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# Augmented Induction of Antigen-Specific Cytotoxic T Cell Responses against Canine Hepatitis by Co-Immunization with pVAX1-CpG-Loop and Adjuvants in BALB/c Mice

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**Abstract:** The objective of this study was to obtain better antigen specific cytotoxic T cell responses *in vivo*. We examined the augmented induction of antigen-specific cytotoxic T cell responses to co-administration of oligonucleotides (CpG-ODN), dimethyl dioctadecyl ammonium bromide (DDA), and Lipofectamine™ 2000 with a DNA vaccine (pVAX1-CpG-Loop) and boosting with pVAX1-CpG-Loop in BALB/c mice. The results show that Loop protein-specific T cell proliferation, cytotoxic T cell activity, and the production of CD8+ T cells and IFN- $\gamma$  were enhanced after co-immunization of mice with adjuvants and pVAX1-CpG-Loop. We demonstrated that significant T cell-mediated immune responses were induced in the mice with the help of DDA, CpG-ODN and Lipofectamine™ 2000.

**Key words:** adjuvants, BALB/c mice, cytotoxic T cell responses, plasmid pVAX1-CpG-Loop

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## Introduction

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Infectious canine hepatitis (ICH) is caused by canine adenovirus type 1 (CAV-1). The increasing prevalence of ICH has increased the need for effective vaccination strategies [21]. The demonstration that CD8+ cytotoxic lymphocytes (CTLs) can mediate immunity against both bacterial and viral pathogens has provided the opportunity to develop vaccine strategies that rely on the generation of CTL-mediated immunity [17]. In order to obtain effective T cell-based vaccines, some obstacles must be overcome. The main obstacle appears to be the

difficulty associated with inducing strong effector T cell responses, especially stronger CD8+ cytotoxic T-cell responses. Prime-boost strategies have thus far been the most effective at triggering potent T cell responses [16]. Moreover, selecting the appropriate adjuvant for use with specific antigenic components would not only enhance the immune response, but also determine the type of immune response. Therefore, an adjuvant is necessary in the vaccine formulation to induce an adequate immune response [4].

Synthetic oligodeoxynucleotides (ODNs), a Toll-like receptor 9 (TLR9) ligand, that contain immunostimula-

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tory CpG motifs can trigger an immunomodulatory cascade that involves B and T cells, natural killer cells, and professional antigen-presenting cells. The response to CpG-ODNs skews the host's immune milieu in favor of T helper 1 (Th1)-cell responses, suggesting their use as a vaccine adjuvant [9]. As an adjuvant, DDA can adsorb most antigens on its surface and promote a strong cell-mediated immune response *in vivo*, which is essential for the induction of protective immunity against most viral infectious diseases [2]. Similarly, Lipofectamine™ 2000, a cationic liposome, has been widely used to enhance DNA vaccines and preferentially activate Th1 responses [12]. Cationic liposomes can interact with the negatively charged cell membrane, and they are widely used for gene therapy and vaccine delivery [1, 6]. Thus, DDA and Lipofectamine™ 2000 as adjuvants for immunization may have potential in vaccination against canine adenovirus type 1 pathogens and infectious canine hepatitis.

In previous studies, we amplified Loop1 and Loop2 from CAV-1 genomic DNA and constructed the plasmid pVAX1-CpG-Loop, which induces cell-mediated and humoral immune responses [25]. In order to obtain better cytotoxic T cell responses in mice, we compared the effects of three types of adjuvants CpG-ODN, DDA and Lipofectamine™ 2000, in mice co-immunized with pVAX1-CpG-Loop. In this study, we show that prime potent CTL responses are induced by the three types of adjuvants upon co-immunization with pVAX1-CpG-Loop. Moreover, the strategy of priming with pVAX1-CpG-Loop (25 µg/mouse) co-administered with adjuvant and boosting with only the pVAX1-CpG-Loop (25 µg/mouse) was equally capable of boosting memory CTL responses in mice.

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## Materials and Methods

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### *Plasmid isolation and purification, Loop protein expression and purification*

The plasmid pVAX1-CpG-Loop was isolated by alkaline lysis and purified by polyethylene glycol precipitation [15]. Prokaryotic expression of recombinant pET28a-Loop was performed as described previously [25]. The recombinant Loop protein containing a 6-histidine tag was expressed and purified by a Ni<sup>2+</sup> affinity

column [15], and stored at -80°C. This protein was used as the stimulating protein to stimulate the cells in the Loop protein-specific lymphocyte proliferation assay, for intracellular staining of interferon-gamma (IFN-γ), and in the Loop protein-specific CTL activity assay.

### *Synthetic CpG-ODN*

CpG-ODN (1826) was synthesized under GMP (Good Manufacturing Practice) conditions (SBS Genetech Co., Ltd., Beijing, China) with a nuclease-resistant phosphorothioate backbone and a sequence of 5'-TCC ATG ACG TTC CTG ACG TT-3' [3, 11]. The CpG oligonucleotide and plasmid pVAX1-CpG-Loop were dissolved in PBS solution prior to immunization.

### *Preparation of DDA solution*

DDA (Sigma, St. Louis, MO, USA) adjuvant was prepared as described previously [17, 23, 27]. Briefly, DDA adjuvant was mixed with 10 mM Tris-buffer at pH 7.4 to a concentration of 1.25 mg/ml and heated to 80°C for 20 min with intermittent shaking until the DDA had completely dissolved to form a gel. The mixture was allowed to cool to room temperature, and was mixed with the pVAX1-CpG-Loop plasmid before immunization.

### *Preparation of Lipofectamine™ 2000 solution*

Forty microliters of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was dissolved in sterile PBS solution prior to immunization. The plasmid was added to the Lipofectamine™ 2000 drop-by-drop, and then cultured at room temperature for 20 min before immunization.

### *Mice*

BALB/c (BALB/cJ) mice were bred and cared for in the Animal Facilities of Hebei Medical University (Shijiazhuang, China), which imported from National Resource Center for Rodent Laboratory Animal (Beijing, China). Only 6–8-week-old female mice were used in the experiments. The mice were used in accordance with the institutional guidelines for animal care.

### *Immunization protocols*

*Experiment 1:* Mice were randomly divided into 4

experimental groups (5 mice/group). For DNA immunizations using pVAX1-CpG-Loop expression plasmids, pVAX1-CpG-Loop plasmid DNA vaccine (25  $\mu\text{g}$ ) was first mixed with various amounts of adjuvant CpG-ODN (20  $\mu\text{g}$ ) or DDA (250  $\mu\text{g}$ ) or Lipofectamine™ 2000 (40  $\mu\text{l}$ ) and then injected intramuscularly (i.m.) in 100  $\mu\text{l}$  of sterile saline divided between the right and left quadriceps muscles. Mice were intramuscularly injected with the pVAX1-CpG-Loop and adjuvant (25  $\mu\text{g}/100 \mu\text{l}$ /mouse) three times on a biweekly schedule.

*Experiment 2:* The grouping plan used was as described in Experiment 1. After three times immunization, mice in all the groups were re-injected on the fourteenth week with pVAX1-CpG-Loop (25  $\mu\text{g}/\text{mouse}$ ) only.

*Experiment 3:* The grouping plan used was as described in Experiment 1. At 2-week intervals the mice were intramuscularly injected three times with the DNA vaccine (100  $\mu\text{g}/\text{mouse}$ ) and CpG-ODN (20  $\mu\text{g}/\text{mouse}$ ) or DDA (250  $\mu\text{g}/\text{mouse}$ ) or Lipofectamine™ 2000 (40  $\mu\text{l}/\text{mouse}$ ) in 100  $\mu\text{l}$  of sterile saline.

#### ELISA

Sera were collected from the immunized mice and specific IgG antibody was detected by indirect ELISA, as previously described [25]. Briefly, dilutions of the serum sample were 1:10, 1:20, 1:40, and 1:80. Purified CAV-1 was used as the antigen, and specific anti-virus IgG (goat anti-mouse IgG-HRP [Horseradish Peroxidase], Zhong Shan Company, Beijing, China) antibody was detected. Sera from non-immunized mice was used as the negative control. Sera from mice immunized with a destroyed-infectivity canine adenovirus vaccine (Fort Dodge Animal Health, Overland Park, KS, USA) were used as a positive control. Absorbance was determined at 492 nm.

#### Splenocyte culture

Two weeks after the final vaccination, the mice were humanely culled and their spleens aseptically removed. The lymphocytes were separated as described previously [18]. Briefly, mouse lymphocyte separating medium (Tian Jin Hao Yang Biological Manufacture, Co., Ltd., Tianjin, China) was used to isolate the lymphocytes. After centrifugation, the supernatant was removed and

the lymphocytes were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS).

#### Specific lymphocyte proliferation assay

The lymphocyte proliferation assay was performed as described previously [18, 25]. Briefly, splenocytes ( $5 \times 10^6$  cells/ml) were incubated with Loop protein (15, 30, 60  $\mu\text{g}/\text{ml}$ ) in triplicate wells of flat-bottom plates. Untreated lymphocytes cultivated in medium alone served as the negative control. Splenocytes ( $5 \times 10^6$  cells/ml) incubated with concanavalin A (—ConA) (5  $\mu\text{g}/\text{ml}$ ) were used as the positive control. The plates were incubated for 72 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Absorbance (A) at 570 nm was determined using a standard 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) method. The results are expressed as the value obtained for the stimulation index (SI). The SI was calculated based on the formula:  $SI = (A_{\text{restimulated}} - A_{\text{blank}}) / (A_{\text{unrestimulated}} - A_{\text{blank}})$ .

#### Surface staining of CD8+ T cells and the staining of intracellular IFN- $\gamma$

The numbers of CD8+ T cells and IFN- $\gamma$ -producing T lymphocytes were determined by flow cytometry, as described previously [18, 25]. For these experiments,  $1 \times 10^7$  spleen cells from immunized mice were cultured in 0.5 ml of complete DMEM in the presence of 30  $\mu\text{g}/\text{ml}$  of recombinant Loop protein and 10 ng/ml of interleukin-2 (R&D, Minneapolis, MN, USA) for 72 h. Untreated lymphocytes cultivated in medium alone served as the negative control. Splenocytes ( $5 \times 10^6$  cells/ml) incubated with ConA (5  $\mu\text{g}/\text{ml}$ ) were used as the positive control. The stimulated lymphocytes were harvested and labeled to detect surface CD8 and intracellular IFN- $\gamma$  using PE (Jingmei Biotech, Shanghai, China) or fluorescein isothiocyanate (BioLegend, San Diego, CA, USA) labeled specific monoclonal antibodies, following the detailed instructions provided by the vendor. Spleen cells were analyzed via flow cytometry (Becton Dickinson, Mountain View, CA, USA).

#### Eukaryotic cell transfection

The CT26 cell line was cultured in DMEM containing 10% fetal bovine serum (FBS) and was transfected with

plasmid pVAX1-CpG-Loop or pVAX1-CpG vector using Lipofectamine™ 2000, according to the manufacturer's protocol [25]. After 48 h incubation at 37°C and 5% CO<sub>2</sub>, CT26 cells were collected and analyzed by RT-PCR and western blot. The total protein obtained from transfected CT26 cells was separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. A 1:50 dilution of monoclonal antibody against the Loop protein and a 1:5,000 dilution of HRP-labeled anti-mouse antibody (Southern Biotechnology Associates, Birmingham, AL, USA) were used as the primary and secondary antibodies, respectively. The membrane was stained using diaminobenzidine (DAB) [25].

#### *Specific CTL activity assay*

In order to test the functional capacity of the induced Ag-specific CD8<sup>+</sup> cells, we performed an *in vitro* CTL assay. CTL stimulation and cell-mediated lymphocyte cytotoxicity were measured by the lactate dehydrogenase (LDH) release assay, as described previously [7, 14]. The splenocytes from primed mice ( $1 \times 10^7$  cell/well) were cultured with Loop protein (30 µg/ml) and recombinant mouse IL-2 (10 ng/ml) in DMEM for 7 days in 6-well tissue culture plates. Cultured splenocytes were used as effectors, and CT26 cells transfected with plasmid pVAX1-CpG-Loop were used as target cells. Untransfected CT26 cells were used as the negative control. Spontaneous and maximum releases were measured by incubating target cells with media and 2% Triton X-100, respectively. In 96-well round-bottom plates, effector cells were incubated with target cells at an effector:target (E:T) ratio of 100:1, 50:1, and 25:1. The mean percentage of specific lysis in triplicate wells was calculated as follows: % specific lysis = [(experimental release - spontaneous release) / (maximum release - spontaneous release)] × 100.

#### *Statistical analyses*

Data from all groups are presented as means ± SD, and were statistically analyzed by the statistical software program System 11.5 (SPSS). Differences between the groups were analyzed by one-way ANOVA and a least significant difference test. Data were considered to be significantly different at  $P < 0.05$ .

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## Results

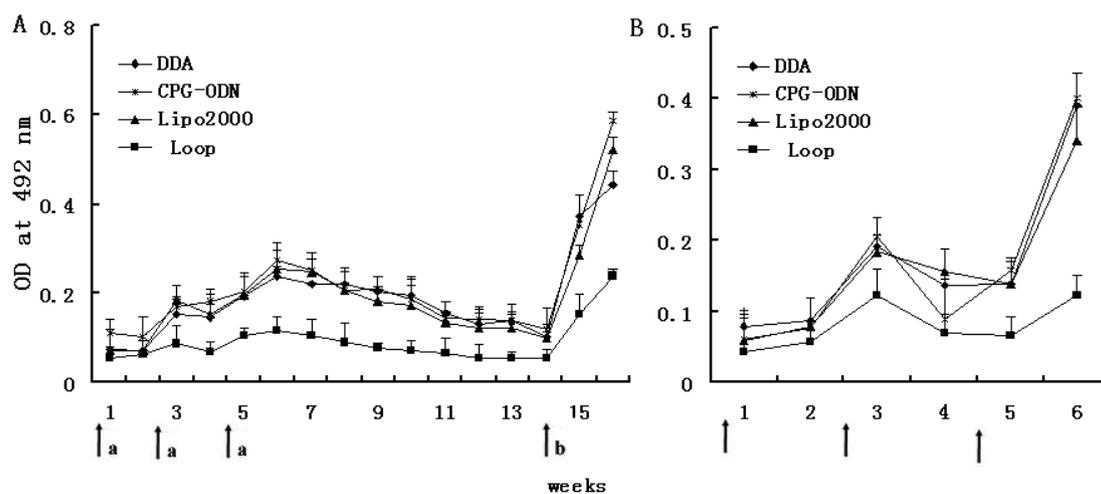
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### *Higher levels of Ag-specific IgG were induced by coimmunization with adjuvant and DNA vaccine*

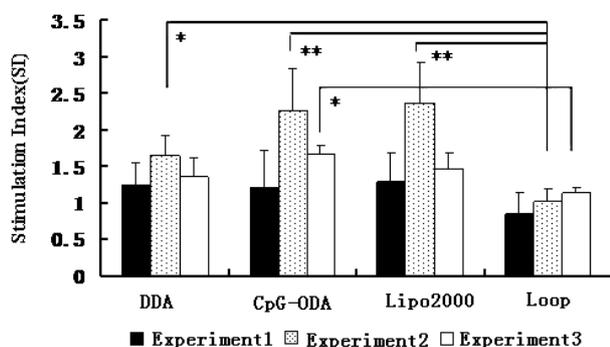
In order to quantitate the efficiency of specific IgG antibodies against Loop protein in different groups co-immunized with DNA vaccine and adjuvant in three experiments, sera from immunized mice were collected and the levels of IgG were detected by ELISA. Representative results are shown in Fig. 1. IgG levels in mice vaccinated with the pVAX1-CpG-Loop (25 µg/mouse) plus the adjuvants in Experiment 1 were lower than the IgG levels in mice vaccinated with a higher dose (100 µg/mouse) of pVAX1-CpG-Loop and adjuvants in Experiment 3 (Fig. 1B). Higher levels of IgG were produced after boosting with pVAX1-CpG-Loop in Experiment 2 (Fig. 1A). In contrast to the control (25 or 100 µg/mouse), higher levels of IgG were elicited in mice immunized with pVAX1-CpG-Loop vaccine plus adjuvants in Experiments 2 and 3 (all  $P < 0.05$ ). However, no significant differences in the levels of IgG were observed in the different adjuvant groups ( $P > 0.05$ ). IgG levels in mice vaccinated in Experiment 1 were lower than those obtained for mice in Experiments 2 and 3.

### *Adjuvant co-administered with pVAX1-CpG-Loop induced a markedly higher specific lymphocyte proliferative response*

Cellular immunity induced by the different formulations was also assessed by an *in vitro* lymphoproliferation assay. Splenocytes obtained from mice immunized with pVAX1-CpG-Loop plus adjuvants showed a markedly higher proliferative response than those from the control in Experiments 2 and 3 (all  $P < 0.05$ ) (Fig. 2). However, splenocytes obtained from the mice in Experiment 1 showed no proliferative response compared to the controls (all  $P > 0.05$ ) (Fig. 2). These results demonstrate that the mice immunized with pVAX1-CpG-Loop plus CpG-ODN induced markedly higher proliferative responses. Moreover, the SI reached 2.26 in Experiment 2 and 1.65 in Experiment 3. Mice immunized with pVAX1-CpG-Loop plus Lipofectamine™ 2000 also presented higher proliferative responses (SI=2.37). However, in comparison to the pVAX1-CpG-Loop plus CpG-ODN group, the SI values of the pVAX1-



**Fig. 1.** Virus-specific IgG responses in mice. Virus-specific IgG was detected by ELISA at a dilution of 1:40 performed in triplicate for all sera collected every week after the first immunization. A: Mice were immunized with the DNA (25  $\mu$ g/ mouse) vaccine and the adjuvants three times (arrows a) and boosted with DNA vaccine (25  $\mu$ g/ mouse) antigen in week fourteen (arrow b) in Experiment 2. B: Mice were immunized with the DNA (100  $\mu$ g/ mouse) vaccine and the adjuvants three times (arrows) in Experiment 3.



**Fig. 2.** Loop protein-specific lymphocyte proliferation from vaccinated mice. All data were obtained in one single representative experiment, but are divided over three panels for clarity (five mice pooled per group). ■, ▨, □ represent the SI of the vaccinated mice in Experiments 1, 2, and 3, respectively.

CpG-Loop plus Lipofectamine™ 2000 group revealed no significant difference ( $P>0.05$ ).

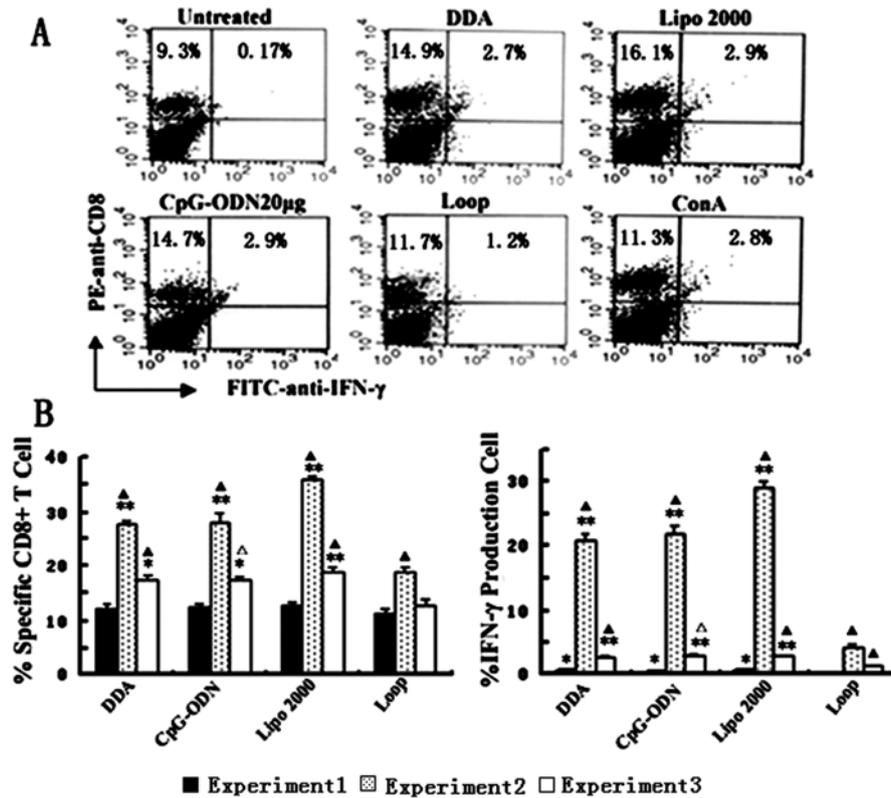
#### *Splenic CD8+ T cells were efficiently elicited by the boosting strategy using lower doses of DNA vaccine*

To test whether splenic CD8+ T cells were efficiently produced in mice, the number of CD8+ T cells seven days after immunization was determined by flow cytometry. In Experiment 1, 11–13% of the splenic CD8+ T

cells were produced by the mice (Fig. 3B). After boosting with pVAX1-CpG-Loop during the fourteenth week in Experiment 2, the percentage of splenic CD8+ T cells reached 19–36% and was significantly higher than before (Fig. 3B). In Experiment 3, 13–19% of the splenic CD8+ T cells were produced by mice immunized with 100  $\mu$ g of DNA vaccine plus a different adjuvant (Fig. 3B). The percentage of splenic CD8+ T cells was significantly higher than that of the control in Experiments 2 and 3,  $P<0.05$  or  $P<0.01$ .

#### *IFN- $\gamma$ in Experiment 3 was produced by the boosting strategy using lower doses of DNA vaccine*

To compare the efficiency of IFN- $\gamma$  production *in vivo*, mice were co-administered pVAX1-CpG-Loop and adjuvant. Mice immunized with pVAX1-CpG-Loop alone were used as controls. Seven days after immunization, we evaluated the Ag-specific T cell activation by intracellular IFN- $\gamma$  production. As shown in Fig. 3B, 21.02–29.17 and 2.65–2.92% of the splenic CD8+ T cells obtained from mice produced IFN- $\gamma$  in Experiments 2 and 3. Compared to the controls, the percentage of IFN- $\gamma$ -producing CD8+ T cells was significantly higher,  $P<0.01$ . However, only 0.26–0.61% of the splenic CD8+ T cells obtained from mice produced IFN- $\gamma$  in Experiment 1 (Fig. 3B),  $P<0.05$ . The splenic CD8+ T cells from mice



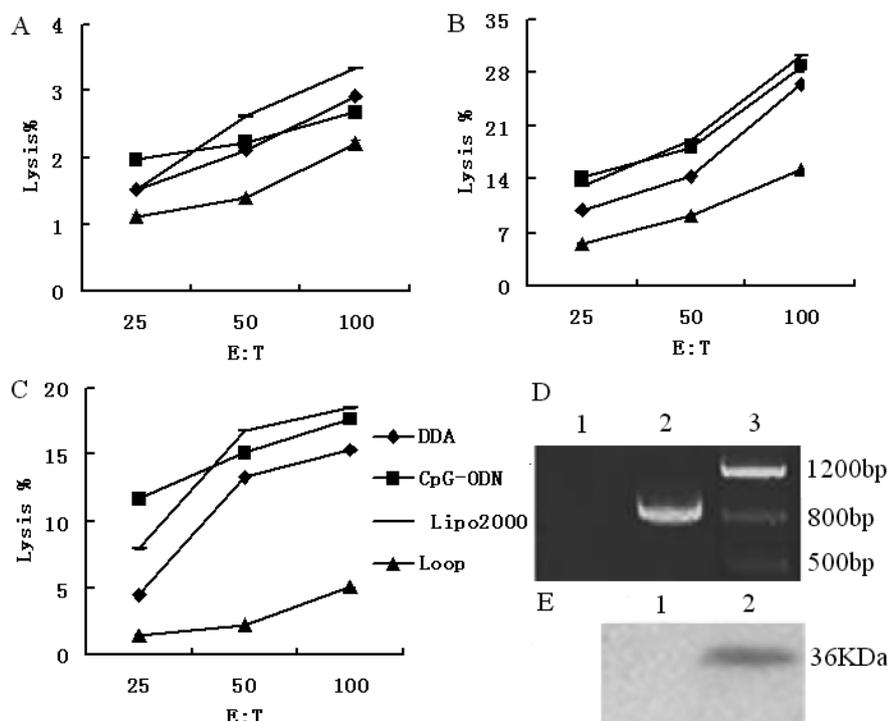
**Fig. 3.** A: Dot plot obtained by flow cytometry for mice immunized three times in Experiment 3. B: The statistical results obtained for CD8<sup>+</sup> T cell frequency and IFN- $\gamma$  production of splenocytes from mice in three experiments by FACS analysis. The data are the means  $\pm$  SD of three experiments performed in triplicate. ■, ▨, □ represent the SI of the vaccinated mice in Experiments 1, 2, and 3, respectively. \* $P$ <0.05, \*\* $P$ <0.01: vs. Mice immunized with pVAX1-CpG-Loop DNA vaccine alone ▲ $P$ <0.01, △ $P$ <0.05: vs. Mice immunized in Experiment 1.

vaccinated with the pVAX1-CpG-Loop plus Lipofectamine™ 2000 produced significantly more IFN- $\gamma$  than any other group based on three independent experiments.

*Significantly higher CTL responses were generated by Lipofectamine™ 2000 co-administration with pVAX1-CpG-Loop*

To determine whether vaccination with the DNA vaccine plus adjuvant could induce CTL responses to the Loop protein *in vitro*, splenocytes from immunized mice were restimulated *in vitro* with the recombinant Loop protein (30  $\mu$ g/ml) and recombinant mouse IL-2 (10 ng/ml), and CTL activity was then assayed by the LDH release assay. Significantly higher CTL responses to the Loop protein were generated by splenocytes from the

mice used in Experiment 2 (Fig. 4B). In Experiment 1, mice vaccinated three times induced weaker CTL responses than those immunized four times in Experiment 2 (Fig. 4A and 4B). Significantly lower CTL responses were generated by splenocytes from mice immunized with 25  $\mu$ g/mouse of pVAX1-CpG-Loop plus adjuvant in Experiment 1 than by those from mice immunized with 100  $\mu$ g/mouse of DNA vaccine plus adjuvant in Experiment 3 (Fig. 4C). These results further reveal that splenocytes from mice immunized with Lipofectamine™ 2000 plus pVAX1-CpG-Loop generated significantly higher CTL responses than those obtained from mice in the three control experiments. Splenocytes from mice injected with a lower dose of pVAX1-CpG-Loop plus adjuvant and boosted with pVAX1-CpG-Loop generated markedly higher CTL responses. In addition, lysis of



**Fig. 4.** LDH-specific lysis of cells obtained from immunized mice in three experiments. A, B, C: % specific lysis from the mice receiving three or four immunizations in Experiments 1, 2, and 3, respectively. D: RT-PCR detection of Loop gene expression. Lane 1: Amplification of Loop in pVAX1-CpG vector-transfected CT26 cells; Lane 2: Amplification of Loop in pVAX1-CpG-Loop vector-transfected CT26 cells; Lane 3: DNA Marker. E: Western blot analysis of total protein obtained from transfected CT26 cells Lane 1: CT26 cells transfected with pVAX1-CpG, Lane 2: CT26 cells transfected with pVAX1-CpG-Loop.

the majority of splenocytes stimulated with Loop protein exceeded 30% at an E:T ratio of 100:1.

## Discussion

Induction of potent CD8<sup>+</sup> T cell responses by DNA vaccines to fight microbes or tumors remains a major challenge, as many animal and human vaccine candidates have been shown to be immunogenically ineffective [19]. In particular, many DNA vaccines against cancers and infectious diseases have failed to induce protective immunity, necessitating a search for more effective vaccine adjuvants to enhance the level of immune activation [3, 18, 27]. A series of adjuvant systems have been included in the formulations of new vaccine candidates to promote faster, stronger and longer protection through induction of large and persistent cell-mediated immune

reactions. The strategy has been to select key adjuvant components, and to identify the best adjuvant combination for the selected antigen(s) to achieve the desired immune responses [13]. In addition, cellular immune responses will likely require tailoring to each pathogen in terms of the different effectors of the cellular immune response induced (e.g., CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells) and the specific antigen(s) or epitope(s) targeted [22]. In the present study, the adjuvant-augmented effects of CpG-ODN, DDA, and Lipofectamine<sup>TM</sup> 2000 on pVAX1-CpG-Loop DNA vaccine were evaluated.

The antibody levels in mice were lower until week 14 after the initial vaccination in Experiments 1 and 2. Vaccinations comprised of plasmid pVAX1-CpG-Loop (25  $\mu$ g/mouse) mixed with adjuvant did not induce an effective immune response in the short-term. However, memory B cells were dramatically enhanced after an

additional vaccination with the pVAX1-CpG-Loop DNA vaccine only (25  $\mu\text{g}/\text{mouse}$ ) in week fourteen (Fig. 1A). In Experiment 3, higher levels of specific antibody were detected shortly after vaccination with plasmid pVAX1-CpG-Loop (100  $\mu\text{g}/\text{mouse}$  Fig. 1B). The results show greater humoral immune responses and immunological memory responses were induced when mice were boosted with pVAX1-CpG-Loop (25  $\mu\text{g}/\text{mouse}$ ) in Experiment 2.

We address here the question of whether a DNA vaccine can be efficiently presented to the T cell repertoire of mice and induce a specific CTL response. There is some evidence that Th1 cell and CTL responses are associated with antiviral cytokines (IFN- $\gamma$ ), which play an important role in viral resolution during natural infection [13]. Interferon- $\gamma$  is a cytokine produced by stimulated T cells and has important effects on immunomodulation [22]. A possible explanation for the ability of DNA vaccine plus adjuvant to elicit significantly higher levels of T cell proliferation and CTL responses than the control group in Experiment 2 could be provided by the data revealing the development of a higher frequency of CD8+ T cells and a higher level of IFN- $\gamma$ . We were further able to demonstrate that DNA vaccine plus adjuvant induced higher IFN- $\gamma$  secretion by responding T cells. The very high specific CTL frequencies induced by boosting long after priming are consistent with the finding that proliferation-competent memory T cells develop slowly over time [16]. This provides an explanation for why the antigen-specific T cell proliferation, higher frequency of CD8+ T cells, IFN- $\gamma$  secretion, and CTL responses in mice in Experiments 1 and 3 did not surpass those of mice boosted with pVAX1-CpG-Loop (25  $\mu\text{g}/\text{mouse}$ ) in Experiment 2. The strategy of co-administering plasmid pVAX1-CpG-Loop (25  $\mu\text{g}/\text{mouse}$ ) with adjuvant and later boosting with DNA vaccine resulted in strong cytotoxic T cell responses in the mice.

The efficacy of CpG-ODN or bacterial DNA containing CpG motifs as immunomodulators has been reported in many studies [2, 5, 8, 11, 17, 24]. According to these studies, CpG-ODN or bacterial DNA containing CpG motifs are known to shift the immune response substantially toward Th1 and modulate the Th1/Th2 immune response [20, 26]. The adjuvant effect of CpG-ODN on pVAX1-CpG-Loop DNA vaccine was evalu-

ated in a mouse model. The results show that splenic lymphocyte proliferation responses, the frequency of CD8+ T cells, intracellular IFN- $\gamma$  production, and CTL activity were markedly increased by CpG-ODN compared to the control (vaccination with only pVAX1-CpG-Loop). These results have direct implications for the use of CpG-ODN as a suitable adjuvant for DNA vaccination (pVAX1-CpG-Loop), as significantly higher CTL responses were generated. The CpG-ODNs contained 11 or 16 CpG motif repeats that were synthesized and inserted into the DNA vaccine, resulting in significant enhancement of the cellular immune response of mice to a conventional virus [28, 29]. However, in the present study, the DNA vaccine (pVAX1-CpG-Loop) contained only three CpG motifs of 1,826 repeats. The results indicate that the CpG motif induced a weaker immune response than DNA vaccine plus CpG-ODN. This is consistent with recent work showing that the number and sequences of CpG motifs can determine the ability of ODN to improve immune responses to vaccines [29].

DDA has been identified as an effective enhancer of the immune response to bacteria and viruses in mammalian models [2, 4, 9, 17, 22]. DDA improved pseudorabies virus-specific cell-mediated immune responses after DNA vaccination against the pseudorabies virus [22]. Moreover, DDA has already been administered to many individuals, including pregnant women and children, with no toxic effects reported [9]. Similarly, DDA has an optimal effect on the efficacy of the pVAX1-CpG-Loop vaccine based on the Loop protein-specific CTL activity assay. In the present study, higher specific lymphoproliferation, and a higher frequencies of specific CD8+ T cells and IFN- $\gamma$ -producing cells were markedly induced by pVAX1-CpG-Loop together with DDA. Furthermore our data also demonstrated that higher specific lymphoproliferation, higher frequencies of specific CD8+ T cells and IFN- $\gamma$ -producing cells and significantly higher CTL responses were observed in mice vaccinated with Lipofectamine™ 2000 and pVAX1-CpG-Loop DNA vaccine than those administered with DNA vaccine plus CpG-ODN or DDA. These studies show that DDA and Lipofectamine™ 2000 augmented the effect of DNA vaccine (pVAX1-CpG-Loop) inducing a strong CTL response in mice. In addition, Lipo-

fectamin™ 2000 may be the best adjuvant in this study for inducing antigen and cellular responses against ICH.

In conclusion, induction of strong cytotoxic T cell responses in mice was achieved by augmenting the effect against ICH with DDA, CpG-ODN, or Lipofectamine™ 2000 delivered together with pVAX1-CpG-Loop. Lipofectamin™ 2000 may be the best adjuvant for augmenting the effect of the DNA vaccine (pVAX1-CpG-Loop) against ICH. Moreover, priming with pVAX1-CpG-Loop (25 µg/mouse) co-administered with adjuvant and boosting with pVAX1-CpG-Loop (25 µg/mouse) alone was capable of boosting memory CTL responses in mice. Thus, this strategy is a potent vaccination strategy for the pVAX1-CpG-Loop DNA vaccine against infectious canine hepatitis.

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