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; 1–12. ©2012 AACR.
tissues and are associated with the antitumor immunity induced by HVJ-E. IL-6 plays a major role in tumor elimination. The antitumor activity of HVJ-E is abrogated by the suppression of IL-6 signaling with anti-IL-6 receptor antibodies in mice bearing CT26 cell-derived tumors (12), and HVJ-E fails to eliminate tumors in IL-6 knockout mice. HVJ-E inhibits Treg-mediated immunosuppression by inducing IL-6 secretion by mature dendritic cells (12). IL-6 most likely inhibits Tregs through increased methylation of the enhancer region of FoxP3, a key transcription factor of Tregs (14, 15). The F protein, one of the HVJ envelope proteins, has been found to be necessary for Toll-like receptor (TLR)–independent IL-6 production in dendritic cells.

Clinical studies to examine the safety and efficacy of HVJ-E have been conducted in patients with melanomas and prostate cancers at Osaka University Hospital (Suita, Japan) since 2009. To achieve more effective cancer immunotherapy, HVJ-E needs to be improved in the following ways. First, the inability of HVJ-E to directly induce IFN-γ production by immune cells must be addressed. IFN-γ is an important factor for antitumor activities, including the activation of CTLs and NK cells (16), the induction of chemokines that mediate T-cell infiltration into the tumor (17, 18), and the upregulation of MHC class I expression in tumor cells (19, 20). Previous reports have shown the importance of IFN-γ in cancer immunotherapy (21), and several clinical trials have shown the positive effect of IFN-γ treatment in cancer therapy (22–25). Second, HVJ-E should be improved to enable systemic administration. The HVJ-E currently used cannot be systemically administered because the HN protein induces hemagglutination in the blood. In this study, we attempted to overcome these limitations by developing a high-performance HVJ-E for more robust cancer immunotherapy.

**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.

IL-12 is a heterodimeric cytokine that is composed of p40 and p35 subunits and exhibits antitumor activity by stimulating IFN-γ secretion and promoting Th1 differentiation (26, 27). It has also been reported that single-chain (sc) IL-12, which connects p40 and p35, maintains the bioactivity of IL-12 (28, 29). Although systemic administration of IL-12 suppresses tumor growth (30), severe side effects are induced by the high serum levels of IFN-γ (31, 32). Thus, systemic administration of IL-12 has not been used clinically. Considering the advantages and disadvantages of IL-12 for cancer therapy, we used a combination of IL-12 and HVJ-E to enhance the antitumor activity of HVJ-E.

In this report, we found that IL-12 and HVJ-E acted synergistically to enhance IFN-γ production in a fusion-independent manner. We then showed that combination treatment with HN-depleted HVJ-E and scL-12 induced much higher levels of IFN-γ secretion from cultured splenocytes than treatment with scL-12 alone and eradicated tumors more effectively than treatment with HVJ-E alone. Systemic administration of scL-12–conjugated HN-depleted HVJ-E successfully reduced the number of pulmonary metastatic foci in murine melanomas by regionally enhancing IFN-γ production in the lungs without elevating the serum IFN-γ levels.

**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.), respectively. All media were supplemented with 10% FBS (BioWest), 100 μg/mL penicillin, and 0.1 mg/mL streptomycin (Penicillin–Streptomycin Mixed Solution, Nacalai Tesque Inc.). B-Mercaptoethanol (4 nM/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

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**Translated**

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H. Taira (Iwate University, Iwate, Japan), was used as a template for amplification. Several HN deletion mutants (ecto-400aa-HN, ecto-300aa-HN, ecto-200aa-HN, ecto-100aa-HN, and ecto-0aa-HN) were generated using PCR. The Fc (CH2–CH3) domain of murine IgG2a cDNA was amplified from murine B cell cDNA, and Fc-HN was generated by connecting Fc and ecto-100aa-HN.

Murine IL-12 p40 and p35 cDNAs were amplified from murine splenocyte cDNA, and scIL-12 was generated by connecting p40 with the 5'-terminus of p35 without including the first 66 nucleotides (the signal peptide sequence) via a (GGGGS)3 linker. ZZ domain cDNA was amplified from pEZZ18 (GE Healthcare UK Ltd.) and fused with an HA-tag sequence at the 5'-terminus (HA-ZZ) using PCR. ZZ-scIL-12 and scIL-12 were generated by introducing the HA-ZZ or HA-tag sequence into the downstream signal peptide sequence of the p40 region.

To construct expression plasmids for the recombinant proteins, the coding regions were introduced into the CAGIpuro Gateway vector.

**Gene transfer**

The recombinant HN (rHN) expression vector was transferred to LLCMK2 cells using Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen) according to the manufacturer’s instructions.

**Stable transformation**

To generate a stable transformant of Fc-HN, LLCMK2 cells were transfected with CAGIpuro/Fc-HN using electroporation. A stable transformant was isolated by adding puromycin (4 μg/mL) to the culture medium. To generate a stable transformant of scIL-12 and ZZ-scIL-12, CHO-K1 cells were transfected with CAGIpuro/scIL-12 and ZZ-scIL-12 using electroporation. The respective stable transformants were isolated by adding puromycin (27 μg/mL) to the culture medium.

**Production of ZZ-scIL-12 and scIL-12**

The culture supernatant of CHO-K1 cells stably expressing scIL-12 and ZZ-scIL-12 was passed through a filter (pore size, 1.2 μm), and protease inhibitor cocktail tablets (Roche) were added to inhibit protein degradation. ScIL-12 and ZZ-scIL-12 were purified from the supernatant with EZview Red Anti-HA Affinity Gel (Sigma).

**Generation of wt-HVJ-E, ΔHN-HVJ-E, and Fc-HVJ-E**

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 was inactivated by UV irradiation (99 mJ/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 antimouse 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**

RHN-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 with hemolysis buffer (Immuno-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. sclL12 or ZZ-sclL12 (10, 20, or 2,000 pg) and HVJ-E [1.5 or 3 × 10^7 particles (F1/F2; 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 × 10^5 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, sclL12-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.

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 sclL12-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 sclL12–HVJ-E with ^{125}I**

HVJ-E (wt-HVJ-E or sclL12–HVJ-E) was labeled with iodine-125 radionuclide (^{125}I; PerkinElmer) using lactoperoxidase, and the ^{125}I-labeled HVJ-E was suspended in saline to a concentration of 6 × 10^8 particles/200 μL. The ^{125}I-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 ^{125}I level in the tissues was measured using γ-scintillation counting.

**Systemic administration of sclL12–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, sclL12–HVJ-E (6 × 10^7 particles/200 μL PBS), ZZ-sclL12 (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.

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

wt-HVJ-E, sclL12–HVJ-E, ZZ-sclL12, 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 ^{51}Cr release CTL assays, the splenocytes and mitomycin C-treated F10 melanoma cells were mixed at a 1:10 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 × 10^5/100 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 (^{51}Cr; PerkinElmer Japan Co., Ltd.; 1.85 MBq/250 μL) for 45 minutes at 37°C and washed 3 times with RPMI-1640. A ^{51}Cr-labeled F10 melanoma suspension (2 × 10^5 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 ^{51}Cr 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 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 × 10^5/100 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 (^{51}Cr; PerkinElmer) using lactoperoxidase, and the ^{125}I-labeled HVJ-E was suspended in saline to a concentration of 6 × 10^8 particles/200 μL. The ^{125}I-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 ^{125}I level in the tissues was measured using γ-scintillation counting.

**Real-time RT-PCR**

On day 5 after the intravenous injection of the F10 melanoma cells, wt-HVJ-E, sclL12–HVJ-E (6 × 10^8 particles/200 μL PBS), ZZ-sclL12 (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.
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).

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 and 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 < 0.05; **, 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-HVJ-E (scIL-12–HVJ-E) that presents scIL-12 on the surface 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 AHN-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 x 10^5 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 (1.5 x 10^7 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–F, 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 scl-12–HVJ-E generated using this system could be functional upon systemic administration in mice. In fact, the Fc-ZZ binding of scl-12–HVJ-E was stable in serum for at least 24 hours (Fig. 4C), and intravenous administration of scl-12–HVJ-E resulted in elevated IFN-γ expression levels in the lungs due to pulmonary accumulation of the virions (Fig. 6A and C). scl-12–HVJ-E and ΔHN-HVJ-E, but not wt-HVJ-E, preferentially accumulated in the lungs (Fig. 6A and Supplementary Fig. S5A). A previous report has indicated that HVJ-E preferentially accumulates in the spleen after intravenous administration (50). This accumulation in the spleen most likely occurs because HN on HVJ-E induces hemagglutination by binding to the sialic acid on the erythrocyte surface, causing the erythrocyte and HVJ-E complex to be transported to the spleen and degraded. Therefore, the accumulation of scl-12–HVJ-E and ΔHN-HVJ-E in the lung may result from the loss of hemagglutinating activity upon HN 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–HVJ-E, which contains approximately 500 pg scl-12, preferentially accumulated in the lungs (Fig. 6A) and mediated the synergistic antitumor effects of HVJ-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-HVJ-E increased the serum IFN-γ level (Supplementary Fig. S6B). Therefore, the conjugation of IL-12 and HVJ-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–HVJ-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.

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Conception and design: Y. Kaneda
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