A juvenile murine model with chronic lung inflammation induced by repeated intratracheal instillation of lipopolysaccharides: a versatile and replicable model

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Background: Recurrent lower respiratory tract infection or chronic pulmonary infection often occur in children with chronic lung diseases (CLDs). By continuous lung inflammation, recurrent and chronic infection could cause irreversible airway structural and lung function damage, which eventually leads to respiratory failure and death.

Methods: In purpose of recapitulating persistent high-intensity lung inflammation caused by recurrent lower respiratory tract infection or chronic infection, we established a juvenile murine model with chronic lung inflammation induced by repeated intratracheal instillations of lipopolysaccharides (LPS) from *Pseudomonas aeruginosa* once a week for 4 weeks. Four-week-old C57BL/6N mice were divided into 4 groups, including $LPS_{0.5}$ group (n=15), $LPS_{1.0}$ group (n=15), Control group (n=15) and Normal group (n=15). Mice in $LPS_{0.5}$ group and $LPS_{1.0}$ group were instilled intratracheally with 0.5 mg/kg LPS and 1.0 mg/kg LPS respectively. Mice in control group were instilled intratracheally with LPS-free sterile 0.9% NaCl, whereas normal group received no treatment. The successful chronic lung inflammation murine model was validated via (I) pathological manifestations of chronic inflammatory mononuclear-cell infiltration and lung parenchyma damage; (II) decreased lung function.

Results: All mice in $LPS_{1.0}$ group died before the third instillation. No death after instillation was observed in Control and $LPS_{0.5}$ group. Histological analysis revealed that in $LPS_{0.5}$ group, 7 days after the third instillation, most bronchus and parabronchial vessels were wrapped by infiltrating monocytes and lymphocyte and alveolar cavities were compressed, which were not observed in control and normal group. Also, ratio of forced expiratory volume in 0.1 second ($FEV_{0.1}$) and forced vital capacity (FVC) in $LPS_{0.5}$ group was significantly lower (P<0.0001) than both control group and normal group, suggesting ventilatory dysfunction developed after repeatedly intratracheal instillation once a week for 4 weeks.

Conclusions: Intratracheal instillation of 0.5 mg/kg LPS once a week for 4 weeks can cause chronic lung inflammation in young mice.

Keywords: Chronic lung inflammation; intratracheal instillation; juvenile murine models

Transl Pediatr 2022;11(8):1292-1300 | https://dx.doi.org/10.21037/tp-22-44

Introduction

Chronic lung diseases (CLDs), characterizing prolonged course and impaired lung function, have great impact on patients' quality of life. In recent years, incidences of CLD with high disability and fatality rates, including interstitial lung disease, bronchiolitis obliterans, primary ciliary dyskinesia, cystic fibrosis and others, have increased in children (1). Apart from primary CLD, secondary CLD is mainly caused by infection, transplantation, toxicants or connective tissue disorders, in which post-infection CLD has become a concern for pediatricians. A meta-analysis revealed that 17% of children with severe pneumonia developed CLD as long-term sequelae, such as bronchiectasis and chronic bronchitis (2). Due to abnormal structures of respiratory tracts and mucus obstruction, children with CLD are more likely to suffer from respiratory infections than healthy children, which have more severe and longer courses accompanied by decreased baseline lung function (3). Recurrent lower respiratory tract infection (RLRI) (4) often occurs in CLD patients. For post-infection CLD, respiratory tract infection and CLD reinforce each other as vicious cycles. RLRI or chronic infection leads to chronic airway inflammatory which provokes persistent lung injury, irreversible structural and lung function damage. Chronic lung inflammation plays a key role in this deterioration process. However, there are some gaps in current studies on CLD and RLRI. (I) Most common animal models of CLD are chemical-induced, such as diacetyl-induced bronchiolitis obliterans (5), cigarette smoke-induced chronic bronchitis (6) and bleomycin-induced pulmonary fibrosis (7), which could not explore the effects of chronic lung inflammation under repeated infections. (II) Instead of studying how chronic inflammation itself alter pulmonary structures, current RLRI or chronic infection animal models focus on specific bacteria. To investigate structural and functional change in children's lungs under sustained and intense inflammation state, a chronic lung inflammation animal model which can recapitulate such real-world situation is in urgent need.

With impaired epithelium and abnormal pulmonary immune status, bacterial colonization often induces RLRI or chronic lung infection in CLD patients (8). Gram-negative (G⁻) bacteria, such as *Haemophilus influenzae* and *Pseudomonas aeruginosa* (PA), are commonly colonized in the airway of CLD patients, among which PA plays important role throughout the course of most cystic fibrosis patients (9). Lipopolysaccharides (LPS), as a pro-inflammatory glycolipid component on the cell wall of G⁻ bacteria (10), are major causative agents during lung injury and pneumonia (11). As purification and production procedure standardized, virulence heterogeneity of LPS obtained by the same manufacturer, extraction method and bacteria source greatly decreased. Therefore, by ensuring relatively stable model quality, LPS have been widely used to induce inflammation *in vitro* and *in vivo* (12), especially a common stimulus for pulmonary inflammatory disease models.

In order to attain chronic lung inflammation in animal models, methods of LPS administration (12) primarily include nebulization and intratracheal instillation. To reproduce RLRI inducing persistent pulmonary inflammation in mice, LPS must access lower respiratory tract, thus intratracheal instillation is preferred. In current models of LPS-induced lung inflammation, the concentrations of LPS are either too high or too low. In LPS-induced acute lung injury models (13), to achieve diffuse alveolar damage, concentration of LPS is higher than 2 mg/kg and animals are usually sacrificed within 72 hours after the instillation. While in LPS-induced chronic bronchitis and chronic obstructive pulmonary diseases models, low concentration of LPS, generally less than 0.5 mg/kg are given for more than 10 weeks to ensure the animals could survive until airway structure changes (14,15). In purpose of recapitulating persistent high-intensity lung inflammation caused by RLRI or chronic infection, we hypothesized that slightly increased the concentration of LPS could trigger greater lung lesions and chronic structural-changed lung inflammation may occur after repeated instillation. Here, we reported a juvenile murine model with chronic lung inflammation induced by repeated intratracheal instillations of *Pseudomonas aeruginosa* LPS. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-44/rc).
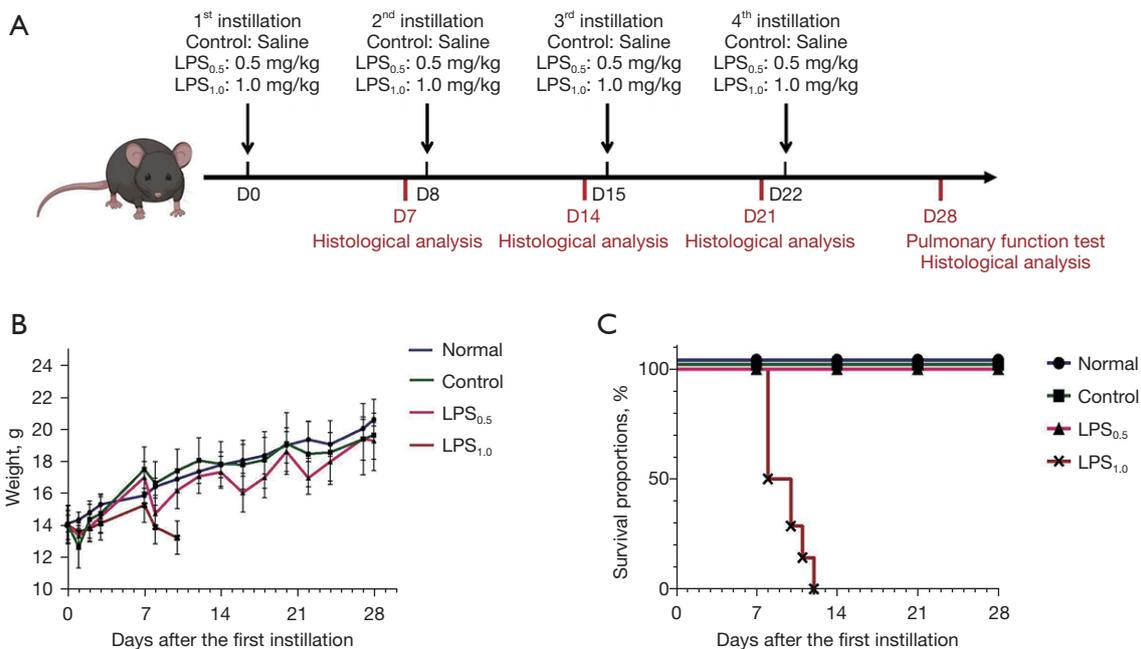
Figure 1 Treatment, weight changes and survival proportions in four groups of mice. (A) Treatment for four groups of mice. (B,C) Kinetic changes of weight and survival proportions in four groups of mice. Number of days after the first instillation are presented on X-axis, in which day 0 represent the day of the first instillation. Weights in units of gram (g) and survival proportions in units of percentage (%) are presented on the y-axis in (B) and (C), respectively. Blue, green, pink and red lines represent normal, control, $LPS_{0.5}$ and $LPS_{1.0}$ group, respectively. LPS, lipopolysaccharides.

Methods

Animals

Four-week-old C57BL/6N female mice (weight 13–15 g) were obtained from Charles River Breeding Laboratories (Beijing, China). Mice were housed and bred under standard conditions of care. Experiments were performed under a project license (No. 2021019) granted by the Animal Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University, in compliance with Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Experimental protocol

Mice were randomly divided into 4 groups, including $LPS_{0.5}$ group (n=15), $LPS_{1.0}$ group (n=15), Control group (n=15) and Normal group (n=15). In order to induce chronic pulmonary inflammation, mice in LPS groups were repeatedly challenged with LPS (*Pseudomonas aeruginosa 10*, L9143, Sigma Chemical Co.) once a week for 4 weeks

by intratracheal instillation. Mice in $LPS_{0.5}$ group and $LPS_{1.0}$ group were instilled intratracheally with 0.5 mg/kg LPS and 1.0 mg/kg LPS respectively. Mice in control group were instilled intratracheally with LPS-free sterile 0.9% NaCl, whereas normal group received no treatment. The administrated volume of LPS and saline were 50 µL, respectively. Mice were anesthetized by intraperitoneal injection of Avertin. A volume of 50 µL LPS or saline was instilled intratracheally via a canule, followed by 0.1 mL of air. After intratracheal treatment, the mice were kept in an upright position for 10 min to allow sufficient spreading of the fluid throughout the lungs. In order to explore the appropriate concentration of LPS, mice were killed on day 7, 14, 21, and 28 after the first instillation to observe the pathological changes of lung tissues. On day 28, 6 mice from each group were taken for lung function testing (*Figure 1A*).

Histological analysis

Lung tissue was inflated with 10% phosphate-buffered formalin (pH 7.4) at a pressure of 20 cmH$_2$O through the trachea for 15 minutes and subsequently fixed in formalin

Table 1 Modified lung inflammation score

Regions	Parameters	Scores			
		0	1	2	3
Perivascular	(I) Inflammatory cell infiltration	Absent	Mild	Moderate	Severe
	(II) Destruction of parenchyma	Absent	Mild	Moderate	Severe
Peribronchial	(I) Inflammatory cell infiltration	Absent	Mild	Moderate	Severe
	(II) Destruction of parenchyma	Absent	Mild	Moderate	Severe
Alveolar	(I) Inflammatory cell infiltration	Absent	Mild	Moderate	Severe
	(II) Compressed alveolar lumens	Absent	Mild	Moderate	Severe
	(III) Alveolar septal thickening	Absent	Mild	Moderate	Severe

for 24 h. After paraffin embedding, 4-μm sections were cut and stained with hematoxylin/eosin (H&E) to evaluate general morphology.

To quantify the extent of inflammatory infiltration and tissue damage, we developed a modified lung inflammation scoring system (*Table 1*) based on previous studies (16-18). Whole-lung sections were scored by two independent observers who were blinded for groups. Degree of inflammatory cell infiltration and destruction of parenchyma were assessed in perivascular regions, peribronchial regions, alveolar septa and lumens. Modified lung inflammation score was expressed as the sum of scores for each parameter. Scores of mice from the same group were pooled and calculated as mean ± standard error of the mean (SEM).

Lung function measurement

FlexiVent^{TM} system was used for lung function measurement. First, calibrate the pulmonary function meter and set up parameters. After intraperitoneal injection of 1.2% avertin to anesthetize the mouse, cut the skin at the thyroid cartilage, bluntly separate the muscles to expose the trachea. Then cut a small opening under the thyroid cartilage and insert canule into trachea. After finishing intubation, move the mouse into plethysmography chamber, intraperitoneally inject muscle relaxant vecuronium (0.3 mL/kg), connect the system and complete lung function assessment.

Statistical analysis

The unpaired, 2-tailed *t*-test was used to determine whether the differences in the lung function parameters between Normal group, Control group and $LPS_{0.5}$ group was statistically significant. Comparison and statistical analysis of modified lung inflammation score between different groups were performed with the Mann-Whitney *U* test. Tests were performed with GraphPad Prism. P values of 0.01–0.05, 0.001–0.01, and 0.0001–0.001 were considered statistically significant, very significant, and extremely significant, respectively.

Results

Weight change and survival proportions

During the first instillation (D0), mice in the Control group were short of breath after control instillation, which recovered after 5 minutes. They woke up after an average of 20 minutes and their actions and reactions recovered as before instillation. In $LPS_{0.5}$ and $LPS_{1.0}$ group, shortness of breath was more pronounced which took averagely 40 minutes for them to recover. On the first day after the first instillation (D1), mice in the LPS group lost an average of 10% in weight. Among them, weight of mice in $LPS_{0.5}$ group gradually recovered as Normal group since D3, while mice in $LPS_{1.0}$ group slowly recovered since D4 (*Figure 1B*). During the second instillation (D8), mice in $LPS_{1.0}$ group had poorer tolerance to anesthetics compared with $LPS_{0.5}$ group. In $LPS_{1.0}$ group, a mouse died after anesthesia and 7 mice died quickly due to dyspnea after instillation. The remaining mice in $LPS_{1.0}$ group died on D9, D10, and D11 (1, 2, and 3 days after the second instillation respectively). All mice in $LPS_{1.0}$ group died before the third instillation on D15 (*Figure 1C*). No death after instillation was observed in Control and $LPS_{0.5}$ group (*Figure 1C*).
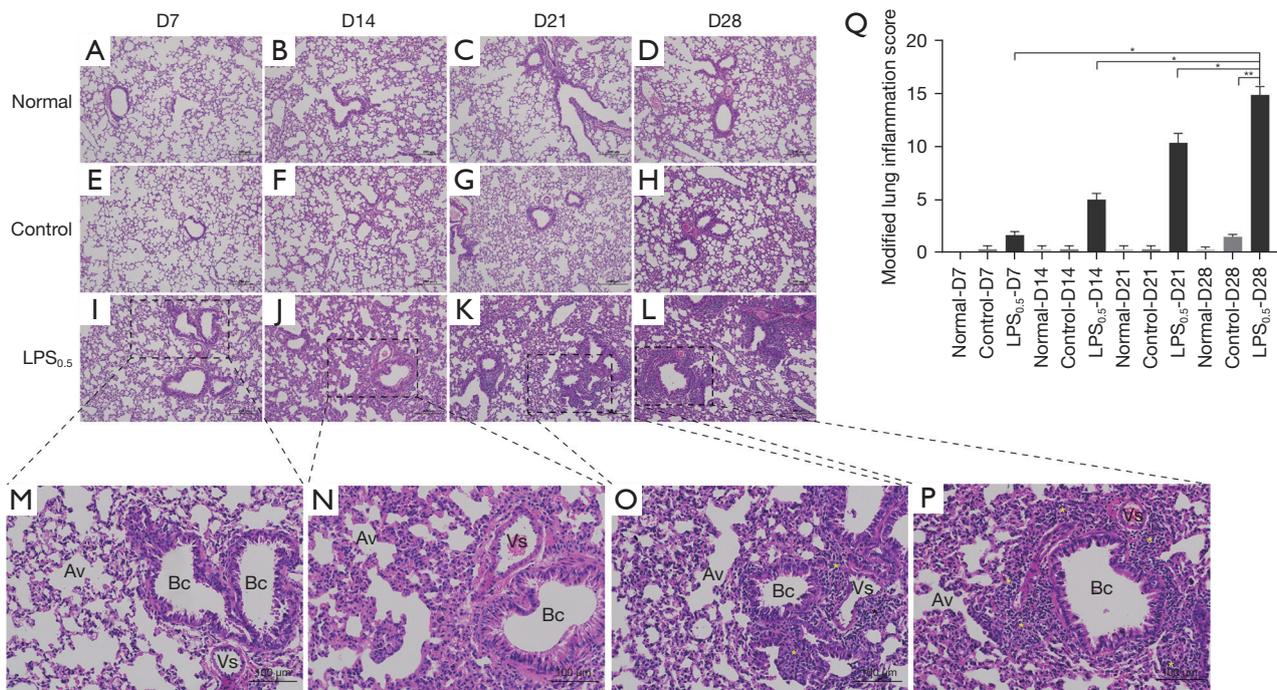
1296

Mai et al. A juvenile murine model of chronic lung inflammation

Figure 2 Morphological analysis of lung tissues of mice in Normal group, Control group and LPS$_{0.5}$ group. (A-D) Lung tissues of mice in Normal group on D7, D14, D21 and D28, respectively. H&E staining, bar =200 µm. (E-H) Lung tissues of mice in Control group on D7, D14, D21 and D28, respectively. H&E staining, bar =200 µm. (I-L) Lung tissues of mice in LPS$_{0.5}$ group on D7, D14, D21 and D28, respectively. H&E staining, bar =200 µm. (M-P) Lung tissues of mice in LPS$_{0.5}$ group on D7, D14, D21 and D28, respectively. Stars denote the mononuclear-cell infiltration and lymphocytic aggregation. H&E staining, bar =100 µm. (Q) Modified lung inflammation score of mice in normal group on D7 (n=3), D14 (n=3), D21 (n=3) and D28 (n=6), control group on D7 (n=3), D14 (n=3), D21 (n=3) and D28 (n=6) and LPS$_{0.5}$ group on D7 (n=3), D14 (n=3), D21 (n=3) and D28 (n=6). Groups were compared using a two-sided Mann-Whitney U test. Error bars represent SEM. *, P<0.05; **, P<0.01. LPS, lipopolysaccharides; Av, alveolus; Bc, bronchus; Vs, vessel.

Dynamic change and characteristics of chronic inflammation during 4 weeks of LPS exposure

For mice in in normal (*Figure 2A-2D*) and control group (*Figure 2E-2H*), no obvious inflammation was observed in lung tissues throughout the experiment. In mice of LPS$_{0.5}$ group: (I) 7 days after the first instillation (D7, *Figure 2I*), few mononuclear cells infiltrated around part of the bronchus, with intact airway epithelium. (II) 7 days after the second instillation (D14, *Figure 2J*), bronchial epithelium thickened with a few neutrophils and mononuclear cells infiltration, while no exudates in the alveoli were observed. (III) 7 days after the third instillation (D21, *Figure 2K*), small amounts of bronchial epithelial cells fell off with few mononuclear cells infiltrating around bronchus and severer lymphocytic infiltration around parabronchial vessels. Local alveolar wall slightly thickened with few mononuclear cells

infiltrated while no exudate in the alveolar cavity were observed. (IV) 7 days after the third instillation (D28, *Figure 2L*), most bronchus and parabronchial vessels are wrapped by lymphocytic aggregation. While alveolar wall thickened with focal infiltration of mononuclear cells and connective tissue hyperplasia, alveolar cavities are compressed and narrowed without exudates in the cavity. Under high-magnification view (*Figure 2M-2P*), we found that agranulocytes constituted the mononuclear-cell infiltration and lymphocytic aggregation in LPS$_{0.5}$ group, which mainly consisted of monocytes, with oval, indented or C-shaped nucleus, and lymphocytes, with rather spherical nucleus. Comparisons of modified lung inflammation scores between mice from different groups (*Figure 2Q*) showed that, there were statistically significant differences between LPS$_{0.5}$ group on D28 and on D7 (P<0.05), D14 (P<0.05), D21 (P<0.05) and Control group on D28 (P<0.01). These
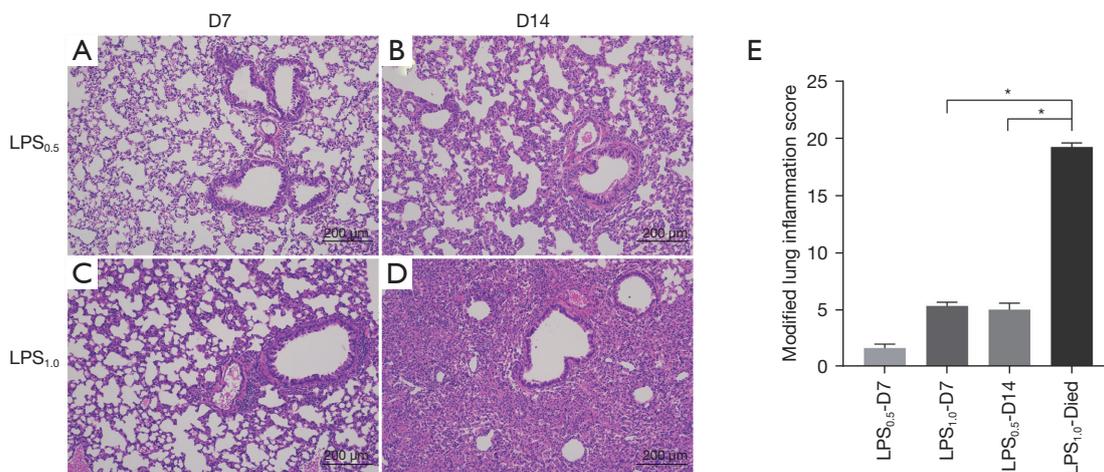
Figure 3 Morphological analysis of lung tissues of mice in $LPS_{0.5}$ group and $LPS_{1.0}$ group (H&E staining, bar =200 µm). (A,B) Lung tissues of mice in $LPS_{0.5}$ group on D7 and D14 respectively. (C) Lung tissues of mice in $LPS_{1.0}$ group on D7. (D) Lung tissues of mice in $LPS_{1.0}$ group died after D7. (E) Modified lung inflammation score of mice in $LPS_{0.5}$ group on D7 (n=3), D14 (n=3), $LPS_{1.0}$ group on D7 (n=3) and mice that died after D7 (n=5). Groups were compared using a two-sided Mann-Whitney U test. Error bars represent SEM. *, P<0.05. LPS, lipopolysaccharides.

Table 2 Ventilatory function of mice in normal, control and $LPS_{0.5}$ groups on D28

Group	$FEV_{0.1}$ (mL, mean ± SD)	FVC (mL, mean ± SD)	$FEV_{0.1}$/FVC (mean ± SD)
Normal (n=6)	1.133±0.233	1.261±0.216	0.896±0.034
Control (n=6)	0.915±0.243	1.066±0.273	0.858±0.018
$LPS_{0.5}$ (n=6)	1.025±0.140	1.462±0.191	0.701±0.040

LPS, lipopolysaccharides; $FEV_{0.1}$, forced expiratory volume in 0.1 second; FVC, forced vital capacity; SD, standard deviation.

indicated that mice of $LPS_{0.5}$ group on D28 had more intense inflammatory pulmonary damage.

All mice in $LPS_{1.0}$ group died before the third instillation (D15) and their lung tissues were taken for morphological analysis. Compared with $LPS_{0.5}$ group (*Figure 3A,3B*), mice in $LPS_{1.0}$ group (*Figure 3C,3D*): (I) on D7, more mononuclear cells were observed around parabronchial vessels, while alveolar walls also thickened with mononuclear-cell infiltration. (II) For mice that died before the third instillation, small amounts of epithelial cells fell off and alveolar cavity was severely compressed by excessively infiltration of neutrophils and mononuclear cells. Modified lung inflammation scores of $LPS_{1.0}$ group (*Figure 3E*) on D14 were significantly higher than D7 (P<0.05) and $LPS_{0.5}$ group on D14 (P<0.05), revealing that mice in $LPS_{1.0}$ group might die of alveolar gas-exchange dysfunction, as consequences of excessive inflammatory infiltration.

Ventilatory dysfunction developed after 4 weeks of LPS exposure

Forced vital capacity (FVC) and forced expiratory volume in 0.1 second ($FEV_{0.1}$) were measured (*Table 2* and *Figure 4*) to evaluate lung function of mice in normal group (n=6), control group (n=6) and $LPS_{0.5}$ group (n=6) on D28 (7 days after the fourth instillation). No statistical differences in $FEV_{0.1}$ were observed between three groups (*Figure 4B*) and FVC of $LPS_{0.5}$ group was higher than that of Control group (*Figure 4C*). Whereas, $FEV_{0.1}$/FVC was significantly lower than Control and Normal group (*Figure 4A*), suggesting ventilatory dysfunction developed after repeatedly intratracheal instillation once a week for 4 weeks.

Pathologic findings of chronic inflammatory cell infiltration, lung parenchyma injury and decreased lung function were observed in all mice in $LPS_{0.5}$ group on D28
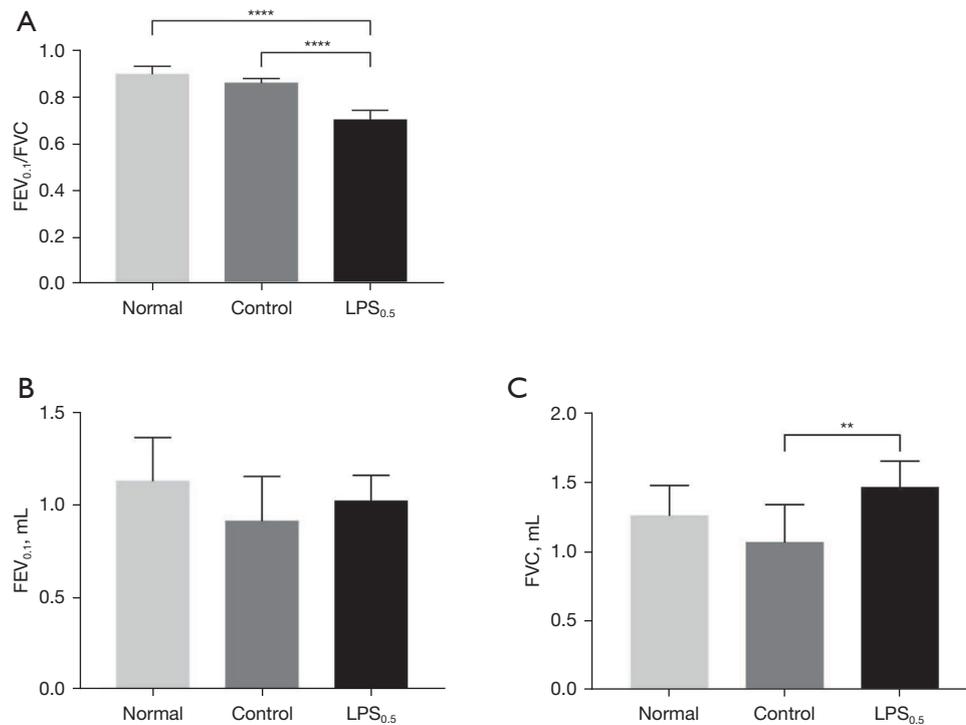
Figure 4 Lung function of mice in normal group, control group and LPS$_{0.5}$ group on D28. FEV$_{0.1}$/FVC, FEV$_{0.1}$ in units of mL and FVC in units of mL are presented on the y-axis in (A), (B) and (C), respectively. Light gray columns represent normal group, deep gray columns represent control group and black columns represent LPS$_{0.5}$ group. **, P<0.01; ****, P<0.0001. LPS, lipopolysaccharides; FEV$_{0.1}$, forced expiratory volume in 0.1 second; FVC, forced vital capacity.

(n=6), reflecting that the success rate of this LPS-induced chronic lung inflammation murine model was 100%.

Discussion

Previous studies (19,20) showed that intratracheal instillation 2 mg/kg of LPS can cause acute lung injury in adult C57BL/6 mice. Acute inflammation peaks 2 days after instillation, and then significantly alleviated 7 days after instillation (19). In the commonly used chronic bronchitis murine model induced by aerosolized low-concentration LPS, infiltration of neutrophils that appeared quickly post-nebulization resolved 1 week after nebulization termination, indicating that acute inflammation began to recover 1 week after LPS exposure (14). Therefore, this study chose 7 days as the instillation interval. In order to ensure the survival rate of mice after repeated LPS instillation, two concentrations of LPS (1.0 and 0.5 mg/kg) were used in this study.

Features of chronic lung inflammation are: (I) clumps of chronic inflammatory cells (mainly lymphocytes and monocytes); (II) destruction of lung parenchyma; (III) connective tissue proliferation and repair (21). In this study, repeated intratracheal instillation of 0.5 mg/kg LPS showed the accumulation process of chronic inflammatory cells in the lung parenchyma, which also infiltrate bronchus and parabronchial vessels. With lung parenchyma excessively infiltrated by inflammatory cells, alveolar cavities are narrowed. Also, although fibrosis is not obvious in this model, a certain extent of connective tissue hyperplasia can be seen in the lung parenchyma. Similar morphological characteristics were also observed in previous studies (14,22), which demand more than 10 weeks to attain chronic lung inflammation. Cavagna *et al.* established a rabbit model of chronic bronchitis by nebulizing LPS dissolved in saline solution. After nebulization for 20 weeks, bronchitis and bronchiolitis manifested as bronchial epithelial cells shed off, endobronchial secretions increased, inflammatory cells infiltrated around bronchus and alveolar walls, accompanied with elevated airway resistance and reduced lung compliance (23). Vernooy *et al.* instilled 5 μg of LPS into the trachea of 12-week-old Swiss mice twice a week.

After 12 weeks, inflammatory cells infiltrated the bronchus, bronchioles, and large vessels. Alveolar infiltration of monocytes and lymphocytic infiltration in bronchial epithelium resulted in local bronchiolitis (14). After aerosol inhalation of LPS for 6 weeks, both wild-type and $Cftr^{-/-}$ mice exhibited expansion of the distal bronchiolar alveolar cavity and destruction of alveolar structure (15). At present, studies on animal models of chronic lung inflammation via repeated instillation of low-concentration LPS into the trachea require at least 6 weeks (15,22). In order to simulate impact of RLRI and subsequent chronic lung inflammation, this study aim to seek the appropriate concentration of LPS which ensure long-term survival rate and maintain the intensity of chronic lung inflammation, which could reduce time required for chronic inflammation formation. Moreover, the significantly reduced $FEV_{0.1}/FVC$ in $LPS_{0.5}$ group suggests that repeated LPS instillation for 4 weeks can make enough structural damages to impair lung function. However, fibrotic remodeling was not observed in our study.

In conclusion, intratracheal instillation of 0.5 mg/kg LPS once a week for 4 weeks can cause chronic lung inflammation in young mice and we expect this model for further use in mechanism exploration and drug development.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-44/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-44/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-44/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 2021019) granted by the Animal Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University, in compliance with Regulations for The Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

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1300

Mai et al. A juvenile murine model of chronic lung inflammation

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