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Research Article

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A Novel Fused Delivery Carrier Fc-Ii Enhances Mucosal and Systemic Immunity through the FcRn

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Abstract

FcRn is a specific receptor for IgG recognition and transportation. Although FcRn has been shown to deliver IgG across the mucosa, it is unable to directly enter the MHC antigen presentation pathway. Conversely, the invariant chain (Ii), a chaperonin of MHC class II molecules, stimulates antigen presentation but is unable to pass through mucosal barriers. An effective strategy to improve immunity would be to target antigenic peptides passing through the mucosa to the MHC molecular pathway. In this study, a novel double carrier (Fc-Ii) was constructed using the COE gene of the porcine epidemic diarrhea virus (PEDV) as a link. The double carrier fusion proteins (Fc-COE-Ii) and single carrier fusion proteins (Fc-COE and COE-Ii) were expressed in E. coli, purified, and refolded. Fc-COE-Ii was used as an immunogen to evaluate the immune response, while COE-Ii, Fc-COE, COE, and PBS were used as controls. Eighty 6–8-week-old female Balb/c mouse were divided into five groups (COE, COE-Ii, Fc-COE, Fc-COE-Ii, and PBS) for intranasal immunization supplemented with the CPG ODN 1826 mucosal adjuvant. The results showed that the single carrier fusion proteins COE-Ii and Fc-COE showed a stronger immune response than the COE protein, and the double carrier fusion protein Fc-COE-Ii exhibited higher immunogenicity than the two single carrier fusion proteins. Therefore, this novel double carrier Fc-Ii may be mediated by FcRn to pass through the mucosal barrier of the respiratory tract to effectively enhance stimulation of the mucosal immunity and systemic immune response.

Keywords: FcRn, IgG Fc, Invariant Chain, Porcine Epidemic Diarrhea Virus COE, Intranasal Immunization

1. Introduction

Research focusing on the neonatal Fc receptor (FcRn) for IgG is increasingly showing promise in vaccine development. This receptor was first identified in the intestinal epithelial cells of a suckling rodent, where it was found to be highly expressed [1]. Furthermore, FcRn is expressed in a variety of cells and tissues, including the mucosal epithelial cells of animals and humans [2]. A normal function of FcRn is to transfer maternal IgG across polarized placental epithelial cells, thereby delivering maternal IgG to the fetus and providing immunity from pathogens before the neonatal immune system develops [3].

The IgG Fc fragment can recognize FcRn on antigen presenting cells [4]. Previous studies suggest that fusion with Fc-tag could facilitate the uptake of antigens and enhance their immunogenicity, and this strategy has been utilized in the design of adjuvant-free vaccines to prevent cancer and virus infections such as HIV-1 and influenza [5,6].

The major histocompatibility complex (MHC) class II-associated invariant chain (Ii) is a non-polymorphic type II transmembrane protein with multiple functional domains. It is highly conserved across mammalian species and widely expressed in different immune cell types [7]. It plays a critical role in antigen presentation and forms MHC class II peptide complexes to generate immune responses [8]. Previous studies evaluating the fusion of Ii to antigens encoded in vector delivery systems have shown that this strategy may enhance immune responses to the encoded antigen [9-11].

Porcine epidemic diarrhea virus (PEDV) is an infectious disease that causes high mortality in piglets and occurs in pig farms in Asia,

Europe, and the United States [12-15]. There are multiple epitopes on the PEDV S protein; among them, the CO-26K-Equivalent (COE) epitopes are highly conserved with good immunogenicity [16,17]. An oral lactic acid bacteria vaccine expressing the COE domain can stimulate the immune response of the small intestine and respond to PEDV [18]. Therefore, the COE domain is an ideal antigen peptide candidate for PEDV vaccine development.

Most pathogenic microorganisms invade the body through mucous membranes. In recent years, based on the discovery of FcRn and its ability to transport IgG, the mucosal immune pathway was explored with an IgG Fc carrier, but the low effect on the immune response restricted its development. An effective strategy to improve immunity would be to target antigenic peptides passing through the mucosa to the MHC II molecular pathway. In this study, we constructed two single carriers (Fc or Ii) and a novel fusion delivery carrier (Fc-Ii), which we fused using the COE domain of PEDV and then expressed in E. coli. The purified and refolded COE, Fc-COE, and COE-Ii proteins, and the double Fc-COE-Ii fusion protein were mixed with PBS supplemented with the CPG ODN 1826 mucosal adjuvant and administered intranasally to immunize female Balb/c mice aged 6–8 weeks. We then estimated the ability to induce an immune response and found that our novel delivery carrier enhanced the immune response; furthermore, since the immunization was effective via the intranasal route, this method could simplify the immunization process and reduce stress on the animal.

2. Materials and Methods

2.1 pET Vector Construction of a Novel Double Carrier (FcIi)

The mouse IgG-Fc and PEDV COE genes were amplified using PCR with the Fc-F/Fc-R and COE-F/COE-R primer pairs, respectively, and linked using splicing overlap extension PCR (SOE PCR) to form a fused Fc-COE gene. The Fc-COE gene was amplified with two pairs of primers (Fc-up/Fc-down; COE-up/COE-down) and linked to the mouse Ii gene using SOE PCR with two pairs of primers (Fc-COE-up/Fc-COE-down; Ii-up/Ii-down) to form a fused Fc-COE-Ii carrier. The fused COE-Ii gene was amplified with the COE-Ii-F/COE-Ii-R primer pair. The fused genes were cloned into a pET-32a vector and confirmed using sequencing, resulting in the following vectors: pET-32a-Fc-COE, pET-32a-COE-Ii, and pET-32a-Fc-COE-Ii. All primers were designed using Primer 5.0 and are listed in Table 1.

Name	Primer sequence (5’-3’)A
Fc-F	5’-CGGGATCCATGGCCAAAACAACAGCCCCATC-3’
Fc-R	5’-CGGAATTCTCATTTACCCGGAGTCC-3’
COE-F	5’-CGGAATTCATGGTTACTTTGCCATCATTTAATG-3’
COE-R	5’-ACGCGTCGACTCATTTACCCGGAGTCTTGG-3’
Fc-up	5’-CGGGATCCATGGAGCCCAGAGGGCCCACAAT-3’
Fc-down	5’-ACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTTTACCCGGAGTCCGGGAGAAGC-3’
COE-up	5’-GGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGTTACTTTGCCATCATTTAATG-3’
COE-down	5’-CGGAATTCTCAAACGTCCGTGACACCTTCAAG -3’
Fc-COE-up	5’-CGGGATCCATGGAGCCCAGAGGGCCCACAAT-3’
Fc-COE-down	5’-AGATCCCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCAACGT-3’
Ii-up	5’-GGATCAGGCGGGGGTGGGTCCGGAGGAGGTGGCTCGGGATCTGATGACCAACGCGACCT-3’
Ii-down	5’-GCGTCGACTCACAGGGTGACTTGACC-3’
COE-Ii-F	5’-CGGAATTCATGGTTACTTTGCCATCATTTAATG-3’
COE-Ii-R	5’-GCGTCGACTCACAGGGTGACTTGACC-3’

[A]The italic bases encode *BamHI* (GGATCC), *SalI* (GTCGAC), and *EcoRI* (GAATTC) restriction sites; the underlined bases encode flexible linker peptides.

Table 1: Primers used for PCR Amplifications and Splicing Overlap Extension PCR

2.2 Expression, Refolding, and Purification of the Fusion Proteins

Each recombinant plasmid pET-32a-Fc-COE, pET-32a-COE-Ii, and pET-32a-Fc-COE-Ii was transformed into *E. coli* BL21, respectively. Each recently transformed *E. coli* BL21 cell colony was cultured in LB broth supplemented with ampicillin (50 mg/mL) at 37 °C and shaking at 200 rpm until the OD_{600} nm reached 0.6. The fusion protein expression was induced by adding IPTG to a final concentration of 0.8 mM and incubating for an additional 6 h. Control cultures containing the empty pET-32a vector were processed in parallel.

The fusion proteins accumulated in the bacteria as inclusion bodies (IBs). The cells were harvested by centrifugation at 5000 g for 10 min and used for the isolation of IBs. The cell suspension was sonicated on ice for 5 s with 5 s intervals for a total of 15 min. After

sonication, the lysate was centrifuged at 5000 g for 10 min, the supernatant was discarded, and the pellet was gently resuspended in Wash buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1 M urea [pH 8.0]). The fusion proteins were refolded using an initial buffer (20 mM NaH2PO4, 0.5 M NaCl, 8 M urea [pH 8.0]) with the following urea concentration gradient dialysis: 8 M, 6 M, 4 M, 2 M, 1 M, 0.5 M, 0 M. The refolded fusion proteins were purified using a Ni^{2+} column as previously described (Zhang et al., 2005). Subsequently, the fusion proteins were resolved on a 12% SDS-PAGE gel under reducing conditions and electrotransferred to a nitrocellulose membrane (Millipore, Germany). The membranes were blocked with 10% calf serum, incubated separately with mouse anti-PEDV COE Ab for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ab (Pierce, Rockford, USA). All blocking, incubation, and washing steps were performed in PBST solution (PBS and 0.05% Tween 20). The prepared fusion proteins were stored at -80 ℃ for immunization of the mice.

2.3 Mice Immunization

6–8-week-old female Balb/c mice were purchased by the Experimental Animal Center of Anhui Medical University. After one week of acclimatization, a total of 80 mice were randomly assigned to 5 groups (n = 16) as follows: COE, COE-Ii, Fc-COE, Fc-COE-Ii, and PBS. Each group was housed separately. The immunization dosage was determined based on preliminary testing. The mice were vaccinated intranasally with 20 µg of fusion protein. The recombinant proteins COE, COE-Ii, Fc-COE, and Fc-COE-Ii, were each diluted in 10 µL of PBS and then emulsified with 10 µL of mucosal immune adjuvant CPG ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3', Shenggong Biological Engineering Co., Ltd., Shanghai, China) for both the first immunization and subsequent booster at an interval of 7 days, with 10 µL PBS used as a negative control. All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of China.

2.4 Sample Collection

On Day 0, 14, 21, 28, and 35 post-vaccinations, the mice were fasted for 12 h, after which 3 mice were randomly selected from each group for sample collection. Anticoagulated peripheral blood samples were collected from the tail vein of the mice. Blood serum was obtained by collecting blood via the retro-orbital collection method followed by centrifugation at 1600 g for 15 min.

The experimental mice were killed by by decapitation under deep anesthesia, and the whole mice were soaked in 75% alcohol for several minutes to disinfect. The trachea was isolated and a circular incision was made. The nose was washed from the trachea incision upward using 1 mL PBS, and the nasal fluid was collected from the nasopharynx. The lung tissue was passively separated and washed three times with 1 mL PBS to collect lung fluid from the lung. The small intestine tissue was separated, the intestinal cavity was washed repeatedly until the fluid was transparent, and intestinal fluid was collected. The collected fluid was centrifuged at 10,000 g for 5 min, and the supernatant was collected and stored at -20 °C for testing.

2.5 Detection of PEDV COE Antibody Responses using Indirect ELISA

Antibody titers of PEDV COE-specific IgG in serum, nasal fluid, lung fluid, and intestinal fluid from immunized mice were measured using a commercial ELISA kit according to the manufacturer's instructions (Meimian Biotechnology Co., Ltd., Yancheng, China). In brief, microtiter plates were coated with 100 µL of COE antigens overnight at 4 °C and blocked with 5% skim milk for 1 h at RT. Diluted samples were added and stored at RT for 1 h, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies for 1 h. The enzymatic activity was detected by adding 3,3',5,5'-tetramethylbenzidine substrate followed by a 2N H2SO4 stop solution, and then the absorbance was read at 450 nm on an Xlement SPR100 microplate reader (Liangzhun Industrial Co. Ltd., Shanghai, China).

2.6 Detection of sIgA and Related Cytokine Levels using Direct ELISA

The serum levels of sIgA, IFN-γ, IL-2, IL-4, and IL-10 were detected using ELISA kits according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

2.7 Statistical Analysis

Data were expressed as mean ± SD, and Analysis of Variance (ANOVA) followed by the post hoc test Duncan's multiple range test (MRT) was performed to analyze the differences among groups using SPSS 17.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Construction, Expression, and Identification of Recombinant Proteins

The PEDV COE, mouse IgG Fc, and mouse Ii genes were first PCR-amplified separately, and then these genes were linked using SOE PCR to form the fused Fc-COE-Ii protein. A schematic diagram of the gene expression cassette and fusion protein is illustrated in Figure 1. The PCR products were cloned using the pET-32a expression vector and sequences were verified. Subsequently, the recombinant plasmids pET-32a-Fc-COE, pET-32a-Fc-COE-Ii, and pET-32a-COE-Ii were transformed into E. coli. After using IPTG at different time points to induce expression, the novel expressed protein bands corresponding to 63, 92, and 60 kDa in the precipitates of the recombinant pET-32a-Fc-COE, pET-32a-Fc-COE-Ii, and pET-32a-COE-Ii transformants were visualized using SDS-PAGE, respectively (Figure 2A). After 6 h of cultivation, the protein was detectable in the precipitate, and the maximum protein concentration occurred at 4 h for each recombinant protein. Western blot analysis was performed with anti-His tag antibody to determine the expression of the target proteins; we observed single reaction bands corresponding to the bands seen in SDS-PAGE, which verifies the expression of the recombinant Fc-COE,

Fc-COE-Ii, and COE-Ii proteins and indicates they have strong reactogenicity to specific antibodies (Figure 2B). After refolding and purification, SDS-PAGE results showed single-protein bands with molecular weights of 60, 63, and 92 kDa (Figure 2C).

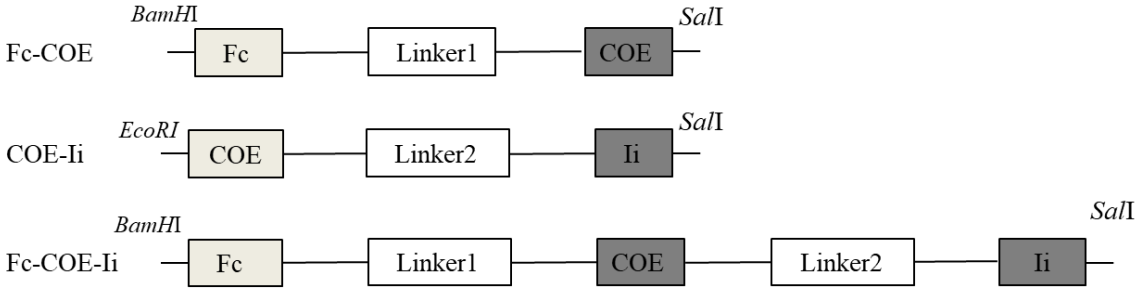

Figure 1: Schematic Diagram of the Fusion Protein

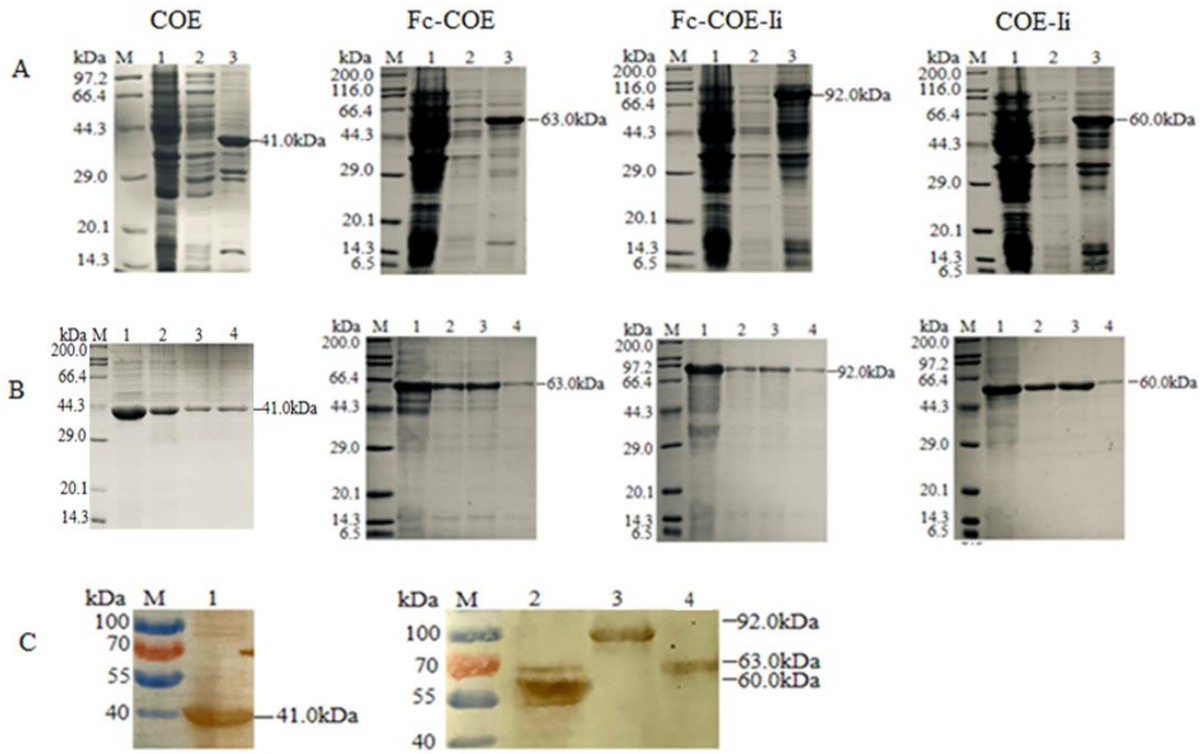

Figure 2: Expression and Purification of the Fusion Proteins in Transformed *E. coli* using pET-32a Expression Vectors

(A) Overall protein expression and solubility identification of the four fusion proteins COE, Fc-COE, Fc-COE-Ii, and COE-Ii using SDS-PAGE. Lane M, Protein molecular weight (MW) marker; Lane 1, E. coli without IPTG treatment; Lane 2, IPTG-treated E. coli; Lane 3, Inclusion bodies isolated from *E. coli*. (B) Identification of refolded and purified fusion proteins COE, Fc-COE, Fc-COE-Ii, and COE-Ii using SDS-PAGE. M, Protein MW marker; Lanes 1–3, Purified fusion protein; Lane 4, Refolded fusion protein. (C) Identification of fusion protein purity using western blot. M, Pre-stained protein MW marker; Lane 1, COE fusion protein; Lane 2, COE-Ii fusion protein; Lane 3, Fc-COE-Ii fusion protein; Lane 4, Fc-COE fusion protein.

3.2 Immune Response based on IgG Titer among Recombinant Protein Groups

Antibody levels induced by intranasal immunization are crucial to examine the effects of delivery strategy of the novel double carrier. The dynamic changes of antibody titers in each group are shown in Figure 3. At day 14 post-immunization, the four groups—COE, Fc-COE, COE-Ii, and Fc-COE-Ii—did not significantly stimulate

IgG against PEDV COE relative to the PBS group. After day 14, the level of IgG was slightly different among groups (P<0.05) with the strengthening of immunity. The serum IgG level was significantly higher in the fused Fc-COE-Ii group than the single carrier Fc-COE and COE-Ii groups (P<0.05). The IgG levels in the single carrier groups were the same as that in the COE group (P>0.05). These results suggest that the recombinant fused protein Fc-COE-Ii has good immunogenicity.

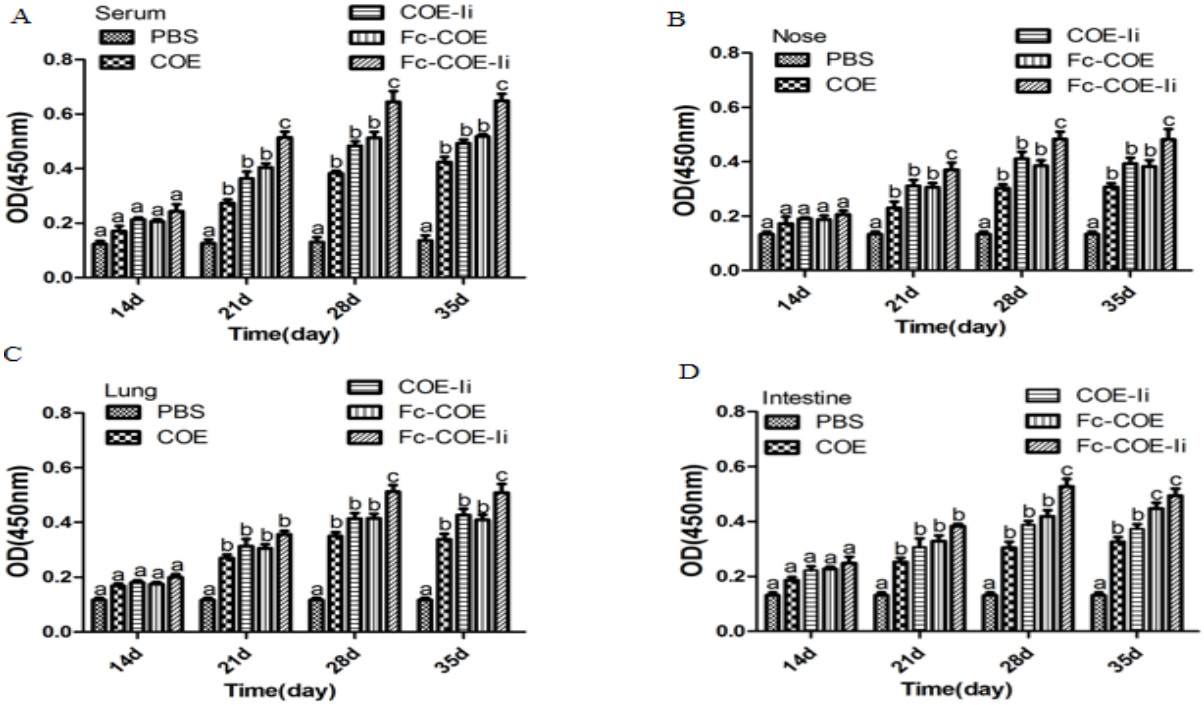

Figure 3: Levels of Anti-PEDV COE in IgG in Immunized Mice

Female Balb/c mice were immunized intranasally with fusion proteins COE-Ii, Fc-COE, or Fc-COE-Ii; or with PBS or COE as controls. The production of anti-PEDV COE IgG from (A) serum, (B) nasal fluid, (C) lung fluid, and (D) intestinal fluid was detected at different time points using ELISA. Data are shown as mean ± SD from three independent experiments. Different lowercase letters indicate significant differences (P < 0.05), as determined using ANOVA followed by Duncan's multiple range test (MRT) post hoc test.

3.3 Evaluation of the sIgA Titer among Recombinant Protein Groups

sIgA is the most abundant immunoglobulin in mucosal tissues and represents the hallmark of the mucosal immune response. The serum and mucosal fluid levels of anti-PEDV COE-specific sIgA were measured using ELISA (Figure 4). Unsurprisingly, starting on day 21, the Fc-COE-Ii and Fc-COE groups had significantly higher titers of anti-PEDV COE-specific sIgA over time than the COE or PBS control groups (P<0.05). On day 35, the serum, nasal fluid, and intestinal fluid sIgA levels in the COE-Ii group were significantly higher than that in the COE group (P<0.05). Notably, the antibody titers in the COE-Ii group were higher than those in the COE control group starting at day 21, but the differences were not significant (P>0.05).

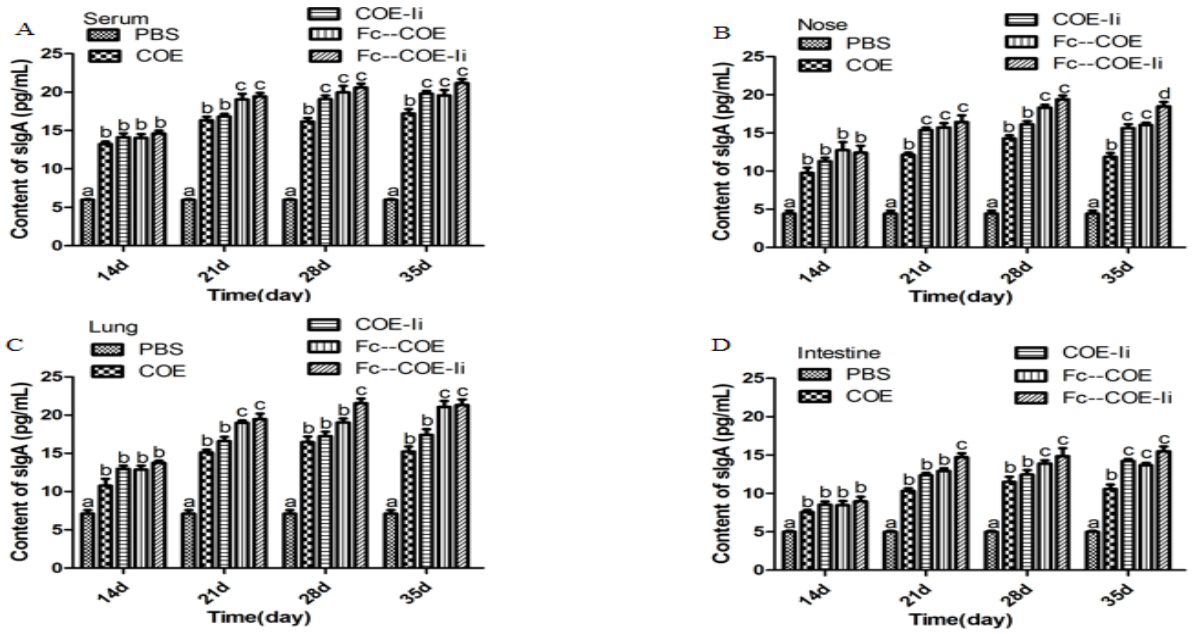

Figure 4: Levels of Anti-PEDV COE sIgA in Immunized Mice

Female Balb/c mice were immunized intranasally with fusion proteins COE-Ii, Fc-COE, or Fc-COE-Ii; or with PBS or COE as controls. The production of anti-PEDV COE sIgA from (A) serum, (B) nasal fluid, (C) lung fluid, and (D) intestinal fluid was detected at different time points using ELISA. Data are shown as mean ± SD from three independent experiments. Different lowercase letters indicate significant differences ($P < 0.05$), as determined using ANOVA followed by Duncan's multiple range test (MRT) post hoc test.

3.4 Cytokine Production

To identify the effect of the novel delivery carrier fusion protein on cytokine secretion, serum cytokine levels were measured using ELISA. The IFN-γ, IL-2, IL-4, and IL-10 levels increased over time with the strengthening of immunity. From days 14–35, the IFN-γ and IL-2 levels produced by lymphocytes from the Fc-COE-Ii and Fc-COE groups were significantly increased relative to the other groups. Starting on day 28, significant increases were also observed for IL-4 and IL-10 levels in the Fc-COE-Ii and Fc-COE groups. However, the COE-Ii group had the same effect on cytokine stimulation as the COE control group, because there were no significant differences between these two groups for all four cytokines at all time points ($P>0.05$) (Figure 5).

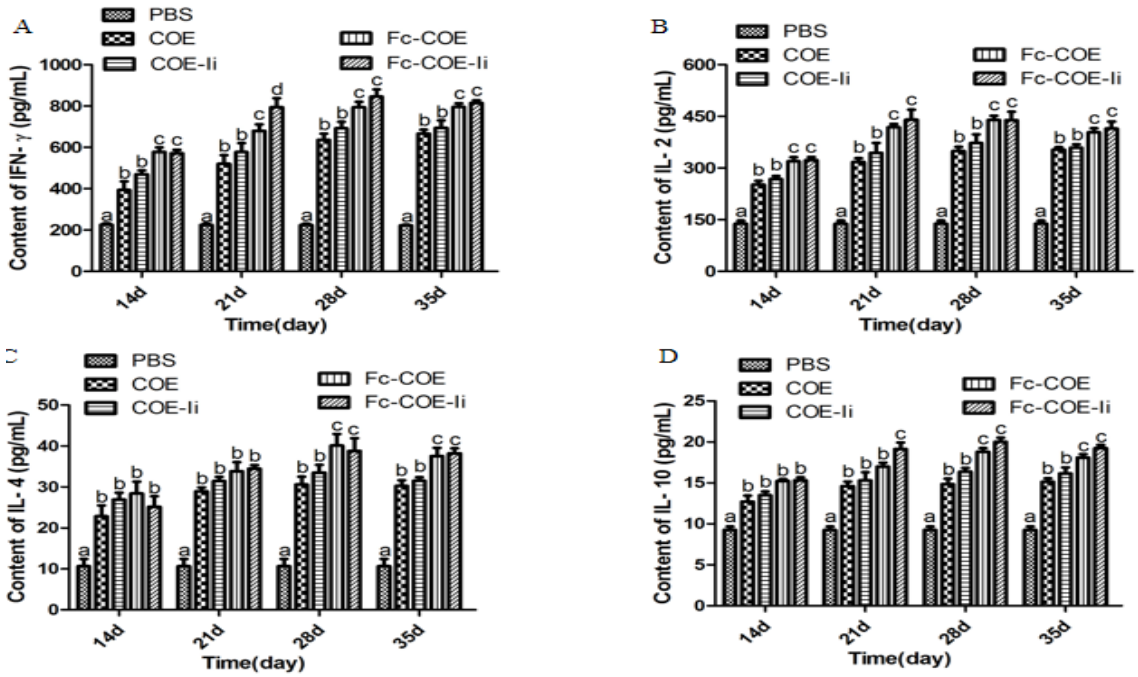

Figure 5: The Double Carrier Fusion Protein Fc-Ii Stimulates Cytokine Secretion in Immunized Mice

Female Balb/c mice were immunized intranasally with fusion proteins COE-Ii, Fc-COE, or Fc-COE-Ii; or with PBS or COE as controls. The production of serum cytokines (A) IFN-γ, (B) IL-2, (C) IL-4, and (D) IL-10 was detected at different time points using ELISA. Data are shown as mean ± SD from three independent experiments. Different lowercase letters indicate significant differences ($P < 0.05$), as determined using ANOVA followed by Duncan's multiple range test (MRT) post hoc test.

4. Discussions

Directing antigenic peptides passing through the mucosa to the MHC molecular pathway is a beneficial strategy to improve immunity. In this study, we evaluate the fusion of IgG Fc and Ii to generate Fc-Ii, a novel double carrier, as a molecular adjuvant in viral vector-based vaccines. This novel delivery vector was used to explore the mucosal route for intranasal immunization to simplify the immunization procedure, reduce stress, and enhance the immune response.

FcRn is a key membrane protein that plays an important role in serum IgG recycling, which extends the antibody's half-life [19,20]. Furthermore, FcRn is known to traffic antigen-bound immunoglobulins, and to interact with immune complexes to facilitate the antigen cross-presentation of peptides derived from the immune complexes in antigen-presenting cells [21,22]. IgG-FcRn molecular interactions have primarily focused on the Fc region. The IgG Fc region is a recruiter and a frontline commander in the combat against infectious diseases [23,24]. The fusion of IgG2a Fc and the HSV-2gD protein after intranasal immunization can stimulate the immune response of B cells and T cells and produce strong mucosal and systemic antibodies [25]. Immunization of chickens with avian metapneumovirus MPV (aMPV) harboring chicken Fc induced higher levels of antibodies and inflammatory cytokines compared to those of aMPV [26]. Our experiments showed that the IgG antibody titers in mice immunized with Fc-COE were higher than those in the PBS control group.

Ii molecules are the chaperone proteins of MHC class II molecules, which play a role in the process of antigen presentation [27]. They can directly present antigen fragments toward the MHC II molecule presentation pathway and significantly increase the antibody response level in immunized animals [28]. (Chen et al., 2012). An FcRn-IgG binding experiment found the presence of Ii molecules, which suggested that the Ii chain may participate in the process of transporting the FcRn-bound IgG [29]. Our results showed that the IgG antibody titers in the mice immunized with COE-Ii was significantly higher than that of the PBS control group ($P<0.05$).

We constructed the novel fused double carrier Fc-Ii and confirmed that its immunostimulatory effect was dependent upon both Ii and FcRn. FcRn specifically transports the Fc fusion protein across the mucosal barrier, which solves the most important problem with mucosal immunity for antigen transmission. Intranasal immunization with the Fc fusion protein can not only effectively stimulate the local mucosal immunity of the body, but also effectively induce the body to produce strong systemic humoral and cellular immunity [30]. Oral vaccination with recombinant L. plantarum expressing 3M2e-Fc has been shown to enhance specific sIgA secretion [31]. We found higher mouse serum and mucosal

fluid sIgA levels in the Fc-COE and Fc-COE-Ii groups than others groups. Additionally, the serum, nasal fluid, and intestinal fluid PEDV COE sIgA levels in the COE-Ii group and the COE group were significantly higher than that in the PBS group ($P < 0.05$). CPG-ODN is a good mucosal adjuvant and stimulates the secretion of sIgA [32]. Furthermore, Ii presented the COE antigen fragments toward the MHC II molecule presentation pathway; therefore, the IgG titers from the Fc-COE-Ii group significantly increased after immunization relative to those from the other groups.

In addition to antibodies, the cellular response to PEDV COE is characterized by T helper cells that support the production of antibodies and cytotoxic T cells that target the infected cells [33]. We investigated systemic T cell activity in vitro by measuring the cytokine levels of IFN-γ, IL-2, IL-4, and IL-10 in serum samples after vaccination. These cytokines, which are produced by T lymphocytes and natural killer cells, are involved in both innate and adaptive cell-mediated immune responses by activating macrophages [7]. Although we observed up-regulation of cytokines in the Fc-COE-Ii group relative to most other groups, there was no significant difference in cytokine level between the Fc-COE and Fc-COE-Ii groups at most test time-points. The Fc region mediates potent immune effector functions by engaging FcRs and serum complement proteins, thereby providing new opportunities for augmenting the immunogenicity of antigens [34].

5. Conclusions

A novel fused delivery carrier Fc-Ii was constructed, which targets antigenic peptides passing through the mucosa to the MHC IImolecular pathway. It effectively enhances the mucosal immunity and systemic immune response, and the intranasal administration provides a simpler method of immunization that reduces the animal's stress response.

Acknowledgments

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Statement of Contribution for Authors

The contributions of Fazhi Xu, Zhengxuan Zhu, Jing Sun, Xuelan Liu, and Qiyun Zhu are equal, and the order is arbitrary.

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