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Efficient induction of cell-mediated immunity to varicella-zoster virus glycoprotein E co-lyophilized with a cationic liposome-based adjuvant in mice

Seo Ri Wui [a,1], Kwang Sung Kim [a,b,1], Ji In Ryu [a], Ara Ko [a], Hien Thi Thu Do [a], Yeon Jung Lee [a], Hark Jun Kim [a], Soo Jeong Lim [a], Shin Ae Park [b], Yang Je Cho [b], Chang-Gyeom Kim [c], Na Gyong Lee [a,*]

[a] Department of Integrated Bioscience and Biotechnology, Sejong University, Seoul 05006, Republic of Korea
[b] R & D Center, EyeGene, Goyang 10551, Republic of Korea
[c] Korea Polytechnic University, Seongnam 13122, Republic of Korea

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ABSTRACT

Varicella zoster virus (VZV) is a neurotropic and lymphotropic alpha herpesvirus that causes varicella and herpes zoster (HZ). At a primary infection, VZV causes varicella in young children. Reactivation of latent VZV in sensory ganglia causes painful HZ in elderly people, occasionally leading to a serious complication, postherpetic neuralgia (PHN). A live attenuated VZV vaccine, the first vaccine licensed for the prevention of HZ and PHN is not very effective, while a recombinant subunit vaccine provides higher and longer protection against HZ. In the present study, we developed a new adjuvant system CIA09A, which is composed of cationic liposomes, the Toll-like receptor 4 (TLR4) agonist de-*O*-acylated lipooligosaccharide, and *Quillaja* saponin fraction QS-21. We then determined its adjuvant activity for recombinant VZV glycoprotein E (gE) in mice. Co-lyophilization of the liposomal adjuvant formulation with gE did not abolish the immune-stimulating activity. In fact, the CIA09A-adjuvanted gE vaccine was highly effective in eliciting both humoral and cellular immune responses to the recombinant gE protein and VZV in a VZV-primed mouse model. Furthermore, the frequency of gE-specific polyfunctional $CD4^+$ T cells expressing interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2 was significantly increased in mice immunized with the adjuvanted vaccine. These data indicate that co-lyophilization of protein antigens with CIA09A enables development of a liposome-adjuvanted vaccine in a single vial to induce strong cell-mediated immunity required for vaccine efficacy. Thus, the CIA09A-adjuvanted gE vaccine warrants further development as a new prophylactic vaccine against HZ.

1. Introduction

Varicella-zoster virus (VZV) is a neurotropic and lymphotropic alpha-herpesvirus that causes two distinct diseases: varicella and herpes zoster (HZ) [1]. HZ occurs upon reactivation of latent VZV in sensory ganglia, causing rashes accompanied by severe pain and occasionally leading to postherpetic neuralgia (PHN) [1]. Inadequate cell-mediated immunity (CMI) due to aging or immunosuppression increases susceptibility to HZ, and thus, CMI is thought to be critical for blocking reactivation of the latent VZV [2,3]. Both $CD4^+$ and $CD8^+$ T cell responses appear to be required for protection against HZ [3,4]. Currently, two HZ vaccines are available for human use [5]. Zostavax, a live attenuated HZ vaccine, was the first vaccine licensed for the prevention of HZ and PHN. This vaccine enhances VZV-specific CMI responses and reduces the incidence of HZ by 70% in individuals aged 50–59 years [6,7]. The protective efficacy, however, decreases with increased age at the time of vaccination, exhibiting only a 41% protection rate in people aged

Abbreviations: APC, antigen-presenting cells; AS01, adjuvant system 01; CAF01, cationic adjuvant formulation; CHO, Chinese hamster ovary; CMI, cell-mediated immunity; DC, dendritic cells; dLOS, de-*O*-acylated lipooligosaccharide; DLS, dynamic light scattering; DMPC, cholesterol, 1,2-dimyristoyl-sn-glycero-3-phospho choline; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammoniumpropane; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; gE, glycoprotein E; FBS, fetal bovine serum; GMT, geometric mean titers; HD, human dose; HPV, human papillomavirus; HRP, horseradish peroxidase; HZ, herpes zoster; ICS, intracellular cytokine staining; IL-2, interleukin-2; IFNγ, interferon-gamma; JEV, Japanese encephalitis vaccine; LAV, live attenuated vaccine; LPS, lipopolysaccharide; mAB, monoclonal antibody; MCP-1, monocyte chemoattractant protein; MIP-1α, macrophage inflammatory protein; MPL, monophosphoryl lipid A; PBS, phosphate-buffered saline; PDI, polydispersity index; pfu, plaque-forming units; PHN, postherpetic neuralgia; QS-21, *Quillaja saponaria Molina* fraction 21; TNFα, tumor necrosis factor-alpha; TLR4, Toll-like receptor 4; VLP, virus-like particles; VZV, varicella-zoster virus.

* Corresponding author at: Department of Integrated Bioscience & Biotechnology, Sejong University, 209 Neungdong-ro, Gwangjin-gu, Seoul 05006, Republic of Korea.
E-mail address: nglee@sejong.ac.kr (N.G. Lee).
[1] These authors equally contributed to the work.

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70 years and older [8]. Recently, a recombinant HZ subunit vaccine, Shingrix, was approved for human use. Shingrix is safe and effective in immunocompromised individuals and provides longer protection compared to the live attenuated vaccine (LAV) [9–11], with 97.2% protective efficacy against HZ in individuals aged 50 years or older and a 91.3% protection rate in those aged 70 years and older [12,13]. Shingrix is composed of recombinant VZV glycoprotein E (gE) antigen and a liposome-based adjuvant system (AS01B).

Liposomes are often used as drug delivery systems and recently have been investigated as vaccine adjuvants [14,15]. Liposomal adjuvants serve as persistent depots of vaccine antigens at the site of injection, facilitate antigen delivery to lymph nodes, and increase antigen uptake and processing by antigen-presenting cells (APCs) [16–18]. Liposomes are generally weak stimulants of the immune system alone but are often used in combination with other immune stimulators to achieve optimal adjuvant activity [19,20]. Unlike neutral or anionic liposomes, however, cationic liposomes can directly activate immune cells, such as dendritic cells (DCs) and macrophages [18,21,22]. Importantly, several liposome-formulated vaccines have proven safe and effective in human clinical trials [14,15]. The cationic adjuvant formulation 01 (CAF01), which is composed of the cationic lipid dimethyldioctadecylammonium and synthetic trehalose-6,6-dibehenate, has been used as an immunostimulant for a tuberculosis vaccine [23]. Adjuvant System 01 (AS01) is a liposome-based adjuvant formulation composed of neutral liposomes and two immunostimulants, the Toll-like receptor 4 (TLR4) agonist monophosphoryl lipid A (MPL) and *Quillaja saponaria Molina* fraction 21 (QS-21) [24]. AS01 is currently used in the malaria vaccine Mosquirix and the herpes zoster vaccine Shingrix [24]. Recently, it was also shown to be effective for a tuberculosis vaccine [25].

We previously investigated the adjuvant activity of CIA06, which is composed of the TLR4 agonist de-*O*-acylated lipooligosaccharide (dLOS) and aluminum hydroxide [26,27]. CIA06 promoted the immunogenicity of several vaccines, including the human papillomavirus (HPV) virus-like particles (VLP) vaccine, influenza split vaccine, anthrax vaccine, *Pseudomonas aeruginosa* vaccine, and Japanese encephalitis vaccine (JEV) [27–32]. The safety and efficacy of CIA06 in the HPV vaccine has been demonstrated in a phase I human trial (unpublished data). Based on this success, we designed a new adjuvant formulation consisting of dLOS and cationic liposomes. Both dLOS-based adjuvant formulations similarly enhanced serum IgG and virus-neutralizing antibody titers, but the liposome formulation was more effective in eliciting CMI as assessed by serum IgG2a isotype antibody titer and antigen-specific interferon (IFN)-γ response [32].

Lyophilization is used to improve the stability of pharmaceuticals with low thermal stability, especially biological products [33]. Thermal stability is an important factor that determines effectiveness in distribution, handling, and storage as well as the efficacy of the drug. Most vaccine antigens are susceptible to thermal stress, suggesting maintenance as frozen or lyophilized molecules is desirable. Liposomes, in spite of several advantages as a vaccine adjuvant, are challenged by low stability and tend to be damaged physicochemically during the freeze-drying and rehydration process, resulting in aggregation and consequent loss of efficacy [34]. For these reasons, vaccines containing liposomes are typically formulated into separate vials of lyophilized vaccine antigen and liquid liposomal adjuvant formulation and mixed together before vaccination.

Here, we prepared a cationic liposome adjuvant formulation CIA09A containing dLOS and QS-21 for use as an adjuvant for VZV gE subunit vaccine. The VZV gE vaccine co-lyophilized with CIA09A was evaluated for its ability to elicit CMI as well as a humoral response in a VZV-primed mouse model. In addition, gE-specific polyfunctional $CD4^+$ and $CD8^+$ T cells that express IFN-γ, tumor necrosis factor (TNF)-α, and/or interleukin (IL)-2 were also determined to evaluate the protective efficacy of the CIA09A-adjuvanted vaccine against HZ.

2. Materials and methods

2.1. Ethics

The animal experimental protocols were reviewed and approved by Sejong University Institutional Animal Care and Use Committee (approval number: SJ-20150301). All mice used for experiments were housed in a temperature- and humidity-controlled chamber with a 12-h light/dark cycle. They were acclimated to the environment for one week before experiments and provided ad libitum access to food and water. Mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture before blood was collected by heart puncture.

2.2. Materials and reagents

Cholesterol, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids and/or NOF. Zostavax, the live attenuated HZ vaccine (Merck, lot no. M005902), was used as live attenuated vaccine (LAV) for animal experiments. Shingrix, a HZ subunit vaccine adjuvanted with AS01B (lot no. XC534) marketed in the USA, was purchased from GlaxoSmithKline through a vendor after obtaining the permission from the Korea Ministry of Food and Drug Safety. MPL was purchased from Avanti Polar Lipids, and aluminum hydroxide (alum, Alhydrogel®) and QS-21 were purchased from Brenntag Biosector and Desert King International, respectively. Goat anti-mouse IgG, IgG1, IgG2a, and IgG2b antibodies were purchased from Jackson ImmunoResearch and Serotec. Anti-VZV gE monoclonal antibody (mAb) and a peptide pool of VZV gE (PepMix™ VZV gE) were obtained from Virusys and JPT Peptide Technologies, respectively. VZV mock antigen was from Microbix Biosystems. Cell culture media and antibiotics were purchased from Welgene, whereas fetal bovine serum (FBS) was from Gibco/Invitrogen. Anti-mouse CD28 and CD49d mAbs were obtained from BioLegend, and fluorochrome-conjugated antibodies for CD4 (FITC), CD8 (FITC), TNF-α (PE), IL-2 (APC), IFN-γ (PE-Cy7) were purchased from eBioscience.

2.3. Preparation of recombinant VZV gE protein

The recombinant VZV gE protein was expressed in Chinese hamster ovary (CHO) cells. The coding sequences for the truncated gE gene lacking the hydrophobic anchor and carboxy-terminal domains were cloned into the pEGC-gE vector containing a CMV promotor and the dihydrofolate reductase gene for CHO cells. The gE protein product secreted into the culture media was purified by sequential anion/cation exchange and immobilized metal-affinity chromatography and ultrafiltration, and analyzed for size, purity, and endotoxin content before use.

2.4. Preparation of vaccine adjuvants

Cationic liposomes were prepared using DOTAP and DMPC at a molar ratio of 1:1 using either a modified dry cake method or thin-film method as previously described [35,36]. For the dry cake method, DOTAP was dissolved in tertiary-butyl alcohol and mixed with an equal volume of DMPC solution in t-butyl alcohol. The mixture was frozen to −80 °C and lyophilized overnight using a freeze dryer. The dry cake was hydrated to a final concentration

of 2 mg/ml phospholipid with a 10% sucrose solution and homogenized using a high-pressure homogenizer (Avestin). Liposome preparations were immediately used to prepare vaccine formulations or lyophilized in sealed glass vials for storage at 4 °C. For experiments, freeze-dried liposomes were rehydrated with deionized water to the original volume. For the thin-film method, a chloroform solution containing the 1:1 DOTAP:DMPC mixture was dried in a round bottom flask using a rotary evaporator (IKA) to form a film. The lipid film was rehydrated with a 10% sucrose solution containing gE (100 μg/ml) and homogenized. The neutral liposome adjuvant formulation containing MPL and QS-21 was prepared as described [37]. The neutral liposome adjuvant formulation is composed of 1.25 mg liposomes (containing DOPC and cholesterol), 50 μg MPL, and 50 μg QS-21 per ml.

The TLR4 agonist dLOS was prepared from an *E. coli* LPS-mutant strain as previously described [38], quantified using the 2-keto-3-deoxyoctonate assay [39], and visualized on a silver-stained SDS-polyacrylamide gel. The adjuvant system CIA06 consists of 500 μg alum and 10 μg dLOS per human dose (HD) [27]. CIA09 was prepared by combining 1 mg cationic liposomes with dLOS at various ratios, while CIA09A was prepared by adding 50 μg QS-21 per mg phospholipid to CIA09.

2.5. Vaccine formulations

To prepare adjuvanted VZV gE vaccines, gE was mixed with liposomes, dLOS, and/or QS-21. The vaccine formulations were either immediately used for experiments or lyophilized in sealed glass vials and stored at 4 °C. Freeze-dried vaccines were reconstituted with deionized water for immunizations.

For the CIA09A-adjuvanted vaccines that used liposomes prepared by the thin-film method, the gE-incorporated liposomes were mixed with dLOS and QS-21 and lyophilized. Two vaccines of different particle sizes (100–300 nm and 300–500 nm) were obtained.

The size and polydispersity index (PDI) of liposomes and vaccine particles were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments). The zeta potential of the particles was determined by laser Doppler micro electrophoresis using a Zetasizer Nano ZSP. Freeze-dried samples were rehydrated with deionized water and diluted 10-fold with a 10% sucrose solution for assays. All measurements were performed in triplicate. Malvern Zetasizer DTS software (version 7.11) was used for data acquisition and analysis.

2.6. Immunization of mice

Six-week-old specific-pathogen-free female BALB/c and C57BL/6 mice (SLC, Japan) were randomly assigned into groups (n = 4–6) and immunized intramuscularly twice at a 2-week interval with an unadjuvanted gE vaccine (2 or 5 μg), adjuvanted vaccines, or phosphate-buffered saline (PBS) in a total volume of 100 μl (Table 1). For Shingrix, gE antigen was reconstituted with AS01B adjuvant according to the manufacturer's instructions, and 1/10 HD (50 μl) was adjusted to 100 μl with PBS before used for immunization. Two or four weeks after the second immunization, blood

Table 1
Study design for animal experiments.

Experiment	Mouse strain	VZV -priming	No. of mice	gE antigen dose/mouse	Treatment*	Sampling day (post-immunization)
Exp. 1	BALB/c	No	6	2 μg	PBS gE gE + CIA09(0.5)** gE/CIA09(0.5)	2 weeks
Exp. 2	C57BL/6	No	6	5 μg	PBS gE gE + CIA09(3) gE/CIA09(3)	4 weeks
Exp. 3	C57BL/6	Yes	4	5 μg	PBS gE gE + CIA06 gE/CIA09(1)	4 weeks
Exp. 4	C57BL/6	Yes	6	5 μg	PBS gE gE + CIA06 gE/CIA09(3) LAV	4 weeks
Exp. 5	C57BL/6	Yes	6	5 μg	PBS gE gE/CIA09(3) gE/CIA09A(3) LAV	4 weeks
Exp. 6	C57BL/6	Yes	6	5 μg	PBS gE gE/LP gE/CIA09(3) gE/LP/QS-21 gE/CIA09A(2) gE/CIA09A(3)	4 weeks
Exp. 7	C57BL/6	Yes	6	5 μg	PBS gE/CIA09A(2) gE + (LP-N + MPL + QS-21)	4 weeks
Exp. 8***	C57BL/6	Yes	6	5 μg	PBS gE/CIA09A(1)-S gE/CIA09A(1)-L Shingrix	4 weeks

* gE antigen was either mixed (+) or co-lyophilized (/) with adjuvants as described in Materials and Methods.
** The doses of dLOS in CIA09 or CIA09A are given in parentheses.
*** The CIA09A-adjuvanted vaccines were prepared by co-lyophilizing gE-incorporated liposomes with dLOS and QS-21, and small (S)- and large (L)-particle vaccines were prepared as described in Materials and Methods.

samples were collected from the animals, allowed to clot at 4 °C overnight, and centrifuged. Sera were divided into aliquots and stored at −70 °C. Spleens were collected from the animals and immediately used for assays.

To evaluate the immunogenicity of the gE vaccine in VZV-positive animals, we adopted a VZV-primed mouse model [40]. C57BL/6 mice were primed with VZV via subcutaneous injection of 1/10 HD of Zostavax. Four or five weeks later, mice were immunized as described above. Immune responses were assessed 4 weeks after the second immunization. For the LAV control group, VZV-primed animals were injected intramuscularly with a single 1/10 HD of Zostavax.

2.7. Measurement of the serum levels of IgG antibody specific for gE and VZV membrane glycoproteins

VZV gE-specific IgG antibody titers in sera from individual mice were determined by end-point dilution enzyme-linked immunosorbent assay (ELISA) as previously described [27]. End-point titers were defined as the highest serum dilutions that resulted in an absorbance value twice as high as that of non-immune serum with a cut-off value of 0.1 and were expressed as group geometric mean titers (GMT) ± SD from 4 to 6 mice.

Serum levels of IgG antibody specific for VZV membrane glycoproteins were also determined using VZV-infected cells as described by Liu et al. [41].

2.8. IFN-γ ELISpot and cytokine ELISA assays

The number of mouse splenocytes producing IFN-γ was determined by ELISpot assays as previously described [27]. Single-cell suspensions were prepared from the spleens of immunized mice and seeded in triplicate in ELISpot plates that had been coated with capture antibodies. Cells were stimulated with gE (5 μg/ml), a pool of gE peptides (2 μg/ml), VZV (100 pfu/ml), concanavalin A (1 μg/ml; positive control), or culture media (negative control) for 24 h at 37 °C under a 5% CO_2 atmosphere. IFN-γ-secreting cells were detected using an ELISpot kit (BD Bioscience), and positive spots

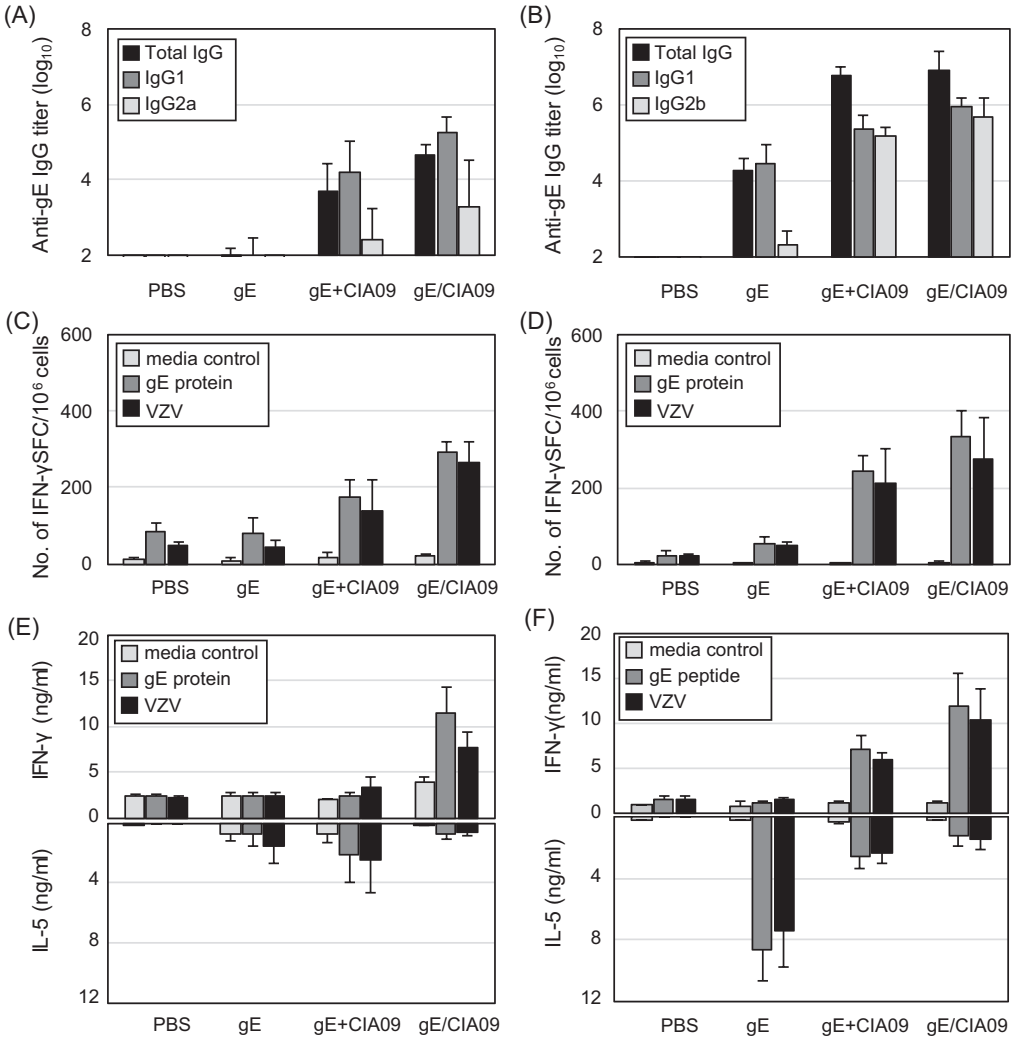

Fig. 1. Immune responses induced by gE mixed or co-lyophilized with CIA09. The adjuvanted gE vaccine was prepared by mixing gE with CIA09 (gE + CIA09) or co-lyophilizing gE with CIA09, which was then reconstituted to the original volume with water prior to use for immunization (gE/CIA09). (A, C, E) In Exp. 1, BALB/c mice ($n = 6$) were immunized twice with a 2-week interval with gE (2 μg) alone or in combination with CIA09 (LP 50 μg, dLOS 0.5 μg), and immune responses were assessed 2 weeks after the second immunization. Control mice were administered PBS. (B, D, F). In Exp. 2, C57BL/6 mice ($n = 6$) were immunized with gE (5 μg) alone or adjuvanted with CIA09 (LP 100 μg, dLOS 3 μg), and immune responses were assessed 4 weeks after the second immunization. The gE-specific IgG titers of individual sera were determined by end-point dilution ELISA (A, B). Splenocytes collected from the immunized mice were restimulated in vitro with gE, a pool of gE peptides, or VZV, and the numbers of IFN-γ-producing splenocytes were determined by ELISpot assay (C, D). Levels of IFN-γ and IL-5 secreted into the culture media were also determined (E, F). Data are expressed as the GMTs ± SD of titers of the sera obtained from six mice in each group (A, B) or the means ± SD of values obtained from triplicate assays using two spleens each (C – F).

were counted using a CTL-ImmunoSpot S5UV Micro Analyzer (Cellular Technology).

Cytokines secreted by mouse splenocytes were measured as previously described [27]. Spleen cells were stimulated with gE (5 μg/ml), a pool of gE peptides (2 μg/ml), or VZV (100 pfu/ml) for 72 h. The culture media were collected and assayed for IFN-γ and IL-5 using a sandwich ELISA in the BD Opt EIA kit (BD Biosciences) or DuoSet ELISA kits (R&D Systems).

2.9. Measurement of the frequency of polyfunctional $CD4^+$ and $CD8^+$ T cells

The frequency of gE-specific polyfunctional $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, and/or IL-2 was determined by intracellular cytokine staining (ICS) of splenocytes of immunized mice followed by flow cytometry as described with slight modifications [40]. Mouse splenocytes were restimulated with a pool of gE peptides (2 μg/ml) for 2 h, and stained with anti-CD4-FITC or anti-CD8-FITC. Intracellular cytokines were labelled with anti-IFN-γ-PE-Cy7, anti-TNF-α-PE, and anti-IL-2-APC and then subjected to flow cytometric analysis using a FACS Canto™ flow cytometer (Becton Dickinson). Acquisition and analysis of samples were performed using FlowJo software. Live cells were gated (FSC/SSC), and 10,000 events of $CD4^+$ or $CD8^+$ T cells were analyzed. The percentages of cells expressing one, two, or three of the cytokines in response to stimulation were calculated by subtracting the number of cells obtained with media stimulation alone. Data were expressed as means ± SD of the percentages of cytokine-expressing $CD4^+$ or $CD8^+$ T cells obtained from triplicate samples from each of two spleens.

2.10. Statistical analysis

SPSS 18.0 software (IBM) was used for statistical analysis. Differences among the experimental groups were analyzed using one-way ANOVA with Tukey's multiple comparison test. The PBS control groups were excluded from statistical analysis. The two-tailed Student's *t*-test was used to compare two experimental groups. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Higher induction of cellular immune responses by the gE vaccine co-lyophilized with CIA09

We prepared the cationic liposome-based adjuvant formulation CIA09A by combining 1 mg of the cationic liposomes, containing DOTAP and DMPC at an equal ratio, with 50 μg QS-21 and 10–30 μg dLOS. The CIA09A-adjuvanted VZV gE vaccine was formulated with 50 μg recombinant gE protein plus 1 mg CIA09A in a volume of 0.5 ml for a putative HD. Upon lyophilization of the adjuvanted vaccine, a white pellet was formed, and reconstitution of the vaccine with the original volume of water yielded a white suspension similar to that observed before lyophilization. The cationic liposomes had a Z-average diameter of 80–120 nm with a zeta potential of +50–60 mV. After mixing gE with CIA09 or CIA09A, the vaccine particles exhibited a *Z*-average diameter of 200–400 nm with a decreased zeta potential of +40–50 mV. After lyophilization and reconstitution, the adjuvanted vaccines tended to become larger with a Z-average diameter of 300–700 nm.

To investigate the impact of lyophilization on the immunogenicity of the liposome-adjuvanted gE vaccine, we prepared gE vaccines either by mixing or co-lyophilizing gE with CIA09 and immunized BALB/c mice (Exp. 1). The adjuvanted vaccines induced serum gE-specific IgG antibody titers significantly higher than the unadjuvanted vaccine did ($P < 0.001$), but the co-lyophilized vaccine showed the highest IgG titer, which was 9-fold higher than the mixed vaccine ($P < 0.01$; Fig. 1A). The number of gE-specific IFN-γ-producing cells, representing CMI responses, induced by the mixed vaccine was 2.3-fold higher than that of the unadju-

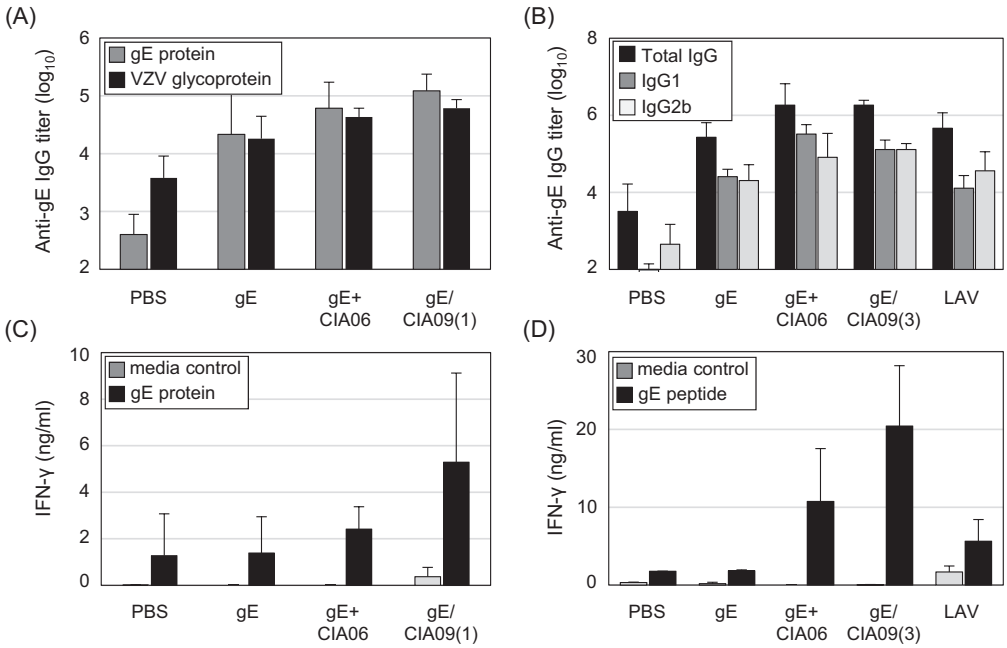

Fig. 2. Comparison of the adjuvant activity of CIA06 and CIA09 with gE. C57BL/6 mice primed with VZV were immunized with gE (5 μg) alone or in combination with CIA06 (1/10 HD), CIA09 (LP 50 μg, dLOS 1 μg; A, C), or CIA09 (LP 100 μg, dLOS 3 μg; B, D). CIA06 was mixed with gE (gE + CIA06) and kept on ice for 2 h, while CIA09 was co-lyophilized with gE (gE/CIA09) followed by reconstitution prior to use for immunization. Control mice were given PBS. Mice administered LAV (1/10 HD of Zostavax®) once were included for comparison. Four weeks after the second immunization, serum IgG titers specific for gE (A, B) or glycoproteins expressed on VZV-infected cells (A) were determined. Splenocytes collected from the mice were restimulated with the gE protein (C) or a pool of gE peptides (D), and IFN-γ levels in the culture media were measured. Data presented in (A) and (B) are expressed as the GMT ± SD of titers of the sera obtained from 4 or 6 mice in each group, respectively. Data presented in (C) and (D) are the means ± SD of values obtained from four spleens for each group or those obtained from triplicate assays using two spleens each, respectively.

vanted vaccine ($P = 0.052$), but induction of IFN-γ secretion was minimal (Fig. 1C and E). In contrast, the co-lyophilized vaccine significantly increased both the number of IFN-γ-producing cells and the cytokine levels by 3.8- and 4.6-fold, respectively, compared to the unadjuvanted vaccine ($P < 0.001$). These results were consistent when using different vaccine preparations (data not shown).

We also examined the immunogenicity of the two adjuvanted vaccines in C57BL/6 mice, which were immunized with vaccine containing higher doses of gE (5 μg) and CIA09 (Exp. 2). Serum gE-specific antibody levels induced by the unadjuvanted vaccine were higher than those observed in Exp. 1, perhaps due to either the higher antigen dose used for immunization or different experimental protocol (Fig. 1B). Unlike in Exp. 1, the gE-specific antibody levels induced by the mixed and co-lyophilized vaccines were similar, whereas the co-lyophilized vaccine was more effective in inducing gE-specific IFN-γ-secreting cells and IFN-γ production, although not statistically significant (Fig. 1D and F). Interestingly, the mice injected with unadjuvanted vaccine exhibited a remarkably high ratio of IL-5/IFN-γ, suggesting induction of Th2-skewed immune responses by immunization with gE alone. Taken together, these data demonstrate that CIA09 promotes both humoral and CMI responses to gE and that lyophilization of the adjuvanted vaccine formulation does not abrogate the adjuvanticity of CIA09 but rather enhances CMI responses to gE.

In a previous study, we compared the adjuvant activity to JEV of CIA09 and CIA06, which is composed of dLOS and alum, and observed that both adjuvant formulations increased antibody response to the vaccine antigen, but only CIA09 promoted CMI response. Here, we compared the adjuvant capability of CIA06 and CIA09 on gE in a VZV-primed mouse model. In Exp. 3, VZV-primed C57BL/6 mice immunized with unadjuvanted gE vaccine, CIA06-adjuvanted vaccine, vaccine co-lyophilized with CIA09, or PBS as a control, all displayed high levels of VZV-specific IgG antibody, reflecting pre-existing antibodies (Fig. 2A). The gE-specific IgG titers induced by the adjuvanted vaccines were higher than that of the unadjuvanted vaccine, although not significantly ($P = 0.189$). Similar results were obtained with serum IgG titers measured against VZV glycoproteins. Additionally, IFN-γ levels were increased only by the CIA09-adjvanted vaccine (Fig. 2C). In Exp. 4, we increased the doses of liposomes and dLOS to 100 μg and 3 μg, respectively, in an attempt to achieve a higher CMI response, and the antibody titers of animals immunized adjuvanted vaccines were significantly higher than with the unadjuvanted vaccine ($P < 0.001$), whereas the antibody titers of animals administered 1/10 HD of LAV as a control were comparable to that of the unadjuvanted vaccine (Fig. 2B). The CIA09-adjuvanted vaccine, but not the CIA06-adjuvanted vaccine, elicited a significantly higher gE-specific IFN-γ secretion compared to the unadjuvanted vaccine ($P < 0.01$; Fig. 2D). Similarly, splenic IFN-γ secretion induced by LAV was minimal. These results demonstrate that the gE vaccine co-lyophilized with CIA09 is more effective in inducing CMI than the CIA06-adjuvanted vaccine or LAV.

3.2. Comparison of immunogenicity of the gE vaccine adjuvanted with different formulations

Next, we examined whether the combination of CIA09 with QS-21 results in a synergistic adjuvant effect. VZV-primed mice were immunized with the unadjuvanted vaccine, LAV, or vaccines co-lyophilized with either CIA09 or a combination of CIA09 and QS-21 (designated as CIA09A) (Exp. 5). Serum titers of gE-specific antibody in the CIA09A-adjuvanted vaccine group were 3.2-fold higher than in the CIA09-adjuvanted vaccine group ($P < 0.05$) and 18.2-fold higher than in the LAV-treated group ($P < 0.001$; Fig. 3A). Similarly, the number of gE-specific IFN-γ-secreting cells and secreted IFN-γ levels were 4- and 2.6-fold higher, respectively, in the CIA09A-adjuvanted vaccine group compared to the CIA09-adjuvanted vaccine group ($P < 0.001$ and $P < 0.005$, respectively; Fig. 3B and C). Stimulation of splenocytes with VZV yielded IFN-γ patterns similar to those obtained with gE peptide stimulation, whereas mock antigen stimulation barely induced IFN-γ response, suggesting that the IFN-γ responses are VZV-specific. These data demonstrate that the combination of CIA09 and QS-21 provides a synergistic adjuvant effect, indicating that CIA09A is highly effective in promoting VZV- and gE-specific humoral and CMI responses.

We further evaluated the adjuvanticity of various combinations of adjuvants (Exp. 6). Cationic liposomes alone increased gE-specific serum IgG antibody responses by 10-fold ($P < 0.05$) compared to unadjuvanted vaccine (Fig. 4A). Addition of dLOS or QS-21 to the liposomes further increased the antibody titers, resulting

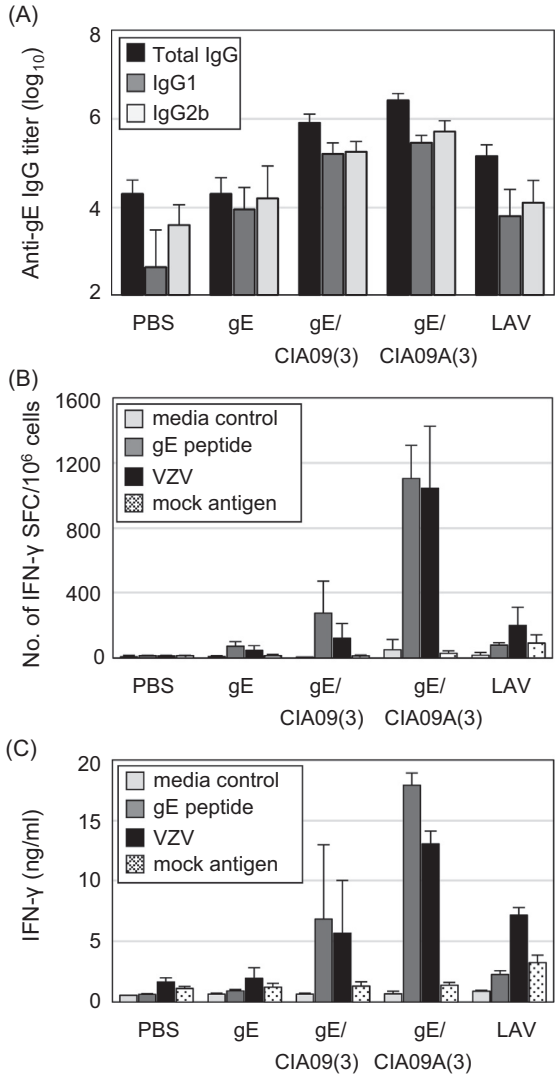

Fig. 3. Evaluation of gE-specific IFN-γ responses by the adjuvanted gE vaccines. C57BL/6 mice ($n = 6$) primed with VZV were immunized with gE (5 μg) alone or co-lyophilized with CIA09 or CIA09A, which contains LP (100 μg) and dLOS (3 μg) with or without QS-21 (5 μg), respectively. A group of mice administered LAV was included for comparison. Four weeks after the second immunization, gE-specific serum IgG titers were determined and expressed as the GMT ± SD of titers of the sera obtained from six mice in each group (A). Splenocytes collected from immunized mice were restimulated with a pool of gE peptides, VZV, or mock antigen, and the number of IFN-γ-secreting cells (B) and the levels of secreted IFN-γ (C) were determined. Data presented are the means ± SD of values obtained from triplicate assays using two spleens each (B, C).

in 2.2- and 5-fold increases, respectively, compared to the liposome-adjuvanted vaccine. To determine the optimal dose of dLOS for vaccine formulation, we prepared two CIA09A-adjuvanted vaccines containing 2 or 3 μg of dLOS. CIA09A elicited the highest antibody titers with a 9-fold increase compared to CIA09A ($P < 0.05$), regardless of the dLOS dose used (Fig. 4C). These data indicate that CIA09A comprising 100 μg cationic liposomes, 2 μg dLOS, and 5 μg QS-21 yields the optimal adjuvant activity for 5 μg gE.

Next, we compared the adjuvant activity of CIA09A with a liposomal adjuvant formulation composed of neutral liposomes, QS-21, and MPL, which is similar to AS01B used in the approved HZ subunit vaccine Shingrix (Exp. 7). The serum levels of gE-specific total IgG antibody titers of the CIA09A-adjuvanted vaccine were comparable to that of the neutral liposome-formulated vaccine ($P = 0.11$; Fig. 4B). Likewise, no significant difference was observed between the CMI responses induced by the two vaccines as determined by IFN-γ levels upon stimulation with either gE peptides or VZV ($P > 0.4$; Fig. 4D). These data suggest that CIA09A may be comparable in adjuvant activity to AS01B for induction of both humoral and CMI responses to gE antigen.

3.3. Efficient induction of polyfunctional $CD4^+$ and $CD8^+$ T cells by the CIA09A-adjuvatned gE vaccine

Next, we determined using splenocytes from the immunized mice in Exp. 6 and 7 whether CIA09A-adjuvanted gE vaccine elicits gE-specific polyfunctional $CD4^+$ T cells expressing IFN-γ, TNF-α, and/or IL-2 cytokines upon restimulation with gE peptides. The frequencies of cytokine-positive $CD4^+$ T cells among experimental groups mirrored their IFN-γ production levels (Fig. 5A). The frequency of gE-specific $CD4^+$ T cells expressing any of the three cytokines was negligible in the mice administered unadjuvanted vaccine and <1% in those administered liposome-adjuvanted or CIA09-adjuanted vaccine. The addition of liposomes and QS-21 to gE significantly increased the $CD4^+$ T cell response, with 2.5% of these cells expressing one or more cytokines. In particular, triple-positive $CD4^+$ T cells increased from 0.4% in animals given unadjuvanted vaccine to 1.6% ($P < 0.01$). The CIA09A-adjuvanted vaccine, containing either 2 or 3 μg of dLOS, yielded the highest frequency of cytokine-positive $CD4^+$ T cells (4.2% and 4.0%, respectively; $P < 0.001$ vs. unadjuvanted vaccine). Moreover, triple-positive and double-positive $CD4^+$ T cells accounted for 66 and 28% of the total number of cytokine-positive $CD4^+$ T cells, suggesting that the vaccines are more effective at eliciting a polyfunctional $CD4^+$ T cell response compared to the neutral liposome-formulated vaccine despite similar IFN-γ levels (Fig. 5B). The mice immunized with CIA09A-adjvanted vaccine also exhibited a higher, but not significantly so, frequency of polyfunctional $CD8^+$ T cells expressing two or three cytokines than those immunized with other vaccines (Fig. 5C).

Finally, we directly compared the immunogenicity of the CIA09A-adjuvanted vaccine prepared using the thin-film method with the licensed HZ subunit vaccine Shingrix (Exp. 8). VZV-

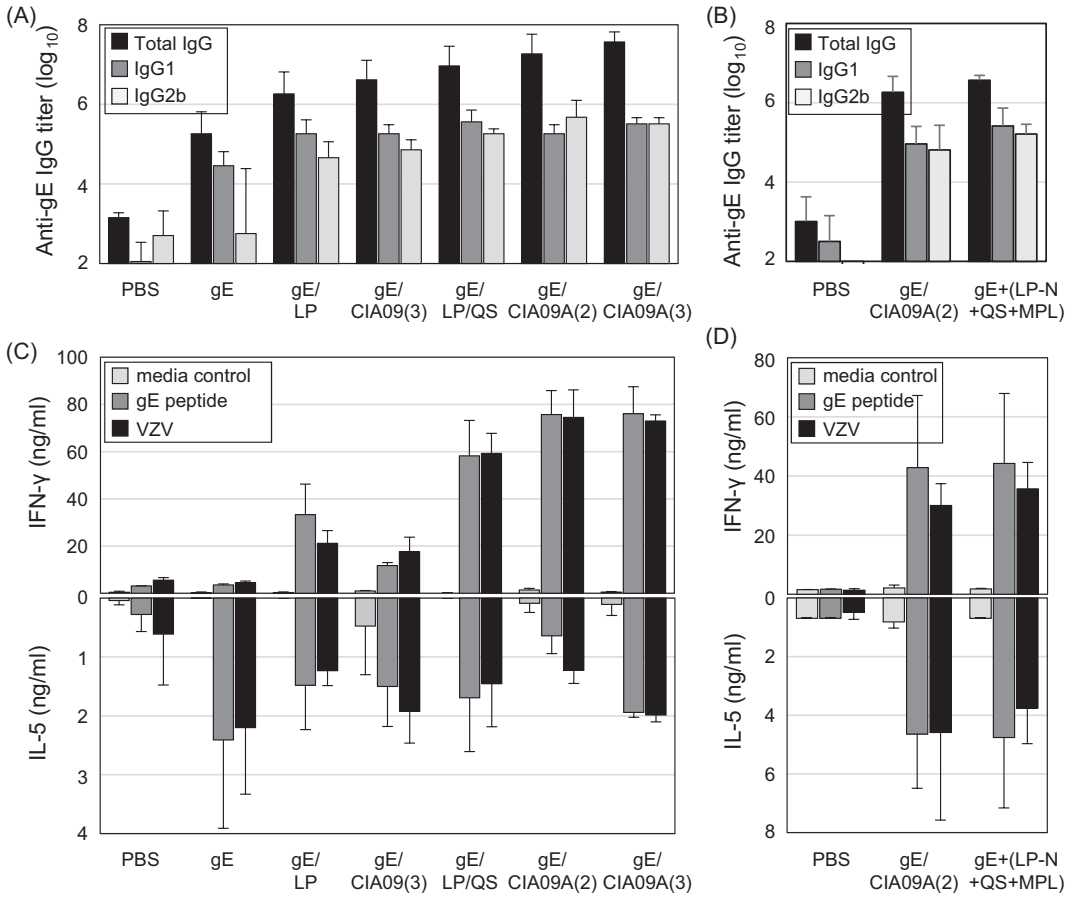

Fig. 4. Determination of the effects of various adjuvant formulations with gE. C57BL/6 mice (n = 6) primed with VZV were immunized with gE (5 μg) alone, mixed (+) or co-lyophilized (/) with various adjuvant formulations. Dose of each adjuvant contained in the vaccine formulations is as follows: LP 100 μg, dLOS 2 or 3 μg, QS-21 5 μg, LP-N 125 μg, and MPL 5 μg. Four weeks after the second immunization, serum gE-specific IgG antibody titers (A, B) and splenic cytokine secretion was assessed (C, D). Results are expressed as the GMTs ± SD of titers of the sera obtained from six mice in each group (A and B) or the means ± SD of values obtained from triplicate assays using two spleens each (C and D).

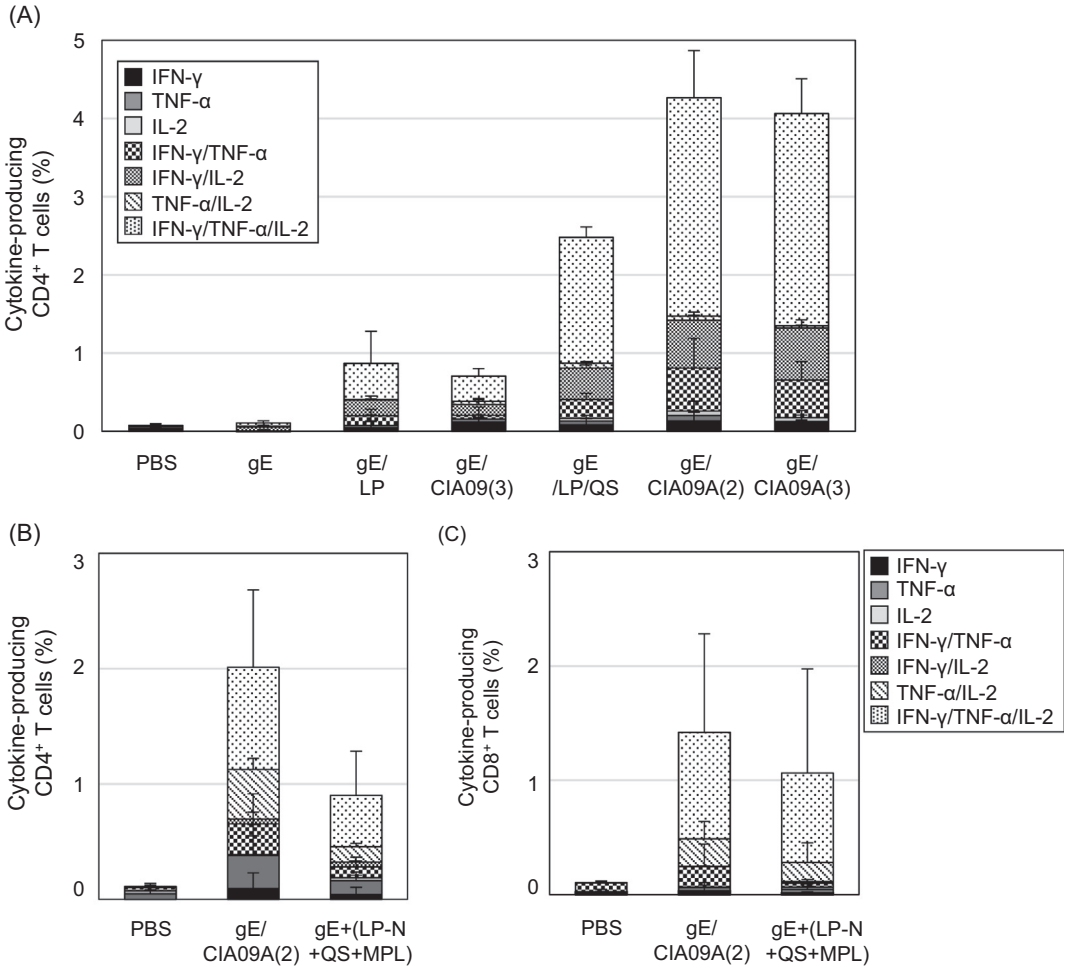

Fig. 5. The frequency of gE-specific polyfunctional $CD4^+$ and $CD8^+$ T cells induced by the adjuvanted gE vaccines. C57BL/6 mice ($n = 6$) primed with VZV were immunized with the unadjuvanted or adjuvanted gE vaccines as described in Fig. 4A and B. Four weeks later, splenocytes harvested from the immunized mice were restimulated with a pool of gE peptides and assayed for the frequencies of gE-specific $CD4^+$ T cells (A and B) and $CD8^+$ T cells (C) expressing IFN-γ, TNF-α, and/or IL-2 by ICS and flow cytometry as described in Materials and Methods. Results are expressed as the means ± SD of values obtained from triplicate assays using two spleens each.

primed mice were administered either of the two particle sizes of CIA09A-adjuvanted vaccines or Shingrix, and no differences in gE-specific serum IgG titers were observed among the three groups (Fig. 6A). The large-particle CIA09A-adjuvanted vaccine and Shingrix elicited higher IFN-γ levels and frequencies of polyfunctional $CD4^+$ T and $CD8^+$ T cells upon stimulation with either gE or VZV than the small-particle CIA09A-adjuivanted vaccine, although the differences were not statistically significant (Fig. 6B–D). These data show that the particle size of the CIA09A-adjuvanted vaccine is an important factor for determining the immunogenicity of the vaccine, particularly with respect to its ability to elicit CMI response.

4. Discussion

Many vaccine adjuvants, including alum, oil-in-water emulsion, and liposomes, are not suitable for lyophilization, and vaccines containing these adjuvants, therefore, are stored in a liquid form or separate vials of freeze-dried vaccine antigen and liquid adjuvant to be mixed together before vaccination. Here, we developed a cationic liposome adjuvant formulation, CIA09A, which contains the TLR4 agonist dLOS and QS-21, and evaluated its potential as an adjuvant for a VZV subunit vaccine to prevent HZ. The VZV gE vaccine prepared by co-lyophilization of gE with the liposome-based adjuvant elicited strong CMI and humoral responses to both gE protein and VZV, demonstrating that co-lyophilization does not abolish the adjuvanticity of the liposomal adjuvant formulation to gE. Furthermore, these results suggest that manufacture of a lyophilized VZV gE vaccine in a single vial is feasible and beneficial for long-term stability, efficacy, handling, and distribution of the vaccine. In fact, stability tests of CIA09A-adjuvanted tuberculosis subunit vaccine revealed that protein antigens in a lyophilized vaccine remained intact even after 3 weeks of storage at 25 °C, while all of the antigens in a liquid vaccine were degraded (unpublished data). These results indicate that lyophilization of the CIA09A-adjuvanted vaccines increases the thermostability of protein antigens. Stability test of the CIA09A-adjuvanted VZV gE vaccine is currently in progress.

Interestingly, the co-lyophilized vaccine was superior to the mixed vaccine in enhancing the immunogenicity of gE, particularly for CMI responses. Although the reason is not clear, we suspect that differences in the particle size of the vaccines are involved, because the *Z*-average diameter of adjuvanted vaccine particles and number of double particles increased after lyophilization as observed by cryo-transmission electron microscopy. Liposome particle size is known to affect the adjuvanticity of liposome adjuvants and, in particular, the balance between Th1- and Th2-type immune responses [15]. Brewer et al. observed that lipid vesicles ≥225 nm preferentially induced Th1-type responses, as characterized by high levels of IgG2a isotype antibody in plasma and IFN-γ secretion by lymph node cells, whereas those <155 nm induced

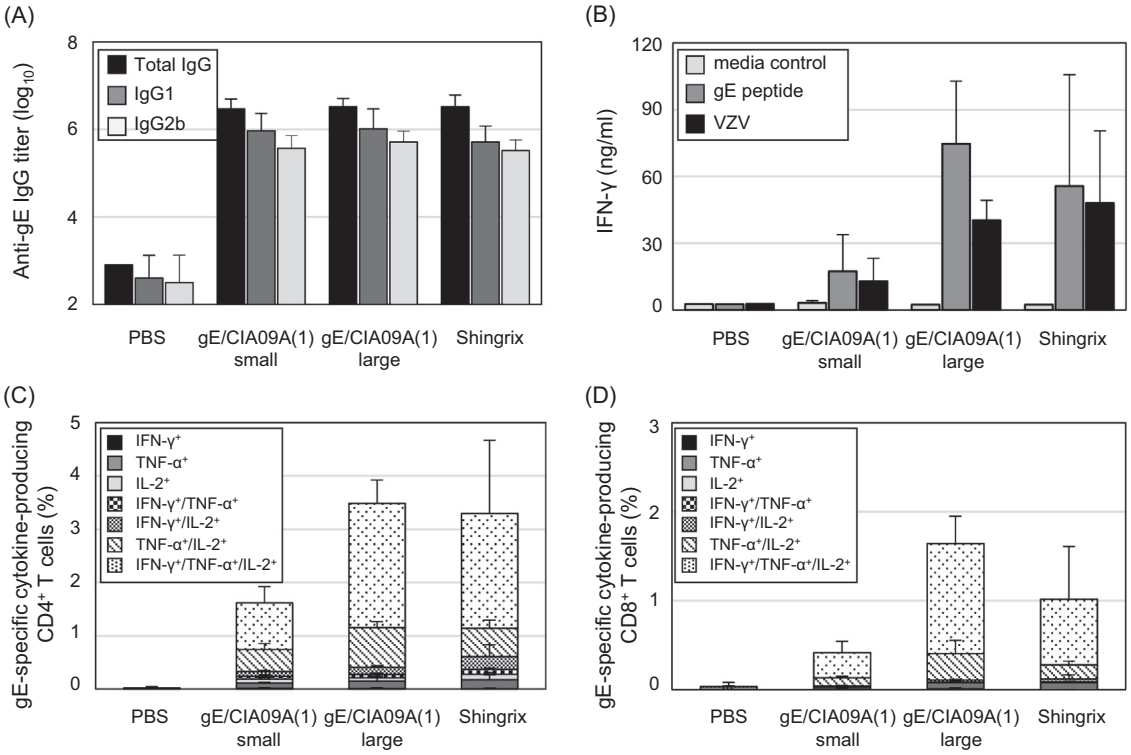

Fig. 6. Comparison of the immunogenicity of the CIA09A-adjuvanted vaccine and Shingrix. C57BL/6 mice ($n = 6$) primed with VZV were immunized twice with the lyophilized (/) CIA09A-adjuvanted vaccines (gE 5 μg, LP 100 μg, dLOS 1 μg, QS-21 5 μg) or 1/10 HD of Shingrix, and four weeks later, serum gE-specific IgG antibody titers were measured (A). Splenocytes harvested from the immunized mice were restimulated with a pool of gE peptides, and the levels of splenic IFN-γ secretion were measured (B). The frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, and/or IL-2 were also determined (C and D, respectively). Results are expressed as the GMTs ± SD of titers of the sera obtained from six mice in each group (A) or the means ± SD of values obtained from triplicate assays using two spleens each (B–D). small, vaccines with a particle size of 100–300 nm; large, vaccine with a particle size of 300–500 nm.

mainly Th2-type responses, as identified by high levels of IgG1 isotype antibody and IL-5 [42]. Badiee et al. showed that liposomes >400 nm were more effective in inducing Th1-type CMI responses to and provided better protection against *Leishmania major* than smaller liposomes [43]. Similarly, Henriksen-Lacey et al. reported higher IFN-γ secretion against tuberculosis antigen following immunization with cationic liposome CAF01 > 500 nm [44]. Brewer et al. reported that vesicle size affects trafficking, processing and presentation of antigen [45]. Antigens prepared in large particles were delivered into early endosome-like, immature phagosomes, whereas those in smaller vesicles were rapidly transported to late endosomes/lysosomes, bypassing the antigen-processing apparatus and resulting in reduced antigen presentation. Our finding that larger particles of the CIA09A-adjuvanted vaccine were more effective at eliciting a response, albeit not significantly, supports our speculation that the increased particle size following lyophilization accounts for the higher immunogenicity. Further studies to determine the effects of lyophilization on the physicochemical characteristics of vaccines containing cationic liposomes are warranted.

The high surface density of positive charges also affects the adjuvant activity of cationic liposomes by facilitating adsorption of antigens to the liposomes and adsorption of liposomes to the surface of negatively-charged cell membranes [17]. Therefore, maintaining a suitable liposome surface charge is important for optimizing the efficacy of liposome-adjuvanted vaccines. The truncated VZV gE antigen is negatively charged (pI 5.5) and therefore strongly binds cationic liposomes (unpublished data). dLOS is also negatively charged due to its phosphate moieties and likely interacts with cationic liposomes. In fact, the particle size of liposomes increased with decreasing zeta potential upon addition of gE and/or dLOS to the liposomes, suggesting co-localization of gE with the immune stimulants, thereby enhancing the immune responses to the antigen.

DOTAP/DOPC-based cationic liposomes, unlike neutral or anionic liposomes, increase CD80 and CD86 expression on the surface of DCs, DC maturation, reactive oxygen species generation, and antigen uptake by DCs [21,22]. Liposomes consisting of DOTAP and DOPC at a 4:1 ratio exhibited greater immune-stimulating activity than those at a 1:4 ratio, indicating that the surface charge density of cationic liposomes directly contributes to their activity on DCs [21]. Although the surface charge of cationic liposomes affected both humoral and cellular responses, it had a greater effect on the balance between Th1- and Th2-type responses. Furthermore, DOTAP upregulated chemokine expression in DCs via the extracellular-regulated kinase (ERK) pathway and increased expression of monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and MIP-1β [46]. Our liposome preparation CIA09 also stimulated MCP-1 secretion and antigen uptake by DCs in vitro, and immune cell recruitment at the site of injection (manuscript in preparation).

Another molecule used in adjuvant formulations is QS-21, a water soluble saponin purified from *Quillaja saponaria*. This adjuvant strongly stimulates both humoral and CMI responses to vaccine antigens by directly activating human monocyte-derived DCs [47], and this molecule exhibits synergy with TLR4 agonists such as MPL [40,48]. In the present study, the addition of QS-21 to CIA09 significantly increased Th1-type CMI responses, in particular the frequency of antigen-specific polyfunctional $CD4^+$ cells that secrete multiple cytokines. QS-21 also has hemolytic activity and is usually co-formulated with cholesterol to reduce the intrinsic toxicity [24]. Thus, we determined that hemolytic activity of

QS-21 in CIA09A decreased inversely to the concentration of cationic liposomes, indicating that cationic liposomes have the ability to quench the hemolytic activity of QS-21 similar to cholesterol (unpublished data).

Previously, we observed that CIA06, a combination of dLOS and alum, promoted not only serum IgG and virus-neutralizing antibody to either HPV L1 VLPs or influenza split vaccine, but also Th1-type CMI responses as determined by serum IgG2a titer and splenic IFN-γ production [27,28]. With the JEV vaccine, however, CIA06 increased only vaccine-specific antibody titers, and not CMI response, whereas the liposome formulation enhanced both antibody and CMI responses, consistent with our results on VZV gE [32]. Both alum and liposomes serve as antigen carriers, but their immunological properties are different. Alum induces a Th2-type-skewed response with Th1 response inversely proportional to the ratio of alum to dLOS [27], whereas cationic liposomes elicit a more balanced Th1/Th2 response (Fig. 4C). Therefore, the physicochemical properties of the vaccine antigen and the type of immunity required for protective efficacy should be considered for vaccine adjuvant formulation.

CMI, but not VZV-specific antibody, plays an important role in restricting reactivation and replication of latent VZV and, thus, is essential for preventing HZ and reducing the severity and incidence of HZ-associated PHN [49,50]. The clinical study of live attenuated HZ vaccine demonstrated that VZV-specific T-cell-mediated immunity, as determined by the frequency of IFN-γ-producing cells, is strongly correlated to the protective efficacy of the vaccine [50]. In particular, $CD4^+$ T cells were implicated in HZ protection. Schub et al. found that $CD4^+$ T cells from healthy individuals predominantly coexpressed IFN-γ, IL-2, and TNF-α, whereas $CD4^+$ T cells from patients with acute herpes zoster were mostly IFN-γ single-positive in spite of elevated levels of VZV-specific antibody and $CD4^+$ T cells, suggesting that polyfunctional $CD4^+$ T cells expressing multiple cytokines prevent VZV reactivation [51]. In fact, polyfunctional $CD4^+$ T cells was used as an indicator of the efficacy of a HZ vaccine in a clinical trial [52]. $CD8^+$ T cells have also been shown to play a role in preventing HZ occurrence, although the correlation between $CD8^+$ T cell induction and protective efficacy of HZ vaccine is less clear than for CD4 T cells [2,3,53].

5. Conclusions

In summary, we demonstrated that CIA09A, a cationic liposome-based adjuvant formulation containing dLOS and QS-21, is effective in enhancing both humoral and CMI responses to both gE antigen and VZV, and that co-lyophilization of VZV gE antigen with CIA09A enables manufacture of a lyophilized subunit vaccine in a single vial.

Acknowledgments

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Conflict of Interests

K.S. Kim, S.A. Park, and Y.J. Cho are employees of EyeGene and inventors of EyeGene-owned patents related to the adjuvant CIA09A and adjuvanted VZV gE vaccine. N.G. Lee is a scientific advisor for EyeGene and inventor of the patents. The other authors have no conflicts of interests.

Authors' contributions

SRW participated in the design of the study, carried out experiments, analyzed the data and drafted the manuscript. KSK and SAP developed the VZV gE expression system and liposome-adjuvanted vaccines, and designed the study and analyzed the data. JIR, AK, HTTD, YJL, and HJK carried out animal experiments and analyzed the data. YJC conceived of the study and participated in its design, data analysis, and coordination. SJL and CGK participated in the design of liposome-based adjuvant and vaccine, and data analysis. NGL conceived of the study and participated in its design, data analysis, coordination and writing of the manuscript. All authors read and approved the final manuscript.

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