

Inhibition or Promotion of Tumor Growth by Granulocyte-Macrophage Colony Stimulating Factor Derived from Engineered Tumor Cells is Dose-dependent

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Abstract. *Background:* Granulocyte-macrophage colony stimulating factor (GM-CSF) has been widely investigated as an adjuvant factor for tumor immunotherapy. However, the results are controversial with antitumor effects in some studies and a tumor growth promotion effect in others. *Materials and Methods:* In order to determine whether there is a dose-dependent effect of GM-CSF on tumor growth, murine GM-CSF-expressing vector was constructed and transfected into TC-1 tumor cells and various clones stably expressing different levels of GM-CSF were obtained. The growth of these clones in vivo was studied. *Results:* Although these clones grow at a similar rate in vitro, their growth in vivo is dramatically different. Clones expressing high levels (>10,000 pg/ml) of GM-CSF grow significantly faster than the control ($p < 0.001$); clones expressing low levels (<100 pg/ml) of GM-CSF grow significantly slower than the control ($p < 0.001$); while clones expressing intermediate levels (1000-2000 pg/ml) of GM-CSF grow at a similar rate as the control ($p > 0.05$). The high levels of GM-CSF secreted by tumor cells induced granulocytosis and lymphopenia. The antitumor growth effect induced by low levels of GM-CSF is not due to the function of lymphocytes. *Conclusion:* The inhibition or promotion of tumor growth by GM-CSF secreted from tumor cells is dose-dependent.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is one of the first cytokines to be characterized and cloned (1). Its function includes activation and augmentation of many of the functions of neutrophils, monocytes or macrophages and dendritic cells (2). Many studies have been performed to utilize GM-CSF to enhance host defenses against a broad

spectrum of invading organisms or as a vaccine adjuvant (3). Antitumor activities have been reported both in animal studies and human trials when GM-CSF was expressed by irradiated tumor cells engineered to express this cytokine alone or with other factors (4-9). However, in some early studies, when live tumor cells expressing high doses of GM-CSF were introduced into mice, fatal toxicities were induced as manifested by profound leukocytosis, hepatosplenomegaly and pulmonary hemorrhage (10). In fact, one recent study has shown that the best antitumor effects were achieved when the GM-CSF-expression level is relatively low (11). From these studies, we suspected that GM-CSF's antitumor effect may be dose-dependent and in order to clarify this, we cloned and transfected murine GM-CSF into TC-1 tumor cells, from which clones expressing different levels of the cytokine were obtained. The tumorigenicity of these clones was investigated in C57BL/6J mice and SCID mice. Clear dose-dependent effects of GM-CSF, ranging from antitumor activity to promoting tumor growth, were observed.

Materials and Methods

Animals and cell line. Female C57BL/6J mice and SCID mice, 6-8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in our pathogen-free animal facilities. Murine tumor cells, TC-1, which were derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes (a kind gift from Dr. T. C. Wu, John Hopkins University), were cultured in RPMI 1640 medium containing 10% FBS, 0.2 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 mg/ml gentamicin, at 37°C with 5% CO₂.

Construction of pcDNA3.1/mGM-CSF/Zeo vectors. A vector, pUMVC1-mGM-CSF, was purchased from Aldevron (Fargo, North Dakota 58104, USA). Then primers for PCR cloning of the mGM-CSF gene were designed to amplify the gene. The sequences of the primers are: 5'-CCAAGCTTCGAATTCACCATGTGGCTGCAG-3' for the 5' primer and 5'-CTGGATCCTCATTTTTGGCTTGG-3'

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for the 3' primer. After PCR amplification, the product was digested with HindIII and BamHI and then ligated with a fragment of pcDNA3.1(+)/Zeo that was treated with the same restriction enzymes. The ligation product was then transformed into one shot TOP 10 cells and plasmid DNAs were prepared from bacterial colonies. The plasmid DNAs were first screened for mGM-CSF using enzyme digestions, which was further confirmed by sequencing analysis. Midi-preps of pcDNA3.1/mGM-CSF/Zeo were made and stored for future use.

Transfection of TC-1 cells and mGM-CSF ELISA. For cell transfection, pcDNA3.1/mGM-CSF/Zeo was first linearized with BglII restriction enzyme and then purified. Transfection of the TC-1 tumor cells was carried out in a 6-well plate using the LipofectAMINE 2000 Reagent from GIBCO (Grand Island, NY, USA) according to the manufacturer's instruction. Single cell clones were selected with 100 µg/ml of Zeocin (Invitrogen, Carlsbad, CA, USA).

To determine the mGM-CSF level in each individual clone, 1 x 10⁶ cells of each clone were seeded in a well of 6-well plates in 3 ml of culture medium and cultured for 48 hours. The supernatants were collected and stored at -20°C. ELISA assay was performed using the Quantikine GM-CSF Immunoassay Kit from R & D Systems (Minneapolis, MN, USA). The sensitivity of this kit is < 1 pg/ml of GM-CSF.

In vitro growth rate of different clones. 2x10⁵ cells of different clones were seeded in T-25 flasks in triplicate. The cells were harvested from each flask and counted 2 days and 4 days after culture.

Animal study with transfected tumor cells. 2x10⁵ tumor cells in 0.2 ml of 1x PBS were intradermally (*i.d.*) injected into female C57BL/6J mice or SCID mice. The tumors were measured with a digital caliper, recorded and statistically analyzed. Tumor volume was calculated as ½ length x width². Each group consisted of eight mice. In order to determine the serum level of GM-CSF in these mice, 200 µl of blood was collected from each mouse through the tail vein. The GM-CSF levels in the blood were detected using the Quantikine kit.

FACS analysis of leukocytes. Two hundred and fifty µl of blood was collected from each mouse. After lysing of the red blood cells, the samples were stained with various FITC/or PE conjugated antibodies including CD3, CD19, Gr-1, Mac-3 and CD11c and analyzed on FACS Calibur with CellQuest software. Each experiment was repeated three times.

Statistical analysis. Two-way ANOVA with Bonferroni post test (Prism 4 software) was used to analyze the tumor growth data and the FACS data.

Results

Individual clones expressing different levels of GM-CSF. After transfection of the TC-1 tumor cells with linearized pcDNA3.1/mGM-CSF/Zeo plasmid DNA, they were cultured in selective medium containing Zeocin. Single colonies resistant to the drug were collected and expanded in selective medium. The GM-CSF expressions by these clones were detected with ELISA and the results are shown

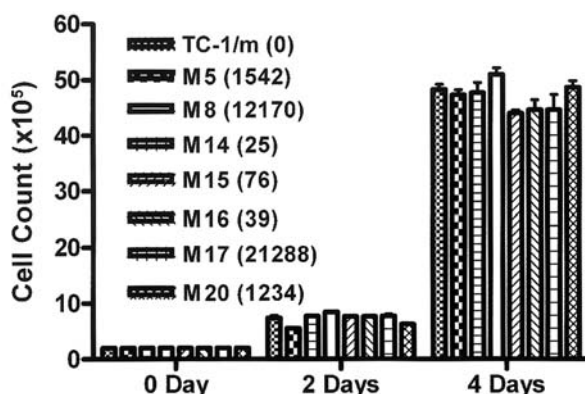


Figure 1. Clones expressing different levels of GM-CSF grow similarly in vitro. 2x10⁵ cells of different clones were seeded in T-25 flasks in triplicate. The cells were harvested from each flask and counted 2 days and 4 days after culture. The numbers in brackets are the GM-CSF expression level in vitro (pg/ml).

in Figure 1. The clones expressed different levels of GM-CSF ranging from 25 pg/ml to 21288 pg/ml. In order to determine whether the expression of GM-CSF at different levels interferes with tumor cell growth *in vitro*, 2x10⁵ cells of each clone were seeded in T-25 flasks in triplicate. Two days and four days after culture, the cells were harvested and counted. Although they expressed different levels of GM-CSF, the clones grew at a similar rate (Figure 1).

Different levels of GM-CSF dramatically affect tumor cell growth in vivo. 2x10⁵ cells of different clones were *i.d.* injected into C57BL/6J mice and tumor growth was measured (Figure 2A). When GM-CSF was expressed at low levels (M16 [39 pg/ml] and M15 [76 pg/ml]), tumor growth was significantly inhibited (*p*<0.001). The growth of clones expressing intermediate levels of GM-CSF (M5 [1542 pg/ml] and M20 [1234 pg/ml]) was similar to control TC-1/m tumor cells (*p*>0.05). However, when the GM-CSF expression was significantly higher (M8 [12170 pg/ml] and M17 [21288 pg/ml]), tumor growth was significantly enhanced compared to control TC-1/m tumor cells (*p*<0.001).

High level GM-CSF induces lymphopenia and granulocytosis in vivo. In order to understand the mechanism behind the dramatic effect of GM-CSF on tumor growth *in vivo*, we first ascertained the serum level of GM-CSF in these mice (Table I). The result correlated very well with the *in vitro* data (Figure 1). Clones that expressed high levels of GM-CSF *in vitro* produced high levels of serum GM-CSF *in vivo*. On the other hand, clones that expressed low levels of GM-CSF *in vitro* produced low levels of GM-CSF *in vivo*. In fact, GM-CSF was not detectable in those mice that received clones M16 and M15.

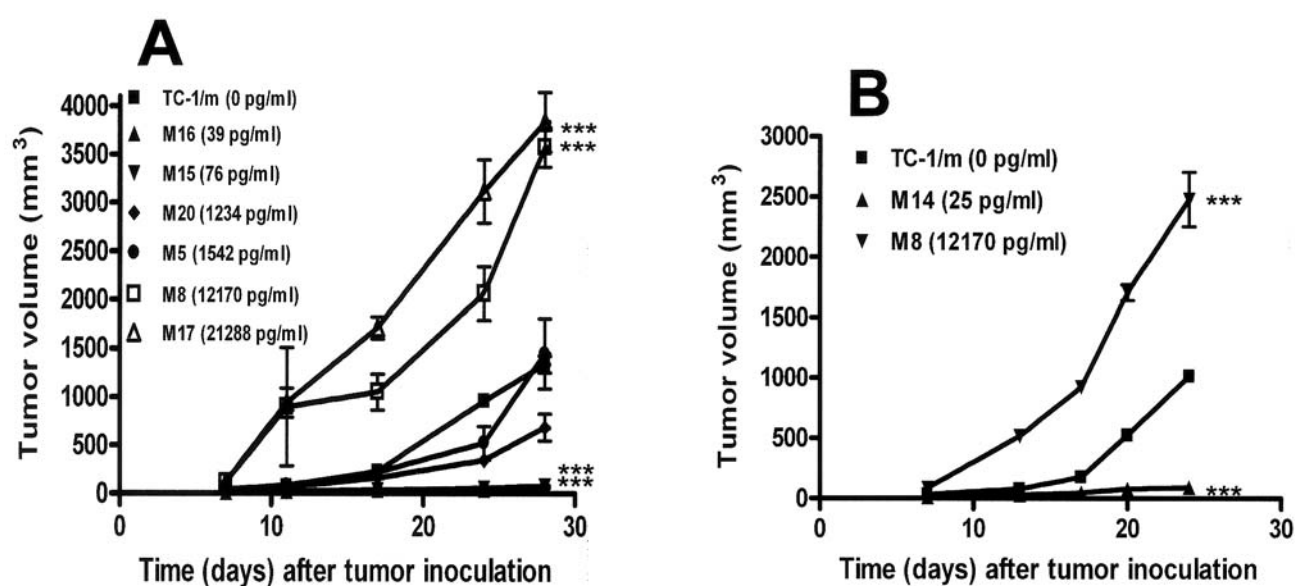


Figure 2. Tumor growth of clones expressing different levels of GM-CSF is different in vivo. 2×10^5 tumor cells in 0.2 ml of 1x PBS were i.d. injected into female C57BL/6J mice (A) or SCID mice (B). The tumors were measured with a digital caliper, recorded and statistically analyzed. Tumor volume was calculated as $\frac{1}{2}$ length \times width². Each group consisted of eight mice. ***: $p < 0.001$

Second, a panel of white blood cells including T cells, B cells, granulocytes, dendritic cells and macrophages from the tumor-bearing mice was analyzed by FACS analysis (Figure 3). On day 26 after tumor cell inoculation, the high level of GM-CSF expression (clone M8) significantly decreased CD3+ T lymphocytes (2.95% vs 29.8%, $p < 0.001$) and CD19+ B cells (0.09% vs 41.6%, $p < 0.001$), but increased Gr-1+ granulocytes (76.25% vs 23.4%, $p < 0.001$), Mac-3+ macrophages (69.9% vs 39.2%, $p < 0.001$) and CD 11c+ dendritic cells (7.05% vs 5.01%, $p < 0.05$). In contrast, a low level of GM-CSF expression (clone M15) decreased macrophages (20.05% vs 36.36%, $p < 0.01$) and dendritic cells (1.6% vs 5.1%, $p < 0.01$), although it did not affect T cells (31.5% vs 26.8%, $p > 0.05$), B cells (46.5% vs 41.6%, $p > 0.05$) and granulocytes (20.5% vs 23.4%, $p > 0.05$).

Tumor inhibition by low level of GM-CSF is not dependent on lymphocytes. In order to determine whether the tumor growth inhibition in the presence of low levels of GM-CSF secreted by tumor cells is due to lymphocyte function, we performed a tumor study in SCID mice which lack functional lymphocytes. 2×10^5 cells of clones M14, M8 and control TC-1/m were i.d. injected into SCID mice and the tumor growth was measured (Figure 2B). To our surprise, a similar effect as in the C57BL/6J mice was observed, i.e. a high level of GM-CSF enhanced tumor growth ($p < 0.001$), while low levels of GM-CSF inhibited tumor growth ($p < 0.001$).

Table I. Average serum level of GM-CSF in mice with different clones.

Time	Serum level of GM-CSF in individual clones (pg/ml)				
	M5	M8	M16	M15	M17
Day 11	ND*	ND	ND	ND	50
Day 17	ND	133	ND	ND	2630
Day 21	33	1242	ND	ND	7515
Day 25	66	1542	ND	ND	8820

*ND: not detectable.

Two hundred μ l of blood was collected from mice bearing tumors of different clones through tail vein. The GM-CSF levels in the blood were detected using GM-CSF ELISA (Quantikine M, R&D Systems). The kit's sensitivity is < 1.0 pg/ml. The data are averages of eight mice.

Discussion

Different TC-1 tumor cell clones expressing various levels of murine GM-CSF were generated by transfection with expression vector encoding GM-CSF and selected. Although these clones express different levels of GM-CSF, they grow at a similar rate *in vitro*, indicating that GM-CSF has no direct effect on tumor cell growth in this TC-1 tumor model. In contrast to this finding, one recent study demonstrated that GM-CSF promotes tumor cell and spleen cell growth *in vitro* (12). We speculate that this different effect may be

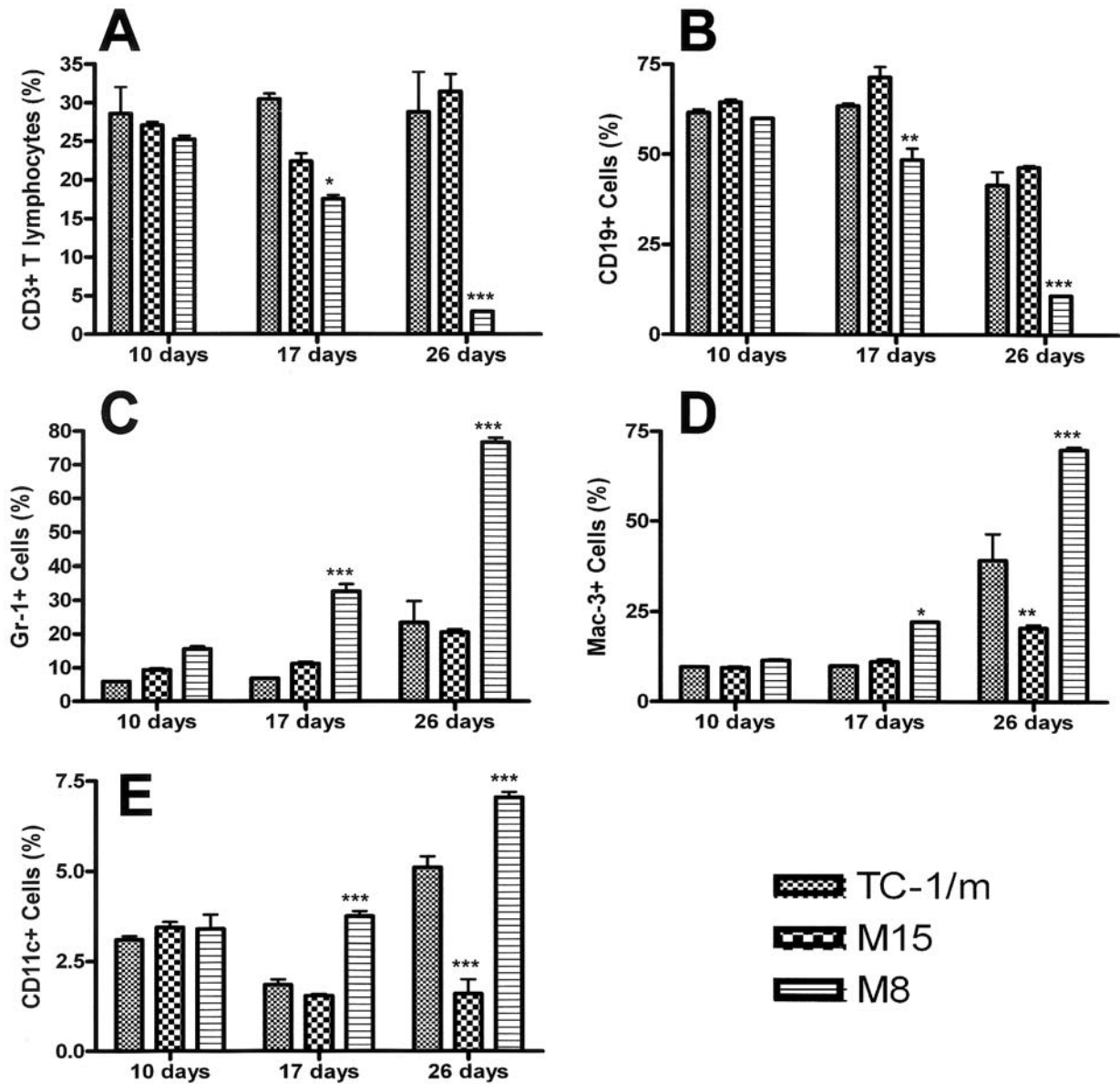


Figure 3. FACS analysis of leukocytes from mice bearing tumors expressing different levels of GM-CSF. Two hundred and fifty μ l of blood was collected from each mouse on days 10, 17 and 26 after tumor cell inoculation. Following lysing of the red blood cells, the samples were stained with various FITC or PE conjugated antibodies including CD3 (A), CD19 (B), Gr-1 (C), Mac-3 (D) and CD11c (E) and analyzed on FACS Calibur with CellQuest software. Each experiment was repeated three times. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

due to different tumor models. However, although the clones in this study grow similarly *in vitro*, the *in vivo* tumor development was dramatically different, ranging from inhibited tumor growth when the GM-CSF expression level is low to promoted tumor growth when GM-CSF expression level is high (Figure 2). This result demonstrates that there is a clear dose-dependent effect of GM-CSF, derived from engineered tumor cells, on tumor growth.

The serum level of GM-CSF mirrored the *in vitro* data (Table I), *i.e.* the serum level of GM-CSF in mice bearing a clone that expressed high levels of GM-CSF was high, and *vice-versa*. Since no GM-CSF was detectable in mice bearing clones that express low levels of GM-CSF *in vitro*, we speculate that the antitumor growth effect of low levels of GM-CSF is restricted locally. In order to confirm this, we injected GM-CSF-expressing tumor cells *i.d.* on one side of

the mice and injected the tumor cells that do not express GM-CSF on the other side. The results showed that the antitumor effect generated by tumor cells expressing low levels of GM-CSF cannot protect growth of tumor cells that do not express GM-CSF at a remote site (data not shown). To understand the mechanism of the dramatically different effect of GM-CSF *in vivo* on tumor growth, a panel of blood cells from the tumor-bearing mice was analyzed by FACS (Figure 3). From this study, we concluded that a high level of GM-CSF secreted by tumor cells induces granulocytosis and lymphopenia, and promotes tumor growth, while a low level of GM-CSF inhibits tumor growth. This observation confirms some previous studies that GM-CSF induces leukocytosis and promotes tumor growth (12, 13), but conflicts with others which showed that GM-CSF induced lymphocyte proliferation and enhanced neutrophil function, which induces cytolysis and tumor regression (12, 14). In line with our observations, Miller *et al.* demonstrated that over-expressed endogenous GM-CSF, although it significantly increased CD11+ DCs *in vivo*, failed to protect against tumor growth in a variety of models including *i.d.* CT26 colorectal cancer, CT26 liver metastases, B16 melanoma liver metastases and *i.d.* EL4.OVA lymphoma (15). In order to determine whether lymphocytes play an important role in the inhibition or promotion of tumor growth in this model, we grew different tumor clones in SCID mice which lack functional lymphocytes. The results are similar to those of normal mice (Figure 2B). Clearly, lymphocytes do not play an important role in this tumor inhibition. We thus hypothesize that the tumor growth inhibition effect by a low level of GM-CSF may be due to an enhanced function of natural killer (NK) cells because, although there is no functional lymphocyte in SCID mice, their NK cells are still functional. This speculation is supported by a recent study (in murine renal carcinoma model (16)) demonstrating that NK cells and granulocytes are predominantly involved in the antitumor action elicited by GM-CSF.

In conclusion, GM-CSF's antitumor effect is clearly dose-dependent. At relatively low level (<100 pg/ml), the cytokine functions as an antitumor growth factor. However, when its expression level is high (>10,000 pg/ml), the cytokine enhances tumor growth. Although the mechanism behind this phenomenon deserves further study, it seems that the antitumor effect of GM-CSF at a low expression level is mediated through NK cells, while the tumor growth enhancement effect is through granulocytosis. This study provides valuable information about the clinical use of GM-CSF.

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References

- 1 Quesniaux VFJ and Jones TC: Granulocyte-macrophage colony stimulating factor. *In*: Thomson A W (eds), The Cytokine Handbook, 3rd edition, pp. 636-670. San Diego, CA: Academic Press, 1998.
- 2 Hamilton JA: GM-CSF in inflammation and autoimmunity. *Trends in Immunol* 23: 403-408, 2002.
- 3 Armitage JO: Emerging applications of recombinant human granulocyte-macrophage colony-stimulation factor. *Blood* 92: 4491-4508, 1998.
- 4 Dranoff G: GM-CSF-secreting melanoma vaccines. *Oncogen* 22: 3188-3192, 2003.
- 5 Chang, EY, Chen CH, Ji H, Wang TL, Hung K, Lee BP, Huang AY, Kurman RJ, Pardoll DM and Wu T: Antigen-specific cancer immunotherapy using a GM-CSF secreting allogeneic tumor cell-based vaccine. *Int J Can* 86: 725-730, 2000.
- 6 Toda M, Martuza RL and Rabkin SD: Tumor growth inhibition by intratumoral inoculation of defective herpes simplex virus vectors expressing granulocyte macrophage colony-stimulating factor. *Mol Ther* 2: 324-329, 2000.
- 7 Parkinson RJ, Mian S, Bishop MC, Gray T, Li G, McArdle SE, Ali S and Rees RC: Disabled infectious single cycle herpes simplex virus (DISC-HSV) is a candidate vector system for gene delivery/expression of GM-CSF in human prostate cancer therapy. *Prostate* 56: 65-73, 2003.
- 8 Loudon PT, McLean CS, Martin G, Curry J, Leigh Shaw M, Hoogstrate C, Verdegaal E and Osanto S: Preclinical evaluation of DISC-GMCSF for the treatment of breast carcinoma. *J Gene Med* 5: 407-416, 2003.
- 9 Couch M, Saunders JK, O'Malley BW Jr, Pardoll D and Jaffee E: Genetically engineered tumor cell vaccine in a head and neck cancer model. *Laryngoscope* 113: 552-556, 2003.
- 10 Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D and Mulligan RC: Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* 90: 3539-3543, 1993.
- 11 Lumniczky K, Desaknai S, Mangel L, Szende B, Hamada H, Hidvegi E and Safrany G: Local tumor irradiation augments the antitumor effect of cytokine-producing autologous cancer cell vaccine in a murine glioma model. *Can Gene Ther* 9: 44-52, 2002.
- 12 Diamant MJ, Stillitani MI, Puricelli L, Bal de Kier-Joffe E and Klein S: GM-CSF secreted by murine adenocarcinoma cells modulates tumor progression and immune activity. *Oncol Rep* 10: 1647-1652, 2004.
- 13 Watanabe M, Ono K, Ozeki Y, Tanaka S, Aida S and Okuno Y: Production of granulocyte-macrophage colony-stimulating factor in a patient with metastatic chest wall large cell carcinoma. *Jpn J Clin Oncol* 28: 559-562, 1998.
- 14 Grote D, Cattaneo R and Fielding AK: Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. *Can Res* 63: 6463-6468, 2003.
- 15 Miller G, Pillarisetty VG, Shah AB, Lahrs S, Xing Z and DeMatteo RP: Endogenous granulocyte-macrophage colony-stimulating factor overexpression *in vivo* results in the long-term recruitment of a distinct dendritic cell population with enhanced immunostimulatory function. *J Immunol* 169: 2875-2885, 2002.
- 16 Maini A, Nishisaka N, Kionshita Y, Jones RF, Wang CY and Haas GP: Combination of radiation and vaccination with autologous tumor cell expressing IL-2, IFN-gamma and GM-CSF for treatment of murine renal carcinoma. *In Vivo* 17: 119-123, 2003.

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