

ORIGINAL ARTICLE

A Recombinant DNA Plasmid Encoding the sIL-4R-NAP Fusion Protein Suppress Airway Inflammation in an OVA-Induced Mouse Model of Asthma

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Abstract—Asthma is a chronic inflammatory airway disease. It was prevalently perceived that Th2 cells played the crucial role in asthma pathogenesis, which has been identified as the important target for anti-asthma therapy. The soluble IL-4 receptor (sIL-4R), which is the decoy receptor for Th2 cytokine IL-4, has been reported to be effective in treating asthma in phase I/II clinical trial. To develop more efficacious anti-asthma agent, we attempt to test whether the *Helicobacter pylori* neutrophil-activating protein (HP-NAP), a novel TLR2 agonist, would enhance the efficacy of sIL-4R in anti-asthma therapy. In our work, we constructed a pcDNA3.1-sIL-4R-NAP plasmid, named PSN, encoding fusion protein of murine sIL-4R and HP-NAP. PSN significantly inhibited airway inflammation, decreased the serum OVA-specific IgE levels and remodeled the Th1/Th2 balance. Notably, PSN is more effective on anti-asthma therapy comparing with plasmid only expressing sIL-4R.

KEY WORDS: asthma; HP-NAP; sIL-4R; Th1/Th2.

INTRODUCTION

Asthma is a common inflammatory respiratory disease characterized by airway hyper-responsiveness, increased infiltration of eosinophils in the lung tissue, and elevated allergen-specific IgE serum level [1,2]. The pathogenesis of asthma is complex and has not been fully

elucidated. It was prevalently perceived that Th2 cells played the crucial role in asthma pathogenesis and the pathway of interleukins derived from Th2 cells has been identified as the important target for anti-asthma therapy [3,4]. IL-4 is a typical Th2 cytokine which involved in inducing the IgE isotype switch, increasing the expression of VCAM-1 on endothelium, enhancing the production of mucus, promoting the activation of 15-lipoxygenase, and leading to the secondary synthesis of IL-4, IL-5, and IL-13 in the progress of asthma [5,6]. Therefore, many biologics were designed to target the IL-4/IL-4 receptor, such as soluble IL-4 receptor (sIL-4R), neutralizing antibodies specific for IL-4, antibodies or chimeric proteins for IL-4 receptor, and so on [7]. sIL-4R is a soluble form of IL-4 receptor α chain, which lacks of transmembrane and intracellular domains. sIL-4R is unable to activate IL-4 receptor-mediated intracellular signaling pathways whereas it reserves high affinity to IL-4 and can antagonize IL-4 as a decoy receptor [8]. The efficiency of sIL-4R in anti-asthma therapy has been assessed in mouse asthma model and two independent early-phase clinical studies. sIL-4R

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significantly reduced late phase pulmonary inflammation, blocked airway eosinophil infiltration, and decreased VCAM-1 expression in murine allergen-challenge asthma model [9]. And in the context of inhaled steroid withdrawal, administration of sIL-4R improved forced expiratory volume in 1 s (FEV1) and reduced asthma symptom scores of patients with moderate persistent asthma, compared to placebo in the randomized controlled trials[6,10].

Recently, the effect of the toll-like receptor (TLR) agonist on anti-asthma therapy attracted more attention from the scientists, since it was able to skew the balance of Th1/Th2 in asthma pathogenesis [11,12]. The *Helicobacter pylori* neutrophil-activating protein (HP-NAP) has been documented as an effective TLR2 agonist [13]. HP-NAP is capable to stimulate neutrophils and monocytes to express IL-12 and IL-23 and can shift antigen-induced T cell lines from a polarized Th2 to Th1 [14,15]. Moreover, HP-NAP has been reported to be a potential immune modulating agent for the treatment of ovalbumin-induced allergic asthma, *Trichinella spiralis* infection, and the growth of tumor by skewing the Th1/Th2 balance [16,17].

To develop more efficacious anti-asthma agent, in our work, we constructed the pcDNA3.1-sIL-4R-NAP plasmid-encoding fusion protein of sIL-4R and HP-NAP, named PSN. Furthermore, the anti-asthma efficacy of PSN was evaluated in an OVA-induced mouse model of asthma. PSN significantly inhibited airway inflammation, decreased the serum OVA-specific IgE levels, and remodeled the Th1/Th2 balance. Notably, PSN was more effective on anti-asthma therapy in comparing with plasmid only expressing sIL-4R (named PS).

MATERIALS AND METHODS

Animals

Female BALB/c mice between 6 and 8 weeks of age were purchased from Vital River Laboratories (Beijing, China) and maintained under specific pathogen-free conditions. Experiments were carried out according to the University guidelines for the care and use of laboratory animals.

Plasmid Construction and DNA Preparation

To construct pcDNA3.1-sIL-4R-NAP plasmid, named PSN (Fig. 1a), first the coding sequence of Linker-HP-NAP gene was cloned into pcDNA3.1(+) vector by using specific primers (sense: 5'-AAGGATCCGGTGGATCAGGAATGAAAACATTT-3', containing a *Bam*HI restriction site and

partial linker peptide GGSG coding sequence; antisense: 5'-AACTCGAGTTAAGCTAAATGGGCTTGCAGC-3', containing a *Xho*I restriction site), then the murine sIL-4R gene was cloned into pcDNA3.1-Linker-NAP plasmid by using specific primers (sense, 5'-ATAAGCTTACCATGGGGCGGCTTTGCACCAAGTTCCT-3', containing a *Hind*III restriction site; antisense: 5'-AAGGATCCGCGCTGTATCAGGGGCGAGCTGGAAGT-3', containing a *Bam*HI restriction site which also served as the partial linker peptide GS coding sequence). To construct the pcDNA3.1-sIL-4R plasmid, named PS, the murine sIL-4R gene was cloned into pcDNA3.1(+) vector by using specific primers (sense 5'-ATAAGCTTACCATGGGGCGGCTTTGCACCAAGTTCCT-3', containing a *Hind*III restriction site; antisense 5'-AAGGATCCCTAGCGCTGTATCAGGGGCGAGCTGGAAGT-3', containing a *Bam*HI restriction site). All clones were sequenced by double-stranded sequencing (Invitrogen, San Diego, CA, USA). Plasmid DNA was prepared using GoldHi EndoFree Plasmid Maxi kits (CWBI, Beijing, China) and resuspended in sterile normal saline.

RT-PCR Analysis

COS-7 cells were plated in a 6-well plate at 2×10^5 cells per well 12 h before transfection. Then transfected with the mixture of 6 μ l FuGENE6 Transfection Reagent (Roche Company, Basel, Switzerland) and 2 μ g plasmid (PSN or empty pcDNA3.1(+) plasmid) according to manufacturer's instructions. Cells were collected at 48 h after transfection and total RNA was isolated using RNAiso Plus reagent (Takara, Dalian, China). First strand cDNA synthesis and touchdown PCR were performed. The primers used in RT-PCR were 5'-ATGGGGCGGCTTTGCACCAAGTTCCT-3' (sense) and 5'-TTAAGCTAAATGGGCTTGCAGCAT CCAAATGG-3' (antisense) for murine sIL-4R-NAP gene (1152 bp); 5'-GCCATCCTGCGTCTGGACCTGGCTG-3' (sense) and 5'-CGGACTCATCGTACTCCT GCTTGCTGATCC-3' (antisense) for murine β -actin gene (577 bp).

Western Blot Analysis

COS-7 cells were transfected with PSN or empty pcDNA3.1(+) plasmid as described above. The culture supernatant and cell lysate were collected at 48 h after transfection. The protein from the culture supernatant and cell lysate were separated by denaturing SDS-PAGE (20 μ g total protein/lane) and then transferred to nitrocellulose membrane (DingGuo Biotech.Co., Zhengzhou, China). The protein bands were probed with antibodies

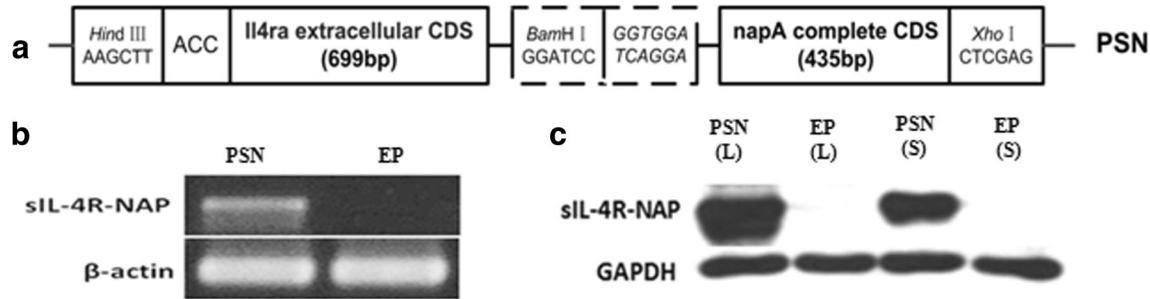


Fig. 1. Plasmid DNA construction and fusion protein expression *in vitro*. Murine sIL-4R gene and HP-NAP gene were linked by a flexible peptide (GSGGSG) coding sequence and cloned into pcDNA3.1(+) vector to generate the fusion protein expression plasmid pcDNA3.1-sIL-4R-NAP, named PSN (a). The sIL-4R-NAP mRNA (1152 bp) expression in COS-7 cells was detected by qRT-PCR; COS-7 cells were transfected with PSN and empty pcDNA3.1(+) plasmid (named EP), respectively (b). The sIL-4R-NAP protein expression was detected by Western blot, the lysates (L), and culture medium supernatant (S) of COS-7 cells transfected with PSN and EP, respectively (c).

against HP-NAP (1:8000) and GAPDH (1:8000), and then visualized using HRP-conjugated secondary antibody (1:8000) and a chemiluminescence detection system. Rabbit anti-HP-NAP polyclonal antibody was manufactured by Zhoushan Tongsheng Biotechnology Company (Zhejiang, China). Antibody against GAPDH and secondary antibody were purchased from BOSTER Biotechnology Company (Wuhan, China).

Asthma Induction and Plasmid Administration

All endotoxin-free DNA constructs (PSN DNA, PS DNA, and empty pcDNA3.1(+) plasmid DNA) for injection were prepared in normal saline at a concentration 100 µg/100 µl. The protocol for asthma induction and plasmid administration were shown in Fig. 2a. On days 0, 7, and 14, mice were sensitized by intraperitoneal (i.p.) injection with 100 µl OVA solution (20 µg OVA with 2 mg alum in 100 µl normal saline). Then OVA-sensitized mice

were divide into three groups randomly (five mice each group) and were administrated with three different DNA constructs (100 µg in 100 µl normal saline) mentioned above by intramuscular (i.m.) injection on days 21 and 28. From day 42 the OVA sensitized mice were challenged by aerosolized 1 % OVA solution (0.3 g OVA in 30 ml normal saline) for 30 min each day over a period of five consecutive days in a plexiglas chamber (10 × 15 × 25 cm). Mice were sacrificed 24 h after the last OVA challenge. The mice sensitized, administrated, and challenged with normal saline were used as normal control.

Bronchoalveolar Lavage and Lung Histology

Mice were anesthetized by i.p. injection of pentobarbital sodium solution (Merck Serono, Beijing, China). Tracheas were surgically exposed and the lungs were lavaged three times with 0.8 ml cold PBS. Bronchoalveolar lavage fluid (BALF) was collected and

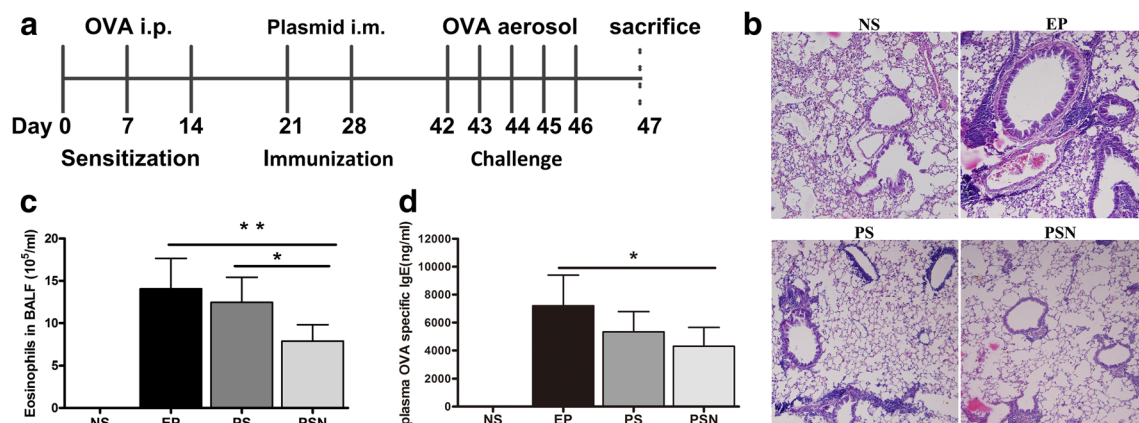


Fig. 2. PSN suppresses lung inflammation and the serum OVA-specific IgE production. Schematic representation of the setting of asthma model and plasmid DNA treatment (a). Histopathological features of lung tissue (b), 200×. The number of eosinophils in BALF (c). OVA-specific IgE levels in serum (d). Values were presented as mean ± SEM. **p* < 0.05, ***p* < 0.01.

centrifuged at 4 °C for 10 min at 1000 rpm. The BALF cell smears were prepared and stained with Wright-Giemsa. At least 300 white blood cells were examined on a smear to obtain the absolutely number of the eosinophils in BALF. For histopathology analysis, the lungs of mice were harvested, fixed in 10 % formalin phosphate buffer, paraffin-embedded, sectioned at 5 µm, and stained with hematoxylin and eosin.

Cytokines and IgE ELISA

IL-4 and IFN-γ levels in the supernatant of BALF were assessed using Mouse ELISA kits (Biolegend, San Diego, CA, USA). OVA-specific IgE level in plasma was detected by ELISA kits (Biolegend, San Diego, CA, USA) according to manufacturer's instructions.

Quantitative Reverse-Transcription PCR

Total RNA was extracted from lung tissues using RNAiso Plus reagent (Takara, Dalian, China). The cDNA was synthesized with the use of 1 µg RNA by PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The quantitative reverse-transcription PCR (qRT-PCR) analysis was performed with the Roche LightCycler 1.5 Real-Time PCR System using SYBR Green Perfect Real Time kit (Takara, Dalian, China) according to manufacturer's instructions. The level of gene expression was normalized against murine β-actin and relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method. A list of the qRT-PCR primers used in the present study was given in table S1.

Statistical Analysis

All the values were presented as mean ± SEM. The statistical significance of differences between experimental groups was analyzed using GraphPad Prism 5.01 (unpaired two-tailed Student's *t* test). A *p* < 0.05 was considered to be statistically significant, and *p* < 0.01 was considered to be very statistically significant.

RESULTS

Expression of the sIL-4R-NAP Gene *In Vitro*

COS-7 cells were transfected with PSN or empty pcDNA3.1(+) plasmid, respectively. The expression of sIL-4R-NAP gene in transiently transfected COS-7 cells was detected. The results of qRT-PCR (Fig. 1b) and Western blot (Fig. 1c) showed that the fusion protein sIL-

4R-NAP was effectively expressed in the cytoplasm of COS-7 cells and can be secreted to extracellular space.

PSN Suppress Lung Inflammation and IgE Production in OVA-Induced Mouse Model of Asthma

The time schedule of sensitization, plasmids administration and challenge in mice was shown in Fig. 2a. The OVA-sensitized mice were divided into three groups randomly, administrated with three different plasmid DNA including pcDNA3.1-sIL-4R-NAP plasmid DNA (named PSN group), pcDNA3.1-sIL-4R plasmid DNA (named PS group), and empty pcDNA3.1(+) plasmid DNA (named EP group), and then challenged with OVA. And the mice sensitized, administrated, and challenged with normal saline were used as normal control, named NS group. The asthma model has been successfully established in OVA-induced mice, which appeared the typical symptoms of asthma attack, such as fast and deep breathing, quietness and stillness, arching back, and so on. After treating with PSN/PS, the symptoms have been significantly alleviated. On day 47, all four groups of mice were sacrificed and the histological assessment of lung tissue, the counts of eosinophils in BALF and the detection of OVA-specific IgE in plasma were carried out. As shown in Fig. 2b, the airway wall thickening and inflammatory cell infiltration in lung tissue were effectively inhibited in PSN group compared to EP group. And the number of eosinophils (Fig. 2c), which was one of the key infiltrated inflammatory cells in the lung of asthma mice [18], were significantly decreased in the PSN group ($7.87 \pm 0.87 \times 10^5/\text{ml}$) compared to the EP group ($14.06 \pm 1.61 \times 10^5/\text{ml}$, *p* < 0.01) and the PS group ($12.46 \pm 1.32 \times 10^5/\text{ml}$, *p* < 0.05). Moreover, the serum OVA-specific IgE levels were dramatically decreased in PSN group comparing with the EP group (*p* < 0.05) (Fig. 2d). It was noteworthy that the PSN was more effective in inhibiting the airway inflammation than PS.

PSN Remodel Th1/Th2 Balance in OVA-Induced Mouse Model of Asthma

In order to further investigate the potential effect of PSN on remodeling of Th1/Th2 cytokine balance in asthma mice, the expression levels of the typical Th2 cytokine IL-4 and the representative cytokine of Th1, IFN-γ, in BALF was measured by ELISA. As shown in Fig. 3, the expression level of IL-4 was substantially increased in all OVA-induced groups of mice, comparing with NS group. After administrating with plasmid, the IL-4 expression was dramatically reduced in the PSN group ($26.63 \pm 2.30 \text{ pg/ml}$), compared to the EP group ($60.74 \pm 9.63 \text{ pg/ml}$, *p* < 0.01).

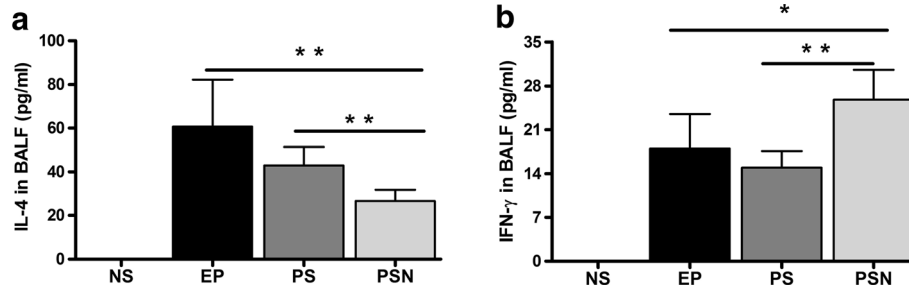


Fig. 3. Th1/Th2 cytokine production in BALF. Amounts of IL-4 (a) and IFN- γ (b) in BALF were measured by ELISA. Values were presented as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$.

Moreover, the expression of IFN- γ in BALF was effectively elevated in the PSN group mice (25.81 ± 2.14 pg/ml), compared with the EP group (18 ± 2.46 pg/ml, $p < 0.05$). Notably, the PSN was more effective on remodeling Th1/Th2 balance in OVA-induced mouse model of asthma than PS.

The Th17/Treg Pathway is not Affected by PSN in OVA-Induced Mouse Model of Asthma

Th17 cells and Treg cells have been demonstrated as the critical player in asthma [19,20]. And the Th17/Treg and Th1/Th2 imbalance coexisted in patients with allergic asthma [21]. Treg cells can downregulate the Th2 cells function by secreting IL-10 through FoxP3-mediated signaling pathway [22]. Th17 cells played a positive role in recruiting the neutrophil to the lung tissue by releasing IL-17, which lead to an increased production of neutrophil chemoattractants, such as CXCL5 [23]. In our work, we further estimated whether the anti-asthma effect of PSN was associated with the Th17/Treg cells function. As shown in Fig. 4, qRT-PCR results showed that the mRNA expression level of IL-10, Foxp3, IL-17, and CXCL5 in the lungs was not affected by administrating of PSN.

DISCUSSION

Asthma is a complex and chronic airway inflammatory disease, which affects more than 300 million people worldwide, and the prevalence of asthma has increased [24]. New drugs are needed because those that are currently in use are not always effective in clinical treatment. Th2 cell-mediated immune response is critically important in the development of allergic asthma. Many anti-asthma agents were developed to target Th2 cytokine signaling

pathways and they would likely be approved for use in the near future [25,26]. sIL-4R was decoy receptor for IL-4, which acted as the effective agent for anti-asthma therapy in phase I/II clinical trial [6,10]. However, the efficacy of sIL-4R has been reported to be limited in further clinical trials, the reason was mainly attributed to the complexity and the redundancy of Th2 cytokine signaling pathways [27,28]. Therefore, in our work, we attempted to use the TLR2 agonist HP-NAP, which is a novel immunomodulator possessing the potential on skewing immune deviation from the Th2 to Th1 phenotype, to improve the efficacy of sIL-4R in anti-asthma therapy [17,29]. In the present study, we constructed the PSN-encoding sIL-4R-NAP fusion protein and studied the anti-asthma effect of PSN on OVA-induced mouse model of asthma. The data showed that with the administration of PSN, the degree of inflammatory cells infiltration in the lung was reduced obviously and the OVA-specific IgE level in serum decreased significantly, comparing with the administration of EP (* $p < 0.05$ for PSN versus EP). Notably, PSN was more effective than PS on reducing eosinophil number in BALF (* $p < 0.05$ for PSN versus PS) and decreasing OVA-specific IgE level in serum. Moreover, the ELISA assay results showed that the level of IL-4 in BALF was dramatically downregulated in the PSN group, comparing with the EP group (** $p < 0.01$) and the PS group (** $p < 0.01$). And the level of IFN- γ in BALF was significantly upregulated in the PSN group, comparing with the EP group (* $p < 0.05$) and the PS group (** $p < 0.01$). These data suggested that PSN was effective in reversing the imbalance of Th1/Th2 in asthma pathogenesis and better than PS.

Recently, the important roles of many more immune cell subtypes in asthma pathogenesis were identified [21]. It has been documented that the moderate and severe asthma phenotypes were associated with increased number of neutrophils infiltration and level of IL-17 [30]. The IL-17, an important Th17 cytokine, is essential for neutrophil

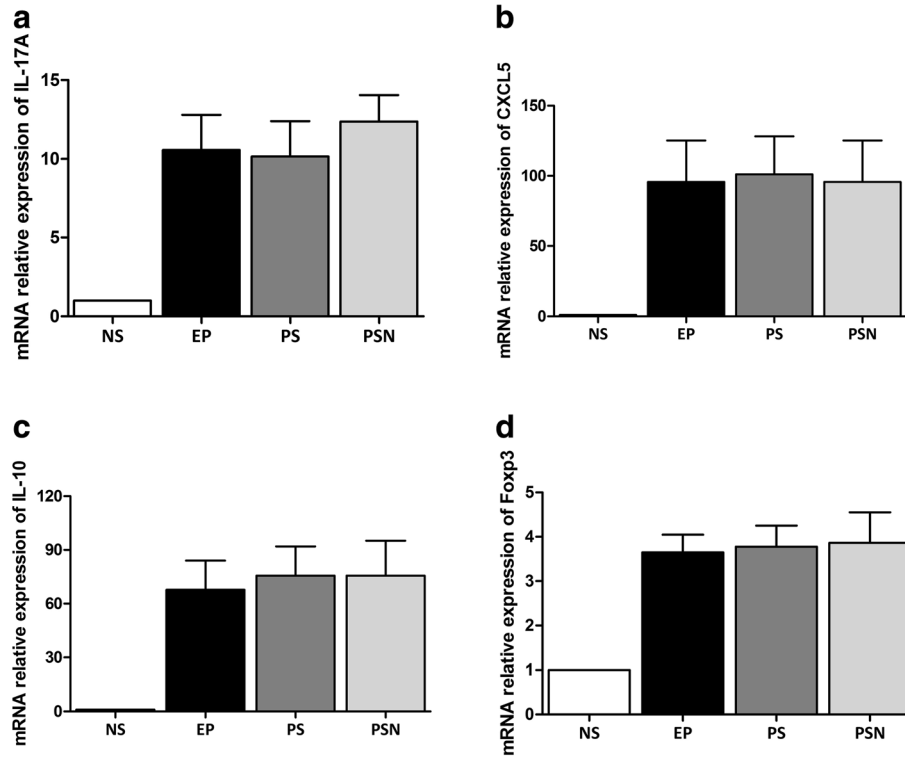


Fig. 4. mRNA levels of Th17/Treg cytokines in lung tissue. The mRNA relative expression of IL-17 (a), CXCL5 (b), Foxp3 (c), and IL-10 (d) in the lungs was analyzed by qRT-PCR.

recruitment and migration to airway mainly through the induction of epithelial-derived neutrophilic CXC chemokines in the lungs, such as CXCL5 [23]. As we known, the agent for anti-asthma therapy targeting Th2 cells may possibly increase IL-17 expression and promote Th17-mediated inflammation in mice asthma model [19]. So we examined the expression of IL-17 and CXCL5 in mice lung tissue after treatment with PSN. The qRT-PCR results showed that there was no obvious difference on the mRNA level of both IL-17 and CXCL5 between the PSN group and EP group. Additionally, the Treg cell has been identified as having the distinct role in asthma pathogenesis, which was strongly associated with the suppression of allergic responses in asthma [31]. Recently, the results of molecular docking and dynamic simulation suggested that HP-NAP probably has interaction with the Tregs [32]. Therefore, in our work, we examined whether the Treg cell would be activated with PSN administration. The qRT-PCR results showed that the expression levels of IL-10 and FoxP3, which are the cytokine and transcription factor characteristically expressed by activated Treg, have no obvious difference between PSN group and EP group. The discrepancy between the computational analysis and

the experiment results should be attributed to multiple reasons, such as the representation of the OVA-induced mouse model of asthma, the sample and method for examination, and so on. The role of Th17/Treg cells in asthma pathogenesis during the administration with PSN still need to be further studied.

In conclusion, the PSN expressing the sIL-4R-NAP can effectively inhibit airway inflammation, decrease the serum OVA-specific IgE levels, and redirect Th1/Th2 balance in OVA-induced mouse model of asthma. Besides the PSN was more effective comparing with PS expressing sIL-4R alone. It indicated that HP-NAP could enhance the efficacy of sIL-4R in anti-asthma therapy and the PSN would probably be a promising therapeutic agent for treating asthma.

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