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Increased Immunogenicity and Protective Efficacy of a *P. aeruginosa* Vaccine in Mice Using an Alum and De-*O*-Acylated Lipooligosaccharide Adjuvant System

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Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that commonly causes fatal infections in cystic fibrosis and burn patients as well as in patients who are hospitalized or have impaired immune systems. *P. aeruginosa* infections are difficult to treat owing to the high resistance of the pathogen to conventional antibiotics. Despite several efforts, no effective prophylactic vaccines against *P. aeruginosa* are currently available. In this study, we investigated the activity of the CIA06 adjuvant system, which is composed of alum and de-*O*-acylated lipooligosaccharide, on a *P. aeruginosa* outer membrane protein (OMP) antigen vaccine in mice. The results indicated that CIA06 significantly increased the antigen-specific IgG titers and opsonophagocytic activity of immune sera against *P. aeruginosa*. In addition, the antibodies induced by the CIA06-adjuvanted vaccine exhibited higher cross-reactivity with heterologous *P. aeruginosa* strains. Finally, mice immunized with the CIA06-adjuvanted vaccine were effectively protected from lethal *P. aeruginosa* challenge. Based on these data, we suggest that the CIA06 adjuvant system might be used to promote the immunogenicity and protective efficacy of the *P. aeruginosa* OMP vaccine.

Keywords: *Pseudomonas aeruginosa*, vaccine, adjuvant, de-*O*-acylated lipooligosaccharide, protective efficacy

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative opportunistic pathogen and one of the most virulent microorganisms to affect immunocompromised patients [1, 2]. Specifically, *P. aeruginosa* is considered a major cause of death in cystic fibrosis (CF) patients and often causes infections in burn patients or individuals with impaired immune systems [3, 4]. *P. aeruginosa* is responsible for approximately 20% of the nosocomial infections caused by gram-negative bacteria, and the risk of infection has been shown to increase with the duration of intensive care unit (ICU) stay [2, 5, 6]. Broad-spectrum antibiotics are widely used to treat *P. aeruginosa* infections, but the frequent emergence of multidrug-resistant bacterial strains is a major concern in clinical settings, accounting for significant morbidity and mortality in ICUs [6, 7]. Accordingly, several efforts have been made to develop vaccines against *P. aeruginosa* infections, but most of the candidates are still in the preclinical or clinical development stages [8, 9].

A primary approach to developing a vaccine against *P. aeruginosa* has involved searching for *P. aeruginosa* virulence factors that generate protective antibodies. Candidate antigens have included lipopolysaccharide (LPS) [10–13], membrane proteins including OprF and OprI, extracted outer membrane protein (OMP) fractions [14–16], extracellular proteins such as Psl [17, 18] and PcrV [18–20], flagellin [21–23], and pilin [23, 24]. A *P. aeruginosa* vaccine generated using the LPS O-antigen was attempted, but was not successful owing to its high toxicity [25]. Furthermore, a

report that 68% of the strains derived from CF patients did not express O-antigen and were nontypeable made the LPS O-antigen less attractive as a *P. aeruginosa* vaccine [26]. Vaccines based on OMPs or extracellular proteins are thought to be attractive candidates because protein antigens are more highly conserved among strains [9, 27]. A *P. aeruginosa* vaccine containing OMPs isolated from four strains was developed 20 years ago [28, 29]. Active and passive immunization with this vaccine conferred protection against systemic *P. aeruginosa* infections in mice [28, 29]. The safety and immunogenicity of the vaccine were also demonstrated in healthy volunteers [30], and the protective efficacy was shown in burn patients [16, 31]. Furthermore, human antibodies specific for *P. aeruginosa* OMPs were highly cross-reactive to and protective against heterologous immunotype *P. aeruginosa* strains [32].

Adjuvants are agents that are used to increase the efficacy of vaccines. Aluminum salts were the first adjuvant introduced in the 1920s and have been widely used for the production of human vaccines. A *P. aeruginosa* vaccine containing alum was developed, but clinical studies of the vaccine did not detect any benefit to using alum in regard to efficacy [14, 33]. Therefore, new adjuvants to develop effective *P. aeruginosa* vaccines are required.

De-*O*-acylated lipooligosaccharide (dLOS) is a Toll-like receptor 4 agonist that is derived from an *Escherichia coli* LPS and contains a core oligosaccharide with N-linked acyl groups on the lipid A moiety [34, 35]. Similar to monophosphoryl lipid A, dLOS induces the secretion of cytokines from murine peritoneal macrophages, but also demonstrates more potent activation of human monocytes and dendritic cells [35]. A combination of dLOS and aluminum hydroxide (alum; designated CIA06) was evaluated for its adjuvant effects on human papillomavirus (HPV) L1 virus-like particles, anthrax protective antigen, and H1N1 influenza vaccines [36–40]. The results indicated that CIA06 significantly increased antibody titers to these vaccines, and that the induced antibodies were effective in neutralizing the HPV pseudovirus, anthrax lethal toxin, and influenza virus, respectively [37–40]. Furthermore, CIA06-adjuvanted vaccines are capable of inducing both Th1- and Th2-type immune responses that persist for an extended period [37, 40]. Accordingly, the safety and immunogenicity of the CIA06-adjuvanted HPV vaccine was subsequently confirmed in a phase I human trial (unpublished data).

In this study, we investigated the adjuvant activity of CIA06 on the *P. aeruginosa* OMP antigen vaccine. The results revealed that the immunogenicity of the CIA06-adjuvanted vaccine was significantly higher and that it provided complete protection against lethal *P. aeruginosa* infections, demonstrating the potential of CIA06 as an effective adjuvant for the *P. aeruginosa* OMP vaccine.

Materials and Methods

Reagents

Tryptic soy broth (TSB) was purchased from BD Difco (USA), and the animal cell culture medium was purchased from Corning (USA). Fetal bovine serum (FBS) and L-alanyl-L-glutamine (GlutaMAX-I) were purchased from Gibco/Thermo Fisher Scientific (USA), whereas the Hyclone defined FBS was obtained from GE HealthCare (USA). Dimethylformamide (DMF), fluorescein isothiocyanate (FITC)-Isomer I, and gelatin were obtained from Sigma-Aldrich (USA), and baby rabbit complement and cell fixation buffer were purchased from Pel-Freez (USA) and eBioscience (USA), respectively. HRP-conjugated goat anti-mouse antibodies (IgG, IgG1, and IgG2a) were purchased from either Jackson ImmunoResearch Laboratories (USA) or Serotec (UK).

P. aeruginosa Strains, Cells, and Culture Conditions

The PA103 (FT2), PAO1 (FT3), and PA5940 (FT6) *P. aeruginosa* strains were purchased from American Type Culture Collection (ATCC, USA). *P. aeruginosa* GN-H3 (FT1) was derived from the GN11189 clinical isolate [41] and was obtained by repeated in vivo passages in this study. The serotypes of the *P. aeruginosa* strains were confirmed using monovalent rabbit anti-*P. aeruginosa* antibodies (Denka Seiken, Japan) and by DNA sequencing of the LPS O-antigen genes [42]. *P. aeruginosa* strains were routinely cultured at 37°C on TSB agar plates. For the experiments, the bacteria were grown in TSB liquid medium to the log phase (OD_{600} = 0.8–1.2), washed, and resuspended in phosphate-buffered saline (PBS) prior to use. The number of cells in each bacterial preparation was determined by viable cell counting. Human HL-60 promyelocytic leukemia cells were obtained from ATCC and maintained in RPMI 1640 medium containing 10% FBS and 2 mM L-alanyl-L-glutamine at 37°C in a humidified 5% CO_2 incubator.

P. aeruginosa OMP Antigens and Adjuvants

The *P. aeruginosa* OMP antigens, which were prepared from the EG-PA01 (FT1) and EG-PA02 (FT2) *P. aeruginosa* strains using the method previously described by Park *et al*. [29], were provided by EyeGene (Korea). The endotoxin levels in the antigen preparations were determined using the Endosafe-PTS test system (Charles River Laboratories, USA) and were confirmed to be lower than 2 EU/μg protein.

The dLOS adjuvant was prepared from an *E. coli* LPS mutant strain as previously described [35], quantified using the 2-keto-3-deoxyoctonate assay [43], and visualized on a silver-stained SDS-polyacrylamide gel. Alum (Alhydrogel) was purchased from Superfos Biosector (Denmark). The CIA06 adjuvant system was

prepared by combining 0.5 μg of dLOS and 25 μg of alum as Al^{3+}. The *P. aeruginosa* OMP antigen was mixed with dLOS, alum, or CIA06 and incubated on ice for 2 h before use for immunization.

Immunization of Mice

Six-week-old specific pathogen-free female BALB/c mice were purchased from SLC (Japan). The design of the animal experiments is detailed in Table 1. Mice were immunized two or three times at a 1-week interval via an intramuscular injection with the *P. aeruginosa* antigen vaccine alone or in combination with dLOS (0.5 μg), alum (25 μg), or CIA06 in 100 μl of PBS (pH 7.4). Control mice were administered PBS or CIA06. Blood samples were collected from the animals at 2 weeks post-immunization. All animal experiments were reviewed and approved by the Sejong University Animal Care and Welfare Committee (SJ-20140803).

Measurement of Antigen-Specific Serum IgG Antibody Titers

Serum IgG antibody levels specific for the *P. aeruginosa* antigen were determined by endpoint dilution enzyme-linked immunosorbent assay (ELISA) using sera from individual mice as previously described [40]. Briefly, each well of a 96-well Nunc-Immuno Plate (Thermo Fisher Scientific) was coated with 100 μl of *P. aeruginosa* antigen (2 μg/ml) and incubated overnight at 4°C. On the following day, the wells were blocked with 1% bovine serum albumin (BSA) in PBS, washed with PBS containing 0.05% Tween 20, and incubated with mouse serum that was serially diluted 2-fold with 1% BSA in PBS. The bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a antibody for 1 h at 37°C followed by incubation with TMB substrate (BD Biosciences). The reaction was stopped, and absorbance was measured at 450 nm using an Infinite M200 microplate reader (Tecan, Switzerland). The endpoint titer was defined as the highest serum dilution with an absorbance value greater than the absorbance value of the non-immune serum, with the cutoff value set at 0.1. The geometric mean titers (GMT) were calculated from individual $\log_{10}$-transformed titers and expressed as GMT ± standard deviation (SD) for each experimental group of mice.

To determine the serum IgG antibody specific for *P. aeruginosa* strains, the bacteria were cultured in TSB and harvested at an OD_{600} of 0.8–1.2. The cells were washed with PBS and resuspended in 0.05 M sodium carbonate buffer (pH 9.6) to an OD_{600} of 0.75, which was used as the coating antigen for the ELISAs.

Opsonophagocytosis Assay

The opsonophagocytic activity of the mouse sera was determined using a fluorescence-based opsonophagocytosis assay as described by Zuercher *et al.* [44] with slight modifications. The bacteria were grown to the log phase, harvested, heat-inactivated at 56°C for 30 min, and labeled with 100 μg/ml of FITC-Isomer I in Hank's Balanced Salt Solution (HBSS) for 1 h at 4°C. After washing with PBS, the bacteria were resuspended in opsonization buffer (5% defined FBS and 0.1% gelatin in HBSS) to an OD_{600} of 0.9, aliquoted, and stored at −20°C until analysis. The labeled bacteria (5×10^6 CFU) were mixed with mouse sera and 10% active or heat-inactivated baby rabbit complement and incubated with shaking at room temperature in the dark for 30 min. Afterward, HL-60 cells that were differentiated in 0.8% DMF for 5 days were added to the mixture (at a 20:1 bacteria:HL-60 cell ratio) and incubated for 30 min. After washing with 0.1% BSA in PBS, the cells were fixed and analyzed by flow cytometry using a FACSCanto II system (BD Biosciences) and FlowJo software (Treestar, USA). The opsonophagocytic activities of the mouse sera were expressed as the geometric mean fluorescence intensity (MFI) of FITC-positive HL-60 cells.

Mouse Protection Assay

To evaluate the protective efficacy of the non-adjuvanted and adjuvanted *P. aeruginosa* vaccines, groups of mice were immunized two or three times at 1-week intervals via an intramuscular injection of the *P. aeruginosa* antigen alone or in combination with the different adjuvants. Two weeks post-immunization, blood samples were collected from the animals. One week later, the mice were challenged via an intraperitoneal injection of *P. aeruginosa* strain PA103 (FT2) or GN-H3 (FT1) at a dose of 10 LD_{50} and monitored for survival and changes in body weight for 8 days. The resulting survival rates were determined by death or >25% loss in body weight, at which point the mice were humanely euthanized.

Statistical Analysis

The SPSS 18.0 software (IBM, USA) was used for the statistical analysis. Differences among the experimental groups were analyzed by one-way ANOVA with Tukey's multiple comparison test. A Student's *t*-test was used for comparisons between two groups. The differences in mouse survival rates among the experimental

Table 1. Study design for the animal experiments.

Antigen type	Experiment	No. of mice per group	Antigen dose	No. of immunizations
FT2	I	5	5 μg	2
	II	6	5 μg	2 or 3
	III	10	5 μg	2
	IV	6	6.5 μg	2
	V	5	5 μg	2 or 3
FT1	VI	5	5 μg	2
	VII	6	2 μg	3

groups were analyzed using a log-rank test. A p value < 0.05 was considered statistically significant.

Results

CIA06 Increases the Serum IgG Antibody Titers to the *P. aeruginosa* OMP Antigen

The effect of the CIA06 adjuvant on the *P. aeruginosa* OMP vaccine was examined using the *P. aeruginosa* FT2 antigen. We immunized mice twice with 5 μg of *P. aeruginosa* FT2 antigen alone or in combination with dLOS, alum, or CIA06 (Table 1, Experiment I), and measured the total IgG antibody titers against the antigen and the PA103 FT2 strain in the immune sera. The addition of dLOS or alum to the antigen increased the antigen-specific IgG antibody titers by 1.8- and 4-fold, respectively (Fig. 1). The co-administration of CIA06 and the antigen further increased the antibody responses, resulting in an IgG titer 21 times higher than the corresponding titer observed following administration of the non-adjuvanted vaccine ($p < 0.001$). The IgG antibody titers of the immune sera against *P. aeruginosa* FT2 bacteria were also determined, which revealed that the mouse antibody titers against FT2 antigen and bacteria were similar. These data suggest the synergistic effects of the two adjuvants on the *P. aeruginosa* OMP antigen.

To determine the effects of the number of immunizations on the immunogenicity of the CIA06-adjuvanted vaccine, we immunized mice two or three times with 5 μg of *P. aeruginosa* FT2 antigen alone or in combination with alum or CIA06 and compared the serum IgG antibody responses to the antigen (Table 1, Experiment II). No significant differences were observed in the total IgG titers following the two immunization schedules in the groups of mice immunized with non-adjuvanted vaccine (Fig. 2). In contrast, mice administered the alum- or CIA06-adjuvanted vaccine exhibited significantly higher IgG antibody titers following three immunizations than the mice that received two immunizations. Furthermore, the IgG titers of the group of mice that received the alum-adjuvanted vaccine were 3.6-fold higher than the titers observed in the non-adjuvanted vaccine group after two immunizations, whereas a 11.2-fold difference was observed between the two groups after three immunizations. Similarly, the IgG titer of the CIA06-adjuvanted vaccine group was 10.2-fold higher than the titer observed in the non-adjuvanted vaccine group after two immunizations, whereas the observed IgG titer was 40-fold higher after three immunizations. In addition, CIA06 enhanced the serum IgG2a antibody responses in

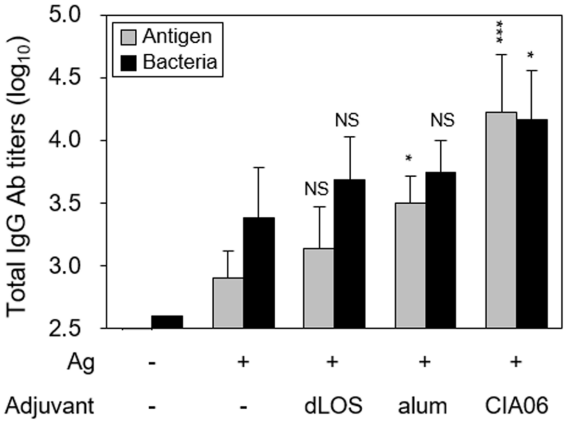

Fig. 1. Serum IgG antibody titers in the mice immunized with an adjuvanted *P. aeruginosa* vaccine.

Mice ($n = 5$) were immunized twice at a 1-week interval with 5 μg of *P. aeruginosa* FT2 vaccine antigen alone or in combination with dLOS, alum, or CIA06. Mice administered PBS were included as a control. Two weeks after the second immunization, blood was collected and individual sera were assayed for IgG antibody titers against the FT2 antigen and FT2 strain PA103. The results are presented as the geometric mean titer ± SD of the values obtained from five mice in each group, and data shown are representative of at least three experiments with similar results. Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. *$p < 0.05$; ***$p < 0.001$; NS, not significant as compared with the group that received antigen alone.

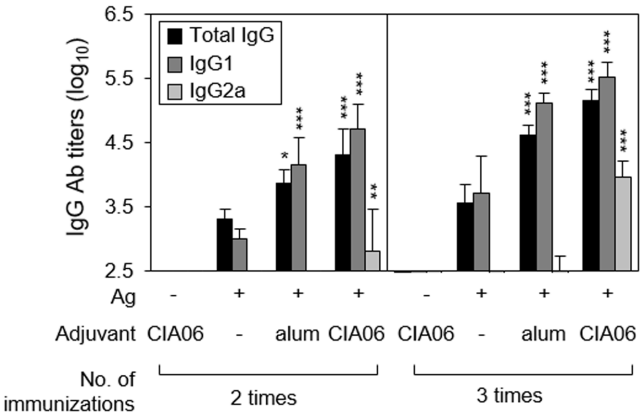

Fig. 2. Effects of the number of immunizations on serum IgG antibody titers specific for *P. aeruginosa*.

Mice ($n = 6$) were immunized two or three times with 5 μg of *P. aeruginosa* FT2 antigen alone or in combination with alum or CIA06. Control mice received CIA06 alone. Two weeks post-immunization, sera were collected and assayed for IgG antibody titers against FT2 strain PA103. Results are presented as the geometric mean titer ± SD of the values obtained from six mice in each group. Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared with the group of mice that received antigen alone.

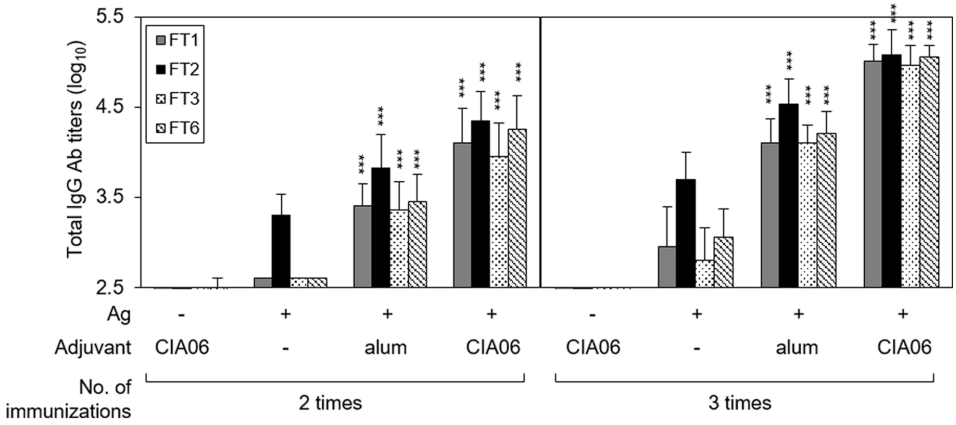

Fig. 3. Cross-reactivity of mouse sera to heterologous serotype strains.
Mice (n = 6) were immunized as described in Fig. 2, and sera were assayed for total IgG antibody titers against *P. aeruginosa* strains GN-H3 (FT1), PA103 (FT2), PAO1 (FT3), and PA5940 (FT6). The results are presented as the geometric mean titer ± SD of the values obtained from six mice in each group. Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. ***$p < 0.001$ compared with the group administered antigen alone.

the mice but the effect was only observed in the mice that received three immunizations. Similar effects were not observed in the mice that received the alum-adjuvanted vaccine.

Antibodies Induced by the CIA06-Adjuvanted Vaccine Exhibit Increased Cross-Reactivity to Heterologous Serotype Strains

To determine the cross-reactivity of the antibody induced by the non-adjuvanted and adjuvanted vaccines to heterologous serotype *P. aeruginosa* strains, we measured the IgG antibody titers against the FT1, FT2, FT3, and FT6 strains in the immune sera from the mice immunized with the FT2 antigen. The immune sera from the mice that were immunized twice reacted weakly to the FT2 strain but not to the other serotype strains (Fig. 3). However, sera from the mice administered the alum- or CIA06-adjuvanted FT2 vaccine exhibited significantly higher reactivity to all of the strains tested, with the highest titer being against the FT2 strain. Three immunizations with the CIA06-adjuvanted vaccine further increased the cross-reactivity of the induced antibodies, and no differences were observed in the reactivity between the homologous and the heterologous serotype strains.

Antibodies Induced by CIA06-Adjuvanted Vaccine Exhibit Increased Opsonophagocytic Activity

Antibody-complement-dependent phagocytosis is one of the crucial defense mechanisms against *P. aeruginosa* infections. Using a fluorescence-based opsonophagocytic assay, we evaluated whether antibodies induced by the CIA06-adjuvanted vaccine exhibited opsonic activity to *P. aeruginosa* [44, 45]. Test sera were obtained from the mice that were immunized twice at a 1-week interval with 6.5 μg of FT2 antigen alone or in combination with alum or CIA06 (Table 1, Experiment IV). As shown in Fig. 4A, only a low level of phagocytosis was observed using the sera from the CIA06 control group, whereas robust phagocytosis was mediated by the sera from the CIA06-adjuvanted vaccine group. Mouse sera were likewise serially diluted and assayed for opsonophagocytic activity (Fig. 4B), the results of which indicated that the opsonic activity of the mouse immune sera was inversely related to the serum dilution factor and that the sera from the group that received the CIA06-adjuvanted vaccine were more effective in mediating the opsonophagocytosis of *P. aeruginosa* than the sera from the group that received the non-adjuvanted or alum-adjuvanted vaccine. As shown in Fig. 4C, the 1:40 diluted mouse sera were assayed for opsonophagocytic activity in the absence or presence of complement. Only a minimal degree of phagocytosis was observed in the absence of complement, confirming that the opsonophagocytosis of *P. aeruginosa* is a complement-dependent process. These results demonstrate that antibodies elicited by the CIA06-adjuvanted vaccine possess increased the opsonophagocytic activity toward *P. aeruginosa*.

CIA06 Promotes the Protective Efficacy of the Vaccine against *P. aeruginosa* Infection

To evaluate the protective efficacy of the adjuvanted

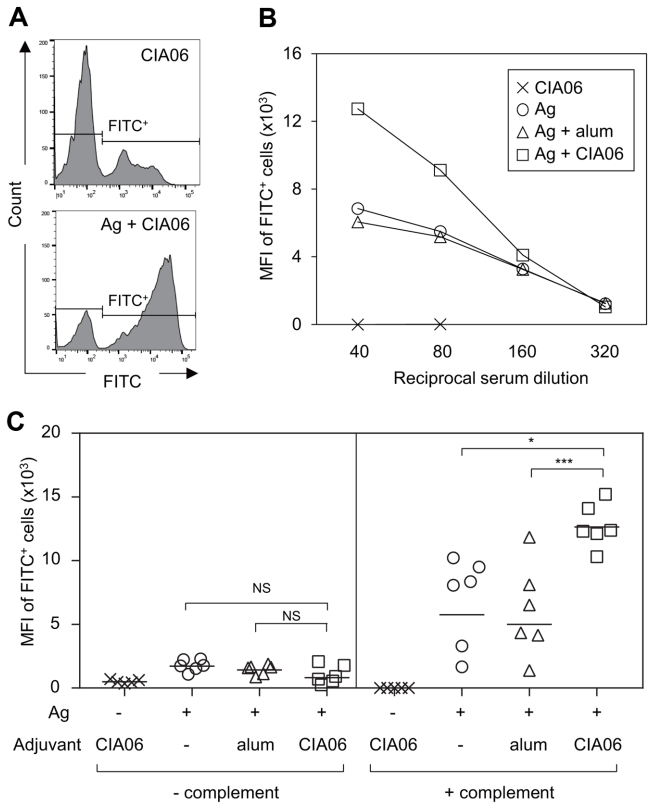

Fig. 4. Opsonophagocytic activity of mouse sera against *P. aeruginosa*.

Mice (n = 6) were immunized twice with 6.5 μg of *P. aeruginosa* FT2 antigen alone or in combination with the different adjuvants. The control mice received CIA06. Two weeks post-immunization, sera were collected and assayed for opsonophagocytic activity against FT2 strain PA103 as described in the Materials and Methods. (**A**) The histograms show phagocytosis of FITC-labeled bacteria by HL-60 cells in the presence of the sera from mice that were immunized with CIA06 alone or with the CIA06-adjuvanted vaccine. (**B**) Mouse sera were serially diluted and assayed for opsonophagocytic activity, and the results are expressed as the geometric mean fluorescence intensity (MFI) of FITC-positive HL-60 cells for each group of six mice. (**C**) The opsonophagocytic activity of the individual sera were determined at a dilution of 1:40 in the absence or presence of complement. The bars indicate the geometric MFI of the values obtained from six mice in each group. *$p < 0.05$; ***$p < 0.001$; NS, not significant.

P. aeruginosa OMP vaccine, we immunized the mice two or three times with the FT2 antigen alone or in combination with the different adjuvants followed by the administration of a lethal dose of a homologous serotype strain PA103, the results of which are summarized in Table 2. None of the control mice receiving PBS or CIA06 alone survived the *P. aeruginosa* challenge. In contrast, the animals immunized twice with 5 μg of the CIA06-adjuvanted vaccine exhibited

Table 2. Protective efficacy of CIA06-adjuvanted *P. aeruginosa* FT2 vaccine against lethal challenges with a homologous strain[a].

Experiment	No. of immunizations	Immunization	Protective efficacy (%)[a]
I	2	PBS	0
		Ag	20
		Ag + dLOS	0
		Ag + alum	20
		Ag + CIA06	80
III	2	CIA06	0
		Ag	40
		Ag + alum	50
		Ag + CIA06	90
IV	2	PBS	0
		Ag	66
		Ag + alum	100
		Ag + CIA06	100
V	2	CIA06	0
		Ag	20
		Ag + CIA06	60
	3	CIA06	0
		Ag	80
		Ag + CIA06	100

[a]Mice were immunized two or three times with *P. aeruginosa* FT2 antigen alone or in combination with the different adjuvants, and blood samples were collected at 2 weeks post-immunization. One week later, the mice were subjected to a lethal challenge with *P. aeruginosa* FT2 strain PA103 and monitored for survival for 8 days.

60–90% survival following *P. aeruginosa* infection, whereas the mice administered the alum-adjuvanted vaccine exhibited 20–50% survival (Experiments I, III, and V). An increase in the antigen dose to 6.5 μg yielded a higher protective efficacy, conferring 100% protection for both the alum- and CIA06-adjuvanted vaccines (Experiment IV). We also compared the protective efficacy of the vaccines following the delivery of two and three immunizations (Experiment V). The results revealed that mice immunized three times with 5 μg of the CIA06-adjuvanted vaccine exhibited improved protection (100% survival) compared with the mice that were immunized twice (60% survival), indicating that three immunizations with the CIA06-adjuvanted vaccine yields better results.

CIA06 Enhances the Immunogenicity and Protective Efficacy of the FT1 Vaccine

To investigate whether CIA06 was able to enhance immune

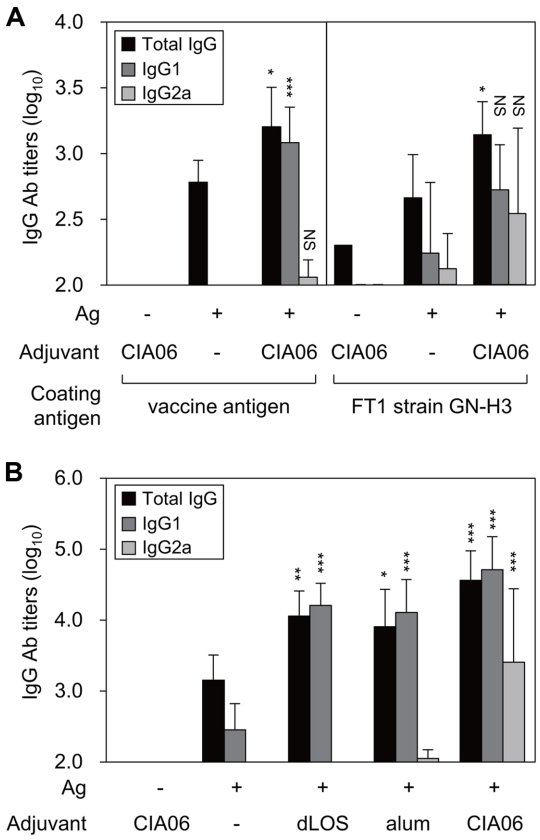

Fig. 5. Adjuvant activity of CIA06 on the *P. aeruginosa* FT1 antigen vaccine.

(**A**) Mice ($n = 5$) were immunized twice with 5 μg of *P. aeruginosa* FT1 antigen alone or in combination with CIA06. Two weeks post-immunization, sera were collected and assayed for IgG antibody titers against *P. aeruginosa* FT1 antigen or FT1 strain GN-H3. (**B**) Mice ($n = 6$) were immunized three times with 2 μg of *P. aeruginosa* FT1 antigen alone or in combination with the different adjuvants. Two weeks later, sera were collected and assayed for IgG antibody titers against the *P. aeruginosa* FT1 antigen. The results were expressed as the geometric mean titer ± SD of the values obtained from five or six mice in each group. Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared with the group administered antigen alone.

responses to other serotype antigens, we combined CIA06 with the *P. aeruginosa* FT1 antigen and evaluated the resulting immune responses (Table 1, Experiments VI and VII). Mice were immunized twice with 5 μg of FT1 antigen, either alone or in combination with CIA06. The mice administered the CIA06-adjuvanted FT1 vaccine exhibited significantly higher serum IgG antibody titers against both the FT1 antigen and the homologous GN-H3 strain than mice that were administered the non-adjuvanted vaccine ($p < 0.05$; Fig. 5A). The protective efficacy of the CIA06-adjuvanted vaccine was also higher (60%) than the efficacy of the non-adjuvanted vaccine (0%) (Table 3). Next, we compared the immune responses of the mice administered two immunizations with 5 μg of antigen and mice that received three immunizations with 2 μg of antigen. Similar to the results observed with the FT2 antigen, the effects of the CIA06 adjuvant were more prominent after three compared with two immunizations in terms of both immunogenicity and protective efficacy (Fig. 5, Table 3).

Table 3. Protective efficacy of CIA06-adjuvanted *P. aeruginosa* FT1 vaccine against *P. aeruginosa* FT1 strain GN-H3.

Experiment	No. of immunizations	Immunization	Protective efficacy (%)[a]
VI	2	CIA06	0
		Ag	0
		Ag + CIA06	60
VII	3	PBS	0
		Ag	100
		Ag + dLOS	83
		Ag + alum	100
		Ag + CIA06	100

[a]Mice were immunized with *P. aeruginosa* FT1 antigen alone or in combination with the different adjuvants, and blood samples were collected at a weeks post-immunization. One week later, the mice were subjected to a lethal challenge with *P. aeruginosa* FT1 strain GN-H3 and monitored for survival for 8 days.

Discussion

Despite significant efforts to develop an effective vaccine against *P. aeruginosa*, no vaccines are commercially available to date. The *P. aeruginosa* OMP vaccine was developed 20 years ago, and its safety and immunogenicity have been demonstrated in healthy volunteers [30]. Moreover, the protective efficacy of the vaccine was demonstrated in a phase II clinical study involving burn patients [16, 31]. In the study, burn patients were immunized three times with 0.5 or 1.0 mg of antigen, which revealed that the 1.0 mg dose was more effective in inducing antibody responses and in providing protection against *P. aeruginosa* infection. In an attempt to improve the *P. aeruginosa* OMP vaccine, we combined CIA06 with the vaccine and evaluated the adjuvant activity of CIA06 against the FT1 and FT2 antigens. The results indicated that CIA06 was highly effective in promoting antibody responses to both the FT1 and FT2 antigens and that the adjuvant effect of CIA06 was more prominent following three immunizations compared with a two-immunization schedule. Three immunizations with 2 μg of FT1 antigen combined with the CIA06

adjuvant yielded significantly higher antibody responses than two immunizations with 5 μg of antigen, and an increase in IgG2a titers was observed only after three immunizations with the CIA06-adjuvanted vaccine. Sera from mice immunized with the CIA06-adjuvanted vaccine also exhibited higher opsonophagocytic activity against *P. aeruginosa* than sera from mice in the non- or alum-adjuvanted group, indicating that the induced antibodies were functionally active. Consequently, the CIA06-adjuvanted vaccine was the most effective in protecting mice from lethal *P. aeruginosa* challenges, confirming the higher protective efficacy of the CIA06-adjuvanted vaccine than the non- or alum-adjuvanted vaccine.

Although the serological variation of OMP or extracellular proteins has not yet been the focus of extensive studies, these antigens are relatively conserved across diverse *P. aeruginosa* isolates [46−48]. The advantage of using these proteins as a vaccine antigen is that the vaccine can induce broader cross-protective immunity in diverse clinical strains. In a previous report by Lee *et al*. [32], anti-*P. aeruginosa* OMP IgG from normal human plasma was able to promote opsonophagocytic killing and protect mice against all seven heterologous serotype strains. The present study showed that CIA06 increased the cross-reactivity of the *P. aeruginosa* OMP vaccine to heterologous strains, suggesting that the CIA06-adjuvanted *P. aeruginosa* OMP vaccine might provide a broader spectrum of cross-protective immunity to various *P. aeruginosa* strains.

Recently, it was proposed that the IL-17 cytokine and opsonophagocytic antibody contribute to host defenses against *P. aeruginosa* infections [49, 50]. Th17 cells are known to be involved in the clearance of extracellular bacteria and fungi, to produce cytokines such as IL-17, IL-21, and IL-22, and to stimulate the secretion of proinflammatory cytokines and chemokines, leading to the recruitment and activation of neutrophils at the site of infection [52, 53]. In addition, Th17 cells play a crucial role in maintaining mucosal immunity and barrier integrity, including the skin, lungs, and intestines [54]. Furthermore, a reduction in the ratio of Th17/Th1 cells in the early stages of injury results in increased susceptibility to extracellular pathogens in burn patients [55]. In the absence of opsonophagocytic antibody, IL-17 plays a crucial role in protecting against acute or chronic pneumonia caused by *P. aeruginosa* [56, 57]. Previously, we have shown that dLOS stimulates Th17 as well as Th1 and Th2 responses [35]. The CIA06-adjuvanted HPV vaccine induced the secretion of antigen-specific IL-17 [36]. In this study, CIA06 increased *P. aeruginosa*-specific IL-17 cytokine secretion from the splenocytes of the immunized mice (data not shown). IL-17 reportedly aids in IgG2a immunoglobulin isotype switching by B cells [58], which is consistent with the finding that mice immunized with the CIA06-adjuvanted vaccine exhibited higher *P. aeruginosa*-specific IgG2a antibody serum titers. This observation might also be related to the drastic increase in serum IgG2a antibody levels in the mice that recovered from *P. aeruginosa* infection (data not shown).

In conclusion, the CIA06-adjuvanted *P. aeruginosa* OMP vaccine was able to efficiently induce *P. aeruginosa*-specific antibodies and increase the opsonophagocytic activity of the serum, thereby conferring protection against systemic *P. aeruginosa* infection. These results suggest that CIA06 is a potentially effective adjuvant for the *P. aeruginosa* OMP vaccine.

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Conflict of Interests

Shin Ae Park, Kwang Sung Kim, and Yang Je Cho are inventors of EyeGene-owned patents and employees of EyeGene. Na Gyong Lee is an inventor of EyeGene-owned patents and a scientific advisor of EyeGene.

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