Inactivated Sendai Virus Particles Eradicate Tumors by Inducing Immune Responses through Blocking Regulatory T Cells

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Abstract

UV-inactivated, replication-defective Sendai virus particles [hemagglutinating virus of Japan envelope (HVJ-E)] injected into murine colon carcinoma (CT26) tumors growing in syngeneic BALB/c mice eradicated 60% to 80% of the tumors and obviously inhibited the growth of the remainder. Induced adaptive antitumor immune responses were dominant in the tumor eradication process because the effect was abrogated in severe combined immunodeficient mice. Murine and human dendritic cells underwent dose-dependent maturation by HVJ-E in vitro. Profiles of cytokines secreted by dendritic cells after HVJ-E stimulation showed that the amount of interleukin-6 (IL-6) released was comparable to that elicited by live HVJ. Real-time reverse transcription-PCR and immunohistochemistry revealed that HVJ-E induced a remarkable infiltration of dendritic cells and CD4+ and CD8+ T cells into tumors. In addition, CT26-specific CTLs were induced with the evidence of enhanced CD8+ T-cell activation in a CD4+CD25+ addition, CT26-specific CTLs were induced with the evidence of enhanced CD8+ T-cell activation in a CD4+CD25+ dependent manner. On the other hand, conditioned medium from dendritic cells stimulated by HVJ-E rescued CD4+CD25+ effector T-cell proliferation from Foxp3+CD4+CD25+ regulatory T cell (Treg)–mediated suppression and IL-6 was presumably dominant for this phenomenon. We also confirmed such rescue in mice treated with HVJ-E in vivo. Moreover, antitumor effect of HVJ-E was significantly reduced by an in vivo blockade of IL-6 signaling. This is the first report to show that HVJ-E alone can eradicate tumors and the mechanism through which it induces antitumor immune responses. Because it can enhance antitumor immunity and simultaneously remove Treg-mediated suppression, HVJ-E shows promise as a novel therapeutic for cancer immunotherapy. [Cancer Res 2007;67(1):227–36]

Introduction

The morbidity and mortality of cancer patients have considerably improved due to progress in surgery, chemotherapy, and radiotherapy. However, some types of cancers, particularly those that are advanced or metastatic, remain refractory to conventional strategies and require novel approaches. Immunotherapy represents one of the most promising of the more recent lines of attack. Considerable basic research and strategies have targeted cancer immunotherapy, but the response rates in preclinical and clinical trials are low (1–5). Thus, not only should tumors be eliminated but also lymphocyte-mediated immunosuppression must be regulated in patients with cancer (6).

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Reagents and antibodies. Recombinant mouse IL-6 (BE84071), GM-CSF (415-ML), human IL-4 (204-IL), GM-CSF (215-GM), anti-mouse IL-6 antibody (M007), anti-mouse IL-6 receptor (IL-6R) antibody (JVM01), anti-mouse CD3e antibody (KXE01), and mouse IFN-γ ELISPOT kit were purchased from R&D Systems (Minneapolis, MN). Anti-mouse CD3e antibody (145-2C11), anti-mouse CD11b antibody (M1/70), anti-mouse CD11c (HL-3), anti-mouse CD40 (3/23), anti-mouse CD80 (16-10A1), anti-mouse CD86 (16-10A1) antibodies, antimouse CCR7 (4B12) were purchased from BioLegend (San Diego, CA). Anti-mouse CCR7 (4B12) was purchased from BioLegend (San Diego, CA). Anti-CD11c antibody was purchased from Abcam Ltd. (Cambridge, United Kingdom). Anti-mouse/rat Foxp3 Staining Set was purchased from eBioscience (San Diego, CA). Alexa Fluor 488–labeled antimasther, antirtar, and antirat immunoglobulin G (IgG) antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). CD11c (N418) MicroBeads, CD8a+ T-Cell Isolation Kit, and CD4+CD25+ Regulatory T-Cell
Isolation Kit were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). [methyl-\(^{3}H\)]Thymidine and chromium-51 were purchased from GE Healthcare Bio-Science Corp. (Piscataway, NJ).

**Preparation of live HVJ and HVJ-E.** HVJ (VR-105 parainfluenza 1 Sendai/S2, Z strain) was purchased from ATCC (Manassas, VA) and prepared as described (7–9). Briefly, 100 μL of 10,000-fold diluted HVJ seed solution were injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Three days later, the allantoic fluid was harvested and the titer of recovered virus (live HVJ) was expressed as hemagglutination units. An HVJ suspension of 1.8 × 10^{10} virus particles was inactivated by UV irradiation (198 mJ/cm²). The inactivated HVJ that was unable to replicate is referred to as HVJ-E. Infectious particles were not produced by HVJ-E injected either into fertilized eggs or in cultured HEK-293, CT26, BHK-21, and LLCMK2 cells (9).

**Adenovirus.** Recombinant replication-defective adenovirus with a deletion of the E1 region was provided by Dr. Kazunori Aoki (National Cancer Center Research Institute, Tokyo, Japan). This vector does not derive from bone marrow were generated as described from BALB/c mice (12). Briefly, bone marrow of the tibia and femur was flushed with culture medium and then the effluent was filtered through a 40-mesh sieve. The filtrate was washed and cells (1 × 10^{6}) sedimented by centrifugation were seeded in 24-well plates in 1 mL of medium supplemented with 10 ng/mL of recombinant mouse GM-CSF. The cultures were nourished every other day by gently aspirating the spent medium and adding fresh medium. Six days later, nonadherent and loosely adherent clusters of proliferating dendritic cells were positive for CD11c and flow cytometry confirmed that their phenotype was immature. These cells were used in subsequent experiments as immature dendritic cells.

**Flow cytometry.** Immature murine or human dendritic cells (1 × 10^{6}) cultured in 96-well plates with the indicated amounts of live HVJ or HVJ-E for 48 h were incubated with respective antibodies. Murine dendritic cells were intracellularly stained with anti-IL-6 antibody (MP5-20F3). The cells were then analyzed by flow cytometry using a FACS-calibur and CellQuest software (BD Biosciences, Mountain View, CA).

**Cytokine measurements.** Immature murine or human dendritic cells (1 × 10^{6}) were cultured in 96-well plates and then live HVJ or HVJ-E were added to the culture medium. Harvested supernatants were evaluated for 48 h later by ELISA. Murine IFN-α, IFN-β (PBL Biomedical Laboratories, Piscataway, NJ), murine or human IL-6, tumor necrosis factor-α (TNF-α), and IL-12p40 (R&D Systems) were measured according to the manufacturer’s instructions.

**Intraduct tumor injection of HVJ-E or adenovirus and measurements of tumor volume.** CT26 cells (5 × 10^{5}) were injected into the intradermal space in the left flank 4 days after inoculation with 5 × 10^{5} CT26 cells into the right flank. Tumor volume was measured in a blinded manner with slide calipers using the formula: V = (D × W)^2/2. To examine the indirect antitumor effect of HVJ-E, 5 × 10^{5} CT26 cells were co-injected into the intradermal space in the left flank 4 days after inoculation with 5 × 10^{5} CT26 cells into the right flank. Three injections of HVJ-E were delivered into the tumors in the right flank as described above. Tumor formation at the left side was monitored to confirm tumorigenesis. For an in vivo blockade of IL-6 signaling, 20 μg of IL-6R antibody were injected into tumors simultaneously with HVJ-E on days 4, 8, and 12.

**Real-time quantitative RT-PCR.** Five micrograms of total RNA extracted from tumors were reverse transcribed into cDNA and amplified by real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) under the following conditions: 40 cycles of denaturation at 94°C for 45 s, annealing at 94°C for 45 s, and elongation at 68°C for 2 min. Mixtures of probes and primer pairs specific for murine CD11c, CD4, CD8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. The concentration of target genes was determined using the comparative CT method (threshold cycle number at the cross-point between amplification plot and threshold) and values were normalized to an internal GAPDH control.

**Immunohistochemistry.** Tumor tissue was isolated at 48 h after HVJ-E or saline injection for 3 successive days. Acetone-fixed frozen section was stained with specific antibodies in combination with fluorescently labeled secondary antibodies. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI).

**CTL assay.** Spleen cells were harvested from mice 10 days after the last HVJ-E or saline injection into tumors as described above. Of the mice treated with HVJ-E, we used only those in which tumors were completely eradicated. Spleen cells (5 × 10^{5} per flask) were stimulated with mitomycin C-treated tumor cells at a ratio of 10:1 in culture medium containing 10 IU/mL of recombinant IL-2 at 37°C in 5% CO₂. The cells including CTLs were harvested on day 7 and used as effector cells in standard 4-h ⁵¹Cr release assays to determine antitumor cytolytic activity. Briefly, target CT26 or Renca cells were labeled with ¹⁰⁶Cr for 90 min at 37°C. Labeled target cells (2 × 10^{4} per well) were incubated with the effector cells at various ratios for 4 h at 37°C in 5% CO₂ plates and the amount of radioactivity in the supernatants was measured. The maximal or spontaneous release was defined as counts from samples incubated with 2% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated using the following formula: % specific ⁵¹Cr release = ([experimental release – spontaneous release] / (maximum release – spontaneous release)) × 100. Cells were assayed in triplicate. Spontaneous release in all assays was <20% of the maximum release.

**Cell purification.** CD4⁺CD25⁺ or CD4⁺CD25⁻ cells were isolated using mouse CD4⁺CD25⁺ regulatory T-Cell Isolation Kits and AutoMACS magnetic sorters (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, spleen and lymph nodes were harvested from mice and then single-cell suspensions were incubated with biotin-antibody cocktail, anti-biotin MicroBeads, and CD25-phycoerythrin antibody to label non-CD4⁺ cells and fluorescently labeled CD25⁺ cells. Non-CD4⁺ T cells were depleted using the AutoMACS. The enriched CD4⁺ T-cell fraction was incubated with anti-phycoerythrin MicroBeads. CD4⁺CD25⁺ Tregs were positively selected using the AutoMACS. The positive and negative fractions were enriched with CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells, respectively. These populations were intracellularly stained with anti-Foxp3 antibody and analyzed by fluorescence-activated cell sorting (FACS). Dendritic cells or CD8⁺ T cells were isolated using mouse CD11c MicroBeads or CD8α isolation kits.

**Immune reconstitution of severe combined immunodeficient mice and ELISPOT assay.** CD8⁺ T cells and CD4⁺CD25⁻ T cells were obtained from naive BALB/c mice. CD8⁺ T cells (1 × 10⁶) with or without 1 × 10⁶ CD4⁺CD25⁻ T cells were injected into severe combined immunodeficient (SCID) mice from tail vein 1 day after CT26 inoculation. HVJ-E treatment was done as described above. CD8⁺ T cells were isolated from spleen 1 week after the last HVJ-E or saline injection. Purified 1 × 10⁶ CD8⁺ T cells were cultured for 24 h with 1 × 10⁶ mitomycin C–treated CT26 or Renca cells. Assay was done using mouse IFN-γ ELISPOT kit (R&D Systems). The number of IFN-γ-secreting CD8⁺ T cells was subsequently counted using a dissecting microscope (Leica, Cambridge, United Kingdom).

**T-cell proliferation assays/Treg suppression assays.** Recombinant mouse IL-6 (1 ng/mL) or 10 μL of supernatant from dendritic cell cultures stimulated with HVJ-E at a multiplicity of infection (MOI) of 300 for 24 h were added to each well at the start of the following experiments. IL-6 neutralizing antibodies (M007; 1 μg/mL) were added to each well where relevant. CD4⁺CD25⁻ T cells (1 × 10⁶) were incubated in 96-well U-bottomed plates with 1 × 10⁵ splenic dendritic cells, 0.1 μg/mL of anti-CD3 antibody, and the indicated number of CD4⁺CD25⁻ T cells for 48 h. T-cell proliferation was determined as [³H]thymidine incorporation during the last 6 h of culture.

**Human dendritic cells.** Frozen normal human dendritic cells derived from peripheral blood mononuclear cells (PBMC) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Human dendritic cells were cultured with 50 ng/mL human IL-4 and GM-CSF for 4 days according to manufacturer's instructions.
Results

HVJ-E eradicated murine colon carcinoma by activating adaptive antitumor immune responses. We examined whether HVJ-E safely stimulates antitumor immunity by injecting $1.5 \times 10^{10}$ HVJ-E virus particles into CT26 tumors growing in the backs of syngeneic BALB/c mice. Three consecutive injections completely eradicated tumors in 60% to 80% of tumor-bearing mice and obviously inhibited growth of the remainder (Fig. 1A and B). Three injections seemed to be necessary for eradication because tumors often relapsed after one or two injections (data not shown). In contrast, the same amount of injected replication-defective adenovirus particles or saline had no effect against CT26 tumors. (Fig. 1A) and the antitumor effect of HVJ-E was minimized when injected into CT26 tumors growing in SCID mice under the same experimental conditions (Fig. 1C). Moreover, tumorigenesis was reduced from 80% to 20% in CT26 cells inoculated into the left flank, distant from the tumors injected with HVJ-E in the right flank (Fig. 1D). These findings showed that HVJ-E eradicated the tumor by inducing antitumor immune responses. The effect was probably not associated with direct HVJ-E cytotoxicity or direct tumor cell killing by activated natural killer cells or macrophages because NK cells and macrophages should function normally in SCID mice (20). Furthermore, HVJ-E is not cytotoxic against CT26 cells in vitro (13).

![Image](https://www.aacrjournals.org)
HVJ-E maintained the ability of live HVJ to induce maturation and IL-6 release from murine and human dendritic cells. We examined how HVJ-E induced host antitumor immunity. The recognition of pathogens by antigen presenting cells (APC) is a pivotal initial step in activating innate immunity and for inducing specific acquired immunity (21, 22). We therefore examined the maturation and cytokine release from murine myeloid dendritic cells induced by HVJ-E and then compared the findings with those of live HVJ. We incubated HVJ-E or live HVJ with dendritic cells in culture medium for 48 h and then stained them to analyze CD40, CD80, CD83, CD86, CXCR4, and CCR7 molecules on the dendritic cell surface by FACS. All markers of dendritic cell maturation were dose-dependently increased by HVJ-E and live HVJ (Fig. 2A). These results suggest that HVJ-E retains the ability of live HVJ to induce dendritic cell maturation. We measured the amounts of type I IFNs (IFN-α and IFN-β) and representative proinflammatory cytokines (IL-6, TNF-α, or IL-12) released into dendritic cell culture medium at 24 h after adding HVJ-E or live HVJ. The secretion of all of these cytokines was decreased by HVJ-E. However, the decrease in the amount of released IL-6 was minimal and comparable to that released in the presence of live HVJ (Fig. 2B and C). Next, we measured CD86 expression and IL-6 secretion in human dendritic cells at 48 h after adding HVJ-E or live HVJ-E to the culture medium. CD86 was dose-dependently increased by HVJ-E as well as by live HVJ. The secretion of IL-6 was enhanced by both live HVJ and HVJ-E as seen in murine dendritic cells (Fig. 2C) although the total amount of IL-6 in human dendritic cells was lower than that in murine dendritic cells (Fig. 2D).

HVJ-E promoted dendritic cell and T-cell recruitment to tumor beds and induced tumor-specific CTLs in vivo. To determine how the host adaptive immune system reacts to HVJ-E in vivo, the mRNA expression of CD11c as markers for dendritic cells and of CD4 and CD8 for effector T cells was measured in CT26 tumors in mice at 24, 48, and 120 h after HVJ-E injection. CD11c and CD4 expression was significantly increased in tumors treated with HVJ-E compared with saline at all time points with a peak at 48 h. CD8 expression was also significantly increased in the group given HVJ-E. Moreover, the expression level remained maximal even at 120 h after treatment (Fig. 3A). Immunohistochemical staining was done to evaluate dendritic cell and T-cell infiltration into tumor tissue after HVJ-E treatment. It revealed that CD11b⁺, CD11c⁺, CD4⁺, or CD8⁺ cells were remarkably infiltrated into tumor by HVJ-E treatment (Fig. 3B). We then did CTL assays and measured standard [3H]thymidine release to determine whether CT26-specific IFN-γ secreting CD8⁺ T cells was counted by ELISPOT assay. As a result, significant increase of CT26-specific IFN-γ secretion from CD8⁺ T cells was observed only when CD8⁺ T cells were transferred with CD4⁺CD25⁻T cells. It revealed that HVJ-E enhanced tumor-specific CD8⁺ T-cell response in a CD4⁺CD25⁻ T-cell–dependent manner (Fig. 3D). All of these findings indicated that HVJ-E induced dendritic cell and CD4⁺ and CD8⁺ T-cell migration to the tumor site and also activated tumor-specific adaptive T cell–mediated immune responses in vivo. It was evident that tumor-specific CD8⁺ T-cell activation was elicited by HVJ-E in a CD4⁺CD25⁻ T-cell–dependent manner.

HVJ-E rescued effector T-cell proliferation from Treg-mediated immunosuppression in vitro and in vivo through the ability to release IL-6 from dendritic cells. Induction of APC maturation is not the only mechanism that controls T-cell activation because Treg also controls T-cell responses (23–26). IL-6 release from dendritic cells is critical for overcoming the CD4⁺CD25⁺ Treg–mediated immunosuppression of effector T cells (27). Because HVJ-E retained the ability of live HVJ to promote IL-6 release from dendritic cells, we examined whether HVJ-E could inhibit Treg-mediated immunosuppression. T-cell proliferation was assayed using various ratios of Tregs (CD4⁺CD25⁺) to responder T cells (CD4⁺CD25⁻) in the presence of splenic CD11c⁺ cells as APCs and anti-CD3 antibodies. Before evaluating T-cell proliferation, we stained the isolated CD4⁺CD25⁺ T cells with anti-Foxp3 antibody because Foxp3 is nowadays considered to be the most specific marker for Treg (25). As a result, the majority of sorted CD4⁺CD25⁺ T cells was confirmed to be Foxp3⁺CD4⁺CD25⁺ Tregs (Fig. 4A). T-cell proliferation determined by incorporation of [3H]thymidine was gradually suppressed as the ratio of Tregs to responder T cells increased. We confirmed that adding mouse recombinant IL-6 to the T-cell culture medium reversed the Treg-mediated suppression and restored T-cell proliferation to near normal levels. This ability of IL-6 was abrogated by IL-6 neutralizing antibodies (Fig. 4B; ref. 27). Next, we examined the reversal of Treg-mediated suppression using conditioned medium from dendritic cells stimulated with HVJ-E (H-DCCM). We found that H-DCCM also reversed Treg-mediated suppression and that IL-6 neutralizing antibodies blocked this restorative effect (Fig. 4C). These results indicated that soluble factors in H-DCCM could block Treg-mediated immunosuppression and that IL-6 was dominant for this blockade. We examined IL-6 secretion from dendritic cells after HVJ-E administration in vivo. HVJ-E was injected into CT26 tumors in the mouse hind footpad for 3 successive days. Dendritic cells were purified from tumor tissues or drainage lymph nodes at 24 h after the last injection. Intracellular IL-6 was positive in the sorted CD11c⁺ cells, which were analyzed by FACS. The ratio (%) of IL-6-positive dendritic cells was significantly increased in tumors and drainage lymph nodes by HVJ-E compared with saline (Fig. 5A). Next, we examined whether HVJ-E indeed contributed to blocking Treg-mediated immunosuppression in vivo. CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ effector cells were purified from tumor drainage lymph.
nodes by magnetic cell sorting at 24 h after HVJ-E injections as described above. The suppressive effect of Treg against effector T cells was significantly reduced in HVJ-E-treated mice compared with saline-treated or naïve mice (Fig. 5B). Finally, we examined whether an in vivo IL-6 neutralization reduces antitumor effect of HVJ-E. Because blockade of IL-6 signaling with IL-6R antibody was reported to recover Treg-mediated immunosuppression in vivo (28), 20 μg of IL-6R antibody were injected into tumors simultaneously with HVJ-E on days 4, 8, and 12. We found that blockade of IL-6 signaling significantly reduced HVJ-E-mediated tumor growth inhibition in vivo (Fig. 5C). These results revealed that HVJ-E was responsible for rescuing effector T-cell proliferation from Treg-mediated immunosuppression. The mechanism through which Treg was suppressed was presumably due to HVJ-E-induced IL-6 secretion from dendritic cells in tumors and drainage lymph nodes.

Discussion

HVJ-E is derived from UV-inactivated HVJ particles. Irradiation with UV breaks the genome of HVJ and removes its ability to

Figure 3. Dendritic cell and T-cell infiltration into tumor beds and induction of tumor-specific CTLs in vivo. A, mRNA expression quantified by real-time PCR in tumors growing in mouse footpads at 24, 48, and 120 h after intratumor injections of 1.5 × 10^10 HVJ-E particles or saline for 3 successive days. CD11c expression was also significantly higher in HVJ-E-treated tumors at any time point with the peak level at 48 h. CD4 expression obviously increased in HVJ-E-treated tumors and remained elevated for 120 h. Expression levels were normalized by GAPDH. Each value was calculated as fold increase relative to value of saline-treated tumors at 120 h. Columns, mean of triplicate samples; bars, SE.

Figure 4. Suppression of regulatory T cells by HVJ-E in vitro. A, intracellular staining of Foxp3 for purified CD4+CD25+ cells or CD4+CD25- cells. FACS analysis revealed that the majority of CD4+CD25+ cells isolated by magnetic microbeads was Foxp3+CD4+CD25+ Tregs. B, CD4+CD25+ effector T-cell proliferation was suppressed by CD4+CD25+ Tregs (open columns, recombinant IL-6(-)). Recombinant IL-6 rescued effector T-cell proliferation from the suppression by Tregs (solid columns, recombinant IL-6(+)). This effect was blocked by IL-6 neutralizing antibody (gray columns, recombinant IL-6 + anti-IL-6 antibody). C, conditioned medium from HVJ-E-stimulated dendritic cells (H-DCCM) rescued CD4+CD25+ effector T-cell proliferation from CD4+CD25+ Treg-mediated suppression. This effect was blocked by IL-6 neutralizing antibody. Open columns, H-DCCM(-); solid columns, H-DCCM(+); gray columns, H-DCCM + anti-IL-6 antibody. Columns, mean T-cell proliferative responses of triplicate samples; bars, SE.
replicate, thus ensuring effective gene transfer and safety. HVJ-E conserves the complete structure of the live HVJ envelope containing E, HN, and M proteins (9). Here, we tested the feasibility of using the HVJ-E virus envelope feature to induce antitumor immunity.

CT26 tumors were eradicated after direct intratumoral injection of HVJ-E. Thus, this strategy alone was sufficient to eliminate established tumors. This property might be unique to Sendai virus particles because the same amount of replication-defective adenovirus had no effect, although the conditions for comparison were limited. We showed that this effect of HVJ-E was due to induced antitumor immune responses because it was abrogated in immunocompromised circumstances such as in SCID mice. None of direct tumor cell killings by activated dendritic cells, macrophages, NK cells, and HVJ-E cell fusion seemed to be predominant because they should be functional in SCID mice (20). Furthermore, the diminished tumorigenesis of CT26 cells co-inoculated distantly from HVJ-E-injected CT26 tumors indicated that the effect of HVJ-E was indirect and that antitumor immunity was enhanced. We also confirmed that cultured CT26 cells were refractory to direct contact with HVJ-E or type I IFNs in vitro (13).

The recognition of pathogen-associated molecular patterns by APC such as dendritic cells through Toll-like receptors or retinoic acid-inducible gene I is important to induce not only the activation of innate immunity but also the development of antigen-specific acquired immunity (21, 22). Because we postulated that the activation of APC by HVJ-E or HVJ-E-fused cancer cell would be a critical initial step in inducing powerful tumor-specific adaptive immune responses, we examined whether HVJ-E activates dendritic cells and induces tumor-specific immunity. We found that dendritic cells were matured by HVJ-E to a level comparable to that induced by live HVJ in vitro. Furthermore, dendritic cell and effector T-cell recruitment was suggested from the significant increase of CD11c, CD4, and CD8 mRNA expression in mouse tumors injected with HVJ-E. Remarkable infiltrations of dendritic cells and CD4+ and CD8+ T cells into HVJ-E-treated tumors were also confirmed in immunohistochemical studies. Moreover, the significant induction of cancer-specific CTL was observed and also confirmed in immunohistochemical studies. The accumulation of Treg in tumors or drainage lymph nodes is evidence of their involvement in immune tolerance in patients bearing tumors (29–33), and Treg depletion contributes to enhanced antitumor immunity (34, 35).

Figure 5. Suppression of regulatory T cells by HVJ-E in vivo. A, ratio (%) of IL-6-secreting CD11c+ cells was significantly increased in tumors and in drainage lymph nodes of mice administered with HVJ-E. CD11c+ cells separated using Macs beads were intracellularly stained with IL-6 and analyzed by FACS at 24 h after HVJ-E was injected into CT26 tumors in mouse footpads for 3 successive days. Columns, mean of triplicate samples; bars, SE. B, CD4+CD25+ Treg-mediated immunosuppression was reduced in HVJ-E-treated mice in vivo. CD4+CD25+ Tregs and CD4+CD25− effector T cells were separated from drainage lymph nodes using Macs beads and then 1 × 10⁶ CD4+CD25− T cells for 48 h. Inhibitory effect of Tregs was significantly reduced in HVJ-E-treated mice in vivo. C, in vivo blockade of IL-6 signaling reduced antitumor effect of HVJ-E. For blocking IL-6 signaling in vivo, 20 μg of IL-6R antibody were injected into tumors simultaneously with HVJ-E on days 4, 8, and 12. Tumor volume was measured as previously described. CT26 tumor growth inhibition by HVJ-E was significantly reduced by local injection of IL-6R antibody. *, P < 0.05; †, HVJ-E + control IgG; ▲, HVJ-E + IL-6R antibody; ●, saline. Arrows, timing of HVJ-E and IL-6R antibody or control IgG injections. Points, mean; bars, SE. Results were statistically analyzed using Student's t test.
Furthermore, tumor cell–licensed immature dendritic cells are critical for the proliferation and accumulation of Tregs in the induction of host immune tolerance against cancer (36). Therefore, overcoming Treg-mediated suppression of antitumor immunoreactions is prerequisite for future cancer immunotherapy. The present study found that H-DCCM rescued effector T-cell proliferation from Treg-mediated immunosuppression and that such rescue was abrogated by IL-6 neutralizing antibody. These results indicated that IL-6 secretion from HVJ-E-treated dendritic cells is key to this inhibitory phenomenon against Treg-mediated suppression. IL-6 is a multifunctional cytokine that regulates immune response, hematopoiesis, acute phase response, and inflammation (37–39), and when released from dendritic cells, IL-6 is critical for overcoming CD4+CD25+ Treg–mediated immunosuppression (27, 40). In addition, blockade of IL-6 signaling contributes to Treg expansion (28). Here, we found that when dendritic cells were cocultured with HVJ-E, IL-6 secretion levels were comparable to those in the presence of live HVJ, whereas levels of many other cytokines were diminished. We also confirmed that the increase in IL-6 secreted by dendritic cells in tumors and their draining lymph nodes was significant in mice administered with HVJ-E, which also reduced CD4+CD25+ Treg–mediated inhibition against CD4+CD25+ effector T-cell proliferation in vivo. Moreover, the reduction of HVJ-E-mediated tumor growth inhibition was confirmed by blocking IL-6 signaling with IL-6R antibody in vivo.

To evaluate the potential side effects by enhanced IL-6 secretion after HVJ-E treatment, the amounts of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, urea nitrogen, and creatinine in serum were measured 1 week after the last HVJ-E injection. There was no significant increase in all these markers of hepatic or renal toxicity in HVJ-E-treated mice. Furthermore, histologic analysis for H&E-stained sections of liver, intestine, and skin showed that no remarkable inflammation or tissue damage was induced by HVJ-E treatment (data not shown).

Lopez et al. reported that CD80 or CD86 costimulatory molecules are normally up-regulated on dendritic cells treated with UV-inactivated Sendai virus in a manner similar to that of live virus, but unlike UV-inactivated influenza, which is another negative-strand RNA virus. However, the secretion of cytokines including IFN-α, IL-1β, IL-6, IL-12, and TNF-α in dendritic cells was considerably diminished by UV inactivation and they suggested that dendritic cell maturation markers and cytokine release are separately regulated (41). Here, we confirmed that HVJ-E induced normal dendritic cell maturation that was comparable to that induced by live HVJ (Sendai virus). Notably, the level of IL-6 secretion was similar, whereas that of other representative cytokines such as IFN-α, IFN-β, and IL-12 was reduced. Because UV inactivation abrogated the infectivity of HVJ-E as described (9), the notion that live HVJ escaped UV-inactivation and contributed to IL-6 production is unlikely. This discrepancy between our results and theirs might be explained by the choice of HVJ strains (Cantell versus Z strain) or the multiplicity of infection (MOI, 0.6–10 versus 3–3,000). Signal transduction in dendritic cells induced by live HVJ stimulation is independent of Toll-like receptor-3, -7, -8, -9 signaling or of the adaptor protein MyD88 (42). Thus, dendritic cells must use another pathway against HVJ-E stimulation that does not require viral replication. Further investigation should address the detailed molecular mechanisms by which HVJ-E induces dendritic cell maturation and retains IL-6 secretion, and explain the immunologic characteristics of HVJ-E that render it advantageous for cancer treatment. Tumor cell–licensed dendritic cells induce Treg activation through transforming growth factor-β secretion, which helps cancer to escape immune surveillance (35). HVJ-E might reverse this mechanism by inducing IL-6 secretion from dendritic cells in the tumor bed or drainage lymph nodes.

As a delivery vector, HVJ-E can incorporate and directly deliver anticancer drugs to cells through its membrane fusion activity. The cytotoxicity of bleomycin to cancer cells is increased >300-fold in the presence, compared with the absence of HVJ-E (13). HVJ-E also delivers small interfering RNA (siRNA) to cells at high efficiency both in vitro and in vivo. Delivering Rad51 siRNA using HVJ-E to cancer cells significantly increases their sensitivity to cisplatin (10). Three injections of HVJ-E-containing Rad51 siRNA to tumor masses in SCID mice significantly inhibited tumor growth in the presence of cisplatin and the present study showed that HVJ-E itself enhanced antitumor immunity via multiple pathways.

All of these findings together indicate that HVJ-E alone could induce vigorous antitumor immune responses, an activity that is simultaneously reinforced by inhibiting Treg-mediated immunosuppression. The IL-6 produced by HVJ-E-stimulated dendritic cells was presumably responsible for this reinforcement. Furthermore, the amount of induced immunity was sufficient to eradicate established tumors. Others have described dendritic cell maturation or cytokine release by Sendai virus (41–43), but we are the first to show the potentially remarkable contribution of HVJ-E to cancer treatment through its ability to enhance host immunity. Moreover, the finding that human dendritic cells matured and released IL-6 after HVJ-E stimulation in vitro indicated that clinical applications are possible. Clinical grade HVJ-E is being produced by our venture company (44). The efficient delivery of anticancer reagents and enhanced antitumor immunity indicate that HVJ-E shows promise as a novel cancer therapeutic.

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References

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