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# Co-immunization with M-CSFR and mM-CSF DNA vaccines is better than M-CSFR-mM-CSF fusion DNA vaccine

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*Background and Objectives*. DNA vaccine against macrophage colony-stimulating factor receptor (M-CSFR) has shown both protective and therapeutic effects. In this study, we explore the possibility of using DNA vaccines against both M-CSFR and membrane-bound macrophage colony-stimulating factor (mM-CSF) to achieve better effects.

*Design and Methods*. Three plasmids were constructed by inserting either extracellular and transmembrane region of mM-CSF (pM), or extracellular region of M-CSFR (pR), or extracellular region of M-CSFR linked with extracellular and transmembrane regions of mM-CSF by a $(Gly\ Gly\ Ser)_2$ flexible linker (pF), into pcDNA3.1. A SP2/0 cell line stably expressing pF (SP2/0-F) was established to evaluate humoral and cytotoxic immune responses as well as therapeutic and preventive effects induced by pM, pR, pF or pM+pR vaccination in BALB/c mice. The mechanisms of these vaccinations were also studied by monitoring the release of interleukin (IL)-4 and interferon (IFN)-$\gamma$ by splenocytes upon activation.

*Results*. Vaccination against two epitopes had better effects than against a single epitope while vaccination by pM+pR had the greatest effects on inducing humoral and cytotoxic immune responses, prolonging survival of mice challenged with SP2/0-F, and inducing IL-4 and IFN-$\gamma$ release by splenocytes.

*Interpretation and Conclusions*. Our results suggest that co-immunization of M-CSFR and mM-CSF DNA vaccines is better than M-CSFR-mM-CSF fusion DNA vaccine.


Key words: membrane-bound macrophage colony-stimulating factor, macrophage colony-stimulating factor receptor, DNA vaccine, co-immunization, fusion DNA vaccine.

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DNA immunization is a novel vaccine technology, which was established in 1990s.[1] Its strategy is that a DNA plasmid encoding a desired protein is inoculated into an animal, in which its polypeptide antigen is synthesized. Once the plasmid-antigen has been processed and presented to the immune system, cellular and humoral immune responses against the antigen are elicited. DNA immunization exhibits advantages over traditional vaccines and accumulating evidence has been published on its ability to induce protective and therapeutic immune effects against infectious diseases and tumors.[2] Naked DNA vectors are relatively easy to engineer and store. So far, it is encouraging that several tumor DNA vaccines have been approved by the FDA for phase I/II clinical trials, including gp100 and TRP-2 DNA vaccines for melanoma,[3,4] HPV E6 and E7 DNA vaccines for cervical carcinoma,[5,6] and MUC1 DNA vaccine for breast cancer.[7]

Macrophage colony-stimulating factor (M-CSF) is an important member in the cytokine-regulating network. Due to alternative splicing, M-CSF can exist in at least three isoforms: soluble (s-M-CSF), membrane-bound (mM-CSF) and extracellular matrix or proteoglycan (PG-M-CSF).[8,9] M-CSF receptor (M-CSFR), the product of *c-fms* proto-oncogene, belongs to the receptor tyrosine kinase family. It has been established that the aberrant expression and/or mutation of mM-CSF and M-CSFR is implicated in the pathogenesis of several solid tumors as well as hematopoietic malignancies.[10-13] Furthermore, co-expression of mM-CSF and M-CSFR by tumor cells implies a more malignant phenotype and poor prognosis.[14] We isolated a membrane-associated factor from the human leukemic cell line J6-1 (formerly designated as MAF-J6-1), which stimulated the growth of J6-1 cells through an auto-juxtacrine mechanism by binding to its receptor (MAF-J6-1R).[15-17] Screening for the expressions of these molecules showed that high level co-expression of these molecules could be found in Hodgkin's disease, leukemias as well as solid tumors such as hepatoma, breast cancer, etc. but not in normal tissues.[18-21] Further work demon-

strated that they were mutant mM-CSF and M-CSFR. Sequence analysis revealed that mutant mM-CSF and M-CSFR from J6-1 cells have 4 and 1 missense mutations, respectively.[22] Hence, they might be potential tumor-associated antigens (TAA) for tumor immunotherapy against certain tumor cells expressing them.

Previously, we constructed an M-CSFR DNA vaccine, which could induce humoral and cellular immunity against M-CSFR bearing SP2/0 cells in a mice model, and markedly prolong the survival of mice challenged with M-CSFR$^+$ tumor.[23] It is well established that vaccines against multi-epitopes have higher specificities and can cause stronger immune responses. In the present study, we explored the possibility of involving both mM-CSF and M-CSFR as targets to construct DNA vaccines. Furthermore, we compared the effects of different immunization procedures involving two epitopes, either co-immunized with two DNA vaccines, each against a single epitope, or immunized with a fusion DNA vaccine against dual epitopes.

## Design and Methods

### *Cell lines, animals and reagents*

Monkey kidney cell line COS-7 and mouse myeloma cell line SP2/0 were maintained in RPMI-1640 (pH 7.2) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C and 5% $CO_2$. Six to 8-week old, specific pathogen-free, female BALB/c (H-2$^d$) mice were bred in the Institute of Hematology, CAMS & PUMC under conventional conditions.

M-MLV reverse transcriptase, lipofect AMINE™, OPTI-MEM medium, pcDNA3.1 and nitrocellulose membrane were purchased from Invitrogen Corporation (CA, USA). Restriction endonucleotidase (Kpn I, Xho I and Not I) were purchased from Takara Co. (Dalian, China). Biotinylated sheep anti-mouse IgG antibody and avidin-peroxidase complex were obtained from the Vector Co. (UK). Mouse anti-M-CSF MAb (B5) and anti-M-CSFR MAb (RE2) were prepared and purified in our laboratory. Endotoxin-free purification kits were purchased from Qiagen Co. (Germany). $^{51}Cr$ was purchased from Perkin Elmer Co. (MA, USA). The mouse IFN-$\gamma$ ELISA kits and mouse IL-4 ELISA kits were products of Diaclone Co. (France) and Jingmei Biotech Co. Ltd. (China), respectively.

### *Construction of plasmids*

The primers used in plasmid construction were synthesized from Sangon Co.(Shanghai, China) and are listed below:

P1 5'AAG GTA CCC CAT GGG CCC AGG AGT (Kpn I);

P2 5'CCG CTC GAG CTC AGA GCT CAA GTT CAA GTA GG (Xho I)

P3 5'GAG GAG GTG TCG;

P4 5'ATA GTT TAG CGG CCG CCT ACA CTG GCA GTT CCA C(Not I);

P5 5' CTC AGA GCT CAA GTT CAA GTA GG;

P6: 5'CGA CAC CTC CTC (CGA TCC TCC)$_2$ CTC AGA GCT CAA GTT CAA GTA GG.

The plasmid pM, encoding signal peptide, extracellular and transmembrane of mM-CSF, has been previously constructed.[22] The plasmid pR was constructed by inserting the fragment encoding the signal peptide and extracellular domain of M-CSFR, which was amplified from pCSFR using P1 and P2 into pcDNA3.1. The M-CSF-R-mM-CSF fusion DNA fragment was constructed by three-round polymerase chain reactions (PCRs).

Briefly, two fragments, either encoding signal peptide and extracellular domain of M-CSF-R or encoding extracellular transmembrane domain but no signal peptide of mM-CSF, were amplified from pM and pR using P1 and P5 or P3 and P4, respectively. Then a second PCR was operated by P1 and P6 using the first-round PCR products as templates. The fusion fragment was generated in the third-round PCR by P1 and P4 using the second PCR product as template. Finally the PCR product was digested and inserted into pcDNA3.1. *E. coli* DH5$\alpha$ were transformed by pR or pF and positive clones were screened by PCR and restriction endonucleotidase digestion. Finally, the successful construction of these plasmids was confirmed by DNA sequencing. Plasmids used to immunize mice were purified by an endotoxin-free purification kit according to its standard protocol. DNA concentrations were determined by the absorbance measured at 260 nm.

### *Western blot*

COS-7 cells were transiently transfected with pR and pF using Lipofect AMINE™. The cell lysates were prepared in modified RIPA buffer (0.15M NaCl, 0.1%SDS, 1%Nonidet P-40, 50 mM Tris-HCl pH 8.0 and 0.5% deoxycholate) before undergoing SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane, which was then blocked by blocking solution [PBS containing 3% bovine serum albumin (BSA) and 0.05% (v/v) Tween20]. The membrane was incubated with either B5 or RE2 for 2h, followed by biotinylated sheep anti-

mouse IgG antibody for 1h and avidin-peroxidase complex for another 1h at room temperature. Finally, the membrane was developed by diaminobenzidine (DAB). Extensive washes with washing buffer [PBS containing 0.05%(v/v) Tween20] were carried out between each two steps.

### *Establishment of cell lines stably expressing M-CSFR-mM-CSF*

To generate stably transfected cell lines expressing M-CSFR-mM-CSF as target cells, the syngenic BALB/c mouse myeloma-derived cell line SP2/0 was transfected with pF using Lipofect AMINE™. In brief, $2\times10^5$ SP2/0 cells were cultured in a six-well culture plate to approximately 50 to 80% confluence. Three micrograms pF or pcDNA3.1, as negative control, were mixed with 10 μL Lipofect AMINE™ in 200 μL of OPTI-MEM medium and incubated for 30min at room temperature, before being added to the cells. After 10h incubation, the complexes were removed and cells were incubated for another 48h. Then cells grew in selection medium containing 800 μg/mL G418 for about 14 days followed by cloning by limiting dilution. Positive clones were screened by ABC immunocytochemical staining[18] and RT-PCR.

### *Reverse transcriptase-polymerase chain reaction assay*

To identify whether pF transfected clones expressed fusion protein, total RNA was isolated using guanidine isothiocyanate. Five micrograms of RNA were reverse transcribed with oligo$(dT)_{12\text{-}18}$ primers and 200U M-MLV reverse transcriptase in 20 μL total volume at 37°C for 1h. Two microliters of the reverse transcribed products were amplified by PCR using P1 and P4 at 94°C for 30 sec, 55°C for 60 sec, and 72°C for 90 sec for 30 cycles.

### *Immunization protocol*

Mice (6-10 mice/group) were immunized weekly three times. The blank pcDNA3.1 and normal saline (NS) were used as controls. In pM, pR, pF and pcDNA3.1 groups 50 μg plasmid in 100 μL NS were injected into the quadriceps, while in the pM+pR group, 50 μg of each plasmid were simultaneously injected bilaterally into the quadriceps. Two weeks after the final immunization, splenocytes and sera were collected to test antibody titers, cytotoxic T-lymphocyte (CTL) activity and release of cytokines.

For tumor protection experiments, one week after the last vaccination mice were challenged s.c. in the right lateral flank with $2\times10^5$ SP2/0-F cells. For tumor treatment experiments, two days before immunization mice were challenged s.c. in the right lateral flank with $1\times10^6$ SP2/0-F cells. Mice were observed each day and the dimension of tumors was measured every three days.

### *ELISA*

Specific serum antibody titers in immunized mice were measured using indirect ELISA essentially as described previously.[17] Briefly, 96-well microtiter plates coated with recombinant M-CSF or M-CSFR protein (1 μg/mL) were incubated at 4°C overnight before being incubated in blocking buffer [PBS containing 3% BSA] for 2h at 37°C. Then, the plate was incubated with test sera diluted (1:50) in the blocking buffer for 2h followed by incubation with biotinylated antibodies against mouse IgG for 1h at 37°C. Finally, avidin-peroxidase complex was added and incubated for another 1h at 37°C, followed by development with O-phenylenediamine dihydrochloride (OPD) and stopped with 2M $H_2SO_4$. Extensive washes were carried out between each two steps. The plate was read with an ELISA reader at 492 nm.

### *Cytokine ELISA assay*

For determination of cytokine release, splenocytes from immunized mice were harvested. Erythrocytes were removed by incubation in 0.75% $NH_4Cl$/0.02 M Tris (pH 7.6) for 5 min at 37°C. Splenocytes were cultured using 6-well plates at $5\times10^6$/mL and stimulated with recombinant fusion M-CSFR-mM-CSF (1 μg/mL). Cell-free supernatant was collected 48h later for IL-4 or 72h later for IFN-γ detection. IL-4 and IFN-γ levels were measured according to the manufacturer's instruction.

### *CTL assay*

Splenocytes ($3\times10^7$) derived from mice two weeks after the last immunization were harvested as described above and cultured with irradiated (9,000 rad) syngenic SP2/0-F cells ($2\times10^6$) with rhIL-2 100 U/mL. Five days later, lymphocytes were harvested as cytotoxic effector cells and SP2/0-F cells were incubated for 1h with 100 μCi of $^{51}Cr$ as target cells. Then assays for CTL activity were performed at lymphocyte E: T ratios of 80:1, 40:1 and 20:1, respectively, using $5\times10^3$ $^{51}Cr$-labeled SP2/0-F/well. After 4h incubation at 37°C, 100 μL of supernatant were removed from each well and counted on a gamma counter. The percentage of specific release was calculated as follows [(experimental release-spontaneous release)]/[(total release–spontaneous release)] × 100%. Total release was measured by resuspending target cells in TritonX-100. Spontaneous release was obtained from target cells incubated with medium alone and is usually <15% of total release.

*Statistical analyses*

ANOVA and an unpaired Student's t test were used. Survival curves were drawn according to the Kaplan-Meier method. Statistical significance was determined by the log-rank test.

## Results

*Expression of pR and pF in mammalian cells*

The construction of the three DNA vaccines is sketched in Figure 1. The $(GGS)_2$ was chosen as the flexible linker in pF to avoid interaction of the two fragments. After screening by PCR and endonucleotidase digestion, the plasmids from positive clones were further verified by DNA sequencing. We chose those plasmids which had the same missense mutations at the corresponding sites as mutant mM-CSF and M-CSFR from J6-1 cells without any additional mutation, as pM, pR and pF, respectively. For a further demonstration of whether the constructed plasmids could express target antigen in mammalian cells, COS-7 cells were transiently transfected with these plasmids. Then ABC immunohistochemistry was done showing that the transfected cells expressed target protein as we expected. Western blot experiments showed that a specific band of 33kDa or 58kDa could be detected in pR or pF transfected COS-7 cells, respectively.

*Establishment of stably transfected SP2/0-F cells*

To generate stably transfected cell lines expressing fusion protein as target cells, SP2/0 cells were transfected with pF. After G418 selection, four clones were obtained. One clone expressing the highest level of fusion protein was selected and designated as SP2/0-F. After having been subcultured for 10 passages, SP2/0-F cells, but not SP2/0 transfected with pcDNA3.1, showed specific transcription as detected by RT-PCR (Figure 2). ABC immunocytochemical assay revealed that specific positive staining could be found in cytoplasma and on membrane of SP2/0-F cells by both RE2 and B5, while negative results was found in SP2/0 cells transfected with pcDNA3.1 blank vector. These results suggested that the SP2/0-F cells stably expressed M-CSFR-mM-CSF fusion protein and could be used as the target cells to evaluate immune responses induced by DNA vaccines.

*Humoral immune responses*

In an attempt to explore the possible mechanism by which anti-tumor activity was induced, mice were immunized as described above with pF, and pM+pR. Blank pcDNA3.1 and normal saline were used as controls. Two weeks after the final immunization, mM-CSF and M-CSFR specific antibodies were detected using ELISA. As shown in Figure 3, specific antibody titers in the pM+pR group were significantly higher than those in the pF group ($p$=0.001 for anti-mM-CSF and $p$=0.038 for anti-M-CSFR, respectively), higher than those in the pcDNA3.1 group ($p$<0.001 and $p$= 0.001, respectively), and higher than those in the normal saline group ($p$<0.001 and $p$<0.001, respectively).

The anti-M-CSFR antibody titer in the pF group was higher than that in the pcDNA3.1 group ($p$=0.038) but no significant difference was found in anti-mM-CSF antibody titer between these two groups ($p$=0.092). No significant difference was found between pcDNA3.1 and normal saline groups. These results suggest that pcDNA3.1 was

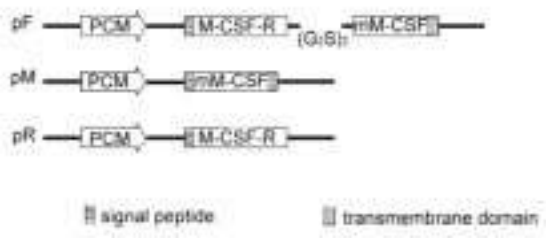


**Figure 1. Construction of DNA vaccines. DNA fragments were inserted into pcDNA3.1 under a Pcmv promoter: pM inserted the fragment containing signal peptide, extracellular and trans-membrane of mM-CSF; pR inserted the fragment containing signal peptide and extracellular domain of M-CSFR; pF inserted the M-CSFR-mM-CSF fusion fragment containing signal peptide and extracellular domain of M-CSFR linked with extracellular and trans-membrane of mM-CSF by a (Gly Gly Ser)$_2$ flexible linker.**

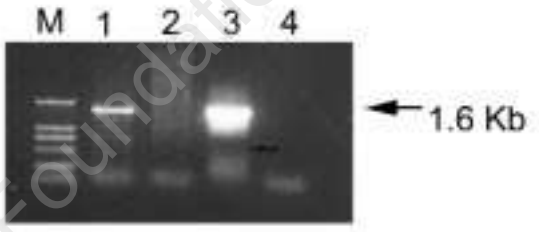


**Figure 2. Establishment of a target cell line (SP2/0-F) expressed M-CSFR-mM-CSF fusion protein. SP2/0 cells were transfected via pF and RT-PCR analysis of M-CSFR-mM-CSF transcription was used for screening and monitoring SP2/0-F clones. RT-PCR methods are detailed in Design and Methods. M: DNA marker; Lane 1: 1.6Kb specific transcription of M-CSFR- mM-CSF fusion DNA was observed in SP2/0-F cells after ten subculture passages. Lane 2: a negative result was found in SP2/0 cells transfected with pcDNA3.1 blank vector. Lane 3: pF was used as template as a positive control. Lane 4: negative control.**

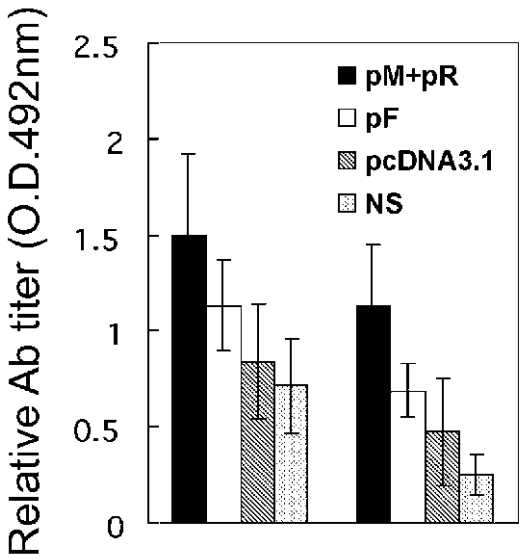


**Figure 3. DNA vaccines induce specific humoral immune responses against M-CSFR and mM-CSF. Mice were immunized with either NS, pcDNA3.1 blank vector, pF or pM+pR. Two weeks after the final inoculation, the relative antibody titers were detected using ELISA. The results are presented as the mean optical densities ($OD_{492}$) of 1:50 diluted serum samples. A: M-CSFR specific antibody titer; B: mM-CSF specific antibody titer.**

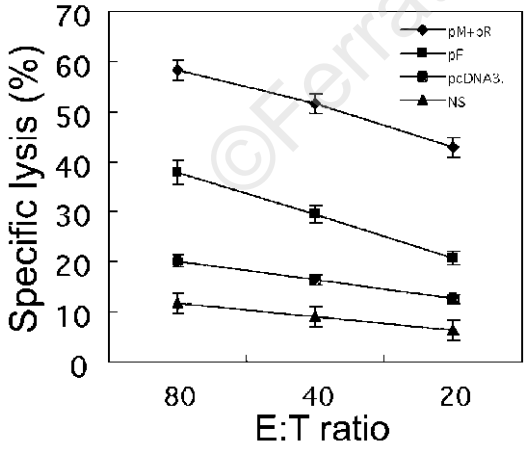


**Figure 4. DNA vaccines induce cellular immune responses against SP2/0-F. CTL activities were determined by $^{51}Cr$ release assay. Splenocytes ($3\times10^7$) derived from mice two weeks after the last immunization were harvested and co-cultured with irradiated (9,000 rad) SP2/0-F cells ($2\times10^6$) for five days. Lymphocyte populations were harvested as cytotoxic effector cells and SP2/0-F cells were incubated for 1 h with 100 μCi of $^{51}Cr$ as target cells. The E:T ratio is the ratio of effector and target cells.**

not able to induce specific antibody, pF has weak potency, while pM+pR has strong potency to induce specific antibodies against both mM-CSF and M-CSFR.

*Cellular immune responses*

Because CTL responses are essential in tumor therapy, we then studied the ability of splenocytes derived from immunized mice with pM+pR and pF to lyse SP2/0-F cells in a $^{51}Cr$ release assay. Blank pcDNA3.1 and NS were used as controls. The CTL activity in the pM+pR group was higher than that in the pF group. When the effector target (E:T) ratio was 80:1, the CTL activities were 58% and 38%, respectively, which were significantly higher than that in pcDNA3.1 or NS group ($p<0.001$ and $p<0.001$, respectively). However, CTL activity in the pcDNA3.1 group reached 20%, which is higher than in the NS group ($p<0.001$). (Figure 4) These results suggest that both pM+pR and pF could induce specific cellular immunity while immunization with two DNA vaccines has greater effects.

*Cytokine assays*

To further explore the possible mechanism by which DNA vaccines induce immune response, we monitored the secretion of IFN-γ and IL-4 by splenocytes from immunized mice by means of ELISA after re-stimulation with fusion protein (Figure 5). The IFN-γ production in the pM+pR group was higher than that in the pF group ($p=0.008$), and the production in both groups was higher than that in the NS group ($p<0.001$ and $p=0.043$, respectively). As compared within the pcDNA3.1 group, IFN-γ production was significantly higher in pM+pR group ($p=0.02$) while no statistical difference was found in the pF group ($p=0.474$). Hence, simultaneous application of both pM and pR had the strongest effect in activating Th1 cells.

IL-4 production was higher in both the pM+pR and pF groups than in the pcDNA3.1 group ($p=0.001$ and 0.005, respectively). Moreover, the production was higher in the pM+pR group than in the pF group($p=0.003$). The above results suggest that vaccination could activate Th1 and Th2 cells to participate in immune responses and the co-immunized vaccination had greater effects.

*Therapeutic and protective antitumor immunity*

In therapeutic experiments (Figure 6), pM+pR vaccination significantly prolonged the survival of mice challenged with SP2/0-F (38±0.82 days) when compared with the survival of the pF group (32.2 ±1.32 days, $p=0.0021$), the pM group (26.67±2.33 days, $p<0.05$), the pR group (27.67±1.58 days,

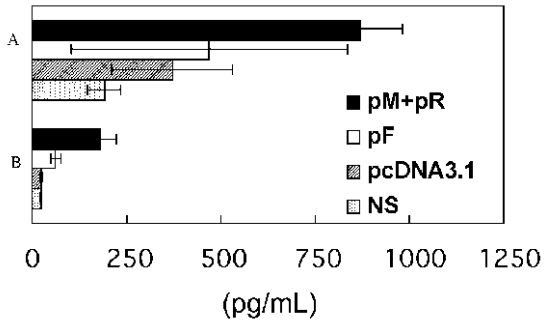


**Figure 5. Secretion of IFN-γ and IL-4 by splenocytes from immunized mice. Splenocytes from mice two weeks after the final immunization were harvested and cultured with recombinant fusion M-CSFR-mM-CSF (1 μg/mL). The production of IFN-α and IL-4 was determined using ELISA according to the manufacturer's instruction. A: production of IFN-γ; B: production of IL-4.**

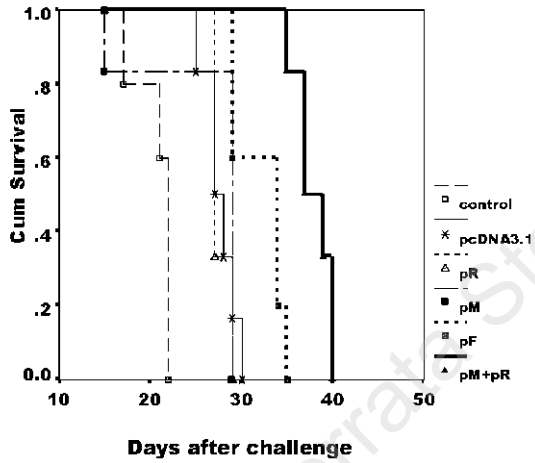


**Figure 6. Therapeutic effects of DNA vaccines against SP2/0-F in a mouse model. Two days before immunization with different DNA vaccines, mice (5-6/group) were challenged s.c. in the right lateral flank with 1×10⁶ SP2/0-F. Mice were observed each day. Survival curves were constructed according to the Kaplan-Meier method.**

$p<0.05$), the pcDNA3.1 group (27.67±0.71 days, $p<0.05$), or the NS group (20.75±1.25 days, $p<0.001$). The pF vaccination was better than vaccination with pM or pR or pcDNA3.1 or NS ($p<0.001$). Vaccination with pM, pR or pcDNA3.1 had similar effects ($p>0.05$) and was better than vaccination with NS.

In preventive experiments, mice were killed two weeks after challenge. Ten percent of mice in either the pM+pR or the pF group were protected from tumors but no statistical difference could be found between the two groups.

Over 150 mice were studied in therapeutic and preventive experiments. Neither accident nor any other disease was found during the period of the experiment. Furthermore, no abnormality was detected in the monocytic-macrophage lineage in mice vaccinated with these DNA vaccines. This implies that these DNA vaccines were safe and caused few side effects.

## Discussion

Nucleic acid vaccines would be one of the most important advances in the history of vaccinology. However, promising results have come mainly from experimental animal models. Only a few human clinical trials have been approved.[24,25] It has been demonstrated that mM-CSF and M-CSFR are tumor-associated antigens for mammary tumor,[26] ovarian cancer,[27-29] hepatoma[30,31] and hematopoietic malignant diseases.[20] In this study, we studied the strategy of DNA vaccination against these molecules in an animal model.

As compared with normal cells, tumor cells express a different spectrum of proteins, some of which are TAAs. Immunization with naked plasmid DNA encoding TAAs has been revealed to be a potent and promising strategy in antitumor immunotherapy from pre-clinical studies in animal models. Both cellular and humoral immune responses can be generated. However, the effects of DNA vaccines are usually not as great as those of protein vaccines. Hence, the improvement of vaccine efficacy has become a critical goal in the development of DNA vaccination. Vaccination against multi-epitopes is one of the strategies used since this is believed to cause more specific, stronger and more effective immune response against tumor cells than that against a single TAA because synergistic effects can be achieved. Polyvalent melanoma-associated antigen DNA vaccine, which could induce an effective systemic immune response, was tested for its prevention and treatment of malignant melanoma in a murine model.[32]

Our results clearly demonstrated that vaccinations against dual epitopes were better than vaccination against a single epitope, and that the effects of two vaccines were better than those of a fusion vaccine. In this model we used SP2/0-F, expressing both epitopes of mM-CSF and M-CSFR, as the target cell. Vaccination by pM+pR or pF caused specific immune responses against both epitopes. Furthermore, these two effects might be additional or even synergetic. Hence their effects were stronger than those against any single epitope. Of the two vacci-

nation methods against dual epitopes, the two-vaccine method might have several advantages: each plasmid is relatively smaller, which means it is easier to transfect cells; the expressed protein fragments are relatively shorter, thus being easier to express; and two epitopes are processed and presented to T-cells by different dendritic cells (DC) at different places rather than by the same DC, in which case T-cells have to compete. So pM+pR had better effects than pF.

In this study DNA immunization prolonged survival of mice challenged with SP2/0-F in tumor therapeutic experiments, however no significant differences were detected in protective experiments. The model in this study, in which we injected $2\times10^5$ tumor cells into mice, was a transplantable tumor model. It demonstrated that our vaccines had little preventive effects under these conditions. It has been well established that DNA vaccines are less effective than traditional vaccines.

Our results suggest that the tumor-forming potency of injected tumor cells in this experiment was stronger than the anti-tumor immunity induced by these vaccines. Weakening the tumor-forming potency, for example by decreasing the amount of injected cells, or enhancing anti-tumor immunity induced by DNA vaccines, for example by increasing the quantity of injected plasmids and/or co-immunizing with adjuvants or adopting prime-boost immune strategy, might increase the protection effect. There are at least three strategies for DNA vaccines involving multi-epitopes in one vaccination. First, immunize with one fusion DNA vaccine with all epitopes fused in one fragment under a single promoter. Fusion protein vaccines have been extensively studied and some have shown advantages over vaccines against a single epitope. Fusion DNA vaccines in literature mainly consist of a target fragment and an adjuvant fragment (GM-CSF, etc.).[33] They showed greater potency in inducing immune responses. Second, immunization with one DNA vaccine with several epitopes under separate promoters. Recently, it was reported that immunization with a DNA vaccine co-expressing adjuvant (GM-CSF) and β-gal into the same plasmid, but under separate promoters resulted in stronger antitumor responses.[34] Third, simultaneously immunization with several DNA vaccines, each targeting a single epitope.[32] Here we show that simultaneous immunization of two separate single-epitope vaccines is better than a fusion vaccine with dual-epitopes in the mM-CSF/M-CSFR model.

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*MHW was responsible for the conception of the study, analysis and interpretation of the data and drafting the article. GGZ was responsible for analysis and interpretation of the data and drafting the article. KFW was responsible for the conception of the study, critical revision of the article for important intellectual content, obtaining funding, and analysis of the data. GL ,YML and QR were responsible for interpretation of the data and critical revision of the article for important intellectual content. YHS was responsible for the design of the study and critical revision of the article for important intellectual content. All the authors approved the article. MHW, GGZ: responsible for manuscript writing: MHW: responsible for the tables and figures.*

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*Disclosures*

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## Peer Review Outcomes

### *Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Dr. Juan Bueren, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Bueren and the Editors. Manuscript received June 3, 2002; accepted August 5, 2002.

### *What is already known on this topic*

Mutated forms of membrane-bound M-CSF and of M-CSF receptor have been described as potential tumor associated antigens.

### *What this study adds*

The current study explores the possibility of using both mM-CSF and M-CSFR as targets to construct DNA vaccines. Different approaches were invstigated, including co-immunization with two different DNA vaccines, each against a single epitope, or immunization with a fusion DNA vaccine against dual epitopes.

### *Potential implications for clinical practice*

Co-immunization with these DNA vaccines, or with other similar, could be potentitally used for the treatment of pathologies such as Hodgkin's disease and leukemias in which tumor cells express these forms of mM-CSF and/or M-CSFR.

*Juan Bueren, Associate Editor (Madrid, Spain)*