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ORIGINAL ARTICLE

Keishi Tanigawa · Hua Yu · Rong Sun
Brian J. Nickoloff · Alfred E. Chang

Gene gun application in the generation of effector T cells for adoptive immunotherapy

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Abstract We utilized the gene gun to transfect subcutaneous D5 melanoma and MT-901 mammary carcinoma tumors in situ with a granulocyte/macrophage-colony-stimulating factor (GM-CSF) plasmid complexed to gold particles. There was diminished tumor growth following bombardment with GM-CSF plasmid, which was apparent only during the period of administration. Transgenic GM-CSF was produced by the skin overlying the tumors and not by the tumors themselves. GM-CSF plasmid bombardment resulted in increased cell yields within tumor-draining lymph nodes (TDLN) with at least a 12-fold increase in the percentage of dendritic cells (8.9%) compared to controls (0.7%). Secondarily activated TDLN cells from animals transfected with GM-CSF demonstrated enhanced cytokine release (interferon γ, GM-CSF and interleukin-10) in response to tumor stimulator cells compared to controls, and had an increased capacity to mediate tumor regression in adoptive immunotherapy. There was a small, but detectable, non-specific immune adjuvant effect observed with gold particle bombardment alone, which was less than with GM-CSF plasmid. The adjuvant effect of GM-CSF plasmid required peri-tumoral transgene expression since gene bombardment away from the tumor was ineffective.

Key words T cells · Gene therapy · Adoptive immunotherapy · Vaccines

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K. Tanigawa · R. Sun · A.E. Chang (✉)
Division of Surgical Oncology, University of Michigan,
3302 Cancer Center, 1500 E. Medical Center Drive,
Ann Arbor, MI 48109-0932, USA
e-mail: aechang@umich.edu
Tel.: +1-734-936-4392
Fax: +1-734-647-9647

H. Yu
H. Lee Moffitt Cancer Center and Research Institute,
University of South Florida, College of Medicine,
Tampa, FL 33612, USA

B.J. Nickoloff
Department of Pathology,
Loyola University Medical Center, Maywood,
IL 00153, USA

Introduction

The adoptive transfer of appropriately sensitized T cells can result in the regression of established, macroscopic disseminated tumor [10, 18, 27]. Our laboratory has focused on isolating immune pre-effector lymphoid cells from either tumor-draining or vaccine-primed lymph nodes [10, 14]. In pre-clinical models, we have demonstrated that these pre-effector cells require further ex vivo activation with anti-CD3 mAb (anti-CD3) followed by expansion in interleukin-2 (IL-2) to mature into competent effector cells. The T cell subpopulations that appear to mediate the antitumor reactivity upon adoptive transfer are those which elaborate type 1 cytokines in response to tumor [4]. In clinical trials, we are utilizing vaccines comprised of autologous tumor cells admixed with bacillus Calmette-Guerin as a means of priming draining lymph nodes [8, 9]. These vaccine-primed lymph nodes are harvested and secondarily activated ex vivo for subsequent adoptive transfer. We have observed significant in vitro tumor-specific reactivity of these vaccine-primed lymph node cells, and durable tumor responses in a subset of patients.

With gene-transfer techniques, the ability to genetically modify tumor cells with immunostimulatory genes has resulted in a number of reports demonstrating that the modified cells are more effective vaccine reagents than are wild-type tumor cells [11, 13, 15, 16]. The majority of these studies have focused on ex vivo transfection of tumor cells with viral vectors. In this report, we have evaluated the use of gene gun technology to transfect growing tumor with granulocyte/macrophage-colony-stimulating factor (GM-CSF) plasmid DNA. We

found the technique simple to use and associated with high levels of transgene expression. Furthermore, the local transfer of GM-CSF plasmid DNA was effective in enhancing tumor-reactive vaccine-primed lymph node cells capable of mediating regression of established metastases in adoptive immunotherapy.

Materials and methods

Mice

Female C57BL/6(B6) and Balb/c mice were purchased from the Jackson Laboratory, Bar Harbor, Me. They were maintained in specific-pathogen-free conditions and were used for experiments 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

D5 is a subclone from B16-BL6 melanoma, which is a tumor of spontaneous origin from the C57BL/6 mouse that has been studied extensively [20]. D5 has been characterized previously by our laboratory [1, 2]. MT 901 is a subline of MT-7, a cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the Balb/c host [7]. Both tumor cell lines were maintained by serial in vitro passage in complete media that consisted of RPMI-1640 medium with 10% heat-inactivated fetal calf serum (FCS), 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Gibco, Grand Island, N.Y.), and 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.). For intravenous (i.v.) and subcutaneous (s.c.) tumor inoculation, adherent tumor cells were trypsinized, washed, and resuspended in Hank's balanced salt solution (HBSS) for administration to animals.

GM-CSF plasmid

The plasmid utilized in this study was obtained from the National Gene Vector Laboratory at the University of Michigan. Briefly, the mGM-CSF gene was cloned by the reverse transcription/polymerase chain reaction using RNA isolated from phorbol-myristate-acetate-stimulated p388 cells. The mGM-CSF cDNA was then inserted into the pNGVL1 backbone digested with *Eco*RI and *Bam*HI. The sequence of the plasmid vector was confirmed by DNA sequencing.

Gene delivery

Gene modification in vivo was performed using the Accel particle delivery device (Agracetus, Middleton, Wis.). Plasmid DNA was precipitated onto gold particles by spermidine and $CaCl_2$. The DNA/gold-particle preparation was then coated onto the inner surface of Tefzel tubing (McMaster-Carr, Atlanta, Ga.) and cut into 1.25-cm segments as DNA cartridges. DNA-particle-mediated DNA delivery was performed with helium pulses of specified pressure to accelerate the DNA-coated gold particles. Balb/c mice were used for optimizing the conditions for gene gun in vivo transfection with murine GM-CSF plasmid DNA. Different conditions were examined by varying the gold particle size (0.6–1.6 mm), DNA concentration (1.0–3.0 μg DNA/mg gold) and shooting pressures (1.4–3.4 MPa). Hair was removed from the abdominal skin of the mice by Nair (Carter Products, New York) and the gene gun was applied three times. After 24 h, an area of skin measuring 0.5×0.5 cm was removed by scissors and then minced into small pieces. The tissue was then put into a microcentrifuge tube with 1.0 ml general extraction buffer, which consisted of 0.24 mg/ml Pefabloc (Boehringer-Mannheim, Indianapolis, Ind.) and 0.05% Triton X (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS). The supernatant of the general extraction buffer was collected 24 h later and assayed for GM-CSF secretion by enzyme-linked immunoabsorbent assays (ELISA: PharMingen, San Diego, Calif.).

Tumorigenicity assessment

Mice were inoculated s.c. in the mid-abdomen with 1×10^6 tumor cells in 0.05 ml HBSS. Starting on day 5, when palpable tumors were present, mice received murine GM-CSF-plasmid-DNA/gold particles applied by the gene gun every other day times three. As controls, mice received gold particles only or no treatment. Tumor size was measured three times a week in two perpendicular dimensions with a Vernier caliper, and the size recorded as area (mm^2). Tumors were measured until they became more than 200 mm^2 or the mice became cachectic, at which time they were humanely killed. Each group had at least six mice and none of the mice were excluded.

Tumor-draining lymph node (TDLN) cells and activation

Samples comprising 1×10^6 tumor cells in 0.05 ml HBSS were inoculated s.c. into the lower flanks of syngeneic mice. Gene delivery was carried out three times on days 5, 7, and 9. TDLN were removed under sterile conditions on day 10 and single-cell lymphocyte suspensions were prepared. This was performed by dissociating lymph nodes with 25-gauge needles and pressing with the blunt end of a 10-ml plastic syringe in RPMI-1640 medium. Single cells were washed by HBSS twice and then resuspended in complete medium. The cells were activated with 1 μg/ml anti-CD3 monoclonal antibody (mAb) immobilized in 24-well culture plates (4×10^6 cells in 2 ml in each well) for 2 days as previously described [33]. Anti-CD3 mAb is a hamster IgG mAb against the CD3ε chain of the murine TCR/CD3 complex produced by the 145-2C11 hybridoma line (Dr. Jeffrey A. Bluestone, University of Chicago, Chicago, Ill.) [22]. Anti-CD3 mAb was produced as ascites by the inoculation of the 145-2C11 hybridoma cells into immunocompromised, pristine-primed DBA/2 mice as previously described [33]. The lymph node cells were subsequently cultured in 60 IU/ml IL-2 for 3 days at 2×10^5 cells/ml in complete medium. Human recombinant IL-2 was a gift from Chiron Therapeutics, Emeryville, Calif. Activated TDLN cells were assessed for immunological reactivity by in vitro cytokine-release assays or by the adoptive transfer into mice with established pulmonary metastases.

Adoptive immunotherapy

Mice were inoculated i.v. with 3×10^5 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were infused i.v. with activated TDLN cells and given i.p. injections of IL-2 (60 000 IU) in 0.5 ml HBSS commencing on the day of cell transfer and continuing twice daily for eight doses. Approximately 14 days after tumor inoculation, all mice were sacrificed for enumeration of pulmonary metastatic nodules. Lungs were removed and the tracheas insufflated with a 15% solution of India ink for enumeration of MT-901 lung nodules or with Fekete's solution for enumeration of D5 lung nodules [31]. Metastatic foci too numerous to count were assigned an arbitrary value of >250. Mice had been previously ear-tagged and the counting of the lung nodules was performed without knowledge of group assignment. The significance of differences in numbers of metastatic nodules between experimental groups was determined using the non-parametric, Wilcoxon rank-sum test. Two-sided P values below <0.05 were considered significant. Each group consisted of at least five mice, and no animal was excluded from the statistical evaluation.

Immunofluorescence and flow cytometry

Between 5×10^5 and 10×10^5 lymph node cells were incubated for 45 min at 4 °C with 100 µl appropriately diluted mAb in PBS containing 2% FCS. The cell-surface phenotype was analyzed by flow cytometry (FACScan; Becton Dickinson & Co., Mountain View, Calif.). Staining was performed with the following fluorescein-isothiocyanate-labeled antibodies: CD3, CD4, CD8, CD45RO, CD11c, and phycoerythrin-labeled antibodies: CD80, CD62L. All antibodies and the appropriate isotype-matched control antibodies were obtained from PharMingen. The fluorescence intensity generated by 10 000 cells was displayed as the logarithm of the intensity plotted against cell number in two dimensions for two-color analysis.

Measurement of in vitro cytokine release by TDLN cells

Samples comprising 1×10^6 activated TDLN cells were co-cultured with 1×10^6 MT-901 tumor cells irradiated to 15 000 cGy in 2 ml complete medium/well of 24-well tissue-culture plates; 60 IU/ml IL-2 was added at the beginning of the cultures, which lasted for 48 h at 37 °C. The culture supernatants were collected and analyzed for cytokine measurements in duplicate by commercially available ELISA kits obtained from PharMingen. If detectable, background cytokine values produced by tumor cells alone were subtracted from the co-culture values in the results reported. For murine GM-CSF, a standard curve starting at 20 ng/ml with serial twofold dilutions performed up to a threshold of approximately 1 pg/ml. For interferon γ (IFNγ) and IL-10, a standard curve starting at 50 ng/ml was employed in a similar fashion (minimal threshold approximately 3 pg/ml). Experimental values were computed by regression analysis.

Histology

Histological and immunohistological findings were examined from the skin that had received gene delivery with GM-CSF plasmid DNA gold particles. As controls, skin samples that had received gold only or no treatment were examined as well. Hair was removed by Nair, and mice underwent gene delivery the next day. Skin samples were removed from mice 24 h later, and then fixed in 10% buffered formalin for staining with hematoxylin and eosin, or frozen for immunohistochemistry. A standard two-step streptavidin/horseradish-peroxidase staining technique was performed on frozen sections. The following primary antibodies were used: rat mAb against the murine CD4 (GK1.5 L3T4) and CD8 (2.43m Lyt-2.2) and hamster mAb against the murine CD11c (N418) and CD80 (1G10). Purified anti-(murine CD80) was obtained from PharMingen and the other primary antibodies were used in ascites, which were obtained from the American Type Culture Collection (Rockville, Md.). Optimal concentrations of each Ab were predetermined by titration assay. At least ten different high-power fields of multiple sections from each sample were evaluated by one pathologist (B. J. N.). The pathologist did not know whether samples were treated with gold particles alone or with GM-CSF/gold particles. He did know which were the untreated control samples since gold particles can be identified in the epidermis.

Results

Optimizing gene transfer

The mGM-CSF plasmid was used to assess various conditions of delivery of DNA to normal skin by gene gun. Preliminary studies evaluating gold particles of different size: 0.6, 1.0 and 1.6 µm were performed and it was apparent that the 1.0-µm and 1.6 µm particles were superior (data not shown). Gene expression was assessed by measurement of the transgenic GM-CSF protein produced from the targeted skin as described in Materials and methods. In a subsequent experiment, the amount of DNA (1.0–3.0 µg/ml) and pressure (1.4–3.4 mPa) used to project the DNA/gold particles was evaluated for particles of 1.0 µm and 1.6 µm (Table 1). For each particle size, a total of 12 different conditions were analyzed. For each condition, three mice were exposed to the gene gun and the treated skin sites excised for transgenic protein measurements. We found that the 1.6-µm particles coated with DNA at 2.0 µg/ml and used to bombard the skin at 2.1 MPa was the optimal condition for in vivo gene transfer. This set of conditions was used for all subsequent experiments.

Tumorigenicity after gene transfer

The tumorigenicity of both MT-901 (Balb/c) and D5 (C57BL/6) tumors was assessed in their respective syngeneic hosts. Samples comprising 1×10^6 tumor cells were inoculated s.c. and gene gun bombardment with gold particles alone or with GM-CSF-DNA/gold particles was performed on days 5, 7, and 9. Growth of the MT-901 tumors, bombarded with GM-CSF-DNA/gold particles was less than that of untreated tumors in control mice or in those animals exposed to gold particles alone when mean tumor areas were compared on days 7 and 9 ($P < 0.05$, unpaired t-test) (Figure 1). However, once the gene gun treatments were discontinued, the tumors grew in all the animals in a similar fashion whether treated or controls. For the D5 tumors, both the group treated with gold particles and the GM-CSF-treated group showed less tumor growth than the control animals on days 7, 9 and 12 ($P < 0.05$, unpaired t-test) (Fig. 1). After these treatments had been discontinued, the tumors resumed growth rates similar to those of tumors present on non-treated control animals.

Table 1 Granulocyte/macrophage-colony-stimulating factor (GM-CSF) transgenic protein expression by skin after gene gun application. Skin was excised 24 h after gene gun application and GM-CSF production assayed as described in Materials and methods. Results represent mean values (SD) from triplicate skin samples at different gold particle pressures. The maximal GM-CSF expression is in bold type

Gold particle Size (µm)	DNA (µg/ml)	GM-CSF expression (ng/ml) at different pressures			
		1.4 MPa	2.1 MPa	2.8 MPa	3.4 MPa
1.0	1.0	3.3 (2.4)	41.3 (5.8)	20.3 (9.6)	13.8 (3.1)
	2.0	10.3 (9.5)	31.0 (2.4)	17.2 (0.5)	20.3 (3.2)
	3.0	1.5 (0.3)	14.8 (7.5)	15.2 (0.4)	13.4 (3.3)
1.6	1.0	8.5 (0)	28.3 (3.5)	53.7 (20.4)	63.6 (0.1)
	2.0	10.1 (3.1)	**74.7 (8.8)**	29.9 (3.4)	28.6 (6.4)
	3.0	7.0 (0.4)	29.0 (10.0)	17.8 (3.8)	42.7 (20.0)

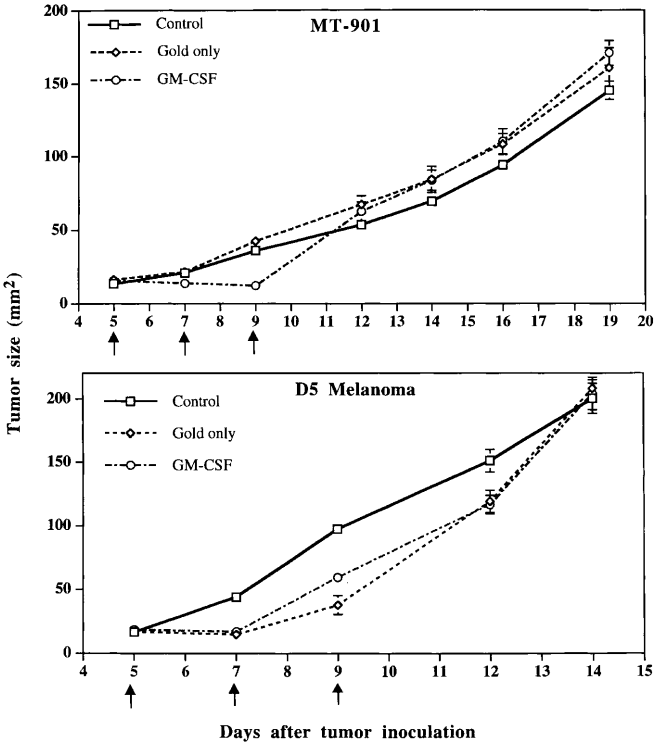


Fig. 1 Tumor growth curves of MT-901 and D5. *Arrows* times when subcutaneous tumors were bombarded with either gold particles or granulocyte/macrophage-colony-stimulating factor (GM-CSF) plasmid coated on gold particles

Histological assessment of tissue

In control skin samples that did not receive any gold particles, the epidermis was intact and only rarely were $CD3^+$ T cells and mononuclear cells found in the underlying dermis (data not shown). There were only occasional scattered $CD11c^+$ dendritic cells (approximately 3–5/high-power field) in the superficial dermis (Fig. 2A).

In skin samples removed from sites receiving gold alone, there was a prominent scale crust with parakeratosis admixed with numerous gold particles. A band of gold particles was also present at the dermo-epidermal interface with increased mononuclear cells in the upper dermis. Occasionally neutrophils and rare $CD3^+$ T cells were also present in the dermis accompanied by clusters of $CD11c^+$ dendritic cells (approximately 8–10/high-power field; Fig. 2B).

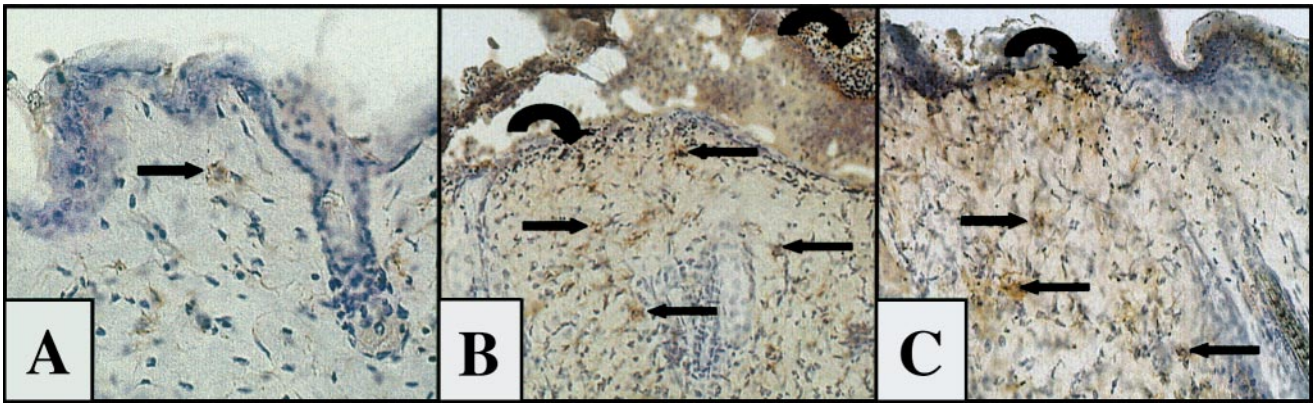
In skin bombarded with GM-CSF-coated gold particles, there was focal scale/crust with gold particles identified throughout the stratum corneum, epidermis and extending into the upper dermis. While $CD3^+$ T cells and neutrophils were rarely present in the dermis, scattered dendritic cells with elongated processes and a stellate appearance were conspicuous across the upper dermis (approximately 10–15/high-power field; Fig. 2C). Qualitatively, we cannot claim that the dendritic cell infiltration of the dermis induced by GM-CSF-DNA/gold particles was greater than that induced by gold particles alone. However, we did observe a greater dermal dendritic cell infiltrate in the GM-CSF-DNA/gold-particle-treated skin than in non-treated control skin.

Immunohistochemical analysis of the tumors beneath the skin sites that had been bombarded with gold particles was performed at the same time. There was no evidence of more dendritic cell infiltration within the tumors from animals treated with gold alone or with GM-CSF-DNA/gold particles than in control animals (data not shown).

Characterization of phenotype and in vitro immunological reactivity of TDLN cells

In one experiment all mice were inoculated with 10^6 D5 tumor cells in the flank. They were then segregated into a group that received gene-gun bombardment with gold particles (gold) or GM-CSF-DNA/gold particles (GM-CSF) to the tumors, a separate group, which received GM-CSF to a non-tumor site over the chest skin, and a

Fig. 2 Color photomicrographs of skin samples. **A** Normal untreated mouse skin stained for $CD11C^+$ dendritic cells when few cells are seen. *Straight arrows* dendritic cells. **B** A skin sample treated with gold particles only. A band of gold particles (*curved arrows*) can be seen at the dermo-epidermal junction accompanied by clusters of dendritic cells. **C** Skin bombarded with GM-CSF-DNA-coated gold particles. There is a greater influx of dendritic cells than in normal untreated mouse skin



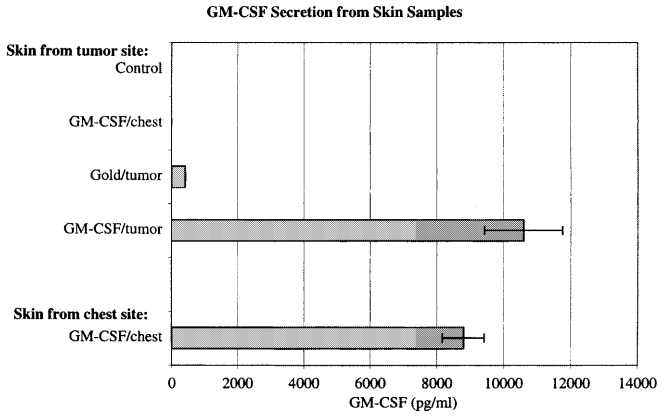


Fig. 3 Transgenic GM-CSF production by skin samples overlying the sites of tumor growth or at a separate site from the chest

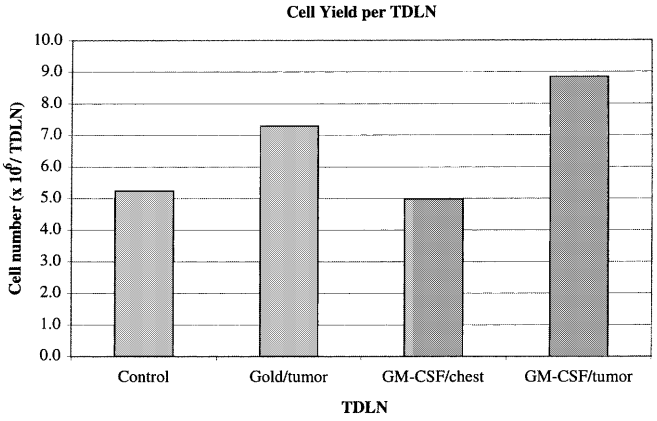


Fig. 4 Cell yield per tumor-draining lymph node (*TDLN*). Groups of animals were inoculated with D5 tumor and treated with the gene gun. TDLN were pooled for each group and the number of cells per TDLN calculated

control group. Bombardment occurred on days 5, 7, and 9. On day 10, samples of skin overlying the tumor, and skin from the chest that had been exposed to GM-CSF, were harvested. Transgenic GM-CSF protein generated by the skin was measured and the results are depicted in Fig. 3. Large amounts of transgenic GM-CSF were produced by skin samples exposed to GM-CSF DNA. The skin samples treated with gold alone had minimal amounts of GM-CSF detectable and were similar to control skin samples. The tumors were also harvested for assessment of GM-CSF production and none was detectable (data not shown).

TDLN was harvested on day 10 and the individual wet weight of each lymph node recorded (six mice per group). The mean weights (SE) of the lymph nodes were 3.5 (0.2) mg, 7.1 (0.6) mg, 7.7 (0.8) mg and 13.4 (1.1) mg for control, GM-CSF/chest, gold/tumor, and GM-CSF/tumor groups, respectively. The mean weight of the lymph nodes from mice receiving GM-CSF-DNA/gold particle treatment to the tumor was significantly greater than that of the other groups ($P < 0.05$, unpaired *t*-test). The lymph nodes were pooled and the number of lymphoid cells per TDLN was calculated; the results are summarized in Fig. 4. The cell yields per TDLN were similar for control animals and animals that received GM-CSF to the chest skin. The cell yield per TDLN of mice that received GM-CSF to the tumor was almost double that of the control group. The group that received the gold to the tumor also had an elevated number of cells per TDLN, but was not comparable to the GM-CSF group.

There were no differences in the percentage of $CD3^+$, $CD4^+$ or $CD8^+$ T cells in the freshly harvested TDLN among the groups (data not shown). Approximately 65%–75% of the TDLN cells were $CD3^+$. The ratio of $CD4^+$:$CD8^+$ cells was 1.3. A previous report has indicated that cells low in L-selectin ($CD62L^{low}$) from TDLN represent a subpopulation of T cells that are highly effective in adoptive immunotherapy [19]. Approximately 10% of the control TDLN cells as well as TDLN cells from GM-CSF-DNA/gold-bombarded mice were $CD62L^{low}$. We carried out double staining for CD11c and CD80 to identify dendritic cells. The percentage of dendritic cells in TDLN harvested from mice exposed to GM-CSF was 8.8%, at least a 12-fold higher than the value for control animals (0.7%) (see Fig. 5). Gold particles administered to the tumor resulted in a modest percentage increase (4.5%) of dendritic cells in the TDLN. Animals given GM-CSF to the chest skin had similar percentages of dendritic cells in TDLN (0.9%) to those of control animals.

Using the MT-901 tumor, we performed a second series of experiments to assess the in vitro antitumor reactivity of TDLN cells after gene gun bombardment with GM-CSF DNA. Gene gun applications were performed as described above. TDLN cells were secondarily activated ex vivo by an anti-CD3/IL-2 protocol and subsequently assessed for cytokine release to MT-901 tumor in an in vitro assay. As illustrated in Fig. 6, release of the cytokines IFNγ, GM-CSF and IL-10 was higher in the group that received GM-CSF at the tumor site than in the other groups.

In vivo immunological reactivity of TDLN cells

The in vivo antitumor reactivity of TDLN cells was assessed in adoptive immunotherapy experiments. Using the different groups of MT-901 TDLN cells described above, animals bearing 3-day established pulmonary metastases were treated with graded doses of effector cells administered i.v. along with low doses of IL-2 given i.p in two separate experiments. As summarized in Table 2, the TDLN cells from the control group, which did not get exposed to the gene gun, mediated no antitumor reactivity at the doses given in experiment 1, but were therapeutically active in experiment 2. This was probably because the mice in the latter experiment had a lower tumor burden, as demonstrated by the untreated control groups in both experiments. TDLN cells from

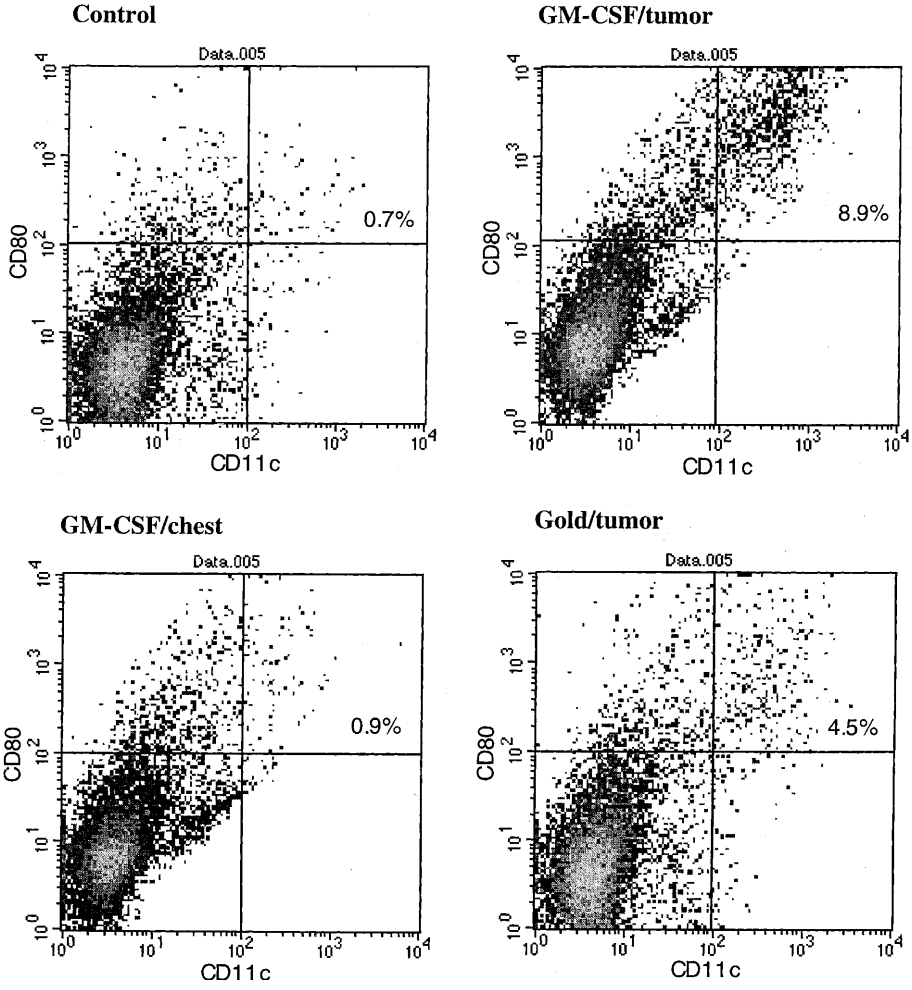


Fig. 5 Two-color fluorescence-activated cell sorting analysis of TDLN cells. Cells were stained for CD80 and CD11c. Dendritic cells were characterized as positive for both markers

the group that had received GM-CSF to the chest did not significantly reduce the number of pulmonary metastases below that found in mice receiving control TDLN cells from animals not treated with DNA bombardment (experiment 1). TDLN from the group that had received gold particles to the tumor caused a significant reduction in pulmonary metastases at the higher cell dose compared to TDLN from the control group that had received no gene gun applications (experiment 1). This non-specific adjuvant effect of gold particles alone was not reproduced in experiment 2, although there were fewer pulmonary metastases in mice treated with gold-particle-treated TDLN cells than in mice receiving control TDLN cells (Table 2). For the group that had received GM-CSF DNA to the tumor, there were significantly fewer pulmonary metastases after treatment with TDLN cells than after treatment with TDLN cells obtained from control mice or from mice treated with gold particles alone in both experiments.

In a separate experiment utilizing the D5 melanoma tumor (Table 3), the in vivo antitumor reactivity of TDLN primed with the gene gun was similarly assessed. D5 TDLN cells were harvested after the different groups had received gold particles or GM-CSF to the tumors. After anti-CD3/IL-2 activation, the cells were adoptively transferred i.v. to mice bearing 3-day established D5 pulmonary metastases and IL-2 was concomitantly administered i.p. As summarized in Table 3, there was a modest, but statistically significant, reduction in pulmonary metastases in the group that received the larger dose of TDLN cells primed by gold particles. By contrast, TDLN cells primed with GM-CSF mediated a significant reduction of pulmonary metastases, which was proportional to the dose of cells transferred.

Discussion

These results expand our previous observations that tumor-draining lymph node cells can be useful in the treatment of established metastatic disease. Furthermore, we and other investigators have demonstrated that the local elaboration of GM-CSF at the site of tumor growth can enhance the induction of tumor-reactive lymphoid cells within TDLN [21]. The unique aspect of this study is the method of establishing local GM-CSF delivery via gene-gun technology.

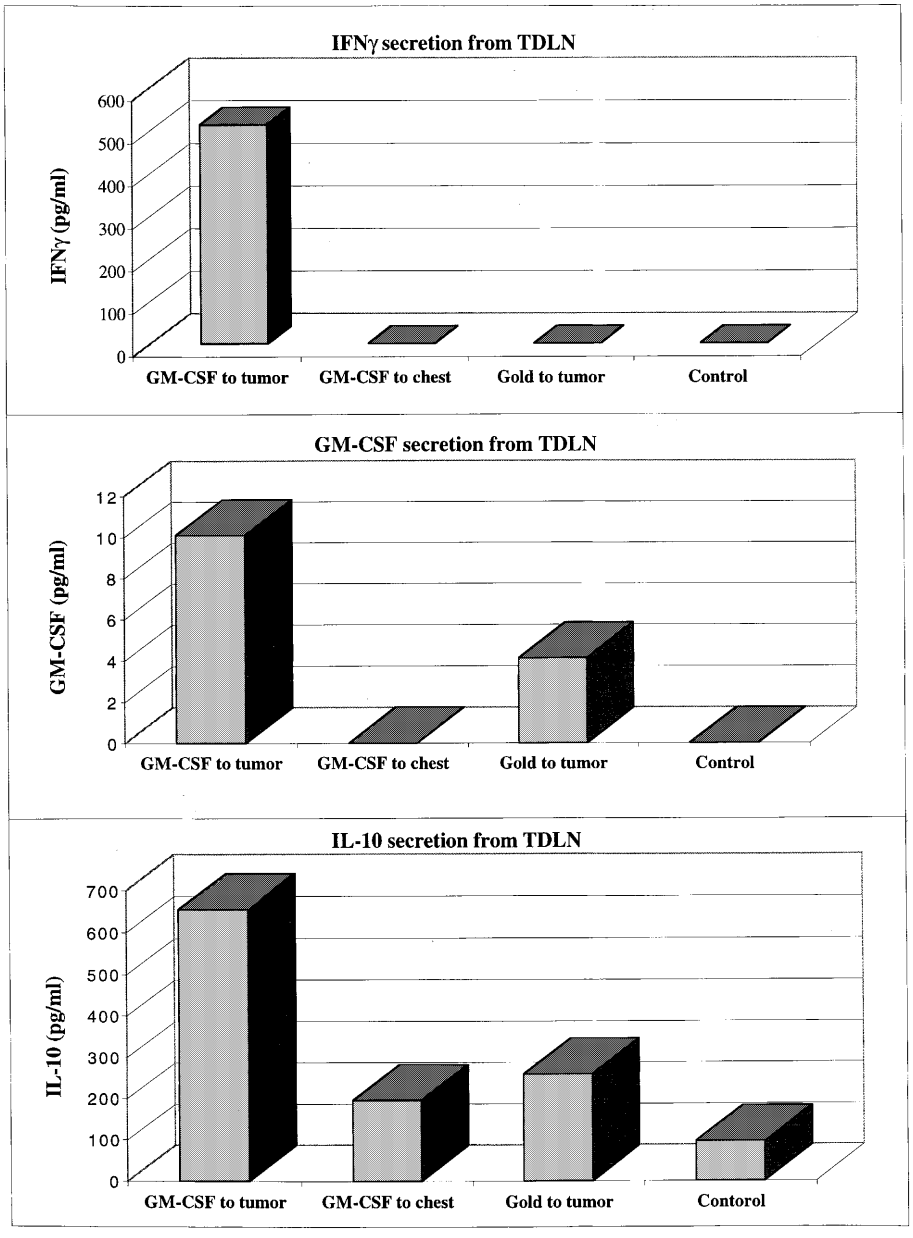


Fig. 6 Cytokine release of interferon γ (*IFNγ*), GM-CSF and interleukin-10 (*IL-10*) by anti-CD3/IL-2-activated TDLN cells in response to irradiated tumor cells. Background cytokine secretion by TDLN cells or tumor cells alone has been subtracted

In prior reports, local elaboration of cytokines by gene transfer has been accomplished via transduction of tumor cells [11–13] or the admixture of tumor cells with fibroblasts that had been previously transduced to secrete cytokines [5, 28, 29]. These approaches required the ex vivo transduction of cultured cells which was labor-intensive and time-consuming. The benefits of utilizing the gene gun for in vivo transfection of DNA is its relative ease of use without the need to culture cells, avoidance of viral vectors, and high transgene expression [26, 32].

We utilized two different animal models: the D5 melanoma and the MT-901 mammary carcinoma. The D5 tumor is poorly immunogenic [2], in contrast to the MT-901 tumor which is weakly immunogenic [7]. The elaboration of GM-CSF by the skin overlying the tumor was capable of up-regulating antitumor reactivity in both models. The D5 tumor does not express class I or II MHC molecules, [30], whereas the MT-901 expresses only MHC class I molecules [7]. We have previously reported that these tumors can induce $CD4^+$ tumor immune T cells when GM-CSF is elaborated locally at the site of a growing tumor [3, 7]. $CD4^+$ T cell depletion after adoptive immunotherapy will abrogate the tumor rejection response in these models. Because of the absence of MHC class II on both tumors, it is apparent that they cannot act as antigen-presenting cells (APC), and therefore must rely on professional APC to sensitize T cells. We found evidence that the application of the gene gun with GM-CSF plasmid resulted in more dendritic cell accumulation within TDLN than in control TDLN, and suggest that these

Table 2 Adoptive immunotherapy of MT-901 pulmonary metastases by tumor-draining lymph node (TDLN) cells primed with the gene gun. TDLN cells were generated in mice inoculated with 10^6 MT-901 tumor cells s.c. in the flank. Gene gun applications on days 5, 7, and 9 were to the tumor site or to a normal separate skin site overlying the chest. TDLN cells were harvested on day 10 for ex vivo activation and transferred i.v. to mice with established 3-day MT-901 lung metastases. Interleukin-2 (*IL-2*; 60 000 IU) was administered i.p. twice daily for eight doses starting after cell transfer. *ND* not done

Therapy			Mean no. of lung metastases (SEM)	
Plasmid	IL-2	No. cells	Expt. 1	Expt. 2
A. None	–	–	248 (1)	127 (25)
B. None	+	–	195 (40)	145 (28)
C. None	+	1×10^7	186 (54)	72 (17)
D. None	+	4×10^7	129 (31)	24 (4)*[5]
E. GM-CSF/chest	+	1×10^7	160 (42)	ND
F. GM-CSF/chest	+	4×10^7	98 (29)*[1]	ND
G. Gold/tumor	+	1×10^7	160 (32)	46 (11)*[6]
H. Gold/tumor	+	4×10^7	25 (6)*[2]	13 (4)*[7]
I. GM-CSF/tumor	+	1×10^7	43 (18)*[3]	11 (2)*[8]
J. GM-CSF/tumor	+	4×10^7	23 (6)*[4]	2 (1)*[9]

*[1] $P < 0.05$ versus A; *[2] $P < 0.05$ versus A–G; *[3] $P < 0.05$ versus A–G; *[4] $P < 0.05$ versus A–G and I; *[5] $P < 0.05$ versus A and B; *[6] $P < 0.05$ versus A–C; *[7] $P < 0.05$ versus A–C and G; *[8] $P < 0.05$ versus A–D and G; *[9] $P < 0.05$ versus A–D, G, H and I

cells play an important role in sensitizing T cells to tumor antigen.

Of interest was the observation that gold particles without plasmid also had a modest effect on increasing dendritic cells in TDLN and was associated with significantly increased ability of TDLN cells to mediate tumor regression in two of three experiments. We hypothesize that this immune adjuvant effect is related to both the local and regional accumulation of dendritic cells observed in the skin samples and lymph nodes from animals treated by gold particles. In tumor-bearing hosts, approaches that have significantly increased dendritic cells in vivo have led to antitumor responses. One example is the use of F1t3 ligand (F1t3L), which has been reported to increase the number of dendritic cells in vivo and induce regression of established tumor [23]. The intratumoral inoculation of normal dendritic cells in established murine tumors has also been associated with tumor regression in animal models (Dr. James Mulé, University of Michigan, personal communication).

It was apparent that the effect of gene transfer on TDLN sensitization required local gene gun application at the site of tumor growth. Skin transfection of GM-CSF plasmid was ineffective at a distant site. This corroborates our previous findings that the systemic administration of soluble GM-CSF did not result in enhanced TDLN sensitization, whereas the local administration of cytokine protein will do so [21]. Furthermore, transgene expression by tumor cells was not required in our model. The advantages of gene transfer with the gene gun as opposed to local administration of soluble protein is the ability to achieve a steady-state level of the cytokine in the tumor microenvironment. In addition, injections of soluble protein may be avoided with gene-transfer approaches, although we did not examine this issue in our studies. An alternative approach in which cytokines were delivered locally over a prolonged period by biodegradable microspheres has been reported [17]. However, the gene-gun technology offers not only the ability to generate cytokines locally, but also permits transfection of dendritic cells with DNA encoding antigen at the local site [25].

Table 3 Adoptive immunotherapy of D5 pulmonary metastases by TDLN cells primed with the gene gun. TDLN cells were generated in mice inoculated with 10^6 D5 melanoma cells and received gene gun applications on days 5, 7, and 9. TDLN cells were harvested on day 10 for ex vivo activation and transferred into mice with established 3-day D5 lung metastases. IL-2 (60 000 IU) was administered i.p. twice daily for eight doses starting after cell transfer

Therapy			Mean no. of pulmonary metastases (SEM)
Plasmid	IL-2	No. cells	
A. None	–	–	>250
B. None	+	–	>250
C. None	+	1×10^7	>250
D. None	+	4×10^7	190 (29)
E. Gold only	+	1×10^7	228 (22)
F. Gold only	+	4×10^7	179 (30)*[1]
G. GM-CSF	+	1×10^7	157 (26)*[1]
H. GM-CSF	+	4×10^7	26 (5)*[2]

*[1] $P < 0.05$ versus A–C; *[2] $P < 0.05$ versus A–G

We are currently exploring the efficacy of autologous tumor cells retrovirally transduced to secrete GM-CSF as a method to stimulate vaccine-primed lymph node cells [9]. One of the advantages of the gene gun is its ability to transfect freshly harvested tumor cells without the need of establishing proliferating tumor cells in vitro for retroviral transduction [24]. Furthermore, combinations of genes encoding immunostimulatory molecules can be used with either additive or synergistic interactions that the gene gun technology would easily be able to accommodate [6]. We plan to utilize this technology in future clinical studies.

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