ORIGINAL

Gene gun-mediated skin transfection with FL gene suppresses the growth of murine fibrosarcoma

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Abstract : Aim : Particle-mediated transfection is known as an efficient method of non-viral gene transfer. Flt3 ligand (FL) is a growth factor for hematopoietic progenitors ; it promotes the growth of dendritic cells (DC). DCs are powerful antigen-presenting cells (APCs) and show a remarkable capacity to stimulate antigen-specific T-cell responses. In this study, we intended to investigate the suppressive effect on tumor growth by gene gun-mediated transfer of FL in a murine model. Methods : C57BL/6J mice were injected intradermally with MCA205 cells. DNA (pNGVL-hFLex)-coated gold particles were delivered to the mouse skin surrounding the target tumor. The expression of FL was determined by RT-PCR. Analyses by immunohistochemistry and fluorescence-activated cell sorter (FACS) revealed an increase in the number of DC after treatment with FL. Results : Gene gun-mediated pNGVL-hFLex transfer significantly inhibited the growth of the MCA205 tumor. FL transfer markedly increased the number of $CD11c^+$ DCs in the tumor tissue. Further, the FL-transfected mice exhibited a significantly higher number of $CD80^+$ MHC-II cells. Conclusion : We successfully performed FL therapy using an *in vivo* gene gun in order to effectively mobilize DCs in situ and induce suppressive immunity. J. Med. Invest. 58 : 39-45, February, 2011

Keywords **:** *fms-like tyrosine kinase 3 ligand, gene gun, dendritic cells*

INTRODUCTION

Flt3 is a member of the type III receptor tyrosine kinase family ; murine and human analogs share 86% structural homology. Fms-like tyrosine kinase 3 ligand (FL), a hematopoietic growth factor, is a specific ligand for Flt3. FL is widely expressed in both murine and human tissues. Murine and human FL are 75% homologous, and both ligands are fully active in cells bearing either murine or human receptors (1). FL plays an important role in the proliferation, survival, and differentiation of early murine and human hematopoietic precursor stem cells.

The majority of common lymphoid precursors express high levels of Flt3 and are highly efficient precursors of dendritic cells (DCs). FL is a growth factor for hematopoietic progenitors, which also promotes the growth of DCs. Administration of FL to mice results in increased numbers of functionally active DCs in the bone marrow, gastrointestinal lymphoid tissue, liver, lymph nodes, lung, peripheral blood, peritoneal cavity, spleen, and thymus (1). Further, administration of FL to healthy human volunteers is known to markedly elevate the number of circulating DCs (2). Following FL administration, an increase in the number of both myeloid and

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lymphoid type DCs in mice and humans has been noted ; moreover, impressive antitumor effects have been induced in several murine models (3-6). It was shown that administration of either tumor cells transfected in vitro with Flt3L vectors or soluble Flt3L fusion protein in a high dose can enhance host antitumor immunity (7). DCs are powerful antigen-presenting cells (APCs) that show a remarkable capacity to stimulate antigen-specific T-cell responses. *In vitro* and *in vivo* antigen-pulsed DC can directly sensitize T-cells and stimulate the development of both protective and therapeutic antitumor immune responses. Several clinical trials have been initiated to evaluate the efficacy of DC-based immunotherapies in cancer (1).

FL has distinct effects on the kinetics of reconstitution of DCs and natural killer (NK) cells with potential implications for the modulation of immune responses (4). NK cells play an important role in the activity of FL-NK depletion results in antitumor activity of FL. Hung *et al.* (5) reported that intradermal delivery (gene gun) of a recombinant chimera of FL in tumor-free animals, linked to a model of antigen increased the frequency of antigen-specific $CD8^+$ T cells. Lynch *et al.* (6) reported that FL-induced fibrosarcoma regression appeared to be specifically mediated, by $CD8^+$ cells.

Previous studies have shown that particle-mediated transfection is an efficient method of non-viral gene transfer. It has been shown that the gene gun can provide a high level of gene expression in a wide variety of cell types, tissues, and mammalian species (8). The particle-mediated method for gene delivery using a gene gun utilizes a shock wave to accelerate DNA-coated gold particles into target cells or tissues. Rakhmilevich *et al*. showed that IL12 cDNA transfer into the tumor by using a gene gun could achieve IL12 expression in the skin surrounding the tumor, causing regression of the established tumor (9). However the effects on tumor growth by the particle-mediated transfer of FL was not reported. In this study, we studied the suppressive effect of gene gun-mediated transfer of FL in a murine model.

MATERIALS AND METHODS

This study was performed in accordance with the guidelines for animal experimentation of Tokushima University.

Mice

Female 8-week-old C57BL/6 mice were purchased from Charles River Laboratories. The animals were housed in a pathogen-free animal facility, 5 mice per cage, under controlled conditions of temperature and humidity.

Tumor cell lines

MCA205 methylcholanthrene-induced fibrosarcoma cells were kindly provided by the University of Pittsburg. They were maintained in an RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate.

Tumor models

Mice were injected intradermally on day 1 with 1×10^5 MCA205 cells in 100 μL saline into a shaved abdominal area. The induced tumors were measured two times a week with calipers. The tumor size represents the product of two perpendicular diameters. For *in vivo* experiments, 5 mice per group were routinely used. We used MCA-induced fibrosarcoma according to the previous paper (6).

Preparation of plasmid DNA

The plasmid pNGVL-hFLex contains the gene for a secreted form of human FLT3 ligand. We used pNGVL as a control vector. The plasmid pNGVL-hFLex was kindly provided by the National Gene Vector Laboratory of the University of Michigan (NGVL-UM). The plasmids were amplified in *Escherichia coli* and purified using the EndoFree Maxi Kit (Qiagen, Japan) according to the manufacturer's instructions.

Gene gun-mediated DNA transfer

Gene gun DNA transfer was performed with a hand-held, helium-driven Helios gene delivery system (Bio-Rad Lab). Plasmid DNA was precipitated onto gold particles with a 1.6-μm average diameter. Overall, 100 μL of the plasmid DNA was coated on 50 mg of gold particles. The DNA-coated gold particles (1 μg DNA/bullet) were delivered to the mouse skin overlying and surrounding the target tumor with a discharge pressure of 300-400 psi on days 4, 5, 7, 8, 10, 11, 13, 14, 16, and 17 after tumor implantation (Figure 1). Gene therapy was performed ten times on the basis of the previous report (1).

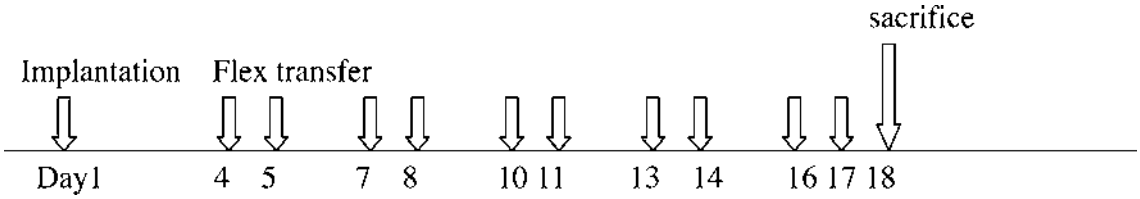

Figure 1. Experimental plan for FL-transfer and tumor challenge.
Experimental setting. Mice were injected i.d. with 1×10^5 cells of MCA205. The DNA-coated gold particles (1 μg DNA/bullet) were delivered to the mouse skin overlying and surrounding the target tumor with a discharge pressure of 300-400 psi on days 4, 5, 7, 8, 10, 11, 13, 14, 16 and 17 after tumor implantation.

Expression of FL mRNA in the tumor by RT-PCR

On day 18 after tumor implantation, mice were sacrificed and the total mRNA was extracted from the tumor with the EASYPrep RNA kit (TAKARA, Japan). The first-strand cDNA synthesis was performed with SuperScript™ III First-Strand Synthesis System (Invitrogen, Japan). cDNA was PCR-amplified using TAKARA Ex Taq (TAKARA) and previously published primers (sense, 5'-ACA ACC TAT CTC CTC CTG CTG-3' ; antisense, 5'-GGC ACA TTT GGT GAC AAA GTG-3'). The housekeeping gene GAPDH (sense, 5'-CAG GTT GTC TCC TGC GAC TT-3' ; antisense, 5'-CTT GCT CAG TGT CCT TGC TG-3') was used as a control. PCR amplification consisted of an initial denaturation step at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min.

Immunohistochemistry

The freshly removed tumor specimens were placed in an embedding medium (Tissue Tek OTC compound, SAKURA), snap-frozen in liquid nitrogen, and stored at -80°C. For the staining of cell differentiation markers, 5-μm thick frozen tissue sections were cut in a cryostat, air-dried, and fixed in acetone for 10 min. The following primary mAbs were used : rat anti-mouse MHC Class II, CD4, CD8a (eBioscience, USA), CD3 (BD Bioscience-Pharmingen, USA), and hamster anti-mouse CD11c (eBioscience). Each primary Ab was applied for 30 min at room temperature. After two washes in PBS, the sections were incubated with the biotinylated secondary rabbit anti-rat IgG Ab or goat anti-hamster IgG Ab for 30 min, and then developed with the VectastainElite ABC kits and Vector DAB substrate (Vector Laboratories, USA). After substrate development, the sections were counterstained with hematoxylin. We counted the number of $CD11c^+$, $CD4^+$, $CD8^+$, and $CD3^+$ cells in 10 high power fields.

fluorescence-activated cell sorter (FACS)

Tumor cells from control mice or from mice treated with FL therapy were obtained on day 18 after tumor implantation. The tumor was harvested and single cell suspensions were prepared by mechanical dissociation and collagenase treatment. The cells were stained with FITC-conjugated rat-antimouse CD80 monoclonal antibody and PE-conjugated rat-antimouse IA-b monoclonal antibody for (BD Bioscience-Pharmingen) for 60 min at 4°C. Propidium iodide (2 μg/mL) was added to stain dead cells, which were subsequently excluded from analysis. Analysis was performed on a Becton Dickinson FACScan with CELLQuest software (Becton Dicknson Immunocytometry System, Mountain View, CA). Data were collected for 10,000 events/sample.

Statistical analysis

All data expressed as mean± S.D. are representative of at least two different experiments. Statistical analysis was performed using the unpaired two-tailed Student's test. The statistical analysis regarding FACS was performed by chi-square test.

RESULTS

A therapeutic effect mediated by gene gun

To study the anti-tumor effect of gene gun-mediated pNGVL-hFLex transfer in the treatment model, we treated a total of 10 mice in dependent experiments. As shown in Figure 2, gene-gun mediated transfer of pNGVL-hFLex significantly inhibited the growth of MCA205 tumor cells (P=0.011).

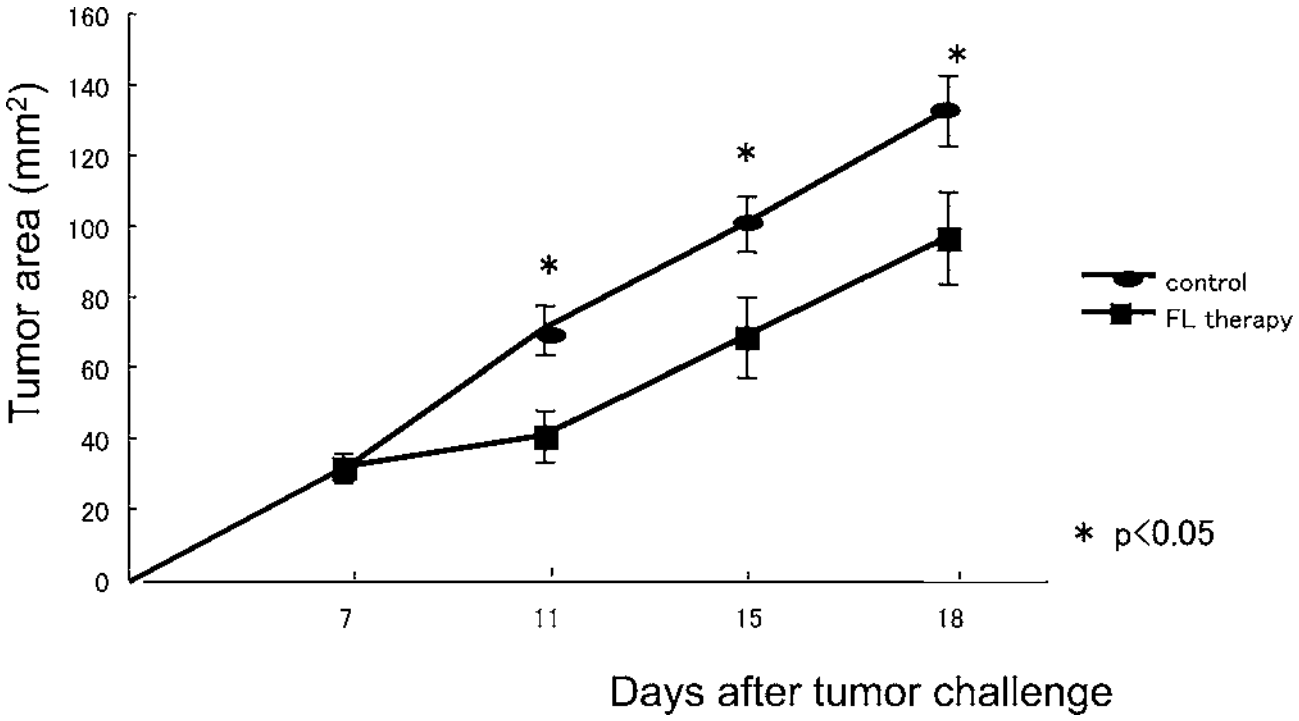

Figure 2. Administration of FL inhibited the tumor growth in MCA205.
Mice were injected i.d. with 1×10^5 cells of MCA205. The DNA-coated gold particles(1 μg DNA/bullet) were delivered to the mouse skin overlying and surrounding the target tumor with a discharge pressure of 300-400 psi on days 4, 5, 7, 8, 10, 11, 13, 14, 16, and 17 after tumor implantation. For each tumor, 2 independent groups of mice were used in the experiment (total n=10 mice). One group was boosted with a plasmid including the FL gene (■). The other group was boosted with a control vector (●). Mean tumor areas for the FL therapy group and the control group are shown. Scale bar=SD. The difference between these 2 curves was statistically significant (P=0.011).

FL expression in the tumor

To examine FL expression, mRNA extracted from the tumor was subjected to RT-PCR analysis. As shown in Figure 3, FL mRNA was expressed in the pNGVL-hFLex-transfected tumor but not in the control vector-transfected tumor.

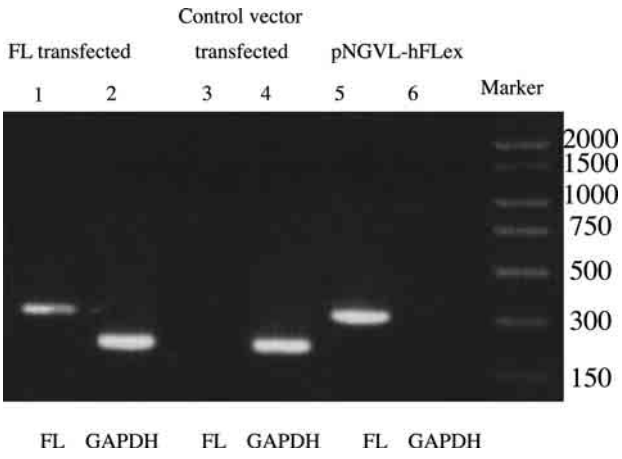

Figure 3. RT-PCR analysis of FL mRNA expression in the tumor after gene transfer.
Lanes 1, 2 : Tumors transfected with FL
Lanes 3, 4 : Tumors transfected with control vector
Lanes 5, 6 : plasmid pNGVL-hFLex alone

To clarify the immune reaction at the tumor site by FL transfer with gene gun, we determined the number of tumor-infiltrating cells by immunohistochemical analysis. Tumors from both control and treated mice were analyzed 18 d after implantation using immunohistochemical techniques. FL transfer increased the number of $CD11c^+$ DCs in the tumor tissues (94/HPF vs 8/HPF). As shown in Figure 4, we also noted a increase in both $CD4^+$, $CD8^+$, and $CD3^+$ cells in the tumors obtained from FL-treated animals as compared with those from the controls (86/HPF vs 26/HPF, 97/HPF vs 12/HPF, 84/HPF vs 32/HPF).

To investigate the impact of FL on DCs *in vivo*, we examined the number of DCs in the tumor using flow cytometry. As shown in Figure 5, FL-transfected mice exhibited significantly higher numbers of $CD80^+$ MHC II cells compared to mice transfected with the control vector. FL therapy markedly increased the percentage of $I\text{-}A^b{+}CD80^+$ cells were significantly increased (3.6% vs. 11.5% : $p<0.05$) in the tumor. $CD80^+$ cells represent a typical phenotypic marker of DC maturation. It showed that mature DC located in the tumor.

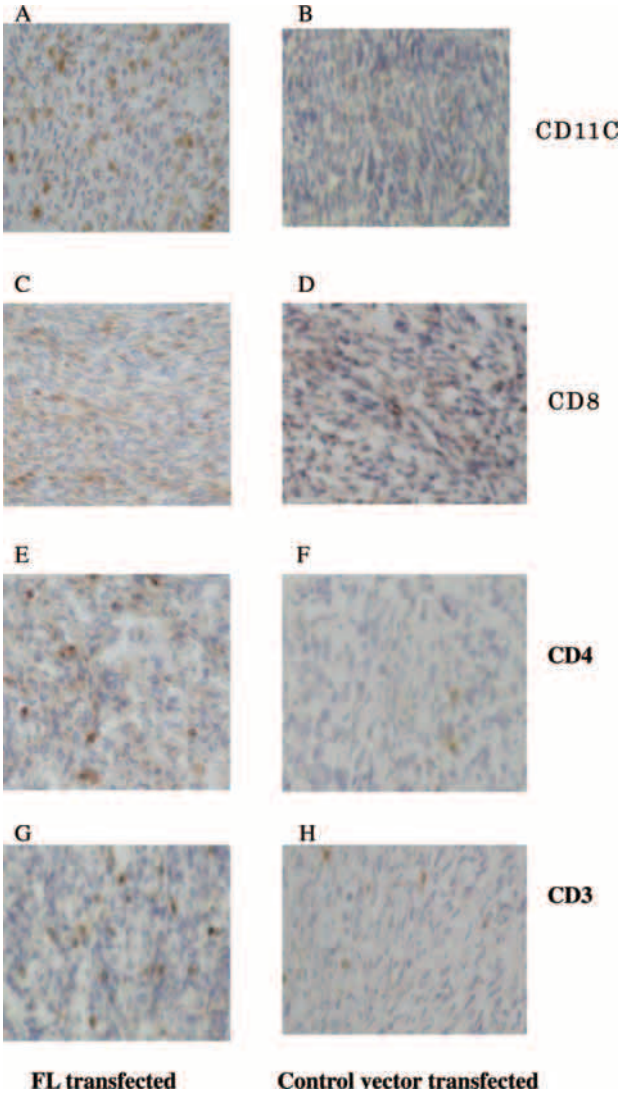

Figure 4. Immunohistochemical staining of CD11c$^+$ CD8, CD4, and CD3 cells in the local tumor site on day 18 after tumor implantation. (FL therapy : A, C, E, and G) (control : B, D, F, and H).

DISCUSSION

Gene gun therapy is one of several non-viral transfection methods that achieve good, but transient, expression of transfected genes (10). A previous paper has described that despite low levels of transgenic IL-12 administered via the gene gun, complete regression of established tumors was achieved in mice. Transfer of IL-12 cDNA into epidermal cells overlying an implanted intradermal tumor resulted in detectable levels (266.0± 27.8 pg) of the transgenic protein at the skin tissue treatment site (9).

The interaction between plasmid DNA and DCs *in vivo* is incompletely understood. Stoecklinger A *et al* described that gene gun immunization is capable of inducing immune reactions independently of epidermal Langerhans cells (10). And gene gun therapy induced skin DC maturation and migration of transfected DC to draining lymph nodes. A small amount of antigen could be traced to the draining lymph nodes in mice immunized with the gene gun.

Gaffal *et al* showed that CD8$^+$ cells stimulated by the gene gun differ phenotypically and functionally from those stimulated by live virus (11). They showed that gene gun stimulated a predominantly regional CD8$^+$ Tcell response and is less effective than intracutaneous injection of virus-transduced DC.

In this study, administration of FL by a gene gun showed inhibition of tumor growth in a murine model of intradermal tumors. We demonstrated an increase in the number of DCs within tumor with

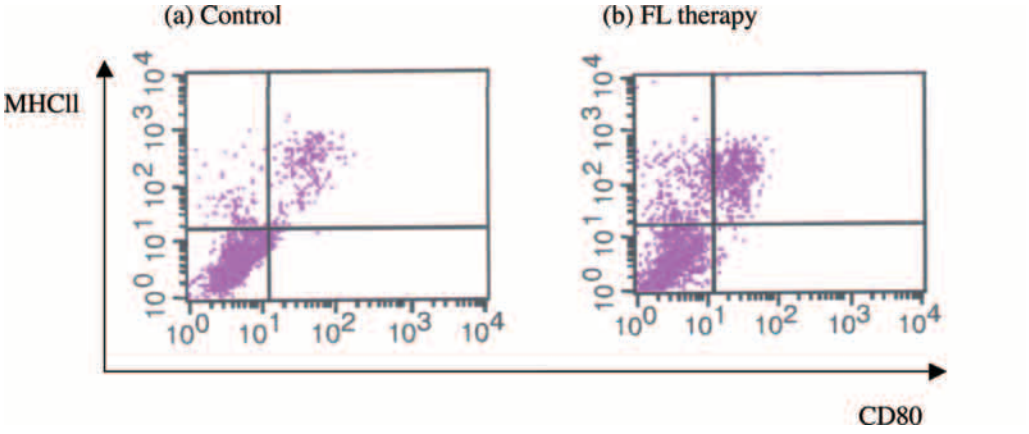

Figure 5. Flow cytometry for CD80 and I-A^b expression in local tumor.
DC expansions induced by Flt-3L (FACS analysis) ; tumors of mice treated with control vectors (a) or pNGVL-FL(b) were analyzed by flow cytometry for CD80 and I-A^b expression. Negative controls included staining with the corresponding isotype for each Ab and staining with the secondary Ab alone. Positive controls included immunostaining of known positive tissues. All sections were lightly counterstained with hematoxylin.

an increased expression of CD80. After gene-therapy, not only the number of $CD11c^+$ DCs but also those of $CD4^+$, $CD8^+$, and $CD3^+$ cells increased. The recruitment of cytotoxic and helper T lymphocytes in the tumor may play a role in the suppressive effects of FL transfer. Taken together, our data suggest that treatment of tumor-bearing mice by intratumoral administration of pNGVL-hFLex gene using the gene gun results in significant inhibition of tumor growth *in vivo*. Overall, treatment with DCs transduced with FL showed an result in a decrease in the MCA205 tumor size. In the present study, we found that tumor growth was inhibited by FL treatment but the suppressive effect was not complete. Hou *et al* showed that administration of soluble FL protein and local injection of adenovirus with insert of gene encoding extracellular domain of mouse Flt3L was effective in eliciting host immune responses, which is able to cure tumors with a small size (12).

The *in vivo* delivery of the FL transgene has been suggested as an alternative to the injection of the FL protein. Shimano *et al*. reported that FL gene transfer using IVE (*in vivo* electroporation) could mobilize and promote the maturation of DCs *in vivo* (13). It has been already reported that administration of FL protein can induce an increase in both lymphoid-related DCs induced to enhance Th1-like responses and myeloid-related DCs that enhance mixed Th1-type responses in murine models (3). The cytokine for the development of the CD8+ DC subset is FL which has major influences on the development of inflammatory and migratory DCs (14). It was shown that administration of soluble FL protein and local injection of Ad-mFL was effective in eliciting host immune response, which is able to cure tumors with a small size (7). Leading to a high production of mFlt3L proteins in association with accumulation of DCs, NK cells and lymphocytes in local tumor regions. In association with the immune responses, DCs and NK cells recruitment to the tumor occurred, which may facilitate tumor necrosis and enhance tumor antigen presentation.

We showed here that FL therapy increased the number of DCs and T cells in the tumor site and delayed the speed of tumor growth. It did not completely eliminate the tumor. Our data suggest that DNA vaccination via the gene gun represents a potent regimen for DNA administration. We suggest that this method may become useful in immunotherapy using DCs. In this study, we performed FL therapy using an *in vivo* gene gun in order to effectively mobilize DCs *in situ*. We considered that the use of other cytokines and/or immunomodulatory molecules together with FL might offer attractive perspectives for immunotherapy.

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