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RESEARCH ARTICLE

Universal Design Multiepitope Vaccines Induces Effective Immunity to *Mycoplasma bovis* in BALB/C Mice and Rabbits

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ABSTRACT

Mycoplasma bovis is one of the primary pathogens associated with bovine respiratory disease. The clinical manifestations of this disease primarily include pneumonia, mastitis, and arthritis, which have resulted in significant economic losses worldwide. Currently, there is no effective vaccine available, and antibiotic treatment often yields unsatisfactory results. Therefore, the development of vaccines with high immunogenicity and effective protective capabilities is crucial for preventing and treating *M. bovis* outbreaks. This study aimed to develop a multiepitope vaccine for *M. bovis* and to evaluate its immunogenicity and protective efficacy in BALB/C mice and rabbits. On the basis of the three dominant antigens, MbovP274, MbovP570, and ENO1, the researchers designed the multiepitope tandem recombinant protein, MbovE3. The vaccine was prepared using prokaryotic expression vectors, baculovirus, and DNA vectors for expressing MbovE3, and BALB/C mice and rabbits were utilized as experimental models to verify vaccine effectiveness. The rMbovE3 vaccine, based on a prokaryotic expression system, induced high levels of specific IgG and cellular immune responses in both the BALB/C mice and rabbit models, significantly reducing *M. bovis* infection and tissue damage in rabbits. Additionally, MbovE3 expressed via baculovirus and the DNA vaccine DNAE3+GM-CSF, expressed by DNA vectors, both exhibited effective protective effects in rabbits. Overall, these findings suggest that rMbovE3, developed via a multiepitope approach, is a promising candidate vaccine against *M. bovis* infection. These findings provide a scientific foundation for the development of effective vaccines against *M. bovis* and will facilitate research on and the development of related vaccine products.

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INTRODUCTION

Mycoplasma bovis (M. bovis) is a cell wall-free bacterium that is recognized as one of the primary pathogens responsible for bovine respiratory disease (BRD). The characteristic symptoms of BRD include low fever, loss of appetite, mild depression, excessive tearing, nasal discharge, dyspnea, shortness of breath, and mild to persistent cough (Ayling *et al.*, 2018). In the United States, BRDs and mastitis caused by *Mycoplasma* result in annual losses exceeding 140 million US dollars; in Europe, the outbreak rate of pneumonia attributed to *M. bovis* in calves ranges from 25 to 33% (Caswell and Archambault, 2007). In 2008, *M. bovis* infection was first identified in newly imported beef cattle in China, characterized by a high incidence rate (over 80%) and a high mortality rate (10%) (Xin *et al.*, 2008). *M. bovis* can form adherent biofilms, a property that facilitates its proliferation within the host and contributes to the development of chronic diseases (Nicholas and Ayling, 2003; Laura *et al.*, 2006; Citti and Blanchard, 2013). Additionally, *M. bovis* can evade the host immune response by modulating the immune mechanisms of host cells (Poumarat *et al.*, 1994; Bürki *et al.*, 2015; Dudek *et al.*, 2016; Dudek *et al.*, 2020). Because it lacks a cell wall, *M. bovis* is naturally resistant to β-lactams and other antimicrobial drugs, which complicates the clinical treatment of *M. bovis* infection (Inna and Ayling, 2016). Typically, antibiotic treatment for *M. bovis* infections requires long-term administration or a combination of multiple drugs. Although vaccines for *M. bovis* have been developed, their protective effects remain suboptimal (Soehnlen *et al.*, 2011). These challenges underscore the urgency and importance of developing novel vaccines to prevent *M. bovis* infection.

Some relatively conserved antigenic proteins of *M. bovis*, such as p48 lipoprotein (Robino *et al.*, 2005), heat shock protein (HSP) (Scherm *et al.*, 2002), pyruvate dehydrogenase E1 (PdhA) (Prysliak and Casal, 2015) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Prysliak *et al.*, 2013), have been confirmed as potential targets for vaccine development. However, Prysliak and Casal, (2015) immunized calves with recombinant proteins such as PdhA, Tuf, PepA, P48, P81, DeoB, OppA, LppB, O256 and PepQ and the results indicated that these proteins did not confer immune protection. Although GAPDH is recognized as an antigenic protein and a potential candidate for BRD diagnosis and vaccine development (Zhang *et al.*, 2012), GAPDH-based vaccines have shown limited protective efficacy in cattle (Prysliak *et al.*, 2013). Consequently, there is an urgent need to identify new specific antigen proteins to facilitate the development of novel vaccine candidates.

The MbovP274 and MbovP570 proteins are potential lipoproteins of *M. bovis*, and they contain T-cell epitopes with high binding affinity for MHC class II molecules (Chong *et al.*, 2015). These proteins are highly antigenic and capable of inducing both innate and acquired immune responses, thereby representing potential protective antigens and subunit vaccine candidates (Dudek *et al.*, 2021). Additionally, MbovP0581, an ATP-binding protein in an ABC transporter, specifically reacts with *M. bovis*-positive serum and has high immunogenicity (Zubair *et al.*, 2020). The O256 protein, a member of the M42 family of metalloproteases, has been shown to significantly increase IgG2 titers when calves are immunized with a subunit vaccine formulated from the recombinant O256 protein and specific adjuvants (Prysliak *et al.*, 2017). Furthermore, the α-enolase (ENO1) protein is highly immunogenic in *Mycoplasma synoviae*(Lucijana BR *et al.*, 2008) and *Mycoplasma capricolum* (Perezcasal and Prysliak, 2007), suggesting that it may also serve as a significant immunogenic protein in *M. bovis*. Finally, the β subunit (PDHB) of *Mycoplasma* pyruvate dehydrogenase complex E1 is a cytoskeleton-like structural phosphoprotein that can be expressed on the surface of *Mycoplasma*, enabling adhesion by binding to host cell surface proteins. This protein has been identified on the surface of various bacteria and is highly antigenic in other *Mycoplasma species* (Pinto *et al.*, 2007; Su *et al.*, 2007; Dallo *et al.*, 2010). Studies have indicated that PDHB can react with *M. bovis*-positive serum and is immunogenic (Sun *et al.*, 2014). However, no studies have investigated the uniformity of the immune response and the protective effects induced by these six potential antigens in immunized animals.

These vaccines consist of multiple distinct epitopes, enabling the stimulation of targeted immune responses against specific epitopes, thereby presenting a powerful alternative immune approach (Saadi *et al.*, 2017; Zhang, 2018). Multiple epitope vaccines have been reported for a range of pathogens, including viruses (Qin *et al.*, 2021), bacteria (Zhang *et al.*, 2022) and parasites (Zhou *et al.*, 2023), and even for cancer (Lennerz *et al.*, 2014). However, in the context of mycoplasmal diseases, there are only a limited number of multiepitope vaccines, such as those targeting *Mycoplasma gallisepticum* (Gao *et al.*, 2022; Zhang *et al.*, 2023), *Mycoplasma hyopneumoniae* (Mugunthan and Harish, 2021), and *Mycobacterium ulcerans* (Nain *et al.*, 2020). Notably, a multiepitope vaccine specific for *M. bovis* has not yet been developed.

The researchers evaluated the immunogenicity of six candidate antigens (MbovP274, MbovP570, MbovP581, O256, ENO1, and PDHB) for the first time by immunizing mice and successfully identified three dominant antigens (MbovP274, MbovP570, and ENO1). On the basis of these identified dominant epitopes, the researchers constructed the first multiepitope tandem antigen, MbovE3, from *M. bovis* and assessed the immune response induced by this antigen in mouse models. The overall aim of this study was to develop a safe and effective multiepitope vaccine against *M. bovis* and evaluate its immunoprotective effects in animal models. The specific objectives were: (1) to identify dominant antigens from six candidates; (2) to construct the first multiepitope tandem antigen (MbovE3) from *M. bovis*; (3) to compare the immune responses induced by three vaccine formulations in mice; and (4) to assess their protective efficacy against *M. bovis* infection in rabbits.

MATERIALS AND METHODS

Ethics statements: All animal experiments were approved by the Biology Ethics Committee of Shihezi University (Approval No. A2025-002) and were conducted in accordance with national and institutional guidelines for animal care and use.

Animals: Female BALB/c mice (6–8 weeks old) were obtained from Charles River Laboratories. Female New Zealand rabbits (2 months old, ~1.5 kg) were obtained from the Shihezi University Experimental Animal Center. All animals were housed under standard conditions (temperature 25±1°C, humidity 60±5%, 12 h light/dark cycle) with free access to standard feed and water. Mice were euthanized by terminal blood collection via tail transection followed by cervical dislocation, and rabbits were euthanized by exsanguination via auricular venipuncture and air embolism.

Strains source: Our laboratory successfully isolated and preserved the *M. bovis* XJ-01 strain (Xiao *et al.*, 2023). *M. bovis* was cultured in complete PPLO medium at 37°C in a 5% CO_2 incubator for 2 to 3 days, following the culture method reported in a previous study (Chao *et al.*, 2019). Additionally, the *BL21* (DE3) *E. coli* strain and the pET-22b (+) expression vector, which were used for protein expression experiments, were obtained from our laboratory stocks.

Recombinant protein expression and purification: The researchers screened six *M. bovis* antigen candidates: MbovP274 (WP_013954681.1), MbovP570

(WP_013954901.1), MbovP581 (WP_014829958.1), O256 (WP_013456256.1), ENO1 (WP_013456555.1), and PDHB (WP_013456437.1). The gene sequences of these strains were codon optimized, His tags were added, and the sequences were subsequently cloned and inserted into the *E. coli* pET-22b(+) expression vector. Recombinant plasmids were obtained through double enzyme digestion and sequence verification. The plasmid containing the recombinant protein was transformed into the *E. coli* BL21 (DE3) strain to induce the expression of the target protein. Following expression, the bacterial cells were collected, resuspended, and lysed, and the lysate was centrifuged to separate the supernatant and precipitate. Recombinant proteins containing His tags were purified from either the supernatant or the precipitate using nickel (Ni-NTA) affinity chromatography. Following purification, the protein samples were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis was completed, the proteins were stained with Coomassie Brilliant Blue or, alternatively, transferred to a polyvinylidene fluoride (PVDF) membrane for subsequent Western blot analysis. Detection via Western blotting was conducted using mouse monoclonal antibodies specific for His-tags, along with polyclonal antibodies targeting the recombinant proteins, to confirm their expression. The absorbance values were measured at a wavelength of 570nM via a BCA protein assay kit (Solarbio, China).

Construction of the multiepitope vaccine rMbovE3: Three dominant antigens, MbovP274, MbovP570, and ENO1, were identified through mouse immunization experiments, followed by the prediction of their B-cell and T-cell epitopes. Linear B-cell epitopes were predicted using the ABCpred, SVMTriP, and BCpred servers, whereas cytotoxic T-cell and helper T-cell epitopes were predicted using the IEDB MHC-I and MHC-II servers. These predicted epitopes, the researchers constructed the multiepitope vaccine MbovE3, with a 6×His-tag incorporated at its C-terminus. The antigenicity of MbovE3 was subsequently predicted using the VaxiJen v2.0 server, and its allergenicity was evaluated using AllergenFP v.1.0. Additionally, the solubility of MbovE3 was predicted using SOLpro, protein properties were assessed with ExPASy ProtParam, the secondary structure was predicted using Sopma, the tertiary structure was simulated with I-Tasser, and visualization was performed using PyMOL. The quality of the tertiary structure was verified using PROCHECK for Ramachandran analysis and ProSA-web. The ElliPro tool was employed to predict B-cell epitopes, and the C-ImmSim server was used to simulate immune responses. The software tools mentioned in this section were all used with default parameters. The codon-optimized MbovE3 gene sequence was cloned and inserted into the Pet-22b (+) vector, expressed, and purified in BL21 (DE3), resulting in the successful production of the recombinant protein rMbovE3.

Immunological evaluation of subunit vaccines in BALB/c mice: BALB/c mice were randomly divided into seven experimental groups, with 10 mice in each group. The experimental groups were immunized with six recombinant proteins in combination with adjuvants, whereas the control group received an equivalent volume of PBS. *Mycobacterium* in Freund's complete adjuvant attracts macrophages and other immune cells to the injection site, thereby enhancing the immune response. Consequently, Freund's complete adjuvant was utilized for primary immunization, and Freund's incomplete adjuvant was employed for secondary immunization to assess the immunogenicity of the vaccine candidates (Harlow and Lane, 1988). The immunization procedure for the second batch of mice mirrored that used for the first batch. The 3H group was vaccinated with a mixture of three recombinant proteins, whereas the rMbovE3 group received the rMbovE3 protein.

To evaluate the levels of IgG, IgG1 and IgG2a antibodies in the serum of immunized mice, the researchers employed ELISA. The purified recombinant protein was added to a 96-well plate at a concentration of 1µg per well. Following overnight incubation, specific antibodies were detected in diluted mouse serum samples, after which HRP-labeled secondary antibodies were introduced. Finally, color development was initiated using TMB substrate solution and the optical density of each well was measured at 450nm using a VersaMax enzymatic tagger (Molecular Devices Corporation, CA, USA).

In addition, the researchers employed the ELISPOT method to measure the secretion of IFN-γ by splenic lymphocytes from immunized mice at 28 and 42 days postimmunization. Following the incubation of the splenic lymphocytes with ConA, PBS, and recombinant protein, the cells were treated with biotinylated anti-IFN-γ antibodies and streptavidin-ALP. Subsequently, BCIP/NBT solution was added for color development, and the resulting spots were quantified using an ELISpot reader (AID, D72479, Germany). The experiment was repeated three times to ensure the reliability of the results. The findings were used to assess the immune responses elicited by the vaccine candidates.

Evaluation of the protective efficacy of immunization with prokaryotic rMbovE3 in rabbits: The researchers conducted immunization and challenge experiments using rMbovE3 in New Zealand white rabbits to assess the protective efficacy of the vaccine. The rabbits were randomly divided into four groups and vaccinated with an adjuvant mixture of MbovP274, MbovP570, and ENO1 (1:1:1); the rMbovE3 protein; an inactivated *M. bovis* vaccine (IMA); or PBS. On the 28th day-postimmunization, all rabbits were challenged with 1.0×10^9 CFU of the *M. bovis* XJ-01 strain via the intranasal route for three consecutive days. Throughout the experiment, the researchers monitored clinical symptoms and changes in body weight and collected nasal swabs and lung tissue samples to determine the *M. bovis* load via RT–PCR. Additionally, the researchers measured the levels of IgG antibodies in the serum of rabbits postimmunization via ELISA and employed ELISPOT to detect IFN-γ secretion by peripheral blood lymphocytes. Furthermore, the researchers performed HE staining and immunohistochemical analysis of lung tissue to evaluate pathological changes and the *M. bovis* load following immunization. The primary antibody utilized for immunohistochemical analysis was a whole-bacterial monoclonal antibody that was prepared and is preserved in this laboratory. The antibody was diluted at a ratio of 1:100 and incubated overnight at 4°C.

Vaccine preparation and immunization with the baculovirus-expressed MbovE3 protein: The MbovE3 gene was subsequently cloned and inserted into the transfer vector plasmid pQB4b, resulting in the construction of the recombinant plasmid pQB4b-E3. SF9 cells were co-transfected with Bacmid DNA (qBac-IIIG, Shaanxi Bacmid Biotechnology Co., Ltd., Shaanxi, China) and pQB4b-E3, leading to the successful generation of the recombinant baculovirus qBac-IIIG-MbovE3. This recombinant baculovirus was subsequently used to infect both SF9 and High Five cells, and the expression of the MbovE3 protein was analyzed via SDS–PAGE following culture. The infected cells were collected and lysed, and the MbovE3 protein was purified from the lysate via Ni-NTA affinity chromatography. After washing and elution, the purified recombinant protein was obtained and verified via SDS–PAGE.

The purified MbovE3 protein (200μg) and Quil-A (50μg, InvivoGen, Toulouse, France) were combined to formulate the vaccine. Similarly, the MbovE3 protein (200μg) and ISA206 (Seppic, Inc., Paris, France) were mixed at a 1:1 ratio according to the manufacturer's instructions. The vaccines were then prepared for administration. The rabbits were immunized via subcutaneous multipoint injection, with the IMA vaccine serving as the positive control.

Preparation of and immunization with the DNA vaccine: The MbovE3 and granulocyte–macrophage colony–stimulating factor (GM–CSF) sequences were subsequently cloned and inserted into the pVAX1 eukaryotic expression vector (Miaoling Bio, Inc., Wuhan, China), which was subsequently transformed into DH5α *E. coli* competent cells. Positive clones were screened, amplified, and cultured in a sterile incubator maintained at 37°C. Following identification via double enzyme digestion, the recombinant plasmids were extracted and sequenced. The correct recombinant plasmids were designated pVAX1-MbovE3 and pVAX1-GM-CSF. A total of 450μg of pVAX1-MbovE3 was utilized to prepare the DNA E3 vaccine, while an equal amount of 450μg of pVAX1-MbovE3 and 450μg of pVAX1-GM-CSF was employed to formulate the DNA E3+GM-CSF vaccine. The rabbits were immunized subcutaneously, with the IMA vaccine serving as the positive control.

Quantification and statistical analysis: All the data presented herein represent the results from three separate experiments and are expressed as the means ± SDs. GraphPad Prism 8.0.2 software was used to generate the figures included in this study, and SPSS software was used for statistical analysis. Comparisons between different groups were conducted using one-way analysis of variance, multivariate analysis of variance, the Student–Newman–Keuls test, and Student's t test. Significance is indicated as follows: Ns indicates not significant ($P>0.05$), *indicates $0.01<P< 0.05$, **indicates $P<0.01$, ***indicates $P<0.001$ and **** indicates $P<0.0001$.

RESULTS

The recombinant protein was successfully expressed and purified: To construct a multiepitope vaccine, the researchers initially aimed to immunize mice with recombinant proteins and subsequently screened for dominant antigens based on levels of humoral and cellular immunity. The researchers selected six candidate antigens (Table S1) and employed a prokaryotic expression system to express and purify the corresponding recombinant proteins. The researchers verified the accuracy of the gene sequences for MbovP274, MbovP570, MbovP581, O256, ENO1, and PDHB using PCR, restriction enzyme digestion, and Illumina HiSeq high-throughput sequencing technology. The recombinant vectors containing these genes were subsequently transformed into *E. coli* BL21 (DE3) for protein expression. Notably, MbovP274, MbovP570, MbovP581, O256, and ENO1 were expressed primarily as soluble proteins, whereas PDHB was expressed in inclusion bodies (Fig. S1). The conditions for inducible expression utilized were 1mmol/L IPTG, with incubation carried out at 16°C for 20 hours. The concentrations of the purified recombinant proteins exceeded 1mg/mL, and the specific data are presented in Table S1. Western blot analysis confirmed that all proteins were recognized by His-tagged monoclonal antibodies and specifically reacted with polyclonal antibodies (Fig. S1). These results indicate that these six recombinant proteins were successfully expressed within the prokaryotic expression system and exhibited specific reactogenicity.

Immunization with recombinant proteins elicited robust cellular and humoral immune responses in BALB/c mice: To evaluate the immunogenicity of the six candidate antigens, the researchers prepared subunit vaccines by mixing six recombinant proteins with Freund's adjuvant for mouse immunization (Fig. 1A). The levels of specific IgG, IgG1, and IgG2a antibodies in the serum of immunized mice were significantly greater than those in the serum of the PBS control group at multiple time points. Notably, at 14 days after the first immunization, the IgG antibody levels in the MbovP274, MbovP570, and ENO1 groups were significantly elevated compared with those in the control group (Fig. 1B-D). Additionally, the MbovP274 and MbovP570 groups exhibited excellent performance in terms of increasing IgG1 antibody levels, with the MbovP274 group presenting the most significant increase in IgG2a antibody levels. These results confirm that the six recombinant subunit vaccines effectively induce the production of specific antibodies, with the MbovP274, MbovP570, and ENO1 groups showing the highest efficacy.

Table S1: Concentration information of recombinant protein after purification

Protein name	Accession number	Molecular weight (kDa)	Protein concentration (mg/mL)
MbovP274	WP_013954681.1	66	1.22
MbovP570	WP_013954901.1	85	1.12
MbovP581	WP_014829958.1	80	1.3
O256	WP_013456256.1	39	1.55
ENO1	WP_013456555.1	49	1.82
PDHB	WP_013456437.1	37	2.17

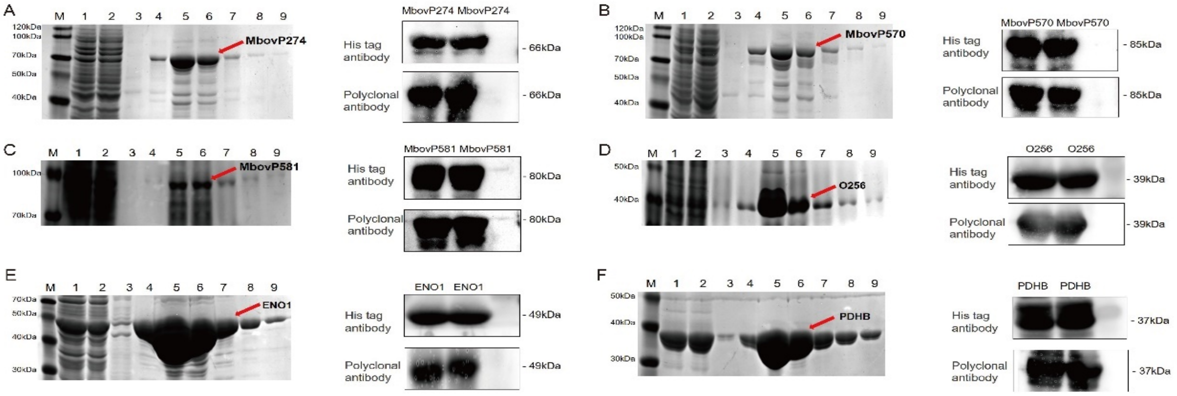


Fig. S1: Induced expression and Western blot identification of six candidate antigens. M: Protein Marker. 1-2: Wash impurity protein buffer. 3-9: Elution purification buffer. The red arrow indicates the position of the recombinant protein. Western blot identification of six recombinant proteins was performed using mouse His tag monoclonal antibodies and rabbit polyclonal antibodies. A: SDS-PAGE and Western blot identification of recombinant protein MbovP274. B: SDS-PAGE and Western blot identification of recombinant protein MbovP570. C: SDS-PAGE and Western blot identification of recombinant protein MbovP581. D: SDS-PAGE and Western blot identification of recombinant protein O256. E: SDS-PAGE and Western blot identification of recombinant protein ENO1. F: SDS-PAGE and Western blot identification of recombinant protein PDHB.

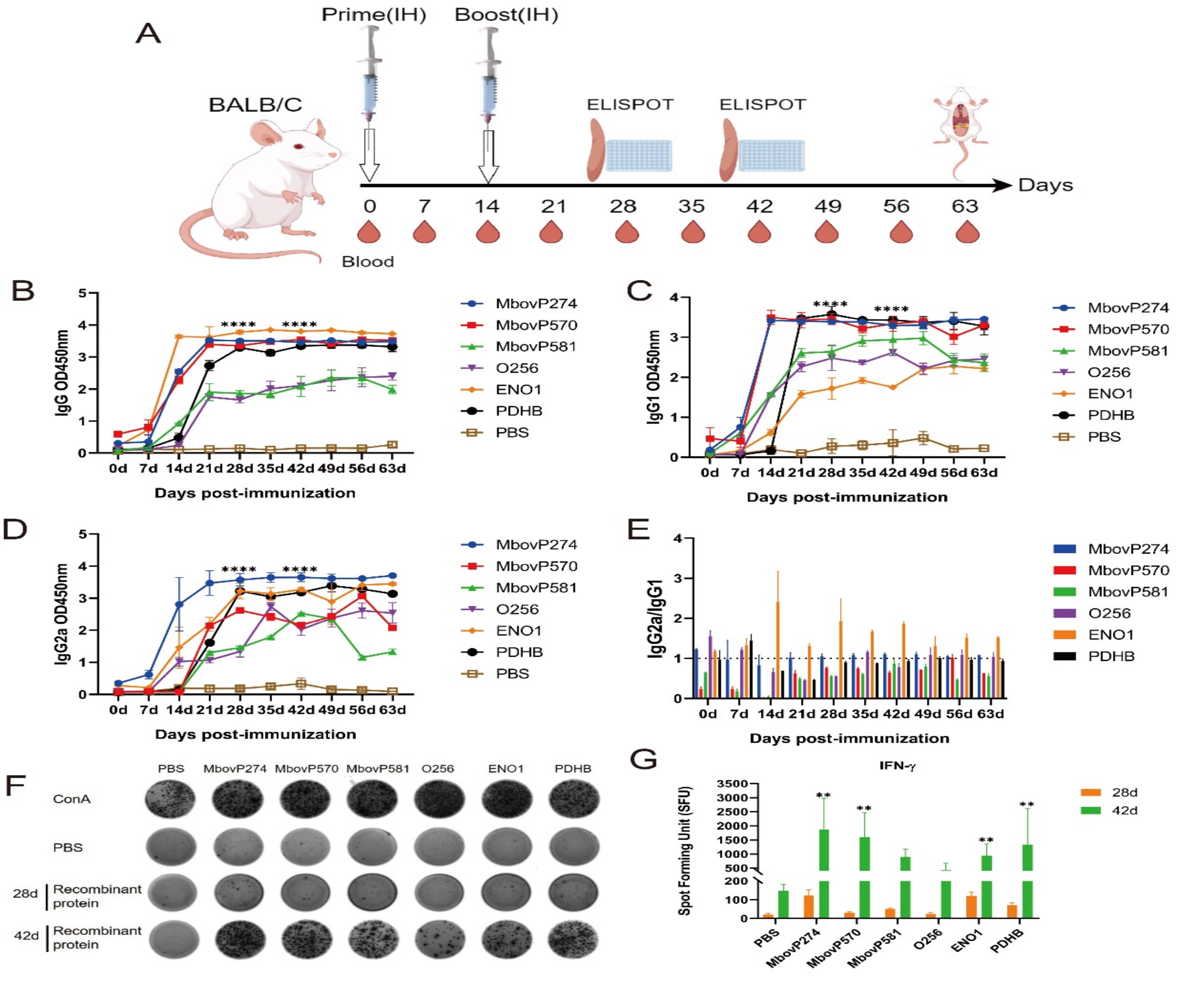
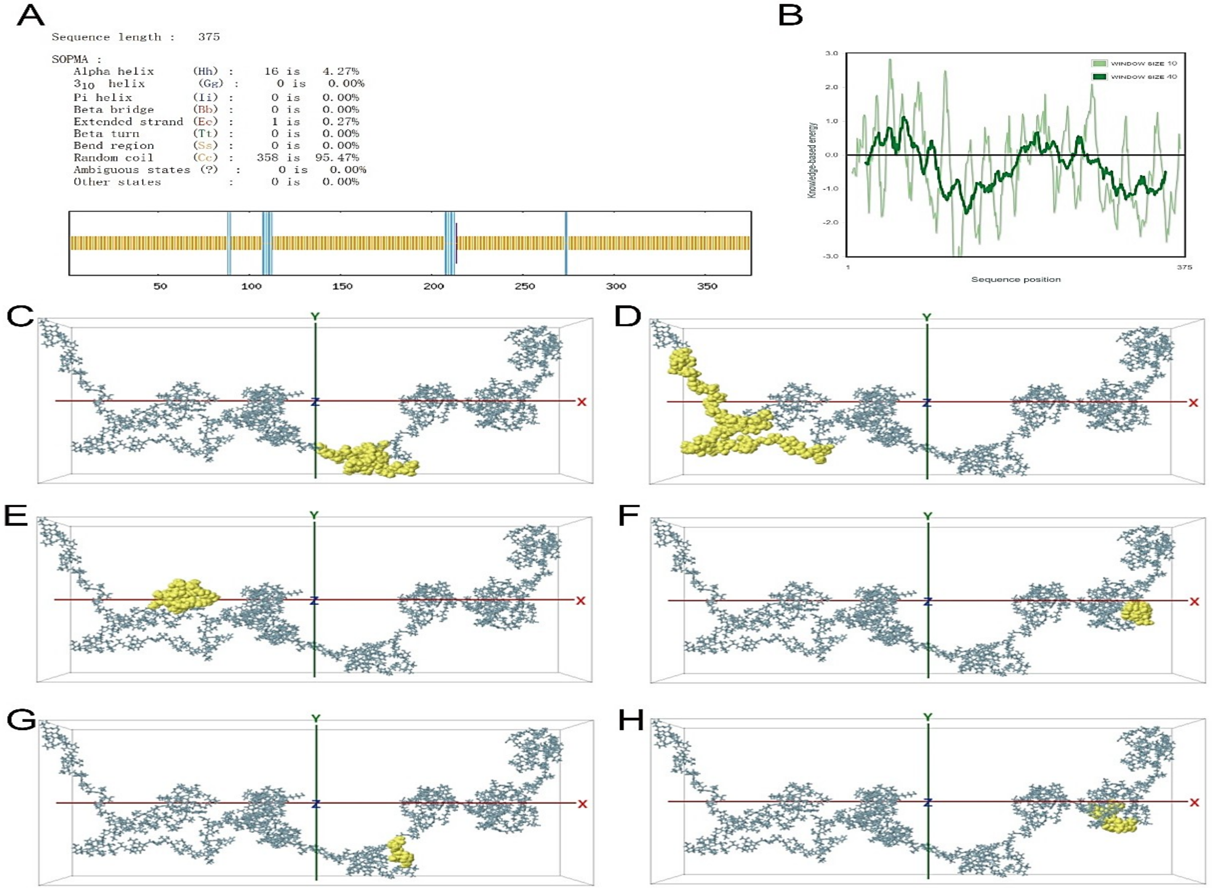


Fig. 1: Six subunit vaccines elicited robust cellular and humoral immune responses in mice. A: The vaccination program and sampling schedule are outlined. Six recombinant proteins were combined with Freund's adjuvant, and the mice were immunized subcutaneously at a dose of 100µg, with a second booster immunization administered two weeks later. The mice were euthanized nine weeks postimmunization. B: Analysis of the OD450 value of IgG was conducted using ELISA. Significance analysis was performed to compare the ENO1 group to the PBS control group. C: The OD450 value of IgG1 was also analyzed by ELISA. Significance analysis was performed to compare the MbovP274 group to the PBS control group. D: The OD450 value of IgG2a was also analyzed. Significance analysis was performed to compare the MbovP274 group to the PBS control group. E: Analysis of the ratio of IgG2a to IgG1 antibodies indicated that the induced immune responses tended to be either Th1- or Th2-type responses. F and G: ELISPOT technology was utilized to quantify IFN-γ-secreting T-cell spots (SFUs) in the groups immunized with the six subunit vaccines. Significance analysis was conducted to compare the 28-day immunization group and the PBS control group and assess the vaccine-induced cellular immune response. Ns indicates not significant (P>0.05), *indicates 0.01<P<0.05, ** indicates P<0.01, *** indicates P<0.001, and **** indicates P<0.0001. All the data are shown as the means ± SEMs from three independent tests.

The IgG2a/IgG1 ratio serves as a crucial indicator for assessing the balance between Th1 and Th2 immune responses. Elevated ratios are indicative of Th1-type responses, whereas diminished ratios suggest Th2-type responses. In the ENO1 group, the IgG2a/IgG1 ratio remained greater than 1 throughout the immunization period, suggesting a predominantly Th1-type immune response. Conversely, in the MbovP274 group, the initial ratio was less than 1, indicating a Th2-type tendency; however, at later time points, the ratio exceeded 1, signifying a shift toward a Th1-type response. The O256 group presented a ratio that was less than 1 at a specific time point, reflecting Th2-type characteristics, followed by a ratio greater than 1 at later time points, which indicated a Th1-type response. The ratios for the MbovP570, MbovP581, and PDHB groups consistently remained below 1, demonstrating a sustained Th2-type tendency (Fig. 1E). These results indicate that the ENO1 group primarily induced Th1-type responses, whereas the other vaccine candidates elicited varying degrees of Th1/Th2 responses.

After the second immunization, the researchers assessed vaccine-induced CD4+ T-cell responses in mouse spleen lymphocytes using the ELISPOT assay. The results indicated that the number of IFN-γ-secreting T cells (SFUs) in each group at 42 days was significantly greater than that in the PBS control group, with the numbers ranging from high to low as follows: MbovP274, MbovP570, PDHB, ENO1, MbovP581, and O256. In contrast, the number of SFUs was lower at 28 days (Fig. 1F and G). These findings demonstrate that the recombinant protein vaccine could significantly enhance the CD4+ T-cell response, particularly in the MbovP274 and MbovP570 groups. On the basis of these results, the researchers concluded that MbovP274, MbovP570, and ENO1 are the dominant antigens capable of inducing increased levels of both cellular and humoral immunity.

Construction and bioinformatics analysis of the multiepitope vaccine MbovE3: To identify the epitopes of the three dominant antigens, the researchers subjected the sequences of MbovP274, MbovP570, and ENO1 to epitope analysis using the ABCpred, SVMTriP, BCpred, and IEDB MHC-I and MHC-II servers. This process resulted in the identification of seven high-scoring B-cell epitopes and twelve T-cell epitopes. The epitopes were connected using GPGPG and GPLS linkers (Fig. 2A), and the MbovE3 protein, which includes a 6×His tag, was constructed, comprising 381 amino acid residues. Secondary structure analysis revealed that MbovE3 is composed predominantly of random coils (95.47%), with α-helices constituting 4.27% of the structure (Fig. S2A). The random coil region serves as a critical site for immune cell recognition (Yano *et al.*, 2005) and plays an important role in recognition by leukocytes and antibodies.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Sequence length :   375

SOPMA :
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   310  helix        (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ec) :     1 is   0.27%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   358 is  95.47%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
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Fig. S2: Prediction results for MbovE3 candidates. A: The secondary structure of MbovE3 was predicted using the Sopma software server. The results indicate that the random coil structure constitutes the largest proportion (95.47%), followed by the α-helical structure (4.27%). B: An energy map of the MbovE3 candidate 3D model was obtained through the ProSA-web server. C-H: The display of conformational B cell epitopes with scores less than 0.8. The conformation of the B cell epitope is represented by a yellow sphere in the figure.

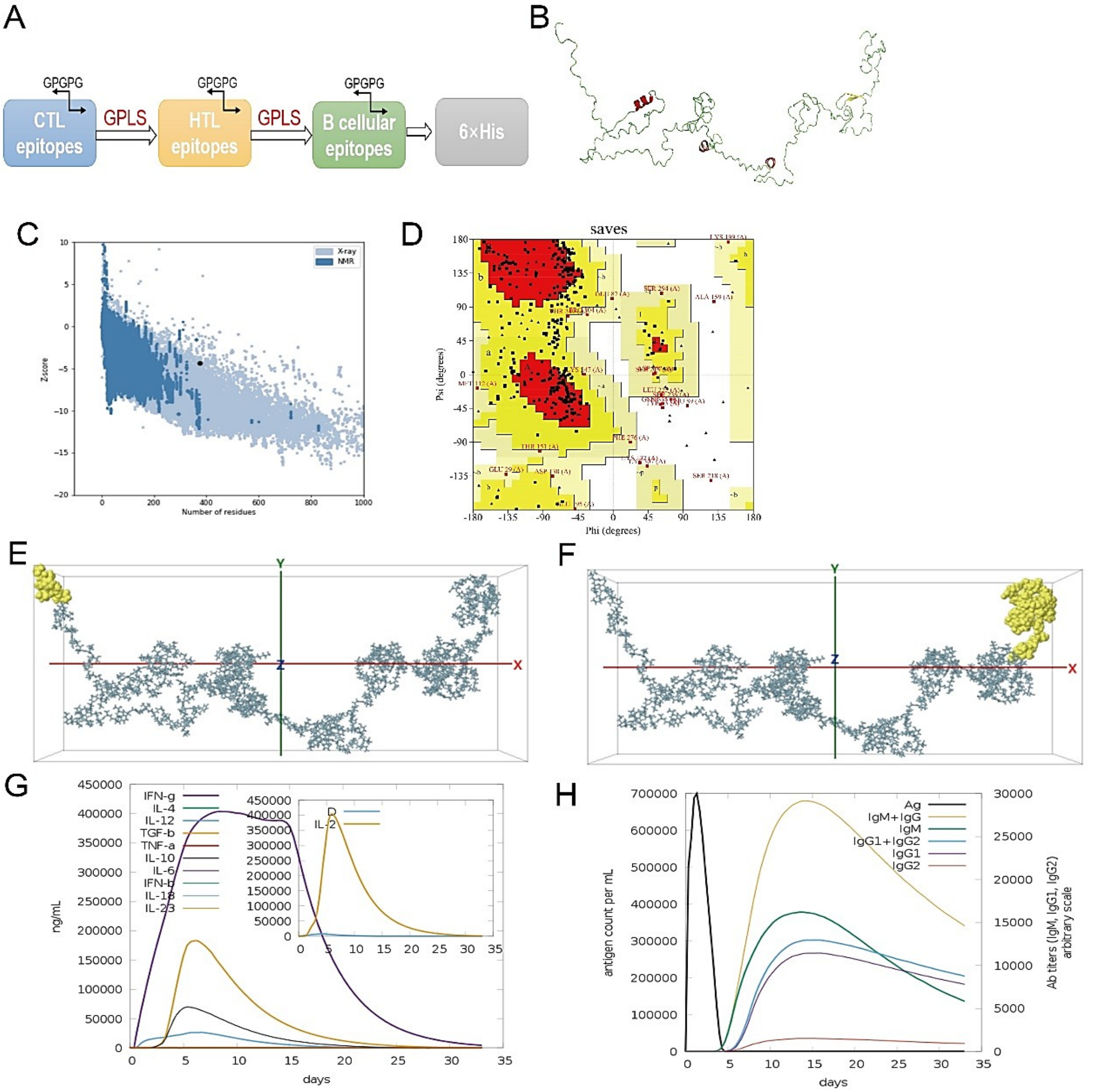

Fig. 2: Construction and bioinformatics analysis of the multiepitope vaccine MbovE3. A: Schematic diagram of the MbovE3 construct, which fuses six cytotoxic T-cell (CTL) epitopes, six helper T-cell (HTL) epitopes, and seven B-cell epitopes and includes a 6xHis tag at the C-terminus to facilitate subsequent purification and detection. B: The candidate 3D model of MbovE3, obtained using the I-TASSER server, provides a preliminary prediction of the protein's spatial structure. C: The Z score of the MbovE3 candidate 3D model, obtained via the ProSA-web server, is -4.31. A negative Z score indicates that the quality of the 3D protein model is superior. D: Analysis of the Ramachandran diagram for the MbovE3 3D model reveals that the preferred area constitutes 49.6% of the total area, the allowed area constitutes 47.4%, and the disallowed area constitutes 3%. These data are critical for assessing the accuracy and reliability of protein models. E and F: Discontinuous B-cell epitopes of the MbovE3 candidates (with a score greater than 0.8). The yellow spheres represent the conformations of highly scored discrete B-cell epitopes that are essential for immune recognition in vaccine design. G: This panel indicates changes in the immunoglobulins produced after antigen immunization (shown in black), while colored peaks represent changes in immune cell subtypes. H: The results demonstrate changes in cytokine secretion, focusing primarily on IFN-γ (indicated in purple) and IL-2 (indicated in yellow). These cytokines are crucial for mediating immune responses.

To ensure the viability of the MbovE3 vaccine candidate, its immunogenicity and allergenicity were evaluated. The results from the VaxiJen v2.0 server indicated that MbovE3 is highly antigenic, with a score of 0.8280, whereas AllergenFP v.1.0 confirmed its nonallergenicity. The SOLpro server predicted a solubility probability of 0.960602 for MbovE3. Analysis via the ProtParam online server revealed that MbovE3 has a molecular weight of 38.4 kDa, is alkaline (with a pI of 8.43), is stable (with an instability index of 17.90), has strong thermal stability (with an aliphatic index of 72.77), and is hydrophilic (GRAVY: -0.403) (Table S2). The tertiary structure of MbovE3 was visualized using PyMOL software (Fig. 2B), and the z score from ProSA-web was -4.31 (Fig. 2C and Fig. S2B), indicating superior model quality. Ramachandran plot analysis revealed that the preferred region of MbovE3 accounted for 49.6% of the structure, the allowed region accounted for 47.4%, and the disallowed region accounted for 3% (Fig. 2D), suggesting that the designed vaccine structure is of high quality and suitable for further research.

Table S2: Antigenic, allergenic and physicochemical assessments of the vaccine construct

Parameter	Result	Remark
Number of amino acids	378	Suitable
Molecular weight (Daltons)	38412.66	Average
Theoretical isoelectric point (Theoretical PI)	8.43	
Estimated half-life (mammalian reticulocytes, in vitro)	0.8 hours	
Estimated half-life (Yeast cells, in vivo)	10 min	
Estimated half-life (Escherichia coli, in vivo)	10 hours	Slightly basic
Instability index (II)	17.9	Stable
Aliphatic index (AI)	72.77	Thermostable
Grand average of hydropathicity (GRAVY)	-0.403	Hydrophobic
Antigenicity	0.828	Antigenic
Allergenicity	Non-allergenic	Non-allergenic
Solubility	0.960602	Soluble

Cell epitopes can be recognized by B-cell receptors or antibodies, thereby triggering humoral immune responses. The researchers utilized the ElliPro server to predict both conformational (discontinuous) and linear (continuous) B-cell epitopes. The ElliPro tool identified eight discontinuous B-cell epitopes (Fig. 2E, F, and Fig. S2C-H) and six linear epitopes (Fig. S3) in MbovE3. The lengths of the discontinuous epitopes ranged from 6 to 58 residues, with scores between 0.567 and 0.988; the linear epitopes varied in length from 5 to 48 residues, with scores ranging from 0.505 to 0.841. Fig. 2E and F illustrate discrete B-cell epitopes with scores exceeding 0.8. To further evaluate the potential of the designed vaccine to induce humoral and cellular immune responses, the researchers employed the C-ImmSim immune simulator to analyze the antibody response elicited by the MbovE3 vaccine. The findings indicate that MbovE3 effectively activates innate immunity and induces immune responses. The number of macrophages peaked on day 3 poststimulation (Fig. S4A), whereas the number of dendritic cells peaked on day 10 (Fig. S4B). C-ImmSim predicts that B lymphocytes primarily engage in antigen presentation during the early stage, with the active B-cell population reaching peak proliferation on the seventh day after stimulation (Fig. S4C). Additionally, MbovE3 significantly promoted CD4+ T-cell proliferation (Fig. S4D), and IgG+IgM antibody production peaked on day 15 (Fig. 2G). Furthermore, MbovE3 induced high levels of IFN-γ and IL-2 expression (Fig. 2H), demonstrating its potential to elicit immune responses. Collectively, these findings suggest that MbovE3 may serve as a promising candidate for vaccine development.

The multiepitope vaccine rMbovE3 elicited an enhanced immune response: The results presented in the previous section were based solely on software predictions, which raised questions regarding whether the MbovE3 designed by the researchers was truly immunogenic? To assess the immunogenicity of MbovE3, the researchers immunized mice with the recombinant protein rMbovE3 and evaluated the levels of both humoral and cellular immune responses in these mice. The researchers successfully expressed and purified the recombinant protein rMbovE3, which has a molecular weight of approximately 38 kDa (Fig. 3A).

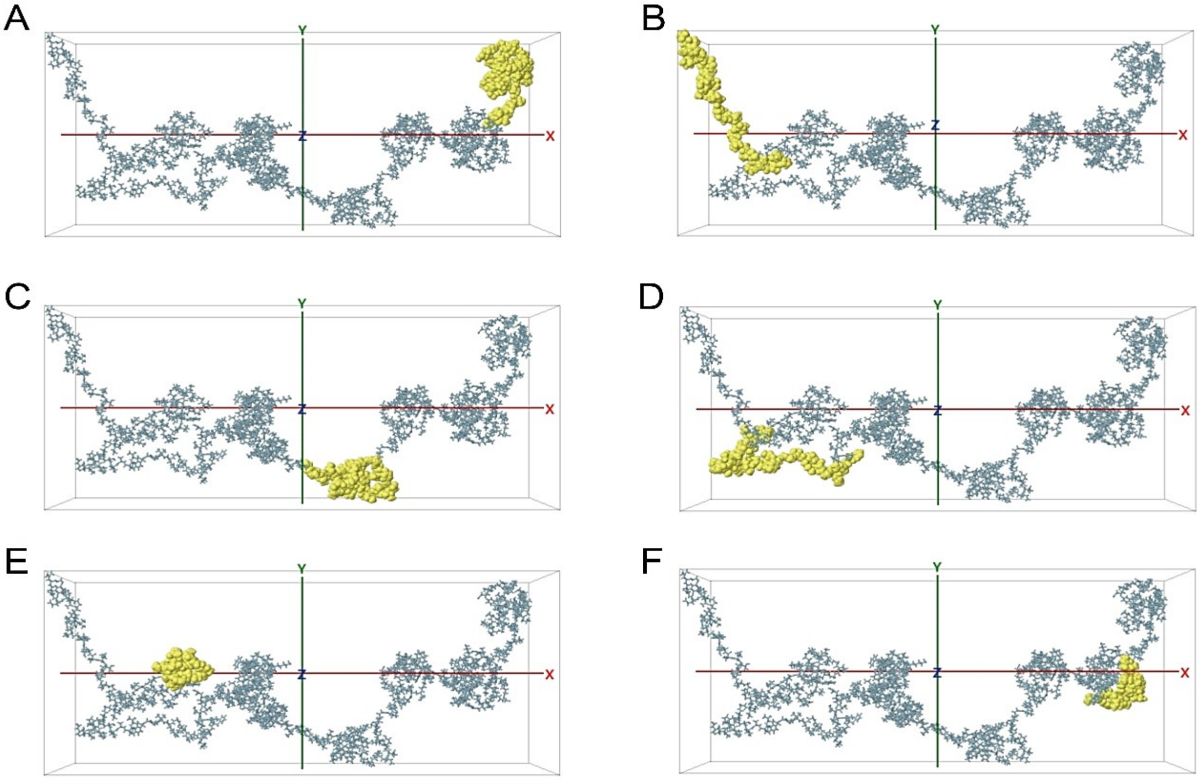

Fig. S3: Linear B cell epitopes of MbovE3 candidates. A-F: Presentation of linear B cell epitopes. The conformation of the linear B cell epitope is represented by a yellow sphere in the figure.

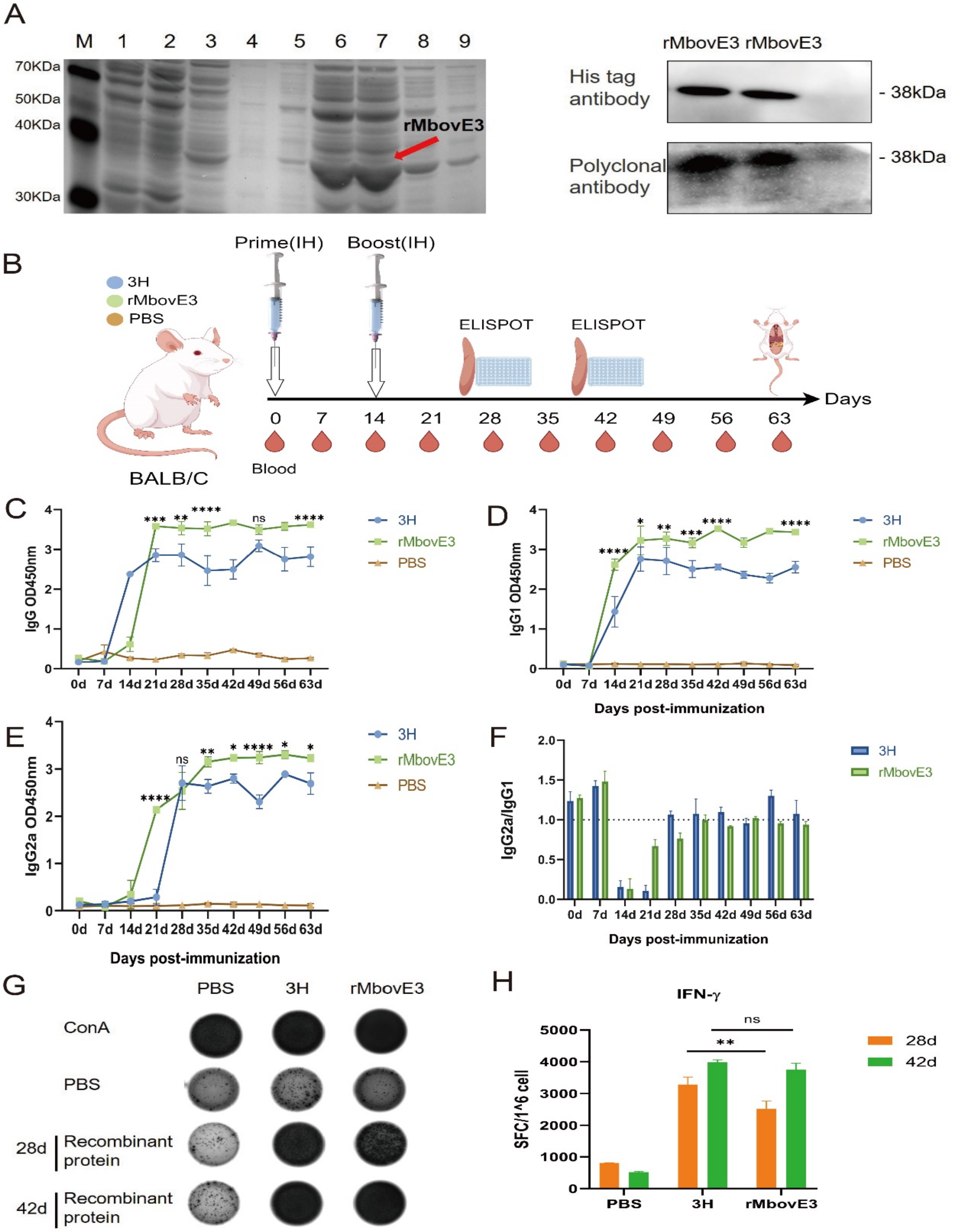

Fig. 3: Expression and purification of rMbovE3 and analysis of the vaccine-induced immune response. A: SDS–PAGE and Western blot identification of the recombinant protein rMbovE3. Lanes 1-2: washes with buffer to remove impurities; lanes 3-9: elution in buffer. The red arrow indicates the position of the recombinant protein rMbovE3. Western blot analysis was conducted using a mouse monoclonal His tag antibody and a rabbit anti-recombinant protein polyclonal antibody to confirm the successful expression and purification of the rMbovE3 protein. B: Vaccination program and sampling schedule. Mice were divided into two groups and immunized on days 0 and 14: one group received the recombinant rMbovE3 protein (designated as the rMbovE3 group), and the other group received a mixture of three dominant antigen proteins (MbovP274, MbovP570, and ENO1) at a 1:1:1 ratio (designated as the 3H group). Mouse spleen lymphocytes were isolated on days 28 and 42 for ELISPOT detection. Additionally, sera were collected weekly for antibody ELISA. C: Serum IgG levels measured by ELISA (OD450). D: Serum IgG1 levels. E: Serum IgG2a levels. F: IgG2a/IgG1 ratio. G and H: SFUs in the immune group were quantified using ELISPOT technology, and the results were compared between the rMbovE3 group and the 3H group. Ns indicates not significant (P>0.05), 0.01 <* P< 0.05, ** P<0.01, *** P<0.001, and **** P<0.0001. All the data are shown as the means ± SEMs from three independent tests.

Immunoreactivity analysis revealed that rMbovE3 exhibited strong reactivity with both monoclonal His-tag antibodies and polyclonal rabbit anti-recombinant protein antibodies (Fig. 3A). rMbovE3, along with a mixture of MbovP274, MbovP570, and ENO1 antigens (3H), was combined with Freund's adjuvant and subsequently used to immunize mice (Fig. 3B). The researchers observed that the levels of specific IgG, IgG1, and IgG2a antibodies in the serum of the mice vaccinated with rMbovE3 were significantly greater than those in the PBS control group. Notably, the IgG antibody titers continued to rise until 7 weeks postbooster immunization (Fig. 3C-E). Furthermore, the antibody levels in the rMbovE3 group from days 21 to 63 were significantly greater than those in the 3H group, indicating a more robust humoral immune response. Analysis of the IgG2a/IgG1 ratio suggested that rMbovE3 immunization predominantly induced Th2-type responses, whereas 3H immunization elicited Th1-type responses (Fig. 3F). These results demonstrate the superiority of rMbovE3 in stimulating specific humoral immunity and reveal differences in immune response type. The researchers evaluated CD4$^+$ T-cell responses in the mice and reported that, on day 28, the number of SFUs in both the 3H and rMbovE3 groups was significantly greater than that in the PBS control group, with the SFU count in the 3H group being greater than that in the rMbovE3 group. On day 42, the SFU counts in both groups remained significantly elevated compared with those in the control group; however, no significant difference was observed between the two groups (Fig. 3G and H). In summary, the rMbovE3 group exhibited stronger humoral and cellular immune responses following immunization than did the 3H group.

The rMbovE3 vaccine could effectively protect rabbits from *Mycoplasma bovis* infection: Research has indicated that rabbits are appropriate models for assessing the efficacy of *M. bovis* vaccines (Zhang *et al.*, 2023). To investigate the immune responses and protective effects induced by the rMbovE3 vaccine in rabbits, the researchers formulated three subunit vaccines by combining rMbovE3 (1mg), recombinant proteins of three dominant antigens (1mg, 3H group), and formaldehyde-inactivated *M. bovis* XJ-01 (IMA group) with Freund's adjuvant. These vaccines were administered via subcutaneous injection at multiple sites in rabbits. On the 28th day postimmunization, the protective efficacy of each vaccine was assessed via nasal instillation of 1.0×10^9 CFU of the *M. bovis* XJ-01 strain for three consecutive days to simulate natural infection conditions (Fig. 4A).

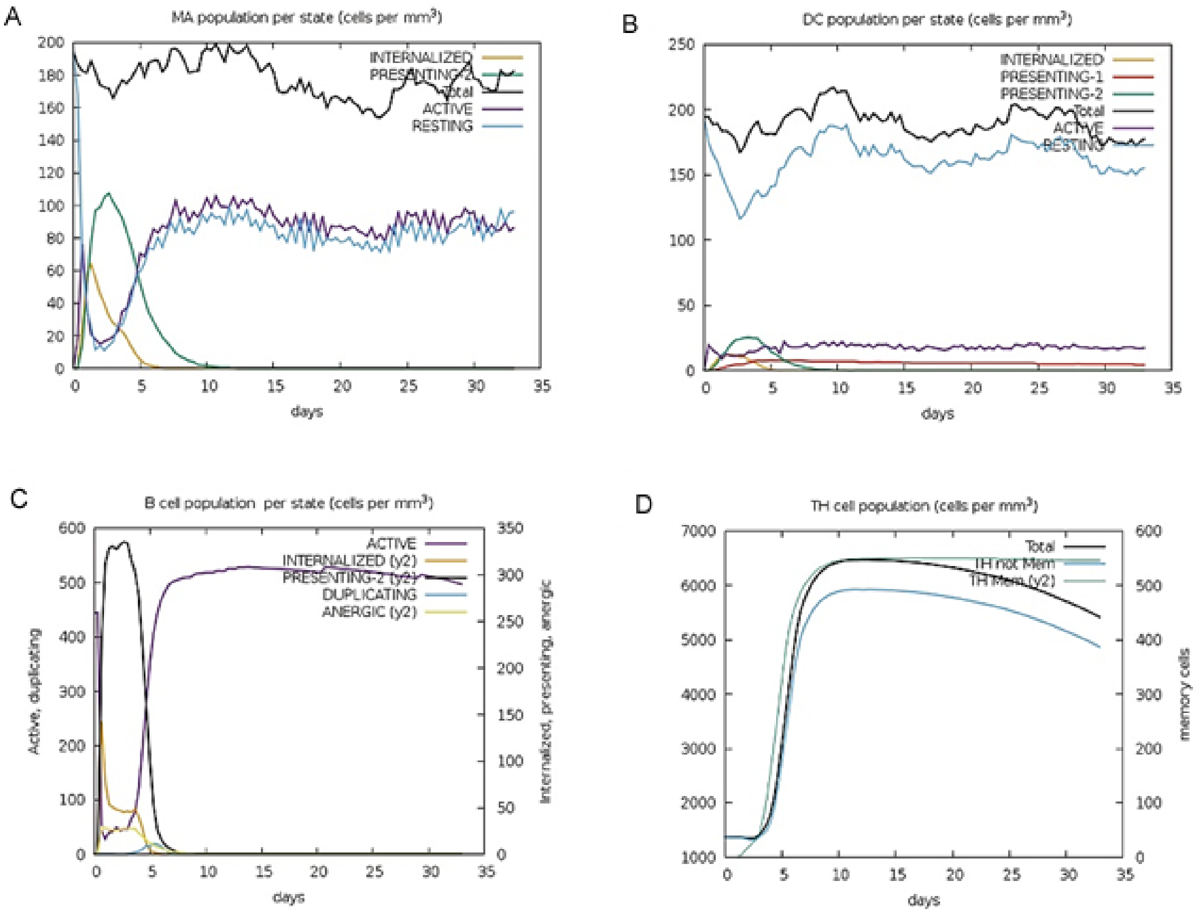

Fig. S4: Analysis of the induction of innate immune response by MbovE3 candidates. A: Illustrates the expression changes in macrophages following stimulation with the MbovE3 antigen (indicated in black). B: Displays the alterations in dendritic cell (DC) levels subsequent to antigen stimulation (indicated in black). C: Represents the changes in the secretion of active B cells after antigen stimulation (indicated in purple). D: Highlights the variations in the secretion levels of helper T lymphocytes (Th) as well as the secretion levels of Th cells of different memory types.

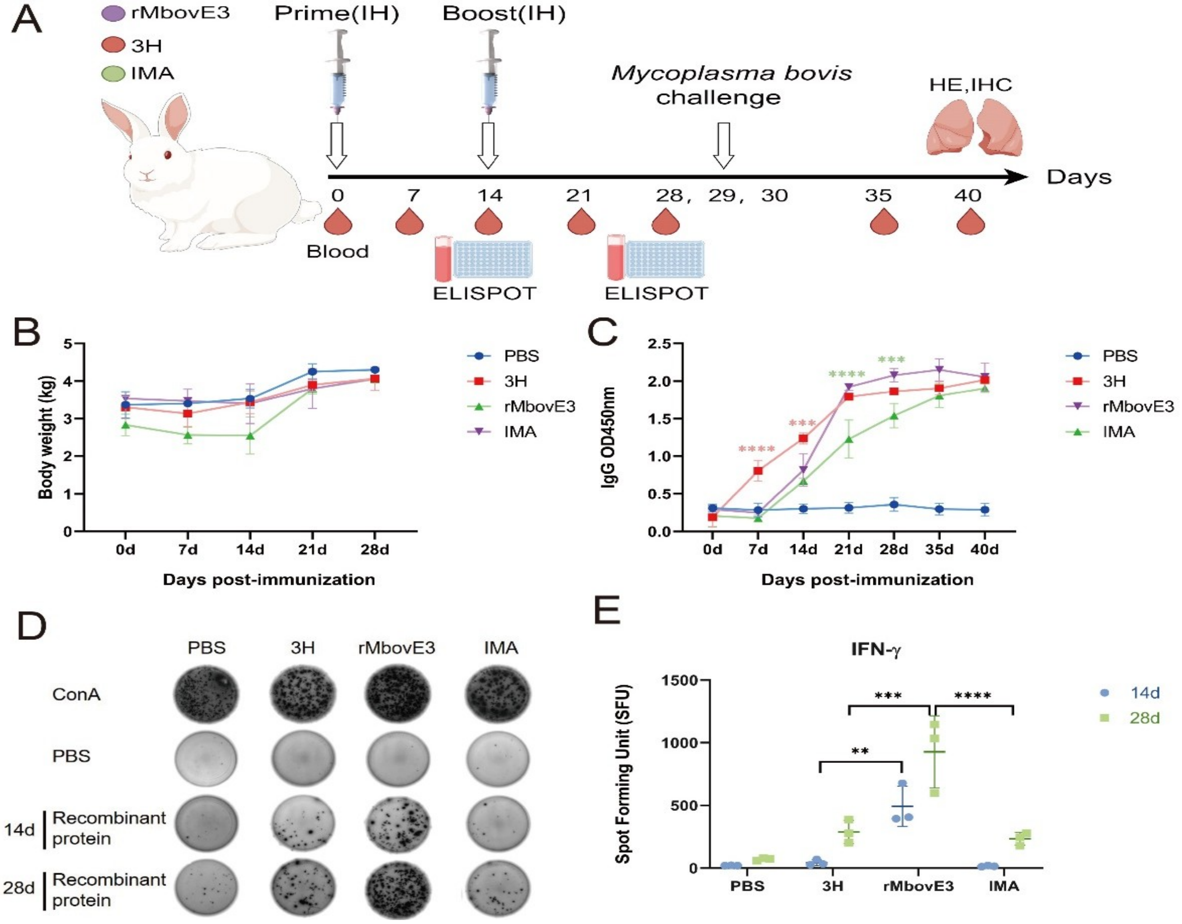

Fig. 4: The rMbovE3 vaccine effectively protected against *M. bovis* infection in rabbits. A: Vaccination program and sampling schedule. Rabbits were immunized subcutaneously with rMbovE3 combined with Freund's adjuvant, three dominant antigens (3H, MbovP274, MbovP570, and ENO1 at a 1:1:1 ratio) combined with Freund's adjuvant, or an inactivated vaccine (IMA). A second booster vaccination was administered two weeks later. The rabbits were euthanized on the 40th day postimmunization. B: Changes in the average weight of the rabbits throughout the experiment. C: Analysis of the OD450 value of IgG by ELISA. The significance results indicated in green in the figure show the comparison of the rMbovE3 group with the IMA group, whereas the significance results marked in red show the comparison of the rMbovE3 group with the 3H group. D and E: ELISPOT technology was employed to quantify SFUs in the vaccine-immunized group to assess the vaccine-induced cellular immune response. ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$. All the data are shown as the means ± SEMs from three independent tests.

At 0 to 7 days postimmunization, the weight of the rabbits in the experimental group slightly decreased, followed by an increase on the 14th day, with no significant differences observed between the groups. Following the challenge, no significant differences in the body weights of the rabbits were noted across the groups (Fig. 4B). In terms of the immune response, the IgG antibody levels in the rMbovE3, 3H, and IMA groups were significantly greater than those in the PBS control group (Fig. 4C). Notably, the IgG antibody levels in the 3H group surpassed those in the rMbovE3 and IMA groups on both the 7th and 14th days postimmunization. After the second immunization, the IgG antibody level in the rMbovE3 group was notably greater, particularly on days 21 and 28, significantly exceeding that in the IMA group. These findings demonstrate that all three vaccine candidates could induce the production of specific IgG antibodies, with rMbovE3 exhibiting strong immunogenicity following the second immunization. After secondary immunization, robust CD4+ T-cell responses were elicited in the rMbovE3, 3H, and IMA groups, underscoring the capacity of these vaccine candidates to activate CD4+ T cells (Fig. 4D and E). The rMbovE3 group displayed a pronounced CD4+ T-cell response in the early stages, with the number of SFUs significantly exceeding that of the 3H and IMA groups. These results suggest that rMbovE3 can effectively induce specific antibody production and CD4+ T-cell responses following immunization, supporting its potential as an effective vaccine candidate. On day 28 postimmunization, the number of SFUs in the rMbovE3 group was the highest among the three groups, further confirming its ability to induce CD4+ T-cell responses.

Following the challenge, rabbit nasal swab samples were collected for eight consecutive days for RT–PCR. The results indicated that the quantity of *M. bovis* in the nasal cavity of the rMbovE3-immunized group on the fourth day post-challenge was significantly lower than that in the IMA immunization group. Additionally, on days four, seven, and eight, the *M. bovis* load was lower than that observed in the control group (Fig. 5A). Bacterial excretion among the rabbits in all the groups was intermittent, with no significant differences noted. DNA was extracted after homogenization of the lung tissue, and RT–PCR revealed that the *M. bovis* load in the lungs of the rMbovE3-, 3H-, and IMA-immunized groups was significantly lower than that in the control group, with the rMbovE3-immunized group exhibiting the lowest load (Fig. 5B). On the tenth day following *M. bovis* infection, the rabbits from all the groups were euthanized, and their lung tissues were collected. Analysis of hematoxylin and eosin (H&E)-stained sections revealed that the PBS+*M. bovis* XJ-01 group exhibited significant inflammatory cell infiltration in the alveolar space and interstitium, widening of the alveolar intervals, alterations in alveolar morphology, and rupture or fusion of some alveoli, resulting in structural damage to the alveoli. Conversely, the rMbovE3-immunized group presented a reduced number of inflammatory cells, normal alveolar spacing and morphology, and only mild damage (Fig. 5C). Compared with those in the PBS+*M. bovis* XJ-01 group, the pathological changes in the 3H and IMA vaccine groups did not significantly improve. Overall, the rMbovE3 vaccine significantly mitigated lung injury induced by *M. bovis* XJ-01. A monoclonal *M. bovis* XJ-01 antibody preserved in our laboratory was used for immunohistochemistry (IHC) of the sections, revealing that *M. bovis* primarily adheres to the cell surface (Fig. 5D). Quantitative analysis of the area of positive staining revealed that rMbovE3 immunization significantly reduced *M. bovis* colonization in the lung tissue (Fig. 5E). The trends observed via RT–PCR analysis of lung tissue align with these findings. Collectively, these results indicate that the rMbovE3 vaccine provided the most effective protection against the adhesion and colonization of *M. bovis* in the lungs.

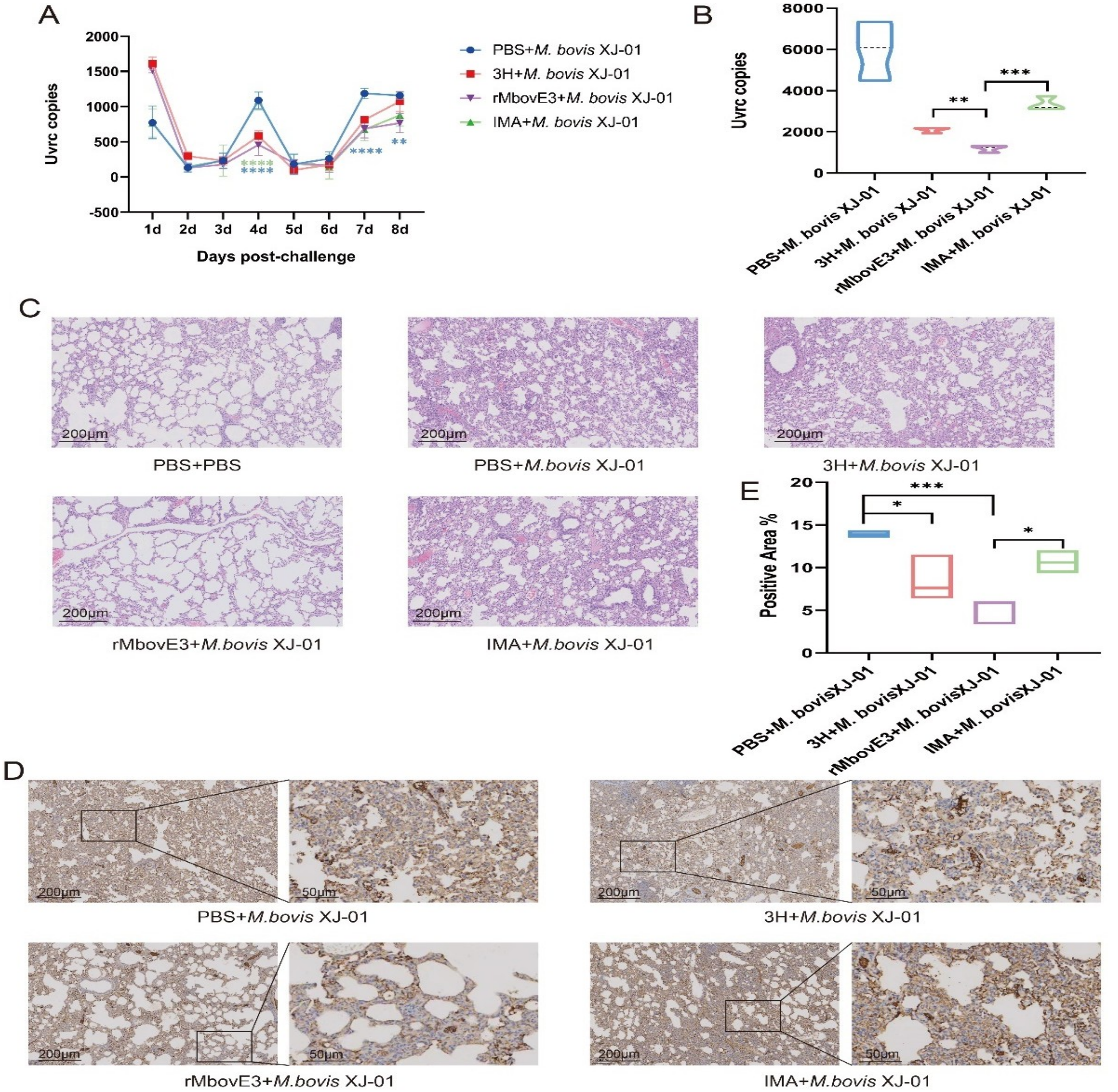

Fig. 5: Evaluation of the protective efficacy of the rMbovE3 vaccine. A: The copy number of the Uvrc gene in rabbit nasal swab samples was determined via RT–PCR to assess the shedding of *M. bovis* in the nasal cavity of rabbits. B: The copy number of the Uvrc gene in rabbit lung tissue was analyzed via RT–PCR to evaluate the *M. bovis* load in the lungs of rabbits. C: Representative images of hematoxylin and eosin (H&E)-stained lung tissue samples collected on day 40. Scale: 200 μm. D: Representative immunohistochemistry (IHC) images of lung tissues obtained on day 40 and stained with a monoclonal *M. bovis* antibody preserved in our laboratory. E: Quantitative data derived from immunohistochemical analysis. ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$. All the data are shown as the means ± SEMs from three independent tests.

The MbovE3 protein derived from the baculovirus expression system elicited protective immune responses in rabbits: To further investigate the immune effects of the MbovE3 protein produced by the eukaryotic expression system, the researchers constructed the recombinant baculovirus qBac-IIIG-MbovE3. The MbovE3 protein expressed in High Five cells presented a structure more like that of the native form (Fig. 6A and Fig. S5A and B). For vaccine preparation, 200 µg of MbovE3 was mixed with Quil-A and ISA206 adjuvants, followed by subcutaneous immunization of the rabbits. On the 28th day of the immunization schedule, the rabbits were challenged intranasally with *M. bovis* for three consecutive days (Fig. 6B).

Following immunization or challenge, there was no significant difference in the body weights of rabbits across the various groups, indicating that the two vaccines are relatively safe (Fig. S5C). The MbovE3+ISA206 group presented the highest specific antibody levels starting at 21 days postimmunization (Fig. 6C). Notably, the IL-4 concentration in the MbovE3+ISA206 group was significantly greater than that in the IMA group seven days after immunization, which aligns with the observed trend in specific antibody levels (Fig. S6A). IL-4, a Th2-type cytokine, facilitates the activation of cytotoxic T cells, enhances the humoral immune response, and promotes antibody production. On days 14 and 28 postimmunization, the MbovE3+ISA206 group demonstrated a robust CD4$^+$ T-cell response, with a significantly greater number of SFUs than observed for both the MbovE3+Quil-A and IMA groups (Fig. 6D). Consistent with this, the TNF-α concentration in the MbovE3+ISA206 group was significantly greater than that in the IMA group (Fig. S6B). These findings indicate that MbovE3+ISA206 immunization effectively induces high-level immune responses during both the early and late stages of immunity, with immune responses appearing to be predominantly IFN-γ-dependent TH1-type cellular responses. Compared with the PBS+*M. bovis* XJ-01 group, both the MbovE3+ISA206 and MbovE3+Quil-A groups, as well as the IMA group, presented significantly reduced nasal shedding and lung colonization of *M. bovis*, although no significant differences were observed among the three groups (Fig. 6E and F). H&E staining revealed that the PBS+*M. bovis* XJ-01 group exhibited significant damage to the lung tissue structure of the rabbits, characterized by pronounced inflammatory cell infiltration in the alveolar cavity and lung interstitium, widening of the alveolar septa, altered alveolar morphology, and fusion. In contrast, the pathological changes were mild in the MbovE3+ISA206, MbovE3+Quil-A, and IMA immunized groups (Fig. 6G). IHC results demonstrated that the protective effects of MbovE3+ISA206 and MbovE3+Quil-A were more pronounced (Fig. 6H and I). These findings suggest that recombinant baculovirus-expressed MbovE3 could confer protection to rabbits against *M. bovis* infection.

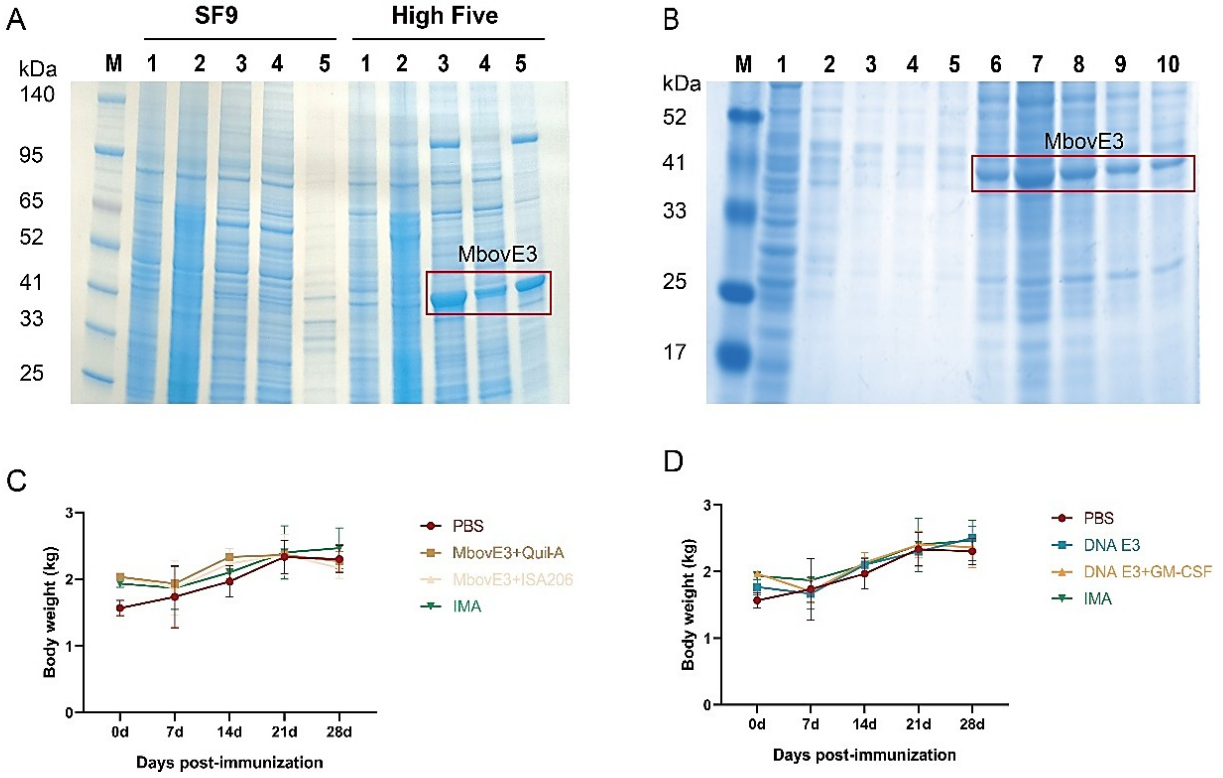

Fig. S5: The expression of MbovE3, facilitated by baculovirus, and the corresponding changes in body weight of rabbits in each group following vaccine immunization were examined. A: Following the infection of SF9 and High Five cells with recombinant baculovirus, the MbovE3 protein is predominantly expressed in High Five cells. M: protein molecular weight marker. 1: Empty cells; 2: Cells infected with the empty virus; 3: Cells infected with the qBac-IIIG-MbovE3 virus; 4: Supernatant from the lysate of qBac-IIIG-MbovE3 virus-infected cells; 5: Precipitate from the lysate of qBac-IIIG-MbovE3 virus-infected cells. B: Purification of MbovE3 protein from the cell lysate supernatant using Ni-NTA affinity chromatography. M: protein molecular weight marker. 1-2: Washing buffer for impurity proteins; 3-10: Elution buffer for purification. The red box highlights the location of the MbovE3 protein. C: Changes in the average body weight of rabbits during the MbovE3 vaccine immunization experiment. D: Changes in the average weight of rabbits during the DNA vaccine immunization experiment.

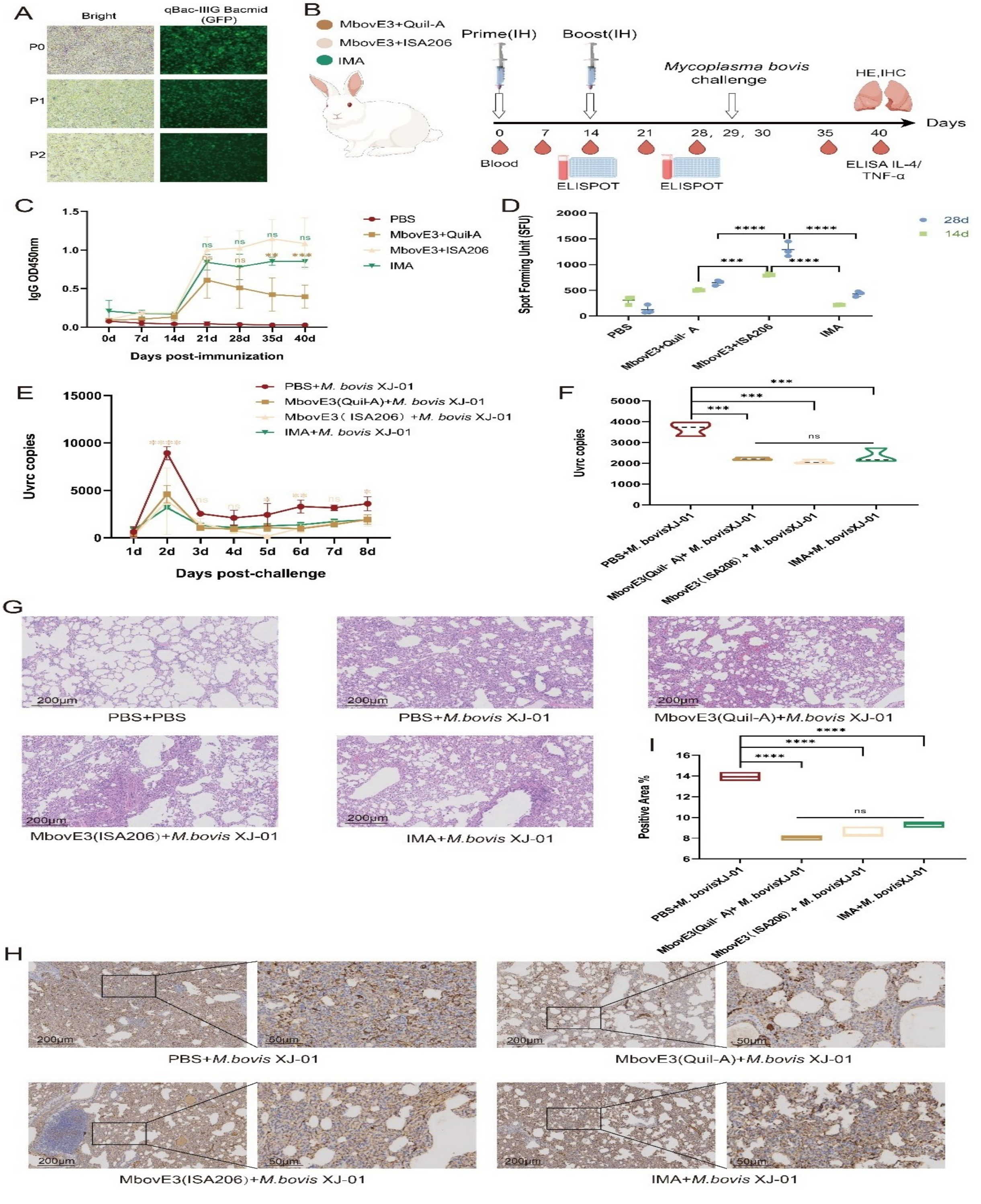

Fig. 6: Evaluation of the immunization effects of a baculovirus expressing MbovE3. A: Construction of the recombinant baculovirus qBac-IIIG-MbovE3. This virus carries the gene encoding green fluorescent protein (GFP), which can be utilized to monitor the infection of Sf9 cells. P0, P2, and P3 in the figure represent generations 0, 1, and 2 of the baculovirus, respectively. B: Vaccination program and sampling schedule. MbovE3 (200 μg) was mixed with Quil-A and ISA206 to formulate a vaccine. The rabbits were immunized subcutaneously and received a booster two weeks later. The inactivated vaccine IMA served as a positive control. The rabbits were euthanized on the 40th day postvaccination. C: The OD450 value of IgG was analyzed using ELISA. The green and brown symbols indicate significant differences between the MbovE3+ISA206 group and the IMA group and between the MbovE3+Quil-A group and the IMA group. D: ELISPOT technology was employed to count SFUs in the vaccine-immunized group, enabling the evaluation of the cellular immune response. E: RT–PCR was used to determine the Uvrc gene copy number in rabbit nasal swab samples to assess the shedding of *M. bovis*. F: RT–PCR was also employed to determine the Uvrc gene copy number in rabbit lung tissue to evaluate the *M. bovis* load in the lungs. G: Images of H&E-stained lung tissue samples collected on day 40. Scale: 200 μm. H: IHC sections of lung tissue that were collected on day 40 and subsequently stained with monoclonal *M. bovis* antibodies that had been stored in the laboratory. I: Quantitative data were obtained from the immunohistochemical analysis. Ns indicates not significant ($P>0.05$), * indicates $0.01<P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, and **** indicates $P<0.0001$. All the data are shown as the means ± SEMs from three independent tests.

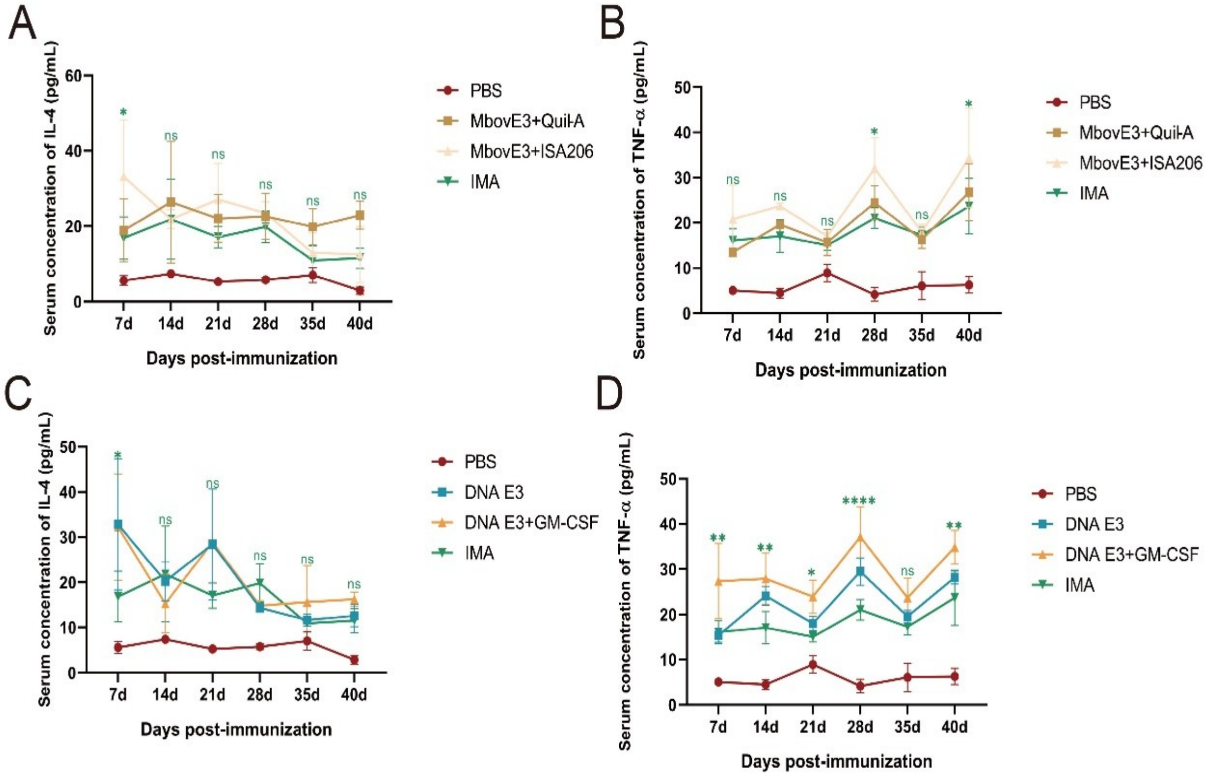

Fig. S6: Changes in the concentrations of IL-4 and TNF-α in the serum of rabbits from each group following immunization with baculovirus-expressed MbovE3 and DNA vaccines. A: The ELISA assay measures the IL-4 concentration in rabbit serum post-immunization with the MbovE3 vaccine expressed by baculovirus. B: The ELISA assay quantifies the TNF-α concentration in rabbit serum after immunization with the MbovE3 vaccine expressed by baculovirus. C: The ELISA assay assesses the IL-4 concentration in rabbit serum following immunization with the DNA vaccine. The green marker indicates the significance analysis between the DNAE3+GM-CSF group and the IMA group. D: The ELISA assay evaluates the TNF-α concentration in rabbit serum after immunization with the DNA vaccine. The green marker indicates the significance analysis between the DNAE3+GM-CSF group and the IMA group. Ns, not significant (P>0.05), 0.01 < * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001. All data are shown as mean ± SEM from three independent tests.

Multiepitope chimeric DNA vaccines can induce only partial protective immunity in rabbits: To thoroughly assess the protective efficacy of recombinant proteins, the researchers developed DNA vaccines incorporating genes encoding recombinant proteins. Given that granulocyte–macrophage colony–stimulating factor (GM-CSF) is a crucial immune regulator that promotes dendritic cell maturation and increases macrophage activity, the researchers inserted the MbovE3 sequence along with the GM-CSF gene. Consequently, two DNA vaccines were constructed: DNA E3 and DNA E3+GM-CSF (Fig. 7A). Their protective effects were subsequently evaluated (Fig. 7B).

At each time point following immunization, there was no significant difference in the body weight of the vaccinated rabbits compared with that of the rabbits in the PBS control group, indicating the safety of the vaccine (Fig. S5D). In terms of the humoral immune response, the level of specific antibodies in the IMA group was significantly greater than that in the DNA E3 and DNA E3+GM-CSF groups, whereas no significant difference was detected between the DNA E3 and DNA E3+GM-CSF groups (Fig. 7C). However, on days 14 and 28 postimmunization, the number of SFUs in the DNA E3+GM-CSF group was significantly greater than those in both the DNA E3 and IMA groups (Fig. 7D). Additionally, the IL-4 concentration in the DNA E3+GM-CSF group was significantly greater than that in the IMA group seven days after immunization (Fig. S6C). The TNF-α concentration in the DNA E3+GM-CSF group was also significantly greater than that in the IMA group (Fig. S6D). These findings indicate that DNA vaccines supplemented with the GM-CSF molecular adjuvant can effectively induce TH1-type cellular immune responses and that GM-CSF enhances the cellular immune effects of DNA vaccines.

The results regarding bacterial loads after challenge demonstrated that, compared with the PBS+*M. bovis* XJ-01 group, the DNA E3, DNA E3+GM-CSF, and IMA groups presented significantly reduced colonization of the nasal cavity and lungs by *M. bovis*. However, no significant differences were observed among the three groups (Fig. 7E and F). The results obtained via analysis of H&E-stained sections indicated that, compared with those in the PBS+*M. bovis* XJ-01 group, the pathological changes in the DNA E3 and DNA E3+GM-CSF immune groups were diminished; however, the difference between these two groups was not statistically significant (Fig. 7G). IHC results demonstrated that the DNA E3 and DNA E3+GM-CSF groups presented the most pronounced protective effects, significantly reducing the colonization of alveoli by *M. bovis* (Fig. 7H and I). These findings confirm that the recombinant protein DNA vaccine can confer substantial protection in rabbits.

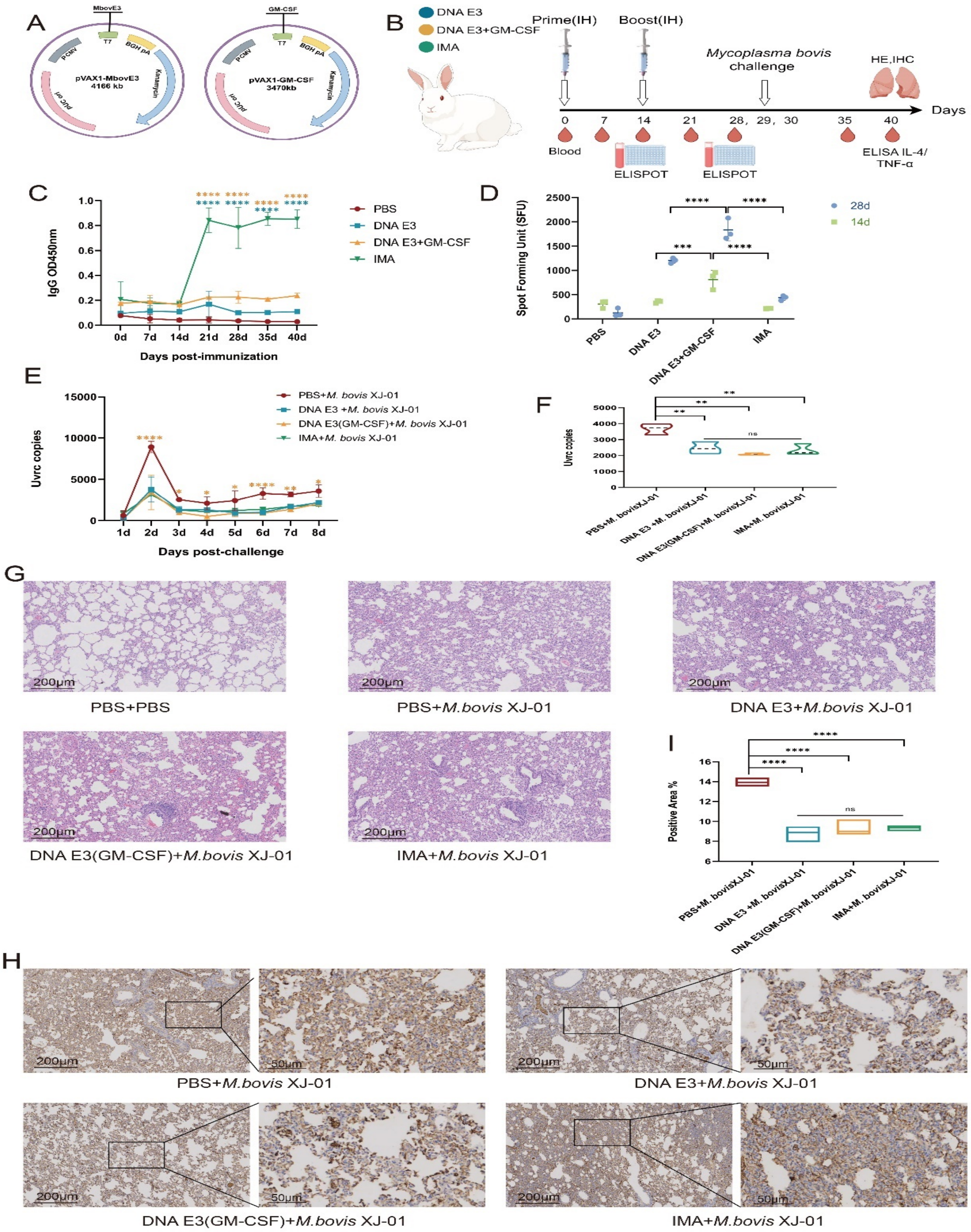

Fig. 7: Evaluation of the immunization efficacy of the DNA vaccine DNAE3. A: Recombinant pVAX1-DNAE3 and pVAX1-DNAE3+GM-CSF plasmids were constructed by cloning the gene sequences of MbovE3 and MbovE3-GM-CSF into the pVAX1 eukaryotic expression vector. B: The vaccination program and sampling schedule are outlined. Rabbits were immunized with pVAX1-DNAE3 (450 µg) and pVAX1-DNAE3+GM-CSF (450 µg) via subcutaneous injection, followed by booster immunization two weeks later. The inactivated vaccine IMA served as the positive control. The rabbits were euthanized on the 40th day postvaccination. C: The OD450 value of the IgG was analyzed using ELISA. The blue and orange symbols indicate significant differences between the DNAE3 group and the IMA group and between the DNAE3+GM-CSF group and the IMA group. D: ELISPOT technology was employed to quantify SFUs in the vaccine-immunized group to assess the cellular immune response. E: RT–PCR was used to determine the Uvrc gene copy number in rabbit nasal swab samples, which was subsequently used to evaluate the shedding of *M. bovis*. F: RT–PCR was also used to determine the Uvrc gene copy number in rabbit lung tissue, allowing assessment of the *M. bovis* load in the lungs. G: Images of hematoxylin–eosin (H&E)-stained lung tissue collected on day 40. Scale: 200 µm. H: Immunohistochemistry (IHC) images of lung tissue collected on day 40 and stained with monoclonal *M. bovis* antibodies that had been stored in the laboratory. I: Additionally, quantitative data from the immunohistochemical analysis were obtained. Ns indicates not significant (P>0.05), * indicates 0.01 <P<0.05, ** indicates P<0.01, *** indicates P<0.001, and **** indicates P<0.0001. All the data are shown as the means ± SEMs from three independent tests.

DISCUSSION

The objective of this study was to develop a multiepitope tandem candidate vaccine targeting *M. bovis* and to assess the vaccine's protective efficacy in a rabbit model. The researchers evaluated the immune response of six candidate antigens of *M. bovis* for the first time using a mouse model and identified three primary antigens: MbovP274, MbovP570, and ENO1. Furthermore, the researchers developed three multiepitope vaccines for the first time, utilizing computational immunology and vaccine informatics tools. These include the rMbovE3 subunit vaccine expressed in a prokaryotic system, the MbovE3 subunit vaccine based on a baculovirus expression system, and the DNA vaccine DNAE3+GM-CSF. All three vaccines were shown to induce high levels of both humoral and cellular immune responses, effectively protecting rabbits from *M. bovis* infection. Among these vaccines, the rMbovE3 vaccine exhibited the most significant protective effect. In rabbit models, rMbovE3 exhibited good safety and effectively induced strong and specific antibody and CD4+ T-cell responses. It also reduced the load of and damage caused by *M. bovis* in the lungs, effectively preventing infection and exerting a significant protective effect. These findings provide a scientific basis for the use of rMbovE3 as a vaccine candidate and highlight its potential in the prevention and control of *M. bovis* infection. Currently, two commercial vaccines against *M. bovis* are available on the American market: MpB Guard and Myco-B ONE DOSE. However, studies have reported that both vaccines fail to effectively prevent colonization of the upper respiratory tract of cattle with *M. bovis*, with their preventive efficacies reported to be 44% and less than 1%, respectively (Soehnlen *et al*., 2011). Other countries and regions currently lack commercially available vaccines against *M. bovis*. Therefore, the development of new vaccines, particularly those capable of inducing stronger immune responses and providing enhanced protection, is of paramount importance for the prevention and control of *M. bovis* infection worldwide.

Research on and the development of *M. bovis* vaccines currently encompass inactivated vaccines, attenuated vaccines, and subunit vaccines. Compared with traditional live attenuated and inactivated vaccines, multiepitope vaccines present an appealing alternative because of their enhanced safety and targeted immune response (Saadi *et al*., 2017). Furthermore, considering that vaccines with single antigens may not offer sufficient protection, subunit vaccines that incorporate multiple antigens represent a more effective option. In this study, we developed the multiepitope vaccine rMbovE3 against *M. bovis* for the first time, utilizing computational immunology and vaccine informatics tools. Inactivated vaccines are costly to produce and may alter *M. bovis* antigen proteins during in vitro culture. Additionally, inactivated vaccines derived from a single isolate cannot provide comprehensive protection against other isolates (Nicholas *et al*., 2002; Dudek *et al*., 2016). In contrast to inactivated vaccines, the rMbovE3 vaccine is more economical, and all its epitopes are highly conserved. Zhang *et al*. (2014) developed an attenuated live *M. bovis* vaccine that failed to completely prevent the spread of *M. bovis* within the host and did not effectively stimulate mucosal immunity. Moreover, attenuated vaccines carry the inherent risk of reverting to virulence, which limits their safety in practical applications. Consistent with the findings of Zhang *et al*. (2014), the rMbovE3 vaccine also did not prevent *M. bovis* infection; however, unlike the attenuated vaccine, rMbovE3 does not pose a risk of restoring virulence, making it a safer alternative.

The limitation of this study lies in the fact that the protective effect of the *M. bovis* vaccine was evaluated solely in rabbits as the model animal, without verification in bovine species. This limitation parallels those of previous studies on *M. bovis* vaccines conducted in mouse models. For example, the research by Prysliak *et al*. (2018) aimed to develop vaccines through a combination of multiple antigens and adjuvants; however, these vaccines ultimately failed to provide effective protection in cattle, despite eliciting significant humoral and cellular immune responses in mice. Similarly, the study by Zhang *et al*. (2023), which included subjects, assessed the vaccine's protective effect in domestic rabbits but did not extend this verification to cattle. These findings underscore that while mouse and rabbit models yield valuable preliminary data for vaccine development, the ultimate efficacy of the *M. bovis* vaccine requires validation in bovine species.

Conclusions: The data presented here clearly demonstrate that the multiepitope vaccine rMbovE3, which incorporates seven B-cell epitopes and twelve T-cell epitopes derived from the MbovP274, MbovP570, and ENO1 proteins, can elicit robust humoral and cellular immunity. Furthermore, the rMbovE3 vaccine mitigated lung damage caused by *M. bovis* in a domestic rabbit model, indicating its potential effectiveness. These findings underscore the use of the MbovE3 vaccine as a promising strategy for the prevention and control of *M. bovis* infections. However, while mouse and rabbit models provide valuable preliminary data for vaccine development, the ultimate efficacy of the *M. bovis* vaccine must be validated in bovine species. Future research should focus on optimizing vaccine formulations, enhancing immunization procedures, and conducting long-term evaluations of vaccine efficacy to develop new strategies for the control and prevention of *M. bovis* infections.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper.

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REFERENCES

Ayling RD, Lysyansky I, Sachse K, *et al.*, 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. Transboundary Emerging Diseases 65:91-109.

Bürki S, Frey J and Pilo P, 2015. Virulence, persistence and dissemination of mycoplasma bovis. Veterinary Microbiology 179:15-22.

Caswell JL and Archambault M, 2007. *Mycoplasma bovis* pneumonia in cattle. Animal Health Research Reviews 8:161-186.

Chao J, Han X, Liu K, *et al.*, 2019. Calves infected with virulent and attenuated mycoplasma bovis strains have upregulated th17 inflammatory and th1 protective responses, respectively. Genes 10:656.

Chong P , Huang J , Leng C , *et al.*, 2015. Recombinant lipoproteins as novel vaccines with intrinsic adjuvant. Advances in Protein Chemical and Structural Biology 99:55-74.

Citti C and Blanchard A, 2013. Mycoplasmas and their host: Emerging and re-emerging minimal pathogens. Trends in Microbiology 21:196-203.

Dallo SF, Kannan TR, Blaylock MW, *et al.*, 2010. Elongation factor tu and e1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in mycoplasma pneumoniae. Molecular Microbiology 46:1041-1051.

Dudek K, Bednarek D, Ayling RD, *et al.*, 2016. An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. Vaccine 34:3051-3058.

Dudek K, Bednarek D, Lisiecka U, *et al.*, 2020. Analysis of the leukocyte response in calves suffered from *Mycoplasma bovis* pneumonia. Pathogens 9:407.

Dudek K, Szacawa E and Nicholas RJ, 2021. Recent developments in vaccines for bovine mycoplasmoses caused by *Mycoplasma bovis* and *Mycoplasma mycoides* subsp mycoides. Vaccines 9:549.

Gao Z, Chen L, Song T, *et al.*, 2022. A candidate multi-epitope vaccine against porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* induces robust humoral and cellular response in mice. Vaccine 40:2370-2378.

Harlow E and Lane D, 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory 10:151.

Inna L and Ayling RD, 2016. *Mycoplasma bovis*: Mechanisms of resistance and trends in antimicrobial susceptibility. Frontiers in Microbiology 7:595.

Laura M, Richard J, Katie M, *et al.*, 2006. Biofilm formation by mycoplasma species and its role in environmental persistence and survival. Microbiology and Molecular Biology Research 152:913-922.

Lennerz V, Gross S, Gallerani E, *et al.*, 2014. Immunologic response to the survivin-derived multi-epitope vaccine emd640744 in patients with advanced solid tumors. Cancer Immunology and Immunotherapy 63:381.

Lucijana BR, Brigita S, Miha L, *et al.*, 2008. Short communication: Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates. Veterinary Microbiology 127:147-154.

Mugunthan SP and Harish MC, 2021. Multi-epitope-based vaccine designed by targeting cytoadherence proteins of *Mycoplasma gallisepticum*. ACS Omega 6:13742-13755.

Nain Z, Karim MM, Sen MK, *et al.*, 2020. Structural basis and designing of peptide vaccine using pe-pgrs family protein of mycobacterium ulcerans-an integrated vaccinomics approach. Cellular and Molecular Immunology 120:146-163.

Nicholas RaJ, Ayling RD and Stipkovits LP, 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis* : Clinical, cultural, serological and pathological findings. Vaccine 20:3569-3575.

Nicholas RJ and Ayling RD, 2003. *Mycoplasma bovis* : Disease, diagnosis, and control. Research in Veterinary Science 74:105-112.

Perezcasal J and Prysliak T, 2007. Detection of antibodies against the *Mycoplasma bovis* glyceraldehyde-3-phosphate dehydrogenase protein in beef cattle. Microbial Pathogenesis 43:189-197.

Pinto PM, Chemale G, Castro LA, *et al.*, 2007. Proteomic survey of the pathogenic mycoplasma hyopneumoniae strain 7448 and identification of novel post-translationally modified and antigenic proteins. Veterinary Microbiology 121:83.

Poumarat F, Solsona M and Boldini M, 1994. Genomic, protein and antigenic variability of *Mycoplasma bovis*. Veterinary Microbiology 40:305-321.

Prysliak T and Casal P, 2015. Immune responses to *Mycoplasma bovis* proteins formulated with different adjuvants. Canadian Journal of Microbiology 62:492.

Prysliak T, Maina T and Perez C, 2018. Th-17 cell mediated immune responses to *Mycoplasma bovis* proteins formulated with montanide isa61 vg and curdlan are not sufficient for protection against an experimental challenge with *Mycoplasma bovis*. Veterinary Immunology and Immunopathology 197:7-14.

Prysliak T, Maina T, Yu L, *et al.*, 2017. Induction of a balanced igg1/igg2 immune response to an experimental challenge with *Mycoplasma bovis* antigens following a vaccine composed of emulsigen, idr peptide1002, and poly i:C. Vaccine 35:6604-6610.

Prysliak T, Merwe J and Perez C, 2013. Vaccination with recombinant *Mycoplasma bovis* gapdh results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *Mycoplasma bovis*. Microbial Pathogenesis 55:1-8.

Qin Y, Tu K, Teng Q, *et al.*, 2021. Identification of novel t-cell epitopes on infectious bronchitis virus n protein and development of a multi-epitope vaccine. Journal of Virology 95:e0066721.

Robino P, Alberti A, Pittau M, *et al.*, 2005. Genetic and antigenic characterization of the surface lipoprotein p48 of *Mycoplasma bovis*. Veterinary Microbiology 109:201-209.

Saadi M, Karkhah A and Nouri HR, 2017. Development of a multi-epitope peptide vaccine inducing robust t cell responses against brucellosis using immunoinformatics based approaches. Infection and Genetic Evolution 51:227-234.

Scherm B, Gerlach GF and Runge M, 2002. Analysis of heat shock protein 60 encoding genes of mycoplasmas and investigations concerning their role in immunity and infection. Veterinary Microbiology 89:141-150.

Soehnlen MK, Aydin A, Lengerich EJ, *et al.*, 2011. Blinded, controlled field trial of two commercially available *Mycoplasma bovis* bacterin vaccines in veal calves. Vaccine 29:5347-5354.

Su HC, Hutchison CA and Giddings MC, 2007. Mapping phosphoproteins in *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. BMC Microbiology 7:63-63.

Sun Z, Fu P, Wei K, *et al.*, 2014. Identification of novel immunogenic proteins from *Mycoplasma bovis* and establishment of an indirect elisa based on recombinant e1 beta subunit of the pyruvate dehydrogenase complex. Plos One 9:e88328.

Xiao Y, Li R and Ma Z, 2023. Isolation, identification and drug sensitivity analysis of *Mycoplasma bovis* in shihezi region of xinjiang. Chinese Animal Husbandary and Veterinary Medicine 50:754-763.

Xin J, Li Y, Guo D, *et al.*, 2008. First isolation of *Mycoplasma bovis* from calf lung with pneumoniae in china. Chinese Journal of Preventive Veterinary Medicine 5:1-8.

Yano A, Onozuka A, Asahi Y, *et al.*, 2005. An ingenious design for peptide vaccines. Vaccine 23:2322-2326.

Zhang G, Han L, Zhao S, *et al.*, 2023. Development and evaluation of a multi-epitope subunit vaccine against *Mycoplasma synoviae* infection. International Journal of Biology of Macromolecules 253:126685.

Zhang K, Lu Z, Zhao P, *et al.*, 2012. Identification of novel immunogenic proteins in *Mycoplasma capricolum* subsp. Capripneumoniae strain m1601. Journal of Veterinary Medical Science 74:1109-1115.

Zhang L, 2018. Multi-epitope vaccines: A promising strategy against tumors and viral infections. Cellular and Molecular Immunology 15:182-184.

Zhang R, Han X, Chen Y, *et al.*, 2014. Attenuated *Mycoplasma bovis* strains provide protection against virulent infection in calves. Vaccine 32:3107-3114.

Zhang S, Zhang Y, Liu G, *et al.*, 2023. The safety and protective efficacy evaluation of an attenuated *Mycoplasma bovis*-bohv-1 bivalent vaccine in rabbits. Vaccines 11:1698.

Zhang Y, Liang S, Zhang S, *et al.*, 2022 Development and evaluation of a multi-epitope subunit vaccine against group b streptococcus infection. Emerging Microbes and Infection 11:2371-2382.

Zhou P, Zhou Z, Huayu M, *et al.*, 2023. A multi-epitope vaccine gile against echinococcus multilocularis infection in mice. Frontiers in Immunology 13:1091004.

Zubair M, Muhamed SA, Khan FA, *et al.*, 2020. Identification of 60 secreted proteins for *Mycoplasma bovis* with secretome assay. Microbial Pathogenesis 143:104135.