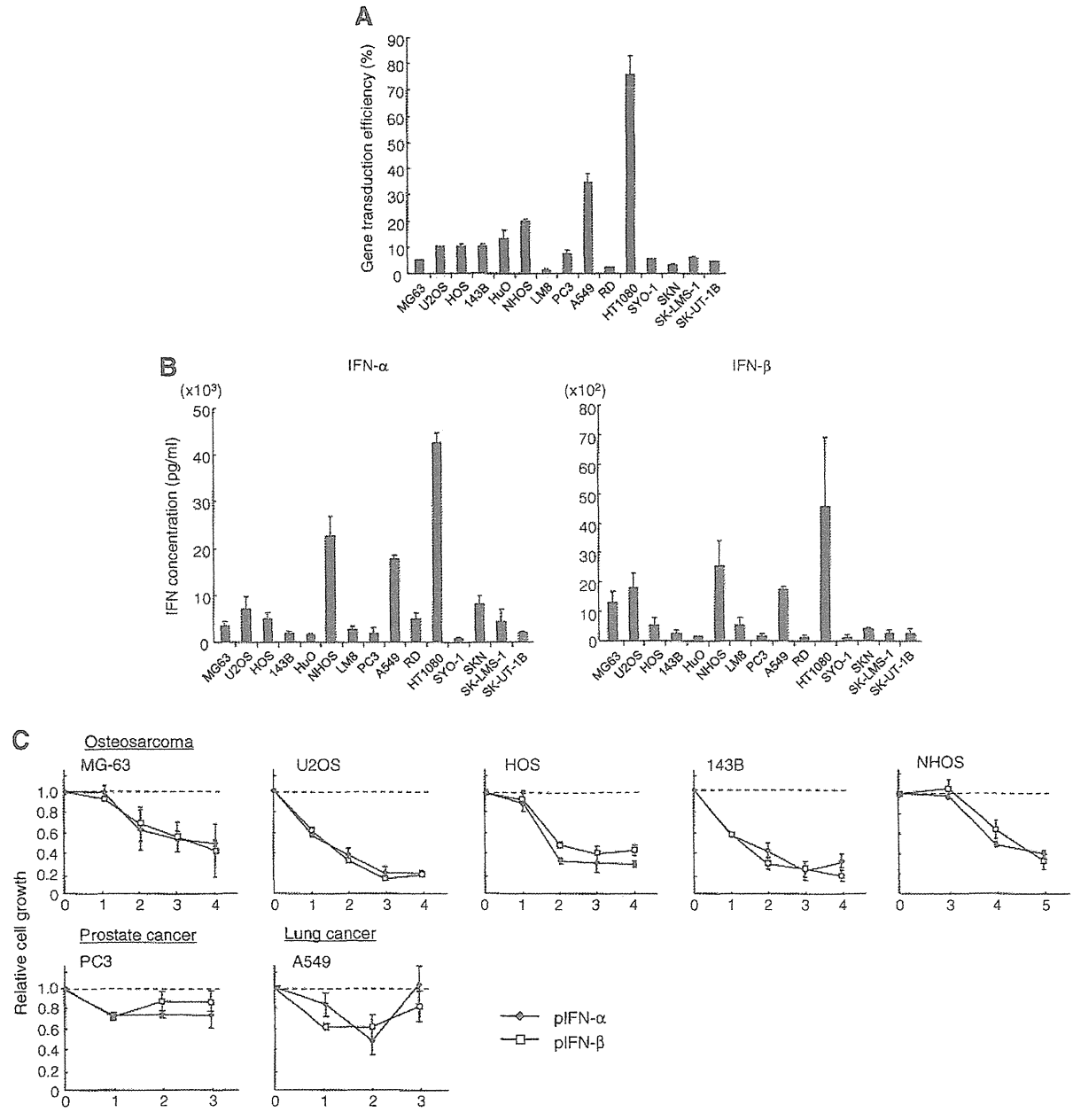

FIG. 1. Cytotoxic effect of type I IFN gene transfer in various sarcoma cell lines. (A) Gene-transduction efficiency in tumor cell lines. The cells were transfected with pEGFP (2 μg/well) in six-well plates, and 48 hr later, the percentage of $EGFP^+$ cells was determined by flow cytometry. (B) IFN concentration in the supernatant of IFN-transduced cells. The cells were transfected with phIFN-α or phIFN-β (pmIFN-α or pmIFN-β for NHOS and LM8) (2 μg/well) in six-well plates, and 48 hr later, the IFN-α and IFN-β concentrations in the culture medium were measured by ELISA (PBL Interferon Source, Piscataway, NJ). (C) Growth suppression of IFN-transduced osteosarcoma cells. The cells were transfected with phIFN-α, phIFN-β (pmIFN-α or pmIFN-β for NHOS), or pEGFP, and cell growth was determined by cell-proliferation assay. (D) Growth suppression of IFN-transduced soft-tissue sarcoma cells. The cells were transfected with phIFN-α, phIFN-β, or pEGFP, and cell growth was determined by cell-proliferation assay. (E) Apoptotic cell death in pIFN-transfected osteosarcoma cells. Cells were transfected with phIFN-α, phIFN-β (pmIFN-α or pmIFN-β for NHOS and LM8), or pLuc, and apoptotic cells were analyzed by Annexin V assay 3 days after transfection. The statistical difference between IFN-α-transduced cells and IFN-β-transduced cells is presented. n.s., not significant. (F) Apoptotic cell death in phIFN-transfected soft-tissue sarcoma cells. Cells were transfected with phIFN-α, phIFN-β, or pLuc, and apoptotic cells were analyzed by Annexin V assay 3 days after transfection. The statistical difference between IFN-α-transduced cells and IFN-β-transduced cells is presented. (G) Proportion of cells in each cell-cycle phase. Cells were transfected with phIFN-α, phIFN-β, or pLuc, and DNA contents were analyzed by staining with PI 3 days after transfection.

(*Figure Continued* →)

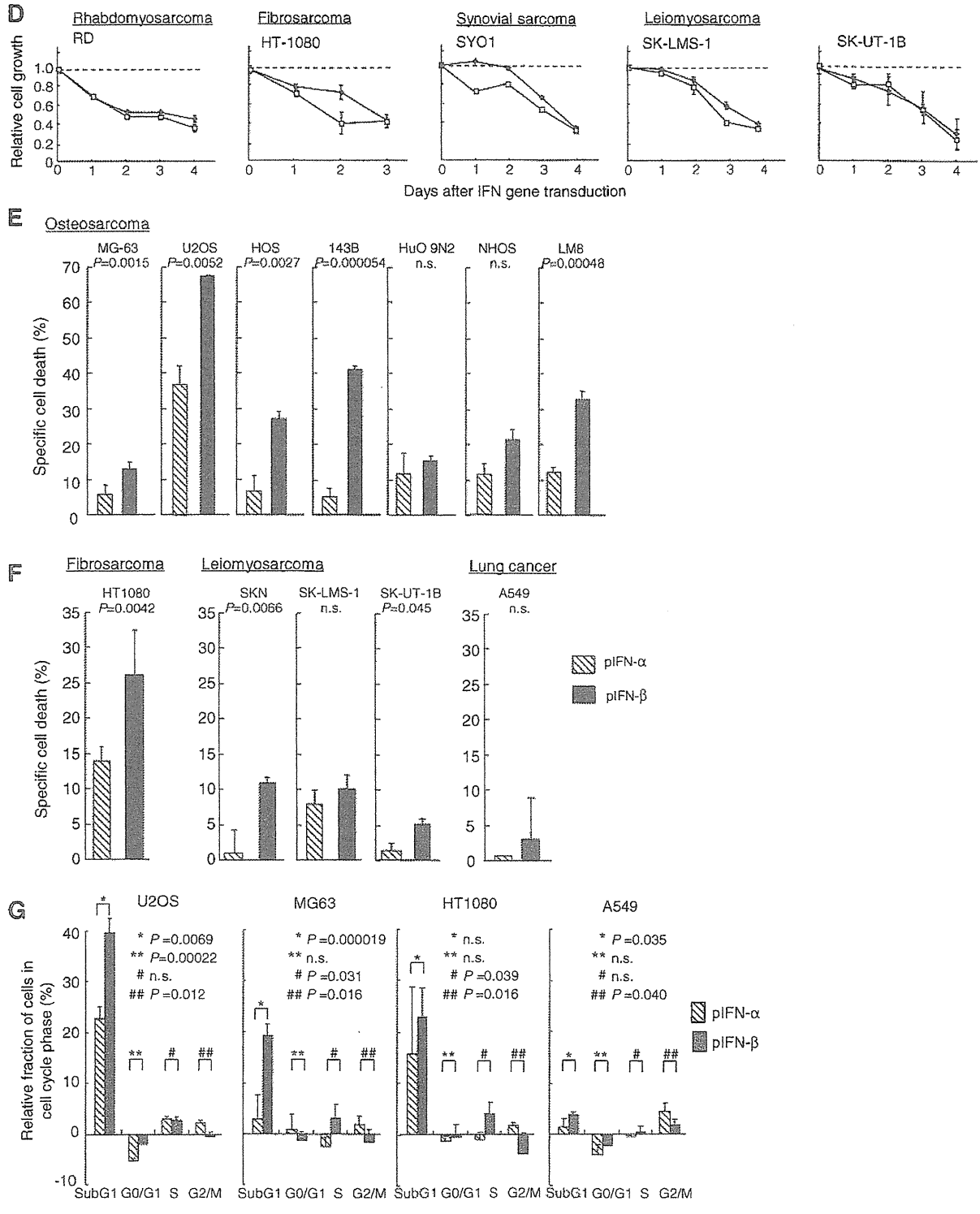

FIG. 1. (*Continued*).

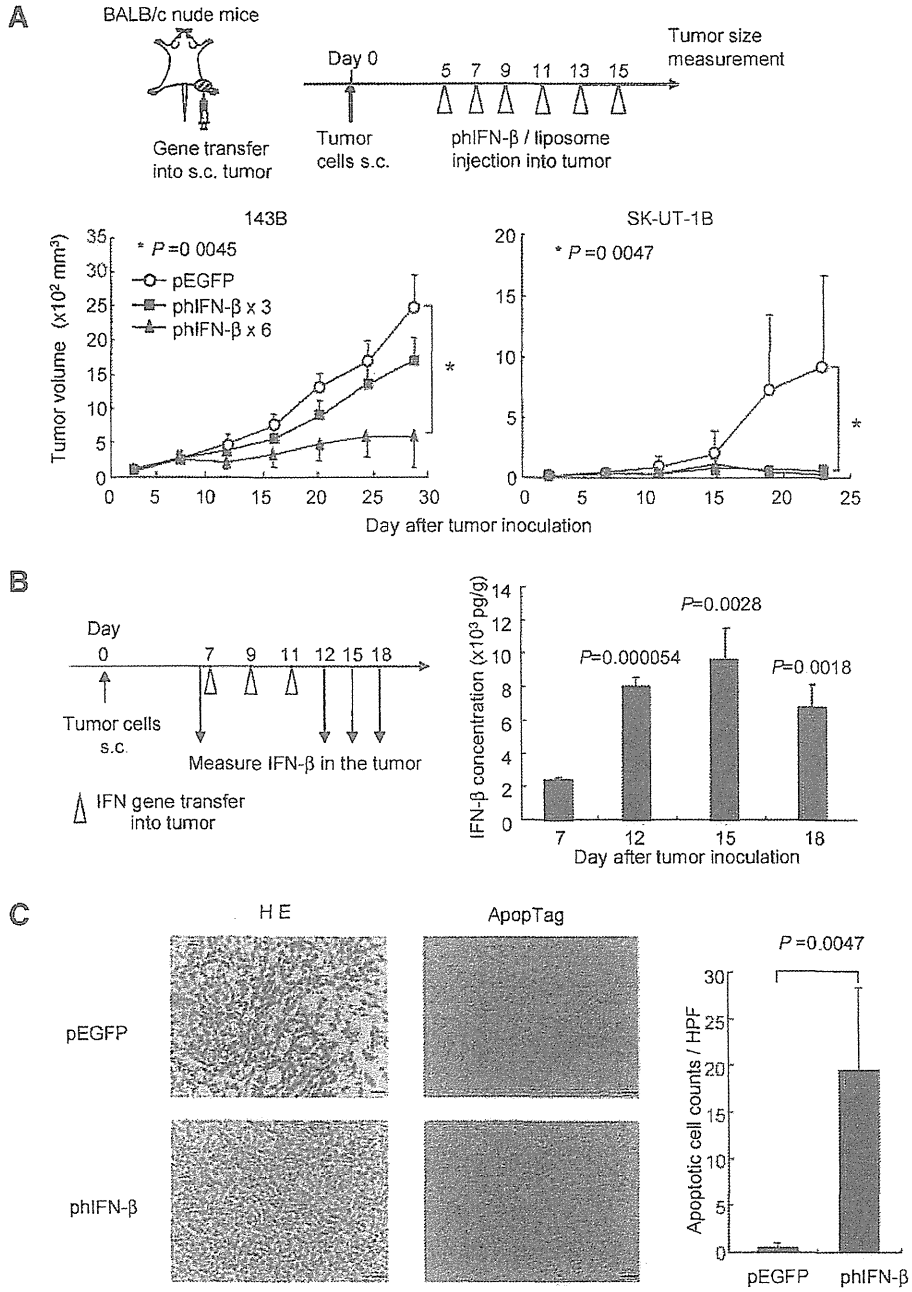

FIG. 2. Growth suppression of human sarcoma xenografts by intratumoral IFN-β gene transfer. **(A)** Direct antitumor effect of human IFN-β gene transfer into 143B or SK-UT-1B subcutaneous tumors. The tumor cells were injected subcutaneously on the right legs of BALB/c nude mice, and 5 days later, phIFN-β-liposome complex was injected three or six times. **(B)** Time course of IFN-β expression in the tumors. The IFN-β levels were measured in the 143B subcutaneous tumors during 7 days after intratumoral injection of phIFN-β (30 μg)–liposome complex at days 12, 15, and 18 ($n=4$). The statistical difference between IFN-β concentration on the indicated days and at day 7 is presented. **(C)** TUNEL staining of treated 143B tumors. Positive cells were counted in 10 representative high-power-view fields (HPF, ×200) under microscope. Scale bars = 50 μm. **(D)** Suppression of contralateral subcutaneous tumors. The 143B or SK-UT-1B cells were injected subcutaneously on both legs of BALB/c nude mice, and 5 days later, pmIFN-β-liposome complex was injected into the right tumors three times. For NK cell-depletion experiments, mice were treated with intraperitoneal injection of anti-asialo GM1 antibody every 6 days (pmIFN-β×3+ab). The percentage of NK cells ($CD49b^+$ cells) was examined by flow cytometry 4 days after the single intraperitoneal injection of anti-asialo GM1 antibody.

(*Figure Continued*→)

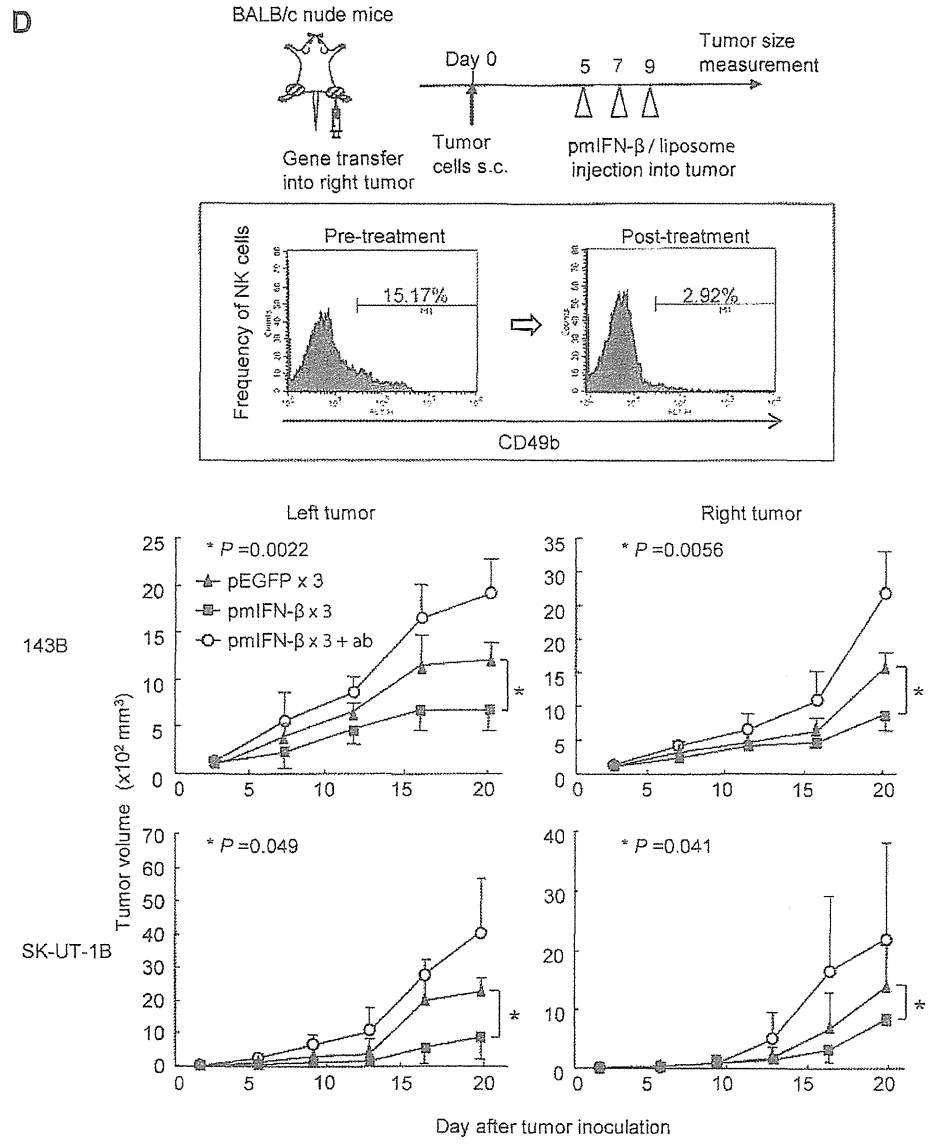

FIG. 2. (*Continued*).

antibody to purge the NK cells. Flow cytometry showed that the treatment with antibody depleted approximately 80% of NK ($CD49b^+$) cells in the spleen (Fig. 2D). Depletion of NK cells canceled the antitumor effect of IFN-β gene transfer and rather increased tumor volume as compared with the control pEGFP-injected group (Fig. 2D); this suggests that NK cells in nude mice have a significant activity to suppress the tumor growth of sarcoma cells and that an intratumoral IFN gene transfer enhances the suppressive effect of NK cells.

Antitumor effect of syngeneic HSCT and intratumoral type I IFN gene transfer against osteosarcoma

To examine whether the antitumor immunity induced by intratumoral type I IFN gene transfer could be enhanced by HP of T cells in lymphopenic hosts, BALB/c mice were injected subcutaneously with NHOS osteosarcoma cells shortly after lethal irradiation, and syngeneic BM and T cells were infused into the mice. Tumor growth was significantly suppressed in the syngeneic HSCT recipients as previously reported (Kobayashi *et al.*, 2007). Furthermore, a combination of intratumoral IFN-α or IFN-β gene transfer and syngeneic HSCT enhanced the antitumor effect against osteosarcoma (Fig. 3A). The activity to induce the antitumor immunity by IFN-α gene transfer may be compatible with that by IFN-β for NHOS tumors. All treated mice appeared healthy during the course of the experiments, and the blood chemistry showed no abnormal values in the treated mice 5 weeks after the HSCT (data not shown).

To examine the immune reaction to intratumoral IFN-β gene transfer in syngeneic HSCT recipients, splenocytes

were extracted from the treated mice and cultured with NHOS cells. An ELISpot assay showed that the average number of IFN-γ-producing splenocytes in response to NHOS cells was slightly increased in the syngeneic HSCT alone group, whereas a combination of intratumoral IFN-β gene transfer and syngeneic HSCT further increased the IFN-γ^+ spots. The spot number in response to control syngeneic splenocytes was not changed in all groups of treated mice (Fig. 3B). An *in vitro* cytotoxic assay also showed that the splenocytes derived from the syngeneic HSCT mice recognized and lysed NHOS cells, and that the IFN-β gene transfer enhanced the cytolysis to NHOS cells (Fig. 3C).

Inhibition of distant metastases by a combination of IFN-β gene transfer and syngeneic HSCT

Lung and liver metastases are among the most frequent causes of mortality in patients with malignant bone and soft-tissue sarcoma. LM8 is a highly metastatic osteosarcoma cell line derived from C3H mouse. Many macroscopic metastatic foci were observed in the liver and lung 20 days after the subcutaneous inoculation of LM8 cells in C3H mice. To evaluate the therapeutic efficacy of IFN-β gene transfer against distant metastases in syngeneic HSCT recipients, LM8-Luc cells, which express the firefly luciferase gene, were inoculated on the right legs of C3H mice after syngeneic HSCT, and 7 days later, a pmIFN-β-liposome complex was injected into the subcutaneous tumor on the leg three times. Intratumoral IFN-β gene transfer alone or syngeneic HSCT alone significantly suppressed tumor growth of subcutaneous tumors on the legs; however, the antitumor effect of an IFN-β gene transfer was clearly enhanced in syngeneic HSCT recipients (Fig. 4A), which was similar to the antitumor effect against NHOS tumors (Fig. 3A).

To examine the suppressive effect for the formation of metastatic tumors, distant metastases in the lung and liver were evaluated by photon counts on the IVIS imaging system in the treated mice (Fig. 4B). Thirty-three days after tumor inoculation, all of the four untreated control mice showed many photon$^+$ spots (one of the four died of cancer progression), whereas three (75%) of the four mice treated with a combination therapy showed no detectable metastatic spots in the lung and liver.

The infiltration of CD4$^+$ and CD8$^+$ T cells was analyzed in the lung tumors of treated mice at day 33 after tumor inoculation. Immunohistochemical staining showed that CD4$^+$ and CD8$^+$ T cells infiltrated into the lung metastases in the group of intratumoral IFN-β gene transfer alone and that of syngeneic HSCT alone, whereas a combination therapy markedly increased the number of these cells in the lung tumors (Fig. 4C). These results indicated that a combination therapy is effective for preventing distant metastases in the liver and lung.

Discussion

In the first part of this study, we showed that type I IFN gene transduction suppressed the cell growth of various sarcoma cells, and that the IFN-β gene was more effective in inducing cell death than was the IFN-α gene. *In vivo* delivery of the IFN-β gene significantly suppressed the growth of osteosarcoma tumors. Then we demonstrated that the integration of two complementary immune therapies, intratumoral IFN gene transfer (which induces tumor-specific immunity) and HSCT (which reconstructs a fresh immune system), significantly enhances the antitumor immunity against osteosarcoma. An autologous HSCT has obvious advantages over allogeneic HSCT, such as a lack of graft-versus-host disease and an independence of donor availability.

Although the underlying mechanisms for the effective induction of cell death by IFN gene transfer in sarcoma cell lines are not clear, it has been reported that the addition of type I IFN protein showed a substantial inhibitory effect on the *in vitro* growth of osteosarcoma cells, as well as various soft-tissue sarcoma cells, and that the administration of IFN protein was also effective in arresting the growth of four different human osteosarcoma xenografts in nude mice (Thulasi *et al.*, 1996; Brodowicz *et al.*, 1999; Strander, 2007; Whelan *et al.*, 2010), suggesting that a sensitivity to type I IFN protein is one of the characteristics of sarcoma cells. Furthermore, Yoshida and co-workers reported that IFN-β gene transfer could induce apoptosis in IFN protein-resistant tumor cells, such as glioma, melanoma, and renal cell carcinoma (Yoshida *et al.*, 2004), demonstrating that an IFN gene transfer has an obvious advantage over IFN protein treatment in the induction of cell death at least in certain types of tumor cells. Sarcoma could be a promising target of IFN gene therapy.

In addition to cell-death induction, IFN gene transfer has an ability to mount an antitumor immunity against osteosarcoma, and this antitumor efficacy was significantly enhanced in syngeneic HSCT recipients (Figs. 3A and 4A). A putative mechanism of inducing an antitumor immunity by a combination therapy is as follows: in the "homeostatic proliferation" condition after syngeneic HSCT, T cells effectively recognize the low-affinity self antigen, including tumor-associated antigens (TAAs), leading to an induction of antitumor immunity. IFN-α/β expression in the tumors causes tumor lysis and exposes an increased quantity of TAAs to DCs. IFNs promote maturation and enhance the antigen-presenting capacity of DCs (Hara *et al.*, 2009), leading to the facilitation of TAA recognition by donor lymphocytes primed during homeostatic proliferation. Furthermore, it was reported that type I IFNs augment the cytotoxicity of T cells and NK cells and enhance *in vivo* proliferation, expansion, and long-term survival of CD8$^+$ T cells in response to specific antigens (Ferrantini *et al.*, 2007; Santini *et al.*, 2009). Last but not least, conditioning of HSCT (by irradiation in our model) destroys the immunotolerance microenvironment developed by sarcoma, contributing to an effective antitumor immunity (our unpublished data). These multiple and supposedly synergistic mechanisms may lead to the development of a strong antitumor effect against sarcoma. The combination of immunostimulatory effects by IFN and the reconstitution of a fresh immune system following HSCT could create an environment mutually supporting the activation of an antitumor response.

The antitumor immunity of gene- and cell-based immunotherapy enhanced by syngeneic HSCT has been shown in

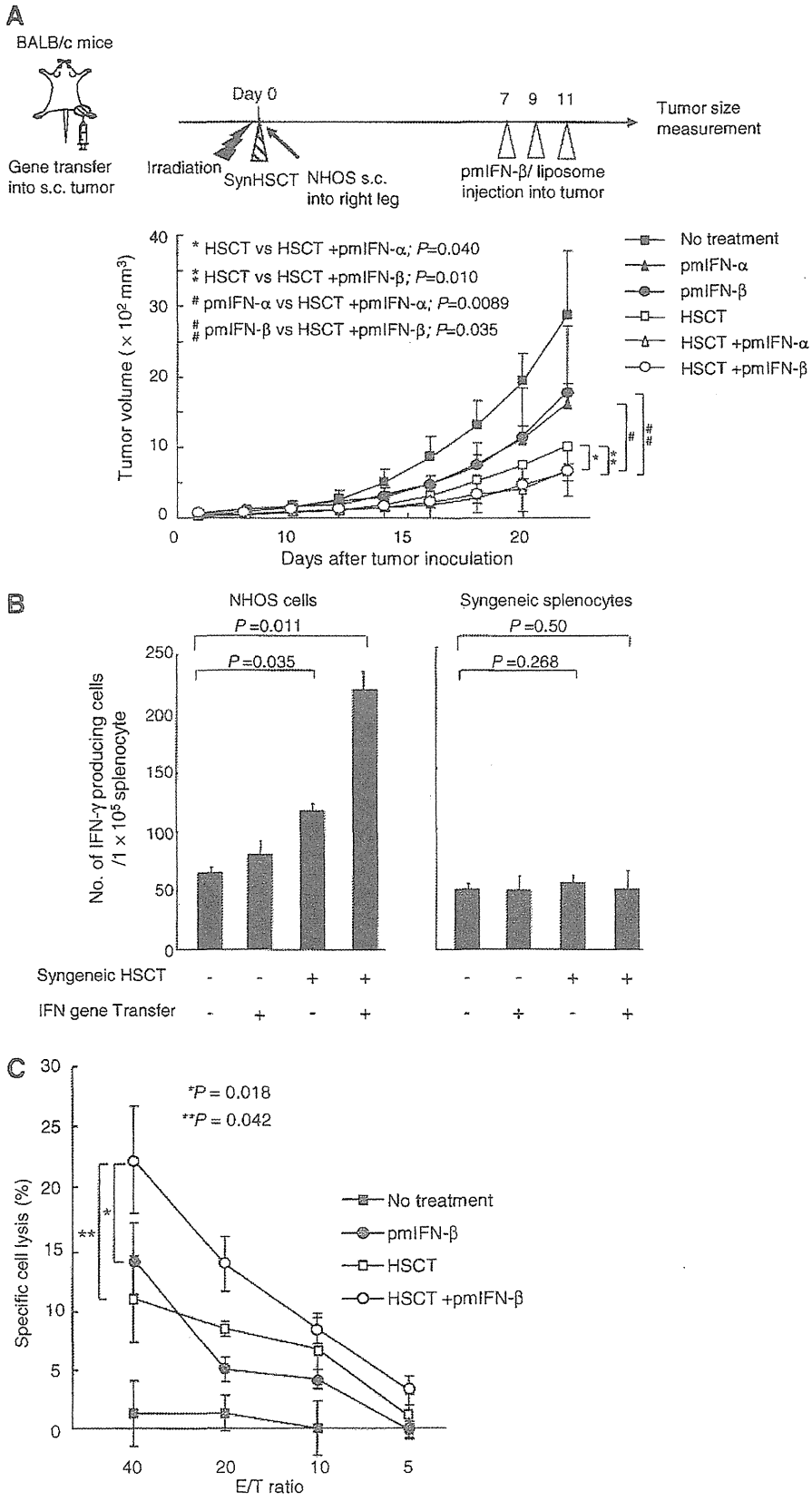

FIG. 3. Type I IFN gene transfer induces a significant antitumor effect in syngeneic HSCT recipients. (A) A combination of syngeneic HSCT and intratumoral type I IFN gene transfer caused marked tumor-growth suppression. The pmIFN-α- or pmIFN-β-liposome complex was injected into the NHOS tumors 7, 9, and 11 days after the tumor inoculation in the syngeneic HSCT mice ($n=6$). The experiments were repeated two times. (B) ELISpot assay of IFN-γ-producing cells in response to stimulation of NHOS cells. Two weeks after IFN-β gene transfer, mouse splenocytes were isolated from treated mice and cocultured with NHOS cells or control syngeneic lymphocytes ($n=3$). The experiments were repeated three times. (C) *In vitro* cytotoxic assay of splenocytes. Splenocytes were isolated from the treated mice, and their cytotoxicity was evaluated in a standard 4-hr ^{51}Cr release assay against NHOS cells ($n=3$).

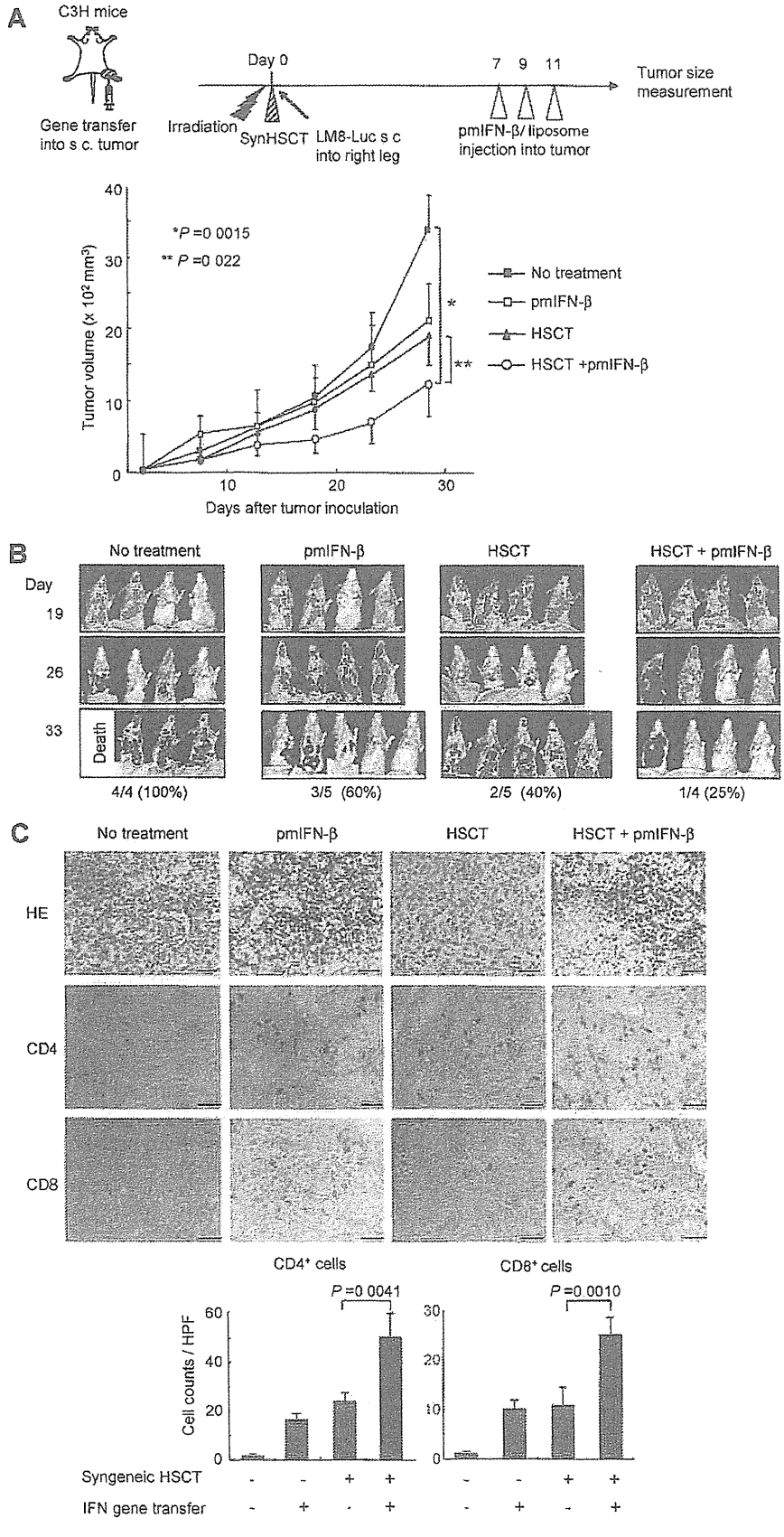

FIG. 4. Suppression of tumors at a distant site by IFN-β gene transfer during immune reconstitution. (A) Intratumoral IFN-β gene transfer into LM8-Luc tumors. LM8-Luc cells were inoculated on the right leg in syngeneic HSCT mice. The pmIFN-β-liposome complex was injected into LM8-Luc subcutaneous tumors at days 7, 9, and 11 ($n=7$). The experiments were repeated two times. (B) Suppression of liver and lung metastasis. After the mouse IFN-β gene transfer, the size of subcutaneous tumors on the legs was measured macroscopically, and the photon count of lung and abdominal tumors was evaluated by the IVIS imaging system. (C) Immunostaining of treated tumors. The lungs were resected at day 33 and stained with CD4 and CD8 antibodies. Positive cells were counted in 10 representative high-power-view fields (HPF, $\times 400$) under microscope. Scale bars = 50 μm.

a variety of animal models and clinical studies. An adoptive transfer of tumor-specific T cells showed a strong antitumor effect in syngeneic HSCT mice (Wrzesinski and Restifo, 2005). In clinical trials, host lymphodepletion followed by autologous tumor-infiltrating lymphocyte transfer and IL-2 administration results in objective response rates of 50–70% in patients with metastatic melanoma refractory to standard therapies (Dudley *et al.*, 2008). As tumor-reactive T cells are mostly polyclonal, and heterogeneous expressions of various TAAs coexist in a tumor mass, the *in vivo* stimulation of multiple tumor-reactive lymphocytes might be critical in the clinical application. Therefore, several laboratories reported that a vaccination with tumor cells expressing immunostimulatory molecules and an immunization with DCs pulsed with whole-tumor cell lysates led to an efficient antitumor response early after syngeneic HSCT (Borrello *et al.*, 2000; Asavaroengchai *et al.*, 2002; Jing *et al.*, 2007; Filatenkov *et al.*, 2009).

Compared with the previous approaches, one of the advantages of the *in vivo* IFN gene transfer is that it does not involve a manipulation and culture of the immune and tumor cells *ex vivo*, making this strategy more feasible for patients with bone and soft-tissue sarcomas. We previously reported that an allogeneic MHC gene transfer could also enhance an effective antitumor immunity in syngeneic HSCT recipients (Kobayashi *et al.*, 2007). The major difference between the allogeneic MHC and IFN gene therapies is in their local effects on tumor sites transduced with the therapeutic genes: IFN gene transfer directly induces cell death, which may enable local control and increased release of TAAs.

The current and previous studies address important points to consider in the clinical feasibility of the combination therapy. First, a liposome-mediated IFN gene transfer effectively suppressed tumor growth in syngeneic HSCT recipients, suggesting that a high concentration of IFN in the tumors is not necessary to induce an effective antitumor immunity. This facilitates a clinical application, because *in vivo* lipofection of an IFN-expressing plasmid is much safer than virus vectors (Rodriguez, 2004). Second, the intratumoral route of the vector administration has a better safety feature compared with an intravenous route, which may distribute and express the IFN gene in various organs. In addition to the lower toxicity, we reported that an intratumoral route of IFN gene transfer is superior to an intravenous administration due to the effective induction of antitumor immunity through the exposure of TAAs to DCs and the maturation of DCs in regional lymph nodes by the intratumoral IFN expression (Narumi *et al.*, 2010). Third, a combination therapy was effective in suppressing not only the vector-injected tumors, but also the vector-uninjected distant tumors in a murine spontaneous metastasis model, simulating a clinical setting. On the other hand, remaining questions include further understanding of the kinetics of homeostatic proliferation, which is the basis for the combination therapy of the autologous HSCT and IFN gene transfer; it may be necessary to establish a method to monitor HP and to maximize the therapeutic effect in individual patients.

In conclusion, a combination of intratumoral IFN-β gene transfer with syngeneic HSCT is a promising new approach for sarcomas, due to the activation of tumor-specific immunity and excellent safety features. This therapeutic strategy deserves an evaluation in future clinical trials for patients with sarcomas.

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Author Disclosure Statement

No competing financial interests exist.

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ORIGINAL ARTICLE

In vivo delivery of *interferon-α* gene enhances tumor immunity and suppresses immunotolerance in reconstituted lymphopenic hosts

K Narumi[1,4], T Udagawa[1], A Kondoh[1], A Kobayashi[1], H Hara[1], Y Ikarashi[1], S Ohnami[2], F Takeshita[3], T Ochiya[3], T Okada[4], M Yamagishi[4], T Yoshida[2] and K Aoki[1]

T cells recognize tumor-associated antigens under the condition of lymphopenia-induced homeostatic proliferation (HP); however, HP-driven antitumor responses gradually decay in association with tumor growth. Type I interferon (IFN) has important roles in regulating the innate and adaptive immune system. In this study we examined whether a tumor-specific immune response induced by IFN-α could enhance and sustain HP-induced antitumor immunity. An intratumoral *IFN-α* gene transfer resulted in marked tumor suppression when administered in the early period of syngeneic hematopoietic stem cell transplantation (synHSCT), and was evident even in distant tumors that were not transduced with the IFN-α vector. The intratumoral delivery of the *IFN-α* gene promoted the maturation of CD11c$^+$ cells in the tumors and effectively augmented the antigen-presentation capacity of the cells. An analysis of the cytokine profile showed that the CD11c$^+$ cells in the treated tumors secreted a large amount of immune-stimulatory cytokines including interleukin (IL)-6. The CD11c$^+$ cells rescued effector T-cell proliferation from regulatory T-cell-mediated suppression, and IL-6 may have a dominant role in this phenomenon. The intratumoral *IFN-α* gene transfer creates an environment strongly supporting the enhancement of antitumor immunity in reconstituted lymphopenic recipients through the induction of tumor-specific immunity and suppression of immunotolerance.
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Keywords: IFN-α; gene transfer; hematopoietic stem cell transplantation; IL-6; regulatory T cells

INTRODUCTION

The development of effective cancer immunotherapy is often difficult because cancer generates an immunotolerant microenvironment against the host immune system.[1] The central objective of cancer immunotherapy is to induce and sustain a tumor-specific immune response; that is, an *in vivo* generation of a large number of highly reactive antitumor lymphocytes that are not restrained by cancer-induced tolerance mechanisms.

It is known that lymphopenia is followed by spontaneous expansion of the remaining T cells in the periphery to restore the original T-cell pool size and maintain homeostasis.[2] Lymphopenia-induced homeostatic proliferation (HP) of T cells following autologous hematopoietic stem cell transplantation (HSCT) is driven by the recognition of self-antigens, and there is an opportunity to skew the T-cell repertoire during the T-cell recovery by engaging tumor-associated antigens (TAAs), leading to a break in tolerance developed by tumors.[2] In fact, a variety of animal tumor models showed that lymphopenic conditions are able to create an environment to mount an efficient antitumor immunity through an HP-induced expansion of T cells.[3–6] However, integration of other immunotherapeutic strategies is necessary to successfully eradicate pre-existing malignant tumors, because HP-driven antitumor responses decay gradually, as they are vulnerable to a development of tolerance.[5,6]

The interferon (IFN)-α protein is a pleiotropic cytokine regulating anti-proliferation, induction of cell death, anti-angiogenesis and immunomodulation, and has been used for treatment in a variety of cancers such as chronic myeloid leukemia, melanoma and renal cancer.[7,8] Although IFN-α was long thought to function mainly by suppressing tumor cell proliferation *in vivo*, more recently it has been established that type I IFNs have important roles in regulating the innate and adaptive arms of the immune system: upregulation of major histocompatibility complex class I gene, promotion of the priming and survival of T cells, enhancement of humoral immunity, increase of the cytotoxic activity of natural killer (NK) cells and CD8$^+$ T cells and activation of dendritic cells (DCs).[9,10] We also reported that in addition to the direct cytotoxicity in the injected site, intratumoral *IFN-α* gene transfer elicits a systemic tumor-specific immunity in several animal models.[11,12] Furthermore, our data showed that, because of the effective induction of antitumor immunity and the lower toxicity, an intratumoral route of the IFN vector is superior to an intravenous administration.[13]

In this study, we examined whether HP-induced antitumor activity can be enhanced by IFN-α gene transfer during a physiological

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immune reconstitution, and investigated mechanisms of the enhancement. From the viewpoint of IFN-α immune therapy also, an autologous HSCT following a preconditioning is expected to introduce a fresh immune system, in which tolerance to tumor cells is not yet induced, and may present a unique opportunity for IFN-α to augment efficacy of the immune therapy.

RESULTS

Adenovirus-mediated *IFN-α* gene transfer induces significant antitumor effect with synHSCT

To examine whether HP of T cells could induce antitumor immunity in lymphopenic hosts, BALB/c mice were injected subcutaneously with CT26 colon cancer cells shortly after lethal (9 Gy) irradiation, and then bone marrow and T cells were infused into the mice. Tumor growth was significantly suppressed in the syngeneic HSCT (synHSCT) recipients (Figure 1a) as previously reported.[6] HSCT with immunodeficient mice did not show the tumor growth suppression as compared with non-transplanted mice (data not shown), indicating that the antitumor effect is not mediated by a nonspecific effect of irradiation or lymphocyte infusion. Then, to examine whether a combination of intratumoral *IFN-α* gene transfer enhances the antitumor effect of synHSCT, 5×10^6 PFU (plaque forming unit) of Ad-mIFN was injected once into the tumor at 5 days after the CT26 inoculation. To detect a synergistic effect, we used a low dose (5×10^6 PFU) of Ad-mIFN in this experiment, although the antitumor effect of intratumoral Ad-mIFN injection is dose-dependent, and a strong antitumor effect is induced by a high dose ($5–10\times10^7$ PFU) of Ad-mIFN alone.[12,13] Although the low dose of Ad-mIFN alone suppressed tumor growth only slightly in naïve mice as expected, a significant growth suppression was observed in the synHSCT recipients compared with the injection of a control Ad-AP (Figure 1b).

Injection of IFN-α-expressing plasmid suppresses tumor growth in synHSCT recipients

We observed a significant growth suppression of colon cancer in a lymphopenic host by an injection of a low dose of Ad-mIFN (Figure 1b). Although the *in vivo* gene transduction efficiency of the plasmid vector is lower than that of the virus vector, the lipofection/

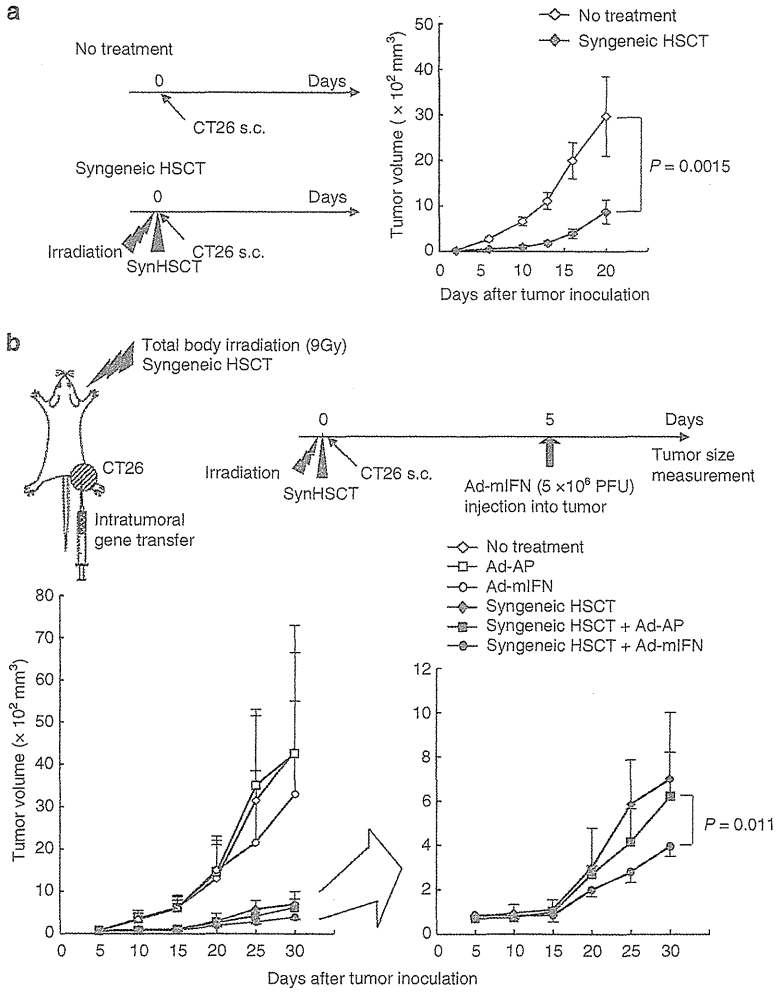


Figure 1 Adenovirus-mediated *IFN-α* gene transfer enhances antitumor effect in synHSCT recipients. (**a**) Growth suppression of subcutaneous tumors in the synHSCT mice. The mice received a lethal dose (9 Gy) of irradiation, followed by a transfusion of bone marrow and splenic T cells, and then CT26 cells were inoculated into right legs. As a control, CT26 cells were inoculated in non-irradiated mice (n=7). (**b**) A combination of syngeneic HSCT and IFN-α adenovirus injection. When CT26 subcutaneous tumors were established, 5×10^6 PFU of Ad-mIFN or control vector (Ad-AP) were injected once into the tumors (n=6–8). The experiments were repeated two times.

polyfection of the plasmid vector has an excellent safety profile.[14] Therefore, we examined whether tumor growth suppression would be observed by an injection of an IFN-α-expressing plasmid (pIFN-α). First, we confirmed that CT26 and Renca cells transfected with the pIFN-α produced significant amounts of IFN-α protein in the culture medium (Figure 2a), and showed growth suppression *in vitro* (Figure 2b). In CT26 tumor-bearing BALB/c mice, the injection of pIFN-α complexed with cationic liposome (DMRIE-DOPE) expressed IFN-α in the tumors in a dose-dependent manner, and an IFN-α concentration by the injections of pIFN-α (30 μg, three times) was comparable with that of Ad-mIFN (5×10^6 PFU, once) (Figure 2c). The plasmid-mediated IFN-α expression continued for more than 10 days after gene transfer and returned to a control level at 14 days after gene transfer (Figure 2d).

Then, to examine the *in vivo* antitumor effect of a plasmid-mediated *IFN-α* gene transfer, a pIFN-α/liposome complex was injected into the CT26 subcutaneous tumors at 7 days after the transplantation. The injection of pIFN-α slightly suppressed tumor growth compared with the non-injected control group, whereas the tumor-suppressive effect by the injection of pIFN-α was significantly enhanced in the lymphopenic mice that received synHSCT (Figure 2e). The results suggested that an *in vivo* injection of pIFN-α induced antitumor immunity in lymphopenic hosts as effectively as the Ad-mIFN. Regarding the timing of intratumoral *IFN-α* gene transfer, the injection of pIFN-α at 8 weeks after transplantation did not enhance the antitumor immunity, whereas a substantial antitumor effect was observed by *IFN-α* gene transfer in the earlier period (2–6 weeks) after the transplantation (Figure 2f), suggesting that intratumoral *IFN-α* gene transfer during immune reconstitution can induce a synergistic antitumor effect. In the mice treated with *IFN-α* gene transfer at 6 weeks after HSCT, tumor growth suppression was still recognized (P=0.016) at day 42 and the survival of the treated mice was significantly prolonged as compared with the injection of the control plasmid (Figure 2g). All treated mice looked healthy during the course of the experiments, and the blood chemistry (albumin, alanine transaminase, total bilirubin, alkaline phosphatase, blood urea nitrogen, creatinine) showed no abnormal values in the treated mice at 5 weeks after the HSCT.

Intratumoral *IFN-α* gene transfer increases tumor-infiltrating lymphocytes after synHSCT

It is known that a large number of tumor-infiltrating lymphocytes (TILs) results in a better prognosis for cancer patients.[15] To examine whether an increase of TILs in treated tumors is related to tumor growth suppression, immunohistochemical staining of CD4 and CD8-positive cells was performed at 1–4 weeks after synHSCT. In this experiment, CT26 cells were inoculated on legs at 1 week before synHSCT, and the number of $CD4^+$ and $CD8^+$ T cells infiltrated into the tumors was examined at the day of synHSCT to evaluate how the number of immune cells increases in the tumors by the combination therapy as compared with the pre-treatment status. In this established tumor model also, a significant antitumor effect was recognized in the synHSCT mice with the *IFN-α* gene transfer (Figure 3a).

The numbers of $CD4^+$ and $CD8^+$ cells gradually diminished in the non-treated tumors, whereas the TILs increased in the tumors of synHSCT mice. Intratumoral *IFN-α* gene transfer further increased the numbers of TILs significantly in synHSCT recipients at 4 weeks (Figure 3b), suggesting that intratumoral *IFN-α* gene transfer enhances and prolongs HP-induced antitumor immunity after synHSCT.

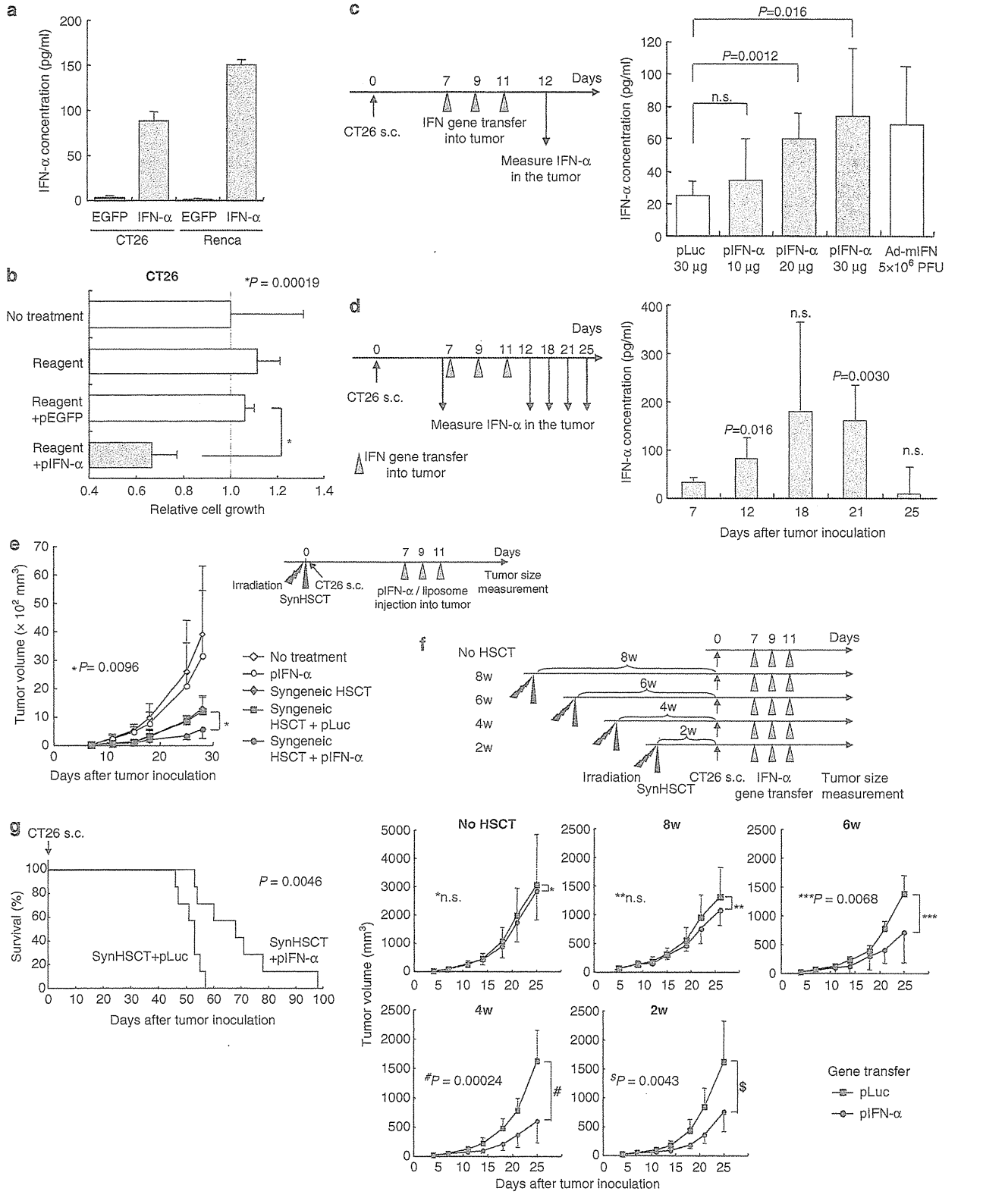
Then, to examine the cytotoxic activity of TILs, $CD8^+$ T cells were isolated from tumors in the treated mice. The flow cytometry showed that the expression of perforin on $CD8^+$ TILs was significantly enhanced in the synHSCT alone group and in the combination therapy-treated group, suggesting that synHSCT creates an environment to enhance the killing activity of TILs in the tumors (Figure 3c).

Tumor-specific lymphocytes are activated by intratumoral *IFN-α* gene transfer in synHSCT recipients

To examine the immune reaction to intratumoral *IFN-α* gene transfer in synHSCT recipients, splenocytes were extracted from the treated mice and cultured with CT26 cells. An enzyme-linked immunosorbent spot assay showed that the average number of IFN-γ-producing splenocytes in response to CT26 cells was slightly increased in the synHSCT alone group, whereas a combination of synHSCT and intratumoral *IFN-α* gene transfer further increased the IFN-γ-positive spots (Figure 4a, left panel). The numbers of the spots in splenocytes co-cultured with syngeneic lymphocytes were not changed in the treated groups (Figure 4a, right panel). To analyze the subset of activated lymphocytes, the frequency of tumor-reactive immune cells was determined by intracellular cytokine staining and flow cytometry. The percentage of $CD4^+$ and $CD8^+$ T cells stimulated to produce IFN-γ in response to CT26 cells increased significantly in the mice treated by a combination of synHSCT and intratumoral *IFN-α* gene transfer, and there was also an increase in the percentage of IFN-γ-positive NK cells (Figure 4b). The results indicated that the numbers of tumor-reactive lymphocytes were increased synergistically by a combination therapy.

An *in vitro* cytotoxic assay showed that the splenocytes derived from the synHSCT mice recognized and lysed CT26 cells, and *IFN-α* gene transfer enhanced the cytolysis to CT26 cells (Figure 4c, left panel). To

Figure 2 Plasmid vector-mediated *IFN-α* gene transfer induces a significant antitumor effect in synHSCT recipients. (**a**) *In vitro* IFN-α concentration in the medium of cancer cells transfected with pIFN-α. CT26 or Renca cells were transfected with pIFN-α, and 48 h later, IFN-α concentration in the culture medium was measured by enzyme-linked immunosorbent assay (ELISA; Immunotech, Marseille Cedex, France). pEGFP was used as a control. (**b**) *In vitro* cytotoxicity of pIFN-α transfection. The cell growth was determined by an *in vitro* cell proliferation assay at 5 days after transfection. The data are expressed as relative cell growth (OD_{450} of indicated cells/OD_{450} of untreated cells). Reagent; Lipofectamine 2000. (**c**) *In vivo* IFN-α concentration in the tumors transfected with pIFN-α. Various amounts (10–30 μg) of pIFN-α complexed with liposome were injected three times into the CT26 tumors at 7, 9 and 11 days after the tumor inoculation. Ad-mIFN (5×10^6 PFU) was injected once into the tumors at day 9. Tumors were collected at day 12, and the IFN-α concentration was measured by ELISA (n=4). (**d**) Time course of IFN-α expression in the tumors. The IFN-α levels were measured in the subcutaneous tumors during 14 days after intratumoral injection of pIFN-α (30 μg)/liposome complex at days 7, 9 and 11 (n=4–5). The statistical difference between IFN-α concentration in the indicated days and at day 7 is presented. (**e**) A combination of syngeneic HSCT and intratumoral *IFN-α* gene transfer caused marked tumor growth suppression. The pIFN-α (30 μg)/liposome complex was injected into the CT26 tumors at 7, 9 and 11 days after the tumor inoculation in the synHSCT mice (n=7). The experiments were repeated two times. (**f**) Intratumoral *IFN-α* gene transfer during an early phase of immune reconstitution enhances the antitumor effects. Syngeneic HSCTs to the BALB/c mice were staggered at intervals of 2 weeks, followed by a subcutaneous injection of CT26 cells and intratumoral *IFN-α* gene transfer at days 7, 9 and 11 (n=6–8). (**g**) Intratumoral *IFN-α* gene transfer significantly extends the survival of synHSCT mice (n=7). The pIFN-α/liposome complex was injected into the CT26 tumors at 6 weeks after the HSCT, and survival of the mice was compared with the mice injected with a control plasmid (n=7).


a
IFN-α concentration (pg/ml)
200
150
100
50
0
EGFP IFN-α EGFP IFN-α
CT26
Renca
b
CT26
*P = 0.00019
No treatment
Reagent
Reagent +pEGFP
Reagent +pIFN-α
0.4 0.6 0.8 1.0 1.2 1.4
Relative cell growth
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0 7 9 11 12 Days
CT26 s.c.
IFN gene transfer into tumor
Measure IFN-α in the tumor
P=0.016
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n.s.
IFN-α concentration (pg/ml)
120
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20
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pLuc 30 μg
pIFN-α 10 μg
pIFN-α 20 μg
pIFN-α 30 μg
Ad-mIFN 5×10^6 PFU
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0 7 9 11 12 18 21 25 Days
CT26 s.c.
Measure IFN-α in the tumor
IFN gene transfer into tumor
IFN-α concentration (pg/ml)
400
300
200
100
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n.s.
P=0.0030
P=0.016
n.s.
7 12 18 21 25
Days after tumor inoculation
e
Tumor volume (× 10^2 mm^3)
70
60
50
40
30
20
10
0
*P= 0.0096
0 10 20 30
Days after tumor inoculation
No treatment
pIFN-α
Syngeneic HSCT
Syngeneic HSCT + pLuc
Syngeneic HSCT + pIFN-α
0 7 9 11 Days
Irradiation
CT26 s.c.
SynHSCT
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Irradiation
SynHSCT
CT26 s.c.
IFN-α gene transfer
Tumor size measurement
g
CT26 s.c.
Survival (%)
100
80
60
40
20
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P = 0.0046
SynHSCT+pLuc
SynHSCT +pIFN-α
0 10 20 30 40 50 60 70 80 90 100
Days after tumor inoculation
No HSCT
8w
6w
4w
2w
Tumor volume (mm^3)
*n.s.
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***P = 0.0068
#P = 0.00024
$P = 0.0043
0 5 10 15 20 25
Days after tumor inoculation
Gene transfer
pLuc
pIFN-α

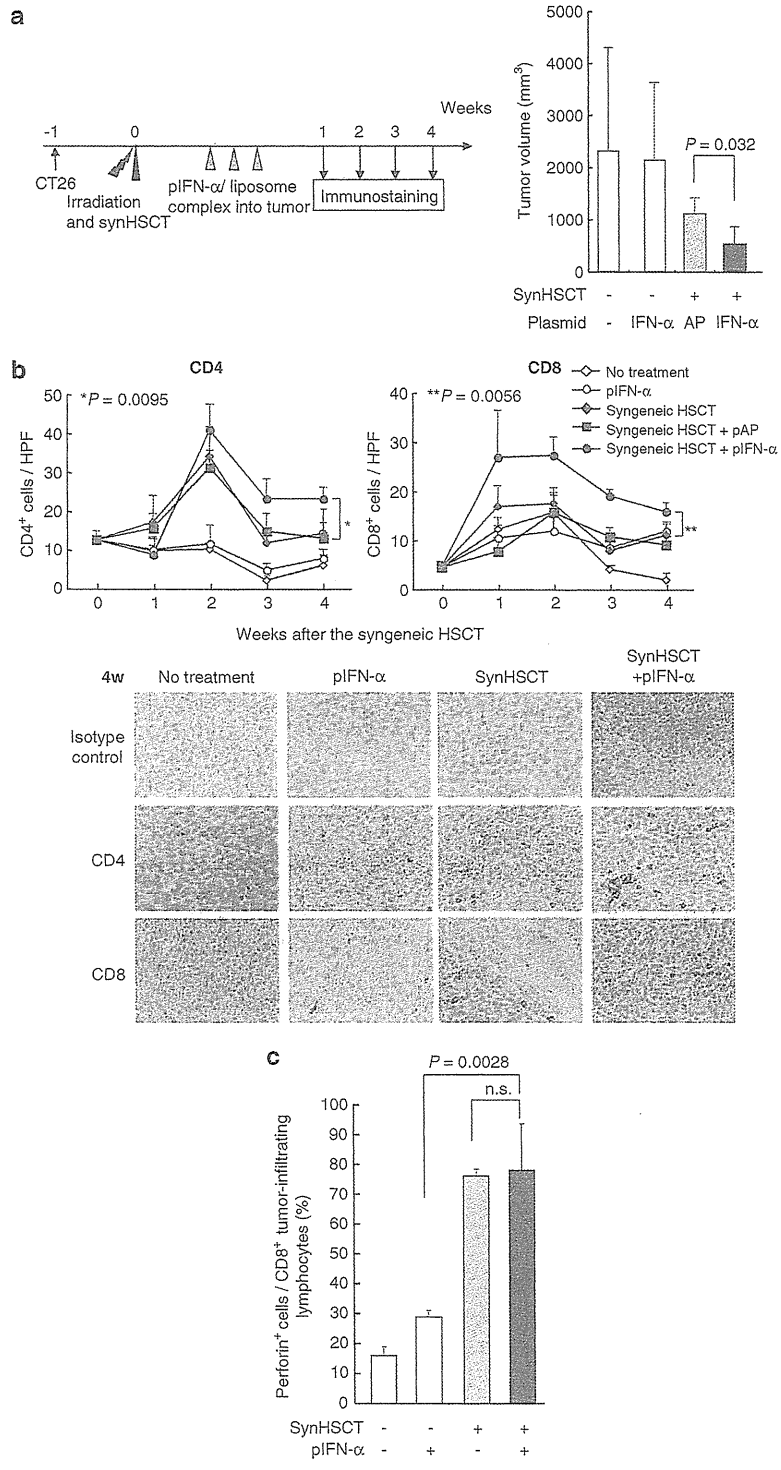


Figure 3 Immunostaining of treated tumors. (**a**) Antitumor effect of *IFN-α* gene transfer for established tumors in the synHSCT recipient mice. CT26 cells were inoculated on the right legs of BALB/c mice at 1 week before the synHSCT, and the pIFN-α/liposome complex was injected into CT26 tumors for three times. The tumor volumes at 3 weeks after the *IFN-α* gene transfer are presented. (**b**) Infiltration of immune cells in the tumors. The subcutaneous tumors were resected at the indicated days (n=3), and stained with CD4 and CD8 antibodies. The $CD4^+$ (upper left) and $CD8^+$ cells (upper right) were counted by microscopy in five high-power fields (×400). Representative photographs of stained cells at 4 weeks after the gene transfer are presented in the lower panel (×400). (**c**) Expression of perforin on $CD8^+$ tumor-infiltrating lymphocytes. The $CD8^+$ T cells were isolated from the tumors using mouse CD8 MicroBeads and AutoMACS magnetic sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The flow cytometry of perforin (eBioOMAK-D; eBioscience, San Diego, CA, USA) was performed on the $CD8^+$ TILs (n=3). The frequency of $perforin^+$ cells per $CD8^+$ cells is presented.

show the major histocompatibility complex class I-restriction of cytotoxicity, lymphocytes were pre-incubated with the anti-CD4 or anti-CD8 antibodies before the cytolysis for CT26 cells. The addition of anti-CD8 antibody markedly inhibited the cell lysis (Figure 4c, right panel).

To explore what kind of lymphocytes contribute to antitumor immunity *in vivo*, the mice were treated with anti-CD4, anti-CD8 and anti-asialo GM1 antibodies intraperitoneally to deplete $CD4^+$ T cells, $CD8^+$ T cells and NK cells, respectively. Depletion of all of the three populations canceled antitumor effect almost completely. Depletion of each $CD4^+$ T-cell, $CD8^+$ T-cell or NK cell showed some growth advantages but still resulted in significant tumor growth inhibition and, in particular, $CD8^+$ T cells appeared to contribute more strongly than $CD4^+$ T and NK cells (Figure 4d).

Intratumoral *IFN-α* gene transfer causes growth suppression in not only treated but also distant tumors

To evaluate the therapeutic efficacy of *IFN-α* gene transfer against tumors at distant sites in synHSCT recipients, the mice were inoculated with CT26 cells on both legs and Renca cells on the back, and an IFN-α plasmid/liposome complex was injected into the CT26 tumor on the right leg. The *IFN-α* gene transfer significantly suppressed the growth of not only the right leg CT26 tumors but also the left CT26 tumors that were not transfected with the *IFN-α* gene, whereas the growth of Renca tumors on the back was not influenced by the *IFN-α* gene transfer into the CT26 tumors (Figure 5a). When we exchanged CT26 and Renca cells, the antitumor effect was *vice versa* (Figure 5b). The results indicated that intratumoral *IFN-α* gene transfer enhances a vector-injected-tumor specific immunity systemically in the synHSCT recipients.

Liver metastasis is one of the most frequent causes of mortality in patients with gastrointestinal cancer such as colorectal cancer. As another model of distant metastasis, CT26-Luc cells were injected beneath the splenic capsule to generate liver metastasis, and CT26 cells were inoculated into the right leg. Intratumoral *IFN-α* gene transfer suppressed tumor growth of subcutaneous tumors on the legs (Figure 5c, upper left panel) as observed in Figure 2e, and the growth of abdominal tumors was also markedly suppressed, which were evaluated by photon counts on the IVIS imaging system (IVIS; Xenogen, Alameda, CA, USA), in the synHSCT mice (Figure 5c, upper right and lower panels). Twenty-one days after tumor inoculation, the mice were killed and the livers were examined by the IVIS imaging. Livers from non-treated mice showed many photon-positive spots, whereas livers from the mice treated by a combination therapy revealed a fewer number of photon-positive spots (Figure 5d, upper panel), which was confirmed by photon counts in those livers (Figure 5d, lower panel). These results indicated that a combination therapy is effective for preventing and regressing liver metastases.

A combination therapy enhances maturation of $CD11c^+$cells and their antigen presentation

To verify whether the intratumoral *IFN-α* gene expression promotes the maturation of DCs in the tumor, we isolated $CD11c^+$ cells from the regional lymph nodes of treated tumors. Flow cytometry showed that expressions of CD40, CD80, CD83 and CD86 were clearly upregulated by the *IFN-α* gene transfer (data not shown and see Narumi *et al.*[13]). Then, the expression of CD83 was examined in the $CD11c^+$ cells isolated from tumors. The frequency of $CD83^+$ cells was increased in IFN/HSCT-$CD11c^+$ cells compared with those in IFN-$CD11c^+$ and HSCT-$CD11c^+$ cells (Figure 6a, left panel), suggesting that IFN-α expression results in the maturation of $CD11c^+$ cells. Then, to examine the antigen-presentation capacity of the $CD11c^+$ cells in the tumors, the lymphocytes isolated from naïve mice were co-cultured with the $CD11c^+$ cells and mitomycin C-treated CT26 cells for 3 days. An enzyme-linked immunosorbent spot assay showed that the IFN/HSCT-$CD11c^+$ cells increased the number of IFN-γ-positive lymphocytes in response to CT26 cells (Figure 6a, middle panel). The production of IFN-γ from the $CD11c^+$ cells *per se* was minimal. Lymphocytes under HP are considered to be primed to TAAs. When lymphocytes isolated from tumor-bearing synHSCT mice were co-cultured with the $CD11c^+$ cells and mitomycin C-treated CT26 cells, the stimulation by IFN/HSCT-$CD11c^+$ cells resulted in a higher number of IFN-γ-secreting lymphocytes than those of IFN-$CD11c^+$ and HSCT-$CD11c^+$cells (Figure 6a, right panel). The results indicated that the antigen-presentation by $CD11c^+$ cells was enhanced by the IFN-α expression in the tumors in synHSCT mice.

$CD11c^+$cells treated by a combination therapy suppresses the activity of regulatory T cells

To analyze the cytokine expression profile of the $CD11c^+$ cells, we collected $CD11c^+$ cells from the treated tumor at 2 weeks after *IFN-α* gene transfer, cultured the cells *in vitro* for 48 h and measured the expression of various cytokines in the medium. The IFN/HSCT-$CD11c^+$ cells produced a large amount of immune-stimulatory cytokines such as interleukin (IL)-1β, IL-6 and IL-12 (Figure 6b), which may enhance the proliferation and activation of lymphocytes in the treated mice. Among the immunosuppressive cytokines (IL-4, IL-10 and transforming growth factor-β), IL-10 production is increased in IFN/HSCT-$CD11c^+$ cells. The enhanced IL-12 production might promote the production of IL-10 in some population of the $CD11c^+$ cells as a negative feedback mechanism.[16] However, the large amount of immune-stimulatory cytokines may overcome the suppressive effect of IL-10 in the tumors treated with the combination therapy.

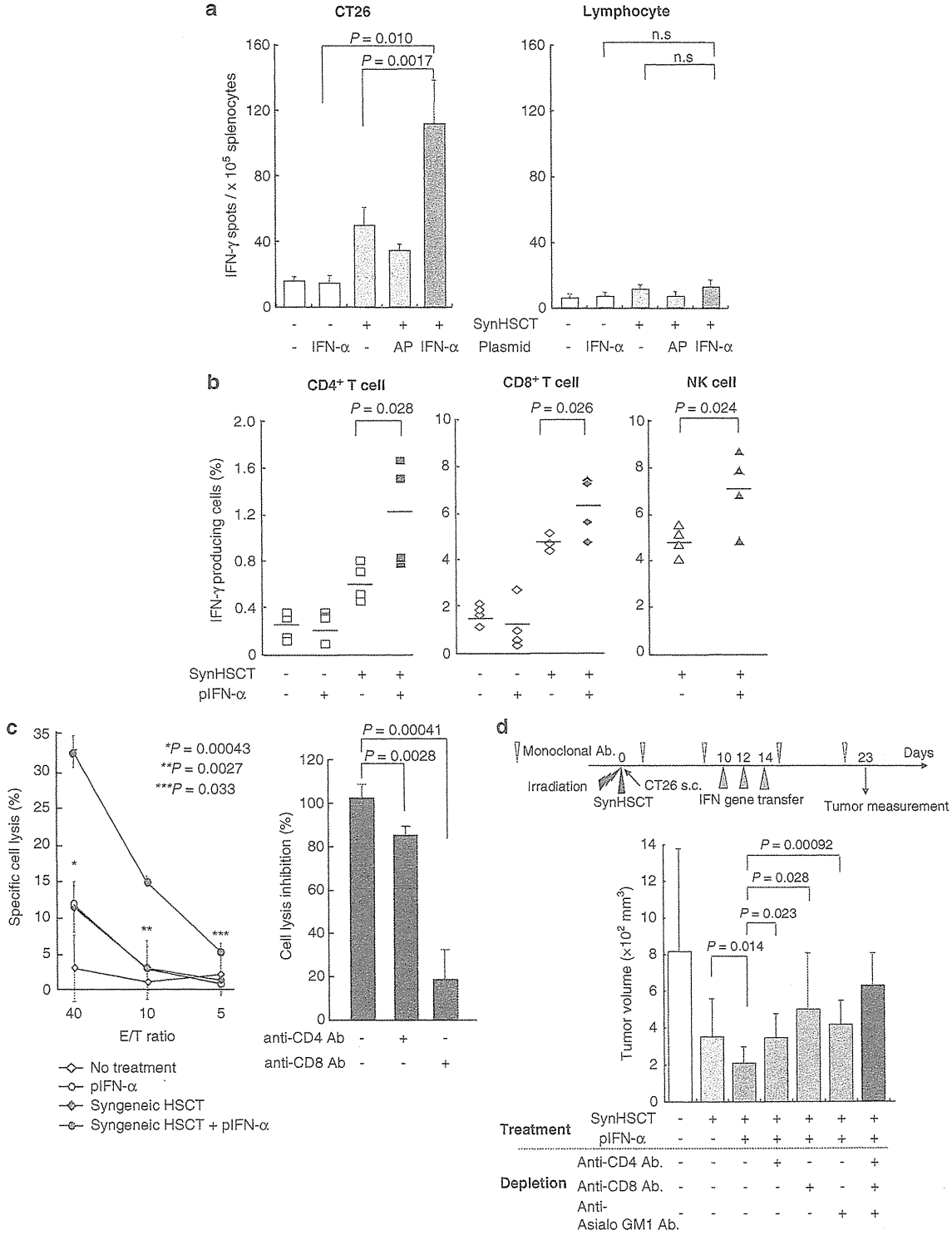
IL-6 is of particular interest, because, when released from DCs, it is critical for overcoming Tregs-mediated immune suppression.[17] When the $CD11c^+$ cells isolated from the spleen of synHSCT and non-HSCT mice were cultured in the medium containing IFN-α protein, the cells produced IL-6 in a dose-response manner (Figure 7a). To examine whether the $CD11c^+$ cells inhibit the suppressive activity of Tregs, $CD11c^+$ cells were isolated from regional lymph nodes and co-cultured with $CD4^+CD25^-$ T cells (target) and $CD4^+CD25^+$ Tregs in mouse anti-CD3 T-cell activation plates. When Tregs were co-cultured with the control $CD11c^+$ cells isolated from non-treated mice, Tregs effectively suppressed the proliferation of target T cells. The co-culture with IFN/HSCT-$CD11c^+$ cells canceled the suppressive activity of Tregs for effector T cells (Figure 7b). The supernatant of IFN/HSCT-$CD11c^+$ cells also inhibited the suppressive activity of Tregs, and the addition of anti-IL-6 antibody in the medium restored the activity of Tregs, indicating that the critical factor of $CD11c^+$ cell-mediated inhibition of Tregs is IL-6, produced from activated $CD11c^+$ cells (Figure 7c).

Then, to examine the *in vivo* effect of intratumoral IFN-α expression on Tregs, we collected lymphocytes from treated tumors in synHSCT mice. Flow cytometry showed that intratumoral *IFN-α* gene transfer decreased the ratio of $Foxp3^+$ cells per $CD4^+$ T cells in the tumors of synHSCT mice, whereas the ratio of $Foxp3^+$ cells in the spleen was same as that in the non-treated mice (Figure 7d). The results suggested that IL-6 produced from $CD11c^+$ cells decreases the ratio of Tregs in the treated tumors. IFN-α expression may result in the extensive infiltration of T cells into the tumors of synHSCT mice (Figure 3b), and the number of $Foxp3^+$ cells also might increase in the

tumors. However, as the antitumor immune response is based on the balance between the effector and regulatory sides of immune cells; the decrease of Treg ratio (Figure 7d) and inhibition of Treg activity (Figure 7b) may lead to a strong antitumor immunity.

DISCUSSION

In this study, we showed that an intratumoral *IFN-α* gene transfer significantly enhances a systemic tumor-specific immunity in the synHSCT recipients. The precise mechanism for the enhancement is



not completely understood, but it should include an effective stimulation of DCs by the expression of IFN-α in the tumors in synHSCT recipients, because (1) intratumoral expression of IFN-α effectively induces cell death of cancer cells and exposes TAAs in large quantity to DCs ($CD11c^+$ cells);[12] (2) IFN-α promotes maturation of $CD11c^+$ cells, which facilitates the presentation of TAAs on $CD11c^+$ cells (Figure 6a); (3) $CD11c^+$ cells in the tumors transduced with the *IFN-α* gene produce a large quantity of immune-stimulatory cytokines such as IL-12 (Figure 6b); (4) the $CD11c^+$ cells in the treated tumors suppress the inhibitory activity of Tregs (Figure 7b). The combination of immune-stimulatory effects by IFN-α and the reconstitution of a fresh immune system following HSCT could create an environment strongly supporting the activation of an antitumor response. We propose a model showing the integrated mechanisms of inducing a strong antitumor immunity by a combination therapy (Figure 8).

Although the conditioning of HSCT with irradiation and/or immunosuppressive reagents can destroy the immunotolerance deployed by the tumor, the tumors restore a tolerant microenvironment by induction of Tregs and the production of immune-inhibitory cytokines,[18] which may be one of the main reasons for the failure to fully sustain HP-mediated antitumor immunity. An analysis of the cytokine profile unexpectedly showed that the IFN/HSCT-$CD11c^+$ cells produce a large amount of IL-6 as well as other immune-stimulatory cytokines (Figure 6b). It has been reported that IL-6 increases methylation of upstream Foxp3 enhancer and represses the Foxp3 transcription in natural Tregs.[19] Our findings demonstrated that $CD11c^+$ cells isolated from the tumors transduced by *IFN-α* gene significantly suppress the activity of Tregs (Figure 7b), which may inhibit or delay the reconstitution of an immunotolerant microenvironment in the tumor. $CD11c^+$ cells seem to have a capacity to produce IL-6 in response to IFN-α in a dose-dependent manner (Figure 7a). The IL-6 production was increased in the HSCT-$CD11c^+$ cells also (Figures 6b and 7b). The intratumoral IFN-α expression and the immune-stimulatory condition by elevated cytokine levels after synHSCT may synergistically influence $CD11c^+$ cells to produce IL-6. It is also reported that IL-6 promotes carcinogenesis through multiple signal pathways.[20] The role of IL-6 in tumors is probably based on the balance between the positive and negative effect of IL-6 on the tumor growth. Although it is known that DCs secrete proinflammatory cytokines such as IL-6 by toll-like receptor stimulation,[17] further research is needed to clarify the critical factor/pathway for the production of IL-6 from DCs in synHSCT recipients.

As HP leads to a break in tolerance against self-antigens, the expression of IFN-α could theoretically promote T-cell response not only against tumor cells but also against host normal cells, which may cause an autoimmune reaction. However, no overt toxicity was observed for the treated mice, including their blood chemistry. The immunogenic DCs at the IFN-α vector-injected tumor site were able to capture both TAAs and normal self-antigens shared by tumor and normal cells, and promote tumor-specific immunity and a local autoimmune reaction, whereas resting host DCs away from the tumor site present only normal self-antigens and may induce tolerance or exhaustion of host-reactive T cells. Alternatively, we recently found that the percentage of $Foxp3^+$ cells per $CD4^+$ T cells in the spleen was clearly elevated at an early phase after syngeneic HSCT (data not shown), which differed from the low frequency of $Foxp3^+$ T cells in the tumor (Figure 7d). The finding suggests that among $CD4^+$ T cells, Tregs rapidly proliferate during HP in the body, which might protect patients against autoimmunity after autologous HSCT.

There have been several animal studies showing the potential efficacy of gene- and cell-based immunotherapy in syngeneic HSCT mice. The vaccination with syngeneic tumor cells expressing granulocyte-macrophage colony stimulating factor showed a strong antitumor effect in the transplanted mice.[5] An immunization with DCs pulsed with whole tumor cell lysates led to efficient antitumor responses in a mouse breast tumor model.[21] Adoptive transfer of tumor-specific T cells has also shown enhanced antitumor immune responses after HSCT in lymphopenic mice,[2] and recently, Morgan *et al.*[22] reported efficacy of a strategy composed of immunodepletion and adaptive cell transfer for patients with metastatic melanoma. As tumor-reactive T cells are mostly polyclonal, and heterogeneous expressions of various TAAs coexist even in a tumor mass, the *in vivo* stimulation of multiple tumor-reactive lymphocytes might be critical in the clinical application. Moreover, compared with the previous approaches, another major advantage of an *in vivo IFN-α* gene transfer is that it does not involve a manipulation and culture of the immune and tumor cells *ex vivo*, making this strategy more feasible for many patients with solid cancers. We previously reported that an allogeneic MHC gene transfer also could enhance an effective antitumor immunity in HSCT recipients.[6] The major difference between the allogeneic MHC and *IFN-α* gene therapies is in their local effects on tumor sites transduced with the therapeutic genes: *IFN-α* gene transfer significantly induces cell death and growth inhibition (Figure 2b and see Hara *et al.*[12]). In addition, IFN-α seems to have direct effects upon DCs such as the maturation of the cells and production of immune-stimulatory cytokines and enhancement of inhibitory activity against Tregs. Therefore, a local *IFN-α* gene therapy is a promising therapeutic strategy, especially in a case in which cancer conditions need strong local tumor control and systemic antitumor activity.

Some of the experiments in this study showed important points to consider in the clinical feasibility of the combination therapy.

Figure 4 A large number of IFN-α-producing cells are induced by intratumoral *IFN-α* gene transfer in synHSCT recipient mice. **(a)** Enzyme-linked immunosorbent spot assay of IFN-γ-producing cells in response to stimulation of CT26 cells. At 2 weeks after plasmid-mediated *IFN-α* gene transfer, mouse splenocytes were isolated from treated mice and co-cultured with CT26 cells or control lymphocytes (n=3). The experiments were repeated three times. **(b)** Intracellular cytokine staining of IFN-γ-producing cells in response to stimulation of CT26 cells. The splenocytes (1×10^6) from treated mice were incubated with CT26 (1×10^5) and stained by allophycocyanin-anti-mouse IFN-γ. The activated cell fractions were analyzed by staining with fluorescein isothiocyanate-anti-mouse CD4, CD8 or CD49b (labeling NK cell) antibody (n=3–4). The experiments were repeated twice. **(c)** *In vitro* cytotoxic assay of splenocytes. Splenocytes were isolated from the treated mice, and their cytotoxicity was evaluated in a standard 4 h ^{51}Cr release assay against CT26 cells (n=3). The statistical difference between cell lysis in the synHSCT mice with *IFN-α* gene transfer and synHSCT alone mice is presented (left panel). Splenocytes isolated from synHSCT mice with *IFN-α* gene transfer were pre-incubated with the anti-CD4 or anti-CD8 antibodies for 1 h before cytolysis for CT26 cells (right panel). The data are expressed as cell lysis inhibition (%) at E/T ratio=40 (cell lysis with co-incubation of antibody/that with no antibody). The experiments were repeated twice. **(d)** Antitumor effect of *IFN-α* gene transfer after *in vivo* depletion of $CD4^+$ T cells, $CD8^+$ T cells and NK cells. A group of transplanted mice were treated with anti-CD4, anti-CD8 or anti-asialo GM1 antibodies (targeting NK cells) to deplete these cell populations, and the CT26 tumors were injected with pIFN-α vector (n=7–9). Tumor volumes at 10 days after *IFN-α* gene transfer are presented.

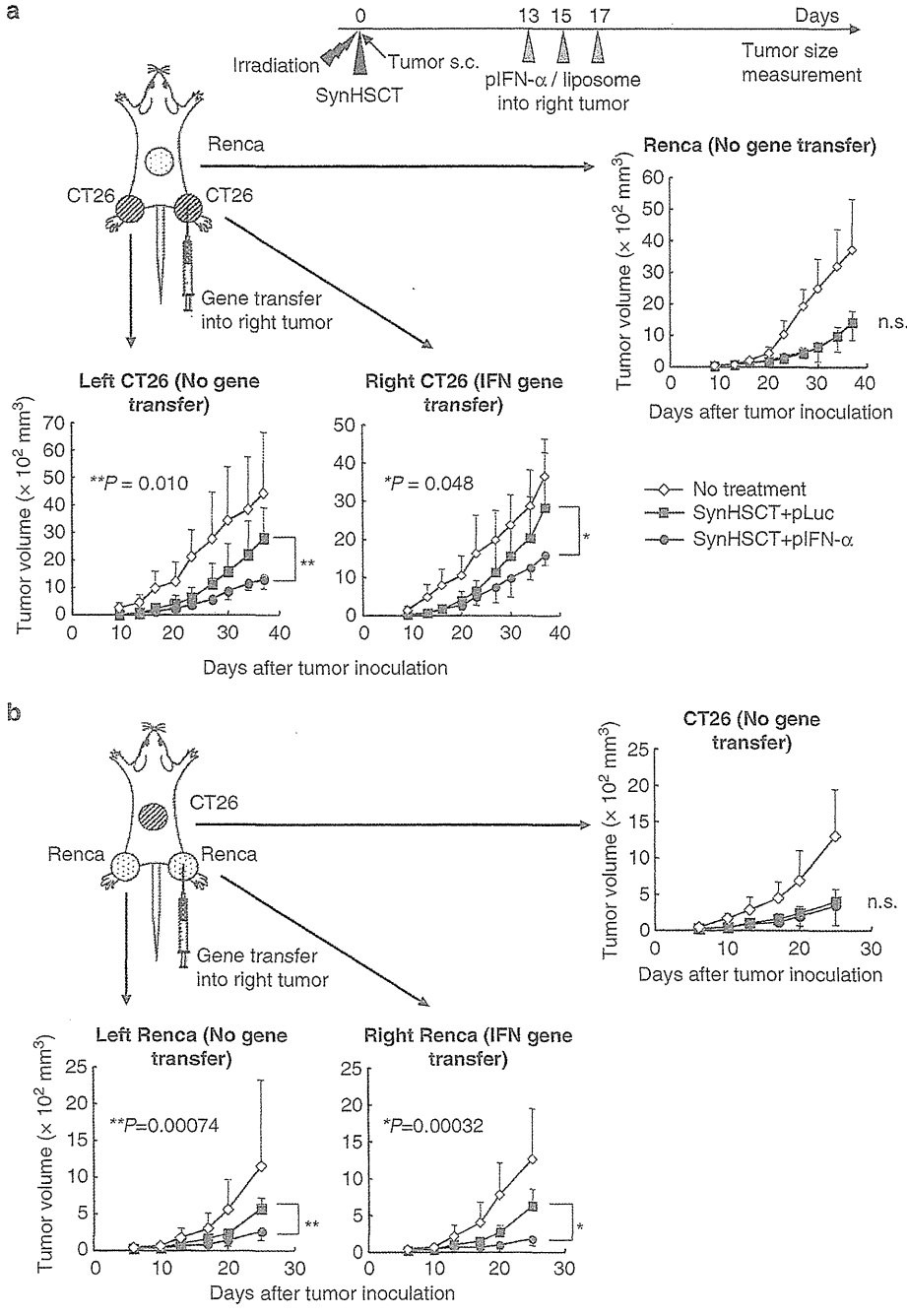


Figure 5 Suppression of tumors at distant site by *IFN-α* gene transfer during immune reconstitution. The experiments were repeated two times. (**a**) Intratumoral *IFN-α* gene transfer into CT26 tumors. CT26 cells were inoculated on both legs and Renca cells were inoculated on the back in synHSCT mice. The pIFN-α/liposome complex was injected into CT26 subcutaneous tumors on the right legs at days 13, 15 and 17 (n=4–8). (**b**) Intratumoral *IFN-α* gene transfer into Renca tumors. Renca cells were inoculated on both legs and CT26 cells on the back in synHSCT mice. The pIFN-α/liposome complex was injected into Renca subcutaneous tumors on the right legs at days 13, 15 and 17 (n=4–8). (**c**) Suppression of liver metastasis. CT26-Luc cells were injected beneath the splenic capsule to generate liver metastasis, and CT26 cells were inoculated on the right leg. After the *IFN-α* gene transfer, the size of subcutaneous tumors on the legs was macroscopically measured, and photon count of abdominal tumors was evaluated by the IVIS imaging system. (**d**) *Ex vivo* imaging of the liver. The livers were resected from treated mice at 21 days after the synHSCT, and photon spots and counts of the livers were evaluated by the imaging system. Arrowheads: liver tumors.

First, a liposome-mediated *IFN-α* gene transfer effectively suppressed tumor growth in synHSCT recipients. Although the peak level of IFN-α expression was not very high, the expression continued for more than 10 days after gene transfer (Figure 2d), suggesting that a high concentration of IFN-α in the tumors is not necessary and that the continuous expression is important to induce an effective antitumor

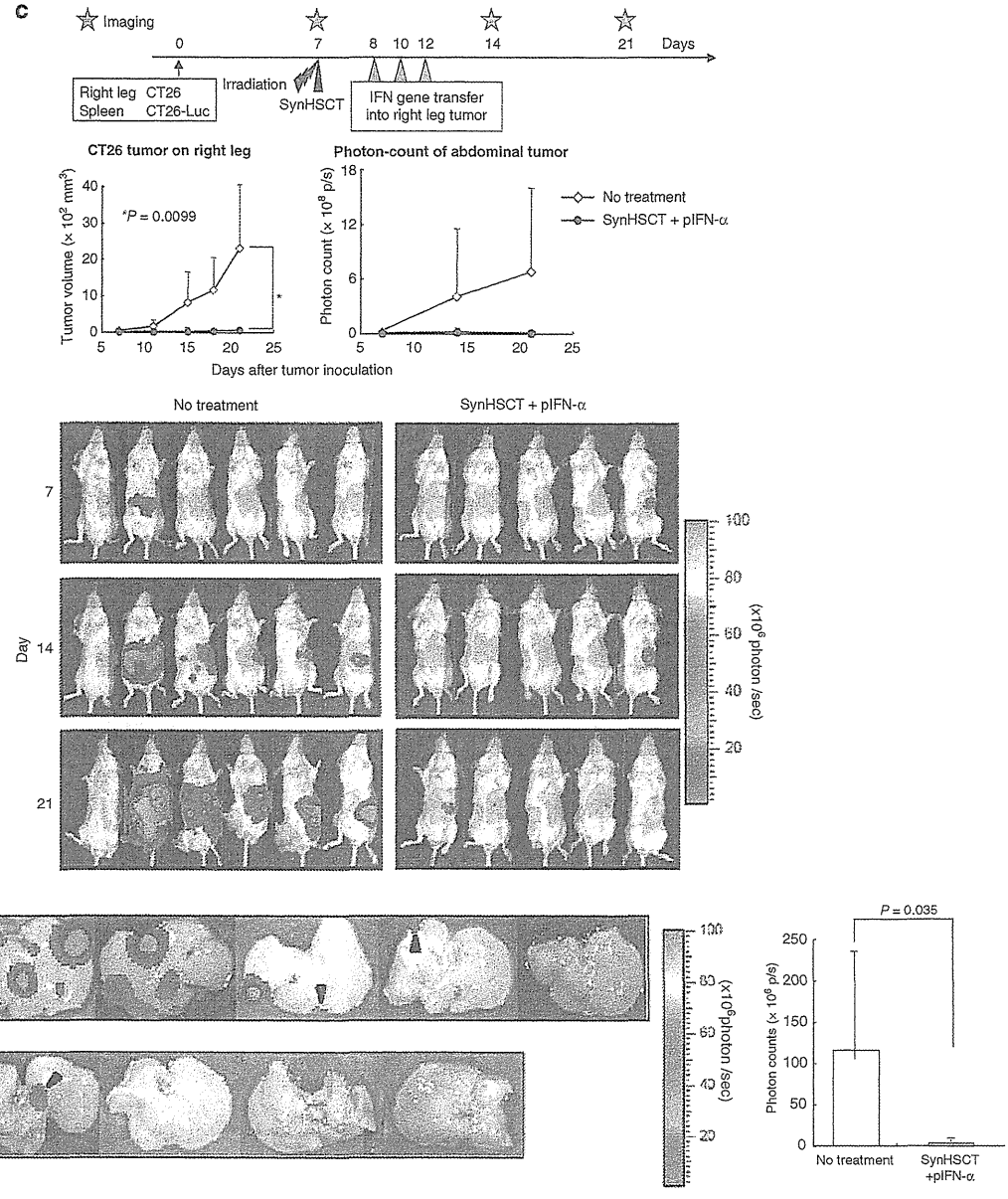


Figure 5 Continued.

immunity. This facilitates a clinical application, because *in vivo* lipofection of an IFN-α-expressing plasmid is much safer than virus vectors, although the latter has a high gene transduction efficacy.[14] Second, the intratumoral *IFN-α* gene transfer at 6 weeks after synHSCT significantly suppressed tumor growth and prolonged the survival of synHSCT mice (Figures 2e and f). At 6 weeks after synHSCT, neutrophil count returned to a normal level (data not shown), which avoids the risk of bacterial infection that accompanies a needle injection for intratumoral gene transfer. Third, a combination therapy was effective in suppressing not only the vector-injected tumors but also the vector-uninjected distant tumors in the liver metastasis model, which resembles a clinical setting. The results indicated that this treatment strategy might be feasible for many patients with solid cancers. The next step in research may include a further elucidation of the main mechanism of IFN-α in inducing tumor immunity and the synergism between IFN-α and synHSCT, including identification of potential key factors other than IL-6 and DC, development of methods to further sustain and control tumor-specific immunity and to predict and monitor HP followed by an individualization of the combination therapy.

In conclusion, a combination of intratumoral *IFN-α* gene transfer with synHSCT is a promising immunotherapy for solid cancers, because of the activation of tumor-specific immunity, suppression of the immunotolerant environment and excellent safety features. This therapeutic strategy deserves an evaluation in future clinical trial for solid cancers.