

# Reduced tumourigenicity of EG7 after RANTES gene transfer and the underlying mechanism

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## Abstract

**Introduction:** Chemokine ligand 5, also known as CCL5 or regulated on activation normal T-cell expressed and secreted (RANTES), is a chemokine expressed in inflamed tissue and capable of inducing migration of immature dendritic cells (DCs) or Langerhans cells. In this study, we explored the effect of RANTES on EG7 cells.

**Material and methods:** *In vivo*, RANTES gene transfer reduced the tumourigenic capacity of EG7 and prolonged the survival of tumour-bearing mice. To reveal the underlying mechanism, we performed the following experiments and provided evidence to support our hypothesis of RANTES gene therapy for EG7. Higher natural killer (NK) cell and cytotoxic T lymphocyte (CTL) activity was induced after RANTES gene transfer, accompanied by higher levels of Th1 type cytokines (IL-2 and IFN- $\gamma$ ).

**Results:** Tumour necrosis was also markedly observed in the tumour tissues after RANTES gene transfer, which was attributed to reduced expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP-2).

**Conclusions:** We draw the conclusion that reduced tumourigenicity of EG7 after RANTES gene transfer can be attributed to higher NK cell and CTL activity, anti-angiogenesis and higher levels of Th1 type cytokines induced by RANTES. These results support the notion that higher chemokine expression in tumour tissue elicits potent anti-tumour immunity.

**Key words:** RANTES, cancer gene therapy, anti-tumour immunity, cytokine gene transfer

## Introduction

Cytokine gene transfer to cancer cells has been recently developed as an attractive mechanism of drug delivery *in situ* without the toxic effects associated with systemic administration.

Chemokines are small polypeptide signalling molecules that bind to and activate a family of seven transmembrane G protein-coupled chemokine receptors and are responsible for the selective recruitment and activation of mononuclear cells [1]. Chemokine ligand 5, also known as CCL5 or RANTES, is a protein which in humans is encoded by the CCL5 gene [2]. CCL5 is an 8 kD protein classified as a chemotactic cytokine or chemokine. Regulated on activation normal T-cell expressed and secreted (RANTES) or CCL5 is a member of the consecutive cysteine

(CC) chemokines that promotes the migration and activation of several types of leukocytes, such as CD4<sup>+</sup>/CD8<sup>+</sup> T-cells, NK cells and DCs, expressing CC-chemokine receptors (CCR1), CCR3 and CCR5 [1, 3-6]. Interestingly, it was reported recently that RANTES plasmid had a potent efficiency for DC recruitment and Th1 bias when it was co-injected with HBV DNA vaccine [3]. Moreover, the vaccine, comprised of an oncolytic adenovirus expressing RANTES (Ad-RANTES-E1A) [7], enhanced tumour infiltration and maturation of antigen-presenting cells *in vivo*. This vaccine recruited DCs, macrophages, natural killer cells, and CD8<sup>+</sup> T cells to the tumour site, and thus enhanced antigen-specific cytotoxic T lymphocyte responses and the NK cell response. This *in situ* immunization strategy could be a potent anti-tumour immunotherapy approach for aggressive established tumours. Consistent with the above results [8], vaccination combination with Fit3L and RANTES in a DNA prime-protein boost regimen also elicits strong cell-mediated immunity and anti-tumour effect.

In view of these findings regarding RANTES' effects on the immune response, we decided to study the mechanism associated with tumour cell growth following expression of RANTES in EG7 cells. Thus we transfected EG7 cells with RANTES gene and established an EG7 cell clone stably expressing RANTES (500 pg/ml every 10<sup>6</sup> cells). We hypothesized that intra-tumoural higher level of RANTES induced local accumulation of T cells and NK cells in the tumour site, which can be activated subsequently by EG7 cells to overcome the occurrence of a hyporesponsive state, and elicited a potent anti-tumour effect.

C57BL/6 mice were inoculated subcutaneously (s.c.) with EG7 cells transfected with RANTES as a model and then we observed the tumorigenicity of EG7/RANTES and investigated the underlying mechanism. Our data illustrated that RANTES gene transfer elicited a significant anti-tumour effect through more efficient induction of a specific and non-specific anti-tumour immune response.

## Material and methods

### Animals

Female C57BL/6 mice (H-2K<sup>b</sup>) aged 6-8 weeks were purchased from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China) and housed in a specific pathogen-free condition for all experiments.

### Cell lines

EG7 cell line is derived from the murine T-cell lymphoma EL-4 transfected with cDNA for ovalbumin (OVA) and lack of B7-1 expression. The

expression vector pKGE839 encoding RANTES was transfected into EG7 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to establish an EG7 cell clone which stably expresses RANTES as described above by us (500 pg/ml every 10<sup>6</sup> cells) (a gift generously provided by Ms. Huiling Diao from Binzhou Medical College). YAC-1, an NK-sensitive lymphoma cell line of A/S (H-2a) origin, and EL4, a T-lymphoma cell line of C57BL/6 origin were obtained from American Type Culture Collection (ATCC, Manassas, VT, USA), maintained in RPMI-1640 medium supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml, 2-mercaptoethanol 50 mmol/l, and 10% fetal calf serum. EG7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 mg/ml), and G418 (500 µg/ml), 50 µg/ml gentamicin, and 2 mM glutamine. All culture media were purchased from Gibco-BRL (Gaithersburg, Md., USA), and fetal calf serum was provided by Shanghai Institute of Biological Products (Shanghai, China).

### Reagents

The expressing vector pKGE839 containing RANTES cDNA and the neomycin resistance gene was also a gift from Active Biotech Research. Recombinant murine interleukin (IL-2) was purchased from Pharmingen (San Diego, CA, USA). IL-2 and interferon-γ (IFN-γ) ELISA kits were purchased from R&D System Incorporation (Minneapolis, MN, USA). G418 was purchased from CalBiochem (Merck, Darmstadt, Germany). Cytotox 96® Non-radioactive Cytotoxicity Assay kit was purchased from Promega (Madison, WI, USA). Trizol Reagent was purchased from Bio Basic Incorporation. RevertAid™ First Strand cDNA Synthesis Kit #K1622 was purchased from Fermentas (Vilnius, Lithuania). Gene Ruler™ 100 bp DNA ladder #SM0241 was purchased from Fermentas (Lot:00014461). Blend TaqE was purchased from Takara Incorporation (Osaka, Japan). dNTPs 10 mM was purchased from Takara Biotechnology Incorporation (Dalian). The primer for VEGF and MMP-2 was synthesized by Shanghai Biotechnology Company.

### Tumorigenicity of EG7 after RANTES gene transfer

C57BL/6 mice were divided into two groups and were s.c. injected into the hind limb with a dose of 5 × 10<sup>5</sup> EG7 cells or EG7/RANTES cells, respectively. The length and width of the tumour mass were measured with calipers every other day after tumour inoculation. Tumour size was

expressed as 1/2 (length + width). Three mice of each group were killed by cervical dislocation three weeks after the tumour inoculation and the tumour in the lateral hind leg was extracted and weighed. An incision was made on the skin of the abdomen using scissors, the entire peritoneal cavity was opened, and finally the spleen was taken out using forceps. Splenocytes isolated from the mice were used for cytotoxic assay of NK cells and CTL and cytokine induction as follows. Seven mice in each group were observed for their survival period. All experiments were performed three times using individual groups of ten mice. Data are representative of three experiments performed. All animal experimental procedures were approved by the Animal Care Committee of Binzhou Medical College Graduate School of Medicine.

#### Cytotoxic assay of NK cells and CTL

Splenic lymphocytes were isolated from the sacrificed mice three weeks after the tumour inoculation. The erythrocytes were depleted with 0.83% ammonium chloride and macrophages were removed by adherence of splenocytes on plastic plates for 2 h. The non-adherent lymphocytes were directly used as NK cells. The lymphocytes were co-cultured with inactivated EG7 cells, EG7/RANTES cells (treated with 50 µg/ml mitomycin for 30 min) for 7 days in the presence of 20 U/ml murine recombinant IL-2 (San Diego, CA, USA) and then collected as CTL. The NK cell activity and CTL activity were determined by lactate dehydrogenase (LDH) release assay with CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) [28]. The target cells (YAC-1 cells, EG7 cells or EG7/RANTES) were washed three times with RPMI 1640 medium containing 5% FCS to remove adherent LDH derived from lysed cells. The cell suspension was diluted with RPMI 1640 medium containing 5% FCS to the final concentration of  $1 \times 10^5$  cells/ml. 100 µl of target cell suspension and 100 µl of different ratios of effector cells were pipetted together into the wells of a round-bottomed microtitre plate. Suspensions containing exclusively effector cells, target cells, or culture medium, respectively, served as controls to estimate the LDH background. The plates were incubated for 4 h in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. After incubation, they were centrifuged for 10 min. Then 100 µl of the supernatant from each well was transferred to the corresponding well of the enzymatic assay plate. 50 µl of reconstituted substrate mix (containing lactate and NAD<sup>+</sup>) was added to each well. The plate was covered and incubated at room temperature (protected from light). Thirty

minutes later, 50 µl of stop solution was added to each well. The reaction was measured in an ELISA reader at a wavelength of 490 nm. Calculations were carried out according to the following formula: % of specific lysis =  $100 \times (\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}) / (\text{target maximum} - \text{target spontaneous})$ .

#### Cytokine induction and measurement of splenocytes

The non-adherent splenocytes were derived from mice three weeks after the tumour inoculation and the cells were washed three times with PBS. For induction of cytokines,  $5 \times 10^6$  cells of splenocytes were incubated with inactivated EG7 cells, EG7/RANTES (treated with 50 µg/ml mitomycin for 30 min) at a 10 : 1 ratio in a total volume of 1 ml at 37°C, 5% CO<sub>2</sub> for 72 h. Culture supernatants were harvested at 24 h (for IL-2 assay) or 72 h (for IFN-γ assay) and stored at -20°C for measurement of cytokines using a standard sandwich ELISA technique with corresponding kits purchased from R&D System Incorporation (Minneapolis, MN, USA).

#### Determination of intratumoural VEGF and MMP-2 mRNA level by semi-quantitative PCR

Subcutaneous tumour nodules were extracted from sacrificed tumour-bearing mice three weeks after the tumour inoculation. Total RNA was extracted from subcutaneous tumour tissues by Trizol Reagent (Bio Basic Inc. Canada), according to the instructions of the manufacturer. For each sample, 1 µg of RNA was reverse-transcribed using a RevertAid™ First Strand cDNA Synthesis Kit #K1622 (Fermentas, Vilnius, Lithuania) in a total volume of 20 µl. cDNA as read-out of the mRNA was quantitated in a competitive PCR using specific primers for VEGF and MMP-2. Primers for amplification of VEGF were 5'-CTG CTC TCT TGG GTG CAC TGG-3' (sense) and 5'-CAC CGC CTT GGC TTG TCA CAT-3' (antisense), with an expected PCR product of VEGF (431 bp). Primers for amplification of MMP-2 were 5'-CAC CAT CGC CCA TCA TCA AGT-3' (sense) and 5'-TGG ATT CGA GAA AAG CGC AGC GG-3' (antisense), with an expected PCR product of 399 bp. Primers for amplification of β-actin were 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' (sense) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (antisense), with an expected PCR product of 348 bp.

PCR products of VEGF, MMP-2 and β-actin were visualized by electrophoresis in 2.5% agarose gel containing 0.5 µg/ml ethidium bromide. The amplified VEGF, MMP-2 and β-actin were photographed using the Kodak EDAS120 digital imaging system version 3 (Gibco-BRL).

### Histological examination

Subcutaneous tumour nodules were extracted from three sacrificed tumour-bearing mice three weeks after the tumour inoculation. The tumour sample was fixed in 10% formalin solution, dehydrated and embedded in paraffin. Thin-sliced sections were stained with haematoxylin and eosin. Finally two slices were prepared from each sacrificed mouse and in total six tissue slices were used to observe necrosis in the tumour tissue for each group. For the evaluation of tumour necrosis, we assigned the following: – no necrosis; + less than one-third of the tumour size; ++ between one-third and two-thirds of the tumour size; +++ more than two-thirds of the tumour size.

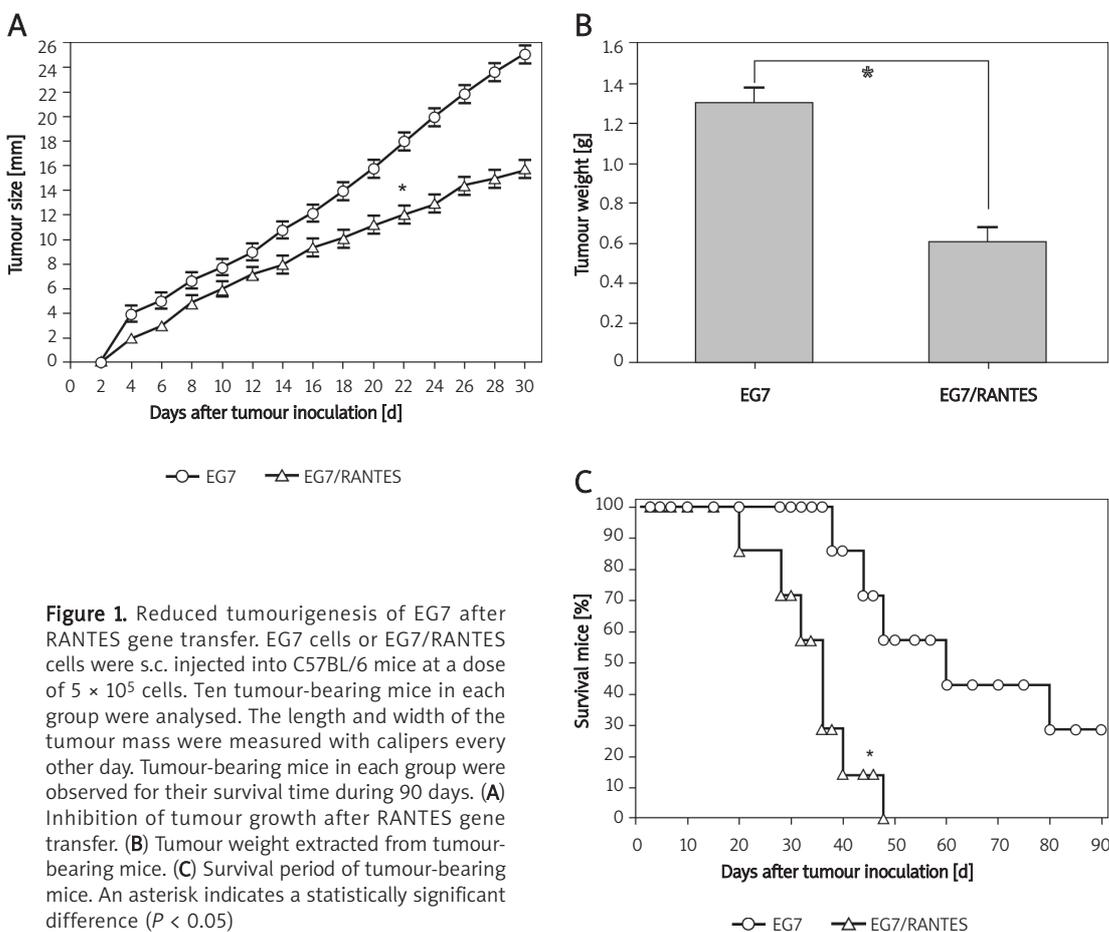
### Statistical analysis

All the experiments were run in triplicate and the results are means ± SD of triplicate determinations (or representative data from one or two independent experiments). Statistical analysis was performed using Student's *t* test and log-rank test (for survival analysis). The difference was considered statistically significant when the *P* value was less than 0.05.

### Results

#### RANTES reduced the tumourigenicity of EG7 cells

The gene encoding RANTES was transduced into EG7 cells. Normal C57BL/6 mice were inoculated s.c. with a dose of  $5 \times 10^5$  EG7 or EG7/RANTES cells, respectively. The results in Figure 1A showed that the tumourigenicity of EG7 cells after RANTES gene transfer was markedly reduced compared with that of EG7 cells ( $P < 0.05$ ). The tumour-bearing mice were killed three weeks after the tumour inoculation and the tumour in the lateral rear leg was extracted and weighed. The results in Figure 1B illustrated that the tumour weight was also less from EG7/RANTES tumour-bearing mice than that of EG7 tumour-bearing mice ( $P < 0.05$ ). Seven tumour-bearing mice in each group were observed for their survival period. The results in Figure 1C showed that EG7/RANTES tumour-bearing mice survived much longer than EG7 tumour-bearing mice ( $P < 0.05$ ). Of seven mice inoculated with EG7/RANTES, two were tumour-free and survived more than 90 days. These data



**Figure 1.** Reduced tumourigenesis of EG7 after RANTES gene transfer. EG7 cells or EG7/RANTES cells were s.c. injected into C57BL/6 mice at a dose of  $5 \times 10^5$  cells. Ten tumour-bearing mice in each group were analysed. The length and width of the tumour mass were measured with calipers every other day. Tumour-bearing mice in each group were observed for their survival time during 90 days. (A) Inhibition of tumour growth after RANTES gene transfer. (B) Tumour weight extracted from tumour-bearing mice. (C) Survival period of tumour-bearing mice. An asterisk indicates a statistically significant difference ( $P < 0.05$ )

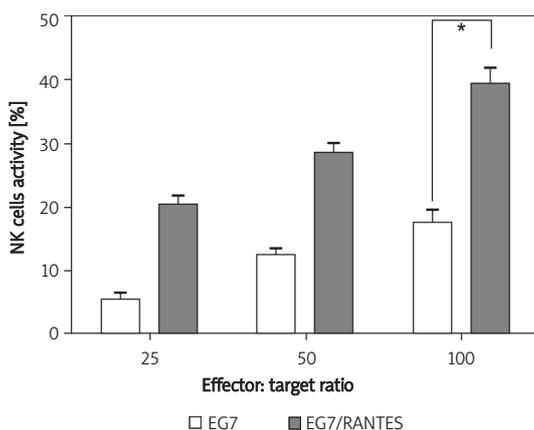
suggested that RANTES gene transfer elicited a more potent and specific anti-tumour effect *in vivo*.

### Increased NK cell activity by RANTES

Three weeks after the tumour inoculation, the splenocytes isolated from sacrificed tumour-bearing mice of each group were used in a cytolytic assay against YAC-1 cells at effector:target (E:T) ratios of 25 : 1, 50 : 1 and 100 : 1 by lactate dehydrogenase (LDH) release assay. As shown in Figure 2, NK cell activity of EG7/RANTES tumour-bearing mice increased when compared with that of EG7 tumour-bearing mice ( $P < 0.05$ ). It suggested that the enhanced non-specific immunity might also be involved in the anti-tumour response of RANTES.

### More potent CTL cytotoxicity induced by RANTES

The splenocytes derived from tumour-bearing mice of each group were re-stimulated *in vitro* with inactivated EG7 or EG7/RANTES cells for seven days in the presence of murine recombinant IL-2 (20 U/ml) and then collected as CTL effector cells. EG7 and EG7/RANTES cells were used as target cells, respectively. The CTL activity was determined at effector:target ratios of 25 : 1, 50 : 1 and 100 : 1 by lactate dehydrogenase (LDH) release assay. Consistent with the protection against the challenge of T lymphoma cells, EG7/RANTES tumour-bearing mice exhibited a T lymphoma-specific CTL response, which was higher than that of EG7 tumour-bearing mice ( $P < 0.05$ ) (Figure 3).



**Figure 2.** Induction of higher NK activity by RANTES. Normal C57BL/6 mice were s.c. inoculated with a dose of  $5 \times 10^5$  EG7 cells or EG7/RANTES cells. Three mice in each group were killed three weeks after the tumour inoculation, and their splenocytes were isolated and used in cytolytic assays against YAC-1 cells. An asterisk indicates a statistically significant difference ( $P < 0.05$ ).

### Enhanced Th1 type cytokine production after RANTES gene transfer

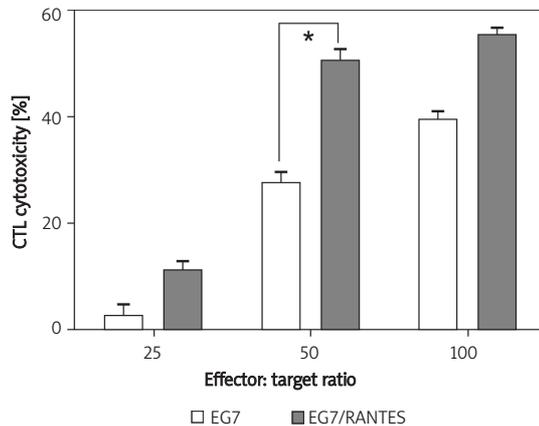
Three weeks after the tumour inoculation, we determined the production of IL-2 (for 24 h) and IFN- $\gamma$  (for 72 h) by splenocytes stimulated *in vitro* with inactivated EG7 or EG7/RANTES cells at a 10:1 ratio in a total volume of 1 ml at 37°C, 5% CO<sub>2</sub>. The data in Figure 4 show that splenocytes from EG7/RANTES tumour-bearing mice secreted higher levels of IL-2 and IFN- $\gamma$  when compared with those from EG7 tumour-bearing mice ( $P < 0.05$ ).

### Reduced expression of VEGF and MMP-2 after RANTES gene transfer

Three weeks after the tumour inoculation, VEGF and MMP-2 mRNA expression was analysed by semi-quantitative polymerase chain reaction (PCR). As shown in Figure 5, it was markedly observed in the tumour tissue from EG7 tumour-bearing mice. The intratumoural expression of VEGF and MMP-2 mRNA from EG7/RANTES tumour-bearing mice was significantly less than that of EG7 tumour-bearing mice. This indicated that RANTES gene could efficiently inhibit angiogenesis in the tumour tissue.

### Manifest necrosis in the tumour tissue after RANTES gene transfer

Subcutaneous tumour nodules were extracted from tumour-bearing mice that were killed three



**Figure 3.** Induction of higher CTL activity against target cells by RANTES. Normal C57BL/6 mice were s.c. inoculated with a dose of  $5 \times 10^5$  EG7 cells or EG7/RANTES cells. Three mice in each group were killed three weeks after the tumour inoculation, and their splenocytes were re-stimulated *in vitro* for seven days with inactivated EG7 cells or EG7/RANTES cells in the presence of murine recombinant IL-2 (20 U/ml) and assayed for their cytotoxicity against EG7 cells or EG7/RANTES cells, respectively. Data are representative of three independent experiments. An asterisk indicates a statistically significant difference ( $P < 0.05$ ).

weeks after tumour challenge. Histological examination of the tumour mass showed that the most obvious tumour necrosis was present inside the tumour from EG7/RANTES tumour-bearing

mice. However, little necrosis was found inside the tumour from EG7 tumour-bearing mice (Table I).

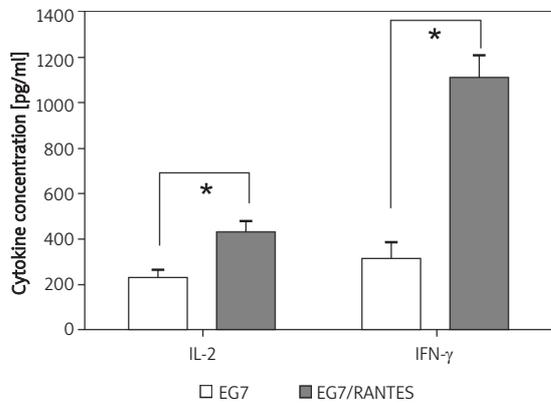
**Discussion**

Cytokine gene transfer to cancer cells has been recently developed as an attractive mechanism of drug delivery in situ without the toxic effects associated with systemic administration. However, the anti-tumour effects of a cancer cell line genetically modified with cytokine gene alone are still far from satisfactory. The reason may be partially due to the failure to recruit lymphocytes of the appropriate specificity. Many studies have demonstrated that chemokine gene transfer elicits potent anti-tumour activity.

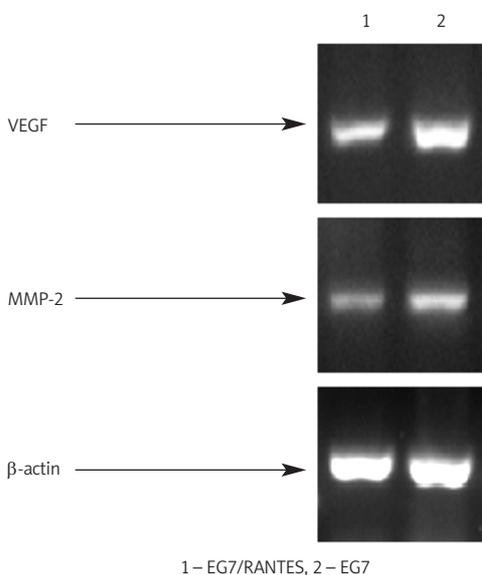
CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. With the help of particular cytokines (IL-2 and IFN- $\gamma$ ) that are released by T cells, CCL5 also induces the proliferation and activation of certain NK cells to form CC-chemokine-activated killer (CHAK) cells [9].

Opportunities exist for manipulating chemokine expression in the tumour microenvironment with therapeutic intent. Transfection of tumour cells to express several chemokines before implantation in mice has shown improved tumour control in several models [10]. In this study, we selected the EG7 cell line (EL-4 transfected with ovalbumin gene). The intra-tumoural higher levels of RANTES caused local accumulation of more NK cells and T cells that can subsequently be activated by EG7 cells, which will contribute to inducing a more potent anti-tumour effect. Our results shown in Figure 1C demonstrated that RANTES gene transfer into EG7 cells could inhibit its tumourigenicity and prolonged the survival of tumour-bearing mice survival (2 of 7 EG7/RANTES tumour-bearing mice were tumour-free and survived more than 90 days).

The mechanism involved in the potent anti-tumour efficacy of RANTES might be related to the following aspects. First, effective induction of a cell-mediated immune response by RANTES might play an important role. Some authors have suggested



**Figure 4.** Higher levels of Th1 type cytokines induced by RANTES. Production of IL-2 and IFN- $\gamma$  by lymphocytes derived from tumour-bearing mice. Three mice in each group were killed three weeks after tumour inoculation.  $5 \times 10^6$  splenocytes were incubated with inactivated EG7 cells or EG7/RANTES cells at a 10 : 1 ratio in a total volume of 1 ml at 37°C, 5% CO<sub>2</sub> atmosphere for 24 h and 72 h, and supernatants were harvested for the detection of cytokines by ELISA. An asterisk indicates a statistically significant difference ( $P < 0.05$ )



**Figure 5.** Intratumoural VEGF and MMP-2 mRNA expression after RANTES gene transfer. Three weeks after the tumour inoculation, total RNA was extracted from subcutaneous tumour tissue by Trizol. The intratumoural VEGF and MMP-2 mRNA expression was analysed by semi-quantitative PCR. PCR products were visualized by electrophoresis in a 2.5% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. One of three independent experiments with similar results is shown

**Table I.** Pathological analysis of tumour mass from tumour-bearing mice. Subcutaneous tumour nodules were extracted from sacrificed tumour-bearing mice three weeks after the tumour inoculation. The tumour sample was used to prepare for paraffin section and then thin-sliced sections were stained with haematoxylin and eosin. The results represent mean assessment of necrosis in tissue samples

Groups	EG7	EG7/RANTES
Necrosis	-	++

Tumour necrosis: (-) no necrosis, (+) less than 1/3 of the tumour size, (++) between 1/3 and 2/3 of the tumour size, (+++) more than 2/3 of the tumour size

that the anti-tumour activity of RANTES observed in animal models might be mainly mediated by NK cells and CTL [1, 3-6]. In our study, the splenic NK and CTL cytotoxicities were markedly enhanced after RANTES gene transfer. The results in Figure 2 showed that NK cell activity of EG7/RANTES tumour-bearing mice (39.5%) was significantly higher than that of EG7 tumour-bearing mice (19.6%) ( $P < 0.05$ ). Similarly, the results in Figure 3 showed that CTL activity of EG7/RANTES tumour-bearing mice (49.5%) was significantly higher than that of EG7 tumour-bearing mice (27.6%) ( $P < 0.05$ ). This suggested that the non-specific and specific anti-tumour immunity were potently activated and might be involved in the anti-tumour response of RANTES. These results suggested that priming of NK cells and CTL by RANTES induced effective anti-tumour immunity.

Secondly, RANTES inhibited angiogenesis *in vivo*. Malignant tumours do not grow beyond 2-3 mm<sup>3</sup> and cannot metastasize unless they stimulate the formation of new blood vessels and thus provide a route for the increased inflow of nutrients and oxygen and outflow of waste products [11]. In our study, the results in Figure 5 showed that expression of VEGF and MMP-2 was reduced in tumour tissues from EG7/RANTES tumour-bearing mice. Moreover, the results of histological examination in Table I also showed that manifest necrosis was markedly observed to be present in the tissue from EG7/RANTES tumour-bearing mice. These results illustrated that RANTES inhibited angiogenesis through inhibiting VEGF and MMP-2 expression, reducing new blood vessels and finally causing manifest necrosis of tumour tissues due to lack of nutrients.

Lastly, it is accepted that the balance between Th1 and Th2 cells plays an important role in the anti-tumour immune response [12]. IL-2 and IFN- $\gamma$ , produced by Th1 cells and NK cells, are critically important for the induction of anti-tumour cellular immunity *in vivo* [13]. Therefore, induction of Th1 type cytokines can be beneficial for tumour eradication. In Figure 4, the results showed that the production of IL-2 and IFN- $\gamma$  from splenocytes increased to 440 pg/ml and 1150 pg/ml after RANTES gene transfer, respectively. This may be related to the fact that more T cells and NK cells were attracted by RANTES and activated by DCs presenting tumour antigens. As a result, more Th1 type cytokines were produced by NK cells and activated T cells.

Several cell types within the tumour micro-environment could potentially produce the chemokines important for recruitment of effector CTL. Our analysis of T lymphoma indicates that a subset of such lines is capable of producing an expanded array of chemokines itself. However,

additional cell types may contribute to chemokine synthesis within the complexity of the tumour microenvironment *in vivo*. Macrophages, endothelial cells and even recruited T cells could theoretically secrete relevant chemokines and positively reinforce recruitment of additional specific T-cell subsets [14]. Further work will be required to identify the cell types producing each chemokine within the metastatic melanoma microenvironment in individual patients using *in situ* assays.

Other previous work has shown that granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1 (MIP-1), and other chemokines can recruit antigen-presenting cells to the site of inoculation and increase vaccine-elicited immune responses in mice [15-19]. The present study extends these observations by demonstrating that the specific chemotactic signal of RANTES results in dramatic and synergistic effects at the host level to resist tumour formation. Ultimately, we hope that the study will provide insights that lead to the development of more effective vaccine-based cancer immunotherapy.

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#### References

1. Maria F, Francesca G, Diego A, et al. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. *Int Immunol* 1999; 11: 979-86.
2. Donlon TA, Krensky AM, Wallace MR, Collins FS, Lovett M, Clayberger C. Localization of a human T-cell-specific gene, RANTES(D17S136E), to chromosome 17q11.2-q12. *Genomics* 1989; 6: 548-53.
3. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J Exp Med* 1999; 189: 169-78.
4. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol* 2001; 122: 83-7.
5. O'Neill DW, Adams S, Bhardwaj N. Manipulating dendritic cell biology or the active immunotherapy of cancer. *Blood* 2004; 104: 2235-46.
6. Foti M, Granucci F, Aggujaro D, et al. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly. *Int Immunol* 1999; 11: 979-86.
7. Lapteva N, Aldrich M, Weksberg D, et al. Targeting the intratumoral dendritic cells by the oncolytic adenoviral vaccine expressing RANTES elicits potent antitumor immunity. *J Immunother* 2009; 32: 145-56.
8. Song S, Liu C, Wang J, et al. Vaccination with combination of Fit3L and RANTES in a DNA prime-protein boost regimen elicits strong cell-mediated immunity and antitumor effect. *Vaccine* 2009; 27: 1111-8.

9. Maghazachi AA, Al-Aoukaty A, Schall TJ. CC chemokines induce the generation of killer cells from CD56+ cells. *Eur J Immunol* 1996; 26: 315-9.
10. Mule JJ, Custer M, Averbook B, et al. RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity in vivo: role of immune cell subpopulations. *Hum Gene Ther* 1996; 7: 1545-53.
11. Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987; 235: 442-7.
12. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; 17: 138-46.
13. Nishimura T, Iwakabe K, Sekimoto M, et al. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* 1999; 190: 617-27.
14. Zlotnik A, Morales J, Hedrick JA. Recent advances in chemokines and chemokine receptors. *Crit Rev Immunol* 1999; 19: 1-47.
15. Shawn MS, Paul FM, Diana MT, et al. Recruitment and expansion of dendritic cell in vivo potentiate the immunogenicity of plasmid DNA vaccines. *J Clin Invest* 2004; 114: 1334-42.
16. Ma K, Xu W, Shao X, et al. Coimmunization with RANTES plasmid polarized Th1 immune response against hepatitis B virus envelope via recruitment of dendritic cells. *Antivir Res* 2007; 76: 140-9.
17. Mwangi W, Brown WC, Lewin HA, et al. DNA-encoded fetal liver tyrosine kinase 3 ligand and granulocyte macrophage colony-stimulating factor increase dendritic cell recruitment to the inoculation site and enhance antigen-specific CD4+ T cell responses induced by DNA vaccination of outbred animals. *J Immunol* 2002; 169: 3837-46.
18. Youssef S, Maor G, Wildbaum G, Grabie N, Gour-Lavie A, Karin N. C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. *J Clin Invest* 2000; 106: 361-71.
19. Kusakabe K, Xin KQ, Katoh H, et al. The timing of GM-CSF expression plasmid administration influences the Th1/Th2 response induced by an HIV-1-specific DNA vaccine. *J Immunol* 2000; 164: 3102-11.