The recombinant protein combination vaccine based on the fragment C of tetanus toxin and the cross-reacting material 197

Running title: Recombinant diphtheria and tetanus vaccine

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Abstract

Tetanus and diphtheria are important public health problems in many parts of the world. Since the diphtheria, tetanus toxoids and acellular pertussis vaccine (DTaP) was generally administered to children, the incidence of diphtheria and tetanus has dropped dramatically. However, with the increase of age, the anti-diphtheria and tetanus antibody in human body will decrease. DTaP is not suitable for boosting vaccine and the recombinant vaccine have more advantages than toxoid vaccines. Therefore, in this study, firstly, we expressed and purified the fragment C of tetanus toxin (TTc) and the cross-reacting material 197 (CRM197) of the diphtheria toxin mutant from *E. coli* BL21(DE3) expression system respectively. Moreover, the characteristics, immunogenicity of the purified protein were analyzed. Lastly, the recombinant TTc and CRM197 combination vaccine (RTCV) was constructed and antibody titers were detected. Our results displayed TTc induced Th2 immune response in mice while CRM197 could induce a mix Th1/Th2 immune response. RTCV was composed of 10 μg/mL TTc, 20 μg/mLCRM197 antigens and aluminum adjuvants (500 μg/mL). We found that the IgG and IgG1 antibody titers produced in mice were similar as those produced by DTaP, except the IgG2a antibody titers. The results above will provide technical support for the future combined recombinant protein vaccine to against diphtheria and tetanus.

Keywords: tetanus, diphtheria, the fragment C, CRM197, a booster vaccine, the recombinant TTc and CRM197 combination vaccine

Introduction

Tetanus is a deadly disease caused by clostridium tetani, a gram-positive bacteria, which infects human wounds, grows and multiplies in an anaerobic environment and produces tetanus toxin [1]. Tetanus toxin , a total length of 1315 amino acids and a molecular weight of 150 KDa [2] , is very toxic, second only to botulinum toxin [3]. Tetanus toxin consists of three fragments: A, B, and C, each with a molecular weight of 50 KDa. The fragment A is a light chain with protease activity, inhibit the release of neurotransmitter [4]. The fragment B is the N-terminus of the heavy chain and associated with the introduction of toxins [5], while the fragment C (TTc) is the C-terminus of the heavy chain and has the function of binding to nerve cells [2]. At present, it has been confirmed that TTc retains many properties: Toxins bind to gangliosides, the immunizing potency is equivalent to that of toxin, non-toxic, and low allergenicity [6-7]. Therefore, TTc can be used to research recombinant protein vaccines to replace existing toxoid vaccines.

Diphtheria is an upper respiratory tract infection disease caused by diphtheria toxin whose molecular weight is 62 KDa. Cross-reacting material 197 (CRM197) is a non-toxic mutant of diphtheria toxin (glutamic acid substituted for glycine at the 52nd amino acid), a molecular weight of 58 KDa [8]. It is composed fragment A and fragment B through a disulfide bridge. The fragment A is the active site of nuclease and can catalyze ADP ribosylation of eukaryotic elongation factor-2 (eEF-2) to inhibit protein synthesis[9]. The fragment B contains a subdomain of the HB-EGF cell receptor and a subdomain of the intracellular translocation associated with toxin entry into the cell [10]. CRM197 protein retains the same inflammation, immunogenicity and nuclease enzyme activity as diphtheria toxin[11-12], which makes it a strong candidate to replace diphtheria toxoid vaccine in the future. Meanwhile, CRM197 can be used to produce polysaccharide vaccine because it contains 39 lysine residues [13].

Since 1940s, tetanus toxoid, diphtheria toxoid and pertussis antigen have been widely used in the world as the binding vaccine - diphtheria, tetanus toxoids and acellular pertussis vaccine (DTaP), and then the incidence of diphtheria and tetanus has dropped dramatically [14]. However, antibody levels for diphtheria and tetanus decrease with age [15-16]. The World Health Organization (WHO) recommends that adults should be vaccinated every ten years to ensure herd immunity [17]. Nevertheless, the DTaP vaccine as an adult booster vaccine can cause side effects, which caused by a variety of factors including the purification level of toxins before detoxification of formaldehyde, the antigen dose in the vaccine and

the health of the vaccinated person and so ne [14].Therefore, it is necessary for the research of new booster vaccines to replace DTaP clinically. The recombinant protein vaccine has the advantages of single component and high safety, which is one of the directions to avoid the above phenomenon. In this study, we first obtained the recombinant protein CRM197 and TTc from *E. coli* expression system respectively, verified the properties of the proteins and analyzed its immunogenicity to determine the antigen dosage in the recombinant TTc and CRM197 combination vaccine (RTCV). Then the recombinant proteins TTc and CRM197 were combined by adding aluminum adjuvant to form RTCV. Finally, we studied the feasibility of RTCV through immunized mice in order to provide technical support for the pre-clinical research of RTCV.

Materials and methods

Expression and purification of CRM197 and TTc in *E. coli* BL21（DE3）

The *CRM197* gene was obtained from the pVAC2035-CRM197 plasmid using PCR. The PCR product was digested with N*de* I and E*coR* I enzymes (Takara) and ligated to the pET26b (+) vector. Then, the recombinant vector was transferred to *E. coli* BL21 (DE3) for expression. The cultivated bacterial suspension (10 mL) was transferred to 500 mL of Luria-Bertani (LB) liquid medium containing 50 μg/mL kanamycin antibiotics. Cells were cultured in shaker at 37 ℃, 220 rpm for hours. Once the optical density at 600 nm (OD_{600}) reached 0.8-1, isopropyl-β-d-thiogalactoside (IPTG, BBI) (a final concentration of 0.4 mM/L) was added to induce expression at 37 ℃ for 3.5 h. After fermentation, cells were harvested by centrifugation at 8000 rpm for 10 min. After washing twice with phosphate buffer (PB, pH7.2), the cells were resuspended in PB with 100 μg/mL PMSF, homogenized at 4 ℃ and 1.4 MPa, centrifuged at 15000 rpm for 20 min. Then the supernatant was discarded, the pellets were washed twice with buffer A (20 mM Tris, 1 mM EDTA, 2 M urea, pH 8.5) and resuspended in buffer B (20 mM Tris, 1 mM EDTA, 8 M urea, pH 8.5) and incubated at 37 ° C for 1 h. Modified liquid was centrifuged at 15000 rpm for 20 min, the supernatant was slowly diluted with 10 times the volume of buffer C (20 mM Tris, 1 mM EDTA, pH 8.5), and refold overnight at 4 ℃. The regenerating liquid was followed to be purified by the Heparin Affinity column (GE Healthcare) using the AKTA Explore 100 system (GE Healthcare). After balancing the column with buffer C, the sample was loaded, and the impurities were washed with 5 column volumes buffer C containing 150 mM NaCl, and then CRM197 protein was eluted with buffer C containing 400 mM NaCl.

The methods for expression and purification of TTc have been reported in our previous studies. To

put it simply, the *TTc* gene fragment (GenBank: *AF154828.1*) was synthesized, whose single base mutation (G-A) with 869 amino acid sequence make its structure stability[18]. The synthetic genes were digested with N*de* I and S*al* I enzymes (Takara), ligated into the pET26b (+) vector, and then transferred to *E. coli* BL21 (DE3) for expression. The expression method of TTc was the same as the protein CRM197, except induced temperature was 16 ℃ for 13 h. After fermentation, cells were harvested by centrifugation at 8000 rpm for 10 min and stored at -20 ℃. The recombinant protein TTc was separated and purified by Q Sepharose XL (GE Healthcare) anion exchange chromatography and Capto SP ImpRes (GE Healthcare) cation exchange chromatography using the AKTA Explore 100 system (GE Healthcare).

SDS-PAGE and Western Blot

SDS-PAGE was used to analyze the expression and purification of the protein, and all purified protein were identified by Western Blot using Rabbit Anti-Diphtheria Toxin antibody (ab151222, Abcam) or Rabbit Anti-Tetanus Toxin antibody (ab53829, Abcam).The methods were same as previously describe [19].

Ganglioside binding assay

The ganglioside binding assay of the purified protein TTc was performed as previously describe [18]. Briefly, ganglioside GT1b (BIOYKSW)was diluted with methanol to 5 μg/mL, coated in the 96-well plate (100 μL/well) at room temperature for overnight until the methanol was completely evaporated. Then wells were washed with PBST. the purified protein TTc diluted with PBST were added, and incubated at 37 ℃ for 1 h. After washed with PBST for 4 times (5 min/each), the plate was incubated with mouse anti-tetanus toxoid polyclonal serum at a 1:800 dilution in PBST at 37 ℃ for 1 h. Then these wells were washed with PBST for 4 times (5 min/each), HRP labeled goat anti-mouse IgG (Southern biotech) was added to react at 37 ℃ for 40 min, follow to be washed with PBST. Finally, substrate TMB (Thermo) was added for color, the absorbance was determined at 450 nm using a microplate reader (ELX800, BIO). CRM197 protein was used as a negative control to repeat the above experimental process.

Immunization program of TTc

6-8 weeks old six female BALB/c mice (Shanghai SLAC Laboratory Animal Co., Ltd) were injected intramuscularly with TTc antigen at 1 μg, 10 μg, and 20 μg with or without aluminum adjuvant. A negative group and a positive control group were necessary with PBS (Hyclone) and DTaP (Wuhan Institute of Biological Products Co., Ltd) respectively. Each mouse of all groups received a total of

immunizations three times on days 0、14th d and 28th d with the same dose. The dose of each groups was 100 μL/mouse, except for the DTaP group (50 μL/mouse), which was one-tenth of the dose for humans. Finally, the mice were euthanized on 42thd, the spleen cells of mouse were isolated for detection of IFN-γ by enzyme-linked ImmunoSpot assay (ELISPOT). And mouse serum was collected and stored at -20℃ for detection of antibody levels. The TTc antigen dose of the combination vaccine was determined based on the principle that the antibody titer was comparable to that of the commercial DTaP.

Immunization program of CRM197

The immunization program of CRM197 protein was similar with the protein TTc, except that the dose of CRM197 protein were 2 μg and 20 μg with or without aluminum adjuvant. The CRM197 antigen dose of the combination vaccine was determined based on the principle that the antibody titer was comparable to that of the commercial DTaP vaccine.

The preparation of RTCV

RTCV was composed of TTc antigen, CRM197 antigen and aluminum adjuvant. In brief, TTc protein and CRM197 protein were dissolved in PBS (pH 7.2) buffer and filtered to sterilize by 0.22 membrane respectively. Then a certain proportion of TTc to CRM197 protein were mixed together, aluminum adjuvant was added (pH 7.2), so that the final concentrations of TTc, CRM197 and aluminum adjuvant was 10 μg/mL, 20 μg/mL and 50 μg/mL respectively. Finally, it was placed at 4 ° C overnight and shocked absorption.

Immunization program of RTCV

Groups of six female BALB/c mice (6-8 weeks old, 6 mice/group) were injected intramuscularly with TTc (1 μg+50 μg aluminum adjuvant/100 μL), CRM197 (2 μg CRM197+50 μg aluminum adjuvant/100 μL) and RTCV (1 μg TTc+2 μg CRM197+50 μg aluminum adjuvant/100 μL). A negative group and a positive control group were necessary with PBS and DTaP respectively. The other immunization programs were same as the protein TTc.

ELISA assays

Indirect enzyme-linked immunosorbent assay (ELISA) was used to detect antibody IgG and antibody subtypes IgG1 and IgG2a in the serum of mice. The method was same as the previously described [20]. Except that ELISA plates were coated with 100 μL/well of TTc protein (2 μg/mL) or CRM197 (1.5 μg/mL) protein in carbonate buffer (Sigma, pH 9.7). The dilution of IgG, IgG1 and IgG2a antibody were 1:20000, 1:20000 and 1:1500 respectively.

ELISPOT assays

ELISPOT assays for IFN-γ were performed by using a commercially BD™ ELISPOT Mouse IFN-ΓELISPOT Set as the previously described [20]. Except that the stimulus was 20 μg/mL of CRM197

protein or TTc protein. The final results were counted on a CTL ImmunoSpot analyzer (Cellular Technology Limited).

Statistical analysis

The data of this paper were expressed as the mean ± SEM. Statistical significance was used to describe differences between groups and analyzed using GraphPad prism 6.0. While $P < 0.05$, the difference was considered significant, while $P < 0.01$, the difference was considered extremely significant.

Results

The expression and purification of the protein CRM197 and TTc

The CRM197 recombinant plasmid was constructed and successfully expressed in the *E. coli* BL21 (DE3) at 30 ℃ under 0.4 mM/L IPTG for 3.5 h. The result showed the recombinant protein existed in the form of inclusion body (See Fig1A). We could see a significant band at 55-70KDa (the theoretical molecular weight was 58 KDa). Western Blot also showed that the recombinant protein could specifically bind to anti-diphtheria toxin antibody (See Fig.1A, lane 7). The purity of the purified CRM197 protein was more than 96 % by SDS-PAGE analysis (Fig.1A, lane 6). At the same time, the expression and purification results of recombinant TTc protein were shown in Fig.1B. SDS-PAGE showed an obvious band between 40 to 55 KDa, consistent with the protein's theoretical molecular weight (50 KDa), TTc was induced to express at 16 ℃ for 13 h under 0.4 mM/L IPTG, and mainly existed with a soluble form. The purity of the TTc protein was more than 96 % by SDS-PAGE analysis finally (See Fig.1B, lane 6) and the purified protein could bind to anti-tetanus toxin antibodies (See Fig.1B, lane 7).

Ganglioside binding assay of TTc

ELISA was used to detect the binding of TTc protein with GT1b receptor. The results were shown in Fig.2. The recombinant protein TTc could still bind to GT1b, while the negative control recombinant protein CRM197 could not bind to GT1b receptor. It showed that our mutation site had not affected the binding of TTc to GT1b.

Immunogenicity of recombinant TTc

To verify the immunogenicity of TTc and determine dose of the RTCV, female BALB/c mice were used to immunized with different dose recombinant TTc protein and DTaP. The specific antibody titer (IgG、IgG1 and IgG2a) were determined by indirect ELISA. As shown in Fig.3, after the last

immunization, there were no significant difference in between 1 μg TTc/adjuvant group, 10 μg TTc/adjuvant group and 20 μg TTc/adjuvant immunized group (See Fig.3A). However, we found the adding of aluminum adjuvant could significantly improve the immune response of TTc ($p < 0.01$). And a stronger antibody titer (IgG) was produced in 1 μg TTc/adjuvant group than the DTaP group ($p<0.05$). Antibody subtype analysis showed that both TTc and DTaP could stimulate the mice to secrete IgG1 and IgG2a. Nevertheless, TTc and DTaP mainly stimulated the body to produce Th2 humoral immune response (See Fig.3B, C). The secretion level of the IFN-γ also confirmed this result by ELISPOT detection (See Fig.3D). These results indicated that TTc stimulated the mice to produce antibodies independent of the dose of antigen. With the increase of immune times, 1 μg TTc/adjuvant elicited a stronger Th2 immune response than DTaP, suggesting that 1 μg recombinant protein TTc could be used as a dose of TTc antigen in RTCV.

Immunogenicity of recombinant CRM197

Female BALB/c mice were immunized with different dose of recombinant CRM197 protein and DTaP to analyze the immunogenicity of recombinant CRM197 protein and determine the CRM197 antigen dosage of RTCV. The level of specific antibody title (IgG, IgG1 and IgG2a) were showed in Fig.4. After the third immunization, the specific antibody (IgG) produced in mice vaccinated with 2 μg CRM197/adjuvant was similar as that of DTaP (See Fig.4 A). The antibody titer of 20 μg CRM197/adjuvant group was higher than that of 2 μg CRM197/adjuvant group. However, comparing the 20 μg CRM197 with the 20 μg CRM197/adjuvant group, there were no different significance (See Fig.4 A). The antibody subtype analysis showed that aluminum adjuvant weakened the production of IgG2a in mice (See Fig.4 B). Moreover, the DTaP and 20 μg CRM197 elicited a mix Th1/Th2 response in mice (See Fig.4 B, C), which were confirmed by IFN-γ ELISPOT assays (See Fig.5D). These results indicated that CRM197 elicited a mix Th1/Th2 response and mice were stimulated to produce IgG and IgG1 antibody titer in the 2 μg CRM197/adjuvant group were similar to those of the DTaP group, suggesting that 2 μg CRM197 can be used as a dose of CRM197 antigen in RTCV

Aluminum adjuvant reduces the Th1 immune response

From the results above, we know that TTc stimulated mice to produce Th2 immune response (See Fig.4) while CRM197 produced a mix Th1/Th2 immune response (See Fig.4). Furthermore, aluminum adjuvant had enhanced the Th2 immune response to TTc antigen ($p<0.01$). However, instead of enhancing the effect of the Th2 immune response, the aluminum adjuvant inhibited the Th1 immune

response to CRM197 antigen ($p<0.01$) These results further support the previous researches results that aluminum adjuvants could heighten humoral immune responses, while restrain cellular immune responses [21].

Feasibility of RTCV

To verify the feasibility of RTCV, we first determined the dose of each antigen for RTCV in the above two immunized experiments. Then we evaluated it by comparing RTCV with a single antigen/adjuvant and commercial DTaP vaccine. Antibody titer and IFN-γ secretory levels of RTCV were used as evaluation indexes (Shown in Fig.5). The anti-CRM197 antibody titer of IgG and IgG1 in RTCV group were not significantly difference between the 2 μg CRM197/adjuvant group and DTaP group (See Fig.5 A, B), but the IgG2a antibody titer of RTCV group was lower than that of the DTaP group（$p<0.01$）(See Fig.5 B). The immune response to CRM197 in RTCV group was biased towards the Th2 immune response (See Fig.5 C, D). As the same time, the anti-TTc antibody titer (IgG、IgG1 and IgG2a) of RTCV group were similar with 1 μg TTc/adjuvant group and DTaP group.(See Fig.5 E, F). And the immune response to TTc in RTCV was dominated by the Th2 immune response (See Fig.5 G, H). which was consistent with the previous conclusion. In summary, the RTCV constructed did not differ from between 1 μg TTc/adjuvant and DTaP in the immune response to TTc, but it was the Th2 immune response to CRM197.

Discussion

Tetanus and diphtheria are important public health problems in many parts of the world where immunization programs are suboptimal, particularly in low income countries [17, 22]. Tetanus can occur at any age and people are generally susceptible to tetanus [23]. Diphtheria was mainly prevalent among children in the past [17]. The recent epidemiological studies of diphtheria have shown that most cases are adolescents and adults. Therefore, the decline in antibody levels between adolescents and adults are urgently needed to boost immunity [24]. The currently commercially DTaP vaccine has good protection for Tetanus and diphtheria, but is not suitable for use as a booster vaccine. Hence, in this study, we focused on recombinant protein to prepare a combined recombinant vaccine as a booster vaccine to ensure herd immunity to tetanus and diphtheria in adults.

There are many factors that restrict the expression of heterologous genes in *E. coli*, including *E. coli* strain, protein toxicity, rare codons and so on [25]. It has been reported that it is difficult for the recombinant CRM197 protein be expressed in *E. coli* BL21 (DE3) [26]. However, in this study, we

successfully expressed CRM197 protein in *E. coli* BL21(DE3) using pET26b (+) vector. Nevertheless, the previous studies have shown that the expression of CRM197 protein in *E. coli* mostly is inclusion bodies, with only a small amount of soluble protein[25, 27]. Our research also confirmed this result, which CRM197 protein could be partially expressed in soluble form at 16 °C (data not shown). In addition, we failed to purify the CRM197 protein in a soluble form. And the addition of tags can help to purify the protein, but it also affected its physiological activity. Although some people think that adding the His tag to the N-terminus does not affect the biological activity of CRM197 *in vitro*, but its purification yield will be reduced by about 50% [25]. Based on these reasons, we mainly performed expression and purification of CRM197 in inclusion bodies form (Fig.1A). After shaking fermentation, the final yield of CRM197 protein reach to 7.88 g/L of the bacterial, which accounted for 19.3 % of the total bacterial protein (data were not shown). The CRM197 protein has HB-EGF binding site[28-29]. Hence, we purified the target protein with a heparin affinity column after denaturation and renaturation. The purity of the target protein was more than 96% (See Fig.1 A). Moreover, by stimulating mice, CRM197 also showed a certain dose-dependent immune response, which was a mix Th1/Th2 immune response (See Fig.4). At the same time CRM197 stimulated mice to produce a certain dose-dependent immune response, and high doses of CRM197 antigen could stimulate the body to produce a mix Th1/Th2 immune response (See Fig.3). However, according to the previous research, the CRM197 protein retains the inflammatory response of diphtheria toxin [11]. In our immune experiment of CRM197, we found that after the initial immunization, the mice of the 20 μg CRM197 group generally lost weight and did not return to normal until the third immunization. In subsequent ELISPOT assays, the spleen of the high-dose group was much larger than PBS group. Therefore, in order to reduce the inflammatory response, the dose of the CRM197 antigen was reduced as much as possible in RTCV. In addition, the DTaP group stimulated lymphocyte T cells to secrete IFN-γ far higher than CRM197 antigen group, However, studies have shown that diphtheria toxoid stimulates the body to produce a Th2 immune response[8], so this phenomenon might be caused by the role of pertussis antigen component in DTaP vaccine as vaccine adjuvant to stimulate the mice to produce cellular immunity.

In our previous study, we successfully achieved the soluble expression of recombinant TTc protein in *E. coli* BL21 (DE3). The presence of ganglioside binding sites are essential to induce the body to produce an anti-tetanus toxin response [30]. TTc has two ganglioside binding sites: Gal4-GalNAc3 and a sialic acid binding site [31-32]. In this paper, we mutated the key amino acid cys to ala at 869 tetanus toxin,

which could stabilize its structure[18]. Therefore, we verified whether the mutant TTc protein had the ability of ganglioside binding, and we found TTc still had the ability of ganglioside binding (See Fig.2). Addition, it is reported that TTc antigen can stimulate mice to produce a stronger immune response than tetanus toxoid [33]. In this study, 1 μg TTc/adjuvant stimulated mice produced antibody titer was higher than DTaP vaccine. Hence, we chose 1 μg as the dose of TTc in RTCV. Last, RTCV was prepared, a certain proportion of recombinant protein TTc was mixed with recombinant protein CRM197, and aluminum adjuvant was added, so that the final concentrations of TTc, CRM197 and aluminum adjuvant were 10, 20 and 500 μg/mL. Then it was placed at 4 ℃ for overnight adsorption. immunization of RTCV was examined, the results displayed that anti-TTc and anti-CRM197 antibody titer (IgG) was similar with DTaP, TTc antigen and CRM197 antigen in RTCV stimulated mice to produce Th2 immune response. However, in the immunization with CRM197 experiment, we found that high-dose CRM197 produced Th1 immune response.

In this experiment, we only used the immune method of intramuscular injection. It has been reported that TTc and CRM197 can stimulate the body to produce better immune response by a variety of routes of administration[34-37]. Therefore, we will focus on the research of vaccine adjuvants and administration routes to improve immunity of the RTCV in the future.

In conclusion, we have successfully constructed a recombinant protein combination vaccine (RTCV) for using an adult booster vaccine. The antibody titer (IgG) of mice stimulated by RTCV was similar to that of DTaP. Meanwhile, High-dose CRM197 antigen could stimulate the mice to produce Th1 immune response. In the future, we will further research vaccine adjuvants and routes of administration.

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Figure legends

Fig.1 SDS-PAGE and Western Blot analysis of the recombinant proteins. (A) the recombinant proteins CRM197. (B) the recombinant proteins TTc. Lane M: the protein marker, lane 1: the cells of E. coli BL21(DE3), lane 2: before the induction cells, lane3: the induction cells, lane4: the supernatant of the induction Cell lysates, lane 5: the precipitate of the induction Cell lysates, lane 6: the finally purified protein, lane7: Western Blot of the finally purified proteins.

Fig. 2 The bind of recombinant protein TTc to GT1b

Fig.3 The level of IgG、IgG1 and IgG2a antibody and IFN-γ produced in mice immunized with TTc recombinant protein. Antibody titers were expressed logarithmically in each group, the bar represent mean ± SEM. (A) IgG antibody titer against TTc antigen. (B) IgG1 and IgG2a antibody titer against TTc antigen. (C) the ratio of IgG1 to IgG2a antibody titer against TTc antigen. (D) the number of T cell secreted IFN-γ.

Fig.4 The level of IgG, IgG1and IgG2a antibody and IFN-γ produced in mice immunized with recombinant CRM197 protein. Antibody titers were expressed logarithmically in each group, the bar represent mean ± SEM (A-D). (A) IgG antibody titer against CRM197 antigen. (B) IgG1 and IgG2a antibody titer against CRM197 antigen. (C) the ratio of IgG1 to IgG2a antibody titer against CRM197 antigen. (D) the number of T cell secreted IFN-γ.

Fig.5 The level of IgG, Ig G1and IgG2a antibody and IFN-γ produced in mice immunized with RTCV. Antibody titers were expressed logarithmically in each group, the bar represent mean ± SEM (A-H). (A) IgG antibody titer against CRM197 antigen. (B) IgG1 and IgG2a antibody titer against CRM197 antigen. (C) the ratio of IgG1 to IgG2a antibody titer against CRM197 antigen. (D) the number of T cell secreted IFN-γ by CRM197 stimulate. (E) IgG antibody titer against TTc antigen. (F) IgG1 and IgG2a antibody titer against TTc antigen. (G) the ratio of IgG1 to IgG2a antibody titer against TTc antigen. (H) the number of T cell secreted IFN-γ by TTc stimulate.

Figures:

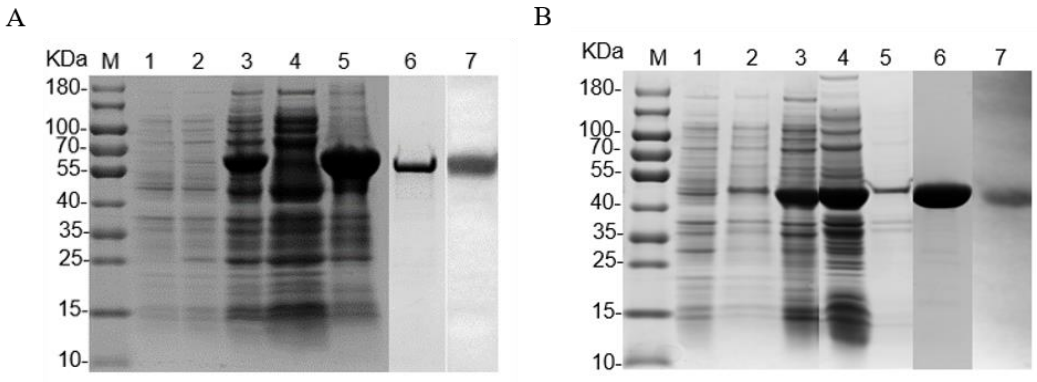

Fig.1 SDS-PAGE and Western Blot analysis of the recombinant proteins.

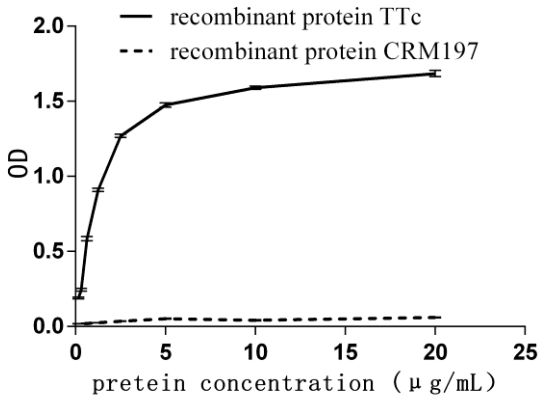

Fig.2 The bind of recombinant protein TTc to GT1b

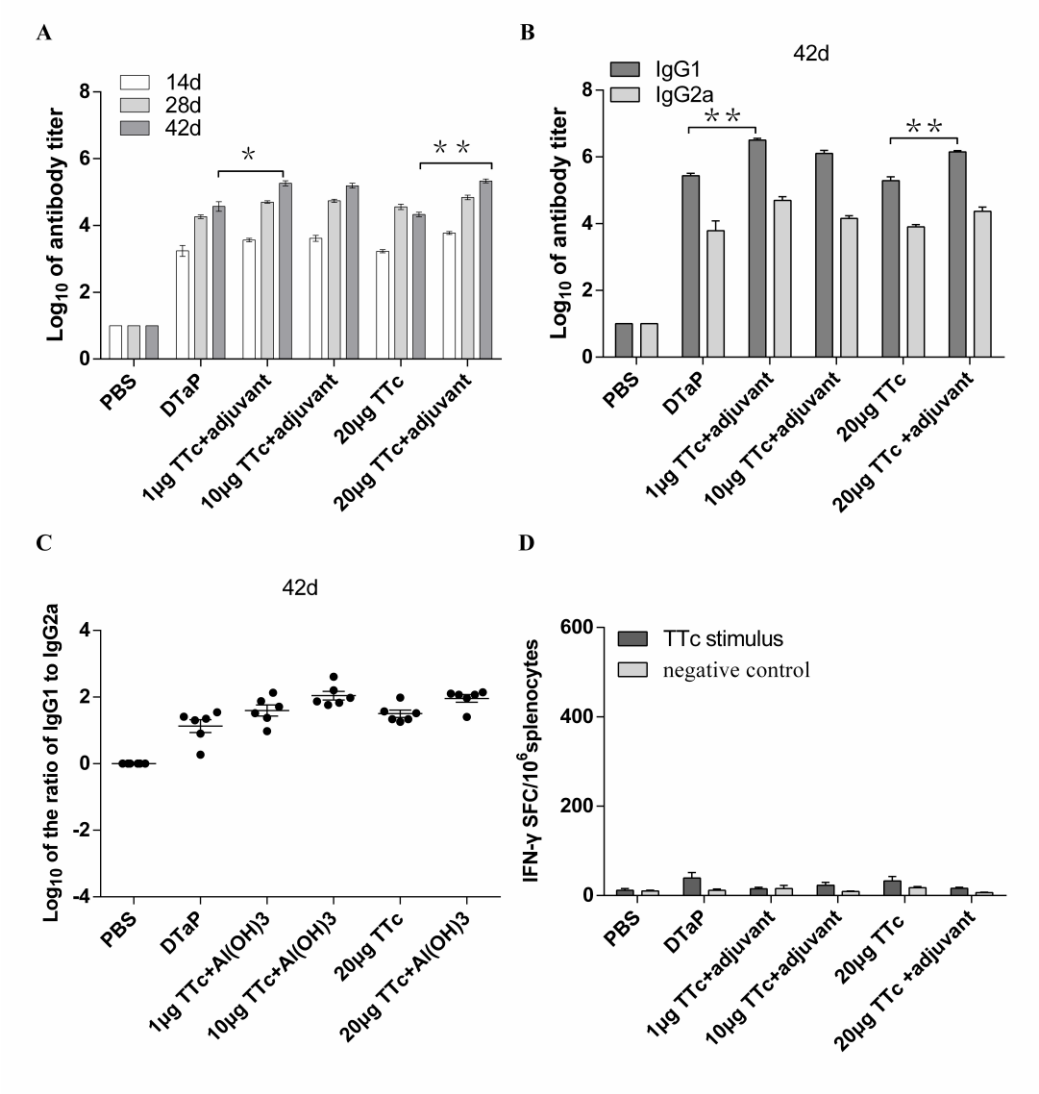

Fig.3 The level of IgG、IgG1 and IgG2a antibody and IFN-γ produced in mice immunized with TTc recombinant protein.

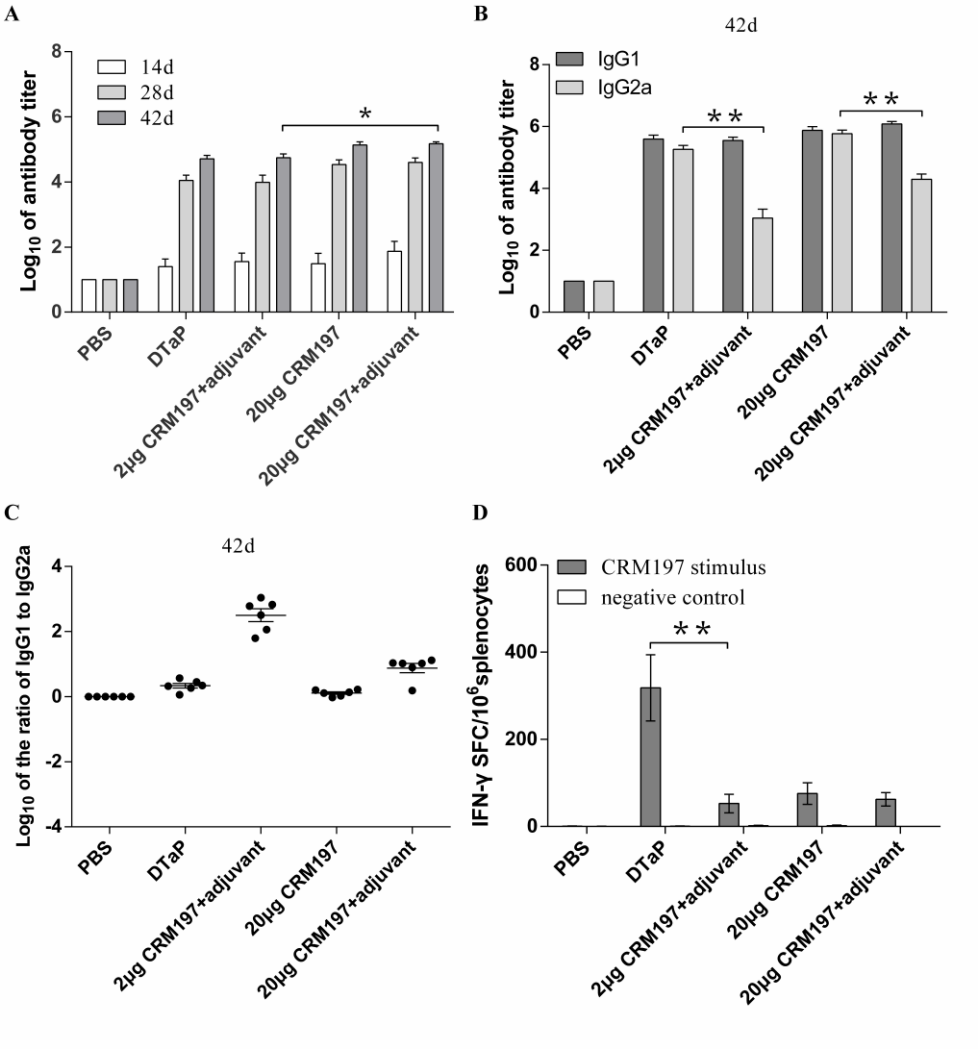

Fig.4 The level of IgG, IgG1and IgG2a antibody and IFN-γ produced in mice immunized with recombinant CRM197 protein

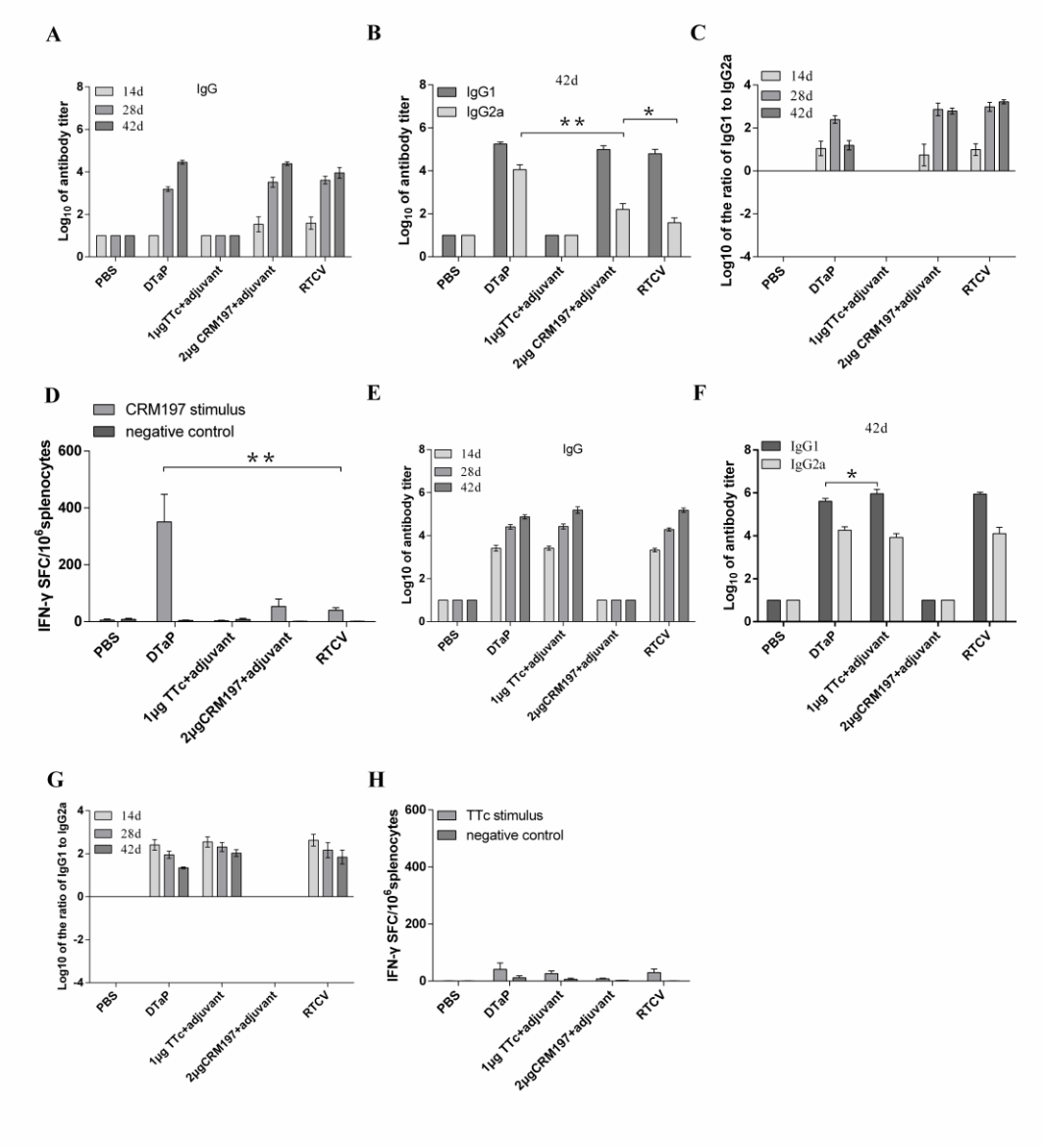

Fig.5 The level of IgG, Ig G1and IgG2a antibody and IFN-γ produced in mice immunized with RTCV

Ethical statement

Following the guidelines of the Animal Ethics Committee at Lanzhou University of Technology. The sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" set by the Ministry of Science and Technology, China. The procedures in the present study had received prior approval from the Experimental Animal Manage Committee of Lanzhou University of Technology.

Conflict of interest

No conflicts of interest to declare.

Author's contribution

PC, JL, XP designed the research; PC and XX performed the experiment; PC analyzed data and wrote the manuscript; JG, WW, SR and AL provided ideas, and revised the manuscript; all authors read and approved the final manuscript.