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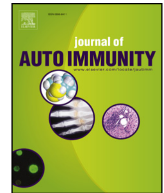

Commensal bacteria aggravate allergic asthma *via* NLRP3/IL-1β signaling in post-weaning mice

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ABSTRACT

Perturbation of commensal bacteria by antibiotic exposure aggravates ovalbumin (OVA)-induced allergic asthma in pre-weaning mice. However, the influence of dysbiosis of commensal bacteria on asthma development in post-weaning mice is still limited. Here, we treated 3-week-old post-weaning mice with antibiotics to disrupt commensal bacteria and then established OVA-induced allergic asthma by peritoneal sensitization using OVA/alum and intranasal challenge with OVA. Contrary to the protective function in pre-weaning mice, commensal bacteria in post-weaning mice aggravated OVA-induced asthma. Commensal bacteria in post-weaning mice promoted OVA-induced allergic asthma through maintenance of NLRP3/IL-1β expression in peritoneal macrophages (pMφ), which promoted recruitment of inflammatory cells, especially inflammatory monocytes, into the peritoneal cavity after OVA/alum sensitization. Further study showed that metronidazole- and vancomycin-sensitive bacteria are involved in maintenance of NLRP3/IL-1β signal in pMφ. Our results suggest that certain species of commensal bacteria in post-weaning mice aggravate OVA-induced allergic asthma through NLRP3/IL-1β signal pathway.

1. Introduction

Allergic asthma is a Th2-cell predominant immune disorder characterized by eosinophilic respiratory inflammation and airway hyperreactivity [1]. In the past two decades, the prevalence of allergic asthma has increased rapidly to afflict > 20% of the population (particularly among children) in industrialized countries. It has been postulated that growing up in an environment with fewer microbiota can lead to an increase in the prevalence of allergic diseases ("hygiene hypothesis" [2]). Also, increased exposure to antibiotics during infancy is perceived to be one of the main causes of the increase in the prevalence of childhood allergic asthma [3,4].

Typically, mechanistic studies analyzing how commensal bacteria affect asthma development are carried out in mouse models. Germ-free mice and antibiotic-treated neonatal mice display increased susceptibility to experimental allergic asthma induced by exposure to OVA or house dust mite [5–10], which are consistent with conclusions from clinical studies. However, mouse studies focus mainly on a "critical window" in early life from birth to weaning (referred to as "pre-weaning mice") [11,12]. The influence of dysbiosis (i.e., microbial imbalance or maladaptation on or within the body) of commensal bacteria in post-weaning mice on asthma development is still limited. The composition and function of commensal bacteria change over time [13]. Hence, the commensal bacteria in different periods of life may have different effects on asthma development.

The NLRP3 inflammasome has been reported to have a role in the aggravation of airway inflammation. Studies have shown that NLRP3-deficient mice display an impaired inflammatory response and lower immunoglobulin (Ig)E level, and that IL-1β- and IL-1R1-deficient mice have significantly reduced expression of the Th2-associated cytokines IL-13, IL-5 and IL-33 in OVA-induced allergic asthma [14–18]. However, how NLRP3 inflammasome execute function in the context of allergic lung disease is currently undetermined.

In the present study, we used the mouse model of OVA-induced allergic asthma and found that, different from the protective function of commensal bacteria in pre-weaning mice, commensal bacteria in post-weaning mice aggravated OVA-induced allergic asthma. NLRP3/IL-1β signaling in pMφ was involved in OVA-induced allergic asthma in post-

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weaning mice. Antibiotic treatment decreased the expressions of NLRP3 and IL-1β in pMφ, which resulted in the decrease of the recruitment of inflammatory monocytes and the reduction of allergic asthma symptoms. Metronidazole- and vancomycin sensitive bacteria played roles in maintenance of NLRP3/IL-1β signaling. Our results suggest that NLRP3/IL-1β signaling in pMφ regulated by commensal bacteria determined the severity of OVA-induced allergic asthma in post-weaning mice.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center (SLAC, Shanghai, China). CD45.1$^+$ mice were obtained from the Jackson Laboratory. *Nlrp3*$^{-/-}$ and *Il-1β*$^{-/-}$ mice were gifts from Dr. Rongbin Zhou (University of Science and Technology of China, Hefei, China). All mice were housed in a specific pathogen-free facility. The study protocol was approved by Ethics Committee for Animal Care and Use at the University of Science and Technology of China (Authorization number: USTCACUC1601003, Hefei, China).

2.2. Mouse treatment with antibiotics

Sex-matched mice were used in experiments. Commensal bacteria were depleted using antibiotics as reported previously [19]. Briefly, the antibiotics ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin sulfate (1 g/L) and metronidazole (1 g/L) were dissolved in sterile water and stored at 4 °C ≤ 1 week before use. This antibiotic-contained water was supplied as drinking water to 3-week-old mice and changed every 3 days. If the body weight of a treated mouse declined to 70% that of control mice, the mouse was removed. Antibiotic treatment continued for the entire experimental period. All antibiotics were purchased from Sangon Biotech (Shanghai, China).

2.3. Protocol for allergen sensitization and challenge

For general intraperitoneal sensitization, mice were injected (i.p.) on day 0 and day 7 with 100 μg of OVA (grade V; Sigma–Aldrich, Saint Louis, MO, USA) in 100 μL of sterile saline and adsorbed in 50 μL of Imject alum (Thermo Scientific, Waltham, MA, USA). At days 14, 15 and 16, mice were anesthetized with sodium pentobarbital (50 μg/g body weight, i.p.; Merck, Whitehouse Station, NJ, USA) and administered (i.n.) with 100 μg of OVA in 50 μL of sterile saline. Mice were harvested at day 21.

2.4. Macrophage depletion in vivo

For macrophage depletion, mice were injected with clodronate liposomes (100 μL, i.p.; Vrije Universiteit, Amsterdam, Netherlands) 2 days before OVA/alum sensitization. For lavage of the peritoneal cavity, mice were anesthetized with sodium pentobarbital (50 μg/g, i.p.) and then injected (i.p.) with 5 mL of sterile saline containing 5 mM of ethylenediamine tetra-acetic acid (EDTA). The abdomen was rubbed gently for 2 min and then lavage fluid collected using an injection syringe with a size-12 needle.

2.5. Parabiosis

CD45.1$^+$ and CD45.2$^+$ mice were connected surgically to generate "parabiotic partners" for 2–3 weeks, as described previously [20]. Briefly, age- and sex-matched mice were anesthetized and shaved. An incision was made along the lateral aspect of each mouse. Then, the mice were sutured together at the elbow and knee as well as at the skin around the incision. Mice were injected with 5% glucose and 0.9% sodium chloride (i.p.) to recover energy and water. Sulfatrim was added to the drinking water for 7 days.

2.6. Collection and analyses of bronchoalveolar lavage fluid (BALF)

BALF was obtained as described previously [21] with slight modifications. For analyses of levels of cytokines and antibody in BALF, 1 mL of phosphate-buffered saline (PBS) containing 5 mM of EDTA was injected into the lungs through the trachea of sacrificed mice; a lavage step was undertaken once. BALF samples were centrifuged at 350 × *g* for 5 min at 4 °C, and the supernatants collected and stored at −80 °C until required. Levels of IL-4, IL-13 and IgE were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Dakewe Biotech (Shenzhen, China) or USCNK (Wuhan, China). For analyses of the cell subsets in BALF, the lavage was repeated three times, and all cell pellets from the four lavages were pooled for flow cytometric analyses.

2.7. Isolation and preparation of cells

For the isolation of lung cells, lungs were minced and digested for 60 min at 37 °C in RPMI-1640 medium containing 0.1% collagenase I (Sigma–Aldrich) and 5% fetal bovine serum. Large lung pieces were removed by filtration through a 200-gauge steel mesh, and the flow-through was collected. Cells were spun down, and the residual lysed with red blood cell (RBC) lysis buffer to remove RBCs. Cell numbers were determined by Countstar® (Shanghai Ruiyu Biotech, China). For the isolation of blood leucocytes, anticoagulant blood was lysed with RBC lysis buffer to remove RBCs. For the isolation of peritoneal leucocytes, mice were sacrificed and then injected (i.p.) with 5 mL of sterile saline containing 5 mM of EDTA. The abdomen was rubbed gently for 2 min and the lavage fluid collected. Lavage fluids were centrifuged and cell pellets were collected.

2.8. Flow cytometry

For staining of cell-surface markers, after blockade of the Fc receptor with rat serum, single cells were incubated with fluorescein-labeled monoclonal antibodies in PBS for 30 min at 4 °C, then washed twice and analyzed. For staining of intracellular cytokines, cells were stimulated with 50 ng/mL of PMA, 1 μg/mL of ionomycin and 10 μg/mL of monensin (all from Sigma–Aldrich) for 4 h at 37 °C. After staining of cell-surface markers, cells were fixed, permeabilized, and stained with fluorescein-labeled monoclonal antibodies or isotype control overnight at 4 °C. Samples were analyzed by flow cytometry using a BD LSRII or LSRFortessa system (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star, Ashland, OR, USA). All antibodies and isotype controls were purchased form BD Biosciences, BioLegend (San Diego, CA, USA), or eBioscience (San Diego, CA, USA) (Supplementary Table 1).

2.9. Cell transfer and real-time polymerase chain reaction (PCR)

A FACSAria II cell sorter (BD Biosciences) was used to purify pMφ, inflammatory monocytes or neutrophils in peritoneal lavage fluid (PLF). The purity of sorted cell populations was > 95%. For the study of cell transfer, purified cells were suspended in physiologic (0.9%) saline and injected (i.p.) into recipient mice. For real-time PCR, total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Then, gene expression was analyzed according to the instructions of the SYBR™ Premix Ex Taq kit (TaKaRa Bio, Kusatsu, Japan) and quantified using the $^{\Delta\Delta}$Ct method. All primers were synthesized by Sangon Biotech (Supplementary Table 2).

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