

RESEARCH LETTER – Biotechnology & Synthetic Biology

Systemic and mucosal pre-administration of recombinant *Helicobacter pylori* neutrophil-activating protein prevents ovalbumin-induced allergic asthma in mice

Shuai Zhou^{1,2}, Yanmei Huang¹, Bingshao Liang¹, Hui Dong¹, Shuwen Yao¹, Yinshuang Chen¹, Yongqiang Xie¹, Yan Long¹, Sitang Gong¹ and Zhenwen Zhou^{1,*}

¹Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 318 Renminzhong Road, Yuexiu, Guangzhou, Guangdong 510120, People's Republic of China and ²Translational Medicine Center, Guangdong Women and Children Hospital, No. 521 Xingnan Avenue, Panyu district, Guangzhou, Guangdong 511400, People's Republic of China

*Corresponding author: Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 318 Renminzhong Road, Yuexiu, Guangzhou, Guangdong 510120, People's Republic of China. Tel: 86208133 0638; E-mail: zzw6248@126.com

One sentence summary: Systemic and mucosal pre-administration of rNAP could suppress the development of OVA-induced asthma in mice.

Editor: Ezio Ricca

ABSTRACT

Purpose: Previous epidemiologic studies have demonstrated an inverse association between *Helicobacter pylori* infection and the frequency of allergic asthma. The neutrophil-activating protein (NAP) of *H. pylori* has been identified as a modulator possessing anti-Th2 inflammation activity. Here, we sought to determine whether systemic or mucosal pre-administration of recombinant *H. pylori* NAP (rNAP) could prevent ovalbumin (OVA)-induced allergic asthma in mice. **Methods:** Mice were exposed to purified rNAP through intraperitoneal injection or inhalation and then sensitized with OVA. Following a challenge with aerosolized OVA, the bronchoalveolar lavage fluid (BALF) cell count, lung tissue histology, BALF cytokines and serum IgE were evaluated. **Results:** Both intraperitoneal injection and inhalation of rNAP prior to OVA sensitization significantly reduced eosinophil accumulation and inflammatory infiltration in lung tissue in OVA-induced asthma mice; eosinophils were reduced in the BALF of rNAP-treated mice. In addition, IL-4 and IL-13 levels were lower ($P < 0.01$), IL-10 and IFN- γ levels were higher ($P < 0.01$) and IgE serum levels were lower ($P < 0.01$) in the treated groups compared to the control group. **Conclusions:** Systemic and mucosal pre-administration of rNAP could suppress the development of OVA-induced asthma in mice; rNAP may be utilized as part of novel strategies for the prevention or treatment of allergic diseases.

Keywords: allergic asthma; cytokines; *Helicobacter pylori*; inflammation; neutrophil-activating protein

INTRODUCTION

Helicobacter pylori is a gram-negative, microaerophilic, rod-shaped bacterium that colonizes the human stomach. *Helicobacter pylori* colonization is strongly associated with increased risk of several diseases such as duodenal ulcer, non-cardia gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Neumeister, Troppan and Raderer 2015). Interestingly, *H. pylori* infection is more prevalent in developing countries than in Western countries (Blaser 2006; Yamaoka 2008).

Helicobacter pylori gastric colonization is typically followed by mucosal infiltration of polymorphonuclear leukocytes, macrophages and T-helper type 1 (Th1) lymphocytes, with active production of interleukin (IL)-12 and interferon- γ (IFN- γ) (D'Elis et al. 1997).

Asthma is a clinical syndrome of intermittent respiratory symptoms characterized by chronic airway inflammation, which has become increasingly prevalent in developed countries over recent decades (Eder, Ege and von Mutius 2006; Usmani 2014). Allergic asthma is characterized by Th2 cytokines responses, Th2 cytokines (IL-4, IL-5, IL-9, IL-13) induce the changes in the airways and lung parenchyma such as IgE secretion, increased production of chemokines that attract mast cell, neutrophils, eosinophils, T cells to the lungs and airway hyperresponsiveness (Finkelman et al. 2010). The Th2 inflammation can be strongly inhibited by Th1 cytokines such as IL-12 and IFN- γ (Holgate and Polosa 2008). Most studies have found that *H. pylori* infection had a protective affect against allergy and asthma (Blaser, Chen and Reibman 2008; Chen and Blaser 2008; Khamechian et al. 2015; Taye et al. 2015). Since the elimination of *H. pylori* is greatly beneficial for curing peptic ulcer disease, gastric MALT lymphoma, and preventing gastric cancer, *H. pylori* infections should be treated, regardless of whether the patient is asthmatic (D'Elis and De Bernard 2010); this situation necessitates the search for alternative allergy prevention strategies.

Helicobacter pylori neutrophil-activating protein (HP-NAP) is a highly conserved protein among geographically distinct *H. pylori* strains (Montecucco and de Bernard 2003). It belongs to a broad superfamily of ferritin-like proteins, whose members mostly share a DNA-protective function under starvation conditions, such as oxidative or nutritional stress (Grant et al. 1998). Members of this family are homopolymers consisting of 12 four-helix bundle subunits containing iron ligands (Tonello et al. 1999; Zanotti et al. 2002). HP-NAP has been identified as a protective antigen against *H. pylori* infection; vaccination of mice with HP-NAP induces protection against subsequent *H. pylori* challenges (Fu 2014). It enhances endogenous IL-12 and IFN- γ responses and exerts potent anti-Th2 activity *in vivo*, targeting both IL-5-induced eosinophilia and IL-4-mediated hyper-IgE responses induced by parasitic infection (Del Prete et al. 2008; D'Elis et al. 2009). These results suggest that HP-NAP might be involved in the molecular mechanism underlying the inverse association between *H. pylori* infection and allergy. In this study, we investigated whether pre-intraperitoneal (IP) injection or pre-inhalation (IN) of recombinant HP-NAP (rNAP) could decrease airway inflammation in ovalbumin (OVA)-sensitized allergic asthma mice.

METHODS

Animals and treatment

This study was carried out in strict accordance with the Guide for Chinese Association for Laboratory Animal Sciences. Four-week-old specific-pathogen-free female BALB/c mice were

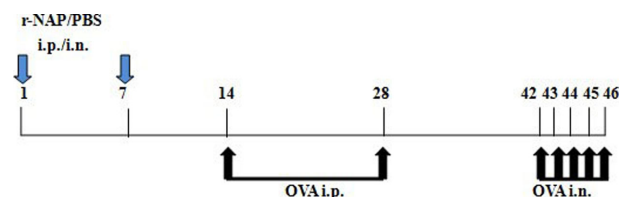


Figure 1. Time course for mouse treatment. rNAP was administered by IP injection or via IN. BALB/c mice (six mice per group) were treated with IP rNAP (50 μ g per mouse) (group ii) or with IN aerosolized rNAP for 20 min (group iv) daily on days 1 and 7. Control animals were subjected to injection (group iii) or IN (group v) with equal volumes of PBS. Mice in groups (i) to (v) were treated with OVA according to a standardized procedure: sensitized with OVA IP (100 μ g per mouse) on days 14 and 28 and then challenged with aerosolized OVA (1% in PBS) IN for 20 min daily on days 42–46.

Table 1. Experimental protocol for animal treatment.

Group	Pretreatment prior to sensitization	Establishment of asthma ma model
i (OVA)	None	Yes
ii (IP rNAP plus OVA)	IP 0.2 mL of NAP	Yes
iii (IP PBS plus OVA)	IP 0.2 mL of PBS	Yes
iv (IN rNAP plus OVA)	IN NAP for 20 min	Yes
v (IN PBS plus OVA)	IN PBS for 20 min	Yes
vi (Naïve)	None	None

Note: rNAP concentration was 0.25 mg/mL in PBS (pH 7.2).

purchased from Guangdong medical laboratory animal center (Guangzhou, China). The mice were housed in the animal care facility with food and water available at all times. Mice were exposed to a 12:12 hour light-dark cycle in room air (21% O₂) at room temperature. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol was approved by the Committee on the Ethics of Animal Experiments of Guangzhou Medical University.

The protocol for pre-administration and asthma induction were shown in Fig. 1. On days 1 and 7, Groups of mice were IP injection of 50 μ g of rNAP in 0.2 mL PBS (group ii), 0.2 mL PBS (group iii), or IN of 50 μ g of rNAP in 0.2 mL PBS (group iv), 0.2 mL PBS (group v); mice were subjected to IP with alum-adjutant OVA (100:1) on days 14 and 28. Subsequently, all animals were exposed to aerosolized OVA (1% in PBS) for 20 min daily from days 42 to 46. Six groups of mice (six mice per group) were pretreated as follows on days 1 and 7: (i) none; (ii) IP injection of rNAP; (iii) IP of phosphate-buffered saline; (iv) IN of rNAP; (v) IN of PBS; (vi) none (Naïve) (Table 1). Groups (ii) and (iv) were treated with 50 μ g of rNAP by either IP or IN for 20 min, respectively. All mice, except those in the naïve group (vi), were subjected to IP with alum-adjutant OVA (100:1) on days 14 and 28.

Bacterial strains and isolation of *Helicobacter pylori* genomic DNA

Helicobacter pylori strain GZCH1 was isolated from the gastric mucosa of a clinical patient (male, 9 years old); it was grown on Columbia blood agar plates plus antibiotics at 37°C for 4–5 days under microaerophilic conditions as previously described (DeLyria, Redline and Blanchard 2009). The *H. pylori* genomic DNA was isolated and purified as previously described (Zhou et al. 2015).

Escherichia coli BL21 (strain DE3) was cultured at 35°C in Lysogeny broth (LB) medium. *Escherichia coli* BL21 (strain DE3)

with plasmid pET28 was cultured in LB medium with 50 $\mu\text{g/mL}$ kanamycin.

Expression and purification of recombinant NAP

The HP-NAP (Genebank ID AF227072) gene was amplified with the following primers: forward primer: 5'-GCG GAATTCATGAAAACATTTGAAATTTTAAACA-3' (EcoRI site is underlined); reverse primer: 5'-AAAGCGCGCCTTAAGCTAAAT GGGCTTCTAG-3' (NotI site is underlined). *Helicobacter pylori* strain GZCH1 genomic DNA was used as the polymerase chain reaction (PCR) template. The PCR conditions were as follows: 94°C for 5 min; 33 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 60 s; and a final extension step of 72°C for 15 min. The purified amplified product was digested with EcoRI and NotI at 37°C for 60 min and ligated with EcoRI and NotI digested pET-28a(+) plasmid (Novagen, Madison, WI, USA) at 16°C overnight. The recombinant plasmid was transformed into *E. coli* BL21 (strain DE3) and verified by DNA sequencing. Expression of the recombinant protein was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37°C for 4 h. Cells were harvested at 5000 $\times g$ for 10 min at 4°C and sonicated. The recombinant protein in the supernatant of sonicated bacterial lysate was then purified with His Bind Purification kit according to the manufacturer's instructions (Novagen). NAP expression and purification efficiency were determined by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue G-250. The final concentration of the purified protein was determined using the bicinchoninic acid assay (Smith et al. 1985). rNAP was subjected to 12% SDS-PAGE and then electrotransferred to a polyvinylidene difluoride membrane for western blot analysis.

NAP antisera were obtained by subcutaneous immunization of a Sprague-Dawley rat with purified rNAP in Freund's adjuvant as previously described (Zhou et al. 2015). The NAP immunoglobulin G (IgG) antisera titer was 1:25 600 as determined by enzyme-linked immunosorbent assays (ELISA).

Blood collection and determination of total IgE levels

Mice were anesthetized 24 h after the final antigen challenge, and peripheral blood was drawn from the excised eyeballs. Total serum IgE levels were quantified by ELISA (Multi-Sciences Biotech Co., Ltd. Hangzhou, China). Serum samples were applied at a 1:40 dilution. Absorbance was measured at 450 nm with 630 nm as the reference wavelength. The final concentrations were determined based on a standard concentration curve ranging from 0 to 1600 ng/mL.

Cellular analysis of BALF

Following blood collection, mice were sacrificed. The right lungs of the mice were ligated and the tracheas were cannulated. The left lungs were washed three times with 0.5 mL of sterile PBS (fluid recovery must be >80%); after centrifugation, the BALF supernatants were aliquoted and stored at -80°C.

The different cell types and numbers in the BALF were determined following cytopsin preparations and Wright-Giemsa staining (BASO, Zhuhai, China). The percentage of eosinophils in the BALF was determined based on eosinophil cell counts per 500 cells according to staining characteristics and morphology.

Lung histopathology

Following the completion of BALF sampling, the right lungs were excised, fixed in 10% formalin and then embedded in paraffin for histological analysis; 5 μm sections were stained with hematoxylin and eosin (H&E) (Tournoy et al. 2000).

Cytokine assays in cell-free BALF supernatants

The levels of IL-4, IL-10, IL-13 and IFN- γ in BALF samples were measured using Mouse IL-4/IL-10/IL-13/IFN- γ ELISA Ready-SET-Go kits (Affymetrix, eBioscience, CA, USA) according to the manufacturer's instructions. Briefly, the ELISA plate was coated overnight with 1 $\times$ specific capture antibody in coating buffer at 4°C. The following day, 200 μL of blocking solution was added to each of the wells and the plate was incubated at 37°C for 1 h. Next, 100 μL of 2-fold serially diluted sample/standard were added to the plate and incubated for 2 h at 37°C, followed by the addition 100 μL of 1 $\times$ specific detection antibody IL4/IL-10/IL13/IFN- γ for 1 h. For detection of IL4/IL-10/IL13/IFN- γ levels, 100 μL of 1 $\times$ avidin-horseradish peroxidase was added to the plate for 30 min, followed by 100 μL of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution for 15 min. The detection reactions were stopped by the addition of 2 M H_2SO_4 , and the absorbance of each well was measured at 450 nm using TMB as the blank. All assays were conducted in duplicate. Cytokine levels were calculated using the standard curves generated with murine cytokines and analyzed using Curve Expert 1.3 software.

Statistical analysis

The results were expressed as mean values $\pm$ SD. Statistical significance among different groups (six mice per group) was calculated by one-way ANOVA to test the differences between the groups, followed by Dunnett's test to compare every experimental group (ii-v) including group Naïve (vi) with group (i). Results were considered statistically significant when P values were <0.05. Data were analyzed using SPSS for Windows version 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Expression and purification of rNAP

As expected, a fragment of approximately 435 bp was visible by agarose gel electrophoresis following amplification of *Helicobacter pylori* strain GZCH1 DNA (Fig. 2). The same size band was obtained following EcoRI/NotI double digestion of the pET-28a-rNAP plasmid. Sequencing of the recombinant plasmid revealed that the NAP open reading frame was in the frame with the protein tag (His) present on the vector. rNAP was expressed as a His-fusion protein in *Escherichia coli* following induction with IPTG (Fig. 3A). The major protein band corresponding to the expected 16.9 kDa recombinant NAP fusion protein was purified from supernatant of an IPTG-induced sonicated bacterial lysate using a Ni^{2+} -NTA purification column (Fig. 3B); rNAP was obtained at a concentration of 1.2 mg/mL from 250 mL of cell culture. Western blot analysis showed that the rat anti-rNAP sera reacted against rNAP extracted from the supernatant of an IPTG-induced sonicated bacterial lysate containing the pET28a-rNAP plasmid (Fig. 3C).

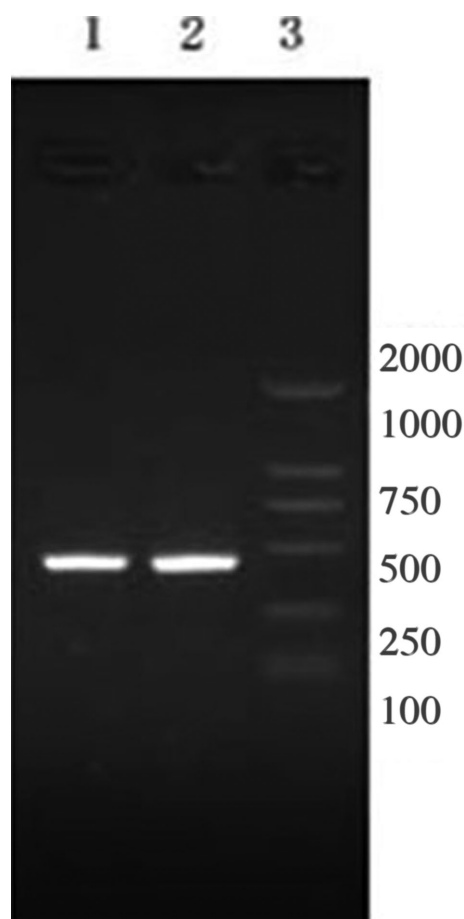


Figure 2. PCR amplification of the NAP gene. Lanes 1 and 2: HP-NAP gene PCR amplification product. Lane 3: DNA ladder, DL2 000.

rNAP inhibits OVA-induced Th2 airway inflammation

We next introduced rNAP into BALB/c mice by either IP injection or IN prior to sensitization with alum-adjuvant OVA and aerosolized OVA challenge. The levels of IL-4, IL-10, IL-13 and IFN- γ in the BALF and total IgE in serum were detected by ELISA. As shown in Fig. 4, most asthma indicators were strongly reduced in rNAP-treated mice. IL-4 and IL-13 levels were significantly lower in the BALF of groups (ii) and (iv) than in that of groups (i), (iii) and (v) ($P < 0.01$); the total serum IgE levels in these animals demonstrated a similar change (Fig. 5A). In contrast, the levels of IL-10 and IFN- γ were significantly higher in the BALF of groups (ii) and (iv) than in groups (i), (iii) and (v) ($P < 0.01$). No significant differences in cytokine and IgE levels were apparent between the IP and IN rNAP pre-treatment groups (ii and iv; $P > 0.05$).

Furthermore, BALF eosinophils were strongly reduced in rNAP-treated mice. The percentage of eosinophils was significantly lower in the BALF of groups (ii) and (iv) than in that of groups (i), (iii) and (v) ($P < 0.01$) (Fig. 5B). The level of eosinophils in systemic rNAP-treated mice (group ii) was similar to that in mucosal rNAP-treated mice (group iv), indicating that both systemic and mucosal pre-administration of rNAP could attenuate OVA-induced eosinophil infiltration in BALF.

Alleviation of pathological changes in the airways

To determine the therapeutic effects of rNAP treatment on OVA-induced lung pathology, the H&E stained lung specimens

were evaluated. Introduction of rNAP significantly alleviated the pathological changes in the murine airway of the mouse model (Fig. 6). Exposure to OVA alone (i) caused marked perivascular and peribronchial eosinophil infiltration and an accumulation of lymphocytes along with scattered bleeding sites in the sub-mucosa of the airway (Fig. 6i). As expected, alleviation of pathological symptoms was found in the lungs of mice in groups (ii) and (iv) (Fig. 6ii, iv). Both systemic and mucosal rNAP treatment caused slight bronchitis along with proliferation of bronchial cells and neutrophilic inflammation. However, groups (i), (iii) and (v) resulted in similar pathological alterations (Fig. 6i, iii, v).

DISCUSSION

Bronchial asthma is characterized by lung infiltration by different leukocytes, including Th2 cells and eosinophils that play a critical role in the development of allergic inflammation (Drazen, Arm and Austen 1996). A number of studies have demonstrated the safety of using recombinant or modified allergens in allergy treatment strategies; these strategies usually involve the use of novel adjuvants or immunomodulators to induce immune redirection and several potential compounds have been tested (Tighe et al. 2000; Freytag and Clements 2005).

The observed HP-NAP-induced immune deviation from the Th2 to Th1 response indicates that rNAP may hinder the pathogenic mechanism underlying allergy. Incubation of antigen-induced T-cell lines with HP-NAP resulted in a remarkable increase in the number of IFN- γ -producing T cells and decrease of IL-4-secreting cells, thus shifting the cytokine profile of antigen-activated human T cells from Th2 to a Th1 cytotoxic phenotype (Amedei et al. 2006). In this study, both pre-IP injection and IN of rNAP significantly increased the IFN- γ level in the BALF of treated mice, suggesting that system and mucosal pre-administration of rNAP in mice can drive Th2 to a Th1 response in the lung.

The recruitment and accumulation of tissue eosinophils during allergic inflammation depend not only on the release of selective chemokines (Lampinen et al. 2004), but also on cell activation induced by cytokines, including IL-4 and IL-13. IL-13, which shares a receptor component and signaling pathways with IL-4, has been shown to be necessary and sufficient for the expression of allergic asthma. IL-13 induces the pathophysiological features of asthma independently of IgE and eosinophils (Wills-Karp et al. 1998; Mabalirajan et al. 2013). Previous studies have reported that an increase in IL-13 activity was sufficient to induce high reactivity in the airway (Wills-Karp et al. 1998). This study shows that IL-4 and IL-13 levels were greatly reduced in the BALF of both rNAP pre-treated groups, suggesting the potential of rNAP for reducing inflammation in the lung.

DNA vaccination of HP-NAP could suppress OVA-induced asthma in mice. Muscle injection of a pcDNA3.1-sIL-4R-NAP plasmid, which encoding fusion protein of the murine soluble IL-4 receptor (sIL-4R) and HP-NAP, significantly inhibited airway inflammation, decreased the serum OVA-specific IgE levels and remodeled the Th1/Th2 balance. Moreover, pcDNA3.1-sIL-4R-NAP plasmid is more effective on antiasthma therapy comparing with plasmid only expressing sIL-4R (Liu et al. 2016), which indicated that HP-NAP could enhance the efficacy of sIL-4R in anti-asthma therapy.

A previous study of rNAP protein on the asthma treatment showed systemic (IP) administration of HP-NAP at the same time of OVA sensitization strongly inhibit the development of airway eosinophilia and bronchial inflammation, and reduce the production of IL-4, IL5 and GM-CSF in BALF (Codolo et al. 2008).

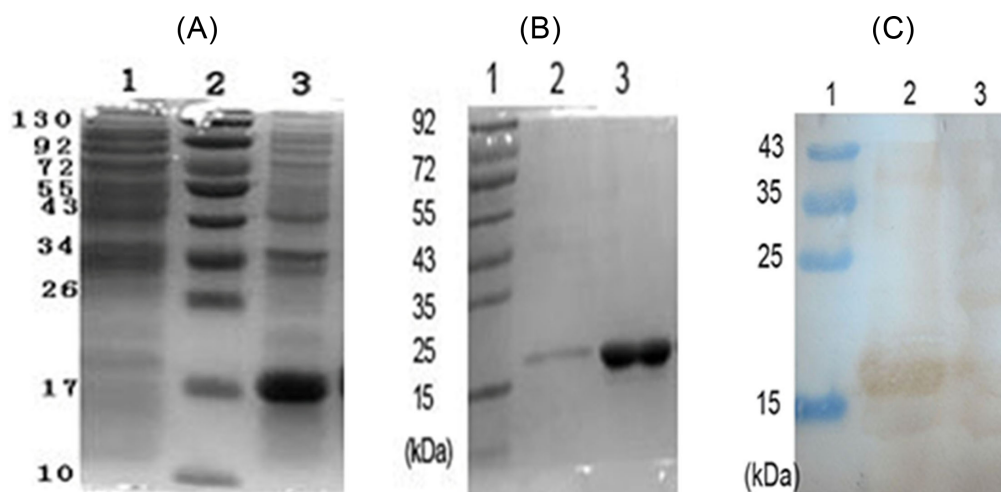


Figure 3. Twelve per cent SDS-PAGE and western blot analysis of recombinant NAP in *E. coli* BL21 following IPTG induction. (A) Coomassie blue stained 12% SDS-PAGE of recombinant proteins. Lane 1: lysate from bacteria transformed with parental plasmid pET-28a(+). Lane 2: protein ladder. Lane 3: lysate from bacteria transformed with plasmid pET-28a-rNAP. (B) Coomassie blue stained 12% SDS-PAGE of purified rNAP. Lane 1: protein ladder, Lanes 2 and 3: purified rNAP. (C) Western blot analysis of recombinant NAP using NAP-specific rat antisera. Lane 1: protein ladder (kDa). Lane 2: HP-NAP reacted with rat anti-NAP sera. Lane 3: HP-NAP reacted with rat pre-immune sera.

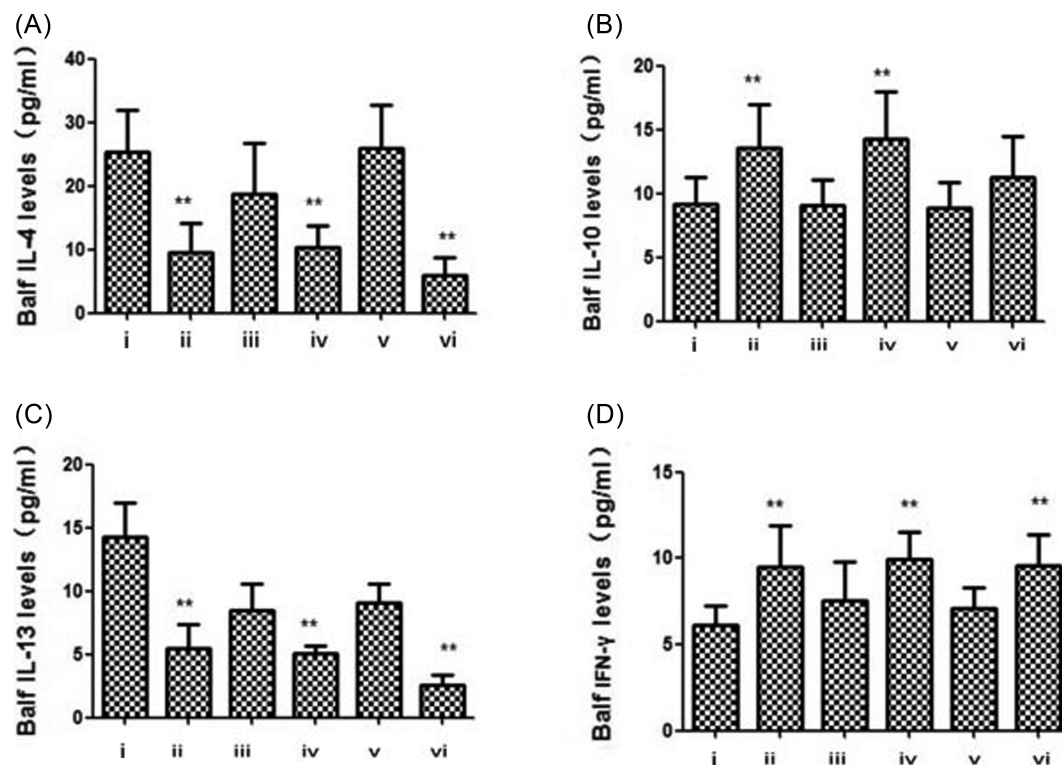


Figure 4. rNAP reduced Th2 cytokine secretion in BALF. Cytokines were assayed in cell-free BALF supernatants by ELISA. Values are presented as mean $\pm$ SD (six mice per group). Statistical significance among different groups was calculated by one-way ANOVA and Dunnett's test. * $P < 0.05$, ** $P < 0.01$ versus group (i). groups: (i) OVA; (ii) IP rNAP plus OVA; (iii) IP PBS plus OVA; (iv) IN rNAP plus OVA; (v) IN PBS plus OVA; (vi) Naïve.

Here, we used different treatment strategies with systemic pre-administration of HP-NAP in mice 2 weeks prior to OVA sensitization, pre-IP injection of rNAP also significantly reduced lung eosinophil accumulation and inflammatory infiltration, decreased IL-4, IL-13 levels and increased IFN- γ levels in BALF, and furthermore, significantly increased the IL-10 level. Mucosal (IN) pre-administration of rNAP could also suppress the development of OVA-induced asthma in mice, suggesting that rNAP could be used not only for the treatment but also for the preven-

tion of allergic asthma. However, future studies will be required to determine whether rNAP treatment results in side effects and toxicity in other animal or human organs.

IL-10 plays an important role in T-cell tolerance to allergens; IL-10-producing T regulatory cells (Tregs; Tr1cells) are linked to an antigen-specific suppressor function (Akdis et al. 1998). The IL-10 levels in BALF determined in this study were significantly higher in the rNAP-treated mice groups than in the control mice, indicating that IP injection or IN of rNAP can increase

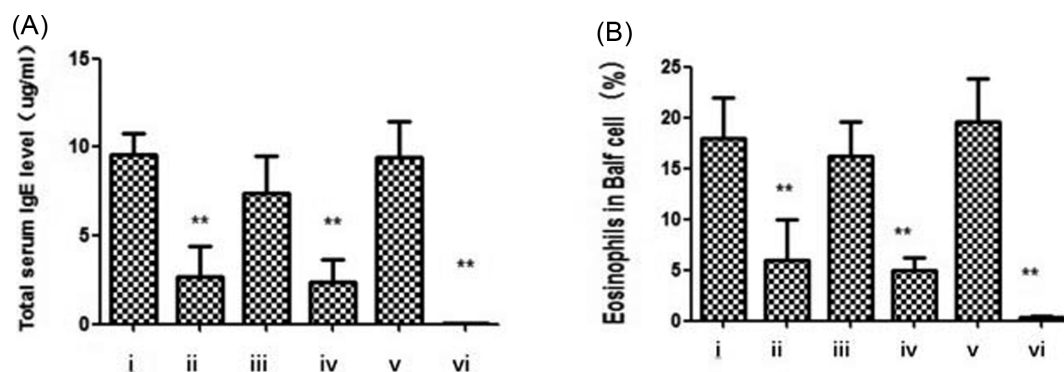


Figure 5. rNAP reduced both serum IgE levels and BALF eosinophils. Blood and BALF were taken from animals following the final OVA challenge. (A) Levels of total serum IgE were assessed by ELISA. (B) The percentage of eosinophils in the BALF was determined based on eosinophil cell counts per 500 cells according to staining characteristics and morphology. Values are presented as mean $\pm$ SD (six mice per group). Statistical significance among different groups was calculated by one-way ANOVA and Dunnett's test. * $P < 0.05$, ** $P < 0.01$ versus group (i). Groups: (i) OVA; (ii) IP rNAP plus OVA; (iii) IP PBS plus OVA; (iv) IN rNAP plus OVA; (v) IN PBS plus OVA; (vi) Naïve.

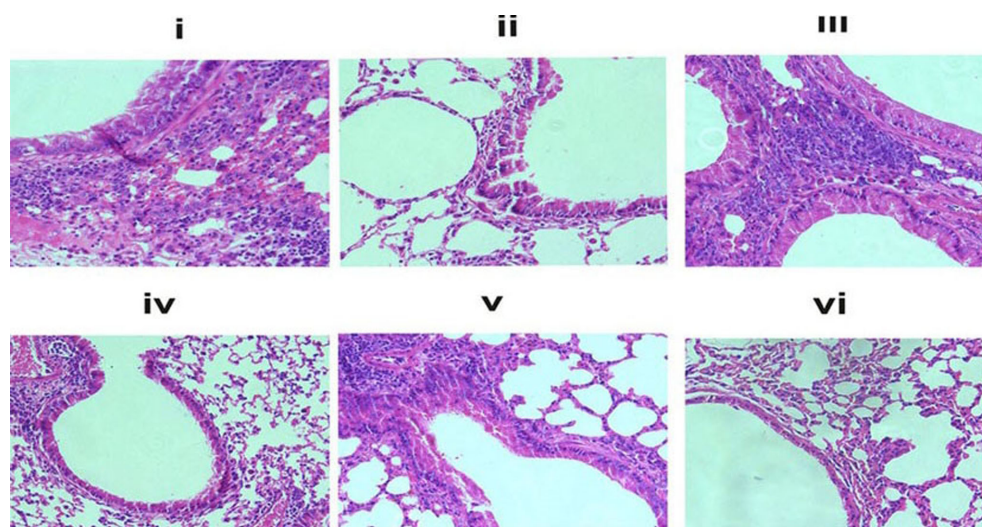


Figure 6. H&E staining at $\times 200$ magnification. Marked perivascular and peribronchial eosinophil infiltration and accumulation of lymphocyte together with scattered bleeding sites are found in the NAP-untreated groups (i, iii, v). Very low infiltration of eosinophils and lymphocytes into the airway submucosa is apparent in the NAP-treated groups (ii, iv). Groups: (i) OVA; (ii) IP rNAP plus OVA; (iii) IP PBS plus OVA; (iv) IN rNAP plus OVA; (v) IN PBS plus OVA; (vi) Naïve.

IL-10 in BALF. HP-NAP is thought to interact with Tregs through the CD25 surface receptor; the signal is then relayed inside the Tregs, which leads to expression and activation of the itch response (Sehrawat, Sinha and Saxena 2015); however, the mechanism underlying this response requires further elucidation. We hypothesize that rNAP treatment might increase CD4+CD25+ Tregs, which in turn increases IL-10 in BALF. Further studies will be required to evaluate the levels of CD4+CD25+ Tregs in rNAP-treated mice by flow cytometry.

Inflammation, particularly eosinophilic inflammation, is a hallmark of asthma. In this study, an OVA-induced asthma model was established; following OVA challenge, the mice exhibited eosinophil infiltration in the lung interstitium and BALF. rNAP pre-treatment could reduce eosinophil infiltration in the lung, as indicated by decreased eosinophil levels in the BALF, and alleviated pathological changes in the murine airway.

Additional questions that need to be addressed include the molecular mechanisms or pathways involved in the rNAP effect and Th2 inflammation in allergic asthma, the underlying mechanism and pathways involved in the reduction of eosinophils in the BALF of rNAP-treated mice, and the possible interaction between rNAP and endogenous NAP present in the animals.

In summary, rNAP demonstrating high immunogenicity and immunoreactivity was expressed and purified using a prokaryotic expression system. Both systemic and mucosal pre-administration of rNAP inhibited lung eosinophil and Th2 inflammation in a mouse model of allergic asthma. We suggest that rNAP could be used as a candidate for novel strategies for the prevention or treatment of allergic diseases.

ACKNOWLEDGEMENTS

We wish to thank Henry Ehrlich for his assistance in revising this manuscript.

FUNDING

This work was supported by funding from the National Natural Science Foundation of China (no. 30801054 and no. 81301758), Natural Science Foundation of Guangdong (no. 8451012001001570 and no. 9151012001000009), Science and Technology Foundation of Guangdong (no. 2014A020212013 and no. 2016A020215013), science and Technology Foundation

of Guangzhou (no. 201605121046328), and the Medical Health Science and Technology Foundation of Guangzhou (no. 201102A212013, 20131A011053 and 2008-YB-067).

Conflict of interest. None declared.

REFERENCES

- Akdis CA, Blesken T, Akdis M et al. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;**102**:98–106.
- Amedei A, Cappon A, Codolo G et al. The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. *J Clin Invest* 2006;**116**:1092–101.
- Blaser MJ. Who are we? Indigenous microbes and the ecology of human diseases. *EMBO Rep* 2006;**7**:956–60.
- Blaser MJ, Chen Y, Reibman J. Does *Helicobacter pylori* protect against asthma and allergy? *Gut* 2008;**57**:561–67.
- Chen Y, Blaser MJ. *Helicobacter pylori* colonization is inversely associated with childhood asthma. *J Infect Dis* 2008;**198**:553–60.
- Codolo G, Mazzi P, Amedei A et al. The neutrophil-activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in ovalbumin-induced allergic asthma. *Cell Microbiol* 2008;**10**:2355–63.
- D'Elios MM, Codolo G, Amedei A et al. *Helicobacter pylori*, asthma and allergy. *FEMS Immunol Med Mic* 2009;**56**:1–8.
- D'Elios MM, de Bernard M. To treat or not to treat *Helicobacter pylori* to benefit asthma patients. *Expert Rev Respir Med* 2010;**4**:147–50.
- D'Elios MM, Manghetti M, De Carli M et al. T helper 1 effector cells specific for *Helicobacter pylori* in the gastric antrum of patients with peptic ulcer disease. *J Immunol* 1997;**158**:962–7.
- DeLyria ES, Redline RW, Blanchard TG. Vaccination of mice against *H. pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology* 2009;**136**:247–56.
- Del Prete G, Chiumiento L, Amedei A et al. Immunosuppression of TH2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* neutrophil-activating protein. *J Allergy Clin Immunol* 2008;**122**:908–13.
- Drazen JM, Arm JP, Austen KF. Sorting out the cytokines of asthma. *J Exp Med* 1996;**183**:1–5.
- Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med* 2006;**355**:2226–35.
- Finkelman FD, Hogan SP, Khurana Hershey GK et al. Importance of cytokines in murine allergic airway disease and human asthma. *J Immunol* 2010;**184**:1663–74.
- Fu HW. *Helicobacter pylori* neutrophil-activating protein: from molecular pathogenesis to clinical applications. *World J Gastroenterol* 2014;**20**:5294–301.
- Freytag LC, Clements JD. Mucosal adjuvants. *Vaccine* 2005;**23**:1804–13.
- Grant RA, Filman DJ, Finkel SE et al. The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol* 1998;**5**:294–303.
- Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008;**8**:218–30.
- Khamechian T, Movahedian AH, Ebrahimi Eskandari G et al. Evaluation of the correlation between childhood asthma and *Helicobacter pylori* in Kashan. *Jundishapur J Microbiol* 2015;**8**:e17842.
- Lampinen M, Carlson M, Hakansson LD et al. Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* 2004;**59**:793–805.
- Liu X, Fu G, Ji ZY et al. A recombinant DNA plasmid encoding the sIL-4R-NAP fusion protein suppress airway inflammation in an OVA-induced mouse model of asthma. *Inflammation* 2016;**39**:1434–40.
- Mabalarajan U, Ahmad T, Rehman R et al. Baicalein reduces airway injury in allergen and IL-13 induced airway inflammation. *PLoS One* 2013;**8**:e62916.
- Montecucco C, de Bernard M. Molecular and cellular mechanisms of action of the vacuolating cytotoxin (VacA) and neutrophil-activating protein (HP-NAP) virulence factors of *Helicobacter pylori*. *Microbes Infect* 2003;**5**:715–21.
- Neumeister P, Troppan K, Raderer M. Management of gastric mucosa-associated lymphoid tissue lymphoma. *Dig Dis* 2015;**33**:11–8.
- Sehrawat A, Sinha S, Saxena A. *Helicobacter pylori* neutrophil-activating protein: a potential Treg modulator suppressing allergic asthma? *Front microbial* 2015;**6**:493.
- Smith PK, Krohn RI, Hermanson GT et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;**150**:76–85.
- Taye B, Enquesselassie F, Tsegaye A et al. Is *Helicobacter Pylori* infection inversely associated with atopy? A systematic review and meta-analysis. *Clin Exp Allergy* 2015;**45**:882–90.
- Tighe H, Takabayashi K, Schwartz D et al. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000;**106**:124–34.
- Tonello F, Dundon WG, Satin B et al. The *Helicobacter pylori* neutrophil-activating protein is an iron-binding protein with dodecameric structure. *Mol Microbiol* 1999;**34**:238–46.
- Tournoy KG, Kips JC, Schou C et al. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 2000;**30**:79–85.
- Usmani OS. Small airways dysfunction in asthma: evaluation and management to improve asthma control. *Allergy Asthma Immunol Res* 2014;**6**:376–88.
- Wills-Karp M, Luyimbazi J, Xu X et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;**282**:2258–61.
- Yamaoka Y. *Helicobacter pylori*: Molecular Genetics and Cellular Biology. Wymondham, UK: Caister Academic Press 2008; 261.
- Zanotti G, Papinutto E, Dundon W et al. Structure of the neutrophil-activating protein from *Helicobacter pylori*. *J Mol Biol* 2002;**323**:125–30.
- Zhou ZW, Gong S, Li XM et al. Expression of *Helicobacter pylori* urease B on the surface of *Bacillus subtilis* spores. *J Med Microbiol* 2015;**64**:104–10.