that shown in Fig. 4 (data not shown). Compared with the control group treated with AdRGD-Luc, in which about 90% of the mice had readily discernable lung metastasis, only one of nine animals treated with AdRGD-IL-12 demonstrated metastasis.

Discussion

Viral vector-encoded chemokines and cytokines are used widely in cancer gene therapy [22,23]. IL-12 has demonstrated remarkable anti-tumor activity when used directly as a recombinant protein or after various viral and non-viral vectors have been used to transfer its genes [24–26]. The development of an efficient vector is pivotal for gene therapy. Because of its high transfection efficiency and because it can transfect both dividing and quiescent cells, Ad vectors are used widely in gene therapy protocols: about 26% of gene therapy clinical trials use Ad vectors as gene carriers [27,28]. However, the lack of Coxsackie adenovirus receptor (CAR), which is an important receptor for conventional Ad vector infection, in many types of malignant cells impairs the tranfection efficiency with Ad vector [29]. Meth-A fibrosarcoma has been confirmed by RT-PCR to be deficient in expression of CAR but with expression of integrin (data not shown). Our previous reports have also shown that insertion of the RGD peptide into the fiber sequences of Ad vectors induces enhanced gene transfection in CT26 and A2058 cells [30,31]. The results of our present study also demonstrate that the fiber-mutant Ad vector induced enhanced expression of the encoded luciferase gene in Meth-A fibrosarcoma cells compared with the expression due to conventional vector (Fig. 2). Furthermore, we confirmed the presence of IL-12 p70 in the supernatant of Meth-A cells transfected with AdRGD-IL-12 (Fig. 3).

Systemic administration of recombinant IL-12 at high doses induces adverse effects associated with high systemic peak concentrations [32,33]. Therefore, gene transfer methods are designed to confine IL-12 production to the tumor environment, thereby preventing systemic toxicity. Tumor cells, dendritic cells, and autologous fibroblasts have been transfected with recombinant adenoviruses or retroviruses to secrete IL-12 locally and have shown favorable efficacy and safety profiles [34,35]. Several groups have shown that intratumoral injection of an Ad vector encoding IL-12 efficiently eradicates experimental gastrointestinal cancer [36,37]. Disadvantages of direct topical administration include tissue damage, and some tumor sites may be inaccessible even to computed tomography-guided percutaneous injection and radiographically directed delivery [38]. However, these limitations favor those types of gene therapy that do not require all tumor cells or tumor masses that express the gene.

Meth-A has shown that it is an IL-12-insensitive tumor cell, in that established tumors could not be treated efficiently via systemic administration of IL-12 and could not even be suppressed effectively (i.e., only 42.5% of mice rejected the tumor) after transfection of an IL-12-containing retroviral vector [12,39]. In our present study, however, a single intratumoral injection of a relatively low dose of AdRGD-IL-12 (2×10^7 PFU) elicited strong anti-tumor activity against established tumors (i.e., diameter of about 10 mm at the beginning of treatment; Fig. 4A). Treatment induced complete tumor regression in about 70% of tumor-bearing mice, and the growth rates of the remaining tumors seem to have been retarded (individual data not shown). Treatment also prolonged the survival of the mice significantly compared with that of the group injected with AdRGD-Luc, a control vector (Fig. 4B). Meanwhile, no detectable IL-12 and IFN-γ existed in the sera after treatment (data not shown)—findings that are consistent with those other reports [40]. Furthermore, intratumoral injection of AdRGD-IL-12 induced a profound long-term specific anti-tumor immunity in mice with complete regression of the initial Meth-A lesion (Table 1).

Studies have shown that IL-12 elicits tumor regression after induction of T-cell migration to tumor sites [41]. The failure of IL-12 therapy in Meth-A via systemic administration is thought to be due to the inability to recruit immune cell migration into tumor cells, and further investigation has indicated a key role of the peritumoral stroma/stromal vasculature in the acceptance of the tumor-infiltrating T cells that are a prerequisite for IL-12-induced tumor regression [12]. Our results similarly demonstrated the accumulation and uniform distribution of $CD3^+$ T cells in the tumor after intratumoral injection, thus supporting the notion that the pronounced anti-tumor effect is related to immune cell infiltration (Fig. 5). However, it remains unclear why intratumoral injection but not systemic administration induces immune cell accumulation in tumor tissue.

We also evaluated the anti-metastasis activity associated with a single intratumoral injection of AdRGD-IL-12. Metastasis is a challenge for cancer treatment, especially because almost all immunotherapy performed in the clinical setting is adjuvant treatment given after surgical reduction of the primary tumor mass for controlling recurrence and metastasis. Interestingly, the single intratumoral injection of AdRGD-IL-12 did induce anti-tumor activity toward disseminated tumors in the lung: histopathology confirmed the complete absence of metastatic tumors in eight of the nine mice tested (and only sporadic residual tumor in the remaining animal). In contrast, all mice that received intratumoral injection of the control vector developed metastases, suggesting that local expression of IL-12 also stimulates the systemic immune response to subsequently affect distant malignant cells.

All the results of our present study indicate that a single intratumoral injection of an IL-12-encoding fiber-mutant Ad vector induces T-cell infiltration into stroma-deficient Meth-A fibrosarcoma and is effective in the treatment of, and protection against challenge with, syngeneic tumors. Our results also suggest that a single intratumoral administration of AdRGD-IL-12 can induce a curative immune response in the face of a micrometastasizing tumor.

Acknowledgments

This study was supported by grants from the Ministry of Health, Labor, and Welfare of Japan and by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Cancer Gene Therapy (2005) 12, 72–83

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Immunological properties and vaccine efficacy of murine dendritic cells simultaneously expressing melanoma-associated antigen and interleukin-12

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Interleukin (IL)-12 is a key factor for inducing cellular immune responses, which play a central role in the eradication of cancer. In the present study, in order to create a dendritic cell (DC)-based vaccine capable of positively skewing immune response toward a cellular immunity-dominant state, we analyzed immunological characteristics and vaccine efficacy of DCs cotransduced with melanoma-associated antigen (gp100) and IL-12 gene (gp100 + IL12/DCs) by using RGD fiber-mutant adenovirus vector (AdRGD), which enables highly efficient gene transduction into DCs. gp100 + IL12/DCs could simultaneously express cytoplasmic gp100 and secretory IL-12 at levels comparable to DCs transduced with each gene alone. In comparison with DCs transduced with gp100 alone (gp100/DCs), upregulation of major histocompatibility complex class I, CD40, and CD86 molecules on the cell surface and more potent T-cell-stimulating ability for proliferation and interferon-γ secretion were observed as characteristic changes in gp100 + IL12/DCs. In addition, administration of gp100 + IL12/DCs, which were prepared by a relatively low dose of AdRGD-IL12, could induce more potent tumor-specific cellular immunity in the murine B16BL6 melanoma model than vaccination with gp100/DCs. However, antitumor effect and B16BL6-specific cytotoxic T-lymphocyte activity in mice vaccinated with gp100 + IL12/DCs diminished with increasing AdRGD-IL12 dose during gene transduction, and paralleled the decrease in presentation levels via MHC class I molecules for antigen transduced with another AdRGD. Collectively, our results suggested that optimization of combined vector dose was required for development of a more efficacious DC-based vaccine for cancer immunotherapy, which relied on genetic engineering to simultaneously express tumor-associated antigen and IL-12.
Cancer Gene Therapy (2005) **12,** 72–83. doi:10.1038/sj.cgt.7700772
Published online 24 September 2004

Keywords: dendritic cell; gene transduction; gp100; IL-12; melanoma

Dendritic cells (DCs) are potent professional antigen-presenting cells (APCs) that play a pivotal role in directing and amplifying the adaptive immune response to pathogens and infected/mutated cells.[1,2] The ability of DCs to stimulate naive T cells has long been thought to be crucial in initiating an effective immune response, and DCs are uniquely situated at the interface between the innate and adaptive immune systems. Because of these immunological properties, DCs are currently under intense scrutiny as potential adjuvants for vaccines in many clinical settings. Studies in healthy volunteers and patients with cancer have shown that tumor-associated antigen (TAA)-pulsed DCs can boost both $CD8^+$ and $CD4^+$ T-cell responses *in vivo*.[3–5]

Although these promising findings encourage the development of DC-based immunotherapy for cancer, the establishment of DC-manipulation capable of supplementing optimal vaccine function is required for the achievement of sufficient efficacy in current therapy. We previously established a highly efficient gene transduction technique into DCs by applying RGD fiber-mutant adenovirus vector (AdRGD),[6,7] and demonstrated that DCs transduced with TAA gene using AdRGD are effective vaccine carriers for induction of tumor-specific immune responses in mice.[8,9] These results suggested that the AdRGD system is very useful for DC-manipulation

Received April 28, 2004.
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through transduction of genes encoding immunofunctional molecules as well as for antigen gene delivery into DCs.

The immune system is roughly classified into cellular immunity and humoral immunity, and optimal immune response is achieved by balanced activation of both systems. Cellular immune response, based on the activation of natural killer (NK) cells and TAA-specific cytotoxic T lymphocytes (CTLs), plays a more important role in elimination of tumor cells by tumor immunity than humoral immune response accompanied by antibody production from B cells.[10–13] Therefore, an approach that biases immune balance toward the cellular immune response would enhance the efficacy of DC-based immunotherapy for cancer. Interleukin (IL)-12 is a 70 kDa (p70) heterodimer protein in which the 40 kDa (p40) and 35 kDa (p35) subunits are connected by one S–S bond.[14,15] IL-12 plays a key role in the induction of cellular immune responses, such as enhancement of proliferation and cytotoxic activity in NK cells and CTLs,[16,17] production of interferon (IFN)-γ from activated cells,[17–19] and promotion of differentiation of helper T-type 1 (Th1) cells from Th0 cells.[17,20,21] IFN-γ is involved in IL-12-mediated tumor regression,[22] and IL-12 also exhibits an antiangiogenic effect that can account for some antitumor activity.[23] Thus, it was strongly predicted that DCs cotransduced with the TAA and IL-12 genes may be efficacious vaccine carriers that can drastically improve effectiveness of DC-based immunotherapy for cancer by positively biasing immune balance toward cellular immunity, the Th1-dominant state, by inducing IL-12 secretion as well as by sensitizing TAA-specific CTLs via TAA-peptide presentation on major histocompatibility complex (MHC) molecules.

In the present study, by using AdRGD, which is superior in gene transduction efficiency to DCs, we created a DC vaccine that simultaneously expressed gp100, a melanoma-associated antigen, and IL-12, and investigated its immunological characteristics and vaccine efficacy.

Materials and methods

Cell lines and mice

The helper cell line, 293 cells, was obtained from JCRB cell bank (Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. CD8-OVA 1.3 cells,[24] a specific T–T hybridoma against ovalbumin $(OVA)^+$ H-$2K^b$ (kindly provided by Dr CV Harding; Department of Pathology, Case Western Reserve University, Cleveland, OH), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol (2-ME), and antibiotics. Murine melanoma B16BL6 cells (H-2^b; JCRB cell bank) were grown in minimum essential medium supplemented with 7.5% FBS and antibiotics. EL4 cells, a T-lymphoma cell line of C57BL/6 origin, and YAC-1 cells, a lymphoma cell line highly sensitive to NK cells, were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, and antibiotics. Female C57BL/6 mice (H-2^b) and female BALB/c mice (H-2^d), ages 7–8 weeks, were purchased from SLC Inc. (Hamamatsu, Japan). All mice were held under specific pathogen-free conditions and the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Vectors

Replication-deficient AdRGD was based on the adenovirus serotype 5 backbone with deletions of regions E1 and E3. The RGD sequence for αv-integrin-targeting was inserted into the HI loop of the fiber knob using a two-step method as previously described.[25] AdRGD-IL12,[26] AdRGD-gp100,[7] AdRGD-OVA,[8] and AdRGD-Luc[25] were previously constructed by an improved *in vitro* ligation method,[25,27,28] and encoded the murine IL-12 gene derived from mIL12 BIA/pBluescript II KS(-) (kindly provided by Dr H Yamamoto; Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan), the human gp100 gene derived from pAx1-CA h-gp100 (kindly provided by Dr H Hamada; Department of Molecular Medicine, Sapporo Medical University, Sapporo, Japan), the OVA gene derived from pAc-neo-OVA (kindly provided by Dr MJ Bevan; Department of Immunology, Howard Hughes Medical Institute, University of Washington, Seattle, WA), and the luciferase gene derived from pGL3-Control (Promega, Madison, WI), respectively. All recombinant AdRGDs were propagated in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C. Titers of infective AdRGD particles were evaluated by the end point dilution method using 293 cells.

Generation and viral transduction of DCs

DCs were prepared according to the method of Lutz et al[29] with slight modification. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 mice were seeded at 0.5–1 × 10^7 cells per sterile 100-mm bacterial grade culture dish in 10 ml of RPMI 1640 containing 10% FBS, 40 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; kindly provided by KIRIN Brewery Co., LTD, Tokyo, Japan), 50 μM 2-ME, and antibiotics. On day 3, another 10 ml of culture medium was added to the dish for medium replenishment. On day 6, 10 ml of the culture supernatant was collected and centrifuged at 1500 rpm for 5 minutes at room temperature, and the pellet was resuspended in 10 ml of fresh culture medium, and then returned to the original dish to conserve unattached cells. On day 8, nonadherent cells were harvested and used as immature DCs. DCs cultured for another 24 hours with media containing 1 μg/ml lipopolysaccharide (LPS; Nacalai Tesque, Inc., Kyoto, Japan) were used as phenotypically mature DCs (LPS/DCs). In transduction using AdRGDs,

DCs were suspended at a concentration of 5×10^6 cells/ml in FBS-free RPMI 1640 and placed in a 15-ml conical tube. Each AdRGD was added at various multiplicity of infections (MOIs), the suspension was mixed well, and the tube was incubated at 37°C for 2 hours with occasional gentle agitation. The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in a suitable solution for subsequent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Transduced DCs and mock DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium for 24 hours. Total RNA was isolated from these cells and LPS/DCs using Sepasol-RNA I Super (Nacalai Tesque, Inc.) according to the manufacturer's instructions. RT proceeded for 60 minutes at 42°C in a 50 μl reaction mixture containing 5 μg total RNA treated with DNase I, 10 μl 5 × RT buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 1 μM random primer (9-mer), 1 μM oligo$(dT)_{20}$, and 100 U ReverTra Ace (TOYOBO Co., LTD, Osaka, Japan). PCR amplification of the gp100, IL-12p35, IL-12p40, and β-actin transcripts was performed in 50 μl of a reaction mixture containing 1 μl of RT-material, 5 μl 10 × PCR buffer, 1.25 U *Taq* DNA polymerase (TOYOBO Co., LTD), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 μM primers. The sequences of the specific primers were as follows: human gp100: forward, 5′-tgg aac agg cag ctg tat cc-3′; reverse, 5′-cct aga act tgc cag tat tgg c-3′; murine IL-12p35: forward, 5′-tgt tta cca ctg gaa cta cac aag a-3′; reverse, 5′-aga gct tca ttt tca ctc tgt aag g-3′; murine IL-12p40: forward, 5′-ctc acc tgt gac acg cct ga-3′; reverse, 5′-cag gac act gaa tac ttc tc-3′; murine β-actin: forward, 5′-tgt gat ggt ggg aat ggg tca g-3′; reverse, 5′-ttt gat gtc acg cac gat ttc c-3′. After denaturation for 2 minutes at 95°C, 20 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 48°C (for IL-12p40), 58°C (for IL-12p35), or 60°C (for gp100 and β-actin), and extension for 30 seconds at 72°C were repeated and followed by completion for 4 minutes at 72°C. The PCR product was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet radiation. EZ Load (BIO-RAD, Tokyo, Japan) was used as a 100 bp-molecular ruler. The expected PCR product sizes were 362 bp (gp100), 334 bp (IL-12p35), 431 bp (IL-12p40), and 514 bp (β-actin).

Intracellular staining method for human gp100 protein in transduced DCs

Transduced DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium for 24 hours. Cells (1×10^6) were fixed by incubation for 10 minutes in 2% paraformaldehyde, and then cell membranes were permeabilized by incubation for 5 minutes in 1% saponin. The cells were incubated with 100 μl staining buffer (PBS containing 0.1% bovine serum albumin and 0.01% NaN_3) containing the anti-FcγRII/III monoclonal antibody (mAb), 2.4G2 (rat $IgG_{2b,\kappa}$; BD Biosciences, San Jose, CA), to block nonspecific binding of the subsequently used mAbs. After 30 minutes, the cells were incubated for 60 minutes with 100 μl staining buffer containing the HMB50 mAb against human gp100 (mouse IgG_{2a}; Neomarkers, Fremont, CA), and then resuspended in 100 μl staining buffer containing fluorescein isothiocyanate-conjugated anti-mouse Ig_κ (187.1; BD Biosciences). After incubation for 30 minutes, 30,000 events of the stained cells were analyzed for human gp100 protein expression by a FACScalibur flow cytometer using CellQuest software (BD Biosciences). Between all incubation steps, cells were washed three times with staining buffer.

Evaluation of IL-12 secretion level in transduced DCs

Transduced DCs, LPS/DCs, and mock DCs were cultured on 24-well plates at 5×10^5 cells/500 μl in GM-CSF-free culture medium for 24 hours. The supernatants were collected and the IL-12 level was measured using a murine IL-12p40 ELISA KIT and a murine IL-12p70 ELISA KIT (Endogen, Rockford, IL).

Analysis of surface marker-expression

All immunoreagents used in this experiment were purchased from BD Biosciences. Transduced DCs, LPS/DCs, and mock DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium for 24 hours. Cells (1×10^6) in 100 μl staining buffer were incubated for 30 minutes on ice with the 2.4G2 mAb. Then, cells were resuspended in 100 μl staining buffer and incubated for 30 minutes on ice using the manufacturer's recommended amounts of biotinylated mAbs: 28-8-6 (anti-H-$2K^b$/D^b), AF6-120.1 (anti-I-A^b), 3/23 (anti-CD40), 16-10A1 (anti-CD80), and GL1 (anti-CD86). The cells were then resuspended in 100 μl staining buffer containing phycoerythrin-conjugated streptavidin at a 1:200 dilution, and nonspecific binding was measured using phycoerythrin-conjugated streptavidin alone. After incubation for 30 minutes on ice, 30,000 events of the stained cells were analyzed for surface phenotype by flow cytometry. Between all incubation steps, cells were washed three times with staining buffer.

Antigen-presentation assay

C57BL/6 DCs were transduced with various combinations of AdRGD-OVA, AdRGD-IL12, and AdRGD-Luc, and then seeded on a 96-well flat-bottom culture plate at a density of 1×10^5 cells/well. These cells were cocultured with 1×10^5 cells/well CD8-OVA 1.3 cells at 37°C for 20 hours. The response of stimulated CD8-OVA 1.3 cells was assessed by determining the amount of IL-2 released into an aliquot of culture medium (100 μl) using a murine IL-2 ELISA KIT (Amersham Biosciences, Piscataway, NJ).

Mixed leukocyte reaction (MLR)

Three kinds of T cells, including allogeneic naive T cells from intact BALB/c mice, syngeneic naive T cells from intact C57BL/6 mice, and syngeneic gp100-primed T cells

from C57BL/6 mice immunized intradermally with 1×10^6 DCs transduced with AdRGD-gp100 at 25 MOI 1 week earlier, were purified from the splenocytes of each mouse as nylon wool nonadherent cells, and were used as responder cells at 1×10^5 cells/well in 96-well plates. Transduced DCs, LPS/DCs, or mock DCs (stimulator cells) were inactivated by 50 μg/ml mitomycin C (MMC) for 30 minutes and added to responder cells in varying cell numbers. Cells were cocultured in 100 μl RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and antibiotics at 37°C and 5% CO_2 for 3 days. Control wells contained either stimulator cells alone or responder cells alone. Cell cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU) during the last 18 hours, and then proliferation of responder cells was evaluated by Cell Proliferation ELISA, BrdU (Roche Diagnostics Co., Indianapolis, IN).

Analysis of Th1/Th2 cytokine secretion from syngeneic T cells stimulated by transduced DCs

C57BL/6 naive T cells were purified from splenocytes as nylon wool nonadherent cells, and were used as responder cells at 5×10^6 cells/well in 24-well plates. Transduced DCs (stimulator cells) were added to responder cells at 5×10^5 cells/well. Cells were cocultured in 1 ml RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 10 U/ml recombinant murine IL-2 (PeproTech EC LTD, London, England) and antibiotics at 37°C and 5% CO_2 for 5 days. The supernatants were collected and the IFN-γ, IL-4, and IL-10 levels were measured using a murine IFN-γ ELISA KIT, a murine IL-4 ELISA KIT, and a murine IL-10 ELISA KIT (Biosource International, Camarillo, CA), respectively.

Tumor protection assay

Transduced DCs were intradermally injected into the left flank of C57BL/6 mice at 2×10^5 cells/50 μl. At 1 week after the vaccination, 2×10^5 B16BL6 melanoma cells were intradermally inoculated into the right flank of the mice. The major and minor axes of the tumor were measured using microcalipers, and the tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) $\times$ (minor axis; mm)$^2 \times 0.5236$. The mice were euthanized when one of the two measurements was greater than 20 mm.

Europium (Eu)-release assay for cytolytic activity of NK cells and CTLs

Transduced or mock DCs were administered once intradermally into C57BL/6 mice at 2×10^5 cells/50 μl. At 1 week after immunization, nonadherent splenocytes were prepared from these mice and directly used as NK effector cells. The splenocytes were restimulated *in vitro* using B16BL6 cells, which were cultured in media containing 100 U/ml recombinant murine IFN-γ (PeproTech EC LTD) for 24 hours and inactivated with 50 μg/ml MMC at 37°C for 30 minutes, at an effector:stimulator ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and antibiotics. After 5 days, the splenocytes were collected and used as CTL effector cells. Target cells (YAC-1 and EL4 cells for NK assay; IFN-γ-stimulated B16BL6 and IFN-γ-stimulated EL4 cells for CTL assay) were Eu-labeled and an Eu-release assay was performed as previously described.[30] Cytolytic activity was determined using the following formula: (% of lysis) = [(experimental Eu-release – spontaneous Eu-release)/(maximum Eu-release – spontaneous Eu-release)] $\times$ 100. Spontaneous Eu-release of the target cells was <10% of maximum Eu-release by detergent in all assays.

Results

Gene expression in DCs cotransduced with gp100 and IL-12

We examined the cytopathic effects of AdRGD-IL12 and AdRGD-gp100 on gene transduction into DCs by using the Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan). At 48 hours posttransduction, viability of DCs transduced with AdRGD-IL12 alone or AdRGD-IL12 plus AdRGD-gp100 remained more than 90% at a vector dose of 200 MOI or less (data not shown). Expression levels of human gp100 and murine IL-12 subunit mRNAs in various transduced DCs were analyzed by RT-PCR at 24 hours after gene transduction (Fig 1). Human gp100-specific PCR products were detected at the same level only in DCs that were transduced with AdRGD-gp100 alone at 25 MOI (lane 1) or the combination of AdRGD-gp100 at 25 MOI and AdRGD-IL12 at various MOI (lanes 3, 4, and 5). PCR

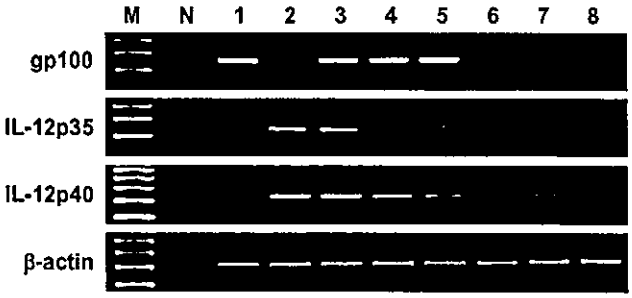


Figure 1 RT-PCR analysis of gp100, IL-12p35, and IL-12p40 in DCs cotransduced with AdRGD-gp100 and AdRGD-IL12. Total RNA was prepared from transduced, LPS-stimulated, or mock DCs, and then RT-PCR was performed as described in the Materials and methods section. The PCR products were electrophoresed through a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Lane M; 100 bp-molecular ruler, lane N; H_2O as template, lane 1; DCs transduced with AdRGD-gp100 alone at 25 MOI, lane 2; DCs transduced with AdRGD-IL12 alone at 25 MOI, lane 3; DCs cotransduced with AdRGD-gp100 at 25 MOI and AdRGD-IL12 at 25 MOI, lane 4; DCs cotransduced with AdRGD-gp100 at 25 MOI and AdRGD-IL12 at 12.5 MOI, lane 5; DCs cotransduced with AdRGD-gp100 at 25 MOI and AdRGD-IL12 at 5 MOI, lane 6; DCs transduced with AdRGD-Luc alone at 25 MOI, lane 7; LPS-stimulated (mature) DCs, lane 8; mock (immature) DCs.

products of IL-12p35 and IL-12p40 mRNA increased in an MOI-dependent manner for AdRGD-IL12 in DCs cotransduced with AdRGD-gp100 and AdRGD-IL12 (lanes 3, 4, and 5), and their levels were equal in DCs transduced with AdRGD-IL12 alone at 25 MOI (lane 2) and DCs cotransduced with AdRGD-gp100 at 25 MOI and AdRGD-IL12 at 25 MOI (lane 3). In addition, DCs transduced with AdRGD-gp100 alone (lane 1) or AdRGD-Luc (control vector) alone (lane 6) exhibited slightly higher IL-12p40 mRNA expression than mock DCs (lane 8), and IL-12p40 mRNA levels of LPS/DCs (lane 7) were comparable with those of DCs cotransduced with AdRGD-gp100 at 25 MOI and AdRGD-IL12 at 5 MOI (lane 5).

In order to confirm cytoplasmic expression of human gp100 protein, we performed flow cytometric analysis by the intracellular staining method using HMB50 mAb (Fig 2a). Under transductional conditions using AdRGD-gp100 at 25 MOI, about 70% of DCs could express gp100 protein in their cytoplasm whether or not the DCs were cotransduced individually with AdRGD-IL12 or AdRGD-Luc at 25 MOI. Gene expression intensity (mean fluorescence intensity; MFI) of gp100 in cotransduced DCs was also comparable to that of DCs transduced with AdRGD-gp100 alone. These data clearly demonstrated that the expression level of endogenous antigen transduced with AdRGD was not affected by cotransduction of AdRGD-IL12 in DCs. Likewise, IL-12 secretion levels in DCs transduced with various combinations of AdRGD-gp100 and AdRGD-IL12 were investigated by ELISA (Fig 2b). MOI-dependent IL-12p70, the biologically active form, and IL12p40 secretion into culture media of DCs cotransduced with AdRGD-gp100 and AdRGD-IL12 was detected at levels equivalent to those of DCs transduced with AdRGD-IL12 alone. Although LPS/DCs could secrete a large quantity of IL-12p40 into culture media, secretion of the active form, IL-12p70, was maintained at low levels, reflecting the low levels of IL-12p35 mRNA expression in RT-PCR analysis.

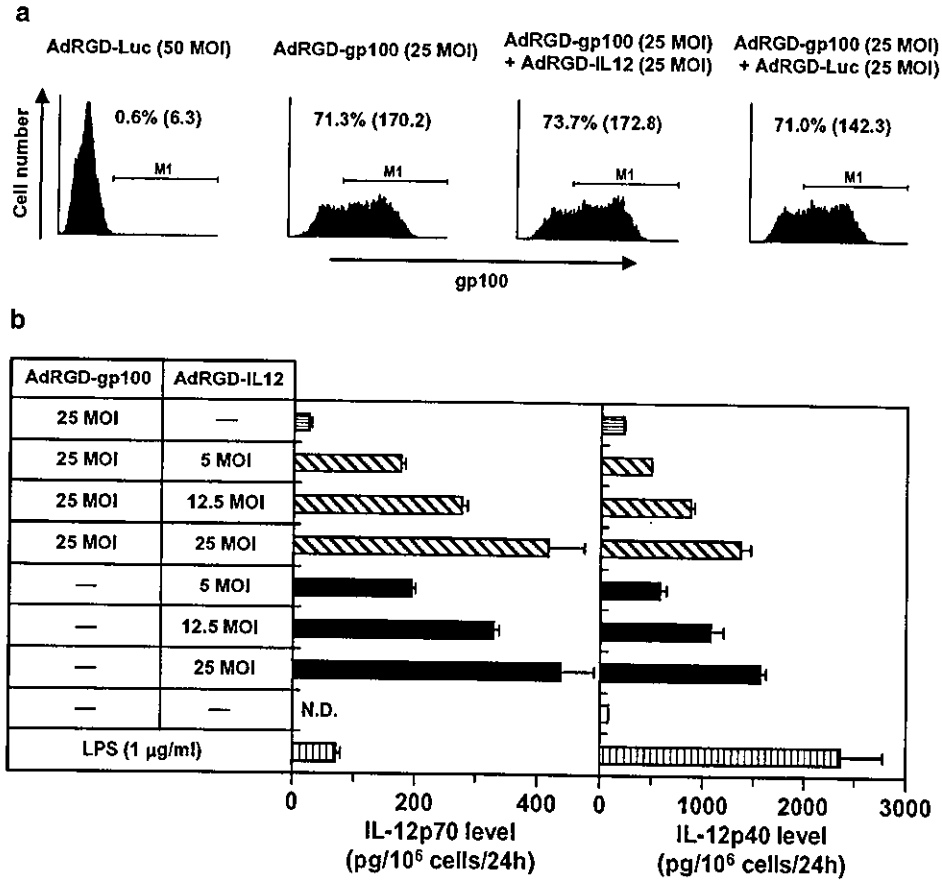


Figure 2 gp100 and IL-12 expression in DCs cotransduced with AdRGD-gp100 and AdRGD-IL12. (a) DCs were transduced with the indicated combinations of various AdRGDs at the indicated MOI for 2 hours. At 24 hours posttransduction, human gp100 gene expression was assessed by flow cytometric analysis. The percentage value and the numerical value in the parenthesis express percentage of M1-gated cells and mean fluorescence intensity (MFI), respectively. The data are representative of two independent experiments. (b) DCs were cotransduced with AdRGD-IL12 and AdRGD-gp100 at the indicated MOI for 2 hours. DCs treated with 1 μg/ml LPS for 24 hours were used as positive controls for phenotypical DC-maturation. After 24 hours cultivation, concentration of murine IL-12p70 and IL-12p40 in culture supernatants was measured by ELISA. The data are presented as mean ± SD of three independent cultures. ND: IL-12p70 secreted from DCs was not detectable.

Immunological characteristics of DCs cotransduced with gp100 and IL-12

We first analyzed the expression levels of MHC/costimulatory molecules by flow cytometry in DCs prepared with various combinations of AdRGD-gp100 and AdRGD-IL12 (Fig 3). In comparison with mock DCs, DCs transduced with AdRGD-gp100 alone exhibited upregulated expression of all tested surface marker molecules, which play critical roles in the sensitization/activation of T cells, as was seen in mature LPS/DCs. This result agreed with our previous report demonstrating that transduction using AdRGD, irrespective of the type of inserted transgene, could enhance the expression of MHC/costimulatory molecules on DCs.[7] In addition, enhanced expression of MHC class I, CD40 and CD86 was observed as a characteristic change in DCs cotransduced with AdRGD-gp100 and AdRGD-IL12 as well as DCs transduced with AdRGD-IL12 alone. As upregulation of these molecules was dependent on MOI of combined AdRGD-IL12, the results suggested that the secreted IL-12 promoted maturation of DCs by an autocrine mechanism.

Next, we compared antigen presentation levels via MHC class I molecules by bioassay using T–T hybridoma, CD8-OVA 1.3 cells, between DCs transduced with various combinations of AdRGD-OVA, AdRGD-IL12, and AdRGD-Luc (Fig 4). In comparison with DCs transduced with AdRGD-OVA alone, DCs cotransduced with AdRGD-OVA and AdRGD-IL12 showed a slight decrease in IL-2 released from CD8-OVA 1.3 cells by vector dose-increase of combined AdRGD-IL12. The OVA-presentation levels in AdRGD-OVA-transduced DCs were not affected by the addition of exogenous recombinant murine IL-12 into culture media during the antigen-presentation assay (data not shown). In addition, an obvious decline in the OVA-presentation level was observed in DCs cotransduced with AdRGD-OVA and AdRGD-Luc, suggesting that competition may occur during a particular step of the MHC class I

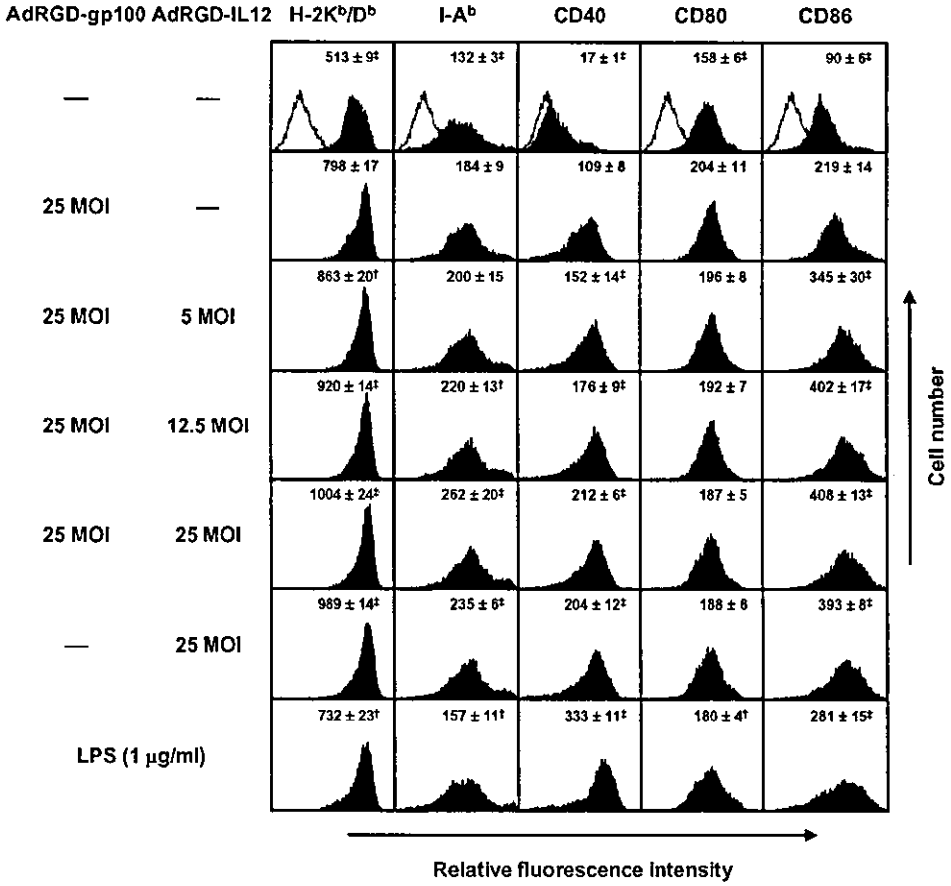
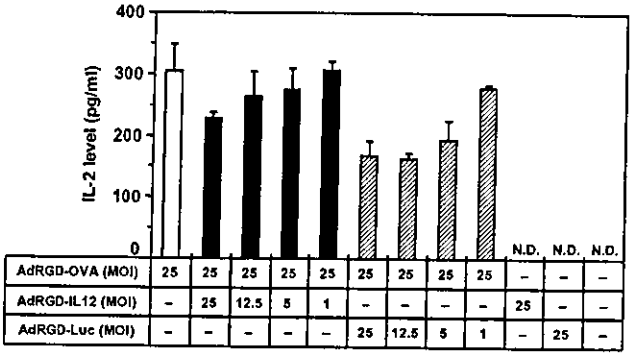


Figure 3 Flow cytometric analysis of surface markers in DCs cotransduced with gp100 and IL-12 gene by AdRGD. DCs were cotransduced with AdRGD-gp100 and AdRGD-IL12 at the indicated MOI for 2 hours. DCs treated with 1 μg/ml LPS for 24 hours were used as positive controls for phenotypical DC maturation. At 24 hours after transduction, cells were stained by indirect immunofluorescence using biotinylated mAbs of the indicated specificities (solid histogram). Dotted histograms represent cells stained by phycoerythrin-conjugated streptavidin alone. The data are representative of three independent experiments, and values indicated in the upper part of each panel represent MFI (mean ± SD) of flow cytometric analysis. The statistical analysis was carried out by Student's *t*-test. †$P<.05$, ‡$P<.01$ *versus* DCs transduced with AdRGD-gp100 alone at 25 MOI.



AdRGD-OVA (MOI)	25	25	25	25	25	25	25	25	25	–	–	–
AdRGD-IL12 (MOI)	–	25	12.5	5	1	–	–	–	–	25	–	–
AdRGD-Luc (MOI)	–	–	–	–	–	25	12.5	5	1	–	25	–

Figure 4 Antigen presentation on MHC class I molecules by DCs cotransduced with OVA and IL-12 gene by AdRGD. DCs were cotransduced with AdRGD-OVA and either AdRGD-IL12 or AdRGD-Luc at the indicated MOI for 2 hours. The levels of OVA peptide presentation via MHC class I molecules by transduced DCs were determined by bioassay using CD8-OVA 1.3 cells. The data represent the mean±SD of three independent cultures. ND: IL-2 secreted from CD8-OVA 1.3 cells was not detectable.

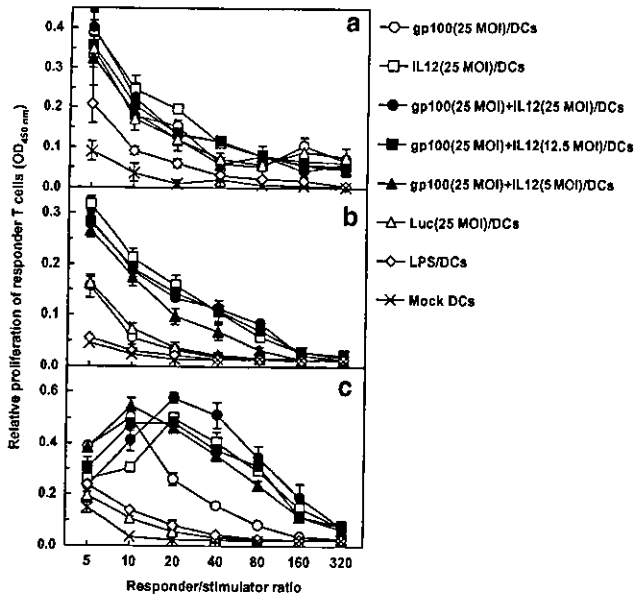


Figure 5 Allogeneic and syngeneic T-cell proliferation-stimulating ability of DCs cotransduced with gp100 and IL-12 gene by AdRGD. C57BL/6 DCs were transduced with the indicated combinations of various AdRGDs at the indicated MOI for 2 hours. Naive BALB/c T lymphocytes (**a**), naive C57BL/6 T lymphocytes (**b**), or gp100-primed C57BL/6 T lymphocytes (**c**), were cocultured with transduced, LPS-stimulated, or mock DCs at different responder/stimulator ratios for 3 days. Cell cultures were pulsed with BrdU during the last 18 hours, and then T-cell proliferation was assessed by BrdU-ELISA. Results are expressed as mean±SE of three independent cultures using T cells prepared from three individual mice.

antigen-presentation pathway in DCs simultaneously expressing distinct proteins due to the presence of multiple AdRGDs. The variation in effects of coexpressed endogenous antigens in DCs might be induced by proteins accumulating in the cytoplasm and secreted to extracellular fluid, such as luciferase and IL-12, respectively. Taken together, antigen-presenting levels via MHC class I molecules on DCs transduced with AdRGD encoding antigen were slightly decreased by increasing dose of combined AdRGD-IL12.

T-cell-stimulating ability of DCs cotransduced with gp100 and IL-12

We performed allogeneic and syngeneic MLR to compare T-cell proliferation-stimulating ability of DCs transduced with AdRGD-gp100 alone or a combination of AdRGD-gp100 and AdRGD-IL12. DCs transduced with AdRGD-gp100 alone at 25 MOI (gp100(25MOI)/DCs), AdRGD-IL12 alone at 25 MOI (IL12(25MOI)/DCs), AdRGD-Luc alone at 25 MOI (Luc(25MOI)/DCs), or various combinations of AdRGD-gp100 and AdRGD-IL12 (gp100 (25MOI) + IL12(25MOI)/DCs, gp100(25MOI) + IL12(12.5 MOI)/DCs, and gp100(25MOI) + IL12(5MOI)/DCs) could equally stimulate proliferation of allogeneic naive T cells used as responder cells (Fig 5a). In addition, T-cell proliferation levels in these groups were higher than those not only in mock DCs but also LPS/DCs. These data indicated that DCs transduced by using AdRGD could sufficiently provide proliferative stimuli to T cells through allogeneic interaction of MHC molecules/T-cell receptors and costimulatory signals, regardless of the quantity of IL-12 secreted from DCs. On the other hand, IL12(25-MOI)/DCs, gp100(25MOI) + IL12(25MOI)/DCs, gp100 (25MOI) + IL12(12.5MOI)/DCs, and gp100(25MOI) + IL12(5MOI)/DCs could more strongly stimulate syngeneic naive T-cell proliferation as compared with gp100(25MOI)/DCs or Luc(25MOI)/DCs (Fig 5b), suggesting that *in vitro* syngeneic naive T-cell proliferation by DCs cotransduced with AdRGD-gp100 and AdRGD-IL12 was greatly influenced by secreted IL-12 rather than antigen-presentation via MHC molecules. Furthermore, in comparison with gp100(25MOI)/DCs, DCs transduced with AdRGD-IL12 alone or in combination with AdRGD-gp100 could induce considerable proliferation of syngeneic gp100-primed T cells, which were purified from C57BL/6 mice vaccinated beforehand with 10^6 gp100(25MOI)/DCs (Fig 5c). However, proliferation levels of syngeneic gp100-primed T cells stimulated by DCs transduced with AdRGD-IL12 alone or in combination with AdRGD-gp100 decreased at high responder/stimulator ratios, and this suppressive effect became remarkable at high AdRGD-IL12 MOI during gene transduction. These observations suggested that excessive IL-12 secreted from transduced DCs might inhibit proliferation or induce cell death in activated T cells.

In addition, we assessed by ELISA the Th1/Th2 cytokine balance in media of syngeneic naive T cells cocultured with various transduced DCs for 5 days at a responder/stimulator ratio of 10 in the presence of 10 U/ml recombinant murine IL-2 (Table 1). IL12(25MOI)/DCs, gp100(25MOI) + IL12(25MOI)/DCs, gp100(25MOI) + IL12(12.5MOI)/DCs, and gp100(25MOI) + IL12(5MOI)/DCs could markedly enhance Th1-skewing IFN-γ secretion from syngeneic naive T cells as compared with mock

Table 1 Cytokine secretion from syngeneic naive T cells cocultured with various transduced DCs

DC treatment				
AdRGD-gp100 (MOI)	AdRGD-IL12 (MOI)	IFN-γ (ng/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
—	—	0.25±0.05	<15	<30
25	—	1.00±0.19	<15	<30
—	25	17.17±0.48	<15	<30
25	25	25.78±1.84	<15	<30
25	12.5	18.61±1.47	<15	<30
25	5	20.23±2.96	<15	<30

Data are expressed as mean±SD of three independent cultures.

DCs, whereas only a slight increase in IFN-γ levels was observed during cocultivation with gp100(25MOI)/DCs. We confirmed that IFN-γ secretion was undetectable in control wells in which only transduced or mock DCs were cultured. On the other hand, secretion of the Th2 cytokines, IL-4 and IL-10, was not detectable in any syngeneic T cells stimulated by transduced or mock DCs. These results suggested that DCs cotransduced with gp100 and IL-12 could more efficiently differentiate sensitized T cells at the Th1-biasing state (the cellular immunity-dominant state), which is required for the induction of efficacious tumor immunity.

Vaccine efficacy of DCs cotransduced with gp100 and IL-12

In order to evaluate the potency of DCs cotransduced with gp100 and IL-12 as vaccine carriers, we investigated protective efficacy against murine B16BL6 melanoma challenge (Fig 6). C57BL/6 mice received a single intradermal injection of 2×10^5 DCs transduced with various combinations of AdRGD-gp100, AdRGD-IL12, and AdRGD-Luc, and then these mice were inoculated with 2×10^5 B16BL6 melanoma cells at 1 week post-immunization. Obvious growth suppression of the challenging B16BL6 tumor was achieved in mice vaccinated with gp100(25MOI)/DCs, as shown in our previous report,[9] whereas the mice immunized with IL12(25-MOI)/DCs or Luc(25MOI)/DCs showed little or no protective effect as compared with vehicle-injected mice. In addition, a more potent inhibitory effect on tumor growth could be observed in mice after vaccination with gp100(25MOI)+IL12(5MOI)/DCs than in mice vaccinated with gp100(25MOI)/DCs. However, vaccine efficacy of DCs cotransduced with gp100 and IL-12 tended to diminish with increasing AdRGD-IL12 MOI during gene transduction, and immunization with gp100(25MOI)+Luc(25MOI)/DCs led to inferior anti-tumor effects compared to those of the gp100(25MOI)/DCs group.

Furthermore, we investigated the cytolytic activities of NK cells and CTLs in mice intradermally immunized with DCs cotransduced with gp100 and IL-12 by Eu-release

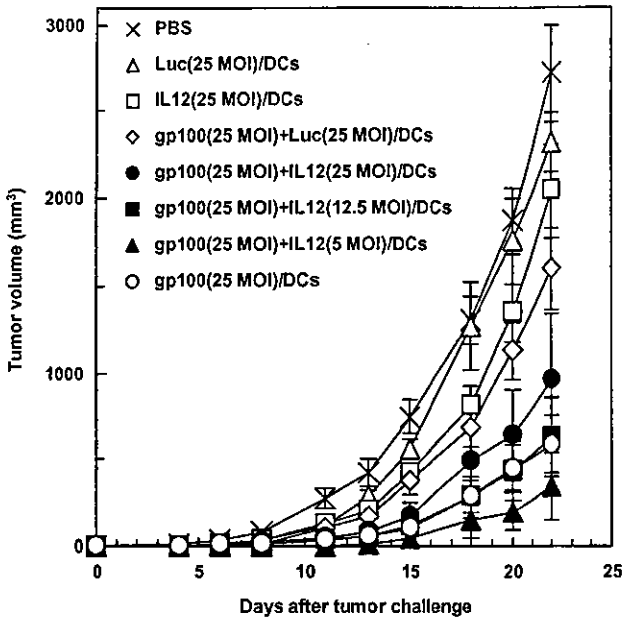
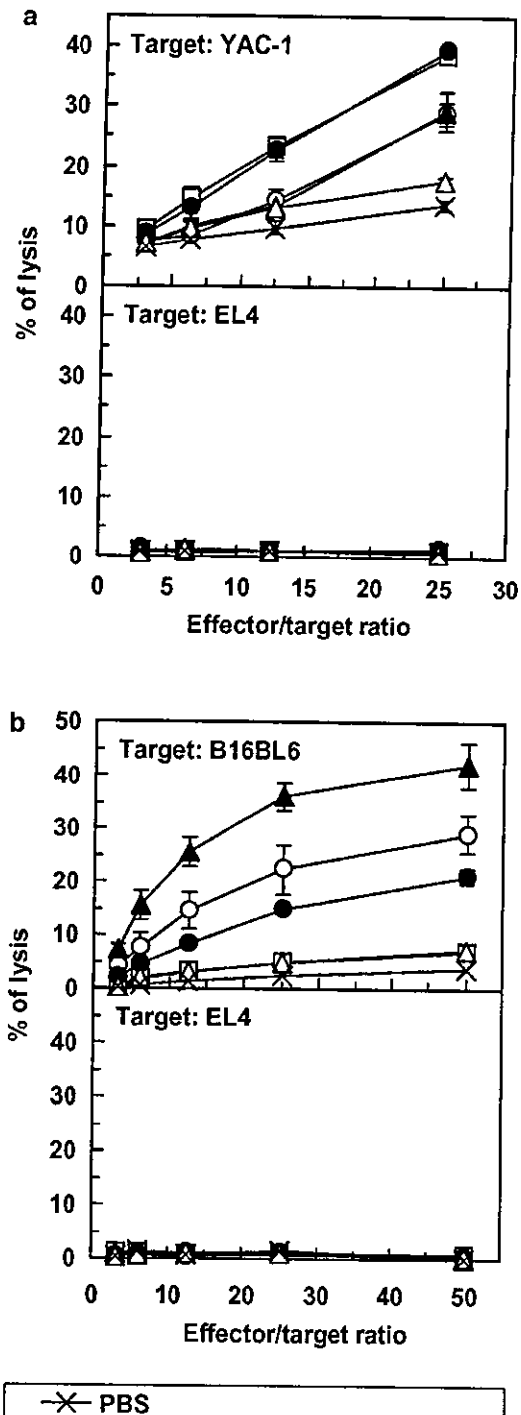


Figure 6 Vaccine efficacy of DCs cotransduced with gp100 and IL-12 gene by AdRGD against B16BL6 melanoma challenge. DCs were transduced with the indicated combinations of various AdRGDs at the indicated MOI for 2 hours. C57BL/6 mice were immunized by intradermal injection of transduced DCs into the left flank at 2×10^5 cells, and then 2×10^5 B16BL6 melanoma cells were inoculated into the right flank of the mice 1 week postvaccination. The size of tumors was assessed using microcalipers three times per week. Each point represents the mean±SE of 6–12 mice. Statistical analysis of tumor volume on day 22 after tumor challenge was carried out by Mann-Whitney *U*-test. $P<.01$ (■, ▲, ○), $P<.05$ (●), not significant (×, □, ◇) *versus* Luc(25MOI)/DCs (△). $P<.01$ (×, △, □, ◇), not significant (●, ■, ▲) *versus* gp100(25MOI)/DCs (○).

assay. At 1 week after immunization of C57BL/6 mice with various DC vaccines, the splenocytes were used in a cytolytic assay against YAC-1 and EL4 cells, and were restimulated *in vitro* with inactivated B16BL6 cells, which were treated with recombinant murine IFN-γ to promote the expression of their MHC class I molecules, for CTL expansion. As shown in Figure 7a, the splenic cytolytic activity against YAC-1 cells markedly increased after immunization with gp100(25MOI)+IL12(25MOI)/DCs as well as IL12(25MOI)/DCs, whereas EL4 cells were not injured by splenocytes prepared from any groups. Effector cells from mice immunized with gp100(25MOI)+IL12(5MOI)/DCs exhibited equivalent NK activity to those from mice immunized with gp100(25MOI)/DCs. These data indicated that the non-specific NK activity involved in the anti-B16BL6 melanoma response was enhanced with the increase in IL-12 secretion from the administered DC vaccine. On the other hand, the cytolytic effects on B16BL6 cells by *in vitro* restimulated effector cells was promoted in mice immunized with gp100(25MOI)+IL12(5MOI)/DCs as compared with mice immunized with gp100(25MOI)/DCs (Fig 7b). This cytolytic activity was caused by B16BL6-specific CTLs because the effector cells prepared from

mice immunized with IL12(25MOI)/DCs or mock DCs did not injure the B16BL6 cells and no cytolytic effects against syngeneic irrelevant EL4 cells were detected in any group. However, consistent with the protective effect against B16BL6 tumor challenge, mice vaccinated with gp100(25MOI) + IL12(25MOI)/DCs showed lower B16BL6-specific CTL activity than mice immunized with gp100(25MOI)/DCs.

Therefore, immunization with DCs genetically modified to express simultaneously gp100 and IL-12 exhibited duplicity for the host's immune response in our experimental model. That is, as compared with DCs transduced with AdRGD-gp100 alone, DCs cotransduced with AdRGD-IL12 at a relatively low ratio to AdRGD-gp100 were equal in inducibility of NK activity, but could more efficiently induce anti-B16BL6 tumor effects and antigen-specific CTL activity. In contrast, DCs combined with a high dose of AdRGD-IL12 during gene transduction could enhance NK activity, but attenuated B16BL6-protective efficacy and CTL activity.



Discussion

Since DCs are the most potent APCs and are uniquely capable of presenting novel antigens to naive T cells to initiate and modulate immune responses,[1,2] various DC-based vaccines for use in immune intervention strategies against cancer have been designed and studied in many research organizations. Antitumor CTLs play a central role in the tumor-specific immune response, and the efficient priming and subsequent activation of antitumor CTLs requires the processing and presentation of TAAs as peptide fragments in the context of appropriate MHC class I molecules by APCs.[31] In addition, a Th1-biased cytokine balance is desirable for sensitization of CTLs specific for TAA by APCs. IL-12 is the key factor that skews the immune balance toward a Th1 response and that can promote a switch from an established Th2 to a Th1 response.[32,33] In fact, potent antitumor effects of DCs genetically engineered with IL-12 have been demonstrated in several murine models by vaccination using TAA-derived peptide pulsed DCs or intratumoral injection using unpulsed DCs.[34–36] Therefore, we believe that, as compared with DCs delivered with TAA gene alone, DCs genetically manipulated to express simultaneously TAA and IL-12 might be a promising vaccine carrier

Figure 7 NK (a) and B16BL6-specific CTL (b) activity in mice immunized with DCs cotransduced with gp100 and IL-12 gene by AdRGD. DCs were transduced with the indicated combinations of AdRGD-gp100 and AdRGD-IL12 at the indicated MOI for 2 hours. Transduced or mock DCs were vaccinated once intradermally into C57BL/6 mice at 2×10^5 cells. At 1 week after immunization, nonadherent splenocytes were prepared from these mice, and directly used in cytolytic assays against YAC-1 and EL-4 cells (a). In addition, the isolated splenocytes were restimulated *in vitro* for 5 days with IFN-γ-stimulated and MMC-inactivated B16BL6 cells. A cytolytic assay using the restimulated splenocytes was performed against IFN-γ-stimulated B16BL6 and EL4 cells (b). Each point represents the mean ± SE of four independent cultures from four individual mice.

capable of generating more efficacious antitumor responses because of their ability to induce Th1-polarized responses.

A vector system, which can effectively deliver a foreign gene to DCs, is required to create a genetically modified DC vaccine having a potential to improve the efficacy of DC-based immunotherapy. We have demonstrated that AdRGD enabled highly efficient gene transduction into murine and human DCs because of the targeting of αv-integrin by the RGD sequence inserted at the HI-loop in their fiber knob.[6–9] When mouse bone marrow-derived DCs were cotransduced with AdRGD-gp100 and AdRGD-IL12, their expression of gp100 and IL-12 was comparable to that in DCs transduced with each AdRGD alone (Figs 1 and 2). IL-12 secreted from these DCs is biologically active because direct intratumoral injection of AdRGD-IL12, used in the present study and whose expression cassette was designed to be transcribed from IL-12p35 cDNA to the internal ribosome entry site sequence to IL-12p40 cDNA under the control of the cytomegalovirus promoter, could induce tumor regression based on promotion of tumor immunity in melanoma-bearing mice.[37] RT-PCR analysis (Fig 1) suggested that LPS-driven maturation or irrelevant AdRGD transduction could moderately enhance expression of the IL-12p40 subunit mRNA in DCs, but not IL-12p35 subunit mRNA. Several reports have demonstrated that the production of the two IL-12 subunits is regulated by different mechanisms, mainly at the level of mRNA expression, and that the level of bioactive IL-12p70 production in APCs in response to LPS and cytokines is determined by the level of IL-12p35 expression.[38,39] Therefore, DCs cannot attain sufficient IL-12p70 productivity, which is based on enhancement of endogenous gene expression of the two subunits, via a maturation signal from LPS or AdRGD transduction alone. Taken together, our results demonstrated that DCs cotransduced with AdRGD-gp100 and AdRGD-IL12 could simultaneously express gp100 and IL-12 at levels equal to DCs transduced with each vector alone, indicating that DCs can obtain several additional functions by using a combination of AdRGDs carrying different genes.

Full analysis and understanding of immunological characteristics of DC vaccine are imperative for the development of DC-based immunotherapy because the polarity of the immune response is greatly influenced by the activated state of DCs during T-cell sensitization. Flow cytometric analysis indicated that the expression of MHC class I/II, CD40, CD80, and CD86 molecules was enhanced only by AdRGD-transduction, and that DCs transduced with the IL-12 gene exhibited considerable upregulation of MHC class I, CD40, and CD86 molecules on their surface in response to autocrine effects of secreted IL-12 as compared with DCs transduced with AdRGD-gp100 alone (Fig 3). On the other hand, DCs cotransduced with OVA and IL-12 by AdRGDs exhibited lower OVA-presentation levels via MHC class I molecules than DCs transduced with AdRGD-OVA alone (Fig 4), although the cytoplasmic expression of endogenous antigen introduced into DCs was not affected by the combination with other AdRGDs (Fig 2a). In addition, OVA-presentation levels in DCs transduced with AdRGD-OVA were markedly decreased by combination with AdRGD-Luc, which expresses luciferase as another endogenous antigen (Fig 4). These inconsistent results suggested that processing machineries in the MHC class I-presentation pathway may compete with multiple proteins transduced by the combination of AdRGDs, and that localization characteristics, such as cytoplasmic accumulation, extracellular secretion, and plasma membrane-specific localization, of other proteins should be considered during the preparation of DCs expressing TAA and other functional proteins by cotransduction with multiple AdRGDs in an attempt to maintain sufficient TAA-presenting capacity. With regard to T-cell-stimulating ability, DCs cotransduced with gp100 and IL-12 could more effectively enhance proliferation of syngeneic naive and gp100-primed T cells than DCs transduced with gp100 alone, although allogeneic T-cell proliferation did not differ between the two types of transduced DCs (Fig 5). Furthermore, we could detect considerable IFN-γ secretion from syngeneic naive T cells stimulated by DCs cotransduced with gp100 and IL-12 (Table 1), as expected. These data from *in vitro* immunological analysis suggested that DCs cotransduced with TAA and IL-12 using AdRGD can function as useful vaccine carriers possessing TAA-presentation ability, sufficient T-cell-stimulating ability, and Th1-driving ability *in vivo*.

We attempted to compare vaccine efficacy of DCs genetically modified with various combinations of AdRGD-gp100, AdRGD-IL12, and AdRGD-Luc using the murine B16BL6 melanoma model. Although mice vaccinated with gp100(25MOI) + IL12(5MOI)/DCs exhibited more effective suppression of B16BL6 tumor growth and efficient induction of B16BL6-specific CTLs than those vaccinated with gp100(25MOI)/DCs, vaccine efficacy of cotransduced DCs diminished with increasing combined AdRGD-IL12 MOI during gene transduction, contrary to our expectation (Figs 6 and 7). We speculated that this adverse effect might be caused by a decrease in antigen presentation in DCs by coexpression of TAA and IL-12 as shown in Figure 4, because the anti-B16BL6 effect of gp100(25MOI) + Luc(25MOI)/DCs was obviously inferior to that by gp100(25MOI)/DCs. An alternative explanation for the negative effect of AdRGD-IL12-cotransduction includes an immunosuppressive effect of excess IL-12 on the host's immune cells. Several studies have demonstrated that IL-12 inhibits cell-mediated immune responses, such as clonal expansion of CTLs, in a dose-dependent manner through IFN-γ-mediated nitric oxide production by macrophages in the murine models.[40–43] The *in vivo* bimodal effect of DCs cotransduced with gp100 and IL-12 in our model might involve immunosuppression based on nitric oxide, because DCs transduced with AdRGD-IL12 could drastically enhance IFN-γ secretion from syngeneic naive T cells in MLR (Table 1). In addition, Piccioli et al[44] reported that an *in vitro* interaction between activated NK cells and DCs at high NK/DC ratios resulted in inhibition of DC functions due to potent killing by NK cells,

whereas this interaction at low NK/DC ratios led to drastic increases in DC cytokine production. Therefore, a remarkable increase in NK activity in mice that were vaccinated with gp100(25MOI) + IL12(25MOI)/DCs, as shown in Figure 7, might suppress the induction of the B16BL6-specific immune response by the administered DC vaccine.

To date, vaccine efficacy of DCs cotransduced with TAA and IL-12 has not been fully clarified because both positive[45] and negative[46] effects of simultaneous expression of IL-12 in DC vaccine have been reported. Based on the results of the present study, we concluded that determination of the specific vector dose capable of optimizing both TAA-presentation levels and IL-12-secretion levels in DC vaccine is essential for improving antitumor efficacy based on active biasing of the immune response toward a cellular immunity dominated state.

Abbreviations

2-ME, 2-mercaptoethanol; AdRGD, RGD fiber-mutant adenovirus vector; APC, antigen-presenting cell; BrdU, 5-bromo-2′-deoxyuridine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; Eu, europium; FBS, fetal bovine serum; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; MMC, mitomycin C; MOI, multiplicity of infection; NK, natural killer; OVA, ovalbumin; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TAA, tumor-associated antigen; Th, helper T cell.

Acknowledgments

We are grateful to Dr Hiroshi Yamamoto (Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan) for providing mIL12 BIA/pBluescript II KS(-), to Dr Hirofumi Hamada (Department of Molecular Medicine, Sapporo Medical University, Sapporo, Japan) for providing pAx1-CA h-gp100, to Dr Michael J Bevan (Department of Immunology, Howard Hughes Medical Institute, University of Washington, Seattle, WA) for providing pAc-neo-OVA, to Dr Clifford V Harding (Department of Pathology, Case Western Reserve University, Cleveland, OH) for providing CD8-OVA 1.3 cells, to Yasushige Masunaga, Masaya Nishida, and Aya Matsui (Department of Biopharmaceutics, Kyoto Pharmaceutical University, Kyoto, Japan) for technical assistance, and to KIRIN Brewery Co., Ltd (Tokyo, Japan) for providing recombinant murine GM-CSF.
The present study was supported in part by the Research on Health Sciences focusing on Drug Innovation from The Japan Health Sciences Foundation; by the Science Research Promotion Fund of the Japan Private School Promotion Foundation; by grants from the Bioventure Development Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by grants from the Ministry of Health, Labour and Welfare in Japan.

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Gene Therapy (2005) 12, 129–139

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RESEARCH ARTICLE

Augmentation of the migratory ability of DC-based vaccine into regional lymph nodes by efficient CCR7 gene transduction

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Although dendritic cell (DC)-based immunotherapy is considered a promising approach for cancer treatment, a large quantity of DC vaccine is required for effective sensitization/activation of immune cells because of the poor migratory ability of administered DCs into regional lymphoid tissue. In this study, we created a DC vaccine sufficiently transduced with CC chemokine receptor-7 gene (CCR7/DCs) by applying RGD fiber-mutant adenovirus vector (AdRGD), and investigated its immunological characteristics and therapeutic efficacy. CCR7/DCs acquired strong chemotactic activity for CC chemokine ligand-21 (CCL21) and exhibited an immunophenotype similar to mature DCs but not immature DCs with regard to major histocompatibility complex/costimulatory molecule-expression levels and allogenic T cell proliferation-stimulating ability, while maintaining inherent endocytotic activity. Importantly, CCR7/DCs injected intradermally into mice could accumulate in draining lymph nodes about 5.5-fold more efficiently than control AdRGD-applied DCs. Reflecting these properties of CCR7/DCs, DC vaccine genetically engineered to simultaneously express endogenous antigen and CCR7 could elicit more effective antigen-specific immune response in vivo using a lower dosage than DC vaccine transduced with antigen alone. Therefore, the application of CCR7/DCs having positive migratory ability to lymphoid tissues may contribute to reduction of efforts and costs associated with DC vaccine preparation by considerably reducing the DC vaccine dosage needed to achieve effective treatment by DC-based immunotherapy.
Gene Therapy (2005) **12,** 129–139. doi:10.1038/sj.gt.3302358
Published online 14 October 2004

Keywords: *dendritic cell-based vaccination; RGD fiber-mutant adenovirus vector; CCR7; migration; melanoma*

Introduction

Immunotherapy using dendritic cells (DCs), which play a critical role in control of both acquired and innate immune responses in the living body, is studied energetically in many research organizations aiming to immunologically eradicate cancer. In addition, several cancer immunotherapy protocols using DC vaccine introduced with tumor-associated antigen (TAA) advanced to the clinical study phase.[1–4] However, since currently available DC-based immunotherapy has not demonstrated exceptional therapeutic effects in these clinical studies, the development of a novel approach capable of improving the efficacy of this promising strategy for cancer treatment is eagerly awaited.

DCs are widely distributed over peripheral tissues, where they catch invading antigens by full endocytotic activity, characteristic of an immature state. The phenotype of DCs internalizing antigens changes to a mature state in response to these inflammatory stimuli. Subsequently, they process the antigens into the peptides presented on major histocompatibility complex (MHC) molecules, migrate into draining lymph nodes (LNs) via afferent lymphatic venules, and induce primary immune responses through antigen presentation to T cells.[5–7] On the basis of these serial immune mechanisms, the degree of administered DC vaccine accumulation in lymphoid tissues is a factor in enhancing or restricting therapeutic effects in DC-based immunotherapy.

In recent years, identification and functional analysis of chemokines/chemokine receptors, which regulate relevant leukocyte migration and invasion into tissues, have progressed remarkably, and the chemokine–chemokine receptor coupling in DC migration from peripheral tissue to lymphoid tissue has been elucidated. Gunn *et al*[8] found that DC migration to secondary lymphoid tissues was inhibited in CC chemokine ligand-21 (CCL21) expression-defective *plt/plt* mice. Likewise, Förster *et al*[9] reported that inhibition of DC migration to secondary lymphoid tissues occurred in CC chemokine receptor-7 (CCR7)-knockout mice. Based on these results, the association between CCL21, which is pro-

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Received 15 April 2004; accepted 5 July 2004; published online 14 October 2004

duced and secreted constitutively in lymphoid tissues and lymphatic vessels, and CCR7, a seven-transmembrane domain G-protein-coupled receptor whose expression is enhanced on the surface of maturing DCs, has been shown to play a central role in control of DC migration from the peripheral tissue to lymphoid tissues. Therefore, DCs, which are not only introduced with antigens but also exhibit enhanced CCR7 expression, may positively migrate to lymphoid tissue and efficiently activate the host's immune system after administration to a living body. Efficient CCR7-gene transduction to DCs is proposed as a preparatory method for this novel 'lymphoid tissue-directivity DC' vaccine.

Efficient gene transduction to DCs is difficult in any conventional vector system including adenovirus vector (Ad), which could transfect in a wide variety of cells and tissues.[10] In this regard, we succeeded in establishing highly efficient gene transduction to DCs by applying RGD fiber-mutant Ad (AdRGD),[11–14] and clarified that vaccination with DCs transduced with TAA gene using AdRGD induced considerable antitumor effect based on activation of TAA-specific cytotoxic T lymphocytes (CTLs) in mice.[12,14] Our results not only revealed that AdRGD is very useful in antigen gene delivery to DCs, but also opened up new potentiality for genetically enhancing the immunological functions of DCs by making use of the predominance of AdRGD in gene transduction efficiency to DCs. Thus, in the present study, we first constructed an AdRGD-carrying CCR7 gene (AdRGD-CCR7), and investigated the immunological properties and vaccine efficacy of murine bone marrow-derived DCs modified with AdRGD-CCR7 in order to create a 'lymphoid tissue-directivity DC' vaccine.

Results

Gene transduction into DCs by AdRGD-CCR7

We examined the cytopathic effects of gene transduction using AdRGD-CCR7 to DCs by MTT assay. AdRGD-CCR7 did not injure DCs with a vector dose of 100 multiplicities of infection (MOI) or less, whereas viability of DCs was slightly reduced by using AdRGD-CCR7 at 200 MOI (data not shown). Thus, CCR7 gene expression was evaluated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis in DCs transduced with AdRGD-CCR7 at 100 MOI or less. The CCR7 mRNA level of lipopolysaccharide-stimulated DCs (LPS/DCs) or Luc/DCs transduced with AdRGD-Luc (luciferase-expressing control vector) at 50 MOI did not show a remarkable change in comparison with that of mock (immature) DCs at 24 h post-treatment (Figure 1a).

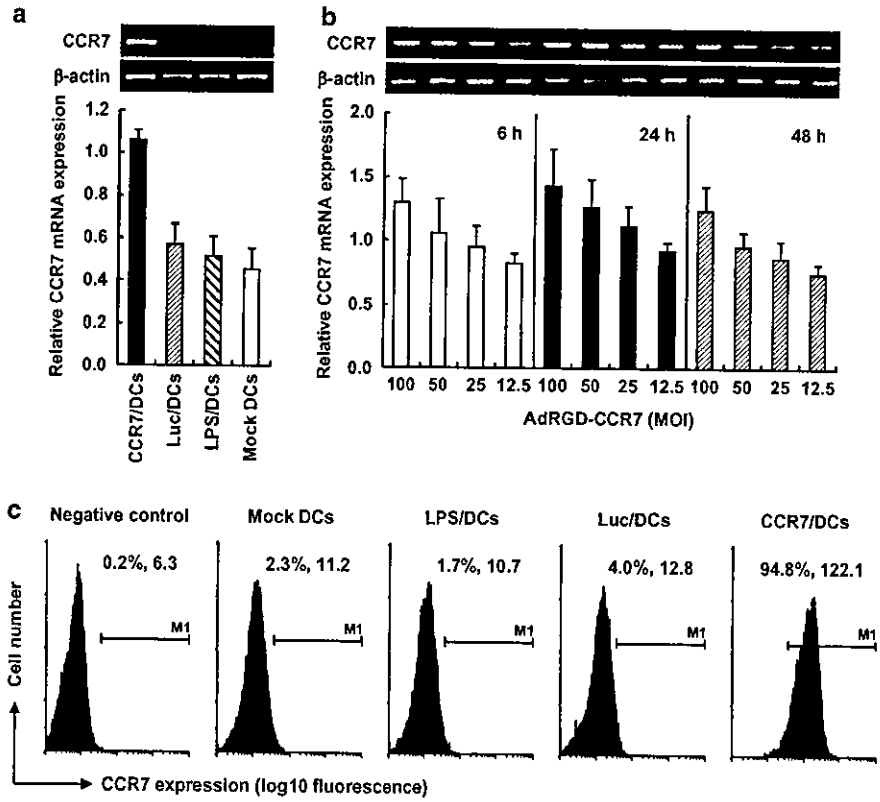

Figure 1 *RT-PCR and flow cytometric analysis for mouse CCR7 expression levels. DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (a and c) or the indicated MOI (b). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h (a and c) or the indicated period (b) in GM-CSF-free medium. (a and b) Total RNA was isolated from these DCs, and then mouse CCR7 mRNA expression was assessed by RT-PCR analysis. Relative CCR7 mRNA expression was calculated as ratio of the densitometric units of PCR products derived from CCR7 transcripts to the densitometric units of PCR products derived from β-actin transcripts. Data are presented as mean±s.d. of results from three independent experiments. (c) Flow cytometric analysis was performed by using anti-mouse CCR7 antibody. Negative control represents mock DCs stained by second antibody alone. The data are representative of two independent experiments, and the % value and the numerical value indicated in the upper part of each panel express % of M1-gated cells and mean fluorescence intensity (MFI), respectively.*

On the other hand, CCR7/DCs prepared with AdRGD-CCR7 at 50 MOI were able to express CCR7 mRNA at a level more than double that of mock DCs. In addition, we analyzed the changes in CCR7 mRNA expression over time in CCR7/DCs transduced at various vector doses (Figure 1b). It was revealed that CCR7 mRNA expression in CCR7/DCs increased in a vector dose-dependent manner, and highest levels were observed at 24 h after gene transduction. Moreover, flow cytometric analysis using anti-mouse CCR7 polyclonal antibody showed that most of the CCR7/DCs, which were prepared with AdRGD-CCR7 at 50 MOI and then cultured for 24 h, expressed CCR7 protein on their surface, whereas few CCR7-positive cells were detected in mock DCs, LPS/DCs, and Luc/DCs (Figure 1c).

Next, we evaluated the chemotactic activity of CCR7/DCs for CCL21 by *in vitro* chemotaxis assay in order to confirm functional CCR7 expression on the cell surface. Under transductional conditions at an MOI of 50, the number of migrating CCR7/DCs cultured for 24 h markedly increased with increasing CCL21 concentration, whereas Luc/DCs, LPS/DCs, and mock DCs remained at low levels despite CCL21 stimulation (Figure 2a). Furthermore, as was observed for RT-PCR analysis in Figure 1b, the chemotactic activity of CCR7/DCs for CCL21 was enhanced with increasing AdRGD-CCR7 dose during transduction (Figure 2b). These results clearly demonstrated that functional CCR7, which could promote migration of DCs in response to a CCL21 concentration gradient, was expressed on the CCR7/DC surface, and that transduction of the chemokine receptor gene by AdRGD could modify chemokine responsiveness of DCs.

Immunological properties of CCR7/DCs

We analyzed the immunological characteristics of CCR7/DCs prepared with AdRGD-CCR7 at 50 MOI. At first, the expression levels of MHC/costimulatory molecules in CCR7/DCs cultured for 24 h were analyzed by flow cytometry (Figure 3a). In comparison with mock DCs, CCR7/DCs and Luc/DCs exhibited upregulated expression of all tested surface marker molecules, which play critical roles in the sensitization/activation of T cells, as is seen in mature LPS/DCs. In particular, the expression levels of CD40 and CD86 were dramatically enhanced by gene transduction using AdRGD. This result agreed with our previous report demonstrating that transduction using AdRGD, irrespective of the type of inserted transgene, could enhance the expression of MHC/costimulatory molecules on DCs.[13] Moreover, CCR7/DCs were able to stimulate proliferation of allogenic naive T cells in mixed leukocyte reaction (MLR) more effectively than mock DCs, and the stimulatory ability of CCR7/DCs, Luc/DCs, and LPS/DCs was equal at a responder/stimulator ratio of 5 (Figure 3b).

The level of fluorescein isothiocyanate (FITC)-dextran uptake in CCR7/DCs was estimated as an index of their endocytotic activity (Figure 3c). Excellent endocytosis for FITC-dextran was observed in mock DCs incubated at 37°C, whereas fluorescence intensity derived from internalized FITC-dextran was drastically decreased by 4°C incubation or LPS-driving maturation. On the other hand, FITC-dextran-uptake levels in CCR7/DCs and Luc/DCs, which were cultured for 24 h after gene transduction, were comparable to those in mock DCs.

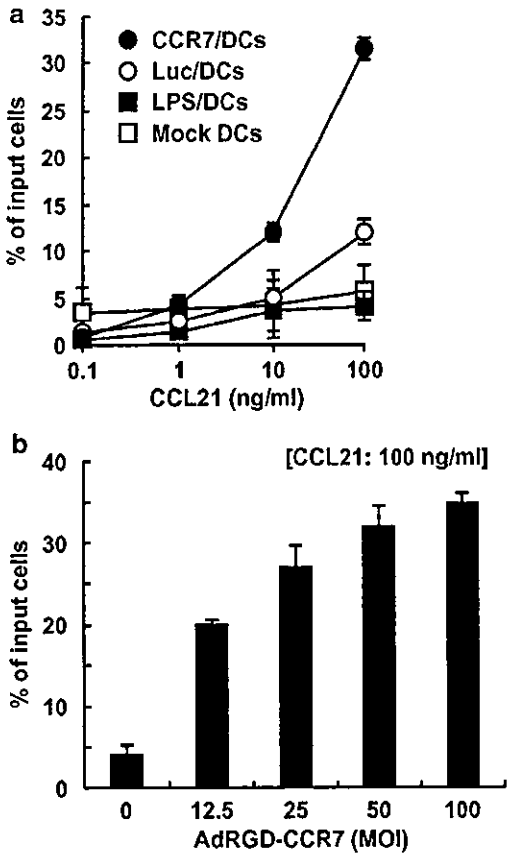

Figure 2 Chemotactic activity of CCR7/DCs in response to CCL21. DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (a) or the indicated MOI (b). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h in GM-CSF-free medium. In vitro chemotaxis assay was performed by a Chemotaxicell-24 installed on a 24-well culture plate. CCL21 solution was added in the lower compartment at the indicated concentration, and DCs were placed in the upper chamber at 10^6 cells. After 4 h incubation, the number of cells that migrated to the lower compartment was counted on a NucleoCounter. Data are presented as mean ± s.d. of four independent cultures.

Taken together, these results demonstrate that gene transduction using AdRGD-CCR7 did not eliminate the antigen-presenting cell (APC) function of DCs; rather, CCR7/DCs acquired an immunophenotype similar to mature DCs while maintaining high endocytotic capacity. Additionally, these immunological characteristics of CCR7/DCs were similar at 48 h post-transduction (data not shown).

Accumulation of CCR7/DCs in regional LN

DCs derived from enhanced green fluorescent protein-transgenic (EGFP-Tg) mice were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI and then cultured for 24 h. These EGFP-positive CCR7/DCs, Luc/DCs, and mock DCs were intradermally injected into the flank of wild-type mice, and their accumulation in the draining inguinal LN was compared at 48 h post-administration by flow cytometric analysis (Figure 4). In all mice, the EGFP-positive DCs were not detected in the inguinal LNs contralateral to the DC-administration site (data not shown). More than double the number of EGFP-positive DCs was detected in regional LN cells prepared from mice injected with Luc/DCs, as compared

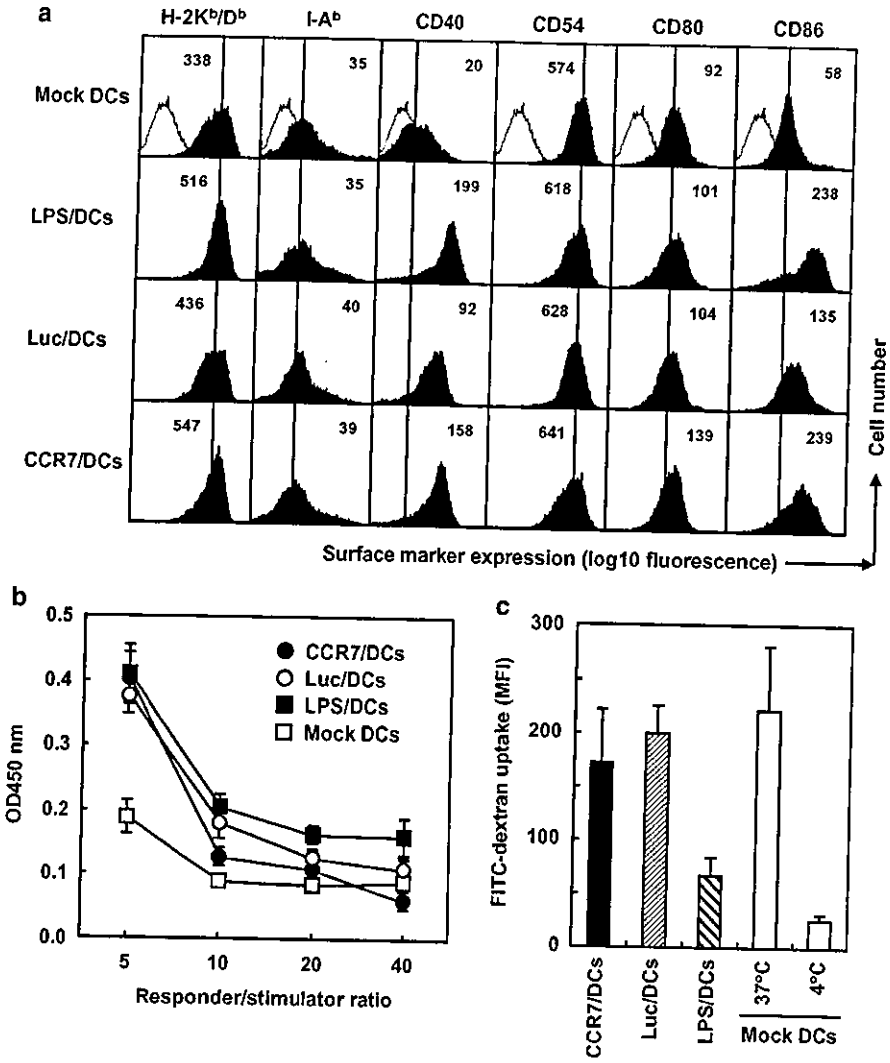

Figure 3 Immunological characteristics of CCR7/DCs. C57BL/6 DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. (a) These transduced cells, LPS-stimulated DCs, and mock DCs were stained by indirect immunofluorescence using monoclonal antibodies of the indicated specificities (solid histogram). Dotted histograms represent cells stained by phycoerythrin-conjugated streptavidin alone. Values indicated in the upper part of each panel represent MFI of flow cytometric analysis. The data are representative of three independent experiments. (b) Naive BALB/c T lymphocytes were co-cultured with CCR7/DCs, Luc/DCs, LPS/DCs, or mock DCs at the indicated responder/stimulator ratio for 3 days. Cell cultures were pulsed with BrdU during the last 18 h, and then T-cell proliferation was assessed by BrdU-ELISA. Results are expressed as mean ± s.e. of three independent cultures using T cells prepared from three individual mice. (c) CCR7/DCs, Luc/DCs, LPS/DCs, and mock DCs were incubated with PBS containing 1 mg/ml FITC-dextran at 4 or 37°C. After 1 h, cells were washed five times with ice-cold PBS and uptake of FITC-dextran was assessed by flow cytometry. MFI of the flow cytometric analysis is presented in the bar chart. Data are presented as the mean ± s.d. of four independent cultures.

with the mock DC-treated group. This phenomenon probably reflected the maturation status of Luc/DCs as shown in Figure 3a and b. Importantly, CCR7/DCs could migrate approximately 5.5- and 15-fold more efficiently into the regional LN than Luc/DCs and mock DCs, respectively, clearly demonstrating that CCR7 gene transduction using AdRGD was useful technology to accelerate the accumulation of DC vaccine in regional LN.

Vaccine efficacy of DCs co-transduced with antigen and CCR7

In order to evaluate the potency of CCR7/DCs as vaccine carriers, we prepared the DC vaccine co-transduced with CCR7 gene and ovalbumin (OVA), model antigen, gene using AdRGD. As shown in Figure 5, DCs combined with AdRGD-OVA and AdRGD-CCR7 could present OVA peptides via MHC class I molecules at levels equal to DCs transduced with AdRGD-OVA alone. On the other hand, the OVA-presentation level in DCs co-transduced with AdRGD-OVA and AdRGD-Luc decreased by half. These data revealed that CCR7 gene transduction did not affect the MHC class I-presentation pathway for antigens endogenously and simultaneously expressed in DCs, and suggested that the proteins accumulating in the cytoplasm and the membrane-localized receptors, such as luciferase and CCR7, respectively, might induce different methods for processing co-expressed endogenous antigens in DCs.

We compared the antitumor effects of DCs co-transduced with gp100, a melanoma-associated antigen, and CCR7 (gp100+CCR7/DCs) and DCs transduced with AdRGD-gp100 alone (gp100/DCs) in the murine

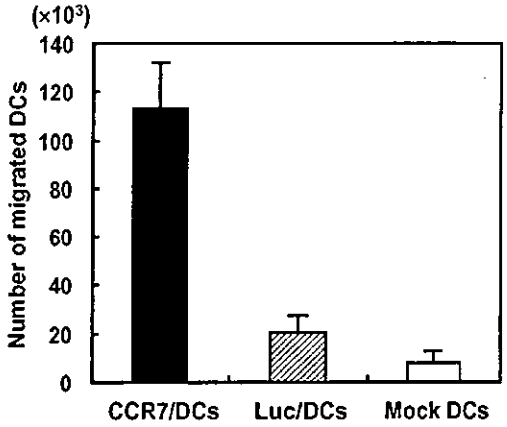

Figure 4 Migration of CCR7/DCs from administration site to draining LN. EGFP-Tg DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. These transduced cells and mock DCs were intradermally injected into the left flank of C57BL/6 mice at 2×10^6 cells/50 μl. After 2 days, the draining inguinal LNs were collected from these mice, and a single-cell suspension was prepared and stained by indirect immunofluorescence using anti-CD11c monoclonal antibody. The abundance of EGFP⁺CD11c⁺ DCs was assessed by flow cytometric analysis acquiring 500 000 events. The number of DCs that had migrated into draining LNs was calculated by multiplying the EGFP⁺CD11c⁺ DC frequency by the total number of isolated LN cells. Data are presented as mean ± s.e. of results from four mice.

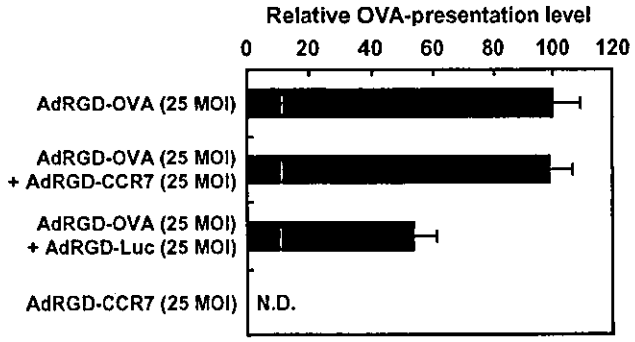

Figure 5 Antigen presentation on MHC class I molecules by CCR7/DCs. DCs were transduced with the indicated combination of AdRGD-OVA, AdRGD-CCR7, and AdRGD-Luc. These cells were co-cultured with CD8-OVA 1.3 cells for 20 h. IL-2 levels released from stimulated CD8-OVA 1.3 cells into culture supernatants were determined by ELISA, and relative OVA-presentation level via MHC class I molecules in each transduced DC was expressed as a percentage of the group using DCs transduced with AdRGD-OVA alone. Data represent the mean ± s.d. of three independent cultures. ND: IL-2 secreted from CD8-OVA 1.3 cells was not detectable.

B16BL6 melanoma model. Obvious growth suppression of B16BL6 tumor challenge was achieved in mice vaccinated with gp100/DCs, as shown in our previous report,[14] whereas immunization with mock DCs or CCR7/DCs was not effective (Figure 6). This vaccine efficacy of gp100/DCs depended on the administered DC dosage. Notably, equal antitumor effects were observed in groups vaccinated with 2×10^5 gp100+CCR7/DCs and with 5×10^5 gp100/DCs, and vaccination with 5×10^5 gp100+CCR7/DCs caused extensive inhibition of B16BL6 tumor growth. Thus, we assessed B16BL6-specific CTL activity in mice vaccinated with gp100+CCR7/DCs or gp100/DCs by europium

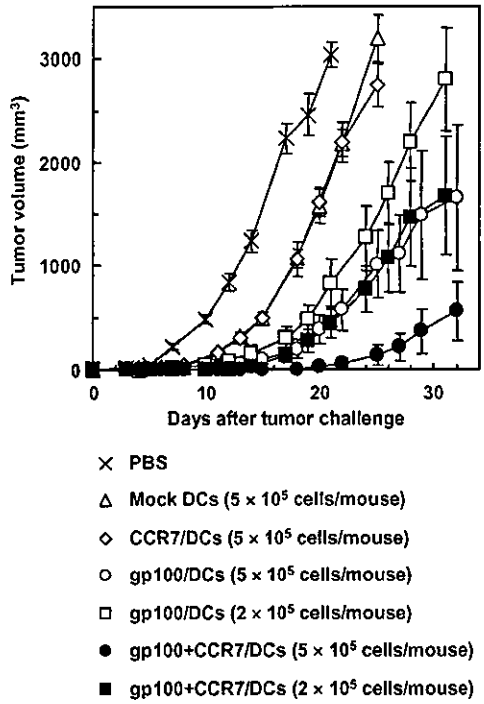

Figure 6 Vaccine efficacy of DCs co-transduced with CCR7 and gp100 gene against B16BL6 melanoma challenge. CCR7/DCs, gp100/DCs, and gp100+CCR7/DCs were prepared using corresponding vectors at 25 MOI, and then cultured for 24 h. C57BL/6 mice were immunized by intradermal injection of transduced DCs into the left flank at the indicated cell dosage, and then 4×10^5 B16BL6 melanoma cells were inoculated into the right flank of the mice at 1 week post-vaccination. The tumor sizes were assessed using microcalipers three times per week. Each point represents the mean ± s.e. of 5–10 mice.

(Eu)-release assay (Figure 7a). The effector cells prepared from mice vaccinated with gp100/DCs could strongly injure B16BL6 cells, as we reported previously.[14] In addition, the effector cells in all groups did not induce lysis of YAC-1 cells, which are highly susceptible to NK activities, and H-2 haplotype-matched irrelevant EL4 thymoma cells (data not shown). B16BL6-specific cytotoxic activity increased in splenocytes from mice vaccinated with gp100+CCR7/DCs, depending on administered DC-dosage, and cytotoxicity exceeded that observed in the group treated with gp100/DCs at same dosage (5×10^5 cells/mouse). Furthermore, re-stimulated splenocytes from mice vaccinated with gp100+CCR7/DCs at 2 or 5×10^5 cells/mouse exhibited higher frequency of interferon-γ (IFN-γ)-producing cells in ELISPOT assay in comparison with those from 5×10^5 gp100/DCs-immunized mice (Figure 7b). Taken together, these data indicated that the induction of a TAA-specific immune response can be potentiated by the improved migration of TAA-loaded DC vaccine from the administration site to lymphoid tissue.

Discussion

The development of a vaccine for cancer treatment aims for sufficient induction of the tumor-specific immune response, in which antitumor CTLs play a central role, to a level capable of tumor rejection and regression. The