Check for updates

Received: 5 January 2022 | Revised: 22 February 2022 | Accepted: 8 March 2022

DOI: 10.1002/iid3.611

ORIGINAL ARTICLE

Immunity, Inflammation and Disease Open Access WILEY

Identification of B-cell dominant epitopes in the recombinant protein P29 from *Echinococcus granulosus*

Yongxue Lv[1,2] | **Shasha Li**[1,2] | **Tingrui Zhang**[1,2] | **Yazhou Zhu**[1,2] | **Jia Tao**[1,2] | **Jihui Yang**[1,2] | **Liangliang Chang**[1,2] | **Changyou Wu**[3] | **Wei Zhao**[1,2]

[1]School of Basic Medicine, Ningxia Medical University, Yinchuan, China

[2]Ningxia Key Laboratory of Prevention and Control of Common Infectious Diseases, Ningxia Hui Autonomous Region, Yinchuan, China

[3]Institute of Immunology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

Correspondence
Wei Zhao, School of Basic Medicine, Ningxia Medical University, Yinchuan 750004, China.
Email: Weizhao@nxmu.edu.cn

Funding information
Key Projects of the Natural Science Foundation of Ningxia Hui Autonomous Region, Grant/Award Number: 2020AAC02039; Natural Science Foundation of Ningxia Hui Autonomous Region, Grant/Award Numbers: 2021AAC03407, NZ15212; Ningxia Hui Autonomous Region Key Research Program, Grant/Award Number: 2018BEG02003; Key R&D Projects (Major Science and Technology Projects), Grant/Award Number: 2019BCG01001

Abstract

Introduction: *Echinococcus granulosus* (*E. granulosus*) causes a hazardous zoonotic parasitic disease. This parasite can occupy the liver and several areas of the body, causing incurable damage. Our previous studies have provided evidence that the recombinant protein P29 (*rEg.P29*) exhibit immune protection in sheep and mice against pathological damage induced by *E. granulosus*, showing its potential as candidate for vaccine development. However, information on the B-cell epitopes of *rEg.P29* has not yet been reported.

Methods: Immunological model was established in mice with *rEg.P29*. SDS-PAGE and Western blot were used to identify protein. Screening for B-cell dominant epitope peptides of *rEg.P29* by enzyme-linked immunosorbent assay (ELISA) and immune serum. Dominant epitopes were validated using ELISA and flow cytometry. Multiple sequence alignment analysis was performed using BLAST and UniProt.

Results: Immunization with *rEg.P29* induced intense and persistent antibody responses, and the epitope of the dominant antigen of B cells are identified as $rEg.P29_{166–185}$ (LKNAKTAEQKAKWEAEVRKD). Anti-$rEg.P29_{166–185}$-specific antibodies lack epitopes against IgA, IgE, and IgG3, compared to anti-*rEg.P29*-specific antibodies. However, anti-$rEg.P29_{166–185}$ IgG showed comparatively higher titers, as determined among those peptides by endpoint titration. In addition, *rEg.P29* and $rEg.P29_{166–185}$ promote B-cell activation and proliferation in vitro. The dominant epitopes are relatively conserved in different subtypes of *the rEg.P29* sequence.

Conclusion: $rEg.P29_{166–185}$ can act as a dominant B-cell epitope for *rEg.P29* and promote cell activation and proliferation in the same way as *rEg.P29*.

KEYWORDS
B-cell epitope, *Echinococcus granulosus*, *rEg.P29*

Yongxue Lv and Shasha Li contributed equally to this study.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2022 John Wiley & Sons Ltd.

1 | INTRODUCTION

Echinococcus granulosus is a zoonotic parasitic disease with an extremely serious economic burden and poses great danger in human health.[1] Western China has a high incidence of hydatid disease.[2] *E. granulosus* grows slowly, and more than 80% of echinococcosis cysts occur in the host liver, causing incurable damage.[3,4] Currently, the main treatment methods for patients with cystic echinococcosis (CE) are surgery and drug treatment[5]; however, drugs generally have side effects. Meanwhile, surgical treatment has a high recurrence rate and results in huge economic pressure and body damage to patients.[6–8]

The massive use of anthelmintics has caused a series of problems, such as drug resistance, drug residues, and environmental pollution.[3] Vaccines are safe and residue-free and are important tools for disease prevention and control.[9,10] However, traditional vaccines are composed of attenuated or inactivated pathogenic microorganisms and may cause unwanted or harmful immune responses in the body.[11] Therefore, the development of an effective and safe vaccine against parasitic diseases is important in animal husbandry and public health.

Despite the wide range of preventative approaches explored, a human vaccine against *E. granulosus* is not yet available. In fact, the *E. granulosus* vaccine has been proposed for a long time, and many candidate proteins have been studied, such as *Eg95*, antigen B, and *rEg.P29*.[12–16] Our previous study found that *rEg.P29* showed 94.5% and 96.6% protective efficacy in sheep and mouse models with secondary infection, respectively, and induced strong cellular and humoral immune responses against *E. granulosus* infection.[16,17] However, the anti-infective mechanism of *rEg.P29* is still unclear.

In recent years, as our understanding of the immune response has improved and research on vaccine production has become more refined, the search for the most precise vaccine components, that is, antigenic epitopes, in recombinant vaccines has begun. These antigenic epitopes represent the smallest immunogenic regions of protein antigens and can induce a specific immune response with the desired effects in the body.[18] Considering the importance of *rEg.P29*, an epitope vaccine containing moderate antigenic peptides of the gene may serve as an efficient vaccine against *E. granulosus* infection.

Studies have shown that B-cell-mediated humoral immune responses play an important role against diseases.[18] Based on this study, we sought to screen for a dominant B-cell epitope of *rEg.P29* and provide a basis for the construction of peptide-based Vaccines for *rEg.P29*.

2 | MATERIALS AND METHODS

2.1 | Animals and immunizations

This study was approved by the Experimental Animal Ethics Committee of the Ningxia Medical University. C57BL/6 female mice (SCXK2016-0006) aged 6–8 weeks were purchased from the Animal Center of Ningxia Medical University and kept for acclimatized feeding for 7 days before the experiment. Immunization protocols per mouse are summarized briefly as follows: 20 μg *rEg.P29* was mixed with 20 μg CpG ODN 1826 (*rEg.P29* + CpG), or 50 μl Freund's complete adjuvant (*rEg.P29* + FCA), and phosphate-buffered saline (PBS) was used as control. Booster immunization was performed using Freund's incomplete adjuvant. For subcutaneous immunization, the mixture was suspended in PBS and injected in the lower quadrant of the abdomen (100 μl/mouse). Immunization was performed three times, 1 week apart (Figure 1A).

2.2 | Amino acid sequence

The complete amino acid sequence of *rEg.P29* was obtained from GenBank (accession number XP_024351425.1).

2.3 | Antigen and adjuvant

Protein purification and expression were performed as described previously.[15] Briefly, the positive strain was induced overnight with 0.05 mg/ml isopropyl-b-D-thiogalactoside (IPTG; Invitrogen) at 37°C to express the recombinant protein P29, which was then purified using an anti-His-tagged nickel purification column (Merck). Purified *rEg.P29* was identified using Western blot analysis. A BCA Kit (KeyGEN Biotech Products) was used to detect the protein concentration. Next, an overlapping peptide library of *rEg.P29* and CpG ODN 1826 (TCCATGACGTTCCTGACGTT) was synthesized with98% purity with the assistance of Shanghai Shenggong Biological Co., Ltd. Complete and incomplete Freund's adjuvants were purchased from Sigma-Aldrich.

2.4 | Sample collection and cell preparation

Blood samples were obtained from the orbit, and the serum was collected and purified via centrifugation at 400 × g at 4°C for 10 min. Splenocytes were isolated from

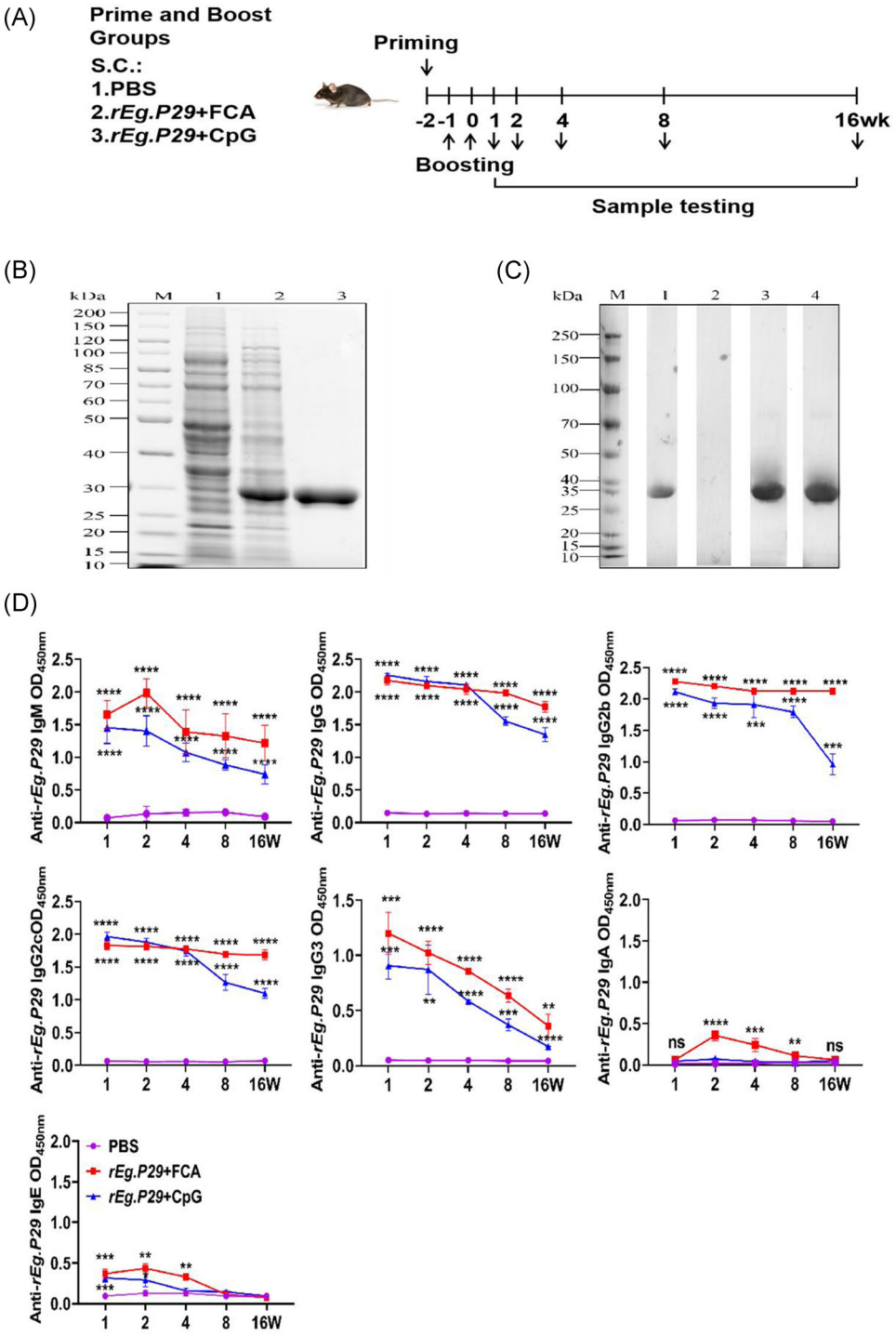


FIGURE 1 *rEg.P29* immunization induced a strong humoral immune response. Mice were primed and boosted with PBS, *rEg.P29*+FCA, or *rEg.P29*+CpG, following the prime-boost protocol. (A) At the indicated time interval, mice were killed and serum was separated by centrifugation. (B) The expressed and purified recombinant P29 protein was detected by SDS-PAGE analysis. Lane M, protein marker with molecular mass indicated on the left; Lane 1, *Escherichia coli* containing pET28a before IPTG induction; Lane 2, *E. coli* containing pET28a-P29 6 h after induction; Lane 3, purified *rEg.P29* using His-affinity chromatography, as indicated by the arrow. (C) Western blot identifies *rEg.P29*. Purified *rEg.P29* was immunoblotted with anti-His tag antibody or postimmunized serum from mice. M, protein marker; Lane 1, anti-his antibody; Lane 2, serum in PBS group; Lane 3, immune serum in *rEg.P29*+CpG group; Lane 4, immune serum in *rEg.P29*+FCA group. Anti-*rEg.P29* specific antibodies were detected by ELISA. (D) ELISA plate was coated with *rEg.P29* (10 μg/ml). Then, serum levels of anti-*rEg.P29* antibodies were measured by ELISA using HRP-labeled anti-mouse antibodies. The absorbance was read at 450 nm. ****$p < .0001$, ***$p < .001$, **$p < .01$, ns, not significant, $p > .05$

the tissue by filtering the tissue through a 70-μm strainer in a sample diluent, and the prepared cell suspension was transferred to a density gradient (Tianjin Haoyang biological products). Spleen lymphocytes were separated via centrifugation at 450×*g* for 20 min.

2.5 | Enzyme-linked immunosorbent assay for antibody production

Enzyme-linked immunosorbent assay (ELISA) plates were coated with individual peptides or *rEg.P29* at 10 μg/ml and incubated overnight at 4°C. The plates were washed five times with 0.05% Tween 20 PBS (PBST) and blocked with 5% skim milk powder in PBST at 37°C for 1 h. After washing five times with PBST, the plates were incubated with primary serum (1:100) in PBS (with 10% FBS) for 2 h and washed five times with PBST for 2–3 min. One hundred microliters each of horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, IgA, IgG1, IgG2a, IgG2b, IgG2c, and IgG3 (Cat nos.: ab97230, ab97023, ab97235, ab97240, ab97245, ab97250, ab97255, and ab97260, respectively; Abcam), and IgE (Cat no.: PA1-84764; Invitrogen) were added enzyme plates and incubated at 37°C for 1 h. After washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added for 8–10 min, and the reaction was stopped by 2 M H_2SO_4. The absorbance was measured at 450 nm within 15 min using an ELISA reader (Thermo Fisher Scientific).

2.6 | Flow cytometry analysis

For the flow cytometry assay, phycoerythrin-Texas Red (PE-CF594) conjugated-CD3, allophycocyanin-Cyanin 7 (APC-Cy7) conjugated-CD19, phycoerythrin (PE) conjugated-CD25, and Alexa Fluor700 conjugated-CD69 were purchased from BD Biosciences, whereas Fixable Viability Dye eFluor™ 506 was obtained from Invitrogen.

Splenic lymphocytes were suspended in complete RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated fetal calf serum (GeminiBio), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (Gibco). B-cell activation was detected using flow cytometry. Briefly, cells were incubated with or without *rEg.P29* or peptide (15 μg/ml) at 37°C, 5% CO_2 for 24 and 72 h. The cells were washed two times with PBS containing 0.1% bovine serum albumin (BSA) and 0.05% sodium azide (Buffer1). Then, the cells were stained with monoclonal antibodies (CD3, CD19, CD25, and CD69) for 30 min at 4°C in the dark. After washing with Buffer1, the cells were suspended in 100 μl of PBS and subjected to FACSCelesta (BD) for analysis and data collection.

As for proliferation, the cells were washed two times with pre-warmed PBS supplemented with 0.1% BSA, carboxyfluorescein diacetate succinimidyl ester at a final concentration of 2.5 μmol/L (CFSE, Invitrogen) was added, and the resulting mixture was incubated for 15 min at 37°C in the dark. The reaction was terminated with precooled RPMI1640 containing 10% FBS, incubated at 4°C for 5 min, and washed two times with precooled RPMI1640 containing 10% FBS. CFSE-labeled cells were then cultured with or without 15 μg/ml *rEg.P29* or peptide for 3 and 5 days at 37°C, 5% CO_2. Cell samples were collected and stained with fluorochrome-conjugated mAbs for phenotyping at 4°C in the dark. The samples were subjected to FACSCelesta™, and data were analyzed using FlowJo software (TreeStar).

2.7 | Multiple sequence alignment

The amino acid sequences of P29 of different subtypes of *E. granulosus* were obtained using BLAST, and the conserved dominant epitopes were analyzed using UniProt (https://www.uniprot.org/). Amino acid sequences obtained from *E. granulosus* were as follows: AHA85389.1 (*E. granulosus s.s.* [G1]), AHA85390.1 (*E. granulosus s.s.* [G1]), AHA85391.1 (*E. equinus*), AHA85392.1 (*E. ortleppi*), AHA85393.1 (*E. canadensis* [G6]), AHA85394.1 (*E. canadensis* [G7]), AHA85395.1 (*E. canadensis* [G10]), AHA85396.1 (*E. multilocularis*), AHA85397.1 (*E. multilocularis*), AHA85398.1 (*E. multilocularis*), and AHA85399.1 (*E. multilocularis*).

2.8 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc.) and SPSS 22.0. Unpaired Student's *t*-test was used for comparisons between two groups, and one-way or two-way analysis of variance was used for comparisons among more than two groups. The least significant difference test or Student–Newman–Keuls test was used to analyze data with normal distribution and homogeneous variance. Dunnett's T3 test or independent sample *t*-test was used to analyze data with normal distribution but had uneven variance. Data are represented as mean or mean ± SD. ****$p < .001$; ***$p < .005$; **$p < .01$; *$p < .05$ were considered statistically significant, whereas $p > .05$ was considered not significant (ns), as stated in figure legends.

3 | RESULTS

3.1 | Expression, purification, and identification of *rEg.P29*

The *rEg.P29* was successfully expressed and purified. SDS-PAGE analysis showed that *rEg.P29* had a molecular weight of 31 kDa (Figure 1B). The protein concentration was 1 mg/ml, and the protein was stored at −80°C). Western blot analysis showed that *rEg.P29* could not be recognized by antibodies in the mouse serum samples from the PBS group but could be recognized by a mouse monoclonal His-tag antibody and mouse sera from the immunization group (Figure 1C).

3.2 | *rEg.P29* induced a sustained antibody response

To assess whether *rEg.P29* induces specific immune responses in mice, we studied the antibody responses to *rEg.P29* immunization in mice sera. Both immunization groups were compared with the control group. Immunization with *rEg.P29* induced a high level of anti-*rEg.P29*-specific antibodies. The antibody responses peaked at Week 2 after boost immunization and were maintained for more than 16 weeks, except for IgA and IgE. Specifically, IgA and IgE levels gradually decreased at Week 4, but IgG remained at high levels. Both immunization groups were compared with the control group (Figure 1D).

3.3 | Immunization induced a polarized Th1 response

Characterization of IgG isotype responses in *rEg.P29* + FCA and *rEg.P29* + CpG group showed the induction of both IgG1 and IgG2a antibody responses, with the IgG2a isotype showing a comparatively higher level than IgG1 (Figure 2).

3.4 | Screening for the immunodominant linear B-cell epitope peptides

The sera of mice immunized with *rEg.P29* and HRP-labeled goat anti-mouse IgM and IgG were used as antibodies to detect the interaction between the antigen peptide (Table S1) and the serum. The dominant epitopes were selected, as shown in Figure 3A,B. Compared to the control group, the synthesized antigen peptide (Table S1) could induce ELISA-specific reactions with the immune serum. ID34-ID36 IgG and IgM levels were significantly higher than those of the other antigenic peptides in both immune groups.

We further assessed IgG specific for ID33, ID34, ID35, ID36, and ID37 using ELISA. All of these epitopes were detected by peptide-specific IgG in the *rEg.P29* + FCA group, but only anti-ID34- and anti-ID35-specific IgG were significantly higher than other antigenic peptides in both immune groups compared to the control group ($p < .0001$) (Figure 3C). To identify the core epitopes of the immunodominant peptide, we synthesized ID34 ($rEg.P29_{166–180}$), ID35 ($rEg.P29_{171–185}$), and their overlap ($rEg.P29_{171–180}$ and $rEg.P29_{166–185}$), as shown in Table 1. The ELISA plate was coated with these four peptides (10 μg/ml, respectively) and the positive control *rEg.P29*. Results indicated that the strongest IgG antibody reactivity was concentrated on a major immunodominant peptide, $rEg.P29_{166–185}$ (LKNAKTAEQKAKWEAEVRKD), of *rEg.P29* ($p < .001$, Figure 3D). In addition, anti-*reg*.$P29_{166–185}$ anti-$rEg.P29_{166-185}$IgG showed comparatively higher titers, as determined among those peptides by endpoint titration. As expected, anti-*rEg.P29* IgG maintained higher titers than anti-$rEg.P29_{166–185}$ IgG (Figure 3E).

3.5 | Detection of antibody subtypes of B-cell epitope core sequences

The ELISA plate was coated with *rEg.P29* and $rEg.P29_{166–185}$ to detect antibody subtypes of B-cell

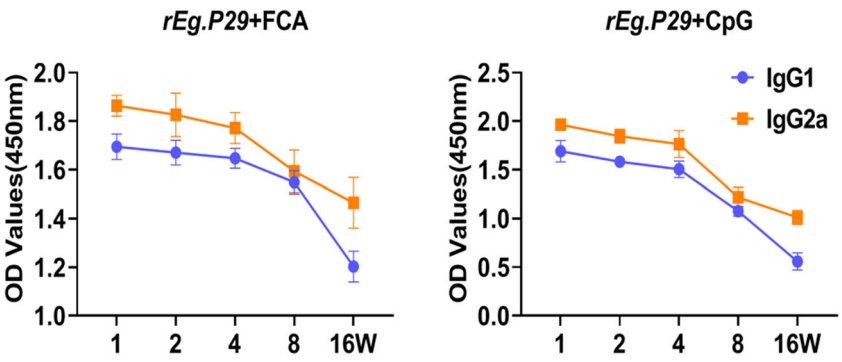


FIGURE 2 Vaccine-induced a polarized Th1 response. Anti-*rEg.P29* specific antibodies were detected by ELISA. IgG1 and IgG2a in serum from immunized mice.****$p < .0001$, ***$p < .001$, **$p < .01$, ns, not significant, $p > .05$

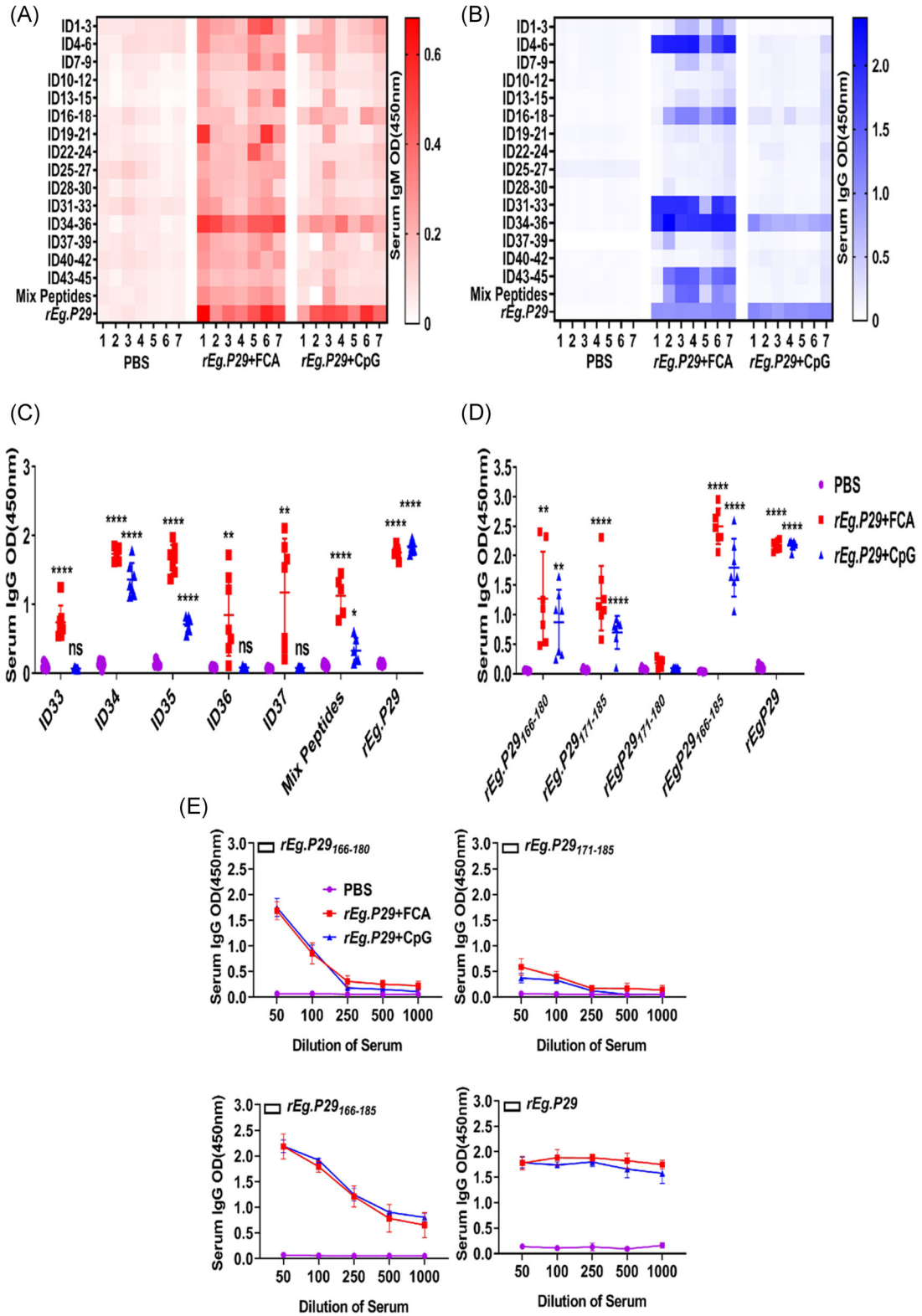


FIGURE 3 Identification of the core epitope of the immunodominant epitope peptide. ELISA detection of B-cell epitope peptides of *rEg.P29*. (A, B) To determine the immunodominant epitope peptide of *rEg.P29*, ELISA plate was coated with synthetic peptides or *rEg.P29*. Then, serum samples from C57 mice that were immunized with PBS, *rEg.P29* + FCA, or *rEg.P29* + CpG were detected. The absorbance was read at 450 nm. (C) ELISA plate was coated with ID33-ID37, mix peptides (ID1–ID45), or *rEg.P29,* specific IgG antibody was detected. (D) To determine the core epitope of immunodominant epitope peptide *rEg.P29*, ELISA plate was coated with four truncated or extended peptides. Then, serum samples from PBS, *rEg.P29* + FCA, or *rEg.P29* + CpG were detected.(E) Titers of IgG in sera from C57.****$p < .0001$, ***$p < .001$, **$p < .01$, ns, not significant, $p > .05$.

TABLE 1 Amino acid sequence of dominant peptides

SEQ ID	Location	Purity (%)	Sequence (N-C)	Number
ID34	*rEg.P29*$_{166–180}$	>98%	LKNAKTAEQKAKWEA	15
ID35	*rEg.P29*$_{171–185}$	>98%	TAEQKAKWEAEVRKD	15
	rEg.P29$_{171–180}$	>98%	TAEQKAKWEA	10
	rEg.P29$_{166–185}$	>98%	LKNAKTAEQKAKWEAEVRKD	20

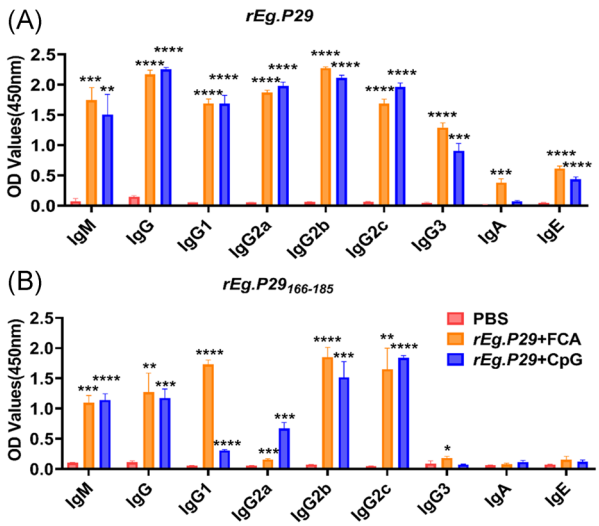


FIGURE 4 Anti-*rEg.P29* or core peptide specific antibodies. (A) ELISA plate was coated with *rEg.P29*. Then, serum samples from C57 mice that were immunized with PBS, *rEg.P29* + FCA, or *rEg.P29* + CpG were detected. (B) ELISA plate was coated with *rEg.P29*$_{166–185}$. Then, serum samples from C57 mice that were immunized with PBS, *rEg.P29* + FCA, or *rEg.P29* + CpG were detected.****$p < .0001$, ***$p < .001$, **$p < .01$, ns, not significant, $p > .05$

epitope core sequences. Results showed that anti-*rEg.P29*$_{166–185}$-specific antibodies lack epitopes against IgA, IgE, and IgG3, compared to anti-*rEg.P29*-specific antibodies (Figure 4A,B).

3.6 | *rEg.P29*$_{166–185}$ and *rEg.P29* promote B-cell activation and proliferation

The expression of CD69 and CD25 was assessed using flow cytometry to study the kinetics of cell activation. Results showed that the expression of CD69 and CD25 from day one was elevated (Figure 5A–D). Gating is shown in Figure S1. Increased CD69 expression in B cells after treatment with *rEg.P29* or *rEg.P29*$_{166–185}$ was significantly different from that in cells cultured without *rEg.P29* or *rEg.P29*$_{86–100}$ (medium) on Day 1 ($p < .001$, $p < .0001$; $p < .001$, $p < .001$). Meanwhile, CD69 expression in B cells decreased on Day 3 ($p < .01$, $p < .001$; $p < .001$, $p < .001$). Additionally, CD25-expressing cells treated with *rEg.P29* or *rEg.P29*$_{166–185}$ were significantly elevated on Day 3 compared to those not treated with *rEg.P29* or *rEg.P29*$_{166–185}$ (all $p < .0001$, $p < .0001$; $p < .0001$, $p < .0001$). These results suggest that most *rEg.P29*-specific B cells had already been activated from Day 1. It is worth noting that *rEg.P29*$_{166–185}$ induced slight increase in CD69 than the corresponding increase for *rEg.P29* ($p < .01$, $p > .05$; $p < .01$, $p > .05$); moreover, there was no difference in the magnitude of CD25 elevation ($p > .05$, $p > .05$; $p > .05$, $p > .05$).

Next, we performed CFSE labeling to estimate cell division (Figure 5E,F). After culturing with *rEg.P29* or *rEg.P29*$_{166–185}$, the CFSE intensity of a portion of B cells was decreased at Day 3 ($p < .01$, $p < .01$; $p < .05$, $p < .05$) and further decreased at Day 5 ($p < .0001$, $p < .0001$; $p < .0001$, $p < .001$) compared to the control group.

3.7 | *rEg.P29*$_{166–185}$ was relatively conserved in different subtypes of P29 sequences

Here, we analyzed the *rEg.P29* sequences of different genotypes of *E. granulosus* and the corresponding regions of the dominant peptides. As shown in Figure S3, the dominant peptide (*rEg.P29*$_{166–185}$) was completely conserved in the P29 sequences of different subtypes.

4 | DISCUSSION

With the improvement in living standards and sanitary conditions, the incidence rate of *E. granulosus* has been declining annually.[4] However, it is still a serious public health problem in pastoral areas. New disciplines, such as bioinformatics, immunoinformatics, and rational vaccine design, have been applied in recent years.[19,20] Among them, the design of a new vaccine composed of multiple epitopes of a single antigen has become a new method to enhance the host's protective immune response at humoral and cellular level. The epitope, also known as the antigenic determinant, is a chemical group in the antigen molecule that determines antigen

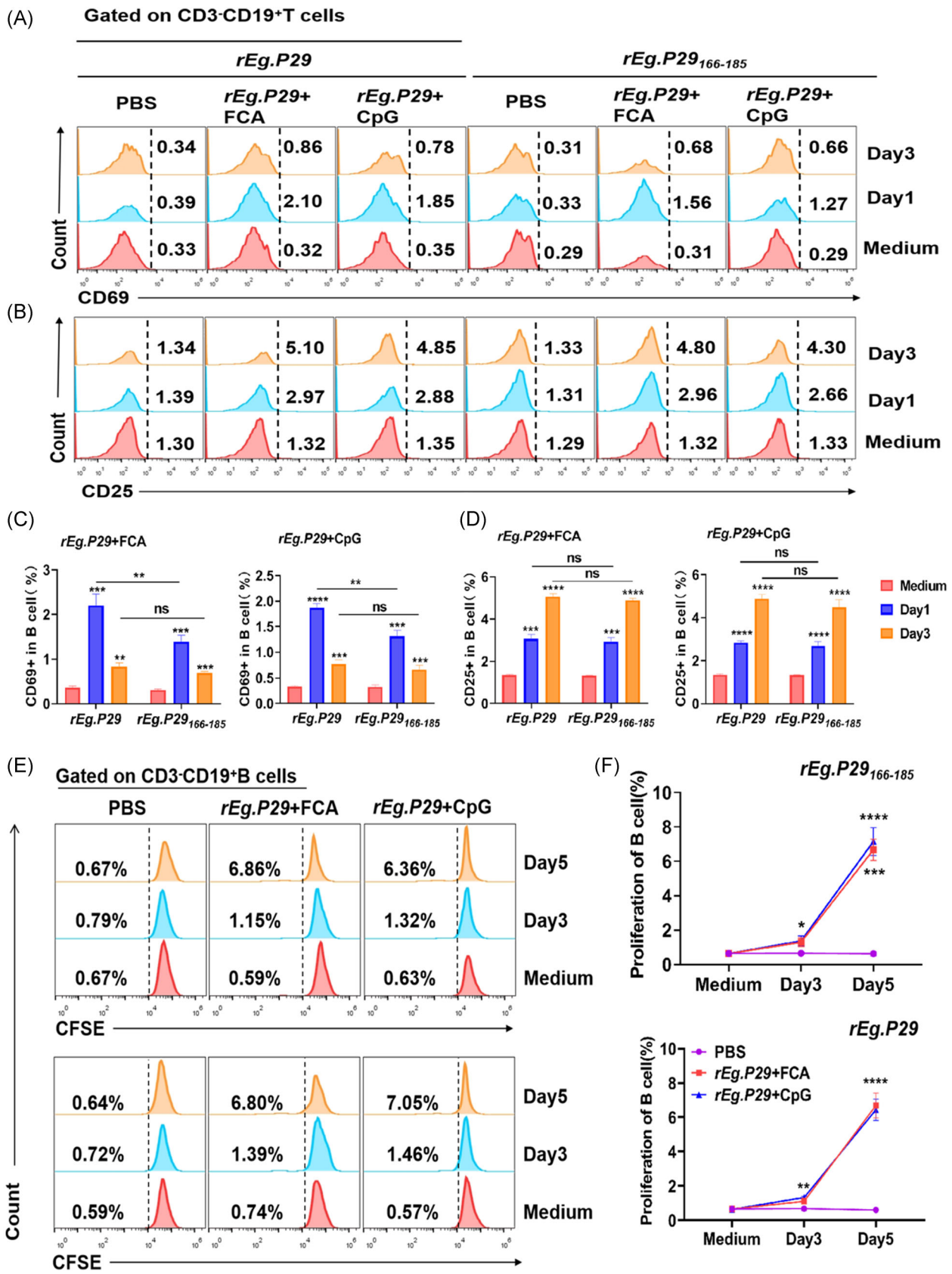


FIGURE 5 (See caption on next page)

specificity.[21] Epitope vaccines are based on the characteristics of the amino acid sequence of antigenic epitopes. They have become a new direction invaccine research because of their highly effective immune protection, safety, and stability.[22,23]

In 2013, Esmaelizad et al.[24] synthesized a multi-T-cell epitope antigen based on five proteins: EgGST, EgA31, Eg95, Eg14-3, and EgTrp. The antigen-stimulated mouse spleen cells to produce IFN-γ and obtained 99.6% protective efficacy in a mouse model. Simultaneously, multiepitope vaccines are also used in other fields.[25–27] The recombinant Bacillus Calmette–Guérin (BCG) vaccine (rBCG) constructed by Mohamud et al.[28] included two peptides, rBCG018 and rBCG032, that exhibited a stronger ability to induce cellular and humoral immune responses than BCG. Compared with traditional vaccines, synthetic peptide vaccines are easily prepared, have stable structure, and do not risk infection; thus, they are used for novel vaccine design at present.

Our previous studies have demonstrated that *rEg.P29* has a favorable protective effect in sheep and mouse models. However, the B-cell epitopes of *rEg.P29* remain unclear. Identification of the B-cell epitopes of *rEg.P29* contributes not only in elucidating the mechanism underlying the B-cell immune process of *E. granulosus* but also to the development of more effective epitope vaccine candidates. Screening epitopes with high immunogenicity is used for epitope development. Here, after the primer and booster doses of *rEg.P29*, the specific antibody levels peaked at Week 2. Immunized mice expressed IgM, IgG, IgG2a, and IgG2b, and few expressed IgA and IgE. Antibody production was maintained for at least 16 weeks, except for IgA and IgE. Studies have shown that IgG, IgG2a, and IgG2b may participate in the protective immune response induced by the vaccine, and IgG1 may be related to susceptibility to hydatidosis. Meanwhile, IgG3 may be associated with the formation of a local inflammatory response early in the host.[29] Th2-related antibodies and cytokines mainly promote the escape of parasites and establish immune tolerance, while Th1 related antibodies and cytokines are closely related to anti-infection.[30,31] Serological antibody tests showed that the immunized mice had a predominantly IgG and IgG2a antibody response against *rEg.P29*, followed by IgG1. Moreover, *rEg.P29* immunization induced a predominantly Th1 immune response. Our results showed that the dominant epitope lacks anti-$rEg.P29_{166–185}$ specific IgA, IgE, and IgG3 antibodies compared to anti-*rEg.P29* specific antibodies. These results suggest that $rEg.P29_{166–185}$ may only induce protective immunity while eliminating allergic reactions.[32,33] However, this difference may be caused by the adjuvant or the weak immunogenicity of the peptide, suggesting that multiple epitopes or vectors should be combined to improve the immunogenicity of the peptide. Furthermore, lymphocyte activation and proliferation levels are indicators that reflect the body's immune function. Here, we screened $rEg.P29_{166–185}$, for its ability to activate and proliferate B cells using flow cytometry. Results showed that $rEg.P29_{166–185}$ and *rEg.P29* promote B-cell activation and proliferation, highlighting that specific B cells can rapidly activate and proliferate in response to infection.

In addition, sequence alignment of the dominant epitope with the P29 protein of different isolates of *E. granulosus* revealed that $rEg.P29_{166–185}$ was highly conserved, suggesting its application as a broad-spectrum vaccine.[34]

Two adjuvants were used in the present study. CpG adjuvant is an oligodeoxynucleotide containing a CpG sequence with strong immune-activating properties; it was reported to show few side effects and can be used in humans.[35] Our results showed that both CpG and Freund's adjuvant enhanced the humoral immune response. However, Freund's adjuvant induces severe local necrotic ulcers and is considered to be toxic for human use. Considering the translational application of the vaccine, we used CpG adjuvant in subsequent experiments. Nonetheless, studies have shown that adjuvants alone do not induce specific antibody responses,[36] as shown in Figure S2.

This study has some limitations. Our study did not validate the immunoprotective effect after immunization of the dominant epitope. Further studies are warranted.

FIGURE 5 $rEg.P29_{166–185}$ and *rEg.P29* promoted specific B-cell activation and proliferation. Mice were primed and boosted with PBS, *rEg.P29* + FCA, or *rEg.P29* + CpG and killed at Week 2 after boosting. Mononuclear cells from the spleen were isolated. (A–D) Cells were stimulated with $rEg.P29_{166–185}$, *rEg.P29,* and medium for 1 and 3 days. The cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies for CD69 and CD25. Data were collected by flow cytometry and analyzed using FlowJo. Representative dot plots in (A) show CD69 expression in B cells, (B) show CD25 expression in B cells. Frequencies of CD69 expression in B cells from *rEg.P29* + FCA and *rEg.P29* + CpG groups are shown in (C). Frequencies of CD25 expression in B cells from *rEg.P29* + FCA and *rEg.P29* + CpG groups are shown in (D). Cells were used for CFSE labeling and stimulated with $rEg.P29_{166–185}$, *rEg.P29,* and medium for 3 and 5 days. The cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies for CD3 and CD19. Data were collected by flow cytometry and analyzed using FlowJo. Representative dot plots in (E) show the identification of proliferative B cells. Frequencies of proliferative B cells from PBS, *rEg.P29* + FCA, and *rEg.P29* + CpG groups are shown in (F). Data from five mice, ****$p < .0001$, ***$p < .001$, **$p < .01$, ns, not significant, $p > .05$

5 | CONCLUSION

In conclusion, our studies show that *rEg.P29* immunization can induce a strong and sustained antibody response, and *rEg.P29*$_{166–185}$ can act as its dominant B-cell epitope. Furthermore, *rEg.P29* and *rEg.P29*$_{166–185}$ stimulation in vitro can promote B-cell activation and proliferation.

ACKNOWLEDGMENTS

The authors thank Ningxia Medical University Science and Technology Center and Ningxia Key Laboratory of Prevention and Control of Common Infectious Diseases of Ningxia Medical University for providing the laboratory environment. This study was supported by the Key Projects of the Natural Science Foundation of Ningxia Hui Autonomous Region (No. 2020AAC02039), the Ningxia Hui Autonomous Region Key Research Program (No. 2018BEG02003), the Natural Science Foundation of Ningxia Hui Autonomous Region (No. NZ15212), Key R&D Projects (Major Science and Technology Projects; No. 2019BCG01001), and the Natural Science Foundation of Ningxia Hui Autonomous Region (No. 2021AAC03407).

ETHICS STATEMENT

All mouse experiments were given permission by the Ningxia Medical University Institutional Review Committee (Permit number: SCXK2016-0006) and carried out in strict accordance with national and institutional guidelines. The data obtained met the appropriate ethical requirements.

AUTHOR CONTRIBUTIONS

Wei Zhao and Changyou Wu were in charge of the project design; Yongxue Lv and Shasha Li were responsible for conducting the experiments, the data analysis, and paper writing; Tingrui Zhang and Jihui Yang were responsible for guiding Flow analysis in the experiment; Jia Tao was responsible for the design of relevant peptides in the experiment; Liangliang Chang and Yazhou Zhu were responsible for animal feeding; Yongxue Lv was in charge of the revision; Wei Zhao and Changyou Wu were responsible for the experimental guidance; all authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Contact the corresponding author for data and material requests.

ORCID

Yongxue Lv http://orcid.org/0000-0002-9095-0469

REFERENCES

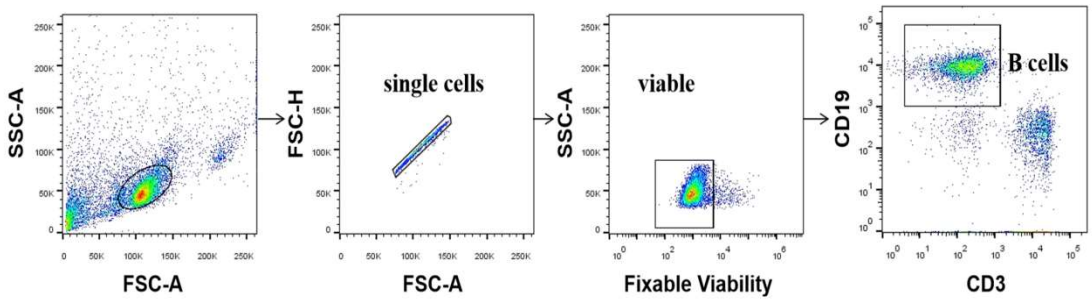
1. Borhani M, Fathi S, Darabi E, et al. Echinococcoses in Iran, Turkey, and Pakistan: old diseases in the new millennium. *Clin Microbiol Rev*. 2021;34:e29020. doi:10.1128/CMR.00290-20
2. Schweiger A, Ammann RW, Candinas D, et al. Human alveolar echinococcosis after fox population increase, Switzerland. *Emerging Infect Dis*. 2007;13:878-882. doi:10.3201/eid1306.061074
3. Sander VA, Sanchez LE, Mendoza ML, Ramos DV, Corigliano MG, Clemente M. Use of veterinary vaccines for livestock as a strategy to control foodborne parasitic diseases. *Front Cell Infect Microbiol*. 2020;10:288. doi:10.3389/fcimb.2020.00288
4. Wen H, Vuitton L, Tuxun T, et al. Echinococcosis: advances in the 21st century. *Clin Microbiol Rev*. 2019;32:e00075-18. doi:10.1128/CMR.00075-18
5. Baumann S, Shi R, Liu W, et al. Worldwide literature on epidemiology of human alveolar echinococcosis: a systematic review of research published in the twenty-first century. *Infection*. 2019;47:703-727. doi:10.1007/s15010-019-01325-2
6. Brunetti E, Kern P, Vuitton DA. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop*. 2010;114:1-16. doi:10.1016/j.actatropica.2009.11.001
7. Nabarro LE, Amin Z, Chiodini PL. Current management of cystic echinococcosis: a survey of specialist practice. *Clin Infect Dis*. 2015;60:721-728. doi:10.1093/cid/ciu931
8. Lissandrin R, Tamarozzi F, Mariconti M, Manciulli T, Brunetti E, Vola A. Watch and wait approach for inactive echinococcal cyst of the liver: an update. *Am J Trop Med Hyg*. 2018;99:375-379. doi:10.4269/ajtmh.18-0164
9. Pourseif MM, Moghaddam G, Nematollahi A, et al. Vaccination with rEGVac elicits immunoprotection against different stages of *Echinococcus granulosus* life cycle: a pilot study. *Acta Trop*. 2021;218:105883. doi:10.1016/j.actatropica.2021.105883
10. Larrieu E, Mujica G, Araya D, et al. Pilot field trial of the EG95 vaccine against ovine cystic echinococcosis in Rio Negro, Argentina: 8 years of work. *Acta Trop*. 2019;191:1-7. doi:10.1016/j.actatropica.2018.12.025
11. Amarir F, Rhalem A, Sadak A, et al. Control of cystic echinococcosis in the Middle Atlas, Morocco: field evaluation of the EG95 vaccine in sheep and cesticide treatment in dogs. *PLoS Neglected Trop Dis*. 2021;15:e9253. doi:10.1371/journal.pntd.0009253
12. Wang L, Gao J, Lan X, et al. Identification of combined T-cell and B-cell reactive *Echinococcus granulosus* 95 antigens for the potential development of a multi-epitope vaccine. *Ann Transl Med*. 2019;7:652. doi:10.21037/atm.2019.10.87
13. Kamenetzky L, Muzulin PM, Gutierrez AM, et al. High polymorphism in genes encoding antigen B from human infecting strains of *Echinococcus granulosus*. *Parasitology*. 2005;131:805-815. doi:10.1017/S0031182005008474
14. Da SE, Cancela M, Monteiro KM, Ferreira HB, Zaha A. Antigen B from *Echinococcus granulosus* enters mammalian cells by endocytic pathways. *PLoS Neglected Trop Dis*. 2018;12:e6473. doi:10.1371/journal.pntd.0006473
15. Wang H, Li Z, Gao F, et al. Immunoprotection of recombinant Eg.P29 against *Echinococcus granulosus* in sheep. *Vet Res Commun*. 2016;40:73-79. doi:10.1007/s11259-016-9656-7

16. Gottstein B, Stojkovic M, Vuitton DA, Millon L, Marcinkute A, Deplazes P. Threat of alveolar echinococcosis to public health—a challenge for Europe. *Trends Parasitol*. 2015;31:407-412. doi:10.1016/j.pt.2015.06.001
17. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov*. 2007;6:404-414. doi:10.1038/nrd2224
18. Conibear AC, Schmid A, Kamalov M, Becker C, Bello C. Recent advances in peptide-based approaches for cancer treatment. *Curr Med Chem*. 2020;27:1174-1205. doi:10.2174/0929867325666171123204851
19. Sabatino D. Medicinal chemistry and methodological advances in the development of peptide-based vaccines. *J Med Chem*. 2020;63:14184-14196. doi:10.1021/acs.jmedchem.0c00848
20. Piel L, Durfee CJ, White SN. Proteome-wide analysis of *Coxiella burnetii* for conserved T-cell epitopes with presentation across multiple host species. *BMC Bioinform*. 2021;22:296. doi:10.1186/s12859-021-04181-w
21. Hajissa K, Zakaria R, Suppian R, Mohamed Z. Epitope-based vaccine as a universal vaccination strategy against *Toxoplasma gondii* infection: a mini-review. *J Adv Vet Anim Res*. 2019;6:174-182. doi:10.5455/javar.2019.f329
22. Liu W, Tang H, Li L, Wang X, Yu Z, Li J. Peptide-based therapeutic cancer vaccine: current trends in clinical application. *Cell Proliferation*. 2021;54:e13025. doi:10.1111/cpr.13025
23. Nelde A, Rammensee HG, Walz JS. The peptide vaccine of the future. *Mol Cell Proteom*. 2021;20:100022. doi:10.1074/mcp.R120.002309
24. Esmaelizad M, Ahmadian G, Aghaiypour K, Shamsara M, Paykari H, Tebianian M. Induction of protective T-helper 1 immune responses against *Echinococcus granulosus* in mice by a multi-T-cell epitope antigen based on five proteins. *Mem Inst Oswaldo Cruz*. 2013;108:408-413. doi:10.1590/S0074-0276108042013003
25. Schumacher T, Bunse L, Pusch S, et al. A vaccine targeting mutant IDH1 induces antitumour immunity. *Nature*. 2014;512:324-327. doi:10.1038/nature13387
26. Li J, Zhao J, Shen J, Wu C, Liu J. Intranasal immunization with Mycobacterium tuberculosis Rv3615c induces sustained adaptive CD4(+) T-cell and antibody responses in the respiratory tract. *J Cell Mol Med*. 2019;23:596-609. doi:10.1111/jcmm.13965
27. Huang HJ, Curin M, Banerjee S, et al. A hypoallergenic peptide mix containing T cell epitopes of the clinically relevant house dust mite allergens. *Allergy*. 2019;74:2461-2478. doi:10.1111/all.13956
28. Mohamud R, Azlan M, Yero D, et al. Immunogenicity of recombinant *Mycobacterium bovis* bacille Calmette-Guerin clones expressing T and B cell epitopes of *Mycobacterium tuberculosis* antigens. *BMC Immunol*. 2013;14(Suppl 1):S5. doi:10.1186/1471-2172-14-S1-S5
29. Zhang W, Wen H, Li J, Lin R, McManus DP. Immunology and immunodiagnosis of cystic echinococcosis: an update. *Clin Dev Immunol*. 2012;2012:101895. doi:10.1155/2012/101895
30. Gottstein B, Soboslay P, Ortona E, Wang J, Siracusano A, Vuitton DA. Immunology of alveolar and cystic echinococcosis (AE and CE). *Adv Parasitol*. 2017;96:1-54. doi:10.1016/bs.apar.2016.09.005
31. Diaz A. Immunology of cystic echinococcosis (hydatid disease). *Br Med Bull*. 2017;124(1):121-133. doi:10.1093/bmb/ldx033
32. Bao J, Zheng H, Wang Y, et al. *Echinococcus granulosus* infection results in an increase in and genera in the gut of mice. *Front Microbiol*. 2018;9:2890. doi:10.3389/fmicb.2018.02890
33. Anvari D, Rezaei F, Ashouri A, et al. Current situation and future prospects of *Echinococcus granulosus* vaccine candidates: a systematic review. *Transbound Emerg Dis*. 2021;68(3):1080-1096. doi:10.1111/tbed.13772
34. Wang J, Dong R, Zou P, et al. Identification of a novel linear B cell epitope on the Sao Protein of *Streptococcus suis* serotype 2. *Front Immunol*. 2020;11:1492. doi:10.3389/fimmu.2020.01492
35. Papagno L, Kuse N, Lissina A, et al. The TLR9 ligand CpG ODN 2006 is a poor adjuvant for the induction of de novo CD8(+) T-cell responses in vitro. *Sci Rep*. 2020;10:11620. doi:10.1038/s41598-020-67704-0
36. Worzner K, Sheward DJ, Schmidt ST, et al. Adjuvanted SARS-CoV-2 spike protein elicits neutralizing antibodies and CD4 T cell responses after a single immunization in mice. *EBioMedicine*. 2021;63:103197. doi:10.1016/j.ebiom.2020.103197

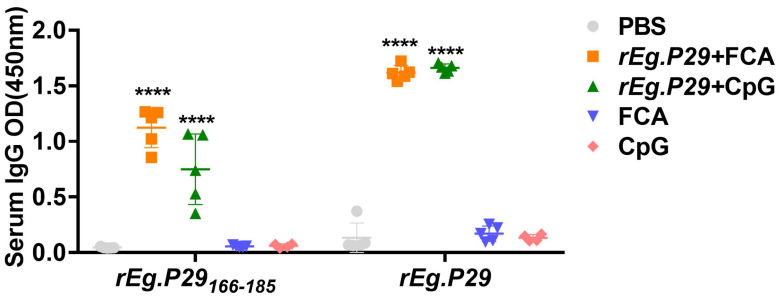
SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lv Y, Li S, Zhang T, et al. Identification of B-cell dominant epitopes in the recombinant protein P29 from *Echinococcus granulosus*. *Immun Inflamm Dis*. 2022;10:e611. doi:10.1002/iid3.611

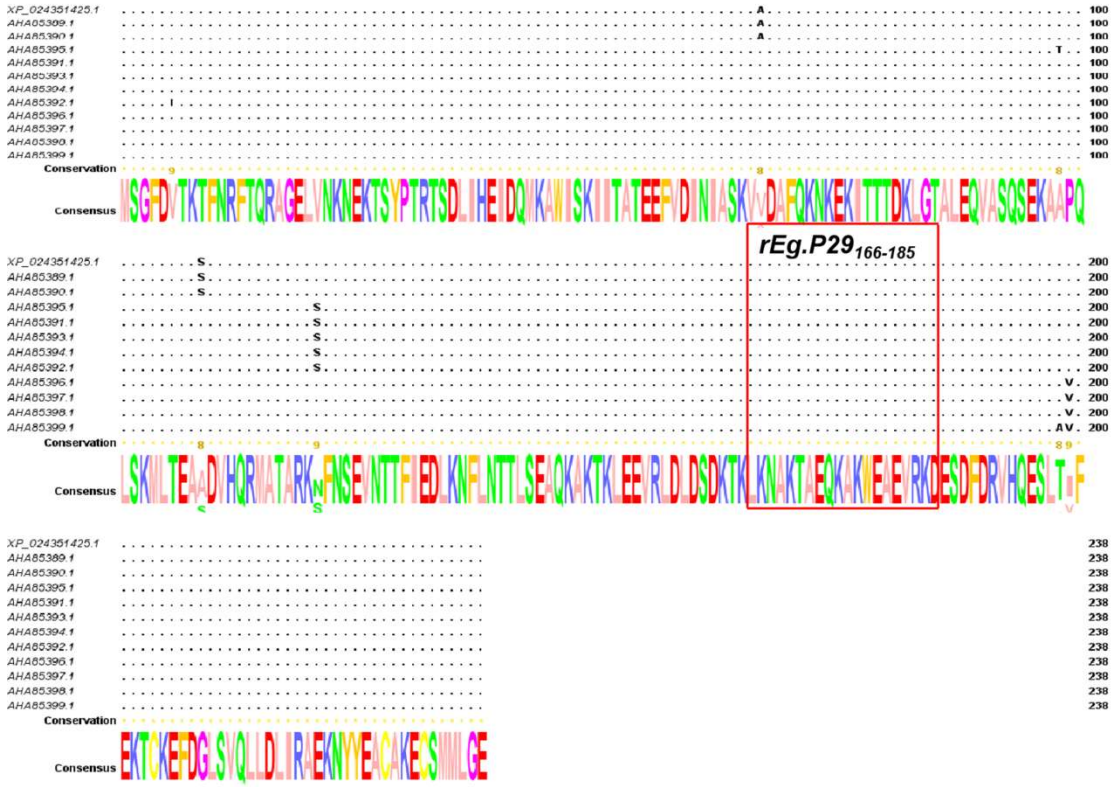


Supplementary Figure 1. After gating on lymphocytes,single cells, live cells, $CD3^{-}CD19^{+}$ B cells were divided based on CD3 and CD19 antibodies.



Supplementary Figure 2. Adjuvants did not induce specific antibody production.

Anti-*rEg.P29* specific antibodies were detected by ELISA.IgG antibody in serum from immunized mice.****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, ns= not significant, $P > 0.05$.



Supplementary Figure 3. Amino acid sequence alignment. Homology was analyzed using BLAST and UniProt.

Supplementary Table1

Amino acid sequences of overlapping peptides

No.		Sequence	number
ID1	*rEg.P29*$_{1\text{-}15}$	MSGFDVTKTFNRFTQ	15
ID2	*rEg.P29*$_{6\text{-}20}$	VTKTFNRFTQRAGEL	15
ID3	*rEg.P29*$_{11\text{-}25}$	NRFTQRAGELVNKNE	15
ID4	*rEg.P29*$_{16\text{-}30}$	RAGELVNKNEKTSYP	15
ID5	*rEg.P29*$_{21\text{-}35}$	VNKNEKTSYPTRTSD	15
ID6	*rEg.P29*$_{26\text{-}40}$	KTSYPTRTSDLIHEI	15
ID7	*rEg.P29*$_{31\text{-}45}$	TRTSDLIHEIDQMKA	15
ID8	*rEg.P29*$_{36\text{-}50}$	LIHEIDQMKAWISKI	15
ID9	*rEg.P29*$_{41\text{-}55}$	DQMKAWISKIITATE	15
ID10	*rEg.P29*$_{46\text{-}60}$	WISKIITATEEFVDI	15
ID11	*rEg.P29*$_{51\text{-}65}$	ITATEEFVDINIASK	15
ID12	*rEg.P29*$_{56\text{-}70}$	EFVDINIASKVADAF	15
ID13	*rEg.P29*$_{61\text{-}75}$	NIASKVADAFQKNKE	15
ID14	*rEg.P29*$_{66\text{-}80}$	VADAFQKNKEKITTT	15
ID15	*rEg.P29*$_{71\text{-}85}$	QKNKEKITTTDKLGT	15
ID16	*rEg.P29*$_{76\text{-}90}$	KITTTDKLGTALEQV	15

ID17	*rEg.P29*$_{81-95}$	DKLGTALEQVASQSE	15
ID18	*rEg.P29*$_{86-100}$	ALEQVASQSEKAAPQ	15
ID19	*rEg.P29*$_{91-105}$	ASQSEKAAPQLSKML	15
ID20	*rEg.P29*$_{96-110}$	KAAPQLSKMLTEASD	15
ID21	*rEg.P29*$_{101-115}$	LSKMLTEASDVHQRM	15
ID22	*rEg.P29*$_{106-120}$	TEASDVHQRMATARK	15
ID23	*rEg.P29*$_{111-125}$	VHQRMATARKNFNSE	15
ID24	*rEg.P29*$_{116-130}$	ATARKNFNSEVNTTF	15
ID25	*rEg.P29*$_{121-135}$	NFNSEVNTTFIEDLK	15
ID26	*rEg.P29*$_{126-140}$	VNTTFIEDLKNFLNT	15
ID27	*rEg.P29*$_{131-145}$	IEDLKNFLNTTLSEA	15
ID28	*rEg.P29*$_{136-150}$	NFLNTTLSEAQKAKT	15
ID29	*rEg.P29*$_{141-155}$	TLSEAQKAKTKLEEV	15
ID30	*rEg.P29*$_{146-160}$	QKAKTKLEEVRLDLD	15
ID31	*rEg.P29*$_{151-165}$	KLEEVRLDLDSDKTK	15
ID32	*rEg.P29*$_{156-170}$	RLDLDSDKTKLKNAK	15
ID33	*rEg.P29*$_{161-175}$	SDKTKLKNAKTAEQK	15
ID34	*rEg.P29*$_{166-180}$	LKNAKTAEQKAKWEA	15
ID35	*rEg.P29*$_{171-185}$	TAEQKAKWEAEVRKD	15
ID36	*rEg.P29*$_{176-190}$	AKWEAEVRKDESDFD	15
ID37	*rEg.P29*$_{181-195}$	EVRKDESDFDRVHQE	15
ID38	*rEg.P29*$_{186-200}$	ESDFDRVHQESLTIF	15
ID39	*rEg.P29*$_{191-205}$	RVHQESLTIFEKTCK	15
ID40	*rEg.P29*$_{196-210}$	SLTIFEKTCKEFDGL	15
ID41	*rEg.P29*$_{201-215}$	EKTCKEFDGLSVQLL	15
ID42	*rEg.P29*$_{206-221}$	EFDGLSVQLLDLIRA	15
ID43	*rEg.P29*$_{211-226}$	SVQLLDLIRAEKNYY	15
ID44	*rEg.P29*$_{216-231}$	DLIRAEKNYYEACAK	15
ID45	*rEg.P29*$_{221-236}$	EKNYYEACAKECSMM	15