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Immunogenicity of a Recombinant Subunit Vaccine Against Feline Coronavirus: A Comparative Study of Three Different Adjuvants

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ABSTRACT

Background: Currently, there is no effective vaccine against feline coronavirus infections. The coronavirus Spike (S) protein plays a critical role in viral binding to cell receptors and contains multiple neutralizing antibody epitopes that trigger the host's immune response to combat infection. Selecting an optimized adjuvant is essential to ensure robust vaccine-induced immunity against pathogenic infections.

Objectives: To produce a recombinant S protein for the development of subunit vaccines and evaluate the immune responses elicited by different adjuvants.

Methods: In this study, we developed three subunit vaccines incorporating distinct adjuvants: Alh, ISA201, and CFA FCoV-SP. BALB/c mice were immunized three times via subcutaneous injections with each vaccine formulation. Serum samples were then analyzed to evaluate S protein-specific IgG levels and cytokine concentrations using enzyme-linked immunosorbent assay (ELISA), assessing the magnitude and nature of the vaccine-induced immune responses.

Results: The ISA201 FCoV-SP vaccine induced significantly higher total IgG levels than those in the Alh or CFA groups. All tested protein concentrations resulted in increased serum IgG antibody levels, with the optimal immune dose of recombinant S protein being 15 μg/dose. Additionally, the ISA201 FCoV-SP vaccine led to increased expression of interferon-γ, interleukin-8, and tumor necrosis factor-α.

Conclusion: Collectively, our findings suggest that ISA201 serves as the most effective adjuvant for a recombinant S protein subunit vaccine against FCoV. Additionally, the subunit vaccine developed in this study exhibited acceptable immune responses in mice.

Keywords: Feline coronavirus, Subunit vaccine, ISA201, Immune response, Adjuvant

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INTRODUCTION

Feline coronavirus (FCoV) is a non-segmented, single-stranded, RNA virus belonging to the *Alphacoronavirus* family (1). Based on pathogenicity, FCoV exists as two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) (2). FECV demonstrates particularly high prevalence among cat populations, with infection rates reaching 90%in stray cat colonies and multi-cat environments (3). While FECV typically causes only mild diarrhea and intestinal discomfort, it often establishes persistent infections with many cats remaining asymptomatic carriers (4). In contrast, FIPV is not transmitted through the fecal-oral route, but is produced by FECV mutations in a small percentage of infected cats, leading to fatal feline infectious peritonitis (FIP) (2). Currently, most treatments for FCoV infection involve appropriate therapies aimed at helping cats overcome the disease by inhibiting viral replication, suppressing inflammatory responses, stimulating the immune system, and reducing the risk of reinfection (5). However, the recurrence rate of the disease is high, and recurrent cases typically present with greater severity. Therefore, vaccination remains an effective strategy for viral control.

Vaccine immunization plays a crucial role in preventing and controlling infectious diseases. However, traditional vaccine development is time-consuming and may not adequately address the current needs for disease prevention and control due to natural variations and the constant emergence of new pathogens. While some progress has been made in creating a vaccine against FCoV with varying success (6), currently, there is no effective vaccine commercially available to protect against FCoV infection. Recombinant protein vaccines involve delivering targeted antigens into cells rather than expressing them in the cytoplasm (7). Antigens can be internalized through endocytosis and subsequently localized to endosomes via the major histocompatibility complex (MHC) II mechanism (8). The endosome then fuses with the lysosome and degrades the antigens into peptide fragments that are limited to MHC classes I and II. MHC class II-specific peptides bind to MHC class II molecules by replacing MHC class II-related invariant chain peptides. This peptide-MHC class II complex is then presented on the cell surface and activates naïve CD4+ T cells (9). Similar to other coronaviruses, FCoV possesses four structural proteins. Among them, the spike (S) protein serves as the primary mediator of host cell entry (10). The S protein facilitates viral invasion by promoting fusion with the host cell membrane through binding to the host cell receptor (11). Additionally, it is a key protein that triggers the host to produce neutralizing antibodies (12). The S protein plays a crucial role in antibody production and shows potential for vaccine development. Notably, an mRNA vaccine based on the SARS-CoV-2 S protein showed promise in a Phase 1 clinical trial, by eliciting an immune response against the pathogen in all patients, without major adverse effects (13). Additionally, FCoV can be classified into type I and type II serotypes based on the S protein variants (14), with type I FCoV strains being highly prevalent in the feline population (15, 16). Therefore, the S protein of FCoV is a prime candidate for the genetic engineering of novel subunit vaccines.

Due to their advantages, such as low cost, high yield and safety, subunit vaccines are used to protect pigs against FCoV infection (17). However, because of their weak immunogenicity and short-lived immune protection, these vaccines are often combined with adjuvants to boost the immune system of treated animals (18). Immune adjuvants are substances that enhance or modify the specific immune responses of the body, thereby increasing antigen immunogenicity (19). Adjuvants can be classified into several categories including aluminum-based compounds, oil-emulsion systems, and liposome formulations. Oil-emulsion

adjuvants are known for promoting high-titer antibody production against different antigens, extending the period of antigen stimulation, and reducing the dose and frequency of antigen inoculations. These properties have led to their widespread use in animal vaccine development (20). ISA201 is a water-in-oil-in-water (W/O/W) mineral oil-based adjuvant emulsion (21). It is designed to reduce the viscosity and side effects, improve the injectability of vaccines, and maintain its capacity to deliver antigens (22).

In this study, we developed a subunit vaccine using recombinant FCoV S protein and evaluated its immunogenicity in mice. In addition, we compared the efficacy of several adjuvants in the subunit vaccine and evaluated immune responses to the ISA201 vaccine in mice. This study provides a research basis to improve preventive strategies for FCoV.

MATERIALS AND METHODS

Recombinant FCoV-SP Protein Production

The FCoV-SP protein was prepared according to previous reports (23). To do this, pET-28a-SP was added to 50 μL of *Escherichia coli* BL21 (DE3) cells and placed in an ice bath for 30 minutes. It was then tranferred to a 42 °C water bath and heated it for 30 s, followed by immediate placement on ice for 2 min. Next, 250 μL of LB medium without antibiotics was added and brought to room temperature. The medium was shaken at 200 rpm/min and 37 °C for 1 h, then evenly spread on solid medium containing ampicillin and incubated at 37 °C for 12 h. Positive colonies were selected, cultured overnight, and induced for FCoV-SP expression using isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1.0 mM.

Vaccine Preparation

Montanide ™ ISA201 VG biphasic oil emulsion adjuvant (ISA201) was produced by Seppic (Paris, France). Freund's complete adjuvant (CFA) was purchased from Sigma (St. Louis, MO, USA), and aluminum hydroxide gel adjuvant (Alh) was purchased from General Chemical Corp. (Ontario, USA). The Alh FCoV-SP vaccine was prepared using a previously reported method (16) with a 1:1 (w/w) ratio of Alh to FCoV-SP in the Alh vaccine. The ISA201 FCoV-SP vaccine was prepared according to the manufacturer's instructions. Briefly, ISA201 was preheated in a water bath (31°C) for 30 min, and then FCoV-SP was slowly added to ISA201 at a final ratio of 1:1 (w/w) and gently shaken to creat the ISA201 vaccine. The CFA FCoV-SP vaccine was prepared by slowly adding CFA to the FCoV-SP antigen at a final ratio of 1:1 (v/v) followed by gentle shaking.

Vaccination Experiments

Thirty female BALB/c mice (eight weeks old) was obtained from Wu's Experimental Animal Trading Co., Ltd. (Fujian, China). They were housed in the animal care facility at Longyan University under standard and ventilated conditions. The mice were randomly divided into five groups, each containing six mice. Three groups of mice were subcutaneously injected in the back with three different adjuvant vaccines (Alh FCoV-SP vaccine, ISA201 FCoV-SP vaccine, and CFA FCoV-SP vaccine) at a dose of 0.2 mL/mouse, with an antigen immune concentration of 15 ug/mouse. Mice in the control group were injected with sterile PBS or FCoV-SP (15 μg/mouse). These injections were administered weekly for three weeks. Caudal artery blood was collected from each mouse, and serum was separated and stored at -80 °C. The animals from each group were then euthanized in a CO_2 chamber. The study was conducted in compliance with the ARRIVE guidelines.

ELISA for Estimating Total Spike-specific IgG

Serum anti-FCoV-SP antibody levels were measured using ELISA, based on previous studies (13). 96-well EIA/RIA plates (Thermo Fisher Scientific) were coated with 4 μg/mL FCoV-SP protein overnight at 4 °C. After

removing unbound protein, the plate was blocked with 5% skimmed milk. The liquid inside the plate was discarded and the plate was then washed with PBST (KH_2PO4, Na_2HPO_4-$12H_2O$, NaCl, KCl and 0.05% Tween-20, 200 µl per well), followed by emptying the PBST residue. The collected serum was diluted at a ratio of 1:400 and 50 µl added to each well of the ELISA plate , and incubated at 37 °C for 1.5 h. After incubation, the ELISA plates were washed with PBST (200 µl/ well), and the PBST residue was emptied. Next, the secondary antibody was diluted at a ratio of 1:20,000, and 50 µl of it was added to each well, and incubated at 37 °C for 1 h. Following incubation, The ELISA plate was washed and 100 µl of 3, 3, 5, 5-tetramethylbenzidine (TMB) substrate solution to was added to each well in the dark for 6 min. After adding the stop solution, the absorbance value at 450 nm (OD450) was measured.

The Optimal Antigen Concentration for Vaccines

Purified FCoV-SP was used as the immunogen, and ISA201 was used as the adjuvant. Subunit vaccines with varying immune concentrations (7.5ug/mouse, 15ug/mouse, 30ug/mouse) were prepared and injected into BALB/c mice (0.2mL/mouse). Mice in the control group were only injected with PBS (0.2 mL/mouse). Serum was collected after three rounds of immunization and total spike-specific IgG was evaluated.

Serum Cytokine Analysis

Serum levels of IFN-γ, IL-8, and TNF-α were assessed using kits purchased from MEIMIAN (Yancheng, China) (17-19). The serum samples were added to ELISA plates pre-coated with monoclonal antibodies for IFN-γ, IL-8, or TNF-α and then incubated at 37 °C for 30 min. Following incubation, the serum concentrations of IFN-γ, IL-8, and TNF-α were determined by incubating with HRP-labeled goat anti-mouse IgG for an additional 30 min, and using standard curves, for reference.

Statistical Analysis

All data were analyzed and visualized using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Serum antibody levels were analyzed using Student's *t*-test and one-way analysis of variance (ANOVA). Statistical significance was set at $p<0.05$.

RESULTS

Spike-specific IgG Titers Varied with Antigen + Adjuvant

First, we evaluated antibody levels (15 µg/mouse) in mice immunized with the FCoV-SP antigen, with or without the Alh, CFA, and ISA201 adjuvants. Compared to the PBS control group, IgG levels were significantly increased in the Alh, ISA201, and CFA groups after the second and final immunizations. Moreover, IgG levels were significantly higher in the ISA201 group than in the other groups (*vs.* PBS [$p<0.0001$], Alh [$p=0.0003$], and CFA [$p=0.0016$]; Fig. 1). Overall, the ISA201-adjuvanted vaccine stimulated the highest titer of antibody production and was selected as the optimal antigen + adjuvant for subsequent experiments.

Determination of Optimal Recombinant Protein Concentration

To determine the optimal antigen concentration for vaccines, we immunized mice with varying concentrations of the ISA201 protein. The results revealed that, compared to the PBS control group, different protein concentrations led to increased levels of serum IgG antibody. Among them, the mice immunized with 15 µg of antigen+ISA201 exhibited the highest levels of IgG antibody levels was, which showed a statistically significant difference compared to the other groups ($p<0.05$). This suggests that a dose of 15 µg per injection is the optimal immune dose (Fig. 2).

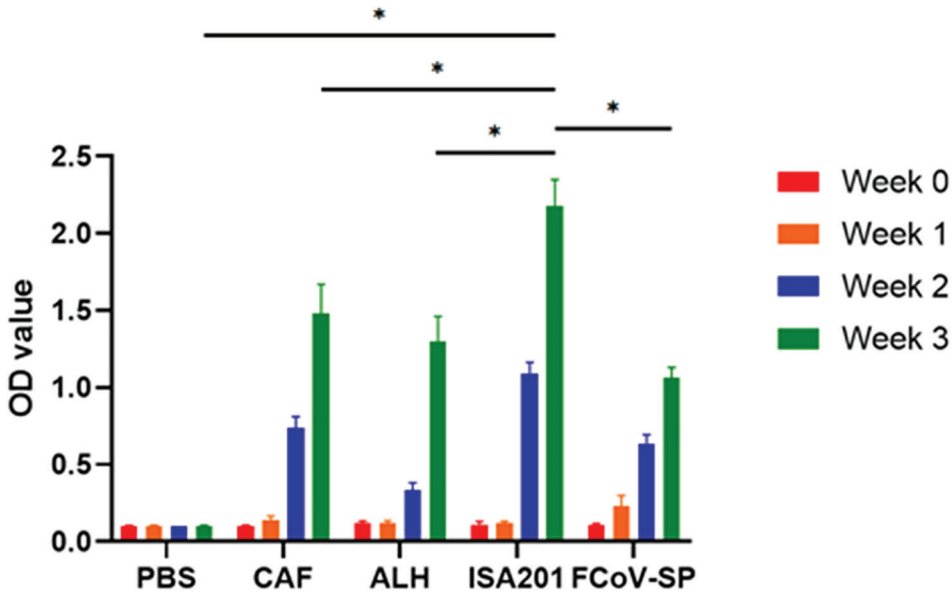

Fig. 1. Adjuvant-antigen integrity tests induced by the feline coronavirus (FCoV) S protein and various adjuvants. BALB/c female mice were randomly divided into 5 groups (n=6 each) and immunized three times via subcutaneous injections with each vaccine formulation. Serum was collected from each mouse and the humoral immune responses of serum spike protein-specific IgG were measured by ELISA. The bars represent mean±SEM. * p<0.05.

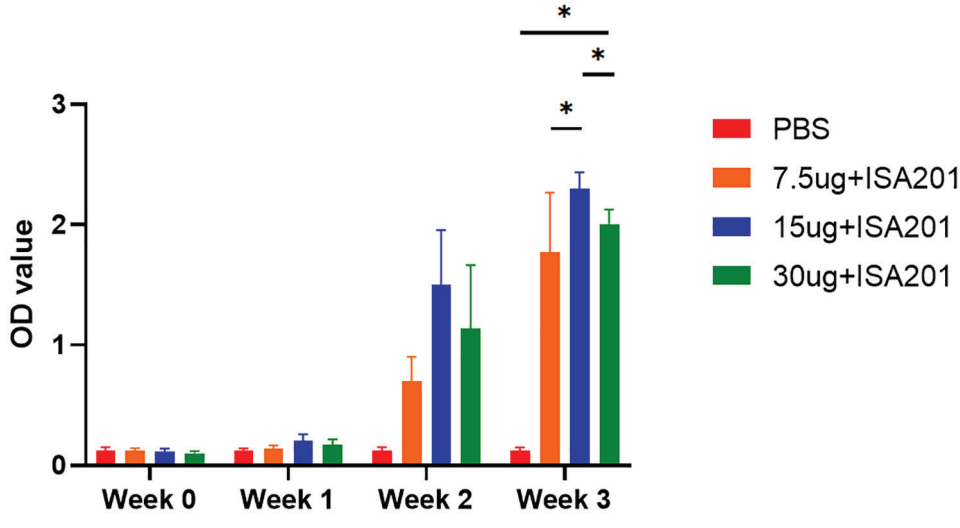

Fig. 2. Optimal vaccine antigen concentration. Mice were immunized with different concentrations of the ISA201 protein with the PBS group serving as the control group (6 mice in each group). Serum was collected from each mouse and the humoral immune responses of serum spike protein-specific IgG were measured by ELISA. The bars represent mean±SEM. *p<0.05.

ISA201 Vaccine Enhanced Serum Cytokine Responses in Mice

Next, we measured serum cytokine responses in mice after the final immunization. The concentration of IFN-γ in the ISA201 FCoV-SP vaccine group was 212.92 ng/L, which was higher than that in the PBS group (139.43 ng/L) (p<0.05). The concentration of IL-8 in the ISA201 FCoV-SP vaccine group was 0.17 pg/mL, which was higher than that in the PBS group (0.13 pg/mL) (p<0.05). The concentration of TNF-α in the ISA201 FCoV-SP vaccine group was 1,403.14 ng/L, which was significantly different from that in the PBS group (638 ng/L) (p<0.01) (Fig. 3).

DISCUSSION

In this study, we evaluated three cost-effective

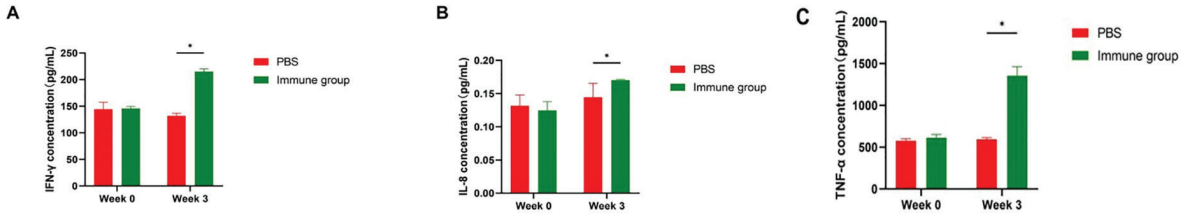

Fig. 3. Serum cytokine levels after vaccination. Mice were immunized with the ISA201 FCoV-SP vaccine, while the PBS group served as the control group (6 mice in each group). Serum was collected from each mouse and serum levels of (A) IFN-γ, (B) IL-8 and (C) TNF-α were assessed using ELISA. The bars represent means±SEM. *p<0.05.

adjuvants, (s Alh, CFA, and ISA201) for their ability to enhance immune responses to FCoV-SP antigen in mice. Analysis revealed that the titer of antigen-specific antibodies in mice immunized with ISA201 was significantly higher than that of Alh and CFA. The ISA201 adjuvant is a type of water/oil/water (W/O/W) dual-phase oil emulsion adjuvant (24), which makes antigens contained in the external water phase easy to recognize by an animal's immune system, while antigens in the internal water phase can undergo delayed release and stimulate rapid and long-term immune responses (25). While both CFA and ISA201 are oil-based adjuvants, clinical observation demonstrated that CFA administration caused notable irritation at the injection site resulting in visible discomfort in immunized mice. Additionally, CFA is not easily emulsified and is hard to absorb after immunization, which lead to ulceration at the immunization site s. Therefore, this study suggests that ISA201 can enhance the immune effects of antigens.

IgG, an antibody produced by B cells during humoral immune responses, facilitates pathogen elimination similarly to phagocytosis (26). In this study, IgG levels increased most significantly within 21 days after initial immunization with the ISA201-adjuvanted vaccine, possibly due to the long-term retention of the ISA201 adjuvant in the body after emulsification with FCoV-SP, thereby stimulating maximum antibody production. After antigenic immunization, cytokines were produced as a result of cellular immune responses. Studies have shown that IFN-γ exerts strong antiviral effects (27). Among interferons, IFN-γ uniquely links the innate and acquired immune responses while facilitating pathogen identification and eliciting specific immune responses (28). IFN-γ is secreted primarily by Th1 cells; therefore, the elevation of IFN-γ levels in this study indicates the degree of immune stimulation by the ISA201 FCoV-SP vaccine, which shifts the immune responses toward the Th1 phenotype.

Th1 cells play an important role in defense against exogenous pathogens. For example, Th1 immune responses specific for coronavirus can offer cross-protection against other viral infections (29). Th1 cells secrete TNF-α, which aids in clearing pathogen and inhibits viral infections (30). A reduction in TNF-α levels can lead to decreased pathogen clearance, increasing the risk of viral infections. Here, increased secretion of IFN-γ and TNF-α indicated that the ISA201 FCoV-SP vaccine induced potent antigen-specific Th1 immune responses in mice. Additionally, IL-8 promotes inflammatory responses and stimulates angiogenesis, making it a valuable target for intervention (31). IL-8 induces the migration of inflammatory cells, such as neutrophils, monocytes, and lymphocytes, to the site of inflammation to facilitate their accumulation and activation, thereby supporting tissue repair and antiviral defense (32). Our findings suggest that the ISA201 FCoV-SP vaccine provides antiviral support by upregulating IL-8 expression.

This study has some limitations. Firstly, the immunological evaluation of the ISA201 FCoV-SP vaccine was conducted only in mice,

rather than in other animals that are more susceptible to FCoV. Therefore, future studies should investigate its specific immune effects in cats. Secondly, only antibody and cytokine levels were measured after immunization with the ISA201 FCoV-SP vaccine, indicating the need for further evaluation of its protective effects against viral infections in future studies.

CONCLUSION

In this study, the ISA201 FCoV-SP vaccine, prepared using 15 μg of recombinant FCoV-SP, effectively stimulated high-titer antibody production in mice. Additionally, serum levels of IFN-γ, IL-8, and TNF-α significantly increased in mice following immunization with the ISA201 FCoV-SP vaccine. Overall, our findings provide valuable insights for developing novel FCoV vaccines.

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No applicable.

AUTHORS' CONTRIBUTION

Bo Dong and Weiming Lin conceived the study and drafted the manuscript. Xiaodong Zhang and Wenqian Hu performed the data analysis. Shuo Zhang, Weijie Zou and Yina Guo performed the immunity in mice. Xiaodong Zhang and Wenqian Hu performed the other experiments involved in this study. All authors read and approved the final manuscript.

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ABBREVIATIONS

FCoV: Feline coronavirus
Alh: adjuvant
CFA: Freund's complete adjuvant

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analyzed in the current study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were conducted after receiving ethics approval from the Committee on the Ethics of Animal Experiments of Longyan University (LY2023002L).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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