

# The recombinant fusion protein of cholera toxin B and neutrophil-activating protein expressed on *Bacillus subtilis* spore surface suppresses allergic inflammation in mice

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**Abstract** The neutrophil-activating protein of *Helicobacter pylori* (HP-NAP) has been identified as a modulator with anti-Th2 inflammation activity, and cholera toxin B (CTB) is a mucosal adjuvant that can also induce antigen tolerance. In this study, we constructed a CTB-NAP fusion protein on the surface of *Bacillus subtilis* spore and evaluate the efficiency of oral administration of the recombinant CTB-NAP spores in preventing asthma in mice. Oral administration of recombi-

nant CTB or CTB-NAP spores significantly decreased serum ovalbumin (OVA)-specific IgE ( $p < 0.001$ ) and increased fecal IgA ( $p < 0.01$ ) compared to the treatment with non-recombinant spores. Oral administration of recombinant CTB or CTB-NAP spores induced IL-10 and IFN- $\gamma$  expression and reduced IL-4 levels in bronchoalveolar lavage fluid (BALF). Moreover, CTB and CTB-NAP spores reduced the eosinophils in BALF and inflammatory cell infiltration in the lungs. Furthermore, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in splenocytes were significantly increased in mice treated with recombinant CTB or CTB-NAP spores. The number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs caused by CTB-NAP was higher than that by CTB alone. Our study indicated that *B. subtilis* spores with surface expression of subunit CTB or CTB-NAP could inhibit OVA-induced allergic inflammation in mice. The attenuated inflammation was attributed to the induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and IgA. Moreover, the fusion protein CTB-NAP demonstrated a better efficiency than CTB alone in inhibiting the inflammation.

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## Introduction

*Helicobacter pylori* is a spiral-shaped microaerophilic gram-negative bacterium that infects the stomachs of over 50% of the world's population. *H. pylori* causes gastric diseases like gastric mucosa-associated lymphoid tissue lymphoma, stomach cancer, chronic gastritis, and duodenal ulcer (Blaser and Atherton 2004). Recently, an inverse relationship between *H. pylori* infection and the incidence of asthma was reported (Blaser et al. 2008; Chen and Blaser 2008; Taye et al. 2015).

Asthma is a long-term inflammatory disease that characterized by bronchospasm and airflow obstruction. It is associated with a T helper type 2 (Th2)-cell immune response, IgE synthesis, and eosinophil recruitment and activation (Zhao et al. 2007). *H. pylori* neutrophil-activating protein (HP-NAP) is a powerful immunomodulator, which can trigger a Th1-biased immune response (Amedei et al. 2006). It induces endogenous IFN- $\gamma$  and IL-12 response and produces anti-Th2 activity, targeting both IL-4-mediated hyper-IgE responses and IL-5-induced eosinophilia (Del et al. 2008; D'Elios et al. 2009). Furthermore, systemic and mucosal HP-NAP delivery markedly reduces eosinophilia, as well as Th2 cytokine and IgE levels, in bronchoalveolar lavage fluid (BALF) in response to ovalbumin (OVA) challenge in mice; the level of IL-5, IL-4, and GM-CSF in the BALF of mice treated with HP-NAP plus OVA was significantly lower than that of mice treated with OVA alone (Codolo et al. 2008). These data suggest that HP-NAP could be used as potential strategies for the treatment and prevention of allergic asthma.

Bacteria-derived adjuvants also act as potent tolerogenic mucosal modulators by inducing IL-10 and regulatory T cells (Tregs) (Basset et al. 2010; Edlmayr et al. 2011). Cholera toxin B (CTB) subunit is a non-toxic component of the *Vibrio cholerae* holotoxin, and it promotes tolerance to mucosally co-administered antigens in autoimmune diseases (Sun et al. 2012; Bergerot et al. 1997; Stanford et al. 2004). Intranasal treatment of mice with CTB fusion with the major birch pollen allergen Bet v 1 increased allergen-specific IgG2a levels in sera, as well as IFN- $\gamma$  production in splenocytes, but markedly decreased serum IgE indicates that antigen fusion to the mucosal adjuvant CTB modifies the immunomodulating properties of the antigen (Bublin et al. 2007).

*Bacillus subtilis* is a gram-positive bacterium which produces endospores when starved of nutrients. Endospores offer unique resistance performances which can survive under extreme circumstances, such as mechanical extrusion, excessive temperature, high pressure, acid, alkali, and other extreme environment (Driks 1999; Nicholson et al. 2000). *B. subtilis* spores are regarded as safe and have been used as probiotics for food supplements and prophylaxis of human and animal gastrointestinal disorders (Lefevre et al. 2015). Because of their safety, good stability in the gastrointestinal environment, and ease of genetic cloning, *B. subtilis* spores have been recently used as efficient oral mucosal carriers for bacterial and parasite vaccines (Zhou et al. 2015; Zhou et al. 2008; Cutting et al. 2009).

With the aim of developing a novel treatment approach for allergic asthma, we used *B. subtilis* spore as a vehicle and a fusion protein of NAP and the mucosal adjuvant CTB. We also evaluated the efficiency of oral administration of the recombinant CTB-NAP spores in preventing asthma in mice.

## Materials and methods

### Animals and ethics statement

The animal study was carried out in accordance with the Guide for the Chinese Association for Laboratory Animal Sciences. Five-week-old specific pathogen-free female BALB/c mice were provided by the Guangdong Medical Laboratory Animal Center (Guangzhou, China), and the mice were housed in the animal maintenance facility at Guangzhou Medical University. The protocol was approved by the Animal Experiments Ethics Committee of Guangzhou Medical University.

### Gene fusion

The strategy for the construction of the gene fusion is shown in Fig. 1. pET-28a-CTB plasmid (Zhou et al. 2015) and pET-28a-NAP plasmid (Zhou et al. 2017) were constructed in our

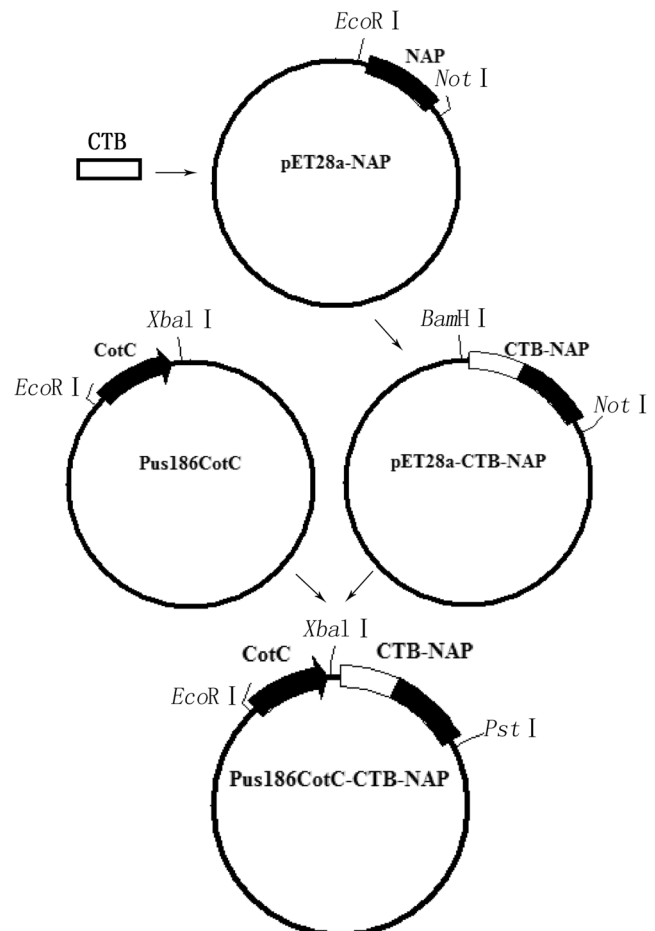


**Fig. 1** Strategy for construction of CotC-CTB-NAP gene fusions. CTB (312 bp) was cloned in frame to the 5' end of NAP in the pET-28a-NAP plasmid, yielding plasmid pET-28a-CTB-NAP, followed by CTB-NAP (753 bp) that was cloned to the 3' end of CotC in the pUS186-CotC plasmid, yielding plasmid pUS186-CotC-CTB-NAP

laboratory. Primers were designed based on the sequences of the target genes *CTB* and *CTB-NAP*. The *CTB* gene was PCR amplified using the constructed pET-28a-CTB plasmid as a template. The forward primer was 5'-gcagatcca cactcaaatattactgattg-3', with a *Bam*HI restriction endonuclease site (underlined), and the reverse primer was 5'-aaagaattcattgccataacttgccgcaa-3', with an *Eco*RI site. The optimized PCR cycle was as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C 60 s; and followed by 72 °C extension for 15 min. Both the purified *CTB* PCR products and the constructed pET-28a-NAP plasmid were digested with the restriction enzymes *Bam*HI and *Eco*RI at 37 °C 1 h. Double digested DNA and pET-28a-NAP were gel purified and ligated at 16 °C overnight. Recombinant pET-28a-CTB-NAP was transformed into *Escherichia coli* BL21. The *CTB-NAP* DNA was amplified by using the recombinant pET-28a-CTB-NAP as a template. The forward primer was 5'-cgctctagacacac tcaaaatattactg-3', with a *Xba*I restriction endonuclease site, and the reverse primer was 5'-aaactgcagttaagctaaatgggcttagcatc-3', with a *Pst*I site. PCR reaction conditions were as follows: 94 °C for 5 min; and 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C 90 s. The purified PCR product was double digested by *Xba*I/*Pst*I and cloned to the carboxy terminal of the *CotC* gene in the constructed plasmid pUS186-*CotC* (Zhou et al. 2008), and then transformed into *B. subtilis* WB600 (Wu et al. 1991) by standard competent cell method (Zhou et al. 2008; Cutting et al. 2009). The recombinant plasmid was identified by DNA sequencing analysis and restriction endonuclease digestion. The NCBI GenBank accession numbers for CTB and NAP were NC\_002505.1 and NC\_000915.1, respectively.

### Production of spores

Difco sporulation medium (DSM) was used to induce spore formation by the exhaustion method (Nicholson and Setlow 1990). Spores were collected after 24-h induction of sporulation. The obtained suspension was treated with lysozyme to breach sporangial bacterium. The suspension was then washed in 1 M NaCl, 1 M KCl, and distilled water (twice) and resuspended in distilled water. Spores were incubated at 68 °C 1 h to kill residual vegetative bacteria. The obtained spore number was counted with a Bürker chamber using an

optical microscope. In this manner, about  $10^{11}$  spores per liter of DSM can be obtained.

### Western blot analysis

Spore coat proteins were extracted using a SDS-DTT solution as previous described (Monroe and Setlow 2006). Extracted spore coat proteins were analyzed by 12% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes. The membrane was blocked with PBS containing 2% (v/v) skimmed milk for 2 h at room temperature. The membrane was then incubated with rat anti-NAP serum (1:500 dilution) at 4 °C overnight and then verified by horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG (1:5000 dilution) (Boster, China) for 2 h. Color was developed by diaminobenzidine substrate solution.

### Immunizations

Forty BABL/c mice (female, 5 weeks old) were randomized into five groups (eight mice per group): (a) naïve, (b) asthma model without treatment, (c) *CotC* spore-treated, (d) recombinant CTB (rCTB) spore-treated, and (e) recombinant CTB-NAP (rCTB-NAP) spore-treated groups (Table 1). The mice in groups c, d, and e were fed by intragastric lavage with  $1.0 \times 10^9$  spores on days 1, 2, 3, 15, 16, 17, 29, 30, and 31, and those in groups a and b lavaged with PBS. Fecal and serum samples were collected on days 0, 14, 28, 42, 56, and 76. All mice, except those in group a, were sensitized by intraperitoneal injections of 10 µg OVA (Sigma, Santa Clara, USA) mixed with aluminum in a total volume of 200 µL on days 43 and 57. Naïve group was injected with equal volumes of PBS. From day 71 to 75, mice were challenged with 1% OVA in PBS for 30 min using a nebulizer (PARI, Wuppertal, Germany) with an airflow rate of 2–8 L/min (Fig. 2). The control groups were challenged with aerosolized PBS. Twenty-four hours after the final aerosol challenge, all mice were humanely sacrificed.

### Bronchoalveolar lavage fluid

After mice were sacrificed, BALF was collected from the right lung with 0.5 mL PBS thrice using a 24-gauge needle inserted

**Table 1** Experimental protocol for animal treatment

| Group                          | Treatment (on days 1–3, 15–17, 29–31) | Sensitization (on days 43 and 57) | Aerosol challenge (on days 71–75) |
|--------------------------------|---------------------------------------|-----------------------------------|-----------------------------------|
| Naïve (a)                      | PBS                                   | PBS                               | PBS                               |
| Asthma (b)                     | PBS                                   | IP OVA                            | 1% OVA                            |
| <i>CotC</i> spore (c)          | <i>CotC</i> spore                     | IP OVA                            | 1% OVA                            |
| <i>CotC</i> -CTB spore (d)     | <i>CotC</i> -CTB spore                | IP OVA                            | 1% OVA                            |
| <i>CotC</i> -CTB-NAP spore (e) | <i>CotC</i> -CTB-NAP spore            | IP OVA                            | 1% OVA                            |

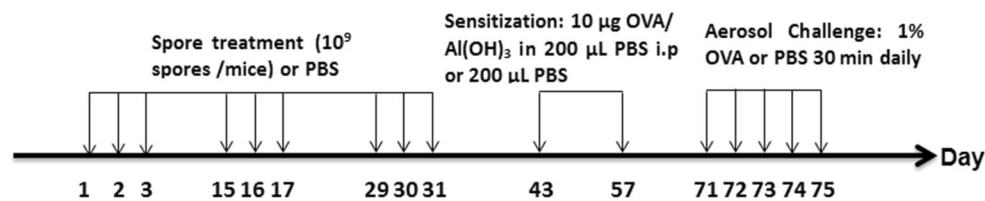


**Fig. 2** Sensitization and challenge protocol for an OVA-induced mice asthma model. Mice were treated with spores and then sensitized by intraperitoneal injections of OVA on days 43 and 57, after which they

were administered the aerosolized OVA challenge on days 71–75 using a nebulizer

into the trachea. The recovery rate of the instilled PBS was >80%. BALF was centrifuged at 4 °C for 10 min with 1500g, and supernatants were used for cytokine analysis by ELISA. Cell pellets were resuspended to count eosinophils. Cytospin preparations of cells were stained with a Wright–Giemsa stain kit (Baso, China) for eosinophil counts. At least 200 cells per slide were counted using an optical microscope (Olympus A1, OCN, Japan) (Zha et al. 2016).

### Lung histopathological analysis

After the completion of BALF sampling, left lung tissue was fixed in 4% paraformaldehyde at 4 °C for 24 h and embedded in the paraffin. For histopathological analysis, 4-µm-thick sections were cut and used for hematoxylin-eosin (H&E) staining to evaluate the inflammatory cell infiltration. The stained slides were analyzed with an optical microscope.

### Measurement of immunoglobulins

Serum OVA-specific IgE was assessed by the standard protocol. ELISA plates were coated with 5 µg OVA at 4 °C overnight, and following this, 100 µL serum samples (1:80 dilution with 1% BSA-PBS) were added and further incubated at 4 °C overnight. This was followed by the addition of 100 µL/well of diluted horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin E (Southern Biotech, USA) and incubation at 37 °C for 1 h. Color was developed by the tetramethylbenzidine (TMB) microwell peroxidase substrate kit (KHB, China). Reactions were stopped by 2 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance at 450 nm was read.

The fecal samples were prepared as previously described (Zhou et al. 2008). Fecal pellets (0.1 g) were suspended in 1% BSA-PBS buffer overnight at 4 °C. The suspensions were centrifuged, and the liquid supernatant was stored at –80 °C until use. This IgA ELISA was conducted similarly to the IgE ELISA mentioned previously. ELISA plates were coated with 2 µg anti-mouse IgA at 4 °C overnight, and 50 µL of fecal supernatant was added and allowed to be incubated at 4 °C overnight. IgA was measured with HRP-goat anti-mouse IgA (Southern Biotech, USA). For OVA-specific IgA measurement, ELISA plates were coated with 5 µg OVA at 4 °C

overnight, and followed by the procedures as above. All assays were conducted in duplicate.

### Measurement of cytokines

Cytokine ELISA kits were purchased from Becton Dickinson and Company (USA). The IL-4, IL-10, and IFN-γ levels in the BALF supernatant were detected by ELISA according to the manufacturer's instructions. Briefly, plates were coated with 100 µL/well of the capture antibody at 4 °C overnight after blocking. Next, 100 µL sample or standard was added to the plates and incubated for 2 h at 37 °C. Working detector (detection antibody + streptavidin-HRP reagent) was added to the microplates and incubated for 1 h, followed by 100-µL substrate solution. Reactions were stopped by 2 M H<sub>2</sub>SO<sub>4</sub>, and the optical density at 450 nm was read. All assays were conducted in duplicate.

### Flow cytometry

Splenocytes were isolated using the standard murine splenocyte preparation method (Lopez-Exposito et al. 2011). The single-cell suspensions were washed with RPMI 1640 and the red blood cells lysed with NH<sub>4</sub>Cl-Tris solution. Then, splenocytes were resuspended in RPMI 1640. Monoclonal antibody combinations marked with different fluorescent substances (CD3-PerCP/CD4-FITC/CD25-APC/Foxp3-PE) were used to detect the regulatory T cells (Morse et al. 2008). Surface phenotyping for CD3, CD4, and CD25 of splenocytes and intracellular phenotyping for forkhead protein 3 (Foxp3) were performed by staining, followed by paraformaldehyde fixation and permeabilization according to the manufacturer's protocol (BD Bioscience). Splenocytes were analyzed using forward and side-scatter properties based on size and granularity by a BD FACSCalibur flow cytometer (Becton Dickinson and Company) with the BD CellQuest Pro software. The percentages of positive cells were calculated.

### Statistical analysis

Results were determined as mean ± S.D; statistical significance of the differences was analyzed by ANOVA with the

SPSS17 software. *P* values less than 0.05 were considered statistically significant.

## Results

### CTB-NAP PCR amplification and cloning

The *CTB* gene was amplified by PCR using a constructed pET28a-CTB plasmid as the template. The band size of the target fragment was as expected (312 bp) (Fig. 3a). The *CTB* gene was cloned into a pET28a-NAP plasmid ahead of *NAP* to form pET28a-CTB-NAP. The recombinant pET28a-CTB-NAP was identified by double enzyme digestion and sequence analysis. The CTB-NAP gene for *B. subtilis* expression was amplified using the pET28a-CTB-NAP construct as the template (Fig. 3b). The expected CTB-NAP band (753 bp) was detected after double enzyme digestion pUS186CotC-CTB-NAP plasmid (Fig. 3c). Sequence analysis indicated that CTB-NAP was in the right open reading frame of pUS186CotC.

### Expression of target fusion proteins in *B. subtilis*

The recombinant pUS186CotC-CTB-NAP plasmid was transformed into competent cell *B. subtilis* WB600. Spore coat protein SDS-PAGE analysis indicated the presence of a 37.4-kDa band in the CTB-NAP recombinant strain, together with CotC (8.8 kDa), CTB (11.6 kDa), and NAP (17 kDa) bands (Fig. 4a). Western blot analysis with NAP-specific rat antiserum also showed the presence of an approximately 37.4-kDa band in the spores of the recombinant strains (Fig. 4b).

### Recombinant spores reduced eosinophils in BALF

To determine the effects of recombinant spores on airway inflammation, the number and differentiation of inflammatory cells in BALF were determined. rCTB-NAP spores markedly reduced eosinophils in BALF compared to the CotC spore-treated group ( $p < 0.001$ , Fig. 5). Moreover, rCTB-NAP spores were more efficient in reducing inflammation than rCTB spores ( $p < 0.001$ ).

### Recombinant spores reduced inflammatory cell infiltration in the lung

Histological changes revealed extensive peribronchial and perivascular inflammation (eosinophil, neutrophil, and lymphocyte infiltration), numerous airway goblet cells, and abundant mucus secreted inside the airway of the lungs of asthma model mice (Fig. 6b). Mucus is largely produced by goblet cells, and goblet cell hyperplasia is a characteristic of airway remodeling. These features resembled those of the asthma model and CotC group lungs (Fig. 6c). Mice in the rCTB spore-treated group had attenuated inflammatory cell infiltration and reduced goblet cell and mucus secretion around and inside the airway (Fig. 6d). In rCTB-NAP spore-treated mice, the inflammation was markedly lower, and goblet cells were almost absent in the airways (Fig. 6e).

### Recombinant spores reduced IgE and increased IgA in mice

CTB and CTB-NAP spores decreased OVA-specific IgE in serum. As shown in Fig. 7a, the IgE in serum was considerably higher in the asthma model group ( $OD_{450} 0.622 \pm 0.092$ )

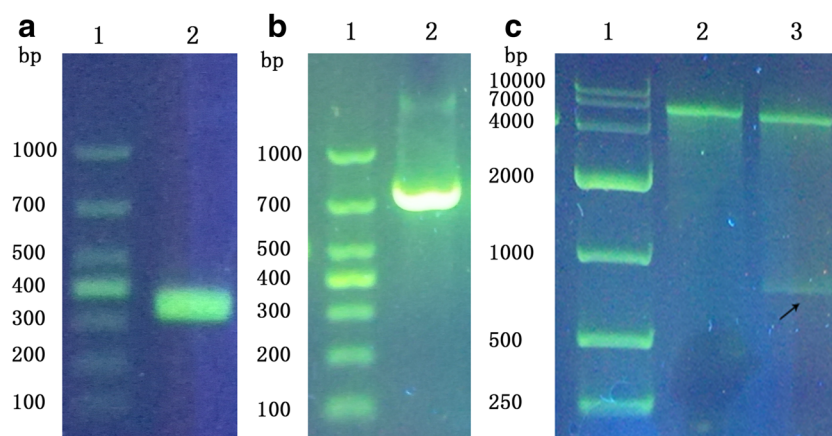


**Fig. 3** PCR amplification of CTB and CTB-NAP and plasmid pUS186-CotC-CTB-NAP double digestion. **a** The target band of CTB gene amplified from pET-28a-CTB plasmid. Lane 1, 1Kb DNA marker; lane 2, the target amplification fragment of CTB gene. **b** The target band of CTB-NAP gene amplified from pET-28a-CTB-NAP plasmid. Lane 1, 1Kb DNA marker; lane 2, the target amplification fragment of CTB-

NAP gene. **c** Identification of CTB-NAP fusion gene in the recombinant plasmid pUS186-CotC-CTB-NAP by *Xba*I and *Pst*II digestion. Lane 1, 10-Kb DNA marker; lane 2, pUS186-CotC plasmid digested by *Xba*I and *Pst*II; lane 3, recombinant plasmid of pUS186-CotC-CTB-NAP digested by *Xba*I and *Pst*II. (arrow points to CTB-NAP fusion gene)

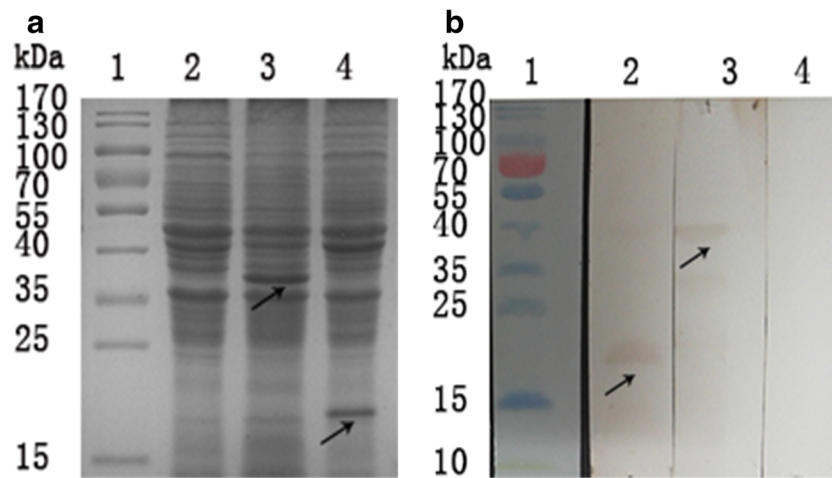


**Fig. 4** Spore coat proteins SDS-PAGE analysis and western blot with specific antibody. **a** Coomassie brilliant blue-stained gels of recombinant strains and the CotC strain. *Lane 1*, markers of protein molecular weight; *lane 2*, CotC strain; *lane 3*, CotC-CTB-NAP strain; *lane 4*, CotC-CTB strain. The *arrow* indicates the fusion protein. **b** Western blot analysis of

spore coat proteins of recombinant strains and the CotC strain using specific antiserum. *Lane 1*, markers of protein molecular weight; *lane 2*, CTB strain with the CTB antibody; *lane 3*, CTB-NAP strain with the NAP antibody; *lane 4*, CotC strain with the NAP antibody. The *arrow* indicates the fusion protein

compared with that in the naïve group ( $OD_{450} 0.0445 \pm 0.013$ ). Oral administration of CTB and CTB-NAP spores significantly decreased the OVA-specific IgE level ( $OD_{450} 0.347 \pm 0.051$ ,  $p < 0.01$ ;  $0.237 \pm 0.048$ ,  $p < 0.001$ ) compared to the CotC group ( $OD_{450} 0.574 \pm 0.158$ ), and the IgE level in CTB-NAP spore-treated mice was significantly lower than that of CTB-treated mice, indicating better performance of CTB-NAP fusion spores in reducing the IgE level than CTB spores alone.

Oral immunization with *B. subtilis* spores expressing CTB and CTB-NAP elicited IgA responses after 2 weeks. Fecal IgA levels were higher in the rCTB and rCTB-NAP spores treated mice than those in the CotC spore-treated mice ( $p < 0.01$ ) (Fig. 7c). Furthermore, the OVA-specific IgA levels before mice were sacrificed were significantly higher in the

rCTB and rCTB-NAP-treated groups compared to those in the non-recombinant CotC spore-treated mice ( $p < 0.001$ ) (Fig. 7b). Between both recombinant spores, rCTB-NAP spores led to significantly higher OVA-specific IgA levels compared to rCTB spores ( $p < 0.05$ ).

#### Recombinant spores reduced IL-4 and increased IL-10 and IFN- $\gamma$ levels in BALF

Immediately after mice were sacrificed, IFN- $\gamma$ , IL-4, and IL-10 were accessed in BALF. IL-4 levels were dramatically reduced in rCTB and rCTB-NAP spore-treated groups compared with those in the non-recombinant CotC spore-treated group ( $p < 0.05$  and  $p < 0.01$ , respectively; Fig. 8a). IL-10 and IFN- $\gamma$  levels were significantly higher in the groups treated

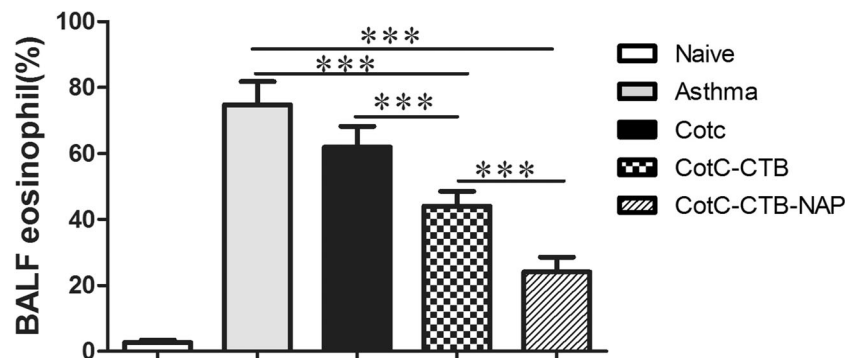


**Fig. 5** Eosinophil count after oral administration of CTB-NAP recombinant spores on OVA-induced asthma in BALF. BALF was collected and examined for the number of eosinophils. The eosinophil

count was obtained by counting at least 200 cells per slide by light microscopy after differential staining with the Wright-Giemsa stain. All values are expressed as means  $\pm$  S.D. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

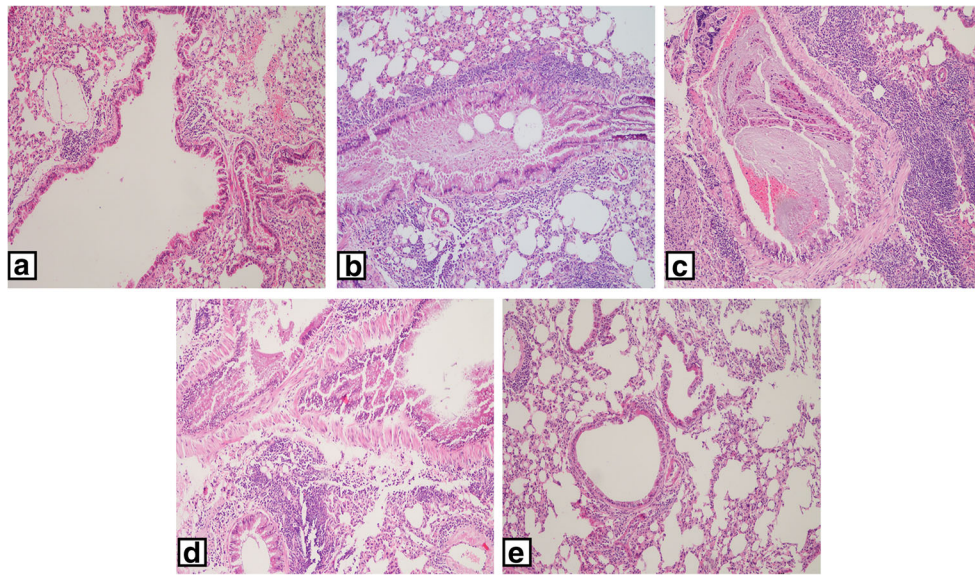


**Fig. 6** Oral administration of CotC-CTB-NAP spores inhibited histopathological changes in the lungs of mice with OVA-induced asthma. (H&E staining of lung sections; 100 $\times$  magnification). Asthma (b) and CotC-treated (c) groups showed severe airway tissue inflammation, mucus hyperproduction around peribronchial and perivascular regions, and goblet cell hyperplasia in the lungs. The CTB

group (d) showed reduced mucus secretion and reduced infiltration of inflammatory cells and goblet cell hyperplasia. The CotC-CTB-NAP group (e) showed very slight infiltration of eosinophils and lymphocytes, decreased mucus secretion, and goblet cell hyperplasia. Groups: naïve (a), asthma (b), CotC spore-treated (c), CTB spore-treated (d), and CTB-NAP spore-treated (e) groups

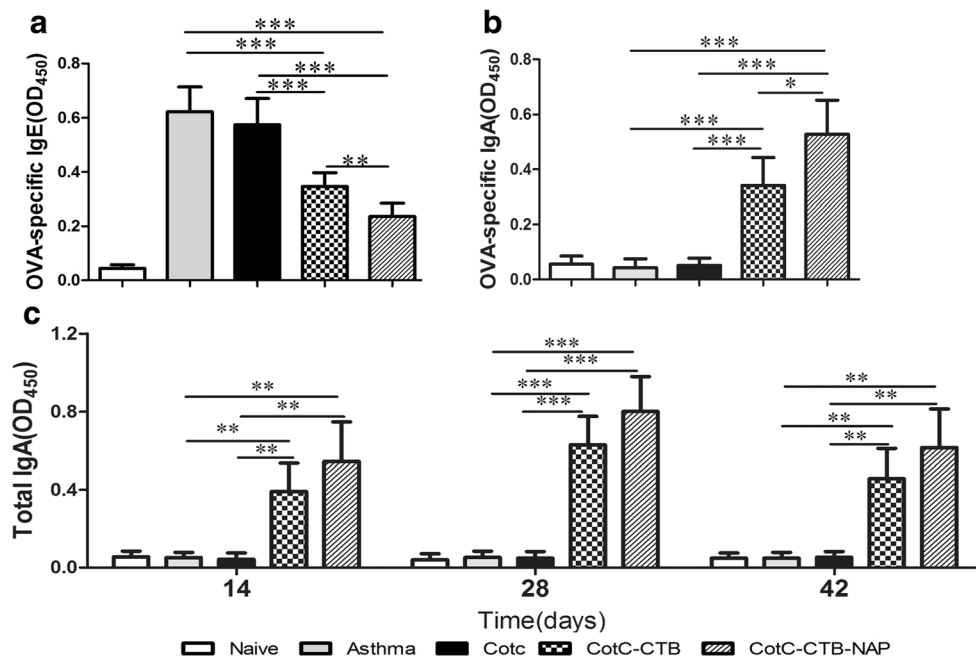


**Fig. 7** ELISA analysis of OVA-specific IgE in serum and total IgA and OVA-specific IgA in fecal. **a** ELISA analysis of OVA-specific IgE in serum (OD<sub>450</sub>). Blood was collected 24 h after the last OVA challenge. Orally administered CotC-CTB and CotC-CTB-NAP spores resulted in significantly decreased serum IgE levels compared to non-recombinant CotC spore-treated mice. Fecal OVA-specific IgA levels (b) and total IgA

levels (OD<sub>450</sub>) (c) following oral administration of recombinant *B. subtilis* spores. Groups of eight mice were oral administered spores expressing CotC, CotC-CTB, or CotC-CTB-NAP. Fecal OVA-specific IgA (b) and total IgA (c) from individual samples were measured by ELISA. All data are expressed as means  $\pm$  S.D. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001

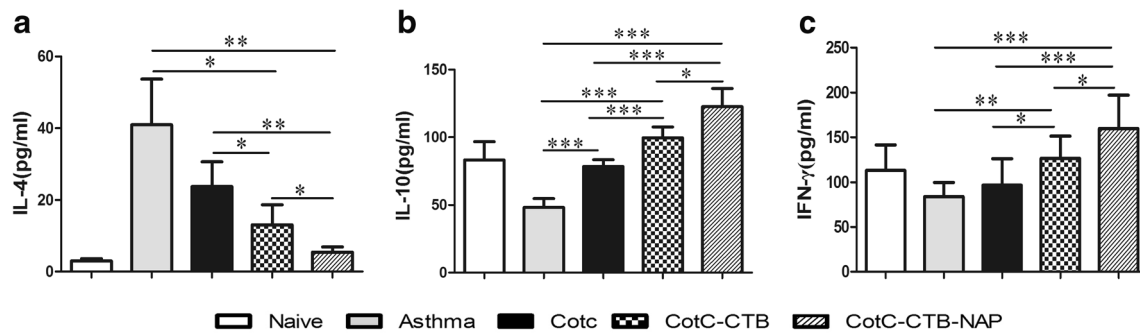


**Fig. 8** Cytokine analysis in BALF. IL-4, IL-10, and IFN- $\gamma$  concentrations in BALF were determined using ELISA kits. **a** Levels of IL-4 in BALF were significantly lower in CotC-CTB and CotC-CTB-NAP spore-treated groups than those in the CotC spore-treated group.

BALF IL-10 (**b**) and IFN- $\gamma$  (**c**) were significantly higher in the CotC-CTB and CotC-CTB-NAP spore-treated groups. All values are expressed as means  $\pm$  S.D. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001

with rCTB or rCTB-NAP spores compared to those in the CotC spore-treated group ( $p$  < 0.001, Fig. 8b;  $p$  < 0.05 and  $p$  < 0.001, respectively; Fig. 8c). In addition, the rCTB-NAP fusion spores were more potent in inducing IL-10 and IFN- $\gamma$  and reducing IL-4 compared to rCTB spores alone ( $p$  < 0.05).

### Recombinant spores increased CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in splenocytes

We investigated the effect of rCTB and rCTB-NAP spores on the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in splenocytes. The fraction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs among the total CD4<sup>+</sup> T cells in splenocytes was higher in the groups treated with rCTB and rCTB-NAP spores ( $4.754 \pm 0.752$ ,  $6.46 \pm 0.744$ ,  $p$  < 0.001) compared to that in the non-recombinant CotC spore-treated group ( $3.472 \pm 1.026$ ; Fig. 9). Moreover, the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg number in rCTB-NAP-treated mice was higher than that in rCTB-treated mice ( $p$  < 0.01).

## Discussion

Asthma associated with imbalance between Th1 and Th2 cell-related responses (Barnes 2008), which induces airway hyper-responsiveness and morphological changes in lung tissues (Kay 2001). Interestingly, there is an inverse association between occurrence of asthma and *H. pylori* infection, particularly in children (Chen and Blaser 2008; Amberbir et al. 2011; Zevit et al. 2012). HP-NAP can induce immune deviation from Th2 to Th1 response, indicating that rNAP might hinder the pathogenic mechanism underlying allergy. Co-culture of neutrophils with recombinant HP-NAP induces significant upregulation of cytokine secretion and mRNA expression, including IL-12p40 and IL-12p35, which assemble into effective IL-23p19 and IL-12. The effect of HP-NAP on cytokine secretion has also been detected in peripheral blood monocytes (Amedei et al. 2006). Thus, HP-NAP induces IL-23 and IL-12 secretion by acting on both monocytes and

neutrophils, which have the potential to drive effective T cells toward a Th1-dominant phenotype (Amedei et al. 2006).

*B. subtilis* has recently been used as a vaccine vehicle to deliver foreign antigens to the intestinal tract (Zhou et al. 2015; Tavares et al. 2014; Lefevre et al. 2015). In an earlier study, we found that oral treatment with *B. subtilis* spores expressing urease B of *H. pylori* induced systemic and mucosal responses and protected mice against *H. pylori* challenge (Zhou et al. 2015). Here, *B. subtilis* spores were used as a vehicle, along with *H. pylori* NAP fused to the mucosal adjuvant CTB, as a novel treatment for allergic asthma in mice. The present study is the first report on the potential of recombinant *B. subtilis* spores to confer immunomodulation to prevent anaphylaxis in allergic mice.

Expression of NAP using *B. subtilis* spore display is effective; using the nine-dose regime (on days 1, 15, and 29, each consecutive 3 days) with  $1.0 \times 10^9$  spores can reduce allergic inflammation in mice. The nine-dose oral regime removes the need to give mice large doses by gavage, solving the viscosity problem of higher concentration spore suspension, but for human trials, we could use a capsule to deliver recombinant spores enabling just three doses or less.

Asthma is associated with lung tissue morphological changes, such as eosinophil infiltration near the vessels and

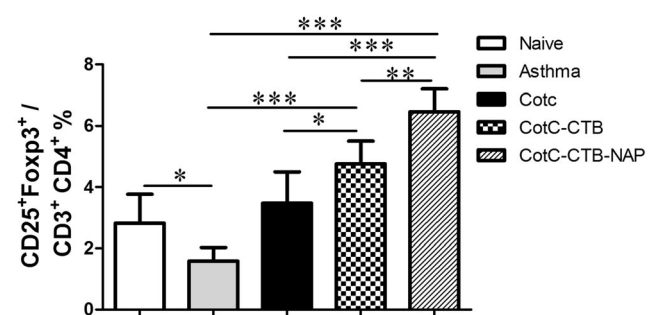


**Fig. 9** The proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs among total CD4<sup>+</sup> T cells in the splenocytes. Splenocytes were stained with fluorescence-labeled CD3, CD4, CD25, and Foxp3 antibodies. Flow cytometry was used to analyze the stained cells. All values are expressed as means  $\pm$  S.D. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001

bronchioles, epithelial cell and goblet cell hyperplasia, and mucus hypersecretion in the bronchioles (Kay 2001). In the present study, oral administration of recombinant rCTB or rCTB-NAP spores markedly reduced inflammation in the lungs and diminished goblet cells in the airways, which indicated that rCTB and rCTB-NAP spores can reduce pathological morphological changes in respiratory organs. Furthermore, mice administered rCTB-NAP spores exhibited markedly reduced inflammation and a near absence of goblet cells in the airways compared to mice treated with rCTB spores.

IgE is known to induce allergic reactions in mice (Finkelman 2007). Here, we demonstrated that oral administration of rCTB and rCTB-NAP spores significantly decreased the serum OVA-specific IgE in mice after OVA challenge. In addition, the IgE level in rCTB-NAP spore-treated mice was even lower than that in rCTB spore-treated mice, suggesting that rCTB-NAP fusion protein has a better efficiency than rCTB alone against allergic asthma.

IgA could neutralize viruses, bacteria, and toxins at the surface of mucosa (Williams and Gibbons 1972; Mazanec et al. 1993) by competing for epithelial adhesion sites and by interfering with their motility, among other mechanisms (Puchelle et al. 1980). Pasquier et al. showed that IgA could reduce inflammatory responses by inhibiting inflammatory cell effector functions (Pasquier et al. 2005). Gloudemans et al. suggested that under homeostatic conditions, secretory IgA promoted the maintenance of mucosal tolerance by inhibiting immune responses. Therefore, secretory IgA might be involved in preventing the induction of hyper-inflammatory responses towards foreign allergens that otherwise could induce allergic inflammation as showed in allergic asthma or rhinitis (Gloudemans et al. 2013). And CTB is known for suppressing allergic inflammation through the induction of secretory IgA (Smits et al. 2009). In this study, rCTB and rCTB-NAP spores significantly increased the fecal total secretory IgA 2 weeks after oral administration, and the OVA-specific IgA before mice were sacrificed was also significantly higher in recombinant spore-treated mice, which suggested that rCTB and rCTB-NAP spores can activate the mucosal immunity system, thereby inhibiting the inflammation of asthma.

Asthma is associated with changes in the expression of Th1- and Th2- related cytokines (Smits et al. 2009), including the decreased expression of IFN- $\gamma$  and IL-12 and the increased expression of TNF- $\alpha$ , IL-13, IL-4, IL-5, and IL-6 (Waserman et al. 2012). We found that the BALF IL-4 level was significantly decreased in rCTB and rCTB-NAP spore-treated mice compared to that in non-recombinant spore-treated mice, but the former mice produced significantly more IFN- $\gamma$ , indicating that rCTB and rCTB-NAP spores can induce Th1-related cytokines. In addition, the level of regulatory cytokine IL-10 in recombinant spore-treated mice was also significantly higher than that in non-recombinant spore-

treated mice. Compared to rCTB spore-treated mice, the rCTB-NAP spore-treated mice showed higher efficiency in increasing IFN- $\gamma$  and IL-10 and decreasing IL-4 in BALF, suggesting the potential of rCTB-NAP spores for the treatment of allergic diseases.

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs play a central role in controlling immune homeostasis and maintaining immune tolerance (Lin et al. 2008). Tregs regulate the development of allergic responses by several pathways. They block effector T cell migration into inflamed tissue, inhibit the activation of Th2 cells, suppress the production of IgE- and Th17- mediated inflammation, and induce IgG4 in B cells (Palomares et al. 2010; Baecher-Allan et al. 2004; Stelmaszczyk-Emmel et al. 2013). In this study, rCTB and rCTB-NAP spores significantly increased CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. Therefore, we hypothesize that this is one of the mechanisms whereby rCTB and rCTB-NAP spores inhibit OVA-induced asthma in mice.

To conclude, we have provided meaningful insights into the ability of *B. subtilis* spores bearing rCTB and rCTB-NAP in preventing OVA-induced asthma through the induction of IgA and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. Spores with the fusion protein rCTB-NAP were found to be particularly efficient in suppressing the inflammatory response in the respiratory system. These findings may provide insights into developing rCTB-NAP *B. subtilis* spores in treating allergic asthma and other allergic diseases. But the effect mechanism remains mostly unclear, which it is necessary to study further.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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