

# 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- $\gamma$ , and TNF- $\alpha$ , suggesting enhanced innate and adaptive immunity. Immunohistochemical analysis of tumor sections after vaccination revealed a more significant contribution of CD4<sup>+</sup> and CD8<sup>+</sup> 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 V $\beta$  chain variables at tumors sites and delayed-type hypersensitivity at vaccination sites with eosinophil infiltration [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 chemottracts 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<sub>2</sub> 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<sup>6</sup> 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 ( $1 \times 10^6$ ) 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  $\mu$ g/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  $^{137}\text{Cs}$  source gamma cell 40 (Atomic Energy of Canada Limited, Ontario, Canada). These irradiated cells were then incubated for 24 h, suspended in 300  $\mu\text{l}$  HBSS (Hank's Buffer Salt Solution; Gibco BRL, NY, US) to  $1 \times 10^7$  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,  $1 \times 10^5$  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,  $3 \times 10^6$  WEHI3B tumor vaccine cells resuspended in 300  $\mu\text{l}$  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  $3 \times 10^6$  irW/GM cells, irW/GM cells alone, or 100  $\mu\text{l}$  of HBSS were injected s.c. into the left flank of mice on days 3, 6, 9 and 12 after inoculation of  $1 \times 10^5$  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  $1 \times 10^5$  WEHI3B cells into the right flank and monitored in the same fashion. Mice with tumor burdens greater than 2,000  $\text{mm}^3$  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  $3.0 \times 10^6$  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 ( $4 \times 10^6$  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  $\mu\text{M}$  2-ME) in 24-well plates at 37°C in 5%  $\text{CO}_2$ . Two days later, 30 U of recombinant human IL-2 (PeproTech EC, London, U.K.) was added to each well in 500  $\mu\text{l}$  fresh culture medium. Splenocytes including CTLs were harvested on day 6 and used as effector cells in a standard 5 h  $^{51}\text{Cr}$  release assay to examine the antitumor cytolytic activity [37]. Briefly, WEHI3B cells as a target tumor and RENCA cells as a nonspecific target ( $1 \times 10^6$  cells) were labeled with 100  $\mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  (PerkinElmer, Boston, MA, US) in 200  $\mu\text{l}$  of RPMI 1640 supplemented with 10% heat-inactivated FBS for 90 min at 37°C. After three washes with PBS, the labeled target cells ( $1 \times 10^4$  cells/well) were incubated with the effector cells for 5 h at 37°C in 96-well round bottomed microtiter plates in 200  $\mu\text{l}$  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  $\gamma$  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  $^{51}\text{Cr}$  release = (experimental release – spontaneous release)  $\times$  100/(maximum release – spontaneous release). Assays were performed in triplicate. The spontaneous release in all assays was <20% of the maximum release.

#### Mouse IFN- $\gamma$ 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,  $3 \times 10^6$  irW/GM, irW/GM + rmTARC 200 ng or irW/GM + rmRANTES 75 ng cells) were tested for secretion of mouse IFN- $\gamma$  and IL-4 using an ELISPOT assay kit (Cytokine ELISPOT Set, BD Pharmingen, NJ, US). ImmunoSpot™ ELISPOT 96-well plates were coated with 5  $\mu\text{l}$ /ml of purified anti-mouse IFN- $\gamma$  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 ( $1 \times 10^5$ ) 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  $\mu$ l at 37°C with 5% CO<sub>2</sub> 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  $\mu$ l/ml of biotinylated anti-mouse IFN- $\gamma$  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- $\gamma$  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- $\alpha$ , and IFN- $\gamma$  production by splenocytes

In a similar fashion to ELISPOT, RBC depleted splenocytes ( $5 \times 10^6$ ) 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- $\alpha$  and IFN- $\gamma$  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  $\mu$ l rabbit anti-asialo GM<sub>1</sub> anti-serum (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 <sup>51</sup>Cr release assay [30].

Immunohistochemistry

Five days after the fourth tumor vaccinations (s.c. injection in the left flank of  $3 \times 10^6$  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  $-80^\circ\text{C}$  until analysis. Serial cryostat (8  $\mu$ m) 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 $\times$  magnification in 10–30 high-power fields. Results are presented as the means  $\pm$  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  $3 \times 10^6$  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  $\mu$ m 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 (CEDAR-LANE, 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- $\gamma$  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 multi-parameter 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**

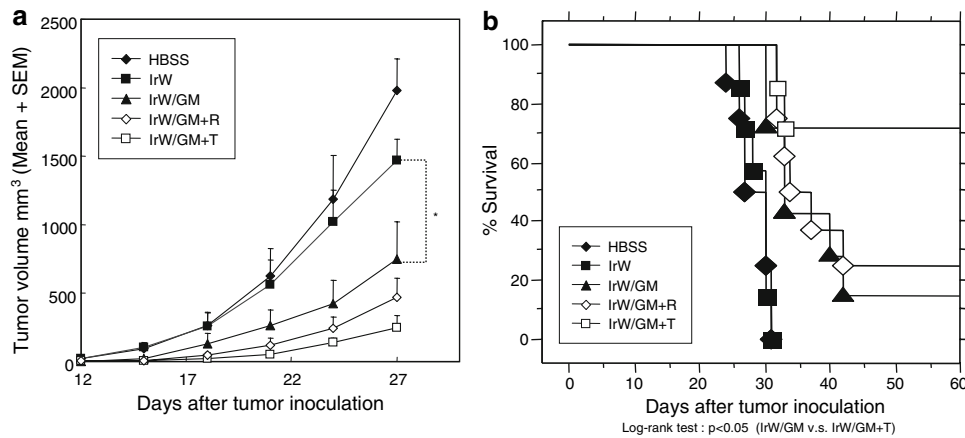
We previously reported that mice inoculated s.c. with non-irradiated WEHI3B cells transduced with GM-CSF

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  $1 \times 10^5$  of parental WEHI3B cells were injected subcutaneously into the right flank of immunocompetent BALB/c mice. On day 3,  $3 \times 10^6$  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  $1 \times 10^5$  parental WEHI3B cells s.c. into the right flank, three of the five 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 transfectant 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 amount of 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  $1 \times 10^5$  WEHI3B cells were inoculated s.c. into the right flank of BALB/c mice on day 0. Next,  $3 \times 10^6$  irW, irW/GM, irW/GM + T, or irW/GM + R cells or 100  $\mu$ l of HBSS were injected s.c. into the left flank of mice on

**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 (%) <sup>a</sup>	Re-rejected mice <sup>d</sup>
HBSS	8	0 (0) <sup>b</sup>	–
IrW	8	0 (0)	–
IrW/GM	7	1 (14.3) <sup>c</sup>	1
IrW/GM + T	7	5 (71.4) <sup>c</sup>	3
IrW/GM + R	8	2 (25.0)	0

<sup>a</sup> Assessed at day 60 after tumor inoculation

<sup>b</sup> Parenthesis shows % of mice that rejected the WEHI3B challenge

<sup>c</sup>  $\chi^2$  test:  $P < 0.05$

<sup>d</sup> 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  $1 \times 10^6$  WEHI3B cells,  $3 \times 10^6$  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

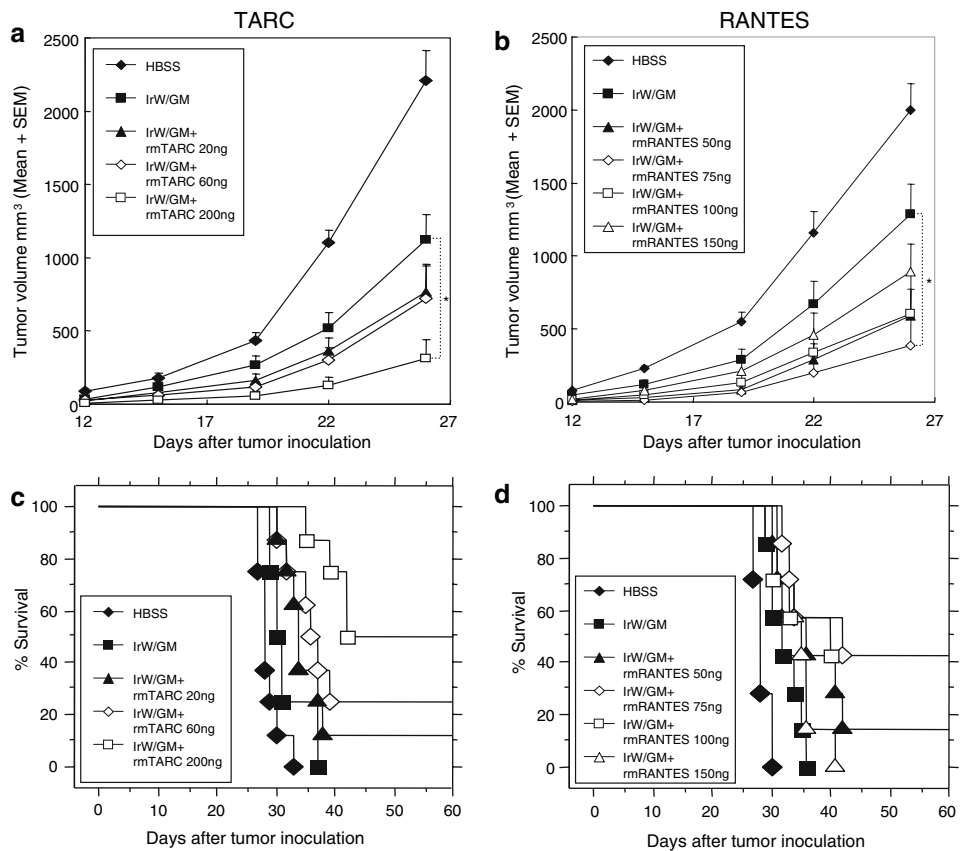
survival compared to control mice vaccinated with or without irW/GM, all of which died within 37 days ( $P < 0.05$ ) (Fig. 2c). Furthermore, 1, 2 and 4 mice vaccinated, respectively, with irW/GM + rmTARC 20, 60 and 200 ng completely rejected tumor formation (Table 3). On day 26, the mice vaccinated with 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 ( $2,338.55 \pm 168.01$  pg/ml) and murine RANTES 2 h ( $713.99 \pm 118.04$  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.

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

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  $1 \times 10^5$  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  $\mu$ l 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 (%) <sup>a</sup>	Re-rejected mice <sup>c</sup>
HBSS	8	0 (0) <sup>b</sup>	–
IrW/GM	8	0 (0) <sup>c,d</sup>	–
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) <sup>c</sup>	2
IrW/GM + rmRANTES 50 ng	7	1 (14.3)	0
IrW/GM + rmRANTES 75 ng	7	3 (52.9) <sup>d</sup>	2
IrW/GM + rmRANTES 100 ng	7	3 (52.9) <sup>d</sup>	1
IrW/GM + rmRANTES 150 ng	7	0 (0)	–

<sup>a</sup> Assessed at day 60 after tumor inoculation

<sup>b</sup> Parenthesis shows percentage of mice that rejected the WEHI3B challenge

<sup>c,d</sup>  $\chi^2$  test :  $P < 0.05$

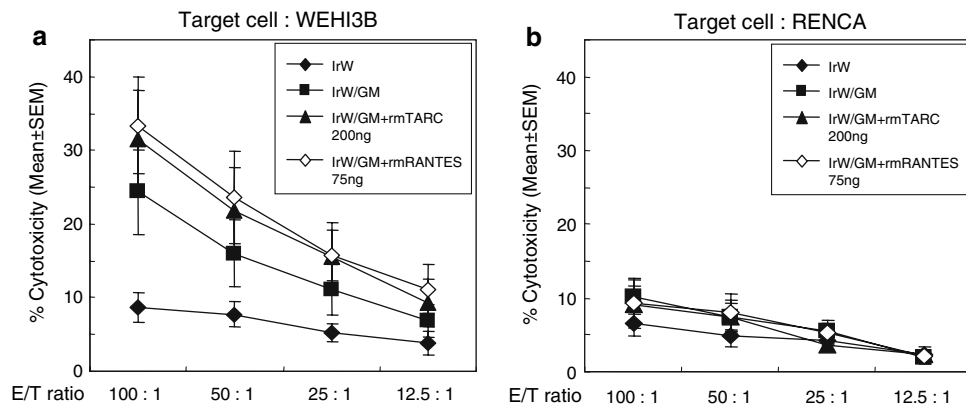
<sup>e</sup> Assessed at day 60 after WEHI3B rechallenge

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- $\gamma$  and IL-4 ELISPOT assays. In the presence of irradiated WEHI3B cells, the numbers of spot-forming IFN- $\gamma$  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  $^{51}\text{Cr}$  release assay as described in “Materials and methods”.  $^{51}\text{Cr}$  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  $\pm$  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- $\gamma$  and TNF- $\alpha$  levels were measured by CBA or ELISA assay. As seen in the ELISPOT assay, IFN- $\gamma$  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- $\alpha$  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

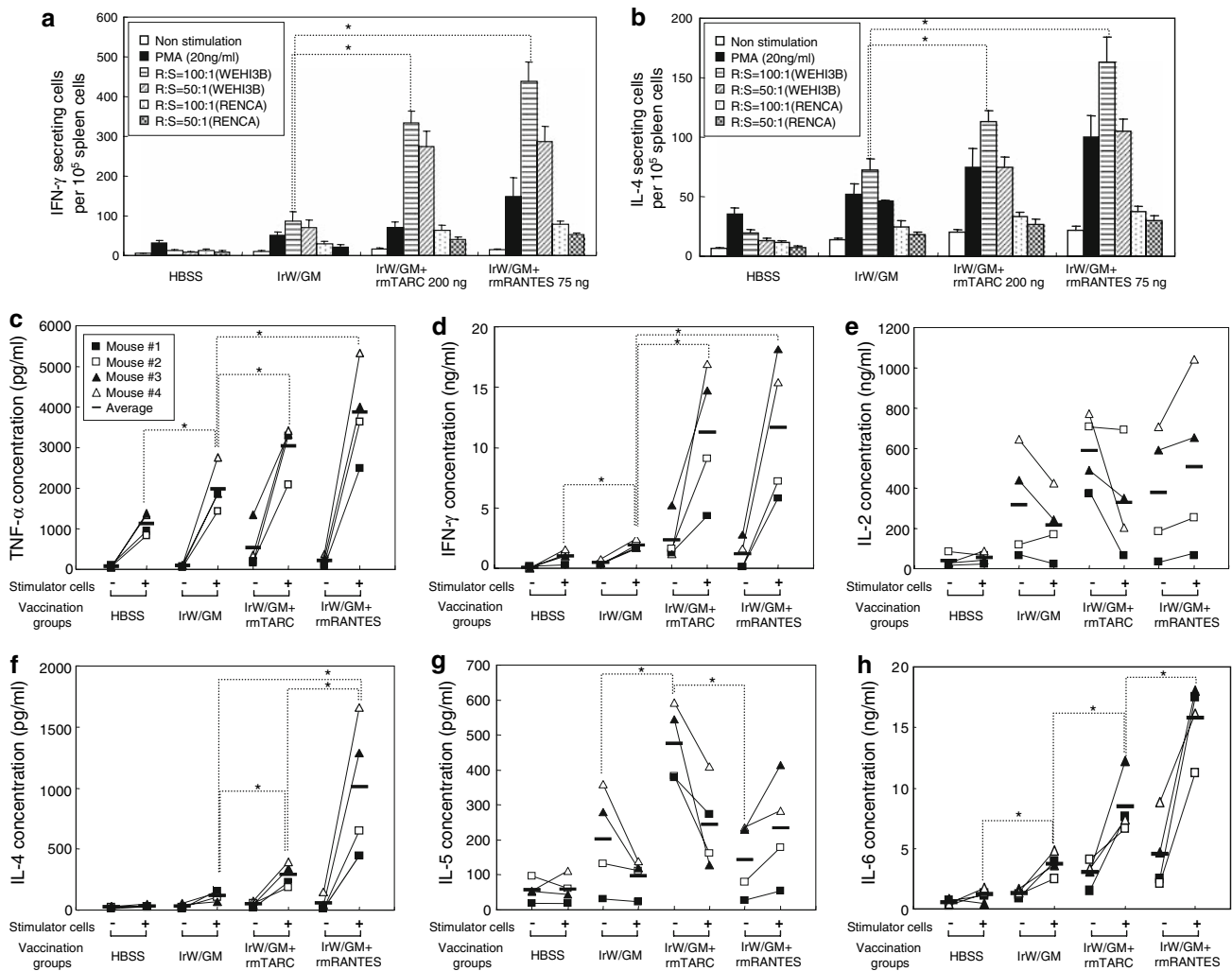
To determine if the additional effects of rmTARC or rmRANTES in combination with GM-CSF in syngeneic immunocompetent mice were mediated by adaptive immunity, we performed in vivo studies using syngeneic BALB/c SCID mice. Three days after subcutaneous injection of  $1 \times 10^5$  of WEHI3B into the right flank of SCID mice,  $3 \times 10^6$  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- $\gamma$  (a) and IL-4 (b) ELISPOT assays. A total of  $1 \times 10^5$  splenocytes from tumor-bearing mice vaccinated with the indicated transfected cells were incubated in 200  $\mu$ l RPMI 1640 with 10% FBS in ImmunoSpot™ 96-well plates coated with anti-IFN- $\gamma$  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- $\gamma$  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- $\alpha$  (c), IFN- $\gamma$  (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

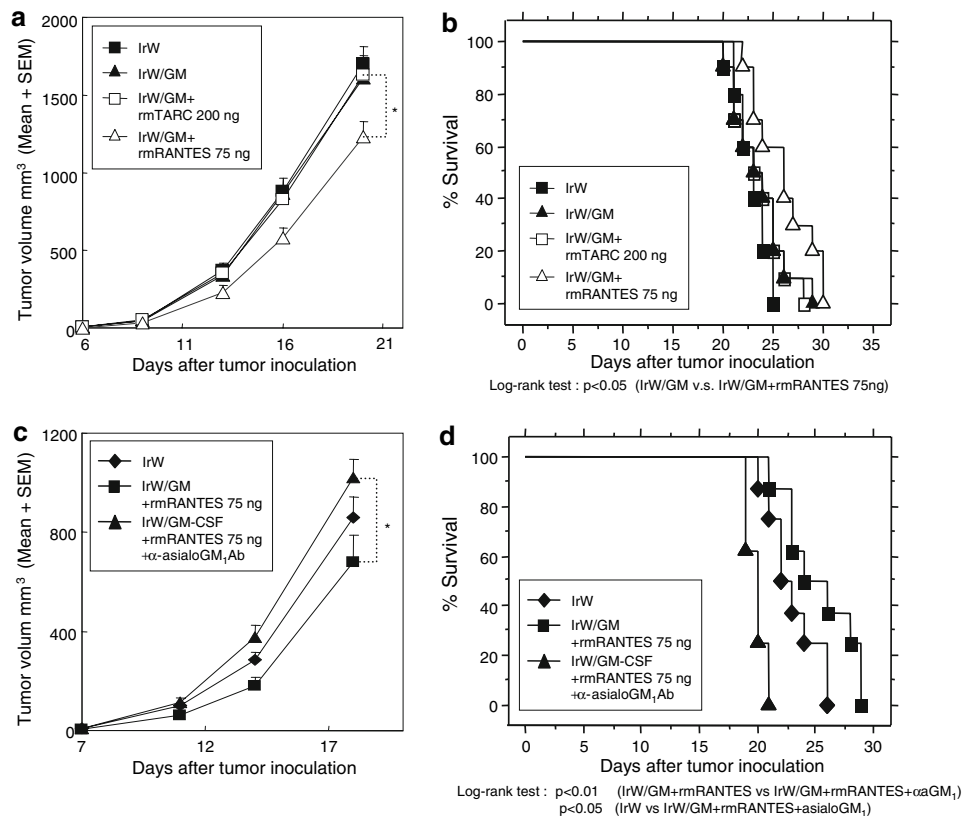
rmRANTES 75 ng than irW/GM ( $P < 0.05$ ). F4/80<sup>+</sup> cell (macrophage) infiltration also increased in the vaccinated groups, but was not statistically different compared with irW (Fig. 6a).

Cytokine secretion profiles of CD4<sup>+</sup> 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<sup>+</sup> TIL subpopulations

from the sites of primary tumor growth and the spleen on the same day as the IHC analysis. Th1 (IFN- $\gamma$ ) 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  $1 \times 10^5$  WEHI3B cells were s.c. injected into the right flank of syngeneic BALB/c SCID mice. Three days later,  $3 \times 10^6$  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<sub>1</sub> 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 ( $*P < 0.05$ )



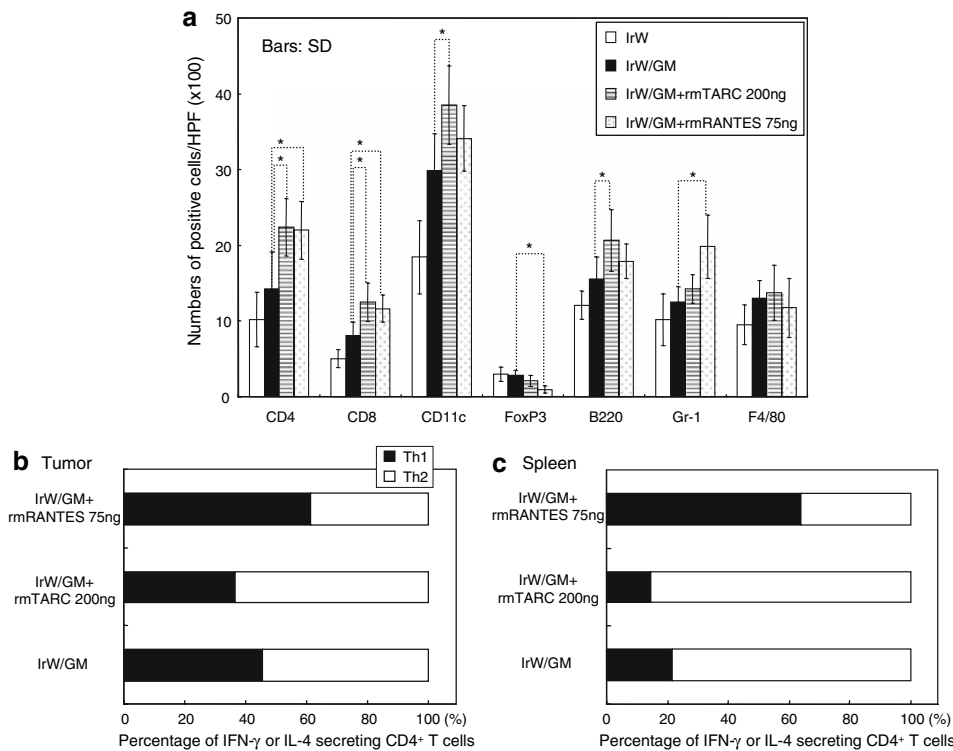
groups was similar to that observed in the TILs, but was even more enhanced (Fig. 6b, c).

## 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- $\gamma$ , IL-4, TNF- $\alpha$ , and IL-6. In addition, our results of immunohistochemical analysis supported these findings by demonstrating a significant increase in CD4<sup>+</sup> and CD8<sup>+</sup> 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 antiviral vaccine study. Sumida et al. [42] demonstrated that coadministration of the chemokine macrophage inflammatory protein-1 (MIP-1 $\alpha$ ) expression plasmid DNA vaccine with



**Fig. 6** **a** Immunophenotypic analysis of tumor-infiltrating cells (TIL) in tumor nodules. Mice were injected s.c. with  $1 \times 10^5$  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<sup>+</sup>, CD8<sup>+</sup>, CD11c<sup>+</sup>, FoxP3<sup>+</sup>, CD45R<sup>+</sup>, F4/80<sup>+</sup> or Ly-6G<sup>+</sup> cells, stained cells were counted microscopically at  $\times 100$  in 10–30 HPF. Results are presented as the means  $\pm$  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 ( $n = 3/\text{experiment}$ ) 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- $\gamma$  and PE-conjugated anti-IL-4. Gates were set on CD4<sup>+</sup> 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- $\gamma$  (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- $\gamma$ , IL-2, IL-4, IL-5, IL-6, and TNF- $\alpha$ . Elevated IL-4 and IL-5 produced from splenocytes treated with IrW/GM + rmTARC suggested that additional rmT-

ARC 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- $\alpha$  and IL-6 than controls, resulting in potent T cell and NK cell activation. Furthermore, TNF- $\alpha$  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<sup>+</sup>CD25<sup>+</sup> regulatory

T cells (Tregs) [32] and to induce hematopoiesis and acute phase inflammation [15, 32], partially by blocking signaling that facilitates Treg 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 micro-environment 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 Treg 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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