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CpG DNA Is a Potent Enhancer of Humoral and Cell-Mediated Immune Responses against *Orientia tsutsugamushi* in C3H/HeN Mice

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Background: Synthetic oligodeoxynucleotide (ODNs) encoding CpG motifs mimic the activity of bacterial CpG motifs that trigger innate immune responses, and their use as an adjuvant has been demonstrated to significantly improve humoral immune response. **Methods:** CpG ODNs were evaluated for their immunostimulatory activities on mice vaccinated with recombinant outer membrane protein P56 (rP56Δ) of *Orientia tsutsugamushi*. **Results:** CpG ODNs significantly enhanced immune responses of mice against rP56Δ post three doses of immunization, with IgG titer ranging from 80,000 to more than 640,000. Furthermore, they also improved the immunization efficiency of rP56Δ by shortening the immunization schedule. Only two doses of immunization of rP56Δ plus CpG ODNs were needed to induce equally high antibody level as the three-dose immunization scheme of rP56Δ plus Freund's adjuvant. Isotypic analysis of antibodies revealed that rP56Δ plus CpG ODNs enhanced mice humoral immune responses as rP56Δ plus Freund's adjuvant did. IgG1, IgG2a, IgG2b and κ light chain were induced significantly. Interestingly, rP56Δ stimulated spleen cells of vaccinated BALB/c mice to produce more IL-12 and gamma interferon than IL-4. In contrast, spleen cells stimulated with ConA, IL-4 was dominantly induced. Furthermore, CpG ODNs could cooperatively elicit memory immune responses in rP56Δ-immunized mice, as demonstrated by quick IgM induction boost shot. **Conclusion:** Results from this study revealed that rP56Δ could stimulate C3H/HeN mice to produce immune responses; and CpG ODN adjuvant enhanced its immunization effect and shortened the immunization time.

Key words: *Orientia tsutsugamushi*, CpG motif, recombinant outer membrane protein, adjuvant, immunization

INTRODUCTION

Scrub typhus is a mite-transmitted rickettsiosis.[1] This disease, as well as the rickettsial diseases, tick typhus and murine typhus, is endemic in the Asia-Pacific region including Korea, Japan, Mainland China, the Philippines, and Thailand.[2,3] *Orientia tsutsugamushi* were classified into eight strains by phylogenetic analyses according to homologies of 56-kDa type-specific antigen genes.[4] Although analytical approaches can be employed to study scrub typhus protein antigens, it still remains technically and economically infeasible to produce large quantities of purified *O. tsutsugamushi* for the isolation of individual antigens for vaccine production. Expression of rickettsial antigens via recombinant DNA technology should overcome this problem to a large extent Previous results demonstrated that recombinant Bor56 (56-kDa protein of Boryong strain)-immunized mice elicited T-cell responses and antibodies that react with *O. tsutsugamushi*.[5] The *O. tsutsugamushi* 56-kDa protein has been shown to induce neutralizing antibodies in mice and rabbits.[6] It has been shown that recombinant Bor56-immunized mice were protected when challenged by the homologous *O. tsutsugamushi* strain.[5] As a result, recombinant 56-kDa protein has become the main candidate for a genetically engineered scrub typhus vaccine.

Since *O. tsutsugamushi* is an obligate intracellular bacterium, which enters mammalian cells through a process of attachment and phagocytosis, there are strong evidences that for tuberculosis, malaria and other parasitic infections, T_H1 and/or $CD8^+$ T-cell responses are the effector mechanisms required for protective immunity.[7] In both mice and humans, CpG ODNs have been shown to stimulate multiple types of immune cell, leading to enhanced T_H1 and $CD8^+$ T-cell responses.[8,9] In fact, unmethylated CpG motifs in bacterial DNA were responsible for their immune stimulatory effects.[10] CpG DNA activates

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directly monocytes, macrophages, and dendritic cells to secrete Th1-like cytokines and expresses increased levels of cell surface costimulatory molecules.[11,12] NK cells are activated by CpG DNA to increase lytic activity and to secrete IFN-γ.[13,14] CpG DNA can also activate B cells and drive them to secrete IL-6, IL-10, and immunoglobulin[2,15] and to proliferate in a polyclonal T-cell independent manner.[10] These results indicate the likelihood of CpG DNA promoting the generation of antigen-specific immune responses.

The discovery that the immune system has apparently developed a detection mechanism for CpG DNA implies that the activation of this mechanism should lead to a useful outcome for the host. In fact, it has been demonstrated that CpG DNA could be an efficient adjuvant for tumor vaccines[16,17] and infectious disease vaccines, such as hepatitis B vaccine[18,19] malaria vaccine[20] and influenza vaccine.[21] CpG DNA is most often given in the form of CpG ODN made with a nuclease-resistant phosphorothioate backbone.[19] Furthermore, it has been suggested that CpG ODN is an effective oral adjuvant that can promote or enhance systemic and mucosal immune responses to protein antigens.[22] In this study, CpG ODN was evaluated for its immunostimulatory activity in mice vaccinated with recombinant outer membrane protein P56 (rP56Δ) of *Orientia tsutsugamushi*.

MATERIALS

Oligodeoxynucleotides

ODN used herein were: CG-ODN1, 5'-AGCTTTCGTCGTTTTGTCGTTTTGTCGTTGG-TAC-3'; CG-ODN2, 5'-CAACGAACAAAACGACGA-CAAAACGACGAA; (CpG dinucleotides underlined for phosphorothioate). All ODNs were synthesized with a nuclease-resistant phosphorothioate backbone by MDBio Inc., and the sodium salts of the ODN were ethanol precipitated and then resuspended in 10 mM Tris (pH 7.0) containing 1 mM EDTA for storage at -20°C. These two ODNs were complementary to each other and were annealed to form double-strand DNA by heating at 95°C for 2 min and then stayed at room temperature for 2 h. It was then stored at -20°C before dilution into saline for injection.

Production of recombinant 56-kDa outer membrane protein of *Orientia tsutsugamushi*

Recombinant protein, rP56Δ, was purified according to the method described previously.[23]

Immunization of mice with rP56Δ of *O. tsutsugamushi*

Immunization with rP56Δ Ag was conducted on 6- to 8-wk-old female C3H/HeN mice (National Science Council, Executive Yuan, Taiwan). Each mouse received three doses (at days 0, 30 and 60) of i.p. injection of a solution containing 5 μg recombinant rP56Δ produced in E. *coli BL21 (DE3)* in a total volume of 100 μl. Control groups (n = 5) received equal volume of phosphate buffer solution (PBS, pH 7.4) plus 50% (v/v) of Fruend's adjuvant (GIBCO BRL, Cat. No. 15720-030). Experimental groups (n = 5) received rP56Δ plus 50% (v/v) of Fruend's adjuvant or rP56Δ plus 50 μg of double-strand CpG ODN. These experiments were performed with CpG ODN in which the backbone was nuclease-resistant (phosphorothioate) to improve cell uptake and in vivo stability.[5] All component solutions were added at the same time, mixed with a vortex, and left on ice for about 30 min before injection.

Ethics Statement

All animal experiments were reviewed by the Institutional Animal Care and Use Committee and approved by the regulatory authorities of Taiwan. The experiments were conducted in accordance with Taiwan's laws on animal experimentation and guidelines set out by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the Office of Laboratory Animal Welfare (OLAW). The IACUC Certificate No. of this study was AN-93-05. Animals were housed according to OLAW and AAALAC guidelines in housing facilities accredited by the Center of Disease Center (CDC) of Taiwan.

Evaluation of *in vivo* humoral response to rP56Δ

Sera were recovered from mice at various times (days 0, 28, 56 and 84) after immunization. Abs specific to rP56Δ were detected and quantified by ELISA assay (in triplicate) on samples from individual animals according to the method illustrated previously.[23] In brief, the 96-well microtiter plates (Falcon #3912, BECTON DICKINSON) were coated with antigens (50 μl/well, 0.2 μg/ml in 0.05 M carbonate buffer, pH 9.6) at 4°C overnight. The contents of the plates were dumped, and the wells were filled with PBST containing 3% skimmed milk (150 μl/well). Plates were then incubated for 1 h at room temperature for blocking. After dumping the contents, the wells were rinsed once with PBST (PBS + 0.05% Tween-20). Sample sera diluted in PBST were then added into wells (50 μl/well), and plates were then incubated at 37°C for 1 h. After dumping the contents, the wells were

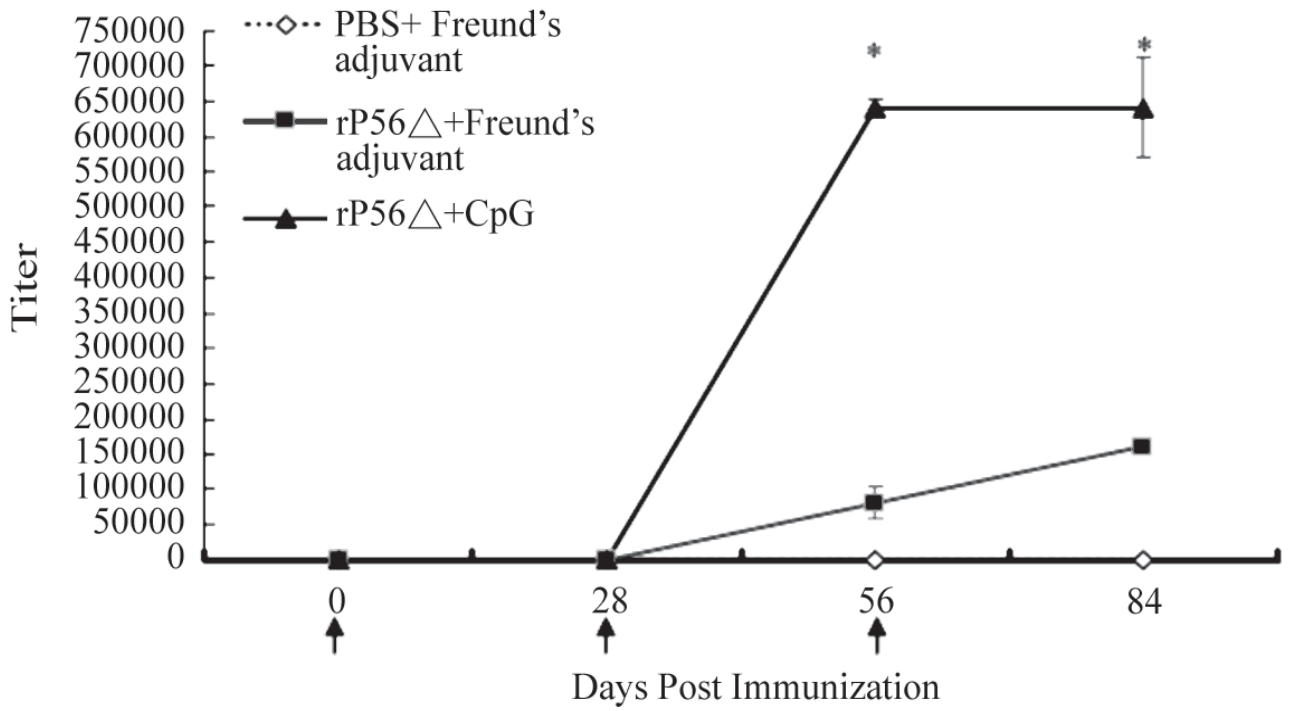

Fig. 1 Characterization of antibody responses to rP56Δ post immunization. Mice (five per group) were immunized with rP56Δ and bled retroorbitally. The titers of antibodies were evaluated by ELISA. Data are representative of two separate experiments. Kinetics of the anti- rP56Δ humoral response in mice immunized with PBS, 5 μg of rP56Δ plus Freund's adjuvant (solid lines) or 5 μg of rP56Δ plus 50 μg of CpG ODN (dashed lines). The squares represent the mean titers±SD (error bars) of antibodies in five animals. The arrows indicate the days of inoculation. Each bar represents the mean titers±SD of antibodies in five animals. The symbol "*" denotes significant difference in IgG titer of mice immunized with rP56Δ compared with that of mice immunized with PBS ($P < 0.05$). *P* values were calculated by the Kruskal-Wallis test.

rinsed five times with PBST. This was followed by incubation with secondary antibodies (goat anti-mouse IgG or goat anti-mouse IgM conjugated with horseradish peroxidase, 1:3000 in PBST containing 3% skimmed milk, 50 μl/well) at room temperature for 1 h. After dumping the contents, the wells were rinsed five times with PBST again. The substrate solution [(TMB (3,3,5,5-tetramethylbenzidine), 50 μl/well with 100 μg/ml in phosphate-citrate buffer, pH 5.0 containing 1/1000 volume of 35% H_2O_2) was added and incubated at room temperature for 10 min. After the reaction was stopped by adding 1 M H_2SO_4 (50 μl/well), the optical densities (ODs) at 450 nm were measured. Sera were always assayed in duplicate. Each plate included an air blank, a negative control (pre-immunized serum in triplicate), as well as a row of different diluted positive controls for establishing a standard curve.

Preparation of splenocyte cells for cytokines studies

Spleens were obtained from 6- to 8-wk-old female C3H/HeN mice that had been maintained under specific pathogen-free conditions in the Institute of Preventive Medicine Animal Care Facility. Splenocyte suspensions were collected, washed in phosphate buffer solution (PBS, pH 7.4), and suspended in complete medium consisting of RPMI 1640 with FCS (fetal calf serum, 10%), penicillin (100 unit/ml), streptomycin (100 unit/ml), L-glutamine (2 mM), and 2-ME (0.05 mM). Cultures were stimulated with 5 μg /ml of rP56Δ, 5 μg /ml of rP56Δ plus CpG ODN (100 μg), or ConA (5 μg /ml). Culture supernatants were harvested after 24 h and 48 h and assayed for cytokine levels.

Isotype-specific Ig ELISA assays

Mouse Hybridoma IsoTyping Kit (Biomeda Corp., cat. no. 22-201) was used for the measurement of total IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, κ light chain and λ chain from mice sera. For each ELISA, 50 μl of diluted

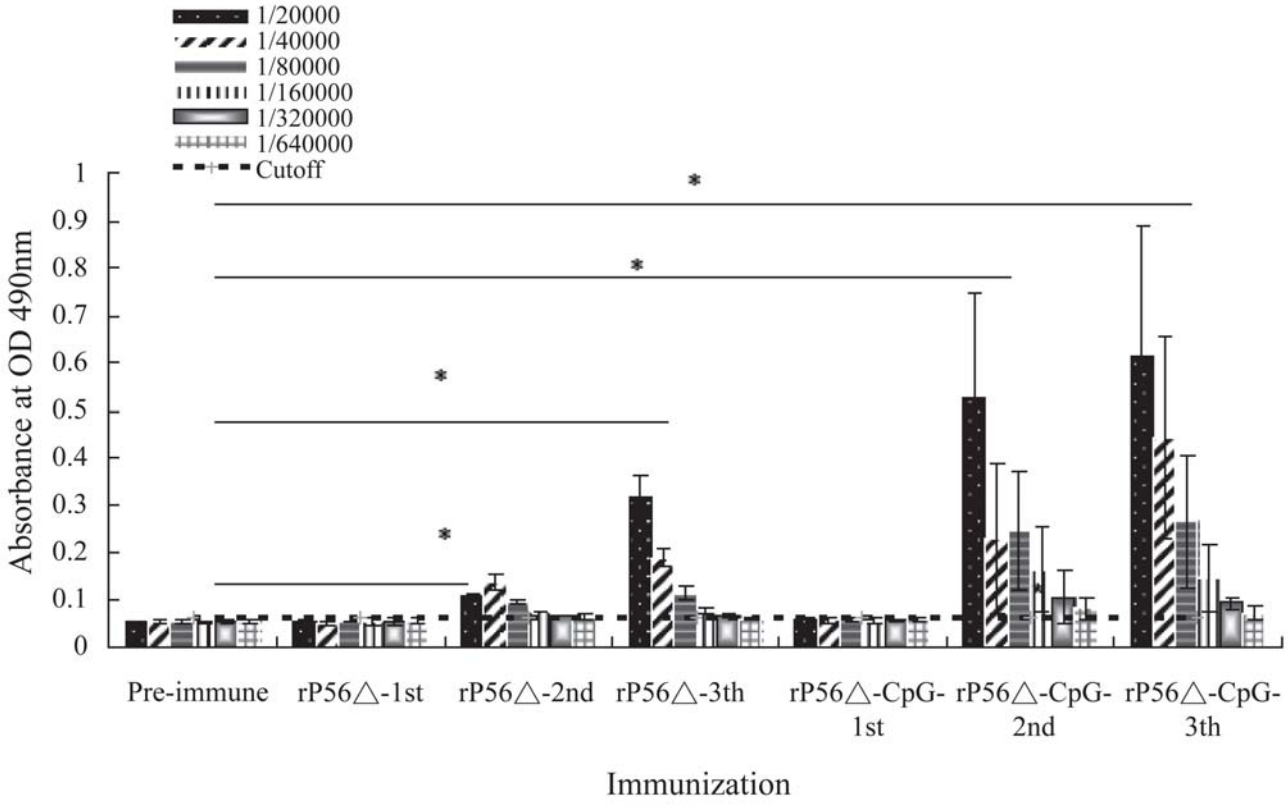

Fig. 2 CpG ODN improved immunization efficiency of rP56Δ. To evaluate the immunostimulatory activity of CpG ODN on mice vaccinated with recombinant outer membrane protein P56 (rP56Δ) of Orientia tsutsugamushi, 50 μg of CpG ODN was used instead of traditional adjuvant. After three doses of immunization, mice sera were assayed for their IgG titer by ELISA as described in Materials and Methods. Each bar represents the mean titers±SD of antibodies in five animals. The symbol "*" denotes significant difference in IgG of mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN compared with that of pre-immunized mice ($P < 0.05$). P value was calculated by the Kruskal-Wallis test.

serum (1/1000 in PBS, PH 7.4) was assayed and quantified according to a standard curve. All experimental procedures followed the instructions of the manual.

Cytokine ELISAs

The levels of IL-4, IL-12, and IFN-γ in culture supernatants were measured by sandwich ELISAs with paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions (Quantikine M Mouse IL-12, IL-4, and IFN-γ Immunoassay; R & D SYSTEM).

Statistical analysis of the data

Significances of differences in antibody responses and cellular responses were determined using the Kruskal-Wallis test and one-way ANOVA, respectively. T-test was used for the comparison of two specific groups in one-way ANOVA. A P value of < 0.05 was considered significant.

RESULTS

Vigorous humoral immune response induction by rP56Δ plus Freund's adjuvant and rP56Δ plus CpG ODN immunization.

The titers of anti-rP56Δ antibodies were measured by ELISA for sera from mice immunized with rP56Δ plus Freund's adjuvant and rP56Δ plus ODN. Immunization with rP56Δ plus Freund's adjuvant elicited a humoral immune response that was detectable 28 days after the first immunization (titer range, 200 to 1,600 with mean of 520) and increased steadily after each immunization to reach a maximum (titer range, 80,000 to 160,000) at day 84 post vaccination (end of the experiment) (Fig. 1). Animals injected with rP56Δ plus ODN also exhibited

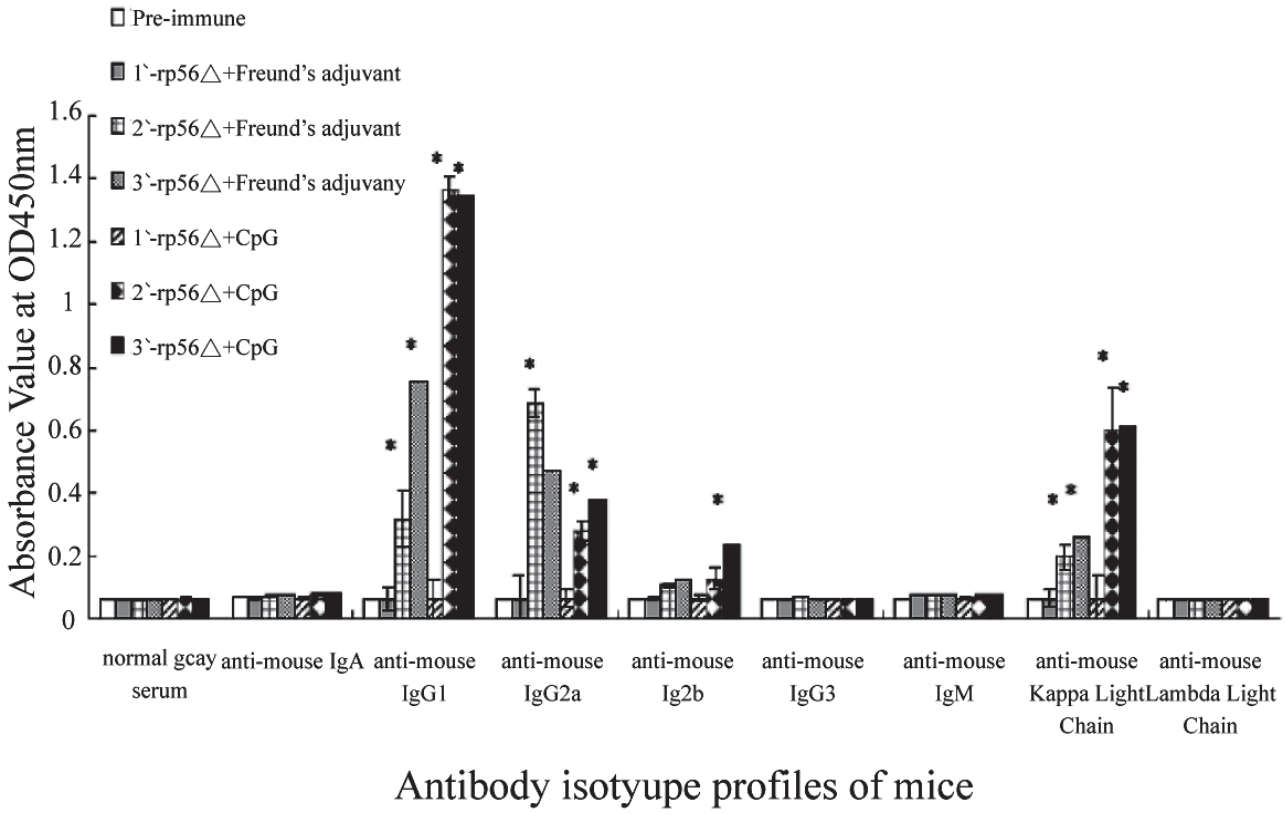

Fig. 3 Freund's adjuvant and CpG ODN induced T_H1 and T_H2 immune responses by rP56Δ. Antibody isotype profiles of mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN were assayed by Mouse Hybridoma IsoTyping Kit (Biomeda Corp., cat. no. 22-201) for the measurement of total IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, κ light chain and λ chain. For each ELISA, 50 μl of diluted serum was assayed and quantified according to a standard curve as described in Materials and Methods. Error bars indicate standard deviations. The symbol "*" denotes significant difference in absorbance value at OD450 nm of antibodies in responses of mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN compared with that in sera pre-immunized mice ($P < 0.05$). P values were calculated by the Kruskal-Wallis test.

a specific antibody response that was detectable 28 days after the first immunization (titer range, 400 to 800 with mean of 720) and increased vigorously and maintained antibody titer at the maximum (titer is equal to 160,000) level to the day 84 post vaccination (end of the experiment) (Fig. 1). Result indicated that rP56Δ plus Freund's adjuvant or rP56Δ plus ODN is capable of eliciting vigorous humoral immune response.

CpG ODN improved immunization efficiency of rP56Δ.

To evaluate the immunostimulatory activity of CpG ODN on mice vaccinated with recombinant outer membrane protein P56 (rP56Δ) of *Orientia tsutsugamushi*, 50 μg of CpG ODN was used instead of traditional adjuvant. After three doses of immunization, CpG ODN significantly enhanced immune response of mice against rP56Δ, with IgG titer ranging from 80,000 to more than 640,000 (Fig. 2). Furthermore, CpG ODN also shortened the immunization schedule; only two doses of immunization with rP56Δ plus ODN (100 μg) were needed to produce higher antibody level than three doses of rP56Δ plus Freund's adjuvant immunization (Fig. 1, 2). Taken together, the results indicated that CpG ODN was capable of improving the immunization efficiency of rP56Δ.

Induction of Th1 and Th2 immune responses by rP56Δ.

Since *O. tsutsugamushi* is an obligate intracellular bacterium, it is anticipated that rP56Δ immunization

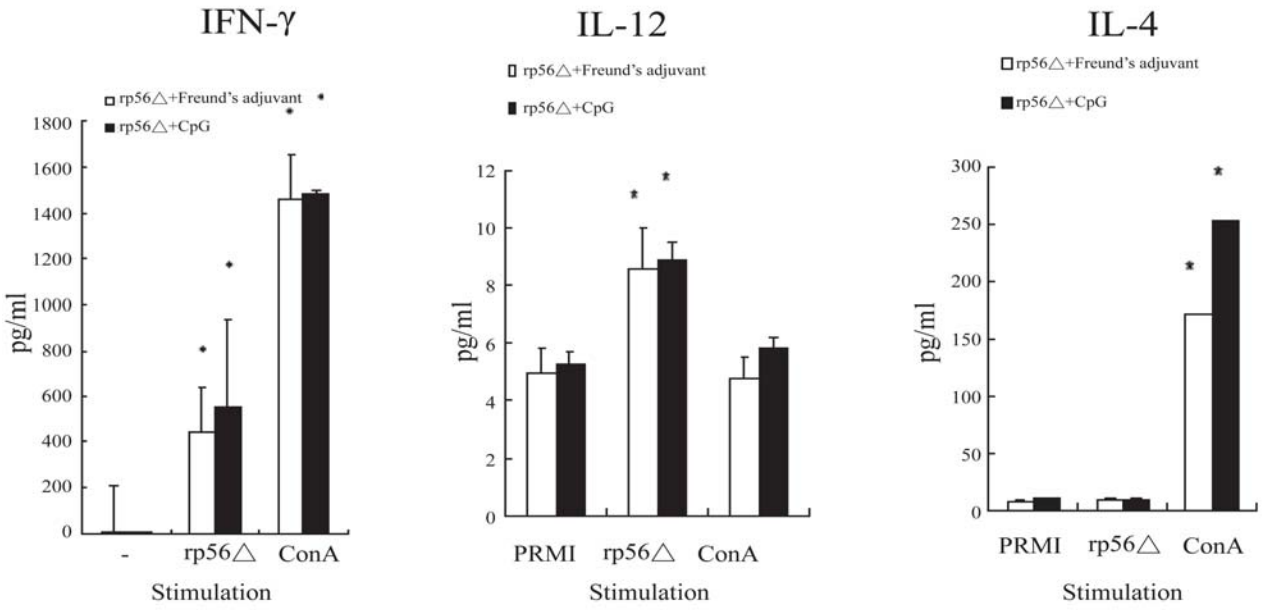

Fig. 4 CpG ODN adjuvant stimulated rP56Δ to induce expressions of IL-12 and IFN-γ. (A) Concentrations of rP56Δ -induced IFN-γ in cells from mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN. Spleen cells (4×10^6/ml) of mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN were stimulated with RPMI 1640, rP56Δ (10 μg/ml), or ConA (5 μg/ml) for 48 h. Levels of IFN-γ in the cell supernatants were quantified by ELISA. Each bar represents the geometric mean±SD (error bar) of the responses in spleen cells from five mice, with experiments run in duplicate. (B) Concentrations of rP56Δ -induced IL-12 in cells of mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN. The levels of IL-12 in culture supernatants were measured as described above. (C) Concentrations of rP56Δ -induced IL-4 in cells from mice immunized with rP56Δ. The symbol "*" denotes significant difference in cytokines responses post rP56Δ stimulation between spleen cells of mice immunized with RPMI 1640- and mice immunized with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN ($P < 0.05$). The data are representative of two separate experiments.

could induce T_H1 and/or $CD8^+$ T-cell responses, an effector mechanism required for protective immunity. As mentioned above, rP56Δ immunization induces a vigorous humoral response. Isotypic analysis of antibodies revealed that both rP56Δ plus Freund's adjuvant and rP56Δ plus ODN-enhanced humoral immunity. The immunized mice developed specific antibodies of isotypes IgG1, IgG2a, IgG2b and κ light chain, but IgA, IgG3, IgM, and λ light chain were not significantly induced (Fig. 3), indicating that both rP56Δ plus Freund's adjuvant and rP56Δ plus ODN induced vigorous humoral immune responses. Administration of CpG ODN promotes type-1 immune responses, characterized by enhanced IL-12 and IFN-γ production.[24-27] Thus, CpG ODN can enhance both innate and humoral immune responses.

CpG ODN adjuvant induced production of IL-12 and IFN-γ.

Since immunization with either rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODN can induce vigorous humoral immune responses, the functional role of CpG ODN adjuvant in eliciting immune responses was evaluated. Isotypic analysis of antibodies revealed that CpG adjuvant could enhance the antibody titer of IgG1, IgG2b, and κ light chain following the immunization schedule ($P < 0.05$) (Fig. 3).

Cytokine secretion from supernatants of spleen cells from rP56Δ-immunized mice (both with Freund's adjuvant and with CpG ODN as adjuvant) was assessed by ELISA. Results showed that rP56Δ (10 μg/ml) induced significantly the production of IL-12 and IFN-γ in cells from mice immunized both by rP56Δ plus Freund's adjuvant and by rP56Δ plus CpG ODN ($P < 0.05$) (Fig. 4, panels A and B); while IL-4 was not significantly induced (Fig. 4, panel C). In contrast, when spleen cells were stimulated with ConA (5 μg/ml), gamma interferon and

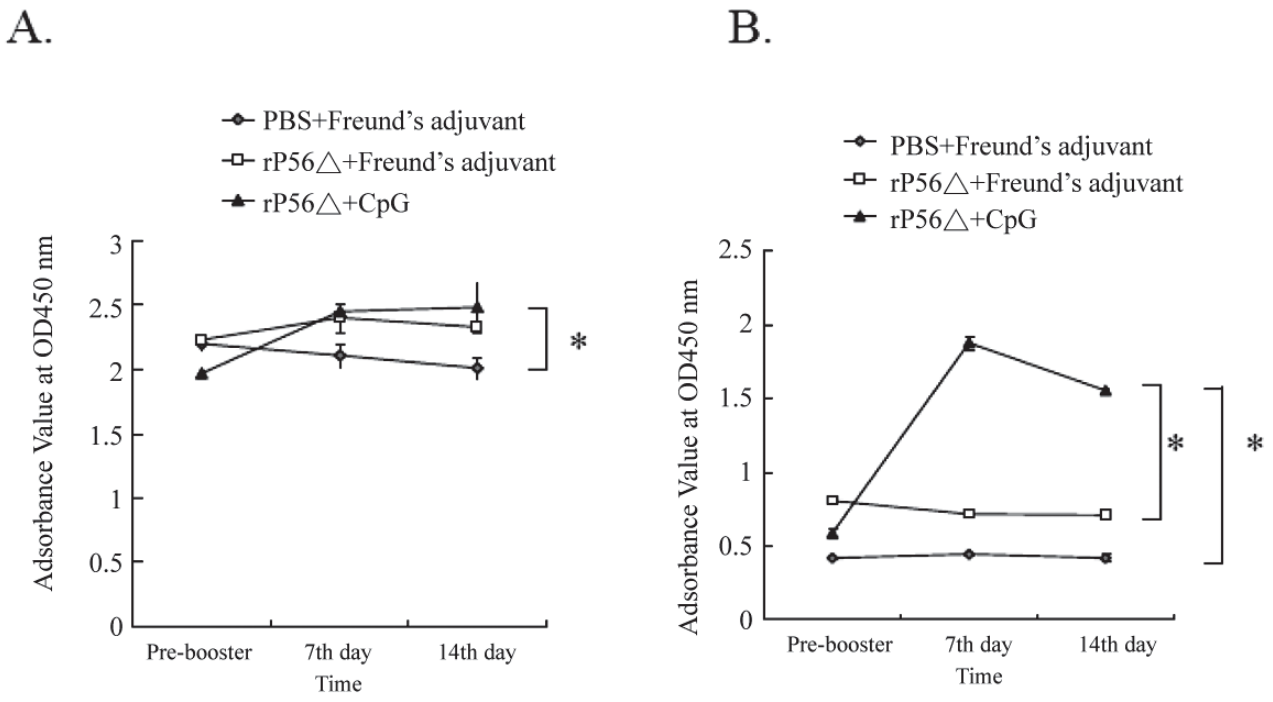

Fig. 5 CpG ODN adjuvant quickly elicited memory immune response of rP56Δ-immunized mice. (A) IgG response of rP56Δ-immunized mice booster shot with different compounds. Mice were fundamentally immunized subcutaneously twice with 5 μg of recombinant truncated protein (rP56Δ) plus Freund's adjuvant at 2nd and 6th weeks after first-time basal serum collection. Immunized mice were booster shot three months later with PBS plus Freund's adjuvant, rP56Δ plus Freund's adjuvant, and rP56Δ plus CpG ODN, respectively. The sera before and after immunization were collected and mixed with 50% glycerol and stored at -20°C until use. Sera were diluted 400 folds in PBST and their IgG immune responses were tested. (B) IgM response of rP56Δ-immunized mice booster shot with different compounds. As described above, sera were diluted 400 folds in PBST and their IgM immune responses were tested. The antibody was assayed for reactivity with rP56Δ by enzyme-linked immunosorbent assay (ELISA) as described in Materials and Methods. Error bars indicate standard deviation. The symbol "*" denotes significant difference in IgG responses between PBS plus Freund's adjuvant and rP56Δ plus CpG (A) or IgM responses between PBS plus Freund's adjuvant or rP56Δ plus Freund's adjuvant and rP56Δ plus CpG (B).

IL-4, but not IL-12, were significantly induced (Fig. 4). Taken together, the results show that rP56Δ could specifically stimulate C3H/HeN mice to produce vigorous immune responses against *O. tsutsugamushi* and promote type-1 immune responses, characterized by enhanced IL-12 and IFN-γ production; and CpG ODN enhanced its immune responses.

CpG ODN adjuvant efficiently induced memory immune response to *Orientia tsutsugamushi.*

As mentioned above, CpG ODN can shorten the immunization schedule and enhance both innate and humoral immune responses. Hence, the capability of CpG ODN adjuvant to efficiently induce the memory immune response to *O. tsutsugamushi* is of concern. To examine such capability, mice were inoculated with rP56Δ plus Freund's adjuvant so as to be highly immunized and then booster shot with rP56Δ plus different compounds three months later (n = 3). As shown in Fig. 5, mice of different immunization groups (panel A) all had high IgG antibody titer. Mice booster shot with rP56Δ plus CpG ODN elicited significant IgM response, while those booster shot with PBS plus Freund's adjuvant and rP56Δ plus Freund's adjuvant did not (Fig. 5, panel B).

DISCUSSION

More than 1 billion people in ~13,000,000 km^2 of land in the Asia-Pacific region are exposed to scrub typhus, but information on disease incidence is very limited.[26]

Scrub fever is currently a challenge to military operations and public health; especially in view of the reported existence of drug-unresponsive scrub typhus in northern Thailand.[28] The suspicion of antibiotic resistance was supported by *in vitro* cell culture and animal studies of *Orientia* isolates from those patients.[26,29] Therefore, development, testing, and certification of an effective scrub typhus vaccine will reduce risk to exposed military personnel and farmers and offer benefits to indigenous populations. For example, recent evidence of reductions in HIV loads in scrub typhus patients suggests other possible benefits from scrub typhus research.[28] There are no FDA-licensed protective vaccines against any of the rickettsioses, although there are military requirements to protect against them.[15] The study on the immunogenicities of antigens and their use in combination with new systems of immunization is very important for the development of better vaccines.

Since *O. tsutsugamushi* is an obligate intracellular bacterium, T_H1 and/or $CD8^+$ T-cell responses seem to be required for protective immunity. Although previous results demonstrated that recombinant Bor56 (56-kDa protein of Boryong strain)-immunized mice elicited T-cell responses and antibodies that react with *O. tsutsugamushi*[30], the detailed immune response, especially the antibody profile of humoral response and cellular immune response of P56 outer membrane protein, needs to be explored.

Synthetic ODNs containing CpG motifs mimic the activity of bacterial DNA motifs[9,31,32], that induce B cells, natural killer (NK) cells, and plasmacytoid dendritic cells to proliferate, mature, and secrete a variety of cytokines, chemokines, and/or Ig.[33,34] The ability of CpG-containing immunostimulatory ODNs (CpG ODNs) to induce both innate and adaptive cellular immune responses has made them a prospective prophylactic and therapeutic vaccine adjuvant for diseases requiring cellular immunity. CpG ODNs have been shown to stimulate macrophages and dendritic cells to synthesize several cytokines, including IL-12, IL-18, tumor necrosis factor alpha, alpha interferon (IFN-α), IFN-β, and IFN-γ, to upregulate costimulatory molecules, such as CD40 and major histocompatibility complex class II, and to enhance the ability of dendritic cells to present soluble protein to class I-restricted T cells.[34,35] In this study, CpG ODN was evaluated for its immunostimulatory activity on mice vaccinated with recombinant outer membrane protein P56 (rP56Δ) of *Orientia tsutsugamushi*.

This study demonstrated that immunization with rP56Δ plus Freund's adjuvant or rP56Δ plus CpG ODNs induced high titers of antibodies. Recombinant protein, rP56Δ, elicited a humoral immune response that was detectable 28 days after the first immunization, and increased steadily after each immunization. Major isotypes of antibodies elicited by rP56Δ included IgG1, IgG2a, IgG2b and κ light chain, implying that immunization with rP56Δ was capable of eliciting both humoral and cellular immune responses. CpG ODN adjuvant enhanced the immunization effect of rP56Δ in humoral immune response, and shortened the immunization schedule from three times of immunization to two times. Antibodies IgG2a, IgG1, IgG2b, and κ light chain were all significantly enhanced by CpG ODN. The reason why CpG ODN fails to enhance IgG2a antibody titer, as compared with rP56Δ plus Freund's adjuvant, is unclear. However, previous studies have demonstrated that the sequence and structure as well as the number and placement of CpG ODNs used as adjuvant might play an important role.[1,17,36,37] Preliminary results of influenza study revealed that CpG ODN can also enhance and direct a Th1-biased immune response to matrix protein (peptide) of influenza (data not shown). Further studies are required for a better understanding of the mechanism of the modulation of CpG ODN on P56 outer membrane protein of *O. tsutsugamushi*.

As with immunity to other intracellular bacteria, immunity to *O. tsutsugamushi* depends on antigen-specific T-cell-mediated activation of macrophages, which are the major effectors mediating the killing of the bacterium. Th1-induced cytokines, like IFN-γ, play an important role in the activation of macrophages *in vivo* and *in vitro*.[25,38] Cells from rP56Δ-immunized mice stimulated *in vitro* with rP56Δ produced IL-12 and IFN-γ but did not produce IL-4 (Fig. 4), indicating that immunization with rP56Δ induced a T_H1 immune response. Although the predominance of IgG1 over IgG2a seems to imply that humoral immune response is dominant, CpG ODN adjuvant tends to enhance both T_H1 and T_H2 immune responses, especially for IgG2b (Fig. 3). In fact, many studies have demonstrated that antibody-mediated immunity is also important for host against intracellular pathogens.[39,40] Tissue examination often revealed that pathogens classified as intracellular, such as *Ehrlichia chaffensis* and *Bartonella grahamii*, could be found in the extracellular space and vice versa.[39,41,42] Furthermore, at some point in the infectious cycle, most intracellular pathogens reside in the extracellular space, where they are vulnerable to antibody action, and Fc receptor cross-linking can have profound effects on the intracellular milieu through signal transduction.[39,43-45] To our knowl-

edge, this is the first report that evaluated the modulation effect of CpG ODN, as an adjuvant, on the immunization of P56 outer membrane protein of *O. tsutsugamushi*. Although results indicated that CpG ODN adjuvant elicited both T_H1 and T_H2 immune responses, it did induce rP56Δ to produce Th1-induced cytokines, such as IL-12 and IFN-γ. In fact, no single ODN was stimulatory in all donors, and optimal Ig, cytokine, and proliferative responses were commonly elicited by different ODNs, even in the same donor.[46] CpG ODN mixtures are active on a broader fraction of the population, suggesting that CpG ODN mixtures may have an improved therapeutic effect and should be tested in further clinical trials.[46] In conclusion, we have shown that inoculation of rP56Δ elicits vigorous antibody immune responses. CpG ODN adjuvant could help rP56Δ enhance immune responses, elicit memory immune response (IgM antibody) quickly, and shorten its vaccination schedule. Results from this study indicate that rP56Δ plus CpG ODN mixture has the potential for immunization against *O. tsutsugamushi*. Further studies are required to improve the efficacy of CpG ODN adjuvant and to better understand the mechanisms of CpG ODNs on the immunostimulatory effect of P56 outer membrane protein of *O. tsutsugamushi*.

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DISCLOSURE

All authors declare that this study has no conflict of interest.

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