Original Article

Dust mite vaccine enhances airway antibacterial host defense by up-regulating beta defensins in asthmatic mice

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Abstract: The airway is always exposed to the microbial environment. It is accepted that allergic airway inflammation inhibits airway antibacterial host defense. This study aimed to test a hypothesis that allergen-specific immunotherapy (ASIT) with a dust mite vaccine could enhance antibacterial host defense in the allergic airway. An asthmatic mouse model was exposed to P. aeruginosa and treated by ASIT with a dust mite vaccine in our study. ASIT with dust mite vaccine could decrease the severity of P. aeruginosa infection in the airway of asthmatic mice, and enhance production of mouse beta defensin 3 (MBD-3) in airway epithelial cells and decrease IL-4 levels in asthmatic mice. Furthermore, IL-4 could down-regulate production of MBD-3 in lung epithelial MLE-12 cells. The results indicate that ASIT with dust mite vaccine can enhance airway antibacterial host defense by up-regulating beta defensin through decreasing IL-4 production in asthmatic mice.

Keywords: Allergen specific immunotherapy, dust mite vaccine, antibacterial host defense, beta defensin 3

Introduction

Allergic asthma is an IgE-mediated chronic inflammatory disease with characteristics of pulmonary eosinophilic granulocyte infiltration and airway hyperreactivity [1, 2]. The airway is always exposed to the microbial environment [3]. Respiratory tract infections are the most common diseases. Previous investigations have indicated that refractory asthma is a risk factor for respiratory tract infection and allergic airway inflammation inhibits airway antibacterial host defense [4, 5].

The innate immune system is the first line of host defense, which consists of naive immune barrier, immune cells and immune molecules [6]. Antimicrobial peptide (AMP) is the most important immune molecules, which has a broad spectrum effect on preventing microbial invasion [7, 8]. Airway epithelial cells are the active part of the innate pulmonary immune system. The cells secret host defense molecules, such as beta defensins, which is a significant kind of AMP [9]. Low levels of AMP expression can increase susceptibility to microbial infections [10]. Treatment with beta defensins in severe combined immunodeficiency mice can decrease bacterial levels in subcutaneous tissue [11]. Th2-type cytokines can inhibit antimicrobial host defense in individuals with allergic diseases [12, 13]. Allergen specific immunotherapy (ASIT) is the only effective approach to cure allergic asthma [14]. Treg cells are important immune regulatory cells in ASIT for asthmatic patients [15]. Active Treg cells can directly inhibit the Th2 responses and the activation of mast cells, basophile granulocytes and eosinophilic granulocytes [16, 17]. However, it is unclear how ASIT with dust mite vaccine affects airway antibacterial host defense.
Therefore, we hypothesize that ASIT with dust mite vaccine can enhance antibacterial host defense in airway epithelial cells by up-regulating AMP through inhibit Th2 immunoreactions. To test this hypothesis, the ability of dust mite vaccine to enhance airway antibacterial host defense was examined in asthmatic mice exposed to \textit{P. aeruginosa}.

\textbf{Materials and methods}

\textbf{Materials}

Female BALB/c mice (Body weight 16 to 22 g, 6 to 8 weeks old) were purchased from the Animal Center of Guangdong Province and maintained in a pathogen-free environment. All experiments were approved by the Animal Ethic Committee at Shenzhen University. The experiments were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. \textit{P. aeruginosa} was provided by the clinical laboratory of Shenzhen Luohu Maternity and Child Healthcare Hospital.

\textbf{Sensitization and challenge protocol}

BALB/c mice were randomly divided into 4 groups (n = 4); the PBS group and PBS/\textit{P. aer} group were treated with phosphate buffered solution (PBS). The other two groups were sensitized intraperitoneally with 50 μg HDM extract absorbed to 2 mg alum on days 0, 7, and 14. From day 28 (every 2 days, 3 administrations in total), mice were sublingually treated with PBS (Der f/\textit{P. aer} group) or 0.1 g HDM extract (Der f/ASIT/\textit{P. aer} group) respectively. Seven days after final immunization, mice were intra-nasally challenged with 50 μg HDM extract daily for 1 week.

\textbf{Acute \textit{P. aeruginosa} pneumonia model}

PBS/\textit{P. aer}, Der f/\textit{P. aer} and Der f/ASIT/\textit{P. aer} group mice were anesthetized using diethyl ether. They were then infected intra-nasally with 1×10^7 CFUs \textit{P. aeruginosa}. The PBS group of mice received equivalent doses of PBS. Mice were euthanized 24 hours after infection.

\textbf{Histopathological analysis and Immunofluorescence histochemistry}

Lung tissue was fixed in 4\% cold formalin solution for 24 hours at room temperature and embedded in paraffin. Five μm serial sections were cut. One part of the sections was stained with hematoxylin-eosin (HE) to examine the histological changes using light microscopy. Then the inflammation index was carried out using color segmentation (ImageJ 1.48v, National Institutes of Health, USA). The other part of the sections was deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3\% hydrogen peroxide and the sections were incubated at room temperature for 10 minutes. Antigen retrieval was performed with citrate (pH = 6) at 95°C in an aqueous bath. The process lasted 40 minutes. The sections were incubated with rabbit polyclonal antibody against MBD-3 (Abcam, Cambridge, MA, USA) at 37°C for 45 min (1:300). The secondary antibody (Envision™, DAKO, Denmark) was applied (1:500) and incubated at 37°C for 45 minutes. Finally, the slides were visualized using DAB immunostaining under a light microscope (Leica, Solms, Germany). The intensity of fluorescence staining of MBD-3 was carried out using color segmentation (ImageJ 1.48v, Wayne Rasband National Institutes of Health, USA).

\textbf{Wright’s stain and gram stain}

The BALF was collected and centrifuged at 400 g at 4°C. Then the supernatant was removed and 100 μL PBS was added to resuspend the sample. One part of the smear of the suspension was measured by Wrights’ stain. The number of inflammatory cells in the smear under high power field was counted using light microscopy. The other part of the smear was measured by gram stain. The number of gram-negative bacilli in the smear under oil immersion field was counted using light microscopy.

\textbf{Quantitation of bacteria}

The lungs were removed, weighed, and homogenized in Roswell Park Memorial Institute 1640 medium (RPMI 1640), and the suspension were inoculated on \textit{P. aeruginosa}-selective plates. Bacterial colonies were counted after incubation at 37°C for 24 hours.

\textbf{Detection of IL-4 and IFN-γ in BALF}

The BALF was collected and centrifuged at 400 g at 4°C. Then the supernatant was lyophilized and stored at -20°C until they were used for
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The levels of BALF IL-4 and IFN-γ were evaluated by sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturers’ instructions.

Cell culture and infection experiments

MLE-12 cells were incubated in 6-well cell culture plates at 37°C and 5% CO₂ in DMEM/F12 culture medium. The cells were pretreated for 48 hours at 37°C until they reached 80% confluence. The medium were removed, then the cells were incubated in serum-free DMEM/F12 culture medium and stimulated by 50 ng ml⁻¹ IL-4 or PBS for 48 hours. Then 10⁷ CFU ml⁻¹ P. aeruginosa was added to MLE-12 cells. After incubation for 1 hour, the medium were removed, the cells were washed with PBS and incubated with serum-free DMEM/F12 culture medium containing polymyxin 100 μg ml⁻¹ for 1 hour to kill extracellular bacteria. The culture media was drew off and plated in LB solid culture medium to confirm that the extracellular bacteria had been killed. The cells were then suspended with 1 ml sterile PBS. 100 μl cell suspensions were spread on LB plates to determine levels of intracellular bacteria. The plates were cultured at 37°C for 24 hours, and colonies were counted. Duplicates were made for each sample and control.

Quantitative real-time PCR

Total RNA was extracted from mouse lung tissue or P. aeruginosa infection MLE-12 cells with Trizol (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. The cDNA was prepared with the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, California). The PCR primers (GeneRay Biotechnology, Shanghai, China) used for RT-PCR were as follows: for MBD-3, sense: 5’-GCATTGGCAACACTCAGC-3’ and antisense: 5’-TGGGAAGAAGAAATTCTGTTGT-3’; for β-actin, sense: 5’-CTCTGAGACTAATGACTGAGA-3’ and antisense: 5’-CAGGACATCCTCTTCCCTAGT-3’. RT-PCR amplification reaction was prepared with the SYBR Green PCR kit (Bio-Rad) and...
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performed using the ABI 7300 Real Time PCR System (Applied Biosystems, Carlsbad, California). PCR products were verified by melting curve analysis. Relative mRNA levels of target genes were calculated by the 2-ΔΔCt method.

Statistical analysis

Data are represented as mean ± SD and analyzed by ANOVA (S-N-K’s post hoc test) or Student’s t-test. P value less than 0.05 was considered significant. The data were analyzed by SPSS 17.0 statistical software.

Results

Effects of dust mite vaccine on lung inflammation in asthmatic mice exposed to *P. aeruginosa*

The mechanism of increased lung infection risk in asthmatic patients exposed to bacteria remains unclear. Twenty-four hours after exposure to *P. aeruginosa*, histological analysis showed there was less inflammatory infiltration in asthmatic mice treated with dust mite vaccine (Der f/ASIT/P.aer) than that in untreated asthmatic mice (Der f/P.aer) (P<0.01) (Figure 1B). Meanwhile, Wright’s staining showed there was less inflammatory cells in the bronchial alveolar lavage fluid (BALF) of asthmatic mice exposed to *P. aeruginosa* treated with dust mite vaccine (Der f/ASIT/P.aer) than that in untreated asthmatic mice (Der f/P.aer) (P<0.01) (Figure 1C, 1D). This indicated that *P. aeruginosa* infection increased lung inflammation in asthmatic mice. However, ASIT with dust mite vaccine could inhibit inflammation in asthmatic mice exposed to *P. aeruginosa*.

Effects of dust mite vaccine on bacterial levels in asthmatic mice exposed to *P. aeruginosa*

To determine whether the dust mite vaccine could decrease the risk of pulmonary infection, bacteria levels in the lungs of the mice infection with *P. aeruginosa* were determined. Lower numbers of bacterial colony forming units (CFU) were observed in Der f/ASIT/P.aer mice than Der f/P.aer mice (P<0.01) (Figure 2A, 2C). The number of CFU in control mice, which received phosphate-buffered saline (PBS) instead of active bacteria, was zero. Lower numbers of bacteria were observed in Der f/ASIT/P.aer...
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**Figure 3.** MBD-3 expression in lungs by immunofluorescence histochemistry and qPCR. A. MBD-3 expression and localization in lung tissues indicated by the white arrow were detected by immunofluorescence histochemistry (original magnification ×200). B. Fluorescence intensity of MBD-3 in lung tissues was analyzed by ImageJ 1.48v software. Fluorescence intensity of MBD-3 in Der f/P.aer mice was significantly lower than PBS/P.aer mice and Der f/ASIT/P.aer mice (*P<0.01, #P<0.05). There was no significant difference of fluorescence intensity of MBD-3 between Der f/ASIT/P.aer mice and PBS/mice (#P>0.05). C. MBD-3 expression in lung tissue was measured by qPCR shown in column configuration. C. Showed that MBD-3 expression in Der f/P.aer mice was sharply lower than in PBS/P.aer mice and Der f/ASIT/P.aer mice (**P<0.01, ###P<0.05). There was no significant difference of MBD-3 expression between Der f/ASIT/P.aer mice and PBS/mice (##P>0.05).

mice than Der f/P.aer mice (P<0.01) (**Figure 2B, 2D**). These data show that ASIT with dust mite vaccine can induce the clearance of P. aeruginosa and decrease pulmonary infection in asthmatic mice.

**Effects of dust mite vaccine on MBD-3 in asthmatic mice exposed to P. aeruginosa**

Airway epithelial cells can be induced to express mouse beta defensin 3 (MBD-3). Whether the dust mite vaccine can enhance the expression of MBD-3 remains unclear. In our study, MBD-3 was expressed in epithelial cells of lungs in normal mice or asthmatic mice ASIT with dust mite vaccine when the mice were exposed to P. aeruginosa (Figure 3A, 3B). MBD-3 expression in Der f/P.aer mice was significantly lower than in PBS/P.aer mice and Der f/ASIT/P.aer mice (P<0.01) (**Figure 3C**). However, there was no significant difference of MBD-3 expression between in Der f/ASIT/P.aer mice and PBS/mice (P>0.05) (**Figure 3C**). Thus, ASIT with dust mite vaccine enhanced production of MBD-3 during antibacterial immune response to asthma.

**Effects of dust mite vaccine on IL-4 and IFN-γ in asthmatic mice exposed to P. aeruginosa**

Levels of IL-4 and IFN-γ in the bronchial alveolar lavage fluid (BALF) of mice exposed to P. aeruginosa were tested to determine the relationship between ASIT with dust mite vaccine and Th1/Th2 immunoreactions for antibacterial host defense during asthma. The levels of IL-4 in BALF were higher in Der f/P.aer mice than those in PBS/P.aer mice (P<0.01). However, the levels of IL-4 in Der f/ASIT/P.aer mice were lower than those in Der f/P.aer mice (P<0.01). IL-4 levels were lower in PBS control mice (PBS) than the other mice (PBS/P.aer, Der f/P.aer and Der f/ASIT/P.aer mice) (P<0.01) (**Figure 4**). The level of IFN-γ in PBS/P.aer mice was not significantly different from that of Der f/ASIT/P.aer mice (P>0.05). IFN-γ levels were lower in Der f/P.aer mice than those in PBS/P.aer and Der f/
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ASIT/P.aer mice (P<0.01). IFN-γ levels were higher in PBS control mice (PBS/P.aer) than the other mice (PBS/P.aer, Der f/P.aer and Der f/ASIT/P.aer mice) (P<0.01) (Figure 4). These data demonstrate that ASIT with dust mite vaccine could decrease IL-4 levels and increase IFN-γ levels in asthmatic mice exposed to P. aeruginosa.

Effects of IL-4 on the antibacterial host defense in MLE-12 cells

Allergic airway inflammation inhibits airway antibacterial host defense [4, 5]. However, whether IL-4 can inhibit the antibacterial host defense in airway epithelial cells remains unclear. In our present studies, MLE-12 cells were pretreated with IL-4 or PBS and then infected with P. aeruginosa. The infected MLE-12 cells were homogenized with sterile PBS and spread on LB plates to determine levels of intracellular bacteria. IL-4 increased the levels of P. aeruginosa in MLE-12 cells (Figure 5A). The total bacterial CFUs were significantly higher in MLE-12 cells pretreated with IL-4 than those pretreated with PBS (P<0.01) (Figure 5B). Infected MLE-12 cells were collected and MBD-3 mRNA expression was analyzed by qPCR. IL-4 was found to be decreased in MBD-3 mRNA expression in MLE-12 cells. MBD-3 mRNA expression was significantly lower in MLE-12 cells pretreated with IL-4 than those pretreated with PBS (P<0.01) (Figure 5C). This indicated that IL-4 might weaken the antibacterial host defense of airway epithelium cells by downregulating MBD-3 (Figure 5D).

Discussion

Allergic asthma is the most common allergic disease in China, which is a complex chronic inflammatory airway disease. Previous research has indicated that allergic airway inflammation inhibits airway antibacterial host defense, meanwhile, respiratory tract infections and bacterial colonization play an important role in refractory asthma [4, 5]. The airway is always exposed to the microbial environment [18]. Respiratory tract infections are the most common diseases. P. aeruginosa is the risk cause of detrimental chronic lung infections, which is also a major determinant of morbidity and mortality [19].

Allergen specific immunotherapy (ASIT) is the only effective approach to cure allergic asthma [14]; it is recommended by world health organization (WHO) as a specific therapy for asthma [20]. Treg cells are important immune regulatory cells in ASIT for asthmatic patients. Insufficient quantity and hypofunction of Treg are the important factors of occurrence of mite allergic asthma [15]. Treg cells can inhibit Th2 responses and activation of mast cells, basophil, granulocytes, and eosinophil granulocytes directly [16, 17]. ASIT with dust mite vaccine can inhibit accentuated Th2-type immune response and up-regulate quantity and function of Treg, which can lessen or release the severity of asthma [21]. However, whether ASIT with dust mite vaccine can affect antimicrobial host defense among asthmatic patients remains indistinct. In our study, lung inflammation and bacterial levels in asthmatic mice exposed to P. aeruginosa were found to die more than those in normal mice exposed to P. aeruginosa. However, when ASIT with dust mite vaccine, lung inflammation in asthmatic mice exposed to P. aeruginosa was found to be less-
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Innate immunity is a natural immune defense system formed during the development and evolution of the body, which is the first line of host defense against microbial invasion. It consists of naïve immune barrier, immune cells and immune molecular [6]. Antimicrobial peptide (AMP) is the most important naïve immune molecular, which has a broad spectrum bactericidal action [7, 8]. Defensins and cathelicidins are primary AMP factors expressed in the lung and secreted by airway epithelial cells, macrophages, neutrophils, and other classical host defense cells [22]. Defensin is an important family of endogeneous antimicrobial peptide, which can kill the intruded pathogens quickly and nonspecifically [23]. As multifunctional effector molecules of innate immunity, defensins are the key components in immune system, which can kill or inhibit most microbes such as bacteria, fungus and envelope virus *in vitro* [24]. Human beta defensin 2 (HBD-2) is the chief component of airway innate immunity, which is the first human defensins inducible expression by inflammation and cytokines. HBD-2 plays a significant role in anti-infection immunity in skin, mucous membrane, and airway mucous membrane especially [25]. In our study, expression of mouse beta defensin 3 (MBD-3) (MBD-3 is a defensin in mice which is the homolog of HBD-2.) was found to be reduced in asthmatic mice exposed to *P. aeruginosa* compared to normal mice exposed to *P. aeruginosa*. Nevertheless, expression of MBD-3 was found to be recovered in lung of mice accepted ASIT with dust mite vaccine. Thus, our study indicates that *P. aeruginosa*...
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Infection inhibits lung MBD-3 expression in asthmatic mice. However, ASIT with dust mite vaccine induced lung MBD-3 expression among asthmatic mice.

Previous research has suggested that the imbalance in Th1/Th2 immunity plays an important role in the pathogenesis of allergic asthma. Increasing quantity and hypofunction of Th2 are the important causes of allergic asthma [26]. Th1 cells produce Th1-type cytokines such as IFN-γ, IL-2, and IL-12, which induce cellular immune response to defense against viruses and bacteria. Th2 cells produce Th2-type cytokines such as IL-4, IL-5, IL-6, IL-9, and IL-13, which induce humoral immune response to participate in allergic inflammation and defense against parasites. Allergic mice showed more viable bacteria in their lungs after infection significantly. Th2-type cytokines can inhibit antimicrobial host defense in individuals with allergic diseases [11, 12]. In our study, compared to normal mice exposed to P. aeruginosa, BALF IL-4 levels of asthmatic mice exposed to P. aeruginosa were found to be increased and BALF IFN-γ levels decreased. However, when ASIT with dust mite vaccine was used, BALF IL-4 levels decreased and BALF IFN-γ levels increased. Th2-induced inflammation was found to down-regulate host defense and reduce AMP expression in the skin [27, 28]. When incubated with IL-4 or IL-13, airway epithelial cells couldn’t kill bacteria efficiently, which indicated that these cytokines inhibit antimicrobial activity of the airway epithelium [29]. Thus, our study indicates that ASIT with dust mite vaccine could be related to the function of up-regulation antimicrobial host defense induced by ASIT with dust mite vaccine.

Conclusions

We show that ASIT with dust mite vaccine could decrease the severity of P. aeruginosa infection in the airway of asthmatic mice and enhance antibacterial host defense in airway epithelial cells by up-regulating MBD-3 through down-regulating IL-4 levels. These findings may suggest that ASIT with dust mite vaccine may enhance airway antibacterial host defense in asthma patients.

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Disclosure of conflict of interest

None.

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