase (HN)-depleted HVJ-E (IL-12–HVJ-E). Although IL-12 has a robust antitumor effect, the systemic administration of IL-12 is prohibited by the critical side effects resulting from systemic elevation in IFN-γ levels. In contrast, IL-12–HVJ-E accumulated in the lung following intravenous administration, induced local IFN-γ expression without increasing the serum IFN-γ level, and significantly reduced metastatic melanoma in the lung. Thus, IL-12–HVJ-E is a promising tool to treat metastatic lung cancers as well as regional cancers.

Translational Relevance
Sendai virus-envelope (HVJ-E) has been shown to induce immunity to multiple types of tumors, and clinical trials to test its safety and antitumor immunity are ongoing in Japan. However, HVJ-E is not able to stimulate the production of IFN-γ, which is important for antitumor immunity. Therefore, to develop a second generation HVJ-E with enhanced antitumor activity, we constructed interleukin (IL)-12-conjugated, hemagglutinin-neuraminidase (HN)–depleted HVJ-E (IL-12–HVJ-E). Although IL-12 has a robust antitumor effect, the systemic administration of IL-12 is prohibited by the critical side effects resulting from systemic elevation in IFN-γ levels. In contrast, IL-12–HVJ-E accumulated in the lung following intravenous administration, induced local IFN-γ expression without increasing the serum IFN-γ level, and significantly reduced metastatic melanoma in the lung. Thus, IL-12–HVJ-E is a promising tool to treat metastatic lung cancers as well as regional cancers.

Materials and Methods

Virus
HVJ (VR-105 parainfluenza1 Sendai/52, Z strain) was purchased from the American Type Culture Collection (ATCC), amplified in the chorioallantoic fluid of 10- to 14-day-old chick eggs and purified using centrifugation, as previously described (12, 13).

Mice
Female C57BL/6N mice were purchased from Japan Clea and maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Osaka University (Suita, Japan).

Cell culture
Monkey kidney cells (LLCMK2) and F10 melanoma cells were purchased from the ATCC, and Chinese hamster ovary cells (CHO-K1) were purchased from the European Collection of Cell Cultures (LLCMK2, CHO-K1, F10 melanoma cells, and murine splenocytes were maintained in minimum essential medium (Gibco-BRL), Ham’s F-12 medium (F-12; MP Biomedicals), Dulbecco’s modified Eagle’s medium (Nacalai Tesque Inc.), and RPMI-1640 medium (Nacalai Tesque Inc.). All media were supplemented with 10% FBS (Biowest), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Penicillin-Streptomycin Mixed Solution, Nacalai Tesque Inc.). β-Mercaptoethanol (4 nL/mL) was added to the media for the culture of splenocytes.

Plasmids
To construct the HN deletion mutants, Fc-HN and full-length HN, HN cDNA, which was kindly provided by...
Generation of wt-HVJ-E, EZview Red Anti-HA Affinity Gel (Sigma), and ZZ-scIL-12 were purified from the supernatant with (Roche) were added to inhibit protein degradation. ScIL-12 (pore size, 1.2 μm) was passed through a filter to precipitate the wt-HVJ particles. wt-HVJ was inactivated by UV irradiation (99 mJ/cm²).

For wt-HVJ-E, the culture medium of HVJ-infected LLCMK2 cells was passed through a filter (pore size, 1.2 μm) and then centrifuged at 100,000 × g for 2 hours at 4°C to precipitate the wt-HVJ particles. wt-HVJ-E was inactivated by UV irradiation (99 ml/cm²).

For ΔHN-HVJ-E, LLCMK2 cells were transfected with 100 pmol/mL HN-siRNA using Lipofectamine and Plus Reagent. Twenty-four hours after the transfection, the cells were infected with HVJ (1.5 particles/cell) for 1 hour. ΔHN-HVJ-E was collected using the same protocol as wt-HVJ-E.

Fc-HVJ-E was collected from LLCMK2 cells stably expressing Fc-HN using the same protocol as ΔHN-HVJ-E.

Generation of F1/F2-formed wt-HVJ-E and F-degraded ΔHN-HVJ-E
F1/F2-formed wt-HVJ-E was prepared by treating wt-HVJ-E with 5 μg/mL trypsin (Nacalai Tesque Inc.) for 30 minutes at 37°C. F-degraded ΔHN-HVJ-E was prepared by treating ΔHN-HVJ-E with 2.5 mg/mL trypsin for 24 hours at 37°C.

Production of scIL-12–HVJ-E
Fc-HVJ-E was treated with Factor Xa (5 μg/mL) for 2.5 hours at 23°C to activate the F protein, and then ZZ-scIL-12 (10 μg) was added. The mixture was placed in a 30% sucrose liquid solution (1.2 mL in a 1.5-mL tube) and centrifuged at 20,000 × g for 1 hour at 4°C. The pellets of scIL-12–HVJ-E were resuspended in PBS.

Sucrose density gradient centrifugation
A 25% to 50% sucrose gradient was created using the Gradient Master system (Towa Kagaku). A mixture of ZZ-scIL-12 and Fc-HVJ-E was placed in the sucrose gradient and centrifuged at 100,000 × g for 11 hours at 4°C.

Coprecipitation of Fc-HN with protein A-Sepharose
LLCMK2 cells transiently expressing Fc-HN and ecto-100aa-HN were solubilized with radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor tablets, and the supernatant was mixed with protein A-Sepharose (GE Healthcare). The mixture was then centrifuged at 2,300 × g for 5 minutes at 4°C, and the protein that coprecipitated with protein A-Sepharose was solubilized with sample buffer for SDS-PAGE.

Western blot analysis
The samples were subjected to SDS-PAGE on 12% gels, and the proteins were transferred to Immobilon-P Transfer Membranes (Millipore Co.). To detect proteins, anti-HN (Scrum Inc.), anti-F (Scrum Inc.), anti-M (Hokkaido System Science Co., Ltd.), anti-Myc tag (Medical & Biological Laboratories Co., Ltd.), anti-HA tag (Sigma), or anti-β-actin (Sigma) immunoglobulin G (IgG) were used as primary antibodies. ECL horseradish peroxidase–conjugated donkey anti-rabbit IgG (GE Healthcare UK Ltd.) was used as a secondary antibody for HN, F, and M detection, and ECL horseradish peroxidase–conjugated sheep anti-mouse IgG (GE Healthcare UK Ltd.) was used as a secondary antibody for Myc tag, HA tag, and β-actin detection. ECL Western Blotting Detection Reagent (GE Healthcare UK Ltd.) was used to detect the signals for each protein.

Immunostaining
HN-transfected LLCMK2 cells were stained with an anti-Myc tag IgG primary antibody and an Alexa Fluor 488–conjugated goat anti-mouse IgG secondary antibody. The cells were mounted in VECTASHIELD mounting medium (Vector Laboratories) and imaged with a confocal laser.
microscope (Radiance 2100: Bio-Rad Japan) equipped with the Laser Sharp 2000 software program.

**Preparation of splenocytes**

Spleens were isolated from female C57BL/6N mice, and the cells derived from the spleens were filtered through a 40-μm mesh sieve. These cells were hemolyzed buffer (Immo-Biological Laboratories Co., Ltd.), and the splenocytes were isolated.

**In vitro measurement of IFN-γ**

Splenocytes (2 × 10^5 cells/100 μL/well) were seeded on 96-well plates. scIL-12 or ZZ-scIL-12 (10, 20, or 2,000 pg) and HVJ-E (1.5 or 3 × 10^7 particles; F0: fusion-competent wild-type, F0: fusion-incompetent wild-type, and ΔHN: HN-deleted type) were added to the splenocytes in each experiment in a total volume of 100 μL of culture medium. The culture medium was collected 24 hours after treatment. The IFN-γ concentration of the culture medium was measured using an IFN-γ ELISA (R&D Systems, Inc.).

**In vivo tumor volume measurement and depletion of CD4, CD8, and NK cells**

Viable F10 melanoma cells (5 × 10^3 cells) were resuspended in 50 μL PBS and intradermally injected into the backs of female C57BL/6N mice. When each tumor had grown to 3 to 5 mm in diameter, the mice were treated with an intratumoral injection of wt-HVJ-E, scIL-12–HVJ-E (3 × 10^8 particles in a total volume of 100 μL) or 100 μL PBS on days 5, 7, and 9. Tumor volume was measured in a blind manner with slide calipers using the following formula: tumor volume (mm^3) = length × (width)^2/2.

Anti-CD4 (clone GK1.5) and anti-CD8 (clone 53-7.62) antibodies were kindly provided by Dr. Murakami (Osaka University, Suita, Japan), and the anti-asialo GM1 antibody was purchased from Wako Pure Chemical Industries, Ltd. To deplete the CD4^+ T cells, CD8^+ T cells, or NK cells, each antibody [anti-CD4 (200 μg), anti-CD8 (500 μg), and anti-asialo GM1 (20 μg)] was administered intraperitoneally on days 4, 5, 6, 7, 9, and 11, and the anti-asialo GM1 antibody (40 μg) was also administered intratumorally at the time of the scIL-12–HVJ-E administration. Rat IgG (Sigma) was used as a control for the anti-CD4 and anti-CD8 antibodies, and rabbit IgG (R&D Systems) was used as a control for the anti-asialo GM1 antibody.

**Labeling of scIL-12–HVJ-E with ^125I**

HVJ-E (wt-HVJ-E or scIL-12–HVJ-E) was labeled with iodine-125 radionuclide (^125I; PerkinElmer) using lactoperoxidase, and the ^125I-labeled HVJ-E was suspended in saline to a concentration of 6 × 10^8 particles/200 μL. The ^125I-labeled HVJ-E (200 μL) was intravenously injected into the tail veins of the mice, and tissues (brain, lungs, heart, liver, kidneys, spleen, muscle, and blood) were harvested from the mice after 24 hours. The ^125I level in the tissues was measured using γ-scintillation counting.

**Systemic administration of scIL-12–HVJ-E**

A viable F10 melanoma cell suspension (5 × 10^5 cells/200 μL PBS) was intravenously injected into the tail veins of female C57BL/6N mice followed by an injection of 300 μL PBS to avoid embolization of the vessels by the F10 melanomas. Beginning 5 days after the F10 melanoma injection, the mice received 3 intravenous injections of wt-HVJ-E, scIL-12–HVJ-E (6 × 10^8 particles/200 μL PBS), ZZ-scIL-12 (500 pg/200 μL PBS), or 200 μL PBS via the tail vein every other day. On day 14, the lungs were isolated from the mice after the last injection was administered, and the number of metastatic foci was counted.

**^51Cr release CTL assays and ELISpot assays**

wt-HVJ-E, scIL-12–HVJ-E, ZZ-scIL-12, or PBS was injected locally into the intradermal tumor masses or systemically via the tail vein in the mice bearing pulmonary metastases. The spleens were isolated from the mice 10 days after the last injection was administered. Splenocytes were isolated from the spleens as described earlier. F10 melanoma cells were treated with mitomycin C (15 μg/mL) for 45 minutes.

For the ^51Cr release CTL assays, the splenocytes and mitomycin C-treated F10 melanoma cells were mixed at a 10:1 ratio and cultured with culture medium that included 10 ng/mL recombinant mouse IL-2 (R&D Systems). Four days later, culture medium containing 5 ng/mL recombinant mouse IL-2 was added to the cultured cells, and the cells were cultured for another 3 days. Nonadherent splenocytes were collected, and serial 2-fold dilutions of splenocytes (20, 10, 5, 2.5, 1.25, and 0.625 × 10^7/100 μL/well) were made in 96-well plates. As positive and negative controls, 1% NP-40 and culture medium were added to the wells, respectively. The F10 melanoma cells were treated with the chromium-51 radionuclide (^51Cr; PerkinElmer Japan Co., Ltd.; 1.85 MBq/250 μL) for 45 minutes at 37°C and washed 3 times with RPMI-1640. A ^51Cr-labeled F10 melanoma suspension (2 × 10^6 cells/100 μL) was added to each well of the 96-well plate, which contained a 2-fold dilution of splenocytes, and the cells were incubated for 4 hours at 37°C. The supernatant of each well was collected after incubation, and the amount of ^51Cr released from the labeled F10 melanoma cells was determined using γ-scintillation counting.

For the ELISpot assay, the splenocytes and mitomycin C-treated F10 melanoma cells were mixed at a ratio of 10:1. Forty-eight hours later, nonadherent splenocytes were collected, and the ELISpot assay was conducted using the Mouse IFN-γ Development Module (R&D Systems) and the ELISpot Blue Color Module (R&D Systems). The number of IFN-γ-secreting cells was subsequently counted.

**Real-time RT-PCR**

On day 5 after the intravenous injection of the F10 melanoma cells, wt-HVJ-E, scIL-12–HVJ-E (6 × 10^8 particles/200 μL PBS), ZZ-scIL-12 (500 pg/200 μL PBS), or 200 μL PBS was intravenously injected into the tail veins of the mice once a day for 3 consecutive days. The lungs were isolated from the mice 24 hours after the last injection was
administered and homogenized in lysis buffer from the RNeasy Mini Kit (Invitrogen) using the Multi-Beads Shocker cell disruption system (Yasui Kikai Co.). Total RNA was isolated from the homogenized lung specimens using the RNeasy Mini Kit, and cDNA was synthesized from the RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan, Ltd.). IFN-γ and NKG2D mRNA were quantified with real-time reverse transcriptase PCR (RT-PCR) using the Real-time PCR Master Mix (Toyobo Co., Ltd.) and TaqMan Probes (Applied Biosystems Japan, Ltd.) for murine IFN-γ and NKG2D. In the same manner, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was quantified as a control.

Statistical analysis
The statistical analyses were conducted using the Tukey–Kramer test or Student unpaired t test, and P less than 0.05 was considered to be statistically significant.

Results
The HVJ-E F protein enhanced the IL-12–induced IFN-γ secretion in a fusion-independent manner

We have described the construction of the murine scIL-12 expression vector in a previous report (28). In this study, we used this vector to produce scIL-12 proteins in CHO cells. Stimulation of splenocytes from normal C57BL/6N mice with HVJ-E or scIL-12 (0.1 ng/mL) induced only a low level of IFN-γ secretion. However, we showed that the combining HVJ-E and scIL-12 dramatically enhanced the secretion of IFN-γ by the splenocytes (Fig. 1A). In addition, administration of a high dose of scIL-12 (10 ng/mL) induced IFN-γ secretion, but scIL-12 was significantly less effective than combined treatment with scIL-12 and HVJ-E (Fig. 1A). Next, we investigated which cells of the heterogeneous splenocyte population produced IFN-γ in response to scIL-12 and HVJ-E and found that IFN-γ was mainly produced by CD3+ T cells (Supplementary Fig. S1).

The envelope of HVJ-E is composed of 2 different membrane proteins, the fusion protein (F) and HN. F exists in either the inactive F0 form on fusion-incompetent HVJ-E or the enzymatically cleaved, active F1/F2 form on fusion-competent HVJ-E (33). Splenocytes were thus treated with either F0- or F1/F2-HVJ-E in combination with scIL-12, and both types of HVJ-E induced robust IFN-γ secretion (Fig. 1B). Therefore, the HVJ-E–mediated enhancement of IFN-γ secretion was observed to be independent of fusion competence. Next, we investigated which membrane glycoproteins, HN or F, were responsible for the enhanced IFN-γ production. When administered with scIL-12, HN-depleted HVJ-E (ΔHN-HVJ-E), which displays increased F expression on the envelope, induced enhanced IFN-γ secretion more potently than wild-type HVJ-E (wt-HVJ-E; Fig. 1C), suggesting that F, but not HN, may be involved in the induction of IFN-γ production. Although both the F and HN proteins are present on the surface of the HVJ-E particle, the F protein is predominant. To generate F-depleted HVJ-E, the F protein of ΔHN-HVJ-E was enzymatically degraded by trypsin over-treatment (2.5 mg/mL for 24 hours at 37°C). F-depleted ΔHN-HVJ-E induced a significantly reduced level of IFN-γ secretion (Fig. 1D), and moreover, the anti-F antibody

Figure 1. The synergistic effect of HVJ-E on IL-12–induced IFN-γ secretion. A–D, scIL-12 and/or various types of HVJ-E [(A) wt-HVJ-E (F1/F2), (B) F1/F2 or F0 wt-HVJ-E, (C) wt-HVJ-E or ΔHN-HVJ-E, and (D) ΔHN-HVJ-E or F-degraded ΔHN-HVJ-E] were added to splenocytes, and 24 hours later, the IFN-γ concentrations of the supernatants were measured by ELISA. To confirm the protein composition of various types of HVJ-E, several viral proteins (HN, F0, F1, and M) were detected by Western blot analysis: (B) F1/F2 and F0 wt-HVJ-E, (C) wt-HVJ-E or ΔHN-HVJ-E, and (D) ΔHN-HVJ-E and F-degraded ΔHN-HVJ-E. All data are presented as the mean ± SD (n = 4); **P < 0.01, Tukey–Kramer test.
inhibited the induction of IFN-γ (Supplementary Fig. S2A), suggesting that the activity of the F protein is required for IFN-γ induction.

**Overall strategy for the presentation of IL-12 on the surface of HVJ-E**

These results prompted us to hypothesize that a close association between HVJ-E and IL-12 in the tumor microenvironment in vivo may synergistically enhance antitumor immunity. To prove this hypothesis, we generated scIL-12–conjugated HVJ-E (scIL-12–HVJ-E) that presents scIL-12 on the surface of δHNV-HVJ-E by binding to the IgG constant region (Fc) and the protein A-Fc binding domain (ZZ; ref. 34) using viral gene engineering technology (35–37). This technology allowed for the manipulation of the HVJ-E membrane proteins by inducing the expression of recombinant envelope proteins on HVJ-infected cells (Fig. 2).

**Generation of Fc-rHN and Fc-HVJ-E**

To generate scIL-12–HVJ-E, we aimed to localize Fc-HN fusion proteins on the surface of δHNV-HVJ-E. We constructed expression plasmids of HN deletion mutants (full length-, ecto-400aa-, ecto-300aa-, ecto-200aa-, and ecto-100aa-HN; Fig. 3A). Although all rHN proteins were expressed in the LLC-MK2 cells transfected with these plasmids (Fig. 3B), only ecto-100aa-HN was incorporated into the cell-derived progeny of HVJ by infecting rHN-expressing cells with live HVJ (Fig. 3C). Therefore, we constructed Fc-rHN, in which murine Fc was fused to the C-terminus of ecto-100aa-HN (Fig. 3D). We confirmed that Fc-rHN had the ability to bind to protein A using a coprecipitation assay with protein...
A-Sepharose (Fig. 3E). We then generated Fc-HVJ-E, which included Fc-rHN with depleted wt-HN (Fig. 3F).

### Generation of ZZ-scIL-12 and scIL-12–HVJ-E

To present scIL-12 on the surface of Fc-HVJ-E, we constructed ZZ-scIL-12 by inserting the ZZ domain into the downstream scIL-12 signal peptide (Fig. 4A). ZZ-scIL-12 and scIL-12 induced IFN-γ secretion by splenocytes (Fig. 4A). Next, we investigated the ability of ZZ-scIL-12 to bind to Fc-rHN on Fc-HVJ-E using sucrose density gradient centrifugation. When analyzed separately, ZZ-scIL-12 was detected in the upper layer, and HVJ-E was detected in the lower layer of the 25% to 50% sucrose gradients (Supplementary Fig. S3). However, ZZ-scIL-12 was shifted to the lower layer in the sedimentation of the ZZ-scIL-12 and Fc-HVJ-E mixture (Fig. 4B), suggesting that ZZ-scIL-12 binds to Fc-rHN on Fc-HVJ-E. Moreover, ZZ-scIL-12–conjugated Fc-HVJ-E (scIL-12–HVJ-E) was incubated in murine serum, and ZZ-scIL-12 was maintained in Fc-HVJ-E for 24 hours (Fig. 4C). We calculated the amount of ZZ-scIL-12 on Fc-HVJ-E by comparing the density of the bands corresponding to ZZ-scIL-12 by Western blot analysis and estimated that approximately 6.83 molecules of ZZ-scIL-12 were loaded onto 1 particle of Fc-HVJ-E (Supplementary Fig. S4). Finally, scIL-12–HVJ-E displayed much stronger IFN-γ–inducing activity on splenocytes and dendritic cells than wt-HVJ-E or scIL-12 alone (Fig. 4D and Supplementary Fig. S2B).

### Antitumor effects against intradermal F10 melanomas induced by the intratumoral administration of scIL-12–HVJ-E

We next investigated the antitumor activity of scIL-12–HVJ-E in vivo. A murine intradermal tumor model was generated through intradermal inoculation of F10 melanoma cells (5 × 10^5 cells) into the backs of female C57BL/6N mice. When each tumor had grown to approximately 3 to 5 mm in diameter, scIL-12–HVJ-E, wt-HVJ-E, or PBS was injected into the tumor for a total of 3 times every other day. In these experiments, scIL-12–HVJ-E caused a much more robust tumor suppression than wt-HVJ-E (Fig. 5A). Moreover, the antitumor immune responses against the F10 melanomas were examined using 51Cr release CTL assays (Fig. 5B) and ELISPOT assays (Fig. 5C). The data revealed that scIL-12–HVJ-E remarkably enhanced CTL activity against F10 melanomas. Next, to investigate the roles of CD4^+ T cells, CD8^+ T cells, and NK cells in the therapeutic effect, scIL-12–HVJ-E–mediated tumor growth inhibition was assessed in mice depleted of CD4^+ T cells, CD8^+ T cells, and NK cells.
and NK cells by administrating a neutralizing antibody specific for each cell type (13, 38). The depletion of CD8+ and NK cells, but not CD4 cells, significantly decreased the scIL-12–HVJ-E–mediated effect (Fig. 5D–F), showing that the antitumor effect of scIL-12–HVJ-E was dependent on CD8+ T cells and NK cells but not CD4+ T cells.

Antitumor effects against metastatic lung F10 melanomas induced by the systemic administration of scIL-12–HVJ-E

We then investigated the tissue distribution of scIL-12–HVJ-E following intravenous injection. 125I-labeled HVJ-E was systemically administered via tail vein injection, and the level of 125I in the tissues (brain, lungs, heart, liver, kidneys, spleen, muscles, and blood) was measured using a γ-scintillation counter 24 hours after injection. Interestingly, scIL-12–HVJ-E and ΔHN-HVJ-E, but not wt-HVJ-E, preferentially accumulated in the lungs (Fig. 6A and Supplementary Fig. S5A), implying that HN depletion likely affected the localization of HVJ-E.

We next investigated the therapeutic effects of intravenous scIL-12–HVJ-E administration in a murine F10 melanoma model of lung metastasis. The mice were inoculated with F10 melanoma cells (5 × 105 cells) by intravenous injection. Multiple metastatic foci appeared on the surface of the lungs 5 days after the F10 melanoma injection (Supplementary Fig. S5B). We began intravenous administration of scIL-12–HVJ-E, wt-HVJ-E, or ZZ-scIL-12 when lung metastasis was confirmed. The injections were repeated for a total of 3 times every other day. As expected, only the scIL-12–HVJ-E–treated mice exhibited a significant reduction in the number of metastatic foci in the lungs (Fig. 6B). On the basis of our microarray analysis of the lung transcripts (data not shown), we focused on IFN-γ and NKG2D, which showed increased expression upon scIL-12–HVJ-E treatment. Real-time RT-PCR analysis confirmed that IFN-γ and NKG2D expression were both increased in lungs treated with scIL-12–HVJ-E (Fig. 6C). Although IFN-γ expression was found to be elevated in the lungs, the serum IFN-γ level was unchanged by scIL-12–HVJ-E treatment (Supplementary Fig. S5B). However, the coadministration of scIL-12 and Fc-HVJ-E increased the serum IFN-γ level (Supplementary Fig. S6B). Moreover, scIL-12–HVJ-E significantly induced NKG2D expression in the lungs, suggesting that NK cells and CD8+ T lymphocytes were activated in the lesions (Fig. 6C). Systemic administration of scIL-12–HVJ-E

Figure 4. The construction of ZZ-scIL-12 and the generation of scIL-12–HVJ-E. A, splenocytes were incubated with 10 ng/mL scIL-12 or ZZ-scIL-12 (2 ng/100 μL for each) for 48 hours, and the IFN-γ concentrations of the supernatants were measured by ELISA. B, scIL-12 or ZZ-scIL-12 was mixed with wt-HVJ-E or Fc-HVJ-E, and each mixture was treated to fractionation by 25% to 50% sucrose gradient. The scIL-12 or ZZ-scIL-12 and wt-HVJ-E or Fc-HVJ-E in each fraction was detected using Western blot analysis. An anti-HA antibody was used to detect scIL-12 and ZZ-scIL-12, and an anti-M antibody was used to detect wt-HVJ-E and Fc-HVJ-E. C, Fc-HVJ-E bound with ZZ-scIL-12 was incubated in murine serum at 37°C for 1, 6, or 24 hours and then subjected to sucrose gradient centrifugation. After isolating the Fc-HVJ-E fraction, ZZ-scIL-12 and Fc-HVJ-E were detected using Western blot analysis. D, wt-HVJ-E, scIL-12–HVJ-E (1.5 × 107 particles), or ZZ-scIL-12 (12.5 pg) in 100 μL PBS was added to the splenocytes for 24 hours, and the IFN-γ concentrations of the supernatants were measured by ELISA. All data are presented as the mean ± SD (n = 4). **, P < 0.01; Tukey–Kramer test.
induced robust activation of CTLs specific for melanoma (Fig. 6D), and no evident adverse effects were observed in the treated mice. The CTL activation was significantly reduced by an anti-IFN-γ antibody, indicating that the CTL activation was dependent on IFN-γ (Supplementary Fig. S7A). Moreover, the CTLs targeted melanoma cells specifically, as the cytotoxic effects were not detected in other cells (Supplementary Fig. S7B).

Discussion

In this study, we showed that HVJ-E dramatically enhances IL-12 activity and induces IFN-γ production in splenocytes. The systemic administration of IL-12–conjugated HVJ-E significantly increases the level of IFN-γ expression in the lungs without elevating the serum IFN-γ level and effectively induces antitumor activity against metastatic lung melanomas.

Previously, our group reported that several components of HVJ-E stimulate tumor cells and dendritic cells to secrete various cytokines. RNA genome fragments of HVJ-E, which are taken into host cells through membrane fusion, are recognized by RIG-I but not TLRs (39), and type-I IFN is induced in tumor cells and dendritic cells. However, F0-formed HVJ-E, which is unable to fuse with host cells, enhances scIL-12–induced IFN-γ secretion (Fig. 1B). Therefore, the activation of RIG-I by RNA fragments is unlikely to be correlated with IFN-γ enhancement. In this study, we showed that F is responsible for enhancing IFN-γ secretion (Fig. 1C and D). Although our previous report shows that F induces IL-6 secretion in dendritic cells in a fusion-independent manner (40), F-mediated IL-6 secretion does not mediate IFN-γ enhancement because IL-6 does not enhance IL-12 activity (41, 42). These results suggest that putative F receptors on...
splenocytes may transmit signals to enhance IFN-γ and IL-12 production.

ΔHN-HVJ-E enhanced IFN-γ induction more robustly than wt-HVJ-E because the expression levels of F in ΔHN-HVJ-E were increased by knocking down HN (37 and Fig. 1C). Because HN induces hemagglutination by binding to sialic acid on the surface receptors of red blood cells (43), the administration of wt-HVJ-E, especially intravenously, has limitations for cancer therapy. ΔHN-HVJ-E shows very low hemagglutinating activity (37) and robustly enhances IFN-γ and IL-12 production in vitro.

In the experiments shown in Fig. 5D–E, although CD8+ T cells and NK cells were required for scIL-12–HVJ-E–mediated antitumor effects, the depletion of CD4+ T cells did not affect the suppression of tumor growth by scIL-12–HVJ-E. Previous reports have indicated that the IL-12–mediated antitumor response is maintained in CD4+ T-cell–depleted mice (44) and that IL-12 stimulates antigen-specific CD8+ T cells in CD4+ T-cell knockout mice (45). Therefore, we suggest that scIL-12–HVJ-E–mediated antitumor immunity was independent of CD4+ T cells. Moreover, in Fig. 5D–F and Supplementary Fig. S7, the peritoneal administration of a large amount of control IgG (more than 200 µg) induced a small decrease in scIL-12–HVJ-E–mediated antitumor immunity. Although the precise mechanism is unclear, this result might be due to the immunosuppressive
effect of the administration of a large amount of IgG (intravenous immunoglobulin; ref. 46). In previous reports, the Fc–ZZ–binding system has been applied to various types of targeting vectors (47–49), and we hypothesized that sclL-12–HV1-E generated using this system could be functional upon systemic administration in mice. In fact, the Fc–ZZ binding of sCL-12–HV1-E was stable in serum for at least 24 hours (Fig. 4C), and intravenous administration of sCL-12–HV1-E resulted in elevated IFN-γ expression levels in the lungs due to pulmonary accumulation of the virions (Fig. 6A and C).

sCL-12–HV1-E and ΔHN-HV1-E, but not wt-HV1-E, preferentially accumulated in the lungs (Fig. 6A and Supplementary Fig. S5A). A previous report has indicated that HV1-E preferentially accumulates in spleen after intravenous administration (50). This accumulation in the spleen most likely occurs because HV1 on HV1-E induces hemagglutination by binding to the sialic acid on the erythrocyte surface, causing the erythrocyte and HV1-E complex to be transported to the spleen and degraded. Therefore, the accumulation of sCL-12–HV1-E and ΔHN-HV1-E in the lung may result from the loss of hemagglutinating activity upon HV1 knockdown and the subsequent escape from the "spleen-trap"; however, the precise mechanism by which this occurs remains unclear.

A previous study has shown that only high doses (~0.5–1.0 μg) of systemic IL-12 suppress tumor growth in mice by inducing a significant elevation in the serum IFN-γ level (30). However, such increases in serum IFN-γ have been shown to induce serious side effects, such as stomatitis, gastrointestinal bleeding, colitis, and diarrhea (31, 32). Therefore, the systemic administration of IL-12 has not been applied for clinical use thus far. We herein showed that systemically administered sCL-12–HV1-E, which contains approximately 500 pg sCL-12, preferentially accumulated in the lungs (Fig. 6A) and mediated the synergistic antitumor effects of HV1-E and IL-12 in the tumor microenvironment (Fig. 6B and C) without elevating the serum IFN-γ levels (Supplementary Fig. S6A). In contrast, the coadministration of sCL-12 and Fc-HV1-E increased the serum IFN-γ level (Supplementary Fig. S6B). Therefore, the conjugation of IL-12 and HV1-E is important to inhibit the elevation of serum IFN-γ, which may cause severe side effects (31, 32). The data shown here provide a basis for the future clinical application of sCL-12–HV1-E, which seems to have a higher anticancer activity and lower toxicity than IL-12 alone.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Kaneda
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Saga
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Saga, K. Tamai, Y. Kaneda
Writing, review, and/or revision of the manuscript: K. Saga, Y. Kaneda
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Saga, T. Yamazaki, Y. Kaneda
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References


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