

The presence of LPS in OVA inhalations affects airway inflammation and AHR but not remodeling in a rodent model of asthma

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Submitted 20 June 2011; accepted in final form 15 April 2012

Tsuchiya K, Siddiqui S, Risse P-A, Hirota N, Martin JG. The presence of LPS in OVA inhalations affects airway inflammation and AHR but not remodeling in a rodent model of asthma. *Am J Physiol Lung Cell Mol Physiol* 303: L54–L63, 2012. First published April 20, 2012; doi:10.1152/ajplung.00208.2011.—Ovalbumin (OVA) is the most frequently used allergen in animal models of asthma. Lipopolysaccharide (LPS) contaminating commercial OVA may modulate the evoked airway inflammatory response to OVA. However, the effect of LPS in OVA on airway remodeling, especially airway smooth muscle (ASM) has not been evaluated. We hypothesized that LPS in commercial OVA may enhance allergen-induced airway inflammation and remodeling. Brown Norway rats were sensitized with OVA on day 0. PBS, OVA, or endotoxin-free OVA (Ef-OVA) was instilled intratracheally on days 14, 19, 24. Bronchoalveolar lavage (BAL) fluid, lung, and intrathoracic lymph node tissues were collected 48 h after the last challenge. Immunohistochemistry for α -smooth muscle actin, Periodic-Acid-Schiff staining, and real-time qPCR were performed. Airway hyperresponsiveness (AHR) was also measured. BAL fluid macrophages, eosinophils, neutrophils, and lymphocytes were increased in OVA-challenged animals, and macrophages and neutrophils were significantly lower in Ef-OVA-challenged animals. The ASM area in larger airways was significantly increased in both OVA and Ef-OVA compared with PBS-challenged animals. The mRNA expression of IFN- γ and IL-13 in lung tissues and IL-4 in lymph nodes was significantly increased by both OVA and Ef-OVA compared with PBS and were not significantly different between OVA and Ef-OVA. Monocyte chemoattractant protein (MCP)-1 in BAL fluid and AHR were significantly increased in OVA but not in Ef-OVA. LPS contamination in OVA contributes to the influx of macrophages and MCP-1 increase in the airways and to AHR after OVA challenges but does not affect OVA-induced Th1 and Th2 cytokine expression, goblet cell hyperplasia, and ASM remodeling.

animal model; airway hyperresponsiveness; Brown Norway rat; endotoxin; ovalbumin

LIPOLYPSACCHARIDE (LPS) is a cell wall component of Gram-negative bacteria and ubiquitous in the environment and is often present in polluted air as well as in household dust. There has been substantial interest in LPS for its effects on respiratory immunity since the development of the hygiene hypothesis (32, 38). Toll-like receptor 4 (TLR4) is a receptor for LPS, and activation of TLR4 is required to prime dendritic cells and epithelial cells for optimal responses to antigen exposure (12). Stimulation of TLR4 is essential for downstream cellular events that are mediated by mitogen-activated protein kinase p38 and c-Jun NH2-terminal kinase, leading to nuclear translocation of NF- κ B, resulting in cytokine mRNA transcription and release of proinflammatory cytokines (37).

Ovalbumin (OVA) is widely used in animal models of asthma to study mechanisms of airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. However, commercial OVA has been reported to be contaminated by LPS and in sufficient quantities to alter the inflammatory response to airway allergen challenge (40). Previous studies have explored the effects of concomitant LPS exposure in conjunction with commercial OVA in animal models of asthma. Exposure to LPS during allergic sensitization attenuates allergen-induced IgE, airway eosinophilia, and Th2 type response, whereas the exposure during allergen challenge exacerbates these responses in OVA-induced experimental asthma (7). Another study has reported that LPS administration concomitantly with OVA challenge suppressed Th2 responses via nitric oxide synthase 2 activity in a murine model of asthma (24).

The effects are dose-dependent; multiple exposures to LPS-free OVA with low-dose LPS are required for induction of airway eosinophilia and Th2 type responses, whereas OVA with high-dose LPS induces airway neutrophilia and an increased Th1 type response (9). On the other hand, LPS inhalation by itself induces airway inflammation, AHR, and even collagen deposition, effects that are inhibited by a TLR4 antagonist (26). Some forms of LPS may also activate the epidermal growth factor receptor (EGFR) on airway epithelium through the release of transforming growth factor (TGF)- α and cause epithelial differentiation to goblet cells (14). We reasoned that TGF- α might be released at the basolateral surface of the epithelial cells, as has been found for another EGFR agonist, heparin-binding epidermal growth factor, in response to cell compression (4), and through EGFR activation lead also to remodeling of airway smooth muscle (ASM). Based on these previous studies, it is possible that LPS contamination in commercial OVA may be involved in the Th2 type reaction after allergen sensitization and challenge and in the subsequent airway remodeling. However, the effects of LPS on OVA-induced airway tissue remodeling, especially ASM, have not been evaluated.

In this study, we examined the role of LPS contamination in OVA in the Th2 type response and airway remodeling, including ASM remodeling induced by repeated OVA challenge of the sensitized Brown Norway (BN) rat. This well-characterized model shows significant proliferation of ASM cells and increase of ASM mass following three intratracheal OVA challenges (15). The effect of LPS on AHR was also studied in tracheotomized and mechanically ventilated animals using the forced oscillation technique for the assessment of respiratory mechanics.

MATERIALS AND METHODS

Animals. Inbred male BN rats, 6–7 wk of age, were purchased from Harlan (Indianapolis, IN) and were maintained in a conventional

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animal facility at McGill University (Montreal, QC, Canada). Experimental procedures were approved by the Animal Care Committee of McGill University.

OVA sensitization. All animals were sensitized with a single subcutaneous injection of 1 mg of OVA (Grade V; Sigma-Aldrich, St. Louis, MO), with 100 mg of aluminum hydroxide in 1 ml of PBS on day 0. Simultaneously, 0.5 ml of *Bordetella pertussis* vaccine containing 2×10^9 heat-killed bacilli was injected intraperitoneally as an adjuvant.

Intratracheal OVA challenge. On days 14, 19 and 24, 0.5 mg of OVA (Grade V; Sigma-Aldrich) or endotoxin-free OVA (Ef-OVA) (endotoxin concentration < 1 EU/mg, EndoGrade Ovalbumin; Hyglos, Regensburg, Germany) in 100 μ l of sterile PBS was directly instilled into the rat trachea under light isoflurane anesthesia. A sample (100 μ l) of sterile PBS was used for challenge in the negative control group.

Study groups. To study the role of the concomitant exposure to LPS and OVA in airway remodeling, rats were divided into three groups: 1) sensitized with OVA and challenged with PBS (OVA/PBS group), 2) sensitized with OVA and challenged with OVA (OVA/OVA group), and 3) sensitized with OVA and challenged with Ef-OVA (OVA/Ef-OVA group). Airway inflammation and remodeling were assessed on different animals from those used for the assessment of AHR.

Measurement of airway responsiveness to methacholine. On day 26, 48 h after the last challenge, animals were anaesthetized with xylazine hydrochloride (14 mg/kg ip) and pentobarbital (30 mg/kg ip) and connected via a tracheostomy tube to a computer-controlled small animal ventilator (Flexivent; Scireq, Montreal, QC, Canada) with the following ventilatory parameters; tidal volume: 8 ml/kg, frequency: 90 breaths/min, and a positive end-expiratory pressure: 2 cmH₂O. Muscle paralysis was induced with pancuronium bromide (1.0 mg/kg ip).

Baseline measurements were recorded after the aerosol administration of saline, followed by the administration of methacholine (MCh) (Sigma-Aldrich) diluted with saline in doubling doses from 4 to 128 mg/ml. MCh was delivered for 10 s at each concentration using the Aeroneb ultrasonic nebulizer (Aerogen, Galway, Ireland) on the Flexivent system. Total respiratory system resistance (Rrs) and elastance (Ers) were assessed using the single-compartment model followed by the assessment of Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H) using the constant-phase model every 15 s for a total of eight times after each MCh dose. Rn provides information concerning the response of large conducting airways, whereas G and H are sensitive to changes in the peripheral airways. The peak response is shown at each concentration of MCh.

Bronchoalveolar lavage. On day 26, 48 h after the last OVA challenge, bronchoalveolar lavage (BAL) was performed with five volumes of 5 ml of PBS via an endotracheal tube. BAL cytospin slides were prepared with 6×10^4 cells/slide (Cytospin4; Shandon, Pittsburgh, PA) and stained with Diff-Quik Stain Set. Total BAL cell numbers were counted, and cells were identified as macrophages, eosinophils, neutrophils, and lymphocytes from a count of at least 400 cells. The percentage and absolute numbers of each cell type were calculated.

The measurement of ASM mass. Left lung tissues were collected 48 h after the last intratracheal OVA challenge and fixed with 10% formalin at 25 cmH₂O overnight. Fixed tissues were embedded in paraffin and sectioned at 5- μ m thickness. The slides were stained for α -smooth muscle actin (α -SMA) to assess the increase in ASM mass. A mouse monoclonal antibody to α -SMA (clone 1A4; Sigma-Aldrich) was used as a primary antibody, and a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories, Burlingame, CA), was used as a secondary antibody. The signal was detected using Vector Red (Vector Laboratories). The ASM mass was estimated from the α -SMA-positive area using the image processing program, ImageJ (National Institutes of Health, <http://rsb.info.nih.gov/ij/>). The ASM mass was normalized for airway size by dividing the ASM area by the

square of the basement membrane perimeter (P_{BM}) (15). The measurements were performed for larger airways ($P_{BM} \geq 1.0$ mm) and smaller airways ($P_{BM} < 1.0$ mm) separately.

The measurement of goblet cell hyperplasia. To evaluate the degree of OVA-induced goblet cell hyperplasia in rat airways, paraffin sections from rat lungs were stained with Periodic-Acid-Schiff (PAS). The number of epithelial cells stained with PAS per unit length of basement membrane (P_{BM}) was determined. The measurements were performed for larger airways ($P_{BM} \geq 1.0$ mm) and smaller airways ($P_{BM} < 1.0$ mm) separately.

Real-time quantitative PCR. The right lungs and intrathoracic lymph nodes were removed and placed in RNA-Later and kept at 4°C overnight. The lung tissues were harvested for the assessment of IL-6, IL-13, IL-17, IFN- γ , cytokine-induced neutrophil chemoattractant-1 (CINC-1), and lymph node tissues for IFN- γ and IL-4 48 h after the third of three OVA challenges. The lung tissues were obtained for assessment of CLCA3, MUC5AC, TGF- α , ADAM17, TNF- α , and lymph node tissues for IL-17 18 h after a single OVA challenge. Tissues were placed in guanidine isothiocyanate-containing buffer (RLT buffer from RNeasy Mini kit; Qiagen, Mississauga, ON, Canada) with 2-mercaptoethanol and homogenized. Total RNA was extracted according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). RNA yield was measured with a spectrophotometer (Biomate3; Thermo Scientific, Waltham, MA). Strand cDNA was made in a 20- μ l reaction, using 1 μ g of total RNA as a template, Oligo(dT)₁₂₋₁₈ primer, dNTP mix, RNaseOUT, and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time quantitative PCR was performed with StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using QuantiTect SYBR Green PCR kit (Qiagen). The mRNA expression for IL-4, IL-6, IL-13, IL-17, IFN- γ , CINC-1, CLCA3, MUC5AC, TGF- α , ADAM17, and TNF- α was examined. Ribosomal S9 was used as a housekeeping gene. The samples were amplified with 15 s of denaturation at 95°C, 20 s of annealing, and 20 s of extension at 72°C. The annealing temperature was 57°C for IFN- γ , 60°C for IL-4, IL-17, CLCA3, MUC5AC, TGF- α , ADAM17, and TNF- α , 62°C for S9 and IL-13, 66°C for IL-6 and CINC-1. Primers for PCR were designed with the Primer3 program (<http://frodo.wi.mit.edu/>). The sequences of each pair of primers are shown in Table 1. The data were corrected for the housekeeping gene, S9, and the ratio of the expression in each sample to the expression in the calibrator was compared among three groups.

Protein levels of chemokines in BAL fluid. For the analysis of the chemokine levels in BAL fluid, animals were killed and BAL was performed on day 15, 18 h after a single OVA challenge. The BAL fluid obtained from the return on the first 5 ml of PBS was retained for analysis. A rat chemokine assay ultrasensitive kit (Meso-Scale Discovery, Gaithersburg, MD) was used to detect the concentrations of monocyte chemoattractant protein (MCP)-1 and CINC-1 in BAL fluid. The plate was read on the MSD detector, and standard curves were drawn using the manufacturer's software.

Cytosolic and nuclear protein extractions from the lung tissues. The middle lobe of right lung was removed 16 h after single intratracheal OVA challenge. Frozen lung tissue was ground in liquid nitrogen to a fine powder, and then cytosolic and nuclear fractions were extracted using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions with slight modifications. Protein concentrations in cytosolic and nuclear extracts were determined by using the Bradford assay. A sample (50 μ g) of protein per lane was used for Western blot analysis.

Western blot analysis. Cytosolic and nuclear extracts were loaded onto 10% SDS-PAGE gels, and electrophoresis was carried out. Proteins were transferred to PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-HCl buffer containing 0.1% Tween 20 (TTBS). Anti-NF- κ B p65 subunit (Upstate Biotechnology, Lake Placid, NY) was diluted 1:1,000 in

Table 1. Primer sequences used for qPCR

	Primer Sequences
IL-4 F	5'- GGATGTAACGACAGCCCTCTG -3'
IL-4 R	5'- CTCCTGGTGTTCCTTCTTG -3'
IL-6 F	5'- GCCACTGCCTTCCCTACTTCAC -3'
IL-6 R	5'- GTGCATCATCGCTGTTTCATACAATC -3'
IL-13 F	5'- GCTTGCCTTGGTGGTCTTGC -3'
IL-13 R	5'- CCATACCATGCTGCTGTTGCAC -3'
IL-17 F	5'- TTCCACTTCACCTGGACTCTG -3'
IL-17 R	5'- TTCTCAGGCTCCCTCTTCAGG -3'
IFN- γ F	5'- TGAAGACAACCAGGCCATC -3'
IFN- γ R	5'- CAGCTTTGTGCTGGATCTGTG -3'
CINC-1 F	5'- GCAGACAGTGGCAGGATTTCAC -3'
CINC-1 R	5'- CGACCATTCTTGAGTGTGGCTATGAC -3'
TNF- α F	5'- TGAAGTTCGGGGTGATCG -3'
TNF- α R	5'- GGGCTTGTCACTCGAGTTT -3'
TGF- α F	5'- TTTGGTGCAGGAAGAGAAGCCA -3'
TGF- α R	5'- ACACAGGTGATAATGAGGACA -3'
ADAM17 F	5'- ATGGGAAAGAGGAAAGCGAGTAC -3'
ADAM17 R	5'- CCTAGAGTCAGGCTCACAA -3'
CLCA3 F	5'- TCTCCTCAGGAAATGCAGTA -3'
CLCA3 R	5'- GGAGATTAACTCCTCTGCTCTCC -3'
MUC5AC F	5'- CTGAAAGGTCAGTGTCTCAA -3'
MUC5AC R	5'- CTCAGGTAGCTGCTGATGTC -3'
ribosomal S9 F	5'- AGGATTTCTTGGAGAGAAGGCTGC -3'
ribosomal S9 R	5'- CTTCTGAGAGTCCAGGCGAACAAT -3'

qPCR, quantitative PCR; F, Forward primer; R, Reverse primer; CINC, cytokine-induced neutrophil chemoattractant.

TTBS containing 1% skim milk and used as a primary antibody. The membranes were incubated with primary antibody overnight at 4°C followed by incubation with secondary antibody (anti-rabbit IgG, H&L chains, HRP) at room temperature for 1 h. Blots were developed by chemiluminescence (Amersham Bioscience, Piscataway, NJ) and visualized using a Fluorchem 8000 Imaging System (Alpha Innotech, San Leandro, CA). Negative controls omitting primary antibody were performed. Densitometry of bands from Western blots was done, and the relative density of the NF- κ B p65 to the β -actin was measured.

Statistical analysis. Data are expressed as the means $\pm$ SE. One-way ANOVA with the Fisher's least-significant-difference test was utilized to make statistical comparisons, and a P value > 0.05 was considered statistically significant.

RESULTS

Inflammatory cell recruitment into the bronchoalveolar compartment. We assessed the inflammatory response to repeated intratracheal OVA challenges by BAL performed 48 h after the last OVA challenge. The total cell count and the counts of macrophages, eosinophils, and lymphocytes in both the OVA/OVA and OVA/Ef-OVA group were significantly greater than in the OVA/PBS group. However, in the OVA/Ef-OVA group, the numbers of total cells, macrophages, and neutrophils were significantly lower than in the OVA/OVA group ($P < 0.001$, $P < 0.001$, $P < 0.01$, respectively), suggesting that the presence of LPS in the OVA preparation affects the recruitment of macrophages and neutrophils into the bronchoalveolar compartment in response to multiple OVA challenges (Fig. 1, A and B).

AHR in vivo. AHR to aerosolized MCh was measured 48 h after the last OVA challenge. Based on the single-compartment model of respiratory system mechanics, the animals in the OVA/OVA group showed significantly higher values of Rrs and Ers at the higher MCh concentrations compared with the

OVA/PBS or OVA/Ef-OVA group ($P < 0.05$, $P < 0.05$, respectively). There were no significant differences in these two parameters between the OVA/PBS and OVA/Ef-OVA group (Fig. 2, A and B). Respiratory system mechanics were also analyzed with the constant phase model to obtain more detail as to the site within the respiratory tree responsible for the differences in airway responsiveness (Fig. 2, C–E). The OVA/OVA group showed significantly higher values of G, a measure of airway and peripheral lung responses, at the higher MCh concentrations than the OVA/PBS group ($P < 0.05$ at 64 and 128 mg/ml of MCh), whereas the OVA/Ef-OVA did not show any differences compared with the OVA/PBS (Fig. 2D). In Rn, there was a strong tendency for the OVA/OVA group to show lower responses to MCh; the difference reached significance compared with the OVA/PBS group at 16 mg/ml of MCh concentration ($P < 0.05$) (Fig. 2C). The H parameter corre-

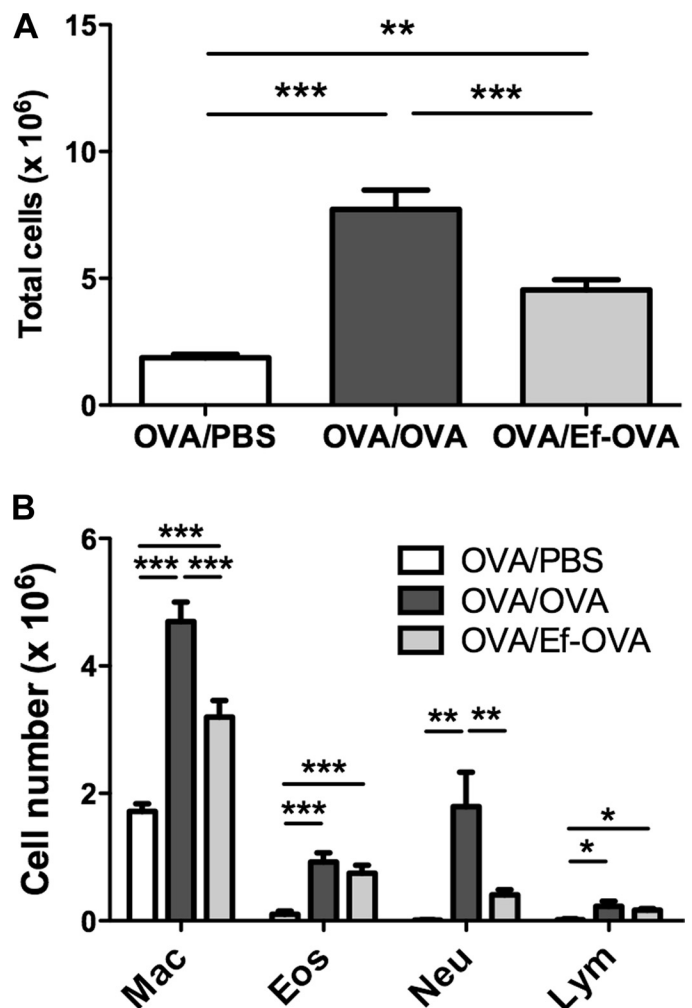


Fig. 1. Total cell count and cell profile in bronchoalveolar lavage (BAL) cells. BAL was performed on day 26, 48 h after the last ovalbumin (OVA) challenge. The total cell count (A) and the numbers of macrophages, eosinophils, neutrophils, and lymphocytes (B) in the OVA-sensitized, OVA-challenged animals (OVA/OVA) group were significantly greater than those in the OVA-sensitized, PBS-challenged animals (OVA/PBS) group. The effects of endotoxin depletion from OVA on the BAL cell count were seen in the reduced number of macrophages and neutrophils. OVA/Ef-OVA, OVA-sensitized, endotoxin-free OVA-challenged animals. Data are expressed as means $\pm$ SE ($n = 5-6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 1-way ANOVA with the Fisher's least-significant-difference (LSD) test was used.

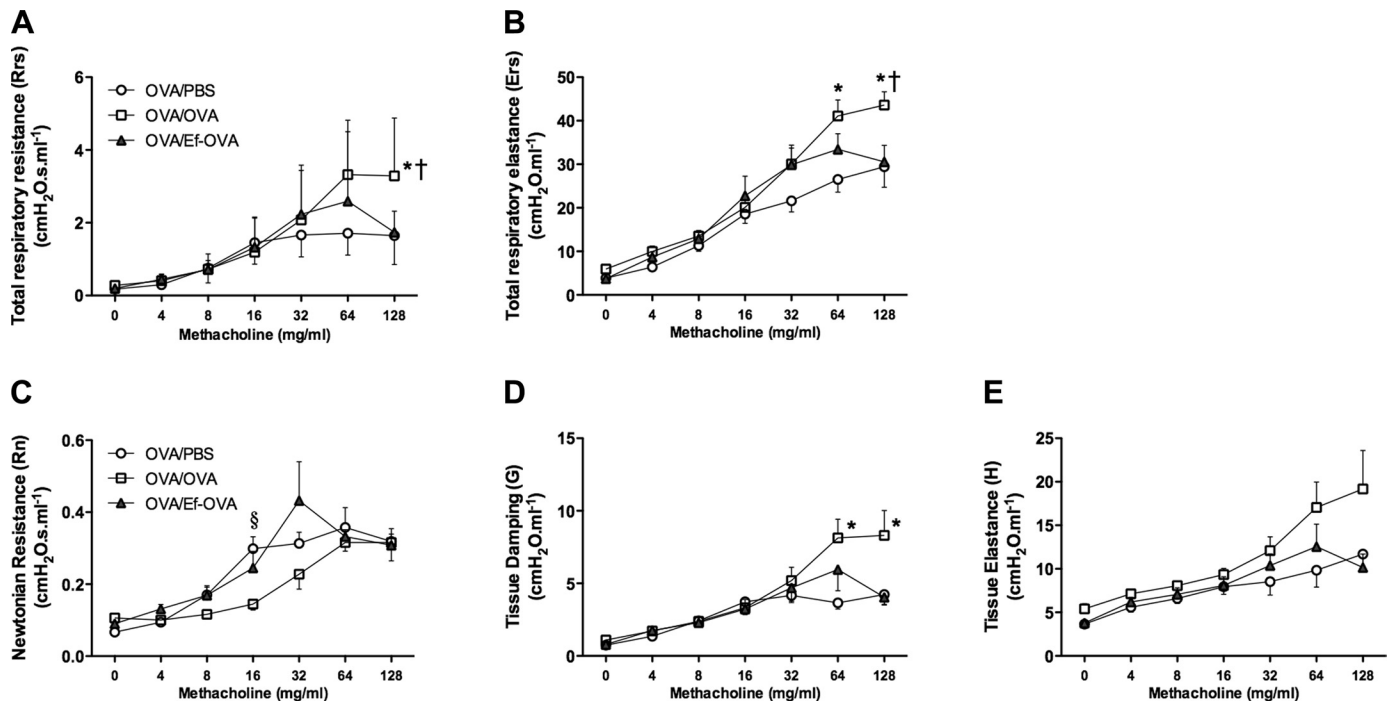


Fig. 2. The effects of endotoxin depletion on allergen-induced airway hyperresponsiveness (AHR). To examine the role of the endotoxin in OVA-induced AHR, animals were challenged with PBS, OVA, or Ef-OVA. AHR was examined 48 h after the last OVA challenge. *A* and *B*: measurement of airway responsiveness to methacholine (MCh) (0–128 mg/ml) using the single-compartment model. The OVA/OVA group showed the significant increases of respiratory system resistance (Rrs) and respiratory system elastance (Ers) compared with the OVA/PBS or OVA/Ef-OVA group. *C–E*: measurement of airway responsiveness to MCh (0–128 mg/ml) using the constant-phase model. The OVA/OVA group showed significantly higher values of *G* (reflecting resistance of the peripheral lung) than the OVA/PBS, whereas the OVA/Ef-OVA did not show any differences compared with the OVA/PBS group. In reflecting resistance of the large airways (*R_n*), the OVA/OVA showed the significant lower response compared with the OVA/PBS group only at 16 mg/ml of MCh concentration. **P* < 0.05; OVA/OVA vs. OVA/PBS, †*P* < 0.05; OVA/OVA vs. OVA/Ef-OVA, §*P* < 0.05; OVA/PBS vs. OVA/OVA. Data are expressed as means + SE (*n* = 7–8). 1-way ANOVA with the Fisher's LSD test was used.

sponding to tissue elastance showed a strong trend for greater responses in the OVA/OVA compared with the OVA/PBS group, but the differences did not reach statistical significance (*P* = 0.059 at 64 mg/ml, *P* = 0.11 at 128 mg/ml) (Fig. 2*E*).

ASM remodeling. We measured the α -SMA-positive area in rat airway tissues to determine ASM remodeling (Fig. 3, *A–C*). The α -SMA-positive area in larger airways ($P_{BM} \geq 1.0$ mm) was significantly greater in the OVA/OVA compared with the

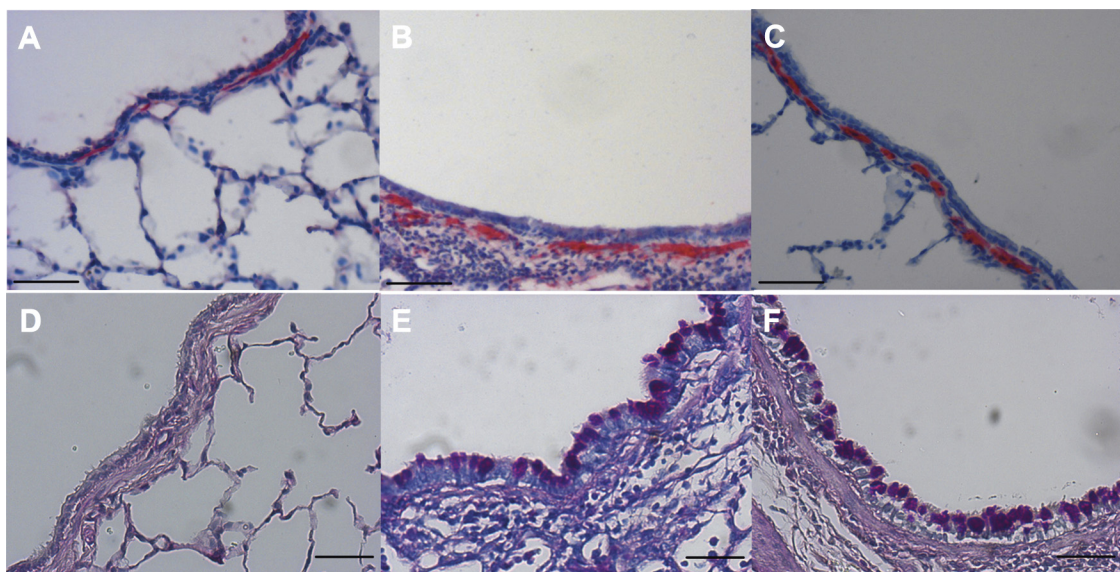


Fig. 3. Histological sections of airways from animals repeatedly challenged with PBS, OVA, or Ef-OVA. Rats were sensitized with OVA on day 0 and challenged with PBS (*A* and *D*), OVA (*B* and *E*), or Ef-OVA (*C* and *F*) on days 14, 19, and 24. Lung tissues were collected on day 26, and paraffin sections were obtained. Immunohistochemistry for α -smooth muscle actin (*A–C*) to detect the airway smooth muscle (ASM) mass increase and Periodic-Acid-Schiff (PAS) staining (*D–F*) to detect the goblet cell hyperplasia in rat airways. Scale bars = 50 μ m (*A–F*).

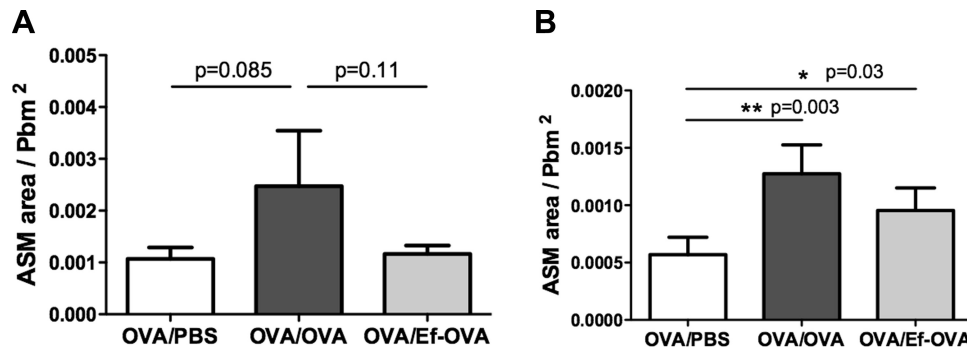


Fig. 4. The effects of endotoxin depletion on OVA-induced ASM mass increase. *A*: ASM mass in smaller airways ($P_{BM} < 1.0$ mm). There were no significant differences among 3 groups. *B*: ASM mass in larger airways ($P_{BM} \geq 1.0$ mm). The OVA/OVA showed the significant increase compared with the OVA/PBS group ($P = 0.003$), and the ASM area in the OVA/Ef-OVA was also significantly greater than the OVA/PBS ($P = 0.03$). There was no significant difference between the OVA/OVA and OVA/Ef-OVA. Data are expressed as means $\pm$ SE ($n = 5$). * $P < 0.05$, ** $P < 0.01$. 1-way ANOVA with the Fisher's LSD test was used.

OVA/PBS group (1.27 ± 0.25 vs. $0.57 \pm 0.15 \times 10^{-3}$, $P = 0.003$). In addition, the ASM area in larger airways in the OVA/Ef-OVA was significantly greater than the OVA/PBS (0.95 ± 0.20 vs. $0.57 \pm 0.15 \times 10^{-3}$, $P = 0.03$) (Fig. 4B). However, there was no significant difference in ASM mass in larger airways between the OVA/OVA and OVA/Ef-OVA (Fig. 4B). In smaller airways ($P_{BM} < 1.0$ mm), there were no significant differences in ASM area among three groups (OVA/PBS 1.07 ± 0.22 vs. OVA/OVA $2.47 \pm 1.07 \times 10^{-3}$, $P = 0.085$; OVA/OVA 2.47 ± 1.07 vs. OVA/Ef-OVA $1.16 \pm 0.16 \times 10^{-3}$, $P = 0.11$) (Fig. 4A).

Goblet cell hyperplasia. We counted the number of PAS-positive epithelial cells in airways for the three experimental groups to evaluate the effects of LPS contamination on goblet cell hyperplasia (Fig. 3, D–F). The number of PAS-positive epithelial cells was significantly higher in both the OVA/OVA and OVA/Ef-OVA compared with the OVA/PBS group ($P < 0.05$, $P < 0.05$, respectively). However, there was no significant difference in goblet cell hyperplasia between the OVA/OVA and OVA/Ef-OVA group (OVA/PBS 7.21 ± 4.34 ; OVA/OVA 19.9 ± 2.93 ; OVA/Ef-OVA 22.0 ± 4.55 /mm) (Fig. 5A).

mRNA expression of cytokines and mucin in the lung tissues. The mRNA expression of IFN- γ and IL-13 in lung tissues was significantly increased in the OVA/OVA compared with the OVA/PBS group (IFN- γ , 2.68 ± 0.60 vs. 0.86 ± 0.09 ; IL-13, 1.70 ± 0.48 vs. 0.48 ± 0.13 , $P < 0.05$, $P < 0.05$, respectively). The OVA/Ef-OVA also showed a significant increase in the mRNA of IFN- γ and IL-13 over that in the OVA/PBS group (IFN- γ , 2.72 ± 0.91 vs. 0.86 ± 0.09 ; IL-13, 1.29 ± 0.30 vs. 0.48 ± 0.13 , $P < 0.05$, $P < 0.05$, respectively). However, there were no significant differences in the mRNA expression of these two cytokines between the OVA/OVA and OVA/Ef-OVA group. The mRNA expression of IL-6, CINC-1, and IL-17 in lung tissues did not show any significant differences among the three groups (IL-17, OVA/PBS vs. OVA/OVA, $P = 0.178$, OVA/OVA vs. OVA/Ef-OVA, $P = 0.383$) (Fig. 6). Furthermore, to evaluate the mucin mRNA expression in these animals, we obtained lung tissues 18 h after the single OVA challenge and performed qPCR for the CLCA3 and MUC5AC. There was a significant difference in the CLCA3 mRNA expression between the OVA/PBS and OVA/OVA ($P < 0.05$), but no significant difference between OVA/OVA and OVA/Ef-OVA was observed (Fig. 5B).

Protein levels of MCP-1 and CINC-1 in BAL fluid. The levels of chemokines in BAL fluid were analyzed using a highly sensitive immunoassay. MCP-1, one of the chemokines for monocytes/macrophages, was increased after single OVA

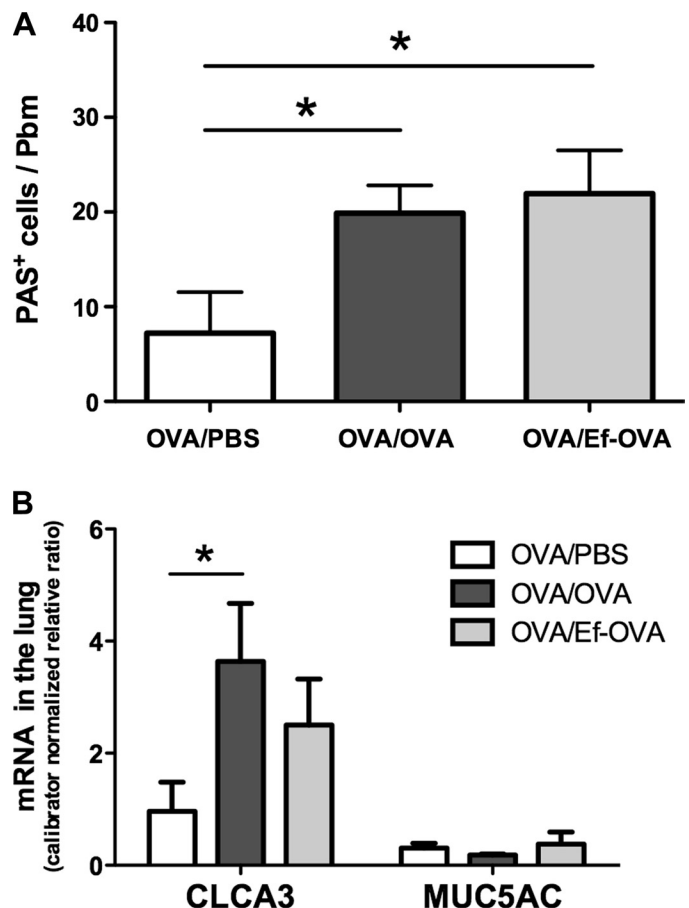


Fig. 5. The effects of endotoxin depletion on OVA-induced goblet cell hyperplasia and mucin mRNA expression. *A*: number of PAS-positive epithelial cells was significantly higher in both the OVA/OVA and OVA/Ef-OVA compared with the OVA/PBS group. There was no significant difference between the OVA/OVA and OVA/Ef-OVA. *B*: to evaluate mucin mRNA expression, lung tissues were obtained 18 h after a single OVA challenge. There was a significant difference in the CLCA3 mRNA expression between the OVA/PBS and OVA/OVA. Data are expressed as means $\pm$ SE (*A*: $n = 6-8$, *B*: $n = 5$). * $P < 0.05$. 1-way ANOVA with the Fisher's LSD test was used.

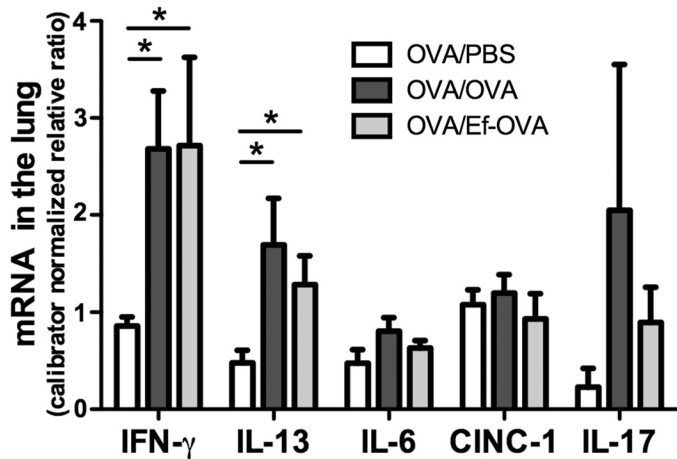


Fig. 6. The effects of endotoxin depletion on cytokine mRNA expression in rat lung tissues. To evaluate cytokine mRNA expression, lung tissues were obtained 48 h after the third OVA challenge. The mRNA expression of IFN- γ and IL-13 in lung tissues was significantly increased in both the OVA/OVA and the OVA/Ef-OVA compared with the OVA/PBS group. However, there were no significant differences between the OVA/OVA and OVA/Ef-OVA group. CINC, cytokine-induced neutrophil chemoattractant. Data are expressed as means $\pm$ SE ($n = 5$). * $P < 0.05$. 1-way ANOVA with the Fisher's LSD test was used.

challenge ($P < 0.05$), and this increase was significantly attenuated by LPS depletion ($P < 0.05$) (Fig. 7). There was no significant difference in CINC-1 among the three groups.

mRNA expression of EGFR pathway-related genes in the lung tissues. We measured mRNA expression of EGFR pathway-related genes in the lung tissues obtained 18 h after single challenge, as it has been reported that LPS stimulation induced EGFR activation through the release of TGF- α from the epithelium. There were no significant differences in mRNA expression of TGF- α , ADAM17, and TNF- α among three groups although there was a strong tendency for ADAM17 to decrease in the OVA/Ef-OVA compared with the OVA/OVA group ($P = 0.055$) (Fig. 8).

Cytokine mRNA expression in the intrathoracic lymph node tissues. The mRNA expression of IFN- γ and IL-4 in the intrathoracic lymph node tissues was significantly increased in the OVA/OVA compared with the OVA/PBS group (IFN- γ , 1.12 ± 0.11 vs. 0.72 ± 0.08 ; IL-4, 1.92 ± 0.48 vs. $1.07 \pm$

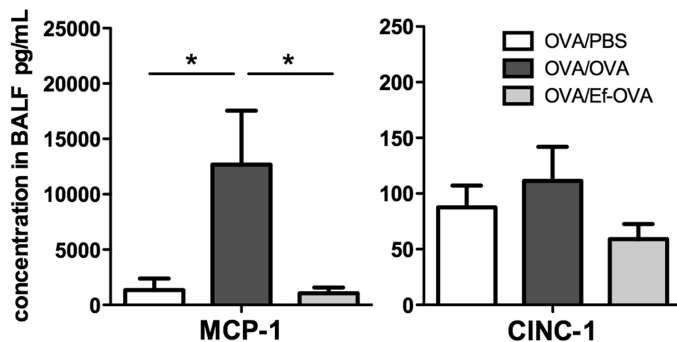


Fig. 7. Determination of monocyte chemoattractant protein (MCP)-1 and CINC-1 levels in BAL fluid (BALF). To measure chemokine levels in BALF, BALF was obtained 18 h after single OVA challenge. MCP-1 level in BALF was significantly increased by OVA challenge and reduced by endotoxin depletion. Data are expressed as means $\pm$ SE ($n = 5-6$). * $P < 0.05$. 1-way ANOVA with the Fisher's LSD test was used.

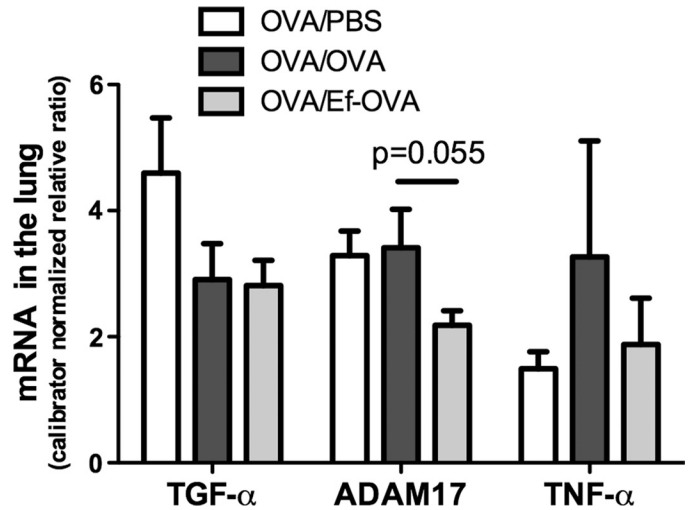


Fig. 8. mRNA expression of epidermal growth factor receptor pathway-related genes in rat lung tissues. Lung tissues were obtained 18 h after single OVA challenge. There were no significant differences in mRNA expression of TGF- α , ADAM17, and TNF- α among 3 groups. Data are expressed as means $\pm$ SE ($n = 5-6$). 1-way ANOVA with the Fisher's LSD test was used.

0.11, $P < 0.05$). The OVA/Ef-OVA group also showed a significant increase in the lymph node IL-4 mRNA expression compared with the OVA/PBS (1.89 ± 0.19 vs. 1.07 ± 0.11 , $P < 0.05$). There were no significant differences in IFN- γ and IL-4 mRNA expression between the OVA/OVA and the OVA/Ef-OVA group. IL-17 mRNA expression in lymph nodes tended to increase in the OVA/OVA compared with the OVA/PBS and decrease in the OVA/Ef-OVA compared with the OVA/OVA group but did not do so significantly ($P = 0.089$ and $P = 0.115$, respectively) (Fig. 9).

Western blot analysis of NF- κ B. To examine whether the effects of LPS on airway inflammation and remodeling are associated with NF- κ B activation, NF- κ B p65 protein expression was measured by Western blot analysis of rat lung tissues using both the cytosolic and nuclear protein extracts. There were no significant differences in the NF- κ B p65 protein expression in either cytosolic or nuclear fractions between the OVA/PBS and the OVA/OVA group. However, the expression of NF- κ B p65 in the cytosolic fractions in the OVA/Ef-OVA was significantly higher compared with the OVA/OVA group (0.38 ± 0.07 vs. 0.09 ± 0.03 , $P < 0.05$) (Fig. 10).

DISCUSSION

There has been substantial interest in the role of TLR4 in sensitization and airway responses to allergen. Both proinflammatory and anti-inflammatory effects of LPS in allergic disease have been described and appear to depend on the dose and timing of administration in relationship to the exposure to allergen. Despite the reported presence of LPS in commercial OVA, the effect this contamination has on allergic airway responses has been little studied, and whether it affects airway remodeling has not been addressed. In the present study we have demonstrated that the repeated challenge of sensitized rats to OVA depleted of LPS decreased the recruitment of macrophages and neutrophils to the airways and OVA-induced AHR to MCh. The depletion of LPS from OVA did not affect the OVA-induced influx of eosinophils and lymphocytes to air-

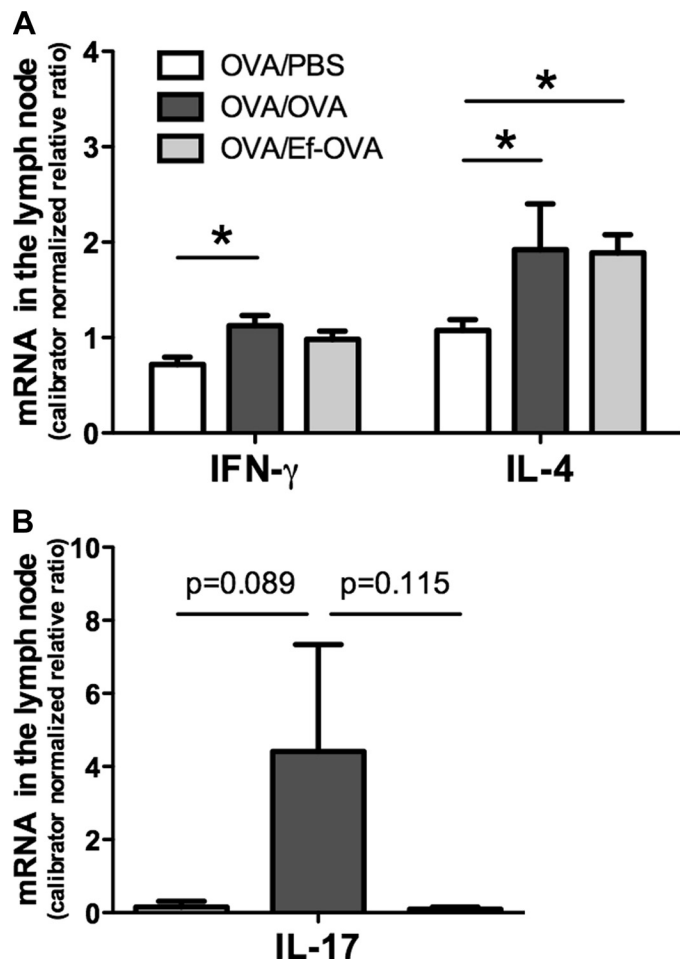


Fig. 9. The effects of endotoxin depletion on cytokine mRNA expression in rat intrathoracic lymph node tissues. Intrathoracic lymph node tissues were obtained 48 h after the third OVA challenge for IFN- γ and IL-4 (A) and were obtained 18 h after single OVA challenge for IL-17 (B). The mRNA expression of IFN- γ and IL-4 in the intrathoracic lymph node tissues was significantly increased in the OVA/OVA compared with the OVA/PBS group. The OVA/Ef-OVA also showed a significant increase in IL-4 compared with the OVA/PBS. There were no significant differences in IFN- γ , IL-4, and IL-17 mRNA expression between the OVA/OVA and the OVA/Ef-OVA group. Data are expressed as means $\pm$ SE ($n = 5$). * $P < 0.05$. 1-way ANOVA with the Fisher's LSD test was used.

ways, increase in ASM mass, goblet cell hyperplasia, and the increase of Th1 and Th2 cytokine expression in lymph nodes and lung tissues.

Consistent with previous findings in this rat model, multiple OVA challenges caused changes in BAL leukocytes, with increases in eosinophils, neutrophils, lymphocytes, and macrophages. Only the increases in eosinophils and lymphocytes were dependent on OVA challenge. The presence of LPS in the OVA appeared to account for the neutrophilia and increases in macrophages. The latter two cells appear to be of importance to the development of AHR (44), which was absent in the Ef-OVA-challenged animals. These data are consistent with previous data that eosinophils may not be essential for the development of AHR in models of asthma or in allergen-challenged human subjects (44, 17). Reduced airway caliber due to structural changes referred to as airway remodeling has been thought to be an important contributor to AHR; however, the

recruitment of inflammatory cells also presumably plays a pivotal role in AHR (5). IL-17-induced neutrophilia was reported to be important for the development of AHR in mice that were sensitized through the airway using OVA with low-dose LPS and challenged with OVA (42). In our study, there was a trend for IL-17 mRNA expression in lymph nodes to increase in the OVA/OVA compared with the OVA/Ef-OVA but not significantly. IL-17 mRNA also did not increase in the lung tissues and so does not seem to be critical for the greater AHR related to the presence of LPS in OVA in this rodent model of airway remodeling.

The depletion of pulmonary macrophages inhibits the prolongation of OVA-induced AHR in BALB/c mice (44), and OVA-induced AHR was reportedly inhibited by the neutral-

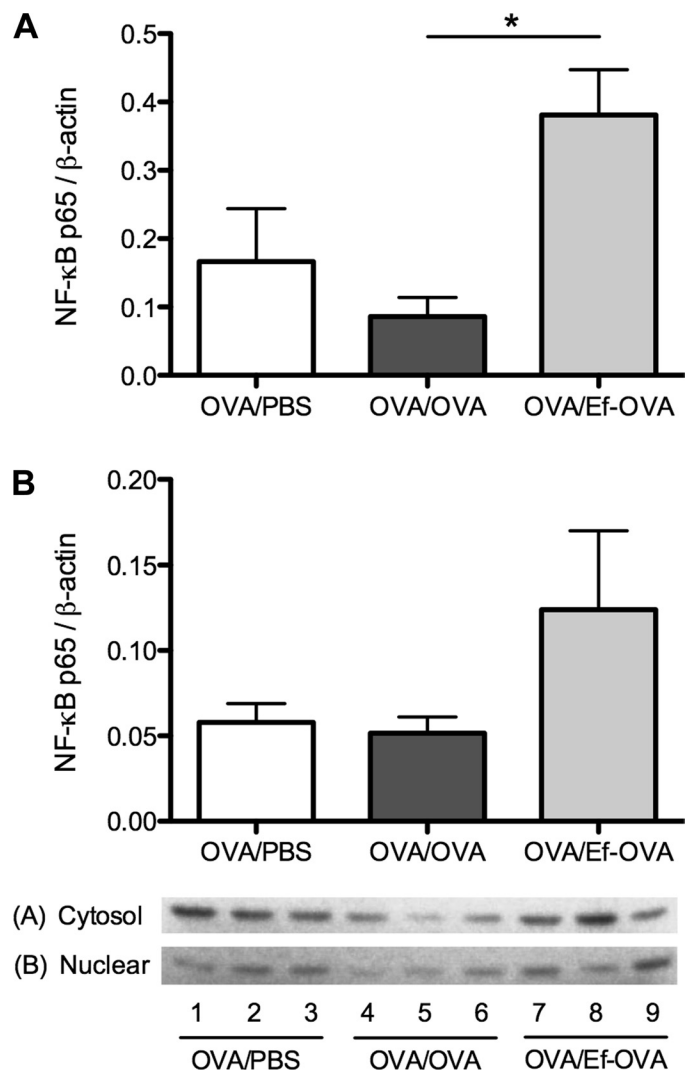


Fig. 10. The effects of endotoxin depletion on NF- κ B p65 protein expression in nuclear and cytosolic extracts from lung tissues. The right lung was removed 16 h after single OVA challenge, and protein was extracted from nuclear and cytosolic fractions. NF- κ B p65 protein expression was assessed by Western blot analysis. A: NF- κ B p65 protein expression in the rat lung cytosolic protein extracts. B: NF- κ B p65 protein expression in the rat lung nuclear protein extracts. There were no significant differences in the NF- κ B p65 protein expression in both cytosolic and nuclear fractions between the OVA/PBS and the OVA/OVA group; however, the NF- κ B p65 in the cytosolic fractions in the OVA/Ef-OVA showed significant increase compared with the OVA/OVA group. Data are expressed as means $\pm$ SE ($n = 3$). * $P < 0.05$. 1-way ANOVA with the Fisher's LSD test was used.

ization of MCP-1 in the mouse (11). MCP-1 (CCL2) is a member of the CC family of chemokines and plays a role in the recruitment and activation of monocytes/macrophages, lymphocytes and basophils, and, in certain states of inflammation, neutrophils (13). MCP-1 is produced by many cell types such as macrophages, lymphocytes, basophils, endothelial cells, fibroblasts, airway epithelial cells, and smooth muscle cells (19, 25) in response to various inflammatory stimuli including IL-1 β , IL-4, -6, -10, IFN- γ , TNF- α , TGF- β , and LPS (10). In our study, the number of macrophages and MCP-1 level in BAL and AHR were significantly decreased in the OVA/Ef-OVA compared with the OVA/OVA group, suggesting that LPS-induced MCP-1 secretion from airway epithelium and/or macrophages may have contributed to or induce AHR in this rat model of asthma. MCP-1 provokes a dose-dependent release of histamine and serotonin from rat peritoneal mast cells (6), and Campbell et al. (3) have demonstrated that intratracheal MCP-1 induces prolonged AHR and the release of histamine and leukotriene C4 in BAL in normal mice. In addition, anti-MCP-1 antibodies inhibit these changes through the pulmonary mast cells in a murine model of cockroach antigen-induced allergic airway inflammation (3). Therefore, the presence of LPS in the OVA may affect the observed AHR mainly through macrophage recruitment and MCP-1 production in the airways. Our data highlight the importance of concomitant stimuli, TLR4 activation, and allergen exposure in the development of AHR.

The mechanism of AHR following repeated allergen challenges is still not clear. It is generally held that increases in ASM account for AHR in asthma (8, 16, 34). However, these outcomes were not associated in the current study. Increase in ASM mass occurred in the larger airways of both OVA- and Ef-OVA-challenged animals. The assessment of respiratory mechanical parameters suggested a lack of association of AHR and remodeling; the remodeling was a large airway phenomenon, whereas the site of AHR was peripheral, as we have previously observed (31). Indeed there was a tendency for the responsiveness of the larger airways reflected in Rn to be reduced. This observation is consistent with previous findings of loss of contractile proteins such as total-myosin and myosin light chain kinase in the central airways in association with myocyte hyperplasia at 48 h after multiple OVA challenges in BN rats (15). ASM remodeling has been usually present in the smaller airways in this model system, but the method of challenge in the present study, namely intratracheal instillation under light anesthesia, is expected to have preferential effects on the larger airways given the likely central pattern of deposition of allergen.

LPS has the potential to induce airway remodeling in the absence of allergen exposure (2, 20). The release of TGF- α from the epithelium has been demonstrated to be mediated by TNF- α -converting enzyme (TACE/ADAM17) following activation of the TLR4 receptor by *Pseudomonas* LPS (29). In our study, however, the mRNA expression of TGF- α , ADAM17, and TNF- α in the lung tissues in the OVA/Ef-OVA were significantly lower than the OVA/OVA group unexpectedly. Furthermore, the nature of the LPS in OVA has been reported to have some features in common with *Salmonella* LPS (40); that may have some account for differences from *Pseudomonas* LPS. To explore the effects of LPS exposure on the respiratory system, a number of studies have used LPS from different

sources such as *Salmonella* (24, 40), *Escherichia coli* (*E. coli*) (7, 9), and *Pseudomonas* (26, 29, 43). Lipid A has been recognized as the active portion responsible for most LPS-induced biological effects. The activity of *Salmonella*-type lipid A is thought to be less than that of *E. coli*-type lipid A (23), and Tanamoto et al. (33) have reported that lipid A preparations from various *Salmonella* strains have very little stimulatory activity for human macrophages despite being potentially active in murine macrophages, suggesting that lipid A has the species-specific actions (33). Whether the activity of LPS in OVA is sufficient to stimulate TLR4 in the rat has not been addressed although the current experiments suggest that it can and does. It has been reported that chronic LPS inhalation triggered the expansion of the subepithelial matrix and AHR in C3H/HeBFeJ mice, and that is dependent on the presence of neutrophils (27).

Most previous studies that showed an effect of LPS exposure on AHR used the evaluation by unrestrained plethysmography (Penh) (27), which is not an accepted measure of airway function (1). The effects of LPS in OVA on AHR should be measured by the method that more faithfully tracks changes in respiratory mechanics, and the present study used a well-characterized small-animal ventilator and forced-oscillation technique (28). Using this system, we demonstrated an important role for LPS contamination of OVA in AHR. Alterations in the responsiveness of the peripheral airways likely accounted for the findings, given the significant response of the G parameter (tissue damping). OVA-induced AHR has been previously reported to affect the peripheral lung in both mice and rats (30, 35). The mechanism of the AHR is not clear. We examined the expression of IL-13 in lung tissue given the important role of this Th2 cytokine in allergen-driven murine models of asthma (41). However, although it was increased in expression at the mRNA level, no differences were detected between the two OVA-challenged groups. In this study, we used OVA depleted of LPS for the allergen challenge phase but not for the allergic sensitization phase, and thus LPS may have had effects on the parameters related to T cell priming or polarization. Th17 cells that produce IL-17, IL-22, and IL-6 have recently been associated with AHR through adoptive transfer of Th17 polarized cells to mice that were subsequently treated with OVA (18). However, we also failed to find any differences in expression of IL-17 or IL-6 between the two OVA-challenged groups. The strong Th2-biased immune response of the BN rat may account for these species differences (39).

LPS causes activation of the NF- κ B pathway in airway epithelium and has substantial effects on the pattern of inflammation generated by airway challenge with LPS (22). We anticipated differences in the activation of NF- κ B between the OVA- and Ef-OVA-challenged animals. We have previously demonstrated activation of NF- κ B at 24 h after a single OVA challenge of the sensitized BN rat (36). There was evidence of activation at 48 h after the third of three Ef-OVA challenges in the current study but not after OVA. These results suggest that the failure of NF- κ B activation is unlikely to be responsible for the lack of AHR in Ef-OVA-challenged rats. Indeed there is no evidence that in the murine model the failure to activate NF- κ B compromises the development of AHR (21).

We conclude that the contamination of commercial OVA by LPS has significant effects on the pattern of inflammation and AHR, perhaps through MCP-1 production in the bronchoal-

veolar compartment following OVA challenge. However, there are no significant effects on either ASM remodeling or goblet cell differentiation. The contrast between our findings and experiments indicating effects of exogenous LPS on goblet cell differentiation suggests that the nature of the LPS contaminating OVA may not be equivalent to LPS derived from either *E. coli* or *P. aeruginosa*. The basis for differences in airway responsiveness induced by OVA and Ef-OVA remains unexplained.

ACKNOWLEDGMENTS

We thank Jamilah Saeed for excellent technical assistance.

GRANTS

This work was supported by a grant from the Canadian Institutes of Health Research (J. G. Martin).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.T., S.S., P.-A.R., and N.H. performed experiments; K.T. and N.H. analyzed data; K.T. prepared figures; K.T. drafted manuscript; K.T. and J.G.M. edited and revised manuscript; J.G.M. conception and design of research; J.G.M. interpreted results of experiments; J.G.M. approved final version of manuscript.

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