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Suppression of Immune Response and Protective Immunity to a Japanese Encephalitis Virus DNA Vaccine by Coadministration of an IL-12-Expressing Plasmid

Hsin-Wei Chen,* Chien-Hsiung Pan,*† Hwei-Wen Huan,* Ming-Yi Liao,** Jen-Ron Chiang,‡ and Mi-Hua Tao*†

IL-12 plays a central role in both innate and acquired immunity and has been demonstrated to potentiate the protective immunity in several experimental vaccines. However, in this study, we show that IL-12 can be detrimental to the immune responses elicited by a plasmid DNA vaccine. Coadministration of the IL-12-expressing plasmid (pIL-12) significantly suppressed the protective immunity elicited by a plasmid DNA vaccine (pE) encoding the envelope protein of Japanese encephalitis virus. This suppressive effect was associated with marked reduction of specific T cell proliferation and Ab responses. A single dose of pIL-12 treatment with plasmid pE in initial priming resulted in significant immune suppression to subsequent pE booster immunization. The pIL-12-mediated immune suppression was dose dependent and evident only when the IL-12 gene was injected either before or coincident with the pE DNA vaccine. Finally, using IFN-γ gene-disrupted mice, we showed that the suppressive activity of the IL-12 plasmid was dependent upon endogenous production of IFN-γ. These results demonstrate that coexpression of the IL-12 gene can sometimes produce untoward effects to immune responses, and thus its application as a vaccine adjuvant should be carefully evaluated.


Interleukin-12 is a heterodimeric cytokine that is produced primarily by APCs and mediates a broad range of effects on both innate and acquired immunity. One of the most important properties of IL-12 is its ability to induce the production of large amounts of IFN-γ from resting and activated T and NK cells (1, 2). Via induction of IFN-γ, IL-12 exerts many immunoregulatory effects on both lymphoid and nonlymphoid cells to promote cell-mediated immunity, thus making IL-12 an effective agent to treat cancer and infectious diseases (3–5). In addition to its potential therapeutic value for established pathogenic conditions, IL-12 has also been proposed as an effective vaccine adjuvant (6). In this regard, IL-12 has been delivered as a soluble product (7), or expressed from plasmid DNA (8–10) or viral vectors (11, 12), for different vaccination strategies. IL-12 generally induces differentiation of type 1 CD4+ and CD8+ Th cells, and thus promotes strong cell-mediated immunity. The adjuvant activity of IL-12 can be further improved by combining it with other cytokines such as IL-2 (13) and IL-18 (14, 15), or with costimulatory molecules such as B7 (16–18).

However, under certain circumstances a high dose of IL-12 has been associated with side effects and immunotoxicities. Administration of high-dose IL-12 to mice infected with lymphocytic choriomeningitis virus resulted in decreasing body weights, higher viral titers, impaired CTL development, and poorer outcome (19, 20). Recombinant IL-12 given to mice vaccinated with irradiated tumor cells enhanced host protection, but only after an early immune suppression. The immune suppression was IL-12 dose dependent and manifested as reduced splenic CTL activity, stimulated cytokine release, and stimulated rejection of tumor cells (21). A high dose of IL-12 was also reported to abrogate the hepatitis C virus-specific cellular immunity induced by an adenoviral vector expressing hepatitis C viral proteins (22). These studies provide clear evidence that the use of recombinant IL-12 or IL-12-expressing vectors as vaccine adjuvant can sometimes generate unexpected and untoward effects.

Japanese encephalitis virus (JEV)

A serious mosquito-borne flavivirus that causes diseases in the human central nervous system (23). We previously showed that a plasmid (pE) encoding the JEV envelope (E) protein produced high titers of E-specific Abs and provided protection against a lethal JEV challenge (24). However, like many other DNA vaccines, pE vaccination only induced substantial amounts of anti-E Ab titers in a large animal model, swine, a natural host of JEV. To seek an approach to increase the efficacy of the pE DNA vaccine, we tested the use of cytokines as immunological adjuvants. It has been previously demonstrated that the magnitude and nature of the immune responses to DNA vaccines can be regulated by coadministration of a broad panel of cytokine genes (25–27). Among these cytokine genes, the IL-12 gene promotes Th1 responses and cell-mediated immunity, and the IL-4 gene favors Th2 cell development and production of Abs. Thus, coadministration of plasmids expressing the IL-4 or IL-12 gene with DNA vaccines offers a simple means to engineer an

*Institute of Biomedical Sciences, Academia Sinica; †Graduate Institute of Life Sciences, National Defense Medical Center; and ‡Center for Disease Control, Department of Health, Taipei, Taiwan

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4Abbreviations used in this paper: JEV, Japanese encephalitis virus; E, envelope; pE, plasmid DNA vaccine encoding the E protein of JEV; GKO, genetically deficient for IFN-γ; pIL-12, IL-12-expressing plasmid; pTCAE, parental plasmid used for construction of pIL-12.
immune response to create the best protection against a particular pathogen. In this study, we show that coinjection of the IL-4 plasmid has little effect on the protective immunity induced by pE DNA vaccine. In contrast, much to our surprise, coinjection of the IL-12 plasmid dramatically suppressed the Ab and T cell responses to the pE DNA vaccine and resulted in decreased protective immunity. This IL-12 gene-mediated immune suppression was dose dependent and was affected by the timing of IL-12 plasmid administration relative to pE vaccination. Using IFN-γ gene-disrupted mice, we demonstrated that the suppressive activity of the IL-12 plasmid was dependent upon endogenous production of IFN-γ.

Materials and Methods

Animals

Female C3H/HeN mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Female C57BL/6 mice were obtained from the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan). C57BL/6 IFN-γ−/− mice were donated by John Kung (Academia Sinica) and maintained as a small breeding colony in our own animal facility. Animal care was provided in accordance with the guidelines approved by the Animal Committee of the Institute of Biomedical Sciences, Academia Sinica.

Plasmids

The plasmid pE encoding the full-length JEV Beijing-1, E protein, and the C-terminal 15 aa of the membrane protein was previously described (24). The cytokine-expressing plasmids pIL-4 (10) and pIL-12 (28) produce biologically active murine IL-4 and IL-12, respectively. Plasmid pTCAE containing the CMV early promoter/enhancer sequence was a parental plasmid used for construction of pIL-12 and served as a control plasmid in this study. Plasmid DNA was purified from transformed Escherichia coli strain DH5α by Qiagen Plasmid Giga Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and was stored at −70°C as pellets. The DNA was reconstituted in sterile saline at a concentration of 2 mg/ml for experimental use.

Immunization and viral challenge

For i.m. DNA immunization, all mice were immunized at 6–8 wk of age as previously described, with some modification (24). In brief, all animals were pretreated with 100 μl of 10 μM cardiotoxin (Sigma, St. Louis, MO) in each quadriceps muscle 1 wk before the first DNA immunization. Groups of mice were then injected i.m. with a mixture of plasmid pE and pTCAE, pIL-4, or pIL-12. Mice immunized with pTCAE alone served as negative controls. The plasmid dose and number of injections are detailed in the figure legends. For some experiments, the pIL-12 plasmid was injected either 3 days before or 3 days after pE immunization at the same location. The JEV strain, Beijing-1, prepared from sucking mouse brain was used to make virus stock for challenge experiments as previously described (24). In brief, mice were i.p. inoculated with JEV Beijing-1 at a dose of 50 times the LD50 for the respective mouse strain (24), followed by a sham intracerebral inoculation. The combination of peripheral inoculation of JEV and sham intracerebral inoculation served to increase the susceptibility of mice to a central nervous system infection. The JEV-challenged mice were observed for symptoms of viral encephalitis and death every day for 30 days.

Ab assays

The ELISA for detection of JEV E-specific Abs was performed as previously described (24). Briefly, serum samples were added to microtiter plates coated with JEV prepared from tissue culture, and the bound Abs were detected with HRP-conjugated goat anti-mouse IgG Fc (1:1000; ICN/Cappel, Aurora, OH). Color was generated by adding ABTS (Sigma), and the absorbance at 405 nm was measured on an ELISA reader. The readings were referenced to a standard serum pooled from mice immunized with inactivated JEV and aluminum hydroxide. The standard curve was generated using the pooled anti-JEV sera, and results were expressed as arbitrary units per milliliter (U/ml; 1 U = 50% maximum OD). The concentration of 1 U/ml is roughly equal to 22 ng of anti-E Ab/ml. For measurement of IgG1 and IgG2a anti-E isotypes, biotin-conjugated rat anti-mouse IgG1 (1:1000; Pharmingen, San Diego, CA) and rat anti-mouse IgG2a (1:1000; Pharmingen) were used as detectors. Avidin-HRP (1:2000; Pharmingen) was then added. Color was developed as described above. End-point titers were defined as the highest serum dilution that resulted in an absorbance value 2 times greater than that of nonimmune serum with a cutoff value of 0.05.

Lymphocyte proliferation assays

To determine whether JEV E protein-specific lymphoproliferative response was induced in immunized animals, spleens were removed 4 days after the last immunization to make single-cell suspensions. We used 24-well plates in this assay to harvest supernatants for cytokine analysis. The splenic T lymphocytes were enriched by nylon wool columns and added to 24-well plates (105 cells in 1 ml/well). Stimulated wells received JEV E protein at a final concentration of 0.25 μg/ml. Transferrin (120 μg/ml; Sigma) served as a negative control Ag, and Con A (10 μg/ml; Sigma) served as a positive mitogenic control. Control wells received cells only. After 3 days in culture, cells were removed from the 24-well plates and added to each well (2 × 105 cells in 200 μl per well) in 96-well U-bottom plates and pulsed with [3H]thymidine (1 μCi per well) for 18 h. Cells were then harvested with FilterMate (Packard, Meriden, CT), and the incorporated radioactivity was determined by TopCount (Packard). The stimulation index was calculated as the mean cpm of the stimulated wells divided by the mean cpm of the control wells.

To examine cytokine secretion from splenic T cells, cell-free supernatants were harvested 3 days after stimulation and assayed immediately or stored at −80°C. These supernatants were screened for the presence of IFN-γ and IL-4 using ELISA detection systems as described in the next section.

Cytokine ELISAs

ELISA systems (Pharmingen) were used to detect and measure the presence of IFN-γ, IL-4, and IL-12 in test samples. The capture Abs for murine IFN-γ, IL-4, and IL-12 were R-4-6A2 (rat IgG1), BVD4-1D11 (rat IgG2b), and C15.6 (rat IgG1), respectively. The detection-biotinylated Abs for IFN-γ, IL-4, and IL-12 were XMG1.2 (rat IgG1), BVD6-2G2 (rat IgG1), and C17.8 (rat IgG2a), respectively. Cytokine ELISAs were performed according to the manufacturer’s instructions and were previously described (10).

Cytotoxicity assays

C3H/HeN mice were immunized i.m. with 100 μg of pE and 100 μg of pIL-12 or pTCAE three times at 3-wk intervals. Mice receiving 200 μg of pTCAE served as negative controls. A sublethal live virus immunization was performed by i.p. injection of 6.0 × 105 PFU of JEV Beijing-1 without a sham intracerebral inoculation and boosted with the same amount of virus 3 wk later. Spleen cells were removed 4 days after the last immunization to measure the CTL activity. Single-cell suspensions of splenocytes were treated with 5 ml per spleen of ACK lysis buffer (0.15 M NaCl, 1 mM EGTA, 1 mM KHC03, 0.1 mM Na2EDTA, pH 7.2) for 5 min at room temperature to remove RBC. To perform the cytotoxic assay, responder splenocytes (2 × 105) were stimulated by incubation with live JEV (6 × 105 PFU) in 2 ml of RPMI 1640 containing 5% FBS (RPMI 5) in 24-well microplates for 5 days at 37°C. Target cells were prepared by infecting L929 cells with JEV Beijing-1 at a multiplicity of infection of 100 PFU/cell or mock infection (16–18 h before the assay). The viable cells (1 × 105 in 0.1 ml RPMI 5) were labeled with 0.1 mCi radiolabeled sodium chromate (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at 37°C, washed three times with RPMI 5, and resuspended at a concentration of 5 × 104 per ml in RPMI 5. A 2-fold serial dilution of 100 μl of stimulated responder splenocytes (starting from 5 × 105 cells) was added to individual wells containing 100 μ1 of labeled target cells (5000 cells). After a 4- h incubation at 37°C, 100 μl of culture supernatant was collected for gamma radiation counting. The percent specific lysis was calculated as [experimental release − spontaneous release]/maximum release − spontaneous release) × 100. Spontaneous release represents the amount of radioactivity released from target cells without the addition of effector cells. Maximum release represents the amount of radioactivity released following lysis of target cells after the addition of Triton X-100 to 1%. The JEV-specific lysis of each group was calculated as the percent specific lysis of infected L929 cells − the percent specific lysis of uninfected L929 cells.
Results

The protective immunity to JEV DNA vaccine is differentially affected by coinjection of the IL-4 and IL-12 genes

We previously showed that plasmid pE encoding the JEV E protein elicited high titers of specific Abs and provide protection against lethal JEV challenge (24). To determine whether the protective immunity of the JEV DNA vaccine can be influenced by preferential induction of Ab or cell-mediated responses, pE was coadministered with pIL-4 or pIL-12 vectors producing murine IL-4 or IL-12, respectively. Groups of C3H/HeN mice were given i.m. injections three times at 3-wk intervals of 100 μg of plasmid pE and 100 μg of pIL-4, pIL-12 or a parental pTCAE vector. Mice that received 200 μg of the pTCAE vector served as negative controls.

Two weeks after the last immunization, animals were challenged with a lethal dose (3 × 10⁷ PFU; 50 LD₅₀) of JEV. As shown in Fig. 1, mice immunized with pTCAE alone were not protected, whereas pE + pTCAE vaccination resulted in 100% long-term survivors (>30 days after viral challenge). Coinjection of plasmid encoding IL-4 with pE had no significant effect on the protection rate, with 80% (8 of 10, p > 0.05) of animals surviving the challenge. Interestingly, vaccination with pE + pIL-12 dramatically suppressed protection against JEV challenge and resulted in only 30% (3 of 10, p < 0.005) long-term survivors. The suppressive effect of the IL-12 gene on pE-induced protective immunity was confirmed in three independent challenge experiments.

Effects of the pIL-12 plasmid on pE-induced Ab responses

The adverse effect of the IL-12 plasmid on JEV DNA vaccine was unexpected. To define the mechanisms leading to the suppressive effect of pIL-12, we analyzed the specific Ab responses in the immunized animals. Mice receiving pIL-12 with the booster immunization hampers the ability of the vaccine to expand an ongoing immune response.

We then analyzed whether coadministration of pIL-12 suppresses an ongoing Ab response induced by pE vaccination. To this end, mice were immunized with plasmid pE at priming and 3 wk later followed by a booster dose of pE with or without pIL-12 coinjection. Fig. 4 shows the result of the specific anti-E titers in the immunized animals. Mice receiving pIL-12 with the booster immunization produced 2-fold less anti-E Ab titer (44 ± 38 U/ml) compared with the animals that were boosted with pE without pIL-12 coinjection (90 ± 62 U/ml, p < 0.05). This result demonstrates that coinjection of the IL-12 gene with a booster pE DNA vaccination hampers the ability of the vaccine to expand an ongoing immune response.
The suppressive effect of pIL-12 is dose and timing dependent

To determine the minimal amount of pIL-12 necessary for suppression, groups of C3H/HeN mice were immunized with 100 μg of pE plus various doses of pIL-12 (100, 30, 10, or 3 μg) three times at 3-wk intervals. The anti-E Ab levels were assayed 2 wk after the last immunization. Animals treated with 100 μg of pE and 100 μg of pTCAE served as controls. Fig. 5A shows that suppression of Ab responses by the IL-12 plasmid was dose dependent; animals treated with higher doses of pIL-12 produced an apparent decrease in anti-E Ab responses. Coadministration of 3 μg of pIL-12 had no effect on pE immunization; the anti-E titer (Fig. 5A) and protective immunity (data not shown) were comparable to those obtained by injection of pE + pTCAE. pIL-12 at doses of 10 and 30 μg inhibited the anti-E titer by ~60 and 80%, respectively, whereas coadministration of 100 μg of pIL-12 almost completely abrogated the specific Ab response.

We then analyzed whether the timing of pIL-12 delivery influences its suppressive effect on pE DNA vaccine. All animals were immunized with 100 μg of pE at day 0. Groups of mice were also inoculated with 100 μg of pIL-12 on day −3, 0, or 3 relative to pE immunization. Following the initial priming, these animals were boosted twice with plasmid pE alone. Sera at different time points were collected and analyzed for the level of anti-E Abs. As shown in Fig. 5B, administration of pIL-12 either before or together with pE significantly decreased the anti-E Ab titers. In contrast, injection of pIL-12 3 days after pE vaccination did not suppress the specific Ab response.

T cell immunity to pE DNA vaccine was suppressed by coinjection of pIL-12

The effect of the IL-12 gene on the T cell immune response elicited by pE vaccination was then examined. Groups of C3H/HeN mice were given injections of pE + pIL-12 or pE + pTCAE three times at 3-wk intervals. Mice that received pTCAE alone served as negative controls. Four days after the last immunization, splenocytes were examined for proliferation in response to specific Ag stimulation. Immunization with pE + pTCAE induced a significant proliferative response to the E protein, with a mean stimulation index of ~5.8 (Fig. 6A). Coadministration of pIL-12 with pE significantly suppressed cellular proliferation, with the mean stimulation index decreased to 2.9 (p < 0.001). Mice vaccinated with the
It is well known that IL-12 can promote specific CTL activity. Thus, we tested whether the CTL activity induced by pE DNA vaccination, cytokine profiles (IL-4 and IFN-γ) released from the E protein-stimulated splenocytes of the different immunized groups were examined. Immunization with pE plus pTCAE or pIL-12 produced exclusively IFN-γ without detectable IL-4. Splenocytes from the pE + pIL-12 group produced much less IFN-γ (374 ± 104 pg/ml) as compared with cells from the pE + pTCAE group (533 ± 177 pg/ml). These results indicate that i.m. immunization with plasmid pE mainly elicited Th1 development, and coadministration of the IL-12 gene at the time of immunization diminished the specific T cell-proliferative responses.

It was previously shown that administration of high-dose recombinant IL-12 produced low JEV-specific CTL activity at high E:T ratios (Fig. 6B). Coexpression of the IL-12 plasmid with pE DNA vaccine failed to enhance CTL activity. We performed the CTL experiments several times and tested different approaches to improve its sensitivity but could only detect inferior amounts of CTL activity induced by pE immunization with or without pIL-12. In contrast, mice immunized with a sublethal dose of live JEV were able to induce a substantial level of JEV-specific lysis (Fig. 6B).

**IFN-γ mediates pIL-12-induced immunosuppression**

It was previously shown that administration of high-dose recombinant IL-12 can result in adverse immunological effects in virus infection (19, 20). Thus, we examined serum levels of IL-12 following pIL-12 administration as the first step to understand the underlying mechanisms leading to immune suppression to the pE DNA vaccine. Groups of C3H/HeN mice were treated with a mixture of pE + pIL-12 or pTCAE, and serum levels of IL-12 were determined at different time points. Treatment of pE + pIL-12 produced 2.3 ± 0.8 ng/ml of serum IL-12, which was significantly higher than the IL-12 concentration of 1.7 ± 0.1 ng/ml present in the control group (p < 0.05) (Fig. 7A). Because induction of IFN-γ was reported to be an important mediator leading to immune suppression by high-dose recombinant IL-12 treatment (29), we then determined serum levels of IFN-γ. Injection of pE + pTCAE produced 0.12 ± 0.02 ng/ml circulating IFN-γ, which was significantly lower than the level of 0.60 ± 0.51 ng/ml IFN-γ present in the pE + pIL-12 group (p < 0.05) (Fig. 7B).

To more clearly define the role of IFN-γ in the observed IL-12-mediated immunosuppression, in the following vaccine studies we used mice that are genetically deficient for IFN-γ (GKO). GKO mice on the C57BL/6 background and their wild-type littermates were immunized with pE + pIL-12 or pE + pTCAE. Serum from each mouse was then analyzed for JEV E-specific Ab titers. As shown in Fig. 8A, coadministration of pIL-12 with the pE DNA vaccine resulted in a dramatic reduction of anti-E titers in wild-type C57BL/6 mice. In contrast, in the GKO mice, similar levels of anti-E Abs were induced by pE vaccination with or without pIL-12. When the immunized animals were lethally challenged with JEV, coadministration of pIL-12 resulted in a significant decrease of the survival rates in the wild-type mice (40 vs 80% of the pE + pTCAE group) but not in the GKO mice (60 vs 70% of the pE + pTCAE group) (Fig. 8B). Taken together, these results demonstrate that induced endogenous IFN-γ is critical for the pIL-12-mediated suppressive effects in this JEV DNA vaccine model.

**Discussion**

IL-12 is a potent cytokine that promotes the development and activation of Th1 cells, and thus facilitates cell-mediated immunity. In addition to its striking therapeutic effects, IL-12 has also been used as a vaccine adjuvant in different vaccination strategies and helps protect against various infectious pathogens (4). However, in this study we found that IL-12 produced adverse effects to the immune responses elicited by DNA vaccines. Coadministration of pIL-12 with a JEV DNA vaccine strongly suppressed both humoral and cellular immune responses. The protective immunity induced by pE DNA vaccination was also significantly suppressed...
pIL-12 (100 μg) in GKO mice and wild-type controls were vaccinated with plasmid pE and/or alone (24) have been reported to induce protective immunity in mids encoding the JEV precursor membrane and E (33, 34) or E nonstructural proteins of JEV, the E protein appears to play the most important role in inducing protective immunity (24). Plasmids encoding the JEV precursor membrane and E (33, 34) or E alone (24) have been reported to induce protective immunity in mice. In this study, we used IL-4, a Th2-promoting cytokine, and IL-12, a Th1-promoting cytokine, to engineer the immune responses elicited by the pE DNA vaccine to determine the best protective immunity against JEV. We found that coinjection of the IL-4 plasmid had little effect on pE-induced protective immunity. In contrast, coinjection of the IL-12 plasmid profoundly decreased the protective rate of pE DNA vaccine from ~90 to 20–30% (Figs. 1 and 8B). The IL-12-mediated suppressive effect was observed in several inbred and outbred mouse strains and lasted for a long period of time. A single dose of pIL-12 treatment at initial priming suppressed two subsequent pE booster immunizations and resulted in a much lower survival rate (20 vs 80% of the pE alone group, Fig. 3). This suppressive effect of IL-12 in the JEV DNA vaccine model is unique and unexpected because many other studies showed that application of IL-12 as a vaccine adjuvant is beneficial on the course of many infectious diseases (4). For example, in a study of HSV-2 DNA vaccine, coinjection of the IL-12 plasmid significantly enhanced Th1 lymphocyte proliferation and resulted in better protection than the DNA vaccination alone (35). Analysis of the underlying mechanism leading to the suppressive effect of IL-12 in our JEV DNA vaccine model could provide important information for general application of IL-12 as a vaccine adjuvant.

We found that coinjection of pIL-12 with pE DNA vaccine led to an almost complete shutoff of JEV E-specific Abs. The Ab titer in mice immunized with pE was dramatically decreased from 189 U/ml to a barely detectable level of 6 U/ml in the presence of pIL-12 (Fig. 2). In addition, 30% of animals in the pE + pIL-12 group remained seronegative after repetitive immunizations. The pIL-12-mediated suppression was noticeable even when it was applied to animals with an ongoing immune response previously induced by pE vaccination (Fig. 4). We found that the Ab suppression by pIL-12 was dose dependent, requiring 30 μg of pIL-12 to achieve a significant suppression (Fig. 5A), but only needed one single pIL-12 treatment at the initial priming to eliminate the specific Ab response (Fig. 3). Previous studies have shown that IL-12 can either enhance or inhibit humoral immunity, depending on the immunoglobulin isotype. Administration of recombinant IL-12 with protein Ag resulted in a significant increase of Th1-associated IgG2a, IgG2b, and IgG3 Ab response (36, 37), whereas the Th2-associated IgG1 response was usually suppressed (38). In most studies of DNA vaccines, codelivery of the IL-12 gene was found to selectively increase IgG2a Ab, but had little effect or even decreased the total Ab titers (8, 39–41). In contrast, in our study with JEV DNA vaccine, we showed that i.m. injection of plasmid pE induced anti-E Ab almost exclusively of the IgG2a isotype (24), and that this Ab response was completely abrogated by codelivery of the IL-12 gene (data not shown). Thus, our data provide evidence that IL-12 treatment can sometimes generate profound suppression on both Th1- and Th2-associated Ab responses.

An even more surprising finding in our study was that IL-12 treatment down-regulated Th1 responses to DNA vaccines. Codelivery of pIL-12 with pE DNA vaccine inhibited the proliferative response of JEV-specific T cells (Fig. 6A) compared with T cells from animals immunized with pE alone. T cells from the pIL-12-treated group also produced significantly less IFN-γ. Mice immunized with pE only produced an insubstantial CTL response, and IL-12 treatment did not help increase CTL activity (Fig. 6B). These results were in contrast to findings from several other DNA vaccine studies that codelivery of the IL-12 gene significantly promoted Th1 cell response and CTL activity (8, 39–41). The cause of the discrepancy between this study and the previous studies is not clear, but may be due to the amount of IL-12 produced in the different studies. In our study, cardiotoxin was applied to the muscle 1 wk before injection of plasmids pE and pIL-12 to facilitate DNA uptake and gene expression. Cardiotoxin pretreatment of mouse muscles was reported to enhance reporter gene expression by >10-fold (42). Indeed, we could detect significant serum levels of IL-12 at day 3 (Fig. 7A) and day 8 (data not shown) after injection of the plasmid pIL-12, indicating that a large amount of IL-12 was produced from the pIL-12-treated muscle tissues. As mentioned above, administration of high doses of IL-12 has been shown to cause immune suppression in a number of infectious and malignant disease models (19–22). Thus, it is likely that the local high concentration of IL-12 produced by pIL-12-transfected muscle tissues in this study suppressed the nearby immune responses to the pE DNA vaccine. It should be noted that the suppressive effect of the DNA-expressing IL-12 was only limited to the local immune responses. Inoculation of plasmids pIL-12 and pE at different locations had no effect on the magnitude of immune responses and protective rate (data not shown). Treatment of pIL-12 also did not significantly alter the body weight and proportions of spleen and lymph node cells of the animals (data not shown). Another interesting finding was that the suppressive effect of pIL-12 was dependent upon the temporal relationship between Ag and cytokine delivery. Administration of pIL-12 before or together with the pE DNA vaccine suppressed anti-E Abs, whereas delayed pIL-12 treatment was not suppressive (Fig. 5B). Other studies also
showed that the relative timing of delivering Ag and cytokine plasmids was an important parameter in determining the overall biological effects of the cytokine. The timing of plasmid cytokine administration could either influence the magnitude of immune responses to a DNA vaccine (43) or determine the phenotype of a DNA vaccine-induced Th cell response (44).

The immune suppression mediated by high doses of IL-12 treatment was recently demonstrated to correlate with its ability to induce IFN-γ production by host lymphocytes. High levels of IFN-γ can activate macrophages and elicit inducible NO synthase activity to generate levels of NO that impair T cell responses (21, 22, 45). In our study, pE + pIL-12 generated a significantly higher serum level of IFN-γ compared with that produced by pE vaccination alone (Fig. 7B), suggesting that IFN-γ might be involved in the observed immune suppression. This hypothesis was confirmed by studies of IFN-γ-deficient GKO mice. In the GKO mice, codelivery of pIL-12 with pE DNA vaccine had no suppressive effect on either the anti-E Ab titer (Fig. 8A) or the overall protective rate (Fig. 8B). Taken together, these results suggest that the JEV E-specific T cells induced by pE vaccination are either eliminated or rendered unresponsive due to the local high concentration of IFN-γ induced by pIL-12 treatment.

In summary, we demonstrated in this study that codelivery of IL-12 plasmid with DNA vaccines can in some cases generate dose- and timing-dependent immune suppression. Therefore, the optimized conditions for delivering the IL-12 plasmid to benefit immune responses to DNA vaccines need to be carefully determined.

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