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Truncated glycoprotein E of varicella-zoster virus is an ideal immunogen for *Escherichia coli*-based vaccine design

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Varicella-zoster virus (VZV) is a highly infectious agent responsible for both varicella and herpes zoster disease. Despite high efficacy, there remain safety and accessibility concerns with the licensed vaccines. Here, we sought to produce a VZV gE immunogen using an *E. coli* expression system. We found that the soluble expression and yield of gE protein could be enhanced via C-terminal truncations to the protein, thereby facilitating a robust and scalable purification process for the purpose of vaccine manufacturing. The lead truncated gE (aa 31–358), hereafter referred to as tgE, was a homogenous monomer in solution and showed excellent antigenicity. Finally, we assessed and compared the immunogenicity of tgE with commercial vOka LAV and Shingrix vaccine. We found that aluminum-adjuvanted tgE was immunogenic as compared with vOka LAV. When adjuvanted with $AS01_B$, a two-dose immunization of tgE showed comparable or better potency in antibody responses and cell-mediated immunity with those of the Shingrix vaccine at the same dosage, especially in terms of the proportion of IFN-γ-expressing $CD4^+$ T cells. In conclusion, this method of *E. coli*-mediate tgE expression offers a cost-effective and scalable strategy to generate an ideal VZV gE immunogen for the development of both varicella and zoster vaccines.

varicella-zoster virus, glycoprotein E, *Escherichia coli*, vaccine

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INTRODUCTION

Varicella-zoster virus (VZV) is a pathogenic, human alpha-herpesvirus that induces two main categories of disease: primary infection with varicella, a highly contagious disease characterized by a blister-like rash on the skin and mild systemic symptoms such as fever and malaise predominantly in children; and herpes zoster (HZ), the latent reactivation of the virus in response to a weakened cellular immune system caused by aging that causes a painful, localized vesicular rash (HZ) with or without several other complications (Cohen, 2013; Heininger and Seward, 2006; Zerboni et al., 2014).

Varicella and HZ can both be prevented by live-attenuated virus vaccines (LAV). The first LAV for the prevention of varicella was developed by Takahashi et al. (1974) based on vOka virus strain, and was later licensed as Varivax for routine use in the United States. This was followed by the development and licensing of Zostavax for the prevention of

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HZ (Tseng et al., 2011). Unfortunately, in the 10 years since inoculations with Varivax began, almost 1/3 of HZ cases have been confirmed to be associated with the vOka strain (Galea et al., 2008), suggesting that vOka can infect and establish latency, like a wild-type virus, and then reactivate to cause HZ. In addition, the efficacy of Zostavax is inversely proportional to the age of the patient at the time of administration, with increased age associated with lower efficacy (Oxman et al., 2005).

Previous studies have shown the VZV glycoprotein E (gE) to be the most abundant viral glycoprotein on the virion envelope and the infected cell surface. gE is also one of the most important VZV protective antigens capable of inducing cellular and humoral immunity during natural varicella infection as well as following vOka vaccination (Arvin, 1992; Oliver et al., 2016). Recently, a recombinant HZ subunit vaccine, Shingrix (HZ/su), was approved for the prevention of HZ and the associated postherpetic neuralgia (PHN) in adults aged≥50 years (Shah et al., 2019; Syed, 2018). In clinical trials, Shingrix induced an efficient, VZV-specific, cell-mediated immunity (CMI), with an overall vaccine efficacy of 97.2% among participants ≥50 years and a 91.3% protection rate in participants ≥70 years. These findings collectively indicate a significantly reduced risk of HZ in vaccinated individuals independent of age, and thus a more potent vaccine option as compared with ZOSTAVAX (Lal et al., 2015; Shah et al., 2019).

Shingrix is designed from a recombinant gE antigen expressed in Chinese hamster ovary (CHO) cells and a liposome-based adjuvant system ($AS01_B$) containing 3-O-desacyl-4′-monophosphoryl lipid A (MPL) and *Quillaja saponaria* Molina, fraction 21 (QS21) (Dendouga et al., 2012). However, recombinant CHO cells require a long production cycle and are associated with a complex purification process that results in a high manufacturing cost and a limited supply. As such, Shingrix is not an ideal vaccination solution for wide-spread use, particularly in developing countries (Kim et al., 2012).

Microbial expression systems offer several advantages over mammalian expression systems, including rapid growth, ease of culture, and lower production costs (Overton, 2014), and are thus widely used in the expression and production of numerous biopharmaceuticals (Adkins and Wagstaff, 1998; Monie et al., 2008; Wu et al., 2012). However, several difficulties during production need to be overcome for the successful expression of recombinant eukaryotic proteins in prokaryotic systems, such as incorrect folding and a lack of post-translational modification (Singh and Panda, 2005). Despite these difficulties, microbial expression systems could offer an ideal system for wide-spread, cost-effective vaccine manufacturing, and it therefore warrants investigating antigens from an *E. coli* expression system for VZV vaccine design.

In previous work, we showed the successful expression of a recombinant gE (rgE) protein in insect cells, and obtained purified proteins for mouse immunization and mAb production (Liu et al., 2015). Building on this work, here we investigate whether the gE protein can be expressed in *E. coli*. We explored the non-fusion soluble expression of gE candidate antigens with a C-terminal truncation (hereafter referred to as truncated gE (tgE) or tgE) in *E. coli* and characterized the purified tgE proteins. We show that the purified tgE is antigenic similar to the native gE, and capable of inducing high antibody titers with vOka-neutralizing activity in mice. In comparing the immunogenicity of the tgE antigen generated in our study with the LAV and Shingrix, we find that our antigen not only exhibits an efficient humoral response but also induces an antigen-specific CMI. Overall, our *E. coli* strategy for the production of tgE protein may pave the way for an alternate industrially produced immunogen for both varicella and HZ vaccination.

RESULTS

Expression and purification of recombinant tgE in *E. coli*

DNA sequences encoding for the VZV gE extracellular domain were prepared as constructs with various N- or C-terminal truncations, and cloned into the pTO-T7 vector for protein expression in *E. coli*. The full-length extracellular domain of gE tended to form inclusion bodies in *E. coli* during expression, and this could be improved with a C-terminal truncation. Of the various truncated proteins, the best solubility was attained with the gE (aa 31–358) construct (Figure 1A), hereafter referred to as truncated gE or tgE.

The candidate tgE was successfully expressed as a soluble recombinant protein and purified from the supernatant of bacteria lysates using three-step chromatography at the lab-scale for further assessment and development as potential vaccine. Strong anionic exchange chromatography was used for capture followed by ceramic hydroxyapatite and hydrophobic chromatography steps (Figure 1B). Successive stages of the purification process saw an increase in the purity of tgE on SDS-PAGE (Figure 1C). tgE migrated as an approximately 40 kD band and showed good reactivity with the specific antibody 1B11 (Figure 1D). The overall yield was about 500 μg of purified tgE per gram of bacteria.

Characterization of the truncated gE

High-performance size-exclusion chromatography (HPSEC) and analytical ultracentrifugation (AUC) were used to analyze the homogeneity and sedimentation coefficients of purified proteins. rgE protein purified from the insect cell

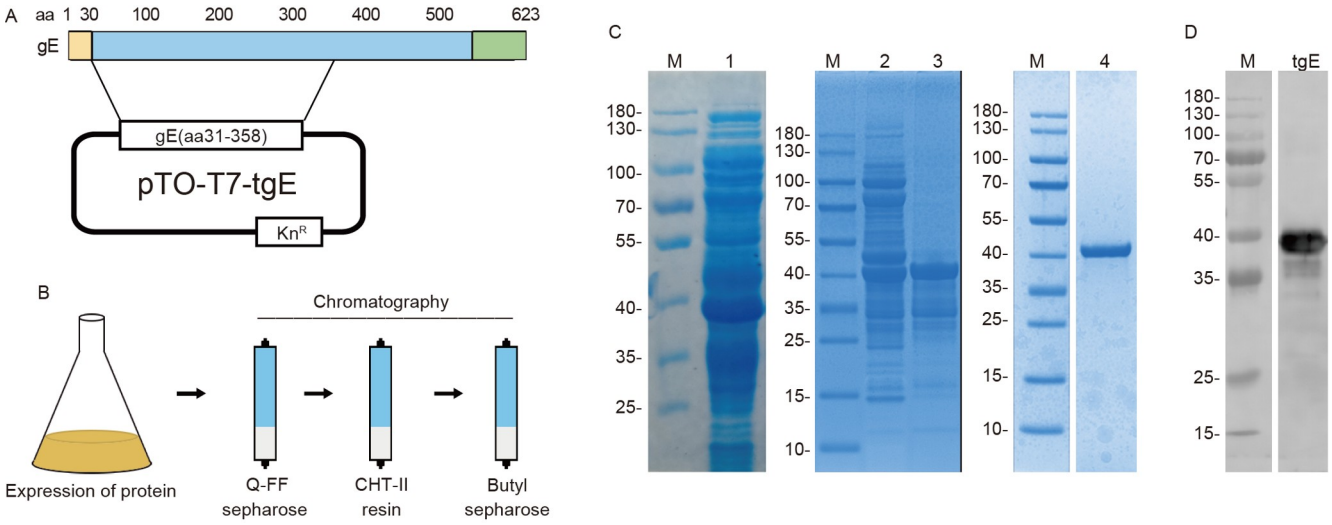


Figure 1 (Color online) Expression and purification of tgE proteins. A, Schematic representation of tgE construct design. *E. coli* cells were transformed with recombinant plasmids carrying the gE (aa 31–358) truncated gene. B, Schematic representation of the tgE purification process. tgE was purified from lysed supernatant using three-step chromatography. C, SDS-PAGE profile of tgE during the purification process. The molecular weight of tgE is approximately 40 kD. M: molecular weight marker. Lane 1: supernatant separated from cell lysate; lane 2: fraction containing tgE from the Q-FF chromatograph; lane 3: fraction containing tgE from CHT chromatograph; lane 4: fraction from the Butyl chromatograph, containing the tgE protein with high purity. D, Immunoblotting of the purified tgE.

system (Liu et al., 2015) was used as a control. In HPSEC, tgE appeared a sharp peak at the predominant retention time of 16.1 min (Figure 2A), reflecting high homogeneity for the purified tgE proteins. In AUC experiments, a predominant sedimentation peak was observed at ~2.3 S (Figure 2B), which equates to ~39.4 kD, indicating that tgE presents as a monomer in solution. Differential scanning calorimetry (DSC) was used to test the thermal stability of the tgE protein by monitoring the heat capacity during the thermal unfolding process. The DSC profiles showed only one thermal transition during protein unfolding at a transition temperature (Tm) value of 63.80°C for tgE, comparable to that of rgE (63.64°C) (Figure 2C).

To investigate the conformational integrity of the purified tgE proteins, we analyzed the antigenicity of tgE using an ELISA assay, with purified rgE proteins expressed in insect cells serving as controls. Nine mAbs that could recognize conformational epitopes (mAb 1B11, 4G4, 14G1) or linear epitopes (4A2, 11B11, 11B12, 6H6, 10H6, 11E3) were used in the ELISA test (Figure 3). We found similar median effective concentration (EC_{50}, ng mL^{-1}) values for tgE and rgE proteins for most of the antibodies except for 1B11, which had an almost 10-fold lower reactivity, suggesting their similar antigenicity. These results suggest that key epitopes on the rgE are well preserved in the tgE protein.

Analysis of immune responses induced by tgE in mice

To compare the immunogenicity of tgE with rgE, we formulated the proteins with two adjuvants commonly used for research work: aluminum-based adjuvant Al-001 and Freund's adjuvant. Mice were immunized thrice at weeks 0, 2, and 4 with a dosage of 1 μg for both adjuvanted tgE and rgE. gE-specific antibody titers and T-cell responses at week 5 post the first immunization were used to determine the immunogenicity of different adjuvant formulations. Mice immunized with the tgE vaccine formulated with Al-001 showed similar antibody profiles to mice in the rgE group, and Freund's adjuvant showed a better stimulation effect when formulated with tgE than with rgE (Figure 4A). Neutralization titers showed no differences in mice immunized with tgE or rgE vaccine, with neutralizing titers of ~10^3 for tgE formulated with the different adjuvants (Figure 4B). However, in the analysis of $CD4^+$ T cells from splenocytes by intracellular cytokine staining (ICS), both tgE and rgE vaccines elicited similar levels of gE-specific IFN-γ as compared with the saline group (Figure 4C, ~0.1% for either vaccines groups or saline group), and only a moderate simulation of gE-specific IL-2 (Figure 4D, ~0.2% for vaccines groups *vs*. ~0.1% for saline group). Taken together, these results show that tgE/Al-001 and tgE/Freund's adjuvant elicit gE-specific immune responses comparable with those elicited by rgE, with a particularly strong humoral response.

Evaluation of tgE varicella vaccine candidate as compared with LAV

We next evaluated antibody production and the persistence of aluminum-adjuvanted tgE proteins in BALB/c mice. Two groups of mice (n=5 per group) were immunized thrice at weeks 0, 2, and 4 with different dosages of 0.5 μg and 5 μg of tgE/Al-001 (Figure 5A and B). The antibody response

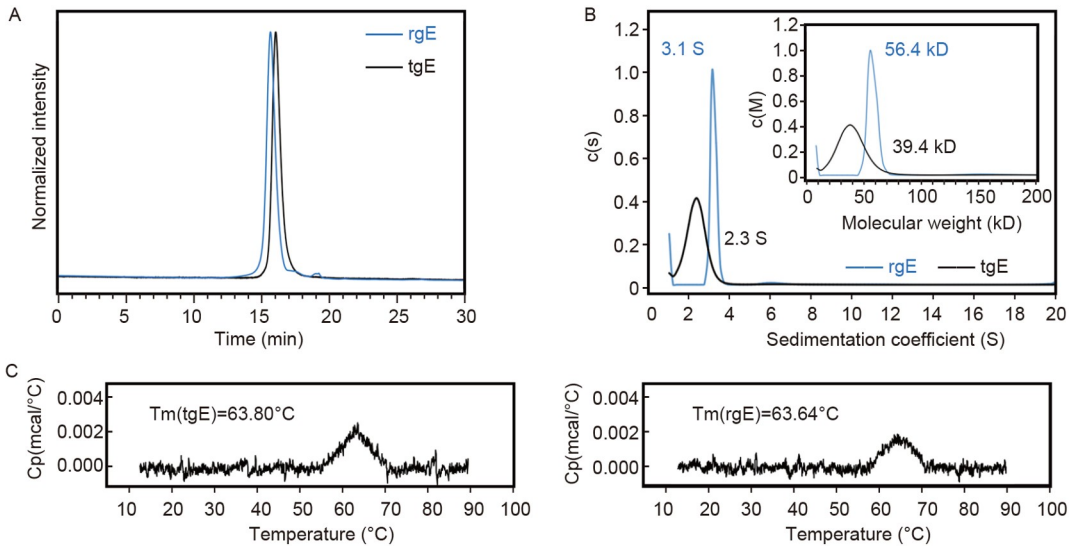


Figure 2 Characterization of tgE and rgE proteins. A, HPSEC profiles of the purified tgE. The retention time of gE is indicated. B, Sedimentation velocity analysis of the purified tgE protein. The sedimentation coefficient and molecular weight of tgE were determined by c(s) and c(M) methods independently. In (A) and (B), tgE is shown in black and rgE in blue. C, Differential scanning calorimetry profiles of the purified tgE and rgE protein.

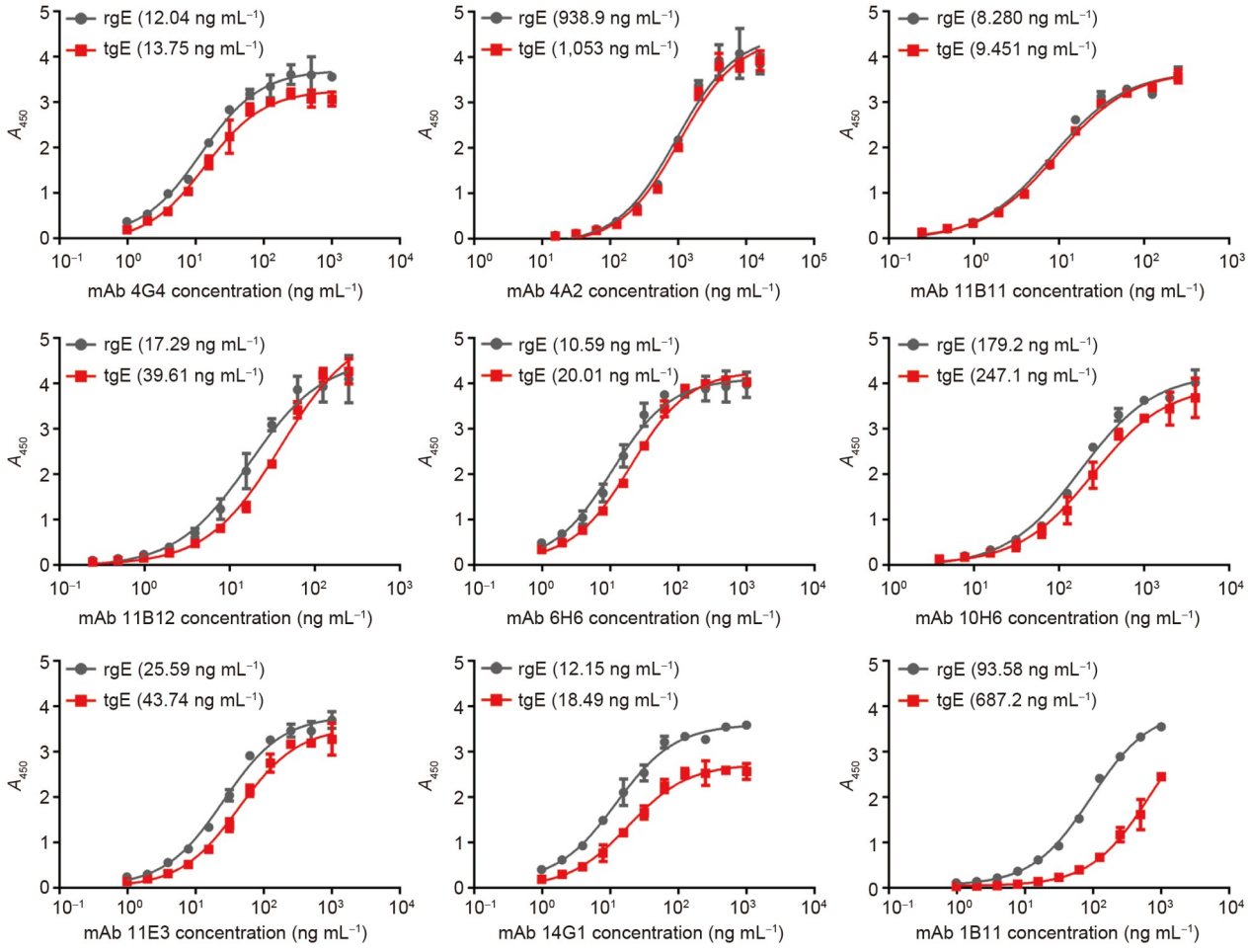


Figure 3 Reactivities of tgE and rgE with mAbs. mAbs were serial diluted and reacted with coated tgE or rgE proteins in an ELISA assay. Reactions were detected using HRP-labeled anti-mouse antibodies. Duplicate wells were measured on the same plate for each dilution. EC_{50} values were calculated by sigmoid trend fitting using GraphPad Prism software.

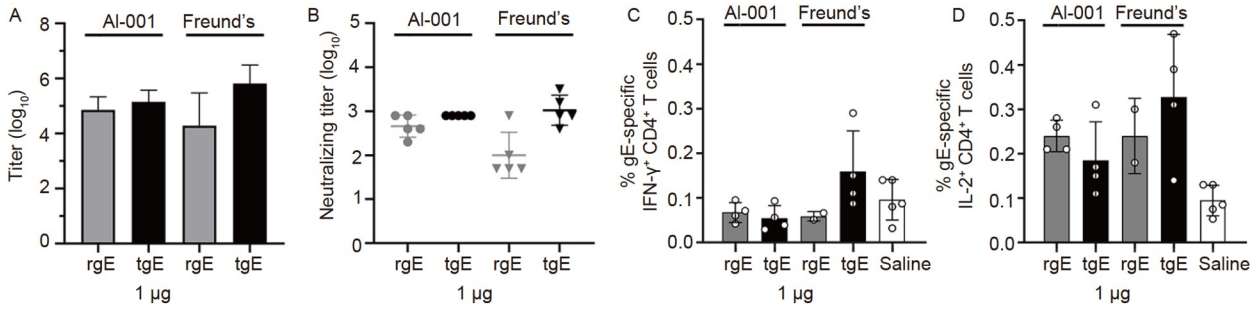
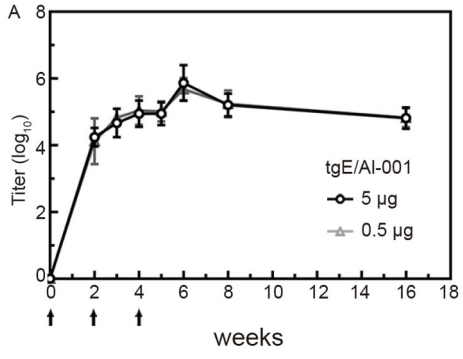
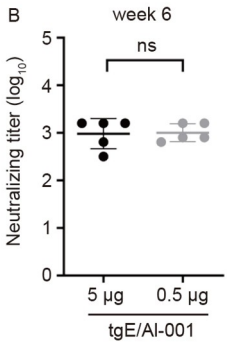


Figure 4 Immunogenicity of tgE and rgE tested in BALB/c mice 1 week after three-dose immunization. A, Antibody production in mice for tgE and rgE formulated with different adjuvants. B, Neutralizing antibody titers in mice for tgE and rgE formulated with different adjuvants. C and D, Evaluations of gE-specific IFN-γ and IL-2 responses induced by tgE or rgE vaccines.





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Antigen	Dosage	Seropositive mice no.	ED_{50}
tgE (μg)	1	5	0.063 μg
	0.5	6	
	0.25	6	
	0.125	6	
	0.0625	3	
	0.03125	1	
vOka (pfu)	1,000	4	449.425 pfu
	500	3	
	250	2	
	125	1	
	62.5	0	

Figure 5 Immunogenicity tests of aluminum-formulated tgE vaccine in BALB/c mice. A and B, Antibody persistence and neutralizing antibody response of tgE/Al-001. Titers were monitored up to 16 weeks. C, ED_{50} in mice for aluminum-formulated tgE and vOka.

profiles showed that the primary inoculation of the tgE protein elicited high antibody titers, with a sustained increase after the two booster immunizations at weeks 2 and 4, and reaching a maximum level at week 6 before slowly dropping to more than 10^4 over the monitoring period. Different dosage groups shared similar antibody titer profiles during the monitoring period, with similar high neutralizing titers at week 6.

The immunogenicity of tgE was evaluated and compared with vOka. The half-effective dosage (ED_{50}) in mice was used as a measure of seroconversion, with a lower ED_{50} indicative of better immunogenicity. Balb/c mice were separated into groups and intraperitoneally immunized with aluminum-adjuvanted tgE (1, 0.5, 0.25, 0.125, 0.0625, or 0.03125 μg; n=6) or vOka vaccine (1,000, 500, 250, 125, or 62.5 pfu; n=6) in serial dilutions. Sera were collected after 4 weeks and tested using ELISA. Seroconversion was analyzed according to the Reed and Muench method (Figure 5C). In the tgE groups, mice had seroconversion rates of 83%, 100%, 100%, 100%, 50%, and 17% for 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 μg dosages, respectively. In the vOka groups, mice had lower seroconversion rates of 67%, 50%, 33%, 17%, and 0% for 1,000, 500, 250, 125, or 62.5 pfu dosages, respectively. The ED_{50} values were 0.063 μg for tgE and 449.425 pfu for vOka, indicating that the immunogenicity of tgE at a lower dosage of 0.3 μg was equivalent to that achieved with a single dose of varicella vaccine containing vOka (usually over 2,000 pfu per dosage).

Evaluation of tgE Zoster vaccine candidate comparing with Shingrix

To compare the immunogenicity of our tgE vaccine with Shingrix, lyophilized tgE protein was formulated with the Shingrix adjuvant, $AS01_B$. C57BL/6 mice were immunized with two doses of tgE/$AS01_B$ or Shingrix intramuscularly in the tibialis muscle at weeks 0 and 4. The antibody levels after the first immunization suggested that immunization with Shingrix was sufficient to induce high levels of anti-gE antibodies, significantly higher than those produced by tgE/$AS01_B$; after the second immunization, however, there was no significant difference in antibody production between the two groups (Figure 6A). As to the neutralizing antibody levels, a single-dose immunization of tgE/$AS01_B$ led to the production of negligible amounts of neutralizing antibody, significantly lower than that produced by Shingrix. However, again, after the booster immunization, we could see no

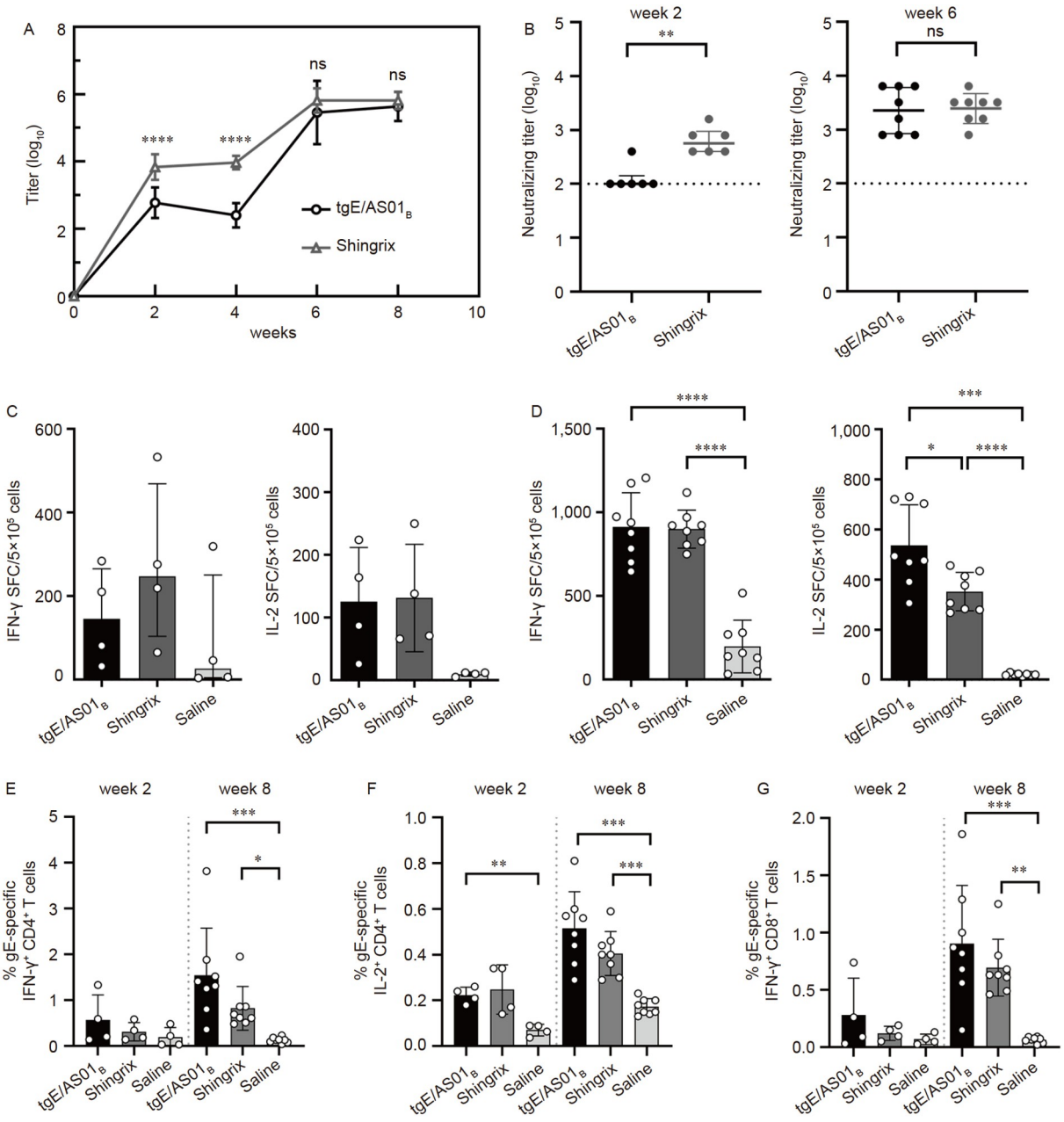

Figure 6 Immunogenicity of tgE in C57BL/6 mice. Doses of 5 μg tgE formulated with 50 μL $AS01_B$ adjuvant were administered. This was 1/10 the dose of the Shingrix vaccine. Immunization was performed at weeks 0 and 4. A, Comparison of antibody production induced by tgE/$AS01_B$ vaccine compared with Shingrix. Serum titers were determined by ELISA assay using rgE-coated plates. B, Comparison of the neutralizing antibody response to vOka following vaccination with tgE/$AS01_B$ vaccine or Shingrix. The neutralization titers of antiserum at week 2 (left panel) and week 6 (right panel) are expressed as the reciprocal of the serum dilution resulting in 50% inhibition. C, Evaluation of gE-specific IFN-γ and IL-2 responses induced by tgE/$AS01_B$ vaccine or Shingrix at week 2. Numbers of IFN-γ- and IL-2-producing splenocytes were calculated from the ELISPOT assay after restimulation with a pool of gE peptides. D, Evaluation of gE-specific IFN-γ and IL-2 responses induced by tgE/$AS01_B$ vaccine or Shingrix at week 8. Numbers of IFN-γ- and IL-2-producing splenocytes were calculated from the ELISPOT assay after restimulation with a pool of gE peptides. E–G, Flow cytometry assays for gE-specific cytokine-expressing $CD4^+$ and $CD8^+$ cells. Proportion of IFN-γ-producing $CD4^+$ T cells, IL-2-producing $CD4^+$ T cells and IFN-γ-producing $CD8^+$ T cells among splenocytes were determined.

significant difference in terms of neutralizing antibody production between the two groups (Figure 6B).

To verify how immunization affects CMI, gE-specific IFN-γ and IL-2 levels were detected after the first and the second immunizations using the enzyme-linked immunosorbent spot (ELISPOT) assay and intracellular cytokine staining. The tgE/$AS01_B$ immunization induced a poor humoral immune response as compared with a single dose of Shingrix, but showed comparable potency in terms of inducing IFN-γ- and IL-2-producing splenocytes (Figure 6C). Indeed, after the second immunization, a significant number of gE-specific cytokine-producing cells were detected in the

tgE/AS01$_B$ group as compared with the saline control group, which were also comparable to or better than that for the Shingrix group (Figure 6D). Similar results were observed in the flow cytometry analysis, with observations of similar proportions of IFN-γ-expressing CD4$^+$ T cells (Figure 6E), IL-2-expressing CD4$^+$ T cells (Figure 6F) and IFN-γ-CD8$^+$ T cells (Figure 6G) between the tgE/AS01$_B$- and Shingrix-immunized mice, regardless of a single or boost-dose immunization. These results demonstrate that the tgE protein derived from the bacterial expression system can induce both humoral and cellular immune responses when formulated with appropriate adjuvants, and thus may be considered as a candidate antigen for the generation of a vaccine against VZV.

DISCUSSION

The VZV gE is a major target of the humoral and cell-mediated immune response during natural varicella infection and following VZV Oka vaccination. Shingrix (GSK) is a recombinant subunit vaccine comprising a VZV gE expressed in CHO cells and the liposome-based adjuvant AS01$_B$. Other work has sought to investigate subunit vaccines against HZ using gE proteins generated from CHO or insect cells formulated with novel adjuvants (Cao et al., 2021; Lee et al., 2020; Luan et al., 2022a, Luan et al., 2022b; Wang et al., 2021; Wui et al., 2019; Wui et al., 2021). Here, we investigated and showed that the gE protein could be solubly expressed and purified from *E. coli*, and then formulated as a subunit vaccine candidate with an appropriate adjuvant to elicit not only a robust neutralizing titer but also a strong CMI response.

E. coli as an expression system offers several advantages: rapid cell growth rate, inexpensive culture conditions, and an efficient and versatile tool for producing recombinant proteins. Herpes virus-associated antigen research has had several successes using the *E. coli*-based expression system. A neutralizing linear epitope gE (aa 121–135) has been fused with 149 aa of the hepatitis B virus core (HBc) protein to produce chimeric VLPs that could induce VZV-specific neutralizing antibodies in mice (Zhu et al., 2016). In another study, a homologous gE protein from Simian varicella virus (SVV) was expressed in *E. coli* utilizing a GST fusion tag for generating gE antiserum (Gray et al., 2001). As yet, there have been no reports of a VZV gE protein without a fusion tag purified from *E. coli* as a vaccine candidate.

gE consists of three regions: a 544-aa hydrophilic ectodomain with a signal peptide (aa 1–30), a 17-aa hydrophobic transmembrane region, and a 62-aa cytoplasmic tail region. We investigated several candidate molecules with different truncations at the N- or C-terminal regions of the gE ectodomain, and found that the exogenous expression of these proteins in *E. coli* was best using the truncated portion (data not shown). An intact gE ectodomain without a fusion tag tends to form inclusion bodies when expressed by *E. coli* and is difficult to purify. Among the truncation candidates tested, gE (aa 31–358) protein showed high soluble expression with minimal difficulties in its purification, as well as high purity and high homogeneity (Figure 1), Thus, this truncation construct was deemed as an ideal candidate vaccine antigen.

This truncation strategy could have had a potential effect on the properties of the protein, particularly its antigenicity. We had previously expressed recombinant gE in insect cells and purified this construct to immunize BALB/c mice, obtaining 70 gE monoclonal antibodies for epitope analysis (Liu et al., 2015). Through our analysis, we identified aa 1–194 as the immunodominant region (data not shown). Similar results were obtained elsewhere, with gE-specific mAbs pointing to aa 109–123 and 160–316 regions being the regions responsible for antibodies stable antibody binding among variant isolates (Vafai, 1994). In another study, using recombinant hybrid gE fragment-VLPs, the authors identified residues 1–134 as the most antigenic region of gE protein, and showed that the C-terminal of gE (residues 303–623) containing several cysteine residues failed to produce particles in yeast (Fowler et al., 1995), suggesting that heterologous expression of gE should consider not only the antigenicity but also the ease of expression. Here, we optimized the length of the gE protein for its efficient soluble expression with minimal reductions in antigenicity. The tgE showed similar reactivity to most of the antibodies in Figure 3 as did rgE produced in insect cells, suggesting that key epitopes are well preserved in the tgE protein purified from *E. coli*. However, one study showed that the immunodominant CD4$^+$ T cell epitopes recognized by Shingrix vaccine donors were dispersed throughout the gE protein (Voic et al., 2020). The immunogenicity of the tgE in our study was identical to that found for gE derived from CHO cells in Shingrix when antigen combined with AS01$_B$ adjuvant, with similarly efficient humoral responses and CMI responses, as shown in Figure 5. These results indicated that the truncated gE derived from the prokaryotic system was as good as the intact gE ectodomain from the eukaryotic system in terms of antigenicity.

There are considerable differences between gE proteins derived from different expression system. The most prominent differences are noted with posttranslational modifications, particularly glycosylation. O-linked glycans from membrane glycoproteins have relevance in terms of T-cell and antibody recognition of viral glycopeptide epitopes, and this recognition is potential of high biomedical significance in our attempts to mediate immune protection against viruses using subunit vaccines. Removal of the glycans in gE produced in CHO-K1 cells resulted in a 17% reduction in reactivity with VZV-positive sera. In contrast, O-glycosylation

may interfere with the immunoreactivity of B cell epitopes: some important epitopes are blocked by additional O-linked glycans (Nordén et al., 2019). In our study, the tgE generated from *E. coli* seems to behave as well as the insect cell-derived rgE in terms of its immunoreactivity and immunogenicity, suggesting that the "naked" tgE protein has promise as a vaccine candidate. Vaccines with tgE as the antigen are likely to induce not only high IgG titers, indicative of its potential as a subunit varicella vaccine without a potential risk of virus latency, but also high CMI, suggestive of its potential as a zoster vaccine as well. Overall, the gE antigen derived from the bacterial expression system in this study offers a much more economical strategy for the development of VZV vaccines.

METHODS

Cells, viruses, and vaccines

Human acute retinal pigment epithelial cells (ARPE-19) and the cell-free virus used for the neutralization assay were prepared as previously described (Liu et al., 2015). The live-attenuated vOka vaccine (LAV) was produced by Changchun BCHT Biotechnology Co. (Changchun, China). Shingrix was produced by Glaxo Smith Kline (USA).

Construction, expression, and purification of proteins

The DNA fragment encoding for gE (aa 31–358) was inserted into the pTO-T7 vector to construct the recombinant plasmid pTO-T7-tgE. The plasmid was transformed into *E. coli* ER2566 cells (Invitrogen, USA) and grown in LB medium at 37°C to an A_{600} of 0.6, with expression subsequently induced by the addition of a final concentration of 0.4 mmol L^{-1} isopropyl-beta-D-1-thiogalactopyranoside (IPTG) for 10 h at 24°C. Cells were harvested by centrifugation and resuspended with cell lysis solution (20 mmol L^{-1} Tris, pH 8.0). The lysate was sonicated and centrifuged, and the supernatant collected and purified by chromatography using Q-FF sepharose (GE Healthcare, USA), CHT-II resin (Bio-Rad, USA) and Butyl sepharose (GE Healthcare). After purification, the concentration of the tgE with high purity was determined using the BCA method. Expression and purification of the rgE from insect cells were carried out as described previously (Liu et al., 2015).

SDS-PAGE and immunoblotting analyses

Samples were obtained during the purification process and the final purified protein samples were subjected to 10% SDS-PAGE. Protein expression was visualized using Coomassie Blue staining, or through immunoblotting using primary mAb 1B11 and an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody.

Analytical ultracentrifugation

The sedimentation coefficient of the purified proteins was estimated by sedimentation velocity using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An60-Ti rotor (Beckman Coulter, USA). Protein samples were prepared at 0.8 mg mL^{-1} with the rotor speed set to 30,000 r min^{-1} (~65,520×*g*). The sedimentation coefficient was calculated using the c(s) method with the Sedifit software, kindly provided by Dr. P. Schuck (NIH, USA). The c(s) was used to estimate protein molar mass (MW).

High-performance size-exclusion chromatography

The distribution of the molecular size of the purified tgE proteins was investigated using ultra-high-performance liquid chromatography (UPLC). Purified proteins were loaded onto TSK Gel G5000PW_{XL} 7.8 mm×300 mm columns (TOSOH, Japan) equilibrated with corresponding sample buffers and subjected to UPLC (Waters, USA). The system flow rate was maintained at 0.5 mL min^{-1} and protein signal was detected at 280 nm.

Thermal stability and differential scanning calorimetry

Protein thermal stability was estimated by DSC using a MicroCal VP-DSC instrument (GE Healthcare). Data were analyzed by MicroCal Origin 7.0 software, baseline subtracted, and fitted with a non-two-state model to yield the Tm.

ELISA

ELISA was performed to determine the antigenicity of gE proteins or sera antibody binding titers. The wells of 96-well microplates were coated with tgE or rgE proteins (100 ng per well in 20 mmol L^{-1} Tris, pH 8.0) at 25°C for 2 h and then blocked with 2% bovine serum albumin (BSA) in PBS overnight at 4°C. The wells were then incubated with 2-fold serial dilutions of the antibody (100 ng in the first well) or serum (the first dilution was 100-fold or higher) for 30 min at 37°C. After 5 washes with PBST, the plates were incubated with HRP-conjugated goat anti-mouse secondary antibody (Abcam, UK) at a 1:5,000 dilution for 30 min at 37°C. The plates were washed five times, and then incubated with 100 μL tetramethylbenzidine (TMB) substrate (Beijing Wantai Biological Medicine Co., Beijing, China) for color development at 37°C for 10 min, followed by the addition of 50 μL/well of 2 mol L^{-1} H_2SO_4 to stop the reaction. Finally, the absorbance was measured at 450 nm.

The EC_{50} is defined as the antibody concentration that

achieves 50% binding with the antigen. The binding titers were defined as the highest serum dilutions that resulted in an absorbance value larger than the cut-off value, which was calculated as the mean of the control A_{450} value plus three times the standard deviation (SD) of the pre-immunization serum samples. Data were analyzed using GraphPad Prism (version 8.0).

Vaccine formulation

Aluminum hydroxyphosphate-based adjuvant (Al-001) was produced in-house in this study. In the final formulation, each antigen was diluted to an appropriate concentration and formulated with various adjuvants. The alum content in the final formulations for the Al-001 groups was 42 μg per dose for immunogenicity evaluations (100 μL per mouse) and 420 μg per dose for ED_{50} assays (1 mL per mouse). The formulated vaccine bulk was stored at 4°C throughout. For Freund's adjuvant formulation, complete Freund's adjuvant (Sigma-Aldrich, USA) (the initial injection) or incomplete Freund's adjuvant (Sigma-Aldrich) (the boost injection) were mixed with equal volume of antigens according to the manufacturer's instruction. Lyophilized LAV vaccine was resolved and diluted using saline before immunization. The vaccine formulations containing $AS01_B$ were used as described by the Shingrix guidelines.

Mice immunization

The experimental protocols were approved by the Xiamen University Laboratory Animal Management Ethics Committee. Female, 6-week-old BALB/c and C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. All animal procedures were approved by the Xiamen University Laboratory Animal Management Ethics Committee. All manipulations were strictly conducted in compliance with animal ethics guidelines and approved protocols.

To estimate the immunogenicity of tgE or rgE, 6-week-old female BALB/c mice were randomly divided into groups (n=5) and immunized intramuscularly at weeks 0, 2, and 4 with tgE or rgE diluted in different adjuvants (100 μL per mouse). The sera were harvested and tgE and rgE were detected using gE-specific antibodies. Mice were sacrificed at the end of the experiment to recover spleens for ELISPOT or ICS measurements.

For ED_{50} calculation, 4-week-old female BALB/c mice (n=6 per group) were vaccinated by intraperitoneal injection with a single dose of tgE in aluminum adjuvant (2-fold serially diluted from 1 to 0.03125 μg mL^{-1}) or vOka in saline (1,000 to 62.5 pfu) (1 mL per mouse). Mice in the control group were immunized with saline only. Serum samples were collected at 4 weeks after immunization, and seroconversion was determined by ELISA using a VZV IgG ELISA kit (Beijing Wantai Biological Medicine Co.). The ED_{50} results were calculated according to the dose-response curve using a Reed-Muench model.

To compare the immunogenicity of tgE and Shingrix, 6-week-old female C57BL/6 mice were randomly divided into groups (n=12) and immunized at weeks 0 and 4 with an intramuscular injection into the tibialis muscle with lyophilized tgE diluted in $AS01_B$ adjuvant or Shingrix (50 μL per mouse). Mice immunized with saline served as a control. Sera were harvested at weeks 0, 2, 4, 6, and 8; and expression was measured using gE-specific antibodies. Four or eight mice were sacrificed at weeks 2 or 8 respectively to recover spleens for ELISPOT or ICS measurements.

In vitro neutralization assay

A neutralization assay was performed as described previously (Zhu et al., 2016). Briefly, inactivated serum samples were serially diluted 2-fold with protection buffer (9 % sucrose, 25 mmol L^{-1} histidine, 150 mmol L^{-1} NaCl, pH 7.35), and then incubated with 100 pfu of cell-free virus in protection buffer with complement (1:10, v/v) at 37°C for 1 h. The mixture was added to ARPE-19 cells pre-seeded into 24-well plates, and incubated at 37°C with 5% CO_2. After 1 h, the media was replaced with fresh media. Cell-free virus (virus only) in suspension was used as a negative control, and mock-infected ARPE-19 cells were used as a positive control. Three days post-infection, an ELISPOT assay was performed, as previously described (Chen et al., 2014). Neutralization titers were determined as the highest serum dilutions that could neutralize half of the virus.

CMI measurement assays

CMI response was measured using ELISPOT or flow cytometry assays with ICS.

For ELISPOT, spleens were collected from immunized mice, and single-cell suspensions were prepared. Splenocytes (5×10^5 cells per well) were seeded into the wells of anti-mouse IFN-γ or IL-2 antibody precoated ELISPOT plates (Dakewe Biotech, Shenzhen, China). Cells were then incubated for 20 h at 37°C and 5% CO_2 with a pool of overlapping 15-mer peptides (1.25 μg mL^{-1}) with an 11-aa overlap that covered the entire sequence of gE (aa 22–537). The detection procedure was conducted according to the manufacturer's instructions. Spots were counted and analyzed by using CTL-ImmunoSpot S5 (Cellular Technology Limited, USA).

For the flow cytometry analysis, splenocytes (2×10^6 cells per well) were seeded into the wells of a 96-well culture plate and stimulated with a pool of gE peptide pool (1.25 μg mL^{-1}) for 18 h at 37°C and 5% CO_2. Splenocytes were then

incubated for a further 6 h in the presence of BD GolgiPlug Protein Transport Inhibitor. After washing with PBS containing 2% fetal bovine serum (FBS), cells were resuspended in a mixture of FITC Rat Anti-Mouse CD4 and PE-Cy7 Rat Anti-Mouse CD8a antibodies (BioLegend, USA) and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen), followed by fixed and permeated step using Fixation/Permeabilization Solution Kit, and further resuspended in a mixture of APC Rat Anti-Mouse IFN-γ and PE Rat Anti-mouse IL-2 antibodies (BD, USA). Finally, the samples were measured using a BD LSRFortessa X-20 Flow Cytometer (BD), with the data analyzed by FlowJo V10.6.0 software.

Statistical analysis

GraphPad Prism (version 8.0) was used to analyze the ELISA data (EC_{50} calculations) and to perform the statistical analysis. A normality test for the data distribution was performed using the Shapiro-Wilk normality test and Kolmogorov-Smirnov test. Based on whether the data showed a normal distribution or not, unpaired *t*-test or Mann-Whitney test, respectively, was used to analyze differences between the two groups. Tukey's test, Brown-Forsythe, and Welch ANOVA tests, or Kruskal-Wallis test with Dunn's method were applied to analyze differences among more than two groups. *P* values in each group are indicated as * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$; "ns" indicates not significant.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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