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Modulation of antigen-specific cellular immune responses to DNA vaccination in rhesus macaques through the use of IL-2, IFN-γ, or IL-4 gene adjuvants

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Abstract

Extensive experiments have shown DNA vaccines' ability to elicit immune responses in vivo in a safe and well-tolerated manner in several model systems, including rodents and non-human primates. As the DNA-based vaccine and immunotherapy approaches are being explored in humans, significant efforts have also been focused on further improving the immune potency of this technology. One strategy to enhance immune responses for DNA vaccines is the use of molecular or genetic adjuvants. These molecular adjuvant constructs (which encodes for immunologically important molecules such as cytokines) can be co-administered along with DNA vaccine constructs. Once delivered, these adjuvants have shown to modulate the magnitude and direction (humoral or cellular) of the vaccine-induced immune responses in rodent models. To date, however, there has been very little data reported from studies in primates. In this study, we examined the effects of cytokine gene adjuvants to enhance the level of cell-mediated immune responses in rhesus macaques. We co-immunized rhesus macaques with expression plasmids encoding for IL-2, IFN-γ or IL-4 cytokines along with the DNA vaccine constructs encoding for HIV env/rev (pCEnv) and SIV gag/pol (pCSGag/pol) proteins. We observed that coadministration of IL-2 and IFN-γ cDNA resulted in enhancement of antigen-specific T cell-mediated immune responses.

Keywords: DNA Vaccines; HIV-1; Cytokines; Adjuvants

1. Introduction

Nucleic acid or DNA inoculation is an important vaccination technique, which delivers DNA constructs encoding specific immunogens directly into the host [1–4]. These expression cassettes transfect host cells, which become the in vivo protein source for the production of antigen. This antigen then is the focus of the resulting immune response. Nucleic acid immunization is being explored as an immunization strategy against a variety of infectious diseases including HIV-1 [3,5,6].

Several groups have been investigating the use of molecular adjuvants as a method of enhancing and modulating immune responses induced by DNA immunogens. Co-delivery of these molecular adjuvants consisting of expression plasmid encoding for immunologically relevant molecules, including costimulatory molecules, cytokines, and chemokines with DNA vaccine constructs led to modulation of the magnitude and direction (humoral or cellular) of the immune responses induced in mice [5,7–9]. It has been reported recently that the modulation of immune responses through this approach could modulate disease progression in several mouse challenge models [9–11]. These results support that disease can be modulated by the use of cytokine adjuvants at least in mice; however, there has been very little data reported to date from studies in primates. Very recently we reported on the use of IL-2 and IL-4 cytokine adjuvants to enhance antigen-specific humoral immune responses in rhesus macaques [12].

In this study, we examined the effects of cytokine gene adjuvants to enhance the level of cell-mediated

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immune responses generated by a multicomponent DNA vaccine in the rhesus macaque primate model. We co-immunized rhesus macaques with expression plasmids encoding for Th1 (IL-2 and IFN-γ) or Th2-type (IL-4) cytokines along with the DNA vaccine constructs encoding for HIV env/rev (pCEnv) and SIV gag/pol (pCSGag/pol) proteins. We observed that antigen-specific cellular immune responses can be modulated in the rhesus macaque model using the cytokine molecular adjuvant approach. Codelivery of IL-2 and IFN-γ cDNA resulted in a positive modulation of antigen-specific T cell-mediated immune responses. These results further support the potential utility of this strategy as an important tool for the development of vaccines and immune therapies.

2. Materials and methods

2.1. DNA plasmids

DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and those expressing SIV gag/pol protein (pCSGag/Pol) were prepared as previously described [6,13,14]. The cytokine genes were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) as previously described [7,8].

2.2. Rhesus macaque immunization

Rhesus macaques (*Macaca mulatta*) were individually housed at the Primedica Mason Labs (Worcester, MA). All animal care and use procedures conformed to the revised Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animals were anesthetized with ketamine HCL for all technical procedures. Macaques were immunized intramuscularly (IM) in the quadriceps with DNA preparations formulated in phosphate buffered saline (PBS) and 0.25% bupivacaine–HCl (Sigma, St. Louis, MO) [7,8].

2.3. Lymphoproliferative assay

Recombinant HIV-1 gp120 and SIV p27 proteins were obtained from ImmunoDiagnostics, Inc. (Bedford, MA). Peripheral blood lymphocytes were prepared as previously described [15]. The isolated cell suspensions were resuspended to a concentration of 5×10^5 cells/ml in a media consisting of RPMI 1640 (Gibco-BRL, Grand Island, NY) with 10% fetal calf serum (Gibco-BRL). A 100 μl aliquot containing 5×10^5 cells was immediately added to each well of a 96-well microtiter round bottom plate. Recombinant p27 or gp120 protein at the final concentrations of 5 μg/ml and 1 μg/ml were added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for 3 days. One μCi of tritiated thymidine was added to each well and the cells were incubated for 12–18 h at 37°C. The plates were harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula: Stimulation Index

(SI) = (Experimental count/Spontaneous count).

Spontaneous count wells (media only) include 10% fetal calf-serum. To assure that cells were healthy, Concanavalin A (Sigma) was used as a polyclonal stimulator positive control.

2.4. Cytotoxic T lymphocyte assay

A standard 5 h ^{51}Cr release CTL assay was performed on PBMCs from the inoculated and control macaques as previously described [15]. Cells for in vitro stimulation of T cells were prepared by infecting autologously transformed B-lymphoblastoid cell lines (LCLs) with a recombinant vaccinia virus which expressed HIV-1 envelope (vMN462) or SIV gag proteins. Prior to use the infected cells were fixed with 0.1% glutaraldehyde and blocked with a 0.1 mM glutamine solution. The fixed cells were incubated with the isolated PBMCs (effectors) for stimulation in CTL stimulator media (RPMI 1640 (Gibco–BRL), 10% fetal calf serum (Gibco–BRL), and recombinant IL-2 (40 U/ml) (Intergen, Purchase, NY)) for 3 weeks. The LCLs infected with specific recombinant vaccinia virus or control recombinant vaccinia virus expressing β-galactosidase (vSC8) were also used as target cells. Cells incubated with the control vaccinia were used as targets to provide background levels of lysis.

2.5. Cytokine expression analysis

Supernatants from effectors stimulated for CTL assay (see above) were collected after specific stimulation and were tested for cytokine profile using ELISA kits for IFN-γ and IL-10 (Biosource International, Inc., Camarillo, CA). Each well values were used to derive the average and the standard deviation values.

3. Results

3.1. Co-delivery of IL-2, IFN-γ, and IL-4 expression cassettes in rhesus macaques

Primates are the most relevant animal models for testing vaccines for potential human use. Previously, it has been reported that DNA immunizations alone in primates may not be sufficient to generate high levels of antigen-specific antibody responses [16]. In this study intramuscular (IM) immunization of an HIV-1 gp120

DNA vaccine construct using a large dose (2 mg of DNA given eight times at 4-week intervals) in rhesus macaques elicited only a low level of antigen-specific binding and no detectable neutralizing antibodies [16]. These observations suggest reduced immunogenecity of DNA vaccines in non-human primates.

Cytokines play a critical regulatory and signaling role in the development of an immune response. Cytokines, which act on lymphocytes, are of special interest because of their role in regulating cells of the immune system. For instance, the presence of IL-2, IFN-γ, and IL-12 activates the T_h0 precursor cell to become a T_h1 inflammatory T cells [17]. On the other hand, the presence of IL-4, IL-5, or IL-10 results in a Th0 precursor becoming an armed Th2 helper cell [17]. IL-2 is produced primarily by stimulated T cells and is critical for the proliferation and clonal expansion of antigen-specific T cells [18]. IL-4 is a prominent Th2 cytokine that plays an important role in the induction of humoral immune responses [19].

We and others have been investigating the use of molecular adjuvants as a method of enhancing and modulating immune responses induced by DNA immunogens [5,7–9,20–22]. Co-delivery of these molecular adjuvants consisting of expression plasmid encoding for immunologically relevant molecules such as cytokines with DNA vaccine constructs led to modulation of the magnitude and direction (humoral or cellular) of the immune responses induced in mice [5,7–9].

We investigated whether the enhancement of immune responses observed in mice through co-immunization with cytokine genes could also be achieved in rhesus macaques.

We chose to examine cytokines (IL-2, IFN-γ, and IL-4) that we had previously studied in mice [8,23].

Four groups of two rhesus macaques each were immunized with various DNA vaccine constructs. The first group was immunized with HIV env/rev (pCEnv) and SIV gag/pol (pCSGag/pol) constructs along with a control vector pCDNA3. The second group was immunized with pCEnv + pCSGag/pol + IL-2 constructs. The third and fourth groups were immunized with pCEnv + pCSGag/pol + IL-4 and pCEnv + pCSGag/pol + IFN-γ, respectively. These macaques were immunized with 200 μg of each DNA at weeks 0, 6, and 12, and boosted with 500 μg of each DNA at weeks 28 and 49. These constructs were mixed prior to injection into the quadriceps muscle.

We had previously reported that rhesus macaques immunized with pCEnv + pCSGag/pol + IL-2 or pCEnv + pCSGag/pol + IL-4 had significant enhancement of the levels of *anti*-envelope or *anti*-gag antibodies [12]. In fact the magnitude of antibody response enhancement in macaques was even greater than the results observed in mice [8,12]. These results demonstrated that antigen-specific antibody responses can be driven to a higher level through the use of cytokine genetic adjuvants in rhesus macaques.

3.2. Cytokine gene co-immunizations modulate cellular responses in rhesus macaques

The effect of cytokine gene adjuvants on the antigen-specific Th cell proliferative and CTL responses in macaques were examined. As shown in Figs. 1 and 2, induction of antigen-specific lymphoproliferative (LPA) responses against gp120 and p27 proteins were observed in all vaccinated animals, but to a varying degree. The groups immunized with pCEnv + pCSGag/pol + pCDNA3 constructs as well as those co-immunized with Th1 cytokines IL-2 and IFN-γ had similar frequency but higher levels of proliferative responses than the group co-immunized with Th2 cytokine IL-4, suggesting a small effect on the resulting immune response at this dose. Overall, the effects of IL-2 and IFN-γ adjuvants on antigen-specific LPA in macaques were less consistent than those effects observed in mice.

We also examined the level of antigen-specific CTL responses in these immunized macaques. We evaluated CTL responses by specifically restimulating the effector T cells in vitro with recombinant vaccinia infected targets (immortalized autologous cell lines) expressing the HIV Env or SIV gag/pol proteins. Antigen-specific (SIV gag/pol) CTL responses in these animals at weeks 42 and 53 are shown in Fig. 3. Overall, the frequency and the consistency of CTL response were low. However, it is interesting that the animals immunized with pCEnv + pCSGag/pol + IL-2 or pCEnv + pCSGag/pol + IFN-γ (especially animals 3, 5, and 6) had strong gag/pol-specific CTL responses at week 53, and in particular the CTL response in animals 3 and 5 were quite high. In fact, these results compare favorably to the results observed in mice.

In addition to these observations, we also analyzed the expression of Th1 and Th2-type cytokine (IFN-γ and IL-10, respectively) expression profiles from individual animals. Cytokines play a key role in directing and targeting immune cells during the development of the immune response. For instance, IFN-γ is produced by Th1 and CD8 + T cells and is intricately involved in the regulation or development of *anti*-viral T cell-mediated immune responses [24,25]. In contrast, IL-10 is produced by many cell types including putative Th2 lymphocytes and has been shown to be a potent Th2-type cytokine [26,27]. In mice, the expression of IFN-γ has been correlated directly with the potency of Th1-type cellular immune responses. Thus, analysis of these cytokines secreted by stimulated T cells may be important in elucidating the extent of cell-mediated responses following DNA immunization in macaques [28].

As shown in Fig. 4, the stimulated T cells from vaccine-immunized rhesus macaques produced higher levels of IFN-γ than the control animals. On the other hand, the level of IL-10 produced by either the protected or unprotected groups was similar. We also

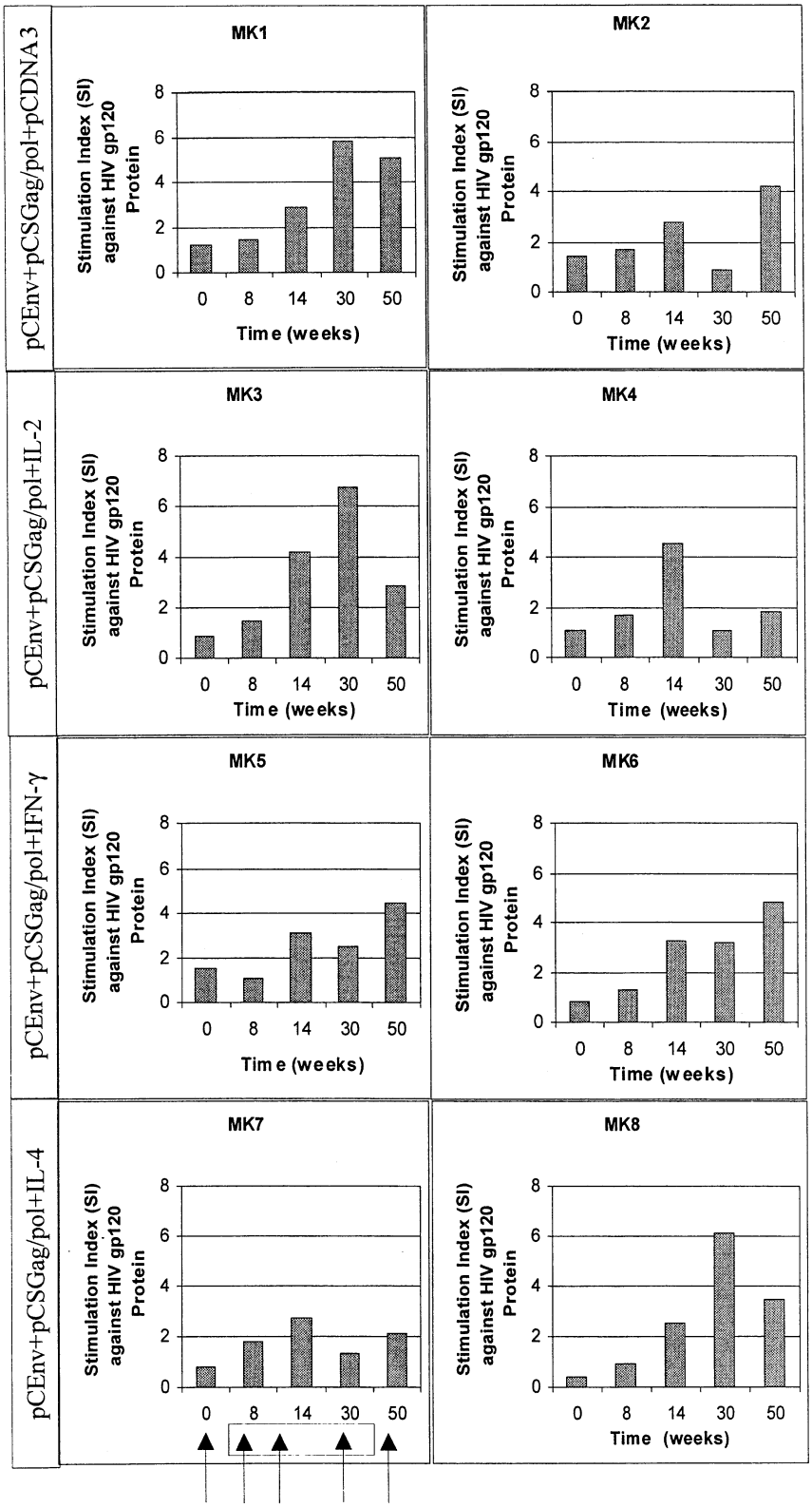

Fig. 1. Envelope-specific lymphoproliferative responses following immunization. Five groups of two rhesus macaques were immunized with 200 μg of each DNA vaccine construct at weeks 0, 6, and 12, and boosted with 500 μg of each DNA at weeks 28 and 49 (as indicated by the arrows). The PBMCs were recovered from the whole blood using the protocol described above. The levels of antigen-specific lymphoproliferative activity (LPA) to HIV gp120 envelope protein (at 5 μg/ml concentration) were examined following immunization using the protocol described above. The control animals were also assayed and were routinely negative.

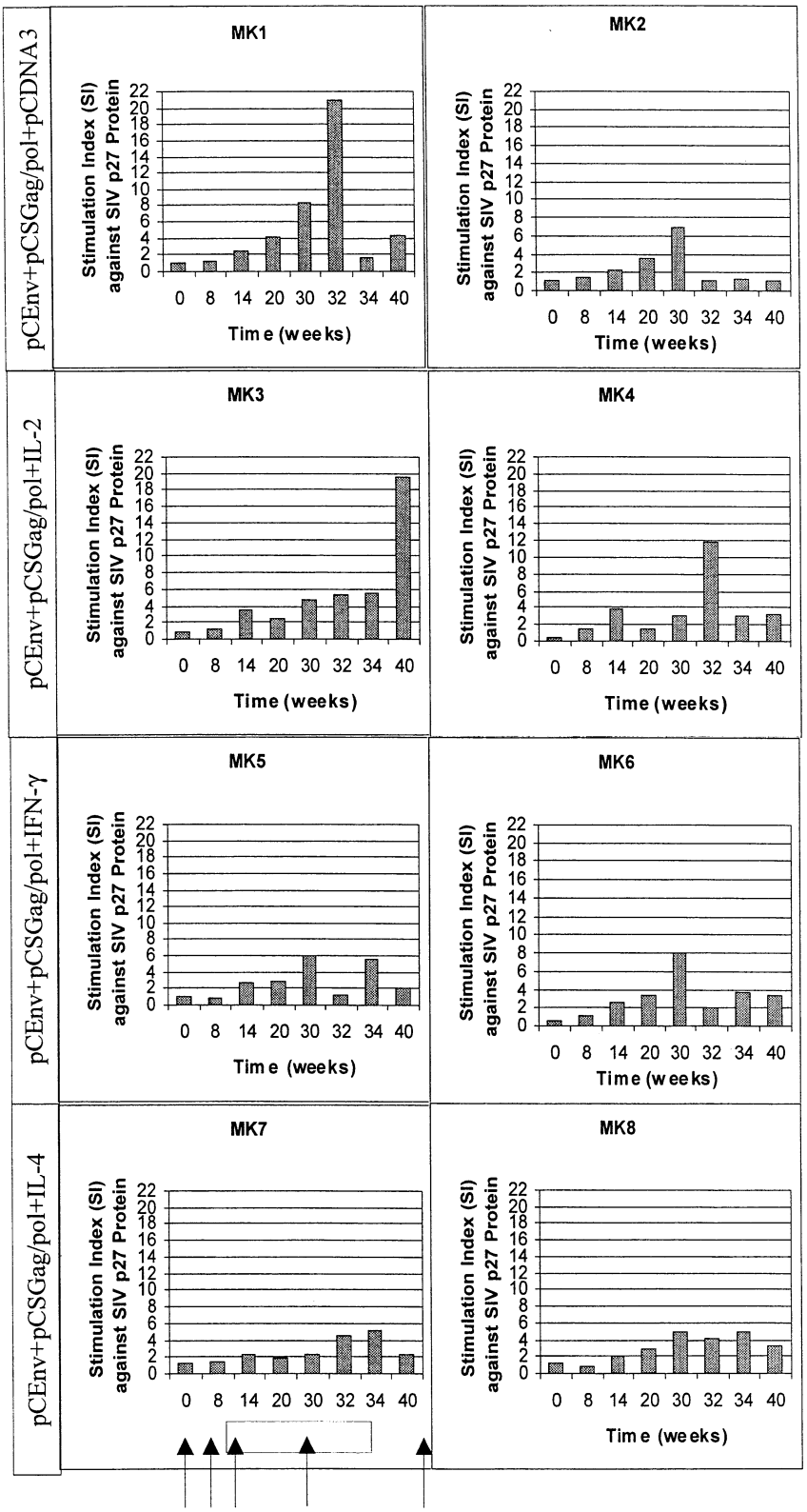

Fig. 2. Gag-specific lymphoproliferative responses following immunization. The levels of antigen-specific LPA to SIV p27 gag protein (at 5 μg/ml concentration) were examined following immunization using the protocol described above. The control animals were also assayed and were routinely negative.

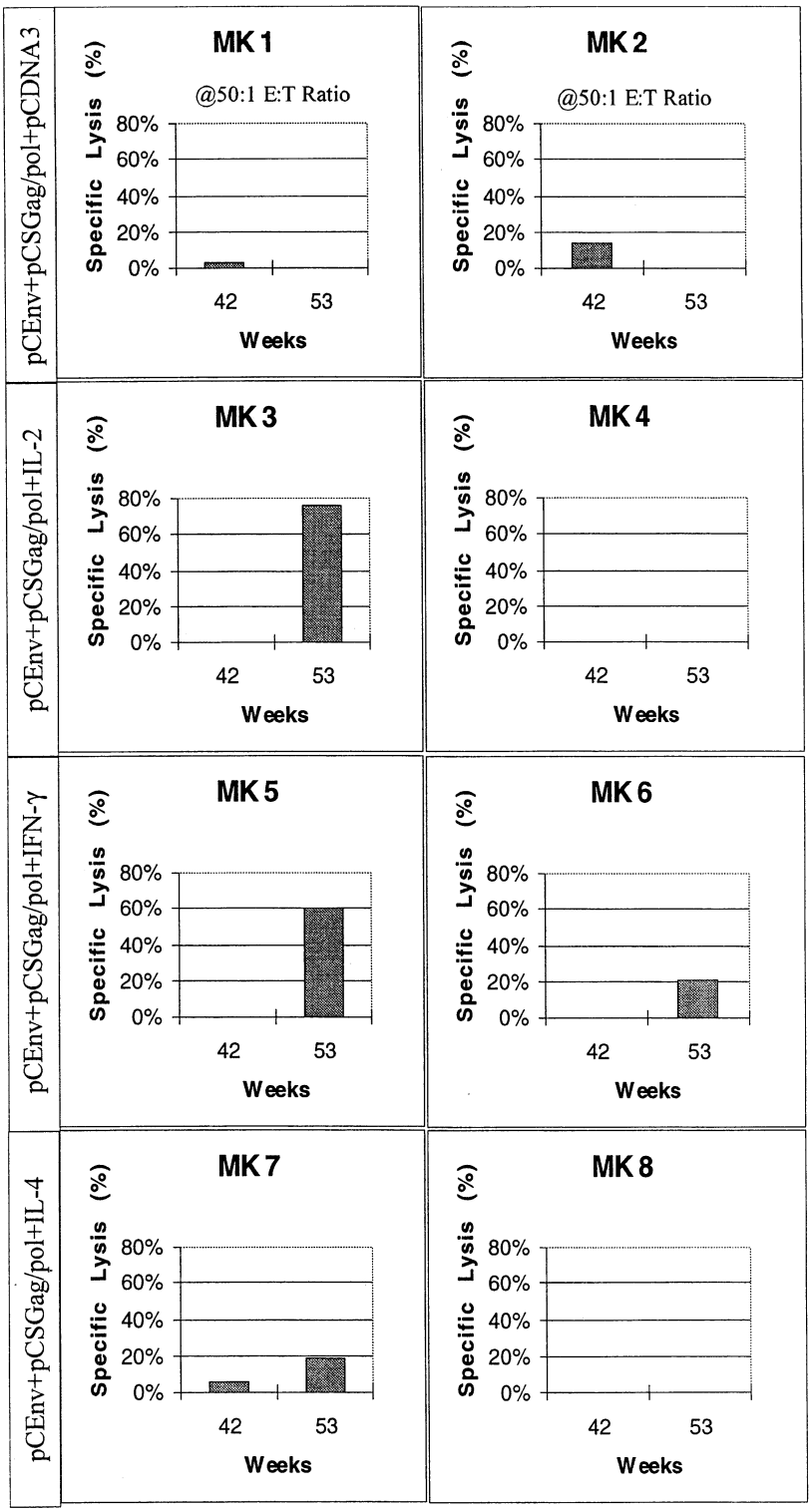

Fig. 3. Cytotoxic T lymphocyte (CTL) response following immunization. The levels of antigen-specific CTL response were examined at week 53. Following 21-day in vitro stimulation of effector cells, the assay was performed by measuring chromium release from specific and non-specific vaccinia infected targets. Vaccinia virus expressing SIV gag/pol proteins were used to infect specific target cells. To calculate specific lysis of targets, the percent lysis of irrelevant targets was subtracted from the percent lysis of specific targets. The figure shows the level of specific lysis against SIV gag/pol expressing target cells at 50:1 Effector:Target ratio.

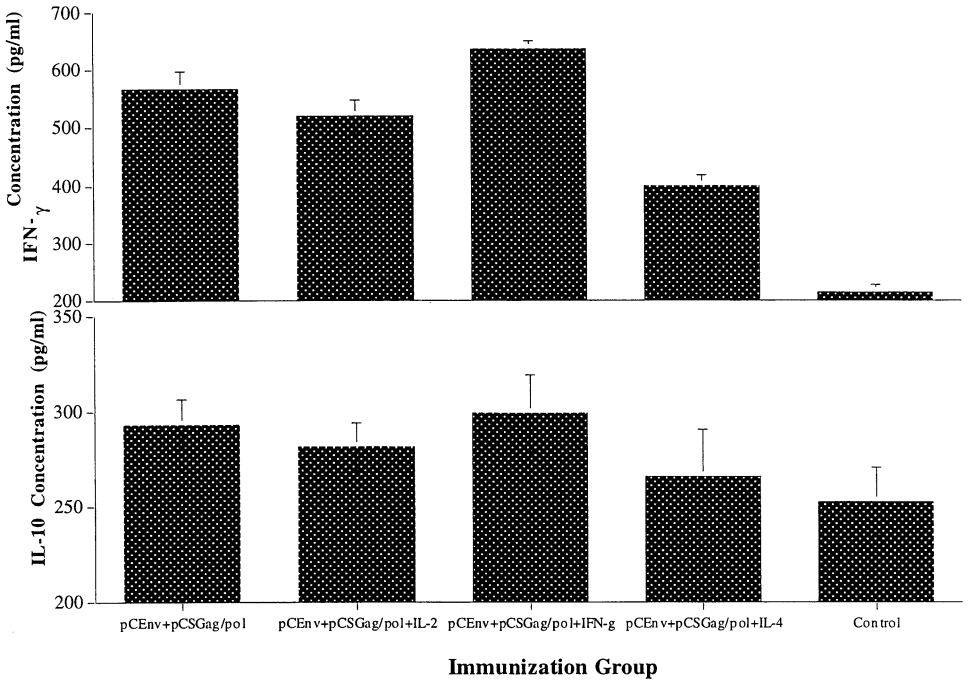

Fig. 4. Variant levels of Th1 and Th2-type cytokine production by immunized animals. Expressions of IFN-γ and IL-10 from animals at week 53 were analyzed by ELISA. The average of each group is represented.

observed that significantly higher levels of T cell-produced IFN-γ in animals immunized with pCEnv + pCSGag/pol + IFN-γ compared to those animals immunized with pCEnv + pCSGag/pol + pCDNA3 constructs. These results seemed to correlate with the level of CTL responses shown in Fig. 3.

4. Discussion

DNA immunization is an important vaccination strategy, which delivers DNA constructs encoding for a specific immunogen into the host [29–31]. These expression cassettes transfect the host cells, which become the in vivo protein source for the production of antigen. This antigen then is the focus of the resulting immune response. This vaccination technique is being explored as an immunization strategy against a variety of infectious diseases including HIV-1. Extensive experiments have shown that DNA vaccines are able to elicit humoral and cellular responses in vivo in a safe and well-tolerated manner in various model systems, including rodents and non-human primates. The first DNA vaccine studies to enter the clinic were DNA vaccines encoding for HIV-1 MN envelope [32]. Fifteen healthy HIV-1 sero-positive volunteers in the trial received three injections each separated by ten weeks with escalating dosage (three dosage groups of five subjects) of envelope vaccine. Preliminary results reveal no significant clinical or laboratory adverse effects measured in all three dosage groups (30, 100, 300 μg). More importantly, the immunized individuals developed an increase in antibody responses to envelope proteins and peptides after receiving the 100 μg dose. Some increases in cellular responses including the lymphoproliferative and CTL responses as well as β-chemokine expression were also observed [32,33]. In addition, phase I trials evaluating a gag/pol construct as a therapeutic vaccine as well as a prophylactic DNA vaccine study for HIV has been undertaken.

These early clinical studies have established the DNA vaccines' ability to elicit immune responses in a safe and well-tolerated manner. However, significant efforts have also focused on improving the immune potency of this technology, as it is unclear if in its present form its potency will be clinically useful. Improving vaccine potency is a central mission of the field of DNA vaccines. One strategy to enhance immune responses for DNA-based vaccines is the use of molecular adjuvants [34]. These molecular adjuvant constructs could be co-administered along with immunogen constructs to modulate the magnitude and direction (humoral or cellular) of the immune responses induced by the vaccine cassettes themselves. Such use of molecular adjuvant constructs results in concurrent kinetics of in vivo expression for both the adjuvant and antigen proteins.

In order to focus the immune responses induced from DNA vaccines, we and others have investigated the co-delivery of cytokine gene adjuvants to modulate vaccine responses [5,7,8,10,20–22,35–37]. We initially reported that co-immunization of GM-CSF cDNA with DNA vaccine constructs increases antigen-specific

antibody and T helper cell proliferation responses while co-immunization with IL-12 cDNA results in weaker antibody responses and enhanced T helper cell proliferation in mice [7,35]. In addition, IL-12 co-immunization resulted in a significant enhancement of CTL responses.

More recently, we observed the modulation of the induction and regulation of immune responses from the co-delivery of proinflammatory (IL-1α, TNF-α, and TNF-β), T_h1 (IL-2, IL-12, IL-15, IL-18, and IFN-γ), and T_h2-type (IL-4, IL-5 and IL-10) cytokines [8,23]. We also observed that codelivery of Th1 or Th2-type cytokine gene adjuvants regulated the immune responses are more important for protection from HSV-2 infection [9]. We co-delivered DNA expression construct encoding for HSV-2 gD protein with the gene plasmids encoding for Th1-type (IL-2, IL-12, IL-15, IL-18) and Th2-type (IL-4, IL-10) cytokines in an effort to drive immunity induced by vaccination. We then analyzed the vaccine modulatory effects on resulting immune phenotype and on the mortality and the morbidity of the immunized animals following HSV lethal challenge. We observed Th1 cytokine gene co-administration not only enhanced survival rate, but also reduced the frequency and severity of herpetic lesions following intravaginal HSV challenge. On the other hand, co-injection with Th2 cytokine genes increased the rate of mortality and morbidity of the challenged mice.

In this study, sought to evaluate whether the enhancement of immune responses observed in mice with co-immunization with cytokine genes could also be achieved in rhesus macaques. DNA vaccines for HIV env/rev and SIV gag/pol alone were evaluated for their immunogenicity and compared to these vaccines which also included IL-2 or IFN-γ (Th1) or IL-4 (Th2) cytokine cDNA constructs.

We had previously observed that macaques immunized with pCEnv + pCSGag/pol + IL-2 or pCEnv + pCSGag/pol + IL-4 had a significant enhancement of the levels of *anti*-envelope or *anti*-gag antibodies while the macaques immunized with pCEnv + pCSGag/pol + IFN-γ had a more moderate response [12]. We also observed that IL-2 and IFN-γ cDNA codelivery resulted in some moderate effects on the levels of antigen-specific LPA responses against gp120 and p27 proteins. The effects of IL-2 and IFN-γ adjuvants on LPA response in macaques were less dramatic than those effects observed in mice. In contrast, the group co-immunized with Th2 cytokine IL-4 seemed to reduce the frequency and extent of antigen-specific LPA responses.

On the other hand, we also observed that the macaques immunized with pCEnv + pCSGag/pol + IL-2 or pCEnv + pCSGag/pol + IFN-γ (especially animals 3, 5, and 6) had strong gag/pol-specific CTL responses at week 53, and in particular the CTL response in animals 3 and 5 were quite high. In fact, the level of *anti*-Gag CTL responses observed in these macaques are actually greater than those levels previously observed in immunized mice [23]. We also observed that stimulated T cells from the pCEnv + pCSGag/pol + IFN-γ immunized animals produced significantly higher levels IFN-γ compared to those animals immunized with pCEnv + pCSGag/pol + pCDNA3 constructs. As in mice, the higher IFN-γ expression levels seemed to correlate with the higher levels of CTL responses [37,38].

Overall, the immunomodulatory effects of cytokine gene adjuvants on cellular immune responses were more moderate compared to those observed in mice. There could be several hypothesis for this observation. One possible cause is that the dosage used in macaques (ranging from 200 to 500 μg per injection) may have been low in comparison to the dosage used in the mouse studies (50 μg per injection). Given the large differences in weights, the dosage used in macaques may have been underestimated. Additional studies using larger dosages could clarify this issue. Another potential cause is that human cytokine cDNA constructs were used in this macaque study while mouse cytokine cDNA constructs were utilized in the mouse studies. Even though human IL-2, IFN-γ, and IL-4 are cross-reactive in macaques, there may be some reduction in potency. Using macaque cytokine cDNA constructs to immunize macaques could further enhance their immunomodulatory effects to the levels observed in mice.

These results further support the use of cytokine gene adjuvant approach and demonstrate that antigen-specific cellular immune responses can be modulated positively in rhesus macaques through the use of IL-12 and IFN-γ gene adjuvants. While additional studies are needed to further improve the potency and consistency of these approaches in primates, these results have important implications for the development of clinically relevant vaccines and immunotherapy approaches using the DNA platform.

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