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Maternal microbiome regulation prevents early allergic airway diseases in mouse offspring

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Editor: Jon Genuneit**Abstract****Background:** Asthma is a serious global health problem, severely affecting the lives of sufferers and their families. An exceptionally hygienic home and reduced microbial exposure can aggravate the incidence of childhood asthma.**Methods:** Specific-pathogen-free BALB/c mice were pre-treated with bacterial lysate (BL; 1 mg/kg) as a high microbial load maternal mouse model, and then, the offspring mice were established as an allergic airway disease (AAD) model. The expression levels of TLR2, TLR4, and HDAC9 in the mother's intestine and the offspring's lungs were detected. Relevant indicators of regulatory T cells (Tregs) were identified in the mother and offspring mice. The changes in the expression of Th1-, Th2-, Th9-, and Th17-related cytokines in the offspring mice were evaluated among different pre-treated groups.**Results:** After augmenting the mothers' intestinal microbiota through oral BL gavage, the expression of TLR2 and TLR4 in the colon mucosa and colon lymphoid tissues was enhanced and that of HDAC9 in the colon mucosa was decreased, and the proportion of spleen Tregs was increased. The offspring showed similar changes in the AAD model compared with the offspring of the control-group mothers: TLR2 and TLR4 expression in the lungs and the proportion of spleen Tregs increased, HDAC9 expression in the lungs decreased, and AAD-induced airway pathologic characteristics were reversed; additionally, Th1/Th2 and Th9 imbalances were rectified.**Conclusions:** This study presents a new framework for the prevention of childhood asthma, elucidating the mechanism of regulating the mother's intestinal microbiome to protect the offspring's early asthma via animal experiments.**KEYWORDS**

allergic airway disease, asthma prevention, microbiome, TLR2, TLR4, Tregs

Jing Liu and Yanlei Li contributing equally to this article

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1 | INTRODUCTION

In recent years, the prevalence of asthma and other allergic diseases has increased significantly and ranges from 1% to 18%, depending on the country. Thus, asthma has become one of the major diseases affecting human health, especially that of children.¹

The incidence of allergic diseases such as asthma is related to various genetic and environmental factors and their interactions; in children with a family history of allergic diseases, the incidence is significantly increased. Hence, for such children at high risk of allergic disease, it is possible to achieve some preventive effect by early adjustment of environmental factors. Recently, many studies on asthma risks and protective factors have found that an excessively hygienic home, which occurs in many cities globally, is a risk factor for childhood asthma.² On the contrary, long-term contact with farm animals during early life³ and a living environment rich in a variety of bacteria and fungi play important roles in preventing asthma and other allergic diseases in children.⁴ Rural environments and increased bacterial endotoxin levels may provide protection against childhood asthma by affecting pregnant mothers.^{3,5} Relevant animal model studies have found that supplementation with bacterial extracts or intestinal bacteria can improve the airway inflammatory response induced by ovalbumin (OVA).^{6,7}

Intestinal microbial disorders affect lung immunity and are associated with lung diseases, such as allergies and asthma.¹ Microorganisms can stimulate pattern recognition receptors on the surface of innate immune cells, especially Toll-like receptors (TLRs), to initiate immediate innate immunity.⁷ In our previous study, we established a high microbial load maternal-offspring mouse allergic airway disease (AAD) model in which bacterial lysate (BL; 1 mg/kg, Broncho-Vaxon®; OM Pharma Ltd.) was used to stimulate safe and adequate microbial load levels during maternal pregnancy to produce offspring mice with reduced pathologic features of AAD.⁸ However, the mechanism of reducing the pathologic features of asthma in offspring is not clear and further study is needed.

TLRs play important roles in regulating T-cell function and homeostasis.⁹ Regulatory T cells (Tregs) are less abundant and less active in adults and children with asthma; furthermore, the inhibitory effect of Tregs on Th2 cells is significantly reduced in asthma.¹⁰ When the T-cell receptor stimulates Tregs, histone deacetylase 9 (HDAC9) is phosphorylated and transported from the nucleus, allowing forkhead box P3 (Foxp3) to be acetylated, enhancing the binding of Foxp3 to a promoter, and optimizing the suppressive function of Tregs.¹¹ Th17/Treg imbalance plays an important role in the pathogenesis of asthma and may be as important as Th1/Th2 imbalance.¹² Th9 cells are characterized by the secretion of large amounts of IL-9 cytokines, which can promote the accumulation of mast cells and eosinophils, produce Th2 cytokines, and cause hypersecretions of mucus.¹³ Herein, we examined the immune transmission from mother to offspring and the protective mechanism of asthma in the high microbial load maternal-offspring mouse asthma model.

2 | METHODS

2.1 | Mice

Specific-pathogen-free (SPF), 11-week-old sexually mature adult female BALB/c mice (weight, 19–22 g) and 12-week-old sexually mature adult male BALB/c mice (weight, 22–25 g) were obtained from the Huafukang Bioscience Company (Beijing, China). All procedures were ethically reviewed and approved by the Animal Experimental Ethics Committee of Jilin University. Animals were maintained in laminar flow cages (with autoclaved wood chips as bedding material; four mice in each cage), kept at 22°C, 45 ± 10% relative humidity, under a 12-hour light-dark cycle, and provided ad libitum access to food and water.

2.2 | Experimental protocols and sampling

As described in a previously published protocol⁸ (Figure A), female mice were randomly divided into two groups: the control group was gavaged with 0.2 mL phosphate-buffered saline (PBS; *n* = 6), and the BL-gavage group was gavaged with 1 mg/kg BL (*n* = 6). Mother mice were euthanized after weaning on the 21st day after delivery (by intravenous injection of pentobarbital sodium 150 mg/kg in the mouse tail); blood (collected from the animals' eye after administration of analgesia), lung, spleen, and colon samples were obtained from each animal. Each mother mouse gave birth (2–6 offspring) only once. Both male and female offspring were used for the AAD model at the 25th day after birth, but were raised in separate cages. The offspring were divided into three groups of six mice each: PBS-challenged offspring of control-group mothers (MP-OP); OVA-challenged (AAD) offspring of control-group mothers (MP-OO); and OVA-challenged (AAD) offspring of BL-gavage mothers (MB-OO).

2.3 | Analysis of 16S ribosomal RNA gene

PCR amplification was performed using general bacterial primers covering the V4–V5 region of the bacterial 16S ribosomal RNA genes. The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline¹⁴ (<https://drive5.com/usearch/manual/>). The OTU representative sequences were taxonomically classified using the Silva 119 database (<https://www.arb-silva.de/>), with a confidence score ≥ 0.8 by the classify.seqs command in mothur (version 1.33.3).

2.4 | Histologic evaluation

Lung sections (3 µm thick) from offspring were stained with hematoxylin and eosin (H&E). The severity of broncho-bronchiolar lesions was evaluated by optical microscopy (Olympus BX51).¹⁵

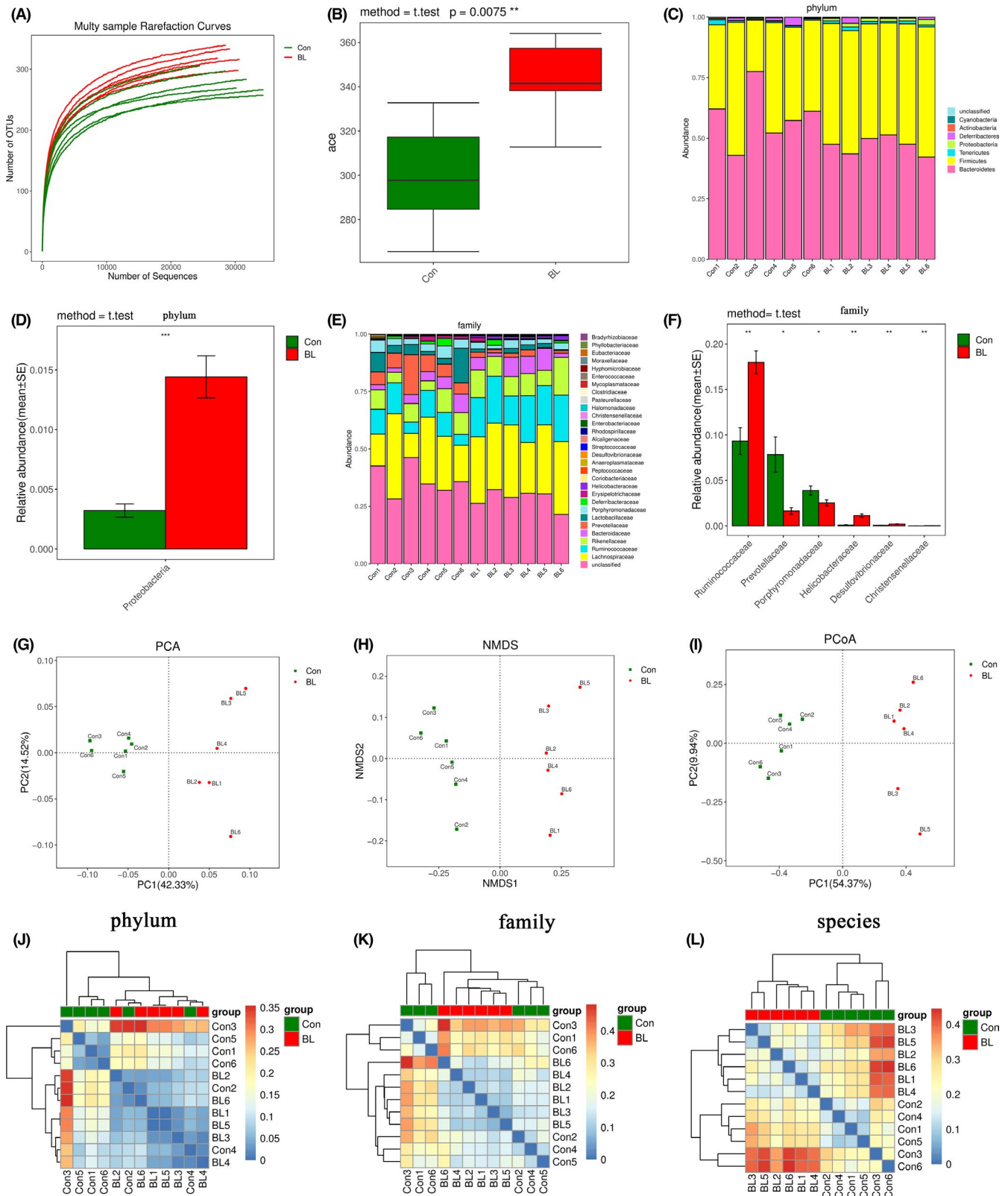


FIGURE 1 Analysis of 16S ribosomal DNA from mother's fecal samples. Rarefaction curves were used to compare the species richness in the two sequencing data groups (A). The abundance-based coverage estimator index was used to calculate bacterial abundance (B). The community structure composition histogram shows a significant difference between the two groups at the phylum (C, D) and family levels (E, F). Principal component analysis (G). Classification for the operational taxonomic unit (OTU) based on beta-diversity analysis, including the non-metric multidimensional scaling (NMDS) analysis (H) and principal coordinate analysis (PCoA) (I). Beta-diversity matrix heat map showing cluster analysis of the samples (J, K, L). Data are expressed as means $\pm$ standard deviation. * $0.01 < P < .05$; ** $0.001 < P < .01$; and *** $P < .001$. Con: PBS-gavage mothers; BL: bacterial lysate-gavage mothers

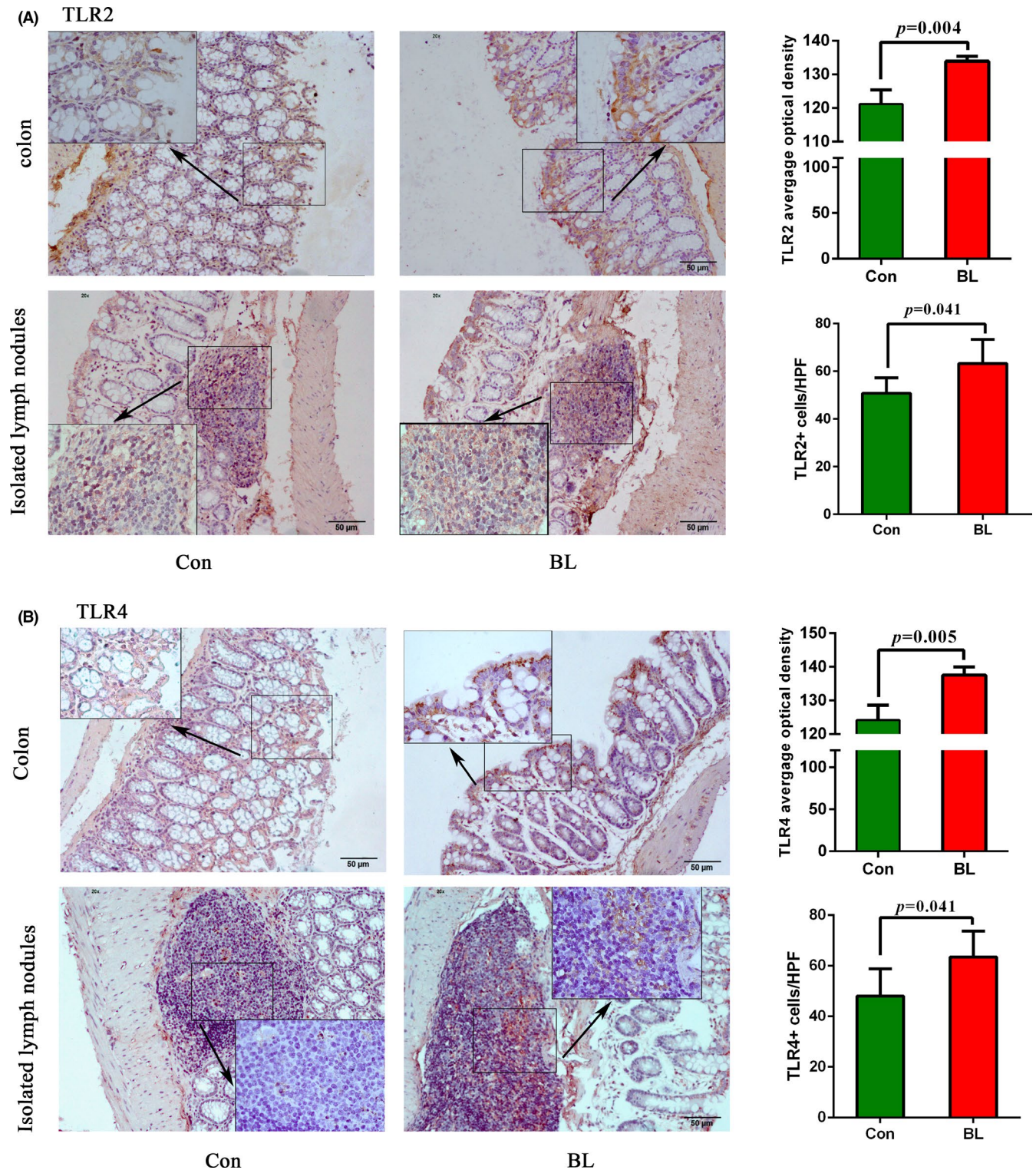


FIGURE 2 TLR2 and TLR4 expressions were affected by maternal oral bacterial lysate (BL) administration in mice. Expression of TLR2 (A) and TLR4 (B) in the intestinal mucosa and intestinal lymphatic tissues (using immunohistochemical staining) in mothers. Expression of TLR2 and TLR4 in the lung was detected by immunohistochemistry (C), and mRNA expression (D), in the ovalbumin-challenged (AAD model) offspring. Average optical density and TLR2/4+ cells/high-power field were used to count positive cells in different tissues. Data are shown as means $\pm$ standard deviation. TLR, Toll-like receptor; AAD, allergic airway disease; Con: PBS-gavage mothers; BL: bacterial lysate-gavage mothers; MP-OP: PBS-challenged offspring of PBS-treated (control-group) mothers; MP-OO: OVA-challenged (AAD) offspring of control-group mothers; MB-OO: OVA-challenged (AAD) offspring of BL-gavage mothers

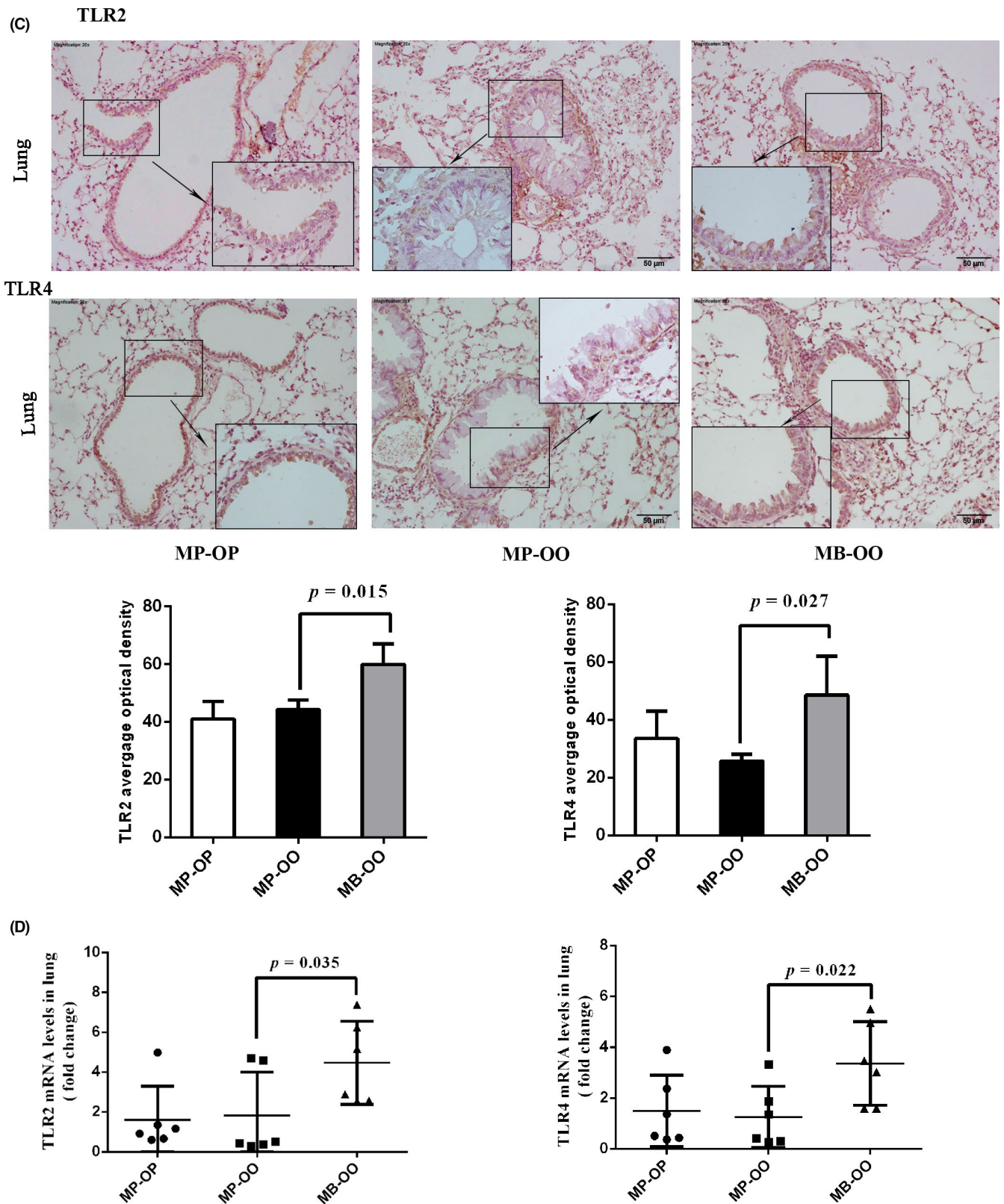


FIGURE 2 (Continued)

After the mice were euthanized and tracheotomized, 1 mL PBS was injected into the lungs, followed by five repeated suction events. The collected bronchoalveolar lavage fluid (BALF) cells were counted using a hemocytometer, excluding epithelial cells and red blood cells. The

BALF cells were pelleted, fixed, and stained with the Wright-Giemsa stain (Baso Diagnostics Inc.) according to the manufacturer's instructions. To classify the BAL cells from each mouse, 200 cells per slide were observed under an oil immersion lens (1000 × magnification).

2.5 | Immunohistochemistry

Samples of the mother's colons and lung tissue of the offspring were fixed in 10% formalin solution, embedded in paraffin, sectioned, and boiled in citric acid buffer (pH6.0, Thermo Fisher Scientific). The samples were incubated with TLR2 (1:100, cat. no. ab213676; Abcam), TLR4 (1:100, cat. no. ab13556; Abcam), and HDAC9 (1:100, cat. no. ab59718; Abcam) overnight at 4°C and subsequently with a secondary antibody (1:200, cat. no. ab205718; Abcam) for 60 min at 37°C. 3,3'-Diaminobenzidine was used as the chromogenic substrate. Images were obtained using the Olympus BX51 microscope and DP2-BSW imaging system (Olympus Corporation). ImageJ IHC software version 1.4.3 (NIH) was used to analyze the average optical density of brown particles in the immunohistochemistry images.¹⁶ The TLR2- and TLR4-positive cells in the lymph nodes were counted by two people, five randomly selected views were analyzed for each sample.

2.6 | ELISA tests

The concentrations of OVA-specific IgE, IL-9, IL-17A, IL-10, IL-4, IL-5, and IFN- γ (BioLegend) in the plasma and BALF were measured using ELISA kits according to the manufacturer's instructions. All samples were tested three times.

2.7 | Quantitative real-time PCR

Total RNA was extracted from the lung samples with a MiniBEST Universal RNA Extraction Kit (Takara Bio Inc.), according to the manufacturer's protocol. A total of 1 μ g RNA was used to perform real-time PCR using the PrimeScript RT Reagent Kit with genomic DNA Eraser (Takara Bio Inc.). The FastStart Universal SYBR Green Master (Rox; Roche Diagnostics) was used for quantitative real-time PCR analysis performed on a LightCycler 480 (Roche Diagnostics).

2.8 | Flow cytometry

The freshly isolated mouse spleen was cut into pieces on ice using scissors. The tissue mass was extruded with a syringe piston, and the tissue suspension was repeatedly aspirated and filtered into a centrifugal tube through a 200-mesh nylon net (mesh diameter 75 μ m). After washing, red blood cells were removed

from the suspended spleen cells using an erythrocyte lysis buffer (BD Biosciences). The spleen cell suspension was incubated with fluorescein isothiocyanate-labeled rat anti-mouse CD4 (cat. no. 553046; BD Biosciences) and APC rat anti-mouse antibodies (cat. no. 557192; BD Biosciences) for CD25. The cells were then fixed, permeabilized (Transcription Factor Buffer Set, BD Biosciences), and incubated with PE rat anti-mouse Foxp3 antibody (cat. no. 563101; BD Biosciences).

2.9 | Statistical analysis

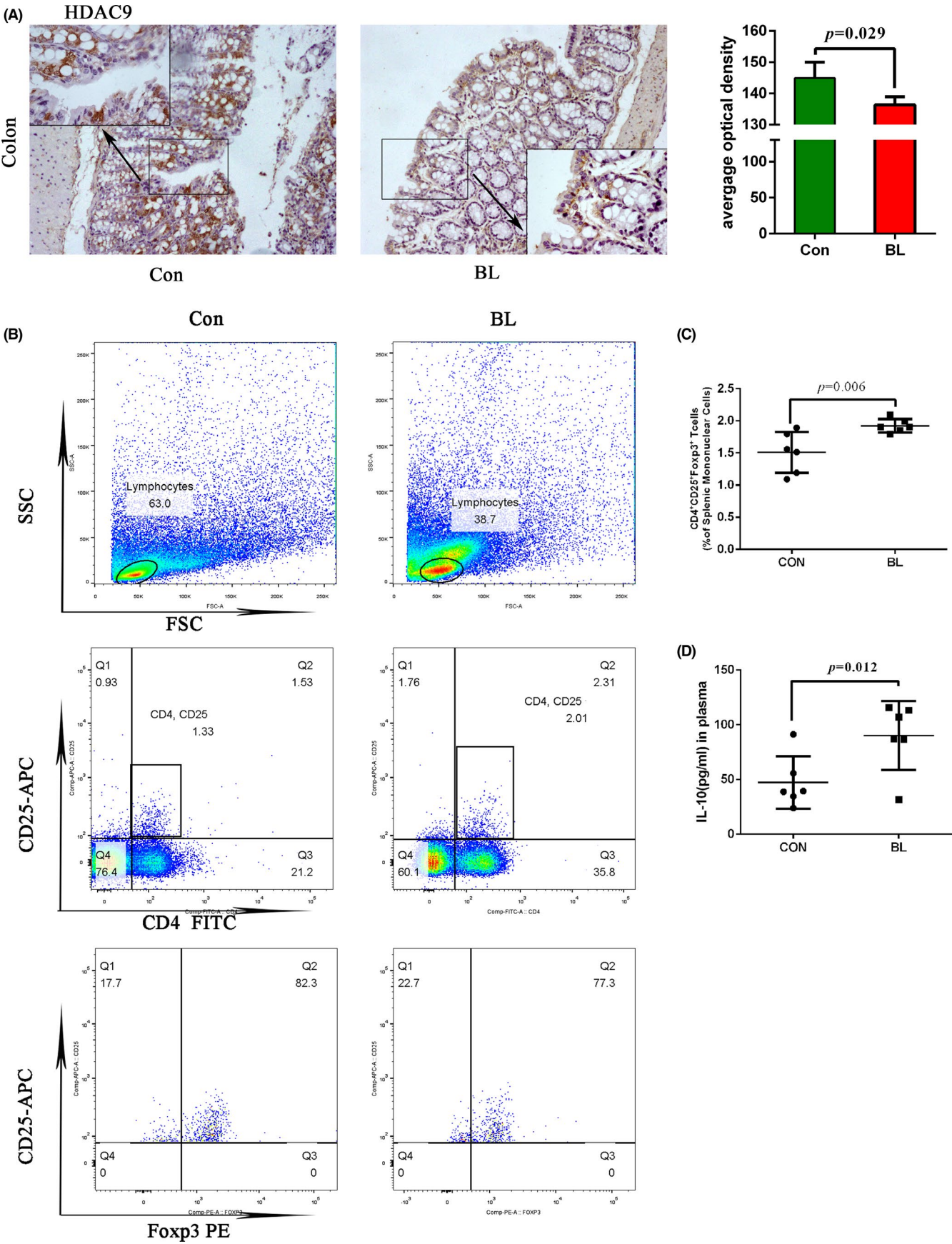
SPSS Statistics ver. 19.0 (IBM Corp.) was used for statistical analyses. GraphPad Prism 5.0 (GraphPad Software Inc.) was used for the layout of data figures. We used the Pearson correlation analysis to test the normal distribution of data. Data are reported as means $\pm$ standard deviation. Student's *t* test was used to determine differences between two groups. One-way analysis of variance, followed by Student-Newman-Keuls test, was used to examine differences between multiple groups. *P* < .05 was considered significant.

3 | RESULTS

3.1 | Maternal oral administration of BL affected the intestinal microbiome of mothers and prevented early AAD in offspring

The rarefaction curve (Figure 1A) shows that species richness differed in the two groups. In terms of alpha diversity, the abundance-based coverage estimator richness index (Figure 1B) showed a higher number of species in the feces of BL-gavage group mothers than in those of control-group mothers. At the phylum level, Firmicutes and Proteobacteria in the BL group were enriched (Figure 1C,D). At the family level, Ruminococcaceae and Helicobacteraceae were richer in the BL-gavage mothers, whereas Prevotellaceae and Porphyromonadaceae were richer in the control-group mothers (Figure 1E,F). In terms of intergroup beta diversity, the principal component analysis showed that the microbial community differed between the two groups (Figure 1G). Clustering was performed with non-metric multidimensional scaling (Figure 1H) and principal coordinate (Figure 1I) analyses, based on OTUs and phylogeny. Clustering became gradually obvious from the phylum to the species level (Figure 1J-L). In the OVA-challenged offspring of control-group mothers, we observed pathologic characteristics of AAD

FIGURE 3 Pre-treating with bacterial lysate upregulates Tregs in mouse mothers and their offspring. Expression of HDAC9 in the intestinal tract of mothers (A, average optical density was used to count HDAC9-positive cells after immunohistochemical staining), proportion of Tregs in the spleen (B, C), and IL-10 production in plasma (D) in mothers. Expression of HDAC9 and Foxp3 mRNAs in the lungs (E), the percentage of CD4+CD25+Foxp3+ cells in the spleen (F, G), and BALF IL-10 production (H) in AAD model offspring. Data are shown as means $\pm$ standard deviation. Tregs, regulatory T cells; HDAC9: histone deacetylase 9; IL-10: interleukin-10; AAD: allergic airway disease; BALF: bronchoalveolar lavage fluid; Con: PBS-gavage mothers; BL: bacterial lysate-gavage mothers; Foxp3: forkhead box P3; MP-OP: PBS-challenged offspring of PBS-treated (control-group) mothers; MP-OO: OVA-challenged (AAD) offspring of control-group mothers; MB-OO: OVA-challenged (AAD) offspring of BL-gavage mothers



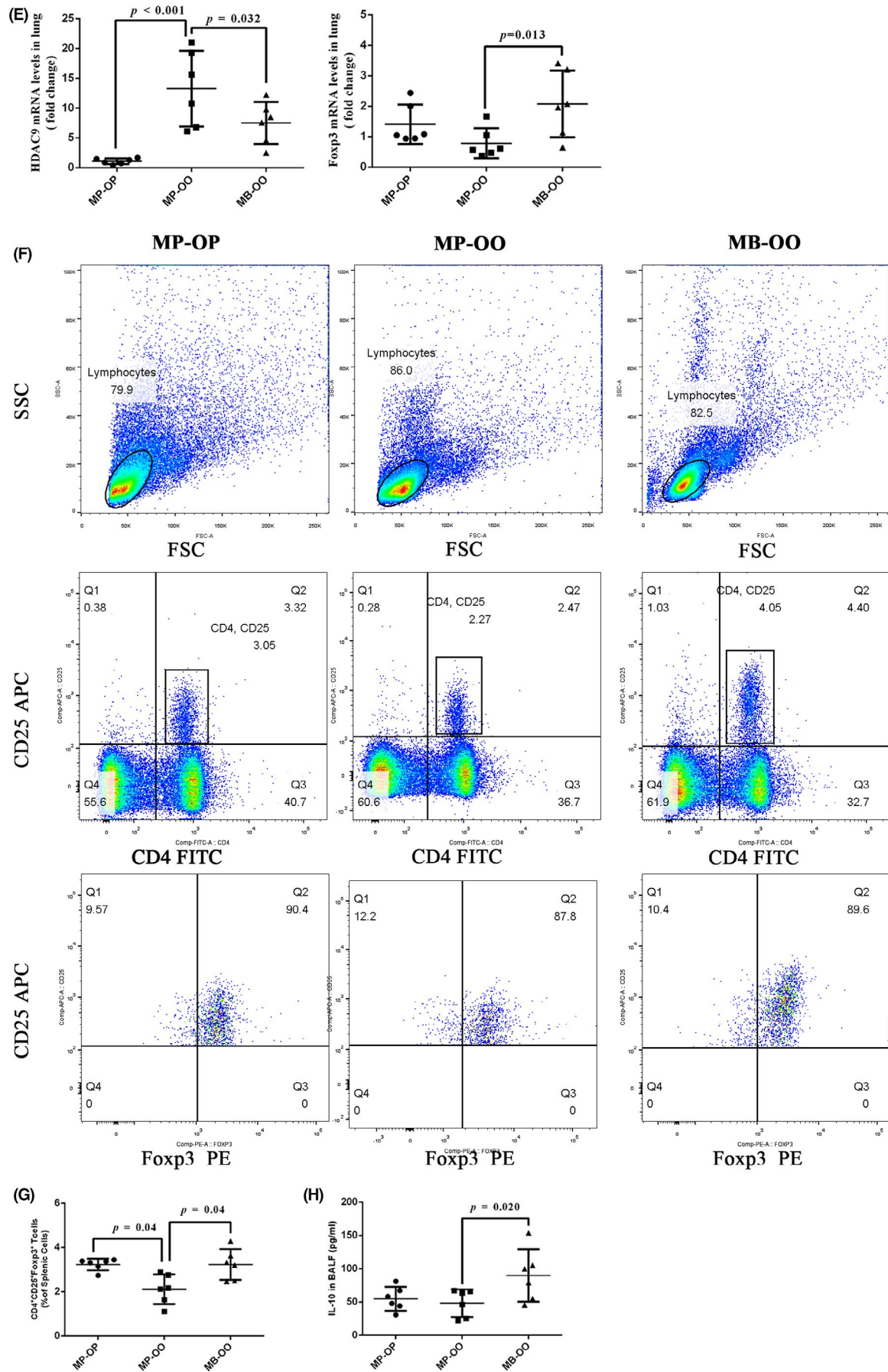


FIGURE 3 (Continued)

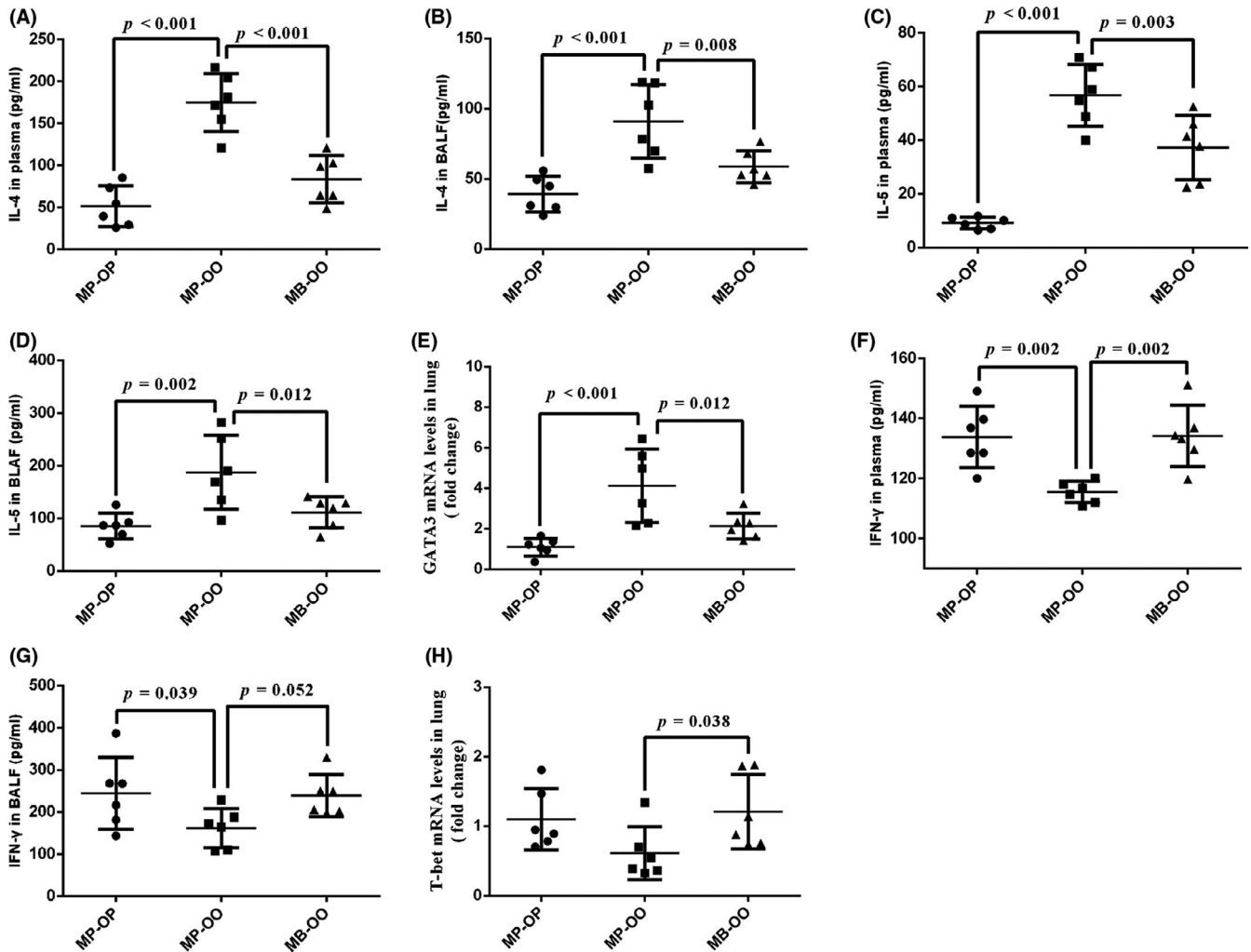


FIGURE 4 Maternal oral bacterial lysate administration reduced the Th1/Th2 cell-axis imbalance in allergic airway disease (AAD) model offspring in mice. Production of Th2-related cytokines IL-4 in the plasma (A) and BALF (B), and of IL-5 in the plasma (C) and BALF (D); the expression of the Th2-related gene, GATA3, in the lungs (E); production of Th1-related cytokine, IFN-γ, in the plasma (F) and BALF (G); and expression of the Th1-related gene, *T-bet*, in the lungs (H) of AAD model offspring. Data are shown as means ± standard deviation. IL: interleukin; BALF: bronchoalveolar lavage fluid; *T-bet*: t-box transcription factor TBX21; GATA3: GATA binding protein 3; IFN-γ: interferon gamma; Th: T-helper cell. MP-OP: PBS-challenged offspring of PBS-treated (control-group) mothers; MP-OO: OVA-challenged (AAD) offspring of control-group mothers; MB-OO: OVA-challenged (AAD) offspring of BL-gavage mothers

(Figure B1,B2), higher total cell counts (Figure B3) and eosinophilic proportions in the BALF (Figure B4), and higher OVA-specific IgE production in the BALF (Figure B5) and plasma (Figure B6) than in the OVA-challenged offspring of control-group mothers. In contrast, all these AAD characteristics were reduced in the OVA-challenged (AAD) offspring of BL-gavage mothers.

3.2 | TLR2 and TLR4 expressions in the mothers' intestines and offspring's lungs were affected by maternal oral BL administration

In the BL-gavage mothers, TLR2 and TLR4 expressions on the intestinal mucosal surface and in the submucosal lymphoid tissue were significantly higher than in the control group (Figure 2A, B). In the

offspring AAD model, TLR2 and TLR4 expressions were significantly higher in the OVA-challenged offspring of BL-gavage mothers than in the offspring control-group mothers (Figure 2C). Furthermore, increased expression of TLR2 and TLR4 genes in the lungs confirmed the above results (Figure 2D).

3.3 | Pre-treating with BL can upregulate Treg expression in mothers and their early AAD model offspring

In BL-gavage mothers, HDAC9 expression in the intestine was lower than in the control-group mothers (Figure 3A), whereas the percentage of CD4 + CD25+Foxp3 + cells in spleen mononuclear cells (Figure 3B,C) and the concentration of IL-10 in the

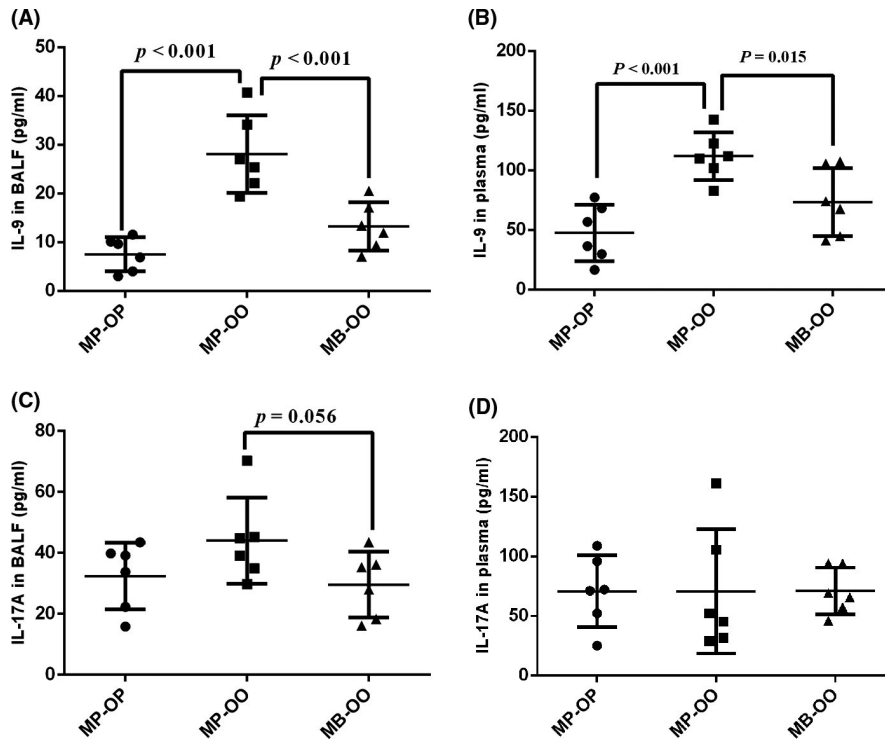


FIGURE 5 Maternal oral bacterial lysate (BL) administration reduced IL-9 production in early allergic airway disease (AAD) model offspring in mice. Production of IL-9 in the plasma (A) and BALF (B), and of IL-17A in the plasma (C) and BALF (D), in AAD model offspring. Data are shown as means $\pm$ standard deviation. IL: interleukin; BALF: bronchoalveolar lavage fluid. MP-OP: PBS-challenged offspring of PBS-treated (control-group) mothers; MP-OO: OVA-challenged (AAD) offspring of control-group mothers; MB-OO: OVA-challenged (AAD) offspring of BL-gavage mothers

plasma (Figure 3D) were higher than in control-group mothers. In the OVA-challenged offspring of control-group mothers, HDAC9 gene expression was higher in the lungs (Figure 3E) and the percentage of CD4 + CD25 + Foxp3 cells was lower in spleen mononuclear cells (Figure 3F,G) than in the PBS-challenged offspring of control-group mothers. In contrast, in the OVA-challenged offspring of BL-gavage mothers, the expression of HDAC9 gene was lower in the lungs (Figure 3E), but the expression of Foxp3 gene in lungs (Figure 3E), the percentage of CD4 + CD25 + Foxp3 cells in spleen mononuclear cells, and IL-10 levels in the BALF were higher (Figure 3F-H) than in the OVA-challenged offspring of control-group mothers.

3.4 | Oral administration of BL to mothers reduced the Th1/Th2 cell-axis imbalance in OVA-challenged (AAD model) offspring

In the OVA-challenged offspring of control-group mothers, the plasma and BALF concentrations of Th2-related cytokines, IL-4 (Figure 4A,B) and IL-5 (Figure 4C,D), and expression of GATA3 (Figure 4E) in the lungs were higher, whereas the concentrations of Th1-related cytokine, IFN- γ , were lower (Figure 4F,G) than in the PBS-challenged offspring of control-group mothers. Except for IFN- γ concentration in the BALF (Figure 4G, $P = .052$), the above changes were reversed in the OVA-challenged offspring of BL-gavage mothers (Figure 4A-F), and Th1-related T-bet expression in the lungs was higher (Figure 4H) than in the OVA-challenged offspring of control-group mothers.

3.5 | Maternal oral BL administration reduced IL-9 production in early AAD model offspring

IL-9 (a Th9-related cytokine) production in plasma and BALF was higher in the OVA-challenged offspring of control-group mothers than in the PBS-challenged offspring of control-group mothers; in contrast, these levels were lower in the OVA-challenged offspring of BL-gavage mothers than in OVA-challenged offspring of control-group mothers (Figure 5A,B). Although maternal oral BL administration may have downregulated the production of IL-17A in the BALF (Figure 5C, $P = .056$), there was no difference in IL-17A production in the three offspring groups (Figure 5C,D).

4 | DISCUSSION

Bacterial DNA has been reported in the amniotic fluid, placenta, and meconium.¹⁷ Studies on animal models have revealed that the mother's immunoregulatory effect on the fetus does not require the presence of live bacteria in the uterus.¹⁸ In this study, BL was used to stimulate the intestinal tract of mothers; the resulting changes in TLR2/4-mediated innate immunity in the mucosa of mothers and offspring were observed, and alterations of Tregs, Th1, Th2, Th9, and Th17 were detected in the offspring AAD model.

Studies have found that BL can affect both innate immunity and acquired immunity and can reduce the number of wheezing episodes in asthmatic children.¹⁹ In this study, oral BL administration to the mother improved species richness and significantly increased the differences in microbial communities compared with the

control group, showing different clustering trends. This may be due to changes in the immune environment caused by BL, which in turn causes changes in bacterial abundance in the intestine; this needs to be investigated further. A prospective study found a long-term change in the differential abundance of intestinal bacteria in children with allergic diseases.²⁰ Intestinal immune homeostasis is affected by the composition of the symbiotic microbial community,²¹ and the mother's microbial environment may affect the early formation of the fetal immune system.²² The use of antibiotics by mothers during pregnancy increases the risk of asthma in their offspring.²³ Hence, some asthma-related immune markers in mothers and their offspring were examined in mice.

In vitro studies have shown that BL components can interact with TLR2 and TLR4 to enhance the activity of macrophages and the ability to fight infection.²⁴ Our data show that oral BL administration can upregulate the expressions of innate immune receptors, TLR2 and TLR4, in the intestinal mucosa and submucosal lymphatic tissues, reduce HDAC9 expression related to Treg regulation in the intestinal mucosa, and promote Treg amplification, thereby stimulating intestinal immunity. Surprisingly, the lungs of the OVA-challenged offspring of BL-gavage mothers also showed consistent changes in TLR2 and TLR4. This supports previous research showing that prenatal application of *Acinetobacter lwoffii* F78 induces TLR signaling in the mother and prevents experimental-induced asthma in adult offspring.⁷ In recent years, it has been found that intestinal microbiota and metabolome are related to lung immunity; this has established a basic understanding of the gut-lung axis in respiratory diseases.²⁵ It is reasonable to speculate that heredity may play a role in determining how changes in intestinal mucosal immunity transfer to the lung mucosa of the offspring.

TLRs can regulate Tregs and other T-cell subsets through their ligands, thereby participating in T-cell development.²⁶ Animal model studies have revealed that oral BL administration can prevent asthma by recruiting Tregs into the airways.²⁷ Human cohort studies also showed that neonatal intestinal microbiota is associated with poly-allergenicity and T-cell differentiation in children,²⁸ whereas most intestinal immunity in newborns is related to the mother, especially in breast-fed newborns.²⁹ Studies have shown that OVA sensitized by *Mycobacterium vaccae* immunization in pregnant mice can inhibit IL-5 production and induce IFN- γ secretion, both in the placenta and in the postpartum.³⁰ In this study, the polarity of Th2 in the offspring was weakened after BL gavage during pregnancy, and the Th1 polarization pathway was correspondingly enhanced. Furthermore, Treg differentiation was enhanced, whereas that of Th9 was weakened. The oral administration of BL during pregnancy had an important effect on the differentiation of CD4⁺ T cells, causing it to deviate from the allergic disease state. However, the BALF IL-17A concentration was not significantly affected by maternal BL gavage.

In summary, oral BL administration regulates the mother's intestinal microbiome, and has protective effects on OVA-challenged offspring, by regulating TLR2/4 and HDAC9, improving the Treg ratio, reducing Th1/Th2 imbalance, and weakening Th9 differentiation. The

microbial transfer mechanisms require further research. Therefore, for children at high risk of allergic diseases, appropriate stimulation of the TLR2/4 immune pathway and differentiation of Tregs in early life (eg, the embryonic stage) may help to prevent allergic diseases later in life.

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CONFLICT OF INTEREST

The authors have declared that they have no conflict of interest. All authors also specifically declare that they have no conflict of interest related to the pharmaceutical company that produces and markets Broncho-Vaxon®.

AUTHOR CONTRIBUTIONS

Jing Liu: Funding acquisition (supporting); Project administration (equal); Writing-original draft (equal); Writing-review & editing (equal). **Changli Tu:** Data curation (equal); Investigation (equal); Methodology (equal). **Jinyan Yu:** Data curation (equal); Formal analysis (equal); Methodology (equal). **Meizhu Chen:** Data curation (equal); Methodology (equal). **Cuiyan Tan:** Methodology (equal); Software (equal). **Xiaobin Zheng:** Methodology (equal); Software (equal). **Zhenguo Wang:** Methodology (equal); Validation (equal). **Yingjian Liang:** Investigation (equal); Methodology (equal). **Kongqiu Wang:** Data curation (equal); Methodology (equal). **Jian Wu:** Data curation (equal); Methodology (equal). **Yanlei Li:** Data curation (equal); Project administration (equal); Writing-original draft (equal); Writing-review & editing (equal).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures were ethically reviewed and approved by the Animal Experimental Ethics Committee of Jilin University.

PEER REVIEW

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APPENDIX

FIGURE A

Female mice underwent gavage (with bacterial lysate or phosphate-buffered saline [PBS]), 10 days before mating with male mice, in a 2:1 ratio. During this period, the female mice underwent gavage until delivery, and the feces were collected before delivery. Infant mice were weaned on the 21st day after birth. From the 25th day after birth, mice in the allergic airway disease (AAD) model group were sensitized by intraperitoneal injection of 20 μ g ovalbumin (OVA; Grade V, Sigma-Aldrich) and 0.4 mg aluminum hydroxide (Beijing Chemical Works, China), whereas those in the control group were sensitized with PBS (pH 7.4). At the 25th, 32nd, and 39th days after the birth of the offspring, the AAD offspring group were challenged with 1% aerosolized OVA (Grade II; Sigma -Aldrich) using an ultrasonic nebulizer (NE-C28; Omron) for 30 min daily from days 46 to 53 after birth. The control offspring group was treated with PBS instead of OVA. Twenty-four hours after the last challenge (the 53rd day of the offspring's life), mice were euthanized (by intravenous injection of pentobarbital sodium 150 mg/kg in the tail); blood, lungs, spleens, and bronchoalveolar lavage fluid (BALF) were obtained.

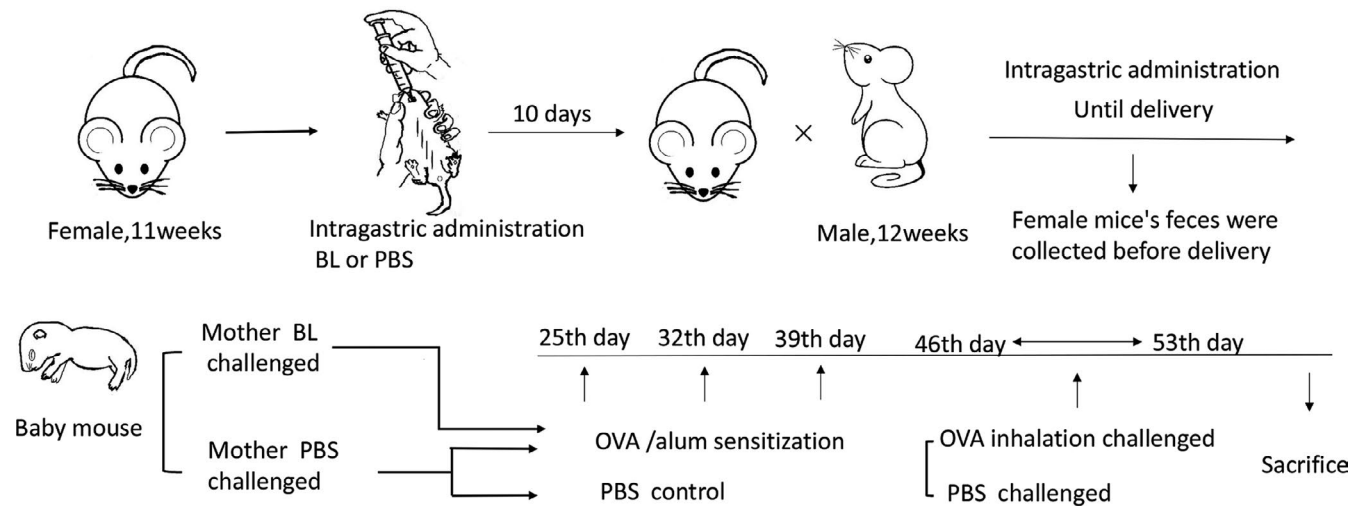


FIGURE B

Maternal oral bacterial lysate (BL) administration prevented early allergic airway disease (AAD) in offspring in mice. Lung tissue (hematoxylin and eosin staining) (1), lung inflammation scores (2), BALF total cell count (3), BALF cell classification (4), and OVA-specific IgE in the plasma (5) and BALF (6), in the AAD model offspring. The data are shown as means $\pm$ standard deviation. OVA: ovalbumin; BALF: bronchoalveolar lavage fluid. MP-OP: PBS-challenged offspring of PBS-treated (control-group) mothers; MP-OO: OVA-challenged (AAD) offspring of control-group mothers; MB-OO: OVA-challenged (AAD) offspring of BL-gavage mothers.

