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Lipopolysaccharide Exposure Alleviates Asthma in Mice by Regulating Th1/Th2 and Treg/Th17 Balance

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Manuscript Preparation E
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Background: It is generally believed that endotoxin exposure exacerbates risk of developing asthmatic symptoms. However, recent studies have indicated that prior bacterial exposure may prevent future symptoms of asthma. Here, we evaluated the influence of pre-exposure to different concentrations of lipopolysaccharide (LPS) to subsequent ovalbumin (OVA) allergen sensitization and challenge.

Material/Methods: Four-week-old Balb/c mice were treated intranasally with varying concentrations of LPS (1 ug, 10 ug, and 100 ug) or sterile PBS for 10 days, then 2 weeks later they were exposed to OVA. Both the molecular and functional airway responses to OVA administration were assessed following prior exposure to different doses of LPS or controls. Additionally, the Th1/Th2 and Treg/Th17 balance was measured.

Results: Airway responsiveness and immune cell recruitment in the bronchoalveolar lavage (BALF) were decreased in animals exposed to a low dose of LPS (1 ug) treatment compared with the asthma group. Moderate-dose (10 ug) and high-dose (100 ug) LPS administration showed no differences from controls. Further, low-dose LPS (1 ug) exposure was associated with increased Th1 cytokines, T-bet, Treg cytokine (IL-10, TGF-β), and Foxp3 expression, but decreased Th2 cytokines (IL-4,5,13), GATA3, Th17, and ROR-γt expression compared with the asthma group. Finally, higher numbers of CD4+CD25+Foxp3+Treg cells, and CD4+INF-γ+T cells, and lower CD4+IL-4+T cells and CD4+IL-17+T cells were observed in the low-dose LPS-treated groups compared to controls.

Conclusions: Our findings suggest that prior exposure to low doses of LPS may protect from OVA-induced airway hyperresponsiveness (AHR) and histopathologic changes through regulation of the Th1/Th2 and Treg/Th17 balance.

MeSH Keywords: Anti-Asthmatic Agents • Antigens, Differentiation, T-Lymphocyte • Endotoxins

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Background

Asthma is characterized by bronchial hyperresponsiveness along with airway inflammation, and is a leading chronic illness among children and adults [1,2]. There are 150–200 million asthma patients worldwide, which poses a huge economic burden on families and society [2]. However, no curative treatment exists for asthma, and all treatment guideline emphasize only clinical control [2]. Thus, methods to prevent or treat asthma are of immense importance.

LPS is found in the cell wall of Gram-negative bacteria which are ubiquitous components of our environment. LPS is a common experimental mimic of bacterial exposure and is one of the most potent microbial stimuli of inflammation [3]. It is widely accepted that airway function is altered by LPS exposure [4–6]. Several studies have shown that LPS exacerbated bronchoconstrictive and inflammatory effects in patients and animal model with allergic asthma, and enhanced antigen-specific allergic responses in the airway, and the severity of asthma is associated with LPS concentration [7–12]. However, recent experiments and epidemiological studies show that LPS protects against asthma [6,13,14]. Explanations for these discrepancies may include: 1) LPS concentration affects the severity of asthma, and 2) different airway responses to endotoxin depend on subject age [15].

Although there is still much debate, there is mounting evidence indicating the protective role of LPS in the development of asthma. Identifying the protective effects of LPS in asthma and mechanisms associated with protection may be useful in developing preventive treatments for asthma. Therefore, we explored whether prior exposure to LPS before OVA induction of asthma protects mice from developing asthma, and whether these effects are concentration-dependent. Furthermore, the mechanisms mediating the protective effects of LPS in asthma were investigated.

Material and Methods

Animals

All animal experiments conducted in this study were strictly designed to adhere to the guidelines provided by the Chinese Counsel on Animal Care and all protocols were submitted to the Chongqing Medical University Institutional Animal Care and Research Advisory Committee and approved before beginning. Four-week-old BALB/c mice were maintained in pathogen-free sterilized cages, under normal conditions, and fed an OVA-free diet. Pathogen-free conditions were used to minimize the effects of other microorganisms on these results and reduce variability based on bioburden differences between mouse cages. Normal conditions included a 12-h light cycle, constant 24°C temperature, and adequate food and water.

LPS preparation, ovalbumin sensitization, and challenge

Mice were exposed to 1 ug, 10 ug, or 100 ug of LPS in 10 ul sterile phosphate buffer (PBS) intranasally for 10 days. Sterile PBS was administered in a similar manner to control groups. After LPS exposure, mice were administered OVA to induce asthma according to the previously published methods [16]. A diagrammatic outline of LPS and OVA administration is provided in Figure 1.

Airway hyperresponsiveness (AHR)

At 24 h after the final OVA challenge, lung resistance (LR) was measured using an invasive lung function test. Briefly, anesthetized mice were intubated and mechanically ventilated using a computer controlled piston ventilator (flexiVent, Scireq). Mice were then challenged with an aerosolized bronchoconstrictor, acetyl-β-methylcholine (Sigma-Aldrich, Saint Louis, MO, USA), at increasing doses: 0, 3.125, 6.25, 12.5, 25, and 50 mg/ml. At each dose, lung resistance was calculated using the single-compartment model.

Histological analysis

After treatments, lungs were harvested, fixed in 10% formalin, and paraffin-embedded. Paraffin blocks were serially sectioned at 4-micron thickness and stained with hematoxylin–eosin (H&E) or processed for immunohistochemical (IHC) analysis. Images were acquired using an inverted light microscope (Nikon Eclipse E200 with a Nikon Coolpix 995 camera, Nikon, Tokyo, Japan).

RNA extraction, reverse transcription, and quantitative PCR (qPCR)

Total RNA from mouse lung tissues was purified, and cDNA synthesis was performed using a PrimeScript RTReagent Kit according to manufacturer's recommendations (Takara, Otsu, Japan). Quantitative PCR (qPCR) was performed using standard techniques [17]. GAPDH was used as endogenous controls. The primer sequences (50>30) of GILZ were 5'-AAA GGA GCA GAT TCG TGA-3' (forward) and 5'-CCA CTT ACA CCG CAG AAC-3' (reverse), T-bet were 5'-AGCAAGGACGGCGAATGTT-'3 (forward) and 5'-GGGTGGACATATAAGCGGTTC-'3 (forward), GATA3 were 5'-CT CGGCCATTCGTACATGGAA-'3 and 5'-GGATACCTCTGCACCGTAGC-'3 (reverse), Rorγt were 5'-CCGCTGAGAGGGCTTCAC-3'(forward) and 5'-TGCAGGAGTAGGCCACATTACA-3' (reverse), Foxp3: 5'-TGGAACCACGGGCACTATCACA-3' (forward) and 5'-GAGG CTGCGTATGATCAGTTATGC-3' (reverse) and GAPDH were 5'-AGCAATGCCTCCTGCACCACCAAC-3' (forward) and 5'-CCGGAGGGGCCATCCACAGTCT-3' (reverse).

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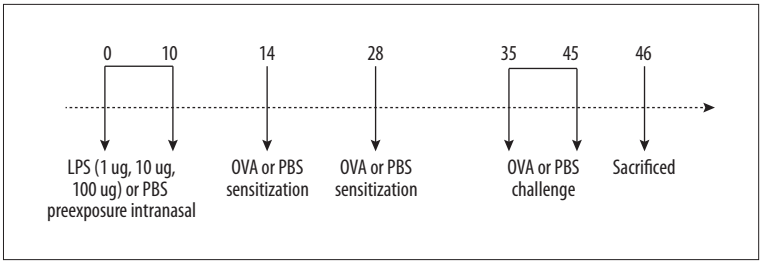

Figure 1. Experimental design and protocols employed in this study. Two LPS pretreatment groups included mice that received intranasal LPS or PBS (Control group) for 10 consecutive days and then were either OVA-sensitized and challenged (LPS/OVA group) or sham-exposed (LPS group). Results for each group display means and SEM of 6 to 8 animals.

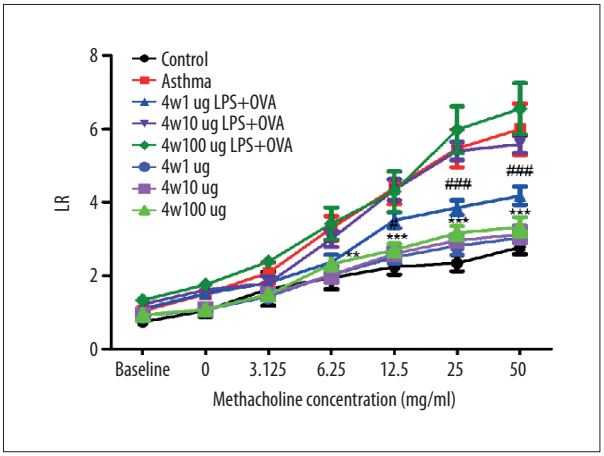

Figure 2. Influence of LPS pretreatment on OVA-driven AHR. AHR in control, allergic asthma (OVA), LPS exposure only (1 ug LPS, 10 ug LPS, 100ug LPS), and LPS exposure followed by OVA sensitization and challenge (1 ug LPS+OVA, 10 ug LPS+OVA, 100 ug LPS+OVA). AHR was conducted 24 h after last OVA or PBS challenge (day 31). Data are reported as mean ± SEM from at least 3 separate experiments (n=6–8 mice/group). AHR was decreased in prior exposure to low-dose of 1ugLPS+OVA group compared with OVA group. 10ugLPS+OVA, 100 ug LPS+OVA showed no difference from the OVA group. * P<0.05, ** P<0.01, *** P<0.001 *vs.* control; # P<0.05, ### P<0.001 *vs.* OVA.

Western blots

Total proteins from mice lung tissues were isolated and protein concentrations determined through a bicinchoninic acid (BCA) assay. Equal amounts of protein were resolved on polyacrylamide gels using SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies using standard techniques [18]. Anti-TNF-α, anti-P38, anti-P-P38, anti-P-P-65, and anti-P65antibodies (1: 2000) (Cell Signaling Technology, Danvers, MA, USA) were used. The band intensity was measured by ImageJ2× 2.1.4.7 (Wayne Rasband, National Institutes of Health, USA).

Collection of BALF, quantifying immune cells, and cytokine levels

BALF was obtained by rinsing lungs with 1 mL of PBS. Light microscopes were utilized to assess total cell numbers. Wright-Giemsa staining was performed to obtain differential cell counts and morphological characteristics were obtained by blinded examiners from 250 cells in each sample. Supernatant was stored at –80°C until cytokine determination. Enzyme-linked immunosorbent assay (ELISA) was performed on BALF supernatant to determine cytokine concentrations. Interferon (IFN)-γ, IL-4, IL-5, IL-13, IL-6, IL-2, transforming growth factor (TGF)-β, IL-10, and IL-17A ELISAs were performed according to kit instructions (Xinbosheng, Shenzhen, China).

Statistical analyses

All studies were performed with at least 2 independent experiments with a minimum of 4 animals per group (n=4). Data are presented as mean ± standard error of the mean (SEM). The GraphPad Prism 4.0 program (GraphPad, San Diego, CA, USA) was used for the statistical analyses. The statistical analyses were performed using two-way ANOVAs or Student's t-test unless otherwise specified. P-values less than 0.05 were considered statistically significant.

Results

Responsiveness of airways and cell numbers in BALF after LPS administration followed by OVA treatment

To investigate the effects of early antigenic stimulation on subsequent immune responses, we exposed 4-week-old animals to various dose of LPS (1 ug, 10 ug, and 100 ug) by local intranasal application (Figure 1). Then, at 6 weeks of age, these animals were sensitized and challenged with the allergen OVA, and airway AHR to methacholine was assessed as described in Figure 2. AHR was abrogated in mice exposed to a low dose of LPS before OVA sensitization and challenge (LPS 1ug/OVA group) compared with the PBS/OVA group, LPS 10 ug/OVA group, and LPS 100 ug/OVA group. Animals in the LPS 1 ug/OVA group showed no inhibition of AHR and behaved similarly to animals in the LPS pre-exposure only group (LPS/PBS) and control group (PBS/PBS) Figure 3.

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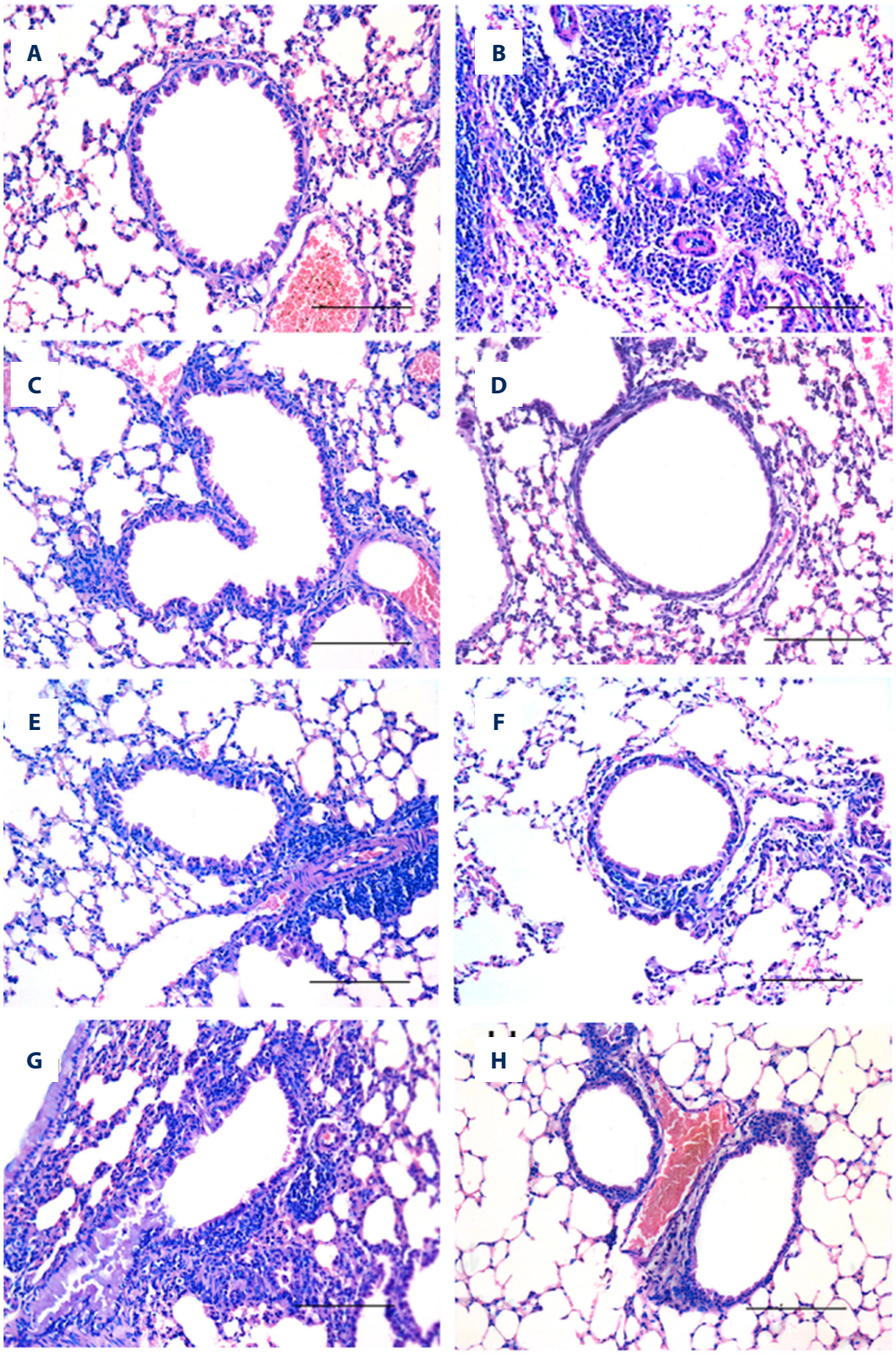

Figure 3. Influence of LPS pretreatment on OVA-driven pathologic change. H&E staining of lung samples from control (**A**), asthma (OVA) (**B**), LPS exposure only (1 ug LPS (**D**), 10 ug LPS (**F**), 100 ug LPS (**H**), LPS exposure followed by OVA sensitization and challenge (1 ug LPS +OVA (**C**), 10ug LPS+OVA (**E**), and 100ug LPS+OVA (**G**). Images were acquired at 200× magnification. Low-dose (1 ug) LPS exposure is associated with decreased pulmonary peribronchiolitis, pulmonary perivasculitis, and pulmonary alveolitis. Moderate- (10 ug) and high- (100 ug) dose LPS showed no protective effect.

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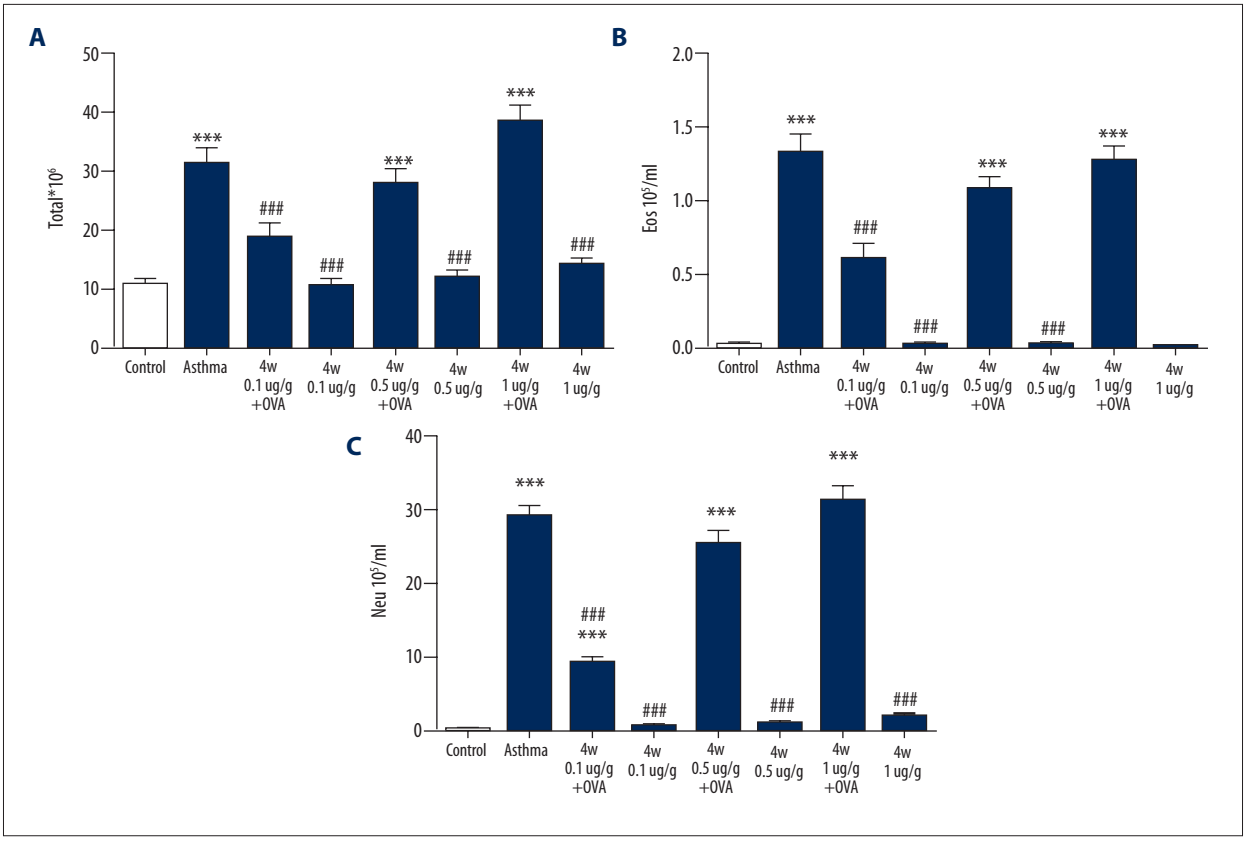

Figure 4. Granulocyte counts of the airways compared with OVA challenge. Total cells (**A**), eosinophils (**B**), and neutrophils (**C**) were counted from BALF in controls, asthma (OVA), pretreatment with different doses of LPS exposure (1 ug LPS, 10 ug LPS, 100 ug LPS), and previous LPS exposure followed by OVA sensitization and challenge (1 ug LPS+OVA, 10 ug LPS+OVA, 100 ug LPS +OVA). Low-dose (1 ug) LPS is associated with decreased total cells, eosinophils, and neutrophils. Moderate- (10 ug) and high- (100 ug) dose LPS showed no protective effect. Data are shown as mean ±SEM from 3 separate experiments (n=6–8 mice/group); *** P<0.001 *vs.* controls; ### P<0.001 *vs.* OVA.

OVA induction of immune cell infiltrate and cytokine levels in BALF along with pathologic gene expression in lungs is significantly reduced by pretreatment with low-dose LPS

Next, we assessed the infiltration of immune cells into the lungs and their release of inflammatory mediators. To test whether pretreatment with LPS decreases the functional and histological severity of OVA-induced asthma through inhibition of immune cell recruitment, we collected BALF from LPA- and/or OVA-treated mice and measured numbers of total cells, eosinophils (Eos), and neutrophils (Neu). A significant increase in total cells, Eos, and Neu were observed after treatment with OVA only (asthma) and OVA along with medium and high doses of LPS pretreatment; there was no significant increase in cell counts in OVA groups pre-treated with low-dose LPS compared to controls (Figure 4). We then measured inflammatory cytokine levels, including: A) IL-4, B) IL-5, C) IL-13, D) interferon (IFN)-γ, E) IL-10, and F) IgE, in BALF (Figure 5). As expected, we observed inhibitory effects of low-dose LPS pretreatment on numerous pro-inflammatory cytokines that were upregulated by OVA administration (Figure 5). There was also a significant effect of low LPS pretreatment on OVA-induction of other cytokines, including Th2 cytokines (IL-4, IL-5, IL-13), Th17 cytokines (IL-17), Th1 cytokines (IFN-γ), and Treg cytokines (TGF-β, IL-10) (Figure 5). Increased expression of a number of genes, including T-bet, GATA3, Foxp3, ROR-γt, and IL-10, in lung tissue have been linked to asthma. Analysis of these genes following OVA insult demonstrated that low-dose LPS pretreatment significantly reduced the expression of these genes in lung tissue compared to OVA treatment alone (Figure 6). Taken together, these data indicate that LPS pretreatment significantly reduces the infiltration of immune cells, expression of cytokines, and lung tissue gene expression changes induced by OVA treatment.

LPS exposure induces $CD4^+$INF-γexpressing T cells, $CD4^+CD25^+Foxp3^+$ T cells, and inhibits the stimulation of $CD4^+$IL-4-expressing T cells

To evaluate the T cell populations derived from OVA-treated animals with or without LPS pretreatment, the phenotype of

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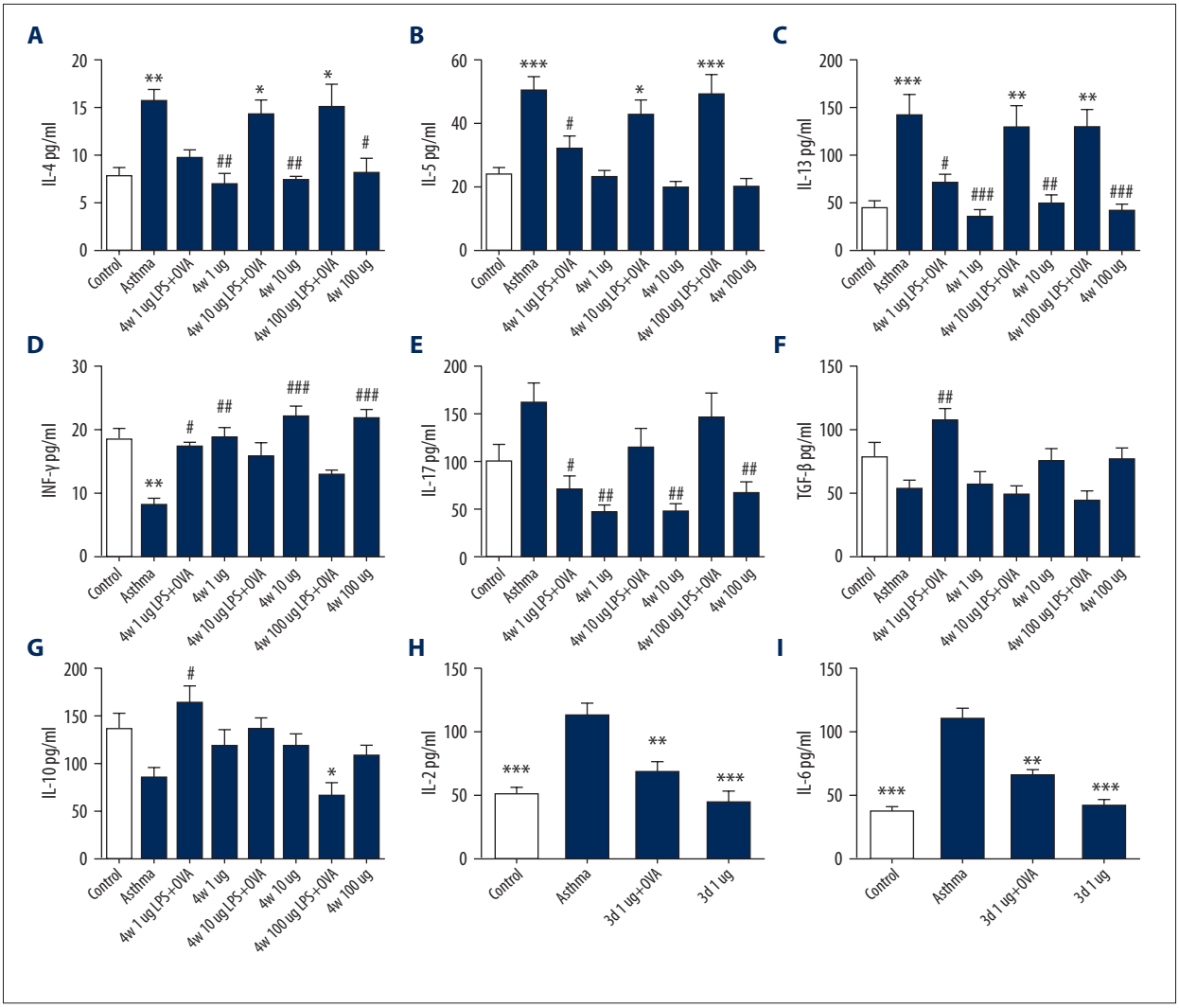

Figure 5. Cytokine levels in BALF. Cytokine levels of interleukin IL-4 (**A**), IL-5 (**B**), IL-13 (**C**), IFN-γ (**D**), IL-10 (**E**), IgE (**F**) in the bronchoalveolar lavage fluid of control, asthma (OVA), and LPS exposure (1 ug LPS+OVA, 10 ug LPS+OVA, 100 ug LPS+OVA) were measured by ELISA. Previous exposure to low dosage (1 ug) LPS decrease Th2 cytokines (IL-4, IL-5, IL-13) and Th17 cytokine (IL-17) production while increasing Th1 cytokine (IFN-γ) and Treg cytokines (TGF-β, IL-10) compared with OVA challenge alone. Data are reported as mean ±SEM from 3 separate experiments (n=6–8 mice/group); * P<0.05; ** P<0.01, *** P<0.001 *vs.* controls; # P<0.05, ## P<0.01, ### P<0.001 *vs.* OVA.

the CD4+ cells from lung tissue were evaluated by flow cytometry. Specifically, CD4+ cells derived from lung tissues were analyzed for intracellular IFN-γ, IL-4, IL-17, and Foxp3 expression. As shown in Table 1, low-dose LPS exposure (LPS/OVA) inhibited the stimulation of IL-4+expressing T cells and resulted in an increase in INF-γ and Foxp3 expression following OVA stimulation. OVA sensitization and challenge after low-dose LPS pre-exposure (LPS1ug/OVA) resulted in an increase in INF-γ and Foxp3 expression along with inhibition of IL-4 and IL-17 expression compared with the asthma group (OVA). However, compared with the OVA group, moderate-dose LPS (10 ug) showed no significant difference from the negative control group (LPS/LPS), and high-dose LPS resulted in increased IL-4 and IL-17 expression and decreased INF-γ and Foxp3 expression (data not shown). Taken altogether, these data suggest that pretreatment with low-dose LPS inhibits the Th2 IL-4-expressing T cells and Th17 cells and results in the development of both Th1 IFN-γ-expressing and Foxp3+ Treg cellular responses to subsequent allergen sensitization (Table 1).

Discussion

Bacterial exposure in infants has been shown to play a protective role in the development of asthma [15]. Additionally, recent studies suggest microbial exposure may protect children

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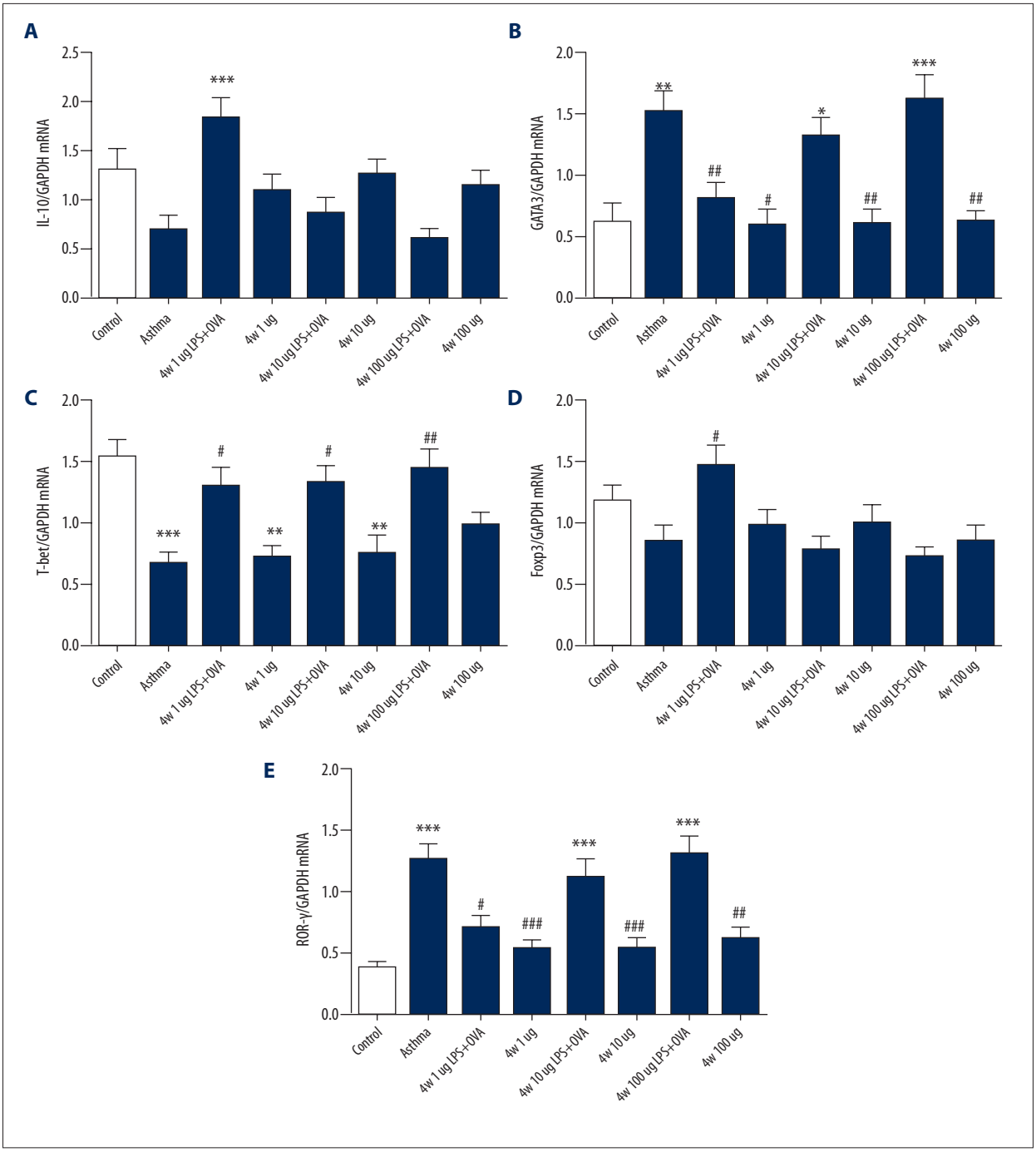

Figure 6. Expression of T-bet, GATA3, Foxp3, ROR-γt, and IL-10 mRNA in lung tissue. Expression of T-bet (**A**), GATA3 (**B**), Foxp3 (**C**), ROR-γt (**D**), and IL-10 (**E**) in lung tissue of control, asthma (OVA), and exposure to different doses of LPS (1 ug LPS+OVA, 10 LPS ug/g+OVA, 100 ug LPS+OVA) mice is presented. Mice with low-dose (1 ug) LPS correlates with lower GATA3 and ROR-γt expression along with higher T-bet and Foxp3 expression. Data are reported as mean ±SEM from 3 separate experiments (n=6–8 mice/group); * P<0.05, ** P<0.01, *** P<0.001 *vs.* controls; # P<0.05, ## P<0.01, ### P<0.001 *vs.* OVA.

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Table 1. Percentages of T cell populations in OVA-induced asthma with or without low dose LPS pre-treatment assessed by flow cytometry.

Group	n	Th1%	Th2%	Treg%	Th17%	Th1/Th2	Treg/Th17
Control	8	2.47±0.17	0.55±0.03	3.9±0.74	1.53±0.22	4.69±0.69	5.19±0.89
OVA	8	0.41±0.97	3.42±3.24	1.41±0.27	9.49±2.17	0.25±0.02	0.24±0.09
LPS 1 ug+OVA	8	4.6±0.35	0.41±0.17	5.4±0.64	2.11±0.06	12.4±1.06	2.8±0.63

against asthmatic responses [19,20]. Furthermore, there are studies demonstrating that children that grow up on farm are less likely to have asthma or atopic disease, as a result of exposure to farm animal or microorganism [14,21]. LPS is an excellent model of bacterial exposure, and has been demonstrated to be one of the most potent microbial stimuli of inflammation [21–26].

In this investigation, exposure to low-dose LPS was effective in protection against OVA-induced AHR, histopathologic change, and the recruitment of neutrophils and lymphocytes compared with OVA sensitization and challenge alone, while higher doses of LPS showed no protective effects or even aggravated asthmatic response. As a result, we conclude low endotoxin administration benefits subsequent asthmatic responses. The fact that only low-dose LPS treatment provided protection to OVA-induced asthma may explain why some previous studies agree with these finding [20,27] while others demonstrate a detrimental role of LPS exposure on development of asthma.

Numerous models exist that propose a mechanism for the protective effects of endotoxin on allergies. One model proposes a complex interplay between T cells, eosinophils, and cytokines, driving the progression of inflammation and AHR [28–30]. There are a number of T cell subsets that secrete distinct profiles of cytokines that may be involved in this process [31–33]. Th2 cells secrete IL-4, IL-13, and IL-5, which are chemoattractants for eosinophils and alter the antibody response [34]. Delayed-type hypersensitivity and cell-mediated immunity are driven by Th1 cells which secrete IFN-γ and IL-12 [35]. It is becoming increasingly clear that the complex interactions between Th1 and Th2 cells and the balance of these cytokines play a critical role in asthma development [31–33,35].

Apart from the role of Th1 and Th2 cells in asthma development, it was recently reported that Treg and Th17 cells play an important role in asthma and atopic disease [36–41]. Foxp3+Treg cells are a distinct subset of CD4+T cells which can suppress effector CD4+T cells responses [38] and have been shown to play a crucial role in allergic diseases, including asthma [36,38,40]. Foxp3+Treg cells can suppress Th2 and Th17 cell-mediated inflammation and prevent airway inflammation and bronchial hyperresponsiveness both in asthmatic patients and in animal models, and the functions of Foxp3+Treg cells are impaired in asthma [36,38,40]. Th17 is a pro-inflammatory CD4+T effector cell population that is different from Th1 and Th2, and Th17-related cytokines play pivotal roles in the pathogenesis of allergic asthma [38]. Th17 responses in chronic allergic airway inflammation abrogate regulatory T cell-mediated tolerance and contribute to airway remodeling. Antigen-specific Th17 cells can promote Th2 cell-mediated eosinophil recruitment into the airways [42–47]. Furthermore, allergen-driven Th17 cells resulted in asthma exacerbations or accelerated tissue damage. AHR in patients with asthma has recently been shown to be exacerbated by IL-17A, and IL-17A induces human bronchial epithelial cells to produce mucus, release cytokines, and recruit immune cells [45,46].

In our study using OVA-challenged mice pre-treated with low-dose LPS, we detected increased Th1 cytokines and Treg cytokines along with decreased Th2 cytokines and Th17 cytokines through ELISAs and flow cytometry. However, higher doses of LPS showed no protective effect and even aggravated asthma in certain settings. Thus, we conclude that low-dose LPS exposure may correct the imbalance of Th1/Th2 and Treg/Th17 cells, promoting Th1 and Treg cells as well as their related cytokines, and also inhibiting harmful effects of Th2 and Th17 and their related cytokines, in the context of OVA-induced asthma. Accordingly, LPS may modify lung function and immune responses during asthma in a dose-dependent manner. Prior exposure of LPS can alter adaptive immune response in adulthood and suppress the development of adult mouse allergic asthma, which suggest a role of the protective immune response induced by low-dose LPS as an immunoregulatory treatment to prevent asthma.

Conclusions

We demonstrated that low-dose LPS pre-exposure suppresses the hallmark features of allergic asthma in mouse models. We found that LPS act as an immunoregulatory factor on innate and adaptive immune cells by enhancing of overall Th1/Th2 and Treg/Th17 balance, and the effects are highly concentration-dependent. As LPS is a component of Gram-negative bacteria, airway homeostasis is affected by bacterial exposure,

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and previous exposure to unsanitary conditions may benefit asthma. The sequence of events leading to AHR in asthma remains elusive and further studies will add to our understanding of these complex and important interactions. The present results were obtained in pathogen-free conditions which do not exactly mimic the human state; therefore, future studies on low-dose LPS exposure in non-sterile conditions may yield results that more accurately recapitulate human asthma.

Conflicts of interest

None.

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