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Conjugation with 8-arm PEG and CRM_{197} enhances the immunogenicity of SARS-CoV-2 ORF8 protein

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ABSTRACT

Safe and effective vaccines are urgently needed to combat the COVID-19 pandemic. However, the SARS-CoV-2 variants raise concerns about the effectiveness of vaccines. As a SARS-CoV-2 antigen target, ORF8 strongly inhibits the IFN-β and NF-κB-responsive promoter, and can be potentially used for the development of SARS-CoV-2 vaccine. However, it is necessary to improve the immunogenicity of ORF8 by adjuvants or delivery systems. CRM_{197} was a carrier protein with the ability to activate T helper cells for antigens. Eight-arm PEG could conjugate multiple antigen molecules in one entity with inherent adjuvant effect. In the present study, ORF8 was conjugated with CRM_{197} and 8-arm PEG, respectively. The cellular and humoral immune responses to the conjugates (ORF8-CRM and ORF8-PEG) were evaluated in the BALB/c mice. As compared with ORF8-CRM and ORF8 administrated with aluminum adjuvant (ORF8/AL), ORF8-PEG induced a higher ORF8-specific IgG titer (2.6×10^4), higher levels of cytokines (IFN-γ, TNF-α, IFN-β, and IL-5), stronger splenocyte proliferation. Thus, conjugation with 8-arm PEG was an effective method to improve the immune response to ORF8. Moreover, ORF8-PEG did not lead to apparent toxicity to the cardiac, liver and renal functions. ORF8-PEG was expected to act as an effective vaccine to provide the immune protection against SARS-CoV-2.

1. Introduction

SARS-CoV-2 is highly pathogenic in human, which leads to the COVID-19 pandemic and poses immeasurable public health challenges to the world [1–3]. The infection of SARS-CoV-2 exhibits a diverse clinical presentation, ranging from mild cases to a respiratory distress syndrome with high morbidity and mortality [4–5]. The importance of effective vaccines is highlighted to control the COVID-19 pandemic. The effectiveness of vaccines based on spike protein or receptor binding domain (RBD) has been established in clinical trials [6]. Recently, several vaccines have been authorized for emergent use against SARS-CoV-2, based on the inactivated viruses, recombinant viral vectors, mRNA, and recombinant proteins [7–9]. However, the emergence of SARS-CoV-2 variants such as Delta and Omicron strains significantly lowered the effectiveness of vaccines [10].

ORF8 is a rapidly evolving accessory protein that has been proposed to interfere with immune responses [11]. ORF8 showed strong inhibition on IFN-β and NF-κB-responsive promoter [12]. ORF8 disrupts antigen presentation and reduces the recognition and the elimination of virus-infected cells by cytotoxic T lymphocytes (CTLs) [13]. Thus, ORF8 is a potential antigen target for the vaccine development and a supplement antigen to S protein, due to its lower mutation rate than S protein [14–16].

However, ORF8 lacks the pathogen-associated molecular patterns and displays low immunogenicity, which necessitates the adjuvants or the delivery system to stimulate the immune system. Alum salts, MF59, AS03, AS04, AF03 and QS-21 have been approved as vaccine adjuvants for clinical use, which have been used for SARS-CoV-2 vaccines [17–18]. However, these adjuvants may lead to the undesirable effects such as inflammation, toxicity, allergy, and immunosuppression [19].

Antigen delivery system with the inherent adjuvant activity is highly desirable for development of SARS-CoV-2 vaccines [20]. In particular, covalent conjugation of a carrier protein with antigens or self-conjugation of antigens is a potent antigen delivery strategy [21–22]. CRM_{197} is a carrier protein possessing T-helper cell epitopes and can activate T helper cells for antigens [23]. Recently, CRM_{197} has been covalently conjugated with bacterial capsular polysaccharide to enhance the activation of B and T cells [24]. Eight-arm polyethylene glycol (PEG) could covalently conjugate multiple antigen molecules in one entity and improve the immunogenicity of antigens [25–26].

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In the present study, SARS-CoV-2 ORF8 was expressed by *E.coli*, denatured and purified with high purity. ORF8 was covalently conjugated with CRM_{197} and self-conjugated with 8-arm PEG to improve the immunogenicity of ORF8, respectively. ORF8 administrated with aluminum adjuvant acted as the control. The cellular and humoral immune responses to the conjugates (ORF8-CRM and ORF8-PEG) were evaluated in the BALB/c mice. The potential toxicity of the conjugates to the cardiac, liver and renal functions was measured. ORF8-CRM and ORF8-PEG were expected to act as an effective vaccine to provide the immune protection against SARS-CoV-2.

2. Materials and methods

2.1. Materials

Eight-arm PEG N-hydroxysuccinimide ester with an Mw of 10 kDa (8-arm PEG), 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS), iminothiolane hydrochloride (IT), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (USA). CRM_{197} was kindly provided by Hualan Biological Engineering, Inc. (China). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (USA). Horseradish peroxidase-conjugated goat anti-mouse IgG Fc antibody (HRP-IgG), IgG1 Fc antibody (HRP-IgG1), IgG2a Fc antibody (HRP-IgG2a) and IgM Fc antibody (HRP-IgM) were ordered from Abcam (USA). IFN-γ and IFN-β ELISA kits were ordered from Legend (USA). TNF-α and IL-5 ELISA kits were ordered from Invitrogen (USA). All other reagents were of analytical grade.

2.2. Construction of ORF8 expression vector

The coding gene of SARS-CoV-2 ORF8 (Amino acid 27894–28259, GenBank Acc. No. NC_045512) was amplified by PCR. The PCR product was then cloned into a *p*ET-28a(+) vector through restriction enzymes BamH1 and XhoI. A start codon and an N-terminal *hexa*-histidine tag were added to the vector. The plasmid consisting of *p*ET-28a(+)-His6-ORF8 was transformed into *E. coli* host TOP10. Restriction digestion and DNA sequence analysis were used to confirm the correct DNA sequences. The resultant plasmid construct was isolated and retransformed into *E. coli* host BL21 (DE3) as an expression strain for ORF8.

2.3. Expression, purification and renaturation of ORF8

The strain (20 mL) was added in 1 L LB medium containing 100 μg/ml kalamycin and cultured for overnight with vigorous shaking at 37 °C. When the strain density was in the range of 0.6–0.8 at 600 nm, IPTG was added at a final concentration of 0.6 mM for incubation at 16℃ for 20 h. The cells were harvested and sonicated in an ice bath.

The inclusion bodies were solubilized by 50 mM Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 10 mM reduced glutathione (buffer A). Then, the sample was loaded on a Ni Sepharose HP column (0.5 cm × 5 cm, GE Healthcare, USA). The column was eluted with 50 mL buffer A and then with 50 mL buffer A containing 0.5 M imidazole. The fractions containing ORF8 were pooled and ORF8 was in denatured state. In order to renature ORF8, the pooled solution was added dropwise to a 50-fold excess of 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione and 0.5 M urea at 4 °C. Finally, the renatured ORF8 was dialyzed against PBS buffer (pH 7.4).

2.4. Preparation of ORF8-CRM and ORF8-PEG

ORF8 (2 mg) was incubated with 80-fold molar excess of IT to introduce the thiol groups in PBS buffer (pH 7.4) at 4 °C for 8 h (Fig. 1). CRM_{197} (2 mg) was incubated with 80-fold molar excess of EMCS to introduce the maleimide groups in PBS buffer (pH 7.4) at 4 °C for 6 h (Fig. 1). The free IT and EMCS were removed by extensive dialysis against PBS buffer (pH 7.4). Then, the ORF8-CRM_{197} conjugate (ORF8-CRM) was obtained by incubating the thiol groups of ORF8 and the maleimide groups CRM_{197} at 4 °C for overnight.

ORF8 (2 mg) was reacted with 10-fold molar excess of 8-arm PEG in PBS buffer (pH 7.4) at 4 °C for overnight to obtain the conjugate (ORF8-PEG).

2.5. Purification of ORF8-CRM and ORF8-PEG

ORF8-CRM and ORF8-PEG were purified by a Superdex 200 column (1.6 cm × 60 cm, GE Healthcare, USA), based on size exclusion chromatography (SEC). The column was equilibrated and eluted by PBS buffer (pH 7.4) at a flow rate of 2.0 mL/min. The effluent was detected at

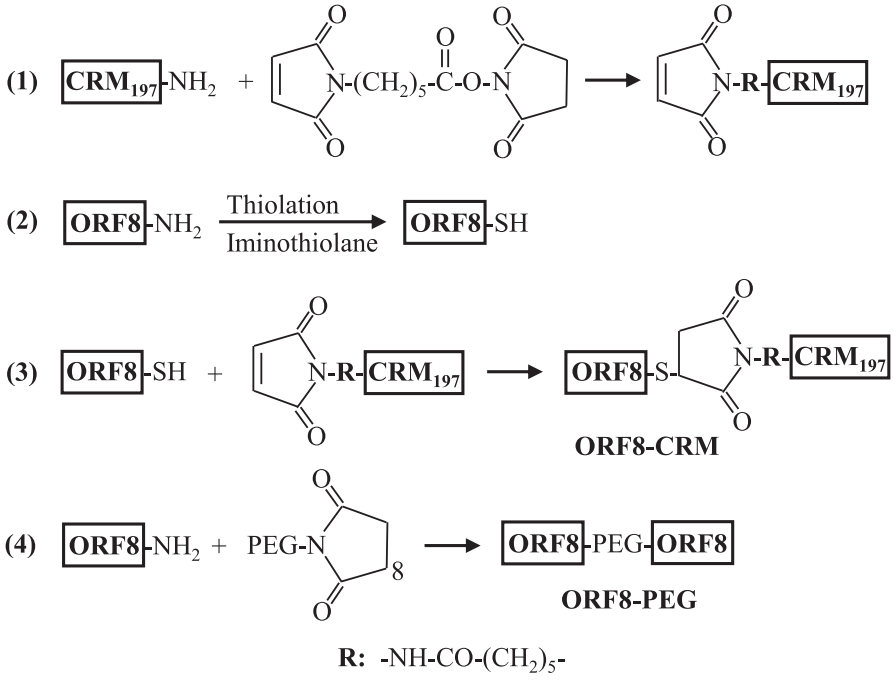

Fig. 1. Preparation scheme of ORF8-CRM and ORF8-PEG.

280 nm. The fractions corresponding to ORF8-CRM and ORF8-PEG were pooled and concentrated by Amicon (Millipore) with 10 kDa cutoff membrane at 4 °C.

2.6. SDS-PAGE analysis

SDS-PAGE was used to analyze the samples. A 12% polyacrylamide gel was utilized under a reducing (5% v/v β-mercaptoethanol) condition. The gel was stained with Coomassie blue R-250.

2.7. Size exclusion chromatography

The samples were analyzed by size exclusion chromatography (SEC), using an analytical Superdex 200 column (1 cm × 30 cm, GE Healthcare, USA). The column was equilibrated and eluted by PBS buffer (pH 7.4) at a constant flow rate of 0.5 mL/min. The effluent was detected at 280 nm.

2.8. Animals

Female BALB/c mice aged six to eight weeks were maintained in the pathogen-free conditions. All procedures of the animal experiments were approved by the Animal Ethical Experimentation Committee of Institute of Process Engineering, Chinese Academy of Sciences (Beijing, China, Permission no. SYXK2019-0004), according to the requirements of the National Act on the Use of Experimental Animals (China).

2.9. Vaccination procedures

Thirty mice were randomly divided into five groups of six mice each. The groups were the PBS, ORF8, ORF8-CRM, ORF8-PEG and ORF8/AL groups, which were defined according to the samples immunized. Mice were immunized subcutaneously in the groin region with the corresponding samples (200 μL) on days 0, 7, and 14. The samples were all at an ORF8 concentration of 100 μg/mL. A mixture of 200 μg aluminum adjuvant and 20 μg ORF8 was used for each mouse of the ORF8/AL group. The blood samples were collected by *retro*-orbital bleeding on days 7, 14 and 21. Mice were sacrificed and the spleens were harvested on day 21.

2.10. ELISA assay

The ORF8-specific antibodies (IgG, IgG1, IgG2a and IgM) titers were measured by ELISA, essentially as described previously [27]. The 96-well plates were pre-coated with ORF8 (0.75 μg/well) in 50 mM $NaHCO_3$ (pH 9.6) at 4 °C overnight. The plates were blocked with 200 μL of 4% skimmed milk in PBS buffer (PBS-milk, pH 7.4) at 37 °C for 1 h, followed by washing 3 times with PBS buffer (pH 7.4). Diluted sera (100 μL) were added the plate, followed by incubation in the wells for 1 h at 37 °C and washing with PBS buffer containing 0.1% Tween 20 (PBST, pH 7.4). The plates were added with 100 μL of the diluted HRP-IgG, HRP-IgG1, HRP-IgG2a or HRP-IgM at 37 °C for 1 h, respectively. After washing 3 times with PBST, 100 μL of 0.015% (w/v) TMB was added and incubated at 37 °C for 30 min, followed by adding 25 μL of 2 M H_2SO_4. The resultant solution was determined at 450 nm. The antibody titers were presented as the highest sample dilution, which led to in an OD value greater than 2.1 times the OD mean of the sera of the PBS group.

2.11. Splenocyte proliferation assay

Spleen cells (2×10^6 cells) from the immunized mice were seeded in the tissue culture plates (Corning, USA) and stimulated with ORF8 (10 μg/mL) at 37 °C with 5% (v/v) CO_2 for 60 h. Cells were stimulated with 1 μg ConA/mL, which served as a positive control. Cells were stimulated with medium alone, which acted as a negative control. Proliferation was determined by CCK-8 vital stain (Dojindo Laboratories, USA). The proliferation index (PI) was calculated as the ratio of the average OD_{450} value of the ORF8-stimulated cells to the one of the medium-stimulated cells.

2.12. Avidity of antibody

The avidity of ORF8-specific IgG was measured using the ammonium thiocyanate elution method [28]. Briefly, the plates were pre-coated with ORF8 and the samples were blocked with PBS-milk, followed by incubation of the diluted mice sera at 37 °C for 1 h. Then, the plate was incubated with 100 μL of ammonium thiocyanate (0–0.75 M) at room temperature for 15 min. After a washing cycle, the determination was repeated. The avidity index (AI) was expressed as ammonium thiocyanate concentration needed to decrease the absorbance by 50%.

2.13. Cytokine production in spleen cell culture

The mouse spleens were grinded and lysed to obtain the splenocytes suspension. Each suspension was resuspended to 5×10^5 cells/mL in RPMI 1640 medium with 10% heat-inactivated FBS, 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were subsequently stimulated with 10 μg/mL of ORF8 and RPMI 1640 medium in 5% (v/v) CO_2 at 37 °C for 60 h, respectively. The culture supernatants were collected at 68 h after stimulation to determine the levels of IFN-γ, IFN-β, TNF-α, and IL-5, using the mouse cytokine ELISA kits as instructed by the manufacturer's protocol. Experiments were performed in triplicate and repeated at least three times independently.

2.14. Toxicity study

Biochemical analysis of the mouse sera was carried out to evaluate the potential toxicity of the samples to the cardiac, hepatic, and renal functions. The serum samples of the five groups were collected on day 21 for toxicity study. Creatine kinase (CK) and lactate dehydrogenase (LDH) in the sera were determined for cardiac function. Alanine aminotransferase (ALT), total protein (TP) and albumin (ALB) were measured for hepatic function. Uric acid (UA) and were assayed for renal function. All analyses were carried out using an Analyzer Medical System (AUTO LAB, Italy).

2.15. Statistical analysis

The differences between the experimental groups were compared by one-way ANOVA. Results were analyzed using GraphPadPrism 5 software (GraphPad Software, CA, USA). The values of $P < 0.05$ (*) and $P < 0.01$ (**) were considered statistically and highly statistically significant between the experimental groups, respectively.

3. Results

3.1. Expression and purification of ORF8

The cell culture without IPTG induction, the one with IPTG induction, the inclusion body and the supernatant were analyzed by SDS-PAGE. As shown in Fig. 2a, a band corresponding to ~15 kDa was apparent in the cell culture with IPTG induction, which was indicated in Lane 2. In contrast, this band was almost absent in the cell culture without IPTG induction (Lane 1). This indicated that IPTG induction could promote the production of ORF8. The inclusion body of the cell culture with IPTG induction displayed several electrophoresis bands (Lane 3). A thick band corresponding to ~15 kDa was apparent and indicated in Lane 3. However, this band was not apparent in the supernatant of the cell culture with IPTG (Lane 4). Thus, ORF8 was expressed in the form of inclusion body.

The inclusion body was dissolved in the buffer containing 6 M guanidine hydrochloride and loaded on a Ni Sepharose HP column (0.5 cm × 5 cm). The column was eluted by the gradient imidazole. Two

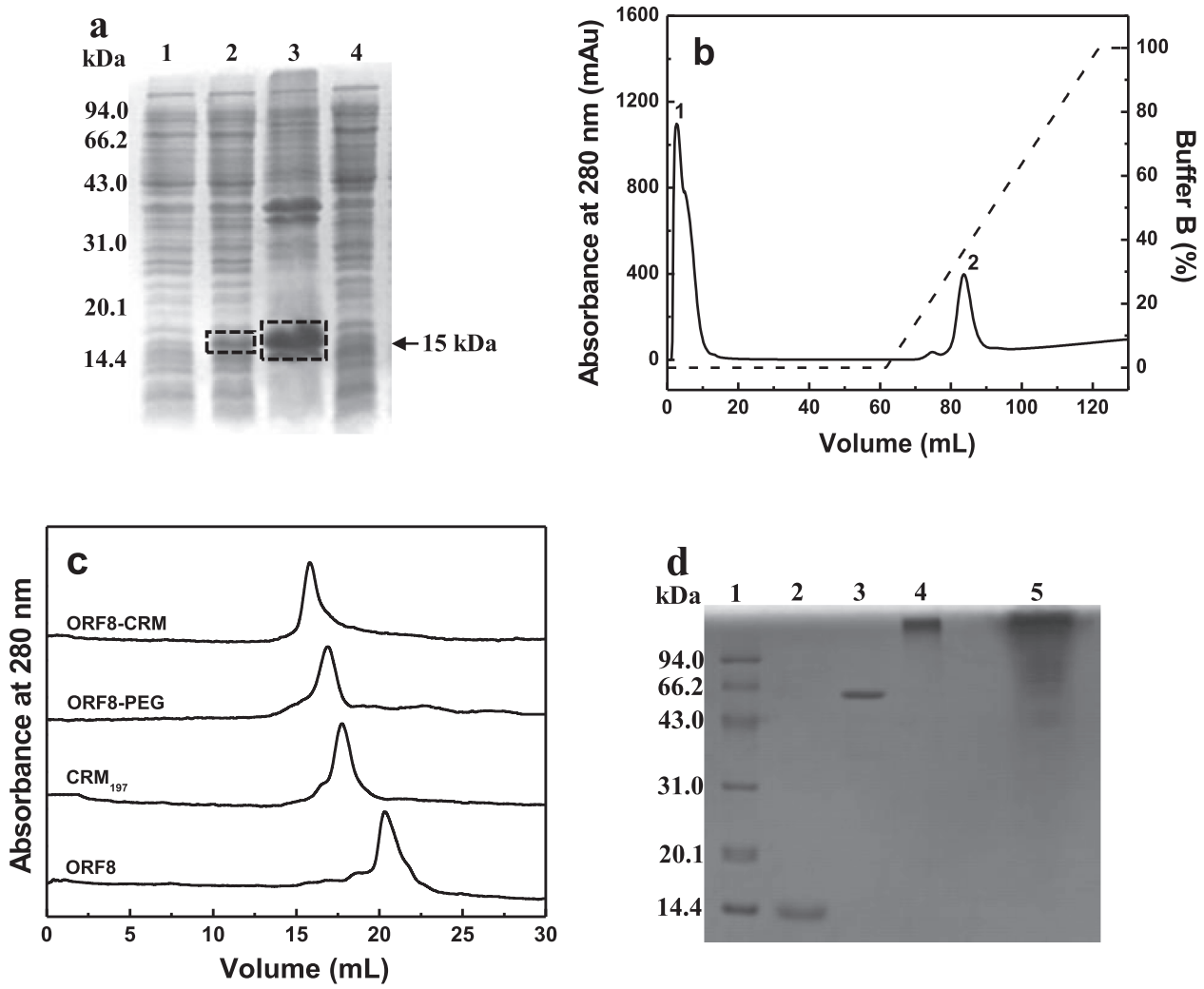

Fig. 2. Purification and characterization of ORF8. The cell cultures were analyzed by SDS-PAGE (a). Lane 1, the cell culture without IPTG induction; Lane 2, the cell culture with IPTG induction; Lane 3, the inclusion body; Lane 4, the supernatant. ORF8 was purified from the cell culture by a Ni Sepharose HP column (b). The two conjugates were analyzed by an analytical Superdex 200 column (c) and SDS-PAGE (d). Lane 1, marker; Lane 2, ORF8; Lane 3, CRM_{197}; Lane 4, ORF8-CRM; Lane 5, ORF8-PEG.

separated major peaks were observed upon the imidazole elution (Fig. 2b). Peak 2 corresponding to ORF8 was fractionated. ORF8 was in denatured and then renatured by dropwise dilution of the solution.

3.2. SEC analysis

ORF8-CRM and ORF8-PEG were analyzed by a Superdex 200 column (1 cm × 30 cm). As shown in Fig. 2c, ORF8, CRM_{197}, ORF8-CRM, and ORF8-PEG were all eluted as a single peak, indicating the high purity. The elution peaks of ORF8 and CRM_{197} were at 20.3 mL and 17.8 mL, respectively. As compared with ORF8 and CRM_{197}, the elution peaks of ORF8-CRM (15.8 mL) and ORF8-PEG (16.9 mL) were both left-shifted. This indicated that conjugation with CRM_{197} and self-conjugation with PEG could both increase the hydrodynamic volume of ORF8.

3.3. SDS-PAGE analysis

As shown in Fig. 2d, ORF8 (Lane 2) and CRM_{197} (Lane 3) both exhibited a single band. In contrast, ORF8-CRM (Lane 4) displayed a single electrophoresis band with slow mobility, corresponding to an Mw of more than 160 kDa. ORF8-PEG (Lane 5) exhibited a disperse band corresponding to an Mw of 100–200 kDa.

3.4. ORF8-specific IgG response

ELISA assay was used to determine the ORF8-specific antibody titers in the BALB/c mice. As shown in Fig. 3a, the ORF8-specific IgG titers of the PBS and ORF8 groups were very low upon the three immunizations, suggesting the intrinsically low immunogenicity of ORF8. The ORF8-specific IgG titers of the ORF8-CRM, ORF8-PEG and ORF8/AL groups were all low at the first dose (day 7). The second (day 14) and the third doses (day 21) progressively enhanced the ORF8-specific IgG titers of the three groups. In particular, the ORF8-specific IgG titer of the ORF8-PEG group (2.6×10^4) was higher than those of the ORF8-CRM group (1.1×10^4) ($P < 0.05$) and ORF8/AL group (1.8×10^4) ($P < 0.05$) at the third dose (day 21). This indicated that the adjuvant effect of administration with aluminum adjuvant was lower than self-conjugation with 8-arm PEG, and higher than that of conjugation with CRM_{197}.

3.5. ORF8-specific IgG subclasses and IgM

The five groups all elicited apparent ORF8-specific Th2-type IgG1 (Fig. 3b) and Th1-type IgG2a titers (Fig. 3c). The ORF8-PEG group showed the highest ORF8-specific IgG1 and IgG2a titers in the four groups on day 21. In particular, the Th1-type ORF8-specific IgG2a titer of the ORF8-PEG group was 1.1×10^4 at the third dose, which was equal

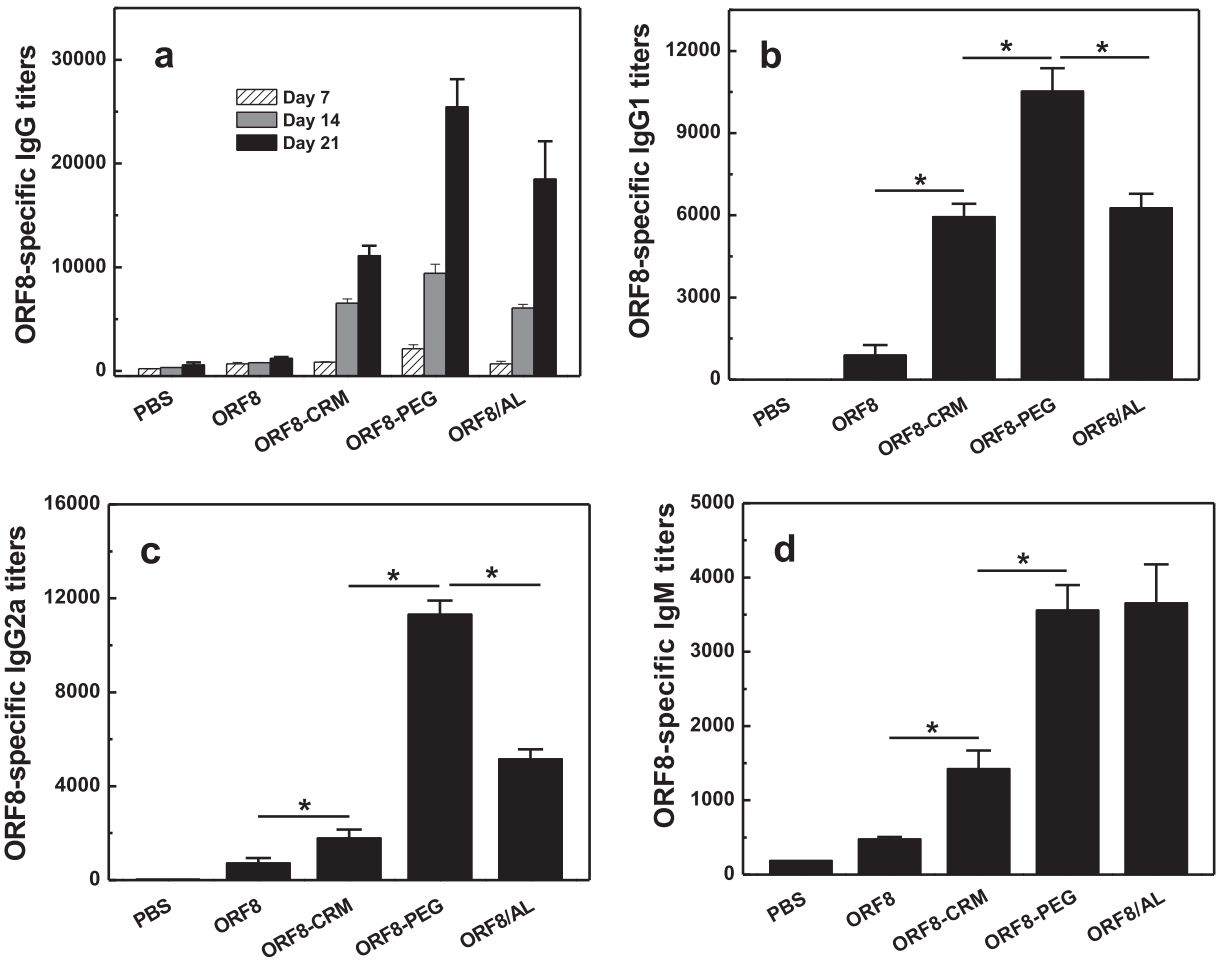

Fig. 3. Antibody response after immunization of the samples. The measurements of ORF8-specific IgG (a), IgG1 (b), IgG2a (c) and IgM (d) were carried out using ELISA. Blood samples after immunization on days 7, 14 and 21 were obtained for antibody measurement. Values represent mean ± S.D. from six mice per group.

to the ORF8-specific IgG1 titer (1.1×10^4). This indicated that self-conjugation with 8-arm PEG induced strong Th1-type and Th2-type immune response to ORF8. Moreover, the IgG1 titers of the ORF8-CRM group at the third dose (6.0×10^3) were approximately equal to that of the ORF8/AL group (6.2×10^3). The IgG2a titers of the ORF8-CRM group at the third dose (1.8×10^3) were lower that of the ORF8/AL group (5.1×10^3). The IgG2a/IgG1 of the ORF8 group was 0.84, whereas the IgG2a/IgG1 values of the ORF8-CRM, ORF8-PEG and ORF8/AL groups was 0.30, 1.00 and 0.82, respectively. Thus, conjugation with CRM_{197} and self-conjugation with 8-arm PEG both altered the

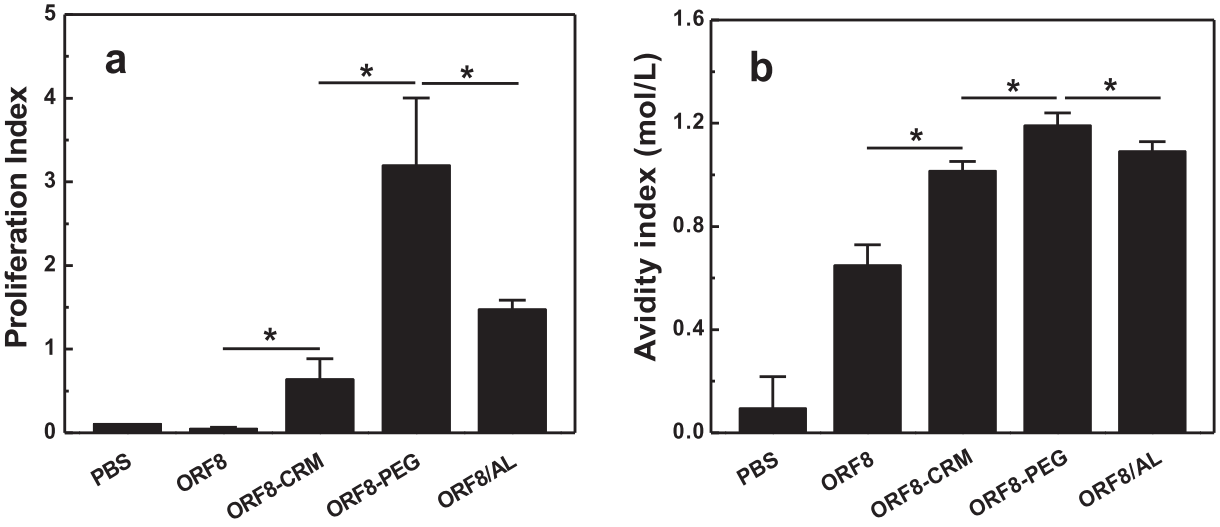

Fig. 4. Mice lymphocyte proliferation and antibody avidity analysis of ORF8-specific IgG. The lymphocyte proliferation of mice spleen suspensions was restimulated by ORF8 (a). The mouse sera on day 21 were used for antibody avidity analysis (b). Bar represents mean ± S.D. from 6 mice per group.

Th1/Th2 bias.

As shown in Fig. 3d, the ORF8 group showed very low ORF8-specific IgM titers (4.8×10^2). The ORF8-CRM group (1.4×10^3) showed higher IgM titers than the ORF8 group ($P < 0.05$). The IgM titer of the ORF8-PEG group (3.6×10^3) was higher than that of the ORF8 group ($P < 0.05$) and comparable to that of the ORF8/AL group (3.7×10^3). Because the IgG/IgM ratio of the ORF8-PEG group was higher than that of the ORF8/AL group, this suggested that self-conjugation with 8-arm PEG shifted more immune response from IgM to IgG than the ORF8/AL group. This was typical for the protein-based vaccines.

3.6. Antigen-specific splenocyte proliferation

Splenocyte proliferation was evaluated after stimulation with ORF8. As shown in Fig. 4a, the PI value of the ORF8-CRM group (0.64) was higher than that of the ORF8 group (0.06) ($P < 0.05$) and lower than that of the ORF8/AL group (1.48) ($P < 0.05$). Thus, conjugation with CRM_{197} could stimulate the ORF8-specific splenocyte proliferation, and the ability was lower than administration with aluminum adjuvant. The PI value of the ORF8-PEG group (3.20) was the highest among the five groups. This indicated that self-conjugation with 8-arm PEG was more effective than aluminum adjuvant and CRM_{197} conjugation to enhance the splenocyte proliferation.

3.7. Antibody avidity

Antibody avidity reflected the bonding degree of the antigen and the antibodies, and the induction of immunological memory. As indicated in Fig. 4b, ORF8-CRM, ORF8-PEG and ORF8/AL all showed higher AI of the ORF8-specific IgG than that of the ORF8 group (0.65) ($P < 0.05$). In particular, the ORF8-PEG group showed the highest AI value (1.20) of the four samples, indicating that self-conjugation with ORF8 significantly increased the avidity of the ORF8-specific IgG.

3.8. Cytokine secretion

The cytokine secretion was important for a vaccine to direct the immune response. IFN-γ, IFN-β, and TNF-α belonged to Th1-type cytokines, while IL-5 was considered as a Th2-type cytokine. These cytokines in the cell supernatants after stimulation with ORF8 were measured by ELISA. As shown in Fig. 5, the levels of the four cytokines in the ORF8-CRM, ORF8-PEG and ORF8/AL groups were all higher than the ORF8 group ($P < 0.05$). The IFN-γ level of the ORF8-PEG group (2675.1 pg/mL) were higher than that of the ORF8-CRM group (443.8 pg/mL) and comparable to that of the ORF8/AL group (2649.0 pg/mL). The TNF-α (3221.4 pg/mL) and IL-5 (17.0 pg/mL) levels of the ORF8-PEG group were higher than those of the ORF8-CRM and ORF8/AL groups. The IFN-β level of the ORF8-PEG group (47.9 pg/mL) were higher than that of the ORF8/AL group (38.2 pg/mL) and comparable to that of the ORF8-CRM group (49.6 pg/mL). Thus, ORF8-PEG showed high ability to increase

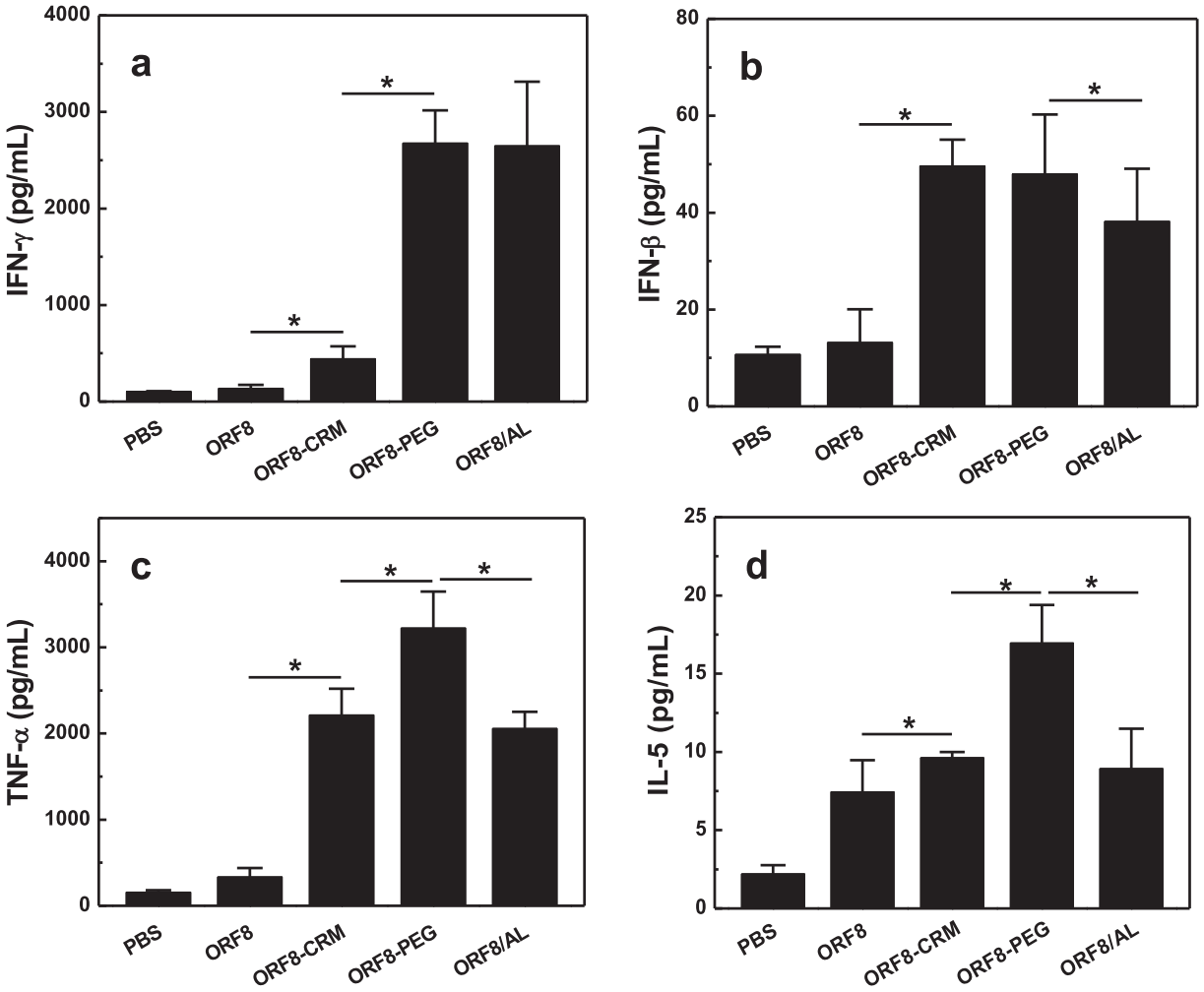

Fig. 5. Determination of the cytokine secretions in the immunized BALB/c mice. IFN-γ (a), IFN-β (b), TNF-α (c) and IL-5 (d) in the culture supernatant were analyzed using the ELISA kits. Values represented the mean value ± S.D. from 6 mice per group.

the Th1- and Th2-type immune responses.

3.9. Toxicity study

CK and LDH indicated the cardiac function; ALT, TP and ALB indicated the hepatic function; UA indicated the renal function. The potential toxicity of ORF8-CRM and ORF-PEG to the cardiac, liver and renal functions of mice was evaluated by measuring these substances in the mice sera. As shown in Table 1, the PBS group displayed certain levels of these substances. As compared with the PBS group, the ORF8 group exhibited no significant differences in these parameters, except a slightly high ALB level; the ORF8-CRM and ORF8/AL groups exhibited no apparent differences except a slightly low LDH and CK levels. These parameters of the ORF8-PEG group were comparable to those of the PBS group. Thus, ORF8-CRM and ORF8-PEG showed no apparent toxicity to the cardiac, liver and renal functions of mice.

Table 1
Toxicity study on the cardiac, hepatic and renal functions of the mice.

Sample	LDH[a] (U/L)	CK[b] (U/L)	ALT[c] (U/L)	ALB[d] (g/L)	TP[e] (g/L)	UA[f] (μmol/L)
PBS	214.6	205.4	13.6	15.7	26.3	95.4
ORF8	215.2	201.1	16.5	21.6	25.4	103.1
ORF8-CRM	187.4	128.6	12.2	14.1	22.4	93.4
ORF8-PEG	174.7	197.6	14.4	16.1	28.0	90.3
ORF8/AL	157.8	110.7	12.6	13.6	21.4	82.1

[a] Lactate dehydrogenase.
[b] Creatine kinase.
[c] Alanine aminotransferase.
[d] Albumin.
[e] Total protein.
[f] Uric acid.

4. Discussion

ORF8 is a rapidly evolving viral accessory protein that has been proposed to interfere with the immune responses [16]. ORF8 can form unique large-scale assemblies potentially mediating unique immune suppression and evasion activities during early viral infection [16]. Moreover, ORF8 modulates the adaptive host immunity through downregulation of MHC-I and innate immune responses by surpassing the host's type I interferon-mediated antiviral response [29]. ORF8 interacts with host factors involved in pulmonary inflammation and fibrogenesis [30]. Thus, ORF8 was used as a potential antigen candidate for SARS-CoV-2 vaccine development. In order to improve the immunogenicity of ORF8, a SARS-CoV-2 vaccine was formulated by covalent conjugation of ORF8 with 8-arm PEG or CRM_{197} in the present study. ORF8 administrated with aluminum adjuvant acted as the control. The immunological properties and preliminary safety of the two conjugates were thus determined.

ORF8 was expressed in the form of inclusion body by *E.coli*. After denaturation of the inclusion body and renaturation process, the resultant protein was loaded on a Ni Sepharose HP column. However, ORF8 could not be bound by the column, due to that the His tag of ORF8 was shielded after the renaturation process (data not shown). As an improved process, the inclusion body was denatured and then directly loaded on the column, which could ensure the full exposure of His tag of ORF8 to the column. ORF8 was then purified with high purity and renatured as the antigen.

Here, ORF8 was self-conjugated with 8-arm PEG and multiple ORF8 molecules were in one entity. ORF8-PEG has a high-loading capacity of ORF8 with the ability to deliver multiple ORF8 molecules, which could facilitate the antigen presenting and the bioavailability to the immune cells. Thus, ORF8-PEG per se showed the inherent adjuvant activity. CRM_{197} is an effective carrier protein possessing T-helper cell epitopes [23]. Conjugation with CRM_{197} can activate T helper cells for ORF8 and strongly improved the immunogenicity of ORF8. Presumably, the T-helper cell epitopes of CRM_{197} could confer on the ORF8-specific immune cells the ability to activate T helper cells. Interestingly, ORF8-PEG showed higher immunogenicity than ORF8-CRM.

The splenocytes of the ORF8-PEG group stimulated with ORF8 could generate high secretion levels of IFN-γ (2675.1 pg/mL), TNF-α (3221.4 pg/mL), IFN-β (47.9 pg/mL) and IL-5 (17.0 pg/mL). IFN-γ, IFN-β and TNF-α were two Th1-type cytokines related to the innate immunity essential to control the viral infection. ORF8-PEG could strongly stimulate the splenocyte proliferation with a PI value of 3.2. Thus, ORF8-PEG elicited a potent cellular immune response. IL-5 was a Th2 -type cytokine related to the humoral immunity and antibody response. ORF8-PEG also elicited high ORF8-specific IgG titers (2.6×10^4). Thus, ORF8-PEG elicited a strong humoral immune response to ORF8. Moreover, the IgG2a and IgG1 levels increased after three immunizations of ORF8-PEG, indicating that booster immunizations led to memory B-cell activation.

In recent years, several vaccines based on spike protein or RBD have launched on the market. However, the emergence of the virus variants (e.g., Delta and Omicron strains) significantly lowered the effectiveness of the vaccines. As a different antiviral target, the vaccine based on ORF8 could be a supplement to the current vaccines, which could provide full immune protection.

In summary, ORF8-PEG induced high ORF8-specific IgG titer, high levels of IFN-γ, TNF-α and IL-5, and strong splenocyte proliferation. Moreover, ORF8-PEG showed higher immunogenicity than ORF8-CRM and ORF8/AL. Thus, conjugation with 8-arm PEG is an effective method to improve the humoral and cellular immune response to SARS-CoV-2 ORF8. Moreover, ORF8-PEG did not lead to apparent toxicity to the cardiac, liver and renal functions. ORF8-PEG was expected to act as a candidate vaccine against SARS-CoV-2.

Author contributions

Ms. Xiaozhao Tang, Dr. Weili Yu and Ms. Lijuan Shen were responsible for the acquisition of data and interpreted the experimental data. Drs. Tao Hu and Jinming Qi designed the study. Dr. Tao Hu was the major contributor in drafting and revising the manuscript. All authors read and approved the final manuscript.

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.

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