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Lipo-pam™ adjuvanted herpes zoster vaccine induces potent gE-specific cellular and humoral immune responses

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Herpes zoster (HZ), also known as shingles, is caused by the reactivation of latent varicella-zoster virus (VZV). Decreased VZV-specific T-cell immune responses significantly contribute to the development of HZ. Shingrix is a recombinant zoster vaccine that is currently used to prevent HZ. However, Shingrix has high reactogenicity and pain at the injection site due to QS21, one of the adjuvant components. In this study, we developed a new herpes zoster vaccine formulation called CVI-VZV-001, containing gE protein and a novel liposome-based adjuvant Lipo-pam™, which consists of two TLR agonists. We evaluated the immunogenicity of CVI-VZV-001 in mouse and rabbit models. CVI-VZV-001 elicited robust gE-specific T-cell immune responses and gE-specific antibody production. Specifically, CVI-VZV-001 induced polyfunctional $CD4^+$ T cell populations that secrete multiple cytokines. Furthermore, CVI-VZV-001 sustained the gE-specific immune responses for up to six months after immunization. To ensure CVI-VZV-001's safety for further development, we conducted a good laboratory practice (GLP) toxicity test, which confirmed that CVI-VZV-001 is safe for use. At present, CVI-VZV-001 is undergoing phase I clinical trials. This study suggests that CVI-VZV-001 can be a potent candidate for the HZ vaccine with high immunogenicity and safety.

Varicella zoster virus (VZV) is an alpha herpes virus that affects the nervous and lymphatic systems. VZV causes varicella, commonly known as chickenpox, and herpes zoster (HZ), also known as shingles[1]. HZ occurs when VZV reactivates from the sensory ganglia and is likely due to insufficient cellular immunity to control reactivation[2–4]. VZV-induced HZ is characterized by a painful, blistering rash that typically appears on one side of the body or face, resulting in nerve damage and a range of other complications[5–7]. Postherpetic neuralgia (PHN) can develop in patients with HZ, causing pain even after the signs and symptoms of HZ have subsided, which can last for months, years, or even a lifetime[7,8]. Therefore, a prophylactic vaccine is essential to reduce the risk of HZ, especially for immunocompromised and elderly individuals who are more vulnerable to HZ.

Currently, there are two types of vaccines used to protect HZ. One type is a live-attenuated vaccine, such as Zostavax (MSD, USA) and SKYZoster (SK bioscience, Korea), while the other is an adjuvanted subunit vaccine, Shingrix (GSK, Belgium). A live-attenuated vaccine contains a weakened live strain of the varicella-zoster virus that stimulates the immune system without causing disease in healthy individuals. However, it is not recommended for individuals with severe immunosuppression due to the risk of adverse reactions to the live virus[9]. Recently, a recombinant zoster vaccine, Shingrix, has replaced a live attenuated vaccine. Shingrix contains gE protein derived from the varicella-zoster virus and an adjuvant called $AS01_B$, composed of liposomes formulated with cholesterol, monophosphoryl lipid A (MPL), and QS21. Although Shingrix is highly effective in preventing HZ, it can cause significant side effects with injection site pain and systemic reactions in some people. In particular, the proportion of people who received Shingrix experienced almost 24 times grade 3 of injection site pain compared to the placebo group[10,11]. This high reactogenicity of Shingrix is likely due to QS21, which is one of the components of adjuvant $AS01_B$. QS21 is a saponin that induces inflammatory cytokines, resulting in T-helper 1 (Th1) responses[12]. However, the high reactogenicity of QS21, including lysis of cell membranes, hemolysis, and injection site pain, are major limiting factors in its use.

We have developed a proprietary adjuvant called L-pampo™, based on Toll-like receptor (TLR) agonists. We have applied it to various vaccine platforms that target infectious diseases and cancers[13–16]. L-pampo™ is a complex of TLR2 and 3 agonists and synergistically induces both humoral and cellular immune responses. Specifically, L-pampo™ effectively induces a

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Th1 response, which is critical for a therapeutic effect. Clinical safety for L-pampo™ has been proven through a phase 1/2a study and a phase 2b study involving up to 200 patients with Hepatitis B chronic infection. We have also developed a liposome-based formulation of L-pampo™ called Lipo-pam™. This formulation serves as an antigen delivery vehicle and immunostimulatory adjuvant. Lipo-pam™ integrates two lipid compositions, 1,2-Dioleoyloxy-3-trimethylammuium propane chloride (DOTAP) and 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to create a liposome bilayer that incorporates the TLR2 agonist. The TLR3 agonist is attached to the liposome surface. This unique structure of Lipo-pam™ facilitates the interaction with the immune cell membrane that leads to the activation of antigen-presenting cells and the induction of robust antigen-specific humoral, cellular immune responses.

In this study, we formulated a recombinant herpes zoster vaccine containing gE antigen adjuvanted with Lipo-pam™, called CVI-VZV-001. We demonstrated that CVI-VZV-001 induced substantial VZV gE-specific cellular and humoral immune responses comparable with Shingrix in animal models. CVI-VZV-001 also increases polyfunctional $CD4^+$ T cells that produce multiple cytokines, such as IFN-γ, IL-2, and TNF, essential for protective immunity. Furthermore, CVI-VZV-001 maintained durable vaccine-induced cellular immune responses for up to six months. Lastly, we evaluated the safety of CVI-VZV-001 in the animal models and observed no signs of toxicity or weight loss. Therefore, our finding suggests that CVI-VZV-001 can be a potent HZ vaccine.

Results

gE/Lipo-pam™ formulations induce gE-specific cell-mediated immune responses

We produced VZV gE proteins, then purified and verified them using Western blotting and size exclusion chromatography (Supplementary Fig. 1a–d). We then formulated Lipo-pam™ and characterized its physicochemical properties, including size, zeta potential, and polydispersity index (PDI) (Supplementary Fig. 2a–c). To examine the immunogenicity of gE/Lipo-pam™ vaccine candidates, we formulated gE/Lipo-pam™ candidates with different ratios of lipid components and the amount of liposome (Lipo-pam™ #1–3). We immunized mice with gE/Lipo-pam™ candidates and analyzed gE-specific T cell responses and VZV-gE antibody titer by using ELISPOT and ELISA assays, respectively (Fig. 1a). We observed that Lipo-pam™ #2 and #3 candidates induced higher number of gE-specific IFN-γ spot formulating cells (SFCs) and gE-specific IgG antibody titer compared to antigen-only and Lipo-pam™ #1 groups (Fig. 1b, c). T helper 1 cell (Th1) and T helper 2 cell (Th2) immune responses are closely associated with vaccine-induced immunity[17]. The isotypes IgG1 and IgG2a/IgG2b/IgG2c represent Th2 and Th1 immune responses, respectively[18]. We measured each isotype's humoral immune responses in the serum (Supplementary Fig. 3a) and calculated their respective IgG2c/IgG1, IgG2b/IgG1, and IgG2a/IgG ratios. We found that the Lipo-pam™ #3 candidate consistently displayed higher IgG2a and IgG2c isotype ratios compared to Lipo-pam™ #1, #2, and antigen-only groups (Supplementary Fig. 3b). This data demonstrates that gE/Lipo-pam™ formulations induce gE-specific cellular and humoral immune responses indicating that gE/Lipo-pam™ can be a vaccine formulation for targeting HZ.

CVI-VZV-001 induces gE-specific immune responses comparable to those elicited by Shingrix in VZV-primed mice

Given the immunogenicity data and Th1-skewed response presented in Fig.1 and Supplementary Fig. 3b, we selected the formulation of Lipo-pam™ #3 as the final vaccine candidate, which we named CVI-VZV-001. Then, we sought to compare the efficacy of CVI-VZV-001 with that of Shingrix. We primed mice with a live attenuated vaccine (SKYZoster) to mimic a clinical

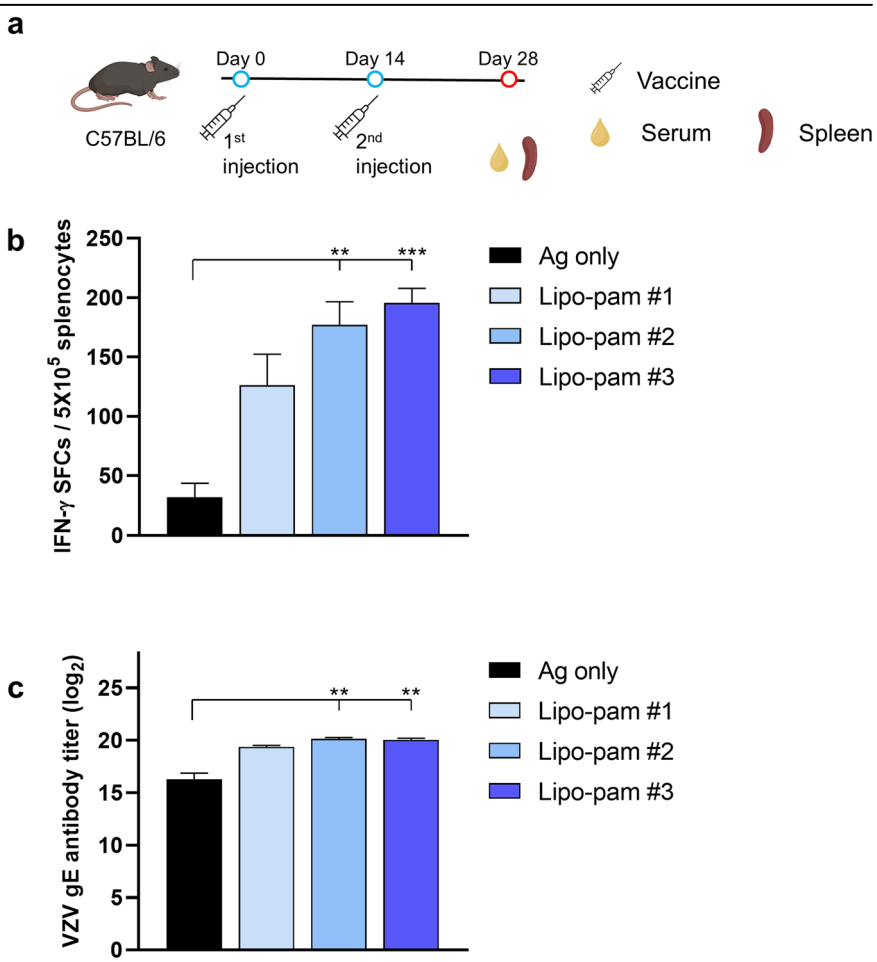

Fig. 1 | VZV gE antigen-specific immune responses induced by the formulations of gE protein adjuvanted with Lipo-pam™. a A schematic of immunization and experimental design. C57BL/6 mice were immunized with recombinant VZV gE antigens with or without adjuvants Lipo-pam™ intramuscularly (i.m.) on days 0 and 14. (Images partly exported from Biorender.com.) **b** SFCs (spot-forming cells) represent antigen-specific IFN-γ secreting cells by ELISPOT assay. **c** Antigen-specific total IgG titer by ELISA. Data are pooled from nine independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; one way ANOVA with the Tukey's test.

environment where most people are vulnerable to HZ and VZV seropositivity. We then immunized the primed mice with different doses of CVI-VZV-001 twice every two weeks (Fig. 2a). The Shingrix group received 1/10 HD that contained 5 μg of the gE antigen. The SKYZoster-immunized group was used as another control (Fig. 2a). We found that CVI-VZV-001 (1X) group increased both gE-specific IFN-γ producing T cell number and IFN-γ production compared to the live attenuated vaccine and low doses of CVI-VZV-001 groups (0.25X and 0.5X) (Fig. 2b, c). IFN-γ related to T cell responses of CVI-VZV-001 (1X) were equivalent to the response of Shingrix (Fig. 2b, c). Polyfunctional $CD4^+$ T cell populations that produce multiple cytokines are critical for the evaluation of immunogenicity targeting HZ. We analyzed gE-specific polyfunctional T cells secreting IFN-γ, IL-2, and TNF cytokines by intracellular cytokine staining (ICS) in splenocytes stimulated with overlapping peptide pools of VZV gE antigen. We found that CVI-VZV-001 (1X) and Shingrix increased the frequency of cytokine-producing $CD4^+$ T cells, especially those producing three cytokines, compared to the live attenuated vaccine and low doses of CVI-VZV-001 groups (0.25X and 0.5X) (Fig. 2d, e and Supplementary Fig. 4). We analyzed $CD8^+$ T cell responses in this model. However, $CD8^+$ T cell immune responses were rarely detected and there were no differences between groups. Next, we analyzed the gE-specific IgG titer and the fluorescent-antibody-to-membrane-antigen (FAMA) titer. A VZV FAMA titer of 1:4 implies a protective immunity against VZV in humans. CVI-VZV-001 (1X), CVI-VZV-001 (0.5X), and Shingrix groups produced higher gE-specific antibodies compared to the live attenuated vaccine group (Fig. 2f). Similar to gE-specific antibody data, CVI-VZV-001 (1X) and Shingrix groups increased 2–3 folds of FAMA titers compared to the live attenuated vaccine group (Fig. 2g). Additionally, we observed that administration of CVI-VZV-001 did not induce significant body weight changes, mortalities, or clinical events (Supplementary Fig. 5a, b). Our results suggest that CVI-VZV-001 is a potent HZ vaccine candidate that elicits gE-specific cellular and humoral immune responses comparable to responses induced by Shingrix, with no severe toxicity.

CVI-VZV-001 elicits gE-specific immune responses in a rabbit model

To verify the immunogenicity of CVI-VZV-001, we analyzed the gE-specific immune responses in rabbits. Our study showed that the second dose of the vaccine significantly induced immune responses comparable to those of the live attenuated vaccine and the Shingrix groups in mouse models (Fig. 2). However, to further validate the immunogenicity of the vaccine in rabbits, we administered low and high doses of CVI-VZV-001 to male and female rabbits aged eighteen weeks three times every two weeks (Fig. 3a). The Lipo-pam™ only group was a control group. We observed that high doses of CVI-VZV-001 significantly induced gE-specific IFN-γ^+ T cell responses in both male and female rabbits, compared to the low doses of CVI-VZV-001 and Lipo-pam™ only groups after the second immunization (Fig. 3b). Interestingly, gE-specific T cell responses were sustained until day 56 (Fig. 3b). Additionally, both doses of CVI-VZV-001 increased sufficient gE-specific IgG antibody production in male and female rabbits compared to Lipo-pam™ only groups after the second immunization (Fig. 3c). The levels of antibody titers were also maintained until day 56 (Fig. 3c). These results suggest that both doses of CVI-VZV-001 effectively induced gE-specific humoral immune responses in rabbits, while high doses of CVI-VZV were more effective in increasing gE-specific cellular immune responses. This study also allows us to proceed to the repeated administration toxicity test in the rabbit model with this immunization scheme.

CVI-VZV-001 maintains long-term immunogenicity in VZV-primed mice

Given the observation of CVI-VZV-001's sustained immunogenicity in the rabbit model, we sought to investigate the longevity of CVI-VZV-001-induced immune responses. We primed mice with a live attenuated vaccine (SKYZoster) and administered one dose or two doses of CVI-VZV-001 (Fig. 4a). The Shingrix group was immunized with 1/10 HD containing 5 μg of the gE antigen. We used the SKYZoster-immunized group as an additional control. We analyzed the gE-specific IgG production in the mouse serum on days 70, 98, 126, and 140 and the gE-specific T cell response on day 140 after the sacrifice (Fig. 4a). Two doses of CVI-VZV-001 and Shingrix groups increased the number of gE-specific-IFN-γ-producing cells and IFN-γ production compared to one dose of CVI-VZV-001 and the live attenuated vaccine groups (Fig. 4b, c). Two doses of CVI-VZV-001 and Shingrix groups also increased gE-specific antibody titers and maintained the levels of antibody production by day 140 compared to one dose of CVI-VZV-001 and the live attenuated vaccine groups (Fig. 4d). To confirm the duration of immunogenicity of CVI-VZV-001, we immunized mice with CVI-VZV-001 and monitored the immune responses by day 224 (Fig. 5a). Consistent with the previous results, two doses of CVI-VZV-001 group increased the number of gE-specific-IFN-γ-producing cells and IFN-γ production compared to one dose of CVI-VZV-001 and the live attenuated vaccine groups on day 224 (Fig. 5b, c). Moreover, we found that the CVI-VZV-001 group increased the frequency of polyfunctional $CD4^+$ T cells producing IFN-γ, IL-2, and TNF cytokines compared to the live attenuated vaccine and low doses of CVI-VZV-001 group (Fig. 5d and Supplementary Fig. 6). The two doses of CVI-VZV-001 induced gE-specific antibody titer compared to one dose of CVI-VZV-001 and live attenuated vaccine groups and maintained the antibody titer by day 224 (Fig. 5e). These data demonstrate that CVI-VZV-001 can sustain cellular and humoral immune responses for at least six months, suggesting that CVI-VZV-001 has the potential to be a vaccine candidate for HZ.

The GLP toxicity test confirms the safety of CVI-VZV-001

We conducted non-clinical toxicity and pharmacology studies to evaluate the safety of CVI-VZV-001, along with Lipo-pam™ (CVI-ADJ-006) (Table 1). All studies complied with good laboratory practice (GLP) guidelines at Biotoxtecha and Charles River Laboratory. In a repeat-dose toxicity study, rabbits were administered Lipo-pam™ or CVI-VZV-001 at four intramuscular doses (25, 50, 70, 90 μg/animal) at weeks 0, 2, and 4. The results showed that CVI-VZV-001 was well-tolerated up to a 70 μg/animal dose. CVI-VZV-001 did not cause any maternal/embryofetal toxicity or post-natal development effects in the reproductive and development toxicity study. In Genotoxicity studies, Lipo-pam™ did not show any genotoxic or clastogenic potential. Safety pharmacology studies were conducted to evaluate the pharmacological effects of CVI-VZV-001 on neurological, respiratory, and cardiovascular parameters. No detrimental effects on vital organs were observed in these studies. The GLP toxicity tests confirmed the safety of CVI-VZV-001.

Discussion

Herein, we demonstrated that the Lipo-pam™ adjuvanted herpes zoster vaccine, called CVI-VZV-001, induced robust gE-specific cellular and humoral immune responses and maintained immunogenicity for up to six months in animal models, indicating its potential for long-term protection. Additionally, we confirmed that CVI-VZV-001 is safe for further development processes.

Cell-mediated immune responses specific to Varicella-zoster virus (VZV) are critical in controlling VZV latency and preventing its reactivation[19]. However, the aging process decreases adaptive immunity, particularly T-cell immune responses in individuals[4,20] who can be more susceptible to Shingles. The aged and individuals with lymphoproliferative malignancies exhibit decreased cell-mediated immunity, leading to a higher proportion of shingles in both groups[21,22]. It is worth noting that globally, about 95% of individuals aged over 50 have been exposed to VZV, putting them at risk of developing shingles[23]. Moreover, the incidence and severity of shingles increase with age, with a significant rise after age 50[24]. Given that the aging global population is rapidly increasing, shingles vaccination has become an essential global health priority[25].

Zostavax (MSD), a commonly used attenuated vaccine to prevent HZ, has limitations. Firstly, its immunity decreases over time, which increases the risk of shingles[26]. Secondly, vaccination can cause chickenpox or HZ due

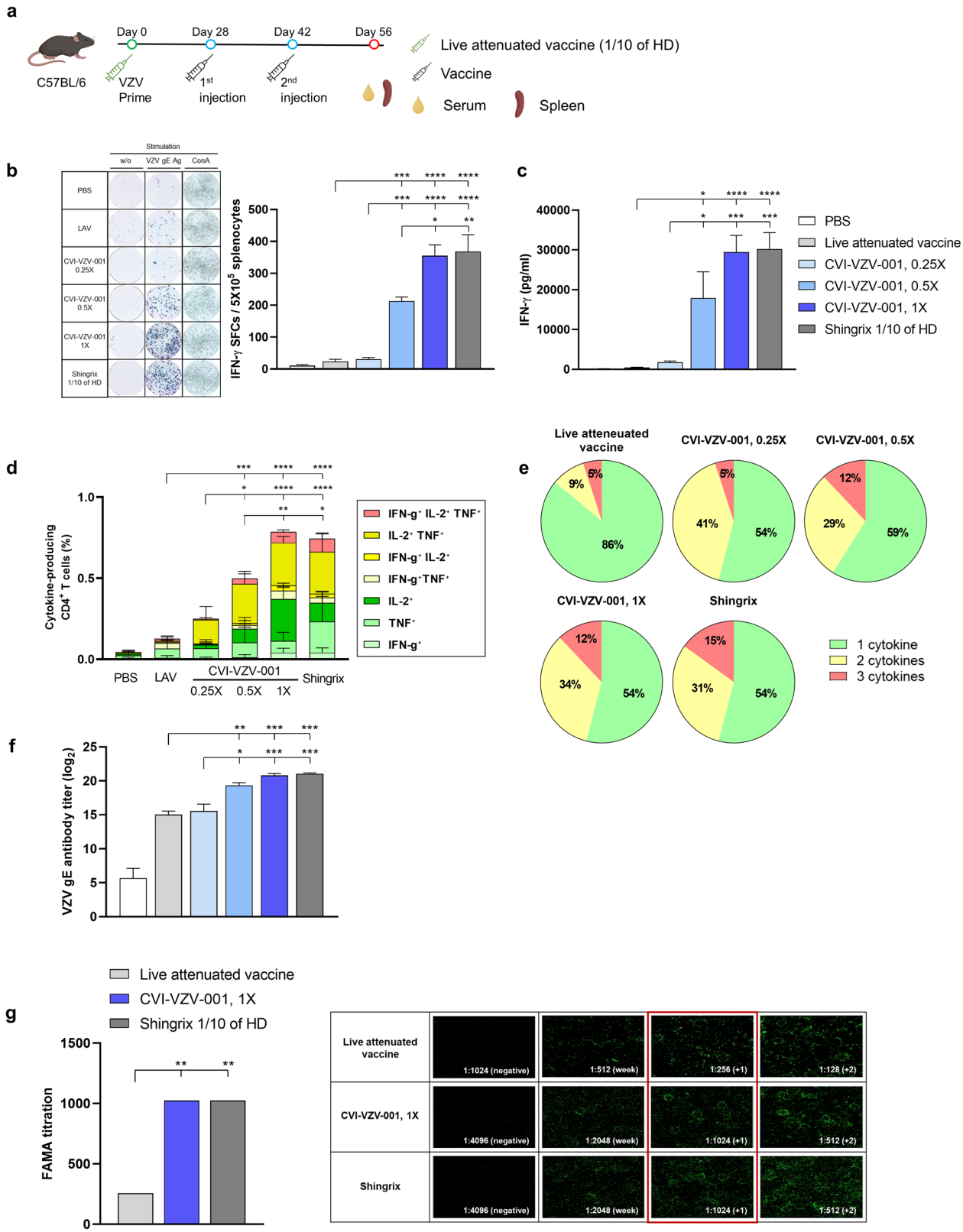

Fig. 2 | VZV gE-specific immune responses induced by dose-dependent CVI-VZV-001 or Shingrix in VZV-primed mice. a A schematic of immunization and experimental design. C57BL/6 mice (n = 5) were primed by subcutaneous administration of the live attenuated varicella vaccine to prepare the VZV-primed mice model that mimics the natural VZV infection. Then VZV-primed mice were immunized with CVI-VZV-001 or Shingrix. (Images partly exported from Biorender.com.) **b** Antigen-specific IFN-γ secreting cells by ELISPOT assay. Each group's representative SFCs (spot-forming cells) were shown in the left panel. **c** Total IFN-γ production by ELISA assay. **d** Measurement of the frequency of cytokine-secreting T cells among $CD4^+$ T cells by intracellular cytokine assay. **e** The pie charts represent the proportions of $CD4^+$ T cells with single (green), double (yellow), and triple (red) functions upon stimulation with the PepMix™ VZV gE peptide pool. **f** Antigen-specific total IgG titer by ELISA. **g** VZV-specific neutralizing antibodies were measured using the FAMA assay. Data are representative of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; one way ANOVA with the Tukey's test (**b**, **f**, **g**) or two way ANOVA with the Tukey's test (**d**).

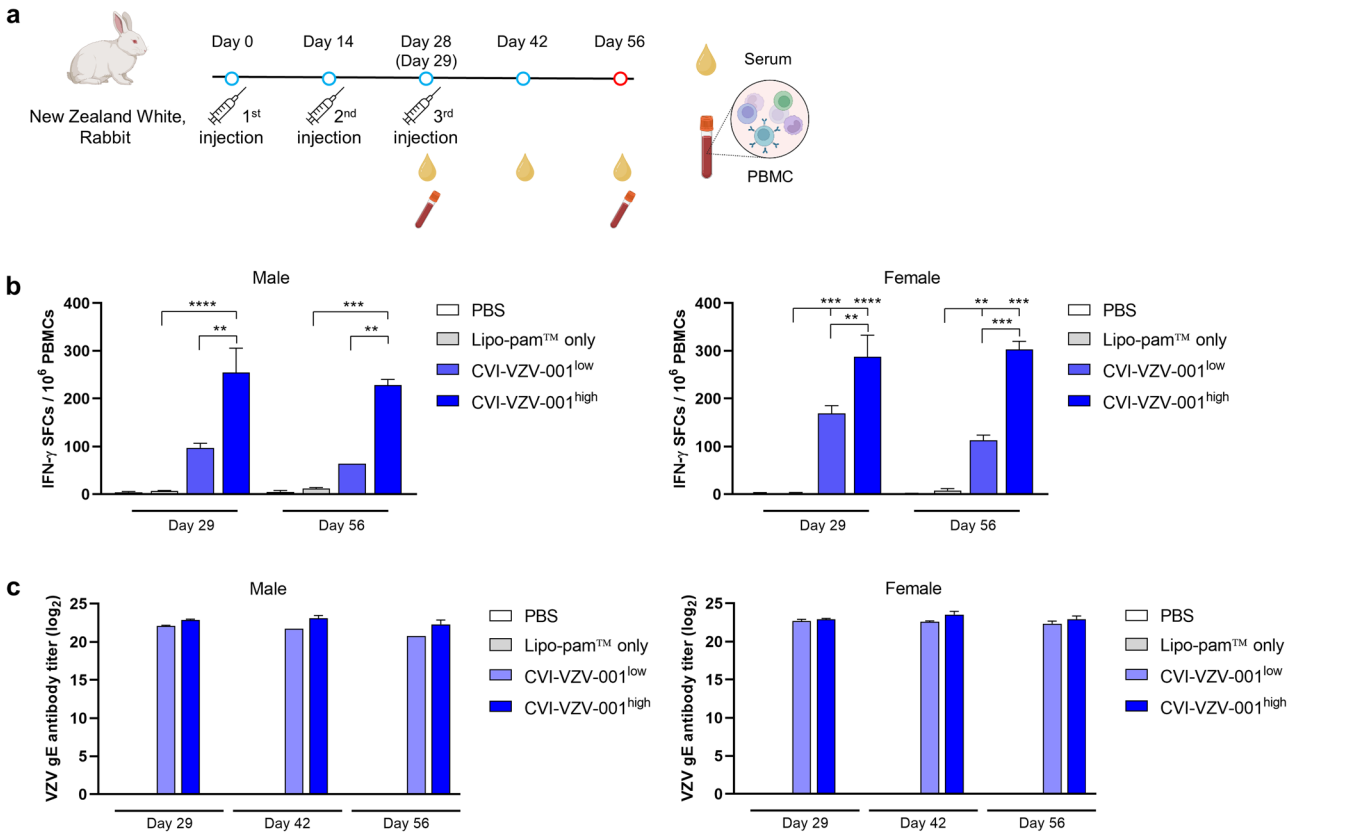

Fig. 3 | VZV gE-specific immune responses induced by CVI-VZV-001 in a rabbit model. a A schematic of immunization and experimental design. New Zealand White Rabbits (n = 5) were immunized with CVI-VZV-001 intramuscularly (i.m.) on days 0,14 and 28. (Images partly exported from Biorender.com.) **b** Evaluation of VZV gE-specific T cell immunity in male and female rabbit PBMC by IFN-γ ELISPOT assay. **c** Antigen-specific total IgG titer by ELISA in both male and female rabbit sera. Data are representative of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; one way ANOVA with the Tukey's test.

to the presence of attenuated live virus[27,28]. Thirdly, it can be challenging for immunocompromised people who need safe and effective vaccination for shingles and hardly receive any live attenuated vaccine for safety reasons[29]. On the other hand, subunit vaccines are safe and effective due to the absence of a virus and the presence of an adjuvant that can induce strong immunity for the HZ subunit vaccine[30,31]. Shingrix, a non-live, recombinant subunit adjuvanted vaccine developed by GSK, is highly effective in preventing HZ in adults over 50, with an effective rate of 97%. In comparison, Zostavax is only 50−64% effective in adults aged 50−70[32,33]. Shingrix also induces more robust VZV- and gE-specific cell-mediated and humoral immune responses than Zostavax[34,35]. However, Shingrix can cause severe symptoms (grade 3) after immunization, which may hinder compliance with one or two shots due to high reactogenicity. Furthermore, the association of Guillain-Barre Syndrome (GBS) with Shingrix is of concern despite the unclear causal relationship[36]. In addition, most countries where Shingrix is licensed have yet to administer it due to significant supply shortages.

Therefore, further development of zoster vaccines is still needed, and our CVI-VZV-001 has shown comparable efficacy to GSK's Shingrix in animal models. Additionally, CVI-VZV-001 has sustained gE-specific cellular immune responses and antibody production for up to 224 days. Recombinant subunit vaccine induces higher VZV-specific $CD4^+$ T-cell responses than the live attenuated vaccines (LAVs), while the $CD8^+$ T cells-mediated cytotoxic response increases in the animal immunized with LAVs[37]. Indeed, the recombinant zoster vaccine with adjuvant generates significantly higher VZV-specific IL-2 and gE-specific IL-2, IFN-γ, and IL-2/IFN-γ-producing $CD4^+$ T cells than the LAVs. Moreover, the recombinant vaccine generates higher memory and effector memory of $CD4^+$ T cells but not $CD8^+$ T cells[34,38]. CVI-VZV-001 also induces the robust VZV-specific $CD4^+$ T cell immune response but not $CD8^+$ T cell responses, consistent with the previous studies.

The dosage of the recombinant vaccine is crucial in determining its effectiveness and durability. Our data demonstrate that the second dose of the CVI-VZV-001 induces sufficient immune responses in both mouse and rabbit models. Moreover, our findings indicate that the third vaccine dose in rabbits does not impact the strength of the cellular and humoral immune responses. This outcome could be attributed to the saturation of immune responses to the CVI-VZV-001 vaccine following the second dose.

We perform the GLP toxicity test and confirm the safety of both CVI-VZV-001 and Lipo-pam™. Currently, we are conducting a phase 1 clinical trial for CVI-VZV-001, a recombinant Herpes Zoster vaccine admixed with Lipo-pam™. So far, none of the 32 participants in the trial have reported any unexpected serious adverse events. These results and the current development process signify that CVI-VZV-001 could be a potent prophylactic vaccine against HZ.

Methods

Preparation of recombinant VZV gE protein

ORF68 (glycoprotein E, strain Dumas) of the entire VZV gE genome was used as a template of VZV gE gene. Additionally, codon-optimization of the genome sequence was conducted to improve the expression efficacy in Chinese hamster ovary cells (CHO DG44). The designed VZV gE gene did not contain the transmembrane and anchor domains of the gE. A restriction enzyme recognition sequence and a Kozak sequence were included in the synthesized VZV gE gene. An expression vector pPGXII-VZV gE plasmid was produced by cloning the synthesized 1.6 kb VZV gE gene into an expression vector pPGXII (PanGen Biotech Inc., Korea). Recombinant VZV

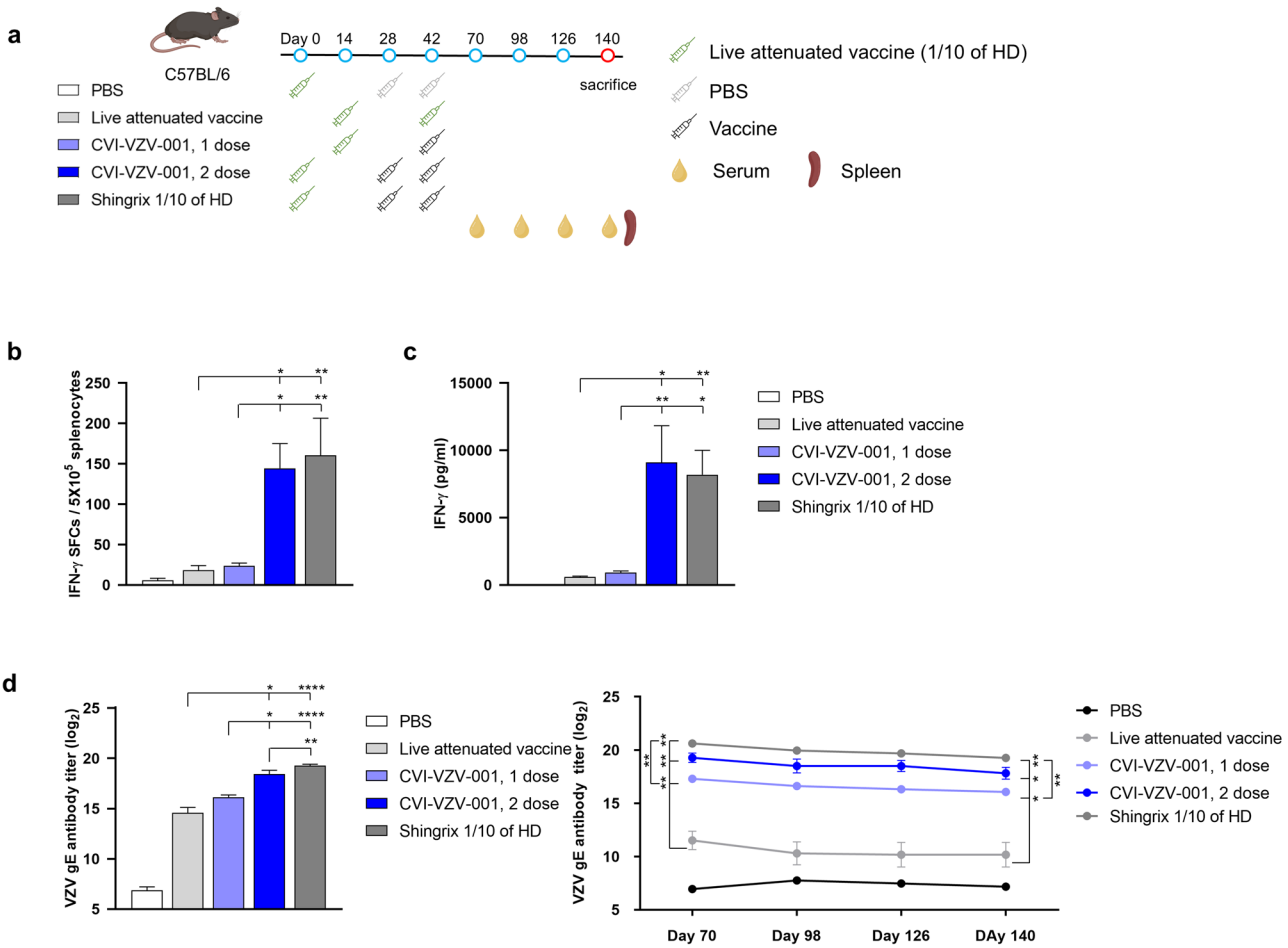

Fig. 4 | The durability of VZV gE-specific immune responses by immunization of CVI-VZV-001 or Shingrix in VZV-primed mice (up to day 140). a A schematic of immunization and experimental design. The VZV-primed mice (n = 5) were administrated by live-attenuated vaccine or CVI-VZV-001 or Shingrix, and cellular and humoral immune responses were measured up to day 140. (Images partly exported from Biorender.com.) **b** SFCs (spot-forming cells) represent antigen-specific IFN-γ secreting cells at day 140 by ELISPOT assay. **c** Total IFN-γ production at day 140 by ELISA assay. **d** Persistence of antigen-specific total IgG titer from day 70 to day 140 (right), and the total IgG titer at day 140 (left). Data are representative of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; one way ANOVA with the Tukey's test (**b**, **c**, **d**-left) or two way ANOVA with the Tukey's test (**d**-right).

gE protein expressed in CHO cells was harvested from culture supernatant and purified through a series of processes such as column chromatography, concentration, diafiltration, and virus filtration. The purified VZV gE protein was analyzed by SDS-PAGE, Western Blotting, and size-exclusion chromatography, and the purity was confirmed as 100%. The protein solution was sterilized by filtration and stored at −70 °C until use.

Formulation of VZV gE/Lipo-pam™

Lipo-pam™ was produced using the lipid-film hydration method. Briefly, DOTAP, DPPC, and TLR2 agonists were dissolved separately in chloroform and mixed at a specific molar ratio. To form a lipid film, the lipid mixture was transferred into a borosilicate glass flask, which was connected to a rotary evaporator, removing the organic solvent and leaving a thin lipid layer at the bottom of the flask. To produce the solution that contained liposomes, the lipid film was hydrated with PBS buffer, followed by vortexed frequently, and produced by Microfluidizer. Lipo-pam™ was formulated by mixing liposomes with TLR3 agonists and final sterilization filtration. The stability of Lipo-pam™ was maintained for two years at 4 °C. Before use, CVI-VZV-001 was formulated with 2.5 μg of recombinant VZV gE antigen mixed with 115 μg of Lipo-pam™.

Animals

Female C57BL/6 (6–8 weeks old) mice were purchased from Orient Bio Inc. (Seong-nam, Korea) and housed in a specific-pathogen-free animal facility at CHA University (Seongnam, Korea). All mice were handled in accordance with the standards approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University (IACUC; #200081). All mice were maintained at a temperature of 22 °C ± 1 °C and a humidity of 50% ± 10%. The light intensity was set at 150−300 lux, and a high-efficiency ventilation system provided 12−18 air changes per hour. Environmental parameters were continuously logged using an automated system, and trained personnel conducted daily checks. They had *ad libitum* access to food and water. Mice were immunized intramuscularly with test articles twice, at weeks 0 and 2 using a 1.0 mL insulin syringe (BD), and sacrificed at the indicated time point. The mice were euthanized by exposure to carbon dioxide (CO_2) overdose, followed by cervical dislocation as a secondary method of euthanasia. The immunogenicity was evaluated in serum and splenocytes. For serum isolation, blood was collected via facial vein or retro-orbital bleeding during the study or abdominal *vena cava* or cardiac puncture after the sacrifice and transferred into a 1.5-mL Eppendorf tube. Blood samples were centrifuged at 2500 rpm for 15 min at 25 °C. The serum (supernatant) was transferred into a new 1.5-mL Eppendorf tube and stored at −20 °C. For isolation of splenocytes, excised spleen was mashed over a 70 μm cell strainer. The RBCs were lysed with Red blood cell lysing buffer Hybri-Max™ (Sigma). Some mouse experiments used a VZV-prime mouse model primed with a live attenuated vaccine (SKYZoster) to mimic a clinical environment where most people are at high risk for HZ and VZV seropositivity.

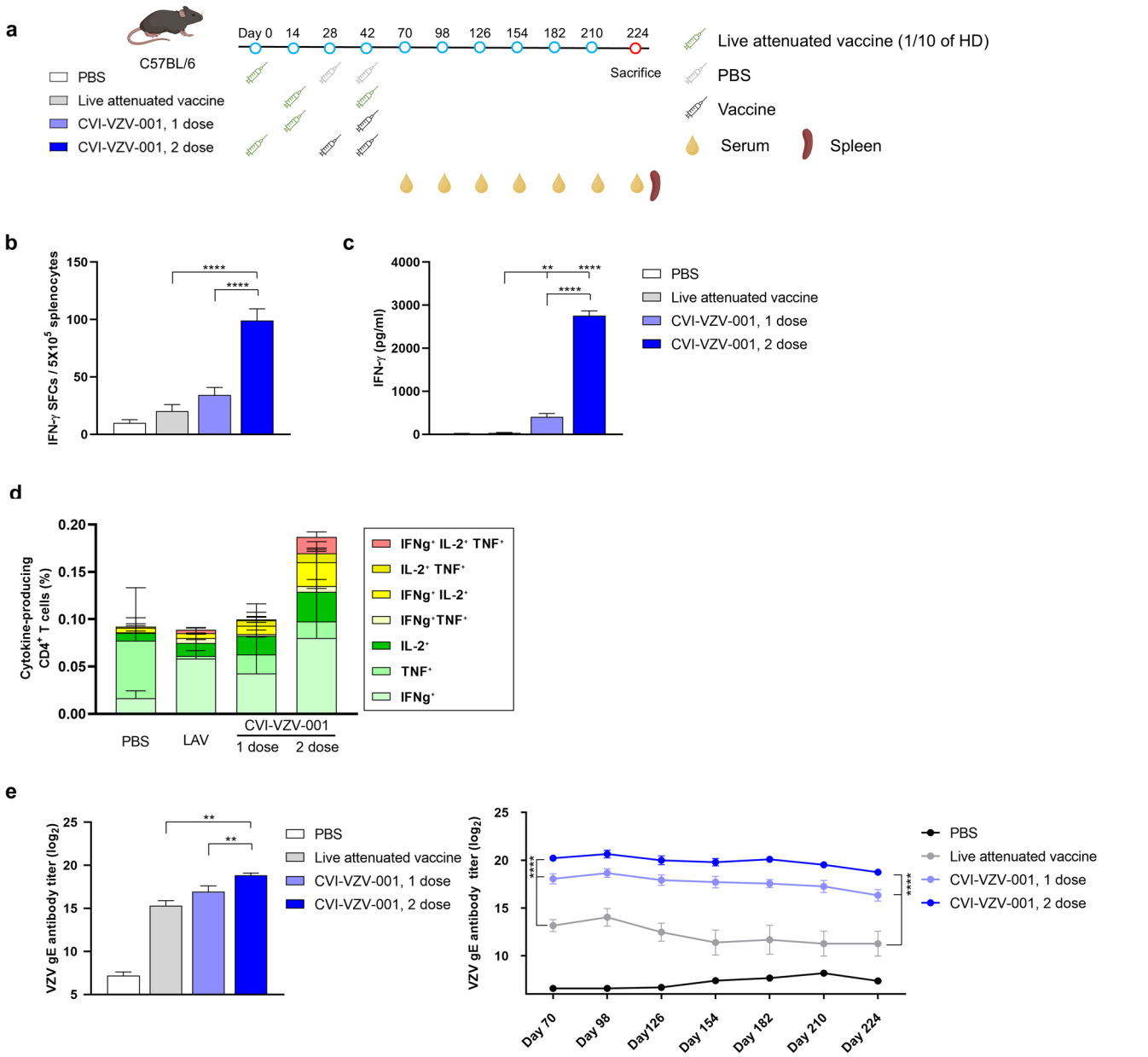

Fig. 5 | The durability of VZV gE-specific immune responses by immunization of CVI-VZV-001 in VZV-primed mice (up to day 224). **a** A schematic of immunization and experimental design. The VZV-primed mice (n = 5) were administrated by live-attenuated vaccine or CVI-VZV-001, and cellular and humoral immune responses were measured up to day 224. (Images partly exported from Biorender.com.) **b** SFCs (spot-forming cells) represent antigen-specific IFN-γ secreting cells at day 224 by ELISPOT assay. **c** Total IFN-γ production at day 224 by ELISA assay. **d** The frequency of cytokine-secreting T cells among $CD4^+$ T cells at day 224 by intracellular cytokine assay. **e** Persistence of antigen-specific total IgG titer from day 70 to day 224 (right) and the total IgG titer at day 224 (left). Data are representative of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; one way ANOVA with the Tukey's test (**b**, **c**, **e**-left) or two way ANOVA with the Tukey's test (**e**-right).

Eighteen-week-old male and female rabbits (New Zealand White) were purchased from Samtako Bio Korea Co. Ltd. (Osan, Korea) and were housed in a facility (Biotoxtech Co. Ltd. Korea) where approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Rabbits were intramuscularly immunized with CVI-VZV-001 on days 0, 14, and 28 using a 1.0 mL insulin syringe (BD). Antibody production and T-cell response were analyzed using serum and peripheral blood mononuclear cells (PBMCs). After the third dose (Day 29, Day 56), 10 mL of blood was collected from the rabbit's ear vein or jugular vein. Out of this, 3 mL was used for serum separation, and approximately 7 mL was mixed into a CBC bottle containing K2EDTA for PBMC isolation. Additionally, about 2 mL of blood was collected from the appropriate vein on Day 42 for serum separation.

Anti-VZV gE-specific IgG ELISA

Ninety-six-well ELISA microplates were coated with VZV gE (1 μg/mL) at 37 °C for 2 h and blocked with bovine serum albumin (1%, w/v) at 37 °C for 1 h. After the microplates were washed, the diluted sera were added to the plates and incubated at 37 °C for 2 h. Then, the bounded antibodies were detected by incubation with HRP-conjugated anti-mouse IgG(KPL), IgG1, IgG2a, IgG2b and IgG2c antibodies (Thermo Fisher, Waltham, MA, USA) at 37 °C for 1 h. The reaction was visualized by adding a TMB–peroxidase solution (Seracare, Milford, MA, USA). The optical density (OD) at 450 nm

Table 1 | Summary of GLP toxicity study of CVI-VZV-001

Test	Animal /Route	Regimen	Results
Repeated-dose toxicity study			
1 dose/2 weeks, a total of 3 doses	Rabbit, New Zealand White (NZW), SPF /Intramuscular	CVI-VZV-001 0, 25, 50, 100 (μg/antigen/animal)	No test substance-related changes were observed in clinical signs, body weight, food consumption, and ophthalmological examination. It is judged that CVI-VZV-001 was tolerated up to 50 μg/antigen/animal.
1 dose/2 weeks, a total of 3 doses	Rabbit, New Zealand White (NZW), SPF /Intramuscular	CVI-VZV-001 0, 25, 50, 70, 90 (μg/antigen/animal)	There were no CVI-VZV-001-related mortalities and no CVI-VZV-001-related clinical observations or effects on body weight, body temperatures, organ weights or macroscopic observations. It is judged that CVI-VZV-001 was tolerated up to 70 μg/antigen/animal.
Reproductive and development toxicity study			
Embryo-Fetal Development Toxicity	Rat, Sprague-Dawley (Crl:CD(SD)), SPF /Intramuscular	CVI-VZV-001 0, 12.5, 25, 50 (μg/antigen/animal)	The no-observed-adverse-effect level (NOAEL) of CVI-VZV-001 for pregnant female rats and embryo-fetal development is considered to be 50 μg/antigen/animal, respectively, under the study conditions.
Genotoxicity			
Bacterial Reverse Mutation Test	Salmonella typhimurium (TA98, TA100, TA1535, TA1537), Escherichia coli (WP2uvrA(pKM101))	CVI-ADJ-006 460, 230, 115, 57.5, 28.8 (μg/plate)	CVI-ADJ-006 does not show any indication of mutagenic potential under the study conditions.
In Vitro Mammalian Chromosomal Aberration Test	Chinese Hamster Lung (CHL/IU) cell line	CVI-ADJ-006 Short-term treatment: 115, 57.5, 28.8, 14.4 (μg/mL) /Continuous treatment: 46.0, 23.0, 11.5, 5.75 (μg/mL)	CVI-ADJ-006 does not induce chromosomal aberrations under the study conditions.
In Vivo Micronucleus Test	Rat, Sprague-Dawley (Crl:CD(SD)), SPF /Intramuscular	CVI-ADJ-006 2.3, 1.15, 0.575 (mg/head)	CVI-ADJ-006 does not have any potential to induce micronuclei in bone marrow cells of Sprague-Dawley rats under the study conditions.
Safety pharmacology study			
Evaluation of Effects on the Central Nervous System	Rat, Sprague-Dawley (Crl:CD(SD)), SPF /Intramuscular	0, 50 (μg/antigen/animal)	A single intramuscular injection of CVI-VZV-001 at a dose level of 50 μg/animal to Sprague-Dawley rats slightly increased the body temperature, but it is judged that it returned to normal range within 24 h post-dose, and no effects of the test substance were observed in other parameters.
Evaluation of Effects on the Respiratory System	Rat, Sprague-Dawley (Crl:CD(SD)), SPF /Intramuscular	0, 50 (μg/antigen/animal)	It is judged that the test substance, CVI-VZV-001, has no adverse effect on the respiratory rate at a dose level of 50 μg/animal after a single intramuscular injection to male Sprague-Dawley rats under the study conditions.
Evaluation of Effects on the Cardiovascular System	Dog, Beagle /Intramuscular	0, 100 (μg/antigen/animal)	A single intramuscular injection of CVI-VZV-001 at a dose level of 100 μg/animal to beagle dogs increased the blood pressure and showed a shortening tendency of the PR interval of ECG parameters, but they all recovered within 24 h post dose. In addition, it was judged that there was no effect of the test substance on other ECG parameters (QRS, and QTc intervals) and heart rate.

was measured with an automatic ELISA plate reader (Thermo Fisher, Waltham, MA, USA).

Fluorescent-antibody-to-membrane-antigen (FAMA) assay

FAMA assay was developed by Williams and others in 1974 and has been used as a "gold standard" method to measure the antibody value that is protective against VZV in cell culture. MRC-5 cells have been used to perform the FAMA test. MRC-5 cells in culture are infected with VZV for 72 h and harvested, then the infected cells are resuspended in PBS and incubated with serial dilutions of mouse sera to bind with VZV-specific antibodies for 30 min. Following washing, cells are incubated with an Alexa Fluor®488 goat anti-human IgG (for positive control human sera) and Alexa Fluor®488 goat anti-mouse IgG for 30 min under the light block. After a washing step, the cells are transferred into small wells on glass slides and incubated for 10−15 min to allow the cells to attach. Drop Vecta Shield® mounting medium with DAPI on each well of the slide with well-dried cells and cover with cover glass. When the viral proteins distributed on the surface of VZV-infected cells bind to their specific antibodies in the serum samples, ring-like fluorescence patterns are typically observed. Depending on the degree of staining of the cell, the grade was read as negative, weak, positive 1+, 2+, and 3+, and dilution multiples corresponding to positive 1+ were used as the result. S-H. B. performed grading that was blinded to sample experimental identity.

Mouse and rabbit IFN-γ ELISPOT assays

Mouse IFN-γ ELISPOT assay (3321-1HP-2, Mabtech, Stockholm, Sweden) and rabbit IFN- γ ELISPOT assay (3110-4HPW-2, Mabtech, Stockholm, Sweden) were performed according to manufacturer's protocol. Briefly, immunized mouse splenocytes (5×10^5 cells /well) and rabbit PBMCs (2.5×10^5 cells /well) were added to a 96-well ELISPOT plate pre-coated with anti-mouse and anti-rabbit monoclonal IFN-γ antibodies. Mouse splenocytes and rabbit PBMCs were incubated with the PepMix™ VZV gE peptide pool (JPT Peptide Technology, Berlin, Germany) (2 μg/mL) at 37 °C for 24 h. Concanavalin A (Sigma, St. Louis, MO, USA) and the medium alone were included as positive and negative controls, respectively. After stimulation, the wells were washed and incubated with the biotinylated anti-mouse IFN-γ antibody or anti-rabbit IFN-γ antibody at room temperature (RT) for 2 h. Subsequently, the plate was incubated with streptavidin-

horseradish peroxidase for 1 h, and spot-forming cells (SFCs) were developed with the TMB substrate. The spots were counted using a AELVIS ELISPOT Reader. To quantify positive peptide-specific responses, mean spot counts for negative controls were subtracted from peptide-stimulated samples. The results were presented as SFCs/5×10^5 and SFCs/1×10^6 for mouse and rabbit model, respectively.

Intracellular cytokine staining

The mouse splenocytes were stimulated at 37 °C for 6 h with or without the PepMix™ VZV gE peptide pool (JPT Peptide Technology, Berlin, Germany) (2 μg/mL) with 1 μg/mL CD28/CD49d (BD). After the stimulation for an hour, 10 μg/mL brefeldin A (BD Biosciences, San Jose, CA, USA) was treated for 5 h to inhibit the cytokines secretion from splenocytes. Cells were stained with a yellow LIVEDEAD fixable dead cell stain kit (BD) and anti-CD3 (Clone 500A2, BD), anti-CD4 (Clone RM4-5, BD), and anti-CD8 (Clone 53-6.7, BD) antibodies. The cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA, USA) and stained with anti-IFN-γ (clone XMG1.2, BD, 1:100), anti-TNF-α (clone MP6-XT22, BD, 1:100) and anti-IL-2 (JES6-5H4, BD, 1:100) antibodies. The cells were acquired on a FACSCelesta (BD) and analyzed with Flowjo software (v 10.8.1) (Tree Star, Ashland, OR, USA). To analyze the polyfunctional $CD4^+$ T cell population, a Boolean gating strategy was utilized. Boolean gating creates gates based on a logical selection of individual gates using operators such as AND, NOT, and OR and can quickly identify cells expressing single, double, and triple cytokines. Briefly, T cells were gated based on the scatter properties typical of lymphocytes and CD3 expression. $CD4^+$ or $CD8^+$ T cells were selected from $CD3^+$ T cells, and each cytokine-producing $CD4^+$ T cell was gated. Next, a cell population that expressed two or three specific gates for the cytokines was created by Boolean gating. This allowed for calculating the number of single, double, and triple cytokine-producing $CD4^+$ T cells (Supplementary Fig. 7).

Isolation of peripheral blood mononuclear cells (PBMCs)

To isolate PBMCs from the blood of rabbits, 7 mL of blood was collected from rabbit ear vein or jugular vein and mixed blood into a CBC bottle containing K_2EDTA. Whole blood was transferred to 50 mL centrifuge tubes and diluted with sterile PBS at a 1:1 v/v ratio. Diluted blood was carefully added into a new 50 mL centrifuge tube containing the same volume of Ficoll (17-1440-03, GE healthcare). 50 mL centrifuge tubes containing the same volume of Ficoll and diluted blood were centrifuged at room temperature for 20 min at 2000 rpm with the brake turned off acceleration 1. The top plasma layer was removed, and the lymphocyte layer was collected without disturbing the Ficoll layer, followed by transferring it to a new 15 mL centrifuge tube. Culture media (RPMI with 10% FBS, 1% penicillin-streptomycin) was added to 10 mL in 15 mL centrifuge tubes and centrifuged for 10 min at room temperature at 2000 rpm. The supernatant was carefully removed from the pellet, and the pellet was resuspended with culture media, followed by repeating the centrifugation. 5×10^6 cells per vial were resuspended with freezing media (FBS: RPMI media: DMSO = 5: 4: 1) and stored at −80 °C before use.

Statistics

Data were analyzed with Graph Prism 9 software. Data are shown as mean ± SEM as noted. For comparisons between more than two groups, one-way ANOVA or two-way ANOVA followed by Tukey's test was performed. No samples were excluded from any experiments performed in this study. Mice were randomized to experimental groups when they were delivered into our mouse facility to avoid caged-based or housing bias. No blinding was used except for FAMA grading of microscopy-based counting as noted above. Differences of $p < 0.05$ were considered statistically significant.

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Author contributions

S.J., B.C.A., and J.S.Y. conceived the concept and designed experiments. S.J., S.H., and S.B. performed the experiments, analyzed the data. H.C. and J.L. established the methods and performed the experiments. E.J. and H.J. provided materials used for the studies. S.J. and E.C. interpreted data and drafted the manuscript. All authors reviewed and approved the paper.

Competing interests

S.J., S.H., S.B., E.J., H.J., B.C.A., and E.C. are CHA Vaccine Institute employees. J.S.Y. is the chief executive officer of CHA Vaccine Institute. CHA Vaccine Institute holds worldwide license to a patent of Lipo-pam™ used in this study. CHA Vaccine Institute partially funded the study. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. No other authors declare any competing interests.

Additional information

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