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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Immune response characterization of mice immunized with *Lactobacillus plantarum* expressing spike antigen of transmissible gastroenteritis virus

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Abstract

The highly infectious porcine transmissible gastroenteritis virus (TGEV), which belongs to the coronaviruses (CoVs), causes diarrhea and high mortality rates in piglets, resulting in severe economic losses in the pork industry worldwide. In this study, we used *Lactobacillus plantarum* (*L. plantarum*) to anchor the expression of TGEV antigen (S) to dendritic cells (DCs) via dendritic cell-targeting peptides (DCpep). The results show that S antigen could be detected on the surface of *L. plantarum* by different detection methods. Furthermore, flow cytometry and ELISA techniques were used to measure the cellular, mucosal, and humoral immune responses of the different orally gavaged mouse groups. The obtained results demonstrated the significant effect of the constructed *L. plantarum* expressing S-DCpep fusion proteins in inducing high expression levels of B7 molecules on DCs, as well as high levels of IgG, secretory IgA, and IFN-γ and IL-4 cytokines compared with the other groups. Accordingly, surface expression of DC-targeted antigens successfully induced cellular, mucosal, and humoral immunity in mice and could be used as a vaccine.

Keywords *L. plantarum* · TGEV · S protein · DCpep · Mucosal immune response

Introduction

Porcine transmissible gastroenteritis virus (TGEV) is classified under the genus *Coronavirus*, family *Coronaviridae*, and order Nidovirales (Jiang et al. 2016). It is one of the important determining causes of acute viral diarrhea in piglets and results in devastating economic losses in the swine industry (Xia et al. 2017b; Yu et al. 2017a). It destructs the intestinal villi epithelium, resulting in a decrease in the surface area and atrophy of the epithelial lining, intestinal disorders, and incomplete food digestion and absorption (Jiang et al. 2016; Xia et al. 2017a).

High morbidity and case fatality rates were recorded in newly born piglets, especially in cases of co-infection with the porcine epidemic diarrhea virus (PEDV) (Yu et al. 2017a). The first report of TGEV was in the USA in 1933 (Doyle and Hutchings 1946), while the first detection in China was in 1970 (Wang et al. 2010).

Four structural proteins, spike (S) proteins, membrane (M) proteins, envelope (E), and nucleoproteins (N), and five non-structural proteins are encoded by the TGEV genome (Zhang et al. 2017). The TGEV spike protein has an approximate size of 250 kDa and contains a highly conserved area (cysteine-rich motif, CRM) near the carboxy-terminal end of the transmembrane region; it combines virus particles and the interaction between them and the M protein (Gelhaus et al. 2014; Nguyen and Hogue 1997; Vennema et al. 1996). Functionally, the spike protein plays an essential role in the tropism of the virus, the binding to the host cell receptor aminopeptide N, and subsequent combination of cellular and viral membranes, as well as the pathogenicity and hemagglutination activity (Reguera et al. 2012; Sanchez et al. 1999). Additionally, the protein has a high immunogenic potency that can stimulate the host immune

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system to generate neutralizing antibodies (Lin et al. 2015). The antigenicity of TGEV was elucidated in different studies, where a successful spike protein-based enzyme linked immune assay (ELISA) was implemented (Lin et al. 2015) and a safe and promising vaccine was developed (Mou et al. 2016).

Dendritic cells (DCs) located in the gut epithelium are the most potent antigen-presenting cells (APCs). They have the unique capability of inducing T cell polarization and differentiation (Subramaniam et al. 2017), the regulation of B cell function, and differentiation into IgG-producing plasma cells (Wang et al. 2016). Furthermore, DCs limit the mucosal penetration of invasive pathogens and encourage the uptake of antigens (Owen et al. 2013) and migration into lymphoid tissues resulting in the presentation of foreign antigens to B and T cells (Mohamadzadeh et al. 2005). A specific DCpep was found to effectively protect against a lethal anthrax disease challenge in a mouse model (Mohamadzadeh et al. 2009). Probiotic feed is beneficial for the host since it prevents infection (Jiang et al. 2016). Different *Lactobacillus* spp. are commonly distributed in nature and are naturally found in the human and animal gastrointestinal tracts (Kaur et al. 2017; Riaz Rajoka et al. 2017). The species have been safely used as heterologous protein antigen delivery vehicles for oral vaccines owing to their characteristics of resistance against gastric secretions, ability to colonize the intestine (Landete et al. 2015; Yu et al. 2017b), relatively simple culture techniques, and suitable manipulations (Wanker et al. 1995).

The poly-γ-glutamic acid synthetase A (pgsA) protein from *Bacillus subtilis*, encoded by the pgsA gene, has a transmembrane region near its N-terminus (Sung et al. 2005), providing the required criteria for the implementation of a pgsA displaying expression system. The expression system is the theoretical basis for the recombination of a recent genetically engineered vaccine (Cai et al. 2016; Narita et al. 2006). For instance, *Lactobacillus plantarum* has been employed to display thymosin α-1 in conjunction with classical swine fever virus E2 antigen (Xu et al. 2015), SO7 of *Eimeria tenella* fusion DC-targeting peptide (Yang et al. 2017a), a porcine epidemic diarrhea virus S gene fused to a DC-targeting peptide (Huang et al. 2018). Surface expression of foreign antigens induced cellular, mucosal, and humoral immunity in animal and could be used as a potential vaccine. Therefore, in the current study, *L. plantarum* was used to express TGEV S protein and was fused to a DCpep to deliver it to mucosal DCs. Its immunogenicity was further investigated at the in vitro and in vivo levels.

Materials and methods

Synthesis of S antigen in *L. plantarum*

L. plantarum NC8 (CCUG 61730) bacteria and *Escherichia coli-Lactobacillus* harboring pSIP409 vector were supplied by Professor A. Kolandaswamy (Madurai Kamaraj University, India) (Sorvig et al. 2005). The pMD19-T-pgsA-S-DCpep or pMD19-T-pgsA-S-Ctrlpep were kept at our lab; the information of DCpep and Ctrlpep was obtained from published literature (Shi et al. 2016). The S gene fragment (GenBank accession numbers KT696544, source 20365 to 22410)/Spike Protein GenBank AMB66488, source 1 to 682) and the pMD19-T-pgsA-S-DCpep or pMD19-T-pgsA-S-Ctrlpep plasmids were digested separately by *Nco*I and *Hin*d III for 4 h at 37 °C. Then, the T4 DNA ligase enzyme was used to ligate either pgsA-S-DCpep or pgsA-S-Ctrlpep into the pSIP409 vector at 4 °C for 12 h. The constructed pSIP409-pgsA-S-DCpep and pSIP409-pgsA-S-Ctrlpep vectors were confirmed and extracted using a plasmid DNA extraction kit (Omega Bio-Tek, Doraville, CA). The pSIP409-pgsA plasmid was constructed using a negative control vector. The *L. plantarum* was transformed with the recombinant plasmid, and positive bacteria were named *Lp*-pSIP-409-pgsA (pSIP409-pgsA in *L. plantarum*), *Lp*-pSIP-409-16 (pSIP409-pgsA-S-Ctrlpep in *L. plantarum*), and *Lp*-pSIP-409-17 (pSIP409-pgsA-S-DCpep in *L. plantarum*).

Identification of the protein anchor expression by flow cytometry and immunofluorescence

For confirmation of S protein expression on the surface of bacteria, the constructed *Lp*-pSIP-409-16 and *Lp*-pSIP-409-17 bacterial strains were cultured in De Man, Rogosa, and Sharpe (MRS) with 10 μg/ml erythromycin as a selective antibiotic. When the OD_{600} of the medium reached a range between 0.3 and 0.4, Sakacin P (SppIP) was added to the culture followed by inducing expression at 30 °C for 10 h. Cells from the different strains were carefully washed using phosphate buffer saline (PBS, contained 1% bovine serum albumin) and resuspended in a concentration of 5×10^5 colony forming unit (CFU) of recombined bacteria. Suspensions were stained for 12 h at 4 °C with polyclonal antibody obtained from mice vaccinated with purified S antigen. After washing twice with PBS containing 0.2% Tween-20 (PBS-T), the recombined bacteria were stained with FITC-conjugated goat anti-mouse IgG (CST, USA) for 1 h at 4 °C in the dark (Yang et al. 2017c). Finally, the cells were washed, resuspended, and tested using a flow cytometer (BD FACS LSR Fortessa™) and a laser scanning confocal microscope (LSM710; Carl Zeiss, Germany).

16S rRNA gene amplification

Bacterial genomic DNA were extracted by bacterial genomic DNA kit according to the manufacturer's instructions (CW Biotech, China). For general bacterial identification, 16S rRNA universal primers were selected (Posman et al. 2017). The most commonly used universal primer was 27F/1492R,

27F: 5′-AGAGTTTGATCCTGGCTCAG-3′, 1492R: 5′-GGTTACCTTGTTACGACTT-3′. Polymerase chain reaction (PCR) amplification was as follows: initial pre-denaturation at 98 °C for 20 s; denaturation at 98 °C for 10 s; annealing at 50 °C for 5 s; extension at 72 °C for 15 s; and 30 cycles. After the end of the last cycle, the final extension was performed at 72 °C for 10 min to allow sufficient amplification of the reaction product. The result of 16S rRNA PCR products was checked by 0.8% agarose gel electrophoresis.

Western blot assay

For further confirmation of S protein expression, induction of expression was carried out as illustrated previously (Shi et al. 2014). Briefly, the different recombined bacteria were resuspended in 10 ml of PBS and lysed by ultrasonic crushing. After separating the protein samples with 13% SDS-PAGE, the gels were then transferred to poly (vinylidene fluoride) (PVDF) membranes. To detect the S-DCpep and S-Ctrlpep antigens, the PVDF membranes were incubated overnight at 4 °C with monoclonal antibody. The secondary antibody used was HRP-conjugated rat anti-mouse IgG (CST, USA). Finally, the samples were developed with the ECL Plus detection kit (Thermo Scientific) to visualize the bounded bands.

Ethics statements for experimental animals

Forty specific-pathogen free (SPF) 6-week-old mice were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. and were kept according to the rules of the Animal Care and Ethics Committees of Jilin Agriculture University. Sterile water and germ-free food were used for mice feeding at Jilin Provincial Engineering Research Center of Animal Probiotics (JLAU06201645), which provided the SPF environment facilities. Hygienic disposal of the carcasses was conducted at the end of the experiments.

Immunizations

Using oral gavages, four groups of 10 randomly selected SPF mice ($n = 10$) were vaccinated with *Lp*-pSIP-409-16, *Lp*-pSIP-409-17, *Lp*-pSIP-409-pgsA, or saline. The experimental protocol was as follows: primary vaccination was administered at days 1 and 2, secondary vaccination was administered at days 15 and 16, and booster immunization doses were given at days 29, 30, and 31. All doses were 1×10^8 CFU/mouse. Furthermore, serum samples were collected from the four groups at 14, 28, and 42 days after the first immunization. Serum samples were taken from mice mail vein at 14, 28, and 42 days after the first immunization. Serum samples were collected before centrifugation at 5000×*g* for 10 min, and serum samples were stored at − 20 °C until detection.

Preparation of single-cell suspensions

Peyer's patch (PPs), lamina propria (LP) cells of the small intestine, and mesenteric lymph nodes (MLNs) were obtained according to a previously published protocol (Kikuchi et al. 2014). Small intestine samples from vaccinated mice were treated with 1X PBS and spliced into pieces of 5 cm in length followed by digestion with a lymphocyte separation medium. Single LP cells were obtained after adding an LP cell digestive mix in an isotonic Percoll solution. A 70-μm sterile filter film was used to filter the cell suspension and was followed by centrifugation at 500×*g* for 10 min at 4 °C. After washing with cold PBS, the cells were resuspended in complete Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, USA) (Shi et al. 2014).

Flow cytometry

We used flow cytometry to detect the expression of costimulatory molecules on DCs and the activation of B cells according to previously published reports (Yang et al. 2016). Briefly, a single-cell suspension was prepared from the small intestine lamina propria of immunized mice and was then diluted to 1×10^6 cells/100 μl. A sample of this single-cell suspension was incubated with APC-conjugated anti-CD11c (clone HL3), fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (clone 16-10A1), and phycoerythrin (PE) CD86 (clone GL1) antibodies to label dendritic cells or isotype control APC-, PE-, and FITC-conjugated antibodies (BD Pharmingen). Another sample was incubated with FITC-conjugated anti-IgA (clone C10-3) and PE-conjugated anti-B220 (clone RA3-6B2) antibodies to label B cells according to the manufacturer's instructions. Next, the samples were incubated for 30 min at 4 °C and then washed twice with fluorescence-activated cell sorting (FACS) buffer (PBS, 1% FCS, and 0.09% sodium azide). Finally, samples were detected by flow cytometry (BD FACS LSR Fortessa™). FlowJo 7.6.1 software was used for data analysis.

ELISA

ELISA was performed by following the method of a previously published paper (Yang et al. 2017b). Specific IgG and sIgA antibodies were tested by ELISA using the purified S protein of TGEV. The end-point titers determined were two and three times higher than the background for the fecal and serum samples, respectively.

Special cytokine assay

The MLN cells were incubated with S protein (5 μg/ml) at 37 °C for 72 h, and the supernatant was collected at 72 h. To check for specific cytokines (IFN-γ and IL-4), ELISA kits (R&D Systems, USA) were used.

Serologic tests

Antibody neutralization assays (micro determination) were carried out to determine the titer of TGEV S neutralizing antibodies of the serum collected from mice immunized with recombinant *L. plantarum* according to a previously published protocol with few modifications (Tian et al. 2014). Briefly, 100 $TCID_{50}$ TGEV was incubated at 37 °C with serially diluted sera samples. After 1 h, the admixture was plated onto Vero cells in 96-well plates. After 48 h, the neutralizing antibody titer PD_{50} was measured using the method detailed by Karber.

Statistical analysis

To analyze the significance of the differences between the means, statistical significance was determined by one-way analysis of variance (ANOVA) using the GraphPad Prism 5.0 graphic pad software. All data are displayed as the means ± SEM, where a p value of < 0.05 was regarded as statistically significant.

Results

TGEV S protein expression on the *L. plantarum* bacteria

The surface expression plasmids were constructed to encode pgsA to fuse to S-Ctrlpep or S-DCpep (Fig. 1). Expression levels of the TGEV S protein were determined by different methods. The S protein expression levels of the *Lp*-pSIP-409-16 and *Lp*-pSIP-409-17 were detected by flow cytometry (Fig. 2a). Using laser confocal microscopy, we show that the distinct green fluorescence in Fig. 2b is an example of the expression of the S antigen at the surface of *Lp*-pSIP-409-16 and *Lp*-pSIP-409-17 compared to the non-fluorescent *Lp*-pSIP-409-pgsA. Furthermore, a positive band was detected using the western blot technique, confirming the expression of the protein and 16S rRNA gene from bacteria as internal controls (Fig. 2c). Therefore, it could be concluded that both *Lp*-pSIP-409-16 and *Lp*-pSIP-409-17 were successfully displayed on the surface of their corresponding bacteria.

Constructed Lp-pSIP-409-17 elicited the activation of DCs

Flow cytometry was carried out to assess the activation degree of the costimulatory molecules induced by DCs in the four compared groups 2 weeks (w) post-booster immunization (Fig. 3a). The mean fluorescence intensity (MFI) of $CD11c^{+}CD80^{+}$ in the small intestinal LP was remarkably enhanced ($p < 0.01$) in the group immunized by *Lp*-pSIP-409-17 compared to the saline and *Lp*-pSIP-409-pgsA-administered groups (Fig. 3b). Additionally, a significant increase ($p < 0.05$) was detected between this group and the *Lp*-pSIP-409-16-immunized group (Fig. 3b). Additionally, the *Lp*-pSIP-409-17-immunized group had the ability to highly activate $CD86^{+}$ production compared to the *Lp*-pSIP-409-16- ($p < 0.05$), *Lp*-pSIP-409-pgsA- ($p < 0.01$), and saline-immunized groups ($p < 0.01$) (Fig. 3c).

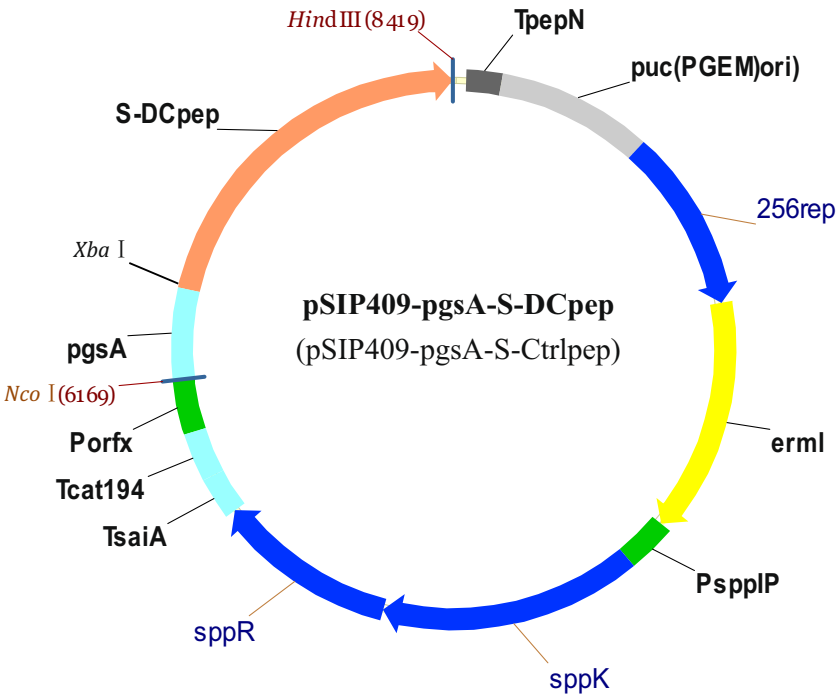
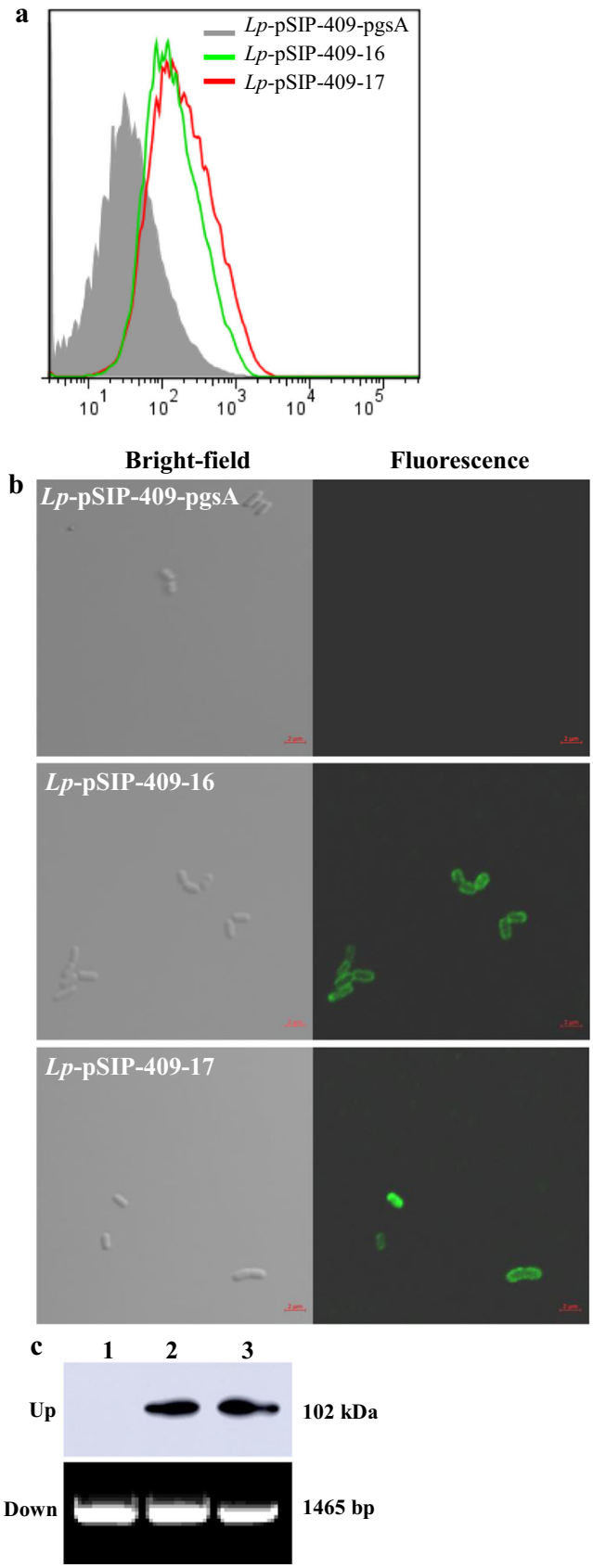


Fig. 1 Pattern diagram of the pSIP409-pgsA-S-Ctrlpep and the pSIP409-pgsA-S-DCpep plasmids



◀ **Fig. 2** Surface displays of the S-Ctrlpep and pgsA-S-DCpep on the *L. plantarum*. **a** The image shows flow cytometric detection of foreign antigen expression after incubating with FITC-conjugated anti-mouse IgG antibodies. **b** The image indicates immunofluorescence observation of foreign antigen expression after incubating with FITC-conjugated anti-mouse IgG antibodies. **c** Western blots of S-DCpep and S-Ctrlpep synthesized proteins (Up) and 16S rRNA gene from bacteria as internal controls (Down). Lane 1: *Lp*-pSIP-409-pgsA. Lane 2: *Lp*-pSIP-409-16. Lane 3: *Lp*-pSIP-409-17

Recombined Lp-pSIP-409-17 induced a B cell immune response

The results obtained by flow cytometry were used to determine the number of $B220^+$ IgA^+ B cells in the PP 2 w after the booster immunization (Fig. 4a). The data showed that the percentage of $B220^+$ IgA^+ B cells markedly increased in the group immunized with *Lp*-pSIP-409-17, compared to the groups immunized with *Lp*-pSIP-409-pgsA, saline ($p < 0.001$), and *Lp*-pSIP-409-16 ($p < 0.05$) (Fig. 4b). Furthermore, $B220^+$ IgA^+ B cells significantly decreased in the *Lp*-pSIP-409-pgsA-immunized group compared to the *Lp*-pSIP-409-16-immunized group ($p < 0.01$) (Fig. 4b). Generally, the obtained data elucidate that immunization with *Lp*-pSIP-409-17 has an important role in the activation of the humoral B cell response in immunized mice.

Recombined Lp-pSIP-409-17 enhanced specific sIgA production

ELISA was used to determine the sIgA titer in intestinal feces collected at 14, 28, and 42 days after the first vaccination (Fig. 5). At 14 days, there was no marked variance between the different groups. However, at 28 days, a remarkable difference ($p < 0.05$) was found only between the group immunized with *Lp*-pSIP-409-17 and both of the *Lp*-pSIP-409-pgsA- and the saline-immunized groups. Importantly, our data show that a significantly high sIgA titer was present in the *Lp*-pSIP-409-16-immunized group at the 42-day mark compared with the saline- ($p < 0.01$) and *Lp*-pSIP-409-pgsA-immunized groups ($p < 0.05$). In contrast, the sIgA production titer was significantly higher in the *Lp*-pSIP-409-17-immunized group compared with the saline- ($p < 0.001$), *Lp*-pSIP-409-pgsA- ($p < 0.001$), and *Lp*-pSIP-409-16-immunized groups ($p < 0.05$). It could be concluded that oral gavage immunization of mice with *Lp*-pSIP-409-17 significantly induced the secretion of sIgA.

Recombined Lp-pSIP-409-17 triggered specific IgG production

Serum samples obtained at 14, 28, and 42 days after the primary vaccination were used to detect the IgG titers

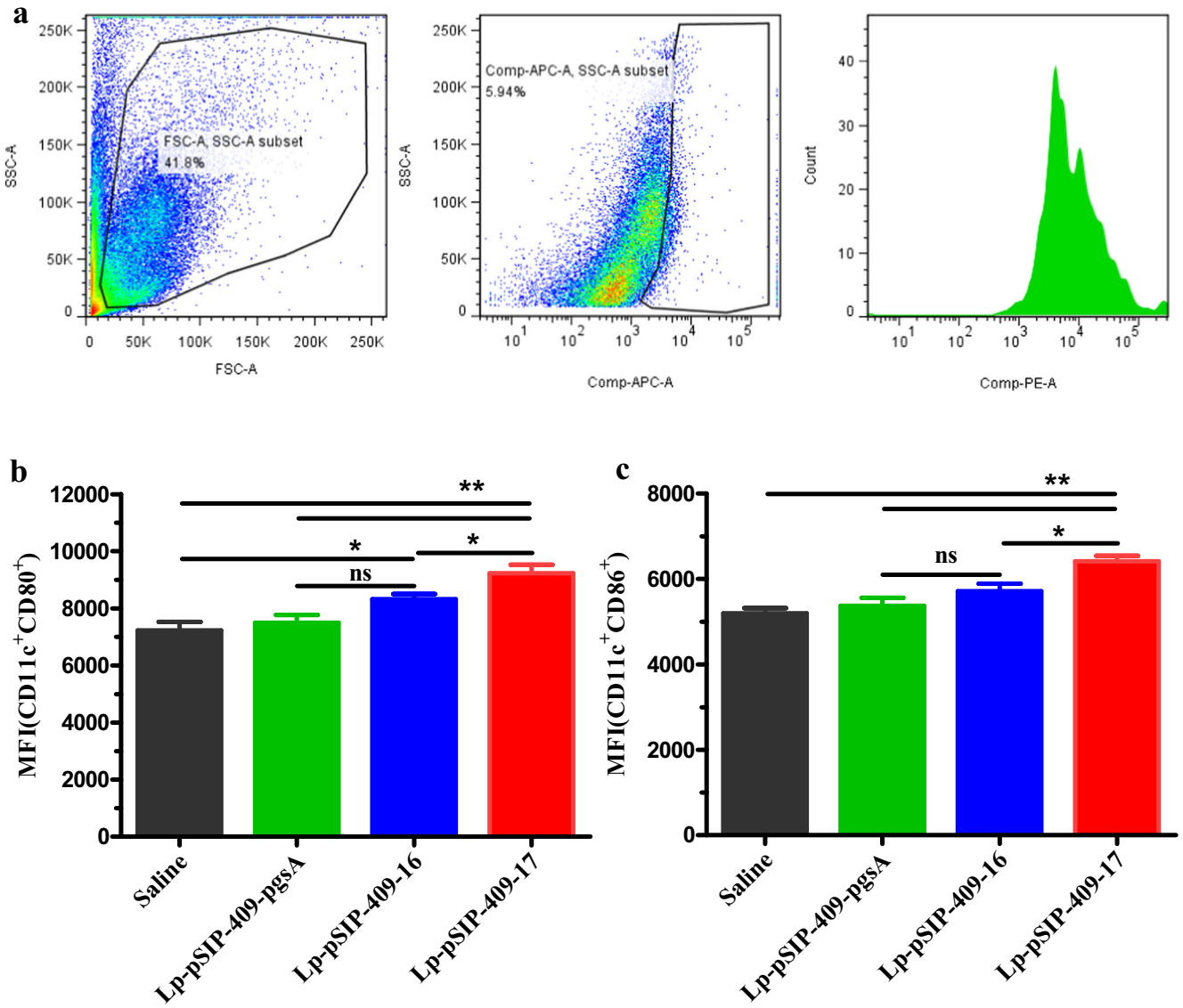


Fig. 3 The activation of the recombinant bacterium on dendritic cell (DCs). Fourteen days after booster immunizations, B7 molecules (CD80/CD86) in the LP cells of the small intestine were tested by flow cytometry. (**a**) A gating strategy of CD86 expression on the DCs was employed. The mean fluorescence intensities (MFI) of $CD11c^+CD80^+$ (**b**) and $CD11c^+CD86^+$ (**c**) are shown. (*$p < 0.05$ and **$p < 0.01$)

using ELISA (Fig. 6). No significant difference was found between the groups immunized with *Lp*-pSIP-409-16 and *Lp*-pSIP-409-17, although there was a significant difference between the *Lp*-pSIP-409-17-immunized group and either the *Lp*-pSIP-409-pgsA- or the saline-immunized groups at 14 days ($p < 0.05$) or 28 days ($p < 0.01$), respectively. In contrast, at 42 days, a significant difference was determined between the *Lp*-pSIP-409-16- and the *Lp*-pSIP-409-17-immunized groups ($p < 0.05$), and a highly significant difference ($p < 0.001$) was recorded between the *Lp*-pSIP-409-17- and both of the *Lp*-pSIP-409-pgsA- and the saline-immunized groups. Taken together, the above data show that immunization with *Lp*-pSIP-409-17 has a great influence on humoral IgG production.

Production of the serum-neutralizing antibodies elicited by the Lp-pSIP-409-17

Oral vaccination of the mice with *Lp*-pSIP-409-17 revealed an increase in the serum antibody titer 42 days after immunization (Fig. 7). The titer was remarkably stronger than the titer induced by *Lp*-pSIP-409-16 ($p < 0.05$) and highly significantly stronger than the titers induced by *Lp*-pSIP-409-pgsA and saline ($p < 0.01$).

Effect of Lp-pSIP-409-17 on production of the cytokine

Fourteen days after the administration of the booster immunization dose, ELISA was carried out to detect the levels of the

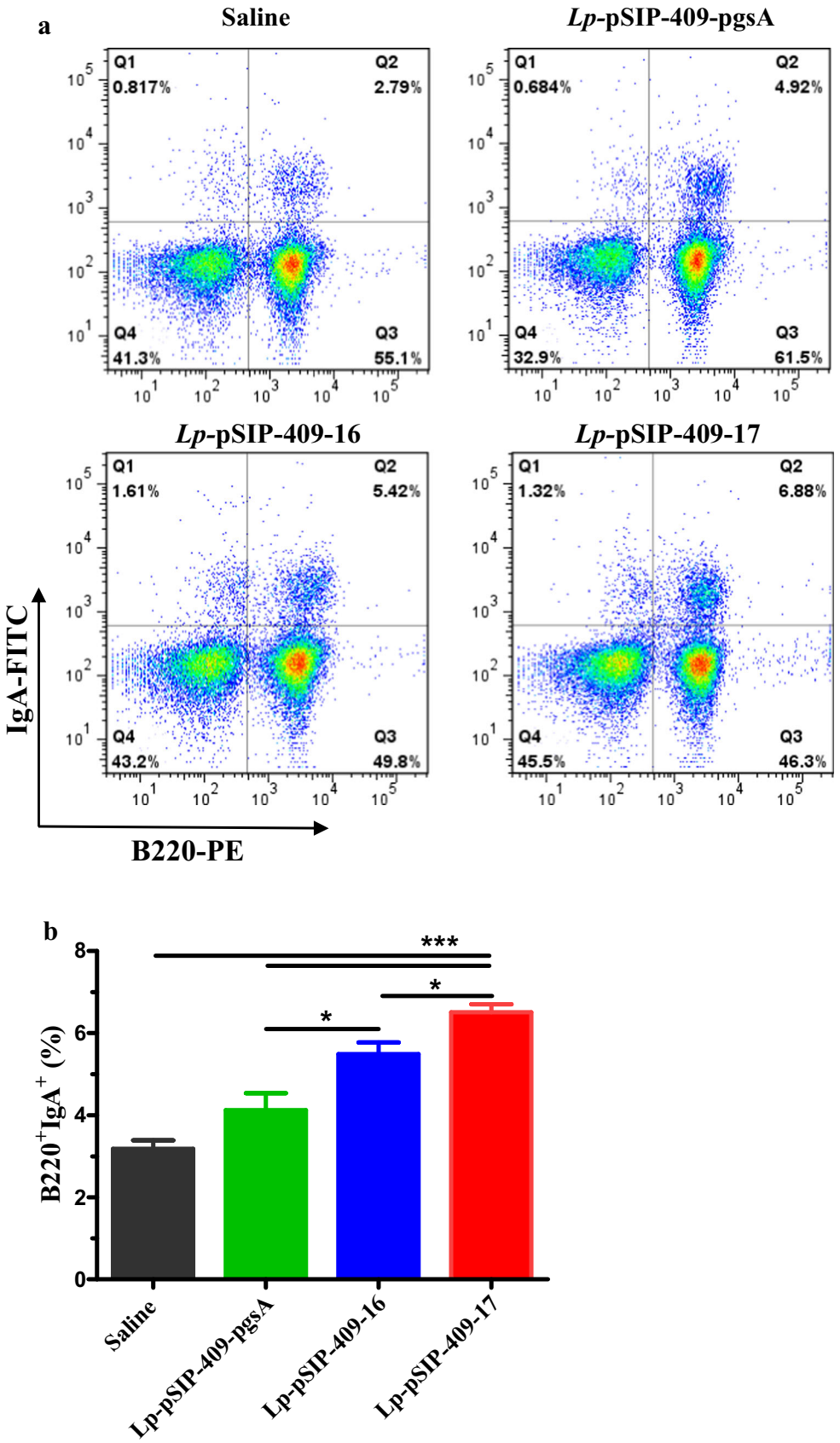


Fig. 4 The recombinant bacterium induced B cell immune responses in the PP of vaccinated mice. Fourteen days after booster immunizations, $B220^{+}IgA^{+}$ in the PP were detected by flow cytometry. The scatter diagram of $B220^{+}IgA^{+}$ (**a**) and histogram (**b**) is shown. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

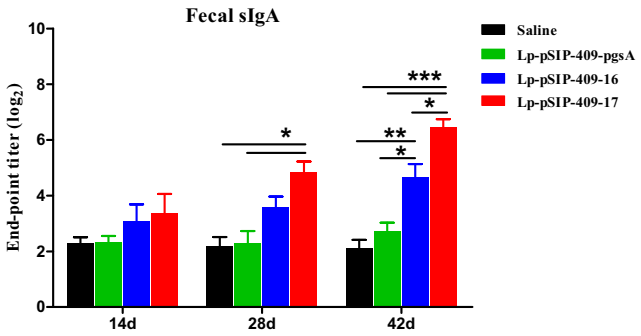


Fig. 5 The recombinant bacterium induced specific sIgA titers. Fourteen, 28, and 42 days after primary immunization, fecal samples were taken from vaccinated mice, and specific antigen sIgA titers were measured with ELISA. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

IFN-γ and IL-4 cytokines in the MLN cell secretions. The production of IFN-γ was significantly ($p < 0.05$) detected in the *Lp*-pSIP-409-17-immunized group compared with the *Lp*-pSIP-409-16- and *Lp*-pSIP-409-pgsA-immunized groups, and the production was highly significant ($p < 0.01$) compared with the saline-immunized group (Fig. 8a). Additionally, a significant ($p < 0.001$) level of IL-4 was detected in the orally vaccinated *Lp*-pSIP-409-17 group when compared with either the saline- or the *Lp*-pSIP-409-pgsA-vaccinated groups, and a marked significant difference ($p < 0.05$) was detected between the *Lp*-pSIP-409-17-immunized group and the *Lp*-pSIP-409-16-immunized group (Fig. 8b).

Discussion

TGEV is a highly infectious coronavirus (CoV) that causes diarrhea in piglets with a high morbidity and mortality rate leading to high economic losses (Zhang et al. 2017). It causes inflammation in intestinal tissues, and usually, the death of the animal is mainly due to a sodium and potassium ion imbalance (Cruz et al. 2013).

So far, there is no effective treatment against the causative agent itself; the prescribed medication acts solely against the

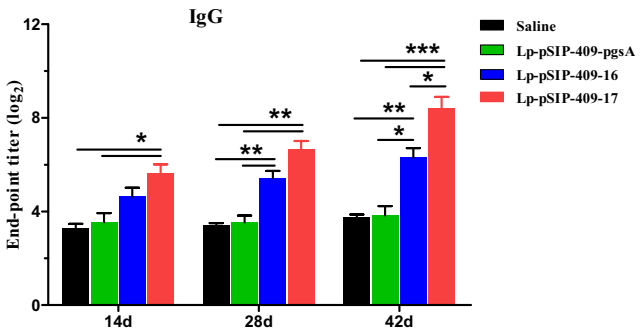


Fig. 6 The recombinant bacterium induced specific IgG titers. Fourteen, 28, and 42 days after primary immunization, serum samples were taken from vaccinated mice, and specific antigen IgG titers were measured with ELISA. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

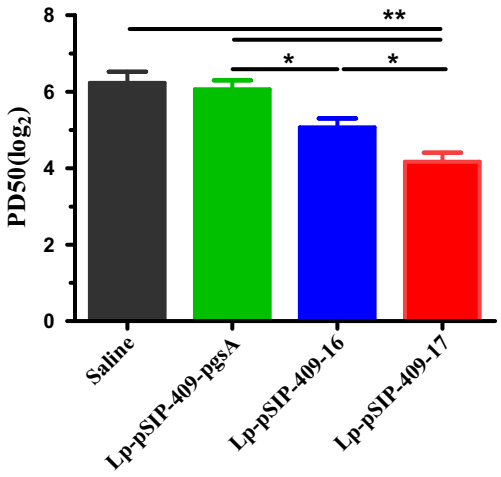


Fig. 7 The recombinant bacterium induced neutralizing antibodies in the serum. Forty-two days after primary immunization, neutralizing antibodies of vaccinated mice were observed in the serum. (*$p < 0.05$ and **$p < 0.01$)

resulting clinical symptoms. This fact necessitates the development of an effective tool for viral control. Effective prevention and control of the TGEV and other coronaviruses can only be achieved through the use of vaccines (Gerdts and Zakhartchouk 2017).

The immune response to swine enteric coronaviruses is based on cytotoxic T cells and secretory antibodies (sIgA), which are produced by antibody-secreting cells in the lamina propria of the mucosal tissues, while systemic antibodies such as IgG and IgM are found in the serum and interstitial tissues and some isotypes can be transported across the mucosal epithelium into the lumen (Chattha et al. 2015; Horton and Vidarsson 2013).

Most present commercial TGEV vaccines are inactivated vaccines or live-attenuated vaccines that are given to the sow during the pregnancy period to provide lactogenic immunity to its offspring (Gerdts and Zakhartchouk 2017). Some parenteral vaccines did not provide effective lactogenic immunity when the vaccinated animals were experimentally challenged with the virus, resulting in a mortality rate that ranged from 44 to 80% in piglets. Furthermore, antibody titers in the milk samples obtained during the first week of lactation rapidly decreased (Voets et al. 1980).

Recent studies showed that *L. plantarum* was successfully used as an exogenous protein vector, adding to its probiotic nature (Yang et al. 2017a). One of the important characteristics of the anchoring protein expressed by the pgsA gene is its ability to position exogenous proteins at the bacterial surface to exhibit their function (Cai et al. 2016; Yang et al. 2017c). Moreover, effective protective immunity was provided by the DCpep constructed vaccine (Yang et al. 2017a). Additionally, the essential role of the S protein in viral tropism (Cruz et al. 2013), mainly in cell fusion and jejunal tissue infection (Almazan et al. 2000) as well as serving as a major target for

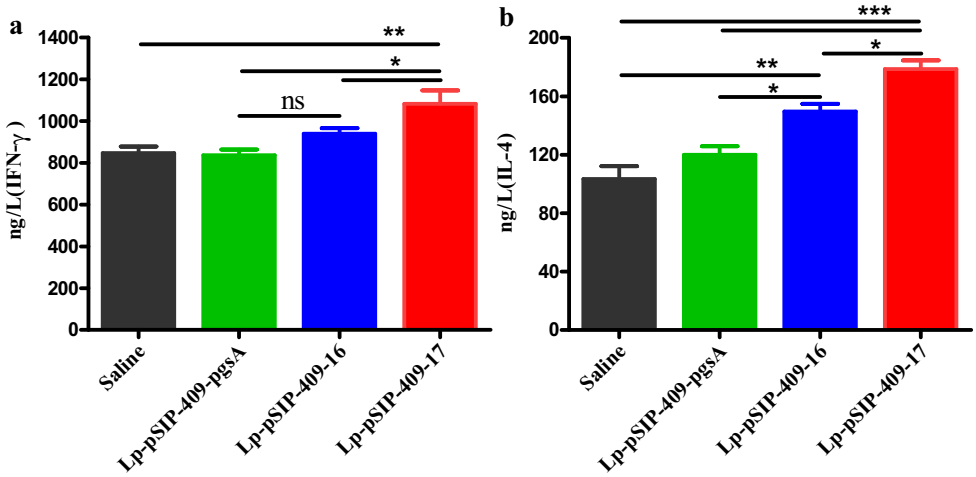


Fig. 8 The recombinant bacterium induced S antigen-specific IFN-γ (**a**) and IL-4 (**b**) in the MLN of vaccinated mice. Two weeks after booster immunizations, single cells from the MLN of vaccinated mice were incubated with specific antigen for 72 h, and supernatants were used to detect IFN-γ (**a**) and IL-4 (**b**). (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

neutralizing antibodies, was recorded. Mice have successfully been used as a model for vaccine studies (Jiang et al. 2017; Yang et al. 2017c; Yu et al. 2017a). Accordingly, the constructed *Lp*-pSIP-409-17 bacteria was developed, and its immunogenicity was tested in mice.

Dendritic cells are a type of antigen-presenting cell, and they have a significant function in delivering antigens to T cells and B cells to improve the effects of vaccines. Studies conducted by our laboratory and others show that recombined bacteria could trigger the activation of DCs at mucosal sites (Mohamadzadeh et al. 2009; Yang et al. 2016). In this study, a clear and significant increase in the production of $CD11c^+CD80^+$ and $CD11c^+CD86^+$ was found in the group immunized with *Lp*-pSIP-409-17 when compared to the other vaccinated groups. Similar observations have been made by other groups (Kathania et al. 2013; Wang et al. 2017). As a whole, all experimental results confirmed that oral administration of recombined bacteria can induce the activation of DCs, in turn enhancing T cell and B cell responses in order to combat pathogens in the host.

Recent studies focused on the importance of mucosal delivery of vaccine and its role in preventing viral diarrheal diseases as it can generate both mucosal and systemic immune responses. This is particularly important since TGEV infections originally arise at the intestinal mucosal surfaces (Jiang et al. 2016). The results from the current study clarify the role of the recombinant *Lp*-pSIP-409-17 bacteria in increasing the mucosal immune response as the number of IgA^+B220^+ B cells increased significantly in mice immunized with this recombinant bacteria when compared with other groups. It is notable that the mucosal immune response is the first barrier to functionally neutralizing TGEV. The obtained results are in agreement with Jiang et al. (2016).

As the neutralizing activity of the produced antibodies is an essential parameter used to evaluate vaccine efficiency and stronger antibody titers reflect higher neutralizing activities, we investigated both in this study. The results of our ELISA analysis showed that the serum IgG titers started to increase at 14 days after vaccination with a level that is significantly different than the other three vaccine groups. Additionally, the titers were markedly increased at 42 days post-primary immunization. A study recorded that the S protein DNA vaccine elicited a humoral response 21–42 days post-immunization (dpi) with the peak of the response observed at day 35 (Meng et al. 2013). Meanwhile, between 35 and 42 dpi, the antibody titers were dramatically reduced. Furthermore, the antibody titer elicited by the recombinant *Lactobacillus casei* oral vaccine was stronger than that triggered by the previously mentioned DNA vaccine (Yu et al. 2017a). The attenuated *Salmonella typhimurium*-based S gene induced an immune-level response that significantly increased at 1 month post-vaccination, peaking at 1.5 months and decreasing at 2 months after immunization (Zhang et al. 2016b). When comparing the results of using a monovalent TGEV S gene vaccine with the bivalent TGEV/PDEV S gene vaccine, the specific IgG antibody titers elicited by the single-gene vaccine were higher than those elicited by the double-gene vaccine (Zhang et al. 2016b). The difference between these results may be attributed to many factors, including the pathogen strain used, the different vector used, and the experimental host.

It has been shown that Th1 and Th2 cells have an important role in immune responses; Th1 cells have been shown to help in cytotoxic T cell differentiation, IFN-γ secretion, and macrophage cell activation (Maldonado-Lopez and Moser 2001), while Th2 cells have a major role in humoral immunity through the stimulation of B cell proliferation and antibody production (Mosmann and Coffman 1989). The ability of monocytes to differentiate into macrophages is widely affected by IL-4 secretion (Lee et al. 2009). In the present study, using specific ELISAs, MLN cell-specific cytokine analysis was used to evaluate the cellular immune response induced by the recombinant *L. plantarum*. Our data show that the production of IL-4 and IFN-γ was remarkably higher in the *Lp*-pSIP-409-17 orally immunized animal group compared with the

Lp-pSIP-409-16 and other groups. These results are in agreement with previous study, which reported that recombinant DNA plasmids expressing TGEV S genes improved the number of T lymphocyte subgroups as well as the proliferation of T lymphocytes, adding to the plasmid's ability to induce a significant production of IFN-γ in the vaccinated animal (Meng et al. 2013). Consistent with other reports, TGEV S recombinant *L. casei* induced IFN-γ production that was stronger than IL-4 in the vaccinated animals, and the Th1/Th2 balance was disturbed (Jiang et al. 2016). It is worth noting that TGEV has developed strategies to evade and interfere with the interferon response and that suppression of this response by many structural and non-structural viral proteins has been documented (Zhang et al. 2016a).

In conclusion, it could be concluded that the successfully constructed *Lp*-pSIP-409-17 shows promising results in inducing both humoral and cellular immune responses in the orally immunized mouse model. This supports the need for further assessment in a porcine model in order to optimize the immunization procedures before it can be used as an easily administered, safe, and protective mucosally delivered vaccine to control TGEV infection.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no competing interests.

Ethical approval All applicable international and national guidelines for the care and use of mice were followed.

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