Enhanced adjuvant effect of granulocyte-macrophage colony-stimulating factor plus interleukin-12 compared with either alone in vaccine-induced tumor immunity

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Using the poorly immunogenic D5 murine melanoma, we examined the adjuvant effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-12 (IL-12) secretion by gene-modified tumor cells inoculated as a vaccine to prime tumor-draining lymph nodes (TDLNs). D5 transfectants that secreted IL-12 or GM-CSF alone were compared with a double transfectant that secreted equivalent amounts of both cytokines. TDLN cells harvested 9–10 days after subcutaneous tumor inoculation were cultured sequentially in anti-CD3 and IL-2 and assessed for antitumor reactivity against wild-type D5 tumor. The double transfectant-induced TDLN effector cells had greater cytotoxicity in a long-term assay than TDLN cells primed by single transfectants. In adoptive immunotherapy, the TDLN cells primed by the double transfectant were significantly better at mediating the regression of established tumors compared with the TDLN cells elicited by the single transfectants. Both IL-12 and GM-CSF had adjuvant effects in promoting tumor-reactive TDLN cells, but the combination was better than either alone. These observations suggest that the immunomodulation roles of IL-12 and GM-CSF are different and complementary.

Key words: Vaccine; granulocyte-macrophage colony-stimulating factor; interleukin-12; immunotherapy; gene therapy; adoptive immunotherapy.

Vaccines comprised of genetically modified tumor cells that secrete specific cytokines reportedly induce systemic immunity in naive hosts.^{1,2} The local elaboration of cytokines into the tumor microenvironment acts as an adjuvant to recruit or stimulate host immune cells to respond to tumor-associated antigens (Ags). It was hoped that this phenomenon in animal studies would lead to the development of vaccines for the therapy of established disease. However, there have been few reports indicating that these gene-modified tumor vaccines can be efficacious in the treatment of established tumors, and only in the setting of minimal tumor burdens.^{3,4} Hence, the real utility of this approach will probably be confined to the treatment of micrometastatic disease as an adjunct to other therapies (i.e., surgery). As an alternative for the treatment of more advanced tumor burdens, we have used these gene-

modified tumor constructs as a means of generating tumor-reactive T cells that can be expanded *ex vivo* and subsequently administered as a form of adoptive immunotherapy. T-cell therapies have been demonstrated to be capable of mediating the regression of advanced tumor burdens in animal models.^{5,6}

In animal studies, we have reported that the intradermal inoculation of viable or irradiated tumor cells induced the development of pre-effector cells in the draining lymph nodes.^{7–9} These pre-effector cells could be activated *in vitro* with anti-CD3 monoclonal antibody (mAb) and expanded in interleukin-2 (IL-2) to mature into effector cells capable of mediating the regression of established tumors in adoptive immunotherapy. Using this model, we have demonstrated that certain cytokine-secreting tumor cells will enhance the *in vivo* induction of pre-effector cells beyond what was capable with wild-type (wt) tumor cells. Cytokines that have proven to be useful adjuvants in our model include granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4.^{10,11} In this report, we have examined IL-12, which was found to enhance pre-effector cell induction to a poorly immunogenic tumor. In addition, we found that there was an additive or synergistic interaction between

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GM-CSF and IL-12 as adjuvants in this vaccine approach.

MATERIALS AND METHODS

Mice

Female B6 mice were purchased from The Jackson Laboratory (Bar Harbor, Me) and maintained in a specific pathogen-free environment. Mice were used at the age of 8 weeks or older. Recognized principles of laboratory animal care were followed, and all animal protocols were approved by the University of Michigan Laboratory of Animal Medicine.

Tumors

The B16-BL6 melanoma is a tumor of spontaneous origin that has been studied extensively.¹² A subclone of the B16-BL6 tumor, termed D5, has been characterized previously by our laboratory.¹⁰ Tumor cells were maintained by serial *in vitro* passage in complete medium (CM), which consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf sera, 0.01 mM nonessential amino acids, 1 μ M sodium pyruvate, 2 mM fresh L-glutamine, 100 μ g/mL streptomycin, 100 international units/mL penicillin, 50 μ g/mL gentamicin, 0.5 mg/mL fungizone (all reagents were obtained from Life Technologies, Grand Island, NY), and 10^{-5} M 2-mercaptoethanol. For intravenous (i.v.) and subcutaneous (s.c.) tumor inoculation, adherent tumor cells were removed from the flask using trypsin and washed in CM. Tumor cells were washed in Hank's balanced salt solution (HBSS) for administration into animals.

Genetic modification of the D5 tumor

A stable transfectant of the D5 tumor was generated using an MFG-based retroviral vector as described previously.¹⁰ This clone, which was termed D5G6, has been characterized and was determined to secrete ~ 280 ng/ 10^6 cells/24 hours of GM-CSF when these studies were performed. D5 and D5G6 were transfected with the IL-12 gene using the cationic liposome *N*-(1-[2,3-dioleoyloxy]propyl)-*N,N*,*N*-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim Corporation, Indianapolis, Ind). The murine IL-12 cDNA expression plasmid in tandem construction was obtained as described previously.¹³ Briefly, 2×10^5 D5 or D5G6 cells were placed into a well of a six-well plate with 5 mL of CM before the day of transfection. After overnight culture, adherent tumor cells were washed twice with RPMI 1640 without sera. A total of 5 μ g of murine IL-12 cDNA and 0.5 μ g of the neomycin resistance gene in 25 μ L of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer was added into 30 μ L of DOTAP solution diluted with 70 μ L of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer for 10 minutes at room temperature. After mixing, 2 mL of sera-free medium was added into the transfection mixture. Next, 1 mL of this mixture was added to the wells previously seeded with tumor cells. The tumor cells with the DOTAP/IL-12 gene mixture were incubated overnight at 37°C, and the medium was subsequently replaced with 5 mL of CM. At 3 days after transfection, tumor cells were cultured with medium containing 0.6 mg of Geneticin (Life Technologies) and incubated further. Visible colonies were plucked, and the IL-12-secreting tumor cell clones were established by limiting dilution methods. The IL-12 secretion of each clone was measured by enzyme-linked immunosorbent assay (kindly provided by Hoffman-La Roche, Nutley, NJ), and some clones were selected for further experiments.

Tumorigenicity assessment

Mice were inoculated s.c. with the indicated number of tumor cells in 0.05 mL of HBSS. Tumors were measured twice per week in two perpendicular dimensions with a Vernier caliper; size was recorded as area.

Tumor-draining lymph nodes (TDLNs)

A quantity of 10^6 parental or modified tumors in 0.10 mL of HBSS was inoculated s.c. in the flanks of syngeneic B6 mice. After 9–10 days, TDLNs were harvested under sterile conditions. Lymphocytes were dissociated into a single-cell suspension by teasing apart the TDLNs using 25-gauge needles and then pressing the tissue fragments with the blunt end of a 10-mL plastic syringe.

In vitro anti-CD3/IL-2 activation

TDLN cells were activated for 2 days in anti-CD3 mAbs (anti-CD3) immobilized in 24-well tissue culture plates at 4×10^6 cells/well as described previously.⁹ Anti-CD3 is a hamster mAb directed against the CD3 ϵ chain of the murine T-cell Ag receptor CD3 complex, which was produced by 2C11 hybridoma cells kindly provided by Dr. Jeffrey Bluestone, (University of Chicago, Chicago, Ill). After anti-CD3 activation, cells were harvested, washed, and resuspended at a concentration of 2×10^5 cells/mL of CM containing 60 μ g/mL of IL-2. Human recombinant IL-2 was kindly supplied by Chiron Therapeutics (Emeryville, Calif) with a specific activity of 18×10^6 U/mg protein. In this report, all units of IL-2 are expressed in international units. Subsequently, cells were harvested, washed, and resuspended in HBSS for adoptive immunotherapy.

Procedure for adoptive immunotherapy

B6 mice were injected i.v. with $1-2 \times 10^5$ D5 cells in 1 mL of HBSS to initiate pulmonary metastases. At 3 days after tumor establishment, anti-CD3/IL-2 activated TDLN cells suspended in 1 mL of HBSS and administered i.v. to mice. Some mice were also given 60,000 U of IL-2 in 0.5 mL of HBSS intraperitoneally twice a day for eight doses to enhance the *in vivo* proliferation and antitumor reactivity of the transferred cells. All control or treatment groups comprised at least five mice per group. Mice were killed at ~ 18 days after tumor establishment, and lungs were insufflated with Fekete's solution.¹⁴ Pulmonary metastases were counted without knowledge of the treatment. For all experiments, lungs with pulmonary nodules too numerous to count were arbitrarily assigned a value of >250 for statistical analysis.

Statistical analysis

Data were evaluated by the Wilcoxon rank-sum test. Two-sided *P* values of .05 were considered statistically significant. No animals were excluded from the statistical analysis.

In vitro cytotoxicity assay

We have reported previously that anti-CD3/IL-2 activation of TDLN cells results in effector cells that are nonlytic in standard 4-hour cytotoxicity assays.^{7,9} Hence, a long-term cytotoxicity assay was established to assess the cytolytic activity of TDLN cells over a 48-hour period, as described previously.¹⁵ Briefly, freshly harvested tumor cells were digested; single cells were cultured in a 96-well flat plate in 100 μ L of CM for 24 hours. After 24 hours, when tumor cells were subconfluent, TDLN cells after anti-CD3/IL-2 activation were added into the well

Figure 1. Tumorigenicity of wt D5 tumor cells and the IL-12-secreting clones. Cells (10^6) were inoculated s.c. into mice. The low-secreting IL-12 clones represent D5/IL-12-A21 cells, and the high-secreting clones are D5/IL-12-A4 cells. SDs of tumor area are indicated.

with 100 μ L of CM containing 4 μ M of IL-2. Triplicates for each effector to target (E:T) ratio were plated. For each TDLN population, E:T ratios consisted of 50, 25, 12.5, and 6.25. After 48 hours, wells were washed three times with phosphate-buffered saline and fixed with fixing solution for 10 minutes. Plates were washed four times with water, and 75 μ L of crystal violet was added for 5 minutes. The plates were then washed several times with water until the blank wells became clear. Plates were dried at room temperature overnight and dissolved with 100 μ L of dissolving solution. The absorbance of each well was read at 570 nm with an enzyme-linked immunosorbent assay plate reader, and cytotoxicity was calculated according to the following equation: percentage of cytotoxicity = $(1 - \text{absorbance of tumor cells treated with effector cells} / \text{absorbance control}) \times 100$.

For statistical analysis, mean values of triplicate determinations at each E:T ratio were calculated and compared by the Student *t* test.

RESULTS

Tumorigenicity of D5 transfectants

The tumorigenicity of the IL-12-secreting transfectant tumor cells was compared with the parental line by inoculating 10^6 cells s.c. into the abdominal skin and measuring the tumors serially. Two different clones were selected that secreted 4 and 64 ng/ 10^6 /24 hours of IL-12; these clones were termed D5/IL-12-A21 and D5/IL-12-A4, respectively. As depicted in Figure 1, IL-12 secretion resulted in reduced tumorigenicity, which was directly proportional to the amount of the transgene protein. At days 4 and 7 after tumor inoculation, both the low- and high-secreting IL-12 clones were significantly decreased in size compared with the parental D5 tumors at *P* values of $< .01$ and $< .001$ (Student's *t* test), respectively. After 25 days, the high-secreting IL-12 tumors were significantly smaller than the low-secreting IL-12 tumors (*P* <

Figure 2. Tumorigenicity of wt D5 tumor cells, the GM-CSF-secreting transfectant cells (D5G6), and the double-transfectant tumor cells that secrete both GM-CSF and IL-12 (D5G6/IL12-A13). Cells (10^6) were inoculated s.c. into mice. SDs of tumor area are indicated.

.001). None of the animals had a complete regression of tumor.

The GM-CSF-secreting tumor cells (D5G6) were not altered in their tumorigenicity compared with parental D5 cells (Fig 2). However, the double-transfectant cell line that secreted both GM-CSF and IL-12 (D5G6/IL-12-A13) was noted to have reduced tumorigenicity (Fig 2). At day 7 after tumor cell inoculation, the double-transfectant tumors were significantly smaller than either the parental D5 or GM-CSF-secreting tumors (*P* < .001, Student's *t* test). The double-transfectant cell line elaborated comparable amounts to the high-secreting IL-12 transfectant and manifested similar growth characteristics when inoculated *in vivo*.

Cytolytic reactivity of lymph node cells draining D5 transfectant tumors

The antitumor reactivity of lymph node cells draining tumor inoculums of the gene-modified cell lines was examined in a long-term cytotoxicity assay. A total of 1×10^6 tumor cells were inoculated s.c. in the flanks of animals and allowed to grow for 10 days, at which time TDLNs were harvested. TDLN cells were cultured by the anti-CD3/IL-2 activation method and then assessed for their ability to lyse parental D5 tumors in a 48-hour cytotoxicity assay. As demonstrated in Figure 3, the cytotoxicity of TDLN cells derived from animals inoculated with the IL-12- or GM-CSF-secreting tumor cells was significantly greater (*P* < .01) than those from animals inoculated with parental tumor cells at all E:T ratios. In addition, the double-transfectant tumor line secreting both IL-12 and GM-CSF resulted in the induction of effector TDLN cells that had significantly greater cytotoxicity (*P* < .01) than TDLN cells induced by the single-transfectant cell lines secreting either IL-12 or GM-CSF alone at E:T ratios of 25, 12.5, and 6.25.

Figure 3. Long-term cytotoxicity of TDLNs derived from mice inoculated with wt D5 tumor cells or gene-modified tumor cells. The tumor target used in this assay was D5.

In vivo antitumor reactivity of lymph node cells draining D5 transfectant tumors

Using a 3-day established pulmonary metastasis model, we assessed the antitumor reactivity of TDLN cells from animals inoculated with the transfectant cell lines. We first evaluated the efficacy of both the low- and high-secreting IL-12 gene-modified tumor cells compared with parental tumor cells to prime TDLNs. TDLN cells were harvested 10 days after s.c. inoculation of 10^6 tumor cells and activated by the anti-CD3/IL-2 culture method. Activated TDLN cells were then adoptively transferred into mice with established lung metastases along with the exogenous administration of IL-2 (Table 1). There was a modest reduction of pulmonary metastases when 3×10^7 parental TDLN cells were administered compared with untreated control animals. By

contrast, there was a significantly greater reduction of pulmonary metastases by the IL-12 transfectant TDLN cells compared with parental TDLN cells. The enhanced antitumor reactivity of the IL-12 transfectant TDLN cells was directly related to the amount of transgenic IL-12 that was elaborated.

The high-secreting IL-12 transfectant was used in a subsequent experiment to evaluate the antitumor reactivity of TDLN cells primed by the double-transfectant tumor line secreting both IL-12 and GM-CSF. The amount of IL-12 secreted by the double transfectant was comparable with the single transfectant-secreting IL-12. Included in this experiment was the D5G6 clone that elaborated amounts of GM-CSF that were comparable to the amounts observed for the double transfectant. Similar to the previous protocol, TDLN cells were harvested 9 days after s.c. inoculation of tumor cells. After anti-CD3/IL-2 culture, the cells were adoptively transferred into mice with established pulmonary metastases. As summarized in Table 2, significantly greater reductions in pulmonary metastases were observed in groups of mice receiving 2×10^7 TDLN cells derived from mice inoculated with IL-12-, GM-CSF-, and IL-12/GM-CSF-secreting tumor cells compared with parental tumor cells. Moreover, at this dose of cells, the antitumor effect of TDLN cells induced with GM-CSF or IL-12/GM-CSF resulted in complete tumor regression, which was significantly better than TDLN cells induced by IL-12 alone. To further assess the potency of the TDLN cells, a reduced dose of cells was transferred in the same experiment. At 0.6×10^7 cells, there was no antitumor reactivity mediated by parental TDLN cells, whereas there were significant reductions in pulmonary metastases mediated by TDLN cells draining inoculums of single-transfectant clones (GM-CSF or IL-12 only). By comparison, the transfer of TDLN cells (derived from mice inoculated with the double transfectant) resulted in a complete regression of lung metastases, which was significantly different than the antitumor effect mediated by either single-transfectant TDLN cells. These results indicate that the combination of GM-CSF and IL-12 elaborated at the site of tumor growth was

Table 1. In Vivo Efficacy of D5/IL-12 TDLNs After Anti-CD3/IL-2 Activation

	TDLNs* primed with	Secretion of IL-12 (ng/ 10^6 cells/24 hours)	Adoptive immunotherapy	
			Cells	Mean no. of pulmonary mets (SEM)
A.	—	—	—	>250
B.	D5	ND	+	162 (26)‡
C.	D5/IL12-A21	4	+	78 (16)§
D.	D5/IL12-A4	64	+	33 (8)¶

*At 10 days after tumor s.c. inoculation, TDLNs were harvested for anti-CD3/IL-2 activation.

†Mice were inoculated with 3×10^5 D5 tumor cells i.v. After 3 days, some groups of mice received 3×10^7 effector cells. All mice received 60,000 U of IL-2 i.p. twice daily for eight doses commencing after cell transfer. Lungs were harvested on day 18.

‡ $P < .01$ compared with group A.

§ $P < .001$ compared with group A; $P < .05$ compared with group B.

¶ $P < .0001$ compared with group A; $P < .01$ compared with B; $P < .05$ compared with C.

||ND, not determined.

Table 2. In Vivo of D5G6/IL-12 TDLN After Anti-CD3/IL-2 Activation

	Source of TDLN cells*	Secretion of		Adoptive immunotherapy†		Mean no. of lung mets (SEM)
		GM-CSF‡	IL-12‡	Cells	IL-2	
A.	—	—	—	—	—	>250
B.	—	—	—	—	+	>250
C.	D5	ND**	ND**	2×10^7	+	126 (24)§
D.	D5	ND**	ND**	0.6×10^7	+	228 (8)
E.	D5/IL-12-A4	ND**	64	2×10^7	+	42 (10)‡
F.	D5/IL-12-A4	ND**	64	0.6×10^7	+	136 (21)§
G.	D5G6	280	ND**	2×10^7	+	0
H.	D5G6	280	ND**	0.6×10^7	+	55 (7)¶
I.	D5G6/IL-12-A13	265	68	2×10^7	+	0
J.	D5G6/IL-12-A13	265	68	0.6×10^7	+	0

*At 9 days after s.c. tumor inoculation, TDLNs were harvested for anti-CD3/IL-2 activation.

†ng/10⁶ cells/24 hours.

‡Mice were inoculated with 3×10^5 tumor cells i.v. After 3 days, some groups of mice received effector cells at the indicated dose. Some groups of mice received 60,000 U of IL-2 i.p. twice daily for eight doses commencing after cell transfer. Lungs were harvested on day 18.

§ $P < .01$ compared with groups A, B, and D.

¶ $P < .001$ compared with groups A, B, and D; $P < .05$ compared with groups C and F.

|| $P < .0001$ compared with groups A, B, and D; $P < .001$ compared with groups C and F; $P < .01$ compared with groups E and H.

**ND, not determined.

either additive or synergistic in priming TDLNs to develop immune pre-effector cells.

DISCUSSION

This report identifies two main observations: (a) the secretion of IL-12 by tumor cells used as a vaccine enhanced the antitumor reactivity of TDLN pre-effector cells; and (b) the combination of IL-12 and GM-CSF secretion was at least as additive as an adjuvant in the priming of TDLN cells. In this same tumor model using the poorly immunogenic D5 tumor, we have reported previously that the secretion of transgenic GM-CSF or IL-4 cytokines up-regulated the priming of TDLN cells, whereas IL-2 or interferon- γ secretion was not effective.¹¹ In this regard, we know that both IL-4 and GM-CSF are requisite growth factors for the *ex vivo* expansion of dendritic cells (DCs), which represent potent Ag-presenting cells.¹⁶ Our observation that the combination of GM-CSF and IL-12 had at least additive effects supports the hypothesis that they have different mechanisms of action as immune adjuvants in a tumor vaccine.

IL-12 is a heterodimeric cytokine that has proinflammatory effects, as well as antitumor properties *in vivo*. As a vaccine adjuvant, the transgene expression of IL-12 by either tumor cells or fibroblasts has been shown to up-regulate the induction of systemic antitumor immunity.¹⁷⁻¹⁹ Our studies would support the premise that IL-12 can play an important role during the afferent arm of the immune response to tumor Ag. IL-12-secreting melanoma tumor cells inoculated s.c. resulted in an increased cytolytic activity of TDLN cells against melanoma targets compared with the inoculation of wt tumor

cells. Furthermore, TDLN cells primed by the IL-12-secreting tumors were more effective in adoptive immunotherapy of melanoma metastases compared with wt tumor-induced TDLN cells. In contrast to our observations regarding IL-12 as an effective adjuvant in the priming of TDLN cells to D5 melanoma, we did not observe this effect with a different tumor, the MT-901 mammary carcinoma, which is syngeneic in the BALB/c host.²⁰ Using the same protocol, IL-12 transfectants of the MT-901 tumor were found to have reduced tumorigenicity *in vivo*. However, unlike the D5 melanoma, the IL-12-secreting MT-901 cells did not enhance the priming of TDLN pre-effector cells. One explanation may be the subset of effector cells responsible for mediating tumor regression between the two tumors. MT-901 is unique compared with all prior murine tumors that we have studied. In our adoptive transfer model, only the transfer of CD4⁺ TDLN effector cells was capable of mediating the regression of MT-901 tumors, whereas CD8⁺ cells were ineffective. We have reported previously that wt D5 pulmonary metastases are susceptible to either CD4⁺ or CD8⁺ TDLN cells given in adoptive transfer.²¹ Based on the differences seen in the two tumor models, we hypothesize that IL-12 enhances the priming of CD8⁺ pre-effector cells in TDLNs, and has no effect on CD4⁺ pre-effector cells. This hypothesis is supported by the observations of Colombo and coworkers, who reported that the transgene expression of IL-12 by the murine CT26 tumor induced CD8⁺ effector responses and not CD4⁺ responses.^{22,23} Moreover, the depletion of CD4⁺ cells in their model enhanced the antitumor response that was up-regulated by the transgenic IL-12.

In a previous report, we have demonstrated that the elaboration of GM-CSF by the D5 melanoma up-regu-

lated the sensitization of both CD4⁺ and CD8⁺ pre-effector TDLN cells.²¹ The modulatory role of GM-CSF in up-regulating CD4⁺ pre-effector cells was corroborated in our MT-901 studies described above.²⁰ We believe that the mechanism of GM-CSF as an immune adjuvant relates to the recruitment or growth of Ag-presenting cells at the site of tumor vaccination and draining lymph nodes. In a previous report, we observed increased Mac-1⁺ and Mac-2⁺ cells in TDLN preparations obtained from animals inoculated with GM-CSF-secreting D5 melanoma tumors compared with wt tumors.¹⁰ In addition, using an antibody specific for DCs, we have observed a dramatic influx of these cells into GM-CSF-secreting MT-901 tumors by immunohistochemical analysis (data not shown). The prevalence of DCs in the vaccine sites of GM-CSF-secreting tumor cells correlates with our observations of analogous tumor cell constructs that are being employed as vaccines in a clinical trial we are currently conducting.²⁴

The different mechanisms of action of IL-12 and GM-CSF to up-regulate immune responsiveness should enable both to be combined with increased effectiveness. We were able to confirm this additive effect in our animal model. In a previous report, we had evaluated the combination of GM-CSF and IL-4 as adjuvants in the priming of TDLN cells.¹¹ Both cytokines secreted singly by gene-modified tumor cells resulted in increased priming of TDLN cells compared with wt tumor; however, the combination of GM-CSF and IL-4 as adjuvants was not greater than either alone. This suggested to us that both cytokines had similar mechanism of action in our model that we hypothesize are related to the up-regulation of Ag presentation. The results in the current study provides a rationale for evaluating both GM-CSF and IL-12 as adjuvants in tumor vaccines using tumor cell preparations or peptide Ags. The adjuvant effect of cytokines appears to be optimal when delivered locally at the site of tumor Ag.²⁵ This could be accomplished as cellular constructs (i.e., tumor cells or fibroblasts) genetically modified to secrete these cytokines; direct *in vivo* gene transfer (i.e., liposome complexes or gene gun delivery); or by direct injection of soluble cytokines. Our data would also suggest that the current interest in using DC-based vaccines may be augmented by the combined use of IL-12 as an adjuvant.

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REFERENCES

1. Miller AR, McBride WH, Hunt K, Economou JS. Cytokine-mediated gene therapy for cancer. *Ann Surg Oncol*. 1994;1:436–450.
2. Gilboa E. Immunotherapy of cancer with genetically modified tumor vaccines. *Semin Oncol*. 1996;23:101–107.
3. Golumbek PT, Lazenby AJ, Levitsky HI, et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science*. 1991;254:713–716.
4. Connor J, Bannerji R, Saito S, Heston W, Fair W, Gilboa E. Regression of bladder tumors in mice treated with interleukin-2 gene-modified tumor cells. *J Exp Med*. 1993; 177:1127–1134.
5. Greenberg PD. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol*. 1991;94:281–355.
6. Chang AE, Shu S. Current status of adoptive immunotherapy of cancer. *Crit Rev Oncol*. 1996;22:213–228.
7. Yoshizawa H, Chang AE, Shu S. Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. *J Immunol*. 1991;147:729–737.
8. Geiger JD, Wagner PD, Shu S, Chang AE. A novel role for autologous tumour cell vaccination in the immunotherapy of the poorly immunogenic B16-BL6 melanoma. *Surg Oncol*. 1992;1:199–208.
9. Geiger JD, Wagner PD, Cameron MJ, Shu S, Chang AE. Generation of T-cells reactive to the poorly immunogenic B16-BL6 melanoma with efficacy in the treatment of spontaneous metastases. *J Immunother*. 1993;13:153–165.
10. Arca MJ, Krauss JC, Aruga A, Cameron MJ, Shu S, Chang AE. Therapeutic efficacy of T cells derived from lymph nodes draining a poorly immunogenic tumor transduced to secrete granulocyte-macrophage colony-stimulating factor. *Cancer Gene Ther*. 1996;3:39–47.
11. Arca MJ, Krauss JC, Strome SE, Cameron MJ, Chang AE. Diverse manifestations of tumorigenicity and immunogenicity displayed by the poorly immunogenic B16/BL6 melanoma transduced with cytokine genes. *Cancer Immunol Immunother*. 1996;42:237–245.
12. Hart IR. The selection and characterization of an invasive variant of the B16 melanoma. *Am J Pathol*. 1979;97:587–600.
13. Rakhmilevich AL, Turner J, Ford MJ, et al. Gene gun-mediated skin transfection with interleukin-12 gene results in regression of established primary and metastatic murine tumors. *Proc Natl Acad Sci USA*. 1996;93:6291–6296.
14. Wexler H. Accurate identification of experimental pulmonary metastases. *J Natl Cancer Inst*. 1996;36:641–645.
15. Aruga A, Aruga E, Chang AE. Reduced efficacy of allogeneic versus syngeneic fibroblasts modified to secrete cytokines as a tumor vaccine adjuvant. *Cancer Res*. 1997; 57:3230–3237.
16. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and downregulated by tumor necrosis factor α . *J Exp Med*. 1994;179:1109–1118.
17. Meko JB, Yim JH, Tsung K, Norton JA. High cytokine production and effective antitumor activity of a recombinant vaccinia virus encoding murine interleukin-12. *Cancer Res*. 1995;55:4765–4770.
18. Tahara H, Zeh HJ, Storkus WJ, et al. Fibroblasts genetically engineered to secrete interleukin-12 can suppress tumor growth and induce antitumor immunity to a murine melanoma *in vivo*. *Cancer Res*. 1994;54:182–189.
19. Tahara H, Zitvogel L, Storkus WJ, et al. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J Immunol*. 1995;154:6466–6474.

20. Aruga E, Aruga A, Arca MJ, et al. Immune responsiveness to a murine mammary carcinoma modified to express B7-1, interleukin-12, or GM-CSF. *Cancer Gene Ther.* 1997;4:157-166.
21. Arca MJ, Krauss JC, Aruga A, Cameron MJ, Chang AE. Concurrent induction of CD4⁺ and CD8⁺ T cells during tumor growth with antitumor reactivity in adoptive immunotherapy. *J Immunother.* 1997;20:138-145.
22. Martinotti A, Stoppacciaro A, Vagliani M, et al. CD4 T cells inhibit *in vivo* the CD8-mediated immune response against murine colon carcinoma cells transduced with interleukin-12 genes. *Eur J Immunol.* 1995;25:137-146.
23. Colombo MP, Vagliani M, Spreafico F, et al. Amount of interleukin-12 available at the tumor site is critical for tumor regression. *Cancer Res.* 1996;56:2531-2534.
24. Chang AE, Sondak VK, Bishop DK, Nickoloff BJ, Mulligan RC, Mulé JJ. Adoptive immunotherapy of cancer with activated lymph node cells primed *in vivo* with autologous tumor cells transduced with the GM-CSF gene. *Hum Gene Ther.* 1996;7:773-792.
25. Kurane S, Arca MJ, Aruga A, Krinock RA, Krauss JC, Chang AE. Cytokines as an adjuvant to tumor vaccines: efficacy of local methods of delivery. *Ann Surg Oncol.* 1997;4:579-585.