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RESEARCH ARTICLE

Brucella Multivalent Antigen-IFN-γ Fusion Protein-Expressing Recombinant Adenoviral Vaccine Induces Th1-Type Immune Responses and Protective Effects Against *Brucella* Infection in Mice

Aodi Wu[2†], Jing Yuan[2†], Andong Wang[6†], Honghuan Li[3], Yuting Zhang[2], Huan Zhang[2], Yalin Gao[1], Rongrong Ni[2], Qimin Dong[2], Yuanwenyu He[4], Nannan Cui[3], Litao Zhu[3], Changsuo Zhang[5], Jinliang Sheng[2], Lu Xiao[7], Jihai Yi[2*], Yuanzhi Wang[3*] and Yueli Wang[1*]

[1]School of Clinical Medicine, Shihezi University, Shihezi, 832003, Xinjiang, China; [2]College of Animal Science and Technology, Shihezi University, Shihezi, 832003, Xinjiang, China; [3]School of Medicine, Shihezi University, Shihezi, 832003, Xinjiang, China; [4]School of Foreign Languages, Shihezi, 832003, Xinjiang, China; [5]Tiankang Biopharmaceutical Co., Ltd., Urumqi, 830032, Xinjiang, China; [6]Shihezi University, Shihezi, 832003, Xinjiang, China; [7]Sichuan Animal Science Academy, Chengdu, 610066, Sichuan, China. [†]These authors contributed equally to this work. *Corresponding author: 724050645@qq.com (JY); wangyuanzhi621@126.com (Yuanzhi W); 577674101@qq.com (Yueli W)

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ABSTRACT

Brucellosis is a widespread zoonotic disease caused by *Brucella* species. Infection leads to chronic disease in both humans and animals, complicating pathogen clearance in affected hosts, and it continues to impose a substantial burden on global health and livestock industries. Although several live attenuated vaccines have been widely used in veterinary practice, their efficacy is limited, and they often fail to induce sterilizing immunity. The persistent transmission of brucellosis underscores the need for more effective vaccines to protect susceptible animals and reduce disease incidence. In this study, we first conducted bioinformatics and structural analysis on three candidate *Brucella* antigens (Cu/Zn-SOD, OMP31, BP26), predicting their stability, hydrophilicity, favorable antigenicity, high solubility, and multiple surface-localized linear B-cell epitopes, which supported their potential as vaccine antigens. We then constructed recombinant adenoviral vaccines expressing Brucella-derived antigens (*Cu/Zn-SOD, OMP31, BP26*) fused with murine IFN-γ; these vaccines were designated rAd-SG, rAd-OG, rAd-BG, and rAd-Mix (a multivalent combination vaccine). Following evaluation in BALB/c mouse models, we found that these vaccines elicited robust immune responses and conferred significant protection against *Brucella melitensis 043* challenge. Collectively, our findings show that the multivalent vaccine (rAd-Mix) induces a Th1-skewed immune response and confers robust protection, representing a promising strategy for developing effective brucellosis vaccines.

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INTRODUCTION

Brucella spp. are Gram-negative, facultative intracellular pathogens that induce abortion in livestock and cause debilitating human diseases characterized by undulant fever, arthritis, and spondylitis (Memish *et al.*, 2004; Ospanov *et al.*, 2024). With an estimated 5–12.5 million new human cases annually worldwide, brucellosis ranks among the most common and debilitating zoonoses (Hull *et al.*, 2018; Mohamed *et al.*, 2025). In goats, brucellosis also exhibits high seroprevalence and specific molecular characteristics (Khan *et al.*, 2025). Controlling *Brucella* in animal reservoirs is critical for reducing human infection. Vaccination is therefore the cornerstone of prevention strategies and is considered the most cost-effective approach for disease control and eradication. However, all licensed vaccines are based on live-attenuated strains (Zhang *et al.*, 2024). Although they provide some protection, their use is complicated by significant limitations: they can cause disease in pregnant animals and

humans, interfere with serological diagnosis, and pose a risk of virulence reversion. Consequently, there is an urgent need to develop safer, more effective vaccines against brucellosis.

Adenoviruses are non-enveloped viruses with a linear double-stranded DNA genome of ~36kb, packaged within an icosahedral capsid 70-100nm in diameter. Their broad host tropism, structural simplicity, high thermostability, and ease of genetic manipulation make adenoviral vectors an attractive vaccine platform, capable of stimulating robust innate, humoral, and cellular adaptive immune responses (Zhu *et al.*, 2007; Watanabe *et al.*, 2021; Yan *et al.*, 2021) .The potency of adenovirus-induced immunity is linked to its ability to activate dendritic cells (DCs), promoting their maturation and enhancing antigen presentation to prime potent T-cell responses (Ding *et al.*, 2022). Most vaccine vectors are based on adenovirus serotypes with deletions in the E1 or E3 genes (Engelhardt *et al.*, 1994), rendering them replication-deficient. Infection begins with binding to the adenovirus receptor (CAR) on host cells, followed by internalization and nuclear translocation of the viral DNA to initiate transcription (Gaden *et al.*, 2004; Wiethoff *et al.*, 2005). Critically, the viral genome remains episomal and does not integrate into the host chromosome. This episomal persistence minimizes the risk of insertional mutagenesis, a key safety advantage for vaccine applications (Ikoma *et al.*, 2004).

Copper-zinc superoxide dismutase (Cu/Zn-SOD), an ~18.5 kDa periplasmic protein encoded by the sodC gene, protects *Brucella* from phagocyte-derived reactive oxygen species (ROS) by neutralizing superoxide anions (O_2^-) and limiting their penetration across the bacterial membrane (Beck *et al.*, 1990). Outer membrane protein 31 (OMP31) is a highly conserved member of the *Brucella* OMP family that is critical for maintaining envelope integrity (Cloeckaert *et al.*, 2002) and mediates host-pathogen interactions (Martin-Martin *et al.*, 2008). Outer membrane protein 26 (BP26) is a soluble, immunogenic protein expressed in all *Brucella* species. Its surface and periplasmic localization, along with its ability to be released from the cell, make it accessible to the host immune system (Xie *et al.*, 2025). The established roles of these proteins in virulence and immunogenicity mark them as promising protective antigen candidates. To further enhance the immune response, we employed interferon-gamma (IFN-γ) as a genetic adjuvant. IFN-γ, a pivotal cytokine produced by activated T cells, NK cells, and macrophages (Lin *et al.*, 2014), can function as a potent molecular adjuvant when co-expressed with antigen genes. Its ability to synergize with and amplify antigen-specific immunity (Kumar *et al.*, 2001; Wang *et al.*, 2013; Bian *et al.*, 2023) provided a rationale for its inclusion in our vaccine design.

To address the critical need for improved *Brucella* vaccines, we developed a novel adenoviral vector platform. We constructed recombinant viruses (rAd-SG, rAd-OG, rAd-BG) co-expressing the *Brucella* antigens Cu/Zn-SOD, OMP31, or BP26 with the adjuvant IFN-γ, and a combination vaccine (rAd-Mix). In mice, these candidates demonstrated stability, robust immunogenicity—eliciting strong antibody and T-cell responses—and conferred significant protection against virulent challenge. This work provides a promising multifaceted strategy and a potent platform for developing safer, more effective brucellosis vaccines.

MATERIALS AND METHODS

Bioinformatics and Structural Analysis of Candidate Antigens: *Brucella* gene sequences *(Cu/Zn-SOD: KF362132.1; OMP31: NC_003318.1; BP26: NC_003317.1)* were obtained from GenBank. ProtParam and Protein-Sol servers assessed physicochemical properties and solubility (Gasteiger *et al.*, 2003; Wiederstein *et al.*, 2007); Antigenicity was predicted via VaxiJen (threshold=0.4); linear B-cell epitopes via IEDB tools (Doytchinova *et al.*, 2007); Tertiary structures were predicted by Phyre2.2, assessed with SWISS-MODEL and visualized using PyMOL (Guex *et al.*, 2009; Powell *et al.*, 2025).

Construction of Recombinant Adenoviruses: Ovis aries IFNG (NM_001009803.1) and His-tag were fused to each candidate gene (General Biosystems, China) to generate fusion genes. Target genes were cloned into shuttle vector VD051-MAX via BamHI/EcoRI sites. Recombinant shuttle plasmids and adenoviral backbone pBHG were co-transfected into HEK293 cells with Lipofectamine 2000 (Thermo Fisher). ZsGreen fluorescence and CPE were monitored to obtain rAd-Cu/Zn-SOD-IFNG (rAd-SG), rAd-OMP31-IFNG (rAd-OG), and rAd-BP26-IFNG (rAd-BG).

Identification of Recombinant Adenoviruses: Total protein was extracted from HEK293 cells infected with recombinant adenoviruses, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with mouse anti-His tag monoclonal antibody (Abcam, UK) and HRP-conjugated goat anti-mouse IgG (H+L) antibody (Abcam, UK). Protein expression was visualized by ECL chemiluminescence. β-actin served as the loading control. Viral morphology was observed via TEM after 2% PTA negative staining. Serial passage PCR verified target gene insertion and genomic stability; primers are listed in Table 1. Primer sequences used in this experiment are listed in Table 1.

Table 1: The primers designed for this experiment.

Primer Name	Primer sequence (5'→3')	Product length/bp	Annealing Temperature/°C
Cu/Zn-SOD-IFNG-F	5'-TCGACAATGGTGCTTATGGC-3'	1002	55
Cu/Zn-SOD-IFNG-R	5'-TTACATTGATGCTCTCCGGC-3'		
OMP31-IFNG-F	5'-GTTGTTTCTGAACCTTCCGC-3'	1158	55
OMP31-IFNG-R	5'-TTACATTGATGCTCTCCGGC-3'		
BP26-IFNG-F	5'-GCTAGCAATTTTCTCGCAGC-3'	1242	55
BP26-IFNG-R	5'-TTACATTGATGCTCTCCGGC-3'		

Amplification, Concentration, Purification, and Titer Determination of Recombinant Adenoviruses: Recombinant adenoviruses were inoculated into HEK293A

cells; CPE and ZsGreen fluorescence were monitored. Cells and supernatant were harvested, freeze-thawed and centrifuged. Virus was concentrated with 8% PEG8000/0.5 M NaCl, purified via discontinuous CsCl gradient ultracentrifugation, and dialyzed to remove CsCl. Titers ($TCID_{50}$/mL) were calculated by the Reed-Muench method after 10-fold dilution and 7-day incubation in HEK293A cells.

Immunization of Mice with Recombinant Adenoviral Vaccines: A total of 72 female BALB/c mice (4–6 weeks old) were randomly divided into 6 groups, with 12 mice per group. The groups included three single-antigen recombinant adenovirus groups (rAd-SG, rAd-OG, and rAd-BG), one multi-antigen mixed recombinant adenovirus group (rAd-SG + rAd-OG + rAd-BG at a 1:1:1 volume ratio), a PBS control group, and a commercial live attenuated vaccine S2 control group. Each mouse in the recombinant adenovirus groups received an immunizing dose of 10^8 $TCID_{50}$ per 100μL via subcutaneous injection, followed by a booster immunization 14 days after the primary immunization. Mice in the S2 control group were immunized with 2×10^5 colony-forming units (CFU) per mouse, with only a single primary immunization (no booster). Mouse serum samples were collected every 7 days after the initial immunization. The animal study period spanned from Day 0 (initial immunization) to Day 56 (euthanasia of all mice post-challenge).

Evaluation of Immunoprotective Efficacy

Detection of IFN-γ-Secreting Splenic Lymphocytes in Mice by ELISpot: Splenocytes were aseptically isolated from mice at 42 days post-immunization using a mouse splenic lymphocyte separation kit (Tbdscience, China), following the manufacturer's instructions. Isolated splenocytes were seeded into 96-well plates at a density of 1×10^6 cells per well. For enzyme-linked immunospot (ELISpot) assay, the protocol provided by the ELISpot Basic kit manufacturer (Mabtech, Sweden) was followed. Concanavalin A (ConA) served as the positive control, PBS as the negative control, and inactivated *Brucella melitensis 043* as the experimental stimulant to trigger splenic lymphocytes. The number of IFN-γ-secreting T cells in splenic lymphocytes of immunized mice was quantified.

Detection of Cytokines in Splenic Lymphocyte Supernatants by ELISA: Splenocytes were aseptically isolated from mice at 42 days post-immunization using a mouse splenic lymphocyte separation kit (Tbdscience, China) according to the manufacturer's guidelines, and seeded into 96-well plates at 1×10^6 cells per well. Inactivated *Brucella melitensis 043* (1×10^5CFU per well) was added to the wells, followed by 24 hours of culture. Meanwhile, unstimulated control wells (containing cells and culture medium only) were set up. Supernatants were collected, and Th1-type cytokines (IFN-γ, IL-2, and TNF-α) in the splenic lymphocyte culture supernatants of immunized mice were detected using a commercial ELISA kit (R&D Systems, USA), following the manufacturer's instructions. The cytokine concentrations of all samples were subtracted by the background values from unstimulated control wells.

Lymphocyte Proliferation Assay: Mouse splenic lymphocytes were seeded into 96-well plates at 1×10^4 cells per 100μL per well. Three groups were set up: Experimental group: Medium, cells, and stimulant added; Negative control group: Medium and cells, no stimulant added; Blank group: Medium only, no cells. After 48 hours of incubation, CCK-8 reagent (Solarbio, China) was added, and the plates were further incubated for 2 hours. The absorbance was measured at 450nm. The stimulation index (SI) was calculated using the following formula:

$$SI=\frac{OD_{450\ nm,Stimulant\ group}-OD_{450\ nm,Blank\ group}}{OD_{450\ nm,Negative\ control\ group}-OD_{450\ nm,Blank\ group}}\times100\%$$

Determination of Antibodies by Enzyme-Linked Immunosorbent Assay (ELISA): Prokaryotically expressed purified proteins (purified and preserved in our laboratory) were coated onto 96-well immunoassay plates using carbonate buffer (pH9.6) at a concentration of 1μg per well. After blocking with 5% non-fat milk, the plates were incubated with appropriately diluted mouse serum (1:1000) at 37℃. Subsequently, the plates were incubated with HRP-conjugated goat anti-mouse antibodies (targeting IgG, IgG1, or IgG2a) (Abcam, China) diluted at 1:50,000, also at 37℃. The chromogenic reaction was initiated using a one-component TMB substrate solution (CWBio, China) and terminated by adding 50μL of stop solution (50mM H_2SO_4). The optical density (OD) of each well was measured at 450nm using a microplate reader.

Determination of Splenic Bacterial Load Post-Infection: At 42 days after the primary immunization, mice were intraperitoneally injected with 100μL of *Brucella melitensis 043* (containing 1×10^5CFU). Fourteen days post-challenge, all mice were euthanized by cervical dislocation. Spleens were aseptically harvested and weighed, then homogenized in PBS. A 100μL aliquot of the spleen homogenate was spread onto tryptic soy agar (TSA) plates (a solid medium for *Brucella* culture) and incubated at 37℃ for 3–5 days. Finally, colony counting was performed to calculate the bacterial load in the mouse spleens.

Histopathological Analysis: Mice were euthanized by cervical dislocation. Livers and spleens were harvested and fixed in 4% paraformaldehyde (Solarbio, China). The fixed tissues were embedded in paraffin, sectioned, and subjected to sequential treatments: drying in an oven, dewaxing, and rehydration. Subsequently, the tissue sections were stained, dehydrated, cleared for transparency, and mounted with neutral balsam, following the manufacturer's instructions of a hematoxylin and eosin (HE) staining kit (Solarbio, China).

Data Processing and Statistical Analysis: GraphPad Prism 9.5.0 was used. One-way and two-way ANOVA were used to analyze overall differences; Dunnett's test or Tukey's for pairwise comparisons. Data are mean±SD; $P<0.05$ was significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

RESULTS

Bioinformatic and Structural Analysis of Candidate Antigens: Results from the physicochemical property

prediction of the three candidate antigens using the Expasy Protparam server revealed the following characteristics: Cu/Zn-SOD had a molecular weight of approximately 18.1kDa, a theoretical isoelectric point (pI) of 6.24, an instability index of 37.94, and an aliphatic index of 73.29; OMP31 had a molecular weight of around 25.3kDa, a theoretical pI of 5.22, an instability index of 8.61, and an aliphatic index of 74.42; BP26 had a molecular weight of roughly 26.5kDa, a theoretical pI of 6.39, an instability index of 27.93, and an aliphatic index of 92.16 (Supplementary Table 1). Predictions via the VaxiJen server indicated that the antigenicity scores of all candidate antigens exceeded the set threshold of 0.4 (Fig. 1A). Similarly, analysis using the Protein–Sol server showed that the predicted solubility values of the candidates were all greater than 0.45 (Fig. 1B). As defined, any scaled solubility value above 0.45 indicates a higher solubility than the average level of soluble *E. coli* proteins in the experimental dataset, whereas values below this threshold correspond to lower solubility. Collectively, these in silico analyses suggest that the candidate antigens are likely stable, soluble, and potentially antigenic, although these predictions require experimental validation. B-cell epitope prediction predicted that Cu/Zn-SOD, OMP31, and BP26 contained 5, 7, and 6 continuous B-cell epitopes, respectively, all with scores exceeding 0.5 (Supplementary Fig. 1). We employed the Phyre2.2 server to model the tertiary structures of the candidate antigens and further assessed the overall quality of these models using Ramachandran plots generated by the Swiss Model server (Supplementary Fig. 2). The Ramachandran plot showed 95.39% (SOD), 81.77% (OMP31), and 85.85% (BP26) residues in favored regions, indicating acceptable stereochemical quality. SOD had excellent geometry, while OMP31 and BP26 showed less favorable regions requiring caution. Finally, we visualized the tertiary structures of the candidate antigens alongside their continuous B-cell epitopes using PyMOL software. The results showed that the majority of these B-cell epitopes were predicted to be localized on the protein surface, consistent with their role as potential antigen recognition sites (Fig. 1). However, linear B-cell epitope prediction has limited accuracy, and the predicted surface exposure of these epitopes requires experimental validation.

Construction and Characterization of Recombinant Adenoviral Vaccines: To develop a vaccine capable of conferring protection against *Brucella*, fusion genes encoding *Brucella*-derived *Cu/Zn-SOD*, *OMP31*, and *BP26* (each fused with IFN-γ) were integrated into adenoviral vectors. As illustrated in Fig. 2A, these fusion genes were inserted into the E1 region of the vector to construct adenoviral shuttle vectors (Supplementary Fig. 3). Subsequently, the shuttle vectors were co-transfected with the backbone plasmid pBHG into HEK293A cells. Within 7–10 days, the first passage of recombinant adenoviruses induced progressive cytopathic effects (CPE), characterized by cell shrinkage and vacuolation. Under fluorescence microscopy, transfected cells exhibited a comet tail-like distribution of green fluorescence; in contrast, no fluorescent signal was detected in the blank cell control group. Concurrently, prominent CPE was observed in the transfected cells under bright-field microscopy (Fig. 2B). After serial passage of adenovirus-infected cells, viral DNA was extracted. PCR analysis confirmed the successful insertion of the *Cu/Zn-SOD-IFN-γ*, *OMP31-IFN-γ*, and *BP26-IFN-γ* genes into the adenoviral genome, as well as their stable maintenance during passage (Supplementary Fig. 4). Furthermore, transmission electron microscopy (TEM) of purified recombinant adenoviruses revealed icosahedral capsid structures with a diameter of 80–100nm, consistent with the expected viral morphology (Fig. 2C). Collectively, these results demonstrate that the recombinant adenoviruses can be stably and correctly expressed in HEK293 cells.

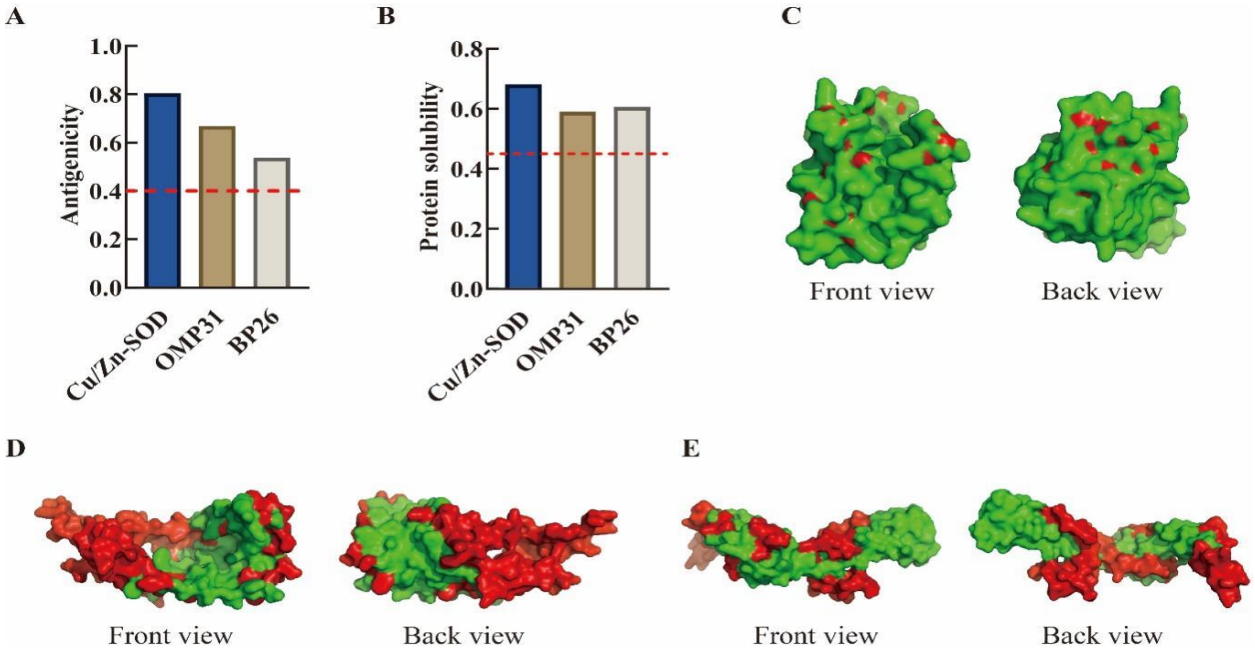

Fig. 1: Bioinformatics analysis of *Brucella* candidate antigens (Cu/Zn-SOD, OMP31, BP26). (A) Antigenicity predicted by VaxiJen (threshold = 0.4, red dashed line). (B) Protein solubility predicted by Protein-Sol (threshold = 0.45, red dashed line). (C–E) PyMOL visualization of 3D structures and B-cell epitopes (red) for Cu/Zn-SOD, OMP31, and BP26.

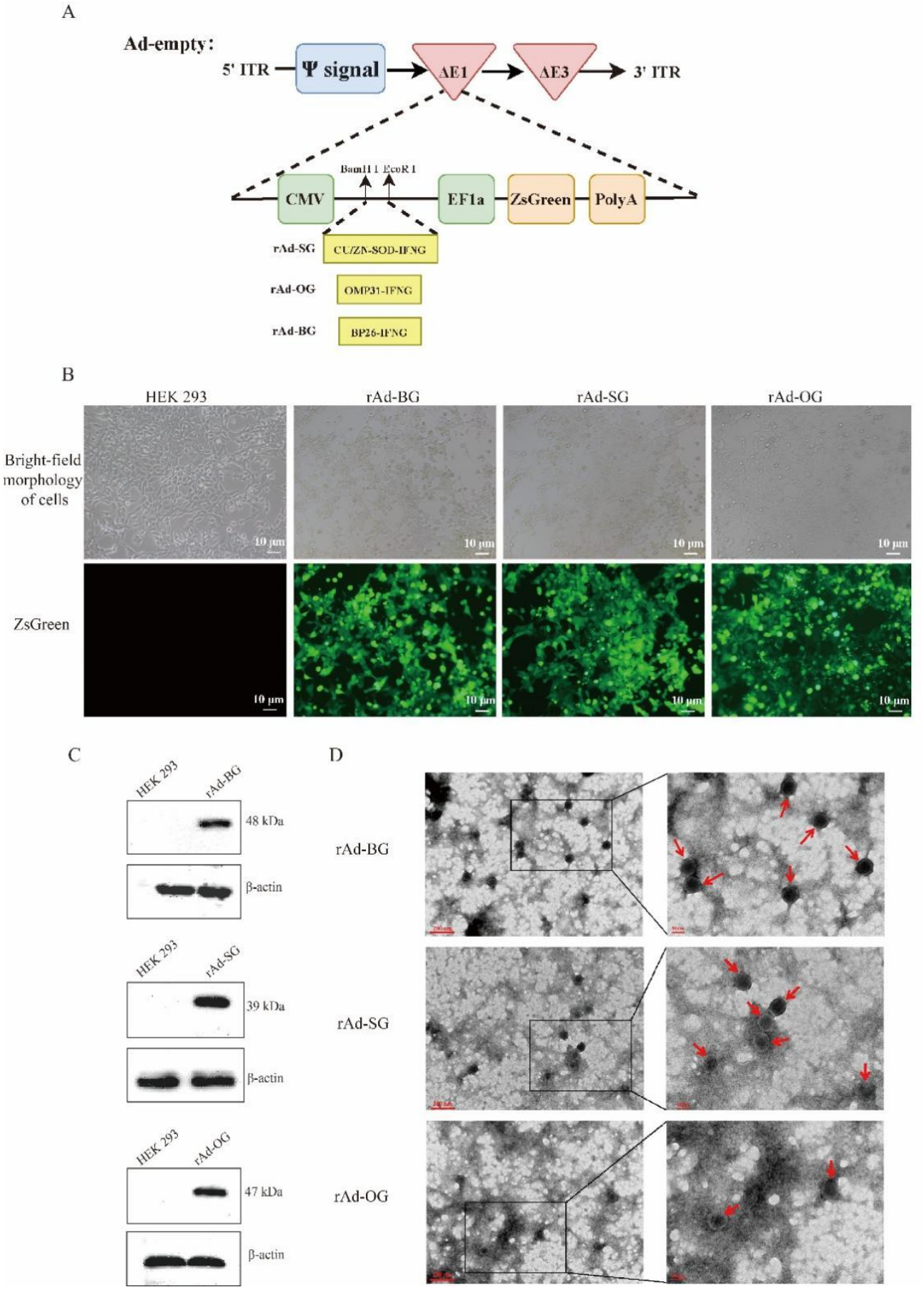

Fig. 2: Construction and characterization of recombinant adenoviral vectors. (A) Schematic diagram of recombinant adenoviral vector construction. (B) Fluorescence microscopy images showing bright-field morphology of cells and ZsGreen fluorescence expression. (C) Western blot was used to detect recombinant protein expression (probed with anti-His tag antibody, with β-actin as the internal reference). (D) TEM images of recombinant adenoviruses (scale bars: 200nm and 50nm; located at the bottom of the figure).

Humoral Immune Responses Induced by Recombinant Adenoviral Vaccines in Mice: To further evaluate the immunoefficacy of the candidate vaccines, a series of mouse experiments were performed (Fig. 3A). Specifically, to investigate the kinetic changes of specific antibodies (IgG, IgG1, and IgG2a) and assess the humoral immune responses induced by the recombinant adenoviral vaccines, indirect ELISA was used to detect antibody levels in mouse serum at 7, 21, 35, and 42 days post-immunization (dpi). As shown in Fig. 3B, the level of total IgG gradually increased from 7 to 35 dpi, peaked at 35 dpi, and remained elevated until 42 dpi. Notably, the IgG responses in all recombinant adenoviral vaccine groups were significantly higher than those in the PBS control group from 21 dpi onward ($P<0.001$). In contrast to IgG, IgG1 levels exhibited a transient increase—peaking between 21 and 35 dpi—followed by a subsequent decline. Only the rAd-Mix group showed significantly higher IgG1 levels than the PBS group at 35 dpi ($P<0.001$), while no significant differences were observed in other vaccine groups ($P\geq0.05$; Fig. 3C). Consistent with the kinetic pattern of total IgG, IgG2a levels increased over time and peaked at 35 dpi. At this time point, the rAd-Mix group also had significantly higher IgG2a levels compared to the PBS group ($P<0.001$; Fig. 3D). Importantly, the IgG2a/IgG1 ratio was greater than 1 in all recombinant adenoviral vaccine groups and the S2 vaccine group. Given that in mice IgG2a is typically associated with Th1-type responses (and IgG1 with Th2-type responses), this ratio indicates that the immune response was skewed toward a Th1-type profile (Fig. 3E), a critical feature for clearing intracellular *Brucella* pathogens (Fig. 3E), a critical feature for clearing intracellular *Brucella* pathogens. Collectively, these results demonstrate that all candidate vaccine groups effectively elicited specific antibody responses.

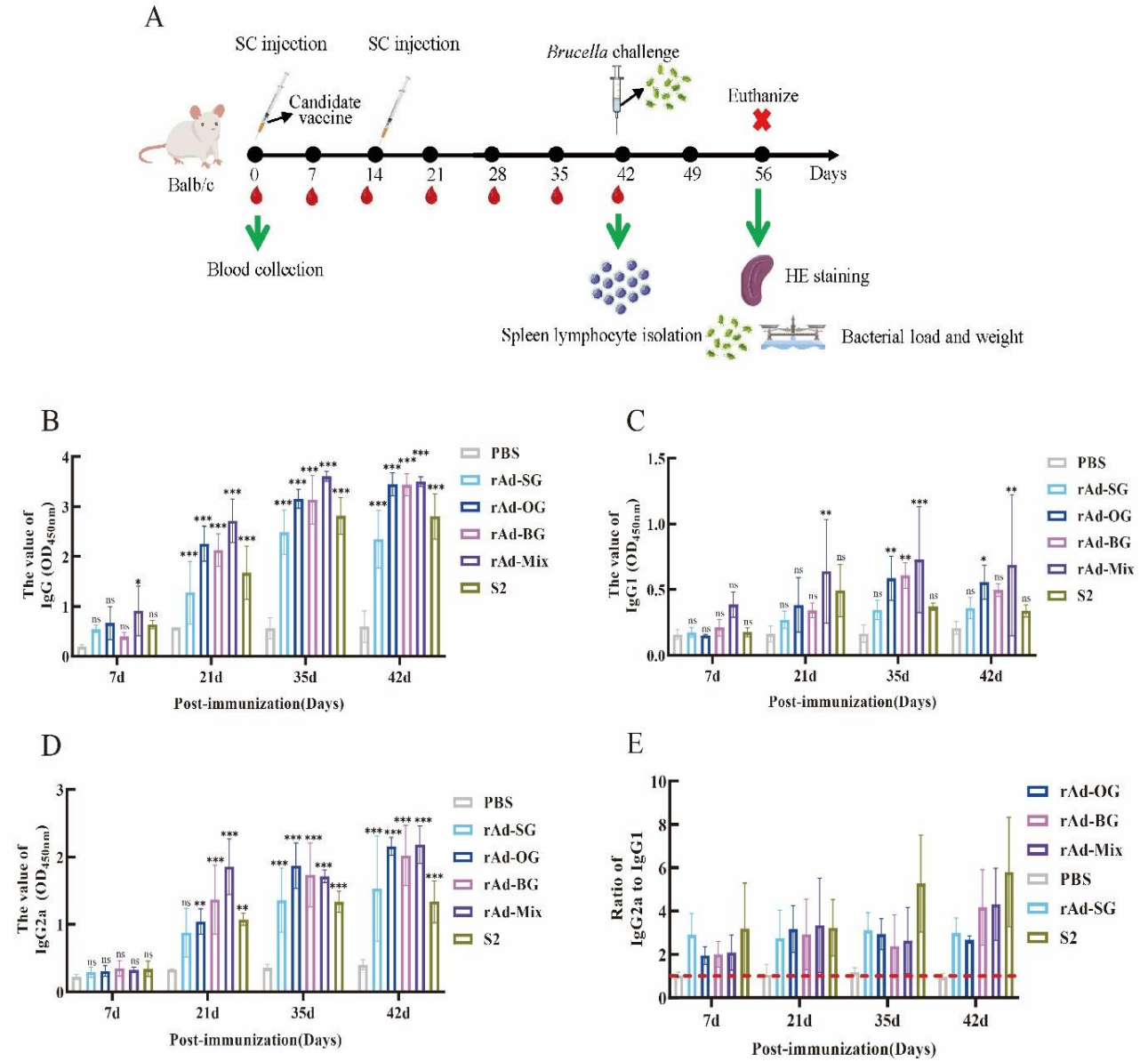

Fig. 3: Analysis of humoral immune responses induced by candidate vaccines. (A) Schedule of immunization and bacterial challenge in mice with candidate vaccines. (B) Determination of total serum IgG antibody levels by ELISA (n=3 for each group). (C) Determination of IgG1 subclass antibody levels by ELISA (n=3 for each group). (D) Determination of IgG2a subclass antibody levels by ELISA (n=3 for each group). (E) Ratio of OD_{450}nm values for IgG2a to IgG1. Data are presented as mean ± standard deviation (SD) and analyzed using Two-way ANOVA followed by Dunnett's multiple comparisons test. ***P<0.001, **P<0.01, *P<0.05, and ns indicates P≥0.05.

Spleen Lymphocyte Proliferation and Cytokine Expression in Vaccinated Mice: To investigate the effect of vaccination on lymphocyte proliferation in mice, the CCK-8 assay was used to determine the proliferation level of splenic lymphocytes in mice immunized with recombinant adenoviral vaccines at 42 days post-immunization (dpi). After 48 hours of stimulation with the target protein, the proliferation levels of splenic lymphocytes were significantly increased in all vaccine groups. Among these, the rAd-SG, rAd-Mix, and S2 groups exhibited extremely significant increases (P<0.001; Fig. 4A). These results indicate that recombinant adenoviral vaccines can effectively activate mouse splenic lymphocytes, enabling them to exhibit robust proliferative activity upon antigen re-stimulation. To further evaluate the cellular immunity levels induced by the recombinant adenoviral vector vaccines, ELISA was employed to detect the secretion levels of IL-2, TNF-α, and IFN-γ from splenic lymphocytes of immunized mice at 42 dpi. For IL-2 expression: all immunized groups showed higher IL-2 levels than the PBS control group, with the rAd-OG, rAd-BG, rAd-Mix, and S2 groups displaying extremely significant increases (P<0.001), and the rAd-SG group showing a significant increase (P<0.01; Fig. 4B). The expression pattern of TNF-α was similar to that of IL-2: all immunized groups had significantly higher TNF-α levels than the PBS control group. Specifically, the rAd-BG and rAd-Mix groups exhibited extremely significant increases (P<0.001); the rAd-SG and S2 groups showed highly significant increases (P<0.01); and the rAd-OG group displayed a significant increase (P<0.05; Fig. 4C). For IFN-γ expression: the rAd-Mix and S2 groups had extremely significant higher levels compared to the PBS control group (P<0.001), while no significant differences were observed between the remaining vaccine groups and the PBS group (P≥0.05; Fig. 4D). Furthermore, ELISpot was used to detect the level of IFN-γ production by antigen-specific T cells in each vaccine-immunized group following antigen stimulation. Consistent with the secretion of IFN-γ in the supernatant, the rAd-Mix and S2 groups showed significantly higher IFN-γ production than the PBS control group (P<0.01); the rAd-OG and rAd-BG groups showed no significant differences compared to the PBS group (P≥0.05), whereas the rAd-SG group exhibited a significant increase relative to the PBS control group (P<0.01; Fig. 4E).

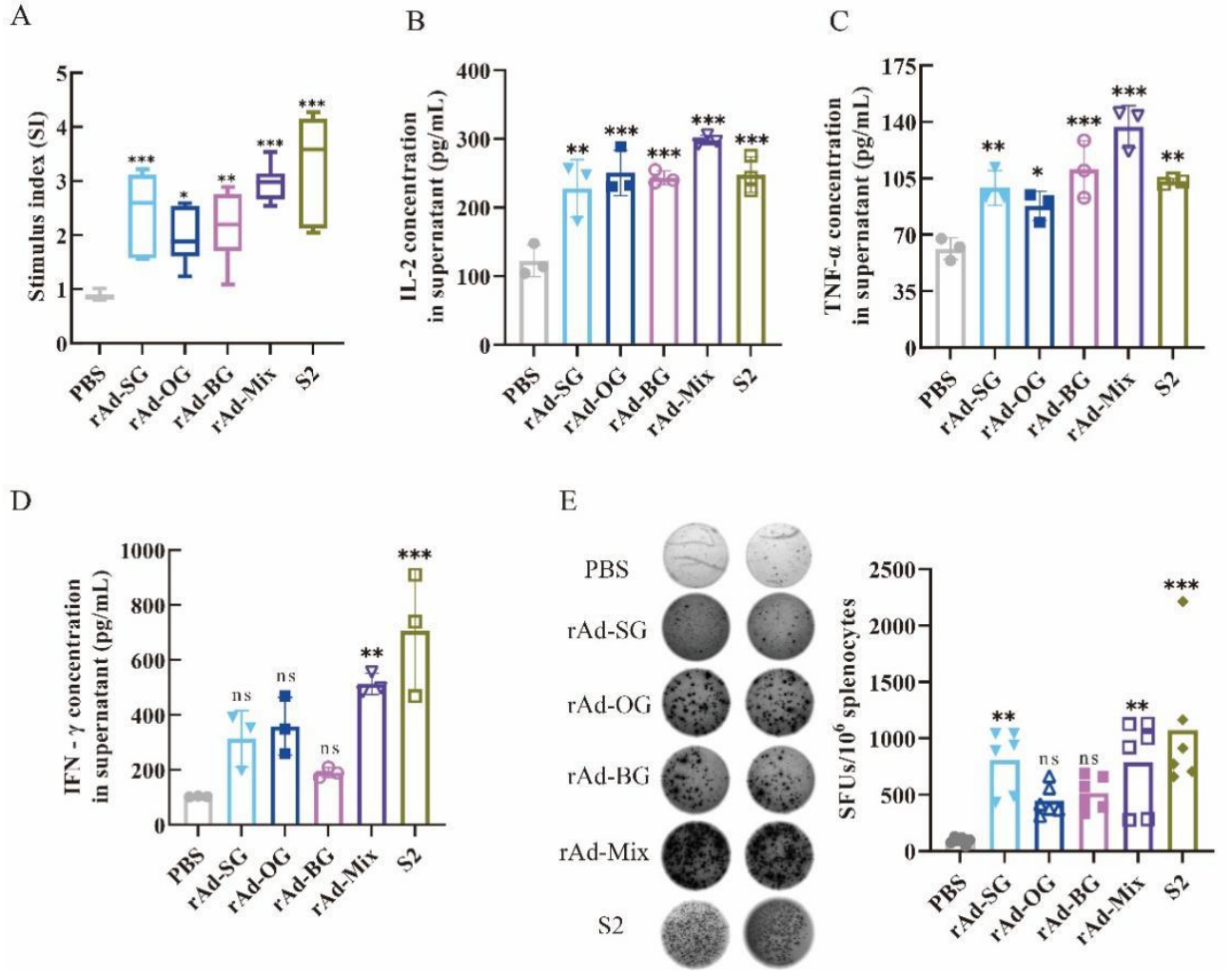

Fig. 4: Analysis of cellular immune responses induced by recombinant adenoviral vaccines. (A) Determination of splenic lymphocyte proliferation levels by the CCK-8 assay. (B) Concentration of IL-2 in the culture supernatant of mouse splenic lymphocytes (n=3 for each group). (C) Concentration of TNF-α in the culture supernatant of mouse splenic lymphocytes (n=3 for each group). (D) Concentration of IFN-γ in the culture supernatant of mouse splenic lymphocytes (n=3 for each group). (E) Assessment of IFN-γ-secreting capacity of antigen-specific T cells by ELISpot assay (n=3 for each group). Data are presented as mean ± standard deviation (SD) and analyzed using One-way ANOVA followed by Dunnett's multiple comparisons test. ***P<0.001, **P<0.01, *P<0.05, and ns indicates P≥0.05.

Protective Efficacy of Recombinant Adenoviral Vaccines Against Bacterial Challenge: To further verify the immunoprotective and anti-damage effects of the recombinant adenoviral vaccines in mice, mice were challenged with *Brucella melitensis 043* at 42 days post-immunization (dpi). Two weeks after challenge, the splenic index, splenic bacterial load, and pathological changes in the liver and spleen were evaluated; histological analysis of the spleen and liver was performed using hematoxylin and eosin (HE) staining. As shown in Fig. 5A, the degree of splenomegaly in all vaccine groups was significantly lower than that in the PBS control group, with extremely significant differences observed in the rAd-Mix and S2 groups compared to the PBS control group ($P<0.001$). For splenic bacterial load (Fig. 5B): the rAd-SG, rAd-OG, and rAd-BG groups showed significantly lower bacterial loads than the PBS control group ($P<0.05$); the rAd-Mix group exhibited a highly significant reduction ($P<0.01$); and the S2 group displayed an extremely significant reduction ($P<0.001$). A further direct comparison between rAd-Mix and S2 revealed that there were no statistically significant differences in spleen index ($P>0.99$) and splenic bacterial load (lgCFU, $P=0.93$), indicating that the protective efficacy conferred by rAd-Mix was comparable to that of S2. Histopathological observations revealed that only a small number of granulomas were present in the liver of all vaccine groups and the S2 group, whereas a large number of granulomas were detected in the PBS control group (Fig. 5C). In the spleen (Fig. 5D): the PBS control group showed extensive infiltration and proliferation of multinucleated giant cells as well as numerous granulomas; in contrast, the rAd-Mix and S2 groups only exhibited mild lymphocyte infiltration and scattered small granulomas; the other recombinant adenoviral groups showed moderate pathological damage. In summary, the *Brucella*-based recombinant vaccines using adenoviral vectors can exert effective immunoprotection in mice against *Brucella melitensis 043* challenge by alleviating splenomegaly, reducing bacterial load, and mitigating hepatosplenic pathological damage.

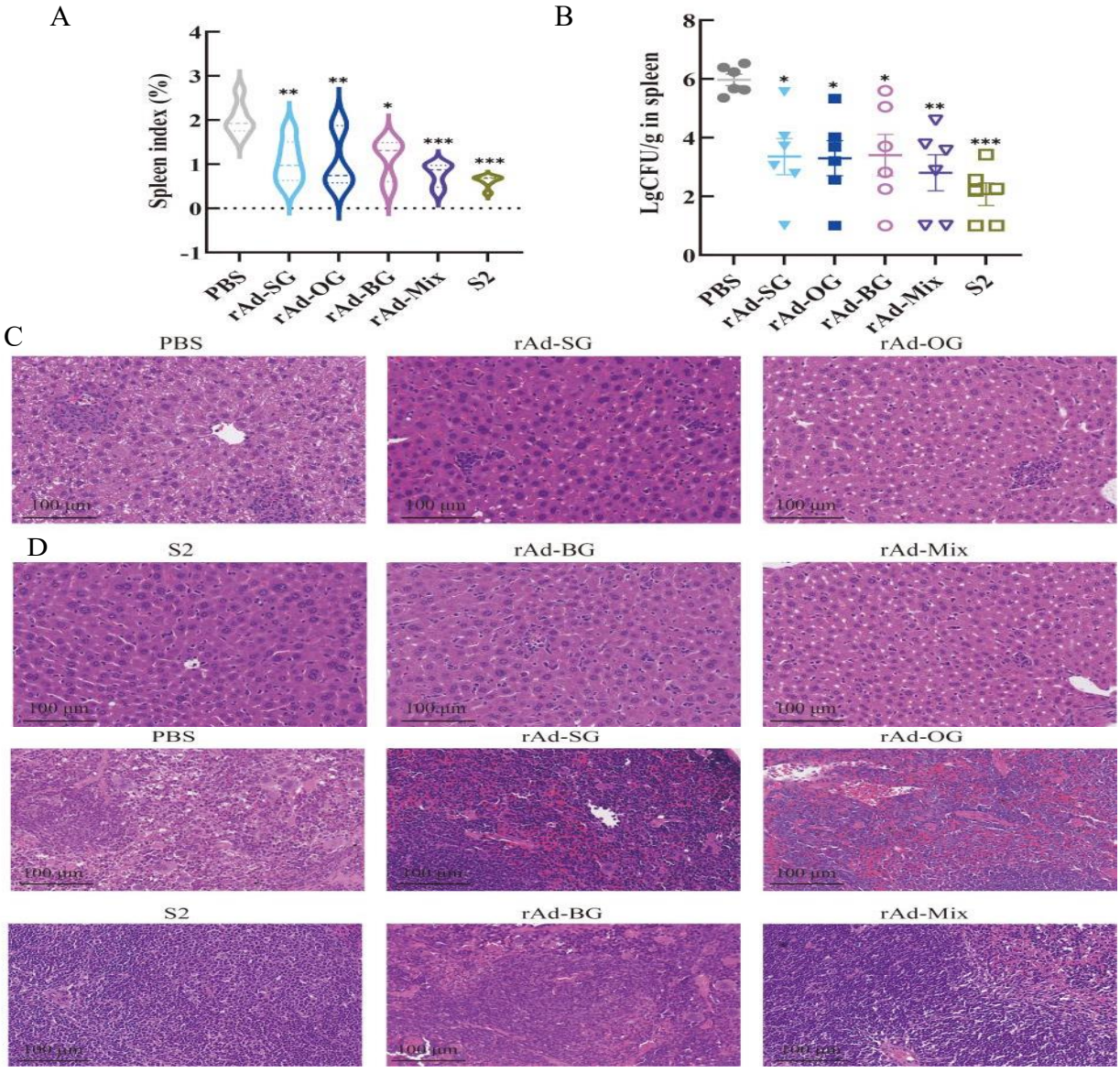

Fig. 5: Analysis of protective efficacy of recombinant adenoviral vaccines against bacterial challenge. (A) Splenic index of mice (n=6 for each group). (B) Splenic bacterial load of mice (n=6 for each group). (C-D) Pathological changes in the liver and spleen of immunized mice post-Brucella infection (n=3 for each group), Scale bar: 100μm (located at the bottom of the figure). Data are presented as mean ± standard deviation (SD) and analyzed using One-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001, **P<0.01, *P<0.05, and ns indicates P≥0.05.

DISCUSSION

Brucellosis, caused by bacteria of the genus *Brucella*, constitutes a major global zoonotic threat, imposing a substantial burden on both public health and livestock economies. Vaccination remains the cornerstone of control strategies (Pandey *et al.*, 2016). While live-attenuated vaccines are widely used, they pose serious drawbacks, including residual virulence, interference with serological diagnosis, and suboptimal efficacy, necessitating the development of safer, more effective alternatives (Gheibi *et al.*, 2018). An ideal vaccine must be cost-effective and elicited robust humoral and cellular immunity (He *et al.*, 2023). Subunit vaccines, though safe, have largely failed to confer protection comparable to live vaccines (Pan *et al.*, 2021; Heidary *et al.*, 2022), primarily because soluble antigens often fail to elicit the potent T-cell responses required to clear intracellular *Brucella*. Replication-deficient adenoviral (Ad) vectors represent a superior platform, combining a strong safety profile with an exceptional capacity to induce cytotoxic T-lymphocyte (CTL) responses critical against intracellular pathogens(Arevalo *et al.*, 2009; Sayedahmed *et al.*, 2020). Their inherent DIVA (Differentiation of Infected from Vaccinated Animals) compatibility further addresses a key limitation of existing vaccines. To harness these advantages, we constructed Ad vectors (rAd-SG, rAd-OG, rAd-BG) co-expressing the molecular adjuvant IFN-γ with the *Brucella* antigens Cu/Zn-SOD, OMP31, or BP26—each implicated in virulence and protection. Predicting that multivalency could synergize and broaden immunity, we also formulated a pooled vaccine (rAd-Mix). We systematically evaluated the immunogenicity and protective efficacy of these candidates in a murine model.

Host immunity against *Brucella* infection involves both cellular and humoral immunity. While humoral immunity plays a critical role in controlling extracellular pathogens, a balanced and Th1-skewed immune response is essential for eliminating intracellular bacteria such as *Brucella* (Vitry *et al.*, 2014; Paul *et al.*, 2018). Specifically, antibody-mediated humoral immune responses are crucial for regulating the levels of circulating *Brucella* in the bloodstream of infected hosts. In the present study, recombinant adenoviral vaccines significantly induced the secretion of total IgG, confirming their ability to elicit a sustained humoral immune response. This effect is attributed to the adenovirus's function as an efficient gene delivery vector: it enables continuous expression of *Brucella* antigens (Cu/Zn-SOD, OMP31, and BP26) in host cells, thereby providing persistent antigenic stimulation for B cell activation (Molinier-Frenkel *et al.*, 2000) and facilitating the gradual production and accumulation of antibodies. Further analysis of antibody subtypes revealed that recombinant adenoviral vaccines significantly induced the secretion of both IgG1 and IgG2a, with an IgG2a/IgG1 ratio > 1—indicating a predominantly Th1-skewed immune response (Paul *et al.*, 2018). This Th1-biased immunity is a prerequisite for combating *Brucella* infection and inducing a fully protective immune response. However, it should be noted that while sustained antigen-specific IgG is induced, future work is needed to characterize the functional properties of these antibodies and their direct contribution to protection.

A critical step in eliminating *Brucella* infection is the induction of Th1-type immune responses and the production of associated cytokines. IL-2, a key driver of T cell proliferation and differentiation, was significantly upregulated in all immunized groups—indicating that recombinant adenoviral vaccines can effectively promote the proliferation and differentiation of naive T cells, which serves as the foundation for initiating adaptive immune responses (He, 2023). This finding suggests that both monovalent antigen recombinant vaccines (rAd-OG, rAd-BG, rAd-SG) and the multivalent mixed vaccine (rAd-Mix) can trigger primary T cell activation via the efficient antigen delivery capability of adenoviral vectors. Notably, IFN-γ—a primary cytokine against *Brucella* infection—plays a pivotal role in the early clearance of intracellular *Brucella* (Luo *et al.*, 2006). It enhances the bactericidal activity of macrophages by activating these cells, thereby effectively inhibiting the replication of intracellular microbes (Dorneles *et al.*, 2015). ELISA results for IFN-γ showed that only the rAd-Mix and S2 groups exhibited extremely significantly elevation in IFN-γ levels. This outcome directly highlights the advantage of synergistic activation by multiple antigens: monovalent antigen vaccines (rAd-OG, rAd-BG, rAd-SG) may only activate a limited subset of T cells specific to a single antigen, resulting in total IFN-γ secretion insufficient to reach statistical significance. In contrast, the rAd-Mix group—prepared by combining the three candidate vaccines (rAd-OG, rAd-BG, rAd-SG)—covers distinct targets of *Brucella* and activates a broader population of antigen-specific Th1 cells, leading to higher IFN-γ production. As a live attenuated vaccine, S2 induces a high-level IFN-γ response, likely because it can efficiently infect host cells and generate multiple endogenous antigens in antigen-presenting cells (APCs) (Luo *et al.*, 2006). On the other hand, our results showed that IFN-γ levels in splenocyte supernatants were higher than those of TNF-α. This specific ratio (high IFN-γ + moderate TNF-α) holds important pathophysiological significance: IFN-γ clears intracellular *Brucella* by enhancing the phagolysosomal fusion efficiency of macrophages; meanwhile, moderate TNF-α secretion avoids tissue damage caused by excessive inflammatory responses (Chadwick *et al.*, 2008). Excessive TNF-α has been associated with tissue damage and immunopathology; therefore, a profile of strong IFN-γ with controlled TNF-α is desirable.

Splenomegaly, a hallmark of murine brucellosis, provides a key index of infection severity (Tsevelmaa *et al.*, 2018). Post-challenge, only mice immunized with rAd-Mix or the live attenuated S2 vaccine exhibited splenic indices comparable to naïve controls, indicating a marked reduction in pathology. The gold standard for vaccine efficacy is the reduction in bacterial burden (Taborska *et al.*, 2021). As expected, the S2 vaccine conferred potent clearance. However, its utility is limited by residual virulence and serodiagnostic interference (Lalsiamthara *et al.*, 2017; Gheibi *et al.*, 2018). Notably, the rAd-Mix vaccine, a replication-deficient vector featuring enhanced theoretical safety and potential DIVA compatibility, elicited protection levels comparable to the S2 benchmark and higher than those of all monovalent vaccines. Nevertheless, formal DIVA validation and safety evaluation in target livestock must be conducted to verify

these merits. (Gomez *et al.*, 2017; Chiuppesi *et al.*, 2020; Huy Tran Xuan *et al.*, 2021). The rAd-Mix group showed bacterial loads and granuloma attenuation equivalent to the S2 group, without evidence of exacerbated inflammation. This establishes rAd-Mix as a highly promising candidate that marries the efficacy of a live vaccine with the safety of a subunit platform. Future studies will delineate the precise mechanisms underpinning this synergistic protection.

The inherent mucosal tropism of adenoviral vectors makes them attractive platforms for non-invasive immunization routes (e.g., oral, intranasal), which can induce localized immune responses and potentially enhance overall immunogenicity (Yao *et al.*, 2018). Notably, adenoviral vectors have been successfully delivered via intranasal or oral routes in other disease models, where they induced potent mucosal and systemic immunity (Jacob-Dolan *et al.*, 2022; Liu *et al.*, 2023). Although the present study focused on subcutaneous delivery, future work comparing mucosal administration will be essential to determine the optimal strategy for inducing protection at the site of pathogen entry.

Moreover, while murine models provide a valuable platform for initial vaccine screening (Bugybayeva *et al.*, 2020; Fadaie *et al.*, 2025; Javed *et al.*, 2025), they cannot fully recapitulate *Brucella* pathogenesis in natural ruminant hosts such as sheep, cattle, and goats. Therefore, evaluating the protective efficacy of our candidate vaccine in these target species represents a critical step toward validating its potential for clinical application.

Conclusions: This study demonstrates that recombinant adenoviral vaccines expressing fusion proteins of *Brucella*-derived Cu/Zn-SOD, OMP31, and BP26 (each fused with IFN-γ) can induce robust Th1-skewed immune responses in mice and provide effective protection against *Brucella* infection. Notably, the multivalent mixed vaccine rAd-Mix exhibits superior immunogenicity and protective efficacy compared to monovalent antigen vaccines, while achieving efficacy comparable to the commercial S2 vaccine. Importantly, rAd-Mix also offers potential safety advantages over traditional live attenuated vaccines (e.g., absence of residual virulence and serological diagnostic interference). These findings support the development of adenoviral-vectored multivalent vaccines as a promising strategy for brucellosis control, holding significant implications for the advancement of both veterinary vaccines and potential human vaccines against brucellosis. Future studies should focus on further optimizing antigen selection, vector design, and delivery approaches to advance this vaccine platform toward clinical application.

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REFERENCES

Arevalo MT, Xu Q, Paton JC, *et al.*, 2009. Mucosal vaccination with a multicomponent adenovirus-vectored vaccine protects against *Streptococcus pneumoniae* infection in the lung. FEMS Immunology and Medical Microbiology 55: 346-351.

Beck BL, Tabatabai LB, and Mayfield JE, 1990. A protein isolated from *Brucella abortus* is a Cu-Zn superoxide dismutase. Biochemistry 29: 372-376.

Bian Y, Walter D,and Zhang C, 2023. Cytometric analysis authenticates adjuvanticity of interferon-γ and suggests potential synergy with toll-like receptor agonists. Journal of Immunology 210:1: 145.03.

Bugybayeva D, Ryskeldinova S, Zinina N, *et al.*, 2020. Development of human vectored brucellosis vaccine formulation: assessment of safety and protectiveness of influenza viral vectors expressing *Brucella* immunodominant proteins in mice and guinea pigs. Biomed Research International 2020: 143892.

Chadwick W, Magnus T, Martin B, *et al.*, 2008. Targeting TNF-α receptors for neurotherapeutics. Trends in Neurosciences 31: 504-511.

Chiuppesi F, Salazar MdA, Contreras H, *et al.*, 2020. Development of a multi-antigenic SARS-CoV-2 vaccine candidate using a synthetic poxvirus platform. Nature Communications 11: 6121.

Cloeckaert A, Vizcaíno N, Paquet JY, *et al.*, 2002. Major outer membrane proteins of *Brucella spp.*: past, present and future. Veterinary Microbiology 90: 229-247.

Ding J, Jiang N, Zheng Y, *et al.*, 2022. Adenovirus vaccine therapy with CD137L promotes CD8+DCs-mediated multifunctional CD8+T cell immunity and elicits potent anti-tumor activity. Pharmacological Research 175: 106034.

Dorneles EMS, Teixeira-Carvalho A, Araujo MSS, *et al.*, 2015. Immune response triggered by *Brucella abortus* following infection or vaccination. Vaccine 33: 3659-3666.

Doytchinova IA and Flower DR, 2007. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics 8: 4.

Engelhardt JF, Litzky L and Wilson JM, 1994. Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a. Human Gene Therapy 5: 1217-1229.

Fadaie M, Esmaeilifallah M, Khalafiyan A, *et al.*, 2025. In vivo immunogenicity assessment of a multiepitope-displayed phage vaccine against *Brucella* species infection in BALB/c mice. Bioengineering & Translational Medicine 2: e70024.

Gaden F, Franqueville L, Magnusson MK, *et al.*, 2004. Gene transduction and cell entry pathway of fiber-modified adenovirus type 5 vectors carrying novel endocytic peptide ligands selected on human tracheal glandular cells. Journal of Virology 78: 7227-7247.

Gasteiger E, Gattiker A, Hoogland C, *et al.*, 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research 31:13: 3784-3788.

Gheibi A, Khanahmad H, Kashfi K, *et al*., 2018. Development of new generation of vaccines for *Brucella abortus*. Heliyon 4: e01079.

Gomez L, Llanos J, Escalona E, *et al.*, 2017. Multivalent fusion DNA vaccine against *Brucella abortus*. Biomed Research International 6535479.

Guex N, Peitsch MC and Schwede T, 2009. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis 30: S162-S173.

He K, 2023. 'IL-2Rα-biased' IL-2 for cancer immunotherapy. Nature Cancer 4: 1222-1223.

He X, Zhang T, Huan S, *et al.*, 2023. Novel influenza vaccines: from research and development (R&D) challenges to regulatory responses. Vaccines 11(10): 1573.

Heidary M, Dashtbin S, Ghanavati R, *et al.*, 2022. Evaluation of brucellosis vaccines: A comprehensive review. Frontiers in Veterinary Science 9: 925773.

Hull NC and Schumaker BA, 2018. Comparisons of brucellosis between human and veterinary medicine. Infection Ecology & Epidemiology 8: 1500846.

Huy Tran Xuan N, Nguyen Trang T, Reyes AWB, *et al.*, 2021. Immunization with a combination of four recombinant *Brucella abortus* proteins Omp16, Omp19, Omp28, and L7/L12 Induces T Helper 1 immune response against virulent *B. abortus* 544 infection in BALB/c Mice. Frontiers in Veterinary Science 7: 577026.

Ikoma T, Takahashi T, Nagano S, *et al.*, 2004. A definitive role of RhoC in metastasis of orthotopic lung cancer in mice. Clinical Cancer Research 10: 1192-1200.

Jacob-Dolan C and Barouch DH, 2022. COVID-19 Vaccines: Adenoviral Vectors. Annual Review of Medicine 73: 41-54.

Javed M, Naseer M, Ibrahim M, *et al.*, 2025. Multi-Epitope based vaccine design and analysis against bovine viral diarrhea virus using immunoinformatics approaches. Pakistan Veterinary Journal 45(2): 783-790.

Khan I, Raza S, Khan AU, *et al.*, 2025. Sero prevalence and molecular characterization of brucellosis in goats. Continental Veterinary Journal 5(2): 207-212.

Kumar M, Behera AK, Hu JN, *et al.*, 2001. IFN-γ and IL-12 plasmid DNAs as vaccine adjuvant in a murine model of grass allergy. Journal of Allergy and Clinical Immunology 108: 402-408.

Lalsiamthara J and Lee JH, 2017. Development and trial of vaccines against *Brucella*. Journal of Veterinary Science 18: 281-290.

Lin F-c, and Young HA, 2014. Interferons: Success in anti-viral immunotherapy. Cytokine & Growth Factor Reviews 25: 369-376.

Liu W, Li H, Liu B, *et al.*, 2023. A new vaccination regimen using adenovirus-vectored vaccine confers effective protection against African swine fever virus in swine. Emerging Microbes & Infections 12(2): 2233643.

Luo D, Bing N, Li P, *et al.*, 2006. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infection and Immunity 74: 2734-2741.

Martin-Martin AI, Caro-Hernandez P, Orduna A, *et al.*, 2008. Importance of the Omp25/Omp31 family in the internalization and intracellular replication of virulent *B. ovis* in murine macrophages and HeLa cells. Microbes and Infection 10: 706-710.

Memish ZA and Balkhy HH, 2004. Brucellosis and international travel. Journal of Travel Medicine 11: 49-55.

Mohamed K, 2025. *Brucella*-host interactions in cattle: zoonotic transmission, immunological insights and non-antibiotic strategies. Pakistan Veterinary Journal 45(2): 527 735.

Molinier-Frenkel V, Le Boulaire C, Le Gal FA, *et al*., 2000. Longitudinal follow-up of cellular and humoral immunity induced by recombinant adenovirus-mediated gene therapy in cancer patients. Human Gene Therapy 11: 1911-1920.

Ospanov Y, Arysbekova A, Kaiyrbek A, *et al.*, 2024. Determination of risks of occurrence and areas of brucellosis infection spread in the territory of the Republic of Kazakhstan. International Journal of Veterinary Science 13(6): 908-913.

Pan C, Yue H and Zhu L, 2021. Prophylactic vaccine delivery systems against epidemic infectious diseases. Advanced Drug Delivery Reviews 176: 113867.

Pandey A, Cabello A, Akoolo L, *et al.*, 2016. The case for live attenuated vaccines against the neglected zoonotic diseases brucellosis and bovine tuberculosis. PLOS Neglected Tropical Diseases 10(8): e0004572.

Paul S, Peddayelachagiri BV, Nagaraj S, *et al.*, 2018. Recombinant outer membrane protein 25c from *Brucella abortus* induces Th1 and Th2 mediated protection against *Brucella abortus* infection in mouse model. Molecular Immunology 99: 9-18.

Powell HR, Islam SA, David A, *et al.*, 2025. Phyre2.2: A community resource for template-based protein structure prediction. Journal of Molecular Biology 437(15): 168960.

Sayedahmed EE, Elkashif A, Alhashimi M, *et al.*, 2020. Adenoviral vector-based vaccine platforms for developing the next generation of influenza vaccines. Vaccines 8(4): 574.

Taborska P, Lastovicka J, Stakheev D, *et al.*, 2021. SARS-CoV-2 spike glycoprotein-reactive T cells can be readily expanded from COVID-19 vaccinated donors. Immunity Inflammation and Disease 9: 1452-1467.

Tsevelmaa N, Narangerel B, Odgerel O, *et al.*, 2018. Anti-Brucella activity of *Caryopteris mongolica* bunge root extract against *Brucella melitensis* infection in mice. BMC Complementary and Alternative Medicine 18: 144.

Vitry MA, Mambres DH, De Trez C, *et al.*, 2014. Humoral immunity and CD4+ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. Journal of Immunology 192: 3740-3752.

Wang YP, Liu D, Guo LJ, *et al.*, 2013. Enhanced protective immune response to PCV2 subunit vaccine by co-administration of recombinant porcine IFN-γ in mice. Vaccine 31: 833-838.

Watanabe M, Nishikawaji Y, Kawakami H, *et al.*, 2021. Adenovirus biology, recombinant adenovirus, and adenovirus usage in gene therapy. Viruses-Basel 13(12): 2502.

Wiederstein M and Sippl MJ, 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Research 35: W407-410.

Wiethoff CM, Wodrich H, Gerace L, *et al.*, 2005. Adenovirus protein VI mediates membrane disruption following capsid disassembly. Journal of Virology 79: 1992-2000.

Xie Y, Lin S, Guo L, *et al.*, 2025. Development and evaluation of the recombinant BP26 protein-based C-ELISA for human brucellosis diagnosis. Frontiers in Microbiology 15: 1516915.

Yan Y, Jing S, Feng L, *et al.*, 2021. Construction and characterization of a novel recombinant attenuated and replication-deficient candidate Human Adenovirus Type 3 Vaccine: "Adenovirus vaccine within an Adenovirus Vector". Virologica Sinica 36: 354-364.

Yao Y, Jeyanathan M, Haddadi S, *et al.*, 2018. Induction of autonomous memory alveolar macrophages requires T Cell help and is critical to trained immunity. Cell 175: 1634-1650.

Zhang G, Jiang H, Zhang G, *et al.*, 2024. Strain-level identification of *Brucella melitensis* reference strain 63/9 using multiplex PCR method by targeting BMEA_B0162 and BMEA_A1238. Pakistan Veterinary Journal 44(1): 183-189.

Zhu J, Huang X and Yang Y, 2007. Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and independent pathways. Journal of Virology 81: 3170-3180.