

RESEARCH ARTICLE

Effects of probiotics, probiotic DNA and the CpG oligodeoxynucleotides on ovalbumin-sensitized Brown-Norway rats via TLR9/NF- κ B pathway

Yan Zhong^{1,2,3}, Juan Huang¹, Wenjing Tang^{2,3}, Bing Chen² & Wei Cai^{1,2,3}

¹Clinical Nutrition Center, Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China; ²Department of Nutrition, School of Medicine, Shanghai Jiao Tong University, Shanghai, China; and ³Shanghai Institute of Pediatric Research, Shanghai, China

Correspondence: Wei Cai, Clinical Nutrition Center, Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University, 1665 Kongjiang Road, Shanghai 200092, China. Tel.: +86 21 25076418; fax: +86 21 65010627; e-mail: caiw204@yahoo.com.cn

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food allergy; immune function; probiotics; toll-like receptor 9/nuclear factor- κ B signal pathway.

Abstract

The aim of the study was to investigate the effect of living probiotics, probiotic DNA and the synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODN) on both immune response and intestinal barrier function in ovalbumin-sensitized rat and the underlying mechanisms. Brown-Norway rats were orally sensitized with ovalbumin, and living probiotics, probiotic DNA extraction, synthetic CpG-ODN or non-CpG ODN control was administered. In the living probiotics, probiotic DNA and CpG-ODN groups, the allergic response was significantly inhibited, the Th1/Th2 cytokine balance was shifted away from Th2 side, the percentage of CD4⁺CD25^{high} Treg cells was increased, and the intestinal barrier function was improved. The levels of toll-like receptor (TLR) 9 mRNA and nuclear factor (NF)- κ B activity, as well as the I κ B- α phosphorylation (p-I κ B- α) was significantly increased in these three intervention groups compared with the OVA-positive group, whereas no such effects were found in the non-CpG ODN control group. These data show that the probiotic genomic DNA and the synthetic CpG-ODN was comparable with living probiotics in preventing food allergic response by immune modulation and intestinal barrier function enhancement, and the activation of TLR9/NF- κ B signal pathway might be involved in this process.

Introduction

The prevalence of food allergy has been increasing worldwide in the last decades. It is suggested to be linked to the reduced microbial exposure during early infancy due to the improved living and sanitary conditions in industrialized countries, called the hygiene hypothesis (Lack, 2008).

Based on the hygiene hypothesis, the immune system of an infant at birth is immature, and moderate microbial exposure is necessary to stimulate the maturation of the immune system. Lack of infections in early childhood is prone to lead to a T-helper 2 (Th2)-dominated immune status, and Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 will up-regulate the production of IgE by B cells and stimulate mast cells and eosinophils, which will promote the allergic symptoms (Bischoff & Ulmer, 2008). On the contrary, the exposure to microbes and their

products might help to shift the Th lymphocyte balance from Th2 domination in newborns to Th1 domination later in life (Ghadimi *et al.*, 2010a, b). Intestinal microflora plays a pivotal role in the induction of immune development in infancy, and differences in the colonic microflora composition between infants who develop allergic diseases and those who do not, have been identified (Bjorksten *et al.*, 2001).

Supplementation of probiotics is considered a beneficial approach for microbial exposure and they may present a promising preventive and therapeutic strategy for the management of allergic diseases (Huang *et al.*, 2010). Administration of probiotics such as lactic acid bacteria has been shown to reduce the severity of food allergy, atopic dermatitis and allergic rhinitis. Although a definitive mechanism of action has not been clearly identified, it is suggested that the anti-allergic activity of probiotics, especially bifidobacteria and lactobacilli, is related to their roles in modulating

the host's immune homeostasis, as well as in maintaining the normal intestinal barrier function (Kim *et al.*, 2008). The effects of *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium longum* BB536 in anti-allergic diseases were previously shown to be promising (Gruber *et al.*, 2007; Odamaki *et al.*, 2007; Sawada *et al.*, 2007). A combination of different probiotics is commonly used in many studies to obtain a strong effect, and therefore a mixture of these two probiotic species was selected for this study.

The active components in probiotics responsible for their anti-allergic activity remain unclear. It was reported that their genomic DNA seemed to contribute about 50% of the immunomodulatory effects of living probiotics (Watson & McKay, 2006; Ghadimi *et al.*, 2008). Unmethylated CpG motif from bacterial DNA is a recognized microbial product with immunostimulatory effects, and the CpG motif derived from probiotic DNA can restore Th1/Th2 bias due to the unique TTTCGTTT motif, skewing the immune response towards Th1 type (Kitazawa *et al.*, 2003; Iliev *et al.*, 2008). The CpG motif is selectively recognized by toll-like receptor (TLR) 9, activating multiple downstream signal pathways including the TLR9/NF- κ B pathway (Kline & Krieg, 2008). NF- κ B is one of the important transcription factors mediating inflammatory response. An *in vitro* study showed that LGG origin DNA attenuated tumor necrosis factor (TNF)- α enhanced NF- κ B activity (Ghadimi *et al.*, 2010a, b), but some other studies showed that probiotics-mediated beneficial effects might require activation of NF- κ B (Pagnini *et al.*, 2010). Until now, the effects of probiotics and their components on the TLR9/NF- κ B pathway, and whether commensal genomic DNA and its contained CpG motif has similar effect as living probiotics in food allergy, is not well studied.

In this study, we extract probiotic DNA from a probiotics mixture and synthesize CpG oligodeoxynucleotide (CpG-ODN) to compare their effects on food allergic responses in ovalbumin (OVA)-sensitized Brown-Norway (BN) rats. Furthermore, we try to determine whether probiotic DNA and synthetic CpG-ODN can function by both modulating immune response and enhancing barrier function, similar to living probiotics, as well as the involvement of the TLR9/NF- κ B pathway.

Materials and methods

Animals

Sixty 5–6-week-old female BN rats were obtained from the Animal Center of the Chinese Academy of Medical Sciences (Beijing, China). The rats were allowed to acclimatize to the laboratory conditions for 7 days under a light-dark cycle of 12 h at constant temperature (22 ± 2 °C) and a relative humidity of 50–70%, consuming an OVA-free rodent diet

and water. The committee of Shanghai Jiao Tong University approved the ethical permission, and all experimental procedures were performed in accordance with the guidelines of the Shanghai Experimental Animal Society, China.

Probiotics mixture and genomic DNA preparation

The probiotics administered were a lyophilized mixture of living LGG (American Type Culture Collection 53103) and *B. longum* BB536 (kindly provided by the Institute of Biology and Pharmacology of Shanghai Jiao Tong University) in a weight ratio of 1 : 1. The mixed probiotics powder was re-suspended in phosphate-buffered saline (PBS; pH 7.2) at a concentration of 1×10^9 colony forming units (CFU) per milliliter before supplementation, and the final concentration for each probiotic was 0.5×10^9 CFU mL⁻¹. The administered dosage was 1.0 mL day⁻¹. Identification of the strains was confirmed by 16S rRNA gene nucleotide sequences.

Genomic DNA from the mixture of LGG and *B. longum* BB536 was purified by repeated phenol extraction followed by chloroform/isoamyl alcohol extraction with a final ethanol precipitation step. Briefly, the probiotics solution was centrifuged at 11000 g for 5 min at 4 °C. The supernatant was discarded and the pellet was washed twice with 10 mL PBS. After further centrifugation, the supernatant was discarded. The pellet was treated with 50 μ L of 100 μ L mL⁻¹ lysis buffer for 1 h at 37 °C. One milliliter of 1 M EDTA was added and incubated for 5 min at room temperature and 400 mL sodium dodecyl sulfate (20% SDS) was then added and the mixture incubated at 65 °C until the solution cleared. The mixture was extracted with equal volumes of phenol and phenol-chloroform-isoamyl alcohol, and this procedure was repeated until no interface was visible. Traces of phenol were removed with chloroform. DNA was precipitated by adding two volumes of cold (-20 °C) 96% ethanol, washed with 70% ethanol, and dissolved in 100 mL sterile double-distilled water. The purity of DNA was confirmed by measuring UV absorbance at 260/280 nm using GeneQuant I (Pharmacia, LKB Biochrom Ltd., Cambridge, UK), and the quality of DNA was further analyzed by conventional gel electrophoresis. All DNA preparations were assayed for endotoxin content using the chromogenic limulus analysis kit (Shanghai Yinhua Medical Technical Ltd Company, China); endotoxin content was less than 0.2 U μ g⁻¹ of DNA.

CpG-ODN and non-CpG ODN control synthesis

The CpG-ODN and non-CpG ODN control was chemically synthesized by Shanghai Sangon Biological

Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences were as follows: CpG-ODN, 5'-TCC ATGACGTTCCCTGACGTT-3', and non-CpG ODN control, 5'-TCCAGGACTTCTCTCAGGTT-3'. The compounds were diluted with PBS to a concentration of $250 \mu\text{g mL}^{-1}$ and stored at 4°C . They were confirmed to be free of detectable endotoxin by using the chromogenic limulus analysis kit according to the manufacturer's manual.

Animal treatment and intervention regimen

The animals were randomly divided into negative control, positive control, living probiotics, probiotic DNA, CpG-ODN, and non-CpG ODN control. The animals in the negative control group were orally gavaged with 1 mL PBS daily for 6 weeks. The animals in the positive control group and the intervention groups were given 1 mg OVA (1 mg mL^{-1} *per os*) (purity > 95%; Sigma-Aldrich, St. Louis, MO) daily for 6 weeks and the animals were deprived of food for 2 h before the oral sensitization. In the living probiotics group, daily oral administration of living probiotics was started 2 weeks prior to the first sensitization until 1 week after the end of sensitization period. OVA and probiotics were gavaged separately, with

an interval of 8 h. Fifty milligram of probiotic DNA, CpG-ODN and non-CpG ODN was injected by intraperitoneal injection on days 0, 3, 7, 14, 28, 42 and 56. Before sacrifice, the rats were challenged orally with 100 mg OVA, and blood and tissue samples were collected for further analysis 1.5 h later (Fig. 1).

Measurement of serum OVA-specific IgE

Serum OVA-specific IgE level was analyzed by means of ELISA. Plates were coated with 0.1 M NaHCO_3 containing $100 \mu\text{g mL}^{-1}$ OVA for 6 h at 37°C . Plates were blocked for 2 h at 37°C with $200 \mu\text{L}$ 3% BSA in PBS, then washed, and $100 \mu\text{L}$ diluted serum was applied and kept overnight at 4°C . The amount of bound antibody was analyzed using biotinylated anti-mouse IgE and avidin-horseradish peroxidase conjugate (BD Pharmingen, San Diego, CA). Plates were read in a microplate auto-reader (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 450 nm. Serum titers for OVA-specific IgE were expressed as relative ELISA units (IU mL^{-1}), referring to a linear standard curve from the laboratory standard sera pool (Shanghai Di Ao Bio-technology Ltd Company, Shanghai, China).

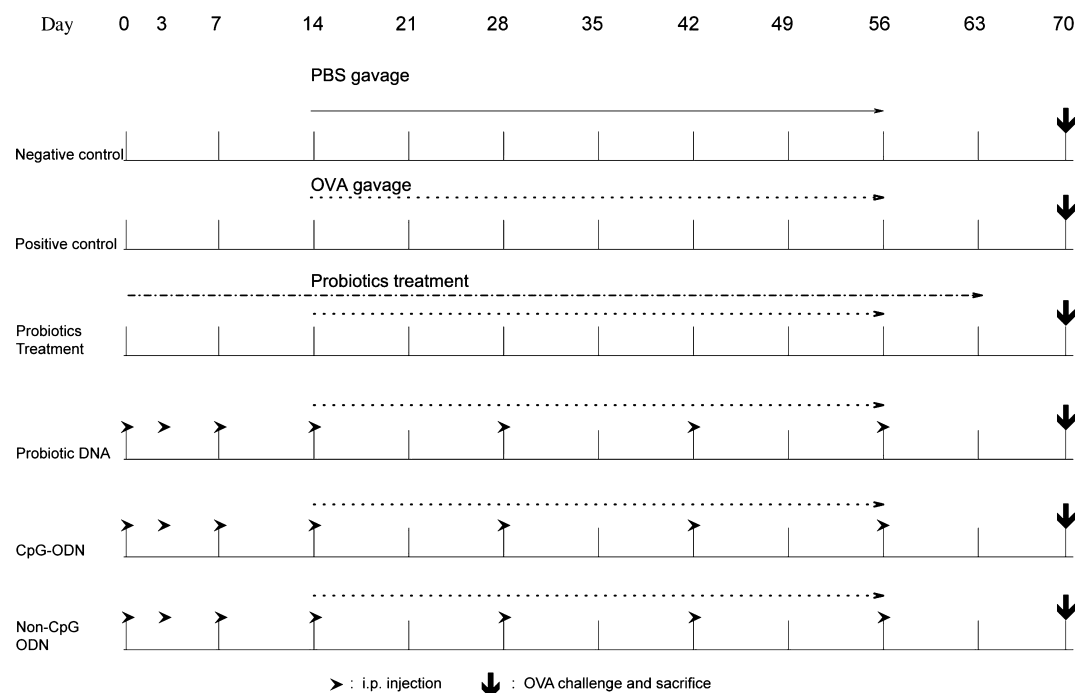


Fig. 1. Experimental protocol shows experimental design and intervention regimen in each group. Negative control: PBS gavage for 6 weeks from day 14; positive control: 1 mg OVA gavage daily for 6 weeks from day 14; living probiotics: oral administration of living probiotics for 9 weeks, from 2 weeks prior to the first sensitization until 1 week after the end of sensitization period; probiotic DNA, CpG-ODN and non-CpG ODN: intraperitoneal injection (i.p.) on days 0, 3, 7, 14, 28, 42, 56.

Measurement of cytokines in mesenteric lymph nodes and splenic cells culture after *in vitro* stimulation

The spleen and the mesenteric lymph nodes (MLNs) were collected, and single cell suspension was prepared and re-suspended in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 2 mM glutamine, 50 μ M 2-mercaptoethanol, 50 IU mL⁻¹ penicillin and streptomycin (complete media). Cells were incubated in 24-well plates (1×10^6 cells mL⁻¹) stimulated with 100 μ g mL⁻¹ OVA at 37 °C in 5% CO₂ atmosphere. The supernatants were collected 48 h later, and the concentrations of interferon (IFN)- γ , IL-4 and IL-10 in the supernatants were determined by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). All procedures were performed using sterile technique, and the measurements were performed in duplicate. The Th1/Th2 balance was assessed using the ratio of IFN- γ /IL-4 according to the description of Shahid *et al.* (2002).

Quantification of CD4⁺CD25^{high} T cells by flow cytometry

Fresh single cell suspension from spleen and MLNs (1×10^6) was stained with fluorescein isothiocyanate (FITC)-labeled anti-rat CD4 monoclonal antibody (MoAb) and phycoerythrin (PE)-conjugated CD25 MoAb (BD Pharmingen) or the corresponding isotype control antibodies for 30 min at 4 °C. Immunostained cells were analyzed with FACSCaliburTM (Becton Dickinson, Mountain View, CA) by acquiring 10 000 events and then the data were analyzed with BD FACSDIVA software. After gating the lymphocyte population, the CD4⁺CD25^{high} population were sequentially gated and analyzed.

Measurement of intestinal mucosal permeability

Intestinal mucosal permeability was assessed by oral administration of FITC-conjugated dextran 4000 (FITC-dextran; Sigma-Aldrich). All animals were gavaged orally with FITC-dextran (20 mg 100 g⁻¹ body weight) 30 min before blood samples were collected. Blood samples were obtained by cardiac puncture and were allowed to clot at 4 °C for 30 min and centrifuged (700 g) for 10 min. Serum FITC-dextran concentration was determined by fluorescence spectrophotometry (HITACHI model F-4010, Tokyo, Japan) at an excitation wavelength of 495 nm and at an emission wavelength of 512 nm using a standard curve performed in duplicate.

Determination of colonic microflora

Fecal samples were collected directly from the colon when the animals were sacrificed and were stored at -80 °C until analysis. Total bacterial DNA was extracted from each fecal sample by a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and was then used as a template for quantitative real-time PCR. The genus-specific 16S rRNA gene-targeted primer sequences used for bifidobacteria were as follows: the forward primer lm26-f (5'-GATTCTGGCTCAGGATGAACG-3') and the reverse primer lm3-f (5'-CGGGTGCTICCCACTTT CATG-3'). The primers for lactobacilli were as follows: the forward primer L159-f (5'-GGAAACAGATGCTAA TACCG-3') and the reverse primer L677-r (5'-CAC CGCTACACATGGAG-3'). Quantitative real-time PCR was performed with the Mx3000P QPCR System (Stratagene, La Jolla, CA). The copy number of 16S rRNA genes of the target bacteria in the template DNA solution was estimated using standard curves made from the known concentrations of the corresponding DNA fragment. The standard curves were prepared in the same PCR assay as for samples. The real-time PCRs were performed in triplicate and average values were used for enumeration.

Detection of secretory IgA (sIgA) in intestinal content

Small intestinal tissues from the ligament of Treitz to the ileocecal valve were excised. The luminal contents were gathered by flushing with 10 mL of ice-cold PBS (pH 7.4) and kept at -80 °C until analysis. The luminal contents were diluted and centrifuged at 10 000 g for 20 min to obtain a clear supernatant, which was used for sIgA measurement. The level of sIgA was quantified with a specific rat-IgA ELISA kit according to the manufacturer's instructions (Bethyl Laboratories Inc., Montgomery, TX).

Inflammatory cell infiltration in small intestine

Small intestinal tissue specimens were fixed in 10% formalin, embedded in paraffin wax on the oriented edge, and cut into 5-mm-thick sections for haematoxylin and eosin staining. Multiple 4-mm sections were stained with o-toluidine blue for mast cell counting. Eosinophil cells were counted in a blinded fashion at 15–20 high-power fields (HPF) at $\times 40$ and expressed as cells per HPF. Mast cell counting was done at $\times 100$ magnification. The slides were analyzed with a Leitz Dialux 20 EB microscope (Wetzlar, Germany).

Detection of TLR9 mRNA expression by real-time PCR

Total RNA was isolated from intestinal tissue using Trizol reagent (Promega, Madison, WI). The RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity was determined by the 260/280 nm absorbance. Single-strand cDNA was synthesized from RNA using TaKaRa RNA PCR kit (AMV, TaKaRa, Dalian, China). The primer sequences (Sangon, Shanghai) used for real-time PCR were as follows: TLR9, 5'-TGGGTGTATAACGAGCTTCGAGTG-3' and 3'-GCCATAGATGGAGGCC CAGA-5'; β -actin, 5'-TGACAGGATGCAGAAGGAGA-3' and 3'-TAGAGCCACCAATCCACACA-5'. The PCR used 2 μ L of first-strand cDNA, 10 μ L of 2 $\times$ SYBR Premix Ex Taq, 0.4 μ L of each specific primer, in a total volume of 20 μ L. The cycling conditions were 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 25 s and 72 °C for 10 s. The TLR9 mRNA level was normalized to the level of β -actin and was represented as fold induction.

Western blot analysis for TLR9, IL-1 receptor-associated kinase (IRAK) 4 and phosphorylation of I κ B- α (p-I κ B- α)

The frozen small intestinal tissue samples were homogenized and lysed for 30 min in lysis buffer (1% Triton X-100, 20 mM Tris, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na₃VO₄, 10 μ g mL⁻¹ leupeptin, 2 μ g mL⁻¹ aprotinin). Samples were then centrifuged at 10 000 g for 15 min, after which the supernatant was collected and the protein concentration determined with the BCA reagent kit (Pierce, Rockford, IL). The proteins were denatured at 95 °C for 10 min in the loading buffer (60 mM Tris, 2.5% SDS, 10% glycerol, 5% mercaptoethanol, 0.1% bromophenol blue). Proteins from each sample were separated in SDS-PAGE gels, followed by transfer of proteins to a polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, UK). After transfer, the membranes were blocked in Tris-buffered saline (TBS) with 5% bovine serum albumin for 60 min at room temperature followed by repeated washing. The membranes were then incubated overnight at 4 °C with antibodies against TLR9, IRAK4, p-I κ B- α (Cell Signaling Technology, Beverly, MA) and β -actin (Shanghai Kangchen Bio-tech Company, Shanghai, China), respectively. After three washes with TBST buffer, membranes were incubated for 1 h with secondary HRP-linked antibody (Shanghai Kangchen Bio-tech Company) at room temperature. And finally, the protein expression was revealed with the enhanced chemiluminescence detection reagent kit (Shanghai Kangchen Bio-tech Company).

Nuclear protein extract and electrophoretic mobility shift assay (EMSA)

Nuclear protein was extracted with NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce, Rockford, IL). Complementary NF- κ B oligonucleotides were 5'-AGTTGAGGGGACTTTCC CAGGC-3' and 3'-GCCTGGGAAAGTCCCCTCAACT-5'. The NF- κ B binding activity was evaluated with a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Oligonucleotides were annealed by mixing equal amounts of the labeled complementary oligonucleotides together and incubating for 1 h at room temperature. Nuclear extracts were incubated with the biotin-labeled DNA probe for 20 min at room temperature, and then the DNA-protein complex was separated from free oligonucleotides on a 5% polyacrylamide gel, transferred to a nylon membrane and cross-linked by UV. The membrane was incubated with streptavidin-horseradish peroxidase conjugate and detected by Enhanced Chemiluminescence Detection kit (Pierce).

Statistical analysis

The results were expressed as mean $\pm$ SD. The bacterial results in fecal samples were expressed in log₁₀ copy numbers per g wet weight. One-way ANOVA test was performed to determine the statistical significance when the data were normally distributed. Nonparametric Kruskal–Wallis test was used among groups when the data did not follow a normal distribution, and a significant difference among groups was found. The differences between the two groups were then determined using the Mann–Whitney *U*-test. A *P*-value < 0.05 was considered to be significant. SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL) was used for data analysis.

Results

Serum OVA-specific IgE antibody level

To investigate the effects of probiotics and their structure components on allergic response, serum OVA-specific IgE was analyzed. After OVA challenge, the OVA-specific IgE concentration in the positive control and non-CpG ODN groups was significantly increased compared with that in the negative control group. The administration of living probiotics, probiotic DNA and CpG-ODN significantly decreased OVA-specific IgE level compared with the positive control or non-CpG ODN group (Fig. 2). These results indicated that the intervention with living

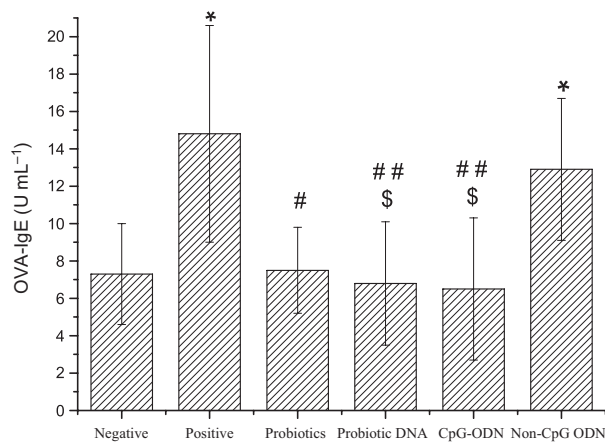


Fig. 2. Effect of living probiotics, probiotic DNA and CpG-ODN administration on serum OVA-specific IgE level. Data represent mean $\pm$ SD of 10 samples in each group, and each sample was measured in duplicate. * $P < 0.05$ vs. negative control. # $P < 0.05$ and ## $P < 0.01$ vs. positive control. \$ $P < 0.05$ vs. non-CpG ODN group.

probiotics, probiotic DNA and CpG-ODN can prevent allergic response in this animal model.

Production of cytokines by splenic and MLNs cells

After *in vitro* culture of splenic and MLNs cells stimulated with OVA for 48 h, the culture supernatant was collected for cytokine analysis. An increased production of IFN- γ , IL-4 and IL-10 was observed in the positive and non-CpG ODN control group compared with the negative group. The administration of living probiotics, probiotic DNA and CpG-ODN inhibited their production compared with the positive control or non-CpG ODN control groups (Fig. 3A–C).

To further investigate the effects of these interventions on the balance of Th1/Th2 cytokines, we analyzed the ratio of IFN- γ /IL-4. A significantly decreased ratio of both splenic and MLNs cells was observed in the positive control and non-CpG ODN groups compared to the negative control group. After interventions with living probiotics, probiotic DNA and CpG-ODN, the ratios were significantly higher than the corresponding values in the positive group or non-CpG ODN group, which suggests that the Th1/Th2 balance was shifted towards the Th1 side (Fig. 3D).

CD4⁺CD25⁺ Treg cells profile

CD4⁺CD25⁺ T cells, particularly CD4⁺CD25⁺ Foxp3⁺ T cells as a main group of Treg cells, are crucial in the initiation and regulation of allergic response. It was iden-

tified that CD4⁺CD25⁺ population cells are generally positive for Foxp3. A significantly decreased percentage of CD4⁺CD25⁺ T cells in spleen and MLNs was observed in the positive control and non-CpG ODN group compared with the negative control group. The administration of probiotic DNA and CpG-ODN significantly increased the percentage of CD4⁺CD25⁺ T cells compared with the positive control and non-CpG ODN control groups. An increasing trend was observed in the living probiotics group compared with the positive control group (Fig. 4).

Colonic microflora

The logarithm numbers of bifidobacteria and lactobacilli in fecal samples were used for comparison. The number of bifidobacteria and lactobacilli genera in the positive group decreased significantly compared with the negative control group. The administration of living probiotics, probiotic DNA and CpG-ODN increased the number of the two bacterial genera compared with the positive or non-CpG ODN control groups, but a significant difference was only detected between the living probiotics and positive control group (Table 1).

Measurement of intestinal mucosal permeability

FITC-dextran 4000 is used to detect the intestinal paracellular permeability, and the higher level of serum FITC-dextran indicates the higher intestinal permeability. The serum FITC-dextran 4000 level was significantly increased in the OVA positive and non-CpG ODN control groups compared with the negative control group. In the living probiotics, probiotic DNA and CpG-ODN groups, serum FITC-dextran 4000 concentration was significantly lower than the positive or non-CpG ODN control group (Table 2).

sIgA concentration in intestinal content

sIgA is the most plentiful immunoglobulin on mucosal surfaces, where it neutralizes harmless food antigens and prevents them from penetrating the epithelium. The difference for the concentration of sIgA among the positive, negative, and non-CpG ODN control groups was not significant. With the administration of living probiotics, probiotic DNA and CpG-ODN, the sIgA levels increased significantly compared with the levels in the positive and negative control group, and the level in the CpG-ODN group was significantly higher than that in the non-CpG ODN control group (Table 2).

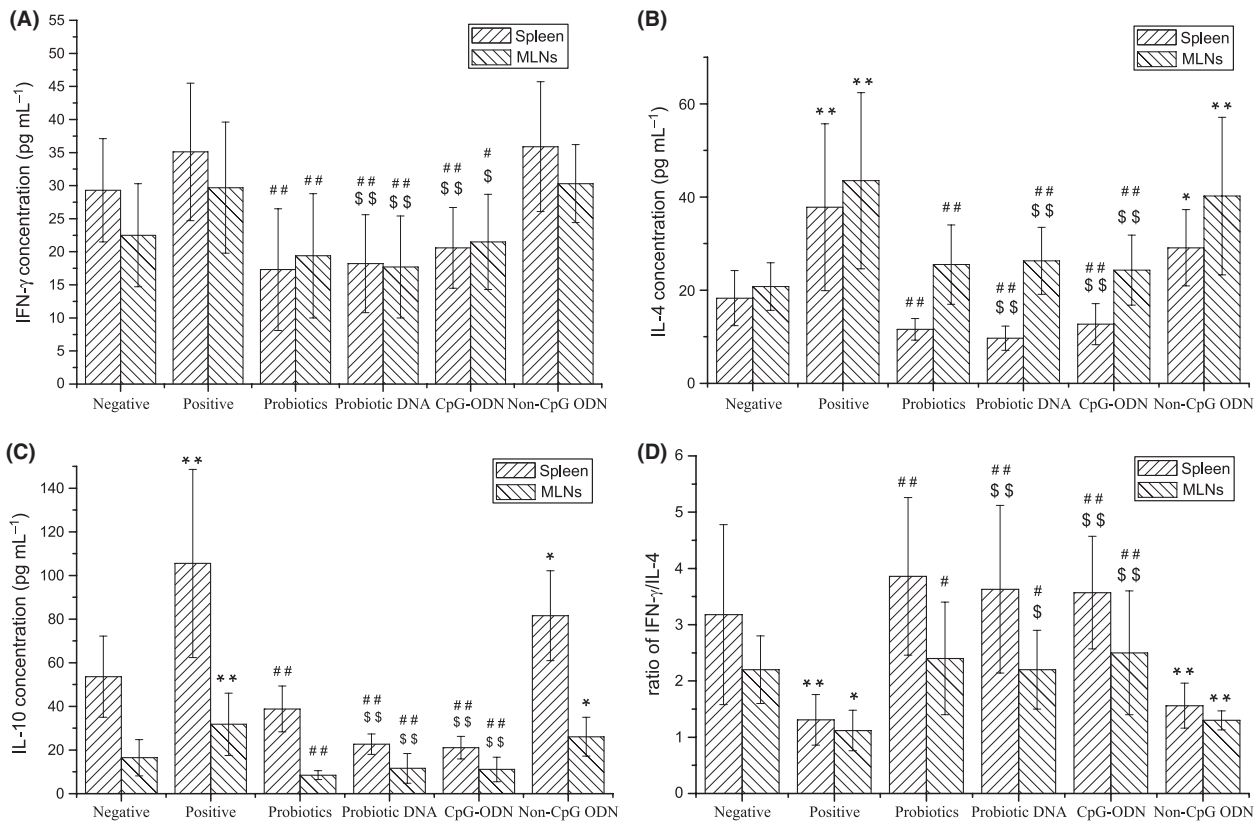


Fig. 3. Effect of living probiotics, probiotic DNA and CpG-ODN administration on the level of cytokines in the culture supernatant of splenic and MLNs cells after re-stimulated with OVA for 48 h (100 μ g mL⁻¹) ($n = 10$ in each group), (A) IFN- γ , (B) IL-4, (C) IL-10, and (D) ratio of IFN- γ /IL-4, which represents the ratio of Th1/Th2. Data present mean $\pm$ SD of 10 samples in each group, and each sample was measured in duplicate. * $P < 0.05$ and ** $P < 0.01$ vs. negative control group. # $P < 0.05$ and ## $P < 0.01$ vs. positive control group. \$ $P < 0.05$ and \$\$ $P < 0.01$ vs. non-CpG ODN group.

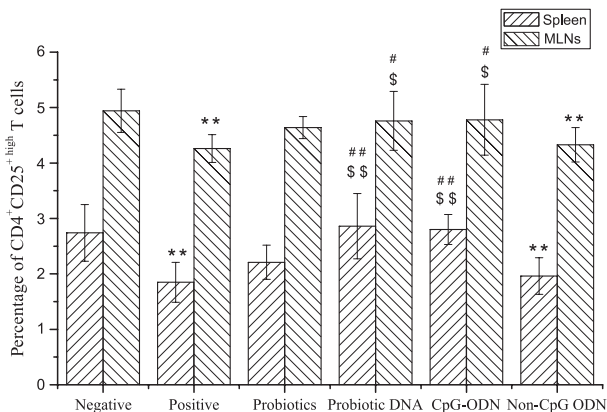


Fig. 4. CD4⁺CD25⁺high T cells in splenic and MLNs cells ($n = 10$ in each group). Single cell suspension from spleen and MLNs was stained with FITC-labeled anti-rat CD4 and PE-labeled anti-rat CD25 monoclonal antibodies and detected by flow cytometry. Data represent mean $\pm$ SD of 10 samples in each group. ** $P < 0.01$ vs. negative control group. # $P < 0.05$ and ## $P < 0.01$ vs. positive control group. \$ $P < 0.05$ and \$\$ $P < 0.01$ vs. non-CpG ODN group.

Inflammatory cells infiltration in small intestine

More eosinophil and mast cell infiltration in small intestinal mucosa was found in the positive and non-CpG ODN control groups than that in the negative control group. In the living probiotics, probiotic DNA and CpG-ODN groups, the eosinophilic and mast cells infiltration was significantly reduced compared with the positive control or non-CpG ODN control group (Table 2).

Expression of TLR9 in small intestine

TLR9 selectively recognizes and responds to bacterial DNA containing unmethylated CpG motif, leading to the activation of a series of down-stream transcription factors. Both TLR9 mRNA and protein were faintly expressed in the positive and non-CpG ODN control group, similar to the level in the negative control group. TLR9 mRNA expression in the living probiotics, probiotic

Table 1. Comparison of the number of bifidobacteria and lactobacillus genera in the colon of different groups

Groups	Negative	Positive	Probiotics	Probiotic DNA	CpG-ODN	Non-CpG ODN
Bifidobacteria	10.7 ± 0.8	9.7 ± 1.1*	11.1 ± 1.2 [†]	10.3 ± 1.1	10.4 ± 1.1	9.9 ± 0.9
Lactobacilli	11.5 ± 0.9	10.5 ± 0.9*	11.6 ± 0.4 [†]	11.3 ± 0.6	11.1 ± 0.8	10.7 ± 0.7

Copy numbers of 16S rRNA genes of bifidobacteria and lactobacilli were transformed into log₁₀ copy numbers per g wet wt, and values are shown as means ± SD, *n* = 10.

**P* < 0.05 vs. negative control group.

[†]*P* < 0.01 vs. positive control group.

Table 2. Comparison of intestinal permeability and inflammatory infiltration in small intestine (mean ± SD)

Groups	Negative	Positive	Probiotics	Probiotic DNA	CpG-ODN	Non-CpG ODN
Serum FD 4000 (μg mL ⁻¹)	3.5 ± 0.7	6.7 ± 2.4*	2.5 ± 0.2 [†]	3.6 ± 1.3 ^{†‡}	3.8 ± 1.2 ^{†‡}	6.4 ± 2.0*
slgA (ng mL ⁻¹)	144.0 ± 34.5	125.1 ± 22.0	226.4 ± 52.1* [†]	220.6 ± 39.8* [†]	233.9 ± 69.6* ^{†‡}	118.9 ± 23.5
Eosinophil (counts per HPF)	30.73 ± 10.3	75.2 ± 13.7*	33.8 ± 9.1 [†]	33.2 ± 10.4 ^{†‡}	35.0 ± 11.9 ^{†‡}	64.6 ± 13.2*
Mast cell (counts per HPF)	14.8 ± 4.5	30.2 ± 9.6*	18.6 ± 8.8 [†]	20.2 ± 5.9 ^{†‡}	16.1 ± 3.6 ^{†‡}	25.9 ± 9.5*

**P* < 0.01 vs. negative control group.

[†]*P* < 0.01 vs. positive control group.

[‡]*P* < 0.01 vs. non-CpG ODN group.

DNA and CpG-ODN groups was significantly increased compared with the positive control or non-CpG ODN group (Fig. 5A). As shown in Fig. 5B, living probiotics, probiotic DNA and CpG-ODN increased TLR9 protein expression (Fig. 5B).

NF-κB activation, IRAK4 and IκB-α phosphorylation in small intestine

NF-κB is one of the important transcription factors mediating inflammatory response. In the negative, positive and non-CpG ODN control groups, the NF-κB activity was comparatively low, which suggested that NF-κB was not activated. Living probiotics, probiotic DNA and CpG-ODN significantly activated NF-κB (EMSA assay) compared with the positive or non-CpG ODN control groups (Fig. 6A and B).

Due to the activation of NF-κB being preceded by the proteolytic degradation/phosphorylation of IκB-α, phosphorylation of IκB-α (p-IκB-α) was analyzed by western blot. The result showed that p-IκB-α was low in the negative, positive and non-CpG ODN control groups. The intervention with living probiotics, probiotic DNA and CpG-ODN significantly increased the expression of IκB-α phosphorylation compared with the positive or non-CpG ODN control group. No significant differences were detected for the protein level of IRAK4 in any of the groups (Fig. 7).

Discussion

Recent studies have suggested that probiotics such as LGG and *Bifidobacteria logum* are good candidates for

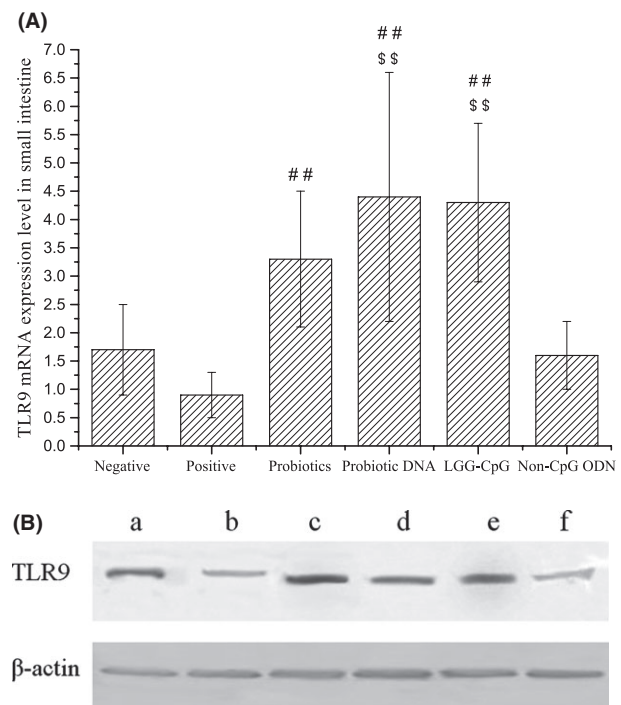


Fig. 5. TLR9 expression in small intestinal tissue. (A) TLR9 mRNA level was analyzed by real-time PCR, and the data present mean ± SD of 10 samples in each group, and each sample was measured in duplicate. (B) TLR9 protein expression was analyzed by western blot, and the results are representative of six independent experiments with similar results. a, negative control group; b, positive control group; c, living probiotics group; d, probiotic DNA group; e, CpG-ODN group; f, non-CpG ODN group. ##*P* < 0.01 vs. positive control group. \$\$*P* < 0.01 vs. non-CpG ODN group.

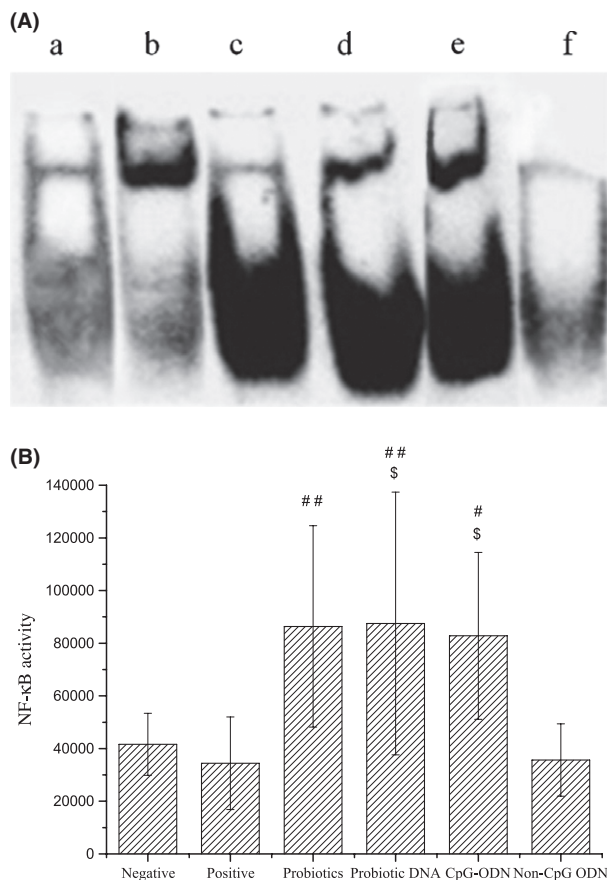


Fig. 6. NF- κ B activity in small intestinal tissues. (A) DNA binding ability was analyzed by EMSA. The results are representative of six independent experiments with similar results. a, negative control group; b, positive control group; c, living probiotics group; d, probiotic DNA group; e, CpG-ODN group; f, non-CpG ODN group. (B) Quantitative analysis of NF- κ B DNA-binding activity, and the results are mean $\pm$ SD of six independent samples in each group. $**P < 0.01$ vs. negative control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. positive control group. $^{\$}P < 0.05$ vs. non-CpG ODN group.

anti-allergy therapy, and their immunostimulatory CpG motif which is contained in DNA sequences may be a crucial factor in the beneficial effects of probiotics (Broide *et al.*, 1998). The main results of the present study demonstrate that, similar to living probiotics, probiotic DNA and synthesized CpG-ODN can also modulate immune responses and enhance intestinal barrier function in an OVA-sensitized animal model, consequently ameliorating allergic response, and the activation of TLR9/NF- κ B signal pathway by CpG motif was demonstrated to be involved in the effects.

To assess the *in vivo* effects of administration of living probiotics, probiotic DNA and synthesized CpG-ODN, we used the OVA-sensitized murine model previously developed in our laboratory (Huang *et al.*, 2009). A decreased

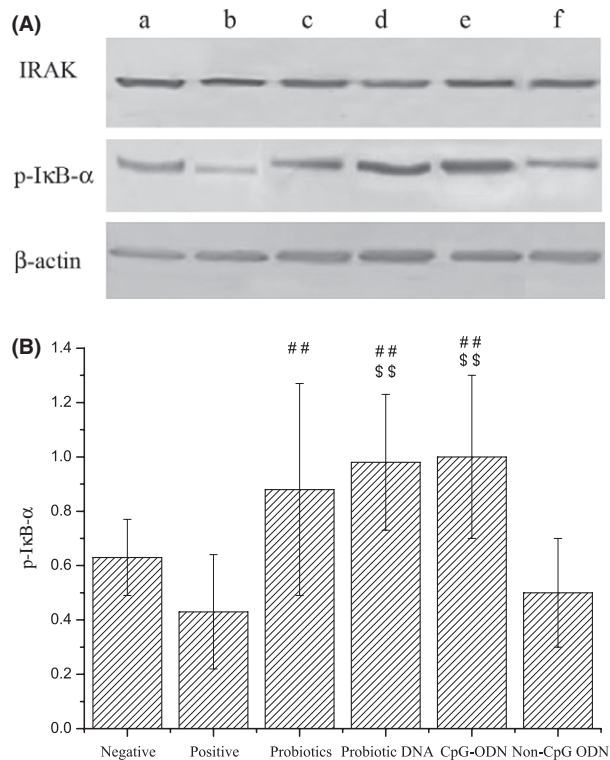


Fig. 7. IRAK expression and I κ B- α phosphorylation (p-I κ B- α) in small intestinal tissue. (A) IRAK and p-I κ B- α protein expression was determined by western blot. β -Actin was used as loading control. a, negative control group; b, positive control group; c, living probiotics group; d, probiotic DNA group; e, CpG-ODN group; f, non-CpG ODN group. (B) Quantitative analysis of p-I κ B- α expression. The results are mean $\pm$ SD of six independent samples in each group. $^{\#\#}P < 0.01$ vs. positive control group. $^{\$\$}P < 0.01$ vs. non-CpG ODN group.

level of OVA-specific IgE was obtained in the living probiotics, probiotic DNA and CpG-ODN intervention groups, but no such effect was detected in the non-CpG ODN group, which suggests that the anti-allergic effects of probiotic DNA and CpG-ODN are not related to the intraperitoneal administration route itself.

Activation of the Th2-dominated immune response is proposed to play an important role in the development of IgE-mediated allergic diseases (Ghadimi *et al.*, 2008). In our study, general suppression effects caused by living probiotics, probiotic DNA and CpG-ODN on IFN- γ , IL-4 and IL-10 were observed; in addition, an improvement of Th1/Th2 balance away from the Th2-like response was found in these intervention groups. It has been suggested that inhibition of Th2 cytokine might be more important than induction of IFN- γ in the restoration of Th1/Th2 balance by probiotics (Perrier *et al.*, 2010). CpG-ODN is known to be an effective Th1 adjuvant which triggers Th1 and B cells to produce the Th1-polarizing cytokine IL-12 and increase IFN- γ secretion (Klinman *et al.*, 1996). It

has been suggested that CpG-ODN may have the potential to treat an ongoing allergic asthma by suppressing the Th2 response during IgE-dependent allergic airway reaction (Sur *et al.*, 1999). Although a number of studies show that probiotics and ODN sequences can induce a strong IFN- γ response in food allergic animals (Takahashi *et al.*, 2006a, b), other studies suggest that the effect on IFN- γ is bacteria-dependent (Pohjavuori *et al.*, 2004). A significant decline in IFN- γ production was observed in allergic rhinitis volunteers given probiotics treatment (Ivory *et al.*, 2008). These controversial results might be related to the different animal disease model induced and the administration pattern of the probiotics. The effect of probiotics on allergic diseases is more complex than modulating the Th1/Th2 balance. Probiotic strains show different capacities to induce regulatory T cells, which are thought to be the master regulators of the immune response (de Roock *et al.*, 2010). The administration of living probiotics, probiotic DNA and CpG-ODN up-regulated the percentage of CD4⁺CD25^{high} cells, which are likely to be regular T cells in our study, but no increased IL-10 level was found in those intervention groups, which is contrary to Sawada *et al.*' (2007) results, suggesting that IL-10 might not be indispensable for induction of Treg cells in this animal model. It was also suggested that the immunomodulatory function of probiotics in atopic dermatitis might be achieved by suppressing inflammatory (IL-17, IFN- γ , and TNF- α) and anti-inflammatory (IL-10) cytokines in both T cells and B cells instead of increasing the immunosuppressive cytokines such as IL-10 or transforming growth factor (TGF)- β (Kwon *et al.*, 2010).

The intestinal barrier function of allergic patients is closely correlated with the severity of their symptoms (Ventura *et al.*, 2006). The effect on improving intestinal barrier function of living probiotics has been well demonstrated (Anderson *et al.*, 2010; Huang *et al.*, 2010). In this study, we found that not only living probiotics, but also probiotic DNA and CpG-ODN administration can improve intestinal barrier function. An *in vitro* study demonstrates that natural commensal-origin DNA can enhance the tight junction integrity of intestinal epithelial cells via TLR9 signaling mediation (Ghadimi *et al.*, 2010a, b). The composition of colonic microflora is regarded as the key determinant for intestinal barrier function, and it is also the main target for probiotic intervention (Stoidis *et al.*, 2010). The intestinal flora can stimulate the mucosal immunity and boost the production of sIgA and tight junction proteins (Patel *et al.*, 2012), and once the intestinal immune environment is regulated, it might influence in turn the microflora composition (Kong *et al.*, 2011), which might explain the increase in the number of bifidobacteria and lactobacillus

detected in probiotic DNA and CpG-ODN administration groups when no living bacteria were given.

Different types of CpG-ODN can significantly affect both the receptor recognition and down-stream cytokine induction in post-TLR9 signaling (Dalpke *et al.*, 2006), and the stimulatory capacity of CpG-ODN is lost in TLR9^{-/-} animals (He *et al.*, 2010). The synthetic CpG-ODN we used in this study has been shown to suppress antigen-specific IgE production *in vivo* and exert its immunostimulatory effect through TLR9, presenting similar activity to LGG-derived CpG motif (Iliev *et al.*, 2008). We further explored the molecular evidence of the involvement of the TLR9/NF- κ B signal pathway in mediating the immunomodulatory effect of probiotics and their structure components on our animal models. TLR9 expression was found to be down-regulated in allergic-related diseases such as allergic conjunctiva (Bonini *et al.*, 2005). In this study, decreased TLR9 mRNA and protein expression was observed in the OVA-positive controls. It is not clear whether oral administration of living probiotics can also directly act on TLR9 in small intestine. Our results confirm that administration of living probiotics can stimulate the expression of TLR9, which suggests that TLR9 may mediate in part the anti-allergic effects of living probiotics. Our study shows that administration of living probiotics, as well as probiotic DNA and synthetic CpG-ODN, can significantly activate NF- κ B, one of the down-stream nuclear factors of TLR9, consequently up-regulating proinflammatory cytokine response. It was shown in a recent study that the proinflammatory response at a certain level induced by some probiotic strains will not result in an inflammatory outcome in the allergic individuals but will induce a higher state of the host innate defence system alertness (Cammarota *et al.*, 2009). The increased phosphorylation of I κ B- α , an important protein involved in the TLR9/NF- κ B pathway observed in the living probiotics, probiotic DNA and CpG-ODN administration groups, supports the involvement of NF- κ B activation in the effects of our interventions. IRAK4 is another key mediator in the TLR signaling pathway, and TLR9 stimulation induced a decrease in IRAK-4 protein level (Hatao *et al.*, 2004). However, in this study we did not find a significant difference in the IRAK4 protein level after interventions. To further understand the role of IRAK4 in TLR9/NF- κ B pathway, we might need to use the inhibitor to decrease IRAK4 and analyze the cleavage product of IRAK4 in a future study, as the cleavage product is suggested to be involved in NF- κ B activation.

In conclusion, administration of living probiotics, probiotic-derived DNA or synthetic CpG-ODN show comparable benefits in enhancing intestinal barrier function and modulating immune response in OVA-sensitized animals,

which might contribute to the reduced severity of allergic response. The activation of the TLR9/NF- κ B signal pathway by the CpG motif might in part mediate these effects.

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Authors' contribution

Y.Z. contributed to the experimental instruction, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; W.C. contributed to conception and design; J.H., W.T. and B.C. contributed to performing experiments and analyzing the samples. All authors read and approved the final manuscript for publication.

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