Intramuscular Immunization with a Plasmid DNA Vaccine Encoding prM-E Protein from Japanese Encephalitis Virus: Enhanced Immunogenicity by Co-Administration of GM-CSF Gene and Genetic Fusions of prM-E Protein and GM-CSF

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Key Words

Encephalitis virus, Japanese • Granulocyte-macrophage colony-stimulating factor • Neutralization test • T lymphocytes • Vaccine, DNA

Abstract

Objective: To investigate the immune responses elicited by pJME with or without various forms of granulocyte-macrophage colony-stimulating factor (GM-CSF) gene. **Methods:** The changes of the T lymphocyte subsets and the levels of Th cell intracellular cytokines IFN- γ and IL-4 were evaluated by flow cytometric analysis. The cytotoxic T lymphocyte kill activity was assessed by lactate dehydrogenase activity release test. An 80% plaque reduction neutralization test was performed to titrate the neutralization antibody before and after viral challenge. **Results:** We demonstrated that simultaneous administration of pJME plus plasmid-encoded GM-CSF (pGM-CSF) activated Th1 immune responses similar to those found by injecting pGM-CSF i.m. into mice 3 days before pJME vaccination, and enhancement of Th2 immunity predominated when the pGM-CSF was injected 3 days after pJME vaccination. Furthermore, the immunization with DNA vaccine encoding precursor membrane envelope/GM-CSF fusion protein was more effective in generating immune responses than that induced by immunization with pJME alone

or in combination with pGM-CSF. **Conclusions:** These observations support the potential of GM-CSF DNA adjuvant for the Th1/Th2 balance and the enhancement of immune responses by showing that the timing of the administration of pGM-CSF and the application of different forms of GM-CSF gene influence the outcome of the resultant immune responses.

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Introduction

A recently described vaccine strategy, termed nucleic acid vaccine or DNA vaccine, which uses DNA instead of protein, opens up a new path for the study of biological vaccines [1]. In animal models direct intramuscular or intradermal injection of plasmid DNA generates potent cell-mediated and humoral immune responses against a variety of pathogens, such as HIV [2], hepatitis B virus [3] and mycobacteria [4]. An important feature of DNA-based immunization is the induction of cytotoxic T lymphocytes (CTLs) that recognize and lyse virus-infected cells, thereby limiting viral spread. Nowadays experimental DNA vaccines have also been evaluated in a number of phase I clinical trials, such as those for colorectal carcinoma [5], B cell lymphoma [6] and melanoma [7].

Japanese encephalitis (JE) is a serious mosquito-borne viral disease caused by Japanese encephalitis virus (JEV) that belongs to the family *Flaviviridae*, genus *Flavivirus*. The JEV genome contains a single positive-stranded RNA of approximately 11 kb in length, containing a single open reading frame encoding a polyprotein, which is proteolytically cleaved into 3 structural proteins [capsid protein, precursor membrane (prM) protein or membrane (M) protein and envelope (E) protein] and at least 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). A number of candidate DNA vaccines against JEV have been developed using plasmids that express various structural or non-structural JEV proteins. In a mouse model, these plasmids were found to provide protection to varying degrees against JEV challenge [8]. Of these, plasmids expressing JEV prME protein induced a better immune effect than other related JEV protein genes [9]. However, JE DNA vaccine still could not attain the expected standard, including the neutralizing antibody production time, antibody titers and titers increasing speed, compared with both inactivated and live-attenuated JE vaccines [10, 11]. It is clear that if plasmid DNA vaccination is to provide effective immunization against JEV infection, enhanced immune responses will need to be generated.

It has been known that granulocyte-macrophage colony-stimulating factor (GM-CSF) has a potent effect on dendritic cell (DC) differentiation and maturation as well as the expression of MHC and co-stimulatory molecules on DCs [12–14]. GM-CSF has also been regarded as genetic adjuvant for the research of JE DNA vaccine immune-enhancing effect, and evidence suggests that precise temporal injection of plasmid-encoded GM-CSF (pGM-CSF) combined with pJME appears to be critical for optimal induction of DNA vaccine-elicited immune responses [15].

At present, the GM-CSF-encoded gene has been used for DNA vaccination studies in 3 kinds of immunization strategies: (1) the GM-CSF gene is linked to an antigen-encoding gene as a bicistronic plasmid [16]; (2) the GM-CSF gene is mixed with the antigen-encoding plasmid as a separate plasmid [17, 18], and (3) the fusion proteins of antigens and GM-CSF and DNA vaccines encoding such fusion proteins have been shown to improve antigen-specific antibody responses and cancer immunotherapy [19–21]. Therefore, in an effort to develop a DNA vaccine candidate for JEV, 2 DNA vaccine candidates have been constructed, and it has been demonstrated that both co-administration of GM-CSF DNA and a prME/GM-CSF fusion construct elicit neutralizing antibodies and a

Th1/Th2 immune response against JEV in mice and that DNA vaccines encoding prME/GM-CSF fusion protein might be a candidate for immune enhancement against JEV.

Materials and Methods

Cell, Virus Strain, Vector and Animals

Chinese hamster ovary (CHO) cells, P815 cells and baby hamster kidney (BHK) cells were purchased from Shanghai Institutes for Biological Sciences, China. CHO cells and P815 cells were maintained at 37° in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco-BRL, Carlsbad, Calif., USA), 100 U/ml penicillin and 100 mg/ml streptomycin. CHO cells were used for the transfection experiment and P815 cells were used as target cells. BHK cells grown at 37° in Eagle's medium with 1% FCS and Beijing-1 strain of JEV [22] were used for the 80% plaque reduction neutralization test (PRNT₈₀). A eukaryotic vector, pcDNA3.1(+), with the strong eukaryotic promoter derived from human cytomegalovirus and T7 bacteriophage promoter was bought from Invitrogen (Carlsbad, Calif., USA) for plasmid construction. Female, 4-week-old BALB/c mice were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, and maintained in sterile cages under specific pathogen-free conditions. The mice were later used directly for the experiment on DNA immunization.

Preparation and Characterization of Recombinant Plasmid DNA Vaccines

The pJME containing JEV gene encoding prME protein was developed and stored in our laboratory. The coding sequence of murine GM-CSF (426 bp) was amplified by nested RT-PCR from RNA isolated from stimulated mouse splenocytes. The reverse transcription primer was 5'-CAGGCACAAAAGCAGCAGTC-3'. The forward orientation primer used in the first PCR reaction was 5'-CAGAGAGAAAGGCTAAGGTC-3', the reverse orientation primer was 5'-CAGGCACAAAAGCAGCAGTC-3'. The forward orientation primer used in the second PCR reaction was 5'-gaattcGGTGGAGGCGGTTTCAGGTGGAGGTGGTTTCAGGAGGAGGTGGATCGATGTGGCTGCAGAATTTAC-3', which contained an *EcoRI* site (lower case letters) and a Gly-linker consisting of 15 aa (underlined letters) at the 5' end. The reverse orientation primer was 5'-gcggccgcTCATTTTGGCCTGGTTTTTTG-3', which contained a *NotI* site (lower case letters) at the 5' end. The RT-PCR product was purified using a DNA Fragment Purification Kit (version 2.0; Takara, Dalian, China) according to the manufacturer's instructions and cloned into the pMD19-T simple (Takara). The pMD19-T simple containing the murine GM-CSF gene was designated pMD19-T simple-GM-CSF. The pMD19-T simple-GM-CSF was subsequently identified with restriction enzymes *EcoRI* and *NotI* and sequenced to be identical to the mouse GM-CSF sequences deposited in GenBank. A 471-bp fragment of murine GM-CSF and Gly-linker was digested with restriction enzymes *EcoRI* and *NotI* from pMD19-T simple-GM-CSF and subcloned into the pcDNA3.1(+) vector at the *EcoRI/NotI* site. The construct containing the murine GM-CSF gene was designated pGM-CSF. To construct a DNA vaccine encoding fusion protein of prME and GM-CSF (pJME/GM-CSF), a fragment of 2,001 bp of JEV prME iso-

lated from pJME by *Bam*HI and *Eco*RI digestion was inserted into the *Bam*HI/*Eco*RI site of pGM-CSF. The backbone plasmid DNA pcDNA3.1(+) was used as the control plasmid throughout the entire study. Competent *Escherichia coli* DH5a cells were transformed with the above recombinant and plated overnight on Luria broth agar plates containing 100 µg/ml ampicillin (Sigma, St. Louis, Mo., USA). Single colonies were picked and inoculated into 2 ml Luria broth liquid cultures containing 100 µg/ml ampicillin. pJME, pGM-CSF and pJME/GM-CSF were purified with Qiagen Mega Plasmid DNA (Qiagen GmbH, Hilden, Germany) kit using the endofree buffer system and dissolved in ddH₂O at a concentration of 1 mg/ml. To identify the recombinant, pJME/GM-CSF and pGM-CSF were digested with restriction enzymes *Bam*HI/*Not*I and *Eco*RI/*Not*I, respectively, and sequenced by T7 promoter primer from pcDNA3.1(+). The endotoxin content from purified plasmid DNA was less than 5 EU/mg.

In vitro Expression of pGM-CSF and pJME/GM-CSF

The CHO cells were transfected using liposome method. Recombinant plasmids of pJME/GM-CSF and pGM-CSF were transfected into CHO cells in order to detect proteins expressed by pJME/GM-CSF and pGM-CSF. Stable transfection was performed using the lipofectamine 2000 reagent (Invitrogen), according to manufacturer's instructions. Briefly, logarithmically growing cells were transfected with 1.5 µg of plasmids and 4 µl of LF2000 reagent. At 48 h after transfection, cells were passaged at 1:10 dilution into fresh medium, and then stable transfectants were isolated by selection with 800 µg/ml G418 (Gibco-BRL, Paisley, UK) for 2 weeks. Pools of geneticin-resistant clones were passaged and expanded for Western blot analysis. Cells transfected with the pcDNA3.1(+) were used as controls.

Indirect Fluorescence Assays

Immunofluorescence microscopy was used to visualize the distribution of GM-CSF or prME/GM-CSF fusion protein in CHO cells. Cultured CHO cells after transfection were allowed to attach overnight to coverslips, washed with PBS 3 times, and fixed with 4% paraformaldehyde at room temperature for 1 h. After being permeated with 0.5% Triton X-100 in PBS for 10 min at room temperature, cells were blocked by 2% bovine serum albumin for 30 min at 37°. The cells were incubated overnight with goat anti-mouse GM-CSF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 1:50 dilution at 4°. Subsequently, they were incubated with FITC-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology) at room temperature in the dark for 1 h. Coverslips were then mounted directly onto glass slides with a tiny drop of 50% glycerol in PBS (9.1 mM Na₂HPO₄/1.7 mM NaH₂PO₄/50 mM NaCl, pH 7.4), and examined by fluorescence microscopy.

Western Blot Analysis

After transfection, all cells were harvested to examine the expression of GM-CSF and fusion protein prME/GM-CSF by Western blot. In brief, samples of GM-CSF and fusion protein prME/GM-CSF were heated for 5 min at 90° in sample buffer and separated on 12% or 10% SDS-polyacrylamide gels, respectively, and then transferred to PVDF membranes (Millipore, Billerica, Mass., USA). After electroblotting, the membranes were blocked with 5% dried skim milk in Tris-buffered saline with 0.05% Tween (TBS-T) overnight at 4°C, followed by incubation for 1 to 2 h at

room temperature with goat polyclonal anti-GM-CSF (1:200; Santa Cruz Biotechnology). The blots were then washed and incubated for 1 h at room temperature with horseradish peroxidase-labeled rabbit anti-goat IgG (1:50,000, Santa Cruz Biotechnology). The blots were washed 5 times for 5 min in TBS-T, followed by incubation (2 min) in Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, Ill., USA) and exposed to Fuji film (Fuji Photo Film, Tokyo, Japan).

Mouse Experiment

We used 7 groups of 4-week-old female BALB/c mice (n = 10 per group) which were as follows: pJME, pJME/GM-CSF, pJME + pGM-CSF(0), pJME + pGM-CSF(-3), pJME + pGM-CSF(+3). For the i.m. immunization, mice were injected with 100 µg pJME/GM-CSF, 100 µg pJME with or without 100 µg of plasmid pGM-CSF into the quadriceps muscle mass of the left hind leg. Numbers in parentheses next to the pGM-CSF indicated the time of injection. Thus, 0 indicated that the pGM-CSF was given at the same time as pJME, -3 indicated that the pGM-CSF was given 3 days ahead of pJME and +3 indicated that the pGM-CSF was given 3 days after pJME delivery. All the mice received 2 booster doses in the same muscle (containing the same amount of plasmid as in the primary dose) 3 and 5 weeks after the primary injection. For the positive control group, each mouse was immunized with inactivated vaccine, a formalin-inactivated mouse brain-derived JEV vaccine (Beijing-1 strain) obtained from Liaoning Province Center of Disease Control and Prevention, and each mouse of the inactivated vaccine group was given an injection of 100 µl (1/5 of a recommended adult dose) of inactivated vaccine and boosted with the same dose 3 and 5 weeks after the first immunization. The pcDNA3.1(+) immunized group was used as the negative control. The volume of vaccine solution injected into each thigh was adjusted to 100 µl per mouse with PBS. For evaluation of cell immunity, spleens were collected from groups of 4 immunized mice 3 weeks after the last immunization. Three weeks after the last immunization, mice were challenged by i.p. injection with 10⁵ PFU/100 µl of Beijing-1 strain of JEV and observed for 3 weeks. Retro-orbital blood was collected successively from each mouse 2 days before challenge and 8 and 21 days after challenge unless otherwise stated. Individual or pooled serum samples were used for evaluation of antibody.

Spleen T Lymphocyte Subsets Measurement

A single-cell suspension of spleen cells was obtained from immunized mice according to the protocol described elsewhere [23]. The fresh spleens removed from the mice were gently squeezed with sterile needles in cold PBS containing 5% FBS and passed through a stainless steel mesh screen and the single-cell suspension was then prepared. Spleen lymphocytes were analyzed using a gate set on forward scatter versus side scatter, and a 3-color flow cytometry to combination reagent of CD3, CD4 and CD8. Anti-mouse monoclonal antibodies CD3-PECP/CD4-FITC/CD8-PE were purchased from BD Pharmingen (San Diego, Calif., USA). The detection was analyzed with the CellQuest software (Becton Dickinson, San Jose, Calif., USA) for each sample. The results were expressed as the percentage of CD3⁺CD4⁺ (short for CD4⁺ below) and CD3⁺CD8⁺ (short for CD8⁺ below) cells found to be positive for the marker antigen in the total T cell population. The handling procedures were performed in strict accordance with the instructions within the reagent kit.

Fig. 1. a Plasmid DNA vaccine construction and characterization. Schematic diagram of the DNA plasmid vaccine encoding GM-CSF or prME/GM-CSF fusion protein. **b** Characterization of pGM-CSF (lanes 4 and 5) and pJME/GM-CSF (lanes 1, 2 and 3) by restriction endonuclease digestion. M = DNA size marker.

Intracellular Cytokine Staining

We used 4-color flow cytometry to analyse the levels of Th cell intracellular cytokines IFN- γ and IL-4 for sorting out Th1 and Th2 subpopulations. The frequency of IFN- γ or IL-4-producing Th cells in spleens of treated BALB/c mice (4 per group) was detected, 3 weeks after the last immunization, using the Cytofix/Cytoperm Plus Kit with GolgiStop (BD Pharmingen). Cells (1×10^6) were cultured for 5 h in 100 ng/ml of phorbol 12-myristate 13-acetate (Sigma), 1 μ g/ml ionomycin (Sigma) and 1 μ l monensin. Then, the cells were harvested, suspended in staining buffer (PBS with 1% FCS and 0.1% sodium azide) and blocked with purified anti-mouse Fc γ RII/III monoclonal antibody (2.4G2; BD Pharmingen) for 15 min at 4 $^\circ$, followed by incubation with PerCP-conjugated anti-CD3 (145-2C11; BD Pharmingen) and PE-conjugated anti-CD8 (53-6.7; BD Pharmingen) for 30 min, at 4 $^\circ$. Intracellular cytokine staining was also carried out by using Cytofix/Cytoperm kit as suggested by the manufacturer (BD Pharmingen) in the presence of FITC-conjugated anti-mouse IFN- γ (XMG1.2; BD Pharmingen), APC-conjugated anti-mouse IL-4 (11B11; BD Pharmingen), or isotype control (BD Pharmingen). Fifty $\times 10^3$ events were acquired by using a 4-color FacsCalibur (Becton Dickinson). The levels of intracellular cytokines IFN- γ and IL-4 were determined with gating of CD3 $^+$ CD8 $^-$ cells. The data were analyzed using Cell Quest software (BD Pharmingen).

In vitro CTL Assays

Cytotoxicity assays using the lactate dehydrogenase (LDH) activity released test were performed at week 8 (3 weeks after the final DNA inoculation), as described previously, with some modifications [22]. Briefly, spleen cells from 4 mice of each group were mixed and stimulated in vitro with 1/20 volume of live JEV (Beijing-1 strain, 10^3 PFU/ml) in 2 ml of RPMI 1640 containing 10% FCS and 10 U/ml rmIL-2 (R&D, Minneapolis, Minn., USA) in 24-well microplates at 37 $^\circ$ for 5 days. The target cells used for this as-

say were JEV-infected P815 cells. The target cells were distributed into quadruplicate wells of a 96-well plate (5×10^3 cells/well) and the effector-to-target cell ratio was adjusted to 10:1. The effector-to-target cells were mixed and incubated in a humidified chamber at 37 $^\circ$, 5% CO $_2$ for 5 h before the supernatant was collected. LDH activity released into the culture medium was measured by using the Cytotox 96 assay kit (Promega Corp., Madison, Wisc., USA), according to the manufacturer's instructions. The percentage of specific lysis was calculated as follows: experimental LDH release – spontaneous LDH release/maximum LDH release – spontaneous LDH release $\times 100$.

PRNT $_{80}$

Neutralizing antibodies elicited in immunized mice were titrated as previously described [22]. The neutralization titer was expressed as the highest serum dilution yielding a 80% reduction in plaque number.

Statistical Analysis

All values were expressed as means $\pm$ SD. Statistical analysis of the experimental data and controls was conducted by 1-way factorial ANOVA, with the levels of significance defined as $p < 0.05$.

Results

Characterization of Recombinant Plasmid DNA Vaccines

The genetic fragment corresponding to murine GM-CSF was amplified using the nested RT-PCR method with appropriate primers. The structure of pGM-CSF and pJME/GM-CSF is illustrated in figure 1a and b, re-

Fig. 2. a Western blot analysis of cell extracts from transfected CHO cells with goat polyclonal anti-GM-CSF antibody. Lane 1 = CHO cell extracts transfected with pGM-CSF; lane 2 = CHO cell extracts transfected with pcDNA3.1(+) (negative control). **b** Western blot analysis of cell extracts from transfected CHO cells with goat polyclonal anti-GM-CSF antibody. Lane 1 = CHO cell extracts transfected with pJME/GM-CSF; lane 2 = CHO cell extracts transfected with pcDNA3.1(+) (negative control).

spectively. Upon restriction enzyme digestion, pGM-CSF was digested with *EcoRI/NotI* into 1 fragment, as predicted, at 471 bp in size (fig. 1b, lane 5), and confirmed that the DNA fragment released from the recombinant plasmid corresponded to the GM-CSF coding sequence from the published murine GM-CSF gene sequence (NM_009969). Meanwhile, the pJME/GM-CSF was cut with *BamHI/EcoRI* and *BamHI/NotI* into 2 fragments as predicted at 2,001 and 2,472 bp in size (fig. 1b, lanes 2, 3), and confirmed that 2,001 bp was the same as that of the JEV prME gene and 2,472 bp was the sum of the murine GM-CSF gene and the JEV prME gene. With sequence analysis using the T7 promoter primer located in pcDNA3.1(+), it was indicated that there was a normal junction of *BamHI* site between vectors and the prME gene of JEV, and *EcoRI* site between vectors and the murine GM-CSF gene.

Expression of GM-CSF and prME/GM-CSF Fusion Protein

The expression of GM-CSF and prME/GM-CSF fusion protein was confirmed by immunofluorescence assay and Western blotting of extracts from stably transfected CHO cells. In Western blots, the specific GM-CSF protein and fusion protein prME/GM-CSF were clearly visible in CHO cells transfected with both pGM-CSF and pJME/GM-CSF, but almost undetectable specificity protein in cells transfected with pcDNA3.1(+). Anti-murine GM-CSF reactive protein bands of about 17 (fig. 2a, lane 1) and 85 kDa (fig. 2b, lane 1) were respectively in correspondence with GM-CSF protein expressed by pGM-CSF and prME/GM-CSF fusion protein expressed by pJME/GM-CSF. As shown in figure 2b, there were some nonspecific bands in lanes 1 and 2. We speculated that these nonspecific bands were related to goat polyclonal anti-GM-CSF antibody used in our study. On the immu-

nofluorescence assay, strong green immunofluorescence (fig. 3a, b) was mainly found in the cytoplasm of CHO cells transfected with pJME/GM-CSF and pGM-CSF, but not much in the membrane of transfected CHO cells. However, very weak or nonspecific immunofluorescence was seen in cells transfected with pcDNA3.1(+) (fig. 3c). The data suggested that the specific GM-CSF protein or fusion protein prME/GM-CSF could be effectively expressed in CHO cells.

Spleen T Lymphocyte Subpopulation Composition

The adjuvant effects of the GM-CSF DNA on the generation of CD4⁺ T cells or CD8⁺ T cells from spleen were compared. The percentage of CD4⁺ T cells in spleen cells was 33.90 ± 0.79% in the pJME/GM-CSF vaccinated group, which significantly increased the relative frequency of CD4⁺ T subpopulations compared with other groups ($p < 0.05$). The percentage of CD4⁺ T cells in the pcDNA3.1(+) vaccinated group was 22.99 ± 1.20%, and lower than in all other groups ($p < 0.05$). GM-CSF DNA co-injection indicated that the percentage of CD4⁺ T cells in spleen cells in pJME + pGM-CSF(0) and pJME + pGM-CSF(-3) vaccinated groups was higher than that in pJME + pGM-CSF(+3) vaccinated group ($p < 0.05$). The average value of CD8⁺ T cell subsets in spleen cells in the pJME + pGM-CSF(0) vaccinated group was 15.80 ± 0.29%, and obviously higher than other immunized groups ($p < 0.05$). The percentage of CD8⁺ T cells in pJME/GM-CSF, pJME + pGM-CSF and pJME vaccinated groups was higher than that in the empty plasmid and JE inactivated vaccine vaccinated groups ($p < 0.05$). However, there was no significant difference in the levels of CD8⁺ T cells between the pJME/GM-CSF, pJME + pGM-CSF(-3), pJME + pGM-CSF(+3) and pJME vaccinated groups ($p < 0.05$; fig. 4).

Fig. 3. Detection of GM-CSF or prME/GM-CSF fusion protein expression by immunofluorescence staining in transfected CHO cells. The pictures show that the strong green immunofluorescence was mainly found in the cytoplasm in CHO cells transfected with pJME/GM-CSF (**a**) and pGM-CSF (**b**), and not much in membranes of transfected CHO cells. However, very weak or non-specific immunofluorescence was seen in cells transfected with pcDNA3.1(+) (**c**).

Percentage of Th Cells with Intracellular Cytokines IFN- γ and IL-4

In order to further analyze the type of immune response, we determined the percentage of IFN- γ and IL-4-producing Th cells in spleens of DNA-immunized mice by flow cytometry. CD3⁺CD8⁻ cells were collected to gate the CD4⁺ Th cells, because membrane CD4 antigen expression could be depleted with the stimulation of PMA. At the end of the eighth week of the experiment, numbers of Th cells producing intracellular cytokine IFN- γ were elevated in all the vaccinated mice except JE inactivated vaccine and pcDNA3.1(+) vaccinated groups, and there was significant difference between 2 random groups ($p < 0.05$). The pJME + pGM-CSF(0) immunized group obtained the highest levels of IFN- γ . The number of IL-4-producing Th cells showed a great decrease in pJME/GM-

CSF, pJME + pGM-CSF(-3) and pJME + pGM-CSF(0) vaccinated groups compared with other groups. The ratio of IFN- γ to IL-4 in spleen cells from the pJME/GM-CSF vaccinated group was the highest in all groups (19.05). The ratios of IFN- γ to IL-4 in spleen cells from both pJME + pGM-CSF(0) and pJME + pGM-CSF(-3) vaccinated groups, respectively, were 12.27 and 7.03, obviously higher than those in the pJME + pGM-CSF(+3) vaccinated group (0.13) and other groups ($p < 0.05$; fig. 5).

Generation of CTLs

We also investigated the generation of JEV-specific CTLs in the immunized mice. As indicated by the percent specific lysis of JEV-infected cells, at an effector-to-target ratio of 10:1, mice immunized with 100 μ g pJME/GM-CSF had higher CTL activity than those immunized

Fig. 4. Expansion of CD3CD4/CD3CD8 positive T cells from mice immunized with pJME with or without GM-CSF gene. **a** Representative dot plots and similar results were obtained in 4 independent experiments. The percentage of CD4⁺ or CD8⁺ T cells is indicated in the upper left quadrant or lower right quadrant of each dot plot.

(For fig. 4.b see next page)

Fig. 4. Expansion of CD3CD4/CD3CD8 positive T cells from mice immunized with pJME with or without GM-CSF gene. **b** Mean percent ($\pm$ SD, $n = 4$) of CD3⁺CD4⁺ (black bars) and CD3⁺CD8⁺ (white bars) T lymphocytes from splenocytes of DNA immunized mice 3 weeks after the last immunization.

with the same amount of other naked DNA ($p < 0.05$). The CTL activities of the spleens of BALB/c mice immunized 3 times with indicated immunogens were as follows: pJME/GM-CSF $51.48 \pm 1.47\%$; pJME + pGM-CSF(0) $47.17 \pm 0.84\%$; pJME + pGM-CSF(-3) $40.40 \pm 2.02\%$; pJME $33.15 \pm 0.90\%$; pJME + pGM-CSF(+3) $25.60 \pm 0.59\%$; JE inactivated vaccine $23.72 \pm 0.82\%$, and pcDNA3.1(+) $8.36 \pm 0.49\%$ (fig. 6).

Titers of Neutralizing Antibody in Mice Vaccinated 3 Times with Plasmid DNA Vaccines and Protection Immunity against JEV

To study the effect of the cytokine-encoding plasmid delivery on JEV neutralization antibody titers and protective immunity, 7 groups of 6 BALB/c mice were challenged with 10^5 PFU/100 μ l of Beijing-1 strain of JE virus 3 weeks after the final immunization. Prechallenge sera were collected 2 days before challenge (defined as day -2) and examined for neutralizing antibody. Following challenge, mice were observed for 3 weeks, and postchallenge sera were collected on days 8 and 21 from all the surviving mice. Table 1 showed that each immunized group had individual antibody titers from 1:10 to 1:20 on day -2.

After challenge, neutralization titers were elevated from 1:40 to 1:80 on day 8 and 1:40 to 1:320 on day 21. The mice immunized 3 times with pJME developed low neutralizing antibody titers (1:10 to 1:40) on days -2, 8 and 21, and obtained 50% protection against JEV challenge. Intramuscular administration of pJME along with pGM-CSF resulted in a large enhancement of JEV-neutralizing antibody titers when compared with those obtained by pJME administration alone [pJME + pGM-CSF(0)/pJME: day minus two 1:20/1:10, day eight 1:80/1:20 and day twenty-one 1:160/1:40], which was equivalent to JEV-neutralizing antibody titers induced by JE inactivated vaccine group. The pJME + pGM-CSF(0) vaccinated group and the JE inactivated vaccine group gave 75 and 91.7% protection, respectively. Administration of pGM-CSF 3 days ahead of pJME delivery, led to approximately 2-fold increase in JEV neutralizing antibody titers when compared with titers obtained in mice immunized with pJME alone, and acquired 66.7% protection. However, co-administration of pGM-CSF 3 days after pJME delivery had no effect on JEV-neutralizing antibody titers induced by pJME and the level of protection did not increase further with co-administration of pGM-CSF. Moreover, we first evaluated the immune adjuvant effects of pJME/GM-CSF in our study. As shown in table 1, the pJME/GM-CSF vaccinated group led to the highest level of JEV-neutralization antibody titers, approximately 8-fold higher JEV-neutralizing antibody titers than those induced by pJME alone and demonstrated 83.3% protection. None of the pcDNA3.1(+) vector immunized mice was protected against virus challenge.

Discussion

CD4⁺ T cells are central to the induction of most immune responses. They are the cells that recognize exogenous-derived antigen presented by professional antigen-presenting cells and they are the major triggering agents to activate other immune effector cells. CD4⁺ T cells are responsible for either provoking or sustaining pathological conditions, such as transplant rejection, graft versus host disease and autoimmune diseases. CD4⁺ effector T cells can develop into Th1 cells or Th2 cells. The former mainly secrete cytokines, such as IFN- γ , IL-2 and IL-12 and TNF- α , and contribute to cellular immunity, while the latter mainly secrete IL-4, IL-5, IL-6 and IL-10 and contribute to humoral immunity. The mode of application of the DNA and the nature of the expressed antigen are factors determining the induction of either a Th1 or

Fig. 5. Flow cytometric analysis of Th intracellular cytokines IFN- γ and IL-4 in gating CD3⁺CD8⁻ splenocytes of DNA immunized mice. **a** Results are shown for a representative mouse from groups of 4, and similar results were obtained in 4 independent experiments. The number in the upper left quadrant of each dot plot represents the percentage of Th2 cells (IL-4⁺, IFN- γ ⁻) and values in the lower right quadrant of dot plots represent Th1 cells (IFN- γ ⁺, IL-4⁻). **b** The summaries of percentage of IFN- γ ⁺CD4⁺ in total CD4⁺ T cells and IL-4⁺CD4⁺ in total CD4⁺ T cells are shown in the histogram. Each column represents the mean $\pm$ SD of 4 independent experiments.

Table 1. Protective immunity against JEV and neutralization titer in BALB/c mice immunized with JE inactivated vaccine, or pJME with or without various forms of GM-CSF gene

Immunogen ^a	Neutralization titer ^b			Protection ^c %
	day -2	day 8	day 21	
pJME	1:10	1:40	1:40	50.0
pJME/GM-CSF	1:20	1:80	1:320	83.3
pJME + pGM-CSF(-3)	1:20	1:40	1:80	66.7
pJME + pGM-CSF(0)	1:20	1:80	1:160	75.0
pJME + pGM-CSF(+3)	1:10	1:40	1:40	50.0
Inactivated vaccine	1:20	1:80	1:160	91.7
pcDNA3.1(+)	<1:10	NA	NA	0.0

^a Groups of six 4-week-old BALB/c mice were immunized i.m. with indicated immunogens with or without GM-CSF gene at 2-week intervals. Mice were challenged 3 weeks after last immunization with JEV Beijing-1 (10^5 PFU/100 μ l i.p.) and monitored for 21 days for survival.

^b The sera of mice in each group were pooled and the neutralization titer was determined. Mice were bled 2 days before (day -2) and 8 and 21 days after i.p. JEV challenge. Individual serum from 6 mice each group were used for neutralization titer determinations. Only 1 of the representative determinations is shown in this table. NA = Serum not available.

^c Results are presented as a summary of 2 independent experiments. The mean percent protection is indicated.

Th2 type immune response [24]. There is growing evidence that induction of a Th1 type response plays a decisive role in the generation of protective immunity against various pathogens such as *Leishmania major* [25] and HIV-1 [26], and the balance of the Th1 versus the Th2 response can also be influenced by combined application of DNA vaccines with plasmid DNAs encoding cytokines such as GM-CSF [27] or IL-12 [28], or co-stimulatory molecules such as B7.2 [29]. However, there are conflicting reports on the role of GM-CSF in the modulation of the Th1/Th2 balance in mice [30–32]. Based on these data that the protective quality of the humoral and cellular immune response is dependent on the type of cytokines secreted by activated Th cells, the levels of the intracellular IFN- γ and IL-4 in Th cells can thus reflect the numbers of Th1 and Th2 cells, respectively. In this study, we investigated the immune enhancing effect of the GM-CSF gene on a DNA vaccine encoding JEV prME protein by detecting the percentage of Th cells with intracellular cytokines IFN- γ and IL-4. Our results demonstrate that co-administration of DNA encoding JEV prME protein and plasmid encoding mouse GM-CSF slightly increased

Fig. 6. Specific lysis of JEV-infected P815 cells by DNA vaccine immunized spleen cells stimulated with JEV virus. Cytotoxic activities against JEV-infected P815 cells were measured at an E:T ratio of 10:1 by using LDH activity as a marker of cell lysis. Data shown are the mean percent ($\pm$ SD, $n = 4$) of specific lysis.

IFN- γ -producing T cells and markedly decreased IL-4-producing T cells in comparison with pJME delivery alone, and induced a Th1-type response. However, the immune enhancing effect induced by co-delivery of GM-CSF was closely related to the timing of plasmid-encoded GM-CSF administration. Our observations revealed that the ratios of IFN- γ to IL-4 that resulted from i.m. administration of pGM-CSF together with pJME or administration of pGM-CSF 3 days ahead of pJME delivery were remarkably higher than those obtained 3 days after pJME administration. Furthermore, we found that co-inoculation of pGM-CSF together with pJME achieved higher ratios of IFN- γ to IL-4 than those caused by administration of pGM-CSF 3 days ahead of pJME. These observations, along with the obvious augmentation of CD4⁺ T cell responses, CTL activity, neutralizing antibody titers and protective immunity against JEV elicited by administration of pGM-CSF together with pJME, suggest that the timing of the adjuvant GM-CSF DNA relative to the antigen is critical in determining the magnitude of immune response, and simultaneous administration of plasmid GM-CSF plus pJME may be desirable for poten-

tial DNA vaccines against JEV. A number of laboratories have reported that the timing of plasmid GM-CSF administration determined the type of immune responses that were augmented. Kusakabe et al. [33] observed that injecting pGM-CSF i.m. into mice 3 days before DNA vaccination primarily induced a Th2 response and simultaneous administration of the DNA vaccine plus pGM-CSF activated both a Th1 and a Th2 response, and injecting pGM-CSF i.m. into mice 3 days after DNA vaccination primarily induced a Th1 response. However, Barouch et al. [34] reported that administering plasmid GM-CSF before or with the gp120 vaccine decreased gp120-specific antibody titers and T cell functional activity, whereas administering plasmid GM-CSF after the gp120 vaccine augmented gp120-specific immune responses. The differences in the findings among these studies are significant and may reflect the differences in antigens, expression vectors and specific assays used.

It has been reported that idiotypic GM-CSF fusion proteins are effective vaccines for lymphoma [19] and the covalent linkage of the antigen and GM-CSF might allow the targeting of APCs expressing GM-CSF receptors, such as DCs. This could improve antigen uptake and presentation and thus also enhance CD8⁺ T cell responses, similar to targeting strategies based on the macrophage mannose receptor or the DEC-205 receptor [35, 36]. Therefore, we detected cellular and humoral immunity induced by DNA vaccines encoding prME/GM-CSF fusion protein to identify optimally immunostimulatory combinations. Our results indicated that immunization with genetic vaccines encoding prME/GM-CSF fusion protein led to an enhanced level of protection in immunized mice against JEV challenge, and dramatically enhanced Th1 immune responses and humoral responses, as manifested by the highest percentage of CD4⁺ T cell and the highest ratios of IFN- γ to IL-4, and stimulated the strongest CTL activities and the highest neutralizing antibody titers of JEV. Previous studies have shown that pGM-CSF results in the accumulation of DCs in vivo, and that DCs stimulated with GM-CSF differentiate and mature into increasingly effective antigen-presenting cells [37, 38]. Thus, it is possible that linking antigen and GM-CSF expression closely in vivo will provide a more conducive microenvironment for coordinated expression of Ag and GM-CSF and maximize Ag presentation and trigger CD4⁺ T cells by these recruited DCs. However, we have found that both DNA vaccine encoding prME/GM-CSF fusion protein and co-administration of separate plasmids expressing GM-CSF or prME protein had a largely selective effect on inhibiting IL-4-secreting spleen

cells, with little effect on activation of IFN- γ -secreting spleen cells in the system used in the present study, in comparison with those elicited by pJME alone and the control groups. All of these results show that further studies are needed to elucidate these mechanisms.

In order to further identify optimal immunostimulatory combinations, we also evaluated the immunomodulatory properties of the GM-CSF gene on CD8⁺ T cell counts. Our observation suggested that there was a lack of correlation between the proportion of CD8⁺ T cells and resultant antigen-specific CTL activities. There was no significant difference in the percentage of CD8⁺ T cells between pJME/GM-CSF and pJME + pGM-CSF(-3) and pJME + pGM-CSF(+3) and pJME vaccinated groups; however, each vaccinated group obtained different JEV-specific CTL activities. In addition, immunization with DNA vaccine encoding prME/GM-CSF fusion protein achieved lower levels of CD8⁺ T cells when compared with simultaneous administration of pJME plus pGM-CSF, whereas DNA vaccine encoding prME/GM-CSF fusion protein induced the highest JEV-specific CTL activities. This disagreement has also been observed in other experimental studies [39, 40]. In view of some reports from other laboratories showing that CD4⁺ T cells played a critical role in CD8⁺ CTL responses to exogenous antigens, our findings also suggest that the proportion of CD4⁺ T cells has a positive relation with the ratios of IFN- γ to IL-4, CTL and protective immunity against JEV in all vaccinated groups. There is still much to be learned about the mechanisms and timing of boosting by GM-CSF gene.

To our knowledge, this is the first study to show the effect of the GM-CSF gene on cellular immune enhancing effect of plasmid DNA encoding prME protein derived from JEV and to compare the levels of cellular and humoral immune responses induced by pJME immunization with or without various forms of GM-CSF DNA. We conclude that DNA vaccines encoding prME/GM-CSF fusion protein create a desirable pattern of immune enhancement against JEV.

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