

ORIGINAL ARTICLE

Clinical Allergy

Oral administration of recombinant *Bacillus subtilis* spores expressing *Helicobacter pylori* neutrophil-activating protein suppresses peanut allergy via up-regulation of Tregs

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Funding information

Guangzhou Institute of Pediatrics / Guangzhou Women and Children's Medical Center, Grant/Award Number: IP-2019-022 and Pre-NSFC-2019-014; Science and Technology Foundation of Guangzhou, Grant/Award Number: 201707010010; Guangdong Science and Technology Foundation, Grant/Award Number: 2016A020215013

Abstract

Background: *Helicobacter pylori* neutrophil-activating protein (NAP) is an immune modulator with anti-Th2 inflammation activity that can be used to prevent IgE-mediated allergic reactions. Cholera toxin B (CTB) is a mucosal adjuvant that can induce antigen tolerance. *Bacillus subtilis* spores are an ideal vehicle for the oral delivery of heterologous antigens.

Objective: We investigated the therapeutic effect of recombinant NAP *B subtilis* spores on peanut allergies in a mouse model.

Methods: Female C3H/HeJ mice were sensitized and challenged with peanut extract by oral administration. Before challenge, recombinant NAP and CTB-NAP (CNAP) spores were orally administered to sensitized mice for 4 weeks. Faecal peanut-specific IgA and serum-specific IgE, IgG1, and IgG2a levels were measured, and the intestinal microbiota was analysed. Mice were intraperitoneally injected with anti-CD25 antibodies for regulatory T cell (Treg) depletion to evaluate the efficacy of Tregs in preventing peanut allergy. After challenge, anaphylactic reactions, plasma histamine, Tregs, and splenocyte interleukin (IL)-10, IL-4, IL-5 and interferon- γ (IFN- γ) levels were evaluated.

Results: After 4 weeks of recombinant spore treatment, faecal IgA levels and serum IgG2a levels were increased, while serum IgG1 and IgE levels were reduced. Intestinal microbiota analysis revealed that CNAP spores increased the taxonomic abundance of *Firmicutes* at the phylum level and *Clostridia* at the class level. After challenge, the administration of NAP or CNAP spores to mice was found to ameliorate anaphylactic reactions and decrease plasma histamine levels. Administration of NAP or CNAP spores also enhanced IL-10 and IFN- γ secretion, and suppressed IL-4 and IL-5 secretion. The protective effect of CNAP spores was more pronounced than that of NAP spores; this therapeutic effect was lost after Treg depletion.

Conclusions and Clinical Relevance: Recombinant NAP spores successfully suppressed Th2 inflammation via the up-regulation of Tregs; this may serve as a novel therapeutic approach for treating food allergies.

KEYWORDS

Bacillus subtilis spores, intestinal microbiota, neutrophil-activating protein, peanut allergy, Treg

1 | INTRODUCTION

Peanut allergy is a life-threatening food allergy and occurs in 0.5%-1% of children under 18 years old.¹ Currently, the most effective strategy to prevent reactions due to peanut allergy is to avoid consuming foods that contain peanuts. Although clinical studies on oral and sublingual immunotherapy have shown some success against peanut allergies, it is difficult to accurately assess the effectiveness and safety of these methods because such studies often exclude participants with a history of severe allergies, and additionally, allergic reactions return in most patients after treatment is stopped.^{2,3} Therefore, it is vital to develop an effective treatment to combat peanut allergy.

Neutrophil-activating protein (NAP) is a major virulence factor of *Helicobacter pylori* and has been identified as a potent toll-like receptor 2 (TLR2) agonist; TLR2 can promote the secretion of IL-12, IL-23, IFN- γ and tumour necrosis factor- α (TNF- α) from human neutrophils and monocytes.⁴ In addition, NAP can suppress T helper 2 (Th2) responses by activating regulatory T cells (Tregs).⁵ The Th2 cell-mediated immune response is critically important in the development of peanut allergies. Although there are no reports on the applications of NAP in the treatment of food allergies, it has been found that NAP can inhibit ovalbumin (OVA)-induced asthma.⁶ Administration of NAP in mice was found to reduce the expression of IgE and IL-4, thereby shifting the antigen-specific T cell response from a Th2 to Th1 phenotype, which is characterized by high levels of IFN- γ . These findings inspired us to investigate the effectiveness of NAP in suppressing peanut allergies.

Oral administration is an inexpensive and needle-free method of delivering antigens to the intestine and eliciting an effective immune response in the intestinal mucosa. However, gastric acids and protease in the gastrointestinal tract can degrade purified proteins and produce a poor immune response.⁷ Therefore, a suitable delivery vector is required. *Bacillus subtilis* spores are novel biological vectors that are non-pathogenic and have been previously used as a probiotic and food additive.^{8,9} Additionally, they show excellent stability. After passing through the gastrointestinal tract, most spores will survive, and even colonize, germinate, reproduce, and produce new spores in the gastrointestinal tract.¹⁰ We previously successfully constructed recombinant *B subtilis* spores expressing NAP or cholera toxin B (CTB)-conjugated NAP (CNAP) in a previous study.¹¹ These recombinant spores showed good immunogenicity and could increase faecal IgA levels. In addition, the co-administration of CTB and the antigen can promote mucosal tolerance in allergic diseases.

Tregs are important regulatory cells in the process of allergic reactions and play a key role in inducing peripheral immune tolerance. Studies investigating the establishment of oral tolerance to ingested antigens indicated that the up-regulation of Tregs is essential.¹² Children with allergic asthma generally show lower Treg production.¹³ Our previous study showed that administration with *B subtilis* spores expressing NAP can induce Treg activation and suppress OVA-induced anaphylactic responses in mice.¹¹ This

study is the first to utilize recombinant NAP *B subtilis* spores for the treatment of a food allergy. We sought to determine whether oral administration of recombinant NAP spores could inhibit allergic inflammation in peanut allergy-induced mice via the up-regulation of CD4⁺CD25⁺Foxp3⁺Tregs.

2 | METHODS

2.1 | Mice

Five-week-old female C3H/HeJ mice were obtained from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Mice were maintained at the Guangzhou Medical University (Guangzhou, China) under specific pathogen-free conditions and fed with peanut-free chow under a 12-hour dark/light cycle. All procedures were performed according to the Ethics Committee of Guangzhou Medical University (approval number: 2016-118).

2.2 | Spore preparation

Bacillus subtilis spore coat protein C (B.s-CotC), B.s-CotC-CTB, B.s-CotC-NAP and B.s-CotC-CNAP were constructed according to the protocol mentioned in our previous study.^{11,14} Recombinant spores were cultured in Difco sporulation medium (DSM; BD Biosciences). After incubation with shaking at 250 rpm for 24 hours, spores were harvested by centrifugation at 8000 g for 10 minutes and then treated with 4 mg/mL lysozyme at 35°C for 30 minutes to disrupt residual sporangial cells. Thereafter, the spores were washed with a solution of 1 mol/L NaCl, 1 mol/L KCl, and distilled water, and finally resuspended in sterile water and incubated at 68°C for 1 hour to remove residual propagules. The outer coat proteins of the spores were extracted using SDS-dithiothreitol (DTT) buffer as previously described.¹⁵ Next, 1.0×10^9 spores were placed in 5 mL SDS-DTT buffer, incubated at 37°C for 2 hours and washed six times with Tris-HCl (pH 8.0). The precipitation of spores was suspended in disrupted buffer (50 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 1 mmol/L PMSF) and ultrasonicated on ice. Then, the outer coat proteins were collected by centrifugation and recombinant proteins were identified by Western blotting.

2.3 | Peanut sensitization/boosting, spore treatment and peanut challenge

Forty-two mice were randomly divided into seven groups, as depicted in Figure 1. Mice were sensitized to peanuts via oral administration according to a previous study.¹⁶ Briefly, each mouse was orally sensitized with 10 mg peanut solution (PN; 100 μ L of 100 mg/mL PN solution) along with 20 μ g cholera toxin and 16.5 μ L vodka (source of food-grade ethanol, Stolichnaya Vodka) for 6 weeks, and this was increased at 6 and 8 weeks to 50 mg PN (250 μ L of 200 mg/mL PN solution) along with 20 μ g cholera toxin and 16.5 μ L vodka. To assess whether the mouse peanut sensitization model was successfully constructed, blood was drawn before treatment with spores to detect

the serum PN-specific IgE levels. Recombinant spore treatment was carried out at 9–12 weeks into the treatment process. Mice were fasted for 2 hours prior to oral administration. PBS, 1.0×10^9 CotC spores, 1.0×10^9 CotC-CTB spores, 1.0×10^9 CotC-NAP spores or 1.0×10^9 CotC-CNAP spores at a volume of 500 μ L were delivered to the mice by oral gavage three times each week. To assess whether treatment with CNAP spores conferred peanut allergy protection by increasing the number of Tregs, Treg depletion experiments were performed in mice treated with CNAP spores for 4 weeks. At week 13, CD4⁺CD25⁺ T cells were depleted *in vivo* by two intraperitoneal injections of 250 μ g anti-CD25 antibody (Thermo Fisher Scientific) at 3-day intervals. Finally, mice were challenged with 1 mL 200 mg/mL PN at week 14.

2.4 | Measurement of peanut-specific serum and mucosal antibodies

Peanut solution-specific IgA in faeces was measured after each treatment. A total of 0.1 g faecal pellets were diluted in 1 mL 1% bovine serum albumin (BSA; Ruishu Biotechnology) as previously described.¹⁷ Following incubation overnight at 4°C, faecal samples were centrifuged at 13 800 g for 10 minutes and the supernatants were used to measure PN-specific IgA levels. Serum samples were collected 1 week after the final treatment (week 13) to measure specific IgE, IgG1 and IgG2a levels. Plates were coated with 10 μ g/mL defatted peanut extract and incubated overnight at 4°C. Plates were washed three times with PBST and blocked with 3% BSA for 2 hours. After washing, the plates were incubated with faecal or serum samples (IgA, 1:4000; IgE, 1:80; IgG1, 1:15 000; IgG2a, 1:5000) overnight at 4°C and developed using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (1:5000), IgE (1:5000), IgG1 (1:5000), or IgG2a (1:5000; SouthernBiotech), as well as 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KHB, Shanghai,

China). The levels of the antibodies were quantified by measuring the optical density at 450 nm (OD₄₅₀).

2.5 | DNA extraction, amplification and sequencing

After 4 weeks of oral spore treatment, mouse faeces were collected, and the total DNA in the faeces of each mouse was extracted.¹⁸ The primers (341F = 5'-CCTACGGGRBGCASCAG-3'; 806R = 5'-GGACTACYVGGGTATCTAAT-3') were designed to sequence the V3-V4 region of the 16S rDNA sequence.¹⁹ Raw data were obtained using the Illumina MiSeq platform (RiboBio); operational taxonomic units (OTU) were identified, and subsequent bioinformatics analysis was performed using Ribosomal Database Project (RDP) Classifier v 2.2.²⁰

2.6 | Plasma histamine detection

Mice were killed by cervical dislocation 30 minutes after challenge, blood was immediately drawn, and plasma was extracted at 4°C and stored at -70°C for subsequent histamine detection. The blood was stored in EDTA tubes on ice. Plasma was collected by centrifugation, and the concentration of histamine was detected with an enzyme immunoassay kit (Jianchen).

2.7 | Measurement of Treg production

Following the final challenge, spleens were isolated under sterile conditions. After washing with PBS, spleens were placed in a 300-m nylon filter and ground with a glass rod to prepare single-cell suspensions. After red blood cells were lysed, spleen cells were adjusted to 1×10^6 cells per tube and stained with fluorescent-conjugated antibodies (eBioscience) for flow cytometry, including anti-CD4-fluorescein isothiocyanate (FITC), anti-CD25-phycoerythrin (PE) and anti-Foxp3-allophycocyanin (APC). Stained cells were analysed

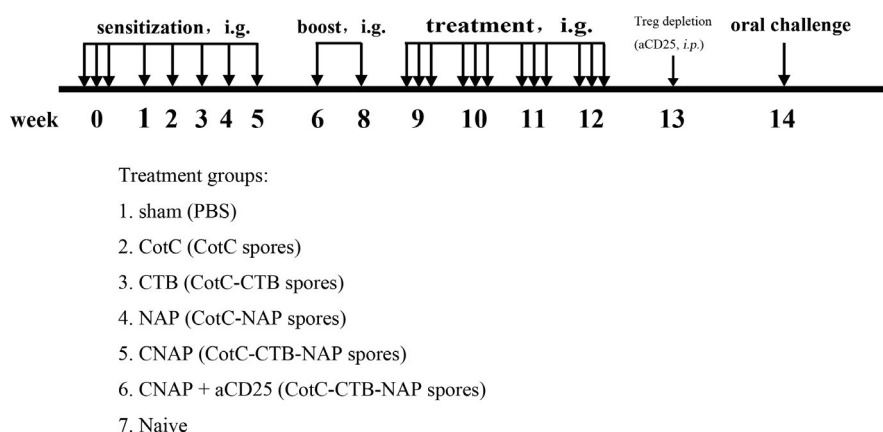


FIGURE 1 Experimental protocol. Mice were sensitized (10 mg peanut solution [PN]/mouse) and boost sensitized (50 mg PN/mouse) with peanut extract from 0 to 8 wk of treatment. Oral spore treatments were administered three times weekly for 4 wk. Anti-CD25 antibodies were intraperitoneally injected twice at 3-d intervals at week 13. All groups were orally administered with 200 mg PN/mouse at week 14. aCD25, anti-CD25 antibody; CotC, spore coat protein C; CTB, cholera toxin B; NAP, neutrophil-activating protein; PBS, phosphate-buffered saline

by BD FACSCalibur, and Treg concentrations were calculated using CellQuest software (BD Biosciences).

2.8 | Cell culture and cytokine measurements

Splenocytes were adjusted to 8×10^6 cells/mL with complete RPMI 1640 cultures (10% FBS, 1% penicillin-streptomycin). Cells were added to 24-well plates at 250 μ L per well and co-cultured with 250 μ L 400 μ g/mL defatted peanut extract (diluted in complete 1640 medium). Cells co-cultured with 5 μ g/mL phytohemagglutinin (PHA; diluted in complete 1640 medium) or complete 1640 medium were used as a positive and negative control, respectively. After 72-hour culture, supernatants were harvested, and the IL-4, IL-5, IL-10 and IFN- γ levels were measured using an ELISA kit (BD Biosciences).

2.9 | Assessment of hypersensitivity reactions

Anaphylactic reactions were evaluated 30 minutes after challenge according to a previously described scoring system¹⁶: 0, no symptoms; 1, scratching and rubbing around the snout and head; 2, puffiness around the eyes and snout, piloerection, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, laboured respiration, and cyanosis around the mouth and tail; 4, no activity after prodding or tremors and convulsions; and 5, death. Rectal temperatures were measured with a rectal probe (OMRON) at 30 minutes after challenge.

2.10 | Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). Parametric data were analysed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. For anaphylactic score data and microbiota variation analysis, Kruskal-Wallis one-way ANOVA on ranks was applied. A value of $P < .05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad).

3 | RESULTS

3.1 | Oral administration of NAP or CNAP spores increased peanut-specific IgA in faeces

After 1 week of treatment, the PN-specific IgA levels in the faeces of CTB, NAP and CNAP spore-treated mice began to increase and were significantly higher than those in the sham group ($P < .05$; Figure 2A). However, the IgA levels in the CTB group were not significantly different from those of the CotC group, while the IgA levels in NAP or CNAP spore-treated mice were significantly higher than those in the CotC group ($P < .05$). After 3 weeks of treatment, IgA levels in the CTB group were significantly higher than those in the CotC group ($P < .05$). After 4 weeks of spore treatment, CNAP spore-induced specific IgA levels were the

highest, at 1.30-fold higher than those of the NAP group, 1.96-fold higher than those of the CTB group and 2.68-fold higher than those of the CotC group. There were no significant differences in PN-specific IgA levels between the sham group and the CotC group. CTB, NAP and CNAP spores could thus effectively induce specific mucosal immunity.

3.2 | CNAP spore treatment reduced serum PN-specific IgE and IgG1 levels, and increased IgG2a levels

To monitor the immune effects of oral administration of NAP and CNAP spores on PN-sensitized mice, mice were treated with spores for 4 weeks before the detection of PN-specific serum IgE (Figure 2B), IgG1 (Figure 2C) and IgG2a (Figure 2D) levels. After sensitization for 8 weeks, serum was collected before spore treatment at week 9 for PN-specific IgE detection, and it was evaluated whether the peanut allergy model was successfully constructed. As shown in Figure S1, the specific IgE levels were significantly increased in sensitized mice compared to those in naïve mice ($P < .001$), while there was no significant difference in the IgE levels of sensitized mice among the different treatment groups. After 4 weeks of treatment, serum PN-specific IgE levels were decreased in the spore treatment group compared to those in the sham group. Mice treated with NAP or CNAP spores showed a significant reduction in PN-specific IgE levels ($P < .05$ vs CotC group). Compared to post-boost sensitization IgE levels, serum PN-specific IgE levels were decreased by 66% and 72% in the NAP and CNAP groups, respectively, after 4 weeks of treatment, while those in the sham group, CotC group and CTB group decreased by 31%, 49% and 56%, respectively. The effects of NAP and CNAP spores on specific IgE were not significantly different. Compared to those in the sham group, mice treated with CNAP spores showed significantly lower serum PN-specific IgG1 levels and higher PN-specific IgG2a levels ($P < .001$). Serum-specific IgG1 levels in the mice treated with CotC, CTB and NAP spores were decreased, but not significantly. The serum PN-specific IgG2a levels in recombinant spore-treated mice were significantly higher than those in the sham group ($P < .05$), but the effects of various spore treatments were different: CNAP spore treatment was the most effective, followed by NAP spore treatment, while the increase in IgG2a levels in mice treated with CotC or CTB spores was minimal. These results suggest that spores can induce an increase in Th1-type (IgG2a) antibodies, but the difference in therapeutic effect was related to the recombinant protein expressed on the spores.

3.3 | NAP and CNAP spore treatment reduced IL-4 levels and induced IFN- γ and IL-10 levels in spleen culture

The levels of the cytokines IL-4, IL-5, IL-10 and IFN- γ after spleen cells were co-cultured with peanut extract for 72 hours are shown in Figure 3. Th2-type cytokines (IL-4 and IL-5) in the spleen cells of

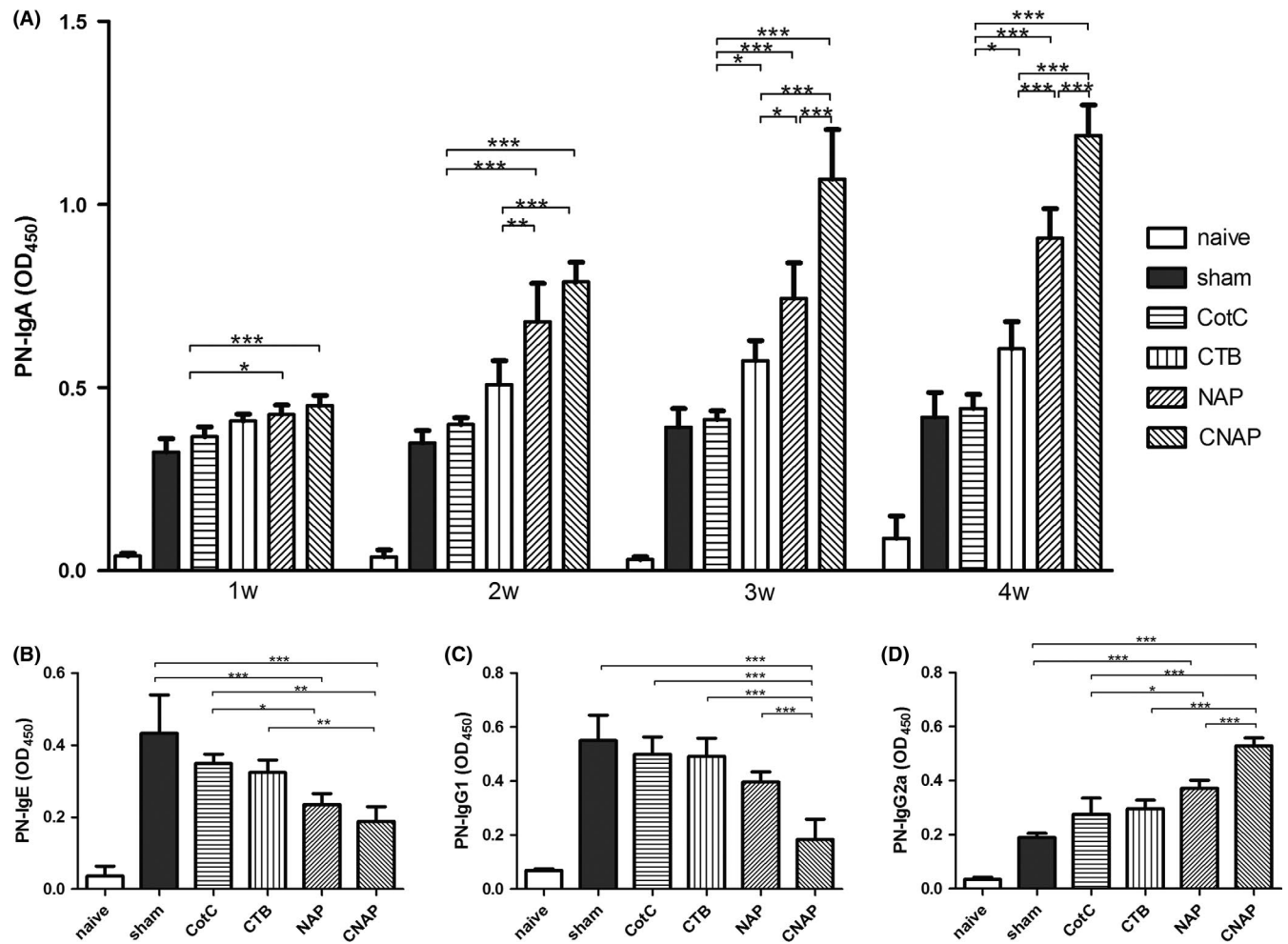


FIGURE 2 Detection of peanut solution (PN)-specific antibodies in mice after oral administration with spores. From 1 wk after the beginning of spore treatment, mouse faeces were collected weekly to monitor PN-specific IgA levels; at 1 wk after the final treatment, serum was collected to detect PN-specific IgE, IgG1 and IgG2a levels. A, Faecal PN-specific IgA levels. B, Serum PN-specific IgE levels. C, Serum PN-specific IgG1 levels. D, Serum PN-specific IgG2a levels. Data are expressed as the mean $\pm$ SD. * $P < .05$, ** $P < .01$, *** $P < .001$

the sham group were significantly increased. Compared to those in the sham group, the IL-4 and IL-5 levels in the CotC group and CTB group were decreased, but not significantly. Oral administration of NAP spores ($P < .01$) or CNAP spores significantly decreased the levels of IL-4 (NAP vs sham, $P < .01$; CNAP vs sham, $P < .001$) and IL-5 (both, $P < .001$) in culture supernatant. In contrast, IFN- γ levels in the NAP and CNAP groups were increased compared to those in the other groups ($P < .001$). Additionally, mice treated with CNAP spores showed higher levels of IFN- γ than those in the NAP group ($P < .001$). Furthermore, the levels of IL-10 were increased in both the NAP and CNAP groups, and the CNAP spore-treated mice showed the highest levels of IL-10 among all the treatment groups ($P < .05$). Mice treated with anti-CD25 antibodies showed significantly reduced IFN- γ and IL-10 levels compared to those in the CNAP group ($P < .001$). Because the ratios of IFN- γ /IL-4 or IFN- γ /IL-5 are often used as an indicator of the Th1/Th2 response, we calculated the ratios of IFN- γ /IL-4 and IFN- γ /IL-5 in the treated mice. As depicted in Figure 4, the ratios of IFN- γ /IL-4 and IFN- γ /IL-5 in the

NAP and CNAP groups were substantially higher than those in the model group, COTC group and CTB group. These results indicated that the oral administration of NAP or CNAP spores induced a Th1 cell-type immune response in mice.

3.4 | Recombinant spores reduced plasma histamine levels after challenge

Histamines are a marker of the degranulation of mast cells or basophils, and are also important mediators of allergic reactions. Therefore, we examined the plasma levels of histamines after challenge. Plasma histamine levels were reduced in CNAP-treated mice compared to those in the other groups ($P < .001$; Figure 4). Mice treated with NAP spores also showed a significant decrease in histamine levels compared to those in the sham treatment group ($P < .001$). After Treg depletion, the histamine plasma concentration was significantly increased compared to that in the CNAP group ($P < .001$).

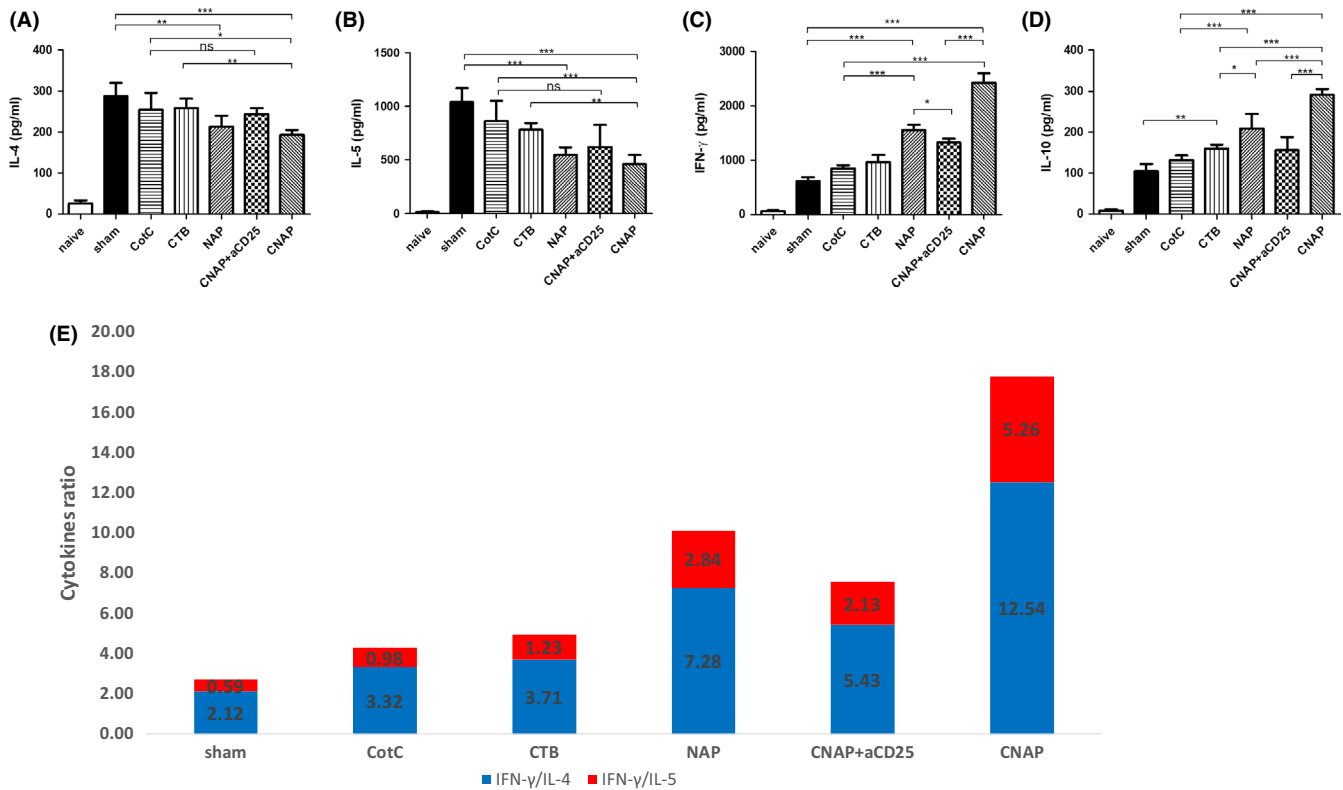


FIGURE 3 Detection of spleen cytokine levels. After peanut challenge, spleen cells (2×10^6 cells/well) were co-cultured with peanut extract (200 μ g/mL) for 72-h in vitro. The supernatants were assayed to determine the levels of (A) IL-4, (B) IL-5, (C) IFN- γ and (D) IL-10. Cytokine ratios involving IFN- γ /IL-4 and IFN- γ /IL-5 were calculated (E). Data are expressed as the mean $\pm$ SD. * $P < .05$, ** $P < .01$, *** $P < .001$

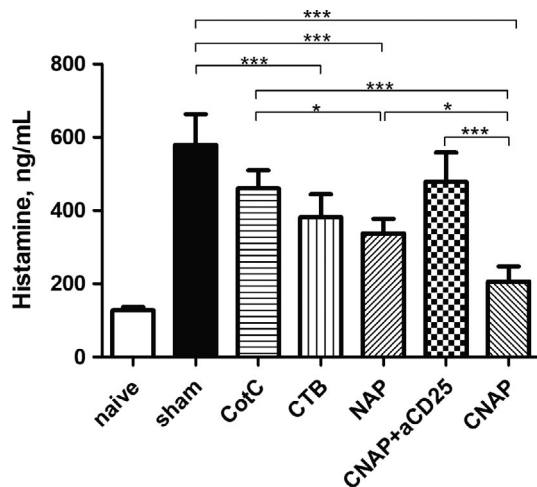


FIGURE 4 Plasma histamine content in mice after peanut challenge. Plasma was collected 30 min after oral peanut challenge, and histamine concentrations were measured by ELISA. Data are expressed as the mean $\pm$ SD. * $P < .05$, ** $P < .01$, *** $P < .001$

3.5 | Tregs in splenocytes were increased in mice treated with CNAP spores

The proportion of CD4⁺CD25⁺Foxp3⁺Treg in spleen cells is shown in Figure 5. The number of CD4⁺CD25⁺Foxp3⁺Tregs in CNAP

spore-treated mice was the highest, 1.7-fold higher than that in the CotC group ($P < .001$). The number of CD4⁺CD25⁺Foxp3⁺ Tregs in the NAP group was higher than that in the sham group, but there were no significant differences compared to the CotC and CTB groups. These data indicate that spores expressing CTB and NAP effectively induced the production of mouse CD4⁺CD25⁺Foxp3⁺ Tregs.

3.6 | Spores modulate faecal microbiota composition in peanut allergy mice

Taxonomic analysis of mouse gut microbiota revealed the differences between the groups (Figure 6A-D). After 4 weeks of treatment, four major bacterial phyla were identified; their concentrations are ranked from high to low as follows: *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*. CNAP spores effectively dysregulated the proportions of dominant bacteria in the gut, increasing the total number of *Firmicutes* and reducing the total number of *Actinobacteria* ($P < .01$ vs sham). The most dominant classes in the treatment groups were *Bacteroidia* and *Clostridia* (Figure 6E,F). In the CTB and CNAP groups, the bacterial abundance of *Bacteroidia* was significantly decreased ($P < .05$), while that of *Clostridia* was significantly increased compared to those in the sham group ($P < .01$). At the genus level, spore treatment reduced the proportion of *Adlercreutzia* and increased that of *Prevotella* compared to that in the sham group (Figure 6G,H).

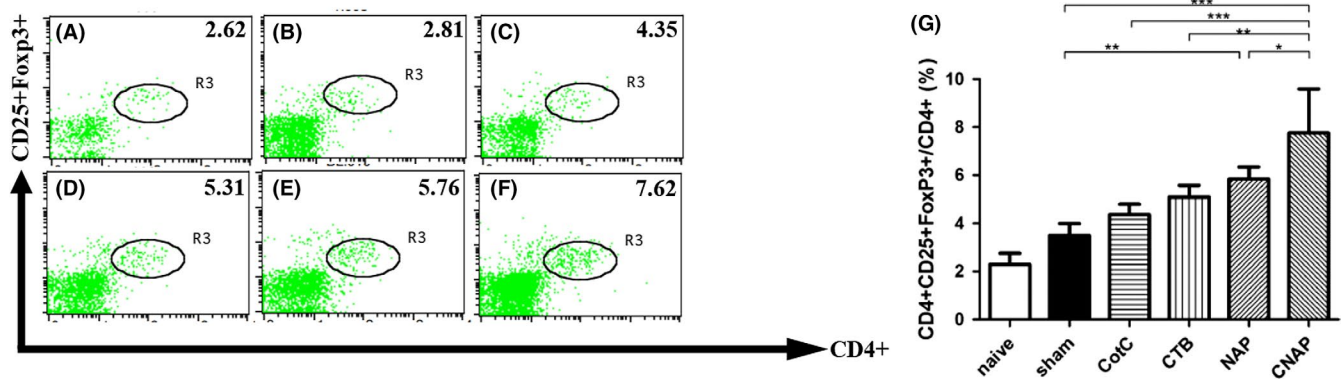


FIGURE 5 Proportion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) in spleen cells. Spleen cells were isolated after mice were killed, and 1×10^6 spleen cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, phycoerythrin (PE)-conjugated anti-mouse CD25, and allophycocyanin (APC)-conjugated anti-mouse Foxp3 in the (A) naïve group, (B) sham group, (C) CotC group, (D) CTB group, (E) NAP group and (F) CNAP group. (G) The proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in all groups. Data are expressed as the mean $\pm$ SD. * $P < .05$, ** $P < .01$, *** $P < .001$

3.7 | Mice treated with CNAP spores were protected from anaphylactic symptoms and anaphylaxis-associated hypothermia

Although NAP and CNAP spores induced an effective immune response in peanut-sensitized mice, their protection against peanut challenge required further exploration. Therefore, the allergic symptoms and body temperatures of mice were observed and scored after peanut challenge. As depicted in Figure 7, compared to the sham, CotC and CTB groups, NAP and CNAP spore treatment alleviated allergic symptoms and decreased body temperatures. The median symptom score in the CNAP group was lower than that in the NAP group, and the body temperature of mice in the CNAP group was significantly higher than that of mice in the NAP group ($P < .05$). The protective effect was therefore the most effective in CNAP group. The body temperature of mice in the NAP group was higher than that of mice in the sham and CotC groups. Although the allergy symptom score was decreased in the NAP group, this difference was not significant compared to those in the sham and CotC groups; however, this may have been because of the low number of mice used in this study. Compared to those in the CNAP group, the allergic symptoms of the mice after intraperitoneal injection of anti-CD25 were aggravated ($P < .01$), and their body temperatures were significantly decreased ($P < .001$).

4 | DISCUSSION

Peanut allergy is an IgE-mediated food allergy that can cause an increase in IL-4, IL-5 and IL-13 expression.^{21,22} Our previous study indicated that CNAP spores could inhibit OVA-induced asthma and reduce IL-4 and IL-5 expressions in a murine model.¹¹ In the present study, recombinant NAP or CNAP spore treatment reduced IL-4 and IL-5 expression and increased IFN- γ expression in the spleen cells of peanut-sensitized mice. Furthermore, increased

serum PN-specific IgG2a levels were observed after the oral administration of NAP or CNAP spores. After peanut challenge, mice treated with NAP spores or CNAP spores experienced mild allergic symptoms, elevated body temperature and reduced histamine release. These changes show encouraging prospects for preventing peanut allergy symptoms. The therapeutic effect of NAP and CNAP spores on peanut allergy can be attributed to multiple mechanisms, and their ability to skew the specific response from a Th2-type to a Th1-type immune response plays a key role in preventing peanut allergy, as the Th2 response plays a critical role in the development of food allergies.²³ When the immune system is exposed to food-derived allergens, elevated IL-4 levels can induce GATA3 expression and promote Th2 cell differentiation.²⁴ The lack of IL-4 in mice is known to lead to a decrease in IgE and IgG1 levels, as well as a reduction in peanut allergic reactions.²⁵ NAP can elicit high rates of IL-12, IFN- γ and IgG2a secretion, while IL-4, IL-5, IgE and IgG1 secretion are inhibited, thereby shifting the Th1/Th2 balance to a Th1-type response.^{26,27} The capability to induce Th1 cytokine secretion by NAP, or other chemical agents such as recombinant IL-12, Chinese herbal medicines and turmeric, is known to be sufficient to inhibit Th2 responses and ameliorate allergic disorders.^{23,28,29} Therefore, the Th1-type immune response caused by NAP and CNAP spores may be a major cause of the weakening of allergic symptoms.

The intestine is an important organ in the pathogenesis of food allergies. It often comes into contact with food-based allergens and has a profound impact on immune tolerance.³⁰ When the gastrointestinal tract is exposed to food allergens, the onset of food allergies or oral tolerance is associated with the mucosal barrier of the gastrointestinal tract. It has been reported that intestinal permeability may increase in food-allergic patients and that cytokines produced by these allergic reactions further exacerbate intestinal permeability.³¹ Studies have found that reduced IgA levels can lead to impaired mucosal immune function, increasing the risk of food allergies.³² Secreted IgA can increase immune tolerance to peanuts

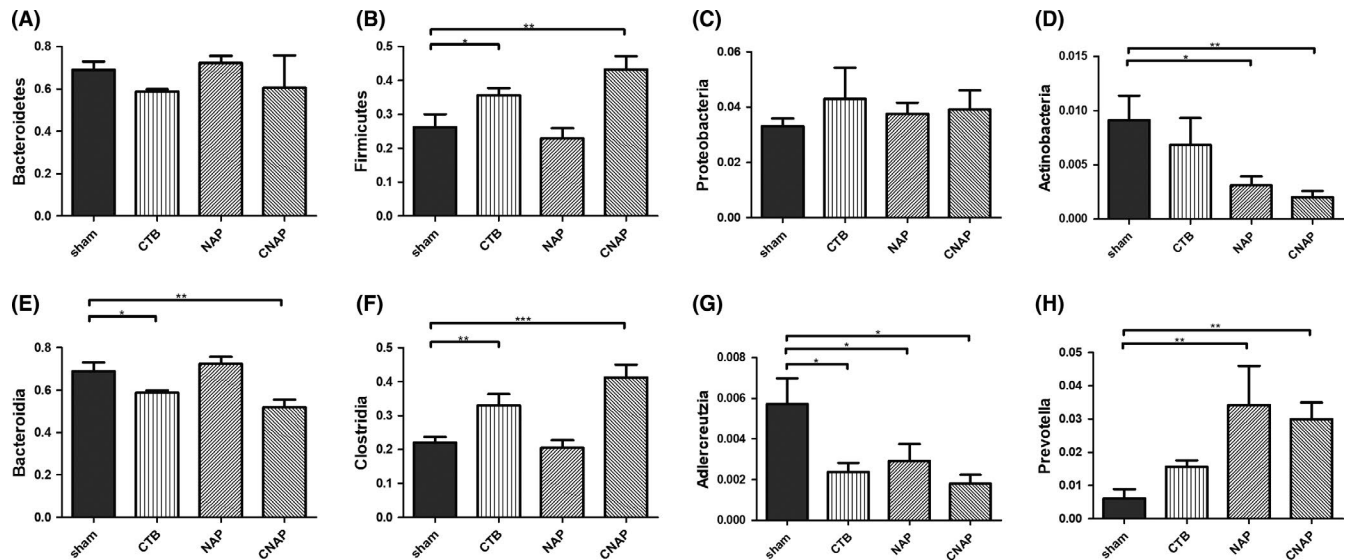


FIGURE 6 Differences in the taxonomic composition distribution of gut bacteria in different phyla (A-D), classes (E, F) and genera (G, H) after treatment with recombinant spores. Faecal samples were collected for intestinal microbiota analysis at 1 wk after the last treatment. Data are expressed as the mean ± SD. Statistical analyses were performed with Kruskal-Wallis one-way ANOVA on ranks. *P < .05, **P < .01, ***P < .001

and promote the maintenance of mucosal tolerance by inhibiting the immune response.^{33,34} Therefore, strong IgA production seems to be related to preventing food allergies. In this study, the oral delivery of CTB spores, NAP spores and CNAP spores effectively stimulated IgA secretion, and the CNAP spores showed the most pronounced stimulation of IgA. CTB is known to induce IgA secretion and ameliorate allergic reactions.³⁵ Although CTB and NAP spores could induce specific IgA production, the experimental results showed that the reduction in allergic reactions was the most obvious when mice were treated with spores expressing the CTB-NAP fusion protein. We found that IgA levels increased as IL-10 levels increased. IL-10 can induce faecal antigen-specific IgA production, which has an impact on the development of tolerance.³⁶ In the present study, a significant increase in IL-10 levels was observed after the administration of NAP or CNAP spores, which may have been associated with increased intestinal IgA secretion and reduced allergic reactions in mice.

Tregs have been shown to play a potential function in protecting the body against food allergies. In the current study, we found that with a decrease in Tregs, mice showed an increase in Th2-type immunity and a decrease in IL-10 levels in splenocytes. We then showed that Tregs were required to maintain the therapeutic effect of recombinant spores, as the inhibition of the peanut allergic reaction by CNAP spores was reversed when Tregs were depleted. Tregs can avoid excessive immune responses by activating T cells expressing IL-2 receptor alpha-chains (CD25) and secreting cytokines such as IL-10 and transforming growth factor- β (TGF- β).³⁷ Smalldini et al³⁸ reported that CD4⁺CD25⁺Foxp3 Tregs and IL-10 expression in gut lamina propria were increased after oral immunotherapy with milk proteins. Bonnet et al³⁹ also found that Treg depletion induced intestinal mastocytosis, which may lead to an allergic reaction. However, we did not analyse Treg and IL-10 tissue levels, or the function of Tregs on the mast cell compartment in this experiment. The

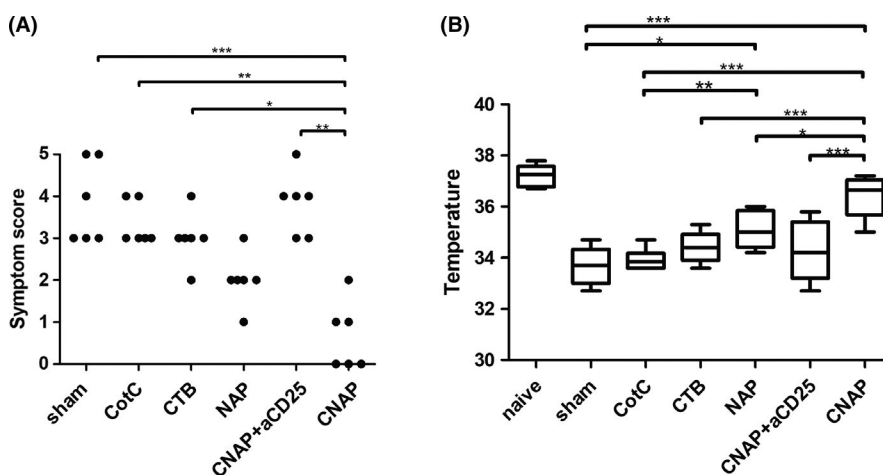


FIGURE 7 Anaphylaxis symptom scores and body temperature after peanut challenge. Anaphylactic signs were visually observed and evaluated at 30 min following oral peanut challenge utilizing the scoring system (ranging from 0 to 5) described in the Methods. Rectal temperatures were measured immediately after scoring using a rectal probe. Boxes (temperature) show the 10% and 90% range of the data in each group. *P < .05, **P < .01, ***P < .001

functional contribution of Tregs to the CNAP spore effect requires further investigation.

Previous studies suggested that intestinal microbes play a major role in allergic disease and that an altered gut microbiota can down-regulate allergic inflammation.^{40,41} In this study, mice treated with recombinant spores showed an increase in *Clostridia* class bacteria, a class of *Firmicutes* that is often found in intestinal epithelial cells. Previous research showed that transferring *Clostridia* to the intestines of mice could increase the expression of IL-22 and Tregs in the intestinal lamina propria, conferring protection to the mucosal barrier and reducing peanut-specific IgE levels.⁴¹ Bunyavanich et al⁴² also found that high gut proportions of *Clostridia* and other *Firmicutes* could increase the chance of milk desensitization in 3- to 6-month-old children with milk allergies. Many genera can be used as potential biomarkers for predicting food sensitization. In this study, mice treated with recombinant spores for 4 weeks showed higher proportions of *Prevotella* compared to those in the sham group. *Prevotella* belongs to the phylum *Bacteroidetes* and includes Gram-negative anaerobic bacteria. Anaerobic bacteria can ferment the intestinal contents and produce short-chain fatty acids, which exert multiple protective effects against food allergies. Chen et al¹⁸ also reported that the abundance of *Prevotella* was significantly decreased in children with food sensitization.

There were some limitations to the present work. The mice were only challenged once in this study, and the duration of the protective effect is therefore unknown. It is important to investigate the long-term protective effect of this treatment through multiple challenges in future work. Mice treated with CNAP spores showed a decrease in plasma histamine concentration after peanut challenge. Histamine is a marker for the degranulation of mast cells and basophils. Decreased expression of affinity IgE receptors (FcεRI) in mast cells was observed in mice receiving oral immunotherapy for peanut desensitization.⁴³ In the present study, peripheral blood basophil numbers, peritoneal or gut mast cell numbers, and FcεRI expression were not analysed; thus, it was not clear whether treatment would reduce mast cell numbers or decrease mast cell activation. Therefore, the mechanism surrounding this requires further study.

In conclusion, our results suggest that oral administration of recombinant *B subtilis* spores expressing NAP or CNAP conferred local and systemic specific immune responses. Recombinant spores could shift the antigen-specific T cell response from a Th2-type to a Th1-type phenotype and increase the production of Tregs, thereby conferring effective protection against peanut allergy.

ACKNOWLEDGEMENTS

This work was supported by the Guangdong Science and Technology Foundation (no. 2016A020215013), the Science and Technology Foundation of Guangzhou (no. 201707010010), and the Guangzhou Institute of Pediatrics/Guangzhou Women and Children's Medical Center (no. Pre-NSFC-2019-014 and no IP-2019-022).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Mai J, Liang B, Xiong Z, et al. Oral administration of recombinant *Bacillus subtilis* spores expressing *Helicobacter pylori* neutrophil-activating protein suppresses peanut allergy via up-regulation of Tregs. *Clin Exp Allergy*. 2019;00:1-10. <https://doi.org/10.1111/cea.13489>

