

LPS binding protein is important in the airway response to inhaled endotoxin

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Background: Inhaled endotoxin is a risk factor for asthma exacerbation, and endotoxin inhalation by itself recapitulates many of the classical features of asthma in mice, including reversible airflow obstruction and inflammation, airways hyperresponsiveness, and airway remodeling.

Objective: Our objective was to determine the importance of LPS binding protein (LBP) in the response to inhaled LPS.

Methods: We challenged LBP-deficient mice (C57BL/6^{LBP-/-}) and C57BL/6 mice with inhaled endotoxin for 4 hours, 5 days, or 4 weeks, followed by 3 days of recovery.

Results: LBP in the lung was significantly increased in LPS-exposed C57BL/6 mice from all 3 groups. Only LPS-exposed C57BL/6 mice had significantly enhanced airway responsiveness to inhaled methacholine. Total lavage cells in LPS-exposed C57BL/6^{LBP-/-} mice were significantly reduced compared with those seen in LPS-exposed C57BL/6 mice; however, the percentage of PMNs was similarly increased in both the C57BL/6 and C57BL/6^{LBP-/-} mice. TNF- α , IL-1 β , and IL-6 protein concentrations in whole-lung lavage fluid from C57BL/6^{LBP-/-} mice were also significantly reduced when compared with those seen in C57BL/6 mice. In C57BL/6^{LBP-/-} mice submucosal cell proliferation was significantly reduced in the 1-week group when compared with that seen in similarly exposed C57BL/6 mice. The 4-week exposed C57BL/6 mice had significantly thickened airway submucosa and significantly increased lavaged TGF- β ₁ protein compared with that seen in C57BL/6^{LBP-/-} mice.

Conclusions: These findings indicate that LBP is one of the critical molecules regulating the acute and chronic airway response to inhaled LPS. (*J Allergy Clin Immunol* 2004;114:586-92.)

Key words: Endotoxin, LPS binding protein, neutrophil

Agricultural workers exposed to grain dust or other bioaerosols experience many of the classical symptoms of asthma: reversible airflow obstruction and airway inflammation, airway hyperreactivity, and accelerated decrease in airflow.^{1,2} Mice exposed to grain dust for a

Abbreviations used

BrdU: Bromodeoxyuridine

EU: Endotoxin unit

LBP: LPS binding protein

period of 2 months also have these same signs, along with persistent airways hyperreactivity and airway remodeling.³ The acute and chronic response to these organic dusts is largely caused by the concentration of endotoxin in the bioaerosol.⁴

The same symptoms caused by subchronic grain-dust inhalation can be recapitulated with endotoxin (LPS) alone.⁵ Although Toll-like receptor 4 is clearly critical to this airway response, the role of other components of the LPS binding complex, such as CD14, LPS binding protein (LBP), and MD-2, have not been investigated. Systemically and *in vitro*, the response to LPS is enhanced by LBP, a 60-kd serum glycoprotein that binds the lipid A portion of LPS molecules to form a high-affinity LBP/LPS complex that potentiates the cellular response to LPS by virtue of its ability to transfer LPS to CD14.⁶

In normal lung LBP is present at low levels.⁷ During segmental allergen challenge in human subjects, local concentrations of LBP can increase significantly within 24 hours.⁸ Furthermore, the concentration of LBP in plasma and bronchoalveolar lavage fluid in patients with acute lung injury can increase 10-fold.⁹ Recently, it has been demonstrated that LBP might be produced by human type II pneumocytes and murine type II-like epithelial cells *in vitro*, suggesting a mechanism for rapid local defense against environmental insult by gram-negative bacteria.¹⁰ However, the role of LBP in the pulmonary innate immune response to inhaled LPS has not been investigated.

Understanding the mode of action of environmental factors, such as endotoxin, in the lung will help us to understand the nature and mechanisms of LPS-induced airway disease. This in turn might help us to understand and manage human asthma by identifying novel sites for pharmacologic intervention. Therefore to test the hypothesis that LBP is critical to the airway response to inhaled LPS, we have compared the response of mice deficient in LBP (C57BL/6^{LBP-/-}) with that of wild-type C57BL/6 mice to subchronic endotoxin exposure. We demonstrate here that LBP substantially influences the acute and chronic physiologic and biologic response to inhaled LPS.

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Supported by grants from the National Institutes of Health (ES07498, ES11375, ES012496, ES011961, HL66611, and HL66604) and the Department of Veterans' Affairs (Merit Review).

Received for publication February 6, 2004; revised April 7, 2004; accepted for publication April 23, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.04.043

METHODS

Experimental animals

Thirty-eight male C57BL/6 mice and 38 male C57BL/6^{LBP-/-} mice (Jackson Laboratory, Bar Harbor, Me) at 8 weeks of age were used. Six C57BL/6 mice and six C57BL/6^{LBP-/-} mice were killed at each time point. Eight C57BL/6 mice and eight C57BL/6^{LBP-/-} mice were killed at 12 weeks of age for use as age-matched control animals. Whole-body plethysmography (described below) was performed on all mice in the 1-day and recovered groups before the beginning of the LPS exposure and again at the indicated time points.

Endotoxin preparation and aerosol exposures

Endotoxin (LPS) was purchased as purified lyophilized powder (25 mg; 30,000,000 endotoxin units [EU]/mg; lot 110K4060) prepared by means of phenol extraction from *Escherichia coli* serotype 0111:B4 from Sigma (St Louis, Mo). LPS was reconstituted with 10 mL of sterile HBSS, and stock aliquots (2.5 mg/mL) were stored at -20°C. Immediately before use, 160 µL of LPS stock (4.7 mg; 7,000,000 EU) was diluted in 75 mL of HBSS for nebulization.

Mice were placed in stainless-steel wire cage exposure racks in 20-L chambers. Animals were exposed for 4 hours. Endotoxin solution was aerosolized with a Collision 6-Jet Nebulizer (model CN-25, BGI, Inc, Waltham, Mass), with all output directed to the exposure chamber. Filtered and dehumidified air was supplied to the nebulizer at 20-psi gauge pressure. The exposure chamber was vented at a flow rate of 28.0 L/min.

Endotoxin assay

The airborne concentration of endotoxin was assessed by sampling 0.30 to 0.40 m³ of air drawn from the exposure chamber through 25-mm, binder-free, glass-fiber filters (Gelman Sciences, Ann Arbor, Mich) held within a 25-mm polypropylene inline air-sampling filter holder (Gelman). Filters were placed in pyrogen-free Petri dishes with 2 mL of sterile PBS containing 0.05% Tween-20 (Sigma) and then placed on a rotating shaker at room temperature for 1 hour. Aliquots of the wash solution were serially diluted in pyrogen-free water and tested for endotoxin by using a chromogenic Limulus amoebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, Md), according to the manufacturer's instructions. The endotoxin concentration averaged 7806 ± 1077 EU/m³ (mean ± SE), which corresponds to 5.25 ± 0.72 µg/m³ according to the Sigma certificate of analysis.

Estimate of airway function

Airway responsiveness to methacholine challenge was estimated in unrestrained nonanesthetized mice by using whole-body plethysmography. Individual mice were placed in 3-inch-diameter chambers (Buxco Electronics, Troy, NY), which were ventilated by means of bias airflow at 1.0 L/min per chamber. In each plethysmograph a pressure signal is generated from the pressure difference of the main chamber containing the unrestrained mouse and a reference chamber, which cancels atmospheric disturbances. Signals were analyzed to derive whole-body flow parameters (SFT3812, BioSystem XA version 2.0.2.48, Buxco Electronics), including respiratory rate, tidal volume, inspiratory time and expiratory time (Te), peak inspiratory flow (PIF) and peak expiratory flow (PEF), and relaxation time. These parameters are used by the program software to calculate enhanced pause, which correlates with lung resistance and reflects changes in pulmonary resistance during bronchoconstriction, by using the following expression: $Penh = ([Te - RT]/RT) \times (PEF/PIF)$.

Lung function was estimated at baseline and after exposure to aerosolized methacholine (0, 5, 10, and 20 mg/mL). Methacholine was aerosolized for 1 minute and then dried for 3 minutes. Recording of breathing parameters began immediately after the end of

methacholine aerosolization and continued for 10 minutes. Average enhanced pause values were determined over the first 3 minutes (early phase) of response to the methacholine aerosol.

Whole-lung lavage

Mice were killed by means of CO₂ inhalation, the chest was opened, the trachea was exposed, and lungs were lavaged through PE-90 tubing with 6.0 mL of sterile saline, 1 mL at a time, at a pressure of 20 cm H₂O. Return volume was recorded and was consistently greater than 4.5 mL. Processing of the lavage fluid has been described previously.¹¹ Briefly, the lavage fluid was centrifuged for 5 minutes at 200g. The supernatant was decanted and stored at -70°C for further use. The cell pellet was resuspended with HBSS (without Ca or Mg), and a small aliquot of this resuspension was used to count total lavage cells per animal with a hemocytometer. One hundred microliters of the cell suspension was spun onto a slide by using a cytocentrifuge (Shandon, Southern Sewickley, Pa). Cells were stained with HEMA-3 (Biochemical Sciences, Inc, Swedesboro, NJ) stain for differential, air-dried, and covered with a cover slip with Cytoseal (Stephens Scientific, Kalamazoo, Mich).

Tissue preparation

After the lavage was completed, the lungs were perfused with saline through the pulmonary artery, and the whole right lung was removed and snap-frozen in liquid nitrogen and stored at -70°C for later use. Freshly prepared ice-cold 4% paraformaldehyde (Fisher Scientific, Pittsburgh, Pa) in 1× PBS (pH 7.4) was instilled through the tracheal cannula into the right lung at a constant pressure of 20 cm H₂O. The trachea was clamped, and the lung was fixed overnight at 4°C in 4% paraformaldehyde. This tissue was embedded in paraffin; 5- to 6-µm thick sections were cut and placed on positively charged slides (Super Frost Plus; Fisher Scientific). Sections were stained with hematoxylin and eosin for semiquantitative histopathology.

Morphometry (quantitative analysis)

Morphometry was performed, applying standard methods previously described.³ Briefly, the area of the submucosa was calculated by using measurements of the perimeter of the airway submucosa and the basement membrane perimeter. Airways profiles were divided into 3 relatively equal groups determined on the basis of airway short diameter: small airways, 0 to 90 µm; medium airways, greater than 90 to 129 µm; and large airways, greater than 129 µm. For every airway measured, submucosal area was normalized to the length of the adjacent basement membrane. To minimize the error that might arise from tangential sectioning, any airway profiles showing a length/width ratio of greater than 2.5 were not used for analysis. The mean value of the submucosal area standardized to the length of the basement membrane was calculated for each airway size for each study animal. These values were used to calculate means ± SEM for each airway size for each study group.

Immunohistochemistry

Bromodeoxyuridine (BrdU) immunohistochemistry was performed as described previously,¹² and a rat monoclonal anti-BrdU antibody at a dilution of 1:100 was applied (Accurate Chemical and Scientific, Westbury, NY).

Quantitative analysis of cell proliferation

One BrdU-stained histologic section from each animal was prepared as described above. Digital images were acquired and categorized with respect to size, as described above, of all airways in each BrdU-stained histologic section from each animal. All airway submucosal cells in each airway image were counted. Separate counts of BrdU-positive submucosal cells were also kept. The mean percentage of positive cells per airway was calculated for each

airway size for each study animal. These values were used to calculate means $\pm$ SEM for each airway size for each study group.

Cytokine analysis

ELISA kits for TNF- α , IL-1 β , and IL-6 were purchased from R&D Systems (Minneapolis, Minn). The ELISA kit for TGF- β was purchased from Promega (Madison, Wis). The ELISA kit for LBP was purchased from Cell Sciences (Norwood, Mass). Kits were used according to the manufacturer's instructions. Standard curves were run with each ELISA. The lower limit of detection for each protein was as follows: 5.1 pg/mL for TNF- α , 3.0 pg/mL for IL-1 β , 10 pg/mL for IL-6, 15.6 pg/mL for TGF- β , and 0.4 μ g/mL for LBP.

Quantitative PCR

Total RNA was isolated from snap-frozen lung tissue by using Trizol (Gibco BRL, Carlsbad, Calif), according to the manufacturer's instructions. Fifty nanograms of total RNA was reversed transcribed into cDNA in a volume of 25 μ L, containing 12.5 μ L 2 $\times$ SYBR Green buffer (Applied Biosystems, Foster City, Calif), 12.5 U of reverse transcriptase, 20 U of RNase inhibitor, and 0.5 μ mol/L LBP-specific primers (forward, CCACAACCAGATCGAGTCGAA; reverse, ACTGTAGTCAATGCCAGGACATT). TFIID primers (forward, ACGGACAACCTGCGTTGATTTT; reverse, ACTTAGCTGGGAAGCCCAAC) were used as an internal control. Each primer set spanned 2 intron-exon boundaries and had melting temperatures of between 58°C and 60°C. The melting temperature of the corresponding primer set did not vary more than 1°C. The reverse transcription reaction was carried out in 1 cycle at 50°C for 30 minutes and 95°C at 10 minutes. The PCR reaction was carried out by using a 2-step amplification cycle (40 cycles): denaturing at 95°C for 15 seconds and annealing-extending at 60°C to 62°C for 2 minutes. A dissociation curve was carried out in 3 steps for 1 cycle as follows: 95°C for 15 seconds, 58°C for 20 seconds, and 95°C for 15 seconds. Each sample was run in triplicate with each primer set, and LBP was normalized to the TFIID internal control.

Statistical analyses

The primary objective of our comparisons was to evaluate the physiologic and biologic differences (airway reactivity, inflammation, and cytokine levels) between C57BL/6^{LBP-/-} and background strain control mice. Total and differential cell counts from bronchoalveolar lavage, sensitivity to inhaled methacholine challenge, and cytokine levels were compared between C57BL/6 and C57BL/6^{LBP-/-} mice. LBP protein and mRNA were compared between exposed and baseline groups of C57BL/6 mice. The differences between variables in each comparison were analyzed by means of ANOVA or the Mann-Whitney *U* test. *P* values of less than .05 (2-tailed) were considered statistically significant. All data are expressed as means $\pm$ SEM.

RESULTS

LBP is induced after LPS inhalation

LPS-exposed C57BL/6 mice in all groups had significantly more LBP in whole-lung lavage fluid compared with pre-exposure values (Fig 1, A). We attempted to determine whether there was a concomitant increase in LBP mRNA expression in the whole lung by using quantitative PCR. mRNA for LBP was significantly increased in C57BL/6 mice from the LPS-exposed 1-week and post (exposed to LPS for 4 weeks followed by 3 days of recovery) groups. As expected, LBP mRNA and protein were not detectable in the C57BL/6^{LBP-/-} mice.

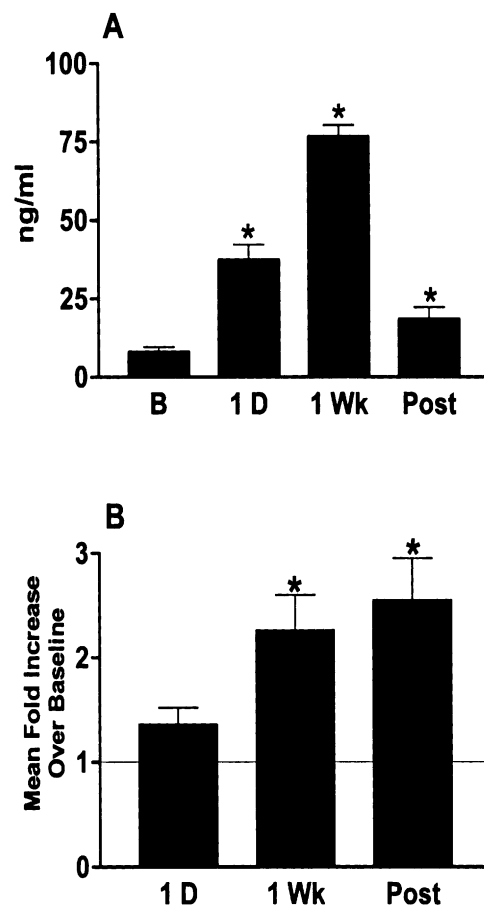


FIG 1. Lung lavage fluid mean concentration of LBP protein expressed as nanograms per milliliter (A) or expression of LBP mRNA in C57BL/6 mice expressed as mean fold increase over baseline (B). B, Baseline; 1D, 1 day; 1 Wk, 1 week; Post, 3 days after the end of the last exposure. Error bars are SEM. **P* < .05, significantly increased over baseline levels.

Estimates of airway hyperreactivity

C57BL/6 and C57BL/6^{LBP-/-} mice were not different in their responsiveness to inhaled methacholine before LPS challenge (Fig 2, A). However, after a single inhalation challenge with LPS, C57BL/6 mice had significantly enhanced responsiveness to inhaled methacholine, whereas C57BL/6^{LBP-/-} mice did not (Fig 2, A). Similarly, the C57BL/6 mice in the LPS-exposed chronic group (Fig 2, B) had significantly increased responsiveness to inhaled methacholine. Again, C57BL/6^{LBP-/-} mice in the LPS-exposed post group did not differ from age-matched control mice in their responsiveness to methacholine (Fig 2, B).

Cellularity of the lower respiratory tract

The concentration of cells in the lower respiratory tract of LPS-exposed C57BL/6^{LBP-/-} mice was significantly reduced at all time points compared with that seen in C57BL/6 mice, although it was significantly increased when compared with pre-exposure values (Fig 3, A). The

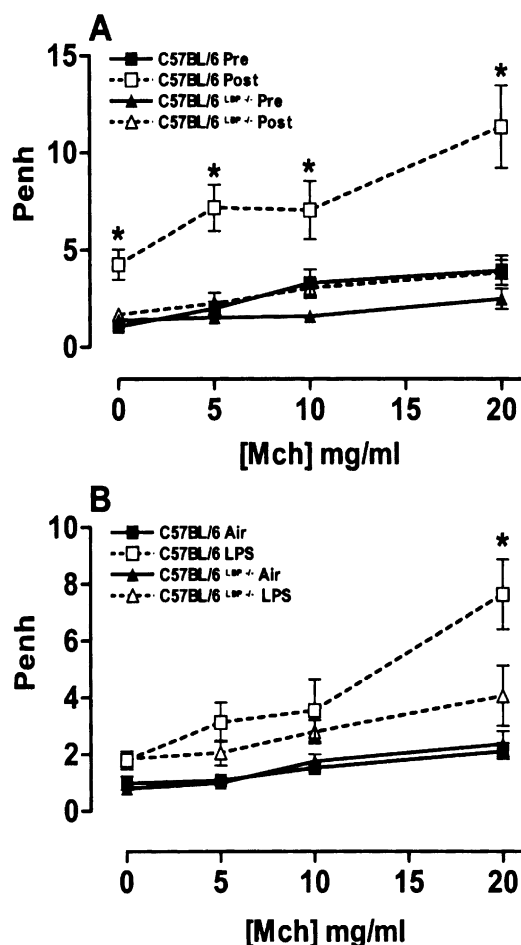


FIG 2. Methacholine responsiveness, expressed as enhanced pause (*Penh*) immediately after a single 4-hour LPS inhalation challenge (A) or 3 days after the end of 4 weeks of LPS inhalation challenge (B). Enhanced pause was measured after each increasing dose of methacholine. Error bars are SE. **P* < .05, significantly increased over pre-exposure and compared with exposed C57BL/6^{LBP-/-}.

inflammatory cells in the lower respiratory tracts of both C57BL/6 and C57BL/6^{LBP-/-} mice were predominantly neutrophils (Fig 3, B). Despite the blunted inflammatory response to inhaled LPS noted in the C57BL/6^{LBP-/-} mice, these mice still had significant increases in the concentration of cells and PMNs in the lung lavage fluid after inhalation of LPS (Fig 3).

Concentration of cytokines in whole-lung lavage fluid

C57BL/6 mice from the 1-day and 1-week groups had significantly increased amounts of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 in whole-lung lavage fluid compared with that seen in C57BL/6^{LBP-/-} mice (Table I). TNF- α , IL-1 β , and IL-6 levels were below the limits of detection in whole-lung lavage fluid at baseline or in the recovered group of exposed animals.

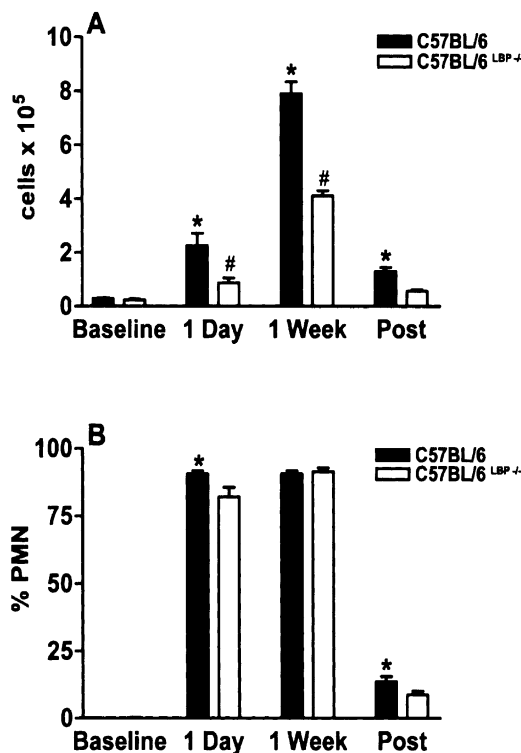


FIG 3. Lung lavage fluid mean concentration of total cells per milliliter (A) and percentage of neutrophils (B). Post, Three days after the end of the last exposure. Error bars are SE. **P* < .05, significantly increased compared with C57BL/6^{LBP-/-} mice and over baseline. #*P* < .05, significantly increased over baseline levels.

Airway morphometry

C57BL/6 mice in the post group had significantly increased submucosal area in all sizes of airways compared with that seen in LPS-exposed C57BL/6^{LBP-/-} mice and both groups of age-matched air control mice (Fig 4). Importantly, no differences were observed between LPS-exposed and air-exposed C57BL/6^{LBP-/-} mice. Moreover, the airway epithelium was not noted to be inflamed or enlarged in the C57BL/6^{LBP-/-} mice after prolonged inhalation of LPS (not shown).

Airway submucosal cell proliferation

There was no difference between LPS-exposed C57BL/6 and C57BL/6^{LBP-/-} mice from the 1-day group in the percentage of BrdU-positive submucosal cells (Fig 5), and these values were not different from baseline levels. Both C57BL/6 and C57BL/6^{LBP-/-} mice in the 1-week LPS-exposed group had BrdU incorporation that was significantly increased over baseline levels. C57BL/6 mice from the 1-week group had BrdU incorporation that was significantly increased over that seen in 1-week exposed C57BL/6^{LBP-/-} mice (Fig 5). In the post group cell proliferation was not different between C57BL/6 and

TABLE I. Lung lavage fluid mean concentration of TNF- α , IL-1 β , and IL-6 protein at 1 day, 1 week, and 3 days after the end of the last exposure

		Baseline	1 d	1 wk	Post
TNF- α	C57BL/6	ND	120.16 $\pm$ 35.99*	703.67 $\pm$ 39.96*	ND
	C57BL/6 ^{LBP-/-}	ND	0.45 $\pm$ 0.45	189.22 $\pm$ 35.22	ND
IL-1 β	C57BL/6	ND	169.06 $\pm$ 61.51*	72.47 $\pm$ 16.64	5.70 $\pm$ 3.59
	C57BL/6 ^{LBP-/-}	ND	37.60 $\pm$ 15.74	40.73 $\pm$ 12.11	1.21 $\pm$ 1.21
IL-6	C57BL/6	ND	66.61 $\pm$ 15.57*	538.89 $\pm$ 36.17*	ND
	C57BL/6 ^{LBP-/-}	ND	24.26 $\pm$ 3.46	186.11 $\pm$ 34.35	ND

Data are presented as means $\pm$ SEM.

Post, Three days after the end of the last exposure; ND, None detected.

* P < .05 compared with C57BL/6^{LBP-/-} mice.

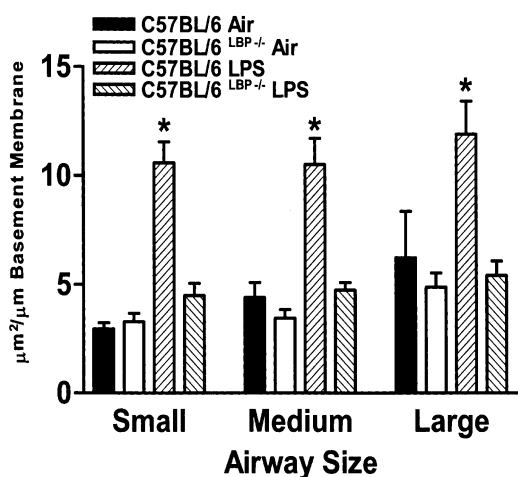


FIG 4. Morphometric measurements of the submucosal area of airways from C57BL/6 and C57BL/6^{LBP-/-} mice exposed to LPS or air for 4 weeks. Error bars are SEM. * P < .05, significantly increased over air controls and compared with LPS-exposed C57BL/6^{LBP-/-} mice.

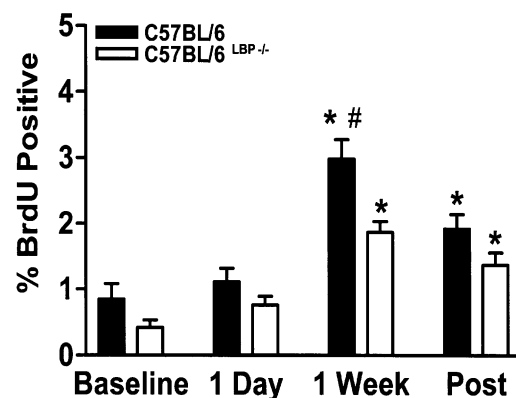


FIG 5. BrdU labeling in the submucosa of all airways combined. Error bars are SEM. * P < .05, significantly increased over baseline levels. # P < .05, significantly increased compared with LPS-exposed C57BL/6^{LBP-/-} mice.

C57BL/6^{LBP-/-} mice (Fig 5), although both groups had BrdU incorporation that was significantly increased to greater than baseline levels.

Lung lavage fluid concentration of TGF- β_1

TGF- β_1 protein concentrations in whole-lung lavage fluid from either C57BL/6 or C57BL/6^{LBP-/-} mice were not different in the LPS-exposed 1-day group (Table II). Although C57BL/6 mice had substantial increases in the concentration of total and active TGF- β_1 in whole-lung lavage fluid after 1 week of LPS inhalation, the C57BL/6^{LBP-/-} mice had only modest increases in this regulatory and profibrotic growth factor (Table II). In the LPS-exposed post groups, both C57BL/6 and C57BL/6^{LBP-/-} mice had significantly increased amounts of total TGF- β_1 protein in whole-lung lavage fluid when compared with baseline levels. Importantly, C57BL/6 mice in the LPS-exposed post group had active TGF- β_1 protein in whole-lung lavage fluid, whereas the LPS-exposed post group C57BL/6^{LBP-/-} mice did not (Table II).

DISCUSSION

Our results demonstrate that LBP is an essential component of the acute and chronic response to inhaled LPS. We have found that both LBP protein and mRNA are increased in the lung after inhalation of LPS and that LPS-exposed C57BL/6^{LBP-/-} mice do not have increased sensitivity to inhaled methacholine, have reduced inflammation of the lower respiratory tract, and do not show a fibrotic response in the subepithelial mucosa. Moreover, cell proliferation and TGF- β_1 protein release are significantly reduced in C57BL/6^{LBP-/-} mice. These data, taken together, suggest that LBP is a critical component of the acute and chronic response to inhaled LPS.

Despite numerous studies supporting the hygiene hypothesis, which suggests that exposure to endotoxin early in life can reduce the risk of later atopy,¹³ a correlation between asthma severity and endotoxin levels in samples of household dust points to the importance of endotoxin as a key risk factor for asthma in atopic children in urban settings.^{14,15} In several studies, analyses of house dust samples revealed that there was a correlation between clinical asthma scores and household endotoxin levels but not dust mite allergen levels.¹⁵ Suggestive evidence that endotoxin might be more important in inducing asthmatic

TABLE II. Lung lavage mean fluid concentration of total and active TGF- β_1 protein

		Baseline	1 d	1 wk	Post
Total	C57BL/6	ND	43.20 $\pm$ 11.36	173.81 $\pm$ 9.95*	266.07 $\pm$ 15.01*
	C57BL/6 ^{LBP-/-}	ND	34.15 $\pm$ 6.41	87.71 $\pm$ 23.91	115.66 $\pm$ 14.05
Active	C57BL/6	ND	6.50 $\pm$ 2.10	58.30 $\pm$ 16.6*	4.30 $\pm$ 4.30
	C57BL/6 ^{LBP-/-}	ND	5.40 $\pm$ 1.90	23.50 $\pm$ 7.40	ND

Data are presented as means $\pm$ SEM.

Post, Three days after the end of the last exposure; ND, None detected.

* $P < .05$ compared with C57BL/6^{LBP-/-} mice.

symptoms than previously thought is provided by a study demonstrating that acute inhalation of LPS by asthmatic patients induced airways hyperresponsiveness.¹⁶ These studies, taken together, suggest that timing and exposure levels are critical to determining the ultimate response to endotoxin. However, avoidance of endotoxin or pharmacologic measures to limit the interaction between LPS and LBP might serve to better control disease in affected individuals.

Independent of the effects of LPS on asthmatic patients, we and others have previously shown that LPS alone can cause persistent environmental airway disease in mice.^{3,5,17} Our data show that LBP enhances responsiveness to LPS in the lung. LBP protein expression in the lung is significantly increased after a single 4-hour exposure (Fig 1, A), whereas mRNA for LBP is not significantly increased until the 1-week time point. This suggests that there is a pool of LBP present in cells in the lung that is available for release, allowing for a rapid and dynamic response to gram-negative insult. LBP protein expression in the post group (Fig 1, A) is returning to baseline levels, whereas mRNA for LBP is still increased, suggesting that after the removal of insult, the lung remains in an increased state of preparedness.

We have found that the response to inhaled LPS is minimal in C57BL/6^{LBP-/-} mice (Table I, TNF- α). Wurfel et al¹⁸ showed that *ex vivo* cytokine responsiveness is highly attenuated in LBP-deficient mice, although *in vivo* there was no difference in response to intravenous LPS injection. In LBP-deficient mice made on a different genetic background (BALB/c),¹⁹ systemic responsiveness to LPS in galactosamine-sensitized mice is LBP dependent and resistance to gram-negative infection is also dependent on the presence of functional LBP. The seeming discrepancies between these 2 studies is most likely attributable to background strain, stimulus, and assay methodology. Interestingly, in the LBP-deficient animals made on the BALB/c genetic background, the oxidative burst in inflammatory cells (an indicator of cell priming and activation) in the peritoneum is LBP dependent.¹⁹ Our results also indicate that the neutrophils in the LBP-deficient mice are not fully activated, suggesting that LPS-induced neutrophil activation is dependent on LBP.

A consistent feature of exposure to inhaled LPS in human subjects and in mice is the production of proinflammatory cytokines in the lung. Proinflammatory

cytokines likely contribute to the initial response to inhaled LPS by increasing LBP expression.¹⁰ IL-6 in particular might also contribute by initiating and sustaining the cell proliferative burst observed in the submucosa. We have shown previously that subchronic inhalation of LPS enhances cell proliferation in submucosal cells of endotoxin-sensitive mice and that this cell proliferation is associated with submucosal thickening.⁵ Additionally, IL-6-dependent cell proliferation has also been associated with inhalation of cigarette smoke or ozone in mice.²⁰ In this set of studies, we show that LBP-deficient mice have IL-6 levels in whole-lung lavage fluid that are significantly reduced compared with those seen in C57BL/6 mice (Table I) and that this is associated with significantly reduced cell proliferation in the submucosa of small- and medium-sized airways in C57BL/6^{LBP-/-} mice compared with that of C57BL/6 mice. This association suggests that one function of the IL-6 elaborated in response to inhaled LPS might be to modulate submucosal cell proliferation.

We have previously shown an association between TGF- β_1 protein in whole-lung lavage fluid and submucosal thickening⁵ and between TGF- β_1 immunoreactivity in the submucosa and submucosal thickening in subchronic LPS inhalation challenge.³ In this study we show significant differences in the levels of TGF- β_1 protein in whole-lung lavage fluid between C57BL/6 and C57BL/6^{LBP-/-} mice (Table II). Given the potent fibrogenic properties of TGF- β_1 and the previous association between TGF- β_1 protein production and airway fibrosis in subchronic endotoxin inhalation, it is likely that the reduced concentration of TGF- β_1 protein in the lungs of LBP-deficient mice accounts for the protected phenotype observed in these animals.

In summary, we have demonstrated that LBP is synthesized *de novo* in the lung in response to inhaled LPS and that LBP is critical to the airway response to inhaled endotoxin. We have also shown that C57BL/6^{LBP-/-} mice are protected from the persistent effects of subchronic LPS inhalation challenge, showing no increased sensitivity to inhaled methacholine challenge or submucosal thickening, despite similar percentages of neutrophils in the pulmonary inflammatory response.

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