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Original Research

Enhancing immune responses of EV71 VP1 DNA vaccine by co-inoculating plasmid IL-12 or GM-CSF expressing vector in mice

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Abstract: Enterovirus 71 (EV71) is a major causative viral agent for large outbreaks of hand, foot, and mouth disease in children and infants, yet there is no vaccine or effective antiviral treatment for severe EV71 infection. The immunogenicity of EV71 VP1 DNA vaccine and the immunoregulatory activity of interleukin-12 (IL-12) or granulocyte-monocyte colony stimulating factor (GM-CSF) were investigated. DNA vaccine plasmids, pcDNA-VP1, pcDNA-IL-12 and pcDNA-GM-CSF were constructed and inoculated into BALB/c mice with or without pcDNA-IL-12 or pcDNA-GM-CSF by intramuscular injection. Cellular and humoral immune responses were assessed by indirect ELISA, lymphocyte proliferation assays, cytokine release assay and FACS. The VP1 DNA vaccine had good immunogenicity and can induce specific humoral and cellular immunity in BALB/c mice, while IL-2 or GM-CSF plays an immunoadjuvant role and enhances specific immune responses. This study provides a frame of reference for the design of DNA vaccines against EV71.

Key words: EV71, VP1, DNA vaccine, IL-12, GM-CSF.

Introduction

Enterovirus 71 (EV71) and coxsackievirus are etiological agents for hand, foot, and mouth disease (HFMD) in children and infants; and EV71 is the major one for large outbreaks of the disease throughout the world, especially in the Asia-Pacific region (1,2). EV71 is a member of the *Enterovirus* genus within the *Picornaviridae* family and is a single-stranded RNA virus with a genomic size of 7500 bp. EV71 has four capsid proteins,VP1 to VP4, of which VP1 to VP3 are responsible for antigenicities among different enteroviruses and VP1 provides for neutralization epitopes.

Since no effective antiviral agents are available, developing vaccines for primary prevention is considered to be the best choice among control strategies against EV71. Several candidates of EV71 vaccines including formalin-inactivated whole virus vaccine, DNA vaccine and recombinant protein vaccine were reported. These vaccine constructs remain promising vaccine strategies that require further refinement, thus further study and development are required (3-5).

DNA vaccines have emerged as an attractive strategy because of their safety, ease of preparation, storage, and administration. However, several problems must be addressed before there is a licensed DNA vaccine (6). Increasing the immunogenicity of DNA vaccines is the most important challenge faced the field. DNA vaccines have been shown capable of stimulating immune responses in small animals, however, the efficacy of the vaccine decreases as the size of the animal increases (7). Cytokines are powerful mediators of innate and adaptive immunity and attractive candidate vaccine adjuvants. Beneficial effects of cytokines as adjuvants include stimulation of T cell-mediated immunity at the level of antigen (Ag) presentation and T-cell proliferation. Therefore, cytokine adjuvants have been co-injected with DNA vaccines to increase the immunogenicity (8).

Interleukin-12 (IL-12) was among the first cytokines tested as molecular adjuvants for a DNA vaccine. IL-12 is a heterodimeric cytokine composed of two disulfide-linked peptides, p35 and p40. It is produced mainly by the Ag-presenting cells, such as dendritic cells and macrophages (9). IL-12 is a key cytokine responsible for polarizing T cells toward the Th1 phenotype (10). Importantly, IL-12 strongly supports the expansion of Ag-specific cytotoxic CD8+ T cells and the expression of cytotoxic mediators such as granzyme B/perforin and interferon-γ (IFN-γ) (11).

Granulocyte-monocyte colony stimulating factor (GM-CSF) is secreted by a variety of cells. It is able to recruit, activate and enhance the function of professional Ag presenting cells, making it as an adjuvant to vaccines. A number of studies in different animal models have clearly shown that plasmid expressing GM-CSF can augment the immune responses generated by DNA vaccines (12,13). Importantly, it has been shown that

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GM-CSF can exert its adjuvant effect without skewing the Th1/Th2 balance, facilitating the generation of both antibody and cell-mediated immune responses.

In this study, a DNA vaccine encoding the VP1 gene of EV71 was designed and constructed. This vaccine candidate was tested *in vitro* for expression of VP1 protein in mammalian cell culture, followed by *in vivo* testing to elicit immune responses in mice. To optimize the immune responses of EV71 DNA vaccine, two immunostimulatory cytokines delivered as plasmid vectors were used to enhance immune responses in mice. Our results showed that EV71 VP1 DNA vaccine induces specific humoral and cellular immunity in mice, while IL-12 and GM-CSF play the immunoadjuvant role. This study provides a new strategy for EV71 DNA vaccine development.

Materials and Methods

Plasmid DNAs

Plasmids pcDNA-VP1, pcDNA-IL-12, and pcDNA-GM-CSF were constructed as follows. A DNA fragment encoding the full-length VP1 gene was amplified by polymerase chain reaction (PCR) from the cDNA of EV71 (GenBank accession No. EU703813) and cloned into a mammalian expression vector, pcDNA3.1/mychisA (Invitrogen, Carlsbad, CA, USA), to derive recombinant plasmid pcDNA-VP1.IL-12 and GM-CSF were each amplified by reverse transcription PCR (RT-PCR) from total RNAs extracted from BALB/c mouse spleen cells and inserted into pcDNA3.1/mychisA vectors to derive pcDNA-IL-12 and pcDNA-GM-CSF. Plasmid pcDNA-IL-12 carries murine IL-12 cDNAs encoding IL-12 subunits p35 and p40 spliced by a hydrophobic polypeptide linker (Gly4Ser)3. All the recombinant plasmids were propagated in *Escherichia coli* DH5α, and their DNAs were extracted with an endotoxin-free plasmid extraction and purification kit (Qiagen, Germany) and verified by restriction digestion and sequencing.

Plasmid pcDNA-VP1, pcDNA-IL-12, or pcDNA-GM-CSF was transiently transfected into Vero cells, and the protein expression was detected by western-bolt and RT-PCR before they were used to immunize the mice. The bioactivities of plasmid-expressed murine IL-12 and GM-CSF were confirmed by their capacities to induce interferon (IFN)-γ secretion in murine splenocytes and proliferation of FDCP1 cells (14), respectively.

DNA immunization of mice

Female BALB/c mice (6-8 weeks old) were purchased from the Songjiang Animal Facility of the Chinese Academy of Sciences of Shanghai and maintained in accordance with our Institutional Animal Care and Research Committee approved protocols. Ninety-one mice were randomly divided into seven groups (13 per group) and intramuscularly (i.m.) immunized three times at 2-week intervals. The mice were immunized three times with a mixture of 100μgof pcDNA-VP1 DNA and 50 μg of either pcDNA-IL-12, pcDNA-GM-CSF DNA, or pcDNA plasmid DNA on days 0, 14, and 28. Control mice were immunized i.m. with 150 μg of parental control vector pcDNA3.1, pcDNA-IL-12,pcDNA-GM-CSF DNA, or 100 μl of phosphate-buffered saline (PBS). All immunizations were delivered into the quadriceps muscles in a total volume of 100 μl PBS. The DNA dosages used in the mice and the immune scheme were optimized through a series of preliminary experiments.

Analysis of humoral immune response

Mice from each group were bled on days 0, 14, 28, 42, and 56. The blood was collected and stored at -70°C before antibody measurement. Anti-VP1 antibody levels in serum were assessed by an indirect sandwich enzyme-linked immunosorbent assay (ELISA). Recombinant VP1 protein expressed in *E. coli* was purified and used as the detection antigen. An optimized concentration (5μg/ml) of antigen was used to coat the 96-well plates (Costar) at 4°C overnight. Plates were washed and blocked with 1% bovine serum albumin (BSA)-buffered solution at 37°C for 1 h prior to incubation with 1:100 diluted mouse sera at37°C for 2h. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, or IgG2a (1:2000; Sigma, USA). Color was developed by the 3,3′, 5,5′-tetramethylbenzidine (Sigma, USA) substrate method, and the absorbance was read at 490 nm using a plate reader (Bio-RAD, USA). All samples were run in triplicates.

Lymphocyte proliferation assay (LPA)

An antigen-specific T-cell proliferation assay was performed as described previously (15). In brief, 2 weeks following the final immunization, mice were euthanized and a single-cell suspension was prepared from spleens. Splenocytes (2×10^5 cells/well) were seeded in a 96-well plate in triplicate and maintained in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS). Cultures were stimulated for 60 h with different reagents: 5μg/ml concanavalin A (ConA, positive control), 5μg/ml purified VP1 antigen (specific antigen), 5μg/ml BSA (irrelevant antigen), or medium alone (negative control). Twenty μl of CellTiter 96 Aqueous One Solution Reagent (Promega, USA) was added into each well according to the manufacturer's protocol. After incubation at 37°C for 4 h, the absorbance was read at 490 nm. The proliferative activity was estimated using the stimulation index (SI), which is the mean optical density at 490 nm (OD_{490}) of antigen-containing wells divided by the mean OD_{490} of wells without antigen.

Cytokine release assay

Splenocytes from immunized or control mice were incubated for 2 days at a concentration of 2×10^6 cells/well in 24-well plates in a total volume of 0.5 ml of RPMI-1640 containing 0.01μg/ml VP1 protein at 37°C in 5% CO_2. The supernatants were collected and assayed to detect IFN-γ and IL-4 using commercially available sandwich-based ELISA kits (BioRad R&D, USA),according to the manufacturer's instructions. All tests were performed intriplicate for each mouse.

Determination of CD4+ and CD8+ cells in peripheral blood mononuclear cells (PBMCs)

Two weeks after the final immunization, blood was collected from the retro-obital venous plexus of each mouse in each group. PBMCs were isolated by the Isopaque-Ficoll (Lymphoprep; Nycomed, Oslo, Norway)

method, according to the manufacturer's instructions. Lymphocytes were isolated from PBMCs and stained with the monoclonal antibodies fluorescein isothiocyanate-labeledanti-mouse CD4 and phycoerythrin-labeled anti-mouse CD8 (eBioscience, SanDiego, CA). Next, the stained lymphocytes were washed three times with PBS containing 4% FBS. A total of 10^5 cells were acquired on a FACSCalibur flow cytometer (Beckton Dickinson, Lincoln Park, NJ, USA), and data were analyzed using the WinMDI software.

Statistical analysis

All data were presented as means± standard deviation (SD). SPSS 13.0 software for windows was used for statistical analysis. Differences in humoral and cellular immune responses between groups were assessed by single factor analysis of variances. The least significant difference t-test was used for between group comparisons. *P*-values <0.05 were considered statistically significant.

Results

Antibody responses to EV71 VP1 protein

To examine the humoral responses elicited by the VP1 DNA vaccination, the EV71VP1-specific antibody was analyzed in sera samples collected on days 0, 14, 28, 42,and 56 after the first immunization of the BALB/c mice by an indirect sandwich ELISA. A significantly higher (P<0.01) anti-VP1 antibody level was found in the pCDNA3.1-VP1, pCDNA3.1-VP1 plus pCDNA3.1-IL12, or pCDNA3.1-VP1 plus pCDNA3.1-GM-CSF immunized mice compared to that in the mice immunized with pcDNA3.1, pcDNA-IL-12, pcDNA-GM-CSF, or PBS (Fig. 1). On day 14, all of the immunized groups showed a small amount of anti-VP1 antibody. Large increases in the VP1 antibody level were observed in the sera of all of the VP1 DNA-immunized groups on day 42 (14 days after the second boost) but not in the pcDNA3.1, pcDNA-IL-12, pcDNA-GM-CSF, or PBS immunized group. The antibody levels of co-administered pcDNA-IL-12 or pcDNA-GM-CSF groups were significantly higher than those in the pcDNA-VP1 alone groups after the second immune boost (P<0.05), and the pCDNA-VP1 DNA plus pCDNA-IL-12 immunized group showed the highest VP1antibody level among all of the immunized groups.

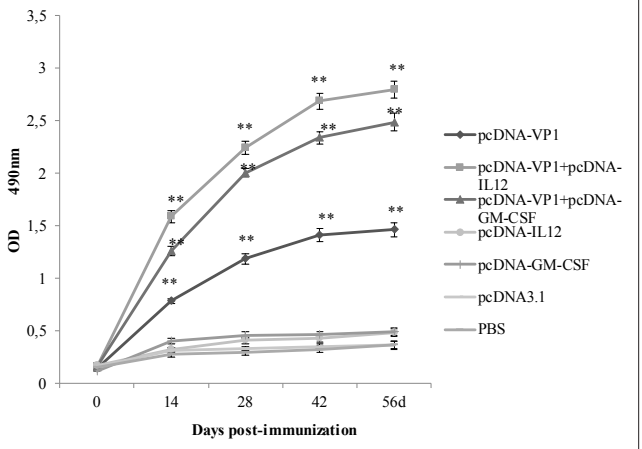

Figure 1. Antibody responses to EV71VP1 protein induced by DNA vaccination intramuscularly in BALB/c mice. Animals were vaccinated three times with pcDNA-VP1 (100 μg) alone,pcDNA-VP1 (100 μg) plus pcDNA-IL12 or pcDNA-GM-CSF (50 μg). Serum samples (13per group) were taken on days 0, 14, 28, 42, and 56 days after the first immunization. **P<0.01, compared to the PBS negative control group.

Subclasses of VP1-specific antibody

As IgG1 is a Th2-dependent subclass and IgG2a is a Th1-dependentsubclass of IgG antibody,IgG1 and IgG2a were measured in order to detect humoral and cytotoxic immune responses, respectively. The isotypes of the specific anti-VP1 IgG induced by DNA vaccination in different groups were measured to investigate modulation of immune responses qualitatively. Specific IgG1 and IgG2a antibody subtypes were measured using specific secondary antibodies. As shown in Fig. 2, anti-VP1-specific IgG subtype profile revealed that both IgG1 and IgG2a were induced in the pcDNA-VP1, pcDNA-VP1 plus pcDNA-IL-12, or pcDNA-GM-CSF immunization regimens. All four groups of control animals did not show any detectable specific antibody response. Antibody levels in the combined pcDNA-VP1 with pcDNA-IL-12 or pcDNA-GM-CSF group showed higher degrees of increase compared to that in pcDNA-VP1group and the pCDNA-VP1 DNA plus pCDNA-IL12-immunized group showed the highest VP1 antibody level among all of the immunized groups, although the antibody subclass profiles were similar in both groups. Co-administration of pcDNA-IL-12 or pcDNA-GM-CSF with pcDNA-VP1 mainly enhanced the antibody response, but it did not change the antibody subclasses.

VP1-specific T-cell proliferation

To study whether T cell proliferation could be influenced by the different immune regimens, 2 weeks after the final immunization, splenocytes from the vaccinated mice were examined for antigen-specific T-cell proliferation. As shown in Fig. 3, all of the VP1 DNA immunization groups exhibited higher levels of T cell proliferative response when stimulated with purified EV71 VP1 protein, while mice vaccinated with pcDNA3.1, pcDNA-IL-12, pcDNA-GM-CSF or PBS did

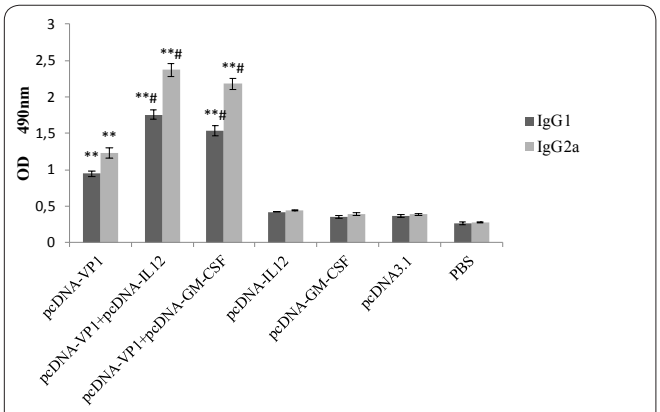

Figure 2. Identification of EV71VP1-specific IgG subclasses in vaccinated mice. Mouse sera (n=13) were collected 2weeks following the final injection, and VP1 protein-specific IgG1 and IgG2a were determined by indirect ELISA. Data are presented as mean ± SD.**P<0.01, compared to the PBS negative control group; #P<0.01, compared to the pcDNA-VP1 group.

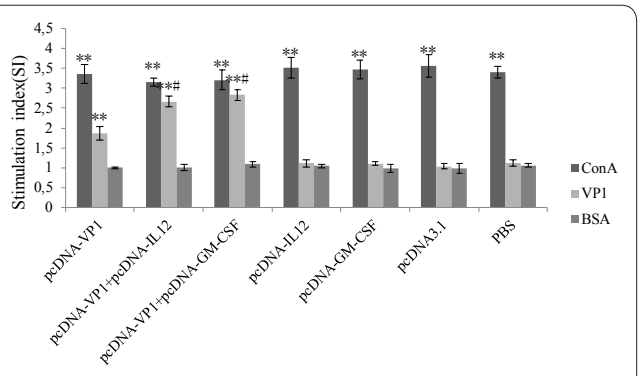

Figure 3. VP1-specific lymphocyte proliferation assay. Two weeks following the final injection, pooled splenocytes were obtained from mice (n = 5) immunized in the different groups. Splenocytes were stimulated *in vitro*, with purified VP1 protein as the test group, ConA as a positive control, and BSA as an irrelevant antigen control. Splenocytes from the control groups (pcDNA-IL12, pcDNA-GM-CSF, pcDNA3.1, or PBS) stimulated with VP1 protein served as the negative controls and the sham control. The stimulation index (SI) was calculated by the following formula: SI = (mean OD of ConA- or antigen-stimulated proliferation)/(mean OD of nonstimulated proliferation). Each bar represents the mean SI ± SD of five mice. ***P*<0.01, compared to the BSA negative control in each group; #*P*<0.01, VP1 in the pcDNA-VP1 plus pcDNA-IL12 or pcDNA-GM-CSF group compared to the pcDNA-VP1 group.

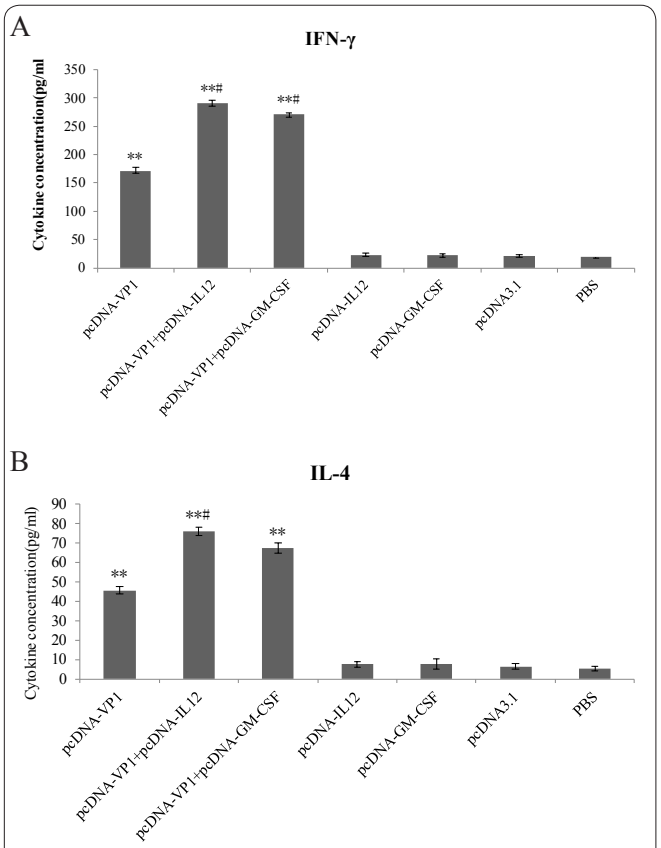

Figure 4. Levels of cytokine production from mouse splenocytes *in vitro*. Spleens of mice were isolated, and the splenocytes were cultured in triplicate for each mouse in the presence of 0.01 μg/ml VP1 protein. After 48 h, the supernatants were collected and analyzed by the sandwich ELISA method to determine IFN-γ (A) and IL-4 (B) titers. Data are the means ±SD of mice in each group. ***P*<0.01, compared to the negative control; #*P*<0.01, the pcDNA-VP1 plus pcDNA-IL12 or pcDNA-GM-CSF group compared to the pcDNA-VP1 group.

not respond to the VP1 protein vaccination (*P*<0.05). Co-immunization of VP1 DNA with pcDNA-IL-12 or pcDNA-GM-CSF induced a substantial increase in the T-cell proliferation response compared to that of the pcDNA-VP1 alone vaccinated group (*P*<0.05), and the VP1 DNA vaccine plus pcDNA-GM-CSF group evoked the strongest T cell response.

VP1-specific Th1- and Th2-type responses

To measure cytokine secretion in the vaccinated mice, splenocytes were cultured as described above and stimulated *in vitro* with EV71-VP1 protein. Collected supernatants were screened for the presence of IFN-γ and IL-4 to determine the type (Th1 versus Th2) of the immune responses. As shown in Fig.4, only low amounts of nonspecific IFN-γ and IL-4 were detected in the pcDNA3.1, pcDNA-IL-12, pcDNA-GM-CSF and PBS control groups. Compared with the negative control groups, significant amounts of VP1-specific IFN-γ and IL-4 were detected in the immunized groups (*P*<0.01). Co-delivery of pcDNA-VP1 with pcDNA-IL-12 or pcDNA-GM-CSF resulted in a significant increase in the IFN-γ and IL-4 secretion by spleen cells, indicating the effect of IL-12 and GM-CSF on enhancing both Th1 and Th2 immune responses. The DNA vaccine plus pcDNA-IL-12 group induced the highest level of VP1-specific IFN-γ. Moreover, in all groups, IFN-γ was induced to a much higher level than IL-4. These results suggest that the VP1-DNA vaccine formulation is more immunogenic, and it likely induces a stronger Th1 bias.

CD8+ and CD4+ lymphocyte responses

Since activated CD4+ and CD8+ T lymphocytes are among the most crucial components of antiviral effectors, CD4+ and CD8+ T lymphocytes in the PBMCs of the vaccinated mice were assessed (Fig. 5). Flow cytometric analysis of unstimulated cells was used to standardize the background responses, and there was little variation in nonimmunized mice. The vaccinations with pcDNA-VP1 or pcDNA-VP1 plus pcDNA-IL-12 or pcDNA-GM-CSF significantly increased the percentages of activated CD4+ and CD8+ cells compared to the percentages for the PBS mock-immunized control group (*P*<0.01). The numbers of activated CD4+ and CD8+ cells increased in all immunized groups except for the pcDNA3.1, IL-12, and GM-CSF groups. The CD8+/CD4+ ratio in the pcDNA-VP1 plus pcDNA-IL-12 or pcDNA-GM-CSF group was higher than that in the pcDNA-VP1 group. The ratio in the groups immunized with pcDNA-VP1 plus pcDNA-IL-12 was the highest of all of the immunized groups, but the difference was not statistically significant (*P*>0.05). These results further reinforced the fact that the VP1 DNA vaccine can elicit a T cell response in mice and that IL-12 expression can enhance the T-lymphocyte activity induced by the VP1 DNA vaccine.

Discussion

In the present study, a novel EV71 DNA vaccine with plasmid VP1 DNA combined with either plasmid IL-2 or GM-CSF DNA was investigated. Our results

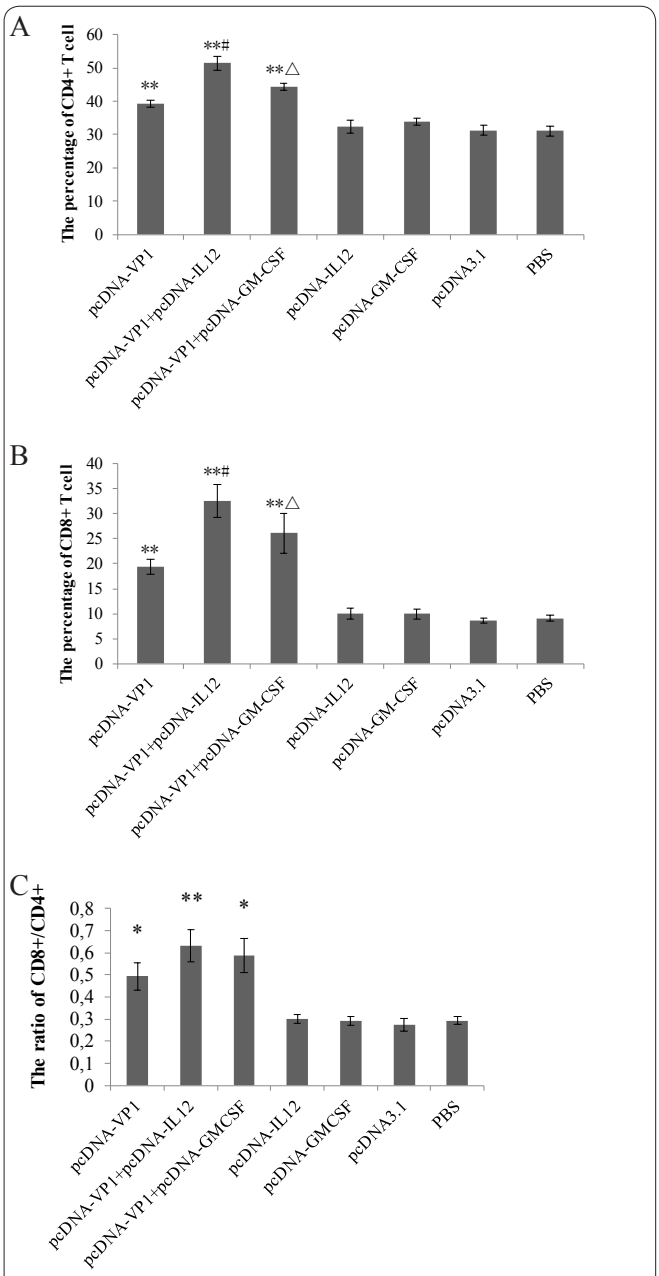

Figure 5. Analysis of CD8+ and CD4+ lymphocytes by flow cytometry.PBMCs were isolated from vaccinated mice (n = 5) 2 weeks after the final immunization. CD4+ and CD8+ T cells from control and immunized BALB/c mice were counted. Values are expressed as means ± SD. **P*<0.05, ***P*<0.01, compared to the PBS negative control group; Δ*P*<0.05,#*P*<0.01, the pcDNA-VP1 plus pcDNA-IL12 orpcDNA-GM-CSF group compared to the pcDNA-VP1 group.

showed that vaccination with EV71 VP1 DNA vaccine elicited EV71 VP1-protein-specific humoral and cellular immune responses were significantly enhanced by the coadministration of IL-12 or GM-CSF expressing vector. Our findings provide basic information for the design of EV71 DNA vaccines.

From our results on the anti-VP1-protein specific antibodies and T-cell proliferation, activated CD4+ and CD8+ cells were shown to be successfully evoked after VP1 DNA vaccination. We also found that antigen-specific specific T cells were capable of secreting high levels of the Th1 cytokine IFN-γ and moderate levels of the Th2 cytokine IL-4 upon *in vitro* stimulation with the EV71 VP1 protein. These results indicate that the VP1 DNA vaccine activates both the Th1 and the Th2 subsets, and the level of activation of the Th1 subset was much higher, which correlated with the tendency for the IgG2a antibody levels to be elevated. These findings suggest that the VP1 DNA vaccine is effective in activating both B and T cells to generate anti-VP1-protein antibodies and cellular immune (mainly Th1) responses in mice.

Several studies have indicated that the codelivery of vectors encoding cytokines, such as IL-2, IL-12, IFN-γ, or GM-CSF, is able to direct the nature of the resulting immune response by augmenting the efficacy of DNA vaccines (16,17). The cytokine IL-12 plays a central role in regulating innate and acquired immune responses and have been shown to exhibit both Th1- and Th2-type properties (18). IL-12 is produced by antigen-presenting cells, indicating its crucial role for protection against intracellular pathogens through the induction of NK cell activity and Th1 cell responses (19). IL-12 has also been adapted as an adjuvant for development of vaccines against intracellular pathogens, such as human immunodeficiency virus (9, 20) and Mycobacterium tuberculosis (21).GM-CSF has been reported to initiate the proliferation, differentiation, and activation of macrophages, neutrophils, and various professional antigen presenting cells (22). Delivered as a protein or a plasmid, GM-CSF has been shown to recruit and activate macrophages and dendritic cells at the site of inoculation (23). In mice, codelivery of pmGM-CSF with a DNA vaccine resulted in the recruitment of macrophages to the site of inoculation and specifically improved vaccine elicited CD4+ T lymphocyte responses (24). It has been shown that codelivery of pGM-CSF with the pDNA prime of a DNA/MVA vaccination regimen in rhesus macaques led to enhanced protection against the acute phase of a SHIV89.6P challenge (25).

In the current study, the immune responses in mice immunized with the EV71 VP1 DNA vaccine alone were compared to those in mice co-immunized with the plasmids encoding IL-12 or GM-CSF by analyzing antibodies, T cell proliferation, T-helper-cell responses, CD4+ and CD8+Tcell responses. The mice which received pcDNA-IL-12 or pcDNA-GM-CSF adjuvant generated higher IgG antibodies than those in mice injected the antigen-encoding plasmid alone. Both IgG1 and IgG2a antibody levels increased in the co-immunized groups, and the dominant isotype did not change by co-injection of the IL-12 or GM-CSF expression vector. A similar pattern was observed in the T cell immune responses measured by LPA, cytokine release assay, and fluorescence-activated cell sorter analysis. Our results showed that immunization with pcDNA-VP1 plus pcDNA-IL-12 or pcDNA-GM-CSF elicited recognizably higher levels of T cell responses compared to those in the groups immunized with pcDNA-VP1 alone. Taken together, our results provide evidence that strategy that includes IL-12 or GM-CSF as the adjuvant can be used to enhance immunity of candidate EV71 vaccines. These results are consistent with previous findings obtained with animal models of chronic viral infection, which showed that the administration of IL-12 or GM-CSF enhances viral antigen-specific Th1 immune responses and improves clinical outcomes (20, 21, 24, 25).

DNA vaccines hold promise for use in humans (26).

However, the immunogenicity of EV71 DNA vaccine in humans has yet to be established. Whether this approach could be applied to other animal models is still unknown, and its immunogenicity in human remains to be established. Development of transgenic mice expressing the appropriate human receptor molecule(s) for EV71 would be the best and promising approach in order to understand the pathogenesis of enterovirus encephalitis and to test the candidate vaccines. This approach has been extremely successful for poliovirus.

Overall, this study has given promising results on genetic immunization with DNA vaccine encoding VP1 gene of EV71 and showed IL-12 and GM-CSF as the promising cytokine adjuvants for EV71 DNA vaccines and perhaps other *Enterovirus* DNA vaccines in the future. With further study and improvement, use of the DNA vaccine might be a potential vaccine strategy against EV71.

Acknowledgments

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