HVJ-E treatment also induced a weak prolongation of mouse survival, but this was not significant (Fig. 2B). Prior studies reported clinical benefits of IL-2 administration in human cutaneous angiosarcoma [8,9]. Thus, the effects of HVJ-E carrying *IL-2* gene (HVJ-E/IL-2) were examined next. HVJ-E/IL-2 more significantly suppressed the growth of ISOS-1 cells than HVJ-E alone (Fig. 2C). Notably, administration of HVJ-E/IL-2 resulted in significantly improved mouse survival (Fig. 2D). Unlike HVJ-E-treated mice, some mice treated with HVJ-E/IL-2 completely eradicated tumors (tumor-free rate, 35.7%, 5 of 14 mice) (Fig. 2E).

3.2. In vitro effects of HVJ-E/IL-2 on ISOS-1 cells

HVJ-E has been demonstrated to directly reduce viability of some human carcinoma cells, such as PC3 cells (human prostate cancer cells) [20]. However, neither HVJ-E nor HVJ-E/iL-2 suppressed the growth of ISOS-1 cells in vitro (data not shown).

3.3. HVJ-E/IL-2 stimulated local accumulation of CD8 (+) T cells and NK cells

To understand the underlying mechanisms of the therapeutic effects of HVJ-E with or without *IL-2* gene, experiments to measure levels of CD8 (+) T cells and NK cells in tumor-infiltrating lymphocytes (TILs) by flow cytometry were performed initially. HVJ-E stimulated accumulation of both these cells (Fig. 3A). Similarly, HVJ-E/IL-2 therapy resulted in increases of CD8 (+) T cells and NK cells, but no trend toward elevated numbers of these cells compared to HVJ-E alone was seen.

3.4. Development of regulatory T cells (Tregs) by HVJ-E/IL-2 treatment

Regional lymph node cells were prepared two days after the last injection of HVJ-E. CD3 (+)/CD25 (+)/Foxp3 (+) regulatory T cells (Tregs) in regional lymph nodes were reduced by HVJ-E treatment. Despite the fact that Tregs express CD25 (IL-2Rα chain), suppression of the development of Tregs was not counteracted by the addition of IL-2 (Fig. 3B, left).

3.5. Myeloid-derived suppressor cells (MDSCs) in HVJ-E/IL-2 treatment

Myeloid-derived suppressor cells (MDSCs) are a group of myeloid cells including granulocytes, macrophages, DCs, and myeloid progenitors. These are phenotypically characterized by Gr-1 (+)/CD11b (+) cells [21]. MDSCs mediate the suppression of the tumor antigen-specific T cell response [22–24]. Unexpectedly, HVJ-E tended to promote the development of MDSCs in spleens despite in vivo anti-tumor effects. Nevertheless, this effect was canceled by the concomitant administration of *IL-2* gene (Fig. 3B, right).

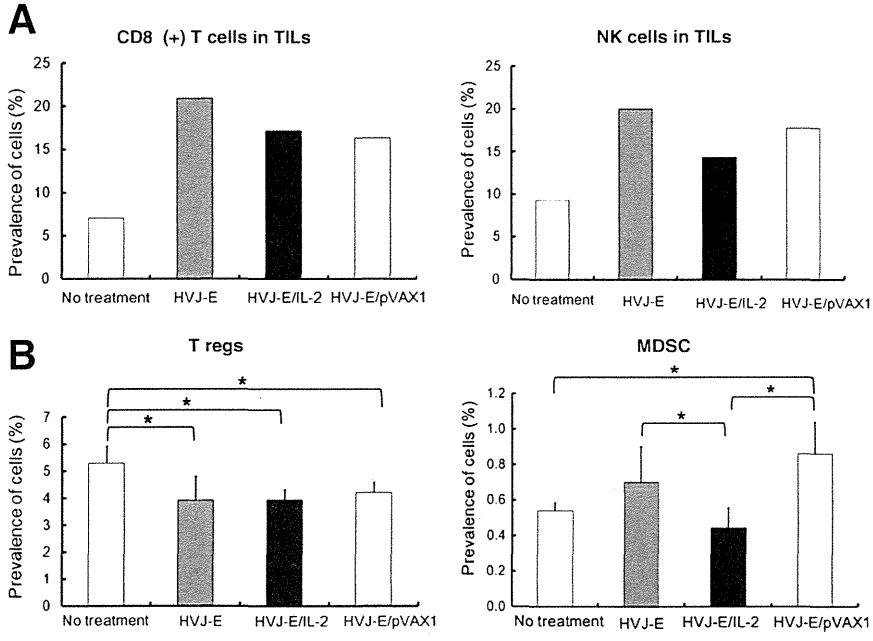

Figure 3 Changes in immune cell populations. A: Tumor-infiltrating lymphocytes (TILs) were collected 2 days after the last HVJ-E injection. CD3 (+)/CD8 (+) T cells and CD3 (−)/CD49b (+) NK cells were assessed by flow cytometry. TILs of each group were a mixture from three mice. Similar results were obtained from additional two independent experiments. B: Regional lymph node cells and spleen cells were prepared 2 days after the last HVJ-E injection. Prevalences of CD3 (+)/CD25 (+)/Foxp3 (+) regulatory T cells (Tregs) in regional lymph nodes and Gr-1 (+)/CD11b (+) myeloid-derived suppressor cells (MDSCs) in spleens were assessed by flow cytometry. Each group consisted of at least 3 mice. Data are presented as means ± S.D Representative results of three independent experiments are shown. $^*P < 0.05$.

3.6. HVJ-E/IL-2 induced maturation of dendritic cells (DCs)

Bone marrow-derived DCs (BMDCs) were stimulated with HVJ-E in vitro. Consistent with a prior report [5], HVJ-E treatment resulted in the enhanced expression of CD86 in BMDCs. HVJ-E/IL-2 also enhanced CD86 expression, but no difference between HVJ-E and HVJ-E/IL-2 was found (data not shown).

3.7. Tumor-specific responses of CD8 (+) T cells are promoted by HVJ-E/IL-2

CD8 (+) cells from mice treated with HVJ-E did not produce IFN-γ in response to ISOS-1 cells in vitro. However, when HVJ-E-fused ISOS-1 cells (ISOS-1/HVJ-E) were used as stimulators, CD8 (+) cells from HVJ-E-treated mice secreted higher amounts of IFN-γ than the control group (Fig. 4). The results in HVJ-E/IL-2-treated mice were in striking contrast to those in HVJ-E-treated mice. CD8 (+) cells from HVJ-E/IL-2-treated mice produced remarkable levels of IFN-γ not only in response to ISOS-1/HVJ-E cells, but also in response to ISOS-1 cells.

3.8. Cytotoxic activity of CD8 (+) cells and NK cells

To further evaluate functional changes in immune cells, cytotoxic activities of CD8 (+) cells and NK cells were examined by flowcytometric analysis with CSFE-labeled target tumor cells, as described in Materials and methods. Treatment with HVJ-E with or without *IL-2* gene did not enhance the cytotoxic activity of CD8 (+) cells against ISOS-1 cells (Fig. 5A), while cytotoxic activity against HVJ-E-fused ISOS-1 cells (ISOS-1/HVJ-E) was weakly enhanced by HVJ-E and HVJ-E/IL-2 (Fig. 5B). On the other hand, unlike CD8 (+) cells, both HVJ-E and HVJ-E/IL-2 treatment resulted in significant promotion of the cytotoxic activity of NK cells against ISOS-1 cells (Fig. 5C). In addition, there was a trend toward a greater increase in cytotoxic activity of NK cells from HVJ-E/IL-2-treated mice than from HVJ-E alone when ISOS-1/HVJ-E cells were used as target cells (Fig. 5D).

3.9. Persistent systemic anti-tumor immunity against ISOS-1 cells is induced by HVJ-E/IL-2

Immune responses induced by intra-tumoral injection of HVJ-E and HVJ-E/IL-2 seemed more effective for ISOS-1/HVJ-E than for ISOS-1 cells alone. These data suggested that these therapies might not be able to exert sufficient inhibitory effects on tumors without HVJ-E-injection, i.e., metastatic tumors. Thus, it was next attempted to assess whether systemic in vivo anti-tumor immunity against ISOS-1 cells was effectively developed. HVJ-E/IL-2-treated mice that completely eradicated inoculated tumors on the back skin were left for up to approximately 130 days to confirm that no recurrence occurred. They were then re-inoculated with ISOS-1 cells (3×10^6 cells/site) on a remote site (nape skin); four of seven (57%) mice were resistant to re-inoculation of ISOS-1 cells without requiring local injection of HVJ-E/IL-2, and they were tumor-free for an additional 50 days. This was somewhat consistent with a prior report of bladder carcinoma mouse model treated with HVJ-E [25]. Spleen cells from these mice (ISOS-1-resistant mice) were then transferred into irradiated naïve mice that subsequently received ISOS-1 inoculation. Notably, none of the mice that received spleen cell transfer from ISOS-1-resistant mice developed tumors (Fig. 6).

3.10. Sunitinib collaboratively enhanced the anti-tumor effects of HVJ-E/IL-2

Sunitinib is a compound which is widely used to treat malignant tumors. Importantly, sunitinib has been shown to inhibit angiogenesis [26] and reduce the development and accumulation of MDSCs [27,28]. To obtain more effective therapeutic outcomes by HVJ-E/IL-2 in vivo, the anti-tumor effects of sunitinib (kindly provided by the Pfizer Inc.) in combination with HVJ-E/IL-2 were verified. Initially, expressions of PDGFRα, PDGFRβ, VEGFR-1, VEGFR-2, and flt3 mRNA were detected in ISOS-1 cells (data not shown), and it was confirmed that the growth of ISOS-1 cells was dose-dependently suppressed by sunitinib (kindly provided by Pfizer Inc., New York City, NY, USA) in vitro as determined by MTS assay (Fig. 7A). Consistent with these findings, oral daily administration of sunitinib (days 12 to 50) suppressed

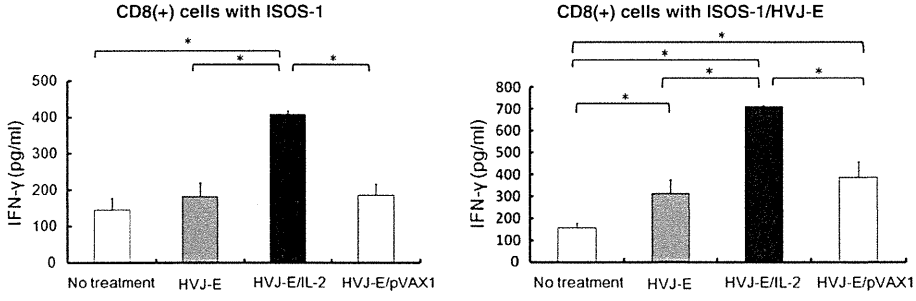

Figure 4 IFN-γ production from CD8 (+) T cells in response to tumor cells. CD8 (+) cells were isolated from spleen as described in Materials and methods. They were incubated with ISOS-1 cells or ISOS-1 cells fused with HVJ-E (ISOS-1/HVJ-E) for 48 h. CD8 (+) cells from HVJ-E-treated mice produced IFN-γ in response to ISOS-1/HVJ-E, but not ISOS-1 cells. On the other hand, CD8 (+) cells from HVJ-E/IL-2-treated mice generated a significant amount of IFN-γ in response to both ISOS-1 and ISOS-1/HVJ-E cells. $^*P < 0.05$. Representative results of at least two independent experiments are shown.

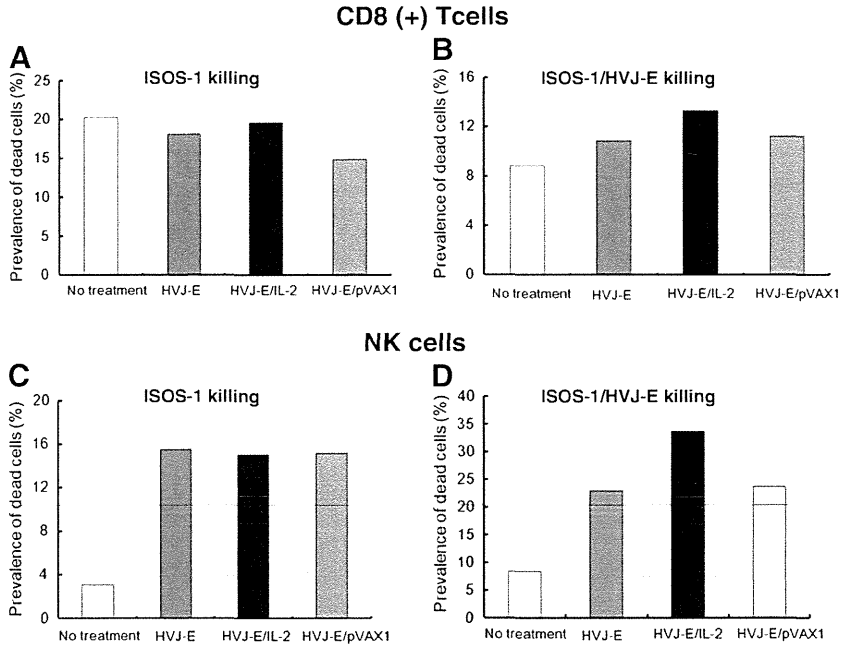

Figure 5 Cytotoxic activity of CD8 (+) cells and NK cells. CFSE-labeled ISOS-1 cells or ISOS-1 cells fused with HVJ-E (ISOS-1/HVJ-E) were incubated with either CD8 (+) T cells (A and B) or CD49b (+) NK cells (C and D). 7-AAD (+)/CSFE (+) cells were analyzed by flow cytometry. Similar results were obtained from additional two independent experiments.

tumor growth in vivo (Fig. 7B). Subsequently, co-administration of sunitinib and HVJ-E/IL-2 showed more marked in vivo therapeutic effects, although this was not significant (Fig. 7C). The tumor-free rate of the HVJ-E/IL-2/sunitinib group, however, was 75.0%, higher than that of the HVJ-E/IL-2 group (35.7%). It was unexpected that sunitinib itself tended to reduce CD8 (+) cells and NK cells in TILs (Fig. 7D), despite its in vivo anti-tumor effects. However, HVJ-E/IL-2 seemed to counteract these effects of sunitinib; no reduction in these cells in mice treated with HVJ-E/IL-2/sunitinib was observed. Mice treated with HVJ-E/IL-2/sunitinib also had lower levels of Tregs than those treated with sunitinib alone. Sunitinib tended to decrease MDSCs, and, notably, this reduction became significant when mice were treated with HVJ-E/IL-2/sunitinib.

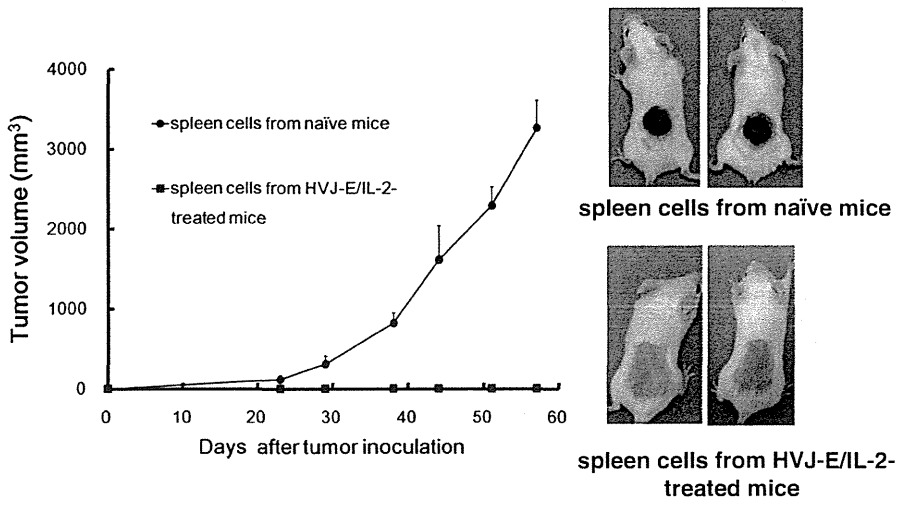

Figure 6 HVJ-E/IL-2 treatment instills systemic persistent immunological memory. HVJ-E/IL-2-treated mice that completely eradicated tumors (ISOS-1) were left up to approximately 130 days. They were then challenged with ISOS-1 cells at a remote site (nape skin). Spleen cells from these mice that did not develop tumors were transferred into irradiated naïve BALB/c mice that were subsequently inoculated with ISOS-1 cells. Mice that received spleen cells from HVJ-E/IL-2-treated mice were entirely resistant to tumors. Each group consisted of at least 4 mice. Data are presented as means ± S.D.

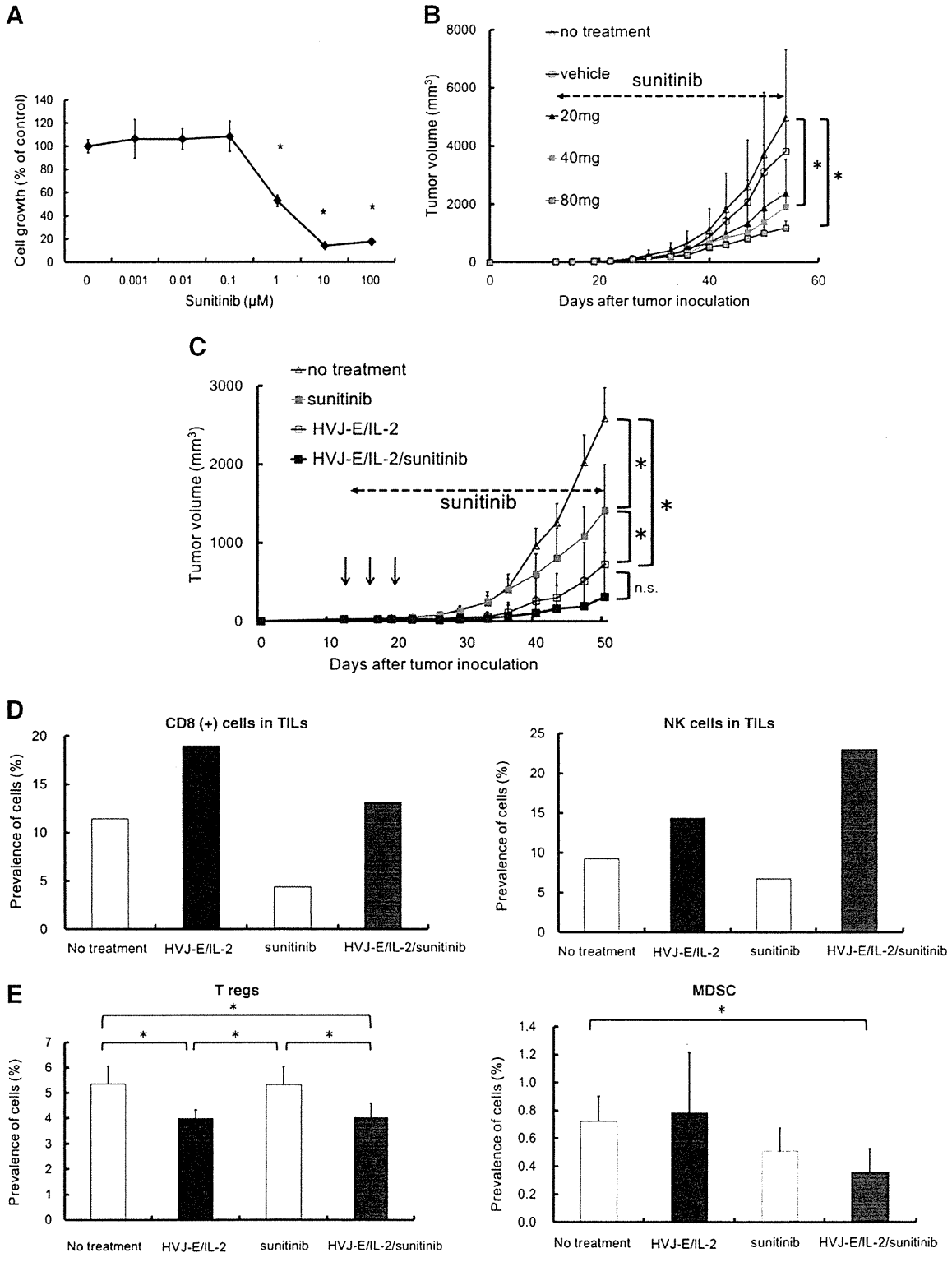

Figure 7 Anti-tumor effects of sunitinib on ISOS-1 cells. A: Sunitinib inhibits growth of ISOS-1 cells in vitro. B: Oral daily administration of sunitinib dose-dependently suppresses tumor growth in vivo. C: Effects of sunitinib (40 mg/day) in combination with HVJ-E/IL-2 on tumor growth. Although no significant differences are observed, mean levels of tumor volumes in mice with HVJ-E/IL-2/sunitinib consistently tend to be smaller than those of HVJ-E/IL-2-treated mice. D: Prevalence of CD8 (+) T cells and NK cells in TILs. Sunitinib tends to reduce CD8 (+) T cells and NK cells, which is reversed by co-administration of HVJ-E/IL-2. Similar results were obtained from additional two independent experiments. E: Sunitinib does not affect HVJ-E/IL-2-induced reduction of Tregs, and HVJ-E/IL-2 promotes sunitinib-induced reduction of MDSCs.

4. Discussion

Despite recent advances in chemotherapy regimens, cutaneous angiosarcoma remains an aggressive malignant tumor with a high mortality rate. In this study, it was demonstrated that intra-tumoral administration of HVJ-E had a therapeutic potential for mouse angiosarcoma, which was more significant when co-administered with *IL-2* gene. HVJ-E/IL-2 therapy resulted in long-lasting, systemic anti-tumor immunity. These results suggested that HVJ-E/IL-2 therapy made mice resistant to both local recurrence and distant metastasis.

HVJ-E promoted local accumulation of CD8 (+) T cells and NK cells in TILs and reduced Tregs in regional lymph nodes, consistent with prior studies with murine models of colon carcinoma (CT26) and renal cell carcinoma [5,6]. Cytotoxic activity of NK cells, but not CD8 (+) cells, against ISOS-1 cells was also promoted by HVJ-E. The addition of *IL-2* gene did not enhance these changes, but IFN-γ production by CD8 (+) cells was markedly enhanced by HVJ-E/IL-2, and there was a trend toward enhancing cytotoxic activity of CD8 (+) cells and NK cells against HVJ-E-fused ISOS-1 cells by IL-2. Immunogene therapies of IL-2 using Sendai virus vectors have also been demonstrated to be effective for models of rat glioma [29] and mouse malignant astrocytoma [16]. In the latter model, the addition of *IL-2* gene significantly enhanced local accumulation of CD4 and CD8 cells compared with HVJ-E alone, which was not observed in the present study.

It could be intriguing to see changes in MDSCs. MDSCs are now known to play a critical role in the suppression of T cell responses, and this appears to account for, at least partly, the limited effectiveness of immunotherapy for malignant tumors [22–24]. MDSCs are closely associated with tumor angiogenesis [30]. Despite in vivo suppressive effects of HVJ-E on tumor growth, therapy with HVJ-E promoted generation of MDSCs, which might be due to the fact that HVJ-E was recognized as a viral antigen. Nevertheless, this increase in MDSCs was blocked by the addition of *IL-2* gene; this could be one of the important advantages of HVJ-E/IL-2 therapy, making it superior to HVJ-E therapy alone. *IL-2* gene did not appear to affect MDSC function, as isolated MDSC from HVJ-E/IL-2-treated mice exhibited comparable suppressive activities on NK cell cytotoxicity against ISOS-1 cells to those from HVJ-E-treated mice (data not shown).

Several lines of prior evidence indicated that local cytokine profiles altered the generation and maturation of MDSCs. Skewing toward Th2, such as increased production of IL-13, was correlated with elevated numbers of MDSCs [31]. On the other hand, a Th1-cytokine-enriched microenvironment, such as generation of IL-2 and IFN-γ, inhibited MDSC development [32,33]. Thus, delivery of *IL-2* gene together with promoted production of IFN-γ from CD8 (+) T cells mediated by the local injection of HVJ-E/IL-2 may account for inhibited generation of MDSCs in HVJ-E/IL-2-treated mice.

Overall, 35.7% of mice treated with HVJ-E/IL-2 had completely eradicated tumors, while none of the mice treated with HVJ-E alone were tumor-free. Intriguingly, 57.0% of HVJ-E/IL-2-treated mice were entirely resistant to re-inoculation of ISOS-1 cells at the remote site even more than 130 days after the first inoculation. This indicated that HVJ-E/IL-2 effectively induced systemic and long-lasting ISOS-1-specific immunity sufficient for tumor eradication. Indeed, mice receiving spleen cells from tumor-free HVJ-E/IL-2-treated mice were entirely resistant to ISOS-1 cells. These might be mediated by HVJ-E-induced generation of tumor-specific CD8 cells and NK cells together with enhanced cytotoxicity of NK cells in donor mice (Fig. 3A and Fig. 5C). Although we may not be able to fully explain the superior therapeutic effects of HVJ-E/IL-2 over HVJ-E by differences in MDSCs, these data strongly suggest that HVJ-E/IL-2 more effectively decreased the risk of local recurrence and distant metastasis of tumors than HVJ-E alone.

Sunitinib is a tyrosine kinase inhibitor that suppresses a variety of tyrosine kinases contributing to tumor growth. In addition, sunitinib has been shown to be an ideal tool for cancer therapy, as it suppressed the development of MDSCs via inhibiting STAT3 signaling [22,28] and tumor angiogenesis. In this study, the direct suppressive effects of sunitinib on the growth of ISOS-1 cells were first confirmed in vitro. Then, the apparent benefits of co-administration of sunitinib and HVJ-E/IL-2 therapy were demonstrated in vivo. This combination therapy promoted the inhibition of tumor growth and increased tumor-free rates. Sunitinib alone paradoxically inhibited accumulations of CD8 (+) cells and NK cells, which was, however, reversed by HVJ-E/IL-2. Notably, addition of HVJ-E/IL-2 to sunitinib more effectively suppressed the development of MDSCs than sunitinib alone. Thus, it seemed plausible that sunitinib did not counteract and/or down-modulate the anti-tumor effects of HVJ-E/IL-2, but rather acted as a promoting agent via direct inhibition of tumor growth, as well as reducing MDSCs.

Collectively, HVJ-E/IL-2 with or without sunitinib would be promising for treating cutaneous angiosarcoma as a novel immunogene therapy.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

Acknowledgments

This work was partly supported by the Japan Society for the Promotion of Science (21791065), and by the grant of the Ministry of Health, Labor and Welfare (H24-008), Japan.

The authors would like to thank C. Miyagishi for the technical assistance.

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Anti-CCR4 mAb selectively depletes effector-type FoxP3$^+$CD4$^+$ regulatory T cells, evoking antitumor immune responses in humans

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CD4$^+$ Treg cells expressing the transcription factor FOXP3 (forkhead box P3) are abundant in tumor tissues and appear to hinder the induction of effective antitumor immunity. A substantial number of T cells, including Treg cells, in tumor tissues and peripheral blood express C-C chemokine receptor 4 (CCR4). Here we show that CCR4 was specifically expressed by a subset of terminally differentiated and most suppressive CD45RA$^-$FOXP3^{hi}CD4$^+$ Treg cells [designated effector Treg (eTreg) cells], but not by CD45RA$^+$FOXP3^{lo}CD4$^+$ naive Treg cells, in peripheral blood of healthy individuals and cancer patients. In melanoma tissues, CCR4$^+$ eTreg cells were predominant among tumor-infiltrating FOXP3$^+$ T cells and much higher in frequency compared with those in peripheral blood. With peripheral blood lymphocytes from healthy individuals and melanoma patients, ex vivo depletion of CCR4$^+$ T cells and subsequent in vitro stimulation of the depleted cell population with the cancer/testis antigen NY-ESO-1 efficiently induced NY-ESO-1–specific CD4$^+$ T cells. Nondepletion failed in the induction. The magnitude of the responses was comparable with total removal of FOXP3$^+$ Treg cells by CD25$^+$ T-cell depletion. CCR4$^+$ T-cell depletion also augmented in vitro induction of NY-ESO-1–specific CD8$^+$ T cells in melanoma patients. Furthermore, in vivo administration of anti-CCR4 mAb markedly reduced the eTreg-cell fraction and augmented NY-ESO-1–specific CD8$^+$ T-cell responses in an adult T-cell leukemia-lymphoma patient whose leukemic cells expressed NY-ESO-1. Collectively, these findings indicate that anti-CCR4 mAb treatment is instrumental for evoking and augmenting antitumor immunity in cancer patients by selectively depleting eTreg cells.

cancer immunotherapy | immunomodulation

Naturally occurring CD25$^+$CD4$^+$ regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (FOXP3) are indispensable for the maintenance of immunological self-tolerance and homeostasis (1, 2). Given that most tumor-associated antigens are antigenically normal self-constituents (3–5), it is likely that natural FOXP3$^+$ Treg cells engaged in self-tolerance concurrently hinder immune surveillance against cancer in healthy individuals and also hamper the development of effective antitumor immunity in tumor-bearing patients. Indeed FOXP3$^+$CD25$^+$CD4$^+$ Treg cells are abundant in tumor tissues (6–10), and their depletion augments spontaneous and vaccine-induced antitumor immune responses in animal models (10, 11). In humans, increased numbers of FOXP3$^+$CD25$^+$CD4$^+$ Treg cells and, in particular, decreased ratios of CD8$^+$ T cells to FOXP3$^+$CD25$^+$CD4$^+$ Treg cells among tumor-infiltrating lymphocytes (TIL) are well correlated with poor prognosis in various types of cancers (6, 7, 10). Some clinical studies have shown the potential of depleting CD25-expressing lymphocytes to augment antitumor immune responses (12, 13); yet other similar studies failed to support the effects (10, 14, 15). Because activated effector T cells also express CD25, and their production of IL-2 is required for the expansion of CD8$^+$ cytotoxic lymphocytes, CD25-based cell depletion may reduce activated effector T cells as well, cancelling the effect of Treg-cell depletion to augment antitumor immunity (10). In addition, it has been demonstrated in animal models that depletion of Treg cells as a whole can trigger autoimmunity (1, 16, 17). Therefore, a current key issue is to determine how Treg cells can be controlled to evoke and enhance antitumor immunity without affecting effector T cells or eliciting deleterious autoimmunity.

Human FOXP3$^+$CD4$^+$ T cells are heterogenous in phenotype and function (2). These cells can be dissected into three subpopulations by the expression levels of FOXP3 and the cell-surface molecules CD45RA and CD25: (*i*) FOXP3^{hi}CD45RA$^-$CD25^{hi} cells, designated effector Treg (eTreg) cells, which are terminally differentiating and highly suppressive; (*ii*) FOXP3^{lo}CD45RA$^+$CD25^{lo} cells, designated naive Treg cells, which differentiate into eTreg cells upon antigenic stimulation; and (*iii*) FOXP3^{lo}CD45RA$^-$CD25^{lo} non-Treg cells, which do not possess suppressive activity but secrete proinflammatory cytokines (18). In principle, these distinct properties of FOXP3$^+$ T-cell subpopulations can be exploited to augment antitumor immunity without inducing autoimmunity, for example, by depleting a particular Treg-cell subpopulation rather than whole Foxp3$^+$-cell population. One of

Significance

Regulatory T (Treg) cells expressing the transcription factor FOXP3 play a critical role in suppressing antitumor immune responses. Here we found that, compared with peripheral blood T cells, tumor-infiltrating T cells contained a higher frequency of effector Tregs, which are defined as FOXP3^{hi} and CD45RA$^-$, terminally differentiated, and most suppressive. Effector Treg cells, but not FOXP3^{lo} and CD45RA$^+$ naïve Treg cells, predominantly expressed C-C chemokine receptor 4 (CCR4) in both cancer tissues and peripheral blood. In vivo or in vitro anti-CCR4 mAb treatment selectively depleted effector Treg cells and efficiently induced tumor-antigen-specific CD4$^+$ and CD8$^+$ T cells. Thus, cell-depleting anti-CCR4 mAb therapy is instrumental for evoking and enhancing tumor immunity in humans via selectively removing effector-type FOXP3$^+$ Treg cells.

Author contributions: H.N. and S.S. designed research; D.S., H.N., Y.M., E.S., and Y.F. performed research; M.N., A.T., I.K., S.E., Y.K., J.K., and E.J. contributed new reagents/analytic tools; D.S., H.N., Y.M., and S.S. analyzed data; D.S., H.N., Y.M., and S.S. wrote the paper; and M.N., A.T., I.K., S.E., Y.K., J.K., and E.J. collected clinical samples and data.

Conflict of interest statement: H.N. received a research grant from Kyowa Hakko Kirin Co., Ltd.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1316796110/-/DCSupplemental.

the candidate molecules for such differential control of Treg-cell subpopulations is chemokine receptors, which allow Treg cells to migrate to a specific inflammation site via sensing specific chemokine milieu (19).

It has been shown that tumor-infiltrating macrophages and tumor cells produce the chemokine (C-C motif) ligand 22 (CCL22), which chemoattracts Treg cells as well as effector T cells expressing C-C chemokine receptor type 4 (CCR4) (6, 10, 20). In this report, we have addressed whether CCR4-targeting treatment is able to selectively reduce a particular Treg-cell subpopulation, rather than whole Treg population, and thereby elicit or augment in vitro and in vivo antitumor immune responses in humans.

Results

Depletion of CCR4$^+$ T Cells Predominantly Depletes eTreg Cells. In peripheral blood mononuclear cells (PBMCs) of healthy individuals, CCR4$^+$ T cells were present in both FOXP3$^+$ and FOXP3$^-$ T-cell fractions, and FOXP3^{hi} cells in particular were CCR4$^+$ (Fig. 1*A*). When FOXP3$^+$ T cells were classified into three populations by the levels of FOXP3 and CD45RA expression (18), FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) predominantly expressed CCR4 at the protein and mRNA level (Fig. 1*A*, and Figs. S1 and S2*A*). In contrast, FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) scarcely expressed the molecule, whereas FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III) exhibited a moderate expression. Among FOXP3$^-$ cells, some CD45RA$^-$CD4$^+$ memory or activated T cells expressed CCR4, whereas CD45RA$^+$ CD4$^+$ naive T cells did not. CD25 expression was well correlated with CCR4 expression with the highest CD25 expression by eTreg cells (Fr. II). Analyses of multiple samples of PBMCs from healthy individuals showed similar patterns of CCR4 expression by FOXP3 subsets (Fig. 1*B*). CD8$^+$ T cells, natural killer (NK) cells, CD14$^+$ monocytes/macrophages, dendritic cells, and B cells hardly expressed CCR4 at the protein and mRNA level (Fig. S2). In vitro depletion of CCR4$^+$ cells from PBMCs by magnet-bead sorting with anti-CCR4 mAb predominantly decreased CD4$^+$FOXP3^{hi} CD45RA$^-$ eTreg cells (Fr. II) and, to a lesser extent, CD4$^+$ FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III), but spared CD4$^+$ FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) and FOXP3$^-$ cells (Fr. IV and V) (Fig. 1*C*). In contrast with anti-CCR4 mAb treatment, similar in vitro cell depletion with anti-CD25 mAb significantly reduced all of the FOXP3$^+$ subpopulations (Fr I, II, and III) and, to a lesser extent, FOXP3$^-$CD45RA$^-$CD4$^+$ activated or memory T cells (Fr. IV), with a relative increase in FOXP3$^-$ CD45RA$^+$CD4$^+$ naive T cells (Fr. V) (Fig. 1*D*). PBMCs of melanoma patients showed similar patterns of CCR4 expression by FOXP3$^+$ subpopulations and similar changes in the composition of FOXP3$^+$ T-cell subsets after in vitro CCR4$^+$ T-cell depletion (Fig. S3).

Taking these data together, we find that CCR4 is predominantly expressed by eTreg cells and depletion of CCR4$^+$ cells results in selective reduction of eTreg cells, while preserving naive Treg cells and the majority of FOXP3$^-$CD4$^+$ T cells.

Tumor-Infiltrating Treg Cells Exhibit the eTreg-Cell Phenotype and Can Be Depleted in Vitro by Anti-CCR4 mAb. Although there is accumulating data that FOXP3$^+$ T cells predominantly infiltrate into tumor tissues (6, 7, 10, 21), their detailed phenotypes remain to be determined. Our analysis of TILs in nine melanoma samples revealed infiltration of a high percentage of CCR4$^+$ T cells, the majority of which were CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II), with only a small number of CD4$^+$FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) (Fig. 2*A*). In vitro depletion of CCR4$^+$ T cells indeed dramatically reduced these tumor-infiltrating eTreg cells (Fig. 2*B*), indicating that anti-CCR4 mAb treatment is able to selectively deplete eTreg cells abundantly infiltrating into tumors.

In Vitro Induction of NY-ESO-1–Specific CD4$^+$ T Cells After CCR4$^+$ T-Cell Depletion from PBMCs of Healthy Donors and Melanoma Patients. With the efficient depletion of the eTreg-cell population by in vitro anti-CCR4 mAb treatment, we next examined

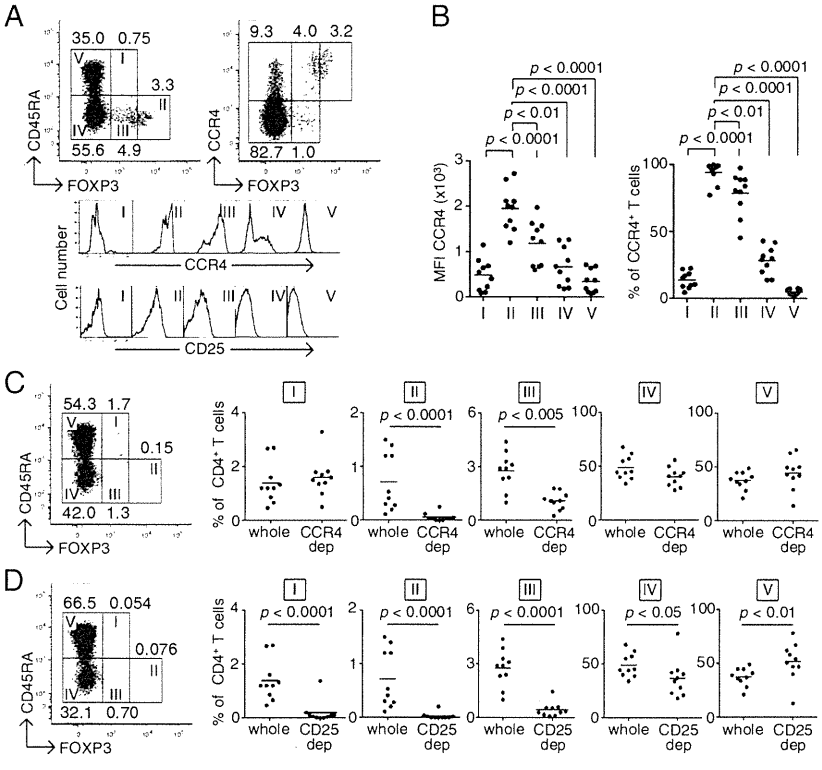

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown. (*B*) Median fluorescence intensity (MFI, *Left*) and frequency (*Right*) of CCR4 expression by each fraction of T cells in PBMCs of healthy donors (n =10). (*C*) Changes in the proportion of T-cell subpopulations after CCR4$^+$ T-cell depletion (CCR4 dep) (n = 10). (*D*) Changes in the proportion of T-cell subpopulations after CD25$^+$ T-cell depletion (CD25 dep) (n = 10). The numbers in *A*, *C*, and *D* indicate the percentage of gated CD4$^+$ T cells. Representative staining profiles in *A*, *C*, and *D* are from the same donor, and the same PBMC samples were analyzed in *B*–*D*.

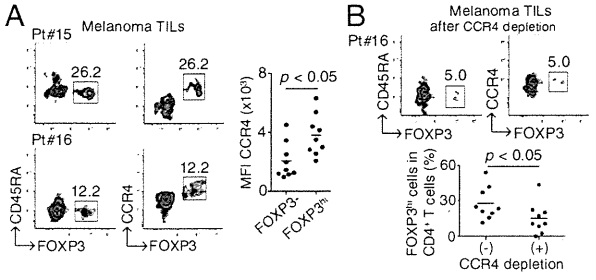

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed. Data from two representative patients are shown. (*Right*) Summary of MFI of CCR4 expression by FOXP3$^-$ or FOXP3$^+$ cells (n = 9). (*B*) CCR4$^+$ CD4$^+$ T cells from melanoma tissues (Pt #16) were depleted of CCR4$^+$ T cells and then analyzed for the proportion of FOXP3^{hi} eTreg cells. (*Lower*) Percentages of FOXP3^{hi} cells among CD4$^+$ T cells after CCR4$^+$ cell depletion or nondepletion (n = 9). The numbers in *A* and *B* indicate the percentage of gated CD4$^+$ T cells.

whether CCR4$^+$ T-cell depletion from PBMCs of healthy donors was able to induce tumor antigen-specific CD4$^+$ T cells. We assessed specific T-cell responses to NY-ESO-1, a cancer/testis antigen, which is normally expressed by human germ-line cells and also by various types of cancer cells (4, 22). CCR4$^-$CD4$^+$ T cells or CD25$^-$CD4$^+$ T cells were cultured with CD4$^-$CD8$^-$ PBMCs as antigen-presenting cells (APCs), which were pulsed overnight with series of overlapping peptides covering the entire sequence of the NY-ESO-1 protein and X-irradiated (35 Gy) before use, as previously described (23, 24). Fifteen to 20 d later, NY-ESO-1–specific CD4$^+$ T cells secreting IFN-γ were enumerated by enzyme-linked immunospot (ELISpot) assay. Significant numbers of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were induced in 7 of 16 healthy donors (43.8%), but only in the cultures with CCR4$^+$ or CD25$^+$ T-cell–depleted T cells (Fig. 3*A*, and summarized in Table S1). Furthermore, the frequencies of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were higher after CCR4$^+$ T-cell depletion compared with CD25$^+$ T-cell depletion in five of seven healthy donors (71.4%) (Table S1). This result could be attributed in part to possible depletion of NY-ESO-1–specific CD25$^+$ activated T cells by anti-CD25 mAb treatment. The NY-ESO-1–specific CD4$^+$ T cells produced IFN-γ and TNF-α (Fig. 3*B*). Those cells induced in vitro after CCR4$^+$ T-cell depletion recognized NY-ESO-1 peptides at the concentration as low as 0.1 μM (Fig. 3*C*), and also NY-ESO-1 peptides produced by natural processing of the NY-ESO-1 protein by APCs, as previously shown with CD25$^+$ T-cell depletion (22, 24) (Fig. 3*D*).

We also attempted to determine whether Treg-cell depletion would evoke anti–NY-ESO-1 responses in apparently nonresponsive melanoma patients. With PBMCs from patients bearing NY-ESO-1–expressing melanomas, but without detectable NY-ESO-1–specific Ab in the sera, in vitro depletion of CCR4$^+$ or CD25$^+$ T cells and subsequent in vitro peptide stimulation induced IFN-γ– and TNF-α–secreting NY-ESO-1–specific CD4$^+$ T cells in three of eight patients (37.5%) (Fig. S4 *A* and *B* and Table S2). These NY-ESO-1–specific CD4$^+$ T cells appeared to express high-avidity T-cell receptors that recognized NY-ESO-1 peptides at a concentration as low as 0.1 μM, as seen with healthy donor T cells (Fig. S4*C*).

Thus, in healthy individuals as well as melanoma patients who had not raised spontaneous NY-ESO-1 immune responses, removal of eTreg cells by CCR4$^+$ T-cell depletion is able to efficiently induce high-avidity NY-ESO-1–specific CD4$^+$ T cells secreting effector cytokines.

CCR4$^+$ T-Cell Depletion Augments in Vitro Induction of NY-ESO-1–Specific CD8$^+$ T Cells from PBMCs of Melanoma Patients. PBMCs from melanoma patients were subjected to in vitro depletion with anti-CCR4 mAb or anti-CD25 mAb, and cultured with NY-ESO-1 peptide capable of binding to HLA class I of each patient. Seven to 10 d later, NY-ESO-1–specific CD8$^+$ T cells were detected by NY-ESO-1/HLA tetramers and analyzed for intracellular cytokine production. NY-ESO-1–specific CD8$^+$ T cells were induced in four of six patients (66.7%), and the responses were markedly augmented after depletion of CCR4$^+$ or CD25$^+$ cells (Fig. 4*A*). In addition, these NY-ESO-1–specific CD8$^+$ T cells recognized an HLA-matched malignant melanoma cell line and secreted IFN-γ and TNF-α (Fig. 4*B*). For example, Pt. #9 (HLA-A*02/29, B*44/27, C*03/04) harbored not only HLA-C*03–restricted NY-ESO-1–specific CD8$^+$ T-cells detected by HLA Cw*0304/NY-ESO-1 tetramers, but also those NY-ESO-1–specific CD8$^+$ T cells that recognized the SK-MEL 37 melanoma line (A*0201$^+$, NY-ESO-1$^+$) in an HLA-A2–restricted manner.

We also examined whether NY-ESO-1–specific CD8$^+$ T cells could be induced by directly adding mAb into cell cultures. Addition of anti-CD25 mAb or anti-CCR4 mAb reduced the frequency of CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) (Fig. S5).

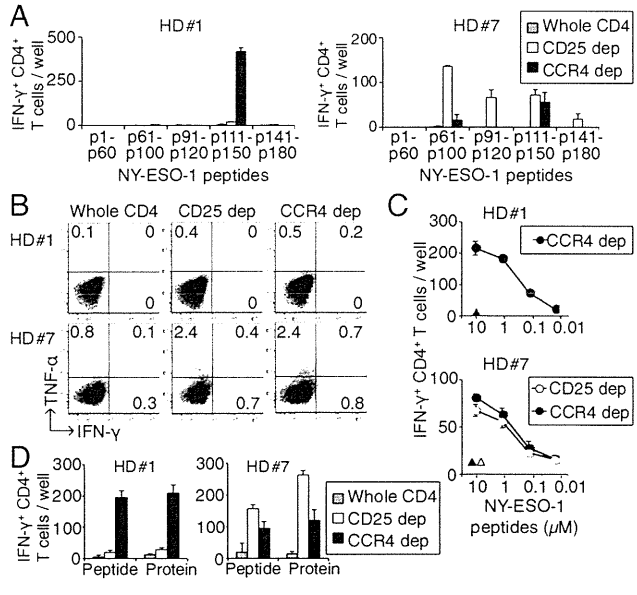

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized with APCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1. Results of 2 (HD#1 and HD#7) among 16 healthy donors are shown. The numbers of IFN-γ–secreting CD4$^+$ T cells were assessed by ELISpot assay. (*B*) Intracellular cytokine secretion of CD4$^+$ T cells shown in *A*. The numbers in figures indicate the percentage of gated CD4$^+$ T cells. (*C*) Peptide dose-dependent recognition of NY-ESO-1–specific IFN-γ–secreting CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T cells derived from CCR4$^+$ or CD25$^+$ T-cell–depleted cells (CCR4 dep and CD25 dep, respectively) were cultured with autologous activated T-cell APCs pulsed with graded amounts of NY-ESO-1 peptides and assessed for the number of IFN-γ–secreting cells as in *A*. Triangles indicate responses to control peptide at 10 μM. (*D*) Recognition of naturally processed NY-ESO-1 protein antigen by NY-ESO-1–specific CD4$^+$ T cells derived from whole CD4$^+$, CCR4$^+$ cell-depleted, or CD25$^+$ cell-depleted cells. NY-ESO-1–specific CD4$^+$ T cells from two healthy donors were cultured with autologous dendritic cells pulsed with NY-ESO-1 or control protein, or with NY-ESO-1 or control peptide. The experiments were independently performed twice with similar results.

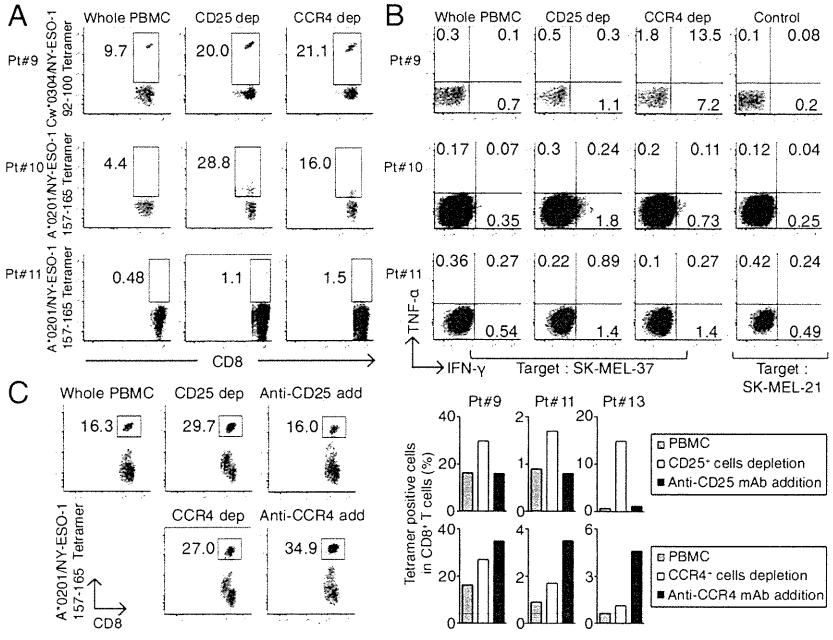

Fig. 4. Augmentation of NY-ESO-1–specific $CD8^+$ T-cell induction in melanoma patients by in vitro $CCR4^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific $CD8^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of $CD25^+$ or $CCR4^+$ cells, were prepared from melanoma patients (n = 6), and presensitized in peptides capable of binding to patients' HLA. NY-ESO-1–specific $CD8^+$ T cells were analyzed with NY-ESO-1/HLA tetramers (Pt. #9: A*02/29, B*44/27, C*03/04, Pt. #10: A*02/11, B*35/44, C*04/05, and Pt. #11: A*02/-, B*13/18, C*06/07). (*B*) Cytokine secretion of NY-ESO-1–specific $CD8^+$ T cells upon recognition of the HLA-A*0201$^+$ melanoma cell line SK-MEL 37 (NY-ESO-1$^+$), or SK-MEL-21 (NY-ESO-1$^-$) analyzed by intracellular cytokine staining. Data from three representative patients are shown. (*C*) Induction of antigen-specific $CD8^+$ T cells by addition (add) of anti-CD25 or anti-CCR4 mAb (KM2160) to cell cultures, or by $CCR4^+$ or $CD25^+$ cell depletion or nondepletion, as shown in *A* (Pt. #13 A02/03, B07/41, C07/17). A representative result (*Left*) and summary of three melanoma patients (*Right*) are shown. The numbers in the panels indicate the percentage of gated $CD8^+$ T cells. These experiments were performed independently at least twice with similar results.

Interestingly, although NY-ESO-1–specific $CD8^+$ T-cell induction was augmented in the cell culture containing anti-CCR4 mAb, the addition of anti-CD25 mAb reduced the frequency of NY-ESO-1–specific $CD8^+$ T cells (Fig. 4*C*), indicating that it might have killed some $CD25^+CD8^+$ activated effector T cells in addition to $CD25^+CD4^+$ Treg cells.

These results indicate that depletion of $CCR4^+$ T cells before in vitro induction or even simple incubation with anti-CCR4 mAb during the induction effectively augments NY-ESO-1–specific $CD8^+$ T-cell responses by selectively reducing eTreg cells.

Anti-CCR4 mAb Administration into Adult T-Cell Leukemia-Lymphoma Patients Reduces $CD4^+FOXP3^{hi}CD45RA^-$ eTreg Cells and Augments NY-ESO-1–Specific $CD8^+$ T-Cell Responses. In adult T-cell leukemia-lymphoma (ATL), which is caused by human T-lymphotropic virus 1 infection, ATL cells are $CD4^+$ and the majority—if not all—of them express FOXP3, CD25, CTLA-4, and CCR4, thus resembling naturally occurring $FOXP3^+$ Treg cells (25–28). Although it is currently difficult to discriminate whether anti-CCR4 mAb reduces ATL cells or normal $FOXP3^+$ Treg cells (29), we examined whether in vivo administration of anti-CCR4 mAb (Mogamulizumab), which has a cell-depleting effect by antibody-dependent cellular cytotoxicity, was able to reduce $FOXP3^+$ cells or a subpopulation thereof. Analysis of PBMCs from ATL patients collected before and after anti-CCR4 mAb therapy revealed that $CD4^+FOXP3^{hi}CD45RA^-$ cells including both ATL cells and eTreg cells were markedly reduced after the therapy (Fig. 5*A*). In addition, in a patient whose ATL cells expressed NY-ESO-1, NY-ESO-1–specific $CD8^+$ T cells producing IFN-γ and TNF-α were induced after several rounds of anti-CCR4 mAb administration (Fig. 5*B*). NY-ESO-1–specific $CD8^+$ T cells producing these cytokines were much higher in frequency than NY-ESO-1–specific $CD8^+$ T cells detected by NY-ESO-1/HLA-B*3501 tetramers, suggesting that this patient additionally possessed $CD8^+$ T cells recognizing other epitopes of NY-ESO-1. These results collectively indicate that anti-CCR4 mAb therapy for ATL is able to selectively deplete eTreg cells as well as ATL cells in vivo, and induce/augment tumor antigen-specific T-cell responses, although it is possible that anti-CCR4 mAb-induced reduction of $FOXP3^+$ ATL cells, which reportedly exhibit a Treg-cell–like in vitro suppressive activity (27, 28), might also contribute to the augmentation of immune responses.

Discussion

Accumulating evidence indicates that effective cancer immunotherapy needs to control $FOXP3^+$ Treg cells naturally present in the immune system and abundantly infiltrating into tumor tissues (10, 11, 30). Here, we have shown that $CD4^+FOXP3^{hi}CD45RA^-$ eTreg cells, which are terminally differentiated and most suppressive, highly express CCR4, that they are predominant among $FOXP3^+$ T cells infiltrating into tumor tissues (e.g., melanoma), and that specific depletion of eTreg cells in vivo or in vitro by anti-CCR4 mAb evoked tumor antigen-specific immune responses mediated by $CD4^+$ and $CD8^+$ T cells in healthy individuals and cancer patients.

Besides high expression of CCR4 in eTreg cells, CCR4 is expressed, although to a lesser extent, in non-Treg $CD4^+$ T-cell fractions [i.e., the $FOXP3^{lo}CD45RA^-$ cells (Fr. III) and $FOXP3^-CD45RA^-$ cells (Fr. IV)]. The former are capable of secreting cytokines, such as IL-4 and IL-17, as previously reported with PBMCs of healthy individuals (18). It has also been shown that Th2 cells and a fraction of central memory $CD8^+$ T cells express CCR4 (31–33). It is thus likely that tumor-infiltrating activated macrophages, and presumably some tumor cells produce CCL22, which predominantly chemoattracts and recruits from peripheral blood both $CCR4^+$ eTreg and $CCR4^+$ effector T cells that recognize tumor-associated antigens (such as cancer/testis antigen) and presumably self-antigens released from tumor cells (6, 10, 21, 34). However, the frequency of IL-4– or IL-17–secreting $CD4^+$ T cells were much lower than eTreg cells among $CCR4^+CD4^+$ T cells in PBMCs and TILs in melanoma tissues of nontreated patients; and CCR4 expression by $CD8^+$ TILs were limited. Moreover, addition of anti-CCR4 mAb into in vitro peptide stimulation more effectively induced antigen-specific $CD8^+$ T cells than $CCR4^+$ T-cell depletion, indicating that anti-CCR4 mAb had reduced eTreg cells but spared $CD8^+$ effector T cells. The result contrasted with the addition of anti-CD25 mAb, which appeared to deplete $CD25^+CD8^+$ T cells and cancel the enhancing effect of Treg-cell depletion. These results taken together indicate that anti-CCR4 mAb treatment to augment antitumor immunity mainly target $CCR4^+$ eTreg cells

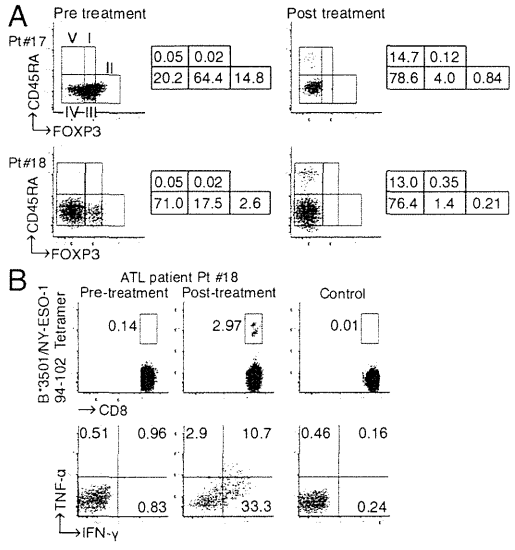

Fig. 5. Reduction of $CD4^+FOXP3^{hi}CD45RA^-$ T cells and augmentation of NY-ESO-1–specific $CD8^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) $FOXP3^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702 and Pt. #18: lymphoma type, HLA-A*0201/3101, B*3501/4002, C*0303/0401) before and after anti-CCR4 mAb therapy. These experiments were performed at least twice with similar results. The numbers indicate the percentage of gated $CD4^+$ T cells. (*B*) Analysis of NY-ESO-1–specific $CD8^+$ T-cell induction before and after anti-CCR4 mAb therapy. PBMCs from Pt. #18 were presensitized in the presence of APCs pulsed with NY-ESO-1_{91-110} peptide corresponding to the patient's HLA. NY-ESO-1–specific $CD8^+$ T cells were detected with NY-ESO-1/HLA tetramers, and cytokine secretion of these NY-ESO-1–specific $CD8^+$ T cells upon recognition of autologous activated T-cell APCs pulsed with NY-ESO-1_{91-110} or control peptide was analyzed by intracellular cytokine staining. The numbers in figures indicate the percentage of gated $CD8^+$ T cells. The result was derived from a single assay because of limited availability of the patient's samples.

in tumor tissues and the regional lymph nodes, as well as peripheral blood, which would otherwise be a reservoir of fresh tumor-infiltrating Treg cells. Further study is warranted to determine whether depletion of $CCR4^+CD4^+$ and $CD8^+$ effector T cells in vivo affects antitumor immunity to a clinically significant extent.

Both NY-ESO-1–specific $CD4^+$ and $CD8^+$ T cells induced by in vitro anti-CCR4 mAb treatment possessed high-avidity T-cell receptors, and responded to dendritic cells processing tumor antigens and histocompatible tumor cell lines, respectively. This finding raises the issue of whether Treg depletion by anti-CCR4 mAb activates and expands already present antigen-primed effector T cells or newly induces effector T cells from a naive T-cell pool. We previously showed that in vitro NY-ESO-1-peptide stimulation following $CD25^+CD4^+$ T-cell depletion could activate NY-ESO-1–specific naive $CD4^+$ T-cell precursors in healthy individuals and in melanoma patients who possessed NY-ESO-1–expressing tumors but failed to develop anti-NY-ESO-1 Ab (23). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in melanoma patients who had spontaneously developed anti–NY-ESO-1 Ab were derived from a memory population and could be activated even in the presence of $CD25^+CD4^+$ Treg cells (23). In addition, following vaccination of ovarian cancer patients with a HLA-DP–restricted NY-ESO-1 peptide, development of NY-ESO-1–specific high-avidity effector T cells from naive T cells was hampered by the presence of $CD25^+CD4^+$ Treg cells, although the vaccination could expand low-avidity NY-ESO-1–specific $CD4^+$ T cells that were apparently present in an effector/memory fraction before the vaccination (24). These results collectively indicate that elimination of eTreg cells by $CCR4^+$ T-cell depletion abrogates Treg cell-mediated suppression on NY-ESO-1–specific high-avidity naive T-cell precursors, allowing their activation and differentiation into high-avidity effector T cells capable of mediating strong antitumor immune responses. This successful induction of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells indicates that the combination of anti-CCR4 mAb administration and vaccination with tumor antigens, such as NY-ESO-1, could be an ideal strategy for immunotherapy of a variety of cancers including ATL, which express NY-ESO-1 (35).

On the other hand, it was noted that not all healthy individuals or melanoma patients developed NY-ESO-1–specific T cells in vitro after Treg depletion for several possible reasons. For example, individuals who do not have a proper HLA haplotype may fail to select NY-ESO-1–reactive T cells thymically (22), hence possessing few NY-ESO-1–specific T-cell precursors. Other types of suppressor cells (such as myeloid-derived suppressor cells, immunosuppressive macrophages, and $Foxp3^-$ Treg cells) might contribute to inhibiting the induction of the responses (30). Alternatively, T cells specific for NY-ESO-1, a cancer/testis antigen, may also be subjected to other mechanisms of immunological self-tolerance—for example, anergy—hence being hyporesponsive to the antigen (36). These possibilities are under investigation to make anti-CCR4 mAb therapy more effective.

Would in vivo anti-CCR4 mAb treatment to deplete Treg cells elicit harmful autoimmunity? It has been shown in animal models that a longer period and a more profound degree of Treg-cell depletion is required to elicit clinically and histologically evident autoimmunity than evoking effective antitumor immunity (37, 38). In humans, naive Treg cells are generally well preserved in peripheral blood in cancer patients, even if they are low in frequency in tumor tissues. Furthermore, $CCR4^+$ T-cell depletion selectively eliminates eTreg cells but spares naive Treg cells. Assuming that effective tumor immunity can be evoked without significant autoimmunity via controlling the degree and duration of Treg-cell depletion, it is likely that, although anti-CCR4 mAb administrations reduce eTreg cells in the immune system during the treatment, the residual $CCR4^-$ eTreg cells (as shown in Fig. 2), including those which have newly differentiated from naive Treg cells, are sufficient to prevent deleterious autoimmunity. Supporting this notion, only a minor population of ATL patients treated with anti–CCR4 mAb experienced severe immune-related adverse events, except skin rashes (29). Anti-CCR4 mAb therapy can therefore be a unique cancer immunotherapy aiming at depleting eTreg cells without clinically serious adverse effects that would be incurred by total Treg-cell depletion or functional blockade (39).

The critical roles of CCR4 in Treg-cell recruitment to tumors have been reported with various types of human cancers, such as malignant lymphomas, gastric, ovarian, and breast cancers (10). $CCR4^+$ eTreg cells abundantly and predominantly infiltrated into gastric and esophageal cancers as observed with melanoma. Although it remains to be determined whether every cancer tissue has predominant infiltration of $CCR4^+$ eTreg cells, it is envisaged that possible combination of anti-CCR4 mAb treatment, tumor antigen immunization, and antibody-mediated immune checkpoint blockade will further increase clinical efficacy of cancer immunotherapy.

Materials and Methods

Donor Samples. PBMCs were obtained from healthy donors, malignant melanoma patients with NY-ESO-1 expression, and ATL patients. To collect tumor-infiltrating T cells, melanoma tissues were minced and treated with gentleMACS Dissociator (Miltenyi Biotec). All healthy donors were subjects with no history of autoimmune disease. All donors provided written informed consent before sampling according to the Declaration of Helsinki. The present study was approved by the institutional ethics committees of Osaka University, Osaka, Japan and Landesarztekammer Hessen, Frankfurt, Germany.

Antibodies and Peptides. The information of antibodies and synthetic peptides is provided in *SI Materials and Methods*.

Preparation of CD25$^-$ or CCR4$^-$ Cells. PBMCs or CD4$^+$ T cells were treated with biotin-anti-CD25 mAb (BC96) or biotin-anti-CCR4 (1G1) mAb (0.01 mg/mL), otherwise specified, for 15 min at 4 °C. Subsequently, anti-Biotin MicroBeads (Miltenyi Biotec) were added as described in the manufacturer's protocol, then washed using PBS containing 2% (vol/vol) FCS. CD25$^-$ or CCR4$^-$ cells were separated on autoMACS Pro Separator (Miltenyi Biotec).

In Vitro Sensitization of NY-ESO-1–Specific CD4$^+$ T Cells. NY-ESO-1–specific CD4$^+$ T cells were presensitized as previously described (23, 24) and in *SI Materials and Methods*.

In Vitro Sensitization of NY-ESO-1–Specific CD8$^+$ T Cells. For in vitro sensitization of NY-ESO-1–specific CD8$^+$ T cells, 1.5–2 × 10^6 cells were cultured with NY-ESO-1 peptides (NY-ESO-$1_{157-165}$ for HLA-A*0201 restricted, NY-ESO-1_{92-100} for HLA-Cw*0304 restricted, NY-ESO-1_{91-110} for HLA-B*3501 restricted, 10 μM) (22, 23) in a 48-well dish or round-bottom 96-well plate. After 8 h, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) and repeated twice per week. In some assays, purified anti-CD25 (M-A251) mAb or anti-CCR4 (KM2160) mAb (1 μg/mL) was included in some wells during the entire period of culture.

ELISpot Assay. The number of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells was assessed by ELISpot assay as previously described (23, 24) and in *SI Materials and Methods*.

Intracellular Cytokine Secretion Assay. The presensitized CD4$^+$ and CD8$^+$ T cells were restimulated with peptide-pulsed autologous activated T-cell APCs, SK-MEL-21 cells (NY-ESO-1$^-$, HLA-A*0201$^+$), or SK-MEL-37 cells (NY-ESO-1$^+$, HLA-A*0201$^+$) for 1 h, after which GolgiStop reagent (BD Biosciences) was added. Subsequently, cells were cultured for another 6–8 h at 37 °C. Cells were stained for cell surface markers and then for intracellular cytokines using BD Cytofix/Cytoperm Buffer and BD Perm/Wash Buffer (BD Biosciences). Results were analyzed by flow cytometry (BD LSRFortessa; BD Biosciences) and FlowJo v9.6.2 software (TreeStar).

Tetramer Assay. Tetramer staining was performed as previously described (35, 40) and in *SI Materials and Methods*.

Preparation of Dendritic Cells. Dendritic cells were prepared as previously described (24) and in *SI Materials and Methods*.

Statistical Analysis. The significance of the difference in each data between two groups was assessed by a Mann–Whitney test using Prism version 6 software (GraphPad). *P* values less than 0.05 were considered significant.

ACKNOWLEDGMENTS. We thank Drs. J. B. Wing and D. O. Adeegbe for helpful discussion and critical reading of this manuscript, and Ms. Y. Tada, K. Teshima and Y. Funabiki for technical assistance. SK-MEL21 and SK-MEL37 were kindly provided by Dr. Lloyd J. Old; anti-CCR4 mAb (KM2160) was a generous gift from Kyowa Hakko Kirin Co., Ltd. This study was supported by Grants-in-Aid for Specially Promoted Research 20002007 (to S.S.) and for Scientific Research (B) 23300354 (to H.N.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Core Research for Evolutional Science and Technology from the Japan Science and Technology Agency (S.S.); Health and Labor Sciences Research Grants, Research on Applying Health Technology H24-Clinical Cancer Research-general-006 and H23-Third Term Comprehensive Control Research for Cancer-general-011 (to H.N.) from the Ministry of Health, Labor, and Welfare, Japan; a Cancer Research Institute Designated grant and CLIP grant (to H.N.); and a research grant from Kyowa Hakko Kirin Co., Ltd. (to H.N.).

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Cancer Gene Therapy (2013), 1–7

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REVIEW

Recent advances and developments in the antitumor effect of the HVJ envelope vector on malignant melanoma: from the bench to clinical application

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Inactivated Sendai virus particles (hemagglutinating virus of Japan envelope; HVJ-E) are considered to be safe and efficient non-viral vectors used for drug delivery, since they can incorporate DNA, RNA, proteins and drugs. We have recently found that HVJ-E has a novel antitumor immune effect using a colon cancer model. HVJ-E has also been shown to have both direct and immune-mediated indirect actions against malignancy. Intratumoral injection of an inactivated HVJ-E solution significantly reduced the tumor volume and prevented spontaneous lung metastasis, leading to an increased overall survival in C57/BL6 mice transplanted with B16/BL6 mouse melanoma cells, and even in immunodeficient mice transplanted with Mewo human melanoma cells. No severe adverse effects including laboratory data abnormalities or anaphylactic reactions were observed. The comprehensive mechanism(s) underlying the immunological effects of HVJ-E appear to include not only enhanced effector T cell- and/or natural killer (NK) cell-mediated immunity, but also rescue from regulatory T cell (Treg)-mediated immunosuppression, presumably through the interleukin-6 secretion from dendritic cells stimulated by HVJ-E. Since a protocol for a clinical study of HVJ-E in malignant melanoma was approved in 2009 by the ethics committee of Osaka University and of the Medical Center for Translational Research in Osaka University Hospital, a phase I/IIa study for advanced malignant melanoma patients was just started. In this review, we show several favorable results regarding the antitumor effects of HVJ-E and describe the novel mechanism underlying this tumor immune response. Since we are conducting a phase I/IIa clinical trial using HVJ-E in advanced melanoma patients on the basis of preclinical results, detailed clinical information and immune-monitoring data are also introduced. The development of new therapeutic modalities for advanced melanoma patients is urgently needed, and we hope that HVJ-E may provide one such treatment.

Cancer Gene Therapy advance online publication, 25 October 2013; doi:10.1038/cgt.2013.61

Keywords: HVJ-E; antitumor effect; malignant melanoma

INTRODUCTION

Malignant melanoma is a highly aggressive malignancy that is resistant to most treatment. Although standard biochemotherapy including dacarbazine and cisplatin, as well as granulocyte macrophage colony-stimulating factor for the activation and maturation of dendritic cells (DCs), has been established for melanoma patients, the response rate is insufficient, at $<25\%$ for advanced malignant melanoma.[1,2] In addition, although many immunotherapies for advanced stage melanoma patients have been evaluated on the basis of their high immunogenic potential, these have generally failed,[3–6] partly because the tumor-specific damage is prevented by a loss of specific antigens caused by the genetic mutation of tumor cells or by the disappearance of human leukocyte antigen-related molecules. Therefore, it is indispensable to look for a novel immunogenic approach that can regulate melanoma growth and metastasis.

Although the transplantation of melanoma antigen-specific T-cell receptor-carrying cytotoxic T lymphocytes (CTLs) can directly attack melanoma cells, the antitumor effect is limited and attenuated because of the deletion of human leukocyte antigen expression and the loss of several inhibitory factors, such as regulatory T cell (Treg) and monocyte-derived suppressor cells via interleukin-10 (IL-10) and transforming growth factor β production. On the other hand, with respect to the examination of direct placement of a tumor antigen genetically modified virus, an antitumor effect was obtained based on a specific immune reaction for a case that was administered a high dosage of IL-2 after immunization with a recombinant fowlpox virus, which was designed to effectively show T-cell epitopes.[7,8] Recently, immunomodulation therapy by adoptive cell transfer of autologous tumor-infiltrating lymphocytes, combined with preceding myelo- or lympho-depletion and subsequent IL-2 administration, was introduced for patients with metastatic solid cancer patients,[9] and the objective response rate was reported to be $>50\%$ in a clinical trial in stage IV melanoma patients.[10]

It is well known that the humanized anti-CTLA-4 (cytotoxic T-lymphocyte antigen 4) antibody, named ipilimumab, which can overcome the negative feedback effect in DCs, was approved by the federal drug and food in 2011 because it provided a significant improvement in the overall survival compared with vaccine therapy using the gp100 peptide.[11] Moreover, the anti-PD-1 (programmed cell death-1) or PD-L1 antibody that restores T-cell inhibition by the PD-L1–CD28 interaction is becoming one of effective tools used for advanced melanoma with regard to immune modulation.[12]

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Received 14 January 2013; revised 23 August 2013; accepted 17 September 2013

Since about 2005, basic and clinical efforts to improve anticancer immunotherapy have shed light on what is needed for sufficient therapy for malignant melanoma.[9–12] Among these promising results in immunotherapy, we have recently shown an augmentation of antitumor immunity and tumor cell death induction by a hemagglutinating virus of Japan envelope (HVJ-E) vector for several carcinomas.[13–16] HVJ, which belongs to *Paramyxovirus* family of *Paramyxoviridae* genus, was originally reported by Ishida *et al.* in 1953.[17] Inactivated HVJ particles have been useful as a drug delivery system based upon their high cell-fusion affinity and ability to deliver DNA, RNA and proteins components.[18] On another front, we have shown and reviewed that HVJ-E has the potential to comprehensively improve the antitumor immunity by performed detailed *in vitro* and *in vivo* analyses.[14–16] In this review, we would like to describe our recent experimental data demonstrating the antitumor effects of HVJ-E in malignant melanoma *in vivo* and introduce the entry information about our first-in-man clinical trial.

The development of HVJ-E

HVJ-E is derived from an inactivated Z strain of HVJ produced from fertilized chicken eggs or human cells cultured in non-serum. Clinical grade HVJ-E is manufactured through the inactivation of cell-derived HVJ with an alkylating agent (β-propiolactone) and ultraviolet irradiation, purification processing by column chromatography and formulation processing by freeze dying. The structure of HVJ-E, as shown in the frame format of Figure 1, is a nanoparticle measuring ~230 nanometers in diameter with an outer membrane, and comprises protein, glycoprotein, a lipid bilayer and residual nucleic acids. HVJ belongs to the *Paramyxovirus* family of *Paramyxoviridae*, which also causes parainfluenza in mice. Following the publication of the report by Ishida *et al.* in 1953,[17] HVJ is otherwise known as the 'Sendai Virus.' Although HVJ is a potential cause of pneumonia in mice, it is not pathogenic in humans due to specific differences in the host enzymes that are necessary to acquire infection.

HVJ is unique in that it demonstrates cell fusion activity through the functions of the F protein and the HN protein that exist in the outer membrane. This phenomenon was reported by Okada *et al.* 1957, based on the results of a study using a Z strain of HVJ[17] which subsequently led to the invention of monoclonal antibodies taking advantage of this property,[19] along with the production of chromosome maps.[20] Furthermore, inactivated HVJ particles (HVJ-E) are being used as a raw material for the delivery of macromolecular substances, by taking advantage of their membrane fusion activity. HVJ is also being developed as a drug delivery system, known as HVJ liposome or HVJ-E, which provided good results in animal experiments.[21,22]

Activation of antitumor immunity by HVJ-E

In recent years, it has been clarified that HVJ-E itself has an antitumor effect[16] against mouse colon carcinoma cell lines subcutaneously transplanted onto the backs of mice. When three doses of 1.5×10^{10} of HVJ-E were administered intratumorally following the development of 5 mm tumors, the growth of the tumors was inhibited compared with mice treated with an adenovirus deactivated by saline or ultraviolet rays in a similar manner. Furthermore, when other cells were transplanted onto the contralateral side of the back of these mice after 4 days, tumor formation was completely prevented in three out of five mice. In other words, HVJ-E treatment also decreased the formation of a new tumor and maintained the antitumor immune reaction, in addition to growth inhibition properties. Furthermore, an analysis of the infiltration of immune-competent cells into the tumor cells was performed. When the CD11c expression was investigated using quantitative RT-PCR to assess the DC infiltration, there were higher levels 24 and 48 h after injection compared with the control

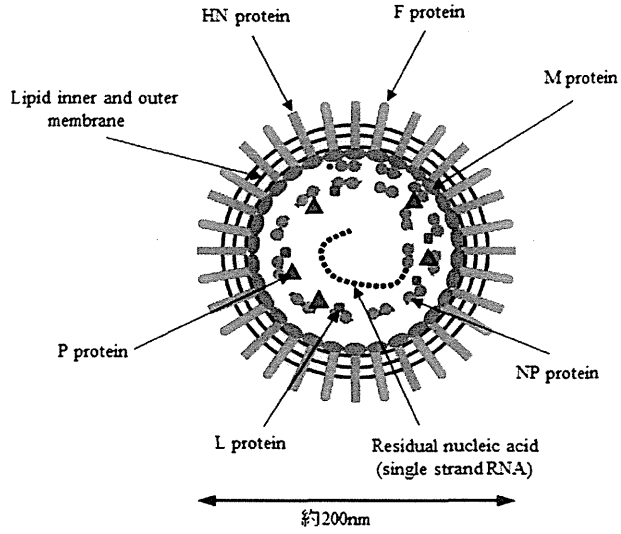

Figure 1. The structure of live hemagglutinating virus of Japan (HVJ). A nucleocapsid containing ~15 kb of the single-stranded viral RNA genome and nucleocapsid protein (NP), as well as polymerases P and L, is located inside, and the F and N proteins penetrating the envelope are associated with cell fusion. Live HVJ becomes the non-amplifying inactive form (HVJ-E; HVJ-envelope) following treatment with ultraviolet radiation or an alkylating agent.

group, and the expression was sustained even after 120 h. As the tumor tissue treated with HVJ-E was found to have increased expression of CD40, CD80 and CD86, which are surface markers indicating the maturation of DCs, it was suggested that mature DCs infiltrated into the tumor tissues injected with HVJ-E (Figure 2a).

Second, a significantly larger number of tumor-infiltrating CD4- and CD8-positive T lymphocytes were observed 48 h after the three consecutive doses of HVJ-E in some of the tumors, indicating the induction of tumor-specific CTLs, which was augmented by HVJ-E (Figure 2b). In fact, systemic activation of CTLs against melanoma was obtained in mice injected with HVJ-E when splenocytes were used for the chrome-release assay in colon cancer cell-bearing mice.[16] On the basis of these results and our previous publications,[14–16,23] it can be concluded that HVJ-E itself has antitumor effects through the activation of CTLs against tumors. For more detailed information about the mechanisms underlying the antitumor effects of intratumoral HVJ-E injection, please see Kaneda,[14,15] Kurooka and Kaneda[16] and Fujiwara *et al.*[23]

Regulatory T cells (hereafter referred to as 'Tregs') have been pointed to inhibit antitumor immunity, with great importance now being placed on how the inhibitory effects of Tregs can be avoided. IL-6 has been reported to be an important cytokine involved in controlling the function and differentiation of Tregs.[24,25] For this reason, we examined whether HVJ-E acted on DCs to accelerate the secretion of IL-6. It was found that HVJ-E acted on DCs to promote them to secrete IL-6 in an amount equivalent to that produced in response to live HVJ. On the other hand, only a small amount of tumor necrosis factor α was secreted following HVJ-E treatment, and while IL-12 was secreted in large amounts in response to adenoviruses, the amount was very small for both HVJ-E and live HVJ virus (Figure 2c).

On the basis of these findings, it was assumed that only IL-6 was secreted in large amounts from DCs by the inactivated HVJ, and that HVJ-E suppressed the function of Tregs via the secretion of IL-6 from DCs, because IL-6 is known to suppress FoxP3 (a transcription factor required by Tregs) expression in DCs.[26] It was also examined whether such production of IL-6 by DCs was in fact happening *in vivo* after the administration of the HVJ-E vector. When CD11c-positive cells were collected from both tumor and

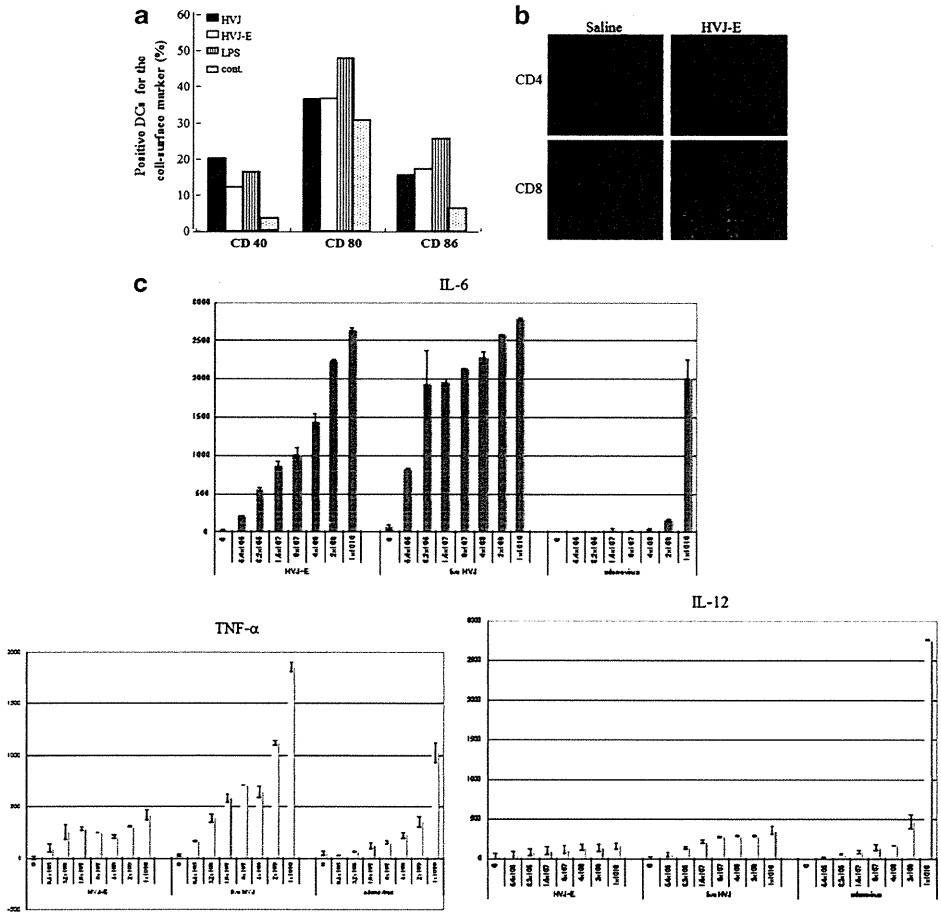

Figure 2. Immunoactivation in the tumor-bearing mice after the treatment with hemagglutinating virus of Japan envelope (HVJ-E). (**a**) The expression of the maturation markers of dendritic cells (DCs) was increased in the implanted tumors after the injection with HVJ. Lipopolysaccharide (LPS) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively. Solid, blank, longitudinal striped and dotted columns indicate the percentages of DCs positive for each maturation marker after the treatment with HVJ, HVJ-E, LPS and PBS, respectively. (**b**) Increased infiltration of CD4+ and CD8+ T cells into the tumors injected with HVJ-E solution. (**c**) Dose-dependent induction of interleukin-6 (IL-6), but not tumor necrosis factor α (TNF-α) or IL-12, secretion from splenocyte-derived dendritic cells following the treatment with HVJ-E. HVJ-E can lead DCs to secrete an equivalent amount of IL-6 as live HVJ.

regional lymph nodes 24 h after the administration of HVJ-E to immunostain the samples for IL-6, the DC-mediated production of IL-6 was found to be significantly increased in both types of CD11c-positive cells. Subsequently, CD4-positive and CD25-positive Tregs, as well as CD4-positive and CD25-negative effector T cells, were separated from the regional lymph nodes of a tumor-bearing mouse into which HVJ-E had been administered, in order to mix the effector T cells that were stimulated with the DCs collected from the spleen, with Tregs in various ratios, and the proliferation was examined in the mixed culture. The results showed that there was a decrease in the proliferation of effector cells associated with an increase in Tregs, and that this was suppressed by the administration of HVJ-E. Furthermore, this suppressive action was cancelled by adding anti-IL-6 antibodies to the mixed culture. On the basis of these results, it was assumed that HVJ-E functioned to avoid the suppression of the T-cell proliferation by Tregs due to the increased secretion of IL-6 from DCs, resulting in the strengthening and maintenance of the antitumor action of CD4- and CD8-positive T cells.

Clinical use of HVJ-E toward the treatment of melanoma

Pre-clinical studies. In the first reported pre-clinical study, mouse melanoma cell lines, B16/BL6, were subcutaneously transplanted onto the backs of mice of the same strain (B16/BL6), and after a total of three injections of HVJ-E beginning on the fourth day, the weight changes in the mice were examined to check for the growth of tumor/metastatic lesions and the systemic safety. With respect to the examination of the tumor size, the transplanted cell lines in the group administered phosphate-buffered saline (PBS) started to form palpable tumors from approximately the eighth day, thus increasing in a logarithmic manner. On the other hand, there were dose-dependent growth suppression effects for 100 mNAU (neuraminidase activity unit), 300 mNAU and 1000 mNAU in the group receiving HVJ-E treatments (Figure 3a). The suppression of tumor proliferation was almost the same for 1000 mNAU and 2000 mNAU, so we elected to administer 1000 mNAU as the maximum dose (Figure 3b). Furthermore, when the survival of each group was examined, it was found that while all the mice that received transplants in the control (no treatment) group had died by the 40th day, a significant improvement in the survival rate was shown in the group receiving HVJ-E, with survival even at the 40th day in the group receiving 1000 mNAU, with a dose-dependent effect again being observed (Figure 3c).

Although transitory hemolysis was expected in HVJ-E-treated animals because it can agglutinate red blood cells due to its membrane structure, no serious side effects were found even in the mice undergoing administration of the maximum dose of

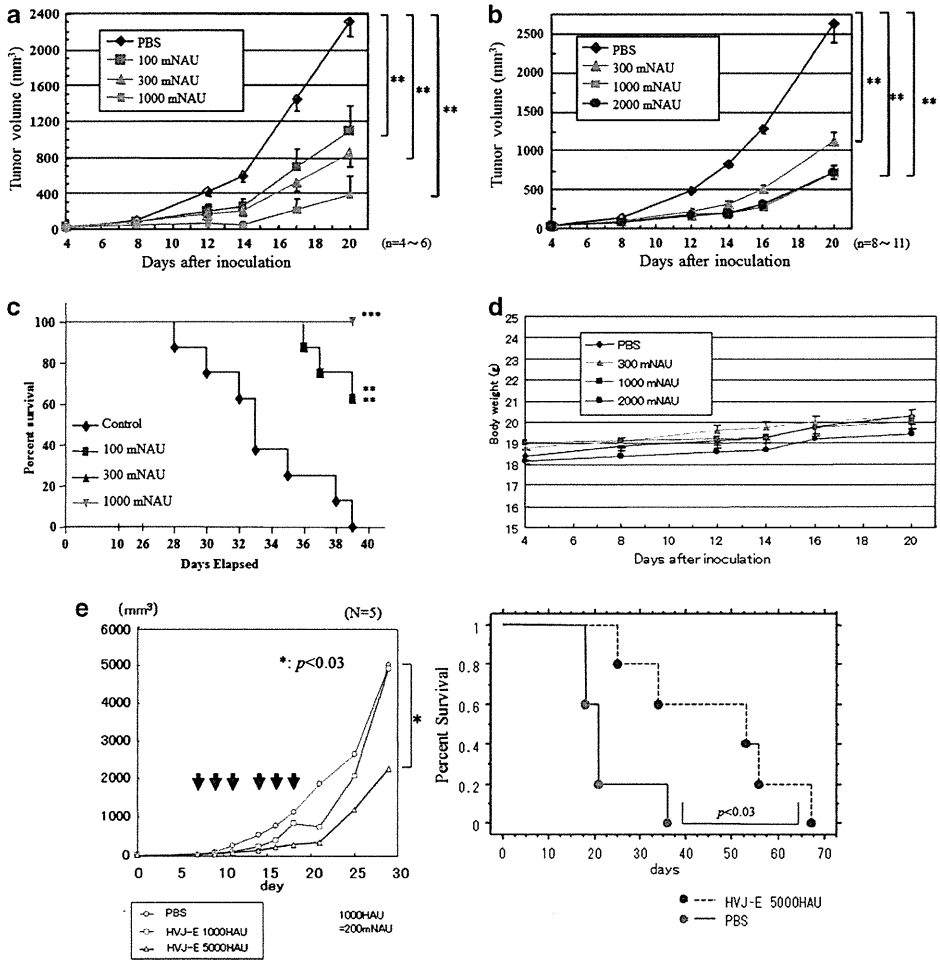

Figure 3. The antitumor effects of hemagglutinating virus of Japan envelope (HVJ-E) in implanted melanoma tissue. (**a**) The tumor growth was significantly decreased in a dose-dependent manner following the treatment with HVJ-E. (**b**) One thousand and 2000 mNAU of HVJ-E resulted in equivalent tumor growth suppression. (**c**) The survival rate was significantly improved in mice treated with HVJ-E compared with control. (**d**) No significant body weight loss was observed in the mice treated with HVJ-E. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively. (**e**) The significant tumor growth suppression (left side) and improved percent survival (right side) in mice bearing Mewo human melanoma cells.

2000 mNAU, and almost no changes in weight were found (Figure 3d). The B16/BL6 mouse melanoma cell line is known to induce spontaneous lung metastasis when intradermally transplanted into C57BL/6 mice.[27] Subsequently, the size of the lung metastasis in these individuals and the ratio of the mice in which metastasis appeared were examined on the 26th day after the tumor cell transplant. It was found that, while some form of metastasis was found in all 10 mice in the group treated with PBS, the rates and the sizes of metastatic lesions decreased in an HVJ-E dose-dependent manner. On the basis of this preliminary experiment, it was proven that HVJ-E also has a suppressive action on the proliferation of malignant melanoma, as well as a suppressive effect on natural distant metastasis *in vivo*. It has been considered that the human leukocyte antigen expression was reduced or absent in B16 melanoma cell lines,[28] and it is believed that natural killer (NK) cells may possibly be involved more than CTLs regarding this effect,[23] although the IL-18 produced by NK cells contradictorily participates in the PD-1-dependent tumor progression during cancer development.[29]

Following these findings, an experiment was implemented using human malignant melanoma (Mewo) cells. The Mewo cells were transplanted onto the backs of immunodeficient severe combined immunodeficiency mice, and intratumoral administration of HVJ-E was carried out every other day from the sixth day with 1000 Hemagglutination Unit (equivalent to 200 mNAU) or 5000 Hemagglutination Unit (1000 mNAU) for a total of six times, to examine the tumor growth and survival rates. It was observed that the HVJ-E administration at 5000 Hemagglutination Unit significantly suppressed the tumor growth and extended the survival of the mice (Figure 3e). On the basis of these observations, it was concluded that non-T cell immunity was involved, in addition to the cell-mediated immunity focusing on T cells, in the antitumor effects of inactivated HVJ-E against malignant melanomas.

To investigate one of the potential mechanisms of action, we conducted a microarray analysis on Renca (mouse renal cancer) tumors that had been removed from severe combined immunodeficiency mice treated with HVJ-E, and found that CXCL10 was activated, together with type I IFN, showing that this chemokine was largely secreted from $CD11c^+$ DCs. Furthermore, it was confirmed through our experiments that CXCR3 was expressed on $DX5^+$ NK cells. Detailed examinations are underway regarding whether the antitumor effect, in which NK cells played the major role, can also be found against malignant melanoma, as expected.[23]

Following these promising preclinical studies of the antitumor effects of HVJ-E *in vitro* and *in vivo*, we have been engaged in the production of GMP grade HVJ-E with the intention of using it for

Table 1. The inclusion and exclusion criteria of our phase I/II trial using the HVJ-E preparation

Inclusion criteria	
(1)	The patient has provided a written informed consent before any study-related procedure
(2)	The patient is at least over 20 years and ⩽90 years old
(3)	The patient has a diagnosis of malignant tumor as confirmed by histopathology or cytology
(4)	The patient has a diagnosis of malignant melanoma progressive melanoma in American Joint Committee on Cancer staging stage IIIC or stage IV
(5)	The patient has one or more administrable lesion of HVJ-E solution on the skin, subcutis or lymph node ($<25\,cm^2$ in size measured by forceps calipers, CT or MRI review)
(6)	The patient has a life expectancy for at least 12 weeks or more.
(7)	The patient meets an Eastern Cooperative Oncology Group Performance Status Scale of 0 or 1
(8)	The patient has measurable lesions (calipers, CT or MRI review)
Exclusion criteria	
(1)	The patient has multiple brain metastases
(2)	The patient shows positive immune response by HVJ-E prick test at screening
(3)	The patient has an uncontrolled serious complication such as active infection
(4)	History of active autoimmune disease
(5)	The patient is a pregnant or a lactating female
(6)	The patient has a history of a transplantation of the allogeneic organ, the autologous organ or tissue
(7)	The patient is inappropriate to be enrolled in this study judged by the doctors in charge

Abbreviations: CT, computed tomography; HVJ-E, hemagglutinating virus of Japan envelope; MRI, magnetic resonance imaging.

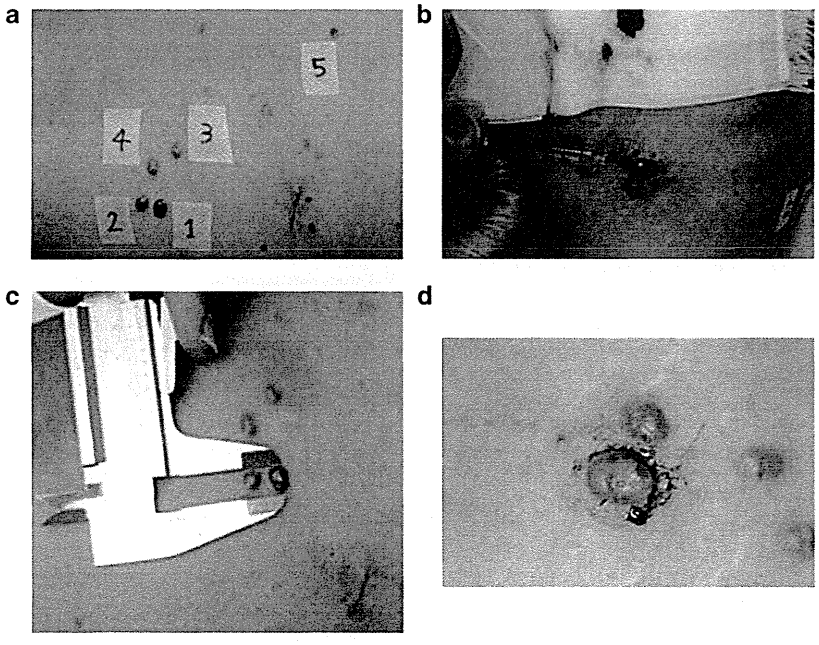

Figure 4. The decisions regarding target lesions and the intratumoral administration of the hemagglutinating virus of Japan envelope (HVJ-E) preparation into target #1.

clinical applications in humans. We established a purification method in 2008 using the bio-reactor production method and three-step column purification, together with the manufacturing of freeze-dried products. Furthermore, it has been confirmed in toxicity tests in mice, rats and monkeys that no serious organ injuries should occur even if the agent is administered in an amount ~18 times 10 000 mNAU (167 mNAU/kg) per dose, when the human body weight is assumed to be 60 kg.

Antibodies to HVJ-E are expected to appear and are expected to increase with the subsequent doses of HVJ-E based on the pre-clinical experiments in mice. However, the induced antibodies in mice did not inversely influence the antitumor immunity and tumor cell apoptosis *in vivo* (data not shown). This may be because HVJ-E is able to fuse to adjacent tumor cells and DCs within 3–5 s, and the subsequent antitumor immunity is dependent on HVJ-E-fused DCs and immunocompetent cells, rather than on HVJ-E itself.[30] In addition, the HVJ envelope vector appears to be much less immunogenic than naïve HVJ, which strongly induces CTLs against virus-infected cells.[30] It is considered to be a major advantage that the antibodies to HVJ-E did not work as neutralizing antibodies, in contrast to most antibody drugs targeting specific molecules.

On the basis of these pre-clinical experiments and results, we prepared a protocol for a clinical study to test the safety and the tolerability of HVJ-E in stage IIIC or IV melanoma patients diagnosed according to the American Joint Committee on Cancer staging. After the protocol was approved by the Medical Ethics Committee at Osaka University in January 2009, we started the clinical study.

Purpose of this clinical study. This clinical study is a phase I/IIa clinical study, the main purpose of which is to confirm the safety

and the tolerability of HVJ-E preparations, and the secondary purpose of which is to measure the antitumor effects, in compliance with the inducibility of tumor immunity/RECIST version 1.1.[31]

Inclusion of subject cases. The detailed information about the study subjects is shown in Table 1. The target patients are those with progressive malignant melanomas, in stage IIIC or stage IV according to the American Joint Committee on Cancer classification, in whom no relapsing/resistance against standard therapy/standard therapy has been noted, or those who refused standard therapy. The subjects were restricted to those cases between the ages of 20 and 90, who were able to provide informed consent describing their intention to participate in the clinical study. Furthermore, to implement the intratumoral administration of HVJ-E, patients must have skin, subcutaneous or lymph-node lesions with more than one administrable location of $<25\,cm^2$ (measured by vernier calipers, computed tomography, or magnetic resonance imaging) as an essential condition. The details of the selection/exclusion criteria are shown in Table 1. With respect to the administration schedule, based on the preclinical safety and efficacy tests, one course is set to have a 4-week rest period after intratumoral administration of the HVJ-E preparation three times a week for 2 weeks, for a total of six injections (Figures 4a–d), and the above purposes will be assessed when two courses are completed. Patients shall be, in principle, hospitalized for 2 weeks while under administration and for the subsequent 2 weeks, to fully observe the general condition of the patients. Decisions regarding the applicability of study entries, final decisions on the effects, or decisions on cancellations due to adverse events, and so on shall be made by the members of the Effect and Safety Assessment Committee which is set outside the hospital.

Study duration and expected number of cases. The duration of the planned study is 30 months after receiving approval in July 2009, and the duration of entry will be 24 months. The expected number of cases is six cases: three cases for the lower dose of HVJ-E and three cases for higher dose of HVJ-E, with a maximum of 12 cases, using a standard design for a gradual increase in the cohort dosage.

General description of the HVJ-E preparation and schedule of the clinical study. Regarding the administration of the HVJ-E preparation, first, 1 ml of distilled water for injection will be added into a vial containing freeze-dried HVJ-E, the raw material, to make a liquid solution (suspension). Then, the suspension will be filtered with a 0.45-micrometer pore size filter. The HVJ-E preparation produced in the hospital in this manner is a white liquid solution (suspension) containing HVJ-E from 8000 mNAU to 10 000 mNAU per 1 ml. In the event that a lower dose is administered, HVJ-E diluted by 3.3-fold will be prepared with saline and used. The HVJ-E preparation will be intratumorally administered using an injectable syringe with an attached 27 Ga needle. With respect to the dosage, the lower dose group will receive 3000 mNAU and the higher dose group will receive 10 000 mNAU.

CONCLUSIONS AND FUTURE PERSPECTIVES

We have just started the world's first clinical study of the administration of inactivated HVJ virus into humans. From this standpoint, it was first planned as an experimental study to fully examine the safety and the tolerability of the HVJ-E preparation; subsequently, the protocol has been revised to assess the comprehensive antitumor immunity as a phase II trial that is expected to include patients with malignant melanomas, through collaboration with other laboratories. On the basis of the results of the initial clinical study introduced here, we are considering developing another clinical study later, in which specific molecules, such as IL-12, that are strong drivers toward the Th1 axis, will be enclosed in HVJ-E. On the basis of the results of this phase I trial, a sequential phase II trial is planned to evaluate the biphasic effect of HVJ-E in terms of the direct and indirect killing of tumors more extensively. On the other hand, recent advances in discoveries of the signaling cascades contributing to tumor development and paradoxical immunosuppression in melanoma have led to the development of immunomodulatory antibody drugs targeting CTLA-4 and PD-1 in T cells.[15,16] The protocol of the phase II study would be composed of a shortened interval of HVJ-E cessation and an increase in the dose to enhance its antitumor action. If the phase II trial is successful, we will then consider the complementary immune-augmentation effects that might be obtained with the combined use of HVJ-E and the new immunomodulatory drugs. Therefore, when the phase I and II clinical trials using HVJ-E alone are finished in the near future, the combination can be administered as part of a phase III trial which includes >100 melanoma patients enrolled worldwide. Further clinical trials for patients with other malignancies, such as prostate cancer and glioblastoma, which showed high sensitivity to HVJ-E, can also be planned. These extended clinical uses are expected to provide a breakthrough for novel antitumor immunotherapy.

ABBREVIATIONS

CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T-lymphocyte antigen 4; HVJ-E, hemagglutinating virus of Japan envelope; PD-1, programmed cell death-1; Treg, regulatory T cell

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Young Scientists (B) (23791268) from the Japanese Ministry of Education, Science, Sports, and Culture and a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 764706, 9 pages
http://dx.doi.org/10.1155/2013/764706

Review Article

Virosome Presents Multimodel Cancer Therapy without Viral Replication

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Received 8 October 2013; Accepted 31 October 2013

Academic Editor: Ryuichi Morishita

A virosome is an artificial envelope that includes viral surface proteins and lacks the ability to produce progeny virus. Virosomes are able to introduce an encapsulated macromolecule into the cytoplasm of cells using their viral envelope fusion ability. Moreover, virus-derived factors have an adjuvant effect for immune stimulation. Therefore, many virosomes have been utilized as drug delivery vectors and adjuvants for cancer therapy. This paper introduces the application of virosomes for cancer treatment. In Particular, we focus on virosomes derived from the influenza and Sendai viruses which have been widely used for cancer therapy. Influenza virosomes have been mainly applied as drug delivery vectors and adjuvants. By contrast, the Sendai virosomes have been mainly applied as anticancer immune activators and apoptosis inducers.

1. Introduction

Currently, general cancer therapies include surgery, chemotherapy, and radiation therapy, but all three have limitations. Applications of surgical and radiation therapy are limited to localized cancer. Chemotherapy is used for a wide range of cancers, including distant metastases, via the systemic administration of anti-cancer drugs; however, it also kills normal cells and induces severe side effects. Therefore, many groups are investigating ways to improve conventional treatments and to develop novel treatments for more effective cancer elimination with fewer side effects.

In recent years, much attention has been paid to cancer immunotherapy, which stimulates anti-cancer immunity, and several cancer immunotherapy systems (Provenge, Ipilimumab and anti-PD1 antibody) have been developed [1–5]. When anti-cancer immunity is systemically activated, it is expected that the primary cancer cells and distant metastases will be eliminated by immune cells. Various tumor-associated antigens (TAAs) have been identified [6–9], for example, HER2/nu, CEA, MAGE, and WT1. TAAs are expressed in cancer cells and are targeted by immune cells, especially cytotoxic T lymphocytes (CTL) [10–13]. Therefore, immunostimulation by TAAs can be applied to cancer immunotherapy. To activate anti-cancer immunity by TAAs, fragments of TAAs should be presented on antigen-presenting cells (APCs) by forming a complex with major histocompatibility complex class I (MHC-I) and II molecules [14]. Generally, cytoplasmic foreign proteins, such as viral proteins expressed in the cytoplasm during viral infection, complex with MHC-I and stimulate $CD8^+$ T cells (CTLs) [14, 15]. However, endocytosed foreign proteins also complex with MHC-II and stimulate $CD4^+$ T cells [14, 16]. Moreover, APCs have a cross-presentation system that presents endocytosed foreign proteins with MHC-I to activate CTLs [17]. Previous reports have shown that the administration of TAA alone does not induce an effective CTL response [18]. Therefore, it is believed that an endocytosed antigen is not sufficient for the activation of MHC-I-restricted CTLs, and, to activate an effective CTL response by TAAs, they should be introduced to the cytoplasm directly.

A new technology, gene therapy, has been developed and applied to cancer treatment. Various cancer gene therapy methods have been reported, such as adoptive immunotherapy using *ex vivo* gene transfer to immune cells [19], intratumoral injection of cytokine genes [20], suicide gene therapy using the herpes virus thymidine kinase gene [21], and intratumoral injection of the p53 gene [22]. To achieve