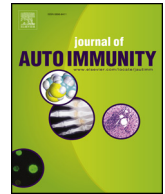




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Commensal bacteria aggravate allergic asthma *via* NLRP3/IL-1 β signaling in post-weaning mice

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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-1 β expression in peritoneal macrophages (pM ϕ), 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-1 β signal in pM ϕ . Our results suggest that certain species of commensal bacteria in post-weaning mice aggravate OVA-induced allergic asthma through NLRP3/IL-1 β 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-1 β - 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-1 β signaling in pM ϕ was involved in OVA-induced allergic asthma in post-

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weaning mice. Antibiotic treatment decreased the expressions of NLRP3 and IL-1 β in pM ϕ , 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-1 β signaling. Our results suggest that NLRP3/IL-1 β signaling in pM ϕ 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-1 β* ^{-/-} 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 $\leq$ 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 μ g of OVA (grade V; Sigma-Aldrich, Saint Louis, MO, USA) in 100 μ L of sterile saline and adsorbed in 50 μ L of Imject alum (Thermo Scientific, Waltham, MA, USA). At days 14, 15 and 16, mice were anesthetized with sodium pentobarbital (50 μ g/g body weight, i.p.; Merck, Whitehouse Station, NJ, USA) and administered (i.n.) with 100 μ g of OVA in 50 μ L 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 μ L, 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 μ g/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 $\times$ 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 μ g/mL of ionomycin and 10 μ g/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 LSR II or LSRFortessa system (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star, Ashland, OR, USA). All antibodies and isotype controls were purchased from 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 pM ϕ , 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).

2.10. Bacterial cultures and identification

Fresh stool was collected and weighed, and bacterial DNA was extracted using the Stool Genome DNA Extraction Kit (YPH-Bio, Beijing, China) according to the manufacturer's instructions. For bacterial titer analysis, fresh stools were homogenized in sterile PBS. The serially diluted homogenates were plated onto blood agar plates (for total cultivable bacterial determination) at 37 °C for 36 h, and then the colonies were counted.

2.11. Histology

Lung tissues were fixed in 10% (v/v) formalin for > 24 h and embedded in paraffin. Sections (thickness, 6 μm) were stained with hematoxylin & eosin or periodic acid-Schiff's solution (Solarbio, Beijing, China). More than six sections from right and left of the lung sections were selected randomly to determine inflammatory injury, and the reviewer was blinded to the treatments. A semi-quantitative scoring system was used to evaluate the degree of the inflammation as previously described [22]. Both peribronchiolar and perivascular inflammation were scored giving a maximum score of 8 as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of more than four cells deep.

2.12. Statistical analysis

All data were expressed as mean ± SEM and analyzed using the Student's two-tailed *t*-test. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. Commensal bacteria aggravate OVA-induced allergic asthma in post-weaning mice

To investigate the effect of dysbiosis of commensal bacteria on allergic asthma in post-weaning mice, we used the mouse model of OVA-induced allergic asthma, an asthma-like allergic airway inflammation, which was widely used in previous studies for exploring the roles of commensal bacteria on allergic asthma in pre-weaning mice [5,7,23]. We administered a combination of antibiotics (ampicillin, vancomycin, neomycin and metronidazole) to 3-week-old post-weaning mice in drinking water (referred to as antibiotic (ATB) mice) to disrupt commensal bacteria as described previously [19], which is one of canonical microbiota deficiency model widely used (Fig. S1). Then, we established allergic asthma by peritoneal sensitization using OVA/alum and intranasal challenge with OVA (Fig. 1A). Compared with non-antibiotic-treated control mice (referred to as ATB-free mice), ATB mice exhibited reduced lung injury (Fig. 1B and C) and infiltration of fewer leukocytes in pulmonary alveoli after asthma establishment (Fig. 1D). Furthermore, the number of asthma-related eosinophils in alveoli was reduced prominently (Figs. S2A and 1E), IgE levels in serum and BALF (Fig. 1F), as well as levels of Th2-type cytokines IL-4 and IL-13 in BALF (Fig. 1G), were decreased significantly in ATB mice compared with ATB-free mice after allergic asthma establishment. These results suggested that, different from the protective function of commensal bacteria in pre-weaning mice [24], commensal bacteria in post-weaning mice aggravated OVA-induced allergic asthma.

3.2. NLRP3/IL-1β signaling plays a critical role in peritoneal macrophages-dependent allergic asthma

Peritoneal macrophages have crucial roles in homeostasis maintenance in the peritoneal cavity and contribute to inflammation during pathogenic infection [25]. Treating WT mice with clodronate liposomes

(CL₂MDP-lip) to clear pMφ (Fig. 2A) could reduce infiltration of leukocytes (including eosinophils and Th2 cells) significantly in alveoli after allergic asthma establishment compared with PBS-lip-treated control mice (Fig. 2B–D). To preclude the possibility that CL₂MDP-lip might enter the blood to deplete phagocytes distributing in other parts of the body and affect the result of OVA-induced allergic asthma, we cleared pMφ directly by peritoneal lavage. We found that, compared with sham-operation mice, mice that underwent peritoneal lavage exhibited less infiltration of eosinophils in alveoli after allergic asthma establishment (Fig. 2E). These results indicated that pMφ were involved in OVA-induced allergic asthma in post-weaning mice and clearance of pMφ attenuated OVA-induced allergic asthma.

Previous studies showed that activation of the NLRP3 inflammasome is critical for induction of a Th2 immune response to alum-absorbed protein antigen [14,15]. To examine if NLRP3/IL-1β signaling had a role in our mouse model, we established OVA-induced allergic asthma in *Nlrp3*^{−/−} and *il-1β*^{−/−} mice. Both types of knockout mice exhibited milder asthmatic symptoms compared with WT control mice (Fig. 3A–D), suggesting that, in addition to the pMφ, OVA-induced allergic asthma also depended on the NLRP3/IL-1β signaling.

Next, to examine if NLRP3/IL-1β signaling plays a critical role in OVA-induced allergic asthma via pMφ, we built a mouse model of parabiosis with WT mice and *Nlrp3*^{−/−} mice (Fig. 3E). In parabiotic mice, pMφ as tissue-resident cells did not exchange between two mice, but other cells in the hematopoietic system, such as T and B cells, exchanged freely (Fig. 3F); this result is helpful to study pMφ function. Then, we established OVA-induced asthma in parabiotic mice by sensitizing one mouse with OVA/alum (i.p.) and challenging the other mouse with OVA (i.n.). We found that sensitization of *Nlrp3*^{−/−} mice (i.p.) with OVA/alum and challenging WT mice (i.n.) with OVA in parabiotic WT/*Nlrp3*^{−/−} mice induced much milder asthmatic symptoms in OVA-challenged mice compared with parabiotic WT/*Nlrp3*^{−/−} mice who had opposite treatment or parabiotic WT/WT mice (Fig. 3G). These results indicated that NLRP3/IL-1β signaling had critical roles via pMφ in OVA-induced allergic asthma.

3.3. Commensal bacteria promotes allergic asthma in NLRP3/IL-1β-dependent manner

As described above, commensal bacteria in post-weaning mice aggravated OVA-induced allergic asthma, and NLRP3/IL-1β signaling in pMφ was involved in OVA-induced allergic asthma. To examine if clearance of commensal bacteria influenced pMφ number and/or function, we measured cell number of pMφ and expressions of NLRP3 and IL-1β in pMφ after antibiotic treatment. The number of pMφ was unchanged after antibiotic treatment in ATB mice compared with ATB-free mice (Fig. 4A), suggesting that clearance of commensal bacteria had no influence on pMφ turnover. However, the mRNA levels of NLRP3 and IL-1β in pMφ from ATB mice were down-regulated (Fig. 4B), but that TLR expressions were unchanged in pMφ from ATB mice at both mRNA and protein levels (Fig. 4C and D). Furthermore, the IL-1β level in the peritoneal cavity was reduced significantly in ATB mice after OVA/alum sensitization compared with ATB-free mice (Fig. 4E). To further test if clearance of commensal bacteria down-regulated the expression of IL-1β in pMφ at protein level, pMφ were separated and stimulated with OVA/alum *in vitro*. pMφ from ATB mice secreted less IL-1β than pMφ from ATB-free mice after stimulation with OVA/alum *in vitro* (Fig. 4F). These results suggested that commensal bacteria in post-weaning mice promoted OVA-induced allergic asthma through maintenance of NLRP3/IL-1β signaling in pMφ.

Next, we explored the mechanism by which NLRP3/IL-1β signaling in pMφ influenced OVA-induced allergic asthma. We monitored the dynamic change of inflammatory cells in the peritoneal cavity of WT mice after OVA/alum sensitization (Fig. S2B). The number of pMφ decreased, along with an increase in the number of inflammatory monocytes and neutrophils, over time after OVA/alum sensitization,

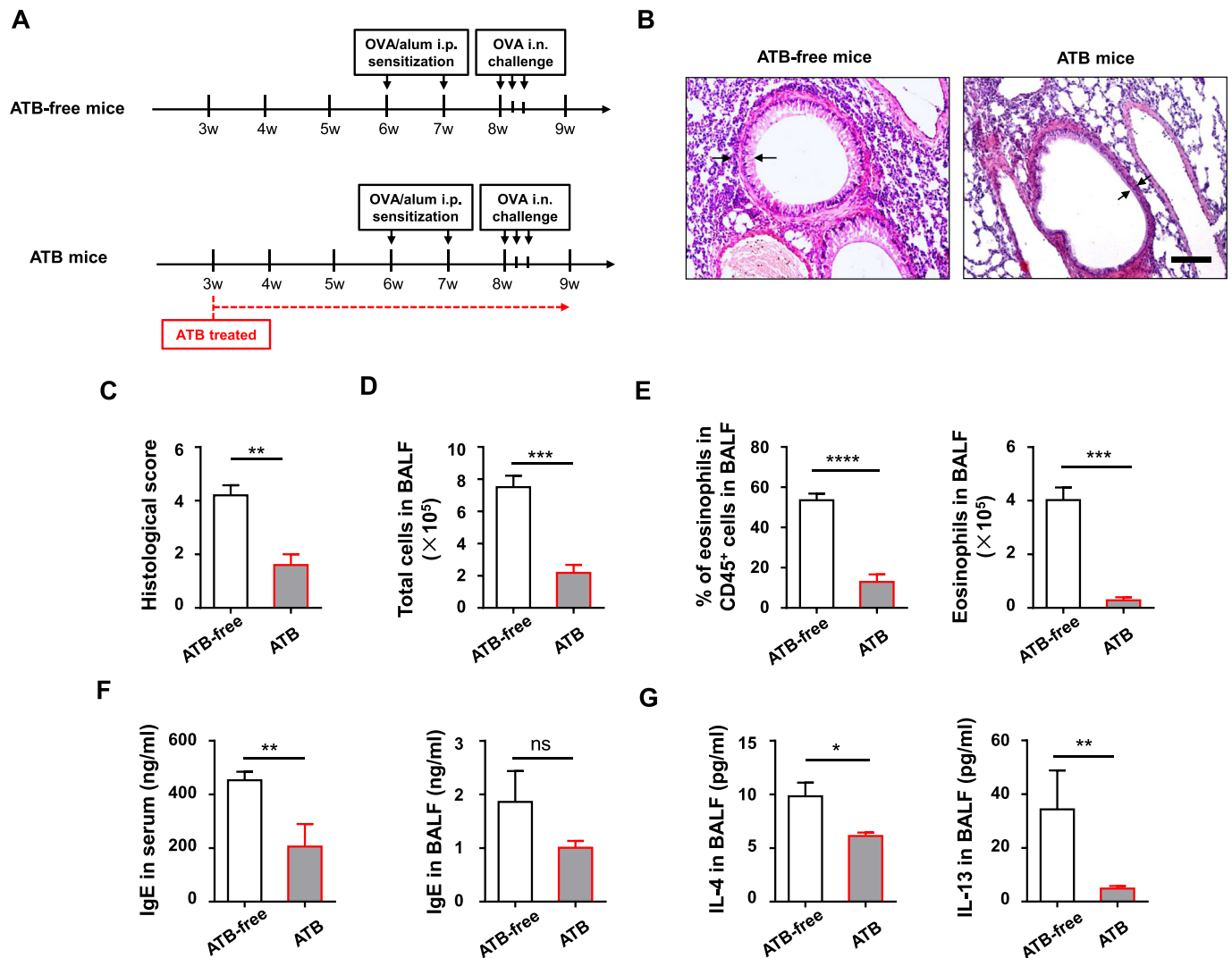


Fig. 1. Antibiotic treatment prevents OVA-induced allergic asthma in post-weaning mice. (A–G) Mouse models of OVA-induced asthma with ATB mice and ATB-free mice. Briefly, mice were treated with combined antibiotic water starting from 3 weeks of age until the end of the experiment (ATB mice), and mice treated with antibiotic-free water were the control (ATB-free mice). All mice were injected (i.p.) with OVA/alum for sensitization at 6- and 7-week-old and then challenged (i.n.) with OVA at 8-week-old. All mice were harvested and evaluated at 9-week-old (A). Histologic sections of lungs from ATB-free and ATB mice were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy. Pictures show representative samples of 4–6 mice/group. Scale bar = 200 μ m (B). (C) Histological scores for assessment of lung injury were shown. (D) Total cells in BALF from different groups of mice were counted. (E) The frequencies and numbers of eosinophils ($CD45^+$ Siglec $^+$ $CD11b^+$ $CD11c^-$) in BALF from different groups of mice were analyzed by FACS. (F) The levels of IgE in serum and BALF were analyzed by ELISA. (G) The levels of IL-4, and IL-13 in BALF were analyzed by ELISA. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.00001$, ns = not significant.

and pM ϕ were almost replaced by recruited inflammatory cells 4 h after OVA/alum sensitization (Fig. 5A). To assess the function of these cell subsets in OVA-induced allergic asthma, we purified pM ϕ , inflammatory monocytes and neutrophils from OVA/alum-sensitized CD45.1 WT mice (Fig. S3) and then transferred them adaptively to CD45.2 recipient mice, respectively. Transfer of inflammatory monocytes could induce asthma in recipient mice after OVA challenge even without OVA/alum sensitization, but transfer of pM ϕ or neutrophils did not have this effect (Fig. 5B and C), suggesting that recruitment of inflammatory monocytes after OVA/alum sensitization had a critical role in OVA-induced allergic asthma. Further study found that there were fewer inflammatory monocytes in the peritoneal cavity of mice that underwent peritoneal lavage after OVA/alum sensitization compared with sham-operation mice (Fig. 5D), and fewer infiltrating inflammatory monocytes were recruited into the peritoneal cavity from peripheral blood in ATB mice after OVA/alum sensitization compared with ATB-free mice (Fig. 5E and F). Altogether, these data suggested

that commensal bacteria in post-weaning mice promote pM ϕ -dependent allergic asthma through maintaining NLRP3/IL-1 β signaling, which promoted the recruitment of inflammatory monocytes to aggravate OVA-induced allergic asthma.

3.4. Metronidazole- and vancomycin-sensitive bacteria are major players in maintenance of NLRP3/IL-1 β signal in pM ϕ

To explore which bacterial species regulated NLRP3/IL-1 β signaling in the mouse model of OVA-induced allergic asthma, we treated WT mice with an individual antibiotic or paired antibiotics in drinking water. The numbers of total cells and eosinophils in alveoli after asthma establishment showed that treatment with ampicillin alone or metronidazole alone attenuated OVA-induced allergic asthma, whereas treatment with vancomycin alone or neomycin alone aggravated asthma (Fig. 6A and B). Hence, ampicillin- or metronidazole-sensitive bacteria might function to maintain NLRP3/IL-1 β signaling. To test

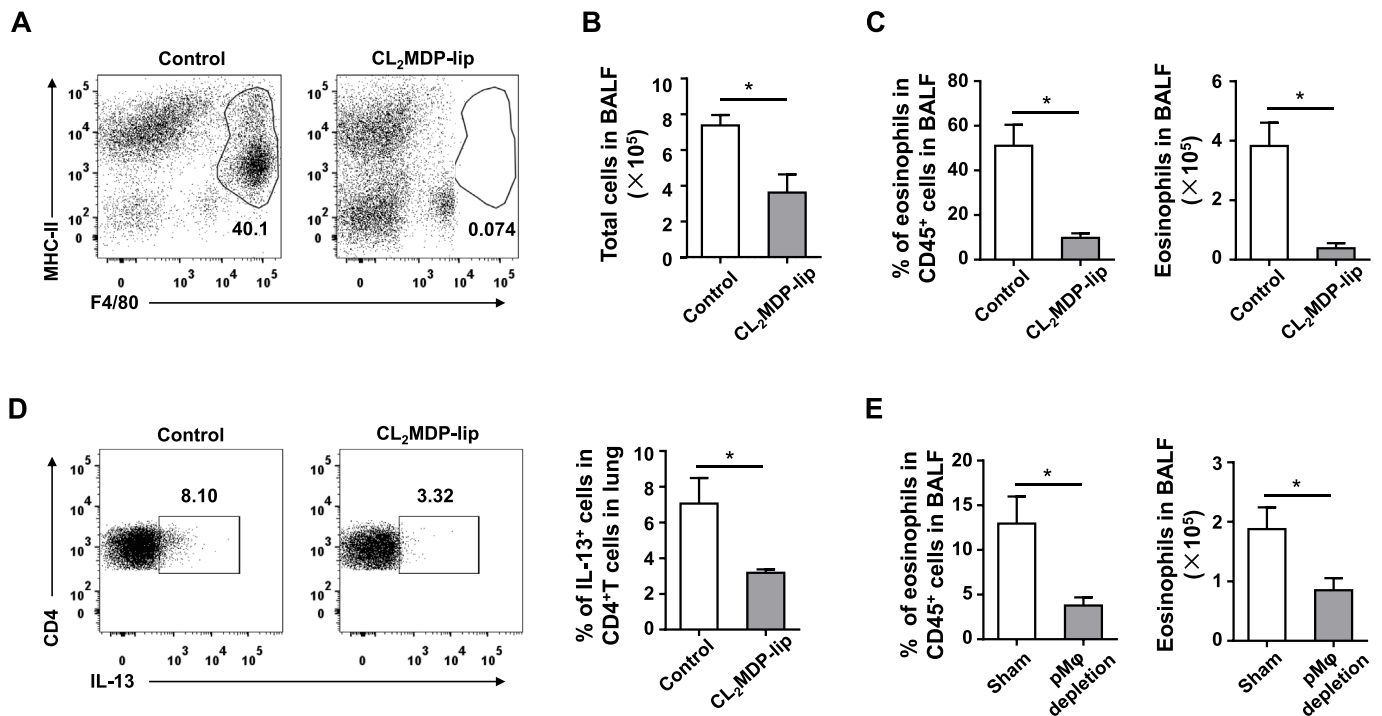


Fig. 2. Clearance of pMφ attenuates OVA-induced allergic asthma. (A–D) WT mice were injected (i.p.) with 100 μ L of clodronate liposomes (CL₂MDP-lip) to clear pMφ before establishing OVA-induced asthma. pMφ (gating on CD45⁺ single cells) in peritoneal cavity were analyzed by FACS 2 days later (A). After asthma establishment, total cells in BALF from control and CL₂MDP-lip-treated mice were counted (B), the frequency and number of eosinophils in BALF were analyzed by FACS (C), and the frequency of Th2 cells in total CD4⁺ T cells (gating on CD3⁺CD4⁺ single cells) from lung was analyzed by FACS (D). (E) WT mice underwent peritoneal lavage or sham operation before establishment of OVA-induced asthma. The frequency and number of eosinophils in BALF were analyzed by FACS after asthma establishment. Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, ns = not significant.

these hypotheses, we first measured expressions of NLRP3 and IL-1 β in pMφ after treatment with an individual antibiotic. Consistent with our hypotheses, we found that treatment with metronidazole alone down-regulated expression of NLRP3 and IL-1 β in pMφ significantly (Fig. 6C). However, unexpectedly, ampicillin treatment alone had no influence on the expression of NLRP3 or IL-1 β in pMφ, but treatment with vancomycin alone displayed the same effect as metronidazole treatment, which contradicted our speculation. Further studies showed that treatment with vancomycin alone also down-regulated TLR expressions in pMφ (Fig. 6D), which could be why treatment with vancomycin alone continued to aggravate asthma even though NLRP3/IL-1 β signaling had been down-regulated, because TLR signaling has been widely reported to protect against allergic asthma in many different mouse models of asthma [26–30]. Thus, metronidazole- and vancomycin-sensitive bacteria were involved in maintenance of NLRP3/IL-1 β signaling in pMφ. In addition, treatment with ampicillin alone or neomycin alone had no influence on NLRP3/IL-1 β , but they could attenuate or aggravate OVA-induced asthma, respectively. These data suggested that, in addition to NLRP3/IL-1 β signaling, commensal bacteria might influence other signaling types to influence OVA-induced asthma.

4. Discussion

The influence of dysbiosis of commensal bacteria on asthma development in pre-weaning mice has been widely studied. Data suggest that commensal bacteria in pre-weaning mice help to protect against OVA-induced allergic asthma. However, the influence of commensal

bacteria in post-weaning mice on asthma development is still limited. Here, we used oral delivery of combined antibiotics to study the influence of commensal bacteria in post-weaning mice on the development of airway allergic inflammation. Unexpectedly, different from the protective function of commensal bacteria in pre-weaning mice, commensal bacteria in post-weaning mice aggravated OVA-induced allergic asthma. Commensal bacteria in post-weaning mice promoted allergic asthma through maintenance of NLRP3/IL-1 β expression in pMφ to recruit inflammatory cells, especially inflammatory monocytes, from peripheral blood into the peritoneal cavity. Finally, the recruited inflammatory monocytes might promote OVA-induced allergic asthma through enhancing Th2 response.

Normally, commensal bacteria cannot remain stable for a long time because they must evolve rapidly to adapt to lifestyle and environmental changes. Modern life, including the use of antibiotics and high-fat diets, can change commensal bacterial communities persistently and, in turn, changes in commensal bacteria increase pathogen susceptibility and the risk of autoimmune disease [31]. It has been demonstrated widely that commensal bacteria are essential for development of the immune system and, in turn, the developed immune system changes the components of commensal bacteria. Miskinyte et al. studied bacterial evolution in the presence of immune cells. They found that the presence of macrophages could impose selective pressure to the commensal bacterium *Escherichia coli* and promote the *E. coli* to become more resistant to the actions of macrophages [32]. In pre-weaning mice, commensal bacteria are mainly from the birth canal and the bacteria in breast milk. However, post-weaning mice start to eat food, and the food becomes the main source of commensal bacteria. Meanwhile,

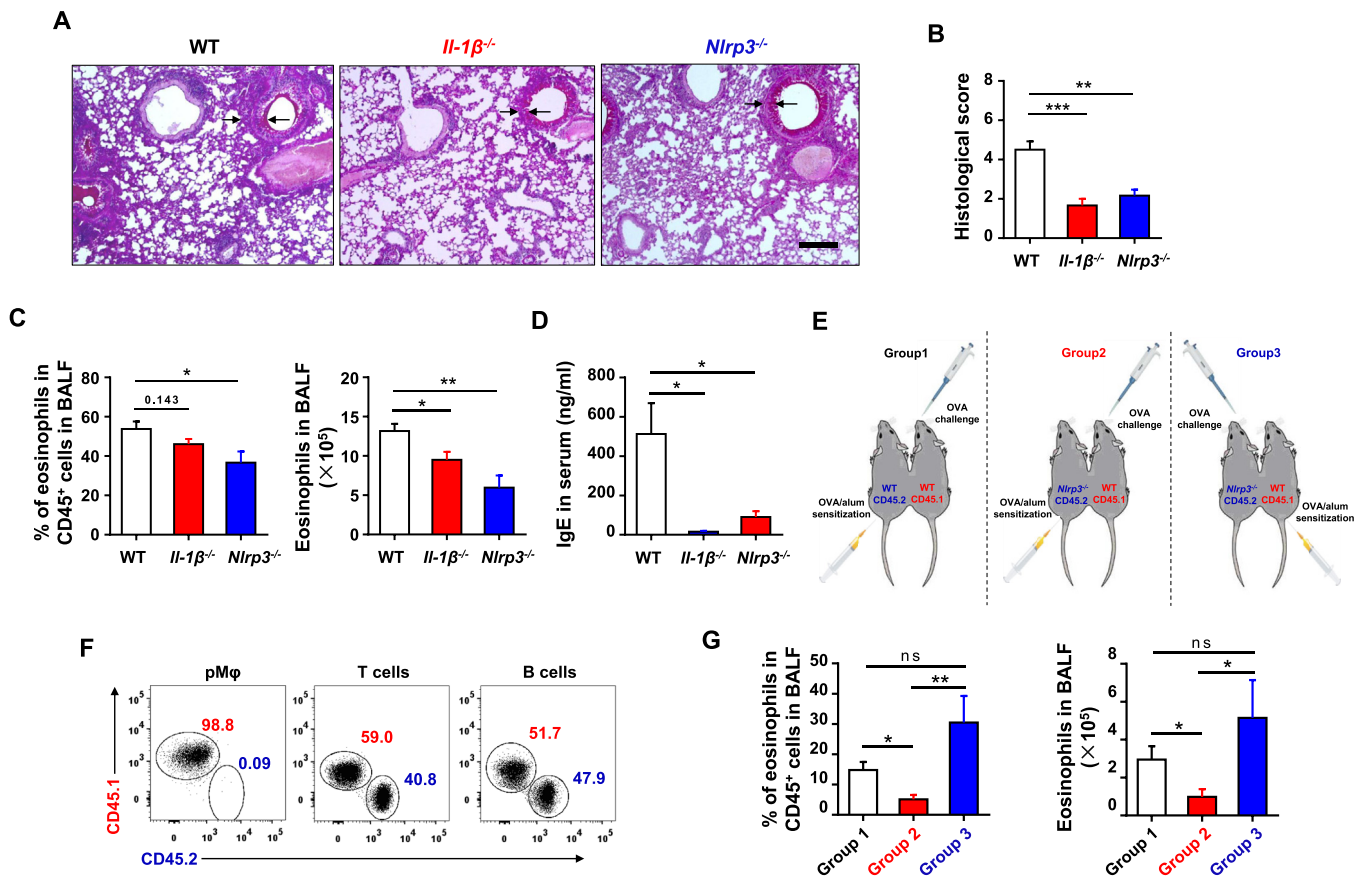


Fig. 3. Deficiency of NLRP3/IL-1 β signaling in pM ϕ attenuates OVA-induced allergic asthma. (A–D) 6-week-old *Nlrp3*^{-/-}, *IL-1β*^{-/-} and control WT mice were treated (i.p.) with OVA/alum and challenged (i.n.) with OVA to induce asthma, and all mice were harvested and analyzed 1 week after OVA challenge. Histologic sections of lungs from each group were stained with Periodic Acid Schiff and analyzed by light microscopy. Pictures show representative samples of 4–6 mice/group. Scale bar = 200 μ m (A). Histological scores for assessment of lung injury were shown (B). The frequency and number of eosinophils in BALF (C) and IgE level in serum (D) were analyzed by FACS and ELISA, respectively. (E–G) Grouping schemes of parabiosis and asthma sensitization and challenge: CD45.1 mice and CD45.2 mice that included WT and *Nlrp3*^{-/-} mice were connected surgically for parabiosis 2–3 weeks before injecting one mouse (i.p.) with OVA/alum for sensitization and then challenging another mouse (i.n.) with OVA. All mice were harvested 1 week after OVA challenge (E). Autologous and xenogenous cells in blood and PLF from CD45.1 mice were analyzed by FACS after 2 weeks of parabiosis (F). The frequency and number of eosinophils in BALF from OVA-challenged mouse were analyzed by FACS (G). Data are representative of three independent experiments except (G). The data in the (G) are pools of four experiments. Data are the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, ns = not significant.

commensal bacteria in post-weaning mice also need to change to adapt to new foods. Thus, the component of commensal bacteria should be different between pre-weaning and post-weaning mice, which might be why commensal bacteria have different functions in OVA-induced allergic asthma in pre-weaning and post-weaning mice.

As described above, commensal bacteria are essential for the development and function of the immune system in local tissues. This effect is explained by the interaction between bacteria (or their products) with innate immune receptors expressed on local barriers (e.g., epithelial cells and resident immune cells) [33]. In addition to local tissue, commensal bacteria also influence distal tissues and even systemic immunity and diseases. For example, intestinal commensal bacteria contribute to the formation of gut-associated lymphoid tissues and intestinal disease, but also have effects on extra-intestinal immune homeostasis and diseases [34,35]. Ichinohe and colleagues found that intestinal commensal bacteria regulate immune defense against infection from influenza viruses in the respiratory tract of C57BL/6 mice through maintenance of an inflammasome signaling pathway at steady state [36]. Consistent with previous studies, we also found that commensal bacteria in post-weaning mice could maintain expression of

NLRP3 and IL-1 β in pM ϕ to promote OVA-induced allergic asthma. With regard to the mechanism by which commensal bacteria regulate expression of NLRP3 and IL-1 β in pM ϕ , we speculated that the components of commensal bacterial might activate the TLRs expressed on pM ϕ surface to maintain expression of NLRP3 and IL-1 β .

PM ϕ are typical tissue-resident macrophages. They are similar to other macrophages in that they have crucial roles in the maintenance of tissue homeostasis but also contribute to inflammatory and repair responses during pathogenic infection and tissue injury [25,37]. After initial recognition of invading pathogens, tissue-resident macrophages activate and secrete chemokines to recruit inflammatory leukocytes, including inflammatory monocytes and neutrophils, from peripheral blood. We also found that pM ϕ depletion reduced the recruitment of blood-derived inflammatory monocytes after OVA/alum sensitization, and that inflammatory monocyte recruitment was dependent upon NLRP3/IL-1 β signaling.

Studies have shown that activation of IL-1 receptors is required for neutrophil recruitment in the immune response against bacterial infection, and that mice deficient in IL-1 β (but not IL-1 α) have reduced recruitment of neutrophils and develop larger lesions with higher

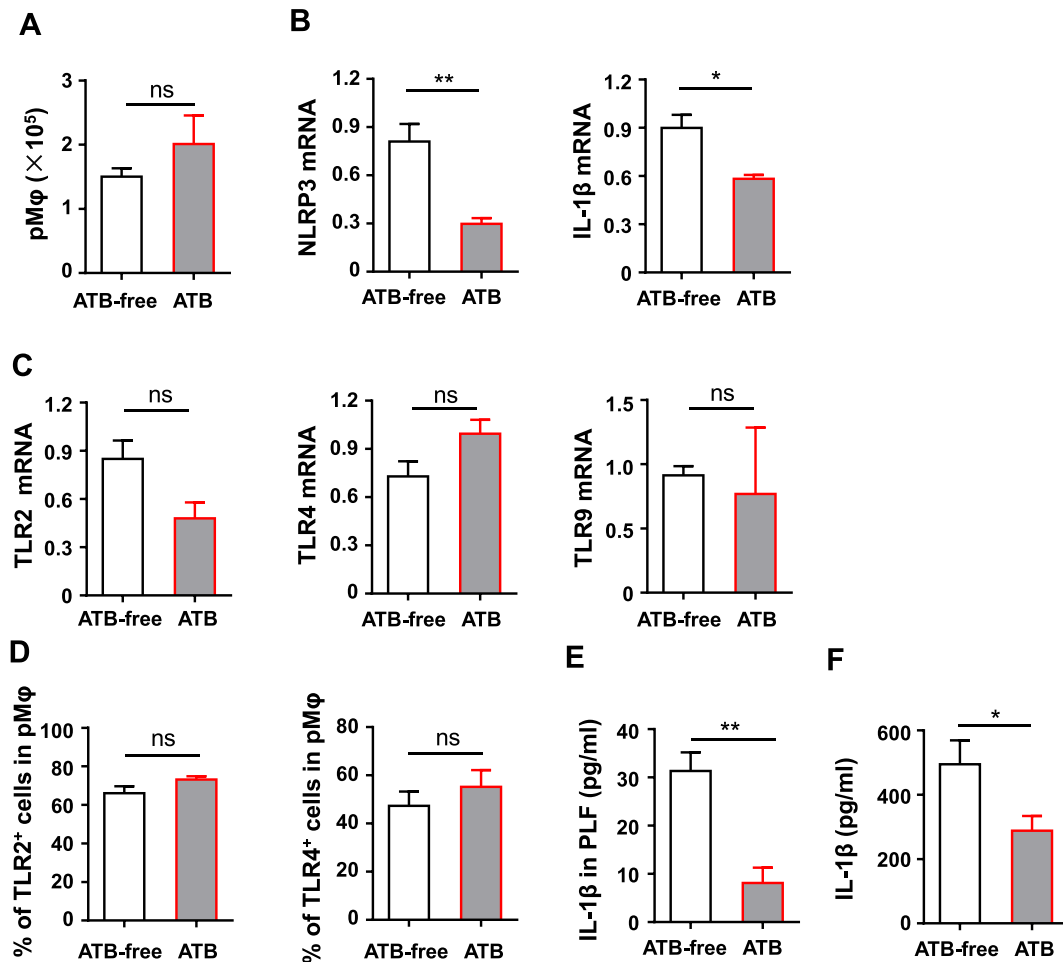


Fig. 4. Antibiotic treatment down-regulates NLRP3/IL-1 β signaling in pM ϕ . (A–F) 3-week-old B6 mice were fed water containing antibiotics for 3 weeks (ATB mice). The number of peritoneal macrophages was counted in peritoneal lavage fluid (PLF) after antibiotic treatment or not (A). NLRP3 and IL-1 β mRNA expression in peritoneal macrophages was analyzed by real-time PCR (B), TLR2, TLR4 and TLR9 mRNA expression in peritoneal macrophages was analyzed by real-time PCR (C), TLR2 and TLR4 expression on peritoneal macrophages was analyzed by FACS (D), and the level of IL-1 β in the peritoneal cavity 6 h after OVA/alum sensitization was analyzed by ELISA (E). PM ϕ from ATB-free and ATB mice were purified and cultured with 20 μ g/mL of OVA and 200 μ g/mL of alum for 14 h *in vitro*. The IL-1 β level in culture supernatants was evaluated by ELISA (F). Data are representative of three or more independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, ns = not significant.

bacterial counts compared with WT mice [38]. Consistent with those studies, we showed that commensal bacteria maintained IL-1 β expression in pM ϕ to promote the recruitment of blood-derived monocytes and neutrophils after OVA/alum sensitization.

Commensal bacteria have critical roles in modulation of many of the genes involved in intestinal barrier function and nutrient absorption, and the interaction between commensal bacteria and the host is dependent mainly upon pattern recognition receptors (PRRs). PRRs include TLRs, nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) and retinoic acid-inducible gene-I-like receptors (RLRs). Different PRRs recognize different pathogen-associated molecular patterns from commensal bacteria and pathogens to activate downstream signaling cascades, including nuclear factor-kappa B, interferon response factors, activator protein 1 or mitogen-activated protein kinases, to execute different functions [39]. TLR signaling, particularly TLR2 signaling, has been reported to maintain intestinal epithelial homeostasis and protect against epithelial injury [19], but NLR signaling has been implicated in the inflammatory response [40]. That is, different bacterial components may mediate different

responses, beneficial or harmful, which depend on PRRs. TLR2 and TLR4 recognize lipopeptides and lipopolysaccharide from Gram-positive and Gram-negative bacteria, respectively. NOD1 and NOD2 can recognize components from *Listeria monocytogenes* [39]. This might be why treating mice with an individual antibiotic had different effects on OVA-induced asthma in our study. However, we do not think that the result from combined antibiotic treatment was an overlay of the effect of individual antibiotic treatment because some results from individual antibiotic treatment contradicted the data from combined antibiotic treatment.

Worldwide, about 230 million people are suffering from asthma according to World Health Organization estimates. However, allergic sensitization in humans is incompletely understood: it is not clear why some people develop asthma but others do not even though both populations are exposed to allergens to the same degree. According to our study, the difference in the constitution of commensal bacteria may contribute to this difference. Thus, it may be possible to answer this question by identifying and comparing commensal bacteria from healthy donors and patients with asthma.

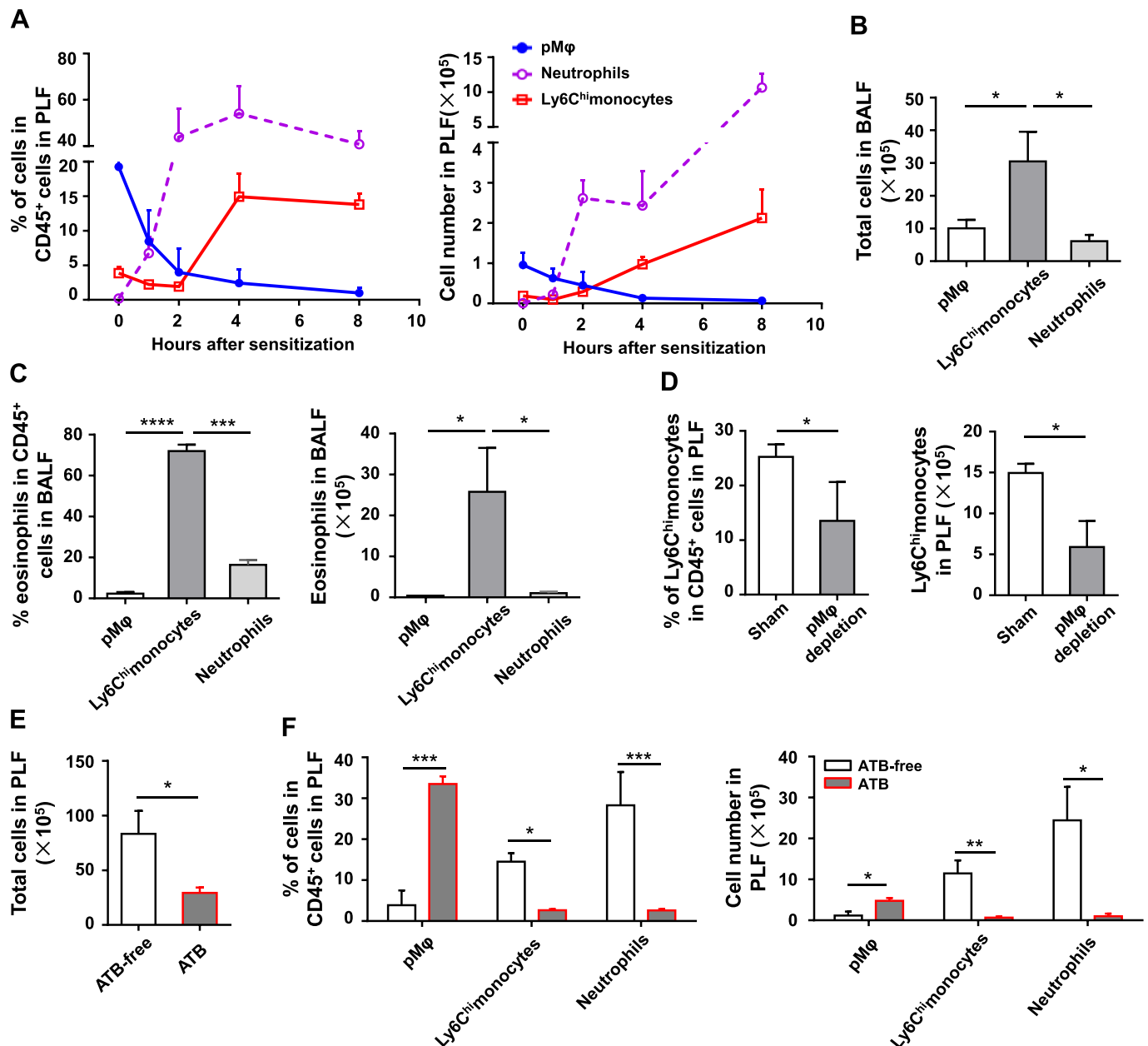


Fig. 5. Antibiotic treatment reduces recruitment of inflammatory monocytes. (A) 6-week-old WT mice were treated (i.p.) with OVA/alum. The frequency and number of pMφ (CD45⁺ F4/80^{hi}CD11b^{hi}), inflammatory monocytes (CD45⁺ F4/80^{lo}Ly6C^{hi}CD11b⁺), and neutrophils (CD45⁺ Ly6G^{hi}CD11b^{hi}) in PLF were analyzed by FACS at the indicated time points after OVA/alum treatment. (B–C) CD45.1 mice were treated (i.p.) with OVA/alum, and PLF were harvested 2 h after treatment to purify pMφ or 4 h after treatment to purify inflammatory monocytes and neutrophils by sorting. 1.5×10^5 of each cell subset were transferred (i.p.) into CD45.2 recipient mice immediately after sorting once a week for 2 weeks, respectively. The CD45.2 recipient mice were challenged (i.n.) with OVA 1 week after the second transfer. All CD45.2 recipient mice were harvested and analyzed 1 week after challenge. Total cells in BALF were counted (B), and the frequency and number of eosinophils in BALF from each group were analyzed by FACS (C). (D) The frequency and number of inflammatory monocytes in PLF from peritoneal lavage-treated mice were analyzed by FACS 24 h after injecting (i.p.) with OVA/alum. (E) Total cells in PLF from ATB and ATB-free mice 24 h after injecting (i.p.) with OVA/alum were counted. (F) The frequency and number of peritoneal macrophages, inflammatory monocytes, and neutrophils in PLF from ATB and ATB-free mice were analyzed by FACS 24 h after injecting (i.p.) with OVA/alum. Data are representative of more than two independent experiments with ≥ 4 mice per group. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.00001$, ns = not significant.

Author contributions

C.H. designed and performed the experiments and analyzed and interpreted the data; R.S. and H.W. established techniques of FACS and histology and interpreted the data; X.Z. and Y.C. assisted with data interpretation; R.Z. supported animal experiments. Z.T. provided strategic planning, conceived the project, and interpreted the data. J.W. supervised the project, provided crucial ideas, and assisted with data interpretation; C.H. wrote the manuscript with J.W. and Z.T.

Conflicts of interest

There is no conflict of interest.

Funding source

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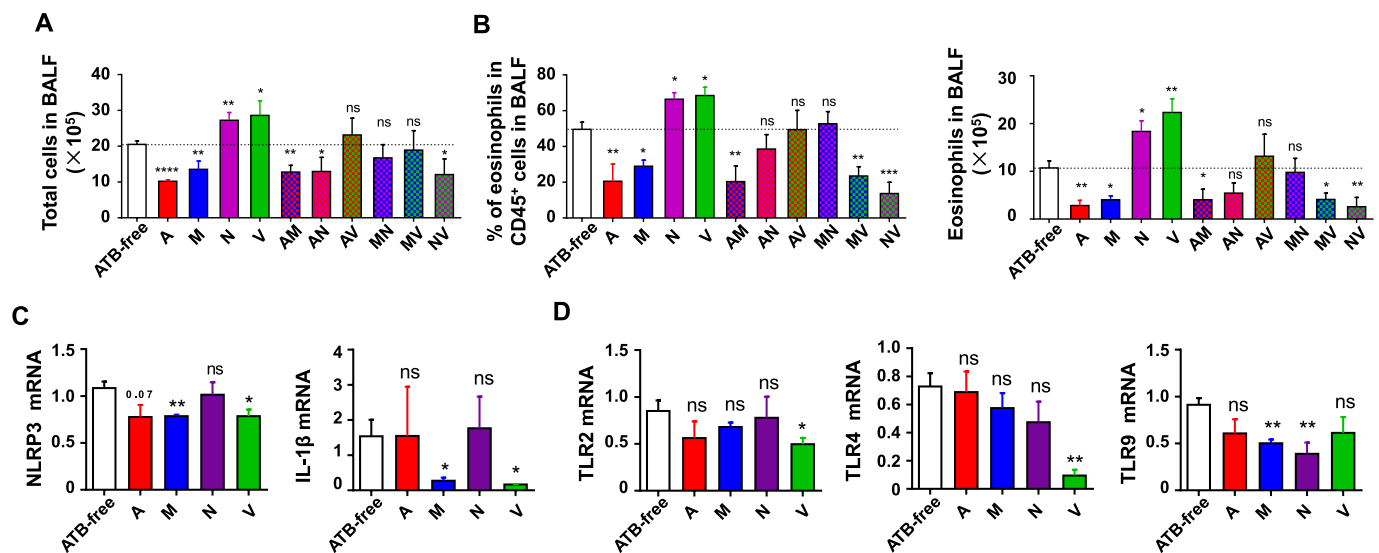


Fig. 6. NLRP3/IL-1 β signaling is regulated by metronidazole- and vancomycin-sensitive bacteria. (A–B) 3-week-old WT mice were treated with an individual antibiotic or paired antibiotics in drinking water until the end of the experiment. All mice were injected (i.p.) with OVA/alum for sensitization at 6- and 7-week-old and then challenged (i.n.) with OVA at 8-week-old. All mice were harvested and analyzed at 9-week-old (A). The frequency and number of eosinophils in BALF from each group were analyzed by FACS (B). (C–D) WT mice were treated with an individual antibiotic. pMφ were purified, and total RNA was extracted from pMφ 3 weeks later. NLRP3 and IL-1 β mRNA expressions in pMφ were analyzed by real-time PCR (C). TLR2, TLR4, and TLR9 mRNA expressions in pMφ were analyzed by real-time PCR (D). Data are pools of three experiments. Data are the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, **** P < 0.00001, ns = not significant. A = ampicillin, V = vancomycin, N = neomycin, M = metronidazole.

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Abbreviations

OVA	ovalbumin
NLRP3	nucleotide-binding domain and leucine-rich repeat protein-3
pMφ	peritoneal macrophages
TLRs	Toll-like receptors
Th	T-helper
Ig	immunoglobulin
BALF	bronchoalveolar lavage fluid
PLF	peritoneal lavage fluid
CL ₂ MDP-lip	clodronate liposomes
ATB-free mice	non-antibiotic-treated control mice
ATB mice	mice treated with antibiotics in drinking water
PRRs	Pattern recognition receptors
NLRs	leucine-rich repeat containing receptors

Appendix A. Supplementary data

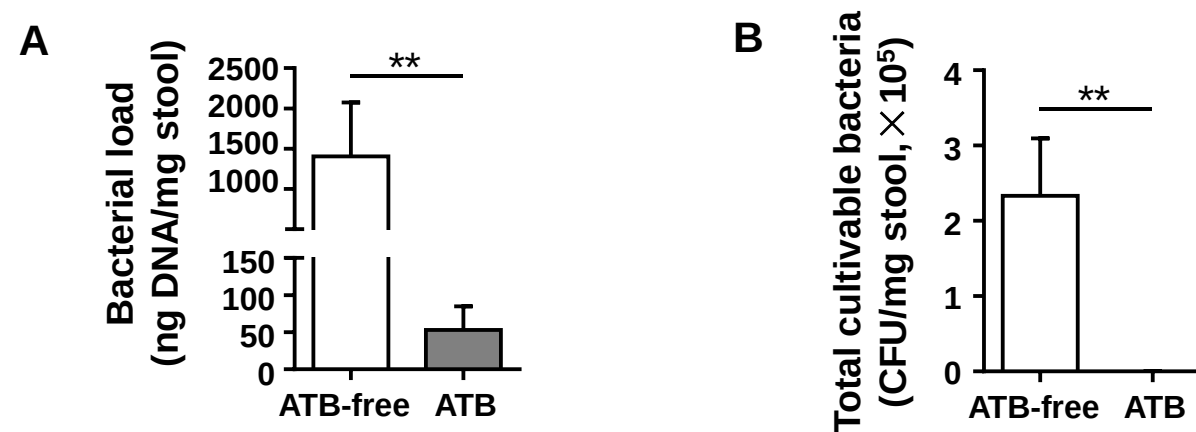
Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jaut.2018.07.003>.

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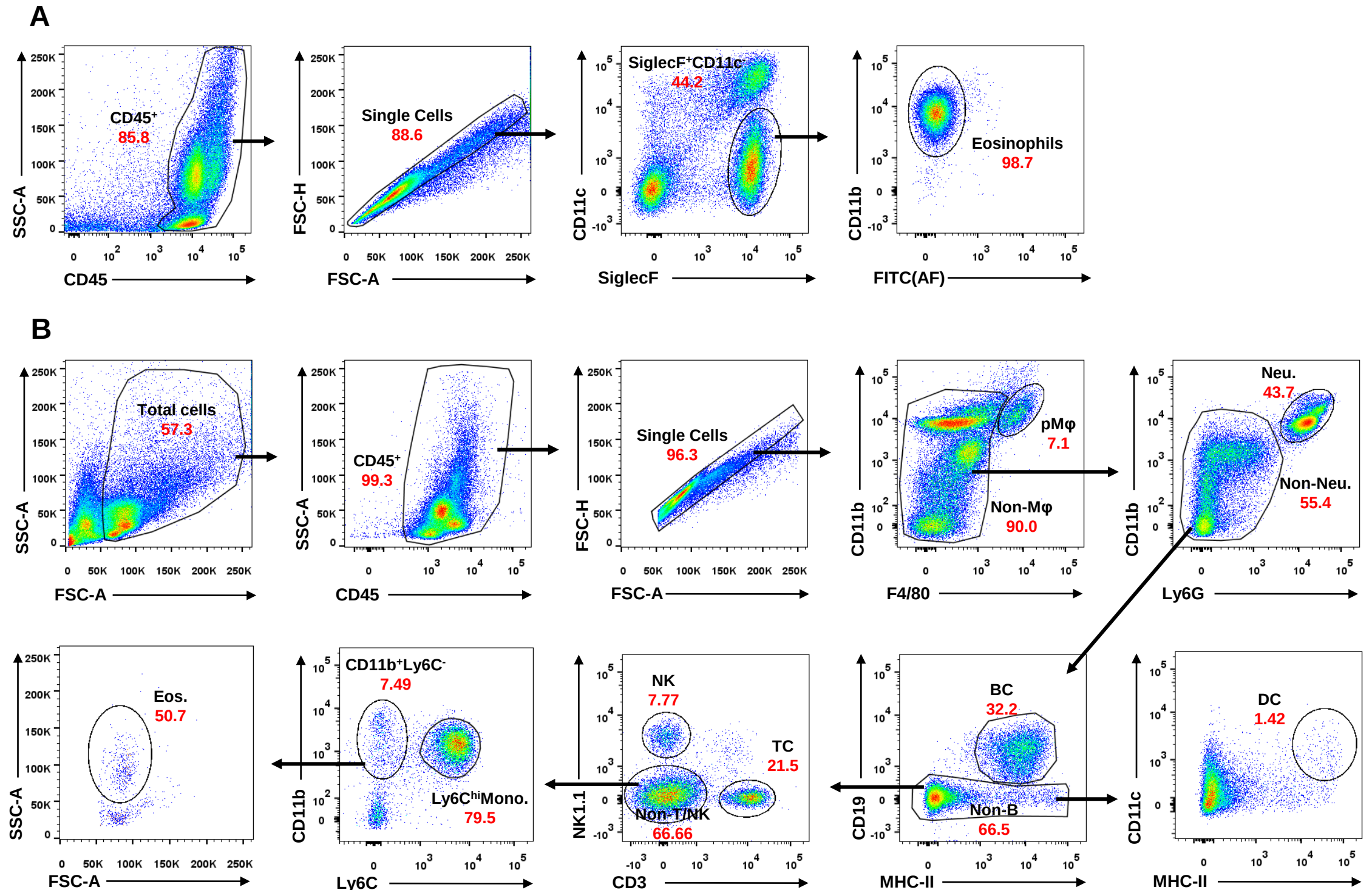
Chao Huang *et al.* Supplementary Figure 1



Supplementary Figure 1. Mouse model of antibiotic-mediated clearance of commensal bacteria.

3-week-old B6 mice were fed on water containing antibiotics (ATB) or not (ATB-free) for 3 weeks. **(A)** Global stool bacterial DNA was extracted and calculated. **(B)** Total cultivable bacteria in fresh stool were planted and counted on blood agar plates. Data are representative of three or more independent experiments with ≥ 3 mice per group. Data are the mean $\pm$ SEM. **P < 0.01.

Chao Huang *et al.* Supplementary Figure 2

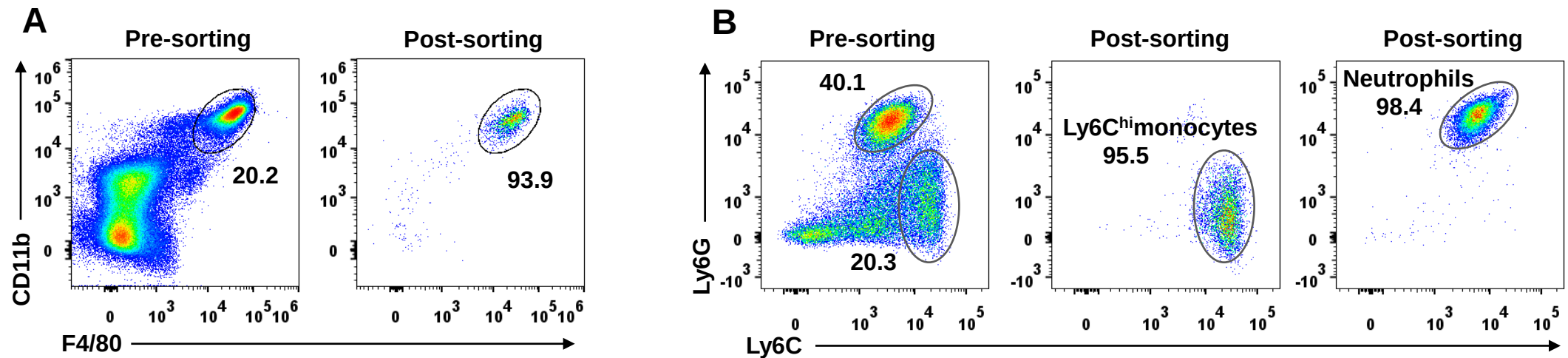


Supplementary Figure 2. Gating strategy used in flow cytometry analysis.

(A) Eosinophils in BALF were gated as CD45⁺SiglecF⁺CD11b⁺CD11c⁻ cells. AF, autofluorescence.

(B) Peritoneal macrophages in PLF were gated as CD45⁺F4/80^{hi}CD11b^{hi} cells, inflammatory monocytes in PLF were gated as CD45⁺F4/80^{lo}Ly6C^{hi}CD11b⁺ cells, and neutrophils in PLF were gated as CD45⁺Ly6G^{hi}CD11b^{hi} cells. Neu., neutrophils; BC, B cells; TC, T cells; DC, dendritic cells; Mono., monocytes; Eos., eosinophils.

Chao Huang *et al.* Supplementary Figure 3



Supplementary Figure 3. The analysis of the purity of cells after sorting.

(A) FACS analysis of the purity of pMφ (gating on CD45⁺ single cells) after cell sorting. Related to Figure 2 and Figure 6.

(B) FACS analysis of the purity of inflammatory monocytes and neutrophils after cell sorting. Related to Figure 5.

Chao Huang *et al.* Supplementary Table 1

Supplementary Table 1. Anti-mouse monoclonal antibodies for flow cytometry

Fluorescence	Antigen	Clone	Company	Catalog number	Isotype control	Dilution
APC	CD4	RM4-4	BioLegend	116014	Rat IgG2a,k	1: 200
APC	CD11c	HL3	BD	550261	ArH IgG1,λ2	1: 200
APC-CY7	CD4	GK1.5	BD	552051	Rat IgG2b,κ	1: 200
APC-CY7	CD19	6D5	BioLegend	115530	Rat IgG2a,k	1: 200
APC-CY7	CD11b	M1/70	BD	557657	Rat IgG2b,κ	1: 200
APC-CY7	CD45.2	104	BioLegend	109824	Ms IgG2a, κ	1: 200
Alexa Fluor® 647	SiglecF	E50-2440	BD	562680	Rat IgG2a, κ	1: 200
BV421	CD11b	M1/70	BioLegend	101236	Rat IgG2b, κ	1: 200
BV510	CD45	30-F11	BD	563891	Rat IgG2b, κ	1: 200
BV510	MHC-II	M5/114.15.2	BioLegend	107635	Rat IgG2b, κ	1: 200
BV510	CD3e	145-2C11	BioLegend	100353	AH IgG1, κ	1: 200
BV605	Ly6C	HK1.4	BioLegend	128035	Rat IgG2c, κ	1: 200
BV605	CD4	RM4-5	BioLegend	100548	Rat IgG2a, κ	1: 200
BV711	F4/80	BM8	BioLegend	123147	Rat IgG2a, κ	1: 200
BV786	CD45	30-F11	BioLegend	103149	Rat IgG2b, κ	1: 200
BV786	CD45.2	104	BD	563686	Rat IgG2a, κ	1: 200
FITC	TLR2	6C2	eB	11-9021	Rat IgG2b,k	1: 200
FITC	Ly6C	AL-21	BD	553104	Rat IgM, κ	1: 200
FITC	CD11b	M1/70	BD	553310	Rat IgG2b, κ	1: 200
FITC	Rat IgG2b,k	A95-1	BD	553988	/	1: 200
Pacific Blue	Ly6G	1A8	BioLegend	127611	Rat IgG2a, κ	1: 200
PE	SiglecF	E50-2440	BD	552126	Rat IgG2a, κ	1: 200
PE	Ly6G	1A8	BD	551461	Rat IgG2a,k	1: 200
PE	IL-13	eBio13A	eB	12-7133	Rat IgG1,κ	1: 100
PE	CD284(TLR4)	UT41	eB	12-9041-80	Mouse IgG1, κ	1: 100
PE	Mouse IgG1, κ	MOPC-31C	BD	550617	/	1: 100
PE	Rat IgG1,k	R3-34	BD	554685	/	1: 100
PE/Dazzle™ 594	CD3	17A2	BioLegend	100246	Rat IgG2b, κ	1: 200
PE-CY7	CD3e	145-2C11	BioLegend	100320	AH IgG	1: 200
PE-CY7	CD45.1	A20	BioLegend	110730	Ms IgG2a,κ	1: 200
PE-CY7	CD45	30-F11	BD	552848	Rat IgG2b,k	1: 200
PE-CY7	NK1.1	PK136	BD	552878	Ms IgG2a,κ	1: 200
Percp-CY5.5	NK1.1	PK136	BioLegend	108728	ms IgG2a,k	1: 200
Percp-CY5.5	F4/80	BM8	eB	45-4801	Rat IgG2a,k	1: 200
Percp-CY5.5	CD3e	145-2C11	BD	551163	AH IgG1,k	1: 200
Percp-CY5.5	CD11b	M1/70	BioLegend	101228	Rat IgG2b,k	1: 200
Percp-CY5.5	CD19	6D5	BioLegend	115534	Rat IgG2a,k	1: 200

Chao Huang *et al.* Supplementary Table 2

Supplementary Table 2. Primers for RT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Il-1β</i>	CTCGTGCTGTCGGACCCAT	CAGGCTTGTGCTCTGCTTGTGA
<i>nlrp3</i>	ACCTCAACAGTCGCTACAC	GTCTCGGGCTCAAACA
<i>tlr2</i>	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
<i>tlr4</i>	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
<i>tlr9</i>	ACTGAGCACCCCTGCTTCTA	AGATTAGTCAGCGGCAGGAA
<i>gapdh</i>	TGCCCAGACATCATCCCTG	TCAGATCCACGACGGACACA