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IFN-α armed gE elicits superior immunogenicity compared to unmodified antigens and flagellin armed gE in mice

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ABSTRACT

Herpes zoster (HZ) induces significant pain and discomfort, which can seriously affect the quality of life of patients. At present, there is no specific treatment for HZ, and the mosteffective HZ control is vaccination. The main obstacle to developing an effective HZ vaccine is poorly induced cellular immune response. In this study, the IFN-α–gE–Fc fusion protein induced higher levels of humoral and cellular immunity compared to the unengineered gE antigen and higher levels of cellular immunity compared to the flagellin–gE–Fc fusion protein in a murine model. Compared with the marketed recombinant herpes zoster vaccine (Shingrix), IFN-α–gE–Fc can replace current used MPL adjuvant. At the same time, the immunogenicity of the IFN-α–gE–Fc + AQ was not weaker than that of the marketed recombinant zoster vaccine. The novel fusion protein provides a candidate entity for the development of a safe and effective novel HZ vaccine.

Introduction

Varicella zoster virus (VZV) infection can induce two different clinical characteristics. The main clinical symptom of VZV infection is chickenpox at the initial infection, and the virus remains latent in the spinal posterior root ganglion or cerebral nerve sensory ganglion for a long time after recovery [1], while the potential VZV reappears in the form of Herpes zoster (HZ) when the body's immunity is weakened [2]. A previous study has shown that the immune response induced by gE antigen can protect animals from virus attack [3]. In addition, the VZV gE monoclonal antibody can mediate antibody-dependent cytotoxicity and neutralize virus infectivity [4]. The current challenge is that gE can only induce low levels of humoral immunity, which cannot meet the requirements of vaccine use. Shingrix®, which is currently on the market, has strong side effects and high prices.

Interferon-α (IFN-α) can stimulate the body to resist viruses. In addition, it can induce the differentiation and activation of human dendritic cells (DCs), promote the antigen presentation ability of DCs, and trigger the activation of T cells [5,6]. Recently, several studies have demonstrated that IFN-α can be used as an immune adjuvant. Fc segment of immunoglobulin can enhance antigen immune presentation and induce antigen-specific immune response [7].

In this study, we evaluated a novel herpes zoster vaccine IFN-α–gE–Fc by expressing outer membrane region of glycoprotein E (gE), fused with interferon-α (IFN-α) and Fc segment of human immunoglobulin (IgG). Then the immunological enhancement effects of IFN-α and flagellin were also compared.

Materials and methods

Ethics statement

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of CanSino Biologics.

Evaluation of the immunization protocol

Female C57BL/6 mice were immunized intramuscularly with gE, IFN-α–gE–Fc, flagellin–gE–Fc, Shingrix, gE + AQ, flagellin–gE–Fc + AQ or IFN-α–gE–Fc + AQ, respectively, and boosted with the same dose 28 days after the initial immunization. All tested vaccines contains 5 μg gE protein. Sodium chloride injection was used as a negative control. Shingrix injection was used as a positive control. Serum was collected on day 28 after the initial immunization and day 14 after the second immunization to determine the levels of gE-specific IgG by enzyme linked

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immunosorbent assay (ELISA). We also evaluated the gE-specific $CD4^+$ and $CD8^+$ T cell responses in spleen cells of the vaccinated animals at 14 days after the second immunization with ICS. The vaccination protocol is described in Fig. 1. The relevant research information of immune dose is shown in Table 1.

Enzyme linked immunosorbent assay (ELISA)

In brief, 96-well plates were coated with 100 μL/well gE (2 μg/mL) overnight at 4 °C. Plates were washed with PBS containing 0.05 % Tween 20 (PBST) and blocked with blocking buffer (PBST containing 1 % bovine serum albumin) on the next day. Immunized animal serum samples were serially diluted and added to the wells, followed by incubation at 37 °C for 1 h. Next, the plates were washed three times with PBST and incubated with goat anti-mouse IgG-horseradish peroxidase (HRP, 1:8000, ZSGB-Bio) at 37 °C for 1 h. Plates were washed six times with PBST, and the HRP substrate TMB was added. The reactions were stopped by 2 M sulfuric acid. A microplate reader (BioTek, Vermont, USA) was used to detect the absorbance value at the wavelength of 450 nm.

Intracellular cytokine staining (ICS)

The ICS assay was performed to evaluate the cytokine expression in antigen-specific T cells according to the reference's protocol [8]. Briefly, Mouse splenocytes were seeded and stimulated by gE peptide library. Then, the cells were harvested and stained with anti-CD3 (Biolegend), anti-CD4 (Biolegend), anti-CD8 (Biolegend), and the LIVE/DEADTM dyes. Subsequently, the cells were fixed and permeated with the fixation/permeation kit (BD Bioscience). Finally, the cells were performed with intracellular staining for TNFα (MP6XT22), IFNγ (XMG1.2), IL-2 (JES6-5H4), IL-4(11B11), IL-5(2E3). All data were acquired using a FACS Fortessa flow cytometer.

Results

Screening of expression vectors

pcDNA3.1-gE-Fc, pCEP4-gE-Fc, pEE12.4-gE-Fc, and pCHO1.0-gE-Fc were expressed individually in CHOS cells and the produced fusion proteins were purified by protein A affinity chromatography. The expression of the vectors pcDNA3.1, pCEP4, pEE12.4 and pCHO1.0 for the fusion protein was detected by the lowry method. 8 % native polyacrylamide gel electrophoresis was used to analyze the dimer formation ability of the expression vectors. The dimerization capability of gE-Fc produced from different expression vectors are shown in Fig. 2. The expression of gE–Fc and the proportion of dimers are shown in Table 2.

The results showed that the expression efficiency of the gE–Fc fusion protein by using expression vectors pcDNA3.1, pCEP4, pEE12.4, and pCHO1.0 was 40.0 mg/L, 81.7 mg/L, 7.3 mg/L, and 15.2 mg/L, respectively, whereas the proportion of dimers was 44.3 %, 85.6 %, 10.1 %, and 2.5 %, respectively. The vector pCEP4 could provide the highest expression of the gE–Fc fusion protein and the highest proportion of dimer formation. Therefore, pCEP4 expression vector was selected for further study.

Table 1
Dosage information for immunogenicity studies.

Vaccine	Containing gE protein	QS21	MPL
gE	5 μg/dose	/	/
flagellin–gE–Fc	5 μg/dose	/	/
IFN-α–gE–Fc	5 μg/dose	/	/
Shingrix	5 μg/dose	5 μg/dose	5 μg/dose
gE + AQ	5 μg/dose	5 μg/dose	/
flagellin–gE–Fc + AQ	5 μg/dose	5 μg/dose	/
IFN-α–gE–Fc + AQ	5 μg/dose	5 μg/dose	/

Linker peptides screening

pCEP4-gE-$(G_4S)_3$-Fc, pCEP4-gE-$(EAAAK)_3$-Fc, pCEP4-gE-$(AP)_8$-Fc, and pCEP4-gE-scFv-Fc were expressed in CHOS cells and fusion proteins were fusion proteins were purified by protein A affinity chromatography. The expression levels of the fusion proteins with the linker peptides $(G_4S)_3$, $(EAAAK)_3$, $(AP)_8$, and scFv was detected by the lowry method. 8 % native polyacrylamide gel electrophoresis and gel imaging analysis software were used to examine the dimer formation ability of the linkers and the fusion proteins. The dimerization capability of gE-Fc produced from different expression vectors are shown in Fig. 3. The expression of gE–Fc and the proportion of dimers are shown in Table 3.

The results showed that the expression levels of the fusion proteins with the linker peptides $(G_4S)_3$, $(EAAAK)_3$, $(AP)_8$, and scFv were 81.7 mg/L, 161.0 mg/L, 12.5 mg/L, and 81.3 mg/L, respectively. The proportion of dimers was 85.6 %, 56.3 %, 61.7 %, and 21.3 %, respectively. The linker peptide $(G_4S)_3$ showed the highest proportion of gE–Fc dimer formation, whereas the linker peptide $(EAAAK)_3$ showed the highest expression level of gE–Fc. Therefore, the linker peptide $(G_4S)_3$ and $(EAAAK)_3$ was selected for further study.

Evaluation of the immunogenicity of fusion proteins

The expression levels of IFN-α–gE–Fc and flagellin–gE–Fc were 54.0 mg/L and 28.3 mg/L, and the proportion of dimer was 72.8 % and 72.2 %.

Binding antibody responses

The titers of the anti-gE IgG antibodies in serum were assessed at day28 post prime immunization (D28) and day14 post booster vaccination (D42) (Fig. 4). After prime immunization (D28), the gE specific IgG response in each group could be stimulated and detected. The elicited IgG titers of IFN-α–gE–Fc and flagellin–gE–Fc groups were significantly higher than unmodified gE group ($P < 0.0001$). The IgG responses of IFN-α–gE–Fc group showed a similar level as that in flagellin–gE–Fc group. The IgG response level of IFN-α-gE-Fc + AQ group was significantly higher than that in Shingrix group ($P < 0.005$).

After booster vaccination (D42), the elicited IgG titers of IFN-α–gE–Fc and flagellin–gE–Fc groups were intensively increased. The IgG responses of IFN-α–gE–Fc + AQ group showed a similar level as that in flagellin–gE–Fc group. After booster vaccination (D42), the elicited IgG

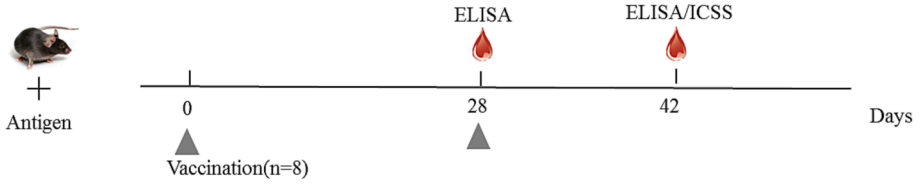

Fig. 1. Schematic diagram of the immunization protocol. Female C57BL/6 mice aged 6–8 weeks (n = 8) were vaccinated with gE, IFN-α–gE–Fc, flagellin–gE–Fc, Shingrix, gE + AQ, flagellin–gE–Fc + AQ or IFN-α–gE–Fc + AQ, respectively via intramuscular injection on day 0 and day 28. The red blood drop symbols indicate sampling time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

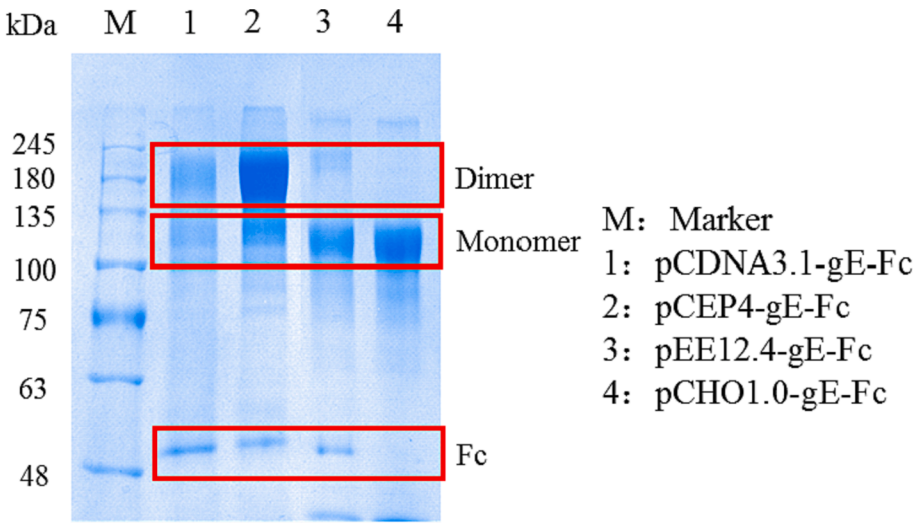

Fig. 2. PAGE analysis of gE-Fc (expression vector screening).

Table 2
Expression of gE–Fc and dimer proportion (expression vector screening).

Expression vector	pcDNA3.1	pCEP4	pEE12.4	pCHO1.0
Expression level (mg/L)	40.0	81.7	7.3	15.2
Dimer proportion (%)	44.3	85.6	10.1	2.5

titers of IFN-α–gE–Fc and flagellin–gE–Fc groups were intensively increased. The IgG responses of IFN-α–gE–Fc + AQ group showed a similar level as that in Shingrix group.

$CD4^+$ and $CD8^+$ T cell responses

We performed gE-specific $CD4^+$ T and $CD8^+$ T cell evaluations in splenocytes at day 14 post booster vaccination (D42). ICS indicated that IFN-α–gE–Fc and IFN-α–gE–Fc + AQ elicited a high frequency of $CD4^+$ T cells and strongly induced the expression of IFNγ, TNFα, IL-2, IL-4 and IL-5 in $CD4^+$ T cells (Fig. 5). Similar results were observed in $CD8^+$ T cell responses (Fig. 6). These results reveal that IFN-α–gE–Fc can stimulate a higher cellular immune response when compared with flagellin–gE–Fc. The cellular immune response of the IFN-α–gE–Fc + AQ group was not weaker than that of the Shingrix group.

Discussion

It has been reported that expression vectors could affect the expression levels of recombinant proteins [9,10]. In this study, we evaluated the expression capability of four commercial mammalian expression vectors, namely pcDNA3.1, pCEP4, pEE12.4, and pCHO1.0, on the expression level of Fc–gE fusion protein. Interestingly, we found that these four vectors indeed performed differently on the expression level as well as the dimer formation when expressing the same recombinant protein in CHOS (Table 2). The expression vector pCEP4 showed the highest expression of the target protein and the highest proportion of dimer formation. Therefore, pCEP4 was selected as the expression vector for the preparation of fusion protein.

Fusion proteins, however, as non-native proteins, may have folding defects due to structural perturbations of different regions [11,12]. To

Table 3
Expression of gE–Fc and dimer proportion (linker peptide screening).

Linker peptide	$(G_4S)_3$	$(EAAAK)_3$	$(AP)_8$	scFv
Expression level (mg/L)	81.7	161.0	12.5	81.3
Dimer proportion (%)	85.6	56.3	61.7	21.3

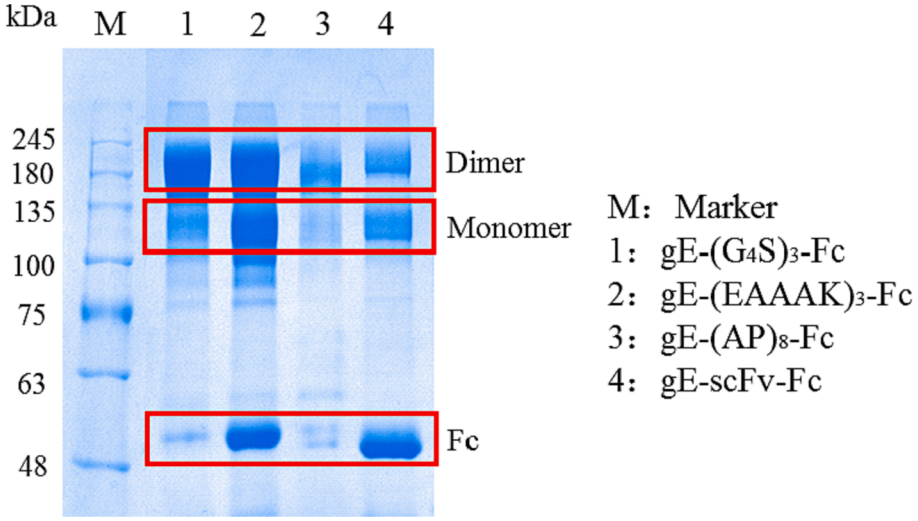

Fig. 3. PAGE analysis of gE-Fc (linker peptide screening).

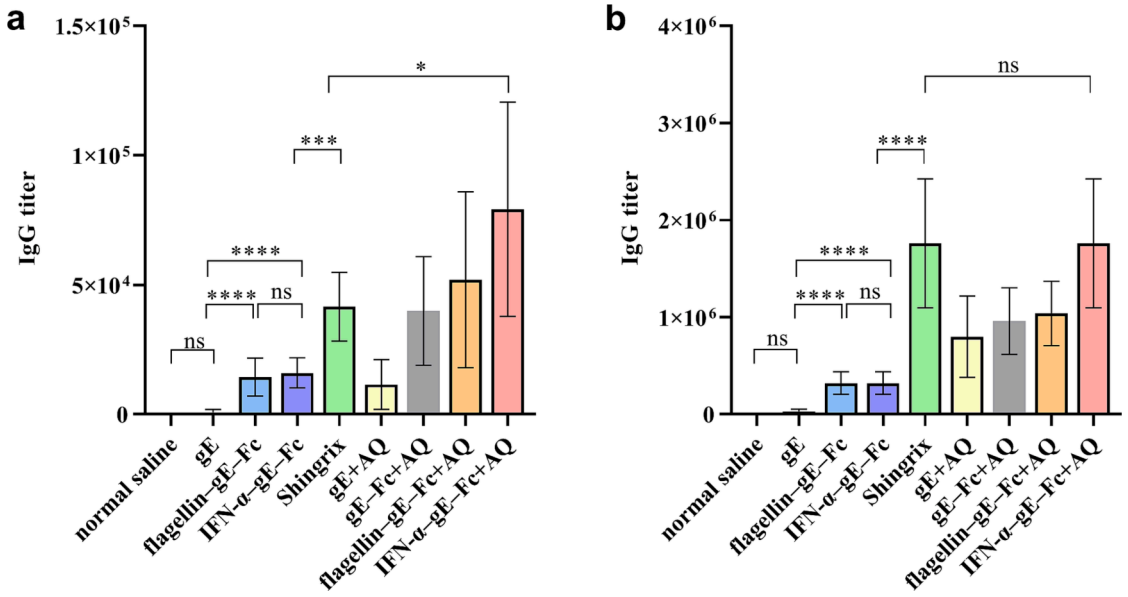

Fig. 4. Assessment of gE-specific binding antibody responses. The gE-specific IgG antibody in serum on D28 (a) and D42 (b) was analyzed by ELISA. Data are shown as mean ± SEM. P-values were calculated by one-way ANOVA with multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: P > 0.05.

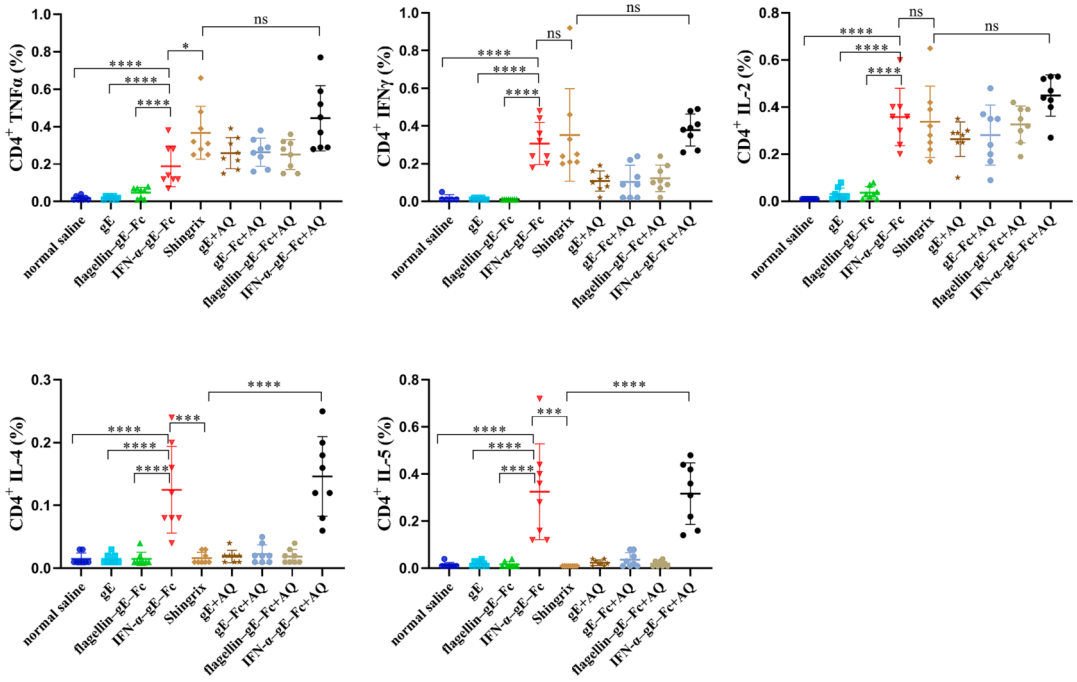

Fig. 5. Assessment of gE-specific $CD4^+$ T cells responses (D42). The percentages of TNFα, IFNγ, IL-2, IL-4, IL-5 $CD4^+$ T cells were determined by intracellular cytokine staining. Data are shown as mean ± SEM. P-values were calculated by one-way ANOVA with multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001****P < 0.0001, ns: P > 0.05.

mitigate this interference, we have inserted linkers between the portions of the fusion protein so that they were separated spatially. We evaluated the effects of different linker peptides, namely $(G_4S)_3$, $(EAAAK)_3$, $(AP)_8$, and scFv on the dimerization of Fc–gE fusion protein. Our results (Table 3) also showed that the proportion of the fusion protein dimer formed by the ligating peptide $(G_4S)_3$ was much higher than that of the fusion proteins $(EAAAK)_3$, $(AP)_8$, and scFv, further confirming that $(G_4S)_3$ interfered with protein domain folding and was conducive to fusion protein dimer formation. The expression of junction peptide $(EAAAK)_3$ was one-fold higher than that of $(G_4S)_3$, $(AP)_8$, and scFv. This indicated that the junction peptide $(EAAAK)_3$ could up-regulate the expression of the fusion protein. As Fc is a key component of dimer formation, the Fc segment of human immunoglobulin (IgG) was fused to the C-terminus of the outer gE membrane segment by the linker peptide $(G_4S)_3$. Human IFN-α was fused to the N-terminus of the gE outer membrane by the ligating peptide $(EAAAK)_3$ to increase the expression of IFN-α–gE–Fc.

Both IFN-α and flagellin have been used as immune modulators [13,14]. We evaluated the immunogenicity of IFN-α–gE–Fc and flagellin–gE–Fc as potential HZ vaccines in the murine model. We found that there was no significant difference for stimulating humoral immune response after immunizing mice with equimolar amounts of IFN-

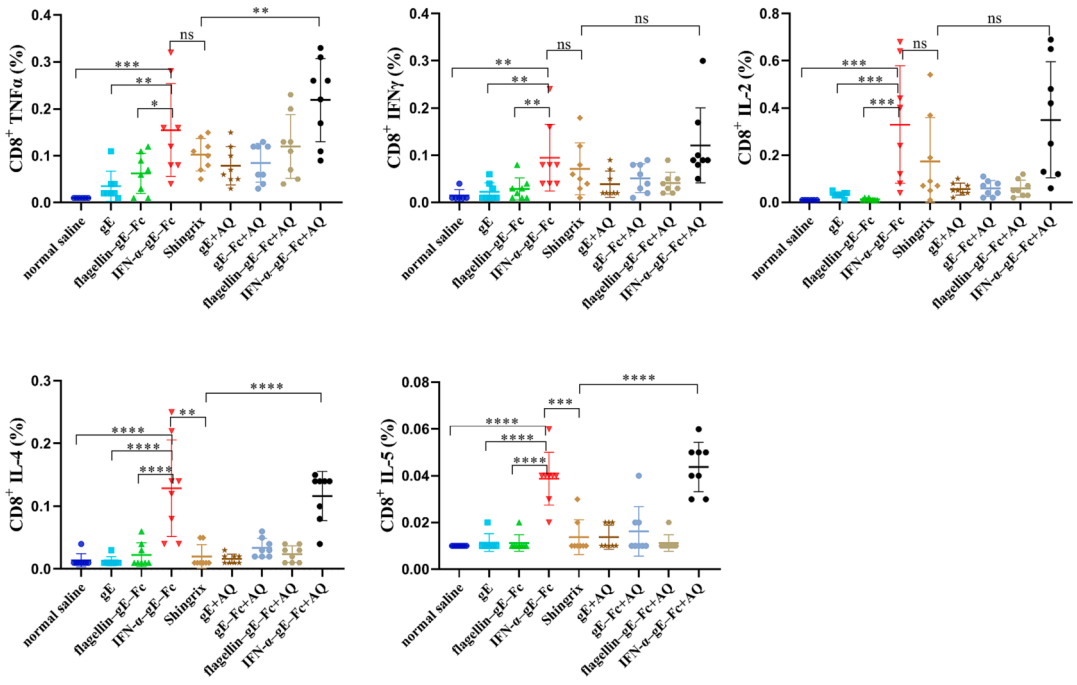

Fig. 6. Assessment of gE-specific $CD8^+$ T cells responses (D42). The percentages of TNFα, IFNγ, IL-2, IL-4, IL-5 $CD8^+$ T cells were determined by intracellular cytokine staining. Data are shown as mean ± SEM. P-values were calculated by one-way ANOVA with multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001****P < 0.0001, ns: P > 0.05.

α–gE–Fc or flagellin–gE–Fc, and both candidates could induced potent gE specific antibody response. Regarding the enhancement of the cellular immunity mediated by T cells. IFN-α–gE–Fc was significantly superior to flagellin–gE–Fc on higher expression levels of TNFα, IFNγ, IL-2, IL-4, and IL-5 in $CD4^+$ and $CD8^+$ T cell. As both $CD4^+$ and $CD8^+$ T cell responses appear to be required for the prevention of HZ infection [15], the fusion protein IFN-α–gE–Fc is a more suitable candidate for the HZ vaccine for a further investigation.

Compared with the marketed recombinant herpes zoster vaccine (Shingrix), IFN-α–gE–Fc can replace current used MPL adjuvant. At the same time, the immunogenicity of the IFN-α–gE–Fc + AQ was not weaker than that of the marketed recombinant zoster vaccine.

CRediT authorship contribution statement

Jiangang Zhang: Writing – original draft. **Shaodan Peng:** Data curation. **Fang Xu:** Writing – review & editing. **Ying Qiao:** Data curation. **Xiaoke Ye:** Methodology. **Yu Guan:** Methodology. **Xiaolong Zhao:** Methodology. **Yueran Wang:** Project administration. **Zhongqi Shao:** Conceptualization. **Tao Zhu:** Conceptualization. **Weixue Si:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2024.100432.

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