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ORIGINAL ARTICLE

TARC and RANTES enhance antitumor immunity induced by the GM-CSF-transduced tumor vaccine in a mouse tumor model

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Abstract
Introduction Transduction of the granulocyte-macrophage colony stimulating factor (GM-CSF) gene into mouse tumor cells abrogates their tumorigenicity in vivo. Our previous report demonstrated that gene transduction of GM-CSF with either TARC or RANTES chemokines suppressed in vivo tumor formation. In this paper, we examined whether the addition of either recombinant TARC or RANTES proteins to irradiated GM-CSF-transduced tumor vaccine cells enhanced antitumor immunity against established mouse tumor models to examine its future clinical application.
Materials and methods Three million irradiated WEHI3B cells retrovirally transduced with murine GM-CSF cDNA in combination with either recombinant TARC or RANTES were subcutaneously inoculated into syngeneic WEHI3B-preestablished BALB/c mice.
Results Vaccinations were well tolerated. Mice treated with GM-CSF-transduced cells and the chemokines demonstrated significantly longer survival than mice treated with GM-CSF-transduced cells alone. Splenocytes harvested from mice treated with the former vaccines produced higher levels of IL-4, IL-6, IFN-γ, and TNF-α, suggesting enhanced innate and adaptive immunity. Immunohistochemical analysis of tumor sections after vaccination revealed a more significant contribution of $CD4^+$ and $CD8^+$ T cells to tumor repression in the combined vaccine groups than controls.
Conclusions TARC and RANTES enhance the immunological antitumor effect induced by GM-CSF in mouse WEHI3B tumor models and may be clinically useful.

Keywords *Gene therapy · Antitumor immunity ·* GM-CSF · TARC · RANTES

Abbreviations

GM-CSF	Granulocyte-macrophage colony stimulating factor
TARC	Thymus and activation-regulated chemokine
RANTES	Regulated on activation, normal T-cell expressed and secreted
APCs	Antigen-presenting cells

Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) matures and differentiates dendritic cells (DCs) into functional antigen-presenting cells (APCs), and enhances their capacity to present tumor-associated antigens, effectively inducing an activation of tumor-specific cytotoxic T lymphocytes (CTLs) and augmenting antitumor immunity in mouse tumor models [11, 14, 17, 22, 26, 28, 34, 45]. Therefore, GM-CSF has been postulated to be one of the most potent immunostimulatory cytokine in the initial tumor immune response [7, 41]. However, the detailed molecular mechanism by which GM-CSF induces antitumor immunity is still unknown. In the past decade, clinical

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trials have shown that autologous GM-CSF-transduced tumor vaccines are feasible, safe, and effective therapies in melanoma [8, 40], renal cell carcinoma (RCC) [38, 44], prostate cancer [19, 39], and non-small cell lung cancer [12, 35]. Our previous clinical studies showed that GM-CSF-transduced tumor vaccines activated acquired immunity, indicated by skewed repertoires of the T cell receptor Vβ chain variables at tumors sites and delayed-type hypersensitivity at vaccination sites with eosinophil infiltation [44]. In spite of these positive immunological findings, the clinical benefits of this therapy alone are insufficient, and additional techniques are required to enhance GM-CSF gene therapy.

We previously analyzed gene expression profiles on temporarily transplanted tumor masses in vivo by serial analysis of gene expression (SAGE) to identify key molecules involved in this antitumor effect [29]. Our results revealed different gene expression levels between the parental and GM-CSF-transduced tumors and identified several molecules involved in the immune response. Among 20 candidate genes, we focused on the chemokine genes of TARC (thymus and activation-regulated chemokine, CCL17) and RANTES (regulated on activation, normal T-cell expressed and secreted, CCL5) [25], which were highly expressed in GM-CSF-transduced tumors. TARC is produced mainly by DCs and specifically chemoattracts Th2 T cells and DCs by binding to the CCR4 surface receptor expressed on Th2 T cells and DCs [16, 36]. RANTES is a CCL5 CC chemokine that is generated by various blood cells including DCs, and binds to the CCR1, CCR3, and CCR5 receptors. As CCR5 is expressed on Th1 cells as well as on macrophages, NK cells and immature DC, RANTES is categorized as both a Th1 cell recruitment mediator and a Th2 cell mediator by activating eosinophils and basophils through CCR3 [9, 20]. Our previous report demonstrated that co-transduction of GM-CSF and TARC/RANTES genes into tumor cells cooperatively inhibited tumor formation in vivo [29]. In this study, we examined whether the therapeutic antitumor effects of irradiated GM-CSF-transduced WEHI3B cells were enhanced by either TARC or RANTES in mouse WEHI3B tumor models to examine its future clinical application.

Materials and methods

Mice

Six- to seven-week-old female BALB/c immunocompetent mice and BALB/c severe combined immunodeficiency (SCID) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Experiments were started with mice 7–8 weeks of age. All animal experiments were approved and performed according to the Guidelines of the Animal Ethics Committees of Kyushu University, Fukuoka, Japan. All animal experiments were performed at least twice to confirm results.

Tumor cells

WEHI3B cells, a murine myelomonocytic leukemia cell line obtained from Dr. D. Metcalf (University of Melbourne), were cultured in 5% CO_2 at 37°C in RPMI 1640 medium (Gibco BRL, NY, US) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics/antimycotics (Gibco BRL). RENCA cells, derived from a spontaneous renal cell carcinoma in a syngeneic BALB/c mouse, were kindly provided by Dr. Miyuki Azuma (Tokyo Medical and Dental University).

Establishment of GM-CSF-producing WEHI3B cells

Recombinant GM-CSF-transduced WEHI3B cell clones were established as previously described. Briefly, the cCRIP-MFG murine GM-CSF retrovirus producing cell line kindly provided by Richard Mulligan [7] was used to produce recombinant retroviruses; isolated retroviral supernatants were then co-cultivated with WEHI3B cells for 5 days. A single clone, producing high levels of GM-CSF (144 ng/24 h/10^6 cells), as measured by ELISA (BD Pharmingen, NJ, US), was obtained by limiting dilution and designated as WEHI3B/GM-CSF (W/GM).

Preparation of tumor vaccine cells and quantification of their cytokine production before and after irradiation

Using an established mouse skin cDNA library, we PCR amplified the chemokine cDNAs using specific primers for murine TARC and RANTES as previously described. These cDNAs were subcloned into the pCR4Blunt TOPO sequencing plasmid (Invitrogen, CA, US) to confirm cloning of the full-length cDNA sequences by nucleotide sequencing. These chemokine cDNAs were then subcloned into a retroviral vector (pMXneo). The recombinant pMXneo vectors were transduced using Lipofectamine (Invitrogen) into the Phoenix A retroviral packaging line, kindly provided by Dr. Toshio Kitamura (The University of Tokyo). Retroviral supernatants (viral solution) were harvested after 2 days of culture. W/GM cells (1×10^6) were then suspended and incubated in 1 ml of viral solution at 37°C for 1 h. After centrifugation, W/GM cells were cultured for two additional days in fresh medium. The medium was replaced with selective medium containing G418 (400 μg/ml) for an additional 7 days. Chemokine-transduced clones were selected by limiting dilution in selective

medium. Cells producing high levels of chemokines were screened by ELISA (R&D systems, MN, US). Clonal W/GM cells containing TARC and RANTES were designated, respectively, as W/GM + T and W/GM + R.

Cloned cells W/GM, W/GM + T and W/GM + R were irradiated at 50 Gy using ^{137}Cs source gamma cell 40 (Atomic Energy of Canada Limited, Ontario, Canada). These irradiated cells were then incubated for 24 h, suspended in 300 μl HBSS (Hank's Buffer Salt Solution; Gibco BRL, NY, US) to 1×10^7 cells/ml, and inoculated subcutaneously (s.c.) into the left flank of mice as a tumor vaccine. The murine GM-CSF, TARC, and RANTES production levels in vitro were measured using the ELISA kits described above.

Tumor vaccination and tumor rechallenge

On the day of tumor challenge, 1×10^5 WEHI3B cells preliminarily cultured in vitro for 1–2 weeks were washed twice in PBS and injected s.c. into the right flank of immunocompetent BALB/c mice ($n = 7$ or 8/group), and the tumor volume was measured twice a week until the end of this experiment. As a treatment, 3×10^6 WEHI3B tumor vaccine cells resuspended in 300 μl HBSS, were injected subcutaneously into the left flank 3 days after tumor challenge then every 4 days for four treatments. The treatment groups included HBSS only, irradiated WEHI3B cells (irW), irradiated W/GM-CSF cells (irW/GM), irradiated W/GM + T cells (irW/GM + T), or irradiated W/GM + R cells (irW/GM + R). In the TARC and RANTES dose escalation study, 20, 60 and 200 ng of recombinant murine TARC (rmTARC) (R&D Systems, MN, US) or 50, 75, 100 and 150 ng of recombinant murine RANTES (rmRANTES) (R&D Systems, MN, US) mixed with 3×10^6 irW/GM cells, irW/GM cells alone, or 100 μl of HBSS were injected s.c. into the left flank of mice on days 3, 6, 9 and 12 after inoculation of 1×10^5 WEHI3B cells into the right flank of BALB/c mice. The products of perpendicular tumor diameters were calculated, plotted, and shown as the mean values and SEM (Bars). Survival was also monitored in WEHI3B-bearing mice and analyzed by a Kaplan–Meier curve. Two bisecting diameters of each tumor were measured with calipers. The tumor volume was calculated using the formula $V = 0.4ab^2$ with "a" as the larger diameter and "b" as the smaller diameter. Changes in tumor growth were monitored twice a week. Mice that responded and completely rejected the tumor with therapy were rechallenged on day 60 with an s.c. injection of 1×10^5 WEHI3B cells into the right flank and monitored in the same fashion. Mice with tumor burdens greater than 2,000 mm^3 were killed and excluded from data for ethical reasons. The serum concentrations of murine TARC and RANTES were measured by ELISA as described above.

CTL assay

Splenocytes were prepared from euthanized tumor-bearing mice 8 days after the fourth tumor vaccinations (s.c. injection in the left flank of 3.0×10^6 irW, irW/GM, irW/GM + rmTARC 200 ng or irW/GM + rmRANTES 75 ng cells), and were depleted of erythrocytes with ammonium chloride. For CTL induction, splenocytes devoid of erythrocytes (4×10^6 cells/well) were co-cultured with mitomycin C-treated WEHI3B cells at a ratio of 10:1 in 1 ml of T cell culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, antibiotics, and 50 μM 2-ME) in 24-well plates at 37°C in 5%CO_2. Two days later, 30 U of recombinant human IL-2 (PeproTech EC, London, U.K.) was added to each well in 500 μl fresh culture medium. Splenocytes including CTLs were harvested on day 6 and used as effector cells in a standard 5 h ^{51}Cr release assay to examine the antitumor cytolytic activity [37]. Briefly, WEHI3B cells as a target tumor and RENCA cells as a nonspecific target (1×10^6 cells) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (PerkinElmer, Boston, MA, US) in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FBS for 90 min at 37°C. After three washes with PBS, the labeled target cells (1×10^4 cells/well) were incubated with the effector cells for 5 h at 37°C in 96-well round bottomed microtiter plates in 200 μl of T cell medium at the indicated E:T ratios. The plates were then centrifuged at 500 rpm for 5 min, and the radioactivity of the supernatants was measured with a(γ counter, Auto Well Gamma System (ALOKA, Tokyo, Japan). The maximum release and spontaneous release were determined from samples incubated with 1% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated using the following formula: percentage of specific ^{51}Cr release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release). Assays were performed in triplicate. The spontaneous release in all assays was <20% of the maximum release.

Mouse IFN-γ and IL-4 ELISPOT

In the course of tumor vaccination as described above, splenocytes from WEHI3B-bearing mice at 5 days after the fourth tumor vaccinations (s.c. injection in the left flank of HBSS, 3×10^6 irW/GM, irW/GM + rmTARC 200 ng or irW/GM + rmRANTES 75 ng cells) were tested for secretion of mouse IFN-γ and IL-4 using an ELISPOT assay kit (Cytokine ELISPOT Set, BD Pharmingen, NJ, US). ImmunoSpot™ ELISPOT 96-well plates were coated with 5 μl/ml of purified anti-mouse IFN-γ or anti-mouse IL-4 monoclonal antibody and incubated overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 and incubated for 2 h with blocking buffer (RPMI 1640 with 10%

FBS) at room temperature. RBC depleted splenocytes (1×10^5) were incubated in the presence or absence of irradiated WEHI3B cells at the indicated splenocyte:WEHI3B cell ratios (100:1, 50:1) in a total volume of 200 μl at 37°C with 5% CO_2 for 20 h. As a positive control, PMA (Phorbol 12-myristate 13-acetate 20 ng/ml; Sigma, St Louis, MO, US), known as mitogen for T cell stimulation, was added to the indicated wells. After the plates were washed, the wells were incubated with 2 μl/ml of biotinylated anti-mouse IFN-γ or anti-mouse IL-4 monoclonal antibody for 2 h at room temperature. Then the plates were washed extensively and streptavidin-HRP solution was added for 1 h at room temperature. After washing the plates twice, Final Substrate Solution (AEC Substrate mixed with AEC Chromogen, BD Pharmingen, NJ, US) was added and spot development was monitored for about 5 min at room temperature. After drying, color spots indicating IFN-γ or IL-4-secreting cells were counted manually under a dissecting microscope and expressed as the mean number of spots+SD of quadruplicated determinations.

CBA and ELISA assay to quantitate mouse IL-2, IL-4, IL-5, IL-6, TNF-α, and IFN-γ production by splenocytes

In a similar fashion to ELISPOT, RBC depleted splenocytes (5×10^6) harvested from mice after the fourth vaccination were incubated in the presence or absence of irradiated WEHI3B cells at a 10:1 ratio in a total volume of 1.0 ml at 37°C for 20 h, and then culture supernatants were collected. The concentrations of mouse IL-2, IL-4, IL-5, TNF-α and IFN-γ in the supernatants were measured by the BD Mouse Th1/Th2 Cytokine CBA (Cytometric Bead Array) Kit (BD Pharmingen, NJ, US) according to the manufacturer's protocol. The concentration of IL-6 was measured using a mouse IL-6 immunoassay kit (R&D Systems, MN, US) following the manufacturer's instructions.

NK cell depletion in vivo

NK cells were depleted from BALB/c SCID mice by peritoneal injection of 200 μl rabbit anti-asialo GM_1 antiserum (freeze-dried anti-serum diluted 1:20 in PBS; Wako, Osaka, Japan), at 1 day before, and 7 and 14 days after tumor inoculation. As with the therapeutic protocol for immunocompetent BALB/c, indicated tumor vaccines were injected subcutaneously into the left flank a total of four times, once on every fourth day, starting 3 days after the tumor challenge (ten mice per group). Tumor growth and survival rates were assessed. Our previous results following the same experimental conditions confirmed NK cells elimination by the absence of splenocyte cytotoxic activity against YAC-1 cells (ATCC) in a standard ^{51}Cr release assay [30].

Immunohistochemistry

Five days after the fourth tumor vaccinations (s.c. injection in the left flank of 3×10^6 irW, irW/GM, irW/GM + rmTARC 200 ng or irW/GM + rmRANTES 75 ng cells), implanted tumor tissues (n = 4/group) were snap frozen by overlaying with OCT compound (Sakura Fine Technical Co., Tokyo, Japan) and stored at −80°C until analysis. Serial cryostat (8 μm) frozen sections were adhered to Superfrost glass slides (Matsunami, Osaka, Japan), fixed in acetone at room temperature for 10 min, air-dried, and rinsed in distilled water to remove embedding medium. Staining was conducted following standard procedures. Briefly, sections were sequentially incubated overnight at 4°C with appropriately diluted primary antibodies, mouse CD4(GK1.5), CD8(53-6.7), CD11c(N418) (DCs), FoxP3(FJK-16s) (Tregs), CD45R/B220(RA3-6B2) (B cells), F4/80(BM8) (macrophages), and Ly-6G/Gr-1(RB6-8C5) (granulocytes) (all from eBioscience, San Diego, CA, USA) following the manufacturer's instructions, followed by a 1-h incubation with biotinylated anti-rat or anti-hamster secondary Abs (eBioscience). After a 30-min incubation with streptavidin-peroxidase (Dako Japan Co. Ltd., Kyoto, Japan), Ag–Ab reactions were developed using 3, 3′-diaminobenzidine (Nakalai tesque, Kyoto, Japan) as a substrate. Slides were washed three times with PBS between each incubation step. Slides were counterstained with Mayer's hematoxylin and dehydrated in sequentially degraded alcohol and xylene prior to mounting. All incubations were conducted in a humidified chamber. Photographs were taken with a Zeiss Axioskop 2 plus microscope (Zeiss, NY, US). The stained cells were counted microscopically at 100× magnification in 10–30 high-power fields. Results are presented as the means ± SD.

Flow cytometric analysis of helper T cells (Th1/Th2 balance) in the tumor and spleen

Five days after the fourth tumor vaccination (s.c. injection in the left flank of 3×10^6 irW, irW/GM, irW/GM + rmTARC 200 ng, or irW/GM + rmRANTES 75 ng), single cell suspensions of primary tumor and splenocytes were obtained from vaccinated mice ($n = 3$/group). Splenocytes and tumor cells were homogenized by mincing and filtered through a 70 μm cell strainer (BD Labware, NJ, US). The homogenized tumor was then digested with collagenase IV (GIBCO) in RPMI 1640 for 90 min at 37°C with continuous stirring. The splenocytes and tumor infiltrating cells (TILs) were then isolated from tumor cells/erythrocytes by centrifuging the cell suspension on a Lympholyte M gradient (CEDARLANE, Ontario, Canada) following the manufacturer's protocol. The resulting single cell suspensions were washed twice with 1% FBS in PBS and stained for flow cytometry. Splenocytes and TILs were stimulated with PMA (40 ng/ml)

(Sigma) and ionomycin (1 μg/ml) (Sigma) for 5 h. Two hours before harvesting, the cells were treated with brefeldin A (50 μg/ml, eBioscience) to retain cytoplasmic cytokines. After washing twice in a fluorescent antibody buffer (FAB) consisting of 0.1% bovine serum albumin and 0.02% sodium azide in 0.01 M PBS (pH 7.2), cells were pretreated with FcR (CD16/32) block followed by an APC-conjugated anti-CD4 mAb for 30 min. Subsequently, cells were fixed with 2% paraformaldehyde and then stained intracellularly with FITC-conjugated IFN-γ and PE-conjugated IL-4 mAb in permeabilization buffer (eBioscience) for 1 h at room temperature. All mAbs were purchased from eBioscience. Stained cells were washed three times in FAB and analyzed by multiparameter flow cytometry using a Becton Dickinson FACS Calibur (San Jose, CA). Dead cells were excluded by the FSC/SCC profiles. Surface marker profiles were analyzed using CELLQUEST Software (Becton Dickinson) and the percentages of positive cells were determined.

Statistical analysis

Except for the animal survival data, statistical analyses were performed using Student's *t* tests. The *P* values were obtained from two-tailed tests of statistical significance. A Kaplan–Meier curve was used to analyze survival rates, and the statistical relevance was determined using a log-rank comparison. A probability value was considered significant when $P < 0.05$.

Results

In vivo antitumor effect of irradiated WEHI3B cells producing GM-CSF plus either murine TARC or murine RANTES

We previously reported that mice inoculated s.c. with non-irradiated WEHI3B cells transduced with GM-CSF plus TARC or RANTES exhibited a significantly better survival rate than mice injected with GM-CSF-transduced WEHI3B cells [29]. In this study, the hypothesis whether irW/GM + T or irW/GM + R provided additional antitumor immunity compared to irW/GM was examined. Prior to vaccination, the production of murine GM-CSF, TARC and RANTES from WEHI3B, W/GM, W/GM + T and W/GM + R cells before and 24 h after irradiation was compared to evaluate the influence of irradiation on the production of transduced cytokine genes. There were no striking differences in cytokine production before and after irradiation (Table 1). A total of 1×10^5 of parental WEHI3B cells were injected subcutaneously into the right flank of immunocompetent BALB/c mice. On day 3, 3×10^6 each of irW, irW/GM, irW/GM + T and irW/GM + R were injected s.c. into the left flank of mice every 4 days for four treatments and monitored periodically for 60 days (Fig. 1). At day 27, posttumor inoculation irW/GM significantly inhibited tumor growth compared to irW ($P < 0.05$). irW/GM + T and irW/GM + R induced a slightly better, although not statistically significant, antitumor effect compared with those induced by irW/GM. Interestingly, the irW/GM + T group had significantly better survival than irW/GM alone ($P < 0.05$). On day 60 postinoculation, some mice in each therapeutic group had completely rejected tumor formation. In the irW/GM + T group, five of the seven mice rejected the tumor development and showed a longer survival compared with that of both the irW/GM and irW/GM + R groups. Furthermore, when rechallenged with 1×10^5 parental WEHI3B cells s.c. into the right flank, three of the fiive irW/GM + T-treated mice that rejected the original WEHI3B tumor, also rejected the second WEHI3B challenge. In contrast, mice treated with either irW/GM or irW/GM + R rejecting the primary tumor challenge died within 25 days after the rechallenge of WEHI3B cells (Table 2).

Table 1 Comparison of cytokine production levels in retrovirally indicated gene transduced WEHI3B cells between before and 24 h after irradiation

Transfectant cells	Amounts of respective cytokines secreted by indicated transfactant cells			
	WEHI3B	W/GM	W/GM + T	W/GM + R
GM-CSF (before)	0	142.47 ± 0.97	104.42 ± 1.35	97.36 ± 12.01
(After)	0	28.42 ± 0.31	47.53 ± 1.62	35.92 ± 0.76
TARC (before)	0	0	6.07 ± 0.12	0
(After)	0	0	6.41 ± 0.35	0
RANTES (before)	0	0	0.259 ± 0.01	18.31 ± 0.14
(After)	0	0	0	28.49 ± 0.43

The amountsof cytokine secreted by indicated transfectant cells were measured by ELISA

Each value is mean ± SD of culture supernatant measurements (per 10^6 cells/24 h)

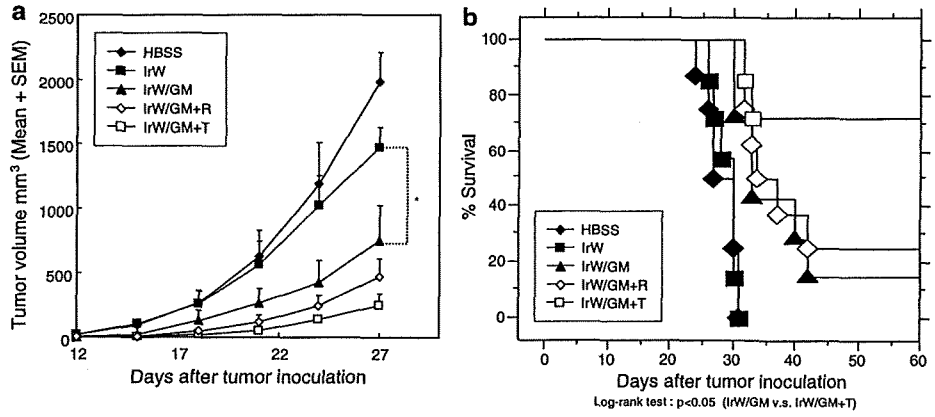

Fig. 1 Therapeutic antitumor effects of irW/GM, irW/GM + T, or irW/GM + R cells on preestablished tumors. A total of 1×10^5 WEHI3B cells were inoculated s.c. into the right flank of BALB/c mice on day 0. Next, 3×10^6 irW, irW/GM, irW/GM + T, or irW/GM + R cells or 100 μl of HBSS were injected s.c. into the left flank of mice on days 3, 6, 9 and 12. The products of perpendicular tumor diameters were calculated and plotted. The mean products and SEM (*bars*) are depicted (**a**). Kaplan–Meier analyses of WEHI3B-bearing mice are shown (**b**) (*$P < 0.05$). Data are representative of two independent experiments, with similar results

Table 2 Antitumor effects induced by s.c. injection of irW/GM, irW/GM + T, and irW/GM + R

Vaccination groups	Challenged mice	Rejected mice (%)[a]	Re-rejected mice[d]
HBSS	8	0 (0)[b]	–
IrW	8	0 (0)	–
IrW/GM	7	1 (14.3)[c]	1
IrW/GM + T	7	5 (71.4)[c]	3
IrW/GM + R	8	2 (25.0)	0

[a] Assessed at day 60 after tumor inoculation

[b] Parenthesis shows % of mice that rejected the WEHI3B challenge

[c] χ^2 test: $P < 0.05$

[d] Assessed at day 60 after WEHI3B rechallenge

Both recombinant murine TARC and RANTES enhanced the antitumor effects of irW/GM in mice with established tumors

We then conducted a dose escalation test of rmTARC or rmRANTES mixed with irW/GM cells against pre-established tumors to determine the appropriate dose of TARC or RANTES to enhance the antitumor effect of irW/GM. Three days after the injection of 1×10^6 WEHI3B cells, 3×10^6 irW/GM cells containing either rmTARC (20, 60, 200 ng/mouse) or rmRANTES (50, 75, 100, 150 ng/mouse) were s.c. inoculated into the left flank every 4 days for four treatments, and tumor growth and survival rate were monitored. On day 26, mice treated with irW/GM + rmTARC suppressed WEHI3B growth in an rmTARC dose dependent manner, with a significant additional antitumor effect compared to irW/GM vaccinated mice ($P < 0.05$) (Fig. 2a). Mice vaccinated with irW/GM + rmTARC at 60 ng and especially 200 ng rmTARC had significantly prolonged survival compared to control mice vaccinated with or without irW/GM, all of which died within 37 days ($P < 0.05$) (Fig. 2c). Furthermore, 1, 2 and 4 mice vaccinated, respectively, with irW/GM + rmTARC 20, 60 and 200 ng completely rejected tumor formation (Table 3). On day 26, the mice vaccinated with irW/GM + rmRANTES 75 ng suppressed WEHI3B growth most significantly among the rmRANTES treated mice, and three mice completely eradicated their tumors. In contrast, none of the eight mice treated with HBSS or irW/GM rejected tumor challenge. Mice vaccinated with irW/GM + rmRANTES 50, 75, and 100 ng, had significantly prolonged survival compared with control mice vaccinated with or without irW/GM ($P < 0.05$) (Fig. 2d). Interestingly, a rmRANTES dose of 75 ng per mouse was the optimal dose that promoted antitumor immunity. Moreover, four of eight and three of seven mice vaccinated with irW/GM + rmTARC 200 ng and irW/GM + rmRANTES 75 ng, respectively, were rechallenged as described above, and two of four, and two of three respective mice with rejected tumors survived for more than 60 additional days without tumors (Table 3). The serum concentrations of murine TARC 4 h (2,338.55 ± 168.01 pg/ml) and murine RANTES 2 h (713.99 ± 118.04 pg/ml) after s.c. tumor vaccination with irW/GM + rmTARC 200 ng and irW/GM + rmRANTES 75 ng, respectively, were measured by ELISA. During the vaccinations, mice were well tolerated without any major adverse events.

Splenocytes from mice vaccinated with irW/GM in combination with either rmTARC or rmRANTES had enhanced antitumor cytotoxicity in vitro

To investigate the mechanism underlying the enhanced specific antitumor immunity by TARC and RANTES, we

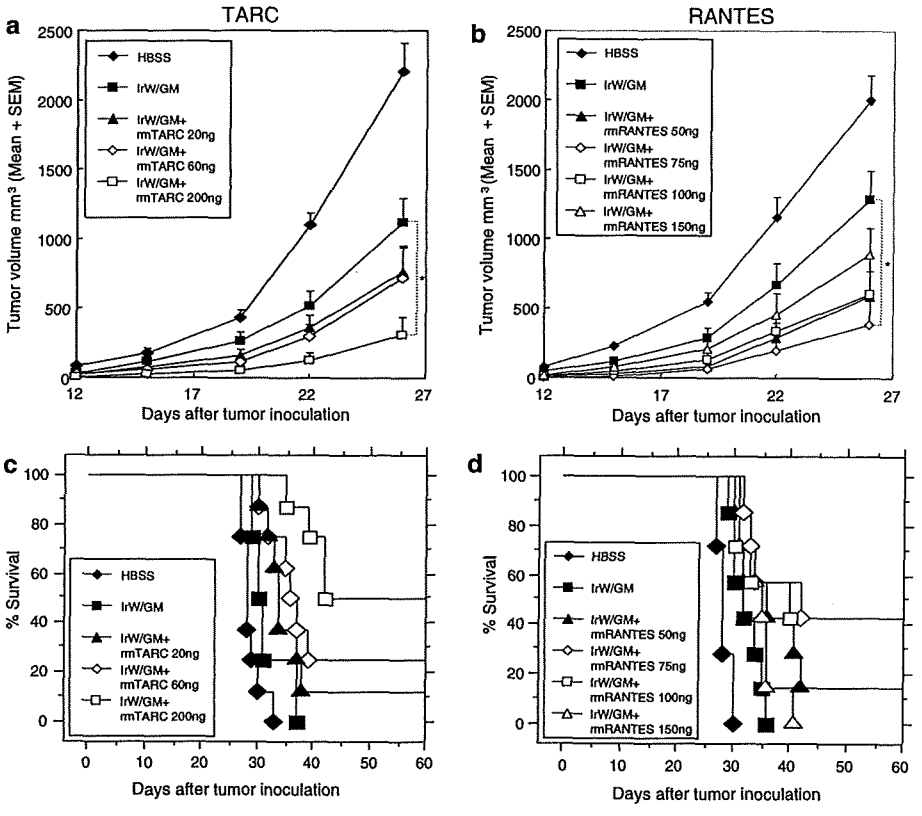

Fig. 2 Therapeutic antitumor effects of irW/GM, irW/GM + rmTARC (20, 60, 200 ng) or irW/GM + rmRANTES (50, 75, 100, 150 ng) cells on pre-established tumors. A total of 1×10^5 WEHI3B cells were implanted s.c. into the right flank of BALB/c mice on day 0. Next, irW/GM, irW/GM + rmTARC (20, 60, 200 ng), irW/GM + rmRANTES (50, 75, 100, 150 ng) cells or 100 μl of HBSS were injected s.c. into the left flank of mice on days 3, 6, 9 and 12. The products of perpendicular tumor diameters were calculated and plotted. The mean products and SEM (*bars*) are depicted (**a**, **b**). Kaplan–Meier analyses of WEHI3B-bearing mice are shown (**c**, **d**) (*$P < 0.05$). Data are representative of two independent experiments, with similar results

Table 3 Antitumor effects induced by s.c. injection of irWGM, irW/GM in combination with either rmTARC or rmRANTES at various doses

Vaccination groups	Challenged mice	Rejected mice (%)[a]	Re-rejected mice[e]
HBSS	8	0 (0)[b]	–
IrW/GM	8	0 (0)[c,d]	–
IrW/GM + rmTARC 20 ng	8	1 (14.3)	0
IrW/GM + rmTARC 60 ng	8	2 (25.0)	0
IrW/GM + rmTARC 200 ng	8	4 (50.0)[c]	2
IrW/GM + rmRANTES 50 ng	7	1 (14.3)	0
IrW/GM + rmRANTES 75 ng	7	3 (52.9)[d]	2
IrW/GM + rmRANTES 100 ng	7	3 (52.9)[d]	1
IrW/GM + rmRANTES 150 ng	7	0 (0)	–

[a] Assessed at day 60 after tumor inoculation

[b] Parenthesis shows percentage of mice that rejected the WEHI3B challenge

[c,d] χ^2 test : $P < 0.05$

[e] Assessed at day 60 after WEHI3B rechallenge

examined the cytotoxic activity of splenocytes from vaccinated mice against WEHI3B cells in vitro. Murine renal carcinoma (RENCA) cells were used as negative control target cells. Splenocytes of mice vaccinated with irW/GM in combination with rmTARC 200 ng or rmRANTES 75 ng demonstrated more than 20% cytotoxicity against WEHI3B cells at 100:1, 50:1, or 25:1 E/T ratios, showing higher cytolytic activity compared to splenocytes from mice vaccinated with irW/GM alone. In contrast, splenocytes from mice vaccinated with irW/GM with or without rmTARC or rmRANTES exhibited no or lower cytotoxicity against RENCA cells at the indicated E/T ratios. Splenocytes from mice vaccinated with irW demonstrated minimal ability to lyse both WEHI3B cells and RENCA cells at each effector/target ratio tested (Fig. 3).

ELISPOT assay and in vitro cytokine production profile of splenocytes from mice vaccinated with irW/GM cells in combination with either rmTARC or rmRANTES

As RANTES and TARC are presumed to recruit or activate Th1 and Th2 T cells in a pathway dependent on the respective CCR4 and CCR5 receptors, we harvested splenocytes from mice receiving HBSS, irW/GM cells with or without rmTARC 200 ng or rmRANTES 75 ng and performed in vitro IFN-γ and IL-4 ELISPOT assays. In the presence of irradiated WEHI3B cells, the numbers of spot-forming IFN-γ or IL-4 secreting splenocytes from tumor-bearing mice treated with irW/GM + rmTARC or rmRANTES were significantly higher than those from mice treated with HBSS or irW/GM ($P < 0.05$) (Fig. 4a, b).

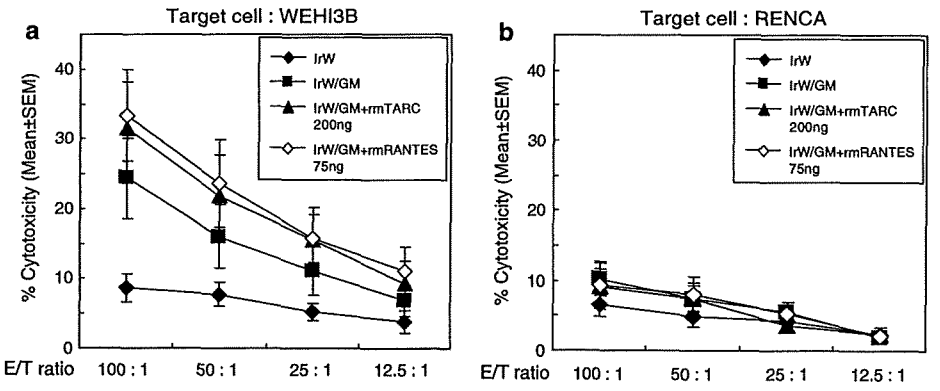

Fig. 3 CTL activity of tumor-bearing mice that received different tumor vaccinations described above. Eight days after the fourth tumor vaccination, splenocytes were harvested from vaccinated mice and used as CTL effector cells in a ^{51}Cr release assay as described in "Materials and methods". ^{51}Cr labeled WEHI3B cells were used as target cells (**a**) and RENCA cells were used as nonspecific target cells (**b**). Cytolytic activity against WEHI3B cells was higher in mice vaccinated with irW/GM plus rmTARC 200 ng or rmRANTES 75 ng compared with mice vaccinated with irW/GM alone (**a**), whereas no cytolytic effect was observed when RENCA cells were used as target cells (**b**). The values represent the mean ± SEM (standard error of the mean) of *percentage cytotoxicity from four independent experiments*

Next, we determined the in vitro cytokine production profiles of mice treated with irW/GM cells in combination with either rmTARC 200 ng or rmRANTES 75 ng Irradiated WEHI3B cells were used as stimulator cells. After 20 h of co-culture, the supernatants were collected and IL-2, IL-4, IL-5, IL-6, IFN-γ and TNF-α levels were measured by CBA or ELISA assay. As seen in the ELISPOT assay, IFN-γ and IL-4 production were significantly higher in the mice treated with irW/GM + rmTARC or irW/GM + rmRANTES than irW/GM ($P < 0.05$) (Fig. 4d, f). Additionally, a significantly higher production of TNF-α and IL-6 was observed in the mice treated with irW/GM + rmTARC or irW/GM + rmRANTES ($P < 0.05$) (Fig. 4c, h). The production of IL-4 and IL-6 was significantly higher in the mice treated with irW/GM + rmRANTES 75 ng than irW/GM + rmTARC 200 ng ($P < 0.05$) (Fig. 4f, h). IL-2 and IL-5 levels were already elevated in the mice treated with irW/GM with or without chemokines and further activation was not induced when splenocytes were co-cultured with restimulator cells (Fig. 4e, g).

In vivo antitumor efficacy of irW/GM cells in combination with either rmTARC or rmRANTES in tumor implanted SCID mice

To determine if the additional effects of rmTARC or rmRANTES in combination with GM-CSF in syngeneic immunocompetent mice were mediated by adaptive immunity, we performed in vivo studies using syngeneic BALB/c SCID mice. Three days after subcutaneous injection of 1×10^5 of WEHI3B into the right flank of SCID mice, 3×10^6 each of irW, irW/GM, irW/GM + rmTARC 200 ng, or irW/GM + rmRANTES 75 ng were subcutaneously injected into the left flank of mice every 4 days for four treatments. Interestingly, only mice treated with irW/GM + rmRANTES 75 ng had significantly retarded tumor growth and revealed better survival than mice treated with irW, irW/GM or irW/GM + rmTARC 200 ng ($P < 0.05$) (Fig. 5a, b). However, all treated mice succumbed to death within 26 days. As SCID mice retain innate immune systems including NK cells and mononuclear cells, we treated SCID mice with intraperitoneal injections of anti-asialo GM_1 anti-serum to abrogate NK cell function and to investigate the contribution of NK cells to the tumor suppression by irW/GM + rmRANTES. The NK-depleted mice treated with irW/GM + rmRANTES had faster tumor growth than controls and SCID mice treated with irW/GM + rmRANTES ($P < 0.05$) (Fig. 5c, d).

Immunohistochemical findings of tumor infiltrating cells in tumor masses during tumor vaccination

During the course of the tumor vaccination studies, we killed four mice with retarded tumor growth in each vaccination group. The distribution of leukocyte subtypes in the tumor infiltrating cells was analyzed by immunohistochemical (IHC) staining. IHC analysis revealed a significantly greater number of infiltrating $CD8^+$ T cells and $CD11^+$ cells in mice vaccinated with irW/GM than irW ($P < 0.05$). The number of $CD4^+$ T cells and $CD8^+$ T cells was significantly increased in mice treated with irW/GM + rmTARC 200 ng and irW/GM + rmRANTES 75 ng compared with irW/GM alone ($P < 0.05$). The number of $CD11c^+$ cells and $CD45R/B220^+$ cells was also significantly increased in mice treated with irW/GM + rmTARC 200 ng than irW/GM ($P < 0.05$). On the other hand, the number of Ly-6G/Gr-1^+ cells was significantly increased, while that of $FoxP3^+$ cells was significantly decreased in mice treated with irW/GM +

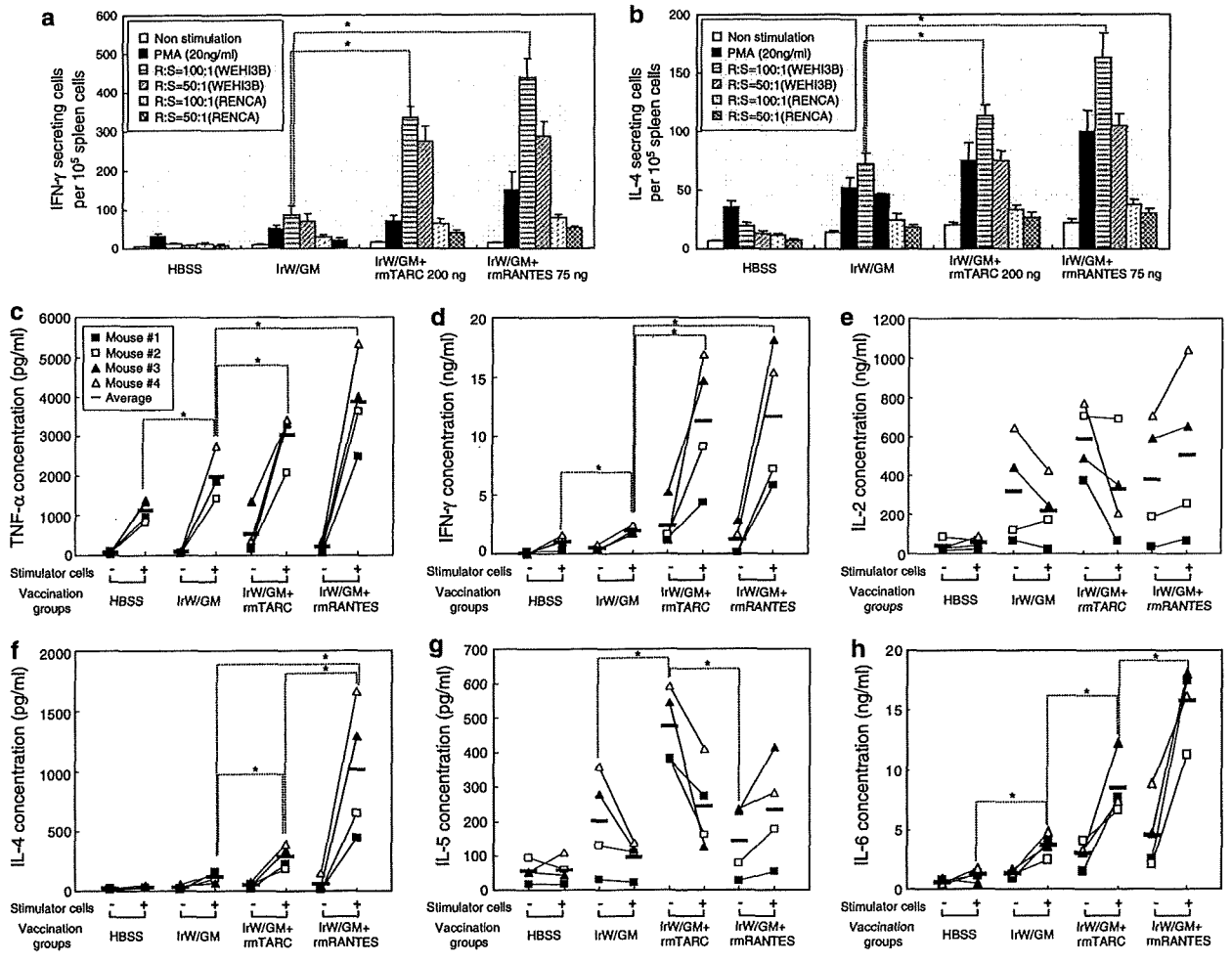

Fig. 4 **a, b** Induction of WEHI3B tumor-specific splenocytes after s.c. injection of irW/GM in combination with either rmTARC 200 ng or rmRANTES 75 ng was evaluated by mouse IFN-γ (**a**) and IL-4 (**b**) ELISPOT assays. A total of 1×10^5 splenocytes from tumor-bearing mice vaccinated with the indicated transfected cells were incubated in 200 μl RPMI 1640 with 10% FBS in ImmunoSpot™ 96-well plates coated with anti-IFN-γ or anti-IL-4 mAb. After a 20 h co-incubation with stimulator cells (RENCA cells were used as negative control) at the indicated ratios, the plates were washed and bound cytokines were visualized by incubating with biotinylated anti-IFN-γ or anti-IL-4 mAb, followed by streptavidin-HRP or premixed peroxidase substrate AEC. Results are expressed as the mean number of spot-forming cells + SD of quadruplicate determinations per 10^6 splenocytes. (**c–h**) In vitro cytokine secretion of splenocytes from mice treated with the tumor vaccines described above. Splenocytes were harvested from mice 5 days after the last tumor vaccine and then cultured with or without irradiated WEHI3B stimulator cells. Twenty hours after a mixed lymphocytes/tumor culture was started, the concentrations of mouse TNF-α (**c**), IFN-γ (**d**), IL-2 (**e**), IL-4 (**f**), IL-5 (**g**) in the culture supernatants were measured. The concentration of IL-6 (**h**) was measured using a mouse IL-6 immunoassay ELISA kit. $^*P < 0.05$ when compared with the indicated group

rmRANTES 75 ng than irW/GM ($P < 0.05$). F4/80$^+$ cell (macrophage) infiltration also increased in the vaccinated groups, but was not statistically different compared with irW (Fig. 6a).

Cytokine secretion profiles of CD4$^+$ T Cells in the tumor and spleen during tumor vaccination

To examine the effect of chemokines (TARC and RANTES) on GM-CSF based tumor vaccination, we next assessed the profiles of cytokines secreted by CD4$^+$ TIL subpopulations from the sites of primary tumor growth and the spleen on the same day as the IHC analysis. Th1 (IFN-γ) and Th2 (IL-4) cell cytokine profiles (recruitment/accumulation of Th1/Th2 in TILs) were analyzed at the single cell level by intracellular staining and flow cytometry, and the percentages of Th1 and Th2 cells in the tumor (TILs) and spleen were calculated. In mice that were treated with irW/GM cells, there were slightly greater numbers of Th2 cells in the TILs, and rmTARC further enhanced the proportion of Th2 cells. In contrast, the addition of rmRANTES shifted the balance to predominantly Th1 cells. In the spleen, the Th1/Th2 balance in the three

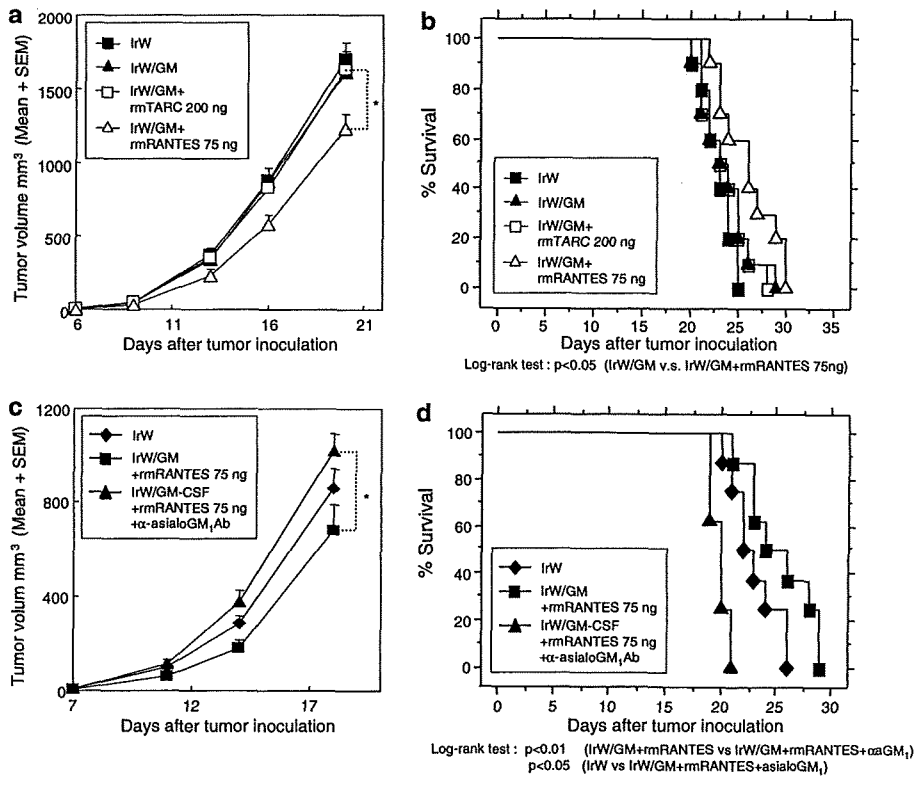

Fig. 5 **a, b** A total of 1×10^5 WEHI3B cells were s.c. injected into the right flank of syngeneic BALB/c SCID mice. Three days later, 3×10^6 each of irW, irW/GM, irW/GM + rmTARC 200 ng, or irW/GM + rmRANTES 75 ng were s.c. injected into the left flank every 4 days for four treatments (n = 10/group). Mice treated with irW/GM + rmRANTES 75 ng had significantly suppressed tumor growth (**a**) and prolonged survival (**b**). (**c, d**) NK cells depletion assay. BALB/c SCID mice received peritoneal injections of rabbit anti-asialo GM_1 anti-serum, at 1 day before, and 7 and 14 days after tumor inoculation (n = 8/group). In NK-depleted mice, the additional antitumor effects including prolonged survival by rmRANTES disappeared. Significant differences are denoted with *asterisks* (*$P < 0.05$)

groups was similar to that observed in the TILs, but was even more enhanced (Fig. 6b, c).

Discussion

This study, along with our previous studies [29], shows that co-administration of the chemokines TARC or RANTES with irW/GM has additional antitumor effects and is considered to be useful possible applications to enhance the antitumor effects of GM-CSF-transduced tumor vaccines. Our results of in vitro CTL, ELISPOT and ELISA assays using splenocytes harvested from mice immunized with irW/GM mixed with TARC or RANTES suggested that the antigen-specific adaptive immunity induced by GM-CSF secreting vaccine cells was mediated by the production of cytokines, including IFN-γ, IL-4, TNF-α, and IL-6. In addition, our results of immunohistochemical analysis supported these findings by demonstrating a significant increase in $CD4^+$ and $CD8^+$ T cell infiltration in tumors of mice treated with both irW/GM + rmTARC and irW/GM + rmRANTES compared with those treated with irW/GM alone.

GM-CSF released from genetically engineered tumor cells enhances tumor antigen presentation by leading to the local accumulation of DCs, the most important APC, in and around the vaccination site. TARC, RANTES or other chemokines and cytokines are thought to act synergistically with GM-CSF to recruit immature DCs to vaccination sites where they uptake tumor antigens. These mediators also present a "danger signal" that activates antitumor immunity by recruiting Th1 and Th2 cells to the vaccination site and draining lymph nodes, contributing to the interaction of effector T cells with tumor cells [13, 31, 43]. Indeed, TARC and RANTES are proinflammatory cytokines involved in the chemoattraction of a number of different effector cell types. Activation of innate and acquired immunity by a combination of cytokines and chemokines would help enhance antitumor immune responses in vivo. This hypothesis is supported by our in vivo NK depletion studies demonstrating that the antitumor effects of rmRANTES with irW/GM were induced by T cells and partially by NK cells. Interestingly, our observations suggested that an optimal amount of RANTES might augment NK cell cytolytic activity on malignant cells [33]. Actually CCR5, one of the RANTES receptors, is expressed on macrophages, NK cells, DCs, and activated Th1 cells [24]. After binding to CCR5, RANTES potently chemoattracts monocytes/macrophages, NK cells, and CTLs, resulting in tumor lysis through the release of granule enzymes [23, 27]. Similar combination effects were also observed in an antivirus vaccine study. Sumida et al. [42] demonstrated that coadministration of the chemokine macrophage inflammatory protein-1 (MIP-1α) expression plasmid DNA vaccine with

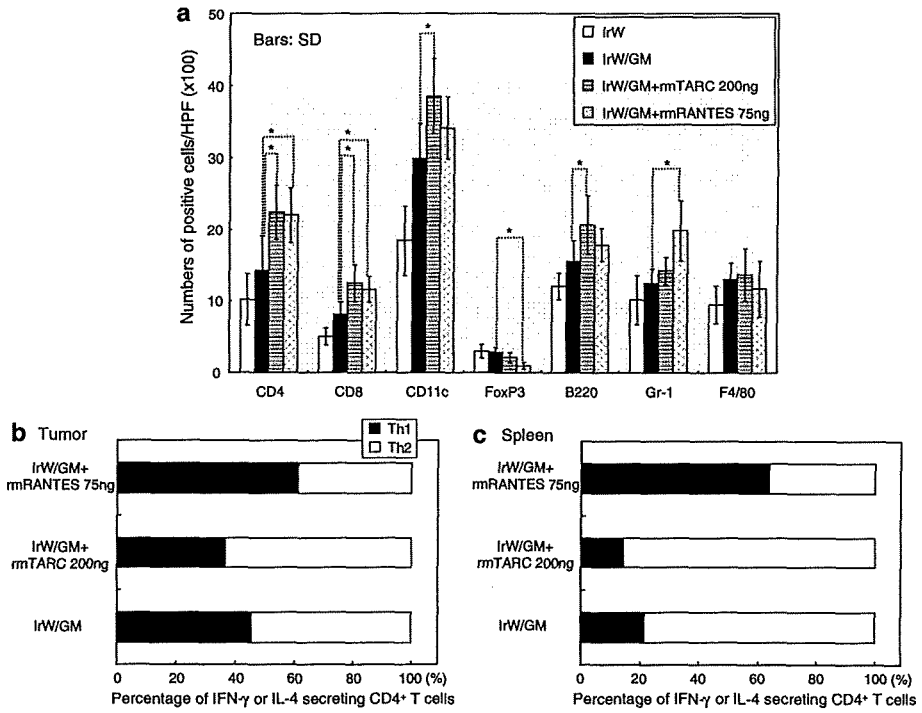

Fig. 6 **a** Immunophenotypic analysis of tumor-infiltrating cells (TIL) in tumor nodules. Mice were injected s.c. with 1×10^5 non-irradiated WEHI3B cells and treated with the indicated tumor vaccine cells as described in "Materials and methods". Excised tumors were subjected to immunohistological evaluation. To determine the proportions of $CD4^+$, $CD8^+$, $CD11c^+$, $FoxP3^+$, $CD45R^+$, $F4/80^+$ or $Ly\text{-}6G^+$ cells, stained cells were counted microscopically at ×100 in 10–30 HPF. Results are presented as the means ± SEM. Significant differences are denoted with *asterisks* ($^*P < 0.05$). **b, c** Th1/Th2 cell cytokine secretion profiles in the tumor (**b**) and spleen (**c**) of mice that received therapeutic tumor vaccination. Tumor-bearing mice ($n = 3$/experiment) were treated with the indicated tumor vaccine cells and killed as described in the immunophenotypic analysis. Single cell suspensions from the primary tumor and spleen were obtained and cultured with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were harvested and labeled with APC-conjugated anti-CD4 followed by FITC anti-IFN-γ and PE-conjugated anti-IL-4. Gates were set on $CD4^+$ T cell populations, and intracellular cytokine staining profiles within these populations were assessed by multicolor flow cytometry. *Numbers* indicate the percentages of specified TILs secreting intracellular IFN-γ (*Th1*) and IL-4 (*Th2*). Data are averages of two independent experiments with similar results

DC-specific growth factor fms-like tyrosine kinase3 ligand resulted in the recruitment, expansion, and activation of large numbers of DCs at the inoculation site and induced remarkable cellular and humoral antiviral immunity. Furthermore, our results on the Th1/Th2 balance in the tumor and spleen demonstrated that RANTES shifted the dominant Th2 balance induced by GM-CSF based tumor vaccination to a Th1 dominant condition through local and systemic Th1 cell accumulation. These results suggested that rmRANTES augmented not only Th1 cellular immunity against WEHI3B cells but also NK cell activation.

Results of our in vitro immunological cytokine assays using splenocytes harvested from mice immunized with irW/GM mixed with rmTARC or rmRANTES also suggested that the antigen-specific adaptive immunity induced by GM-CSF secreting cells was potentiated through the production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and TNF-α. Elevated IL-4 and IL-5 produced from splenocytes treated with irW/GM + rmTARC suggested that additional rmTARC might enhance systemic Th2 dominant responses through the activation of eosinophils, which are putatively involved in GM-CSF antitumor responses [3, 4, 40]. Indeed, our analysis of the Th1/Th2 balance supported the hypothesis. Since the combination of GM-CSF with IL-4 was efficient in generating DCs from hematopoietic precursors in vitro [2], localized production of GM-CSF and IL-4 induced by either rmTARC or rmRANTES may potentiate the antitumor effects induced by GM-CSF alone in vivo [10]. Miller et al. [26] reported that GM-CSF-recruited DCs expressed higher levels of both TNF-α and IL-6 than controls, resulting in potent T cell and NK cell activation. Furthermore, TNF-α enhanced the capacity of bone marrow-derived DCs to generate antitumor effects and CTL responses [1].

IL-6 enhanced an autologous tumor cell vaccine that secreted GM-CSF in a mouse RCC therapeutic model [21]. A recent report showed that IL-6 is essential to overcome immune suppression mediated by $CD4^+CD25^+$ regulatory

T cells (Tregs) [32] and to induce hematopoiesis and acute phase inflammation [15, 32], partially by blocking signaling that facilitates Tregs expansion [5]. Accordingly, our results suggest that both TARC and RANTES in combination with GM-CSF may interfere with the suppressive effects of Tregs, by enhancing IL-6 secretion and creating a microenvironment that does not support tumor growth. However, it is still possible that TARC attracts CCR4+ Treg cells [18]. Our immunohistochemical analysis showing less $FoxP3^+$ positive cells in tumors from mice treated with both irW/GM + rmTARC and irW/GM + rmRANTES further suggests that IL-6 is necessary to suppress Tregs expansion.

We have demonstrated the vaccine efficacy of GM-CSF-transduced tumor cells (auto-GVAX) in preclinical and clinical settings. Particularly, two of four patients had long-term survival greater 5 years with low dose IL-2 and one patient survived more than 8 years in PS0. The combination of auto-GVAX with other immune therapies is thought to potentiate the tumor specific immunity of auto-GVAX. The combination of GVAX with a neutralizing antibody to cytotoxic T lymphocyte antigen-4 (CTLA-4) is one of the strong candidates [6]. In this study, we demonstrated that TARC and RANTES enhanced antitumor immunity induced by GM-CSF, most likely by augmenting dominant Th2 and Th1 T cell immune responses, respectively, in mouse WEHI3B tumor models. These results also suggest that these chemokines have a clinical application in tumor vaccination. Namely, GVAX vaccination with the addition of either recombinant RANTES and/or TARC expression vectors is a promising second-generation GVAX gene therapy candidate. This latter novel gene therapy is currently under investigation using several viral vectors. In this regimen, GVAX may effectively enhance immune recognition of tumor antigens via DC activation and subsequent migration of activated T cells into the tumor tissue, leading to tumor eradication. Further preclinical investigations using different malignant cells types are required to prove this hypothesis.

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Non-transmissible Sendai virus encoding granulocyte macrophage colony-stimulating factor is a novel and potent vector system for producing autologous tumor vaccines

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The recent clinical application of granulocyte macrophage colony-stimulating factor (GM-CSF)-transduced autologous tumor vaccines revealed substantial antitumor activity and valuable clinical results. However, for these vaccines to be optimally effective, the antitumor efficacies must be improved. Recently, Sendai virus (SeV) vectors, which are cytoplasmic RNA vectors, have emerged as safe vectors with high gene transduction. In the current study, the *in vivo* therapeutic antitumor efficacies of irradiated GM-CSF-transduced mouse renal cell carcinoma (RENCA) vaccine cells mediated by either fusion gene-deleted non-transmissible SeV encoding mouse GM-CSF (SeV/dF/G) or adenovirus (E1, E3 deleted serotype 5 adenovirus) encoding mouse GM-CSF (AdV/G) (respectively described as irRC/SeV/GM or irRC/AdV/GM) were compared in RENCA-bearing mice. The results showed that the antitumor effect was equivalent between irRC/SeV/GM and irRC/AdV/GM cells, even though the former produced less GM-CSF *in vitro*. The cell numbers of activated ($CD80^+$, $CD86^+$, $CD80^+CD86^+$) dendritic cells in lymph nodes from mice treated with irRC/AdV/GM or irRC/SeV/GM cells were increased significantly compared with those of mice treated with the respective controls, at both the earlier and later phases. In an *in vitro* cytotoxicity assay, splenocytes harvested from mice treated with both irRC/SeV/GM and irRC/AdV/GM cells showed tumor-specific responses against RENCA cells. The restimulated splenocytes harvested from mice treated with irRC/SeV/GM or irRC/AdV/GM cells produced significantly higher levels of interleukin-2, interleukin-4, and interferon-γ compared with their respective controls ($P < 0.05$). Furthermore, vaccination with irRC/AdV/GM or irRC/SeV/GM cells induced significantly enhanced recruitment of the cytolytic effectors of $CD107a^+CD8^+$ T cells and $CD107a^+$ natural killer cells into tumors compared with those induced by their respective controls ($P < 0.05$). Taken together, our results suggest that the SeV/dF/G vector is a potential candidate for the production of effective autologous GM-CSF-transduced tumor vaccines in clinical cancer immune gene therapy. (*Cancer Sci* 2008; 99: 2315–2326)

Several studies have evaluated the capacity to augment antitumor immunity using various mouse models and have shown that GM-CSF is one of the most potent immunostimulatory cytokines.[1–3] GM-CSF is an important maturation and differentiation factor for DC,[4] including Langerhans cells to APC,[5] enhancing their capacity to present tumor-associated antigens to activate CTL effectively.[6] Moreover, the cytotoxic activity of NK cells or CTL can be enhanced or induced by GM-CSF-recruited DC. Therefore, GM-CSF has been postulated to be a critical mediator of the initial antitumor immune response.[7] In the past decade, clinical trials have shown that autologous GM-CSF gene-transduced tumor vaccine therapy is feasible, safe, and has effective antitumor immunomodulating activity against melanoma,[8,9] RCC,[10,11] prostate cancer,[12] pancreatic cancer[13] and non-small-cell lung cancer.[14] To generate effective GM-CSF-transduced tumor vaccines, it is essential to efficiently transduce tumor cells and to obtain appropriate expression of the induced gene. Serotype 5 E1, E3 gene-deleted adenovirus encoding human GM-CSF is one of the most promising vectors for tumor vaccines. However, adenoviral gene transduction is limited because the receptors for adenovirus serotype 5, including Coxsackie adenovirus receptor, integrin $\alpha v\beta 3$, and integrin $\alpha v\beta 5$, are not expressed on many tumor cells. SeV, a member of the *Paramyxoviridae* family, has a non-segmented negative-strand RNA genome and infects via sialic acid residues on surface glycoproteins or asialoglycoproteins, which are present on most mammalian cells.[15,16] Because of the ubiquitous expression of the SeV receptor and high gene transduction capacity, SeV is emerging as a promising gene therapy tool. As SeV possesses a cytoplasmic transcription system, it can transfer exogenous genes to the cytoplasm, where genomic replication and translation are carried out by virally encoded RNA polymerase. This replication system reduces the risk of malignant transformation due to genomic integration of the vector into the host-cell chromosome and increases the safety of this viral vector.[17–20] To further improve the safety of the SeV vector, we used a newly developed genetically modified temperature-sensitive mutant recombinant vector of non-transmissible SeV (SeV/dF). This vector can self-replicate but can not be transmitted to adjacent cells due to the lack of the F, thereby increasing the clinical application of this system.[21,22]

In the present study, we successfully transduced GFP, mouse GM-CSF, and human GM-CSF cDNA using SeV/dF encoding GFP (SeV/dF/GFP), mouse GM-CSF (SeV/dF/mGM), and human GM-CSF (SeV/dF/hGM) cDNA, respectively, into various tumor-cell lines *in vitro*. Subsequently, the antitumor efficacies of irradiated SeV-mediated GM-CSF-transduced RENCA (irRC/SeV/GM)

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Abbreviations: APC, antigen-presenting cell; CM, complete medium; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DMEM, Dulbecco's modified Eagle's medium; DLN, draining lymph node; F, fusion; FBS, fetal bovine serum; GFP, green fluorescent protein; GM-CSF, granulocyte macrophage colony-stimulating factor; HBSS, Hank's buffered salt solution; IL, interleukin; IFN, interferon; LLC, Lewis lung carcinoma; MHC, major histocompatibility complex; MOI, multiplicity of infection; NK, natural killer; PBS, phosphate-buffered saline; RCC, renal cell carcinoma; SeV, Sendai virus; TIL, tumor-infiltrating leukocyte; TNF, tumor necrosis factor.

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cells and irradiated AdV-mediated GM-CSF-transduced RENCA (irRC/AdV/GM) cells were compared in a RENCA-bearing mouse model, demonstrating that irRC/SeV/GM cells and irRC/AdV/GM cells had equivalent antitumor effects.

Materials and Methods

Mice. Six to eight week old female immunocompetent BALB/c and C57BL/6 mice were purchased from Clea Japan, (Tokyo, Japan) and housed in the Animal Maintenance Facility at Kyushu University under specific pathogen-free conditions. All animal experiments were approved by the Committee of the Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University and carried out following the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, Fukuoka, Japan and The Law and Notification of the Government. Mouse experiments were carried out at least twice to confirm results.

Tumor cell lines. WEHI-3B, a mouse myelomonocytic leukemia cell line, was kindly provided by Dr D. Metcalf (University of Melbourne), and RENCA, a mouse renal cell carcinoma cell line, was kindly provided by Dr M. Azuma (Tokyo Medical and Dental University). Mouse LLC and EL4 (lymphoma) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The human non-small-cell carcinoma cell lines PC9, H1299, H460, and LK87, were kindly provided by Dr K. Takayama (Kyushu University). The human RCC cell lines Caki-1, Caki-2, and A498 were purchased from the American Type Culture Collection. OSRC-2 and VMRC-RCW cells were purchased from the Riken BioResource Center (Ibaraki, Japan) and the Japanese Collection of Research Bioresources (Osaka, Japan), respectively. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2. All tumor-cell lines except for LLC cells (which were maintained in DMEM [Gibco, New York, NY, USA]) were cultured in tissue flasks or Petri dishes containing RPMI-1640 (Gibco) supplemented with 10% heat-inactivated FBS and penicillin (100 units/mL), streptomycin (0.1 mg/mL), and 2 mmol/L glutamine (CM).

Preparation of non-transmissible recombinant Sendai virus vectors. Preparation and recovery of recombinant temperature-sensitive non-transmissible SeV vectors harboring GFP, mouse GM-CSF, or human GM-CSF (SeV/dF/GFP, SeV/dF/mGM, and SeV/dF/hGM, respectively) were constructed as described previously.[21–23] A series of SeV/dF vectors were prepared using recombinant LLC-MK_2 cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used to induce the F protein into LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying a T7 RNA polymerase was inactivated with psoralen and long-wave ultraviolet irradiation, and then used to recover the ribonucleoprotein complex. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units. These vectors were kept frozen at –80°C until use (Fig. 1a).

Preparation of recombinant adenovirus vectors. The replication-defective recombinant adenovirus serotype 5 vectors that lack the E1A, E1B, and E3 genes and harbor the GFP or mouse GM-CSF genes (AdV/GFP and AdV/G, respectively) were constructed as described previously[24] and kindly provided by the Riken BioResource Center. The recombinant virus vectors were propagated in 293 cells (American Type Culture Collection), and titers were determined by a plaque-forming assay on 293 cells ($TCID_{50}$ method). The adenovirus solution was stored at –80°C until use. The recombinant adenovirus vector was used as a control to compare its antitumor activity with that of the recombinant SeV vectors in the present study (Fig. 1b).

SeV/dF/GFP-mediated green fluorescent protein transduction efficiency. One million cells of the various mouse (five) and human (nine) cell lines were seeded in six-well plates and transduced with SeV/dF/GFP when monolayers reached 60–80% confluence. As the standard inoculation procedure for vaccination, monolayers were washed twice with PBS and overlaid with serum-free medium containing SeV/dF/GFP at a MOI of 0, 1, 10, 50, 100, or 300. After a 90-min incubation at 37°C, nonadsorbed virus was removed, medium containing 10% FBS was added, and the cells were incubated for over 48 h at 37°C. The transduction studies were carried out in triplicate for each MOI. Microscopy was used to detect transduced cells by GFP fluorescence. At 48 h after tranduction, the GFP-transduced cells were analyzed for GFP expression using a FACS Calibur (BD Pharmingen, Franklin Lakes, NJ, USA).

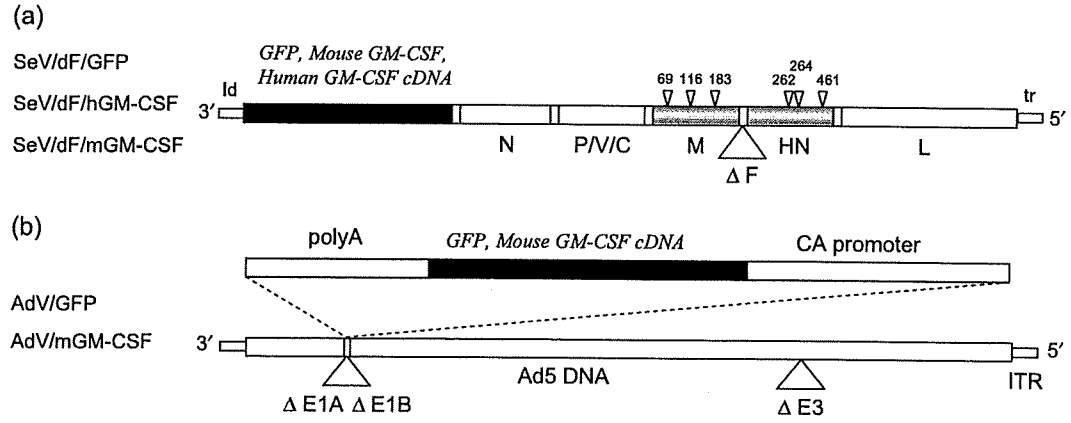

Fig. 1. Schematic representation of the viral vectors used in the present study. (a) The three recombinant Sendai virus (SeV) vectors (SeV/dF/GFP, SeV/dF/mGM, and SeV/dF/hGM) were based on the Z-strain of SeV. The SeV genome is delimited by two promoter regions: the leader (Id) and the trailer (tr) regions. The respective exogenous genes (green fluorescent protein [GFP], mouse granulocyte macrophage colony-stimulating factor [GM-CSF], and human GM-CSF) were inserted between the Id and the open reading frame of the N gene. The SeV genes encode the envelope-related proteins M, F, and HN, and the negative-stranded genomic ribonucleotide-protein complex (RNP) proteins N, P/V/C, and L. Temperature-sensitive recombinant SeV/dF vectors lose expression of the envelope-related M and HN genes, and have ribonucleotide substitutions in the M, HN, and L genes, as indicated by the arrowheads.[23] (b) The recombinant adenovirus vectors containing the GFP or mouse GM-CSF cDNA expression cassettes (AdV/GFP or AdV/mGM-CSF) were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome.[24] The expression of these genes was driven by a CAG promoter. These replication-defective adenovirus serotype 5 (Ad5)-based vectors have deletions in the E1A, E1B, and E3 regions. ITR, internal terminal repeat.

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Gene transduction and preparation for tumor vaccine cells. *Adenovirus-mediated gene transduction.* Tumor cells seeded in 10-cm Petri dishes were washed with PBS, and 1000 μL viral solution containing 5–10% FBS was added to each dish. After a 60-min incubation at 37°C, CM was added.

Sendai virus-mediated gene transduction. Tumor cells seeded in 10-cm Petri dishes were washed with PBS, and 1 mL FBS-free viral solution was added to each dish. After a 90-min incubation at 37°C, CM was added.

These adenovirus or SeV genetically modified tumor cells were incubated for an additional 24 h in CM, and then irradiated with 50 Gy (for mouse tumor cell lines) or 100 Gy (for human cell lines) using a ^{137}Cs-source γ cell 40 (Atomic Energy of Canada, Missisauga, ON, Canada). These irradiated cells were incubated for an additional 1–2 days (for RENCA vaccine cells, a 2-day incubation), and trypsinized cells were subjected to the following tumor vaccine experiments.

Quantification of granulocyte macrophage colony-stimulating factor production levels from granulocyte macrophage colony-stimulating factor-transduced tumor cells. The *in vitro* levels of mouse or human GM-CSF produced from adenovirus- or SeV-mediated GM-CSF-transduced tumor cells at the indicated MOI and times, with or without irradiation, were measured using mouse GM-CSF and human GM-CSF enzyme-linked immunosorbent assay kits (BD Pharmingen), respectively.

***In vitro* viability of tumor cells after non-transmissible Sendai virus transduction.** Cell viability was determined by trypan blue exclusion. Two million parental RENCA or LLC cells were seeded onto 100-mm Petri dishes, transduced with SeV/dF/GFP (MOI = 100) or SeV/dF/mGM (MOI = 100) for 90 min in serum-free RPMI-1640 or DMEM, cultured in CM for 48 h, and then trypsinized, diluted, and stained with 0.4% (w/v) trypan blue (Gibco). The number of trypan blue-positive and -negative cells was counted under a light microscope. The percentage of cells excluding trypan blue was taken as an index of cell viability. Cell morphology was also visualized under the light microscope.

***In vitro* proliferation assay.** For the proliferation assay, RENCA or LLC cells were cultured separately in 96-well microplates at a concentration of 1×10^4 cells/well. After a 4–5-h incubation with CM to promote cell adhesion, the tumor cells were washed with PBS, transduced with SeV/dF/GFP (MOI = 1, 10 or 100) or SeV/dF/mGM (MOI = 1, 10 or 100) for 90 min in serum-free RPMI-1640 or DMEM, and incubated for 1–4 days. At each time point (days 0, 1, 2, and 4 after SeV transduction), the number of viable cells was estimated spectrophotometrically by the incorporation of tetrazolium dye using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan). The reagent was added, and the cells were incubated for an additional 1 h, after which an optical density value at 450 nm was determined using a microplate reader. All assays from three independent experiments were carried out in triplicate.

Experimental design of granulocyte macrophage colony-stimulating factor-transduced tumor vaccines. On the day of tumor challenge, RENCA cells that had been thawed from frozen stores and cultured *in vitro* for 1–2 weeks were trypsinized, washed twice in HBSS, and inoculated subcutaneously into the right flank of BALB/c mice (day 0, 1×10^6 cells/mouse, $n = 9$). Tumor volume was monitored two or three times per week. RENCA vaccine cells were inoculated subcutaneously into the left flank three times weekly, starting 7 days after tumor inoculation. The treatment groups included HBSS, irRC cells, irRC/AdV/GFP cells (MOI = 300), irRC/AdV/GM cells (MOI = 300), irRC/SeV/GFP cells (MOI = 100), or irRC/SeV/GM cells (MOI = 100).

In all tumor-implantation experiments, each injection was diluted in 100 μL HBSS using a 1-mL tuberculin syringe with a 27-gauge needle. Two bisecting diameters of each tumor were measured with calipers. The tumor volumes were calculated using the formula: volume = $0.4ab^2$, where a is the larger diameter and b is the smaller diameter. Changes in tumor growth were monitored two or three times per week.

Immunofluorescence analysis for costimulation-related molecules on dendritic cells. RENCA-bearing mice were treated with the tumor vaccinations described above. The two left axillary (vaccination side) DLN were dissected on day 2 after the first tumor vaccination and on day 7 after the third tumor vaccination. All single-cell suspensions from DLN ($n = 3$/group) were prepared by mechanical homogenization. The number of cells was determined by counting crushed DLN with a hemocytometer under a light microscope. For phenotype profiles of DC, the cells were washed and blocked with antimouse CD16/32 FcR antibody for 15 min and then analyzed by triple immunostaining using the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-CD86, phycoerithrin (PE)-conjugated anti-CD80, APC-conjugated anti-CD11c, and isotype controls (all from eBioscience, San Diego, CA, USA) for 30 min at room temperature. Analysis was carried out using a FACS Calibur with CellQuest software (BD Pharmingen). Data were collected on 40 000 viable cells.

***In vitro* cytotoxicity assay.** Splenocytes were prepared from dead RENCA-bearing mice 7 days after the third indicated tumor vaccination as described above. The vaccination groups included irRC/AdV/GFP, irRC/AdV/GM, irRC/SeV/GFP, and irRC/SeV/GM cells. To generate RENCA-specific effector cells, splenocytes (4×10^6 cells/well) depleted of erythrocytes with ammonium chloride were cocultured with mitomycin C (100 μg/mL, 90 min, 37°C)-treated RENCA cells at a ratio of 10:1 in 1 mL CM in 24-well plates at 37°C in 5% CO_2. Two days later, recombinant human IL-2 (PeproTech EC, London, UK), at a concentration of 30 U in 500 μL fresh CM, was added to each well. Splenocytes were harvested on day 6 and used as effector cells in a standard 5-h ^{51}Cr release assay to examine antitumor cytolytic activity.[25] Briefly, both RENCA cells as the tumor target and WEHI-3B cells as the cold target (1×10^6 cells) were labeled with 3.7 MBq 100 μCi $Na_2{}^{51}CrO_4$ (PerkinElmer, Boston, MA, ISA) in 200 μL CM for 90 min at 37°C. After three washes with PBS, the labeled target cells (1×10^4 cells/well) were incubated with the effector cells for 5 h at 37°C in 96-well round-bottomed microtiter plates at the indicated effector:target ratios. The plates were then centrifuged at 50g for 5 min, and the radioactivity of the supernatants was measured with a γ counter from Auto Well Gamma Systems (Aloka, Tokyo, Japan). The maximum and spontaneous release were determined from samples incubated with 1% Triton X-100 and medium alone, respectively. Cytolytic activity was calculated using the following formula: specific ^{51}Cr release (%) = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release). Assays were carried out in triplicate. The spontaneous release in all assays was <10% of the maximum release.

Detection of splenic cytotoxic T lymphocyte activity using the CD107a mobilization assay. To monitor the cytolytic activity of putative tumor-specific CTL ($CD3^+CD8^+$ T), the CD107a mobilization assay was carried out to detect CTL degranulation.[26–28] Briefly, splenocytes (1×10^4 cells/well) harvested as described previously were cocultured in CM with RENCA cells at a ratio of 20:1 for 72 h. The cell suspension was then collected and restimulated with or without RENCA cells or WEHI-3B cells at the indicated ratio for an additional 5–6 h in the presence of phycoerithrin (PE)-conjugated antimouse CD107a antibody or isotype IgG controls. Cells were washed and blocked with antimouse CD16/32 FcR antibody for 15 min and then surface stained with fluorescence-conjugated antimouse CD3, CD8, and CD107a markers (all from eBioscience).

Interferon-γ and interleukin-4 ELISPOT assay for splenocytes of mice immunized with tumor vaccine cells. On day 6 after the second tumor vaccination in the therapeutic model described above, mice were killed and their splenocytes were tested for mouse IFN-γ and IL-4 secretion using an ELISPOT assay kit (Cytokine ELISPOT Set; BD Pharmingen). ImmunoSpot ELISPOT 96-well plates were

coated with 5 μg/mL purified antimouse IFN-γ or antimouse IL-4 monoclonal antibody and incubated overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 and incubated for 2 h with blocking buffer (RPMI-1640 with 10% FBS) at room temperature. Red blood cell (RBC)-depleted splenocytes (1×10^5) were incubated for 20 h at 37°C with 5% CO_2 in the presence or absence of irradiated RENCA cells or WEHI-3B cells at the indicated splenocyte:irradiated tumor cell ratios (100:1 and 50:1) in a total volume of 200 μL. As a positive control, 20 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, St Louis, MO, USA), known as a mitogen for T-cell stimulation, was added to the indicated wells. After the plates were washed, the wells were incubated with 2 μg/mL biotinylated antimouse IFN-γ or antimouse IL-4 monoclonal antibody for 2 h at room temperature. The plates were then washed extensively, incubated with streptavidin–horseradish peroxidase solution for 1 h at room temperature, washed twice, incubated with Final Substrate Solution (AEC substrate mixed with AEC Chromogen; BD Pharmingen), and then monitored for spot development for 5 min at room temperature. After drying, spots indicating IFN-γ- or IL-4-secreting cells were enumerated manually under a dissecting Axiovert microscope (Zeiss, Jena, Germany) and expressed as the mean number of spots + SD of quadruplicated determinations.

Cytometric bead array and enzyme-linked immunosorbent assays for the quantification of mouse cytokines produced from splenocytes of mice immunized with tumor vaccine cells. Similar to the ELISPOT analysis, RBC-depleted mouse splenocytes (5×10^6) harvested on day 6 after the second tumor vaccination were incubated in the presence or absence of irradiated RENCA cells at a 10:1 ratio in a total volume of 1.0 mL at 37°C for 20 h. Cell supernatants were collected, and the concentrations of mouse IL-2, IL-4, IL-5, TNF-α, and IFN-γ were measured using the BD Mouse Th1/Th2 Cytokine Cytometric Bead Array Kit (BD Pharmingen) according to the manufacturer's protocol. The concentration of IL-6 was measured using a mouse IL-6 immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's procedure.

Immunohistochemistry. On day 6 after the second indicated tumor vaccination in the therapeutic RENCA model described above, established RENCA tumors ($n = 3$/group) were snap frozen by overlaying with OCT compound (Sakura Fine Technical, Tokyo, Japan). All samples were stored at –80°C until analysis. Serial cryostat (8–10-μm) frozen sections were adhered to Superfrost slide glasses (Matsunami, Osaka, Japan), fixed in acetone at room temperature for 10 min, air-dried, and rinsed in distilled water to remove the embedding medium. Staining for TIL was conducted following standard procedures. Briefly, sections were incubated sequentially overnight at 4°C with the appropriately diluted primary antibodies mouse CD4 (GK1.5), CD8 (53–6.7), CD11c (N418), and FoxP3 (FJK-16s) (all from eBioscience) following the manufacturer's instructions, followed by a 1-h incubation with biotinylated antirat or antihamster secondary antibody (eBioscience). After a 30-min incubation with streptavidin–peroxidase (Dako Japan, Kyoto, Japan), antigen–antibody reactions were developed using 3,3′-diaminobenzidine (Nakalai Tesque, Kyoto, Japan) substrate. Slides were washed three times with PBS between each incubation step, counterstained with Mayer's hematoxylin, and dehydrated in a sequentially graded alcohol and xylene series prior to mounting. All incubations were conducted in a humid chamber. Photographs were taken with an Axiovert microscope. The stained cells were visualized in a series of high-power fields and counted microscopically at ×200 magnification in 30–70 high-power fields. The percentages of positive cells were calculated, and the results are expressed as the mean ± SD.

Immunofluorescence analysis for tumor-infiltrating cytolytic effector cells. At the same time as the *in vitro* cytotoxicity assay, established RENCA tumors ($n = 3$/group) were dissected. For flow cytometric analysis, we placed tumors in six-well plates and minced them finely. They were transferred to 15-mL tubes, incubated for 90 min under continuous rotation in RPMI-1640-containing collagenase (Gibco), and passed through a 70-μm strainer, washed and resuspended in PBS. Viable lymphocytes were enriched and collected using centrifugation over Lympholyte-M (Cedarlane Laboratories, Barlington, ON, Canada) at 1000× for 20 min and counted using a hemocytometer. Subsequently, to quantify the cytolytic effector cells in TIL, the cell suspensions were stained with fluorescence-conjugated antimouse CD8, anti-DX-5, and antimouse CD107a antibodies for 30 min. The cells were washed twice in staining buffer and analyzed on a FACS Calibur.

Statistical analysis. For statistical analysis, a two-tailed Student's *t*-test was used. The *P*-values were obtained from two-tailed tests of statistical significance. Survival was plotted using Kaplan–Meier curves and statistical relevance was determined by a log-rank comparison using Statview software. A probability value was considered significant when $P < 0.05$.

Results

Transduction efficiency of the SeV/dF/GFP vector into various human and mouse tumor-cell lines. Nine human and five mouse tumor-cell lines propagated *in vitro* were collected, transduced by SeV/dF/GFP, and examined for gene transduction efficiency. Flow cytometric analyses showed dose-dependent GFP expression, and optimal expression was obtained at MOI of 10–100; >90% GFP-positive tumor-cell lines were detected at MOI over 10 (Fig. 2a,b).

Continuous *in vitro* granulocyte macrophage colony-stimulating factor expression was obtained with SeV/dF/mGM- and SeV/dF/hGM-transduced tumor-cell lines. Next, we quantified that levels of GM-CSF produced from mouse or human GM-CSF-transduced tumor cell lines (by SeV/dF/mGM or SeV/dF/hGM, respectively) at the indicated MOI. As shown in Figure 3a, mouse GM-CSF levels produced from four histologically different mouse tumor cell lines that were SeV/dF/mGM tranduced were maximized to more than 300 ng/10^6 cells/24 h, at MOI over 50. Likewise, four human cell lines (two non-small-cell lung cancer and two RCC cell lines) that were transduced with SeV/dF/hGM produced sufficiently high GM-CSF levels in a MOI-dependent manner for at least 7 days after transduction (Fig. 3b). Taken together, these findings demonstrate that SeV/dF/GFP, SeV/dF/mGM, and SeV/dF/hGM vectors have highly successful and continuous gene transduction in various tumor cell lines.

SeV/dF vectors did not inhibit the proliferation or viability of transduced tumor cell lines. To exclude the possibility that the SeV-transduced exogenous genes and constitutive SeV viral components affected the survival and growth of tumor cells, parental RENCA or LLC tumor cells transduced with either SeV/dF/GFP or SeV/dF/mGM at the indicated MOI were cultured *in vitro* under the same conditions, and cell viability and proliferation were evaluated. SeV transduction (MOI = 100) had little effect on RENCA and LLC cell survival on day 2 when RC/SeV/G cells had rather significantly greater viability than non-transduced RENCA and RC/SeV/GFP cells ($P < 0.05$) (Fig. 3c,d). Furthermore, after SeV/dF/GFP transduction at various MOI, both RENCA and LLC cells had the same proliferation rates as non-transduced cells (control) over 4 consecutive days ($P < 0.05$) (Fig. 3e,f).

Effects of irradiation on granulocyte macrophage colony-stimulating factor production from SeV/dF/G-transduced tumor cells *in vitro*. To determine the effects of irradiation on the transgene expression of SeV/dF/G-transduced tumor cells, we measured GM-CSF production levels from SeV/dF/G-transduced RENCA (mouse) or A549 (human) cells, with or without irradiation on day 1 (50 Gy and 100 Gy, respectively), at the indicated MOI and times. As shown in Figure 4a,b, irradiated A549 cells produced significantly higher levels of GM-CSF than non-irradiated A549 cells, whereas irradiated RENCA cells produced lower (day 2) or similar (days 3 and 5) levels of GM-CSF than non-irradiated RENCA cells. The different observations between RENCA and A549 cells seemed

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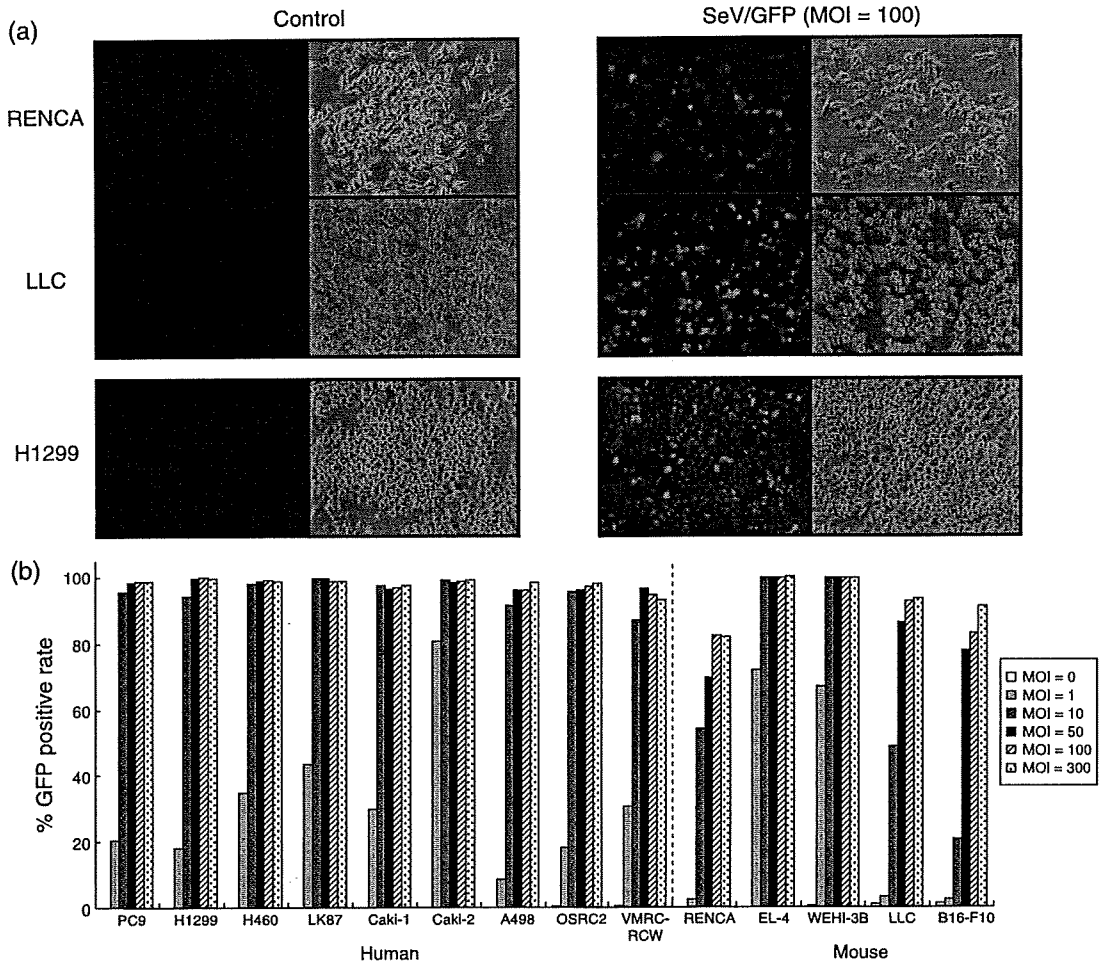

Fig. 2. Transduction of various mouse and human cell lines with the SeV/dF/GFP vector. (a) RENCA (row 1), Lewis lung carcinoma (LLC) (row 2), and H1299 cells (row 3) were transduced with SeV/dF/GFP at a multiplicity of infection (MOI) of 100. Fluorescence microscopy of transduced cells was carried out 48 h later (green fluorescent protein [GFP] phases are displayed in column 3). The background fluorescence of transduced cells (control; column 1) was determined in non-transduced cultures. Phase contrast pictograms are displayed in columns 2 and 4. (b) Nine mouse and five human cell lines were transduced with SeV/dF/GFP at MOI of 0, 1, 10, 50, 100, and 300. The percentage of GFP-expressing cells was determined by flow cytometric analysis. The bar graph depicts the percentage of GFP-positive cells at 48 h after transduction with or without SeV/dF/GFP.

to be negligible, and tumor vaccine cells continued to produce abundant GM-CSF even on day 5 after SeV transduction.

Therapeutic vaccination with irradiated SeV- or adenovirus-mediated granulocyte macrophage colony-stimulating factor-transduced RENCA cells retarded established tumor development *in vivo*. We determined the optimal MOI of adenovirus or SeV vectors for tumor vaccination in preliminary experiments. Irradiated RENCA cells transduced with AdV/mGM or SeV/dF/mGM at a MOI of 300 or 100 produced the highest levels of mouse GM-CSF *in vitro* (1250 ± 15.9 ng/10^6 cells/48 h and 643.98 ± 57.61 ng/10^6 cells/48 h, respectively), and showed the most effective antitumor efficacies in therapeutic experiments in RENCA-bearing mice (data not shown). Next, we directly compared the *in vivo* antitumor therapeutic effects of tumor vaccination between irRC/AdV/GM and irRC/SeV/GM cells. Immunocompetent BALB/c mice were inoculated subcutaneously into the right flank with parental RENCA cells (day 0). On days 7, 14, and 21, 1×10^6 cells of irRC, irRC/AdV/GFP, irRC/SeV/GFP, irRC/AdV/GM, or irRC/SeV/GM were inoculated subcutaneously into the left flank as a tumor vaccination. On day 24, the growth of established RENCA tumors was significantly retarded in mice treated with irRC/AdV/GM or irRC/SeV/GM cells compared with control mice (irRC/AdV/GFP or irRC/SeV/GFP, respectively) ($P < 0.05$) (Fig. 4c), although tumor development was not eliminated in all treated mice. Mice treated with irRC/SeV/GM cells survived longer than those treated with irRC/AdV/GM cells ($P < 0.05$) (Fig. 4d), whereas the antitumor effect of irRC/SeV/GM cells was not statistically significant on day 24 compared with that of irRC/AdV/GM cells, suggesting that irRC/SeV/GM vaccination may prevent primary metastases.

Therapeutic vaccination with irradiated SeV- or adenovirus-mediated granulocyte macrophage colony-stimulating factor-transduced RENCA cells enhanced the expression of the costimulatory markers CD80 and CD86 on dendritic cells *in vivo*. We next examined the expression levels of the costimulatory markers CD80 (B7-1) and CD86 (B7-2) on DC ($CD11c^+$) in DLN during therapy with tumor vaccination. As shown in Figure 5a,b, the total numbers of $CD80^+$, $CD86^+$, and $CD80^+CD86^+$ DC in DLN from mice treated with irRC/AdV/GM or irRC/SeV/GM cells were increased significantly compared with those of mice treated with their respective controls, at the earlier phase on day 2 after first tumor vaccination. The only exception was the $CD80^+$ DC comparison between irRC/SeV/GFP and irRC/SeV/GM therapy ($P < 0.05$). Furthermore, at the later

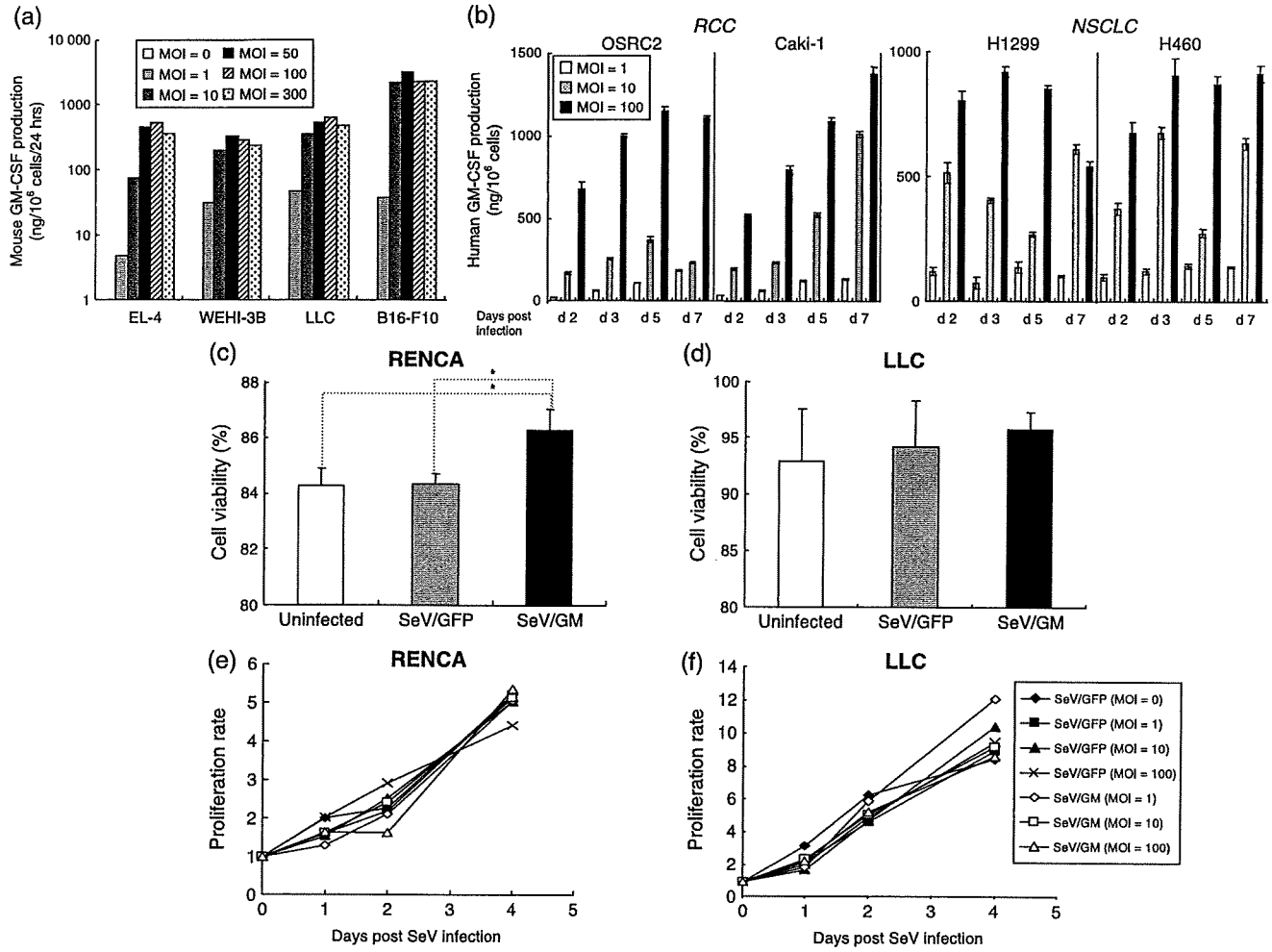

Fig. 3. Granulocyte macrophage colony-stimulating factor (GM-CSF) production from mouse or human tumor cell lines transduced with SeV/dF/mGM or SeV/dF/hGM, and the viability and proliferation of Sendai virus (SeV)-transduced cells. (a) One million cells from four mouse tumor cell lines were transduced with SeV/dF/mGM at multiplicities of infection (MOI) of 0, 1,10, 50, 100, and 300 for 90 min in serum-free RPMI-1640 and incubated for 10% fetal bovine serum (FBS)/RPMI in 6-well plates for 24 h. Mouse GM-CSF levels produced in each supernatant were measured by enzyme-linked immunosorbent assays. (b) Human GM-CSF levels produced by four human cell lines (two for non-small-cell lung cancer [NSCLC] and two for renal cell carcinoma [RCC]) transduced with SeV/dF/hGM at MOI of 1, 10, and 100 on days 2, 3, 5, and 7 after transduction were measured by enzyme-linked immunosorbent assays. Cell viability after SeV infection was evaluated by trypan blue exclusion. (c,d) Two million parental RENCA or Lewis lung carcinoma (LLC) cells were transduced with SeV/dF/GFP (MOI = 100) or SeV/dF/mGM (MOI = 100) for 90 min, and cultured for 48 h. The number of trypan blue-positive and -negative cells was counted under a light microscope, and the percentage of cells excluding trypan blue is represented as an index of cell viability. (e,f) RENCA and LLC cells were cultured separately in 96-well microplates at 1×10^4 cells/well. They were transduced with SeV/dF/GFP (MOI = 1, 10, or 100) or SeV/dF/mGM (MOI = 1, 10, or 100) for 90 min in serum-free medium, and cultured for 1, 2, and 4 days in RPMI-1640 with 10% FBS or Dulbecco's modified Eagle's medium with 10% FBS, respectively. At each time point (day 0, 1, 2, or 4 after SeV transduction), the number of viable cells was estimated spectrophotometrically by the incorporation of tetrazolium dye. Representative data from three independent experiments are shown.

phase, on day 7 after the third tumor vaccination as a booster, the total numbers of $CD80^+$, $CD86^+$, and $CD80^+CD86^+$ DC in DLN from mice treated with irRC/AdV/GM or irRC/SeV/GM cells were increased significantly, amplified almost 10 times compared with those observed at the earlier phase and those treated with their respective controls ($P < 0.05$). Similar to these results, the percentages of $CD80^+$-, $CD86^+$-, and $CD80^+CD86^+$-stained cells on DC from mice treated with irRC/AdV/GM or irRC/SeV/GM cells were also higher than those of mice treated with their respective controls at the same two time points (data not shown). Collectively, these results suggest that the costimulatory markers were markedly upregulated by GM-CSF-transduced RENCA vaccines.

Splenocytes from mice treated with irRC/AdV/GM or irRC/SeV/GM cells showed tumor-specific cytotoxicitiy against RENCA cells. To compare the *in vitro* cytolytic activity against RENCA cells, we next carried out a ^{51}Cr-release assay using splenocytes from each immunized mouse. Prior to the cytotoxicity assay, we evaluated MHC class I ($H\text{-}2K^d$) surface expression on RENCA and WEHI-3B tumor cells by flow cytometry, as MHC class I expression on tumor cells is required for CTL recognition in cancer immunotherapy.[29] MHC class I expression was high on RENCA cells and moderate on WEHI-3B cells (data not shown). Mice were killed 7 days after the last-indicated tumor vaccination and splenocytes were harvested. Prepared splenocytes were restimulated *in vitro* with mitomycin (MMC)-treated RENCA cells for 6 days, and cytolytic activity was measured. The results showed that cytotoxicity against RENCA cells from mice treated with irRC/AdV/GM or irRC/SeV/GM cells were elevated and superior to those from

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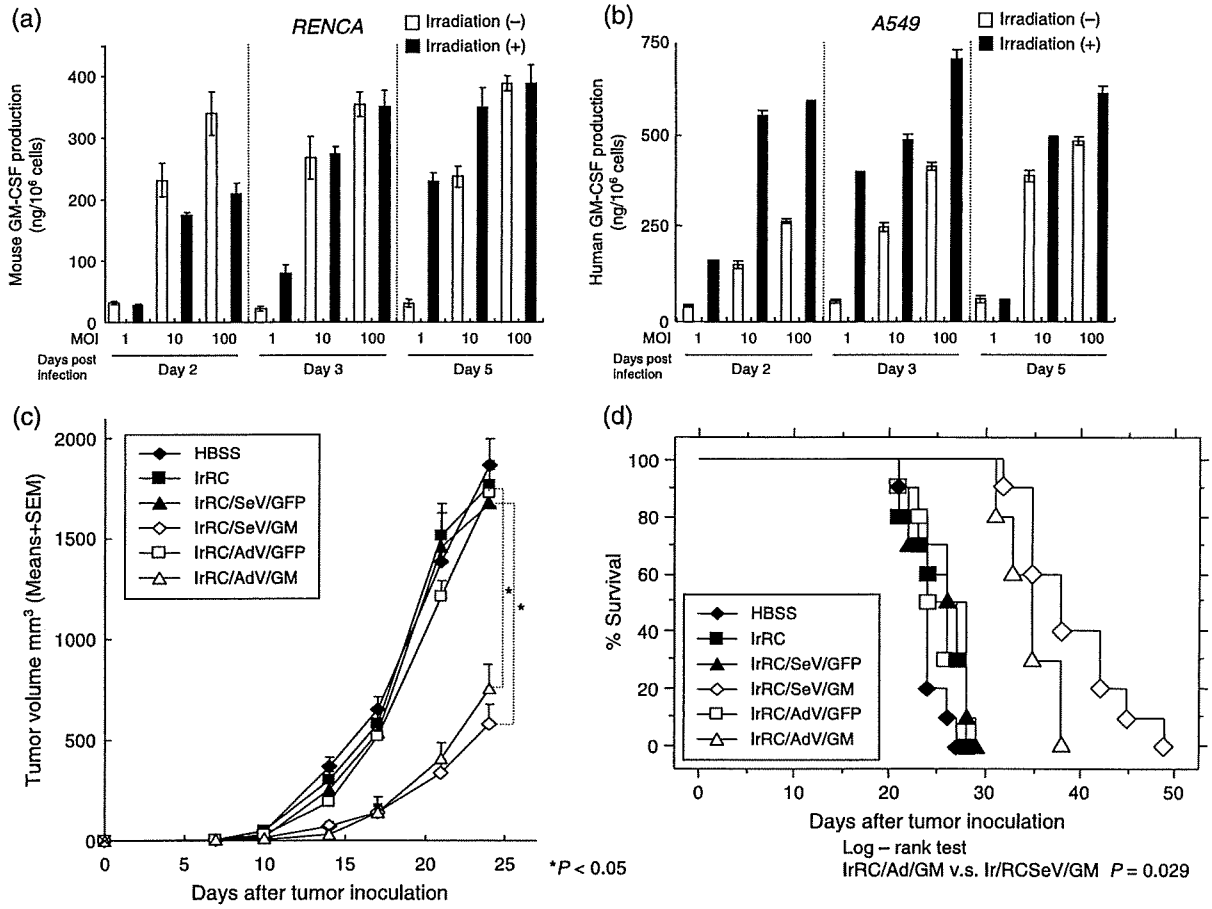

Fig. 4. *In vitro* effects of irradiation on granulocyte macrophage colony-stimulating factor (GM-CSF) production from SeV/dF/G-transduced tumor cells and *in vivo* effects of irradiated Sendai virus (SeV)- or adenovirus-mediated GM-CSF-transduced RENCA vaccine cells against established tumors. (a,b) Levels of GM-CSF produced from SeV/dF/mGM-transduced mouse RENCA cells or SeV/dF/hGM-transduced human H1299 cells with or without irradiation (on day 1) were measured comparatively at multiplicities of infection (MOI) of 1, 10, and 100 on days 2, 3, and 5 after transduction by enzyme-linked immunosorbent assays. (c) One million of the parental RENCA cells were inoculated subcutaneously into the right flank of BALB/c mice ($n = 9$), followed by subcutaneous inoculation of 1×10^6 cells of the indicated RENCA vaccine in the left flank weekly for three times (on days 7, 14, 21). The treatment groups included Hank's buffered salt solution (HBSS) only, irRC, irRC/AdV/GFP, irRC/AdV/GM, irRC/SeV/GFP, and irRC/SeV/GM cells. For adenovirus- or SeV-mediated transduction for preparing tumor vaccine cells, RENCA cells were transduced with adenovirus or SeV at a MOI of 300 or 100, respectively. Tumor volume was monitored twice or three times per week. (d) Survival curve of the RENCA-bearing mice treated with tumor vaccination as described above. Bar graphs depict the means ± SEM. Significant differences are denoted with asterisks (*$P < 0.05$).

mice treated with their respective controls. Intriguingly, a relatively low level of cytotoxicity was observed in mice treated with irRC/SeV/GFP cells (Fig. 6a). In contrast, when control syngeneic WEHI-3B cells were used as a target, they showed negative results (Fig. 6b). These results indicate that GM-CSF but not GFP substantially contributes to the induction of RENCA-specific antitumor activity.

In order to examine whether the cytolytic effector cells consisted mainly of $CD8^+$ T cells (CTL), a CD107a (lysosomal membrane glycoprotein 1) mobilization assay (a surrogate for lytic degranulation)[26–28] was carried out using restimulated splenocytes with RENCA cells *in vitro*. CD107a mobilization of $CD8^+$ T cells against RENCA cells was increased significantly in mice treated with irRC/AdV/GM and irRC/SeV/GM cells compared with those treated with their respective controls, whereas the mobilization of $CD8^+$ T cells incubated with WEHI-3B cells remained at basal levels ($P < 0.05$) (Fig. 6c). The results suggest that tumor-specific $CD8^+$ T cells were generated *in vivo* and possessed the capacity to release an abundant amount of cytolytic granules, including perforin and granzyme B.

***In vitro* inflammatory cytokine production profile of splenocytes from mice treated with granulocyte macrophage colony-stimulating factor-transduced RENCA vaccine cells.** For characterization of the immunomodulatory effects of GM-CSF-transduced RENCA vaccination, we examined *in vitro* inflammatory cytokine production of splenocytes cocultured with or without irradiated RENCA cells using immunocompetent mice immunized with RENCA vaccine cells. First, to quantify the number of IL-4- or IFN-γ-producing splenocytes, splenocytes harvested from mice either untreated or treated with irRC/AdV/GFP, irRC/SeV/GFP, irRC/AdV/GM, or irRC/SeV/GM cells were subjected to an *in vitro* ELISPOT assay for IFN-γ and IL-4. When cocultured in the presence of irradiated RENCA cells, the number of splenocytes from RENCA-bearing mice treated with either irRC/AdV/GM or irRC/SeV/GM cells that produced both IFN-γ and IL-4 was significantly higher than those from each control group (irRC/AdV/GFP or irRC/SeV/GFP) ($P < 0.05$). In addition, the number of splenocytes from mice treated with irRC/SeV/GM cells that produced IFN-γ was significantly higher than those from mice treated with irRC/AdV/GM cells ($P < 0.05$) (Fig. 7a). These enhanced IFN-γ-producing cells were