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CpG DNA facilitate the inactivated transmissible gastroenteritis virus in enhancing the local and systemic immune response of pigs via oral administration

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ABSTRACT

Transmissible gastroenteritis virus (TGEV) replicates in the small intestine and induces enteritis and watery diarrhea. Establishment of local immunity in the intestine would thus prevent TGEV transmission. CpG DNA has been reported as a promising mucosal adjuvant in some animals. The effects of oral immunization of CpG DNA together with inactivated TGEV (ITGEV) were investigated in this study. Pigs (6 weeks old) were orally immunized with ITGEV plus CpG DNA. The TGEV-specific IgA level in the intestinal tract and the TGEV-specific IgG level in serum significantly increased following immunization with ITGEV plus CpG DNA ($P \leq 0.05$). Moreover, populations of IgA-secreting cells, CD3+ T lymphocytes and intraepithelial lymphocytes (IELs), in the intestine increased significantly after immunization with ITGEV plus CpG DNA ($P \leq 0.05$). Furthermore, the expression of IL-6, IL-12 and interferon-γ (IFN-γ) in ligated intestine segments increased significantly after injection with ITGEV plus CpG DNA ($P \leq 0.05$). Taken together, these data suggest that oral immunization of ITGEV plus CpG DNA elicits a local immune response. Further studies are required to determine whether this immunity provides protection against TGEV in pigs.

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1. Introduction

Transmissible gastroenteritis (TGE), caused by transmissible gastroenteritis virus (TGEV), induces severe, fatal diarrhea in newborn pigs (Lin et al., 2015) and has caused serious economic losses worldwide for many years (Wang et al., 2013). Initial infection with TGEV occurs at the intestinal mucosal epithelium, and so local intestinal immunity plays an important role in the prevention of TGEV infection (Tuboly et al., 2000). Traditional parenteral vaccination with virulent or attenuated virus does not induce sufficient mucosal immunity in the intestinal tract (Gu et al., 2012). Oral vaccination, a promising alternative, elicits strong local and systemic immune responses (Otczyk and Cripps, 2010), which have been shown to block viral attachment and colonization, and prevent localized infections of the intestinal tract (Otczyk and Cripps, 2010). However, few reports regarding the oral immunization of large animals have been published (Cox et al., 2002).

The inactivated vaccine has excellent safety and tolerance profiles (Beyer et al., 2002; Cox et al., 2004) with few adverse reactions reported. However, oral immunization with inactivated virus does not result in effective immune protection (Petrovsky and Aguilar, 2004; Scheerlinck et al., 2006). Thus, the immune response to inactivated virus needs to be enhanced. CpG DNA, as an adjuvant, can induce Th1-like cytokine responses by stimulating antigen-presenting cells via toll-like receptors (McCluskie and Krieg, 2006; Zhang et al., 2007b). CpG DNA as a mucosal adjuvant elicits a strong immune response in mice following intranasal immunization (McCluskie and Davis, 1999; McCluskie et al., 2000). Our previous study suggested that CpG DNA could markedly enhance the mucosal and systemic immune responses to inactivated H9N2 avian influenza viruses when administered to ducks (Kang et al., 2012, 2013). Therefore, the aim of this study was to determine whether CpG DNA could improve the efficiency of oral immunization with inactivated TGEV and so prevent the development of TGE.

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Table 1
Experimental groups and administration strategies.

	Groups	Treatments
Oral administration	(A) Control	PBS (20 ml per head, $n = 4$)
	(B) CpG DNA	CpG DNA (20 ml of 25 μg ml^{-1} per head, $n = 4$)
	(C) ITGEV	ITGEV (20 ml of 1×10^7 TCID$_{50}$ ml^{-1} per head, $n = 4$)
	(D) ITGEV + CpG DNA	ITGEV (20 ml of 1×10^7 TCID$_{50}$ ml^{-1} per head) CpG DNA (20 ml of 25 μg ml^{-1} per head) ($n = 4$)
Hypodermic injection	(E) ITGEV	ITGEV (2 ml of 1×10^7 TCID$_{50}$ ml^{-1} per head, $n = 4$)
Intestinal ligation	(a) Control	PBS (1 ml per segment, $n = 3$)
	(b) ITGEV	ITGEV (1 ml of 1×10^7 TCID$_{50}$ ml^{-1} per segment, $n = 3$)
	(c) CpG DNA	CpG DNA (1 ml of 25 μg ml^{-1} per segment, $n = 3$)
	(d) ITGEV + CpG DNA	ITGEV (1 ml of 1×10^7 TCID$_{50}$ ml^{-1} per segment) CpG DNA (1 ml of 25 μg ml^{-1} per segment) ($n = 3$)

2. Materials and methods

2.1. Vaccine strain and adjuvant

TGEV [SHXB strain (Zhao et al., 2014), $1 \times 10^{8.4}$ TCID$_{50}$ (50% cell tissue infectious dose)/1 ml] was provided by Jiangsu Academy of Agricultural Sciences (JAAS). TGEV was exposed to ultraviolet light (UV) for 4 h to prepare inactivated virus, which had been tested for complete loss of the infectivity by inoculation into the Swine Testicle (ST) cells for three passages. CpG DNA (20 μg/μl; kept in our lab) was extracted from *Escherichia coli* and *Poephogus grunniens* strain and dissolved in Tris–EDTA buffer. The CpG DNA was analyzed by measurement of the A260/A280 ratio (A260/A280 = 1.82), gel electrophoresis, and free endotoxin detection (Kang et al., 2013).

2.2. Proliferation assays

Lymphocytes were isolated from the spleen of six-week-old healthy pigs maintained in JAAS (Nanjing, China). Cells were cultured in 96-well plates and stimulated with CpG DNA (30 μg/ml), ITGEV (1×10^5 TCID$_{50}$/ml) or together at 37 °C for 48 h. CpG-ODN D19 (30 μg/ml, 5′-gggTGCATCGATGCAGggggg-3′ synthesized at Life Technologies, China) and Concanavalin A (100 ng/ml; Sigma, USA) were used as two positive controls, while the medium was used as negative control. Cell proliferation assays were performed with WST-8Cell Counting Kit-8 (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Absorbance of each well was quantified at 450 nm by ELISA reader. The stimulation index (SI) was calculated with the following formula: $SI = (OD_{samplewell} - OD_{blankwell})/(OD_{negativewell} - OD_{blankwell})$.

2.3. Animal experimental

Six-week-old cross-bred pigs were bred and maintained in a unique high sanitary state characterized by freedom from a large range of porcine pathogens, e.g., TGEV, PEDV, PCV-2 and PRRSV. All animal experiments were approved by the Ethical Committee of Animal Experiments of the College of Veterinary Medicine, Nanjing Agricultural University, and all animal care and use was conducted in strict accordance with the Animal Research Committee guidelines of the College of Veterinary Medicine, Nanjing Agricultural University.

Animal experiment (first): three pigs were starved 24 h before anesthetized with pentobarbital sodium and a midline incision was made anterior to the navel (Nielsen and Sautter, 1968). Ileum segments, including Peyer's Patches (PPs) part, were divided into 4 groups (see Table 1). Pigs were kept warm on a 37 °C warming pad for 6 h and then the post-ligated intestine segments were removed and immediately frozen in liquid nitrogen for extraction of total RNA.

Animal experiment (second): twenty pigs were randomly divided into 5 groups (Table 1) and immunized as Fig. 1. Serum were separated by centrifugation and stored at −70 °C for specific IgG detection, while feces (0.3 g) were suspended into PBS and stored at −70 °C for specific IgA detection. Ileum tissues were triturated in a mortar with liquid nitrogen, and the homogenate was dissolved in PBS. Concentrations of lavage and homogenate supernatants were measured by BCA protein assay kit (Thermo Scientific Pierce). Other ileum tissue were fixed in Bouin's liquid for histological and immunohistochemistry staining.

2.4. RNA isolation and RT-qPCR analysis

Total RNA of intestinal segments was extracted with TRIzol Reagent (Life Technologies, USA) and subjected to reverse transcription with cDNA with PrimeScript RT kit (Takara, China). Then, qPCR was carried out on ABI7500 instrument with specific primer (listed in Table 2). Data were reported as values normalized to the housekeeping gene (β-Actin) account for repeated measures and calculated

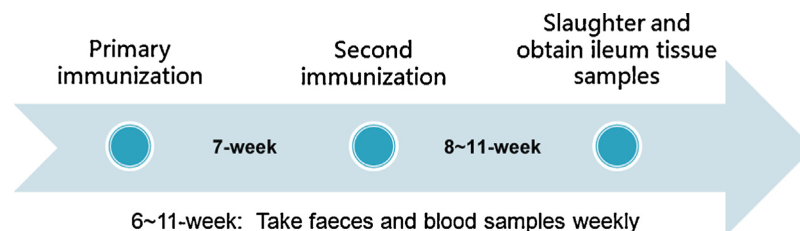

Fig. 1. Flow chart of animal immunization and sample collection.
Twenty pigs were divided randomly into five groups of four pigs and were immunized at 6 and 7 weeks of age. Feces and blood samples were obtained weekly. Serum was separated by centrifugation and stored for the detection of specific IgG. Feces were suspended in PBS and stored for the detection of specific IgA. Pigs were euthanized and ileum tissue removed after maintained 12 weeks. Ileum tissues were triturated in a mortar with liquid nitrogen and the homogenate was dissolved in PBS. The protein concentrations of lavage and homogenate supernatants were measured using a BCA protein assay kit. Other ileum tissue was fixed in Bouin's liquid for histological and immunohistochemical evaluation.

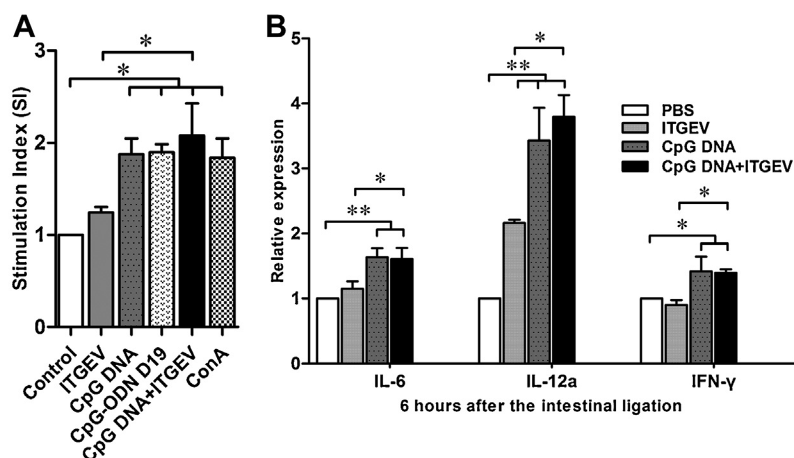

Fig. 2. Proliferation and cytokine secretion of splenic lymphocytes.
(A) Proliferation of splenic lymphocytes. Lymphocytes were obtained from the spleen and stimulated with ITGEV (1×10^5 TCID50/ml), CpG DNA (30 μg/ml), or CpG DNA plus ITGEV at 37 °C for 48 h. Proliferation results are shown. CpG ODN D19 (30 μg/ml) and concanavalin A (Con A) were used as positive controls, and the medium was used as the blank control. Significance was determined by one-way ANOVA with Tukey's multiple comparison test. (B) IL-6, IL-12 and IFN-γ mRNA levels in the ileum at 6 h after ligation and injection. IL-6, IL-12 and IFN-γ data are the means ± SD of three samples. $^{**}P < 0.01$, $^{*}P < 0.05$ in comparison with the blank group. All results are representative of three independent experiments. Significance was determined by one-way ANOVA with Tukey's multiple comparison test.

as the following formula. Relative quantification = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{\beta\text{-actin}})_{treatedgroup} - (Ct_{targetgene} - Ct_{\beta\text{-actin}})_{untreatedgroup}$.

2.5. Histological examination of the intraepithelial lymphocytes (IELs)

Fixed samples were embedded in paraffin and sectioned at 5 μm thickness. Hematoxylin–eosin staining was applied in the paraffin sections and then sealed with a cover slip. Tissue sections were checked with 40× stage objectives, an Olympus BH2 microscope. For each tissue section (10 sections per sample), the number of IELs at ten different fields of ileum in each animal was counted for the statistical analysis of the data. The data expressed as the average number of cells.

2.6. Immunohistochemical detection of CD3+ T lymphocytes and IgA secreting cells

Paraffin sections were dewaxed and rehydrated in PBS. The endogenous peroxidase activity was neutralized by 3% H_2O_2 in PBS for 10 min, and then rinsed in PBS for 5 min. All sections were treated with 5% BSA for 30 min to block non-specific binding and then stained with rabbit anti-pig CD3 antibody (Abcam, USA), or goat anti-pig IgA antibody (Bethyl, USA) overnight at 4 °C. Subsequently, the slides were five times rinsed with PBS and then incubated for another 30 min at 37 °C with an ABC-based system (biotinylated goat anti-rabbit or rabbit anti-goat IgG was used as the secondary antibody with diaminobenzidine, Boster, Wuhan, China). All incubation were performed in a moist chamber. The negative control was performed simultaneously in which the first antibody was replaced with PBS. The number of CD3+ T lymphocytes is counted with the above method of IELs. The regions that contained IgA secreting cells were counted by the Image Pro-plus analysis program (Cambridge, UK) and data expressed as the average area of cells.

2.7. ELISA detection of IgA and IgG

Anti-TGEV specific IgG and specific sIgA antibody levels in serum, feces and small intestine tissue were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (Kang et al., 2012; Li et al., 2012). In brief, ELISA plates were coated with purified TGEV S-AD protein (kept in our lab). HRP conjugated rabbit anti-pig IgG and goat anti-pig IgA (Bethyl, USA) were then used. The absorbance was measured at 450 nm, using an automated ELISA reader. Endpoint titers were defined as the maximum dilutions showing a reaction.

2.8. Statistical analyses

Data were evaluated by unpaired two-tailed Student's *t*-test using GraphPad Prism 5 (http://www.graphpad.com) (CSSN), with P value < 0.05 considered to be statistically significant. The significance of the data was also determined by one-way ANOVA, followed by Tukey's multiple comparison tests.

Table 2
Sequences of primers used for quantitative real-time PCR.

Gene	Primer sequence (5′–3′)	Product size	Accession no.
β-Actin	F: AGATCAAGATCATCGCGCCT R: ATGCAACTAACAGTCCGCCT	170 bp	XM_003124280.3
IL-6	F: ACCCTGAGGCAAAAGGGAAAG R: CAGGTGCCCCAGCTACATTA	195 bp	JQ839263.1
IL-12A	F: TGCCTCAACCACTCCCAAAA R: TGCTAAGGCACAGGGTTGTC	268 bp	NM_213993.1
IFN-γ	F: TCAGCTTTGCGTGACTTTGTG R: TCACTCTCCTCTTTCCAATTCTTCA	162 bp	NM_213948.1

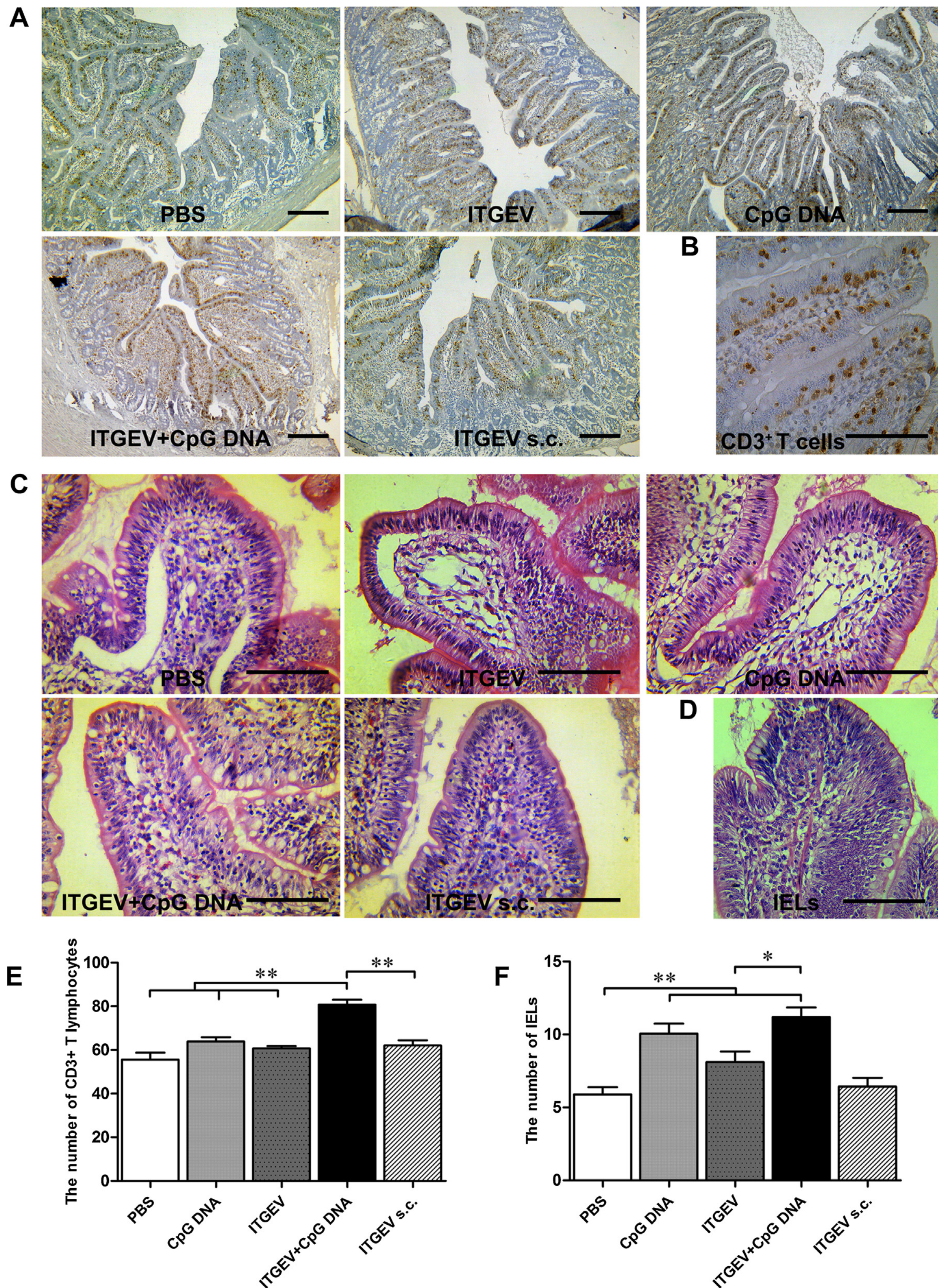

Fig. 3. Distribution and area of IELs and CD3+ T lymphocytes in small intestinal villi.
Fixed samples were embedded in paraffin and sectioned at 5 μm thickness. Haematoxylin–eosin staining was performed on the paraffin sections, which were then sealed with a cover slip. Tissue sections were visualized using the 40× stage objective. For each tissue section (10 sections per sample), IELs were enumerated in 10 separate fields of the ileum of each animal. Data are average numbers of cells. (A) Distribution of CD3+ T lymphocytes in small intestinal villi (bars: 200 μm). (B) Change in the area in which CD3+ T lymphocytes are distributed in small intestinal villi (bars: 100 μm).(C) Distribution of IELs in small intestinal villi (bars: 100 μm). (D) Change in the area in which IELs are distributed in small intestinal villi (bars: 100 μm). (E) Areas of CD3+ T lymphocytes in small intestinal villi. (F) Change in the area in which IELs are distributed in small intestinal villi. Data are means ± SEM. *$P < 0.05$; **$P < 0.01$. ITGEV, inactivated transmissible gastroenteritis coronavirus; s.c., subcutaneous injection.

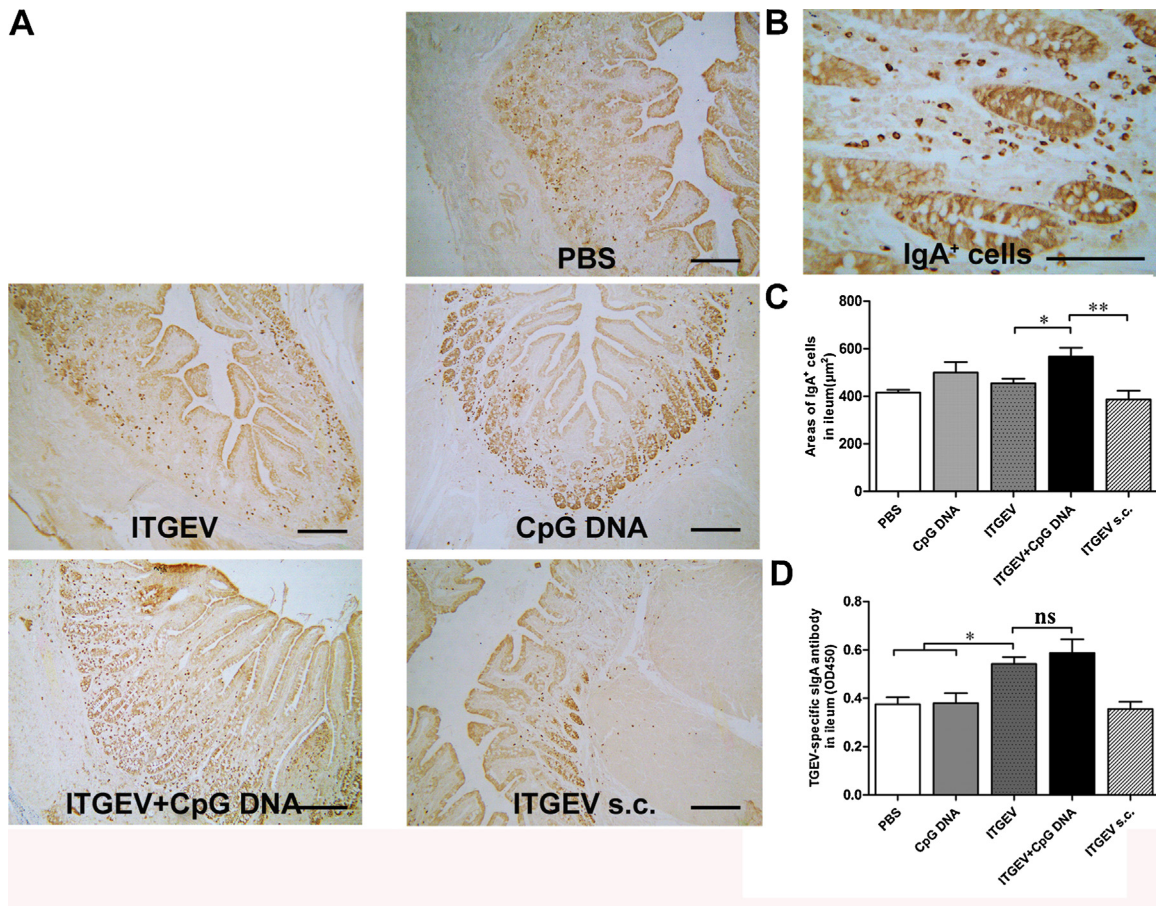

Fig. 4. Numbers of IgA-secreting cells and TGEV-specific IgA levels in the intestinal tract.
(A) Distribution and area of IgA-secreting cells in the ileum (bars: 200 μm). (B) IgA-secreting cells were identified as lymphocytes based on their characteristic morphology: rounded, with a nucleus surrounded by a ring of yellow-brown cytoplasm (bars: 100 μm). (C) Changes in the area of IgA-secreting cells in the lamina propria (40× stage objective). (D) Antigen-specific mucosal IgA levels in the ileum of pigs immunized 42 days after the first vaccination were determined by ELISA. Data are means ± SEM. $^{*}P<0.05$; $^{**}P<0.01$. OD, optical density; ITGEV, inactivated transmissible gastroenteritis coronavirus; s.c., subcutaneous injection. Bars: (a) 200 μm; (b) 100 μm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

3. Results

3.1. Cellular proliferation in vitro

To evaluate the effects of adjuvant CpG DNA, we assessed the in vitro proliferation of lymphocytes derived from the spleen. Co-incubation of naïve spleen lymphocytes with CpG DNA or CpG DNA plus ITGEV resulted in significant cellular proliferation ($^{**}P<0.01$) (Fig. 2A), while stimulation with ITGEV did not induce significant cellular proliferation.

3.2. IL-6, IL-12 and IFN-γ mRNA levels in the intestine

To assess cellular immunity induced by CpG DNA, we measured the mRNA levels of IL-6, IL-12 and IFN-γ by qRT-PCR in intestinal ligation experiments. As shown in Fig. 2B, CpG DNA stimulation significantly increased levels of IL-6, IL-12, and IFN-γ mRNA in intestinal segments ($^{**}P<0.01$). In contrast, only the IL-12 mRNA level was significantly increased in the ITGEV-stimulated group ($^{**}P<0.01$), while those of IL-6 and IFN-γ were unchanged. Compared with the group that had been stimulated with TGEV alone, the ITGEV and CpG DNA co-stimulated group exhibited marked increases in IL-6, IL-12 and IFN-γ mRNA levels in the intestine ($^{**}P<0.01$).

3.3. CD3+ T lymphocytes and IELs in the intestine

The results of histological assessments suggested that the IELs were located between epithelial cells, beneath the intercellular tight junctions and stay at the basal of the epithelium (Fig. 3C and D). Immunohistochemical staining indicated that CD3+ T lymphocytes were located mostly in the lamina propria of the mucosa and submucosa (Fig. 3A and B). Further, the number of CD3+ T lymphocytes and IELs in the ileum significantly increased after stimulation with CpG DNA or ITGEV plus CpG DNA ($^{*}P<0.05$, $^{**}P<0.01$) (Fig. 3E and F). However, the number of CD3+ T lymphocytes and IELs in the ITGEV group or the ITGEV s.c. group increased only slightly compared with the PBS-treated group ($P>0.05$).

3.4. IgA-secreting cells in the intestine

Intestinal IgA-secreting cells were detected by immunohistochemistry. As shown in Fig. 4A and B, the IgA-secreting cells were located mainly in the submucosa and their membranes were stained a deep yellow-brown. The IgA-secreting cell areas in the ileum increased significantly after stimulation with CpG DNA and ITGEV, either together or separately ($^{**}P<0.01$) at 12 weeks (Fig. 4C).

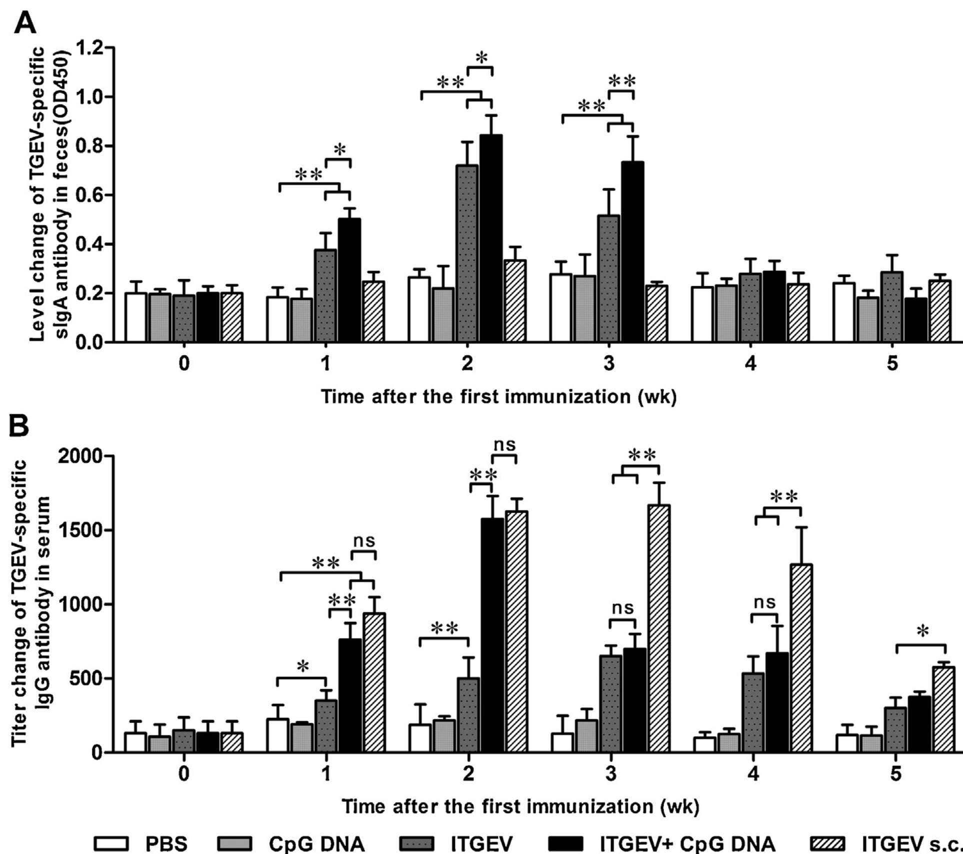

Fig. 5. TGEV-specific sIgA levels in feces and IgG levels in serum.
(A) TGEV-specific sIgA levels in feces sampled weekly after the primary immunization. Data are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. OD, optical density; ITGEV, inactivated transmissible gastroenteritis coronavirus; s.c., subcutaneous injection.(B) IgG levels in serum collected weekly after the primary immunization. Data are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. OD, optical density; ITGEV, inactivated transmissible gastroenteritis coronavirus; s.c., subcutaneous injection.

3.5. Detection of local anti-TGEV-specific IgA

Because the area of IgA-secreting cells increased significantly following immunization with ITGEV and CpG DNA, we next investigated the levels of TGEV-specific sIgA in the ileum by ELISA. The level of local anti-TGEV specific sIgA increased significantly following immunization with ITGEV and CpG DNA, either together or separately ($^{**}P < 0.01$; Fig. 4D). However, there was no significant difference between the CpG DNA- and PBS-stimulated groups.

3.6. Detection of TGEV-specific sIgA and IgG

TGEV-specific sIgA levels in feces were determined by ELISA at weekly intervals after the first immunization. As shown in Fig. 5A, the TGEV-specific sIgA level increased significantly in the first 3 weeks after immunization with ITGEV and CpG DNA ($^{**}P < 0.01$). In contrast, the level of TGEV-specific sIgA in the ITGEV and CpG DNA stimulated group decreased markedly at weeks 4 and 5. Consistent with the increment in TGEV-specific sIgA, systemic immunity in the ITGEV plus CpG DNA group was also enhanced after oral immunization. As shown in Fig. 5B, the TGEV-specific IgG level in serum significantly increased and peaked at week 2 after immunization with ITGEV plus CpG DNA ($^{**}P < 0.01$). However, with the exception of week 2 ($^{*}P > 0.05$), the efficacy of oral immunization was lower than that of ITGEV hypodermic immunization.

4. Discussion

CpG-ODN has been used widely as an adjuvant for oral immunization (Blaas et al., 2006; McCluskie and Davis, 1999). However, the use of synthetic CpG-ODN is limited, particularly in the animal industry, by its high cost (Kang et al., 2013). Natural CpG DNA exhibited immunostimulatory activities similar to that of synthetic CpG-ODN (McCluskie et al., 2000). Moreover, large-scale production of natural CpG DNA is less expensive (Li et al., 2012). In this study, we evaluated the immune-stimulatory activity of natural CpG DNA using porcine splenic lymphocytes. Consistent with the report by Kamstrup, we found that CpG stimulation induced a significant increase in PBMC proliferation (Kamstrup et al., 2001). Several works have suggested that CpG DNA exerts a number of effects on pigs (Kamstrup et al., 2001; Yang and Parkhouse, 1996; Zhang et al., 2007a). In vivo animal experiments were performed to further evaluate the immune-stimulatory effects of CpG DNA on local immunity in pigs.

CpG DNA directly targets dendritic cells, macrophages, and B cells via TLR9-mediated recognition to promote the secretion of Th1 cytokines (Cope et al., 2011; Heeg and Zimmermann, 2000), which enhances mucosal and systemic immune responses (Liang et al., 2013). Our findings demonstrated that the local Th1 cytokines IL-12 and IFN-γ were induced early after immunization. These results agree with those obtained in other animal models where CpG preferentially induced Th1 responses due to activation of DCs and secretion of IL-12 (Chu et al., 1997; Zimmermann et al., 1998). Moreover, IL-6 levels increased after oral immunization with ITGEV and CpG, either together or separately. IL-6 induces the maturation and activation of B cells, which increases the secretion of antigen-specific IgA (Burdin et al., 1995; Neurath and Finotto, 2011). The increased levels of IL-6, IL-12 and IFN-γ in our study suggested boosting of cellular immunity early after stimulation with ITGEV and CpG DNA, either together or separately.

Intestinal intraepithelial lymphocytes (IELs) are the first line of epithelial defense against invading enteric viruses. IELs have a cytotoxic activity, secrete cytokines, and are involved in immune surveillance and modulation of epithelial cell death and regeneration (Gobel et al., 2001; Qiu et al., 2015). 90% of IELs are CD3+ T lymphocytes. The increased numbers of IELs and CD3+ T lymphocytes in the ITGEV and CpG-immunized group suggest enhanced protection against TGEV. This is consistent with our previous report that CpG DNA markedly increased the number of IELs and CD3+ T lymphocytes in the trachea after intranasal immunization with inactivated H9N2 influenza in chickens (Xiaowen et al., 2009), and with *Mycoplasma hyopneumoniae* in pigs (Li et al., 2012).

Oral delivery of vaccine induces a mucosal IgA response, where TGEV-specific sIgA plays a crucial role in protecting pigs against TGEV infection (Hooper and Haelterman, 1966; Tuboly et al., 2000). The presence of TGEV-specific sIgA in the colostrum and milk of sows provides effective protection to new-born piglets. However, infection can still occur after the cessation of breast feeding (Gu et al., 2012). In contrast, parenteral immunization with inactivated TGEV seldom resulted in the generation of neutralizing antibody in the small intestine (VanCott et al., 1994). Previous studies have reported enhanced local mucosal immunity after immunization with CpG DNA (Zanvit et al., 2005). In this study, oral immunization resulted in a high level of TGEV-specific sIgA in feces, likely due to administration of CpG DNA (Kovacs-Nolan et al., 2009). This result is similar to that obtained with intranasal immunizations with porcine reproductive and respiratory syndrome virus together with a porcine-specific CpG (Zhang et al., 2007b). Interestingly, sIgA levels in feces peaked at week 2 and decreased gradually thereafter. Thus, improvement of oral vaccine dosage forms may be an effective strategy to enhance the sustained release of antigens. We found that sIgA levels in feces decreased over time while TGEV-specific IgA levels in the ileum remained high up to week 6. This indicated that sIgA levels in feces were not correlated with those in other tissues.

5. Conclusions

We demonstrated that oral immunization with ITGEV and CpG DNA induced effective immune responses in the local intestinal tract and circulatory system of pigs. CpG DNA, as an oral adjuvant for ITGEV, markedly enhanced the local mucosal immune response and systemic humoral response. Our data indicate that oral immunization with ITGEV and CpG DNA may be used to prevent TGE in pigs.

Conflicts of interest

The authors of this editorial have no conflicts of interest/funding to declare.

Acknowledgments

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