Lipopolysaccharide Binding Protein Potentiates Airway Reactivity in a Murine Model of Allergic Asthma

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The development of allergic asthma is influenced by both genetic and environmental factors. Epidemiologic data often show no clear relationship between the levels of allergen and clinical symptoms. Recent data suggest that bacterial LPS may be a risk factor related to asthma severity. Airborne LPS is typically present at levels that are insufficient to activate alveolar macrophages in the absence of the accessory molecule LPS binding protein (LBP). LBP levels are markedly elevated in bronchoalveolar lavage fluids obtained from asthmatic subjects compared with those in normal controls. We hypothesized that LBP present in the lung could augment the pulmonary inflammation and airway reactivity associated with allergic asthma by sensitizing alveolar macrophages to LPS or other bacterial products and triggering them to release proinflammatory mediators. We compared wild-type (WT) and LBP-deficient mice using a defined Ag immunization and aerosol challenge model of allergic asthma. Immunized LBP-deficient mice did not develop substantial Ag-induced airway reactivity, whereas WT mice developed marked bronchoconstriction following aerosol Ag sensitization and challenge with methacholine. Similarly, production of NO synthase 2 protein and the NO catabolite peroxynitrite was dramatically higher in the lungs of WT mice following challenge compared with that in LBP-deficient mice. Thus, NO production appears to correlate with airway reactivity. In contrast, both mice developed similar pulmonary inflammatory cell infiltrates and elevated mucin production. Thus, LBP appears to participate in the development of Ag-induced airway reactivity and peroxynitrite production, but does not seem to be required for the development of pulmonary inflammation. The Journal of Immunology, 2001, 166: 2063–2070.

The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall with mast cells, lymphocytes, and eosinophils (reviewed in Ref. 1). Although asthma is multifactorial in origin, a number of predisposing factors have been identified. Atopy, the genetic predisposition for the development of an IgE-mediated immune response to aeroallergens, is probably the most well-recognized factor. A critical clinical and immunologic feature associated with asthma is pulmonary inflammation. The development of airway reactivity is distinct from the development of pulmonary inflammation, although inflammation is generally believed to be required for airway reactivity to occur. The accumulation of allergen-specific CD4⁺ T cells leads to the production of cytokines that promote the activation of B cells and eosinophils. Rapid mucosal edema, airway narrowing, and mast cell degranulation characterize the early asthmatic response. The binding of IgE produced by allergen-reactive B cells triggers the degranulation of mast cells. The late asthmatic response is characterized by the migration of eosinophils and lymphocytes from the blood into the lung parenchyma and airway epithelium. In both the early and late responses, the production of vasoactive mediators, chemotactic factors, and cytokines that promote a Th2-type immune response (e.g., IL-4, IL-5, IL-13) all contribute to the development of airway reactivity and airflow obstruction.

Many studies have focused on the roles of lymphocytes, mast cells, and eosinophils in the development of pulmonary inflammation in asthma. Although these cells do play critical roles in the pathogenesis of this disease, resident macrophages may also contribute to pulmonary inflammation. For example, activated alveolar macrophages can secrete a variety of proinflammatory mediators found in the lungs of asthma patients. These include proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines (e.g., RANTES, eotaxin, macrophage inflammatory protein-1, monocyte chemoattractant protein-1), bioactive lipids (e.g., PGs, leukotrienes), and NO. Unlike lymphocytes, alveolar macrophages do not respond directly to allergens, and epidemiological data have shown that there is often no clear relationship between the levels of allergen and clinical symptoms. These macrophages can be activated by a variety of airborne particles that are inhaled along with aeroallergens. These airborne particles often contain LPS and/or other bacterial products. Recent studies suggest that LPS may be a risk factor related to asthma severity (reviewed in Ref. 2). In several studies, analyses of house dust samples revealed that there was a correlation between clinical asthma scores and household LPS levels, but not dust mite allergen levels (2–4). More direct evidence for a link between LPS and asthma comes from a recent study demonstrating that acute inhalation of LPS by asthmatics induced bronchial obstruction and hyperresponsiveness (5). Furthermore, this LPS-induced bronchial obstruction was associated with nonspecific responsiveness, but not with atopy (6). Together, these data suggest that LPS exposure may be able to aggravate the already existing inflammation among asthmatics.
Cellular activation by LPS is typically mediated by LPS binding protein (LBP), an acute phase protein that binds the lipid A portion of LPS with high affinity (reviewed in Ref. 7). LBP facilitates the catalytic transfer of LPS monomers from aggregates to the cell surface receptor CD14 (8), thereby enhancing the sensitivity of cells to LPS (9). Evidence of a role for LBP and LPS in pulmonary inflammation comes from several observations. First, inhalation of LPS causes an inflammatory response in the lungs (10), and airway reactivity is significantly increased after LPS inhalation in asthmatics (11). Second, the levels of soluble CD14 and LBP have been shown to be markedly elevated in the bronchoalveolar lavage fluid (BALF) of asthma subjects following segmental allergen challenge (12). These findings led to the proposal that elevated levels of soluble CD14 and LBP in the bronchoalveolar compartment after allergen exposure may enhance the capacity of inhaled LPS to activate an inflammatory cascade and thereby amplify the inflammatory response to inhaled allergen in some asthmatics. Third, LPS has been shown to be the active component in grain dust that causes airway inflammation following aerosol challenge (13). Intratracheal administration of the LPS antagonist Rhodobacter sphaeroides diphosphyl lipid A to mice before exposure to LPS or corn dust extract resulted in a significant reduction of the inflammatory response compared with that in mice pretreated with saline (14). Lastly, epidemiological evidence has shown that environmental levels of LPS correlate positively with the incidence and severity of asthma (2, 3, 15). Although these observations predicted an important role for LBP in LPS-induced models of pulmonary inflammation, a role for LBP in allergic asthma has been only indirectly demonstrated. Here we directly examined the role of LBP in allergic asthma in vivo using LBP-deficient mice. Our data demonstrate that LBP may serve as a mediator of Ag-induced asthma symptoms. Although wild-type (WT) mice developed a robust bronchoconstriction in response to aerosol allergen sensitization and acetyl-β-methylcholine chloride (MCh) challenge, LBP-/- mice were resistant to the development of airway reactivity. Both strains of mice developed quantitatively and qualitatively similar pulmonary inflammation, but NO synthase 2 (NOS2) expression level was severely reduced in the lungs of LBP-/- sensitized with aerosolized OVA compared with OVA-sensitized WT controls.

Materials and Methods

Animals

The generation and phenotype of LBP-/- mice were previously described (16). The mouse colony was maintained within a barrier facility in microisolation cages. Mice were backcrossed against BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) for at least four generations before use. Allergen model of airway reactivity

Six-week-old LBP-/-, hemizygote littermate LBP+/−, and BALB/cJ mice were immunized by two i.p. injections (100 μl each) of 10 μg of OVA (Sigma, St. Louis, MO) dissolved in PBS mixed 1:1 with Imject alum adjuvant (Pierce, Rockford, IL), spaced 12 days apart. Sera collected 1 wk after the final injection were used to confirm the development of an immune response, as judged by the presence of anti-OVA Ab. Two weeks after the final OVA-alum injection, mice were exposed to nebulized unfiltered OVA (1% OVA in PBS) or PBS alone for 20 min in a sealed microisolation cage daily once every 3 days. The day following the last challenge, mice were exposed to aerosolized MCh (Sigma) in saline at increasing doses (5 min/dose), and noninvasive measurement of airway responsiveness was determined by barometric plethysmography (Bu xo & Sharan, CT) using established protocols (4). This noninvasive method calcu lates enhanced pause (Penh) as an index of airway resistance (4). Mice were euthanized the next day; their lungs were fixed using paraformaldehyde and then processed for histological analysis.

Total IgE and OVA-specific Ig ELISAs

OVA-specific IgG levels were assayed by ELISA. ELISA plates were coated overnight at 4°C with OVA (10 μg/ml), and then blocked with 2% BSA in PBS for 2 h before adding sera and anti-OVA IgG standard (PharMingen, San Diego, CA). The plates were incubated overnight, washed, and a biotinylated primary goat-anti-mouse IgG F(ab')2 Ab (1: 5000; PharMingen) was added for 1 h at room temperature. The final incubation was with HRP-conjugated streptavidin (1:7500; Sigma) for 1 h at room temperature. After overnight incubation with streptavidin, the plates were washed, and 0.03% 3-ethylbenzthiazoline-6-sulfonic acid (Sigma) in 0.1 M citric acid was added. OD at 405 nm was measured periodically in an ELISA plate reader. All data presented were obtained from the 15 min point. General levels of IgE were assayed using the protocol described above with slight modifications. Briefly, plates were coated at 4°C overnight with an anti-IgE mAb (2 μg/ml; PharMingen), washed, and blocked with 1% BSA at room temperature for 30 min. Sera and Ig standards (PharMingen) were applied overnight. Plates were then incubated with biotinylated anti-mouse IgE (PharMingen) for 1 h at room temperature, followed by streptavidin-HRP at room temperature for 30 min. ELISA plates were developed as described above. OVA-specific IgE levels were assayed by coating plates overnight with an anti-mouse IgE capture mAb (4 μg/ml; PharMingen), followed by overnight incubation with sera. After washing the plates, OVA (20 μg/ml) was added for 1 h at room temperature, followed by peroxidase-conjugated rabbit anti-OVA at 1 μg/ml was added in BSA-PBS (Bioscience International, Saco, ME) and incubated 30 min. ELISA plates were developed as described above.

Processing of mouse lung tissue sections

Mice were killed by cervical dislocation, and the lungs were fixed by intratracheal inflation with fresh 4% paraformaldehyde in PBS, processed, embedded in paraplast, and sectioned at 6 μm using common histological techniques. For hematoxylin/eosin (H/E) staining, sections were dewaxed using hemo D (Fisher, Pittsburgh, PA), hydrated, then incubated in a 1:10 dilution of hematoxylin Gill no. 3 solution (Sigma) for 60 s. The slides were then washed in ddH₂O and dipped in a 10% hydrochloric acid/70% alcohol wash for 10 s. After washing in ddH₂O again, cells were stained with eosin Y (Sigma) for 4 min, rinsed, then dehydrated and coverslipped. For the periodic acid-Schiff (PAS) stain, dewaxed sections were stained using a commercial PAS staining system kit (Sigma) according to the manufacturer’s directions.

Detection of nitrotyrosine and NOS2 in tissue sections

Generation of nitrotyrosine in the tissues was assessed by immunohistochemistry by measuring the presence of 3-nitrotyrosine, the stable end product of nitric oxide interaction with cellular protein tyrosyl residues, using a rabbit polyclonal anti-3-nitrotyrosine IgG (Upstate Biotechnology, Lake Placid, NY), as previously described (17). Briefly, dewaxed tissue sections were blocked with 8% BSA in PBS for 30 min at room temperature, then the primary anti-nitrotyrosine Ab was added at 1:10 dilution of 5 μg/ml overnight at 4°C. Sections were then incubated with a goat anti-rabbit IgG Ab coupled to HRP (Vectastain ABC kit, Vector, Burlingame, CA). The presence of bound secondary Abs was detected using the chromogenic peroxidase substrate 3,3'-diaminobenzidine (DAB). To detect NOS2 protein, dewaxed tissue sections were first blocked overnight with 8% BSA at 4°C, then blocked again using a commercial reagent (Vector M.O.M. Immunodetection Kit). Primary staining of the tissue sections was performed using an anti-mouse NOS2 IgG2a mAb (clone 6, Transduction Laboratories, Lexington, KY) and detected using a commercial kit as recommended by the manufacturer (Vector M.O.M. Immunodetection Kit). In these experiments, bound Ab was detected using DAB.

Results

LBP-deficient mice develop a normal anti-OVA immune response

LBP-/-, LBP+/−, and WT BALB/cJ mice were immunized with OVA as described above. Each strain mounted a comparable immune response to the Ag, as judged by the titers of anti-OVA IgE Abs present in their sera 6 days after the second immunization (Fig. 1). Sera collected from preimmune mice did not possess any significant levels of anti-OVA Abs. Similarly, identical titers of
anti-OVA IgG Abs and total IgE Abs were found in the sera of all three immunized mice (data not shown).

WT, but not LBP-deficient, mice developed airway reactivity

OVA-immunized LBP<sup>−/−</sup>, LBP<sup>+/−</sup>, and WT mice were then sensitized to either aerosolized OVA or PBS during a 3-day period. Subsequently, the mice were challenged with increasing doses of MCh to induce airway reactivity. Before the initial exposure and after each dose of MCh, noninvasive measurement of airway responsiveness was determined by barometric plethysmography. Data were collected for 10 min following each 5-min challenge, and the mice were then rechallenged with the next higher dose of MCh. As shown in Fig. 2, Penh values were significantly higher in WT mice sensitized with OVA, compared with mice exposed to PBS (shown as percentage above the baseline measurement taken before MCh exposure). WT mice that were not initially immunized with OVA had similar airway reactivity as OVA-sensitized PBS-challenged mice (data not shown), demonstrating that aerosolized OVA alone did not affect airway reactivity. As an additional control we examined the responses of hemizygous littermates of the LBP-deficient mice. These LBP<sup>−/−</sup> mice exhibited airway reactivity comparable to that of WT mice (Fig. 2). Finally, we compared these responses to the responses observed using MCh-challenged LBP-deficient mice. Unlike the WT and LBP<sup>−/−</sup> mice, OVA-sensitized LBP<sup>−/−</sup> mice did not develop significant airway reactivity following MCh challenge. This experiment was performed four additional times using four mice per group, and similar results were obtained. Our data demonstrate that the absence of LBP had a profound influence on airway reactivity in the LBP<sup>−/−</sup> mice compared with WT controls.

Both LBP-deficient and WT mice developed similar features of lung inflammation

The finding that LBP<sup>−/−</sup> mice did not develop airway reactivity following MCh challenge prompted us to determine whether they also developed lung inflammation as a consequence of OVA immunization and aerosol sensitization. Tissue sections were prepared from the lungs of immunized WT and LBP<sup>−/−</sup> mice that were sensitized to either aerosolized OVA or PBS. These tissue sections were then stained with H&E to detect inflammatory cells and with PAS stain to detect mucus. As shown in Fig. 3A, inflammatory cells were present in the lungs of both WT and LBP<sup>−/−</sup> mice that were sensitized with aerosolized OVA. In comparison, mice exposed to aerosolized PBS did not exhibit lung inflammation (Fig. 3A). Although both WT and LBP<sup>−/−</sup> mice contained inflammatory foci within the lungs, there were subtle differences in the location of these foci. In WT mice, inflammatory cells were localized to the perivascular regions, whereas inflammatory cells in lungs of LBP-deficient mice were localized to both perivascular and airway regions (data not shown). In both mouse strains the inflammatory cells observed at the sites of inflammation were predominantly lymphocytes and eosinophils, as judged by histochemical staining (data not shown). Although the OVA stock solution used for aerosol challenges did contain some contaminating LPS (210 ng/ml), no inflammatory infiltrates were observed in the lungs of nonimmunized mice that were sensitized with OVA (data not shown). This demonstrates that LPS contamination alone did not induce the observed inflammation and airway reactivity. Regardless of whether LPS exposure arises more from contamination of the aeroantigen vs airborne LPS arising from the cage litter and feces, our data clearly show that the presence of LBP dramatically affects the development of airway reactivity (but not inflammation) in this mouse model.

Another feature associated with allergic asthma is the copious expression of mucus in the airways. As shown in Fig. 3B, both WT and LBP<sup>−/−</sup> mice that were sensitized with aerosolized OVA expressed mucus along the proximal airways, as detected using PAS stain. The lungs of WT mice contained more sites of mucus production (~50% more) than the LBP-deficient mice, and those mucus-expressing sites also appeared to be more extensive in the WT mice. Mice exposed to PBS exhibited no PAS-positive staining. Regardless of these modest differences, it is clear that both OVA-sensitized mouse strains produced mucus even though they differed dramatically in MCh-induced airway reactivity.

Lastly, bronchoalveolar lavage cells were obtained from the lungs of both WT and LBP<sup>−/−</sup> mice that were sensitized with aerosolized OVA or PBS. These cells were analyzed using flow cytometry and histological staining. As shown in Table I, total BALF cell numbers were significantly higher in both WT and LBP-deficient mice that received aerosolized OVA compared with mice that received aerosolized PBS. Enumeration of the distinct inflammatory cell types, based on histological staining, revealed that there was a marked and specific increase in the numbers of

**FIGURE 1.** LBP<sup>−/−</sup> mice exhibit a normal humoral response following OVA immunization. An anti-OVA IgE ELISA was performed as described in Materials and Methods. Serial 1:1 dilutions of preimmune and immune mouse sera were aliquoted into wells of a 96-well plate, with dilution 1 being a neat solution. □ and ■, WT mice; △ and ▲, LBP<sup>+/−</sup> littermates; ○ and ●, LBP<sup>−/−</sup> mice. Solid symbols represent sera from preimmune mice, and open symbols are from sera collected 6 days after the second immunization. Data are the average of at least eight mice per condition ± SEM.

**FIGURE 2.** LBP predisposes mice to developing an allergic asthma response. WT, LBP<sup>−/−</sup>, and LBP<sup>+/−</sup> mice were immunized with OVA as described in Materials and Methods. Half the mice were exposed to aerosolized OVA in PBS, while the remaining mice received PBS alone as indicated. The airway reactivity of the mice was then measured by plethysmography. Although WT and LBP<sup>+/−</sup> mice exhibited an increased Penh response to MCh, LBP-deficient mice exhibited responses that were essentially identical with their aerosolized PBS-treated controls. These data are representative of five experiments using at least four mice per group in each experiment.

| LBP Phenotype | Lungs | MCh-Induced Airway Reactivity | Mucin-Expressing Sites |
|--------------|-------|------------------------------|-----------------------|
| WT           | WT    | WT                           | WT                    |
| LBP<sup>−/−</sup> | LBP<sup>−/−</sup> | LBP<sup>−/−</sup> | LBP<sup>−/−</sup> |
| PBS          | PBS   | PBS                          | PBS                   |

**FIGURE 2A.** LBP predisposes mice to developing an allergic asthma response. WT, LBP<sup>−/−</sup>, and LBP<sup>+/−</sup> mice were immunized with OVA as described in Materials and Methods. Half the mice were exposed to aerosolized OVA in PBS, while the remaining mice received PBS alone as indicated. The airway reactivity of the mice was then measured by plethysmography. Although WT and LBP<sup>+/−</sup> mice exhibited an increased Penh response to MCh, LBP-deficient mice exhibited responses that were essentially identical with their aerosolized PBS-treated controls. These data are representative of five experiments using at least four mice per group in each experiment.
eosinophils in the BALF from both strains of mice that were sensitized with aerosolized OVA compared with those exposed to aerosolized PBS. For comparison, a forward and side scatter profile of BALF cells obtained using flow cytometry is also shown (Fig. 4). Cells labeled in Fig. 4 were identified based on previous studies using a panel of fluorescently labeled mAbs (anti-CD3, CD-4, CD-8, CD14, CD18, CD25). No significant differences were observed in the numbers and types of BALF cells recovered from WT and LBP−/− mice that were sensitized to OVA. The apparent paucity of alveolar macrophages in the LBP-deficient mice challenged with OVA (lower right panel) compared with that in control animals (upper right panel) was neither significant nor consistently observed.

**FIGURE 3.** Both WT and LBP−/− mice develop lung inflammation. A, H/E staining (×40 magnification). The top panels show sections from WT mice (WT/PBS, WT/OVA), and the bottom panels display the LBP−/− mouse (KO/PBS, KO/OVA) sections. Mouse lung tissue sections were prepared from MCh-challenged mice as described in Materials and Methods and then stained with H/E. Arrowheads in the left panels indicate the sites of inflammation. These data are representative of two independent experiments involving multiple lung lobe sections. B, PAS staining for mucins (×40 magnification). Tissue sections were first stained using PAS, then counterstained with hematoxylin. The bright magenta color indicates sites of mucin production along the airways. These data are representative of two independent experiments involving multiple lung lobe sections.

Peroxynitrite and NOS2 are present in the lungs of WT, but not LBP-deficient, mice

Recent studies have shown that NO production is a prominent feature of the asthmatic lung (18). We subsequently sought to determine whether NO was differentially expressed in the lungs of WT and LBP-deficient mice. NO can be produced in the lung and airway tissues by both activated macrophages and epithelial cells, and exhaled NO levels have been shown to correlate with disease severity in asthma (reviewed in Ref. 19). In the lung, NO can combine with reactive oxygen intermediates to form the highly reactive product peroxynitrite (ONOO−). Interaction of peroxynitrite with tissue proteins causes rapid formation of protein nitrotyrosine residues, which, in turn, can be used as an indicator of the existence of peroxynitrite. To a lesser extent, nitrotyrosine formation can also result from the direct modification of tyrosine by the enzymatic action of eosinophil peroxidase, which uses the NO catabolite nitrite (NO2) as a substrate for nitration of protein tyrosyl residues (20). It has been reported that nonasthmatic human lungs showed little or no nitrotyrosine staining, whereas asthmatic lungs showed significantly more staining in both the airways and lung parenchyma (21).
We used a specific anti-nitrotyrosine Ab to stain tissue sections from WT and LBP-deficient mice for the presence of peroxynitrite. As shown in Fig. 5, substantial nitrotyrosine staining was observed in tissue sections prepared from OVA-immunized WT, but not LBP−/− mice that were sensitized with aerosolized OVA. Minimal nitrotyrosine staining was observed in sections prepared from non-immunized mice sensitized with aerosolized OVA (data not shown) or from OVA-immunized mice sensitized with aerosolized PBS (Fig. 5). Interestingly, we found that the basal levels of peroxynitrite generation were higher in WT mice than in LBP-deficient mice sensitized with PBS (Fig. 5). These same tissue sections were stained with an anti-NOS2 mAb, and a staining pattern highly similar to that shown for peroxynitrite was observed (Fig. 6). Thus, inducible NOS2 expression in the lung correlates with peroxynitrite generation in situ. NOS2 protein was only detected in the lungs of WT mice that were both immunized and sensitized with OVA. OVA immunization and aerosol sensitization did not induce the expression of NOS2 in LBP-deficient mice. Together, these data demonstrate that the differential airway reactivity observed in WT and LBP−/− mice correlates with differential generation of peroxynitrite and expression of NOS2 in the lung. Whether NO (or its catabolite, peroxynitrite) directly contributes to the development of airway reactivity or is a consequence of the same initial pathophysiological event(s) remains to be determined.

Conclusions

The importance of LBP in cellular responses to LPS has clearly been established in vitro (22) and in vivo using LBP-deficient mice (16, 23). Several investigators have reported that airborne LPS may be a risk factor related to asthma severity (reviewed in Ref. 15). Here we sought to determine whether LBP was a mediator of Ag-induced asthma symptoms. Both LPS−/− and WT mice could develop a robust Ag-specific systemic immunity to the OVA allergen. Surprisingly, we found that LBP−/− mice were resistant to the development of airway reactivity in a defined murine model of allergic asthma. Unlike LBP-deficient mice, WT mice exhibited substantial bronchoconstriction following aerosol allergen sensitization and MCh challenge. Notably, both strains of mice developed quantitatively and qualitatively similar pulmonary inflammation. This was characterized by a large influx of leukocytes, especially eosinophils, and the production of mucus in the airway spaces. Thus, we were able to observe a separation between the development of inflammation and airway reactivity in the LBP−/− mice. Lastly, we also observed that peroxynitrite production was severely reduced or absent in the lungs of LBP−/− mice sensitized with aerosolized OVA compared with that in OVA-sensitized WT controls. In the WT lungs, large amounts of peroxynitrite production and NOS2 protein expression were observed, suggesting that NO production was a significant feature of disease in this mouse model. Whether the absence of NO production in the LBP−/− mice is a feature of the mechanism by which these mice also fail to develop airway reactivity or whether the absence of NO is an unrelated consequence of the same mechanism will be discussed below.

Asthma is characterized by a Th2-like immune response, enhanced IgG and IgE production, reversible airway narrowing, and episodic inflammatory cell infiltration of the bronchial wall (24). Although airborne LPS has been implicated as a contributing factor in the development of asthmatic symptoms (3, 25), no direct role for LPS in allergic asthma has been reported. LBP is an important mediator of LPS responsiveness in vivo (16, 23), and while it is largely expressed in the liver, LBP can also be expressed in the lung (26). In vitro, proinflammatory cytokines have been shown to

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**Table I.** Eosinophils are significantly increased in mice with allergic asthma*

|          | Total Cells (×1000) | Macrophages | Lymphocytes | Neutrophils | Eosinophils |
|----------|---------------------|-------------|-------------|-------------|-------------|
| WS       | 25 ± 5              | 94.1 ± 0.2  | 2.6 ± 1.5   | 3.3 ± 1.5   | 0.0 ± 0.0   |
| WO       | 388 ± 64            | 41.3 ± 6.9  | 4.4 ± 1.2   | 0.2 ± 0.2   | 54.0 ± 5.8  |
| HS       | 64 ± 31             | 86.5 ± 7.2  | 4.0 ± 2.1   | 0.6 ± 0.7   | 8.8 ± 5.4   |
| HO       | 520 ± 180           | 59.9 ± 9.7  | 5.6 ± 1.1   | 1.5 ± 0.9   | 33.1 ± 10.0 |
| KS       | 105 ± 9             | 97.0 ± 1.2  | 2.8 ± 1.2   | 0.0 ± 0.0   | 0.2 ± 0.3   |
| KO       | 726 ± 115           | 36.6 ± 11.3 | 5.5 ± 1.1   | 0.6 ± 0.7   | 57.3 ± 12.3 |

* The day following plethysmography, mice were subjected to bronchoalveolar lavage. The recovered cells were counted, a portion of the cells were centrifuged onto microscope slides by cytospin, the slides were stained with Wright stain, and differential cell counts were obtained. Total cells recovered in the lavage are recorded above in the first column. Mice exposed to aerosolized PBS contained macrophages predominantly in their lavage fluid (WT = WS, LBP−/− = HS, and LBP−/− = KS), shown as percent of total cells. After exposure to aerosol OVA, the ratio of cells shifted due to the influx of eosinophils (WT = WO, LBP−/− = HO, and LBP−/− = KO). Data are presented as mean values ± SEM, using BALF cells from least four mice per condition. The data are representative of four separate experiments.

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**FIGURE 4.** Eosinophils were present in BALF from both LBP−/− and LBP+/+ mice. One day following plethysmography mice were subjected to bronchoalveolar lavage. The recovered cells were counted, a portion was used for differential histological staining, and the remainder were pooled and examined by flow cytometry. As indicated, an increase in eosinophils (designated NE because neutrophils and eosinophils exhibit similar forward and side scatter) was observed in both the WT (WT/OVA) and LBP−/− (KO/OVA) mice exposed to aerosolized OVA. Mice exposed to aerosolized PBS (WT/Sal and KO/Sal) did not develop lung eosinophilia. Alveolar macrophages (M) were present in BALF from all four mice, whereas T lymphocytes (L) were only present in BALF from immunized and OVA-sensitized mice. The relative numbers of each cell type are shown in Table I. These data are representative of six independent experiments.
FIGURE 5. WT, but not LBP-deficient, mice generate peroxynitrite in the lungs. The top panels show sections from WT mice (WT/PBS, WT/OVA), and the bottom panels show sections from LBP^{-/-} mice (KO/PBS, KO/OVA) photographed at ×40 magnification. Mouse lung tissue sections were prepared from MCh-challenged mice, and then stained to detect 3-nitrotyrosine as described in Materials and Methods. The binding of the anti-3-nitrotyrosine peroxidase-conjugated Ab was detected by using the chromogenic substrate DAB (brown color). These data are representative of two independent experiments involving multiple lung lobe sections.

FIGURE 6. WT, but not LBP-deficient, mice express NOS2 protein in the lungs. The top panels show sections from WT mice (WT/PBS, WT/OVA), and the bottom panels show sections from LBP^{-/-} mice (KO/PBS, KO/OVA) photographed at ×40 magnification. Mouse lung tissue sections were prepared from MCh-challenged mice and then stained to detect NOS2 protein, as described in Materials and Methods. The binding of the anti-NOS2 mAb was detected by using the chromogenic substrate DAB (brown color). These data are representative of two independent experiments involving multiple lung lobe sections.
induce LBP expression by pulmonary vascular smooth muscle cells (27) and type II epithelial cells (28). In vivo, LBP levels were previously reported to be >100-fold higher in BALF obtained from asthmatic subjects following segmental allergen challenge compared with those in normal controls (12). We hypothesized that LBP present in the lung could augment the pulmonary inflammation and airway reactivity associated with allergic asthma by sensitizing alveolar macrophages to airborne LPS and triggering them to release proinflammatory mediators. Thus, concomitant Ag-dependent and LPS-dependent cellular responses might synergize to worsen the course of disease. The source of airborne LPS in these studies, and the amount of LPS actually inhaled by the mice, are not currently known. Although the OVA aerosolantigen contained some contaminating LPS, this was not sufficient to induce an inflammatory response on its own. It is not possible to determine the volume of OVA actually inhaled by the mice during the course of the experiment, and therefore how much contaminating LPS they were actually exposed to. Regardless of whether LPS exposure arises more from contamination of the aeroantigen vs airborne LPS arising from the cage litter and feces, our data provide the first evidence that the presence of LBP in the lung dramatically affects the development of airway reactivity, but not inflammation, in this mouse model.

One of the most significant observations in our studies was the difference in levels of peroxynitrite and NOS2 protein in the lungs of OVA-sensitized LBP−/− and WT mice. Peroxynitrite is the product of the reaction of NO with reactive oxygen intermediates. The effects of NO in asthma are currently controversial (reviewed in Ref. 29, 30). Studies using NOS inhibitors in vivo have suggested that NO promoted pulmonary inflammation (31). Moreover, asthmatic patients exhale elevated levels of NO compared with healthy individuals (32), and NO synthesis is similarly elevated in the lungs of asthmatics (33, 34). Studies in murine cells suggest that the Th2 cytokine response may be favored by NO, which promotes inflammation by altering the balance between Th1 and Th2 cells (35). Most recently, Guo et al. reported that NOS2 mRNA and protein are abundantly expressed in the asthmatic airway, with the high output production of NO fueled by an increase in the NOS substrate, L-arginine, in airway epithelial cells of asthmatics (18). Thus, differences in NO production or its metabolism could affect the airway reactivity seen in asthmatic mice. Our finding that the lungs of LBP-deficient mice showed little evidence of peroxynitrite generation and NOS2 protein production suggests that NO and/or the redox state of the lung plays a direct role in mediating airway hyper-reactiveness in WT mice. It should be noted that our data cannot exclude the possibility that NO production may be triggered by the same factors that trigger airway reactivity and thus is simply an unrelated consequence of the same mechanism that leads to airway reactivity. It has been recently reported that mice lacking NOS2 have significantly reduced inflammatory responses to allergen challenge in the lung, with little difference in the levels of airway reactivity (36). This supports the possibility that reduced peroxynitrite production and reduced airway hyper-reactiveness in the LBP−/− mouse may be unrelated responses. Whether the production of other mediators, such as cytokines and bioactive lipids, differ in WT and LBP−/− mice is currently under investigation.

Using LPS-hyporesponsive LBP−/− mice we have provided experimental evidence to implicate LPS in the development of allergic asthma. Although we hypothesize that the function of LBP is to mediate LPS responsiveness in the lung, other airborne bacterial products may be equally capable of activating alveolar macrophages in an LBP-dependent manner. LPS is now known to activate cells via binding to the GPI-linked cell surface receptor CD14, and engagement of the signal-transducing protein Toll-like receptor 4 (reviewed in Ref. 37). A variety of other bacterial products have been shown to activate cells via the homologous signal-transducing protein, Toll-like receptor 2. In several cases these bacterial products were also found to depend on the presence of both CD14 and LBP for cellular activation (38–40). A bacterial product other than or in addition to LPS may be acting in a CD14- and LBP-dependent manner to augment the development of airway reactivity in this mouse model. Alternatively, the phenotype of the LBP-deficient mice may arise from a previously unknown function of LBP that is independent of LPS or bacterial products. Future studies using knockout mice that lack genes that mediate cellular activation by bacterial products, such as CD14 and Toll-like receptor knockout mice, will be needed to discriminate between these possibilities.

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