

## Neutrophils play a critical role in development of LPS-induced airway disease

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**Savov, Jordan D., Stephen H. Gavett, David M. Brass, Daniel L. Costa, and David A. Schwartz.** Neutrophils play a critical role in development of LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 283: L952–L962, 2002; 10.1152/ajplung.00420.2001.—We investigated the role of neutrophils in the development of endotoxin-induced airway disease via systemic neutrophil depletion of C3H/HeBFeJ mice and coincident inhalation challenge with lipopolysaccharide (LPS) over a 4-wk period. Mice were made neutropenic with intraperitoneal injections of neutrophil antiserum before and throughout the exposure period. Experimental conditions included LPS-exposed, antiserum-treated; LPS-exposed, control serum-treated; air-exposed, antiserum-treated; and air-exposed, control serum-treated groups. Physiological, biological, and morphological assessments were performed after a 4-wk exposure and again after a 4-wk recovery period. After the 4-wk exposure, LPS-induced inflammation of the lower airways was significantly attenuated in the neutropenic mice, although airway responsiveness (AR) to methacholine (MCh) remained unchanged. After the recovery period, LPS-exposed neutrophil-replete mice had increased AR to MCh when compared with the LPS-exposed neutropenic animals. Morphometric data indicate that the 4-wk exposure to LPS leads to a substantial expansion of the subepithelial area of the medium-sized airways (90–129  $\mu\text{m}$  diameter) in nonneutropenic mice but not neutropenic mice, and this difference persisted even after the recovery period. Expression of bronchial epithelial and subepithelial transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) was diminished in the challenged neutropenic mice compared with the neutrophil-sufficient mice. These studies demonstrate that neutrophils play a critical role in the development of chronic LPS-induced airway disease.

lipopolysaccharide; polymorphonuclear leukocytes; endotoxin; subepithelial fibrosis; hyperreactivity; remodeling; transforming growth factor- $\beta$ 1

INFILTRATION OF AIRWAY MUCOSA with inflammatory cells is thought to be an important factor in the pathogenesis of a broad spectrum of airway disorders including acute and chronic bronchitis and asthma. Neutrophils [or polymorphonuclear leukocytes (PMNs)] contribute

to the immune response of the airway to infectious and noninfectious irritants. Although it is usually protective and beneficial, this inflammatory response also has the potential to cause tissue injury. Neutrophils that are recruited to the sites of inflammation (airway epithelial and subepithelial regions) can cause tissue damage via the production and release of oxygen radicals, proteases, and soluble mediators of inflammation (e.g., cytokines and chemokines).

Inhaled irritants such as cigarette smoke, ozone, and bacterial endotoxin can produce inflammation through nonallergic mechanisms. Endotoxin [or lipopolysaccharide (LPS)], which is a component of the cell wall of gram-negative bacteria, is ubiquitous in the environment and is often present in high concentrations in organic dusts (40) as well as air-pollution particulate matter (2). Several studies have demonstrated that inhalation of air that is contaminated with endotoxin is associated with the classic features of asthma including reversible airflow obstruction and inflammation and persistent airway hyperreactivity and remodeling (35). Epidemiological studies have shown that the concentration of inhaled endotoxin in the bioaerosol is strongly and consistently associated with reversible airflow obstruction among cotton workers (25), agricultural workers (44), and fiberglass workers (31). Our previous studies have shown that the concentration of endotoxin in the bioaerosol is the most important occupational exposure associated with the development (43) and progression of airway disease in agricultural workers (44). Experimentally, inhalation of endotoxin can cause reversible airflow obstruction and airway inflammation in previously unexposed healthy study subjects (39). In fact, healthy study subjects challenged with dust from animal-confinement buildings develop airflow obstruction and an increase in the serum concentration of neutrophils and interleukin-6 (IL-6), all of which are most strongly associated with the concentration of endotoxin (not dust) in the bioaerosol (50). In endotoxin-sensitive (C3H/HeBFeJ) but not endotoxin-

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resistant (C3H/HeJ) mice, subchronic inhalation of grain dust causes persistent airway hyperreactivity and remodeling, which suggests that endotoxin is one of the principal components of grain dust that causes the development of chronic airway disease (17).

Although thickening of the subepithelial region of the airway is a consistent histological feature of asthma, cystic fibrosis, and chronic obstructive lung disease and is directly related to the clinical severity of these diseases, the biological factors that lead to a localized fibrotic response following chronic airway inflammation have not been well defined. To elucidate whether PMNs are essential to the development of chronic LPS-induced airway disease, we used PMN antiserum to produce neutrophil-depleted mice and examined the LPS-induced changes in those animals compared to similarly exposed mice that were not neutropenic. We hypothesized that antiserum to PMNs would substantially minimize the acute inflammatory response to inhaled LPS and in doing so would substantially inhibit subepithelial thickening and alter the development of chronic LPS-induced airway disease.

## MATERIALS AND METHODS

To determine whether neutrophils are essential to the development of LPS-induced airway disease, we investigated the impact of systemic neutrophil depletion on chronic LPS-induced airway hyperresponsiveness, inflammation, and remodeling. Physiological, biological, and morphological measures were performed at three time points: before the inhalation challenge, immediately after completion of the 4-wk inhalation challenge, and 4 wk after completion of the 4-wk inhalation challenge. Airway responsiveness (AR) to methacholine (MCh) aerosol was determined, lung inflammation was assessed in whole lung lavage fluid, and airway remodeling was estimated by light microscopic morphometry, Masson-Trichrome staining, and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) immunohistochemistry.

**Animals.** Forty-four male, 8-wk-old, C3H/HeBFeJ mice (a strain sensitive to endotoxin) from Jackson Laboratories (Bar Harbor, ME) were used in this study. To determine the effects of neutrophil depletion on LPS-induced airway disease, we randomly assigned the 44 mice to two experimental groups ( $n = 22$  animals per group): one group received rabbit anti-mouse PMN antiserum, and the second group received normal rabbit serum [both sera were from Accurate Chemical and Scientific (Westbury, NY)]. In each treatment group, 16 animals were exposed to LPS and 6 were used as controls, which were exposed to filtered air. Mice in each group were evaluated before the exposure (baseline group;  $n = 22$ ), immediately after the 4-wk exposure (4-wk-LPS group;  $n = 11$ ), and 4 wk after the end of exposure (recovered group;  $n = 11$ ). To minimize the rate of infection especially in neutropenic animals, all mice in this experiment were housed in the pathogen-free portion of the vivarium. The study protocol was in accordance with guidelines set forth by the Duke University Animal Care and Use Committee.

**Generation of neutropenia.** To deplete circulating neutrophils, mice assigned to the antiserum group received rabbit anti-mouse neutrophil antiserum as described previously (1, 16). Each dose was 0.5 ml of diluted antiserum (1:10 dilution in sterile saline) administered by intraperitoneal injection. The dosage and regimen of antiserum administration were

chosen based on preliminary studies designed to determine optimal dosing of intraperitoneal serum to achieve functional prolonged neutropenia ( $>75\%$  depletion of peripheral PMNs). Initial injections were administered 72 h before the inhalation exposure. The second dose was administered in the morning before the exposure started. The neutrophil antiserum was then administered three times a week for 4 wk for the 4-wk-LPS group of mice. The recovered group received the antiserum for an additional week (an additional three doses) after the exposure was completed. Concurrently, the control group of mice followed the same schedule and received intraperitoneal injections of 0.5 ml of diluted normal rabbit serum.

**Endotoxin preparation and LPS aerosol exposures.** LPS (*Escherichia coli* serotype 0111:B4 from Sigma, St. Louis, MO) was reconstituted with 10 ml of sterile PBS, and stock aliquots (2.5 mg/ml) were stored at  $-20^{\circ}\text{C}$ . Stock aliquots were thawed once and stored at  $4^{\circ}\text{C}$  thereafter. Immediately before use, 0.7 ml of LPS stock [1.75 mg; 2,625,000 endotoxin units (EU)] were diluted in 100 ml of PBS for nebulization. Mice were placed in individual compartments of stainless steel wire-cage exposure racks in two 135-l chambers: one was for LPS exposure and the other was for filtered air. Animals were exposed for 4 h per day and 5 days per week over a 4-wk period. Endotoxin solution was aerosolized with a constant-output atomizer (model 3076, TSI, Minneapolis, MN), and all of the output was directed into the exposure chamber. Filtered, dehumidified air was supplied to the nebulizer at a gauge pressure of 30 psi and a flow rate of  $\sim 3.3$  l/min. The chamber was exhausted at a flow rate of 35.0 l/min.

**Endotoxin assay.** The airborne concentration of endotoxin was assessed by sampling  $0.3\text{--}0.4\text{ m}^3$  of air drawn from the exposure chamber through 25-mm binder-free glass-fiber filters held within a 25-mm polypropylene in-line air-sampling filter holder (Gelman Sciences, Ann Arbor, MI). Filters were placed in pyrogen-free petri dishes with 2 ml of sterile PBS that contained 0.05% Tween 20 (Sigma) and were then placed on a rotating shaker at room temperature for 1 h. Aliquots of the wash solution were serially diluted in pyrogen-free water and tested for endotoxin using a chromogenic *Limulus* amoebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) according to the manufacturer's instructions. The endotoxin concentration during exposures averaged  $9,477 \pm 704$  EU/ $\text{m}^3$  (means  $\pm$  SD) over the 4-wk period, which corresponds to  $6.32 \pm 0.47\text{ }\mu\text{g}/\text{m}^3$  according to the Sigma certificate of analysis.

**Blood collection and assessment of circulating neutrophils.** Peripheral blood was first collected from all 44 mice before treatment and exposure. Additional blood samples were collected after the first couple of intraperitoneal serum (antiserum or control serum) injections before the exposure. Neutrophil concentrations were assessed once every week throughout the treatment and exposure periods. Mice were anesthetized with Metofane methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL) inhalation, and  $\sim 0.2$  ml of blood was drawn from the periorbital sinus of each mouse. The number of nucleated cells per cubic millimeter of blood was counted with a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA). Differential counts of leukocytes were performed by counting 200 nucleated white blood cells from blood smears stained with HEMA-3 stain (Biochemical Sciences, Swedesboro, NJ).

**Airway reactivity assessment.** The AR to MCh challenge was estimated in unrestrained unanesthetized mice using whole body plethysmography. Individual mice were placed in 3-in-diameter chambers (Buxco Electronics, Troy, NY) that

were ventilated by bias airflow at a rate of  $0.5 \text{ l} \cdot \text{min}^{-1} \cdot \text{chamber}^{-1}$ . In each plethysmograph, a pressure signal is generated from the pressure difference between the main chamber that contains the unrestrained mouse and a reference chamber, which cancels atmospheric disturbances. Signals were analyzed to derive whole body respiratory parameters (SFT3812, BioSystem XA version 2.0.2.48, Buxco), including respiratory rate, tidal volume, inspiratory and expiratory times ( $T_I$  and  $T_E$ , respectively), peak inspiratory and expiratory flows (PIF and PEF, respectively), and relaxation time ( $T_R$ ). These parameters are used by the software to calculate enhanced pause ( $P_{\text{enh}}$ , unitless), which strongly correlates with lung resistance (18) and reflects changes in pulmonary resistance during bronchoconstriction using the following expression:  $P_{\text{enh}} = [(T_E - T_R) / T_R] \times (PEF / PIF)$ . Lung function was evaluated at baseline and after exposure to increasing doses of aerosolized MCh (0, 5, 10, and 20 mg/ml). MCh was aerosolized for 1 min and then dried for 2 min. Recording of breathing parameters began immediately after the end of MCh aerosolization and continued for 10 min. Average  $P_{\text{enh}}$  values were determined over the first 3 min (early phase) of response to the MCh aerosol. To analyze the reactivity of the animals to inhaled MCh, the concentration required to induce a twofold increase (up to 200%) in control (saline-induced)  $P_{\text{enh}}$  was estimated in each animal by graphical interpolation from the dose-response curve as reported by others (23). This parameter was designated as the effective dose ( $ED_{200}$ ) of MCh and was used as an index of AR. Mean  $\pm$  SE values of the  $ED_{200}$  were determined for each experimental group and were compared.

**Whole lung lavage.** Mice were euthanized by  $\text{CO}_2$  inhalation, and the trachea was exposed. The lungs were lavaged with 6.0 ml of sterile saline through a polyethylene (PE)-90 tube. The saline was infused 1 ml at a time at a pressure of 25 cmH<sub>2</sub>O. Return volume was recorded and was consistently  $>4.5$  ml. Processing of the lavage fluid has been described previously (42). Briefly, the lavage fluid was centrifuged for 5 min at 200  $g$ . The supernatant was decanted, and the cell pellet was resuspended with Hanks' balanced salt solution (without Ca or Mg) and washed twice. A small aliquot of resuspended cells was used for counting cells using a hemocytometer. The cell suspension ( $\sim 100 \mu\text{l}$ ) was spun onto a slide using a cytocentrifuge (Shandon, Southern Sewickley, PA) and was stained with HEMA-3 stain.

**Tissue preparation.** Lungs were perfused with saline through the pulmonary artery. Freshly prepared ice-cold 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in  $1 \times$  PBS (pH 7.4) was instilled through the tracheal cannula into the left lung at a constant pressure of 25 cm H<sub>2</sub>O and was fixed in 4% paraformaldehyde at  $4^\circ\text{C}$  overnight. Tissue was embedded in paraffin, and 5- $\mu\text{m}$  sections were cut and collected from the same depth in all of the tissue blocks. Sections were mounted onto positively charged slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), stained with hematoxylin and eosin or Masson-Trichrome (IMEB, San Marcos, CA), and examined with an Optiphot-2 microscope (Nikon, Tokyo, Japan).

**Morphometry: quantitative analysis.** Stereology was performed as previously described (17) based on the earlier principals developed by Cruz-Orive and Weibel (11) and Hogg et al. (21). Airway perimeters and wall areas were examined by capturing all conducting airway images with a SPOT Jr. digital camera (Diagnostic Instruments, Sterling, MI) and were analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) computer software. Measurements used in the study have been previously defined (17). Briefly, internal and external airway perimeters and basement mem-

brane perimeter were outlined and measured at  $\times 200$  magnification. The areas of airway epithelial and subepithelial tissue were calculated using these measurements. All airway profiles were divided into three groups based on airway diameter: small ( $\leq 90 \mu\text{m}$ ), medium ( $>90$ – $129 \mu\text{m}$ ), and large ( $>129 \mu\text{m}$ ). For every profile, the epithelial and subepithelial areas were normalized to the length of the adjacent basal membrane. The length-normalized area for every airway profile as well as the mean for each airway size was calculated for each animal. Means  $\pm$  SE for each experimental group were determined. To minimize the error that might arise from tangential sectioning, any airway profiles that showed a length-to-width ratio  $>2.5$  were not used for analysis. The presence or absence of lymphoid nodules was also taken into account and recorded for each bronchiolar profile. Three sections, which represented approximately the same depth of each tissue block, were stained and analyzed by morphometry. For each mouse, 10–12 airways were measured (mean, 10.7), and these were relatively equally distributed between the three airway sizes (3–5 images from each airway size contributed to the average data per animal). A total of 474 airway profiles was obtained and measured. The epithelial and subepithelial areas were traced and calculated over  $\sim 9$  mm of the basal lamina for each animal.

**Immunohistochemical analysis.** Immunohistochemical detection of TGF- $\beta 1$  was performed on paraffin sections using rabbit polyclonal affinity purified TGF- $\beta 1$ (V) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a standard avidin-biotin protocol (Vector Laboratories, Burlingame, CA). Two sets of slides per animal from all of the animals ( $2 \times 44$ ) were stained following the protocol with the same concentrations of all the solutions and equal incubation times for each slide. Negative-control sections were prepared with normal rabbit IgG and were stained simultaneously. Sections were stained with 3,3'-diaminobenzidine (DAB; Vector Laboratories) and hydrogen peroxide and then counterstained with hematoxylin. The patterns and intensities of TGF- $\beta 1$  immunohistochemical reactions were assessed semiquantitatively using a four-tier scoring system: 0, no reactivity; 1, weak reactivity (light-brown staining); 2, moderate reactivity (medium-brown staining); and 3, strong reactivity (dark-brown to black staining). All slides were scored in a blinded fashion. The TGF- $\beta 1$  signal was evaluated in both the bronchial epithelial region and the subepithelial area, and the score was given after assessment of the entire section. A mean value for the TGF- $\beta 1$  signals was determined for each animal (from both sets of slides), and a mean for each animal group was then calculated.

**Statistical analyses.** All data are expressed as means  $\pm$  SE. We primarily compared the physiological and biological (inflammatory and airway morphometry) responses in PMN-depleted and control mice after a 4-wk exposure and again after a 4-wk recovery. The differences between the two variables were analyzed using the Mann-Whitney  $U$ -test (14). To analyze the data as a function of the time point after the exposure (early vs. late) and the treatment with PMN antiserum, a two-way ANOVA was performed for our outcomes of interest, which included the plethysmography data (values for  $ED_{200}$  of MCh), bronchoalveolar lavage (BAL) fluid cell counts and differentials, and morphometry mean values. Probability values of  $P < 0.05$  (two-tailed) were considered statistically significant.

## RESULTS

**Circulating blood neutrophils.** The intraperitoneal injection of rabbit anti-mouse PMN antiserum signifi-

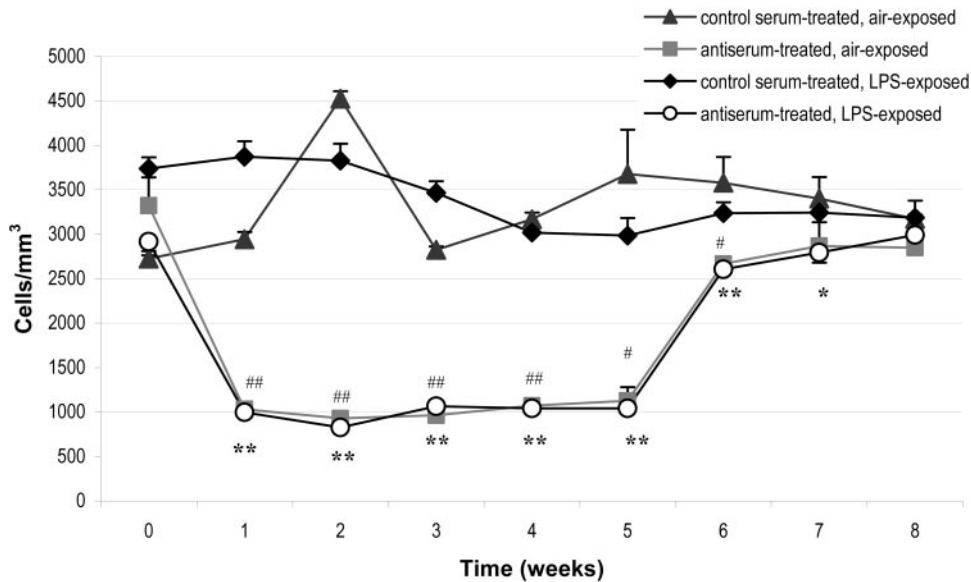


Fig. 1. Effect of antiserum treatment on the number of circulating blood neutrophils. Values are group means  $\pm$  SE. Time point 0 represents the baseline count from all 44 animals. Exposure was performed during the first 4 wk [ $n = 16$  mice/group in lipopolysaccharide (LPS)-exposed groups;  $n = 6$  mice/group in filtered air-exposed groups]. Data for the last 4 wk represent the recovered groups of mice ( $n = 8$  mice in LPS-exposed groups;  $n = 3$  mice in filtered air-exposed groups). \*\* $P < 0.005$ , \* $P < 0.05$ , significant differences within the LPS-exposed group, antiserum vs. control serum treated; ## $P < 0.005$ , # $P < 0.05$ , significant differences within filtered air-exposed group, antiserum vs. control serum treated.

cantly and consistently decreased the numbers of circulating neutrophils in the antiserum-treated group of mice compared with the control serum-treated animals (Fig. 1). Mice exposed to LPS and treated with neutrophil antiserum had neutrophil concentrations that were 21–34% (average, 27%) of those in control serum-treated mice. Similarly, in air-exposed mice, neutrophils from PMN antiserum-treated mice were at circulating levels of 24–36% (average, 31%) below the levels in air-exposed mice treated with control serum. The total neutrophil count decreased from  $3,366 \pm 105$  cells/mm<sup>3</sup> in the control serum-treated animals to  $1,009 \pm 27$  cells/mm<sup>3</sup> in the antiserum-treated group ( $P < 0.005$ ). Antiserum treatment also resulted in a younger population of circulating neutrophils; in control serum-treated mice, bands constituted 2–3% of all neutrophils, whereas in the antiserum-treated group, bands constituted 20–24% of all neutrophils. There was no significant difference in the number of circulat-

ing neutrophils between LPS- and air-exposed animals injected with the same type of serum (control serum or antiserum) at any time point. In the recovered group of animals, the number of circulating neutrophils returned to the baseline level 1 wk after antiserum treatment was discontinued. The number of circulating lymphocytes in the antiserum-treated mice was also reduced significantly ( $P < 0.005$ ) to 40% (e.g.,  $1,059 \pm 137$  cells/mm<sup>3</sup>) compared to the control serum-treated group of animals.

**Airway physiology.** To evaluate differences in the response to inhaled MCh and provide longitudinal comparisons, ED<sub>200</sub> of MCh was calculated for each animal and a mean was determined for every group of mice (Fig. 2). Before exposure, there were no significant differences in AR to aerosolized MCh between experimental groups. AR to MCh was assessed at two time points after completion of 4 wk of LPS inhalation, within 12 h after completion of the exposure, and at

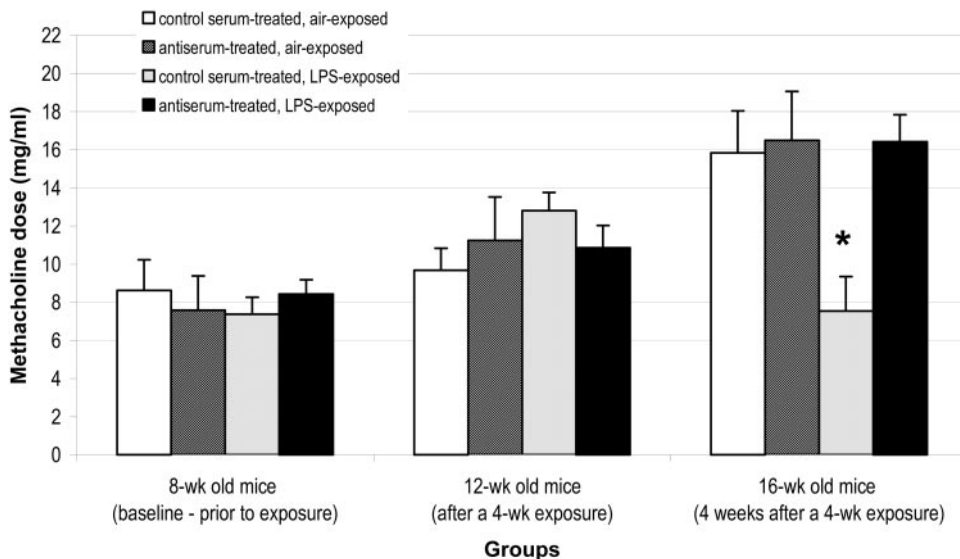


Fig. 2. Airway responsiveness to methacholine (MCh) dose measured with whole body plethysmography. Sensitivity to MCh is expressed as the dose required to provoke a 200% increase in baseline enhanced pause (ED<sub>200</sub> of MCh). Before exposure, data are from all 44 animals ( $n = 16$  in LPS-exposed groups;  $n = 6$  in filtered air-exposed groups); 4-wk exposure data are from the same number of animals 72 h after completion of exposure; 4-wk postexposure data are from 22 mice ( $n = 8$  in LPS-exposed groups;  $n = 3$  in filtered air-exposed groups). Air-exposed controls showed that within the age range of 8–16 wk, sensitivity to MCh decreases. \* $P < 0.005$ , significant increase in MCh sensitivity in LPS-exposed, control serum-treated group compared to LPS-exposed, antiserum treated group.

72 h after completion of the exposure. No consistent significant differences in airway reactivity to MCh between antiserum- and control serum-treated animals were determined at either the 12- or 72-h time points after completion of the inhalation challenge. Figure 2 shows the results at the 72-h time point. However, after the 4-wk recovery period, control serum-treated, LPS-exposed mice showed significantly increased ( $P < 0.005$ ) AR to MCh (as reflected by a decrease in ED<sub>200</sub> of MCh) compared with antiserum-treated, LPS-exposed mice (Fig. 2). The ANOVA comparisons show significant ( $P < 0.002$ ) time/age-dependent changes (8 vs. 12 vs. 16 wk of age) as a decrease of the estimates of AR to MCh within each exposure group. Further ANOVA confirmed the univariate analyses that the only significant difference ( $P < 0.001$ ) defined from the serum treatment was found for the LPS-exposed group after the recovery period.

**Inflammation in lower respiratory tract.** Three days after the 4-wk inhalation challenge with LPS, both control serum-treated and antiserum-treated mice demonstrated a pronounced pulmonary inflammatory response. LPS exposure induced a significant increase ( $P < 0.05$ ) in total BAL fluid cell number in both control serum-treated (>25-fold) and antiserum-treated (>5-fold) mice compared with corresponding air-exposed control animals (Fig. 3A). Changes in total BAL fluid cell numbers in the control serum-treated, LPS-exposed group were due predominantly to increases in the number of macrophages (representing 60% of total BAL fluid cells), neutrophils (15%), and lymphocytes (22%). In antiserum-treated, LPS-exposed animals, the neutrophil influx was relatively smaller (7% of total BAL fluid cells), whereas macrophages (75%) and lymphocytes (14%) were more abundant. At this same time point, the LPS-exposed, antiserum-treated mice showed a significant reduction ( $P < 0.005$ ) in the total number of BAL fluid cells and in the counts of macrophages, neutrophils, and lymphocytes per milliliter of BAL fluid compared with LPS-exposed animals that received control serum. Antiserum treatment had no effect on BAL fluid cell numbers in air-exposed mice. After the completion of the 4-wk recovery period, the significant difference ( $P < 0.05$ ) in total BAL fluid cell counts in LPS-exposed vs. air-exposed animals treated with the same two types of serum still persisted (Fig. 3B). However, the cellular pattern was different than the response observed acutely after exposure. Macrophages, airway epithelial cells, and lymphocytes now comprised up to 99% of the total BAL fluid cell count, and neutrophils contributed to <1% of the BAL fluid cell population for all treatment groups. No significant differences in BAL fluid cell count in antiserum-treated vs. control serum-treated animals within exposure groups were found at the end of the recovery period. The ANOVA results show that all univariate analyses were significant.

**Airway architecture: histopathology.** At the end of the 4-wk exposure period, control serum-treated and antiserum-treated mice exposed to air exhibited normal lung architecture (Fig. 4A). After LPS inhalation

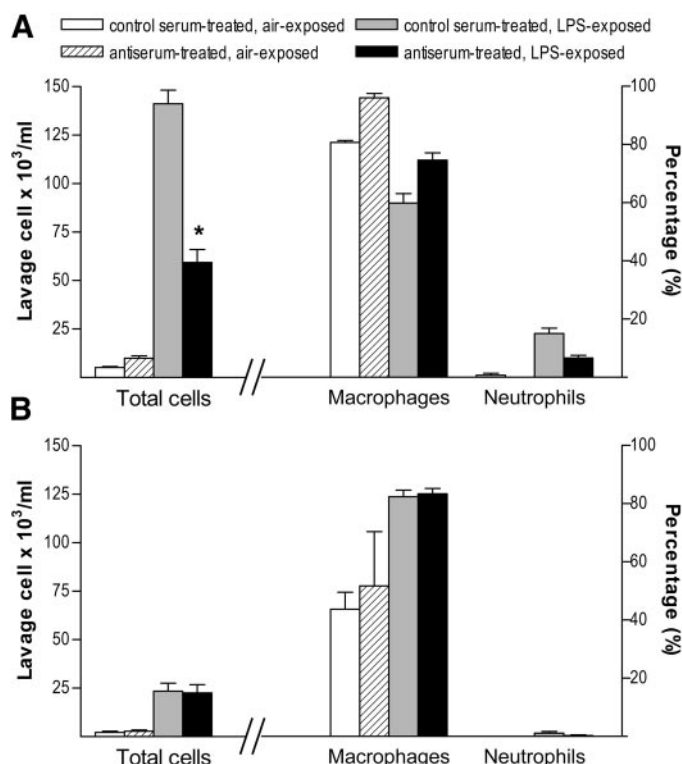


Fig. 3. Bronchoalveolar lavage (BAL) fluid cell counts (total cells per milliliter of lavage fluid, percent macrophages, and percent neutrophils). A: At 72 h after a 4-wk exposure, the increase in percentage of neutrophils and decrease in percentage of macrophages after LPS exposure are greater in control serum-treated than in antiserum-treated mice. \* $P < 0.005$ , significant difference in total cell count between antiserum-treated, LPS-exposed and control serum-treated, LPS-exposed mice. B: after the 4-wk recovery period, there was a significant difference only in the LPS-exposed vs. air-exposed animals. No significant difference in BAL fluid cell count in antiserum-treated vs. control serum-treated similarly exposed animals was recognizable.

challenge, lungs from the nonneutropenic mice showed an intense cellular infiltration (predominantly neutrophils) of the interstitium and bronchiolar walls (Fig. 4B). This effect was considerably attenuated in the neutropenic (antiserum-treated) animals (Fig. 4C). Our morphometric analysis demonstrated that the subepithelial area (calculated per length of basal membrane) of the medium-size airways in the LPS-exposed, control serum-treated group was significantly increased ( $P < 0.05$ ) compared with corresponding air-exposed control animals (Fig. 5A). Inflammatory cells and edema fluid clearly contributed to the subepithelial area expansion, but excluding inflammatory cells from area tracing was unavoidable. At the same time point, mice that were LPS exposed but antiserum treated had significantly reduced ( $P < 0.005$ ) expansion of the subepithelial area for the medium airways compared with the control serum-treated, LPS-exposed mice.

After the 4-wk recovery period, LPS-exposed neutrophil-sufficient animals showed moderate thickening of the lung interstitial areas and much less cellular infiltration compared with the samples prepared immedi-

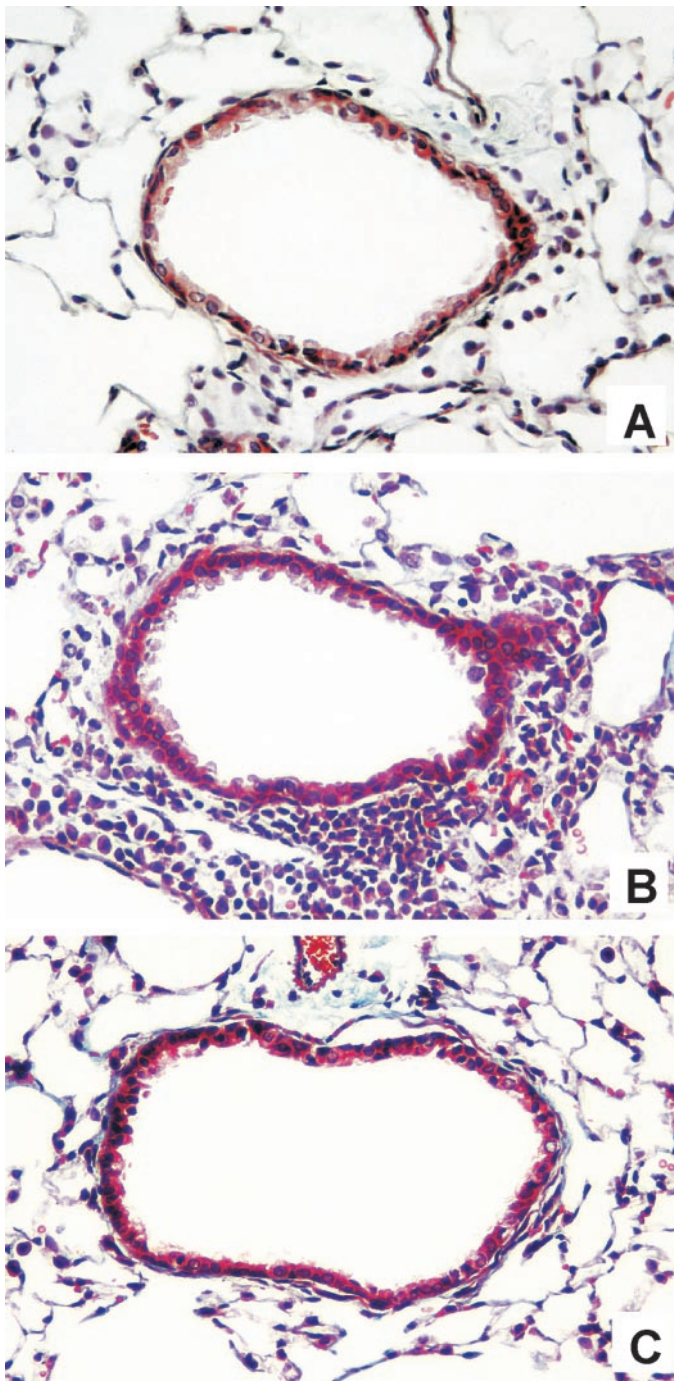


Fig. 4. Representative histological lung sections (with Masson-Trichrome staining) from 4-wk-exposure groups. A: control serum-treated mouse exposed to air. B: LPS-exposed, control serum-treated (nonneutropenic) mouse. C: LPS-exposed, antiserum-treated (neutropenic) mouse. Original magnification for all photomicrographs,  $\times 200$ .

ately after LPS challenge. Chronic inhalation of LPS resulted in expansion of the subepithelial region with the subepithelial area of the medium-size airways in LPS-exposed, control serum-treated mice showing significant increases ( $P < 0.05$ ) compared to the corresponding air-exposed animals (Fig. 5B). However, reduction in the concentration of neutrophils attenuated

this response; LPS-exposed, antiserum-treated animals showed a significantly reduced ( $P < 0.05$ ) length-normalized subepithelial area compared with the control serum-treated, LPS-exposed mice. Figure 6 illustrates the collagen deposition in the airway wall that was observed after the recovery period in animals receiving normal rabbit serum during the 4-wk LPS exposure compared with sections obtained from animals treated with antiserum during the inhalation challenge period.

ANOVA demonstrated no significant differences in small- and large-airway subepithelial areas within each exposure group. There was a significant change ( $P < 0.004$ ) in the normalized subepithelial area of the medium airways with the LPS exposure. This subepithelial change was also significantly altered by the antiserum treatment both immediately after exposure and 4 wk postexposure.

**Immunohistological analysis.** Table 1 summarizes the average scores for TGF- $\beta 1$  staining of epithelial and subepithelial compartments for all of the experimental groups. At 72 h after a 4-wk LPS exposure, antiserum-treated mice showed staining for TGF- $\beta 1$  with moderate intensity that was localized in the cytoplasm of the bronchiolar epithelial cells and absent or

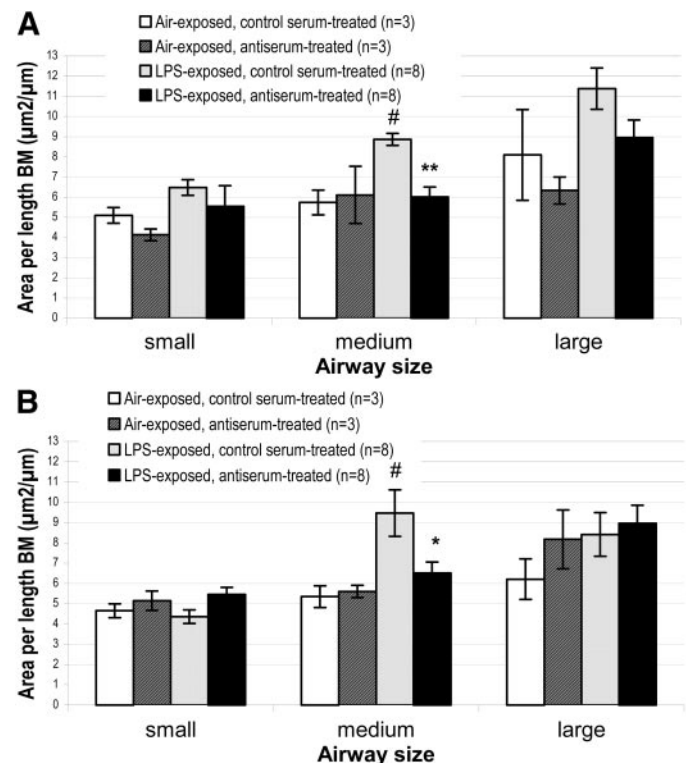


Fig. 5. Values obtained by morphometric analysis for three sizes of airways: small (diameter,  $\leq 90 \mu\text{m}$ ), medium (diameter,  $>90$ – $129 \mu\text{m}$ ), and large (diameter,  $>129 \mu\text{m}$ ). A: subepithelial area per length of basal membrane after completion of a 4-wk exposure. <sup>#</sup> $P < 0.05$ , compared with corresponding air-exposed controls; <sup>\*\*</sup> $P < 0.005$ , compared with corresponding nonneutropenic mice. B: normalized measurements for the subepithelial area from recovered group of animals; <sup>\*</sup> $P < 0.05$  vs. corresponding nonneutropenic mice; <sup>#</sup> $P < 0.05$  vs. corresponding air-exposed controls.

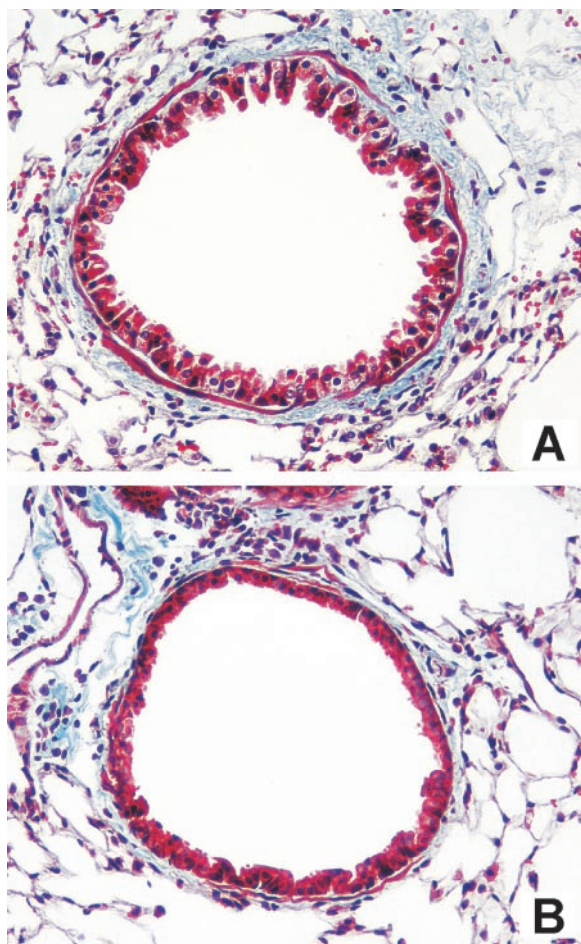


Fig. 6. Representative light micrographs of lung sections from recovered group used for morphometry. Masson-Trichrome staining demonstrates collagen (blue). A: LPS-exposed control serum-treated mouse and a medium-size airway profile that illustrates the collagen deposition in the airway wall. B: LPS-exposed antiserum-treated mouse and a medium-size airway profile that presents a relatively thinner collagen layer. Original magnification for both photomicrographs,  $\times 200$ .

very weak subepithelial staining (Fig. 7). At the same time point, the LPS-exposed, control serum-treated group demonstrated weak epithelial TGF- $\beta 1$  reactivity and strong staining of the subepithelial area with the latter significantly different ( $P < 0.005$ ) compared to

the LPS-exposed, neutrophil-depleted group (Fig. 8A). After 4 wk of recovery, a prominent TGF- $\beta 1$ -positive reaction in bronchiolar epithelium and an almost lack of reactivity in the subepithelium were observed in the sections from the lung of the LPS-exposed, control serum-treated mice. In contrast, LPS-exposed, antiserum-treated mice showed substantially less intense staining ( $P < 0.005$ ) of the bronchiolar epithelium compared to the neutrophil-replete, LPS-exposed animals (Figs. 7 and 8B). As expected, staining for TGF- $\beta 1$  in the epithelial cells of bronchioli of the air-exposed animals was considerably weaker than in the LPS-treated mice.

## DISCUSSION

The results of the present study indicate that the development of chronic LPS-induced airway disease is dependent on the presence of neutrophils. Mice that were depleted of circulating neutrophils had markedly less lung inflammation and demonstrated little evidence of LPS-induced airway hyperreactivity or airway remodeling (expansion of the subepithelial matrix). In contrast, neutrophil-replete mice developed airway inflammation, hyperreactivity, and remodeling after a prolonged exposure to LPS. These results provide support for the hypothesis that chronic airway disease in individuals exposed to dust contaminated with LPS may be largely mediated by PMNs and suggest a direct association between airway inflammation and remodeling.

Our study demonstrates that a 4-wk exposure to LPS by inhalation causes development of chronic airway disease in LPS-responsive mice. In the evolution of this condition, two phases could be recognized: the early or direct phase (during and immediately after the exposure), which was induced by the consistent and repetitive presence of the stimulus and during which infiltration and activation of inflammatory cells (mostly neutrophils) took place without accompanying enhancement in airway reactivity; and the late or indirect phase (4 wk after the exposure), which was characterized by fibrotic airway remodeling and hyperresponsiveness. Our results demonstrate that inhibition of neutrophil recruitment in the early phase mitigates the chronic inflammatory response to in-

Table 1. Semiquantitative evaluation of TGF- $\beta 1$  immunohistochemical staining

Air-Exposed Group	Staining Score		LPS-Exposed Group	Staining Score	
	Epithelium	Subepithelium		Epithelium	Subepithelium
Neutrophil sufficient, 72 h after a 4-wk exposure	0.83 $\pm$ 0.17	0.00 $\pm$ 0.00	Neutrophil sufficient 72 h after a 4-wk exposure	1.14 $\pm$ 0.17	2.64 $\pm$ 0.15*
Neutrophil sufficient, 4 wk after a 4-wk exposure	0.83 $\pm$ 0.17	0.00 $\pm$ 0.00	Neutrophil sufficient, 4 wk after a 4-wk exposure	2.59 $\pm$ 0.15*	0.05 $\pm$ 0.05
Neutrophil depleted, 72 h after a 4-wk exposure	0.75 $\pm$ 0.14	0.00 $\pm$ 0.00	Neutrophil depleted, 72 h after a 4-wk exposure	1.93 $\pm$ 0.17	0.36 $\pm$ 0.18
Neutrophil depleted, 4 wk after a 4-wk exposure	0.75 $\pm$ 0.14	0.00 $\pm$ 0.00	Neutrophil depleted, 4 wk after a 4-wk exposure	1.44 $\pm$ 0.13	0.00 $\pm$ 0.00

Values are means  $\pm$  SE;  $n = 8$  in each lipopolysaccharide (LPS)-exposed group;  $n = 3$  in each filtered air-exposed group; \* $P < 0.005$  compared with neutrophil-depleted, LPS-exposed mice. TGF- $\beta 1$ , transforming growth factor- $\beta 1$ .

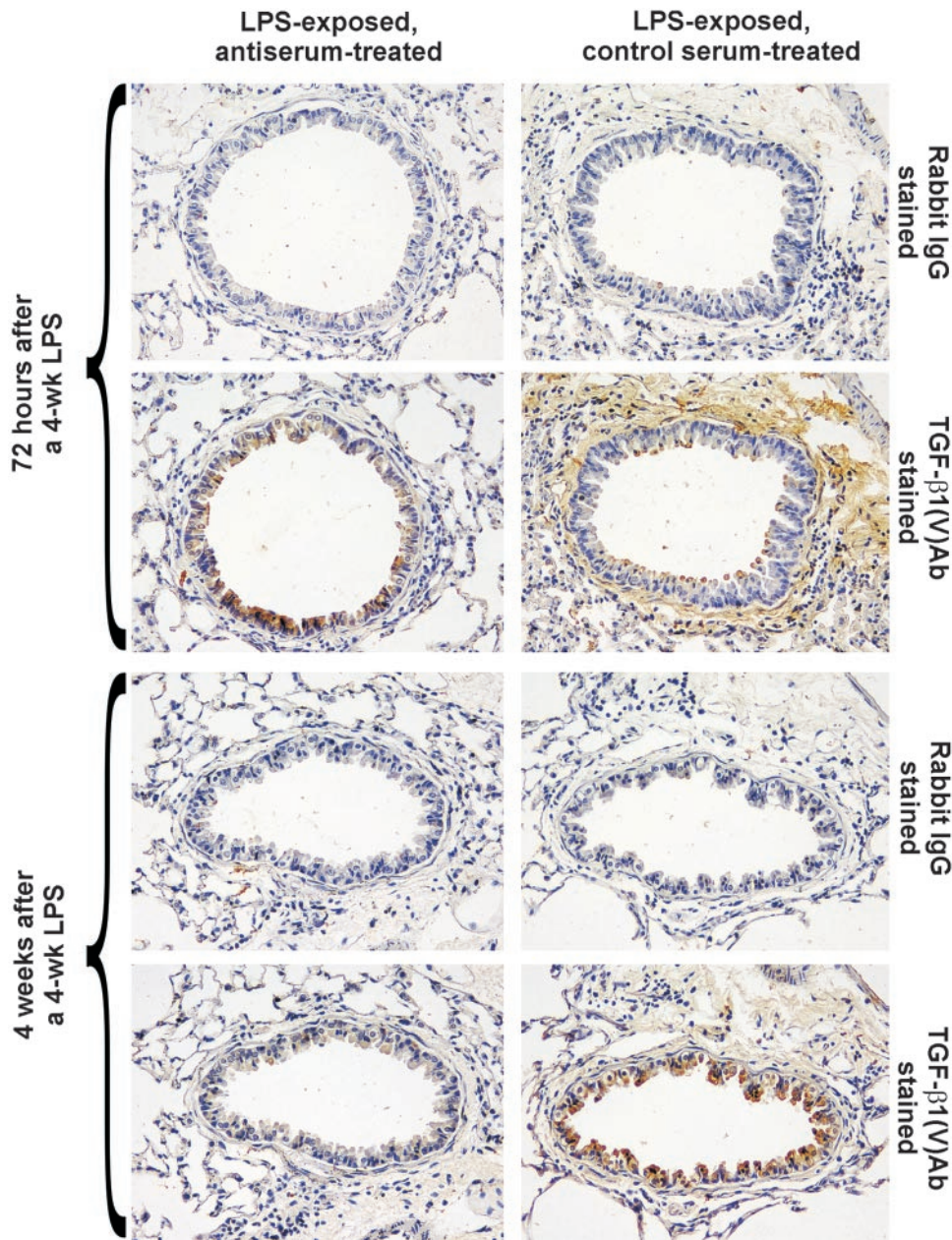


Fig. 7. Transforming growth factor (TGF)- $\beta$ 1 expression in LPS-exposed mice as detected by immunohistochemistry. Positive staining is visualized as a brown color (3,3'-diaminobenzidine reaction product). Rabbit IgG instead of TGF- $\beta$ 1(V) antibody was used as a negative control for TGF- $\beta$ 1 staining. At 72 h after a 4-wk LPS exposure, antiserum-treated mice showed expression of TGF- $\beta$ 1 in bronchial epithelium, whereas in the control serum-treated group the positive TGF- $\beta$ 1 staining was mainly located around the airway epithelium layer underneath the basement membrane. After a 4-wk recovery period, a much higher intensity of TGF- $\beta$ 1 staining was shown in the airway epithelium of control serum-treated animals compared with antiserum-treated animals. All photomicrographs have the same original magnification,  $\times 200$ .

haled endotoxin (LPS) and subsequently attenuates the late-phase airway hyperreactivity and remodeling in LPS-responsive mice. To our knowledge, the data presented here are the first to show a temporal and causative relationship between neutrophil recruitment in the lung provoked by repetitive stimuli and the later development of airway hyperreactivity and remodeling. However, because the PMN antiserum that we used was effective but not completely selective for neutrophils, our results do not rule out the possibility that in addition to neutrophils, lymphocytes might also be involved in the development of the chronic LPS-induced airway disease.

The mechanism of action of LPS has been studied extensively. LPS is not directly chemotactic for PMNs (10, 45); therefore, endogenous factors must be re-

leased to facilitate the extravasation of PMNs after inhalation of LPS. Toll-like receptor 4 (TLR-4) is an essential receptor for LPS signaling and is also the main protein involved in recognition of gram-negative bacteria (20). Additionally, CD14, a plasma membrane protein, assists monocyte/macrophage recognition of LPS binding protein (LBP) complexes and enhances the response of TLR-4 to endotoxin (7). Stimulation of TLR-4 results in activation of proinflammatory transcriptional factors and production and release of proinflammatory cytokines (46). In fact, in previous studies, we have demonstrated that TNF- $\alpha$ , IL-6, and macrophage inflammatory protein-2 (MIP-2) are released within hours of the exposure and may persist for up to 48 h after the inhalation challenge (12). We have also shown that recruited neutrophils are the primary cells

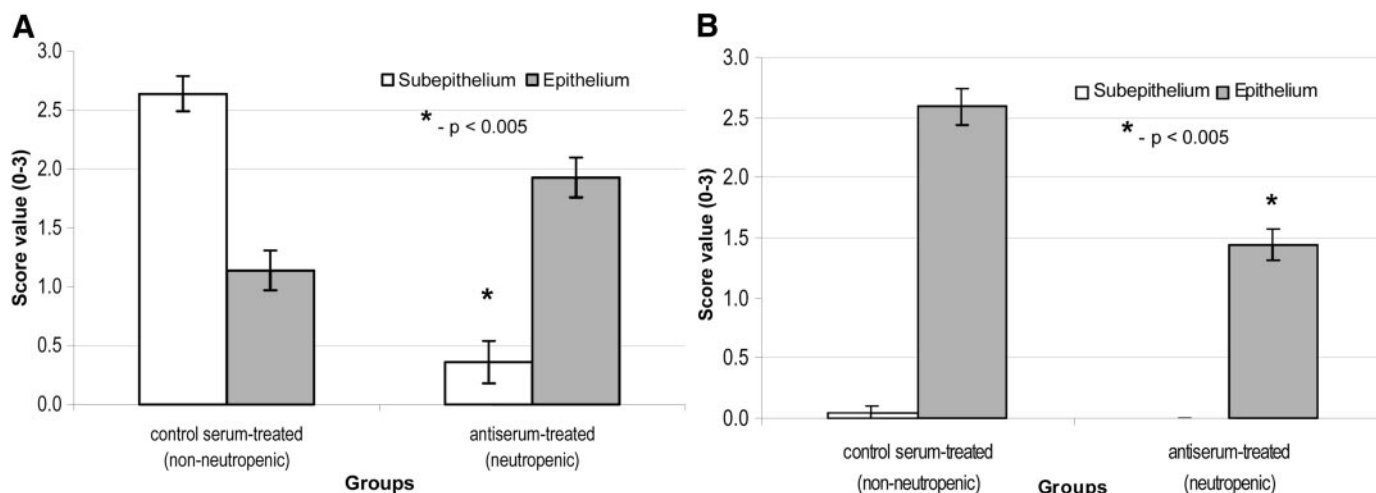


Fig. 8. TGF- $\beta$ 1 immunohistochemical reactivity by semiquantitative analysis. A: at 72 h after a 4-wk LPS exposure, there was a significant difference in the intensity of the signal in the subepithelial zone between neutrophil-depleted and neutrophil-sufficient mice. B: after a 4-wk recovery period, significant differences in the scores for the epithelium in neutrophil-replete vs. neutropenic LPS-exposed groups.

that produce these proinflammatory cytokines (48). The findings presented in this report suggest that prolonged neutrophilic inflammation of the airway wall may be an important initial step in the development of chronic LPS-induced airway disease. This step may initiate a chain of events that results in subsequent subepithelial extracellular matrix deposition.

The relationship between nonallergic airway inflammation and airway hyperreactivity is poorly understood. Acute airway exposure to LPS causes airway neutrophilia and induces airflow obstruction accompanied by short-lived airway hyperreactivity to aerosolized MCh (30, 41). Although neutrophils persist 24 h after the exposure, enhancement of airway hyperreactivity to MCh was not observed (30), which suggests that neutrophils were not sufficient to cause airway hyperreactivity. This is supported by experiments that have shown that inhaled LPS, although associated with neutrophil recruitment, results in bronchial hyporesponsiveness to histamine (15). These findings suggest that the presence of neutrophils in lung compartments (air spaces, interstitium, airway and alveolar walls, and vascular bed) is insufficient to mediate increased AR to bronchoconstrictors. However, although our results demonstrate that prolonged neutrophil infiltration is also not sufficient for the development of airway hyperreactivity to MCh, the presence of neutrophils is involved in airway remodeling that eventually leads to an enhanced response to MCh. Another relevant observation in our study is the gradual decrease of airway sensitivity to inhaled MCh in mice within 8–16 wk of age. This is clearly evident in the sentinel mice included in this experiment. We are not aware of a longitudinal study on age-related changes in airway reactivity in mice to validate these findings independent of our experimental system. However, in other rodent models such as the guinea pig, the effect of age on muscarinic agonist-induced contraction of airway smooth muscle (ASM) has been

demonstrated (47). In addition, in humans, it is well documented that during adolescence (6–16 yr of age) there is a natural decrease in airway reactivity to inhaled MCh (33, 34). This is true not only for normal (nonasthmatic) individuals but also for asthmatics. One of the possible mechanisms that might contribute to this phenomenon is the maturation (decrease in activity) of the Na<sup>+</sup>/K<sup>+</sup> pump in ASM cells that occurs throughout the pubertal period (37). Those age-related changes in the function and properties of ASM cells, including changes in responsiveness to bronchoconstrictors, are reported for different species. Alternatively, this could simply reflect a change in receptor density along the airway and/or the growth of the airways.

Airway-tissue remodeling in asthma, chronic obstructive lung disease, and cystic fibrosis involves a fibrotic response that alters the structure and function of the airway. Although thickening and fibrosis of the subepithelial region beneath the basement membrane is a consistent (4, 13, 24, 29, 36, 38) histological feature of these diseases with the magnitude of this response directly related to clinical severity (6, 32), the biological factors responsible for this localized fibrotic response have not been well defined. Immunohistochemical staining reveals that the subepithelial fibrosis predominantly consists of types I, III, and IV collagen, which probably originate from fibroblasts and myofibroblasts (5, 38) rather than epithelial cells (which produce branched collagens of types V and VII; Ref. 22). In this study, we have demonstrated that the medium-size airways of nonneutropenic mice exposed to a 4-wk LPS inhalation develop, in the early phase, expansion of the subepithelial tissue area. This expansion was not observed in the neutropenic, LPS-exposed mice. Changes persisted 1 mo after the end of the exposure and were characterized by enhanced subepithelial deposition of collagen.

TGF- $\beta$ s are pleiotropic factors that strongly influence processes such as somatic tissue development and repair, inflammatory diseases, tumorigenesis, wound healing, and fibrosis (3). TGF- $\beta$ 1 is member of a family of several similar molecules (including TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3) with genes that are highly conserved across species and thought to modulate the development of fibrosis as well as other biological processes (3). Recent studies have shown that TGF- $\beta$  secreted by airway epithelia and inflammatory cells may play a role in the development of subepithelial fibrosis. TGF- $\beta$ 1, which is abundant in the lung, can be demonstrated in BAL fluid and appears to play an important role in modulating inflammation and fibrosis in several forms of lung disease (27, 28). The cellular sources of TGF- $\beta$ 1 in the lung (especially at sites of injury) include inflammatory cells such as lymphocytes, macrophages (26, 49), eosinophils in the airway wall of asthmatics (32), epithelial cells (27), and platelets (19). Our data suggest that during the early phase (the end of the 4-wk LPS exposure) in the neutrophil-replete mice, cells in the airway subepithelium were the predominant source of TGF- $\beta$ 1. This expression of TGF- $\beta$ 1 in the subepithelial matrix was not observed in the neutropenic, LPS-exposed mice. However, after 4 wk of recovery, the bronchial epithelium of nonneutropenic mice showed strong expression of TGF- $\beta$ 1 whereas much less was present in the epithelium of neutropenic mice. TGF- $\beta$ 1 may be produced by a variety of cells that includes ASM cells, lymphocytes, tissue macrophages, and even neutrophils (8). Once released, TGF- $\beta$ 1 could function in an autocrine manner (to induce ASM cells to synthesize procollagen I) or in a paracrine fashion as a mitogen to immature fibroblasts (9). In aggregate, these findings suggest that expression of TGF- $\beta$ 1 in the mouse conducting airways increases after chronic LPS inhalation challenge and is dependant on neutrophilic infiltration and associated with subsequent collagen deposition. Diminishing PMNs in the bronchial wall with antiserum treatment reduces TGF- $\beta$ 1 expression and collagen formation. We hypothesize that the increased TGF- $\beta$ 1 activity induced by LPS exposure and mediated by neutrophils recruited into the tissue stimulates extracellular matrix production that contributes to the expansion of the bronchial subepithelial region and is likely to be critical to airway remodeling.

PMNs are phagocytic cells, and it is fully recognized that neutrophil infiltration of the airway as a part of the acute or chronic inflammatory response to microbial stimuli is beneficial, protective, and important in resolving injury to the lung. Once activated, PMNs generate free radicals and release lytic enzymes from cytosolic granules. This ability to become activated and release damaging intermediates is believed to mediate neutrophil-dependant host-tissue injuries. Our findings indicate that after chronic endotoxin exposure, neutrophils can contribute to airway remodeling and hyperreactivity. TGF- $\beta$ 1 may play an important role in this development. This suggests that control of neutrophil recruitment in the airway or the neutralization of

TGF- $\beta$ 1 may limit the extent of chronic LPS-induced airway disease.

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