Interleukin-12-Independent Down-Modulation of Cockroach Antigen-Induced Asthma in Mice by Intranasal Exposure to Bacterial Lipopolysaccharide

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Several studies have shown that exposure to bacterial lipopolysaccharide (LPS) can either prevent or inhibit asthma in humans and laboratory rodents. Much emphasis has been placed on the role of cytokines and chemokines in the establishment and maintenance of allergic airway disease. Therefore, it is of interest to study the role of LPS in affecting airway pathology and lung cytokine and chemokine responses in the maintenance phase of asthma. Increasing doses of LPS were administered into the airways of mice pre-sensitized with cockroach allergen (CRAg), then allergic airway disease parameters were assessed after CRAg challenge. Airway hyperresponsiveness after antigen challenge decreased at the highest dose of LPS tested, which was accompanied by a decrease in airway and lung eosinophils. However, a dramatic increase in lung inflammation because of neutrophil influx was observed. Measurement of cytokines in lungs of LPS-treated, CRAg-sensitized mice indicated that interleukin (IL)-12 levels were increased by LPS treatment in a dose-dependent manner, as were levels of several inflammatory chemokines. In contrast, levels of IL-4, IL-13, IL-5, and IL-10 were reduced in whole lung homogenates only of high-dose LPS-treated mice. Intranasal administration of neutralizing anti-IL-12 at the time of high-dose LPS challenge reduced lung IL-12, interferon- γ , CXCL9, and CXCL10 but did not affect levels of the other chemokines or Th2-type cytokines, and did not restore AHR. These findings suggest that the amelioration of airway hyperresponsiveness observed in LPS-treated, CRAg-sensitized mice is coincident with an immune deviation of the lung inflammatory response, independent of IL-12. (*Am J Pathol* 2003, 163:1961–1968)

The incidence and severity of asthma has risen dramatically throughout the past few decades, with the greatest increases being evident in the well-developed nations of North America and Europe.¹ The disease has been primarily associated with allergens derived from plant pol-

lens or household pests such as dust mites and cockroaches, which are fairly evenly distributed throughout the world. Inhalation of aerosolized allergen leads to inflammation of the major airways of the lung, which is mediated by cytokines and chemokines of the innate and Th2-type adaptive immune response.^{2,3} Asthma is characterized by production of allergen-specific IgE, mast cell activation, influx of eosinophils into the lung and airway hyperproduction of mucus, and increased sensitivity of airway smooth muscle to neurotransmitters leading to bronchoconstriction and decreased air capacity.

Increased sanitation and antibiotic usage and decreased environmental exposure to immunogenic organisms including bacteria have been linked to increasing incidence and severity of asthma in developed countries leading to the hygiene hypothesis.^{4–7} A recent epidemiological report clearly demonstrated an inverse correlation between several markers of allergy and atopy of a large cohort of children and the amount of endotoxin [lipopolysaccharide (LPS)] found in their bedding material.⁸ This study is in agreement with several other studies showing decreased incidence of asthma in children raised in a farming environment and associations based on household size and birth order.^{9–11} However, a significant amount of data has shown that exposure to endotoxin in the workplace or as a component of cigarette smoke leads to exacerbation of pre-existing asthma.^{12–15} Thus, it remains unclear what role exposure to endotoxin plays in modulation of human allergic airway disease.

Studies in animal models of asthma using ovalbumin (OVA) as the allergen have also been contradictory, with nearly as many investigators reporting that endotoxin inhibits asthma-associated airway inflammation as those reporting enhancement.^{16–21} Eisenbarth and colleagues²² have shown that the allergic response to inhaled OVA is dependent on activation through toll-like receptor 4 by low-dose contamination with LPS, but that a higher dose of LPS inhibited development of asthma. The difference in response was associated with drastic changes in leukocyte migration, immunoglobulin isotype switching, and cytokine expression, such that the highly

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Th2-type response elicited by low-dose LPS was reversed to a predominant Th1-type pattern at the higher dose. These data suggested that there could be a therapeutic dose of endotoxin that would be effective at treating asthma.

In the current study, we hypothesized that intranasal endotoxin exposure would cause immune deviation and reverse asthmatic disease in mice presensitized to cockroach antigens (CRAg). Increasing doses of intranasal LPS were administered to CRAg-sensitized mice before a final intratracheal challenge with CRAg and measurement of asthma parameters. The lowest dose of LPS tested appeared to decrease airway hyperreactivity (AHR). As the dose of LPS increased, AHR was progressively reduced and the pattern of cytokine expression changed from a high-Th2/low Th1-type to a high-Th2/high-Th1-type, then to a low-Th2-type/high-Th1-type response. Alterations in lung chemokine expression, a dramatic influx of neutrophils and CD4⁺ T lymphocytes, and a decrease in lung eosinophilia were also observed in LPS-treated mice. Neutralization of interleukin (IL)-12 by intranasal anti-murine IL-12 treatment caused a reduction in Th1-cytokine expression but did not lead to increased Th2 cytokine expression or reconstitution of AHR.

Materials and Methods

Animals and Reagents

Female CBA/J mice (8 to 10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in covered boxes (five mice/box) in the Unit for Laboratory Animal Medicine (ULAM) facility of the University of Michigan. Clinical skin-test grade cockroach antigen extract was purchased from Hollister-Stier (Spokane, WA), small molecular weight components were removed by centrifugation through an Amicon 3000 column, and batches were tested for the absence of endotoxin by limulus amoebocyte assay (detection limit 6 pg/ml; BioWhittaker, Walkersville, MD) before use. Incomplete Freund's adjuvant and bacterial LPS (*Escherichia coli* serotype 0111:B4, lot no. 70K4108) were purchased from Sigma (St. Louis, MO). LPS was diluted to 1 mg/ml in sterile saline and sonicated for 1 hour before aliquoting and freezing. A fresh aliquot was thawed and vigorously shaken before the start of each experiment. Neutralizing anti-murine IL-12 antibodies were collected from polyclonal anti-serum of rabbits immunized and rechallenged with recombinant murine IL-12. Normal rabbit serum was collected and used to control for the presence of rabbit immunoglobulins. The rabbit antibodies were purified over a protein A column and diluted to a total protein concentration of 10 mg/ml in sterile saline before use.

Antigen Sensitization and Challenge

See Figure 1 for a schematic timeline of the sensitization protocol. Cockroach antigen (20,000 PNU/ml) was mixed at a ratio of 1:1 with incomplete Freund's adjuvant and 0.1 ml of emulsion was injected both intraperitoneally and subcutaneously at the nape of the neck. Fourteen days

Figure 1. Female CBA/J mice were sensitized on day 0 by intraperitoneal and subcutaneous immunization with CRAg emulsified in IFA. An intranasal challenge with undiluted CRAg was given on day 14, followed by four alternate-day intranasal exposures to LPS or saline starting on day 15. Some mice received intranasal treatments with normal rabbit serum or neutralizing anti-IL-12 antibodies at the time of LPS exposure. Mice received a final intratracheal challenge with undiluted CRAg on day 21, followed by assessment of AHR and collection of tissue samples 18 to 20 hours after intratracheal challenge. The CRAg used in these studies tested negative (<6 pg/ml) for LPS by limulus amoebocyte assay.

later, mice were anesthetized with ketamine and xylazine, then 15 μ l of undiluted CRAg was injected intranasally. A 15- μ l dose of the indicated amount of LPS or sterile saline diluent was given intranasally to anesthetized mice on days 15, 17, 19, and 21 after CRAg sensitization. Anti-body-treated mice received an intranasal dose of anti-murine IL-12 or normal rabbit serum (10 μ l) at the same time as LPS administration. Immediately after the final dose of LPS, the trachea was exposed by incision, 40 μ l of undiluted CRAg was injected down the airway, and the wound was closed with wound clips.

Measurement of AHR and Harvest of Tissues

Mice were anesthetized with sodium pentobarbital (3.3 mg/mouse) 18 to 20 hours after intratracheal CRAg challenge, trachea were exposed, an airway tube was inserted, and mice were connected directly to a plethysmograph (Buxco, Troy, NY). A baseline of airway resistance was established for each mouse, then methacholine (6.25 μ g in 0.1 ml) was administered intravenously and airway resistance was measured again. Peak airway resistance after methacholine challenge was divided by baseline to determine the fold increase in AHR. Bronchoalveolar cells and fluid were collected by lavage with 1.0 ml of sterile saline. Blood was obtained by orbital bleed, followed by sacrifice and removal of the lungs.

Flow Cytometry

Total right lobe CD4⁺ T cells were determined by collagenase dispersion of cells from the whole right lobe of each mouse, followed by a total cell count with trypan blue. Dispersed cells were stained with FcBlock (BD Biosciences, San Diego, CA), followed by staining with phycoerythrin-conjugated anti-murine CD4 (BD Biosciences), and analyzed on a Beckman Coulter Epics XL flow cytometer (Brea, CA). The total number of cells in the right lobe was multiplied by the percentage of CD4⁺ cells to determine total CD4⁺ T-cell content.

Histology

Lungs were removed after analysis of AHR and inflated with 10% neutral buffered formalin before paraffin embedding. Tissue morphology and lung eosinophil content

Figure 2. Anesthetized mice were analyzed for airway hyperresponsiveness before and after methacholine challenge at 18 to 20 hours after intratracheal challenge. Data are the fold change in airway resistance after methacholine administration $\pm$ SE for three to five mice from two separate experiments. The cumulative data from four experiments using the 15- μ g/ml dose of intranasal LPS ($n = 11$ mice) indicated a statistically significant decrease in AHR ($P < 0.05$) compared to saline control mice ($n = 16$ mice).

were assessed on hematoxylin and eosin-stained sections from paraffin-embedded, right lobes. Lung neutrophil, eosinophil, monocyte/macrophage, and lymphocyte content was determined from Diffquick (Dade Behring, Newark, DE)-stained cytopspins of the cells dispersed for flow cytometry. Bronchoalveolar lavage (BAL) cells were centrifuged and resuspended in 1.0 ml of ACK red blood cell lysis buffer for 3 minutes before immobilizing 0.2 ml of the cell suspension on a microscope slide by cytopspin and staining with Diffquick. Cell differentials were counted using an Olympus BX40 microscope at $\times 1000$ magnification and photographs were taken with a Spot RT color camera (Diagnostic Instruments, Sterling Heights, MI).

Measurement of Cytokines

The left lobe of each lung was snap-frozen and homogenized in 1.0 ml of 0.1% Triton X-100:phosphate-buffered saline containing protease inhibitors before analysis of cytokine content by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) following manufacturer's instructions. Total protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA). The cytokine content of each lung was normalized to total

protein and then mean and SE were determined within each treatment group.

Statistical Analysis

Multiple pairwise comparisons were performed using the analysis of variance portion of the Prism software. Individual pairwise comparisons were performed using Student's *t*-test.

Results

Bronchoconstriction is a well-established marker of asthma and is tested for clinically by measurement of airway capacity after administration of methacholine. Mice sensitized with CRAg responded to methacholine with a sevenfold to eightfold increase in airway hyperresponsiveness (AHR) compared to baseline airway resistance before methacholine challenge (Figure 2). Repeated intranasal exposure to increasing doses of LPS during the course of CRAg sensitization led to an apparent dose-dependent decrease in airway hyperresponsiveness.

Serum levels of IgE were higher in CRAg-sensitized mice than in unsensitized animals but treatment with LPS did not significantly affect systemic IgE levels (Table 1). The lungs of LPS-treated mice were highly inflamed (Figure 3) with an increase in the presence of total leukocytes and CD4⁺ T cells within the lung tissue and a dramatic increase in the presence of neutrophils in the BAL fluid (Table 1). The number of eosinophils in the BAL and surrounding the bronchi was sharply reduced with the highest dose of LPS, but was not significantly affected at the lower doses (Table 1).

Treatment with LPS led to a dose-dependent increase in IL-12 production in the mouse lung 18 hours after airway exposure (Figure 4), however, neither interferon (IFN)- γ nor tumor necrosis factor- α levels in the lung were increased by intranasal treatment with LPS (data not shown). Levels of the Th2-associated cytokines, IL-4, IL-13, IL-5, and IL-10 were not decreased by the low-

Table 1. Increased Neutrophilia and Decreased Eosinophilia in LPS-Expressed CRAg-Sensitized Mice

Group	n	Total serum IgE (μg/ml)*	Lung (Rt. lobe)		Peribronchial eosinophils (cells/20 HPF)	Bronchoalveolar lavage			
			Leukocytes (×10 ⁶)	CD4 ⁺ (×10 ⁵)		Total leukocytes (cells/HPF)	PMN (%)	Eosinophils (%)	Lymphs (%)
Exp. 1									
No LPS	4	5.4 ± 1.4 [†]	6.7 ± 0.7	4.9 ± 0.6	N.D. [‡]	15.5 ± 2.5	48.8 ± 4.8	4.3 ± 1.5	2.4 ± 0.4
LPS, 0.3 μg	5	6.6 ± 1.9	9.0 ± 0.8 [§]	6.2 ± 0.7	N.D.	21.1 ± 2.0 [§]	70.2 ± 6.0 [§]	6.3 ± 2.4	1.4 ± 0.6
LPS, 15 μg	3	6.4 ± 1.7	20.9 ± 1.0 [§]	10.6 ± 0.5 [§]	N.D.	47.1 ± 3.1 [§]	81.2 ± 0.4 [§]	0.0 ± 0.0 [§]	0.9 ± 0.1 [§]
Exp. 2									
No LPS	4	4.8 ± 1.2	N.D.	N.D.	139 ± 59	14.3 ± 2.4	44.9 ± 10.6	9.2 ± 5.9	2.2 ± 0.3
LPS, 0.6 μg	4	3.6 ± 1.2	N.D.	N.D.	77 ± 20	27.1 ± 2.8 [§]	82.3 ± 2.4 [§]	1.2 ± 0.4 [§]	0.9 ± 0.3 [§]
LPS, 3 μg	4	4.5 ± 2.3	N.D.	N.D.	62 ± 24	25.8 ± 2.3 [§]	82.1 ± 3.6 [§]	3.1 ± 1.4	1.8 ± 0.5
LPS, 15 μg	3	4.2 ± 1.5	N.D.	N.D.	18 ± 6 [§]	62.3 ± 5.4 [§]	93.1 ± 2.8 [§]	0.1 ± 0.1 [§]	0.8 ± 0.5 [§]

*Serum IgE level in non-CRAg-sensitized mice was 1.0 $\pm$ 0.4 μ g/ml of serum.

[†]Data are mean $\pm$ standard error.

[‡]N.D. = not determined.

[§]Statistically significant difference ($P < 0.05$) from no LPS control.

Figure 3. Lungs were removed 18 to 20 hours after intratracheal CRAg challenge and paraffin-embedded. Sections (5 μ m) were stained with H&E and photographed at the indicated magnification. Lungs of CRAg-sensitized mice exposed to intranasal saline had modest levels of inflammation with evidence of peribronchial eosinophilia. Lungs of CRAg-sensitized mice given intranasal LPS (15 μ g $\times$ 4 doses) had large areas of severe inflammation and airways surrounded by cellular infiltrates consisting primarily of polymorphonuclear neutrophils and monocytes.

dose LPS treatment that caused progressive elevation of IL-12, but the levels of all of these Th2 cytokines were diminished at the highest dose of LPS (Figure 4).

Leukocyte recruitment to the lung during inflammation is partially dependent on the release of chemokines from T cells, leukocytes, and structural cells. Treatment with LPS caused a dose-dependent increase in the level of CXCL8/MIP-2, CCL2/JE, and CCL20/MIP-3 α , that have been associated with the innate inflammatory response (Figure 5). Similarly, increases in Th1-associated chemokines, CCL3/MIP-1 α , CCL5/RANTES, CXCL9/MIG, and CXCL10/IP-10 were noted after airway exposure to increasing doses of LPS. Although lung levels of the Th2-associated chemokines, CCL11/Eotaxin, CCL17/TARC, and CCL22/MDC were not significantly reduced by LPS exposure (data not shown), CCL1/TCA-3 was reduced by high-dose LPS treatment, similar to the findings for the Th2-associated cytokines (Figure 5).

A similar increase of innate and Th1-associated cytokines and chemokines was observed in the BAL fluid of intranasal LPS-exposed mice (Figure 6). Unlike the sustained levels of IL-10 observed in the whole lung after intermediate doses of LPS, the levels of IL-10 in BAL fluid dropped progressively in a LPS dose-dependent manner.

The dose-dependent increase in Th1-associated chemokines prompted interest in the role of IL-12 to the modulation of AHR and inflammation after LPS exposure.

Neutralization of airway IL-12 by intranasal exposure at the time of each high-dose LPS administration did not affect the down-regulation of AHR (Figure 7) despite decreasing BAL levels of IL-12, CXCL9, and CXCL10. Although whole lung levels of IL-12, IFN- γ , CXCL9, and CXCL10 levels were significantly reduced after anti-IL-12 treatment (Figure 8), no reciprocal increase in the Th2-associated cytokines IL-4, IL-5, IL-13 (Figure 8); or the Th2-associated chemokines CCL1, CCL11, CCL17, or CCL22 (data not shown) was detected in the lungs of antibody-treated mice. Further, the chemokines CCL2, CCL3, CCL5, and CXCL8, that were all induced by LPS exposure, were not significantly affected by airway installation of anti-IL-12 antibody.

Intranasal IL-12 neutralization at the time of LPS exposure did not lead to a significant change in total serum IgE. (Table 2) However the number of leukocytes in the BAL fluid was significantly reduced and an apparent increase in peribronchial eosinophilia was also observed after IL-12 neutralization, but did not reach statistical significance (Table 2).

Discussion

In the current study, bacterial LPS was shown to deviate the inflammatory response in a cockroach allergen

Figure 4. Lungs of CRAg-sensitized, untreated, and LPS exposed mice were removed after analysis of AHR and snap-frozen. Whole lung homogenates were analyzed by sandwich enzyme-linked immunosorbent assay for the cytokines listed. Additional analysis was done for IFN- γ , tumor necrosis factor- α , and transforming growth factor- β but no significant differences were detected in any experiment (not shown). Data are the mean cytokine concentration $\pm$ SE for three to five mice/group in this representative experiment of four performed. The increase in IL-12 was significant ($P < 0.05$) at the 3 μ g dose compared to the untreated control. Decreased IL-4, IL-5, and IL-10 showed significance at the 15- μ g dose ($P < 0.05$, 0.02, and 0.05, respectively). Although the decrease in IL-13 did not reach statistical significance in this experiment, it was consistently reduced in all experiments at the 15- μ g dose, and did reach significance in several experiments.

(CRAg)-induced model of asthma. Repeated intranasal exposure to LPS led to a dose-dependent decrease in AHR after methacholine administration. This finding is in agreement with several epidemiological studies that have shown an inverse relationship between human asthma and environmental exposure to LPS.^{8–10,23} Several studies in animal models have shown a similar decrease in AHR when LPS exposure was combined with airway allergen challenge. Cochran and colleagues¹⁶ demonstrated that a single intranasal dose of 1 μ g of LPS given to young mice decreased AHR when the mice were subsequently challenged with OVA. In a study more similar to our current protocol, Tulic and colleagues¹⁹ demonstrated that LPS exposure of rats that were presensitized to OVA led to a LPS dose-dependent decrease in sensitivity to methacholine.

Figure 5. Whole lung homogenates were analyzed for chemokine expression by sandwich enzyme-linked immunosorbent assay. Data shown are mean chemokine concentration $\pm$ SE for three to five mice/group in this representative experiment of four performed. Levels of CXCL8, CCL2, CXCL10, CXCL9, CCL3, and CCL5 were significantly increased after exposure to the lowest dose of LPS compared to untreated controls ($P < 0.05$, 0.02, 0.01, 0.02, 0.01, and 0.01, respectively). Increased CCL20 reached significance at the 15- μ g dose of LPS ($P < 0.001$). Decreased CCL1 ($P < 0.01$) was detected at the 15- μ g dose of LPS. Whole lung levels of CCL22/MDC were unchanged by LPS exposure, but CCL11/eotaxin and CCL17/TARC were nonsignificantly reduced at the 15- μ g dose of LPS in all experiments (not shown).

A well-established mechanism of induction of AHR in asthma is the release of leukotrienes from mast cell granules after crosslinkage of antigen-specific IgE on the mast cell surface. The current study showed that serum IgE levels were not significantly affected by intranasal exposure to LPS. A recent study by Gerhold and colleagues¹⁷ showed that systemic exposure to LPS before OVA sensitization led to prevention of OVA-specific IgE production, however, that study also indicated that intranasal LPS treatment did not affect total or antigen-specific IgE production, in agreement with the current findings. However, in contrast to our current findings, Gerhold and colleagues¹⁷ did not detect any change in

Figure 6. BAL was performed by injection of 1 ml of sterile saline into a tracheal tube followed by aspiration. Cells were removed by centrifugation and the supernatant was frozen until tested by enzyme-linked immunosorbent assay for the indicated cytokines and chemokines. Total protein content in BAL was determined with the Bradford reagent and cytokine levels were normalized to protein content for each sample. Data are mean concentration $\pm$ SE for three to five mice/group in this representative experiment of four performed. A significant increase in total BAL protein ($P < 0.02$) was detected with high-dose LPS exposure. IL-10 levels were reduced ($P < 0.05$) and IL-12, CCL5, CXCL8, and CXCL10 levels were increased by the lowest dose of LPS ($P < 0.01$, 0.001, 0.001 and 0.02, respectively). The increase in CXCL9 was significant ($P < 0.02$) after high-dose LPS exposure. IL-4, IL-5, IL-13, and IFN- γ were below detectable levels in the BAL fluid of these mice.

Figure 7. AHR and BAL cytokine levels were tested as described in Figures 1 and 5 after intranasal exposure of CRAG-challenged mice (18 hours) with sterile saline/normal rabbit serum (**white bars**), high-dose LPS/normal rabbit serum (**gray bars**), or high-dose LPS/anti-IL-12 (**striped bars**). Data are mean $\pm$ SE for five mice/group in this representative experiment of two performed. AHR was unchanged by anti-IL-12 treatment compared with mice receiving high-dose LPS alone. Reductions in BAL IL-12, CXCL10 ($P < 0.01$), and CXCL9 ($P < 0.05$), were evident after anti-IL-12 treatment. No statistically significant changes in other cytokines or chemokines were detected (CXCL8/MIP-2 shown as an example).

AHR after local exposure to LPS. Plausible explanations of these conflicting results include the difference in allergens and strains of mice used in these studies. Another recent study showed that low-level contamination of OVA by LPS (0.1 μ g/dose) was necessary to elicit a Th2 cytokine response, OVA-specific IgE and allergic disease, whereas a high dose of LPS (100 μ g/dose) was inhibitory of allergen sensitization.²² The CRAG used in our study contained less than 6 pg/ml of LPS and was sufficient to elicit an asthmatic response in our model. The lowest dose of LPS used in the current study (0.3 μ g/dose) did not exacerbate AHR induced by CRAG alone, but rather caused a slight decrease in hyperreactivity. This result suggests that CRAG does not require LPS contamination to induce asthma and that even low levels of LPS exposure are inhibitory in this model.

Airway exposure of CRAG-sensitized mice with LPS led to a dramatic influx of neutrophils into the lung paren-

Figure 8. Lung cytokine levels were measured in anti-IL-12-treated, LPS-exposed, CRAG-sensitized mice (**striped bars**) and compared to controls that were CRAG-sensitized and received sterile saline (**white bars**) or intranasal LPS (**gray bars**) and normal rabbit serum control antibody. Significant decreases in IL-12, IFN- γ , CXCL9 ($P < 0.05$), and CXCL10 ($P < 0.02$) were observed. No changes in the Th2-associated cytokines IL-4, IL-5, or IL-13 were detected after airway neutralization of IL-12 at the time of LPS exposure. Data are mean $\pm$ SE for five mice/group in this representative experiment of two performed.

chyma and the airway itself. An influx of CD4⁺ Th cells was also detected in the lung but a reduced number of total lymphocytes and CD4⁺ Th cells was detected in the BAL. At low doses of LPS, eosinophils were still present in the lung and BAL, but mice that received the highest dose of LPS displayed a marked reduction in airway and lung eosinophilia. These data, which agree with previous studies, indicate a drastic change in the inflammatory cellular response dependent on LPS.²²

The decreased hyperreactivity was more closely associated to increased levels of IL-12 and several inflammatory and Th1-associated chemokines in the lung and BAL than to decreased levels of Th2-associated cytokines and chemokines. In fact, significant reductions of the Th2-associated cytokines were only detected at the highest dose of LPS. These findings supported the hypothesis that a diversion of the immune response from a Th2- to a Th1-type response caused the decrease in AHR. To test this hypothesis, localized neutralization of IL-12 was performed because IL-12 is a critical cytokine in the establishment of Th1-associated inflammation. Although levels of IL-12 and other Th1-associated cytokines and chemokines were reduced after anti-IL-12 instillation, the treatment had no effect on the decreased AHR resulting from LPS exposure. Surprisingly, the neutralization of lung IL-12 was not effective at restoring expression of any of the Th2-associated cytokines or chemokines tested in this study, indicating that another mechanism of Th2 suppression was in effect. Yet, it seems unlikely that the suppression of the local Th2 response alone can explain decreased AHR because decreased AHR was apparent at doses of LPS that did not suppress Th2 cytokine production in the lung.

The findings of this study shed some light on some of the currently held hypotheses of the effects of LPS on AHR.⁵ Th1 immune deviation has been shown to decrease AHR, therefore, it has been suggested that LPS-induced immune deviation toward a Th1 response inhibits the Th2 response that leads to asthma.^{24–30} The current findings do not support that hypothesis because neither IFN- γ nor tumor necrosis factor- α were elevated in the lungs of LPS-treated mice, and inhibition of the classical Th1 response by neutralization of IL-12 did not reverse the blockade on AHR.

Over-production of IL-10 in response to LPS has been put forward as a plausible mechanism of LPS-mediated down-regulation of airway inflammation.^{5,19,31,32} Some studies have shown that local increases in the levels of IL-10 in the lung, have an anti-inflammatory effect on

Table 2. Blockade of IL-12 Decreases LPS-Induced Leukocyte Infiltration

Group	n	Total serum IgE (μ g/ml)	Peribronchial eosinophils (cells/20 HPF)	Bronchoalveolar lavage			
				Total leukocytes (cells/HPF)	PMN (%)	Eosinophils (%)	Lymphs (%)
No LPS + NRS	7	4.1 $\pm$ 0.7*	125 $\pm$ 44	17 $\pm$ 1	48.3 $\pm$ 6.1	5.9 $\pm$ 2.6	1.7 $\pm$ 0.4
15 μ g LPS + NRS	7	3.8 $\pm$ 0.4	40 $\pm$ 12	70 $\pm$ 4 [†]	86.8 $\pm$ 5.6 [†]	0.4 $\pm$ 0.2	1.0 $\pm$ 0.3
15 μ g LPS + aIL-12	9	5.5 $\pm$ 0.9	70 $\pm$ 13	46 $\pm$ 3 ^{†‡}	79.6 $\pm$ 4.6 [†]	1.0 $\pm$ 0.2	1.2 $\pm$ 0.4

*Data are combined mean $\pm$ standard error from two separate experiments.

[†]Statistically significant difference ($P < 0.05$) compared to no LPS control.

[‡]Statistically significant difference compared to LPS + NRS group.

airway inflammation.^{28,33} In the current study, there was no evidence of IL-10 induction by LPS, and in fact, the levels were markedly reduced in the BAL at low doses of LPS and in the whole lung at higher doses. To ensure that the discrepancy between our findings regarding IL-10 and those put forward by others was not because of timing of detection, we used neutralizing antibodies to IL-10 in a similar manner as for blocking IL-12. Airway neutralization of IL-10 at the time of LPS exposure had no effect on AHR, cellular infiltration, or cytokine/chemokine production in our model (data not shown).

In addition to these more commonly held hypotheses, we had entertained a role for LPS-activated, B-1a cells as a source of regulatory cytokines or as inducers of CD4⁺ T-cell apoptosis.³⁴ However, studies to test the latter hypothesis in Xid, B-1 cell-deficient mice indicated no role for B-1 cells in affecting the LPS-mediated events.

Thus, it remains unclear what is the direct effector mechanism of LPS exposure on reducing AHR. There were several chemokines elevated by LPS exposure in this study that were not neutralized by our anti-IL-12 treatment. These chemokines may have been responsible for directing cellular influx and activation changes that led to decreased AHR. Another possibility is that LPS had an effect on other mediators of airway smooth muscle cell contraction. It has been suggested that changes in nitric oxide production induced by LPS are mediators of decreased AHR.^{35–37} In addition, LPS may have had direct effects on airway mast cells that led to early spontaneous degranulation before the time that AHR was tested in this study.¹⁹ Prolonged exposure to LPS could eventually lead to desensitization of mast cells, alveolar macrophages, smooth muscle cells, or other cells involved in the asthmatic response.

AHR is one of the most severe and life-threatening symptoms of asthma, therefore, LPS or one of its derivatives would seem to be a good candidate for therapy of asthmatic patients. However, a note of caution is in order. The mice exposed to high doses of LPS in this study displayed severe inflammation in their lungs, high levels of potentially dangerous Th1-associated cytokines, and an increasing mortality rate (40% at the highest dose tested). A better approach to developing therapies based on the natural effects of LPS may be to target the specific cytokines, chemokines, cellular interactions, or other inflammatory mediators involved in the decreased airway hyperreactive response. To that end, further study of the complex nature of the airway response to LPS exposure is warranted.

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