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rMBP-NAP Suppresses OXA-induced Allergic Dermatitis by Regulating the Th1/Th2 Balance

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ABSTRACT

Background: Allergic dermatitis (AD) is an inflammatory skin disease that arises from abnormal T lymphocyte activation. A recombinant fusion protein comprising *Helicobacter pylori* neutrophil-activating protein and maltose binding protein, rMBP-NAP, has been documented as a novel immunomodulatory TLR agonist.

Objective: To explore the effect of the rMBP-NAP on the OXA-induced AD in a mouse model and clarify the possible action mechanism.

Methods: The AD animal model was induced by repeated administration of oxazolone (OXA) in BALB/c mice. H&E staining was used to analyze the ear epidermis thickness and the number of infiltrating inflammatory cells. TB staining was used to detect mast cell infiltration in the ear tissue. ELISA was used to analyze the secretion of cytokines IL-4 and IFN-γ in peripheral blood. qRT-PCR was used to determine the expression levels of IL-4, IFN-γ, and IL-13 in ear tissue.

Results: OXA induced the establishment of an AD model. After the rMBP-NAP treatment, the thickness of the ear tissue and the number of mast cells infiltrated in AD mice reduced, and the serum and ear tissue levels of IL-4 and IFN-γ increased, but the ratio of IFN-γ (rMBP-NAP group)/IL-4 (rMBP-NAP group) was greater than the ratio of IFN-γ (sensitized group)/IL-4 (sensitized group).

Conclusion: The rMBP-NAP improved the disease symptoms including skin lesions in AD, alleviated the inflammation in ear tissue, and restored the Th1/2 balance by inducing a shift from the Th2 to the Th1 response. The results of our work support the use of rMBP-NAP as an immunomodulator for AD treatment in future investigations

Keywords: Allergic dermatitis, Immunomodulation, rMBP-NAP, Th1/Th2

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INTRODUCTION

Allergic dermatitis (AD) is a recurrent immunological skin disease caused by abnormal T lymphocyte activation. AD is characterized by local skin inflammation and itching and often leads to a series of complications such as allergic rhinitis, conjunctivitis, and asthma [1]. The pathogenesis of AD is the result of a complex interaction between genetic factors, environmental factors, skin barrier function defects, and immune abnormalities [2, 3]. AD occurs in infants and presents different manifestations at different ages. It is easy to recur and difficult to cure completely, having a great impact on the health and normal daily life of AD patients [4]. At present, the World Health Organization (WHO) has listed allergy-related diseases (such as AD) as one of the three major diseases in the 21st century [3].

AD pathogenesis is very complex and has not been elucidated completely. T helper 2 (Th2) cells are mainly activated immune cells in the acute phase of AD. The mainly characterized acute AD skin lesions are epidermal thickening, the dermal thickening, $CD4^+$ T cells and eosinophils infiltration in dermal, and enhanced local expression of Th2 cytokines [5]. Interleukin (IL)-4, a typical Th2 cytokine, reportedly plays a critical role in certain pathogenesis of skin disorders. IgE induced by IL-4 combines with the FcεRI receptor on mast cells to stimulate the release of histamine and other related inflammatory mediators [6]. Moreover, the inflammatory mediators can promote T cell activation and migration to the skin lesions, as well as the polarization of Th2 cells [7]. Therefore, the abnormal and excessive activation of Th2 cells causes the imbalance of the Th1/Th2 immune response, which further aggravates the lesion of the skin barrier and leads to the vicious circle of disease, even seriously affecting the immune homeostasis of patients [8]. Currently, emollients, anti-histamine drugs, glucocorticoids, immunosuppressants, and immunomodulators that can maintain moisture and alleviate the inflammation level of the skin are commonly used in the treatment of AD [9]. However, the long-term use of glucocorticoids would lead to drug resistance, and even other side effects, including skin erythema, pruritus, edema, pain, etc. [10]. In addition, these immunosuppressive drugs, including tacrolimus, cyclosporine, and pimecrolimus have been used in a limited way for AD therapy due to the systemic side effects. Therefore, it is greatly needed to explore anti-AD immunomodulators and drugs with greater therapeutic effects [11].

HP-NAP is an important virulence factor expressed by *Helicobacter pylori* [12]. HP-NAP is a potential immunomodulator, which can enhance the Th1 immune response and induce IFN- γ and IL-12 expression to inhibit the activation of Th2 cells. Recent studies have reported that HP-NAP protein possesses a certain therapeutic effect on OVA-induced allergic asthma model mice [13]. After the HP-NAP treatment, the production of cytokines, such as IL-5, IL-4, GM-CSF, and infiltration of eosinophils in bronchoalveolitis significantly decreased. Meanwhile, HP-NAP can significantly reduce the amount of IgE in the serum of asthma model mice, reverse or inhibit the polarization of Th2 cells, and regulate the balance of Th1/Th2 cells [14]. *E. coli* maltose binding protein (MBP) is a member of the bacterial maltose transport system, which can induce the activation of dendritic cells (DCs) and activate the NF-κB signaling pathway as a TLR4 agonist. MBP also induces the activation of Th1 cells, NK cells, and macrophages. Recent studies have shown that MBP activates the Th1 immune response through the MyD88-dependent pathway triggered by TLR2 and the TRIF/TRAF3-dependent pathway triggered by TLR4. In addition, the synergistic effects of MBP and BCG result in an increased expression of INF-γ, TLR2/4/9 and regulation of the Th1 immune response [15]. Therefore, MBP can enhance the Th1 cells immune response, presenting a new idea for the AD treatment. The rMBP-NAP is the fusion product of MBP and HP-NAP by genetic

engineering technology. In our previous study in the subcutaneous transplanted tumor mouse model of liver cancer, the growth of the tumor was significantly inhibited after the administration of the rMBP-NAP [12]. The rMBP-NAP also increased IFN-γ secretion in splenocytes and significantly enhanced Th1 type immune response. We also documented that in the melanoma B16-F10 lung metastasis model, the rMBP-NAP led to enhanced Th1-type immune responses, the modulation of immune imbalance, and inhibition of tumor metastasis. It is suggested that the rMBP-NAP could be an effective potential immunoregulatory agent.

In this work, the anti-AD effects of the rMBP-NAP in OXA-induced AD mouse models were explored. To estimate the therapeutic effect and clarify the action mechanism of the rMBP-NAP on AD, the ear epidermal thickness, mast cell infiltration, Th1/Th2 cytokine (IFN-γ/IL-4) levels in serum, and mRNA expression level of pathogenic-related cytokines in the ear tissues were examined.

MATERIALS AND METHODS

Experimental Animals

Six-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Technology Co. Ltd.) weighing 18 to 20 g were used for the experiments. All the mice were maintained in a pathogen-free environment (23±3 ℃, 55±5% humidity) with a 12 h light/dark cycle. The animals had free access to food and water in animal cages. All the project procedures in this work were approved by the Zhengzhou University's Committee for Animal Experiments (Certificate No. SYXK (Yu) 2019-0002, Henan, China).

Reagent Preparation

OXA (Sigma, USA) was dissolved in acetone: olive oil (4:1) first. Then the OXA solution was prepared to a concentration of 5% (w/v) for initial sensitization and 0.3% (w/v) for subsequent application to the ear [16]. Dexamethasone (DEX) was dissolved in PBS to a concentration of 1.5 mg/mL.

Expression and Purification of the rMBP-NAP

The expression and purification of the rMBP-NAP were according to the previous studies with some modifications [17]. The neutrophil-activating protein gene of H. pylori (Hp-napA) was subcloned from pET-24b-napA (restored in our laboratory) into the pMAL-C2X vector. The sequence-confirmed pMAL-C2X-napA plasmids were transformed into the *E.coli* strain TB1. The *E.coli* TB1 (pMAL-C2X-napA) were cultured overnight in LB medium containing 0.1 g/L kanamycin at 37 ℃ and then in expanded culture before the logarithmic growth phase. Then, IPTG (final concentration 0.3 mmol/L) was added to induce the expression of the fusion proteins rMBP-NAP for 3 hrs at 37 ℃. The strain cells were harvested, sonicated (ice-water bath), centrifuged (4000g for 30 min at 4 ℃), and purified by amylose affinity chromatography. Then, the agarose bed columns containing immobilized polymyxin B (Thermo Scientific, USA) were used to remove endotoxin. The rMBP-NAP protein was dissolved in PBS at a concentration of 1.5 mg/mL for a subsequent experiment.

Mouse AD Model

The mice were randomly divided into four groups of five mice as follows: (i) sensitization group: mice sensitized and challenged with OXA and treated with PBS (IP injection); (ii) DEX group: the positive control group, mice sensitized and challenged with OXA and treated with 10 mg/kg DEX (IP injection); (iii) the rMBP-NAP group: mice sensitized and challenged with OXA and treated with 10 mg/kg rMBP-NAP protein (IP injection); (iv) the control group: the normal control group, sensitized, challenged and treated with PBS.

On day -7, we shaved the dorsal skin of mice and sensitized them with 20 μL of OXA (5% w/v). From day 0, 20 μL of 0.3% OXA

were used to challenge the mice, and the mice were treated with 10 mg/kg of rMBAP-NAP (IP injection) ten times in 3 weeks by equal time interval and 10 mg/kg DEX was used as a positive control. The schematic diagram of the experiment is shown in Figure 1A.

Histological Observations

The ear tissues were collected, fixed with 4% paraformaldehyde (Sigma, USA), and embedded in paraffin. Thin sections (6 µm) were prepared and stained with hematoxylin and eosin staining (H&E). Ear tissue sections were stained with toluidine blue (TB) for the evaluation of mast cell infiltration. Samples were imaged and examined under light microscopy (Olympus, Japan). All clinical and histological evaluations were performed in a blinded manner.

RNA Isolation and qRT-PCR

The total RNA was isolated from the ear tissue using Trizol reagent (Carlsbad life science company, USA). An aliquot (1 mg) of total RNA from each sample was reverse transcribed using a cDNA synthesis Kit (Otsu Plateau Biology Co., Ltd., Japan). Obtained cDNA was used for qRT-PCR with the Light Cycler 480 System II (Roche Diagnostics GmbH, Germany) using Fast SYBR Mixture (Low Rox) according to the manufacturer's instructions (Table 1).

ELISA Analysis in Serum

According to the manufacturer´s instructions, we allowed blood samples to clot for two hrs at room temperature before centrifugation for 20 min at 1000 g. Assay freshly prepared serum immediately or store samples in an aliquot at -80 °C for later use. The concentrations of cytokines were quantified using IL-4 and IFN-γ ELISA kit (BD Bioscience, USA) and calculated as pg/mL total protein. The optical density was measured at 450 nm using a microplate reader (Bio-Rad Model1680, USA).

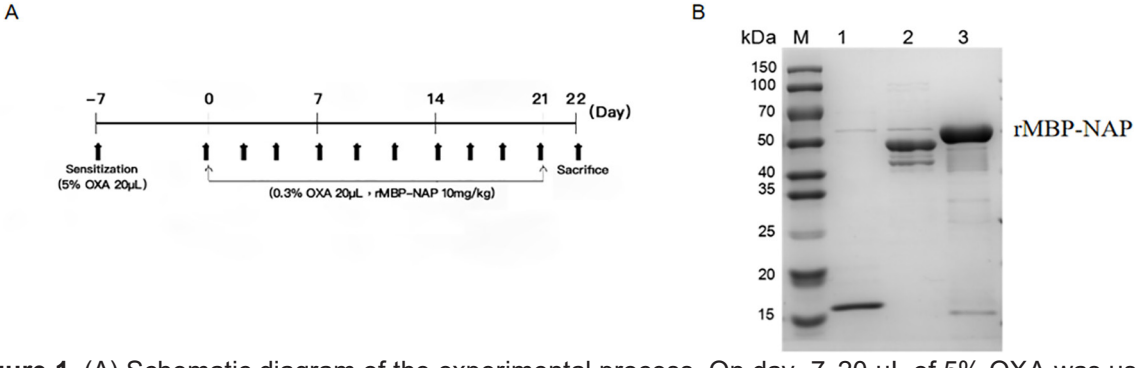

Figure 1. (A) Schematic diagram of the experimental process. On day -7, 20 µL of 5% OXA was used to sensitize the mice. From day 0, 20 µL of 0.3% OXA was used to challenge the mice, and the mice were treated with 10 mg/kg of the rMBAP-NAP (IP injection) ten times in 3 weeks by equal time interval. (B) The rMBP-NAP protein was purified and analyzed by SDS-PAGE. Lane 1: HP-NAP; Lane 2: MBP; Lane 3: rMBP-NAP; M: protein marker

Table 1. Primer sequences.

No	Primers	Sequences
1	β-actin	5'GGCATAGAGGTCTTTACGG 3' 5' GTGGCATCCATGAAACTACAT3'
2	IL-4	5'GCTAT-TGATGGGTCTCACCC3' 5'CAGGACGTCAAGGTA-CAGGA3'
3	IL-13	5'CTGGATTCCCTGACCAACAT3' 5'GGTTACAGAGGCCATGCAAT3'
4	IFN-γ	5'TGGCTCTGCAGGATTTTCATG3' 5'TCAAGTGGCATAGATGTGGAAGAA3'

Organ Index Analysis

The spleens and livers were removed after the mice were sacrificed and weighed respectively to calculate the organ indexes according to the following equation:
Organ index=organ weight (mg) /mouse weight (g)

Statistical Analysis

Data are presented as the mean±standard error of mean (SEM). For more than three groups, the One-way ANOVA was employed to analyze the significance. Statistical analyses were performed using GraphPad software and the differences were considered significant at $P<0.05$.

RESULTS

Experiment Schematic and SDS-PAGE Analysis

The plasmids pET-24b-napA, pMAL-C2X, and pMAL-C2X-napA were constructed, and the protein expression was induced by IPTG. After that, the strain was collected to obtain the total protein. The HP-NAP, MBP, and the rMBP-NAP proteins were purified by the Ni column and amylose column, respectively. And the protein expression was verified by SDS-PAGE. As shown in Figure 1B, the rMBP-NAP protein was successfully prepared and used in subsequent experiments.

Effects of the rMBP-NAP on OXA-Induced AD Mouse Symptoms

As shown in Figure 1B, the OXA-induced AD mouse model was successfully established for the estimation of the effect of the rMBP-NAP. In Figure 2, it is shown that during days 0-7, there was no significant difference in ear swelling between the control group and other groups. On day 14, the sensitization group showed the ear thickness exhibited significant thickening compared with the control group ($P<0.01$). On day 16, the rMBP-NAP and DEX significantly decreased the ear thickening ($P<0.01$) (Figure 2B). Meanwhile, the disease

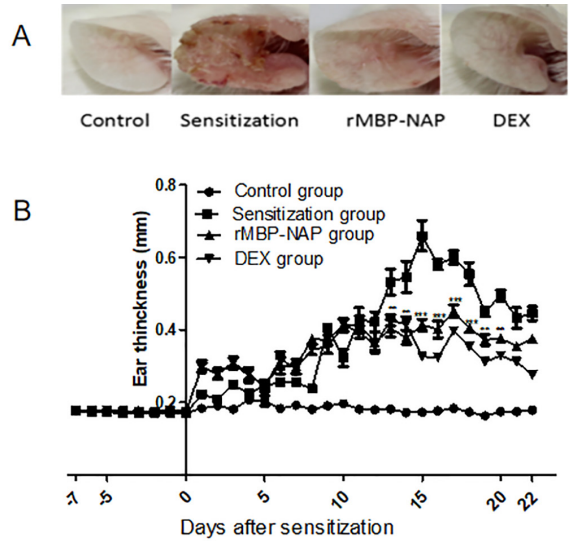

Figure 2. Treatment effects of the rMBP-NAP protein on AD mice symptoms. (A) The ear skin lesions of the mice were imaged on day 16. (B) The ear thickness of mice was measured 3 times a week for 4 weeks. The statistical analyses were conducted with One-way ANOVA followed by the Student–Newman–Keuls multiple comparison tests (**P<0.01, *** P<0.001 vs. sensitization group)

characteristics in the ear notably alleviated after treatment with the rMBP-NAP and DEX (Figure 2A).

Effect of the rMBP-NAP on Ear Pathology in AD Mice

The H&E staining images showed that the epidermal thickness of ear skin after OXA-challenged dramatically increased compared with the control group. Meanwhile, the inflammatory cells infiltrated markedly in ear tissue (Figure 3A). After treatment with the rMBP-NAP or DEX, the number of inflammatory cells in ear tissue notably reduced in AD mice. In addition, the epidermal thickness reduced by the rMBP-NAP or DEX treatment ($P<0.01$) (Figure 3B).

Effect of the rMBP-NAP on Mast Cells Infiltration in Ears of AD Mice

Mast cells play a key role in the pathogenesis of AD as both effector and regulatory cells [18]. Infiltration of mast cells in ear tissue was detected with TB staining. The results showed that there were a large number of mast cells

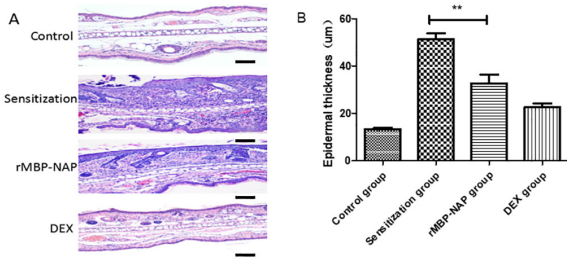

Figure 3. The epidermal thickness of ear tissue in AD mice after the rMBP-NAP protein treatment. (A) H&E staining images of ear sections. Bar, 100 μm. (B) The epidermis thickness of ear tissue was measured on the H&E stained sections with Image Pro-Plus 5.1 software and statistically analyzed with One-way ANOVA followed by the Student–Newman–Keuls multiple comparison tests (**P<0.01 vs. sensitization group).

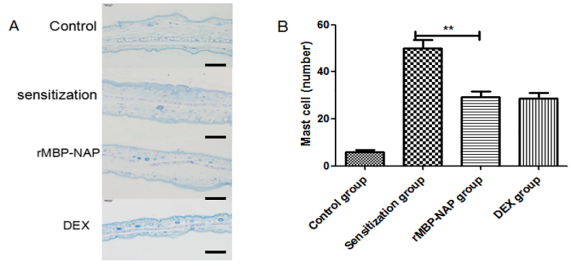

Figure 4. Mast cells infiltration in ears tissue of AD mice after the rMBP-NAP protein treatment. (A) TB staining images of ear sections. Bar, 100 μm. (B) The amount of infiltrating mast cells was counted on the TB staining sections using Image Pro-Plus 5.1 software and statistically analyzed with One-way ANOVA followed by the Student–Newman–Keuls multiple comparison test (**P<0.01 vs. sensitization group).

near the ear epidermis of the sensitization group (Figure 4). The amount of infiltration of mast cells in the rMBP-NAP and DEX groups were lower than in the sensitization group significantly (P<0.01).

Effects of the rMBP-NAP on Th1/2 Balance in AD Mice

The pathogenesis of AD is mainly due to the Th2 cells abnormal activation which causes the imbalance of the Th1/Th2 immune response aggravates the lesion of the skin barrier, and leads to the vicious circle of further disease. For further research the potential effect of the rMBP-NAP protein was conducted on the reversing of Th1/2 balance in AD mice. The expression levels of IL-4 (Th2 type cytokine) and IFN-γ (Th1 type cytokine) in peripheral blood were determined by ELISA assay. Figure 5 shows that compared with the sensitization group, the expression level of IL-4 and IFN-γ significantly increased in the rMBP-NAP group (P<0.05). And the ratio of IFN-γ (rMBP-NAP group)/IL-4 (rMBP-NAP group) was greater than the ratio of IFN-γ (sensitized group)/IL-4 (sensitized group) (P<0.01). Therefore, the rMBP-NAP may play a certain role in the treatment of AD mice by enhancing Th1 cell immunity and regulating the Th1/2 balance.

Effect of the rMBP-NAP on Expression Levels of Cytokines in the Ears of AD Mice

The mRNA expression levels of cytokines IL-4, IL-13, and IFN-γ were detected. The results showed that IL-13, a cytokine produced by mast cells, significantly reduced after the rMBP-NAP treatment (P<0.01). At the same time, the cytokines IL-4 and IFN-γ of mice

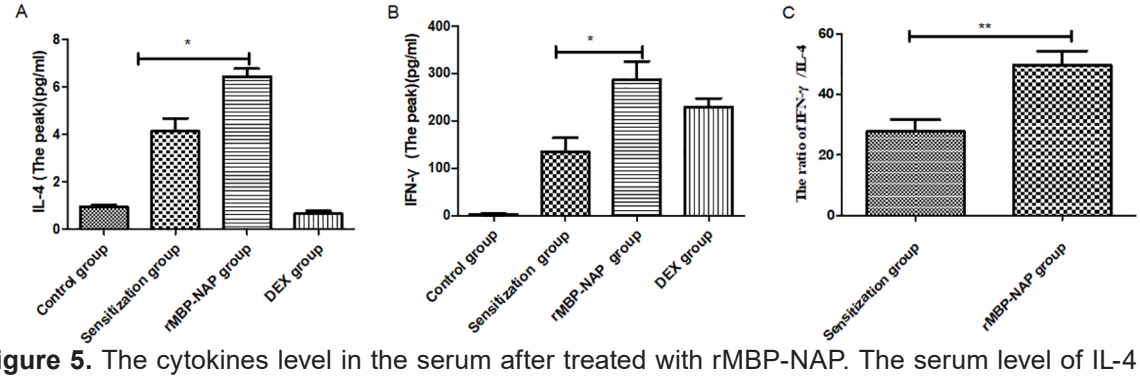

Figure 5. The cytokines level in the serum after treated with rMBP-NAP. The serum level of IL-4 (A) and IFN-γ (B) by ELISA assay. (C) The ratio of IFN-γ /IL-4. The statistical analyses were conucted with One-way ANOVA followed by the Student–Newman-Keuls multiple comparison tests (*P<0.05, ** P <0.01 vs. Sensitization group)

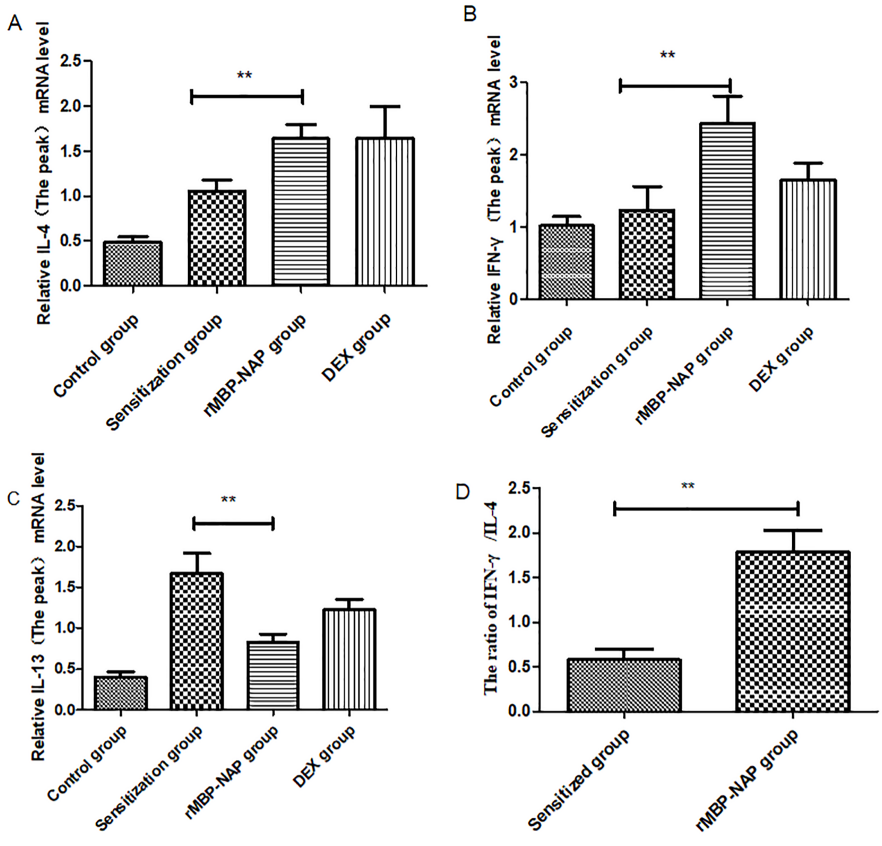

Figure 6. The mRNA expression level of cytokines in ear tissues after being treated with the rMBP-NAP. The expression of IL-4 (A), IFN-γ (B), and IL-13(C) were quantified by qRT-PCR. (D) The ratio of IFN-γ/IL-4. The statistical analyses were conducted with One-way ANOVA followed by the Student–Newman-Keuls multiple comparison tests (**P<0.01 vs. sensitization group)

in the rMBP-NAP administration group increased to a certain extent (P<0.01). And the ratio of IFN-γ (rMBP-NAP group)/IL-4 (rMBP-NAP group) was greater than the ratio of IFN-γ (sensitized group)/IL-4 (sensitized group) (P<0.01) (Figure 6).

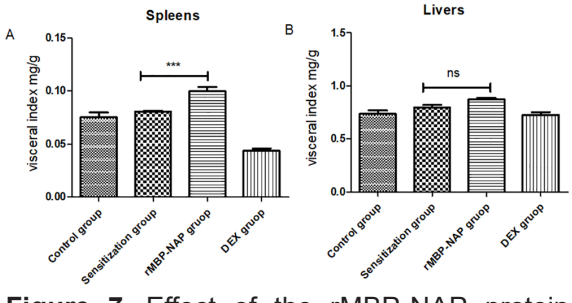

Figure 7. Effect of the rMBP-NAP protein on spleen and liver weight of AD mice. (A) The change of spleens tissue weight; (B) The change of livers tissue weight. The statistical analyses were conducted with One-way ANOVA followed by the Student–Newman–Keuls multiple comparison tests (***P<0.001 vs. sensitization group).

Effect of the rMBP-NAP Protein on Organ Weight of AD Mice

As Figure 7 shows, the spleens weight increased more in the rMBP-NAP treatment group than in the control group (P<0.001). While there was no significant change in livers weight after being treated with the rMBP-NAP.

DISCUSSION

AD is a complex immune-mediated inflammatory skin disease that seriously affects the quality of normal daily life of patients. The abnormal and excessive activation of Th2 cells play an important role in AD pathogenesis. Although it has made great progress in drug development for AD therapy, as we know, steroids and glucocorticoids are the main drugs for AD

treatment so far. As it would lead to a variety of side effects with long-term administration of these drugs, new agents for AD treatment are urgently needed.

In this work, the potential of the rMBP-NAP as an anti-AD drug was estimated for OXA-induced AD mice. When OXA was topically applied repeatedly, ear swelling significantly aggravated and ear thickness increased in AD mice. In addition, OXA induced remarkable AD lesions, such as hemorrhage, edema, excoriation, and scaling. DEX is a commonly used drug in the clinical treatment of skin inflammation-related diseases, used as a positive control drug for treating AD in our work. When treated with 10 mg/kg DEX, during the whole experimental period, we can observe the continuous and significant suppression of ear swelling, callus, and mosses in the AD mice model. Although the rMBP-NAP administration did not act effective and on time as DEX in improving dermatitis symptoms at the early stages of the model, it can significantly decrease atopic dermatitis symptoms at the peak period (day 16) in the AD disease process (Figure 2A).

In our previous study, the rMBP-NAP was reported as an effective immunomodulator in treating tumor mice by enhancing T cell immune response and promoting the secretion of Th1 cytokines. It was previously perceived that the imbalance of Th1/Th2 cells was the pathogenesis basis in AD. The regulation of Th1/Th2 balance in AD is crucial for the remodeling of Th cell homeostasis. In our work, we found that the expressions of IL-4 (Th2 type cytokine) and IFN-γ (Th1 type cytokine) in serum both increased after the rMBP-NAP treatment. Notably the ratio of IFN-γ (rMBP-NAP group)/ IL-4 (rMBP-NAP group) was greater than the ratio of IFN-γ(sensitized group)/IL-4 (sensitized group). It suggested that the rMBP-NAP can stimulate immune response and reverse the imbalance between Th1 and Th2 in AD mice. After being treated with the rMBP-NAP, the spleen index significantly increased, which may also implicate that the rMBP-NAP had an immune-enhancing effect. However, the liver index showed no significant change, indicating that the rMBP-NAP has no obvious toxicity.

CONCLUSION

The rMBP-NAP improved the disease symptoms of skin lesions, alleviated the inflammation in ear tissue, and restored the Th1/2 balance by inducing the shift from the Th2 response to the Th1 response. Our work documented that the rMBP-NAP is probably therapeutically valid as an immunomodulator for AD treatment.

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Conflict of Interest: None declared.

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